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Major Article

β-Glucan of *Candida albicans* Cell Wall Extract Inhibits *Salmonella* Typhimurium Colonization by Potentiating Cellular Immunity (CD8⁺ and CD4⁺ T Cells)

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Abstract

Introduction: Antimicrobial resistance has been reported in the drugs used for the treatment of typhoid fever. The immunomodulatory substance β-glucan can be used as an alternative therapy as it potentiates host immunity. The aims of this study are to observe the effect of *Candida albicans* cell wall (CCW) extract towards host immunity (TCD8⁺ and TCD4⁺ cells in spleen, intestinal sIgA) and its capacity to kill *Salmonella* in the intestine and liver of typhoid fever mice models. **Methods:** Typhoid fever mice models were created by infecting mice with *S.* Typhimurium orally. Mice were divided into four groups: the Non-Infected, Infected, CCW (infected mice treated with 300 µg CCW extract/mouse once a day), and Ciprofloxacin groups (infected mice treated with 15 mg/kg BW ciprofloxacin twice a day). **Results:** Secretory IgA (sIgA) concentrations of mice in the CCW group remained unchanged. However, their TCD4⁺ and TCD8⁺ cells increased substantially compared to those in the Non-Infected group. In the Ciprofloxacin group, sIgA concentrations increased markedly compared to those in the Non-Infected and CCW groups; TCD4+ and TCD8+ cells also increased significantly compared to those in the Infected Group, but not significant compared to those in the CCW group. Colonization of *S.* Typhimurium in the intestine and liver decreased significantly in the CCW and Ciprofloxacin groups compared to that in the Infected group, with the lowest reduction being found in the Ciprofloxacin group. **Conclusions**: The inhibition of *S.* Typhimurium colonization by CCW is associated with the increase in TCD4⁺ and TCD8⁺ cells.

Keywords: β-glucan. *Candida albicans* cell wall extract. CD4⁺. CD8⁺. *Salmonella.* sIgA.

INTRODUCTION

Typhoid fever, which is caused by *Salmonella* Typhi, is still considered a global health burden. Approximately 21 million cases occur worldwide each year, causing 200 thousand deaths;[1] almost 80% of these cases are from Asia.[2] Antimicrobial medication for typhoid fever kills *S*. Typhi even if the bacteria survive inside phagocytes;[3] however, *S*. Typhi has developed resistance to antimicrobials.[4] Because of this phenomenon, many countries like Indonesia and China have faced Multidrug-Resistant Typhoid Fever (MDRTF) for the last two decades.[2] On the other hand, drug development takes 12 to 15 years to complete and costs around 802 million USD.[5] This situation has encouraged scientists to discover new therapies other than conventional antimicrobials to circumvent the development of resistance by these bacteria.

There are alternative approaches to overcome antimicrobial resistance and one of them is immunostimulation, which can indirectly kill pathogens by enhancing the host immune system. β-glucan is an example of a potent immunostimulant.[6] It is contained in the cell wall of yeasts such as *Candida albicans*, along with other components.[7] *C. albicans* cell wall (CCW) can be easily collected in a microbiology laboratory as residue of laboratory specimens.[8]

Salmonella Typhimurium infects hosts orally. When it reaches the intestine, *S.* Typhimurium invades M cells and quickly penetrates the Peyer's patch.[9] Afterwards,

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S. Typhimurium may survive within both macrophages and non-macrophage cells and disseminates to various organs, including the liver and spleen. *S.* Typhimurium is classified as an intracellular pathogen that is eliminated mainly by cellular immunity composed of macrophages, $CD4^+$ T cells, and $CD8^+$ T cells.[10,11,12] Secretory IgA (sIgA) composes 80% of total mucosal antibodies and is located at the intestinal mucosa to encounter pathogens entering through the oral route.[13]

This study used *S.* Typhimurium, which has been broadly utilized in research models of typhoid fever in mice due to the similarity in its pathogenesis with that of human typhoid fever.[14,15] The aim of this research is to examine the effect of CCW extract in modulating the host's immune response (intestinal sIgA, $CD4^+$ T cell, and $CD8^+$ T cell) against *Salmonella* infection in relation to its colonization pattern in the intestine and liver.

