



Characterization of Vaginal *Lactobacillus* Strains and Their Potential Antagonistic Effects on *Candida albicans*

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Authors' contributions

This work was carried out in collaboration between all authors. Author SW did most of the experiments and data analyses. Author QW made the table and figures. Author BX provided *Lactobacillus* strains. Author RZ performed the statistical analysis. Author BW managed the literature searches. Author QL participated in the design of this study. Author HZ proofread the manuscript and gave critical comments. Author TL designed the study and wrote the first draft of this manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To characterize *Lactobacillus* strains for potential beneficial effects, especially for inhibition of *Candida albicans* growth.

Materials and Methods: Twelve vaginal *Lactobacillus* strains belonging to five species from our laboratory collection were studied. The acid production and hydrogen peroxide (H₂O₂) production were determined. The biofilm formation ability was measured by a semi-quantitative microtiter plate

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assay. Besides the detection of autoaggregation and coaggregation capacities with *C. albicans* using a spectrophotometric assay, aggregating clusters were observed by light microscopy. The spot inhibition, agar overlay and Oxford Cup methods were used to test antagonistic activities of *Lactobacillus* strains against *C. albicans*.

Results: Seven strains of *L. fermentum* (A011, A024, A025, A035, A041, A044 and A081) and *L. salivarius* A023 were revealed to decrease the medium pH below 4.5 just within six hours due to the rapid growth. *L. jensenii* A083 and *L. gasseri* A054 were the best biofilm formers in polystyrene plates, whereas the other ten strains could form biofilm with varying affinities. In addition to significant H₂O₂ production found in *L. jensenii* A083, *L. crispatus* A014 and A055, these strains showed strong autoaggregation and coaggregation abilities with *C. albicans* (ATCC 90029 and Z03). *L. salivarius* A023, *L. fermentum* A025 and *L. crispatus* A014 displayed strong activities against *C. albicans* in spot inhibition and agar overlay assays.

Conclusion: The potential beneficial properties of *Lactobacillus* spp. were strain specific. Among the tested strains, *L. salivarius* A023, *L. fermentum* A025 and *L. crispatus* A014 might be better candidates for further investigation on the inhibition of *C. albicans* growth.

Keywords: Vaginal *Lactobacillus*; biofilm; aggregation; antagonistic activities; *Candida albicans*.

1. INTRODUCTION

The female vagina has been increasingly recognized as a dynamic and complex microbial ecosystem, where lactobacilli are the most prevalent and often numerically dominant organisms in the healthy women. More than 20 *Lactobacillus* species have been reported in the human vagina, among which *L. crispatus*, *L. iners*, *L. gasseri* and *L. jensenii* have been shown to be the most predominant species [1,2].

The production of antimicrobial compounds by lactobacilli has been suggested to be important in maintaining a healthy vaginal microbial ecosystem. Lactic acid as the common antimicrobial compound could help to maintain the vaginal pH below 4.5 thereby affecting the growth of vaginal pathogens. In addition, different *Lactobacillus* strains may produce a variety of antimicrobial compounds, such as hydrogen peroxide (H₂O₂), bacteriocin and biosurfactants, limiting the pathogen colonization of and growth in the vagina [3,4]. However, the production of antimicrobial compounds has been reported to be a species/strain specific property. For example, the production of H₂O₂ was reported to vary even within the same species of *Lactobacillus* [4]. In addition, the competitive adherence to form biofilms or aggregates has been suggested to be an important mechanism for lactobacilli to exclude potential pathogens [5]. Hence, lactobacilli can be used as probiotics for prevention and treatment of urogenital infections, including bacterial vaginosis, urinary tract infections and vulvovaginal candidiasis (VVC), which are the most common reasons for women to visit a doctor [6].

