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Exploring the influence of endometritis diagnostic criteria on uterine involution, milk yield and fertility in dairy cows

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Abstract

Background We investigated the impact of different classification schemes for postpartum uterine disease on genital tract involution, milk yield, and reproductive performance in dairy cows. The reproductive tracts of 223 Polish Holstein cows were examined between 21 and 29 days postpartum (median 24 days). Transrectal ultrasonography was employed to assess reproductive tract dimensions, vaginoscopy was used for visual scoring of vaginal discharge, and endometrial cytology with the cytobrush was used to calculate the percentage of polymorphonuclear cells (PMN). The health status of the cows was classified using two diagnostics models. Model 1 categorized cows as healthy (H; <5% PMN and absence of purulent vaginal discharge [PVD0]), subclinical endometritis (SE; ≥5% PMN and PVD0), and further subdivided PVD into three categories: flecks of pus in vaginal discharge (PVD1), mucopurulent discharge (PVD2), or purulent discharge including red-brownish watery fetid discharge (PVD3), irrespective of endometrial PMN%. Model 2 classified cows as healthy (H; <5% PMN and PVD0), subclinical endometritis (SE; ≥5% PMN and ≤ PVD1), and clinical endometritis (CE; ≥5% PMN and > PVD1).

Results Cows with ≥ PVD2 (Model 1) and CE (Model 2) had larger cervical and uterine horn diameters and were less likely to resume ovarian activity by the fourth week postpartum in comparison to H and SE. For Model 1, the milk yield to 120 days postpartum was lower in PVD3 in comparison to H while for Model 2, CE produced less milk to 120 days postpartum when compared to H or SE. For both models, SE had a lower first service conception rate than H cows, but the pregnancy risk to 210 days postpartum did not differ. When compared to H, PVD2 (Model 1), and CE (Model 2) had the lowest first service conception rate and pregnancy risk to 210 days postpartum.

Conclusion Model 1 provides detailed PVD severity assessment, which is valuable for potential targeted treatment and management but adding complexity due to multiple categories. Model 2 offers simpler categorization for clear management decisions but may overlook disease nuances. Though labor-intensive and less practical for routine use, these protocols are useful for population-level decision-making in dairy farm reproductive performance.

Keywords Uterine disease, Subclinical endometritis, Vaginal discharge, Clinical endometritis, Fertility

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Introduction

Reproductive tract inflammatory diseases are common causes of impaired fertility in dairy cows, which significantly impact farming profitability and animal welfare. Most cows can effectively eliminate bacteria present in their reproductive tracts in the initial five weeks after parturition [1]. However, uterine bacteria dysbiosis that results in persistent endometrial inflammation and infection may result in postponed resumption of ovarian cyclicity and prolonged luteal phases [2]. These conditions decrease conception rates, extend calving intervals, increase the number of services per pregnancy, and ultimately lead to uneconomical culling [3–5].

Over the past few decades, the definition of clinical endometritis (CE) has undergone several adjustments. Initially, it was described as the presence of purulent discharge presumptively originated in the uterus but present in the vagina after 21 days postpartum. However, other studies have suggested that additional criteria, such as the diameter of the cervix based on transrectal palpation, may also be useful in diagnosing CE in dairy cows [1, 6]. Thus, LeBlanc (2002) proposed that the presence of mucopurulent or purulent vaginal discharge (PVD) after 21 days of delivery or the diameter of the cervix being ≥7.5 cm are adequate criteria to diagnose CE in dairy cows [1]. While these criteria have been useful in identifying cows with CE, recent studies have indicated that the presence of PVD alone may not be an accurate indicator of endometritis. This is because not all cows with PVD have high counts of polymorphonuclear cells (PMN) in their uterine lumen diagnosed via cytology [7–9]. Therefore, evaluation of both vaginal discharge and endometrial cytology are necessary for accurate diagnosis of CE.

Williams (2005) introduced a 4-point vaginal discharge score scale for the diagnosis and characterization of PVD in dairy cows. This scale defines PVD0 as clear or no mucus, PVD1 as clear mucus with flecks of pus, PVD2 as < 50% pus in mucus, and PVD3 as > 50% pus in mucus diagnosed ≥ 21 days after parturition [10]. Decreased fertility was reported predominantly at the diagnosis of PVD ≥ 2 in the fifth week postpartum [7]. However, Giuliodori (2017) demonstrated that a PVD ≥ 1 diagnosed between 28 and 35 days postpartum was associated with a decreased probability of pregnancy and an extended calving-to-pregnancy interval when compared to healthy cows. These results suggest that the full 4-point PVD score scale could be a useful tool for on-farm identifying and managing reproductive problems in postpartum dairy cows [9], however, the exact cut-off point for PVD diagnosis may be adjustable due to managerial and environmental conditions.

Subclinical endometritis (SE) refers to a chronic postpartum uterine inflammation not accompanied by obvious clinical signs (invisible to the naked eye). The most recognized approach for SE diagnosis is by collecting an endometrial cytological sample in which the percentage of PMN to epithelial cells is assessed microscopically in stained smears. The diagnostic threshold for PMN percentage varies considerably across studies, typically ranging from 5 to 18%, reflecting ongoing debates in the field. This variability is largely attributed to the timing of sample collection relative to calving, expressed as Days in Milk (DIM) (11). Research indicates that the optimal PMN cut-off point tends to decrease as DIM increases, mirroring the gradual resolution of physiological postpartum inflammation [4, 11]. As a consensus, Madoz (2013) and Wagener (2017) suggested using the general threshold of 5% PMN for the diagnosis of SE between 21 and 62 days postpartum [11, 12]. While many reports confirm the negative impact of SE on the reproductive performance of dairy cows, such as a reduced pregnancy risk, and negative impact on embryo survival and quality [13–16], some studies do not confirm the negative impact of SE on dairy cow fertility [17–19]. Additionally, diagnosing SE requires extra laboratory procedures such as staining and subsequent microscopic evaluation which cannot be routinely performed on-site by field veterinarians.

While literature abounds with works elucidating the definitions of reproductive tract inflammatory diseases, offering extensive academic discourse on the subject, there is an observable discrepancy. Those explorations have enhanced our understanding of the underlying pathophysiology, risk factors, and possible frameworks for diagnosis. However, despite the increasing body of knowledge surrounding reproductive tract inflammatory diseases, there is a visible gap in the form of a limited number of practical reports that offer possible implementations. Such reports, which depict the real-world scenarios encountered in veterinary practice, are crucial for bridging the gap between theoretical research and the practical realities faced by veterinarians and farmers. This lack of case-based literature underscores the need for studies that not only thoroughly examine the scientific aspects of these diseases but also provide insights into their practical management and implications in the field.

The objectives of this study were to: (1) compare two diagnostic models for classifying postpartum uterine diseases in dairy cows, (2) evaluate the impact of these classification schemes on uterine involution, ovarian activity, milk yield, and reproductive performance, and (3) determine which model provides more clinically relevant information for farm management. We hypothesized that the detailed classification system (Model 1) would offer greater insights into varying degrees of uterine health and their impacts on productivity and fertility compared to the simplified system (Model 2). Additionally, we expected both models to show significant associations between severe uterine health issues and impaired reproductive performance, with each model offering different predictive nuances. Understanding these differences could help in developing more effective management strategies for dairy farms.

Materials and methods

All procedures of this observational cohort study were carried out in accordance with the Polish Animal Protection Law (Journal of Laws of 21 February 2005, No. 33, item 289) and after obtaining approval from the Local Ethics Committee for Animal Experiments in Olsztyn (decision No. 49/2016).

Sample size calculation

The sample size for each reproductive health status was calculated based on two key parameters: milk yield and fertility. This approach ensured a focused and detailed examination of critical aspects of dairy cow productivity with an α level of 0.05 and 75% power for all calculations, corresponding to a $1 - Z_{1-\beta}$ value of 0.675.

For milk production, we focused on the effect of either reproductive tract inflammatory disease on the mean milk yield to 120 days postpartum. Assuming a normal distribution, with an expected difference of 4 kg and a standard deviation of 4 kg [20]. The following formula applied was:

$$n = 2 \cdot \left(\frac{Z_{1-\frac{\alpha}{2}} + Z_{1-\beta}}{\delta} \right)^2 \cdot \sigma^2$$

where δ is the expected difference in means, and σ is the standard deviation. This resulted in a requirement of at least 16 cows per group.

