Interactions of *Candida albicans* with non-pathogenic gut bacteria

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„Wir können den Wind nicht ändern, aber die Segel anders setzen."

Aristoteles

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Summary

The yeast *Candida albicans* is a commensal colonizer of mucosal surfaces in humans. Under certain circumstances, the fungus can cause superficial infections like oropharyngeal or vulvovaginal candidiasis, but also invasive and disseminated infections. Predispositions for this opportunistic pathogen to cause disease include antibiotic treatment, immunosuppression, and a compromised epithelial barrier. The intestine is the main reservoir from which the fungus can translocate across the intestinal barrier, invade the bloodstream, and disseminate when predisposing conditions permit. Since intestinal colonization with *C. albicans* is a prerequisite for infection, research on the fungus in this biological niche is of specific importance to understand the pathogenesis of candidiasis and provide fundamental knowledge for novel therapeutic strategies.

As the natural microbiota in the healthy intestine limits fungal overgrowth, this thesis aimed to investigate interactions between *C. albicans*, intestinal epithelial cells, and intestinal bacteria and to dissect the fungal commensal-to-pathogen shift. Therefore, a static *in vitro* gut model was established that includes intestinal epithelial cells and mucus-secreting goblet cells, which were colonized with *Lactobacillus* spp. as a single species artificial microbiota before *C. albicans* infection. Using this model, *Lactobacillus* spp. colonization was observed to mediate a time-, dose-, and species-dependent protection against *C. albicans* pathogenicity. Particularly *L. rhamnosus* was identified to exhibit a potent protection compared to other tested *Lactobacillus* species. The bacterial antagonism was related to a reduction of *C. albicans* cytotoxicity, hypha-length, and translocation. These results were verified in a perfused intestine-on-chip model that contains two compartments separated by a porous membrane to mimic the gut lumen with intestinal epithelial cells and the vasculature with endothelial cells.

Further, the physical separation of *C. albicans* from the intestinal epithelium *via L. rhamnosus*-induced shedding was identified as a potential protective mechanism in this thesis. Mechanistic insights into the complex interplay between *C. albicans*, intestinal epithelial cells, and *L. rhamnosus* were achieved on a molecular level using transcriptomics and metabolomics, which were supported by *in silico* predictions. The growth and metabolic activity of *L. rhamnosus*, as a prerequisite for its protective effect, was identified to be dependent on the presence of intestinal epithelial cells. Metabolites, like citric acid, gamma-glutamyl amino acids, and carnitine, which were secreted by intestinal epithelial cells, fostered *L. rhamnosus* growth. When growing on intestinal epithelial cells, *L. rhamnosus* released metabolites with known antifungal potential. In addition to this, new candidates of secreted molecules like cytosine were investigated concerning their antifungal effects. Colonization with *L. rhamnosus* altered the availability of carbon sources in the environment. Consequently, the fungus responded to these alterations by extensive changes in the gene expression profiles, in particular of genes associated with metabolism. Corresponding mutants lacking selected genes associated with metabolic functions were tested for their growth- and damage potential. These data showed that genes downregulated in the presence of *L. rhamnosus* (*PRN4*, *PTP3*, *RIM13*, *AHR1*, *ACE2*, and *orf19.4292*) are indispensable for virulence. Taken together the results of this thesis provide novel insights into the tripartite interactions between *C. albicans*, intestinal epithelial cells, and *Lactobacillus* spp. that could serve as a steppingstone for novel therapeutic strategies and underline the importance of intestinal homeostasis to prevent disease.

Zusammenfassung

Der Hefepilz *Candida albicans* kommt sowohl als natürlicher Kommensale auf menschlichen Schleimhäuten vor, als auch als Erreger der oberflächlichen oropharyngealen oder vulvovaginalen Candidose. Zudem kann *C. albicans* auch invasive und systemische Infektionen hervorrufen. Prädisponierende Faktoren, die zur Krankheitsentwicklung durch diesen opportunistisch pathogenen Pilz beitragen, sind zum Beispiel: Antibiotikatherapien, Immunsuppression und eine geschädigte Epithelbarriere. Der Darm stellt die Hauptquelle für invasive disseminierte *C. albicans* Infektionen dar. Unter den entsprechenden Bedingungen kann der Pilz dort die intestinale Barriere überwinden, in den Blutstrom gelangen und sich von dort im Körper ausbreiten.

Da die intestinale Kolonisierung als Voraussetzung für systemische *C. albicans* Infektionen gilt, ist die Erforschung des Pilzes in dieser biologischen Nische von besonderer Bedeutung, um die Pathogenitätsmechanismen zu verstehen und mögliche Ansätze zur Behandlung zu finden.

Die natürliche Mikrobiota des gesunden Darms verhindert im Normalfall das Überwachsen von *C. albicans*. Darauf aufbauend lag das Ziel dieser Doktorarbeit darin, die Interaktionen zwischen *C. albicans*, intestinalen Epithelzellen und Darmbakterien zu untersuchen, sowie den Wechsel von *C. albicans* vom Kommensalen zum Pathogen zu charakterisieren. Dafür wurde ein statisches *in vitro* Modell entwickelt, welches eine intestinale Barriere aus Epithelzellen sowie Mukus-produzierende Becherzellen beinhaltet. Dieses Modell wurde vor der *C. albicans* Infektion mit einer *Lactobacillus* Monokultur kolonisiert, um eine artifizielle Mikrobiota einzubringen. Durch die *Lactobacillus* Kolonisierung konnte ein spezies-, zeit-, und dosisabhängiger protektiver Effekt gegenüber der *C. albicans*-induzierten Schädigung gezeigt werden. Dabei war der protektive Effekt durch *L. rhamnosus* im Vergleich zu den anderen getesteten *Lactobacillus* Arten am größten. Der bakterielle Antagonismus ging mit einer

Reduzierung der Zytotoxizität, Hyphenlänge sowie Translokation des Pilzes einher. Diese Ergebnisse konnten in einem perfusionierten Darm-Chip Model reproduziert werden, welches über zwei, per Membran voneinander getrennte, Kammern verfügt, die sowohl das Darmlumen mit intestinalen Epithelzellen als auch den Blutstrom mit Endothelzellen imitiert. Zudem wurde ein weiterer möglicher antimykotischer Mechanismus von *L. rhamnosus* identifiziert, welcher auf der physikalischen Separierung von *C. albicans* und dem intestinalen Epithel beruht.

Detailliertere mechanistische Einblicke in die komplexen Interaktionen zwischen *C. albicans*, intestinalen Epithelzellen und *L. rhamnosus* wurden durch die Anwendung verschiedenster molekularbiologischer Methoden wie Transkriptions- und Metabolomanalysen erreicht, welche zusätzlich durch *in silico* Analysen unterstützt wurden. Voraussetzung für den protektiven Einfluss von *L. rhamnosus* war dessen Wachstum und metabolische Aktivität, welche nur in Gegenwart von intestinalen Epithelzellen gewährleistet war. Von den Epithelzellen sekretierte Metaboliten wie Zitronensäure, Gamma-Glutamyl-Aminosäuren und Carnitin unterstützten das Wachstum des Bakteriums. Während des Wachstums von *L. rhamnosus* auf intestinalen Epithelzellen, sekretierte das Bakterium Metaboliten mit bereits beschriebener antimykotischer Wirkung. Ferner wurden Metaboliten wie Cytosin nachgewiesen und dessen antimykotischer Effekt untersucht. Die Kolonisierung mit *L. rhamnosus* führte zur veränderten Verfügbarkeit von präferierten Kohlenstoffquellen im *in vitro* Modell, was zu erheblichen Veränderungen in der (Metabolismus-assoziierten) Genexpression des Pilzes führte. Mit Hilfe von Gendeletionsmutanten, welche bezüglich Wachstumsverhalten und Zytotoxizität gegenüber intestinalen Epithelzellen untersucht wurden, konnte ein Zusammenhang zwischen der transkriptionellen Anpassungen und dem protektiven Effekt durch *L. rhamnosus* gezeigt werden. Dabei wurde eine durch *L. rhamnosus*

induzierte Herunterregulierung von *C. albicans* Genen (*PRN4*, *PTP3*, *RIM13*, *AHR1*, *ACE2*, und *orf19.4292*) gezeigt. Zudem wurde belegt, dass diese Gene zur Virulenz von *C. albicans* beitragen. Zusammenfassend ermöglichen die Ergebnisse dieser Dissertation neue Einblicke in die komplexe, dreigliedrige Interaktion von *C. albicans*, intestinalen Epithelzellen und *Lactobacillus* Arten. Die etablierten Modelle und die damit gewonnenen Erkenntnisse unterstreichen die Bedeutung der intestinalen Homöostase zur Vermeidung von Krankheiten und könnten als Grundlage für neue therapeutische Ansätze dienen.

1. Introduction

1.1. *Candida albicans* - an opportunistic fungal pathogen causing severe infections

Candida albicans is a polymorphic fungus, belonging to the phylum of Ascomycota and the class of Saccharomycetes. This yeast is an opportunistic pathogen that can cause superficial infections of oral and vaginal mucosal tissues, which are associated with a high impact on quality of life. One of the most prominent examples is vulvovaginal candidiasis (VVC), which affects around 70 % of women during their reproductive age, independently of their health status (Gonçalves *et al.* 2016; Sobel 2007). Oropharyngeal candidiasis is a common problem in HIV/AIDS and diabetic patients, but can also occur under antibiotic-, immunosuppressive-, or chemotherapeutic treatment (Swidergall and Filler 2017). Invasive candidiasis occurs less frequently but is associated with a mortality rate of up to 71 % (Falagas *et al.* 2006a), thereby posing a major threat to patients with underlying disease, for example, in intensive care units (Kett *et al.* 2011; Méan *et al.* 2008). In general, *Candida* species and especially *C. albicans*, *C. glabrata*, *C. tropicales*, and *C. parapsilosis* are responsible for the majority of hospital-acquired fungal infections (Perlroth *et al.* 2007). During invasive candidiasis, fungal dissemination *via* the bloodstream (candidemia) occurs, from where it can spread potentially to every organ of the human body. Invasive candidiasis only occurs in patients with major predisposing factors such as severe illness, gastrointestinal perforation, or sepsis (Pappas *et al.* 2018). Infection of these susceptible patients occurs through the direct entry of *C. albicans* into deeper tissue and the bloodstream *via* central venous catheters, transplants, or major surgery. Nevertheless, the population of *C. albicans* in the intestinal mycobiome serves as a reservoir for disseminated candidiasis (Gouba and Drancourt 2015; Pappas *et al.* 2018) and invades the intestinal epithelium when predisposing conditions allow it (Albac *et al.* 2016; Dalle *et al.* 2010).

C. albicans possess a multiplicity of pathogenicity mechanisms. Due to its morphological flexibility, *C. albicans* can grow as yeast, hyphae, or pseudohyphae and thereby adapt to environmental changes. In the intestine, various environmental conditions like a temperature of 37°C, 5% CO_2, microaerophilic conditions, N-acetylglucosamine (Sudbery 2011), as well as the contact to host cells (Moyes *et al.* 2015) can trigger the yeast-to-hyphae transition. Hyphae are crucial for tissue invasion and damage, proved by an avirulent yeast locked mutant *in vivo* (Iranzo *et al.* 2003; Jacobsen and Hube 2017; Lo *et al.* 1997). Traditionally, hyphal morphology is linked to pathogenicity of *C. albicans*, whereas yeast cells are more connected to commensalism, yet disseminated candidiasis requires both, yeast and hyphae (Chin *et al.* 2014; Fradin *et al.* 2005).

When grown *in vitro* on intestinal epithelium, *C. albicans* unopposed invades and damages the host cells by inducing necrotic cell death. In this process, the first crucial step is the adhesion of the fungus to the intestinal epithelium, mediated by adhesins like Als3 and Hwp1 (Phan *et al.* 2007; Ponniah *et al.* 2007; Zhao *et al.* 2004). After the adhesion to the intestinal epithelium is established, the fungus can invade the tissue *via* active penetration (Dalle *et al.* 2010; Wächtler *et al.* 2012). This fungal-driven process is mediated by physical forces mediated by hyphal growth combined with the release of hydrolases like secreted aspartic proteases (Saps) (Naglik *et al.* 2003; Schaller *et al.* 2003)**.** Additionally, the toxin candidalysin is released by hyphae and causes necrotic lysis of intestinal epithelial cells and paves the way for transcellular translocation (Allert *et al.* 2018; Moyes *et al.* 2016). Therefore, candidalysin is the missing link between hypha formation and damage (Wilson *et al.* 2016)**.**

Interestingly, for other epithelial cell types, such as oral and vaginal cells, induced endocytosis is another translocation route for *C. albicans*. Induced endocytosis is a host-driven process,

which is induced by fungal invasins like Als3 or Ssa1 that bind host ligands and trigger the fungal uptake (Phan *et al.* 2007; Sun *et al.* 2010).

1.2. The commensal lifestyle of *Candida albicans*

Bearing in mind that *C. albicans* damages host cells *in vitro* extremly efficient, and that this yeast causes infections with high mortality and morbidity, it is surprising that this yeast colonizes humans as a harmless commensal. The majority (60 %) of the population is colonized by *C. albicans* (Pappas *et al.* 2018) and the intestine is the major source of invasive and disseminated candidiasis. In the gastrointestinal tract, *C. albicans* is the most prevalent fungus in the mycobiome (Hallen-Adams and Suhr 2017). *C. albicans* colonization seems to be dependent on the availability of specific nutrients. For example, a positive correlation has been observed between colonization and carbohydrate-rich diets (Hoffmann *et al.* 2013) as well as reduced colonization in diets rich in proteins, amino acids, and fatty acids (Gunsalus *et al.* 2016). In general, the intestinal mycobiome composition is underlying more timely and individual changes compared to the bacterial microbiome (Nash *et al.* 2017).

Even when yeast and hyphae have been detected during *C. albicans* colonization *in vivo* (Witchley *et al.* 2019), the yeast morphology is assumed to be dominant during commensalism in the intestine. This theory is supported by studies that demonstrate a colonization benefit of *C. albicans* when the yeast-to-hyphae transition is suppressed (Witchley *et al.* 2019) or the adaption of *C. albicans* during intestinal evolution with the loss of filamentation (Tso *et al.* 2018). Moreover, a morphological and functional adapted GUT (gastrointestinally induced transition) yeast phenotype promoted commensalism after passage through the mammalian gut (Pande *et al.* 2013). Next to its neutral colonization, *C. albicans* can even have beneficial effects in the intestine, due to the induction of a protective immune response that is effective against invasive fungal diseases such as candidiasis (Shao *et al.* 2019; Tso *et al.* 2018) and

aspergillosis (Tso *et al.* 2018) and even bacterial *S. aureus* (Shao *et al.* 2019; Tso *et al.* 2018), *P. aeruginosa* (Tso *et al.* 2018), and *C. difficile* infection (Markey *et al.* 2018) *in vivo*.

As colonization with *C. albicans* is regarded as a prerequisite for *C. albicans* infections originating from the intestine (Miranda *et al.* 2009), studies on the commensal state of *C. albicans* are an important research field. It is advantageous to dissect the host-fungal interactions in the commensal state of *C. albicans* to understand the mechanisms leading to the commensal-to-pathogen shift and potential options to prevent it. To achieve the homeostasis that keeps *C. albicans* in its commensal state, three players have to interact in a balanced way: the intestinal epithelium, the immune system, and the microbiota. Whereas an imbalance in one player can often be settled by the two other opponents, disturbance in all three players is connected to an increased risk for candidiasis **(Fig.1)**. This is specifically illustrated by *in vitro* models that lack any form of microbiota or immune components. Under such conditions *C. albicans* unresisted invades and damages intestinal epithelial cells unless it is genetically deficient in key virulence attributes (Allert *et al.* 2018). In mice, the combination of immunosuppression and mucosal disruption was shown to result in dissemination and mortality of *C. albicans* (Koh *et al.* 2008). Likewise, in patients, severe predisposition like the combination of immune suppression, broad-spectrum antibiotics, and mucosal damage are necessary to favor invasive Candidiasis (da Silva *et al.* 2019; Ostrosky-Zeichner *et al.* 2007).

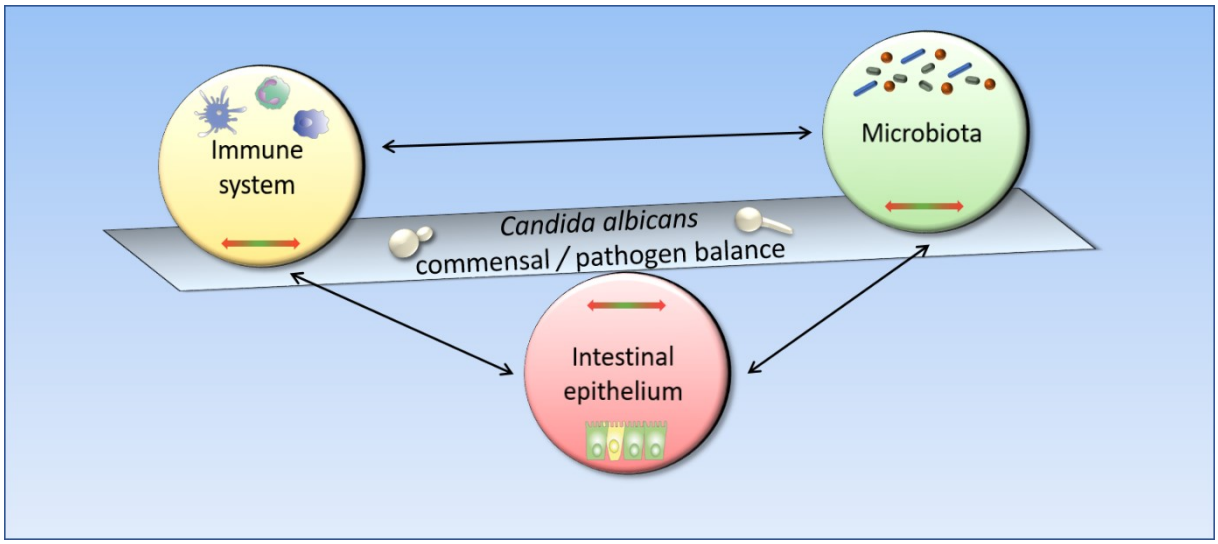

Fig.1: Schematic overview of the complex interactions between the immune system, the microbiota, and the intestinal epithelium to maintain *C. albicans*' commensalism. It is assumed that imbalance of one of the three key players (in size or direction) can be compensated by the others, but a strong imbalance in the system (e.g. through dysfunctions in all three players) leads probably to a commensal-to-pathogen shift of *C. albicans*.

1.2.1. The intestinal barrier

The intestinal epithelium creates a physical barrier between the external environment and the sterile tissues of the human body. It is responsible for nutrient- and water absorption as well as for the protection against harmful dietary substances and pathogens, by being the crosstalk center between the microbiota and the immune cells (Allaire *et al.* 2018; Peterson and Artis 2014; Soderholm and Pedicord 2019). The intestinal epithelium is composed of a single layer of different cell types like enterocytes, goblet cells, enteroendocrine cells, paneth cells, and microfold cells. The cells are kept together by tight and weak junctions and organized in villi and crypts (Okumura and Takeda 2017; Peterson and Artis 2014). Microvilli on top of the epithelial cells increase their surface for optimized nutrient uptake. The intestinal epithelium is comprised of the *lamina propia* underneath and a mucus layer above. The water-based mucus contains glycosylated mucin proteins, electrolytes, and small proteins, forming a gel-like structure that creates an important barrier against pathogens (Bansil and Turner 2018;

Martens *et al.* 2018). Mucins are also known for their protective role against virulence traits of *C. albicans* like adhesion, filamentation, and biofilm formation due to the downregulation of virulence-related genes like *ALS3*, *HWP1*, *EFG1,* and *ECE1* (Arevalo and Nobile 2020; Basmaciyan *et al.* 2019; Kavanaugh *et al.* 2014). *C. albicans* can overcome the mucus barrier by adherence to mucins (de Repentigny *et al.* 2000) or secretion of mucinolytic enzymes (Colina *et al.* 1996). Another way of intestinal self-protection is shielding the deeper tissue from pathogens by the constant renewal of the mucosal surface (Cone 2009). Nevertheless, general disturbance of the intestinal barrier by iatrogenic impairments like surgery or chemotherapy, or by trauma fosters *C. albicans* translocation and dissemination (Pasqualotto *et al.* 2006; Pfaller and Diekema 2007).

1.2.2. The immune system

The immune system cooperates closely with the intestinal epithelium and the microbiota to maintain *C. albicans* commensalism. Pathogen associated molecular pattern (PAMPs) of *C. albicans* that are mainly cell wall components like chitin, glucans, and mannans are recognized by pattern recognition receptors (PRRs) of the intestinal epithelium (Cheng *et al.* 2012; Naglik *et al.* 2017). Compared to the yeast morphology that is associated with commensalism, the immune system mounts stronger responses when PRRs are sensing hyphae that are associated with invasion (Schirbel *et al.* 2018). A certain level of the toxin candidalysin activates the innate immune response *via* MAP kinase signaling, which results in the release of antimicrobial peptides (Moyes *et al.* 2016). Additionally, it induces the secretion of cytokines and chemokines that mediate the recruitment of innate immune cells (Pott and Hornef 2012; Richardson *et al.* 2019). The recruited myeloid cells of the innate immune system engulf and kill fungal cells, and can activate adaptive immune responses (Richardson and Moyes 2015; Tong and Tang 2017).

Whereas an intact immune system can clear the fungal infection, a compromised immune system due to chemotherapy (Teoh and Pavelka 2016) or immunosuppressive therapy (Lionakis and Kontoyiannis 2003; Monneret *et al.* 2011) are known risk factors contributing to the development of *C. albicans* infections.

1.2.3. The intestinal microbiota

The third important player that supports the commensal state of *C. albicans* is the intestinal microbiota (Hillman *et al.* 2017). The microbial composition varies between individuals, is different in every part of the gastrointestinal tract, and strongly dependent on diet (Donovan 2017; Rinninella *et al.* 2019). In synergy with the host, the intestinal microbiota has versatile functions (Rinninella *et al.* 2019). Next to digestion and processing of food (Sonnenburg and Bäckhed 2016), the microbiota provides vitamins (LeBlanc *et al.* 2013), hormones (Neuman *et al.* 2015), and neurotransmitters (Strandwitz 2018) that affect appetite and health. The microbiota produces short chain fatty acids (SCFAs) which are a source of energy for the intestinal epithelial cells (Koh *et al.* 2016). Besides its metabolic role, the microbiota also protects against exogenous pathogens, for example, by preventing colonization of potentially harmful pathogens; termed as colonization resistance. This resistance is mediated through indirect mechanisms like the stimulation of antimicrobial peptide production, bile acid production, or maintenance of the intestinal barrier integrity and direct mechanisms like competition for adhesion sites and nutrients or the secretion of antimicrobial compounds (Kim *et al.* 2017; Pickard *et al.* 2017; Ubeda *et al.* 2017). In contrast to most humans, who are colonized with *C. albicans*, most strains of laboratory mice display colonization resistance, while gnotobiotic mice can be colonized with *C. albicans* (Böhm *et al.* 2017). In many mice strains with an intact microbiota, colonization resistance can only be overcome with antibiotic treatment that disturbs the microbiota composition (Koh 2013; Koh *et al.* 2008; Wiesner *et al.*

2001). While antibiotics are crucial life-saving drugs for the treatment of bacterial infections, these drugs disturb the microbial balance, leading to an altered intestinal microbiota (Pérez-Cobas *et al.* 2013), and even dysbiosis. Collectively, this microbiota disturbance can favor the overgrowth of opportunistic bacterial (Becattini *et al.* 2016; Iacob and Iacob 2019; Kim *et al.* 2017) as well as fungal pathogens such as *C. albicans* (Weiss and Hennet 2017). To regulate the homeostasis of the microbiota and to counteract opportunistic pathogens, microbiota-derived and external bacteria are used for prevention and therapy. These "*biological products that contain live organisms, such as bacteria; are applicable to the prevention, treatment, or cure of a disease or condition of human beings; and are not a vaccine"*, are defined as "live biotherapeutic microorganisms (LBM)" by the Food and Drug Administration (FDA 2016). Compared to LBMs the term "probiotic" is connected to the preventive use of living microorganisms in adequate amounts to confer health benefits (Hill *et al.* 2014). Known probiotics belong to the genera *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, as well as the species *Saccharomyces boulardii* and *Escherichia coli* Nissle 1917 (Azad *et al.* 2018; Sanders *et al.* 2019). This thesis will focus on the antagonistic potential of *Lactobacillus* spp.

1.2.3.1. Lactobacillus spp.

Lactobacilli are aerotolerant anaerobic or microaerophilic gram-positive bacteria, belonging to the phylum *Firmicutes*. Due to their ability to convert sugars into lactic and butyric acid, they are grouped to lactic acid bacteria, and can counteract (opportunistic) pathogens (Fayol-Messaoudi *et al.* 2005; Maudsdotter *et al.* 2011; Ogawa *et al.* 2001; Tachedjian *et al.* 2017). In the human vaginal tract, lactobacilli dominate the bacterial microbiota (Ravel *et al.* 2011; Witkin and Linhares 2017) and contribute to the acidification of the environment through lactic acid production. In the intestine, lactobacilli are less dominant and can be original (autochthones) members or introduced (allochthonous) by fermented food (Duar *et al.* 2017;

Tannock 2004). However, *Lactobacillus* spp. can colonize the intestinal tract, forming stable populations, and can be detected in feces (Alander *et al.* 1999; Tannock *et al.* 2000). The species *L. rhamnosus*, *L. casei*, *L. paracasei*, *L. brevis*, *L. fermentum*, and *L. salivarius* are common members of the human intestinal microbiota (Rossi *et al.* 2016). In this thesis, these species are used to mimic a part of the commensal microbiota. Among them, *L. rhamnosus* is one of the best-characterized probiotics with the capacity to antagonize bacterial (Bertuccini *et al.* 2017; Campana *et al.* 2017; De Keersmaecker *et al.* 2006) and fungal pathogens (Gerbaldo *et al.* 2012; Mailander-Sanchez *et al.* 2017; Matsubara *et al.* 2016; Ribeiro *et al.* 2017).

1.3. Studying host-fungal interactions

To understand the complex interactions between *C. albicans*, the microbiota, the immune system, and the intestinal epithelium, appropriate model systems are indispensable. Usually, *in vivo* systems like mouse models are the first choice for the study of such complex interactions. *C. albicans* is naturally not colonizing the intestine of most laboratory mice and *in vivo* models have limitations in terms of molecular readouts. At the same time, there is a social consensus to reduce animal experiments to the minimum possible. Therefore, this thesis is focused on *in vitro* models to study the impact of bacteria on fungal-host interactions. These *in vitro* models were used to make observations on the effect of antagonistic bacteria on *C. albicans* pathogenicity mechanisms and how this is mediated on a transcriptional and metabolic level. *In vitro* models offer advantages such as easy handling, high throughput screening, and ample flexibility to modify experimental conditions, like including different cell types, immune cells, or microorganisms. Due to varying levels of complexity between different *in vitro* models, it is possible to adapt experiments in accordance to the research question and to choose the model that fits best to answer this question.

1.4. Aims of the study

C. albicans exists as a harmless commensal on mucosal surfaces, but can also cause superficial and invasive infections. Life-threatening systemic dissemination of the fungus is possible after translocation through the intestinal barrier and into the bloodstream (Koh *et al.* 2008; Miranda *et al.* 2009). Certain predispositions like a compromised immune system, the loss of epithelial barrier functions, and an imbalance of the intestinal microbiota are known, to favor *C. albicans* dissemination (Basmaciyan *et al.* 2019). A compromised immune system and loss of barrier function are parameters that cannot be prevented during urgent and life-saving therapies. In addition, the microbial balance is often affected due to prophylactic broad-spectrum antibiotic treatment, required to prevent life-threatening bacterial infections. For that reason, the interactions between *C. albicans* and commensal bacteria in the background of the intestinal epithelium seem to be crucial events regarding the prevention or development of candidiasis.

This thesis aimed to dissect interactions of *C. albicans* with intestinal bacteria focusing on their potential to reinforce the commensal state of *C. albicans* and inhibit the switch to pathogenicity. The literature was explored and the described synergistic and antagonistic interactions between *C. albicans* and gram-positive bacteria summarized in **Manuscript I** (Förster *et al.* 2016) of this thesis. **Manuscript II** (Graf *et al.* 2019) describes our study that aimed to establish an *in vitro* model that can simulate the commensal state of *C. albicans.* By increasing the biological complexity of this model *via* the inclusion of mucus-producing goblet cells and an artificial microbiota, we were able to reduce *C. albicans*-induced damage to a minimum. Particularly, the colonization of intestinal epithelial cells with lactobacilli showed species-, dose-, and time-dependent protective effects against *C. albicans* inflicted damage. Among the tested *Lactobacillus* species, *L. rhamnosus* was most protective. In addition to the

reduction of *C. albicans* proliferation and hyphal growth, bacterial driven shedding was discovered as a potential protective mechanism. To gain deeper insights into the molecular interplay between *L. rhamnosus* and *C. albicans* that result in antagonism of pathogenicity, transcriptional and metabolic analysis, supported by *in silico* predictions were performed and described in **Manuscript III.** These in-depth molecular and metabolic studies revealed how intestinal epithelial cells support *L. rhamnosus* growth and consequently how *L. rhamnosus* modulates *C. albicans* pathogenicity by regulating the expression of virulence factors and the fungal metabolism. **Manuscript IV** (Maurer *et al.* 2019) describes the verification of the antagonistic potential of *L. rhamnosus* additionally in an intestine-on-chip model that has increased physiological relevance due to its near-physiological tissue architecture, perfusion, and presence of immune cells. When this model was colonized with *L. rhamnosus*, reduced growth and translocation of *C. albicans* was demonstrated. The experiences made with different *in vitro* models to study pathogenicity and potentially commensalism of *C. albicans* were the foundation for a literature summary of *in vitro* models to study opportunistic fungal infections described in **Manuscript V**. In this review, light is shed on the myriad of *in vitro* models available to mimic fungal infections and how these can be applied to answer specific research questions.

2. Manuscripts

2.1. Manuscript I: Förster *et al*., Cellular Microbiology, 2016

Enemies and brothers in arms: *Candida albicans* and gram-positive bacteria

Toni M. Förster, Selene Mogavero, **Antonia Dräger**, Katja Graf, Melanie Polke, Ilse D. Jacobsen, and Bernhard Hube

Cell Microbiol. 2016 Dec;18(12):1709-1715. doi: 10.1111/cmi.12657.

Summary:

As an opportunistic fungal pathogen and commensal, *C. albicans* can be found in different niches of the human body, like the oral cavity, the intestinal, and the vaginal tract. In all these niches *C. albicans* interacts with the human host and the bacterial microbiota. The balance or dysbalance of this microverse is crucial for the potential development of mucosal candidiasis and invasion of host tissues. This review provides an overview of the interactions between *C. albicans* and gram-positive bacteria. Specifically, it describes the interactions between *C. albicans* and Streptococci in the background of biofilm formation that synergistically damage the host on the one hand. On the other hand, interactions between *C. albicans* and lactobacilli that are antagonistic towards *C. albicans* are described with a detailed explanation of the underlying mechanisms.

Own contribution:

Antonia Last (née Dräger) performed literature research and wrote parts of the review.

Estimated authors' contributions:

Toni M. Förster	60%
Selene Mogavero	10%
Antonia Last (née Dräger)	5%
Katja Graf	5%
Melanie Polke	5%
Ilse D. Jacobsen	5%
Bernhard Hube	10%

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MICROREVIEW

Enemies and brothers in arms: *Candida albicans* and gram-positive bacteria

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Abstract

Candida albicans is an important human opportunistic fungal pathogen which is frequently found as part of the normal human microbiota. It is well accepted that the fungus interacts with other components of the resident microbiota and that this impacts the commensal or pathogenic outcome of *C. albicans* colonization. Different types of interactions, including synergism or antagonism, contribute to a complex balance between the multitude of different species. Mixed biofilms of *C. albicans* and streptococci are a well-studied example of a mutualistic interaction often potentiating the virulence of the individual members. In contrast, other bacteria like lactobacilli are known to antagonize *C. albicans*, and research has just started elucidating the mechanisms behind these interactions. This scenario is even more complicated by a third player, the host. This review focuses on interactions between *C. albicans* and gram-positive bacteria whose investigation will without doubt ultimately help understanding *C. albicans* infections.

1 | INTRODUCTION

Mucosal surfaces of the human body, like in the oral cavity, the gut, and the vaginal tract, are frequently colonized by *Candida albicans* (Williams et al., 2013). In all these niches *C. albicans* interacts with other resident colonizers, especially bacteria, where they coexist in harmony (Neville, d'Enfert, & Bougnoux, 2015). In contrast, predisposing factors like antibiotic or chemotherapeutic treatment, or immunosuppression, can induce a commensal-to-pathogen shift of the fungus, resulting in local or systemic infections (Kennedy & Volz, 1985; Ampel, 1996). Both the commensal and pathogenic facets of *C. albicans* are influenced by direct and indirect interactions with bacteria.

It is generally acknowledged that the gastro-intestinal tract (GIT) microbiota controls GIT colonization by *C. albicans* (Kennedy & Volz, 1985; Neville et al., 2015) and, therefore, may act antagonistically on the fungus. Similar scenarios are likely also true for the oral cavity and the vagina (Falagas, Betsi, & Athanasiou, 2006). However, some bacteria exert commensal relationships, and some act even synergistically during pathogenesis. In general, unraveling the mechanisms behind microbiota-*C. albicans* interactions could provide important information for prevention and treatment of *C. albicans* infections. In this review, we focus on the interactions between *C. albicans* and gram-positive bacteria, choosing two representative examples, streptococci for synergistic and lactobacilli for antagonistic interactions, and how these affect virulence.

2 | BROTHERS IN ARMS: SYNERGISTIC INTERACTIONS WITH STREPTOCOCCI IN MIXED BIOFILMS

The occurrence of polymicrobial biofilms containing *C. albicans* together with a variety of different bacteria is a commonly observed phenomenon in the clinical setting (Thein, Seneviratne, Samaranayake, & Samaranayake, 2009). These mixed biofilms are often associated with diseases such as denture stomatitis (Baena-Monroy et al., 2005), cystic fibrosis (Valenza et al., 2008), or burn-wound infections (Gupta, Haque, Mukhopadhyay, Narayan, & Prasad, 2005). Moreover, mixed biofilms harboring *C. albicans* on catheters are one of the main causes of fungal dissemination into the bloodstream and fungal sepsis (Ramage, Martinez, & Lopez-Ribot, 2006). In general, >20% of all candidemia cases involve a bacterial coinfection and are more lethal than *Candida* mono-infections (Klotz, Chasin, Powell, Gaur, & Lipke, 2007). Several bacterial species have been identified in mixed biofilms with *C. albicans*, most notable

are streptococci, including *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus sanguinis*, or *Streptococcus gordonii*, which are part of the normal oral microbiota.

2.1 | Get together and attach to the host: a little help from friends

Biofilms dominated by, or exclusively consisting of, *C. albicans* have a complex architecture with yeast, pseudohyphal, and hyphal morphologies embedded in an extracellular matrix. The ability to form true hyphae is characteristic for *C. albicans*, and several bacteria adhere to this cell type. For example, *S. mutans* displayed a strong affinity to *C. albicans* hyphae, as shown by electron microscopy on both human teeth and hydroxyapatite substrate samples (Metwalli, Khan, Krom, & Jabra-Rizk, 2013). Several factors are known to facilitate binding between *C. albicans* and streptococci, such as the adhesin Als3 and the aspartic protease Sap9 on the fungal side or the adhesin SspB and glucosyltransferases on the bacterial side (Phan et al., 2007; Silverman, Nobbs, Vickerman, Barbour, & Jenkinson, 2010; Gregoire et al., 2011; Ricker, Vickerman, & Dongari-bagtzoglou, 2014; Dutton, Jenkinson, Lamont, & Nobbs, 2016). Binding is additionally influenced by O-mannosylation affecting accessibility of Als3 on the fungal surface (Dutton et al., 2014).

Interactions between *C. albicans* and bacteria also influence the formation of biofilm extracellular matrix. *S. mutans* and *C. albicans* have an enhanced ability to form biofilms when grown together *in vitro* and *in vivo*, characterized by higher production of exopolysaccharide-matrix (Falsetta et al., 2014). This matrix, produced by bacterial glucosyltransferases and consisting of fungal mannans and β-glucans, is a key mediator of mixed biofilm development.

Overall, strong cohesion of bacterial and fungal cells stabilizes the overall structure of mixed biofilms and allows for a more stable colonization by the individual members. Also, the adhesion capacity to host epithelia differs between *C. albicans* and bacteria: *S. mutans* has a high tropism for teeth and less for the oral epithelium, unlike *C. albicans*, which adheres robustly to mucosal surfaces (Williams et al., 2013). The capacity of streptococci to attach to *C. albicans* promotes their biofilm formation on mucosal epithelia (Diaz et al., 2012). Similarly, colonization and persistence of *C. albicans* in the oral cavity can be promoted by binding to bacteria (Cannon & Chaffin, 2001).

2.2 | Communication in mixed biofilms: scratching each other's back

Both synergistic and antagonistic interactions between fungal and bacterial species involve chemical and metabolic communications. For instance, *C. albicans* can induce the quorum-sensing system of *S. mutans*, which improves its survival and genetic adaptation (Sztajer et al., 2014). Similarly, the communication signal AI-2 (auto-inducer 2) and H_2O_2, produced by *S. gordonii*, increase biofilm formation and filamentation of *C. albicans* (Bamford et al., 2009). In contrast, the competence stimulating peptide (CSP) of *S. gordonii* seems to affect *C. albicans* by inhibiting fungal biofilm formation, but not hyphal growth (Jack et al., 2015), while the CSP of *S. mutans* and the fatty acid signaling molecule trans-2-decenoic acid inhibit hyphal formation (Jarosz, Deng, van der Mei, Crielaard, & Krom, 2009; Vílchez et al., 2010). Therefore, bacterial molecules can increase or decrease fungal growth, filamentation, and biofilm formation. Additionally, the antifilamentous effect of farnesol—*C. albicans*' own quorum sensing molecule—is inhibited in mixed biofilms with *S. gordonii* (Bamford et al., 2009). Likely, a combination of different pro- and antifilamentation signals contributes to a balance of yeast and hyphae within biofilms and prevents overgrowth of *C. albicans* in the community. Transcriptomes of mixed biofilms containing *C. albicans* and bacteria show an upregulation of fungal genes associated with filamentation, proteinase genes, and genes encoding cell wall proteins (Dutton et al., 2015). For *S. gordonii*, it was found that a number of genes associated with fructose-metabolism were differentially regulated in mixed biofilms with *C. albicans* and deletion of these genes led to a less robust mixed biofilm (Jesionowski, Mansfield, Brittan, Jenkinson, & Vickerman, 2015). Therefore, it is conceivable that *S. gordonii* metabolizes fructose-compounds possibly derived from *C. albicans* indicating the occurrence of metabolic interactions. In the same way, *C. albicans* can use lactate produced by streptococci as a carbon source in mixed biofilms (Metwalli et al., 2013).

2.3 | Advantages: enhanced drug resistance and virulence

One of the general characteristics of biofilms is an enhanced drug resistance of the embedded microbial community, a feature which can even be increased in mixed-species biofilms. It has been shown that a *C. albicans* containing mixed biofilm limits the diffusion of antibiotic compounds by matrix components (e.g., β-1,3-glucans), thereby enhancing drug resistance (Harriott & Noverr, 2009; De Brucker et al., 2015). Of note, this relationship does not go one way. Bacteria are also able to actively protect *C. albicans* from antifungals, such as in mixed biofilms with *S. epidermidis*, where the slime produced by the bacterium seems to be protective against the action of fluconazole on *C. albicans* (Adam, Baillie, & Douglas, 2002).

In general, *C. albicans* exerts a higher pathogenic potential when growing in mixed biofilms with oral bacteria. This is reflected by higher invasion rates, increased damage of host cells and higher expression of virulence genes (Diaz et al., 2012; Cavalcanti et al., 2015). A similar synergy has been observed *in vivo*, in a murine oral coinfection model of streptococci and *C. albicans*, where coinfections augmented oral thrush lesions and deep organ dissemination of the fungus (Xu et al., 2014). This virulence synergy is of clinical relevance as the cariogenic development is accelerated by the interaction of *C. albicans* and *S. mutans* (Falsetta et al., 2014).

In conclusion, the interaction between *C. albicans* and bacteria can lead to great benefits for both players, for example, in terms of better adhesion to diverse environments or exchange of protective substrates. This mutuality leads to synergy, that is, the individual potentiation of critical features such as drug resistance or virulence.

3 | NEIGHBORHOOD WATCH: ANTAGONISTIC INTERACTIONS BETWEEN *C. albicans* AND LACTOBACILLI

Lactobacilli are a diverse group of gram-positive bacteria that exist both in the environment and as part of the microbiota of animals (Felis & Dellaglio, 2007). In humans, lactobacilli comprise the main part of the vaginal microbiota; however, within the GIT, it seems that most lactobacilli are not autochthonous but only transient inhabitants (Walter, 2008). All lactobacilli produce lactic acid from sugar metabolism and those colonizing the GIT use a variety of complex sugars such as oligosaccharides (O'Donnell, O'Toole, & Ross, 2013) to produce short chain fatty acids (SCFAs), important for the function of enterocytes and the intestinal immune system (Macfarlane & Macfarlane, 2011).

Interactions between lactobacilli and *C. albicans* occur naturally, such as in the human vagina, where lactobacilli are dominant and *C. albicans* is frequently isolated (van de Wijgert et al., 2014). Further interactions can occur in the GIT, especially after ingestion of probiotics containing lactobacilli. Several *in vitro* and *in vivo* studies and some clinical trials investigated the effect of different lactobacilli on *C. albicans* (Wagner et al., 1997; Strus et al., 2005; Manzoni et al., 2006; Gil, Martinez, Gomes, Nomizo, & De Martinis, 2010; Köhler, Assefa, & Reid, 2012; Nyanzi, Awouafack, Steenkamp, Jooste, & Eloff, 2014; Sharma & Srivastava, 2014; Parolin et al., 2015). Different studies investigated different species or strains, making a direct comparison between studies difficult. Nevertheless, all of them reported an inhibitory effect of lactobacilli on *C. albicans*: lactobacilli have been shown to reduce adhesion of *C. albicans* to host cells, inhibit hyphae formation, and exhibit a fungicidal/fungistatic effect. Supernatants of lactobacilli cultures are sufficient to mediate some of these effects, indicating that the inhibiting property might belong to the secretome (Nyanzi et al., 2014; Sharma & Srivastava, 2014).

3.1 | Molecular armory—mechanisms of *C. albicans* virulence inhibition

The molecular mechanisms responsible for lactobacilli mediated inhibition of *C. albicans* are not completely understood. However, lactobacilli can compete for nutrients (Basson, 2000) or for the same adhesion sites (Rizzo, Losacco, & Carratelli, 2013; Donnarumma et al., 2014; Parolin et al., 2015), thereby inhibiting fungal invasion (Mayer, Wilson, & Hube, 2013).

In addition, lactobacilli produce metabolic by-products of fermentation like SCFAs, including lactic acid, acetic acid, propionic acid, or butyric acid (Hove, Nordgaard-Andersen, & Mortensen, 1994). These can reach concentrations of 20–140 mM in the intestine or the vagina (Topping & Clifton, 2001; O'Hanlon, Moench, & Cone, 2011). In general, a low pH generated by the SCFAs is restricting filamentation of *C. albicans*, therefore preventing invasion (Buffo, Herman, & Soll, 1984). Lactate is a known carbon source for *C. albicans*; however, it only allows slow growth and cell walls of lactate-grown cells have a reduced immunogenic potential (Ene, Cheng, Netea, & Brown, 2013). Additionally, at acidic pH (<4,5), lactate is protonized into lactic acid, which can cross the cell membrane of *C. albicans* and acidifies the cytoplasm thereby impairing cellular functions (Köhler et al., 2012). A similar mode of action is known for acetic acid, which causes apoptosis or necrosis (Lastauskiene, Zinkeviciene, Girkontaite, Kaunietis, & Kvedariene, 2014). Furthermore, butyric acid was shown to be a potent inhibitor of *C. albicans* filamentation at physiological concentrations and reduced fungal colonization of mice *in vivo* (Noverr & Huffnagle, 2004; Fan et al., 2015). Interestingly, butyric acid is also a key metabolite for colonic cells, serving as fuel, regulating differentiation, and improving the gut barrier and immunity (Macfarlane & Macfarlane, 2011). *C. albicans* reacts to SCFAs by a specific transcriptional stress response with Mig1 being a key transcription factor (Cottier, Tan, Xu, Wang, & Pavelka, 2015).

Lactobacilli are also producers of antimicrobial substances such as H_2O_2, biosurfactants, and bacteriocins. H_2O_2 generation by lactobacilli has been shown in several studies, but probably only plays a minor role in the inhibition of *C. albicans* (Strus et al., 2005; Kaewsrichan, Peeyananjarassri, & Kongprasertkit, 2006; Parolin et al., 2015). Biosurfactants are amphiphilic molecules with an emulsifying activity and are also used for industrial applications (Santos, Rufino, Luna, Santos, & Sarubbo, 2016). The biosurfactants of lactobacilli exhibit an anticandidal activity, which is based on their reduction of surface tension that decreases adhesion and acts cytotoxic (Gomaa, 2013). Bacteriocins are classified as small bactericidal peptides, and they are often used for food preservation (Cotter, Ross, & Hill, 2013). Their production by lactobacilli was shown in several studies, including gassericin A or salivaricins (Messaoudi et al., 2013; Pandey, Malik, Kaushik, & Singroha, 2013), and for some it is already known that they are active against *Candida* spp. (Kaewsrichan et al., 2006).

Finally, lactobacilli can prevent invasion of *C. albicans* via indirect mechanisms involving the host tissue or immune system. Lactobacilli strengthen the epithelial barrier by stimulation of mucin production (Da Silva et al., 2014; Wang et al., 2014) and tight junction preservation (Yu, Yuan, Deng, & Yang, 2015; Zhao et al., 2015). Furthermore, lactobacilli and SCFAs can stimulate epithelial production of antimicrobial peptides (AMPs) like human β-defensins (Rizzo et al., 2013; Donnarumma et al., 2014) or cathelicidins like LL-37 (Schauber et al., 2003), which are toxic to *C. albicans* (Feng et al., 2005). Even the mucosal immune system is modulated by lactobacilli, for example, by increasing the expression of toll-like receptors that recognize *C. albicans* components (Saegusa, Totsuka, Kaminogawa, & Hosoi, 2004; Rizzo et al., 2013) and dampening *C. albicans*-induced inflammation (Plantinga et al., 2012; Fong, Kirjavainen, Wong, & El-Nezami, 2015). Interestingly, an additional link between the host tryptophan metabolism, lactobacilli, and the IL-22-mediated immune response to *C. albicans* in mice has been described (Zelante et al., 2013). Figure 1 summarizes the mechanisms of *C. albicans* inhibition by lactobacilli.

3.2 | Striking back: influence of *C. albicans* on lactobacilli

Whereas the positive influence of lactobacilli on the immune defense against *C. albicans* and their antagonistic effects on *C. albicans* colonization have clearly been demonstrated *in vivo* in mice (Wagner et al., 1997; Villena, Salva, Aguero, & Alvarez, 2011), much less is known about the influence of *C. albicans* on lactobacilli. Mason et al.

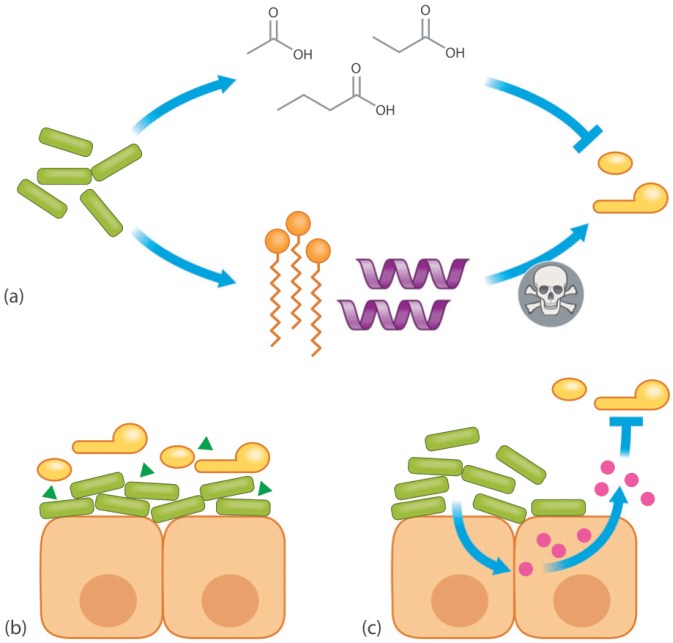

FIGURE 1 Mechanisms of *C. albicans* inhibition by *Lactobacillus* spp. (a) Production of inhibitory compounds by lactobacilli. This comprises short chain fatty acids (SCFAs) that acidify the medium and directly inhibit hyphae formation or biosurfactants and bacteriocins that are toxic to *C. albicans*. (b) Competition for adhesion sites and nutrients. (c) Indirect effects of lactobacilli on *C. albicans* through host cells, for example, by stimulation of antimicrobial peptide production.

demonstrated that *C. albicans* prevents the regrowth of lactobacilli after antibiotic treatment in mice (Mason et al., 2012). While the mechanisms behind this antagonism remain unclear, Iwakura and coworkers recently discovered another related scenario. The *C. albicans* cell wall component β-glucan can activate the host receptor Dectin-1 leading to the production of AMPs, which in turn suppress *L. murinus* in the murine gut (Tang et al., 2015). The authors also speculate that this scenario may be transferred to the human situation involving *L. salivarius*. Furthermore, within the vagina, *C. albicans* can degrade host-derived hemoglobin to hemocidins, which are toxic to bacteria such as lactobacilli (Bochenska et al., 2013).

In summary, lactobacilli are able to interfere with *C. albicans*, inhibiting its colonization and reducing virulence and vice versa. This is not only a prime example for an antagonistic interaction of competing microbes, but also a good example of coevolution of competing microbes in association with their host as they exploit host mechanisms to influence each other and end up in a balance that is tolerated, and potentially beneficial for the host.

4 | NOT SYNERGISM, NOR ANTAGONISM, BUT COMMENSALISM

We have discussed interactions between *C. albicans* and gram-positive bacteria with a focus on antagonistic versus synergistic interactions, but it should be noted that there are further distinct interactions of a different nature. For example, anaerobic, commensal bacteria such as *Escherichia coli*, *Enterococcus faecalis*, or *Klebsiella pneumoniae* cause an upregulation of the *C. albicans* master regulator gene of phenotypic switching, *WOR-1*, in mixed biofilms (Fox et al., 2014). This induces a switch of *C. albicans* from the white to the opaque state indicating an influence of bacteria on another distinct morphological program, which is associated with mating, biofilm formation, and the induction of a distinct phenotype called "Gastrointestinal IndUced Transition"—GUT (Pande, Chen, & Noble, 2013). The GUT phenotype is well adapted to gut conditions and favors commensal colonization in mice. Therefore, bacteria can contribute to a stage of fungal commensalism on the molecular level by modifying the expression of key regulatory genes.

Similarly, Cruz et al. identified that *E. faecalis* and *C. albicans* inhibit each other's virulence in a nematode model (Cruz, Graham, Gagliano, Lorenz, & Garsin, 2013). Whereas in mono-infections these species caused severe damage, both species coexist as commensals during co-infections in nematodes. This interesting commensal coexistence between enterococci and *C. albicans* is also supported by other studies. Mason et al. investigated the post-antibiotic microbiota of mice and detected higher levels of enterococci, especially *E. faecalis*, when *C. albicans* was introduced (Mason et al., 2012). Clinical data provide additional evidence for such an interaction, as reported in critically ill patients that harbor ultra-low diversity pathogen communities (Zaborin et al., 2014). Thus, interactions that appear to be primarily antagonistic may be mutualistic in the setting of the host, if they promote survival of both species as commensals, thereby preventing host damage and subsequent inflammation which otherwise might ultimately lead to clearance of the microorganisms by the immune system.

The examples described in this review demonstrate that pathogens, the host, and the microbial community influence each other in a complex network. Thus, it appears possible to utilize these interactions for prophylactic and therapeutic interactions aiming at

maintaining or restoring a balanced microbial ecosystem within the host. As it becomes increasingly clear that the human microbiota influence multiple physiological processes and diseases (Backhed et al., 2012; Robles Alonso & Guarner, 2013), further research on the interactions between different microorganisms is needed to understand what factors influence the microbiota. While much of the previous research has focused on bacteria, recent evidence shows that cross-kingdom interactions between fungi and bacteria needs to be taken into account when aiming at a better understanding of the microbial ecosystem within the human body (Xu & Dongari-Bagtzoglou, 2015).

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CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

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2.2. Manuscript II: Graf and Last *et al.*, Disease Models & Mechanisms, 2019

Keeping *Candida* commensal: how lactobacilli antagonize pathogenicity of *Candida albicans* in an *in vitro* gut model

Katja Graf*, **Antonia Last***, Rena Gratz, Stefanie Allert, Susanne Linde, Martin Westermann, Marko Gröger, Alexander S. Mosig, Mark S. Gresnigt, and Bernhard Hube

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Summary:

Candida albicans can colonize the human intestine as a harmless commensal, but in immunocompromised patients, the gut serves as the main entry route for systemic and disseminated *Candida* infections. Whereas *C. albicans* is usually studied in its pathogenic state, this publication aimed to focus on the commensal state of *C. albicans*. To prevent the commensal-to-pathogen shift of the fungus, an *in vitro* gut model consisting of different intestinal cell lines and colonized with antagonistic lactobacilli was established. Using this *in vitro* model, a time-, dose- and species-dependent effect of lactobacilli was shown to antagonize the pathogenic state of *C. albicans*. Among different *Lactobacillus* species, *L. rhamnosus* was identified as the most efficient in antagonizing *C. albicans* pathogenicity mechanisms. Besides the reduction of hyphal elongation and fungal growth, bacterial-driven shedding from the host epithelium was discovered as a potential protective mechanism.

Own contribution:

Antonia Last performed most of the experiments, including growth, adhesion, damage, translocation, and apoptosis studies, and contributed to the establishment of the *in vitro* model. She was involved in consultations and coordination with cooperation partners. She wrote parts of the manuscript, was involved in structuring the paper, created several figures, and was responsible for the revision.

Estimated authors' contributions:

Katja Graf	35 %
Antonia Last	35 %
Rena Gratz	6 %
Stefanie Allert	4 %
Susanne Linde	1 %
Martin Westermann	1 %
Marko Gröger	1 %
Alexander S. Mosig	1 %
Mark S. Gresnigt	6 %
Bernhard Hube	10 %

Supplement:

Antonia Last and Katja Graf have been invited to a first person interview by the journal *Disease Models & Mechanisms.* (see appendix 5.1.)

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RESEARCH ARTICLE

Keeping *Candida* commensal: how lactobacilli antagonize pathogenicity of *Candida albicans* in an *in vitro* gut model

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ABSTRACT

The intestine is the primary reservoir of *Candida albicans* that can cause systemic infections in immunocompromised patients. In this reservoir, the fungus exists as a harmless commensal. However, antibiotic treatment can disturb the bacterial microbiota, facilitating fungal overgrowth and favoring pathogenicity. The current *in vitro* gut models that are used to study the pathogenesis of *C. albicans* investigate the state in which *C. albicans* behaves as a pathogen rather than as a commensal. We present a novel *in vitro* gut model in which the fungal pathogenicity is reduced to a minimum by increasing the biological complexity. In this model, enterocytes represent the epithelial barrier and goblet cells limit *C. albicans* adhesion and invasion. Significant protection against *C. albicans*-induced necrotic damage was achieved by the introduction of a microbiota of antagonistic lactobacilli. We demonstrated a time-, dose- and species-dependent protective effect against *C. albicans*-induced cytotoxicity. This required bacterial growth, which relied on the presence of host cells, but was not dependent on the competition for adhesion sites. *Lactobacillus rhamnosus* reduced hyphal elongation, a key virulence attribute. Furthermore, bacterial-driven shedding of hyphae from the epithelial surface, associated with apoptotic epithelial cells, was identified as a main and novel mechanism of damage protection. However, host cell apoptosis was not the driving mechanism behind shedding. Collectively, we established an *in vitro* gut model that can be used to experimentally dissect commensal-like interactions of *C. albicans* with a bacterial microbiota and the host epithelial barrier. We also discovered fungal shedding as a novel mechanism by which bacteria contribute to the protection of epithelial surfaces.

This article has an associated First Person interview with the joint first authors of the paper.

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INTRODUCTION

The gut epithelium is a barrier between the sterile host environment and gut microbiota. Intestinal epithelial cells (IECs) represent the first line of defense against microbial invasion by being a passive physical barrier that prevents translocation (Peterson and Artis, 2014). In addition, goblet cells within the intestinal epithelium produce a protective mucus layer (Maynard et al., 2012; Yan et al., 2013). This layer serves as an anchor for the attachment of microbes and represents a nutrient source for mutualistic bacteria living within the gut (Cockburn and Koropatkin, 2016) that produce metabolites, nourishing IECs (Maynard et al., 2012). The gut microbiota is crucial for the development and maintenance of mucosal host defense by improving the physical barrier, competition for nutrients and adhesion sites with potential pathogens, and by developing the immune system (Sekirov et al., 2010; Bischoff et al., 2014). However, when the balance of the microbiota is impaired, opportunistic pathogens may outgrow the beneficial microbiota. The host's immune system and a functional intestinal barrier are generally sufficient to prevent infection. However, cytostatic therapy for the treatment of cancer targets fast-dividing cells. As a result, not only malignant cells are targeted, but also cells of the immune system and intestinal epithelial lining. In this immunocompromised state, patients are predisposed to develop opportunistic infections with otherwise harmless commensals of the microbiota. For example, the yeast *Candida albicans* exists as a commensal in the gastrointestinal tract of approximately 50% of the western population (Bougnoux et al., 2006), but can cause severe systemic infections under certain predisposing factors. Use of broad-spectrum antibiotics and a compromised immune status are such factors that can lead to *C. albicans* overgrowth and a switch from commensalism to pathogenicity (Bassetti et al., 2011; Mason et al., 2012), potentially resulting in translocation through the intestinal barrier and disseminated infections (Koh et al., 2008). Indeed, the main reservoir of *C. albicans* that causes systemic candidiasis is the gut (Gouba and Drancourt, 2015; Miranda et al., 2009; Nucci and Anaissie, 2001).