VVC is defined as signs and symptoms of inflammation caused by overgrowth of *Candida* spp., most commonly *C. albicans*. It affects 70%~75% of women at least once during their lives, and is most frequently observed in young women of childbearing age [7]. The studies on the interactions of various vaginal *Lactobacillus* strains with *C. albicans* are very important in terms of understanding natural control of and artificial intervention for *Candida* overgrowth using lactobacilli, especially today when antibiotic resistance of *Candida* is presenting as a severe clinical problem. Some researchers have evaluated the beneficial properties of *Lactobacillus* strains against *Candida* [8, 9]. However, the probiotic use of autochthonous *Lactobacillus* strains has not been well characterized and only very few probiotic strains are effective against *Candida* in the vagina. Therefore, studying potential beneficial effects of lactobacilli candidates in this context is needed.

The aims of this study were to characterize the vaginal *Lactobacillus* strains from our laboratory collection in order to identify novel *Lactobacillus* strains that harbor potential beneficial effects on vaginal health by determining bacterial growth, acid and H₂O₂ production, biofilm formation, autoaggregation/coaggregation abilities and antagonistic activity against *C. albicans*.

2. MATERIALS AND METHODS

2.1 Microbial Strains and Growth Conditions

Twelve *Lactobacillus* strains from our laboratory collection were used in this study. They were

originally isolated from vaginal secretions of fertile women, and belonged to *L. fermentum* (A011, A024, A025, A035, A041, A044 and A081), *L. crispatus* (A014 and A055), *L. gasseri* (A054), *L. jensenii* (A083) and *L. salivarius* (A023). The strains were initially identified by Gram's stain and carbohydrate fermentation, and confirmed by 16S rDNA sequencing analyses. The primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTACGACTT-3') were used for amplification of the 16S rDNA internal fragment (~1400 bp) and commercial sequencing (BGI, Beijing, China) [10]. The 16S rDNA sequences were analyzed using the EzTaxon-e database (<http://eztaxon-e.ezbiocloud.net>) [11]. The De Man, Rogosa and Sharpe (MRS; BD, Sparks, MD, USA) medium and anaerobic GasPak™ EZ Gas Generating Container Systems (BD, Sparks, MD, USA) were used for cultivation.

Candida albicans ATCC 90029 and the Clinical strain of *C. albicans* Z03 was kindly provided by Dr. JH Hu (National Center for Clinical Laboratory, Beijing, China) and Dr. XY Hu (Nanchang University). *C. albicans* was cultured in yeast extract-peptone-dextrose (YPD) medium at 37°C for 24 h.

2.2 Growth and Acidification Measurements

Bacterial growth was determined by measurement of optical density at 630nm (OD_{630nm}) wavelength using a microplate reader (Model 680, Bio-rad, Hercules, CA, USA). The pH was monitored by using a pH meter (PHS-3E, INESA Scientific Instrument, Shanghai, China). MRS broth (pH 6.5) was inoculated with pre-cultured bacterial suspension to give an initial bacterial concentration of ~10⁵ CFU/mL and incubated anaerobically at 37°C for 24 h. The OD_{630nm} and pH values were recorded at 2 h intervals.

2.3 Semi-quantitative Determination of H₂O₂ Production

The H₂O₂-producing ability was determined as previously reported [12,13]. In brief, following 48 h of anaerobic incubation on the TMB-MRS plates [MRS agar containing 250 µg/mL 3, 3', 5, 5'-tetramethylbenzidine (TMB; Amresco, Solon, OH, USA) and 50 µg/mL horseradish peroxidase (HRP; Biodee, Beijing, China)], the colonies of H₂O₂-producing strains turned blue after 30 min air exposure. The color intensity was scored

visually, with white, pale blue and dark blue colonies indicating no, poor and high H₂O₂ production, respectively. *L. acidophilus* CGMCC 1.1878 (= ATCC 4356) purchased from the China General Microbiological Culture Collection Center was used as a positive control, and graded as a strong H₂O₂ producer.

2.4 Biofilm Formation Assay

The biofilm-forming ability of lactobacilli was monitored as described with some modifications [14]. Biofilm formation was induced in sterile 96-well round-bottomed polystyrene plates (Costar 3799, Corning, NY, USA). The broth culture in mid-exponential phase was inoculated into fresh MRS broth at a dilution of 1:100, from which a 200 µL aliquot was added to each well and incubated anaerobically at 37°C for 24 h, 48 h and 72 h. The MRS broth was used as a negative control. Each strain was tested in six replicates. At the sampling time point, the wells were washed three times with pH 7.2 phosphate-buffered saline to remove planktonic bacteria. The remaining attached bacteria were fixed with 200 µL methanol for 15min, stained with 200 µL 0.2% (w/v) crystal violet for 20 min, and then washed with distilled water to remove the excess crystal violet. The bound crystal violet was released by 200 µL of 33% acetic acid, from which 150 µL was removed for measuring the OD_{630nm} using microplate reader. The experiment was performed independently for three times.

