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CORRELATION BETWEEN MUCOSAL AND SYSTEMIC ADAPTIVE IMMUNE RESPONSE AFTER PROBIOTIC ADMINISTRATION IN MOUSE MODEL

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ABSTRACT

Background: Several previous studies were conducted to explain probiotic mechanism of action, especially associated to immune system. Probiotic was proved to induce adaptive immune response in mucosa, providing a hope if it presents evidence to affect the systemic immune response.

Objective: To investigate the correlation of adaptive immune response in intestinal mucosa to systemic immune response after probiotic administration.

Methods: Thirty-two male Balb/c mice were divided into 4 groups of treatment, including: LPS + probiotic, LPS, probiotic and control group. LPS were tested in the first day, while probiotic was administered in later 7 days. The ileum and blood were collected and analyzed to measure the number of cells that produce various cytokine indicating the T_H subset.

Results: Significant findings were found in all treatment groups, indicating similar patterns of cytokine-produced cell detection, found more in mucosal ileum than in blood. In the control group, the pattern was irregular. There was no correlation between immune response evoked in mucosal ileum and systemic immune response. Probiotics presents various mechanisms to modulate adaptive immune response. Mixture of probiotics could increase all subsets of T_H, indicating the variety. In short period, cell number of each subset was higher in intestine mucosa than in blood, negating that immune modulation effect of probiotics only acts locally on intestinal mucosa, not on systemic immune response.

Conclusions: Probiotics was found to have immunomodulation effect to adaptive immune response on intestinal mucosa. In sum, there is insignificant correlation between the two.

1. Introduction

In recent years, probiotics has gained popularity due to its beneficial attribute, utilized by healthy person as prevention, and also by unhealthy person as adjuvant therapy. Probiotic has been consumed as part of treatments, specifically for gastrointestinal diseases, such as: diarrhea, infection, inflammatory bowel disease, irritable bowel syndrome, and for other diseases including: allergic treatment and atopic dermatitis (Canani *et al*, 2007; Floch *et al*, 2008). Protection effect of probiotic in gastrointestinal lumen has well known and explained from several mechanisms of pathways, such as: to increase antimicrobial activity, to decrease pH of gut's lumen, secreting antimicrobial peptide, inhibit bacterial infection, blockage bacterial adhesion on epithelial wall, to increase the barrier defence by enhancing mucous production (bacteriosin / defensin), to modulate immune system, etc (Isolauri *et al*, 2001; Galdeano *et al*, 2006; Kim *et al*, 2006;

Saavendra *et al*, 2007; Hart *et al,* 2009). Probiotic also modulate innate and adaptive immunity by producing cytokine pro and anti-inflammation (Delcenserie *et al*, 2007; Galdeano *et al*, 2007; Dharma *et al*, 2009; Iskandar *et al*, 2009).

In addition to clinical research, several supporting studies were also conducted related to in-vitro research on immune response after oral probiotic administration at animal study in some research (Isolauri *et al*, 1995; Perdigon *et al*, 2001; Perdigon *et al*, 2002; Asahara *et al*, 2004; Bauer *et al*, 2004; Rastall *et al*, 2005; Madsen, 2006; Corthesy *et al*, 2007). Previous studies also present attempt to reveal the immune response after probiotic treatment from gastrointestinal, innate, adaptive, celluler or even humoral immune response, from blood serum. Thus this raises questions proving: any relationship between mucosal gastrointestinal immunity and systemic immune response inside the blood; the "connection" or "link" between mucosal immune response inside gastrointestinal and systemic immune response inside the blood,; and the rational connection from scientific research approach. The relationship may be explained by the patterns of mucosal and systemic immune respond, after administration of probiotic. It opens into other research interests: (1) if probiotic treatment could necessarily activate immune response simultaneously, identically with gastrointestinal mucosa and blood serum, or (2) if immune response at gastrointestinal will start series of the next systemic response, or (3) if the immune response in gastrointestinal will inhibit systemic immune response.

The aim of this research is to compare between adaptive immune response inside gastrointesinal mucosa and immune response outside gastrointestinal, after administration of probiotic by using mice (*Mus musculus Balb/C*) as experimental animal.

2. Materials and methods

The method of experimental *Randomized Post Test to Control Group Design* is utilized to discover any correlation between immune response in mucosal ileum and in blood by induction of probiotic. Immune response was measured by detecting cells producing certain biomarker such as cytokine by flowcytometry procedure. This research was conducted at Biomedical Laboratory and pharmacology Laboratory in Faculty of Medicine, at Brawijaya University Malang.