For fertility, we focused on the effect of reproductive tract inflammatory disease on first service conception rate (FSCR). We expected a to detect a 20% decrease in conception rate in affected cows (either reproductive tract inflammatory disease condition) from a baseline of 50% in healthy animals [21]. The following formula was used:

$$n = \left(\frac{Z^2_{1-\frac{\alpha}{2}} + Z^2_{1-\beta}}{(p_1 - p_2)^2} \right) \cdot \left(p \cdot (1-p) \cdot \left(\frac{1}{p_1} + \frac{1}{p_2} \right) \right)$$

where $p1$ is the baseline proportion, $p2$ is the expected increased proportion. This calculation indicated at least 18 cows per group.

The decision to select a general sample size of around 250 cows from a single farm was underpinned by both statistical and operational considerations. This sample size was determined based on the assumption of a 10% prevalence for each category of PVD [22] and at least a 10% occurrence of CE and SE [23] in the studied population. The larger sample size suggested by calculations accommodated for potential dropouts and ensuring adequate statistical power across various study groups in both models [24]. The single-farm research approach allowed for greater control over variables like feeding, housing, and management practices, which are key in accurately assessing the impact of the studied factors without the confounding effects of differing farm management practices. By limiting variability inherent in multi-farm studies, such as differences in management practices, environmental conditions, and genetic variations, the study could produce more accurate and reliable statistical interpretations, effectively reducing the risk of Type I and Type II errors [24].

Animals

The study was conducted on a commercial dairy farm in the northeastern region of Poland (Warmian-Masurian Voivodeship) and comprised 253 year-round calving Polish Holstein cows. The cows were housed in a free-stall system and fed a partially mixed ration following the recommendations of the German Agricultural Society (Deutsche Landwirtschafts-Gesellschaft, DLG). The average 305-day milk yield for the entire cohort was 9,200 kg, with an average of 8,680 kg for first lactation and 9,458 kg for multiparous cows. The parity of the cows ranged from 1 to 6. Milk production data were collected using a voluntary milking system (VMS; DeLaval®, Tumba, Botkyrka, Sweden).

The study was conducted from calving until 210 days postpartum or until the cow was culled. All cows that calved between March and November 2017 were included, resulting in 253 eligible animals. Thirty cows were culled earlier than 21 days postpartum, while data of the remaining 223 cows were used in further analyses.

Cows were bred using artificial insemination (AI) after visual estrus detection by a single trained observer. Heat detection was performed three times per day for 30 min (in the morning, at noon, and late afternoon), and animals in heat were inseminated between 8 and 14 h after the observed heat period. The voluntary waiting period was set at 60 days postpartum. Pregnancy was confirmed by transrectal ultrasonography (Honda HS-1500 Ultrasound, Toyohashi, Japan) starting at 30 days after AI.

Postpartum clinical diseases

The health condition of the cows was monitored on a weekly basis by a team of trained personnel and veterinary professionals. Throughout the complete experimental period, the occurrence of postpartum diseases such as milk fever, lameness, and mastitis, as well as the administration of all veterinary treatments, were meticulously documented for each individual cow. In order to evaluate the presence of lameness, a rating scale ranging from 1 to 5, as proposed by Flower and Weary (2006) was employed [25]. Cows that received a score of 4 or 5 were classified as lame and underwent specialized hoof trimming tailored to their unique condition. Clinical cases of mastitis were diagnosed utilizing the Pinzón-Sánchez and Ruegg (2011) scale [26]. Cows exhibiting symptoms of abnormal milk accompanied by swelling or redness of the mammary gland were diagnosed with mastitis. Upon confirmation of mastitis, the cow received a local antibiotic treatment based on antimicrobial resistance test results. Clinical hypocalcemia (milk fever) was diagnosed when symptoms such as recumbency, severe lethargy, and a progressive state of unresponsiveness to external stimuli, including touch and sound [27]. Other peripartum events were meticulously recorded according to standardized criteria. These included twin births and dystocia, defined as requiring assistance from multiple individuals, veterinary intervention, or a cesarean section for calf delivery [28]. Retained placenta, defined as fetal membranes not expelled within 24 h postpartum, was also monitored as described by Kimura et al. (2002). Metritis was characterized by an enlarged uterus upon palpation, fetid watery red-brown uterine discharge diagnosed within 21 days postpartum, depression, body temperature above 39.5 °C, and loss of appetite, following the definition by Sheldon et al. (2006) [6]. Treatment protocols were established for the observed conditions. Cows diagnosed with milk fever received intravenous treatment with Tetanusan 24% (240 mg/ml Ca, Kon-Pharma, Vechta, Germany). Retained placenta was treated by administering intrauterine tablets containing ampicillin trihydrate (577.5 mg) and cloxacillin sodium monohydrate (545.0 mg) (Aniclox®, Livisto, Senden, Germany). Metritis cases received a subcutaneous injection of ceftiofur (Excenel® 50 mg/ml, Zoetis, Parsippany, New Jersey, U.S). It is important to note that this study did not specifically diagnose or record cases of displaced abomasum, pneumonia, or non-specific digestive problems. The focus was on the most common postpartum diseases that could potentially affect reproductive performance. We acknowledge that this approach may have resulted in some uncontrolled confounding.

Reproductive tract evaluation

Ultrasonographic assessments of the diameter of the cervix and both uterine horns were transrectally performed using a Honda HS-1500 ultrasound (Honda HS-1500 Ultrasound, Toyohashi, Japan) between 21 and 29 days postpartum. The measurements were performed using a slight modification of the method described by Heppelmann (2013). Briefly, to examine the cervical diameter, the linear transducer was placed transversally halfway along the length of the cervix. To examine the diameter of the uterine horns, the probe was placed transversally, approximately 2 cm cranially from the uterine bifurcation. The diameter of the cervix and uterine horns was calculated as the mean of horizontal, vertical, and diagonal measurements of its cross-Sect. [29]. Both ovaries were scanned and all follicles and corpora lutea (CL) were counted and measured at the time of ultrasonography. Follicles larger than 2.5 cm on one or both ovaries in the absence of CL on both ovaries were classified as cysts. An inactive ovary was defined as an ovary with follicles smaller than 0.5 cm in the absence of a CL. The remaining cows were divided into those with a CL on at least one ovary and cows with no CL on either ovary in presence of follicles bigger than 0.5 cm (CL and NON-CL groups).

Vaginoscopy was conducted utilizing a metallic duckbill type speculum (KRUUSE Vaginal speculum, Langeskov, Denmark) after ultrasound scanning. The vaginal discharge score was classified on the following categories: PVD0—absence of mucus or presence of clear mucus, PVD1—clear mucus containing small particles of pus, PVD2—mucus with less than 50% pus content, or PVD3—mucus with greater than 50% pus content.

After vaginoscopy, endometrial sampling was conducted using the cytobrush technique (Cervical Brush, Zarys International Group, Poland). The cytological brush was affixed to a mandrel and introduced into a sterile stainless-steel catheter. To mitigate the likelihood of contamination from the vagina and cervix, an additional safeguard in the form of a glove for rectal palpation was utilized. The entire apparatus was inserted into the vaginal canal and passed through the cervix, with the glove being punctured at the cranial end of the cervix. The sample was acquired by diligently rotating the brush clockwise on the endometrium, caudal to the uterine bifurcation. Each cytobrush was rolled onto duplicate slides. Subsequently, the slides were allowed to air-dry and were subjected to a Romanowski-type staining. Each slide was meticulously examined under a light microscope at a magnification of 400×, and the proportion of PMN was determined based on the examination of 300 cells [4].

Reproductive data were collected for each cow from calving to 210 days postpartum or until culling. The dataset included records of observed estrus, artificial inseminations, and pregnancy diagnoses. At 21–29 days postpartum (median 24 days), uterine involution was assessed by measuring cervical and uterine horn diameters via ultrasonography. Ovarian structures, including corpus luteum, follicles, and cysts, were evaluated to determine cyclicity resumption.

Heat Detection Risk, defined as the probability of a cow being detected in heat for the first time postpartum, was analyzed up to 150 days postpartum using survival analysis. First Service Conception Rate (FSCR), the proportion of cows that conceived from their first post-calving insemination, was calculated based on outcomes of initial breeding attempts from 60 to 210 days in milk (DIM).

Pregnancy diagnoses were conducted at 30 days post-insemination. Pregnancy Risk by 210 days postpartum, representing the proportion of cows confirmed pregnant by 210 days post-calving, was determined through survival analysis. Cows that did not conceive following insemination were monitored and re-inseminated at the next observed estrus.

