Evaluation of the inhibitory effects of vaginal microorganisms on sperm motility *in vitro*

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Abstract. Female infertility usually occurs as a result of ageing, physical impairment, hormone disturbances and lifestyle or environmental factors. However, the potential role of *Lactobacillus* in female infertility has remained largely unexplored. In the present study, high-throughput sequencing, real-time PCR, bacterial adherence assays and sperm motility assays were used to evaluate the microbial diversity, adherence properties and effect on sperm motility of sperm bacteria, vaginal bacteria and vaginal bacteria that had been co-cultured with sperm. The results indicated that in the co-culture group, *Lactobacillus* adhered to sperm cells in numbers that were 332-fold higher than those of control species *Enterococcus* and analysis of sequencing data using the Kyoto Encyclopedia of Genes and Genomes indicated that adhered microbes reduced sperm cell motility. Vaginal isolates, as well as bacterial strains used as controls, were co-cultured with sperm and it was indicated that all strains were able to adhere to sperm cells in large numbers. The probiotic *Lactobacillus (L.)* strains *L. crispatus*, *L. acidophilus*, *L. helveticus* and *L. gasseri* significantly reduced sperm motility (based on measurements of general, straightforward and non-straightforward progressive motility, total motility and average path velocity; $P<0.05$). Furthermore, *L. crispatus*, *L. acidophilus*, *L. salivarius*, *L. helveticus* and *L. gasseri* markedly reduced sperm penetration in a viscous medium. Based on these results, it may be hypothesized that the weakening effect of *Lactobacillus* on sperm motility may be beneficial for healthy couples to prevent the combination of abnormal sperms and eggs, but may be detrimental for males with severe asthenospermia, oligospermia or aspermia.

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Introduction

Infection and inflammation of the urogenital tract caused by microbiological factors accounts for >12% of cases of male infertility, while sexually transmitted infections are the leading cause of female infertility (1). For years, microbiological studies have focused on how infection and inflammation of the urogenital tract induce infertility, but little attention has been paid to the possible impact of the normal reproductive microbiome on infertility (2).

The vaginal microbiota of healthy females consists of a wide variety of anaerobic and aerobic bacterial genera (two to five genera at any one time) (3,4), and the vaginal microbiomes have been classified as *Lactobacillus (L.) crispatus* type, *L. gasseri* type, *L. iners* type and *L. jensenii* type, based on the most dominant species of *Lactobacillus* present (4,5). Depletion of *Lactobacillus* has been associated with several adverse conditions, including ectopic pregnancy, pelvic inflammatory disease and infertility (3,4).

In previous studies, *Anaerococcus, Corynebacterium*, *Gardnerella*, *Lactobacillus*, *Prevotella*, *Pseudomonas*, *Streptococcus* and *Veillonella* have been identified as the most abundant bacteria in semen (6-10), and the predominance of *Anaerococcus*, *Prevotella*, *Pseudomonas* and *Lactobacillus* are typically associated with semen health and fertility (6-9). Levels of infertility are predicted to increase in the future (7), and infectious agents, including bacteria (*Escherichia coli* in particular), yeasts and viruses may directly impair sperm motility (12). However, the role of other types of pathogenic bacteria or vaginal probiotic bacteria on sperm motility has remained largely unexplored.

In the present study, vaginal secretions from 60 healthy human females were used to isolate vaginal bacteria. These bacteria were used to evaluate bacterial capacity to adhere to sperm, to study bacterial effect on human sperm function and to study the potential role of *Lactobacillus* in fertility.

Materials and methods

Sample collection and treatment. A total of 100 reproductive-aged females were recruited for the present study between June 2016 and November 2016. After health screening 60 of these females were selected for collection of vaginal secretions. Subjects with pathogen or HIV infection were excluded. The subjects were known to be free of hysteromyoma, adenomyosis, endometriosis, salpingemphraxis vaginal inflammation, severe pelvic adhesion, any acute inflammation, cancer and endocrine as well as autoimmune disorders. None of the subjects reported any recent use of hormones, antibiotics or vaginal medications. The subjects had not received any cervical treatment within one week and were instructed not to perform any douching within 5 days or sexual activity within 48 h. None of the subjects were pregnant, lactating or during menses at the time of sampling.

Samples of total semen were collected from donors in The Second Affiliated Hospital of Nanchang University, China (n=10; age, 22-38 years; without leukocytospermia) between October 2016 and December 2016 after informed consent was obtained. Donors with normal sperm parameters according to the 2010 World Health Organization criteria, 5th edition (13) (seminal volume, sperm count, progressive motile spermatozoa, motile spermatozoa, non-motile spermatozoa and sperm morphology) were recruited. Subjects with abnormal karyotype and those who had suffered injury to the genitals were excluded. Prior to sampling, the donors washed the glans of their penises using soap and water. Semen was obtained by masturbation, ejaculated into a sterile collection tube and incubated at 37˚C for 25-45 min for liquefaction. Basic semen parameters (including seminal volume and sperm count) and leukocytospermia were measured immediately.

Microbial diversity in sperm, vaginal fluid and co-cultures. Vaginal fluid from all 60 donors were pooled together in a 50 ml tube. Bacteria were isolated from the mixture of vaginal fluids using a plate separation method and identified using 16S ribosomal (r)RNA gene sequencing, as previously described (13).

