

# Identification of and Hydrogen Peroxide Production by Fecal and Vaginal Lactobacilli Isolated from Japanese Women and Newborn Infants

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Received 8 March 1999/Returned for modification 22 May 1999/Accepted 5 June 1999

**We identified *Lactobacillus* isolates from Japanese women and newborn infants by a DNA-DNA hybridization method. The predominating lactobacilli were *Lactobacillus crispatus* and *Lactobacillus gasseri* in the women's vaginas and the newborns' intestines and *L. gasseri* and *Lactobacillus fermentum* in the women's intestines. All *L. crispatus* strains were exclusively strong H<sub>2</sub>O<sub>2</sub> producers.**

The human intestinal tract and vagina harbor a number of microorganisms which form complex and finely balanced ecosystems with their environments. Among these microbes, *Lactobacillus* spp. are believed to play an important role in stabilization of the microflora by providing an important microbial defense against vaginal and intestinal colonization by exogenous pathogenic microorganisms. Classification studies have resulted in recent taxonomic changes of human-related lactobacilli; members of the *Lactobacillus acidophilus* group have been divided into six species—*L. acidophilus*, *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, and *Lactobacillus johnsonii* (7, 11)—and *Lactobacillus vaginalis* has been recently classified (4). Few studies adapted to the recent classification of *Lactobacillus* have been performed for investigation of fecal and vaginal lactobacilli (1, 2).

Although previous reports have suggested that production of H<sub>2</sub>O<sub>2</sub> by lactobacilli may represent an important nonspecific antimicrobial defense mechanism in the vaginal ecosystem (5, 9, 10, 14, 16), the H<sub>2</sub>O<sub>2</sub>-producing lactobacilli have been seldom identified to the species level.

In this study, using a DNA-DNA hybridization method, we identified to the species level *Lactobacillus* strains which had been isolated from stool specimens of mothers and infants and vaginal swabs of women to understand the precise ecology of intestinal and vaginal lactobacilli, and we investigated their abilities to produce H<sub>2</sub>O<sub>2</sub>.

Reference strains used were 26 *Lactobacillus* species or subspecies (Table 1). Eighty-five fecal lactobacilli were isolates from 49 healthy mothers and 36 infants born by normal vaginal delivery at Gifu University Hospital in Gifu between 1995 and 1996; 91 vaginal lactobacilli from healthy women were isolated from 27 of the mothers mentioned above and from 64 pregnant women who visited a prenatal clinic in Gifu (16); and 6 vaginal lactobacilli from women with bacterial vaginosis (BV) were isolates from 6 pregnant women (16). MRS agar (Becton Dickinson and Company, Cockeysville, Md.) was used for anaerobic culture of isolates. Lactobacilli were identified as catalase-

negative, non-spore-forming, microaerophilic gram-positive rods producing abundant lactate as an end product with or without small amounts of acetate (12).

Bacterial DNA was extracted by the method described previously (13). DNA was labeled with PHOTOPROBE biotin (Vector Laboratories Inc., Burlingame, Calif.), according to the manufacturer's instructions. DNA-DNA hybridization was

TABLE 1. Reference strains of *Lactobacillus* species used for DNA hybridization studies

Group and species	Strain
<i>L. delbrueckii</i> group (obligately homofermentative) <sup>a</sup>	
<i>L. acidophilus</i> .....	JCM 1132
<i>L. amylophilus</i> .....	JCM 1125
<i>L. amylovorus</i> .....	JCM 1126
<i>L. crispatus</i> .....	JCM 1185
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> .....	JCM 1002
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i> .....	JCM 1012
<i>L. delbrueckii</i> subsp. <i>lactis</i> .....	JCM 1248
<i>L. gallinarum</i> .....	JCM 2011
<i>L. gasseri</i> .....	JCM 1025
<i>L. helveticus</i> .....	JCM 1120
<i>L. jensenii</i> .....	JCM 1146
<i>L. johnsonii</i> .....	JCM 2012
<i>L. delbrueckii</i> group (facultatively heterofermentative) <sup>a</sup>	
<i>L. acetotolerans</i> .....	JCM 3825
<i>L. casei</i> - <i>Pediococcus</i> group (obligately homofermentative) <sup>a</sup>	
<i>L. salivarius</i> subsp. <i>salicinius</i> .....	JCM 1150
<i>L. salivarius</i> subsp. <i>salivarius</i> .....	JCM 1231
<i>L. casei</i> - <i>Pediococcus</i> group (facultatively heterofermentative) <sup>a</sup>	
<i>L. casei</i> .....	JCM 1134
<i>L. paracasei</i> subsp. <i>paracasei</i> .....	JCM 1181
<i>L. paracasei</i> subsp. <i>tolerans</i> .....	JCM 1171
<i>L. plantarum</i> .....	JCM 1149
<i>L. rhamnosus</i> .....	JCM 1136
<i>L. casei</i> - <i>Pediococcus</i> group (obligately heterofermentative) <sup>a</sup>	
<i>L. brevis</i> subsp. <i>brevis</i> .....	JCM 1059
<i>L. buchneri</i> .....	JCM 1115
<i>L. fermentum</i> .....	JCM 1173
<i>L. fructivorans</i> .....	JCM 1117
<i>L. reuteri</i> .....	JCM 1112
<i>L. vaginalis</i> .....	JCM 9505

