Sains Malaysiana 54(7)(2025): 1677-1685
http://doi.org/10.17576/jsm-2025-5407-03

Study on the Mechanism of miRNA9718 Inhibition of the STAT3 Pathway Exacerbating *Candida albicans*-Induced Intestinal Epithelial Cell Damage

(Kajian tentang Mekanisme miRNA9718 Perencatan Laluan STAT3 Memburukkan Kerosakan Sel Epitelium Usus Aruhan *Candida albicans*)

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Received: 6 September 2024/Accepted: 27 December 2024

ABSTRACT

Candida albicans is a commensal yeast pathogen that frequently colonizes the human gastrointestinal (GI) tract, and this capacity to efficiently invade allows it access to deeper tissues leading to disease. Probing this resistance is necessary to develop effective treatment strategies and requires further understanding of how intestinal epithelial cells resist invasion by *C. albicans*. In this study, we performed miRNA transcriptomic analysis combined with experiments to screen differentially expressed miRNAs under *C. albicans* exposure in intestinal epithelial cells. We also identified a subset of miRNAs that were notably deregulated by *C. albicans* invasion, largely within the context of targeting STAT3. Taken together, we provide new insights on how intestinal epithelial cells counteract *C. albicans* invasion. The STAT3 pathway and its regulation of the dysregulated miRNAs are evidenced by our studies. These results further the progress in intestinal host-pathogen interaction and has implications for possible *C. albicans* directed therapeutics approaches.

Keywords: *Candida albicans*; intestinal epithelial cells; mechanism of resistance; miRNA omics analysis; STAT3 pathway

ABSTRAK

Candida albicans adalah patogen kulat oportunistik yang biasa dikenali kerana keupayaannya untuk menyerang dan menyebabkan jangkitan gastrointestinal. Memahami mekanisme apabila sel epitel usus menolak serangan oleh *C. albicans* adalah penting untuk membangunkan strategi rawatan yang berkesan. Untuk mengenal pasti miRNA yang berubah ekspresinya sebagai tindak balas kepada pendedahan *C. albicans* dalam sel epitel usus, kami melakukan analisis transkriptomik miRNA dan mengesahkan hasil tersebut melalui uji kaji. Analisis kami menunjukkan satu kumpulan miRNA yang mengalami disregulasi secara signifikan semasa serangan *C. albicans*, terutamanya yang menjejaskan laluan STAT3. Kajian kami memberikan pandangan baharu tentang mekanisme apabila sel epitel usus menolak serangan oleh *C. albicans*. Kami tunjukkan keterlibatan laluan STAT3 dan peranan kawalan miRNA yang disregulasi dalam proses ini. Penemuan ini menyumbang kepada pemahaman kita tentang interaksi hos-patogen dalam saluran gastrointestinal dan mungkin mempunyai implikasi dalam pembangunan pendekatan terapeutik baharu yang menumpukan kepada jangkitan *C. albicans*.

Kata kunci: Analisis omik miRNA; *Candida albicans*; laluan STAT3; mekanisme perlindungan; sel epitel usus

INTRODUCTION

Candida albicans is a commensal fungus in the human gut microbiota but can be pathogenic in opportunistic infections in immunocompromised people (Mayer, Wilson & Hube 2013). Therefore, a healthy intestinal epithelial barrier is vital to prevent *C. albicans* invasion and subsequent systemic infections. In addition, the STAT3 pathway is involved in controlling intestinal barrier and immune response. However, the underlying mechanism of miRNA-STAT3 interaction remains unclear (Butturini, Carcereri De Prati & Mariotto 2020; Zhang et al. 2014; Zou et al. 2020). Moreover, epithelial barrier, the first line of defense against invading microbial pathogens, maintains gut microbe-host homeostasis, and significant breach of this barrier is linked to systemic bloodstream infections, such as 'leaky gut' and resultant microbial translocation. Shifting microorganisms including *C. albicans* from the oral cavity into the blood circulation continues to be a key driver of many disorders (Cao et al. 2013; Othumpangat et al. 2021; Servais et al. 2019; Zhang et al. 2017; Zhu et al. 2021).

