Publication information

Beneficial Microbes: ISSN 1876-2883 (paper edition); ISSN 1876-2891 (online edition)

Subscription to 'Beneficial Microbes' (4 issues, calendar year) is either on an institutional (campus) basis or a personal basis. Subscriptions can be online only, printed copy, or both. Prices are available upon request from the Publisher or from the journal's website (www.BeneficialMicrobes.org). Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Subscriptions will be renewed automatically unless a notification of cancelation has been received before the 1st of December. Issues are send by standard mail. Claims for missing issues should be made within six months of the date of dispatch.
Further information about the journal is available through the website www.BeneficialMicrobes.org.

Paper submission

http://mc.manuscriptcentral.com/bm

Editorial office

Bastiaanse Communication
Leading in life science communication

P.O. Box 179
3720 AD Bilthoven
The Netherlands
editorial@BeneficialMicrobes.org
Tel: +31 30 2294247
Fax: +31 30 2252910

Orders, claims and back volumes

Wageningen Academic Publishers

P.O. Box 220
6700 AE Wageningen
The Netherlands
subscription@BeneficialMicrobes.org
Tel: +31 317 476516
Fax: +31 317 453417

Beneficial Microbes, 2015; 6(5): 747-751

Wageningen Academic Publishers

Comparison of detection methods for vaginal lactobacilli

I. Smidt[1,2], R. Kiiker[2,3], H. Oopkaup[1], E. Lapp[1,2], T. Rööp[1,2], K. Truusalu[1], J. Štšepetova[1,2], J. Truu[2,3] and R. Mändar[1,2*]

[1]Department of Microbiology, Faculty of Medicine, University of Tartu, Ravila 19, Tartu 50411, Estonia; [2]Competence Centre on Health Technologies, Tiigi 61b, Tartu 50410, Estonia; [3]Faculty of Science and Technology, University of Tartu, Ravila 19, Tartu 50411, Estonia; reet.mandar@ut.ee

Received: 28 October 2014 / Accepted: 21 February 2015

RESEARCH ARTICLE

Abstract

Vaginal lactobacilli offer protection against microbiota imbalance and genitourinary tract infections. We compared vaginal lactobacilli in 50 Estonian women of child-bearing age applying culture-based methods, quantitative PCR and next-generation sequencing (NGS). The culture-based methods found three different lactobacilli: *Lactobacillus crispatus, Lactobacillus jensenii* and *Lactobacillus gasseri*. Using NGS revealed the presence of *L. crispatus* in 76%, *Lactobacillus iners* in 52%, *L. jensenii* in 47% and *L. gasseri* in 33% of the samples. According to qPCR, *L. iners* was present in 67% and *L. crispatus* in 64% of the samples. The proportions of *L. crispatus* revealed by qPCR and NGS were in good correlation (R=0.79, $P<0.001$), while that of *L. iners* correlated poorly (R=0.13, $P>0.05$). Good concordance for *L. crispatus* was also found between the results of the culture-based method and qPCR. Finally, good overlap between the results of the culture-based method and NGS was revealed: in case of a positive NGS result for *L. crispatus,* the same species was isolated in 95% of samples. The corresponding percentages were 82% for *L. jensenii* and 86% for *L. gasseri*. Our data indicate fairly general concordance of the three methods for detecting vaginal lactobacilli, except for *L. iners*. This points out the importance of standardisation of techniques, and the respective studies should involve cultures applying a medium suitable for the fastidious *L. iners*.

Keywords: vaginal lactobacilli, *Lactobacillus iners,* next generation sequencing, qPCR

1. Introduction

Lactobacilli comprise the most important part of the indigenous vaginal microbiota. They offer protection against microbiota imbalance as well as genital and urinary tract infections by maintaining the low vaginal pH, producing lactic acid and various other antimicrobial compounds. *Lactobacillus acidophilus* was considered as a predominant species for many years, but more advanced molecular methods indicated that other species of the same fermentation group (mostly *Lactobacillus iners, Lactobacillus crispatus, Lactobacillus jensenii,* and *Lactobacillus gasseri*) prevail in most women. Recently, the use of next-generation sequencing (NGS) techniques has provided a high-throughput method to determine detailed taxonomic and abundance information regarding to the microbes present in diverse microbial communities (Liu *et al.*, 2013; Van Oostrum *et al.*, 2013; Verstraelen *et al.*, 2009; Zhou *et al.*, 2004, 2010).

