Symposium review: The uterine microbiome associated with the development of uterine disease in dairy cows*

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ABSTRACT

Until 2010, our knowledge of the uterine microbiome in cows that developed uterine disease relied almost exclusively on culture-dependent studies and mostly included cows with clinical endometritis (i.e., with purulent uterine discharge). Those studies consistently found a strong positive correlation between *Trueperella pyogenes* and clinical endometritis, whereas other pathogens such as *Escherichia coli*, *Fusobacterium necrophorum*, *Prevotella melaninogenica*, and *Bacteroides* spp. were also commonly cocultured. In contrast, *Streptococcus* spp., *Staphylococcus* spp., and *Bacillus* spp. were usually isolated from healthy cows. Starting in 2010, culture-independent studies using PCR explored the microbiome of cows with metritis and clinical endometritis, and observed that *E. coli* was a pioneer pathogen that predisposed cows to infection with *F. necrophorum*, which was strongly associated with metritis, and to infection with *T. pyogenes*, which was strongly associated with clinical endometritis. Starting in 2011, culture-independent studies using metagenomic sequencing expanded our knowledge of the uterine microbiome. It has been shown that cows have bacteria in the uterus even before calving, they have an established uterine microbiome within 20 min of calving, and that the microbiome structure is identical between cows that develop metritis and healthy cows until 2 d postpartum, after which the bacterial structure of cows that developed metritis deviates in favor of greater relative abundance of *Bacteroidetes* and *Fusobacteria* and lesser relative abundance of *Proteobacteria* and *Tenericutes*. The shift in the uterine microbiome in cows that develop metritis is characterized by a loss of heterogeneity and a decrease in bacterial richness. At the genus level,

Bacteroides, *Porphyromonas*, and *Fusobacterium* have the strongest association with metritis. At the species level, we observed that *Bacteroides pyogenes*, *Porphyromonas levii*, and *Helcococcus ovis* were potential emerging uterine pathogens. Finally, we have shown that the hematogenous route is a viable route of uterine infection with uterine pathogens. Herein, we propose that metritis is associated with a dysbiosis of the uterine microbiota characterized by decreased richness, and an increase in *Bacteroidetes* and *Fusobacteria*, particularly *Bacteroides*, *Porphyromonas*, and *Fusobacterium*.

Key words: metritis, uterine microbiota, dairy cow

INTRODUCTION

The transition to lactation is a challenging period for a high-producing dairy cow. This period is characterized by a sharp decrease in immune function (Kehrli and Goff, 1989; Cai et al., 1994). At the same time, physical barriers such as the cervix are breached at parturition, which allows for rapid colonization of the uterus by bacteria (Elliott et al., 1968; Sheldon and Dobson, 2004). Cows defend themselves against invading pathogens using tolerance and resistance mechanisms. Tolerance is the ability to limit the disease severity induced by a given pathogen burden, and there is evidence that high milk yield impairs disease tolerance (Sheldon et al., 2019). Resistance is the ability to limit the pathogen burden and is usually a function of immunity. However, the system is overwhelmed by a decrease in immune function, a large bacterial challenge, and reduced tolerance in high-producing dairy cows, and uterine diseases such as metritis, clinical endometritis, and subclinical endometritis are established in a large proportion of cows in early postpartum. Metritis and clinical endometritis have a prevalence of ~20%, ranging from 8% to >40% in some farms (LeBlanc, 2008; Galvão, 2012). We recently completed a survey of 16 farms in 6 regions of the United States, in which 11,733 cows were checked within the first 14 d postpartum (DPP) and 11,129 were checked from 30 to 36 DPP.

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We observed a prevalence of metritis of 25% (range: 14 to 44%) and a prevalence of clinical endometritis of 26% (range: 12 to 35%; Gonzalez-Pena et al., 2016). Of the cows evaluated for both diseases, 29.3% had one or the other and 10.6% had both; therefore, 39.9% of the cows had uterine disease postpartum. Numerous studies have demonstrated both direct and indirect negative effects of metritis on overall dairy herd performance and profitability (Goshen and Shpigel, 2006; Overton and Fetrow, 2008).