The isolated bacteria were co-cultured with purified and capacitated sperm at 37˚C and 5% CO_2 for 2 h and the initial sperm: Bacteria ratio was 1:10; the non-adhered bacteria were removed by washing three times using PBS. Subsequently, the mixed samples were centrifuged at 400 x g for 5 min at room temperature, and the supernatant was removed. Extraction of bacterial DNA from the sperm [sperm mixture (SM) group; repeated 3 times], vaginal fluid (vaginal fluid mixture (VM) group; repeated 3 times) and co-cultures [mixture of the bacteria adhered to sperm (AM) group; repeated 3 times] was performed using a TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd) combined with bead-beating (using 0.5 mm diameter glass beads; Bio Spec Products, Inc.) by vortex mixer (model, MXF; SCILOGEX, LLC) according to the manufacturer's protocol. The concentration and quality of extracted genomic DNA was tested prior to sequencing using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Extracted genomic DNA was used as the template to amplify the V4 region of 16S rRNA genes using the forward barcoded 515F/806R primer pair (GenBank accession no. PRJNA517276) (13). PCR, pyrosequencing of the PCR amplicons and quality control of raw data were performed as described previously (13). Paired-end reads from the original DNA fragments were merged using Fast Length Adjustment of Short Reads when there was a certain overlap with the read generated from the opposite end of the same DNA fragment and paired-end reads were assigned to each sample according to the unique barcodes (13).

Sequence analysis was performed with UPARSE software version 7.0.100 (http://drive5.com/uparse/) using the UPARSE-operational taxonomic units (OTU) and UPARSE-OTUref algorithms (http://www.drive5.com/usearch/manual/uparseotu_algo.html). Pre-existing Perl scripts were used to analyse alpha and beta diversity (within and among sequences respectively). Sequences with ≥97% similarity were assigned to the same OTUs. A sequence was picked as representative for each OTU and the Ribosomal Database Project classifier (http://rdp.cme.msu.edu/; Michigan State University) was used to annotate taxonomic information (14). Weighted UniFrac distance analysis was performed using the Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.9.1 (http://qiime.org/; QIIME Development Team) and cluster analysis was then performed. Differentially abundant species were identified and subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/; Kanehisa Laboratories) pathway analysis and they were characterised for their metabolic capacity by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, version 1.0.0 (http://picrust.github.io/picrust/; The PICRUSt project) (13,15). Unweighted Pair-Group Method with Arithmetic Mean method (UPGMA, https://www.sequentix.de/gelquest/help/upgma_method.htm; SequentiX) was used to build a phylogenetic tree and the LEfSe (Linear discriminant analysis effect size) method was used to analyse the bacteria with significant differences among AM, SM and VM groups (16).

Real time-quantitative PCR. DNA extraction was as described above. The primers for PCR were designed using Primer 5.0, (http://www.premierbiosoft.com/; Premier Biosoft International) and real-time PCR amplification was performed using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) (13). The PCR mixture contained 10 μl SYBR® Primer Ex Taq II (Takara Biotechnology, Co., Ltd), 0.4 μl ROX Reference Dye (50X; Takara Biotechnology, Co., Ltd), 1.0 μl template DNA, 0.8 μl each of the primers (final concentration, 0.4 μM) and 7 μl milli-Q H_2O. The amplification was programmed to start at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. Relative levels (fold change) of the target bacteria were analysed using the $2^{-\Delta\Delta Cq}$ method using data from the comparative quantification cycle (Cq) (16). The primers are listed in Table I. *Enterococcus* was used as a control species due to its similar numbers across all groups.

Adherence assay. Single bacterial strains or the bacterial mixture isolated from vaginal fluid were co-cultured with sperm in HEPES saline (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid and 1 mM Na-pyruvate at pH 7.4) at 37˚C with

Table I. Primers for analysis of microbiota via PCR.

Target bacteria	Forward primer (5'-3')	Reverse primer (5'-3')
Bacteroides	GGTGTCGGCTTAAGTGCCAT	CGGA(C/T)GTAAGGGCCGTGC
Bifidobacterium	TCGCGTC(C/T)GGTGTGAAAG	CCACATCCAGC(A/G)TCCAC
Enterococcus	CCCTTATTGTTAGTTGCCATCAT	ACTCGTTGTACTTCCCATTGT
Enterobacteriaceae	CATGACGTTACCCGCAGAAGAAG	CTCTACGAGACTCAAGCTTGC
Clostridium perfringens	CGCATAACGTTGAAAGATGG	CCTTGGTAGGCCGTTACCC
Lactobacillus	CACCGCTACACATGGAG	AGCAGTAGGGAATCTTCCA
Fusobacterium spp.	CCCTTCAGTGCCGCAGT	GTCGCAGGATGTCAAGAC

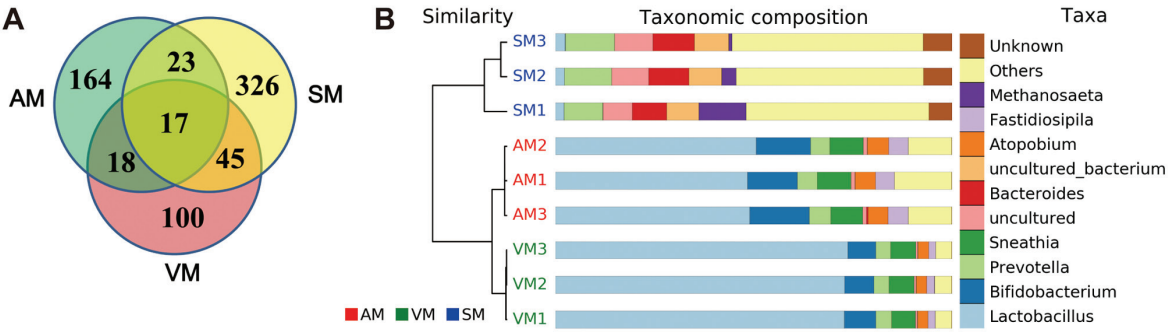

Figure 1. Comparison of microbial diversity between the AM, SM and VM groups using high-throughput sequencing. A total of 222, 411 and 180 OTUs were identified from the AM, SM and VM group, respectively. A similar microbiota was obtained from groups AM and VM, of which *Lactobacillus* was identified as dominant bacteria. (A) Analysis of the shared and unique operational taxonomic units presented as a Venn diagram. (B) Cluster analysis of the bacterial similarity of samples in the AM, SM and VM groups. VM, vaginal bacteria; SM, sperm bacteria; AM, vaginal and sperm bacteria combined.