Reproductive tract case disease definition

The disease definition for each case was classified using two distinct criteria, as illustrated in Fig. 1. The first criterion, referred to as Model 1, cows were classified as healthy (H; <5% PMN and PVD0), SE (≥5% PMN and PVD0), PVD1 (irrespective of PMN%), PVD2 (irrespective of PMN%), and PVD3 (irrespective of PMN%). The second criterion, known as Model 2, cows were classified as H (<5% PMN and PVD0), SE (≥5% PMN and ≤PVD1), and CE (≥5% PMN and >PVD1). In Model 2, cows exhibiting <5% PMN and greater than or equal to ≥PVD1 in number (a total of 3 cows) were excluded. This removal was due to the limited number of cows that could be allocated to a distinct group for the purpose of statistical modeling.

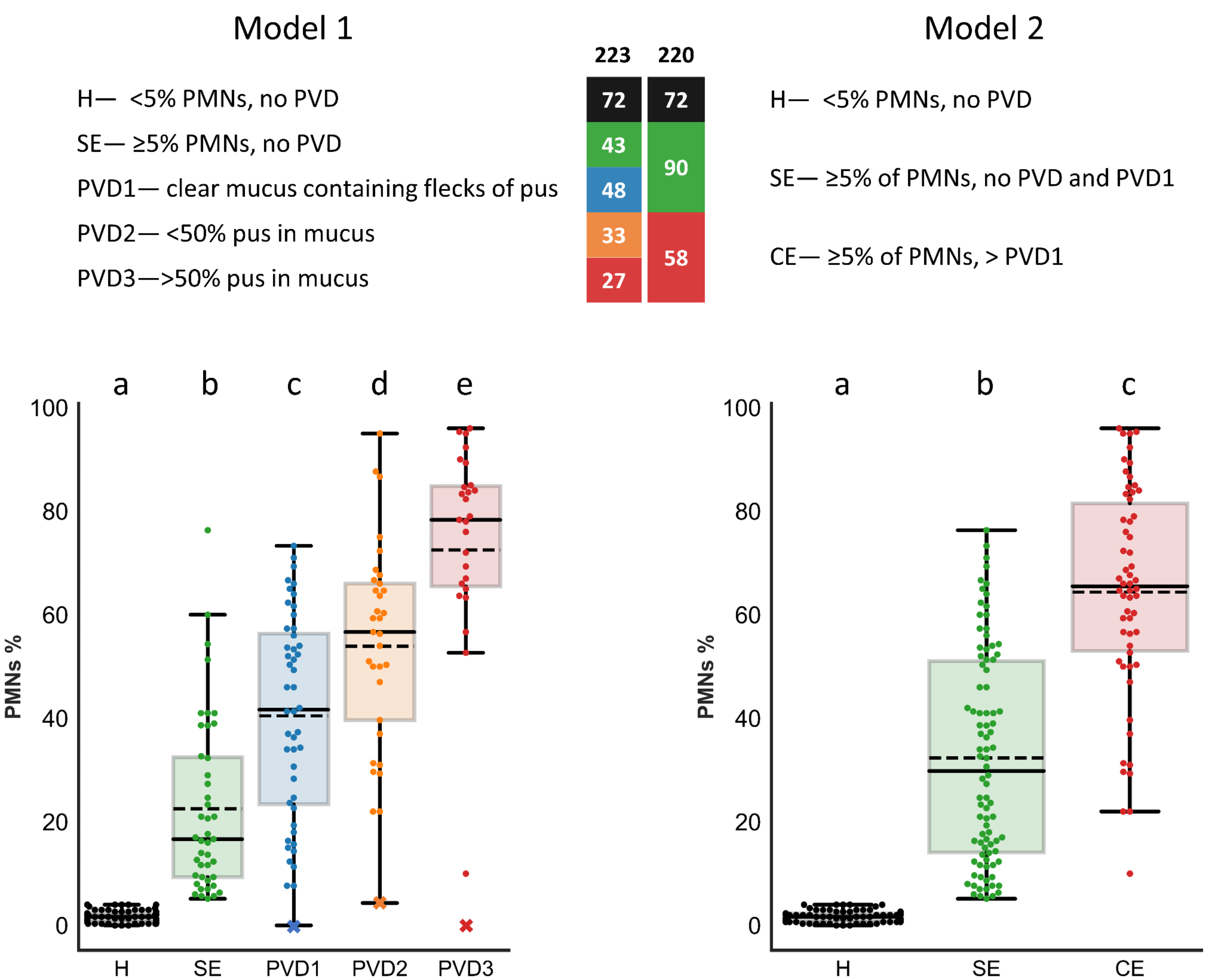

Fig. 1 Distribution of purulent vaginal discharge (PVD) scores and percentages of polymorphonuclear leukocytes (PMNs) in endometrial smears from Polish Holstein cows, classified according to two distinct diagnostic criteria (Models 1 and 2) in the fourth week postpartum. In Model 1, cows were classified as healthy (H; <5% endometrial PMN and no PVD), subclinical endometritis (SE; ≥5% PMN and PVD0), and into distinct PVD categories (PVD1 with flecks of pus, PVD2 with mucopurulent discharge, or PVD3 with purulent discharge, irrespective of endometrial PMN%). In Model 2, cows were categorized as H (<5% PMN and PVD0), SE (≥5% PMN and ≤PVD1), and clinical endometritis (CE; ≥5% PMN and >PVD1) Different superscripts (a to e) indicate a significance level of $p<0.05$

In this study, therapeutic interventions for PVD, CE, or SE were deliberately omitted, and the farm owner remained uninformed about the animals' specific diagnoses. This approach was strategically adopted to eliminate treatment-induced biases, thus preserving the natural disease progression. This methodology enhances the validity of our findings by providing an unaltered representation of these conditions in dairy cows within conventional farming environments, crucial for ensuring the study integrity and the applicability of its results to practical dairy management scenarios.

Statistical analyses

Data were gathered from the VMS and stored in Excel files. Data wrangling and analyses were carried out utilizing Python (Version 3.7, Rossum G, Drake FL. Python 3 Reference Manual. Scotts Valley, CA: CreateSpace; 2009), R (Version 4.1.0, R Development Core Team, Vienna, Austria, 2008), and JASP (JASP Team, Version 0.16.1, 2022). The assumption of normality for continuous parameters was evaluated using the Shapiro–Wilk test, whereas the equality of variances was assessed using the Levene's test. The frequencies of diseases and ovarian structures underwent analysis through the utilization of contingency tables and Chi-Squared tests. The examination of diameters pertaining to the reproductive tract was performed via the Kruskal-Wallis test, followed by the application of the Bonferroni correction. The mean daily milk yields were calculated as the average of all kilograms of milk obtained by the VMS within 30-, 60-, 90-, and 120-days postpartum within each group being studied. The determination of peak milk yield involved the computation of the highest mean over a span of seven consecutive lactation days. Linear regression and the Kruskal-Wallis test with Bonferroni correction were employed to analyze the mean daily milk yields for 30, 60, 90, and 120 days, and peak milk yield. The proportion of cows with no heat detected and the proportion of open cows were analyzed by the Kaplan-Meier survival analysis using the Tarone-Ware test to calculate hazard ratios (HR). Censor criteria were cows that left the herd for any reason before confirmation of pregnancy or cows remaining open at 210 days postpartum. The Pearson correlation coefficient was used to assess the correlation between the size of the left and right uterine horns in all studied groups. Logistic regression analysis was employed to compute the odds for FSCR. Initially, we considered factors such as dystocia and the occurrence of clinical diseases during lactation. We weighed the possibility of including diseases as a fixed or random value in the model, individually and combined as a binomial value. Ultimately, the final models included only the significant factor of dystocia and parity, while excluding the effect of diseases during lactation as it was non-significant. Models were tested using Akaike Information Criterion (AIC), McFadden R^2, Nagelkerke R^2, Tjur R^2, and Cox & Snell R^2. A significance level of $p \le 0.05$ was set for all analysis.

