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Full length article

The urinary, vaginal and gut microbiota in women with genital lichen sclerosus – A case-control study

Sofie Nygaard [a,b,1], Katrine Gerlif [a,b,1], Caspar Bundgaard-Nielsen [a,b], Jean Saleh Media [d], Peter Leutscher [a,b,c], Suzette Sørensen [a,b,c], Annemarie Brusen Villadsen [a,b], Louise Thomsen Schmidt Arenholt [a,b,e,*]

[a] Centre for Clinical Research, North Denmark Regional Hospital, Hjoerring, Denmark
[b] Department of Clinical Medicine, Aalborg University, Aalborg, Denmark
[c] Steno Diabetes Center North Denmark, Aalborg, Denmark
[d] Department of Obstetrics and Gynecology, Aalborg University Hospital, Aalborg, Denmark
[e] Department of Obstetrics and Gynecology, North Denmark Regional Hospital, Hjoerring, Denmark

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ABSTRACT

Background: Lichen sclerosus (LS) is a chronic, autoimmune skin disease predominantly located in the anogenital region in women. In recent years, the role of the human microbiota in the pathogenesis of autoimmune diseases, including LS, has received interest.
Objectives: The study aimed to evaluate and compare the composition of the urinary, vaginal and gut microbiota in women with LS versus non-affected controls.
Study design: Women diagnosed with LS (n = 16) and matched controls (n = 14) were enrolled in the study. From each participant, midstream urine, upper and lower vaginal swabs, as well as faecal samples, were collected. The microbiota composition was assessed using 16S ribosomal RNA (rRNA) gene sequencing of the V4 hypervariable region.
Results: We observed no LS-specific clustering in either of the four anatomic niches, using either hierarchical cluster analysis or weighted beta diversity metrics. However, for unweighted UniFrac, significant differences in the urinary and lower vaginal microbiota were observed when comparing women with LS to controls. These findings indicate that while the two groups have microbiota dominated by the same bacteria, variations do occur amongst less abundant bacteria. The LEfSe analysis revealed a higher relative abundance of the genus *Streptococcus* in the urinary and lower vaginal microbiota in women with LS compared to controls. Additionally, a higher relative abundance of phylum Euryarchaeota was observed in the gut microbiota in women with LS compared to controls.
Conclusion: In this study, we demonstrated several differences amongst less abundant bacteria in the urinary, lower vaginal and faecal microbiota when comparing women with LS to controls. However, further research is required to assess whether these microbiota differences are causative or merely a result of the underlying LS disease.

Introduction

Lichen sclerosus (LS) is a chronic, inflammatory skin disease predominantly located in the anogenital region [1,2]. The disease is more common in women and the incidence is increasing [3].

LS is characterized by anogenital irritation and pain, sexual difficulties, as well as histological and anatomical changes to the vulvar region. However, some cases are asymptomatic [4].

Although the cause of LS is unknown [4], an autoimmune aetiology has been suggested as patients with LS have detectable autoantibodies in

Abbreviations: ASV, Amplicon sequence variants; LEfSe, Linear discriminant analysis-effect size; LS, Lichen Sclerosus; MSU, Midstream urine sample; PCoA, Principal coordinate analyses; PERMANOVA, Permutational multivariate analysis of variancer; RNA, Ribosomal Ribonucleic Acid; VAS, Visual analogue scale.

* Corresponding author at: Center for Clinical Research, Bispensgade 37, 9800 Hjoerring, Denmark.
E-mail address: ltsa@rn.dk (L. Thomsen Schmidt Arenholt).

[1] Shared first authorship.

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their sera and are more frequently diagnosed with other autoimmune disorders [5].

Recently, the relationship between the composition of the human microbiota and the development of autoimmune disorders has received increased focus [6]. An imbalance in the microbiota composition (dysbiosis) may lead to perturbed immune homeostasis thus resulting in disease manifestation [7]. A coherence between dysbiosis of the gut microbiota and the development of autoimmune diseases, such as arthritis, psoriasis and multiple sclerosis is well known [8]. However, the urogenital microbiota is less adequately investigated but is likely to be equally important, especially in the development of LS. The urinary and vaginal niches are closely related, and it is known that disturbances in the vaginal microbiota can alter the urinary microbiota [9].