The causes of uterine disease are multifactorial. Of the 3 components of the disease triangle, there is good evidence for the susceptibility of the host and the role of pathogenic bacteria but less evidence for the effect of the environment (Sheldon and Dobson, 2004), although it cannot be disregarded (Schuenemann et al., 2011). The major risk factors for uterine disease development, the role of the host immune response in the development of uterine diseases, and the consequences of uterine disease have been previously reviewed (Sheldon et al., 2009; Galvão and Santos, 2014; Sheldon et al., 2019). Hence, this review will focus on the role of the uterine microbiome in the development of metritis in dairy cows. We pay special attention to recent advances in the use of metagenomic sequencing to characterize the uterine microbiome of cows that develop metritis, a comparison of the uterine microbiome in cows with and without a fever at the time of metritis diagnosis, the progression of the uterine microbiome after metritis diagnosis, particularly in cows that cure and fail to cure metritis, and the establishment of hematogenous route as a possible route of uterine infection with uterine pathogens.

UTERINE MICROBIOME IN COWS THAT DEVELOP METRITIS: CULTURE-DEPENDENT STUDIES

Until 2010, our knowledge of the postpartum uterine microbiome in dairy cows that developed uterine disease relied almost exclusively on culture-dependent studies (Griffin et al., 1974; Studer and Morrow, 1978; Ruder et al., 1981; Olson et al., 1984; Bonnett et al., 1991; Noakes et al., 1991; Dohmen et al., 1995; Huszenicza et al., 1999; Dohmen et al., 2000; Williams et al., 2005). Moreover, most studies evaluated cows with clinical endometritis (i.e., presence of pus in the uterine discharge) or pyometra (i.e., presence of pus in uterus in the presence of a corpus luteum), with the exceptions being Huszenicza et al. (1999) and Drillich et al. (2001), who evaluated cows with metritis within 14 DPP. Thus, the caveat is that a clear definition of metritis and clinical endometritis only became available in the early 2000s (LeBlanc et al., 2002a; Sheldon et al., 2006). Based on the previous literature, Sheldon

et al. (2002) classified bacteria according to their expected pathogenicity, and they considered *Trueperella* (formerly *Arcanobacterium*) *pyogenes*, *Escherichia coli*, *Prevotella melaninogenica*, and *Fusobacterium necrophorum* as recognized uterine pathogens, although the literature was not always consistent on the associations of these 4 pathogens with uterine disease, particularly in regards to the involvement of *E. coli* and *Prev. melaninogenica*. Nonetheless, this classification was widely disseminated and became the accepted dogma (Sheldon and Dobson, 2004; Williams et al., 2005; Sheldon et al., 2009).

Williams et al. (2005) sampled cows from 21 to 28 DPP and reported that *T. pyogenes* and *E. coli* were the most prevalent recognized uterine pathogens isolated from the uterus, followed by *Prev. melaninogenica* and *F. necrophorum*. They observed that cows diagnosed with endometritis (i.e., purulent vaginal discharge) had greater growth density of recognized and potential uterine pathogens but not of opportunists. Cows with *T. pyogenes* were more likely to be diagnosed with endometritis and more likely to have fetid vaginal mucus. Cows with *E. coli* were more likely to have fetid vaginal mucus. Cows with *F. necrophorum* were more likely to be diagnosed with mucopurulent vaginal discharge but not purulent vaginal discharge; *Prev. melaninogenica* was not associated with mucopurulent or purulent vaginal discharge.

Trueperella pyogenes has long been known capable of causing endometritis in animals (Lamming et al., 1955). Reviewing the previous literature, we found that Griffin et al. (1974) were among the first to report the association of *T. pyogenes* with endometrial inflammation and infertility in cows. Others (Studer and Morrow, 1978; Bonnett et al., 1991) later confirmed the association between isolation of *T. pyogenes* and endometrial inflammation. Studer and Morrow (1978) also reported that cows with *T. pyogenes* were more likely to be diagnosed with clinical endometritis (i.e., purulent vaginal discharge) and had the most severe histological lesions. They also reported that coliforms (*E. coli* or *Aerobacter* spp.) were associated with clinical endometritis and endometrial histological changes. In contrast, Bonnett et al. (1991) reported no association between *E. coli* and endometrial inflammation. Dohmen et al. (1995) reported that *T. pyogenes*, *F. necrophorum*, and *Bacteroidetes*, particularly *Porphyromonas levii* but also *Prev. melaninogenica*, were correlated among themselves, and were isolated with higher frequency as the uterine discharge score worsened (i.e., from flecks of pus to hemorrhagic and fetid). Previous studies commonly reported the isolation of *F. necrophorum* in combination with *T. pyogenes* (Ruder et al., 1981; Olson et al., 1984; Noakes et al., 1991; Dohmen et al.,

1995) and other bacteria such as *Prev. melaninogenica* (Olson et al., 1984; Noakes et al., 1991) from cows with endometritis or pyometra. Hence, based on the findings by Olson et al. (1984) and Noakes et al. (1991), *T. pyogenes*, *F. necrophorum*, and *Prev. melaninogenica* alone or in combination were used to induce pyometra in dairy cows (Farin et al., 1989), which showed that these bacteria can cause uterine disease. More recent studies have shown that infusion of *T. pyogenes* alone (Lima et al., 2015) or in combination with *E. coli* (Pier-santi et al., 2019) can cause uterine inflammation.

