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Molecular characterisation of the uterine microbiome of dairy cows suffering from endometritis, metritis, and pyometra

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VET-PHD-2014

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Preface

The work described in this PhD thesis tells the tale of three years of focus on endometritis in dairy cows, metagenomic and metatranscriptomic technologies. When I started at the university for my bachelors, I studied agricultural science with a special interest in sheep and cows, however, I quickly realised that biotechnology and laboratory work was a path more suited to me, and changed my direction to accommodate this. From September 1st 2011 and August 31st I have enjoyed the privilege of focussing on a project that combines animal agriculture with modern biotechnological technologies.

This project is a collaboration between the Section of Bacteriology, Pathology and Parasitology at National Veterinary Institute at the Technical University of Denmark and the Veterinary Reproduction & Obstetrics group at the Department of Large Animal Sciences at Copenhagen University.

My Supervisors were Kirstine Klitgaard Schou, Tim Kåre Jensen, and Øystein Angen from the Norwegian Veterinary Institute was my co-supervisor. Initially, Lars Mølbak was also my supervisor, but when he changed his job he did not have time to continue his supervision responsibilities. I collaborated with fellow PhD student Cecilia Christensen Karstrup, Professor Jørgen Agerholm, Associate Professor Hanne Gervi Pedersen and Eva Láadal Rasmussen.

Part of the metatranscriptomics was performed at the University of Minnesota in Professor Timothy Johnson's group in the Department of Veterinary and Biomedical Sciences, and the data for the RNA sequencing data analysis, I have had the fortune to collaborate with Paolo Marcatili from the Center for Biological Sequences Analysis at the Technical University of Denmark.

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The samples were taken from a commercial dairy herd in Arden in Northern Jutland, and we are grateful that farmer Bøje Petersen let us examine his cows. We are also grateful that Kenneth Krogh, the local veterinarian assisted us in the sampling and performed the uterine scoring.

I would like to thank my supervisor Kirstine Klitgaards Schou for choosing to work with me. I have enjoyed every meeting we have had, you always listen and give honest feed-back, which is very much appreciated. You are also a good role model for a scientist, and I wish everyone could have as conscientious a supervisor as you.

A big thank you to Tim Kåre Jensen, Øystein Angen, Cecilia Christensen Karstrup, Hanne Gervi Pedersen, Jørgen Agerholm, and Eva Láadal Rasmussen for good collaboration and discussions about the project and the practicalities of the sampling.

I would like to thank the wonderful technicians here in the Microbial Ecology group for good technical assistance and invaluable help with DNA and RNA extraction marathons, many PCR reactions with almost as many primers, patience with stubborn PhD students and excellent company in the lab.

A warm thank you to all of my colleagues in the Microbial Ecology group. Without you the daily life would not have been anywhere near as productive, fruitful or fun. You guys are the best.

I would like to thank Paolo Marcatili from the Center for Biological Sequence Analysis, Technical University of Denmark for a interesting debates and good collabor-

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And finally my friends and family, without whom none of this would have been possible. You always put up with the amount of time I spend on work, support me in everything I do and, make me laugh and feel good and light at heart. Special gratitude to my boyfriend, James, for always being there for me.

Summary

Postpartum uterine disease is a problem in dairy herds. Approximately 90% of dairy cows experience postpartum bacterial contamination of the uterus. Most of the cows are able to clear the infection within 8 weeks in the process of involution, but up to 20% of the cows develop metritis, which is infection throughout the uterine wall; and in some herds, 30-50% of cows develop endometritis, which is infection in the inner lining of the uterus. Pyometra is a related postpartum uterine disease, which is thought to occur when a cow with endometritis ovulates, and the cervix closes. The diseases are negatively correlated to reproductive performance, and in combination with the high incidence rate, they are costly for the farmers. Traditional culture-based studies are biased towards bacteria that thrive in a laboratory environment. In this project the bacterial flora were investigated by molecular microbiology methods, primarily 16S rRNA PCR and next generation sequencing.

The study included uterine flush samples from the lumen as well as endometrial samples, to evaluate the correlation between the uterine flush samples, which are commonly used sample type in the area, and the bacteria found adhering to the mucosal layer of the uterus, the endometrium. It was hypothesised that pathogenic bacteria in the uterus initially adhere to the endometrium to cause disease, and that the chance of identifying pathogens is higher in examinations of endometrial biopsies than in uterine flush samples. In order to investigate the expression patterns of the bacteria in the endometritic uterus, a metatransgenomic study was performed. This method is based on mRNA sequencing, and provides a snapshot of the expression profile of the bacteria at the time of sampling. Previous studies of virulence factors have been performed with quantitative PCR, which requires prior knowledge of gene sequence.

It was found that there was an association between the *Fusobacteriaceae* and *Porphyromonadaceae* families and metritis in week 1 postpartum. For endometritis in weeks 4 and 7, there was not a bacterial family consistently associated with the disease across time points and sample types. There were large differences between the uterine flush samples and the endometrial biopsies, and although the sample types were correlated, the diversity of the microbiota in the biopsy samples was higher than the diversity of the microbiota of the uterine flush samples. Furthermore, the bacterial families that made out the majority of the population were the same over time.

The most abundant family observed in cows with pyometra was the *Fusobacteriaceae* family, which contain *F. necrophorum*, a pathogen previously known to be associated with pyometra, whereas evidence of the association of *T. pyogenes* with pyometra was less convincing. The previously unidentified Gram-negative bacteria observed in other studies of pyometra are likely to belong to the *Porphyromonadaceae*, *Pasteurellaceae*, and *Mycoplasmataceae* families identified.

It was found that the 50 most up-regulated transcripts of the microbiota from the uterus of cows with metritis and endometritis were primarily involved in DNA replication, transcription, translation, and metabolic processes. This indicates an active multiplication phase in the infection, and an adaption to the host environment. Furthermore, an up-regulation was observed of genes potentially involved in the synthesis of LPS, lipid A, haemagglutinin, and several genes that code for proteases. These genes are putative virulence genes. The majority of the most differentially expressed transcripts mapped most closely to proteins from the *F. necrophorum* and *P. levii* species. This indicates that these species were the most metabolically active in the uterus of the cows with uterine disease, and that these may be the primary pathogens of uterine disease. Transcripts from other species were also observed to be highly expressed in the uterus of cows with uterine disease, among others from *M. bovigenitalium*.

The results in this thesis underline the high number of bacteria found in the bovine postpartum uterus. *F. necrophorum* and *P. levii*, were observed to be the most important pathogens in uterine disease, but the association of other bacterial species, perhaps contained within the *Mycoplasmataceae*, *Pasteurellaceae*, *Ruminociccaceae*, *Bacteroidaceae*,*Leptotrichiaceae* families, and an uncharacterised family that belong to class *Bacteroidia* seems likely. The data presented in this thesis does not support a role of the *E. coli* and *T. pyogenes* species, that have been identified as possible pathogens with traditional culture methods.

Resume

Postpartum uterine sygdomme er et problem i malkekvægsbesætninger. Omtrent 90% af alle malkekøer bliver kontaminerede af bakterier i uterus efter kælvning. De fleste køer kommer selv af med infektionen indenfor otte uger i involutionsprocessen, men op mod 20% of alle køer udvikler metritis, hvor hele uterusvæggen er inficeret; og i nogle besætninger udvikler 30%-50% af køerne endometritis, som er infektion i det inderste lag af uterus. Pyometra er en relateret postpartum uterin sygdom, som forekommer når en ko med endometritis får ægløsning, og cervix lukkes om infektionen. Sygdommene er negativt korrelerede med reproduktions ydelsen, og dette i tillæg til den høje prævalens gør sygdommene dyre for landmændene. Traditionelle dyrkningsmetoder til identifikation af bakterier er tilbøjelige til at indføre bias mod bakterier, der trives i et laboratoriemiljø. I dette projekt blev den bakterielle flora undersøgt ved molekylære mikrobiologiske metoder, primært 16S rRNA PCR og næste generations sekventering. Studiet inkluderer skylleprøver fra uterus lumen, såvel som endometrielle biopsier, for at evaluere korrelationen mellem de bakterier der observeres i de uterine skylleprøver, som hyppigst anvendes i feltet, og de bakterier som adhærer til det mucosale lag i uterus, endometriet. For at undersøge ekspressionsmønstret af bakterier tilstede i en uterus med endometritis blev et metatranskriptom studie udført. Denne metode er baseret påmRNA sekventering, og giver et øjebliksbillede af ekspressionsprofilen fra bakterier påprøveudtagnings tidspunktet. Tidligere studier af virulensfaktorer i postpartum uterine sygdomme har været baseret påkvantitativ PCR, hvilket kræver forhåndsviden om gensekvens.

Der blev fundet en signifikant association mellem *Fusobacteriaceae* og *Porphyromonadaceae* familierne og metritis i uge 1 postpartum. For endometritis i uge 4 og 7 var der ingen bakterielle familier der var signifikant associeret med sygdommen konsistent over prøvetidspunkter og prøvetyper. Der var store forskelle mellem de uterine

skylleprøver og de endometrielle biopsier, og selvom prøvetyperne var korrelerede, såvar diversiteten af mikrobiotaen i uterus højere i biopsierne end i skylleprøverne.

Den mest observerede bakterielle familie i uterus fra køer med pyometra var *Fusobacteriaceae* familien, som indeholder *Fusobacterium necrophorum*; en kendt patogen, der allerede er associeret med pyometra. Derimod støtter vores data ikke sammenhængen mellem pyometra og *Trueperella pyogenes*, det andet tidligere identificerede patogen. De uidentificerede Gram-negative bakterier som tidligere studier har observeret i pyometra, tilhører formentlig de Gram-negative familier *Porphyromonadaceae*, *Pasteurellaceae* og *Mycoplasmataceae*, som er identificeret i dette studie.

Det blev observeret at de 50 mest opregulerede transkripter i uterus mikrobiotaen fra køer med metritis of endometritis primært var involveret i DNA replikation, transkription, translation og metaboliske processer. Dette indikerer en aktiv multiplikationsfase i infektionen, og en tilpasning til værtens miljø. Derudover blev en opregulering observeret i gener, der potentielt er involverede i syntese af LPS, lipid A, hæmagglutinin samt flere gener, der koder for proteaser. Disse gener er mulige virulens gener. Størstedelen af de mest differentielt udtrykte transkripter havde størst similaritet med proteiner fra *F. necrophorum* og *P. levii* arterne. Dette indikerer at disse arter er de mest metabolisk aktive i uteri fra køerne med metritis og endometritis, og at de måske er de primære patogener i uterine sygdomme. Transkripter fra andre arter blev ogsåobseveret, blandt andet fra *Mycoplasma bovigenitalium*.

Resultaterne i denne afhandling understreger diversiteten i den bovine mikrobiota i postpartum uterus. *F. necrophorum* og *P. levii* blev identificeret som de mest vigitge patogener i uterine sygdomme, men en association med andre bakterie arter synes sandsynligt, muligvis indenfor familierne *Mycoplasmataceae*, *Pasteurellaceae*, *Ruminococcaceae*, *Bacteroidaceae* og *Leptotrichiaceae*. Den rolle i uterine sygdomme, som ved tidligere studier, der benyttede traditionelle dyrkningsmetoder, er blevet tilskrevet *Escherichia coli* og *T. pyogenes* kunne ikke understøttes af data i denne afhandling.

Publication list

Manuscript 1: Lif Rødtness Vesterby Knudsen*, Cecilia Christensen Karstrup, Hanne Gervi Pedersen, Øystein Angen, Jørgen Steen Agerholm, Eva Láadal Rasmussen, Tim Kåre Jensen, Kirstine Klitgaard. An investigation of the microbiota in uterine flush samples and endometrial biopsies from dairy cows during the first 7 weeks postpartum. Submitted to Veterinary Microbiology.

Manuscript 2: Lif Rødtness Vesterby Knudsen*, Cecilia Christensen Karstrup, Hanne Gervi Pedersen, Jørgen Steen Agerholm, Tim Kåre Jensen, Kirstine Klitgaard. Revisiting pyometra in postpartum cows - new insights into the disease using a culture-independent deep sequencing approach. Submitted to Veterinary Microbiology.

Manuscript 3: Lif Rødtness Vesterby Knudsen*, Cecilia Christensen Karstrup, Paolo Marcatili, Kirstine Klitgaard. In vivo mRNA profiling of bacteria recovered from the uteri of dairy cows with postpartum uterine disease revealed high prevalence and metabolic activity of Fusobacterium necrophorum. Manuscript in preparation.

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Abbreviations

CFU	Colony Forming Units
CL	Corpus luteum
DGGE	Denaturing Gradient Gel Electrophoresis
E_2	Estradiol
FISH	Fluorescent *in situ* Hybridization
FSH	Follicle stimulating Hormone
GnRH	Gonadotrophin releasing hormone
IL	Interleukin
LH	Lutenising hormone
MIC	Minimum Inhibitory Concentration
nr	non-redundant
OTUs	Operational Taxonomic Units
OD	optical density
PG_4	Progesterone
PVD	Purulent Vaginal Discharge
qPCR	quantitative PCR
454	Roche 454 GS-FLX
T-RLFP	Terminal Restriction Fragment Length Polymorphism
TLR	Toll-like receptors
TNF-α	Tumor Necrosis Factor-α

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Chapter 1

Introduction

1.1 Postpartum uterine diseases

The postpartum uterine diseases endometritis, metritis and pyometra are a major concern for dairy farmers, due to both welfare and economical reasons. Endometritis, metritis, and pyometra are related uterine diseases [1]. They are a result of the contamination of the uterus that occur during and after calving [2] [3]. Diagnosis of the uterine diseases is done by evaluating the uterine discharge, the clinical signs of the illness, and by investigating the cytology, and by taking into account the number of days since parturition [4].

Endometritis can be clinical or subclinical. Clinical endometritis is defined as an inflammation of the inner lining of the uterus [5]. It is characterised by purulent or mucopurulent uterine exudate occurring 21 days or more postpartum, with no signs of systemic illness [4]. Subclinical endometritis is an infection in the endometrium in the absence of purulent exudate. Subclinical endometritis is diagnosed with cytology by a neutrophil count in uterine cytology samples above 18% at days 21-33, or above 10% at days 34-47 [6] [4]. Subclinical endometritis is estimated to affect 30-50% of cows, whereas clinical endometritis affects 5-25% of cows [7] [8]. An alternative definition has also been proposed, where Purulent Vaginal Discharge (PVD) is diagnosed in the presence of purulent or mucupurulent discharge, and

cytological endometritis is diagnosed when a high percentage of neutrophils in the cytological samples is observed [9] [7].

Metritis is inflammation in all layers of the uterus, from the endometrium through the submucosa, the muscularis and the serosa [5]. It is characterised by an abnormally enlarged uterus and fetid brown uterine discharge [4]. Affected cows often have systemic illness, with fever, loss of appetite, weakness, decreased milk yield, elevated heart rate and depression [5] [4]. Metritis affects 5-20% of cows [8], and is the most severe of the uterine diseases discussed here, as it is can lead to septic-toxic metritis, which is life-threatening [1].

Pyometra is defined as inflammation in the inner lining of the uterus in the presence of a persistent corpus luteum, and with accumulation of purulent exudate in the uterus [5] [4]. Affected cows are not systemically ill and they do not exhibit estrus, and are infertile [5] [4] [10]. Pyometra account for less than 5% of the clinical uterine disease cases [11].

1.1.1 Impact of the uterine diseases

The diseases reduce the welfare of affected cows by causing discomfort and may cause them to be eliminated from the herd [3] [10] [12]. It has been shown that acute phase proteins in the blood of postpartum dairy cows increase in concentration in association with uterine bacterial contamination [13], which indicates some degree of discomfort and pain to the cow [14]. While the lack of systemic illness in endometritis and pyometra makes it difficult to assess the discomfort or pain experienced by affected cows [15], cows with metritis do have systemic illness with fever, and exhibit loss of appetite, weakness, and depression [4].

Uterine diseases have a negative impact on the reproductive performance. Cows with endometritis have a 17-20% lower conception rate [15] [10] and take approximately 27% longer to become pregnant than cows without endometritis [15]. Cows with metritis have a 6-20% lower conception rate and a 12-21 days longer calving to conception interval [16] [1]. Cows with clinical endometritis are less fertile even after successful treatment [3] [10]. Furthermore, infertility is registered as one of the most prevalent reasons for culling [1] [17].

The fertility of high producing dairy cattle is declining [18] [19]. In a study performed over the two periods 1975-1982 and 1995-1998, it was found that the interval from calving to first service increased with 1% per year, the calving rate decreased with 0.45% per year, and the total amount of cows with a persistent corpus luteum in the first cycle postpartum rose from 7.3% to 18.5% [20]. The reasons for the declining

fertility may be many. The most important reason is probably the negative genetic correlations between reproductive traits and a high milk yield, fat yield, and protein yield [21] [19] [22] [23]. The breeding goals have been focussed on the milk yield traits at the cost of the reproduction traits [23]. Other important reasons for the declining fertility are negative energy balance [18] [19] [24], reduced immune system function [24], and postpartum uterine infections [25] [24]. Some of these factors are interrelated; for example, negative energy balance and reduced immune function are correlated to postpartum uterine disease [8]. This study focusses on the bacteria associated with postpartum uterine infections metritis, endometritis and pyometra.

The negative effect that the postpartum uterine diseases have on the reproductive performance [25] [24] in addition to the high prevalence of these diseases make them economically important for the farmers. PVD is the third most expensive disease for the dairy farmer, after mastitis and lameness [26]. The extra days without conception is the main cost for the farmers, estimated to $3 per cow per day. The extra artificial inseminations also add cost, as does culling [26] and the reduced milk yield caused by metritis [4]. The direct cost of each case of metritis was set to £70.81 in 1997, but with indirect costs added, the total cost of each metritis case was £161.58 per affected cow [26]. In 2009 the average total cost per animal was estimated to €292, which was approximately £219 [11]. The annual cost of metritis with a prevalence of 20% was calculated to €1.411 billion in the EU and $650 million in the US [11].

1.2 The bovine uterus

The bovine uterus is bicornuate, as seen in Figure 1.1. There are three layers to the uterine wall, the perimetrium, which is the outer layer of the uterus; the myometrium is the middle layer, which itself has three layers: a circular and a longitudinal muscle layer, and a vascular layer; and the endometrium, which is the inner layer of the uterus that consists of a mucosa and a submucosa [27].

The healthy bovine uterus is assumed to be sterile between parturitions [3] [10] [7]. The anatomical features that help the uterus avoid contamination from the external environment are the vulva, a muscular sphincter, the vestbule, and the cervix [5]. The pH of the uterus is between 6.8 and 8.3 depending on the composition of the diet and whether the cow is in estrus [28], and the environment expected to be anaerobic [29] [30]. In the pregnant uterus, the endometrium forms caruncles. Caruncles are fibrous thickened protrusions of the endometrium, which have deep villi, folds and lamellae, extensive blood supply, and a large surface area, which serves as the contact point for the cotyledons, which also have deep villi [27] [31]

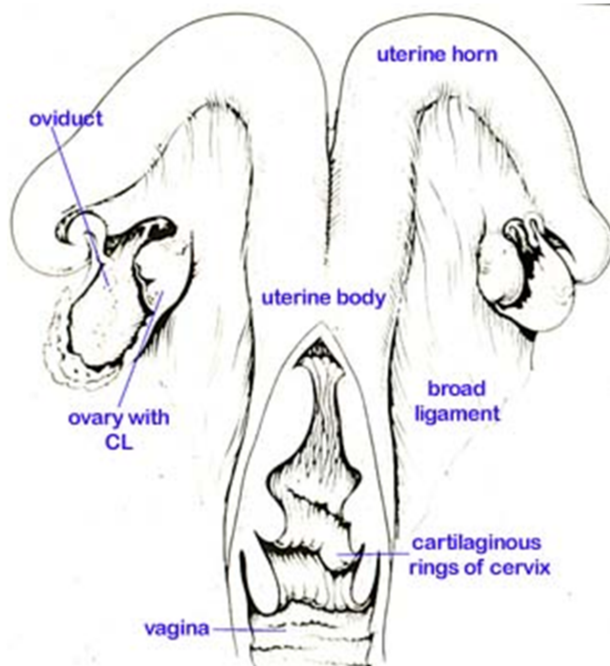

Figure 1.1: Diagram of the female bovine reproductive organs. From the University of Pennsylvania School of Veterinary Medicine's Computer Aided Learning program. http://cal.vet.upenn.edu/projects/repropath/FReview/06cowtrctdiacopy.jpg

[32]. Together, the caruncles and the cotelydons form the placentome, and the large surface area and extensive blood ensure exchange of nourishment between the maternal and the foetal blood [27] [31].

95 After parturition, the uterus undergoes involution. Involution is a process in which the uterus is reduced in both size and mass over a period of approximately 20-25 days [33], although complete involution has been reported to last up to 47-50 days [34]. The caruncles are reduced within 10-13 days of the parturition, starting with the retraction of the blood vessels and the caruncle pedicles. The decidual layer of 100 the caruncles undergoes necrobiosis and is shed five to eight days after parturition [33]. Lochia is the thick uterine fluid that is made of the shed decidua, blood, uterine exudates, and foetal membranes and fluids [33], [10]. The lochia is expelled from the uterus by contractions of the myometrium [35], and during the expulsion from the uterus, it changes colour and consistency during the course of 13-18 days

[33] [34]. The lochia can serve as growth medium for contaminating bacteria [5] [35] [14].

The postpartum uterus is contaminated by bacteria from the external environment [2] [3]. For the majority of cows, the bacterial contamination is present the first 10-14 days postpartum [3]. Healthy cows clear the bacterial contamination, regenerate the endometrium, and return to cyclic ovarian function within the process of involution [35] [11].

1.2.1 The endocrine system

In the postpartum period, an important hormone that works to re-establish normal cyclic ovarian function is Gonadotrophin releasing hormone (GnRH), which in turn releases the Gonadotrophins Follicle stimulating Hormone (FSH) and Lutenising hormone (LH). The concentration of FSH and LH fluctuate, and causes several follicles to grow. The follicles release estrogens, most notably Estradiol (E_2) [36]. The Progesterone PG_4 produced by the Corpus luteum (CL) keeps the E_2 waves below the concentration threshold that induces ovulation. After approximately 17 or 21 days post ovulation, the endometrium causes the CL to lyse, and the subsequent large wave of E_2 causes the dominant follicle to ovulate [5] [36].

The mucosal immune system in the uterus is sensitive to the hormonal fluctuations in the postpartum cow [5]. During the periparturient period, for example, the immune system of the cow is subdued [37].

1.2.2 The immune system

In the postpartum period, where the majority of cows experience an influx of bacteria into the uterus [3], a lack of balance between the uterine infection and the cows intrauterine self-defence mechanisms can lead to complications such as puerperal metritis, clinical or subclinical endometritis, and pyometra [35] [8]. Especially the innate immune system is pivotal in the defence against bacteria from the external environment [38] [24]. The endometrium express Toll-like receptors (TLR), that recognise pathogen associated molecular patterns. TLR1, TLR2, and TLR6 recognise bacterial lipids, TLR3, TLR7, TLR8, and TLR9 recognise nucleic acids, often viral nucleic acids, and TLR4 recognises lipopolysaccharide from Gram-negative bacteria [39] [10]. The endometrium is also protected by antimicrobial peptides, such as defensis, and acute phase proteins [13] [10]. When recognising a pathogen, the TLR receptors release cytokines and chemokines such as interleukine-1 (IL-1), IL-6

and Tumor Necrosis Factor-α (TNF-α) [35] [39] to start an inflammatory response, where particularly neutrophils migrate to the site of infection [5] [7] [24] [8]. The
140 neutrophils adhere to and ingest the pathogens [1]. Later follows an influx of macrophages, eosinophils [5] and leukocytes [35]. An adaptive immune response may also be triggered by the pathogens, attracting lymphocytes such as B-cells and T-cells [1] [5].

The immune response is decreased by the stress of calving and the initiation of
145 lactation [37] [40] [41]. Likewise, the feed intake of the cow also plays a role in the immune system, as fat mobilisation also produces pro-inflammatory cytokines, such as TNFα and IL6. Additionally, non-esterified fatty acids can bind to the TLR4 receptors, and release further cytokines. These metabolism-induced systemic inflammatory responses may result in a prolonged or severe inflammatory response
150 in the postpartum uterus, and may influence the uterine infections negatively [8]. Certain hormones also influence the immune system, for example, PG_4 has immunosuppression properties, and is essential for the maintenance of pregnancy [42].

1.3 The microbiota

A microbiota is the community of microbes that inhabit one environment, or a
155 niche of an environment. There are diverse communities of microbes found in various body sites of animals and humans. As only part of a microbiota can usually be cultivated, non-culture dependent methods such as high-throughput sequencing are usually applied in the study of microbial populations. In cows, it is primarily the microbiota of the digestive system which has been investigated by these methods
160 [43][44] but also the vaginal microbiota and the hoofs have been studied by next sequencing methods[45] [46]. Cows have a symbiotic relationship with the bacteria and other microorganisms that inhabit the rumen. In the bovine rumen, there are $10^8 - 10^{10}$/g viable bacteria [47], and the rumen makes out 9-13% of the cows total body volume [48]. The microbiota in the rumen act as a fermentation chamber
165 for the cow, and besides harvesting energy from the normally indigestible parts of the plants, the microbiota produces volatile fatty acids, vitamins, and bacterial proteins that are important for the cow [48]. Alteration in the feed influences the pH, the concentration of gasses, and volatile fatty acids produced but not the total amount of bacteria in the rumen [47]. Generally, different species of
170 bacteria are adjusted to very particular environments. For example, it has been shown that when fed different diets cows produce faecal samples with different bacterial compositions [49]. So while the microbiota influences the environment it inhabits, for example by the volatile fatty acids and gasses produced by the rumen microbiota, the environment also affects the microbiota. It is therefore possible

175 that certain factors in the uterine environment will effect the composition of the microbiota that thrives there postpartum. It has been suggested that a highly diverse microbiota contributes more to the ecosystem, here the cow, because more highly specific niches will be filled, allowing more nutrients to be utilised [49]. In humans, the gut microbiota has also been linked to diseases such as obesity, inflammatory 180 bowels disease, Crohn's disease, and malnutrition [50].

In the bovine uterus, it is doubtful that there should be a stable microbiota over time, since the uterus is supposed to be sterile between parturitions [3] [10] [7]. However, it is of interest how the transient postpartum microbiota affect the host, and in particular, how the transient postpartum microbiota of the uterus develop 185 into a uterine infection for some cows.