The association between candidiasis and the use of broad-spectrum antibiotics is believed to relate to the eradication of bacteria that antagonize *C. albicans* pathogenicity. Although numerous bacterial species interact with *C. albicans* (Bamford et al., 2009; Cruz et al., 2013; Fan et al., 2015; Förster et al., 2016), *Lactobacillus* species are the most widely known for their antagonistic potential. Most studies that aimed at investigating the mechanisms by which lactobacilli can counteract *C. albicans* were performed in host-free environments (Köhler et al., 2012; Strus et al., 2005) or on human (vaginal, oral or cervical) epithelial cells (do Carmo et al., 2016; Donnarumma et al., 2014; Mailänder-Sánchez et al., 2017; Rizzo et al., 2013). Lactobacilli have been

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shown to counteract *C. albicans* through inhibition of fungal growth (Coman et al., 2015; de Barros et al., 2018; Hasslöf et al., 2010; Köhler et al., 2012; Ribeiro et al., 2017; Strus et al., 2005), inhibition of hyphal morphogenesis (Allonsius et al., 2019), prevention of adhesion (Donnarumma et al., 2014; Mailänder-Sánchez et al., 2017; Rizzo et al., 2013), competition for nutrients (Mailänder-Sánchez et al., 2017) or by influencing immune responses (Marranzino et al., 2012; Plantinga et al., 2012; Rizzo et al., 2013). Among other lactobacilli, *L. rhamnosus* can reduce *C. albicans* hyphal induction and biofilm formation via cell-cell interactions and the secretion of exometabolites (James et al., 2016; Matsubara et al., 2016a). Exopolysaccharides of *L. rhamnosus* GG interfere with hyphal formation and adhesion to vaginal and bronchial epithelial cells (Allonsius et al., 2017). *L. rhamnosus* GG also protects oral epithelial cells against *C. albicans*-induced damage by competing for adhesion sites as well as depleting nutrients (Mailänder-Sánchez et al., 2017). Furthermore, lactobacilli can produce compounds such as hydrogen peroxide, lactic acid, bacteriocins and biosurfactants, which inhibit the growth of potential pathogens (reviewed by Förster et al., 2016 and Matsubara et al., 2016b). The importance of the microbiota in preventing damage to epithelial cells by *C. albicans* is demonstrated by the fact that any epithelial cell layer exposed to *C. albicans in vitro* is rapidly and efficiently invaded and damaged via necrotic cell death and unable to prevent translocation in the absence of a microbiota (Allert et al., 2018).

Here, we studied whether we could achieve a 'commensal' model in which the gut epithelial barrier is subjected to minimal *C. albicans*-induced damage and translocation by increasing biological complexity. Using a model consisting of intestinal epithelial cells, mucus-producing goblet cells and lactobacilli, we investigated how lactobacilli antagonize *C. albicans*-induced necrotic cytotoxicity.

RESULTS

C. albicans-induced epithelial damage is reduced by colonization with *Lactobacillus* species

Our study aimed to establish an *in vitro* model, which mimics the commensal phase of *C. albicans* in the gut, in order to dissect commensal-like scenarios. First, we reduced *C. albicans*-induced damage to a minimum by manipulating the composition of the epithelial barrier. As mucus can dampen virulence attributes of *C. albicans* (Kavanaugh et al., 2014), the mucus-producing goblet cell line HT29-MTX, which has been extensively validated for compatibility and functional properties with C2BBe1 enterocytes (Ferraretto et al., 2018), was introduced in an existing model for *Candida*-gut translocation (Allert et al., 2018). In comparison to C2BBe1 enterocytes alone, the presence of HT29-MTX cells, at a ratio of 70:30 respectively, reduced adhesion and translocation of *C. albicans* by approximately 30% (Fig. 1A,B). However, the potential for *C. albicans* necrotic cell damage, as determined by the concentration of epithelial cytosolic lactate dehydrogenase (LDH) in the culture supernatants (Chan et al., 2013), as well as *C. albicans*' hyphal length, remained comparable to the C2BBe1 monoculture model (Fig. 1C,D).

To introduce another level of complexity, an artificial 'microbiota' was included. Various *Lactobacillus* species with known *Candida*-antagonizing potential were investigated for their impact on *C. albicans* pathogenicity. The gut model was either colonized (pre-incubated; Pre-Inc.) with lactobacilli overnight followed by fungal infection or simultaneously colonized during *C. albicans* infection (w/o Pre-Inc.) (Fig. 1E). Colonization with *Lactobacillus* species alone did not induce necrotic epithelial cell damage (Fig. 1F). However, colonization with *L. paracasei*, *L. rhamnosus*, *L. salivarius* or *L. casei* before *C. albicans* infection reduced *C. albicans*-induced necrotic cytotoxicity (Fig. 1F). Colonization with *L. fermentum* and *L. brevis* or simultaneous colonization of lactobacilli with *C. albicans* infection did not influence *Candida*-induced damage. As lactobacilli were successful in protecting the epithelial barrier, we systematically investigated the factors that might contribute to this effect.

Lactobacillus-mediated damage protection is dose-, time- and species dependent

When colonized during *C. albicans* infection, *L. rhamnosus* dose-dependently reduced damage (Fig. 2A). A 250-fold excess of bacteria to *Candida* cells decreased LDH release almost to the degree of uninfected epithelial cells, yet colonization of IECs before *C. albicans* infection required significantly lower numbers of bacteria (5- to 50-fold excess of bacterial cells) to achieve damage protection. This may be because of the capacity of *L. rhamnosus* to multiply during colonization (Fig. 2B), meaning that a 50-fold excess of bacterial cells is sufficient to reduce *C. albicans*-induced damage when time for replication is given and the bacterium is able to grow on host cells. Conversely, the inability of *L. fermentum* and *L. brevis* to protect against *Candida*-induced damage (Fig. 1F) correlated with their inability to replicate in the model (Fig. 2B). Therefore, *Lactobacillus*-mediated damage protection relies on the ability of the bacteria to proliferate and an interaction of a high quantity of bacteria with *C. albicans*. In subsequent experiments, colonization of 50 bacterial cells per yeast cell was used to study the protective effects mediated by lactobacilli.

Lactobacilli do not interfere with fungal adhesion, but suppress filamentation and translocation

Prerequisite processes for *C. albicans* pathogenicity are adhesion to host cells and a subsequent morphological transition from yeast to hyphae, enabling the fungus to invade host cells, cause damage and translocate across barriers (Allert et al., 2018; Dalle et al., 2010). Therefore, the impairment of *C. albicans* adhesion and/or filamentation could be a potential mechanism for damage protection by lactobacilli (Allonsius et al., 2017; Coman et al., 2015; Donnarumma et al., 2014; James et al., 2016; Kang et al., 2018; Niu et al., 2017; Parolin et al., 2015; Santos et al., 2016; Tan et al., 2017; Wang et al., 2017). Although colonization of the model by *Lactobacillus* species did not interfere with *C. albicans*' adhesion to IECs and hyphal formation per se (Fig. 2C), *L. rhamnosus* significantly reduced hyphal length (42% reduction in length) (Fig. 2D). *L. brevis*, as a less-protective *Lactobacillus* species, did not reduce hyphal length. Interestingly, lactobacilli only grew in the host cell culture medium when host cells were present. Therefore, they did not reach the necessary number of bacterial cells to gain influence on fungal filamentation in the absence of host cells. As a result, *Lactobacillus*-mediated protection relied on host cells to sustain bacterial growth. The reduced *C. albicans* filamentation in the presence of *L. rhamnosus* was accompanied by reduced translocation (Fig. 2E). Consequently, *L. brevis*, which did not reduce filamentation, also failed to reduce *C. albicans* translocation.

Despite reduced hyphal length and translocation, a loss of epithelial barrier integrity [quantified via measurement of transepithelial electrical resistance (TEER)] was observed in both scenarios: *C. albicans* single infection and when the model was colonized with *L. rhamnosus* (Fig. 2F). Even *L. rhamnosus* alone decreased epithelial integrity after 24 h of colonization. *C. albicans*-induced loss of epithelial integrity is accompanied by decreasing

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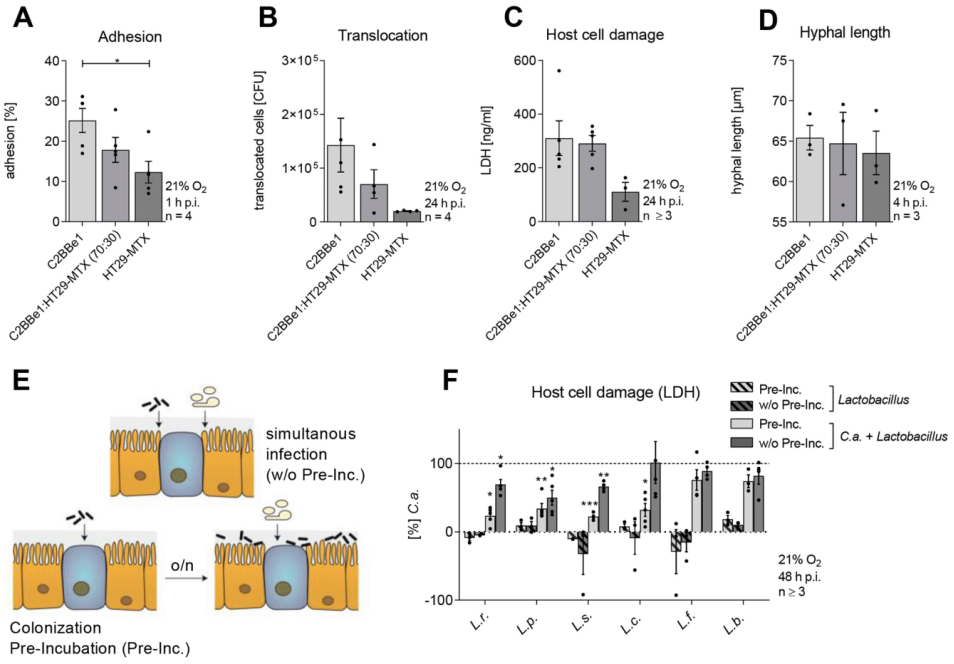

Fig. 1. Influence of combined host cell types on *C. albicans* adhesion, translocation and cytotoxicity and the protective effect of *Lactobacillus* species. (A-D) Percentage of *C. albicans* adhered to IECs at 1 h post-infection (A), translocation of *C. albicans* across IECs at 24 h post-infection (B), LDH release of IECs at 24 h post-infection (C) or *C. albicans* hyphal length at 4 h post-infection (D) on C2BBe1 (enterocyte) and HT29-MTX (mucus-secreting goblet cell) monocultures or in co-culture (C2BBe1:HT29-MTX). (E) Schematic of the two different infection regimens. IECs C2BBe1 (orange) and HT29-MTX (blue) were infected with *C. albicans* (white) and lactobacilli (black) simultaneously (w/o Pre-Inc.) or IECs were colonized (Pre-Inc.) with lactobacilli 18 h (o/n) before *C. albicans* infection. (F) LDH release of IECs colonized or not with *L. paracasei* (*L.p.*), *L. rhamnosus* (*L.r.*), *L. salivarius* (*L.s.*), *L. fermentum* (*L.f.*) or *L. brevis* (*L.b.*) (MOI 50) 48 h post *C. albicans* (*C.a.*) infection (MOI 1). Results were normalized to *C. albicans* single infection. Data are mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.005 (*t*-test).

epithelial E-cadherin (CDH1) levels induced by increasing levels of μ-calpain (CAPN1), a proteolytic enzyme that targets E-cadherin (Rouabhia et al., 2012; Xu et al., 2016). E-cadherin degradation was observed when IECs were infected with *C. albicans* for at least 12 h (Fig. 2G). Although *L. rhamnosus* reduced fungal translocation, E-cadherin breakdown still occurred. Nevertheless, stable E-cadherin levels during *L. rhamnosus* colonization indicate that the bacteria do not affect the epithelial E-cadherin level.

Damage protection by lactobacilli is associated with reduced IEC damage and stress response

For oral, vaginal and intestinal epithelial cells, *Candida*-induced damage correlates with the induction of mitogen-activated protein kinase (MAPK) and NF-κB responses (Böhringer et al., 2016; Moyes et al., 2011, 2010). Therefore, key mediators of this 'danger' response were assessed for their activation during the *Lactobacillus*-*Candida* interaction on IECs.

The *cFOS* gene, which encodes a subunit of the heterodimeric transcription factor AP-1, was highly expressed in IECs 12 h after *Candida* infection (Fig. 3A). The dual-specificity phosphatases are primary targets of AP-1 (Patterson et al., 2009) and inactivate stress-related MAPKs such as p38, ERK and JNK. This provides a negative feedback on MAPK activation. The *DUSP1* and *DUSP5* genes, encoding two of these phosphatases, were upregulated by *C. albicans* infection (Fig. 3B,C). Activation of the NF-κB signaling pathway induces expression of several NF-κB transcriptional target genes, including NFKB inhibitor alpha (*NFKBIA*), which functions as a negative feedback regulator of NF-κB activation (Jacobs and Harrison, 1998). *NFKBIA* gene expression was observed 12 h after *Candida* infection (Fig. 3D). *L. rhamnosus* colonization alone or combined with *C. albicans* infection resulted in a marginal induction of *cFOS*, *DUSP1*, *DUSP5* and *NFKBIA* expression at early time points, but *L. rhamnosus* colonization before infection downregulated these genes at later stages of infection. These data indicate that *L. rhamnosus*, on a molecular level, counteracts the damage response to *C. albicans*.

Lactobacillus-mediated damage protection is independent of oxygen levels

As the gastrointestinal tract has distinct hypoxic niches, with low oxygen tension in the intestinal lumen and higher oxygen tension towards the crypts (Zheng et al., 2015), hypoxic conditions were applied to further work towards a model in which *C. albicans* exhibits a commensal-like co-existence. As lactobacilli preferably

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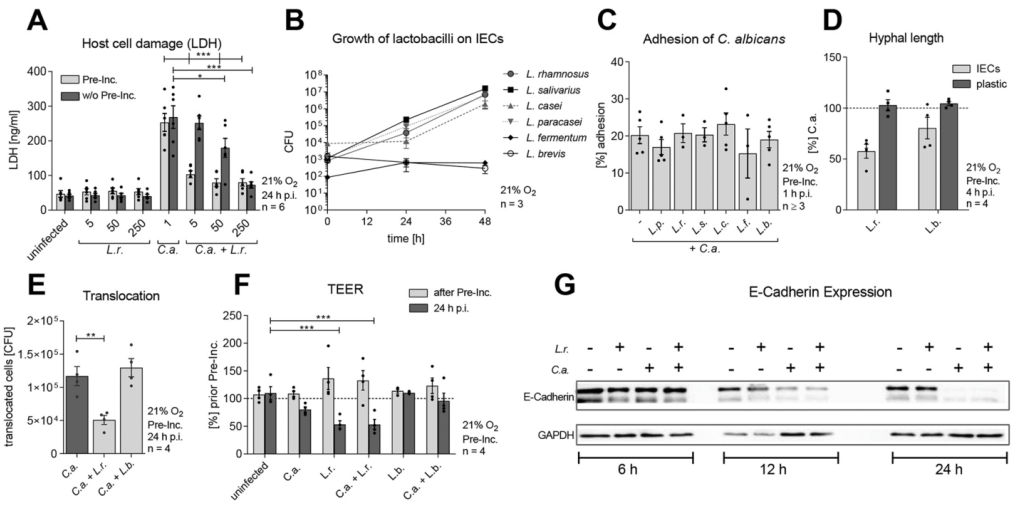

Fig. 2. Growth of lactobacilli on IECs and their influence towards *C. albicans* cytotoxicity, adhesion, hyphal length and translocation. (A) LDH release of IECs colonized (Pre-Inc.) or simultaneously colonized (w/o Pre-Inc.) with *L. rhamnosus* (*L.r.*) at different MOI (5, 50 or 250) and infected or not with *C. albicans* (*C.a.*) (MOI 1) and measured at 24 h post-infection. (B) Growth of *L. paracasei* (*L.p.*), *L. rhamnosus*, *L. salivarius* (*L.s.*), *L. fermentum* (*L.f.*), and *L. brevis* (*L.b.*) on IECs. (C) Percentage of *C. albicans* adhered to IECs colonized with different *Lactobacillus* species (MOI 50) at 1 h post-infection. (D) *C. albicans* hyphal induction on IECs or on plastic colonized with *L. rhamnosus* or *L. brevis* (MOI 50) at 4 h post-infection. Results were normalized to *C. albicans* single infection. (E) Translocation of *C. albicans* (MOI 1) across IECs colonized with *L. rhamnosus* or *L. brevis* (MOI 50) at 24 h post-infection. (F) Assessment of epithelial barrier integrity measured as the loss of transepithelial electrical resistance (TEER) in response to *L. rhamnosus* or *L. brevis* (MOI 50) colonization and *C. albicans* infection in the presence or absence of *Lactobacillus* colonization at 24 h post-infection. Data are TEER loss in percentage of uninfected host cells (before pre-incubation). (G) E-Cadherin protein expression analyzed by western blot compared to GAPDH in IECs that were left uninfected or colonized with *L. rhamnosus* (MOI 50) and infected with *C. albicans* for 6, 12 and 24 h. Data are mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.005 (*t*-test).

grow under anaerobic conditions, we speculated that hypoxia might augment *Lactobacillus*-mediated damage protection. A minimal oxygen level of 2% was selected, as lower levels reduced IEC viability. Compared to 21%, 2% oxygen diminished *C. albicans*-induced cytotoxicity by more than 50% (Fig. 4A). This correlated with reduced hyphal formation during hypoxia (Fig. 4B), which was further reduced when the model was colonized with *L. rhamnosus*. Protection against *C. albicans*-induced cytotoxicity was more pronounced in the presence of protective *Lactobacillus* species; however, not significantly different from damage protection at 21% oxygen. To further investigate the mechanism associated with *Lactobacillus*-mediated damage protection, experiments were continued at 21% oxygen.

Lactobacillus-mediated damage protection is contact-dependent

To determine whether damage protection was mediated by secreted bacterial compounds (James et al., 2016; Parolin et al., 2015; Santos et al., 2018; Tan et al., 2017; Wang et al., 2017), we analyzed the ability of *L. rhamnosus* to counteract *C. albicans*-induced cytotoxicity in a contact-independent manner. Supernatants (SN) from *Lactobacillus*-colonized gut models (Pre-Inc. SN) were unable to reduce *Candida*-mediated epithelial damage, whereas supernatants from *Lactobacillus* grown in MRS broth (MRS-SN) resulted in a trend of reduced damage (Fig. 5A). Supernatants obtained after *Lactobacillus* colonization or after 24 h of infection also did not influence hyphal length (Fig. 5B).

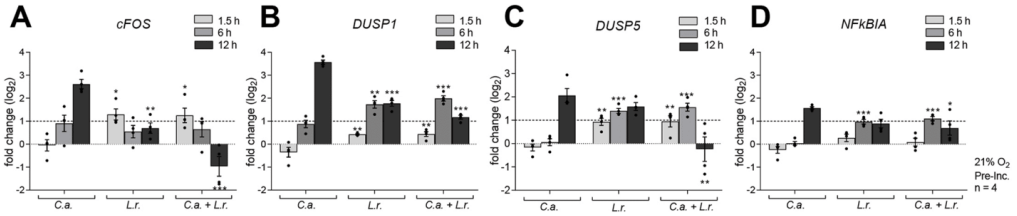

Fig. 3. Lactobacilli suppress the expression of damage- and stress-related host factors by *C. albicans*. (A-D) The relative mRNA expression of *cFOS* (A), *DUSP1* (B), *DUSP5* (C) and *NFKBIA* (D) in IECs either left untreated or colonized with *L. rhamnosus* (*L.r.*) (MOI 50) and subsequently infected or not with *C. albicans* (*C.a.*) (MOI 1) for 1.5, 6 or 12 h. Expression levels were normalized to the reference genes *ACT1* and *GAPDH*. Data are mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.005 (one-way ANOVA).

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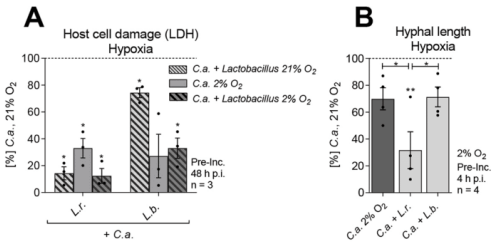

Fig. 4. The protective effect of lactobacilli under hypoxia. (A) LDH release of IECs colonized with *L. rhamnosus* (*L.r.*) and *L. brevis* (*L.b.*) (MOI 50) and infected or not with *C. albicans* (*C.a.*) (MOI 1) at 48 h post-infection at an oxygen level of 21% or 2%. (B) Hyphal length of *C. albicans* on IECs colonized with *L. rhamnosus* or *L. brevis* (MOI 50) at 4 h post-infection. Results were normalized to *C. albicans* single infection at 21% O_2. Data are mean±s.e.m. **P*<0.05, ***P*<0.01 compared with *C. albicans* single infection at 21% oxygen (*t*-test).

Full damage protection requires viable lactobacilli

As high numbers of bacteria mediated protection against *C. albicans*-induced damage (Fig. 2A), we investigated whether *L. rhamnosus* needs to be alive and metabolically active or whether just sheer biomass and physical presence are sufficient to exert its protective effect. Neither heat- nor formaldehyde-killed lactobacilli were able to reduce *Candida*-mediated damage (Fig. 5C). As killed bacteria do not multiply on host cells, they may not reach the required biomass. Therefore, simultaneous infections of *C. albicans* with increasing numbers of killed lactobacilli were performed (Fig. 5D). Only extremely high numbers [multiplicity of infection (MOI) 500] of killed *L. rhamnosus* demonstrated a trend towards damage protection. However, even *L. brevis*, which did not mediate damage protection in other assays, induced a similar damage reduction at such high numbers. This indicates that the protection observed at these concentrations is likely due to different mechanisms from those of viable bacteria at lower numbers. Supporting this, viable *L. rhamnosus* cells at the same inoculum achieved almost 100% damage protection.

Exopolysaccharides of *L. rhamnosus* are not involved in damage protection

Protection against *C. albicans* pathogenicity by lactobacilli was previously attributed to the presence of exopolysaccharides of the outer carbohydrate layer of *L. rhamnosus* GG (Allonsius et al., 2017). However, exopolysaccharide-deficient *L. rhamnosus* inhibited *C. albicans*-induced cytotoxicity to the same extent as wild-type *L. rhamnosus* (Fig. S2).

Glucose consumption and lactate production are not responsible for *Lactobacillus*-mediated damage protection

Previous studies suggested that glucose consumption and lactate production by lactobacilli may be critical for the reduction of fungal damage potential (Hasslöf et al., 2010; Hütt et al., 2016; Köhler et al., 2012; Mailänder-Sánchez et al., 2017).

Although glucose levels dropped slowly when the model remained uninfected or was colonized with *L. brevis*, glucose was consumed within 12 h during *C. albicans* infection (Fig. 6A). Colonization with *L. rhamnosus* (in the presence or absence of *C. albicans* infection) already led to increased glucose consumption within the first 6 h of the experiment (Fig. 6A). As rapid glucose consumption is a mechanism by which *C. albicans* can cause damage to macrophages (Tucey et al., 2018), we speculated that the reduced glucose levels caused by *L. rhamnosus* might affect the potential of *C. albicans* to cause damage. However, when glucose was supplemented after colonization with lactobacilli, to compensate for the reduced glucose levels, no effect on *L. rhamnosus*-mediated damage protection was observed (Fig. 6B).

Lactate levels slowly increased in all conditions, but this effect was significantly enhanced by colonization with *L. rhamnosus* (Fig. 6C). As lactate was previously described to be a key mediator of *Lactobacillus* protection (Buffo et al., 1984; Hasslöf et al., 2010; Hütt et al., 2016; Köhler et al., 2012; Maudsdotter et al., 2011; Noverr and Huffnagle, 2004), we validated whether lactate itself affected filamentation of *C. albicans* in the medium used in our model. The influence of lactate on the extracellular pH was excluded in our model by buffering the cell culture medium, an essential requirement for the fitness of the intestinal cells. Increasing lactate concentrations in this setting did not affect hyphal length (Fig. 6D). In addition, lactate supplementation did not induce damage protection (Fig. 6E).

Lactobacillus-mediated damage protection involves the shedding of *C. albicans*

In order to invade and damage epithelial cells, *C. albicans* requires physical contact with the cells (Dalle et al., 2010; Wächtler et al., 2011). As we did not observe an influence of lactobacilli on adhesion

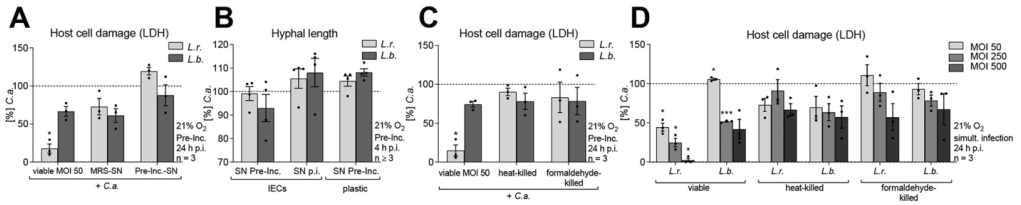

Fig. 5. Damage protection requires the presence of viable lactobacilli. (A) LDH release at 24 h post-infection of IECs either colonized with viable *L. rhamnosus* (*L.r.*) or *L. brevis* (*L.b.*) (MOI 50), co-incubated with MRS supernatant (MRS-SN) or colonized (Pre-Inc. SN) (see Materials and Methods) and infected with *C. albicans* (*C.a.*) (MOI 1). The results were normalized to *C. albicans* single infection. (B) *C. albicans* hyphal length after 4 h of infection on IECs or on plastic co-incubated with Pre-Inc. supernatant (SN Pre-Inc.) or post-infection supernatant (SN p.i.) from an *L. rhamnosus* or *L. brevis* colonization setting (see Materials and Methods). (C) LDH release at 24 h post-infection of IECs colonized by viable or inactivated (heat- or formaldehyde-treatment) *L. rhamnosus* or *L. brevis* (MOI 50) followed by infection with *C. albicans* (MOI 1). The results were normalized to *C. albicans* single infection. (D) LDH release at 24 h post-infection of IECs colonized by viable or inactivated (heat- or formaldehyde-treatment) *L. rhamnosus* or *L. brevis* (MOI 50) followed by infection with *C. albicans* (MOI 1) or added simultaneously at various MOI (50, 250, 500) with *C. albicans* infection (MOI 1). Single infections with *C. albicans* or lactobacilli were performed as controls. Results were normalized to *C. albicans* single infection. Data are mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.005 (*t*-test).

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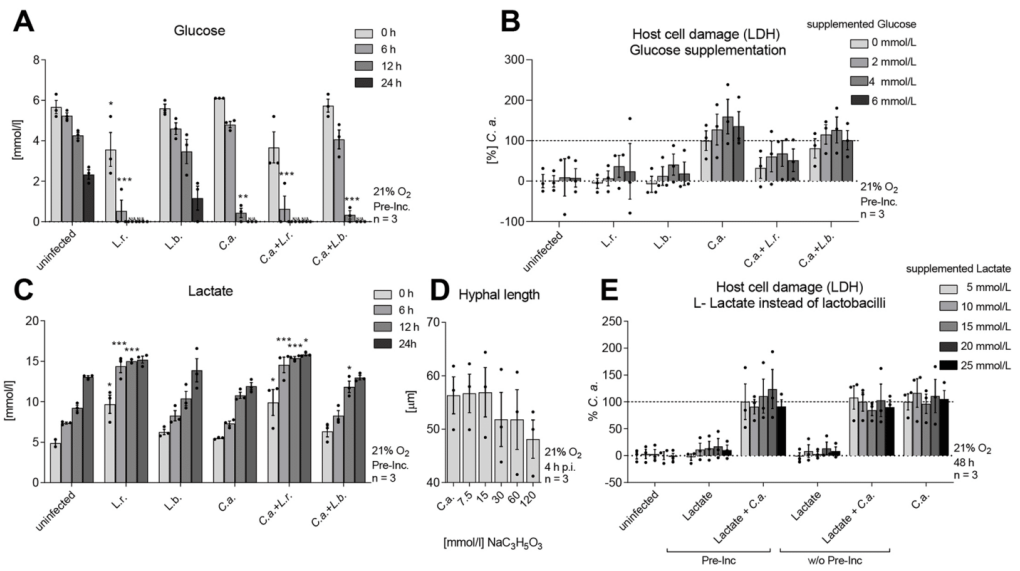

Fig. 6. Reduced glucose and increased lactate levels do not mediate *Lactobacillus*-driven damage protection. (A,C) IECs were colonized with *L. rhamnosus* (L.r.) or *L. brevis* (L.b.) (MOI 50) or left uncolonized. Subsequently, cells were challenged or not with *C. albicans* (C.a.) (MOI 1). The amount of glucose (A) and lactate (C) was evaluated after colonization (0 h) and at 6, 12 and 24 h post-infection. (B) LDH release at 24 h post-infection of IECs colonized with *L. rhamnosus* and *L. brevis* and glucose supplementation (0-6 mmol/l) simultaneous to *C. albicans* (MOI 1) infection. (D) The effect of increasing concentrations of sodium L-lactate (0-120 mmol/l) on the hyphal length of *C. albicans* was measured microscopically after 4 h of incubation in KBM cell culture medium. (E) LDH release at 48 h post-infection of IECs either colonized or co-incubated with lactate (5-20 mmol/l) and infected with *C. albicans* (MOI 1) or not. The results were normalized to *C. albicans* single infection. Data are mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.005 (one-way ANOVA).

of *C. albicans*, we speculated that damage protection could involve displacement of already bound fungal cells. Therefore, we performed displacement assays in which *C. albicans* was used to infect the model and, subsequently, viable lactobacilli were added 1, 3 or 6 h post-infection (Fig. 7A). The more bacterial cells were added, the stronger the damage was reduced. In addition, the longer the inoculation of lactobacilli was delayed, the less protection was achieved. Still, *L. rhamnosus* added 6 h after *C. albicans* infection reduced host cell damage by 60% at the highest inoculum, compared to 90% reduction when added 1 h post-infection.

An accumulation of debris was observed in the supernatant at later stages of infection, with more macroscopically observable debris when the model was colonized by *L. rhamnosus* (Fig. 7B). Thus, we quantified and analyzed the localization of lactobacilli and *C. albicans* cells during infection (Fig. 7C-G). The most substantial fraction of *L. rhamnosus* colony forming units (CFUs) was retrieved from the culture supernatant (Fig. 7C), and 1 h post-infection the number of CFUs doubled compared to the starting inoculum. Nevertheless, *L. rhamnosus* CFUs continued to increase drastically during *C. albicans* infection to almost 200-fold the initial inoculum (Fig. 7D). *L. brevis* appeared to be more localized at the epithelial barrier (Fig. 7C) and did not proliferate during the course of infection (Fig. 7D).

On the fungal side, independent of lactobacilli colonization, approximately half of the fungi were present in the supernatant 1 h post-infection, whereas the other half were localized within or attached to the epithelial barrier (Fig. 7E). During the course of infection, increasing percentages of fungi were associated with the gut epithelium layer over time, with 99% of fungal cells attached to host cells after 24 h (Fig. 7E). Colonization of the model with *L. rhamnosus* caused slightly decreased fungal association with the gut epithelial barrier over time, but 24 h post-infection the majority of fungal cells (62%) were found to be in the supernatant (Fig. 7E). Similar numbers were observed with gut models consisting of only C2BBe1 cells. Thus, this shedding was independent of the presence of HT29-MTX mucus-secreting goblet cells (Fig. 7F). In line with this, *L. rhamnosus* colonization also did not significantly influence gene expression or protein levels of mucin genes known to be expressed by intestinal tissue (Fig. S3). In terms of absolute numbers, *C. albicans* CFUs significantly increased at 24 h post-infection, yet colonization with *L. rhamnosus* also prevented *C. albicans* outgrowth and kept the number of viable fungi constant during the course of infection (Fig. 7G).

Lactobacilli induce aggregation of *C. albicans* hyphae, bacteria and host cells

To investigate the cell-cell interactions in our model, we visualized the aggregation potential of microbial species to either themselves (auto-aggregation) or each other (co-aggregation) using scanning electron microscopy. Aggregation of both *Lactobacillus* species with *C. albicans* was observed (Fig. 7H-M). Six hours after infection of colonized IECs, almost all *C. albicans* hyphae were covered with *L. brevis* cells (Fig. 7H). Interestingly, bacterial cells were only found in contact with fungal cells; they did not adhere to host cells. *L. rhamnosus* formed large masses via auto-aggregation, which also stuck to *C. albicans* hyphae (Fig. 7K-M) and were rarely found on host cell layers.

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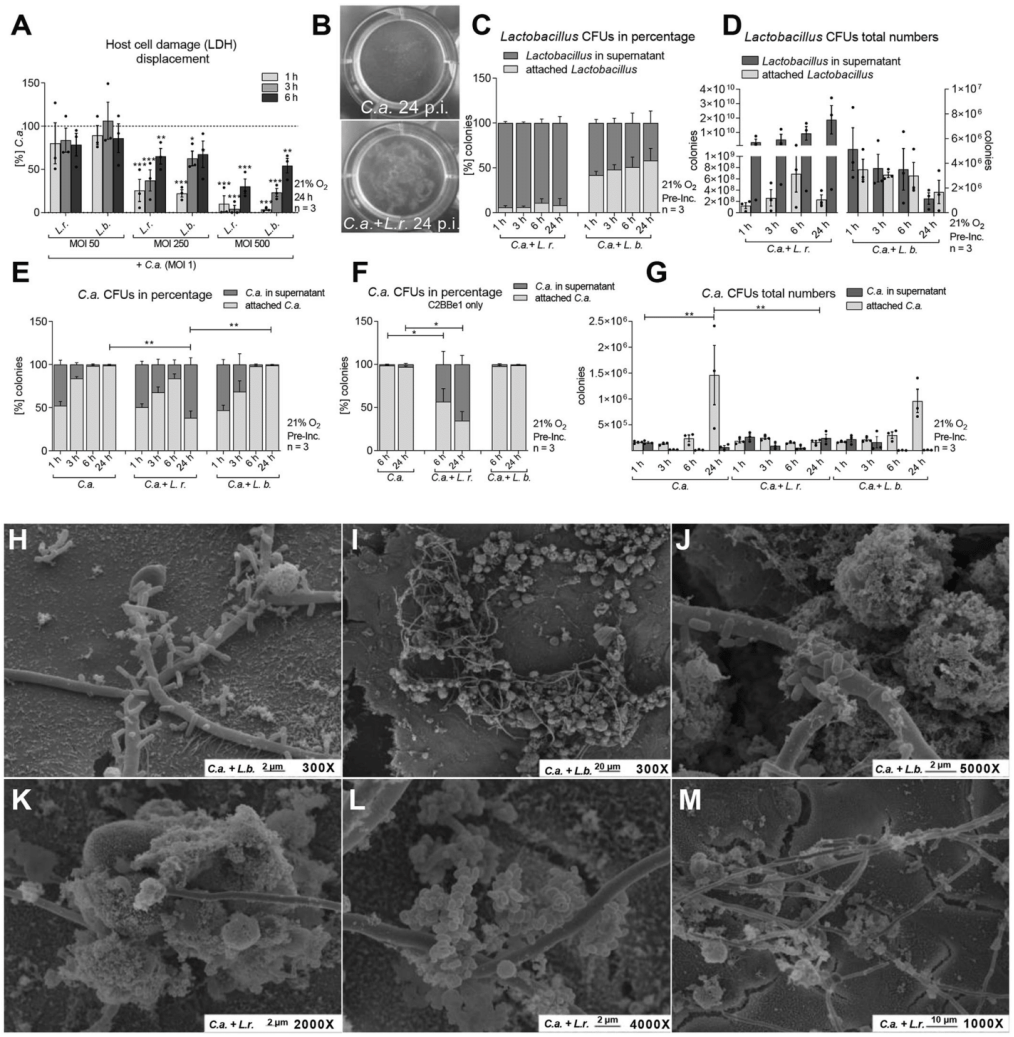

Fig. 7. Lactobacilli induce shedding of *C. albicans* and host cells. (A) LDH at 24 h post-infection of IECs infected with *C. albicans* (C.a.) (MOI 1) for 1, 3 or 6 h and subsequently colonized with *L. rhamnosus* (L.r.) or *L. brevis* (L.b.) at various MOI (50, 250, 500). The results were normalized to *C. albicans* single infection. (B) Macroscopic observation of shedding at 24 h post-infection in a model of IECs colonized with *L. rhamnosus* or not and infected with *C. albicans* (MOI 1). (C,D) *L. rhamnosus* or *L. brevis* supernatant and cell-associated CFUs measured during the course of *C. albicans* infection at 1, 3, 6 and 24 h; data shown as relative percentages (C) or absolute numbers (D). (E-G) *C. albicans* supernatant and cell-associated CFUs measured during the course of *C. albicans* infection in untreated and *L. rhamnosus*- or *L. brevis*-colonized (MOI 50) IECs at 1, 3, 6 and 24 h post-infection (E,G) or 6 and 24 h post-infection (F); data shown as relative percentages (E,F) or absolute numbers (G). Data are mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.005 (A: *t*-test; D-G: one-way ANOVA). (H-M) Scanning electron micrographs of IECs colonized with *L. brevis* (H-J) or *L. rhamnosus* (MOI 50) (K-M) and infected with *C. albicans* for 6 h.

Shedding of *C. albicans* is associated with host cell apoptosis

To maintain tissue homeostasis, the intestinal epithelium induces apoptosis and shedding of senescent cells, while the renewal of cells conserves barrier integrity. As we observed that *C. albicans* is shed in association with apoptotic host cells, live-cell imaging was used to investigate the influence of *L. rhamnosus* on host cell apoptosis as determined by extracellular phosphatidylserine exposure stained with annexin-V (ANXA5; Fig. 8A,B) and caspase 3/7 (CASP3/7) activity (Fig. 8C). Although *L. rhamnosus* and *L. brevis* alone did

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not induce host cell apoptosis (Fig. 8A-C), *C. albicans* infection did induce host cell apoptosis 16 h post-infection to some degree; however, colonization with *L. rhamnosus* significantly increased this induction (Fig. 8A-C). With *L. brevis*, the induction of apoptosis was similar to that of *C. albicans* alone (Fig. 8C). To verify that apoptotic cells were localized within the shed material, caspase 3/7 activity of epithelial cells within this material was quantified. When *L. rhamnosus* was used to colonize the model, significantly more epithelial cells with caspase 3/7 activity were observed in the shed material (Fig. 8D). To elucidate whether increased apoptosis in the presence of *L. rhamnosus* was a prerequisite for shedding, apoptosis was inhibited. Despite efficient inhibition of apoptosis through a selective caspase 3/7 inhibitor, no inhibition of shedding (Fig. 8E) or restoration of *C. albicans*-induced damage was observed (Fig. 8F).

DISCUSSION

Epithelial cells exposed to *C. albicans in vitro* are rapidly and severely damaged indicating a high pathogenic potential in this *in vitro* context (Allert et al., 2018; Dalle et al., 2010; Wächtler et al., 2011). Here, we established a novel *in vitro* gut model in which we systematically explored the mechanisms that can prevent pathogenicity and help to achieve a commensal state. The inclusion of mucus-producing goblet cells reduced the adhesion of *C. albicans* to the epithelial barrier and its translocation, but not damage or hyphal length. To achieve protection against damage, a protective artificial 'microbiota' in the form of *Lactobacillus s*pecies with *Candida*-antagonizing potential needed to be co-cultured in the model. The presence of these live bacteria prevented *C. albicans* overgrowth and caused shedding of *C. albicans* from the host cells in a contact-dependent manner, which temporally correlated with increased host cell apoptosis. However, apoptosis was not required for *Lactobacillus*-induced shedding and protection against *C. albicans*-mediated damage. Although less potent than the presence of a protective microbiota, hypoxia also reduced *C. albicans*-mediated damage. Still, the antagonistic potential of *L. rhamnosus* was not augmented by hypoxic conditions. In this three-partite gut model of intestinal epithelial cells, a bacterial member of the gut microbiota and *C. albicans*, the pathogenic potential of *C. albicans* could be reduced.

Current *in vitro* models used to study *C. albicans* pathogenicity mostly rely on a monoculture of epithelial cells (Allert et al., 2018; Donnarumma et al., 2014; Mailänder-Sánchez et al., 2017; Rizzo et al., 2013). Here, a mixture of enterocytes and mucus-producing goblet cells was used to more closely mimic the intestinal epithelium (Beduneau et al., 2014; Ferraretto et al., 2018). Using this setup, adhesion and translocation of *C. albicans* were decreased, presumably owing to the presence of mucus. Nevertheless, epithelial damage as well as hyphal length remained unchanged. Therefore, an additional level of complexity was introduced in the form of an artificial microbiota. As microbiota, *Lactobacillus* species were selected for several reasons. First, they are found throughout the gastrointestinal tract of healthy humans (Finegold et al., 1977). Second, they are not pathogens themselves or fast-growing and, therefore, do not pose a threat to the epithelial cells. Third, they are widely described to have antagonistic properties against *C. albicans* (reviewed by Matsubara et al., 2016a and Förster et al., 2016). Fourth, *Lactobacillus* species are the most widely used probiotics to prevent or treat *Candida*-infections in clinical trials. *C. albicans* colonization of preterm neonates treated with probiotic *L. rhamnosus* was reduced

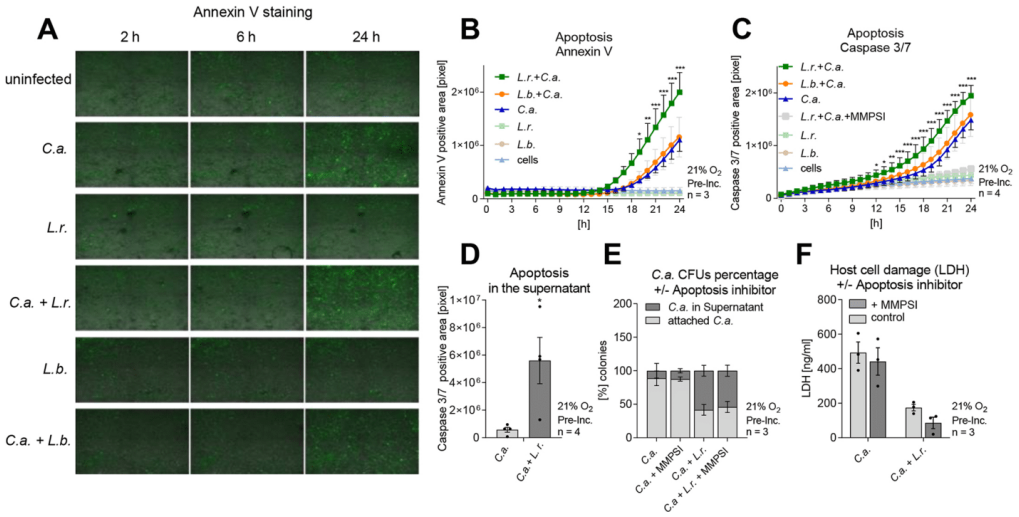

Fig. 8. *Lactobacillus*-induced shedding of *C. albicans* correlates with host cell apoptosis. (A-C) Apoptosis of IECs tracked during the course of *C. albicans* (C.a.) infection by live-cell imaging of annexin V expression (A,B) and caspase 3/7 activity (C). Apoptosis was followed for uninfected cells, *L. rhamnosus*- and *L. brevis*-colonized cells as well as *C. albicans*-infected cells in the presence and absence of *L. rhamnosus* (L.r.) or *L. brevis* (L.b.) colonization. Calculated area of annexin V-expressing cells (B) or cells with caspase 3/7 activity (C). (D) Caspase 3/7 activity in shed material at 24 h post *C. albicans* infection of IECs that were colonized with *L. rhamnosus* or left uncolonized. (E) relative percentages of *C. albicans* supernatant and cell-associated CFUs measured during the course of *C. albicans* infection in untreated and *L. rhamnosus*-colonized (MOI 50) IECs in the presence or absence of a caspase 3/7 inhibitor (MMPSI, 50 µM) at 24 h post-infection. (F) LDH release at 24 h post-infection of IECs colonized with *L. rhamnosus* or not infected with *C. albicans* (MOI 1) in the presence or absence of a caspase 3/7 inhibitor (MMPSI, 50 µM). Data are mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.005 (B,C: two-way ANOVA; D: *t*-test).

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(Manzoni et al., 2006). Furthermore, *L. reuteri* colonization was as effective as antifungal Nystatin prophylaxis in this patient group (Oncel et al., 2015) and can reduce late-onset sepsis (Romeo et al., 2011). A mixture of different *Lactobacillus* spp. strains, *Bifidobacterium* spp., and *Saccharomyces* spp. reduced gastrointestinal *C. albicans* colonization as well as candiduria as a result of disseminated infections in critically ill children receiving broad-spectrum antibiotics (Kumar et al., 2013). Of note, clinical data are limited on the use of probiotics in immunocompromised patients treated with broad-spectrum antibiotics, the major risk group for systemic candidiasis. Yet, despite the extensive evidence that lactobacilli antagonize *C. albicans*, direct interactions on mucosal surfaces mimicking the gastrointestinal tract have not been described.

Out of six tested *Lactobacillus* species, *L. rhamnosus*, *L. paracasei*, *L. casei* and *L. salivarius* were capable of preventing *C. albicans*-induced cytotoxicity. Antifungal activity for these *Lactobacillus* species was also described in Allonsius et al. (2019). For a more in-depth analysis of the reduced damage, protective *L. rhamnosus* was compared with the less protective species *L. brevis*. The reduced damage following *L. rhamnosus* colonization correlated with reduced hyphal elongation, fungal translocation and shedding of fungal filaments in conglomerates consisting of apoptotic host cells, hyphae and bacteria. Reduced induction of damage- and stress-related host responses (MAPK and NF-κB signaling), which were previously shown to be part of the damage response to *C. albicans* in epithelial cells (Moyes et al., 2010), validated the improved condition of the epithelial barrier on a molecular level.

Many studies demonstrated that *Lactobacillus* supernatants restrict growth, yeast-to-hyphal-transition and biofilm formation (James et al., 2016; Parolin et al., 2015; Santos et al., 2018; Tan et al., 2017; Wang et al., 2017). In contrast, we observed that damage protection exerted by *L. rhamnosus* was contact dependent. Of note, these studies used a variety of *Lactobacillus* strains, and the production of antimicrobial metabolites is highly strain specific. In agreement with Felten et al. (1999), who showed that *L. rhamnosus* is not an H_2O_2 producer, no hydrogen peroxide production was detected for any of the *Lactobacillus* species tested in our study (data not shown). Likewise, the production of biosurfactants and bacteriocins, which are anti-candidal and decrease adhesion and biofilm formation (Fracchia et al., 2010; Hütt et al., 2016; Kaewsrichan et al., 2006; Morais et al., 2017; Parolin et al., 2015; Strus et al., 2005; Zakaria Gomaa, 2013), played only a minor role, if any, in our study, as *Lactobacillus* supernatants did not reduce damage. Even though colonization of *L. rhamnosus* resulted in lactate accumulation, its supplementation at concentrations comparable to those in *L. rhamnosus*-colonized models did not inhibit *C. albicans*' cytotoxicity or hyphal elongation. This can likely be attributed to the buffered KBM cell culture medium (required to keep the epithelial cells healthy in our model), which kept the pH neutral throughout the experiment and thus dampened any potential antimicrobial effect of lactic acid shown in other studies at acidic pH (Buffo et al., 1984; Hasslöf et al., 2010; Hütt et al., 2016; Köhler et al., 2012; Maudsdotter et al., 2011). Nevertheless, acidification via lactate production may contribute to protection against *C. albicans* pathogenicity *in vivo*. However, our data suggest the necessity of physical interaction between *L. rhamnosus* and *C. albicans* for damage protection in the setting of our gut model.

A crucial aspect of our study is the observation that live *L. rhamnosus* cells are required for reduced *C. albicans* pathogenicity. Nevertheless, an extreme biomass of killed bacteria, even the non-protective *L. brevis* strain, mediated protection. The four protective *Lactobacillus* species (*L. rhamnosus*, *L. paracasei*, *L. casei* and *L. salivarius*) proliferated on host cells. Thus, overnight colonization of a low bacterial cell number yielded the required protective biomass. As *L. brevis* and *L. fermentum* did not grow under the given conditions, the two species are only protective at higher inocula, as shown in simultaneous infections and displacement experiments. This means that the ability to replicate during colonization before infection with *C. albicans* distinguishes the protective from less-protective *Lactobacillus* species. In other words, the reduced growth of *L. brevis* and *L. fermentum* in our model appears to be the key reason why these species have a limited protective potential, as they otherwise have similar attributes to the protective species. Interestingly, the growth of lactobacilli required the presence of host cells, as growth in KBM medium alone was not observed for any species. Adhesion to mucin-coated surfaces can shift lactobacilli to a more protease-active phenotype, thereby degrading mucin as a nutrient source for the bacteria (Wickström et al., 2013). Another possibility is the formation of hypoxic microniches when intestinal cells are present, resulting in the growth of microaerophilic bacteria that can bear oxygen, but prefer environments containing lower levels of oxygen for growth. *L. rhamnosus* even showed increased proliferation during *C. albicans* infection, which could suggest that *C. albicans* may additionally support *Lactobacillus* growth. A potential explanation could be hypoxic microniches that are formed by metabolically active *C. albicans* hyphae (Lambooij et al., 2017). The *Lactobacillus* species used in the study differ in their ability to utilize environmental conditions for replication. Reaching a critical biomass of ∼200 bacteria per *C. albicans* cell was indispensable for *Lactobacillus*-mediated inhibition of *C. albicans*. Our data show a significant expansion of the *L. rhamnosus* population during colonization and over the course of infection, which easily reaches this biomass of viable bacteria. Nevertheless, killed *L. rhamnosus*, and even *L. brevis*, reduced fungal-induced damage to some extent, but not in a manner akin to viable bacteria. It is likely that steric hindrance influences the cytotoxicity of *C. albicans*. Incidentally, this was also observed when using killed *C. albicans* cells instead of killed bacteria (Fig. S4). Still, for potent damage protection, viable lactobacilli were essential.

It can only be speculated whether a ratio of 200:1 can be reached in patients treated with *L. rhamnosus* probiotics. Clearly, colonization of the gastrointestinal tract in the stomach, ileum and colon (Alander et al., 1997, 1999; Valeur et al., 2004) can be achieved. However, the extent of colonization by lactobacilli varies drastically between studies with various formulations, treatment regimens and colonization readouts (Alander et al., 1999; Piano et al., 2012; Valeur et al., 2004). Nevertheless, the studies mentioned above show that the current probiotic treatment strategies are efficient in pre-term neonates, though knowledge is lacking regarding the exact quantitative interactions in such clinical settings, as well as the effect in an adult patient population.

Scanning electron microscopy revealed the ability of *L. rhamnosus* to co-aggregate with fungal cells. Therefore, the bacteria may reside in microniches that enable close contact cell-cell interactions. Within such an interface, *L. rhamnosus* may form a hostile microenvironment that mediates anti-*Candida* activities. Antimicrobial factors (mentioned above) were likely produced by the lactobacilli in our study, but were ineffective in the supernatant, probably because such factors did not reach minimum inhibitory concentrations. However, the same factor may inhibit *C. albicans* when higher concentrations are achieved in microniches. The concentration of short-chain fatty acids and other metabolites may

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be even higher, whereas nutrient availability may be even lower within these niches. Competition for nutrients could be one explanation. In agreement with Mailänder-Sánchez et al. (2017), damage prevention in the presence of *L. rhamnosus* was accompanied by glucose depletion. Despite the deprivation of the primary carbon source of *C. albicans*, supplementation of glucose at levels similar to those amounts consumed by *L. rhamnosus* during the colonization phase did not restore *C. albicans*' damage potential. However, we cannot exclude that nutrient competition in microniches within the bacteria–*Candida* interface may contribute to growth inhibition and shedding of *C. albicans*.

Several studies indicate an association between co-aggregation of protective bacteria with pathogens with the interference of adhesion to host cells. This involves bacterial surface molecules such as peptidoglycan, teichoic acids, (glyco)proteins and polysaccharides (Allonsius et al., 2017; Coman et al., 2015; Kang et al., 2018; Kleerebezem et al., 2010; Lebeer et al., 2012, 2010; Malik et al., 2016; Niu et al., 2017; Parolin et al., 2015; Santos et al., 2016; Zivkovic et al., 2015). In the case of IECs, we observed striking differences to data obtained in many other studies. For example, exopolysaccharides of *L. rhamnosus* GG were shown to interfere with hyphal formation and *C. albicans* adhesion to vaginal epithelial cells (Allonsius et al., 2017; Donnarumma et al., 2014). However, the presence of lactobacilli in our model did not influence the binding efficiency of the fungus. Accordingly, an exopolysaccharide-deficient *L. rhamnosus* GG mutant (Allonsius et al., 2017) conveyed protection to a similar extent as the wild type in our study. The fact that *C. albicans* adhesion remained unaffected in our model and the finding that exopolysaccharides were not involved in damage reduction suggests mechanisms different from those shown in other studies.

Filamentation is one of the most essential virulence attributes of *C. albicans* through which the fungus can invade tissue and is associated with cytotoxicity (Dalle et al., 2010; Gow et al., 2011; Höfs et al., 2016; Moyes et al., 2016). Lactobacilli-influenced hyphal length is potentially supported by the mucus-producing goblet cells (Allonsius et al., 2019; Kavanaugh et al., 2014) and likely contributes, in part, to the reduced cytotoxicity. However, we propose that the actual mode of action of *Lactobacillus*-mediated damage protection is likely inhibition of fungal overgrowth and shedding of fungal cells, which already takes place after 6 h of infection. After 24 h, the majority of *Candida* cells were located in the supernatants, spatially restricting fungal cells from host cells and preventing invasion and hyphal-associated damage of the epithelial cell layer. Indeed, the presence of *L. rhamnosus* reduced *C. albicans* translocation. To our knowledge, inhibition of adhesion was mainly studied on vaginal and oral epithelial cells (Allonsius et al., 2017; Mailänder-Sánchez et al., 2017). This means that beneficial effects of lactobacilli, which are often considered species- and even strain-specific, likely also depend on the epithelial environment within the human body.

Lactobacillus-induced mucus production correlates with the inhibition of attachment of various pathogens to epithelial surfaces (Hafez, 2012; Kim et al., 2008; Li et al., 2008; Zivkovic et al., 2015). This was also considered as a mechanism that could have contributed to the detachment and shedding of *Candida* hyphae. However, no influence of *L. rhamnosus* on mucin gene or protein expression was observed and it can, therefore, be assumed that an increased mucus production has little, if any, contribution to the detachment of *C. albicans* from *Lactobacillus*-colonized intestinal epithelial cells. Consistent with this, shedding was also induced in the absence of mucus-secreting goblet cells.

Besides protective mucus, the intestinal epithelium is known for its very high turnover rate. Enterocytes have a short lifetime and are continuously shed into the lumen of the gut, completely renewing the epithelium every 5-7 days. In humans, this daily loss has been estimated at 10^{11} cells (Potten, 1990). Fully differentiated intestinal epithelial cells experience detachment-induced, caspase-dependent apoptosis (a programmed cell death termed 'anoikis') (Beauséjour et al., 2012; Bullen et al., 2006; Patterson and Watson, 2017). This process is accompanied by perturbations of tight junctions, which are rapidly reassembled by neighboring cells to maintain barrier integrity (Guan et al., 2011; Williams et al., 2015). We observed that the shed *Candida*-bacteria aggregates contained host cells with apoptotic characteristics such as phosphatidylserine expression and caspase 3/7 activity. Furthermore, we observed that *L. rhamnosus* increased expression of apoptotic markers of epithelial cells, without increasing LDH release. Apoptotic cells, in contrast to necrotic cells, retain their membrane integrity (Zhang et al., 2018). Therefore, the observation of apoptotic markers in the absence of LDH release points towards induction of apoptosis by *L. rhamnosus* during infection with *C. albicans*. In the literature, contrasting results were described concerning *Lactobacillus*-mediated apoptosis induction. Some studies demonstrate the ability to prevent pathogen-induced apoptosis (Li et al., 2008; Santos et al., 2016), whereas others state the opposite (Sungur et al., 2017; Zielinska et al., 2018). Interestingly, the timing of the apoptosis induction coincided with the observed shedding. Nevertheless, blockade of apoptosis via inhibition of caspase 3/7 did not reduce shedding nor did it restore *C. albicans*-induced damage in the presence of *L. rhamnosus*. This could suggest that apoptosis might be a consequence of shedding rather than the mechanism causing it. We therefore propose that the *L. rhamnosus*-induced shedding of *C. albicans* hyphae may result in detachment of epithelial cells to which the hyphae are attached, resulting in induction of apoptosis due to a loss of anchorage.

Although phosphatidylserine exposure and caspase 3/7 activity were also observed during infection with *C. albicans* alone, this was paired with increased LDH release, suggesting predominantly necrotic cell death (Allert et al., 2018; Chan et al., 2013). Furthermore, the epithelial integrity measured by TEER showed a decrease when exposed to *C. albicans* alone. Allert et al. (2018) stated that loss of epithelial integrity and *C. albicans*-induced damage can be independent processes – meaning for the current study that colonization with *L. rhamnosus* can lead to TEER loss (presumably by shedding of apoptotic cells and renewal of the epithelial barrier) and a simultaneous inhibition of *C. albicans* translocation. Nevertheless, it remains unknown why TEER is reduced upon colonization of *L. rhamnosus* alone, as no induction of apoptosis was observed under these conditions. This reduction of TEER in the presence of *L. rhamnosus* contrasts the other protective effects imposed upon *C. albicans* pathogenicity. This also contrasts with previous studies demonstrating that *Lactobacillus* species can even improve barrier integrity (Barnett et al., 2018).

Although the molecular mechanism behind the shedding observed in this study remains unknown, it appears to be clear that this is the result of a tripartite interaction of bacteria, *C. albicans* and epithelial cells rather than due to dual interactions between two of the players in this model. Whether shedding is an exclusive feature of *L. rhamnosus* or is also induced by other *Candida*-antagonizing bacteria, remains to be investigated. Similarly, shedding may also be a protective feature of other epithelial tissues colonized by *Candida*-antagonizing bacteria. Even though supernatants of *L. rhamnosus* cultured within the model were not capable of inhibiting *C. albicans* pathogenicity, yet undiscovered

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metabolic interactions may play a role in *Lactobacillus*-mediated damage protection.

We are convinced that our model, which is to our knowledge the first *in vitro* gut model to mimic a more commensal (non-damaging) state of *C. albicans*, can provide crucial insights to understanding the interplay between host cells and microbiota in preventing pathogenicity. The analysis of *C. albicans* as a commensal using *in vivo* models is hampered by the fact that most laboratory animals (including mice) are not natural hosts of *C. albicans* and colonization requires germ-free animals or an antibiotic-induced dysbiosis (Fan et al., 2015; Koh, 2013; Naglik et al., 2008). The model developed here could facilitate the study of *C. albicans* commensalism and pathogenesis with human cells. Increasing our current knowledge on the commensal state of *C. albicans* is crucial to understand how it gained its pathogenic potential (Hube, 2004) and to explain biological effects observed in *in vivo* clinical trials, which, for example, showed the usefulness of lactobacilli for the prevention and treatment of vulvovaginal and oral candidiasis (Hatakka et al., 2007; Kumar et al., 2013; Manzoni et al., 2011, 2006; Martinez et al., 2009). We believe that our experimental model and refined future modifications, e.g. containing immune cells, for *C. albicans* commensalism can be widely applied for future studies investigating the interaction between *C. albicans* and other members of the microbiota in a more biologically relevant context.