METHODS

Animal

Twenty four male BALB/c mice aged 6–8 weeks were divided randomly into four groups: Non-Infected group (non-infected mice that were only given standard animal nursing care), Infected group (untreated infected mice), CCW group (infected mice treated with 300 µg *C. albicans* cell wall extract/mouse once a day), and Ciprofloxacin group (infected mice treated with 15 mg/kg BW ciprofloxacin twice a day). Ciprofloxacin therapeutic regimen in mice was based on standard therapy of laboratory animal. Mice were orally infected with 300 µL of *S.* Typhimurium at a concentration of 10^8 cells/mL orally on days 1 and 3. Mice in the Ciprofloxacin and CCW groups were treated from days 5 to 9. Mice were sacrificed at day 10. The experiment was conducted according to the regulation of animal laboratory care of Universitas Brawijaya Indonesia as stated in the Ethical Clearance of this study (No. 427/EC/KEPK/08/2015 03 Aug 2015).

C. albicans Culture and Identification

C. albicans used in this study was isolated from a clinical vaginal specimen. It was cultured in Sabouraud Dextrose Agar (SDA) and incubated at 37°C for 18–24 h. Gram staining and observation under a microscope at 1000× magnification, and germ tube test were performed to identify *C. albicans*.

The *C. albicans* culture obtained was soft, beige, and had the typical odor of yeast. To ensure that *C. albicans* was correctly cultured, microscopic analysis was performed by observing under a microscope at 1000× magnification; the presence of budding cells were noted. Gram staining and germ tube test of the *C. albicans* culture were also performed and observed under 1000× magnification. It was found to be gram positive and pseudohyphae were also observed.

C. albicans Cell Wall Extraction

C. albicans culture was harvested and collected by centrifugation at 3000×*g* with phosphate-buffered saline (PBS), pH 7.4. The cell-pellet was collected and washed with lysis buffer (10 mM Tris-HCl combined with 1 mM phenylmethylsulfonyl fluoride [PMSF]) five times, and then centrifuged at 3000×*g* for 10 min. The sediment was again collected and suspended with lysis buffer at 4°C, and then mechanically lysed with 1-mm glass beads using an Omni Mixer Homogenizer at 6000×*g* for 10 min. The lysed cells were separated subsequently by centrifugation at 3000×*g* for 10 min. The precipitate obtained was the cell wall fraction.

β-glucan Isolation

The cell wall fraction of *C. albicans* was washed once with distilled water at 4°C, 5 times with 1 mM PMSF containing 5% NaCL, 2% NaCl, and 1% NaCl, respectively, then centrifuged at 4°C at 6000×*g* for 10 min.[8] Then, 1 M NaOH (1:1) was added to the sediment, and the suspension was incubated at 90°C for 2 h and centrifuged thereafter. H_3PO_4 was added to the sediment, mixed using a vortex mixer and incubated at room temperature for 2 h before being centrifuged and washed with distilled water 3 times. Afterwards, 96% ethanol was added to the sediment and it was homogenized using a vortex mixer. The mixture was strained with paper strainer, and β-glucan was obtained as a solid matter on the filter.[16]

β-glucan Identification

Fourier Transformed Infrared Spectroscopy (FTIR; Shimadzu FTIR-8400) was used to analyze the β-glucan extracted from the *C. albicans* cell wall. KBr was mixed with the extract and then it was ground until it appeared fine and homogenous. This mixture was then pressed using a hand press until it was thin enough to be measured. The same procedure was also applied to the β-glucan standard. Baseline was determined by measuring KBr only. The measurement was done by inserting the mixture into the sample holder of the FTIR to obtain the spectra.[17] The spectra of β-glucan in CCW and the β-glucan standard were compared.

S. Typhimurium Identification

This study used an *S.* Typhimurium reference strain obtained from the National Collection of Type Cultures with the catalogue number NTC12023. *S.* Typhimurium was cultured in Bismuth Sulfite Agar (BSA) medium and incubated at 37°C overnight. Identification of *S.* Typhimurium was performed using Gram staining and Microbact System.

Ciprofloxacin Sensitivity Test in Vitro

Ciprofloxacin was used as a control to compare the effect of β-glucan on typhoid fever. The Kirby-Bauer disk diffusion method was used to test the sensitivity of *S.* Typhimurium toward ciprofloxacin. Based on the inhibition zone obtained (32 mm), the *Salmonella* strain used in this study was sensitive.

