Assessment of Safety of Lactobacillus Strains Based on Resistance to Host Innate Defense Mechanisms

Takashi Asahara,1 Masatoshi Takahashi,1 Koji Nomoto,1* Hiroo Takayama,1 Masaharu Onoue,1 Masami Morotomi,1 Ryuichiro Tanaka,1 Teruo Yokokura,1 and Naoya Yamashita2

Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo 186-8650,1 and Department of Pediatrics of the School of Medicine, Keio University, 35 Shinanomachi, Shinjuku, Tokyo 160-8582,2 Japan

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Seven Lactobacillus strains belonging to four species were evaluated for pathogenicity as well as for in vitro sensitivity to the bactericidal mechanisms of macrophages in a rabbit infective endocarditis (IE) model. Two bacteremia-associated strains, L. rhamnosus PHLS A103/70 and L. casei PHLS A357/84, as well as the L. rhamnosus type strain and the probiotic L. rhamnosus strain ATCC 53103, showed moderate infectivity, and the virulence of the probiotic L. casei strain Shirota and type strains such as L. acidophilus ATCC 43566 and L. gasseri DSM 20243 in the model was negligible. The strains that showed pathogenic potential in the rabbit IE model (PHLS A357/84, PHLS A103/70, and ATCC 53103) were more resistant than strain Shirota to intracellular killing activity by mouse macrophages in vitro and also to bactericidal nitrogen intermediates, such as nitric oxide and NO2− ions. These results suggest that resistance to host innate defense systems, which would function at inflammatory lesions, should be considered in the safety assessment of Lactobacillus strains.

The widespread use of lactic acid bacteria (LAB) in fermented foods and dairy products has a long history, and most strains are considered commensal microorganisms with no pathogenic potential. Members of the genus Lactobacillus in particular have been shown to inhabit the human gastrointestinal tract, and the long history of safe use of many species of lactobacillus gave them generally-recognized-as-safe status (13, 23).

However, some cases of local or systemic infections, including septicemia, meningitis, and endocarditis, due to LAB have been reported, but they account for only a very small number of bacteremia cases (the incidence of causation is 5 to 15% for enterococci and 0.1% for lactobacilli) (12, 21, 22). Moreover, infective endocarditis, meningitis, and endocarditis, due to LAB have been reported, but they account for only a very small number of bacteremia cases (the incidence of causation is 5 to 15% for enterococci and 0.1% for lactobacilli) (12, 21, 22). Moreover, most LAB strains linked to clinical cases belong to the species Enterococcus faecium and E. faecalis, but a few belong to L. rhamnosus, L. casei or L. paracasei, and L. plantarum (1, 8). Infective endocarditis (IE) is the most common infection to be associated with lactobacilli, and recent reports show that relatively restricted species of Lactobacillus, such as L. casei and L. rhamnosus, are the most commonly associated with IE, although the frequency of occurrence of Lactobacillus endocarditis is very low, as evidenced by previous studies (for reviews, see reference 8). A European Union-sponsored workshop organized by the Lactic Acid Bacteria Industrial Platform examined these infections and judged that in all cases reported thus far, only patients with abnormal heart valves or immunocompromised states appeared to be infected with the patients’ own indigenous lactic acid bacteria. The workshop concluded that the risk of infection due to all LAB, excluding enterococci, is very low when they are ingested (1, 17). Nevertheless, the workshop also proposed that the safety of each strain should be checked by appropriate methods.

In the present report, we describe the results of the assessment of the safety of seven Lactobacillus strains belonging to four species. Infectivity in a rabbit experimental IE model (3, 6, 7), in vitro resistance to intracellular killing by mouse macrophages, and in vitro resistance to nitrogen free radicals were tested.