Extensive research has been conducted so far to study the intricate relationship amid the gut microbiota, intestinal epithelial barrier, and immune responses (Zheng et al. 2021). The STAT3 pathway, which significantly mediates such a role, has by far attracted considerable attention. STAT3 signaling is a critical signaling molecule that is implicated in certain epithelial cell proliferative activity and differentiation and immune response throughout various tissues, including the intestine (Arkowitz & Bassilana 2019; Kinashi & Hase 2021). The perpetration of the STAT3 pathway has also been linked to numerous diseases, including gastrointestinal diseases and IBD. Although much has been achieved to decipher the role miRNAs play in counteracting the candidal invasion and maintaining intestinal barrier integrity through the STAT3 pathway, an extensive understanding of the exact mechanisms of action involved is still a gap (Basmaciyan et al. 2019).

To date, extensive research has focused on understanding the complex interplay between the gut microbiota, the intestinal epithelial barrier, and immune responses. The role of the STAT3 pathway in regulating these processes has gained considerable attention. STAT3 acts as a key signaling molecule involved in mediating epithelial cell proliferation (Han et al. 2022; Liu et al. 2018; Yan et al. 2011), differentiation, and immune responses in various tissues, including the intestine (Fang et al. 2019; Nguyen et al. 2013; Wang et al. 2017; Xiu et al. 2018). Dysregulation of the STAT3 pathway has been implicated in multiple diseases, including gastrointestinal disorders and inflammatory bowel diseases (Cao et al. 2013; El-Daly et al. 2019; Qin et al. 2017; Rescigno 2011; Zou et al. 2020).

Therefore, the current study aimed to bridge this gap by studying the miRNA mediated STAT3 pathway in defense of *C. albicans* invasion and maintenance of intestinal barrier function. Extensive Assay approach including the cell culture models, Western blot analysis, transcriptome sequencing, Quantitative PCR, and LDH assay was employed to achieve the study objective cognitive focus. The study aims to provide insight into how miRNA9718 regulates the STAT3 signaling pathway and intestinal that underlies its role in defending against *C. albicans* invasion (Lei et al. 2022; Qiu et al. 2017).

Knowledge of these complex pathways required for sustaining the intestinal epithelial barrier and blocking translocation through *C. albicans* is necessary to design new therapeutic approaches. The identification of miRNA9718 as a regulator of STAT3 pathway not only brings new cues about potential therapeutic targets to combat *C. albicans* infections, but also creates boundaries for future investigation in this realm.

This study aims to clarify the mechanism that miRNA–mediated regulation of STAT3 pathway, which played a protecting role against *C. albicans* invasion via modulating intestinal epithelial barrier permeability. We aim to fill this critical research void, and our findings could serve as a basis for new experimental therapies that would be used to maintain the integrity of gut lining, inhibiting *C. albicans* colonization.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT

The *in vitro* study was performed using the *Candida albicans* strain ATCC 90028 (American Type Culture Collection, Rockville, MD) as the test microorganism for biofilm adhesion inhibition on Caco-2 cells. The CO_2 fincubator was cultured at 37 °C, and the experimental groups were divided into: (1) *C. albicans* group; Caco-2 cells treated with logarithmic growth phase culture of candida vegetable tree within Fermi *Mycobacterium* pstraws, (2) miRNA 100 nM group Were plated in a six-well plate and transfected using final consent. miRNA mimics have advanced capacity for obtaining N M60 pmol; hitting korpi net until Farnesolsipurpygeon treatment FC2935000 μft※Particle beings configured up to cell.

miRNA SEQUENCING METHOD

Total RNA was isolated using the mirVana miRNA Isolation Kit from Ambion, adhering to the supplier's guidelines. The concentration of the extracted RNA was measured with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and its integrity was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). For the preparation of small RNA sequencing libraries, each sample provided 1 μg of total RNA, which was processed with the NEBNext Small RNA Library Prep Set for Illumina (Catalog Number NEB#E7330S, New England Biolabs, USA) following the protocol. In summary, the RNA was subjected to adapter ligation at both the 5' and 3' ends, reverse transcribed into cDNA and amplified via PCR. The PCR products, specifically those within the size range of 140–160 bp, were selected and purified to form the small RNA libraries. The libraries' quality was evaluated using the Agilent 2100 Bioanalyzer, and they underwent sequencing on an Illumina Novaseq 6000 instrument, producing 150 bp paired-end reads.

