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# Screening on anti-inflammatory property of 45 Thai human probiotics from Biodiversity Research Centre of TISTR

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**Abstract:** Probiotics are defined as living microorganisms which can provide various health benefits. They can inhibit overgrowth of pathogenic bacteria by antibacterial substance production and stimulating the health function in gut system. Moreover, probiotics can promote the affect on innate immunity such as phagocytosis, and anti-inflammatory activity. The anti-inflammatory effect of probiotics is attributed to the released cytokines from immune cells such as lymphocytes, mast cells and macrophages. The aim of this study was to determine the anti-inflammatory activity of 45 TISTR human probiotic strains on lipopolysaccharide-induced macrophage RAW 264.7 cell line. Heat-killed supernatants of these 45 probiotic strains (Med1 to Med45) prepared and tested at 100% concentration. Lipopolysaccharides (LPS, 10 µg/ml) of 100 µl was used to induce inflammation. Results were collected based on the measurements of nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) productions using ELISA Kit assays. The results revealed that 20 out of 45 TISTR probiotics yielded various degrees of their anti-inflammatory capacity with %NO inhibition ranging from 19.91±1.50 to 82.04±3.71% compared with β-glucan (83.93±0.07%, positive control). Stimulation of RAW 264.7 macrophages with LPS induced a high production of NO by 378.61 pg/ml. The reduction in TNF-α levels (% TNF-α inhibition) was seen in 16 TISTR probiotics with the highest activity at 52.46% (Med 8 strain) and the lowest at 5.69% (Med 9 strain). Considering on % IL-6 inhibition, among 45 TISTR probiotic strains, 18 strains expressed positive results by 5.79 to 68.79%. The results suggest that many TISTR probiotic strains are potential of anti-inflammatory activity on macrophage RAW 264.7 cells. These findings can support the utilization of TISTR probiotics in preventing and down-regulating inflammation.

**Keywords:** Anti-inflammation, interleukin, macrophages, nitric oxide, probiotics, tumor necrosis factor-α

## Introduction

Thailand Institute of Scientific and Technological Research (TISTR) by Biodiversity Research Centre (BRC) is responsible for culture collection of bacteria, yeasts, molds and seaweeds which are beneficial to industrial and environmental usage. At the moment, BRC has more than 500 human probiotics collected and isolated from Thai population kept in the bank. Probiotics have been promoted the affecting on innate immunity such as phagocytosis, bactericidal activity and anti-inflammatory activity. The anti-inflammatory effect of probiotics is attributed to the released cytokines from immune cells such as lymphocytes, mast cells and macrophages (Thomas and Versalovic, 2010). Macrophages express a variety of surface receptors that including cytokine receptors (CRs), the interleukin-1 receptor (IL-1R) and complement receptor for pathogen associated molecular pathways (PAMPs) such as lipopolysaccharide (LPS). The activated macrophages also produce and secrete cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-12 and IFN)-γ, that are important for inflammatory and acquired immune response (Guan et al., 2011; Ariel et al., 2012). Rodes et al., (2013) reported that the pro-inflammatory cytokine concentration of TNF-α, IL-1beta was reduced by probiotics *Lactobasilus* and *Bifidobacterium* but anti-inflammatory cytokine was increased on lipopolysaccharide induced-RAW 264.7 macrophages. Therefore, macrophages behave as critical play in the initiation, maintenance, and resolution of inflammation through the release of variety of factors in response to an activating stimulus (Moro et al., 2012). The aim of the present study was to determine the anti-inflammatory mediator effect of 45 TISTR human probiotic strains on lipopolysaccharide-induced macrophage RAW 264.7 cell line.

