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Analysis of Vaginal Lactic Acid Producing Bacteria in Healthy Women

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Vaginal lactic acid-producing bacteria of 80 pre-menopausal women were studied by isolation on Blood and DeMan-Rogosa-Sharpe agar, PCR with group-specific primers for Lactobacillus-denaturing gradient gel electrophoresis (DGGE), and PCR with specific primers for V3 region in 16S rRNA-temporal temperature gel electrophoresis (TTGE). Conventional isolation method on media detected only one lactobacillus (*Lactobacillus brevis*) while TTGE detected only *Lactobacillus* sp. DGGE detected seven *Lactobacillus* species; *L. coleohominis*, *L. crispatus*, *L. iners*, *L. reuteri*, *L. rhamnosus*, *L. vaginalis*, and *Leuconostoc lactis*. *L. acidophilus* and *L. gasseri,* which are prevalent in Western women, were not detected in Korean women. Furthermore, *L. rhamnosus*, *Leuc. lactis*, *L. coleohominis*, and *Weissella cibaria*, which were not previously reported in the vaginal microbiota of Korean women, were detected. The five most prevalent LABs in vaginal microbiota in Korean women were *L. iners*, *Enterococcus faecalis*, *L. crispatus*, *Leuc. lactis*, and *W. cibaria.*

Keywords: vaginal microbiota, lactic acid-producing bacteria, denaturing gradient gel electrophoresis, temporal temperature gradient gel electrophoresis

Lactic acid-producing bacteria (LABs) are believed to be the dominant members of normal post-pubertal and pre-menopausal vaginal microbiota (Coolen *et al.*, 2005). LABs are required to maintain good health by producing acid, hydrogen peroxide, and bacteriocins, thus preventing bacterial vaginosis, yeast infections, sexually transmitted diseases and urinary tract infections (Boskey *et al.*, 1999; Martin *et al.*, 1999; Song *et al.*, 1999; Wilks *et al.*, 2004). Using isolation techniques, *Lactobacillus acidophilus* has been reported to be the most frequently isolated *Lactobacillus* species recovered from the vagina (Onderdonk and Wissemann, 1993). However, using genotypic methods, studies conducted in many countries, including Korea, have reported that *L. crispatus*, *L. fermentum*, *L. jensenii*, *L. iners*, *L. gasseri*, *L. pentosus*, and *L. vaginalis* are the most common *Lactobacillus* species in the vagina, with *L. crispatus* and *L. iners* being the most prevalent (Chang *et al.*, 2002; Burton *et al.*, 2004; Zhou *et al.*, 2004). The phenotypic methods commonly used for the identification of *Lactobacillus* species have poor concordance with genomics-based tests (Boyd *et al.*, 2005).

Women whose vaginal microbiota lacks appreciable numbers of lactobacilli, apparently maintain normal vaginal ecosystems. Clearly, several microbial populations other than lactobacilli are dominant in a rather large proportion of normal vaginal microbiota, and they either alone or in combination suppress the growth of pathogens (Redondo-Lopez *et al.*, 1990; Coolen *et al.*, 2005).

To understand the composition of vaginal LABs, we analyzed the vaginal LABs of 80 healthy pre-menopausal women using genotypic methods such as PCR-temporal temperature gradient electrophoresis (TTGE) and PCR-denaturing gradient gel electrophoresis (DGGE), which have been successfully used to analyze various microbiota (Muyzer *et al.*, 1993; Walter *et al.*, 2001; Ogier *et al.*, 2002) in addition to isolation on media.

