# Oral administration of *Lactobacillus acidophilus* alleviates exacerbations in *Pseudomonas aeruginosa* and *Staphylococcus aureus* pulmonary infections

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**Abstract**: *Staphylococcus aureus* and *Pseudomonas aeruginosa* are largely the cause of morbidity and mortality in both hospital and community settings. These pathogens remain the important cause of pulmonary infections in patients with cystic fibrosis with a worldwide prevalence. Although, antibiotics are efficient measures of treating bacterial lung infections, the occurrence of antibiotic resistant bacteria has been encouraging the researchers to explore novel therapeutic approaches. It has been discovered that certain lactic acid bacteria possess protective effects against bacterial and viral respiratory infections. The aim of present study was to investigate the capability of orally administered *L. acidophilus* to ameliorate *S. aureus* and *P. aeruginosa* pulmonary infections. Animals were exposed to aerosol of pathogenic suspension. After 24 hours of infection, *L. acidophilus* treatment was administered orally for 7 consecutive days. Evaluation of tissue bacteriology, histopathology and serum cytokinomics were performed. In parallel, human alveolar A549 cells were utilized to determine possible role of probiotic on pulmonary infections. Oral administration of *L. acidophilus* significantly ($P<0.05$) alleviate lung bacterial load and severity of infection as depicted by our histopathological studies. Results obtained from cytokinomics revealed that pro-inflammatory cytokines induced due to lung infection were suppressed in oral probiotic treatment groups. In addition, treatment with *L. acidophilus* induced murine lung anti inflammatory, IL-10 cytokine level. Current work suggests that orally administered *L. acidophilus* in mice is able to attenuate *S. aureus* and *P. aeruginosa* induced lung cytotoxicity by modulation of host immune response.

**Keywords**: Host immune response, lung infection, lactic acid bacteria, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

## INTRODUCTION

*Staphylococcus aureus* and *Pseudomonas aeruginosa* are major opportunistic and nosocomial pathogens. They are largely the cause of morbidity and mortality in both hospital and community settings. These pathogens remain the important cause of pulmonary infections in patients with cystic fibrosis with a worldwide prevalence. Although, antibiotics are efficient measures of treating bacterial lung infections, the occurrence of antibiotic resistant bacteria has encouraged the researchers to explore novel therapeutic approaches (Nordmann *et al.* 2007; Gould, 2008; Kumarasamy, 2010). Thus, there is a need for the development of potent antimicrobial for the effective treatment of lung infections. Herein we focused on two great medical important human pathogenic bacteria, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. About 30% of the human population is the carriers of *S. aureus* (Graham *et al.*, 2006). It can colonize several niches of the human body and cause a multitude of diseases like pneumonia, osteomyelitis, septicaemia and endocarditis. While normal carriage is asymptomatic, *S. aureus* may invade tissues where it causes diseases ranging from scalded skin syndrome to life threatening conditions such as septicemia (Krut *et al.*, 2003).

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*P. aeruginosa* causes systemic acute infections in patients with weakened immune systems (Lyczak *et al.*, 2000), establishes its chronic infection in the lungs of Cystic fibrosis patients (Lyczak *et al.*, 2002) and initiates chronic inflammation that leads to destruction of the lungs (Pier and *Ramphal*, 2004). *P. aeruginosa* wide range of infections are largely due to the many virulence factors that it produces.

Probiotic lactic acid bacteria are regarded as live micro-organisms which when administered in certain amounts bestow a health benefit on the host (FAO/WHO, 2002). It has been discovered that certain lactic acid bacteria do possess protective effects against bacterial and viral infections in the gastrointestinal and respiratory systems (Goldin and Gorbach, 2008). Administration of probiotics has been associated with lower incidence of ventilator associated pneumonia (Morrow *et al.*, 2010) and reduced respiratory infections. In addition, the immuno-modulatory effects of these probiotics have also been reported in an another study (Meydani and Ha, 2000).

This study was initiated because of the continuously growing problem of antibiotic resistance in these major pathogens of cystic fibrosis. The point of present work was to investigate the capability of orally administered *L. acidophilus* to ameliorate pulmonary infections caused by *S. aureus* and *P. aeruginosa*. Since, limited studies have

demonstrated the connection amongst gut and respiratory tract in animal models.

## MATERIALS AND METHODS

***Microorganisms and growth conditions***

For infection, human bacterial pathogens *(Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853) were obtained from American Type Culture Collection (ATCC), cultured in Luria-Bertani (LB) broth, incubated at 37°C for 24 hours and adjusted to $10^{10}$ CFU/mL. For treatment, *Lactobacillus acidophilus* CMCC 878 were acquired from China Medical Culture Collection (CMCC) cultured in de Mann Rogosa Sharpe (MRS) broth and incubated at 37°C and adjusted to $10^{10}$ CFU/mL.