1.4 The bacteria associated with postpartum uterine disease

There is not a very clear relationship between infection with one pathogen and postpartum uterine disease but some bacteria are commonly linked to uterine disease 190 or to a healthy uterus, while others are ubiquitous. The majority of the studies addressing bovine postpartum uterine diseases have used traditional culture methods. The bacteria that have been detected in the uterus of cows with a postpartum uterine infection by traditional culture studies are summarised in Table 1.1.

In recent years, pyrosequencing studies have been applied to investigate the bovine 195 postpartum microbiota. An overview of the bacteria associated with endometritis and metritis is given in Table 1.2. Pyometra does not appear in the table, because no metagenomic analysis of this disease were found.

The metagenomic studies on endometritis and metritis also observed some bacteria that were associated with the postpartum uterus of healthy cows. *Lactobacillus* 200 spp. from phylum *Firmicutes* and *Propionibacter* spp. from phylum *Actinobacteria*appear to be associated with good reproduction performance [55]. The phyla *Proteobacteria* and *Tenericutes* have been associated with the uterine microbiomes of healthy cows [55] [57].

A recent paper, which used next generation sequencing and Denaturing Gradient Gel 205 Electrophoresis (DGGE), found that the phyla *Bacteroidetes*, *Fusobacteria*, *Firmicutes*, *Proteobacteria*, and *Tenericutes* are present in the uterine microbiome regardless of uterine health status [54].

Table 1.1: Bacteria observed in the uterus of cows with endometritis and metritis in studies using traditional culture methods

	Species
Endometritis	*Trueperella pyogenes* [1] [51] [3] [52] [11]
	Prevotella melaninogenicus [3] [52]
	Escherichia coli [51] [3] [52] [11]
	Fusobacterium necrophorum [1] [51] [3] [52] [11]
	Prevotella spp. [11]
	$\alpha-$haemolytic *streptococci* [51]
	Bacteroides spp. [1] [51]
	Pasteurella spp. [51]
	Proteus spp. [51]
Metritis	*E. coli* [35] [2] [34]
	T. pyogenes [35] [2]
	F. necrophorum [35] [2]
	Streptococcus spp. [2]
	Stapholycoccus spp. [2]
	Pseudomoas spp. [2]
	Prevotella spp. [35]
	Bacteroides spp. [35]
pyometra	*F. necrophorum* [29] [30]
	T. pyogenes [53] [30] [29]
	P. melaninogenicus [29]

Some of these studies have also focussed on the timing of the bacterial observations, and have sampled the cows at different time points. *Fusobacterium*, *Bacteroidetes*
210 and *Tenericutes* were found in normal cows in the first week post partum in a study using next generation sequencing, and *Bacteroidetes*, *Firmicutes* and *Fusobacterium* were found in cows with metritis at the same time point [54]. Approximately one month post partum in the same study, *Firmicutes*, *Proteobacteria* and to some degree *Bacteroidetes* were the most abundant phyla observed in healthy cows, and
215 *Tenericutes*, *Synergistes* and *Proteobacteria* were most abundantly observed in cows with endometritis [54]. In a Terminal Restriction Fragment Length Polymorphism (T-RLFP) study, *F. necrophorum*, genera *Pseudomonas/Acetinobacter* and genera *Bacteroides/Sphingobacterium/Prevotellaceae* were the most abundantly found species at both 1 week and 1 month post partum irrespective of uterine health [58].
220 The rapid changes in the uterine microbiome post partum, and clearance of infection followed by re-contamination of the uterus was also demonstrated by Griffin *et al.* (1974) in a culturing study. They concluded that culturing the bacteria present

Table 1.2: Bacteria observed in the uterus of cows with endometritis and metritis in studies using next generation sequencing

	OTU
Endometritis	*Bacteroidetes* [54]
	Fusobacteria [54]
	Firmicutes [54]
	Proteobacteria [54]
	Bacteroides spp. [55]
	Ureaplasma spp. [55]
	Fusobacterium spp. [55]
	Peptostreptococcus spp. [55]
	Sneathia spp. [55]
	Prevotella spp. [55]
	Trueperella spp [55]
Metritis	*Fusobacteria* [56] [54]
	Bacteroidetes [56] [54]
	Proteobacteria [54]
	Firmicutes [54]
	Bacteroides spp. [55]
	Ureaplasma spp. [55]

in the uterus the first seven weeks postpartum is not predictive of future fertility of the cows [59].

225 ## 1.5 Next generation sequencing

In 1996, pyrosequencing was invented by Ronaghi and coworkers [60], allowing for much deeper sequencing than with the chain termination Sanger method, with the possiblity of sequencing millions of sequences at the same time [61]. The technology has been developed much since 1996, and the cost of sequencing has decreased to
230 affordable prices [61].

The next generation sequencing technology called Roche 454 GS-FLX (454) is based on sequencing by synthesis. The DNA fragments to be sequenced are suspended and amplified in an emulsion and captured by nucleotide adaptors on small beads that are distributed in the wells of a picotiter plate. In a stepwise enzymatic synthesis

235 of the DNA fragment, just one nucleotide is added at a time. A chemiluminescent technique converts the pyrophosphate released by the incorporation of nucleotides to a light signal, and the light is captured by a camera, which in turn calculates the amount of nucleotides incorporated [62] [63] [61].

The Illumina technology is also based on sequencing by synthesis. The DNA fragments are hybridised to adaptors in a flowcell, and through bridge amplification they form clusters of the DNA fragments. The incorporation of nucleotides into the growing DNA strand is monitored by fluorescent dye in the incorporated nucleotides [63] [61]. A removable blocking group on the nucleotide ensures that only one nucleotide is incorporated at a time [64].

The yield from 454 sequencing is typically 1 million reads of high quality with a read length of approximately 500 bp [65]. An inherent weakness of the 454 technology is the inaccuracy in the reads of homopolymers. The light signal emitted if for example six nucleotides are incorporated in the growing DNA strand at one time is not exactly six times brighter, as there is not a perfectly linear relationship between the amount of pyrophosphate released and the light signal emitted. This increases the chance of sequencing mistakes in homopolymeric regions of the target DNA [61]. With Illumina sequencing, approximately 450 million reads are obtained of high quality of up to 120 bp [65]. Newer versions of the Illumina technology, the HiSeq and the MiSeq platforms, allow for 250 bp reads. In paired end sequencing, the DNA fragments are read from both ends [66], and if the primers sites are placed so far from each other that there is only one overlapping bp, the read length can be up to 499 bp.

The 16S rRNA gene has been used to identify bacteria based on their DNA sequence since the invention of the PCR reaction [67] [68]. The 16S rRNA gene has several features which makes it good for identifying bacteria. The gene codes for the small subunit of the ribosomes, which translate mRNA into proteins [69]. This function is essential for the bacteria and the gene is thus highly conserved [67]. Furthermore, the gene has several regions of high variability, the hypervariable regions [70] [68]. By designing primers that bind to conserved regions of the gene, and letting the amplicon span a hypervariable region, 16S rRNA sequencing is a powerful tool for identifying bacteria based on their DNA sequence [67]. Furthermore, the general usage of the 16S rRNA gene to identify bacteria have given rise to large databases with reference sequences. For example, the Ribosomal Database Project (RDP) contained 643,915 16S rRNA entries in September 2008 [71] and 3,019,928 16S rRNA entries in September 2014. However, sequencing a small part of the 16S rRNA gene, such as one or two hypervariable regions, may not provide sufficient resolution to distinguish between closely related bacteria with very similar 16S rRNA gene sequences [72]. Furthermore, the copy number of the 16S rRNA gene can vary between bacterial species, so a direct quantification of the number of bacteria based

275 on the 16S rRNA observed may be inaccurate [73].

RNA sequencing is next generation sequencing methods applied to cDNA in order to investigate the transcriptome of cells [74] [75]. The transcriptome is the collected transcripts of a given cell, and it conveys information of the functional elements of the genome [75]. RNA sequencing of bacteria has been used to investigate in-
280 teraction with host and regulation of fitness and pathogenesis [76]. For example, the transcriptomes of *Listeria monocytogenes* [77] [78], *Salmonella enterica* serovar *Typhi* [79], and *Chlamydia trachomatis* [80] have been investigated with RNA sequencing technology.

1.6 Metatransgenomics

285 A metatranscriptomic study is a study of the collected pool of transcripts produced by a community of e.g. bacteria. By applying next generation sequencing techniques to RNA fragments, it is possible to obtain a snapshot of the genes transcribed by a bacterial population such as the microbiome of the bovine postpartum uterus. By observing which genes are up-regulated in the uterus from a cow with a post-
290 partum disease in comparison to a cow without a postpartum disease, it is possible to further the understanding of functions needed by the pathogens to develop or maintain an infection. Some of these up-regulated transcripts may be virulence factors, while others are household genes needed in the uterine environment. Metatranscriptomic studies have been performed on environmental samples [81] [82],
295 human faecal samples [83], and human dental samples [84] [85]. In cattle, metatranscriptomic studies have been performed on the ruminal samples [86] and on milk samples from cows with mastitis [87]. There is not much literature on the bovine metatranscriptome.

1.6.1 Potential virulence factors of uterine disease

300 There is not complete consensus on the definitions of pathogenicity and virulence, and they are often used as synonymously [88]. According to Thomas and Elkinton (2004) [88], virulence is the inherent ability of a microorganism to cause injury or disease to the host, while pathogenicity is a broader term, that includes virulence in addition to the speed and the transmission ability of the microorganism. Further-
305 more, pathogenesis is the mechanism by which a pathogen causes disease to the host, and a virulence factor is a gene that when expressed contributes to virulence [89]. Pathogenesis is seen from the point of view of the host, while virulence is seen

from the point of view of the bacteria [90].

Virulence factors in endometritis and metritis have been studied previously by quantitative PCR (qPCR). qPCR relies on primers for already known targets. In Table 1.3, the known virulence factors from the uterine pathogens *E. coli*, *T.pyogenes* and *F. necrophorum* are shown, and are described below.

Table 1.3: Putative virulence factors of major uterine pathogens

Pathogen	Virulence factor	Reference
E. coli	*fimH*	[12] [91] [92]
	astA	[91]
	cdt	[91]
	kpsMII	[91] [92]
	ibeA	[91]
	hlyA	[91]
	fyuA	[92]
T. pyogenes	*plo*	[12] [93] [94] [95]
	cbpA	[12][93] [95]
	fimA	[12][93] [95]
	fimG	[93]
	nanH	[95]
	nanP	[95]
F. necrophorum	*lktA* (plus *lktB* and *lktC*)	[12] [96][97][98]
	hly	[98] [99] [97]
	Haemagglutinin	[100] [101]
	Outer membrane proteins	[102]
	Endotoxin	[98]
	Adhesin	[98]
	Dermonecrotic toxin	[98]
	Platelet aggregation factor	[98]
	Proteases	[98]

1.6.2 *Eschericha coli*

E. coli is a Gram-negative, facultative anaerobic bacteria that belongs to the *Enterobacteriaceae* family, the *Enterobacteriales* order, the *Gammaproteobacteria* class, and the *Proteobacteria* phylum. There are both commensal strains and pathogenic strains of *E. coli*, and they cause both intestinal diseases such as diarrhoea and extraintestinal diseases such as urinary tract infections [103] [104].

The virulence factors from *E. coli* that most references agree upon is associated with uterine disease is a type 1 pilus. One of the subunits is coded for by the gene *fimH* [12] [91]. *fimH* codes for the adhesive subunit of the protein, and it has been found that the pilus with the fimH adhesin in uropathogenic *E. coli* is responsible not only for adhesion to the epithelial cells, but also for invasion [105]. In a study of six different virulence genes from different bacteria isolated from cows with postpartum uterine disease, *fimH* was the only gene that had a negative correlation with reproduction [12]. The results were dependent on the number of days postpartum, and the presence of *fimH* 1-3 days postpartum increased the likelihood 16 times of finding *F. necrophorum* at days 8-10 postpartum [12]. This supports the notion that *E. coli* facilitates the infection of *F. necrophorum*. A different study [91] tested the presence of 32 different putative *E. coli* virulence factors in relation to postpartum uterine disease. Eleven of the tested virulence genes were detected and there was found significant association with metritis for six of them: *fimH* (type 1 pili), *astA* (gene coding for endotoxin), *cdt* (cytolethal distending toxin), *kpsMII* (gene coding for capsule protein), *ibeA* (gene coding for factor that fascilitates invasion), and *hlyA* (gene coding for toxin) [91]. This experiment also found that *fimH* was the virulence factor most closely associated with metritis, and notes that in the intrauterine *E. coli*, they found an 87% prevalence of the *fimH* gene. Finally, the ferric yersiniabactin uptake receptor *fyuA* gene, which is involved in iron uptake, was found to be associated with endometritis [92].

E. coli isolated from cows with uterine disease have increased adhesion and invasion properties to bovine endothelial cells in comparison to diarrhoeagenic and extraintestinal pathogenic *E. coli* isolated from cows without uterine disease and lack a number of virulence factors normally associated with virulence in the diarrhoeagenic and extraintestinal pathogenic *E. coli* [92]. These *E. coli* strains may be specific for bovine uterine diseases.

1.6.3 *Trueperella pyogenes*

T. pyogenes is a Gram-positive, facultative anaerobic bacteria. *T. pyogenes* belongs to the *Actinomycetaceae* family, the *Actinomycetales* order, the *Actinobacteria* class, and the *Actinobacteria* phylum. *T. pyogenes* is an opportunistic pathogens that often cause infections after damage to mucosal surface or a different microbial trauma [95]. They are frequently isolated from mucosal surfaces in healthy production animals such as cattle and swine. When they are pathogenic, they are associated to conditions such as mastitis, pneumonia, abscesses, uterine infections et.c. [95].

Pyolysin, a haemolytic exotoxin coded for by the *plo* gene is mentioned as the dominant *T. pyogenes* virulence factor [106] [95]. A knock-out experiment showed that *plo* mutants were non-hemolytic and virulence attenuated [94]. A dose of 10^8 of *plo* mutants was not enough to cause infection, though the same dose of wild type *T. pyogenes* caused infection in 7 out of 8 animals [94].

A neuraminidase is also one of the *T. pyogenes* virulence factors. It is involved in adhesion to the host, cleaving sialic acid from the host to mediate adhesion [95]. The neuraminidase is coded for by the *nanH* gene. In a knock-out experiment, a *NanH* mutant still kept 20% neuraminidase function, indicating redundancy [107]. This discovery lead to the identification of *nanP*, a second neoraminidase, which is not present in all *T. pyogenes*, but seem to be found primarily in *T. pyogenes* from bovine hosts [108]. The double mutant *nanH, nanP* showed no neuraminidase activity, and had 53% reduction in ability to bind to HeLa cells. The single mutants showed no change in their ability to bind to HeLa cells [108].

T. pyogenes also have virulence factors that allow them to bind to collagen, fibrinogen and fibronectin [95]. CbpA, a collagen binding protein coded for by *cbpA* is the best described of these [95]. Two studies [93] and [12] tested for presence of *cbpA* with PCR and did not find a correlation between the presence of *cbpA* and uterine disease, but both of these studies are based on flush samples, where the host collagen might not be as exposed as in biopsies where tissue invasive *T. pyogenes* are present.

In a study from 2010, the putative *T. pyogenes* virulence factor genes *plo*, *cbpA*, *fimA* and *fimG* were tested for by PCR. *fimA*, which codes for a fimbrial subunit, was the only one that was significantly more present in cows with endometritis than in cows without endometritis [93]. A different paper tested *fimA*, *cbpA* and *plo*, and found that *fimA* and *plo* were associated with both metritis and endometritis [12].

1.6.4 *Fusobacterium necrophorum*

F. necrophorum is a Gram-negative, obligate anaerobic bacterium, which belongs to the *Fusobacteriaceae* familiy, the *Fusobacteriales* order, the *Fusobacteria* class, 385 and the *Fusobacteria* phylum. There are two subspecies of *F. necrophorum* with distinct morphology, growth and biochemical properties. *F. necrophorum* subspecies *necrophorum* is most often found in liver abscesses in cattle, and *F. necrophorum* subspecies *funduliforme* is most often found in the rumen of cattle [109] [110] [98] [97]. *F. necrophorum* is an opportunistic pathogen. In humans, *F. necrophorum* is 390 known to cause Lemierres disease, peritonsillar abscesses and tonsillitis [111], and in other domestic animals, *F. necrophorum* is associated with foot rot, calf diphtheria, and necrotic lesions in the oral cavity [96]. The bacteria can be carried in the blood stream and cause necrosis and abscesses in internal tissues [96].

The most important virulence factor produced by *F. necrophorum* is leukotoxin, 395 which is an extracellular protein that is cytotoxic to neutrophils and macrophages [96] [112] [98] [101] and helps the pathogen evade the host defence. Leukotoxin is coded for by the *lkt* operon, consisting of three genes: *lktA*, *lktB* and *lktC* [97]. The *lktA* gene is the primary target for PCR detection of leukotoxin [12] [96] [97] [98]. Leukotoxin is considered the most important virulence factor in *F. necrophorum*[97] 400 [98].

Along with the leukotoxin, there are other secreted toxins: A haemolysin, which creates an anaerobic environment and releases iron through lysis of erythrocytes and is coded for by the gene *hly* [99] [97]; a haemagglutinin that enables adhesion [100], an adhesin, a dermonecrotic toxin, a platelet aggregation factor, and proteases 405 [98]. A recent study found that out of six virulence genes tested in cows with uterine disease with PCR, the *F. necrophorum lktA* gene was the most prevalent gene in dairy cows with endometrits together with the *fimH* gene from *E. coli*[12].

F. necrophorum has outer membrane proteins that mediate adhesion to bovine endothelial cells [102]. A study found that removal of the outer membrane proteins 410 reduced the attachment of *F. necrophorum* to bovine endothelial cells with approximately 25% compared to non trypsin-treated *F. necrophorum*. The four most prominent outer membrane proteins were of the sizes 17, 24, 40 and 74 kDa [102].

Chapter 2

Hypotheses

This Ph.D. project was performed in collaboration with fellow Ph.D. student, Cecilia Christensen Karstrup, from the Section for Veterinary Reproduction and Obstetrics, Department of Large Animal Sciences, Faculty of Health and Medical Sciences, Veterinary Reproduction & Obstetrics, University of Copenhagen. Cecilia is performing the clinical investigations and the histological examinations of the samples.

The present Ph.D. project used molecular methods to investigate the microbiota in the uterus of dairy cows with endometritis, metritis, and pyometra. Until recently, studies of this kind relied on traditional cultivation techniques, which require that the bacteria in the uterus thrive in the laboratory environment used, although not all bacteria are easily detected using this method. The methods used in this project are based on DNA sequencing for detection of bacteria and are more likely to detect fastidious bacteria.

In the primary part of this project, the microbiota of healthy bovine postpartum uterus was compared with the microbiota of cows affected by endometritis and metritis. The second part of the study investigated the microbiota in the uterus of cows with pyometra. These studies were based on 16S rRNA PCR, which in combination with next generation sequencing enables taxonomic identification of the bacteria present in the samples.

Additionally, a metatransgenomic study was performed on samples from healthy cows and cows with postpartum disease in order to identify mRNA transcripts that were up-regulated in the uterus of cows with an ongoing infection compared to seemingly healthy cows. This elucidates which proteins are expressed by bacteria present in the uterus, and offers a more functional insight into the development of endometritis and metritis.

The aim of manuscript 1 was to identify bacteria or groups of bacteria that are associated with endometritis and metritis. The hypotheses used for the study were:

- There is a difference between the microbiota of the postpartum uterus of normal cows and cows with endometritis and metritis.
- The bacterial flora that invades the endometrium is taxonomically different from the bacterial flora in the lumen of the uterus.

The aim of manuscript 2 was to describe the microbiota associated with pyometra. The hypothesis used for paper two were:

- Very few families of bacteria dominate the microbiota in the uterus of cows with pyometra. These bacterial families are assumed to be associated with the disease.
- The bacterial flora that invades the endometrium is taxonomically different from the bacterial flora in the lumen of the uterus.

The aim of manuscript 3 was to describe the most differentially expressed transcripts between healthy cows and cows with metritis or endometritis. The hypothesis used for the paper was:

- The expression pattern differs between bacteria observed in the healthy bovine uterus postpartum and the uterus affected by endometritis and metritis. The most up-regulated transcripts in cows with a uterine disease are assumed represent species of importance for the pathogenicity of uterine disease.
- The expression pattern differs between the microbiota of the endometrium and the microbiota observed in the uterine lumen.

Chapter 3

Methodology

3.1 Bias

The traditional way of investigating the microbiota of any environment is by culture
465 methods. However, in most bacterial populations only part of the bacteria are cultivable on nutrient media and therefore go unnoticed by traditional culturing methods. Next generation sequencing now allows us to investigate the diversity of the not-yet-cultivable part of microbial communities [113] [114] [76].

While the next generation sequencing technology opens up many new opportunities,
470 it is important to keep in mind that there are still rich sources of bias in next generation sequencing, and every time the sample is handled, it seems to be at the peril of introducing more bias. Some of these biases can be reduced through careful vigilance when handling the samples, and others are inherent in the method, for example the preference of certain sequences by the polymerase, the selectivity
475 of the universal primers, and wrong base-calls by the sequencing machine. New sequencing methods (third generation sequencing) are being designed, that do not rely on synthesis, which will be a step nearer a good representation of the tested microbiotas [115].

3.2 Sampling

3.2.1 Sampling for manuscripts 1 and 3

Both hypothesis from manuscripts 1 and 3 were tested on material from the same samples. The first hypothesis was that if compared, the observed uterine bacteria and their expression pattern would differ between the uterus of healthy cows and cows affected by endometritis or metritis. The other hypothesis was that the observed uterine bacteria and their expression pattern would differ between the endometrial biopsies and the uterine flush samples.

Both research questions for manuscripts 1 and 3 were addressed using samples from 68 Holstein cows from a dairy farm in Denmark. The cows were sampled at three different time-points, in weeks 1, 4, and 7 postpartum and we obtained both uterine flush samples and endometrial biopsies. The cows were grouped in week 1 according to their uterine score postpartum and in weeks 4 and 7 by the ratio of neutrophils to endometrial cells observed during the microscopy of smears. The uterine score was based on vaginal exploration performed by the herd veterinarian, in accordance with the Danish scale for uterine (Table 3.1) [58]. The 'normal' group, indicating that the cows in this group were free of disease at the sampling time, consisted of cows with a uterine score of 3 or less in week 1, whereas cows with a uterine score of 4 or higher in week 1 were considered to have uterine disease (puerperal metritis or clinical metritis). This cut-off was based on the treatment threshold of the farm. The 'normal' group in weeks 4 and 7 consisted of cows with a neutrophil to endometrial cell count below 18% and 10%, respectively, as suggested by Kasimanickam et al. (2004) [6]. Cows were considered to have endometritis if they had neutrophil count above 18% in week 4 or 10% in week 7.

Small group sizes of cows with endometritis and metritis were a risk, since the diagnosis was retrospective and the prevalence is 5-25% for endometritis and up to 20% for metritis [7] [8]. In order to obtain a large enough group size of cows with endometritis and metritis, 2-3 cows with a uterine score of 7 or more were included in the study. The rest of the cows were chosen randomly among cows that were within one week after parturition.

The sampling procedure was the point in the study which posed the highest risk of contamination. First of all, the samples were taken from live cows, and a stable is not a sterile environment. Secondly, it is difficult to sample the uterus without contaminating the sample with the microbiota of other parts of the cow. This means that great care must be taken when moving the sampling instruments from the outside, through the vulva, vagina, cervix, and into the uterus. The perineum

Table 3.1: Danish scale for uterine score, routinely used on cows in Denmark 5-20 days postpartum. Translated from danish by Elkjær *et al.* (2013) [58]

Score	Clinic
0	None or a very small amount of clear mucous secretion - no smell.
1	A very small amount of bloody mucous secretion - no smell.
2	Small amount of bloody mucous/gray secretion - no smell.
3	Plenty amounts of bloody seromucous/gray-yellow secretion - scabs on the tail - no smell.
4	Plenty amounts of gray/yellow seromucous secretion - no abnormal smell.
5	Poor/plenty amounts of purulent secretion - difference in moisture and color - smells abnormal.
6	Increasing amounts of secretion - difference in texture and color - smells abnormal.
7	Increasing amounts of secretion - beginning to look red-brownish - stinks.
8	Plenty amounts of grayish secretion - stinks.
9	Large amounts of brown-yellow/brown secrete - typically a retained placenta - very foul smell.

515 was washed with soap to minimise faecal contamination, and the biopsy instrument was sheathed by a plastic sleeve, that was broken in the cervix before entering the uterus. The biopsy instrument was designed so that the biopsy itself would be enclosed within the instrument, protecting it from contamination upon retraction (Figure 3.1A). However, the embryo flush catheter used to take the flush samples
520 was made of latex, and although it was possible to fit the catheter with a plastic sleeve, it was not possible to break the sleeve in the cervix before entering the uterus. The plastic sleeve was not used, and the uterine flush sampling can be seen in Figure 3.1B. The risk of contaminating the uterus with bacteria from the vagina was therefore higher with the uterine flush samples than for the endometrial
525 biopsies. In order to preserve the DNA and the RNA, the samples were stored in RNA*later* [116].

Figure 3.1: Uterine sampling. A: Endometral biopsy, photograph by Eva Láadal Rasmussen. B: Uterine flush, phototograph by Julius Kemna.

3.2.2 Sampling for manuscript 2

The first hypotheses for manuscript 2 was that very few families of bacteria dominate the microbiota in the uterus of cows with pyometra. These bacterial families are assumed to be associated with the disease. This assumption is made because the cervix is closed in pyometra [4] [11] [40], and the re-contamination of the uterus from the environment is less likely to occur. The second hypothesis was that the bacterial flora of the endometrium would be taxonomically different from the bacterial flora in the lumen of the uterus. Both these research questions were addressed with the investigation of the uteri of 21 cows slaughtered at the Danish Crown slaughterhouse, Tønder, Denmark. The uteri were sent whole at 4 °C, and samples were taken within 72 hours of slaughter. The risk of contaminating the samples in the sampling process was much less, as an incision was made directly into the uterus. The outside of the uterus was wiped with ethanol and flamed before the incision was made to minimise contamination from the outside of the uterus.