SMALL RNA SEQUENCING ANALYSIS

Low-quality sequences were eliminated, including those with 5' primer contaminants and poly(A) tails. Reads lacking a 3' adapter or insert tag, as well as those shorter than 18 nucleotides (nt) or exceeding 36 nt, were also removed from the raw dataset to yield high-quality reads. The length distribution of these clean reads was assessed within the reference genome. Subsequently, the reads were aligned using Bowtie against miRBase (http://www.mirbase.org/) for annotation and further filtration. Additionally, cDNA sequences and species-specific repeat sequences from the Repbase database were identified with Bowtie. Mature

miRNAs were determined by alignment with miRBase v22, and their expression profiles across various samples were examined. Unannotated reads were subjected to miRDeep2 for the prediction of novel miRNA candidates. The miRNA star sequences, and mature sequences were identified based on the pre-miRNA hairpin structure and miRBase data.

DETERMINATION OF miRNA EXPRESSION BY QPCR

Total RNA was extracted using Trizol (Invitrogen, USA) and changed into cDNA by Reverse transcriptase kit (Fermentas, USA). QPCR was performed as routine protocol using the primers as outlined below:

Gene	Forward sequences	Reverse sequences
ZO-1	GTCTGCCATTACACGGTCCT	TGCTTGCTGCTTACCTGTTG
STAT3	GACACCGTAAGTGGCTTCCT	GCTTCCAACCTTTGGCAGATT
Occludin	CTCGTCTGCGTCATCCATCA	TTGACACTGGCCTACAGGAA
GAPDH	AAGGTCGGTGTGAACGGATTT	AACTTGCCGTGGGTAGAGTC

The thermal cycling protocol began with a preliminary denaturation phase at 95 °C for 5 min, succeeded by 35 cycles of 95 °C for 10 s for denaturation, 50-60 °C for 35 s for annealing, and 72 °C for 30 s for extension. A concluding extension phase was performed at 72 °C for 5 min. The RT-qPCR analysis was executed on the Light Cycler 480 RT-qPCR System (Roche, Basel, Switzerland). Gene expression fold-changes were calculated using the 2^-ΔΔCT method, with GAPDH as the reference gene. The CT values were determined by comparing the tested samples to control samples as follows: ΔCt = Ct (tested samples) - Ct (GAPDH); ΔΔCt = ΔCt (tested samples) - ΔCt (control samples); fold change = 2^(-ΔΔCT) {REF}.

WESTERN BLOTTING

In the Western blot analysis, total protein samples were separated on 10% SDS-PAGE gels and subsequently transferred onto PVDF membranes (Millipore, USA). Primary antibodies were employed, comprising mouse polyclonal GADPH antibody (1:1000; Sigma, China), rabbit polyclonal ZO-1 antibody (1:20000; Sigma, USA), rabbit polyclonal STAT3 antibody (1:20000; Sigma, USA), and rabbit polyclonal Occludin antibody (1:20000; Sigma, USA). Subsequently, the reactive bands were visualized using the ECL kit (Millipore, USA) on a GIS-2009 Western blotting detection system (Shanghai Tianneng Biotechnology Company, China) after incubation with HRP-linked IgG {REF}.

LACTATE DEHYDROGENASE(LDH) ASSAY

A total of 5000 Caco-2 cells per well in a 96-well plate were seeded to maintain a cell density of 80-90% at the time of testing. Various drugs were applied for treatment, with appropriate controls in place. Following drug exposure, the plate was spun at 400 g for 5 min using a multi-well centrifuge to pellet the cells. The supernatant was removed, and 150 µl of the LDH release reagent from the kit, diluted with PBS (1:10 ratio), was added to each well. The plate was gently mixed and returned to the incubator for 1 h. Afterward, the plate was centrifuged again at 400 g for 5 min. Then, 120 µl of the supernatant was transferred to a new 96-well plate for analysis. This plate was kept at room temperature in the dark for 30 min before measuring the absorbance at 490 nm {REF}.

STATISTICAL ANALYSIS

In this research, experimental data were expressed as the mean ± standard deviation (SD). Prior to analysis, the data underwent assessments for variance homogeneity and normal distribution. Comparative analyses between groups were performed using either the t-test or one-way ANOVA, depending on the data characteristics. All statistical computations were handled using SPSS software, version 19.0 (SPSS Inc., Chicago, IL, USA) {REF}, with statistical significance defined as $P < 0.05$.