To assort local probiotic strains for Thailand Institute of Scientific and Technological Research (TISTR) probiotic bank, we randomly collected various sources of flowers and fruits grown in the northern part of Thailand to isolate bacterial strains. All isolated bacterial strains were screened for probiotic properties according to a notification of Thai Food and Drug Administration (FDA), Ministry of Public Health (MOPH) and World Health Organization (WHO) starting from gram straining, catalase-oxidase activity, hemolytic activity, antimicrobial activity against potentially pathogens, antibiotic susceptibility, gastric acidity resistance (pH 2 and 3), bile salt tolerance, adherence to human epithelial cell line, and immunomodulatory activity in macrophage function. They were gram

positive, rod shape, catalase-negative, and negative hemolytic activity bacteria. All of them have antimicrobial activity against pathogens such as *Escherichia coli, Staphylococcus aureus.* The antibiotic susceptibility, gastric acidity and bile salt resistance and adherence to human epithelial cell lines of them were already proved. After probiotic properties screening, we got 45 probiotic strains in total. Therefore, this study was carried out to evaluate anti-inflammation activity of these 45 TISTR probiotics.

## Materials and Methods

### Probiotic strains and growth condition

A total of 45 TISTR human probiotic strains (namely Med 1 to Med 45) from the TISTR culture collection bank of Biodiversity Research Centre (BRC) were cultivated in de Man Rogosa and Sharpe (MRS) broth (Merck, Germany) with 0.05% L-cysteine (Merck, Germany) under anaerobic condition at 37°C for 48 hr. They were adjusted their turbidity to the concentration to match that of McFarland No. 0.5 (approximate $10^8$ CFU/ml). Cell pellets were obtained by centrifugation at 8,500 rpm for 5 min at 25°C and washed 3 times with 0.85% NaCl. After washing, they were resuspended with 10 ml of Dulbecco's Modified Eagle Medium (DMEM) high glucose (Biowest, USA) without antibiotics before heating at 110°C for 10 min. The supernatants of each probiotic strain was kept at -20°C until use.

### Macrophages culture

RAW 264.7 macrophage cell line (ATCC TIB-71™) was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured as adherent in Dulbecco's Modified Eagle Medium (DMEM) (Biowest, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (GIBCO®, USA) and 1% (v/v) penicillin-streptomycin (GIBCO®, USA) in humidified atmosphere incubator with 5% $CO_2$ at 37°C. The cells were collected by centrifugation (Kobota, Japan) at 1500 rpm, 4°C for 5 min, aspirated and fresh culture medium was added before dispensing into new culture flasks. All steps of culturing and maintaining of RAW 264.7 macrophage cells were conducted in aseptic condition using the Biological Safety Cabinet Class II (SANYO, Japan).

### Determination of the anti-inflammatory activity in RAW 264.7 macrophage cells.

### Nitric oxide measurement by Griess reagent assay

Griess reagent system is based on the chemical reaction which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NEDD) under phosphoric acid conditions. When sulfanilamide is added, the nitrites ($NO_2^-$) form a sulphonamide. Then, the NEDD was added, the sulphonamide was formed a pink colour of diazonium. To determine the anti-inflammatory activity, 200 µl of RAW 264.7 macrophage cells in DMEM were seed onto 96-well plates at a density of $2x10^4$ cells/well and incubated for 24 hr at 37°C in humidified atmosphere incubator with 5% $CO_2$. The overnight culture media were removed and RAW 264.7 macrophage cells ($2x10^6$ cells/well) were separately treated with 100 µl of probiotics (Med1-Med45) or β-glucan at 100 µg/ml (positive control) and incubated for 24 hr. Culture media were replaced by 100 µl (10 µg/ml concentration) of lipopolysaccharide (LPS) from *Escherichia coli* (Sigma, USA) and incubated at 37°C in humidified atmosphere incubator with 5% $CO_2$ for 24 hr. After incubation, the culture media was analyzed for nitric oxide (NO) production using the Griess reaction. Briefly, the culture media was mixed with 100 µl of Griess reagent (1% sulfanilamide, 5% phosphoric acid and 1% Naphthyletylenediamine dihydrochloride) and incubated at room temperature for 10 min. Evaluation of the concentration of NO in the supernatants of the RAW 264.7 cell culture was carried out with enzyme-linked immunosorbent assay (ELISA) kit (Thermo, USA) according to the manufacturer's instruction. Then, the absorbance at 540 nm was measured using microplate reader (Tecan Infinite 200 Pro, Austria). The amount of nitrite presented in the samples was measured with the sodium nitrite serial dilution standard curve.

