Effects of the sampling time on the vaginal microbiota in healthy pregnant women: a prospective observational study

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BACKGROUND: Studies using 16S rRNA gene sequencing have extensively examined the vaginal microbiota changes of pregnant women. However, no study has examined these changes considering the time of day at which vaginal fluid samples were collected from near-term pregnant women.
OBJECTIVE: To describe the vaginal microbiota of Japanese near-term pregnant women with normal pregnancy outcomes and potential vaginal microbiota changes from wake-up to bedtime.
STUDY DESIGN: In this prospective observational study, vaginal swab specimens were obtained from healthy near-term pregnant women twice on the same day, after waking up and before bedtime. All specimens were examined for total bacterial cell count per gram of vaginal fluid, Nugent score, pH, and vaginal microbiota analysis using 16S rRNA gene sequencing. Initially, the wake-up and bedtime samples of all participants were analyzed at the genus level using next-generation sequencing. Subsequently, all samples were analyzed at the genus and species levels using Sanger sequencing.
RESULTS: Sixteen pregnant women were enrolled in this study. The median age of the participants was 32.5 years, and the median gestational age was 38 weeks. Median bacterial counts in vaginal fluids at wake-up and bedtime were 3.9×10^9/g and 3.6×10^9/g, respectively, with no significant difference. Vaginal microbiota analyses based on 16S rRNA genes showed that the vaginal microbiota in pregnant women with no abnormalities during pregnancy was limited to a single flora dominated by *Lactobacillus* spp. and included pregnant women with highly diverse vaginal microbiota. Genus-level analysis using next-generation sequencing showed that the vaginal microbiota differed between wake-up and bedtime in more diverse samples but not in less diverse samples. However, these differences were small compared to individual differences. The dominant genera in each sample had similar relative abundances in both wake-up and bedtime samples. However, the non-dominant genus *Streptococcus* spp. was significantly more frequently detected in bedtime samples. In species-level analyses, the proportions of dominant and non-dominant species showed little change between wake-up and bedtime.
CONCLUSIONS: The vaginal microbiota of pregnant women with normal pregnancy outcomes was not necessarily dominated by *Lactobacillus* spp. Further studies are required to define the vaginal microbiota in healthy pregnant women. When vaginal fluid samples were collected from the same pregnant women at wake-up and bedtime under the same conditions, the differences between wake-up and bedtime samples were greater for women with high diversity of the vaginal microbiota than for those with low diversity. However, these differences were not sufficiently large to exceed individual differences, and almost no change in the abundances of the dominant genera was observed. Since the relative abundance of *Streptococcus* spp., a non-dominant species of the vaginal microbiota tends to change between wake-up and bedtime, it might be necessary to collect samples before bedtime to detect group B *Streptococci*.

Key words: circadian rhythm, cluster analysis, *Lactobacillus*, microbiota, phylogeny, pregnancy, *Streptococcus*

Introduction

Many studies examined the vaginal microbiota (VM) in women with sexually transmitted diseases,[1,2] gynecological cancer,[3,4] among others, and analyzed the relationships between VM and pregnancy-associated complications like preterm deliveries,[5–8] gestational diabetes mellitus,[9,10] and chorioamnionitis.[11,12] In most studies, *Lactobacillus* spp. dominance in the VM is associated with positive pregnancy outcomes.[1–12] Factors other than diseases that affect the VM in pregnant women include pregnancy history, diet, sexual activity, race and ethnicity, and gene polymorphisms.[13] Furthermore, many studies assessed

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Tweetable statement: The diurnal changes in the vaginal microbiota have not been described yet. We collected two samples on the same day after waking up and bedtime. The dominant genera did not differ between the two, but *Streptococcus* spp. was more abundant at bedtime.

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Why was this study conducted?

Recently reports described, diurnal variations in the gut, oral, and skin microbiota of humans, and in the gut microbiota of animals.

However, the extent of VM fluctuations in pregnant women from wake-up to bedtime remains unknown, and no study has considered the time of collection of vaginal fluid samples.

Here, we focused on the time of sample collection and compared vaginal fluids from the same participants twice on the same day at wake-up and bedtime.

What are the key findings?

Vaginal microbiota analyses based on 16S rRNA genes showed that the vaginal microbiota in pregnant women with no abnormalities during pregnancy was not limited to a single flora dominated by *Lactobacillus* spp. and included pregnant women with highly diverse vaginal microbiota.

Genus-level analysis using next-generation sequencing showed that the vaginal microbiota differed between wake-up and bedtime in more diverse samples but not in less diverse samples. However, these differences were small compared to individual differences. The dominant genera in each sample had similar relative abundances in both wake-up and bedtime samples. However, the non-dominant genus *Streptococcus* spp. was significantly more frequently detected in bedtime samples.

Species-level analyses using Sanger sequencing, the proportions of dominant and non-dominant species showed little change between wake-up and bedtime.

What does this study add to what is already known?

Many studies assessed VM changes during pregnancy, and there is general agreement that the VM partially changes or does not change during the three trimesters of pregnancy. This study showed that VM partially changes within a day in pregnant women of high-diversity samples and is stable in women of low-diversity samples.

VM changes during pregnancy, and there is general agreement that the VM partially changes during the three trimesters of pregnancy.[14–16] However, few studies have investigated the VM exclusively in healthy Japanese women with term pregnancies who gave birth to healthy newborns.