Therefore, we aimed at investigating the composition of the urinary, vaginal and gut microbiota in women with LS to study if a possible variation in microbiota occurs between women with LS and controls.

Materials and methods

Study participants

This study was a pilot study aiming to include 20 women with genital LS and 20 women without. The women were recruited at gynaecological outpatient clinics at the North Denmark Regional Hospital and Aalborg University Hospital. The study was conducted according to the principles expressed in the Declaration of Helsinki, approved by the North Denmark Region Committee on Health Research Ethics (N-20190060). Informed consent was obtained from all women.

To be included the women with LS needed to have objective signs of LS together with symptoms. These were evaluated using a questionnaire measuring the frequency and bother of vulvar symptoms. Age-matched controls consisted of women referred to the clinics due to other general gynaecological symptoms such as irregular bleeding or abdominal pain. Exclusion criteria for both groups included age under 18 years, current pregnancy, symptoms of infection in the bladder, vagina or uterus, suspicion of vulvar dysplasia/cancer or treatment with antibiotics and/or vaginally applicated medicine, such as topical steroids, within the last three months. For the women without LS known chronic disease in the vagina or vulva was also considered an exclusion criterion.

Questionnaire

To assess the frequency and bother of vulvar symptoms, a modified version of a clinical scoring system [10] was used. Four items were chosen: burning sensation, pruritus, pain and dyspareunia. Frequency of "rare" and "never" was merged. Bother was measured using a visual analogue scale (VAS) with "0" being no bother (supplementary material 1).

Gynaecological examination

A gynaecological examination including a thorough inspection of the anogenital region was performed by an experienced physician. One or more objective signs should be present: atrophy, white hyperkeratosis, fissures and/or vulvar anatomical changes. Vulvar biopsy was not performed since this is not a standard diagnostic procedure according to the national guideline. Hence, the lack of objective signs precludes LS.

Collection of samples

During the gynaecological examination, two vaginal samples were obtained by a swab (FLOQSwabs, Copan) from the posterior vaginal fornix and the lower 5 cm of the posterior vaginal wall. The women collected a midstream urine sample (MSU) after cleaning the genital area. Vaginal and urine samples were placed in sterile collection tubes with no medium and immediately stored at −80 °C until further analysis. Lastly, each participant received a stool kit and instructions for sample collection at home. The faecal samples were stored in sterile collection tubes and placed in a domestic freezer at −20 °C in the women's homes until it was collected within one week. The samples were then transported to the laboratory in cooling bags with ice and immediately stored at −80 °C until further analysis.

DNA extraction and 16S rRNA gene sequencing

The procedures for DNA extraction and 16S rRNA gene sequencing used in this study, are described briefly. For more information, see supplementary (supplementary material 2). Briefly, DNA was extracted from 10 mL urine, vaginal swabs and 200 mg ± 50 mg faecal samples. Location controls were included for urine and vaginal samples using sterile water instead of urine or a clean swab. A reagent control was included by performing the DNA extraction on nuclease-free water.

The resulting DNA was analysed by 16S rRNA gene sequencing targeting the V4 region, utilizing the primers 515F [11]/806R [12]. The resulting reads were investigated using the Usearch V11 pipeline for preprocessing, and QIIME2 v. 2020.8 [13] for error correction, amplicon sequence variants (ASVs) clustering and assignment of taxonomy.