<sup>a</sup> Data from the review by Vandamme et al. (17).

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TABLE 2. *Lactobacillus* species isolated from stool and vaginal specimens of Japanese women and newborn infants

Species	No. of specimens (%)			
	Stool		Vagina	
	Mothers	Infants	Mothers and women without BV	Women with BV
<i>L. crispatus</i>	4 (8.2)	6 (16.7)	48 (52.7)	2 (33.3)
<i>L. fermentum</i>	9 (18.4)	2 (5.6)	5 (5.5)	1 (16.7)
<i>L. gasseri</i>	11 (22.4)	12 (33.3)	19 (20.8)	2 (33.3)
<i>L. paracasei</i> subsp. <i>paracasei</i>	5 (10.2)	3 (8.3)	0	0
<i>L. paracasei</i> subsp. <i>tolerans</i>	3 (6.1)	2 (5.6)	0	0
<i>L. plantarum</i>	4 (8.2)	0	3 (3.3)	0
<i>L. reuteri</i>	2 (4.1)	0	0	0
<i>L. rhamnosus</i>	2 (4.1)	1 (2.8)	0	0
<i>L. salivarius</i> subsp. <i>salicinii</i>	3 (6.1)	3 (8.3)	1 (1.1)	0
<i>L. salivarius</i> subsp. <i>salivarius</i>	3 (6.1)	6 (16.7)	1 (1.1)	0
<i>L. vaginalis</i>	0	0	8 (8.8)	1 (16.7)
Unidentified	3 (6.1)	1 (2.8)	6 (6.6)	0
Total	49	36	91	6

carried out as described elsewhere (6) with modifications. Genomic DNA was adjusted to 100 µg/ml with distilled water. DNA diluted 10-fold with phosphate-buffered saline (pH 7.4) containing 0.1 M MgCl<sub>2</sub> was distributed into a microtiter plate (100 µl/well). Hybridization of DNA from reference strains with denatured, biotinylated sample DNA was carried out for 4 to 5 h at 45°C.

H<sub>2</sub>O<sub>2</sub> production by *Lactobacillus* strains was tested with MRS agar supplemented with 0.25 mg of tetramethylbenzidine (Sigma, St. Louis, Mo.) per ml and 0.01 mg of horseradish peroxidase (Sigma) per ml (5). Inoculated plates were anaerobically incubated for 2 days at 37°C. H<sub>2</sub>O<sub>2</sub> production was ranked as strongly positive, weakly positive, or negative according to the intensity of blue color development.

Ten species and subspecies from mothers' stools and eight species from stools of infants were detected, whereas three strains from mothers and one from an infant remained unidentified (Table 2). *L. gasseri* was the most commonly found species in both mothers and infants, but the second most predominant species differed between the mothers and infants studied: *Lactobacillus fermentum* in the mothers and *L. crispatus* and *Lactobacillus salivarius* subsp. *salivarius* in the infants.

The predominant species found in the vaginas of 91 healthy women included *L. crispatus*, *L. gasseri*, and *L. vaginalis* (Table 2). In women with BV, *L. crispatus* and *L. gasseri* were common.

A total of 172 strains identified by DNA hybridization were tested for H<sub>2</sub>O<sub>2</sub> production (Table 3). All *L. crispatus* strains were strongly positive for H<sub>2</sub>O<sub>2</sub> production, and all *L. gasseri* and *L. vaginalis* strains were strongly or weakly positive, while *L. paracasei* and *L. plantarum* strains were all negative. Of six isolates from women with BV, two were *L. crispatus*, two were *L. gasseri*, one was *L. vaginalis*, and one was *L. fermentum*; all but the *L. fermentum* strain were positive for H<sub>2</sub>O<sub>2</sub> production.