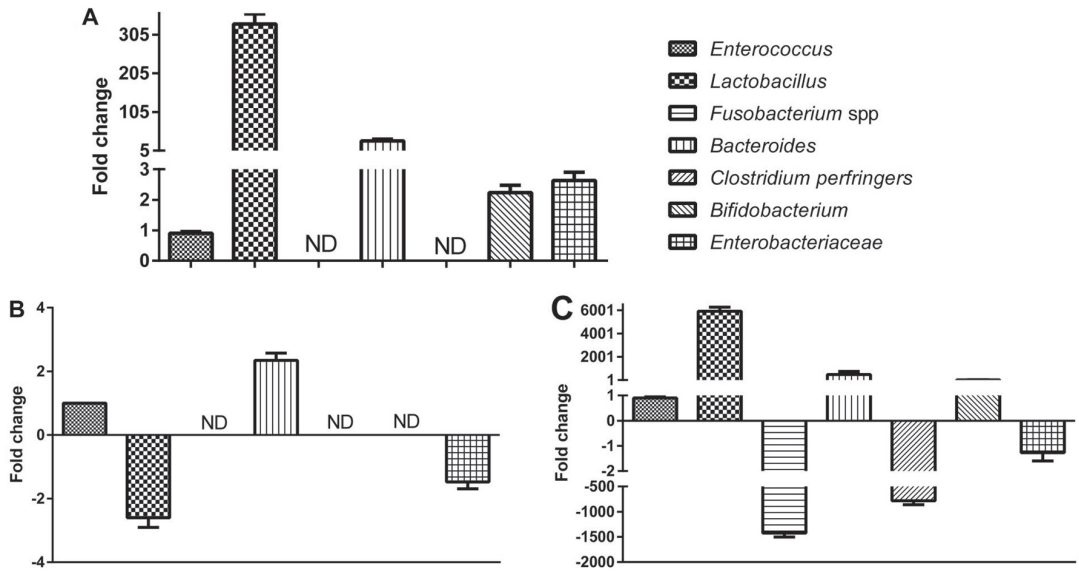

Figure 2. PCR analysis of bacterial strains. The relative amounts (fold change relative to *Enterococcus*) of *Lactobacillus*, *Fusobacterium* spp., *Bacteroides*, *Clostridium perfringers*, *Bifidobacterium* and *Enterobacteriaceae* in the AM, SM and VM groups. *Enterococcus* was selected as the reference strain due to its conserved numbers between the groups (A) AM, (B) SM and (C) VM. The relative numbers of *Lactobacillus* in the AM group and VM group were higher than those in the SM group. VM, vaginal bacteria; SM, sperm bacteria; AM, vaginal and sperm bacteria combined; ND, not detectable.

5% CO_2. After 2 h of incubation, cultures were centrifuged at a speed of 400 x g for 5 min at room temperature, to separate sperm from any non-adhered bacteria. The remaining sperm were washed with sterile PBS and centrifuged at 400 x g for 5 min at room temperature and this procedure was repeated 4 times. The cells were then fixed with methanol for 30 min, Gram-stained and examined microscopically (Olympus BX63 optical microscope; Olympus Corp.). An adherence index, defined as the number of adherent bacteria per 100 sperm, was determined from 20 random microscopic fields. Each

adherence assay was performed in triplicate. Control bacterial strains (purchased from BeNa Culture Collection; Beijing Beina Chuangian Biotechnology Institute) of *Salmonella typhimurium*, *Escherichia coli* O157:H7, Group β-H *streptococcus*, *Candida albicans*, *Salmonella enteritidis*, *Shigella flexneri*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Pediococcus acidilactici* were also used for adhesion assays.

Determination of sperm motility. Sperm motility was examined after incubation with bacteria using a computer-assisted sperm analysis (CASA) system (WLJY-9,000; WeiLi Co., Ltd.). Parameters associated with progressive motility (PR), progressive straight-line motility (PRA), progressive non-linear motility (PRB), total sperm motility (TM) and average path velocity (VAP) were recorded. A minimum of 200 sperm cells were analysed for each assay.

Sperm penetration of a viscous medium. The ability of human sperm to penetrate viscous media is a comprehensive indicator for the evaluation of sperm motility in the viscous environment of the female reproductive tract. In the present study, methylcellulose solution was used to mimic the viscous environment encountered in the female reproductive tract (17). Methylcellulose (1% w/v) was dissolved in human tubal fluid (HTF) medium and introduced into 7.5-cm flattened capillary tubes with 1.0 mm inner diameter (Elite Medical Co., Ltd.). One end of the tube was sealed with plasticine. Human sperm was incubated in HTF medium for 1 h at 37˚C and 5% CO_2, as sperm motility was commonly observed to increase to hyperactivity after incubation. Next, the open ends of the capillary tubes were inserted into the incubation mixture. After 1 h, the tubes were removed, wiped and the penetration of the methylcellulose by the sperm was analysed using the CASA system (WLJY-9,000; WeiLi Co., Ltd.). Three fields (x10) were counted at 1 and 2 cm from the base of the tube and the average number of cells per field were calculated. The cell numbers were normalised to the values of untreated controls.

Statistical analysis. Data analysis was performed using Graphpad Prism version 7.0 (GraphPad Software, Inc.). Values are expressed as the mean ± standard deviation. Differences between the controls and the various samples were assessed by one-way analysis of variance followed by Dunnett's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Molecular identification of the vaginal bacteria adhered to sperm. A total of 100 females donated their vaginal fluids and 60 samples were identified as healthy samples using clinical examinations. As presented in Fig. 1A, 222, 411 and 180 OTUs were identified from the groups AM, SM and VM, respectively. Only 17 OTUs were determined to be common OTUs among all groups, and *Lactobacillus*, *Enterococcus* and *Prevotella* accounted for three, one and three of the common OTUs, respectively (Table II). Analysis of the top 10 genus populations using the UPGMA method indicated that the microbial populations in groups AM and VM were highly similar (Fig. 1B). *Lactobacillus* accounted for 49 and 73% of the total OTUs in the AM group and VM group, respectively, but only 2.3% of the total OTUs in the SM group (Fig. 1B).

