Does exposure to different types of menstrual protections affect the vaginal environment?

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Abstract

The vaginal ecosystem is a key component of women's health. It also represents an ideal system for ecologists to investigate the consequence of perturbations on species diversity and emerging properties between organisational levels. Here, we study how exposure to different types of menstrual protection products is linked to microbial, immunological, demographic, and behavioural measurements in a cohort of young adult women who reported using more often tampons ($n = 107$) or menstrual cups ($n = 31$). We first found that cup users were older and smoked less than tampon users. When analysing health indicators, we detected potential associations between cups use reporting fungal genital infection. Multivariate analysis confirmed that, in our cohort, reporting using cups over tampons was associated with the higher odds ratio to report a fungal genital infection diagnosis by a medical doctor within the last 3 months. We did not detect significant differences between groups in terms of their bacterial vaginal microbiota composition and found marginal differences in the level of expression of 20 cytokines. However, a multivariate analysis of these biological data identified some level of clustering based on the type of menstrual production preferred (cups or tampons). These results suggest that exposure to different types of menstrual products could influence menstrual health. Larger studies and studies with a more powered setting are needed to assess the robustness of these associations and identify causal mechanisms.

Keywords: vaginal microbiota; fungal infections; immunity; epidemiology; women's health

Introduction

There is a long history of considering organisms as ecosystems, and *vice versa* [1]. This parallel has been particularly exploited to study within-host parasite dynamics [2]. With the advent of metagenomics, there is a renewed interest in applying ecological theory to understand microbiota dynamics [3, 4]. Beyond parasites or even the microbiota, the importance of encompassing the diversity of these ecosystems, e.g. including immune cells, is increasingly acknowledged, especially since the border between self and non-self is tenuous in this context [5].

Some general questions pertaining to ecosystem functioning can be studied by considering an individual host as an ecosystem. For instance, it is challenging to clearly define properties that emerge at a higher level of organisation in large-scale ecological systems. However, the definition and measurement of individual health outcomes (i.e., systems-level outcomes) is routine in clinical research. In contrast to free-living ecosystems, individual hosts are also smaller, more numerous, and readily replicable, making empirical ecosystem ecology research more accessible and reproducible [6].

The vaginal ecosystem is an ideal study system to understand how within-host interactions shape host health. First, the vaginal microbiota is a key component of women's health throughout their lives [7], modulating the risk of diseases such as fungal genital infections, urinary tract infections, and sexually transmitted infections [8], such as human immunodeficiency virus [9]. Contrary to its gut counterpart, the vaginal microbiota is characterised by relatively few, clearly-defined, community state types (CST), most of which are dominated by one species of *Lactobacillus* bacteria [10]. Second, the microbes in the vaginal environment, including bacteria, fungi, and viruses, are involved in a tight cross-talk with the local innate and adaptive immune response, the components of which are known to be adapted to this highly variable environment [11]. In particular, the interaction between the immune response and sex hormones throughout the menstrual cycle is well documented [12]. Finally, the vaginal ecosystem is perturbed by factors including menses, sexual activities, and antibiotic treatments [13]. Longitudinal follow-ups demonstrate that such perturbation can lead to shifts from one CST to another [14]. Perturbations that jeopardise the balance between immunity, hormones, and the vaginal

microbiota could also hinder the efficiency of the immune system to control bacteria, fungal, and viral pathogens [13].

Here, we explore whether exposure to a particular type of menstrual protection product, namely menstrual cups or tampons, is associated with differences in the vaginal environment. To this end, we analyse the vaginal microbiota, immune response (i.e., cytokines and antibodies), and, more generally, the emerging property of this ecosystem, which revolves around menstrual health [15].

