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# Immunomodulatory mechanism of *Bacillus subtilis* R0179 in RAW 264.7 cells against *Candida albicans* challenge

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ABSTRACT

This study was aimed to explore the immunomodulatory and anti-*Candida* mechanisms of *Bacillus subtilis* (*B. subtilis*) R0179 in macrophages. RAW 264.7 cells were first challenged with *B. subtilis* R0179. *B. subtilis* R0179 was found to down-regulate the signals of Dectin-1, Card9, P-Iκ-Bα, Iκ-Bα, and NF-κB. Meanwhile, it reduced the levels of cytokines interleukin (IL)-1β, IL-6, IL-12, and tumor necrosis factor (TNF)-α, but increased the level of cytokine IL-10. Then RAW 264.7 cells were pretreated with *B. subtilis* R0179 before challenged with *Candida albicans* (*C. albicans*) or RAW 264.7 cells were co-treated with *B. subtilis* R0179 and *C. albicans*. In the presence of *C. albicans*, *B. subtilis* R0179 also showed the similar immunomodulatory effects on RAW 264.7 cells. Hence, this study provides the first insight into the immunomodulatory mechanisms of *B. subtilis* R0179 on the Dectin-1-related downstream signaling pathways in macrophages, which may prevent tissue damage caused by excessive pro-inflammatory response during the infection of *C. albicans*.

## 1. Introduction

*C. albicans* is a commensal fungus, which can be isolated from the oral mucosa, vaginal mucosa, and gastrointestinal tract of many healthy individuals [1]. Nowadays with the widespread use of antibiotics, glucocorticoids, and immunosuppressants, *C. albicans* can lead to superficial, deep invasive, as well as systemic candidiasis as an opportunistic pathogenic fungus [2]. Due to long-term use of conventional antifungals, such as nystatin and fluconazole, *C. albicans* develops drug resistance [3]. Meanwhile, the overuse of antibiotics and corticosteroids can increase the risk for candidiasis. Hence, alternative and complementary therapies are urgently needed and probiotics provide a possible resolution [4].

Probiotics have been defined by the World Health Organization as live microorganisms that provide health benefits when applied in suitable amounts [5,6]. There is accumulated evidence that appropriate use of probiotics has the potential of preventing or treating candidiasis in animal and human [7–9]. In immunosuppressed mice, *Lactobacillus rhamnosus* ATCC 7469 was able to avoid or decrease the progress of oral candidiasis [10]. In a clinical trial, *Lactobacillus plantarum* P17630 was reported to prevent the recurrence of vulvovaginal candidiasis [11]. Besides, in our earlier randomized clinical trial, *Streptococcus salivarius* K12 also exhibited efficacy as an adjuvant treatment in oral candidiasis [12].

The anti-*Candida* mechanisms of probiotics have been widely researched, which can be indirectly through metabolites, such as $H_2O_2$ and acids, or directly through competition adhesion sites and nutrients with *Candida* [13–17]. In addition, probiotics stimulated innate and adaptive immune system [18,19]. The innate immune system is the first line of defense during microbial infection and macrophages are the links between the innate and adaptive immune system [20,21]. The pattern-recognition receptors (PRRs) on the surface of macrophages, such as C-type lectin receptors (CLRs), recognize the pathogen-associated molecular patterns (PAMPs) in *Candida* cell wall.

Dectin-1, a primary C-type lectin receptor, recognizes beta-glucans in *Candida* cell wall, and induces the activation of Nuclear factor-kappaB (NF-κB) through Caspase-recruitment domain 9 (Card9), which is reported to essential to protect against the invasion of *Candida* [22]. And then these signals induce Spleen tyrosine kinase (Syk) phosphorylation and Recombinant inhibitory subunit of Nuclear factor kappa B alpha

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(Iκ-Bα) phosphorylation [23]. During the onset of the inflammatory process, the activated macrophages secrete pro-inflammatory cytokines, such as Interleukin (IL)-6, Tumor necrosis factor (TNF)-α, and IL-12 to promote T-helper 1 (Th1) response, which generates reactive oxygen and nitrogen intermediates to kill *Candida*. But when the inflammation is severe enough, excessive activation and secretion of pro-inflammatory cytokines lead to cell destruction and death [24]. Hence, macrophages are polarized from M1 macrophages (mainly involved in pro-inflammatory responses) to M2 macrophage (mainly involved in anti-inflammatory) to balance the immune response during the infection of *C. albicans* [25,26].

The effects of probiotics were strain-specific, which changed the process of *Candida* recognition by macrophages and played a dual role in terms of the physiopathological context [27]. *Lactobacillus rhamnosus* LR32, *Lactobacillus casei* L324 m, and *Lactobacillus acidophilus* NCFM down-regulated the expression of Dectin-1, reduced pro-inflammatory cytokine such as IL-12, and increased anti-inflammatory cytokine such as IL-10 [28]. However, *Lactobacillus gasseri* LA806 and *Lactobacillus helveticus* LA401 increased the expression of Dectin-1 and increased pro-inflammatory cytokines such as IL-12, accompanied with the induction of anti-inflammatory cytokine such as IL-10. These results showed that LA401 and LA806 strains oriented the macrophages towards both a fungicidal pro-inflammatory phenotype to defense against *Candida* and an anti-inflammatory phenotype to control the pernicious inflammatory response [29]. Based on these inconsistent conclusions, further research is required to confirm the beneficial effects of specific probiotics before application.