Table II. Taxonomy of 17 common OTUs among the three groups (vaginal bacteria, sperm bacteria and their combination).

OTU ID	Taxonomy (at genus level)
OTU11315	*Lactobacillus*
OTU14528	*Gluconacetobacter*
OTU15433	*Lactobacillus*
OTU37354	*Ureaplasma*
OTU46791	*Leuconostoc*
OTU46844	*Enterococcus*
OTU47147	*Finegoldia*
OTU51860	*Porphyromonas*
OTU56305	*Bacillus*
OTU71236	*Prevotella*
OTU74227	*Streptococcus*
OTU74838	*Prevotella*
OTU75162	*Anaerococcus*
OTU77493	*Lactobacillus*
OTU79751	*Prevotella*
OTU85858	*Peptoniphilus*
OTU96990	*Mobiluncus*

OTU, operational taxonomic units.

Real-time PCR analysis was performed to evaluate the relative numbers of *Enterococcus*, *Lactobacillus*, *Fusobacterium* spp., *Bacteroides*, *Clostridium perfringens*, *Bifidobacterium* and *Enterobacteriaceae* in groups AM, SM and VM, and the relatively stable genus of *Enterococcus* was set as the control. As presented in Fig. 2, the numbers of *Lactobacillus* in the AM group and VM group were 332- and 5917-fold higher than those of *Enterococcus*, respectively, while the number of *Lactobacillus* was 2.6-fold lower than that of *Enterococcus* in the SM group. The numbers of *Enterobacteriaceae* were 2.7-fold higher than those of *Enterococcus* in the AM group, but 1.3- and 1.5-fold lower in the SM group and the VM group, respectively. The prevalence of *Bacteroides*, *Prevotella*, *Methanosaeta*, *Pseudomonas*, *Blautia* and *Bacillus* strains was significantly higher in the SM group than that in the AM group or the VM group (Fig. 3; $P<0.05$).

A bioinformatics analysis based on high-throughput sequencing was then performed and the KEGG metabolic pathway variance analysis revealed that adhered vaginal bacteria were significantly associated with the terms promotion of sperm motility and reduced drug resistance and rates of infection, as compared with non-adherent bacteria ($P<0.05$; Fig. 4A). Conversely, the bacteria in the AM group were associated significantly with the terms increased drug resistance, infection, cell growth and death, as well as reduced motility, as compared with those in the SM group ($P<0.05$; Fig. 4B).

Adhesion of selected bacteria to sperm. Isolation of bacteria from vaginal fluid yielded the following species: *Staphylococcus*