### Cytokines measurement

RAW 264.7 macrophage cells were prepared in 96-well plate as previously described in NO measurement assay. Following removal of overnight culture medium, cells ($2x10^6$ cells/well) were treated with 100 µl of each TISTR probiotics (Med1-Med45) or β-glucan (Sigma, USA) at 100 µg/ml which was served as a positive control. Cells were incubated at 37°Cin humidified atmosphere incubator with 5% $CO_2$ for 24 hr. Then, culture media were replaced with 100 µl of LPS (Sigma, USA) at 10 µg/ml and incubated for 24 hr. The supernatants of treated-RAW 264.7 macrophage cells were collected by centrifugation at 1500 rpm, 4°C for 5 min and analyzed for two cytokine measurements including interleukin-6 (IL-6) and tumor necrosis- alpha (TNF-α) by Human IL-6 ELISA kit (Thermo, USA) and Human TNF-α ELISA kit (Thermo, USA), respectively. The secreted cytokine concentrations were quantified on the basis of a linear dose-response standard curve.

**Statistical analysis**

All data were expressed as mean ± standard error (Mean ± SE) of triplicated determination. The significance of difference was used to compare mean ($p<0.05$). All analyses were performed using One-way ANOVA and Tukey's Honestly Significant Difference test.

## Result & Discussion

In this study, the anti-inflammatory capacity of 45 TISTR probiotic strains was determined on % inhibition of NO, TNF-α and IL-6 productions. Following treatments, the morphology of RAW 264.7 macrophage cells was examined under the inverted microscopy as demonstrated in Figure 1. It was observed that 25-26 TISTR probiotic strains exhibited cytotoxic property (cell viability less than 50%) against RAW 264.7 macrophage cells when tested at 100% concentration (undiluted heat-killed supernatants). This observation was confirmed by cell viability and proliferation test using WST-1 assay whose principle is based upon a conversion of tetrazolium salts to colored formazan compounds by mitochondrial succinate-tetrazolium reductase which exists in viable cells. The results of WST-1 assay were analyzed by spectrophotometric quantification. However, results of WST-1 assay suggested that at least 27 out of 45 TISTR probiotic strains were non-cytotoxic to RAW 264.7 macrophage cells. Among them, 10 strains demonstrated a cell-proliferation promoting activity with cell survival rate greater than 100% compared with the untreated cells.

Macrophages are a type of phagocytic cells of the innate immune system. They play a crucial role and function as one of the first line of host defense mechanism against invading pathogen, foreign materials and inflammation (Flannagan et al., 2007; Ariel et al., 2012). Macrophages are activated in the inflammatory process including cytokines, bacterial lipopolysaccharide (LPS), extracelluar matrix proteins, and other chemical mediators. Activated macrophages release several different chemical mediators, including ROS, NO, IL-6, TNF-α, and $PGE_2$, that perpetuate the pro-inflammatory response (Shaikh 2011; Soromou et al., 2012). To determine the effect of TISTR probiotics on NO and pro-inflammatory mediators (TNF-α, IL-6) productions, RAW 264.7 cells were treated with 45 heat-killed TISTR probiotic strains (Med1 - Med45) at 100 % concentration for 24 hr prior to stimulate with LPS at a concentration of 10 µg/ml for 24 hr. Then, concentrations of NO, TNF-α and IL-6 mediators in medium were determined in cell supernatants. Under the stimulus with LPS which was a pro-inflammatory molecule, RAW 264.7 macrophage cells exhibited high concentration levels of NO (up to 126.17±5.33 µM), TNF-α (up to 378.61±0.52 pg/ml) and IL-6 (up to 149.73±1.52 pg/ml). Overall of 45 heat-killed TISTR probiotic strains, 19 strains were able to reduce the production of NO demonstrating as "% NO inhibition" (Figure 2). Overall, Med2, Med6 and Med10 showed the best anti-inflammatory activity on RAW264.7 macrophage cells. Their anti-inflammatory capacity was close to β-glucan (83.93±0.07%, positive control). The decrease of NO production was not related to viability of cells, which was not affected by LPS stimulation (data not shown).