In our previous study, upon screening, *B. subtilis* R0179 directly inhibited *C. albicans* in an engineered human oral mucosa model probably by secreting the antifungal agent Iturin A to disrupt the plasma membrane of *C. albicans* [30]. The inhibitory activity of *B.subtilis* R0179 against *C.albicans* could be explained by iturin A, however, the immunomodulatory mechanism remains unknown. Hence, in this study, macrophages were used to evaluate the possible immunomodulatory effects of *B. subtilis* R0179 on macrophages against *C. albicans*.

## 2. Materials and methods

### 2.1. Microbial culture

*C. albicans* ATCC 90028 was cultured at 37 °C on Sabouraud Dextrose Agar (AOBOXing Product, China) medium for 18 h. *B. subtilis* R0179 was separated from the product of Medilac-Vita (Hanmi Pharm. Co., Ltd., China) and propagated on LB agar (Solarbio, China) at 37 °C for 18 h.

### 2.2. 16S rRNA gene sequencing analysis of isolated monoclonal bacteria in the product of Medilac-Vit

The DNA of isolated monoclonal bacteria in the product of Medilac-Vit was extracted with Bacterial Genomic DNA Extraction Kit (Solarbio, China). The forward primer of 27F (AGAGTTTGATCCTGGCTCAG) and the reverse primer of 1492R (TACGGCTACCTTGTTACGACTT) were used for polymerase chain reaction (PCR) amplification. The temperature procedure of the PCR assay was set at 94 °C for 4 min, 94 °C for 1 min for 35 cycles for denaturation, 57 °C for 1 min for primer annealing, 72 °C for 1 min for strand elongation, and 72 °C for 10 min for the final elongation [31]. The products of amplified PCR were electrophoresed on 1.0% agarose gel and visualized under a gel documentation system (Syngene, United States). Amplified DNA bands were then cut from the agarose gel and processed for Sanger nucleotide sequencing. Then the obtained nucleotide sequences were searched for homology analysis with the nucleotide sequences of *B. subtilis* R0179 in the GenBank using NCBI BLAST (NCBI, United States) computer program [32].

### 2.3. Cell culture

Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (HyClone, United States), enriched with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, United States) was used for the growth of RAW 264.7 cells in an atmosphere containing 5% $CO_2$ at 37 °C without any penicillin or streptomycin. And $5 \times 10^6$ cells were inoculated into a fresh 10-cm cell-culture dish (Corning-Costar, United States). Upon 80% confluence, RAW 264.7 cells were sub-cultured serially to be used for the following experiments.

### 2.4. Co-culture of macrophages and B. subtilis R0179

To evaluate the immunomodulatory effects of *B. subtilis* R0179 on macrophages. RAW 264.7 cells ($2 \times 10^6$ cells/well) were plated in 6-well plates (Corning-Costar, United States) without FBS or antibiotics, and incubated at 37 °C with 5% $CO_2$ for adhesion. Then RAW 264.7 cells challenged with *B. subtilis* R0179 ($2 \times 10^7$ CFU/well) for 0 h, 3 h, and 12 h were collected for the detections of Dectin-1 and Card9 by quantitative real-time polymerase chain reaction (qPCR) and for the detections of Dectin-1, Syk, P-Syk, Card9, P-Iκ-Bα, Iκ-Bα, and NF-κB by Western blot. Besides, the cell culture supernatants after RAW 264.7 cells challenged with *B. subtilis* R0179 for 0 h, 12 h, and 24 h were collected for the detections of IL-6, IL-1β, IL-12, TNF-α, and IL-10 by Enzyme-linked immunosorbent assays (ELISA).

### 2.5. Co-culture of macrophages with B. subtilis R0179 and C. albicans

To evaluate the effects of *B. subtilis* R0179 on macrophages during the infection of *C. albicans*. The experimental groups were set as follows: RAW 264.7 cells ($2 \times 10^6$ cells/well) alone as control; RAW 264.7 cells were challenged with *C. albicans* ($6 \times 10^5$ CFU/well); RAW 264.7 cells were challenged with *B. subtilis* R0179 ($2 \times 10^7$ CFU/well) and *C. albicans* ($6 \times 10^5$ CFU/well) together (co-treatment group); RAW 264.7 cells were pretreated with *B. subtilis* R0179 overnight (about 12 h) before challenged with *C. albicans*. The stimulation time of *C. albicans* in different groups was consistent, and these groups were finally incubated after addition of *C. albicans* for 3 h to detect the mRNA levels of Dectin-1 and Card9 by qPCR, 30 min, 3 h, and 12 h to detect the protein levels of Dectin-1, Syk, P-Syk, Card9, P-Iκ-Bα, Iκ-Bα, and NF-κB by Western blot, and 24 h to detect the cytokine levels of IL-6, IL-1β, IL-12, TNF-α, and IL-10 by ELISA. All tests were conducted in triplicate in three independent experiments.

