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Modulation of Immune Response from FiberCreme-VCO Based Supplementation in Immunosuppressed Rats

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Immunomodulators are substances that have the ability to influence the human immune system. FiberCreme is a commercial non-dairy food high in oligosaccharides, which are difficult to digest. Combination of FiberCreme and VCO (FC-VCO) considered to possess high value of bioavailability in body. This study was aimed to determine immunomodulation effect of FiberCreme-VCO against immunosuppressed rats. This study used male rat (Rattus novergicus) which was induced by doxorubicin twice a week for seven days. Treatment by FC-VCO was then administered orally for 14 days. Whole blood and spleen were collected and analyzed based on immunomodulator parameters such as spleen weight, body weight, IL-6, TNF-α, and INF-? levels, the CD4+/CD8+ ratio and the percentage of FOXP3. Statistical analysis was determined by GraphPad Prism software (version 9: San Diego, CA, USA). Regardless of dosage, FC-VCO did not improve body weight or lymphatic weight appreciably. In contrast to the negative group, FC-VCO supplementation at a dose of 6 mg/kgBW was able to raise CD4+ levels, and this difference was statistically significant ($p<0.05$). IFN-? levels were also increased by FC-VCO at a dose of 9 mg/kgBW, and these differences were statistically significant ($p <0.05$) when compared to the negative group. Since FC-VCO affects the roles and responsibilities of CD4+ and IFN-? in immunosuppressive situations, it can strengthen the immune system.

Keywords: Doxorubicin; FiberCreme; Healthy Life; Immunomodulator; Virgin Coconut Oil.

The global frequency of viral infections has increased during the previous decade. This infection has become a serious concern in many countries which cause catastrophe in health, social, economic, and financial sectors[1]. In 2021, there have been 100,455,529 confirmed cases worldwide,

with 2,166,440 deaths recorded[2]. Viral infection can result in an immunological response in the host known as cytokine storm with overproduction of cytokines. This immune response promotes inflammation and fluid accumulation in the lungs which can make respiratory disorder[3]. Various drug discovery is also being pursued, including the development of therapeutic options such as reuse of existing drugs or combination between drugs and supplements to prevent inflammation[4]. Viral infection is a self-limiting disease, yet immunomodulation activity in body remains crucial for well-being[5]. Immunomodulatory dose regimens for viral infections are a preventive strategy which will lower resistance against viral infection affecting human immune system[6].

Previous research has shown that Doxorubicine (Dox) has harmful effects on the rat hematopoietic system, resulting in a decrease in the number of granulocytes, lymphocytes, and monocytes as well as red blood cells (RBCs), white blood cells (WBCs), and WBCs. According to reports, DOX inhibited IL-10 downregulation, lymphocyte proliferation, macrophage capability, phagocytosis activity, and CD8+ cytotoxic T cell production in rats. In spleenocytes from tumor-bearing mice, Dox lowered the production of IL-2 and INF-, as well as lymphocyte proliferation, the CD4+/CD8+ ratio, and NK cell cytotoxicity[7]. It also affects the cellular components that trigger immunological responses, leading to immunosuppression with a higher risk of microbial infection and a slower rate of wound healing. Growing interest is being given to the immunomodulatory properties of plants with a broad spectrum of therapeutic properties to develop potential immune-enhancing agents which can be used as components of functional foods, as plant-based therapeutic agents are linked to relatively low toxicities. Silalahi et al. (2018) demonstrated that giving mice VCO once a day for 7 days with a dose of DOX 4.67 mg/kg body weight on day 1 and day 4 improved the decrease in TCD4+ and TCD8+ (20.18% and 16.00%) caused by Dox administration. Dox decreases lymphocyte proliferation, suppresses phagocytosis macrophage activity and ability, TCD4+ suppression, and TCD8+ and IL-10 downregulation in Dox-treated animals[8].