3.3 Pilotstudy for 16S rRNA sequencing method

Initially, a pilot study was performed to optimise the DNA extraction procedure, to test whether the PCR reaction worked satisfactorily, and to estimate the required sequencing depth. The biopsies obtained from the live animals were approximately 0.3 x 0.3 cm, and it was important to extract sufficient amounts of DNA, and to introduce as little bias as possible. The samples for the pilot study were obtained from the Danish Crown slaughterhouses in Holstebro and the Mogens Nielsen Kreaturslagteri A/S in Herlufsmagle. Samples were obtained from the uterus horn of 50 cows and heifers.

3.4 Optimisation of the DNA extraction

To obtain high quality DNA, we used the samples from the pilot study to test a number of DNA extraction methods. Besides the sampling process, this is the part of the process where substantial bias can be introduced to the samples. Extracting DNA from a composite environment while still preserving the DNA of vastly different bacteria posed a challenge. Generally, Gram-positive bacteria are thought to be more resistant to lysis in the DNA extraction process, due to their thick cell wall, and Gram-negative bacteria may be overrepresented if many Gram-positive bacteria are missed in the DNA extraction process [117] [118]. Reversely, DNA from Gram-

negative bacteria might shear if subjected to harsh lysis conditions of Gram-positive
560 bacteria [119].

Two different commercial DNA extraction kits were tested. In the testing of one of the procedures, the cell walls of both the bacteria and the host cells in case of the endometrial tissue samples were disrupted by mortal and pestle in liquid nitrogen, and in another by mechanical disruption through a spin column. Good results were
565 obtained with beat-beating but it was a concern that the Gram-positive bacteria would escape the cell rupture, so we added an enzymatic step with Lysozyme. The final protocol included both a beat-beading step and a Lysozyme step where the concentration of Lysozyme was doubled and the incubation time halved to limit the time that the samples would be subjected to 37 °C in a lysozme buffer. Of
570 commercial extraction kits, the Allprep kit from QIAGEN and the Maxwell ® LEV Blood DNA Purification Kit (Promega) were tested, and higher DNA concentrations were obtained with the Maxwell blood kit (Figure 3.2). The DNA extraction efficiency was determined based on the concentration of the DNA extracted, and on the 260/280 nm and 260/230 nm ratios (Figure 3.3). The DNA extractions from
575 the Maxwell blood kit also displayed better 260/280 and 260/230 nm ratios than the Allprep kit with samples of this type, and the Maxwell blood kit was therefore chosen for all the DNA extractions in the study.

To minimise the risk of contaminating the samples in the DNA extraction step, personal protection equipment such as laboratory coat and gloves, was worn at al
580 times when handling the samples. All disposable lab equipment that would come into contact with the samples would be UV treated for at least 30 minutes before use.

3.5 The PCR reaction

The universal primers applied in this project are designed to target all eubacteria and
585 are therefore very sensitive to contamination in the PCR set up. Negative controls were included for each primer set. To avoid contamination, the environment was kept as clean as possible. The reaction was set up in a room dedicated solely to PCR set up. The template DNA was added in a different room, and the PCR cyclers were situated in a third room. Personal protection equipment was worn at
590 all times in the PCR room, including plastic covers for foot-wear, laboratory coats and disposable gloves.

The PCR step can potentially introduce considerable bias into the samples. PCR conditions differ widely between commercial providers of PCR kits. Some poly-

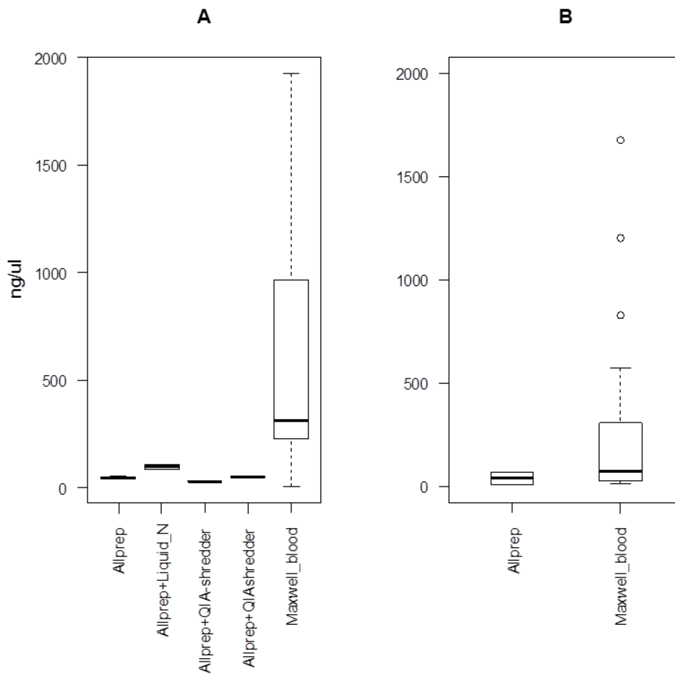

Figure 3.2: Concentration of DNA extracted with different methods. A: DNA extraction from endometrial tissue samples. Allprep: n = 3, Allprep + QIAshredder: n = 3, Allprep + liiquid N: n = 2, Maxwell blood: n = 19. B: DNA extraction from uterine flush samples. Allprep: n = 6, Maxwell blood: n = 22.

merases have preferences for certain nucleotide sequences [120], and different poly-
595 merases require different temperatures or buffer conditions for optimal function, which may also influence the primer to template annealing. Furthermore, 16S rRNA primers can target the nine different hypervariable regions of the 16S rRNA ribosomal gene. The performance of the 16S rRNA primers differs depending on the hypervariable region targeted [121], and some bacteria have genomic sequences
600 flanking the 16S rRNA gene that inhibits PCR [122].

Twelwe different 16S rRNA primers were tested in ten different combinations. To simulate the conditions in the uterine samples, we made a small mock samples consisting of *E. coli*, *T. pyogenes*, *F. necrophorum*, *P. levii*, *Streptococcus epidemicus*, and *Clostridium perfringens*. The primer set that came closest to producing one
605 distinct DNA fragment without too much smear was the V1F and V2R primer combination. This primer set was used for all subsequent PCR reactions.

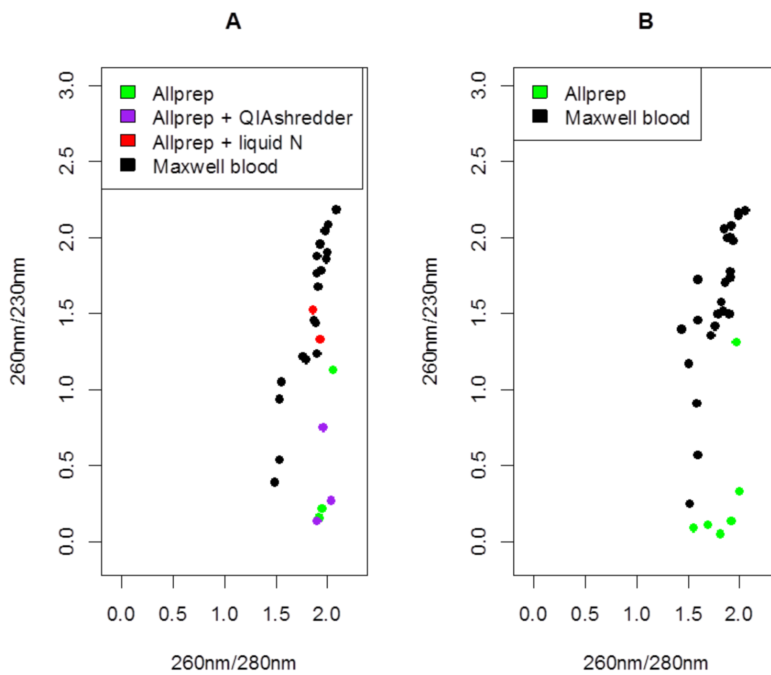

Figure 3.3: Purity of the DNA extracted with different methods. A: DNA extraction from endometrial tissue samples. B: DNA extraction from uterine flush samples.

3.6 Next generation sequencing

Amplicons from 33 pilot study samples were sequenced at the GATC facilities (GATC, Gernamy). The Roche 454 sequencing method was chosen because of the
610 longer reads that this type of sequencing provides. The results of the pilot study showed a visible difference between the microbiota from cows without apparent uterine disease and the microbiota from cows with apparent endometritis. This was found in both endometrial tissue samples (Figure 3.4A) and uterine flush samples (Figure 3.4B). The rarefaction curves from the pilot study approached plateau at
615 approximately 2000 reads per sample except for a few samples with steeper rarefaction curves (Figure 3.5). Because of the low complexity of the samples, as indicated by the rarefaction curves, more samples could be included in study without loosing information about many species present in those samples. In both the manuscripts 1 and 2, Illumina Miseq sequencing technology was used instead of 454, because
620 paired-end technology enabled read lengths that would cover the approximately 360

Table 3.2: 16S rRNA primers targeting different hypervariable regions of the 16S rRNA gene. The IUPAC nucleotide code was used for ambiguous nucleotides.

Primer	Sequence	Hypervariable region
V1F	5'-AGAGTTTGATCCTGGCTCAG-3'	V1 [123] [121]
V2R	5'-CTGCTGCCTYCCGTA-3'	V2 [124] [121]
V2F	5'-AGYGGCGNACGGGTGAGTAA-3'	V2 [125] [121]
V3R	5'-ATTACCGCGGCTGCTGG-3'	V3 [126] [121]
V3F	5'-ACTCCTACGGRAGGCAGCAG-3'	V3 [121]
V4R	5'-TACNVGGGTATCTAATCC-3'	V4 [121]
V4F	5'-AYTGGGYDTAAAGNG-3'	V4 [121]
V5R	5'-CCGTCAATTYYTTTRAGTTT-3'	V5 [121]
V5F	5'-RGGATTAGATACCC-3'	V5 [121]
V6R	5'-GYACWCACCGCCCGTC-3'	V6 [127] [121]
V7F	5'-GYAACGAGCGCAACCC-3'	V7 [121]
V8R	5'-GACGGGCGGTGWGTRC-3'	V8 [121]

bp long amplicons for a lower cost.

3.7 Dataanalysis

The pilot study data analysis was performed with the open source software QIIME [128], while the data for manusripts 1 and 2 were analysed using the open source software BION (https://app.box.com/bion). The BION software was faster than QIIME at performing the analysis, and the output was easier to subject to a statistical analysis of choice. The database which the sequences are compared against can influence which sequences are annotated. The database used in this study was the RDP database (http://rdp.cme.msu.edu), which is a large, curated database [129]. The classification tool used does not seem to introduce significant bias, except for when handling reads below 250 bp [65]. The OTUs that were unclassified at the family level were given at the next possible taxonomical level, such as class or phylum. A few sequences could not be classified beyond the kingdom level.

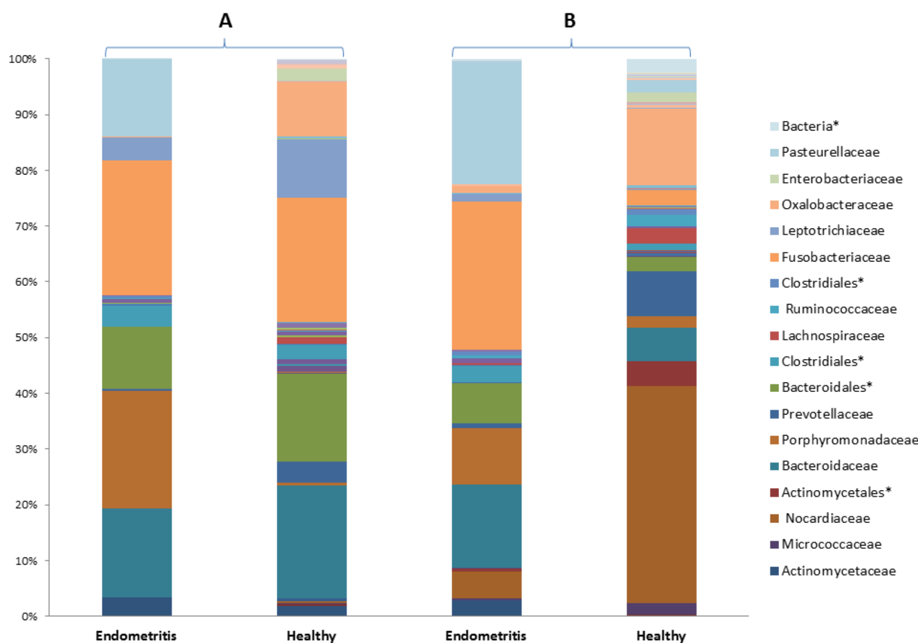

Figure 3.4: Colour-coded histogram representing the distribution of Operational Taxonomic Units (OTUs) observed in the uterus of cows from the slaughterhouse. A: Tissue samples. Samples from cows with uterine disease: n = 14, samples from cows with no apparent uterine disease: n = 3. B: Uterine fluid samples. samples from cows with uterine disease: n = 9, samples from cows with no apparent uterine disease: n = 7.

3.8 Culturing and tests for antibiotic susceptibility

As part of this Ph.D. project the level of antibiotic susceptibility of the bacteria isolated from the postpartum uterus of Danish dairy cattle was to be investigated. Aliquots of the uterine flush samples that were collected for manuscripts 1 and 3 were saved in collection tubes without RNA*later*. Within 12 hours, these uterine flush samples were streaked onto MacConkey agar plates, VL agar plates and veil blood agar plates and incubated. The MacConkey plates were incubated at 37 °C aerobically for 16 hours, the veil blood agar plates were incubated at 37 °C in 10% CO_2 for 16 hours, and the VL agar plates were incubated anaerobically at 37 °C for

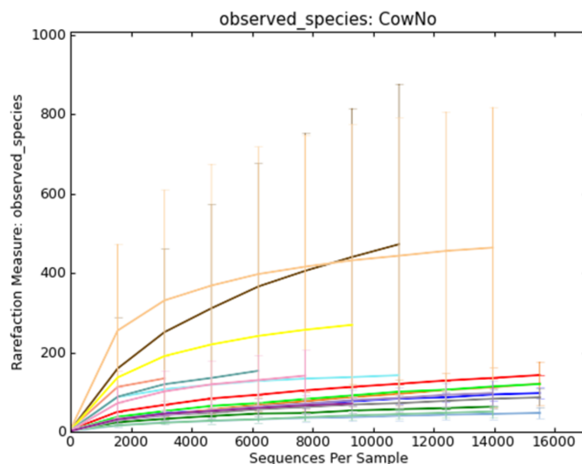

Figure 3.5: Rarefaction curves from the pilot study

five days. It was decided to culture for *E. coli*, *T. pyogenes*, *F. necrophorum* and *P. levii*, however, pure cultures of *P. levii* proved unattainable, despite many attempts. Obtaining clean isolates was based on morphological observations. The antibiotic susceptibility was tested using VetMic GP-mo (version 2) (Art. No. 395102 from Statens veterinärmedicinska anstalt, SVA) and VetMic GN-mo (version 4) plates (Atr. No. 395103 from SVA).

The VetMic system is based on liquid cultures of the bacteria, so the optical density (OD) to Colony Forming Units (CFU) relationship for the different bacteria were tested. This test indicated that *T. pyogenes* did not distribute evenly in liquid cultures. The remaining isolates to be tested were 33 *F. necrophorum* strains and 34 *E. coli* strains isolated from the uterus of the 68 cows from paper 1. Then 90 μl Mueller Hinton broth (Mueller-Hinton broth, Magnesiumchlorid-Hexahydrat, Calciumchlorid-Dihydrat, Tes, pH 7.4) was loaded in each VetMic well, and 10^6 bacteria in 10 μl was added to each well, except *E. coli*, of which 10^5 cells were added. The plates were incubated at 37°C in anaerobic conditions.

Following incubation, the Minimum Inhibitory Concentration (MIC) was determined

660 by localising the well with the concentration of antibiotics at which the 10^6 bacteria showed no growth. The control strains we are using a strain of *E. coli*, *Enterococcus faecalis* and *F. necrophorum*. There were no official breakpoints from the European Committee for Antimicrobial Susceptibility Testing (EUCAST) for the bacteria we were testing, so unless two populations with differing susceptibility were observed,
665 conclusions of susceptibility could not be made.

The susceptibility test was performed once with the *E. coli* strains. However, the reference strain was not behaving in a similar manner between plates, and because of time limitation, the susceptibility tests were abandoned.

3.9 Pyometra study

670 As mentioned above, the aim of manuscript 2 was to describe the microbiota associated with pyometra, and to test if the microbiota of the endometrial biopsies were comparable to the microbiota of the uterine flush samples. The research questions were addressed using samples from uteri of cows obtained from a slaughterhouse. The inclusion criteria were an inflamed uterine mucosa, an enlarged uterus owing
675 to accumulated pathological exudates, and the presence of a corpus luteum. The samples were discarded if a corpus luteum was not present, if the uterus was not inflamed, or if the cow was less than 21 days postpartum. The latter information was retrieved from the Central Husbandry Register, an elaborate system of animal identification and registration, which is owned by the Danish Ministry of Food,
680 Agriculture, and Fisheries. Having already optimised the DNA extraction method, the same methods as described above were applied. *E. coli*, *F. necrophorum* and *T. pyogenes* were isolated for antibiotic susceptibility testing, but the antibiotic susceptibility tests were abandoned for the same reasons as described previously.

3.10 Metatranscriptomic study

685 The hypotheses for manuscript 3 was that the expression pattern would differ between bacteria observed in the uterus of cows with endometritis and metritis and the bacteria observed in the uterus of healthy cows. Additionally, the expression pattern between the lumen and the endometrium was expected to differ.

The research question were addressed with a subset of 27 samples from manuscript
690 1, in which 68 cows were sampled at weeks 1, 4, and 7 postpartum. Both endo-

metrial biopsies and uterine flush samples were obtained. The subset was chosen before the final classification of uterine health status was made. Inclusion was based on the uterine scores as described in Table 3.1 and inclusion of both healthy cows and cows with a uterine disease was attempted. The final classification of samples into 'normal' and 'uterine disease' groups was based on both uterine score and the neutrophil to endometrial cell ratio in the smears, as described in section 3.2. Due to the small sample size of the subset, the metritis and endometritis samples were joined in one group, named 'uterine disease' but a cow was only represented once in a group. None of the cows in the subset were sick at all three time points nor healthy at all three time points.

3.11 RNA extraction

The commercial RNA extraction kits tested were the RNeasy mini kit (QIAGEN), the Allprep DNA/RNA mini kit(QIAGEN), and the SV total RNA Isolation System (Promega). The RNA concentration and integrity was analysed on an Agilent 2100 Bioanalyzer (Agilent). The two ribosomal peaks, representing 16S rRNA and 23S rRNA, are usually used to assess the integrity of the extracted RNA. However, when visualised on the Agilent 2100 Bioanalyser, most of the uterine flush RNA extractions had unusual large and wide peaks that masked the two ribosomal peaks (Figure 3.6), thus making it difficult to assess the RNA integrity of the uterine flush samples. All the commercial RNA extraction kits showed the same results with the flush samples, and eventually, the Allprep kit was chosen because of a slightly higher RNA concentrations of the RNA extracted from the biopsy samples.

3.12 Enrichment

The samples were enriched for bacterial RNA using MICROBEnrich^{TM} kit (Ambion), which is based on the binding and subsequent removal of 18S and 28S sequences as well as polyadenylated RNA, thus binding and discarding most of the eukaryotic RNA. The main part of the bacterial ribosomal RNA was then removed from the samples by use of the MICROBExpress^{TM} bacterial mRNA enrichment kit (Ambion), which is based on binding and removing the 16S and 23S sequences. The danger of enriching the RNA is that each time the RNA is handled, there is a risk of degradation of the RNA, and transcripts are lost in each washing step too [130]. In spite of these disadvantages, the enrichment will reduce the vast amount of host RNA and ribosomal RNA, that are irrelevant in an expression study. Host RNA and rRNA would most likely be the most prevalent RNA present in the samples,

725 and the relatively under represented bacterial mRNA would be harder to detect [130]. Finally, a reverse transcription of the mRNA to cDNA before sequencing is performed, and this procedure may also introduce bias because the use of random hexamer primers may favour certain sequences [131].

3.13 Dataanalysis

730 The data analysis of expression data was performed on open source software, as described in the draft for manuscript 3. The assembled reads were mapped first to the *Bos taurus* genome and then to the 16S rRNA gene to remove host mRNA and ribosomal RNA that was not removed by the enrichment procedure. The samples differed in how much of the mRNA mapped to the cow genome and the 735 16S rRNA gene but on average, approximately 2% of the total RNA was bacterial non-ribosomal RNA. These 2% of reads were subject to an analysis using the open source package EdgeR to compare transcripts from cows with either endometritis or metritis with transcripts from animals with no apparent uterine disease. The 50 most differentially expressed transcripts were annotated by performing a BLAST 740 search against both the non-redundant (nr) database.

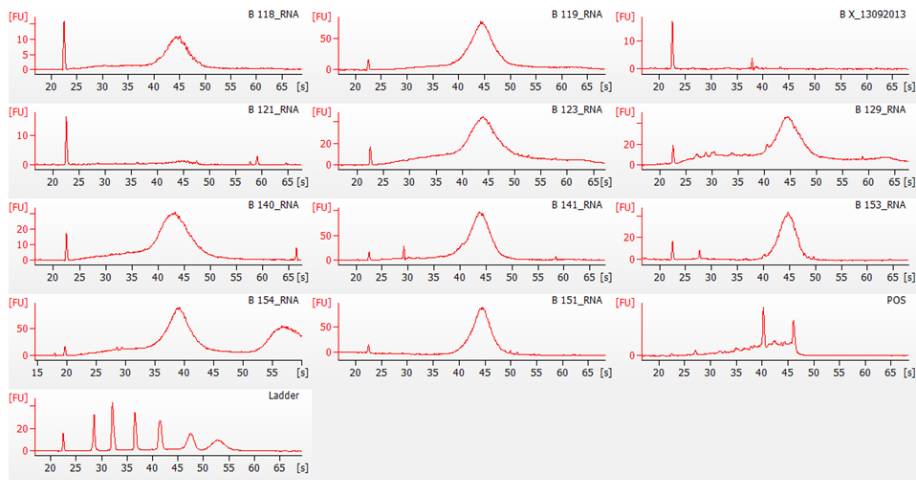

Figure 3.6: Total RNA extracted from uterine flush samples. The ribosomal peaks can be seen in the positive control (lower right corner, marked 'POS'). In this run, the ribosomal peaks appear at 40 s and 46 s, approximately. In many of the other samples, a large, wide peak stretches from 40 s to 50 s, thus masking the ribosomal peaks.

Chapter 4

Results and discussion

The first hypothesis of manuscript 1 was that there is a difference between the microbiota of the postpartum uterus of normal cows and cows with endometritis and 745 metritis. The hypothesis was confirmed for metritis, as the operational taxonomic units (OTUs) mapping to the *Fusobacteriaceae* and *Porphyromonadaceae* families were significantly associated with metritis in week 1 postpartum. No OTUs were found to be significantly associated with endometritis, so the hypothesis could not be confirmed. There were larger differences between the endometrial biopsies and 750 the uterine flush samples in the endometritis samples than was observed in the metritis samples. In week 4 postpartum, OTUs mapping to *Mycoplasmataceae* and *Leptotrichiaceae* families were abundant in the uterine flush samples, and in week 7 the OTUs mapping to the *Mycoplasmataceae* family made out the majority of the OTUs in the uterine flush samples from cows with endometritis. In the 755 endometrial biopsies from weeks 4 and 7, the most observed OTUs mapped to the *Ruminococcaceae* and *Bacteroidaceae* families, and an unclassified family that belong to the *Bacteroidia* class. *E. coli* and *T. pyogenes* are considered important bacteria in the development of metritis and endometritis. Less than 1% of the observed OTUs mapped to the *Enterobacteriaceae* (*E. coli*) or *Actinomycetaceae* 760 (*T. pyogenes*) families at any of the investigated time points. Therefore, the data presented in this thesis does not support the association of these pathogens with endometritis and metritis.

The first hypothesis for manuscript 2 was that very few families of bacteria would

be observed to dominate the microbiota in the uterus of cows with pyometra. These bacterial families were assumed to be associated with the disease. The hypothesis was supported by the finding that a low complexity was observed in the microbiota of the uterus from cows with pyometra. The OTU mapping to the *Fusobacteriaceae* family accounted for 27% of the total reads in the endometrial biopsies from cows with pyometra, and 35% of the total reads from the uterine flush samples. The Gram-negative families *Bacteroidaceae*, *Pasteurellaceae*, and *Porphyromonadaceae* accounted for 19%, 13%, and 15%, respectively, of the uterine flush samples, and 19%, 23%, and 14% of the endometrial biopsies. Previous studies, which relied on traditional culture methods, have observed *F. necrophorum*, *T. pyogenes*, and unidentified Gram-negative bacteria to be the pathogens of pyometra. This study confirmed that the family *Fusobacteriaceae*, in which *F. necrophorum* is contained, is associated with pyometra. On the other hand, the OTU mapping to the *Actinomycetaceae* family, which harbours *T. pyogenens*, accounted for less than 1% of the total amount of OTUs. Based on this data, it can therefore not be confirmed that *T. pyogenes* is associated with pyometra. The *Bacteroidaceae*, *Pasteurellaceae*, and *Porphyromonadaceae* families were also observed in the uterus from cows with pyometra in this study, and these may contain the unidentified Gram-negative species that were found to be associated with pyometra in the studies based on traditional culture methods.

In manuscript 3, the main hypothesis was that there is a difference in the expression pattern between the bacteria observed in the uterus of normal cows and the microbiota observed in the uterus of cows with endometritis and metritis. The endometritis and metritis groups were pooled to increase group sizes, but the subset observed was too small for statistical analysis. A descriptive approach was chosen, and the 50 most differentially expressed transcript between normal cows and cows with endometritis or metritis were compared for both endometrial biopsies and uterine flush samples. Despite the lack of statistical analysis, up to 13 log2 (fold change) were observed between normal cows and cows with uterine disease, which indicates that the hypothesis cannot be rejected.

In the uterine flush samples, 66.7% of the 50 most differentially expressed transcripts either exhibited low homology with potential hits in the non-redundant database at NCBI, or had closest homology with genes from the *Bos taurus* genome. These transcripts were not annotated. The high percentage of non-annotated transcripts indicates low quality of the sequences, despite filtering the reads for quality in the data analysis. These difficulties could be a result of the difficulties experienced with the RNA extraction, as described in section 3.11. As a consequence of the high percentage of non-annotated transcripts, the results from the uterine flush samples were not compared with the results from the endometrial biopsies.