RESULTS

DIFFERENTIALLY EXPRESSED (DE) miRNAs IN SEQUENCING

In comparison to the control group, a total of 162 differentially expressed (DE) miRNAs were identified, comprising 99 known DE miRNAs (45 upregulated and 54 downregulated) and 63 novel DE miRNAs (45 upregulated and 18 downregulated). Figure 1(a) presents the Volcano Plot depicting the differentially expressed known miRNAs between samples, while Figure 1(b) shows the Cluster Heatmap of these miRNAs.

FUNCTIONAL PREDICTION OF TARGET GENES OF DE miRNAs

The most significant enrichment in the biological processes of the target genes of differentially expressed (DE) miRNAs included the anterior/posterior pattern, specification, cardiac chamber morphogenesis, cardiac ventricle development, outflow tract morphogenesis, atrioventricular valve, mesenchyme morphogenesis, and heart valve morphogenesis, as depicted in Figure 1(c). The enriched KEGG pathways shown in Figure 1(d) included MicroRNAs in cancer, human T-cell leukemia virus-1 infection, human papillomavirus infection, cell cycle, PI3K-Akt signaling pathway, TGF-beta signaling pathway, and endocrine resistance.

RESULT OF miRNA EXPRESSION BY QPCR

In Figure 2, we analyzed the mRNA levels of STAT3, ZO-1, and Occludin using qPCR. The overexpression of miRNA9718 resulted in a significant decrease in the expression of STAT3, ZO-1, and Occludin compared to the

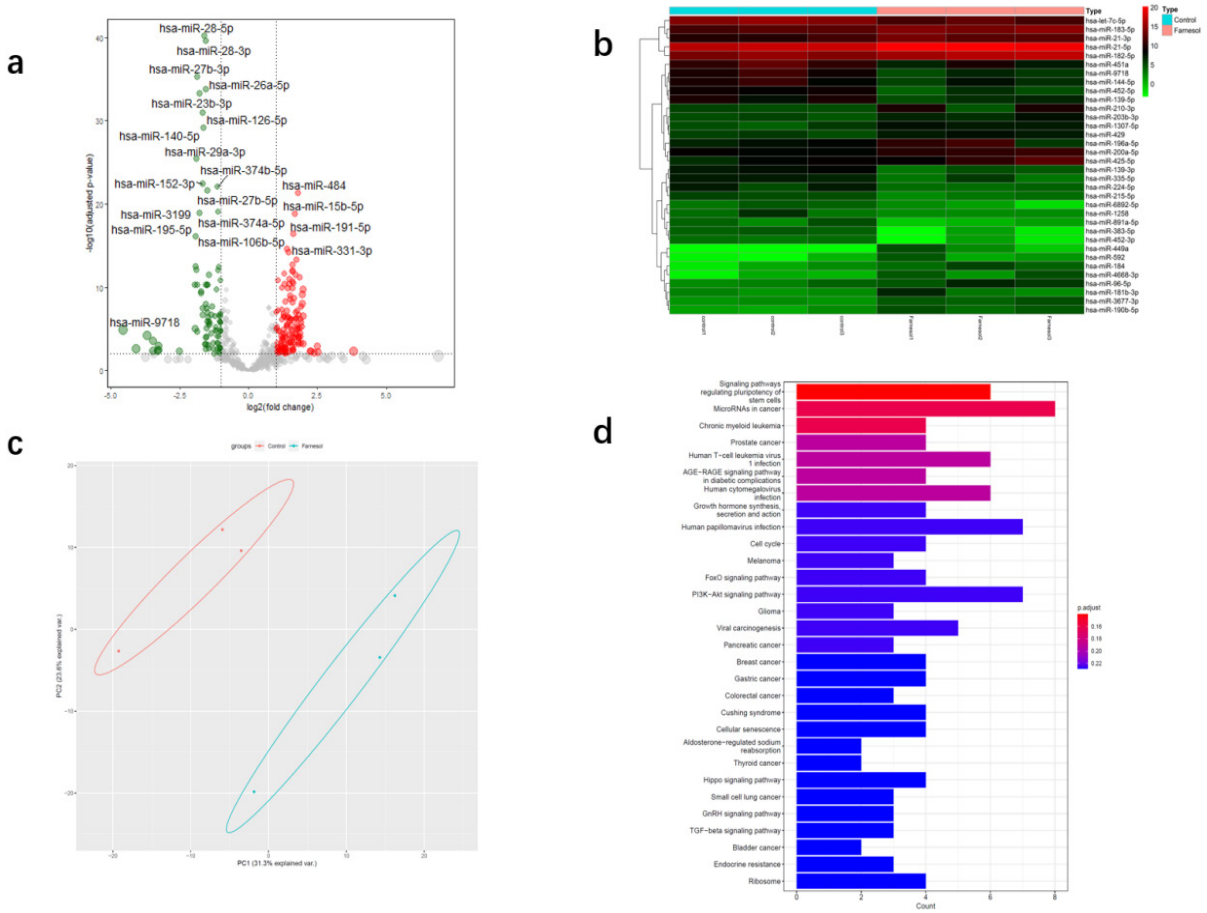
control group. This suggests that miRNA9718 treatment had an impact on the expression of STAT3, ZO-1, and Occludin, as well as on the relative signaling transcription. Moreover, the combined treatment of miRNA9718 and farnesol effectively reversed the effect of miRNA9718 and restored the mRNA expression of STAT3, ZO-1, and Occludin. This further confirms that miRNA9718 influenced the expression of STAT3, ZO-1, and Occludin, ultimately leading to alterations in cell permeability.