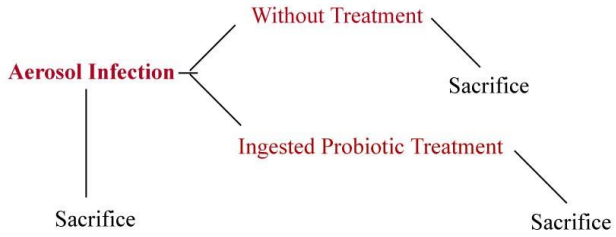



**Fig. 1**: Schematic representation of study outline

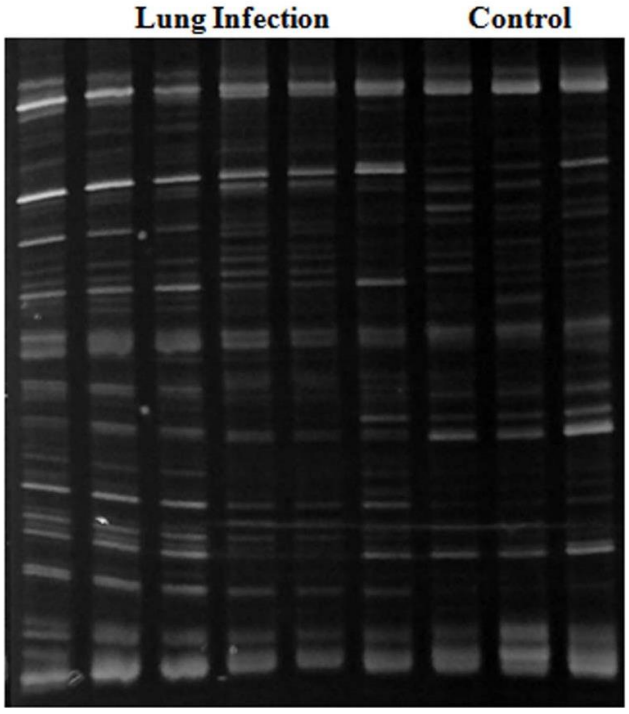
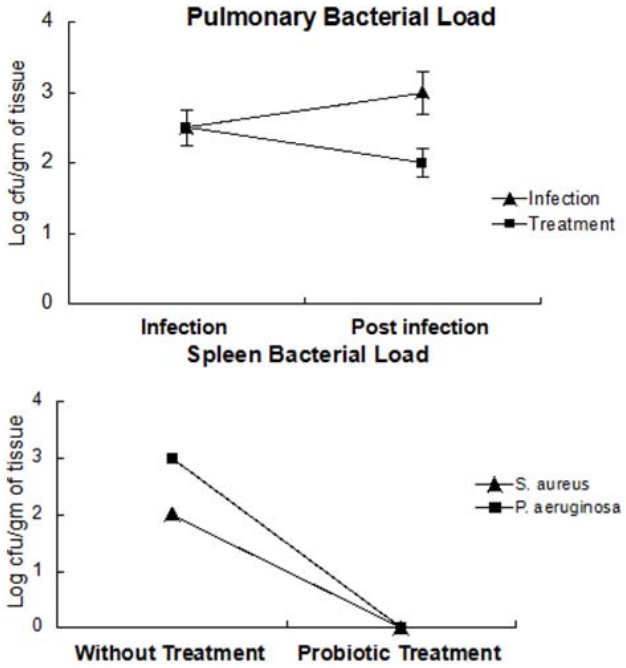



**Fig. 2**: Denaturing Gradient Gel Electrophoretic Analysis (Biodiversity) following *P. aeruginosa* lung infection

***Experimental animals***

30 male C57BL6 mice weighing 200±2g of SPF grade were supplied by Animal Lab Center of Dalian Medical University. All mice were housed in a standard facility, allowed unrestricted access to water and food and distributed into five groups: Control, *S. aureus* infected group, *S. aureus* infected followed by probiotic treatment group, *P. aeruginosa* infected group and *P. aeruginosa* infected followed by treatment group.




(Level of significance $p<0.05$)

**Fig. 3**: Detection of tissue bacterial load

***Infection model***

For single aerosol infection, mice were placed in a nebulizing chamber and exposed to the aerosol of bacterial suspension ($10^{10}$ CFU/mL saline) for 30 min with 5 min intervals. Stool sample from each animal was collected and DNA was extracted to perform Denaturing Gradient Gel Electrophoresis (DGGE) for the analysis of intestinal microbiome diversity. Probiotic treatment with *L. acidophilus* was started at second day post infection and was administered orally at a concentration of $10^{10}$ CFU/mL for 7 consecutive days as exhibited in fig. 1.

***Tissue bacteriology***

Mice left lung and spleen from all groups were excised aseptically, homogenized in saline, serially diluted and plated onto Mannitol salt agar (for *S. aureus*) and Cetrimide agar (for *P. aeruginosa*). Plates were incubated at 37°C for 48 to 72 hours and CFU/gm of tissue was determined.

***Histopathological studies***

Randomly selected right lungs from the mice were used for lung histopathology, fixed in formalin buffer (4% formaldehyde, pH 7.0) and then embedded in paraffin wax and cut into 5µm sections. Hematoxylin and eosin staining was used to stain sections as previously described (Liu *et al.*, 2011); (Zhu *et al.*, 2012).

**Table 1**: Sequences of primers used in the study

| Gene | Sequence (5' - 3') |
|---|---|
| β-actin | CCAAGGCCAACCGCGAGAAGATGAC |
| | AGGGTACATGGTGGTGCCGCCAGAC |
| TNF-α | ATGAGCACTGAAAGCATGATCCGG |
| | GCAATGATCCCAAAGTAGACCTGCCC |
| iNOS | TGGAATTCACTCAGCTGTGC |
| | GATGTTGTAGCGCTGGACG |
| IL-6 | GTCTCCTCATTGAATCCAGATTGG |
| | AGCTCAGCTATGAACTCCTTCTC |
| IL-10 | TTGCCAAGCCTTGTCTGAGAT |
| | TTCTCCCCCAGGGAGTTCAC |
| IL-12 | TGGAGCACTCCCCATTCCT |
| | TGCGCTGGATTCGAACAA |