Anti-inflammatory activity of 45 heat-killed TISTR probiotic strains was confirmed by inhibition of productions of pro-inflammatory cytokines focusing on TNF-α and IL-6. RAW264.7 cells were first pre-treated with TISTR probiotic samples (Med1 - Med45) and then stimulated with LPS for verifying their protective anti-inflammatory activity. The known anti-inflammatory compound, β-glucan at concentration of 100 µg/ml was used as a positive control. β-glucans are naturally occurring polysaccharides that are produced by bacteria, yeast, fungi and many plants (Han et al., 2008). Results on % inhibition of TNF-α and IL-6 productions were shown in Figures 3 and 4, respectively. It was found that 19 out of 45 TISTR probiotic strains yielded various degrees of their anti-inflammatory capacity against LPS-induced TNF-α and IL-6 mediators in RAW264.7 macrophage cells. The reduction in TNF-α levels (% TNF-α inhibition) was found in 18 TISTR probiotics with the highest activity by 52.46±2.98% (Med 8) and the lowest at 5.56±32.66% (Med 9) (Figure 3). Regarding % IL-6 inhibition, among 45 TISTR probiotic strains, 18 strains expressed positive results by 5.79±2.80% (Med40) to 68.53±1.63% (Med1) - 68.79±2.79% (Med41) (Figure 4). However, the anti-inflammatory capacity was not found in 26 TISTR probiotic strains tested including Med12, Med13, Med15, Med17, Med18, Med19, Med20, Med21, Med22, Med23, Med24, Med25, Med26, Med27, Med28, Med29, Med32, Med33, Med34, Med35, Med36, Med37, Med38, Med43 and Med45 strains. This might be due to their cytotoxic property when tested at 100% concentration as described in WST-1 assay results.

## Conclusion

Besides increasing inflammation and stimulating the immune system, macrophages play an important anti-inflammatory role and can decrease immune reactions through the release of cytokines. The results generated by this study suggest that many TISTR probiotic strains (positive results found in 18-20 strains among 45 strains tested) potentially exhibit anti-inflammatory activity on RAW 264.7 macrophage cells. These findings can support the utilization of TISTR probiotics in preventing and down-regulating inflammation. They can be utilized as ingredients for functional foods and dietary supplement products.

