

# Non-Viable *Lactobacillus Casei* Beneficially Modulates Poly I:C Immune Response in Co-Cultures of Human Cells

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## ABSTRACT

**Background:** Polyinosinic:polycytidylic acid (Poly-IC) has been used as a viral stimulus to mimic *in vivo* and *in vitro* infection induced by some viruses. **Objective:** To determine whether non-viable *Lactobacillus casei* CRL431 (LcM) can modulate the immune response induced by Poly I:C in co-culture models of peripheral blood mononuclear cells (PBMC) and A549 cells. **Methods:** T and NK cell activation was evaluated by flow cytometry and levels of TNF- $\alpha$ , IFN- $\gamma$ , IL-10, IL-29, and IL-17 by ELISA. Cells in direct contact with A549 (PBMC-A549) and cells with no contact with it (PBMC//A549) were used for this purpose. PBMCs alone and both co-culture systems were stimulated for 24 h with the following stimuli: LPS (10  $\mu$ g/ml), LcM (10<sup>6</sup> UFC/ml), Poly I:C (2  $\mu$ g/ml), Poly I:C+LcM, and LcM (3 h)+Poly I:C. Moreover, unstimulated cells were used as a control. **Results:** Poly I:C and LcM (3 h)+Poly I:C in PBMC-A549 showed a significant increase in the percentage of CD8<sup>+</sup> expression ( $p < 0.05$ ). All stimuli induced significant activation from T CD4<sup>+</sup>, CD8<sup>+</sup> cells compared with unstimulated PBMCs in both co-culture cells system. However, activation percentages were higher in direct co-culture. Poly I:C induced a higher level of pro-inflammatory TNF- $\alpha$  and IFN- $\gamma$  cytokines as well as IL-17 and IL-29 with lower IL-10 levels in both co-culture systems while LcM induced a beneficial pattern of cytokines that would regulate Poly I:C effect. **Conclusion:** This *in vitro* model allowed us to highlight the potential of LcM as a modulator of anti-viral immune response and suggest its potential use in formulations against RNA respiratory viruses.

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**Keywords:** A549 Cell, Immunomodulation, Non-Viable *Lactobacillus*, Poly I:C, PBMC

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## INTRODUCTION

Viruses are the most common pathogens responsible for acute infectious diseases worldwide and respiratory viruses are the main cause of morbidity and mortality (1,2). When cells are infected with RNA viruses, a substantial amount of double-stranded RNA (dsRNA) is released from infected cells into the extracellular space. dsRNA is a common viral replication intermediate of RNA viruses and a potent indicator of viral infection (3,4,5). This dsRNA is able to provide an early signal for immune cells to establish an antiviral response prior to their own infection (6). Polyinosinic: polycytidylic acid (Poly-IC), an agonist of TLR3 (a pathogen-associated molecular pattern (PAMP) receptor for dsRNA), is a synthetic mimic of dsRNA that induces characteristic inflammatory responses associated with respiratory viral infection such as increased production of inflammatory cytokines, excessive mucus secretion, loss of epithelial integrity, and impaired ciliary function that can occur as a result of airway injury (7). The lung epithelium forms an impenetrable barrier that protects upper airways with a combined effort between ciliated and secretory cells. This physical barrier, together with an adequate mucociliary clearance, inflammatory and regulatory cytokine release, and immune cell activation, represents the defense mechanisms that protect the lung (8). Stimulation of airway epithelial cells with Poly I:C has been found to closely mimic inflammatory responses associated with viral infection. Also, Poly I:C challenge in peripheral blood mononuclear cells (PBMC) can induce gene expression changes similar to acute viral infections, involving TLR3, INF, and NF- $\kappa$ B-dependent pathways (9). Moreover, Poly I:C is able to induce a pulmonary dysfunction similar to a respiratory syncytial virus in a mouse model (10).

Based on these data, in the present work, Poly I:C was used as a viral stimulus. Respiratory viruses target the epithelium of conducting airways, cause cytopathic effects, and replicate themselves using the host. Airway epithelial cells (AECs) of the mucosa actively prevent the entry of respiratory pathogens and are the first barrier against inhaled microorganisms. AECs participate in host defense mechanisms by producing cytokines and chemokines in response to antigenic stimuli (11). In addition, PBMCs is an important factor in both the defense against infection and the modulation of the immune response in the lung (12).

A topic of interest in the fight against respiratory viruses is the development of strategies to stimulate the immune system of the host through vaccines or adjuvants through antiviral drugs. In this sense, it was demonstrated that certain lactic acid bacteria can favorably modulate the host upper respiratory tract immune system in a pneumococcal infection model. *Lactobacillus casei* CRL 431 has been proved effective as orally delivered mucosal adjuvant in a mice model (13). In addition, LcV and LcM nasally co-administered with a specific pneumococcal antigen in experimental vaccines (14,15,16) were effective in reducing pneumococcal lung colonization and preventing the passage of pathogens to the blood after pneumococcal challenge in infant mice. In a previous report, the immune response induced by combinations of LcV and LcM as adjuvants associated with pneumococcal protective A protein (PppA) in PBMC was evaluated (17). LcV and LcM stimulated beneficial Th1, Th2, and Th17 cytokine profiles and exerted an important adjuvant effect when associated with PppA (17).

In the present work, the effect of LcM on the modulation of the immune response induced by Poly I:C in co-culture models of human PBMC and pulmonary epithelial cells (A549) is evaluated, considering activation of T and NK cells and cytokine

production. The potential of LcM as an immunomodulator and/or adjuvant in vaccines for the defense against RNA respiratory viruses was discussed. Finally, we analyzed the effect of the interrelation between PBMCs and A549 in direct and transwell co-cultures on the immune response induced after stimulation.

## MATERIALS AND METHODS

***Lactobacillus Casei* Culture Conditions.** *Lactobacillus casei* CRL 431 (Lc) (14), obtained from the CERELA culture collection, was cultured for 18 h at 37°C (final log phase) in Man-Rogosa-Sharpe broth (Oxoid), harvested, and washed with sterile 0.01 mol/L phosphate buffer saline (PBS), pH 7.2. Non-viable *L. casei* (LcM) was prepared by heating bacteria in a water bath at 80°C for 30 min and lack of bacterial growth was confirmed using MRS agar plates.