### 2.6. RNA extraction and qPCR assays

The relative expression of Dectin-1 and Card9 was assessed by qPCR assay. Briefly, macrophages were lysed with TRIzol (Thermo Fisher Scientific, United States), and the total RNA of macrophages was extracted. The purity and concentration of RNA samples were detected using a NanoDrop One (Thermo Fisher Scientific, United States). The RNA was reverse transcribed into cDNA using ABScript II RT Master Mix (ABclonal, China). The conditions of reverse transcription were set at 25 °C for 5 min, 42 °C for 12 min, 85 °C for 5 s, and then maintained at 12 °C. TaqMan primers (Sangon Biotech, China) used in the experiment

**Table 1**
Primers used in real-time PCR experiment.

| Gene name | Primer sequence 5′→3′ | GenBank |
|---|---|---|
| GAPDH | F GGT TGT CTC CTG CGA CTT CA<br>R TGG TCC AGG GTT TCT TAC TCC | CM000999.2 |
| Dectin-1 | F GCC AGG CTC CAT CTT CAC CTT G<br>R TCC AAT TAG GAA GGC AAG GCT GAG | CM000999.2 |
| Card9 | F GCT CCA AGG ACG ACT TCA TCA AGG<br>R CGC ACT GCT CAG CTC ACA CTC | CM000995.2 |

are shown in Table 1. Universal SYBR Green Fast qPCR Mix (ABclonal, China) was used to evaluate the relative expressions of Dectin-1 and Card9. All data were processed uniformly using GAPDH as the reference.

### 2.7. Western blot analysis

Macrophages were lysed in lysis buffer with RIPA (Huaxingbio Science, China), protease (Huaxingbio Science, China), and phosphatase inhibitors (Huaxingbio Science, China). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Sigma, United States). The membranes were incubated with primary antibodies overnight at 4 °C. These primary antibodies including anti-Dectin-1 (1:2000), anti-Syk (1:5000), anti-phospho-Syk (1:1000), anti-phospho-Iκ-Bα (1:10000), anti-Iκ-Bα (1:10000) were purchased from Abcam. Besides, anti-Card9 (1:1000), anti-NF-κB (P65) (1:5000) were purchased from CST, and anti-β-actin (1:3000) were purchased from ZSGB-BIO. Secondary antibody including anti-rabbit IgG HRP-conjugated antibodies (1:1000, Proteintech, China) were used to incubate with the membranes for 1 h in room temperature. Finally, the membranes were visualized with a chemiluminescence kit. Image J software for image processing was used to verify the band intensities as a result of Western blot [33].

### 2.8. ELISA assays

The secretion of pro-inflammatory and anti-inflammatory cytokines, including IL-6, IL-1β, IL-12, TNF-α, and IL-10, were investigated using cell-free supernatants. The cell-free supernatants in different groups were collected by centrifugation at 3000 rpm for 30 min at 4 °C, then they were subjected to membrane filtration (0.45 μm) and stored at −80 °C until use. ELISA kits were used to assess the concentrations of IL-6, IL-1β, IL-12, TNF-α, and IL-10 secreted by macrophages according to the manufacturer's protocols (BD Biosciences, United States).

### 2.9. Statistical analysis

Unless indicated otherwise, all data are shown as median ± standard deviation (SD) of three independent trials. Analysis of Variance (ANOVA) test followed by Tukey's test was used for statistical analysis. $P < 0.05$ was considered statistically significant (GraphPad PrismR v 8.0.1.244, United States).

## 3. Results

### 3.1. Identification results of the isolated monoclonal bacteria in the product of Medilac-Vit

The nucleotide sequences of the isolated monoclonal bacteria in the product of Medilac-Vit were shown in supporting information (Fig. S1), which showed 100% homology with the nucleotide sequences of *B. subtilis* R0179 in NCBI. Hence, we confirmed that the strain we have isolated was *B. subtilis* R0179.

### 3.2. B. subtilis R0179 regulated the immune responses in macrophages

#### 3.2.1. Immunomodulatory effects of B. subtilis R0179 on macrophages in mRNA transcription of Dectin-1 and Card9

qPCR was used to ascertain the effect of *B. subtilis* R0179 on mRNA transcription of Dectin-1 and Card9 in macrophages (Fig. 1). There were no significant changes on mRNA transcription of Dectin-1 (Fig. 1A) and Card9 (Fig. 1B) at 3 h compared with 0 h. However, macrophages challenged with *B. subtilis* R0179 down-regulated the mRNA transcription of Dectin-1 (Fig. 1A) and Card9 (Fig. 1B) at 12 h compared with 0 h or 3 h.

#### 3.2.2. Immunomodulatory effects of B. subtilis R0179 on macrophages in protein levels of Dectin-1, Card9, P-Iκ-Bα, Iκ-Bα, and NF-κB

To better explain the downstream molecular signaling pathways of Dectin-1, the Western blot analysis was performed (Fig. 2). The quantitative graphs made by Image J software are shown in supporting information (Fig. S2). *B. subtilis* R0179 did not make significant changes about the protein levels of Dectin-1 and Card9 in macrophages in 3 h compared with 0 h, which was consist with the results in qPCR. However, *B. subtilis* R0179 down-regulated the protein levels of Dectin-1, Card9, P-Iκ-Bα, and NF-κB in 12 h as compared to 0 h or 3 h. Besides, *B. subtilis* R0179 down-regulated the protein level of Iκ-Bα in 12 h as compared to 0 h (Fig. 2). At the same time, there were no significant changes about the signals of P-Syk and Syk in 3 h and 12 h compared with 0 h.