Collectively, we established a commensal-like model to study *C. albicans* interactions in the presence of antagonistic *Lactobacillus* species and intestinal epithelial cells including mucus-producing goblet cells (Fig. S1). Mechanistically, these bacteria induce shedding of invading *C. albicans* in aggregates with bacteria and host cells, thereby spatially restricting *C. albicans* from the epithelial barrier. This novel *in vitro* model may provide a stepping stone for more complex models aimed at studying the commensal and pathogenic states of *C. albicans* and could serve as a tool to study novel therapies aiming at preventing *C. albicans* pathogenicity.

MATERIALS AND METHODS

Microbial strains and culture conditions

The *C. albicans* wild-type strain SC5314 was grown on YPD plates (2% peptone, 1% yeast extract, 2% glucose, 2% agar) at 30°C. For use in experiments, *C. albicans* cells were grown overnight (o/n) in YPD medium (2% peptone, 1% yeast extract, 2% glucose) at 30°C and 180 rpm in a shaker incubator. Before infection, yeast cells from an o/n culture were collected by centrifugation at 20,000 ***g*** and washed twice with phosphate-buffered saline (PBS). The cell number was determined using a Neubauer chamber system, and the concentration adjusted to 4×10^5 cells/ml in serum-free Keratinocyte Basal Medium (KBM, Lonza). For the killing of *C. albicans*, fungal cells were treated for 10 min at 70°C and the viability was proven by the absence of growth on YPD plates. *Lactobacillus* strains [*L. brevis*: ATCC, 14869; *L. casei*: ATCC, 393; *L. fermentum*: ATCC, 14931; *L. paracasei*: ATCC, 11578; *L. rhamnosus*: ATCC, 7469; *L. salivarius*: ATCC, 11741; *L. rhamnosus*: CMP5351 (Lebeer et al., 2009); *L. rhamnosus* GG: ATCC, 53103 (Lebeer et al., 2009)] were grown on Man, Rogosa and Sharpe (MRS) agar plates (Carl Roth) at 37°C and 1% O_2. For use in experiments, bacterial cells were grown at 37°C without agitation in MRS broth (Carl Roth). Before experiments, lactobacilli were collected by centrifugation at 20,000 ***g***, washed twice in PBS and diluted to an optical density OD_{600} of 1 in KBM medium equaling a cell number of $\sim1\times10^8$ CFU/ml. For the killing of lactobacilli, bacterial cells were either treated for 30 min in Histofix (Carl Roth) or for 10 min at 90°C. Viability was proven by the absence of growth on MRS agar plates.

Cell culture

The human intestinal epithelial Caco-2 subclone Caco2 brush border expressing 1 (C2BBe1; ATCC, CRL2102™; Peterson and Mooseker, 1992) and the human intestinal goblet cell HT29-MTX (ATCC, HTB-38; CLS, Lot No. 13B021) were routinely cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS) (Bio&Sell), 10 µg/ml Holotransferrin (Calbiochem, Merck) and 1% non-essential amino acids (Gibco, Thermo Fisher Scientific) at 37°C and 5% CO_2 for no longer than 15 passages. Cell lines have been authenticated via commercial STR profiling (Eurofins Genomic) and checked for contaminations using a PCR mycoplasma test kit (PromoKine) according to the manufacturer's instructions. For detachment, C2BBe1 cells and HT29-MTX cells were treated with Accutase or 0.05% Trypsin-EDTA solution (Gibco, Thermo Fisher Scientific), respectively. For cell culture maintenance, cells were seeded in fresh 75 cm^2 culture flasks containing supplemented DMEM at a 1:4 split ratio. For use in experiments, the cell numbers were determined using a Neubauer chamber system, and a mixture of C2BBe1:HT29-MTX cells at a 70:30 ratio was seeded in collagen I-coated [10 µg/ml collagen I for 2 h at room temperature (RT); Invitrogen, Thermo Fisher Scientific] well plates at an initial cell density of 2×10^4 cells/well in a 96-well plate, 1×10^5 cells/well in a 24-well plate and 4×10^5 cells/well in a 6-well plate. IECs were cultured with regular medium exchange for 14 days post-seeding for differentiation before experimental use.

Infection of IECs

For infection experiments, IECs were cultivated in serum-free KBM. Monolayers were either colonized (pre-incubated; pre-Inc.) with lactobacilli for 18 h before infection with *C. albicans*, or bacterial and fungal cells were added simultaneously (Fig. 1E). For displacement experiments, the sequence of inoculation was reversed. In this case, IECs were infected with *C. albicans* 1, 3 or 6 h before the addition of lactobacilli. Incubation was then continued for a total infection period of 24 h. For the exact number of cells (host, lactobacilli, and *C. albicans*) and incubation periods used in the respective experiments see Table S1. In addition to the coinfection of *C. albicans* and lactobacilli, single infections were carried out as controls.

Quantification of cytotoxicity (LDH)

The influence of lactobacilli on *C. albicans*-mediated host cell damage was investigated by measuring the release of cytoplasmic LDH (Chan et al., 2013) as a proxy for loss of membrane integrity, a hallmark of necrosis (Zhang et al., 2018). LDH was quantified in the supernatant of infected IEC monolayers 24-48 h post-infection using the Cytotoxicity Detection Kit (Roche) according to the manufacturer's instructions. LDH from rabbit muscle (5 mg/ml, Roche) was used to generate a standard curve for the determination of LDH concentrations. The background control level of uninfected IECs was subtracted from the experimental LDH release and usually compared to 100% *C. albicans* single infection.

Adhesion and filamentation of *C. albicans*

Upon colonization with lactobacilli, adhesion of *C. albicans* to epithelial cells was determined 1 h post-infection (for infective doses see Table S1). Non-attached *C. albicans* cells were removed by washing twice with PBS. Samples were fixed with Histofix for 15 min at RT or o/n at 4°C and subsequently rinsed three times with PBS. Adherent fungi were stained with Calcofluor White [10 µg/ml in 0.1 M Tris-HCl (pH 9.0), Sigma-Aldrich] for 20 min at RT in the dark. After washing three times with water, samples were mounted on glass slides with ProLong mounting medium (Thermo Fisher Scientific) and analyzed using fluorescence microscopy. The number of adherent *C. albicans* cells was determined in random fields of a defined size, allowing calculation of the adhesion percentage versus inoculated *C. albicans* cells.

Hyphal length of *C. albicans* was analyzed using differential staining according to Wächtler et al. (2011), with the following minor modifications. Briefly, after 4 h of *C. albicans* infection, IECs were washed three times with PBS and fixed with Histofix. Extracellular non-invasive fungal components were stained by incubation with a primary antibody against *C. albicans* (1:2000 in PBS, rabbit anti-*Candida*, BP1006, Acris Antibodies) for 1 h at 30°C, washing three times with PBS and incubating with a secondary antibody (1:5000 in PBS, goat anti-rabbit AlexaFluor488,

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A-11008, Thermo Fisher Scientific) for 1 h at 30°C. After rinsing three times with PBS, epithelial cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 10 min at RT and washed again three times with PBS. Entire *C. albicans* hyphae were stained with Calcofluor White [10 µg/ml in 0.1 M Tris-HCl (pH 9.0)] for 20 min at RT in the dark followed by washing three times with water. Stained samples were mounted on glass slides and visualized using fluorescence microscopy. The total hyphal length was also recorded for at least 100 hyphae per condition.

Hyphal length influenced by sodium L-lactate

A *C. albicans* o/n culture was adjusted to 4×10^5 cells/ml in KBM media. Sodium L-lactate (Sigma-Aldrich) was adjusted to a concentration of 0-120 mmol/l in a total volume of 375 µl per well in a 24-well plate (see Table S1). Cells were fixed with Histofix 4 h post-infection with *C. albicans*, and analyzed using a Cell Discoverer 7 (Carl Zeiss) with a 10× magnification. At least 100 hyphae per condition were measured to estimate average hyphal length.

In vitro translocation

For measuring translocation, IECs were cultivated for 14 days on collagen I-coated transwell inserts with a 6.5 mm diameter and 5 µm pore size (Corning). Following colonization with lactobacilli, cells were infected with 2×10^4 *C. albicans* cells per transwell for 24 h at 37°C and 5% CO_2 (see Table S1). TEER values were measured using a voltmeter (World Precision Instruments) before and after colonization and 24 h post-infection. The resistance of a blank insert (120 Ω) was subtracted from each value. The translocation rate 24 h post-infection was measured using the following procedure. Zymolyase (260 U/ml in serum-free DMEM; Amsbio) was added to the lower compartment to a final concentration of 20 U/ml and incubated for 2 h at 37°C and 5% CO_2. The detached *C. albicans* hyphae were then collected and plated at appropriate dilutions on YPD agar plates. Plates were incubated at 30°C for 1-2 days until adequate growth for determining colony-forming units (CFUs) was reached.

Effects of lactobacilli cell-free supernatants

Lactobacilli were grown in MRS broth for 24 h at 37°C. The MRS-SN was prepared by centrifuging the culture at 4700 ***g*** for 5 min at 4°C, filtering through 0.22 µm filters (Millipore) and rebuffering against KBM medium within a 3K Amicon Ultra centrifugal filter device (Millipore) (final pH 7.3) according to the manufacturer's instructions. Moreover, IECs differentiated in 6-well plates were colonized with lactobacilli for 18 h. The culture supernatant (Pre-Inc. SN) was filtered through 0.22 µm filters (Millipore). Both supernatants were stored at 4°C until use in experiments the same day (see Table S1). Host cell damage induced by *C. albicans* infection in the presence of lactobacilli supernatants was measured 24 h post-infection by quantification of LDH (for format and volume see Table S1).

Glucose and lactate measurements

Glucose and lactate were measured in supernatants of the *in vitro* model (KBM media) in a 6-well format (see Table S1). We collected 120 µl of the supernatant after 0, 6, 12 and 24 h and analyzed using the Abbott Architect ci8200 Integrated System (Abbott Laboratories) according to the manufacturer's protocol. Basal levels of the KBM medium were 9 mmol/l glucose and <0.17 mmol/l lactate.

Quantification of shedding of *C. albicans* and lactobacilli – CFU determination

Shedding of *C. albicans* and lactobacilli was measured upon infection of *Lactobacillus*-colonized IECs for the respective time periods (see Table S1). Supernatants were collected and vortexed thoroughly. IECs were treated with 0.2% Triton-X-100 (Sigma-Aldrich) to lyse the host cells and release adherent fungal and bacterial cells. Supernatant and lysate samples were diluted appropriately with PBS. To follow the growth of *C. albicans*, the diluted samples were plated on YPD plates with 1× PenStrep (Gibco, Thermo Fisher Scientific) and incubated at 30°C for 1-2 days until adequate growth for determining the CFUs was reached. For lactobacilli, MRS plates with Nystatin (50 µg/ml; Carl Roth) were used for plating and incubated at 37°C and 1% O_2 for 2 days.

Scanning electron microscopy (SEM)

IECs differentiated for 14 days in a 24-well plate on glass coverslips were colonized with lactobacilli for 18 h and infected with *C. albicans* for 6 h (see Table S1). The SEM samples were fixed by incubating them in cacodylate-buffer containing 2.5% glutaraldehyde o/n at 4°C. Afterwards samples were washed three times in cacodylate buffer and dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, 100%, 100% each 15 min), followed by critical point drying in a Leica EM CPD300 Automated Critical Point Dryer. The samples were sputter-coated with platin (layer thickness 20 nm) in a CCU-010 Compact Coating Unit (Safematic). Finally, samples were analyzed at different magnifications using a Zeiss (LEO) 1530 Gemini field emission scanning electron microscope (Carl Zeiss) at 7 kV acceleration voltage and a working distance of 5 mm using an InLens secondary electron detector.

Isolation of human RNA

At the indicated time points uninfected, single infected and mixed infected IECs were harvested by removing supernatants and adding RLT buffer (Qiagen) to the host cell lawn. The cell lysates were further processed with the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Human RNA quantity was determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and RNA quality was verified with a 2100 Bioanalyzer (Agilent Technologies).

Reverse transcription quantitative real-time PCR (RT-qPCR)

We reverse transcribed 500 ng of high-quality DNase I-treated RNA samples into cDNA using oligo-dT primers and SuperScript™ III Reverse Transcriptase (Life Technologies). Subsequently, 1 µl of diluted cDNA was used for gene expression analyses with EVAGreen® qPCR Mix (Bio&Sell) and a C1000 thermocycler (Bio-Rad). Expression levels of biological triplicates were normalized to the reference genes *ACT1* and *GAPDH*. Primers used for qPCR analyses are listed in Table S2.

Mucin ELISAs

Differentiated IECs were colonized with lactobacilli and infected with *C. albicans* for 24 h (see Table S1). For the measurement of released and membrane-bound mucin proteins, supernatant samples were collected and IECs were lysed by treatment with 75 µl RIPA-buffer (Millipore) containing protease inhibitors (cOmplete Protease Inhibitor Cocktail, Roche). Supernatant and lysate samples were mixed and centrifuged at 1000 ***g*** for 10 min at 4°C. Mucin concentrations were quantified using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Human MUC 2, 3, 4, 13 and 17, ELISA Kit, DlDevelop; Human MUC5AC, ELISA Kit, Elabscience).

Apoptosis

Differentiated IECs were colonized with lactobacilli or not and infected with *C. albicans* or not (see Table S1). Simultaneously with *C. albicans* infection, IECs were stained for phosphatidyl serine expression using annexin V (pSIVA™ Real-Time Apoptosis Kit; Bio-Rad) and caspase 3/7 activity (CellEvent™ Caspase 3/7 detection reagent; Invitrogen, Life Technologies) according to the manufacturer's instructions. Apoptosis was followed by live-cell imaging of the fluorescence at an excitation maximum of 488 nm (annexin V or caspase 3/7) every 1 h using a Cell Discoverer 7 (Carl Zeiss) with a 10× magnification. Images were processed using the Fiji software (ImageJ). After conversion to binary images, the total area of positive cells was determined using the Particle Analyzer tool. To inhibit apoptosis, the caspase 3/7 inhibitor 1 MMPSI (Abcam) dissolved in ethanol with a final concentration of 50 µM was used.

Quantification of shedding of apoptotic cells

Differentiated IECs were colonized with lactobacilli or not and infected with *C. albicans* or not (see Table S1). Supernatants were collected, placed in a new 24-well plate and stained for caspase 3/7 activity (CellEvent™ Caspase 3/7 detection reagent; Invitrogen), subsequently the entire wells were

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imaged at an excitation maximum of 488 nm at 10× magnification in a Cell Discoverer 7 (Carl Zeiss). Images were processed using the Fiji software (ImageJ) and cells were quantified using the Particle Analyser tool.

Western blot analysis

Differentiated IECs were colonized with *L. rhamnosus* or *L. brevis* and infected with *C. albicans* for 6, 12 and 24 h (see Table S1). Following incubation, supernatants were removed and cells were scraped off in PBS followed by centrifugation for 1 min at 500 ***g***. The pellet was resuspended in 100 µl modified RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS] containing protease inhibitors (cOmplete Protease Inhibitor Cocktail, Roche) and phosSTOP phosphatase inhibitor cocktail tablets (Roche). Lysates were cleared by centrifugation for 5 min at 20,000 ***g*** at 4°C and protein concentration was quantified using a BCA assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Diluted protein extracts were denatured in 1× Laemmli Sample Buffer [125 mM Tris-HCl (pH 6.8), 50% glycerol, 4% SDS, 0.02% Bromphenol blue, 0.1% 14 M 2-mercaptoethanol] for 5 min at 95°C and centrifuged for 5 s at 5000 ***g***. For SDS-PAGE, 30 µg of sample was separated on 10% SDS PAGE gels using a Mini-PROTEAN® Tetra Cell system (Bio-Rad) and separated proteins were transferred to a nitrocellulose membrane (Amersham™ Proton™ 0.45 µM NC, GE Healthcare). The membranes were blocked with 5% I-Block protein-based blocking reagent (Life Technologies), solved in TBS-T [50 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 0.05% Tween-20] and then incubated with primary antibodies E-cadherin (1:500, goat anti-human, AF648, R&D Systems) and GAPDH (1:500, rabbit anti-human, NB300-327, Novus) o/n at 4°C. After washing three times with TBS-T, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (E-Cadherin: anti-goat, 1:2000, SC-2020, Santa Cruz Biotechnology; GAPDH: anti-rabbit, 1:500, P0217, Dako) followed by three washing steps. Immunoreactivity was detected using enhanced chemiluminescence (ECL Plus Western Blotting Substrate, Thermo Fisher Scientific). Coomassie staining of membrane or gel was used to ensure equal loading of supernatant samples to the gel (not shown).

Statistical analyses

Experiments were performed in biological triplicates ($n \geq 3$) unless otherwise stated. Data are mean of biological replicates±s.e.m. Data were analyzed using GraphPad Prism 7. For significance testing, lognormal ratio values were log transformed and tested against deviation from zero using a two-tailed *t*-test or by means of a one-way analysis of variance (ANOVA) test with a follow-up test for multiple comparisons (Tukey's correction). Other values were tested using two-tailed *t*-tests against the reference condition. Normality of individual distributions were ascertained by Shapiro-Wilk normality tests (threshold $P>0.05$) before claiming significance by *t*-tests, one-way ANOVA or two-way ANOVA. Statistical significance is indicated in the graphs: $*P \leq 0.05$, $**P \leq 0.01$ or $***P \leq 0.001$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.G., A.L., B.H.; Methodology: K.G., A.L., R.G., S.A., S.L., M.W.; Software: S.L., M.W., M.S.G.; Validation: A.L.; Formal analysis: K.G., A.L., R.G., S.A., M.G., A.S.M., M.S.G.; Investigation: K.G., A.L., R.G.; Resources: B.H.; Writing - original draft: K.G., A.L.; Writing - review & editing: K.G., S.A., A.S.M., M.S.G., B.H.; Visualization: K.G., A.L.; Supervision: K.G., M.S.G., B.H.; Project administration: K.G., B.H.; Funding acquisition: B.H.

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Supplementary information

Supplementary information available online at
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Supplement

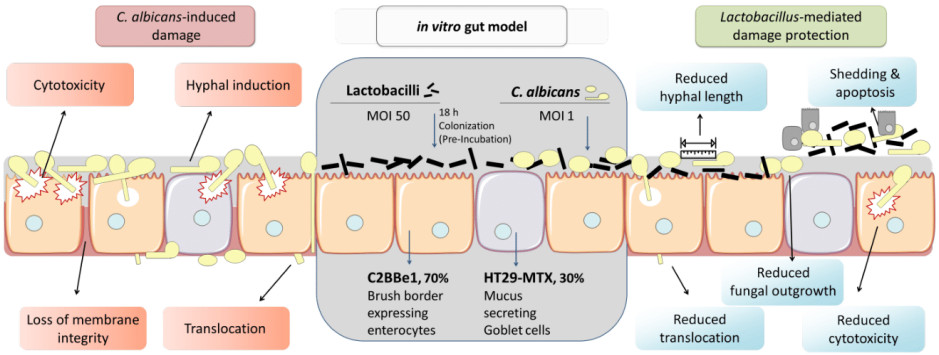

Figure S1: graphical abstract to visualize the written abstract.

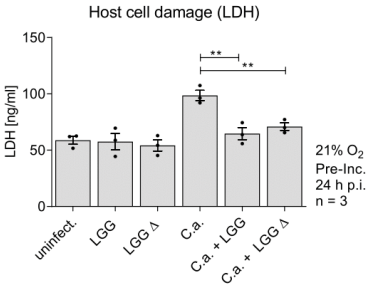

Figure S2: The protective effect of *L. rhamnosus* is independent of exopolysaccharides. LDH release at 24 h post-infection of IECs left untreated, colonized either with the *L. rhamnosus* GG mutant ((LGGΔ) lacking exopolysaccharides), or the corresponding WT strain (LGG) and infected with *C. albicans* (MOI 1) or not. The results were normalized to *C. albicans* single infection. Results shown are the mean values ± SEM, $*p < 0.05$, $***p < 0.005$.

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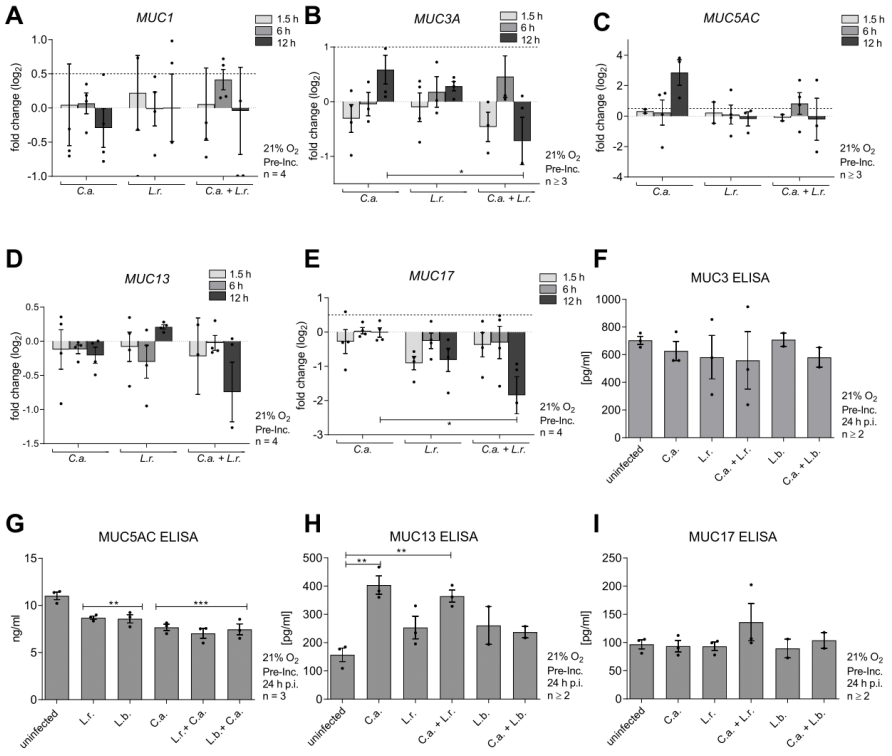

Figure S3: The protective effect of lactobacilli does not correlate with altered mucus secretion. The relative mRNA expression of (A) *MUC1*, (B) *MUC3A*, (C) *MUC5AC*, (D) *MUC13*, and (E) *MUC17* in IECs either left untreated or colonized with *L. rhamnosus* (MOI 50) and infected or not with *C. albicans* (MOI 1) for 1.5, 6, or 12 h. Expression levels were normalized to the reference genes *ACT1* and *GAPDH*. Results shown are the mean values ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.005. (F-I) Protein concentrations of (F) MUC3, (G) MUC5AC, (H) MUC13, and (I) MUC17 quantified in whole cell lysate of IECs left untreated or colonized with lactobacilli (*L. rhamnosus* or *L. brevis*; MOI 50) and infected or not with *C. albicans* for 24 h. Results shown are the mean values ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.005 compared to uninfected host cells.

Disease Models & Mechanisms: doi:10.1242/dmm.039719: Supplementary information

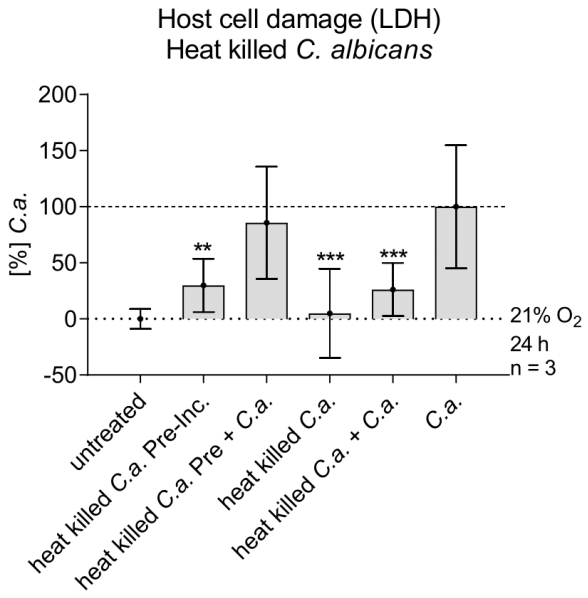

Figure S4: Protective effect through high biomass of heat-killed *C. albicans*. LDH release at 24 h post-infection of IECs colonized or not with heat-killed *C. albicans* (MOI 100) and infected with *C. albicans* (MOI 1) or not. The results were normalized to *C. albicans* single infection.

Disease Models & Mechanisms: doi:10.1242/dmm.039719: Supplementary information

Experiment	*Format*	*initial No of IECs/well (volume)*	*CFU C.a./well (volume)*	*CFU lactobacilli/well [excess compared to C.a.] (volume)*	*total medium*	*incubation period*
Cytotoxicity (LDH assay)	96 well plate	2×10^4 (50 µl)	2×10^4 (50 µl)	1×10^5 [5x] (50 µl) 1×10^6 [50x] (50 µl) 5×10^6 [250x] (50 µl)	150 µl	24 or 48 h
Adhesion of *C. albicans*	24 well plate coverslips	1×10^5 (125 µl)	5×10^4 (125 µl)	2.5×10^6 [50x] (125 µl)	375 µl	1 h
Hyphal length	24 well plate coverslips	1×10^5 (125 µl)	5×10^4 (125 µl)	2.5×10^6 [50x] (125 µl))	375 µl	4 h
Translocation, TEER	transwell inserts	2×10^4 (50 µl)	2×10^4 (50 µl)	1×10^6 [50x] (150 µl)	250 µl	24 h
cell-free supernatants	96 well plate	2×10^4 (0 µl)	2×10^4 (10 µl)	- undiluted SN 140 µl	150 µl	24 h
killed lactobacilli (pre-incubation)	96 well plate	2×10^4 (50 µl)	2×10^4 (50 µl)	1×10^6 [50x] (50 µl)	150 µl	24 h
killed lactobacilli (simult. infection), displacement	96 well plate	2×10^4 (50 µl)	2×10^4 (50 µl)	1×10^6 [50x] (50 µl) 5×10^6 [250x] (50 µl) 1×10^7 [500x] (50 µl)	150 µl	24 h
Growth of lactobacilli in IECs	24 well plate	1×10^5 (125 µl)	5×10^4 (0 µl)	2.0×10^3 [-10x] (1000 µl]	1000 µl	24 h
Western Blot	6 well plate	4×10^5 (1300 µl)	4×10^5 (1300 µl)	2.0×10^7 [50x] (1300 µl)	3900 µl	6 h, 12 h, 24 h
Glucose- and Lactate measurement	6 well plate	4×10^5 (1300 µl)	4×10^5 (1300 µl)	2.0×10^7 [50x] (1300 µl)	3900 µl	0 h, 6 h, 12 h, 24 h
C.a. shedding	6 well plate	4×10^5 (1300 µl)	4×10^5 (1300 µl)	2.0×10^7 [50x] (1300 µl)	3900 µl	1 h, 3 h, 6 h, 24 h
SEM	24 well plate coverslips	1×10^5 (125 µl)	5×10^4 (125 µl)	2.5×10^6 [50x] (125 µl)	375 µl	6 h
ELISA	24 well plate	1×10^5 (125 µl)	5×10^4 (125 µl)	2.5×10^6 [50x] (125 µl)	375 µl	24 h
RNA isolation	6 well plate	4×10^5 (1300 µl)	4×10^5 (1300 µl)	2.0×10^7 [50x] (1300 µl)	3900 µl	1.5 h, 6 h, 12 h
Apoptosis	96 well plate	2×10^4 (50 µl)	2×10^4 (50 µl)	1×10^6 [50x] (50 µl)	150 µl	0 h-24 h

Table S1: Overview of the exact number of host cells, *Lactobacillus* species, *C. albicans*, and incubation periods used in the respective experiments.

target gene	sequence	target gene	sequence
β-ACTIN	F: AAATGCTTCTAGGCGGACTAT R: AAGGGACTTCCTGTAACAACG	*MUC13*	F: GAGCTTGCAACCTAGCCTCA R: AGAGTGCACCCCATAGTGGA
GAPDH	F: TTGCCCTCAACGACCACTTT R: GGTGGTCCAGGGGTCTTACT	*MUC17*	F: TTGTCAGCACCACACTTCCA R: GGCAGTGTCAGTAGAAGGGG
MUC1	F: CAGTGCCGCCGAAAGAACTA R: TAGGGGCTACGATCGGTACT	*cFOS*	F: GGAGGAGGGAGCTGACTGAT R: GCTGCCAGGATGAACTCTAGT
MUC2	F: CACTGCCCTCGACAGCTTTA R: TGGAAAGCAAGGACTGAACAA	*DUSP1*	F: CTGCAGTTTGAGTCCCAGGT R: AGACGGGGAAGTTGAACACG
MUC3A	F: TCACATCCTGGTCCCTAGCA R: TGAATGGACGGGCTCAGAAG	*DUSP5*	F: TGGAAGACAGCCACACGG R: GAGATCCCAGCCTCACAGTG
MUC4	F: CAGCTCCTGGGCTGAACATT R: CTCGTGTGAAGTCCGATGCT	*NFκB-IA*	F: ACCTCACCTTTGTGGGGTTT R: ACAGGATACCACTGGGGTCA
MUC5AC	F: GTACTCGCTCGAGGGCAACA R: TTCCACCTCGGTGTAGCTGA		

Table S2: Primers used for gene expression analyses

2.3. Manuscript III: Last *et al*., Manuscript in preparation, 2021

Intestinal epithelial cells foster bacteria that antagonize *C. albicans* pathogenicity through metabolic and transcriptional reprogramming (Manuscript in preparation)

Antonia Last, Mohammad H. Mirhakkak, Raquel Alonso-Roman, Jakob Sprague, Peter Großmann, Katja Graf, Rena Gratz, Gianni Panagiotou, Slavena Vylkova, Sascha Schäuble, Bernhard Hube, Mark S. Gresnigt

Summary:

Lactobacillus rhamnosus antagonizes *Candida albicans* by inducing shedding and thereby reducing the number of *C. albicans* cells in contact with the host cell surface. However, similar *C. albicans* cell numbers in contact with the intestinal epithelium cause more damage in the absence of *L. rhamnosus* as compared to co-cultures. Therefore, the reduced damage in the presence of *L. rhamnosus* must be attributed to additional molecular mechanisms. Furthermore, the antagonistic activity of *L. rhamnosus* requires the presence of intestinal epithelial cells. Transcriptional and metabolic analyses and *in silico* metabolic modeling followed by *in vitro* validation experiments showed that epithelial cells support growth of *L. rhamnosus* by providing metabolites consumed by the bacteria. In turn, *L. rhamnosus* produces potential antifungal metabolites and its presence causes metabolic and transcriptional reprogramming and the repression of genes of *C. albicans* that are essential for the full damage capacity of the fungus.

Own contribution:

Antonia Last structured the paper. She performed damage and growth assays, RNA isolation, microarrays, and prepared the samples for the metabolome study. Antonia coordinated the work with the cooperation partners. She wrote the paper draft and generated several figures.

Estimated authors' contributions:

Antonia Last	33 %
Mohammad H. Mirhakkak	10 %
Raquel Alonso-Roman	10 %
Jakob Sprague	4 %
Peter Großmann	1 %
Katja Graf	1 %
Rena Gratz	1 %
Gianni Panagiotou	1 %
Slavena Vylkova	1 %
Sascha Schäuble	10 %
Bernhard Hube	6 %
Mark S. Gresnigt	22 %

Intestinal epithelial cells foster bacteria that antagonize *C. albicans* pathogenicity through metabolic and transcriptional reprogramming

Key words: *Candida albicans*, *Lactobacillus rhamnosus*, fungal-host interactions, transcriptome, metabolome, *in silico* genome scale metabolic modelling

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Abstract

Removal or imbalance of the intestinal bacterial microbiota by antibiotic treatment can initiate fungal overgrowth – a significant predisposing factor for disseminated candidiasis. *Lactobacillus rhamnosus* is a well-characterized species of bacteria known for their ability to antagonize the pathogenicity of *Candida albicans*, the most frequent cause of disseminated candidiasis.

Using an *in vitro* gut model, we dissected the complex interplay between *C. albicans*, *L. rhamnosus,* and intestinal epithelial cells (IECs). In this scenario, *L. rhamnosus* relies on IECs to sustain its growth, which is a prerequisite for its protective potential. By applying transcriptional and metabolic profiling combined with *in silico* metabolic modelling, we thus investigated the molecular mechanisms by which IECs support bacterial proliferation and by which *L. rhamnosus* protects IECs.

In silico analyses and metabolomics showed that *L. rhamnosus* cells, supported by IECs, mediate protection *via* modifications of the metabolic environment. This includes a shift from energetically favored to less favored carbon sources for *C. albicans*. Accordingly, *C. albicans* altered the expression of metabolic genes and downregulated several genes (*AHR1, ACE2, PTP3, PRN4, RIM13, and orf19.4292*) that are required for virulence. Moreover, we identified bacterial metabolites which antagonize *C. albicans* suggesting that combined and interwoven activities of antagonistic bacteria and ICEs are required to counteract the virulence potential of *C. albicans*.

Introduction

Candia albicans is usually a harmless commensal on mucosal surfaces of humans. However, predisposing conditions can favor superficial infections on mucosal surfaces. In severe cases, the fungus can cause invasive, disseminated infections, mostly *via* translocation from the intestine into the bloodstream [1,2]. In healthy individuals, this translocation is prevented by the epithelial barrier, the immune system, and the commensal microbiota [3,4]. Bacteria of the genera *Bifidobacterium* and *Lactobacillus* have been studied extensively concerning their antagonistic effects against *C. albicans* [5,6]. *Lactobacillus* species are well known for their protection and application against vulvovaginal candidiasis [7-9]. Their protective potential was also demonstrated *in vitro* on oral [10] and intestinal [11,12] epithelial cells. Underlying mechanisms for this protection are the reduction of *C. albicans* proliferation [7,11,13,14], hypha formation [11,15-17], and biofilm formation [13,18]. This is associated with reduced adhesion to, invasion into, and damage of epithelial cells by the fungus [11]. Secreted metabolites from gut bacteria, such as short-chain fatty acids (SCFAs; e.g. lactic acid, acetic acid, butyric acid), possess health-promoting properties [19,20] and potentially attenuate *C. albicans* virulence [21,22]. Antibiotic treatment, causing a reduction in bacterial cell numbers and a reduced variety of bacterial species, has been demonstrated to lower the levels of SCFAs, which contributes to increased *C. albicans* colonization rates in mice [21]. Similarly, antibiotic treatment or a diet that is unfavorable for antagonistic bacteria like *Lactobacillus* species were shown to promote *C. albicans* colonization in mice [23]. *Lactobacillus* species also indirectly affect *C. albicans* through modulation of proinflammatory cytokine responses [24-27]. Previously, we dissected the *C. albicans*-antagonizing effects of *L. rhamnosus* in two independent *in vitro* models. *L. rhamnosus* colonization prevented *C. albicans* outgrowth on intestinal epithelial cells (IECs) and reduced the number of host cell associated *C. albicans* through shedding [11]. These findings were validated in a near-physiological intestine-on-chip-model, where *L. rhamnosus* colonization reduced the fungal burden in the “intestinal lumen” and host cell damage, resulting in improved epithelial barrier integrity and reduced fungal translocation into a bloodstream-like compartment [12].

The observed protective effects were proposed to depend on a reduced contact of fungi with host cells, which consequently are incapable of invading and damaging the IECs [11]. However, the underlying molecular mechanisms driving the reduced growth and shedding of *C. albicans* remain unresolved. Furthermore, it is unclear whether a reduced number of *C. albicans* cells

in contact with the epithelium is sufficient to mediate protection or whether additional molecular mechanisms are at play in such a scenario. Here, we investigated the protection mediated by *L. rhamnosus* on a molecular level *via* multi-omics profiling and *in silico* metabolic modeling. This included transcriptional profiling of the fungus and investigating the metabolic cross-talk between host cells, *L. rhamnosus*, and *C. albicans* to elucidate the role of the metabolic environment in the protection against *C. albicans*-induced damage.

Results

Reduced host-cell associated C. albicans is insufficient for damage protection

We recently showed that *L. rhamnosus* not only reduced the proliferation of *C. albicans* in an intestinal epithelial infection model but also physically separated the fungus from host epithelial cells through shedding [11]. As a consequence, reduced numbers of *C. albicans* are in contact with the epithelial barrier and reduced invasion of the tissue occurs. To investigate whether a reduced number of *C. albicans* cells associated with IECs would be sufficient to substantially prevent damage, we performed infections of IECs with a reduced *C. albicans* inoculum ($1x10^4$ cell/ml) alone (without lactobacilli) to match the number of host-associated fungi remaining 24 hours post infection (hpi) in the presence of *L. rhamnosus* **(Fig.1A)**. This reduced fungal burden in the absence of *L. rhamnosus* did not result in a significant damage reduction **(Fig.1B)**, showing that reduced numbers of host-cell associated *C. albicans* alone are insufficient to mediate protection.

L. rhamnosus modulates transcriptional reprogramming of C. albicans

Next, we aimed at elucidating the potential molecular mechanisms associated with *L. rhamnosus*-mediated protection. We performed transcriptional profiling of *C. albicans* during infection to investigate differential regulation of genes in the presence of *L. rhamnosus* that may explain the observed protective effects. Unsupervised hierarchical clustering **(Fig.2A)** and principal component analysis (PCA) **(Fig.2B)** of the expression profiles display a distinct gene expression pattern depending on the presence of *L. rhamnosus* at both 6 and 24 hpi. At 6 hpi, 947 genes were differentially expressed in response to the presence of *L. rhamnosus*; among them, 486 (51.3 %) were downregulated and 461 (48.7 %) were upregulated **(Fig.2C)**.

At 24 hpi a total of 541 differentially expressed genes (DEGs) were identified with 308 (56.9 %) downregulated genes and 233 (43.1 %) upregulated genes in response to the presence of *L. rhamnosus* **(Fig.2D)**. The analysis of overlapping gene sets revealed only a limited overlap of the up- and downregulated genes at different time points. Only 9 genes are upregulated and 13 genes are downregulated at both time points. In contrast, increased expression at 6 hpi with decreased expression at 24 hpi and *vice versa* were observed for 77 and 39 genes, respectively **(Fig.2E)**. Overall, this demonstrates a clear bacterial influence on the overall transcriptional profiles of the fungus during IEC colonization.

Antibiotics suppress the L. rhamnosus-induced transcriptional changes in C. albicans

Previously we observed that *L. rhamnosus* has to be physically present during infection and alive to protect IECs against *C. albicans* induced damage [11]. Unsupervised hierarchical clustering **(Fig.2F)** and PCA **(Fig.2G)** analysis revealed that killing of *L. rhamnosus* with antibiotics (Gentamicin, Streptomycin, Penicillin) at 4 hpi strongly influenced *C. albicans*' gene expression pattern as compared to fungal cells exposed to untreated *L. rhamnosus*. Likewise, damage inflicted to IECs was induced when *L. rhamnosus* cells were treated with antibiotics **(Fig.2H)**, underlining that the presence of live and metabolically active *L. rhamnosus* mediates the protective effect and that the observed transcriptional changes with live *L. rhamnosus* are likely to play a role in the observed protection.

Mutant screen to identify growth- and virulence-relevant DEGs of C. albicans

We assumed that genes differentially regulated in response to live *L. rhamnosus* may be critical for the level of damage caused by *C. albicans*. We thus screened mutants from a *C. albicans* gene deletion mutant library [28] lacking corresponding genes for their growth and damage potential. Out of the 70 selected mutants, four (*pga7Δ/Δ*, *prn4Δ/Δ*, *ptp3Δ/Δ*, *ywp1Δ/Δ*) showed reduced growth in KBM (Keratinocyte basal medium) **(Fig.3A)**, seven (*prn4Δ/Δ*, *kre5Δ/Δ*, *rim13Δ/Δ*, *ptp3Δ/Δ*, *orf19.4292Δ/Δ*, *ahr1Δ/Δ*, *ace2Δ/Δ*) were attenuated in IEC damage potential, and 14 (*ypt7Δ/Δ*, *aro80Δ/Δ*, *orf19.4459Δ/Δ*, *hyr1Δ/Δ*, *ycp4Δ/Δ*, *opt7Δ/Δ*, *fcr1Δ/Δ*, *cyb5Δ/Δ*, *rbe1Δ/Δ*, *pdk2Δ/Δ*, *orf19.7328Δ/Δ*, *pwp1Δ/Δ*, *zcf27Δ/Δ*, *rgs2Δ/Δ*) showed a hyper-damaging phenotype **(Fig.3B)**. The comparison of gene expression, growth,

and induced host cell damage **(Fig.3C)** shows a clear correlation between the mutants with a reduced damage capacity and downregulation of the corresponding genes at 6 hpi (exception: *kre5Δ/Δ*), and two (*prn4Δ/Δ*, *ptp3Δ/Δ*) showed reduced growth in KBM. Conversely, nine of the 14 hyper-damaging mutants corresponded to genes that were significantly upregulated at 6 hpi by *L. rhamnosus,* which could hint towards induction of potential anti-virulence genes [29]. Collectively, this supports the hypothesis that colonization with *L. rhamnosus* alters the expression of *C. albicans* virulence and anti-virulence genes.

L. rhamnosus induces C. albicans metabolic reprogramming on a transcriptional level

To identify further processes associated with protection, the transcriptional response of *C. albicans* to the presence of *L. rhamnosus* was investigated globally by searching for common molecular functions represented by enriched GO-terms. Upregulated genes at 6 hpi revealed terms associated to transport-, aggregation-, and response mechanisms **(Fig.4A),** while the downregulated GO-terms consisted of a wide range of metabolic processes **(Fig.4B)**; suggesting a metabolic response to the presence of *L. rhamnosus* and potentially related environmental metabolic changes. While several metabolic processes were downregulated 6 hpi, the opposite was observed at 24 hpi **(Fig.4C)**. Nevertheless, genes involved in carbohydrate utilization and metabolism were downregulated at 24 hpi **(Fig.4D).** This could hint towards a limited carbohydrate availability, which forces *C. albicans* to use alternative carbon sources and induce adaptive metabolic processes during infection in the presence of *L. rhamnosus.*

Metabolic interactions between C. albicans, L. rhamnosus, and IECs

The metabolome of the supernatants in the model was characterized to obtain a detailed insight into the complex metabolic interplay between *C. albicans*, *L. rhamnosus,* and IECs. The supernatant of all members alone and in combination with each other was analyzed using untargeted metabolomics 6 and 12 hpi. The supernatant metabolic profile slightly differed between uninfected IECs and *C. albicans*-infected IECs **(Fig.5A I)**. In line with the transcriptional data, clear differences were seen depending on the presence or absence of *L. rhamnosus* on IECs, pointing towards a substantial influence of the bacterial colonization on

the metabolic environment **(Fig.5A II)**. Interestingly, in the presence of *L. rhamnosus* the metabolic profile was indistinguishable between *C. albicans* infected and uninfected IECs **(Fig.5A III)**. The unsupervised hierarchical clustering of the metabolic profiles allowed us to characterize distinct clusters of metabolites in the model at 6 hpi **(Fig.5B, Tab.S1A, Tab.S1B)** and 12 hpi **(Fig. S1, Tab.S2A, Tab.S2B)**, which associate with specific roles**.** The metabolites in cluster 4 at 6 hpi **(Fig.5B, Tab.S1A)** were present in the medium, not consumed by *C. albicans* or *L. rhamnosus*, but consumed when IECs were in the model. Cluster 2 and 3 represent metabolites being released by IECs **(Tab.S1A)**. The metabolites in cluster 3, however, disappeared during *L. rhamnosus* colonization. Since *L. rhamnosus* relies on IECs to sustain its growth in KBM [11], these metabolites may nourish *L. rhamnosus* and allow proliferation on IECs. Metabolites in cluster 7 were present in the medium but not consumed in any situation, except when *L. rhamnosus* colonized IECs **(Tab.S1B)**. These metabolites may be expected to be only consumed when *L. rhamnosus* is actively growing on IECs. Similar clusters have been identified 12 hpi **(Fig. S1, Tab.S2A, Tab.S2B)**. The metabolites in cluster 8 were secreted by *L. rhamnosus* independent of whether they are growing on IECs or not, whereas the metabolites in cluster 1 were exclusively present when IECs and *L. rhamnosus* are cultured together. The metabolites specifically produced (cluster 1) or consumed by *L. rhamnosus* on IECs (cluster 3) may play a role in modifying the metabolic environment that forces transcriptional adaptation in *C. albicans*.

Intestinal epithelial cells foster L. rhamnosus growth

L. rhamnosus growth is essential for its protective potential, yet it cannot grow in KBM medium unless IECs are present, which suggests that essential metabolites are absent in KBM. *L. rhamnosus* cells in transwells physically separated from IECs were still able to grow **(Fig 5C)**, indicating that epithelial-mediated changes in the metabolic environment may be responsible. Likewise, KBM medium conditioned by IECs supported *L. rhamnosus* growth to similar rates as the control in direct contact with IECs **(Fig. 5C)**, supporting the hypothesis that the IECs nourish *L. rhamnosus* through the release of essential metabolites. Thus, the metabolites that are released by IECs and disappear as soon as *L. rhamnosus* is in the model (cluster 3 6 hpi **(Fig. 5B, Tab.S1A),** cluster 4 12 hpi **(Fig. S1, Tab.S2A)** may be the metabolites that support bacterial growth. By investigating biomass formation with flux balance analysis [30], *in silico*

simulations of the genome-scale metabolic modeling of *L. rhamnosus* predicted that this bacterium can grow on IEC-conditioned media, while there is no theoretical growth rate on KBM media alone **(Fig.5D)**. Further, *in silico* analysis of the pathway activity levels revealed a strong influence of IECs on *L. rhamnosus* metabolism, especially at 12 hpi **(Fig. 5E)**.

Different types of metabolites, including sugars, amino acids, and fatty acids were represented in the metabolomics analysis (**Fig. 5B**; cluster 3) of potential nourishing metabolites **(Tab.S1A)**. From each type of metabolite, a representative was selected to study the effect on *L. rhamnosus'* growth. Fructose, xanthine, nicotinamide, nicotinic acid, 3-methyl-2-oxobutyrate, 3-methyl-2-oxovalerate, N-acetylgalactosamine as well as amino acids were unable to promote *L. rhamnosus* growth individually **(Fig.5F)**. Supplementation of the metabolites carnitine, fatty acids, and citric acid slightly supported *L. rhamnosus* growth **(Fig. 5F)**. However, the combination of citric acid + gamma-glutamylalanin or citric acid + carnitine supported *L. rhamnosus* growth in unconditioned KBM medium to a similar extent as IEC-conditioned KBM medium **(Fig. 5G).** The addition of all three metabolites (citric acid, gamma-glutamylalanin, and carnitine) exceeded the control growth rate, validating that the release of these metabolites by IECs were likely responsible for fostering *L. rhamnosus* growth.

L. rhamnosus induces an unfavorable metabolic environment for C. albicans pathogenicity

The *in vitro* results that showed reduced *C. albicans* growth in the presence of *L. rhamnosus* [11] were also reflected by *in silico* flux balance analysis [30] of the genome-scale metabolic model of *C. albicans* **(Fig. S2A)**. We additionally simulated reconstructed genome-scale metabolic models (GEMs) in IECs or *L. rhamnosus* [31,32], to predict metabolic secretion or uptake of metabolites alongside biomass production by flux variability analysis (FVA) [33]. The simulated media consisted of metabolites available in the supernatant of IECs. FVA allows determining feasible metabolic reaction fluxes that support the yield of a target reaction, e.g. biomass formation. We cross-checked these predictions with available phenotypic growth data of *C. albicans* [34] and metabolomics fold changes for different conditions **(Fig.S2B).** Interestingly**,** our data show reduced growth of *C. albicans* with amino acids as sole carbon sources. In contrast, the fungus grows better on amino acids, when provided as the sole nitrogen source, which might be due to the additional presence of glucose as carbon source in the phenotypic microarray data [34]. Except of histidine and glycine, amino acids were significantly less

abundant compared to blank media. *In silico* GEM based analyses predicted that these molecules can be taken up and also, at least in parts, secreted by IECs or *L. rhamnosus* cells in the majority of cases **(Fig.S2B)**. Alternative carbon sources like lactate, glycerol, or malate were also predicted to be both secreted and taken up by IECs and *L. rhamnosus* cells and were highly abundant in media with IECs or *L. rhamnosus* **(Fig.S2B)**. Here, the lactate concentration was higher, while the glycerol concentration was lower in IEC supernatants with *L. rhamnosus* than in IEC supernatants without bacteria. Interestingly, the additional presence of *C. albicans* with IECs and *L. rhamnosus* resulted in low fold changes for lactate and increased concentrations of glycerol **(Fig.S2B)**. *L. rhamnosus* was predicted to be capable of secreting succinate **(Fig S2B)**, an important intermediate of the TCA cycle, which is beneficial to *C. albicans* growth according to the phenotypic microarray data, but is also the reduction product of fumarate, a common terminal electron acceptor for anaerobic respiration in the CO_2 rich gut environment [35]. This metabolite was present at higher levels in supernatants of IECs and *L. rhamnosus* **(Fig S2B)**, hinting that *C. albicans* might make use of this abundant metabolite in the gut environment if more favorable carbon resources are not present or depleted. Next to carbon compounds, nucleotides, particularly cytosine, also showed an interesting abundance pattern. As shown by phenotypic microarray analysis, *C. albicans* grows better when cytosine is available as a nitrogen source compared to the respective negative control and is substantially more abundant in supernatants of both IECs and *L. rhamnosus*. These data suggest that IECs take advantage of the increased cytosine concentration presumably provided by *L. rhamnosus*. The *in silico* analysis, however, predicted that IECs can take up cytosine, but cannot secrete it. This suggests that cytosine is essential for reaching an optimal *in silico* biomass formation, which prevents the production of any excess cytosine levels. Taken together, our complementary *in silico* and metabolomics analyses show that several metabolites can be secreted and potentially "presented" as alternative carbon or nitrogen sources by either IECs and/or *L. rhamnosus* to *C. albicans*, which in turn is forced into a suboptimal growth milieu.

In silico GEM analyses can also provide clues on the activity of individual metabolic pathways such as glycolysis, amino acid or nucleotide pathways, and thus the potential mechanism underlying a metabolic shift. We investigated the GEMs used for IECs and *L. rhamnosus* as well as a recently reconstructed GEM for *C. albicans* [34] for flux shifts in metabolic pathways across different conditions (**Fig.5E; Fig.S3**). We screened for pathways with a notable number of

metabolic reactions that show a shifted feasible flux range when simulated on different metabolome profiles by FVA analysis (**Fig5E; Fig.S3**). When comparing pathway activity across conditions for all three GEMs, we identified *L. rhamnosus* to change a particularly high number of metabolic pathways as soon as IECs contributed to the simulated growth media based on the respective supernatant **(Fig.5E)**. Altered pathway activity levels like pyrimidine or purine synthesis, or different amino acid metabolic pathways hint to an increased activity of growth relevant pathways. In contrast, we did not observe substantially altered metabolic pathway activity in IECs, showcasing that *L. rhamnosus* might change its metabolic activity as soon as IECs provide an available metabolite pool. Nevertheless, several pathways show subtle activity changes and comprise carbon metabolic pathways like the pentose phosphate pathway, amino acid, or fatty acid metabolism, hinting to a subtle change in the metabolism of IECs once *C. albicans* potentially impacts the available metabolite pool **(Fig. S3)**. Interestingly, simulations of the *C. albicans* GEM also predict subtle to moderate changes in carbon metabolite pathways, like the TCA cycle, but also in many amino acid and fatty acid relevant metabolic pathways **(Fig.S3).** Oxidative phosphorylation was substantially altered in the *C. albicans* GEM, if simulated on spent medium from IECs. The additional presence of *L. rhamnosus* also impacts the oxidative phosphorylation predicted activity for *C. albicans*, hinting that energy metabolic processes might be perturbed in the fungus as soon as *L. rhamnosus* colonizes the IECs.

In summary, these data predict that integral carbon utilization pathways are affected. To investigate this in more detail predictions for central carbon and energy metabolic pathways, including glycolysis, the pentose phosphate pathway, the TCA cycle, and oxidative phosphorylation were crosschecked against available data for metabolite availability and transcriptomic expression of *C. albicans* in the presence of IECs and the optional presence of *L. rhamnosus* **(Fig. 6)**. The *in silico* analysis based on the availability of metabolites at 12 hpi predicted less glycolytic activity of *C. albicans* in the presence of *L. rhamnosus*. This is in line with the downregulation of corresponding genes at 24 hpi in the fungus, showing a sustained shift in glycolytic activity. Interestingly, the pentose phosphate pathway was also predicted to have decreased activity at 12 hpi. However, the gene expression profile at 24 hpi revealed an upregulation of the corresponding genes, suggesting a counter-reaction by the fungus initiated to compensate for e.g. decreased amounts of energy co-factors such as NADH. *In silico* prediction also reflects most parts of the available transcriptional and metabolic data

concerning the TCA cycle. Our untargeted metabolome data shows low levels of the metabolites citrate and isocitrate when *L. rhamnosus* is also present. In contrast, TCA cycle components show increased concentration profiles on the metabolomic and transcriptomic levels after isocitrate, the branching point to the glyoxylate shunt, a two-step metabolic pathway (isocitrate lyase, and malate synthase) that serves as an alternative route in the TCA cycle. This is also reflected by the *in silico* analysis except for the succinate dehydrogenase, which might be due to its additional role in the electron transport chain. Finally, several parts of the oxidative phosphorylation pathway were predicted to be less active in the presence of *L. rhamnosus*. This is supported by the available omics data, where particularly the transcriptomic data for 24 hpi hint that later stages of the oxidative phosphorylation are downregulated, which might be a reaction towards potentially critical low levels of NADH that fuels complex I, the entry point to the electron transport chain **(Fig. 6)**. This again supports the hypothesis of a perturbed energy metabolism, which potentially inhibits fungal pathogenicity.

L. rhamnosus secretes metabolites that antagonize C. albicans pathogenicity

To assess the protective effect of *L. rhamnosus* independently of contact with *C. albicans*, bacteria were separated from IECs and *C. albicans* using a transwell system. Cytotoxicity measurements showed that *L. rhamnosus* reduced *C. albicans* pathogenicity contact-independently **(Fig. 7A)**, which suggests that the effect is mediated by metabolic changes in the environment. When *L. rhamnosus* was grown on IEC-specific metabolites, metabolites with potential *C. albicans* perturbing effects were identified **(**cluster 1 **Fig.5B**, **Tab.S1A)**. Several of the metabolites secreted by *L. rhamnosus* on IECs like phenyllactic acid [36], mevalonolactone [37], 2-hydroxyisocaproic acid (HICA) [38,39], and 3-hydroxyoctanoate [40], are known to have antifungal potential.

Additionally, a screening of some of the compounds in cluster 1 revealed that cytosine has a protective effect against *C. albicans*-induced cytotoxicity. When *C. albicans* infection was performed in presence of 50 mM cytosine, both LDH release and translocation were reduced **(Fig. 7B, C)**. However, the total number of CFUs increased **(Fig.7D)**. Live cell imaging revealed that cytosine induces a hypha-to-yeast switch in *C. albicans*. As a result, hypha formation is almost completely halted after approximately 8 hpi and cells continue to proliferate as yeasts without further filamentation **(Fig. 7E)**.

Discussion:

In this study, we investigated the molecular and metabolic aspects of protection against *C. albicans*-induced damage mediated by *L. rhamnosus* colonization. Colonization of IECs with *L. rhamnosus* reduces the number of *C. albicans* in contact with the epithelium through shedding and reduced growth [11]. Since reduced inocula were still able to damage significantly, this hints at additional mechanisms mediating the protective effect. Using transcriptional and metabolic profiling coupled with *in silico* predictions we identified a complex transcriptional and metabolic interplay that underlies the *L. rhamnosus*-mediated reduction of damage caused by *C. albicans*. We identified the metabolic profiles in this scenario and characterized individual metabolites that foster *L. rhamnosus* growth on IECs, a prerequisite for its protective potential [11]. Colonization with viable, and thus metabolically active, but not killed *L. rhamnosus* cells caused clear changes in the transcriptional pattern of *C. albicans* during infection. The differentially expressed genes included key genes of metabolic pathways and transcriptional pattern suggested major metabolic adaptations of the fungus. In fact, differentially expressed genes included genes that were shown to be essential for *C. albicans* growth and full damage potential. *In silico* analysis of the metabolic environment upon *L. rhamnosus* colonization suggested a shift to less favored metabolites and the release of metabolites with known or validated antagonistic effects.

We observed that *L. rhamnosus* has to proliferate to render its antagonistic effect towards *C. albicans* in our infection model [11], yet the culture medium alone was unable to sustain growth. Untargeted metabolomics and *in silico* analysis suggested that IECs provide metabolites that permit growth of *L. rhamnosus*. Likewise, IEC-conditioned medium and medium supplemented with metabolites produced by IECs fostered *L. rhamnosus* growth. Collectively, this provides strong evidence that a bacterial-host metabolic interplay is required for *L. rhamnosus* growth in our model and that this interplay is a prerequisite for the antagonistic effects towards *C. albicans*. Such interactions are also common in the human gut, where the intestinal epithelium produces specific metabolites to sustain beneficial members of the microbiota. For example, some bacteria of the microbiota can use mucus glycan as a carbon source [41,42]. Other members of the intestinal microbiota can break down complex carbohydrates, proteins, and fats into metabolites usable for other bacteria and the human host [43-45].

We observed that treatment of *L. rhamnosus* by antibiotics blocked protection and prevented the dramatic transcriptional changes of *C. albicans* caused by viable bacteria. This suggests a potential correlation between the transcriptional changes and protection.

The potential impact of these alterations on the transcriptional level on *C. albicans* growth and damage was validated for selected genes using corresponding mutants from a gene deletion mutant library [28]. Deletion mutants of the six genes *PRN4, PTP3, RIM13, AHR1, ACE2,* and *orf19.4292,* which were downregulated by *L. rhamnosus,* exhibited an attenuated damage potential. For *prn4*Δ/Δ and *ptp3*Δ/Δ, the decreased damage was additionally associated with a reduced growth capacity. *PTP3* encodes a protein tyrosine phosphatase and *PRN4* expresses a pirin-like protein, but its function remains unknown. Ahr1 was recently described as a transcription factor regulating the virulence genes *ALS3* and *ECE1* [46], as well as a repressor of the white-to-opaque switch [47] in a complex transcriptional network [48].

