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Original Research

Insight Into the Complex Dynamics of Diverse Endometrial Microbial Communities in Dairy Cows During the Transition Period: Normal Condition Versus Pathology

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Abstract

Background: Dysbacteriosis of the reproductive tract is a risk factor for the development of postpartum endometritis in cows. Understanding the complex dynamics of diverse endometrial microbial communities in dairy cows during the transition period, when healthy and compared to with a pathology, is crucial for developing the appropriate measures to control postpartum endometritis depending on the livestock farming system used. The aim of this study was to analyse the endometrial microbiome changes in cows during the transition period in two separate experiments on an industrial (IF) and an organic farm, or eco-farm (EF). **Methods:** Samples were taken on Days 20 (D−20) and 10 (D−10) before calving, at calving (D0), and on Days 3 (D+3), 5 (D+5) and 20 (D+20) postpartum. On IF, the samples were divided into three groups: healthy (IF1H), subclinical (IF2S) and purulent-catarrhal endometritis (IF3E) animals. To investigate the abundance of microbial operational taxonomic units (OTUs) and dynamics of endometrial microbiota composition, quantitative real–time PCR was used. The obtained macroecological time series data was additionally analyzed using the Hurst exponent approach. **Results:** In Group IF0, one of the total bacterial count peaks was observed on D0, reaching an average of 5.0 × 106 DNA copies/mL, which was 100-fold higher than on D–20 ($p < 0.001$) and 38.4-fold higher than on D–10 ($p < 0.01$). Abundance of *Fusobacterium*, *Sneathia* and *Leptotrichia* were 125 times higher in Group IF3E compared to Group IF1H. On IF, a high concentration of *Aspergillus* was also observed. On EF, these micromycetes were present only at the time of calving. Variability in the number of micromycetes *Aspergillus* and *Fusarium* spp. differed depending on the state of the reproductive system. The Hurst exponent-based analysis suggested a somewhat more persistent tendency in the short-time shifts observed in endometrial microbial communities in cows from EF. **Conclusions:** During the transition period, the endometrial microbiome on both farms underwent macroecological changes associated with the state of reproductive health and temporal fluctuations in microbiota abundances. The results of the study can be used to develop strategies for the prevention and treatment of postpartum endometritis in cows.

Keywords: endometrial microbiota dynamics; dairy cows; transition period; endometritis; lactobacilli; micromycetes; quantitative real–time PCR (qRT-PCR); microbiota imbalance

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1. Introduction

Postpartum reproductive system diseases, such as endometritis, are one of the main causes of decreased reproductive function, milk production and productive longevity of cows (*Bos taurus*), causing significant economic losses to dairy farming [1]. Postpartum endometritis etiology is multifactorial and includes both physiological changes occurring in the cow's body and the impact of environmental and herd maintenance factors [2]. The transition period, covering the time from three weeks before calving to three weeks after it, is a critical stage in the dairy cow production [3]. During this time, an animal undergoes significant physiological changes associated with fetal growth, preparation for lactation and postpartum recovery [4]. These changes, including metabolic/inflammatory imbalances, hormonal shifts and immunosuppression, make cows more susceptible to reproductive tract infections [5].

The role of microbiota in maintaining animal health and reproductive function is important [6]. The bovine reproductive tract microbiota is a complex microbial community and a dynamic ecosystem, including such taxa as Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria and Tenericutes [7]. This microbiome plays a pivotal role in supporting reproductive homeostasis, immunomodulation, metabolism and defense against pathogens [8]. Imbalance of the reproductive tract microbiota, or dysbiosis, can lead to the development of various reproductive diseases, including postpartum endometritis in cattle and endometriosis in humans [9,10]. Changes in the endometrial microbiota composition and function can influence the uterine immune response, promote adhesion and colonization of pathogenic bacteria and impair postpartum endometrial recovery [10]. Therefore, understanding the mechanisms of interactions between the microbiota and the cow's body is an important step in developing effective strategies for the prevention and treatment of postpartum diseases. Hereby, researchers have recently turned their focus to the uterine and vaginal healthy microbiomes in an effort to emphasize prevention over treatment [1].

The influence of various factors on the ruminant reproductive tract microbiota composition and function is actively explored using metagenomic and functional genomic approaches [11]. These factors include physiological state [12], species [7], health status and antibiotic use [13]. However, most bovine reproductive tract microbiota studies focus on animals kept in industrial farms (IFs) [14]. Pronounced dysbiotic changes in the microbiota in industrial conditions are associated with antibiotics use, high stress levels and other unfavorable factors [7]. On the other hand, organic livestock farming implies the refusal to use synthetic chemicals, antibiotics and hormones, as well as providing animals with the opportunity to roam freely and have access to pastures [15]. To date, there have been virtually no studies on the uterine microbiota of cows kept on organic farms, or eco-farms (EFs) [16]. The importance of tracing differences in the endometrial microbiome depending on farming practices is a vital area of livestock reproduction research. It has previously been shown [17,18] that differences in animal reproductive health are determined primarily by genetic factors and diet. However, comparative studies of the endometrial microbiome can demonstrate how farming practices (industrial vs. organic livestock farming) influence the endometrial microbiome and, consequently, the reproductive health of cows.

Complex system modeling to comprehend the crucial factors influencing bacteriotherapy response is one of the challenges facing microbiome research [19]. Previously, Ji et al. [20] employed the Hurst exponent [21] as an auxiliary metric in describing the macroecological dynamics of human and mouse gut microbiota. The Hurst exponent is used to identify the trendiness (persistence) of changes in biology, medicine and other areas [22,23,24,25,26,27,28,29,30]. Ji et al. [20] used macroecological quantitative relationships, such as the Hurst exponent [21] and time series data [31,32], to understand the temporal fluctuations in short- and long-term changes in gut microbiota abundances. Utilizing time series data in metagenomics analysis to its fullest potential is made possible by specialized analytic tools, the Hurst exponent among them. Based on data from Ji et al. [20], the Hurst exponent enables to identify characteristic patterns in microbiome dynamics, allowing to classify them as stable processes. The study by Ji et al. [20] established that the gut microbiota obeys universal laws of macroecology, which brings it closer to other ecosystems, and demonstrated that the Hurst exponent is a useful tool for analyzing microbiome dynamics. Such tools might quantify abnormalities that make community behavior unpredictable, or they can help create prediction models or uncover periodic patterns. Multiple stable states or changing environments might cause microbial populations to shift suddenly in response to minor disturbances [31]. When modelling complex microbial ecosystems, fractal approaches can prove to be very informative [33].

Previously, we examined the endometrial and rectal microbiota in dairy cows by analyzing metagenomic composition and predicted metabolic pathway when introducing of a complex feed additive [34]. Also, we preliminarily performed a bioinformatic data analysis based on the metagenomic whole genome sequencing of endometrial microorganisms in cows with normal and pathological conditions [35] and compared the EF cows' endometrial microbiome in the early and late dry periods [36]. The aim of this study was to analyze the macroecological endometrial microbiome dynamics in cows during the transition period, considering the incidence of endometritis. Herewith, these dynamic processes were investigated on an IF characterized by high risks of postpartum endometritis and on an EF where low postpartum endometritis rates are observed. We acknowledge that selecting farms solely based on their

current endometritis risk status may introduce bias and distort the study results. Therefore, when selecting farms, we not only considered their current endometritis risk status but also analyzed data for the previous three years. Furthermore, in the current experiment, we attempted to standardize the study conditions, including endometritis diagnostic methods and animal selection criteria. We believe this helped reduce the influence of confounding factors.