Among the variety of products used during menses, menstrual cups are perceived as a safe, practical, economical, and ecologically friendly alternative to tampons and sanitary pads [16]. The majority of women using them report wanting to continue to use them, both in high and low-income settings, showing a good level of acceptability [17, 16]. Nevertheless, there have been some reported cases of toxic shock syndrome, renal colic, and allergies linked with menstrual cup usage [16]. For example, higher levels of *Staphylococcus aureus* growth have been reported in menstrual cups compared to tampons [18]. In 2019, a systematic review identified 12 clinical trials and 23 observational studies comparing this type of menstrual protection to others [16]. In the vast majority of existing studies, the outcome studied was the practicality of the cups implementation, e.g. acceptability or risk of leakage. Therefore, we know little about the potential microbiological and immunological implications of cup usage compared to other menstrual hygiene products.

In the present study, we analysed biological, demographic, and behavioural longitudinal data from 138 women. We compared two populations of women defined by the type of menstrual protection they reported using most often (tampons or menstrual cups) and analysed microbiological, immunological, and clinical data while statistically accounting for demographic and behavioural differences. Using statistical modelling, we identified profile differences depending on the type of menstrual product used.

Materials and methods

Cohort description and data curation

Women included in the present study were enrolled in the PAPCLEAR longitudinal clinical study [19], which followed 149 women between 2016 and 2020 to study human

papillomavirus (HPV) infections [19]. The women enrolled were between 18 and 25 years old, lived in the area of Montpellier (France), and reported at least one new partner within the last 12 months. Their status for HPV and other genital infections, immunological responses (antibodies and cytokines), and behaviours were followed for up to two years (see [20] for details about the study protocol).

We selected participants who reported using tampons or cups as menstrual products and for whom detailed cytokine profiles, microbiota metabarcoding data, and antibody data at the inclusion visit were available. We assigned tampon or menstrual cup categories when a participant reported using either type of menstrual product over 75% of the time over the whole duration of the study (see Figure S1 for details on how the assignment was performed). Women using menstrual cups more frequently than tampons are denoted 'menstrual cups users' for convenience throughout the manuscript. There was no difference in follow-up duration between women using mostly cups or mostly tampons (Table 1). Other analyses of demographic, behavioural, and biological data were performed on the first visit of the participant (inclusion visit). Overall, our analysis includes data from 138 women.

Since the PAPCLEAR study was designed to understand the ecology of HPVs and their interaction with immune responses and vaginal microbiota, the data from this cross-sectional study is not perfectly balanced in terms of exposure to each type of menstrual product. However, given the current lack of studies, it greatly improves our understanding of women's menstrual health.

Biological analyses

HPV infections were assessed from cervical smears using the LiPA_{25} genotyping assay, which discriminates between 25 genotypes [21]. Further details on HPV detection and prevalence in this cohort can be found elsewhere [20]. A 'focal' infection was defined as an infection by the same HPV genotype at the first and second visits.

The microbiota metabarcoding was performed on 200 µL of vaginal swabs specimen stored at -80° in Amies medium. DNA extraction was performed using the MagAttract PowerMicrobiome DNA/RNA Kit (Qiagen). Next-generation sequencing of the V3-V4 region of the 16S gene [22] was performed on an Illumina HiSeq 4000 platform

(150 bp paired-end mode) at the Genomic Resource Center at the University of Maryland School of Medicine. Taxonomic assignment was performed using the internal software package SpeciateIT (https://github.com/Ravel-Laboratory/speciateIT) and the community state type (CST) was determined using the VALENCIA software package [23].

Antibodies were analyzed using a multiplex Luminex assay [24], as already described in the context of HPV infections [20]. Cytokine data were obtained using MesoScale discovery technology from vaginal secretions collected using ophthalmic sponges placed directly on the cervical os for approximately one minute, as described in a previous study which analyses this data in the context of HPV infections [25]. We used the same protocol to obtain values that were normalised per total protein concentration in the sample.

Statistical analyses

To study the difference between the main characteristics of our two populations of interest (Table 1 and Table 2), we used χ^2-test or Kruskal-Wallis rank sum tests when applicable.

We then performed multivariate analyses using generalised linear models (GLMs) with a binomial distribution for the response variable for the models shown in Figure 1A, Table 3, and Supplementary Tables S4 to S8. In the first series of models, the response variable was the type of menstrual protection (cups or tampons). In the second series, the response variable was being diagnosed or not with a fungal genital infection within the last three months by a medical doctor.