Statistical analyses

Data analysis was performed using R version 4.0.3 through the Rstudio IDE. 16S rRNA data were analysed using the ampvis2 package v.2.6.6 and phyloseq v1.32.0. Alpha diversity was determined using ASV richness, Shannon diversity index, and Pielou's evenness index. Beta diversity was evaluated using principal coordinate analyses (PCoA), based on Bray-Curtis dissimilarity, weighted, and unweighted UniFrac, as well as through dendrograms and heatmaps. For continuous data, distribution and variance were assessed using Shapiro-Wilks test and Bartlett's test, respectively. Parametric data were expressed by a mean value and compared using two-sample *t*-test. Non-parametric data were expressed by a median value and compared using the Wilcoxon-Mann-Whitney *U* test or Kruskall-Wallis test with Dunns Post-Hoc test and Benjamini-Hochberg's procedure to adjust for false-discovery rate. Differences in beta diversity were evaluated using permutational multivariate analysis of variance (PERMANOVA), while the linear discriminant analysis-effect size (LEfSe) method was used to identify the bacteria most likely to describe the difference between LS and controls. A p-value < 0.05 or multiple hypothesis-corrected p-value < 0.01 was considered significant.

Results

In total, 16 women with LS and 14 controls were included in the study. There were no significant differences in age, BMI, parity, smoking status, or previous vaginal surgeries between the two groups (Table 1).

We found a significant difference in frequency of vulvar pruritus, burning sensations, and pain in the anogenital region between the two groups. The VAS scores related to the symptoms were found to be significantly different as well (supplementary material 3).

Several bacterial genera and families of the urinary microbiota are more abundant in women with LS

No significant difference in ASV richness (Fig. 1C, $p = 0.956$), Shannon Diversity Index (Fig. 1G, $p = 0.993$) or Pielou's evenness (Fig. 1K, $p = 0.861$) were observed between the groups.

Conversely, the bacterial composition of urinary samples differed between women with and without LS for unweighted UniFrac (Fig. 2J, $p = 0.032$).

Hierarchical cluster analysis did not reveal any LS-specific clustering (Fig. 3C). This indicated that the two groups of women had a urinary microbiota dominated by the same bacterial genera. The most abundant

Table 1
Demographic of included study participants.

Participants N = 30 (100%)	LS N = 16	Non-affected controls N = 14	Statistical significance p-value
Age (years) ***Mean (SD)***	46.6 (14.6)	41.6 (12.3)	p = 0.33
BMI (kg/m^2) ***Median***	27.9	25.2	p = 0.30
Smoking status ***N(%)***			
Never	6 (37.5%)	7 (50.0%)	p = 0.76
Previously	9 (56.3%)	6 (42.9%)	
Currently	1 (6.3%)	1 (7.1%)	
Vaginal births ***Median***	2.0	2.0	p = 0.83
Caesarean section ***Median***	0.0	0.0	p = 0.79
Previous vaginal surgery **N(%)**	1 (6.3%)	3 (21.4%)	p = 0.32