**Cell Culture.** PBMCs were isolated from the heparinized peripheral blood of 5 healthy volunteers (median age 28 years, range 25-33 years) after obtaining their written consent, as described in a previous report (17). The Ethics Committee of the Postgraduate Department of the Facultad de Bioquímica, Química y Farmacia approved this study. Briefly, PBMCs were isolated by centrifugation over a Ficoll density gradient (SIGMA, NJ, USA), washed with RPMI-1640 (Gibco, Invitrogen, Buenos Aires, Argentina), adjusted to  $1 \times 10^6$  cells/ml in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) (Natacor, Córdoba, Argentina), 2 mM l-glutamine, 100 mg/ml streptomycin and 100 U/ml penicillin (Sigma). PBMCs were incubated in 24-well flat-bottomed polystyrene microtiter plates (Corning, GBO, Argentina) in the presence or absence of different stimulants in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C for 24 h. Human A549 cells, a type II alveolar epithelial cell line, were kindly provided by Dr. M Rumbo's Group-Institute of Immunological and Pathophysiological Studies (CCT-La Plata). These cells were grown in cell culture flasks in the RPMI 1640 culture medium. Then, A549 were harvested, plated at  $2 \times 10^5$ /ml in 24-well plates in a culture medium, and grown for 24 h to the confluence. PBMCs obtained as explained above and A549 cells were co-cultured by adding  $1 \times 10^6$  PBMC/ml to the plated A549 cells; this co-culture was defined as PBMC-A549. In addition, PBMCs were added to transwells (Costar) in the lower compartment, while A549 cells were localized in up inserts; this co-culture was defined as PBMC//A549. PBMCs alone and in co-culture cell systems were stimulated for 24 h with the following stimuli: 1) LPS (10 µg/ml), 2) LcM (10<sup>6</sup> UFC/ml), 3) Poly I:C (2 µg/ml), 4) Poly I:C+LcM, and 5) LcM added 3 h before Poly I:C (LcM (3h)+Poly I:C). The PBMCs stimulation period (24 h) was selected based on previous reports (9,17). Non-stimulated PBMCs alone and from co-cultures were also evaluated as controls of basal cytokine production and cell-surface marker expression. All reagents were tested by the E-toxate test for LPS (Sigma) and shown to be below the detection limit (2 pg/ml). Each stimulus was tested in duplicate. At the end of the stimulation period, PBMCs culture supernatants were collected, fractionated in aliquots and stored at -20°C until cytokines were analyzed. In addition, PBMCs from co-cultures were harvested for cytometry analysis as described below.

**Cytokine Assays.** Cytokine concentrations of supernatants were measured using human TNF- $\alpha$ , IFN- $\gamma$ , IL-10, IL-17, and IL-29 enzyme-linked immunosorbent assay (ELISA) Ready SET Kit (BD Bioscience, San Diego, CA, USA). Detection procedures were performed according to the manufacturer's instructions. The sensitivity of the assays for

each cytokine was as follows: 4 pg/ml for IFN- $\gamma$ , IL-29, and IL-17, and 2 pg/ml for TNF- $\alpha$  and IL-10.

**PBMCs Phenotyping by Flow Cytometry.** In order to evaluate the effects of different stimuli on PBMCs surface antigen expression from PBMC-A549 and PBMC//A549, cells were stained with appropriate fluorescently labeled monoclonal antibodies (mAbs). These antibodies were fluorescein isothiocyanate (FITC)-labeled anti-CD3 and anti-CD25; phycoerythrin (PE)-labeled anti-CD56, anti-CD25, anti-CD4, and peridinin chlorophyll-protein (PerCP) anti-CD8. In addition, triple CD4-CD8 and CD3 mAbs were used to enumerate T cells (all mAbs from BD Pharmingen). Cells were stained with labeled mAbs in PBS + 2% fetal bovine serum (PBS-FBS) for 30 min at 4°C, washed, and re-suspended in ice-cold PBS-FBS. Then, they were acquired by a Partec Pas-II flow cytometer (BD Bioscience). Data were analyzed using the Flomax software.

**Statistical Analysis.** Experiments were performed in triplicate and results were expressed as the mean  $\pm$  standard deviation (SD). Statistical analyses were carried out with Statgraphics plus 5.1 software (Manugistics, Rockville, MD, USA). Data were evaluated using one-way or two-way ANOVA tests. Tukey's test (for pairwise comparisons of the mean values of different groups) was used to test differences between the groups, which were considered significant at  $p < 0.05$ .

