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Retained Placenta as a Potential Source of Mastitis Pathogens in Dairy Cows

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Featured Application: This study identifies potential mastitis pathogens, in the uterine fluid of dairy cows presenting placental retention, that may be widespread in confined farm environments.

Abstract: (1) Background: Retained placenta (RP) and mastitis are relevant diseases in dairy cows. This study mainly aimed to evaluate the contamination of uterine fluid by mastitis pathogens in dairy cows presenting RP. (2) Methods: Uterine fluids were sampled at RP diagnosis (89 ± 15 h after calving) from 5 primiparous and 10 (parity: 2–5) multiparous cows. The real-time PCR methodology was used to identify 15 mastitis and uterine pathogens. Results were analyzed using multivariate logistic regression, including the factors fever and parity. (3) Results: The prevalence of *Escherichia coli* was 93.3% (95% CI: 70.2–98.9%); *Staphylococcus* spp. (93.3%; 95% CI: 70.2–98.9%), yeasts (92.9%; 95% CI: 68.5–98.7%), *Trueperella pyogenes*/*Peptoniphilus indolicus* (80.0%; 95% CI: 54.8–93.0%), *Streptococcus uberis* (78.6%; 95% CI: 52.4–92.4%) and *Streptococcus dysgalactiae* (57.1%; 95% CI: 32.6–78.6%) comprised the largest proportions of pathogens in uterine contamination. *Strep. uberis* was related to the presence of fever (relative risk: 1.6; 95% CI: 1.0–2.8; $p = 0.05$). (4) Conclusions: Dairy cows with RP can be a relevant source of mastitis pathogens in farms. Only *Strep. uberis* was linked to clinical signs of infection. A high proportion of yeasts was observed in uterine fluids. Further research is needed to evaluate the real impact of RP on mastitis prevalence in dairy herds.

Keywords: retained fetal membranes; mastitis; dairy bovine farms; bacterial contamination; *Streptococcus uberis*

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1. Introduction

Bovine uterine infections and mastitis are two of the most important causes of economic losses in dairy farms [1,2]. Environmental bacteria play an important role in the contamination of uterine lumen from the vagina through to the cervical canal in the immediate postpartum period, and can lead to uterine infections [3]. Bacteria causing uterine infections after calving come from the feces and from a contaminated environment, with the establishment of an infection depending on the animal's immune status, bacterial load and the pathogenicity of the invading microorganisms [4–6].

Retained placenta (RP) is a reproductive–metabolic disease [7], defined as a failure to expel fetal membranes within 12–24 h after calving. The persistence of placental membranes and lochia is a source of nutrients for bacterial growth. The economic consequence of RP, in addition to direct veterinary costs and impaired milk production, is the increased risk for further metabolic diseases and enhanced incidence of metritis and endometritis [8]. RP

enhances the severity of infection, as it is the perfect medium for bacterial growth [6]. The interactions among pathogenic, opportunist and microbiome species form a complex and highly dynamic process [2,9–11].

Uterine microbiota is indeed more diverse than once was thought, and due to the significant impact of the related reproductive disorders, its characterization has long been a focus of research. The techniques have included traditional culture-based techniques and more advanced molecular ones, such as real-time polymerase chain reaction (RT-PCR), denaturing gradient gel electrophoresis (DGGE) or ribosomal RNA clone libraries [12–14]. More recently, next-generation sequencing (NGS) technologies (sequencing of the rrs gene encoding 16S rRNA), allowing researchers to conduct in-depth sequencing and facilitating the analysis of thousands of sequences per sample, permit the description of previous unculturable bacteria of the complex uterine bacterial community [15–18].

The establishment of uterine microbiota is a normal and dynamic postpartum process. Less uterine microbial diversity and microbial dysbiosis with an increased abundance of several bacterial species such as *Bacteroides*, *Fusobacterium*, *Trueperella* and *Porphyromonas* contributes to uterine infection [19]. *Trueperella pyogenes*, *Escherichia coli*, *Bacillus* spp. and *Streptococcus uberis* are the bacteria most frequently isolated from the uterine content in cows presenting clinical endometritis [2]. Most of these bacteria are also mastitis pathogens. The etiological agents of mastitis include a variety of Gram-positive and Gram-negative bacteria, and can be environmental (e.g., *E. coli*, *Enterococcus* spp., coagulase-negative *Staphylococcus* (CNS), *Strep. uberis*, *Serratia marcescens*) or contagious (e.g., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma* spp.) [1]. In a previous study, *Strep. uberis* was simultaneously observed in both the genital tract (vaginal and uterine secretions) and udder, in 82.6% of cases [20]. The carriage of *Mycoplasma bovigenitalium* from the lochia of postpartum cows to their udder was also reported [21].