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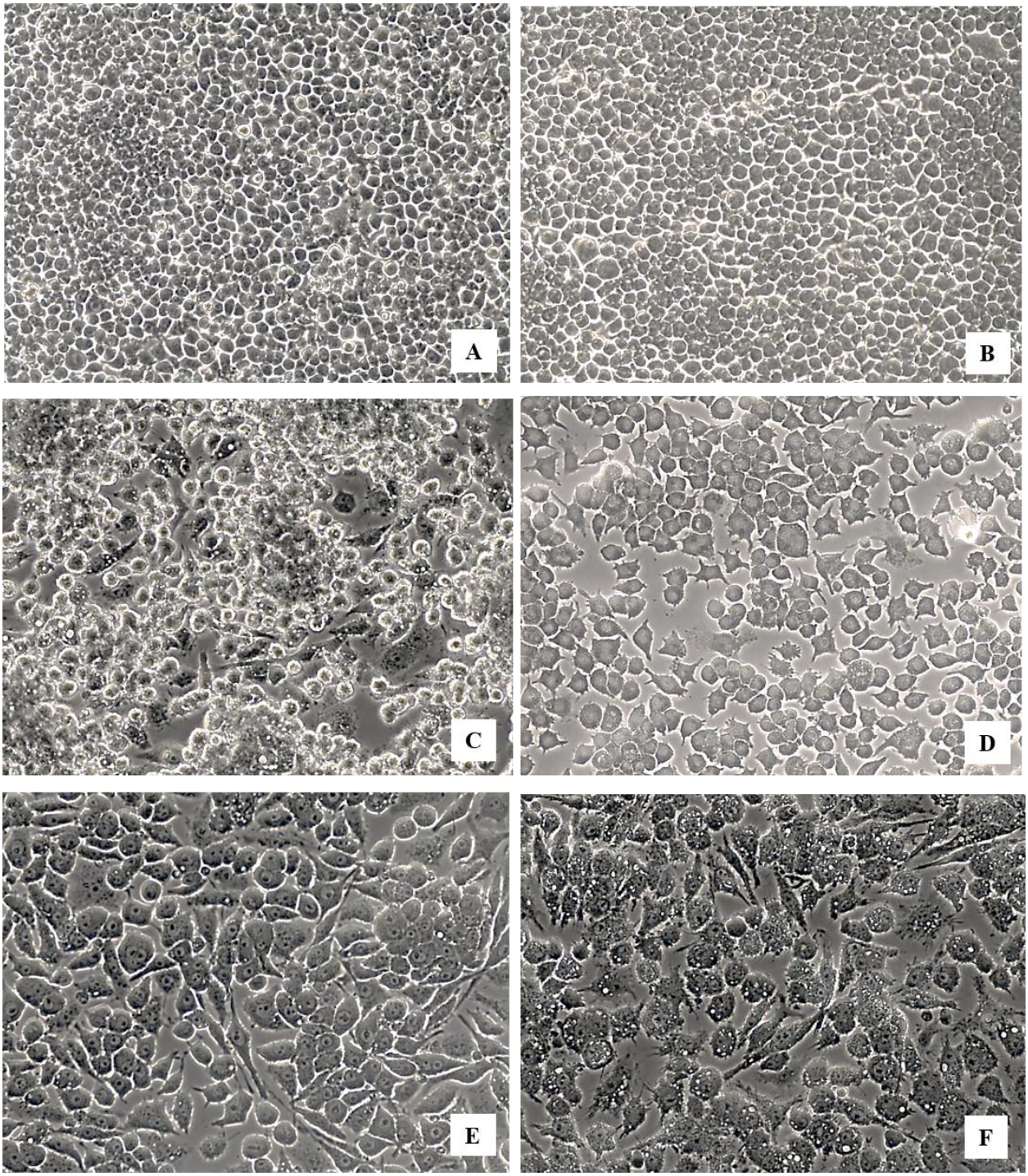


**Figure 1.** Morphology of RAW 264.7 macrophage cells after treated with TISTR probiotic strains. (A) Control (100% cell viability), (B) Probiotics (80-100% cell viability), (C) Probiotics (60-79% cell viability) (D) Probiotics (40-59% cell viability), (E) Probiotics (20-39% cell viability), (F) Probiotics (1-19% cell viability).