2. Materials and Methods

2.1 Animals and Farms

In 2024, a study was conducted at two livestock farms in Leningrad Oblast and the Republic of Karelia to analyze the endometrial microbiome dynamics in dairy cows during the transition period. The following were selected as research sites: (1) SPK Polyany IF (Vyborg District, Leningrad Region), characterized by a high risk of developing postpartum endometritis, and (2) Valaam Monastery EF (Valaam Island, Sortavala District, Republic of Karelia) that has low postpartum endometritis rates. On IF, second-lactation Holstein cows kept on a tether were examined. Their body weight during the dry period varied within 580–600 kg and during the fresh period within 510–530 kg. Mean milk yield for 305 days of the previous lactation was 10,000–11,000 kg. Animal feeding and housing were provided following generally accepted zootechnical standards and recommendations [37]. Second-lactation Ayrshire cows were studied on EF. Their body weight was 600 kg during the dry period and 550 kg during the fresh period. Mean milk yield was 7930 kg in the previous lactation, fat content in milk was 4.5% and protein content was 3.1%. Their feeding and housing conditions conformed to the organic animal husbandry principles that implied no use of synthetic chemicals and drugs, loose housing, regular exercise, etc. [37,38,39]. It should be noted that our study does not claim to establish a direct cause-and-effect relationship between the farming management system and the endometrial microbiome due to the combined influence of a number of factors. Cows' diet composition in the dry and postpartum periods is presented in **Supplementary Table 1**.

2.2 Experimental Groups and Sampling

During the experiment, gynecological examination of cows was performed as described elsewhere [40,41,42]. Selection of animals into experimental groups was made on the basis of the criteria presented in Table 1.

Sampling of endometrial surface scrapings was conducted in the following periods: on Days 20 (D–20) and 10 (D–10) before calving, at calving (D0) and on Day 3 postpartum (D+3) in Stage I of the experiment, as well as on Days 5 (D+5) and 20 (D+20) postpartum in Stage II of the experiment. On IF in Stage II, sampling was performed separately in three groups: IF1H, clinically healthy postparturient cows without complications during and after calving, signs of postpartum endometritis, pathologies and factors that can affect the microbiota composition of the reproductive organs; IF2S, animals diagnosed with postpartum subclinical endometritis; and IF3E, animals with postpartum purulent-catarrhal endometritis. To divide the cows into groups throughout the entire experimental period, diagnostic procedures were performed in cows from each group of the industrial livestock farm and organic farm to analyze the health of their reproductive organs. For this, we used standard clinical diagnostic methods, i.e., external genital examination, thermometry, cervical palpation, colposcopy, endometrial cytology (polymorphonuclear neutrophil count), and ultrasound examination of the internal genital organs. Data were recorded in a veterinary log. The exact dates of disease detection and recovery were also recorded.

The sample sizes were relatively small, which may limit broad conclusions. However, this study was designed with the use of a traumatic sampling method for animals, which can provoke inflammatory diseases, postpartum complications, and even lethal outcomes. This forced us to minimize the number of replicates in the samples, as otherwise, doing so would have violated ethical requirements of animal research. The low number of replicates was partially offset by a relatively high number of sampling points over time. In this respect, this investigation should be considered as preliminary in nature, requiring caution in interpreting its results. The obtained data should be viewed as a pilot study output, and their confirmation requires further large-scale studies, which we plan to conduct in a future research cycle in this area.

The sampling procedure was conducted under strict aseptic conditions using a cytobrush [43]. To prevent contamination, the animal's tail was covered with a sterile gauze, and the perineal and vulval areas were thoroughly washed with soapy water and then treated with an antiseptic. The procedure for collecting endometrial scrapings before calving, when the risk of complications significantly increases, required special precautions. We developed the scraping procedure based on the principle of minimizing risk to animals and approved it by the ethics committee. The protocol underwent a thorough review by the Bioethical Commission of the L.K. Ernst Federal Research Center for Animal Husbandry (Protocol No. 2023-10/1, dated October 31, 2023), taking into account all aspects of animal safety and welfare. The procedure was based on the recommendations of leading veterinarians and scientists in the field of bovine reproductive medicine. Specifically, the procedure was performed under the strict supervision of a qualified specialist with many years of experience working with heavily pregnant animals. We organized training in advance for the personnel performing the procedure to ensure that all participants understood the importance of adhering to safety protocols and caring for the welfare of the cows. Each animal underwent a medical examination to rule out

Table 1. Experimental design for monitoring the reproductive system microbiomes and criteria for selecting cows in the experimental groups.

Experimental groups [1]	Livestock farming system	Sampling points [2]	Criteria for selection of cows into groups
Experiment stage I: period from 20 days before calving to 3 days postpartum			
Group IF0 (n = 7)	Industrial farm	D−20, D−10, D0 and D+3	Clinically healthy cows in the period from the beginning of the transit period to 3 days postpartum
Group EF0 (n = 20)	Eco-farm		
Experiment stage II: postpartum period			
Group IF1H (n = 5)	Industrial farm	D+5 and D+20	Clinically healthy postparturient cows
Group IF2S (n = 5)			Cows diagnosed with postpartum subclinical endometritis
Group IF3E (n = 5)			Cows diagnosed with postpartum purulent-catarrhal endometritis
Group EF1H (n = 5)	Eco-farm		Clinically healthy postparturient cows

[1] n, number of animals in a group. [2] D−20 and D−10, 20 and 10 days before calving; D0, on the calving day; D+3, D+5 and D+20, 3, 5 and 20 days postpartum.

possible contraindications. After the procedure, all animals were continuously monitored by a veterinarian to identify possible complications.

2.3 DNA Extraction and Microbial Community Analysis

The produced endometrial samples were immediately frozen at −20 °C and transported to the laboratory for further analysis. Total DNA was isolated from endometrial microbiota samples using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions.

The microbial community composition at the levels of total bacterial count (TNB) and operational taxonomic units (OTUs) was assessed by quantitative real–time PCR (qRT-PCR) using a detecting cycler DT Lite-4 624 (LLC NPO DNA-Technology, Moscow, Russia) and a reagent kit for qRT-PCR in the presence of the intercalating dye EVA Green (Syntol, Moscow, Russia). Amplification was performed according to the following protocol: initial denaturation at 95 °C for 3 min (1 cycle), 40 cycles including denaturation at 95 °C for 1 min, annealing at 57.6 °C for 1 min and elongation at 72 °C for 1 min, with a final elongation at 72 °C for 5 min (1 cycle). Primer sequences (5′–3′) [44,45,46,47,48,49,50,51,52,53,54] are presented in **Supplementary Table 2**. To confirm the specificity of the primers, a Basic Local Alignment Search Tool (BLAST; [55]) analysis was performed. The BLAST analysis results showed that the primers had a high degree of homology with the sequences of the micromycetes and bacteria analyzed in the study and did not exhibit significant homology with the sequences of other taxa that may be present in the endometrial microbiota of cows.

The selection of specific OTUs was based on a combination of factors. A number of the OTUs we selected have long been known to be widely represented in the microbiome of the reproductive system of mammals, including ruminants (e.g., [7]). Some of the selected OTUs are associated with the development of endometritis. For example, bacteria of the genus *Fusobacterium* are known pathogens associated with endometritis in cattle (e.g., [10]). Some OTUs (*Lactobacillus* spp.) are present in the reproductive tract of women, but are virtually absent in cows (e.g., [10]). Micromycetes are rarely analyzed in the reproductive tract of cows, but a number of factors indicate their possible presence there (e.g., [56]).

2.4 Statistical Analyses

Using Microsoft Excel XP/2003 (Version 14.0.4750.1000; Microsoft Corporation, Redmond, WA, USA) and RStudio (Version 1.1.453; Posit Software, PBC formerly RStudio, PBC, Boston, MA, USA), multivariate analysis of variance (MANOVA; [57]) was used to process the results statistically. The means (M) and standard errors of the mean (± SEM) were displayed as the results. Differences between groups were estimated using the Student's t-test (https://www.graphpad.com/quickcalcs/ttest1/?format=sem (Accessed: 26 July 2025)).

To analyze the macroecological dynamics of the endometrial microbiota in cows, we turned to the Hurst exponent [21]. The latter implies the computation of cumulative sums [21] and can be used for developing models and algorithms for fractal analysis of chaotic time series in real time [58]. The Hurst exponent for each microorganism was calculated using Eqn. 1:

$$H = \frac{log\left(\frac{R}{S}\right)}{log(a \times N)} \quad (1)$$

where H is Hurst coefficient; S is the standard deviation of the time series levels; R is the value of the accumulated

deviation; N is the number of observation periods; a is a given constant, i.e., a positive number that was empirically calculated for series for a short period of time and accepted here as 1.