The cut-off OD (OD_c) was defined as three standard deviations (SD) above the mean OD of the negative control and calculated separately for each microtiter plate. Final OD value of a tested strain is expressed as a mean OD value of this strain reduced by OD_c value [15]. Statistical analyses were performed using SPSS software (version 20.0).

2.5 Spectrophotometric Aggregation Assay

A quantitative spectrophotometric assay was used with some modifications [16]. The exponential-growth-phased microorganisms were harvested by centrifugation, washed twice with aggregation buffer (0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.15 M NaCl and 3.1 mM NaN₃ dissolved in 1 mM Tris buffer adjusted to pH 7.0) [17], and then suspended in the same buffer (approximately 10⁸ CFU/mL for *Lactobacillus* spp. and 10⁷ CFU/mL for *C. albicans*). Measurement of the autoaggregation percentage of each organism

was evaluated as follows: the 1.4 mL cell suspension was vortexed for 10 s and incubated at 37°C for 4 h without agitation. The OD_{630nm} of upper suspension (0.2 mL) was measured before and after incubation. The autoaggregation percentage was expressed as: %Autoaggregation = (OD_{initial} - OD_{final})/OD_{initial} × 100.

The method for preparing the cell suspensions for coaggregation was the same as that for autoaggregation assay. Equal volumes (0.7 mL) of each cell suspension were mixed together in pairs by vortexing for 10 s. The OD_{630nm} of upper suspension was measured before and after 4 h of incubation at 37°C. The coaggregation percentage was calculated as follows: %Coaggregation = [(OD_A + OD_B) - 2 × OD_{mix}]/(OD_A + OD_B) × 100, where OD_A and OD_B represented the OD_{630nm} of strain A (*Lactobacillus* spp.) and strain B (*C. albicans*) before incubation, respectively; and OD_{mix} represented the OD_{630nm} of the coaggregation mixture after incubation.

2.6 Observation of Coaggregation Using an Optical Microscope

Cells were prepared as described above. The 0.1 mL of each *Lactobacillus* suspension was mixed with 0.1 mL of *Candida* suspension by vortexing for 10 s and then incubated in a 96-wellplate for 4 h at 37°C with gentle shaking (50 rpm). A droplet (5 µL) of each suspension was then put on a glass slide and stained with crystal violet for visual observation of aggregates by light microscopy.

2.7 Antagonistic Activities of *Lactobacillus* Strains against *C. albicans*

To detect antagonistic activities of *Lactobacillus* strains against *C. albicans*, the spot inhibition, agar overlay and Oxford Cup methods were used. The spot inhibition assay was performed as follows, about 10⁷ CFU/mL of *C. albicans* were spread over MRS agar plates using a sterilized cotton swab, and then each of 5 µL of overnight cultures of *Lactobacillus* strains were spotted onto the surface of MRS agar, respectively. The plates were incubated anaerobically at 37°C to allow the development of *Lactobacillus* spots. After 48 h of incubation, the plates were incubated aerobically at 37°C for 24 h and inhibition zones were read. The agar overlay method was a modification of that described by Xu et al. [13]. The tested

Lactobacillus strains were spotted onto the surface of MRS agar plates and incubated anaerobically for 48 h. These plates were the overlaid with a 10 mL of YPD agar (containing 0.5% agar) inoculated with about 10⁷ CFU/mL of *C. albicans*. After aerobic incubation for 24 h, the plates were checked for the inhibition zones.