Nonetheless, bacterial data from uterine samples of primiparous and multiparous dairy cows presenting RP are limited (e.g., [4,22,23]). There is also scarce information on the relationship among bacterial species present in the uterine lumen during RP and the clinical signs observed, such as fever. We hypothesized that dairy cattle with RP may be a source of mastitis pathogens. Therefore, we aimed at evaluating the presence of mastitis pathogens in dairy cows with RP, using RT-PCR, and determining if the severity of the disease in terms of the presence of fever related to certain species. The agreement between RT-PCR and bacterial culture was also assessed.

2. Materials and Methods

2.1. Animals, Study Design and Sample Collection

Between 2020 and 2021, uterine fluid samples were taken, at 89 $\pm$ 15 h after eutocic calving, from 5 primiparous and 10 (parity: 2–5) multiparous Holstein-Friesian cows presenting with RP from seven dairy farms located in North Portugal. RP was diagnosed via a visualization of fetal membranes hanging from the vulva >24 h after calving and their unsuccessful removal after a brief tracking procedure conducted to confirm cotyledon–caruncle attachment. Transrectal palpation was avoided to minimize the risk of further contamination for sampling. Prior to sampling, rectal temperature was measured. Fever was considered if cows had a rectal temperature $\geq$39.5 °C, depression and anorexia. The cows presented healthy udders, and no antimicrobial treatment was carried out, at least from calving to sample collection.

Uterine fluid samples were collected at the time of diagnosis of RP using a sterilized uterine swab (Ref.: 17214/2950; Minitub Ibérica S.L., Barcelona, Spain). Prior cleaning and disinfection of the vulvar area were carried out. A pipette and pre-perforated cap were introduced into the uterine lumen in accordance with the manufacturer's instructions. These samples were transferred to a sterile AMIES transport medium to avoid dehydration until they arrived at an accredited (NP EN ISO/IEC 17025) laboratory (Segalab SA, Porto, Portugal). Each sample was immediately refrigerated and delivered to the laboratory within 24 h after collection.

2.2. Gene Detection by Polymerase Chain Reaction

Samples were analyzed by RT-PCR for *S. aureus*, *Staphylococcus* spp. (including all major coagulase-negative staphylococci), *Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*, *E. coli*, *Enterococcus* spp. (including *E. faecalis* and *E. faecium*), *Klebsiella oxytoca/K. pneumoniae*, *Serratia marcescens*, *Corynebacterium bovis*, *T. pyogenes/Peptoniphilus indolicus*, *Mycoplasma* spp., *Mycoplasma bovis*, yeasts, *Prototheca* spp. and the beta-lactamase (BlaZ) gene.

Briefly, microorganisms were characterized using Applied Biosystems™ VetMAX™ MastiType Multi Kit (ThermoFisher Scientific Inc., Waltham, MA, USA), which enables the detection of mastitis-causing pathogens using RT-PCR amplification using the DNA methodology, unique to each pathogen, based on the extraction of its DNA, in four separate PCR reactions. The results were interpreted, reported, and stored using Animal Health VeriVet Software available on ThermoFisher Cloud. All procedures were carried out in accordance with the manufacturer's instructions [24].

2.3. Bacterial Culture

Upon laboratory arrival, the uterine fluid samples were immediately and individually inoculated into three different mediums, Columbia Agar containing 5% sheep blood, Columbia CNA Agar containing 5% sheep blood (Gram-positive bacteria) and MacConkey Agar (Gram-negative bacteria), and then subsequently incubated at 37 °C for 24 h (max. 48 h) in aerobic conditions. Obtained isolates were identified through the VITEK®2 Gram Positive (GP) or Gram Negative (GN) identification card (BioMérieux S.A., Marcy l'Étoile, France) [25]. All procedures were carried out in accordance with the manufacturer's instructions.

2.4. Statistical Analyses

Results were analyzed via multivariate logistic regression to determine the association between the presence/absence of each pathogen and the factors fever and parity. Differences between groups were evaluated using a likelihood ratio test. Relative risk (RR) was determined.