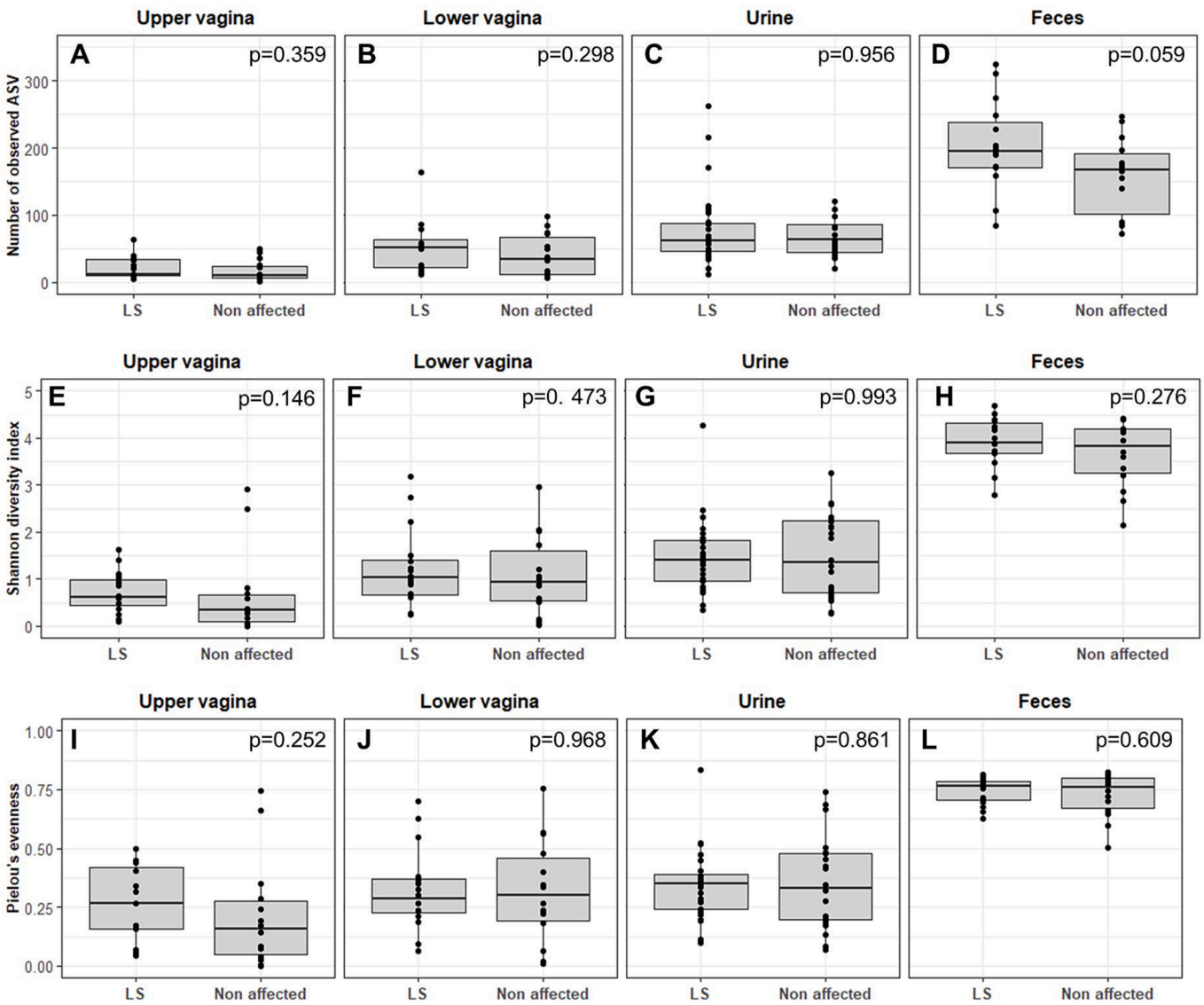

Fig. 1. Alpha diversity, depicting the within-sample diversity, as indicated by ASV richness A) upper vagina, B) lower vagina, C) urine D) faeces, Shannon Diversity Index E) upper vagina, F) lower vagina, G) urine, H) faeces and Pielou's evenness index I) upper vagina, J) lower vagina, K) urine, I) faeces. Statistics were tested using either Mann-Whitney *U* test or students *t* test.