Huszenicza et al. (1999) reported findings similar to Williams et al. (2005) in cows with metritis; namely, a greater prevalence of *T. pyogenes* and *E. coli* in cows with metritis. Additionally, they reported that cows with metritis had greater prevalence of gram-negative anaerobes, particularly *Bacteroides* spp., but interestingly, they reported a low prevalence of *F. necrophorum* and black-pigmented *Bacteroides* such as *Por. levii* and *Prev. melaninogenica*. In contrast, Drillich et al. (2001) sampled 15 cows with metritis and reported that *F. necrophorum* and *Por. levii* were the most prevalent (67%) bacteria isolated. Interestingly, the role of *Por. levii* in the development of uterine disease has been largely overlooked (Sheldon et al., 2002; Williams et al., 2005).

In contrast to observations by Williams et al. (2005), Dohmen et al. (1995) observed that *E. coli* was isolated less frequently as uterine discharge worsened. Furthermore, *E. coli* was negatively associated with the presence of *T. pyogenes* at 14 DPP. Bonnett et al. (1993) reported that cows with *T. pyogenes* at 40 DPP had poor reproductive performance, which was later confirmed by others (Huszenicza et al., 1999; Gilbert and Santos, 2016). Bonnett et al. (1993) also reported that cows with *E. coli* tended to have good reproductive performance (i.e., conceived by 119 DPP), which supports the findings by Dohmen et al. (1995). de Boer et al. (2015) reported that cows with *E. coli* at calving were more likely to have *E. coli* or any other bacteria at 21 DPP and to be diagnosed with endometritis at 21 DPP. Ultimately, cows with *E. coli* at 21 DPP tended to take longer to conceive after the start of the breeding season (de Boer et al., 2015). Others have reported that cows with *E. coli* in the first week postpartum were more likely to have *T. pyogenes* and gram-negative anaerobes (i.e., *F. necrophorum* and *Bacteroides* spp.) in the second (Dohmen et al., 2000) and third (Gilbert and Santos, 2016) weeks postpartum. Therefore, although the association between *E. coli* and uterine disease and fertility has been conflicting, the findings by Dohmen et al. (2000), de Boer et al. (2015), and Gilbert and Santos (2016) support a long-held view that *E. coli* is a pioneer organism that sets up the environment for

other pathogens such as *T. pyogenes* and gram-negative anaerobes to become established (Sheldon and Dobson, 2004).

In summary, the results from culture-dependent studies indicate that *T. pyogenes* is a critical pathogen involved in the development of clinical endometritis, and that gram-negative anaerobes such as *F. necrophorum*, *Por. levii*, and *Prev. melaninogenica* may act synergistically with *T. pyogenes* to cause clinical endometritis. Culture-dependent studies also support the involvement of *E. coli* either as a main pathogen or as a pioneer pathogen that sets up stage for *T. pyogenes* and gram-negative anaerobes. Although data were scarcer for cows with metritis, the available data also point to an involvement of *T. pyogenes*, *E. coli*, and gram-negative anaerobes in the development of metritis.

In regards to bacteria associated with uterine health, Bonnett et al. (1991) reported that isolation of α -hemolytic streptococci was negatively correlated with isolation of *A. pyogenes* and with inflammation. Dohmen et al. (1995) reported that streptococci were isolated less frequently as the uterine discharge worsened, and that streptococci were negatively associated with the presence of *T. pyogenes* at 14 DPP. Williams et al. (2005) observed that cows with α -hemolytic streptococci, the most common opportunist, and coagulase-negative staphylococci were less likely to be diagnosed with endometritis. Huszenicza et al. (1999) also reported a lower prevalence of streptococci and staphylococci in cows with metritis compared with healthy cows. Gilbert and Santos (2016) reported that cows with α -hemolytic streptococci at 7 DPP had improved fertility. Therefore, culture-based studies indicate that streptococci, or more specifically, α -hemolytic streptococci, can promote uterine health. There were also some indications that *E. coli* detected later in lactation could promote uterine health (Bonnett et al., 1993; Dohmen et al., 1995) but these findings were inconsistent (de Boer et al., 2015).