FiberCreme is a commercial non-dairy food high in oligosaccharides which are difficult to digest[9]. Prebiotics are nondigestible oligosaccharides (e.g., inulin, oligofructose, isomaltose, raffinose, palatinose, and lactose) which can encourage the growth of probiotic bacteria such as *Lactobacilli* and *Bifidobacteria*. Prebiotics affect local immune system in the gut and systemic immune system. Moreover, prebiotics help maintain intestinal permeability and regulate inflammation[10]. Dietary oligosaccharides can impact the immune system directly by binding to particular sugar receptors on human cells and alter systemic immunological responses[11]. The elimination of pro-inflammatory cytokines by anti-inflammatory drugs is one of intervention mechanisms[12]. An innovation was created in this study by replacing vegetable oil in FiberCreme with virgin coconut oil (VCO). Virgin coconut oil is vegetable oil derived from kernel juice of fresh and ripe coconuts (*Cocos nucifera* L.) and has been widely utilized as food component in food additive[13]. Moreover, VCO has high concentration of bioactive substances such as tocopherols, sterols, and polyphenols[14]. Virgin coconut oil has ability to disintegrate viral envelope, hinder final maturation stage of viral replication, and prevent viral proteins from attaching to the host cell membrane. Furthermore, VCO could decrease C-reactive protein levels to recover from COVID-19[4].

Numerous studies showed immunomodulation potency from VCO, but still lack of information about combination with FiberCreme. This study aimed to determine the effect of FiberCreme and VCO on immunomodulatory effect in immunosuppressed rat.

MATERIALS AND METHODS

Materials

The key ingredient in this study was FC-VCO which was purchased from PT. Lautan Natural Crimerindo (LNK), Mojokerto, Indonesia. Doxorubicin as immunosuppressive drug and Stimuno® (PT. Dexa Medica) as immunomodulator were obtained from Faculty of Pharmacy, Universitas Surabaya, Surabaya, Indonesia. Characterization of FC-VCO showed that it contains protein, fat and ash components (Table 1).

Animals

This study used 36 male Wistar rats (*Rattus norvegicus*), 10 weeks old and weighing 180-200 g. They were obtained from Integrated Research and Testing Laboratory (LPPT) in Universitas Gajah Mada, Yogyakarta, Indonesia. The animal experimental procedure was authorized by Universitas Surabaya with ethics committee (212/KE/XI/2021). Acclimatization was done for 15 days. Rats were housed in 20 x 30 x 40 cages, three rats of each with free access to food and water *ad libitum*. Room temperature and humidity were set at 22-24°C and 65-70%, respectively.

Experimental Design

The animals were separated into six groups after acclimatization.

Normal Groups : This group used 6 rats that were not stimulated by doxorubicin or FC-VCO and were just given vehicle treatment.

Negative Groups : These groups used 6 mice that were given doxorubicin 4.67 mg/kg BW intraperitoneally on day 1 and 4 and then treated with a vehicle.

Positive Groups : This groups used six mice that were administered doxorubicin 4.67 mg/kg BW intraperitoneally on day 1 and 4 and Stimuno® orally 0.005 g/kg BW every day for 14 days.

FC-VCO Groups : This groups used 18 mice that were given doxorubicin 4.67 mg/kg BW intraperitoneally on day 1 and 4, then divided into three groups. Three dose levels of FC-VCO were administered to each group: 3 kg/mg BW, 6 mg/kg BW, and 9 mg/kg BW. For 14 days, all FC-VCO treatments were initiated orally each day.

Sample Collection

The measurement of body weight was done at 12 hours after the last administration of treatment. Afterwards, blood sample was collected from intracardiac under anesthetic conditions with chloroform and the spleen was removed immediately for flow cytometry analysis. The mice were killed, and the spleen was weighed to calculate spleen index (spleen weight (g)/body weight (g)). Blood serum was collected at 4°C for 15 minutes at 3000 RPM.

Analysis of Pro-inflammatory Cytokines

Serum IL-6, TNF-α and INF-ã levels were measured by enzyme-linked immunosorbent assay (ELISA) methods according to protocol standard kit (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China).