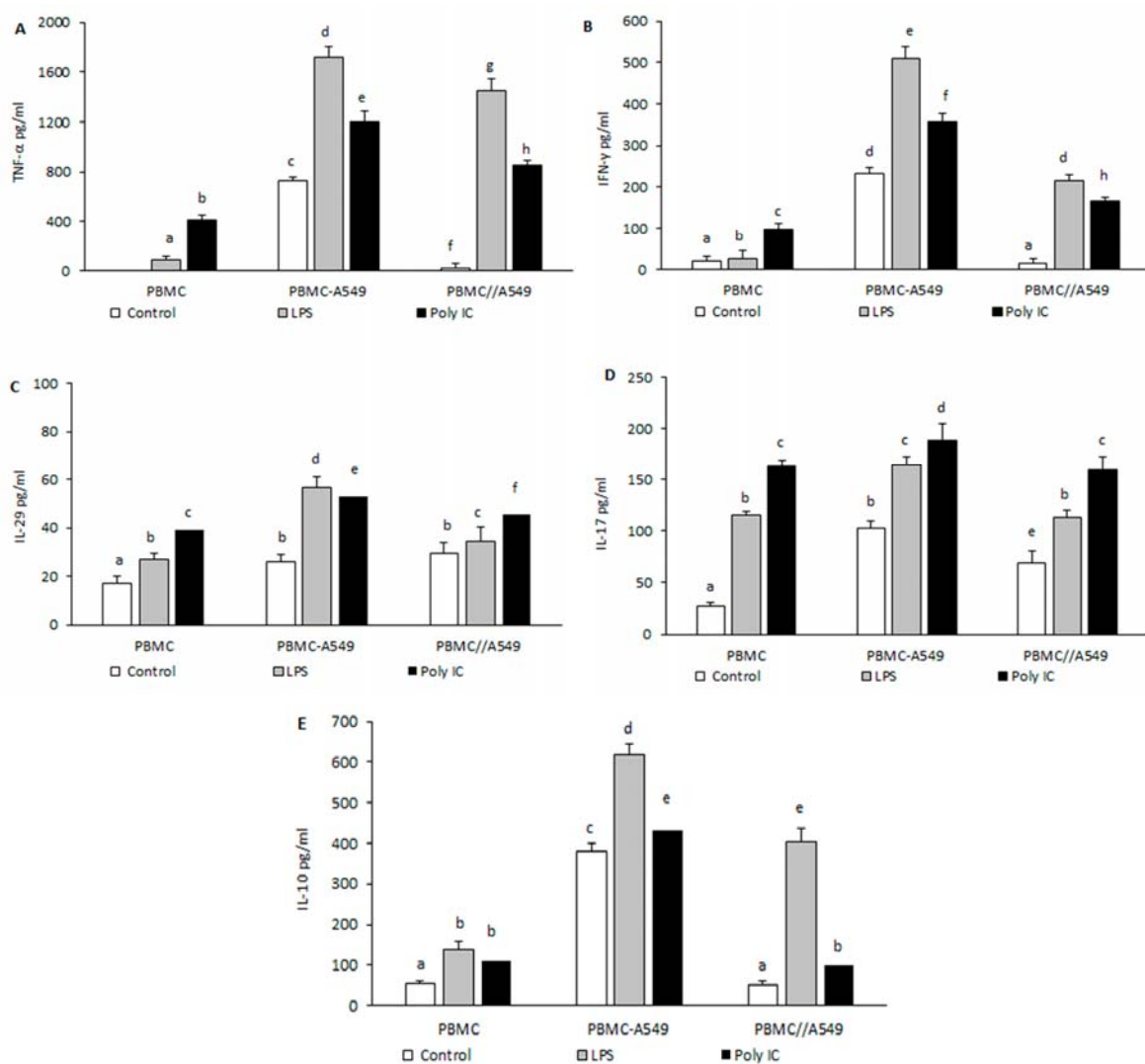
## RESULTS

### **Cytokine patterns induced in PBMCs by stimulation with Poly I:C and evaluation of LcM capacity to modulate this response.**

TNF- $\alpha$ , IFN- $\gamma$ , antiviral IL-29 (type III IFN), IL-17, and regulatory IL-10 cytokines were evaluated in supernatants of PBMCs alone and in supernatants of PBMCs from co-culture systems PBMC-A549 and PBMC//A549. LPS and RPMI were used as positive and unstimulated controls, respectively. Results of cytokines profiles induced by cells stimulation with Poly I:C are shown in Figs. 1A, 1B, 1C, 1D, and 1E. LPS induced high levels of the 5 cytokines mentioned above. Poly I:C induced significantly high levels of inflammatory TNF- $\alpha$ , IFN- $\gamma$ , IL-17, and anti-viral IL-29 cytokines compared with the unstimulated control. While IL-10 levels were different depending on the culture system considered, Poly I:C induced IL-10 values that were only slightly but significantly higher than the unstimulated control.

Overall, the highest cytokine level was obtained in the co-culture PBMC-A549, where cells had a direct contact. Then, in order to determine the modulatory capacity of LcM in our systems of culture cells, stimulation with this non-viable lactobacillus was evaluated (Figs. 2A, 2B, 2C, 2D, and 2E). Results showed that LcM stimulation alone, together with Poly I:C (LcM+Poly I:C) or previous to Poly I:C (LcM (3h)+ Poly I:C), induced lower values of TNF- $\alpha$  and IFN- $\gamma$  in three culture systems compared with those induced by Poly I:C, except LcM+Poly I:C for IFN- $\gamma$  in PBMCs. LcM, LcM+Poly I:C, and LcM (3h)+ Poly I:C induced an increase in antiviral IL-29 in the three culture systems, with the last two stimuli showing the highest IL-29 levels.

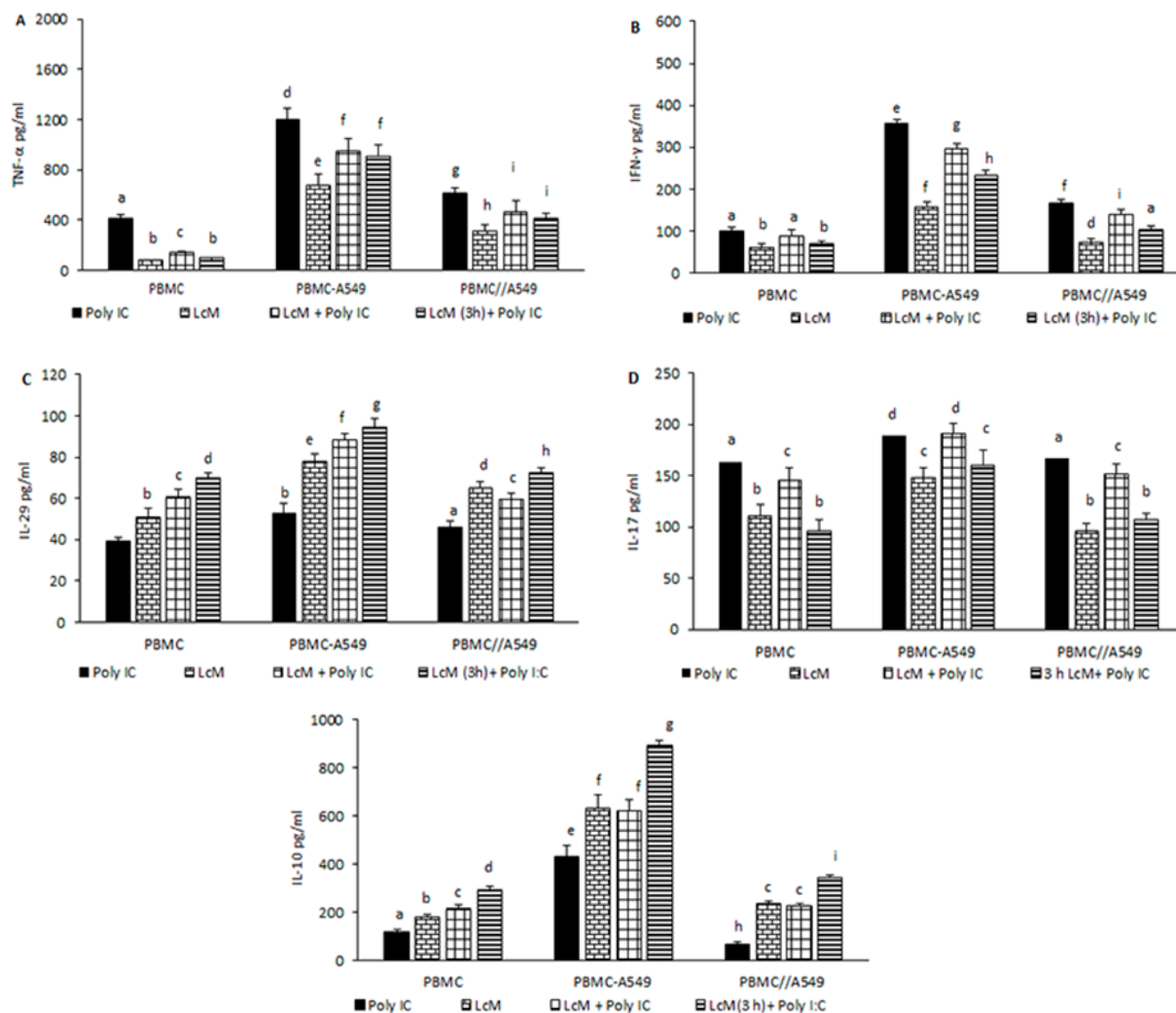
IL-17 showed a different behavior depending on the cell culture system considered. Thus, LcM induced lower IL-17 values than Poly I:C in the three culture systems and LcM+Poly I:C induced a significantly higher level of this cytokine compared with LcM (3h)+ Poly I:C ( $p < 0.05$ ). An important result was observed for IL-10 because of LcM and their combinations with Poly I:C was able to induce the highest levels of this