Supporting our results, an *ace2*Δ/Δ mutant showed slightly decreased virulence in a model of disseminated candidiasis *in vivo* [49]. Ace2 is a multifunctional transcriptional regulator that activates the glycostress response of *C. albicans* to restore damaged glycostructures of the cell wall [50]. Additionally, it influences hypha formation under low oxygen conditions and regulates metabolism [51]. This supports the notion of a connection between the transcriptional and metabolic changes induced by *L. rhamnosus,* which includes genes that are relevant for *C. albicans* virulence.

Other studies have observed similar changes in the *C. albicans* transcriptional profile in the presence of lactobacilli. The vaginal commensal *L. crispatus,* reduced *C. albicans* growth and hypha formation, which was associated with suppression of the hypha-specific genes *ALS3*, *HWP1*, and *ECE1*, and induction of the repressor gene *NRG1* [52]. A mixture of *L. plantarum* and *L. helveticus* similarly downregulated the hypha-associated genes *ALS3, SAP5, EFG1,* and *HWP1* [53]. As these genes were not affected in our model system, we hypothesize different modes of action for the diverse *Lactobacillus* species depending on the environmental context.

A close look at the differentially regulated genes of *C. albicans* highlighted an enrichment of metabolic responses, presumably to the metabolic environment altered by *L. rhamnosus.* GO-term enrichment analysis revealed significant changes in *C. albicans* metabolic pathways during infection of IECs colonized by *L. rhamnosus*. These metabolic changes could influence

C. albicans in several ways. First, we hypothesize that the consumption of preferred metabolites by *L. rhamnosus* induced a shift towards the utilization of alternative carbon sources by *C. albicans.* Secondly, we propose that the production of antifungal metabolites reduced *C. albicans* pathogenicity.

The alternative carbon source hypothesis is supported by the observation that several genes, which were downregulated, especially in the late phase of infection, were associated with carbohydrate metabolism. The utilization of different carbon sources can drastically influence *C. albicans* fitness and pathogenicity as reviewed in [54]. Comparative analysis of the metabolic profiling in the current study and previously published metabolic phenotyping of *C. albicans* [34] revealed that preferred carbon sources were removed, while less-favored carbon sources remained available. The transcriptional data show that the gene coding for Mig1, an essential regulator in the glucose repression pathway [55], was significantly upregulated 6 hpi and therefore could repress the use of alternative carbon sources, but was downregulated 24 hpi when only alternative carbon sources were available. In parallel, genes that regulate glycolysis (*TYE7*, *GAL4*) were downregulated 24 hpi. Interestingly, a *tye7*Δ/Δ deletion mutant has attenuated damage potential in different *in vivo* models [56]. A closer look into the carbohydrate catabolism revealed the downregulation of glycolosis-relevant genes and a simultaneous upregulation of genes for the pentose phosphate pathway as well as the TCA cycle. Interestingly, this transcriptional pattern mimics the phenotype of the *ace2*Δ/Δ mutant [57]. As we observed that the *ace2*Δ/Δ mutant has an attenuated damage potential, it is possible that *C. albicans* is less able to cause damage when it has to cope with changing carbohydrate utilization. This suggests that factors like Ace2, which are crucial for the gene regulation metabolism, also affect *C. albicans* pathogenicity.

Our results strongly support a limitation of glucose in the *in vitro* system. Similarly, an *in vitro* model with oral epithelial cells and *L. rhamnosus* revealed an induction of genes associated with fatty acid catabolism, the glyoxylate cycle, and gluconeogenesis and suppression of genes contributing to glycolysis and ergosterol biosynthesis [10]. Potentially, this can cause a change in the fungal cell wall composition, which in turn can alter diverse phenotypes relevant for pathogenicity [58,59].

Lactobacilli are well-characterized in terms of their antibacterial and antifungal properties in connection to the production of antimicrobial metabolites. We identified several metabolites

with known antifungal potential that are released when *L. rhamnosus* colonizes IECs. Phenyllactic acid was shown to be an anti-*Candida* metabolite and its production by *Lactobacillus* spp. was improved by the use of prebiotic galactosyl polyols [36]. Among antimicrobial metabolites of *L. plantarum*, mevalonolactone was identified to slightly reduce fungal growth and exert a strong anti-bacterial potential, especially in combination with other *Lactobacillus*-produced metabolites like benzoic or lactic acid [37]. Further *in vitro* studies revealed antifungal effects of 2-hydroxyisocaproic acid (HICA) against *A. fumigatus* [39] and *C. albicans* [38,39]. An anti-*Candida* effect was also described for 3-hydroxyoctanoate [40]. Additionally, we identified cytosine to exert strong anti-virulence properties including the reduction of *C. albicans* hypha formation, epithelial damage, and translocation. Even though the cytosine-induced mechanism is not yet understood, it shows a functional interplay between *L. rhamnosus* and *C. albicans* in the *in vitro* system.

Next to the influence of *L. rhamnosus* to the carbohydrate utilization network of *C. albicans* proposed by GO enrichment analysis, the *in silico* pathway activity analysis suggested oxidative phosphorylation as another affected pathway. The transcriptional analysis revealed a *L. rhamnosus* dependent suppression of genes that are relevant for this pathway, e.g. *ADH1* was downregulated 6 hpi. This could hint toward limited ATP production in *C. albicans* and consequently limited energy for its growth, including hypha formation, and virulence. A recent study showed a correlation between *C. albicans* oxidative phosphorylation and pathogenicity mediated by *ADH1*, as an *adh1*Δ/Δ mutant showed both reduced oxidative phosphorylation and decreased damage and hypha formation *in vitro* and *in vivo* [60].

The current metabolic and transcriptional insights into the antagonistic potential of a single member of the microbiota underline the importance and complexity of a balanced intestinal microbiota that keeps *C. albicans* in its commensal state. Antibiotics were shown to create a shift from mutualism to competition in the human gut and that the fungal community requires a longer recovery from antibiotic treatment than bacteria [61]. Our study revealed novel insights that could be exploited for the development of anti-*Candida* therapies. One avenue might be a direct supplementation of anti-*Candida* metabolites or a provision of a diet rich in carbon sources that are less favored by *C. albicans*. Alternatively, the growth of *L. rhamnosus* in the intestine could be promoted by special diets including citric acid and gamma-glutamylalanine or carnitine. Along these lines, *C. albicans* colonization of laboratory mice strains, which are colonization resistant, was possible with diets that do not promote *Lactobacillus* spp.

growth [23]. Interestingly, next to the preventative impact of lactobacilli, a first study also showed their potential use as therapeutics for already existing *C. albicans* infections [62].

Our results combined both *in silico* and omics data analysis on both the transcriptional and metabolomics level, as well as genetic validation of predictions. Our data provide fundamental new insights into the mechanisms by which *C. albicans* pathogenic activity can be controlled or prevented.

Materials and Methods

In vitro model and infection

An *in vitro* intestinal *C. albicans*-infection model was used to perform the experiments as previously described [11]. C2BBe1 (ATCC®CRL2102) and HT29-MTX (ATCC HTB-38; CLS, Lot No. 13B021) cells were seeded in collagen I coated (10 µg/ml, 2 h at room temperature [RT]; Thermo Scientific, Waltham, USA) 6-well or 96-well plates at a ratio of 70:30 with a total cell density of 4×10^5 cells/well (6-well) and 2×10^4 cells/well (96 well). Cells were used for experiments after 14 days of differentiation in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Thermo Scientific, Waltham, USA) supplemented with 10% fetal calf serum (FCS; Bio & Sell), 10 µg/ml Holotransferrin (Calbiochem Merck, Darmstadt, Germany), and 1% non-essential amino acids (Gibco, Thermo Scientific, Waltham, USA) with medium exchange twice per week. Cell lines have been authenticated *via* commercial STR profiling (Eurofins Genomic) and checked for mycoplasma contaminations using a PCR mycoplasma test kit (PromoKine) according to the manufacturer's instructions.

For infection, DMEM was exchanged by 1.3 ml (6 well) or 50 µl (96 well) serum-free Keratinocyte Basal Medium (KBM) (Lonza, Basel, Switzerland) and monolayers were colonized with 1.3 ml (6-well) or 50 µl (96 well) *Lactobacillus rhamnosus* ATCC 7469 (OD 0,2 in KBM) for 18 h prior to infection with 1.3 ml (6 well) or 50 µl (96 well) *C. albicans* WT SC5314 (4×10^5 cells/ml in KBM). For the samples with antibiotic treatment, 500 µg/ml Gentamicin (Merck, Darmstadt, Germany) and 1× PenStrep (Gibco, Thermo Scientific, Waltham, USA) were added 4 hpi. For infection in presence of cytosine, a solution of 50 mM cytosine in KBM was prepared fresh and sterile filtered. Then, 150 µl (96-well, transwells) of the solution with *C. albicans* (MOI 1) were used to infect the cells for 24 h. Wells with only medium, *L. rhamnosus*, or *C. albicans* in the presence or absence of the host cells served as controls. Infected cells and controls were incubated at 37 °C with 5 %CO_2.

C. albicans CFU quantification

To determine how many *C. albicans* cells were present 24 hpi, CFU quantification was performed in 96-well plates. Supernatants were collected and IECs were treated for 5 min with 0.2% Triton-X-100 (Sigma-Aldrich) to lyse the host cells and release adherent fungal cells. After detaching adherent host cells *via* scraping with a pipette tip, the lysate was added to the respective supernatant. Wells were washed twice with PBS. The final samples were diluted

appropriately with PBS and plated on YPD agar with 1× PenStrep (Gibco, Thermo Fisher Scientific) and incubated at 30°C for 24 h until adequate growth for CFU quantification was reached.

Quantification of cytotoxicity (LDH)

The necrotic cell damage induced by a low *C. albicans* infection dose, *C. albicans* mutants, and after antibiotic treatment was determined by measuring the release of cytoplasmic LDH [63] **(Fig.S4)**. LDH release was quantified in the supernatant of infected IEC monolayers in 96 well plates 24 hpi using the Cytotoxicity Detection Kit (Roche) according to the manufacturer's instructions. LDH from rabbit muscle (5 mg/ml, Roche) was used to generate a standard curve for the determination of LDH concentrations. The background control level of uninfected IECs was subtracted from the experimental LDH release.

Mutant screen: growth and damage

C. albicans mutants from the [28] deletion mutant library were cultivated in YPD broth in 96 well plates and incubated overnight at 30°C with shaking at 180 rpm. The overnight cultures were adjusted to an OD_{600} of 0.0025 in KBM. For growth, the diluted overnight cultures were diluted 1:2 in KBM in 96 well plates and incubated for 24h at 37°C with 5% CO_2 in a microplate reader (Tecan Infinite M200; i-control software). Growth was monitored with OD_{600} measurements every 30 min over 24 h. For damage, the diluted overnight cultures were diluted 1:2 in KBM in 96 well plates with a confluent layer of differentiated intestinal epithelial cells (C2BBe1:HT29-MTX, 70:30). Damage was measured *via* LDH (see Quantification of cytotoxicity (LDH)).

***Lactobacillus rhamnosus* growth**

*L. rhamnosus*was grown in MRS Broth for 48 h at 37°C with 5% CO_2 and 1% O_2. Afterwards, cells were washed and 3000 cells/ml were inoculated in the different media and IECs were colonized. At 0, 24, and 48 h independent wells were resuspended and the content was appropriately diluted and plated on MRS agar. Plates were incubated for 48 h at 37°C with 5% CO_2 and 1% O_2 until CFU quantification. Tested metabolites are shown in **Tab.1**.

Tab.1: Tested metabolites to improve *L. rhamnosus* growth

Compound name	Experimental concentration	Company
Citric acid	5 mM	Roth
Carnitine	5 mM	Sigma
Palmitic acid/Lauric acid (faty acids)	1 g/L	Sigma/Roth
Gamma-glutamyl-alanine	5 mM	Sigma
Xanthine	0.05 mM	Sigma
3-methyl-2-oxovalerate	5 Mm	Sigma
3-methyl-2-oxobutyrate	5 mM	Sigma
Non Essential Aminoacids + L-Glutamine	1 mM	Gibco
Fructose	1 mM	Sigma
Nicotinamide	0.001 mM	Fluka
N-acetyl-glucosamine	1 mM	Sigma

Live cell imaging

C. albicans ($1x10^4$ cell/ml) in KBM medium supplemented with 50 mM cytosine was incubated for 24 h at 37°C with 5% CO_2 inside the Cell Discoverer 7 microscope (Zeiss), in which a bright field picture was taken every hour.

Translocation assay

To determine translocation through the epithelial barrier, infections were performed in Transwell inserts (Sarstedt). 24 hpi, zymolyase (260 U/ml) was added to the bottom compartment and incubated for 2 h at 37°C with 5% CO_2. Afterwards, samples were diluted in PBS, plated on YPD agar and incubated at 30°C for 24 h.

Transcriptome analysis

After 6 and 24 h of *C. albicans* infection, RNA isolation of *C. albicans* was performed **(Fig.S4)**. At the appropriate time points, 650 µl RLT buffer was added to the wells and the plates were frozen in liquid nitrogen immediately. After thawing, fungal and host cells were collected *via*

scraping. The collected material was centrifuged and fungal RNA isolation was performed on the pellet according to a previously described protocol [64]. RNA quantities were determined with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and RNA quality was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was subsequently converted into Cy5-labeled cRNA (Cy5 CTP; GE Healthcare, United Kingdom) using a QuickAmp labeling kit (Agilent). Samples were cohybridized with a common Cy3-labeled reference (RNA from mid-log-phase-grown *C. albicans* SC5314 [65]) on Agilent arrays (*C.a.*: AMADID 026869), scanned in a GenePix 4200AL with GenePix Pro 6.1 (Auto PMT; pixel size, 5 µm). Differentially expressed genes (*p*-value: 0,05; $\log_2$ fold change) were analyzed with GeneSpring 14.9 (Agilent) and the Candida Genome Database (CGD; http://www.candidagenome.org). Out of the 6130 *C. albicans* genes, 5125 genes were used for the analysis after filtering on the minimal fluorescent signal and subtraction of the background signal. Genes were considered differentially regulated when they had a Bonferroni-corrected *p*-value of < 0.05 and a $\log_2$ fold change of more than 1 or less than -1.

Metabolome analysis

Supernatants for untargeted metabolomics were collected 6 and 12 h after *C. albicans* infection **(Fig.S4)**. 500 µl of the supernatant was collected, centrifuged, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Samples were analyzed and interpreted by Metabolon (Morrisville, USA). Experiments included 3 technical replicates and 5 independent experiments have been performed in total.

Data analysis

Gene expression data were exported from GeneSpring and metabolome data were received from Metabolon (Morrisville, USA). Raw metabolome data was rescaled to set the median equal to 1, and the missing values imputed with the minimum. Data were loaded in R version 1.2.5019, rows were normalized and Euclidian distances were calculated. The heatmap was generated with the pHeatmap package v1.0.12 and used to manually obtain the optimal number of clusters. The distance matrix was subjected to hierarchical cluster analysis using the complete linkage agglomeration method and metabolites were classified according to their cluster. Color bars indicating cluster or condition were added to the dendrograms using the R package “dendextend”. The Principal Components Analysis (PCA) was calculated using the R function *prcomp*. Graphs were generated using the ggbiplot package v 0.55 and rgl

v0.100. A proportional Venn diagram of the differentially expressed genes was drawn using the eulerAPE application v2.0.3 [66].

GO-term enrichment was analyzed with the Candida genome database GO Term Finder and overlapping and redundant GO-terms were removed with the Revigo program (similarity: Tiny (0,4); database: whole Uniprot; semantic similarity measure: SimRel).

Data from *in vitro* growth and damage assays was analyzed using Graphpad prism version 8. Data of n≥3 was analyzed for statistical significance using a one-way ANOVA with multiple comparisons. Statistical significance is depicted in the figures: * = $p \leq 0.05$, ** = $p \leq 0.01$, or *** = $p \leq 0.001$.

Bioinformatics

Genome scale metabolic models (GEMs) for *C. albicans*, human, and *L. rhamnosus* metabolism were used to simulate and analyze different growth scenarios *in silico*. Specifically, the recently published model for *C. albicans* [34], *Lactobacillus rhamnosus* LMS2-1 for *L. rhamnosus* , and Recon3D 3.01 for intestinal epithelial cells were used [31,67]. The GEMs *Lactobacillus rhamnosus* LMS2-1 and Recon3D 3.01 were downloaded from www.vmh.life. Metabolomics data were used to modulate feasible nutrition uptake for each model *via* respective exchange reactions as defined by each GEM. In brief, feasible uptake rates for available metabolites were adapted from the metabolome measurements across all investigated conditions. Any maximum allowed uptake rate was transformed into the model defined allowed uptake range between 0 and 1000 mmol/g(DW)h with the maximum observed metabolite concentration set to 1000. Flux balance and flux variability analysis were used to identify changed reaction activity levels considering an anaerobic environment across different conditions [30,33]. All GEM analyses were done in COBRApy [68] using Python 3.6.4 and the IBM ILOG CPLEX Optimizer (version 12.8).

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Figures:

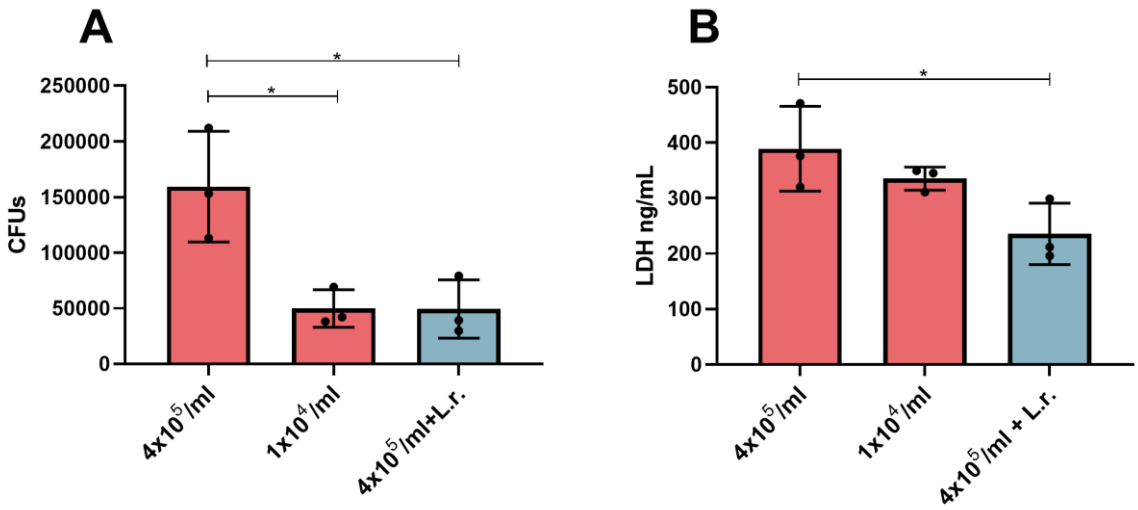
A
CFUs
250000
200000
150000
100000
50000
0
*
*
4x10^5/ml
1x10^4/ml
4x10^5/ml+L.r.
B
LDH ng/mL
500
400
300
200
100
0
*
4x10^5/ml
1x10^4/ml
4x10^5/ml + L.r.

Fig. 1: Reduced *C. albicans* inocula do not reduce damage to the extent of *L. rhamnosus*

A) Fungal burden assessed by quantification of *C. albicans* CFUs and **(B)** the induced necrotic damage of IECs quantified by release of LDH at 24 hpi. Cells were infected with a normal *C. albicans* infection dose ($4x10^5$/ml) in the presence and absence of *L. rhamnosus* as well as the reduced infections dose ($1x10^4$/ml) in the absence of *L. rhamnosus*. Bars represent the mean and standard error of the mean (SEM) of n=3 independent experiments, dot plots show the mean of the technical replicates of the individual experiments, data were compared for significance using a one-way ANOVA with multiple comparisons, * = $p \leq 0.05$.

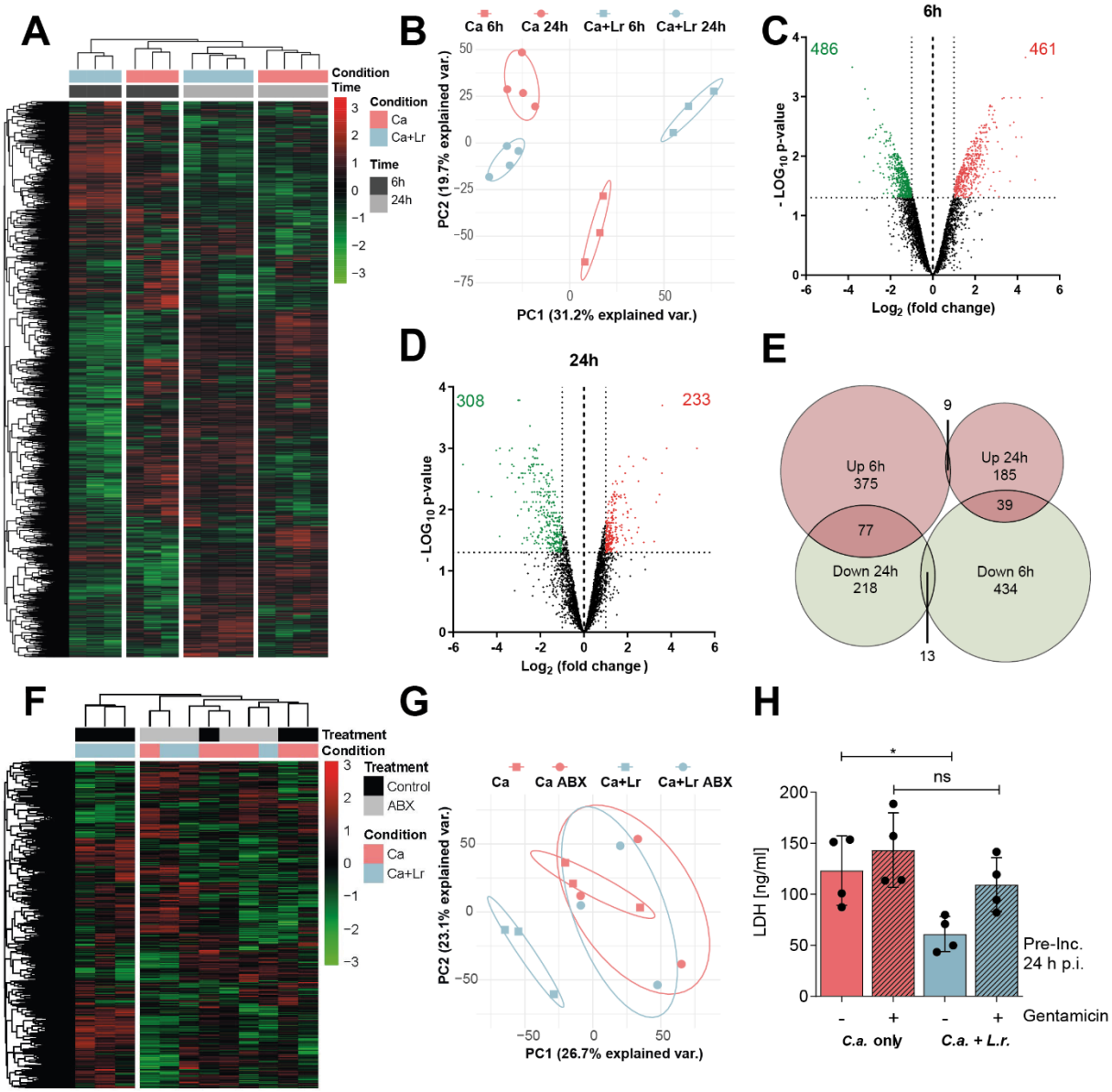
A
Condition
Time
Condition
Ca
Ca+Lr
Time
6h
24h
B
Ca 6h
Ca 24h
Ca+Lr 6h
Ca+Lr 24h
PC2 (19.7% explained var.)
PC1 (31.2% explained var.)
C
6h
486
461
- LOG10 p-value
Log2 (fold change)
D
24h
308
233
- LOG10 p-value
Log2 (fold change)
E
Up 6h
375
Up 24h
185
77
39
Down 24h
218
Down 6h
434
9
13
F
Treatment
Condition
Treatment
Control
ABX
Condition
Ca
Ca+Lr
G
Ca
Ca ABX
Ca+Lr
Ca+Lr ABX
PC2 (23.1% explained var.)
PC1 (26.7% explained var.)
H
*
ns
LDH [ng/ml]
Pre-Inc.
24 h p.i.
Gentamicin
C.a. only
C.a. + L.r.

Fig. 2: *L. rhamnosus* manipulates *C. albicans* transcriptional response during infection

(A) Hierarchal clustering based on Euclidean distance and **(B)** Principal component analysis of *C. albicans* gene expression at 6 and 24 h during *in vitro* infection of IECs in the presence and absence of *L. rhamnosus* colonization. Data represents n=3 and n=4 independent experiments at 6 and 24 hpi respectively. **(C, D)** Volcano plots showing differentially regulated *C. albicans* genes at 6 and 24 hpi as a result of *L. rhamnosus* colonization prior to infection based on the criteria of a Log_2 fold change of >1 or <-1 and a Bonferroni corrected *p*-value of <0.05 **(E)** Venn diagram analysis of the overlap in differentially expressed genes (*C.a.* + *L.r.* vs *C.a.*) at 6 and 24 h. **(F)** Hierarchal clustering based on Euclidean distance, **(G)** Principal component analysis of *C. albicans* gene expression. Data represents n=3 at 24 hpi. **(H)** Necrotic cell damage of IECs quantified by LDH release at 24 hpi after *in vitro* infection of IECs in the presence and absence of *L. rhamnosus* colonization and antibiotic treatment with Gentamicin and Penicillin/Streptomycin 4 hpi. Bars represent the mean and standard error of the mean (SEM) of n=3 independent experiments, dot plots show the mean of the technical replicates of the individual experiments, data were compared for significance using a one-way ANOVA with multiple comparisons, * = $p \leq 0.05$.

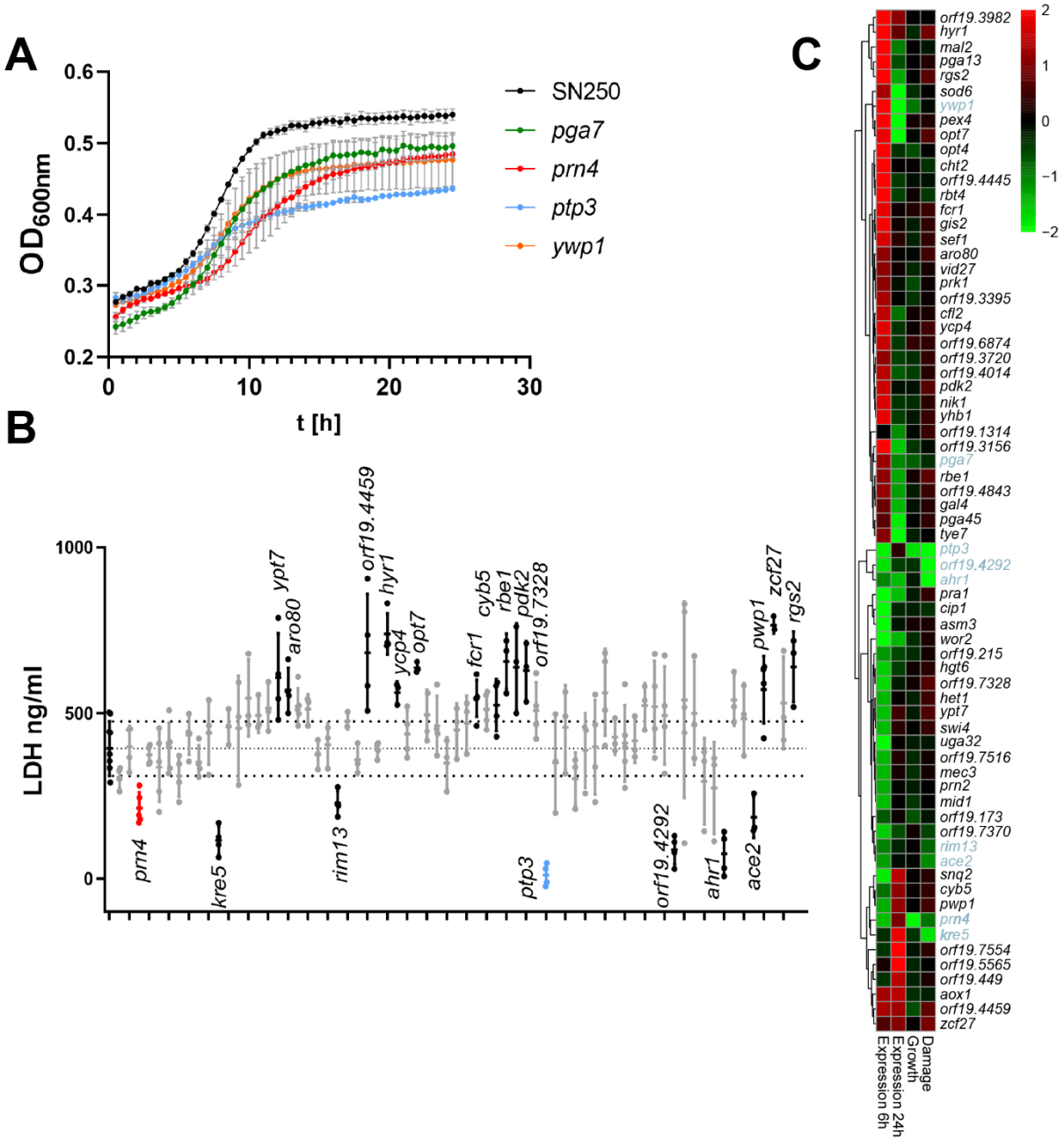
A
OD600nm
t [h]
SN250
pga7
prn4
ptp3
ywp1
B
LDH ng/ml
prn4
kre5
rim13
ptp3
orf19.4292
ahr1
ace2
ypt7
aro80
orf19.4459
hyr1
ycp4
opt7
fcr1
cyb5
rbe1
pdk2
orf19.7328
pwp1
zcf27
rgs2
C
Damage
Growth
Expression 24h
Expression 6h

Fig. 3: Screening of *C. albicans* deletion mutants

(A) Growth rates of deletion mutants with significantly reduced growth (colored lines) compared to the parental strain (black line) in KBM. Lines represent the mean and standard error of the mean (SEM) of n=3 independent experiments, and were compared for significance using an ANOVA with Bonferroni-adjusted post-hoc analysis, * = $p \leq 0.05$. **(B)** Ability of deletion mutants to induce necrotic cell damage of IECs assessed by the release of LDH. Data is shown as the mean and standard error of the mean (SEM) with dots showing the individual replicates. Deletion mutants were compared to the wild type control using a one-way ANOVA and Dunnett's Multiple Comparison posttest. Mutants with a significantly increased or decreased damage potential (p-value < 0.05) are shown in bold. **(C)** Clustering of the differentially expressed genes ($\log_2$ fold change >1 or <-1 and p<0.05) in the presence of *L. rhamnosus* 6 and 24 hpi and the phenotype of the corresponding gene deletion mutants in growth in KBM media and damage inflicted to IECs in KBM media.

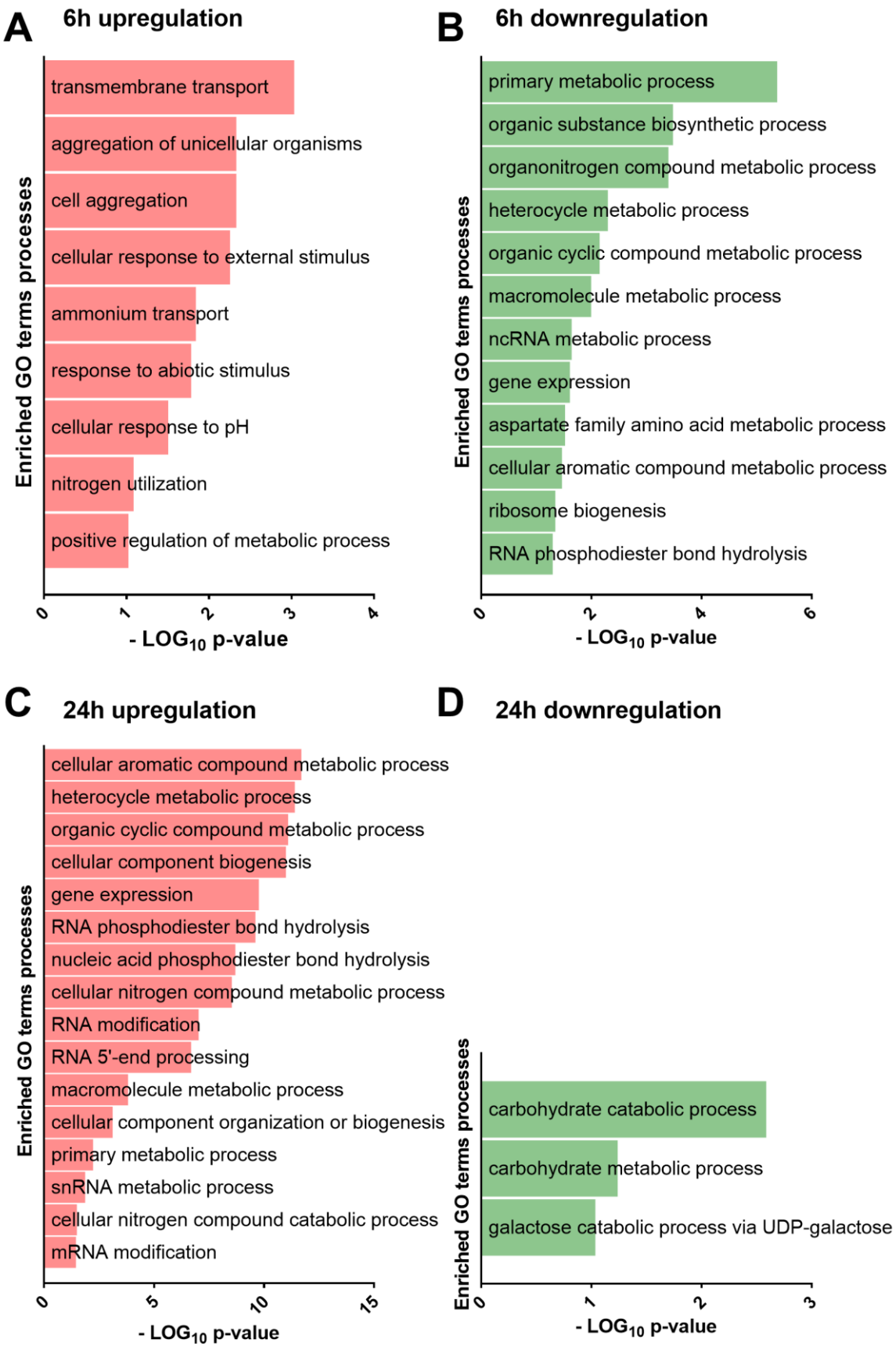
A
6h upregulation
transmembrane transport
aggregation of unicellular organisms
cell aggregation
cellular response to external stimulus
ammonium transport
response to abiotic stimulus
cellular response to pH
nitrogen utilization
positive regulation of metabolic process
Enriched GO terms processes
0
1
2
3
4
- LOG10 p-value
B
6h downregulation
primary metabolic process
organic substance biosynthetic process
organonitrogen compound metabolic process
heterocycle metabolic process
organic cyclic compound metabolic process
macromolecule metabolic process
ncRNA metabolic process
gene expression
aspartate family amino acid metabolic process
cellular aromatic compound metabolic process
ribosome biogenesis
RNA phosphodiester bond hydrolysis
Enriched GO terms processes
0
2
4
6
- LOG10 p-value
C
24h upregulation
cellular aromatic compound metabolic process
heterocycle metabolic process
organic cyclic compound metabolic process
cellular component biogenesis
gene expression
RNA phosphodiester bond hydrolysis
nucleic acid phosphodiester bond hydrolysis
cellular nitrogen compound metabolic process
RNA modification
RNA 5'-end processing
macromolecule metabolic process
cellular component organization or biogenesis
primary metabolic process
snRNA metabolic process
cellular nitrogen compound catabolic process
mRNA modification
Enriched GO terms processes
0
5
10
15
- LOG10 p-value
D
24h downregulation
carbohydrate catabolic process
carbohydrate metabolic process
galactose catabolic process via UDP-galactose
Enriched GO terms processes
0
1
2
3
- LOG10 p-value

Fig. 4: GO term enrichment

(A-D) GO term enrichment of differentially regulated genes (log_2 fold change >1 or <-1 and p<0.05) analyzed with the GO term finder on the Candida Genome Database website and reduced with the Revigo program (http://revigo.irb.hr/) (similarity: Tiny (0.4)). Significantly enriched GO terms are plotted based on the -10log p-value.

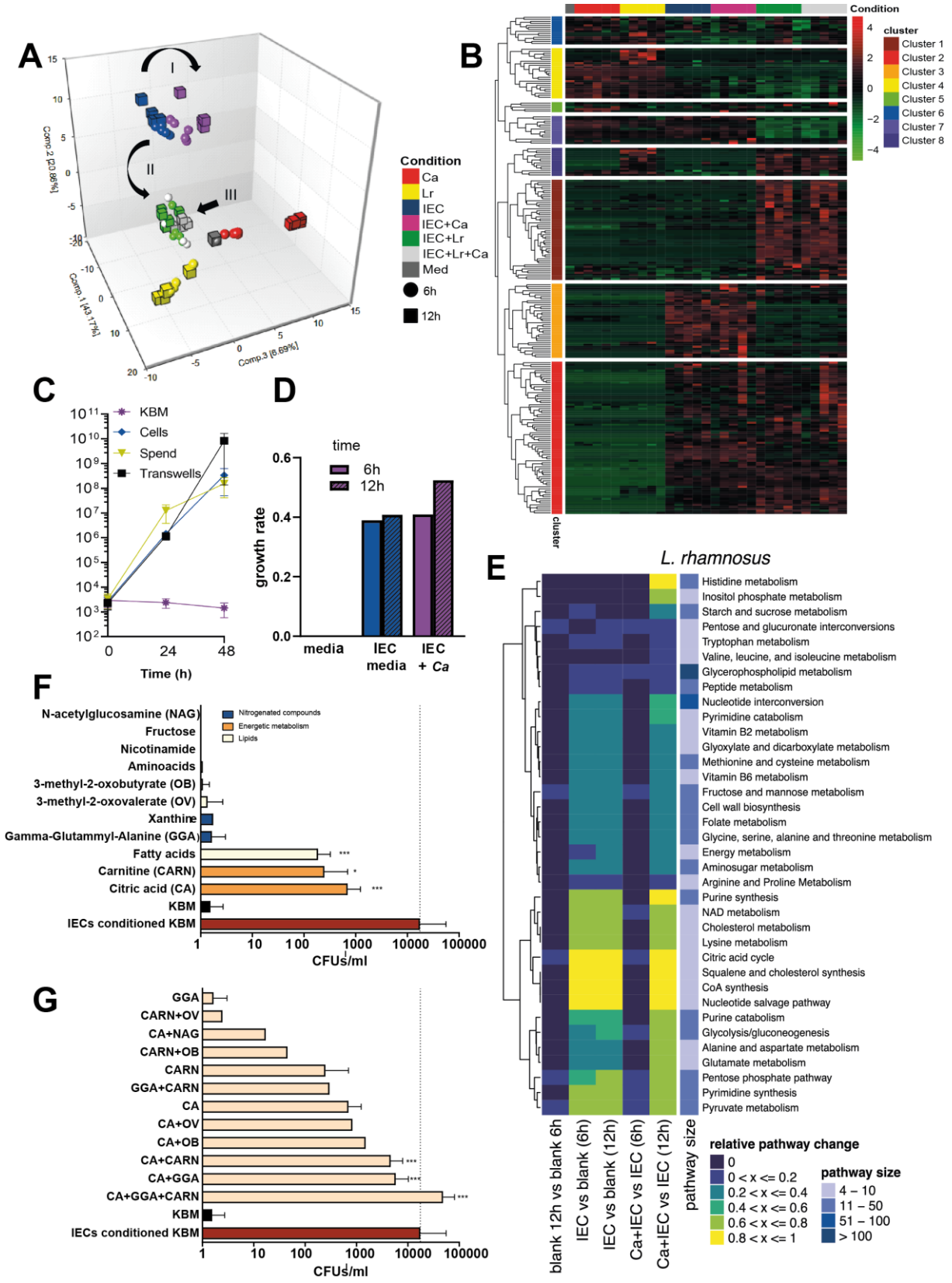
A
Condition
Ca
Lr
IEC
IEC+Ca
IEC+Lr
IEC+Lr+Ca
Med
6h
12h
B
Condition
cluster
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
C
KBM
Cells
Spend
Transwells
Time (h)
D
time
6h
12h
growth rate
media
IEC media
IEC + Ca
E
L. rhamnosus
Histidine metabolism
Inositol phosphate metabolism
Starch and sucrose metabolism
Pentose and glucuronate interconversions
Tryptophan metabolism
Valine, leucine, and isoleucine metabolism
Glycerophospholipid metabolism
Peptide metabolism
Nucleotide interconversion
Pyrimidine catabolism
Vitamin B2 metabolism
Glyoxylate and dicarboxylate metabolism
Methionine and cysteine metabolism
Vitamin B6 metabolism
Fructose and mannose metabolism
Cell wall biosynthesis
Folate metabolism
Glycine, serine, alanine and threonine metabolism
Energy metabolism
Aminosugar metabolism
Arginine and Proline Metabolism
Purine synthesis
NAD metabolism
Cholesterol metabolism
Lysine metabolism
Citric acid cycle
Squalene and cholesterol synthesis
CoA synthesis
Nucleotide salvage pathway
Purine catabolism
Glycolysis/gluconeogenesis
Alanine and aspartate metabolism
Glutamate metabolism
Pentose phosphate pathway
Pyrimidine synthesis
Pyruvate metabolism
blank 12h vs blank 6h
IEC vs blank (6h)
IEC vs blank (12h)
Ca+IEC vs IEC (6h)
Ca+IEC vs IEC (12h)
pathway size
relative pathway change
pathway size
F
Nitrogenated compounds
Energetic metabolism
Lipids
N-acetylglucosamine (NAG)
Fructose
Nicotinamide
Aminoacids
3-methyl-2-oxobutyrate (OB)
3-methyl-2-oxovalerate (OV)
Xanthine
Gamma-Glutammyl-Alanine (GGA)
Fatty acids
Carnitine (CARN)
Citric acid (CA)
KBM
IECs conditioned KBM
CFUs/ml
G
GGA
CARN+OV
CA+NAG
CARN+OB
CARN
GGA+CARN
CA
CA+OV
CA+OB
CA+CARN
CA+GGA
CA+GGA+CARN
KBM
IECs conditioned KBM
CFUs/ml

Fig. 5: Metabolomics in the *in vitro* model

(A) Principal component analysis of metabolomics samples 6 and 12 hpi. **(B)** Hierarchical clustering based on Euclidean distance of metabolites in the supernatants in the *in vitro* model at 6 hpi. **(C)** Growth of *L. rhamnosus* assessed by counting CFUs in transwells physically separated from IECs or grown in IEC-conditioned medium. Data is shown as the mean and standard error of the mean (SEM). **(D)** *In silico* prediction of *L. rhamnosus* growth in the presence and absence of IECs. **(E)** Analysis of pathway activity levels compared between different setups as indicated. Relative pathway change was determined by identifying the number of pathway-specific reactions for which feasible flux ranges differ according to flux variability analysis. **(F, G)** Growth of *L. rhamnosus* assessed by CFUs after 24 and 48 h incubation in KBM supplemented with single metabolites **(F)** or combinations of metabolites **(G).** Data are shown as the mean and standard error of the mean (SEM). Data were compared for significance using a t-test, * = $p \leq 0.05$.

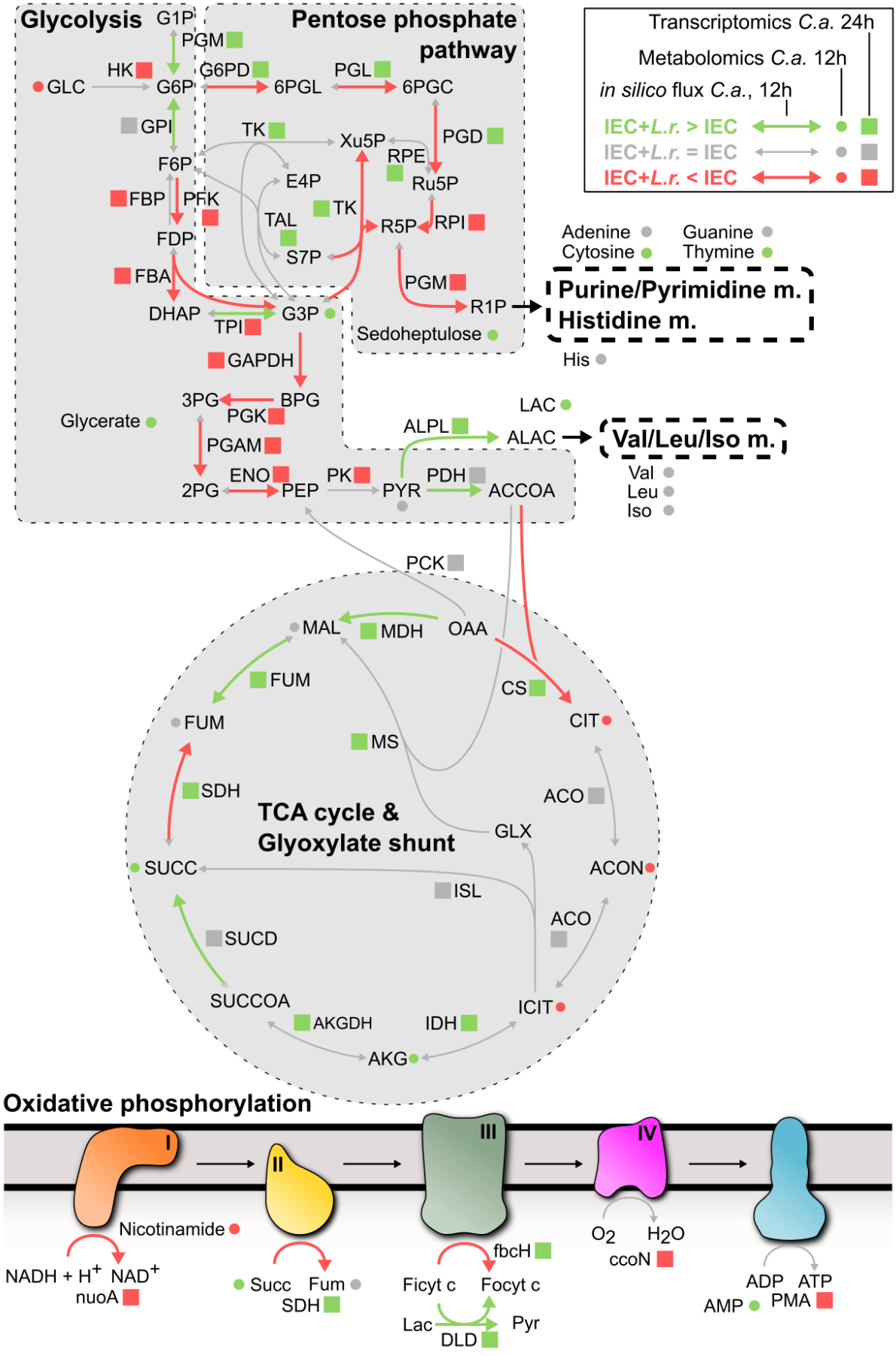
Glycolysis
Pentose phosphate pathway
G1P
PGM
HK
G6PD
PGL
GLC
G6P
6PGL
6PGC
GPI
TK
Xu5P
PGD
F6P
RPE
E4P
Ru5P
FBP
PFK
TK
TAL
R5P
RPI
FDP
S7P
FBA
PGM
R1P
DHAP
TPI
G3P
Sedoheptulose
GAPDH
3PG
BPG
Glycerate
PGK
PGAM
2PG
ENO
PEP
PK
PYR
PDH
ACCOA
ALPL
LAC
ALAC
Transcriptomics C.a. 24h
Metabolomics C.a. 12h
in silico flux C.a., 12h
IEC+L.r. > IEC
IEC+L.r. = IEC
IEC+L.r. < IEC
Adenine
Guanine
Cytosine
Thymine
Purine/Pyrimidine m.
Histidine m.
His
Val/Leu/Iso m.
Val
Leu
Iso
PCK
MAL
MDH
OAA
FUM
CS
FUM
CIT
MS
SDH
ACO
TCA cycle &
Glyoxylate shunt
GLX
SUCC
ACON
ISL
ACO
SUCD
SUCCOA
ICIT
AKGDH
IDH
AKG
Oxidative phosphorylation
I
II
III
IV
Nicotinamide
NADH + H+ NAD+
nuoA
Succ Fum
SDH
fbcH
Ficyt c Focyt c
Lac
Pyr
DLD
O2 H2O
ccoN
ADP ATP
AMP
PMA

Fig.6: Central metabolic metabolism of *C. albicans* including data overlay and *in silico* metabolic flux predictions. Reactions associated to glycolysis, the pentose phosphate pathway, Krebs cycle, and oxidative phosphorylation are indicated. Information on metabolome (12 h) and transcriptomic (24 h) data are combined with *in silico* genome-wide metabolic flux predictions (12 h). Reaction abbreviations: **(Tab.S3)**.

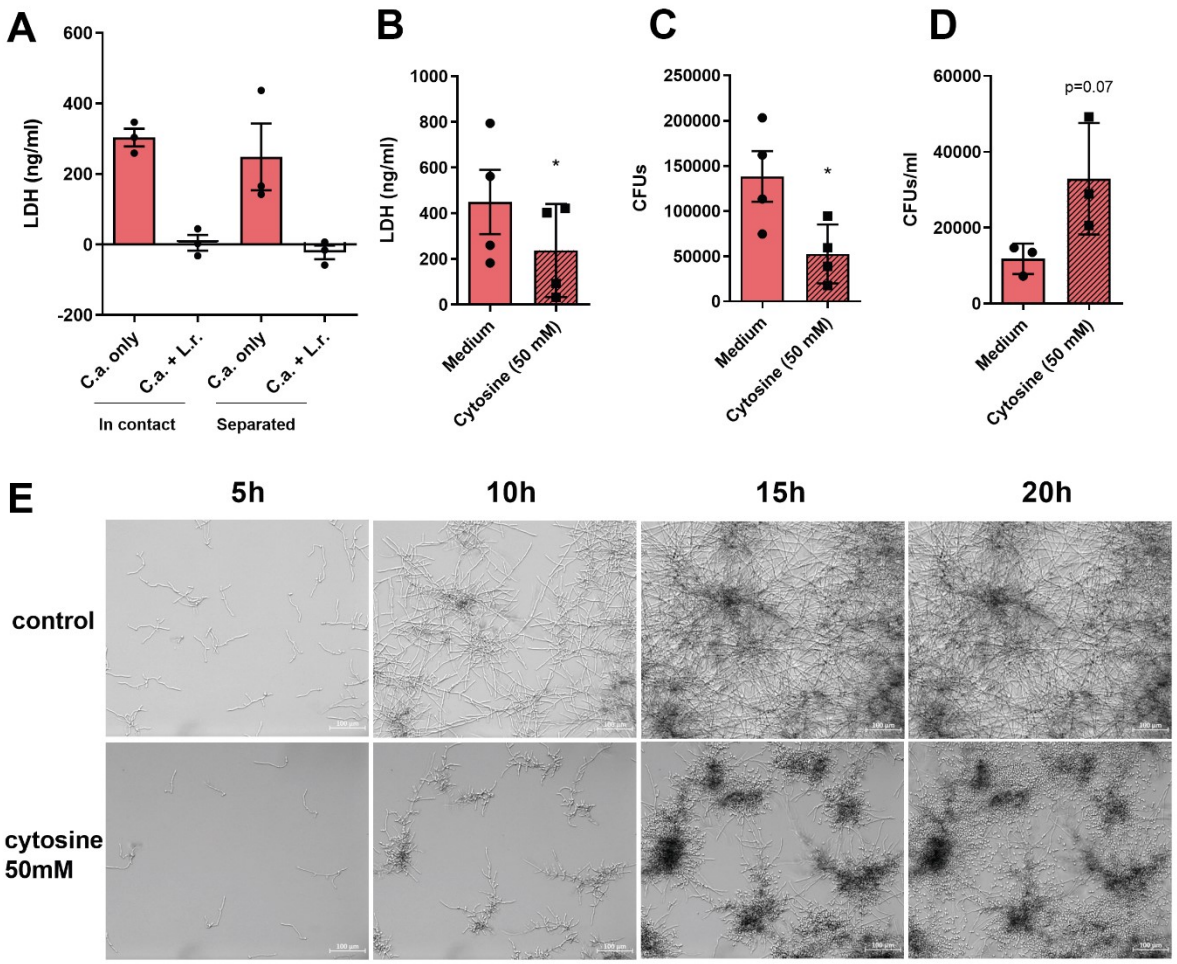
A
LDH (ng/ml)
600
400
200
0
-200
C.a. only
C.a. + L.r.
C.a. only
C.a. + L.r.
In contact
Separated
B
LDH (ng/ml)
1000
800
600
400
200
0
*
Medium
Cytosine (50 mM)
C
CFUs
250000
200000
150000
100000
50000
0
*
Medium
Cytosine (50 mM)
D
CFUs/ml
60000
40000
20000
0
p=0.07
Medium
Cytosine (50 mM)
E
5h
10h
15h
20h
control
cytosine 50mM

Fig. 7: Cytosine produced by *L. rhamnosus* shows *C. albicans*-perturbing activity

A) Necrotic cell damage of IECs quantified by LDH release at 24 hpi after *in vitro* infection of IECs in the presence and absence of *L. rhamnosus* colonization. *L. rhamnosus* was either directly in contact with the cells or physically separated by a transwell insert with a 0.4 µm pore size. **(B)** Necrotic damage of IECs measured LDH release induced by *C. albicans,* **(C)** *C. albicans* translocation across the epithelial barrier, **(D)** and *C. albicans* fungal burden assessed in the presence or absence of cytosine at 50 mM. Bars represent the mean and standard error of the mean (SEM) of n≥3 independent experiments, dot plots show the mean of the technical replicates of the individual experiments, data were compared for significance using a t-test, * = $p \leq 0.05$. **(E)** Representative images at selected time points of *C. albicans* morphology changes in presence or absence of cytosine at 50 mM, incubated at 37°C with 5% CO_2.

Supplement:

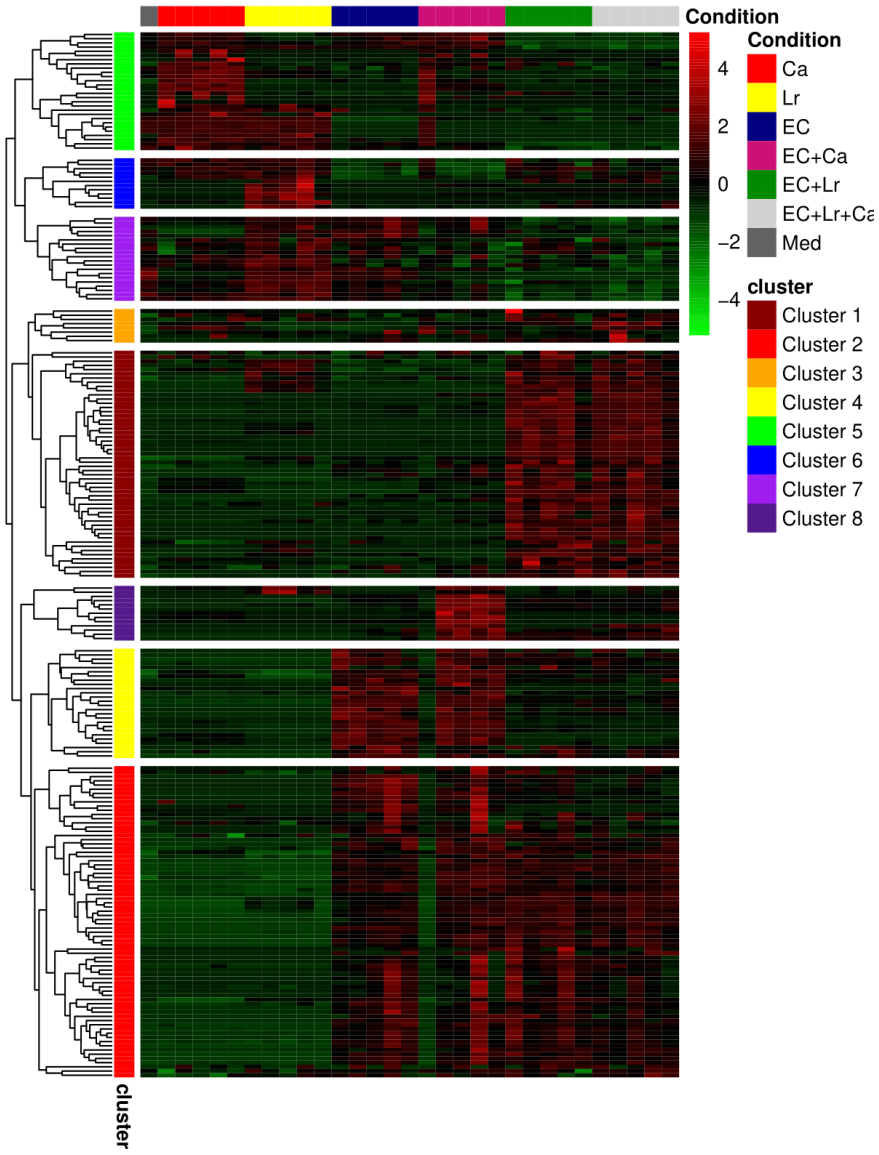

Fig. S1: Hierarchical clustering of the presence and absence of metabolites in the *in vitro* model 12 hpi.

