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# Bovine mastitis is a polymicrobial disease requiring a polydiagnostic approach

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## ABSTRACT

Bovine mastitis, an inflammation of the udder, is associated with increases in milk somatic cell count usually resulting from bacterial infection. We analysed 50 mastitic milk samples via cultivation, 16S rRNA sequencing and a combination of the two (culturomics) to define the complete microbial content of the milk. Most samples contained over 10,000 cfu mL$^{-1}$ total bacterial counts including isolates that were haemolysin positive ($n = 36$). Among colonies isolated from blood agar plates, *Streptococcus uberis* was dominant (11/50) followed by *Streptococcus dysgalactiae* (6/50), *Pseudomonas* (6/50), *Enterococcus faecalis* (6/50), *Escherichia coli* (6/50), *Staphylococcus argenteus* (4/50), *Bacillus* (4/50) and *Staphylococcus aureus* (2/50). 16S rRNA profiling revealed that amplicons were dominated by *Rhodococcus*, *Staphylococcus*, *Streptococcus* and *Pseudomonas*. A higher inter-sample diversity was noted in the 16S rRNA readouts, which was not always reflected in the plating results. The combination of the two methods highlights the polymicrobial complexity of bovine mastitis.

## 1. Introduction

Mastitis is an inflammation of the cow's udder and is a disease of high frequency and economic significance due to depleted milk production, discarded milk, premature culling and treatment costs (Bar et al., 2008; Halasa, Huijps, Osteras, & Hogeveen, 2007; Hertl et al., 2010). A large volume of milk is processed to a variety of dairy products and apart from the risk of bacterial contamination, alterations in the composition of mastitic milk can negatively affect the quality of these products (Merin et al., 2008). For example, it is known that the somatic cell count (SCC) level negatively correlates with cheese yield due to slower coagulation properties of the milk (Le Maréchal, Thiéry, Vautor, & Le Loir, 2011).

Mastitis can be classified into clinical or subclinical subgroups, with the latter being indicated by an escalation in SCC in the absence of overt symptoms (Vanderhaeghen et al., 2015). Milk is classified as being clinical or subclinical based on SCC, with a SCC of 200,000 cells mL$^{-1}$ generally being accepted as an indicator of the presence of mastitis infection (IDF, 1997) and the SCC threshold for milk purchasers being 400,000 cells mL$^{-1}$ according to EU regulations (Regulation (EC) No. 853 of 2004). Furthermore, mastitis-causing bacteria have been grouped as contagious or environmental based on their distribution and interplay with the teat and teat duct (Smith & Hogan, 1993). The disease is normally the result of bacterial intramammary infection, and the most commonly associated causative agents are staphylococci, streptococci and coliforms (Bradley, Leach, Breen, Green, & Green, 2007; Vanderhaeghen et al., 2015). However, up to 200 different microbial species have been documented in mastitic cases. These are primarily bacteria, but can include fungi or even monocellular achlorophylic algae (Cvetnić, Samardžija, Habrun, Kompes, & Benić, 2016).

Identification of the microbe driving the disease is of critical importance for clinical resolution. The gold standard method used for the characterisation of microorganisms responsible for mastitis is bacterial culture. Nevertheless, restrictions of culture-dependent techniques include a delay of 24–48 h to acquire results and the fact that roughly 25% of milk samples from clinical mastitis cases are culture negative (Taponen, Salmikivi, Simojoki, Koskinen, & Pyörälä, 2009). This highlights the

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importance of evaluating culture-independent techniques for mastitis diagnosis. It has been suggested that all mastitis treatments should be evidence-based, which primarily requires the identification of the mastitis-causing organism(s) (Milkproduction.com, 2007).

Sequencing and analysis of hypervariable regions within the 16S rRNA gene can furnish comparably expeditious and cost-effective methods for appraising bacterial diversity and abundance and has proved an effective tool for pathogen discovery and identification (Oikonomou, Machado, Santisteban, Schukken, & Bicalho, 2012). These technologies have enabled the investigation of microbial communities in milk without some of the limitations of culture methods (Ganda et al., 2016; Jimenez et al., 2015; Oikonomou et al., 2012). It should be noted that the resulting datasets are compositional (Gloor, Macklaim, Pawlowsky-Glahn, & Egozcue, 2017), failing to provide resolution to species/strain level and do not differentiate between living and dead microorganisms.

We employed both culture-dependent and culture-independent methods to identify the major pathogenic species found in milks collected from diseased animals.