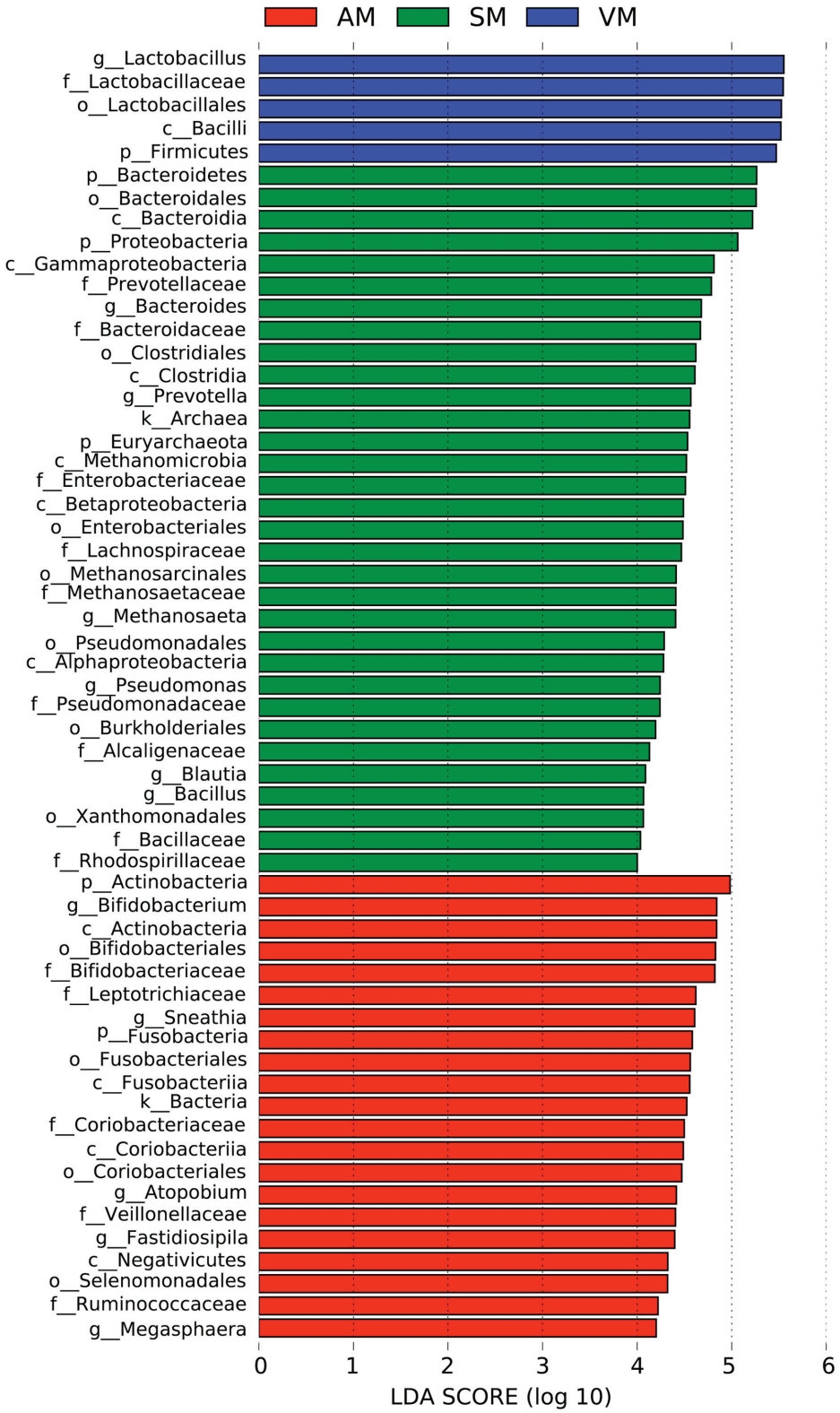

Figure 3. Analysis of the bacteria with significant differences among groups AM, SM and VM using tLEfSe. The results indicated that the prevalence of *Bacteroides*, *Prevotella*, *Methanosaeta*, *Pseudomonas*, *Blautia* and *Bacillus* strains was markedly higher in the SM group than in the AM group or VM group. VM, vaginal bacteria; SM, sperm bacteria; AM, vaginal and sperm bacteria combined.

epidermidis, *L. crispatus*, *L. acidophilus, L. salivarius*, *L. helveticus*, *Pseudomonas stutzeri*, *Staphylococcus capitis*, *L. gasseri* and *Enterococcus faecalis*. Of note, all of the tested bacteria, whether probiotic or pathogenic, adhered to sperm in large numbers. The number of adhered *L. crispatus, L. gasseri* and *E. faecalis* cells was 565, 806 and 881 per 100 sperm, respectively (Fig. 5).

Effect of adhered bacteria on human sperm motility. The vaginal bacteria *E. coli* O157:H7, *L. crispatus*, *L. acidophilus*,

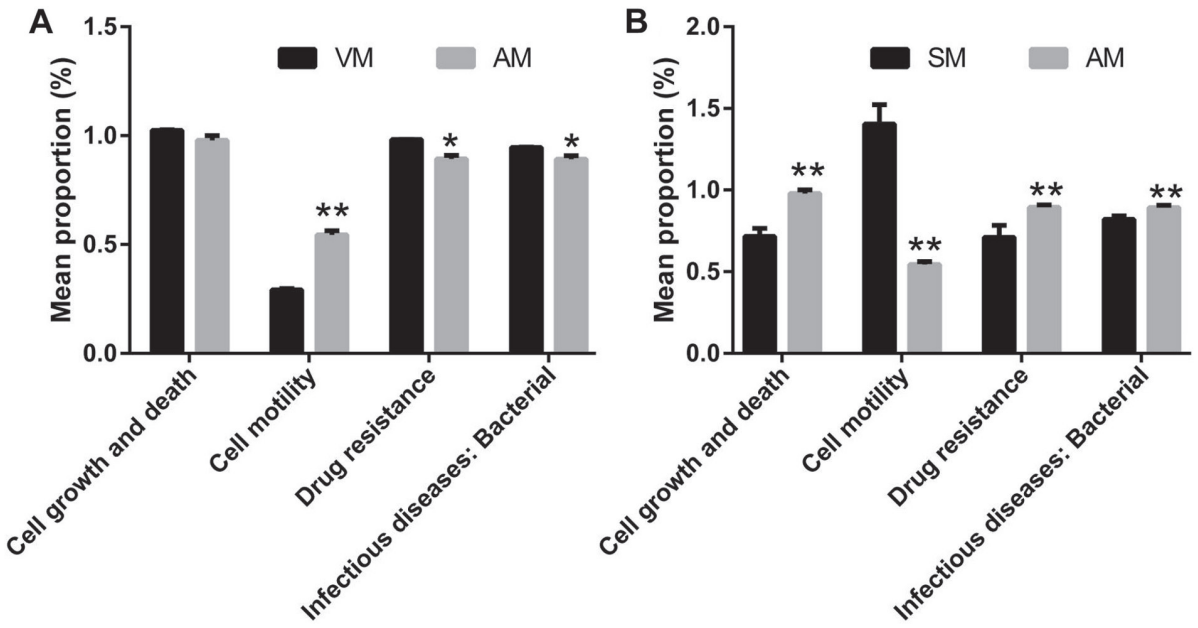

Figure 4. Kyoto Encyclopedia of Genes and Genomes metabolic pathway variance analysis was used to predict the effects of bacteria on the physiological functions of sperm. (A) VM vs. AM; (B) SM vs. AM. Adherent vaginal bacteria significantly enhanced sperm motility. The drug resistance of the bacteria and rate of bacterial infection were markedly reduced in the SM group compared with that in the AM group. *P<0.05; **P<0.01 vs. VM or SM group. VM, vaginal bacteria; SM, sperm bacteria; AM, vaginal and sperm bacteria combined.

β-H. streptococcus, *L. salivarius*, *L. helveticus*, *C. Albicans*, *S. aureus*, *L. gasseri* and *E. faecalis* were selected for evaluation of their effects on human sperm motility. As presented in Figs. 6 and 7, nearly all tested bacteria caused a statistically significant reduction in all sperm motility parameters, including PRA, PRB, RP, TM and VAP, while *E. faecalis* had a reduced affect on these parameters, though it adhered to sperm in high numbers. The effects of the adhered bacteria on sperm motility in a viscous medium were a better indicator of sperm motility in the female reproductive system. Comparison of the sperm numbers at 1 and 2 cm from the base of the tube revealed that *L. crispatus*, *L. acidophilus*, *β-H. streptococcus*, *L. salivarius*, *L. helveticus*, *C. Albicans*, *S. aureus* and *L. gasseri* significantly reduced sperm motility, while *E. coli* O157:H7 and *E. faecalis* did not (Fig. 7B and C). Of note, the probiotic bacteria *L. crispatus, L. acidophilus, L. helveticus* and *L. gasseri* caused a marked reduction in all of the motility parameters of human sperm, while the effects of *L. salivarius* were less pronounced compared with those of the other *Lactobacillus*.

