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Interaction of *Candida albicans* with human gut epithelium in the presence of Live Biotherapeutic Products (LBPs)

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Abstract. *Candida albicans* is a semi-ubiquitous pathobiont that is known to significantly impact human health and wellbeing, causing a significant financial strain on the medical system. Due to increasing antifungal resistance, there is a growing need for novel fungal therapeutics to treat diseases caused by this fungus. The development and use of Live Biotherapeutic Products (LBPs) is an innovative and novel approach to potentially treating Candidiasis and other comorbidities associated with *C. albicans* infection. To evaluate their anti-pathogenic efficacy, it is necessary to understand the underlying mechanisms involved, via the use of biomimetic cell models. In this study, six LBPs were chosen to investigate their competitive inhibitory effect against *C. albicans* using a co-culture of Caco-2 cells and mucous-secreting HT29-MTX cells to mimic human gut epithelium. The LBP strains were supplied by Servatus Biopharmaceuticals and identified as SVT 01D1, SVT 04P1, SVT 05P2, SVT 06B1, SVT 07R1 and SVT 08Z1. Five out of the six LBPs showed a significant reduction in the adhesion of *C. albicans* and all six LBPs reduced *C. albicans* invasion in the co-culture cells to varying degrees. There was no significant difference between co-inoculation of *C. albicans* with the LBPs or pre-inoculation of LBPs before the addition of *C. albicans*. The potential of these LBPs as novel anti-fungal therapeutics for the treatment of *C. albicans* diseases can be further documented in clinical trials.

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Introduction

The gut microbiome is arguably one of the most mysterious ‘organs’ of the human body and is vital to all aspects of human health and our sense of wellbeing. Over the past few decades significant research has been conducted to understand the dynamics involved in microbe-to-microbe and microbe-to-gut interactions and how these impact on overall human health. One microbe that has been the subject of ongoing investigation is *Candida albicans,* an opportunistic pathogenic yeast found in about 70% of people[1]. As a polymorphic fungus, it is generally considered to inhabit the body as a commensal, kept under control by the host’s beneficial microbiota. However, in certain circumstances, such as in immunocompromised individuals[2], and during prolonged antibiotic therapy[3], *C. albicans* is able to overgrow within its local environment, that is, within the gastrointestinal (GI) tract or translocate across the gut epithelium leading to systemic Candidiasis. This disease has a high morbidity and mortality ranging from 20% to 49%[4–6]. Studies suggest that *C. albicans* adversely affects inflammatory bowel disease (IBD) exacerbating inflammation in the gut and delaying healing of ulcerative colitis, for example in humans and mice[7–9]. An increased abundance of *C. albicans* has been observed in patients with IBD compared with healthy subjects suggesting that fungi may play a role in its pathogenesis[10,11].

Current gaps and future direction

Scientific investigations have identified various fungal genes known to play a role in *C. albicans* pathogenicity. However, there are still gaps in current knowledge of atypical virulence mechanisms, particularly in understanding the ability of *C. albicans* to invade gut epithelial cells. The majority of studies have focused on the interaction of *C. albicans* with oral/vaginal epithelial cells as opposed to gastrointestinal (GI) epithelial cells[12]. While antibiotics are still the drug of choice to treat bacterial and fungal infections in clinic, Live Biotherapeutic Products (LBPs) have been suggested as an alternative for treating infections. LBPs are defined by the Food and Drug Administration (FDA) Centre for Biologic Evaluation and Research (CBER) as ‘a live biological product that: (1) contains live organisms, such as bacteria; (2) is applicable to the prevention, treatment

or cure of a disease or condition of human beings; and (3) is not a vaccine'[13]. They are further described as 'medicinal products containing live micro-organisms (bacteria or yeasts) for human use' by the European Pharmacopoeia (Ph. Eur.) (which excludes faecal microbiota transplants and gene therapy agents from this category)[14]. The investigation of LBPs in treating inflammatory, autoimmune and even malignant conditions is accelerating at an astonishing rate, being recognised as novel drug candidates that aim to change the medical paradigm in treating human illness[15]. In this study we investigated the competitive inhibitory effects of six LBPs on adhesion and invasion of *C. albicans* using a co-culture of Caco-2:HT29-MTX cells as a model of human gut epithelium to provide insight into the potential of these LBPs for managing Candidiasis.