Bacteria Culture from Intestine and Liver

Mouse intestine was longitudinally cut until its lumen was exposed. Mucus from the lumen was collected (for sIgA measurement) and stored in 1 mM PMSF containing PBS and anti-protease to prevent antibody degradation. The remaining intestine and liver were ground and diluted in 0.9% NaCl solution. Each suspension of intestine and liver were divided into

three dilution groups (10^{-1}, 10^{-2}, and 10^{-3}). Bacterial culture was performed by the pour plate method using BSA medium. One mL of each specimen in every dilution concentration was mixed with 9 mL of liquid BSA. Bacterial cultures was incubated at 37°C for 48 h. Colonies were counted with a colony counter.

sIgA Measurement

Intestinal mucus sample in PBS was centrifuged at 1000×*g* for 5 min. The supernatant was collected and centrifuged at 7000×*g* for 5 min. Supernatant was collected and its sIgA was measured by indirect ELISA (Biolegend, USA). Whole cells of *S.* Typhimurium to be used as antigens were obtained by sonication (SONICA ETH series) at 20 kHz, 10 times, 3 min each. This was used to coat the wells overnight before ELISA was performed.

CD4$^+$ T Cell and CD8$^+$ T Cell Measurement

CD4$^+$ and CD8$^+$ T lymphocytes were measured using flow cytometry.[18] Spleen was ground and diluted in 2 mL PBS. The homogenate was transferred to a 1.5-mL microtube and then centrifuged at 1500×*g* at 4°C for 5 min. The sediment was washed twice with PBS (pH 7.2). Then, 50 µL of staining solution (FITC anti-mouse CD4 and PE anti-mouse CD8; 1:100 dilution) and 350 µL of buffer were added and mixed into 50 µL of suspension. The mixture was incubated in a dark room at room temperature for 30 min and analyzed using the flow cytometer (BD FACS Calibur).

Data Analysis

Statistical analysis was performed using IBM SPSS Statistics, version 21.0 with 95% confidence interval ($\alpha = 0.05$). Homogeneity of variances was tested using the Levene statistic, and normality was tested using the Shapiro-Wilk normality test. In the case of homogenous equal variance data, one-way ANOVA was performed. In the case of unequal variances or non-homogenous data, the Kruskal-Wallis test was performed.

RESULTS

β-glucan Identification

Two similar spectra were obtained for both substances. The specific characteristics of β-glucan include peak transmittance patterns at 1160, 1078, 1044, and 890 cm^{-1} wavelength bands.[19] CCW extraction was able to produce a β-glucan yield of around 90%.

S. Typhimurium Colonization in Intestine and Liver

Intestinal specimen was obtained from the mucus scraped from the gut. Hence, only bacteria that survived the sIgA response was cultured. There were significant differences between the intestinal *S.* Typhimurium colony numbers among groups ($p = 0.000$). The mean intestinal colony number of *S.* Typhimurium in the CCW group was substantially lower than that in the Infected group ($p = 0.000$), but it was higher than that in the Ciprofloxacin group ($p = 0.000$) (see **Figure 1A**).

In the liver, the colony count of the CCW group was lower than that of the Infected group, but it was not statistically significant ($p = 0.631$); however, it was substantially higher ($p = 0.006$) than that of the Ciprofloxacin group, which had the lowest colony count ($p = 0.001$ compared to Infected group) as shown in **Figure 1B**.

Splenic CD4$^+$ T Cell and CD8$^+$ T Cell Count

Figures 1C–1E depict CD4$^+$ T cell and CD8$^+$ T cell counts obtained by flow cytometry. There was a substantial difference in the number of CD4$^+$ T cells among groups ($p = 0.001$). Administration of ciprofloxacin resulted in a higher number of CD4$^+$ T cells than those in the Non-infected ($p = 0.001$) and Infected groups ($p = 0.038$). The CCW group also had a higher number of CD4$^+$ T cells than the Non-infected group ($p = 0.011$).

The CD8$^+$ T cell counts of the Ciprofloxacin and CCW groups were higher than those of the Non-infected ($p = 0.000$) and Infected groups ($p = 0.000$). There was no significant difference between the CD8$^+$ T cell counts of the Non-infected and Infected groups ($p = 0.170$). There was also no significant difference between the CD8$^+$ T cell counts of the CCW and Ciprofloxacin groups ($p = 0.971$).

sIgA Concentration

There was substantial difference in the sIgA concentrations among groups ($p = 0.017$). The sIgA concentration of the Ciprofloxacin group was higher than those of the Non-infected ($p = 0.036$) and CCW groups ($p = 0.006$), but the sIgA concentration of the CCW group was lower than that of the Infected group ($p = 0,036$) (see **Figure 1F**).

