Original Article

Nonopsonic phagocytosis of Lactobacilli by mice Peyer's patches' macrophages

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The ingestion of lactobacilli is of great importance for the probiotic effect of host gut Peyer's patches (PPs) macrophages. The present study is in time focus on the investigation of the factors determining the ingestion of lactobacilli by PPs macrophages. Physicochemical properties of cell surface and adhesive property of nine *Lactobacillus* strains were examined in the present work. The association of the bacteria with PPs macrophage was checked with macrophage monolayers on coverslips. The influence of lactobacilli on macrophages phagocytic capacity was also investigated with a neutral red uptake assay *in vitro*. The results show that the macrophages could ingest lactobacilli in a strain dependent manner, and the most ingested strain is *L. plantarum* Lp6 compared to other tested strains, which displayed strain specific enhancement on the phagocytic activity of PPs macrophages. And there is no correlation between the physicochemical or adhesive properties of the cell surface and the ingestion. The association of *L. plantarum* Lp6 with PPs macrophage could be decreased by Protease K treatment. Surface proteins of *L. plantarum* Lp6 could promote the ingestion of fluorescent latex beads by PPs macrophages. In conclusion, the hydrophobicity of the cell surface might not be the key factor determining the association of lactobacilli with PPs macrophages. Cell surface proteins are the media for the binding L. plantarum Lp6 to macrophages.

Key Words: lactobacilli, immune-modulating effect, Peyer's patches, macrophages

Introduction

Lactobacilli are considered as nonpathogenic commensals of human gut flora and are believed to be beneficial to human health. Stimulation of the immune system is one of the health promoting or probiotic effects of lactobacilli [1]. Some of the probiotic dairy products have been demonstrated to possess the effect of enhancing immune functions and thus reducing the risk of infection.[1]

The components of lactobacilli cell with immune modulating properties are lipoteichoic acids (LTA), CpG-DNA and peptidoglycans, named commensal associated molecule pattern (CAMP). These molecules could interact with toll like receptors (TLRx) on the surface of antigen presenting cells (APCs).[2] Lactobacilli-APC contacts are important for inducing immune response through CAMP-TLRx signal pathway and maintaining host immune homeostasis. However, it is relative low for the affinity of CAMP with particular TLRx.[3] Other molecule on APC or bacteria might help to increase the affinity of CAMP for a particular TLRx. Luminal antigens gain access to the mucosal lymphoid tissues via the Peyer's patches (PPs) in the small intestine.[4] Macrophages are key antigen presenting cells (APCs) that concentrated within the follicular epithelium and subepithelial zone of PPs.[5] Interaction of lactobacilli with PPs macrophages might be essential for the induction of immune response.

It has found that the mucosal immunostimulation induced by lactobacilli varied with the strain being studied [6] or their growth phases.[7] This might be related to different interaction of lactobacilli with PP macrophages. However, it is still obscure for the relationship between particular bacterial features and the biological activity. In a preliminary study, we found that it is strain specific for the efficiency of PP macrophages to uptake lactobacilli. This study is therefore aimed to determine the factors influencing the interaction of *Lactobacillus* strains with PPs macrophages.

Methods

Strains, culture media and incubation conditions

The *Lactobacillus* strains used in the study (Table 1) were maintained as glycerol stocks stored at -70°C. Bacteria were incubated in Mann Rogosa Sharpe (MRS) broth for 18 h at 37 °C, harvested by centrifugation (10000 × g), and washed twice with phosphate buffered saline (PBS, pH 7.3).

Microbial adhesion to hexadecane (MATH) test

The MATH assay was performed as described previously.[8] The fraction of bacteria adhering to the hexadecane/water interface (θ) was calculated as:

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$$\theta = \frac{A_0 - A_1}{A_0 - A_b} \times 100$$

where A_0, A_1, and A_b were the optical densities at 600 nm of the initial bacterial suspension, the extracted solution, and the blank, respectively.

Determination of zeta potentials

Zeta potentials of the *Lactobacillus* strains were determined with a Zetasizer 3000 (Malvern Instruments Ltd., Malvern, United Kingdom) as described previously.[9]

Preparation of rat small intestinal mucus

All the animal studies reported here adhered to the accepted standards (local and national regulations) of humane animal care. Twenty healthy female Sprague-Dawley rats (Shanghai slac Co., Shanghai, China) weight 100-125 g were anaesthetized with diethyl ether and killed by cervical dislocation. Small intestinal mucus was released by gently scraping the mucosa, homogenized and centrifuged at 12000 × g for 30 min (4°C). The mucus was precipitated twice from the clear supernatant with pre-cooled ethanol, suspended in ultra pure water and lyophilized. Mucus was dissolved in 50 mM Na_2CO_3 buffer (pH 9.7) to 1 mg/mL, centrifuged for 15 min (5000× g, 4°C), and the supernatant was pooled for further use.