We identified 15 covariates of interest (Table S1). Given the exploratory nature of the study, we built models with all the possible combinations of covariates as explanatory variables. We then selected the best models using the Akaike Information Criterion corrected for small sample sizes (AICc). We used a lowest AICc +5 interval to identify the most probable best models [26]. For each model, we computed the odds ratios associated with each predictor along with a 95% confidence interval.

For clustering, factor analysis of mixed data was used when combining both factor and numeric data (Figure 3B and S2), and multiple correspondence analysis was used when analysing categorical data (Figures S2 and S4) [27]. Non-overlapping 95% confidence

ellipses indicate a statistically significant difference between cups and tampons users. The covariates used for the socio-economic analysis using a multiple correspondence analysis are listed in Table S2.

Statistical analyses were performed in R 4.1.3 [28] with the with the `kruskal.test` function for Kruskal-Wallis rank sum tests (in Tables 1 and 2 for numeric data), `chisq.test` for χ^2 test (in Table 1 and Figure 2B for proportional data), `wilcox.test` function for Mann-Whitney test (Figure 2C) and the `glmulti` function for binomial regressions [29] (in Figure 1A and Table 3). The factor analysis of mixed data and the multiple correspondence analysis (in Figures 1B, 3B, S2, S3, and S4) were processed and represented using the `FactorMineR` and the `FactoExtra` (https://github.com/kassambara/factoextra) packages [27].

Results

The 138 women included in the analysis were aged from 18 to 25 years old and primarily university students (119/138, 86%). We stratified the population according to the most frequent type of menstrual product used, *i.e.* either tampons, $n = 107$, or menstrual cups, $n = 31$. Analysis of the main demographic characteristics (shown in Table 1) using a generalised linear model (GLM) selection approach identified significant differences between these two populations in terms of age and smoking status: cups users were older and reported smoking less than tampon users (Figure 1A and Table S3). On the other hand, a multiple correspondence analysis on 12 covariates associated with socio-economic status and listed in Table S2 did not show any difference between the two populations (Figure 1B).

We then explored associations between the type of menstrual product used and microbiological covariates. First, we analysed vaginal microbiota diversity using 16S metabarcoding data (Figure 2A). We found no significant difference in community state types (CST) composition (Figure 2B), although there were some qualitative differences. For instance, among our menstrual cup users, we found no occurrence of CST II, which is dominated by *L. gasseri*, but slightly more CST I and V, which are dominated by *L. crispatus* and *L. jensenii* [10]. We also did not find any significant differences in microbiota diversity, assessed using Shannon diversity index, between women using menstrual cups or tampons (Figure 2C).

We then analysed six health-related covariates, most of which correspond to infections (Table 2). The only significant difference was that women using cups also reported more often having been diagnosed with fungal genital infections within the last 3 months. To further investigate this association, we used our GLM selection method to identify the covariates associated with this fungal genital infection. We found that reporting using cups is the only predictor with a significant odds ratio (OR of 4.73, 95% CI: 1.44-16.1) associated with the risk of reporting a fungal genital infection (the number of lifetime partners is also present in the model, but not significant). Similar models for the other five health-related covariates are shown in Appendix (Tables S4 to S8).

To investigate the potential effect of menstrual cups on the local immune response,

we analysed cytokine and chemokine relative concentrations in cervical samples. Among the 20 analytes measured, IL-10, MIP-1α and TNF-α appeared to be significantly lower in women using cups (IL-10: $p = 0.012$, MIP-1α: $p = 0.049$ and TNF-α: $p = 0.036$), although these associations did not withstand correction for multiple testing comparisons ($p = 0.24$, $p = 0.32$, and $p = 0.32$ respectively) (Figure 3A).