DISCUSSION

Typhoid fever antimicrobial resistance has encouraged scientists to develop alternative therapies to conventional antimicrobials. β-glucan extracted from the cell wall of *C. albicans* was studied in this research as a potential alternative. According to FTIR analysis, β-glucan composed about 90% of CCW. β-glucan is a substance recognized by dectin-1 receptor, complement 3 receptor, and Toll-like receptor (TLR), which are present on immune cells including monocytes, macrophages, dendritic cells, neutrophils, eosinophils, and natural killer cells; it is capable of inducing both innate and adaptive immunity.[20,21]

Our study found that oral administration of CCW reduced *Salmonella* colonization substantially in the intestine and slightly in the liver. Although the reduction in colonization was not as good as that by ciprofloxacin, the inhibition of colonization in intestine by CCW denoted its potency to interfere with the growth of *Salmonella*.[22] The potency of β-glucan to inhibit the colonization of intracellular pathogens (*Mycobacterium bovis*) has been previously demonstrated as it stimulates respiratory burst and cytokine production in macrophages, as well as the secretion of antimicrobial substances that inhibit the growth of *M. bovis*, but not *M. tuberculosis*.[23]

Significant increments in the splenic and CD4$^+$ T cell counts of the CCW and Ciprofloxacin groups were followed by the raise in CD8$^+$ T cell counts of both groups. Intracellular pathogens are eliminated mainly through CD8$^+$ T cell-mediated immune response, in which the macrophage used as a reservoir of the bacteria is lysed to enable pathogen elimination.[24] A study