Discussion

It has been indicated that changes in the vaginal microbiome may affect the risk of gynaecological cancers (19). A healthy vaginal microbiome is dominated by species of *Lactobacillus* that have a protective effect and may have therapeutic potential (4,5). As with the intestinal microbiome, disruption of the vaginal microbiota may affect immunity or lead to increased growth of pathogens, potentially resulting in the occurrence of diseases (20). It has been suggested that vaginal microbiota with abundant *Lactobacillus* may also reduce the risk of HIV transmission, particularly female-to-male transmission, and reduce the rate of preterm birth (4,5). The ability of the human vagina and its microflora to affect vaginal discharge, menses, neonates and the overall health of individuals is currently not sufficiently recognized (133).

Motility is the key factor of sperm function and is predictive of its fertilisation potential *in vitro*. In previous studies, the adhesion of *E. coli* and *P. aeruginosa* to sperms has been extensively studied (4,5), while the effects of *Lactobacillus* adhesion on sperm function have remained to be evaluated, to the best of our knowledge. In preliminary study by our group, vaginal bacteria were isolated and it was indicated that most isolates were able to adhere to HeLa cells (data not shown); therefore, the present study was performed to assess whether these isolates are able to adhere to sperms also.

Considering the challenges of assessing the effects of microorganisms on sperm motility *in vivo*, an *in vitro* model was used in the present study. First of all, microbial diversity was compared between vaginal secretions and semen, and a lower bacterial number was identified in the vagina, possibly due to the harsh environment (low pH of 3.8-4.4) in the vagina. In total, only 17 common OTUs were identified from the groups AM, SM and VM, and *Lactobacillus*, *Enterococcus* and *Prevotella* were identified from these OTUs.

The top 10 genus populations were then further analysed using the UPGMA method, revealing that *Lactobacillus* accounted for only 2.3% of the total OTUs in the SM group, while it was as high as 49% in the AM group, indicating *Lactobacillus* was able to effectively adhere to sperms. The real-time PCR analysis further confirmed that the number of *Lactobacillus* in the AM group was 332-fold higher than that of *Enterococcus* (set as the control strain due to its constant number in all groups), while the number of *Lactobacillus* in the SM group was 2.6-fold lower than that of *Enterococcus*.

Subsequently, KEGG metabolic pathway variance analyses were performed and the results indicated that adhered microbes

