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ORIGINAL ARTICLE

Effects of probiotics on the enhancement of the innate mucosal immune response against pathogenic bacteria

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ABSTRACT

Background and Objectives: Probiotics have been widely used for host immune system enhancement but with limited knowledge regarding the immunomodulation mechanisms by which they assist the mucosal innate immune response. We investigated the effects of probiotics on the modulation of the innate mucosal immune response particularly in association with Toll-like receptor (TLR)-2, TLR-4 and nuclear factor-kappa B (NF-κB) p65 and p105.

Materials and Methods: We randomized 24 male BALB/c mice into four groups. Two groups were administered probiotics for 21 consecutive days; one of these groups was challenged with Lipopolysaccharide (LPS) on day 15. The third group was challenged with only LPS. The fourth group remained untreated. All mice were sacrificed after 21 days. An immunohistochemistry procedure on the ileum was performed and monoclonal antibodies specific for TLR-2, TLR-4 and NF-κB p65 and p105 were used for the analysis of innate lymphoid cells.

Results: In the LPS-only treated group, there was a significant decrease in p105, indicating an alternative transcription pathway for the process of pro-inflammatory cytokine production. In the probiotics-only treated group there was significant enhancement of TLR-2 and TLR-4 and NF-κB p65 and p105. When mice treated with probiotics were exposed to LPS, there was a significant decrease in NF-κB p65 and p105, indicating employment of the classical pathway for pro-inflammatory cytokine production.

Conclusion: Probiotics can enhance the innate mucosal immune response in healthy mice and can maintain the homeostasis of the gut mucosal immune response against LPS through the activation of the classical NF-κB pathway.

Keywords: Probiotics; Lipopolysaccharides; Toll-like receptor; Nuclear factor-kappa B; Innate mucosal immune response

INTRODUCTION

The mucosal immune system plays a very important role in the immune system as it is broadly exposed to the outside world and is essential for resistance against infection (1). Failure of the intestinal mucosal immune system to regulate an immune response results in an imbalance of immunity and tolerance. Impaired immunity facilitates the occurrence of infectious disease via the intestinal mucosa, and regulational disruption of the mucosal immune system is an important factor in the pathogenesis of several diseases (2, 3).

Probiotics as living microbiota can benefit the health of a host if prescribed in sufficient quantities (4). Several probiotic working mechanisms are known, which include increasing the ratio of Bifido-

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bacteria and Lactobacilli to pathogenic microbes (5), competition for obtaining nutrients and inhibiting adhesion from pathogenic germs (6), increasing mucin production (6), anti-microbial activity (7) and influencing the immune response within intestinal mucosa (8). Probiotics research for multiple pathological conditions has resulted in good efficacy (9), but whether or not probiotics can also induce alertness in the mucosal immune response in response to pathogenic exposure if administered to healthy individuals is not deeply understood (10, 11).

A primed mucosal immune response involves immunocompetent cells within intestinal mucosa that are in a condition prepared to face pathogen exposure, requiring a sensor system capable of distinguishing not only self and non-self, but also pathogenic and non-pathogenic bacteria, all while maintaining both lumen and epithelial cell intestinal homeostasis (12).

The role of Toll-like receptors (TLRs), along with the non-TLRs that were discovered later (13), is very important for the mucosal response sensor system. Together with nuclear factor-kappa B (NF-κB), serving as a transcription factor with a central regulatory role in the processes of transcription and translation, TLRs play a key role in regulation of mucosal immune responses in maintenance of intestinal homeostasis through mechanisms related to cross-pathogenic non-pathogenic bacteria or intestinal commensal microbiota (8, 14).

The role of probiotics within various studies of healthy individuals concerning the alertness of the mucosal immune response explains more about the immune response activity within the mechanisms of adaptive immunity, and namely related to the increase of secretory immunoglobulin A, which exhibits both reactive and preventive properties in the intestinal mucosa following pathogen exposure (15-18). However, another study clearly states that the alert mechanism of mucosal immune responses as modulated by probiotics is via innate immunity pathways (8, 19). Thus the effect of probiotics for the priming of mucosal innate immune responses should be further investigated, especially concerning the role of TLR2, TLR4 and NF-κB in response to pathogenic exposure.