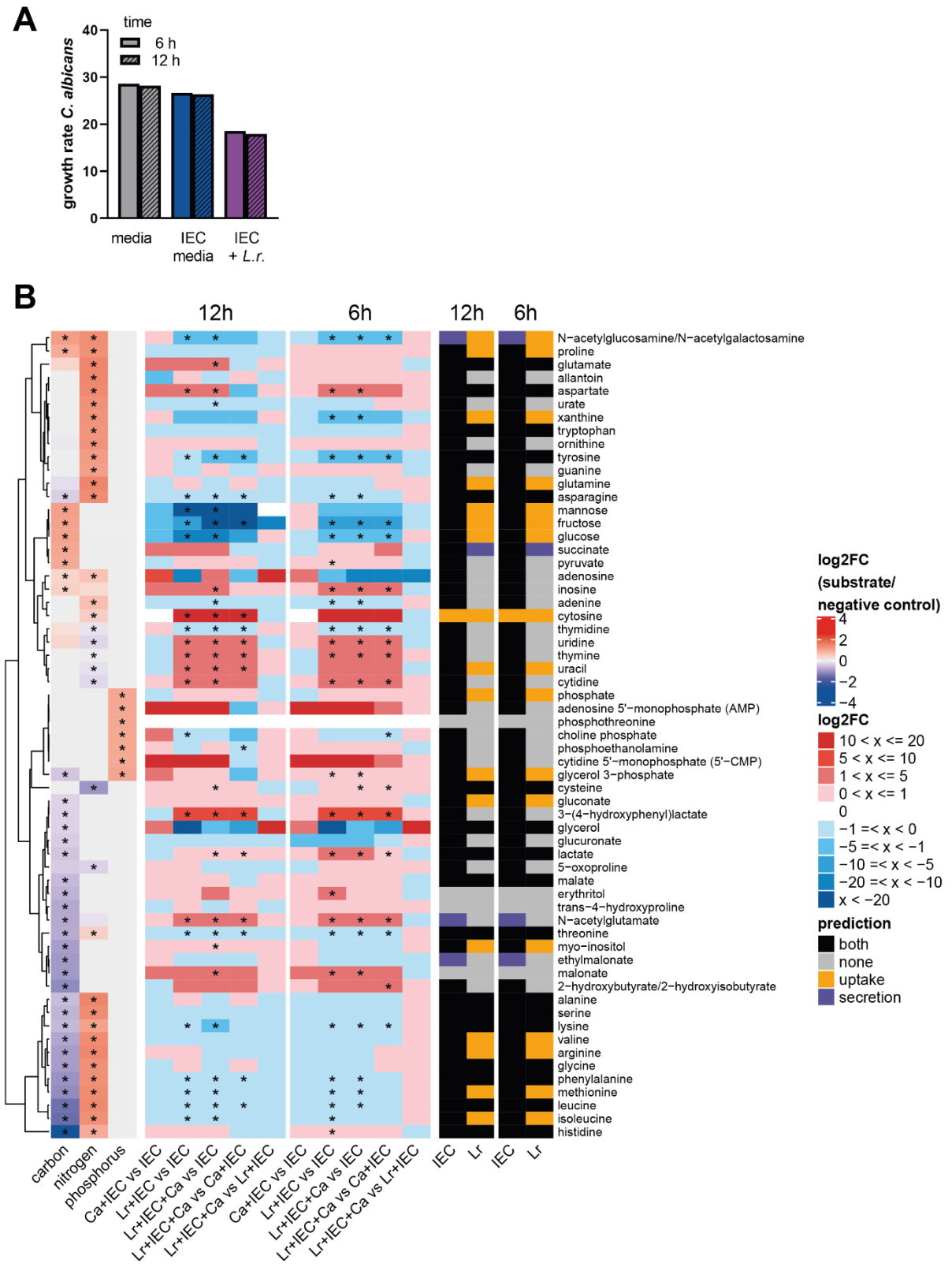

Fig.S2: **(A)** *In silico* prediction of *C. albicans* growth in the presence and absence of *L. rhamnosus*. **(B)** Phenotypic microarray growth experiments for wild-type *C. albicans* [34], metabolome data measured at 6 and 12 h as well as metabolic modeling predictions are indicated. For metabolic modeling, media was adapted from metabolome data derived from

IECs spent media or blank. Uptake or secretion was determined by identifying feasible flux ranges for metabolite-specific exchange reactions alongside optimization for biomass.

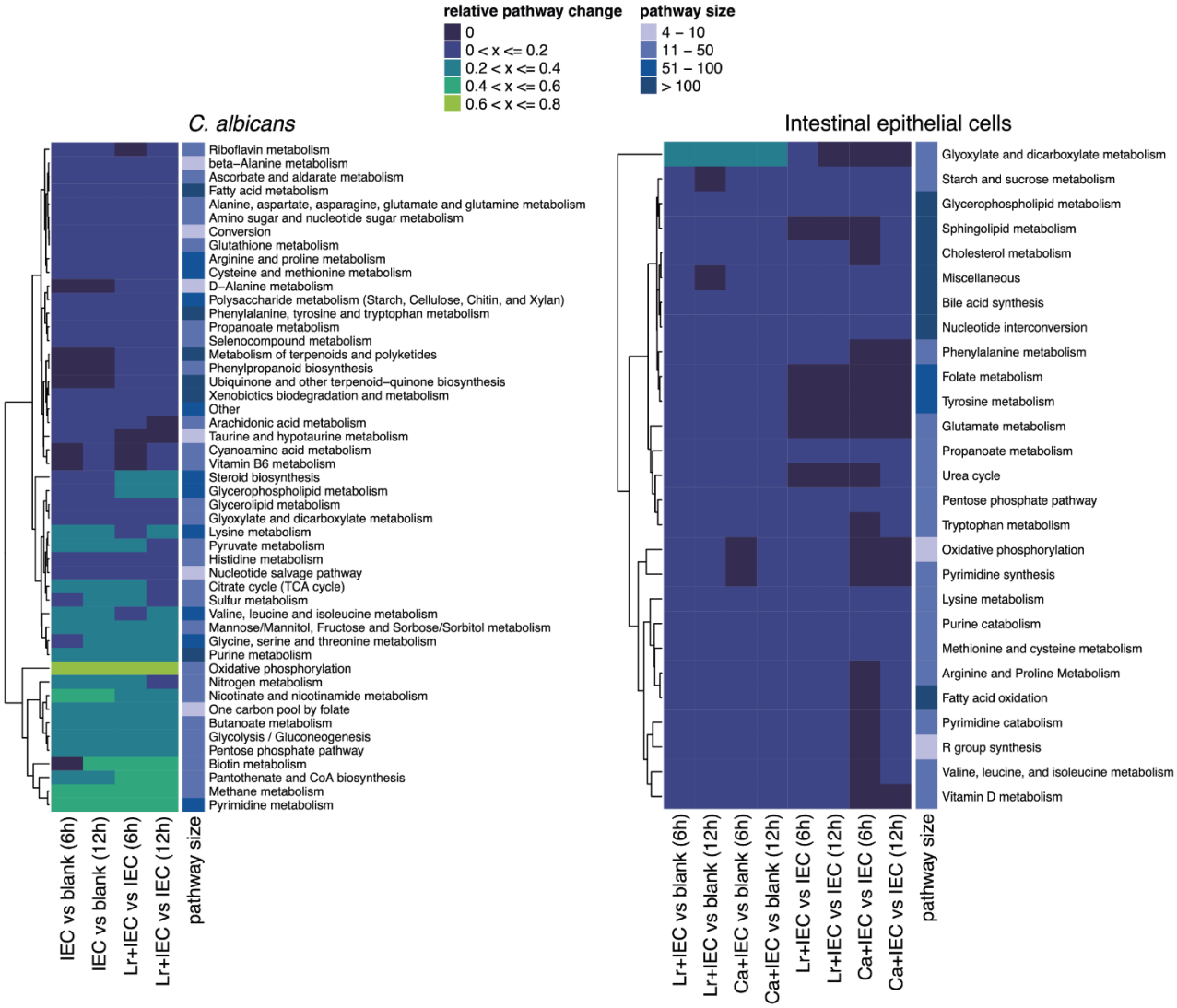

Fig.S3: Analysis of pathway activity levels compared between different setups as indicated. Relative pathway change was determined by identifying the number of pathway-specific reactions for which feasible flux ranges differ according to flux variability analysis.

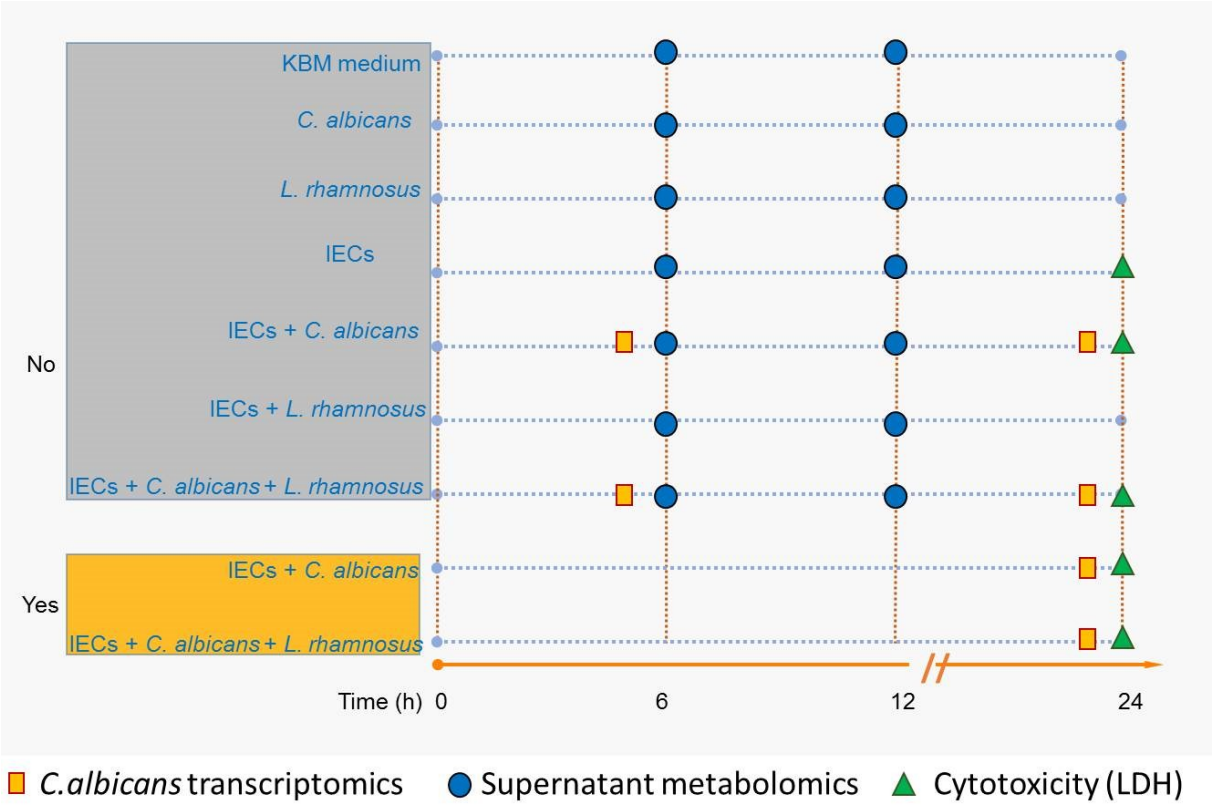

Fig. S4: Study design

Overview of the samples, taken at different time points for transcriptional, metabolic, and cytotoxicity analysis in the *in vitro* model.

Tab.S1A: Metabolites of the clusters 1-4 shown in Fig. 5B (6 hpi).

Cluster 1 Secreted by *L. rhamnosus* and IECs in synergy	**Cluster 2** Secreted by IECs	**Cluster 3** Secreted by IECs consumed by *L. rhamnosus*	**Cluster 4** Consumed by IECs
(N(1) + N(8))-acetylspermidine	1-carboxyethylisoleucine	1-ribosyl-imidazoleacetate	2'-deoxyadenosine
1-carboxyethyltyrosine	1-carboxyethylleucine	2'-O-methyluridine	3-sulfo-L-alanine
2'-deoxycytidine	1-carboxyethylphenylalanine	3-methyl-2-oxobutyrate	adenosine
2'-deoxyinosine	1-carboxyethylvaline	3-ureidopropionate	alpha-lipoate
2-hydroxy-3-methylvalerate	1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	5-methylthioadenosine (MTA)	arginine
2-hydroxy-4-(methylthio)butanoic acid	1-stearoyl-2-oleoyl-GPC (18:0/18:1)	acetylcarnitine (C2)	aspartate
2-hydroxybutyrate/2-hydroxyisobutyrate	2'-deoxyuridine	carnitine	cysteine s-sulfate
2-hydroxyglutarate	2'-O-methylcytidine	carnosine	cysteine sulfinic acid
2R,3R-dihydroxybutyrate	3-hydroxy-3-methylglutarate	cysteinylglycine disulfide	cystine
3-(4-hydroxyphenyl)lactate	3-hydroxybutyrate (BHBA)	deoxycarnitine	gluconate
3-formylindole	3-hydroxyisobutyrate	fructose	glutamate
3-hydroxyoctanoate	4-imidazoleacetate	gamma-glutamylalanine	glutamine
3-indoleglyoxylic acid	7-methylguanine	gamma-glutamylglutamine	inosine
4-methylthio-2-oxobutanoate	alanine	gamma-glutamylhistidine	isoleucine
5-methyluridine (ribothymidine)	alpha-ketoglutarate	gamma-glutamylisoleucine	leucine
allantoin	arabonate/xylonate	gamma-glutamylleucine	mannonate
alpha-hydroxyisocaproate	beta-alanine	gamma-glutamylmethionine	methionine
alpha-hydroxyisovalerate	cholesterol	gamma-glutamylserine	methionine sulfoxide
benzoate	choline phosphate	hippurate	N-acetylarginine
cysteine	creatine	N-acetylglucosamine/N-acetylgalactosamine	N-acetylglutamine
cytidine	creatinine	nicotinamide	phenylalanylhydroxyproline
cytosine	cystathionine	S-1-pyrroline-5-carboxylate	phosphoethanolamine
dihydroorotate	dimethylarginine (SDMA + ADMA)	xanthine	phosphothreonine
glycerate	erythritol	4-methyl-2-oxopentanoate	serine
glycerophosphoglycerol	erythronate	aconitate [cis or trans]	valine
histidine	ethylmalonate	citrate	

hypoxanthine	fumarate	isocitrate	
imidazole lactate	glycerol 3-phosphate	N-acetylmethionine	
indolelactate	glycerophosphoethanolamine		
lactate	glycerophosphoinositol		
N-acetylglutamate	glycerophosphorylcholine (GPC)		
N-acetylproline	glycerophosphoserine		
N-acetylthreonine	gulonate		
N-acetyltryptophan	homoarginine		
N-acetyltyrosine	hypotaurine		
N-carbamoylaspartate	malate		
N-formylmethionine	methionine sulfone		
nicotinamide riboside	N1-methyladenosine		
nicotinate ribonucleoside	N6,N6,N6-trimethyllysine		
orotate	N-acetylalanine		
phenyllactate (PLA)	N-acetyl-isoputreanine		
phenylpyruvate	N-acetylneuraminate		
phosphate	N-monomethylarginine		
pipecolate	ornithine		
p-toluic acid	palmitoyl sphingomyelin (d18:1/16:0)		
sedoheptulose	pantothenate		
thioproline	p-cresol sulfate		
thymine	phenol sulfate		
trimethylamine N-oxide	pseudouridine		
uracil	pyridoxal		
uridine	ribonate		
1-palmitoyl-2-linoleoyl-GPC (16:0/18:2)	sulfate		
	taurine		
	trans-4-hydroxyproline		
	2'-deoxycytidine		
	allantoin		
	benzoate		
	glycerophosphoglycerol		
	hypoxanthine		
	lactate		
	nicotinamide riboside		
	1-methylnicotinamide		
	argininosuccinate		
	betaine		
	gamma-glutamylthreonine		
	glucuronate		
	mannitol/sorbitol		

	N1-methylinosine		
	N-acetylleucine		
	pyridoxamine		
	pyridoxate		
	urate		
	4-acetamidobutanoate		
	4-guanidinobutanoate		
	gamma-glutamyl-alpha-lysine		
	glycine		
	guanine		
	kynurenine		
	nicotinamide ribonucleotide (NMN)		
	phenol red		
	phenylacetylglycine		
	proline		
	pyridoxine (Vitamin B6)		
	succinate		
	thiamin (Vitamin B1)		
	threonate		

Tab.S1B: Metabolites of the clusters 5-8 shown in Fig. 5B (6 hpi).

Cluster 5 Produced by *C. albicans*	**Cluster 6** Inconclusive	**cluster 7** Consumed by *L. rhamnosus* when on IECs	**Cluster 8** Secreted by *L. rhamnosus* alone
2,3-dihydroxyisovalerate	2-oxoarginine	adenine	N-acetylleucine
4-hydroxyphenylpyruvate	3-hydroxybutyroylglycine	arabitol/xylitol	alpha-ketoglutaramate
folate	5-oxoproline	asparagine	argininate
glutarate (C5-DC)	adenosine 5'-monophosphate (AMP)	cysteine-glutathione disulfide	diacetylspermidine
kynurenate	biotin	fructosyllysine	glutamine conjugate of C6H10O2 (2)
glycerol	choline	gamma-glutamylvaline	malonate
	cytidine 5'-monophosphate (5'-CMP)	glucose	methylsuccinate
	HEPES	lysine	mevalonate
	myo-inositol	phenylalanine	mevalonolactone
	N-acetylasparagine	ribitol	N-acetylisoleucine
	N-acetylaspartate (NAA)	riboflavin (Vitamin B2)	N-acetylphenylalanine
	N-acetylhistidine	threonine	N-acetylputrescine
	pyroglutamine	thymidine	N-acetylserine
	tryptophan	tyrosine	pyruvate

Tab.S2A: Metabolites of the clusters 1-4 shown in Fig. S1 (12 hpi).

Cluster 1 Secreted in synergy between IECs and *L. rhamnosus*	**Cluster 2** Secreted by IECs	**Cluster 3** Inconclusive	**Cluster 4** Secreted by IECs and consumed by *L. rhamnosus*
(N(1) + N(8))-acetylspermidine	1-carboxyethylisoleucine	1-palmitoyl-2-linoleoyl-GPC (16:0/18:2)	1-ribosyl-imidazoleacetate
1-carboxyethyltyrosine	1-carboxyethylleucine	3-hydroxybutyroylglycine	3-methyl-2-oxobutyrate
2'-deoxyinosine	1-carboxyethylphenylalanine	benzoate	3-methyl-2-oxovalerate
2-hydroxy-3-methylvalerate	1-carboxyethylvaline	cholesterol	4-methyl-2-oxopentanoate
2-hydroxy-4-(methylthio)butanoic acid	1-methylnicotinamide	imidazole lactate	5-methylthioadenosine (MTA)
2-hydroxybutyrate/2-hydroxyisobutyrate	1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	methylsuccinate	acetylcarnitine (C2)
2-hydroxyglutarate	1-stearoyl-2-oleoyl-GPC (18:0/18:1)	N-acetylaspartate (NAA)	aconitate [cis or trans]
2R,3R-dihydroxybutyrate	2'-deoxyuridine	N-acetylproline	carnitine
3-(4-hydroxyphenyl)lactate	2'-O-methylcytidine		carnosine
3-formylindole	3-hydroxy-3-methylglutarate		citrate
3-hydroxyoctanoate	3-hydroxybutyrate (BHBA)		deoxycarnitine
3-indoleglyoxylic acid	3-hydroxyisobutyrate		fructose
3-ureidopropionate	4-imidazoleacetate		gamma-glutamylalanine
5-methyluridine (ribothymidine)	7-methylguanine		gamma-glutamylglutamine
alpha-hydroxyisocaproate	alanine		gamma-glutamylhistidine
alpha-hydroxyisovalerate	allantoin		gamma-glutamylisoleucine
cytidine	alpha-ketoglutarate		gamma-glutamylleucine
cytosine	beta-alanine		gamma-glutamylmethionine
dihydroorotate	betaine		gamma-glutamylserine
glycerate	creatine		gamma-glutamylvaline
indolelactate	creatinine		hippurate
mevalonate	cystathionine		isocitrate
mevalonolactone	dimethylarginine (SDMA + ADMA)		N-acetylglucosamine/N-acetylgalactosamine
N-acetylglutamate	erythronate		N-acetylmethionine
N-acetylthreonine	ethylmalonate		nicotinamide
N-acetyltryptophan	fumarate		S-1-pyrroline-5-carboxylate
N-acetyltyrosine	gamma-glutamylthreonine		xanthine
N-carbamoylaspartate	gulonate		

nicotinate ribonucleoside	homoarginine		
phenyllactate (PLA)	hypotaurine		
pipecolate	lactate		
sedoheptulose	malate		
thymine	methionine sulfone		
uracil	N1-methyladenosine		
uridine	N6,N6,N6-trimethyllysine		
	N-acetylalanine		
	N-acetyl-isoputreanine		
	N-acetylneuraminate		
	ornithine		
	palmitoyl sphingomyelin (d18:1/16:0)		
	pantothenate		
	p-cresol sulfate		
	phenol sulfate		
	pseudouridine		
	pyridoxal		
	pyridoxamine		
	pyridoxate		
	ribonate		
	sulfate		
	taurine		
	trans-4-hydroxyproline		
	urate		
	choline phosphate		
	glycerol 3-phosphate		
	glycerophosphoethanolamine		
	glycerophosphoinositol		
	glycerophosphorylcholine (GPC)		
	glycerophosphoserine		
	2'-deoxycytidine		
	arabonate/xylonate		
	argininosuccinate		
	benzoate		
	cholesterol		
	erythritol		
	glucuronate		
	glycerophosphoglycerol		
	hypoxanthine		
	mannitol/sorbitol		
	N1-methylinosine		
	N-acetylleucine		
	nicotinamide riboside		
	N-monomethylarginine		

Tab.S2B: Metabolites of the clusters 5-8 shown in Fig. S1 (12 hpi).

Cluster 5 Secreted by *C. albicans*	**Cluster 6** Secreted by *L. rhamnosus* alone	**Cluster 7** Consumed by *L. rhamnosus* alone when on IECs	**Cluster 8** Present when *C. albicans* infects IECs
2'-deoxyadenosine	adenosine	fructosyllysine	indoleacetate
3-sulfo-L-alanine	argininate	glucose	isovalerate (i5:0)
4-methylthio-2-oxobutanoate	diacetylspermidine	adenine	cysteine-glutathione disulfide
arabitol/xylitol	gluconate	arginine	nicotinamide ribonucleotide (NMN)
kynurenate	glutamine conjugate of C6H10O2 (2)	asparagine	
phenylalanylhydroxyproline	inosine	biotin	
2,3-dihydroxyisovalerate	malonate	cystine	
benzoate	N-acetylisoleucine	glutamine	
glycerol	N-acetylphenylalanine	HEPES	
imidazole lactate	N-acetylserine	isoleucine	
N-acetylleucine	phosphothreonine	leucine	
N-acetylputrescine		lysine	
ribitol		mannitol/sorbitol	
adenosine		mannose	
alpha-lipoate		methionine	
aspartate		phenylalanine	
cysteine s-sulfate		serine	
		threonine	
		tryptophan	
		valine	

Tab.S3: Abbreviations used in Fig.6

Abbreviation	Full name
ACO	Aconitate Hydratase
AKGDH	alpha-Ketoglutarate Dehydrogenase
ALPL	2-Acetolactate Pyruvate-Lyase
ccoN	Cytochrome c Oxidase
CS	Citrate Synthase
DLD	D-lactate dehydrogenase
ENO	Enolase
FBA	Fructose-Bisphosphate Aldolase
fbcH	Ubiquinol-Cytochrome c Reductase
FBP	Fructose-1,6-Bisphosphatase
FUM	Fumarate Hydratase
G6PD	Glucose-6-phosphate 1-Dehydrogenase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GPI	Glucose-6-Phosphate Isomerase
HK	Hexokinase
IDH	Isocitrate Dehydrogenase
ISL	Isocitrate Lyase
MDH	Malate Dehydrogenase
MS	Malate Synthase
nuoA	NADH-Quinone Oxidoreductase
PCK	Phosphoenolpyruvate Carboxykinase
PDH	Pyruvate Dehydrogenase
PFK	6-Phosphofructokinase
PGAM	2,3-Bisphosphoglycerate-dependent Phosphoglycerate Mutase
PGD	6-Phosphogluconate Dehydrogenase
PGK	Phosphoglycerate Kinase
PGL	6-Phosphogluconolactonase
PK	Pyruvate Kinase
PMA	Plasma Membrane ATPase
RPE	Ribulose-Phosphate 3-Epimerase
RPI	Ribose 5-Phosphate Isomerase
SDH	Succinate Dehydrogenase
SUCD	Succinyl-CoA Synthetase
TAL	Transaldolase
TK	Transketolase
TPI	Triosephosphate Isomerase

2.4. Manuscript IV: Maurer *et al*., Biomaterials, 2019

A three-dimensional immunocompetent intestine-on-chip model as *in vitro* platform for functional and microbial interaction studies

Michelle Maurer, Mark S. Gresnigt, **Antonia Last**, Tony Wollny, Florian Berlinghof, Rebecca Pospich, Zoltan Cseresnyes, Anna Medyukhina, Katja Graf, Marko Gröger, Martin Raasch, Fatina Siwczak, Sandor Nietzsche, Ilse D. Jacobsen, Marc Thilo Figge, Bernhard Hube, Otmar Huber, and Alexander S. Mosig

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Summary:

Host-microbe interactions are mainly studied *in vitro* in models that can provide a detailed insight into interactions but do not represent physiological tissue, whereas *in vivo* models provide physiological conditions but lack many advantages of *in vitro* models. To expand the field of *in vitro* models and to mimic the physiological situation of the human intestine, a near-physiological intestine-on-chip model was developed and described in this publication. The model consists of a two-chamber chip system, separated by a porous membrane. The luminal side includes intestinal epithelial cells that differentiate to a three-dimensional cell layer and form microvilli under flow. The vascular side is mimicked by an endothelial cell layer, and additional mucosal macrophages and dendritic cells create an immunocompetent environment. A treatment with lipopolysaccharides on the luminal side resulted in immune tolerance. To emulate the natural microbiota of the intestine artificially, the model was colonized with the commensal bacterium *Lactobacillus rhamnosus.* The bacterium was tolerated by the immune cells and exhibited its antagonistic potential towards the opportunistic fungal pathogen *Candida albicans* by reducing the fungus' growth and its translocation from the luminal to the vascular side in the intestine-on-chip model.

Own contribution:

Antonia Last performed intestine-on-chip experiments with *L. rhamnosus* and *C. albicans* for the publication and wrote parts of the manuscript.

Estimated authors' contributions:

Michelle Maurer	51 %
Mark S Gresnigt	5 %
Antonia Last	5 %
Tony Wollny	3 %
Florian Berlinghof	1 %
Rebecca Pospich	1 %
Zoltan Cseresnyes	6 %
Anna Medyukhina	6 %
Katja Graf	1 %
Marko Gröger	3 %
Martin Raasch	1 %
Fatina Siwczak	1 %
Sandor Nietzsche	1 %
Ilse D Jacobsen	1 %
Marc Thilo Figge	1 %
Bernhard Hube	1 %
Otmar Huber	2 %
Alexander S Mosig	10 %

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A three-dimensional immunocompetent intestine-on-chip model as *in vitro* platform for functional and microbial interaction studies

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ABSTRACT

Alterations of the microbial composition in the gut and the concomitant dysregulation of the mucosal immune response are associated with the pathogenesis of opportunistic infections, chronic inflammation, and inflammatory bowel disease. To create a platform for the investigation of the underlying mechanisms, we established a three-dimensional microphysiological model of the human intestine. This model resembles organotypic microanatomical structures and includes tissue resident innate immune cells exhibiting features of mucosal macrophages and dendritic cells. The model displays the physiological immune tolerance of the intestinal lumen to microbial-associated molecular patterns and can, therefore, be colonised with living microorganisms. Functional studies on microbial interaction between probiotic *Lactobacillus rhamnosus* and the opportunistic pathogen *Candida albicans* show that pre-colonization of the intestinal lumen of the model by *L. rhamnosus* reduces *C. albicans*-induced tissue damage, lowers its translocation, and limits fungal burden. We demonstrate that microbial interactions can be efficiently investigated using the *in vitro* model creating a more physiological and immunocompetent microenvironment. The intestinal model allows a detailed characterisation of the immune response, microbial pathogenicity mechanisms, and quantification of cellular dysfunction attributed to alterations in the microbial composition.

1. Introduction

Commensal microorganisms of the intestinal microbiota support the digestion and absorption of nutrients by the gut. Microbial colonization is supported by the host *via* a mucus layer secreted by epithelial cells organized in a complex tissue comprising villi and crypts that form a tight and protective barrier between the microbiota and the circulation. A physiological communication between the members of the intestinal microbiota and their host is crucial for the maintenance of homeostasis in the human body. Thus, dysregulation and imbalance of these interactions known as dysbiosis are directly associated with the development of human diseases, including diabetes [1], obesity [2], inflammatory bowel disease (IBD) [3], cancer [3], depression [4] and non-infectious inflammatory diseases caused by opportunistic pathogenic fungi [5].

However, current knowledge on the impact of the microbiota on health is based solely on descriptive and correlative studies. So far, the lack of suitable experimental models prevented mechanistic studies on the complex cellular and molecular signalling processes within the microbiota under physiological conditions. Current *in vitro* models lack

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the required tissue complexity with major limitations for the co-culture of living bacteria with human intestinal cells. On the other hand, animal models have limitations in the transferability to the human situation, since the composition of the microbiota, as well as immune system considerably varies between men and mice and even between individual mouse strains [6]. Established cell lines, such as Caco-2 cells, cultured as monolayers on extracellular matrix-coated standard plastic dishes or transwell inserts are frequently used to study human intestinal epithelial cells *in vitro*. Although these two-dimensional culture techniques have elucidated numerous aspects of intestinal epithelial cell biology, it should be noted that they do not recreate typical microanatomical structures of the human intestine.

The intestine is the primary site for interaction of the gut microbiota consisting of commensal microorganisms and opportunistic pathogens [7]. The continuous exposure to microbial-associated molecular patterns (MAMPs) in the intestinal lumen requires the immune system to weigh tolerogenic and protective responses continuously. It is becoming increasingly clear that dysregulation of this tolerant immune response and alterations in the gut microbiota contribute to chronic inflammatory diseases and breakdown of the gut barrier [3,8]. Although three-dimensional organoid cultures allow the *in vitro* differentiation of microanatomical structures in high detail [9], these models have limitations in their ability to reflect a tolerant immune response to the colonising microbiota. Recently a microfluidically perfused model of the human intestine has been described that allows circulation of peripheral blood mononuclear cells (PBMCs) and effectively recapitulates structural features of intestinal microanatomy and physiology, including a polarised intestinal tissue differentiation [10,11]. Although this system represents a major advancement in emulating the human intestine, models are still required that are capable of stably cultivating commensal microbiota in an immune responsive environment and consider the balanced response to the microbiota and its metabolites.

The Toll-like receptor 4 agonist lipopolysaccharide (LPS) derived from gram-negative bacteria is a ubiquitously present MAMP in the human intestinal lumen [12]. However, upon dysfunction of the intestinal barrier, LPS can translocate into the circulation and in more substantial amounts eventually triggers a systemic inflammatory response syndrome associated with multiple organ failure [13]. Phagocytes play a critical role in gatekeeping MAMPs between the intestinal lumen and the circulation. In mice, it has been shown that tissue-resident phagocytes such as mucosal macrophages (mMphs) and dendritic cells (DCs) are central players in the release of inflammatory cytokines [14]. Both cell types populate the normal human intestinal mucosa but play distinct complementary roles in the selective local immune response and tissue homeostasis with mMphs as the most abundant mononuclear phagocytes in the intestinal lamina propria [15]. They account for most of the uptake of microbes that cross the epithelial barrier and are required to maintain an anti-inflammatory milieu in the mucosa by scavenging of apoptotic and damaged cells [16,17]. The expression of G protein-coupled chemokine receptor CX3CR1 on mMphs has been demonstrated as a central receptor in regulating intestinal barrier integrity [18,19]. The homeostatic function of $CX3CR1^+$ mMphs is complemented by $CD103^+$ DCs that represent another primary antigen-presenting cell type of the intestine [20]. These cells continually sample their environment for antigens derived from food, microbiota, and self-antigens [21] by extension of transepithelial dendrites into the intestinal lumen [22]. During homeostasis, intestinal DCs are considered to be tolerogenic, whereas mMphs are actively involved in tissue remodelling.

To resemble these conditions *in vitro*, we developed a three-dimensional model of the human intestine composed of endothelial and epithelial cell layers forming organotypic microanatomical villus- and crypt-like structures. For the first time, we demonstrate physiological interactions between epithelial and endothelial cells in an immunocompetent environment. This is created by tissue resident mMphs and DCs in a microphysiological model resembling essential conditions within the human intestine. We studied the biological intestinal homeostasis, barrier functionality and immunotolerance to stimulation with LPS at the intestinal luminal side of the model. To emulate dysregulation, imbalance and inflammation, the endothelial side was stimulated with LPS in a model of endotoxemia. Furthermore, we colonized the intestinal model with non-damaging living bacteria (*Lactobacillus rhamnosus*). Similar to *in vivo,* where the intestinal microbiota antagonizes microbial associated pathogenicity by limiting overgrowth and translocation through the intestinal epithelial barrier, we validated *L. rhamnosus'* antagonistic interactions on the opportunistic pathogenic fungus *Candida albicans* in our intestinal model.

2. Material and methods

2.1. Cell isolation and culture

Endothelial cells: Human umbilical cord vein endothelial cells (HUVECs) were isolated and seeded at a density of 2.5×10^4 cells/cm^2 in Endothelial Cell Medium (ECM) (Promocell, Heidelberg, Germany) up to passage 4 as described previously [23]. The study was approved by the ethics committee of the Friedrich Schiller University Jena (2018–1052), and all donors were informed about the aim of the study and gave written consent.

Caco-2 cells: The human epithelial colorectal cell line Caco-2 was kindly provided by the Institute for Laboratory Medicine, Clinical Chemistry and Pathobiochemistry, Charité - University Medicine Berlin. Cells were cultured in DMEM (GIBCO Darmstadt, Germany) supplemented with 10% foetal calf serum (FCS, Life Technologies), 20 ng/ml gentamicin (Invitrogen), 1% MEM non-essential amino acids (GIBCO). 5×10^5 Caco-2 cells are seeded in a 25 cm^2 flask and cultured in DMEM medium (Life Technologies) containing 10% FCS. The medium was changed every three to four days and cells used from passage 30 to 50.

Peripheral blood mononuclear cells and primary macrophages: PBMCs of three different healthy donors were isolated by Ficoll density gradient centrifugation as described previously [66] and seeded in 6- well plates with a density of 1.0×10^6 cells/cm^2 in X–VIVO 15 medium (Lonza, Cologne, Germany) supplemented with 10% (v/v) autologous human serum, 10 ng/ml human granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, Hamburg, Germany), 10 ng/ml M-CSF (PeproTech), 100 U/ml penicillin and 100 µg/ml streptomycin (Pen/Strep) (Thermo Fisher, Darmstadt, Germany). After 1 h incubation in a humidified cell incubator at 5% CO_2 and 37 °C the cells were washed twice with X–VIVO 15 medium and cultivated for 24 h before subcultured with endothelial cells in the biochip were terminal macrophage differentiation was performed in the supplemented X–VIVO 15 medium.

2.2. Biochip cell culture

MOTiF biochips made from polystyrol (PS) were obtained from microfluidic ChipShop GmbH (Jena, Germany). Biochips were manufactured by injection moulding as described elsewhere [23]. The chamber above the membrane has a height of 700 µm; the chamber under the membrane has a height of 400 µm. The width of the afferent and efferent channels is 0.8 mm and 2 mm, respectively. The height of these channels is 0.6 mm and 0.4 mm, respectively. Upper and the lower chamber including channel systems have a volume of 220 µl and 120 µl, respectively. A 12 µm thin polyethylene terephthalate (PET) membrane with a pore diameter of 8 µm and a pore density of 1×10^5 pores/cm^2 (TRAKETCH Sabeu, Radeberg, Germany) was integrated. An area of 1,1 cm^2 is available for cell culture. Chips and channels structures were sealed on top and bottom side with an extruded 140 µm thin PS foil using a low-temperature proprietary bonding method. Gas permeable silicon tubing was used for perfusion allowing oxygen equilibration during experiments. Additionally, PS bulk material and 140 µm thin PS bonding foil allowed re-diffusion of oxygen. Ramping

structures have been introduced into the chip bulk for prevention of unfavourable flow conditions and trapping of stationary air bubbles. For hydrophilization of the whole surface, the biochips were treated with oxygen plasma.

Intestine-on-chip models were assembled by staggered seeding of endothelial and epithelial cell layers. HUVECs were seeded with a density of $1.5 \times 10^5\,cm^{-2}$ in the upper chamber of a sterilised biochip in EC medium. Following 48 h of culture, macrophages were seeded on top of the confluent HUVEC cell layer with a density of $5 \times 10^4\,cm^{-2}$ in M199 medium (Life Technologies) supplemented with 10% FCS), 10% (v/v) autologous serum, 68×10^{-7} M L-glutamine (Sigma-Aldrich), 25 µg/mL heparin (Sigma- Aldrich), 7.5 µg/ml endothelial mitogen (Thermo Fisher), 5 µg/ml ascorbic acid (Sigma-Aldrich), 10 ng/ml GM-CSF, 10 ng/ml M-CSF and Pen/Strep. After 72 h of static culture and daily medium exchange, Caco-2 cells were seeded in the bottom chamber of the biochip with a density of $4{,}5 \times 10^5\,cm^{-2}$ and cultured in DMEM. The inlets of each biochip were sealed and the whole intestinal model cultured upside down for 24 h under static conditions. Subsequently, the fully assembled organ-on-chip model was used in experimental series as described. The endothelial side of the model was perfused with M199 medium with a flow rate of 50 µl/min (shear stress: 0.07 Pa). The luminal side of the model was perfused with 50µl/min (shear stress: 0.01 Pa). For LPS stimulation cells were treated with 100 ng/ml LPS (Sigma-Aldrich, Taufkirchen, Germany). Supernatants were collected and replaced with fresh medium containing 100 ng/ml LPS after 24 h. Supernatants were collected again after an additional 24 h. Cells were cultured in a humidified cell incubator at 5% CO2 and 37 °C.

2.2.1. Microbial strains and culture conditions

L. rhamnosus ATCC 7469 was grown on Man, Rogosa, and Sharpe (MRS) agar plates (Carl Roth, Karlsruhe, Germany) at 37 °C and 1% O_2. For use in experiments, bacterial cells were grown at 37 °C without agitation in MRS broth (Carl Roth, Karlsruhe, Germany). Prior to experiments, lactobacilli were collected by centrifugation, washed twice in PBS and diluted to an optical density OD_{600nm} of 0,5 in DMEM.

The *C. albicans* wild-type strain SC5314 was grown on YPD plates (2% peptone, 1% yeast extract, 2% glucose, 2% agar) at 30 °C. For use in experiments, *C. albicans* cells were grown overnight (o/n) in YPD medium (2% peptone, 1% yeast extract, 2% glucose) at 30 °C and 180 rpm. Prior to infection, yeast cells from an o/n culture were collected by centrifugation, washed two times with phosphate-buffered saline (PBS), the cell number was determined using a Neubauer chamber system, and adjusted to 5×10^3 cells/120 µl in DMEM.

Host cells were cultured in antibiotic-free medium for 72 h. After 24 h of LPS treatment, the bacterial suspension was inoculated onto the epithelial layer and incubated for 30 min to allow attachment to the epithelium. Subsequently, unattached cells were removed and PBMCs inoculated. The endothelial layer was cyclically perfused with a flow-rate of 50µl/min (shear stress: 0.07Pa) while the luminal chamber was linearly perfused with 25µl/min (shear stress: 0.03Pa). After 24 h of culture in the chip, supernatants were plated on MRS agar (Carl Roth). The membrane was lysed in 0.2% Triton-X 100 for 15 min at RT and removed before the suspension was pelleted, washed with PBS and plated.

2.3. Immunofluorescence staining

Cells were fixed with either 4% paraformaldehyde for 10 min at room temperature or methanol for 20 min at −20 °C. For permeabilization and blocking of unspecific binding sites, cells were incubated in PBS including 0.1% saponin (Sigma Aldrich) and 3% goat serum (Dianova, Hamburg). Staining was performed with antibodies against CD68, CD103, E-cadherin, VE-cadherin, β-Catenin (BD Biosciences, Heidelberg, Germany), occludin, ZO-1 (Invitrogen, Karlsruhe, Germany), CEACAM-1, CX3CR1, CYP3A4 (Merck-Millipore, Schwalbach, Germany), α-defensin (abcam, Cambridge, UK), mucin 2 (Acris, Herford, Germany), villin (Santa Cruz Biotechnology, Heidelberg, Germany), von Willebrand factor (Dako, Hamburg, Germany) and secondary antibodies goat-anti-mouse-Cy3, goat-anti-rabbit-AF488, goat-anti-rabbit-AF647, DAPI (Invitrogen) and Phalloidin (ThermoFisher). Samples were embedded in fluorescent mounting medium (Dako). Imaging was performed with an AxioObserver Z1 fluorescence microscope equipped with an ApoTome-2 (Carl Zeiss AG, Jena, Germany). Images were analysed with ImageJ2 software (Fiji).

2.4. Scanning electron microscopy

Cells were fixed with 2.5% (v/v) glutaraldehyde in cacodylate buffer for 120 min. Afterwards, the samples were washed twice with cacodylate buffer for 10 min and dehydrated in ascending ethanol concentrations (30, 50, 70, 90 and 100%) for 10 min each. Subsequently, the samples were critical-point dried using liquid CO_2 and sputter coated with gold (thickness approx. 4 nm) using a SCD005 sputter coater (BAL-TEC, Liechtenstein) to avoid surface charging. Finally, the specimens were investigated with a field emission (FE) SEM LEO-1530 Gemini (Carl Zeiss NTS GmbH, Oberkochen, Germany).

2.5. Image analysis and quantification

Images were acquired as Z-stacks utilising a Zeiss Apotome microscope and were saved in the Zeiss native image format „CZI". The Apotome images were first processed by Zeiss' ZEN software to provide optical sectioning. Further preprocessing included deconvolution using Huygens Professional (SVI, Hilversum, Holland), applying the „Spinning disk" deconvolution module by setting the pinhole spacing at 4 µm, according to SVI guidelines. The deconvolved images were analysed using Imaris 9.2.1 (Bitplane, Zürich, Switzerland). Automated quantification of confocal fluorescence microscopy data was performed by customised software, which was implemented in the programming language "python" (https://www.python.org/) and is available upon request.

Cell-based analysis: The three-dimensional image data were used to identify individual cells applying nuclear staining as guidance. Here the „Cells" module of Imaris was applied, where the DAPI channel was selected to create the cell nuclei, whereas the E−(VE-) cadherin or the ZO-1 labelling channel provided information about the membrane location. The segmented nuclei served as seeds to assist the search for the cell membranes. After successful segmentation, the area between the nuclei and the cell membrane was identified as cytoplasm. Particle analysis was applied when necessary, where the intensity and morphometry of additional components (for example the von Willebrand factor) was characterised in the cytoplasmic area.

Membrane-based analysis: The 3D image stacks were preprocessed in Zen and Huygens Professional as described above. In analysing the (V) E-cadherin and ZO-1 distribution, cell membranes were segmented using the Surfaces module of Imaris 9.2.1. The deconvolved Z-stacks were locally thresholded using the Otsu algorithm with a 2-µm neighbourhood diameter preceded by Gaussian blurring with a spatial filter size of 0.4 µm. The segmented membrane objects were limited to items with sphericity in the range of 0.0–0.9 and volume above 100 voxels in order to exclude artefacts. Macrophages were segmented as surfaces objects in Imaris. Here the Gaussian blurring was executed with a filter size of 0.645 µm, and the local Otsu thresholding was applied at a 2.4-µm neighbourhood diameter. The macrophage volume was limited to the 60–1200 µm [3] range in order to avoid detecting fragments or large clusters.

Quantification: Statistical information was extracted from the segmented images in Imaris 9.2.1. Here we measured the mean and the standard deviation of the fluorescence intensity for all channels within all regions of the segmented surfaces and cells; the area, the volume and

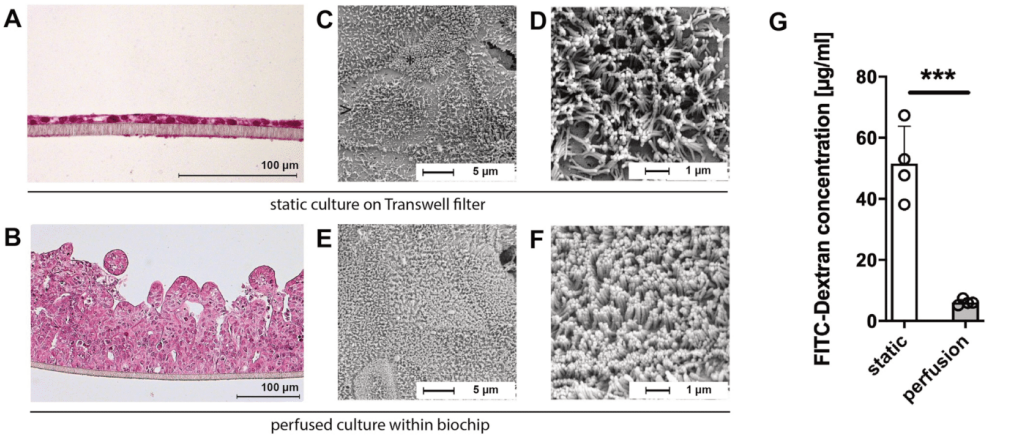

Fig. 1. Caco-2 cells form crypt and villus-like structures under perfusion conditions in the biochip. A, B) Histological H&E staining of Caco-2 cell layers cultured A) statically in the transwell, and B) under perfused conditions in the biochip. C-F) Scanning electron microscopy of Caco-2 cell layers cultured for 7 days under C, D) static conditions on transwell filters, and E, F) under perfusion conditions in the biochip. G) Permeability of epithelial cell layers for FITC-labelled dextran beads (3–5 kDa) under static and perfused conditions in the biochip. A-F) microscopy images are representative of three independent experiments, G) data from four independent experiments, data presentation shows the mean ± standard deviation., Statistical test was made using the Student's t-test, ****$p < 0.0001$.

the sphericity of the entire cells, the cell compartments and the cell membranes; the number of cells per Z-stack, etc. These analysis results were saved in Excel spreadsheets and used for statistical analysis and plotting.

Ellipticity index: The reconstructed surfaces were assigned a best-fitting ellipsoid in Imaris. According to the relationship amongst the main axis of the ellipsoid (a, b, c), we characterized the surfaces as prolate (a = b < c; cigar-shaped) and oblate (a < b = c; disk-shaped), by using the following indices:

$$e_{prolate} = \frac{2a^2}{a^2 + b^2} * \left(1 - \frac{a^2 + b^2}{2c^2}\right),$$

$$e_{oblate} = \frac{2b^2}{b^2 + c^2} * \left(1 - \frac{2a^2}{b^2 + c^2}\right).$$

2D analysis: Automated quantification of VE-cadherin staining in 2D was performed by customized software, which was implemented in the programming language Python. Prior to the analysis, images were preprocessed with a median filter (size 3 pixels) to remove spike noise. Then each image was thresholded at 100 intensity levels (out of 255, 8-bit) to obtain a binary mask of the cellular junctions. After that, connected regions were identified and regions with an area less than 20 µm [2] were assigned to a separate class of small particles. To evaluate the integrity of the cellular junctions, we computed the total area of cellular junctions, as well as the mean and maximum intensity of the cellular junctions. For computing the mean intensity, only those pixels were used that belonged to the foreground after the thresholding step. Moreover, we computed the total area and area fraction of the small particles. In the latter case, the area of the small particles was normalized by the total area of the cellular junctions.

2.6. Cytokine profiles

Supernatants were collected after indicated time periods and immediately frozen at −80 °C. Cytokines were detected using CBA assay (BD Biosciences) according to the manufacturer's protocol. Enhanced sensitivity flex set was used for the measurement of TNF and IL-1β release. Secretion of IL-6, IL-8 and IL-10 was analysed using standard CBA flex sets. The analysis was performed on a BD FACS-Canto II cytometer with FACSDiva software. Data analysis was performed using FCAP Array V3 software (Softflow, Pecs, Hungary).

2.7. Permeability assay

Fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich) with an atomic mass of 3–5 kDa was used. The medium in both chambers is replaced by preheated PBS. Subsequently, 150 µl FITC-dextran solution is added in the lower chamber containing Caco-2 cells and is incubated for 30 min at 37 °C. The biochip is turned upside down during incubation allowing FITC-dextran to permeate through the cell layer under static conditions. After washing of the lower chamber with PBS, the solution was collected from the upper chamber. All steps were performed protected from light.

2.8. Statistics

For each experiment replicates have been performed as indicated in the figure legend. Statistical analysis has been performed with GraphPad Prism 6.05 (GraphPad Software, La Jolla, CA, USA). For analysis of statistical significance, the tests indicated in the figure legend have been performed. A p-value < 0.05 was considered statistically significant.

3. Results

The epithelial cell layer of the intestinal model is formed by Caco-2 cells cultured on a 10 µm thin porous polyethylene terephthalate (PET) membrane suspended in the biochip and continuously perfused with cell culture medium. During cell culture, microfluidic perfusion ensures a constant removal of metabolic waste products and a continuous resupply with nutrients [23]. In contrast to conventional cell culture approaches such as transwell filters (Fig. 1 A), Caco-2 cells cultured in microfluidically perfused biochips form a self-organised three-dimensional cell layer with columnar epithelium (Fig. 2 B) and increased microvilli formation at the apical cell surface within 7 days of culture (Fig. 1C - F). Permeability measurements confirmed an increased barrier function of the three-dimensional cell layer compared to conventional cell culture under static conditions where only two-

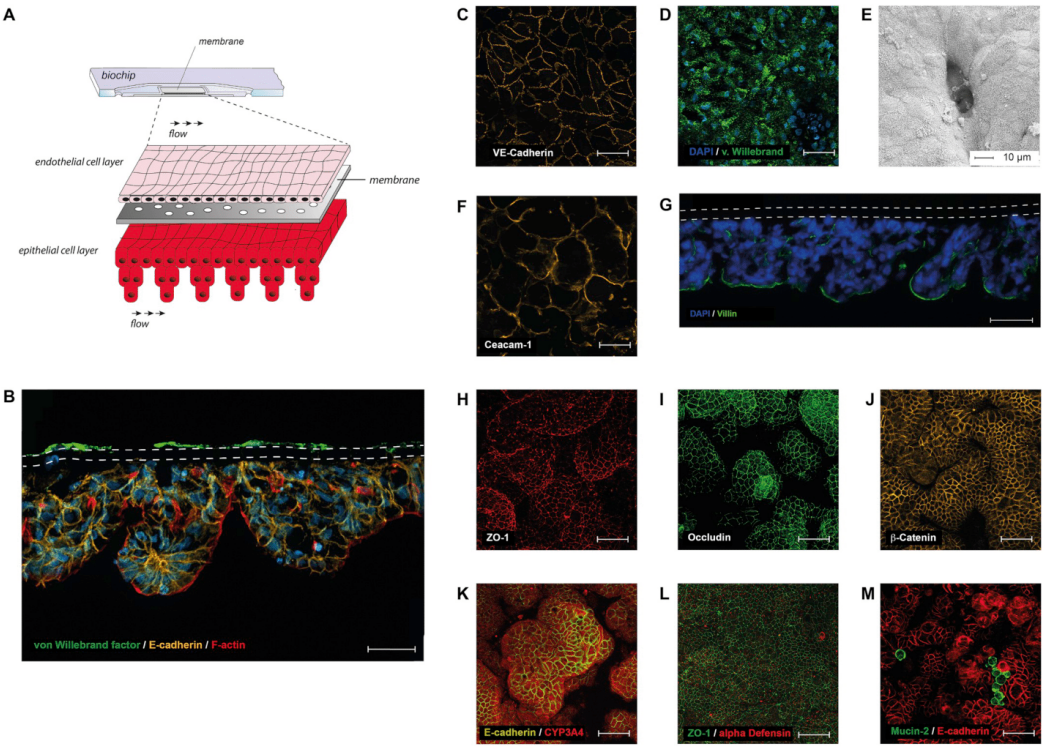

Fig. 2. Design of the intestine-on-chip model featuring organotypic microanatomy and expression of endothelial and intestinal epithelial cell type markers. A) A porous membrane suspended in the biochip serves as a scaffold for the multi-layered intestinal model composed of endothelial and epithelial cells. Microchannels integrated into the biochip separately perfuse both epithelial and endothelial layers at 50 µl/min. B) Cross-section of the three-dimensional intestinal model: endothelial cells express von Willebrand factor (green), epithelial cells express E-cadherin (orange) and F-actin (red). Both cell layers are separated by a porous membrane (dashed line). Actin filaments are stained with phalloidin (red). Scale bar 100 µm. Nuclei were stained with DAPI (blue). C-D) Endothelial cells form a confluent monolayer and express C) VE-cadherin (orange) and D) von Willebrand factor (green). E-N) Epithelial cell layer: E) Scanning electron microscopic image of the crypt-like structures. Expression of F) CEACAM-1 (orange); G) villin (green) (DAP blue, dashed lines marks membrane); H) ZO-1 (red); I) occludin (green); J) β-catenin (orange); K) E-cadherin (orange); CYP3A4 (red); L) α-defensin (red); ZO-1 (green); M) mucin-2 (green); E-cadherin (red); C-D, F-J) Scale bar 50 µm. Nuclei were stained with DAPI (blue). C-M) Representative images of four independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

dimensional Caco-2 monolayers are formed (Fig. 1G).

The multi-layered tissue model was separately perfused by two microchannels at the endothelial and the luminal side of the model (Fig. 2A). The vasculature of the intestine was mimicked by co-culture of HUVECs on the membrane opposite to the epithelial layer. Endothelial cells formed a confluent lining in the biochip that express endothelial cell marker proteins VE-cadherin (Supplementary Video S1) and von Willebrand factor (vWF) (Fig. 2B–D). In the epithelial cell layer, we observed the cellular self-organisation into three-dimensional structures with morphological features of crypts and villi that fully cover the membrane serving as cell substrate (Fig. 2 E-J, Supplementary Video S2). The formation of a three-dimensional Caco-2 cell layer upon microfluidic perfusion has also been reported by Ingber and colleagues, demonstrating the differentiation of these cells into a polarized columnar epithelium that contains cells with markers of absorptive, mucus-secretory, enteroendocrine, and Paneth cell populations [11]. Also in our model, villus-like structures appear polarised and express at the outer cell borders the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1, Fig. 2 F), a protein expressed by intestinal epithelial cells (IEC) *in vivo* at the apical surface of intestinal villi [24]. Further, villin, a protein controlling the shape and motility of IEC by interconnecting the brush borders to the cytoskeleton [25] was found expressed at the tip of villus-like structures (Fig. 2 G). These structures present themselves in a well-defined organisation expressing tight junction proteins ZO-1 and occludin (Fig. 2H, I), both critically involved in the maintenance of barrier function [26]. Further, β-catenin (Fig. 2 J), an essential regulator of epithelial cell proliferation and differentiation [27], and cytochrome P450 3A4 (CYP3A4, Fig. 2 K), a major CYP enzyme responsible for metabolizing over fifty percent of prescribed drugs [28] are stably expressed by the epithelial cell layer. In contrast to static culture, Caco-2 cells differentiate under perfusion conditions into cell-subtypes expressing specific cell type markers such as α-defensin, a marker of Paneth cells (Fig. 2 L) and Mucin-2, a marker protein of Goblet cells (Fig. 2M).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.biomaterials.2019.119396

The lack of tissue-resident mMphs and DCs is a limitation of most currently available *in vitro* gut models. These two cell types are required to initiate immune responses causing inflammation and to prevent pathogen dissemination, yet also for mediation of immunotolerance to

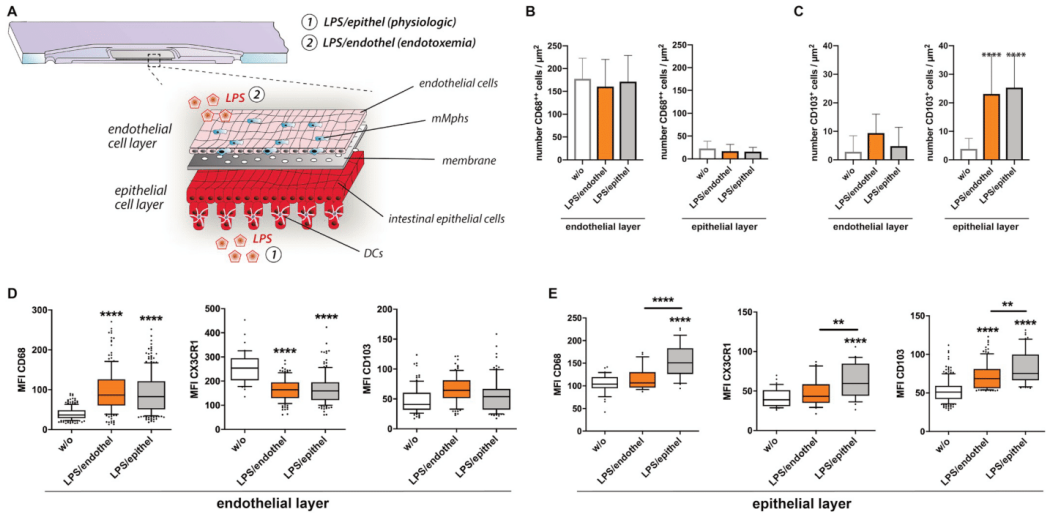

Fig. 3. Mobility and phenotype marker expression of mPCs upon LPS stimulation. A) The distribution pattern of mPCs with the majority of mMphs populating the endothelial cell layer and DCs residing in the epithelial cell layer. Two different LPS stimulation conditions were tested for recreating 1) physiological contact of epithelial cells with LPS; and 2) conditions of endotoxemia, with LPS exposed to the endothelial cell layer. B-C) Total number of B) $CD68^{++}$ cells or C) $CD103^{+}$ cells per µm [2] within the endothelial or epithelial cell layer of the non-stimulated model (w/o), and the model stimulated at the endothelial side (LPS/endothel) or the epithelial side (LPS/epithel). D-E) Mean fluorescence intensity (MFI) of CD68, CX3CR1 and CD103 expression by mPCs in the D) endothelial and E) epithelial cell layer. Whisker plots: 10th – 90th percentile, the box represents the 25th and 75th percentile with the line in the box marking the median. Data points outside whiskers mark outliers. A-D) Statistical testing with one-way ANOVA and Tukey's correction, $^{**}p < 0.01$, $^{****}p < 0.0001$ vs. non-stimulated condition (w/o); Data of five independent experiments is shown.

commensal bacteria and derived MAMPs in the gut lumen. To simulate these functions *in vitro*, human primary monocytes were seeded on the endothelial cell layer and differentiated in the presence of macrophage colony stimulating factor (M-CSF), and granulocyte-monocyte colony stimulated factor (GM-CSF). After 7 days of culture, monocytes differentiated into two phenotypically distinct monocyte-derived phagocyte subsets (mPCs) resembling features of mMphs and DCs, respectively. The functionality of these mPCs was studied in the presence of LPS, both at the endothelial and epithelial cell layer (Fig. 3 A). Expression of CX3CR1 with a high level of CD68 (CD68++) defined mMph surrogates, whereas DCs were defined by expression of lower CD68 levels ($CD68^{+}$) and the presence of the DC marker integrin alpha E (CD103). It has been reported that mMphs depend on CX3CR1 to be maintained within the lamina propria and to prevent translocation of commensal bacteria to mesenteric lymph nodes [29]. In accordance with these reports, we found that LPS exposure to the intestinal luminal side of the model triggered mMphs and DCs to self-organise within the tissue. LPS stimulation at the intestinal luminal side supported the formation of $CD68^{+}/CD103^{+}$ DCs and triggered DC invasion into the epithelial cell layer (Fig. 3C, D, E) with some of these DCs forming dendrites through the epithelial cell layer contacting the luminal space (Supplementary Figure 1). In mice, lamina propria-resident $CD103^{+}$ DCs were shown to migrate into the epithelium during homeostasis to facilitate capture of bacterial antigens [21]. In the intestinal model, a significantly increased expression of CD103 by DCs was observed at the luminal side upon epithelial cell stimulation (Fig. 3 D, E, Supplementary Figures 2 and 3). Thus, a physiological contact of epithelial cells with LPS contributed to improved DC maturation within the epithelial cell layer. LPS stimulation also upregulated CD68 and CX3CR1 in mMphs that predominately populate the endothelial cell layer (Fig. 3 B, D, E).