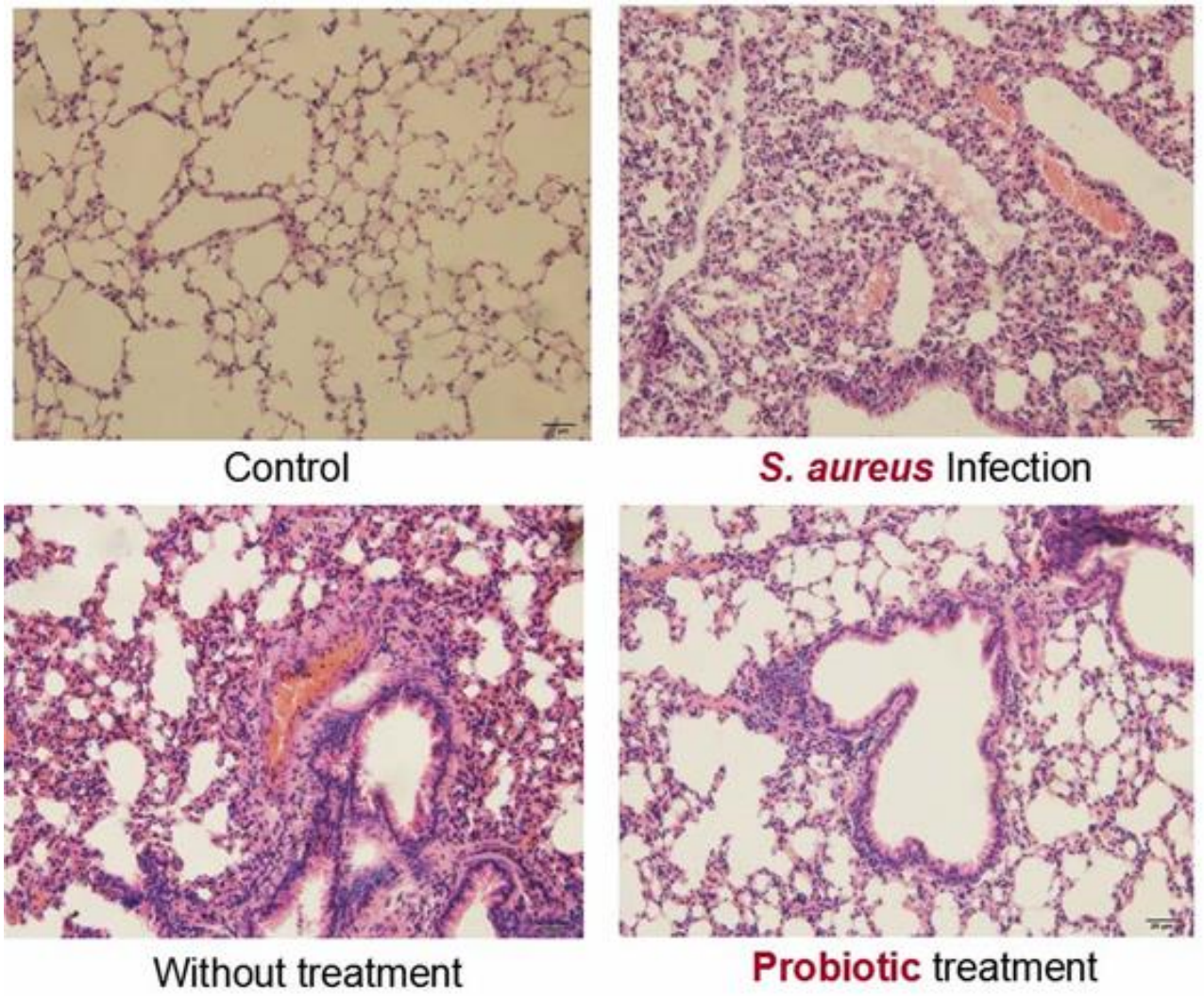



**Fig. 4A**: Histopathology of *S. aureus* pulmonary infection (HnE was used to stain sections, Bar indicates 20μm)

To demonstrate the expression and localization of TNF-α, immunohistochemistry was used as described earlier (Liu *et al.*, 2011). Lung sections were incubated with TNF-α, (anti-mouse, 1:200, Abcam) and detected with the secondary antibody (Bioscience, China).

***Nitric oxide (NO) detection***

Lung nitric oxide content was determined by using NO detection kit (Nanjing Jiancheng Bioengineering Institute) according to manufacturer protocol.