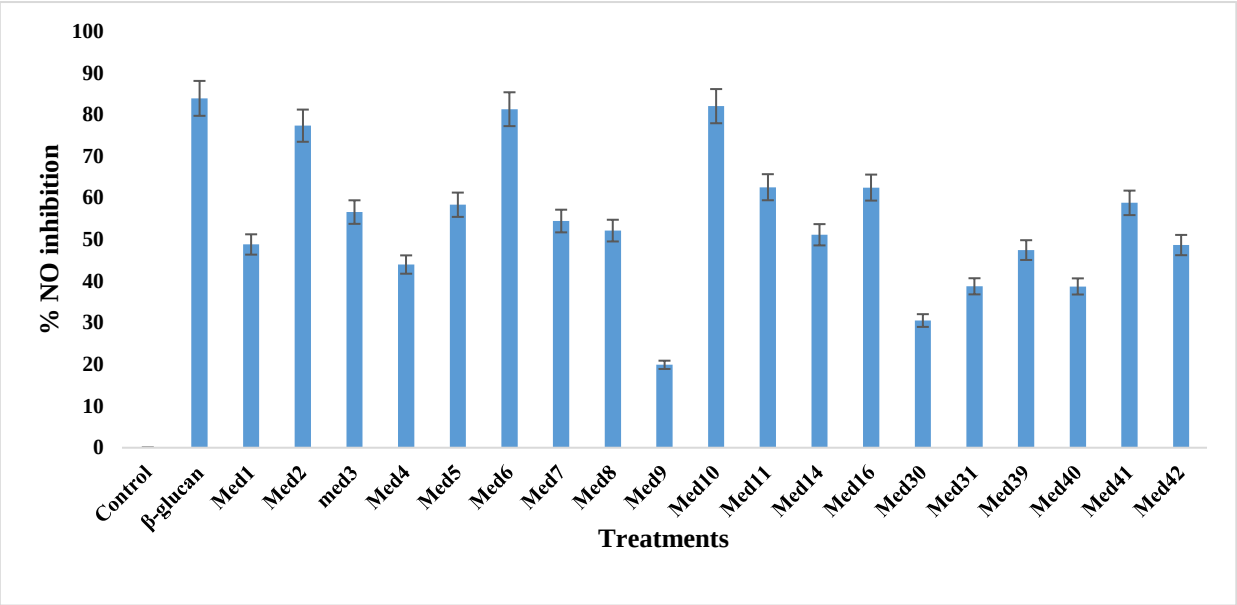


**Figure 2.** Inhibitory effect on LPS-induced nitric oxide (NO) production in RAW 264.7 macrophage cells by heat-
killed TISTR probiotic strains at 100% concentration. Beta-glucan (100 µg/ml) was served as positive control.