Recently reports described, diurnal variations in the gut,[17] oral,[18] and skin[19] microbiota of humans, and in the gut microbiota of animals.[17,20,21] Previous studies on gut microbiota have associated various factors, including light-dark cycles and food intake timing, with diurnal microbiota variations.[22] Labor is a physiological event in pregnant women and is believed to have a circadian rhythm.[23] Perinatal care workers generally agree that labor, childbirth, and membrane rupture are more likely to occur at specific periods of the day. Natural births are more frequent at night and less frequent at dawn because of various factors, including light-dark rhythms and endocrinological factors.[24] However, the extent of VM fluctuations in pregnant women from wake-up to bedtime remains unknown, and no study has considered the time of collection of vaginal fluid samples.

Here, we focused on the time of sample collection, which may explain differences in human VM studies, and compared vaginal fluids from the same participants twice on the same day at wake-up and bedtime. Diurnal VM changes were assessed using sequence-based analysis and conventional methods for diagnosing bacterial vaginosis (BV) including Nugent scores, Amsel criteria, and bacterial cell counts. In clinical practice, BV is diagnosed when anaerobic bacteria other than *Lactobacilli* are dominant using the Nugent score or Amsel criteria. As next-generation sequencing (NGS) on the Illumina platform provides credible genus-level but poor species-level identification, we used NGS to perform genus-level analyses.[25,26] The clone library method, i.e., Sanger sequencing (SS), was used to analyze the VM at the species level by sequencing longer reads with higher accuracy than that obtained from short sequencing reads using NGS.

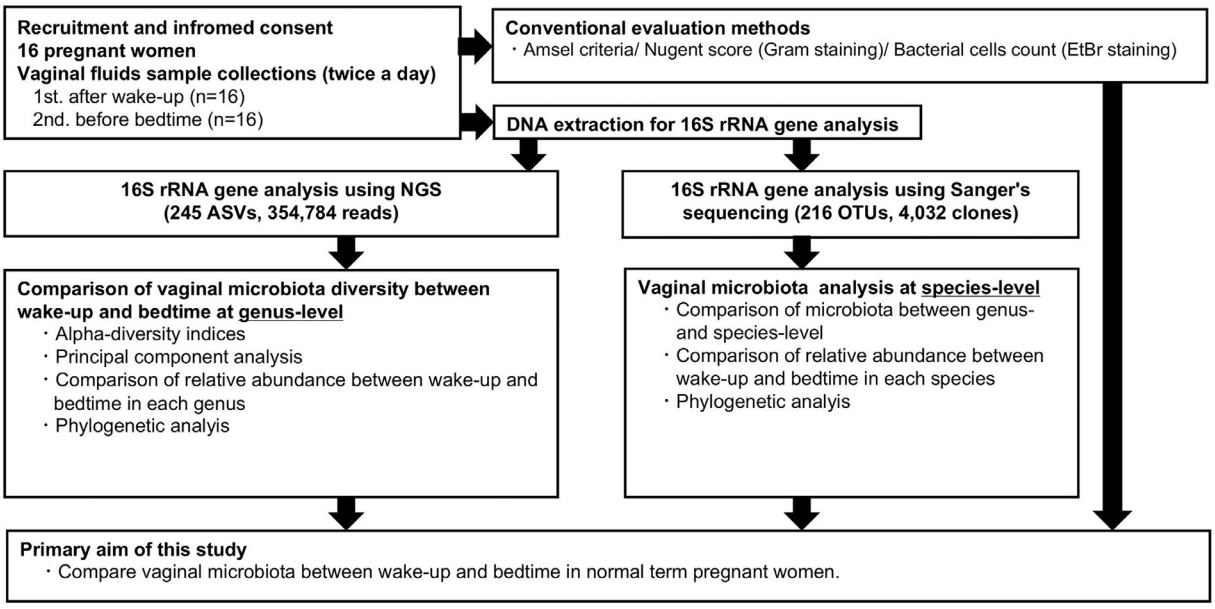
This study aimed to describe the VM in Japanese near-term pregnant women with normal pregnancy outcomes and to determine potential VM changes from wake-up to bedtime.

Materials and methods

Study design and participants

We conducted a prospective observational study of healthy pregnant women who attended the University of Occupational and Environmental Health, Japan Hospital between May and September 2022. Verbal and written explanations of the study were provided to all participants, and written informed consent was obtained from all participants. This study was approved by the Ethics Committee of the University of Occupational and Environmental Health, Japan (approval number: UOEHCRB21-087, approval date: 17 August 2021), according to the Japanese ethical guidelines of the Ministry of Health, Labor and Welfare. All procedures were conducted according to the relevant guidelines and regulations. We recruited 20 to 40-year-old healthy pregnant women over 37 weeks of gestation with normal pregnancy course and fetal growth who met the eligibility criteria described in the Supplemental Materials. We collected vaginal samples from these participants twice on the same day. The characteristics and background data of the participants at the time of sample collection and delivery were obtained from medical records. Figure 1 shows the study flowchart. Details on materials, methods, inclusion criteria, and sample collection are described in the Supplemental Materials.

FIGURE 1
Flowchart of the overall study design.



ASV, amplicon sequence variant; EtBr, ethidium bromide; *NGS*, next-generation sequencing; *OTU*, operational taxonomic unit.