Materials and Methods

Sample collection

Mid-vaginal swabs from 80 pre-menopausal, non-pregnant women between the ages of 28 and 44 years old were obtained during an annual visit at the clinic between April to September 2006. Based on clinical examinations and the lack of self-reported abnormalities, it was concluded that the women were apparently healthy. Bacterial cells retrieved on swabs were inoculated into transport media (OTS Transport Medium, Yuhan Lab. Tech., Korea) and transferred to the laboratory. Bacterial cells on each swab were suspended in sterile saline and inoculated on Blood (BD, USA) agar and DeMan-Rogosa-Sharpe (MRS) agar (BD) containing 0.002% bromophenol blue. After 24 h of incubation at 35°C, each well-isolated colony was suspended in MRS and observed under a light microscope after Gram-staining. The remainder of the suspension was mixed with glycerol (20% final concentration) and stored at -70°C until use.

Identification of bacteria

Each colony selected on Blood or MRS agar was identified according to the Bergey manual of determinative bacteriology using an appropriate API kit (bio-Mérieux, France), such as 20STREP for Enterococcus or 50CHL for Lactobacillus. Sequencing of 16S rRNA was performed according to the

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methodology described by Sanger *et al.* (1977) using 27F; AGA GTT TGA TCC TGG CTC AG (O'Sullivan *et al.*, 1992) and 1492R; GGT TAC CTT GTT ACG ACT T (Lane, 1991) in an ABI prism 310 Genetic Analyzer (Perkin-Elmer, USA). Sequences were compared with the data in Genbank (http://www.ncbi.nlm.nih.gov/).

Temporal temperature gradient gel electrophoresis (TTGE)
Sterile water (400 μl) was added to 100 μl glycerol stock and bacterial cells were collected by centrifugation. The bacterial cell pellet was dispersed in 10 μl sterile water and the DNA was extracted by heat treatment at 100°C for 10 min. The first step PCR was performed with W01; 5'-AGA GTT TGA TC[AC] TGG CTC-3' and W012; 5'-TAC GCA TTT CAC C[GT]C TAC A-3' specific to 16S rDNA designed by Ogier *et al.* (2002) with pre-denaturation at 96°C for 4 min, 30 cycles of 96°C for 10 sec, 50°C for 30 sec, and 72°C for 1 min, followed by final extension at 72°C for 2 min. After fragments with 700 bp were confirmed on a gel, the second step PCR to amplify the V3 region of 16S rRNA was performed with HDA1-GC; 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3' and HDA2; 5'-GTA TTA CCG CGG CTG CTG GCA-3', designed by Ogier *et al.* (2002) with pre-denaturation at 94°C for 4 min, 30 cycles of 94°C for 10 sec, 58°C for 30 sec, and 68°C for 1 min followed by final extension at 68°C for 7 min. DNA products were loaded on a 10% acrylamide gel with 6 M (final concentration) urea in 1.25× TAE (40 mM Tris-acetate, 1 mM EDTA) after which electrophoresis was carried out at 50 V for 16 h with a temperature gradient ranging from 62°C to 70°C with a 0.5°C increase every hour according to the manual of Dcode universal mutation detection system (Bio-Rad, USA). Following electrophoresis, unidentified bands were extracted from a gel with QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced by Bionics Co. (Korea).

Denaturing gradient gel electrophoresis (DGGE)
After the first step, PCR was performed as described above-for TTGE. The second step PCR for DGGE was performed with group-specific primers for Lactobacillus, Lac1; 5'-AGC AGTAGGGAATCTTCCA-3' and Lac2GC; 5'GC-Clamp-AT TYCACCGCTACACATG-3', designed by Walter *et al.* (2001) with pre-denaturation at 94°C for 2 min, 35 cycles of 94°C for 30 sec, 61°C for 1 min, and 68°C for 1 min, followed by final extension at 68°C for 7 min. The resulting DNA fragments were separated on a 10% polyacrylamide gel containing a 30-60% gradient of denaturing solution [40% (v/v)