Finally, we performed a profile analysis using a factor analysis of mixed data approach involving CST data, Shannon diversity index, and cytokine and chemokine relative concentrations. Women who use tampons and women using menstrual cups fall into two largely non-overlapping clusters (Figure 3B and Supplementary Figure S2), suggesting that the type of menstrual product used could be associated with a different immunological and microbial environment. Conversely, a similar multiple correspondence analysis approach using blood seropositivity status for immunoglobulins G (IgG) and immunoglobulins M (IgMs) of several sexually transmitted infections, including HPVs, detected no clustering (Supplementary Figure S3), hinting that the women in these two groups have similar exposure risks to sexually transmitted infections.

Discussion

The vaginal ecosystem is an essential part of women's health and several 'perturbations' such as menses, sexual intercourse, or drug treatment can cause pronounced shifts [7]. In this study, we analysed whether exposure to different types of menstrual products, namely menstrual cups or tampons, could be associated with differences in health indicators or, more generally, in the vaginal environment.

Menstrual cups are gaining in popularity as an environmentally sustainable and affordable type of menstrual protection, but the data surrounding their use are limited, especially outside low-income countries [16]. Therefore, we first analysed the profiles of the two populations of women defined based on the type of menstrual protection they reported using most. In terms of demographic covariates, we found that the main factors that were associated with preferential use of cups over tampons were age (cup users were older) and smoking status (tampon users smoked more). In terms of socio-economic status, a multiple correspondence analysis did not suggest any differences between the two populations, which could be due to the fact that the majority of the cohort member was

university students. A similar multiple correspondence analysis based on serological data for a variety of sexually transmitted infections also did not find any difference, further suggesting that these two cohorts are comparable in terms of their general lifestyle.

On the microbiological side, we did not find a significant difference between the bacterial community compositions of the vaginal environment depending on the type of menstrual product used. A recent preprint [30] studied vaginal microbiota in a large cohort of $3,345$ women aged 18 to 98 with a median age of 32 years old. Their results show that women who reported using menstrual cups are more often associated with a *Lactobacillus crispatus* cluster (which also includes *L. jensenii*, and could be described as encompassing both CST-I and CST-V); which is consistent with the trend detected in our dataset.

Moving to health-related factors, we detected a strong association between reporting using menstrual cups and a recent diagnosis of a fungal genital infection. None of the other five health-related variables that we analyzed, such as reporting urinary tract infections or the diagnosis of chlamydia infections, were significantly different. To further investigate this trend, we used a generalised linear model selection approach. In almost all the best models, reporting using cups more than tampons was significantly associated with a higher risk of reporting fungal genital infections. It is noteworthy that the number of partners was present in most of the best models (98.75% of the time), although never significant.

Finally, we investigated the host component of the vaginal environment by analyzing local cytokine and chemokine concentrations. Three cytokines or chemokines were found to exhibit potentially lower concentrations in women using cups (IL-10, MIP-1α, and TNF-α), although significance did not withstand correction by multiple hypothesis testing. Furthermore, the joint analysis of microbiota and immunological data suggests that women tend to segregate into two clusters based on the type of menstrual product they use most suggesting general profile differences in terms of their vaginal environment.

Our study has several limitations, the strongest being the relatively small sample size of the cohort used ($n = 138$), which was originally designed to study HPV infections. This may hinder our ability to detect moderate or subtle changes induced by menstrual cups in the vaginal environment. For example, we included the use of lubricants in the

analysis, but more detailed studies could also include details on contraception methods or probiotic use. Another limitation of the study lies in its cross-sectional nature. Further longitudinal analyses would be helpful to establish long-term potential impacts of the use of menstrual cups on the local environment, for example, on the vaginal microbiota composition, which can be highly variable over time for some women [31]. In particular, prospective or retrospective cohort studies, where participants are stratified based on the type of menstrual products used, could be a powerful means to study the occurrence of a particular negative outcome, i.e. here fungal infections.

Regarding these infections, we cannot provide any further details regarding the fungal species or their abundance with the current data. A quantitative polymerase chain reaction approach targeting the ITS locus coupled with a metagenomics approach could provide a more accurate picture, especially thanks to existing gene catalogs for the vaginal microbiota, such as VIRGO which already includes more than 10,000 fungal genes [32].