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Conventional VM evaluation methods

We evaluated all samples using conventional methods of vaginal fluid collection, including Nugent scores, Amsel criteria, and bacterial cell counts. Details are described in the Supplemental Materials.

Microbiota analysis using bacterial 16S rRNA gene

After DNA extraction, all samples were analyzed at the genus level using two different sequencing methods, NGS and SS. Next, SS was used to analyze all samples at the species level. A phylogenetic tree analysis was performed for each representative sequence obtained using NGS and SS. The details of DNA extraction and 16S rRNA gene sequencing are described in the Supplemental Materials.

Statistical analyses

Bacterial cell counts, relative abundances of genus or species, and alpha diversity were compared between wake-up and bedtime using Mann-Whitney-Wilcoxon tests, Bray–Curtis dissimilarity indices between samples with low and high alpha diversity (a Shannon index of ≥0.6 and a Simpson index of ≥0.3 indicated high alpha diversity) were also evaluated using the Mann-Whitney-Wilcoxon test. Statistical significance was set at $P<.05$. All statistical analyses were performed using the Bell Curve for Excel software v.4.06 (Social Survey Research Information Co, Ltd Tokyo, Japan).

Results

Participants and backgrounds

Eighteen healthy Japanese near-term pregnant women were enrolled in this study. Two participants (No. 1 and 4) were excluded because samples were not collected on time, and data from the remaining 16 women were analyzed. Table 1 shows their characteristics and birth-related information.

Comparison of the VM in wake-up and bedtime samples using conventional BV evaluation methods

Table 2 shows the results of Nugent scoring (score and grade), Amsel criteria (the number of positive parameters out of four parameters), and the pH value of the vaginal fluid from the 16 participants (32 samples). In all women, both vaginal fluid pH and Nugent grade did not differ between wake-up and bedtime. In two participants (No. 8 and 10) with intermediate Nugent grades, the wake-up and bedtime results of the Nugent score and Amsel criteria differed.

Comparison of bacterial cell counts between wake-up and bedtime

Supplemental Figure 1 shows in a logarithmic graph the changes in bacterial cell counts per gram of vaginal fluid in wake-up and bedtime samples. Bacterial cell counts ranged between 10^8 and 10^{10} per gram of vaginal fluid. The bacterial cell counts in wake-up and bedtime samples did not significantly differ ($P=.68$). The number of bacterial cells increased from wake-up to bedtime in eight women but decreased in the remaining eight women. When analyzed separately by Nugent grade (normal and intermediate groups), bacterial cell counts did not significantly differ between wake-up and bedtime samples (Supplemental Table 1).

NGS analysis of the VM in wake-up and bedtime samples at the genus level

We used NGS for comprehensive genus-level analyses. Using NGS,

TABLE 1
Characteristics of participants

	n=16 median (range), number (%)
race, ethnicity	Asian, Japanese (100%)
age, years	32.5 (26–40)
gravida	3 (1–5)
para	1 (0–4)
primipara	3 (19%)
BMI, kg/m^2	28 (17.9–34.6)
weight gain, kg	9.25 (2–18.9)
gestational age at sample collection, weeks	38 (37–38)
delivery, weeks	38 (37–39)
baby weight, g	3183 (2481–3404)
apgar score 1 min	8 (8-8)
apgar score 5 min	9 (9-9)
smoking	2 (13%)
alcohol use	0
pre-pregnancy smoking	5 (31%)
pre-pregnancy alcohol	3 (19%)
average sleeping time, hours	7 (5–8)
defecation	
everyday	4 (25%)
every 2 days	7 (44%)
every 2–3 days	4 (25%)
every 4–5 days	1 (6.2%)
once a week	0

BMI, body mass index.

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765,016 reads were obtained from 32 samples and clustered into 254 amplicon sequence variants (ASVs). After their rarefication with vegan software, we obtained 354,784 reads and 245 ASVs (Supplemental Figure 2A). The rarefaction curves of all samples were nearly saturated, suggesting that our results provided coverage of the VM (Supplemental Figure 3A).

Figure 2A shows the relative abundances of ASVs across all samples. Genus-level analyses (Figure 2B) identified four dominant genera: *Lactobacillus* spp. (64.2%), *Atopobium* spp. (13.2%), *Gardnerella* spp. (12.9%), and *Bifidobacterium* spp. (4.7%) accounting for 94.9% of total reads. The remaining 16 genera had relative abundances of <1% each. In all samples, except from those of participant No. 5, the relative abundances of the genera *Lactobacillus* spp. and *Atopobium* spp. were lower at bedtime than at wake-up, whereas the relative abundances of the genera *Prevotella* spp., *Sneathia* spp., *Metamycoplasma* spp. (previously *Mycoplasma* spp.), and *Parvimonas* spp. increased. The dominant genera did not significantly differ in relative abundances between wake-up and bedtime, whereas *Streptococcus* spp., a non-dominant genus, showed significant changes (Table 3). Alpha diversity was not significantly different between wake-up and bedtime for Shannon or Simpson indices (Figure 2C).