However, overlap of the results obtained by different methods may be moderate due to several reasons and limitations. For example, one of the most frequent species, *L. iners* is uncultivable using conventional media, like MRS and Rogosa agar (Falsen *et al.*, 1999; Vásquez *et al.*, 2002). On the other hand, molecular methods may give incorrect results due to different reasons ranging from the DNA extraction method used or primer selection to software errors.

We aimed to detect and compare vaginal lactobacilli in Estonian women of child-bearing age by applying culture-based methods, quantitative PCR and next-generation sequencing.

2. Material and methods

Study group

The study was carried out at the Tartu University Hospital and Competence Centre on Health Technologies (Tartu, Estonia) from 2009 to 2013. Fifty women of childbearing age consulting the physician for couple infertility or prophylactic reasons were sampled. Exclusion criteria included anti-microbial therapy within 3 months and anti-inflammatory medications for at least 2 weeks before evaluations. Participation in the study was voluntary. All subjects were at least 18 years old. Written informed consent was obtained from all study subjects. The study was approved by the Ethics Review Committee on Human Research of Tartu University, Estonia.

Specimens

Vaginal secretion was collected using three swabs. The swabs were inserted 6 to 8 cm into the vagina. The swab with transport medium was stored at +4 °C until culturing on the same day and as soon as possible. Dry swabs were stored at -80 °C until DNA extraction. The third swab was used to prepare a smear for Gram staining and Nugent scoring.

Cultures

Vaginal swabs were suspended for 30 seconds in 1 ml of phosphate-buffered saline (PBS) in an anaerobic glove box (the gaseous environment consisted of 85% nitrogen, 10% carbon dioxide and 5% hydrogen). Cultures were made onto 2 freshly prepared MRS agars (Oxoid, Basingstoke, UK), one plate was incubated in CO_2-thermostate and the second plate in anaerobic glove box for 48-72 h. Three colonies from each positive culture plate were isolated and identified by 16S rDNA fragment sequencing. The PCR products were obtained using two 16S rDNA primers: CO1 (5'-AGTTTGATCCTGGCTCAG-3') and CO2 (5'-TACCTTGTTACGACT-3') as described by Simpson *et al.* (2003). Thereafter, the PCR products were sequenced and the lactobacilli were identified using the BLAST program (http://blast.ncbi.nlm.nih.gov).

DNA extraction

Vaginal swabs were suspended in 1 ml of PBS and collected by centrifugation at 16,000×*g* for 20 min. The supernatant was discarded, and the remaining pellet was resolved in PBS. DNA was extracted with the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA).

Quantitative PCR

Amplification reactions were assayed in a total volume of 10 µl containing Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The thermocycling conditions used were as follows: 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles consisting of denaturation at 95 °C for 15 s, annealing at 58 °C (*L. iners*) or 60 °C (*L. crispatus*) for 30 s and elongation at 72 °C for 30 s. InersFw (5'-GTCTGCCTTGAAGATCGG-3') and InersRev (5'-ACAGTTGATAGGCATCATC-3') primers were used for *L. iners* with a final concentration of 0.8 µM (De Backer *et al.*, 2007). LcrisF (5'-AGCGAGCGGAACTAACAGATTTAC-3') and LcrisR (5'-AGCTGATCATGCGATCTGCTT-3') primers were used for *L. crispatus* with a final concentration of 0.4 µM (Byun *et al.*, 2004). All the reactions were performed in triplicate using 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Illumina sequencing

The samples were characterised by profiling the microbial community on the basis of the 16S rRNA gene by using the Illumina HiSeq2000 (Illumina, San Diego, CA, USA) sequencing combinatorial sequence-tagged PCR products. Forward (5'-CAACGCGARG AACCTTACC-3') and reverse (5'-ACAACACGAG CTGACGAC-3') primers were used to amplify the bacterial-specific V6 hypervariable region of the 16S rRNA gene (Gloor *et al.*, 2010). The PCR mixture for each sample contained a unique primer pair combination that differed from the rest of the reactions by a 6-base pair long barcode sequence at the 5' end (Parameswaran *et al.*, 2007).