**Figure 1. TNF-α, IFN-γ, IL-17, IL-29 and IL-10 cytokines production by peripheral blood mononuclear cells stimulated with Poly I:C.** LPS was used as positive control and unstimulated cells were also included: Control (RPMI) in a direct co-culture with A549 (PBMC-A549) and transwells (PBMC//A549). Panel A: TNF-α production, Panel B: IFN-γ production, Panel C: IL-17, Panel D: IL-29 and Panel E: IL-10. Results are expressed as mean ± SD of duplicate measurements determined in four independent experiments. Tukey's test was used to test for differences between the groups. Means in the figure with different letters (a–h) were significantly different (p<0.01).

regulatory cytokine compared with Poly I:C. Moreover, LcM (3h)+Poly I:C induced the highest IL-10 level in all culture systems used. Overall, these results showed an important immunomodulatory activity of LcM on the immune response induced by Poly I:C.

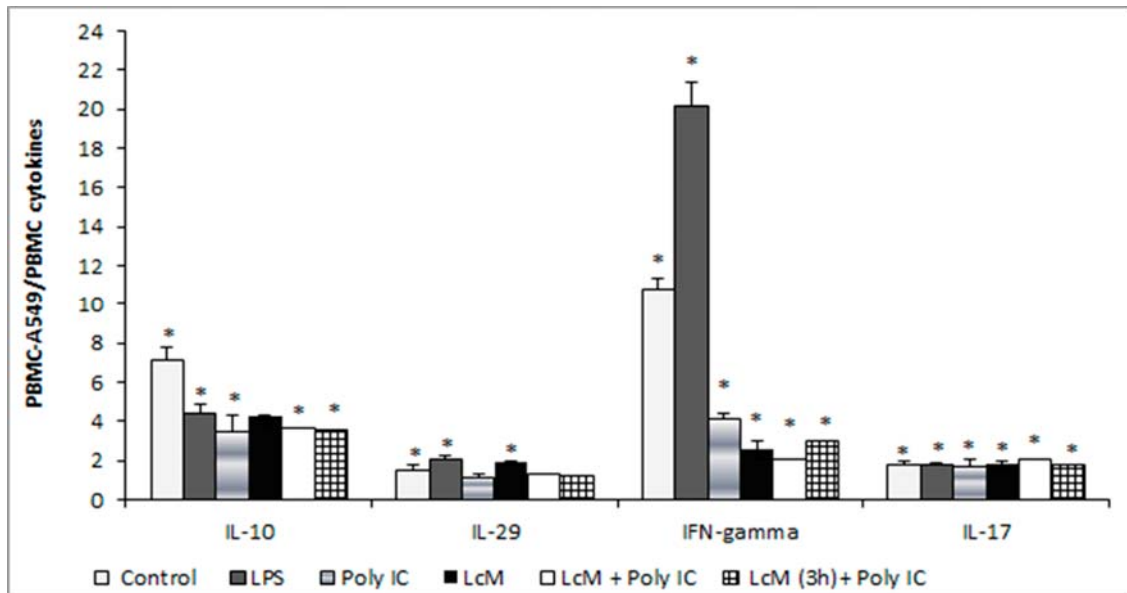
Taking into account the influence of direct interaction of PBMCs and A549 cells (PBMC-A549) on the levels of cytokine release, the ratio for each cytokine was evaluated between PBMC-A549 and PBMCs. Results were expressed as the ratio of cytokines produced by a stimulated co-culture to cytokines produced by stimulated PBMCs



**Figure 2. TNF- $\alpha$ , IFN- $\gamma$ , IL-17, IL-29 and IL-10 cytokines production by peripheral blood mononuclear cells stimulated with A) Poly I:C, B) LcM, C) LcM+Poly I:C and D) LcM (3h)+Poly I:C.** LPS was used as positive control and unstimulated cells also was included: Control (RPMI) in a direct co-culture with A549 (PBMC-A549) and transwells (PBMC//A549). Panel A: TNF- $\alpha$  production, Panel B: IFN- $\gamma$  production, Panel C: IL-17, Panel D: IL-29 and Panel E: IL-10. Results are expressed as mean  $\pm$  SD of duplicate measurements determined in four independent experiments. Tukey's test was used to test for differences between the groups. Means in the figure with different letters (a-i) were significantly different ( $p < 0.01$ ).