2.1.Research Sample

Thirty two white mice *Mus musculus* (BALB/c mice) which age 10-12 weeks with weight of between 30-40 grams and male gender were applied in this research, divided into 4 groups, including: LPS + probiotic, LPS, probiotic, and control group. The mice were taken from Veterinarian Centre Farma at Ahmad Yani Street Surabaya.

Research sample will be excluded if the tested animal was found sick tracked from change of activity (change of food/drink pattern, and animal activity) and other important clinical signs (decrease of body weight, breathing pattern, diarrhea, vomitting, and so forth). The tested animal which dies due to physical or mental stress, and damage to organ or tissue was also applied for sampling to examination of flowcytometry. The mice underwent acclimatization for a week before the treatment was started.

All the protocols in this research has already been approved by the Ethical Committee of Faculty of Medicine, Brawijaya University, Malang Indonesia.

2.2.Lipopolysacharide (LPS) administration

LPS is derived from *Escherichia coli serotype 055:B5* bacteria, with dosage of 250 µg/kg BB, thus each mice will get average of 7,5 µg. LPS will be dilluted with NaCl of 0,9% with comparation of 10:1, and will be given with orogastric tube at the first day of treatment for LPS and LPS+probiotic group mice.

2.3.Probiotic administration

Probiotic is derived from Mix bacteria with the composition of *Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus*

acidophilus, Lactobacillus bulgaricus, Bifidobacteria breve, Bifidobacteria infantis, and *Streptococcus thermophilus*, in which every sachet got the amount of living bacteria (*total viable count*) of 1,00x10^9 CFU. Probiotic is derived from dosage of 10^9/kgBB/day, thus each mice will get average dosage of 3 x 10^7 CFU. Probiotic will then be dilluted in D5% media with volume of 0.5 cc administered with orogastric tube (once a day) for 7 days. In LPS+probiotic group mice, the probiotic solution was administered in the following day after LPS induction.

2.4.Collection of samples

Samples of mucosal ileum and blood were taken from each mice. After all the treatment was completed, each mice underwent euthanasia by ether, to withdraw sample of ileum and blood. The ileum mucosa was homogenized, then diluted and analyzed by flowcytometry procedure. The plasma was separated from the cell by centrifugation to be analyzed by flowcytometry procedure. The number of mucosal and blood cells that expressed specific cytokine (IL-2 , IFN-γ, IL-4, IL-5, TGF-β, IL-10, IL-17, IL-22) were detect and counted by flowcytometry machine.

2.5.Flowcytometry

Flowcytometry was performed to measure the amount of cell producing cytokine to mark the subsets of immune response, including: IL-2 & IFN-γ indicate T_H1 subset, IL-4 & IL-5 indicate T_H2 subset, IL-10 & TGF-β indicate Treg subset, and IL-17 & IL-22 indicate T_H17 subset. Each cell which produced certain cytokine was detected by antibody of each cytokine from the flowcytometry kit (BioLegend, USA), according to manufacturer protocol.

2.4.Collection of samples

Data are presented in mean ± SD. Paired t-test was performed to reveal the differences between mucosa and systemic. If the data was not distributed normally, Wilcoxon test would be applied. Pearson Correlation test was performed to find correlation between adaptive and systemic immune response, taken from intestine mucosa and blood. If the data was not distributed normally, Spearman test will be utilized. The statistical calculation was performed by employing SPSS 21 software (SPSS Inc.). The differences is considered statistically significant at $p \leq 0.05$.

3. Results and discussions

In the first group (LPS + probiotic), there were some significantly different results between mucosal and systemic immune response, which was IL-2 with P=0.002, IFN-γ with P=0.000, IL-5 with P=0.000, TGF-β with P=0.004, IL-10 with P=0.000, and IL-22 with P=0.000. In contrast, IL-4 and IL-17 presented no significant difference (P=0.078 and 0.159 respectively) of adaptive immune response in ileum mucosa compared to systemic, as depicted in figure 1.

In the second group (LPS only), some significantly different results were found from adaptive immune response in ileum mucosa, which was IL-2 with P=0.05, IFN-γ with P=0.000, IL-5 with P=0.000, TGF-β with P=0.012, IL-10 with P=0.000, IL-17 with P=0.000, and IL-22 with P=0.000. Meanwhile, IL-4 presents insignificant difference (P=0.843) of adaptive immune response in ileum mucosa compared to systemic, as illustrated in figure 2.