The annotation of the transcripts that are most differentially expressed between

normal cows and cows with endometritis and metritis revealed that majority of the
805 annotated transcripts were from the species *F. necrophroum* and *P. levii*. This was unexpected because of the diversity observed in the metagenomic investigation of the same cows in manuscript 1. In the endometrial biopsies, 75% of the 50 most diversely expressed transcripts were from the *F. necrophorum* species, 10% were from *P. levii*, 6% were other species just represented by one transcript, and 2%
810 were not annotated. In the uterine flush samples only 2% of the 50 most diversely expressed transcripts were from the *F. necrophorum* species, while 16% were from *P. levii*, 12.5% were other species just represented by one transcript, and 66.7% were not annotated, as mentioned above. In both sample types, 4% of the transcripts were from the genus *Porphyromonas*, excluding *P. levii*.

815 The 50 most differentially expressed transcripts in the postpartum uterus reveal a microbiota in an active multiplication phase during the infection. This is shown by the up-regulation of genes involved in DNA replication as well as protein transcription and translation. The functional groups "DNA replication and repair" and "Transcription/translation" contained 9% and 19% of the identified ORFs, respect-
820 ively. The functional group "Metabolic processes" contained 16% of the identified ORFs. In order to survive in the host environment, the bacterial pathogens must adapt. For example, they may adapt their metabolism to the available nutrients. It was found that particularly the *F. necrophorum* gene regulation displayed signs of host interaction with induced cell wall metabolism, altered carbon metabolism,
825 and activation of classical chaperones and transport systems. Furthermore, we observed an up-regulation of a number of potential virulence genes in *F. necrophorum*, including genes potentially involved in synthesis of LPS, lipid A, and haemagglutinin as well as several protease-coding genes. The functional group "pathogenesis" contained 2% of the identified ORFs.

830 The results from the three manuscripts support each other. In manuscript 1, OTUs mapping to the *Fusobacteriaceae* and *Porphyromonadaceae* families were significantly associated with metritis. No OTUs were significantly associated with endometritis, and depending on the sampling time and the sample type, the *Mycoplasmataceae*, *Leptotrichiaceae*, *Ruminococcaceae*, *Bacteroidaceae*, or an unclassified
835 family from class *Bacteroidia* were observed in the largest amounts. In manuscript 2, the *Fusobacteriaceae* family is associated with pyometra, and the *Bacteroidaceae*, *Pasteurellaceae*, and *Porphyromonadaceae* families were also observed in large quantities. In manuscript 3, *F. necrophorum* and *P. levii* were found to be the most likely candidates of uterine disease. *F. necrophorum* and *P. levii* are contained
840 in the *Fusobacteriaceae* and *Porphyromonadaceae* families, respectively, which are the two families significantly associated with metritis, as observed in manuscript 1, and two of the families observed in manuscript 2. However, only one of the cows sampled in manuscript 3 was diagnosed with metritis, the remaining samples were from cows diagnosed with endometritis, which was not significantly associated with

845 any OTU. The 16S rRNA sequencing method used in manuscripts 1 and 2 does not distinguish between live or dead bacteria, while the RNAsequencing method only detects metabolically active bacteria. It is therefore possible that although the *Fusobacteriaceae* and *Porphyromonadaceae* families were not among the most observed families in the 16S rRNA sequencing experiment, they were the most metabolically 850 active bacteria at the sampling times. Our expression analysis focussed on the 50 most differentially expressed transcripts between the uterus of normal cows and cows with endometritis and metritis. This excludes bacteria that do not differ much in metabolism between the normal cows and the cows with endometritis and metritis. Many bacteria have been observed to be present in the postpartum uterus regardless 855 of the uterine health status, and these bacteria would not be visible in the results from manuscript 3. Nevertheless, the results indicates that *F. necrophorum* and *P. levii* are the two most likely pathogens of endometritis, metritis, and pyometra.

In all three manuscripts, the second hypothesis was that there was a difference between the microbiota observable in the endometrial biopsies and the uterine flush 860 samples. In manuscript 3, however, the comparison was abandoned because the majority of the transcripts from the fluid samples were not annotated due to poor homology to anything in the nr database. Our results indicate that endometrial biopsies are better suited than uterine flush samples for expression analysis of the uterine microbiota due to technocal difficulties. Although it was found in both 865 manuscripts 1 and 2 that the microbiota of the two different sample types were statistically correlated, there were large discrepancies between some of the most abundant OTUs observed between sample types in manuscript 1. For example, in week 7 postpartum, the *Fusobacteriaceae* and *Leptotrichieaceae* families made out 20% and 13%, respectively, of the microbiota observed in the uterine flush 870 samples, whereas they were not detected in the endometrial biopsies at the same time. Furthermore, the *Mycoplasmataceae* family made out 43% and 52% of the total number of OTUs of the uterine flush samples but only 12% and 4% of the endometrial biopsies in weeks 4 and 7, respectively. Interestingly, one of 50 most differentially expressed transcripts in the endometrial biopsies, as observed in ma- 875 nuscript 3, were from *Mycoplasma bovigenitalium*, which is contained within the *Mycoplasmataceae* family. This transcript was not observed in the 50 most differentially expressed transcripts of the uterine flush samples in manuscript 3, although OTUs mapping to the *Mycoplasmataceae* family accounted approximately half of the OTUs observed in the flush samples in manuscript 1. This further illustrates 880 that the RNA sequencing results from the uterine flush samples should be regarded with caution.

Despite the statistical correlation between the sample types in manuscript 1, large discrepancies were observed for some bacterial families, and the hypothesis was not categorically rejected. It was concluded that the endometrial biopsies were a 885 valuable addition to the uterine flush samples. In manuscript 2, microbiota of the

endometrial biopsies and the uterine flush samples were statistically correlated, and large differences between single bacterial families were not observed. The hypothesis was thus rejected. A possible explanation of the difference in these results could be that there was a difference in sampling method. In manuscript 1, the samples were collected from live animals through a latex embryo flush catheter, and in case the uterine fluid was not readily obtainable, 20-40 ml of sterile saline solution was flushed into the uterus and then aspired. In manuscript 2, the samples were collected from uteri of slaughtered cows, and the samples were taken directly from the uterine horn through an incision. The dilution of the uterine samples with sterile saline solution may add to a bias in the collected sample to a higher degree than when the samples are taken directly from the uterus.

In manuscrits 1 and 2, a low Shannon index score has been observed in the samples from the cows with endometritis, metritis and pyometra. The Shannon index score reflects the number of different species found in the samples, and the evenness of their distribution. Recent metagenomic studies have also shown a reduced species richness in uterine samples from cows with uterine disease compared to uterine samples from helthy postpartum cows [56] [54]. In manuscript 3, the majority of the 50 most differentially expressed transcripts were from only two different species. It is possible that the diversity observed in the healthy cows in comparison to the cows with a uterine disease is a reflection of the diversity in the contaminating bacteria, most likely originating in the environment, while a bloom in pathogenic bacteria in the uterus of cows with a uterine disease might affect the diversity negatively.

One of the findings of this Ph.D. study was that the families that harbour *E. coli* and *T. pyogenes* only accounted for 1% of the OTUS in manuscripts 1 and 2. In the pilot study, *Enterobacteriaceae* was observed to account for up to 2% of the OTUs in the samples from the uteri that were apparently free of uterine disease, and less than 1% of the OTUs observed in the samples from the cows with endometritis. A higher observance rate of *E. coli* and *T. pyogenes* in the uterus of cows with metritis and endometritis was expected because previous studies based on traditional culture methods have found that *E. coli* are often present early in the uterus of dairy cows that developed endometritis [132] [133] [92], often followed by *T. pyogenes* and other Gram negative bacteria [132] [133]. Although the traditional culture methods did introduce bias towards cultivable bacteria in the investigations of the postpartum microbiota of the bovine uterus [113], and the next generation sequencing method does not introduce this bias, the next generation sequencing methods may introduce other kinds of bias. These are discussed in section 3. In order to investigate whether the DNA from the Gram-negative *E. coli* was degraded in the DNA extraction step, sample material from the pilotstudy was spiked with *E. coli* (ATTC 25922) and extracted using the same protocol that had been used for manuscripts 1 and 2. The resulting DNA was subjected to a PCR reaction with the 16E1 and 16E2 primers

[134] that amplify a fragment of the *E. coli* 16S gene. This reaction produced DNA fragments of the expected size (data not shown), and it was concluded that the DNA from *E. coli* was not degraded in the DNA extraction process. The observation that *E. coli* does not account for many of the observed OTUs in the bovine postpartum uterus is in accordance with recent similar studies [54] [135]. Recent studies show that *Bacteroidetes* and *Firmicutes* make out the majority of the bovine ruminal microbiota; *Proteobacteria* account for 5-6% and *Actinobacterium* for approximately 1% [43], and that only 3 out of 20 dairy cows had *E. coli* in their faecal samples [136]. These findings suggest that *E. coli* and *T. pyogenes* are not present in high abundance in the stable environment, where the postpartum uterine contamination may originate [35] [4].

As mentioned above, this Ph.D. thesis was performed in collaboration with a Ph.D. student, Cecilia Christensen Karstrup, from the University of Copenhagen, Faculty of Health and Medical Sciences, Department of Large Animal Sciences, Veterinary Reproduction & Obstetrics. Cecilia student is performing the histological examinations of the samples and Fluorescent *in situ* Hybridisation (FISH), and those results might elucidate the extent of tissue invasion and validate some of the results found in this thesis. The general absence of large amounts of *E. coli* has been confirmed (personal communication). A histological examination such as the one that is taking place at the University of Copenhagen is of great importance, as it visualises the extent of the bacterial infection and invasion, potentially reveals individual location of pathogens, and verifies the presence of intact bacteria. The 16S RNA gene sequencing method does not discriminate between dead and live bacteria, and although this method gives a very good overview of the postpartum microbiota, it does not necessarily indicate which of the bacteria are causal for the disease. With histological methods it is possible to stain for dead or live bacteria. Using RNA sequencing technology, it is also possible to observe only the metabolically active bacteria, and in addition, it is possible get a functional inside on the infecting bacteria. However, there may be a difference between the mRNA expressed and the mRNA that is translated into proteins. To obtain a more accurate overview of the proteins needed by the pathogenic bacteria to sustain an infection, a proteomics analysis must be performed, like it has been for cows with retained membranes [32]. Future works, besides the histological examinations should include an RNA analysis of a larger dataset from more than one herd, with distinct sample groups of metritis and endometritis. It would also be very interesting to observe the pyometra sample with the RNA sequencing method to investigate the extent of metabolically active bacteria in the pyometric uterus. Such an analysis may identify more potential virulence genes. In addition, it might be possible to use the RNA sequencing data to identify vaccination targets. For this purpose, it should be verified that the relevant genes are translated into proteins.

The species *F. necrophorum* and *P. levii* were identified as the two species with the

strongest association with metritis, endometritis and pyometra. *F. necrophorum* is a known pathogen of uterine disease, but it has not yet been investigated which
970 subspecies is most dominant in uterine diseases. This should be investigated in a molecular manner, either using FISH, PCR or qPRC with specific primers against both subspecies. Furthermore, not much is known about the role of *P. levii* in uterine disease. This species should be characterised by performing a full genome sequencing.

975 Finally, the antibiotic susceptibility tests should be repeated. Given the difficulties of obtaining clean cultures of *P. levii*, as found in the abandoned antibiotic susceptibility test, perhaps a qPCR method could be used, designed to detect already known antibiotic resistance genes.

Chapter 5

Conclusion

In manuscript 1, it was found that there was an association between the *Fusobacteriaceae* and *Porphyromonadaceae* families and metritis in week 1 postpartum. For endometritis in weeks 4 and 7, no bacterial family was consistently associated with the disease across time points and sample types. There were large differences between the uterine flush samples and the endometrial biopsies, and although the sample types were correlated, the diversity of the microbiota in the biopsy samples was higher than the diversity of the microbiota of the uterine flush samples. Furthermore, the bacterial families that made out the majority of the population in the biopsies were the same over time. The data from this paper did not support the association of *E. coli* and *T. pyogenens* with uterine disease.

In manuscript 2, it was found that the most abundant family observed in cows with pyometra was *Fusobacteriaceae*, which contain *F. necrophorum*, a pathogen previously known to be associated with pyometra, whereas evidence of the association of *T. pyogenes* with pyometra was less convincing. The previously unidentified Gram-negative bacteria observed in other studies of pyometra are likely to belong to the *Porphyromonadaceae*, *Pasteurellaceae*, and *Mycoplasmataceae* families identified.

In manuscript 3, it was found that the 50 most up-regulated transcripts in the uterus of cows with metritis and endometritis were primarily involved in DNA replication, transcription, translation, and metabolic processes. This indicates an active multi-

1000 plication phase in the infection, and an adaption to the host environment. Furthermore, an up-regulation was observed of genes potentially involved in the synthesis of LPS, lipid A, haemagglutinin, and several genes that code for proteases. These genes are putative virulence factors. In addition, it was noted that the majority of the most differentially expressed transcripts mapped most closely to proteins from 1005 the *F. necrophorum* and *P. levii* species. This indicates that these species are the most metabolically active in the uterus of cows with uterine disease, and that these may be the primary pathogens of uterine disease. Transcripts from other species were also observed to be highly expressed in the uterus of cows with uterine disease, most notably a transcript from *M. bovigenitalium*.

1010 The results in this thesis underline the high number of bacteria found in the bovine postpartum uterus. *F. necrophorum* and *P. levii*, were observed to be the most important pathogens in uterine disease, but the association of other bacterial species, perhaps contained within the *Mycoplasmataceae*, *Pasteurellaceae*, *Ruminociccaceae*, *Bacteroidaceae*,*Leptotrichiaceae* families, and an uncharacterised family 1015 that belong to class *Bacteroidia* seems likely. The data presented in this thesis does not support a role of the *E. coli* and *T. pyogenes* species, that have been identified as possible pathogens with traditional culture methods.

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Manuscript 1

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An investigation of the microbiota in uterine flush samples and endometrial biopsies from dairy cows during the first 7 weeks postpartum

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1 **Abstract**

2 Metritis and endometritis often develop in dairy cows after calving but although numerous studies
3 have been performed to identify the pathogens, a complete overview has not been obtained.
4 Metagenomic studies have analysed the bacterial populations of uterine flush samples from
5 postpartum dairy cows but the microbiota in the uterine lumen may differ from the microbiota of
6 the endometrium itself, and important putative pathogens may therefore have been overlooked. In
7 the present study, we investigated and compared the microbiota of both the uterine lumen and the
8 endometrium of healthy, metritic and endometritic cows. Samples were collected from 68 Holstein
9 dairy cows at 1, 4, and 7 weeks postpartum, and the data was analysed by deep sequencing of the
10 V1 and V2 hypervariable regions of the 16S rRNA gene.

11 The results showed that *Porphyromonadaceae*, *Fusobacteriaceaea*, *Leptotrichiaceae*, and
12 *Mycobacteriaceae* may be associated with uterine disease. The microbiota of the uterine flush
13 samples and the endometrial biopsies were correlated, but the microbiota of the biopsies was more

diverse. Furthermore, *Fusobacteriaceae* and *Leptotrichiaceae* were not observed in the biopsies in week 7, whereas they accounted for 20% and 13%, respectively, of the bacterial populations in the flush samples. In addition, the *Mycoplasmataceae* family was observed in much higher quantity in the flush samples than in the biopsies of the endometritis groups in weeks 4 and 7. Our findings support the observations of previous metagenomic studies but also illustrate the importance of including endometrial biopsies to obtain a more complete picture of the postpartum uterine microbiota.

Keywords: Metritis, Endometritis, dairy cow, metagenomics, microbiota

Introduction

The uterus is generally considered to be a sterile environment between parturitions, apart from the postpartum period. During the first 10-14 days after calving, an estimated 90% of all dairy cows have intrauterine bacterial contamination (Sheldon and Dobson 2004). Within 8 weeks postpartum, a sterile environment should be re-established in the healthy uterus through the process of involution (Földi et al., 2006). However, bacterial infection may cause endometritis in 5-25% of cows, develop into a subclinical infection within 4-8 weeks postpartum in 30-50% of cows, or progress into metritis in up to 20% of cows (Dubuc 2011; LeBlanc 2014). Metritis is a profound inflammation of the uterine wall with signs of systemic disease that occurs up to 21 days postpartum but often within 10 days (Sheldon et al., 2006). Endometritis is characterised by the presence of uterine purulent or mucopurulent uterine exudates 21 days or more postpartum (Sheldon et al. 2006), and subclinical endometritis is a postpartum uterine infection diagnosed by cytology in the absence of purulent or mucopurulent uterine exudates (Sheldon et al. 2006).

37 Postpartum uterine infection in dairy cows is negatively correlated with reproductive performance,
38 which, in combination with a high prevalence, makes this disease costly for farmers (Gilbert, 2011;
39 LeBlanc et al., 2011, 2002).

40 A variety of bacteria contaminate the bovine uterus shortly after calving but the relationship
41 between individual bacterial species or groups of bacteria and the development of uterine infection
42 is poorly understood. The bacteria that contaminate the uterus postpartum most likely originate
43 from the environment (Bicalho et al., 2011), which may explain why some phyla such as
44 *Bacteroidetes*, *Fusobacteria*, *Firmicutes*, *Proteobacteria*, and *Tenericutes* have been observed in
45 the postpartum uterus, regardless of the uterine health status of the cows (Santos and Bicalho,
46 2012).

47 In conventional culture studies, *Escherichia coli*, *Trueperella pyogenes*, *Prevotella* spp., and
48 *Fusobacterium necrophorum* have commonly been detected in the uterus of cows with a postpartum
49 uterine infection (Königsson et al., 2001; Sheldon et al., 2006; Williams et al., 2005). Metagenomic
50 studies have found an association between *Fusobacterium* spp., *Trueperella* spp., *Ureaplasma* spp.,
51 *Prevotella* spp., and *Bacteroides* spp. and uterine disease 35 days postpartum (Machado et al.,
52 2012). An association was also found between endometritis and *Bacteroidetes*, *Firmicutes*, and
53 *Fusobacteria* (Santos and Bicalho, 2012). Other bacteria, such as members of the phylum
54 *Proteobacteria*, may be related to maintaining the uterine health of postpartum dairy cows
55 (Machado et al., 2012); furthermore, *Lactobacillus* spp. and *Propionibacter* spp. appear to be
56 associated with good reproductive performance (Machado et al., 2012).

57 Profound insight into the diversity and composition of the microbiota of both the diseased and
58 healthy uterus of dairy cows is a prerequisite for developing comprehensive treatments for these
59 reproductive disorders. Although a number of potential causative microorganisms have been

60 identified for bovine postpartum metritis and endometritis, the complete pathogenesis of these
61 disorders is still unclear. Previous culture-independent studies of uterine flush samples have
62 demonstrated that multiple bacterial species are associated with metritis and endometritis and that
63 the bovine uterine microbiota is much more diverse than previously recognised based on traditional
64 cultivation (Machado et al., 2012; Peng et al., 2013; Santos and Bicalho, 2012; Santos et al., 2011).
65 Tissue-invasive bacteria, however, may not be predominant in the lumen but must adhere to and
66 invade the endothelium to proliferate and cause disease. We therefore hypothesise that potentially
67 pathogenic bacteria will be more prevalent in the endometrium than in the lumen of the uterus. The
68 purpose of this study was to identify metritis- and endometritis-related bacteria by performing in-
69 depth analysis of the postpartum microbiota, including samples from both the uterine lumen and the
70 endometrium of postpartum dairy cows. We applied next generation sequencing of the V1-V2
71 variable region of the 16S rRNA gene, which enables bacterial identification (Cho and Blaser,
72 2012; Jarvie, 2005).

73

74 **Materials and methods**

75 **Animals**

76 Samples were collected from 68 cows from a dairy herd in Denmark of approximately 1200 Danish
77 Holstein cows. The cows were kept in loose housing, fed *ad libitum* with total mixed ration, and
78 milked twice daily with an average herd milk yield of 9700 kg ECM.
79 The cows calved between April and July 2012. Their parity ranged from 1-5. The herd used
80 artificial breeding with semen from proven sires and the herd was free of bovine virus diarrhea virus
81 infection and a number of other infections officially eradicated from Denmark (FVST, 2013).

82

83

84 **Uterine sampling procedures**

85 Samples were taken at week 1 postpartum (days 4-12 postpartum), week 4 (days 24-32) and week 7
86 (days 46-53). To increase the group size of cows with a postpartum uterine infection, 2-3 cows with
87 a high uterine score were included in the week 1 sampling. Cows were not sampled after
88 insemination, thus fewer cows were sampled in week 7. After washing the perineum with Lactacyd
89 soap (Sanofi-Aventis, Paris, France) and drying with paper towels, a latex embryo flush catheter
90 was inserted the uterus; the uterine fluid was then aspirated. If no fluid was obtained by aspiration,
91 20-40 ml of sterile saline was flushed into the uterine lumen and then aspirated into a syringe.
92 Approximately 1 ml of uterine fluid was added to prepared tubes containing 4 ml of RNA*later*
93 (Ambion, Austin, TX, USA). A smear of the uterine fluid was made on a microscope slide for
94 cytology. The slides were stained with Hemacolor® (Merck Millipore, Darmstadt, Germany), and
95 mounted with Pertex® (Histolab, Göteborg, Sweden). An endometrial biopsy was taken using a
96 dividable biopsy instrument (Kruuse, Langeskov, Denmark). The biopsy instrument, protected by a
97 plastic sleeve, was inserted into the cervix. The biopsy instrument was unsheathed in the cervix and
98 advanced into the uterus where the biopsy was taken. The biopsy was then enclosed within the
99 instrument. The surface of the instrument was wiped with ethanol prior to opening the instrument to
100 remove the biopsy. The biopsy was transferred into 2 ml of RNA*later* with a sterile needle. Samples
101 in RNA*later* were stored at room temperature for approximately 24 h and then stored at -20 °C.

102

103 **Ethics statement**

104 All animal procedures were approved by the Danish Animal Experiments Inspectorate under the
105 Ministry of Justice, and the animal experiments were conducted in strict accordance with their
106 guidelines.

107

108 **Classification**

109 To obtain a larger group size, cows with either clinical or subclinical endometritis from weeks 4 and
110 7 were retrospectively merged into a group called ‘endometritis’. For simplicity, the group of cows
111 classified with uterine disease (puerperal or clinical metritis) from week 1 was renamed ‘metritis’.

112 The cows were grouped in week 1 according to their uterine score postpartum and in weeks 4 and 7
113 by the ratio of neutrophils to endometrial cells observed during microscopy of smears. The uterine
114 score was based on vaginal exploration performed by the herd veterinarian, in accordance with the
115 Danish scale for uterine scoring (Elkjær et al., 2013). Briefly, scores of 0-4 characterise cows with
116 odourless, mucoid uterine discharge in varying amounts, while scores of 5-9 characterise cows with
117 smelly uterine discharge in increasing amounts. Cows were classified as ‘normal’, indicating no
118 uterine disease, if they had a uterine score of 3 or less in week 1, and cows with a uterine score of 4
119 or higher in week 1 were considered to have uterine disease (puerperal metritis or clinical metritis).
120 The cut-off was based on the treatment threshold of the farm. In weeks 4 and 7, cows were
121 classified as normal if they had neutrophil counts below 18 and 10%, respectively, as suggested by
122 Kasimanickam *et al*. (2004). Cows were considered to have clinical endometritis if they had
123 purulent exudate and a neutrophil count above 18% in week 4 or 10% in week 7. Cows were
124 classified as having subclinical endometritis if they did not have purulent discharge but had a
125 neutrophil count above 18% in week 4 and 10% in week 7.

126

127 **DNA extraction from endometrial biopsies**

128 After thawing, approximately 10 mg of endometrial tissue was placed in 300 µl lysozyme buffer
129 (20 mM Tris-HCL, 2 mM EDTA, 1.2% Triton X and 5 mg Lysozyme per 100 ml). The samples

were then incubated at 37 °C for 30 min. In total, 350 µl lysis buffer from the Maxwell 16 LEV blood DNA kit (Promega, Madison, WI, USA) and a 5 mm stainless steel ball (QIAGEN, Hilden, Germany) were added before the tissue was bead-beaten at 20 Hz for 2 min in a TissueLyser II (QIAGEN). Next, 30 µl Protease K was added and the sample was incubated at 56 °C for 1 h. DNA was extracted on a Maxwell® 16 Research Instrument System using a Maxwell® LEV Blood DNA Purification Kit (Promega). Afterwards, the DNA concentration and quality was measured on a NanoDrop 1000 machine (Saveen Werner, Limhamn, Sweden).

DNA extraction from uterine flush samples

A volume of aspirated uterine fluid in RNA*later* (Ambion) predicted to produce a pellet of approximately 10 mg (between 200 µl and 1800 µl) was measured, and PBS was added to a total volume of 1900 µl. The samples were then centrifuged at 13000 *g* at 4 °C for 30 min. The pellet was resuspended in 100 µl lysozyme buffer (see above). DNA was extracted from the pellet in the same manner as DNA was extracted from the endometrial biopsies (see DNA extraction from endometrial biopsies).

Next generation sequencing

16S rRNA PCR was performed with primers targeting the V1 and V2 regions (Wilmotte et al., 1993). The forward primer sequence was: 5'-AGAGTTTGATCCTGGCTCAG-3', and the reverse primer sequence was 5'-CTGCTGCCTYCCGTA-3'. Both the forward and reverse primers had a hexamer barcode in the 5' end. A total of 51 primers with barcodes were used. The PCR conditions were 5 µl 10x PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 3 µl 25 mM dNTP, 2 µl 20 mM primers, 0.5 µl 5 U/µl Taq gold polymerase (Applied Biosystems) and nuclease-free water was added to a volume of 50 µl. The PCR amplification was performed as follows: an initial

154 denaturation step at 94 °C for 3 min, then 30 cycles of 94 °C for 45 sec, 57 °C for 45 sec, and 72 °C
155 for 90 sec, followed by 10 min at 72 °C. A negative control for each primer set was included. The
156 amplicons were analysed with the Agilent DNA 1000 Reagents kit on the Agilent 2100 Bioanalyzer
157 (Agilent Technologies, Waldbronn, Germany) for concentration and purity. Amplicons with a
158 concentration below 5 ng/μl were discarded, resulting in 153 endometrial biopsies and 135 uterine
159 flush samples. These were divided equimolarly into eight pools of up to 51 samples. Primer dimers
160 and PCR reagents were removed using the MinElute PCR Purification Kit (QIAGEN). The samples
161 were sent for Illumina MiSeq paired-end sequencing at the National High-throughput DNA
162 Sequencing Centre, University of Copenhagen, Denmark.
163
164 **Data analysis**
165 The sequences were analysed using BION software (https://app.box.com/bion). Paired sequences
166 were trimmed with a 97% quality minimum in a 10 bp window; one mutation was allowed in the
167 primers, and the minimum length of the reads was set to 260 bp after trimming. The reads were de-
168 replicated, and reads with a chimera score above 30 were removed. The consensus sequences were
169 mapped against the RDP database (http://rdp.cme.msu.edu) with a match minimum of 86%, and
170 taxonomical classification was based on the best 1% of the similarities from the RDP database.
171 Each sample was normalised to 100,000 reads. Samples were excluded if the log10-transformed
172 amount of read numbers in the samples was less than the overall average of reads in the experiment
173 minus two standard deviations.
174 A two-tailed Fisher's exact test was performed on the normalised reads. For each taxonomic unit,
175 the metritis and the endometritis groups were tested against the normal group with a threshold value
176 of 5% of the total normalised reads. A Spearman rank test was performed on the average number of

normalised reads per operational taxonomic unit (OTU) to investigate the correlation between the biopsy and flush samples.