## 2. Materials and methods

### 2.1. Sample collection

Fifty mastitic milk samples were collected from 46 cows which had elevated SCC ($\geq$200,000 cells mL$^{-1}$) during the period of November 2016 to April 2018. Samples were taken after the first streams of milk were discarded and stored below 4 °C, overnight until they were further processed. Aliquots of 1 mL of fresh milk were subject to cultivation within 24 h of donation. Remaining aliquots were immediately frozen at −20 °C for subsequent DNA extraction.

### 2.2. Determination of SCC

Milk samples were analysed for SCC using a Somacount 300 (Bentley Instruments, Inc., Chaska, MN, USA) according to the International Dairy Federation (IDF) guidelines (IDF, 1981).

### 2.3. Microbiological analysis

Clotted samples with high SCC (>10,000,000 cells mL$^{-1}$) were homogenised for thirty minutes with the use of a stomacher machine (IUL Instruments, SA) whereas the remaining samples were directly processed. Aliquots of milk sample, 1 mL, were mixed with 9 mL of maximum recovery diluent (Oxoid, Basingstoke, UK) to make an initial 10$^{-1}$ dilution. Serial dilutions were enumerated by the spread plate method in duplicate onto: (i) de Man, Rogosa, Sharpe (MRS) agar (Oxoid) at 37 °C (pH 5.5) for 3 days in anaerobic jars (gas-pack plus anaerobic system, BBL; BD Diagnostics, USA), which selects for lactobacilli; (ii) blood agar base (Oxoid) supplemented with 7% (v/v) defibrinated sheep blood (Cruinn Diagnostics, Ireland) at 37 °C for 48 h aerobically, which is a non-selective medium; (iii) Baird Parker agar (Oxoid) supplemented with 50 mL egg yolk tellurite emulsion (Oxoid) at 37 °C for 48 h aerobically, which selects for staphylococci; (iv) MacConkey agar (Oxoid) at 37 °C for 24 h aerobically, which selects for enterobacteria; (v) plate count agar (Oxoid) at 30 °C for 72 h, aerobically in which total mesophilic bacteria were counted. Plates were assessed for growth and colony morphology characteristics and the blood agar plates were subsequently analysed for haemolytic characteristics.

### 2.4. Species determination by Sanger sequencing

Colony PCR was performed on forty isolated colonies from blood agar plates and forty isolated colonies from Baird Parker plates per sample based on different morphology in the analysed samples (Supplementary material, Table S1). Cells were lysed in 10% Igepal 630 (Sigma–Aldrich, Germany) at 95 °C for 10 min. PCR was performed in a total volume of 25 μL using 10 μL Phusion Green Hot Start II High Fidelity PCR master mix (ThermoFisher Scientific, Waltham, MA, USA), 10 μL PCR-grade water, 1.5 μL of the nonspecific primers 27F and 1495R (primer stocks at 0.1 ng μL$^{-1}$) (Sigma–Aldrich) and 2 μL of DNA template from lysed cells. Amplification was carried out with reaction conditions as follows: initial denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s with a final extension step at 72 °C for 10 min. Five microlitres of the resulting amplicons from each reaction were electrophoresed in a 1.5% (w/v) agarose gel. A Gene-Genius Imaging System (Syngene, Cambridge, UK) was used for visualisation. The PCR products were purified using the GeneJet Gel Extraction Kit (ThermoFisher Scientific). DNA sequencing of the forward strand was performed by Source BioScience (Tramore, Ireland). The resulting sequences were used for searching sequences deposited in the GenBank database using NCBI BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) and the identity of the isolates was determined on the basis of the highest scores (>98%).