Endotoxemia represents a disease condition in which LPS leaks into the bloodstream. This condition was simulated by exposing the endothelial lining to LPS. Although we observed an upregulation of CD68 in mPCs, we were unable to find increased upregulation of CX3CR1 in mMphs within the epithelial cell layer as observed upon intestinal luminal LPS stimulation. Further, CD103 expression by DCs was significantly decreased in the epithelial barrier compared to LPS stimulation at the luminal side (Fig. 3 B - E). These results demonstrate guided mobility and adapted activation pattern of mPCs depending on the microenvironment created by the endothelial and epithelial cell layers with their individual responsiveness to LPS exposure.

Subsequently, we studied the responsiveness of mPCs depending on their differentiation and distribution pattern induced by the different LPS stimulation sites. In general, no adverse effects were observed on barrier functionality despite the presence of mPCs in the model, irrespective of the side of LPS exposure (Fig. 4 A). Under simulated physiological conditions with LPS present at the luminal side of the model, we observed immunotolerance reflected by the absence of a release of pro-inflammatory cytokines. However, emulated endotoxemia with LPS exposure to the endothelial layer induced a robust inflammatory response characterized by release of TNF, IL-1β, IL-6, and IL-8 (Fig. 4B–E).

The proinflammatory cytokines TNF, IL-1β, IL-6, and IL-8, are essential mediators of human intestinal inflammation and LPS-induced cell damage [30]. Moreover, these cytokines are critically involved in the loss of intestinal barrier function frequently observed in IBD [31,32]. A decrease in the expression of endothelial VE-cadherin, and E-cadherin/ZO-1 expressed by IECs is directly associated with intestinal permeability in IBD [33]. Similar observations were made in our intestinal model, were direct exposure of the endothelial lining to LPS significantly reduced expression of VE-cadherin with a diffuse and disrupted expression pattern along the cell borders. In addition, an analysis of endothelial cells by quantification of the cellular ellipsoid index (0 = line, 1 = circle) revealed significant morphological

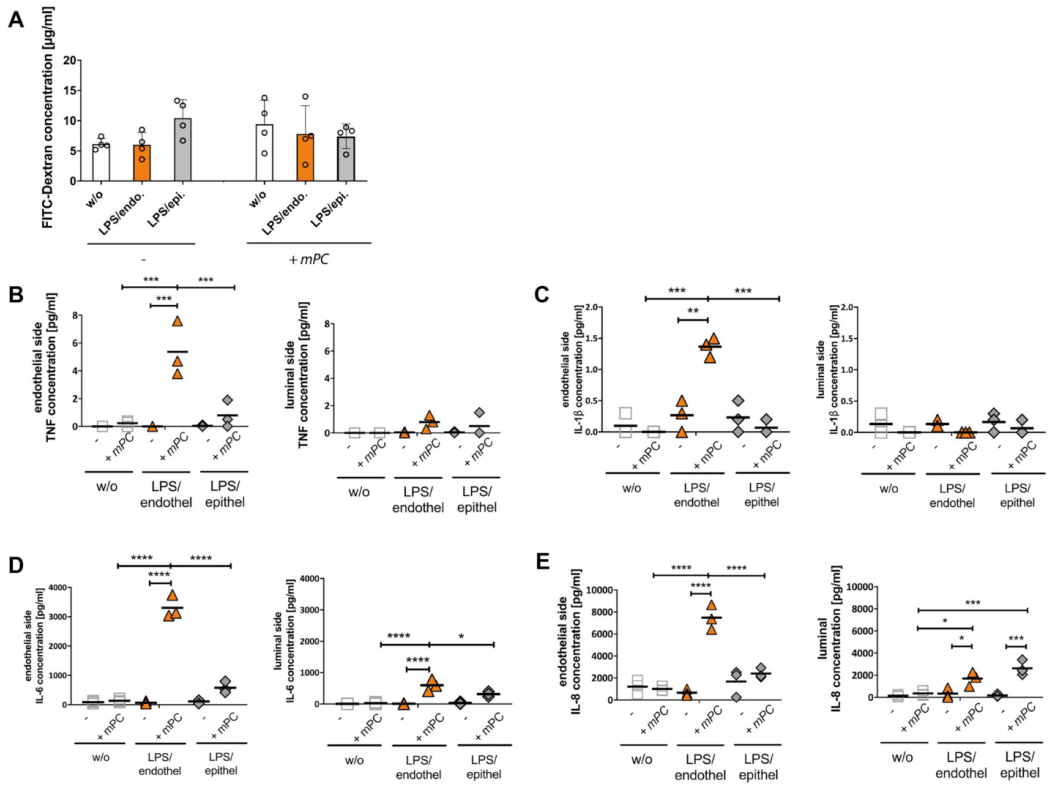

Fig. 4. Permeability and cytokine release upon LPS stimulation. A) Permeability of the intestinal model for FITC-dextran, and B-E) release of proinflammatory cytokines B) TNF, C) IL-1β, D) IL-6, and E) IL-8 at the endothelial or luminal side of the intestinal model without LPS stimulation (w/o), and upon LPS stimulation at the endothelial cell layer (LPS/endothel) or the epithelial cell layer (LPS/epithel) in absence (−) or presence (+) of mPCs. B-E) data is shown as scatter plots with mean values ± standard deviation, statistical significance was calculated by two-way ANOVA with Sidak's multiple comparisons test (****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05). Data of three independent experiments are shown.

alterations of endothelial cells directly exposed to LPS. Endothelial cells exposed to LPS appeared elongated with a diffuse VE-cadherin expression, indicating endothelial dysfunction [34]. In contrast, we observed an even increased VE-cadherin expression and no alterations of endothelial morphology when LPS was perfused at the intestinal lumen of the model (Fig. 5A and B; Supplementary Figure 4). Similar observations were made for E-cadherin and ZO-1 expression in the epithelial cell layer. For both proteins, we found an increased expression level when the epithelial cell layer was stimulated with LPS. However, the expression of E-cadherin and ZO-1 was significantly reduced when the endothelial cell layer was exposed to LPS (Fig. 5C, D; Supplementary Figs. 4B and C).

The observed immunotolerance at the luminal side represents a crucial physiological feature required for stable colonisation with living bacteria in the presence of functional mPCs. Therefore, we next colonised the luminal side with the probiotic bacterium *L. rhamnosus* in the presence of LPS at the luminal side resembling physiological conditions. Alternatively, LPS was added to the endothelial layer mimicking endotoxemia. We observed that independent from the side of LPS stimulation, *L. rhamnosus* stably colonised the epithelial cell layer without translocating through the tissue barrier (Fig. 6 A). Further, a trend towards an improved barrier function was observed upon luminal colonisation with *L. rhamnosus* (Fig. 6 B). Infection of the intestinal tract is often accompanied by infiltration of the intestine by leukocytes [35]. We therefore introduced circulating PBMCs into the vascular perfusion circuit of the endothelial compartment to investigate the potential immunomodulatory effects of *L. rhamnosus* in the intestinal model. Measurement of the cytokine release profiles for IL-1β, IL-6, IL-8, IL-10, and TNF in intestinal models colonised with *L. rhamnosus* showed a significant increase of cytokine release and PBMC adhesion upon LPS stimulation of the endothelial cell layer compared to LPS stimulation at the luminal side (Fig. 6C, D). Though, we observed only slightly increased IL-1β and IL-6 levels and no alterations of IL-8, IL-10 and TNF release associated with the luminal colonisation with *L. rhamnosus*.

L. rhamnosus was described among other probiotic bacteria to reduce enteric colonisation and infection with opportunistic pathogens. Multiple mechanisms, including nutritional competition, reduction of barrier permeability, and the modulation of the immune response have been discussed in this context [36,37]. We found that *L. rhamnosus* colonisation significantly increased the expression of E-cadherin and ZO-1 even in the presence of PBMCs (Fig. 6 E, F) and improved cell viability at the epithelial cell layer indicated by lower LDH release (Fig. 6 G). Lactobacilli have been shown to counteract infections of the gastrointestinal tract (GI) by opportunistic pathogens [38,39]. The

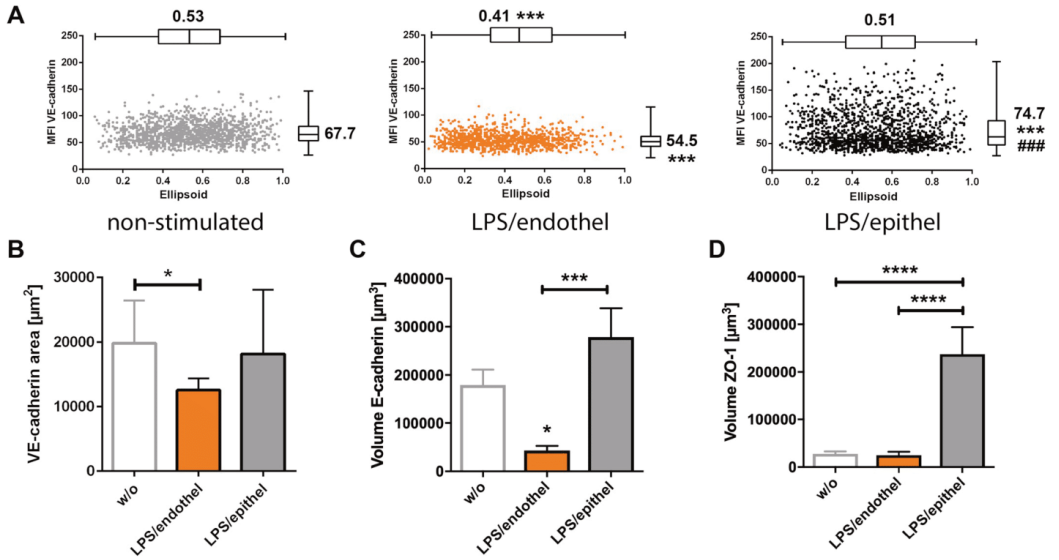

Fig. 5. Expression of VE-cadherin, E-cadherin and ZO-1 upon LPS stimulation. A) Expression pattern of VE-cadherin and endothelial cell morphology under non-stimulated conditions, and upon exposure of the endothelial layer to LPS (LPS/endothel) or the epithelial cell layer (LPS/epithel). Mean fluorescence intensity (MFI) of VE-cadherin expression is plotted for each individual cell analysed against its ellipsoid index (0 = line, 1 = circle). B) Quantification of VE-cadherin expression area in endothelial cells. C) Expression of E-cadherin and D) ZO-1 in the epithelial cell layers by z-stack image analysis and quantification as the volume of expressed protein levels. Statistical testing performed by one-way ANOVA with Tukey's correction; *p < 0.05, ***p < 0.001, ****p < 0.0001 vs. non-stimulated condition (w/o) or between indicated conditions. Data of four independent experiments are shown.

yeast *C. albicans* usually exists as a harmless commensal in healthy individuals [40]. However, it can also become a pathogen causing severe systemic infections as part of ultra-low diversity microbial communities in the gut, i.e. after antibiotic treatment of critically ill patients with suppressed immune function [41]. *L. rhamnosus* has been shown to interfere with colonization of *C. albicans* in the gut by limiting fungal growth [38,42]. However, most of these studies were performed in absence of epithelial cells [43,44] or in monolayered epithelial cell cultures [45–47]. We therefore studied the interaction of *C. albicans* with *L. rhamnosus* a more complex and immunocompetent microenvironment reflected by the intestine-on-chip model. The luminal side of the intestinal model was infected with *C. albicans*, and the impact of *L. rhamnosus* pre-colonization was analysed for the resulting fungal burden after 24 h of co-culture. We found that *L. rhamnosus* colonisation not only limit the growth of *C. albicans* at the luminal side of the model (Fig. 6H), but also reduces the translocation of the fungus over the intestinal barrier into the endothelial compartment emulating the vasculature (Fig. 6 I, J, K).

4. Discussion

The intestinal epithelium is one of the most dynamic and rapidly renewing tissues in the body, which requires continuous remodelling as well as disposal of effete cells. In order to simulate the human intestine as a stable and immunocompetent ecosystem, we developed a microfluidic organ-on-chip model forming a three-dimensional epithelial cell layer that can be colonised by living bacteria. We could demonstrate that the model, in contrast to monolayer cultures, exhibits near physiological 3D tissue architecture. Similar to normal intestinal epithelial our model demonstrates immunotolerance of resident mMphs and DCs to MAMPs (LPS) present in the intestinal lumen, whereas the presence of LPS in the vascular compartment elicited strong inflammatory responses. Further, we were able to include an artificial microbiota consisting of living lactobacilli that was shown to protect against invasion of the opportunistic pathogen *C. albicans* by lowering fungal burden overgrowth in the intestinal lumen and limiting fungal translocation through the gut barrier. Our observations are in agreement with reports from *in vivo* studies [38] and demonstrate the usefulness of the intestine-on-chip model as a platform for functional studies on microbial communication and host-microbe interaction *in vitro*.

A potential limitation of our model is the use of the intestinal epithelial Caco-2 cell line, isolated from a colorectal tumour [48]. However, the cell line was shown to exhibit features more similar to human small intestine [10,11] and to retain stem cell-like capabilities able to recreate microanatomical structures of the human intestine *in vitro* [11]. Caco-2 cells cultured under microfluidically perfused conditions can self-organise and differentiate into a polarised columnar epithelium containing cells with markers of absorptive, mucus-secretory, enteroendocrine, and Paneth cell populations [11], which is not observed in Caco-2 cells cultured under static conditions [49]. The use of perfused conditions is thus imperative to evolve a more physiological microenvironment facilitating proper cell differentiation and increased mucus secretion to provide a suitable substrate layer that could be efficiently populated by living bacteria. Moreover, microfluidic perfusion and related shear forces on cell layers have been recently shown to prevent detrimental bacterial overgrowth *in vitro* [50].

Recently the HuMiX system, a multichannel intestine chip consisting of Caco-2 cells has been described [51]. This system allowed substantial advances in modelling host-microbe interactions *in vitro* but lacks endothelial cells and an immunocompetent environment created by mPCs. Further, microorganisms cultured in the chip are separated by a nanoporous membrane from the epithelial cells preventing their direct interaction. To fully mimic host-microbe interaction direct interactions of epithelial cells, immune cells, and the microbes are required.

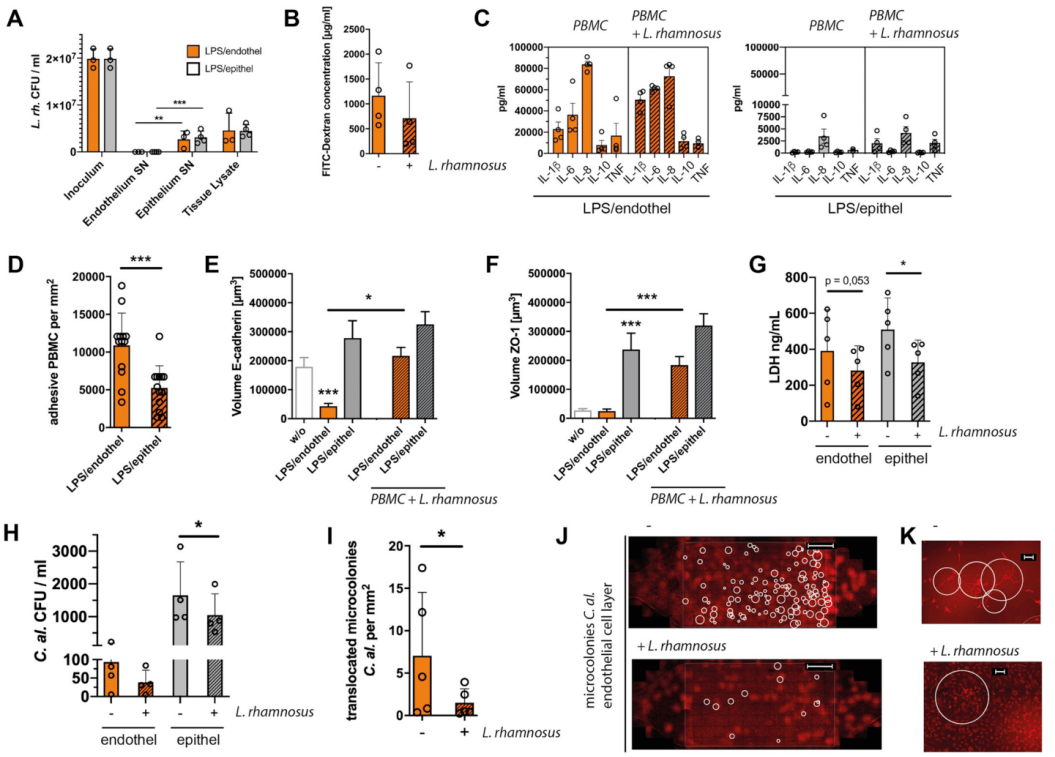

Fig. 6. Colonisation of the intestinal model with *L. rhamnosus* and *C. albicans*. A) Colony forming units (CFU) of *L. rhamnosus* from inoculated medium (Inoculum), in the supernatant at the endothelial side (Endothelium SN) or the luminal side (Epithelium SN), and in both cell layers after tissue lysis (Tissue Lysate). Colonisation was performed with LPS exposed to the endothelial side (LPS/endothel, orange bars) or LPS exposed to the epithelial cell layer (LPS/epithel, grey bars). B) Permeability assays with FITC-dextran in the model without *L. rhamnosus* (−) or *L. rhamnosus* colonised at the luminal side (+). C) Cytokine release in the supernatant at the endothelial side with endothelial cells stimulated with LPS (LPS/endothel) or epithelial cells stimulated with LPS (LPS/epithel). PBMCs were circulated at the endothelial side without *L. rhamnosus* (PBMC), and with *L. rhamnosus* colonising the luminal side of the model (PBMC + *L. rhamnosus*). D) Adhesive PBMCs in the presence of *L. rhamnosus* at the luminal side upon stimulation with LPS at the endothelial (LPS/endothel) or epithelial cell layer (LPS/epithel). E-F) Expression of E) E-cadherin and F) ZO-1 quantified as the volume in the epithelial cell layer. G) Release of LDH in the supernatant of the intestinal luminal side and vascular side with and without *L. rhamnosus* colonisation. H) Colony forming units (CFU) of *C. albicans* (*C. a.*) co-cultured without and with *L. rhamnosus* at the epithelial and endothelial side. I-K) C. Microcolonies formed by *C. albicans* upon translocation from the luminal side in absence or presence of *L. rhamnosus*. I) Quantified results of microcolony formation. J) low (scale bar 2 mm) and K) higher magnification (scale bar 100 µm) of formed microcolonies. J, K) Representative images of four independent experiments. Circles indicate identified microcolonies, fuzzy red background is caused by endothelial cells. E-F) A-C) Statistical testing with one-way ANOVA and Tukey's correction, *p < 0.05, ***p < 0.001 vs. non-stimulated condition (w/o) or between indicated conditions. D, G, H, I) Two-tailed ratio paired *t*-Test, *p < 0.05, ***p < 0.001 between indicated conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The self-formation of the three-dimensional epithelial cell layer in our model allows the colonisation of living microorganisms directly at the luminal side of the intestinal model. Further, the organotypic microenvironment supports the self-organization of mMphs and DCs guided by physiological LPS gradients ranging from the luminal to the vascular side. Already present at birth, mMphs share many characteristics with their monocyte-derived counterparts in the adult intestine including high-phagocytic activity [52]. These findings support the emerging view that instead of their origin, the location and the surrounding microenvironment determines the functions of tissue-resident macrophages [53,54]. After their arrival in the mucosa, invading monocytes undergo a process of local cell differentiation and acquire the expression of CX3CR1 and MHCII [55]. During homeostasis, resident intestinal mMphs are replenished continuously by circulating monocytes with up to 50% of mature mMphs [56]. Similar to the *in vivo* situation these cells were found to express high levels of CX3CR1 upon LPS stimulation of the intestinal lumen. These mMphs primarily populated the endothelial side of the model, whereas CD103 expressing DCs were mainly recruited to the luminal side of the epithelial cell layer [19,21]. *In vivo* CX3CR1^{+} mMphs initiate innate and adaptive immune responses to fungi in the intestine via expression of c-type lectin receptors [57]. Furthermore, it has been recently shown that CX3CR1^{+} mMphs are critically involved in limiting microbiota-induced intestinal inflammation. They promote tissue homeostasis by limiting expansion of microbe-specific T helper 1 (Th1) cells and support the generation of microbiota specific regulatory T-cells. Interestingly, this study also demonstrated that colonization with microbes that adhere to the epithelium can compensate for intestinal microbiota loss and activate homeostatic immunoregulatory mechanisms [19]. Under physiological conditions *in vivo* mMphs do not respond to inflammatory triggers such

as LPS allowing them to act as efficient scavengers without inducing inflammation that usually ensues upon encounter of MAMPs and would compromise intestinal homeostasis [58]. In the intestinal model, we could demonstrate that LPS is well tolerated at the luminal side of the model with physiological homeostasis in the presence of commensal *L. rhamnosus*.

The maintenance of epithelial integrity has been shown to rely on the function of mMphs and DCs that reduce epithelial susceptibility to inflammatory insults and drive intestinal epithelial cell renewal and differentiation [59,60]. Further, mMphs stimulate the differentiation of regulatory T-cells and thereby contribute to intestinal homeostasis [61]. Recruited monocytes can differentiate into mature mMphs under inflammatory conditions exerting repair functions as has been described for monocytes infiltrating the liver [62,63]. We were able to demonstrate that by integration of mMphs and DCs tissue homeostasis can by mediated and LPS is tolerated at the luminal model side. Further, primary leucocytes (PBMCs) can be perfused in the model in the presence of LPS without detrimental effects on immune cell activation.

Colonisation of the intestine with *L. rhamnosus* did not induce inflammatory responses by release of proinflammatory cytokines. Instead, colonization improved cell viability of the intestinal model and was associated with increased E-cadherin and ZO-1 expression in the epithelial layer under simulated conditions of endotoxemia. These observations are in agreement with recent *in vivo* studies in mice, were an improved E-cadherin expression was also induced by lactobacilli [36]. Although the mechanism of the barrier improvement remains speculative, a potential explanation might be cytoprotective induction of heat shock proteins that were reported to contribute to cell protection by modulation of tight junction protein expression [64]. Stable colonisation of an intestinal model with *L. rhamnosus* was also shown by Kim *at al* [10]. However, PBMCs circulated in the model caused injury of villi and the intestinal barrier through the release of IL-8 induced by LPS present at the luminal side [10]. Here we demonstrate that in presence of mMphs and DCs physiological immunotolerance to LPS can be established, without detrimental effects of immune cell activation. This allows endothelial PBMC perfusion and a stable co-culture of living bacteria and LPS at the luminal side of the model. A pro-inflammatory immune response associated with tissue damage was only observed in the endotoxemia model, with *L. rhamnosus* colonisation ameliorating inflammation-associated tissue damage.

In vivo, continuous checks and balances between immune tolerance and the induction of inflammatory responses upon translocation of microorganisms are required. We observed increased leukocyte recruitment to the endothelial cell layer when LPS was present at the vascular side of the model creating an inflammatory environment that resembles conditions of IBD. The number of adhesive PBMCs under these simulated disease conditions was significantly higher compared to the more physiological conditions when LPS was present at the luminal side of the model. These observations are in agreement with clinical reports from IBD patients where blockade of recruitment of leucocytes into the intestine represents a promising treatment option for the patient [65]. The model has proven a suitable platform to elucidate mechanisms of host-microbe interactions in an immunocompetent environment in studies on leucocyte recruitment, immune cell differentiation, and the establishment of an adapted immune response to commensal and pathogenic microorganisms. Nevertheless, further improvements will be performed in follow up studies. These improvements should consider the contribution of other immune cells on immune surveillance of microorganisms in the gut, i.e. B- and T-cells organised in lymphoid follicles of the Peyer's patches and within gut-associated lymphoid tissue.

We could further demonstrate the feasibility of our model to investigate microbial interactions between commensal and opportunistic microorganisms with functional consequences for the intestinal barrier under near-physiological and well-controlled conditions *in vitro*. We here show for the first time that *L. rhamnosus* colonisation can diminish invasion of *C. albicans* into the “bloodstream” in a microphysiological environment created by our intestine-on-chip system. Similar to the *in vivo* situation where the bacterial microbiota protects against *C. albicans* overgrowth [38,39], we could demonstrate a protective effect of probiotic *L. rhamnosus* limiting the overgrowth and translocation of *C. albicans* to the endothelial cell compartment. It is tempting to speculate that also luminal exposure to LPS contributes to a reduced translocation of pathogenic microbes into the vascular compartment by improving the intestinal barrier.

Our intestinal model has proven to be a valuable tool to systematically explore the underlying mechanisms of microbial communication, host-microbe interactions, microbial pathogenicity mechanisms, and immune cell activation under physiologically relevant conditions *in vitro*. Thus, it represents a powerful platform for the investigation of disease mechanisms, driven by (opportunistic) pathogens, i.e. under well-defined conditions of dysbiosis created in synthetic microbiomes that are composed of a limited number of microbial community members. Further, it allows the screening and development of novel treatment strategies for IBD by supporting and maintaining physiological conditions of the human microbiota that keep opportunistic pathogens in their commensal state and prevent the onset of related inflammatory diseases.

Author contributions

M.M., T.W., F.B., M.G., M.R., F.S. contributed experiments for organ-on-chip development. M.M., M.S.G., A.L., R.P., K.G. contributed microbiological experimentation and functional interaction studies. Z.C., A.M., M.T.F. contributed image analysis and quantification strategies. S.N. contributed electron microscopy. I.D.J., B.H., O.H., A.S.M. contributed design and supervision of experiments. B.H., A.S.M. designed the study. A.S.M. wrote the manuscript. I.D.J., B.H., O.H., A.S.M. contributed funding acquisition. All authors read and approved the manuscript.

Conflicts of interest

M.R. and A.S.M. hold equity in Dynamic42 GmbH. M.R. is CEO of Dynamic42 GmbH. A.S.M. consults to the company.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

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Appendix A. Supplementary data

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In vitro infection models to study fungal-host interactions

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Summary:

In vitro models are essential tools to gain vital insights into the mechanisms of biological interactions. In the field of medical mycology, *in vitro* models are fundamental to study pathogenic fungi and their interactions with the human host and its microbiota. This review provides an overview of the interactions of the opportunistic fungal pathogens *Aspergillus fumigatus*, *Candida* spp., *Cryptococcus neoformans*, and *Histoplasma capsulatum* with the human host in different niches and how these interactions can be modeled using *in vitro* systems. The *in vitro* models covered in this review range from cell monolayers to 3D cultures and complex organ-on-chip systems. Details concerning their handling, applications, advantages, and limitations are discussed. Next to the description of commonly used *in vitro* models, the review gives insights into new fields of potential new models like multi-organ-on-chip systems and the use of human-induced pluripotent stem cells, to expand the toolbox for investigating host-pathogen interactions in the future.

Own contribution:

Antonia Last planned the structure of the review, conducted literature research, wrote most of the article and integrated all contributions of the co-authors. She was involved in the figure corrections and conducted the revision process.

Estimated authors' contributions:

Antonia Last	35 %
Michelle Maurer	35 %
Alexander S. Mosig	5 %
Mark S. Gresnigt	20 %
Bernhard Hube	5 %

In vitro infection models to study fungal-host interactions

Summary sentence: From basic to complex: *in vitro* models to study interactions between human fungal pathogens and their host.

Keywords: *in vitro* model, fungal-host interaction, *Aspergillus*, *Candida*, *Histoplasma*, *Cryptococcus*

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Abstract:

Fungal infections (mycoses) affect over a billion people per year. Approximately two million of these infections are life-threatening, especially for patients with a compromised immune system. Fungi of the genera *Aspergillus*, *Candida*, *Histoplasma,* and *Cryptococcus* are opportunistic pathogens that contribute to a substantial number of mycoses. To optimize the diagnosis and treatment of mycoses, we need to understand the complex fungal-host interplay during pathogenesis, the fungal attributes causing virulence, and how the host resists infection *via* immunological defenses. *In vitro* models can be used to mimic fungal infections of various tissues and organs and the corresponding immune responses at near-physiological conditions. Furthermore, models can include fungal interactions with the host-microbiota to mimic the *in vivo* situation on skin and mucosal surfaces. This article reviews currently used *in vitro* models of fungal infections ranging from cell monolayers to microfluidic 3D organ-on-chip (OOC) platforms. We also discuss how OOC models can expand the toolbox for investigating interactions of fungi and their human hosts in the future.

Introduction

Human fungal infections lead to approximately 1.5 million deaths worldwide each year, but receive little attention compared to malaria or tuberculosis, which kill a similar number of people on an annual basis (Bongomin *et al.* 2017; Brown *et al.* 2012). Over 70% of deaths resulting from fungal infections can be attributed to fungi of the genera *Aspergillus*, *Candida*, *Cryptococcus,* and *Histoplasma* (Brown *et al.* 2012). These opportunistic fungal pathogens are either normal commensals of the human microbiota or reside in the environment, resulting in constant exposure to pathogenic fungi for humans. Even in immunocompetent human hosts, superficial fungal infections are widespread. Among them, fungal skin diseases are the most common health complications (Vos *et al.* 2012), and vulvovaginal candidiasis (VVC) affects approximately 70% of women (Gonçalves *et al.* 2016; Rosati *et al.* 2020). Such infections are often connected to an imbalance of the bacterial microbiota, for example, after the use of antibiotics that favor fungal overgrowth (Weiss and Hennet, 2017). In addition to superficial infections, opportunistic fungal pathogens can also cause severe life-threatening systemic infections under certain predispositions, like surgery, stem cell transplantation, chemotherapy, or HIV/ AIDS (Perlroth *et al.* 2007; Polvi *et al.* 2015; Vallabhaneni and Chiller 2016). Considering their clinical significance, suitable models to study opportunistic fungal infections are essential for obtaining insights into disease pathogenesis**.** Ideally, these models would allow the dissection of the molecular details of host-pathogen interactions under physiologically relevant conditions. They should provide sufficient complexity to mimic the different types and stages of infections and predispositions of the host. These models should also be suitable to test experimental therapeutic interventions and allow the evaluation of clinically relevant biomarkers. Here we review currently used *in vitro* models to study molecular mechanisms of fungal infections caused by common fungal pathogens including *Aspergillus fumigatus*, *Candida* spp., *Cryptococcus neoformans,* and *Histoplasma capsulatum* and provide an outlook about models that will likely expand our toolbox to study fungal-host interactions in the near future.

Disease modeling

To study fungal pathogens and their related diseases, a wide range of models can be used. Commonly, host-pathogen interactions are investigated in models based on animals such as mice, rats, fish, insects, or worms. *In vivo* models offer the advantage to study host-pathogen

interactions in a whole organism, providing the most complex interactions that can be achieved experimentally. However, in addition to critical ethical issues associated with the use of animal models (Robinson *et al.* 2019), the translation of results from animal experiments to human disease can be hampered by differences in physiology. Another approach is the use of tissue samples or organs from living organisms and their culture in an *ex vivo* environment that resembles *in vivo* conditions. These *ex vivo* models offer the advantage that conditions can be easily manipulated and are often easier to handle than living organisms. A broad overview of *ex vivo* models to study fungal infection is given by Maciel Quatrin *et al.* (2019). *In vitro* experiments are also performed outside of the natural biological environment. Primary cells isolated from tissues and biopsies can be cultured for a limited time or can be immortalized and cultured as cell lines. *In vitro* models may lack the complexity of *in vivo* models, but allow ample control over external growth conditions of cells concerning O_2 and CO_2 saturation, temperature, pH, and nutrients. Moreover, it is relatively easy to manipulate as well as to quantitatively and qualitatively assess the metabolism, transcription, and protein function of cells, making it possible to work in and test conditions that cannot be studied in *in vivo* models. It is also possible to introduce or omit different cell types to study the individual impact of different kinds of cells within the system. *In vitro* models **(Fig. 1)** range from monolayers in well plates, to transwell systems**,** 3D tissue structures, and complex organ-on-chip (OOC) models (Mosig 2017), which are able to mimic several organs such as the liver (Groger *et al.* 2016; Jang *et al.* 2019), lung (Benam *et al.* 2016; Deinhardt-Emmer *et al.* 2020), and gut (Maurer *et al.* 2019; Shin and Kim 2018). OOC models represent the smallest functional entity of an organ and as well as a versatile and promising resource to study host-pathogen interactions (Ahadian *et al.* 2018). However, each type of model has its specific advantages and disadvantages. The most suitable model is the one that meets the actual needs with high predictability and robustness, depending on the pathogen, the host, and the questions to be answered.

We discuss the fungal-host interactions in different biological niches **(Fig. 2)**. We review *in vitro* models used to mimic infection routes and highlight relevant findings that contributed to expand our knowledge on fungal infections. Because the immune system plays a major role during fungal infections, the interplay of fungi and immune cells are discussed in the first part, followed by sections covering the respiratory tract, the gastrointestinal tract, the vaginal tract, the bloodstream, and the blood-brain-barrier.

Studying fungal interactions with the immune system

A properly functioning immune system is crucial for resistance against infections with fungal pathogens. Individuals with a compromised immune system are more susceptible to invasive fungal diseases, whereas detrimental, improper, or hypersensitive immune reactions can also contribute to disease (Romani 2004; Wheeler *et al.* 2017). Thus, a protective host response against opportunistic fungal pathogens has to be specific, tightly regulated, and effective. However, pathogenic fungi have evolved a series of mechanisms to deal with and evade the immune system. Knowledge of both aspects is crucial for the design of therapeutic strategies aiming to strengthen appropriate responses and suppress detrimental ones (Armstrong-James *et al.* 2017). We will discuss (I) the different immune cells involved in antifungal host defense, (II) the different roles these cells play in antifungal immunity, and (III) different models and readouts that can be used to study the efficiency of the host response to pathogenic fungi.

Immune cells involved in antifungal host defense

A healthy and efficient immune system is fundamental to cope with the environmental fungi we encounter on a daily basis and to deal with the fungi we harbor as commensals. This antifungal immunity relies on the innate immune system represented by cells such as macrophages, neutrophils, and natural killer (NK) cells as well as the adaptive immune system, in particular on T helper cell responses. The importance of these different types of immune cells becomes apparent when they are dysfunctional or absent. For example, a compromised innate immune system due to immunosuppressive therapy predisposes to invasive candidiasis (Lionakis 2014), but also aspergillosis (Herbrecht *et al.* 2012). While the innate immune system plays a role in host defense against cryptococcosis (Voelz and May 2010), patients with a compromised adaptive immune response due to HIV infections are particularly susceptible (Warkentien and Crum-Cianflone 2010). In contrast to *Candida, Aspergillus*, and *Cryptococcus* species, *Histoplasma* species more commonly cause infections in healthy individuals (Köhler *et al.* 2017). Nevertheless, a compromised innate as well as adaptive immune response increases the susceptibility to histoplasmosis (Akram and Koirala 2020).

Tissue-resident macrophages and monocyte-derived macrophages especially play an essential role against invasive candidiasis (Austermeier *et al.* 2020), whereas alveolar macrophages (AMs) are essential for clearance of fungi like *Aspergillus*, *Cryptococcus*, or *Histoplasma* species that enter our body *via* the airways (Newman 2005; Xu and Shinohara 2017).

Neutropenia is a common risk factor for aspergillosis and invasive candidiasis, showing the crucial role of neutrophils in antifungal host defense (Herbrecht *et al.* 2000). Dendritic cells (DCs) are crucial for activation of the adaptive immune system. Dysfunctions of the adaptive immune system like the reduced CD4+ T cell function in AIDS patients increase the susceptibility for infections with *C. albicans*, *A. fumigatus*, *C. neoformans*, or *H. capsulatum* (van de Veerdonk and Netea 2010). Interestingly this predisposition manifests as mucosal *C. albicans* infections, in particular oropharyngeal candidiasis (OPC), but systemic *C. albicans* infections are also observed under such conditions (Fidel 2011). This is believed to be closely connected to the crucial roles of T helper responses in orchestrating oral mucosal resistance to infection (Gaffen and Moutsopoulos 2020; Scheffold *et al.* 2020). Antifungal immunity in the brain is connected to microglia that are the resident macrophage-like cells of the central nervous system (CNS), which show strong responses to fungal species like *C. albicans* (Blasi *et al.* 1991) and *C. neoformans* (Barluzzi *et al.* 1998). The C-type lectin receptor signaling adaptor CARD9 is expressed by microglia cells and its deficiency is associated with fungal brain infections (Drummond and Lionakis 2019). NK cells also exhibit antifungal effects (Schmidt *et al.* 2017) and a delayed NK cell reconstitution (e.g. after allogeneic stem cell transplantation) is associated with a higher risk of invasive aspergillosis (Weiss *et al.* 2020).

Antifungal effector functions during host defense against fungal pathogens

After the recognition of pathogen-associated molecular patterns (PAMPs) *via* pathogen recognition receptors (PRRs), supported by opsonization, innate immune cells mount responses to counteract the invading fungi. At early stages of infection, macrophages detect and engulf fungal pathogens (Gilbert *et al.* 2014) **(Fig. 2A).** In addition, through the release of cytokines and chemokines they recruit and activate other immune cells. When neutrophils migrate to the site of infection, they act against fungal pathogens through phagocytosis, oxidative bursts, and NETosis (Gazendam *et al.* 2016; Urban and Nett 2019). The production of reactive oxygen species (ROS) by phagocytes can kill fungal pathogens, like *C. albicans*, directly (Grondman *et al.* 2019) or impact LC3-mediated phagocytosis during defense against *A. fumigatus* (Sprenkeler *et al.* 2016). This is highlighted, for example, by the fact that chronic granulomatous disease (CGD) patients, incapable of producing ROS, are highly susceptible to aspergillosis (Segal *et al.* 2000). DCs represent the bridge to activate the adaptive immune system *via* antigen processing and presentation to T-cells (LeibundGut-Landmann *et al.* 2007).

T-cell differentiation can influence infection in different ways. Th1 cells augment the innate immune function through the release of IFNγ (Lionakis and Levitz 2018), which increases the microbicidal capacity of macrophages (Netea *et al.* 2015). Th17 cells release proinflammatory cytokines such as IL-17 and IL-22, which mediate recruitment of neutrophils and induce production of antimicrobial peptides (Conti *et al.* 2016; Khader *et al.* 2009) **(Fig. 2A)**. The importance of these T-cell types for antifungal defense is evident in corresponding knock-out mice that have an increased susceptibility to disseminated *C. albicans* infections (Balish *et al.* 1998; Huang *et al.* 2004), but also show a striking susceptibility to mucosal infections. Th2 responses can result in a detrimental immune response, manifesting in a higher susceptibly to disseminated *C. albicans* infections (Haraguchi *et al.* 2010) or an aberrant immune response to *A. fumigatus* spores connected to allergic bronchopulmonary aspergillosis (ABPA) (Knutsen and Slavin 2011). T regulatory cells can suppress inflammatory responses and are highly beneficial to prevent immunopathology in the case of ABPA (Montagnoli *et al.* 2006), but also allow *C. albicans* persistence in the gastrointestinal tract (De Luca *et al.* 2007).

In vitro models to study interactions between fungi and immune cells

The interactions between fungi and the different effector functions of the immune system can be easily studied *in vitro* using cell lines **(Tab. S1A)** and primary immune cells **(Tab. S1B)**. Cell lines have the advantage of easy handling and provide highly reproducible results. The availability of many reporter cell lines and the possibilities to generate transgenic/knockout cell lines represent valuable resources that allow the study of highly conserved mechanisms in the immunology against fungal infections. Nevertheless, central cellular processes such as pyroptosis, apoptosis, and autophagy are considerably different or modified in cancer cell lines. Over the past few years the essential role of these processes in shaping antifungal immunity has become increasingly clear (Dominguez-Andres *et al.* 2017; Evans *et al.* 2018; Gonçalves *et al.* 2020; Kanayama and Shinohara 2016; O'Meara and Cowen 2018; Sprenkeler *et al.* 2016; Thak *et al.* 2020; Weerasinghe and Traven 2020). Therefore, primary cells offer the highest similarity to the physiological situation. Primary immune cells are commonly isolated from peripheral human blood. By density gradient centrifugation, peripheral blood mononuclear cells (PBMCs) can be separated from erythrocytes and granulocytes (Munoz and Leff 2006). An important aspect to consider when using primary cells is that strong donor variation and even seasonal differences can influence experimental outcomes (Ter Horst *et al.*

2016). However, genetic differences between donors can also be exploited to analyze the impact of a given genotype on the antifungal immune response (Gresnigt *et al.* 2018b; Jaeger *et al.* 2019a; Jaeger *et al.* 2019b; Lionakis *et al.* 2013; Matzaraki *et al.* 2017; Smeekens *et al.* 2013). In these functional genomic approaches, immune cells of large cohorts of volunteers are screened for variation in specific immunological effectors such as cytokine release, ROS release, or fungal killing. After genotyping the donors, the results of immunological phenotypes can be stratified based on the corresponding genotype. This knowledge on the influence of common genetic variations on the antifungal host response can provide valuable information about the role of certain genes in antifungal host defense. Combined with genetic association studies, functional genomics can be used to validate the impact of identified variants on immune pathways and susceptibilities to infections. In this way, crucial roles have been identified for CX3CR1 and its role in host defense against of systemic candidiasis (Lionakis *et al.* 2013), as well as for the SIGLEC15 receptor in the susceptibility to vulvovaginal candidiasis (Jaeger *et al.* 2019b). Conversely, knowledge about genetic variations that influence critical antifungal host defense pathways can lead to the discovery of genetic susceptibilities. In this way NOD2 variants were found to increase resistance to invasive aspergillosis (Gresnigt *et al.* 2018b).

Macrophages

Interactions between macrophages/macrophage-like cells and fungal pathogens have been studied using cell lines like J774A.1, RAW, Ana-1, U937, BV-2, and THP-1 **(Tab. S1A).** Such cell lines allow the generation of reporter constructs that can be used to monitor the activation of specific immune pathways. In this way, the importance of RAB-GTPases for maturation of *C. albicans*-containing phagosomes has been demonstrated (Bain *et al.* 2014; Okai *et al.* 2015). Another option is the use of macrophages derived from murine bone marrow cells and differentiated *in vitro* (BMDMs) **(Tab. S1B)**. A major advantage of this approach is the possibility to isolate BMDMs from mice with different genetic backgrounds (e.g. gene knockout or transgenic mice), thus providing a toolbox to obtain in-depth knowledge about the key players of the host immune response during fungal infections. Such cells from knockout mice have been widely used to investigate, for example, inflammasome activation in the response to *C. albicans* (Kasper *et al.* 2018)*, C. neoformans* (Guo *et al.* 2014), and *A. fumigatus* (Karki *et al.* 2015). In addition to BMDMs, human monocyte-derived macrophages

(MDMs) can be used for *in vitro* studies. In such experiments, monocytes are isolated from PBMCs or whole blood and can be differentiated *in vitro* into a wide range of functionally different MDMs (Xue *et al.* 2014). MDMs have been used in numerous studies to dissect cytokine release, inflammasome activation, oxidative bursts, phagocytosis, and phagosome maturation after confrontation with fungi, but also escape and survival mechanisms of fungi during these interactions (Friedrich *et al.* 2019; Gresnigt *et al.* 2018b; Kasper *et al.* 2018; O'Meara *et al.* 2018; Smith *et al.* 2015) **(Tab. S1B).**

However, undifferentiated monocytes are also used, for example, to investigate how these cells are differentially activated (Camilli *et al.* 2018; Dominguez-Andres *et al.* 2017; Halder *et al.* 2016; Klassert *et al.* 2017; Leonhardt *et al.* 2018). The stimulation of monocytes using PAMPs such as β-glucan can induce epigenetic reprogramming, which alters the response to secondary *C. albicans* stimulation (Quintin *et al.* 2012), a concept known as innate immune memory or "trained immunity". In contrast, the response to *C. albicans* can also be hampered by the induction of innate immune tolerance by PAMPs such as lipopolysaccharide (Grondman *et al.* 2019). Over the past years it has also become increasingly evident that cell metabolism is linked with immune cell functionality. Global as well as targeted profiling of metabolic pathways in primary immune cells, especially monocytes and macrophages, have been used to uncover immunometabolism in response to fungi (Dominguez-Andres *et al.* 2017; Gonçalves *et al.* 2020; Weerasinghe and Traven 2020).

Since pathogenic fungi often colonize and infect specific organs, the corresponding tissue macrophages offer the highest physiological relevance. For example, specific cells lines such as the murine alveolar macrophage cell lines MH-S (Mattern *et al.* 2015) and AMJ2-C11 (Pitangui Nde *et al.* 2015) are used to study fungal pathogens that cause pulmonary infections **(Tab. S1A)**. Alternatively, primary alveolar macrophages can be used to study the immune response of pulmonary fungal infections *ex vivo.* Though, the limited availability of these cells makes it challenging to obtain sufficient numbers for experiments. Nevertheless, protocols are available to obtain large numbers of AMs from bronchoalveolar lavage (BAL) (Busch *et al.* 2019) or resected lung tissue (Nayak *et al.* 2018). Similarly, peritoneal macrophages have been used to study the interactions with *Candida* spp. (Ifrim *et al.* 2016; Shimamura *et al.* 2019). Because peritoneal macrophages are easier to obtain in great quantities than AMs, they have also been used for interaction studies with *H. capsulatum* (primarily infecting the lung) (Huang

et al. 2018; Shen *et al.* 2018; Youseff *et al.* 2012) **(Tab. S1B)**. To dissect fungal interactions with immune cells in the brain, BV-2 microglia cells (Blasi *et al.* 1990) **(Tab. S1A)** were co-cultured with astrocytes to demonstrate that candidalysin induces IL-1β release, which in turn mediates neutrophil recruitment (Drummond *et al.* 2019) **(Fig. 1B)**.

Interaction studies with macrophages revealed mechanisms enabling fungal cells to evade macrophage phagocytosis or to escape from phagosomes. Masking of cell wall epitopes can prevent the detection of *A. fumigatus*, *C. albicans*, and *H. capsulatum* by macrophages (Aimanianda *et al.* 2009; Ballou *et al.* 2016; Rappleye *et al.* 2007). Morphological changes such as titan cell formation by *C. neoformans* (Okagaki and Nielsen 2012) or filamentation by *A. fumigatus* and *C. albicans* influence phagocytosis efficiency (Erwig and Gow 2016; Lewis *et al.* 2012; Maxson *et al.* 2018). Additionally, these fungi can inhibit phagosome acidification or phagosome maturation to prevent intracellular killing. These processes are reviewed in detail by Gilbert *et al.* (2014); and Seider *et al.* (2010).

Irrespectively of the immune cell type used, there are numerous readouts to study interactions between fungi and cells of the immune system. Transcriptional profiling has provided indispensable insights into the interplay between immune cells and fungal pathogens. Specifically, dual-species transcriptional profiling has helped to elucidate key features of the adaptations of fungal cells in response to immune cells and *vice versa* (Munoz *et al.* 2019; Niemiec *et al.* 2017). Given the crucial role of phagocytes in fungal clearance, protocols established to investigate phagocytosis and phagosome maturation are common **(Fig. 2A)**. Using live-cell microscopy, phagocytosis and viability dynamics can be studied on a kinetic scale involving multiple phagocytes (Gresnigt *et al.* 2018a; Guimaraes *et al.* 2019; Kasper *et al.* 2018; Lim *et al.* 2018; Seoane *et al.* 2020; Smith *et al.* 2015). For example, a struggle for glucose availability between macrophages and *C. albicans* was demonstrated to be crucial in dictating inflammasome activation (Tucey *et al.* 2020). Phagocytosis and phagosome maturation can also be examined in detail on a single-cell level (Bain *et al.* 2014; Okai *et al.* 2015; Westman *et al.* 2018). Such studies have contributed to the understanding of the role of phagosome-lysosome fusion in maintaining phagosome integrity while fungal cells filament inside the phagosome (Westman *et al.* 2020). Apart from live cell imaging, phagocytes can also be fixed at specific time-points to investigate the co-localization of specific proteins to the phagosome using immunofluorescence staining. In this way, LC3-

associated phagocytosis has been investigated as a crucial pathway to improve phagocytosis efficiency of *H. capsulatum* and *A. fumigatus* (Huang *et al.* 2018; Kyrmizi *et al.* 2018). Using a similar approach, a key role has been shown for flotillin-dependent microdomains or lipid rafts in phagosome formation for efficient host defense against *A. fumigatus* (Schmidt *et al.* 2020).

Natural Killer (NK) cells

Primary Natural Killer (NK) cells can be obtained from PBMCs by different isolation kits (Wang *et al.* 2017). NK cells have been studied alone or in co-culture with other immune cells and have been observed to have direct antifungal capacity against *C. neoformans* through the release of perforins (Wiseman *et al.* 2007). The recognition of β1,3-glucan through the NKp30 receptor was identified to trigger and enhance the killing of *C. albicans* and *C. neoformans* by NK cells (Li *et al.* 2018). Other *in vitro* studies revealed an exhausted phenotype of NK cells, when they degranulate in contact with *A. fumigatus* (Santiago *et al.* 2018). NK cell activation in response to *Candida* species has been observed to occur indirectly by cross talk with monocytes (Marolda *et al.* 2020). Similarly, for *A. fumigatus,* crosstalk between NK cells and DCs was found to mediate DC activation (Weiss *et al.* 2018). Further, direct antifungal effects of NK-cells against *A. fumigatus* have been associated with release of IFNγ (Bouzani *et al.* 2011) **(Tab. S1B)**.

Neutrophils

Using hypotonic lysis of erythrocytes or other gradient solutions like PolymorphPrep© (Degel and Shokrani 2010), primary neutrophils can be isolated from PBMCs to investigate their interaction with fungi. Neutrophils can act as phagocytes, but can also form neutrophil extracellular traps (NETs) and release cytokines in the presence of fungal cells. These features were studied intensively *in vitro* (Bruns *et al.* 2010; Dasari *et al.* 2018; Rocha *et al.* 2015; Sun and Shi 2016; Thompson-Souza *et al.* 2020; Urban *et al.* 2006). By studying phagocytosis, killing, NETosis, and cytokine release, Spleen Tyrosine Kinase (Syk) was identified as a crucial mediator for inducing antifungal effector mechanisms against various *Candida* species (Negoro *et al.* 2020). Another aspect is to monitor how these phagocytes migrate to the site of infection. Chemotaxis assays using specialized *in vitro* systems (Chen 2005; Richards *et al.* 2004; Thunström Salzer *et al.* 2018) can be used to elucidate this process in the context of fungal infections (Coenjaerts *et al.* 2001; Drummond *et al.* 2015; Rieber *et al.* 2016) **(Tab. S1B)**.

ROS release or oxidative bursts in response to fungal pathogens can be assessed in neutrophils (Boyle *et al.* 2011; Liu *et al.* 2018), but also in monocytes (Brunel *et al.* 2018; Wellington *et al.* 2009) and macrophages (Arce Miranda *et al.* 2019; Sun *et al.* 2014; Wolf *et al.* 1987; Youseff *et al.* 2012) **(Fig. 2A)**, and **(Tab. S1B)**. Using a modified model, in which *C. albicans* cells are grown in clusters on poly-L-lysine coated glass slides, neutrophils were observed to form "swarms" to efficiently use oxidative stress mechanisms to attack *C. albicans* (Hopke *et al.* 2020). *C. albicans* cells however, can filament thereby complicating clearance through phagocytosis (Erwig and Gow 2016).

Dendritic cells, T-cells, and whole blood models

Virtually all immune cell types are being employed to study transcriptional responses to fungal pathogens (Hellwig *et al.* 2016; Niemiec *et al.* 2017; Smeekens *et al.* 2013; Van Prooyen *et al.* 2016) as well as cytokine and chemokine responses (Becker *et al.* 2016; Coady and Sil 2015; Marischen *et al.* 2018) to fungal pathogens **(Fig. 2A)**. Often such studies involve crosstalk between different immune cell types such as antigen-presenting cells and cells of the adaptive immune system. PBMCs are frequently used due to their composition of innate and adaptive immune cells and allow the study of innate host responses (Alvarez-Rueda *et al.* 2020; Becker *et al.* 2016), but also T-cell mediated responses such as Th1, Th17, Th2, and Tregs (Becker *et al.* 2015; Gresnigt *et al.* 2013; Page *et al.* 2018; Raijmakers *et al.* 2017; Vogel *et al.* 2018; Zielinski *et al.* 2012) **(Fig. 2A)**. For example, using PBMCs, the type I interferon pathway was identified to play a crucial role in *C. albicans* defense (Smeekens *et al.* 2013). Interactions between DCs and T-cells were used to investigate how the adaptive immune response is polarized through antigen presentation, co-stimulation, and the cytokine environment (Stephen-Victor *et al.* 2017; van der Does *et al.* 2012). DC maturation can be examined in transwell systems (Lother *et al.* 2014) or by profiling maturation features *via* flow cytometry (Hefter *et al.* 2017; Pietrella *et al.* 2005; Vivas *et al.* 2019). For interaction studies including a wide range of immune cell types, whole blood models were used to gain information about fungal killing (Hunniger *et al.* 2014), transcriptional responses (Dix *et al.* 2015; Kämmer *et al.* 2020), cytokine release (Oesterreicher *et al.* 2019), and platelet interactions (Eberl *et al.* 2019; Fréalle *et al.* 2018) **(Tab. S1B)**.

Studying respiratory tract infections with *Aspergillus*, *Histoplasma*, and *Cryptococcus* spp.

The respiratory tract serves as a niche where fungal pathogens such as *A. fumigatus*, *H. capsulatum,* and *C. neoformans* can cause infections in predisposed hosts. Since the major biological niche of these fungi is the environment, fungal elements (mostly conidia or yeast) are frequently inhaled by the human host. The healthy immune system can clear these inhaled fungal elements, whereas immunocompromised individuals or patients with pre-existing pulmonary conditions may fail to clear fungi and have a higher risk to develop aspergillosis, histoplasmosis, or cryptococcosis. The clinical manifestations of these fungal diseases, however, are very diverse. Infections with pathogenic *Aspergillus* species can develop differently, depending on the immune reaction and underlying lung pathology (Soubani and Chandrasekar 2002; van de Veerdonk *et al.* 2017). While a compromised immune response can result in invasive pulmonary aspergillosis, pre-existing lung injury can lead to the development of an aspergilloma and a chronic or hyper inflammatory response. Such responses can also provoke allergic bronchopulmonary aspergillosis (Kosmidis and Denning 2015). In immunocompromised patients, specifically patients suffering from AIDS, *C. neoformans* can cause either pulmonary cryptococcosis or can disseminate into other organs after an (asymptomatic) pulmonary infection (Setianingrum *et al.* 2019). *C. neoformans* cells can be engulfed by AMs and DCs and can survive within the phagolysosome, proliferate, and eventually escape *via* non-lytic exocytosis (vomocytosis). **(Fig. 2C I)**. Vomocytosis was also observed for *C. albicans* (Bain *et al.* 2012), *C. krusei* (García-Rodas *et al.* 2011), *A. nidulans* and *A. fumigatus* (Gresnigt *et al.* 2018a). Intracellular survival is one key strategy of *C. neoformans* to disseminate from the respiratory tract (Coelho *et al.* 2014). Another translocation route involves fungal cells crossing the epithelial border *via* transcytosis **(Fig. 2C II)** or a direct migration through areas where the epithelial lining has been compromised **(Fig. 2C III)** (Denham and Brown 2018). *H. capsulatum* can cause pulmonary histoplasmosis, and similar to *C. neoformans*, it can evade the immune system by hiding inside AMs (Ray and Rappleye 2019). Following growth and replication, it can induce apoptosis facilitating further dissemination within the bloodstream and lymphatic organs **(Fig. 2C IV)** (Long *et al.* 2003; Mihu and Nosanchuk 2012; Pitangui Nde *et al.* 2015). In contrast to *H. capsulatum* and *C. neoformans*, which grow as yeast during infection, *A. fumigatus* proliferates as hyphae in the lung, allowing deep tissue invasion **(Fig. 2C V)**.

Simple in vitro models mimicking lung infections

To mimic the alveolar environment, the pulmonary epithelial cell line A549, originating from a human alveolar cell carcinoma (Lieber *et al.* 1976), is frequently used to study pathogenicity attributes including adhesion (Gravelat *et al.* 2010; Pitangui *et al.* 2012; Teixeira *et al.* 2014), endocytosis (Liu *et al.* 2016), epithelial detachment (Bertuzzi *et al.* 2014; Kogan *et al.* 2004), and epithelial damage (Bertuzzi *et al.* 2014; Ejzykowicz *et al.* 2010). These studies revealed crucial roles for the *A. fumigatus* transcription factors PacC (Bertuzzi *et al.* 2014) and DvrA (Ejzykowicz *et al.* 2010) to mediate tissue invasion and damage. In addition, A549 cells were used to dissect pulmonary epithelial IL-8 responses to *C. neoformans* and *H. capsulatum* (Alcantara *et al.* 2020; Barbosa *et al.* 2007), and shed light on how different *A. fumigatus* isolates differentially regulate gene expression of epithelial cells (Watkins *et al.* 2018) **(Tab. S2)**. To examine the fungal translocation through the pulmonary epithelium, transwell models with different modifications have been employed **(Fig. 1A)**.

Complex in vitro models mimicking lung infections

Models that combine A549 cells with DCs (Morton *et al.* 2018) or a bilayer of human pulmonary artery endothelial cells (HPAECs) with (Morton *et al.* 2014) or without DCs (Belic *et al.* 2018; Hope *et al.* 2007) were utilized to model the cellular complexity in the alveolus and the cellular cytokine response to fungal infections. The translational capacity of such a model was reflected in a study that validated the measurement of galactomannan as a biomarker of fungal infection and antifungal efficacy *in* vitro (Hope *et al.* 2007). These models have also been employed for microscopy-based analyses, gene expression analysis, and analysis of immune activation to gain insights into the host-*Aspergillus* interactions at the alveolar epithelial interface **(Tab. S2)**.