Although Hurst empirically calculated the constant for relatively short-term time series of natural phenomena to be around 0.5, Eqn. 1 still tends to overestimate the H values. Therefore, for small time series, Nayman [23,24] modified the Eqn. 1 for a sample of random variables represented by a small number of observations and also proposed to use $a = \pi/2$ as follows:

$$H = \frac{log\left(\frac{R}{S}\right)}{log\left(\pi \times \frac{N}{2}\right)} \times (-0.0011 \times ln(N) + 1.0136) \quad (2)$$

Hurst coefficient values vary in the range from 0 to 1. The Hurst exponent value range $0 \leq H < 0.5$ is called 'pink noise' and mean that the time series has anti-persistent properties, for which a change in the previous direction is more likely. The H index value close to 0.5 is called 'white' noise and means Brownian motion, i.e., there is a stochastic time series for which any change in direction is possible, observations are random and current values do not affect future ones. The index values in the range $0.5 < H \leq 1$ are called 'black noise', mean the presence of fractal properties and indicate the trend stability of the series [25]. That is, in the latter case, there is a persistent time series for which a change in the previous direction is least likely [59,60]. Finally, a cumulative value of H was computed for each group of cows by summing Hurst coefficients for all microbial taxa studied.

3. Results

3.1 Overall Endometrial Microbiota Composition and Its Changes in the Transition Period

Our endometrial microbiota study in cows during the transition period revealed significant short-term changes and fluctuations in the composition of various microbial OTUs, i.e., groups of bacteria, yeasts and micromycetes, on IF and EF (**Supplementary Table 3** and Table 2, respectively).

In Group IF0 (**Supplementary Table 3**), one of the TNB peaks was observed on D0, reaching an average of 5.0×10^6 DNA copies/mL that was 100-fold higher than on D–20 ($p < 0.001$) and 38.4-fold higher than on D–10 ($p < 0.01$). On D+3, a 63.2-fold decrease in TNB was observed (to 7.9×10^4 DNA copies/mL; $p < 0.01$). However, by D+5 and even in Group IF1H, an increase in bacterial contamination by 12.7 times ($p < 0.01$) was again observed. In Group IF3E, TNB was a record high on D+5, reaching 3.2×10^7 DNA copies/mL that was 32-fold higher than that in healthy animals at the same sampling time ($p < 0.01$). By D+20, TNB in Group IF3E decreased 64-fold ($p < 0.01$), but remained significantly higher than that in Group IF1H at the same time point ($p < 0.01$). In Group IF2S, a decrease in TNB by D+20 was also observed compared to D+5 ($p < 0.01$).

On EF (Table 2), TNB changed more abruptly compared to IF. Relatively low bacterial load was observed on D−20, D−10 and D0. However, by D+3 there was a pronounced jump in TNB, reaching 1.3×10^8 DNA copies/mL ($p < 0.001$). By D+5, a 185.7-fold decrease in bacterial load was observed ($p < 0.001$), but by D+20 TNB increased again, reaching 7.9×10^8 DNA copies/mL.

3.2 Abundance Shifts of Bacterial Taxa in the Cow Endometrium

As for individual OTUs, representatives of *Prevotella* and *Porphyromonas* spp. demonstrated dynamics similar to TNB. Their high concentration was observed on D0 (up to 1.0×10^6 DNA copies/mL). The maximum values were identified in Group IF3E on D+5 (1.0×10^7 DNA copies/mL). In Group IF1H, these bacteria were practically undetected on D+20.

Enterobacteriaceae family representatives demonstrated a significant increase in abundance on D+3 in Group IF0 (1.6×10^5 DNA copies/mL) compared to the dry period ($p < 0.001$). An elevation in the abundance of this family representatives was also observed on D+5 in Group IF3E (3.2×10^5 DNA copies/mL) compared to Group IF1H ($p < 0.01$).

Similarly, the number of bacteria from the genera *Fusobacterium*, *Sneathia* and *Leptotrichia* group enlarged essentially in Group IF3E on D+5 (to 7.9×10^6 DNA copies/mL, i.e., 125-fold) compared to Group IF1H ($p < 0.001$). Herewith, these bacteria were undetected in Group IF2S. In addition, actinomycetes (*Mobiluncus* and *Corynebacterium* spp.) also increased significantly (by three orders of magnitude) in Group IF3E on D+5 compared to Groups IF1H and IF2S, reaching a concentration of 1.0×10^6 DNA copies/mL ($p < 0.001$).

Eubacterium spp. also showed an increased abundance in Group IF3E on D0 compared to D−20 and D−10 ($p < 0.001$), reaching peak values of 2.0×10^6 DNA copies/mL on D+5. In Group IF1H on D+20, an abrupt decrease in their abundance was observed ($p < 0.001$).

The abundance of the genera *Lachnobacterium* and *Clostridium* remained relatively stable throughout the study period, with some growth in Groups IF1H and IF2S on D+5 compared to D+3 ($p < 0.05$).

Lactobacillus spp. also demonstrated fairly stable values (from 2.5×10^3 to 3.2×10^4 DNA copies/mL) throughout the study during the transition period, without significant changes.

The abundance of most of the bacterial OTUs on EF demonstrated a relative similarity with IF. For example, on D+3, D+5 and D+20, the dynamics of the opportunistic bacteria *Fusobacterium*, *Sneathia* and *Leptotrichia* spp. in healthy EF cows was similar to Group IF3E.

Table 2. Composition of endometrial microbiota in cows from the eco-farm using qRT-PCR (in DNA copies/mL).