For Oxford Cup method, the supernatant of the 48 h-culture of lactobacilli were obtained by centrifugation (10000 × g, 10 min), then sterilized through a 0.22 µm Millipore filter. *C. albicans* strains (about 10⁷ CFU/mL) were spread over YPD agar by cotton swabs and the stainless steel Oxford Cup were then placed on the surface of the inoculated medium. An aliquot of 200 µL of the supernatant was added into the Cup. The plates were incubated aerobically for 24 h, and the inhibition zones were read. The inhibitory effect of MRS was tested as a negative control on each plate.

All assays were independently performed at least three times. The growth inhibitory activity (GIA) was calculated by subtracting a circle diameter (CD) of a *Lactobacillus* spot or a Cup (outside diameter 8 mm) from the inhibition zone diameter (IZD) and expressed as follows: GIA = (IZD - CD)/2 [18].

2.8 Statistical Analyses

Statistical analyses were performed using SPSS software (version 20.0). Bonferroni's test was used to determine significant differences in biomass at the different time points for biofilm formation. Student's t-test was used to compare coaggregation percentage between *Lactobacillus* with *C. albicans* ATCC 90029 and with *C. albicans* Z03. A *P*-value of < 0.05 was considered statistically significant.

3. RESULTS

3.1 Identification of *Lactobacillus* by 16S rDNA Sequence Analysis

By comparing the 16S rDNA sequences to those held in EzTaxon-e database, the twelve *Lactobacillus* strains were identified as *L. fermentum* (7 strains), *L. gasseri* (1 strain), *L. crispatus* (2 strains), *L. jensenii* (1 strain) and *L. salivarius* (1 strain) (similarity > 99.8%), respectively. The obtained sequences were deposited in GenBank with the following accession numbers: KF649202, KF661284, KF661285, KF661286, KF661287, KF740707,

KF661289, KF661290, KF661291, KF661292, KF661293 and KF661294.

3.2 Growth and Acidification Properties

In this study the pH decline, an indicator of organic acid production, was found to parallel the bacterial growth (Fig. 1). After 24 h incubation the medium pH was found to be ranging from 3.7 to 4.3. The results revealed that *L. fermentum* and *L. salivarius* were found to take the shortest time (6 h) to achieve pH 4.5. In addition, the results revealed that an $OD_{630nm} > 1.0$ of bacterial culture was critical to achieve $pH < 4.5$ for most strains except for *L. crispatus*, in which an $OD_{630nm} > 0.8$ was sufficient.

3.3 H₂O₂ Producing Abilities

The results showed *L. acidophilus* CGMCC 1.1878 (positive control) and *L. jensenii* A083 as strong, *L. crispatus* (A014 and A055) as moderate and *L. salivarius* A023 as weak H₂O₂ producers, while all strains of *L. fermentum* and *L. gasseri* A054 were not found to produce H₂O₂.

3.4 Biofilm Formation Abilities

All the tested strains except for *L. crispatus* A014 could form a biofilm on the polystyrene surface throughout the whole experimental period

(Fig. 2). In addition, two major dynamic patterns of biofilm formation were found. Some strains displayed a mountain-like biofilm formation pattern (*L. fermentum* A011, A044, A024, A035, A041 and A025; *L. jensenii* A083), in which the biofilm biomass at 48 h was the largest; whereas others showed a time-dependent biomass decreasing pattern (*L. crispatus* A055, *L. fermentum* A081 and *L. gasseri* A054).

3.5 Aggregation Abilities

The spectrophotometric assay showed that all the tested *Lactobacillus* strains had autoaggregation abilities, but the autoaggregation percentages varied significantly from 20.2% of *L. salivarius* A023 to 92.1% of *L. jensenii* A083 (Fig. 3A). The strains with the best autoaggregation abilities were *L. crispatus* A014, *L. crispatus* A055 and *L. jensenii* A083, whose autoaggregation percentage were 85.2%, 88.6%, and 92.1%, respectively. More importantly, these three strains exhibited relatively high coaggregation abilities (> 80.0%) as well. The tested strains showed higher coaggregation percentages with *C. albicans* ATCC 90029 than with *C. albicans* Z03, and the difference was statistically significant ($P < 0.05$).