The introductions between PBMCs and A549 cells upregulated cytokine production with all stimuli (Fig. 3). LPS stimulation showed the highest ratio for IFN- $\gamma$  production, indicating that stimulation of PBMC/A549 with LPS induced higher levels than LPS stimulation of PBMCs alone. Compared to other stimuli, ratios of IFN production were similar among different stimuli, except for Poly I:C where the IFN ratio was higher than with the other stimuli. The influence of direct contact between two cell types was also observed in TNF- $\alpha$  levels, with a large increase in ratios such as LPS=  $20.4 \pm 4.6$ , Poly I:C=  $2.9 \pm 0.3$ , LcM=  $8.8 \pm 0.9$ , LcM+Poly I:C=  $6.7 \pm 0.6$ , and LcM (3h)+Poly I:C=  $9.5 \pm 1.1$  fold, respectively ( $p < 0.05$ ), with the highest rate for the unstimulated control

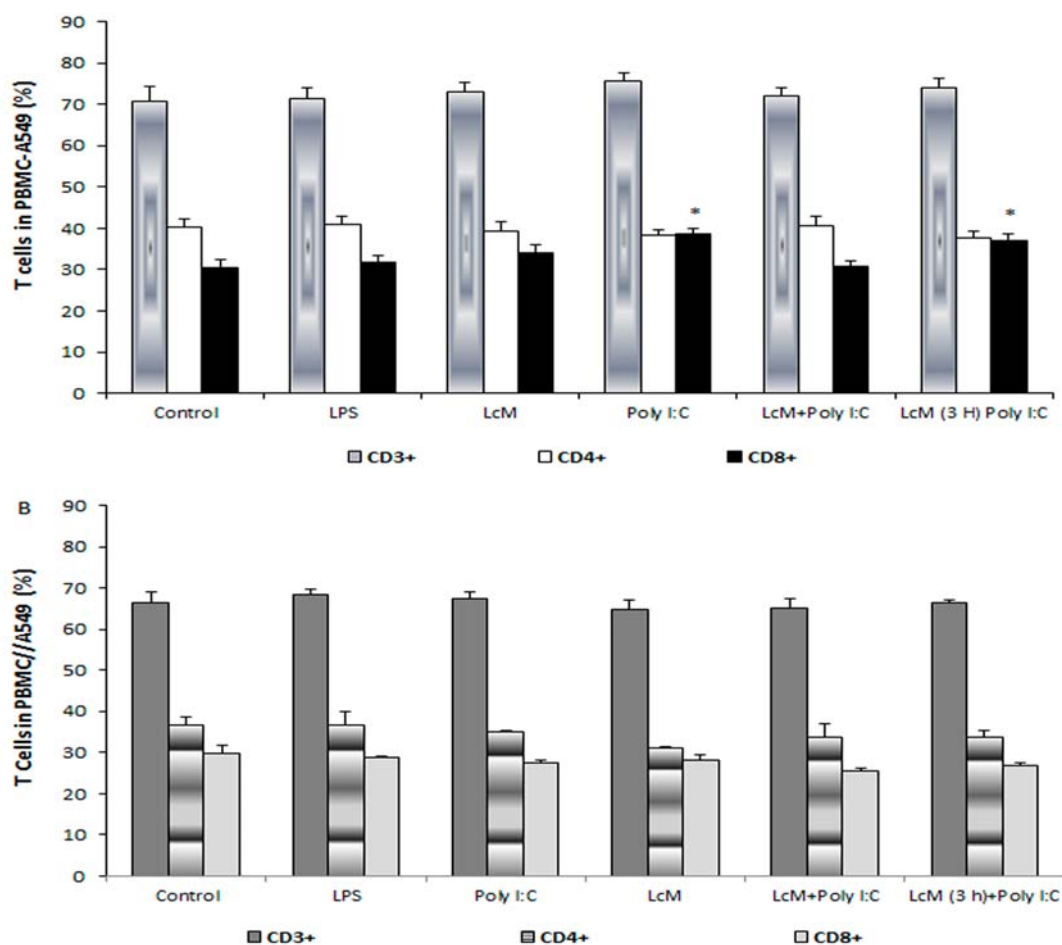
because TNF- $\alpha$  production in PBMCs was not detectable. Accordingly, the ratios for TNF- $\alpha$  were not included in Fig. 3. IL-17 ratios were even higher in unstimulated controls than in stimulated cells. The ratio for regulatory IL-10 cytokine was also higher in unstimulated culture cells. The ratio for IL-29 was higher for LPS stimulation.



**Figure 3.** Comparison of cytokine levels in co-cultures of PBMC and pulmonary A549 cells with PBMCs in response to LcM, Poly I:C, Poly I:C+LcM; Poly I:C (LcM (3h)+Poly I:C) and LPS and RPMI in a direct co-culture with A549 (PBMC-A549) and transwells (PBMC//A549). The y axis represents the ratio of cytokines produced in co-cultures to those produced by PBMC stimulated with the same agent. Values are the means of ratios  $\pm$  standard deviations obtained from five donors. A ratio of 1 corresponds to equal amounts of cytokine in co-cultures and PBMC. Tukey's test was used to test for differences between the groups. The significant differences in rate PBMC-A549/PBMC of each cytokine in function of stimuli were indicated with letters (a-b, a'-b', a $^{\circ}$ -d $^{\circ}$ , a\*-c\*). Means in the figure with different letters were significantly different ( $p < 0.05$ ).