The Phusion Hot Start High Fidelity Polymerase (Thermo Fisher Scientific) reaction mixture was used to perform PCRs, according to the manufacturer's instructions. The following PCR program was used: 3 min of denaturation at 98 °C, 6 thermal cycles of denaturation at 98 °C for 5 s, annealing at 62 °C for 30 s with a reduction of 1 °C each cycle, and extension at 72 °C for 10 s, followed by 19 cycles of denaturation at 98 °C for 5 s, annealing at 57 °C for 30 s, and extension at 72 °C for 10 s. The final extension step was performed at 72 °C for 5 min. For each sample, three replicates of PCR products (20 µl each) were prepared and pooled. The PCR product concentrations of each composite sample were determined on a 2% agarose gel using a MassRuler Express DNA Ladder (Fermentas, Waltham, MA, USA) and Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Finally, the PCR products of all samples were pooled in equal proportions, and the mixture was cleaned and concentrated (4.5 times) with the NucleoSpin Extract II kit (Macherey-Nagel GmbH & Co., Düren, Germany). The final

concentration of the PCR product mixture was measured with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Preparation of the paired-end DNA library was performed with the NEXTflex PCR-Free DNA Sequencing Kit (BIOO Scientific Corp., Austin, TX, USA). Sequencing was performed with the Illumina HiSeq2000 system.

Sequence data preparation, taxonomic assessment and data analysis

Paired-end reads were assembled into composite reads with SHERA (Rodrigue *et al.*, 2010). Sequences with a SHERA quality score less than 0.5 were discarded. Custom Perl scripts were used to sort reads to samples according to barcodes, while also removing barcodes and primers. Assembled reads were processed with the program Mothur v. 1.27 (Schloss *et al.*, 2009), following the standard operating procedure guidelines.

Sequences were discarded if they met any of the following criteria: the average sequencing quality score dropped below 35 over a 25-bp sliding window, the sequence was shorter than 70 bp, had any ambiguous bases, or had homopolymers longer than 6 bp. Remaining sequences were aligned to the SILVA-compatible reference alignment (Pruesse *et al.*, 2007). Only sequences that overlapped in the same alignment space (in total, 176,358 effective reads) were analysed. Within the samples, the number of effective reads was between 268 and 7,177.

The Greengenes reference database (McDonald *et al.*, 2012) was trimmed to the 16S rRNA V6 region by using V-Xtractor v. 2.0 (Hartmann *et al.*, 2010). This trimmed database was used with the 6-nearest neighbour algorithm for taxonomic assignment. De-noised sequences were clustered into operational taxonomic units (OTUs) using CROP v. 1.33 with 95% similarity level (Hao *et al.*, 2011). OTUs with less than 10 sequences were discarded to reduce false diversity because of sequencing errors (Werner *et al.*, 2012).

Statistical analysis

For statistical analyses, SigmaStat (Systat Software, Chicago, IL, USA) and MS Excel (Microsoft, Redmond, WA, USA) software programs were used. The differences between the groups were calculated with Chi-square test. Spearman correlation was used to determine correlations between the parameters. Statistical significance was assumed at $P<0.05$ for all parameters.

3. Results

Culture-based methods were performed in all women resulting in 135 lactobacilli strains. All the isolated strains belonged to 3 species: the majority of the strains appeared to be *L. crispatus* (55%), while 27% of the strains were *L. jensenii* and 18% *L. gasseri*. The strains of *L. crispatus* were isolated from 64% of the women, *L. jensenii* from 42% and *L. gasseri* from 24% (Figure 1).

The two molecular methods (qPCR and NGS) were thereafter applied to the vaginal swabs of 21 women, a proportion 0.1% of total microbial count in sample was used as a limit to separate positive and negative cases. Illumina NGS revealed *L. iners* in 52% of the samples while *L. crispatus* was detected in 76%, *L. jensenii* in 47% and *L. gasseri* in 33% of the samples. Most of the women were colonised by more than one species: 4% of the women harboured 4 species, 24% of the women 3 species, 43% of the women 2 species and only 30% of the women had 1 species according to the NGS method. We found significant negative correlations between *L. crispatus* and *L. gasseri* (R=-0.47, $P=0.008$) and *L. iners* and *L. gasseri* (R=-0.55, $P=0.001$), while borderline positive correlation was detected between *L. iners* and *L. crispatus* (R=0.35, $P=0.052$). We also found negative correlation between Nugent score and *L. iners* (R=-0.38, $P=0.039$) as well as borderline negative correlation between Nugent score and *L. crispatus* (R=-0.34, $P=0.065$).