#### 3.2.3. Immunomodulatory effects of B. subtilis R0179 on macrophages in terms of cytokines level

The levels of cytokines IL-6, IL-1β, IL-12, TNF-α, and IL-10 were detected to evaluate the immunomodulatory effects of *B. subtilis* R0179 on macrophages after co-culture for 0 h, 12 h, and 24 h (Fig. 3). *B. subtilis* R0179 significantly down-regulated the levels of cytokines IL-1β (Fig. 3A), IL-12 (Fig. 3C), and IL-6 (Fig. 3E) in 12 h and 24 h, which were more significant at 12 h than 24 h. Besides, *B. subtilis* R0179 also down-regulated the level of TNF-α (Fig. 3D) but up-regulated the level of IL-10 (Fig. 3B) at 12 h and 24 h.

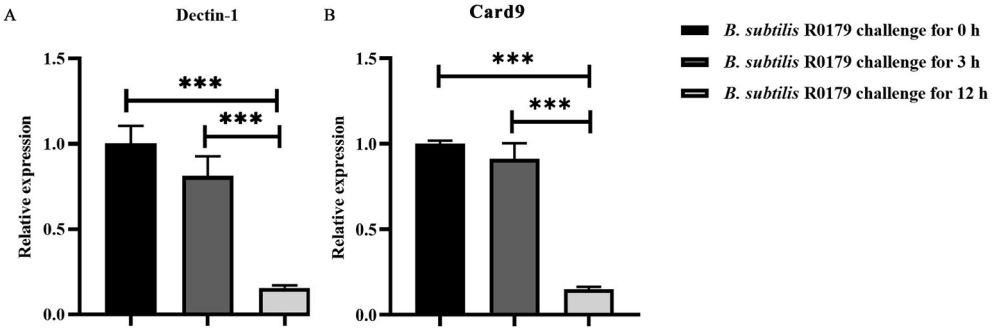


**Fig. 1.** Relative transcription of Dectin-1 (A) and Card9 (B) in macrophages challenged with *B. subtilis* R0179 for 0 h, 3 h, and 12 h was tested with qPCR. Transcription of Dectin-1 and Card9 was normalized using GAPDH as the reference gene. Macrophages challenged with *B. subtilis* R0179 for 0 h was set as 1. Data are presented as median ±SD. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$.

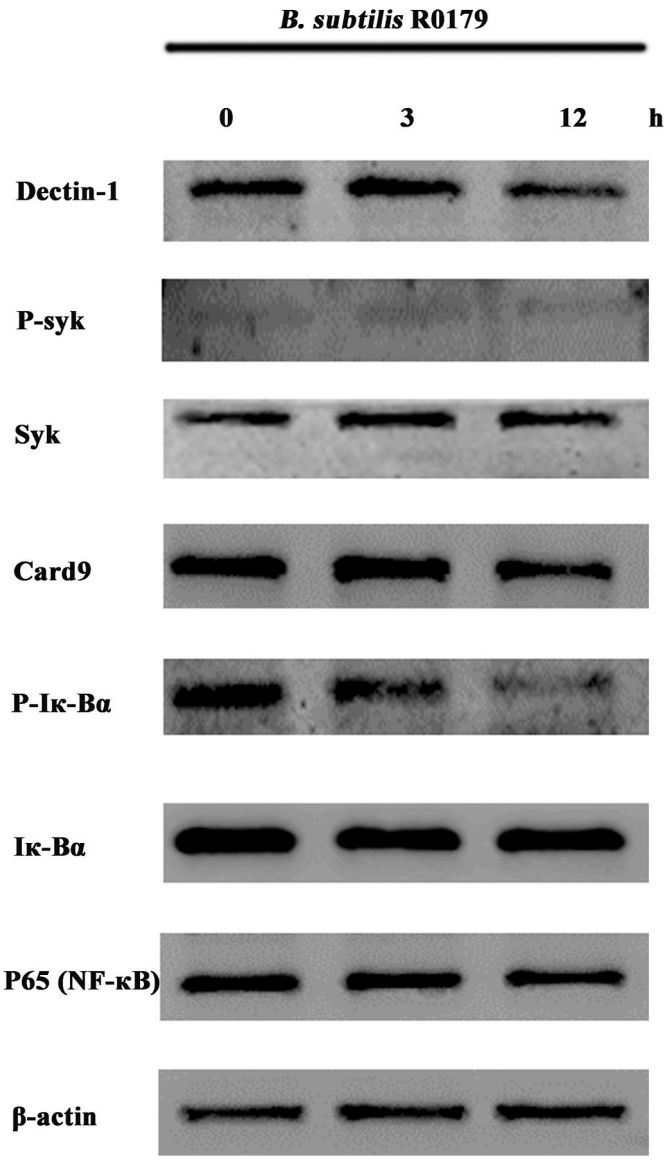


**Fig. 2.** Effects of *B. subtilis* R0179 on the protein levels of Dectin-1, P-Syk, Syk, Card9, P-Iκ-Bα, Iκ-Bα, NF-κB, and β-actin were analyzed by Western blot in 0 h, 3 h, and 12 h.

