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Vaginal microbiota: age dynamic and ethnic particularities of Algerian women

Ouarabi L. et al.

## Human Microbiome

# Vaginal Microbiota: Age Dynamic and Ethnic Particularities of Algerian Women

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## Abstract

The composition of the vaginal microbiota is a key element for maintaining gynecological and reproductive health. With the aim of obtaining an accurate overview of the vaginal microbiota of Algerian women, in terms of their age and ethnic group, we conducted a 16S rRNA gene targeted metagenomic analysis of 100 vaginal samples taken from healthy childbearing and menopausal women. These data were used to establish the pattern of the vaginal microbiota during reproductive and postreproductive phases. Hormone levels were correlated to changes in microbial composition for menopausal women. The ethnic comparison revealed a particular microbiota profile for Algerian women, with a dominance of CST III and CST I. A rapid qPCR method developed by the authors was successfully used to identify the vaginal bacterial pattern for a customized gynecological management.

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## Keywords

Vaginal microbiota

Metagenetics

Menopause

Probiotics

Ethnicity

## Electronic supplementary material

The online version of this article (<https://doi.org/10.1007/s00248-020-01606-6>) contains supplementary material, which is available to authorized users.

## Introduction

Analyses of high-throughput sequencing have enabled researchers to characterize the composition and variation of species across different microbiota samples and explain the correlations between diseases and microbes and how these microbial species can interact with their host. Because of the crucial role of its different microbiota, human beings have been defined as multi-species individuals [1], in which the sum of the host genome and traits of the microbiome constitute together an evolutionary individual or holobiont [2]. From the different human microbial ecosystems, the vaginal microbiota accounts for 9% of the total human microbiota [3] and remains under-studied despite its importance for women's health and future generations. The health-promoting effects of the vaginal microbiota are reflected in reproductive fitness of the host through direct and indirect antipathogenic mechanisms. Moreover, the vaginal microbiota exerts a beneficial influence on fertility, conception, and healthy pregnancy and also on infant seeding and prevention of premature birth [4].

Metagenomic analyses revealed the presence of a high proportion of *Firmicutes* and a low percentage of *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Actinobacteria* in the vaginal microbiota [3], which also contain fungi (*Candida* sp., *Pichia kudriavzevii*) and viruses belonging to the *Papillomaviridae*, *Herpesviridae*, *Anelloviridae*, and *Polyomaviridae* families [5, 6, 7]. Of note, the fungi were identified in the absence of any pathology [8]. Regarding the bacterial composition, a large body of reports has confirmed the dominance of *Lactobacillus* species in the vaginal microbiota of healthy women [9]. Notably, five community state types (CSTs) were described throughout the world (I-V), the majority of which (types I, II, III, and V) were dominated by one or more species of *Lactobacillus* [10]. The dominance of lactobacilli in the vaginal microbiota has been linked to the production of estrogen and the accumulation of glycogen in the upper layers of the stratified vaginal epithelium [11]. Besides production of lactic acid, the beneficial properties of lactobacilli are

associated with (i) production of bacteriocins and hydrogen peroxide as antimicrobial mechanisms, (ii) adherence to vaginal epithelia, and (iii) ability to compete for available nutrients [12].

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The vaginal microbial community is in a dynamic equilibrium, in which fluctuations occur in response to hormonal changes during the menstrual cycle, combined with a plethora of disruptive behavioral factors [13]. The menopause is also characterized by a multitude of changes, which affect both the anatomical structure and the microbiota of the lower female reproductive tract [14]. An ethnic variability in the composition of the vaginal microbiota has also been described [10, 15], though so far not fully understood. The host genome may play a major role via the regulation of the innate and adaptive immune systems as well as the composition and quantity of vaginal secretions [16]. The role of the genome in directing the composition of vaginal communities cannot be clearly dissociated from various environmental and cultural factors that are specific to each ethnic group [10].

The study aimed at identifying, for the first time, the particularities of the vaginal microbial ecosystem in healthy Algerian women of childbearing age and also menopausal women. A metagenomic approach based on 16S rRNA gene sequencing was used to register the relative abundance of bacterial operational taxonomic units and thereby identify vaginal community state types (CSTs).

## Material and Methods

### Study Design and Sample Collection

A cohort of 100 healthy women consulting the gynecology service of a private health care unit in Bejaia city (Algeria) was enrolled in this study. A written informed consent was obtained from all participants in this study. A detailed medical case history related to previous gestational situations, gynecological symptoms, infection status, and antecedent of antibiotic or hormonal therapy was obtained from each participant. Pregnant women, those consulting for vaginal infections or taking hormonal replacement therapy, were all excluded from this study. A group of 50 young women of childbearing age (mean age was  $35.1 \pm 7.2$  years) and another of 50 menopausal women (mean age was  $57.7 \pm 8.2$  years) were retained (Online Resource 1). Vaginal sampling was performed by the gynecologist in charge of the service using a sterile speculum without prior antiseptic cleaning of the exocervix. A swab was inserted into the vagina using a rotational movement to recover a sufficient amount of vaginal secretions in a sterile falcon tube. Samples of the vaginal swab were immediately frozen and stored at  $-80\text{ }^{\circ}\text{C}$  until DNA extraction. In parallel, a blood sample was taken from each participant in order to determine the amount of estradiol.

### DNA Extraction

Total DNA from the frozen vaginal swabs was extracted using the QIAamp PowerFecal DNA Kit (Qiagen), following the manufacturer's instructions. DNA amounts were quantified using a BioSpectrometer (Eppendorf), and the DNA quality was checked using a 2100 Bioanalyzer (Agilent).