Cohen's Kappa coefficient was used to test the range agreement between both methods of bacterial determination (RT-PCR and bacterial culture) and interpreted according to the level of agreement reported by McHugh (2012) [26].

A significance level of 0.05 was used to evaluate differences between groups. JMP® version Pro 16 statistical software was used for all analyses.

3. Results

3.1. Identification of Mastitis Pathogens and BlaZ Gene via RT-PCR and Their Association with Parity and Fever

The microorganisms identified using RT-PCR are described in Table 1. At least three species of mastitis pathogens or groups were detected in each cow. No significant association was observed between parity and fever with any pathogen, or with the blaZ gene ($p > 0.05$), except for *Strep. uberis*.

The following mastitis pathogens, *M. bovis*, *S. aureus*, *Prototheca* spp., *C. bovis*, *K. oxytoca/K. pneumoniae* and *S. marcescens*, were not detected via RT-PCR.

Fever was detected in 54.5% of the 11 cows with *Strep. uberis* in their uterine fluid. In the absence of this bacterium, no animal had fever (Figure 1). Therefore, the presence of *Strep. uberis* in the uterine lumen of cows with RP was revealed as a significant risk factor for fever occurrence (RR = 1.6; 95% CI: 1.0–2.8; $p = 0.05$).

Table 1. Association between parity and fever in terms of the prevalence of microorganisms and the beta-lactamase (BlaZ) gene isolated with RT-PCR from uterine fluid samples of cows with RP.

Bacteria Identified by RT-PCR	% Infected Cows (x/X)	95% CI	Parity	Fever
Escherichia coli	93.3 (14/15)	70.2–98.9	0.12	0.28
Staphylococcus spp.	93.3 (14/15)	70.2–98.9	0.12	0.28
Yeasts *	92.9 (13/14)	68.5–98.7	0.34	0.18
Trueperella pyogenes/*Peptoniphilus indolicus*	80.0 (12/15)	54.8–93.0	1.00	0.30
Streptococcus uberis *	78.6 (11/14)	52.4–92.4	0.85	0.05
Streptococcus dysgalactiae *	57.1 (8/14)	32.6–78.6	0.62	0.30
Enterococcus spp.	46.7 (7/15)	24.8–69.9	0.71	0.83
Mycoplasma spp.	40.0 (6/15)	19.8–64.3	0.26	0.50
BlaZ gene *	28.6 (4/14)	11.7–54.7	0.60	0.75
Streptococcus agalactiae	6.7 (1/15)	1.2–29.8	0.11	0.14

95% CI: 95% confidence interval. * The result was inconclusive in one cow.

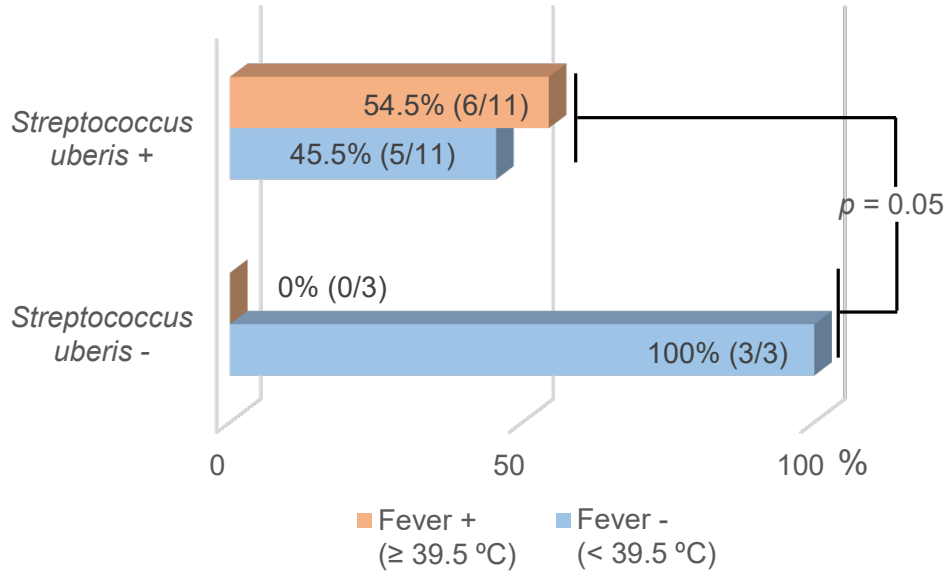

Figure 1. Relationship between *Strep. uberis* and presence of fever according to logistic regression (n = 14).