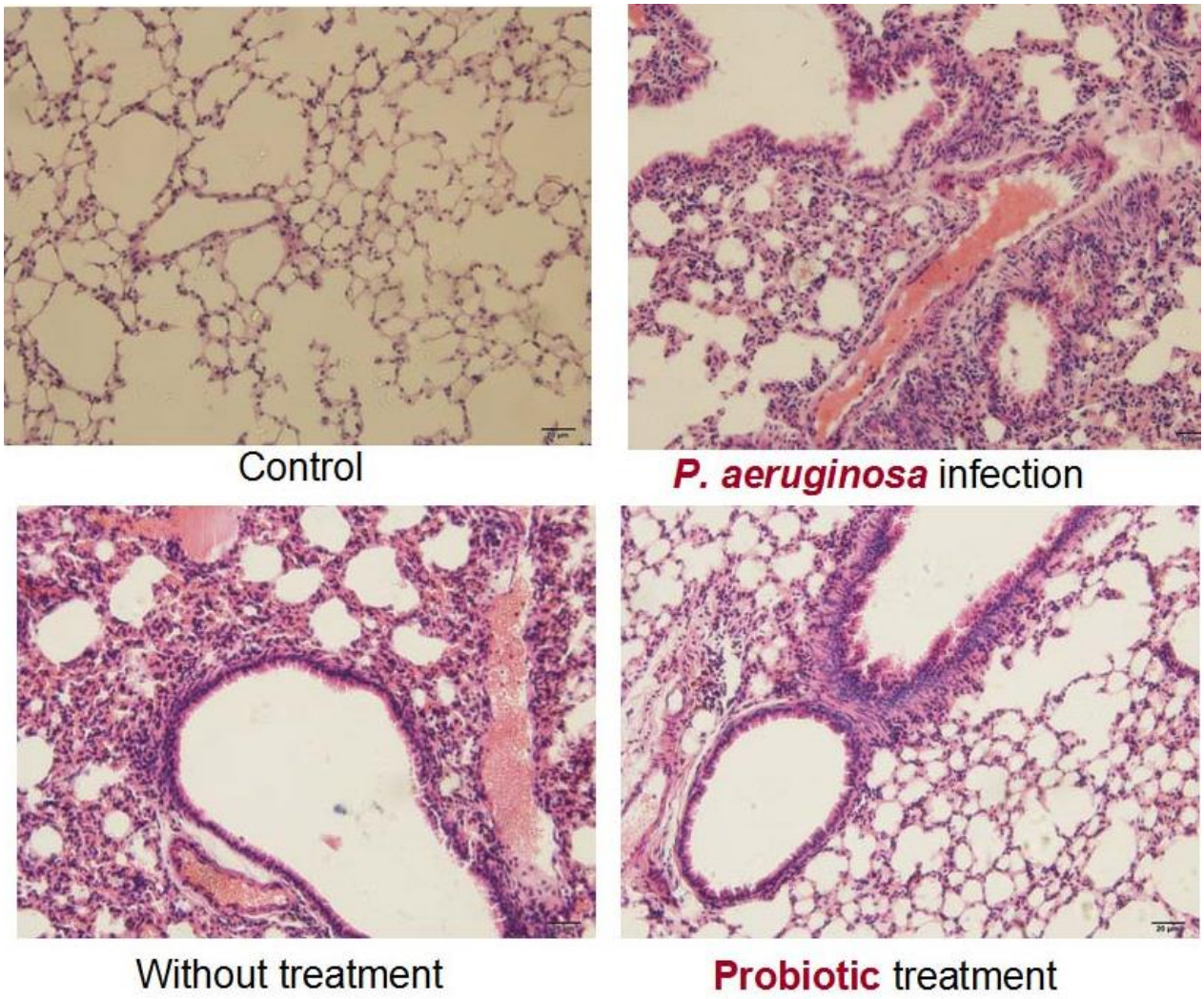



**Fig. 4B**: Histopathology of *P. aeruginosa* pulmonary infection (HnE was used to stain sections, Bar indicates 20µm)

***Measurement of serum cytokines***

Serum was extracted from the peripheral blood, centrifuged at 3000 rpm for 15 min at 4 $^0$C and stored at -80$^o$C until analysis. Levels of TNF-α, IL-6 and IFNγ were measured using cytometric bead assay kit (Becton, Dickinson and Company) in accordance with the manufacturer instructions.

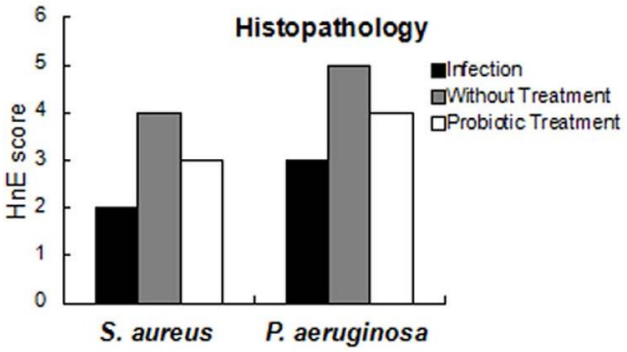



**Fig. 4C**: Histopathology score

***Cultivation of A549 cells***

The A549 human type II alveolar epithelial cell line (CCL-185) was used in this study. Epithelial cells were maintained in DMEM medium (Thermofisher Scientific) supplemented with 10% heat inactivated fetal bovine serum and 100 µg/mL of penicillin-streptomycin (Invitrogen) to 75% confluency in 5% $CO_2$ incubator at 37°C. Cells were washed in PBS (3×) and transferred into the culture medium without serum-antibiotic for 2 hours before infection and then used for adhesion experiments.

***Bacterial adherence***

Bacterial pellets were suspended in serum-antibiotic free DMEM and adjusted to $10^8$ CFU/mL prior to the adherence assay. A549 cells were infected with pathogens in the presence or absence of *L. acidophilus* in 24-well plate for 24 hours. Trypan blue dye exclusion determined the average number of viable cells per well, and this was used to obtain the multiplicity of infection (MOI) of 1:10. Following incubation, non adherent cells were washed with PBS (3×) and then adherent cells were detached by