Table 1 Cervix measurement. measurement.of cervix diameter and diameters of previously pregnant and non-pregnant uterine horns, based on ultrasound examinations performed on Polish Holstein cows in the fourth week postpartum

	Cervix diameter	Previously not pregnant uterine horns diameter	Previously pregnant uterine horns diameter
Model 1	mm ± SD	mm ± SD	mm ± SD
H	35.47 ± 4.72 [a]	28.57 ± 5.22 [a]	30.94 ± 5.16 [a]
SE	36.16 ± 5.07 [a]	28.70 ± 4.57 [a]	31.47 ± 4.58 [a]
PVD1	36.65 ± 4.75 [a]	29.27 ± 5.27 [a]	32.06 ± 5.38 [a]
PVD2	37.85 ± 3.99 [ab]	32.94 ± 4.70 [b]	36.03 ± 5.41 [b]
PVD3	40.41 ± 5.20 [b]	34.00 ± 4.83 [b]	37.96 ± 5.47 [b]
Model 2			
H	35.47 ± 4.72 [a]	28.57 ± 5.22 [a]	30.94 ± 5.16 [a]
SE	36.42 ± 4.91 [a]	28.96 ± 4.94 [a]	31.73 ± 5.01 [a]
CE	39.05 ± 4.78 [b]	33.57 ± 4.69 [b]	37.12 ± 5.42 [b]

In Model 1 ($n = 223$), cows were classified as healthy (H; <5% endometrial polymorphonuclear cells [PMN] and no purulent vaginal discharge [PVD]), subclinical endometritis (SE; ≥5% PMN and PVD0), and various PVD categories (PVD1 with flecks of pus, PVD2 with mucopurulent discharge, or PVD3 with purulent discharge, irrespective of endometrial PMN%). In Model 2 ($n = 220$), cows were categorized as H (< 5% PMN and PVD0), SE (≥ 5% PMN and ≤ PVD1), and clinical endometritis (CE; ≥5% PMN and > PVD1). Different superscripts (a and b) within columns indicate a significance level of $p < 0.05$

Results

Prevalence of diseases

A total of 223 cows (85 primiparous and 135 multiparous) were subjected to examination within the time frame of 21 to 29 days after parturition (with a median of 24 days). The prevalence of reproductive tract inflammatory diseases classified as Models 1 and 2 are shown in Fig. 1. Primiparous cows experienced a greater incidence of SE (23.5%) and PVD3 (17.6%) but exhibited a lower prevalence of PVD1 (20.0%) and PVD2 (14.1%) in comparison to multiparous cows (SE—16.7%, PVD1—22.5%, PVD2—15.2%, PVD3—8.7% ($p = 0.01$)).

Additionally, it is noteworthy that the prevalence of clinical diseases within the studied cow population was considerable. The numerical breakdown for each condition was as follows: 24 cows (10.8%) milk fever, 10 cows (4.5%) lameness, 45 cows (20.2%) mastitis, 36 cows (16.1%) dystocia, 9 cows (4%) retained placenta, and 15 cows metritis (6.7%). Notably, all cows with metritis had a previous history of dystocia.

Reproductive ultrasonography

Model 1. as presented in Table 1, shows the uterine horn and cervix diameters across all groups. It was observed that the diameters of both the cervix and the previously pregnant and non-pregnant uterine horns were greater in the PVD3 group compared to the H and SE cows ($p < 0.001$). The correlation coefficients, for the diameters of both the previously pregnant and non-pregnant horns were found to be the highest in the H group ($r = 0.93$), lowest in PVD3 ($r = 0.78$), and similar in SE ($r = 0.85$), PVD1 ($r = 0.89$), and PVD2 ($r = 0.89$) ($p < 0.001$ for all).

Model 2. also presented in Table 1, shows the uterine horn and cervix diameters across all groups. It was noted that the diameters of both the cervix and the previously pregnant and non-pregnant uterine horns all were greater in the CE group compared to the H and SE cows ($p < 0.001$). The correlation coefficients for the diameters of both the previously pregnant and non-pregnant horns were observed to be the highest in the H group ($r = 0.93$), followed by SE ($r = 0.88$), and the lowest in the CE group ($r = 0.84$) ($p < 0.001$ for all).

Between 21 and 29 days postpartum, a total of 48% of the cows displayed a CL on either ovary. The incidence of ovarian structures among groups of cows, as per the criteria outlined in Models 1 and 2, is illustrated in Table 2.

Reproductive tract inflammatory disease and milk yield

Model 1. Figure 2 illustrates the mean milk yield of each health status clustered by parity. Multiparous cows with SE produced more milk from 60 to 120 days postpartum in comparison to cows with PVD3 ($p < 0.05$). In primiparous cows, H and SE cows produced more milk than PVD1 and PVD3 cows ($p < 0.05$) in first 30 days postpartum. The peak milk yield was not different among groups in multiparous cows while primiparous PVD1 animals had a lower peak milk yield than H cows ($p < 0.05$) (Fig. 2).

Table 2 Assessment of ovarian activity. Criterium based on the presence of corpus luteum (CL), absence of CL with follicles larger than 0.5 cm (NON-CL), presence of ovarian cysts, and inactive ovaries with minimal follicular activity

		CL	NON-CL	Cysts	Inactive ovaries
Model 1	p-value	0.046	0.045	0.174	0.899
Total	N (%)	107 (48.0)	91 (40.8)	13 (5.8)	12 (5.4)
H	n (%)	41 (56.9) [a]	21 (29.2) [a]	7 (9.7)	3 (4.2)
SE	n (%)	13 (30.2) [b]	25 (58.1) [b]	2 (4.7)	3 (7.0)
PVD1	n (%)	20 (41.7) [ab]	21 (43.8) [ab]	4 (8.3)	3 (6.2)
PVD2	n (%)	18 (54.5) [ab]	14 (42.4) [ab]	0 (0.0)	1 (3.0)
PVD3	n (%)	15 (55.6) [ab]	10 (37.0) [ab]	0 (0.0)	2 (7.4)
Model 2	p-value	0.022	0.019	0.053	0.780
Total	N (%)	105 (47.7)	91 (41.3)	12 (5.5)	12 (5.5)
H	n (%)	41 (56.9) [a]	21 (29.2) [a]	7 (9.7)	3 (4.2)
SE	n (%)	33 (36.7) [b]	46 (51.1) [b]	5 (5.5)	6 (6.7)
CE	n (%)	31 (53.4) [ab]	24 (41.4) [ab]	0 (0)	3 (5.2)

In Model 1 ($n = 223$), cows were categorized into: healthy (H; <5% endometrial polymorphonuclear cells [PMN] and no purulent vaginal discharge [PVD]), subclinical endometritis (SE; ≥5% PMN and PVD0), and various PVD categories (PVD1 with flecks of pus, PVD2 with mucopurulent discharge, or PVD3 with purulent discharge, regardless of endometrial PMN%). Model 2 ($n = 220$) classified cows as H (<5% PMN and PVD0), SE (≥5% PMN and ≤PVD1), and clinical endometritis (CE; ≥5% PMN and >PVD1). Different superscripts (a and b) within columns indicate a significance level of $p < 0.05$

Model 2. Figure 3 illustrates the mean milk yield of each health status clustered by parity. Healthy and SE multiparous cows produced more milk than cows with CE from 30 to 120 days postpartum ($p < 0.001$). For primiparous cows, the mean milk yield in the SE group was comparable to the CE group. However, healthy primiparous cows maintained a consistently higher level of milk production up until day 120 in comparison to the other groups ($p < 0.05$). Contrastingly, in multiparous cows, no differences in milk yield were observed between H and SE within 120 days postpartum. However, both primi- and multiparous cows suffering from CE had lower milk yield ($p < 0.05$) than H and SE throughout the entire observation period. Furthermore, in primiparous H cows, the peak milk yield was higher than in those with SE and CE ($p < 0.05$), a difference that was not observed in multiparous cows ($p = 0.21$) as shown in Fig. 3.

Reproductive tract inflammatory disease and reproductive performance

Hazard ratio for the risk of heat detection for 150 days postpartum

Model 1. Figure 4 illustrates the HR for heat detection of Model 1. The SE group had a HR for heat detection of 0.77 (95% confidence interval (CI): 0.48–1.25, $p = 0.3$), indicating a 23% increased risk of delayed first heat detection compared to H cows. The PVD1 group had a HR for heat detection of 0.69 (CI: 0.44–1.09, $p = 0.13$), indicating a 31% lower risk of first heat detection compared to H cows. The PVD2 and PVD3 groups had HR for heat detection of 0.52 (CI: 0.31–0.89, $p = 0.01$) and 0.80 (CI: 0.47–1.35, $p = 0.40$), respectively, indicating a 48 and 20% lower risk of delayed first heat detection when compared to H animals. Comparing the groups based on their median of first heat detection, PVD2 was observed to have the highest median of 83 days (CI: 69–99), followed by PVD3 with a median of 78 days (CI: 52–93), and SE with a median of 77 days (CI: 58–97). Healthy cows have the lowest median of first heat detection of 49 days (CI: 46–66), followed by PVD1 with a median of 56 days (CI: 49–84).