Adhesion assay

Microtiter wells were coated with mucus. 200 μL of bacterial suspended in PBS containing 0.01% Tween 20 (PBST) (1×10^8 CUF/mL) was added to each well and incubated overnight at 4°C. The wells were washed with PBST and stained with safranin after the wells were dried. A_{490nm} was measured in an ELISA plate reader (Labsystems, Helsinki, Finland).

Peyer's patches macrophages collection

Peyer's patches macrophages were prepared as described previously.[10]

Bacteria-macrophage association assay

Macrophages were incubated for 4 h in 35-mm-diameter tissue culture dishes, each containing a glass coverslip (22 by 22 mm) attached to the bottom, at a ratio of 5×10^5 macrophages per dish. Culture medium was replaced with 1 mL of bacteria suspension (bacterium/macrophage=50:1) and incubated for 45 min. Coverslips were gently washed with PBS and air-dried. Macrophages were fixed and stained for direct microscopy counts in monolayers. The assay was also conducted for protease K (1 mg/mL in PBS, 1 h) treated *L. plantarum* Lp6.

Phagocytosis of fluorescent latex beads by PPs macrophages

Cell surface proteins of *L. plantatum* Lp6 were extracted with 5 M LiCl as previously described [11]. Cell surface proteins were added to green-fluorescent latex beads (Sigma) suspension (1×10^8 beads per mL in PBS) to 50 μg/mL, gently agitated 3 h, washed twice with PBS and stored on ice. Aliquots of 100 μL beads suspensions (10^6 beads/mL) were added into 96-well plates wells inoculated with macrophages (2×10^4 cells/well). The interaction were conducted for 1 h at 37°C in 5% CO_2, stopped on ice (5 min) and the cells were centrifuged (300×g, 5 min, 4°C). Non-cell-associated beads were removed and the cells were washed twice with ice-cold PBS. The fluorescence was measured in a microplate multimode detector (Anthos-Mikrosysteme, Austria) at 485 nm excitation and 530 nm emission wavelengths and also determined using fluorescence microscopy (Olympus, Hamburg, Germany).

Uptake of neutral red by PPs macrophages

Phagocytic capacities of PPs macrophages were assayed as described previously [12]. The result was recorded with an ELASA reader using a test wavelength of 540 nm.

Statistical analysis

The data were expressed as mean ± SD (n = 6). Results were analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison using SPSS version 10.0 (SPSS Inc., Chicago, Illinois, USA) with a significance level of $p<0.05$. Unpaired t test was performed to analyze the difference between macrophages uptake of surface protein-coated bead samples and the controls; protease K treated bacteria and the control.

Results

Table 1 shows that the ingesting efficiency was dependent on the tested *lactobacillus* strains. *L. plantarum* Lp6 was ingested most greatly among test strains. Lactobacilli show strain specific activity in enhancing the phagocytic activity of PPs macrophages. The activity was significantly positively correlated with the ingestion of the bacteria by PPs macrophages (r=0.83013, p=0.00561).

All tested strains show medium hydrophobicity, among which the smallest and highest value was determined on *Lactobacillus sp* strain 1 and *L. plantarum* Lp6, respectively (Table 1). However, the statistical difference among strains is not significant. Except for *L. plantarum* Lp5, all strains have high negative surface charge. *L. plantarum* Lp5 and *L. sp.* strains 1 had the highest and lowest net hydrophobic attractive force, but the macrophages did not ingest them in the highest and lowest level, respectively. *L. plantarum* Lp6 is the strain that shows the highest uptake by PPs macrophages, although this strain shows medium net hydrophobic attractive force. Therefore, there is no direct relationship between physicochemical surface properties and macrophage ingestion of lactobacilli in the present study.

Adhering ability of nine strains of lactobacilli to rat small intestinal mucus was studied. Table 1 shows adhesion is specific for distinct strain. *L. plantarum* Lp6 has best adhesion. No relationship was found between adhesion and the macrophage ingestion of tested lactobacilli.