Seven Lactobacillus strains were used. Two strains (L. casei PHLS A357/84 and L. rhamnosus PHLS A103/70) which had been isolated from patients with IE were obtained from the United Kingdom Public Health Laboratory Service (PHLS) (10). Two probiotic strains, L. casei strain Shirota and L. rhamnosus strain ATCC 53103, were used. Three type strains of Lactobacillus, namely, L. rhamnosus ATCC 74697, L. acidophilus ATCC 43568, and L. gasseri DSM 202437, were used. The species of Lactobacillus strains was determined by DNA-DNA hybridization technique. Staphylococcus aureus strain IE-1 and Streptococcus mitis strain IE-2, which had been isolated from IE patients at Keio University hospital, Tokyo, Japan, were used. Listeria monocytogenes EGD was used as the resistant control against intracellular killing by macrophages. Lactobacilli were cultivated in MRS broth (Difco Laboratories, Detroit, Mich.) at 37°C for 24 h in an anaerobic atmosphere of 7% H2 and 5% CO2 in N2. S. aureus IE-1, S. mitis IE-2, and L. monocytogenes EGD were incubated in brain heart infusion (BHI) broth (Difco) at 37°C for 24 h. Cultures were washed three times by centrifugation in phosphate-buffered saline (PBS) (pH 7.3) and resuspended to an optical density at 600 nm of 0.9 ± 0.1 (approximately 109 CFU/ml), which was used in all assays.
Specific-pathogen-free male Japanese white rabbits (Kita-
yama Rabesu Co. Ltd., Ina, Japan) weighing approximately 2.5
kg were anesthetized, and the external jugular artery on the
right side of the neck was exposed. A polyethylene catheter
with an external diameter of 1.0 mm (Atom Medical Co. Ltd.,
Tokyo), filled with sterile saline, was passed down the artery
and tied in place when the tip reached the level of the left side
of the heart. The cervical end of the catheter was sealed and
buried when the wound was closed with silk sutures. The final
position of the catheter tip was in the left ventricle. The rabbits
were not further disturbed for 7 to 10 days, during which time
small sterile vegetations composed of platelets and fibrin
formed on the tricuspid valve or the endocardium at points of
contact with the catheter. Each Lactobacillus strain, S. aureus,
or S. mitis (inoculum doses are shown in Table 1), in a 1.0-ml
volume, was injected into the marginal ear vein of rabbits with
nonbacterial endocarditis at 24 h after catheterization. Blood
was drawn from the opposite marginal ear vein at intervals as
required, and 0.1-ml portions were spread on MRS agar plates
(Difco) for lactobacilli or BHI agar plates (Difco) for S. aureus
and S. mitis. Then the animals were killed by intravenous
injection of sodium pentobarbital (Dinabot Co. Ltd., Osaka),
and small pieces of liver, spleen, and vegetation at the heart
valve were removed, weighed, and then homogenized in 1.0 ml
of sterile saline solution. A 0.1-ml volume of the organ homo-
genates was spread on the agar plates.

Both S. aureus and S. mitis, which had been isolated from IE
patients, induced lethal infection when only 2 \times 10^9 and 3 \times 10^9
cells of the bacteria, respectively, were injected into pre-
catheterized rabbits (Fig. 1E to F). The challenge injection
killed four out of five rabbits (S. aureus) and three out of four
rabbits (S. mitis) within 2 and 7 days after the challenge, re-
spectively. Both strains colonized at the vegetation at a con-
centration of 10^9 CFU/g, and bacterial concentrations of
around 10^5 to 10^6 CFU/g were detected in the organs, such as
the liver and spleen (Table 1). After a parenteral injection of
L. rhamnosus ATCC 53103, L. casei PHLS A357/84, L. rham-
nosus PHLS A103/70, or L. rhamnosus ATCC 7469^T, the lac-
tobacilli at the lower detection levels of less than 10^3 CFU/ml
of peripheral blood were maintained in the peripheral blood of
most of the rabbits tested (Fig. 1B to D and G), but no animals
were killed by the injection of any strain by day 14 after the
challenge. The Lactobacillus strains, however, were found to
colonize at the vegetation at a concentration of 10^5 to 10^6
CFU/g, and the bacteria were detected at concentrations of
around 10^5 to 10^6 CFU/g in liver and spleen (Table 1). His-
topathological analysis of the rabbit heart indicated that the
vegetations that formed with fibrin clot and bacterial colonies
were on the endocardium at the heart valve, and infiltration of
inflammatory cells, such as macrophages and pseudoeosino-
philis, and granulomatous reactions by these cells were ob-
served at the infected vegetations (data not shown). On the
other hand, L. casei strain Shirota, L. acidophilus ATCC 4356^T,
and L. gasseri DSM 20243^T showed no infectivity in this
model: viable bacterial cells were not detected either in the
peripheral blood after the 7th day of the challenge or from
the vegetation of any animal on the 14th day of the chal-
genue (Fig. 1A, H, and I; Table 1), and no histopathological
changes due to any infection were observed in the groups
(data not shown).