Analysis of $CD4^+$ and $CD8^+$ levels

The spleen was extracted, cleaned twice with PBS, and put in a petri dish containing 5 mL of PBS to assess the ratio of $CD4^+$ and $CD8^+$ T cells ($CD4^+$/CD8+). The spleen was washed, crushed, and filtered using a Millipore filter before being placed in a propylene tube and centrifuged at 2500 RPM for 5 min at 4°C to extract the pellets. Afterwards, 1 mL of PBS was added into the pellets and then homogenized by pipetting. 100 ìL was removed and placed in fresh microtube, followed by the addition of 500 ìL of PBS. The mixture was then centrifuged for 5 minutes at 4°C at 2500 RPM. Subsequently, 50 ìL of extracellular antibodies ($CD4^+$ and $CD8^+$) were added. Furthermore, samples were placed in flowcytometry to analyze the percentage of $CD4^+$ and $CD8^+$ cells.

Statistical Analysis

All of data were analyzed with GraphPad Prism software (version 9: San Diego, CA, USA). Shapiro-Wilk test was used to determine normality test. One-way ANOVA test was also used to analyze significancy from FC-VCO treatment. Each group was determined for significancy against normal group and negative group by Tukey test. Significancy value was displayed when $p<0.05$.

RESULTS

Effects of FiberCreme-VCO on body weight and spleen weight

The body weight and spleen weight did not show significant difference between negative group and FC-VCO group (Table 2). FiberCreme-VCO did not increase body weight after being induced by doxorubicin, whereas Stimuno® did lead to body weight gain. In addition, the decrease in spleen weight in the negative group compared to the normal group showed improvement in FC-VCO treatment, although the increase in spleen weight in the FC-VCO group was not significantly different.

Effects of FiberCreme-VCO on $CD4^+$ and $CD8^+$ percentage

The $CD4^+$ percentage significantly decreased after doxorubicin induction compared to normal group. Administration of Stimuno® and FC-VCO aimed to stimulate $CD4^+$ percentage

(Figure 1). The findings of this study revealed that the CD4+ percentage in the Stimuno® therapy group was higher than in the control group, with a significant difference ($p<0.05$). FC-VCO at 6 mg/kg BW had a higher percentage CD4+ than the control group. However, as compared to the negative group, FC-VCO at 3 mg/kg BW and 9 mg/kg BW did not demonstrate a significantly different increase in percentage CD4+ ($p>0.05$). The $CD8^+$ percentage from spleen was also measured in this study. Negative group showed elevated percentage than normal group but not significantly different ($p>0.05$). Furthermore, Stimuno® and FC-VCO therapy also not significantly ($p>0.05$) increasing $CD8^+$ percentage than negative group. The ratio between $CD4^+$ and $CD8^+$ percentage were checked to understand the correlation with the treatment

Table 1. Characterization analysis in FiberCreme-VCO

Characteristic	Content (%)
Protein	2.3 – 2.4
Fat	31 – 37
Ash	2.7 – 2.9

Table 2. Observation of body weight and spleen weight in rats after FiberCreme-VCO induction

Group	Body Weight (g)	Spleen Weight (g)
Normal	210.67 ± 13.61	1.08 ± 0.15
Negative	172 ± 29.51	1.03 ± 0.23
Positive	175.33 ± 25.03	0.78 ± 0.26
FC-VCO3	176 ± 34.22	2.01 ± 1.25
FC-VCO6	168 ± 25.87	1.19 ± 0.07
FC-VCO9	173 ± 31.88	1.47 ± 0.25