### *3.3. B. subtilis R0179 regulated the immune responses in macrophages challenged with C. albicans*

#### *3.3.1. Immunomodulatory effects of B. subtilis R0179 on macrophages challenged with C. albicans in the mRNA transcription of Dectin-1 and Card9*

In order to evaluate the effects of *B. subtilis* R0179 on macrophages challenged with *C. albicans* for 3 h, the mRNA levels of Dectin-1 and Card9 were measured by qPCR (Fig. 4). Macrophages challenged with *C. albicans* for 3 h as compared to untreated macrophages significantly up-regulated the mRNA level of Dectin-1 (Fig. 4A) and Card9 (Fig. 4B). Meanwhile, the pretreatment or co-treatment with *B. subtilis* R0179 significantly reduced the mRNA expressions of Dectin-1 (Fig. 4A) and Card9 (Fig. 4B) in macrophages challenged with *C. albicans*. Moreover, the pretreatment with *B. subtilis* R0179 showed stronger regulatory effect on the mRNA levels of Dectin-1 (Fig. 4A) and Card9 (Fig. 4B).

#### *3.3.2. Immunomodulatory effects of B. subtilis R0179 on macrophages challenged with C. albicans in the protein levels of Dectin-1, Card9, P-Iκ-Bα, Iκ-Bα, and NF-κB*

To further explore the effects of *B. subtilis* R0179 on the Dectin-1-activated signaling pathway in macrophages challenged with *C. albicans* for 30 min, 3 h, and 12 h, the signaling molecules were assessed at the protein levels by Western blot (Fig. 5). There were no significant changes about the signals of P-Syk and Syk in different groups (Fig. 5). And the quantitative graphs made by Image J software are shown in supporting information (Fig. S3).

Macrophages challenged with *C. albicans* significantly increased the protein levels of Dectin-1 and Card9 as compared to unchallenged macrophages at 30 min (Fig. 5A). In the presence of *C. albicans*, however, the pretreatment with *B. subtilis* R0179 down-regulated the protein levels of Dectin-1, Card9, P-Iκ-Bα, Iκ-Bα, and NF-κB (P65) at 30 min, which was not found in the group co-treated with *B. subtilis* R0179 (Fig. 5A).

Macrophages challenged with *C. albicans* significantly increased the protein levels of Dectin-1, Card9, P-Iκ-Bα, Iκ-Bα, and NF-κB as compared to unchallenged macrophages at 3 h (Fig. 5B). In the presence of *C. albicans*, however, the pretreatment or co-treatment with *B. subtilis* R0179 down-regulated the protein levels of Dectin-1, Card9, P-Iκ-Bα, Iκ-Bα, and NF-κB, moreover, the pretreatment with *B. subtilis* R0179 showed stronger regulatory effects (Fig. 5B).

Macrophages challenged with *C. albicans* significantly increased the protein level of NF-κB as compared to unchallenged macrophages at 12 h (Fig. 5C). In the presence of *C. albicans*, the pretreatment or co-treatment with *B. subtilis* R0179 down-regulated the protein levels of Card9, Iκ-Bα, and NF-κB, moreover, the pretreatment with *B. subtilis* R0179 showed stronger down-regulation effects (Fig. 5C). However, in the presence of *C. albicans* for 12 h (Fig. 5C), the pretreatment with *B. subtilis* R0179 significantly up-regulated the protein level of P-Iκ-Bα (addressed in the discussion).

In conclusion, these results suggested *B. subtilis* R0179 down-regulated the signals of Dectin-1, Card9, Iκ-Bα, and NF-κB in macrophages challenged with *C. albicans*, especially in the pretreatment method.

#### *3.3.3. Immunomodulatory effects of B. subtilis R0179 on macrophages challenged with C. albicans in terms of cytokines level*

In Fig. 6, after macrophages were challenged with *C. albicans* for 24 h, the levels of cytokines IL-1β, IL-12, TNF-α, IL-6, and IL-10 were shown. Compared with macrophages challenged with *C. albicans*, the macrophages pretreated or co-treated with *B. subtilis* R0179 effectively down-regulated the levels of cytokines IL-1β (Fig. 6A), IL-12 (Fig. 6C), TNF-α (Fig. 6D), and IL-6 (Fig. 6E), but up-regulated the level of cytokine IL-10 (Fig. 6B), especially in the groups of macrophages pretreated with *B. subtilis* R0179.

## 4. Discussion

*B. subtilis* R0179 was found to inhibit the growth of *C. albicans* in vitro [30]. However, the profound mechanisms are poorly understood, especially from the perspective of fungi-host immunity. Hence, we investigated the mechanism of *B. subtilis* R0179 in balancing the inflammatory responses of macrophages in the presence of *C. albicans*. RAW 264.7 cell (mouse macrophages) line can phagocytose, deliver signals, and produce cytokines, which directly reflects the role of the innate immune system and is frequently used in vitro study [34]. During infection, macrophages recognize microorganism via cytoplasmic pattern recognition receptors (PRRs), which consists of mannose receptor (MR), Toll like receptor (TLR), C-type lectins receptor, such as Dectin-1 [35].