Phagocytes have been reported to have a protective effect
during the induction and/or course of IE caused by gram-
positive bacteria such as Staphylococcus epidermidis and Strept-
tococcus sanguinis in rabbits (15, 24). Electron microscopic

### TABLE 1. Induction of infective endocarditis in rabbits by various bacterial strains

| Strain                             | Inoculum (CFU/rabbit) | Vegetationa | Incidence of infection | Log_{10} bacteria/g of organ (mean ± SD)b,c  |
|-----------------------------------|-----------------------|-------------|------------------------|---------------------------------------------|
|                                   |                       | Wt (mg)     | (no. infected/total no. inoculated) | Vegetation | Liver | Spleen |
| Probiotic strains                  |                       | (mean ± SD) |                        |                             |      |      |
| L. casei strain Shirota           | 1.3 × 10^9            | 15 ± 9      | 0/5                    | <1.0          | <1.0 | <1.0  |
|                                  | 6.7 × 10^7            | 11 ± 8      | 0/5                    | <1.0          | <1.0 | <1.0  |
| L. rhamnosus ATCC 53103           | 1.1 × 10^9            | 78 ± 30     | 5/5                    | 8.0 ± 0.9     | 2.2 ± 0.8 | 1.9 ± 0.7 |
|                                  | 3.4 × 10^7            | 32 ± 19     | 5/5                    | 9.0 ± 0.7     | 4.1 ± 0.6 | 4.3 ± 0.5 |
| Endocarditis clinical isolates    |                       |             |                        |                             |      |      |
| L. casei PHLS A357/84            | 4.1 × 10^8            | 76 ± 37     | 5/5                    | 7.7 ± 1.0     | 1.8 ± 0.5 | 1.5 ± 0.5 |
| L. rhamnosus PHLS A103/70        | 6.0 × 10^8            | 126 ± 53    | 5/5                    | 8.6 ± 1.2     | 2.1 ± 1.0 | 2.1 ± 0.9 |
| Streptococcus mitis IE-1^T        | 2.0 × 10^8            | 61          | 1/1                    | 9.1           | 6.4    | 6.0    |
| Streptococcus mitis IE-2^d        | 3.5 × 10^3            | 50          | 2/2                    | 9.5           | 4.9    | 5.3    |
| Type strains                      |                       |             |                        |                             |      |      |
| L. rhamnosus ATCC 7469^T         | 3.0 × 10^9            | NT^a        | 6/6                    | 8.3 ± 0.5     | NT    | NT    |
|                                  | 6.0 × 10^7            | NT          | 4/5                    | 8.7 ± 1.5     | NT    | NT    |
| L. acidophilus ATCC 4356^T        | 1.5 × 10^9            | 9 ± 7       | 0/4                    | <1.0          | <1.0  | <1.0  |
| L. gasseri DSM 20243^T            | 2.4 × 10^9            | 18 ± 13     | 0/3                    | <1.0          | <1.0  | <1.0  |

a Three to six rabbits per group were injected with the bacterial strains and were killed for bacteriological examination on the 14th day after the injection except for those infected with S. aureus IE-1 and S. mitis IE-2.

b Viable counts of the injected bacteria in the organ homogenates were examined by plating appropriate dilutions after incubation on the agar plates.

c The rabbit was dissected on the second day after the challenge, because four out of five rabbits died within 2 days after the challenge.

d Rabbits were dissected on the seventh day after the challenge, because three out of five rabbits died by day 7 after the challenge.

e NT, not tested.