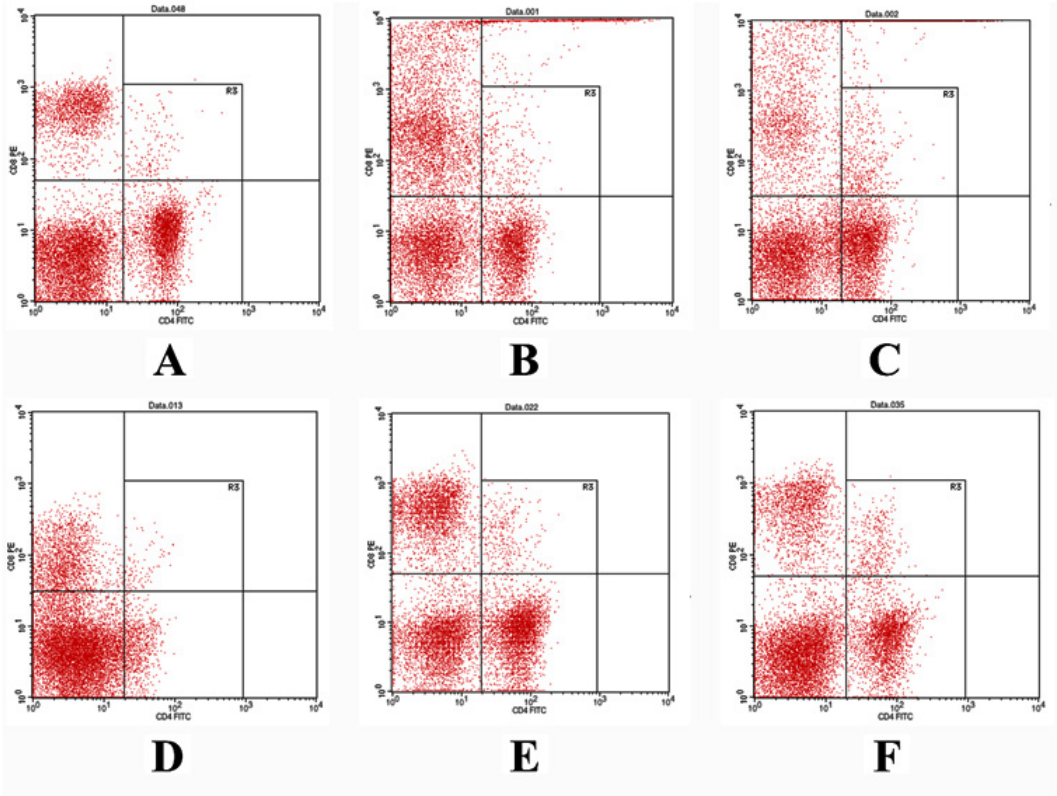

Fig. 1. CD4+ and CD8+ double staining analysis by flow cytometry. A: Normal group, B: Negative group, C: Positive group, D: FC-VCO3 group, E: FC-VCO6 group, F: FC-VCO7 group. The Y-axis displayed CD8+ percentage and X-axis displayed CD4+ percentage. Lower left position showed no CD4+ and CD8+ expression, Lower right position showed CD4+ expression, Upper right position showed CD4+ and CD8+ expression, Upper left position showed CD8+ expression

given. The value yielded by negative group was found to be lower than normal group yet still not significant ($p>0.05$). Furthermore, Stimuno® and FC-VCO with dose 6 mg/kg BW possessed higher ratio than negative group. However, the result was also found to be not significant ($p>0.05$) ~~too~~. The Foxp3 percentage after doxorubicin induction was also display lower value than normal group eventhough not significant ($p>0.05$) (Figure 2). A combination of Stimuno® and FC-VCO treatment was used to increase Foxp3 in spleen cells. Both could increase Foxp3 percentage although not significantly ($p>0.05$) than negative group (Figure 3).

Effects of FiberCreme-VCO on pro-inflammatory cytokines

Pro-inflammatory cytokines in this study were important to measure the suitability of FC-VCO as immunomodulator. TNF-α levels from negative group showed higher value than normal group. However, this increasing was not significant ($p>0.05$) (Figure 4). Inducing with Stimuno® and FC-VCO treatment has elevated the value higher than negative group albeit not significantly ($p>0.05$). The IFN-γ levels of negative group displayed significantly lower value than normal group ($p<0.05$). Positive and FC-VCO9 groups displayed significantly elevated ($p<0.05$) IFN-γ levels than negative group. Meanwhile, FC-VCO3 and FC-VCO6 groups did not show a significant increase ($p>0.05$) than negative group. Measurement of IL-6 levels displayed decreasing value in positive group when compared to normal group, but also not significant ($p>0.05$). Inducing with Stimuno® could significantly increase ($p<0.05$) IL-6 levels than negative group. Moreover, FC-VCO treatment could elevate IL-6 levels higher than negative group, although insignificantly ($p>0.05$).