In the third group (probiotic only), there were significantly different results of all cytokine level from adaptive immune response between in ileum mucosa and in systemic immune response, which was IL-2 with P=0.001, IFN-γ with P=0.001, IL-4 with P=0.003, IL-5 with P=0.001, TGF-β with P=0.002, IL-10 with P=0.000, IL-17 with P=0.008, and IL-22 with P=0.000 as depicted in figure 3.

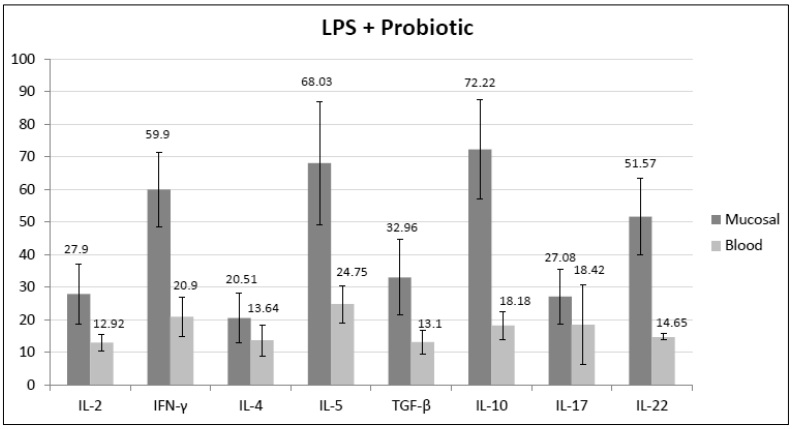

Figure. 1. Mean response of adaptive immune system in ileum mucosa and in systemic immune response in mice, administered with probiotic and LPS. The number of all mucosal cell that expressed specific cytokines taken from intestinal mucosa were significantly higher than blood cell, except for IL-4 and IL-17

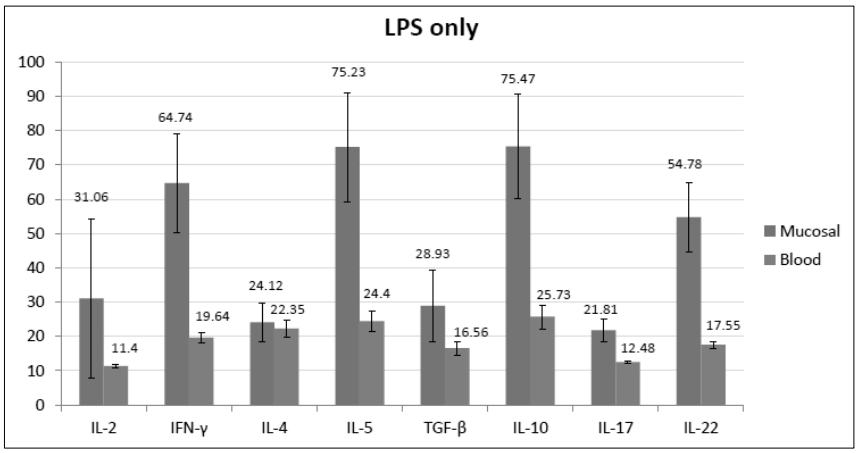

Figure. 2. Mean response of adaptive immune system in ileum mucosa and in systemic immune response in mice administered with LPS. Overall, the number of all mucosal cell that expressed specific cytokines taken from intestinal mucosa were significantly higher than blood cell, except for IL-4