MATERIALS AND METHODS

Animals. Twenty-four BALB/c male mice aged 10-12 weeks, weighing approximately 30-40 grams each, were obtained from the Ethics Committee (Animal Care and Use Committee) of the Veterinary Medicine School of Airlangga University Surabaya, and were fed as according to standard protocol with free access to water. The mice were then divided randomly to four groups (I–IV). The first group of six mice were administered probiotics for 21 consecutive days, with lipopolysaccharide (LPS) being administered on day 15 (group I). The second group consisted of six mice that were not given probiotics, but only LPS on day 15 (group II). The third group consisted of six mice with probiotics administered for 21 days without administration of LPS (group III) and the final group included six mice that received neither probiotic nor LPS (group IV). Mice were examined daily for morbidity and other symptoms of illness, such as reduced activity level, abnormal evacuation, and decreased body weight. All mice were sacrificed on day 21 of the experiment. Immunohistochemistry procedure on ileum was performed and monoclonal antibodies specific for TLR-2, TLR-4 and NF-κB p65 and p105 were utilized for examination of the mucosal immune response within the gut.

Probiotics and LPS. The probiotic used in this study contained a 1×10^9 CFU probiotic combination, namely *Lactobacillus acidophilus* PXN 35, *L. casei* subsp. *casei* PXN 37, *L. rhamnosus* PXN 54, *L. bulgaricus* PXN 39, *Bifidobacterium breve* PXN 25, *B. infantis* PXN 27 and *Streptococcus thermophilus* PXN 66. The administered dose was 10^9/kg animal weight/day and was dissolved in 0.5 ml of D5% liquid and was administered via gastric tube once daily for 21 consecutive days for groups I and III.

LPS was derived from *Escherichia coli* O55:B5 (L2880; Sigma-Aldrich, St. Louis, MO, USA), administered at a dose of 250 μg/kg animal weight and was diluted in 0.9% NaCl at a 10:1 ratio. LPS was orally administered via a gastric tube on day 15 of the study for groups I and II.

Histological sample and mucosal immune response detection. At the conclusion of the study, mice from all groups were surgically dissected under ether anesthesia. Ileum sections were excised and cleaned, then fixed with a 10% formalin buffer solution. This process was followed by dehydration, clearing and embedding. Obtained ileal tissues were probed with monoclonal anti-TLR2 and anti-TLR4

mouse antibodies (SAB1404474 and SAB1404475, respectively; Sigma-Aldrich, St. Louis, MO, USA), NF-κB p65 monoclonal antibody (33-9900; Thermo Fisher Scientific, Waltham, MA, USA), and NF-κB p105/p50 monoclonal antibody (GTX60465; GeneTex, Inc, Irvine, CA, USA). Samples were observed under a light microscope (CX21; Olympus, Tokyo, Japan) and photographed with an ILCE6000 camera (Sony, Tokyo, Japan). The number of immunopositive cells was determined by counting the mean number of cells in 20 random fields at 450× magnification, and results were expressed as number of cells within the field vision.

Statistical analysis. Differences between groups were analyzed by the independent samples t-test for data with normal distribution or by the Mann–Whitney test for data with abnormal distribution. Results were considered significant if p values were <0.05.

RESULTS

In this study, we aimed to analyze the ability of probiotics to modulate the murine immune response, which was represented by TLR-2, TLR-4, NF-κB p65 and NF-κB p105. LPS was used in this study as a representation of pathogenic exposure. The mean values of the TLR-2, TLR-4, NF-κB p65 and NF-κB p105 counts in each group are represented in Figs. 1 and 2. To meet inferential requirements, the data was analyzed by parametric test after being tested by a