WESTERN BLOTTING

As shown in Figure 3(a), key proteins including STAT3, ZO-1, and Occludin were examined to assess changes in the STAT3 signaling pathways across various experimental groups: *C. albicans* group, *C. albicans* + miRNA9718 mimic group, *C. albicans* + miRNA9718 inhibitor group, and *C. albicans* + miRNA9718 mimic + farnesol group. Comparative to the control group, our findings indicate that excessive treatment with miRNA9718 led to a significant decrease in the expression of STAT3, ZO-1, and Occludin, thereby demonstrating the impact of miRNA9718 on the signaling pathways of these proteins and its influence on cell permeability. Additionally, when comparing the miRNA9718 treatment group to the miRNA9718 treatment + farnesol group, it was observed that the latter significantly reversed the effects of miRNA9718 on the protein expression of STAT3, ZO-1, and Occludin, thus confirming that miRNA9718 can reduce the expression of these proteins and consequently alter cell permeability. Furthermore, the data were analyzed, as illustrated in Figure 3(b).

LDH ASSAY

The levels of LDH were evaluated in the experimental groups, which included: the *C. albicans* group, the *C. albicans* + miRNA9718 mimic group, the *C. albicans* + miRNA9718 inhibitor group, and the *C. albicans* + miRNA9718 mimic + phanolol group. The results are depicted in Figure 4.



a) Volcano plot of differentially expressed known miRNAs between Samples, b) Cluster heatmap of differentially expressed known miRNAs between samples, c) PCA analysis result plot, and d) KEGG analysis plot of differentially expressed target genes of known miRNAs