Beta diversity, based on the ASVs of all samples was evaluated using principal component analysis (PCA). In the PCA results (Figure 2D), spots with intermediate Nugent scores and high vaginal pH were distributed in the positive direction of the first principal component (PC1), whereas spots with normal Nugent scores and low vaginal pH samples were scattered in the negative direction of PC1. The representative eigenvectors that significantly influenced the negative direction of PC1 were ASV0 and ASV1 (Figure 2E). The genera of each ASV are listed in Supplemental Table 2. Moreover, the wake-up and bedtime spots of samples in the positive direction of PC1 were far apart, whereas those of the samples in the negative direction overlapped. To further understand how beta diversity differed between samples with high and low alpha diversity, we assessed Bray–Curtis dissimilarity (Figure 2F). We observed a significant increase in dissimilarity among samples with high alpha diversity (P=.0104). *Lactobacillus*-dominant VMs were stable, whereas the VMs in which *Lactobacillus* spp. were not dominant differed between wake-up and bedtime. However, these differences were not beyond individual differences.

SS analysis of the VM in wake-up and bedtime samples at species and genus levels

SS was used to analyze the VM at the species level. After rarefying operational taxonomic units (OUTs) using vegan software, we obtained 4032 reads and 216 OTUs (Supplemental Figure 2B). The relative genus-level abundances were similar in SS and NGS analyses. The rarefaction curves of almost all samples showed that 126 reads per sample were near saturation, and some samples were insufficient to cover all highly diverse specimens (Supplemental Figure 3B). Therefore, alpha and beta diversity analyses using SS were not subsequently performed. SS detected fewer genera than NGS (Figures 2B and 3A). Figures 3A and 3B show the relative abundances at genus and species levels using SS, demonstrating the presence of multiple species in the same genus. Figures 3C

TABLE 2
Conventional evaluation of all vaginal microbiota of 16 pregnant women

Case no	2		3		5		6	
	wake-up	bedtime	wake-up	bedtime	wake-up	bedtime	wake-up	bedtime
Nugent score	5	5	1	1	4	4	0	0
Nugent grade	Inter-mediate	Inter-mediate	normal	normal	Inter-mediate	Inter-mediate	normal	normal
LAC grade	IIb	IIb	I	I	IIb	IIb	I	I
Amsel criteria	2	2	0	0	1	1	0	0
vaginal pH	4.4	4.4	4.1	4.1	4.4	4.4	3.6	3.6

Case no.	7		8		9		10	
	wake-up	bedtime	wake-up	bedtime	wake-up	bedtime	wake-up	bedtime
Nugent score	0	0	6	5	1	1	5	4
Nugent grade	normal	normal	Inter-mediate	Inter-mediate	normal	normal	Inter-mediate	Inter-mediate
LAC grade	I	I	IIb	IIa	I	I	IIa	IIb
Amsel criteria	0	0	2	1	0	0	2	2
vaginal pH	3.6	3.6	4.4	4.4	4.1	4.1	4.1	4.1

Case no.	11		12		13		14	
	wake-up	bedtime	wake-up	bedtime	wake-up	bedtime	wake-up	bedtime
Nugent score	6	6	0	0	0	0	0	0
Nugent grade	Inter-mediate	Inter-mediate	normal	normal	normal	normal	normal	normal
LAC grade	III	III	I	I	I	I	I	I
Amsel criteria	4	4	0	0	0	0	0	0
vaginal pH	4.7	4.7	4.1	4.1	3.6	3.6	4.1	4.1

Case no.	15		16		17		18	
	wake-up	bedtime	wake-up	bedtime	wake-up	bedtime	wake-up	bedtime
Nugent score	1	1	1	1	4	4	1	1
Nugent grade	normal	normal	normal	normal	Inter-mediate	Inter-mediate	normal	normal
LAC grade	I	I	I	I	IIa	IIa	I	I
Amsel criteria	0	0	0	0	2	2	0	0
vaginal pH	3.6	3.6	4.1	4.1	4.1	4.1	4.1	4.1

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and 3D show that some *Lactobacillus* spp. and *Prevotella* spp. samples contained more than one species in the same sample. The relative species-level abundances did not significantly differ between wake-up and bedtime (Supplemental Table 3).

Phylogenetic tree analysis using NGS and SS

A phylogenetic tree analysis was performed for each genus using both SS and NGS. In this analysis, SS had a higher resolution than NGS for species-level identification of *Lactobacillus* spp. and *Gardnerella* spp. (Figures 4 and 5). However, for many other genera, the high-accuracy NGS used in this study had the same resolution as SS (Supplemental Figure 5−10).

Comments

Principal findings

This study mainly aimed at contributing to our understanding of the importance of VM changes within a day. In this study, VM samples with relatively high alpha diversity showed changes between wake-up and bedtime of the same day, but these diurnal differences did not exceed individual differences. By contrast, VM with low alpha diversity showed little change within a day. If the genera with relative abundances exceeding 1% of the total read counts, i.e., *Lactobacillus* spp., *Atopobium* spp., *Gardnerella* spp., and *Bifidobacterium* spp. were dominant, the relative abundances of these genera varied slightly in

FIGURE 2
Genus-level comparison of the vaginal microbiota in wake-up and bedtime samples.