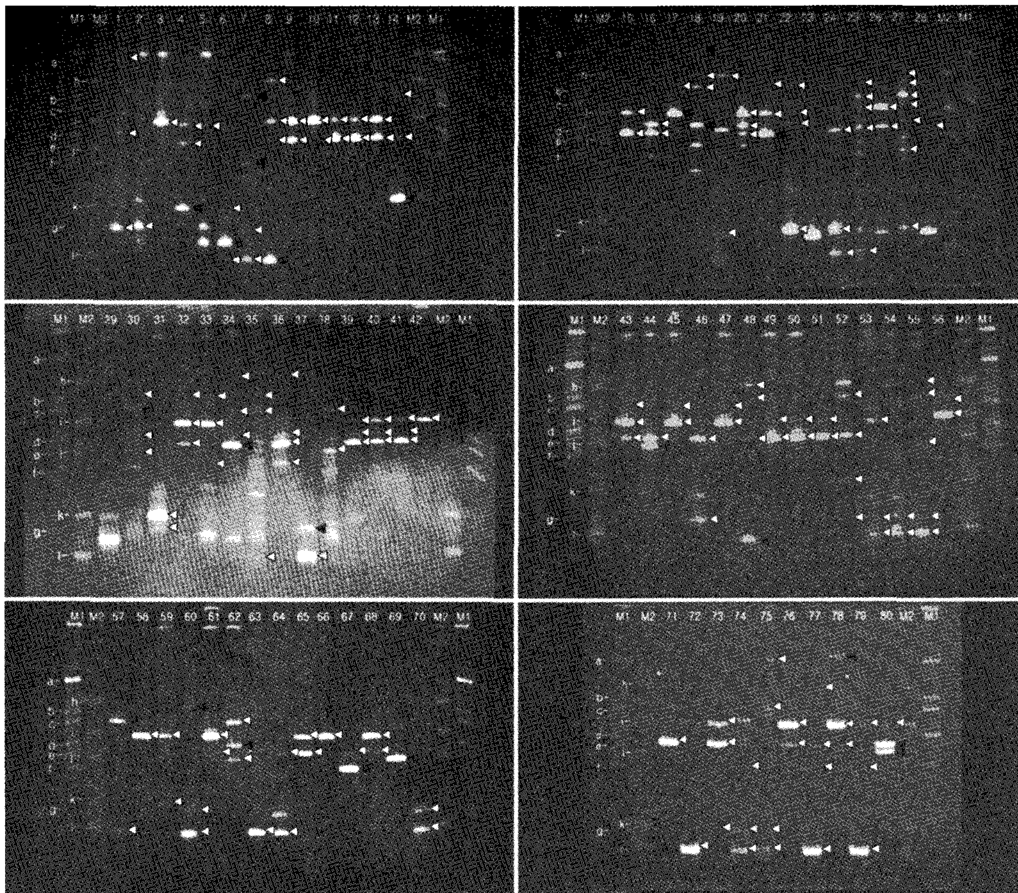

Fig. 1. TTGE of vaginal microbiota from a group of 80 women. M1 and M2, control bands; a, *W. cibaria*; b, *Staph. epidermidis*; c, *En. durans*; d, *Strep. agalactiae*; e, *En. faecalis*; f, *Strep. infantarius*; g, *Staph. xylosus*; h, *Atop. vaginae*; i, *E. coli*; j, *Lactobacillus* sp.; k, *Anaerococcus* sp.; l, *Streptococcus* sp.; ◀, bands identified with sequencing; ◁, bands identified with comparison to controls. 1~80, sample no.

Table 1. Detected bacterium in Korean women, with isolation on media, TTGE, and DGGE