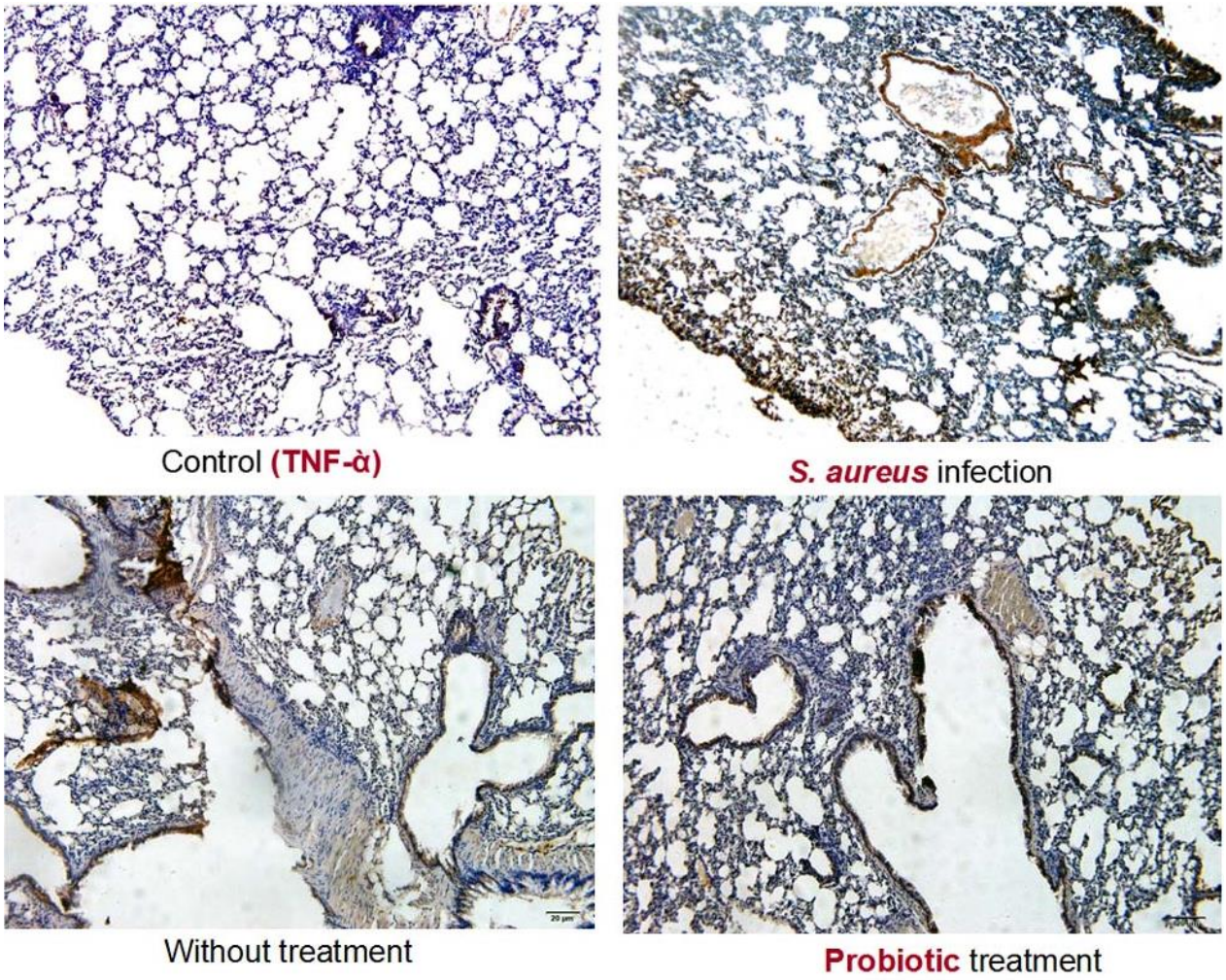



**Fig. 5A**: Expression and localization of TNF-α of murine lung infected by *S. aureus* (Localization of TNF-α was detected by using immunohistochemistry, Bar indicates 20μm)

using trypsin-EDTA solution (0.5% porcine trypsin and 0.2% EDTA in PBS, Sigma, USA) along with 0.2% Triton X-100. The mixture of bacterial cells was diluted and plated onto LB plates to achieve number of adhered bacteria (Hawdon *et al.* 2010).

***RNA extraction and RT-PCR***

Total RNA was extracted from A549 cells by using RNAiso plus. The concentration of RNA was determined by nano drop and cDNA was synthesized by using cDNA kit (Transgene) according to manufacturer protocol. The mRNA expression of TNF-α, IL-6, IL-10, IL-12 and iNOS was determined by RT-PCR (Schnupf and Sansonetti, 2012). Table 1 exhibits the sequences of the primers used for RT-PCR.

***Measurement of cytokines by ELISA***

Cell culture medium was removed following infection with *S. aureus* and *P. aeruginosa* strains in the presence and absence of *L. acidophilus* at concentrations of $10^8$ CFU/mL. Centrifugation was performed at 10,000 rpm for 10 min at 4°C and supernatant was stored at -80 $^{o}$C until analysis.

Levels of IL-10 and IL-12 were measured using an ELISA kit (R&D Systems) in accordance with the manufacturer instructions.

***Ethical approval***

The animals were supplied by the Animal Lab Center of Dalian Medical University. They were housed in a standard facility, allowed unrestricted access to water and food The study was approved by Ethical Committee of Dalian Medical University.

## STATISTICAL ANALYSIS

Data are presented as mean standard deviation. For bacterial load, student *t* test and for ELISA and CBA studies, one way ANOVA was utilized by using SPSS software 19.0 to evaluate differences between means. $P< 0.05$ was taken as the level of significance.