Dectin-1 is one of the primary PRRs during the infection of *Candida* [36]. Dectin-1 can recognize β-glucan on the cell wall of *C. albicans*, thus triggering a battery of antifungal responses [37]. The activation of

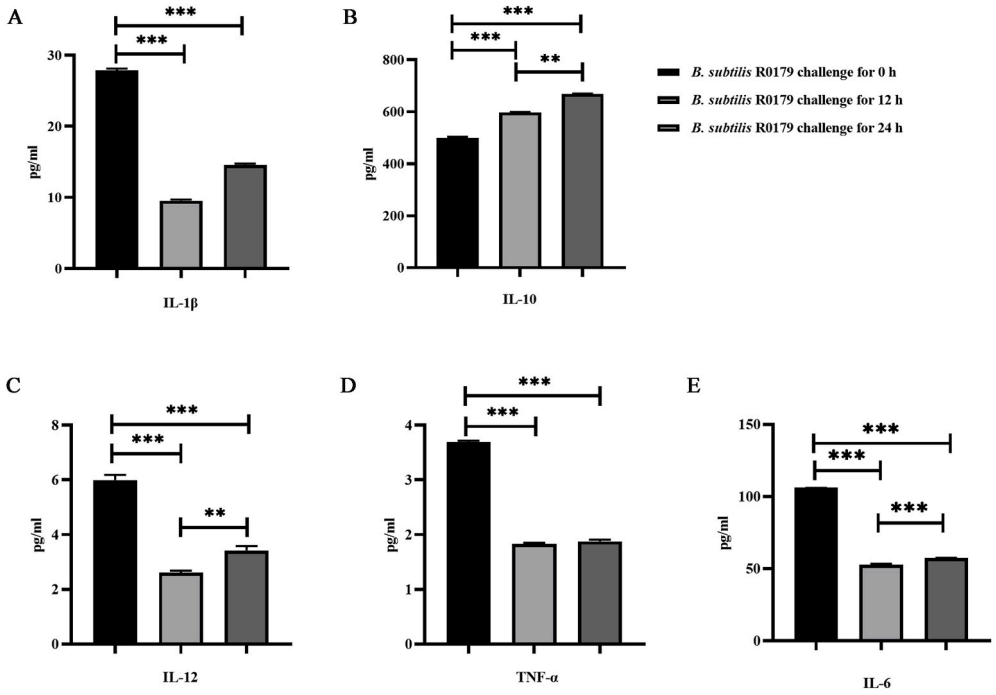


**Fig. 3.** The effects of *B. subtilis* R0179 on the productions of IL-1β (A), IL-10 (B), IL-12 (C), TNF-α (D), and IL-6 (E) were measured by ELISA at 0 h, 12 h and 24 h. Data are presented as median ±SD for three separate trials. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$.

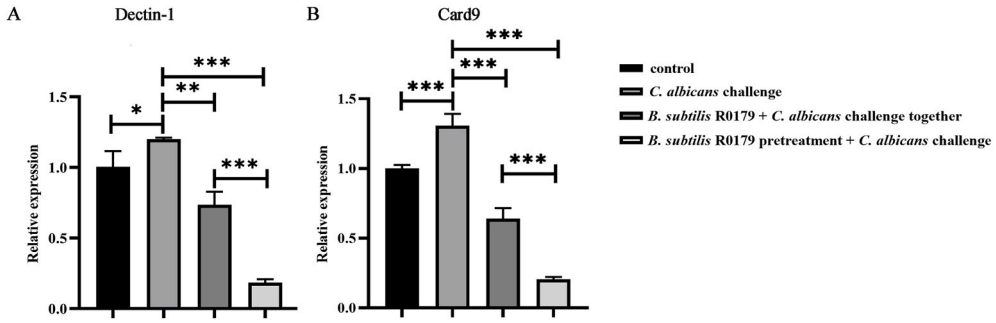


**Fig. 4.** The effects of pretreated or co-treated with *B. subtilis* R0179 on relative transcription of Dectin-1 (A) and Card9 (B) in macrophages challenged with *C. albicans* for 3 h were tested with qPCR. And they were normalized using GAPDH as the reference gene. The control group was set as 1. Data are presented as median ±SD. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$.

Dectin-1 signals recruits Syk and induces a scaffold to assemble, which is made of recruited CARD9 protein and the adaptor protein complex of Bcl-10 and MALT1 [38]. Then Iκ-Bα is phosphorylated and targeted for degradation through the ubiquitin proteasome pathway, which induces nuclear translocation of the NF-κB subunit p65-C-Rel and activation of NF-κB [39]. Consequently, macrophages produce cytokines like TNF-α, IL-1β, IL-6, IL-12, and IL-10 [40].