Results

A total of 20,128,922 paired-end reads in eight pools were obtained. After demultiplexing the reads according to barcodes and primers, 14,646,852 reads remained. The paired end reads were joined, leaving 6,939,462 reads, which were trimmed at the 5' and 3' ends of the sequences according to quality. Sequences with a quality below 97% or a trimmed length of less than 260 bp were discarded. This left 5,641,274 reads that were rarefied to 2,795,873 unique sequences. After the chimera filter, 2,575,771 reads over 288 samples were tested against the RDP database for taxonomic classification. Of these reads, 1,851,529 reads were matched to entries in the database, and 724,242 were not matched against any entries in the database. The rarefaction curves were not steep, but only several of the curves plateaued (data not shown). This indicates that a deeper sequencing would most likely have revealed more OTUs.

The obtained OTUs were identified to the family level, as the majority of the OTUs were unclassified below that taxonomic level. OTUs that were unclassified at the family level are given at the nearest taxonomical level possible.

The samples were collected at three time points: weeks 1, 4, and 7 postpartum. The 288 sequenced samples were classified according to the uterine health status of the cows from which they originated. We used 162 samples for the analysis, and the number of samples per group is shown Table 1. In total, 126 samples were removed because they were derived from cows that had received treatment with antibiotics, or were classified as neither normal nor a member of one of the groups with metritis or endometritis. The samples from week 7 were excluded from the statistical analysis because of small group size.

201

202 **Diversity**

203 We measured diversity using the Shannon index (Figure 1), which takes into account both the
204 number of species found in each category and the evenness of their distribution. The Shannon index
205 scores showed that the bacterial diversity was higher in the endometrial biopsies compared with the
206 uterine flush samples. The Spearman rank test is a non-parametric test that we applied to compare
207 the ranked normalised abundance of each OTU between the endometrial biopsies and the uterine
208 flush samples. It showed that the uterine flush samples and the endometrial biopsies were correlated
209 ($p < 0.01$).

210

211 **Metritis**

212 The relative distribution of OTUs in week 1 is shown in Figure 2A. Table 2 is presented to easily
213 discern the most abundant OTUs found per group. Three families constituted 59 and 62% of the
214 microbiota in uterine flush samples and endometrial biopsies from cows with metritis, respectively.
215 The uterine flush samples and the endometrial biopsies exhibited similar taxonomic compositions,
216 and both showed that the metritic flora was dominated by bacteria within the *Fusobacteriaceae*,
217 *Porphyromonadaceae*, and *Streptococcaceae* families.
218 Rather than doing a statistical analysis on the average count of OTUs in each group, we applied
219 Fisher's exact test because the group sizes presented in Table 1 were smaller than expected. Our
220 application of the Fisher's exact test investigates the ratio of cows where the number of
221 observations of a given OTU exceeds 5% of the total amount of reads in the sample. In the metritis
222 group, *Fusobacteriaceae* was found at a significantly higher frequency ($p = 0.03$) in endometrial
223 biopsies and *Porphyromonadaceae* in uterine flush samples ($p = 0.03$) compared with normal cows.

224 In week 1, an abundance of *Streptococcaceae* was observed in all sample types (Table 2). The
225 application of Fisher's exact test did not show any difference in frequency between the normal and
226 the metritic cows with respect to the presence of *Streptococcaceae*.
227

228 **Endometritis**

229 The microbiota in cases of endometritis in week 4 differed from the microbiota in metritis cases in
230 week 1, as none of the bacterial families that made up the major portion of the sequences in the
231 metritis samples were among the three most abundant OTUs in the endometritis samples in week 4
232 (Figure 2B). Furthermore, the uterine flush and endometrial biopsy samples displayed a more
233 divergent pattern. In the uterine flush samples from endometritic cows, *Mycoplamataceae* was the
234 dominant family, which together with *Leptotrichiaceae*, accounted for 60% of the OTUs in week 4.
235 This observation was not mirrored in the endometrial biopsies in week 4, for which the three most
236 dominant families, *Ruminococcaceae* followed by *Mycoplamataceae* and *Bacteroidaceae*, together
237 constituted only 43% of the total OTUs (Table 2). Regardless of sample type, no OTUs were
238 observed at a significantly higher frequency in cows with endometritis compared with normal cows.

239 The relative distribution of OTUs in week 7 is shown in Figure 2C. At this time point,
240 *Mycoplasmataceae* still dominated the uterine flush samples of cows with endometritis where this
241 bacterial family accounted for 52% of the OTUs observed (Table 2).
242 Sequences affiliated with *Fusobacteriaceae* were observed at a relatively high abundance in uterine
243 flush samples from both normal cows and cows with endometritis in week 7. However, these
244 sequences only accounted for 5% of the OTUs observed in the endometrial biopsies from
245 endometritic cows and appeared to be absent from the endometrial biopsies of normal cows.

The three most abundant families in the endometrial biopsies from cows with endometritis in week 7 observed were as follows: *Ruminococcaceae*, *Bacteroidaceae*, and an unclassified family that belongs to class *Bacteroidia* (Table 2). These three families were also the most abundant families in endometrial biopsies from normal cows in weeks 4 and 7. As a similar pattern could not be observed in the uterine flush samples, the microbial population of the endometrial biopsies appeared to be more stable over time and uterine health status than the microbial population of the uterine flush samples.

Cows with no apparent uterine disease

In the normal group, differences were also observed between uterine flush samples and endometrial biopsies. In the uterine flush samples, the most abundant families changed between the time points, but were most often dominated by *Mycoplasmataceae, Leptotrichiaceae* and *Bacteroidaceae* families. In the endometrial biopsies, OTUs representing the *Ruminococcaceae* and *Bacteroidaceae* families, and an unclassified family that belongs to class *Bacteroidia* were the most abundant in weeks 4 and 7.

Although *Ruminococcaceae* was not among the most abundant OTUs found in the endometrial biopsies in week 1, Fisher's exact test revealed that this OTU was present at a significantly higher frequency in endometrial biopsies from normal cows than in cows with metritis ($p = 0.003$). *Ruminococcaceae* also occurred in a relatively high quantity and at a significantly higher frequency ($p = 0.0005$) in uterine flush samples from normal cows in week 4 compared with endometritic cows. Although this indicates a link between *Ruminococcaceae* and a normal uterine microbiota, *Ruminococcaceae* was also observed in high abundance in endometrial biopsies from cows with endometritis in weeks 4 and 7.

268 *Lachnospiraceae* (p = 0.002) and *Porphyromonadaceae* (p = 0.03) were observed at a significantly
269 higher frequency in uterine flush samples from normal cows in week 4 compared with endometritic
270 cows. In the endometrial biopsies, *Porphyromonadaceae* and an unclassified family that belongs to
271 phylum *Bacteroidetes* were also found at a significantly higher frequency in normal cows compared
272 with endometritic cows. These observations were not repeated in week 7, except for the abundance
273 of the ubiquitous bacteria that belong to phylum *Bacteroidetes*.

274 *Bacteroidaceae* and an unclassified family that belongs to class *Bacteroidia* were observed in high
275 abundance in week 7 in both uterine flush samples and endometrial biopsies from normal cows.
276 *Bacteroidaceae* was generally abundant in samples from both normal cows and cows with
277 endometritis. *Fusobacteriaceae* and *Leptotrichiaceae* were observed in relatively high quantities in
278 the uterine flush samples but not in the endometrial biopsies in week 7.

279

280 **Discussion**

281 Until now, the cultivation-independent studies published on the bacterial diversity in the uterus after
282 parturition have all analysed uterine flush samples (Machado et al., 2012; Peng et al., 2013; Santos
283 and Bicalho, 2012; Santos et al., 2011). Examinations of samples solely from the uterine lumen risk
284 underestimating or overlooking potential tissue-invading bacteria that are mainly present in the
285 endometrium. Both types of uterine samples were therefore included in the present investigation,
286 where 68 postpartum dairy cows were sampled three times over a period of 7 weeks. This approach
287 allowed us to compare over time the microbial composition and relative bacterial abundance in the
288 uterus of healthy cows and cows with postpartum uterine infection and to investigate whether the
289 microbiota found in the uterine lumen corresponds to the bacterial population of the endometrium.

A distinct pattern was observed regarding the bacterial diversity. Differences in the Shannon indexes between endometrial biopsies and uterine flush samples showed that biopsies harboured a higher number of OTUs that were distributed more evenly than in the uterine flush samples.

Similar to previous culture-independent studies (Santos et al. 2012, Machado et al. 2012) most of the >1.8 M sequence reads were affiliated with the phyla *Bacteroidetes* (*Bacteroidaceae* and unclassified families that belong to class *Bacteroidia*), *Fusobacteria* (*Fusobacteriaceae* and *Leptotrichiae*), *Firmicutes* (*Ruminococcaceae* and *Streptococcaceae*), and *Tenericutes* (*Mycoplasmataceae*) regardless of the uterine health status of the cow. Differences in the phylogenetic composition and bacterial abundance of both normal and diseased cows were observed over time (Figure 2).

A relatively clear pattern emerged of the OTUs present in the metritis group. OTU mapping showed that the *Fusobacteriaceae* and *Porphyromonadaceae* families were more abundant and more frequently observed in samples from cows with metritis than in samples from normal cows in week 1. It is therefore likely that bacteria from these families play a causal role in the development of metritis in dairy cattle during the first week after calving. *F. necrophorum* and *P. levii*, which are members of the *Fusobacteriaceae* and *Porphyromonadaceae* families, respectively, have been identified in culture-independent studies of postpartum disease. *F. necrophorum* has previously been isolated from cases of bovine metritis (Bicalho et al., 2011) and is a well-known pathogen that causes profound necrotising inflammation in cattle (Nagaraja et al., 2005). *P. levii* has previously been associated with metritis (Santos et al., 2011) and bovine necrotic vulvovaginitis (Elad et al., 2004).

By contrast, bacteria from the *Porphyromonadaceae* family occurred more frequently in normal cows than in cows with endometritis both from week 4 uterine flush samples and endometrial

313 biopsies. The role of bacteria from *Porphyromonadaceae* in the development of postpartum uterine
314 infections in dairy cows needs further investigation.

315 *E. coli* and *T. pyogenes* are considered important bacteria in the development of metritis and
316 endometritis. The presence of *E. coli* in the uterus at an early time point after parturition has been
317 linked with the development of endometritis in culture-based studies (Dohmen et al., 2000; Sheldon
318 et al., 2010; Williams et al., 2007), often followed by *T. pyogenes* and other Gram-negative bacteria
319 (Dohmen et al., 2000; Williams et al., 2007). The present data do not support the hypothesis that *E.*
320 *coli* and *T. pyogenes* are important aetiological factors for the development of metritis or
321 endometritis in dairy cattle. Furthermore, less than 1% of the sequence reads could be assigned to
322 the *Enterobacteriaceae* (*E. coli*) or *Actinomycetaceae* (*T. pyogenes*) families at any of the
323 investigated time points. These results support the findings of Santos and Bicalho (2012), Peng *et*
324 *al*. (2013) who also did not observe *E. coli* or *T. pyogenes* to be present in a significant amount in
325 their culture-independent studies.

326 Based on the present data, we did not detect any OTUs that occurred more frequently in the samples
327 from cows with endometritis than in the samples from normal cows. As will be described later, the
328 most notable differences in week 4 and 7 were the increasing discrepancies between the most
329 abundant OTUs observed in the uterine flush samples and the endometrial biopsies. In addition, the
330 bacterial populations of the endometrial biopsies appeared more stable from week 4 to week 7 than
331 the microbiota of the flush samples.

332 The normal cows were characterised by a high diversity and a stable microbiota in the endometrial
333 biopsies, most notably *Ruminococcaceae*, *Bacteroidaceae*, and unclassified families that belong to
334 class *Bacteroidia*. The bacterial population observed in the uterine flush samples was less stable,
335 and mainly dominated by the *Mycoplasmataceae*, *Leptotrichiaceae*, and *Bacteroidaceae* families.

336 The normal postpartum cows had a very diverse uterine microbiota. We did not find any OTU that
337 was consistently associated with a healthy bovine postpartum uterus without also being observed in
338 samples from cows with endometritis or metritis. For example, OTUs mapping to
339 *Ruminococcaceae*, *Lachnospiraceae, Porphyromonadaceae*, and unclassified families that belong
340 to phylum *Bacteroidetes* were significantly associated with normal cows in different sample types
341 and at different time points in the dataset. However, all these families were also found either in high
342 frequency or in high abundance in samples from cows with metritis or endometritis. Likewise,
343 although *Ruminococcaceae*, *Bacteroidaceae*, and unclassified families that belong to class
344 *Bacteroidia* were found in high abundance in the endometrial biopsies from normal cows in weeks
345 4 and 7, these families were also present in abundance in samples from cows with endometritis in
346 week 7. The diversity observed in the normal cows, coupled with several frequently occurring
347 families that are also found in high quantities in bovine faecal matter (Dowd et al., 2008) most
348 likely reflects the environmental contamination of the uterus. This could be viewed as a part of a
349 temporary core microbiota; a microbiota that will most likely disappear when the postpartum uterus
350 undergo involution, during which bacterial contamination is cleared (Földi et al., 2006).

351 We hypothesised that tissue-invasive species would more likely be identified from endometrial
352 biopsies than from uterine flush samples and tested if the microbiota of the two specimen types
353 were comparable. According to the Spearman rank test, there was a correlation between the two
354 types of specimens. Despite the correlation between sample types, considerable differences could be
355 observed among the most abundant bacterial families of the endometrial biopsies and uterine flush
356 samples. The most surprising of these differences was the apparent absence of *Fusobacteriaceae*
357 and *Leptotrichiaceae* in the biopsy samples in week 7, whereas they accounted for approximately
358 20 and 13%, respectively, of the OTUs observed in the flush samples (Table 1). The
359 *Mycoplasmataceae* family was also observed in much higher amounts in the uterine flush samples

than in the endometrial biopsies. The difference is striking in the endometritis groups in weeks 4 and 7, where this OTU accounted for 43 and 52% of the flush samples but only 12 and 4% of the endometrial biopsies, respectively (Table 1). Differences in sample handling may cause some variation between sample types, and the vast differences we observed for the mentioned bacterial families indicate that the uterine flush samples are more easily skewed than the endometrial biopsies. A surprisingly rich bacterial flora was observed in the endometrium, and we must highlight that pinpointing invasive species is not an easy task in this sample type. Fluorescent in situ hybridisation or similar methods are needed to make conclusions about the tissue invasiveness of the bacteria observed in the biopsies.

Conclusions

We found that the microbial environment in the uterus postpartum is complex. There was an association between metritis in week 1 postpartum and *Fusobacteriaceae* and *Porphyromonadaceae*. However, in weeks 4 and 7, none of the OTUs that were observed frequently in samples from cows with endometritis had an unequivocal association with the disease. Additional studies are needed to completely understand the role of these bacteria in postpartum uterine disease. In the comparison between the endometrial biopsies and the uterine flush samples, we found that although they were correlated, the microbial population found in the biopsies had a higher diversity and was more stable over time. To the best of our knowledge, this is the first study that applies next generation sequencing to tissues samples from the bovine uterus, and the results provide important details about the postpartum uterine microbiota.

Nucleotide sequences

383 Nucleotide sequences obtained in this project were submitted to GenBank. Accession numbers to

384 follow.

385 **Acknowledgements**

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389 Pedersen, for his hospitality and for giving us access to his cows.

390

391 **Conflicts of interest**

392 The authors declare that there are no conflicts of interest.

393

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479

480 *Table1: Number of samples analysed at each time point for both uterine flush samples and*
481 *endometrial biopsies. Samples taken at week 1 represent cases of metritis while samples taken*
482 *during weeks 4 and 7 originate from cows with endometritis.*

Sample type	Uterine status	Week 1	Week 4	Week 7
Flush	Normal	32	7	3
	Metritis/endometritis	13	19	6
Biopsy	Normal	31	8	4
	Metritis/endometritis	11	22	6

483

484 *Table 2: The three most abundant operational taxonomic units (OTUs) observed in the uterine flush*
485 *samples and the endometrial biopsies (measured in percent within groups). Grey colouration*
486 *indicates that the OTU was not among the three most abundant in the group but is displayed to*
487 *make comparisons between sample types more straightforward. OTUs marked with an asterisk **
488 *were unclassified at the family level, and are given at class level.*

Week 1	**OTU**	**Flush**	**Biopsy**
Normal	*Streptococcaceae*	20%	12%
	Mycoplasmataceae	17%	16%
	Pasteurellaceae	14%	5%
	Oxalobacteraceae	0%	8%
Metritis	*Fusobacteriaceae*	22%	26%
	Porphyromonadaceae	19%	16%
	Streptococcaceae	18%	20%
Week 4			
Normal	*Ruminococcaceae*	20%	21%
	Mycoplasmataceae	13%	2%
	*Bacteroidia**	9%	13%

1470

	Bacteroidaceae	9%	9%
Endometritis	*Mycoplasmataceae*	43%	12%
	Leptotrichiaceae	17%	6%
	Ruminococcaceae	4%	18%
	Bacteroidaceae	8%	10%
Week 7			
Normal	*Bacteroidaceae*	28%	9%
	Fusobacteriaceae	21%	0%
	Ruminococcaceae	11%	20%
	Leptotrichiceae	13%	0%
	*Bacteroidia**	5%	11%
Endometritis	*Mycoplasmataceae*	52%	4%
	Ruminococcaceae	2%	20%
	Bacteroidaceae	16%	15%
	Fusobacteriaceae	13%	5%
	*Bacteroidia**	1%	13%

489

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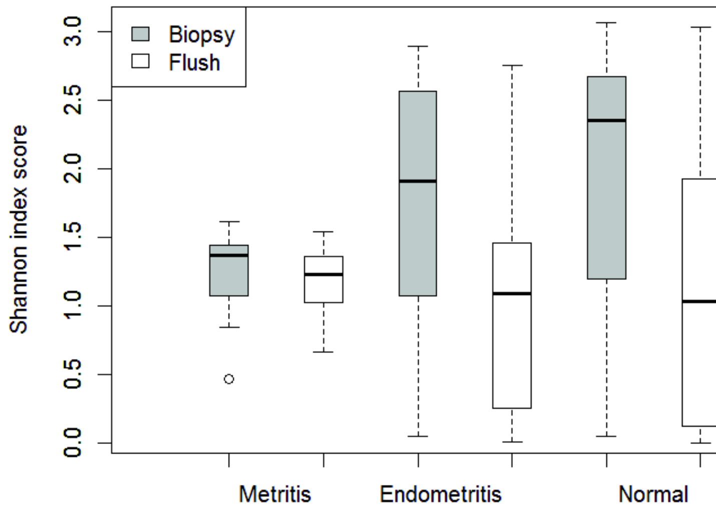
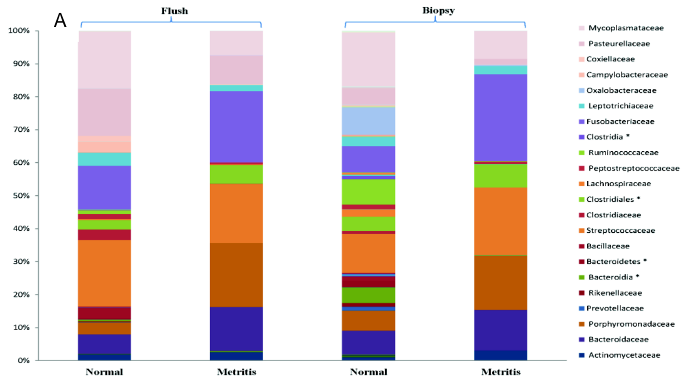


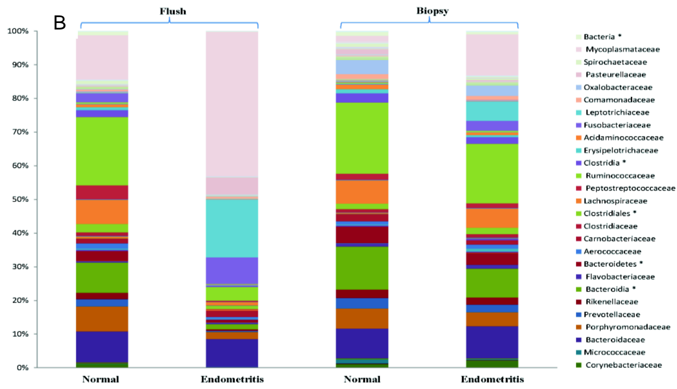
491

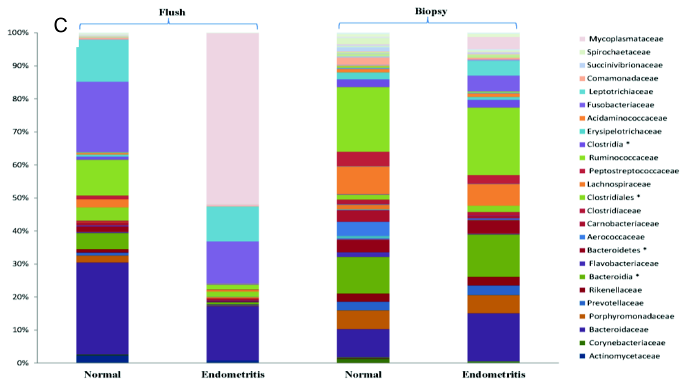
492 *Figure 1: Box plot of the distribution of Shannon index scores for each sample in the metritis,*

493 *endometritis and normal groups.*

494







495

496 *Figure 2: Colour-coded bar plot showing the relative uterine bacterial operational taxonomic unit*

497 *(OTU) composition in uterine flush samples and endometrial biopsies from normal cows and cows*

498 *with metritis and endometritis.* ***A****: Week 1 postpartum, uterine flush samples from normal cows.* ***B****:*

499 *Week 4 postpartum, uterine flush samples from normal cows.* ***C****: Week 7 postpartum, uterine flush*

500 *samples from normal cows. The colour code is given for families that constitute ≥ 1% of the relative*

501 *abundance of all the OTUs observed within each group. The OTUs are determined to the family*

502 *level. OTUs that are unclassified at the family level are given at the nearest taxonomical level*

503 *possible. These are indicated with an asterisk *.The number of cows in each group can be seen in*

504 *Table 1.*

505

Manuscript 2

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Revisiting pyometra in postpartum cows - new insights into the disease using a culture-independent deep sequencing approach

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20 **Abstract**

21 The bacteria present in the uterus during pyometra have previously been studied using
22 bacteriological culturing. These studies identified *Fusobacterium necrophorum* and *Trueperella*
23 *pyogenes* as the major contributors to the pathogenesis of pyometra. However, an increasing
24 number of culture-independent studies have demonstrated that the bacterial diversity in most
25 environments is underestimated in culture-based studies. Consequently, fastidious pyometra-
26 associated pathogens may have been overlooked. Therefore, the primary purpose of this study was
27 to investigate the diversity of bacteria in the uterus of cows with pyometra by using culture-
28 independent 16S rRNA PCR combined with next generation sequencing.

29 We investigated the microbial composition in the uterus of 21 cows with pyometra, which were
30 obtained from a Danish slaughterhouse. Similar to the observations from the culture studies,
31 *Fusobacteriaceae,* the family that *F. necrophorum* belongs to, was the operational taxonomic unit
32 (OTU) observed in the largest quantities. By contrast, the *Actinomycetaceae* family, which includes
33 *T. pyogenes*, constituted only 1% of the total number of reads. Thus we cannot confirm the
34 previously reported role of species from this family in the pathogenesis of pyometra. Finally, we
35 identified a large number of sequences representing three families of Gram-negative bacteria in the
36 pyometra samples: *Porphyromonadaceae, Mycoplasmataceae*, and *Pasteurellaceae*. It is likely that
37 these families comprise potential pathogenic species of a fastidious nature, which have been
38 overlooked in previous studies. Our results increase the knowledge of the complexity of the
39 pyometra microbiota and suggest that pathogens in addition to *F. necrophorum* may be involved in
40 the aetiology of pyometra.