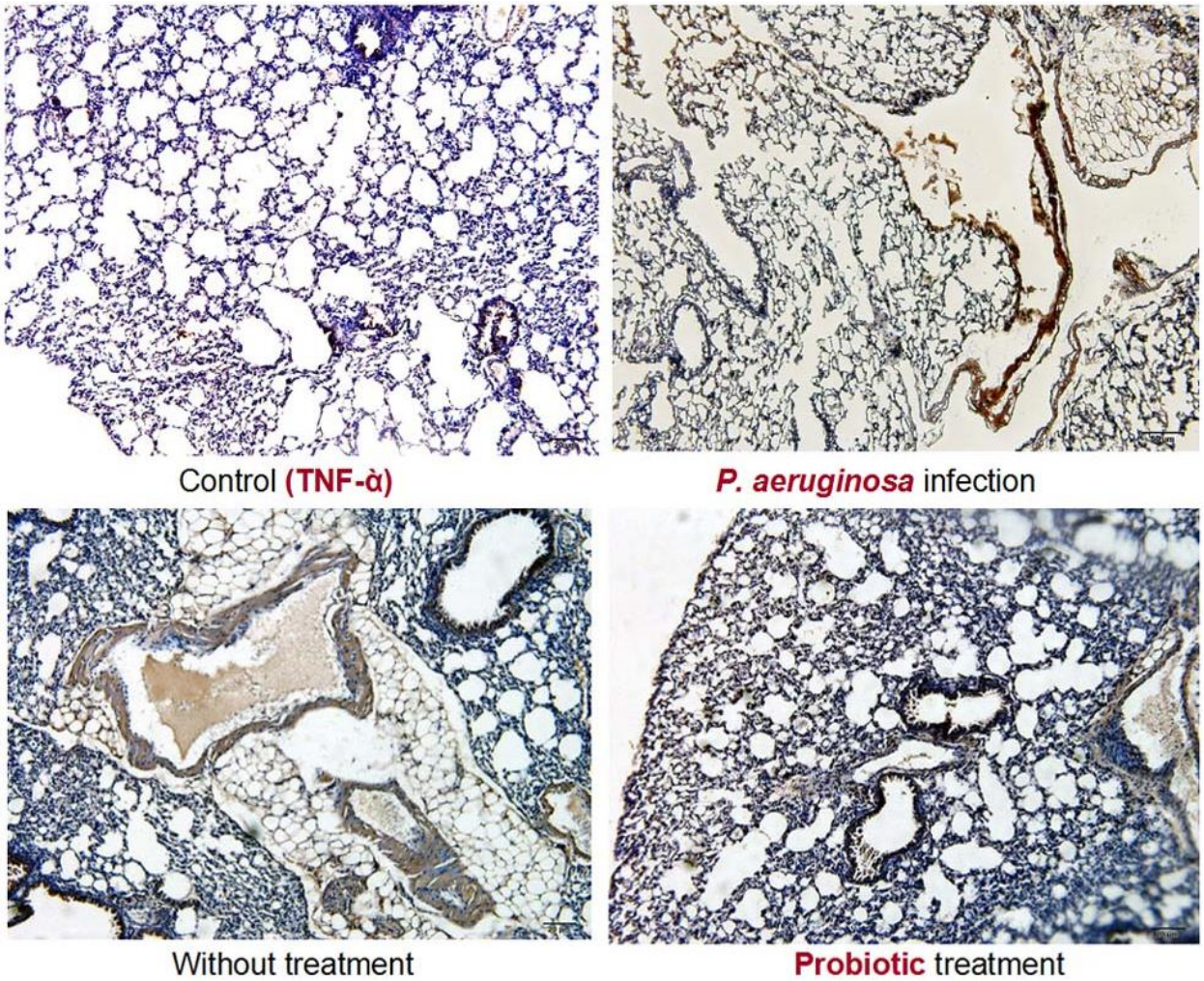



**Fig. 5B**: Expression and localization of TNF-α of murine lung infected by *P. aeruginosa* (Localization of TNF-α was detected by using immunohistochemistry, Bar indicates 20μm)

## RESULTS

***Animal general status***

Changes in the general state and weight can somehow indicate the disease state and recovery of animal. Normal and treated mice were agile and active, and had natural breathing. Feces were hard and dark brown. It was found that infected animals had reduced activity, their feces gradually became softer and intestinal microbial imbalance was observed as depicted in fig. 2. After *S. aurues* lung infection, stool samples were also positive for this bacterium showing bacterial translocation.

***L. acidophilus alleviates pathogenic count***

Oral introduction of *L. acidophilus* significantly ($P<0.05$) alleviates pulmonary pathogenic count more than in group that did not receive any treatment. Notably, after infection, we found the presence of pathogen in spleen showing bacterial translocation. But the treatment group was devoid of pathogens in the spleen as shown in fig. 3.

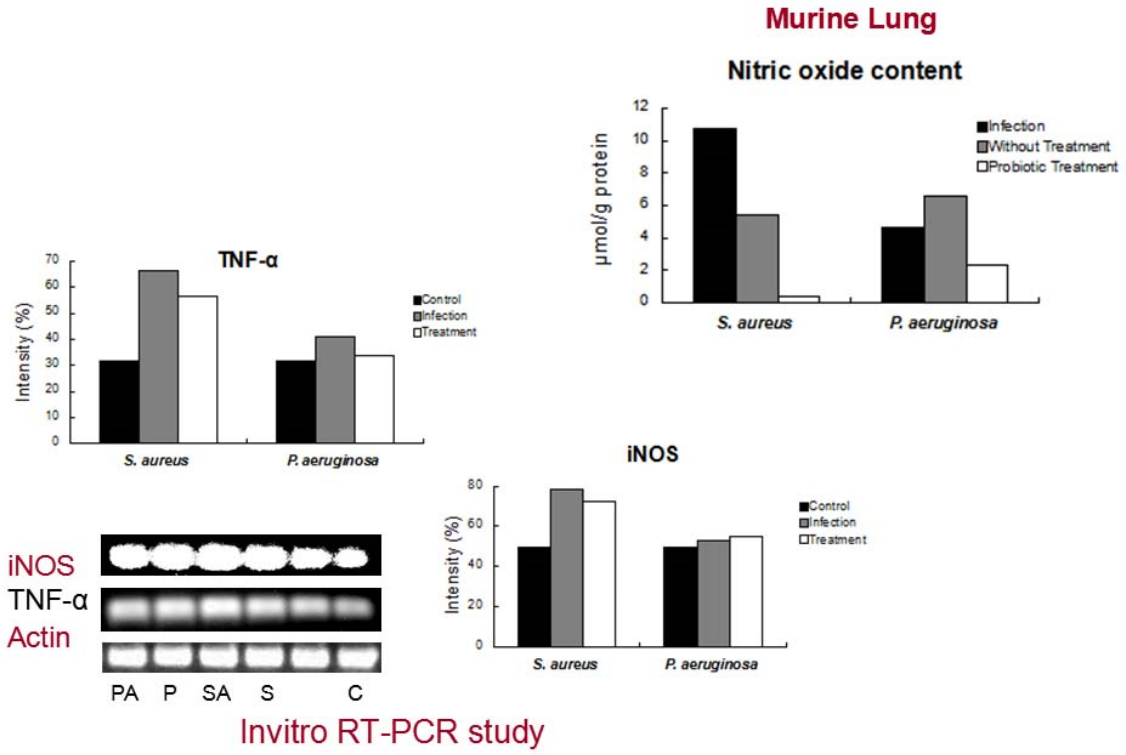
***Oral probiotic treatment reduces severity of infection***

Results obtained from histopathology indicated that *P. aeruginosa* pulmonary infection was more destructive and severe as compared to *S. aureus* infection. Alveolar walls became widen; edema and interstitial pneumonia were observed. Severity of infection was reduced in the groups that received oral probiotic treatment as depicted by histopathology score (fig. 4).

TNF-α is the hallmark of infection and inflammation. fig. 5 exhibits localization of TNF-α in murine infected lung sections. These results are concordant with our *in vitro* RT-PCR results that demonstrated reduce % intensity of TNF-α in the presence of *L. acidophilus* treatment (fig. 6).

***Levels of NO and iNOS expression***

Reduced pulmonary nitric oxide content was observed in groups that received oral probiotic treatment. Further, our *in vitro* RT-PCR results also exhibited lower iNOS expression in case of treatment with *L. acidophilus* (fig. 6).