Good overlap between culture-based method and NGS was revealed: in case of a positive NGS result for *L. crispatus,* the same species was isolated in 95% of samples by cultures. The corresponding percentages were 82 and 86% for *L. jensenii* and *L. gasseri,* respectively. qPCR was additionally used to count the two most prevalent species, *L. iners* and *L. crispatus*. According to qPCR, *L. iners* was detected in 67% of the samples, and if present, counts tended to be high (median proportion in positive samples 58%). *L. crispatus* was present in 64% of the samples, with a median proportion of 31% among positive cases. The proportions

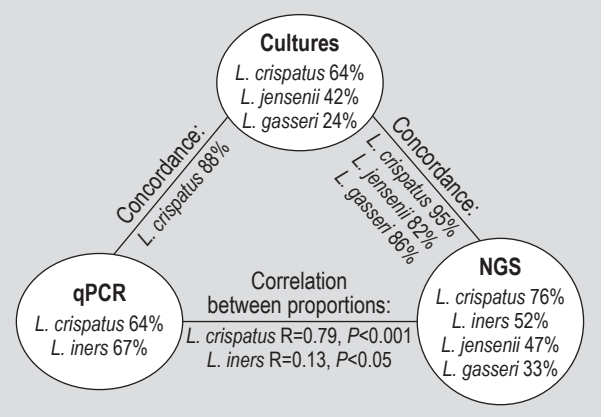

Figure 1. Comparison of different methods in detection of vaginal lactobacilli. Culture-based methods were used with 50 women, while molecular methods were used (qPCR, NGS) on 21 women. Correlations and concordances were calculated using only on the 21 samples that were tested by all three methods.

of *L. crispatus* revealed by qPCR and 16S rRNA sequencing were in good correlation (R=0,79, $P<0.001$), while that of *L. iners* were poorly correlated (R=0.13, $P>0.05$). Good concordance between culture-based methods and qPCR was revealed for *L. crispatus*: in case of a positive qPCR result, the same species was also isolated from 88% of the samples.

4. Discussion

Our study revealed four *Lactobacillus* species (*L. crispatus, L. iners, L. jensenii, L. gasseri*) from vaginal samples of Estonian women. Good concordance between cultures and NGS was found for all three cultivable species (*L. crispatus, L. jensenii, L. gasseri*). Good concurrency between cultures and qPCR, and between NGS and qPCR was found in the case of *L. crispatus*.

Under normal conditions, the lower female genital tract harbours a mutualistic microbiota that primarily consists of lactobacilli, that confer antimicrobial protection to the vagina as a critical port of entry for local, ascending and systemic infectious disease. The lactobacilli-driven defence of the vaginal niche is in its essence seized as a principle of colonisation resistance, i.e. the vaginal lactobacilli prevent colonisation of the vaginal epithelium by other microorganisms, through a variety of mechanisms (Verstraelen *et al.*, 2009).

Previous studies have shown that most of the vaginal lactobacilli tend to be obligately homofermentative species, mostly *L. crispatus* and *L. iners,* but also *L. jensenii, L. gasseri* and some other species (Drell *et al.*, 2013; Ravel *et al.*, 2011; Verstraelen *et al.*, 2009). This was also confirmed by the results of our study. We could confirm the inverse relationship between *L. iners* and *L. gasseri* as shown earlier (DeBacker *et al.*, 2007).

Previous studies have mostly used a single method to detect vaginal lactobacilli, either culture-based or a selected molecular method. We compared the results obtained by three different – culture-based and molecular – methods and achieved some interesting results. Although we only picked up three colonies from each medium plate, we revealed good concordance between cultures (a gold standard) and Illumina sequencing, as well as between cultures and qPCR. At the same time, concordance between qPCR and a novel Illumina sequencing was good for *L. crispatus,* but not for *L. iners*. *L. iners* is the smallest *Lactobacillus* discovered to date. Due to special nutrient requirements it does not grow on common *Lactobacillus* media (MRS or Rogosa) and little is known about its characteristics. Recently, it was found that this species is able to produce a cytolytic toxin called inerolysin (Petricevic *et al.*, 2014; Rampersaud *et al.*, 2011). In the present study, we used carefully designed specific primers described by De Backer *et al.* (2007) to detect *L. iners* by qPCR. For Illumina sequencing, we amplified the V6 region of 16S rRNA. The same region and/or primers have been used by other researchers as well (Ghartey *et al.*, 2014; Hummelen *et al.*, 2011; Xu *et al.*, 2013). A study by Zhang *et al.* (2012) has also revealed that different techniques, such as species-specific PCR and comparison against a 16S rRNA gene clone library may result in different findings regarding *Lactobacillus* species prevalence in the vagina. In case of NGS, the broad-spectrum primers may miss whole phyla or fail to amplify some targets for unknown reasons; and rare species may be missed entirely in the presence of overwhelmingly dominant species (Lambert *et al.*, 2013). In addition, software for high-quality interpretation of NGS data is still under development (Fournier *et al.*, 2014). These findings highlight the importance of standardisation of techniques used for evaluation of vaginal bacterial communities.