Model 2. Figure 5 illustrates the HR for heat detection of Model 2. The SE group had a HR for heat detection of 0.72 (CI: 0.59–1.16, $p = 0.27$), indicating a 28% increased risk of delayed first heat detection compared to H cows. The CE group had a HR for heat detection of 0.63 (CI: 0.42–0.93, $p = 0.02$), indicating a 37% lower risk of delayed first heat detection. Comparing the groups based on their median of first heat detection, H has the lowest median of 49 days (CI: 46–66), followed by SE with a median of 68 days (CI: 50–81), and CE with a median of 77 days (CI: 69–90).

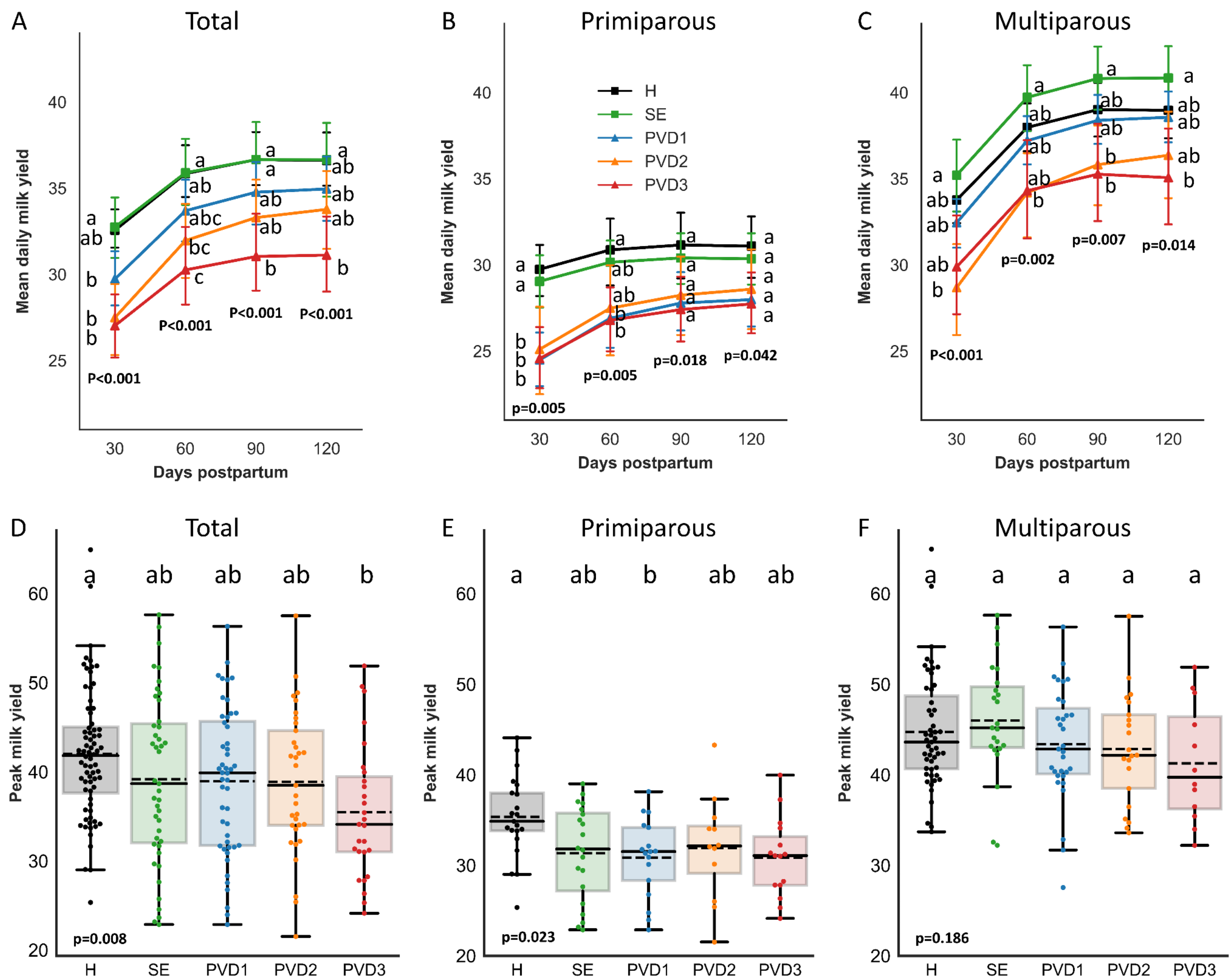

Fig. 2 Milk production trends in Polish Holstein cows classified by Model 1. Trends in milk production for Polish Holstein cows classified by Model 1 from a single dairy, shown overall ($n = 223$) and categorized by parity ($n = 85$ primiparous and $n = 138$ multiparous). Cows were examined in the fourth week post-partum and classified as healthy (H; <5% endometrial polymorphonuclear cells [PMN] and no purulent vaginal discharge [PVD]), subclinical endometritis (SE; ≥5% PMN and PVD0), and into distinct PVD categories (PVD1 with flecks of pus, PVD2 with mucopurulent discharge, and PVD3 with purulent discharge, regardless of endometrial PMN%). Different superscripts (a and b) indicate a significance level of $p < 0.05$

Hazard ratio of pregnancy risk up to 210 days postpartum

Model 1. Figure 4 illustrates the HR for pregnancy of Model 1. The HR of pregnancy differed between the PVD2 and H cows, with an HR of 0.53 (CI: 0.31–0.89, $p = 0.01$), indicating a 47% increased risk of delayed pregnancy in PVD2 cows. The SE group had an HR of 0.78 (CI: 0.48–1.25, $p = 0.30$), representing a 22% lower risk of pregnancy in comparison to H cows. The PVD1 and PVD3 groups had HR of pregnancy of 0.69 (CI: 0.44–1.09, $p = 0.11$) and 0.80 (CI: 0.47–1.35, $p = 0.40$), respectively, indicating a 31 and 20% lower risk of pregnancy than H cows. Comparing the groups based on their median calving to pregnancy intervals, it was observed that PVD2 exhibited the highest median interval of 167 days (CI: 155–179), whereas PVD3 followed closely with a median interval of 157 days (CI: 104–209). In contrast, H and SE displayed the lowest median intervals of 131 days (CI: 104–152) and 126 days (CI: 111–126), respectively. Notably, PVD1 recorded a median interval of 160 days (CI: 126–206), which was found to be intermediate between the values for H and PVD2.

Model 2. Figure 5 illustrates the HR for pregnancy of Model 2. The HR of pregnancy differed between the CE and H groups, with an HR of 0.63 for CE (CI: 0.41–0.95, $p = 0.02$), indicating a 37% increased risk of delayed pregnancy in CE in comparison to H cows. The SE group had a HR of 0.73 (CI: 0.50–1.06, $p = 0.09$), representing a 27% lower risk of pregnancy in comparison to H cows. Comparing the groups based on their median calving to pregnancy intervals, we can see that H has the lowest median interval of 131 days (CI: 104–152), followed by SE with a