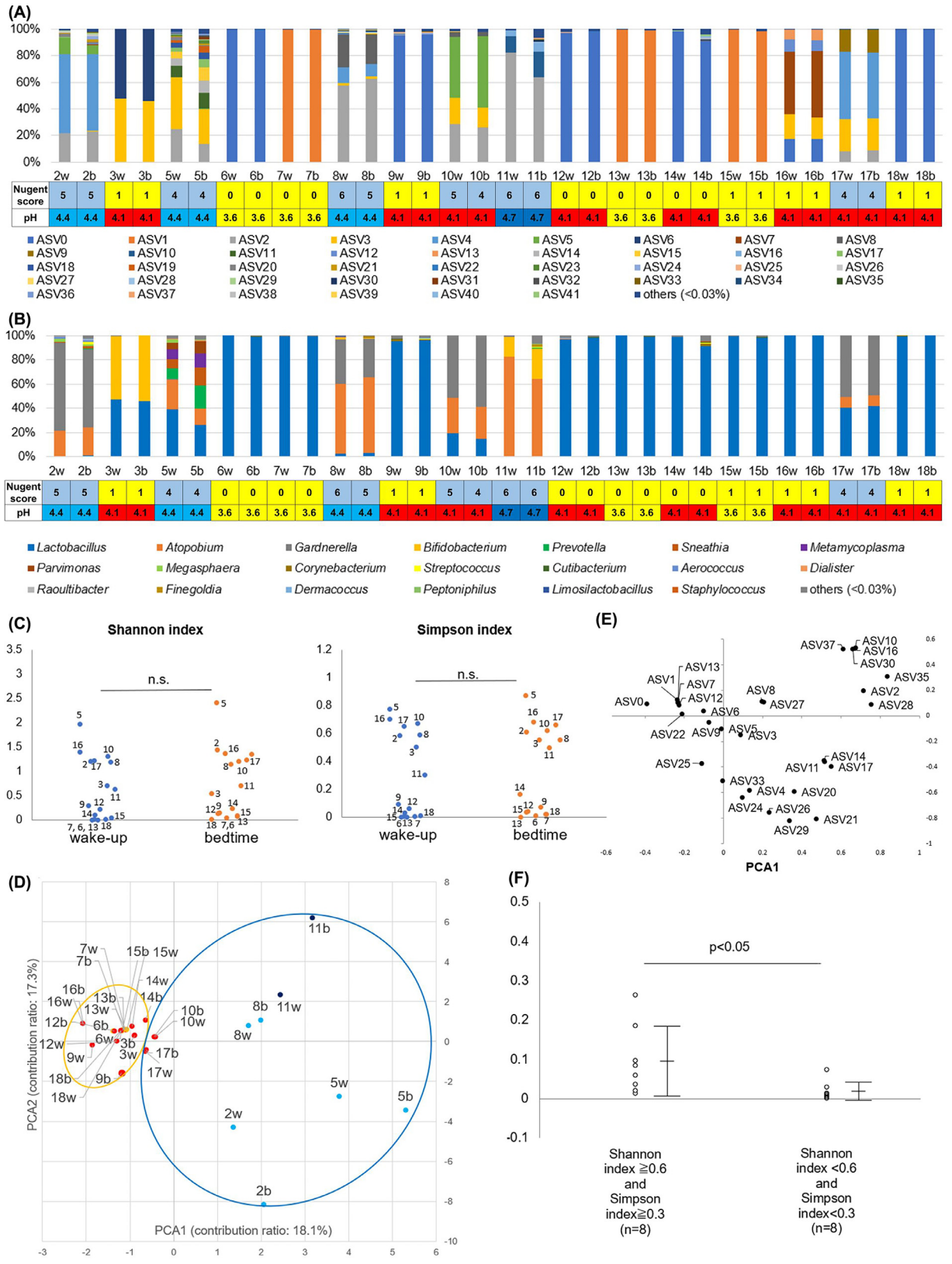

TABLE 3
Comparison of relative abundance between wake-up and bedtime at the genus level

genus	wake-up	bedtime	*P*-value
Lactobacillus	96.1	93.7	.64
Atopobium	8.4	8.6	.77
Gardnerella	0.41	0.23	.59
Bifidobacterium	1.04	0.97	1
Prevotella	0.099	0.18	.89
Sneathia	0.5	2.01	.19
Corynebacterium	0.05	0.1	.32
Streptococcus	0.02	0.97	.029[a]
Cutibacterium	0.03	0.14	.3
Dialister	0.07	0.29	.79
Finegoldia	0	0.12	.095
Dermacoccus	0	0.05	.52

[a] <0.05.

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all VM samples with high alpha diversity, but the order of relative abundance did not change between wake-up and bedtime. In VM samples with low alpha diversity, *Lactobacillus* spp. was the dominant genus. This suggests that it may be less necessary to consider the timing of specimen sampling during the day in studies that primarily examine dominant genera. However, 11 non-dominant genera detected by NGS, which accounted for 0.8% of all readings, were detected only during wake-up or bedtime. Among the non-dominant genera, *Streptococcus* spp. was significantly more likely to be detected only at bedtime. This suggests that, although this was a small study with very few participants, clinical studies examining non-dominant VM genera may better collect multiple specimens over a day, rather than just one specimen. Although the sequences of *Streptococcus* spp. detected in this study using NGS were inferred from phylogenetic analysis and were not strains of *Streptococcus agalactiae* (group B *Streptococcus*: GBS), a pathogenic bacterium that is closely associated with neonatal health during the perinatal period,[27] it is noteworthy that the relative abundances of *Streptococcus* spp. varied significantly between wake-up and bedtime. GBS is known to be colonized not only in the vagina but also in the gut,[27] and the diurnal changes of this VM may be influenced by the gut-derived microbiota. However, since the dominant genera of the gut microbiota such as *Bacteroides* spp. and *Ruminococcus* spp., were rarely detected in this study, the changes in the relative abundance of *Streptococcus* spp. may be related to the specific properties of *Streptococcus* spp. in the vagina rather than to the effect of contamination by intestinal contents.