In conclusion, our data indicated fairly general concordance of the three methods for detecting vaginal lactobacilli. However, further standardisation of techniques for counting *L. iners* is required, and the respective studies should involve cultures applying a medium suitable for the fastidious *L. iners*.

Acknowledgements

The authors are thankful to Jens-Konrad Preem, Kristjan Oopkaup and Signe Oolep for technical help and Natalja Borovkova, Margus Punab, Paul Korrovits, Kristo Ausmees and Kadri Poolak for sample collection. This work was supported by Estonian Science Foundation (grant No. 5701), Estonian Ministry of Education and Research (target financing No. SF0180132s08, IUT 34-19 and scientific collection financing KOGU-HUMB), University of Tartu (grant No. SARMBARENG) and Enterprise Estonia (grant No. EU30020).

References

Byun, R., Nadkarni, M.A., Chhour, K.L., Martin, F.E., Jacques, N.A. and Hunter, N., 2004. Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. Journal of Clinical Microbiology 42: 3128-3136.

De Backer, E., Verhelst, R., Verstraelen, H., Alqumber, M.A., Burton, J.P., Tagg, J.R., Temmerman, M. and Vaneechoutte, M., 2007. Quantitative determination by real-time PCR of four vaginal *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae* indicates an inverse relationship between *L. gasseri* and *L. iners*. BMC Microbiology 7: 115.

Drell, T., Lillsaar, T., Tummeleht, L., Simm, J., Aaspõllu, A., Väin, E., Saarma, I., Salumets, A., Donders, G.G. and Metsis, M., 2013. Characterization of the vaginal micro- and mycobiome in asymptomatic reproductive-age Estonian women. PLoS ONE 8: e54379.

Falsen, E., Pascual, C., Sjoden, B., Ohlen, M. and Collins, M.D., 1999. Phenotypic and phylogenetic characterization of a novel *Lactobacillus* species from human sources: description of *Lactobacillus iners* sp. nov. International Journal of Systematic and Evolutionary Microbiology 49: 217-221.

Fournier, P.E., Dubourg, G. and Raoult, D., 2014. Clinical detection and characterization of bacterial pathogens in the genomics era. Genome Medicine 6: 114.

Ghartey, J.P., Smith, B.C., Chen, Z., Buckley, N., Lo, Y., Ratner, A.J., Herold, B.C. and Burk, R.D., 2014. *Lactobacillus crispatus* dominant vaginal microbiome is associated with inhibitory activity of female genital tract secretions against *Escherichia coli*. PLoS ONE 9: e96659.

Gloor, G.B., Hummelen, R., Macklaim, J.M., Dickson, R.J., Fernandes, A.D., MacPhee, R. and Reid, G., 2010. Microbiome profiling by Illumina sequencing of combinatorial sequence-tagged PCR products. PLoS ONE 5: e15406.

Hao, X., Jiang, R. and Chen, T., 2011. Clustering 16S rRNA for OTU prediction: a method of unsupervised Bayesian clustering. Bioinformatics 27: 611-618.

Hartmann, M., Howes, C.G., Abarenkov, K., Mohn, W.W. and Nilsson, R.H., 2010. V-Xtractor: an open-source, high-throughput software tool to identify and extract hypervariable regions of small subunit (16S/18S) ribosomal RNA gene sequences. Journal of Microbiology Methods 83: 250-253.

Hummelen, R., Macklaim, J.M., Bisanz, J.E., Hammond, J.A., McMillan, A., Vongsa, R., Koenig, D., Gloor, G.B. and Reid, G., 2011. Vaginal microbiome and epithelial gene array in post-menopausal women with moderate to severe dryness. PLoS ONE 6: e26602.

Lambert, J.A., Kalra, A., Dodge, C.T., John, S., Sobel, J.D. and Akins, R.A., 2013. Novel PCR-based methods enhance characterization of vaginal microbiota in a bacterial vaginosis patient before and after treatment. Applied and Environmental Microbiology 79: 4181-4185.