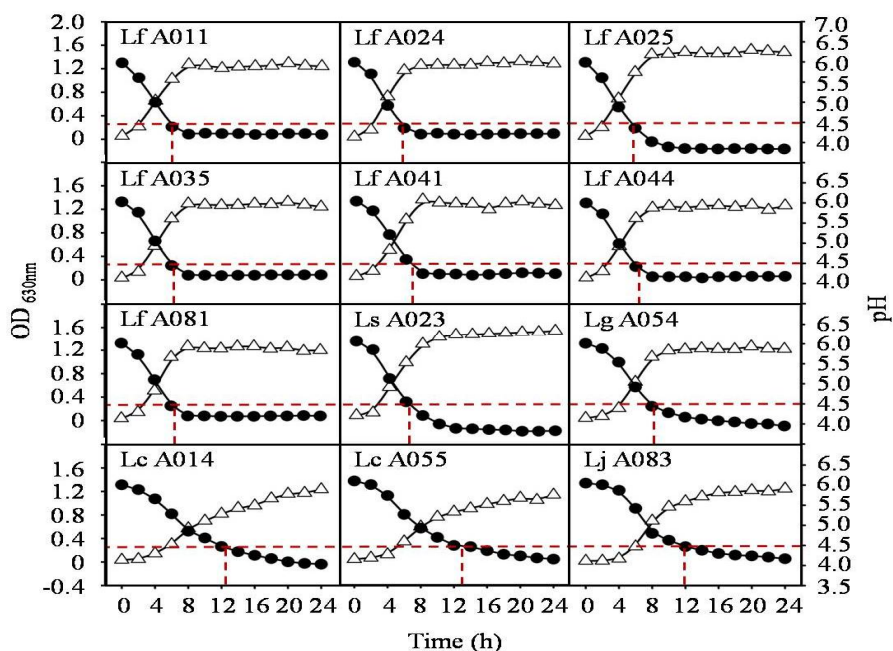


Fig. 1. Growth and pH curves of vaginal *Lactobacillus* isolates. The figure shows one representative experiment of three independent replicates. Lf: *L. fermentum*, Ls: *L. salivarius*, Lg: *L. gasseri*, Lc: *L. crispatus*, Lj: *L. jensenii*. Symbols: OD_{630nm} (Δ) and pH (\bullet)

In addition, the coaggregation abilities of *Lactobacillus* strains with *C. albicans* were observed by light microscopy. The coaggregating clusters formed by *C. albicans* ATCC 90029 and three *Lactobacillus* strains with high coaggregation percentages are shown in Fig. 3B-D. The coaggregates of each pair had a different appearance.

3.6 Antagonistic Activities of *Lactobacillus* Strains against *C. albicans*

The abilities of *Lactobacillus* strains to antagonize the growth of *C. albicans* were evaluated by using the spot inhibition, agar overlay and Oxford Cup assays, (Table 1). Using the spot inhibition assay, besides *L. gasseri* A054, all of the tested *Lactobacillus* strains showed inhibitory activities; The *Lactobacillus* strains showed stronger inhibitory activity against *C. albicans* ATCC 90029 than against *C. albicans* Z03. *L. salivarius* A023 displayed the highest inhibitory effect against *C. albicans* ATCC 90029 (GIA: 5.50 ± 1.00 mm) and *C. albicans* Z03 (GIA: 4.67 ± 1.04 mm). In the agar overlay assay, only *L. salivarius* A023, *L. fermentum* A025 and *L. crispatus* A014 showed growth inhibitory activity against both *C. albicans* ATCC 90029 and *C. albicans* Z03. In contrast, using the Oxford Cup method, no growth inhibitory activity of

supernatants from any *Lactobacillus* strains was observed.

4. DISCUSSION

Lactobacillus species significantly influence vaginal microbiota. The use of probiotic lactobacilli, such as *L. crispatus* CTV05, *L. rhamnosus* GR-1 and *L. reuteri* RC-14, to prevent urogenital infections has a good rationale and safety record [19-22]. However, different reports describe the controversial results. Jurden et al. [23] reported that a treatment with probiotic suppositories containing *Lactobacillus* could not reduce the recurrences of either BV or VVC compared with the placebo treatment. This might be due to the strain-specific effects of probiotics or inter patient differences. Considering the limited amount of probiotic strains for clinical use, more probiotic lactobacilli candidates should become available. This could be of particular interest in China, where many women suffer from the recurrent BV and VVC. In addition, the domestic lactobacilli strains have not been well characterized for the potential probiotic use. Therefore, it is worth exploring and characterizing autochthonous *Lactobacillus* strains in order to obtain novel probiotic candidates to improve vaginal health.