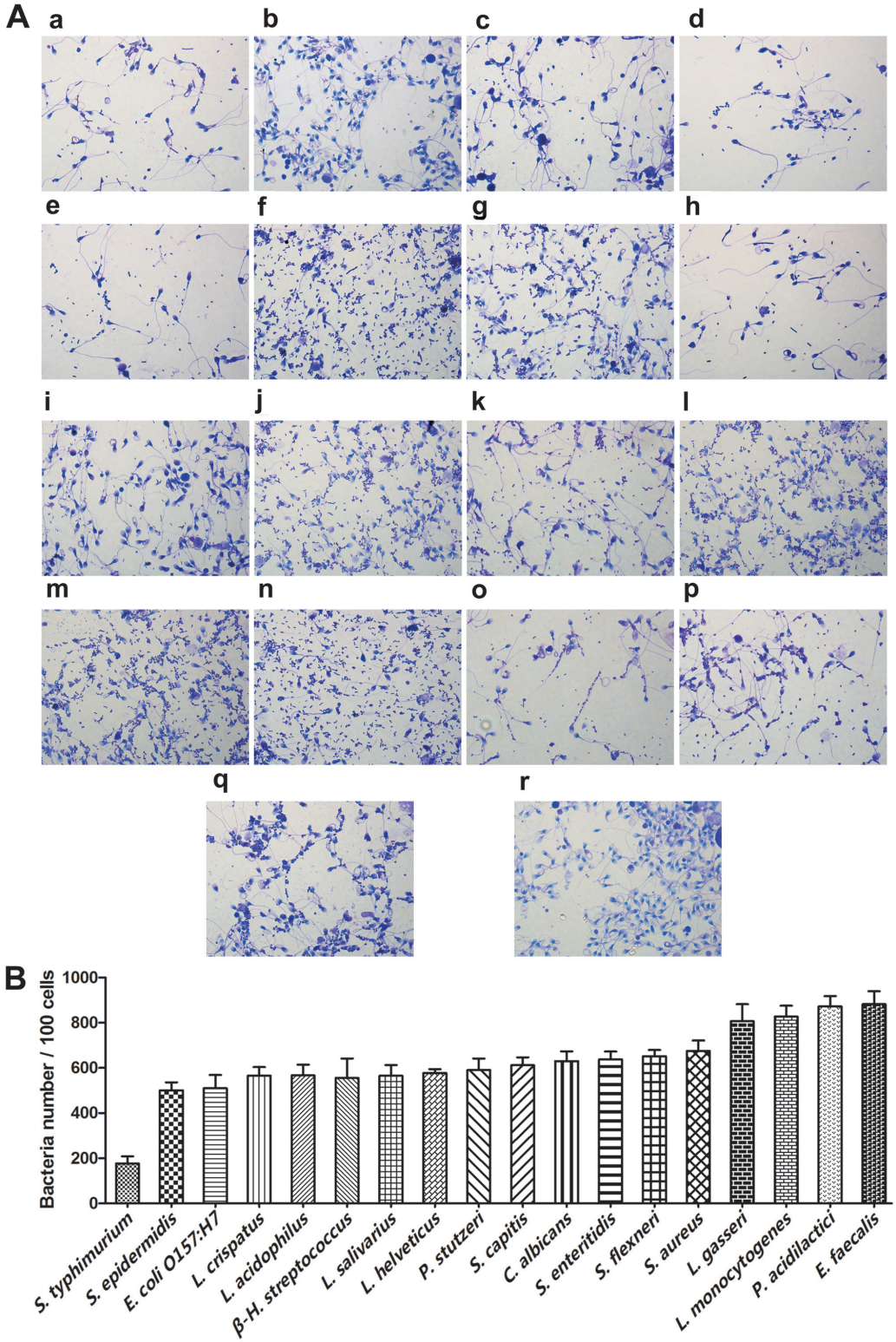

Figure 5. Adhesion of the selected bacteria to sperm cells. (A) The gram stain results (magnification, x100) and (B) adhesion numbers of *S. typhimurium*, *S. epidermidis*, *E. coli* O157:H7, *L. crispatus*, *L. acidophilus*, *β-H. streptococcus*, *L. salivarius*, *L. helveticus*, *P. stutzeri*, *S. capitis*, *C. Albicans*, *S. enteritidis*, *S. flexneri*, *S. aureus*, *L. gasseri*, *L. monocytogenes*, *P. acidilactici* and *E. faecalis*. The initial sperm/bacteria ratio was 1:10 and *S. epidermidis*, *L. crispatus*, *L. acidophilus*, *L. salivarius*, *L. helveticus*, *P. stutzeri*, *S. capitis*, *L. gasseri* and *E. Faecalis* were isolated from vaginal secretions of healthy females, while the others were available in-house. Statistical analysis was not performed due to the lack of an obvious control strain. (B) Adherence index is defined as the number of adherent bacteria per 100 sperm and was determined from 18 random microscopic fields. Each adherence assay was performed in triplicate. All of the tested bacteria, whether probiotic or pathogenic, adhered to sperms in large numbers.

markedly reduced sperm cell motility, and enhanced bacterial cell growth and death, drug resistance and bacterial infection compared to seminal bacteria (P<0.05). Therefore, the effect of the vaginal isolates and certain in-house strains on human sperm motility was evaluated. The results revealed that all tested bacteria were able to adhere to sperms and nearly all of

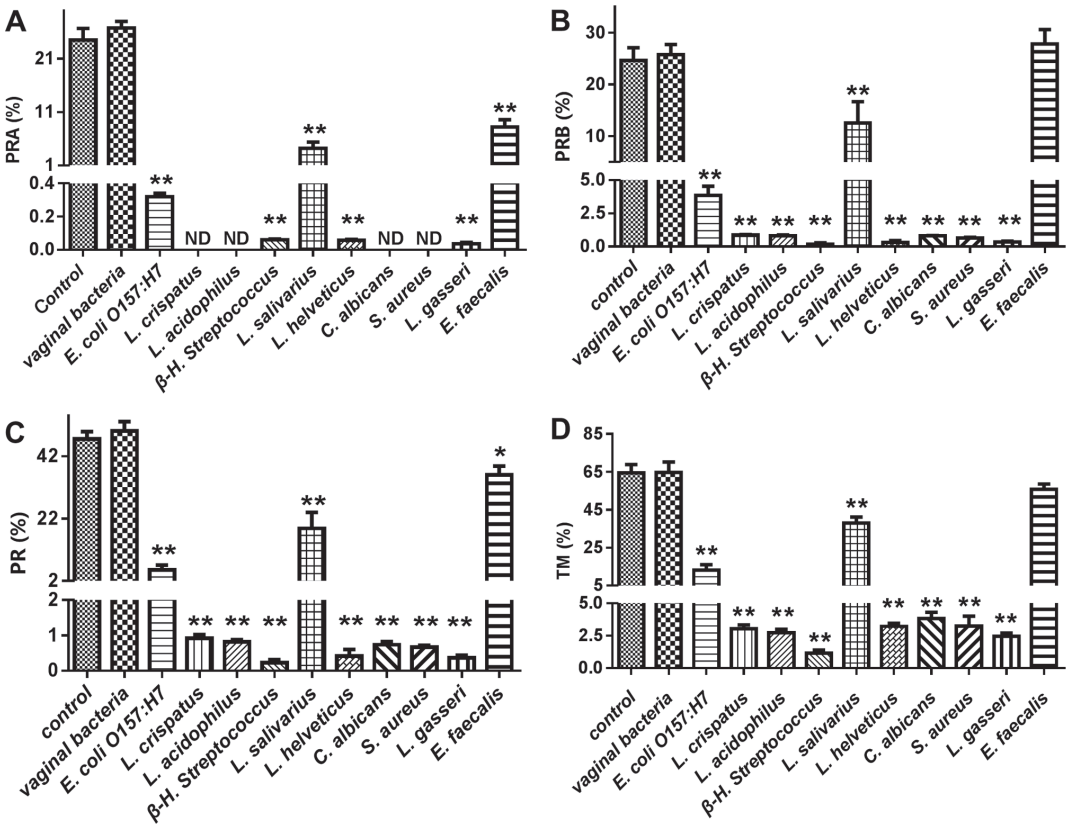

Figure 6. Effects of the selected bacteria on sperm motility *in vitro*. Human ejaculated sperm was incubated with a single species of bacteria in human tubal fluid medium at 37˚C and 5% CO_2 for 2 h. The sperm progressive motility parameters (A) PRA, (B) PRB and (C) PR, as well as (D) TM were analysed by computer-assisted sperm analysis. The initial sperm/bacteria ratio was 1:10. All tested bacteria caused a statistically significant reduction in all sperm motility parameters regarding PM and TM. Analysis of variance was applied to determine significant differences among groups. *P<0.05; **P<0.01 vs. control. PRA, progressive straight-line motility; PRB, progressive non-linear motility; PR, progressive motility; TM, total sperm motility; ND, not detectable.