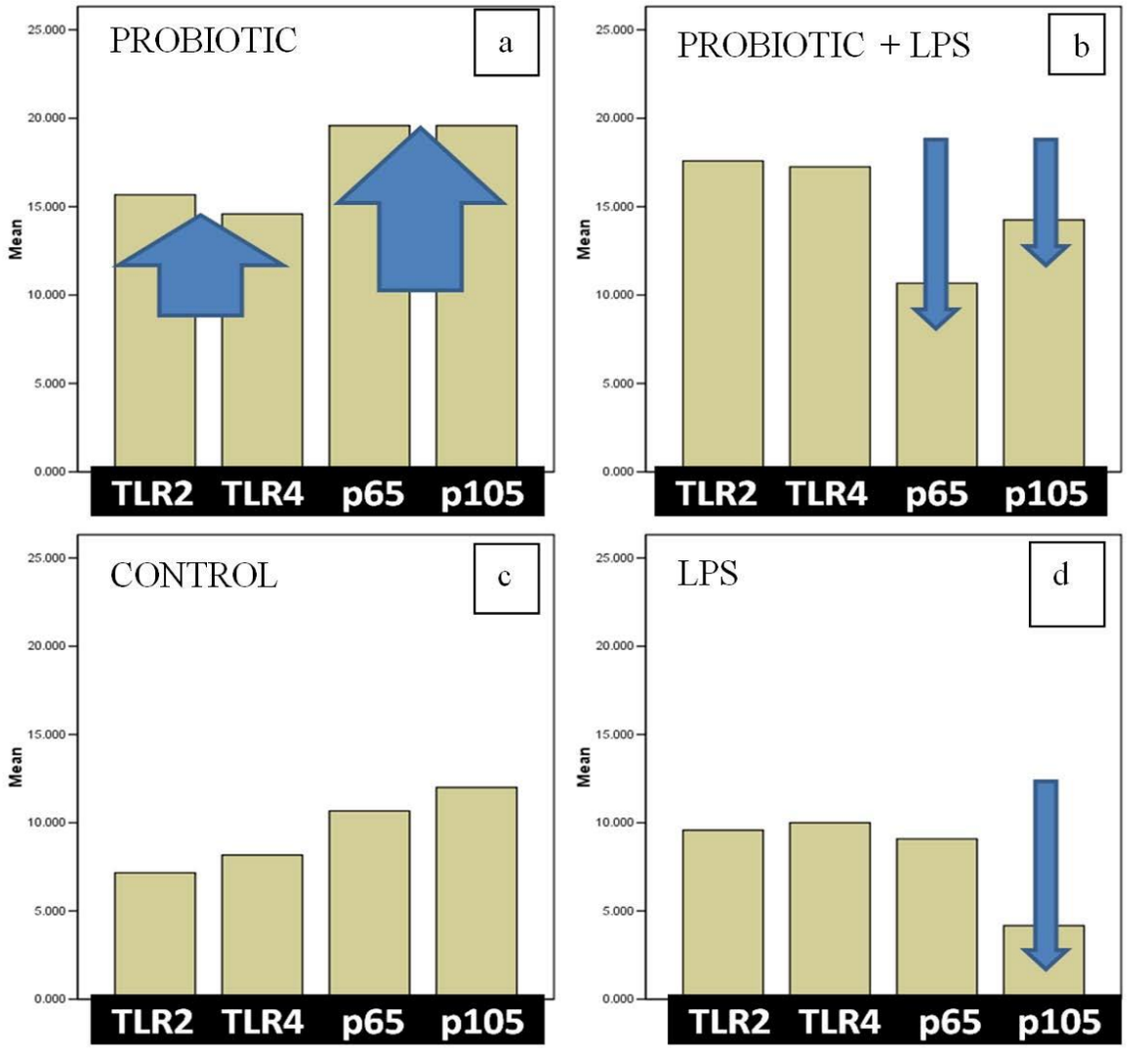

Fig. 1. Mean values for TLR-2; B) TLR-4; C) NF-κB p65; D) NF-κB p105

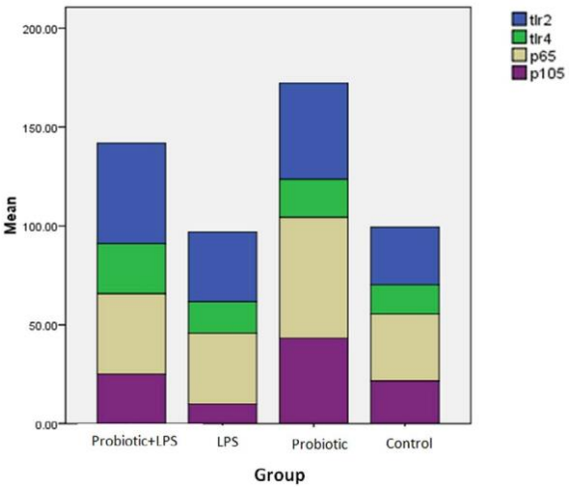

Fig. 2. Mean sum values for TLR-2, TLR-4, NF-κB p65 and NF-κB p105

Kolmogorov–Smirnov Test. The latter normality test results showed that the data for all four groups were normally distributed, and therefore parametric tests could be performed.

We compared the probiotic immune response with the control group. Differences in TLR-2, NF-κB p65 and NF-κB p105 levels between both groups were significant. However, the differences within TLR-4 levels between the groups were not significant (Table 1).

To determine the alertness of innate mucosal immune response within probiotic and non-probiotic groups in response to LPS exposure, we compared group I, (probiotic treatment plus LPS exposure), and group II (only LPS). TLR-4 and p105 levels were found to differ significantly between both groups (Table 2).