The existing literature on the usage of menstrual cups focuses on availability and acceptability, yet its health implications remain understudied. A recent systematic review reported nine cases of urinary tract complaints [16], which we did not detect as being significantly more frequent among menstrual cups users than for tampons users. Conversely, the primary adverse health outcome associated with menstrual cup use in our study, i.e. fungal infections, was not included in the systematic review. Our results are consistent with another recent study, which does not show adverse effects of menstrual cups on the vaginal microbiota, and even potentially an increased proportion of some *Lactobacillus*-dominated communities, which are considered beneficial for maintaining the vaginal health [30]. These findings can help inform public health policies regarding the use of menstrual cups and underline the need for larger, balanced cohort studies.

Health is partly the result of dynamic interactions between hosts and their microbes that have a long coevolutionary history. The human vaginal microbiota is an ideal system for studying these questions for several reasons. First, its composition is a known health moderator — *Lactobacillus*-dominated communities tend to decrease the risk of acquisition of sexually transmitted infections [10]. Second, the composition of the vaginal microbiota can vary over time as a result of menstrual cycles or age, as well as a variety of 'perturbations' including sexual intercourse and chemical interventions [14]. These

two points are not unique to the vaginal microbiota but contrarily to other microbiota, especially in the gut, the diversity is limited (both between and within women) and the variability better understood. From an evolutionary point of view, human vaginal microbiota are unique for the high prevalence of *Lactobacillus*-dominance: Lactobacilli are rarely dominant in other mammals, including non-human primates [33]. This human specificity complicates experimental studies using animal model systems. However, three-dimensional cell culture models now offer new opportunities for manipulative experiments, for instance to test how the microbiota composition affects the interaction between a parasite and host cells [34]. As illustrated by our study, integrating knowledge across scales, i.e. going from the microbiological and immunological variables to individual health is data-intensive and requires multidisciplinary knowledge from molecular biology to public health. Nonetheless, it is a necessary and worthwhile challenge to bridge the gap between molecular mechanisms and health outcomes.

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Disclosure of Interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: TW serves on advisory boards for MesoScale discovery (Merck) Sharp & Dohme.

Contributions

NT, CLM, NBo, and SA designed the study. NT, CLM, BE, BR, TK, IGB, and SA designed the experiments. CB, VB, SGro, MR, and NBe performed experiments. CH, JRa, and TW contributed reagents, materials, and analysis tools. CB, VB, SGra, SGro, MR, MB, CG, VT, VF, CLM, JRe, IGB, MSe, NBo, and SA contributed to study design, patient recruitment, and clinical data acquisition. NT, IBU, BE, BR, CS, TK, CLM,

TK and SA performed data analyses. NT, IBU, IGB, CLM, TK and SA wrote the initial version of the manuscript. All authors approved the final version of the manuscript.

Ethics

The PAPCLEAR trial was promoted by the Centre Hospitalier Universitaire (CHU) de Montpellier and has been approved by the Comité de Protection des Personnes (CPP) Sud Méditerranée I on 11 May 2016 (CPP number 16 42, reference number ID RCB 2016-A00712-49); by the Comité Consultatif sur le Traitement de l'Information en matière de Recherche dans le domaine de la Santé on 12 July 2016 (reference number 16.504); and by the Commission Nationale Informatique et Libertés on 16 December 2016 (reference number MMS/ABD/AR1612278, decision number DR-2016–488). This trial was authorised by the Agence Nationale de Sécurité du Médicament et des Produits de Santé on 20 July 2016 (reference 20160072000007). The ClinicalTrials.gov identifier is NCT02946346. All participants provided written informed consent.

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Data availability

Table 1, 2, 3, 4, Figures 1, 2, 3, Supplementary Tables S1, S2, S3, S4, S5, S6, S7, S8 and supplementary figures S1, S2, S3 have associated raw data. The data that support the findings of this study are available from the corresponding author upon request, and data are available in the Zenodo public repository (10.5281/zenodo.6913875).