## Metabarcoding Analysis

The sequencing was carried out at the Faculty of Veterinary Medicine of Liège University (Belgium). For sequencing, the amplification of the V1-V3 region of the 16S rDNA and the library preparation were performed with these primers: direct (5'-GAGAGTTTGATYMTGGCTCAG-3') and inverse (5'-GAGAGTTTGCTCAG-3'). Each PCR product was purified with the Agencourt AMPure XP Ball Kit (Beckman Coulter, Pasadena, USA) and subjected to a second round of PCR for indexing, using Nextera XT index 1 and 2 primers. After purification, the PCR products were quantitated using the Quant-IT PicoGreen (Thermo Fisher Scientific, Waltham, USA) and diluted to 10 ng/ $\mu$ L. A final qPCR quantification of each library sample was performed using the KAPA SYBR FAST qPCR Kit (KapaBiosystems, Wilmington, USA) before standardization, pooling, and sequencing on a MiSeq sequencer using v3 reagents (ILLUMINA, USA). Data processing was performed using, respectively, the MOTHUR v1.35 package and the UCHIME algorithm for alignment, clustering, and chimera detection. Data obtained from NGS analysis were analyzed for bacterial biodiversity, richness, and evenness. The principal component analysis (PCA) using the FactoMineR package in R version 3.5.2 (r-project.org) and the Venn diagrams platform (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>) were used to cross-check the data. Graphical representations were performed using the GraphPad Prism version 8.00 for windows, GraphPad Software.

## Quantitative PCR

DNA samples were amplified with species-specific primer sets targeting 16S rDNA fragments (Table 1). The quantitative PCR (qPCR) was carried out in the iCycler iQ real-time PCR detection system (Bio-Rad), in 96-well microplates. Amplifications were carried out in a final volume of 20  $\mu$ L containing 0.3  $\mu$ M of each primer, 10  $\mu$ L qPCR Core Kit for SYBR Green I (Eurogentec), and either 1  $\mu$ L of DNA or water (no-template control). The thermal cycling conditions were as follows: an initial denaturation step at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 65 °C for 30 s, extension at 72 °C for 30 s, and a fluorescence acquisition step at 72 °C for 10 min. DNAs extracted from vaginal samples were amplified in triplicate for each primer set, and the mean values were used for statistical analysis. Data were expressed as nanograms of DNA of the targeted species per microgram of total DNA extracted from the vaginal sample. For each pair of primers, a melting curve was made to verify the specificity of the amplification. The efficiency and threshold cycle (Ct) were calculated with the CFX Manager software (Bio-Rad). Total bacteria is a broad-range primer that identifies the conserved region of the 16S rRNA encoding gene for a wide range of bacteria. Data obtained by this qPCR reaction gives the amount

of total bacteria present in the sample. Then, the relative quantification of each target bacterium or group was normalized with the total bacteria content in the sample, by the comparative C(T) method [20]. Data were calculated as  $2^{-\Delta Ct}$  means  $\pm$  SD.

**Table 1**

Primer sequences for qPCR

Specificity	Primer	Sequence 5'-3'	Annealing temperature	Reference
<i>Lactobacillus crispatus</i>	Forward	TCTTGACATCTAGTGCCATTTGT	63 °C	[17]
	Reverse	TGCACCACCTGTCTTAGC		
<i>Gardnerella vaginalis</i>	Forward	ACCTGGGCTTGACATGTGCCT	63 °C	
	Reverse	CATGCACCACCTGTGAACCTG		
<i>Atopobium vaginae</i>	Forward	GGTGAAGCAGTGGAAACACT	62 °C	
	Reverse	ATTCGCTTCTGCTCGCGCA		
<i>Lactobacillus gasseri</i>	Forward	TGGAACAGRTGCTAATACCG	60 °C	
	Reverse	CAGTTACTACCTCTATCTTTCTTCACTAC		
<i>Lactobacillus jensenii</i>	Forward	CCTTAAGTCTGGGATACCATT	54 °C	[18]
	Reverse	ACGCCGCCTTTTAAACTTCTT		
<i>Lactobacillus iners</i>	Forward	GTCTGCCTTGAAGATCGG	65 °C	
	Reverse	ACAGTTGATAGGCATCATC		
	Reverse	TTCACTGCTCAAGTAATCATC		
Total bacteria	Forward	ACTCCTACGGGAGGCAG	55 °C	
	Reverse	GTATTACCGCGGCTGCTG		

## Statistical Analyses

The analysis of statistical significance was performed by one-way ANOVA and the post hoc Tukey test ( $p < 0.05$ ).

## Results

16S rDNA targeted metagenomics analysis was performed in individual vaginal samples of 50 young women of childbearing age (Y) and 50 menopausal women (M) and allowed comparison of the overall bacterial richness and phylogenetic composition of the vaginal microbiota. After sequence processing, a total of 176 different operational taxonomic units (OTUs) were identified in the Y and 413 OTUs in the M dataset, respectively. All sequences can be found at the US-National Center for Biotechnology Information (NCBI) BioProject repository (PRJNA630844).