Our results showed that macrophages purely challenged with *B. subtilis* R0179 significantly down-regulated the mRNA levels of Dectin-1 and Card9 at 12 h, which was consistent with the changes in protein levels of Dectin-1 and Card9 by Western blot. Besides, *B. subtilis* R0179 mainly down-regulated the protein level of P-Iκ-Bα and slightly induced Iκ-Bα degradation, suggesting that *B. subtilis* R0179 may mainly alter the Iκ-Bα phosphorylation instead of its stability. Reduced phosphorylation of Iκ-Bα resulted in decreased activation of NF-κB, which led to a subsequent downregulation of IL-6, IL-12, IL-1β, and TNF-α (pro-inflammatory cytokines), but up-regulated IL-10 (anti-inflammatory cytokine). Hence, *B. subtilis* R0179 showed immunomodulatory effects through down-regulating Dectin-1-related downstream signaling pathways, reducing pro-inflammatory cytokines, and increasing anti-inflammatory cytokines, which was not reported before.

Dectin-1 on the surface of macrophages binding to β-glucans on *C. albicans* cell wall can also induce classical Syk-dependent NF-κB signaling pathways [41]. Then we further evaluated if the immunomodulatory effects of *B. subtilis* R0179 on macrophages played a role during the infection of *C. albicans*. The challenge of *C. albicans* in macrophages up-regulated the signals of Dectin-1, Card9, P-Iκ-Bα, Iκ-Bα, and NF-κB. However, *B. subtilis* R0179 provided 'reprogramming' stimulation to macrophages, and it downregulated the signals of Dectin-1, Card9, Iκ-Bα, and NF-κB, which was consistent with previous studies on the regulation of NF-kB signaling by probiotics, *Lactobacilli plantarum* 59 and *Lactobacillus rhamnosus* GR-1(R) were reported to have anti-inflammatory effects against *C. albicans* by down-regulating the NF-κB pathway [42,43]. Besides, *B. subtilis* R0179 had a stronger regulating effect in pretreatment method, which was supported by a previous study, it showed that preventive intake of *Lactobacillus rhamnosus* ATCC 7469 could prevent or reduce the progress of candidiasis in mice than the intake of it during *Candida* infection [10].

Interestingly, in the presence of *C. albicans* for 12 h, the pretreatment with *B. subtilis* R0179 significantly up-regulated the protein level of P-Iκ-

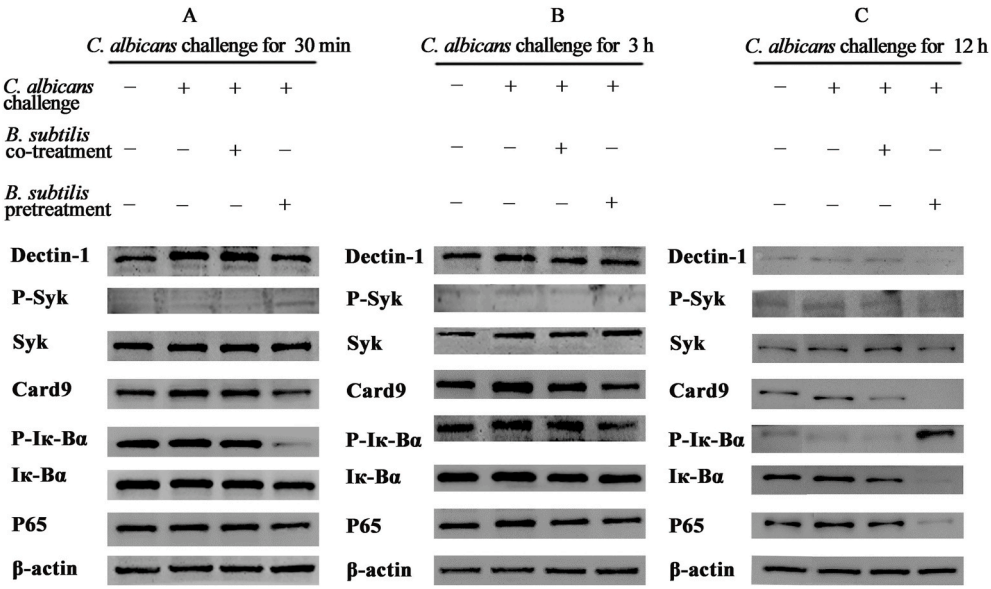


**Fig. 5.** The effects of pretreated or co-treated with *B. subtilis* R0179 on the protein levels of Dectin-1, P-Syk, Syk, Card9, P-Iκ-Bα, Iκ-Bα, NF-κB, and β-actin were analyzed by Western blot after macrophages were challenged with *C. albicans* for 30min (A), 3 h (B), and 12 h (C).

