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Full Length Research Paper

Vaginal lactobacillus distributions and functions in pregnant women

Li Lin*, **Hain-ing Yang and Jianrong Su**

Department of Obstetrics and Gynecology, Beijing Friendship Hospital–affiliated to Capital Medical University of Medical Sciences, Beijing 100050, China.

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***Lactobacillus* plays an important role in keeping the vaginal micro-ecological balance and inhibiting the growth and reproduction of pathogens. However, the knowledge of *Lactobacillus* distributions and functions in pregnant women remains unclear. The study was undertaken the prevalence of *Lactobacillus* and the specific strains of *Lactobacillus* present in vaginal discharge from women at different stages of pregnancy. This study was performed between October 2009 and January 2010 at the Department of Obstetrics and Gynecology, Beijing Friendship Hospital, Affiliate of Capital University of Medical Sciences, China. pH value of vaginal discharges was determined, vaginal discharges were collected and *Lactobacillus* were isolated and cultured, hydrogen peroxide generation of *Lactobacillus* was performed on 120 pregnant women. Statistical analysis was carried out using SPSS 11.5 software. Among the 120 samples of vaginal discharge, *Lactobacilli* was isolated in 104 samples (86.7%), 80 were *Lactobacillus acidophilus (*76.9%), 80 were LB+ (76.9%), 64 were *L. acidophilus.* Most *L. acidophilus* isolates could produce hydrogen peroxide. pH gradually decreased with ongoing pregnancy, but there were no significant differences among the three trimesters (*P*>0.05). Our results indicated that *L. acidophilus* is the dominant bacterial strain in terms of the vaginal *Lactobacillus* distributions and functions in pregnant women.**

Key words: Lactobacillus, vaginal, pregnant women.

INTRODUCTION

Lactobacillus constitutes the main bacterial flora in the normal vagina. It can inhibit the growth of other bacteria by competing for vaginal epithelial cell adhesion and secreting organic acids, hydrogen peroxide, bacteriocin or lactobacillin to maintain an acidic vaginal environment, which is important to keep the vaginal micro-ecological balance and inhibit the growth and reproduction of pathogens, and thus reduce the incidence of vaginal infection (Lazăr et al., 2009; Koyama et al., 2010; Eun et al., 2011). By generating hydrogen peroxide, *Lactobacillus* plays a key role in maintaining the health of the vagina by virtue of its self-cleaning and anti-infection activities (Muench et al., 2009; Kiose et al., 2010). There is a certain degree of correspondence between the vaginal and rectal microflora (El Aila et al., 2009). The presence of different *Lactobacillus* species with the normal vaginal microflora is a major determinant to the stability of this microflora in pregnancy (Verstraelen et al., 2009). Thus, the comprehensive understanding of vaginal *Lactobacillus* states and functions in pregnant women is essential for a better understanding of the factors involved in maintaining the vaginal micro-ecological balance and preventing and treating vaginal infections during pregnancy. However, until now there are no reports to assess the roles of *Lactobacillus* during pregnancy, and even worse, to our knowledge, no cohort studies having been conducted to investigate the changes of vaginal *Lactobacillus* during ongoing pregnancy.

In this study, we collected vaginal discharge from women in the first (10 to 12 weeks), second (26 to 28

*Corresponding author. E-mail: linli488@126.com. Tel: +8610-63138385. Fax: +8610-63138385.