The analysis of beta diversity showed that compared to VM samples in which *Lactobacillus* spp. were dominant, VM samples in which genera other than *Lactobacillus* spp. were dominant showed greater VM changes between wake-up and bedtime samples, but these changes did not exceed individual differences. Nugent score and vaginal pH, which are conventionally used in clinical practice for the assessment of VM, were also associated with diversity in 16S rRNA gene-based VM analyses. Although this has been reported by several studies,[28–30] the present study further showed that the aforementioned conventional assessment methods may be an indicator of VM, which is prone to changes between wake-up and bedtime.

Results in the context of what is known

It is known that the VM, mainly dominated by *Lactobacillus* spp., is stable throughout the gestation period.[14,16] The present study further shows that the VM is also stable within one day. Conversely, the present study found that a more diverse VM was more likely to fluctuate during a day than a *Lactobacillus*-dominant VM. This result is similar to previous findings showing that women with more diverse flora are more likely to show VM fluctuations during pregnancy.[14,16] The VM of healthy Japanese pregnant women who gave birth at term has not been previously analyzed using 16S rRNA genes. Several studies examined the VM of pregnant Japanese women, but the gestational weeks at the time of sampling

(A) Relative abundances of ASVs across all samples by NGS. (B) Relative abundances of the genera across all samples by NGS. (C) Comparison of alpha-diversity indices between wake-up and bedtime using NGS. The number next to each dot indicates the participant number. (D) PCA based on ASVs. The blue circle indicates bound samples with an intermediate Nugent grade, and the yellow circle indicates bound samples with a normal Nugent grade. The color of each dot indicates the pH value of the sample: yellow for 3.6, red for 4.1, light blue for 4.4, and dark blue for 4.7. (E) The eigenvector of PCA based on ASV. (F) Comparison of Bray—Curtis dissimilarity indexes between samples with low and high alpha diversity. The scatter plot also includes mean values and SD error bars. *ASV,* amplicon sequence variant*; NGS,* next-generation sequencing; *n.s.*, not significant; *PCA*, principal component analysis.

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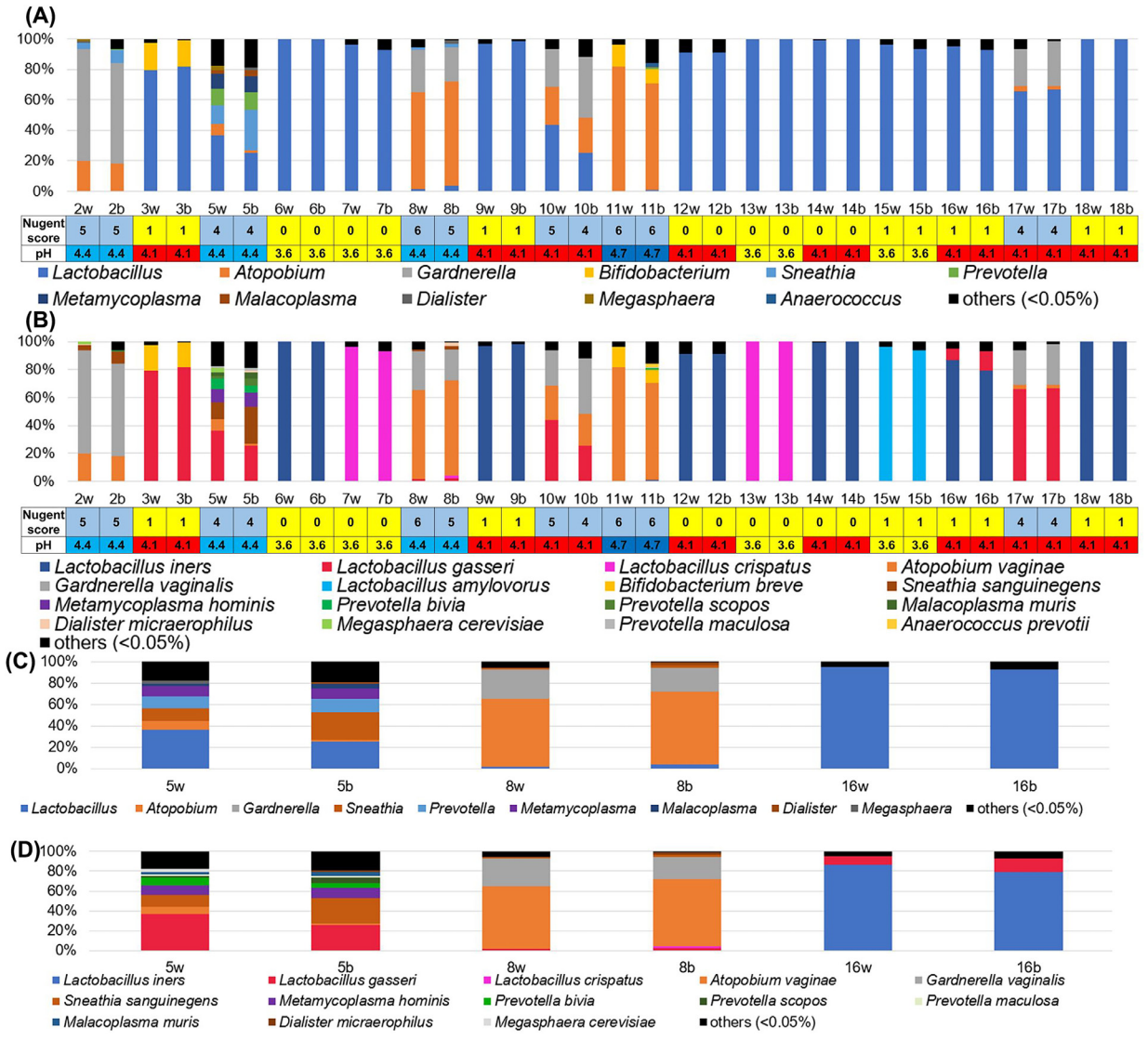
FIGURE 3
Species-level comparison of the vaginal microbiota in wake-up and bedtime samples