FIGURE 1. Analysis of differentially expressed known miRNAs

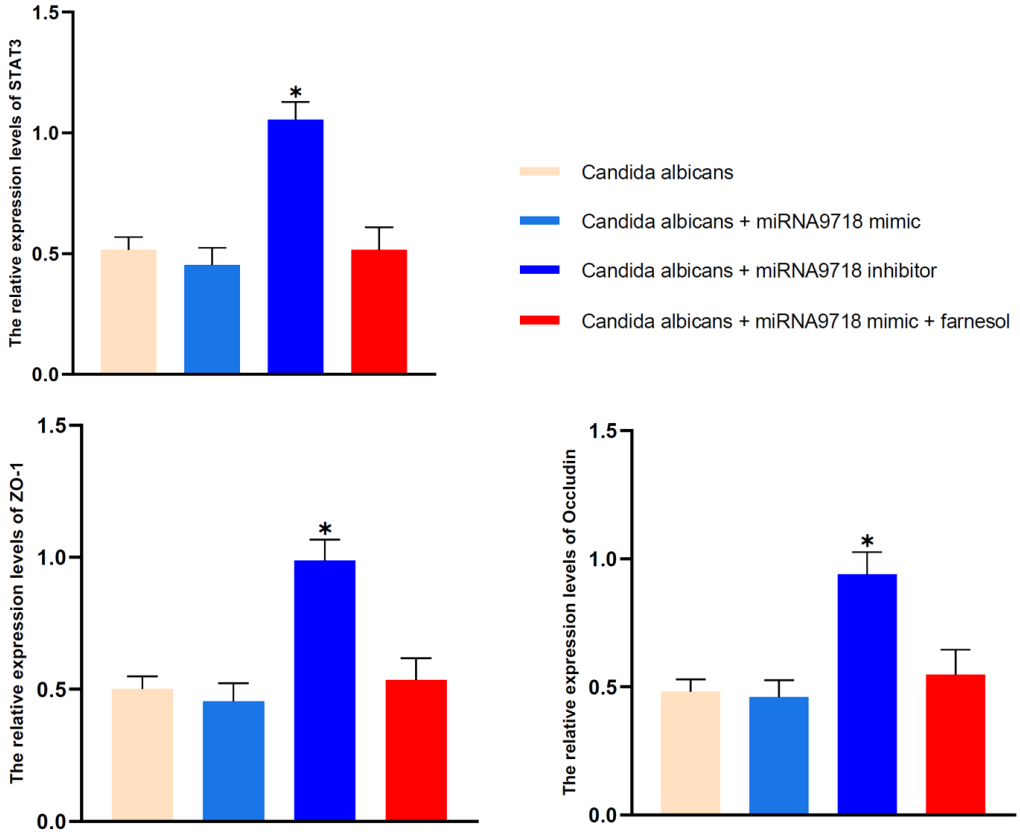


FIGURE 2. Data of mRNA expression analysis for STAT3, ZO-1, and Occludin using qPCR. *P < 0.05

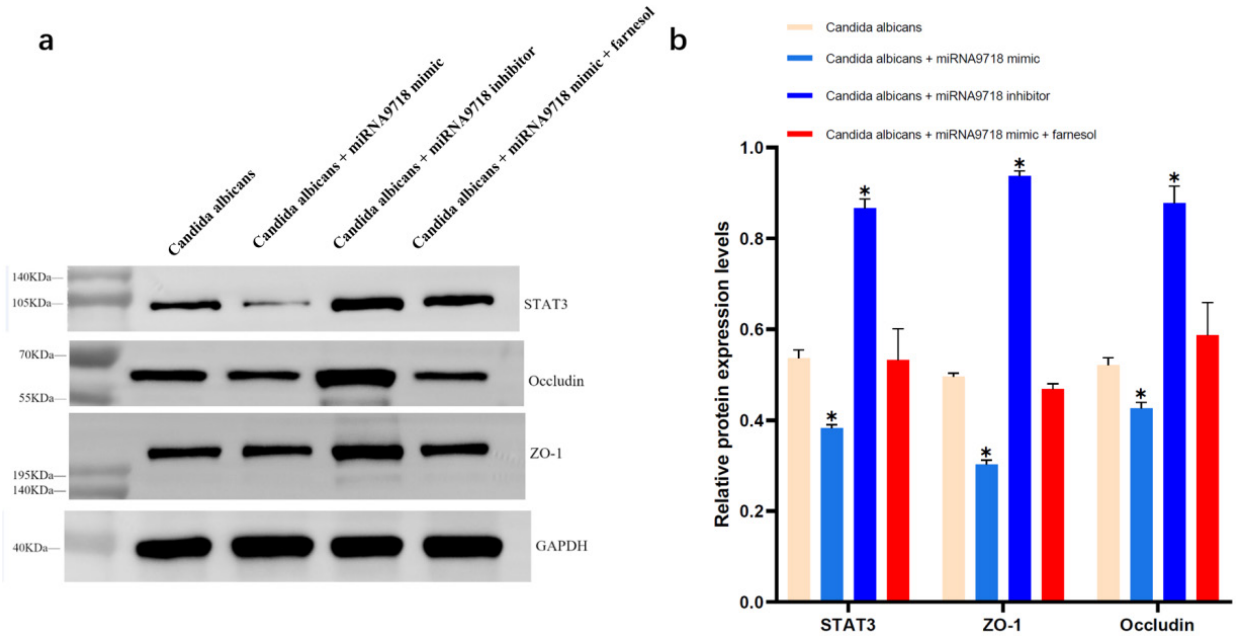
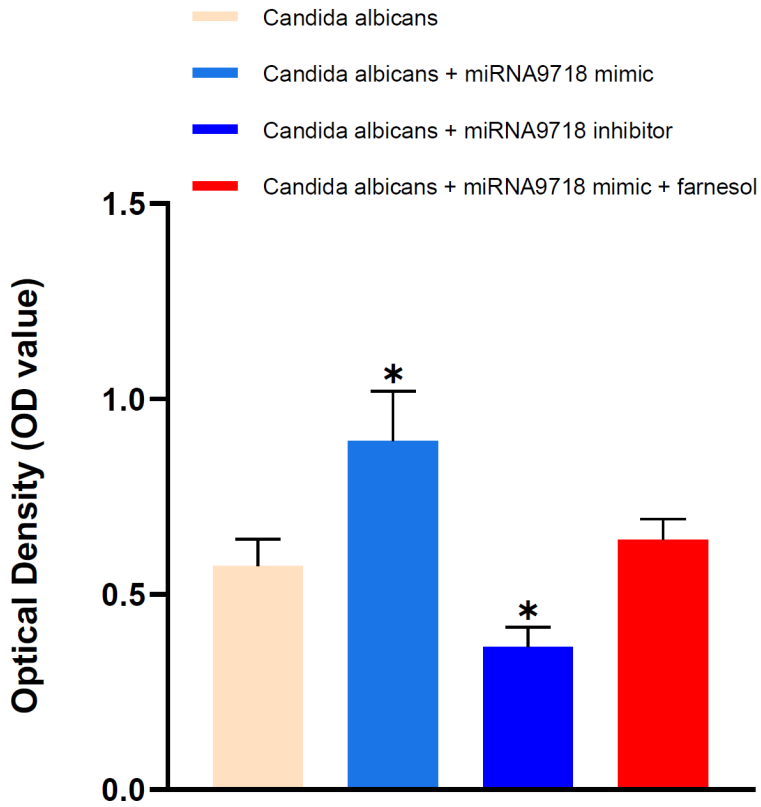