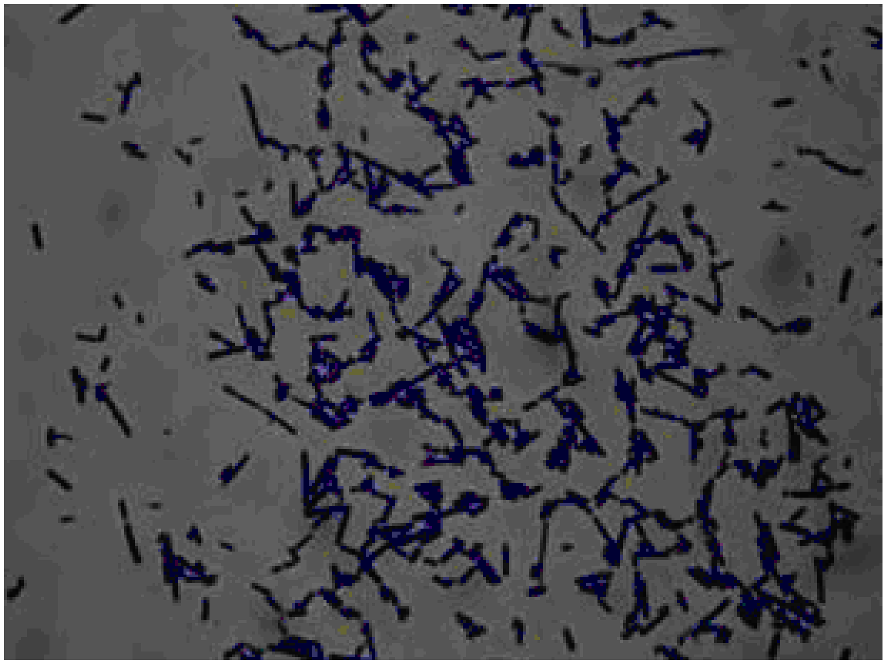

Figure 1. Gram staining of *Lactobacillus acidophilus* under an oil objective Lens (1000×).

weeks) and third (34 to 36 weeks) trimesters to study the states and functions of *Lactobacillus*.

MATERIALS AND METHODS

Subjects

A total of 128 pregnant women between October 2009 and January 2010 were selected for collection of vaginal discharge at three consecutive stages of pregnancy, that is, the first (10 to 12 weeks), second (26 to 28 weeks) and third (34 to 36 weeks) trimesters, with 24, 22 and 22 cases at each time point, respectively. B-ultrasound showed a single live intrauterine fetus. Before collecting the samples, vaginal symptoms were surveyed and women with vaginal hemorrhage, class-III cleanliness, and vulvovaginal candidiasis or trichomonal vaginitis were excluded. And then vaginal discharge smears were examined by microscopy, and cases with bacterial vaginosis (determined by a BV Blue diagnostic kit) were also excluded.

Collection of vaginal discharges and pH determination

Vaginal discharges were collected at the vaginal lateral wall, 4 cm from the vaginal orifice with a sterile cotton swab under a vaginal speculum, without lubricants used, placed into *Lactobacillus* medium, and then sent to a laboratory for cultivation within 4 h after collection. pH values were determined using pH test papers (pH 3.8 to 5.4) from Beijing Chemical Factory. The test papers were directly placed on the vaginal lateral wall, 4 cm from the vaginal orifice. 30 min later, the pH values were detected.

Isolation and culture of *Lactobacillus*

Vagina posterior fornix discharge was directly inoculated onto a modified MRS agar plate, placed in an anaerobic incubator, and cultured at 37℃ for 48 to 72 h. The typical colonies were picked for Gram staining. After being dried and fixed, the smears were stained with crystal violet for 1 min. Excess color was removed with water, and samples were stained with iodine for 1 min. Samples were then washed and bleached with 95% alcohol, and stained with the carbol-fuchsin solution for 0.5 min, and then washed again. The colonies were observed under a 1000× microscope, and initially identified as *Lactobacillus spp*. (Figure 1). *Lactobacillus* is a gram-positive bacterium with rod-shaped, fence-shaped or chain-like appearance without spores or capsules, and it is generally not motile. The catalase test was negative. The bacteria were isolated and cultured by the plate-streaking method. After 3 to 4 rounds of separation, pure strains were obtained.

Identification of bacteria strains

The API 50 CHL *Lactobacillus* strain identification kit was used to identify the *Lactobacillus* strains present in the samples. After isolation and purification, the colonies on the MRS agar plate were scratched with a sterile cotton swab and placed into 2 ml of sterile water, and then shaken for the concentrated strain solution. Afterwards, the solution was pipetted with a sterile pipette into 5 ml of sterile water with the turbidity compared with 2 McB units by turbidimetry, and the number of drops was recorded as n. After that, 2 n drops of bacteria solution were added into 7 ml of the *Lactobacillus* strain identification reagent in a micro-tube, and shaken. The resulting solution was added into the API 50 CHL *Lactobacillus* identification reagent kit with a fluid infusion apparatus, sealed with sterile liquid paraffin, and then cultured at 37℃ for 48 h (Figure 2). API 50 CHL identification software was used to identify the bacteria strains.