3.2. Identification of Mastitis Pathogens with Bacterial Culture and Agreement between Methodologies

In total, 28 bacterial isolates were obtained, from which 46.4% (13/28) were confirmed as *E. coli* through conventional bacterial culture, whereas 25.0% (7/28) were identified as *Strep. uberis*, and only 3.6% (1/28) were identified as *Strep. dysgalactiae* and *Enterococcus faecalis*. Additionally, *Strep. mutans*, *Strep. porcinus*, *Strep. pseudoporcinus*, *Hafnia alvei*, *Proteus vulgaris and E. fergusonii* were isolated.

Except for *E. coli* (substantial agreement; $p < 0.01$), the agreement between RT-PCR and the bacterial culture was fair (*Strep. uberis*; $p < 0.05$) or not significant (*Enterococcus* spp. and *Strep. dysgalactiae*; $p > 0.05$; Table 2).

Table 2. Agreement in bacterial identification between RT-PCR and bacterial culture methods.

Bacteria	Agreement between Methods	Cohen's Kappa Coefficient	Number of Isolates BC- and RT-PCR+ (1)
E. coli (***n*** = 15)	93.3%	0.64 (substantial) **	1
Strep. uberis (***n*** = 14)	64.3%	0.34 (fair) *	5
Enterococcus spp. (***n*** = 15)	60.0%	0.15 (slight)	6
Strep. dysgalactiae (***n*** = 14)	50.0%	0.11 (slight)	7

** $p < 0.01$; *: $p < 0.05$. (1) Number of isolates negative on bacterial culture (BC) but positive on RT-PCR. The inverse occurrence (RT-PCR—and bacterial culture +) was not observed.

4. Discussion

In this study, 60% (9/15) of the mastitis pathogens that could be identified via RT-PCR MastiType Multi Kit were detected in the uterine fluid of cows with RP. Of these pathogens, 40% (6/15) were isolated in more than half of the cows. The microorganisms were mainly (66.7%; 6/9) environmental mastitis pathogens (*E. coli*, *Staphylococcus* spp., *Strep. uberis*, *Enterococcus* spp., *T. pyogenes*/*P. indolicus*, yeasts). As previously mentioned, when the cervix opens at calving, environmental bacteria and yeast (e.g., vaginal and fecal) contaminate the uterus, usually up to two weeks after calving [27].

Strep. dysgalactiae, *Strep. agalactiae* and *Mycoplasma* spp. are considered contagious mastitis pathogens [28,29], i.e., their main route of transmission occurs from udder (mammary gland) to udder through milking procedures carried out with contaminated milking equipment. However, environmental spread also occurs, and these pathogens can be classified as both environmental and contagious pathogens (see the review by Cobirka et al. [29]). *Mycoplasma* spp., e.g., *M. bovigenitalium* and *M. californicum* (9.3%; 3/32), have also been detected previously in vaginal [30] and milk samples [31] from dairy cows.

In cattle, uterine microbiota is established within 20 min after calving [17]. At that time, the decomposing placental membrane and lochia provide an optimal intrauterine environment for microorganisms' colonization and growth [32,33]. In turn, the discharge of uterine secretions contributes to the spread of (normal and pathogenic) bacteria. It was observed by Bicalho et al. [34] that the total bacteria load in the vagina was similar at the day of calving in cows with or without RP. Nonetheless, a tendency ($p = 0.06$) on day 3 postpartum and on day 7 postpartum of a significantly larger ($p < 0.001$) total bacteria load (bacterial/archaeal 16S rDNA gene), in cows with RP rather than in healthy cows, was observed in their study [34]. These differences highlight the relevance of environmental bacteria spreading to RP animals.

In our study, *E. coli* and *Staphylococcus* spp. were the bacteria most frequently detected in the uterine fluid. Likewise, *E. coli* has been reported by Dohmen et al. [4] as the most prevalent bacteria in the uterus, in early postpartum in cows with or without RP. Moreover, it was observed by Bicalho et al. [35] that cows with RP were 45 times more likely to present *E. coli* during the first three days postpartum than cows without it (odds ratio = 44.8; $p < 0.01$). *E. coli* is also the most frequently found Gram-negative pathogen causing acute clinical mastitis during early lactation in high-producing dairy cows. It invades the udder through the teats, proliferates, and induces a local and systemic acute phase response that may cause irreversible tissue damage in the mammary gland, and severe clinical symptoms, sometimes even leading to the animal's death [1,36].