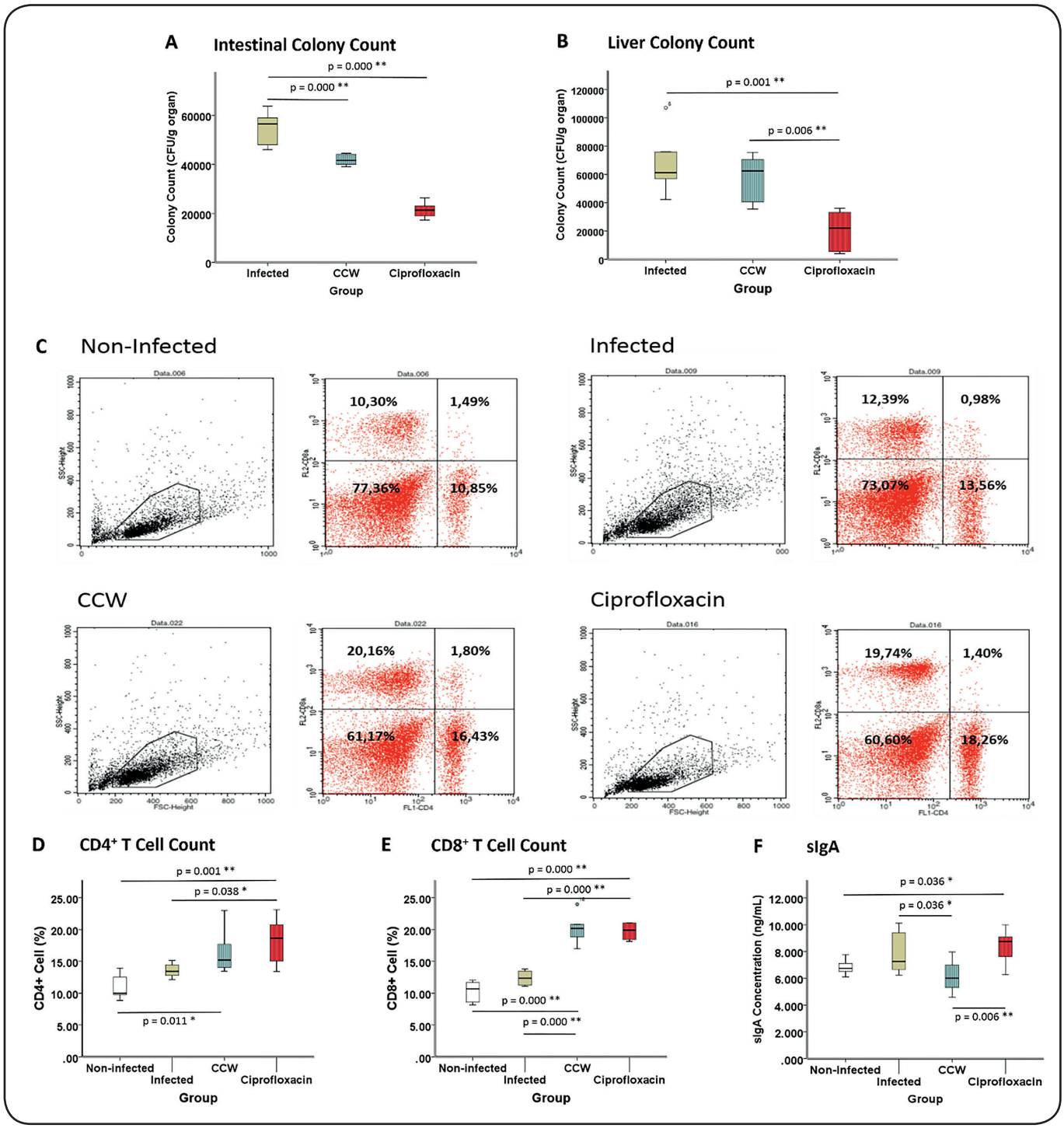

FIGURE 1: CCW Administration Enhances Cellular Immunity in Treating *Salmonella*. (A) Colony count of *Salmonella* in the intestine of Infected, CCW, and Ciprofloxacin groups. (B) *Salmonella* colony count in the liver of Infected, CCW, and Ciprofloxacin groups. (C) Flow cytometry profile of spleen cells. Gating was performed in lymphocyte area. Upper left quadrant: $CD4^- CD8^+$; upper right quadrant: $CD4^+ CD8^+$; lower left quadrant: $CD4^- CD8^-$; lower right quadrant: $CD4^+ CD8^-$. (D) $CD4^+$ splenic T cells of Non-Infected, Infected, CCW, and Ciprofloxacin groups based on $CD4^+ CD8^-$ percentage. (E) $CD8^+$ splenic T cell count from $CD4^- CD8^+$ percentage. (F) sIgA concentration from mice gut mucus. *$p < 0.05$; **$p < 0.005$.

reported that β-glucan was able to enhance $CD8^+$ and $CD4^+$ T cells by promoting dendritic cell maturation in Hepatitis B DNA vaccination via Th1.[25] Interestingly, sIgA, the main antibody of mucosal organs, declined with CCW treatment, indicating that the Th1 response was potentiated in this study. Th1 and Th2 antagonize each other, while sIgA antibody synthesis is mediated mainly by Th2 cytokines.[26] This study observed that an increase in $CD8^+$ T cell was accompanied by a decline in sIgA concentration, indicating that the cellular immunity pathway was potentiated via Th1.

This study found a notable result wherein the immune response ($CD4^+$ T cell, $CD8^+$ T cell, and sIgA) was also modulated

by ciprofloxacin. To this end, several studies have demonstrated the immunomodulatory activity of fluoroquinolones, including ciprofloxacin, even though the underlying mechanism remains unclear and needs to be elucidated further. A study by Bode *et al.* (2014) using moxifloxacin showed a decrease in inflammatory cytokines (IL-1β, IL-6) modulated by TLR signaling in immune cells.[27] Another study also found that moxifloxacin and ciprofloxacin were able to reduce interferon-gamma and IL-4 in helper T cells with an unchanged Th1/Th2 ratio.[28] Another study reported a contrasting result wherein ciprofloxacin in human peripheral blood lymphocytes caused an increase in T cell function and macrophage-T cell interactions.[29]

In summary, our study indicates that CCW treatment reduced *Salmonella* colonization mainly in the intestine, likely through the potentiation of cellular immunity by $CD8^+$ T cells via Th1 pathway, as the decline in sIgA was also observed. The colonization inhibition ability of ciprofloxacin was superior to that of CCW, and this may be due to the synergistic dual role of ciprofloxacin (anti-microbial and immunomodulatory) that was observed in this study. These findings indicated that the immunomodulatory activity of CCW may contribute towards eliminating *Salmonella* during infection.

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Conflict of Interest: The authors state that there was no conflict of interest to declare.

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