### Microbial Diversity

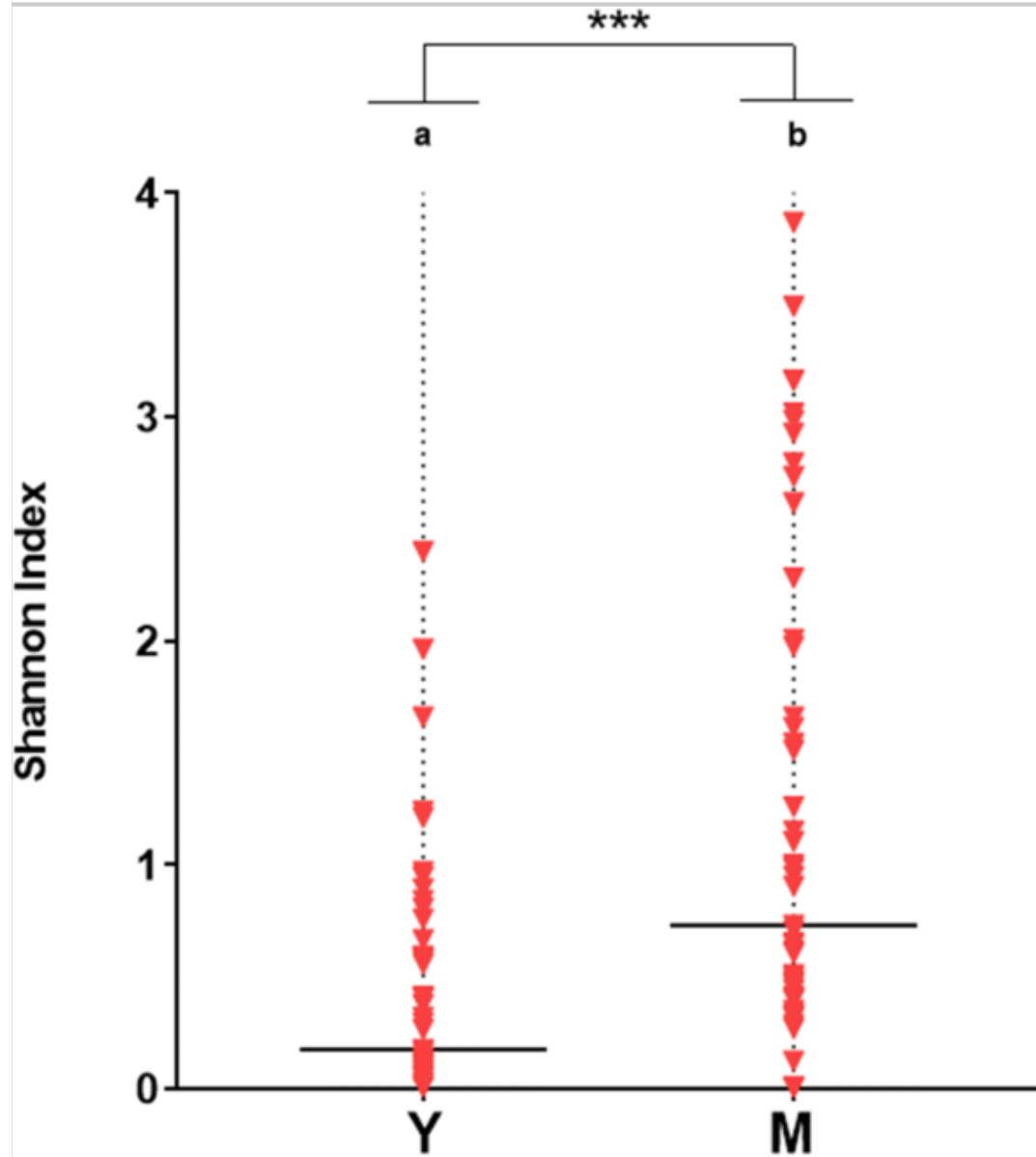
The average number of OTUs was about 429 which is of the same order of magnitude as other studies of vaginal microbiota [21]. The Shannon Index (SI) indicated a significant increase of the alpha-diversity for M group with a  $p < 0.0001$  (Fig. 1). The PCA of the beta-diversity in the vaginal microbiota composition revealed also an important increase of the taxon diversity for M subjects (Fig. 2).

#### Fig. 1

Shannon Index of bacterial diversity of vaginal microbiota in young (Y) and menopausal (M) women. M subjects presented a significantly increased diversity compared with Y subjects. Significant differences are indicated with letters (a, b); triple asterisks indicate  $p < 0.0001$

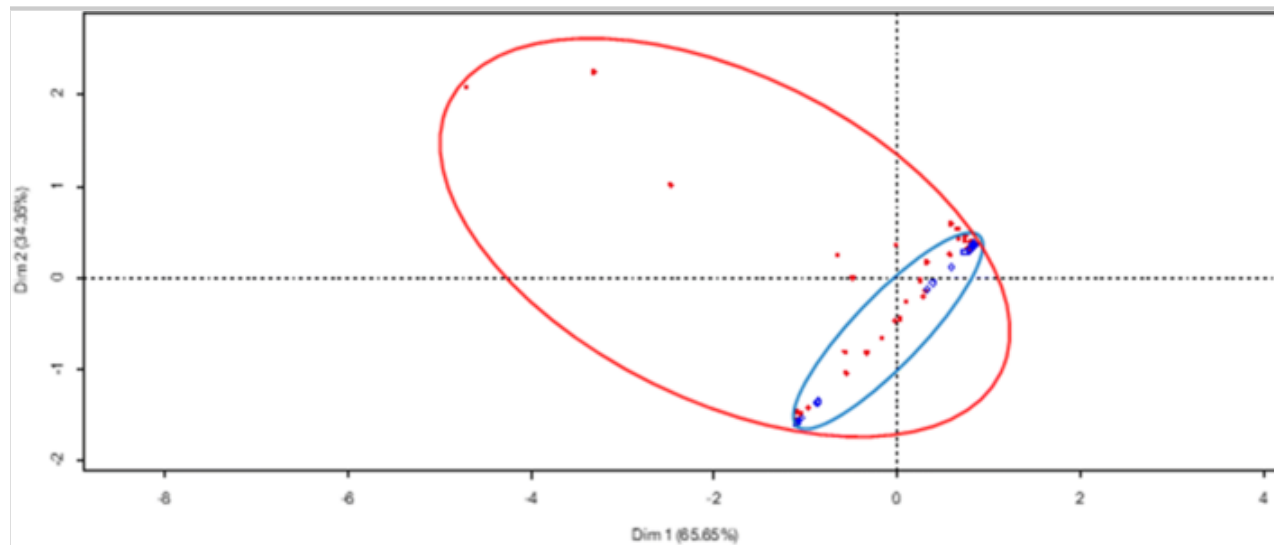
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**Fig. 2**  
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Principal component analysis of the vaginal microbiota collected from young (Y) and menopausal (M) women ( $n = 50$  per group). The two sets of samples are not similar as the Y subjects (blue circle) present a restrained taxon diversity comparing with M subjects (red circle)