Proteinase K treatment could significantly decrease macrophage ingestion of *L. plantarum* Lp6 ($p<0.01$) (Fig 1, Fig 2), suggesting that the bacterial surface proteins might mediate the association of *L. plantarum* Lp6 with PPs macrophages. This was further confirmed by the phenomenon that the surface protein of *L. plantarum* Lp6 could enhance the interaction of latex beads with PPs

Table 1. Adhesion, physico-chemical surface properties and ingestion by mice small intestinal PPs macrophages (PM) of lactobacilli †.

Strains	Source	hydrophobicity (%)	Zeta potential (mV)	Adhesion (A_{492})	Ingestion (/100 PM)	Phagocytic capacity (%) ‡
L. sp. strain 1	Human feces	7.52±4.40	-15.8±1.3	0.10±0.01*	23.64±6.57	123.38±22.09
L. sp. strain 2	Sauerkraut	13.32±7.18	-10.7±2.2	0.02±0.01	22.08±12.77	120.77±21.35
L. plantarum Lp1	Sauerkraut	10.89±7.42	-16.4±1.0	0.07±0.01	18.74±3.82	106.49±4.69
L. plantarum Lp2	Sauerkraut	14.60±6.12	-16.86±2.0	0.04±0.02	17.19±4.54	110.39±3.97
L. plantarum Lp3	Sauerkraut	10.91±9.66	-16.4±2.1	0.10±0.01	28.41±2.15	109.09±9.09
L. plantarum Lp4	Yoghourt	14.54±2.60	-16.9±2.3	0.03±0.02	28.53±5.13	112.99±13.08
L. plantarum Lp5	Yoghourt	15.15±3.56	-0.46±2.9	0.09±0.01	39.39±4.77	114.29±2.60
L. plantarum Lp6	Yoghourt	16.51±0.55	-15.24±1.2	0.14±0.03*	53.00±11.10**	149.35±26.53*
L. Bulgaricus	Yoghourt	13.01±2.89	-13.3±1.86	0.05±0.02	48.70±7.28**	150.65±2.60*

† Values are means ± SD of three separate experiments. ‡ The result was expressed as relative uptake of neutral red by lactobacilli stimulated PP macrophages. The mean value obtained from the control (PBS) was defined as 100%. * $p < 0.05$, significantly different after Tukey's multiple comparison after One-way ANOVA analysis. ** $p < 0.001$, significantly different after Tukey's multiple comparison after One-way ANOVA analysis.

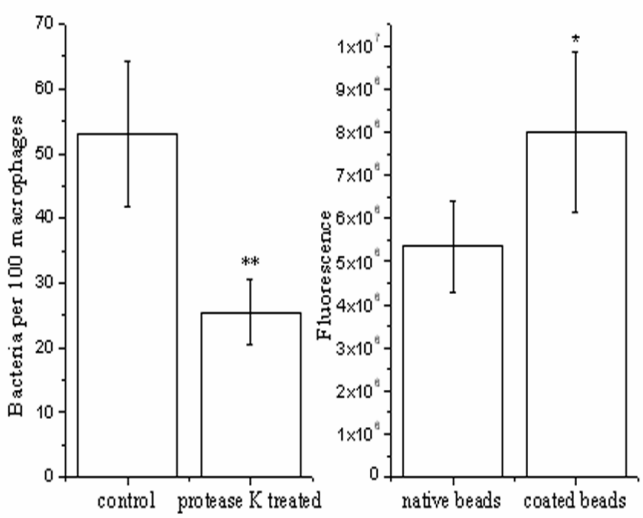

Figure 1. Role of surface protein in ingestion of *Lactobacillus plantarum* Lp6 by mice small intestinal PPs macrophages. Left: number of bacteria ingested per 100 PPs macrophage were significantly decreased after the bacteria were treated with Protease K for 1 h. Right: ingestion of fluorescent latex beads was significantly increased after the beads were coated with surface protein or *L. plantarum* Lp6 extracted with 5 M LiCl. * $p < 0.05$, significantly different from control (unpaired t test). ** $p < 0.01$, significantly different from control (unpaired t test).