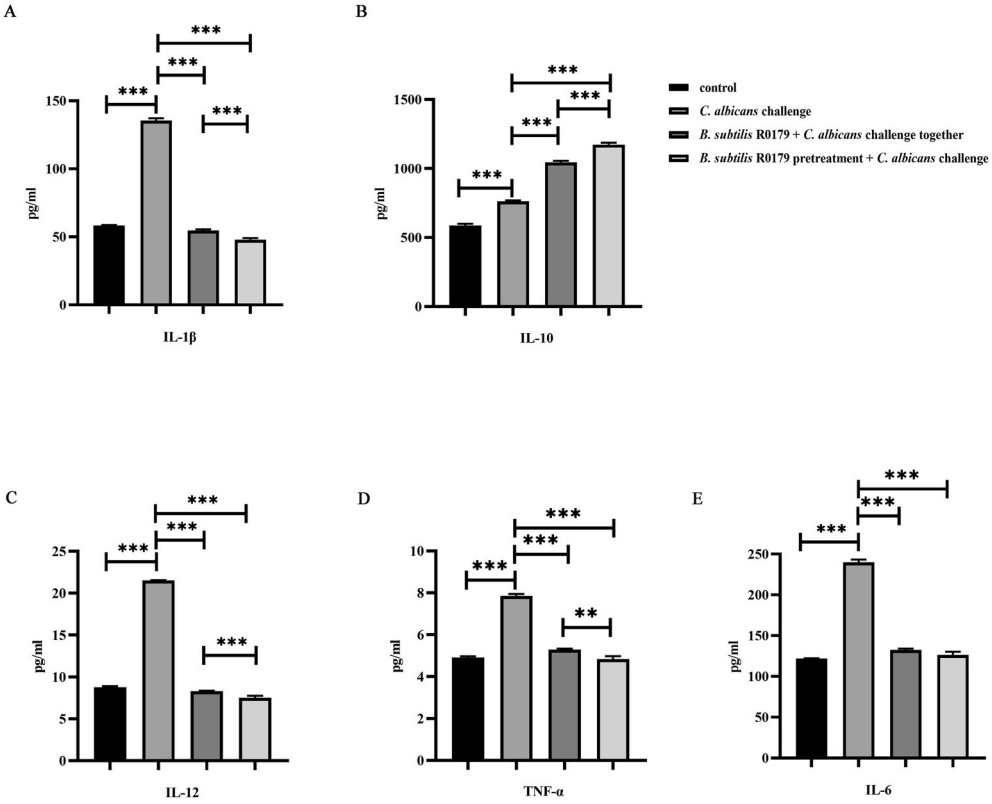


**Fig. 6.** The effects of pretreated or co-treated with *B. subtilis* R0179 on the production of IL-1β (A), IL-10 (B), IL-12 (C), TNF-α (D), and IL-6 (E) in macrophages challenged with *C. albicans* for 24 h were measured by ELISA. Data are presented as median ±SD. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$.

Bα, which was different from the trends of Dectin-1, Card9, Iκ-Bα, and NF-κB regulated by *B. subtilis* R0179. According to previous researches, the up-regulation of phosphorylation of Iκ-Bα (P-Iκ-Bα) was involved in cell starvation and apoptosis. Hence, in our experiment, the possible reasons for the different trend of P-Iκ-Bα were cell starvation and apoptosis [44–47], which were caused by the absence of serum in the medium and persistent infection by *B. subtilis* R0179 and *C. albicans*. Further study needs to be performed to explore the underneath mechanism of this phenomenon.

Furthermore, after the activation of NF-κB signal, *C. albicans* increased the levels of cytokines IL-6, TNF-α, IL-12, TNF-α, and IL-10 in macrophages. It was reported that macrophages balanced polarization to control the status of inflammation [48]. When the infection of *C. albicans* was severe enough, macrophages first released TNF-α, IL-1β, IL-12, and IL-6 against the infection, but, if this state continued, it may lead to tissue damage. Therefore, macrophages would secrete high

amounts of IL-10 to suppress the inflammation and keep homeostasis [49]. In our study, upregulation of TNF-α, IL-1β, IL-12, IL-6, and IL-10 after challenge with *C. albicans* suggested the macrophages was activated as a defense against the infection [50].

However, in the presence of *B. subtilis* R0179, the cytokines induced by *C. albicans* infection in macrophages were altered, which showed that IL-10 (anti-inflammatory cytokine) was up-regulated, but TNF-α, IL-1β, IL-12 and IL-6 (pro-inflammatory cytokines) were down-regulated. Because IL-10 initiated the formation of Th2-associated protective immune responses, and Th2-associated cytokines inhibited IL-12 synthesis, which led to the onset of Th2 cells rather than Th1 cells [51,52]. Hence *B. subtilis* R0179 may balance the cellular responses of Th1 and Th2 and maintained immune homeostasis [53]. In summary, this study indicates that *B. subtilis* R0179 regulates cellular immunity in macrophages through dectin-1 pathway, which may prevent tissue damage caused by excessive pro-inflammatory response during the infection of *C. albicans*.

Despite these findings, the study had its own limitations. For instance, Dectin-1 is a type of PRR of macrophages, whether the immune recognition mechanism of *B. subtilis* R0179 involves any other PRRs, such as TLR or MR, is not revealed, and needs to be further investigated. In addition, the immunomodulatory mechanism of *B. subtilis* R0179 needs to be confirmed in animal models, and other kinds of probiotics on *Candida*-host interactions also need to be uncovered. Hence, further studies are necessary to clarify these hypotheses.

## 5. Conclusion

*B. subtilis* R0179 may have immunomodulatory effects on macrophages by down-regulating the signals of Dectin-1, Card9, Iκ-Bα, and NF-κB, decreasing the levels of cytokines IL-12, IL-6, IL-1β, and TNF-α, but increasing the level of cytokine IL-10, which may prevent tissue damage caused by excessive pro-inflammatory response during the infection of *C. albicans*.

## Author agreement

We certify that all authors have seen and approved the final version of the manuscript being submitted. We warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

## Declaration of competing interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2021.104988.

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