To more closely resemble the physiological situation, primary human bronchial or small airway epithelial (HBE, SAE) cells were used to study proinflammatory epithelial cytokine responses to *C. neoformans* infections (Guillot *et al.* 2008). These cells differentiate when cultured at an air-liquid interphase (ALI) into lung epithelium and were also used to assess the host response to *A. fumigatus* conidia. Transcriptome and proteome analyses revealed the upregulation of apoptosis, autophagy, translation, and cell cycle pathways as well as the downregulation of complement and coagulation pathways (Toor *et al.* 2018). The combination of differentiated pulmonary epithelial cells with DCs and macrophages provides an even more complex model,

which allows the study of the interplay between fungal cells, the epithelium, and the immune system (Chandorkar *et al.* 2017). As an alternative strategy to investigate *Aspergillus* spp. infections, bronchial mucosal tissue resected from cancer patients was used. Using this *ex vivo* model, adhesion, invasion, damage, and structural changes of the epithelium were investigated (Amitani and Kawanami 2009). Although the latter model represents human physiology, its applicability is limited by the difficulty of obtaining patient material. Besides confounding factors, such as therapies and medication, inter-individual differences may impact the validity of this model and the ability to obtain reproducible results.

Lung-on-chip models

In most lung models used so far cells were cultured statically and thus not subject to shear stress. Further, these models rarely consider the impact of additional members of the microbial community, such as the lung microbiota in the infection process. A number of lung-on-chip models have been established that reflect additional physiological key features of the lung. A “breathing” alveolus-on-chip is mimicked by stretching and contraction of a membrane using a vacuum, which leads to an increased uptake of nanoparticles of the epithelium and transport to the vasculature (Huh *et al.* 2010; Stucki *et al.* 2018). Mechanostimulation represents an important biophysical cue since the stretching of the lungs influences repair mechanisms in damaged epithelial cells and might also play a significant role during fungal invasion (Desai *et al.* 2008). Deinhardt-Emmer and colleagues established an alveolus-on-chip model that harbored immune cells and consisted of two compartments. In the upper compartment, lung epithelial cells differentiated into the two types of alveolar epithelial cells and were separated by a porous membrane from an endothelial lining, subjected to flow in the lower compartment (Deinhardt-Emmer *et al.* 2020) **(Fig. 1A)**. Although this model was not used to dissect fungal-host interactions so far, it revealed new insights about the interplay of *S. aureus* and influenza virus at the alveolar-capillary interface. During co-infection, increased inflammatory responses were observed including cytokine expression and loss of barrier function similar to severe clinical outcomes of patients with bacterial-viral superinfections (Deinhardt-Emmer *et al.* 2020). Other platforms have used human alveolar epithelial cells (hAEpCs), and also integrated neutrophils (Benam *et al.* 2016; Huh *et al.* 2010; Jain *et al.* 2018; Zhang *et al.* 2018). Future models can be colonized with (additional) members of the pulmonary microbiome to investigate the interplay with fungi, which can contribute to

progression of pulmonary fungal infections (Kolwijck and van de Veerdonk 2014). Taken together, current lung-on-chip models can produce a microenvironment resembling the *in vivo* physiology by imitating an ALI, mechanical strain, and immune responses. This can facilitate the establishment of sophisticated pulmonary-infection models.

Studying colonization and infection of the oral cavity, the intestinal and vaginal tract by *Candida* spp.

In the oral cavity, the intestinal-, and vaginal tract, *Candida* spp. normally live as harmless commensal yeasts. However, some opportunistic *Candida* spp. can cause infections. These range from mucocutaneous infections such as oropharyngeal candidiasis (OPC) (Millsop and Fazel 2016) and vulvovaginal candidiasis (VVC) (Rosati *et al.* 2020) to invasive candidiasis (Pappas *et al.* 2018). Diverse predispositions, like immunosuppression, an impaired barrier function, and an imbalanced microbiota are prerequisites to enable infection of *Candida* species. However, both predisposition and protection by an adjusted immune response differ between the specific types of infections. In the following sections we discuss current *in vitro* models used to study *C. albicans* and *C. glabrata* interactions with the host in three different niches of the human body.

Studying *Candida* spp. infections of the oral cavity

OPC occurs mostly in combination with the use of broad-spectrum antibiotic therapy and immune suppression, e.g. through HIV/ AIDS, chemotherapy, or radiation therapy. Further, neonates, diabetic, and elderly individuals are more susceptible (Patil *et al.* 2015). *C. albicans* is the most prevalent species, but also other *Candida* species like *C. glabrata, C. dubliniensis, C. krusei, C. kefyr, C. parapsilosis, C. stellatoidea,* and *C. tropicalis* can be found in oral lesions (Millsop and Fazel 2016). *C. albicans* mainly interacts with the oral epithelium by invading cells *via* active penetration **(Fig. 2E I)** and/or induced endocytosis **(Fig. 2E II)** (Dalle *et al.* 2010; Naglik *et al.* 2017; Phan *et al.* 2007; Sheppard and Filler 2014; Wachtler *et al.* 2011a), or invasion of the tissue by degradation of E-cadherin, thereby disrupting the epithelial barrier **(Fig. 2E III)** (Villar *et al.* 2007). *In vivo*, the uppermost layer of the oral epithelium consists of stratified squamous epithelium, followed by a basal membrane and fibroblasts in the lamina propria.

Simple in vitro models mimicking oral infections

To study *Candida*-host interactions of the oral cavity, oral epithelial cells are commonly used. TR146 cells are derived from a squamous cell carcinoma of the buccal mucosa (Rupniak *et al.* 1985) and used to investigate invasion (Puri *et al.* 2019), damage (Meir *et al.* 2018; Wilson *et al.* 2014), and gene expression (McCall *et al.* 2018; Meir *et al.* 2018; Schaller *et al.* 1998). The TR146 model has contributed significantly to the understanding of *C. albicans* pathogenicity by showing that the peptide toxin candidalysin is responsible for the capacity of *C. albicans* hyphae to cause damage (Moyes *et al.* 2016). The same model was used to demonstrate that candidalysin also activates epithelial proinflammatory responses through the Epithelial Growth Factor Receptor (EFGR) (Ho *et al.* 2019) and its synergistic signaling with IL-17 (Verma *et al.* 2017). Immortalized oral mucosal cells (OKF6/TERT-2) (Dickson *et al.* 2000) have also been used to study epithelial transcriptional responses (Liu *et al.* 2015), to visualize *C. albicans* invasion (Wollert *et al.* 2012), and to demonstrate that invasion is, in part, mediated through endocytosis (Solis *et al.* 2017; Swidergall *et al.* 2018). The same cell line was used to show that damage is mediated through white cells in contrast to opaque cells (Solis *et al.* 2018). Furthermore, Epha2 was identified as an epithelial cell pattern recognition receptor for fungal β-glucans, activating a signal cascade that results in a proinflammatory and antifungal response (Swidergall *et al.* 2018).

Tongue cells derived from a squamous cell carcinoma (SCC15) represent a third cell type used to dissect interactions of *C. albicans* with the oral epithelium (Lindberg and Rheinwald 1990). Similar to the studies discussed above, SCC15 cells were used to investigate epithelial damage (Kumar *et al.* 2015), invasion (Villar *et al.* 2007), and cytokine release (Dongari-Bagtzoglou and Kashleva 2003) **(Tab. S3A)**.

Complex in vitro models mimicking oral infections

In addition to monolayer models **(Fig. 1A)**, organotypic 3D models known as reconstituted human oral epithelium (RHOE) are commonly used to study oral *Candida* spp. infections due to its histological similarity to physiological oral epithelium. In these RHOE models, TR146 cells are cultured on a polycarbonate filter at an ALI with culture medium on the basal side, resulting in a multilayer model with differentiated cells **(Fig. 1B)**. This model has been used to study epithelial damage (Mailander-Sanchez *et al.* 2017; Silva *et al.* 2011) and fungal (Spiering *et al.* 2010) or host cell gene expression (Wagener *et al.* 2012) **(Tab. S3A)**. In addition, the

model was used to show enhanced invasion and tissue damage during co-infection of *C. albicans* and *C. glabrata* (Silva *et al.* 2011). Because fungal biofilm formation is crucial for the development of caries and OPC, the RHOE model has also been used to analyze the expression of *C. albicans* virulence genes associated with biofilm formation (Nailis *et al.* 2010). Similar RHOE models exist, containing collagen embedded fibroblasts from mice and oral mucosal cells OKF6/TERT-2 cells, differentiated at an ALI (Dongari-Bagtzoglou and Kashleva 2006a; Dongari-Bagtzoglou and Kashleva 2006b). Since the interplay with the oral microbiota plays an essential role for the maintenance of a commensal state of *C. albicans* or for development of OPC (Montelongo-Jauregui and Lopez-Ribot 2018), the organotypic 3D models were also used to study interactions between *C. albicans* and bacteria. For example, antagonistic interactions between *Lactobacillus rhamnosus* and *C. albicans* were dissected (Mailander-Sanchez *et al.* 2017). Furthermore, a fungal-induced dysbiosis after chemotherapy (Bertolini *et al.* 2019) and synergistically increased tissue damage during interactions with *S. mutans* (Diaz *et al.* 2012) were observed. Additionally, biofilm formation of *C. albicans* and *C. glabrata* after chemotherapeutic treatment was examined in the latter organotypic 3D model (Sobue *et al.* 2018). The model was further "humanized" by using human fibroblasts and spontaneously immortalized keratinocytes to analyze interactions between *C. albicans* and *S. aureus* (de Carvalho Dias *et al.* 2018) **(Tab. S3A)**.

In vitro modeling of C. albicans stomatitis

C. albicans mediated stomatitis, an inflammatory reaction of the oral mucosa, is a major complication for users of removable dental prostheses, but also common in smokers or patients suffering from diabetes mellitus (Alzayer *et al.* 2018; Javed *et al.* 2017; Salerno *et al.* 2011). To model this type of oral infection, primary human palate epithelial cells (HPECs) were used to study the host response to *C. albicans* in terms of apoptosis, nitric oxide production (Casaroto *et al.* 2019), and mucosal gene expression (Offenbacher *et al.* 2019). Similarly, a combination of TR146 cells and primary fibroblasts was used for adhesion and gene expression studies (Morse *et al.* 2018) **(Tab. S3A)**.

Mucosa-on-chip models

Monolayer or multilayered mucosal models commonly feature a perpendicular configuration. This vertical culture arrangement hampers the individual monitoring of different cell layers by microscopy, and resolution decreases in deeper layers. A horizontal organization of cell layers

was applied in a mucosa-on-chip model (Rahimi *et al.* 2018) consisting of microchambers, which were aligned in parallel and interconnected by pores. A central subepithelial chamber harbored a collagen hydrogel with gingival fibroblasts, while keratinocytes were seeded into the pores connecting the luminal and subepithelial compartment. The luminal chamber can be microfluidically perfused to imitate saliva and saliva flow, which is an important contributor to epithelial barrier integrity. A further refinement for both static and microfluidic models can include an endothelial lining and immune cells such as dendritic Langerhans cells, which are almost exclusively found in stratified squamous epithelium and have been shown to react to *Candida* species (Upadhyay *et al.* 2013).

Studying *Candida* spp. colonization of the intestinal tract and intestinal translocation

Both *C. albicans* and *C. glabrata* colonize the human intestinal tract (Hallen-Adams and Suhr 2017). The gut represents the main reservoir of fungi, especially *C. albicans*, that can cause disseminated and systemic infections (Gouba and Drancourt 2015). In these life-threatening infections, the fungus overcomes the intestinal epithelium, which forms a barrier between the intestinal lumen and the sterile tissues of the human body. During this process, termed translocation, the fungus employs several mechanisms including active penetration **(Fig. 2F I)**, paracellular translocation **(Fig. 2F II)**, or migration through the intestinal epithelial layer without damaging the host cells **(Fig. 2F III)** (Allert *et al.* 2018; Basmaciyan *et al.* 2019). Certain predispositions favor fungal overgrowth and translocation: antibiotics that induce an imbalance of the microbiota and cytostatic therapy or abdominal surgery, which compromise the barrier function (Pfaller and Diekema 2007). To better understand the conditions that keep *C. albicans* commensal or drive the commensal-to-pathogen shift, the interactions between *C. albicans* and the intestinal barrier are studied extensively to find ways to prevent or reverse this shift (Kumamoto *et al.* 2020).

Simple in vitro models mimicking intestinal infections

Monolayers of cell lines originating from colorectal adenocarcinomas are widely used **(Fig. 1A)**. The most common cell lines are Caco-2 and HT-29. Caco-2 cells differentiate spontaneously into a polarized monolayer with characteristic villi and tight junctions after 12 days of culture (Fogh *et al.* 1977). These cells were used to demonstrate that damage to the intestinal epithelium induced by *C. albicans* relies on a combination of adhesion mediated

contact-sensing, tissue invasion through hyphal extension, and damage by the expression of pathogenicity factors (Wachtler *et al.* 2011a). Interactions with non-pathogenic yeast cells that can antagonize *C. albicans* pathogenicity were examined (Kunyeit *et al.* 2019; Lohith and Anu-Appaiah 2018). Furthermore, receptor signaling pathways (Mao *et al.* 2019), induction of defensins (Gacser *et al.* 2014), impact on tight junctions (Goyer *et al.* 2016), and the potential of epithelial cells to discriminate between yeast and hyphal morphologies (Schirbel *et al.* 2018) are processes that can be analyzed in this model. A subclone of the Caco-2 cell line, C2BBe1, was often used in *in vitro* systems due to its more homogeneous brush boarder expression (Peterson and Mooseker 1992). C2BBe1 cells cultured in transwell systems **(Fig. 1A)** were instrumental to elucidate important virulence requirements of translocation through the epithelial barrier and revealed a key role for candidalysin by mediating necrotic cell damage that allowed transcellular translocation (Allert *et al.* 2018). Additionally, using this model, a MAPK/NFκB mediated epithelial response to *C. albicans* infection was shown to increase epithelial resistance (Bohringer *et al.* 2016) **(Tab. S3B)**.

Essential features of *C. albicans* pathogenicity like adhesion, invasion, and damage were also studied using the HT-29 cell line (Deng *et al.* 2015; Garcia *et al.* 2018). A methotrexate treatment of HT-29 cells, transformed these cells into mucus-secreting goblet cells (HT-29-MTX) (Lesuffleur *et al.* 1990). These mucus-secreting cells were instrumental in demonstrating the role of mucus in suppressing virulence-associated attributes of *C. albicans*, such as hypha formation (Kavanaugh *et al.* 2014).

Complex in vitro models mimicking intestinal infections

As the intestinal epithelium consists of a myriad of cell types, combinations of different cell lines have been employed to more accurately mimic the *in vivo* situation. For example, a combination of Caco-2 cells and Raji B cells (human Burkitt's lymphoma) was used to study the interaction of *C. albicans* with an epithelial barrier including M-cells, which demonstrated M-cells as a preferred cell type for translocation *via* induced endocytosis **(Fig. 2F IV)** (Albac *et al.* 2016). In general, most *in vitro* models investigate *C. albicans* in its pathogenic state. To limit the pathogenicity of *C. albicans* and mimic commensalism, a mixture of C2BBe1 cells and the mucus-producing HT-29-MTX cells were colonized with *L. rhamnosus* in a basic "commensal" model **(Fig. 1B)**. Using this model, a damage reduction was observed in the presence of mucus and bacteria, both antagonizing *C. albicans* pathogenicity by reducing

filamentation, proliferation, and inducing shedding that physically separates hyphae from host cells (Graf *et al.* 2019) **(Tab. S3B)**.

Intestine-on-chip models

Although 2D intestinal models mimic the fundamental physiological structures of the intestinal tissue such as mucus production, M-cells, and brush border epithelium, they do not reflect the unique 3D architecture of the intestinal epithelial tissue consisting of villi and crypts. Cells in these models are cultured statically and are not subjected to the peristaltic movement characteristic for the intestine. In addition, *in vitro* models often lack immune cells, which convey tolerance towards commensals and trigger inflammatory responses when pathogens inflict damage to the intestinal lining. A number of intestine-on-chip models have been developed that recapitulate some of these key physiological features (Bein *et al.* 2018). In these models, Caco-2 cells grow out and form villi-like structures when grown on a membrane and exposed to shear stress (Kim and Ingber 2013). Microfluidic intestine models often include endothelial cells adjacent to epithelial cells in an individually perfused compartment. The luminal and the vascular compartment are separated by a porous membrane to facilitate transmigration of cells and cell communication. Innate immune cells such as monocytes can be implemented in the endothelial layer and differentiated into macrophages and DC-like cells, which tolerate inflammatory triggers in the intestinal lumen, but elicit a strong inflammatory response when a systemic infection is mimicked (Maurer *et al.* 2019). In this model, *C. albicans'* invasion of the epithelial layer and subsequent invasion of the bloodstream compartment in the presence and absence of the commensal bacterium *L. rhamnosus* was investigated. Patient-derived colon epithelial cells are difficult to access, but can sufficiently be maintained in microfluidic platforms and produce a mucus layer resembling the *in vivo* thickness (Sontheimer-Phelps *et al.* 2020). 3D intestine-on-chip models will be valuable tools to uncover the role of commensals and their products, as well as host immune responses in the yeast-to-hypha transition of *C. albicans* in the future **(Tab. S3B)**.

Intestinal Organoids

Apart from intestine-on-chip models, human intestinal organoids (HIOs) have emerged as a valuable disease-modeling tool. HIOs can be grown from adult stem cells (ASCs) extracted from intestine biopsies or induced pluripotent stem cells (iPSCs) (Rahmani *et al.* 2019) to form three-dimensional organotypic structures by self-organization and resemblance of key

embryonic signaling *in vitro* (Clevers 2016) **(Fig. 1A)**. Intestinal organoids show a villus and crypt-like architecture with epithelial cells facing inwards, creating a lumen as an enclosed space (Sato *et al.* 2009; Spence *et al.* 2011). Organoid models face similar challenges like organ-on-Chip (OOC) platforms, such as additional cell types, immune cells, endothelial cells, and extracellular matrix components that need to be incorporated to create a physiological microenvironment for cell differentiation and tissue development. However, mesenchymal cells and neural crest cells have already been successfully implemented in these models (Workman *et al.* 2017). Unlike microfluidic OOC models, stem cell-derived organoids currently lack perfusion and therefore deprive epithelial cells of shear stress and removal of metabolites. An idea has emerged that aims at combining self-assembling organoids with microfluidic OOC techniques, termed "Organoids-on-a-Chip" (Park *et al.* 2019) **(Fig. 1A)**. The technique encompasses the maturation of organoids within a dynamic culture environment allowing the control of nutrient supply, establishment of biochemical gradients vital for self-organization of the organoids, and the introduction of additional cell types.

Studying *Candida* spp. infections of the vaginal tract

The vaginal tract represents another commensal niche of *Candida* spp. in the human body. Vulvovaginal candidiasis (VVC) affects 70-75% of women in their reproductive age (Sobel 2007). Antibiotic treatment is a strong predisposing factor for VVC (Shukla and Sobel 2019), most likely due to the induced dysbiosis of the vaginal microbiome. *C. albicans* is the most prominent species isolated from VVC, followed by *C. glabrata* (Makanjuola *et al.* 2018). The interactions between *Candida* spp. and the vaginal epithelium, as well as the vaginal microbiota are complex (Kalia *et al.* 2020; Pekmezovic *et al.* 2019), and invasion of the epithelium occurs through active penetration **(Fig. 2G I)** and induced endocytosis **(Fig. 2G II),** while neutrophils are attracted simultaneously.

Simple in vitro models mimicking vaginal infections

The VK2/E6E7 cell line originates from healthy human vaginal mucosal tissue and was immortalized by retroviral transduction (Fichorova *et al.* 1997). This cell line was used to demonstrate synergistic interactions between *C. albicans* and streptococci (Pidwill *et al.* 2018) and a role for autophagy machinery in the survival of epithelial cells during *C. albicans* infection (Shroff and Reddy 2018). In addition, Type-I IFN signaling was elucidated to increase

resistance of the epithelium to *C. albicans* infection (Li *et al.* 2017). By introducing high glucose conditions, this model has been used to demonstrate that the association of VVC in diabetes patients might be related to increased adhesion of *C. albicans* through a potential interaction with ICAM-1 (Mikamo *et al.* 2018). Another cell line, A431, originates from a vaginal epidermoid carcinoma. This cell line was used to investigate inflammatory cytokine responses and damage of A431 cells induced by candidalysin (Richardson *et al.* 2018). Additionally, the cell line was utilized to evaluate the impact of azole antifungal treatment on damage induced by *C. albicans* spp. (Wachtler *et al.* 2011b). **(Tab. S3C)**.

Complex in vitro models mimicking vaginal infections

A reconstituted vaginal epithelium (RHVE) is available as an alternative model. RHVE is based on A431 cells, cultivated at an ALI, similar to the previously described RHOE **(Fig. 1B)**. RHVE was used to demonstrate that *C. albicans* facilitates interactions of *C. glabrata* with the vaginal epithelium by increasing fungal colonization, invasion, and damage of epithelial cells during co-infection (Alves *et al.* 2014). Furthermore, the adaptation of *C. glabrata* to an acidic vaginal environment was investigated using RHVE (Bernardo *et al.* 2017) **(Tab. S3C)**.

Organ-on-chip models mimicking vaginal infections

Several OOC models for the female reproductive tract are available, predominantly to mimic the physiology of the endometrium, the uterus, or the placenta (Mancini and Pensabene 2019). Possible OOC models of the vaginal mucosa should comprise stratified squamous epithelium and perfused endothelial cells, separated by a porous membrane. Immune cells can easily be integrated to recapitulate relevant inflammatory responses during hyphal invasion of the epithelium such as neutrophil recruitment.

In vivo, the vaginal tract harbors a microbiota that consist to a large extent of *Lactobacillus* species. Although predicted, it is not entirely clear whether the microbiota actually has a protective effect against *Candida* spp. infection and if so, whether diversity among microbial communities leads to a higher degree of protection (Cassone 2015).

Studying fungal bloodstream infection and crossing of the blood-brain barrier (BBB)

Vascular infection models

Fungal dissemination into the bloodstream is a major driver for the development of multi-organ infections or sepsis. *A. fumigatus*, *H. capsulatum,* and *C. neoformans* can enter the bloodstream after crossing the pulmonary alveolar epithelium **(Fig. 2C)**, whereas *C. albicans* reaches the bloodstream mostly *via* the intestinal tract **(Fig. 2F)**. Central venous catheters, surgery, and parenteral nutrition represent additional entry routes, especially for *Candida* species (Hashemi Fesharaki *et al.* 2018). To exit the blood circulation and invade other organs, fungi interact with the endothelial lining of the blood vessels **(Fig. 2D)**, which can be simulated by human umbilical vein endothelial cells (HUVECs) (Jaffe *et al.* 1973). Although access to umbilical cords is limited, high amounts of cells can be isolated from a single umbilical cord and stored frozen for several experiments (Crampton *et al.* 2007). HUVECs were used to dissect *C. albicans*' adhesion to the endothelial lining **(Fig. 2D I)**, for example, it was shown that a certain hyphal length is crucial for adhesion in a circulatory *in vitro* model that simulated physiological capillary blood pressure (Wilson and Hube 2010) **(Fig. 1B)**. Following adhesion, three mechanisms to pass the endothelial barrier were discovered. Attached *Candida* cells can be endocytosed by endothelial cells (Liu *et al.* 2016; Phan *et al.* 2005) **(Fig. 2D II)**, a process which depends on a complex formation including endothelial cell septin 7 (SEP7) and N-cadherin (Phan *et al.* 2013). Endocytosis was also described for *A. fumigatus,* independent of its morphology (Kamai *et al.* 2006) **(Fig. 2D II).** In addition, *Candida* spp. can cross the endothelial barrier *via* paracellular translocation **(Fig. 2D III)** or *via* leucocytes following engulfment **(Fig.2D IV)** (Filler and Sheppard 2006; Grubb *et al.* 2008). It is likely that similar Trojan horse transport mechanisms following engulfment by mononuclear cells are exploited by intracellularly persistant *H. capsulatum* (Gilbert *et al.* 2014) **(Fig. 2D IV)** as it already has been shown for *Cryptococcus neoformans* (Coelho et al. 2019)**.**

The ability of different *C. albicans* mutants to damage HUVECs was leveraged to identify virulence factors that are important for fungal dissemination (Sanchez *et al.* 2004). Similarly, the transcription factor DvrA was identified as crucial for endothelial damage induced by *A. fumigatus* (Ejzykowicz *et al.* 2010). Besides, the proteome profile of HUVECs was investigated during infection with *A. fumigatus* (Neves *et al.* 2017) and *C. neoformans* (Wang *et al.* 2011),

indicating alterations that contribute to fungal invasion. Transcriptional profiling of HUVECs revealed the upregulation of genes involved in chemotaxis, stress response, angiogenesis, and inhibition of apoptosis in response to *C. albicans* (Barker *et al.* 2008). A proinflammatory immune response associated with the release of TNFα in HUVECs was reported after infections with *C. albicans* (Orozco *et al.* 2000) and *A. fumigatus* (Kamai *et al.* 2009; Neves *et al.* 2017). In addition, it was shown that neutrophils protect endothelial cells against *C. albicans*-induced damage in a co-culture model with HUVECs and neutrophils (Edwards *et al.* 1987) **(Tab. S4)**.

Blood-brain barrier

Whereas cerebral infections with *Candida* spp. (Drummond *et al.* 2015), *Aspergillus* spp. (Rieber *et al.* 2016), or *Histoplasma* spp. (Schestatsky *et al.* 2006) are rare, meningitis is the most prominent complication during cryptococcosis (Srikanta *et al.* 2014). Cerebral infections are induced when fungi cross the blood-brain barrier (BBB), a part of the neurovascular unit (NVU). Other than the endothelial lining, the NVU consists of pericytes, forming a scaffold for endothelial cells together with the basal lamina. Endfeet of astrocytes provide a connection to neurons and microglia (van der Helm *et al.* 2016). A physical barrier between the blood circulation and the brain tissue is maintained by an intact NVU *via* zona occludens proteins and claudins.

Simple in vitro models mimicking the blood-brain barrier

Immortalized human brain vascular endothelial cells (HBMEC and HCMEC/D3) are commonly used for BBB models, whereas primary cells are not frequently used due to drawbacks such as insufficient availability and loss of phenotype during culturing (Oddo *et al.* 2019). The HBMEC and HCMEC/D3 cell lines are especially suitable to model the BBB because of their expression of tight junction proteins, receptors, and transporters (Oddo *et al.* 2019; Weksler *et al.* 2013). They can be cultured as monolayers on transwell inserts or cell culture plates and infected with *C. albicans* (Jong *et al.* 2001), *A. fumigatus* (Patel *et al.* 2018), or *C. neoformans* (Aaron *et al.* 2018) and used for transcytosis (Aaron *et al.* 2018), gene expression (Lahiri *et al.* 2019), and barrier integrity studies (Patel *et al.* 2018). For example, it was demonstrated that *C. neoformans* and *C. albicans* can pass the BBB *via* transcytosis **(Fig. 2B I)**. True hyphae of *C. albicans* are associated with endocytosis by endothelial cells (Liu *et al.* 2011) **(Fig. 2B I).** *C.*

neoformans, however, was shown to also translocate paracellularly **(Fig. 2B II)** and use macrophages as a shuttle to cross the BBB using the Trojan horse mechanism mentioned above (Charlier *et al.* 2009; Santiago-Tirado *et al.* 2017) **(Fig. 2B III).** This mechanism was visualized and analyzed in detail using a co-culture model of HCMEC/D3 cells and THP-1 cells or primary monocytes (He *et al.* 2016; Santiago-Tirado *et al.* 2017) **(Tab. S4)**.

BBB-on-chip models

2D transwell models of the BBB can be valuable tools to gain insights into how fungi invade the central nervous system (CNS). However, current models lack some key properties of the NVU. For example, endothelial cells need to experience shear stress to trigger the establishment of a barrier that limits Na^+ and Cl^- ions efflux and influx (Oddo *et al.* 2019). Furthermore, to mimic the physiological situation more closely, the model should contain multiple cell types of the NVU such astrocytes, pericytes, and neurons since their communication influences each other's growth, differentiation, and permeability (Abbott *et al.* 2006). A range of microfluidic BBB-on-Chip models has recently been developed, recapitulating the blood flow by perfusion of the endothelium in realistic dimensions and geometry and integration of various NVU cell types (Griep *et al.* 2013; Maoz *et al.* 2018; Raasch *et al.* 2016). In models using one cell type, HUVECs in astrocyte conditioned medium or HCMEC/D3 cells have been cultured in a single perfused channel (Englert *et al.* 2016; Griep *et al.* 2013; Yeon *et al.* 2012). Using a CNS angiogenesis model comprising endothelial cells, pericytes, astrocytes, and lung fibroblasts, it was demonstrated that a low vascular permeability can be achieved by co-culturing the different NVU cell types (Lee *et al.* 2020). These microfluidic BBB models can contribute to investigating the role of additional cell types of the NVU and shear stress in the transmigration of fungi across the BBB. Moreover, the implementation of innate immune cells would enable the simulation of inflammatory responses in the brain tissue following fungal invasion **(Tab. S4)**.

Future directions

Interconnecting Organ-on-chip systems to study fungal dissemination

Although the multiple infection models reviewed here have been and will be very useful tools to study fungal infections, we can expect a new generation of complex *in vitro* system based

on OOC platforms. In fact, individual OOC systems can be combined to recapitulate multi-organ cross communication in an enclosed microfluidic network (Luni *et al.* 2014). These platforms have the potential to investigate fungal infections not only at a single-organ level, but also at the multi-organ level, including systemic immune responses **(Fig. 1A)**. The complexity of systemic immune reactions was only addressed in animal models until recently. Multi-organ-on-chip (MOC) models expand the toolbox with systems having a purely human genetic background to circumvent the problem of inter-species transferability. A range of MOC platforms have been developed that connect two or more organs such as the liver and intestine (Chen *et al.* 2018; Ramme *et al.* 2019; Zhang *et al.* 2009). MOC models provide the opportunity to study the dissemination of fungi throughout the body. It will allow (to mimic) tracking dissemination of *Candida* spp. from the intestine to the liver and kidney, one of the key target organs of disseminated candidiasis and the main target organ in mice (Lionakis *et al.* 2011), or dissemination of *A. fumigatus*, *C. neoformans*, and *H. capsulatum* from the lung to the brain, which has not been possible *in vitro* so far. An additional aspect to be elucidated using MOC models is the relationship between dysbiosis in the intestine resulting in overgrowth of *C. albicans* and concomitant biochemical changes in the brain or the liver (gut-brain axis and gut-liver axis, respectively) (Burrus 2012; Yang *et al.* 2017). However, MOC systems are still in their infancy and there are many obstacles to overcome. A current challenge is to scale the organs to their relative physiological size (Lee and Sung 2017; Rogal *et al.* 2017). Current MOC systems are mostly used for toxicity screening of drugs and chemicals and are constructed in a way to be suitable for this particular application (Rogal *et al.* 2017). MOC models dedicated for fungal studies may take into account other criteria, e.g. the distance between distinct tissues, the number of integrated immune cells, and possibilities to prevent adherence of fungi to tubing and subsequent clogging, to be applicable as tools.

Human induced pluripotent stem cells as another cell source for fungal *in vitro* systems

The *in vitro* models discussed in this review rely on primary cells and cell lines. Human induced pluripotent stem cells (hiPSC) are an alternative source of cells and are highly relevant for biomedical research (Raasch *et al.* 2019). hiPSC can be generated by reprogramming adult tissue cells, such as fibroblasts, to an embryonic-like pluripotent state (Takahashi and Yamanaka 2006). Once reprogrammed, they can be differentiated into virtually all cell types except extra-embryonic cell types. Therefore, they offer the opportunity to establish OOC

systems containing various cell types originating from a single donor. However, current models often combine hiPSC with primary cells and cell lines. Taking the BBB as an example, Brown and colleagues cultured HBMEC, glutamatergic neurons differentiated from iPSC, primary pericytes and astrocytes in a two-chamber model. The resulting system consisted of a brain compartment, which is separated from perfused vasculature by a porous membrane (Brown *et al.* 2015).

hiPSC are also utilized for the establishment of "patient-on-chip" models to mimic genetic predispositions. Aspergillosis is a common complication of patients suffering from asthma and cystic fibrosis (CF) (Knutsen and Slavin 2011) or CGD (Leiding and Holland 1993), CARD9 and STAT1 mutations predispose for *C. albicans* CNS (Drummond *et al.* 2019) and mucocutaneous infections (van de Veerdonk *et al.* 2011) respectively, and diabetes mellitus is a common predisposition for histoplasmosis (Lockhart and Guarner 2019). Furthermore, intestinal fungi have been tightly connected to inflammatory bowel diseases (Leonardi *et al.* 2018). Future OOC models might be able to reflect these predispositions by implementing hiPSC generated from patients bearing these diseases. Alternatively, specific mutations associated with the disease can be reproduced in hiPSCs. For example, they have been successfully differentiated into macrophages and lung epithelial cells that carry mutations associated with CF (Pollard and Pollard 2018) and CGD (Brault *et al.* 2017). Although there has been substantial progress in OOC systems incorporating hiPSC, caution should be exercised: Protocols for differentiation require optimization and standardization, especially the understanding of factors promoting differentiation need improvement. Differentiation might differ under static and dynamic conditions (Luni *et al.* 2014; Rogal *et al.* 2017). Standardization of these aspects is crucial to guarantee reproducibility of findings from different labs.

Concluding remarks

To study human fungal infections on a higher level of complexity, expertise of fungal infection biology and the OOC platforms needs to be combined. This will ensure studies in the most suitable *in vitro* model, providing conditions akin to the *in vivo* situation. For example, 3D Intestine-on-chip models will be valuable tools to uncover the role of microbial commensals and their products, as well as host immune responses to a local yeast-to-hypha transition of *C. albicans*. In the future, it would be favorable to make use of experience gained with MOC

systems to mimic and follow fungal dissemination throughout the body and evaluate novel therapeutic strategies addressing fungal infections.

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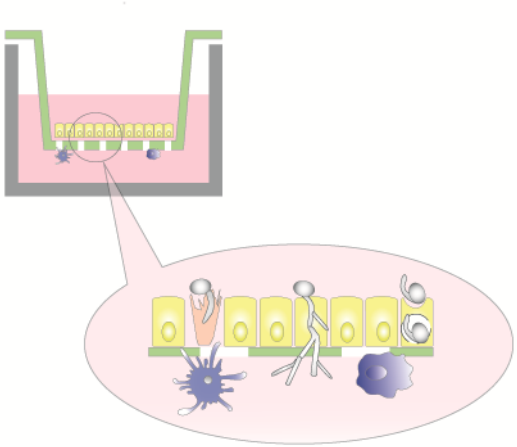
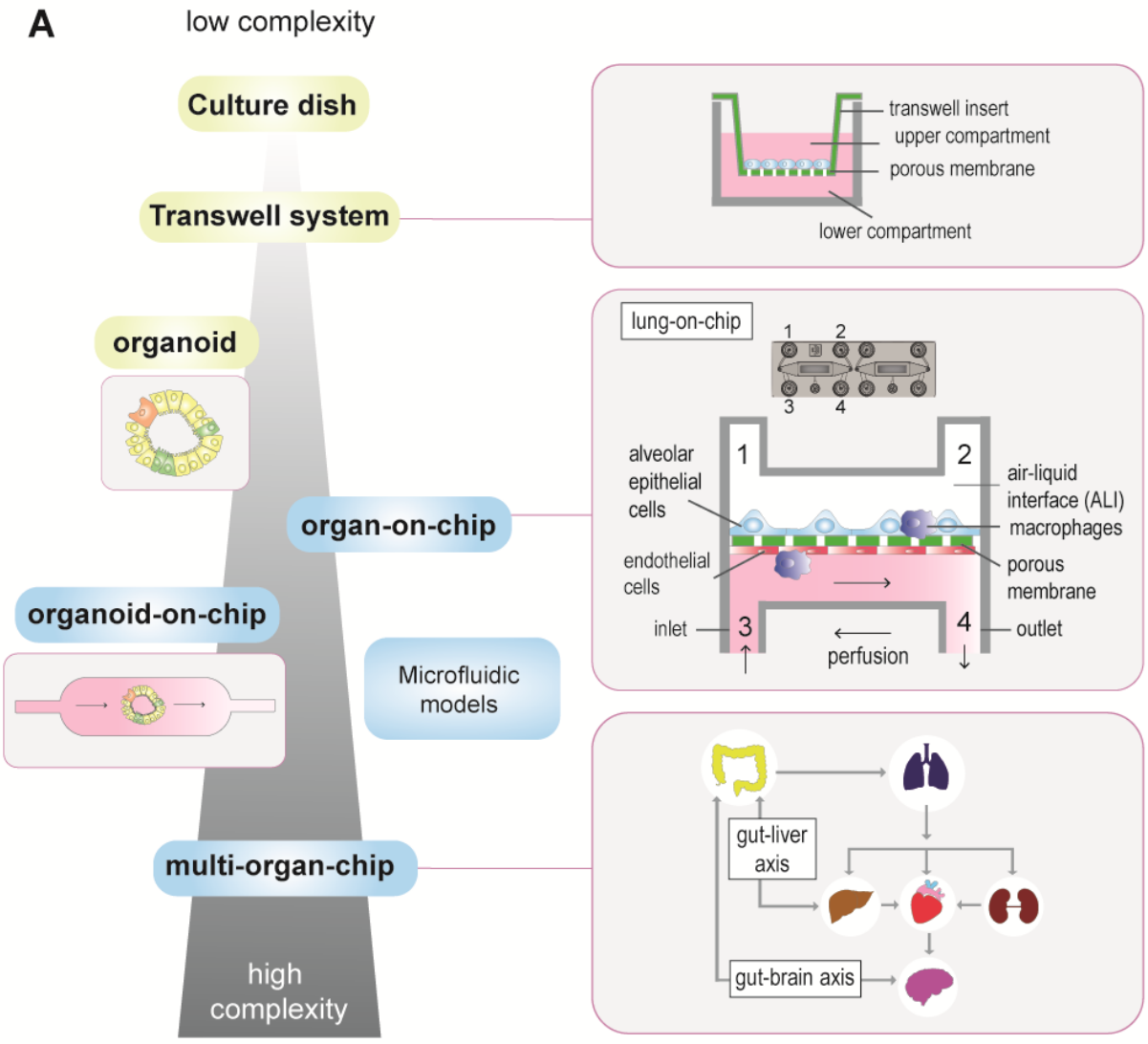
Figures:



Grafical abstract

Summary sentence: From basic to complex: *in vitro* models to study interactions between human fungal pathogens and their host.


A
low complexity
Culture dish
Transwell system
organoid
organ-on-chip
organoid-on-chip
Microfluidic models
multi-organ-chip
high complexity
transwell insert
upper compartment
porous membrane
lower compartment
lung-on-chip
1
2
3
4
alveolar epithelial cells
air-liquid interface (ALI)
macrophages
endothelial cells
porous membrane
inlet
outlet
perfusion
gut-liver axis
gut-brain axis

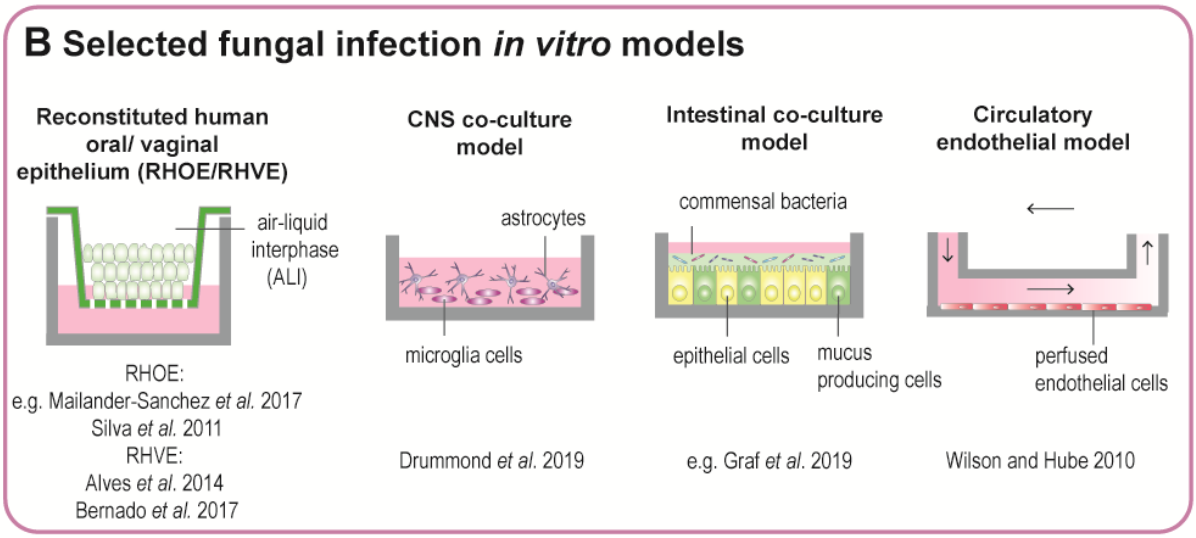

B Selected fungal infection *in vitro* models
Reconstituted human oral/ vaginal epithelium (RHOE/RHVE)
air-liquid interphase (ALI)
RHOE:
e.g. Mailander-Sanchez *et al.* 2017
Silva *et al.* 2011
RHVE:
Alves *et al.* 2014
Bernado *et al.* 2017
CNS co-culture model
astrocytes
microglia cells
Drummond *et al.* 2019
Intestinal co-culture model
commensal bacteria
epithelial cells
mucus producing cells
e.g. Graf *et al.* 2019
Circulatory endothelial model
perfused endothelial cells
Wilson and Hube 2010

Figure 1. (A) Evolution of *in vitro* models from low to high complexity. Culture dish: one cell type cultured in media. **Transwell system**: transwell inserts separate the culture area into an upper and lower compartment; cells are cultured under static conditions on a porous membrane allowing apical-basal polarization. **Organoid**: 3D miniature organ generated out of intestinal stem cells. **Organ-on-chip** (example): 3D lung on-chip model on a microfluidic biochip holding a porous membrane and two individually accessible channels with one inlet and outlet each; pulmonary epithelial cells are cultured in the upper compartment in an air-liquid interface; endothelial cells in the lower compartment are perfused with cell culture medium enabling removal of metabolites. **Organoid-on-chip**: maturation of organoids within a dynamic culture environment. **Principle of a multi-organ-on-chip**: Interconnected organ-on-chip models of gut and liver or gut and brain or other combinations of lung, intestine, liver, brain and/or kidneys. Such combinations can, for example, mimic certain steps of fungal dissemination throughout the body. The intestine and lung serve as primary infection sites. **(B) Selected *in vitro* models to study host-fungal interactions.** 3D reconstituted human oral (RHOE) or vaginal (RHVE) epithelium grown at an air-liquid interface. CNS co-culture model including microglia cells and astrocytes. Intestinal co-culture model including epithelial cells, goblet cells, and bacteria. Circulatory model with perfused endothelial cells.

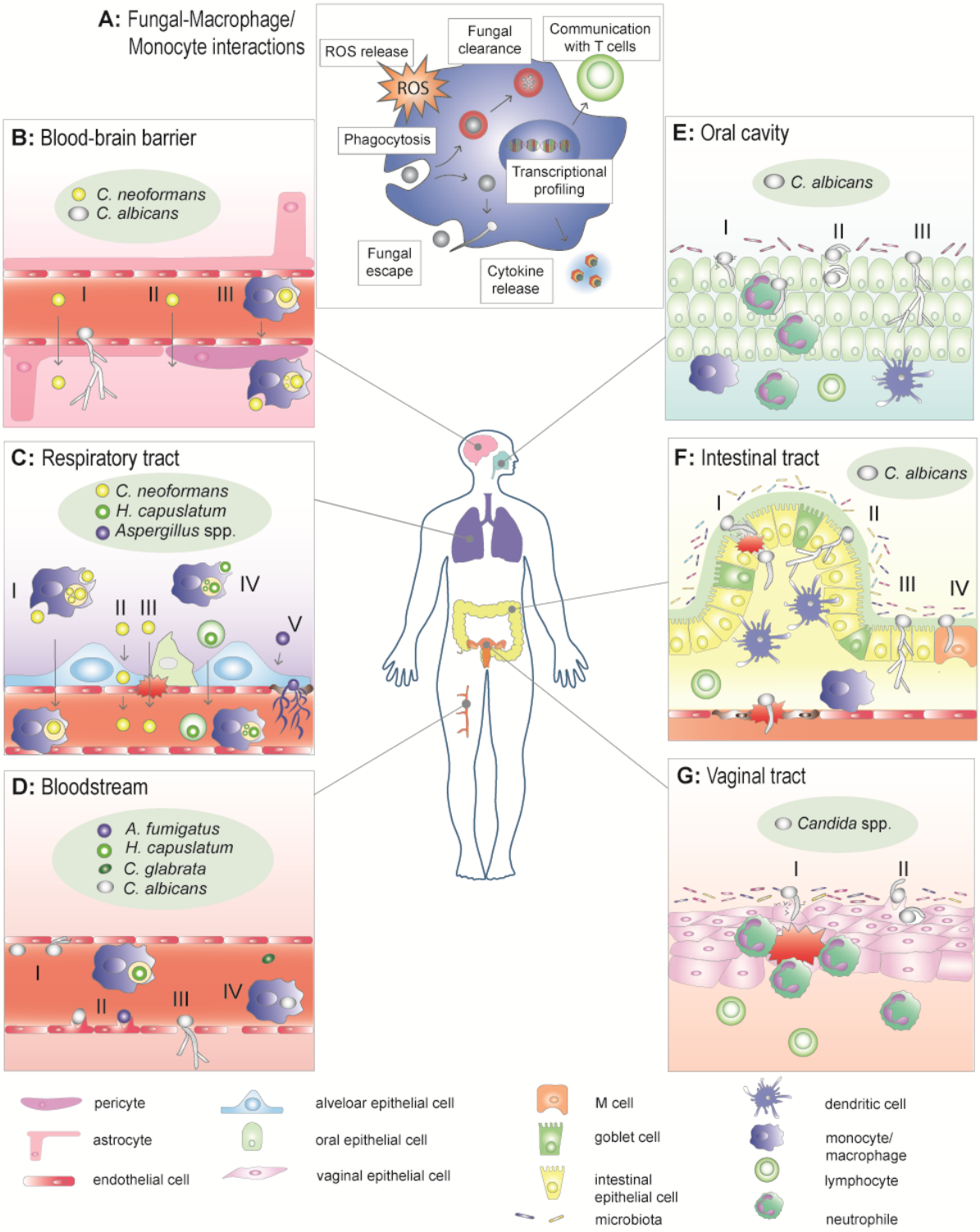

A: Fungal-Macrophage/ Monocyte interactions
ROS release
Fungal clearance
Communication with T cells
ROS
Phagocytosis
Transcriptional profiling
Fungal escape
Cytokine release
B: Blood-brain barrier
C. neoformans
C. albicans
I
II
III
E: Oral cavity
C. albicans
I
II
III
C: Respiratory tract
C. neoformans
H. capuslatum
Aspergillus spp.
I
II III
IV
V
F: Intestinal tract
C. albicans
I
II
III
IV
D: Bloodstream
A. fumigatus
H. capuslatum
C. glabrata
C. albicans
I
II
III
IV
G: Vaginal tract
Candida spp.
I
II
pericyte
astrocyte
endothelial cell
alveloar epithelial cell
oral epithelial cell
vaginal epithelial cell
M cell
goblet cell
intestinal epithelial cell
microbiota
dendritic cell
monocyte/ macrophage
lymphocyte
neutrophile

Figure 2. Fungal – host interactions during fungal diseases which are mimicked by *in vitro* infection models discussed in this review (A) Fungal - monocyte/macrophage interactions resulting in several effector mechanisms that contribute to immunity against fungal infections (ROS: reactive oxygen species). **(B)** *C. neoformans* and *C. albicans* can cross the blood-brain barrier *via* transcytosis **(I);** *C. neoformans* can overcome the barrier paracellularly **(II)**, or use macrophages as shuttles (macrophages as "Trojan horse") **(III)**. **(C)** In the lung, *C. neoformans* and *H. capsulatum* induce their own phagocytosis by innate immune cells, they can replicate intracellularly and use host cells as a shuttles to reach the blood stream and subsequently escape **(I** and **IV)**; evasion of *C. neoformans via* transcytosis **(II)** or crossing of *C. neoformans* through a compromised epithelium **(III)**. *Aspergillus* spp. form hyphae, can invade endothelial cells and enter the bloodstream **(V)**. **(D)** *Candida* spp. can escape the blood circulation after adhesion to endothelial cells **(I)**. *Candida* spp. and *A. fumigatus* can be endocytosed **(II)**; *Candida* spp. can also use fenestrated endothelium as an escape route **(III)** or use leukocytes as shuttles **(IV)**. **(E)** In the oral cavity, *C. albicans* hyphae can actively penetrate the epithelium **(I)** and /or invade *via* induced endocytosis **(II)**, or translocate paracellularly **(III). (F)** In the intestine, *C. albicans* can actively penetrate the epithelium by hyphal growth **(I)**, translocate paracellularly **(II)**, invade without damaging the host cell **(III)** or translocate *via* M cells by inducing endocytosis **(IV)**. **(G)** In the vaginal tract, *C. albicans* hyphae can actively penetrate the epithelium **(I)** or invade *via* induced endocytosis **(II)** thereby attracting neutrophils.

Supplement:

Table S1: Overview about the different immune cell *in vitro* models, their structure, possible read outs and applications for the different fungi

Model	Structure	Readout	Reference
A) Macrophage (like) cell lines			
J774A.1 (Mouse cell line)	Monoculture	Fungal killing	*A. fumigatus*: (Gresnigt *et al.* 2018a)
		Phagocytosis	*A. fumigatus*: (Gresnigt *et al.* 2018a) *C. albicans*: (Cottier *et al.* 2019; Duvenage *et al.* 2019; Loureiro *et al.* 2019; Rudkin *et al.* 2018) *C. neoformans*: (Bryan *et al.* 2014)
		Cytokine release	*A. fumigatus:* (Cho *et al.* 2016)
RAW (Mouse cell line)	Monoculture	Fungal killing	*C. glabrata:* (Chew *et al.* 2019)
		Proteomics	*A. fumigatus*: (Schmidt *et al.* 2018)
		ROS production	*C. albicans*: (Arce Miranda *et al.* 2019)
		Phagosome maturation	*C. albicans:* (Bain *et al.* 2014; Okai *et al.* 2015)
Ana-1 (Mouse cell line)	Monoculture	Apoptosis	*C. albicans*: (Jiang *et al.* 2019)
THP-1 (Human cell line)	Monoculture	Phagocytosis	*C. albicans* : (Liu *et al.* 2019; Vaz *et al.* 2019)
		Proteomics	*C. albicans*: (Vaz *et al.* 2019)
		Cytokine release	*A. fumigatus:* (Oya *et al.* 2019) *C. albicans*: (de Albuquerque *et al.* 2018)
		ROS release	*A. fumigatus:* (Sun *et al.* 2014)
U937 (Human cell line)	Monoculture	Adhesion	*C. albicans:* (Lopez et al. 2014) *C. glabrata:* (Kuhn and Vyas 2012)
		Internalization	*H. capsulatum:* (Scott and Woods 2000)
		Cytokine release	*C. albicans:* (Kaya et al. 2011)
MH-S (Mouse alveolar macrophage cell line)	Monoculture	Phagocytosis	*A. fumigatus:* (Mattern *et al.* 2015)
AMJ2-C11 (Mouse alveolar macrophage cell line)	Monoculture	Intracellular yeast arrangement	*H. capsulatum:* (Pitangui Nde et al., 2015)
BV-2 (Mouse microglial cell line)	Monoculture	Cytokine release	*C. albicans:* (Wu et al. 2019b) *C. neoformans*: (Barluzzi *et al.* 1998)

BV-2 (Mouse cell line) **+** **C8-D1A Astrocytes** (Mouse cell line)	Co-Culture	Cytokine release	*C. albicans:* (Drummond et al. 2019)
B) Primary cells			
Bone marrow derived macrophages BMDMs (Murine primary cells)	Monoculture	Phagocytosis	*A. fumigatus:* (Gresnigt *et al.* 2018a) *C. albicans:* (Haider *et al.* 2019; Okai *et al.* 2015) *H. capsulatum*: (Baltazar *et al.* 2018; Guimaraes *et al.* 2019)
		Cytokine release	*A. fumigatus*: (Rubino *et al.* 2012) *C. albicans*: (Alsina-Beauchamp *et al.* 2018; Thompson *et al.* 2019; Wang *et al.* 2019) *C. neoformans*: (Veloso Júnior *et al.* 2019)
		Exocytosis	*C. neoformans*: (Stukes and Casadevall 2014)
		Transcriptomics	*C. albicans:* (Muñoz et al. 2019)
Alveolar macrophages (Murine/Human primary cells)	Monoculture	Phagocytosis	*A. fumigatus:* (Grimm *et al.* 2014; Wu *et al.* 2016) *C. neoformans*: (Hansakon *et al.* 2019; Walsh *et al.* 2017) *H. capsulatum*: (Pereira *et al.* 2018; Tagliari *et al.* 2012)
		Autophagy	*A. fumigatus:* (Dai *et al.* 2018)
		Apoptosis	*H. capsulatum:* (Deepe and Buesing 2012)
		Cytokine release	*A. fumigatus:* (Zhang *et al.* 2017a) *H. capsulatum*: (Coady and Sil 2015)
Peritoneal macrophages (Murine primary cells)	Monoculture	Phagocytosis	*H. capsulatum*: (Huang et al., 2018)
		Autophagy	*C. albicans:* (Ifrim et al. 2016) *C. glabrata*: (Shimamura *et al.* 2019)
		Cytokine release	*H. capsulatum*: (Shen *et al.* 2018)
		ROS release	*H. capsulatum:* (Youseff *et al.* 2012)
Monocyte derived macrophages MDMs (Human primary cells)	Monoculture	Phagocytosis	*C. albicans:* (Behrens *et al.* 2019; Munawara *et al.* 2017)
		Phagosome maturation	*C. neoformans*: (Smith *et al.* 2015)

		Cytokine release	*A. fumigatus:* (Gresnigt *et al.* 2018b) *C. albicans:* (Kasper *et al.* 2018; O'Meara *et al.* 2018) *H. capsulatum*: (Friedrich *et al.* 2019)
		ROS release	*A. fumigatus:* (Gresnigt *et al.* 2018b) *H. capsulatum:* (Wolf *et al.* 1987)
		Immuno-metabolism	*A. fumigatus:* (Gonçalves et al. 2020)
Monocytes (Human primary cells)	Monoculture	Phagocytosis	*A. fumigatus:* (Brunel *et al.* 2017; Kyrmizi *et al.* 2018) *C. albicans:* (Camilli *et al.* 2018; Halder *et al.* 2016) *C. neoformans*: (Charlier *et al.* 2009)
		Metabolomics	*C. albicans:* (Grondman *et al.* 2019)
		Cytokine release	*C. albicans:* (Dominguez-Andres *et al.* 2017; Leonhardt *et al.* 2018)
		ROS release	*A. fumigatus:* (Brunel *et al.* 2018) *C. albicans*: (Camilli *et al.* 2018; Wellington *et al.* 2009)
Monocytes (Human primary cells) **+** **DCs** (Human primary differentiated cells)	Co-culture	Phagocytosis Cytokine release	*C. neoformans*: (Alvarez *et al.* 2009; Kelly *et al.* 2005)
Neutrophils (Human primary cells)	Monoculture	Fungal killing	*A. fumigatus*: (Dasari *et al.* 2018; Gazendam *et al.* 2016; Jones *et al.* 2019) *C. albicans*: (Essig *et al.* 2015; Gazendam *et al.* 2016; Jones *et al.* 2019; Salvatori *et al.* 2018)
		Phagocytosis	*A. fumigatus:* (Brunel *et al.* 2017) *C. neoformans*: (Sun *et al.* 2016)
		NET formation	*A. fumigatus*: (Bruns *et al.* 2010; Clark *et al.* 2018; Ellett *et al.* 2017; Röhm *et al.* 2014; Silva *et al.* 2019) *C. albicans*: (Campos-Garcia *et al.* 2019; Guiducci *et al.* 2018; Johnson *et al.* 2017; Negoro *et al.* 2020; Urban *et al.* 2006; Wu *et al.* 2019a; Zawrotniak *et al.* 2019)

			C. neoformans: (Rocha *et al.* 2015) *H. capsulatum*: (Thompson-Souza *et al.* 2020)
		Transcriptomics	*C. albicans*: (Niemiec *et al.* 2017)
		ROS release	*A. fumigatus:* (Boyle *et al.* 2011) *C. albicans:* (Liu *et al.* 2018; Miramón *et al.* 2012; Salvatori *et al.* 2018)
	Monoculture on poly-L-lysine coated glass slides	Swarming	*C. albicans:* (Hopke et al. 2020)
	Monoculture on Transwell (top: neutrophils Bottom: chemoattractant)	Chemotaxis	*A. fumigatus:* (Rieber *et al.* 2016) *C. albicans*: (Drummond *et al.* 2015) *C. neoformans:* (Coenjaerts *et al.* 2001)
Dendritic cells DCs (Human primary differentiated cells)	Monoculture	Phagocytosis	*A. fumigatus*: (Lother *et al.* 2014) *H. capsulatum*: (Gildea *et al.* 2001; Nguyen *et al.* 2018)
		Maturation	*A. fumigatus*: (Fliesser *et al.* 2016; Hefter *et al.* 2017; Lother *et al.* 2014) *C. albicans:* (Roudbary *et al.* 2009; Vivas *et al.* 2019) *C. glabrata:* (Bazan *et al.* 2018) *C. neoformans:* (Pietrella *et al.* 2005)
		Transcriptomics	*A. fumigatus*: (Srivastava *et al.* 2019) *H. capsulatum*: (Van Prooyen *et al.* 2016)
		Cytokine release	*C. albicans*: (Maher *et al.* 2015) *H. capsulatum*: (Chang *et al.* 2017; Garfoot *et al.* 2016)
Natural killer cells NK cells (Human primary cells)	Monoculture	Fungal killing	*A. fumigatus:* (Weiss *et al.* 2018) *C. albicans*: (Li *et al.* 2018) *C. neoformans:* (Ma *et al.* 2004; Wiseman *et al.* 2007)
		Transcriptomics	*C. albicans*: (Hellwig *et al.* 2016)
		Chemokines	*A. fumigatus:* (Marischen *et al.* 2018)

		Cytokine release	*A. fumigatus:* (Bouzani *et al.* 2011; Santiago *et al.* 2018) *C. albicans:* (Marolda *et al.* 2020; Voigt *et al.* 2014)
NK cells (Human primary cells) **+ DCs** (Human primary differentiated cells)	Co-culture	NK-DC cross talk	*A. fumigatus:* (Weiss *et al.* 2018)
DCs (Human primary differentiated cells) **+ T-cells** (Human primary cells)	Co-culture	Maturation	*A. fumigatus:* (Stephen-Victor *et al.* 2017)
		Cytokine release	*C. albicans:* (van der Does *et al.* 2012)
		Cytokine release	*A. fumigatus:* (Becker *et al.* 2016; Page *et al.* 2018) *C. albicans*: (Alvarez-Rueda *et al.* 2020; Estrada-Mata *et al.* 2015; Li *et al.* 2016) *C. neoformans:* (Mora *et al.* 2017; Siddiqui *et al.* 2006)
Whole blood model (Human/Mouse)	Multiculture	Fungal killing	*C. albicans*: (Duggan *et al.* 2015; Hunniger *et al.* 2014)
		Transcriptomics	*A. fumigatus:* (Dix *et al.* 2015) *C. albicans:* (Fradin *et al.* 2005; Kämmer *et al.* 2020)
		Cytokine release	*A. fumigatus:* (Oesterreicher *et al.* 2019)
		Platelet interaction	*A. fumigatus:* (Fréalle *et al.* 2018) *C. albicans:* (Eberl *et al.* 2019)

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Table S2: Overview about the different lung *in vitro* models, their structure, possible read outs and applications for the different fungi

Model	Structure	Readout	Reference
Alveolar epithelial cells A549 (Human cell line)	Monoculture	Adhesion	*A. fumigatus*: (Gravelat *et al.* 2010; Xu *et al.* 2012) *C. neoformans*: (Choo *et al.* 2015; Ganendren *et al.* 2006; Teixeira *et al.* 2014) *H. capsulatum*: (Pitangui *et al.* 2012)
		Endocytosis	*A. fumigatus:* (Amin *et al.* 2014; Liu *et al.* 2016; Xu *et al.* 2012)
		Damage	*A. fumigatus:* (Bertuzzi *et al.* 2014; Dasari *et al.* 2019; Ejzykowicz *et al.* 2010)
		Cell Detachment	*A. fumigatus:* (Bertuzzi *et al.* 2014; Kogan *et al.* 2004)

		Transcriptomics	*A. fumigatus:* (Jepsen *et al.* 2018; Takahashi-Nakaguchi *et al.* 2018; Watkins *et al.* 2018)
		Proteomics	*A. fumigatus:* (Margalit *et al.* 2020; Voltersen *et al.* 2018)
		Metabolomics	*C. neoformans*: (Liew *et al.* 2016)
		Cytokine release	*C. neoformans:* (Barbosa *et al.* 2007) *H. capsulatum*: (Alcantara *et al.* 2020; Maza and Suzuki 2016)
Human bronchial epithelial or small airway epithelial Cells **HBE, SAE** (Human primary cells)	Monoculture	Cytokine release	*A. fumigatus*: (Sun *et al.* 2012) *C. neoformans:* (Guillot *et al.* 2008)
	Monoculture differentiated in an ALI	Transcriptomics Proteomics	*A. fumigatus*: (Toor *et al.* 2018)
A549 (Human cell line) **+ DCs** (Human primary differentiated cells)	Co-culture on Transwell (top: A549 + DCs)	Damage Membrane integrity Cytokine release	*A. fumigatus*: (Morton *et al.* 2018)
Human pulmonary artery endothelial cells HPAECs (Human cell line) **+ A549** (Human cell line)	Co-culture on Transwell (top: A549 bottom: HPAECs)	Microscopy Kinetics	*A. fumigatus*: (Hope *et al.* 2007)
		Cytokine release	*A. fumigatus*: (Belic *et al.* 2018)
HBE, SAE (Human primary cells) **+ DCs** (Human primary differentiated cells) **+ Macrophages** (Human primary cells)	Co-culture differentiated in an ALI	Cytokine release	*A. fumigatus*: (Chandorkar *et al.* 2017)
HPAECs (Human cell line)	Co-culture on	Transcriptomics	*A. fumigatus*: (Morton *et al.* 2014)

+ A549 (Human cell line) **+ monocyte-derived DCs and myeloid DCs** (Human primary differentiated cells)	Transwell (top: A549 + DCs bottom: HPAECs)		
Lung-on-chip model alveolar epithelium **+** microvascular endothelium	Bilayer with ALI on one side and flow on the other side	Model establishment	(Deinhardt-Emmer et al. 2020)

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Table S3: Overview about the different *in vitro* models for the gastrointestinal tract and vaginal tract, their structure, possible read outs and applications for *Candida* spp.