Hydrogen peroxide generation of *Lactobacillus*

According to the qualitative method (Eschenbach et al., 1989), the

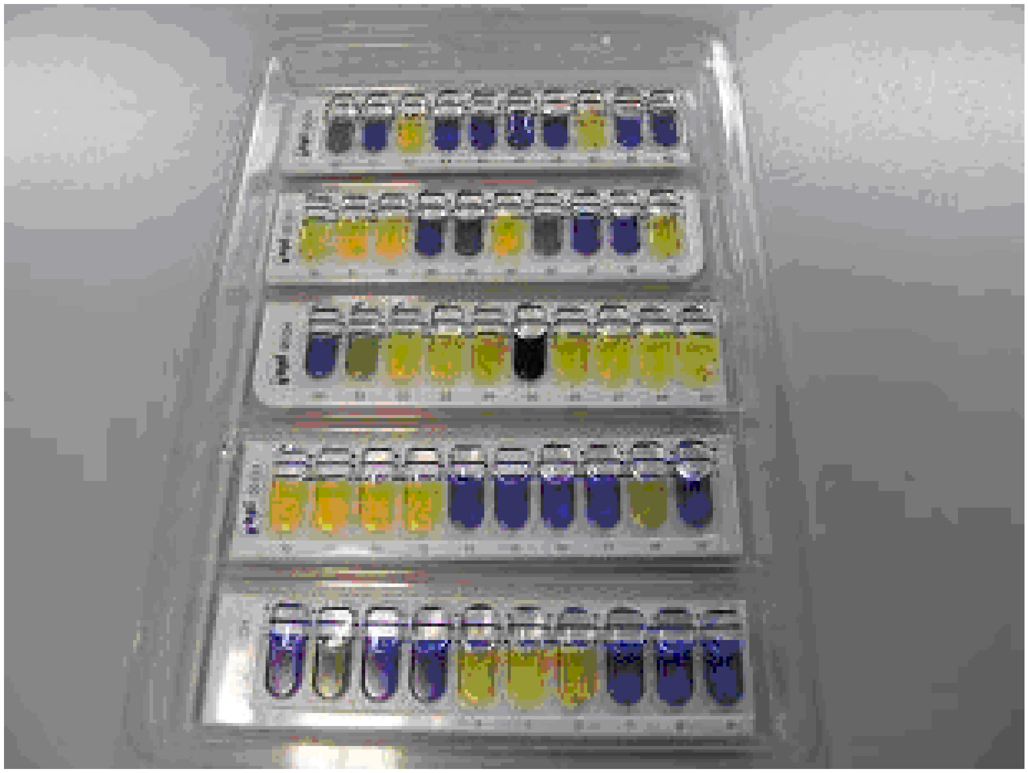

Figure 2. Color reaction of *Lactobacillus acidophilus* on the API50 CHL *Lactobacillus* identification kit.

pure *Lactobacillus* strains after isolation were directly inoculated into MRS culture medium supplemented with 0.25 mg/ml of tetramethylbenzidine (TMB) and 0.01 mg/ml horseradish peroxidase (HRP), placed in an anaerobic incubator, and cultured at 37°C for 48 h. Then, the culture medium was removed, and bacteria were exposed to the aerobic environment for 30 min. *Lactobacillus* (LB +) colonies producing hydrogen peroxide would develop a blue color in that the hydrogen peroxide generated by *Lactobacilli* would be decomposed by peroxidase to generate oxygen, which, in turn, would oxidize TMB to form a blue adduct (Figure 3).

Statistical analysis

SPSS 11.5 software was used to conduct the statistical analysis on the data. χ^2 test was carried out. P-value <0.05 was considered to be significant difference.

RESULTS

Subject characteristics

A total of 120 pregnant women were eligible, participated in the study, and the results were obtained for all tests. The average age was (28.86±3.49) years old. The average gestational age at the first examination was (11.2±0.4) weeks, (26.8±0.7) weeks at the second and (34.6±0.5) weeks at the third.

Vaginal pH

pH gradually decreased with ongoing pregnancy, but there were no significant differences among the three trimesters (*P*>0.05) (Table 1).

Rate of *Lactobacillus* isolation

Among the 120 samples of vaginal discharge, *Lactobacilli* were isolated in 104 samples (86.7%), including 80% in the first trimester and 90% in both the second and the third trimesters. Accordingly, the rate of vaginal *Lactobacilli* isolation was lower in the first trimester than that in the second or the third, although this was not statistically significant (P>0.05) (Table 2).