Scope of this project

Current methods to investigate pathogenic interactions of microbes on gut epithelium rely on using cell lines that resemble biomimetic synthetic intestines, mainly Caco-2 or HT-29 cell lines[16–18]. Caco-2 cells are differentiated in culture medium to form a polarized cell monolayer with tight junctions and microvilli to resemble important characteristics of human intestinal mature enterocytes. The main drawback of this cell line is that it does not produce a sufficient mucus layer. HT29, with methotrexate (MTX) adaptation, differentiates in culture media to secret mucin[19], an essential component of the gut epithelium. We used a co-culture of Caco-2 and HT29-MTX cells to investigate the interaction of *C. albicans* ATCC 10231 with the gut epithelium. Six LBP candidates were selected and provided by Servatus Biopharmaceuticals: SVT 01D1, SVT 04P1, SVT 05P2, SVT 06B1, SVT 07R1 and SVT 08Z1.

Interaction of *C. albicans* ATCC 10231 at a final concentration of 10^6 CFU/mL with the co-culture Caco-2:HT29-MTX (9:1) alone and in the presence of each of the LBPs (10^6 CFU/mL) was assessed by measuring reduction in *C. albicans* colonisation when co-inoculated with LBP strains, and when pre-inoculated for 60 min with LBP strains prior to inoculation of *C. albicans*. The number of adhering

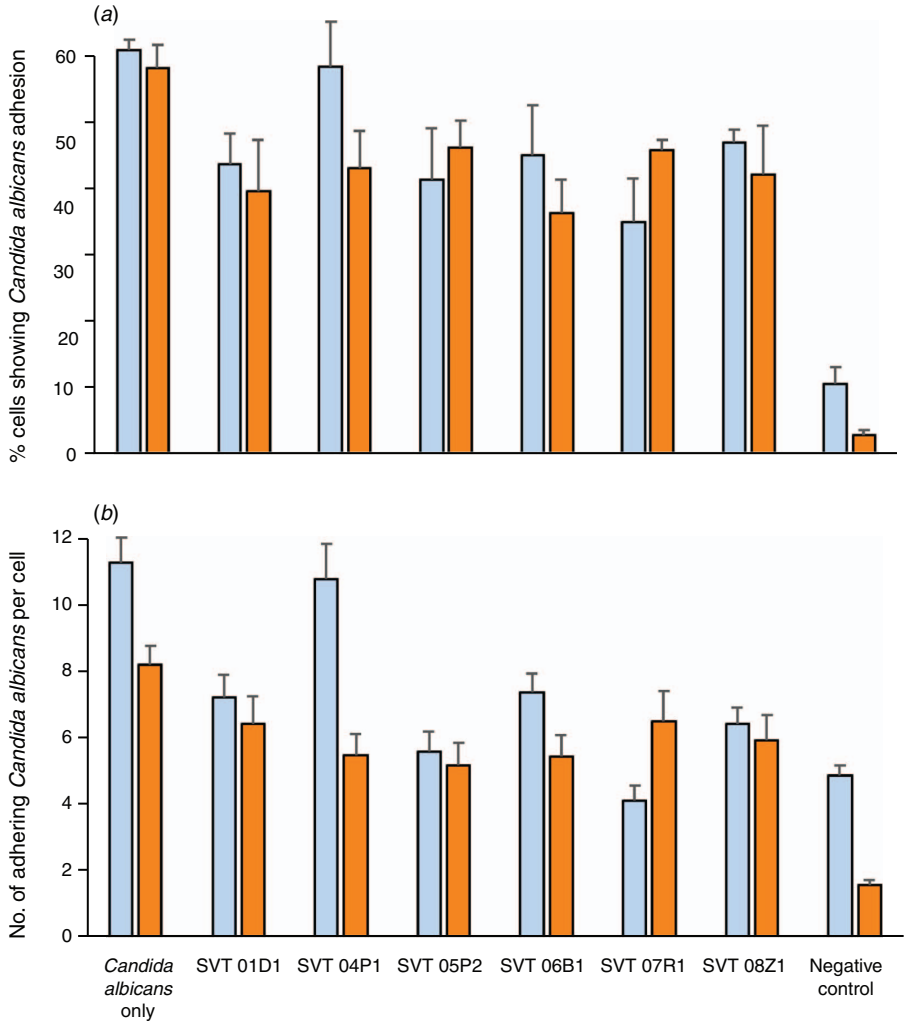

Figure 1. Percent colonisation on co-culture of Caco-2:HT29-MTX by *C. albicans* ATCC 10231 (*a*) and number of adhering *C. albicans* per cell (*b*) when co-inoculated with LBPs (blue) and following pre-inoculation with LBPs (orange). *E. coli* strain 46-4 was used as a negative control. Error bars represent SEM.

C. albicans per cell was recorded to identify the competitive ability of LBP strains to inhibit adherence of *C. albicans* per cell. The results indicated that the LBP strains (except SVT 04P1) reduced the colonisation of *C. albicans* on the co-culture cells by 21–43% (Figure 1*a*) and adhesion per cell by 21–64% (Figure 1*b*) both in co-inoculation and pre-inoculation assays.