Model	Structure	Readout	Reference
A) Oral cavity			
Keratinocyte cells TR146 (Human cell line)	Monoculture	Invasion	*C. albicans:* (Puri *et al.* 2019)
		Damage	*C. albicans:* (Wilson *et al.* 2014)
		Transcriptomics	*C. albicans:* (McCall *et al.* 2018; Schaller *et al.* 1998)
		Cytokine release	*C. albicans:* (Ho *et al.* 2019; Verma *et al.* 2017; Verma *et al.* 2018)
Tongue cells SCC15 (Human cell line)	Monoculture	Invasion	*C. albicans:* (Villar et al. 2007)
		Damage	*C. albicans:* (Kumar et al. 2015)
		Cytokine release	*C. albicans:* (Dongari-Bagtzoglou and Kashleva 2003)
Immortalized oral mucosal cells OKF6/TERT-2 (Human cell line)	Monoculture	Invasion	*C. albicans:* (Solis et al. 2017; Swidergall et al. 2018; Zhu et al. 2012)
		Damage	*C. albicans:* (Liu et al. 2014; Solis et al. 2018)
		Transcriptomics	*C. albicans:* (Liu *et al.* 2015)
		Live cell imaging	*C. albicans:* (Wollert *et al.* 2012)
Human palate epithelial cells HPECs (Human primary cells)	Monoculture	Apoptosis	*C. albicans:* (Casaroto *et al.* 2019)
		Host gene expression	*C. albicans:* (Offenbacher *et al.* 2019)
TR146 (Human cell line) **+ Fibroblasts** (Human primary cells)	Co-culture	Adhesion Gene expression	*C. albicans:* (Morse *et al.* 2018)
Reconstituted human oral epithelium RHOE (cell line based)	3D structure Multiculture model with differentiated cells	Damage	*C. albicans* / *C. glabrata:* (Silva *et al.* 2011) *C. albicans:* (Cavalcanti *et al.* 2015; Mailander-Sanchez *et al.* 2017)
		Transcriptomics	*C. albicans:* (Nailis *et al.* 2010; Spiering *et al.* 2010)
		Cytokine release	*C. albicans:* (Wagener *et al.* 2012)

RHOE + Fibroblasts (Human primary cells)	3D structure Multiculture model with differentiated cells	Bacterial interactions	*C. albicans:* (Bertolini *et al.* 2019; de Carvalho Dias *et al.* 2018; Diaz *et al.* 2012)
		Biofilm formation	*C. albicans:* (Sobue *et al.* 2018)
Oral mucosa-on-chip Keratinocytes Gie-No3B11 (Human cell line) **+ gingival fibroblasts** (Human cell line)	Collagen embedded fibroblast and Keratinocytes on a porous membrane under flow	Model establishment	(Rahimi *et al.* 2018)
B) Intestinal tract			
Colorectal adenocarnima cells Caco-2 (Human cell line)	Monoculture	Damage	*C. albicans:* (Wachtler *et al.* 2011a)
		Invasion	*C. albicans:* (Goyer *et al.* 2016; Wachtler *et al.* 2011a)
		Interactions antagonistic yeasts	*C. albicans*: (Lohith and Anu-Appaiah 2018; Murzyn *et al.* 2010) *C. glabrata*: (Kunyeit *et al.* 2019)
		Cytokine release	*C. albicans:* (Gacser *et al.* 2014; Mao *et al.* 2019; Schirbel *et al.* 2018)
Colorectal adenocarnimo cells HT-29 (Human cell line)	Monoculture	Adhesion Invasion Damage	*C. albicans:* (Deng *et al.* 2015; Garcia *et al.* 2018)
Mucus secreting goblet cells HT-29-MTX (Human cell line)	Monoculture	Gene expression	*C. albicans:* (Kavanaugh *et al.* 2014)
Subclone of Caco-2 C2BBe1 (Human cell line)	Monoculture in transwell	Translocation	*C. albicans:* (Allert *et al.* 2018)
		Transcriptomics	*C. albicans:* (Bohringer *et al.* 2016)
Caco-2 (Human cell line) **+ Raji B cells** (Human cell line)	Co-culture	Adhesion Invasion	*C. albicans:* (Albac *et al.* 2016)
C2BBe1 (Human cell line)	Co-culture	Adhesion	*C. albicans:* (Graf *et al.* 2019)

+ HT-29-MTX (Human cell line)		Damage	
Intestine-on-Chip C2BBe1 (Human cell line) **+ HUVECs** (Human primary cells) **+ Monocytes** (Human primary cells)	Bilayer under flow (top: C2BBe1 Bottom: HUVECs + Monocytes)	Damage Translocation	*C. albicans:* (Maurer *et al.* 2019)
C) Vaginal tract			
Human immortalized vaginal mucosal cells VK2/E6E7 (Human cell line)	Monoculture	Adhesion	*C. albicans:* (Luan *et al.* 2020; Mikamo *et al.* 2018)
		Bacterial interactions	*C. albicans:* (Pidwill *et al.* 2018)
		Autophagy	*C. albicans:* (Shroff and Reddy 2018)
		Cytokine release	*C. albicans:* (Li *et al.* 2017)
Vaginal epidermoid carcinoma cells A431 (Human cell line)	Monoculture	Damage	*C. albicans:* (Wachtler *et al.* 2011b)
		Cytokine release	*C. albicans:* (Richardson *et al.* 2018)
Reconstituted vaginal epithelium RHVE (cell line based)	3D structure Multiculture model with differentiated cells	Invasion Damage	*C. albicans:* (Alves *et al.* 2014)
		Transcriptomics	*C. glabrata:* (Bernardo *et al.* 2017)
		Cytokine release	*C. albicans:* (Schaller *et al.* 2005)

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Table S4: Overview about the different endothelial and blood-brain barrier *in vitro* models, their structure, possible read outs and applications for the different fungi

Model	Structure	Readout	Reference
Human umbilical vein endothelial cells HUVECs (Human primary cells)	Monoculture	Adhesion	*C. albicans:* (Citiulo *et al.* 2012; Lopez *et al.* 2014; Phan *et al.* 2013) *C. neoformans*: (Ibrahim *et al.* 1995)
		Invasion	*A. fumigatus:* (Liu et al. 2016) *C. albicans:* (Phan *et al.* 2013; Phan *et al.* 2005; Phan *et al.* 2007)
		Damage	*A. fumigatus:* (Ejzykowicz et al. 2010) *C. albicans:* (Rotrosen et al. 1985; Sanchez et al. 2004)
		Proteomics	*A. fumigatus:* (Neves *et al.* 2016; Zhang *et al.* 2017b) *C. neoformans*: (Wang *et al.* 2011)
		Transcriptomics	*C. albicans:* (Barker et al. 2008) *C. neoformans*: (Coenjaerts *et al.* 2006)
		Cytokine release	*A.fumigatus:* (Kamai *et al.* 2009; Neves *et al.* 2017) *C. albicans*: (Orozco *et al.* 2000)

	Monoculture under flow	Adhesion	*C. albicans:* (Wilson and Hube 2010)
HUVECs (Human primary cells) + **Neutrophils** (Human primary cells)	Co-culture	Damage	*C. albicans:* (Edwards et al. 1987)
Human brain microvascular endothelial cells HMBEC HCMEC/D3 (Human cell line)	Monoculture on Transwell	Adhesion Invasion	*C. albicans*: (Liu *et al.* 2011)
		TEER Permeability assay	*A. fumigatus*: (Patel *et al.* 2018) *C. neoformans:* (Stie and Fox 2012)
		Traversal	*C. neoformans:* (Aaron *et al.* 2018; Chang *et al.* 2004; Huang *et al.* 2011; Kim *et al.* 2012; Na Pombejra *et al.* 2018; Na Pombejra *et al.* 2017; Vu *et al.* 2013; Vu *et al.* 2009; Zhu *et al.* 2017)
		TEM	*C. neoformans*: (Chen *et al.* 2003)
		Transcriptomics	*C. neoformans*: (Lahiri *et al.* 2019)
HMBEC HCMEC/D3 (Human cell line) **+ THP-1** (Human cell line) **or Monocytes** (Human primary cells)	Co-culture on transwell	Model development	*C. neoformans*: (Santiago-Tirado *et al.* 2019)
		Traversal	*C. neoformans*: (He *et al.* 2016; Santiago-Tirado *et al.* 2017; Sorrell *et al.* 2016)
BBB -on-Chip HUVECs (Human primary cells)	Monoculture under flow	Model establishment	(Yeon *et al.* 2012)
BBB -on-Chip HCMEC/D3 (Human cell line)	Monoculture under flow	Model establishment	(Griep *et al.* 2013)
BBB -on-Chip HCMEC/D3 + astrocytes (Human primary cells) **+ pericytes** (Human primary cells)	Multiculture under flow	Model establishment	(Lee *et al.* 2020)

+ fibrobasts (Human primary cells)			

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3. Discussion

3.1. Model *C. albicans* as a commensal

Most available *in vitro* models only allow studying the pathogenicity mechanisms of *C. albicans* in the state where the fungus behaves like a pathogen (Reviewed in Manuscript V). These models are often characterized by a relatively low biological complexity, absence of immune cells as well as bacteria of the microbiota. Consequently, these models are ideal to model certain steps of diseases caused by the fungus in the context of immunocompromised and antibiotic-treated patients. The research presented in this thesis aimed to distinguish from existing models by establishing and studying an *in vitro* model that rather mimics commensalism of *C. albicans* in the intestine. A systematic approach was taken to mimic the physiological features of the intestine and increase the biological complexity of the model on various levels.

Mucus-secreting goblet cells were included next to intestinal epithelial cells (IECs) since mucins have been observed to reduce *C. albicans* pathogenicity (Kavanaugh *et al.* 2014). This co-culture reduced *C. albicans* adhesion and translocation to a certain extent (Manuscript II (Graf *et al.* 2019)). The intestinal microbiota is one of the most complex players that supports the commensal state of *C. albicans in vivo* (Manuscript I (Förster *et al.* 2016)). Consequently, colonization of the model with *Lactobacillus* spp. to model a single-species artificial intestinal microbiota had a significant impact on *C. albicans* pathogenicity in the *in vitro* model (Manuscript II (Graf *et al.* 2019)). To mimic the varying oxygen availability in the intestine (Glover *et al.* 2016; Zheng *et al.* 2015) and because of the preferred growth of many microbiome members under anaerobic conditions, the influence of different oxygen levels was tested in the model. Low oxygen levels reduced cytotoxicity of *C. albicans* (Manuscript II

(Graf *et al.* 2019)), but not to a greater extent than colonization with *Lactobacillus* species. Further investigations were conducted under normoxia to exclude the influence of hypoxia and for ease of model handling. The protection against *C. albicans* induced cytotoxicity by *L. rhamnosus* was further validated in a more complex intestine-on-chip model that mimics the intestinal epithelium to near-physiological conditions and includes resident tissue immune cells (Manuscript IV (Maurer *et al.* 2019)). These models provided a solid base for the further dissection of the molecular interactions between *L. rhamnosus* and IECs that foster the near-commensal state of *C. albicans* (Manuscript III). One could debate whether the phenotype that was achieved in the novel *in vitro* models mimics commensalism or is just suppressed pathogenicity. As (invasive) candidiasis can only originate from the intestine under severe clinical predispositions, it is a general question of how commensalism of *C. albicans* can be defined. Interestingly, the observations made in this thesis show several parallels to the *in vivo* situation.

The reduction in *C. albicans*-induced host cell damage in the presence of *L. rhamnosus* colonization was observed over 48h in the static *in vitro* model (Manuscript II (Graf *et al.* 2019)). The limitation of this static model is related to the depletion of the culture medium as well as accumulation of metabolic end products that could be prevented by perfused models as used in Manuscript IV (Maurer *et al.* 2019). The colonization with *L. rhamnosus*, which growth is dependent on metabolites secreted by IECs (Manuscript III), represents another similarity to the complex interactions between the microbiota and the intestinal epithelium (Guzman *et al.* 2013). In line with the proven connection between antibiotic treatment and candidiasis (Weiss and Hennet 2017), the protective effect of *L. rhamnosus* colonization was abolished by antibiotic treatment (Manuscript III). Although tissue-resident immune cells have been included in the intestine-on-chip model (Manuscript IV, (Maurer *et al.* 2019)), we have

not investigated to what extent these cells contribute to reducing *C. albicans* pathogenicity. Future studies could aim to investigate the role of immune cells in fungal damage and translocation or the role of bacterial colonization on immune responses to *C. albicans* infection.

Although the yeast morphology is commonly associated with commensalism, it should be noted that filamentation is observed in the intestine of mice during colonization (Witchley *et al.* 2019). A repression of filamentation is observed when *C. albicans* infects *L. rhamnosus* colonized IECs, yet under certain conditions that are connected to *L. rhamnosus* colonization also a hyphae-to-yeast switch can be observed (Manuscript III). It should be noted though that many *in vitro* conditions, especially culture media for mammalian cells, strongly induce filamentation of *C. albicans*.

Compared to the limited number of *in vitro* studies that investigate *C. albicans* commensalism, this topic is being extensively studied using *in vivo* models. An important role was demonstrated for commensal bacteria like *Bacteroidetes* spp. that maintain colonization resistance in mice by inducing the release of antimicrobial peptides (Fan *et al.* 2015). Different fungal morphologies can be recognized by intestinal epithelial cells as well as T-cells, resulting in different reactions (Schirbel *et al.* 2018). IL-9 and mast cells have also been identified to play a crucial role in the discrimination between commensal and pathogenic *C. albicans* (Renga *et al.* 2018).

In this thesis, the shedding of *C. albicans* hyphae was observed as a novel potential protective mechanism induced by *L. rhamnosus* (Manuscript II (Graf *et al.* 2019)). On top of that, reduced *C. albicans* proliferation was observed when the fungus infected *L. rhamnosus*-colonized IECs. Collectively, these two mechanisms substantially reduced the number of *C. albicans* cells in contact with the epithelial barrier, thereby potentially reducing fungal invasion. As a reduced

fungal burden did not reduce *C. albicans*' cytotoxicity to the same extent as the presence of *L. rhamnsosus*, the damage protection could not exclusively be explained by reduced contact between fungal cells and epithelial cells. Transcriptional and metabolic profiling was performed, which revealed complex molecular interactions underlying the protective effect mediated by *L. rhamnosus* colonization (Manuscript III).

One requisite for protection of *Lactobacillus* species against *C. albicans* induced damage was active proliferating of the bacteria on IECs (Manuscript II (Graf *et al.* 2019)). We observed that the protective *Lactobacillus* species proliferated in co-culture with IECs, but not in the medium alone. Metabolic profiling of the *in vitro* model as well as *in silico* modeling of the metabolism of all three cell types involved revealed that *L. rhamnosus* is nourished by metabolites released by IECs, which was validated by supplementing culture medium with essential metabolites (Manuscript III). The identification of essential metabolites for *L. rhamnosus* growth in the model conditions allowed the dissection of the mechanisms directly mediated by *L. rhamnosus* as well as the tripartite interaction with epithelial cells. When metabolically active and proliferating on IECs, *L. rhamnosus* cells consumes favored carbon sources of *C. albicans*, while in parallel secreting metabolites such as phenyllactic acid, mevalonolactone, 2-hydroxyisocaproic acid, and 3-hydroxyoctanoate with known antifungal potential (Lipinska-Zubrycka *et al.* 2020; Nieminen *et al.* 2014; Niku-Paavola *et al.* 1999; Radivojevic *et al.* 2016; Sakko *et al.* 2014) (Manuscript III). The changed environment by *L. rhamnosus* colonization was associated with major changes in expression of *C. albicans* genes, which were validated to be required for pathogenicity in this setting by using corresponding deletion mutants (Manuscript III). Static *in vitro* models support the depletion of preferred carbon sources and the accumulation of antifungal compounds, due to the absence of medium exchange. The consumption of glucose was also shown in antagonistic interaction studies between

C. albicans and *L. rhamnosus* GG on oral epithelial cells (Mailander-Sanchez *et al.* 2017). Additionally, another study showed that oral bacteria, including *L. casei,* outcompeted *C. albicans* in terms of glucose consumption (Basson 2000). Nevertheless, reduced damage and translocation of *C. albicans* was similarly observed in an intestine-on-chip model that was colonized with *L. rhamnosus.* In this model, the perfusion with fresh medium replenishes carbon sources and removes potential antifungal metabolites continuously (Manuscript IV (Maurer *et al.* 2019)). To study this in detail, it would be very valuable to measure the metabolic composition directly in the compartment that mimics the gut lumen. Commercially available tools, such as sensor plugs allow real-time measurements of metabolite concentrations. Sensors to measure oxygen, pH, glucose, and lactate as well as reactive species (ROS/RN) are available (Kieninger *et al.* 2018), but have not yet been implemented in organ-on-chip systems for fungal-bacteria-host interactions. A possible explanation for the accumulation of metabolites despite the perfusion could be given by the three-dimensional epithelial structure in the intestine-on-chip model. IECs form distinct crypts and villus-like structures *in vitro* as a result of perfusion (Manuscript IV (Maurer *et al.* 2019)). These structures potentially provide preferential attachment sites for *C. albicans* and/or *L. rhamnosus*, which can be protected from the flow. Thus these site can be used by the microbes to establish microniches (Klotz *et al.* 2007), which may allow the accumulation and depletion of specific metabolites.

3.2. Antagonistic mechanisms of *L. rhamnosus* and other bacteria against *C. albicans*

Apart from *Lactobacillus* species, many antagonistic interactions between bacteria of the microbiota and *C. albicans* are well characterized. A comparison of the antagonistic mechanisms discovered in this thesis with these known mechanisms, reveals similarities but also highlights that we have identified novel protective mechanisms.

3.2.1. Inhibition of adhesion

Adhesion is the first crucial step during *C. albicans* infection, which is highlighted by the fact that compromised adhesion is associated with reduced virulence (Phan *et al.* 2007; Tsai *et al.* 2011). In this thesis, the *C. albicans* adhesion to host cells was not directly inhibited at early stages of infection, but the *L. rhamnosus*-induced shedding reduced the cell number of *C. albicans* attached to the intestinal epithelium (Manuscript II (Graf *et al.* 2019)). Other studies with *Lactobacillus* spp. also revealed reduced adhesion to intestinal (Kang *et al.* 2018; Poupet *et al.* 2019), vaginal (Allonsius *et al.* 2017; He *et al.* 2020; Niu *et al.* 2017) and oral (Mailander-Sanchez *et al.* 2017) epithelium. This can be explained by a competition for adhesion sites on the epithelial surface (Mailander-Sanchez *et al.* 2017; Parolin *et al.* 2015), which may also be a mechanism by which the intestinal microbiota maintains *C. albicans* commensalism (Kennedy and Volz 1985). Another explanation could be given by a change in the expression of adhesin genes like *ALS3* or *HWP1*, which also play a role in *C. albicans* ability to form biofilms (James *et al.* 2016; Matsuda *et al.* 2018; Ribeiro *et al.* 2017). Interestingly, these genes were not downregulated in the presence of *L. rhamnosus* and therefore not relevant for the observed protection in this thesis (Manuscript III). In general counteracting *C. albicans* adhesion can effectively prevent invasion of the epithelium by the fungus. Consequently, targeting *C. albicans* adhesion *via* small molecules was recently described as a promising tool for the devolpment of new antifungal agents (Martin *et al.* 2020).

3.2.2. Reduction of C. albicans growth

The reduction of fungal growth is another mechanism by which *Lactobacillus* species antagonize *C. albicans* (Köhler *et al.* 2012; Matsubara *et al.* 2016; Rizzo *et al.* 2013; Wang *et al.* 2017). *C. albicans* growth was reduced during infection of epithelial cells which were

colonized by *Lactobacillus* spp. *in vitro* (Manuscript II (Graf *et al.* 2019)). In line with this, *in silico* predictions of *C. albicans* growth in the metabolic environment created by *L. rhamnosus*-colonized epithelium, suggest that reduced biomass can be achieved compared to the metabolic environment of epithelial cells alone (Manuscript III). A likely explanation, especially for static *in vitro* models, could be a competition for favored nutrients, especially carbon sources. Additionally, a metabolic imbalance in *C. albicans* due to drastic changes in key metabolic and metabolic regulatory genes was observed (Manuscript III). Other studies revealed the secretion of lactic acid and the resulting pH decrease as the driving force for inhibition of *C. albicans* growth by *Lactobacillus* spp. (Köhler *et al.* 2012; Noverr and Huffnagle 2004). In contrast, recent results showed no growth reduction for *C. albicans* in the presence of lactic acid under low pH (Lourenço *et al.* 2018). Similarly, in this thesis, the growth reduction was observed in highly buffered media (KBM) with a stable pH and independent on the presence of lactate (Manuscript II (Graf *et al.* 2019)) and thereby supports a pH/lactate-independent mechanism.

3.2.3. Inhibition of hypha formation

Filamentation is essential for *C. albicans* to translocate across the intestinal barrier (Allert *et al.* 2018), and can be influenced by the presence of bacteria (Manuscript II (Graf *et al.* 2019)). Several studies demonstrated that core-filamentation genes (Martin *et al.* 2013), including *ALS3*, *HWP1*, *SAP5,* and *ECE1* are downregulated in the presence of *Lactobacillus* species such as *L. crispatus* (Wang *et al.* 2017), or *L. plantarum* (James *et al.* 2016). Similarly, other intestinal bacteria including *Escherichia coli* (Park *et al.* 2014), *Proteus mirabilis* (Lee *et al.* 2017), as well as bacterial mixtures from stool samples (García *et al.* 2017) reduce filamentation. This supports the hypothesis that bacteria play a crucial role in repressing pathogenicity attributes during *C. albicans* commensalism in the intestine (Kumamoto *et al.* 2020). Interestingly, in this

thesis, the reduction of *C. albicans* hypha-length (Manuscript II (Graf *et al.* 2019)) was not associated with a downregulation of the core-filamentation genes (Manuscript III). Nevertheless, hypha-length measurements and transcriptional profiling were performed at different time points, which could explain the lack of an association. As filamentation was only reduced, but not absent, it is possible that this difference was not reflected on a transcriptional level. Alternative methods like proteomics could help to achieve valuable insights at specific time points.

Certain *Lactobacillus* species and strains express the chitinase Msp1, which breaks down chitin in the fungal cell wall, thereby inhibiting hyphal growth (Allonsius *et al.* 2019). The quorum-sensing molecule autoinducer-2 (AI-2) from bacteria of the oral cavity like *Aggregatibacter actinomycetemcomitans* (Bachtiar *et al.* 2014) or *Streptococcus gordonii* (Bamford *et al.* 2009) was also observed to inhibit *C. albicans* filamentation. *Salmonella enterica* serovar *thyphimurium* can inhibit filamentation through a SopB effector molecule secreted by a type 3 secretion system (Kim and Mylonakis 2011; Tampakakis *et al.* 2009). Interestingly, *Serratia marcescens* injects antifungal effectors (Tfe1, Tfe2) *via* a type 6 secretion system, resulting in a direct killing of *C. albicans* (Trunk *et al.* 2018).

These results illustrate a variety of mechanisms by which filamentation, fitness or viability can be influenced by bacteria. Especially for systemic disease originating from the intestine, *C. albicans* hyphae mediate the invasion of the intestinal barrier to enter the bloodstream subsequently (Pappas *et al.* 2018). For this reason, the inhibition of the yeast-to-hyphae transition is considered as a potential strategy to prevent invasive candidiasis (Jacobsen *et al.* 2012; Vila *et al.* 2017). Several chemicals capable of inhibiting filamentation have been described (Bar-Yosef *et al.* 2017; Pierce *et al.* 2015; Shareck and Belhumeur 2011), but also natural products such as bacterial toxins like EntV from *Enterococcus faecalis* (Graham *et al.*

2017) can be a base for potential prevention and treatment approaches. In this thesis, cytosine was discovered, which is secreted upon colonization of IECs with *L. rhamnosus* and induces a shift from hyphal growth to yeast (Manuscript III).

3.2.4. Modulation of the host immune system and epithelial resistance to infection

Interactions of bacteria with the host are another mechanism by which *C. albicans* pathogenicity can be influenced indirectly. Several studies revealed that *Lactobacillus* colonization can improve the epithelial barrier function (Blackwood *et al.* 2017; Pan *et al.* 2019; Wang *et al.* 2018; Yu *et al.* 2015), which increases resistance to *C. albicans* infections (Tsata *et al.* 2016). In this thesis, *L. rhamnosus* colonization increased trans epithelial electrical resistance (TEER) at an early time point, but *C. albicans* infection similarly reduced TEER as on uncolonized epithelium (Manuscript II (Graf *et al.* 2019)). These data, contrast the expectation of an increased TEER after infection but are in line with the discovery that loss of epithelial integrity and epithelial damage are independent processes (Allert *et al.* 2018).

Antimicrobial peptides are another important epithelial defense mechanism against candidiasis (Höfs *et al.* 2016). Increased production of β-defensin 2/3 by HeLa cells has been observed in the presence of *L. crispatus*, which inhibited *C. albicans* growth (Rizzo *et al.* 2013). In the models presented in this thesis, the role of antimicrobial peptides has not been investigated, but this could represent a promising direction for future research. Nevertheless, the epithelial response has been studied to some extent in this thesis. A decreased expression was revealed for several epithelial damage-and stress-related genes like *cFOS, DUSP1, DUSP5* and NFKB inhibitor alpha (*NFKBIA*) in *L. rhamnosus* colonized epithelial cells with *C. albicans* (Manuscript II (Graf *et al.* 2019)). A possible explanation for this limited activation of the danger response pathway (Moyes *et al.* 2010; Naglik *et al.* 2011) could be the reduced hypha-contact to the intestinal epithelium during shedding (Manuscript II (Graf *et al.* 2019)) and

reduced expression of genes required for *C. albicans* virulence (Manuscript III). *Lactobacillus* spp. can also prevent epithelial barrier dysfunctions resulting from inflammatory responses (Han *et al.* 2019). It is therefore tempting to speculate that similarly *Lactobacillus* spp. could neutralize detrimental inflammatory responses by *C. albicans*.

No consistent observations were made in terms of cytokine (e.g. IL-8) release by *L. rhamnosus*-colonized IECs infected with *C. albicans* (Graf e*t al*., unpublished data). Better insights into the epithelial responses to bacterial colonization and fungal infection could be obtained by transcriptional profiling of IECs. This would be also a possible starting point for comparative analysis with the responses of other epithelial cell types, like oral (Liu *et al.* 2015; Moyes *et al.* 2014) and vaginal cells (Bruno *et al.* 2015), to achieve insights into the complex interactions of *Lactobacillus* spp. and *C. albicans* in different biological niches.

The recognition of *C. albicans* by macrophages can be influenced by *Lactobacillus* spp. by altering expression of the pattern recognition receptors dectin-1, TLR2, and TLR4 and modulating cytokine secretion (Matsubara *et al.* 2017). The secretion of proinflammatory cytokines, including IL-2 and IL-6, by vaginal epithelial cells, was observed to be upregulated by *Lactobacillus* spp. (Niu *et al.* 2017). Contrasting data exist on the influence of *Lactobacillus* spp. on secretion of IL-17, a crucial cytokine for mucosal host defense against *C. albicans* (Gaffen *et al.* 2011). While some studies showed that lactobacilli increased levels of IL-17 (de Oliveira *et al.* 2017; Niu *et al.* 2017) others observed a decrease (Liao *et al.* 2019; Plantinga *et al.* 2012). These different results could be due to the use of diverse host cells and experimental setups. In different settings also anti-inflammatory characteristics are associated with *Lactobacillus* spp., such as the downregulation of IL-8 in vaginal cells (Niu *et al.* 2017; Wagner and Johnson 2012). Overall, these results show the important influence of the host and its

versatile interactions with *Lactobacillus* spp. and *C. albicans* during commensalism and pathogenicity.

3.3. Synergistic interactions of oral and intestinal bacteria with *C. albicans*

Apart from the variety of bacterial species that antagonize *C. albicans*, other species can benefit from the interaction with *C. albicans* without impairing fungal virulence capacity. A well-known example is the interaction with *Streptococcus mutans* in the oral cavity. The bacteria form stable biofilms with *C. albicans* that are protected from environmental stress and enable improved adhesion to the epithelium (reviewed in Manuscript I (Förster *et al.* 2016)). Obligate anaerobes in the oral cavity can survive in aerobic environments by growing in close proximity to *C. albicans* hyphae, which extensily use oxidative respiration thereby creating hypoxic microniches (Lambooij *et al.* 2017).

In the stomach, the opportunistic pathogen *Helicobacter pylori* can use *C. albicans* as a shuttle to invade the epithelium and be protected from stressful environmental conditions like low pH or antibiotics (Sánchez-Alonzo *et al.* 2020; Saniee *et al.* 2013). *E. coli* EHEC (entero-haemorrhagic *E. coli*) variants induce increased and more rapid epithelial damage in the presence of *C. albicans* (Yang *et al.* 2016). In general, the invasion of *C. albicans* hyphae into the epithelial barrier is believed to provide a way for bacteria to invade the tissue. Taken together, these examples represent a fraction of the complex bacteria-fungus interactions taking place in humans during health and disease.

3.4. Clinical relevance of live biotherapeutic microorganisms (LBMs) against candidiasis, candidemia, and sepsis

3.4.1. LBMs and oral candidiasis

Live biotherapeutic microorganisms (LBMs) and probiotics (please see the introduction for detailed information) find versatile (potential) applications against *Candida* spp. infections. Due to the potent antagonistic effects of *Lactobacillus* species against *C. albicans* pathogenicity these bacteria are being explored for probiotic and therapeutic applications. Protective effects of *L. rhamnosus* against oral candidiasis have been shown in a mouse model (Leão *et al.* 2018). Although clinical studies are rare, some revealed that probiotics protect against colonization and oral infection by *Candida* spp. in susceptible individuals like head- and neck radiotherapy patients (Doppalapudi *et al.* 2020), elderly (Ai *et al.* 2017; Hatakka *et al.* 2007), and denture wearers (Ishikawa *et al.* 2015). HIV/AIDS patients are another risk group for the development of oral candidiasis. Since HIV patients have an altered oral mycobiota (Hager and Ghannoum 2018), the use of probiotics to counteract the dysbiosis is an attractive approach. The inhibitory effect of *Lactobacillus* spp. against oral *Candida* spp. isolates from HIV patients showed an inhibition of growth for most *Candida* spp., except for *C. albicans* (Salari and Ghasemi Nejad Almani 2020). Probiotics are considered as a strategy to prevent oral candidiasis in HIV patients (Ramírez-Amador *et al.* 2020), but solid clinical data are still needed to validate their efficacy.

3.4.2. LBMs and VVC

In vitro (Jang *et al.* 2019; Kang *et al.* 2018) and *in vivo* (De Gregorio *et al.* 2019; Li *et al.* 2019) studies have proven major beneficial effects of *Lactobacillus*-based therapies for VVC. Although clinical data show short-term effects of LBMs against VVC, their efficacy is not fully proven (reviewed in Buggio *et al.* 2019; Falagas *et al.* 2006b). Nevertheless, there is evidence

that probiotics can support the prevention of VVC (Murina *et al.* 2014). Further, probiotic co-therapy next to conventional VVC treatment demonstrated beneficial effects (Ehrström *et al.* 2010; Kovachev and Vatcheva-Dobrevska 2015). Probably, probiotics or LBMs are a viable therapeutic option to prevent VVC, for example due to the improvement of the microbial composition, the reduction of *C. albicans* colonization, or the promotion of antifungal therapy.

3.4.3. LBMs and intestinal diseases, and critical ill patients

In the clinical therapeutic arsenal against intestinal diseases, probiotics and LBMs are being broadly applied. For example, probiotics and LBMs are used to prevent and palliate antibiotic-related diarrhea (Guo *et al.* 2019; Johnston *et al.* 2011), *Clostridium difficil*e infections (Goldenberg *et al.* 2017; Pattani *et al.* 2013), irritable bowel syndrome (Baştürk *et al.* 2016; Didari *et al.* 2015; Guglielmetti *et al.* 2011; Principi *et al.* 2018), and necrotizing enterocolitis (AlFaleh and Anabrees 2014; Olsen *et al.* 2016). In contrast, no efficacious effects of probiotics and LBMs were found in the context of Crohn's disease (Abraham and Quigley 2017; Limketkai *et al.* 2020).

Concerning invasive *C. albicans* infections originating from the intestine, clinical data on the application of probiotics mainly focus on preterm neonates in ICUs. In this patient group, the intestinal microbiome is not fully developed and is often disturbed by antibiotics (Gasparrini *et al.* 2016; Gibson *et al.* 2015). Due to the underdeveloped microbiome the colonization with *Candida* spp. is often associated with an uncontrolled overgrowth, resulting in translocation and infection (James *et al.* 2020; LaTuga *et al.* 2011). *Candida* species are the third most frequent cause of sepsis under these circumstances (Stoll *et al.* 2002). Clinical studies revealed beneficial effects of probiotics in reducing the gastrointestinal colonization with *C. albicans* and thereby minimizing the risk of invasive candidiasis (Kumar *et al.* 2013; Manzoni *et al.* 2006; Romeo *et al.* 2011; Roy *et al.* 2014). Detrimental side effects are usually less compared to

conventional antifungal therapies, but rarely probiotic bacteria have been observed to cause bacteremia and sepsis in immunocompromised patients (Kumar and Singhi 2013; Singhi and Kumar 2016). Interestingly, probiotics containing *L. rhamnosus* were used in most of the studies (Manzoni *et al.* 2006; Romeo *et al.* 2011). Therefore, it is tempting to speculate that the identified antifungal properties of *L. rhamnosus* in this thesis (Manuscript II (Graf *et al.* 2019), III, IV (Maurer *et al.* 2019)) may underlie the efficacious antifungal properties *in vivo*. A potential approach to improve the impact of *L. rhamnosus* probiotics in preterm neonates could be the supplementation of nutrients (like citrate, carnitine, and gamma-glutamyl-amino acids) that improve *Lactobacillus* growth, that were identified in this thesis (Manuscript III). Moreover, the clinical data support the *L. rhamnosus* protection that was observed in a simple *in vitro* model (Manuscript II (Graf *et al.* 2019)) and a complex intestine-on-chip system (Manuscript IV (Maurer *et al.* 2019)), confirming the translational capacity of results from *in vitro* models to the clinical situation.

The microbiota and especially its dysbiosis play a crucial role in the development of candidiasis (Pappas *et al.* 2018); not only in preterm neonates but also in critically ill patients. A healthy microbiome is usually dominated by Firmicutes and Bacteroidetes (Arumugam et al. 2011). In ICU patients, a switch to a pathobiome can occur, which has an altered relation of these phyla and an increased number of pathogenic bacteria (Alverdy and Krezalek 2017; Krezalek *et al.* 2016; Ojima *et al.* 2016; Otani *et al.* 2019). This can be explained by the use of antibiotics and the altered nutrient availability in the intestinal environment due to (par)enteral nutrition (Fay *et al.* 2017; Iapichino *et al.* 2008; Krezalek *et al.* 2017). Not only pathogenic bacteria increase in abundance, but also *C. albicans* and *C. glabrata* were detected with increased abundances in stool samples of ICU patients (Zaborin *et al.* 2014). On top of changes in the microbiome composition, the gut barrier in ICU patients is often compromised as a result of cytostatic

therapy, catheters, or surgeries (Fink 2003; Yoseph *et al.* 2016). The dysregulation and/or suppression of the immune system is another comorbidity in ICU patients that predisposes to secondary fungal infections (Monneret *et al.* 2011). Collectively, the predispositions in ICU patients can lead to fungal overgrowth, translocation of *Candida spp.* and dissemination through the bloodstream (Zhai et al 2020). Even when LBMs alone are not effective enough to treat invasive candidiasis in these patients, their beneficial effects could counteract several predisposing factors. This could expand the time frame for other therapeutic applications. General positive aspects of probiotics in critically ill patients like a reduced incidence of infections such as ventilator-associated pneumonia, have been described (Manzanares *et al.* 2016; Shimizu *et al.* 2018).

3.5. Conclusion and future directions

The data described in this thesis provides fundamental insights into the interactions between *C. albicans*, intestinal epithelial cells, and *Lactobacillus* species. These mechanistic insights obtained using *in vitro* models, paired with collected knowledge on fungal-bacterial and fungal-host interactions, revealed the relevance of the fungal-host-microbiota interplay to keep *C. albicans* commensal. Several antagonistic mechanisms of *L. rhamnosus* including shedding, the downregulation of virulence and metabolic genes, and the production of metabolites that are effective against *C. albicans* were observed to contribute to reduced hypha formation, proliferation, and cytotoxicity of the fungus. These potent effects that antagonize *C. albicans* virulence have been, and could be further, exploited for potential preventive therapeutic approaches.

3.5.1. Modeling of vaginal C. albicans infections in vitro

Different from systemic disease originating from the intestine, infections of the oral and vaginal mucosa are very common. Especially vulvovaginal candidiasis (VVC) is of interest, as it can occur in immunocompetent women (Denning *et al.* 2018). *Lactobacillus* spp. are predominantly present in the vaginal microbiota of reproductive-age women (Ravel *et al.* 2011; Witkin and Linhares 2017). As a dysbalance of this *Lactobacillus*-dominated microbiota is associated with VVC, it is obvious to investigate other *Lactobacillus* species for their antifungal properties in this niche that can be exploited for therapy. In a model setup similar to Manuscript II (Graf *et al.* 2019), but with vaginal epithelial cells, *L. rhamnosus* colonization was similarly observed to protect against *C. albicans*-induced damage. The protection was dependent on *L. rhamnosus*, but shedding as on IECs was not observed (Valentine *et al.*, unpublished data). Interactions between vaginal *Lactobacillus* spp. and *C. albicans* are also well-studied (De Gregorio *et al.* 2019; do Carmo *et al.* 2016; Jang *et al.* 2019; Li *et al.* 2019; Parolin *et al.* 2015). In this context, it would be interesting to investigate whether naturally appearing lactobacilli like *L. crispatus* or *L. iners* (Lamont *et al.* 2011; Ravel *et al.* 2011) are exhibiting similar effects on a transcriptional and metabolic level as the mechanisms unraveled in this thesis (Manuscript III). It would be also interesting to study various *C. albicans* strains and isolates, especially from VVC patients, for their damage capacity in the presence and absence of *Lactobacillus* spp. In principle, VVC has been studied in a wide range of *in vitro* model systems as explained in Manuscript V and also *in vivo* (Bozó *et al.* 2016; Carrara *et al.* 2010; Fidel *et al.* 1997). Nevertheless, these models often lack biological complexity in terms of the presence of neutrophils, which are crucial players in the pathogenesis of VVC (reviewed in Yano *et al.* 2018). A VVC-on-chip model mimicking the

vaginal epithelium and the underlying vasculature could help to study the interactions between *C. albicans*, the vaginal epithelium, the vaginal microbiota, and innate immune cells.

3.5.2. Complex bacterial mixtures to protect against and treat candidiasis

The microbiota is a complex composition of bacterial species; more physiologically relevant insights could be obtained by evaluating defined microbial compositions for their potential synergistic antifungal effects. In our hands, a combination of two *Lactobacillus* species did not exhibit additional beneficial effects (Last *et al*., unpublished data). Based on the data discussed in this thesis, showing that *Lactobacillus* spp. mediated effects are species-dependent and discussing a wide range of different mechanisms for different bacterial species exist, it is likely to achieve better and broader protective effects when a mixture of bacteria is used to combine their diverse advantages. For example, other bacteria may provide essential nutrients that foster *Lactobacillus* spp. growth. Even when this question cannot be answered for *C. albicans* yet, for other diseases, such as irritable bowel syndrome, diarrhea, or respiratory tract infections the use of mixture-based probiotics was proven to be more efficient than single bacterial strains (reviewed in Chapman *et al.* 2011). To mimic the natural situation, first interaction studies with fecal extracts or transplants were performed. A reduced *C. albicans* colonization was observed in mice after fecal microbiota transplantation (FMT) in antibiotic-treated mice (Li *et al.* 2020; Matsuo *et al.* 2019). In ulcerative colitis patients, *C. albicans* colonization before FMT was related to an increased bacterial diversity afterward (Leonardi *et al.* 2020). In contrast, a high abundance of *C. albicans* in donor stools reduced the FMT efficiency (Zuo *et al.* 2018), hinting towards a significant impact of *C. albicans* to the intestinal microbiome homeostasis.

These results support the essential function of a healthy intestinal microbiota that helps to keep *C. albicans* in its commensal state. By transferring human intestinal microbiota in germ-

free mice a “humanized” mouse model of the human microbiota can be established. Such a model has been used for interaction studies with *Clostridium difficile* (Collins *et al.* 2015) and would be a promising tool to validate and extend the received results of this thesis in a suitable *in vivo* model.

3.5.3. Therapeutic microbes

Next to the use of natural bacteria and yeast and their antimicrobial properties another research field is dealing with “therapeutic microbes”. These bacteria are genetically engineered to achieve therapeutic effects. For example, an *E. coli* strain was modified to sense hydroxyphenyl acetic acid (HPA) released by *C. albicans* and subsequently secrete a substance that inhibits filamentation (Tscherner 2019). Next to direct interactions with intestinal pathogens, genetically engineered *E. coli* strains also have been used to detect inflammation-related metabolites in the gut for long-term diagnostics of IBD (Riglar *et al.* 2017). The use of genetically engineered microorganisms seems to be promising for the detection and treatment of intestinal infections. Concerning our studies, it would be a theoretical option to produce the identified anti-*Candida* metabolites (Manuscript III) in high amounts in *E. coli* to achieve a strong antifungal effect. Nevertheless, the transfer of genetically engineered therapeutic microbes into the environment could provoke social opposition.

In conclusion, the establishment of a static *in vitro* model was used to dissect complex interactions between *C. albicans*, IECs and *Lactobacillus* spp. and to reach a near-commensal state of the fungus. The broad insights into antagonistic effects of colonization with *Lactobacillus* spp. provide fundamental insights into fungal-host-microbiota interactions that mediate *C. albicans* commensalism. The mechanistic insights suggest potential targets and candidate metabolites that can be exploited to prevent a shift to pathogenicity.

4. Literature

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5. Appendix

5.1. First person Interview

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FIRST PERSON

First person – Katja Graf and Antonia Last

First Person is a series of interviews with the first authors of a selection of papers published in Disease Models & Mechanisms (DMM), helping early-career researchers promote themselves alongside their papers. Katja Graf and Antonia Last are co-first authors on 'Keeping *Candida* commensal: how lactobacilli antagonize pathogenicity of *Candida albicans* in an *in vitro* gut model', published in DMM. Katja is a postdoc and Antonia is a PhD student in the lab of Bernhard Hube at the Hans Knöll Institute (HKI) investigating molecular pathogenicity mechanisms.

Antonia Last and Katja Graf

How would you explain the main findings of your paper to non-scientific family and friends?

AL+KG: We work with the fungus *Candida albicans*, which is better known as the cause of vaginal or oral yeast infections (thrush). These infections affect millions of individuals all over the world. However, this fungus is found in most people on mucosal surfaces, including the human gut, living in harmony with harmless and beneficial gut bacteria, together forming a complex community of microorganisms – the microbiota. Under certain circumstances this balanced microbiota can be disrupted, for example by the use of broad-spectrum antibiotics, which remove most bacteria, both beneficial and disease-causing, but not the fungi. This allows *C. albicans* to overgrow everything else and potentially trigger an infection (candidiasis). In rare worst cases, this fungus can enter the bloodstream, causing systemic infections and sepsis with an often fatal outcome. Lactobacilli are well-known protectors of mucosal barriers that help to maintain microbial balance, but the reasons for that are not fully understood. In our model, we proved that *Lactobacillus rhamnosus* protects gut barriers against *C. albicans* damage and obtained interesting insights into the mechanism. When both *C. albicans* and lactobacilli are present, some of the epithelial cells lining the gut commit a form of 'cellular suicide' (apoptosis). These dead human cells, the fungal cells and the bacteria clump together and are thereby removed from the remaining healthy human cells. We assume that this physical separation can protect the gut barrier from invasion by the fungus and prevent a disease at the earliest stage.

What are the potential implications of these results for your field of research?

AL+KG: We established an *in vitro* model based on intestinal epithelial cells and mucus-secreting goblet cells. In this model, we added an artificial microbiota in the form of lactobacilli to mimic the *in vivo* situation. This more complex model is closer to reality than many other models and a good basis for all kinds of research into interactions of gut cells with commensal or pathogenic members of the microbiota. Our study shows an unexpected interaction between the intestinal epithelial cells, probiotic bacteria and the opportunistic fungal pathogen *C. albicans*, which relies on physical separation induced by the presence of the bacteria. This model and its read-out can now be used to test other bacteria or antifungals for their protective potential against *Candida*-induced damage. Even pathogenic bacteria could be tested, to model polymicrobial infections. Given the renewed interest in probiotics and the role of the microbiota in health, such easily accessible *in vitro* models are, in our opinion, urgently needed.

What are the main advantages and drawbacks of the model system you have used as it relates to the disease you are investigating?

AL: One big advantage of our *in vitro* model is the fact that we are working *in vitro*, which is much easier to plan and to work with compared to *in vivo* models, and is generally more reproducible and ethically preferred. Personally, I really enjoyed the fact that the cell culture cells had to differentiate for two weeks before we could use them, as that gave me enough time to prepare my experiments thoroughly and re-think everything before conducting the experiment. On the other hand, it is extremely frustrating and time consuming if something goes wrong; a contamination in your cell culture can delay your next experiment by three to four weeks! All in all, it was a good exercise for me to plan experiments in advance.

"Working *in vitro* is much easier to plan and to work with compared to *in vivo* models, and is generally more reproducible and ethically preferred." – *Antonia Last*

KG: Our *in vitro* model is more complex than most established cell line-based models, which usually consist of only one host cell type. We included two different gut cell types and additional probiotic bacteria to come closer to the real *in vivo* situation. This gives us the chance to study the complex cell-bacteria-fungal interactions in a more life-like manner. However, one important part is still missing in our model: immune cells, which also play a major role during commensalism and pathogenicity in the gut. We plan to include these cell types, although it will make it more complicated to work

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Disease Models & Mechanisms

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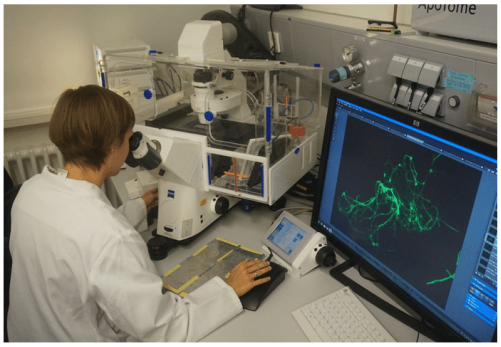

Antonia Last studying *C. albicans* at a fluorescent microscope.

with. As is so often the case, we could lose the benefits of our reductionist approach by adding complexity. At the end, every *in vitro* model is, to varying degrees, artificial, and we are happy that we were able to establish this more complex model to study fungal infection biology.

What has surprised you the most while conducting your research?

KG: In our publication we explain that the protective effect of lactobacilli against *C. albicans*-induced damage is due to shedding of fungal, bacterial and apoptotic host cells. We saw this shedding by eye at the beginning of our studies, but were not really considering it as relevant. It was only when we saw aggregation of the microbes and human cells via electron microscopy, that we gave this original observation a second thought and based a new hypotheses on it. I am frequently surprised that such seemingly irrelevant observations can turn out to be the most important hints in science, so I can only recommend keeping your eyes open!

AL: I was extremely surprised how many new and useful results you can get during the revision process after submission! It was quite a lot of work for us, but through the reviewers' helpful comments and suggestions, we were able to improve our publication significantly. This is probably one of the more underestimated factors in science.

"Seemingly irrelevant observations can turn out to be the most important hints in science, so I can only recommend keeping your eyes open!" – *Katya Graf*

Describe what you think is the most significant challenge impacting your research at this time and how will this be addressed over the next 10 years?

KG: The number of (nosocomial) *C. albicans* infections and related sepsis cases is increasing. This puts fungal infections more in focus nowadays. Even though *C. albicans* research is already comparatively well established (for a fungal pathogen), many pathogenicity mechanisms of the fungus remain unknown. Its interaction with the human host, and especially its complex interplay with the microbiota (an increasingly recognized contributor to health and disease), requires much more research. The main challenge here is probably the 'black box' gut, as we still don't have a complete picture of the resident microbiota, and especially not of the myriad of interactions within this unique biosphere. If we want to prevent and cure life-threatening *C. albicans* infections, we need to know much more about the natural habitat of the fungus, its neighbours and the human gut in general.

What changes do you think could improve the professional lives of early-career scientists?

AL: From my point of view, it is very helpful to be part of a graduate school as a PhD student. I have the opportunity to join soft-skill courses, scientific courses and seminars as well as national and international conferences. All these experiences help me to get a broader view into the life of a researcher and allow me to improve the skills necessary for this kind of work. Moreover, collaborations can be very fruitful, as they give new input and broaden the spectrum of equipment and expertise. But next to all these, the most important thing to have for a successful PhD is the supervision. I am really thankful that in our department every PhD student has an experienced postdoc on their side. To have someone to discuss problems and ideas with is not only extremely useful, but absolutely necessary in this phase of the career.

What's next for you?

AL+KG: We would like to get deeper insights into the described protective mechanism of lactobacilli against *C. albicans*-induced damage. In order to further increase the biological complexity of our model we have teamed up with the research group of Alexander Mosig at the University Hospital in Jena and are working on establishing our model in their intestine-on-chip model (Maurer et al., 2019). In addition, we are currently conducting experiments in our *in vitro* set up, in which we perform transcriptional and metabolic profiling. We hope the new results will answer some questions about the gene regulation involved during the interactions between lactobacilli, *C. albicans* and intestinal epithelial cells. Another aim is the introduction of immune cells in our model, to investigate the contribution of another important player in the gut.

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5.2. Abbreviations

AI-2	autoinducer-2
AIDS	acquired immune deficiency syndrome
C2BBe1	Caco-2 brush border expressing 1
EHEC	entero-haemorrhagic *E. coli*
FDA	Food and Drug Administration
FMT	fecal microbiota transplantation
GUT	gastrointestinally induced transition
HIV	human immunodeficiency virus
HPA	hydroxyphenyl acetic acid
IBD	inflammatory bowel disease
ICU	intensive care unit
IEC	intestinal epithelial cell
IFN	interferon
IL	interleukin
KBM	keratinocyte growth basal medium
LBM	live biotherapeutic microorganisms
MAP	mitogen-activated protein
PAMP	pathogen associated molecular pattern
PRR	pattern recognition receptors
RNS	reactive nitrogen species
ROS	reactive ocygen species
SAP	secreted aspartic protease
SCFA	short chain fatty acid
spp.	species
TEER	trans epithelial electrical resistance
TLR	toll-like receptor
VVC	vulvovaginal candidiasis

5.3. Publications and Manuscripts

2021 **Last A,** Mirhakkak M, Alonso-Roman R, Sprague J, Großmann P, Graf K, Gratz R, Panagiotou G, Vylkova S, Schäuble S, Hube B, Gresnigt MS (2021) **Intestinal epithelial cells foster bacteria that antagonize *C. albicans* pathogenicity through metabolic and transcriptional reprogramming** (Manuscript in preparation)

Last A, Maurer M, Mosig A. S., Gresnigt M.S., Hube B (2021) ***In vitro* infection models to study fungal-host interactions.** *FEMS Microbiology Reviews*

2019 Graf K, **Last A**, Gratz R, Allert S, Linde S, Westermann M, Gröger M, Mosig AS, Gresnigt MS, Hube B (2019) **Keeping *Candida* commensal - How lactobacilli antagonize pathogenicity of *Candida albicans* in an *in vitro* gut model**. *Dis Model Mech* 12(9), pii: dmm039719.

Maurer M, Gresnigt MS, **Last A**, Wollny T, Berlinghof F, Pospich R, Cseresnyes Z, Medyukhina A, Graf K, Gröger M, Raasch M, Siwczak F, Nietzsche S, Jacobsen ID, Figge MT, Hube B, Huber O, Mosig AS (2019) **A three-dimensional immunocompetent intestine-on-chip model as *in vitro* platform for functional and microbial interaction studies.** *Biomaterials*

2016 Förster TM, Mogavero S, **Dräger A**, Graf K, Polke M, Jacobsen ID, Hube B (2016) Enemies and brothers in arms: ***Candida albicans* and gram-positive bacteria**. *Cell Microbiol* 18(12), 1709-1715. (Review)

5.4. Scientific presentations

Talks

2019 Sepsis Update, Weimar, Germany, Title: "How lactobacilli antagonize pathogenicity of *Candida albicans*: Lessons learned from *in vitro* gut models and a dynamic organ-on-chip model "

71. Annual conference of the german society for hygiene and microbiology DGHM (Deutsche Gesellschaft für Hygiene und Mikrobiologie), Göttingen, Germany, Title: "Keeping *Candida albicans* commensal: How lactobacilli protect intestinal cells against cytotoxicity"

2016 5th CESC FEMS Central European Summer Course on Mycology, Szeged, Hungary, Title: "Investigating the interactions between *Candida albicans* and lactobacilli using a commensal *in vitro* gut model"

2. FunComPath-Infect Era Meeting, Gothenburg, Denmark, Title: "From colonization to infection: dissection of the commensal-to-pathogen shift of *Candida albicans*"

Statusworkshop Eukaryontische Krankheitserreger (DGHM), Aachen, Germany, Title: “Establishment of a commensal gut model to study interactions between *C. albicans* and lactobacilli”

Poster

2019 Sepsis Update, Weimar, Germany, Title: “How lactobacilli antagonize pathogenicity of *Candida albicans*: Lessons learned from *in vitro* gut models and a dynamic organ-on-chip model “

2018 MYK 2018 (Deutschsprachige Mykologische Gesellschaft e.V. (DMykG)), Innsbruck, Austria, Title: “Keeping *Candida albicans* commensal: How lactobacilli protect intestinal cells against cytotoxicity in an *in vitro* commensal gut model”

2016 Annual conference of the Association for General and Applied Microbiology (VAAM), Jena, Germany, Title: “Establishment of a commensal *in vitro* gut model to study *Candida*-lactobacilli interactions”

5.5. Grants

2020 Candida and Candidiasis Access Bursary

2019 Travel grant of the DGHM

5.6. Sebständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbst verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Mir ist die geltende Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bekannt. Personen, die mich bei den Experimenten, der Datenanalyse und der Verfassung der Manuskripte unterstützt haben, sind als Ko-Autoren auf den entsprechenden Manuskripten verzeichnet. Personen die mich bei der Verfassung der Dissertation unterstützt haben, sind in der Danksagung der Dissertation vermerkt. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Es haben Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die vorliegende Arbeit wurde in gleicher oder ähnlicher Form noch bei keiner anderen Hochschule als Dissertation eingereicht und auch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung verwendet.

Jena, den 05.07.2021

Antonia Last