41

42 **Keywords**

43 Bovine pyometra, Cattle, Metagenomics, Microbiota

44

45 **Introduction**

46 After calving, the uterine lumen is usually contaminated by bacteria from the external environment
47 (Sheldon et al., 2006). Most cows are able to clear the contamination; however, up to 20% of cows
48 develop metritis, 2-25% develop endometritis, and up to 5% develop pyometra (Földi et al., 2006;
49 Sheldon et al., 2008; Gautam et al., 2010; Dubuc, 2011; LeBlanc, 2014). Postpartum uterine
50 infectious diseases, which include metritis, endometritis and pyometra, have a major impact on the
51 health and productivity of dairy cows and are among the most prevalent and costly diseases in high
52 producing dairy cows (Kossaibati and Esslemont, 1997; McDougall, 2001; Sheldon et al., 2008).
53 Pyometra is a disease in the uterus, where fluid and purulent exudate accumulate in the uterine
54 lumen while there is a persistent corpus luteum (Sheldon et al., 2006, 2008). The presence of
55 endometrial infection when the cow returns to ovarian cyclic function after calving is a prerequisite
56 for the development of pyometra (Olson et al., 1984). This infection inhibits the release of
57 prostaglandin from the endometrium, thus preventing the usual regression of the corpus luteum. The
58 progesterone production by the persistent corpus luteum causes closure of the cervix thus leading to
59 the enlargement and distension of the uterus due to the accumulation of exudates in the lumen
60 (Olson et al., 1984; Bondurant, 1999). There are very few, if any, signs of systemic illness. An
61 affected cow will not exhibit oestrus and is infertile (Bondurant, 1999; Sheldon et al., 2006).
62 The aetiopathogeneses of metritis and endometritis are of interest for the study of pyometra. A
63 considerable number of studies have been conducted on the microbiota of metritis and endometritis.
64 Traditional culture methods have identified *Escherichia coli*, *T. pyogenes*, *F. necrophorum*, *P.*
65 *melinogenica*, *Bacteriodetes* spp., *Pseudomonas* spp., *Proteus* spp. *Streptococcus* spp., and
66 *Staphylococcus* spp. (Königsson et al., 2001; Sheldon et al., 2002; Williams et al., 2005, 2007;

67 Miller et al., 2007). Recently, these studies have been supplemented by culture-independent
68 molecular methods, and a range of additional potential pathogens have been detected, mainly in the
69 five major phyla of *Proteobacteria*, *Firmicutes*, *Fusobacteria*, *Bacteroidetes*, and *Tenericutes*
70 (Machado et al., 2012; Santos and Bicalho, 2012).
71 It is known that the parasite *Tritricomonas foetus* is associated with pyometra in cattle (Rae and
72 Crews, 2006). *T. foetus* is not prevalent in Denmark due to decades of the widespread use of semen
73 from sires tested negative for trichomonosis (FVST et al., 2013). Pyometra in Danish cattle is
74 therefore considered to be primarily due to bacteria.
75 Few studies have investigated the bacterial flora associated with bovine pyometra. It has been
76 proposed that *Trueperella pyogenes*, the Gram-negative anaerobic bacteria *Fusobacterium*
77 *necrophorum*, *Prevotella melaninogenica*, and other unidentified anaerobic Gram-negative bacteria
78 act synergistically to cause pyometra in the uterus (Hartigan et al., 1974; Ruder et al., 1981; Olson
79 et al., 1984). The hypothesis was supported by a challenge study, where pyometra was induced in
80 lactating Holstein cows by a uterine infusion with *T. pyogenes* either alone or in combination with
81 other anaerobic bacteria (Farin et al,. 1989). These previous investigations of pyometra in dairy
82 cows were all based on traditional culture methods of uterine exudates and may have excluded
83 potential pathogens in bacteria that do not grow under standard culture conditions.
84 In this study, we re-evaluated the standing hypothesis that a pathogen consortium consisting of *T.*
85 *pyogenes*, *F. necrophorum*, and *P. melaninogenica* leads to pyometra by applying modern
86 molecular methods. More specifically, we used 16S rRNA PCR combined with next generation
87 sequencing (NGS) to investigate the bacteria found in the uterus of slaughtered cows with
88 pyometra. The NGS method is culture-independent and is therefore capable of detecting bacteria
89 that do not grow under standard culture conditions (Zoetendal et al., 2004; Jarvie, 2005).
90 Furthermore, we analysed and compared samples from both the uterine exudate and uterine

91 mucosae, as the microbial populations of the lumen may differ from the microbiota of the

92 endometrium.

1480

93

94 Materials and methods

95

96 Sampling

97 In the period from September 2012 to September 2013, uteri from cows with pyometra were sent to
98 us by the Danish Crown slaughterhouse, Tønder, Denmark and arrived within 72 h of slaughter. The
99 uteri were cooled to 4 °C at the slaughterhouse and during transport. The uteri were examined upon
100 arrival to confirm the diagnosis, and information on calving dates was retrieved from the Central
101 Husbandry Register, an elaborate system of animal identification and registration, which is owned
102 by the Danish Ministry of Food, Agriculture, and Fisheries. A case was included in this study if the
103 uterine mucosa was inflamed, if the uterus was enlarged owing to accumulated pathological
104 exudates, and if a corpus luteum was present. Uteri from cows that were 21 days or less postpartum
105 were discarded. Specimens containing macerated foetuses were also omitted. In total, 21 uteri were
106 sampled.

107 The surface of the uterus was wiped with alcohol and briefly flamed before an incision was made
108 into the uterine body. Approximately 1 ml of exudate was aspired and added to pre-prepared tubes
109 containing 4 ml of RNA*later* (Ambion, Austin, TX, USA). A specimen of approximately 0.5x0.5
110 cm was retrieved from just after the uterine bifurcation in the left uterine horn and transferred into 2
111 ml of RNA*later*. Samples were kept at room temperature for approximately 24 h and then stored at -
112 20 °C.

113

114 DNA extraction from endometrial biopsy samples

115 Approximately 10 mg of thawed biopsy tissue was incubated in 300 µl lysozyme buffer (20 mM
116 Tris-HCL, 2 mM EDTA, 1.2% Triton X and 5 mg Lysozyme per 100 ml) at 37 °C for 30 min. Next,
117 a 5 mm steel ball (QIAGEN, Hilden, Germany) was added with 350 µl lysis buffer from the
118 Maxwell 16 LEV blood DNA kit (Promega, Madison, WI, USA), and the tissue was disrupted
119 through bead-beating in a Tissuelyser II (QIAGEN) at 20 Hz for 2 min. Then, 30 µl Protease K was
120 added, and the samples were incubated at 56 °C for 1 h. DNA was extracted on a Maxwell® 16
121 Research Instrument System using a Maxwell® LEV Blood DNA Purification Kit (Promega). The
122 concentration and quality of the DNA was measured on a NanoDrop 1000 (Saveen Werner,
123 Limhamn, Sweden).
124
125 **DNA extraction from uterine fluid samples**
126 A volume of uterine fluid in RNA*later* (Ambion) estimated to produce a pellet of approximately 10
127 mg (200-1800 µl) was measured. PBS was added to a total volume of 1900 µl, and the samples
128 were centrifuged at 13000 *g* at 4 °C for 30 min. The pellet was resuspended in 100 µl Lysozyme
129 buffer (see above), and DNA was extracted as described above (see DNA extraction from biopsy
130 samples).
131
132 **Next generation sequencing**
133 The DNA was amplified by PCR with primers that target the V1 and V2 hypervariable regions of
134 the 16S rRNA gene (Wilmotte et al., 1993). The forward primer sequence was: 5'-
135 AGAGTTTGATCCTGGCTCAG-3', and the reverse primer sequence was 5'-
136 CTGCTGCCTYCCGTA-3'. The amplicons were expected to have a length of approximately 350
137 bp. Both oligonucleotide primers had a hexamer barcode in the 5' end. A total of 26 primers with
138 barcodes were used. The PCR conditions were as follows: 0.5 µl 5 U/µl Taq gold polymerase

139 (Applied Biosystems, Branchburg, NJ, USA), 5 µl 10x PCR buffer (Applied Biosystems), 1 µl 10
140 mM dNTP, 2 µl 20 mM primers, 3 µl 25 mM MgCl, and nuclease-free water were added to a
141 volume of 50 µl; initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 45 sec, 57 °C for 45
142 sec, and 72 °C for 90 sec, followed by a final elongation for 10 min at 72 °C. A negative control
143 was included for each primer set. The resultant amplicons were assessed for concentration and
144 purity with the Agilent DNA 1000 Reagents kit on the Agilent 2100 Bioanalyzer (Agilent
145 Technologies, Waldbronn, Germany). The amplicons were pooled in equal amounts in two pools,
146 purified with the MinElute PCR Purification Kit (QIAGEN), and sent for MiSeq paired-end
147 sequencing at the National High-throughput DNA Sequencing Centre, University of Copenhagen,
148 Denmark.
149
150 **Data analysis**
151 The sequences were analysed using the BION sequence analysis tool (https://app.box.com/bion).
152 The 350 bp paired-end sequences were demultiplexed based on primer and barcode sequences. The
153 paired-end sequences were joined and then trimmed with a 98% quality minimum in a 10 bp
154 window, in which one mutation was allowed, and the minimum length of the reads after trimming
155 was set to 260 bp. The reads were rarefied to only the unique sequences, and reads with a chimaera
156 score above 30 were discarded. The unique sequences were mapped against the RDP database
157 (http://rdp.cme.msu.edu/index.jsp) with a match minimum of 86%, and taxonomic classification
158 was based on the best 1% of the similarities from the RDP database. To make the comparison
159 between samples straightforward, all samples were normalised to 100,000 reads.
160 Samples were excluded if the log 10-transformed amount of read numbers in the samples was
161 below two standard deviations from the overall average of reads in the experiment.

162 In-house R scripts used log 10-transformed data to compare the means of the groups in a one-way
163 ANOVA test and the individual means in Tukey's test. Šidák correction was used to correct for
164 multiple comparisons. An ANOSIM multivariable test was used to test for similarities between the
165 endometrial biopsies and the uterine fluid.

166

167 **Results**

168 In total, 22,822,468 paired-end reads were obtained. The reads were demultiplexed according to
169 barcodes and primers, leaving 14,930,152 reads. The majority of the reads that did not match a
170 primer or a barcode were from the *PhiX* library, which was used to spike the library before
171 sequencing because low complexity was expected. The paired-end reads were joined, and the
172 resultant 7,400,654 reads were quality filtered at the 3' and 5' ends at a 98% quality threshold.
173 Reads with a length below 260 bp after quality filtering were discarded. After the quality filtering,
174 6,133,061 reads remained, and these were rarefied to 905,295 unique sequences, which were then
175 subjected to a chimaera filter, in which all reads with a chimaera score above 30 were discarded.
176 The resultant 808,920 reads were compared with the RDP database (http://rdp.cme.msu.edu), and
177 690,714 matches were found. In total, 118,206 reads did not map to any sequence within the
178 database. The operational taxonomic units (OTU) that were obtained were identified to the family
179 level, as the majority of the OTUs were unclassified below that taxonomic level. The OTUs that
180 were unclassified at the family level were assigned to the closest possible taxonomic level.

181 The relative distribution of OTUs in the pyometra samples is shown in Figure 1A, and the five most
182 abundant OTUs are listed in Table 1. The complexity in the samples was low, as indicated by the
183 observation that *Fusobacteriaceae*, *Mycoplasmataceae*, *Bacteroidaceae*, *Porphyromonadaceae*, and
184 *Pasteurellaceae* accounted for approximately 85% of the OTUs observed in the uterine fluid
185 samples and 88% of the endometrial specimens. The low complexity was also reflected through

186 relatively low Shannon index scores. The Shannon index measures diversity by the amount of
187 different OTUs in the samples and the evenness with which these OTUs are distributed in the
188 samples. The Shannon index scores were on average 1.00 for tissue samples and 0.94 for fluid
189 samples. In an ANOSIM multivariable test, no significant difference was found between the uterine
190 fluid samples and the endometrial tissue samples ($p = 0.98$). The similar OTU distribution within
191 cows is shown in figure 1B. The endometrial tissue samples and the uterine fluid samples from the
192 same cow are positioned next to each other, and with the exception of cows identified by 8, 12, and
193 15, the OTU distribution within the same cow is very similar.
194

195 **Discussion**

196 In the present study, the *Fusobacteriaceae*, *Mycoplasmataceae*, *Bacteroidaceae*,
197 *Porphyromonadaceae*, and *Pasteurellaceae* families constituted 85% and 88% of the bacterial
198 communities in the lumen and endometrium, respectively, thus indicating a low diversity of bacteria
199 in the uterus of cows with pyometra. The low diversity in the pyometra samples was also reflected
200 in the Shannon diversity index, which was approximately 1 for both the uterine fluid samples and
201 endometrial tissue. In a recent study, we examined the uteri of 22 healthy dairy cows in weeks 4 and
202 7 postpartum with the same methods as those used in this study. In these cows, the five most
203 abundant OTUs observed in the uterus comprised 53% of the bacterial community in the uterine
204 flush samples and 55% of the endometrial biopsies; the Shannon index score was approximately 2.5
205 (data not shown). The reduced richness of microbiota in the uterus of cows with pyometra may be
206 caused by an overgrowth of bacteria. Recent studies of metritis and endometritis have also revealed
207 reduced bacterial diversity compared to healthy cows (Santos et al., 2011; Santos and Bicalho,
208 2012).

209 According to cultivation-based studies, the most important pathogens of pyometra are *F.*
210 *necrophorum*, *T. pyogenes* (Ruder et al., 1981; Olson et al., 1984; Farin et al., 1989) and several
211 unclassified Gram-negative bacteria (Olson et al., 1984; Farin et al., 1989). *Fusobacteriaceae*,
212 which is the family that harbours *F. necrophorum*, and which is associated with endometritis and
213 metritis (Santos et al., 2011; Machado et al., 2012; Santos and Bicalho, 2012), was present in large
214 quantities in the present dataset. This supports the association between species from the
215 *Fusobacteriaceae* family and pyometra. However, *Actinomycetaceae,* which harbours *T. pyogenes*,
216 only accounted for 1% of the OTUs found in both the uterine fluid samples and the endometrial
217 tissue. Based on this dataset, we cannot confirm the importance of *T. pyogenes* in pyometra and
218 additional studies are needed to clarify the role of this bacterium.
219 Very few OTUs mapped to the *Enterobacteriaceae* family, which harbours *E. coli*. This bacterium
220 is often associated with metritis and endometritis in traditional culture studies but is mainly thought
221 to be important during the first week postpartum (Dohmen et al., 2000; Bicalho et al., 2010;
222 Sheldon et al., 2010). In addition, recent culture-independent papers on metritis and endometritis
223 also did not observe *E. coli* in significant numbers (Santos and Bicalho, 2012; Peng et al., 2013) and
224 also question the role of this bacterium in the development of uterine disease.
225
226 Sequences that could be ascribed to the *Porphyromonadaceae, Pasteurellaceae*, and
227 *Mycoplasmataceae* families were present in large quantities in the samples. These families have not
228 previously been associated with pyometra, but *Pasteurellaceae* and *Porphyromonadaceae* may be
229 among the unclassified Gram-negative bacteria found in the uteri with pyometra in culture studies
230 (Olson et al., 1984; Farin et al., 1989). Their relatively high quantities indicate that they may play a
231 role in the pathogenesis of pyometra. For example, a possible candidate on the species level could
232 be *Porphyromonas levii* from the *Porphyromonadaceae* family. This pathogen has previously been

233 identified in the uterus of metritic cows and has been associated with cases of bovine necrotising
234 vulvovaginitis (Elad et al., 2004; Santos et al., 2011). The OTUs that mapped to the *Bacteroidaceae*
235 family, which harbours *P. melaninogenica*, another suspected pathogen of pyometra, were also
236 observed in relatively large quantities. *Bacteroides* spp. have previously been isolated from the uteri
237 of cows with pyometra (Olson et al., 1984), and species from the *Bacteroidetes* phylum are
238 associated with endometritis and metritis (Santos et al., 2011; Machado et al., 2012; Santos and
239 Bicalho, 2012; Peng et al., 2013).
240 The bacterial families identified in this study as associated with pyometra were members of the
241 *Fusobacteria*, *Bacteroidetes*, *Tenericutes*, and *Proteobacteria* phyla, which are also among the most
242 prominent groups observed in postpartum metritis and endometritis (Santos et al., 2011; Machado et
243 al., 2012; Santos and Bicalho, 2012; Peng et al., 2013), thus underlining that these disease
244 complexes are closely interrelated.
245
246 To obtain a complete overview of the bacteria present in the uterus of cows with pyometra, we
247 examined samples from both the lumen and the endometrium. Although three cows seemed to have
248 a differing OTU distribution between the uterine flush sample and the endometrial specimen
249 (Figure 1B), the ANOSIM test found no significant difference between the endometrial tissue and
250 the uterine fluid samples ($p = 0.97$). Based on these results, we conclude that the microbiota of the
251 uterine fluid is similar to that of the endometrium.
252 One drawback of the 16S rRNA gene sequencing method applied here is that it does not
253 discriminate between live and dead bacteria. Future efforts should be directed towards more
254 detailed phylogenetic analyses and deep sequencing approaches that target the transcriptome of the
255 uterine microbial ecosystem and the local immune response of the host to uterine disease.
256

257 **Conclusions**

258 In this study, new information was obtained regarding the aetiology of pyometra. The most
259 abundant family comprises *F. necrophorum*, a pathogen previously known to be associated with
260 pyometra, whereas evidence of the association of *T. pyogenes* with pyometra was less convincing.
261 The previously unidentified Gram-negative bacteria observed in other studies are likely to belong to
262 the *Porphyromonadaceae, Pasteurellaceae*, and *Mycoplasmataceae* families identified in this study.

263

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267 time to send us uteri from cows with suspected pyometra. We would also like to thank Anastasia
268 Isbrand from the National Veterinary Institute, the Technical University of Denmark for technical
269 assistance.

270

271 **Availability of supporting data**

272 The data set supporting the results of this article were submitted to GenBank. Accession number
273 and link to dataset to follow.

274

275 **Competing interests**

276 The authors declare that they have no competing interests

277

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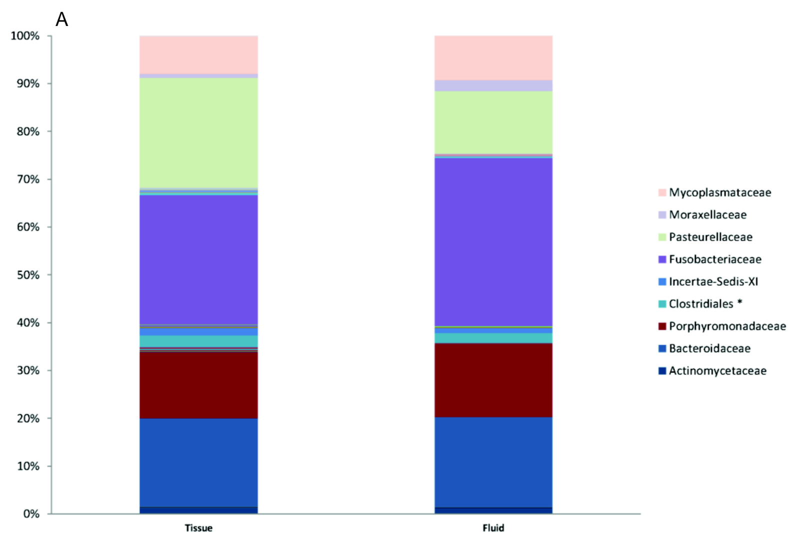
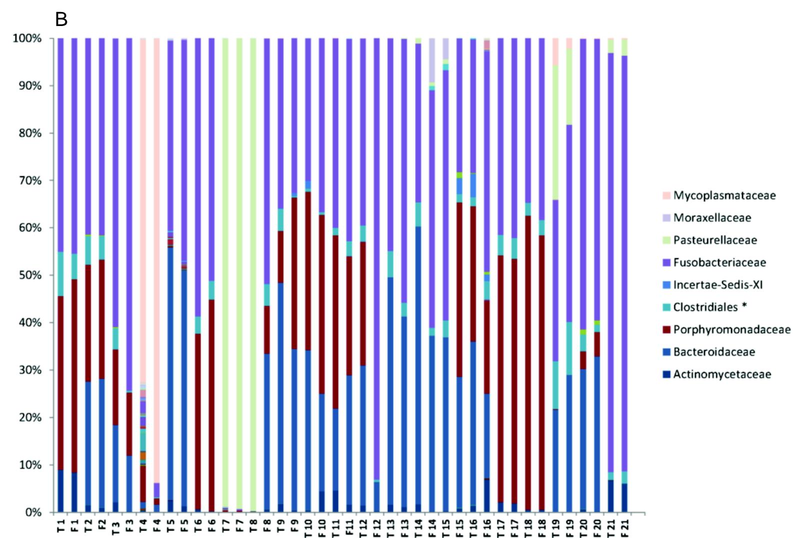
364

365 ***Figure 1:*** *Colour-coded histogram representing the relative distribution of operational taxonomic*
366 *units (OTUs) between the endometrial tissue samples and the uterine fluid samples from cows with*
367 *pyometra. The colour code is given for families that constitute ≥ 1% of all of the OTUs observed*
368 *within each group. The asterisk (*) indicates that the OTU was unclassified at the family level but is*
369 *given at the closest possible taxonomic level.* ***A****: The overall distribution of OTUs in the*
370 *endometrial tissue and the uterine fluid.* ***B****: The distribution of OTUs in each sample. T:*
371 *endometrial tissue sample, F: uterine fluid sample. The numbers 1-21 refer to the cow number.*





372

373 ***Table 1:*** *The five most abundant operational taxonomic units (OTUs) observed in uterine fluid*
374 *samples and endometrial tissue samples from cows with pyometra, given as the percentage of the*
375 *total number of OTUs within the sample type group.*

OTU	Uterine fluid	Endometrium
Fusobacteriaceae	35%	27%
Bacteroidaceae	19%	19%
Pasteurellaceae	13%	23%
Porphyromonadaceae	15%	14%

376

Manuscript 3

1495 Manuscript in preparation

1 ***In vivo* mRNA profiling of bacteria recovered from the uteri of dairy cows with**

2 **postpartum uterine disease revealed high prevalence and metabolic activity of**

3 ***Fusobacterium necrophorum***

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4 **ABSTRACT**

5 Bacterial pathogens adapt to the host environment by altering their pattern of gene expression. To
6 the best of our knowledge, this is the first metatranscriptomics study of the uteri of dairy cows with
7 postpartum uterine disease. Massively parallel cDNA sequencing was used to compare the
8 differences in expression patterns between the microbiomes of uteri from healthy cows and from
9 cows with postpartum uterine disease. In the uterine biopsies, 75% and 10% of the 50 most
10 differentially expressed microbial transcripts were homologous to proteins from *Fusobacterium*
11 *necrophorum* and *Porphyromonas levii*, respectively. From these transcripts, 158 open reading
12 frames were identified. The data presented here show that the postpartum microbiota is in an active
13 multiplication phase during the infection, as revealed by the high expression of genes involved in
14 DNA replication as well as protein transcription and translation. *In vivo* survival of bacterial
15 pathogens in the host environment requires adaption of their metabolism to the available nutrients
16 and physical conditions. Particularly the *F. necrophorum* gene regulation displayed signs of host
17 interaction with induced cell wall metabolism, altered carbon metabolism and, activation of
18 classical chaperones and transport systems. Similarly, we observed an up-regulation of a number of
19 potential virulence genes in *F. necrophorum*, including genes potentially involved in synthesis of
20 LPS, lipid A, and haemagglutinin as well as several protease-coding genes. The results presented
21 here support the hypothesis that *F. necrophorum* and possibly also *P. levii* are important pathogens
22 in postpartum uterine disease, and provide novel insight into the genetic requirements for a
23 successful bacterial infection.

24

25 **INTRODUCTION**

26 An estimated 90% of all dairy cows have intrauterine contamination with bacteria in the period after
27 calving (Azawi, 2008; Földi et al., 2006; Sheldon and Dobson, 2004). Whether the cow develops a
28 uterine disease following the uterine contamination depends on several factors, most importantly
29 the immune status of the animal, the extent of the negative energy balance, as well as the species

30 composition and load of the invading bacteria (Dubuc et al., 2010; Gautam et al., 2010; LeBlanc,
31 2014; Sheldon et al., 2006). Most cows are able to clear the bacteria within eight weeks (Földi et al.,
32 2006), but in up to 20% of cows the bacterial invasion results in metritis, and between 30-50% of
33 cows develop endometritis or subclinical inflammation in the cervix 4-8 weeks postpartum (Dubuc,
34 2011; LeBlanc, 2014). In endometritis, the inflammation is limited to the endometrium, while the
35 entire uterine wall is affected in metritis (Sheldon et al., 2006). Postpartum uterine disease has a
36 negative effect on the reproductive performance of the dairy cows (Gilbert, 2011; LeBlanc et al.,
37 2002).
38 The bacteria present in the postpartum uterus have traditionally been determined by conventional
39 culture studies. The species *Escherichia coli*, *Trueperella pyogenes*, *Prevotella* spp. and
40 *Fusobacterium necrophorum* have been identified by this method as possible pathogens of
41 postpartum uterine infection (Königsson et al., 2001; Sheldon et al., 2006; Williams et al., 2005).
42 Recently, with the advent of molecular methods, which are not biased towards the bacteria that
43 thrive in standard laboratory conditions, *Fusobacterium* spp., *Trueperella* spp., *Ureaplasma* spp.,
44 *Prevotella* spp., and *Bacteroides* spp. have also been affiliated with postpartum uterine disease
45 (Machado et al., 2012). Likewise, an association was found between endometritis and phyla
46 *Bacteroidetes*, *Firmicutes*, and *Fusobacteria* (Santos and Bicalho, 2012).
47 In order to cause a uterine infection, the pathogenic organism must first be able to adhere to the
48 mucosa and subsequently to colonise or penetrate the uterine epithelium (Falkow, 2004). Previous
49 studies have used cloning, PCR and quantitative PCR methods to investigate possible virulence
50 mechanisms of bacteria associated with postpartum disease. So far these studies have been limited
51 to virulence factors with already known DNA sequences (Kanoe and Iwaki, 1987; Langworth,
52 1977; Miao et al., 2010; Nagaraja et al., 2005; Narayanan and Stewart, 2002; Tadepalli et al., 2008;
53 Tan et al., 1994), and no studies have yet been performed that investigate the transcriptome of the
54 bacteria in postpartum uterine infections.

55 In recent years, however, the development of high-throughput sequencing methods has facilitated a
56 more comprehensive study of bacterial transcriptomes during the course of colonisation and
57 infection (Bielecki et al., 2014; Febrer et al., 2011; Mandlik et al., 2011; Turner et al., 2014). In this
58 study massively parallel cDNA sequencing (RNA-seq) analysis was employed to gain insight into
59 which bacteria were most active during infection and which proteins were expressed in order to
60 survive and establish an infection in the host. Identifying functions in uterine samples from cows
61 with postpartum disease may elucidate why certain bacteria, such as the ubiquitous *F.*
62 *necrophorum*, cause disease in some cows but not in others. Metatranscriptomics can also help
63 identify virulence factors needed for the bacteria to establish an infection in the host, and may be
64 used to identify vaccine targets.
65 In the present study, we made a quantitative comparison of the uterine microbial expression in
66 postpartum dairy cows with no apparent uterine disease versus cows diagnosed with a uterine
67 disease. Eight dairy cows were sampled at three time-points postpartum to follow the development
68 in the microbiome. Thirteen uterine flush samples and fourteen endometrial biopsies were attained
69 to investigate the role of tissue-invasive bacteria. This is, to the best of our knowledge, the first
70 metatrascriptomic investigation of the postpartum bovine uterus. This first analysis of the in vivo
71 expression of the infecting uterine microbiota enhances our understanding of the bacteria involved
72 in the development of uterine disease.
73
74 **MATERIALS AND METHODS**
75 **Sampling procedures**
76 The samples in this study originated from a Danish dairy herd of approximately 1200 Holstein
77 cows. Uterine flush samples and biopsies of the uterine wall from nine cows were included in the
78 study. Samples were taken at week 1 postpartum (days 4-12 postpartum), week 4 (days 24-32) and
79 week 7 (days 46-53). The samples in this study came from a larger set of samples from 68 cows
80 collected for a previous investigation (Knudsen *et al*. manuscript submitted). The present specimens

81 were chosen before the final classification of the uterine health status of the cows was performed.