### PBMCs phenotyping by flow cytometry.

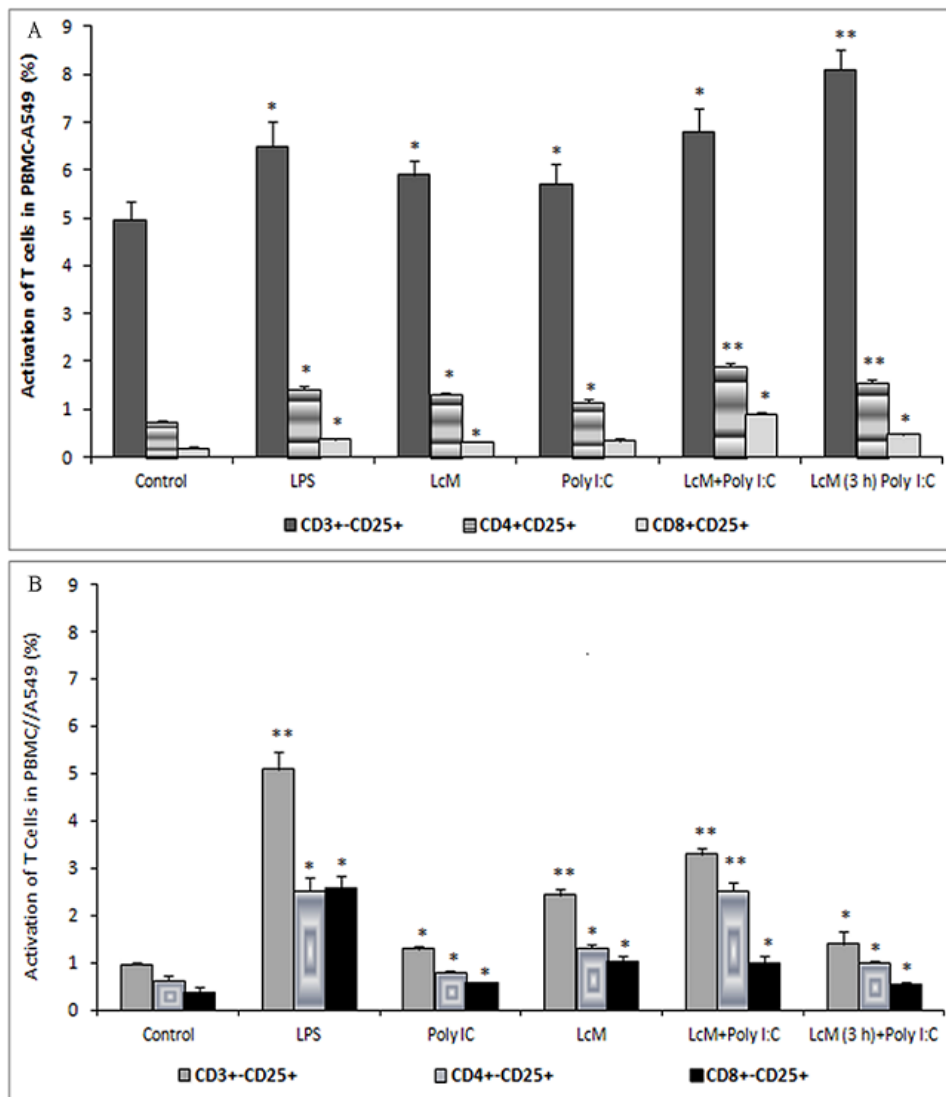
T and NK surface marker expressions and activation induced by different stimuli in PBMCs. In order to evaluate the effect of Poly I:C, LcM and their combinations on the expression of CD3 $^{+}$ , CD4 $^{+}$ , and CD8 $^{+}$  T surface markers and their activation through the CD25 $^{+}$  marker, PBMCs from PBMC-A549 and PBMC//A549 cultures were analyzed. Results showed that Poly I:C, LcM or combinations of both did not induce any changes in the expression of cell surface marker CD3 of PBMCs from PBMC-A549 or PBMC//A549 co-cultures. In this regard the total T population in all treatments showed expression values similar to those of the control (Fig. 4A and B). Similar results were observed for the CD4 $^{+}$  marker. We also determined whether the expression of CD8 (CD8 $^{+}$  T cell=cytotoxic T lymphocytes) was modified. In PBMCs from PBMC-A549, Poly I:C and LcM (3h)+Poly I:C showed a significant increase in the percentage of CD8 $^{+}$  expression ( $p < 0.05$ ).



**Figure 4. Effect of LcM, Poly I:C, combinations of both (Poly I:C+LcM; Poly I:C (LcM (3h)+Poly I:C), LPS and Control (RPMI) in a direct co-culture with A549 (PBMC-A549) and transwells (PBMC//A549) on the expression of surface markers CD3+, CD4+, CD8+. The graph indicates the percentage of cells for each of these subsets. Panel A: CDs expression in PBMC-A549; Panel B: CDs expression in PBMC//A549. Data are expressed as mean  $\pm$  SD values of 5 healthy donors. Duplicate assays were performed for each donor and each stimulus (\* $p$ <0.05, \*\* $p$ <0.01).**

In contrast, no significant differences were found with any of the stimuli in PBMCs when evaluating the CD8+ marker from PBMC//A549 co-culture. In addition, CD25+ was evaluated as an activation marker in T CD3+, CD4+, and CD8+ cells (Figs. 5A and 5B). All stimuli induced significant activation of T CD4+ and CD8+ cells compared with unstimulated PBMCs in both PBMC-A549 and PBMC//A549 co-culture systems and these results were reflected in the activation levels of TCD3+ total population. However, activation percentages were higher in the direct co-culture PBMC-A549 than in PBMC//A549. The influence of direct contact of PBMCs with A549 in the activation of T cells is unquestionable but A549 would also induce activation of PBMCs, possibly through soluble factors; however, further studies should be performed on the subject.