E. coli, as with other microorganisms, is a ubiquitous and opportunistic pathogen that depends on intrinsic virulent factors [37], bacterial load [38], and immune, metabolic and oxidative status to cause disease [6,34]. During the transition period and mainly around parturition, the increased energy demand favors the incidence of metabolic diseases, compromising immunity and resistance to infections by pathogenic and opportunistic agents. The condition of RP increases the occurrence of postpartum infectious diseases via uterine colonization and the development of *E. coli* and other microorganisms [9,39]. In the same way, the negative energy balance during this period impairs the immune response in

the mammary gland of lactating dairy cows, increasing the susceptibility to mastitis [40–42] and RP [39]. Furthermore, oxidative stress has also been associated with negative energy balance [43,44] and metabolic disruptions [45] in dairy cows. It was suggested that the link between RP and mastitis may be related to the weakening of the immune system [46], through peripheral leukocyte impairment [47]. Moreover, the chronological conjugation of these factors with bacterial load, including bacterial virulent factors [35], can have a relevant impact on the incidence of these diseases. Nonetheless, the common possible source for RP and mastitis does not rule out the possibility that RP plays a key role in aggravating mastitis through causing an increased level of contamination in the environment of the udder of affected cows, regarding *E. coli* and other bacteria.

Suriyasathaporn et al. [48] observed a tendency for cows with RP to have an increased risk of presenting clinical mastitis (risk ratio = 2.88; 95% CI = 0.96–8.64; $p = 0.06$) than cows without RP. The risk of developing severe mastitis was higher (RR = 5.4) in cows with RP than that in healthy cows. Also, cows with RP up to five days after calving were more likely to present clinical mastitis up to 120 days postpartum (odds ratio = 9.45; 95% IC = 8.62–10.27; $p < 0.001$) than cows without RP [49]. A large epidemiological study addressing environmental, bacterial and host factors is required to evaluate the relationship between RP and mastitis in more depth.

The *Staphylococcus* spp. group mainly comprises CNS with more than 50 bacterial species, with *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans* and *S. xylosus* being the most common CNS milk pathogens [50]. Large numbers of this species are also found in the environment and can partially explain the high proportion of *Staphylococcus* spp. (93.3%) observed in our study as a result of contamination through the vagina in the early postpartum period. On the other hand, in recent years, CNS have been considered the most prevalent microorganisms identified in subclinical mastitis in dairy cows [51].

A high proportion of yeasts (92.9% of the cows) were detected in our study. However, detection does not mean that colonization occurs. We hypothesize that the birth channel is just the route of transmission from the environment (or vagina) to the udder. Yeats are common in the environment, on the skin, in feces and even in the vagina microbiota. They are also opportunist microorganisms (e.g., *Candida albicans*, *C. krusei*, *C. tropicalis*), and uterine colonization is usually related to cows with specific conditions such as infertile cows, repeat breeders, cows with persistent endometritis, prolonged intra-uterine antibiotherapy, and immunosuppression [33,52]. Nonetheless, a yeast prevalence between 21 and 33% from cervicovaginal fluids of Holstein cows ($n = 176$) was previously reported by Garoussi et al. [53]. In that study, no statistically different prevalence was observed between cows with reproductive disorders (including those with RP) and non-pregnant or pregnant healthy cows. A significant prevalence of yeast contamination from the mammary glands of dairy cows has been reported in some countries such as Mexico (25.8%; 282/1095; [54]) and Algeria (10.2%; 114/1121; [55]). In a recent and large study (Japan) [56], from 18,915 bulk tank milk samples in 1,088 bovine farms, 184 isolates of yeast species were detected. *Pichia kudriavzevii* ($n = 97$ isolates), *Kluyveromyces marxianus* ($n = 28$), *C. akabanensis* ($n = 21$), *C. parapsilosis* ($n = 13$) and *P. cactophila* ($n = 12$) were isolated, highlighting their relative importance (1% of the samples) in mastitis prevalence. However, contamination of bulk milk by environmental saprophytes yeasts can also occur. Therefore, more attention is required to establish the impact of yeasts on reproductive health in dairy cows.