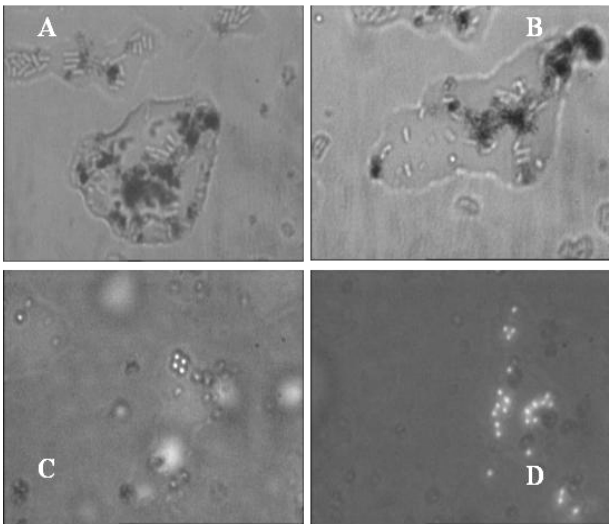

Figure 2. Ingestion of *Lactobacillus plantarum* Lp6 or fluorescent latex beads by mice small intestinal PPs macrophages. Mice small intestinal peyer's patches macrophages on microscope slides were incubated for 1 h with *L. plantarum* Lp6 (A) or the bacteria pre-incubated with protease K for 1 h (B) and stained with Wright-Giemsa and examined under oil-immersion microscopy (1000×). The macrophage were also incubated with fluorescent latex beads (C) or the beads coated with surface protein of *L. plantarum* Lp6 extracted with 5 M LiCl (D), Cells were washed and cytocentrifuged onto microscope slides, and were examined under fluorescence microscope (400×).

macrophages.

Discussion

The interaction may take place between consumed or commensal lactobacilli with PPs macrophages in M-cell pockets, possibly via limited bacterial translocation through the epithelial barrier.[13] The present study reveals that Lactobacillus strain with better macrophage binding ability could enhance phagocytic capacity of the latter much better, which might attribute to the stronger interaction of bacterial CAMP with their receptor on macrophages. It has found that numerous TLRx ligands specifically enhance phagocytosis of macrophage. Binding of lactobacilli to macrophages might be essential for interaction of CAMP with TLRx. In mammals, phagocytosis is vital for a variety of biological events, including tissue remodeling and the continuous clearance of dying cells and invaded pathogenic bacteria. Bacteria ingested by macrophage are able to trigger the release of antimicrobial agents to kill extracellular bacteria. This implicates the importance of efficiency that PP macrophage ingests lactobacilli.

Association of lactobacilli with PPs macrophages might depend on either nonspecific or specific mechanism.

The former is determined by bacterial cell surface physico-chemical properties. The hydrophobicity is related to nonopsonophagocytosis of several bacteria, and a high level of hydrophobicity facilitates phagocytosis.[14-15] Whereas, negative charges on bacteria cell surface would induce electrostatic repulsion between the bacteria and phagocytic cells.[16] Therefore, the net hydrophobic attractive force between the bacteria and macrophages might be more important. However, physicochemical properties was not related to association of PPs macrophages with the tested lactobacilli in the present study. Therefore, other factors might be more important such as specific interaction. The ingestion might also be related to the surface property of macrophages because the ingestion was not related to adhesion ability of the lactobacilli.

Considerable evidence shows that specific recognition between phagocytes and their targets may be accomplished by the interaction of carbohydrate-binding proteins, e.g. lectins, on the surface of one type of cell that combine with complementary sugars on the surface of another in a lock-and-key manner.[17] *L. plantarum* Lp6 was the strain that showed an excellent adhesion and a good feature for being ingested by macrophage. We previously found that this strain adheres to mucus through specific recognition of mannose residues by its lectin like protein.[18] It was also found this protein mediated the interaction of the bacteria with PP macrophages in the present study. This reveals there are some specific mannose residues on surface of PP macrophages that could interact with bacterial lectins. On the other hand, macrophages have been observed to possess specific carbohydrate receptors that mediate phagocytosis.[19-20] Among these receptors GlcNAc/Man lectins specific for N-acetyl-glucosamine, mannose, glucose, and L-fucose are widely expressed on tissue macrophages. Lectin typing study revealed that lactobacilli express different surface carbohydrate.[21] The glycocalyx of *L. plantarum* have mannose, glucose and N-acetyl-D-glucosamine. These might also interact with GlcNAc/Man lectins in ingestion of *L. plantarum* Lp6.

In conclusion, different ingestion of lactobacilli might be one of the reasons that lactobacilli share strain dependent immune modulating activity. Ingestion of *L. plantarum* Lp6 was related to the bacterial surface proteins. More related study would provide a basis for understanding the different behaviors exhibited by lactobacilli.

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