UTERINE MICROBIOME IN COWS THAT DEVELOP METRITIS: CULTURE-INDEPENDENT STUDIES

Study of the Uterine Microbiome Using PCR-Based Techniques

Culture-dependent methods have an inherent limitation of only being able to detect bacteria for which a culturing and identification method has been established. Indeed, it has been estimated that less than 1% of bacteria can be cultured in the laboratory (Kaeberlein et al., 2002). To overcome this limitation, culture-independent techniques such as PCR, clone library sequencing, 16S rRNA metagenomic sequencing,

and whole-metagenome sequencing have been used to elucidate the role of the main uterine pathogens identified in culture-dependent studies. Nonetheless, these techniques are not without drawbacks. Limitations include the following: (1) bacterial viability cannot be evaluated; (2) sequencing depth and coverage are low when using clone library sequencing; (3) operational taxonomic unit classification may change depending on the variable region of the 16S rRNA gene that is targeted for sequencing; (4) classification based on 16S rRNA gene sequencing is not accurate at the species level; and (5) cost, which tends to be higher, particularly for whole metagenome sequencing (Chakravorty et al., 2007). The choice of variable region of the 16S rRNA gene to be targeted has been extensively studied because it may affect taxonomic classification and diversity estimates. Nonetheless, different studies have arrived at different conclusions, with recommendations including one or more of the V2, V3, V4, V6, or V3/V4 regions (Mizrahi-Man et al., 2013).

Starting in 2010, several studies using culture-independent techniques helped us expand our understanding of the uterine microbiome associated with uterine disease. Using multilocus sequence typing, Sheldon et al. (2010) identified specific strains of *E. coli* from animals with endometritis. These strains were more adherent and invasive to endometrial epithelial and stromal cells than *E. coli* isolated from healthy cows. Although they could not identify a specific virulence factor responsible for this increased adherence and invasion, a mannose-binding assay pointed to the involvement of *fimH*. The *fimH* gene is expressed in more than 90% of uropathogenic *E. coli* and encodes type 1 fimbrial D-mannose specific adhesion, which is central to its pathogenicity in the urogenital tract of women (Tchesnokova et al., 2011). Bicalho et al. (2010) screened for the presence of 32 virulence factors in *E. coli* isolated from the uterus of dairy cows within the first week postpartum and observed that prevalence of metritis was higher in cows with *E. coli* possessing *fimH* alone or in combination with other virulence factors such as *hlyA*, *cdt*, *kpsMII*, *ibeA*, or *astA*. Later, the same group observed that cows positive for *fimH* at 1 to 3 DPP were more likely to be diagnosed with metritis at 8 to 10 DPP and with endometritis at 34 to 35 DPP (Bicalho et al., 2012). Cows positive for *fimH* at 1 to 3 DPP were also more likely to be positive for *F. necrophorum lktA* at 8 to 10 DPP, which increased the likelihood of having metritis at 8 to 10 DPP. Furthermore, cows positive for *F. necrophorum lktA* at 34 to 36 DPP were also more likely to be positive for *T. pyogenes fimA*, which in turn had the greatest positive correlation with clinical endometritis at 34 to 36 DPP. Ultimately, cows positive for *fimH* at 1 to 3 DPP had decreased hazard

of pregnancy. Interestingly, being positive for *E. coli* or *T. pyogenes* at 8 to 10 DPP was not associated with a diagnosis of metritis at 8 to 10 DPP. We have observed that abundance of *E. coli* and *T. pyogenes* at the time of metritis diagnosis, determined by quantitative PCR, was low and not different between metritic and healthy cows, whereas metritic cows had greater abundance of *F. necrophorum*, *Por. levii*, and *Prev. melaninogenica* compared with healthy cows (Cunha et al., 2018). Furthermore, being positive for *E. coli* at 34 to 36 DPP was not associated with a diagnosis of clinical endometritis at 34 to 36 DPP. These findings support the idea of *E. coli* being a pioneer pathogen and highlight the synergism between *E. coli*, *F. necrophorum*, and *T. pyogenes* in causing metritis and clinical endometritis (Bicalho et al., 2012). Therefore, our interpretation of these data is that *E. coli* predisposes to uterine colonization by *F. necrophorum*, which is the main pathogen associated with the development of metritis, and *F. necrophorum* predisposes to colonization by *T. pyogenes*, which act synergistically with *F. necrophorum* to cause clinical endometritis.