Identification of *Lactobacillus* strains

Among the 104 isolated strains of *Lactobacillus*, 80 were *L. acidophilus*. 16 were *Lactobacillus delbrueckii ssp* and 8 were *Lactobacillus crispatu*. Accordingly, the dominant vaginal *Lactobacillus* strains in pregnant women were *L. acidophilus* (76.9%), followed by *L. delbrueckii ssp delbrueckii* (15.4%) and *L. crispatu* (7.7%). The proportion of samples containing *L. acidophilus* was significantly higher in the third trimester than that in the

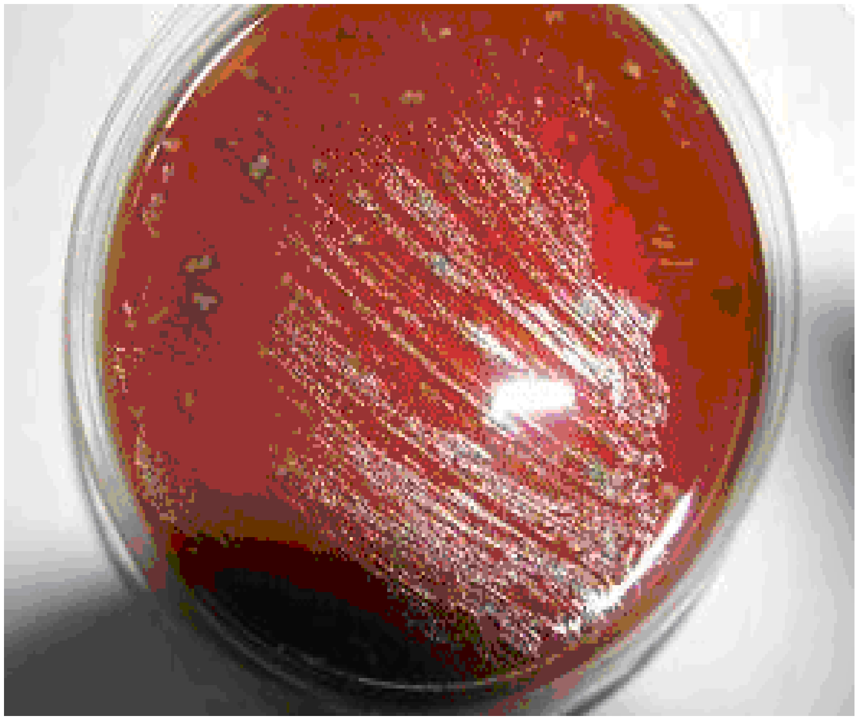

Figure 3. Hydrogen peroxide production by *Lactobacillus acidophilus* (blue colonies indicate colonies capable of producing hydrogen peroxide).

Table 1. pH of vaginal discharge from pregnant women.

Trimester	Number of cases (n)	pH value (mean ± SD)
First	40	4.10±0.20
Second	40	4.01±0.25
Third	40	3.92±0.21

(χ^2 test; χ^2 = 1.676, *P*>0.05).

Table 2. Rates of *Lactobacillus* isolation in vaginal discharge from pregnant women.

Trimester	Positive		Negative		Total (n)
	n	Isolation rate (%)	n	Isolation rate (%)	
First	32	80	8	20	40
Second	36	90	4	10	40
Third	36	90	4	10	40
Total	104	86.7	16	13.3	120

(χ^2 test; χ^2 = 2.308, *P*>0.05).

first or the second trimester (*P*<0.05). However, there were no statistically significant differences in the German distribution of *Lactobacillus* subspecies among three trimesters (*P*>0.05). Curly *Lactobacillus* was only identified in the second trimester, rather than in the first or second trimester (Table 3).

Isolation of hydrogen peroxide-producing *Lactobacilli* (LB+) strains

Of the 104 isolated *Lactobacilli* strains, 80 were LB+ (76.9%), including 62.5% in the first trimester, 77.8% in the second trimester and 88.9% in the third trimester.

Table 3. Distribution of vaginal *Lactobacillus* strains in pregnant women.

Trimester	*Lactobacillus acidophilus*		*Lactobacillus delbrueckii ssp delbrueckii*		*Lactobacillus crispatu*		Total (n)
	n	Isolation rate (%)	n	Isolation rate (%)	n	Isolation rate (%)	
First	28	26.9	4	3.8	0	0	32
Second	20	19.2	8	7.7	8	7.7$^{\$}$	36
Third	32	30.8*	4	3.8$^{\#}$	0	0	36
Total	80	76.9	16	15.4	8	7.7	104

(χ^2 test; $^{*}\chi^2$ = 14.18, *P*<0.05; $^{\#}\chi^2$ = 2.003, *P*>0.05; $^{\$}\chi^2$ = 16.37, *P*<0.05).