FIGURE 3. Results and analysis of Western blot for STAT3, ZO-1, and Occludin. *P < 0.05



LDH detection of *Candida albicans* group, *Candida albicans* + miRNA9718 mimic group, *Candida albicans* + miRNA9718 inhibitor group, *Candida albicans* + miRNA9718 mimic + farnesol group. *P < 0.05

FIGURE 4. Lactate Dehydrogenase(LDH) detection results in the supernatant of Caco-2 cells from different groups

DISCUSSION

Candida albicans, a commensal fungus in the human gut microbiota, has the potential to cause opportunistic infections in immunocompromised individuals (Ho et al. 2021). The key to preventing the invasion and subsequent systemic infection of *C. albicans* lies in stabilizing the intestinal epithelial barrier (Gibson et al. 2010; Grivennikov et al. 2009; Mao et al. 2021; Pang et al. 2021). The STAT3 signaling pathway is involved in the regulation of other immune responses and seems to play a role in cellular permeability through maintenance the integrity of intestinal barrier function (Cao et al. 2013; Xu et al. 2021). However, the molecular mechanisms by which miRNAs inactivate STAT3 to resist against *C. albicans* are still unclear.

We conducted this study to explore the involvement of miRNA- mediated STAT3 signaling regulation in host defense against *C. albicans* infection and for maintenance of intestinal barrier integrity. Using a panel of cell culture models, coupled with Western blot analyses, transcriptome sequencing and quantitative PCR as well lactate dehydrogenase (LDH) assays, we set out to define the functional effects underlying miRNA9718-mediated regulation on STAT3 signaling pathway activation and cellular permeability.

The results presented showed the suppressive effects of miRNA9718 on STAT3 ordering by lower expression levels of p-STAT3 and subsequently inactivation of downstream pathways leading to reduced cell permeability (Figure 5). Although this seems logical pursuant to our hypothesis of miRNA targeting STAT3 pathway during *C. albicans* infection, it requires further investigation which is currently underway in the lab. Here we hypothesized that regulation of the STAT3 pathway could decrease intestinal epithelial barrier permeability and enhance mucosal immunity against *C. albicans* invasion.