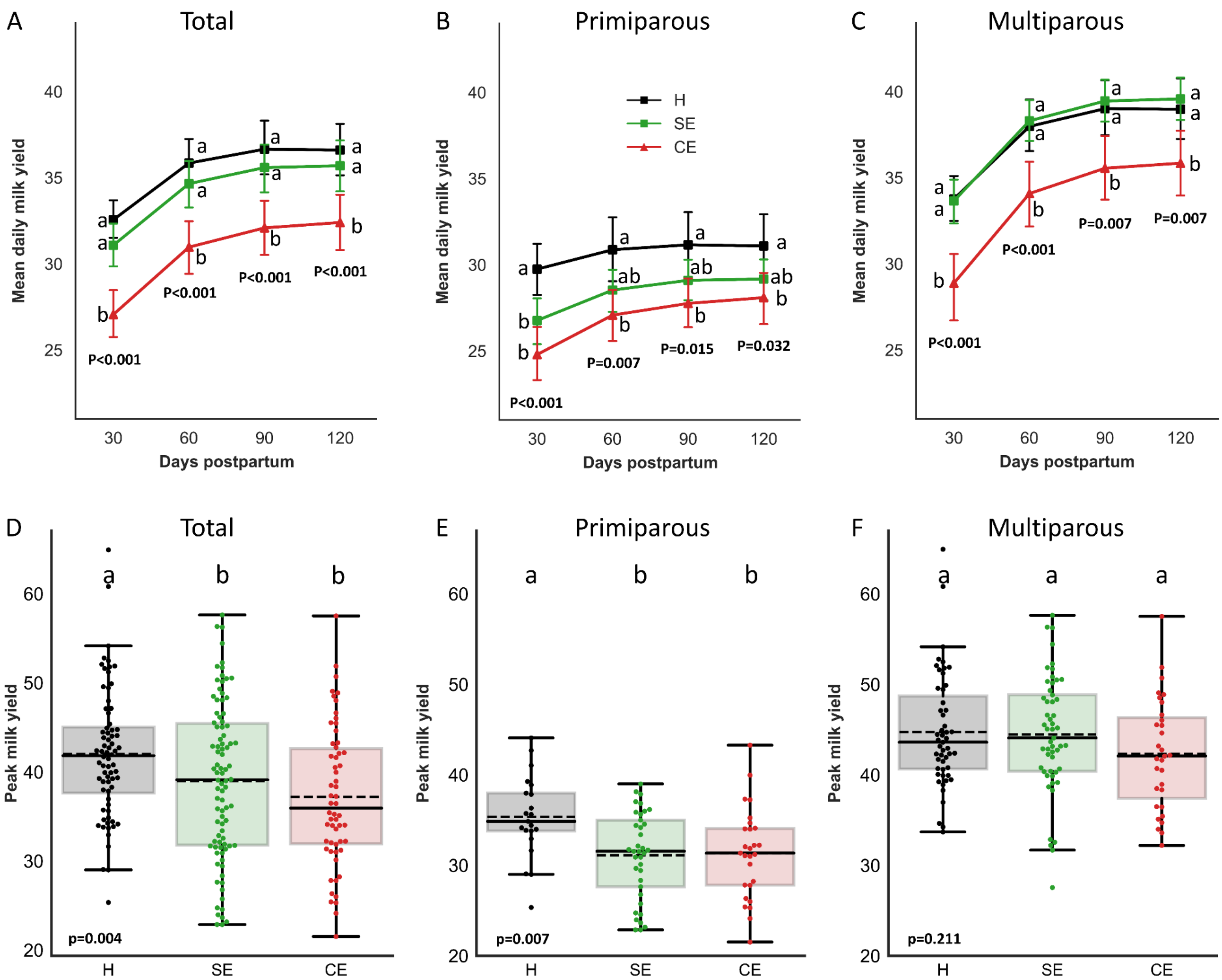

Fig. 3 Milk production trends in Polish Holstein cows classified by Model 2. Trends in milk production for Polish Holstein cows classified by Model 2 from a single dairy, shown overall ($n = 220$) and categorized by parity ($n = 85$ primiparous and $n = 135$ multiparous). Cows were examined in the fourth week postpartum and classified as healthy (H; <5% polymorphonuclear cells [PMN] and no purulent vaginal discharge [PVD]), subclinical endometritis (SE; ≥5% PMN and ≤ PVD1), and clinical endometritis (CE; ≥5% PMN and > PVD1). Different superscripts (a and b) indicate a significance level of $p < 0.05$

median interval of 139 days (CI: 119–186), while CE has the highest median interval of 162 days (CI: 150–174).

First service conception rate

Model 1. The FSCR was lower in SE (OR = 0.37, $p = 0.02$), PVD1 (OR = 0.31, $p = 0.007$), and PVD2 (OR = 0.37, $p = 0.03$) than in H cows. Interestingly, the FSCR was not lower in PVD3 (OR = 0.51, $p = 0.17$) in comparison to H cows. Multiparous cows had lower FSC than primiparous (OR = 0.51, $p = 0.02$). All the other comparisons clustered by parity are shown in Table 3.

Model 2: The FSCR in SE (OR = 0.34, $p = 0.002$) and CE (OR = 0.45, $p = 0.09$) were lower than in H cows. Multiparous cows had lower FSCR than primiparous (OR = 0.51, $p = 0.03$). All the other comparisons clustered by parity are shown in Table 3.

Discussion

We assessed the impact of reproductive tract inflammatory disease classification criteria on the productive and reproductive performance of Polish Holstein cows in a single dairy farm. For this purpose, we developed two analytical models. In the first model, cows were categorized as H, SE, and different intensities of PVD including PVD1, PVD2, and PVD3. The second, more traditional model, classified cows as H, SE, and CE. Our analysis revealed that cows classified under PVD2, PVD3, and CE exhibited notably larger cervical and uterine horn diameters compared to those in the SE and H categories. This was accompanied by a reduced rate of ovarian activity. An interesting observation was that cows with PVD3 and those categorized as CE showed a decrease in milk production. Interestingly, while the PVD2 classification adversely impacted fertility, PVD3 did not demonstrate

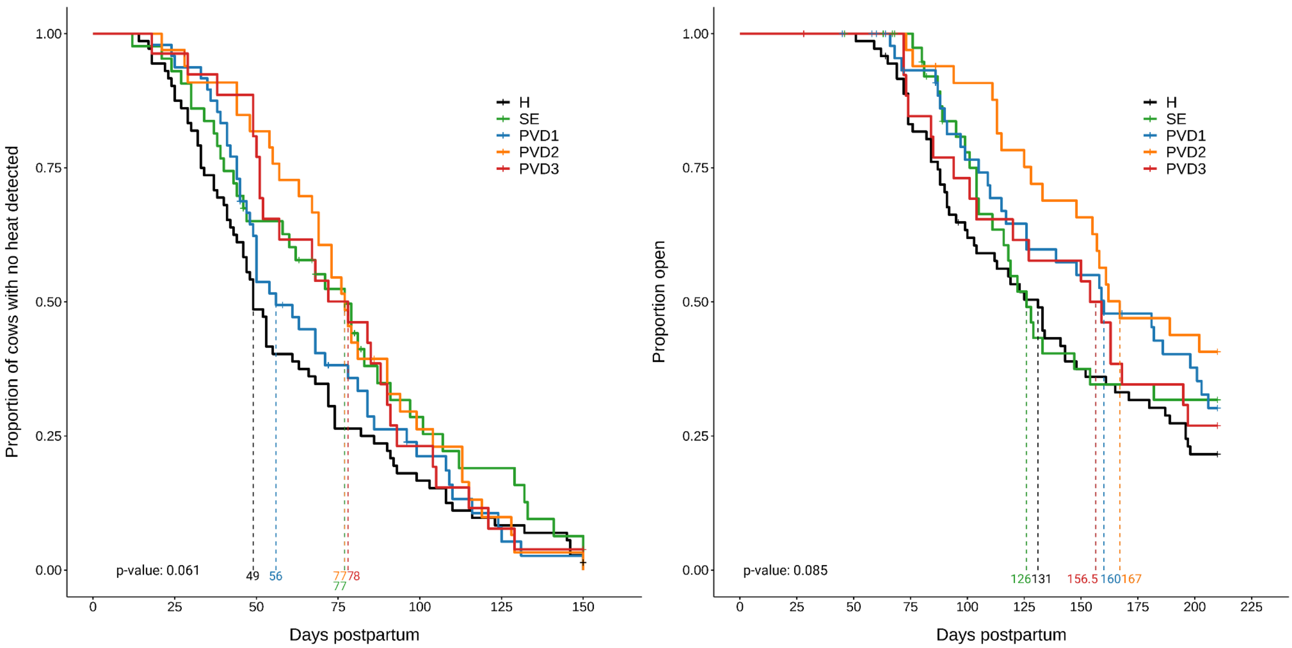

Fig. 4 Kaplan-Meier curves for heat detection and pregnancy risks (Model 1). Two hundred and twenty-three Polish Holstein cows were examined in the fourth week postpartum and classified as healthy (H; <5% endometrial polymorphonuclear cells [PMN] and no purulent vaginal discharge [PVD]), subclinical endometritis (SE; ≥5% PMN and PVD0), and into distinct PVD categories (PVD1 with flecks of pus, PVD2 with mucopurulent discharge, or PVD3 with purulent discharge, irrespective of endometrial PMN%)