Table 4. Isolation of LB+ strains in pregnant women.

Trimester	LB+		LB–		Total (n)
	n	Isolation rate (%)	n	Isolation rate (%)	
First	20	62.5	12	37.5	32
Second	28	77.8	8	22.2	36
Third	32	88.9	4	11.1	36
Total	80	76.9	24	23.1	104

(χ^2 test; χ^2 = 6.669, *P*<0.05).

Therefore, with ongoing pregnancy, the isolated LB+ strains in the vagina gradually increased. The result showed that, with ongoing pregnancy, the vaginal LB+ separation rate gradually increased. These differences among the groups were statistically significant (P<0.05) (Table 4).

Distributions of LB+ strains

Of the 80 LB+ strains, 64 were *L. acidophilus.* Eight were *L. delbrueckii ssp delbrueckii* and eight were *L. crispatu.* Accordingly, the dominant LB+ strain in the vaginas of pregnant women was *L. acidophilus* (80%). *L. acidophilus* LB+ was more commonly found in the third trimester than in the first or second trimester (P<0.05). There were no statistically significant differences in the distribution of *Lactobacillus* LB+ German subspecies among three trimesters (P>0.05). Lactobacillus LB+ curl was only found in the second trimester (Table 5).

Hydrogen peroxide-producing capacities of the three kinds of *Lactobacillus* strains

All *L. crispatu* isolates were capable of producing hydrogen peroxide (8/8, 100%), while most *L. acidophilus* isolates produced hydrogen peroxide (64/80, 80%), and only half of the *L. delbrueckii ssp delbrueckii* (8/16, 50%) isolates produced hydrogen peroxide. Accordingly, the three kinds of *Lactobacilus* were expressed, according to their different abilities in producing hydrogen peroxide, as follows: *L. crispatu>L. acidophilus>L. delbrueckii ssp delbrueckii*. There were significant differences in terms of the hydrogen peroxide-producting capacity among the three kinds of *Lactobacillus* (*P*<0.05) (Table 6).

DISCUSSSION

During pregnancy, glycogen synthesis increases in vaginal epithelial cells because of the effects of high estrogen levels with enhanced production of lactic acid from glycogenolysis. Therefore, the vaginal pH is lower in pregnant women than that in non-pregnant women (McClelland et al., 2008, 2009). However, till now few studies have investigated the changes in vaginal pH during pregnancy. In this study, we measured vaginal pH in pregnant women during the first, second and third trimesters. We found that there was a gradual decline in vaginal pH during pregnancy, which may be related to the gradual increase in estrogen level over time. However, these findings were not significantly different. Nevertheless, the results were consistent with the previous report (Ma et al., 2010). During pregnancy, the changes of the estrogen level lead to the changes in the vaginal micro-ecological structure. The most obvious change is the increased amount of *Lactobacilli* in the vagina. As study showed, in which the vaginal discharge of 102 women in the early stage of pregnancy were

Table 5. Distribution of vaginal LB+ strains of pregnant women.

Trimester	*Lactobacillus acidophilus*		*Lactobacillus delbrueckii ssp delbrueckii*		*Lactobacillus crispatu*		Total (n)
	n	%	n	%	n	%	
First	20	25	0	0	0	0	20
Second	16	20	4	10	8	$20^{\$}$	28
Third	28	35*	4	$10^{\#}$	0	0	32
Total	64	80	8	20	8	20	80

(χ^2 test; $^*\chi^2 = 15.268$, $P<0.05$; $^{\#}\chi^2 = 3.016$, $P>0.05$; $^{\$}\chi^2 = 16.508$, $P<0.05$).

Table 6. Capacity of *Lactobacilli* strains to produce hydrogen peroxide.