It is essential to foster an understanding of the delicate mechanisms that contribute to intestinal epithelial barrier function, thus, defense against *C. albicans* translocation in order for the development novel therapeutic strategies aimed at protection from *C. albicans* infections. Our results showed how miRNA9718 regulates the STAT3 pathway point to a potential therapeutic target for maintaining intestinal barrier function and thereby prevent *C. albicans* invasion. These findings not only show a potential mechanism by which the intestinal epithelial barrier is regulated to restrict *C. albicans* infection but also serve as basis for further studies in this angle.

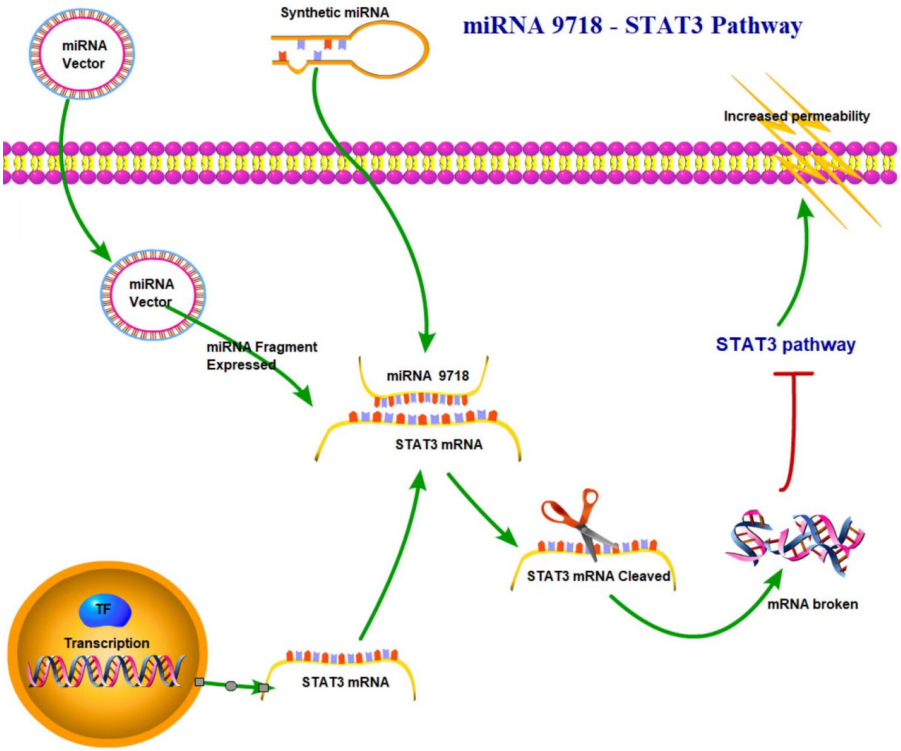


FIGURE 5. Schematic Diagram of miRNA9718 and the STAT3 Signaling Pathway. miRNA9718 is depicted as a regulatory molecule that exerts its influence on the STAT3 pathway. Through overexpression, miRNA9718 modulates the expression of STAT3, leading to the inhibition of the STAT3 signaling cascade. This inhibition subsequently results in an increase in cellular permeability

In summary, we are unearthing the mechanism that miRNA modulates intestinal epithelial barrier permeability and protects against *C. albicans* invasion through STAT3 pathway regulation. This study could provide new ideas for therapeutics whereby the protection of intestinal integrity may prevent *C. albicans* infections.

ACKNOWLEDGEMENTS

The research was supported by Minhang District Science and Technology Commission Natural Science Foundation (No.2021MHZ098), Key Medical Specialty funded by Shanghai Fifth People 's Hospital Fudan University (No.2020WYZDZK06). We the authors declare no competing interests in this study. Our contributions are as follows: Jinying Zhao performed the majority of experiments, analyzed the data, and drafted the manuscript. Jinying Zhao, Jianguo Tang and Xue Yin designed the research. Jinying Zhao, Liping Yin, Linxin Jiang and Sijia Shen conducted the molecular biology assays and assisted in writing the manuscript. Jinying Zhao collected and analyzed the data. Jianguo Tang and Xue Yin provided critical revision of the manuscript for important intellectual content. All authors reviewed the manuscript.

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