Microorganisms	Groups of cows (n = 3) and sampling points [1]					
	Group EF0			Group EF1H		
	D−20	D−10	D0	D+3	D+5	D+20
Total number of bacteria	$2.0 \times 10^5 \pm 1.9 \times 10^4$ [a]	$5.0 \times 10^5 \pm 2.2 \times 10^3$ [b]	$5.0 \times 10^5 \pm 1.0 \times 10^4$ [b]	$1.3 \times 10^8 \pm 2.6 \times 10^7$ [c]	$7.0 \times 10^5 \pm 3.1 \times 10^4$ [b]	$7.9 \times 10^8 \pm 6.5 \times 10^7$ [d]
Prevotella and *Porphyromonas* spp.	$4.0 \times 10^3 \pm 7.7 \times 10^2$ [a]	$1.0 \times 10^4 \pm 3.0 \times 10^3$ [b]	$3.2 \times 10^4 \pm 2.4 \times 10^3$ [b]	$7.9 \times 10^7 \pm 4.1 \times 10^6$ [c]	$2.0 \times 10^4 \pm 3.5 \times 10^3$ [b]	$6.3 \times 10^8 \pm 8.0 \times 10^7$ [d]
Eubacterium spp.	$1.0 \times 10^4 \pm 6.9 \times 10^2$ [a]	$4.0 \times 10^4 \pm 2.1 \times 10^3$ [b]	$5.0 \times 10^4 \pm 2.5 \times 10^3$ [b]	$2.5 \times 10^6 \pm 4.1 \times 10^5$ [c]	$2.5 \times 10^3 \pm 2.0 \times 10^2$ [d]	$2.5 \times 10^7 \pm 3.8 \times 10^6$ [e]
Lachnobacterium and *Clostridium* spp.	$6.3 \times 10^4 \pm 3.4 \times 10^3$ [a]	$1.6 \times 10^5 \pm 1.1 \times 10^4$ [b]	$2.0 \times 10^5 \pm 1.7 \times 10^4$ [b]	$1.3 \times 10^7 \pm 2.3 \times 10^6$ [c]	$6.3 \times 10^5 \pm 3.9 \times 10^4$ [d]	$3.2 \times 10^7 \pm 1.5 \times 10^6$ [c]
Lactobacillus spp.	$8.9 \times 10^4 \pm 3.6 \times 10^3$ [a]	$1.6 \times 10^3 \pm 1.0 \times 10^2$ [b]	–	$1.0 \times 10^5 \pm 2.1 \times 10^4$ [c]	$6.0 \times 10^5 \pm 3.1 \times 10^4$ [d]	$1.6 \times 10^4 \pm 6.7 \times 10^2$ [e]
Megasphaera, *Veillonella* and *Dialister* spp.	$1.0 \times 10^3 \pm 1.7 \times 10^2$ [a]	$1.0 \times 10^3 \pm 1.4 \times 10^2$ [a]	$6.3 \times 10^3 \pm 2.3 \times 10^2$ [b]	$2.5 \times 10^6 \pm 9.7 \times 10^4$ [c]	$3.2 \times 10^3 \pm 2.0 \times 10^2$ [a]	$5.0 \times 10^6 \pm 4.8 \times 10^5$ [c]
Peptostreptococcus spp.	$1.3 \times 10^4 \pm 1.0 \times 10^3$ [a]	$5.0 \times 10^4 \pm 3.4 \times 10^3$ [b]	$5.0 \times 10^4 \pm 2.7 \times 10^3$ [b]	$2.5 \times 10^6 \pm 5.1 \times 10^5$ [c]	$3.2 \times 10^5 \pm 1.2 \times 10^4$ [d]	$4.0 \times 10^6 \pm 7.1 \times 10^5$ [c]
Enterobacteriaceae	$4.0 \times 10^3 \pm 1.5 \times 10^2$ [a]	$1.3 \times 10^4 \pm 1.4 \times 10^3$ [b]	$2.5 \times 10^4 \pm 4.7 \times 10^3$ [a]	$1.0 \times 10^6 \pm 2.0 \times 10^5$ [a,c]	$2.0 \times 10^3 \pm 9.0 \times 10^2$	$5.0 \times 10^5 \pm 2.3 \times 10^4$ [d]
Mobiluncus and *Corynebacterium* spp.	$1.3 \times 10^4 \pm 4.4 \times 10^3$	$6.3 \times 10^4 \pm 3.0 \times 10^3$	$5.0 \times 10^4 \pm 2.9 \times 10^3$	$5.0 \times 10^4 \pm 1.8 \times 10^3$	$1.3 \times 10^6 \pm 1.0 \times 10^5$	$3.2 \times 10^3 \pm 1.5 \times 10^2$
Atopobium spp.	$1.3 \times 10 \pm 1.5$ [a]	$5.0 \times 10 \pm 2.3$ [b]	$1.0 \times 10^2 \pm 1.0 \times 10$ [c]	$2.5 \times 10^3 \pm 4.3 \times 10^2$ [d]	$4.0 \times 10^2 \pm 1.6 \times 10$ [c]	$1.0 \times 10^3 \pm 7.2 \times 10^2$ [d]
Fusobacterium, *Sneathia* and *Leptotrichia* spp.	–	–	–	$3.2 \times 10^3 \pm 1.1 \times 10^2$ [a]	$7.9 \times 10^6 \pm 3.8 \times 10^5$ [b]	$6.3 \times 10^3 \pm 2.5 \times 10^2$ [c]
Streptococcus spp.	$2.5 \times 10^3 \pm 1.2 \times 10^2$ [a]	$5.0 \times 10^3 \pm 2.6 \times 10^2$ [a,b]	$6.3 \times 10^3 \pm 3.1 \times 10^2$ [b]	$2.5 \times 10^3 \pm 1.1 \times 10^2$ [a]	$5.0 \times 10^5 \pm 7.9 \times 10^4$ [c]	$4.0 \times 10^4 \pm 2.1 \times 10^3$ [d]
Staphylococcus spp.	$2.0 \times 10^3 \pm 1.3 \times 10^2$ [a]	$1.3 \times 10^4 \pm 1.9 \times 10^3$ [b]	$6.3 \times 10^3 \pm 2.9 \times 10^2$ [c]	$2.5 \times 10^4 \pm 4.7 \times 10^4$ [b]	$1.0 \times 10^4 \pm 5.5 \times 10^3$ [b]	$1.6 \times 10^4 \pm 6.4 \times 10^3$ [b]
Mycoplasma spp.	$1.3 \times 10^4 \pm 1.5 \times 10^3$	–	–	–	–	–
Ureaplasma spp.	$3.2 \times 10^4 \pm 4.9 \times 10^3$	–	–	–	–	–
Candida spp.	$3.2 \times 10^3 \pm 2.1 \times 10^2$ [a]	$1.6 \times 10^4 \pm 2.1 \times 10^3$ [b]	–	$2.0 \times 10^4 \pm 1.5 \times 10^3$ [b]	$3.2 \times 10^4 \pm 1.2 \times 10^3$ [b]	$4.0 \times 10^4 \pm 2.3 \times 10^3$ [b]
Aspergillus spp.	–	–	$2.0 \times 10^2 \pm 14.0$	–	–	–
Fusarium spp.	–	–	–	–	–	–

[1] Means and standard errors of the mean, with n denoting the number of cows from one group sampled and analyzed for microbial community composition. [a–e] Differences within a row are significant between values with different letter superscripts and insignificant between those with the same letter superscript. Groups: EF0, experiment stage I; EF1H, EF healthy, experiment stage II. Sampling points: D−20 and D−10, 20 and 10 days before calving; D0, at calving; and D+3, D+5 and D+20, 3, 5 and 20 days postpartum.

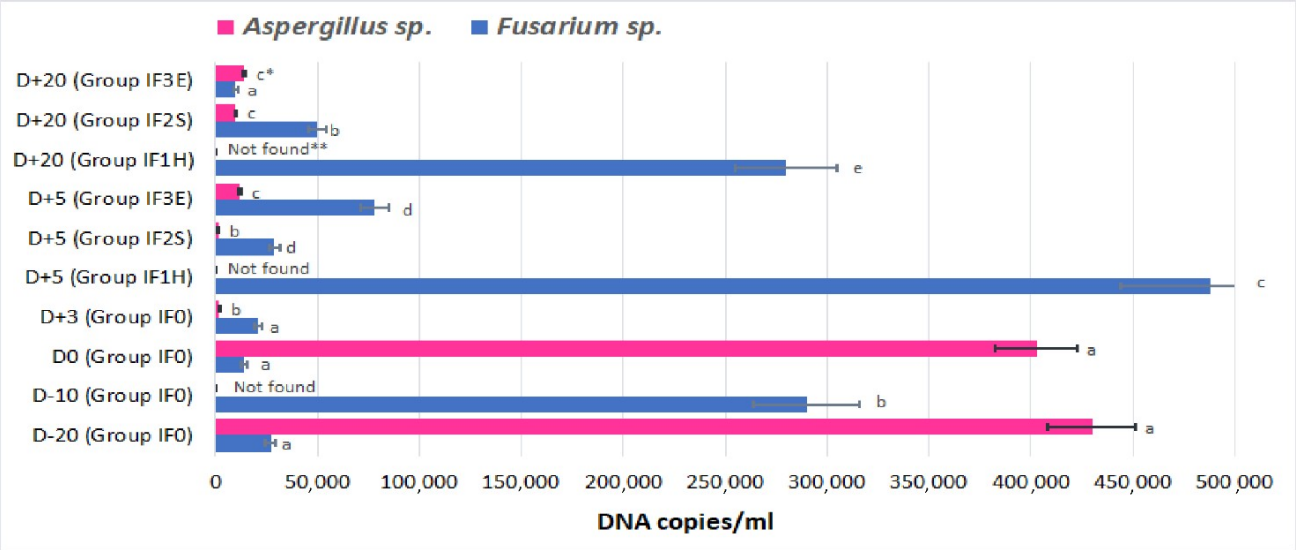


Fig. 1. Content of micromycetes *Aspergillus* and *Fusarium* spp. (DNA copies/mL) in the endometrial microbiota of cows on the industrial farm (IF) using qRT-PCR (n = 3). * $^{a–e}$ Differences within one sampling point are significant between values with different letter superscripts and insignificant between those with the same letter superscript; indicates a statistically significant difference between groups within one sampling point (at $p < 0.05$) according to the Student's t-test. Letter indices (a–e) were calculated when comparing parameters between groups of cows (i.e., between different collection dates). The * symbol is used only as a footnote marker and not to indicate significant differences between groups. ** Not found, *Aspergillus* and *Fusarium* microorganisms were undetected in endometrial scraping samples. The ** symbol means that *Aspergillus* and *Fusarium* spp. were not detected (DNA content below detection limit) and does not indicate the level of statistical significance. Sampling points: D−20 and D−10, 20 and 10 days before calving; D0, at calving; and D+3, D+5 and D+20, 3, 5 and 20 days postpartum. Groups: IF0, experiment stage I; IF1H, IF healthy; IF2S, subclinical endometritis; and IF3E, endometritis. The x-axis is the content of micromycetes *Aspergillus* and *Fusarium* spp., and the y-axis is the sampling points relative to calving.