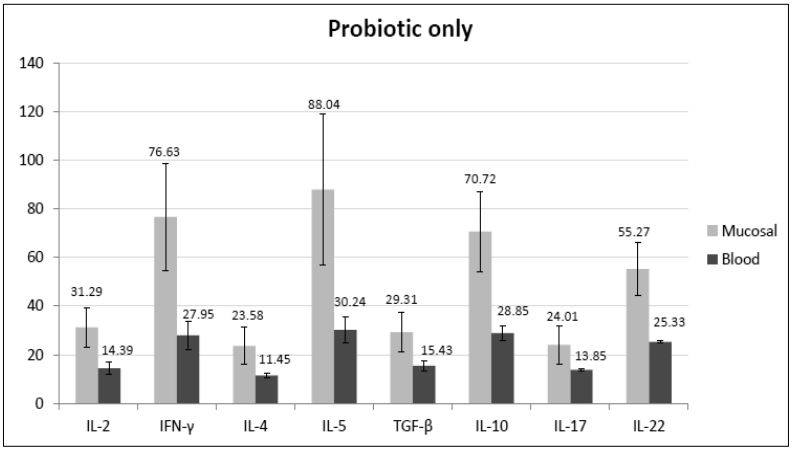

Figure. 3. Mean response of adaptive immune system in ileum mucosa and in systemic immune response in mice administered with probiotic. The number of all mucosal cell that expressed specific cytokines taken from intestinal mucosa were significantly higher than blood cell

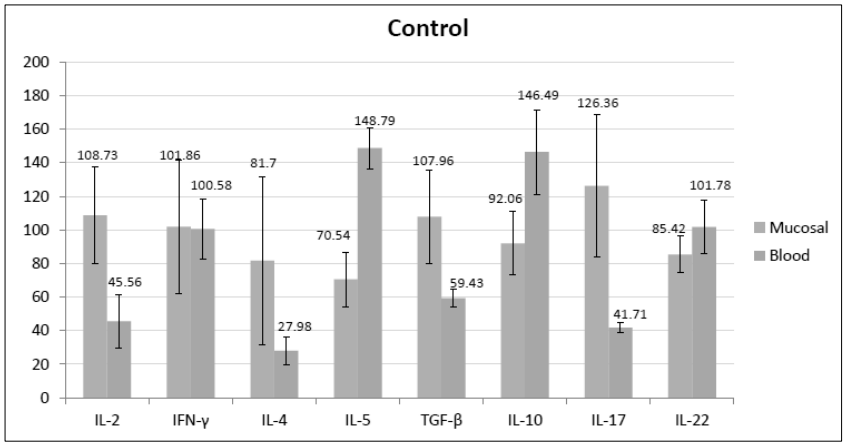

Figure. 4. Mean response of adaptive immune system in ileum mucosa and in systemic immune response in control group mice. The number of most mucosal cell that expressed specific cytokines taken from intestinal mucosa were significantly higher than blood cell, but there higher number count on cell that expressed IL-5, IL-10, and IL-22 obtained from blood than from mucosal ileum.

Table 1. Correlation of adaptive immune response in ileum mucosa and blood serum. There was no significant relationship in adaptive immune response in ileum mucosa and in systemic immune response at number of cell that expressed IL-2, IFN-γ, IL-4, IL-5, IL-10, TGF-β, IL-17, and IL-22

Parameter	Mucosal ileum	Blood	p	Correlation Coefficient	P
IL-2	27.9 ± 9.18	12.92 ± 2.47	0.002	0.213	0.613
IFN-γ	59.9 ± 11.54	20.9 ± 5.96	0.000	-0.192	0.648
IL-4	20.51 ± 7.67	13.64 ± 4.74	0.078	-0.102	0.810
IL-5	68.03 ± 18.95	24.75 ± 5.64	0.000	-0.055	0.897
TGF-B	32.96 ± 11.58	13.10 ± 3.67	0.004	-0.425	0.294
IL-10	72.22 ± 15.33	18.18 ± 4.39	0.000	0.551	0.157
IL-17	27.08 ± 8.4	18.42 ± 12.23	0.159	-0.103	0.808
IL-22	51.57 ± 11.75	14.65 ± 0.95	0.000	-0.106	0.803

Table 2. Correlation of adaptive immune response in ileum mucosa and blood serum. There was no significant relationship in adaptive immune response in ileum mucosa and in systemic immune response at number of cell that expressed IL-2, IFN-γ, IL-4, IL-5, IL-10, TGF-β and IL-22. There was only significant relationship in IL-17

Parameter	Mucosal ileum	Blood	P	Correlation Coefficient	P
IL-2	31.06 ± 23.33	11.4 ± 0.4	0.05	-0.551	0.157
IFN-γ	64.74 ± 14.39	19.64 ± 1.38	0.000	-0.155	0.713
IL-4	24.12 ± 5.56	22.35 ± 2.57	0.843	0.210	0.618
IL-5	75.23 ± 15.99	24.4 ± 3.08	0.000	0.514	0.193
TGF-B	28.93 ± 10.51	16.56 ± 1.93	0.012	0.106	0.803
IL-10	75.47 ± 15.14	25.73 ± 3.49	0.000	0.427	0.292
IL-17	21.81 ± 3.19	12.48 ± 0.36	0.000	-0.741	0.035