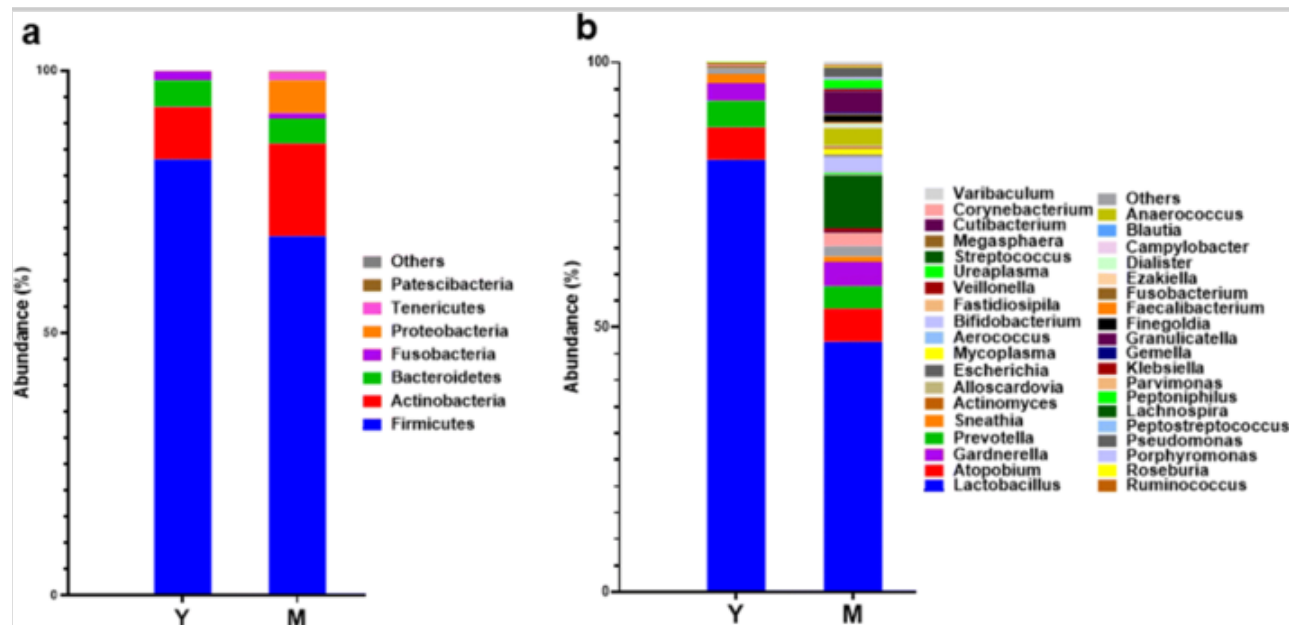
## Microbial Characteristics and Community State Types

The community composition of each sample, representing the bacterial phyla, genus, and species level ranking for major OTU, is depicted in Figs. 3 and 4 and Online Resource 2. The most abundant phyla across all young subjects were *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria*. The relative abundance of these phyla appeared to change for menopausal women, where *Firmicutes* abundance has significantly diminished (83.04% in Y vs 69.36% in M subjects,  $p < 0.01$ ), whereas abundance has increased for *Actinobacteria* (10.04% in Y vs 18.19% in M subjects) (Online Resource 3). Of note, two other phyla containing mainly pathogenic bacteria, *Proteobacteria* and *Tenericutes*, became more important in microbiota of menopausal women (Fig. 3a) and passed in average from 0.07% and 0.05% in Y to 4.92% and 1.27% in M subjects, respectively ( $p > 0.9$ ). This tendency was confirmed at the genus level where the decrease of *Firmicutes* abundance was reflected in lactobacilli dynamics. *Lactobacillus* was the upmost representative genus in vaginal microbiota of Y subjects representing 82.45% and only 47.32% for M subjects ( $p < 0.0001$ ) (Fig. 3b). Other genera belonging to *Actinobacteria* such as *Bifidobacterium*, *Gardnerella*, and *Corynebacterium* increased from 0.02%, 3.32%, and 0.22% in Y to 2.96%, 4.59%, and 2.56% in M subjects, respectively. Likewise, *Mycoplasma* and *Ureaplasma* belonging to *Tenericutes* increased significantly ( $p > 0.9$ ) in abundance, from 0.01% and 0.04% in Y to 1.06% and 0.27% in M subjects respectively. Nevertheless, *Pseudomonas* belonging

to *Proteobacteria* appeared only in M subjects (1.86%). Species abundance revealed a more detailed profile of individual microbiota when CSTs were clearly identified (Fig. 4). For Y subjects, 34% were found to belong to CST I dominated by *Lb. crispatus*, 8% to CST II, dominated by *Lb. gasseri*, 38% to CST III dominated by *Lb. iners*, 18% to CST IV dominated by anaerobes (such as *Atopobium vaginae*, *Prevotella* sp., and *Sneathia amnii*), and only 2% to CST V, dominated by *Lb. jensenii*. For M subjects, 20% presented a CST I profile, 2% CST II, 24% CST III, and the majority (54%) a CST IV profile.