Differences in the abundance of some other microorganisms were also found between EF and IF. For instance, *Fusobacterium*, *Sneathia* and *Leptotrichia* spp. were absent on EF on D−10 and D0. In contrast, these bacteria were present at these time points on IF at 5.0×10^3 and 6.3×10^4 DNA copies/mL, respectively.

In addition, there was a generally increased ($p < 0.05$) content of Enterobacteriaceae and actinomycetes *Mobiluncus* and *Corynebacterium* spp. on EF (up to 1.0×10^6 and 1.3×10^6 DNA copies/mL, respectively) compared to IF (up to 1.6×10^5 and 5.0×10^3 DNA copies/mL, respectively).

Among the normal microbiota representatives, a higher content of *Lactobacillus* spp. (up to 6.0×10^5 DNA copies/mL) was noted on EF than on IF ($p < 0.05$).

3.3 Abundance Changes of Yeasts and Micromycetes

A mainly higher *Candida* yeast content was found on EF compared to IF during the transition period ($p < 0.05$). Micromycetes analysis showed that high concentrations of *Aspergillus* and *Fusarium* spp. were observed on IF, reaching a maximum of 4.3×10^5 and 4.9×10^5 DNA copies/mL, respectively (Fig. 1, **Supplementary Table 3**). In contrast, on EF, *Aspergillus* spp. were detected only on D0 and in significantly lower quantities (up to 2.0×10^2 DNA copies/mL), while *Fusarium* spp. were completely absent (Table 2).

The dynamics of Aspergillus abundance varied significantly in the normal situation and in cows with a pathology (Fig. 1, **Supplementary Table 3**). These micromycetes were undetected in Group IF1H on D+5 and D+20, suggesting their absence in the norm postpartum. In Group IF2S, a rise in the Aspergillus spp. to 1.3×10^3 DNA copies/mL on D+5 was observed, indicating an increase in fungal colonization. In Group IF3E, a significant elevation in their abundance to 1.2×10^4 DNA copies/mL was noted ($p < 0.01$). At the same time, the dynamics of Fusarium spp. abundance had a different trend. It consisted of completely different short-term temporal fluctuations in abundance. For example, in Group IF3E, the content of Fusarium spp. was significantly lower than in Group IF1H ($p < 0.01$).

3.4 Dynamics Analysis of Endometrial Microbiota Shifts

To proceed with the endometrial microbiome macroecological dynamics analysis, we normalized the microflora composition data using the respective $\log_{10}$ values. Next, we supplemented the time intervals D−20, D−10, D0 and D+3 with D+5 and D+20 respectively within three groups

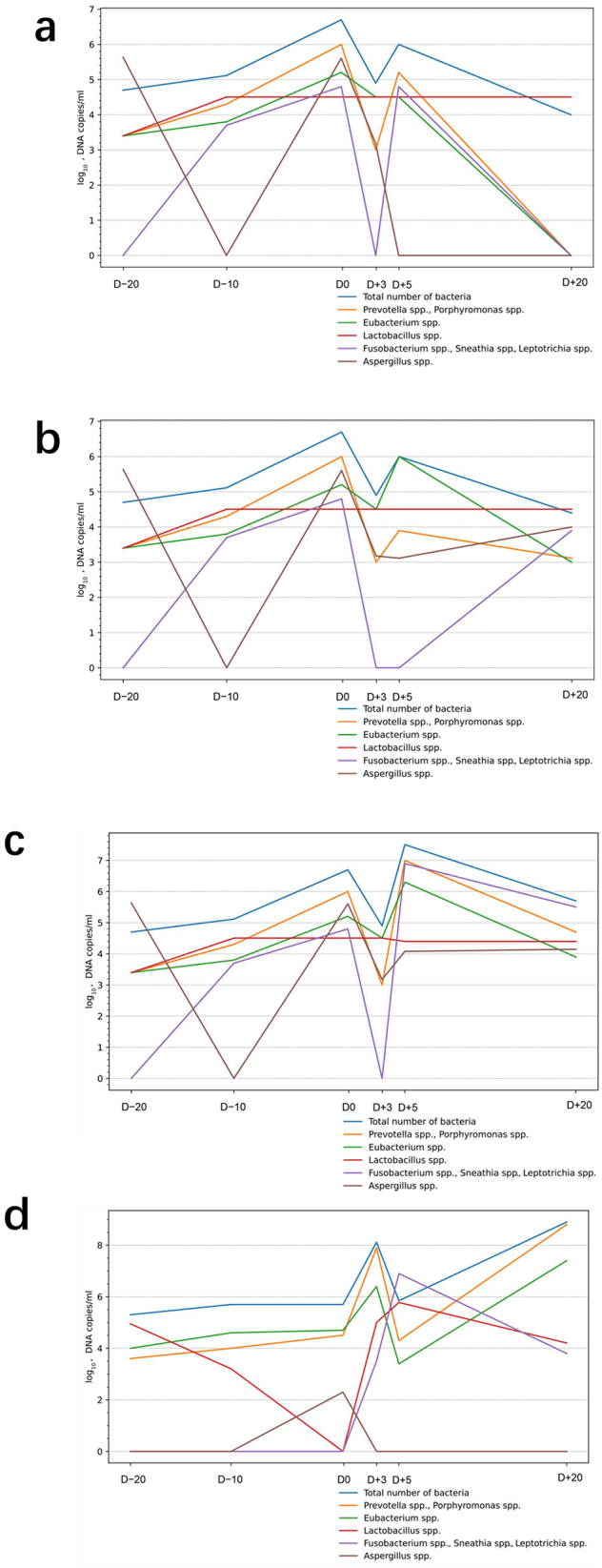


Fig. 2. Graphs of time series data for complex fluctuations and changes in the balance of selected microorganisms in the reproductive system in different physiological periods (D−20, D−10, D0, D+3, D+5, D+20) depending on the health status of cows on the industrial farm (IF; a, b, c) and on an eco-farm (EF; d). Groups: (a) IF1H, IF healthy; (b) IF2S, IF subclinical; (c) IF3E, IF endometritis; and (d) EF1H, EF healthy. Sampling points: D−20 and D−10, 20 and 10 days before calving; D0, at calving; and D+3, D+5 and D+20, 3, 5 and 20 days postpartum. Values on the *y*-axis are expressed as $\log_{10}$ DNA copies/mL.

Table 3. Comparison of the Hurst exponent values based on two formulae for the time series data and calculated for the number of microorganisms in each cow group on the industrial (IF) and eco- (EF) farms.

Microorganisms	Eqn. 1				Eqn. 2			
	IF1H	IF2S	IF3E	EF1H	IF1H	IF2S	IF3E	EF1H
Total number of bacteria	0.37	0.37	0.27	0.40	0.30	0.30	0.21	0.32
Prevotella and *Porphyromonas* spp.	0.34	0.43	0.22	0.38	0.28	0.34	0.18	0.31
Eubacterium spp.	0.39	0.49	0.46	0.24	0.32	0.40	0.37	0.19
Lachnobacterium and *Clostridium* spp.	0.34	0.36	0.31	0.47	0.28	0.29	0.25	0.38
Lactobacillus spp.	0.40	0.40	0.39	0.43	0.32	0.32	0.32	0.35
Megasphaera, *Veillonella* and *Dialister* spp.	0.33	0.35	0.21	0.39	0.26	0.28	0.17	0.31
Peptostreptococcus spp.	0.39	0.38	0.39	0.51	0.31	0.31	0.31	0.41
Enterobacteriaceae	0.34	0.48	0.52	0.27	0.28	0.39	0.42	0.22
Mobiluncus and *Corynebacterium* spp.	0.43	0.36	0.31	0.37	0.34	0.29	0.25	0.3
Atopobium spp.	0.40	0.40	0.40	0.50	0.32	0.32	0.32	0.4
Fusobacterium, *Sneathia* and *Leptotrichia* spp.	0.33	0.36	0.35	0.51	0.27	0.29	0.28	0.41
Streptococcus spp.	0.40	0.40	0.40	0.47	0.32	0.32	0.32	0.38
Staphylococcus spp.	– [1]	0.40	–	0.33	–	0.32	–	0.27
Mycoplasma spp.	–	–	0.40	0.40	–	–	0.32	0.32
Ureaplasma spp.	–	–	0.40	0.40	–	–	0.32	0.32
Candida spp.	0.4	0.37	0.40	0.38	0.32	0.30	0.32	0.31
Aspergillus spp.	0.38	0.35	0.29	–	0.31	0.28	0.24	–
Fusarium spp.	0.31	0.30	0.33	0.40	0.25	0.25	0.27	0.32
Mean ± standard error of the mean	0.37 ± 0.009	0.39 ± 0.013	0.36 ± 0.020	0.40 ± 0.019	0.30 ± 0.008	0.31 ± 0.010	0.29 ± 0.017	0.33 ± 0.015

[1] –, not available or not a number (i.e., coefficients could not be calculated). Groups: IF1H, IF healthy; IF2S, IF subclinical; IF3E, IF endometritis; and EF1H, EF healthy.