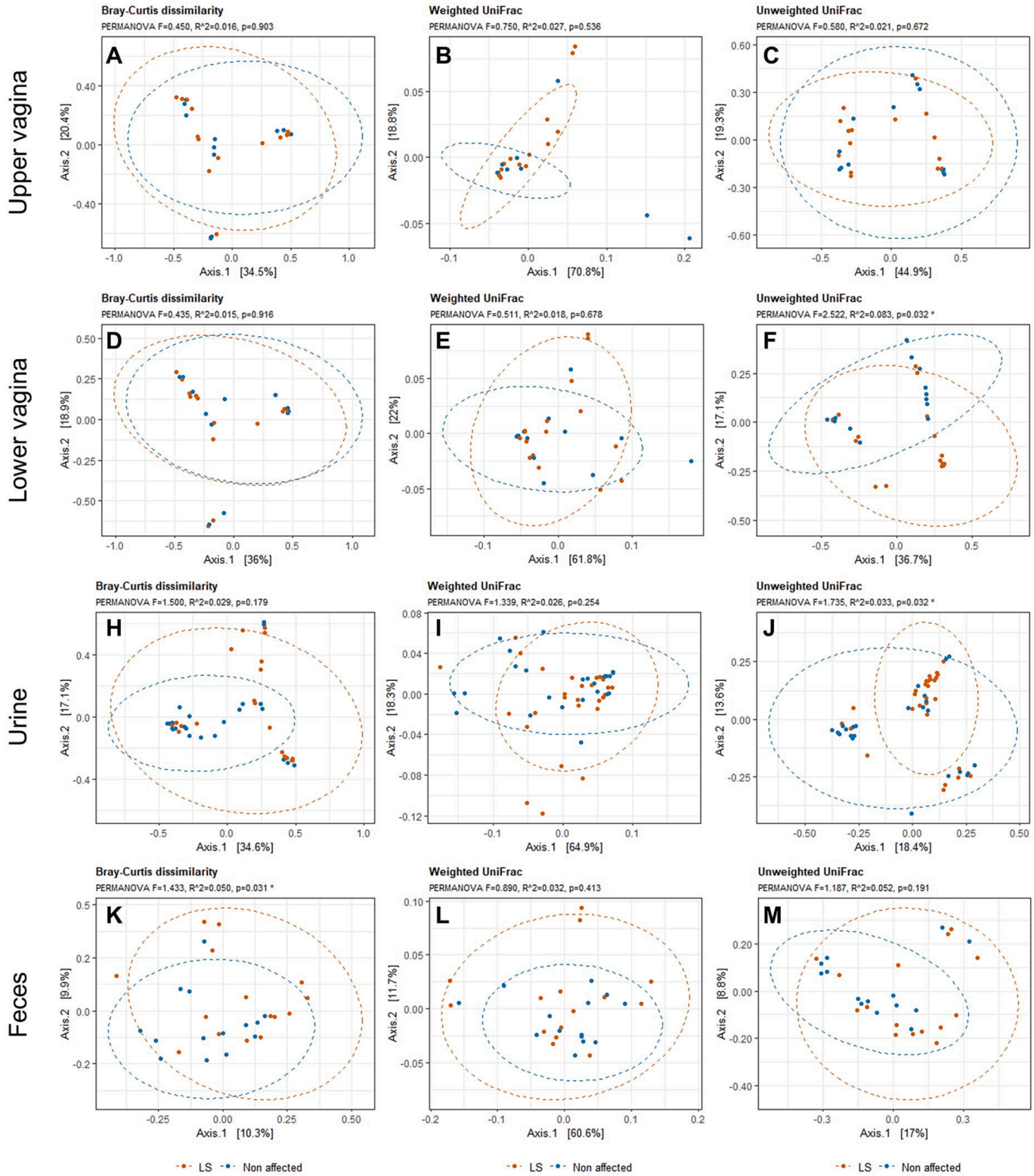

Fig. 2. Beta diversity of women with LS and controls presented using Principal Coordinate Analysis, with either PCoA of Bray-Curtis dissimilarities (A, D, H and K), weighted UniFrac (B, E, I and L) or unweighted UniFrac (C, F, J and M). Microbiota originating from the upper vagina (A, B and C), lower vagina (D, E and F), urine (H, I and J) and faeces (K, L and M) were compared between women with LS and controls, respectively. Ellipses depict 95 confidence intervals. PERMANOVA results are indicated with adjusted p values and represent overall differences in beta diversity.

bacterial genera overlapped across the two groups as expressed by the heatmaps. For both groups, *Lactobacillus, Gardnerella* and *Prevotella* were the most abundant genera (Fig. 3C).

Using LEfSe analysis (Fig. 4C) several highly diverse bacterial genera and families were significantly more abundant in women with LS compared to controls, including the order Bifidobacteriales, the families

Fig. 3. Dendrograms, depicting clusters of women with LS and controls based upon their microbiota composition. A) upper vagina, B) lower vagina, C) urine and D) faeces. Heatmaps of the 20 most abundant bacterial genera in women with LS and controls. In the columns, orange indicates a higher relative abundance of the bacteria, while blue indicates a lower relative abundance of the bacteria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Streptococcaceae, Bifidobacteriaceae and *Alcanivoracaceae* as well as the genus *Streptococcus*. Conversely, the family *Hungateiclostridiaceae*, the genus *Fastidiosipila* and several members of the phylum Synergistota were more abundant in the control group.