82 The choice was based on the uterine score (Elkjær et al., 2013) given to the cow in week 1

83 postpartum by a local veterinarian. It was attempted to obtain an equal number of 'normal' cows

1500

84 and cows with 'uterine disease'. The final classification of samples into 'normal' and 'uterine

85 disease' groups was based on both uterine score from week 1 and the neutrophil to

86 endometrial cell ratio in a smear of uterine fluid on microscope glass that was investigated for

87 cytology. Cows with a uterine score of 3 or less were grouped as 'normal', and cows with a

88 uterine score between 4 and 9 were grouped as 'uterine disease' (puerperal or clinical

89 metritis). Cows with a neutrophil to endometrial cell ratio above 18% in week 4, and above

90 10% in week 7 were considered to have a 'uterine disease' (clinical or subclinical

91 endometritis) (Kasimanickam et al., 2004). Cows with neutrophil ratios below 18% and 10%

92 in weeks 4 and 7, respectively, were grouped as 'normal'. To increase group sizes, no

93 distinction was made between metritis and endometritis, and furthermore, separate groups

94 for each sample time point were not made. If the same cow was represented more than once

95 in each group, the sample from the latest time point would be discarded. None of the cows in

96 the subset were sick at all three time points or healthy at all three time points.

97

98 Approximately 1 ml of uterine fluid was added to prepared tubes containing 4 ml of RNA*later*

99 (Ambion, Austin, TX, USA). The biopsies were transferred into 2 ml of RNA*later* (Ambion) with a

100 sterile needle. Samples were stored at room temperature for approximately 24 h and then stored at -

101 20 °C. The details of the herd and sampling procedures were described in detail in Knudsen *et al*.

102 (Knudsen *et al*. manuscript submitted). A smear of the uterine fluid was made on a microscope slide

103 for cytology. The slides were stained with Hemacolor® (Merck Millipore, Darmstadt, Germany),

104 and mounted with Pertex® (Histolab, Göteborg, Sweden). All animal procedures were approved by

105 the Danish Animal Experiments Inspectorate under the Ministry of Justice, and the animal

106 experiments were conducted in strict accordance with their guidelines.

107

108 **RNA extraction and sequencing**

109 From the endometrial biopsies the starting material was 10 mg tissue, and for the uterine flush

110 samples, a volume of aspired uterine fluid in RNA*later* predicted to produce a pellet of 10 mg

111 (between 200 µl and 1800 µl) was measured. PBS buffer was added to a total volume of 1900 µl.

112 The flush samples were centrifuged at 13000 *g* at 4 °C for 15 min, and the supernatant was

113 discarded. A 5mm stainless steel ball (QIAGEN, Hilden, Germany) was added to both kinds of

114 samples, and they were bead-beaten in a TissueLyser II at 20 hz for 2 min in buffer RLT from the

115 AllPrep DNA/RNA miniprep kit (QIAGEN) with 10 µl per ml β-Mercaptoethanol. Then 30 µl

116 Proteinase K was added and the samples were incubated for 1h at 56°C before continuing with the

117 total RNA extraction with the AllPrep DNA/RNA miniprep kit (QIAGEN) following the

118 manufacturer's instructions. Then the RNA was enriched and amplified using the MICROBEnrich

119 and MICROBExpress (Ambion) kits according to the manufacturer's instructions.

120 Finally, 38 mRNA samples collected from 9 cows over three time points were reverse transcribed to

121 cDNA and sequenced on an Illumina Hiseq™ 2000 (Illunima Inc., San Diego, CA, USA) in single

122 reads with 100 cycles and 10 million reads per sample. The read length was 101 bp and the insert

123 length was on average 200 bp. The cDNA synthesis and sequencing were performed at the

124 University of Minnesota Genomics Center (UMGC), 28 Snyder Hall, 1475 Gortner Avenue, St.

125 Paul, MN 55108.

126

127 **Data analysis**

128 The FastQC software version 0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)

129 was used to analyse the quality of the sequences. Any sequence exceeding an 3 bp overlap with the

130 adapters sequences at its 3'end were trimmed using the software Cutadapt version 1.2.1

131 (https://code.google.com/p/cutadapt/) (Martin, 2011). Left and right reads that overlapped with a

132 minimum of 10 bp were merged in a single-end read using the software Flash version 1.2.9

133 (http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011). Then 8 bp were trimmed from
134 the 5' end of both the single-end reads and the remaining non merged paired-end reads, and all
135 bases with a quality score below 10 were trimmed from the 3' end. Finally, sequences shorter than
136 25 bp and sequences with a mean quality score below 20 were discarded, and an additional FastQC
137 analysis was performed.
138 The mapping was performed against the SILVA database of rRNA sequences (http://www.arb-
139 silva.de/)(Quast et al., 2013) using the SortMeRNA software version 1.99-beta
140 (http://bioinfo.lifl.fr/RNA/sortmerna/) (Kopylova et al., 2012). Initially, the mapping was performed
141 against eukaryotic rRNA sequences, and any read that mapped was discarded. The remaining reads
142 were mapped against bacterial rRNA.
143
144 After the rRNA filtering, the TopHat 2 software (http://ccb.jhu.edu/software/tophat/index.shtml)
145 (Kim et al., 2013) with default parameters was used to map the remainder of the reads against the
146 *Bos taurus* genome and annotation (build UMD_3.1, version 75). The reads that did not map on the
147 cow genome were collected and added to the unmapped single-end reads. These reads were joined
148 and assembled into transcripts using the Trinity software (http://trinityrnaseq.sourceforge.net/)
149 (Grabherr et al., 2011). Each single sample was then mapped back on the transcripts in order to
150 estimate the sample-specific expression level of each transcript. Comparison of expression level
151 was performed in the statistic software R environment with the EdgeR package
152 (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html) (Robinson et al., 2010).
153 The transcripts were submitted to BLASTX analysis against the National Center for Biotechnology
154 Information's (NCBI) non-redundant protein sequence database (http://www.ncbi.nlm.nih.gov/).
155 Every transcript with a positive hit (e-value <10e-5) was annotated using taxonomical description
156 down to the species level. Functional analysis of protein sequences encoded by the predicted ORFs
157 was performed by classifying them into families and predicting the presence of domains and
158 important sites using the InterPro resource (http://www.ebi.ac.uk/interpro/) (Hunter et al., 2012).

159

160 RESULTS

161 To gain an understanding of which bacteria were metabolically active during the infection and the
162 adaptions of these bacteria to the host environment, RNA-seq techniques were used to characterise
163 the uterine microbial transcriptome in postpartum dairy cows with metritis or endometritis
164 compared with cows with no apparent uterine disease.
165 Sequencing was performed on 38 samples from 9 cows. When unclassifiable samples and samples
166 from treated cows were discarded, 27 samples from 8 cows remained. These were distributed
167 among normal cows and cows with a uterine disease as shown in Table 1.
168
169 Across the 38 samples, 1.3 billion paired-end reads were obtained, which approximates to 17
170 million reads per sample when accounting for the paired-end sequencing. Subsequently, the
171 adaptors and the low quality bases were trimmed, and the paired-end reads were joined. Between
172 97% and 99% of reads were merged.
173 The joined reads were mapped against the 18S and 28S databases to filter eukaryotic ribosomal
174 RNA. For the biopsy samples, between 55% and 82% of the reads mapped to the reference
175 sequences and were discarded. For the flush samples, between 13% and 82% mapped to the
176 eukaryotic ribosomal RNA reference sequences and were discarded.
177 The remaining reads were mapped first to the 16S and 23S databases to filter bacterial ribosomal
178 RNA and then to the *Bos taurus* genome (Build 3.1, version 75), to filter bovine mRNA. After these
179 steps, on average 400,000 reads remained in each sample, which neither mapped to the cow
180 genome, the eukaryotic ribosomal RNA, nor the bacterial ribosomal RNA. These reads contain
181 bacterial mRNA but also cover unannotated bovine genes and viral genes. The reads were
182 assembled into a total of 368,000 different transcripts.
183

184 Although the endometritis and the metritis groups were pooled across time points to increase group
185 sizes, the subset observed was too small for statistical analysis. Consequently, a descriptive
186 approach was chosen, and the 50 most differentially expressed transcript between normal cows and
187 cows with postpartum disease were compared for both endometrial biopsies and uterine flush
188 samples. In the biopsies, the 50 most differentially expressed transcripts were up-regulated between
189 6.3 and 13.0 log2 (fold change) times in the disease-transcriptome when compared to the healthy
190 transcriptome. In the flush samples, the differential expression between disease and healthy
191 transcriptomes ranged from 13.6 log2 (fold change) up-regulation to 9.7 log2 (fold change) down-
192 regulation.
193
194 Using BLASTX analysis, we were able to identify open reading frames in 46 of the transcripts
195 originating from the endometrial biopsies – in most cases down to species level (Table 2); 75% of
196 the 50 most diversely expressed transcripts were from the *F. necrophorum* species, 10% were from
197 *Porphyromoas levii*, 6% were from other species just represented by one transcript, and 2% were
198 not annotated. In the uterine flush samples, however, 66.7% of the 50 most differentially expressed
199 transcripts either exhibited low homology with potential hits in the non-redundant (nr) database at
200 NCBI, or had closest homology with genes from the *Bos Taurus* genome (Table 3). These
201 transcripts were not annotated. Due to the apparent low quality of the flush samples, they were not
202 further analysed in this study.
203 For the biopsy samples, the coverage of know open reading frames (ORFs) in the transcripts ranged
204 between 59% and 100% (Table 2), when excluding the 4 transcripts where no ORFs could be
205 identified. In total, 158 ORFs were identified, which displayed high homology with proteins from
206 known species (Figure 1). BLASTX analysis resulted in the identification of 129 ORFs that were
207 between 59% and 100% identical to proteins identified in *F. necrophorum*. Of these, 123 ORFs
208 displayed between 98% and 100% identity to this bacterium on the protein level. Seventeen ORFs
209 encoded proteins which were between 83% and 100% homologous to proteins from *P. levii*. Six

210 ORFs were between 89% and 99% identical to *Mycoplasma bovigenitalium* on the protein level
211 (Table 4).
212
1505
213 **Functional analysis of the ORFs identified in the biopsy samples**
214 To identify genes potentially implicated in virulence, an analysis of function was performed in
215 Interpro on all the potential proteins that were identified among the 50 most up-regulated in the
216 microbiota of the infected postpartum uterus. An overview of all the predicted proteins can be
217 found in Table 4, and the functional categories that contain the up-regulated ORFs from the uterus
218 of cows with uterine disease are described below and depicted in Figure 2.
219
220 ***DNA replication and repair***
221 Approximately 9% of the up-regulated ORFs coded for proteins predicted to be involved in DNA
222 replication, recombination and repair. In addition, approximately 19% of the ORFs most highly
223 expressed in the uterus of cows with metritis or endometritis included genes that code for ribosomal
224 proteins (although most of these had probably been removed in the mRNA enrichment process), as
225 well as genes involved in protein transcription and translation (Figure 2 and Table 4). The high
226 expression of genes involved in the above mentioned processes most likely indicate that the bacteria
227 in the uterine wall were in an active multiplication phase during infection.
228
229 ***Metabolic processes***
230 ORFs that encode metabolic functions accounted for 16% of the 50 most differentially expressed
231 genes in the infecting microbiota (Table 4). Many of the up-regulated genes from this metabolic
232 functional group were predicted to be involved in carbohydrate metabolism, which indicates a shift
233 in utilisation of carbon sources for the bacteria that successfully colonise the host. Both enzymes
234 from the Gluconeogenesis (orf_23), the glycolysis pathway (orf_53, orf_54) and its parallel, the
235 pentose phosphate pathway (orf_33, orf_106), were represented among the highly up-regulated

236 genes identified in the diseased state uterus. Similarly, the activity of a classical amino sugar
237 pathway was indicated by the up-regulation of the enzyme Glucosamine-fructose-6-
238 phosphate aminotransferase (glmS; orf_97) in the infecting bacteria. GlmS is the first and rate-
239 limiting enzyme of the hexosamine biosynthetic pathway. The final product of the
240 hexosamine pathway, UDP-N-acetyl glucosamine, is the precursor for numerous macromolecules,
241 and an essential structural building block for the bacterial peptidoglycan and the lipopolysaccharide
242 of Gram-negative bacteria (Durand et al., 2008).
243 The β-oxidation spiral of fatty acid degradation consists of four enzymatic transformations that
244 reduce the acyl chain length by two carbons in each cycle, resulting in the formation of acetyl-CoA
245 as the by-product in each cycle (Agnihotri and Liu, 2003). Three of the four enzymes that drive this
246 cycle (orf_44, orf_94, and orf_95**)** were among the highly up-regulated genes found in the uterine
247 microbiome of cows with metritis and endometritis. The presence of these genes suggests that the
248 bacteria infecting the uterus are able to use other carbon sources than glucose; these carbon
249 substrates may derive from host cell lipids and phospholipids or triglycerides (Eisenreich et al.,
250 2010)**.**
251
252 ***Genes implicated in stress response during infection***
253 We identified four genes from the functional group "protein folding". These included three classical
254 chaperones, *dnaK* (orf_83), *GroEL* (orf_60), and *clpB* (orf_81), and a trigger factor (TF) (orf_90)
255 which were all highly expressed in animals with postpartum disease. *DnaK* and its co-chaperones
256 are the most potent cellular defences against environmental insults such as heat or oxidative stress
257 (Genevaux et al., 2007). At high temperatures, *dnaK* prevents protein aggregation and assist protein
258 disaggregation in cooperation with *clpB* and other chaperones (Haslberger et al., 2007). In concert
259 with the ribosome bound chaperone trigger factor, *dnaK* protects nascent proteins from misfolding
260 and aggregation. At temperatures above 30 degrees, the combined absence of *dnaK* and TF is lethal,
261 underpinning their importance for de novo protein folding (Genevaux et al., 2004).

262

263 ***Transport***

264 The cell membrane functions as a permeability barrier that regulates the passage of substances into
265 and out of the cell; and bacteria therefore have multiple transport systems for the uptake and export
266 of most nutrients. Secretion of proteins across the inner membrane in some Gram-negative bacteria
267 occurs via the preprotein translocase pathway encoded by the *sec* genes (Duong and Wickner,
268 1997). The *sec*-encoded proteins, SecA (orf_20) and SecY (orf_158), which are essential for cell
269 viability and for the preprotein translocation *in vivo* (Oliver and Beckwith, 1982), were highly
270 expressed in the microbiome of cows with uterine disease.
271 In the bacteria invading the uterine wall, we observed a high expression of genes potentially coding
272 for magnesium and cobalt transport (orf_120) as well as a heavy metal translocating P-type ATPase
273 (orf_99). As an imbalance in bacterial metal homeostasis is deleterious, part of the host defence
274 either consists of metal starvation by sequestration or toxicity by the highly concentrated release of
275 metals (Porcheron et al., 2013). Consequently, in order for the bacteria to survive in the host, the
276 bacteria need to ensure a metal uptake which is in accordance with availability and physiological
277 needs. For this purpose, the infecting agent employs a variety of metal uptake and export systems,
278 allowing them to adapt to changing concentration of metal ions in the environment. As a
279 consequence, iron, zinc, manganese, and copper uptake systems significantly contribute to the
280 virulence of many pathogenic bacteria (Porcheron et al., 2013).
281 ORFs putatively coding for ABC transporter proteins, were highly up-regulated in *F. necrophorum*
282 (orf_27, orf_119) and *M. bovigenitalium* (orf_5-7). The ABC transporters cover a functionally
283 diverse family of proteins which shuttle a great variety of substrates across different cellular
284 membranes. They are involved in the uptake and export of nutrients, and play a role in the
285 elimination of waste products and toxins from the cell. They are essential for export of cell wall
286 polysaccharides and for polypeptides required outside the cell e.g. toxins and proteinases. And
287 finally, they are essential for antibiotic resistance of bacteria (Higgins, 2001; Procko et al., 2009).

288

289 ***High expression of cells encoding cell wall metabolism proteins during infection***

290 Peptidoglycan (or murein) is a major component of the cell wall of almost all eubacteria. It is a
291 complex heteropolymer that is composed of long glycan chains that are cross-linked by short
292 peptides. The stepwise assembly of the peptide stem of peptidoglycan is ensured by a series of four
293 essential enzymes, known as the Mur ligases (MurC, D, E and F) (Barreteau et al., 2008). Putative
294 ORFs, *murCDF* (orf_10-12) were up-regulated in the microbiota of cows with postpartum disease.
295 Lipopolysaccharide (LPS) is the major glycolipid of the outer membrane of Gram-negative bacteria.
296 Lipid A, or endotoxin, the lipid portion of LPS, is a potent immunostimulant (Emptage et al., 2013;
297 Garrett et al., 1997). Lipid A in *E. coli* is synthesised on the cytoplasmic surface of the inner
298 membrane by a conserved pathway of nine constitutive enzymes. As lipid A is essential for the
299 viability of most of the Gram-negative bacteria, the pathway is an attractive target for the
300 development of novel antimicrobials (Raetz and Reynolds, 2007). In the uterine wall of the cows
301 with postpartum disease, an enzyme from the lipid A synthesis pathway, lipid A 4′-kinase
302 (orf_123), was highly expressed by *F. necrophorum*. Similarly, an up-regulated hypothetical ORF
303 in *P. levii* was detected, which possibly encodes a zinc-dependent metalloamidases (orf_116) that
304 catalyses the second and committed step in the biosynthesis of lipid A (Coggins et al., 2005).

305

306 ***Proteolysis***

307 Proteases are enzymes that can break down peptides and proteins of the infected host and are an
308 important part of the arsenal of bacterial virulence factors (Hoge et al., 2010). Seven *F.*
309 *necrophorum* ORFs identified in the uterine wall of the diseased dairy cows were characterised as
310 coding for putative proteases. One was a methionine aminopeptidase (orf_51). Aminopeptidases has
311 proven important for the survival of *F. nucleatum* in the subgingival environment of the mouth of
312 humans (Rogers et al., 1998). Another was protease IV (WP_005952730.1) which has been

313 connected with virulence in the opportunistic pathogen *Pseudomonas aeruginosa* (Mochizuki et
314 al., 2014).
315
316 ***Virulence factors***
317 In InterPro, one category is designated "pathogenesis" which is defined as **"**the set of specific
318 processes that generate the ability of an organism to cause disease in another". In this investigation,
319 the putative protein products encoded by three ORFs in *F. necrophorum* fell into this category. No
320 particular function could be ascribed to these proteins. All three were most likely outer membrane
321 proteins and one of them (orf_144) a possible autotransporter adhesin. The ability to adhere to host
322 cells or surfaces is a vital part of a successful bacterial invasion (Henderson et al., 2001; Zhuge et
323 al., 2013).
324 Among the ORFs that could not be designated any functional group were a couple of genes that
325 might code for proteins involved in virulence. An ORF from *F. necrophorum* was 100% identical to
326 a putative RNA binding protein S1 *(*orf_28), which according to Interpro included a conserved
327 virulence factor B (CvfB_fam). The ribosomal protein S1 is a key mRNA-binding protein in Gram-
328 negative bacteria (Hajnsdorf & Boni 2012); the *cvfB* gene is conserved among various pathogenic
329 bacteria; it regulates exoprotein gene expression and is responsible for virulence in e.g.
330 *Staphylococcus aureus* (Matsumoto et al., 2007).
331 Also among the putative proteins encoded by an up-regulated ORF in *F. necrophorum* during
332 infection of the uterus was a filamentous haemagglutinin protein (EYD69863.1). The findings by
333 Kanoe *et al*. 1998 suggest that filamentous haemagglutinin adhesin (HA) is a components of the
334 cell surfaces of *F. necrophorum* subsp, *necrophorum* (Kanoe *et al*., 1998). Haemagglutinin may
335 play a significant role in adherence to and invasion of ruminal epithelial cells by *F. necrophorum*
336 (Chukwu et al., 2014; Nagaraja et al., 2005; Tadepalli et al., 2009; Tan et al., 1994). In a study by
337 Smith *et al*. 1993 (Smith and Thornton, 1993), animal isolates of *F. necrophorum* subsp.

338 *necrophorum* with haemagglutinin were more virulent than animal and human isolates of *F.*
339 *necrophorum* subsp. *funduliforme*, which lacks haemagglutinin (Smith and Thornton, 1993).
340
1510

341 **DISCUSSION**

342 *E. coli*, *T. pyogenes*, *F. necrophorum* and *Prevotella* species are all commonly associated with the
343 postpartum diseases endometritis and metritis in dairy cows but the role of each bacterium in the
344 pathogenicity is still unclear, and many aspects of the aetiology of these diseases remain
345 unresolved. However, in polymicrobial infections, estimation of the metabolic activity of the
346 bacteria at the site of infection may identify the most important of the pathogenic species as well as
347 their infection strategy. Therefore, to address the aetiology and pathogenesis of metritis and
348 endometritis, the gene expression was compared between bacteria isolated from the postpartum
349 uterus of normal cows and cows with metritis and endometritis. It was assumed that the most
350 differentially expressed genes between the two groups would be important for bacterial fitness *in*
351 *vivo*. In the analysis, the endometritis and metritis groups were pooled to increase group sizes and
352 the 50 most differentially expressed transcript between normal cows and cows with postpartum
353 disease were compared for both endometrial biopsies and uterine flush samples. Due to the apparent
354 low quality of the uterine flush sample sequences, further analysis of the flush samples was
355 abandoned.

356 The 50 most differentially expressed transcripts identified in the endometrial biopsies revealed a
357 surprisingly low diversity of bacterial species, as 75% and 10% were highly homologous to proteins
358 from *F. necrophorum* and *P. levii*, respectively. Among the few other species identified were *M.*
359 *bovigenitalis* and *Streptobacillus moniliformis*, represented by one transcript only. As we have
360 chosen to exclusively focus on a subset of differentially expressed transcripts, we cannot rule out
361 that the actual biodiversity of the infected site was higher, and that many other bacteria were
362 represented in the remaining transcripts. Nevertheless, we assumed that the bacteria most important

363 for disease development were the ones exhibiting the most diverse expression at the site of
364 infection.
365

366 The links between *P. levii* and *M. bovigenitalium* and postpartum disease are scarce but nonetheless
367 present (Bekana et al., 1997; Ghanem et al., 2013; Santos et al., 2011). *P. levii* has also been
368 associated with bovine necrotic vulvovaginitis (Elad et al., 2004) and bovine foot rot (Walter, 2001)
369 while *M. bovigenitalium* has been connected with infertility in heifers (Saed *et al*. 1984).
370 *Fusobacterium* species have previously been connected with postpartum diseases in dairy cows,
371 both by 16S rRNA phylogenetic studies and cultivation studies of uterine postpartum disease
372 (Bekana et al., 1997; Machado et al., 2012; Santos and Bicalho, 2012; Santos et al., 2011; Williams
373 et al., 2007). It is an opportunistic pathogen that causes a wide variety of infections in animals and
374 humans (Chukwu et al., 2014; Nagaraja et al., 2005). In cattle it is associated with the necrotic
375 conditions bovine hepatic abscesses and ruminant foot rot (Nagaraja et al., 2005). *F. necrophorum*
376 pathogenesis is complex and not very well defined. Virulence factors implicated in the pathogenesis
377 of this bacterium are factors which contribute to entry, colonisation, and proliferation in the host.
378 Among these are leukotoxin, which is considered the major virulence factor of *F. necrophorum*,
379 endotoxic lipopolysaccharide (LPS), haemolysin, haemagglutinin, capsule, adhesins or pili, and
380 several extranuclear enzymes including proteases and deoxyribonuclases (Nagaraja et al., 2005;
381 Oelke et al., 2005).
382

383 The two subspecies of *F. necrophorum*, subsp. *necrophorum* (Type A) and subsp. *funduliforme*
384 (Type B) differ morphologically, biochemically and biologically. Type A produces more leucotoxin
385 than type B and is generally considered to be the more virulent of the two subspecies (Nagaraja et
386 al., 2005; Oelke et al., 2005). The expression of *F. necrophorum* leukotoxin has been significantly
387 associated with metritis (Bicalho et al., 2011). In this study we were not able to detect high

388 expression of leucotoxin, and one might speculate that it could be due to an overrepresentation of
389 subsp. *funduliforme* in the infected uterine samples.
390 We identified 158 differentially regulated ORFs likely to encode functions required by the bacteria
391 for colonisation and survival in the host; most of them were homologous to proteins from *F.*
392 *necrophorum.* The *F. necrophorum* isolated from the uteri affected by postpartum disease appeared
393 to be in an active phase of multiplication, as could be deducted from the relatively high number of
394 genes involved in replication, protein transcription and translation. Another dominating group was
395 ORFs encoding proteins involved in various aspects of energy metabolism, especially carbohydrate
396 and fatty acid metabolism, which may indicate that a shift in utilisation of carbon sources is needed
397 for survival in the host. Other genes which seemed to be important for adaption and survival in the
398 host were the classical chaperones, *dnaK*, GroEL ,*clpB*, and a number of transport systems,
399 including the sec-pathway for protein secretion, metal uptake and export systems, and ABC
400 transporters. Likewise, we observed signs of active remodelling of the bacterial envelope through
401 activation of genes responsible for cell wall components. In *F. necrophorum*, host contact may have
402 induced the observed differential regulation of genes coding the peptidoglycan biosynthetic
403 pathway (*murCDF*).
404
405 Besides the changes just described, we also observed differential up-regulations of a number of
406 genes that might correspond to some of the virulence factors of *F. necrophorum*, which are
407 described in the literature (Chukwu et al., 2014; Nagaraja et al., 2005). These included an endotoxin
408 coding gene (lipid A 4'kinase); endotoxins stimulates pro-inflammatory cytokines, and are
409 responsible for inflammatory and toxic effects (Tan et al., 1996); a number of protease genes, which
410 may participate in facilitating penetration of the ruminal epithelium (Nagaraja et al., 2005); and
411 finally, a putative autotransporter adhesion gene, and a putative filamentous haemagglutinin gene,
412 which may facilitate adhesion to host epithelial cells.
413

CONCLUDING REMARKS

The method applied in this study, RNA-seq, seems to combine the best features of bacterial cultivation and non-culture dependent methods (e.g. 16S rRNA sequencing), for the analysis of pathogenic microbiota: it detects both cultivable and not-yet-cultivable bacteria and differentiates between live and dead bacteria, as it only monitors the genes that are actively expressed. By comparing the microbial *in vivo* genomic expression of bacteria that reside in healthy versus diseased uteri of postpartum cows, we were able to derive new detailed information regarding the microbial composition of the active trancriptome and its adaptions to the host environment. Based on these results, we cannot rule out the effect of other pathogenic agents such as *T. pyogenes* and *E. coli*, in the pathogenicity of postpartum disease. Nevertheless, our result do indicate that in the dairy cows included in this particular study, *F. necrophorum* and most likely also *P. levii* contribute to the development of metritis and endometritis. In the future, large scale studies including different herds and several time-points during disease development will be needed to confirm these results.