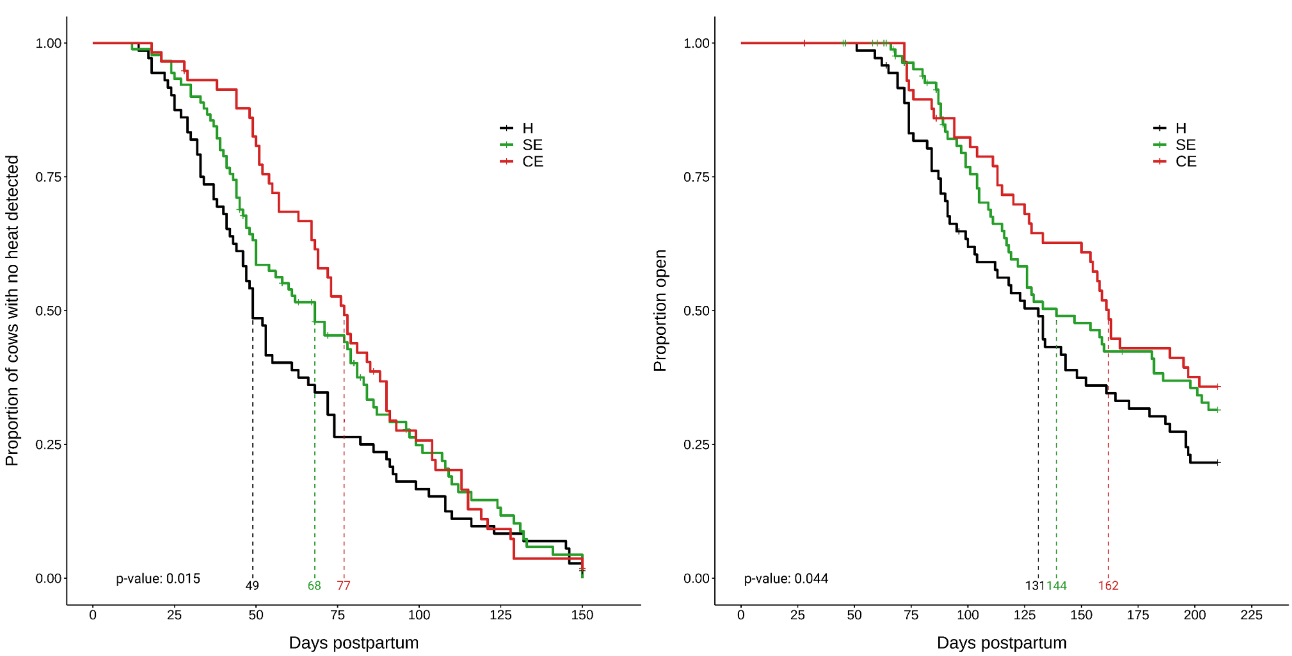

Fig. 5 Kaplan-Meier curves for heat detection and pregnancy risks (Model 2). Two hundred and twenty Polish Holstein cows were examined in the fourth week postpartum and classified as healthy (H; <5% polymorphonuclear cells [PMN] and no purulent vaginal discharge [PVD]), subclinical endometritis (SE; ≥5% PMN and ≤ PVD1), and clinical endometritis (CE; ≥5% PMN and > PVD1)

a negative effect on fertility metrics. In Model 2, CE was associated with impaired heat expression, a lower FSCR, and diminished pregnancy risk to 210 days postpartum. In both models, SE had a lower FSCR, although this was not accompanied by a lower heat expression to 150 days postpartum nor pregnancy risk to 210 days postpartum in comparison to H cows.

Our results revealed an unexpected pattern in the relationship between the severity of vaginal discharge and reproductive performance. Despite having the most

Table 3 Results from logistic regression analyses predicting the first service conception rate (FSCR) in dairy cows

	Estimate	SE	Odds Ratio	*p*-value	95% CI of OR	
Model 1					Lower	Upper
Primiparous	ref					
Multiparous	-0.68	0.31	0.51	0.029	0.28	0.93
H	ref					
SE	-0.99	0.43	0.37	0.023	0.16	0.87
PVD1	-1.18	0.43	0.31	0.007	0.13	0.72
PVD2	-0.99	0.48	0.37	0.038	0.15	0.95
PVD3	-0.67	0.49	0.51	0.173	0.20	1.34
Model 2						
Primiparous	ref					
Multiparous	-0.67	0.31	0.51	0.030	0.28	0.94
H	ref					
SE	-1.07	0.35	0.34	0.002	0.17	0.68
CE	-0.79	0.38	0.45	0.039	0.21	0.96

In Model 1 ($n=223$), Polish Holstein cows were classified into three groups: healthy (H; characterized by <5% endometrial polymorphonuclear cells [PMN] and absence of purulent vaginal discharge [PVD]), subclinical endometritis (SE; marked by ≥5% PMN and PVD0), and various PVD categories (including PVD1 with flecks of pus in the vaginal discharge, PVD2 with mucopurulent vaginal discharge, and PVD3 with purulent vaginal discharge, irrespective of endometrial PMN%). In Model 2 ($n=220$), the classification included healthy (H; <5% PMN and PVD0), SE (≥5% PMN and ≤PVD1), and clinical endometritis (CE; ≥5% PMN and >PVD1)

severe form of vaginal discharge and showing higher levels of inflammation, cows with PVD3 did not have significantly lower first service conception rates compared to healthy cows, while PVD1 and PVD2 groups did. This counterintuitive finding extends to heat detection timing as well, with PVD3 cows showing less delay than PVD2 cows. These results appear to contradict biological expectations and warrant careful consideration.

Several factors might contribute to this unexpected outcome. The visible severity of PVD3 likely prompted more intensive monitoring and supportive care, potentially mitigating negative impacts on fertility and enabling earlier heat detection. There may also be a survivor effect, where only the most resilient PVD3 cows remained in the study until breeding. This effect could be particularly pronounced given that PVD3 was the smallest group, containing only 27 cows. Additionally, it's worth noting that 30 animals were culled before the study commenced, which might suggest that these were cows experiencing the most severe post-partum complications. Consequently, the PVD3 group in our study may represent a subset of animals that, while exhibiting severe clinical signs, were robust enough to survive the immediate postpartum period. This selection bias could partly explain the anomalous results observed in this group. Furthermore, the intense inflammatory response in PVD3 cows, while initially more severe, might have been more effective at clearing the uterine infection, potentially resulting in a healthier uterine environment by the time of breeding and resumption of cyclicity. These factors combined could contribute to the counterintuitive reproductive performance observed in the PVD3 group.

We acknowledge that these explanations require further investigation to be fully validated. While we initially considered the incidence of concurrent diseases in our statistical analyses, we found that they did not significantly influence the outcomes we were measuring. As a result, these factors were not included in the final forms of our models. This finding is noteworthy in itself, as it suggests that the effects of uterine health status on reproductive performance may be robust even in the presence of other health challenges. However, it's important to note that the complex nature of reproductive physiology in dairy cows means that there may be other, unidentified factors at play.

The PVD-based model (Model 1) provides a detailed assessment of the severity of reproductive tract inflammatory disease, which is valuable for specific treatments and managerial interventions. However, it might introduce complexity in decision-making processes due to its multiple categories. In contrast, Model 2 offers a more straightforward approach to categorization, aiding in clear-cut management decisions, but it may overlook the nuances within the disease spectrum, potentially leading to over-generalized treatment strategies. It should be noted, diagnosing SE and CE is inherently more complex due to the necessity of endometrial cytology. This evaluation cannot be conducted immediately cow-side at the farm, nor does it provide quick results. The latter diagnostic method, while accurate, demands resources and expertise, which could be a limiting factor in some farm settings. Thus, the reliance on cytological evaluation for SE and CE can be seen as both an advantage as well as a disadvantage as it allows for a more precise diagnosis but also adds complexity and potential delays in the treatment process. Therefore, researchers and field veterinarians must weigh the benefits of detailed diagnostics against the practicality and resource requirements of such approaches when choosing an endometritis classification scheme.

We found that the SE prevalence was similar to PVD1, whereas the other two PVD scores occurred less frequently. In previous studies, the prevalence of SE varied from 11.1 to even 75.4% [5, 11, 14, 17]. Since in our study the prevalence of SE was 19.3%, our results are ranked on the lower end of prevalence findings. The prevalence of CE found in the present study was 26.3%, which is also at the lower end of previously reported results (between 25 and 47.5%) [22, 30–32]. Around half of the cows (48.4%) had ≥ PVD1 in the present study. Some discrepancies may reflect the ongoing debate about the definitions of CE and PVD. Additionally, not all studies differentiated PVD severity levels based on visual scoring of the discharge, and some even exclude PVD1 cases from their prevalence estimates [33]. Importantly, Model 1 included three degrees of PVD severity, determined through visual assessment at the external cervical os via vaginoscopy. Adopting this approach in future research could influence the findings, potentially leading to a higher number of confirmed PVD cases, which is crucial for consistent comparisons of CE/PVD prevalence across studies.