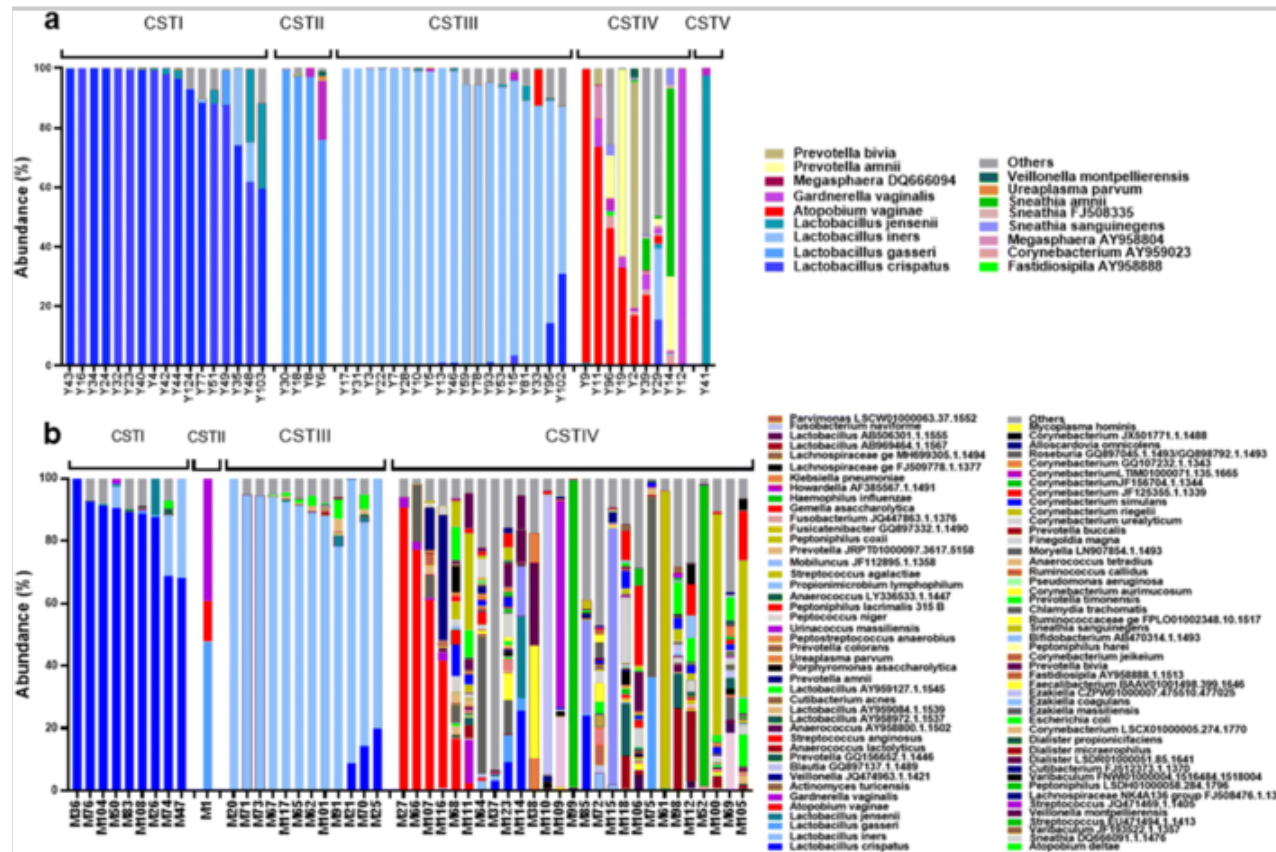
**Fig. 3**

Relative abundance of bacterial phyla (a) and genera (b) in the vaginal microbiota. Y, young women; M, menopausal women



**Fig. 4**

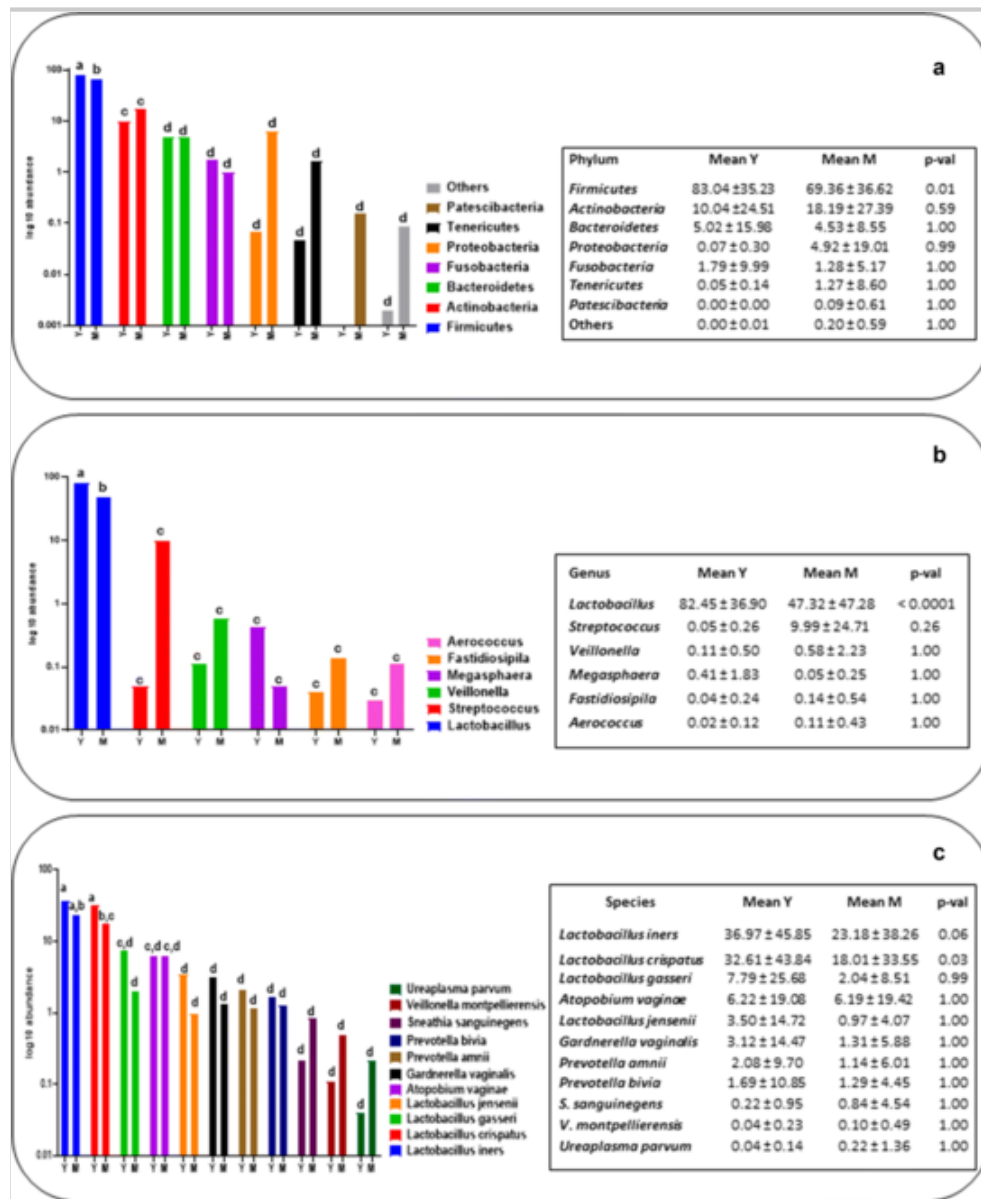
Relative abundance of bacterial species in the vaginal microbiota of young (a) and menopausal (b) women. Y, young women; M, menopausal women. CST profiles are indicated above



All these variations and statistical meanings are represented for different taxonomic levels (phylum, genera, species) by comparing young and menopausal women (Fig. 5).