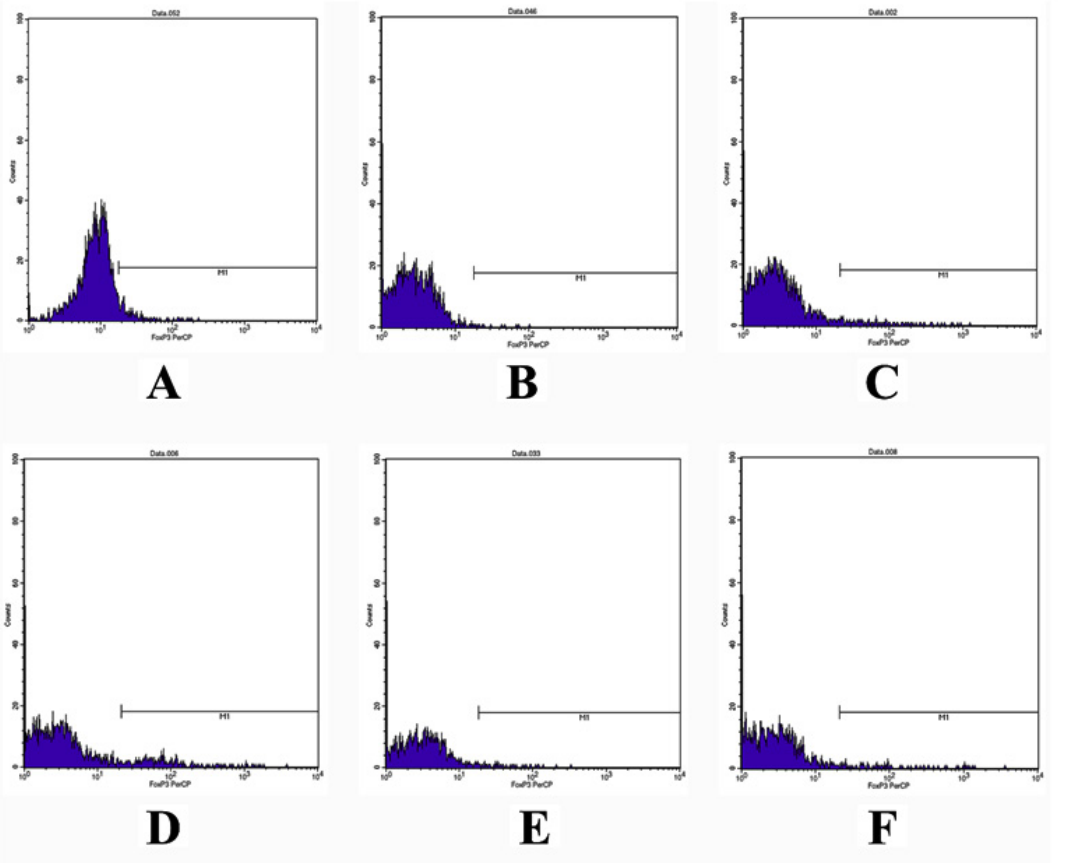

Fig. 2. Foxp3 staining analysis by flow cytometry. A: Normal group, B: Negative group, C: Positive group, D: FC-VCO3 group, E: FC-VCO6 group, F: FC-VCO7 group. The X-axis (M1) displayed Foxp3 percentage

DISCUSSION

Immunomodulators are substances which assist in regulating immune system[15]. Doxorubicin is one of the chemotherapy agents to treat various cancers including breast, pulmonary, prostate, skeletal, and bone. However, long-term use of doxorubicin leads to immunosuppression. Doxorubicin has non-selective effect on cell formation that are actively dividing such as bone marrow, lymphocytes, hair, and various organ toxicities[16,7]. Adding FC-VCO as a treatment could increase body and spleen weight due to nutrition from FC-VCO such as protein would elevate