In primiparous cows, we observed a higher prevalence of SE and PVD3, whereas in multiparous cows, PVD1 was more common. It is established that parity impacts dairy cows' reproductive physiology, including uterine involution, ovarian function, and general metabolism, which encompasses milk production [34]. This physiological difference between primi- and multiparous cows may contribute to the varying prevalence of SE/PVD observed in our study. Furthermore, the development of SE/PVD might also result from delivery-related trauma to reproductive tract tissues and subsequent stress [35]. This stress can facilitate bacterial infection and chronic inflammation [36], potentially delaying the growth of the first postpartum dominant follicle and reducing the likelihood of successful insemination [37].

Comparative analysis with other studies reveals different prevalence rates for both SE and CE in postpartum dairy cows [5, 11, 14, 17]. Differences may be due to multiple factors, including the timing of examinations in the postpartum period, the PMN threshold applied, the health status of the animals examined, and differences in herd environment and management [34].

LeBlanc (2002) identified that a cervical diameter exceeding 7.5 cm > 20 days postpartum is indicative of CE and correlates with reduced fertility. However, they observed that the presence of a CL or dominant follicle was not associated with future fertility results [1]. Interestingly, another study found a higher percentage of PMNs in cows with a pre-ovulatory follicle at the 5th week postpartum, compared to those which had already resumed ovarian activity, as evidenced by the presence of a CL [38]. The latter study also focused on uterine involution, particularly on the degree of uterine tension and fluid accumulation in the uterine lumen, without considering the dimensions of the cervix or uterine horns. In contrast, Ernstberger (2019) established a link between visible vaginal discharge and reduced fertility in CE cows but found no correlation among ultrasonographic assessments of intrauterine content, cervical diameter, and pregnancy risk in CE cows [39]. Poock (2020) further explored the relationship between the dimensions of the cervix, uterus, and horns, and fertility in Jersey cows, revealing similar findings to our study. They concluded that larger cervical or uterine horn sizes at day 28 postpartum, negatively impact the likelihood of first service pregnancy and the resumption of ovarian activity [40]. In the present study, ultrasound examinations revealed that healthy cows more frequently possessed CL compared to those diagnosed with SE. Furthermore, Model 1 indicated that cervical and uterine horn diameters in PVD2 and PVD3 cows were significantly larger than in H and SE cows. A notable finding was the proportionally lower correlation between the diameters of the previously pregnant and non-pregnant horns in cows with higher scores of PVD. These results suggest that measurements of uterine horns could be a crucial factor in determining the severity of PVD and its subsequent impact on reproductive performance.

We found no substantial differences in milk yield between H and SE cows. However, our data indicates that multiparous SE cows had a numerically higher mean milk yield than healthy animals. This observation aligns with the report by Fourichon (2000) that SE does not invariably lead to a reduction in milk yield [41]. In contrast, Cheong (2011) found an association between higher milk production and the onset of SE in primiparous cows, a relationship that was not observed in multiparous cows [17]. McDougall (2011) reported lower milk yield in SE cows compared to healthy counterparts during the first 42 days of lactation [42]. In the present study, primiparous cows with PVD1 to 3 and multiparous cows with PVD2 and PVD3 exhibited a decrease in mean milk yield during the first 30 days postpartum in comparison to healthy cows. This aligns with Galvão (2010), who reported decreased milk yield in primiparous cows with CE. However, in contrast to their findings, our study showed an increase in milk yield in multiparous cows with CE. Interestingly, Galvão (2010) suggested that factors like a greater degree of negative energy balance and lower intracellular PMN glycogen levels are factors influencing milk production [43].

Subclinical endometritis cows had impaired FSCR but the moment of first visible heat and the pregnancy risk to 210 days postpartum were not affected. This is in contrast with the general consensus as observed in most studies, reporting a negative relationship between SE and reproductive performance in dairy cows [4, 5, 18]. While some research using lower SE thresholds reported adverse effects on reproductive performance [14], other studies highlighted the need for higher thresholds to assess the negative impact on reproduction [3, 43–45]. For instance, Galvão (2010) noted that using thresholds of 6.5 and 4% PMNs at 35- and 49-days postpartum respectively, led to increased time to pregnancy in SE cows. This aligns with findings by Cheong (2011), where multiparous cows with SE had a median of 44 additional days open compared to unaffected cows [17]. Similarly, Prunner (2014) observed that a diagnosis of SE resulted in extended periods between calving and conception [19]. The PVD classification employed in Model 1 demonstrated clearer results and more accurate predictions regarding reproductive outcomes. The strength of this approach lies in the ability to differentiate between various degrees of PVD, allowing for a more targeted approach in managing these conditions.

While our study focused on diagnostic criteria and their relationship to reproductive outcomes, it is crucial to consider the broader context of ongoing research into treatments for improving fertility in cows suffering from uterine diseases such as metritis, clinical and subclinical endometritis. Recent studies have explored various innovative approaches to enhance reproductive performance in dairy cows with uterine diseases.

For instance, Escandón (2020) demonstrated the effectiveness of intrauterine ozone therapy (IUTO) in reducing subclinical endometritis and improving reproductive performance in postpartum dairy cows. This study highlighted the significant reduction in polymorphonuclear leukocytes and subclinical endometritis percentages, alongside improved conception rates, when using IUTO as a treatment method [46]. Haimerl et al. (2017) conducted a meta-analysis on the use of antibiotics for treating metritis in dairy cows, emphasizing the benefits of ceftiofur in reducing metritis prevalence. While antibiotics remain a cornerstone in managing uterine infections, the emergence of antibiotic resistance necessitates the exploration of alternative treatments. In this context, the use of probiotics has emerged as a promising alternative for treating uterine diseases [47]. Genís (2018) conducted a study using intravaginal lactic acid bacteria (LAB) as a preventive measure against metritis. They found that precalving administration of LAB reduced metritis prevalence and modulated the immune response in dairy cows. Furthermore, Peter (2018) investigated the influence of intrauterine administration of Lactobacillus buchneri on cows with subclinical endometritis. Results showed improved reproductive performance and a reduction in pro-inflammatory endometrial mRNA expression, suggesting that targeted probiotic treatments could be effective in managing subclinical endometritis [48].

Conclusion

Our study elucidated key relationships between uterine involution, ovarian status, and the diagnoses of SE, PVD, and CE, highlighting their impact on the reproductive status of dairy cows. We found that the presence of a CL in the fourth week postpartum was more common in H cows, suggesting quicker reproductive cyclicity resumption. Additionally, a positive correlation was observed between the diameters of the cervix and uterine horns and the severity of reproductive tract inflammatory diseases, indicating that larger diameters might signal more severe conditions. The evaluation of two diagnostic models showed that Model 1, which utilizes the number of PMNs in smears and the presence of vaginal discharge, provided a detailed assessment of uterine health and was predictive of milk yield and fertility outcomes. Our findings underscore the importance of precise anatomical and clinical assessments for managing reproductive health, suggesting that detailed evaluations of vaginal discharge and reproductive tract measurements can significantly aid in making informed decisions about treatment and management of dairy cows, thereby enhancing reproductive outcomes and overall herd productivity.

Abbreviations

CL	Corpus Luteum
NON-CL	Absence of Corpus Luteum with follicles larger than 0.5 cm
PVD	Purulent Vaginal Discharge
PMN	Polymorphonuclear Cells
CE	Clinical Endometritis
SE	Subclinical Endometritis

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Author contributions

D. T. conceptualized the study and curated the data along with W. B. D. T. performed the formal analysis and developed the software. W. B. secured the funding and managed resources. D. T. and W. B. jointly developed the methodology. Project administration was handled by W. B., D. T., Z. P., and M. K. Supervision was provided by W. B., D. T., G. O., and O. B. P. D. T. validated and visualized the data. D. T. wrote the original draft, and W. B., D. T., G. O., O. B. P., Z. P., and M. K. reviewed and edited the manuscript.

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Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All procedures of this observational cohort study were carried out in accordance with the Polish Animal Protection Law (Journal of Laws of 21 February 2005, No. 33, item 289) and after obtaining approval from the Local Ethics Committee for Animal Experiments in Olsztyn (decision No. 49/2016).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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