Study of the Uterine Microbiome Using Metagenomic DNA Sequencing Techniques

Although PCR is a great tool to investigate specific pathogens, it provides only a narrow view of the whole microbial community. Therefore, to overcome this limitation, recent studies have used metagenomic sequencing to identify the bacterial community of cows with and without uterine disease (Santos et al., 2011; Machado et al., 2012; Jeon et al., 2015). Using clone library sequencing of composite samples from 2 farms, Santos et al. (2011) observed higher prevalence of *Fusobacteria* (particularly *F. necrophorum*) and *Bacteroidetes* (particularly *Por. levii*) in metritic cows and higher prevalence of *Proteobacteria* and *Tenericutes* in healthy cows. Interestingly, neither *E. coli* nor *T. pyogenes* sequences were detected in any of the 4 libraries. This was the first study using metagenomic sequences that pointed to the importance of *Fusobacteria* and *Bacteroidetes* at the time of metritis diagnosis, confirmed the importance of *F. necrophorum*, raised the profile of *Por. levii* as an emerging pathogen, and questioned the relevance of *E. coli* and *T. pyogenes* at the time of metritis diagnosis. Nonetheless, this study had limitations, including the use of composite samples, the lack of longitudinal sampling, and the use of clone library sequencing, which has a low depth of coverage.

To overcome some of the limitations in the study by Santos et al. (2011), we used 16S rRNA gene sequencing to characterize the uterine microbiota progression from calving until establishment of metritis in dairy cows

(Jeon et al., 2015). We observed 28 phyla and 824 genera of bacteria in our uterine samples. We showed that all cows had an established uterine microbiota within 20 min of calving, and that the uterine microbiome changed rapidly from calving to 6 ± 2 DPP (Figure 1A, 1B) with a reduction in the relative abundance of *Proteobacteria* (particularly *Coxiella*) and an increase in the relative abundance of *Bacteroidetes* (particularly *Bacteroides* and *Porphyromonas*) and *Fusobacteria* (particularly *Fusobacterium*; Jeon et al., 2015). Additionally, we showed that the uterine microbiome structure was identical between cows that later developed metritis and healthy cows until 2 DPP, after which the bacterial structure deviated in favor of greater relative abundance of *Bacteroidetes*, particularly *Bacteroides* and *Porphyromonas* (Jeon et al., 2015). This dysbiosis in the microbial community in metritic cows was characterized by consolidation of the uterine microbiome (Figure 2A-F) and a loss of microbial richness and diversity (Jeon et al., 2015, 2016), while at the same time the total bacterial load was increased (Figure 1C). Similar dynamics in bacterial load have been observed by others (Bicalho et al., 2017b).

Interestingly, the relative abundance of *Fusobacteria* was similar between healthy and metritic cows (Jeon et al., 2015). Nonetheless, at the genus level, discriminant analysis, correlation among bacteria, and correlation between bacteria and uterine discharge score pointed to a synergism among *Bacteroides*, *Porphyromonas*, and *Fusobacterium* in the development of metritis (Jeon et al., 2015). We also observed that failure to cure metritis was associated with an increase in the relative abundance of *Bacteroides*, *Porphyromonas*, and *Fusobacterium*, and a further decrease in bacterial diversity (Jeon et al., 2018). Later, we observed that the microbiome of metritic cows with or without a fever was virtu-

ally identical, and that independent of the presence of fever at the time of diagnosis, metritic cows had greater prevalence of *Bacteroides* and *Porphyromonas* and numerically greater prevalence of *Fusobacterium* (Jeon et al., 2016). Greater abundance of *Bacteroides*, *Porphyromonas*, or *Fusobacterium* in metritic cows has since been observed by others (Knudsen et al., 2016; Bicalho et al., 2017a; Sicsic et al., 2018; Galvão et al., 2019), but for this review, we compiled a large data set with 80 metritic and 31 healthy cows to be able to infer with more certainty what pathogens are associated with metritis at the time of diagnosis. Using this data set, we performed linear discriminant analysis effect size (LEfSe) to determine the phyla and genera most likely to explain differences between healthy and metritic cows (Segata et al., 2011), in addition to ordination using non-metric multidimensional scaling (Figure 3A-D). With this large data set, we confirmed the loss of richness and consolidation of the uterine microbiome (Figure 3A,B) observed in our previous studies (Jeon et al., 2015, 2016). We also observed that *Bacteroidetes* (39.4 vs. 15.2%) and *Fusobacteria* (30.4 vs. 9.5%) were the only 2 phyla that were differentially more abundant in metritic cows than in healthy cows. At the genus level, *Fusobacterium* (29.8 vs. 11.8%), *Bacteroides* (21.4 vs. 7.0%), and *Porphyromonas* (16.9 vs. 5.3%) were the most prevalent genera more abundant in metritic cows than in healthy cows (Figure 3C,D). Similar to our original data (Jeon et al., 2015), this large data set supports the involvement of other less prevalent (<5%) genera, such as *Peptostreptococcus*, *Filifactor*, *Prevotella*, *Helcococcus*, *Peptoniphilus*, and *Arcanobacterium*. Interestingly, with the exception of *Prevotella*, these other genera are gram-positive. Finally, we determined Spearman correlations among these main bacteria and propose an updated picture of the interactions among