(Control = C, infections of *S. aureus* = S and *P. aeruginosa* = P, treatment with *L. acidophilus* in case of *S. aureus* infection = SA and in case of *P. aeruginosa* infection= PA)

**Fig. 6**: Pulmonary pathological studies

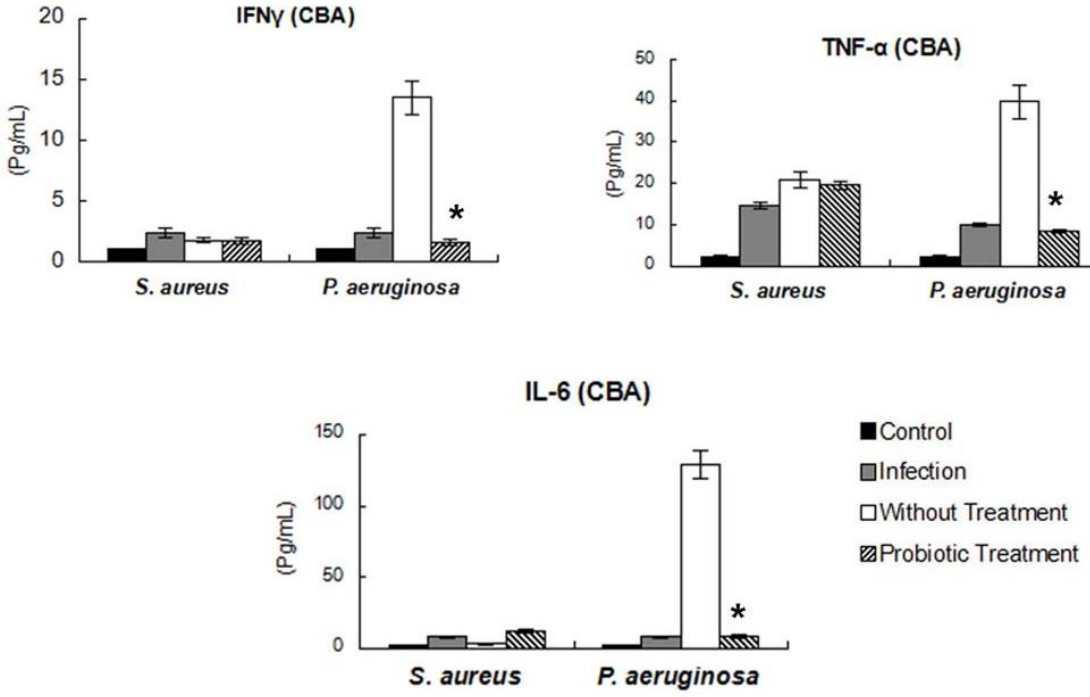



**Fig. 7**: Serum cytokinomics using cytometric bead assay (CBA)

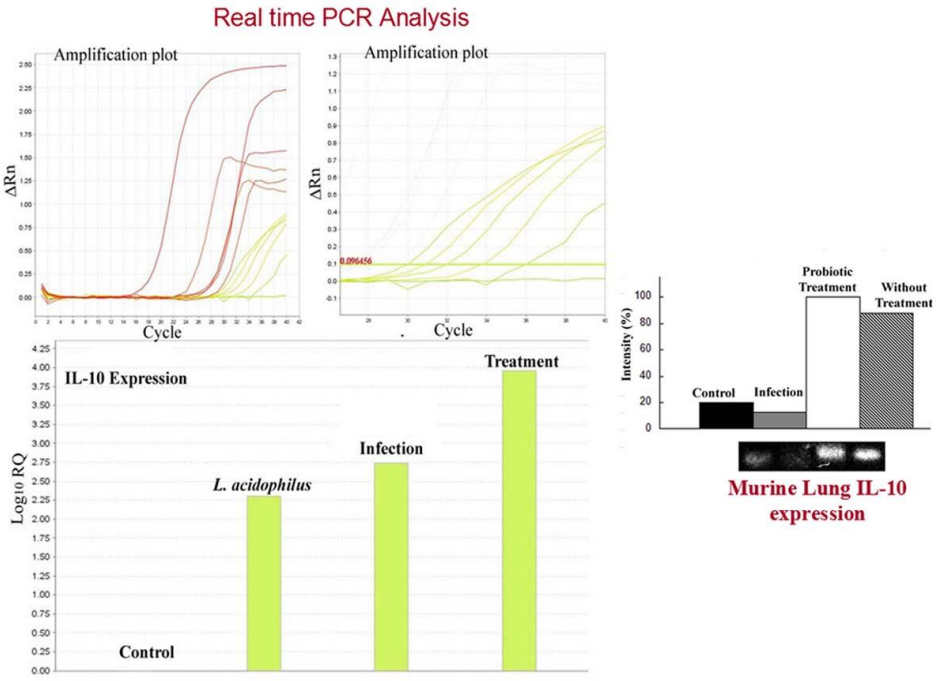
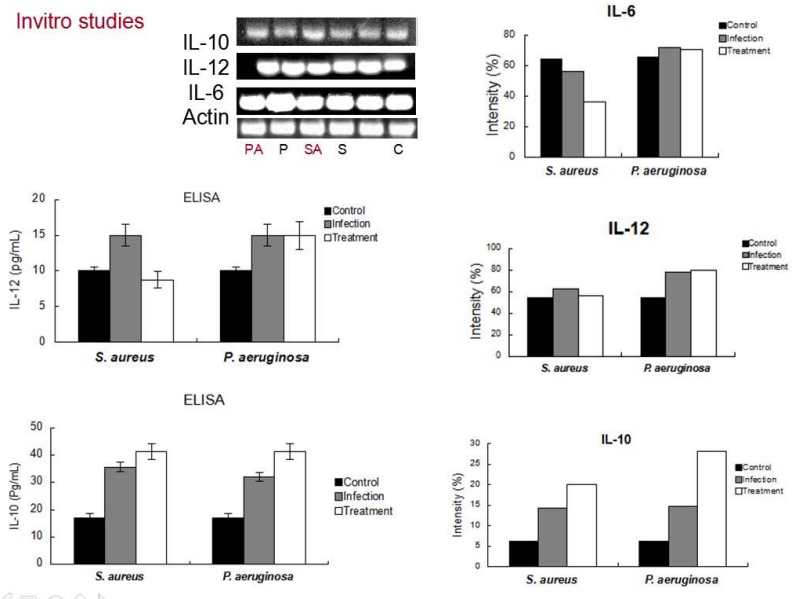