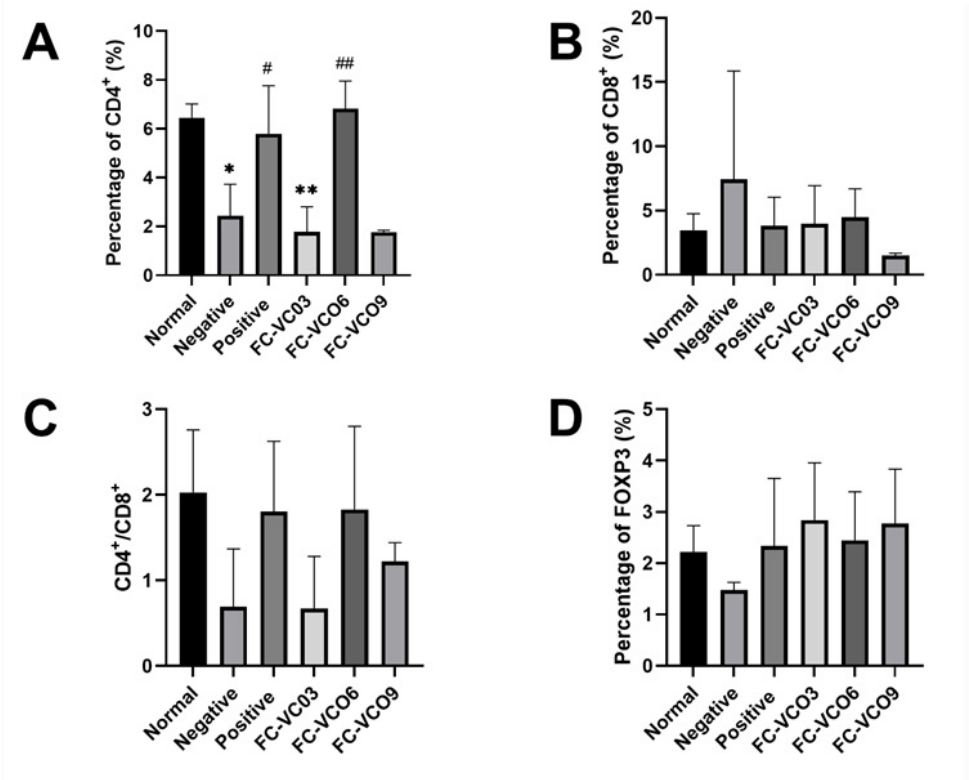

Fig. 3. $CD4^+$ and $CD8^+$ percentage after inducing by doxorubicin for 14 days. A: $CD4^+$ percentage, B: $CD8^+$ percentage, C: Ration of $CD4^+$ and $CD8^+$, D: Foxp3 percentage. *p<0.05 com-pared with normal group, **p<0.01 compared with normal group, #p<0.05 compared with negative group, ##p<0.01 compared with negative group

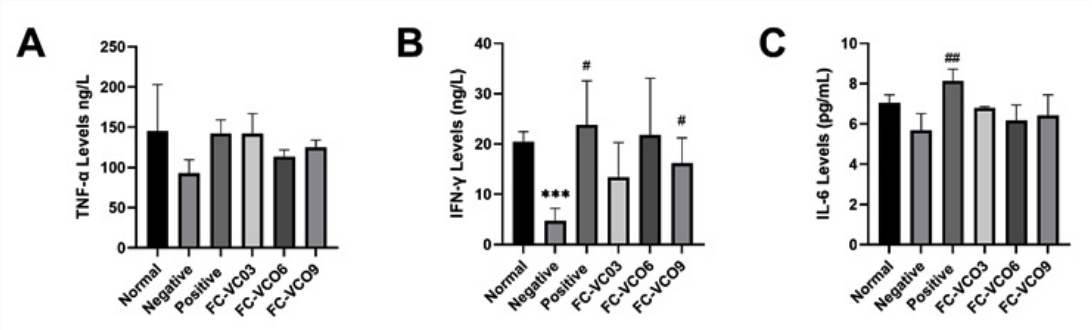

Fig. 4. pro-inflammatory cytokine levels after inducing by doxorubicin for 14 days. A: TNF-α levels; B: IFN-γ levels; IL-6 levels. #p<0.05 compared with negative group, ##p<0.01 com-pared with negative group. TNF-α: Tumor necrosis factor-α. IFN-γ: Interferon-γ, IL-6: Inter-leukin-6

regeneration rate in cells. Numerous studies have shown that decreased T cell proliferation affect in low cytokine levels such as TNF-α, IFN-γ, IL-2 and IL-12. Immune enhancers have recently been developed as components of functional meals by immunomodulatory activities from natural foods or food additive[17,18]. The findings of this study provide preclinical evidence of the promising effects of FC-VCO on Dox-induced immune system modulation in rats, further supporting the potential utility of FC-VCO as an immune-enhancing functional food agent. These results could be attributed to the ability of FC-VCO to reverse Dox-induced changes in the parameters of body weight, spleen weight, inflammatory response, and T cell balance.