Method	*Actinobacterium*	*Anaerococcus*	*Atop. vaginae*	*C. albicans*	*Citro. freundii*	*E. coli*	*Enterobacter aerogens*	*Enterococcus durans*	*Enterococcus faecalis*	*Enterococcus faecium*	*L. brevis*	*L. coleohominis*	*L. crispatus*	*L. iners*	*L. reuteri*	*L. rhamnosus*	*L.* sp.	*L. vaginalis*	*Leuc. lactis*	*Pan. agglomerans*	*Staph. aureus*	*Staph. epidermidis*	*Staph. haemolyticus*	*Staph. hominis*	*Staph. lugdunensis*	*Staph. salivarius*	*Staph.* sp.	*Staph. xylosus*	*Staph. simulans*	*Staph. warneri*	*Strep. agalactiae*	*Strep. anginosus*	*Strep. infantarius*	*Strep.* sp.	*W. cibaria*
A	1	0	0	7	1	9	1	0	9	4	1	0	0	0	0	0	0	0	0	2	3	16	2	4	2	1	1	0	1	3	7	2	1	0	4
B	0	6	10	0	0	42	0	13	41	0	0	0	0	0	0	0	5	0	0	0	0	16	0	0	0	0	0	20	0	0	13	0	9	21	4
C	0	0	0	0	0	0	0	0	0	0	0	2	38	51	2	4	0	4	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

A, isolation on media; B, PCR-universal primers-TTGE; C, PCR-group specific primers to lactobacillus-DGGE

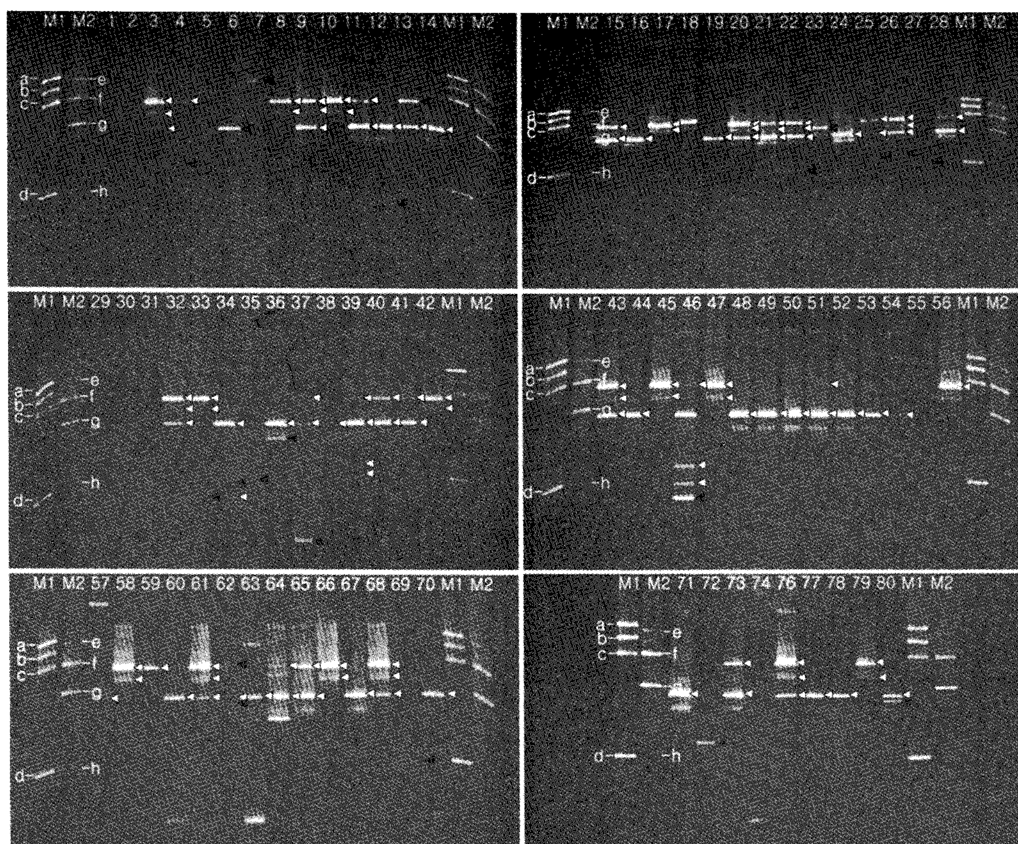

Fig. 2. DGGE of vaginal microbiota from a group of 80 women. M1 and M2, control bands; a, *L. brevis* KCTC 3498^T; b, *L. gasseri* KCTC 3163^T; c, *W. confusa* KCTC 3499^T; d, *L. acidophilus* KCTC 3111^T; e, *W. koreensis* KCCM 41516^T; f, *W. kimchii* (*W. cibaria*) CHJ3 KCTC 3746^T; g, *Leuc. mensenteroides* KCTC 3652^T; h, *L. plantarum* KCTC 3108^T; ◀, bands identified with sequencing; ◁, bands identified with comparison to controls. 1~80, sample no.