### 2.5. DNA extraction and MiSeq sequencing

DNA was purified from milk samples using the PowerFood Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) with slight modifications. Four mL of the milk samples were centrifuged twice at 4000 ×g for 30 min. The top fat layer was removed with a sterile cotton swab. The pellet was washed twice with sterile phosphate-buffered saline (PBS), re-suspended in 90 μL of 50 mg mL$^{-1}$ lysozyme (Sigma–Aldrich) and 25 μL of 10 KU mL$^{-1}$ mutanolysin (Sigma–Aldrich) and incubated at 55 °C for 15 min. Subsequently, 28 μL of proteinase K (Sigma–Aldrich) was added and the pellet was incubated at 55 °C for 15 min. The supernatant was removed after centrifugation at 13,000 ×g at 4 °C. The remaining steps were performed using the PowerFood Microbial DNA Isolation Kit according to manufacturer's instructions with the bead-beating time reduced to 3 min to limit DNA shearing. The microbiota composition of the samples was established by amplicon sequencing of a ~460 base pair (bp) fragment of the V3–V4 hypervariable region of the bacterial 16S rRNA gene following the Illumina 16S Metagenomic Sequencing Library Preparation guide. PCR amplification of V3–V4 region was performed using the forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and reverse primer 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAAC-3′. Each 30 μL PCR reaction contained up to 5 ng μL$^{-1}$ microbial genomic DNA, 6 μL of each primer (1 μM) and 15 μL Phusion High-Fidelity PCR Master Mix (ThermoFisher Scientific). The PCR conditions were as follows: initial denaturation for 30 s at 98 °C; 25 cycles of 10 s at 98 °C, 15 s at 55 °C and 20 s at 72 °C; and 72 °C for 5 min for final extension. The Agencourt AMPure XP system (Beckman Coulter, UK) was used to purify the amplicons. A subsequent limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters. Amplicons were quantified, normalised and pooled using the Qubit® dsDNA HS Assay Kit (Life Technologies, Carlsbad, California, USA). Library preparation was carried out by GATC Biotech prior to 2 × 300 bp sequencing on the Illumina MiSeq platform.

### 2.6. Bioinformatic analysis of high throughput sequencing data

Read quality was assessed using FastQC (v0.11.5) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) both before and after quality filtering with Trimmomatic (v0.32) (Bolger, Lohse, & Usadel, 2014) where a Phred quality threshold of 20 in a sliding window of size 4 was employed. The leading 15 bases of each read was removed followed by a crop at base 270, all reads greater than or equal to 50 bases in length were retained. Read pairs were merged using FLASH (v1.2.11) (Magoc & Salzberg, 2011) on default settings before being processed using VSEARCH in QIIME2 (v2018.8) (Bolyen et al., 2018). To do this, reads were dereplicated and clustered de novo at 97% forming OTUs. Chimeric reads were removed in two successive steps, both de novo and reference based against the ChimeraSlayer Gold database. Taxonomic classification was determined using mothur (v.1.38.0, bootstrap $\geq$ 80) (Schloss et al., 2009) and SPINGO (Allard, Ryan, Jeffery, & Claesson, 2015) (v1.3, bootstrap $\geq$ 0.8, similarity score $\geq$ 0.5) using the RDP v11.4 database. Analysis was performed using the R programming language (v3.5.1) (Ihaka & Gentleman, 1996) and visualised using ggplot2 (v3.1.0) (Wickham, 2009). Raw data has been made publicly available in the NCBI's Sequence Read Archive under the accession number: PRJNA509157.

## 3. Results and discussion

The aim of the study was to define the microbial composition of milks from mastitic cows using both cultivation and high throughput sequencing approaches. Fifty bovine milk samples with elevated SCC were analysed in this study, fluctuating between 221,000 and > 10,000,000 cells mL$^{-1}$ (Table 1). Based on microbiological cultivation, the majority of the samples contained isolates with haemolytic patterns with α-haemolysis being dominant (70% of the samples). β-Haemolytic bacteria were also detected in 40% of the samples, while γ-haemolysis was less common and found in 20% of the samples. Total mesophilic bacteria were enumerated at an average population of 5.92 log cfu mL$^{-1}$ on PCA, with four culture negative samples (M29, M32, M33, and M45). Comparable mesophilic counts were demonstrated by Dobranić, Kazazić, Filipović, Mikulec, and Zdolec (2016), who found up to 5.39 log cfu mL$^{-1}$ total mesophilic counts in bovine milk samples from animals cured of mastitis. The average population of presumptive lactic acid bacteria (LAB) grown on MRS was 4.30 log cfu mL$^{-1}$, similar to that reported by Qiao et al. (2015) who enumerated lactobacilli using quantitative PCR (qPCR) in 12 mildly subclinical milk samples and 28 severely subclinical milk samples. In the mild subclinical group (SCC <500,000 cells mL$^{-1}$), the mean counts were 4.83 log cfu mL$^{-1}$ whereas in the severely subclinical group (SCC >500,000 cells mL$^{-1}$), the mean counts for lactobacilli were 4.74 log cfu mL$^{-1}$.