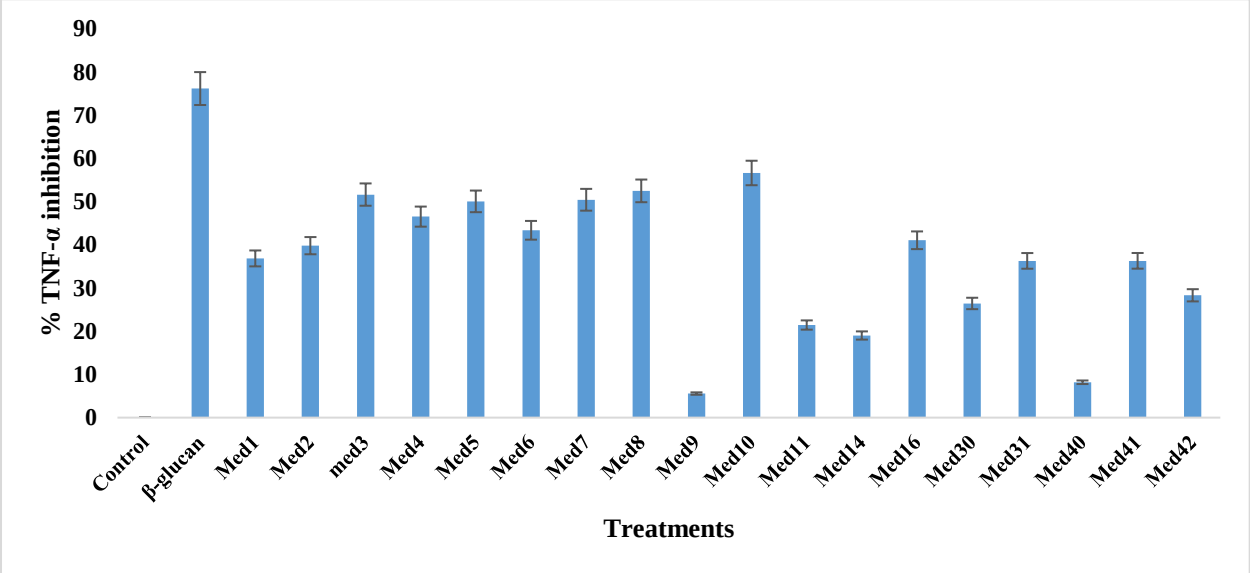


**Figure 3.** Inhibitory effect on LPS-induced tumor necrosis-alpha (TNF-α) production in RAW 264.7 macrophage cells by heat-killed TISTR probiotic strains at 100% concentration. Beta-glucan (100 µg/ml) was served as positive control.

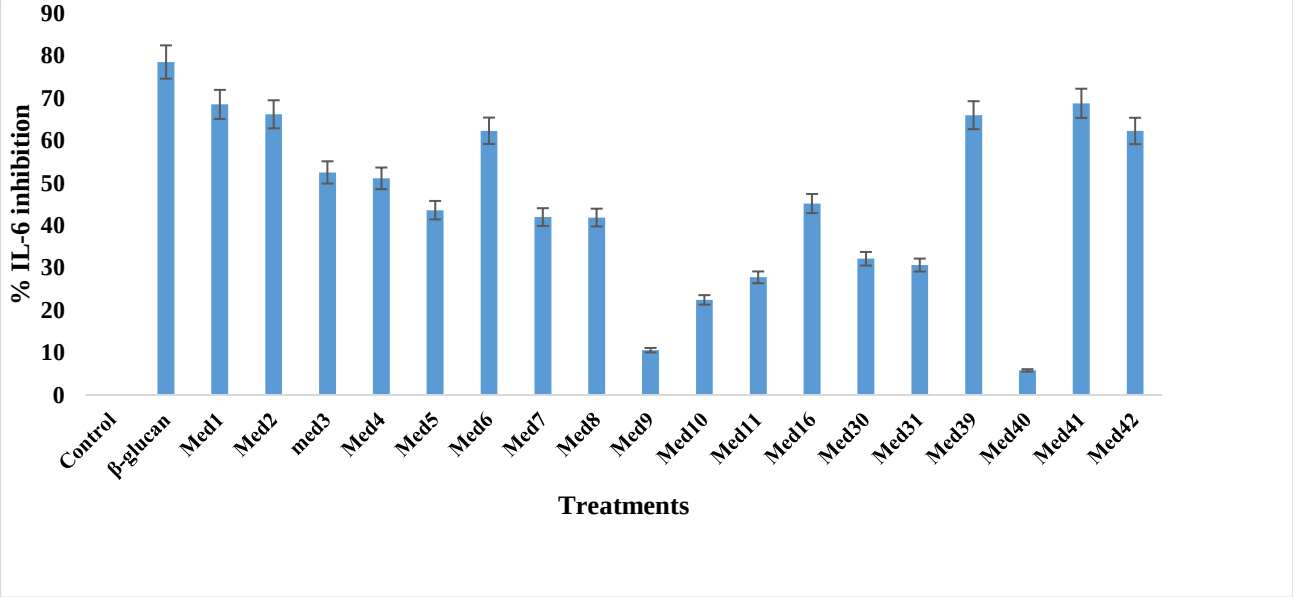


**Figure 4.** Inhibitory effect on LPS-induced Interleukin-6 (IL-6) production in RAW 264.7 macrophage cells by heat-killed TISTR probiotic strains at 100% concentration. Beta-glucan (100 µg/ml) was served as positive control.