formamide, 7.0 M urea]. DGGE was conducted for 6 h with a constant voltage of 130 V at 60°C. The sequencing of the unidentified bands was performed.

Differentiation of Weissella cibaria from W. confusa with amplified 16S ribosomal DNA restriction analysis (ARDRA)
Since the identification of *W. confusa* has recently been changed to *W. kimchii* (Choi *et al.*, 2002), also known as *W. cibaria* (Ennahar and Cai, 2002), *W. confusa* isolated on media was re-identified with ARDRA following a previously reported procedure (Jang *et al.*, 2002). The PCR reaction mixture (50 μl) contained 2 μl of genomic DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1.25 U Taq polymerase, 0.5 μM of a forward primer S-G-Wei-0121-a-S-20; 5'-CGTGGGAAAC CTACCTCTTA-3', and a reverse primer S-G-Wei-0823-a-A-18; 5'-CCCTCAAACATCTAGCAC-3' designed by Jang *et al.* (2002), in addition to 5 μl of 10× reaction buffer. The PCR reaction consisted of one cycle of denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 sec, 61°C for 30 sec, and 72°C for 1 min, and final extension at 72°C for 7 min. PCR

products were treated with *Mnl*I (MBI Fermentas, USA) at 37°C for 2 h and electrophoresed on a 2% agarose gel.

Results

Bacteria isolated on media

Colonies with the same morphology on each plate were considered as one species. A total of 92 colonies were isolated from blood and MRS agar, after which 22 species were identified with Gram-staining, API kits, and 16S rRNA sequencing. *Staphylococcus epidermidis* was the most frequently detected bacterium (observed in 16 women), followed by *En. faecalis* (observed in 9 women), and *Escherichia coli* (observed in 9 women). Conversely, the least prevalent species was *L. brevis,* detected only in one woman (see Table 1).

Bacterial species detected with TTGE

PCR with specific primers for V3 region of 16S rRNA-TTGE separated a total of 161 DNA bands in 12 species (Fig. 1 and Table 1). Even though TTGE could detect *Lactobacillus* sp. and *Staph. xylosus,* which were not detected on media, it was not able to differentiate each individual *Lactobacillus* species or detect the majority of *Staphylococcus* sp. isolated on the media. DNA fragments from PCR with group specific primers for Lactobacillus could not be separated with TTGE.

Bacterial species detected with DGGE

A total of seven different species of LAB were detected in 72 women using PCR with group-specific primers for Lactobacillus-DDGE (Fig. 2 and Table 1). *L. iners* was found in 51 women, *L. crispatus* in 38 women, *Leuc. lactis* in 23 women, *L. rhamnosus* and *L. vaginalis* in four women, and *L. coleohominis* and *L. reuteri* in two women, respectively. LAB was not detected in 7 women. DNA fragments produced from PCR with primers specific to V3 region of 16S rRNA could not be separated from each other by DGGE.