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Phagocytes have been reported to have a protective effect during the induction and/or course of IE caused by gram-positive bacteria such as Staphylococcus epidermidis and Streptococcus sanguinis in rabbits (15, 24). Electron microscopic
observation of the macrophage at the vegetation infiltrated with lactobacilli (\textit{L. casei} strain Shirota (inoculum, 1.3 \times 10^9 CFU) (A), \textit{L. rhamnosus} strain ATCC 53103 (1.1 \times 10^9 CFU) (B), \textit{L. casei} PHLS A357/84 (4.1 \times 10^9 CFU) (C), \textit{L. rhamnosus} PHLS A103/70 (6.0 \times 10^8 CFU) (D), \textit{S. aureus} IE-1 (2.0 \times 10^6 CFU) (E), \textit{S. mitis} IE-2 (3.5 \times 10^9 CFU) (F), \textit{L. rhamnosus} ATCC 7469^T (3.0 \times 10^8 CFU) (G), \textit{L. acidophilus} ATCC 4356^T (1.5 \times 10^9 CFU) (H), and \textit{L. gasseri} DSM 20243^T (2.4 \times 10^9 CFU) (I).} The results are expressed as the means and the standard deviations of results from three to six rabbits. The fractions show the numbers of samples in which lactobacilli were detected out of the number of samples tested.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Comparison of the clearance of bacterial cells from peripheral blood after intravenous injection of various \textit{Lactobacillus} strains in a rabbit IE model. The viable counts of bacteria in peripheral blood were determined. The panels show results for the following strains and inocula: \textit{L. casei} strain Shirota (inoculum, 1.3 \times 10^9 CFU) (A), \textit{L. rhamnosus} strain ATCC 53103 (1.1 \times 10^9 CFU) (B), \textit{L. casei} PHLS A357/84 (4.1 \times 10^9 CFU) (C), \textit{L. rhamnosus} PHLS A103/70 (6.0 \times 10^8 CFU) (D), \textit{S. aureus} IE-1 (2.0 \times 10^6 CFU) (E), \textit{S. mitis} IE-2 (3.5 \times 10^9 CFU) (F), \textit{L. rhamnosus} ATCC 7469^T (3.0 \times 10^8 CFU) (G), \textit{L. acidophilus} ATCC 4356^T (1.5 \times 10^9 CFU) (H), and \textit{L. gasseri} DSM 20243^T (2.4 \times 10^9 CFU) (I). The results are expressed as the means and the standard deviations of results from three to six rabbits. The fractions show the numbers of samples in which lactobacilli were detected out of the number of samples tested.}
\end{figure}

bacteria. After the cells were washed again with PBS three times, the cells were resuspended in medium containing 4 \( \mu \)g of streptomycin sulfate/ml, which was added to prevent extracellular replication. Then, the cells with internalized bacteria were incubated in triplicate tubes for 0, 12, or 24 h. After incubation, the cells were centrifuged once to remove the medium and then lysed by the addition of 1 ml of 0.25% Triton X-100 in water; the number of intracellular bacteria was determined by plating the serial dilution of the lysate on MRS agar plates (for \textit{L. monocytogenes}, on BHI agar plates) and counting CFU on triplicate plates. The viable numbers of each strain at the beginning of the incubation were 2 \times 10^5 to 3 \times 10^5 CFU. The intracellular parasite \textit{L. monocytogenes} EGD completely resisted intracellular killing: the survival ratio of the pathogen in the macrophages after incubation for 24 h was 125% (data not shown). On the other hand, macrophages showed time-dependent killing of \textit{Lactobacillus} strains, and the two clinical isolates and \textit{L. rhamnosus} ATCC 53103 had higher resistances to intracellular killing than \textit{L. casei} strain Shirota (Fig. 2B).

It has been suggested that nitric oxide and related reactive
nitrates exert microbistatic and microbicidal effects against bacteria and that nitric oxide plays an important role in the killing of invading bacteria by macrophages (2, 4, 9, 16). We used N-ethyl-(1-ethyl-2-hydroxy-2-nitroso-hydrazino)-ethanamine (NOC12) (Wako Chemical Co. Ltd., Osaka, Japan) as a spontaneous NO donor to examine the sensitivity of lactobacilli to NO (11). The Lactobacillus suspensions that were prepared as described above were incubated at 37°C for 12 h with the presence of NOC12 at concentrations ranging from 0 to 50 μg/ml in Hanks balanced salt solution at pH 7.3. More than 50% of the initial counts of the Lactobacillus clinical strains and probiotic L. rhamnosus ATCC 53103 survived the treatment with 500 μg of NOC/ml for 12 h, whereas strain Shirota was sensitive to 125 μg of NOC/ml (Fig. 2C). Because NO is known to be oxidized easily to NO₂⁻ in solution and because NO₂⁻ has been reported to exert bactericidal activity (20), we also tested the sensitivity of lactobacilli to NO₂⁻ ions. The clinical isolates and L. rhamnosus ATCC 53103 were more resistant to NO₂⁻ than L. casei strain Shirota (data not shown).