High SCC does not always correlate with a high bacterial load. For example, while samples M7, M21, M37 and M49 had a SCC in excess of 5 million and were clotted in appearance, their total mesophilic counts were only 2.5, 2.4, 4.2 and 3.9 log cfu mL$^{-1}$, respectively. This could be due to a high load of uncultivable microorganisms in these samples. The identity of microorganisms isolated from blood agar and Baird Parker plates was determined by Sanger sequencing (Fig. 1, Supplementary Material Table S2 and Fig. 2, Supplementary Material Table S3, respectively). Colonies from blood agar plates were dominated by the genus *Streptococcus* (31.6%) and more specifically by *Streptococcus uberis* (18.2%) followed by *Streptococcus dysgalactiae* (11.1%), *Streptococcus agalactiae* (2.2%) and *Streptococcus urinalis* (0.1%). *St. uberis, St. dysgalactiae* and *St. agalactiae* are well-known mastitic pathogens (Klaas & Zadoks, 2018), while *St. urinalis* belongs to a subgroup of streptococci which cause urinary tract infections in humans and has not been associated with bovine mastitis until now (Peltroche-Llacsahuanga, Frye, & Haase, 2012).

Of the isolates from blood agar plates, 18.3% were staphylococci with a relatively even distribution of *Staphylococcus aureus* (3.9%), *Staphylococcus argenteus* (4%), *Staphylococcus sciuri* (3.3%) and *Staphylococcus chromogenes* (2.9%). *S. aureus* is a well-established mastitis pathogen both in cows and humans while *S. argenteus* is a relatively novel species (Tong et al., 2015) that has been isolated from human infections (Jiang et al., 2018), but not from bovine mastitis until now. The results identifying strains as *S. argenteus* were inconclusive as to whether they were *S. argenteus* or *S. aureus*. *S. sciuri* and *S. chromogenes*, both coagulase-negative staphylococci, have been previously isolated in bovine mastitis studies (dos Santos et al., 2016; Hosseinzadeh & Dastmalchi Saei, 2014). Members of *Escherichia*, *Enterococcus* and *Pseudomonas* were identified at comparable frequencies of 9.7%, 8.9% and 8.2%, respectively. All *Escherichia* isolates were *Escherichia coli* while almost 98% of the *Enterococcus* belonged to *Enterococcus faecalis*. *E. coli* has been identified as one of the major mastitis-causing pathogens (Luoreng, Wang, Mei, & Zan, 2018; Vasquez et al., 2019), while enterococci have also been frequently isolated from mastitic cows (Gomes, Saavedra, & Henriques, 2016). Thirteen species of *Pseudomonas* were detected (see Fig. 1), but none was identified as *Pseudomonas aeruginosa*, a microbe that is often detected in mastitis (Park et al., 2014). *Pseudomonas lactis*, *Pseudomonas paralactis* (von Neubeck et al., 2017) and *Pseudomonas weihenstephanensis* (von Neubeck et al., 2016) have been previously isolated from cows' milk. *Kocuria* (3.2%), which is usually found in skin and mucous membranes of humans and animals and is an emerging cause of infection (Kandi et al., 2016), was also detected on blood agar plates together with *Trueperella pyogenes* (2.2%), which has been associated with summer mastitis (Pyörälä, Jousimies-Somer, & Mero, 1992).

Due to the semi-selective nature of the media, isolated colonies from Baird Parker agar plates were predominantly identified as *Staphylococcus*, particularly as *S. argenteus* (19.5%), *S. aureus* (19.5%), *S. chromogenes* (11.8%), *Staphylococcus epidermidis* (8.3%) and *Staphylococcus haemolyticus* (7.4%) (Fig. 2, Supplementary Material Table S2).

It is broadly acknowledged that many bacteria are not cultivable on standard microbiological media under standard conditions (Kamagata & Tamaki, 2005; Sekiguchi, 2006) and so in parallel we applied high throughput sequencing to characterise uncultivated microbiota (DeLong, 2005).

MiSeq sequencing of 16S rRNA amplicons from bovine mastitic milk samples yielded a total of 14,319,524 quality filtered reads, with a median read length of 234 ± 53 bases. Following quality control, we recorded an average of 286,391 reads per sample. At phylum level, Actinobacteria had the highest relative abundance in 38% of the bovine mastitic milk samples, while Firmicutes which were most abundant in 36% and Proteobacteria in 24% (Fig. 3).

At genus level *Rhodococcus* was the most abundant in most samples (38%), followed by *Pseudomonas* (16%), *Streptococcus* (12%) and *Staphylococcus* (8%) (Fig. 4, Supplementary Material Table S4).