Figure 1. Non-metric multidimensional scaling (NMDS) of uterine bacterial communities based on Bray-Curtis dissimilarity at the (A) phylum and (B) genus levels, and (C) quantitative PCR of total bacteria from calving (d 0) until 6 ± 2 d postpartum (DPP). Error bars indicate SEM.

the main bacteria associated with metritis at the time of diagnosis (Figure 4).

It is worth noting that many of these pathogens have never been recognized by conventional culture studies, and therefore, are potential emerging uterine pathogens. Indeed, we investigated the leads from our previous 16S rRNA gene sequencing studies using quantitative PCR, and confirmed that metritic cows had greater copy numbers of *Bacteroides pyogenes* and *Helcococcus ovis*, which were the main species from the genera *Bacteroides* and *Helcococcus*, respectively (Jeon et al., 2016; Cunha et al., 2018).

Healthy cows maintain a much more heterogeneous and rich uterine microbiome compared with metritic cows (Jeon et al., 2015, 2016; Figure 2A-F; Figure 3A,B). We observed that *Proteobacteria* and *Tenericutes* were numerically more abundant in healthy cows, and that several genera were associated with uterine health including *Escherichia*, *Serratia*, *Mycoplasma*, *Ureaplasma*, and *Ruminococcus*, among others (Jeon et al., 2015). Herein, we confirmed that the uterine microbiome of healthy cows is more complex than that of metritic cows. *Proteobacteria*, *Tenericutes*, and several

other phyla were more abundant in healthy cows than in metritic cows (Figure 3C). At the genus level, *Tralbulsiella*, *Escherichia*, *Mycoplasma*, and *Ureaplasma*, among several others, were more abundant in healthy cows than in metritic cows (Figure 3D). Although some genera reported herein as being associated with uterine health, such as *Tralbulsiella*, differ from those in our first report, others were confirmed, such as *Escherichia*, *Mycoplasma*, *Ureaplasma*, and *Ruminococcus* (Jeon et al., 2015). It is worth mentioning that higher relative abundance of several genera in healthy cows is likely a result of maintaining a heterogeneous, diverse microbiome that is not overtaken by *Fusobacteria* and *Bacteroidetes*, as most of these genera are not found in all healthy cows, with the exception of *Escherichia*, *Mycoplasma*, and *Ureaplasma*, and have low prevalence ($\leq 7\%$) in healthy cows. In 2017, we showed that cows that remain healthy and cows that develop metritis had sub-communities of bacteria that led to the development of metritis and sub-communities that were associated with uterine health (Jeon et al., 2017). However, in cows that develop metritis, there is a dysbiosis in the microbial community in which bacteria such as *Fu-*

Figure 2. Non-metric multidimensional scaling (NMDS) of uterine bacterial communities based on Bray-Curtis dissimilarity at the (A, B, C) phylum and (D, E, F) genus levels from calving (d 0) until 6 ± 2 d postpartum (DPP). Metritis d 0 = 9 cows; d 2 = 12 cows; d 6 ± 2 = 11 cows; no metritis d 0 = 8 cows; d 2 = 9 cows; d 6 ± 2 = 11 cows.

Figure 3. Non-metric multidimensional scaling (NMDS) of uterine bacterial communities based on Bray-Curtis dissimilarity at the (A) phylum and (B) genus levels, and linear discriminant analysis (LDA) effect size at the (C) phylum and (D) genus levels at the time of metritis diagnosis. Metritis = 80 cows; no metritis = 31 cows.