IL-22	54.78 ± 10.18	17.55 ± 0.98	0.000	0.144	0.734

Table 3. Correlation of adaptive immune response in ileum mucosa and blood serum. There was no significant relationship in adaptive immune response in ileum mucosa and in systemic immune response at number of cell that expressed IL-2, IFN-γ, IL-4, IL-5, IL-10, TGF-β, IL-17, and IL-22

Parameter	Mucosal ileum	Blood	P	Correlation Coefficient	P
IL-2	31.29 ± 8.15	14.39 ± 2.58	0.001	-0.166	0.695
IFN-γ	76.63 ± 22.18	27.95 ± 5.68	0.001	-0.165	0.696
IL-4	23.58 ± 7.73	11.45 ± 0.8	0.003	0.274	0.512
IL-5	88.04 ± 31.07	30.24 ± 5.32	0.001	-0.020	0.963
TGF-B	29.31 ± 8.21	15.43 ± 2.1	0.002	0.178	0.674
IL-10	70.72 ± 16.54	28.85 ± 2.9	0.000	-0.323	0.436
IL-17	24.01 ± 7.84	13.85 ± 0.31	0.008	0.116	0.785
IL-22	55.27 ± 10.94	25.33 ± 0.5	0.000	0.073	0.864

Table 4. Correlation of adaptive immune response in ileum mucosa and blood serum. There was no significant relationship in all adaptive immune response in ileum mucosa and in systemic immune response at number of cell that expressed specific cytokines.

Parameter	Mucosal ileum	Blood	P	Correlation Coefficient	P
IL-2	108.73 ± 28.68	45.56 ± 15.82	0.000	-0.166	0.695
IFN-γ	101.86 ± 39.67	100.58 ± 18.04	0.91	-0.165	0.696
IL-4	81.7 ± 49.92	27.98 ± 8.43	0.026	0.274	0.512
IL-5	70.54 ± 16.22	148.79 ± 12.15	0.000	-0.020	0.963
TGF-B	107.96 ± 27.72	59.43 ± 5.37	0.002	0.178	0.674
IL-10	92.06 ± 18.83	146.49 ± 25.24	0.001	-0.323	0.436
IL-17	126.36 ± 42.37	41.71 ± 2.97	0.001	0.116	0.785
IL-22	85.42 ± 10.97	101.78 ± 16.1	0.05	0.073	0.864

In the fourth group (control group), there were significant differences of almost all cytokine levels from adaptive immune response between ileum mucosa and systemic, but the pattern was random, as presented in figure 4. There was significant higher concentration on mucosal ileum of IL-2, IL-4, TGF-β & IL-17. In the other hand, higher concentration of IL-5, IL-10, and IL-22 obtained from blood than from mucosal ileum.

In this study, probiotic treatment could induce all of the subset of T_H cell, whether preceded by induction of LPS or standing alone. This result is consistent with previous statement of variety effect on T_H cell by probiotics. The amount of all cytokine illustrate the same pattern, which was cytokine produced locally in intestinal mucose had a higher level compared to cytokine level in serum. This result definitely shows that probiotic had a significant effect of immunomodulation locally, not systemic. LPS known to be an inducer of innate immunity, by attached to TLRs of macrophage or dendritic cell. Then, macrophage as an APC produces some cytokine that could induce all of T_H subset, according to its environment (Abbas et al, 2007).

Previous result from Nunez et al. showed that effect of probiotic to immune response dominantly on intestinal environment. Probiotic treatment could improve vili condition, increase IgA mucosal production, and maintain activity of macrophage intestinal whether probiotic couldn't increase IgG in the serum, which indicate locally scope of probiotic effect (Nunez *et al*, 2014).

Probiotic used as a prevention of intestinal pathogen, which could be shown from result that probiotic could increase the amount of cell producing IL-2 and IFN-γ significantly, indicate activation of T_H1 subset. Previous studies reported that *L.casei* in probiotic could increase activation of transcription factor NF-κB in macrophage induced by LPS. This activation lead to increased IL-12 production, which has the activity to induce differentiation form T_H naïve to T_H1 (Ishida *et al*, 2007). Probiotic also could potentiate semi-mature dendritic cell, to produce more pro-inflammatory cytokine, such as IL-6 and TNF-α to fortify intestinal lining from pathogen invasion (Rizzello *et al*, 2011).