In our study, similar intrauterine prevalence was observed for *T. pyogenes*/*P. indolicus* (80.0%; 95% CI: 54.8−93.0%) and *Strep. uberis* (78.6%; 95% CI: 52.4−92.4%). It has been previously observed by Ballas et al. [10] that the *T. pyogenes* prevalence correlates ($p < 0.001$) with that of *Peptoniphilus* spp. on day 3 postpartum in uterine fluid, with *T. pyogenes* prevalence reaching its maximum (52.5%; 64/122) on day 15 [10]. A similar pattern was also found by Wagener et al. [2]. Regarding the differences in *T. pyogenes* prevalence between both studies, it is necessary to investigate if the presence of RP increases the risk of *T. pyogenes* contamination during the first days postpartum, and if the physical presence of the placenta membrane enhances that risk. Mastitis caused by this bacterium has been

related to summer mastitis [28,57]. This may be explained by the increased number of vectors (*Hydrotaea irritans*) able to transmit *T. pyogenes* [58].

Strep. uberis is considered a major mastitis pathogen with high genetic variability in the environment and animal udders [59]. It is also a bacterium that frequently colonizes the uterus postpartum, and a dynamic interaction between *Strep. uberis* and *T. pyogenes* has been described [2]. *Strep. uberis* is also a commensal microorganism of the bovine gut [60]. Thus, transmission to the udder via the digestive tract and feces is considered one of the most relevant routes of udder contamination [61], and the same is probably true for uterine contamination. Interestingly, 54.5% of the cows positive for *Strep. uberis* presented fever, whereas this clinical sign was not observed in any other cow. We did not find any study on this issue. *Strep. uberis* mastitis is often sudden in terms of its onset, and produces a hard, swollen quarter, with large white clots in milk, and sometimes with a high, or very high, body temperature [62]. In fact, this infection in dairy cows causes major signs of clinical mastitis, in some cases associated with systemic reactions [63]. *Strep. uberis* is recognized as an inducer of fever due to its involvement of systemic cytokines [64,65]. Apparently, according to our study, similar pathogenesis may occur when the infection is intrauterine.

Strep. dysgalactiae is other relevant contagious mastitis pathogen with previous scarce detection in the uterus, during the early postpartum period [66,67]. In contrast, we detected it in a high proportion of cases (57.1%; 8/14) in our study. There is evidence that *Strep. dysgalactiae* can persist for more than one year on the environment in dairy farms [63]. Hence, the importance of *Strep. dysgalactiae* in the puerperal uterus should be evaluated.

Finally, as expected, except for *E. coli*, a fair (*Strep. uberis*; k = 0.34; $p < 0.05$) or non-significant agreement (*Enterococcus* spp., *Strep. dysgalactiae*; $p > 0.05$) was observed between RT-PCR and bacterial culture methodologies, confirming the latter as useful especially for challenging bacterial cultures (e.g., *Mycoplasma*). In fact, the better performance of a multiplex PCR assay than that of bacterial cultures considering mastitis pathogens has previously been reported [68,69]. Despite its high sensitivity and specificity, RT-PCR can cause an increase in false positives due to the contamination of samples. Moreover, false negatives can appear due to small changes in the primer sites of new strains [14]. The substantial agreement (K = 0.64; $p < 0.01$) between RT-PCR and bacterial culture methods for *E. coli* observed in the present study was probably due to the high prevalence of this bacteria in the uterus detected via both methodologies (14/15 and 13/15 contaminated cows, respectively).

A limitation of this study was the reduced number of animals studied and that we only worked with cows with RP, without previous or posterior udder health evaluation. It was therefore not possible to associate RP with mastitis. However, overall, a large proportion of mastitis pathogens were detected in uterine fluids, supporting our hypothesis that cows with RP may serve as sources of intramammary infection, indirectly. More in-depth and larger comparative studies are required to evaluate the real impact of uterine secretions on mastitis prevalence during the postpartum period. Factors such as the environmental bacterial load on the animal surface and facilities (e.g., milking room and machines; calving parks), bacterial virulence and animal susceptibility should be taken into consideration for a holistic approach.

5. Conclusions

We found the same bacterial species that cause mastitis in the uterine fluid of cows presenting RP, which suggests that uterine secretions from cows with RP may be a relevant contamination source of mastitis pathogens. Therefore, although further studies should be carried out to better understand the extent of this relationship, specific care should be implemented for the udders of dairy cows with RP.

Author Contributions: Conceptualization, methodology, formal analysis, and data curation were performed by D.R., S.A., A.F.-N. and J.S. writing—original draft preparation; writing—review and editing were performed in equal proportions by D.R., S.A., A.F.-N., G.M. and J.S. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all farmers and the laboratory involved in the study.

Data Availability Statement: The raw data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

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