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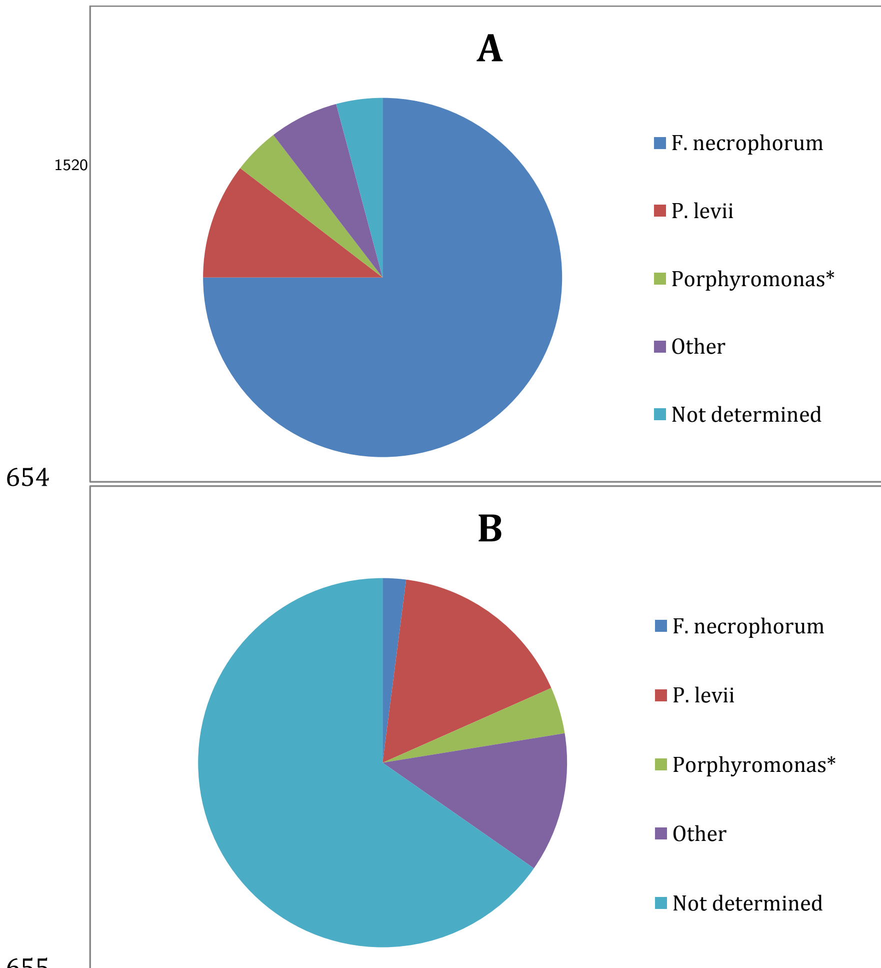

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656 **Figure 1. Bacterial species distribution of identified transcripts in A) uterine biopsies**
657 **and B) uterine flush samples as determined by BLASTX analysis.**
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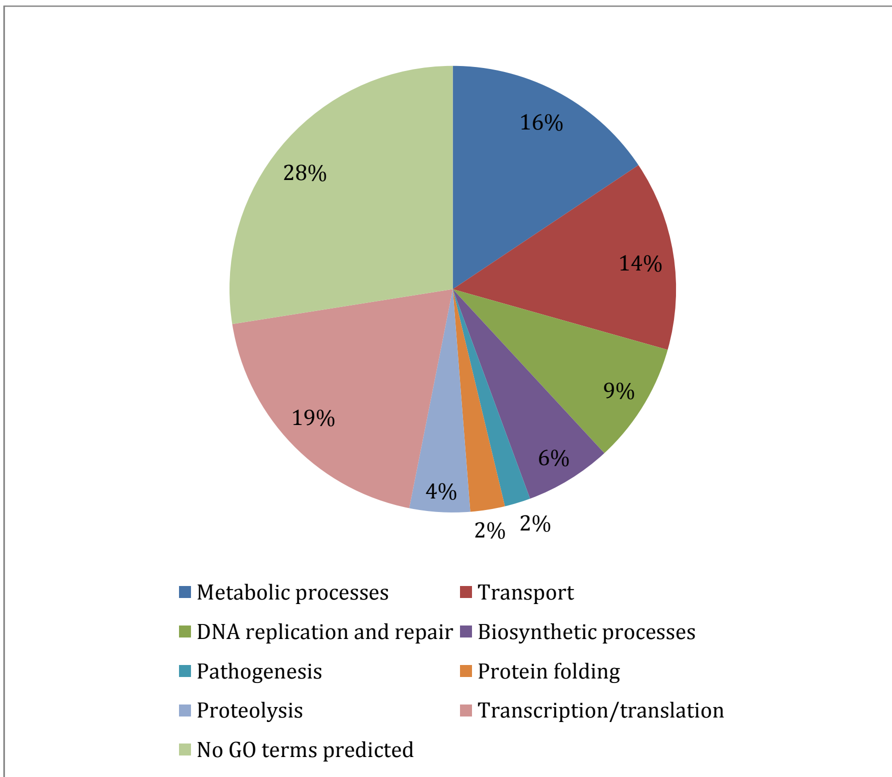

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660 **Figure 2. Overview of functional distribution (GO terms) of the 158 ORFs identified in**
661 **the biopsy samples.**
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665 **Table 1: Number of samples obtained per uterine health status and specimen type.**

	Uterine disease	**Normal**
Endometrial biopsy	7	7
Uterine flush	7	6

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670 **Table 2. Coverage of identified ORFs in transcripts in biopsy samples**

Sequence id	**logFC**	**Transcript length (bp)**	**Length of identified ORFs (bp)**	**Total match length %**
c70395_g1_i2	13,0	27458	8103	30
c128788_g1_i1	11,4	9056	8583	95
c70918_g1_i1	11,3	27426	6474	24
c70922_g1_i1	10,8	29656	16536	56
c70909_g1_i1	10,8	22040	7503	34
c234666_g1_i1	10,7	8775	3468	40

c70813_g1_i1	10,6	11497	10008	87
c64514_g1_i2	10,4	1355	924	68
c69962_g2_i1	10,3	3880	2829	73
c70670_g1_i1	10,2	12578	8400	67
c69362_g1_i1	10,2	1469	582	40
c70545_g2_i2	10,1	5026	2358	47
c70866_g1_i1	10,1	11199	5676	51
c70890_g1_i1	10,0	18136	6729	37
c70378_g2_i1	9,9	2962	1617	55
c66822_g1_i1	9,8	10183	3249	32
c67506_g1_i2	9,8	4283	2151	50
c70809_g1_i2	9,8	8462	5541	65
c68953_g1_i1	9,7	9092	5886	65
c70573_g2_i1	9,7	7198	5994	83
c70874_g1_i1	9,7	13774	9438	69
c70904_g1_i2	9,7	19189	9105	47
c70649_g1_i1	9,6	11899	5487	46
c70632_g1_i1	9,6	3701	1824	49
c70514_g1_i1	9,6	6762	4206	62
c70816_g1_i1	9,5	7889	6156	78
c32449_g1_i1	9,5	402	294	73
c70832_g1_i1	9,5	6242	4161	67
c70885_g1_i1	9,5	9907	6339	64
c69486_g1_i1	9,4	7003	2403	34
c70657_g1_i1	9,4	5457	2910	53
c70044_g1_i1	9,4	4705	3630	77
c70779_g1_i1	9,4	10877	8343	77
c69739_g3_i2	9,4	18348	6030	33
c70676_g1_i1	9,4	6137	4875	79
c70903_g1_i1	9,3	22586	11025	49
c69428_g1_i1	9,3	3073	2478	81
c70551_g1_i1	9,2	3581	3579	100
c70678_g1_i1	9,2	5394	3051	57
c66324_g1_i1	8,9	12963	9528	74
c70870_g1_i1	8,7	11506	5154	45
c70483_g1_i3	8,6	5372	3693	69
c67025_g2_i1	8,6	5981	4602	77
c70876_g1_i1	8,5	12327	6729	55
c70873_g1_i1	8,2	13802	5289	38
c68776_g2_i1	6,3	9004	1785	20

671

672 **Table 3. Coverage of identified ORFs in transcripts in flush samples**

Sequence id	logFC	Transcript length (bp)	Length of identified ORFs (bp)	Total match length %
c332921_g1_i1	13,6	6865	6288	92
c125390_g1_i1	12,7	2711	2484	92
c21214_g1_i1	12,6	5879	4233	72

c21537_g1_i1	12,5	4110	3675	89
c70395_g4_i1	12,4	9470	6012	63
c76025_g1_i1	12,1	506	0	0
c63265_g1_i1	12,1	4290	3306	77
c62307_g1_i1	11,9	9615	5577	58
c68942_g1_i1	11,4	14161	4932	35
c333069_g1_i1	11,4	3555	1938	55
c1910_g1_i1	11,1	454	0	0
c68723_g1_i2	11	5277	1494	28
c67025_g2_i1	10,9	5981	990	17
c68776_g2_i1	10,7	9004	0	0
c70798_g1_i1	9,3	8902	5367	60
c70286_g1_i1	6	8514	1512	18
c18735_g1_i1	4,1	593	0	0
c38821_g1_i1	2,8	1980	0	0
c52152_g1_i1	2,6	1022	0	0
c67874_g2_i1	-2,6	1021	0	0
c70023_g1_i1	-2,7	2524	0	0
c70668_g1_i2	-2,8	307	0	0
c70849_g2_i1	-3,1	3349	0	0
c18573_g1_i1	-3,2	964	0	0
c61944_g1_i1	-3,4	1276	0	0
c51508_g1_i1	-3,4	295	0	0
c70692_g1_i2	-3,4	779	0	0
c69183_g1_i1	-3,4	1010	0	0
c57624_g1_i1	-3,4	921	0	0
c70535_g2_i1	-3,5	353	0	0
c69414_g8_i1	-3,6	377	0	0
c70626_g10_i1	-3,6	656	0	0
c70535_g3_i1	-3,7	1556	0	0
c70626_g2_i2	-3,7	1748	0	0
c67172_g1_i1	-3,8	724	345	48
c69146_g1_i2	-3,8	1440	0	0
c70196_g1_i1	-3,9	1089	0	0
c70626_g9_i2	-4	874	0	0
c70114_g4_i3	-4,1	631	0	0
c15143_g1_i1	-4,1	959	0	0
c255685_g1_i1	-4,2	773	0	0
c136851_g1_i1	-4,5	344	0	0
c141013_g1_i1	-4,6	288	0	0
c64669_g1_i1	-4,6	345	0	0
c70804_g2_i2	-4,7	347	0	0
c70404_g1_i1	-4,9	1433	0	0
c180088_g1_i1	-4,9	807	0	0
c63554_g1_i1	-5,1	904	0	0
c71312_g1_i1	-9,7	1435	357	25

673
674 **Table 3. Coverage of identified ORFs in transcripts in flush samples**

Sequence id	logFC	Transcript length (bp)	Length of identified ORFs (bp)	Total match length %
c332921_g1_i1	13,6	6865	6288	92
c125390_g1_i1	12,7	2711	2484	92
c21214_g1_i1	12,6	5879	4233	72
c21537_g1_i1	12,5	4110	3675	89
c70395_g4_i1	12,4	9470	6012	63
c76025_g1_i1	12,1	506	0	0
c63265_g1_i1	12,1	4290	3306	77
c62307_g1_i1	11,9	9615	5577	58
c68942_g1_i1	11,4	14161	4932	35
c333069_g1_i1	11,4	3555	1938	55
c1910_g1_i1	11,1	454	0	0
c68723_g1_i2	11	5277	1494	28
c67025_g2_i1	10,9	5981	990	17
c68776_g2_i1	10,7	9004	0	0
c70798_g1_i1	9,3	8902	5367	60
c70286_g1_i1	6	8514	1512	18
c18735_g1_i1	4,1	593	0	0
c38821_g1_i1	2,8	1980	0	0
c52152_g1_i1	2,6	1022	0	0
c67874_g2_i1	-2,6	1021	0	0
c70023_g1_i1	-2,7	2524	0	0
c70668_g1_i2	-2,8	307	0	0
c70849_g2_i1	-3,1	3349	0	0
c18573_g1_i1	-3,2	964	0	0
c61944_g1_i1	-3,4	1276	0	0
c51508_g1_i1	-3,4	295	0	0
c70692_g1_i2	-3,4	779	0	0
c69183_g1_i1	-3,4	1010	0	0
c57624_g1_i1	-3,4	921	0	0
c70535_g2_i1	-3,5	353	0	0
c69414_g8_i1	-3,6	377	0	0
c70626_g10_i1	-3,6	656	0	0
c70535_g3_i1	-3,7	1556	0	0
c70626_g2_i2	-3,7	1748	0	0
c67172_g1_i1	-3,8	724	345	48
c69146_g1_i2	-3,8	1440	0	0
c70196_g1_i1	-3,9	1089	0	0
c70626_g9_i2	-4	874	0	0
c70114_g4_i3	-4,1	631	0	0
c15143_g1_i1	-4,1	959	0	0
c255685_g1_i1	-4,2	773	0	0
c136851_g1_i1	-4,5	344	0	0
c141013_g1_i1	-4,6	288	0	0

c64669_g1_i1	-4,6	345	0	0
c70804_g2_i2	-4,7	347	0	0
c70404_g1_i1	-4,9	1433	0	0
c180088_g1_i1	-4,9	807	0	0
c63554_g1_i1	-5,1	904	0	0
c71312_g1_i1	-9,7	1435	357	25

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Table 4. Overview of all predicted proteins.

Transcript id	log2 FC	Genbank ID*	Homology aa(%)	ORF id	GO term prediction
c69486_g1_i1	9.4	WP_027640232.1	63	orf_100	Metabolic process
c70918_g1_i1	11.3	EYD68623.1	99	orf_9	tRNA aminoacylation for protein translation, leucyl-tRNA aminoacylation
		WP_005953560.1	99	orf_10	Regulation of cell shape, biosynthetic process, cell division
		WP_005956648.1	100	orf_11	Regulation of cell shape, biosynthetic process, cell division
		WP_005953553.1	100	orf_12	Regulation of cell shape, biosynthetic process, cell division
c70922_g1_i1	10.8	WP_005959271.1	99	orf_13	Metabolic process
		KDE61285.1	98	orf_14	Metabolic process
		EYD70261.1	99	orf_15	NP
		WP_005959270.1	99	orf_16	NP
		KDE62880.1	100	orf_17	Peptidoglycan biosynthetic process, response to antibiotic
		EYD70255.1	99	orf_18	NP
		KDE65428.1	99	orf_19	NP
c70909_g1_i1	10.8	WP_005959567.1	99	orf_20	Protein targeting, intracellular protein transport, protein import
		KDE64167.1	99	orf_21	Glycerol metabolic process
		WP_005959572.1	100	orf_22	Proteolysis
c70813_g1_i1	10.6	KDE65259.1	99	orf_25	Dicarboxylic acid transport
		WP_005962671.1	100	orf_26	NP
		WP_027132273.1	100	orf_27	Transport
		WP_005958057.1	100	orf_28	NP
		WP_005955408.1	99	orf_29	Pathogenesis
		WP_005958034.1	100	orf_30	GPI anchor metabolic process, intracellular protein transport
		WP_005955404.1	99	orf_31	NP
		WP_005955402.1	100	orf_32	Regulation of transcription, DNA-templated
		WP_005962898.1	99	orf_33	Carbohydrate metabolic process, pentose-phosphate shunt, metabolic process
		WP_027132279.1	100	orf_34	NP
		EYD69176.1	99	orf_35	NP
c64514_g1_i2	10.4	EYD68480.1	100	orf_37	NP
c69962_g2_i1	10.3	KDE67855.1	100	orf_38	Proteolysis
		WP_005955929.1	100	orf_39	NP
		KDE66412.1	100	orf_40	NP
c70670_g1_i1	10.2	WP_005959392.1	100	orf_41	NP
		KDE61562.1	99	orf_42	NP

		WP_005959390.1	100	orf_43	NP
		WP_005961083.1	100	orf_44	Metabolic process, oxidation-reduction process
		KDE62155.1	99	orf_45	Biosynthetic process
		WP_005957939.1	100	orf_46	NP
		WP_005955752.1	100	orf_47	Transport
		WP_005957927.1	100	orf_48	Amino acid transport
c69362_g1_i1	10.2	WP_005953509.1	100	orf_49	Regulation of transcription, DNA-templated, regulation of DNA-templated tran elongation
c70545_g2_i2	10.1	WP_005961168.1	100	orf_50	Nucleobase-containing compound metabolic process
		WP_005956157.1	100	orf_51	Proteolysis
		WP_005956146.1	99	orf_52	Transcription, DNA-templated
c70866_g1_i1	10.1	WP_005959434.1	98	orf_53	Glycolytic process
		WP_005960309.1	99	orf_54	Glycolytic process
		WP_005954914.1	100	orf_55	NP
		WP_005960307.1	99	orf_56	NP
c70890_g1_i1	10.0	WP_005958217.1	100	orf_57	Metabolic process, electron transport chain, oxidation-reduction process
		WP_005962844.1	100	orf_58	DNA topological change
		WP_005958234.1	100	orf_59	DNA repair, nucleotide-excision repair, SOS response
c70378_g2_i1	9.9	WP_005958293.1	100	orf_60	Protein folding, protein refolding, cellular protein metabolic process
c67506_g1_i2	9.8	WP_005952617.1	100	orf_62	Metabolic process, oxidation-reduction process
		WP_005959556.1	100	orf_63	NP
c70809_g1_i2	9.8	WP_027132294.1	99	orf_64	NP
		WP_005967080.1	99	orf_65	tRNA aminoacylation for protein translation, threonyl-tRNA aminoacylation, tR aminoacylation
c70573_g2_i1	9.7	EYD69863.1	99	orf_70	DNA repair
c70874_g1_i1	9.7	WP_005954969.1	100	orf_71	Nucleobase-containing compound metabolic process, DNA repair
		WP_027132354.1	100	orf_72	Signal transduction
		WP_005960467.1	99	orf_73	tRNA aminoacylation for protein translation, isoleucyl-tRNA aminoacylation
		WP_005954960.1	100	orf_74	Glycyl-tRNA aminoacylation
c70904_g1_i2	9.7	WP_009005918.1	100	orf_75	Proteolysis
		WP_005952626.1	100	orf_76	DNA replication
		WP_005964540.1	100	orf_77	DNA replication
		WP_005959526.1	100	orf_78	Phosphorelay signal transduction system, regulation of transcription, DNA-tem
		KDE69846.1	97	orf_79	NP
		WP_027131908.1	100	orf_80	NP
c70649_g1_i1	9.6	WP_027131996.1	100	orf_81	Response to heat, protein processing, protein metabolic process
		KDE63316.1	99	orf_82	NP
c70632_g1_i1	9.6	WP_005956122.1	100	orf_83	Protein folding
c70514_g1_i1	9.6	WP_005954590.1	99	orf_84	NP
		WP_005954597.1	100	orf_85	Folic acid-containing compound biosynthetic process
		EYD68913.1	100	orf_86	Pathogenesis
c70816_g1_i1	9.5	WP_005960091.1	100	orf_87	Regulation of DNA-templated transcription, termination
		WP_005955137.1	100	orf_88	Translational initiation
		WP_005964872.1	100	orf_89	DNA repair, DNA recombination
		WP_005960104.1	99	orf_90	Protein folding, protein transport
c32449_g1_i1	9.5	AOJP01000007.1	84	orf_91	NP
c70832_g1_i1	9.5	KDE63247.1	99	orf_92	DNA repair, DNA recombination
		EYD68473.1	99	orf_93	RNA binding, GTP binding
		WP_005963708.1	99	orf_94	Metabolic process

		WP_005958146.1	100	orf_95	Fatty acid metabolic process, oxidation-reduction process
c70885_g1_i1	9.5	WP_005964660.1	100	orf_96	NP
		WP_005952588.1	99	orf_97	Carbohydrate metabolic proces, carbohydrate biosynthetic process
		WP_005959551.1	99	orf_98	NP
		EYD69596.1	98	orf_99	Cation transport, metal ion transport
c70657_g1_i1	9.4	KDE64031.1	97	orf_101	GTP catabolic process, tRNA modification
		EYD68568.1	100	orf_102	tRNA wobble uridine modification, tRNA processing
c70044_g1_i1	9.4	WP_005956783.1	100	orf_103	NP
		WP_005956709.1	100	orf_104	NP
		WP_005956821.1	99	orf_105	NP
		WP_005954706.1	99	orf_106	Carbohydrate metabolic process, pentose-phosphate shunt
		KDE61227.1	99	orf_107	Metabolic process
c70779_g1_i1	9.4	WP_005963339.1	99	orf_108	Cation transport, potassium ion transport, transmembrane transport
		EYD69324.1	99	orf_109	RNA processing
		KDE62449.1	99	orf_110	tRNA aminoacylation for protein translation
		WP_005957767.1	100	orf_111	tRNA aminoacylation for protein translation, histidyl-tRNA aminoacylation
		WP_027132210.1	100	orf_112	DNA replication, DNA repair, DNA recombination
		KDE68336.1	99	orf_113	DNA repair, base-excision repair
c70676_g1_i1	9.4	WP_005964434.1	99	orf_117	NP
		WP_005953514.1	100	orf_118	NP
		WP_005953516.1	100	orf_119	transport
		WP_005957436.1	100	orf_120	cobalt ion transport, magnesium ion transport, metal ion transport, transmem transport
		WP_005957517.1	100	orf_121	NP
c70903_g1_i1	9.3	WP_005957131.1	99	orf_122	sister chromatid cohesion, chromosome condensation, chromosome organizat
		KDE62787.1	99	orf_123	lipid A biosynthetic process
		KDE63388.1	100	orf_124	Protein methylation, methylation
		WP_005957067.1	99	orf_125	Queuosine biosynthetic process
		WP_005957245.1	99	orf_126	Proteolysis
		KDE65557.1	99	orf_127	NP
		WP_005952778.1	100	orf_128	SRP-dependent cotranslational protein targeting to membrane
c69428_g1_i1	9.3	WP_005956675.1	100	orf_129	NP
		WP_027131651.1	99	orf_130	metabolic process, oxidation-reduction process
c70551_g1_i1	9.2	EYD69863.1	86	orf_131	DNA repair
c70678_g1_i1	9.2	WP_005956339.1	100	orf_132	NP
		WP_005956336.1	99	orf_133	Transport, electron transport chain
		WP_005961121.1	100	orf_134	NP
c70870_g1_i1	8.7	WP_005964657.1	98	orf_141	tRNA aminoacylation for protein translation, methionyl-tRNA aminoacylation
		WP_005952730.1	98	orf_142	Proteolysis
		KDE62288.1	99	orf_143	Proteolysis
c70483_g1_i3	8.6	WP_005954849.1	96	orf_144	Pathogenesis
		WP_005954851.1	59	orf_145	Transmembrane transport
c70876_g1_i1	8.5	WP_005958711.1	99	orf_149	Phenylalanyl-tRNA aminoacylation, tRNA processing
		WP_005958668.1	99	orf_150	DNA metabolic process, DNA topological change
		WP_005961114.1	100	orf_151	DNA topological change
c70873_g1_i1	8.2	WP_005958006.1	100	orf_152	Pseudouridine synthesis, RNA modifications
		KDE61382.1	100	orf_153	Translation

		WP_005958016.1	100	orf_154	Translation
		WP_005956206.1	100	orf_155	Translation
		WP_005956200.1	100	orf_156	Translation
		WP_005956189.1	100	orf_157	Translation
		WP_005958012.1	100	orf_158	Protein transport
c128788_g1_i1	11.4	WP_004418948.1	89	orf_3	NP
		WP_004418947.1	97	orf_4	NP
		WP_004418945.1	99	orf_5	Transport
		WP_004418943.1	99	orf_6	Transport
		WP_004418941.1	97	orf_7	Transport
		WP_004418937.1	96	orf_8	NP
c70395_g1_i2	13.0	WP_018357753.1	100	orf_1	Transcription, DNA-templated
		WP_026215555.1	99	orf_2	Transcription, DNA-templated
		WP_018357743.1	83	orf_24	Metabolic proces, oxidation-reduction process
c66822_g1_i1	9.8	WP_026215665.1	99	orf_61	Nitrogen compound metabolic process
c68953_g1_i1	9.7	WP_018357579.1	100	orf_66	NP
		WP_026215531.1	100	orf_67	Cation transport, transmembrane transport, potassium ion transmembrane tra
		WP_018357574.1	99	orf_68	Potassium ion transport
		WP_026215530.1	99	orf_69	Metabolic process, terpenoid biosynthetic process
c69739_g3_i2	9.4	WP_018358647.1	99	orf_114	Transport
		WP_018358646.1	100	orf_115	NP
		WP_018358636.1	99	orf_116	TonB-linked outer membrane proteins (OMP), lipid A biosynthetic process
c66324_g1_i1	8.9	WP_018357362.1	99	orf_135	Metabolic process
		WP_018357360.1	99	orf_136	Isoprenoid biosynthetic process
		WP_018357359.1	99	orf_137	DNA repair, DNA recombination
		WP_018357358.1	99	orf_138	tRNA wobble uridine modification, tRNA processing
		WP_018357357.1	99	orf_139	DNA repair
		WP_018357353.1	99	orf_140	NP
c234666_g1_i1	10.7	WP_025837040.1	89	orf_23	Gluconeogenesis
c68776_g2_i1	6.3	WP_018358196.1	83	orf_159	NP
c67025_g2_i1	8.6	WP_012858122.1	59	orf_146	Transport
		WP_007779376.1	59	orf_147	Peptide transport
		WP_012858118.1	78	orf_148	Transport

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