Strain	Total cases (n)	LB+ (n)	%
Lactobacillus acidophilus	80	64	80
Lactobacillus delbrueckii ssp delbrueckii	16	8	50
Lactobacillus crispatu	8	8	100

(χ^2 test; $\chi^2 = 9.36$, $P<0.05$).

investigated, that *Lactobacillus* was found in 81.37% of the samples, which was higher than that in non-pregnant women of childbearing age (57.62%) (Corret al., 2007; Lombardo, 2008). However, to our knowledge, there are no reports on changes of vaginal *Lactobacillus* in pregnant women with a cohort study design involving subjects at different stages of pregnancy until now. A longitudinal study is expected to represent the actual conditions in view of its adopted methodology. In this study, we observed the changes of vaginal *Lactobacilli* in pregnant women during the first, second and third trimesters. And the overall detection rate of *Lactobacillus* in the 60 pregnant women was 86.7%, based on rates of 80% in the first trimester, and of 90% in both the second and third trimesters, suggesting a slight increase in the second trimester, which was maintained in the third trimester. However, these changes were not statistically significant.

Though the debate around distribution of vaginal *Lactobacillus* strains in non-pregnant women is heated nowadays, till now only a few studies have investigated the distributions of dominant vaginal *Lactobacillus* strains in pregnant women, among which, one study reported that the dominant vaginal *Lactobacillus* strain in pregnant women was *L. acidophilus* (Wylie and Henderson, 1969). However, another study reported that the dominant strains in pregnant women in the first trimester were *L. crispatu* and *Lactobacillus casei* (Kiss et al., 2007).

In our study, we used biochemical methods to identify the vaginal lactobacilli and found that the rate of vaginal *Lactobacillus acidophilus* (76.9%) was significantly higher than that of *L. delbrueckii ssp delbrueckii* (15.4%) or *L. crispatu* (7.7%). Thus, in our study, the dominant vaginal Lactobacillus strain was *L. acidophilus*, suggesting that *L. acidophilus* may play an important role in maintaining the vaginal micro-ecological balance in pregnant women. As early as the 1950s, studies on the antibacterial activity of hydrogen peroxide were performed. Hydrogen peroxide alone or in combination with peroxidase and halides had a toxic effect on vaginal pathogenic microorganisms, and this toxicity could be inhibited by catalase (Klebanoff et al., 1991). Hydrogen peroxide-producing *Lactobacillus* (LB+) was cytotoxic on microbes that did not produce or produce low levels of catalase, and proliferation of catalase-negative microbes such as vaginal *Gardnerella vaginalis* (an obligate anaerobe) might occur in the absence of vaginal LB+. Vaginal *Lactobacillus* can also reduce the vaginal pH, and an acidic vaginal environment can facilitate the production of hydrogen peroxide by *Lactobacillus*. Hydrogen peroxide is relatively stable in an acidic environment in that the activity of hydrogen peroxide will decrease when the concentration of hydrogen ions decreases. LB+ is the dominant vaginal bacterium in healthy pregnant women of childbearing age, and appears to play a key role in maintaining the self-cleaning effect of the vagina and preventing vaginal infections. Different strains of *Lactobacillus* have different capacities for hydrogen peroxide production. It was reported that *L. crispatu* (95%) and *L. jensenii* (94%) showed the greatest capacities to produce hydrogen peroxide (Antonio et al., 1999). In our study, the rate of vaginal LB+ gradually increased with ongoing pregnancy, which might be related to the concurrent decrease in vaginal pH. The dominant vaginal LB+ in our pregnant women was *L. acidophilus,* which was found in 80% of samples. All *L. crispatu* isolates (100%), most *L. acidophilus* isolates (80%) and half of the *L. delbrueckii ssp delbrueckii* isolates (50%) produced hydrogen peroxide.

These results indicate that *L. acidophilus* is the dominant vaginal LB+ strain in pregnant women. Notably, LB+ strains appear better suited to form colonies in the vagina of pregnant women than LB– strains, suggesting that *L. acidophilus* may play a key role in maintaining the vaginal micro-ecological balance and preventing vaginal infections for pregnant women. Although the relatively small number samples may affect the statistical comparisons of the different stages of pregnancy, we found that, with ongoing pregnancy, there was a gradual decline in vaginal pH. Similarly, irrespective of the different stages of pregnancy, the most commonly isolated strain of *Lactobacillus* was *L. acidophilus*, which was significantly more frequently found than *L. delbrueckii ssp delbrueckii* or *L. crispatu*. In terms of the capacity of *Lactobacilli* strains in producing hydrogen peroxide, of the three kinds of vaginal *Lactobacilli* isolates, all *L. crispatu* isolates and 80% of the *L. acidophilus* isolates produced hydrogen peroxide. This indicates that the dominant vaginal bacteria strain is *L. acidophilus*, which seems to be the main bacterial strain involved in maintaining vaginal micro-ecological balance and protecting against vaginal infections for pregnant women.

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