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Table 1: **Key characteristics of participants included in the study.** n indicates the number of individuals, p-value refers to the outcome of a Kruskal-Wallis Rank Sum Test (`kruskal.test` function in R), IQR is the interquartile range, SD the standard deviation.

	Tampon	Menstrual cup	p
n	107	31	
PAPCLEAR follow-up duration (days, median [IQR])	220.00 [116.50, 374.50]	270.00 [74.00, 437.50]	0.740
Lifetime number of partners (median [IQR])	8.00 [4.00, 15.00]	11.00 [4. 00, 16.50]	0.396
Age (years, median [IQR])	21.00 [20.00, 23.00]	23.00 [22.00, 24.00]	0 .021
Age at menarchy (years, median [IQR])	13.00 [12.00, 14.00]	13.00 [12.00, 14.00]	0.695
Age at sexual debut (years, median [IQR])	17 [15.00, 18.00]	16 [15.00, 17.00]	0.338
Body Mass Index (BMI, median [IQR])	21.15 [19.78, 23.40]	21.62 [20.56, 23.22]	0.483
Smoking (%)			0.129
No	64 (59.8)	24 (77.4)	
Occasionally	15 (14.0)	4 (12.9)	
Often	28 (26.2)	3 (9.7)	
Antibiotics (last two weeks) = Yes (%)	7 (6.5)	3 (9.7)	0.842
Lubricant (last two weeks) (%)	14 (13.1)	5 (16.1)	0.891
Intercourse with a regular partner (last two weeks) (%)	63 (58.9)	18 (58.1)	1.000
Intercourse with an occasional partner (last two weeks) (%)	15 (14.0)	4 (12.9)	1.000
Stress level (past week) (%)			0.168
0 (Min)	20 (18.7)	5 (16.1)	
1	45 (42.1)	13 (41.9)	
2	30 (28.0)	13 (41.9)	
3 (Max)	12 (11.2)	0 (0.0)	
Menses (last two weeks) (%) (Yes)	58 (54.2)	20 (64.5)	0.416
HPV vaccinated (%)	55 (51.4)	19 (61.3)	0.443
HPV positive (focal) (%)	49 (45.8)	10 (32.3)	0.256
HPV positive (multiple HPVs) (%)	33 (30.8)	9 (29.0)	1.000