### Fig. 5

Variations in taxa abundances. Significant differences in abundance were observed at phylum level (a) for only *Firmicutes*, at genera level (b) for only *Lactobacillus* and at species level (c) for *Lactobacillus crispatus*. Means of relative abundances are represented for young (Y) and menopausal (M) women. Means without a common letter are different ( $p < 0.05$ ) using one-way ANOVA with Tukey's test for pairwise comparisons

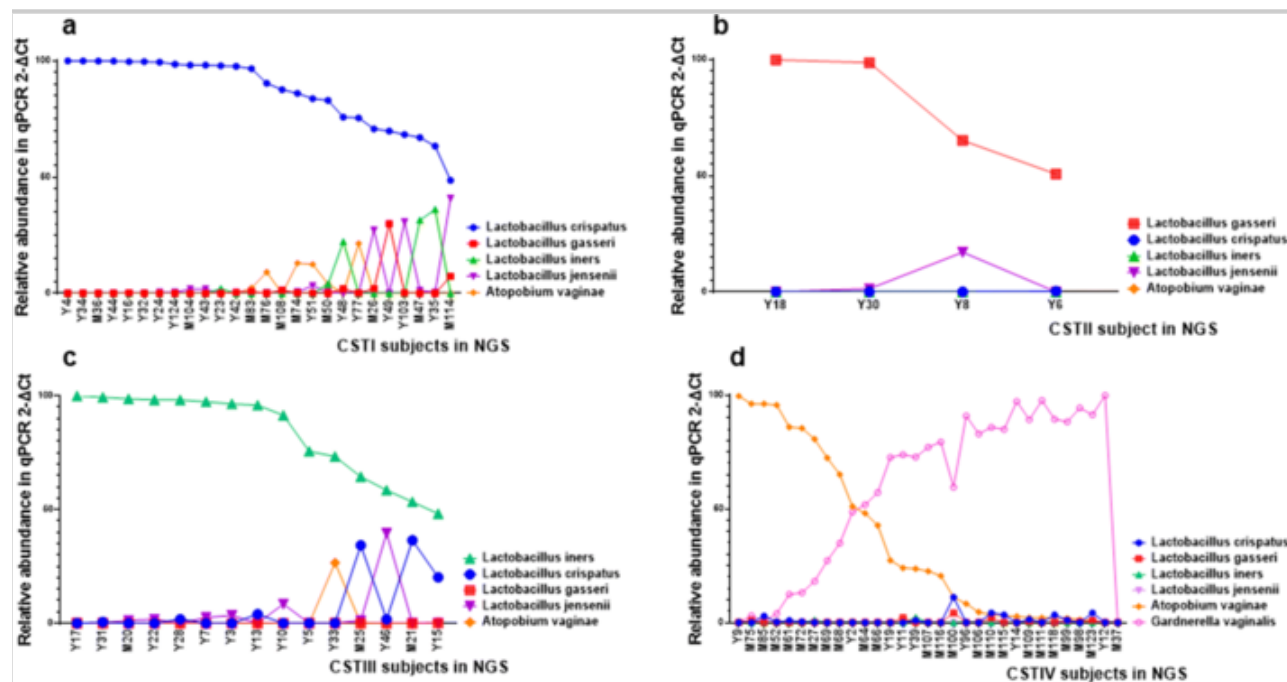


### qPCR Method for CST Identification

The abundance of the main species in each CST was checked by the qPCR technique. Thus, a specific amplification was performed for *Lb. crispatus* (that is dominant in CST I), *Lb. gasseri* (dominant in CST II), *Lb. iners* (dominant in CST III), *Lb. jensenii* (dominant in CST V), *Atopobium vaginae*, and *Gardnerella vaginalis* (the most representative for the CST IV). The resulting qPCR data were in good agreement with the metagenomic analysis and confirmed the dominant taxon for each CST (Fig. 6).

**Fig. 6**

Rapid qPCR method for CST identification. Relative abundance of six bacterial taxa in vaginal swab of young (Y) and menopausal (M) women were calculated by qPCR assay for each CST detected by NGS. The dominant taxon confirmed the same major abundance for 96.3% of subjects with CST I (a), 80% of subjects with CST II (b), 50% of subjects with CST III (c), and 88.5% of subjects with CST IV (d)



## Discussion

This study was conducted to analyze the composition of the vaginal microbiota of a cohort of 100 Algerian women, 50 of whom were of reproductive age and 50 at the confirmed menopause period. An analysis of the dynamics of this composition was carried out to intercept

age-related changes, and an assessment of the ethnic specificity of this composition was discussed.

## Age Dynamics of Vaginal Microbiota

According to Gajer et al. [22], the stability of the vaginal microbiome is not necessarily expressed by changes in taxa composition, but rather by CST consistency. Then, it has been suggested that the vaginal microbiota changes from one CST to another in response to the modifications brought about by the transition to menopause. Although the longitudinal follow-up was not possible for this study, it is important to point out the dramatic change in the CST pattern observed in menopausal women, suggesting that all CST profiles changed in their composition as 70% of CST I, 75% of CST II, or 36% of CST III are modified towards the anaerobe dominated CST IV, whose abundance increased from 18% in Y subjects to 54% in M subjects.

All physiological, medical, or historical parameters collected from participants were analyzed with the aim to explain these changes. Of note, only estrogen levels were found to be correlated with microbial fluctuations across the age groups. Thus, all menopausal women were characterized by a dramatic decrease in estrogen levels ( $29 \pm 40$  pg/mL) compared with young women ( $126 \pm 95$  pg/mL) (Online Resource 3). It has also been reported that changes in the vaginal microbiota composition were associated with the noticeable hormonal shifts taking place throughout a woman's life. With menopause, estrogen levels and the glycogen content in the vaginal epithelium decreased significantly, thereby causing a decrease in lactobacilli prevalence [23]. Thus, this hypoestrogenism related to menopause must have induced a decline of glycogen level on the vaginal epithelium [24]. As glycogen is the preferred substrate for lactobacilli, the first consequence is a significant decrease in numbers of all lactobacilli, with a direct impact on CST composition. This situation was already observed in menopausal women where a decrease of lactobacilli, caused a vaginal pH enhancement, and only 39 to 55% of menopausal women carried a *Lactobacillus*-dominant vaginal microbial community [25]. Concerning individual estrogen variations, no correlation with the microbiota composition has been observed. This is in accord with the sampling realized at approximately the same time of the reproductive cycle in Y subjects. Other previously reported studies showed that the different fluctuations in vaginal microbiota composition during estrous cycle did not, overall, change the CST profiles [22, 26, 27].