(IF1H, IF2S and IF3E). In this way, we combined experiment stage I and experiment stage II and formed a total of three groups of time intervals, i.e., IF1H, IF2S and IF3E, with time series data for six sampling points. The fourth group (EF1H) was transformed similarly. Fig. 2 and **Supplementary Fig. 1** show the short-term fluctuations of diverse endometrial microbial communities observed in the four studied groups based on the above time series data.

Table 3 comprises Hurst exponents using Eqns. 1,2 in order to demonstrate both variants of the dynamics analysis of endometrial microbiota shifts for the available time series data. In Hurst's Eqn. 1, the coefficient $a = 1$ was adopted, and in the modified Nayman's Eqn. 2, $a = \pi/2$. Table 3 also shows Hurst coefficients averaged over all microbial OTUs for each cow groups and presented as M ± SEM.

The dynamics analysis of the short-term time series for the endometrial microbiota shifts enables further comparison of the means between groups. To assess the overall contribution of microorganisms to the macroecological dynamics in each group, Hurst coefficients (Table 3) were summed up, i.e., their cumulative sums were obtained (**Supplementary Table 4**). This seems to express more clearly the effect of using Hurst coefficients, showing higher values for Group EF1H, lower values for Group IF1H, and intermediate values for Groups IF2S and IF3E.

4. Discussion

4.1 Macroecological Changes in Bacterial Communities

In this study, we examined the endometrial microbiome composition in cows from IF, a dairy farm characterized by a high risk of postpartum endometritis, and EF, a dairy farm characterized by low rates of postpartum endometritis. We recognize that choosing farms purely on the basis of their present endometritis risk status could introduce bias and skew the study's findings. Therefore, we examined data for the preceding three years in addition to the farms' present endometritis risk status when choosing them. Additionally, we made an effort to standardize the study settings in this experiment, including the criteria for animal selection and the diagnostic techniques for endometritis. We believe this lessened the impact of confounding variables.

The qRT-PCR method used in our study was instrumental in analyzing the abundance of preselected taxa. Although this approach is straightforward and cost-effective, it has certain limitations that should be considered when interpreting the results (e.g., [61]). This leaves open questions about possible unaccounted pathogens or representatives of the normal microbial community, which may lead to an underestimation of microbiome diversity. The endometrial microbiome is undoubtedly a complex ecosystem comprising thousands of microbial species (e.g., [10]).

Our study of the endometrial microbiota in transition cows suggests dynamic changes in the microbial community composition, especially in relation to calving and the development of endometritis. Differences between breeds and management practices may introduce potential bias into our research results. Holstein and Ayrshire breeds have distinct genetic traits that may impact microbiome composition [62,63]. The geographic location of the farms is also significantly dissimilar. Also, industrial farms differ from organic farms in many ways, including feeding, sanitation, and exercise, which may impact microbiome composition [64]. These disparities may complicate the interpretation of the results.

A greater TNB at calving in IF cows is likely due to a weakened immune system and hormonal changes that facilitate bacterial adhesion to the endometrium [65]. The subsequent decrease in bacterial counts may be due to activation of innate immune mechanisms. The re-increase in bacterial counts on D+5, especially in cows with endometritis, may be associated with lochial discharge and endometrial microtrauma [66].

Cows with clinical endometritis on D+5 had a record high TNB, which probably reflects the peak of the inflammatory process, when microorganisms actively multiply because of a weakened immune system and damaged endometrium. Despite the decreased TNB by D+20, their level remained significantly higher than in healthy animals, suggesting that complete microbiota restoration and elimination of pathogens takes longer in clinical endometritis. A decreased TNB by D+20 in cows with subclinical endometritis indicates the body's ability to partially self-regulate even in the presence of an inflammatory process. Perhaps, in cases of subclinical endometritis, the immune system and defense mechanisms are more effective in controlling bacterial contamination.

On EF, TNB in the endometrium demonstrated more pronounced dynamics with abrupt fluctuations. Relatively low bacterial levels during the dry period and at the time of calving were replaced by a noticeable jump in abundance on D+3. This peak was likely associated with postpartum physiological changes in the uterus, such as placental abruption, tissue rupture and endometrial regeneration onset, which creates a favorable nutrient medium for microorganisms [67]. The subsequent decrease in bacterial load was followed by a new increase on D+20, suggesting ongoing endometrial restoration and colonization processes, possibly involving different microbial communities at different regeneration stages.

High levels of *Prevotella* and *Porphyromonas* spp. in cows with endometritis under IF conditions support their role in the pathogenesis. These bacteria often dominate the microbiome of cows with endometritis [10]. In clinically healthy IF cows on D+20, these bacteria were virtually undetectable, likely indicating an effective immune system functioning. In addition, normal microbiota restoration, including bacteria competing with *Prevotella* and *Porphyromonas* spp. for resources, may also contribute to their reduction. These bacteria are the dominant anaerobes in the rumen of ruminants and play an important role in car-

bohydrate fermentation [68]. Their detection in the endometrium, especially in the postpartum period, suggest translocation of bacteria from the intestine or contamination from the environment (e.g., fecal contamination).

The detection of Enterobacteriaceae, *Fusobacterium*, *Sneathia*, *Leptotrichia* and actinomycetes (*Mobiluncus* and *Corynebacterium*) in high concentrations in cows with clinical endometritis indicates their involvement in the inflammatory process development. Enterobacteriaceae, e.g., *E. coli*, are capable of causing inflammatory processes and tissue damage in bovine uterine inflammatory diseases [10]. 16S rRNA-based screening in vaginal and uterine samples showed that high prevalence of *Fusobacterium* and *Corynebacterium* bacteria is usually associated with metritis, endometritis and infertility [69,70]. *Mobiluncus* and *Corynebacterium* spp. are also often associated with purulent inflammation and may worsen the endometritis course [10]. Also, *Porphyromonas*, Enterobacteriaceae and *Fusobacterium* can interact with other bacteria in the uterine microbiome, enhancing each other's pathogenicity [71]. They form a symbiotic relationship with each other, promoting endometrial colonization and immune evasion [72].

The relatively stable abundance of *Lachnobacterium*, *Clostridium* and *Lactobacillus* spp. suggests that they may belong to the normal endometrium microbiota. Our findings confirm previous studies [10] that the cows' endometrial and vaginal microbiome contains relatively few lactobacilli.

Our results on bacteria abundance in IF conditions did not reveal an unambiguous cause of cows' postpartum endometritis. Presumably, the disease is triggered by an imbalance in the uterine microbiota resulted to a change in the ratio of normal microbiota. Judging from our data, clinical endometritis is characterized by an increased content of *Porphyromonas*, Enterobacteriaceae, *Fusobacterium*, *Mobiluncus*, *Corynebacterium* and *Eubacterium* compared to healthy animals. However, the absence of a specific pathogen suggests that endometritis is most likely a consequence of a complex interaction of various microorganisms present in the norm and not the result of infection with a specific type of bacteria. Perhaps a key role is played by a violation of the natural balance in the uterine microbiota that creates favorable conditions for the proliferation of opportunistic microorganisms and leads to the development of an inflammatory process. Further research is needed to understand fully the mechanisms of endometritis development and develop effective prevention and treatment strategies. Next-generation sequencing applications [73], such as 16S rRNA sequencing or whole genome sequencing [74], may allow for a comprehensive microbial community characterization and detection of new or rare microorganisms potentially involved in the endometritis pathogenesis.