Vaginal microbiota detected with three methods

Among 80 women examined in the present study, 7 women (10%) did not have any LAB (Table 2). Among LABs, *L. iners* (51 women, 63.8%) was the most frequently detected, followed by *L. crispatus* (38 women, 47.5%), *En. faecalis* (43 women, 53.8%), *Leuconostoc* (23 women, 28.8%), and *W. cibaria* (7 women, 8.75%). Seventy-three women had more than one species of LAB and 60 women had more than two species of LAB. The most frequently detected LABs with all three methods were *L. iners*, fol-

Table 2. Vaginal lactic acid-producing bacteria detected in a group of 80 women

Sample no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
L. brevis																																								
L. coleohominis																																							○	
L. crispatus			○	○				○	○	○	○		○		○		○			○	○	○			○	○		○				○	○				○		○	○
L. iners			○			○			○		○	○	○	○	○	○			○	○	○	○		○	○	○		○				○		○		○	○	○	○	○
L. reuteri																																								
L. rhamnosus																											○								○				○	
Lactobacillus sp.																														○								○		
L. vaginalis												○																					○	○						
Leu. lactis			○					○	○	○							○			○	○	○	○			○						○	○							
W. cibaria	○					○												○								○														
En. faecalis				○				○	○	○	○	○	○	○	○	○				○	○			○	○	○		○				○		○		○			○	○
En. faecium																					○																		○	

Sample no.	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
L. brevis																																							○	
L. coleohominis																																○								
L. crispatus	○	○	○		○	○	○				○					○		○	○		○			○	○	○		○					○			○			○	
L. iners	○		○	○		○		○	○	○	○	○	○	○			○			○	○	○	○	○	○		○	○		○	○		○			○	○	○		○
L. reuteri						○																							○											
L. rhamnosus						○																																		
Lactobacillus sp.																						○							○											○
L. vaginalis						○																																		
Leu. lactis		○	○		○		○									○		○			○					○		○								○			○	
W. cibaria																													○						○			○		
En. faecalis	○	○	○	○		○		○	○	○	○	○									○	○		○	○		○	○			○		○			○	○	○		○
En. faecium									○																				○											

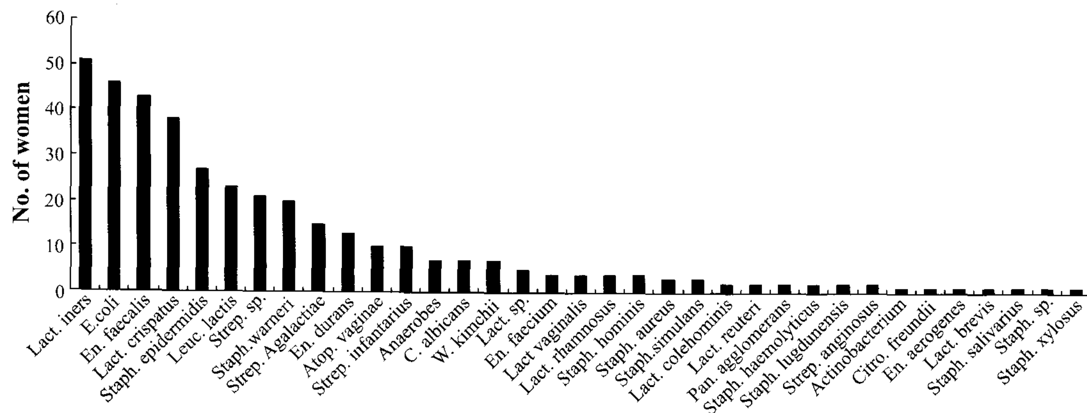

Fig. 3. Bacterium frequency in the vaginal microbiota of Korean women.

lowed by *L. crispatus*, and *En. faecalis* (Fig. 3). No bacterium was detected in one woman with all three methods. Various bacteria were detected in addition to LABs, while the order of prevalence of non-LAB was *E. coli*, *En. faecalis*, *Staph. epidermidis*, *Streptococcus* sp., *Staph. warneri*, *Strep. agalactiae*, *En. Durans*, and *Atopobium vaginae.* Each of these bacteria was observed in more than 10 women.