**Fig. 8**: RT-PCR analysis of IL-10 expression




(Control = C, infections of *S. aureus* = S and *P. aeruginosa* = P, treatment with *L. acidophilus* in case of *S. aureus* infection= SA and in case of *P. aeruginosa* infection = PA, * $P < 0.05$)

**Fig. 9**: In-vitro RT-PCR and ELISA studies

***Host cytokinomics***

With a specific end goal to decide the mechanism of action of *L. acidophilus*, we prepared animal serum and *in vitro* cell culture supernatant to evaluate host immune response against infection. It was found that pro-inflammatory cytokines induced due to pulmonary infection were suppressed in the presence of oral probiotic treatment (fig. 7). Moreover, treatment with *L. acidophilus* induced anti-inflammatory, IL-10 cytokine tissue levels (fig. 8) which was further confirmed by our *in vitro* studies as depicted in fig. 9.

## DISCUSSION

*S. aureus* and *P. aeruginosa* remain the important cause of respiratory tract infections in patients with cystic fibrosis with an overall pervasiveness. Lung infection by these

pathogens also caused gastrointestinal microbial dysbiosis as displayed by our DGGE analysis. The result of this study demonstrates that ingested *L. acidophilus* can weaken lung cytotoxic impact initiated by *S. aureus* and *P. aeruginosa*.

Innate immune mechanisms are essential in determining the outcome of infections caused by many bacterial pathogens. Peptidoglycan and lipoteichoic acid (LTA) of Gram positive bacteria induce cell activation mediated by TLR 2, followed by production of pro-inflammatory cytokines (Shwandner *et al.* 1999). While lipopolysaccharide (LPS) of Gram negative bacteria is associated with TLR 4, which regulates the expression of various cytokines and chemokines that are involved in immune responses (Zhang and Ghosh, 2001). Lactic acid bacteria modulate the immunity of the host by interacting with epithelial cells and modulating the secretion of anti-inflammatory cytokines which results in clearance of pathogens and alleviation of inflammation. Previous murine study indicates that ingested *L. casei* or yogurt has been shown to stimulate the activity of alveolar macrophages and increase clearance of respiratory *P. aeruginosa* (Alvarez *et al.* 2001).

The characteristic feature of inflammatory response to bacterial pulmonary infection is the migration of PMNs towards site of damage (Baltimore *et al.* 1989), resulting in the up-regulation of pro-inflammatory cytokines. The levels of IL-6 in serum have been found to be elevated in a number of inflammatory diseases (Kishimoto, 2010) and together with TNF α, it has been well known inflammatory marker. Moreover, pulmonary infections with *P. aeruginosa* and *S. aureus* are hallmarked by recruitment of neutrophils. This recruitment is mostly due to the generation of IL-8 chemokine. In present study, suppression of these pro-inflammatory cytokines by probiotic bacteria would result in a reduction of recruitment of innate immune cells towards site of infection, which could impact the level of exacerbations.

Level of nitric oxide is regarded as an important mediator of physiological and pathophysiological processes (Chen *et al.* 2003). Obtained results from current work suggest that the elevated pulmonary NO content is attributed to enhance expression of iNOS detected by RT-PCR which is associated with severity of lung damage. Further, iNOS expression was reduced in case of probiotic treatment. Previous study reported that iNOS knockout or wild type mice treated with selective iNOS inhibitor are protected against liver injury (McKim *et al.* 2003).

IL-10 is an important regulator of the immune system and it has been shown to be anti-inflammatory in many model systems (Asadullah *et al.* 2003). *Lactobacilli* are reported to induce regulatory IL-10 production (Zeuthen *et al.*, 2006). Current study clearly indicates raised levels of this cytokine which may have contributed to anti-inflammatory response. These results are in agreement with previous murine study which demonstrates intra-gastric administration of *L. lactis* to secrete IL-10 causes 50 % reduction in colitis (Steidler *et al.* 2000).

Data obtained from our IL-12 cytokine study shows that there is no significant impact of *L. acidophilus* on *S. aureus* and *P. aeruginosa* infections. These results are contradictory to some previous studies which demonstrate that orally administered *Lactobacilli* can affect the production of IL-12 (Blobe *et al.* 2000); (De Moreno *et al.* 2005). Exactly how oral administration of *L. acidophilus* enables to protect murine lungs from developing *S. aureus* and *P. aeruginosa* infections remain unclear. However, stimulation of host immune function by lactic acid bacteria may have contributed to the increased clearance of these resistant pathogens. Recent studies suggest that probiotics may possess anti-inflammatory properties. Likewise, treatment with probiotic in case of *H. pylori* induced enteric inflammation was improved (Canducci *et al.* 2000).

Taken together, our results with murine study exhibit that ingested *L. acidophilus* significantly improved pulmonary bacterial clearance, reduced bacterial translocation and lung pathology, probably by modulation of host immune status.

## CONCLUSION

*S. aureus* and *P. aeruginosa* are considered as major pathogens of cystic fibrosis lung. Our work suggests that orally administered *L. acidophilus* in mice is able to attenuate lung cytotoxic effect induced by *S. aureus* and *P. aeruginosa* probably by modulation of host immune response.

Oral lactic acid bacterial treatment could be useful for the respiratory patients since probiotic bacteria possess many beneficial effects and have no known side effects. However, further studies with extended group and controlled clinical trials are required to take asset from this group of bacteria.

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