The microbiota of the lower, but not upper, vagina in women with LS differs from that of non-affected controls

Assessing the alpha diversity of upper and lower vaginal swabs (Fig. 1A, B, E, F, I and J), no significant differences were observed for ASV richness (Fig. 1A, $p = 0.359$ upper vagina, Fig. 1B, $p = 0.298$ lower vagina), Shannon Diversity Index (Fig. 1E, $p = 0.146$ upper vagina, Fig. 1F, $p = 0.473$ lower vagina) or Pielou's evenness (Fig. 1I, $p = 0.252$ upper vagina, Fig. 1J, $p = 0.968$ lower vagina), between women with LS and controls.

The microbiota from the lower vagina in women with LS clustered significantly differently from controls in PCoA, when using unweighted but not weighted UniFrac, indicating that variations occurred amongst less abundant bacteria (Fig. 2F, $p = 0.032$).

Using hierarchical cluster analysis, no obvious clustering in women with LS and controls was observed (Fig. 3A and B). In the microbiota from the upper and lower vagina, the most abundant bacterial genera overlapped across the two groups as expressed by the heatmap (Fig. 3A and B). For both groups, *Lactobacillus, Gardnerella* and *Atopobium* were the dominant genera in the upper vagina (Fig. 3A), as opposed to *Lactobacillus, Gardnerella* and *Bifidobacterium* in the lower vagina (Fig. 3B).

LEfSe analysis revealed no significant differences between the microbiota from the upper vagina in women with LS and controls (Fig. 4A). However, several differences in the microbiota of the lower vagina between the two groups were observed (Fig. 4B). The relative abundance of the order Pseudomonadales, the families *Pseudomonadaceae* and *Streptococcaceae* and the genera *Pseudomonas* and *Streptococcus,* as well as the genus *Ezakiella,* were higher in women with LS compared to controls.

The gut microbiota in women with LS is associated with a higher bacterial richness, as well as a higher abundance of diverse bacterial genera and families

No significant difference in faecal ASV richness between the groups was found, however, a tendency was observed for the women with LS to have a significantly higher ASV richness compared to controls (Fig. 1D, $p = 0.059$). Additionally, no significant differences were observed for the Shannon Diversity Index (Fig. 1H, $p = 0.276$) or Pielou's evenness (Fig. 1L, $p = 0.609$), between women with LS and controls.

Assessing the beta diversity using PCoA an overall difference was observed for Bray Curtis when comparing the gut microbiota in women