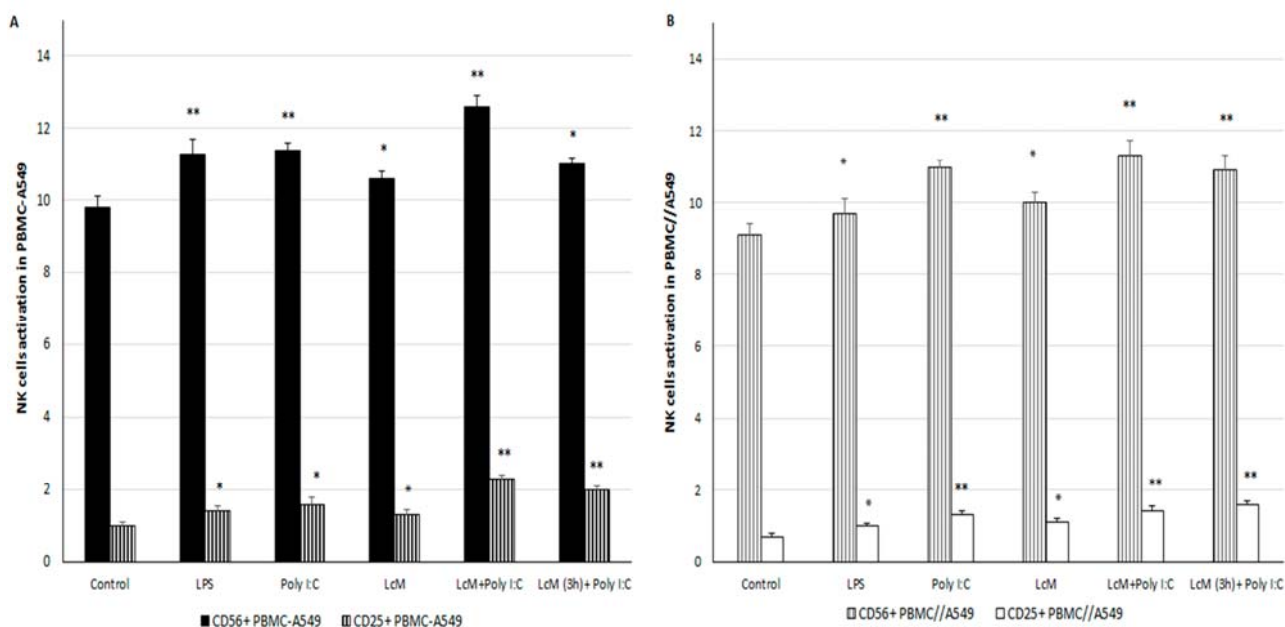




**Figure 5.** Effect of LcM, Poly I:C, combinations of both (Poly I:C+LcM; Poly I:C (LcM (3h)+Poly I:C), LPS and Control (RPMI) in a direct co-culture with A549 (PBMC-A549) and transwells (PBMC//A549) on activation marked CD25+ in peripheral blood mononuclear cells. Panel A: CD25 expression in T cells from PBMC-A549; Panel B: CD25 expression in T cells from PBMC//A549. Data are expressed as mean  $\pm$  SD values of 5 healthy donors. Duplicate assays were performed for each donor and each stimulus (\* $p$ <0.05, \*\* $p$ <0.01).

Since NK cells are the nexus between innate and adaptive immunity and play an important role in antiviral defense, these cells were also evaluated (Fig. 6A and 6B). Results showed that all stimuli induced an increase in CD56+ cells population compared with control in both co-culture systems. NK activation was also evaluated through the CD25+ marker. Results showed that all stimuli increased NK activation in both culture systems, highest activation levels being observed in PBMCs from PBMC-A549. Direct contact between PBMCs and A549 cells influenced CD25+ expression in NK cells (Figs. 6A and 6B). Poly I:C induced a higher NK activation than LcM. Also, a combination of both (LcM+Poly I:C) was able to induce highest CD25 expression in

this population in both co-culture systems. In addition, stimulation with LcM(3h)+Poly I:C induced lower NK activation than LcM+Poly I:C ( $P<0.05$ ) in both systems, suggesting modulation exerted by LcM on the effect of Poly I:C when this viral RNA analog is added to PBMCs.



**Figure 6. Effect of Poly I:C, Heat-killed *Lactobacillus casei* (LcM) and combinations of both (LcM+Poly I:C+, LcM(3h)+ Poly I:C) and LPS on expression of surface markers of NK (CD56+) cells and activation marker (CD25+) in peripheral blood mononuclear cells from PBMC-A549 and PBMC//A549 co-culture. A) Total NK cells (CD56+) and NK activated (CD56+CD25+) of PBMCs from PBMC-A549, B) Total NK cells (CD56+) and NK activated (CD56+CD25+) of PBMCs from PBMC//A549. Graphic indicates the percentage of cells for each of these subsets. Data are expressed as mean  $\pm$  SD values of 5 healthy donors. Duplicate assays were performed for each donor and each stimulus (\* $p<0.05$ , \*\* $p<0.01$ ).**

## DISCUSSION

In this work, we performed an *in vitro* study of some of the main components of the immune system involved in the response induced by stimulation of PBMCs with Poly I:C, evaluating the modulation of this response exerted by non-viable *Lactobacillus casei* (LcM). In addition, the potential of LcM as an adjuvant in mucosal vaccines against RNA respiratory viruses was considered. Two co-culture systems with A549 cells were evaluated. Poly(I:C) challenge in PBMCs can induce changes in the immune response that mimics an acute viral infection (9). Poly(I:C)-induced inflammation was also studied in airway epithelial cell lines including A549, BEAS-2B, and 16HBE14 (18,19,20). Considering that interactions between PBMCs and A549 cells play a key role in the defense against respiratory pathogens (11,12), evaluation of the immune response was performed in a co-culture of PBMCs with A549. Cytokines response was also assessed in PBMCs alone. Direct contact between A549 and PBMCs is extremely