Discussion

The vaginal microbiota of healthy women of child-bearing age is believed to be dominated by *L. acidophilus* and *L. fermentum*, followed by *L. brevis, L. jensenii, L. casei,* and other species (Onderdonk and Wissemann, 1993; Lachlak *et al.*, 1996). More recently, molecular methods have shown that *L. crispatus, L. iners,* and *L. jensenii* are the most common LAB isolates (Zhou *et al.*, 2004; Coolen *et al.*, 2005). Our results are in close agreement with the results from these studies. In addition to these species, other studies have reported *L. gasseri*, *L. cellobiosus*, *L. fermentum*, and *L. vaginalis* as the most abundant species (Giorgi *et al.*, 1987; Steyn and Holzaphel, 1991; Reid *et al.*, 1996; Silvester and Dicks, 2003). Furthermore, *L. plantarum, L. brevis, L. casei, L. delbrueckii, L. reuteri,* and *L. salivarius* were detected in the vaginal fluid (Redondo-Lopez *et al.*, 1990). In comparison with other studies, *L. acidophilus* and *L. gasseri* were not detected while *W. cibaria*, *Leuconostoc*, *L. rhamnosus,* and *L. coleohominis* detected in this study have not been previously reported in Western women. In particular, *L. coleohominis* has rarely been reported (Nikolaitchouk *et al.*, 2001) and has not been previously reported in the vaginal fluid. In the case of *Weissella* spp., *W. viridescens,* and *W. confusa* have been previously detected in the vaginal fluid (Silvester and Dicks, 2003), while *W. cibaria* (formerly *W. kimchii*) has yet to be detected. *W. cibaria* has recently been separated from *W. confusa* (Ennahar and Cai, 2002) and present in large amounts in kimchi, (Korean traditional fermented vegetables). Interestingly, the varieties of LABs found in women have been shown in be region specific. For example, *L. plantarum* and *W. viridescens* were reported in South Africa (Silvester and Dicks, 2003), while *L. cellobiosus* has been reported in Namibia (Steyn and Holzaphel, 1991) but never before in Western women.

LABs cannot be differentiated from each other with PCR using primers for V3 region-DGGE or PCR with group-specific primers for Lactobacillus-TTGE. Thus, we decided to employ PCR with primers for V3 region-TTGE for non-LABs and PCR with group-specific primers for Lactobacillus-DGGE to detect LABs. *En. durans* could not be detected using a conventional method or PCR-DGGE, but were with PCR-TTGE. Seven species of LAB could not be detected with conventional isolation methods and could not be differentiated from each other using TTGE, but were with DGGE.

Results in this study showed that LABs constituting vaginal microbiota in Korean women are quite different from those in Western women in terms of species and prevalence. The same is also true for other bacteria.

This may be due to different characteristics of vaginal epithelial cells or LABs in fermented foods, a possible origin of human LABs. For example, LABs in kimchi (Korean fermented vegetables) are very different from those existing in fermented foods in Western countries such as yoghurt or sausage. The link between diet and vaginal microbiota has already been demonsrated (e.g. Hoesl and Altwein, 2005), where orally administered probiotics have appeared in vaginal fluid. Therefore, we suggest that LABs present in fermented foods as one of the sources of vaginal microbiota. This study has demonstrated that non-culture-based techniques, such as DGGE, are useful adjuncts for studies of the vaginal microbiota, especially for LABs when group specific primers for Lactobacillus are used. However, PCR with group specific primers to Lactobacillus could not detect other LABs including *Enterococcus.* sp. and *Weissella* sp. PCR with primers specific to V3 region in 16S rRNA-TTGE could detect other bacteria such as *Lactobacillus*. However, it could only identify *Lactobacillus* down to a Genus level. Conventional isolation methods are also necessary to identify the entire members of the microbiota other than LABs. They detected various kinds of bacterium other than TTGE or DGGE, except lactobacillus. On media, other bacteria grow much faster than LABs making it impossible to isolate slower growing LABs. Therefore, we suggest that all three methods should be used to study the entire vaginal

microbiota, while the development of additional primers is necessary to detect various LABs and bacteria.

Acknowledgments

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