sobacterium, *Bacteroides*, and *Porphyromonas* overtake others such as *Escherichia*, *Mycoplasma*, *Ureaplasma*, and *Ruminococcus*, hence decreasing microbial richness (Jeon et al., 2015, 2016; Sicsic et al., 2018; Galvão et al., 2019). Therefore, studies of the microbiome show that all cows have all the uterine pathogens; however, cows that remain healthy are better able to prevent the shift toward greater abundance of *Fusobacteria* and *Bacteroidetes*. This is illustrated in Figure 5. In that sense, bacterial resistance seems to be an important component of maintaining uterine health. Nonetheless, total bacterial load and the load of specific pathogens also increase postpartum (Bicalho et al., 2017b), which indicates a component of bacterial tolerance in maintaining uterine health. At this point, it is unclear what factors contribute the most to differences in bacterial

resistance and tolerance and the dysbiosis that leads to uterine disease. It is likely that major risk factors such as parity, dystocia, twins, stillbirth, retained placenta, and metabolic stress affect bacterial resistance and tolerance. Indeed, cows with dystocia, twins, stillbirth, and retained placenta have been shown to have decreased bacterial resistance postpartum (Bicalho et al., 2010, 2017b).

Given the fact that healthy cows have an abundance of bacteria in the uterus, including pathogenic bacteria, what is the usefulness to antibiotic treatment for cows with uterine disease? We just showed that treating cows for metritis with ceftiofur increased cure rates, did not decrease culling in the first 60 d postpartum, but increased milk yield and fertility up to 300 d postpartum (Oliveira et al., 2019). Others have shown that treating

Figure 4. Microbial interactions associated with metritis. Bacteria in red boxes are the most prevalent (>15%) genera that were more abundant in the metritis group in the linear discriminant analysis effect size analysis. Bacteria in the white boxes were more abundant in the metritis group but had low prevalence (<5%). Arrows indicate positive correlations ($P \leq 0.02$).

cows with clinical endometritis with cephalirin sodium also improves fertility (LeBlanc et al., 2002b; Denis-Robichaud and Dubuc, 2015); therefore, treatment of cows with uterine diseases with antibiotics should take into account welfare, economics, and antibiotic resistance considerations.

Interestingly, we saw that a sub-community of uterine pathogens that included *Fusobacterium*, *Bacteroides*, and *Porphyromonas* was also present in blood shortly after calving, which indicate that a hematogenous route of uterine infection is feasible in cows (Jeon et al., 2017). Therefore, uterine pathogens could be transferred to the uterus shortly after calving or even before calving. Indeed, a study showed that bacterial translocation to extra-intestinal sites was more common in late gestation and shortly after parturition than in the nonpregnant state or in early or mid lactation mice (Perez et al., 2007). Furthermore, recent studies using fluorescence in situ hybridization reported the presence of *F. necrophorum*, *Por. levis*, and *T. pyogenes* within the endometrium, on the endometrial surface, and in the caruncular stroma of pregnant cows, which indicates that bacterial pathogens could be in the uterus even before parturition (Karstrup et al., 2017). Using massive parallel sequencing, the same study reported that *Porphyromonadaceae* was the most prevalent family of bacteria (Karstrup et al., 2017). Interestingly, the presence of bacteria in the uterus prepartum was not associated with inflammation, which indicates that there is increased bacterial resistance and tolerance during pregnancy. This is likely a result of inadequate conditions for bacterial proliferation, such as an in-

tact endometrium, adequate oxygen perfusion, lack of substrate for bacterial growth, and a switch from a T-helper (Th)1 to a Th2 response during pregnancy. Nonetheless, bacterial proliferation and inflammation (placentitis) is likely a cause of pregnancy loss in cattle. During routine pregnancy diagnosis using ultrasonography between 60 and 90 d after breeding, the authors (K. N. Galvão and R. C. Bicalho) have observed several cows with echogenic fluid, characteristic of pus, between the endometrium and the chorioallantois. These changes invariably led to pregnancy loss. Unfortunately, the causative agents of these early-gestation abortions are not usually investigated. In contrast, mid- to late-gestation abortions are investigated, and the causative agents are varied and include uterine pathogens such as *T. pyogenes* (Anderson, 2007). Pregnancy loss due to placentitis is a well-documented problem in mares and is believed to be caused by ascending infection (Macpherson and Bailey, 2008). In cows, deep-seated infections or hematogenous translocation may be the most likely cause. Some pathogens are more likely to be deep-seated such as *Tritrichomonas foetus* and *Campylobacter fetus*, which spread venereally, whereas others are more likely to translocate from blood such as *Brucella abortus* and *Leptospira* spp., which transmit by direct contact. Regarding uterine pathogens such as *T. pyogenes*, both routes may be possible. Moore et al. (2017) also observed that the uterine endometrium from virgin heifers and pregnant uteri harbored a microbiome, in which *Firmicutes* and *Bacteroidetes* were the most prevalent phyla. Although bacteria could already be in the uterus or arrive there shortly after

calving through the hematogenous route, it is likely that ascending contamination from the vagina or the environment through the vagina is also possible.