We found that *L. crispatus* was the predominant vaginal lactobacillus, followed by *L. gasseri*, in Japanese women. Giorgi et al. (8), who used DNA homology techniques, reported that *L. crispatus* and *L. jensenii* were the predominating lactobacilli in healthy women. These data suggest that *L. crispatus* is a common vaginal lactobacillus in healthy women in both Japan and Western countries.

The present study showed that the predominant species in stools were *L. gasseri*, *L. fermentum*, and *L. paracasei* subsp.

*paracasei* for women and *L. gasseri*, *L. crispatus*, and *L. salivarius* subsp. *salivarius* for infants. Benno et al. (2) reported that *L. gasseri* was the dominant species among lactobacilli isolated from the intestinal tracts of elderly Japanese people. Meanwhile, in the studies in Western countries, the *L. acidophilus* group, *L. salivarius*, and *L. fermentum* were usually recovered from stools of adults and infants (3), and the largest taxa in the rectal mucosa of healthy adults were *L. plantarum*, *L. rhamnosus*, and *L. paracasei* subsp. *paracasei* (1). These results indicate that the intestinal lactobacilli may be different between Japanese and Western people. Although the reasons for this dissimilarity are unclear, it may be speculated that the inhabiting *Lactobacillus* species in stools and in the intestinal mucosa are different and that differences between Japanese and Western diets may influence the *Lactobacillus* species resident in the intestine.

We found that all *L. crispatus* strains were strong H<sub>2</sub>O<sub>2</sub> producers, while *L. paracasei* and *L. plantarum* strains were negative for H<sub>2</sub>O<sub>2</sub> production, and that there was relatedness between *Lactobacillus* species and H<sub>2</sub>O<sub>2</sub> production regardless of whether or not the isolates were from women with BV. Nagy

TABLE 3. H<sub>2</sub>O<sub>2</sub> production by lactobacilli isolated from stool and vaginal specimens

Species	No. of strains tested	H <sub>2</sub> O <sub>2</sub> production	No. of isolates			
			Stool		Vagina	Total (%)
			Mother	Infant		
<i>L. crispatus</i>	60	Strongly positive	4	6	50	60 (100)
		Weakly positive	0	0	0	0 (0)
		Negative	0	0	0	0 (0)
<i>L. gasseri</i>	44	Strongly positive	6	6	6	18 (40.9)
		Weakly positive	5	6	15	26 (59.1)
		Negative	0	0	0	0 (0)
<i>L. fermentum</i>	17	Strongly positive	4	0	1	5 (29.4)
		Weakly positive	2	1	1	4 (23.5)
		Negative	3	1	4	8 (47.1)
<i>L. paracasei</i> subsp. <i>paracasei</i>	8	Strongly positive	0	0	0	0 (0)
		Weakly positive	0	0	0	0 (0)
		Negative	5	3	0	8 (100)
<i>L. paracasei</i> subsp. <i>tolerans</i>	5	Strongly positive	0	0	0	0 (0)
		Weakly positive	0	0	0	0 (0)
		Negative	3	2	0	5 (100)
<i>L. plantarum</i>	7	Strongly positive	0	0	0	0 (0)
		Weakly positive	0	0	0	0 (0)
		Negative	4	0	3	7 (100)
<i>L. reuteri</i>	2	Strongly positive	1	0	0	1 (50)
		Weakly positive	0	0	0	0 (0)
		Negative	1	0	0	1 (50)
<i>L. rhamnosus</i>	3	Strongly positive	0	0	0	0 (0)
		Weakly positive	1	1	0	2 (66.7)
		Negative	1	0	0	1 (33.3)
<i>L. salivarius</i> subsp. <i>salicinii</i>	7	Strongly positive	1	0	0	1 (14.3)
		Weakly positive	1	2	0	3 (42.9)
		Negative	1	1	1	3 (42.9)
<i>L. salivarius</i> subsp. <i>salivarius</i>	10	Strongly positive	0	3	0	3 (30)
		Weakly positive	0	3	1	4 (40)
		Negative	3	0	0	3 (30)
<i>L. vaginalis</i>	9	Strongly positive	0	0	4	4 (44.4)
		Weakly positive	0	0	5	5 (55.6)
		Negative	0	0	0	0 (0)

et al. (15), who identified lactobacilli by phenotypic characteristics, showed that the ability to produce H<sub>2</sub>O<sub>2</sub> was more likely to be associated with the origins of strains (BV or non-BV) than with the *Lactobacillus* species themselves. Since the methods used for detection of the ability to produce H<sub>2</sub>O<sub>2</sub> were almost the same between our study and that of Nagy et al., differences in lactobacillus identification might be responsible for the contradictory results.

Y.-L.S. is a recipient of a Nihon Monbusho Scholarship.

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