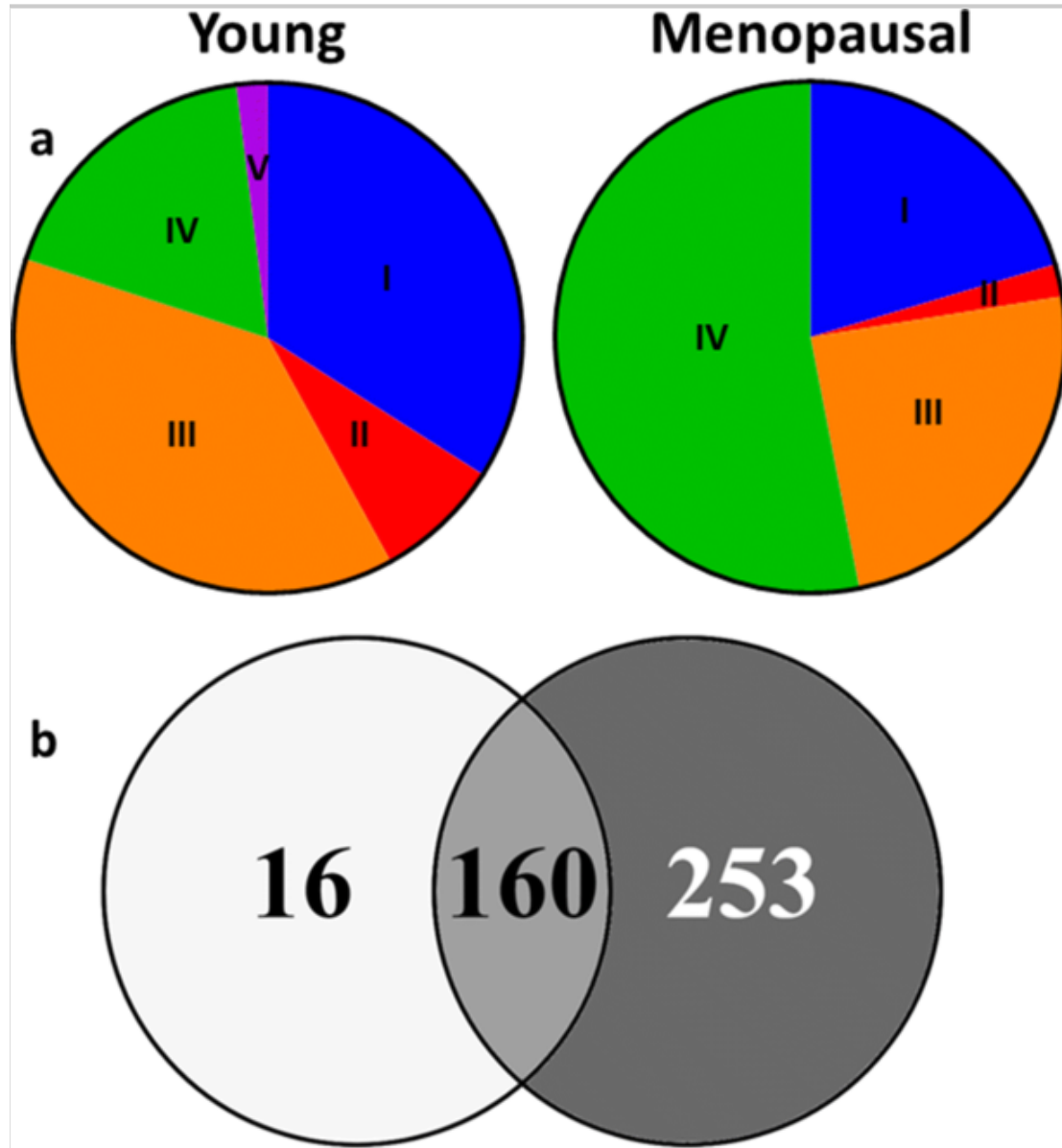
Taking into account all these observations, we can consider that the vaginal microbiome of Algerian women keeps a normal dynamic with age. Nevertheless, this age-dependent imbalance in the vaginal microbiota is known as a risk-factor for vaginal infections in postmenopausal women. To estimate the risks for bacterial vaginosis (BV) for Algerian postmenopausal women, a comparison of CST profiles and a qualitative comparison of the microbiota composition were performed on Y and M subjects (Fig. 7; Online Resource 4). Lactobacilli dominated CST (I, II, III, and V), either diminished or disappeared in M subjects (Fig. 7a), and sixteen species seem completely lost in menopausal women (Fig. 7b). These included *Actinomyces naeslundii*, identified as an antagonist of *Candida albicans* with protective effect against mycosis [27], and *Carnobacterium maltaromaticum* that is also a beneficial bacterium used in the dairy

industry for lactic acid and bacteriocin production [28]. On the other hand, the rate of anaerobe-dominated CST IV increased in M subjects (Fig. 7a) and 253 new species occupied the vaginal microbiome of menopausal women (Fig. 7b). Of note, *Prevotella*, *Dialister*, or *Bacteroides* species were reported to be involved in bacterial vaginosis [29, 30], and *Actinomyces* species can lead to actinomycosis [31]. A new equilibrium may operate in the vaginal microbiota of menopausal women due to hormonal changes. Nevertheless, the risk of vaginosis remains considerably increased in menopausal women [32]. In our work, the clinical case history of 5 postmenopausal subjects presenting the CST IV profile (M111, M109, M106, M118, and M69) also suggested this risk (Online Resource 1). For a personalized monitoring of CST profile evolution, especially in menopausal transition period, the qPCR method could be very useful as a rapid method of detection of the CST in a vaginal swab.