Regarding T_H17 subset, it was shown that IL-17 production on intestinal mucosa in this study, was not significantly differs from serum. Source of IL-17 dominantly from T_H17 and a very little amount from γδT cell, so it needs significant activation of T_H17 to increase IL-17 production significantly, which couldn't achieved by short term induction of probiotics (Jin and Dong, 2013). But, as consistent to previous statement, probiotic could induce development of T_H1/T_H17 subset locally on intestinal mucosa.

Activation of macrophage could also induce T_H2 subset, and this effect could be increased by certain type of microorganism. Increased number of cell producing IL-4 and IL-5 indicate activation of T_H2 to further activate B lymphocyte to produce Immunoglobulin. This result consistent with previous studies that report probiotic (*L.reuteri, B. longum*) could reduce TNF-α and increase IL-4 production by LPS-induced macrophage (Rodes *et al*, 2013). The variety of immune modulation by probiotic not only in the scene of pro-inflammatory T_H subset, even in Treg development. Another study of probiotic, using mixture of probiotic showed that probiotic could interact with regulatory Dendritic Cell (rDC) to produce IL-10 and TGF-β (Kwon *et al*, 2009). These two cytokine are necessary for differentiation of naïve T_H cell to Treg. Similar result gained from our study that probiotic also increase number of cell to produce IL-10 and TGF-β. This result completely shows us again that probiotics have a variety effect to immune system. One probiotics, which contain *Lactobacillus reuteri*, could induce IL-10 production by macrophage and dendritic cell to activate Treg subset (Hemarajata, 2013). Another probiotics which contain *Lactobacillus rhamnosus*, could induce production of TNF-α, IL-12, and IL-6 by dendritic cell that induce activation of T_H1 and T_H17 subset (Evrard *et al*, 2011).

Probiotic well reported as a prevention of allergy, although the evidences still not convincing. This indicate that probiotic preferential is to suppress T_H2 subset, which mainly causes allergy reaction (Cuello *et al*, 2015). In this study, on the contrary, the difference between IL-4 in mucosal ileum and blood seems to be significant on probiotic only group. This result likely indicate that probiotic actually activating T_H2 subset response. This contrary can be explained by the real amount of cells that produce cytokine. In probiotic only group, it was actualy the amount of cytokine produced by decreasing, made the differences to significant statistically. This decrease due to absence of LPS induction which can elicit systemic immune response. The mean amount of cell producing IL-4 was similar between all three treatment group, indicate the action of probiotic less dominant than the LPS induction. There were any similar pattern between treatment group, which was any cytokine produced much higher in mucosal ileum than systemically produced, which indicate dominance of immune response occurred locally, whether elicited by LPS or probiotic. In control group, the pattern was inconsistent between mucosal ileum and blood.

Correlation analysis performed to measure if there are any correlation between effect of probiotic on mucosal immunity to the systemic one in circulation. From the correlation result, it was showed that no significant correlation performed by probiotics. It means that the effect of probiotic to modulate immune system only happened locally on intestinal mucosa. This result similarly consistent with previous study by Galdeano et al. when probiotic was given as an adjuvant to re-nutrition diet on protein-energy malnutrition mice. Probiotic effectively induce local immunity, shown by increase of DC and various cytokine such as IFN-γ, IL-2, and IL-6. In systemic scope, IgG production increased after probiotic and re-nourishment therapy, which indicate that probiotic doesn't has direct correlation effect to systemic immunity (Galdeano *et al*, 2011).

4. Conclusions

The importance of this study is to clarify usefulness of probiotic to various disease on local or systemic base. The result from present study showed that probiotic has a variety effect on intestinal mucosa and systemic immunity, whether it is pro- or anti-inflammation. There are significant difference number of cell that produce cytokines taken from intestinal mucosa compare with cell that taken from blood. The number of cell that taken from intestinal mucosa significantly higher than blood. Furthermore, using correlation test, there were no correlations between the changing number of cell in ileum and blood. It indicates that adaptive immune respon after administrastion of probiotics, in short periode, seems only act locally on intestinal mucose, but not systemically. There is no correlation between probiotic treatment with activation of systemic immune response. Further necessarily studies of probiotic to identify how commensal bacteria interact with immune cell on the molecular base.

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