Liu, M.B., Xu, S.R., He, Y., Deng, G.H., Sheng, H.F., Huang, X.M., Ouyang, C.Y. and Zhou, H.W., 2013. Diverse vaginal microbiomes in reproductive-age women with vulvovaginal candidiasis. PLoS ONE 8: e79812.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R. and Hugenholtz, P., 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME Journal 6: 610-618.

Parameswaran, P., Jalili, R., Tao, L., Shokralla, S., Gharizadeh, B., Ronaghi, M. and Fire, A.Z., 2007. A pyrosequencing-tailored nucleotide barcode design unveils opportunities for large-scale sample multiplexing. Nucleic Acids Research 35: e130.

Petricevic, L., Domig, K.J., Nierscher, F.J., Sandhofer, M.J., Fidesser, M., Krondorfer, I., Husslein, P., Kneifel, W. and Kiss, H., 2014. Characterisation of the vaginal *Lactobacillus* microbiota associated with preterm delivery. Scientific Reports 4: 5136.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J. and Glöckner, F.O., 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35: 7188-9196.

Rampersaud, R., Planet, P.J., Randis, T.M., Kulkarni, R., Aguilar, J.L., Lehrer, R.I. and Ratner, A.J., 2011. Inerolysin, a cholesterol-dependent cytolysin produced by *Lactobacillus iners*. Journal of Bacteriology 193: 1034-1041.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., McCulle, S.L., Karlebach, S., Gorle, R., Russell, J., Tacket, C.O., Brotman, R.M., Davis, C.C., Ault, K., Peralta, L. and Forney, L.J., 2011. Vaginal microbiome of reproductive-age women. Proceedings of the National Academy of Sciences of the USA 108: 4680-4687.

Rodrigue, S., Materna, A.C., Timberlake, S.C., Blackburn, M.C., Malmstrom, R.R., Alm, E.J. and Chisholm, S.W., 2010. Unlocking short read sequencing for metagenomics. PLoS ONE 5: e11840.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB *et al.* (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75: 7537-7541.

Simpson, P.J., Stanton, C., Fitzgerald, G.F. and Ross, R.P., 2003. Genomic diversity and relatedness of bifidobacteria isolated from a porcine cecum. Journal of Bacteriology 185: 2571-2581.

Van Oostrum, N., De Sutter, P., Meys, J. and Verstraelen, H., 2013. Risks associated with bacterial vaginosis in infertility patients: a systematic review and meta-analysis. Human Reproduction 28: 1809-1815.

Vásquez, A., Jakobsson, T., Ahrne S., Forsum U. and Molin G., 2002. Vaginal *Lactobacillus* flora of healthy Swedish women. Journal of Clinical Microbiology 40: 2746-2749.

Verstraelen, H., Verhelst, R., Claeys, G., De Backer, E., Temmerman, M. and Vaneechoutte, M., 2009. Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are more conductive to the occurrence of abnormal vaginal microflora. BMC Microbiology 9: 116.

Werner, J.J., Zhou, D., Caporaso, J.G., Knight, R. and Angenent, LT., 2012. Comparison of Illumina paired-end and single-direction sequencing for microbial 16S rRNA gene amplicon surveys. ISME Journal 6: 1273-1276.

Xu, S., Zong, L., Liu, M., He, Y., Huang, X. and Zhou, H., 2013. Illumina sequencing 16S rRNA tagging reveals diverse vaginal microbiomes associated with bacterial vaginosis. Nan Fang Yi Ke Da Xue Xue Bao 33: 672-657.

Zhang, R., Daroczy, K., Xiao, B., Yu, L., Chen, R. and Liao, Q., 2012. Qualitative and semiquantitative analysis of *Lactobacillus* species in the vaginas of healthy fertile and postmenopausal Chinese women. Journal of Medical Microbiology 61: 729-739.

Zhou, X., Bent, S.J., Schneider, M.G., Davis, C.C., Islam, M.R. and Forney, L.J., 2004. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiology 150: 2565-2573.

Zhou, X., Hansmann, M.A., Davis, C.C., Suzuki, H., Brown, C.J., Schütte, U., Pierson, J.D. and Forney, L.J., 2010. The vaginal bacterial communities of Japanese women resemble those of women in other racial groups. FEMS Immunology and Medical Microbiology 58: 169-181.