However, it should be noted that our study design had limitations related to the inability to predict endometritis before calving and the lack of long-term data on the microbiome before the disease developed. Endometritis as a pathological condition after calving cannot be diagnosed until the early postpartum period. Without long-term data obtained from cows with endometritis before their disease, definitive statements cannot be made. The division into IF1H (healthy), IF2S (subclinical endometritis), and IF3E (clinical endometritis) groups was indeed based on postpartum diagnosis. This means that the differences in the microbiome observed on D+5 and D+20 may be both a cause and a consequence of endometritis. Previously, researchers have also raised the issue that, despite a number of studies, it has not been fully confirmed that bovine endometritis is caused by microorganisms, rather than by uncontrolled inflammation in the uterus caused by other (non-microbiological) factors (e.g., [10]). Indeed, an imbalance in the composition of the microbiota (dysbacteriosis) disrupts homeostasis that triggers an inflammatory response and alters the physiological state of the body. Vaginal dysbacteriosis has been repeatedly associated with endometritis, infertility, or adverse pregnancy outcomes in all studied mammalian species. However, Carneiro et al. [75] hypothesized that, in cows that develop endometritis, microorganisms present in the uterus after parturition may be both etiologic agents and secondary infections that develop against a background of reduced resistance. Thus, the observed change in the composition of the reproductive tract microbiota associated with endometritis may simply be a consequence of changes in hormonal status. It is known that cows with endometritis in the early postpartum period exhibit elevated serum progesterone levels accompanied by low estradiol levels (e.g., [1]). Therefore, the question of cause and effect remains open. Our data only reveal associations between microbiome composition and the risk of developing endometritis, but do not prove that microbiome changes are the cause or consequence of the disease.

The abundance of most of the studied bacterial OTUs in the EF cows' endometrium showed a relative similarity with the abundance indicators for IF. In particular, the abundance dynamics of opportunistic bacteria, such as *Fusobacterium*, *Sneathia* and *Leptotrichia* spp., during the postpartum period turned out to be similar in healthy EF animals and IF cows with endometritis. This observation, despite breed differences and differences in the reproductive system health, feeding and housing systems, is quite unexpected. The obtained data may contradict the classical concepts about the role of opportunistic microorganisms in the development of endometritis and raises the question of other factors that can protect animals from the disease (e.g., [10,76]). In our opinion, the results may indicate the role of other factors, such as breed or the body's immune response, in shaping the microbiome and its relationship with the development of endometritis. There is a possibility that differences in the immune response between breeds (Holstein and Ayrshire) may play a key role in the reaction to

colonization by *Fusobacterium*, *Sneathia* and *Leptotrichia* spp. For example, the Ayrshire breed may have stronger local immunity or other adaptive mechanisms that allow it to avoid the development of endometritis despite the presence of pathogens. This assumption is consistent with the ideas of other researchers about the influence of genetic factors on the microbiome (e.g., [77]). However, due to the simultaneous influence of other factors (different geographical locations of farms, types of farming, etc.), it is not possible to give a definitive answer at this stage of research.

Along with a certain abundance similarity, there were also significant differences between the endometrial microbiota on EF and IF. In particular, *Fusobacterium*, *Sneathia* and *Leptotrichia* spp. were undetected on EF during the late dry period and at calving, while these bacteria were present in noticeable quantities on IF. This may be due to a more balanced microbiome in EF cows during this period, perhaps due to higher immune status of animals, limited antibiotics use and other factors. Frequent antibiotics use can lead to beneficial bacteria destruction and resistant microorganisms' selection [78], including *Fusobacterium*, *Sneathia* and *Leptotrichia* spp., which contributes to their colonization.

Another interesting aspect is significantly higher content of Enterobacteriaceae and actinomycetes *Mobiluncus* and *Corynebacterium* spp. in EF cows compared to IF animals. Increased levels of enterobacteria and actinomycetes are associated with endometritis [79], but are difficult to explain in the uterus of healthy EF cows. Importantly, uterine microbiome is a complex and dynamic system influenced by many factors [80]. These factors are likely to shape unique microbiological profiles on each farm studied. Breed factor and different ecogeographic location of the farms may partly explain the variation in the endometrial microbiome between the farms. Additionally, differences in the quantitative microbiome composition between the farms may reflect the microorganisms' adaptation to different environmental conditions and faming systems. On EFs, cows tend to have greater access to the environment, coming into contact with a diverse soil and plant microbiota represented by the above bacteria groups [81]. This may lead to a more frequent uterus colonization with various microorganisms. Herewith, because of a higher immune status due to natural feeding and housing conditions, the cows' body can effectively control the number of some microorganisms, preventing the development of pathological processes. Also, EFs use bedding made of natural materials (straw, hay), i.e., a favorable environment for the development of diverse microbiota, including enterobacteria and actinomycetes [82]. IFs often use synthetic materials that can create a different microbiota composition. However, differences in breeds (Holstein vs. Ayrshire) and regions (geographical location of the farms) can have a significant impact on the microbiome, independent of the farming method itself. These factors cannot be completely excluded from the analysis, making a direct comparison of the impact of farming methods on the endometrial microbiome problematic.

A higher *Lactobacillus* content was found in the EF cows' endometrium. High concentrations of lactobacilli can help normalize the microbiota, improving the immune response and reducing the inflammatory disease risk in the uterus. In contrast, a lower content of lactobacilli in the reproductive system is explained by total dysbiosis of the microbiome in intensive livestock farming conditions [83].

4.2 Macroecological Shifts in Micromycetes Communities

On IF, high concentrations of *Aspergillus* and *Fusarium* micromycetes were identified. This observation, along with large short-term temporal fluctuations in abundance, may indicate an imbalance in the reproductive tract microbiota caused by different factors. In the case of *Fusarium* representatives, this is likely due to their widespread distribution in soil and the environment [84], as well as contact of the cows' genital tract with contaminated surfaces in industrial conditions [85]. The concentration of *Fusarium* spores is often elevated in industrial premises containing large amounts of dust and contaminants [86]. Micromycetes can infiltrate the reproductive system from various anatomical locations. When the physical cervical barrier is compromised due to calving, these microorganisms can enter the genital system through the vagina, feces, animal skin, or the environment [85,87]. Additionally, important sources of contamination can be forages [85]. Perhaps, this situation is observed exclusively in IF conditions, where contact with potential contamination sources is inevitable. On EF, where the influence of industrial factors is reduced, such a situation was unnoted. Therefore, on EF, a more favorable situation was observed with respect to micromycetes.

4.3 Association Between Endometrial Microbiome and Endometritis Occurrence in Cows

Cattle endometritis etiology and pathogenesis remain the subject of ongoing debate [10]. Overall, bacterial agents are considered as the main causative endometritis agents [88]. However, no consistent correlation has been found between the presence of a specific bacterial taxon and the cows' reproductive disease development [10]. The reproductive organs of both healthy and endometritis-affected animals are characterized by the presence of the same dominant taxa, while the differences between the groups are reduced mainly to variations in their quantitative ratio [10]. This fact calls into question the concept of a direct causal relationship between certain bacterial species and the endometritis occurrence.

Antibiotics can inhibit the growth of bacteria that compete with fungi for nutrients and space, thereby facilitating their proliferation [89]. Inhibition of commensal bacteria by antibiotics in intensive cow rearing conditions likely creates favorable conditions for active proliferation and colo-

nization of ascomycetes, among which *Aspergillus*, *Fusarium* and *Candida* are often predominant in the healthy cows' reproductive tract [85]. Endometrium tissue damage leading to the necrotic foci formation also represents an ideal environment for the fungal infection development [90]. The immunosuppressed state caused by of intensive animal husbandry factors (stress, metabolic disorders and tethered housing) weakens the body's defense mechanisms and enhances susceptibility to fungal infections [91].