Table 1. Comparison of mucosal innate immune response between probiotic and control groups

	Probiotic Group (mean ± SD)	**Control Group (mean ± SD)**	**P**
TLR 2	48.7 ± 16.75	29.2 ± 8.61	0.025*
TLR 4	19.2 ± 4.87	14.7 ± 2.73	0.154
p65	61.2 ± 14.43	34.0 ± 8.07	0.002*
p105	43.2 ± 14.54	21.5 ± 9.35	0.001*

*Significant if $p < 0.05$; SD, standard deviation

Table 2. Comparison of mucosal innate immune response between probiotic + LPS and LPS groups

	Probiotic + LPS Group mean ± SD	**LPS Group mean ± SD**	**P**
TLR 2	50.8 ± 15.37	35.2 ± 13.52	0.065
TLR 4	25.3 ± 8.62	16.0 ± 2.28	0.006*
p65	40.7 ± 17.00	35.8 ± 12.64	0.540
p105	25.0 ± 7.01	9.8 ± 1.47	0.011*

*Significant if $p < 0.05$; SD, standard deviation

DISCUSSION

The role of probiotics within immune system modulation has been demonstrated both *in vitro* and *in vivo* (20). Although not much is known about the mechanism of probiotics in modulating innate mucosal immune responses, it is hypothesized that TLRs play a role in the innate mucosal immune response with probiotic administration (21, 22). This is consistent with the results of this study, in which TLR-2 levels in the group that received probiotics was significantly higher than that of the control group.

The upregulation of TLR-2 expression within the probiotic group is in line with several previous studies (16, 19). One of these studies examined the response presented within BALB/c, mice which were given *Lactobacillus casei* CRL431 and exhibited increased expression of TLR-2 within an immunofluorescence assay performed on the small intestine, which indicated that probiotics primarily modulate the mucosal immune response through an innate immunity pathway reflected by TLR-2. Probiotics, which are mostly Gram-positive bacteria, do employ the TLR-2 pathway (19).

However, TLR-4 did not increase significantly in the probiotics group within this study, which was also reported by another group that found that probiotics increased immune responses through innate immunity pathways, especially via upregulation of TLR-2 and NOD, but not TLR-4 (23). Another explanation could be that TLR-4 expression is negatively regulated by IL-4 (24), and BALB/c mice have a genetic bias toward $CD4^+$ T cells leading to Th2 cell responses, as indicated by a rapid increase in IL-4 and IFN-γ levels (25, 26). Thus, there was no significant increase of TLR-4 in either the probiotic-treated or LPS-treated groups in this study.

Here we found significant increases in the levels of transcription factors related to an immune response (NF-κB p65 and p105), similar to that reported by a previous study, which showed that probiotics increased the immune response through upregulation of NF-κB. However, little research has been performed on the effect (s) of probiotics on increasing p65 and p105 transcription factors (27). NF-κB p65 is a transcription factor with a trancriptional activation domain (TAD) in which the p65 dimer, as either a homodimer or heterodimer, is the active form of NF-κB. Conversely, p105 is a form of the NF-κB precursor that is responsible for maintaining NF-κB in the cytosol before it is activated and enters the nucleus. According to another study an increase of p105 can escalate the p50 subunit so that it can increase the rate of transcription. Furthermore, p105 can also bind to and inhibit free NF-κB dimers, especially those containing p50-dimers, and retains NF-κB in the cytoplasm. We assume that within the probiotic-treated group there was a balanced increase of p65 and p105, which would reflect a balanced increase of transcription NF-κB TAD (p65) and inhibitor factors (p105) (28).

The combination of probiotic treatment with LPS administration resulted in significantly increased TLR-4 and P105. LPS has a direct impact on p105 without going through the TLR-2 or TLR-4 pathways, but instead via utilization of a non-TLR pathway or alternative route. Whereas TLR-4 was shown to be significantly increased with probiotic administration, another study using male C57BL/6 mice with administration of alcohol and probiotics showed that alcohol increased TLR-4 activity in liver tissue (29), but probiotics decreased TLR-4 activity by increasing NF-κB activity (30). When compared to previous studies displaying different results, this variability may be due to different research objectives and uncertain mechanisms that resulted in a lack of research within this scope.

In order to understand priming of the mucosal innate immune response between probiotic- and non-probiotic-treated groups when facing LPS exposure, we compared mice within a probiotic-LPS group with mice in an LPS-only treated group. We obtained significant differences within TLR-4 ($p = 0.006$) and p105 ($p = 0.011$) levels but not for TLR-2 and p65 levels.

In conclusion, it can be inferred from this study that multispecies probiotics may regulate the innate immunity response through dendritic cells but not through NK-cells in a BALB/c mice animal model.

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