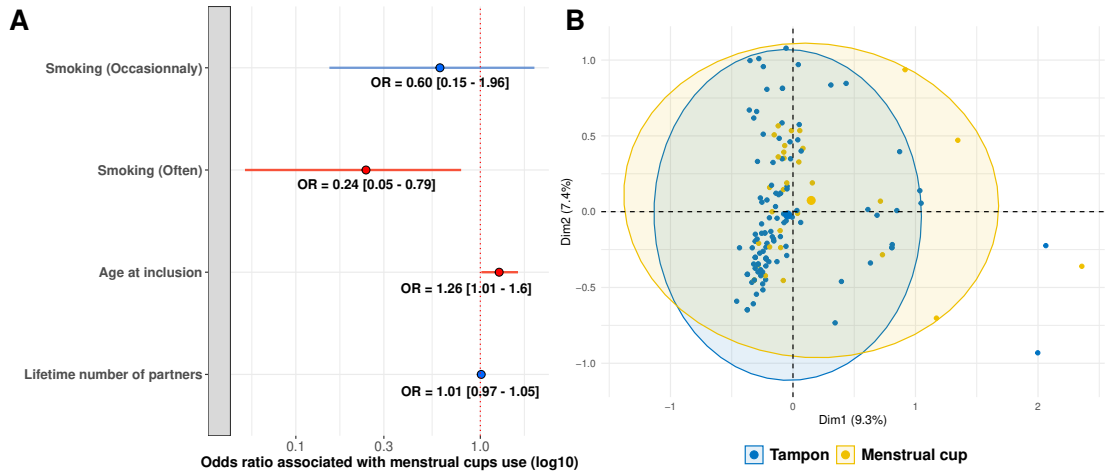

Figure 1: **Profile of menstrual cup users** A) Odds ratio of covariates associated with the use of menstrual cups. Results show the odds ratio (OR) of the factors selected in the best generalised linear model against menstrual cup as the response variable and using an Akaike Information Criterion corrected for small sample size (AICc). All covariates included in the model selection process are listed in Table S3. 95% confidence intervals are shown in brackets. B) Multiple Correspondence Analysis of socio-economic variables clustering participant using tampons or menstrual cups. Ellipses indicate 95% confidence intervals. Blue indicates tampon users, whereas yellow indicates women using menstrual cup.

Table 2: **Values of the main health metrics included in the study.** We show the number of occurrences and associated percentages (in parentheses) for each metric in the two study populations of size n. 'p-value' indicates the outcome of a t-test.

	Tampon	Menstrual cup	p-value
n	107	31	
Fungal genital infection (last 3 months) (%)	7 (6.5)	7 (22.6)	0.023
Genital infection (last 3 months) (%)	5 (4.7)	1 (3.2)	1.000
Chlamydia infection (last 3 months)(%)	4 (3.7)	1 (3.2)	1.000
Vaginosis (last 3 months)(%)	1 (0.9)	2 (6.5)	0.248
Urinary tract infection (last 3 months)(%)	12 (11.2)	6 (19.4)	0.378
HPV infection (focal) (%)	49 (45.8)	10 (32.3)	0.256

Table 3: **Factors associated with the reporting of a fungal genital infection.** Results show the odds ratios (OR) of the factors selected in the best generalised linear model with fungal genital infection as the response variable and using an Akaike Information Criterion corrected for small sample size (AICc). All covariates included in the model selection process are listed in Table 4. SE stands for standard 'error'. CI 2.5% and CI 97.5% indicate the lower and upper bounds of the 95% confidence interval.

Response = Fungal genital infection	OR	OR SE	CI 2.5%	CI 97.5%	p.value	
Menstrual cup	4.731	2.857	1.440	16.058	0.010	*
Lifetime number of partners	0.998	0.027	0.938	1.047	0.952	

* : $p < 0.05$

Table 4: **Frequency of occurrence of the covariates among the 80 best GLM models with fungal genital infection as a response variable (Model 2).** The models were selected using AICc, assuming an AICc + 5 cutoff. The columns indicate the number and percentage of models that contain each covariate, as well as the percentage of models where the associated p-value is marginally significant (lower than 10%) or significant (lower than 5%).