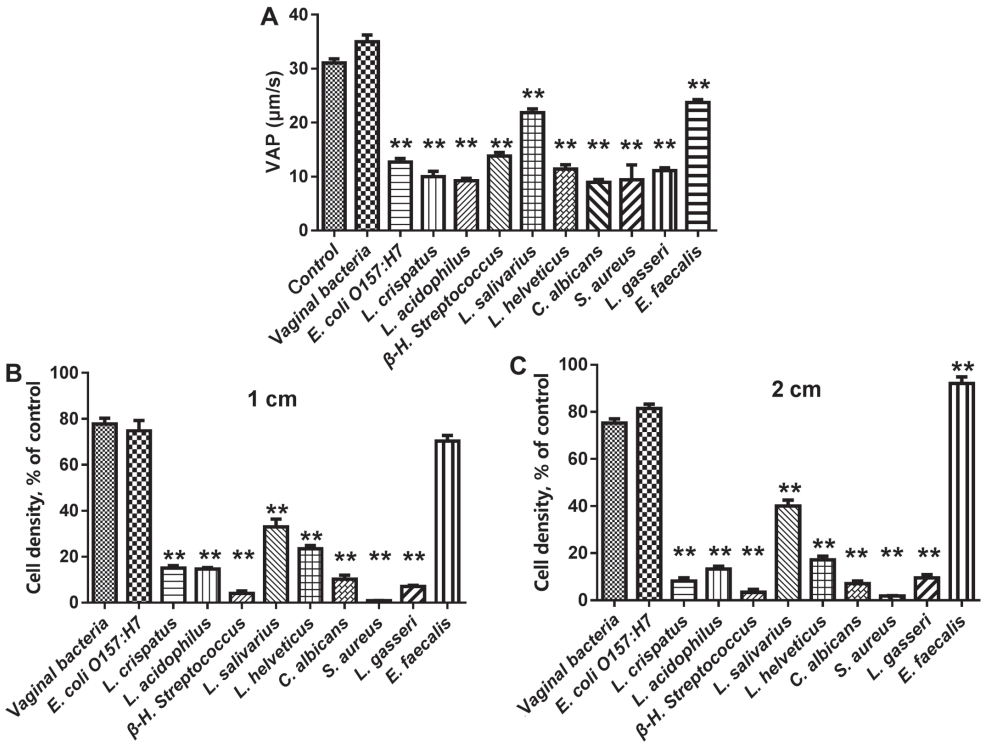

Figure 7. Effects of the selected bacteria on sperm motility of (A) VAP, and the penetrating ability of sperm into a viscous medium was assessed at (B) 1 cm and (C) 2 cm by determining the cell density as a percentage of the control. The initial sperm/bacteria ratio was 1:10. All tested bacteria caused a statistically significant reduction of the average path velocity of sperms. analysis of variance was applied to determine the significant differences among groups. **P<0.01 vs. control. VAP, average pathway velocity.

them caused significant decreases in sperm motility parameters and decreased sperm motility in a viscous medium, indicating their potential negative effect on sperm function.

It is known that the predominance of vaginal *Lactobacillus* has important health-promoting effects, which may maintain the reproductive fitness of females through direct and indirect anti-pathogenic mechanisms (21). Certain bacteria, fungi, viruses and parasites are known to interfere with reproductive functions in either sex and infections of the genitourinary tract account for 15% of male infertility cases (3,4). The results of the present study revealed that all of the *Lactobacillus* assessed (*L. crispatus*, *L. acidophilus*, *L. salivarius*, *L. helveticus* and *L. gasseri*) were able to effectively adhere to sperm and markedly reduce sperm motility. As one of the most important *Lactobacillus* species, *L. crispatus* appears to dominate the vaginal microbiota of most healthy females (4). This species is positively associated with anti-inflammatory cytokines [e.g. interleukin (IL-10)], potentially decreasing the production of pro-inflammatory cytokines, includings IL-6, IL-8 and tumour necrosis factor-α (22). However, the high adhesion of *Lactobacillus* species to sperms significantly reduced the sperm functions, showcasing the dual character of *Lactobacillus* in vaginal health and reproduction.

Of note, the present results indicated that *E. faecalis* had relatively minor negative effects on the PR, TM or VAP of sperm or the ability of sperm to penetrate a viscous medium, although this strain was able to adhere well to sperm cells. *E. faecalis* are Gram-positive cocci that may survive harsh conditions and certain strains are the cause of serious human and animal infections (23-25). It is therefore worthwhile studying the role of *E. faecalis* in the human vagina.

In conclusion, the present study evaluated the effect of bacterial adhesion on sperm motility. All bacteria that were tested adhered effectively to sperm and reduced their motility, which was particularly obvious for the probiotic strains of *Lactobacillus*. It may be postulated that the reduction in motility caused by adhesion of *Lactobacillus* may be beneficial for healthy couples attempting to conceive, as it may reduce the chance of poor-quality sperm combining with eggs. However, this effect may be detrimental for males with severe asthenospermia, oligospermia or aspermia, as the bacteria may eliminate the potential of the sperm combining with the egg. Therefore, the high adhesion capability of vaginal bacteria should be considered in the development of therapeutics, and the use of probiotics in the treatment of gynaecological diseases should be investigated. However, considering the small number of samples used and the challenges of accurately controlling and quantifying bacterial adherence *in vitro*, animal experiments, which may provide a better model for the interaction of bacteria and sperms, may be required in future work to obtain more robust conclusions.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TC and HX designed the experiment. HW, YC, TL, HC and BT performed the experiments. TC analysed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Ethics approval and consent to participate

This study was approved by the institutional review boards of Jiangxi Maternal and Child Health Hospital (Nanchang, China). Patients signed a written informed consent form for the use of their samples for scientific research and for the publication of their data. All participants in the study were adults.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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