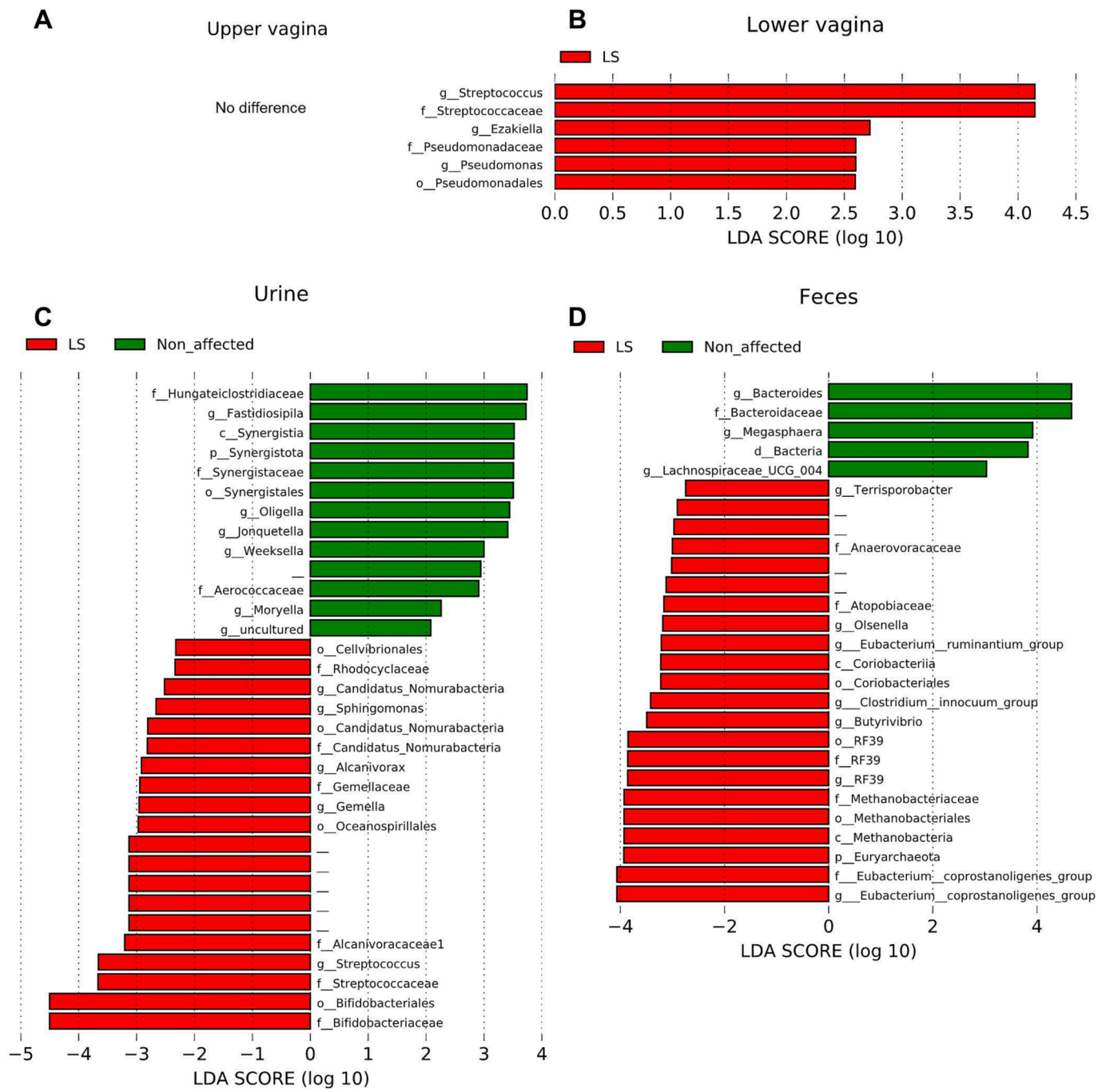

Fig. 4. LEfSe analysis, depicting differences in the microbiota composition between the women with LS and controls (LDA score > 2.0, $p < 0.05$). The results are presented using a-d) barplots, representing the LDA score of each genus. Microbiota originating from the upper vagina (A), lower vagina (B), urine (C) and faeces (D) were compared between women with LS and controls, respectively.

with LS to controls (Fig. 2K, $p = 0.031$).

Using hierarchical cluster analysis, no obvious clustering was observed (Fig. 3D). For both groups, *Bacteroides*, *Prevotella* and *Faecalibacterium* were the dominant genera (Fig. 3D).

LEfSe analysis revealed several differences between the gut microbiota in women with LS and controls (Fig. 4D). The genus *Eubacterium-coprostanoligenes*, the phylum Euryarchaeota as well as several members of the order Methanobacteriales were found to have a significantly higher relative abundance in women with LS compared to controls. Conversely, the family *Bacteroidaceae* and the genera *Bacteroides* and *Megasphaera* had an increased relative abundance in the control group.

Discussion

In our study, we found no LS-specific microbiota in urine, lower or upper vagina, or gut compared to controls. However, we found variations amongst less abundant bacteria in the urinary, lower vaginal and faecal microbiota when comparing women with LS to controls.