Micromycetes may likely contribute to dysbiotic disturbances leading to uterine inflammation. *Aspergillus* fungi are a group of ubiquitous saprophytic microorganisms [92]. Some *Aspergillus* spp. are opportunistic pathogens that can cause invasive diseases in mammals, especially in the presence of a weakened immune system [93]. They play a key role in the etiology of various respiratory diseases, including fatal ones [94,95], and can also cause other systemic infections [96,97,98]. Our data suggested the dynamic endometrial colonization by *Aspergillus* spp. and their relationship with the pathological process development. In healthy cows, *Aspergillus* spp. were undetected in the postpartum period. However, in cows with subclinical endometritis, a greater abundance of *Aspergillus* spp. was observed, indicating the activation of colonization. The most pronounced rise in the *Aspergillus* abundance was identified in cows with clinically expressed endometritis, suggesting a close relationship between the micromycetes proliferation and the pathological process. Therefore, *Aspergillus* spp. can play a significant role in the cows' endometritis pathogenesis, contributing to the inflammation development and normal microbiological environment disruption in the postpartum endometrium. Increased content of *Aspergillus* spp. can serve as a marker of fungal infection in endometritis.

Fusarium fungi are ubiquitous in microbial communities of soil, plants and air [99] and can cause fusariosis [100,101,102]. *Fusarium* spp. manifest themselves as opportunistic microorganisms; their pathogenic potential realization require certain predisposing factors, i.e., traumatic injuries causing integrity disrupt of the skin and mucous membranes, and immunodeficiency of various etiologies [56]. This indicates a key role of the immune system in restraining the invasion of *Fusarium* spp. and maintaining them in a commensal state. When the body's protective barriers are disrupted, conditions are created for the pathogenic potential realization and invasive infection development. We found high levels of *Fusarium* spp. in both healthy and sick IF animals, which is presumably explained by the widespread distribution of spores in industrial conditions.

4.4 Complex Dynamics of Diverse Endometrial Microbial Communities

In the analyzed data on complex changes in endometrial microbial communities, certain time series could be traced. Consequently, macroecological endometrial microbiota dynamics was described applying the Hurst exponent [21]. When using the Hurst exponent, one can conclude about the fractal similarity of the observed microbial communities that is expressed through the relationship between the fractal and the Hurst exponent. In theory [20,32,102,103,104,105], short-term prediction of macroecological dynamics may rely on the Laplace distribution, and long-term forecasting on the Hurst exponent. The use of the Hurst exponent to analyze endometrial microbiota dynamics is rather scarce; its application to the microbiome research represents a novel approach previously not widely used in similar studies (e.g., [20]). However, we considered it appropriate to introduce this exponent due to its potential to reveal underlying patterns and likely trends in complex and dynamic systems, such as the endometrial microbiota of cows during a transition period. The Hurst exponent, originally developed for time series analysis in ecology and economics, is currently finding application in various fields of science, including biology and medicine [106,107,108,109,110,111,112]. We found it worthwhile and innovative to explore this approach in a new context—endometrial microbiome studies—which may provide new insights into the dynamics of microbial communities. On the other hand, this approach was directly inapplicable in relation to our study results, since we had small data and a fairly short time series of 40 days, which may result in a distorted (underestimated) the Hurst exponent estimate accuracy. Therefore, we made comparative calculations using the original Hurst's [21] Eqn. 1 and the modified Eqn. 2 [23]. In the second case, the results seem more plausible and appropriate, since the Nayman's [23] formula is adjusted for short-term time series using correction factors.

The produced H values did not allow us to determine the prediction horizon; it was not practicable to establish a long-term dependence ("memory") for these data at $H < 0.5$. It is known [27] that the closer the H value to zero, the more variable the series (in contrast to a random series), which is more consistent with our observations for the cows' endometrial microbial communities. The range $0.5 < H < 1.0$ corresponds to persistent, or trend-stable, time series, which, according to our calculations, was not the case in our experiments. Nevertheless, it can be assumed that the presented data on complex macroecological dynamics can still reflect some time series validity, while noting that there is a data lack to provide a complete picture. Fig. 2 and **Supplementary Fig. 1** graphs for the examined cows' groups to some extent reflect a general dynamics of microbial communities, which is confirmed by the computed Hurst coefficients. Our results suggest that Group EF1H tended to have a more stable genital tract microflora than IF animals. The latter had a greater tendency to change the microflora balance. Although IF1H animals were clinically healthy, this does not rule out a chance that they might be more prone to urogenital tract diseases than EF cows.

5. Conclusions

During the transition period, significant dynamic endometrial microbiota changes in cows on both IF and EF were revealed, correlating in IF conditions with the postpartum endometritis development. Pathogenesis in the postparturient IF cows' uterus was accompanied by an increased total bacterial load and the dominance of opportunistic taxa, such as *Prevotella*, *Porphyromonas* and *Eubacterium* spp., Enterobacteriaceae family representatives, *Fusobacterium* spp., actinomycetes and *Aspergillus* fungi. However, it is currently impossible to determine whether these changes were a cause or a consequence of endometritis. Further studies using a long-term experimental design with repeated microbiome measurements in the same cows (healthy and with endometritis) before and after calving would be needed. Cows kept on EF were characterized by a fairly balanced microbiological profile with a *Lactobacillus* predominance and a significant decrease in the proportion of pathogenic microorganisms. Endometrial microbiome dynamics was explored on two farms that differed in both the housing/feeding systems and the ecogeographic location. This implies a possible influence of many factors on the microbiome formation, including different climatic conditions, soil and vegetation composition on pastures, etc. Additionally, it was not practicable to compare the endometrial microbiome in animals of the same breed in both farming systems. Genetic/breed factors can affect physiological processes in the animal body, including immune response, metabolism and microbiota composition [113,114]. Thus, the influence of breed differences on the observed endometrial microbiome dynamics variation cannot be completely ruled out. Therefore, the obtained results do not allow us to assert definitively a direct cause-and-effect relationship between the farming system (organic vs. industrial) and the endometrial microbiome composition. We urge caution in attempts to link directly differences in the microbiome to farm type. This issue requires further study, and any interpretations should be approached with caution. Only by strictly controlling breed and geographic factors can reliable conclusions be drawn to explain the results.

Nonetheless, ecological farming practices may create conditions conducive to the development of a diverse and stable endometrial microbiome, which, in turn, may have a positive effect on the cows' reproductive health [115]. Intensive farming practices on IFs may have a negative impact on the microbiome, leading to a reduction in its diversity and a greater risk of developing dysbiotic conditions [116]. The Hurst exponent application to the analysis of short-term temporal fluctuations in the microorganisms' abundance in endometrial communities observed here, to some extent, reflects the different trends in the microbiome formation and the risk of cows' reproductive tract diseases under different livestock farming systems. Further studies of various factors involved in the microbiome composition formation are needed to verify our hypotheses. This investigation significantly expands the understanding of the reproductive system microbiome composition, functions and dynamics in cattle and emphasizes the importance of developing comprehensive preventive and therapeutic strategies aimed at maintaining endometrial microbiological homeostasis. Of course, using qRT-PCR alone is a limitation for analyzing all microbiome components. Further functional metagenomic and metabolomic analyses will provide much more in-depth insights into the interactions between the microbiome and the animal's body.

Availability of Data and Materials

This article contains a presentation of all the experiments and findings from the study. Further details can be obtained upon request from the corresponding authors.

Author Contributions

Conceptualization, EAY and GYL; methodology, EAY, VAF, LAI, EAB and EAK; software, EAB and ESP; validation, EAY and ESP; formal analysis, EAY, EAB, ESP, DKG and MNR; investigation, KAS, IAK, VAZ, EAK and VNB; resources, VNB and EAK; data curation, EAY, DGT and NIN; visualization, EAY, EAB and ESP; supervision, GYL and DKG; project administration, EAY, GYL and MNR; funding acquisition, EAY and VAF; writing—original draft preparation, EAY, EAB and MNR; writing—review and editing, EAY, EAB, MNR and DKG. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The current investigation was performed in accordance with the guidelines set forth by the European Convention for the Protection of Vertebrate Animals used for Research and Other Purposes (ETS No. 123, Strasbourg, 1986). Also, the study received ethical approval from the Bioethical Commission of the L.K. Ernst Federal Research Center for Animal Husbandry (Protocol No. 2023-10/1, dated October 31, 2023). The study was carried out in accordance with the ARRIVE guidelines.

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Conflicts of Interest

The authors declare no conflicts of interest. Elena A. Yildirim, Georgi Yu. Laptev, Daria G. Tiurina, Valentina

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBE46742.

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