All LBP strains (except SVT 04P1) demonstrated a significant reduction in colonisation and adhesion per cell of *C. albicans* ($P < 0.01$ in both co- and pre-inoculation). Overall, reduction in number of adhering *C. albicans* ATCC 10231 was seen for both co-inoculation and pre-inoculation, with SVT 07R1 showing the highest reduction ($P = 0.0005$).

In scanning electron microscopy (Figure 2) of the co-culture assay, *C. albicans* ATCC 10231 was shown to be highly invasive in that the *C. albicans* hypha was seen penetrating the epithelial cell monolayer. A similar procedure to the adhesion assay was used for co-inoculation and pre-inoculation in the invasion assay. A suspension of *C. albicans* was inoculated into 96-well plates at a final concentration of 10^7 CFU/well. After 90 min the wells were inoculated with nystatin (24 µg/mL) for 60 min to kill any extracellular *C. albicans*, followed by incubation with 0.1% Triton-X-10 (Sigma-Aldrich) for 15 min to lyse the monolayer releasing invading pathogens and enumerating them. The results showed a reduction in invasion of *C. albicans* in the presence of LBP strains that demonstrated variable efficacy (Figure 3) with SVT 01D1 showing the highest reduction overall.

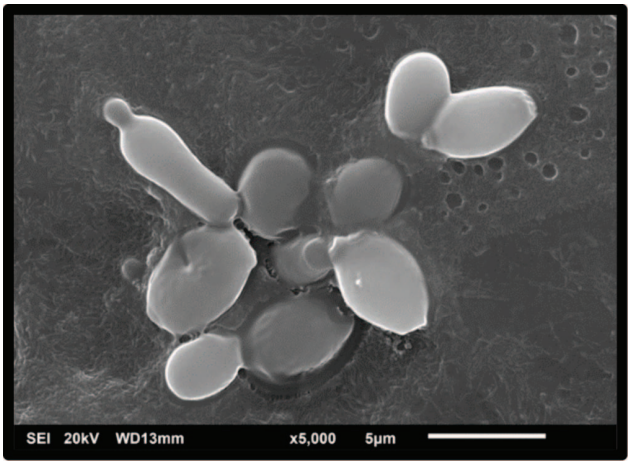

Figure 2. Scanning electron micrograph showing invasion of *C. albicans* strain ATCC 10231 into Caco-2/HT29-MTX cells after a 20-min incubation. Scale bar = 5 µm.

Discussion

The escalating need to develop alternative approaches for managing *C. albicans* infection has highlighted the potential for the use of LBPs as anti-fungal therapeutics. We showed that most LBPs used in this study showed a significant reduction in the adhesion and invasion of *C. albicans* in our human gut epithelium cell culture model, although these effects varied among the LBPs. The potential use of these LBPs as a therapeutic or as a prophylactic measure was also tested using co-inoculation and pre-inoculation models of the LBPs against the *C. albicans*. While there was a significant reduction in colonisation and invasion of the cells by *C. albicans* in the presence of LBPs, we did not observe a significant difference between co-inoculation and pre-inoculation of LBPs one hour before the addition of *C. albicans*. Poupet *et al*. studied the curative effect of LBP *L. rhamnosus* Lcr35® on *Caenorhabditis elegans* survival after *C. albicans* exposure, and found that the 2-h and the 4-h pre-inoculation periods were most protective against *C. albicans* infection[20]. Future studies to investigate the effect of longer incubation periods of LBPs used in our study can provide a better understanding