In both the urinary and lower vaginal microbiota, we demonstrated a significant difference in the relative abundance of *Streptococcus* in women with LS compared to controls. An increased relative abundance of *Streptococcus* may be a part of the autoimmune etiology of LS. This is supported by Feito-Rodrigues *et al.* [14] and Powell *et al.* [15] who both described *Streptococcus* to be one of the most common infective

organisms in girls with LS. A study by Ganju *et al.* [16] observed an increased relative abundance of *Streptococcus* in skin lesions of the autoimmune skin disorder vitiligo compared to unaffected skin, thus suggesting, in combination with our results, a possible association between increased *Streptococcus* abundance and autoimmune skin diseases.

An association between dysbiosis of the gut microbiota and the pathophysiology of inflammatory skin disorders has been proposed by Salem *et al.* [17]. In our study, we found a significant difference in the relative abundance of *Eubacterium-coprostanoligenes* and *Euryarchaeota* in the gut microbiota, in women with LS when compared to controls. The study by Picchianti-Diamanti *et al.* [18] observed a direct correlation between the presence of *Euryarchaeota* in the gut microbiota and disease activity in patients with rheumatoid arthritis. Furthermore, an increased relative abundance of *Euryarchaeota* is also reported in the gut microbiota of patients with multiple sclerosis [19]. As both diseases display autoimmune pathogenesis and are associated with LS [20] it is reasonable to suggest an inflammatory role of *Euryarchaeota*, thus providing a possible explanation for the increased presence of the phylum in the gut microbiota of women with LS. However, more studies are needed to investigate such an association.

In general, most autoimmune disorders display a decrease in diversity when investigating gut microbiota. A study by Chen *et al.* [21] described a decreased gut microbial diversity in patients with rheumatoid arthritis compared to controls. Studies by Abrahamsson *et al.* [22,23] also observed low intestinal microbial diversity in patients with infantile asthma and atopic eczema, respectively. This is, however, in contrast to our observations.

Few other studies have also found differences in the microbiota between patients with LS and controls. Watchorn *et al.* [24] observed a higher relative abundance of *Fusobacterium* in both the balanopreputial sac and urine in male genital LS patients compared to controls, thus indicating an association between dysbiosis and LS. The study by Chattopadhyay *et al.* [25] found significant differences in the cutaneous microbiota between the labia majora and minora, perineum as well as the gut microbiota in girls with LS compared to controls.

This study has several strengths. To the best of our knowledge, this is the first study to investigate both the urinary, vaginal, and gut microbiota in women with LS, allowing greater knowledge of the microbiota in LS. Additionally, collection and handling of samples was managed identically for each participant to avoid any confounding variables affecting the bacterial composition.

However, some limitations need to be addressed as well. First, the study had a small sample size, limiting the generalizability of the results. Second, we did not collect data regarding intercourse, which could act as a possible confounder as Price *et al.* [26] observed an association between sexual activity and increased variability in both the urinary and vaginal microbiota. The same applies to menopause status, hormonal contraception as well as menstrual cycle [27], which according to Ammitzbøll *et al.* [28] and Fosch *et al.* [29] affect the urinary as well as vaginal microbiota, respectively. Additionally, we did not measure if the participants had detectable autoantibodies in their sera. This could potentially have strengthened our theory that the microbiome does have an impact in the development of LS.

Furthermore, the inclusion of vulvar biopsies from the women with LS could have confirmed the diagnosis. Finally, 16S rRNA gene sequencing has its limitations as it lacks accuracy for species identification compared to whole-genome sequencing [30].

Conclusion

In conclusion, we demonstrated several differences amongst less abundant bacteria in the urinary, lower vaginal and faecal microbiota when comparing women with LS to controls. These findings suggest that the bacterial composition of the urinary, vaginal and faecal microbiota may play a role in the aetiopathogenesis of genital LS. However, due to the small sample size, it is not possible to draw any definitive conclusions and further research is required to assess whether these microbiota differences are causative or merely a result of the underlying LS disease.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejogrb.2023.08.004.

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