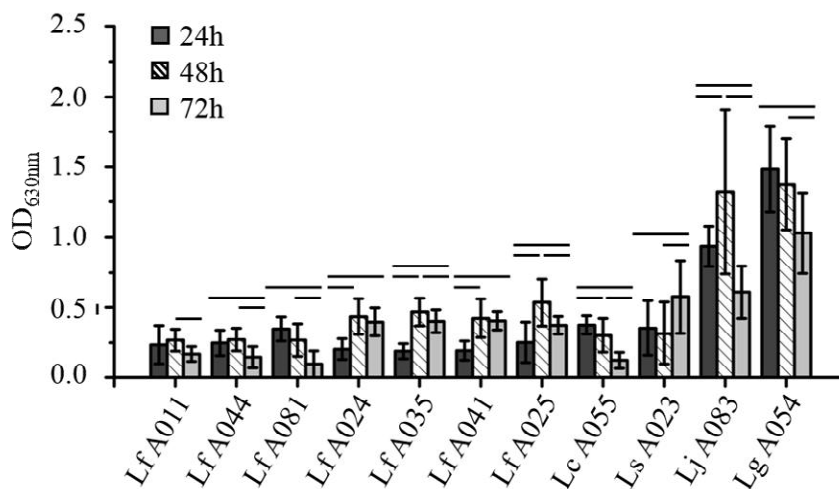


Fig. 2. Biofilm formation by vaginal *Lactobacillus* isolates on 96-well polystyrene plates at different incubation time periods. *L. crispatus* A014 was not found to form biofilm and not shown here. The data were expressed as the final $OD_{630nm} \pm SD$ of acetic acid-solubilized, crystal violet-stained cultures from microplates. All assays were repeated in triplicate. Lc: *L. crispatus*, Lf: *L. fermentum*, Ls: *L. salivarius*, Lj: *L. jensenii*, Lg: *L. gasseri*. “—”: significant difference ($P < 0.05$)