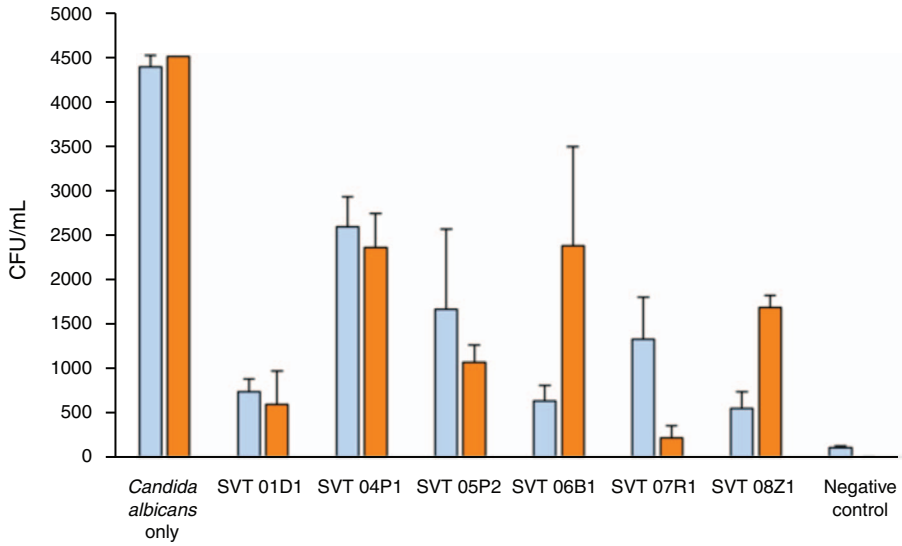

Figure 3. The number of invading *C. albicans* ATCC 10231 cells in a co-culture of Caco-2:HT29-MTX cells when co-inoculated with the LBPs (blue) and following pre-inoculation with LBPs (orange). Error bars represent SEM.

of the impact of pre-inoculation time in clinical studies aiming to assess the prophylactic effect of LBPs against *C. albicans*. Furthermore, it could prove insightful to explore the efficacy of various other LBP strains against *C. albicans*. Similarly, the use of further *Candida* strains in future studies for comparison would strengthen our findings.

Although the most reliable model to establish the impact of LBPs against *C. albicans* and other enteric pathogens is clinical trials in humans, *in vitro* studies utilising a co-culture of Caco-2 and HT29-MTX cells lines as used in this study, provide a suitable model to mimic the human gut epithelium. Caco-2 cells can be differentiated in the culture medium to form a polarized cell monolayer with tight junctions and microvilli that resemble important characteristics of human intestinal mature enterocytes. The other cell line, HT29, with methotrexate (MTX) adaptation, differentiates in culture media to secret mucin. In this study we used this co-culture model to investigate the efficacy of the LBPs against *C. albicans*, however, to achieve a far more reliable and robust gut epithelium model which resembles biomimetic molecular mechanisms in the intestinal niche, further improvements of this model such as the use of secretory IgAs and/or various other crucial antibodies/cytokines necessary for managing gut microbiome homeostasis are necessary[21].

This fascinating field of research has significant potential for determining the link in a chain of events involving interactions between *C. albicans* and the gut epithelium where LBPs are used to treat the invading pathogens.

Future studies

We are currently investigating the cellular response of the gut epithelial cells to *C. albicans* colonisation by comparing global gene expression (using RNA sequencing) with and without co-inoculation of LBPs. The RNAs will represent a snapshot of interaction/non-interaction of *C. albicans* with gut epithelium cells following the competitive adhesion of *C. albicans* with and without LBPs and identify genes that play a major role during these interactions. This will further our understanding of the mechanisms associated with using LBPs to treat invading pathogens.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

Born in South Africa, **Bronwyn Smit** is a naturalised New Zealand citizen, currently residing in Queensland. She completed her undergraduate degree in Biomedical Science at the University of Auckland, followed by a master's degree in forensic science. Aside from briefly working in environmental science, Bronwyn explored career opportunities in the arts and hospitality industries before developing a fascination with the gut microbiome and its overwhelming impact on human health and disease. This led to her current PhD project in Microbiology at University of the Sunshine Coast, investigating the competitive inhibition of *Candida albicans* by live biotherapeutic products in the human gut.

Dr Anna Kuballa obtained a Bachelor of Biomedical Science degree (Hons) majoring in microbiology from the James Cook University. She graduated with a Doctor of Philosophy in the field of molecular biology from the University of Queensland, Brisbane in 2007. She continued her research as a post-doctoral research fellow at the University of the Sunshine Coast where she currently holds an academic research and teaching position. Dr Kuballa's published contributions centre around the molecular pathways involved in inflammation and microbial infection, with a special interest in the microbiome of inflammatory bowel disease and the breast milk microbiome.

Samantha Coulson joined Servatus in 2018 as Head of the Clinical Research Department and holds a PhD in Medicine from the University of Queensland. With over 15 years' experience, Samantha has a diverse background in both academia and industry with extensive knowledge of the human microbiome. She is adept in designing, initiating, leading and completing multidisciplinary research projects and also in managing product research, innovation and development programs. As Head of Clinical Research Samantha manages all aspects of Servatus' human clinical trial projects and preclinical studies, together with a small but highly experienced research team and global collaborators.

Associate Professor Mohammad Katouli obtained his PhD in 1980 from University of Ulster in UK. He then joined the Research and Development Department of DP Pharmaceuticals. In 1985, he took the position of the Head of Microbiology Department at the Pasteur Institute in Tehran. Between 1988 and 1998, he worked as a senior research fellow at the Microbiology and Tumor Biology Centre of the Karolinska Institute, Stockholm, Sweden. In 1998, he took a teaching and research position at University of the Sunshine Coast. His current interest is gut microbiota and the role of *E. coli* in pathogenesis of IBD.