Previous research has shown that medium-chain fatty acids in VCO improve phagocytic activity[19]. In this study, immunosuppression could be reversed by FC-VCO which specifically reduced splenocyte cell proliferation, cytokine and cytotoxic T cell lymphocyte (CTL) activity, $CD4^+$ percentage, $CD8^+$ percentage, and CD4/CD8 ratio[20]. The cytokines produced by diverse immune cells are crucial for immunological responses like host defense against bacterial infection, cell survival, and control of inflammation. Cell function is determined by cytokines which are crucial in the humoral immune response[21]. Enhancement in proliferation of lymphocytes, neutrophils, $CD8^+$ cells, and $CD4^+$ cells were exhibited by FC-VCO's immunostimulatory impact, especially from VCO and isomalto-oligosaccharides (IMOSs) in FC-VCO[22].

T cells are classified into two primary subsets; helper T cells, and killer T cells, which express $CD4^+$ and $CD8^+$, respectively. Helper T cells are recognized as cytokine makers, whereas killer T cells exhibit cytotoxicity to infections. Depending on the cytokines that they express, helper T cells are further divided into Th1 and Th2 subtypes. When necessary, Th1 cytokines activate additional immune cells, including neutrophils, lymphocytes, and macrophages. Th1-type cytokines cause proinflammatory responses by releasing Th1 cytokines such as IL-2, IFN-γ, and TNF-α. IFN-γ regulates macrophages, which are secreted by immune cells such T cells, macrophages, and NK cells. It is also identified as a Th1 T cell representative marker. Th1 and Th2 cells actively interact in an optimal immune system to produce balance through a complimentary connection. Our findings reveal that FC-VCO at dose 9 mg/kgBW supplementation increased the expression of Th1 cytokines IFN-γ, showing that FC-VCO has a strong immune-boosting impact. The isomalto-oligosaccharide content of FC-VCO may be the mechanism underlying the involvement of IFN, a cytokine released by Th cells that has the ability to influence the immune system [23,24].

Isomalto-oligosaccharides as one of commercially prebiotic sub-stances, are created by enzymatic conversion of starch. Moreover, IMOSs are generally utilized as functionalized food in Asia and are made of á(1-6)- and á(1-4)-linked glucose oligomers which enhance gut microbiota and encourage the growth of 'good bacteria'. Prebiotics can be used to modulate immune system in both humans and animals. Improving immune function in prebiotic-treated hosts is primarily due to increasing in population of beneficial bacteria and their products in gut. The gut microbiota plays major role in the immune system of host[25,26,27].

Treatment with FC-VCO restored IFN-γ along with improvement in $CD4^+$ levels in immunosuppressed splenocyte model induced by doxorubicin. The cytokine is crucial for growth and maintenance of T regulatory (Treg) cells as well as activation-induced cell death, which regulates non-essential immune responses. These results collectively imply that FC-VCO contribute to stimulate ~~of~~ humoral and cell-mediated immune responses and boosts immunostimulatory activity by protecting immune cells from doxorubicin-induced damage. Therefore, FC-VCO has the potential to be used as a functional food alternative to boost immunity, particularly for immunosuppressed patients.

CONCLUSIONS

In conclusion, FC-VCO at dose 9 mg/kgBW supplementation increased the expression of Th1 cytokines IFN-γ ($p<0.05$) along with increased $CD4^+$ levels ($p<0.05$) in immunosuppressed splenocyte model caused by doxorubicin, showing that FC-VCO has a strong immune-boosting impact and has the potential to be used as a functional food alternative to boost immunity.

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Competing Interests

There is no competing interest between author.

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