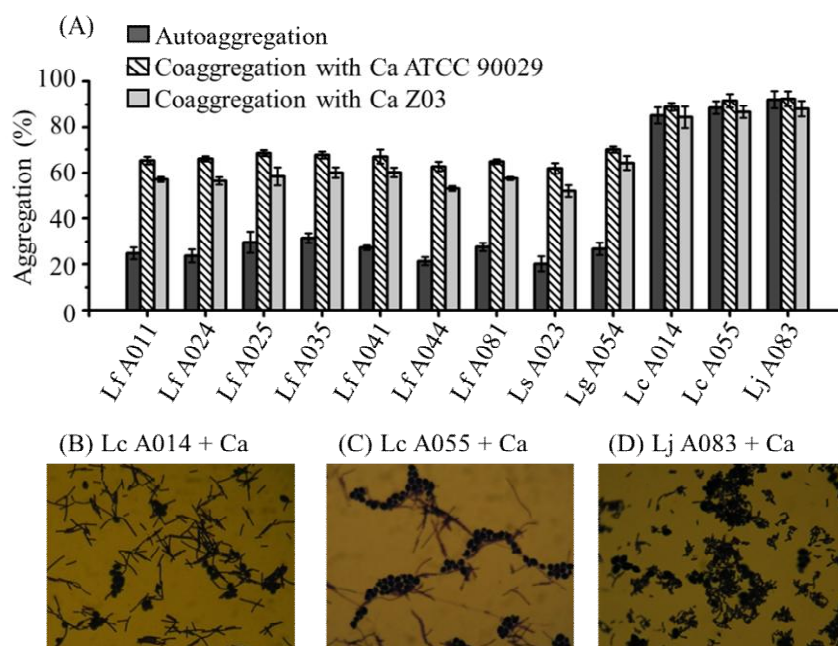


Fig. 3. Autoaggregation and coaggregation abilities of *Lactobacillus* strains. (A) comparison of autoaggregation and coaggregation percentage of *Lactobacillus* strains after 4h incubation. Each value shown is the mean \pm SD from three experiments. Lf: *L. fermentum*, Ls: *L. salivarius*, Lg: *L. gasseri*, Lj: *L. jensenii*, Lc: *L. crispatus*, Ca: *C. albicans*. (B-D) observation of coaggregating clusters formed by *C. albicans* ATCC 90029 and three *Lactobacillus* strains with high coaggregation abilities (by light microscopy, originally 1000 \times)

Table 1. Growth inhibitory activity of *Lactobacillus* strains against *C. albicans*

Strains	<i>C. albicans</i> ATCC 90029			<i>C. albicans</i> Z03		
	Spot inhibition	Agar overlay	Oxford cup	Spot inhibition	Agar overlay	Oxford cup
<i>L. fermentum</i> A011	+	-	-	+	+	-
<i>L. fermentum</i> A024	+	-	-	+	-	-
<i>L. fermentum</i> A025	+++	+++	-	++	++	-
<i>L. fermentum</i> A035	++	-	-	+	-	-
<i>L. fermentum</i> A041	++	+	-	+	-	-
<i>L. fermentum</i> A044	++	-	-	+	-	-
<i>L. fermentum</i> A081	+	-	-	+	-	-
<i>L. crispatus</i> A014	++	++	-	++	+	-
<i>L. crispatus</i> A055	++	+	-	+	-	-
<i>L. salivarius</i> A023	+++	++	-	+++	++	-
<i>L. gasseri</i> A054	-	-	-	-	-	-
<i>L. jensenii</i> A083	+	-	-	+	-	-

Note: The growth inhibitory activity (GIA) was calculated by subtracting a circle diameter (CD) of a *Lactobacillus* spot or a cup (outside diameter: 8 mm) from the inhibition zone diameter (IZD) and expressed as follows: $GIA = (IZD - CD)/2$ [18]. Symbols: -, $GIA < 0.5$ mm; +, 0.5 mm \leq GIA < 2 mm; ++, 2 mm \leq GIA < 3.5 mm; +++, GIA ≥ 3.5 mm

In this study, a total of 12 vaginal *Lactobacillus* strains belonging to *L. fermentum*, *L. gasseri*, *L. crispatus*, *L. jensenii* and *L. salivarius* confirmed by their 16S rDNA sequences were characterized for their potential beneficial effects in the context of vaginal infections. These *Lactobacillus* species have been reported to be commonly found in healthy women [2,24].

Lactic acid and other fatty acid production are basic probiotic properties of lactobacilli [25]. Fig.1 shows that *L. fermentum* and *L. salivarius* could achieve pH 4.5 within 6 h of incubation. At this pH the proliferations of pathogens, such as *Escherichia coli*, *Neisseria gonorrhoeae*, *Prevotellabivia*, *Gardnerella vaginalis* and *C.albicans*, can be inhibited [3,25]. Thus, their

primary contributions in keeping vaginal health might be the creation of an acidic environment as soon as possible during the initial colonization most likely by rapid growth.

The development of vaginal infection has been reported to be associated with a reduction in the prevalence and amount of H₂O₂-producing bacteria [26]. Production of H₂O₂ by *Lactobacillus* species is considered to represent an important nonspecific antimicrobial defense mechanism of the normal vaginal ecosystem [27]. The data generated in this *in vitro* study show that the relatively fast-growing strains (*L. fermentum*, *L. salivarius* and *L. gasseri*) were not found to produce H₂O₂, while the relatively slow-growing strains (*L. crispatus* and *L. jensenii*) were moderate to strong H₂O₂ producers. These results seem to indicate that mainly fast-growing lactobacilli might contribute to a rapid lactic acid accumulation resulting in a pH decrease in a H₂O₂-safe environment during the initial colonization period, when the potential antagonistic effect of H₂O₂ against non-producers could be minimized. Then the initially slow-growing H₂O₂ producers would gradually take over the vaginal ecosystem and benefit the host at least by lactic acid and H₂O₂ productions. However, more investigations are needed to verify this hypothesis.