(A) Relative abundances of the genera across all samples by Sanger sequencing. (B) Relative abundances of the species across all samples by Sanger sequencing. (C) Relative abundances of the genera in samples with multiple species of the same genus. (D) Relative abundances of the species in samples with multiple species of the same genus.

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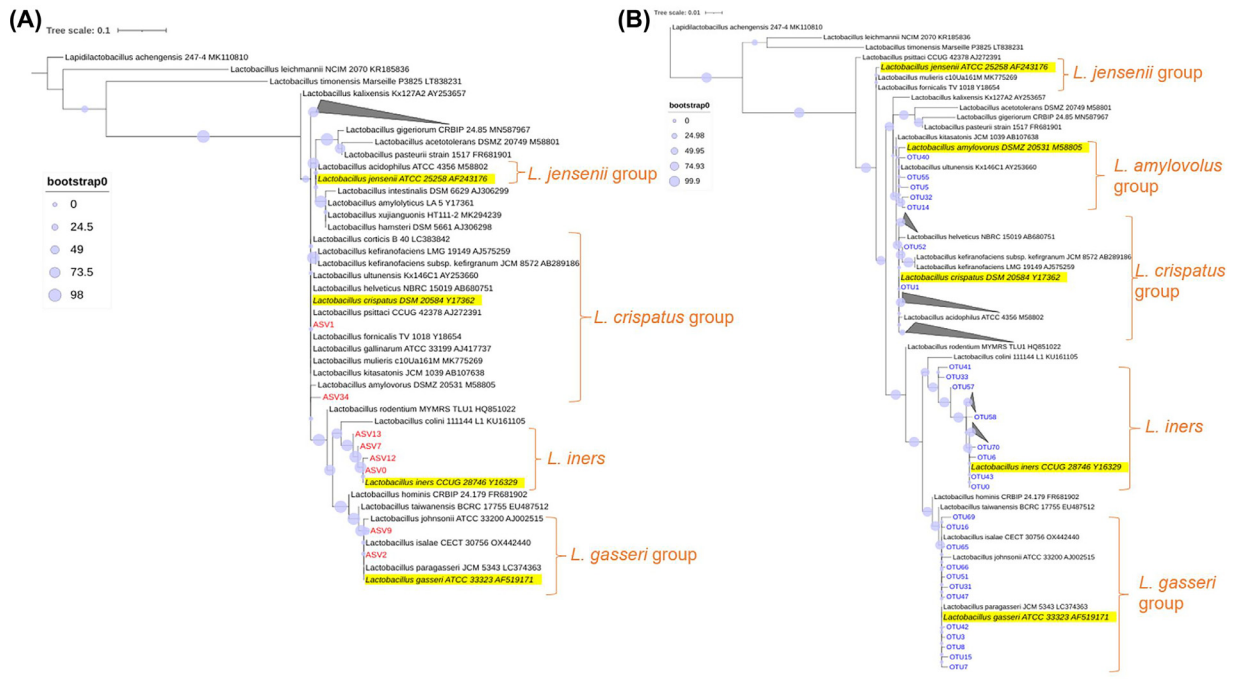
were either not clearly described or within 37 weeks of gestation.[31,32] In these studies, the proportion of women with non-dominant *Lactobacillus* flora was approximately 25%, whereas in the present study, which included only healthy-pregnant women, this proportion was as high as 50%. None of our participants were diagnosed with BV. These results contradict those of previous studies showing that *Lactobacillus*-dominant VM is associated with positive perinatal outcomes. We have no definitive information on the reason for this discrepancy, although it may be specific to pregnant Japanese women or be caused by the small sample size.

Clinical implications

Currently, in most countries, including Japan, universal screening for GBS is recommended once at approximately 35 weeks of gestation by collecting and culturing a specimen from the rectovaginal area of pregnant women.[27,33] A problem with this screening approach is that many infants with early-onset GBS diseases are born to mothers with negative results in this antenatal GBS screening.[34] Many guidelines recommend using a selective culture medium for GBS because of the increased number of false negatives in conventional presumptive tests using a non-selective culture medium.[33,35] The present findings suggest that taking

FIGURE 4
Phylogenetic tree analysis of *Lactobacillus* spp



(A) Phylogenetic tree of ASVs assigned to *Lactobacillus* spp. (NGS) (B) Phylogenetic tree of OTUs assigned to *Lactobacillus* spp. (Sanger sequencing)
ASV, amplicon sequence variant; *NGS*, next-generation sequencing; *OTU*, operational taxonomic unit.