Very few studies have examined the virulence of Lactobacillus strains in experimental animal models (25; C. Pelletier, C. Bouley, P. Bourlioux, and C. Carbon, Abstr. Soc. Microbiol. Ecol. Dis. Paris Meet., 1996). It should be noted that a probiotic L. rhamnosus ATCC 53103 showed infectivity in the rabbit IE model at almost the same level as those exerted by the two clinical isolates, whereas the virulence of another probiotic strain Shirota in this model was found to be negligible (Fig. 1; Table 1). A recent report showed such clinical cases as liver abscess due to probiotic L. rhamnosus organisms (19) and endocarditis due to swallowing a probiotic preparation or eating of large quantities of probiotic yogurt containing L. rhamnosus (14, 18). Taken together, it appears to be necessary to reassess the safety of probiotic Lactobacillus strains in suitable in vivo experimental models such as the rabbit IE model (3, 6, 7), because the animal studies to date indicate an absence of infectivity of probiotic strains, and specific toxicity studies have shown no signs of toxic or harmful effects even at extremely high dose levels (5, 25). Moreover, the present results suggest that resistance to host innate defense systems such as macrophage bactericidal activity, which would function at inflammatory lesions, should be considered in the safety assessment of Lactobacillus strains.

FIG. 2. Differences in the sensitivity of Lactobacillus strains to intracellular killing by mouse macrophages in vitro. (A) Electron microscopy of a macrophage in the vegetation of the rabbit that was treated with L. casei PHLS A357/84 (inoculum, 3.6 × 10⁵ CFU). A lot of rod-shaped bacteria (lactobacilli, arrow) were incorporated in the lysosome of the macrophage. N, nucleus. (B) Macrophage intracellular killing. Macrophages at a concentration of 2.5 × 10⁶ cells/ml and incorporating L. casei strain Shirota (2.3 × 10⁵ CFU) (○), L. rhamnosus strain ATCC 53103 (2.0 × 10⁵ CFU) (●), L. casei PHLS A357/84 (2.9 × 10⁵ CFU) (□), or L. rhamnosus PHLS A103/70 (2.6 × 10⁵ CFU) (■) at time zero were incubated at 37°C in an atmosphere of 5% CO₂ and air for 12 or 24 h. The viable counts of intracellular bacteria were determined. The results are expressed as the mean survival ratios and the standard deviations of results from the triplicate cultures. Representative data of three repeat experiments are shown. Lowercase letters indicate significant difference: a, significantly different from

- L. casei PHLS A357/84 (P < 0.01) and L. casei strain Shirota (P < 0.01); b and c, significantly different from L. casei PHLS A357/84 (P < 0.05) and L. casei strain Shirota (P < 0.01); c, significantly different from L. casei strain Shirota (P < 0.05); d, significantly different from L. rhamnosus PHLS A103/70 (P < 0.05), L. casei PHLS A357/84 (P < 0.01), and L. casei strain Shirota (P < 0.01); and f, significantly different from L. casei strain Shirota (P < 0.01). (C) Differences in the concentration of NOC-12 (μg/ml)

- L. casei PHLS A357/84 (P < 0.01) and L. casei strain Shirota (P < 0.01); b and e, significantly different from L. casei PHLS A357/84 (P < 0.05) and L. casei strain Shirota (P < 0.01); c, significantly different from L. casei strain Shirota (P < 0.05); d, significantly different from L. rhamnosus PHLS A103/70 (P < 0.05), L. casei PHLS A357/84 (P < 0.01), and L. casei strain Shirota (P < 0.01); and f, significantly different from L. casei strain Shirota (P < 0.01). (C) Differences in the sensitivity of Lactobacillus strains to nitric oxide in vitro. Lactobacillus strains at a concentration of 10⁷ CFU/ml were suspended in Hanks balanced salt solution (pH 7.3) in the presence of NOC12 at various concentrations and incubated at 37°C for 12 h. The results are expressed as the mean values of results from the duplicate cultures, and the representative data of three repeat experiments are shown. Symbols: ○, L. casei strain Shirota; ●, L. rhamnosus strain ATCC 53103, □, L. casei PHLS A357/84; ■, L. rhamnosus PHLS A103/70.
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