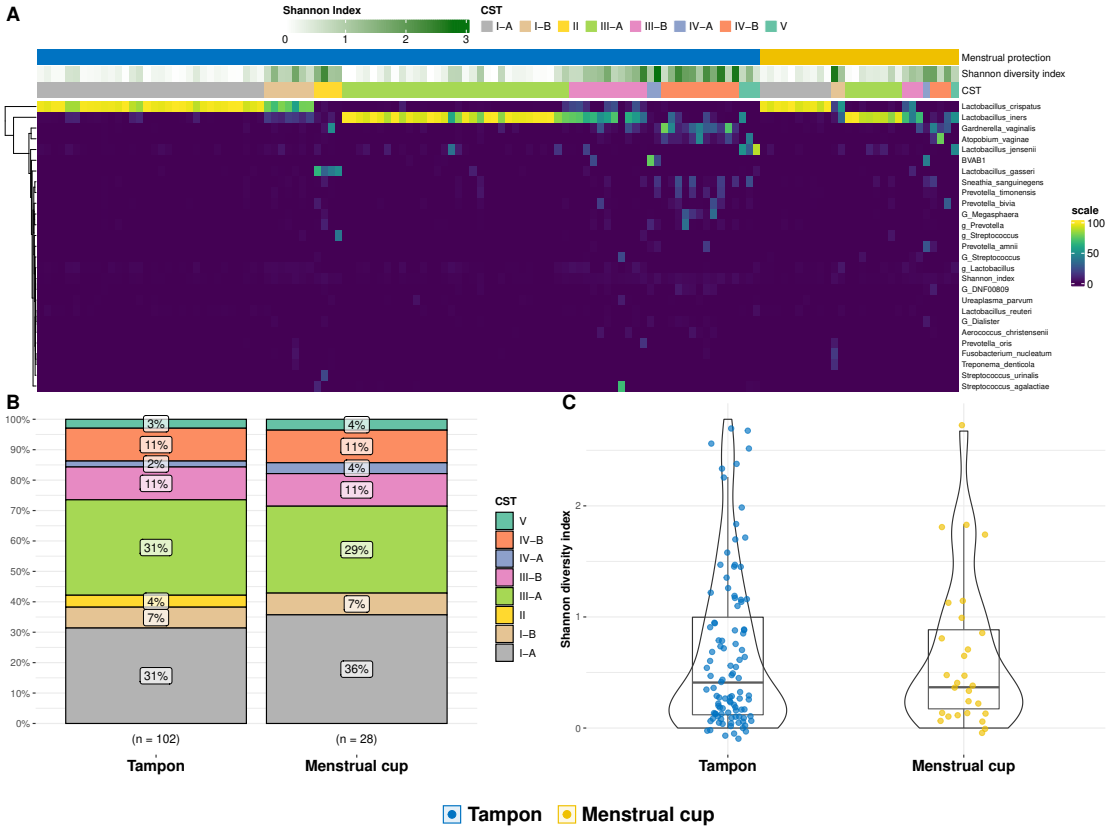

Figure 2: **Metabarcoding differences between women using mainly menstrual cups or tampons.** A) Abundance and diversity of the top 1% most abundant bacterial species found in participants, B) Community State Types (CST) distribution, C) Shannon diversity index. In A and B, colours show the type of menstrual product used (tampons in blue and cups in yellow).

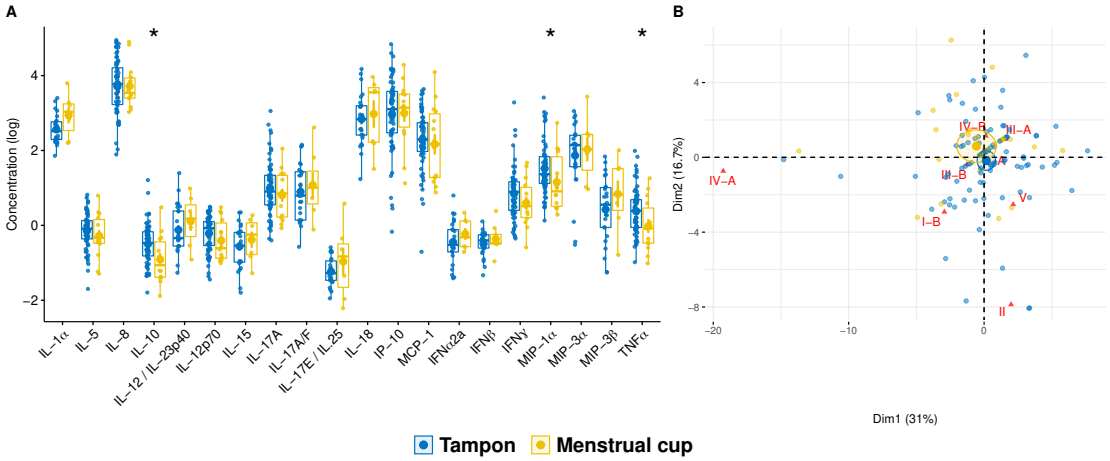

Figure 3: **Immunological differences between women using mainly menstrual cups or tampons.** A) Cytokines local concentrations (on a log scale), and B) Outcome of a multi-parametric clustering analysis using factor analysis of mixed data. In A, the presence of a star indicates significance ($p < 0.05$). Ellipses indicate 95% confidence intervals. In A and B, colours show the type of menstrual product used (tampons in blue and cups in yellow). In B, the vaginal Community State Types (CSTs) are shown in red.