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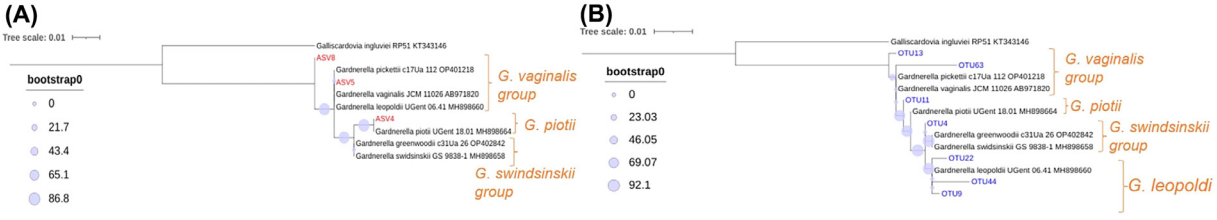
FIGURE 5
Phylogenetic tree analysis of *Gardnerella* spp



(A) Phylogenetic tree of ASVs assigned to *Gardnerella* spp. (NGS); (B) Phylogenetic tree of OTU assigned to *Gardnerella* spp. (Sanger sequencing)
ASV, amplicon sequence variant; *NGS*, next-generation sequencing; *OTU*, operational taxonomic unit.

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vaginal samples at night may also effectively reduce false negative results. Although issues such as the cost of testing and the physical burden on patients remain, this is considered a future research topic worth pursuing.

Research implications

First, in studies of the VM in pregnant and non-pregnant women, the vector of the relative abundance of bacterial phylotypes in a sample is called community state type (CST), and clustering of CSTs into groups with similar bacterial composition and abundance is often used.[29,36,37] The cluster status is usually classified into the five CSTs proposed by Ravel et al[29] Four of the five CSTs are dominated by *Lactobacillus* spp. and are classified at the species level as CST Ⅰ (*L. crispatus*), CST Ⅱ (*L. gasseri*), CST Ⅲ (*L.iners*), and CST Ⅴ (*L. jensenii*). The community of the VM, which is composed of anaerobes other than *Lactobacillus* spp., e.g., *Atopobium* spp., *Gardnerella* spp., and *Prevotella* spp., *is* CST Ⅳ. The dominant genus in the study population is also an important indicator when conducting studies based on such community state classifications, and there may be no need to consider the sampling time, regardless of its alpha diversity. However, studies that primarily investigate non-dominant genera, such as GBS, may need to consider study designs with multiple

samplings over a day, because these genera may not be detected depending on the sampling timing within a day.

Second, microbiota analyses using NGS cannot be classified at the species level, as pointed out in previous studies using non-vaginal samples.[25,26] It can be argued that studies using only NGS to sequence vaginal samples should be limited to a discussion at the genus level and that analysis at the species level should be carried out using methods capable of obtaining longer read lengths, particularly for *Gardnerella* spp. and *Lactobacillus* spp.

Strengths and limitations

The strength and novelty of this study are that vaginal specimens from pregnant women were collected twice on the same day, under the same conditions, by the same healthcare provider. No clinical trials have been conducted using such a design. Another strength is that we sequenced the same samples using two sequencing methods, NGS and SS, to analyze the genus and species levels. No previous study has used two different high-precision 180 bp and 550 bp sequence lengths for vaginal fluid samples.

This study has some limitations. First, because vaginal samples were not collected on multiple days, it is unknown whether the changes in wake-up and bedtime VM observed in this study were cyclical. Second, the sampling frequency was low with only two samples on the same day. We took vaginal samples only twice because frequent vaginal sampling can be physically and mentally demanding for participants, and we were interested in VM changes within the same day. Third, only a small number of participants were enrolled because they were recruited prospectively, targeting only healthy pregnant women during the study period. This study revealed that some VM are more prone to change than others, but the mechanisms behind these changes remain unclear. It would be valuable in the future to see how the results extend to sampling over various days, other populations, and given demographic and lifestyle characteristics.

Conclusions

Approximately half of the healthy Japanese near-term pregnant women had *Lactobacillus* spp. non-dominant VM. When vaginal fluid samples were collected from the same pregnant women at wake-up and bedtime under the same conditions, the diurnal differences were greater for pregnant women with high-diversity VM of high diversity than for those with low-diversity VM. However, these differences were not significantly large to exceed individual differences, almost no change in the abundance of the dominant genera was observed. *Streptococcus* spp., a non-dominant genus, was substantially more likely to be detected in bedtime samples. Studies investigating pathogenic bacteria, especially of the *Streptococcus* genus, might better collect samples at night than in the morning. Future studies should enroll large-scale multi-center cohorts to validate the findings of this study in more diverse populations. ■

Conflicts of Interest

The authors report no conflict of interest.

CRediT authorship contribution statement

Hiroshi Mori: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Eiji Shibata:** Supervision, Conceptualization. **Emi Kondo:** Supervision, Data curation. **Mitsumasa Saito:** Validation, Supervision. **Kiyoshi Yoshino:** Validation, Supervision, Conceptualization. **Kazumasa Fukuda:** Supervision, Formal analysis, Data curation, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xagr.2025.100460.

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