Machado et al. (2012) explored the uterine microbiome of cows with clinical endometritis using pyrosequencing of the 16S rRNA gene, and observed that cows with clinical endometritis had higher relative abundance of *Bacteroidetes*, particularly *Bacteroides*; *Tenericutes*, particularly *Ureaplasma*; *Fusobacteria*, particularly *Fusobacterium*; and *Actinobacteria*, particularly *Trueperella*, whereas healthy cows had higher relative abundance of *Firmicutes*, particularly *Geobacillus*. These data showed that the uterine microbiome of

cows with clinical endometritis was very similar to that of cows with metritis, except for the higher relative abundance of *Tenericutes* in cows with clinical endometritis. Higher relative abundance of *Bacteroidetes* and *Fusobacterium* in cows that developed clinical endometritis was later confirmed by others (Wang et al., 2018; Miranda-CasoLuengo et al., 2019).

The advent of metagenomic tools such as 16S rRNA gene sequencing, allowed researchers to characterize the bacterial sub-communities associated with uterine health and disease in cows. Whereas culture-dependent studies pointed to *T. pyogenes* and *E. coli* as the most prevalent uterine pathogens, metagenomic studies

Figure 5. Microbial shift at the phylum level from calving until establishment of metritis in dairy cows. Arrows following phylum names indicate relative abundance comparison among cows with metritis and healthy cows; a dash indicates no difference in relative abundance. DPP = days postpartum.

showed that *Fusobacterium* (particularly *F. necrophorum*), *Porphyromonas* (particularly *Por. levii*), and *Bacteroides* (particularly *B. pyogenes*) are the most prevalent bacteria in the uterus of cows with uterine disease. Metagenomic studies allowed us to recognize emerging pathogens such as *B. pyogenes* and *H. ovis*. Additionally, metagenomic studies allowed us to better characterize the complex interactions among bacteria, even in tissues where culture is usually unproductive such as the pregnant uterus and blood. Hence, we now have a much better understanding of the major bacteria associated with metritis and clinical endometritis, which is the first step to studying the microbiome. The next step is to perform integrative studies that involve whole-metagenome sequencing and metabolome and immune function analysis to better understand the complex interactions that lead to immunosuppression, microbial dysbiosis, and disease establishment, so we can work on establishing causation rather than just associations (Segata et al., 2013; Aguiar-Pulido et al., 2016; Schmidt et al., 2018). Additionally, understanding the web of causation will allow development of new prebiotics, probiotics, and therapeutics to prevent and treat uterine disease in dairy cows. This leap has been taken on the human side with the integrative human microbiome project (iHMP; <https://www.hmpdacc.org/ihip/>); a similar approach in the animal side is needed to advance our understanding of factors that affect animal health and disease.

CONCLUSIONS

Cows had bacteria in the uterus even before calving, they had an established uterine microbiome within 20 min of calving, and the microbiome structure was identical between cows that developed metritis and healthy cows up until 2 d postpartum, after which the bacterial composition of cows that developed metritis deviated in favor of greater relative abundance of *Bacteroidetes* and *Fusobacteria* and lesser relative abundance of *Proteobacteria* and *Tenericutes*. The shift in the uterine microbiome in cows that developed metritis is characterized by a loss of heterogeneity and a decrease in bacterial richness. At the genus level, *Bacteroides*, *Porphyromonas*, and *Fusobacterium* seem to act synergistically to cause metritis. At the species level, we identified *B. pyogenes*, *Por. levii*, and *H. ovis* as potential emerging uterine pathogens, and confirmed *F. necrophorum* as one of the species mainly associated with uterine disease. We also observed that failure to cure metritis was associated with an increase in the relative abundance of *Bacteroides*, *Porphyromonas*, and *Fusobacterium*, and a further decrease in bacterial diversity. The structure of the uterine microbiome was similar between cows that

did and did not have a fever at the time of diagnosis. Finally, we showed that the hematogenous route is a viable route of uterine infection with uterine pathogens such as *Bacteroides*, *Porphyromonas*, and *Fusobacterium*. Herein, we propose that metritis is associated with dysbiosis of the uterine microbiota characterized by decreased richness, and an increase in *Bacteroidetes* and *Fusobacteria*, particularly *Bacteroides*, *Porphyromonas*, and *Fusobacterium*.

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