important for cytokine induction and CD marker expressions because the response to stimulation in PBMCs from the PBMC//A549 system was lower for almost all stimuli. With respect to cytokines, cells that produce these mediators play a key role in the modulation of pathological processes in tissue injury and in the pathogenesis of the disease. TNF- $\alpha$ , an inflammatory cytokine, is important in the fight against pathogens; however, its release must be controlled since high TNF- $\alpha$  levels would be harmful to the host. In this study, direct contact between PBMCs and A549 induced highest TNF- $\alpha$  values with all stimuli. A recent study showed that when Respiratory Syncytial Virus (RSV), an RNA virus, infects human bronchial epithelial cells, the heat shock protein HSP72 secreted binds to TLR4 on neutrophils and leads to an increase in IL-8 and TNF- $\alpha$  production (21). Although TNF- $\alpha$  contributes to viral clearance during the early stages of RSV infection, its continued production exacerbates illness and tissue injury during the late stages of infection (22). LcM was able to reduce TNF- $\alpha$  levels when it was employed previous to and simultaneously with Poly I:C in PBMC alone and also in both co-culture systems. This effect is in line with previous reports showing that antiviral respiratory defenses are beneficially modulated by heat-killed immunobiotics (23). Thus, LcM would be useful to reduce damage in lung tissue in ongoing viral infections and would be an advantage when used as an adjuvant in vaccines or as a modulator of the immune response against viral respiratory pathogens in prophylactic formulations. In this sense, although some reports have shown that Poly I:C can be used as an adjuvant in viral vaccines (24), in view of its inflammatory properties, the use of LcM as an adjuvant would be another valuable and safe option to favor immunity against pathogens with the beneficial regulation of the inflammation. With respect to IL-29, the expression of type III IFN such as IL-29 is restricted mainly to epithelial cells, which would account for the higher IL-29 values induced in PBMC-A549 than in PBMCs. Given its production by epithelial cells, IL-29 would contribute to the prevention of viral invasion through skin and mucosa. In addition, type III IFN contributes to the suppression of virus replication and inflammatory cytokine induction in RSV-infected nasal epithelial cells (25,26), which are the first sites of exposure to respiratory viruses. Another cytokine evaluated, IFN- $\gamma$ , is a pleiotropic cytokine able to enhance cellular immune responses through a variety of mechanisms. LcM was able to modulate the levels of IFN- $\gamma$  induced by Poly I:C in both culture systems considered in this work. IFN- $\gamma$  is mainly induced in response to the recognition of infected cells by activated T lymphocytes and natural killer (NK) cells (27). On the other hand, LcM was able to modulate NK activation induced by Poly I:C in PBMC-A549 and PBMC//A549 systems while keeping activation levels higher than control. This effect is important in vaccines because recent works have shown the ability of NK cells to contribute to the effector response after vaccination and to vaccine-induced immunity in elderly individuals (28). Failure to generate a robust NK-cell response, one of the problems observed in studies of infants severely infected with RSV (29), is an important factor to consider in vaccine formulations and in adjuvant selection (30,31). In addition to their direct anti-viral properties, NK cells also play a key role in the priming of adaptive immune responses against a broad range of viral infections. Indeed, the recruitment and activation of IFN- $\gamma$ -producing NK cells to the inflammation site is critical in the subsequent development of effector CD4 Th1 and cytotoxic T lymphocytes (CTLs) response (32). In this sense, LcM also induced activation of CD4<sup>+</sup> and CD8<sup>+</sup> cells, which are key cells in the development of adaptive immunity. T cells, in particular, CD8 T cells, play a central role in the defense response against viral respiratory infections such as RSV (33). Thus,

stimulation of T cells by LcM is another interesting effect that supports its potential use as a mucosal adjuvant in vaccines against RNA viruses.

Th17 cells that produce IL-17 are some T helper (Th) cells that play a key role in the immune response against pathogens. LcM was able to modulate the IL-17 induced by Poly I:C in both systems evaluated. Although IL-17 can be involved in autoimmune diseases, it also plays an important role in host protection against specific pathogens (34,35). The role of IL-17 in antiviral protection has remained controversial so far since it depends on the specific virus in question. In viral infection by RNA viruses such as RSV, IL-17 plays a role in pathogenesis through the modulation of three important mechanisms: hyperfocus production in the airways, alteration of effector CD8<sup>+</sup> T cell responses, and viral clearance (36). In a mouse model of RSV infection, treatment with anti-IL-17 reduced inflammation decreased the viral load and increased RSV-specific CD8<sup>+</sup> T cells compared with antibody-treated control mice. In addition, neutralization of IL-17 was able to reduce mucus production and Th2 cytokines, with decreased viral proteins (36). IL-17 induces the enhancement of chemokine production and granulopoiesis, a fact that might lead to a significant increase in the migration of neutrophilic granulocytes to the inflammation site with damage to the host tissue. In a mouse model of Influenza virus, IL-17 was involved in acute lung injury. IL-17 was critical for weight loss and neutrophil migration and increased in tissue myeloperoxidase (MPO) after influenza infection; however it did not affect the recruitment of CD8 T cells specific for influenza and was not required for viral clearance (37). In this work, LcM and LcM (3h)+Poly I:C induced lower IL-17 levels than Poly I:C in both co-culture systems. Also, the LcM+Poly I:C group showed a tendency to reduce IL-17 values. This fact is important for the potential use of LcM as an adjuvant in mucosal vaccines against viral respiratory pathogens such as RSV and Influenza virus. Within the context of the pro-inflammatory cytokines analyzed above, IL-10 plays a crucial role in the modulation of the immune response induced after infection and after immunization. IL-10 is typically an anti-inflammatory cytokine with regulatory functions on immune cells that also promotes humoral immunity. IL-10 enhances proliferation and differentiation of B cells, prevents their apoptosis, and stimulates MHCII expression in these cells (38), a desirable aspect in vaccine formulations. In addition, IL-10 is able to stimulate NK activity and increase IL-2-1 induced proliferation of CD56<sup>+</sup> cells (39). In this sense, LcM, alone and combined with Poly I:C was capable of increasing IL-10, highest levels being reached with direct contact between A549 and PBMCs. A tight regulation of defense mechanisms against pathogens is vital and the host must balance adequate immune responses to each pathogen while limiting immunopathological responses. IL-10 is able to inhibit disease and inflammation in mice infected with RSV, especially during recovery from infection (40), an important and desirable effect in the design of effective vaccines against this virus. The immune response was induced after vaccination depends on a multiplicity of factors such as preexisting immunity, nutritional status, and genetic and environmental factors. In addition, adjuvant capacity is strain-dependent when lactic acid bacteria are considered. Further studies are underway to evaluate its adjuvanticity against viral proteins in animal models. However, *in vitro* assays using human PBMCs that implicitly include the above aspects and A549 cells, human lung cells, would be useful models to evaluate an experimental mucosal vaccine that includes a non-viable lactobacilli or lactic acid bacteria strains as adjuvants associated with specific antigens. Epithelial cells mediate a wide variety of processes with the goal of limiting airway

inflammation (41), a function that is critical to maintain lung function. The assays conducted in this work demonstrate that cell interaction between PBMCs and pulmonary epithelial cells can be bidirectional and that the differential modulation of cytokines and chemokines is stimulus-dependent. In this context, LcM would contribute to the regulation of the inflammatory response associated with viral respiratory infections. Poly I:C is able to impair host defense against Gram-positive bacterial pathogens, an effect that would be mediated by type I IFNs (42,43). Further studies are necessary to determine if formulations containing LcM would be useful as preventive or therapeutic supplements capable of improving the protective immune response against certain RNA viruses in children. In addition, the use of non-viable *L. casei* presents an important advantage compared to viable bacteria because it would allow the administration of potential vaccines even in elderly people and immunocompromised hosts without the risks associated with live bacteria.

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