Vaginal adherence is an important virulence factor of *C. albicans*; thus, reducing its adherence may prevent VVC. As demonstrated by many studies, biofilm formation and autoaggregation, which are bacterial surface structure/molecule-mediated properties, have been suggested as the desired properties for colonization of probiotic bacteria [28]. Coaggregation of lactobacilli with *Candida* may also be important for the prophylaxis against vaginal infections by preventing the binding of *Candida* to the receptors of the vaginal epithelium [29]. Our results suggest that adherence to a surface most likely is an essential trait for the tested *Lactobacillus* spp., but the differences might exist in the mechanisms of biofilm formation and responses to the environmental changes (e.g., H₂O₂ and lactic acid productions) among different species/strains [30-32]. The dynamic observation of biofilm formation (Fig. 2) and pH changes (Fig. 1) revealed that lactobacilli in the biofilm could survive for at least three days in this *in vitro* very acidic environment, where many pathogens would hardly survive.

In addition, Fig. 2 and 3 demonstrate that the ability of biofilm formation not always parallels with the ability of autoaggregation or coaggregation with *C. albicans*, suggesting that different bacterial surface structures/molecules might be involved in these processes [30,33]. Further investigations are needed to characterize the surface structures/molecules of those *Lactobacillus* strains with potential probiotic use, such as *L. jensenii* A083, which had very strong biofilm formation, autoaggregation and coaggregation abilities.

Another interesting finding is that three good H₂O₂ producers (*L. crispatus* A014, *L. crispatus* A055 and *L. jensenii* A083) are also very proficient in autoaggregation and coaggregation with *C. albicans*, whereas those strains with low autoaggregation abilities were found to produce no or small amounts of H₂O₂ (*L. fermentum*, *L. gasseri* A054 and *L. salivarius*) (Fig. 3). It is known that most lactobacilli do not have catalase, an enzyme converting the toxic H₂O₂ to H₂O and O₂. The survival mechanisms of the H₂O₂ producing *Lactobacillus* strains without catalase have not been well characterized. To confirm that the aforementioned findings truly indicate that autoaggregation provides a shelter for the survival of H₂O₂ producing strains in an environment rich in H₂O₂, experiments using more strains and in depth studies on the underlying molecular mechanisms are required. To study the antagonistic activities of *Lactobacillus* against *C. albicans*, various experimental protocols have been described. In this study we used three assays adopted or modified from others [34-36]. We did not treat lactobacilli supernatants with NaOH or catalase prior to testing; thus, the anti-*Candida* activities of organic acids and H₂O₂ were not excluded. We observed that different assays give different results, which has also been reported by Verdenelli et al. [35]. The results indicate that *L. salivarius* A023, *L. fermentum* A025 and *L. crispatus* A014 have high antagonistic activities against *C. albicans* in the spot inhibition and agar overlay methods, however their supernatants did not exhibit the same activities. The results hint that the inhibition activities found in the spot inhibition and agar overlay method cannot be attributed to lactic acid and H₂O₂ production. The results indicate that the observed anti-*Candida* activities of *Lactobacillus* strains might depend on the production of extracellular, diffusible inhibitory compounds, which might be induced by the presence of *Candida*, rather than the production of H₂O₂ and organic acids. Our results

suggest that the spot inhibition assay could be used as a rapid screening method for growth inhibitory activity of lactobacilli, but the other assays were also needed to clarify the observations.

5. CONCLUSION

In summary, *L. jensenii* A083 might exclude the vaginal pathogens through H₂O₂ production, coaggregation and biofilm formation; *L. fermentum* (A011, A024, A025, A035, A041, A044 and A081) might play an important role in rapidly lowering the vaginal pH; *L. salivarius* A023, *L. fermentum* A025 and *L. crispatus* A014 were shown to display potential abilities to inhibit *C. albicans* growth. Whether these *Lactobacillus* strains alone or in combination with other strains will effectively prevent *C. albicans* from colonization or overgrowth in the vaginal environment, more investigation will have to be carried out.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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