### **Fig. 7**

Age dynamics of vaginal microbiota. **a** CST ratio in young and menopausal women (I-IV). **b** Venn diagram representing the number of species found in young (white circle) and menopausal women (black circle)



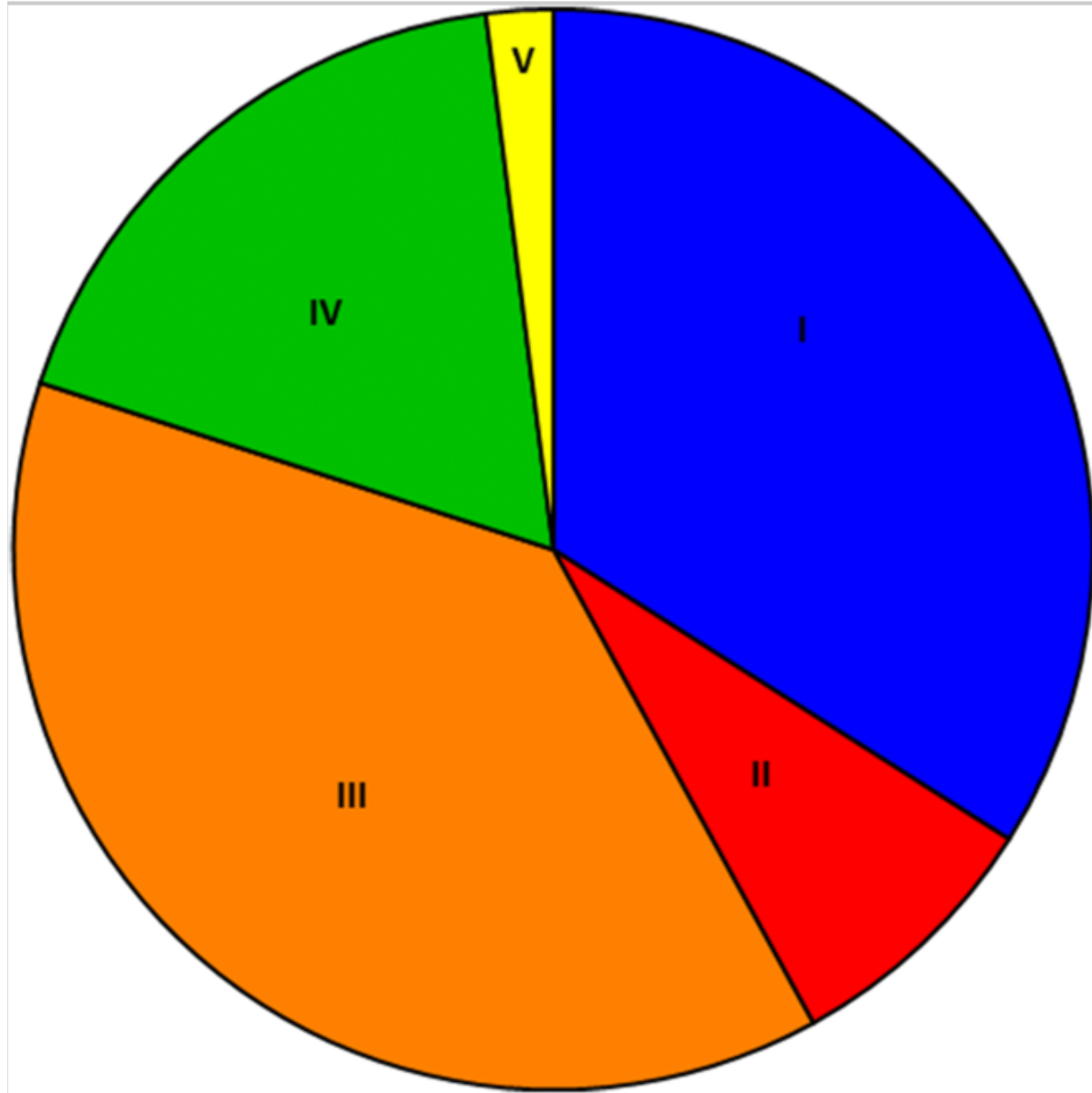


### Ethnic Framework

To determine the vaginal microbiota profiles of Algerian women, in an ethnic context, we compared the CST patterns of the Y subjects with the results of similar studies carried out in different parts of the world such as a large North American cohort comprising four ethnic groups (Caucasian, African, Hispanic, and Asian) [10], as well as with cohorts of Nigerian [33], British [34], Dutch, Turkish, and Moroccan women [15] (Online Resource 5). Interestingly, regardless of the geographical relocation of the women, the ethnic particularities of the CST composition are maintained. The CST I was predominant for Caucasian groups, CST IV for African and Hispanic, and CST III for Asian, Turkish, and Moroccan cohorts. Turkish and Moroccan groups stand out also for their high proportion of CST II and V. Algerian group presented the CST III (38%) and CST I (34%) as major groups, followed by CST IV (18%), CST II (8%), and CST V (2%) (Fig. 8). The reasons for these differences within ethnic groups are unknown and possibly can be linked to genetic differences such as their innate and adaptive immune systems [35], the composition and quantity of their vaginal secretions, and ligands on epithelial cell surfaces [10]. These factors certainly shape vaginal communities, but in addition, human habits and practices, including personal hygiene, birth methods, and sexual behaviors, can also exert strong influences [36].

**Fig. 8**

Algerian CST profiles. For a cohort of 50 women in reproductive age, the CST III and CST I were found the major groups in vaginal microbiota (38 and 34%, respectively), followed by CST IV (18%), CST II (8%), and CST V (10%)



## Conclusion

In this study, we have examined for the first time an Algerian cohort of 50 young women of childbearing age and 50 menopausal women by performing a 16S rRNA gene targeted metagenomic analysis of the individual vaginal microbiota. The bacterial composition and CSTs were identified for these two healthy states, and a normal age dynamic was observed. The transition from childbearing to menopausal status was accompanied by an increasing of the anaerobe community that became dominant and was estimated to be 3 times more important. Hormonal changes were correlated with low lactobacilli abundance. These changes predispose menopausal women to BV, which is why we developed the monitoring system of CST profile evolution by a rapid qPCR method reported here. The ethnic comparison revealed a particular microbiota profile for Algerian women, with a dominance of CST III and CST I, and this finding should be taken into consideration for different diagnostics, clinical investigations, or treatments.

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## Compliance with Ethical Standards

*Conflict of Interest* The authors declare that there is no conflict of interest.

## Electronic Supplementary Material

**Online Resource 1.**

List of subjects included in the study. Y - young women, M – menopausal women, BMI - body mass index. (XLSX 23 kb)

**Online Resource 2.**

List of bacterial species in the vaginal microbiota of Algerian young (Y) and menopausal (M) women (PDF 1253 kb)

**Online Resource 3.**

Oestradiol variations and microbiota composition (PDF 758 kb)

**Online Resource 4.**

Age dynamics of bacterial species in vaginal microbiota of young and menopausal women (PDF 151 kb)

**Online Resource 5.**

CST composition (%) of the vaginal microbiota in different ethnic groups around the world (PDF 294 kb)

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