The culture-based and metagenomics approaches displayed considerable divergence in their output. For example, approximately 50% of Miseq samples had high levels of *Rhodococcus* while three samples had high levels of *Acinetobacter,* but we did not detect either genus using culture-dependent methods (discussed in detail below). Based on the results of both methods, we categorised our samples in four groups: Group 1 (M7, M9, M10, M13, M15, M22, M25, M30, M34 and M40) consisted of ten samples for which both 16S rRNA sequencing and 16S Sanger sequencing from blood agar plates resulted in the detection of the same dominant genus. More specifically, samples M7, M9 and M13 were dominated by

**Table 1**
Heat map of the microbial load in fifty bovine mastitic milk samples.[a]

| Samples | SCC | Blood agar | | | McC | BP | PCA | MRS |
|---|---|---|---|---|---|---|---|---|
| | | α-haemolysis | β-haemolysis | γ-haemolysis | | | | |
| M1 | >10,000,000 | 4.6 | | | 4.6 | 5.5 | 6.0 | 4.4 |
| M2 | >10,000,000 | 5.0 | | | | 4.9 | 5.4 | 5.0 |
| M3 | >10,000,000 | 5.4 | | | 4.9 | 2.0 | 5.6 | 5.3 |
| M4 | 3,343,000 | 2.8 | | | 1.8 | 1.5 | 3.7 | 1.9 |
| M5 | 324,000 | 2.5 | | | 1.6 | 1.0 | 4.2 | 2.6 |
| M6 | 3,917,000 | | | 2.0 | 2.0 | | 4.0 | 3.0 |
| M7 | 5,707,000 | | 1.7 | 1.5 | 2.4 | | 2.5 | 2.8 |
| M8 | 346,000 | 3.5 | | | 2.5 | 3.0 | 3.8 | 2.5 |
| M9 | 700,000 | | 2.0 | | 4.4 | 1.5 | 3.5 | 2.8 |
| M10 | 9,422,000 | 5.0 | | | 4.2 | 3.8 | 4.3 | 5.7 |
| M11 | 8,115,000 | 5.7 | | | | | 5.2 | |
| M12 | 644,000 | | | | 5.3 | 5.0 | 5.2 | 7.2 |
| M13 | 221,000 | 5.2 | | 1.0 | 4.6 | 5.5 | 6.3 | 3.9 |
| M14 | 4,330,000 | 3.0 | 1.0 | 1.2 | 1.4 | 1.5 | 7.4 | 6.6 |
| M15 | 2,502.000 | 3.1 | | 4.5 | 4.1 | | 4.6 | 3.5 |
| M16 | 9,999,000 | 0.5 | 2.0 | 4.4 | 0.6 | 5.0 | 5.0 | 1.9 |
| M17 | 809,000 | | | | 3.1 | 5.2 | 6.3 | 4.0 |
| M18 | 1,247,000 | 8.3 | | | 8.3 | 5.5 | 9.5 | 1.8 |
| M19 | 2,607,000 | 9.0 | | | 7.3 | 4.6 | 8.3 | 5.0 |
| M20 | >10,000,000 | 6.5 | | | 5.2 | 4.1 | 9.5 | 4.0 |
| M21 | >10,000,000 | 2.8 | 2.3 | | 1.0 | 2.4 | 4.9 | |
| M22 | >10,000,000 | 8.1 | | | | | 7.7 | |
| M23 | >10,000,000 | 4.5 | | | 4.5 | | 4.1 | |
| M24 | >10,000,000 | 7.1 | | | 4.0 | | 6.8 | |
| M25 | >10,000,000 | 8.9 | | | 3.7 | 6.1 | 8.9 | |
| M26 | >10,000,000 | 5.6 | 5.4 | | 5.5 | 4.5 | 5.2 | |
| M27 | >10,000,000 | 5.0 | 4.6 | | 4.4 | 4.5 | 5.9 | |
| M28 | >10,000,000 | 6.0 | | 5.1 | 4.6 | 3.0 | 6.1 | |
| M29 | 592,000 | | | 3.0 | | 3.0 | | |
| M30 | >10,000,000 | 5.9 | | | 4.9 | | 5.7 | |
| M31 | >10,000,000 | | | 7.2 | 6.9 | | 7.9 | 6.4 |
| M32 | >10,000,000 | 8.8 | | | 4.5 | 3.5 | | 5.1 |
| M33 | 8,181,000 | | 6.3 | | | 3.0 | | |
| M34 | >10,000,000 | | 5.8 | | | | 6.1 | 3.3 |
| M35 | 934,000 | 4.6 | | | 4.7 | 5.3 | 4.1 | 3.6 |
| M36 | 1,061,000 | | 4.1 | | 4.6 | 4.1 | 4.3 | 4.1 |
| M37 | >10,000,000 | | | | 3.1 | | 4.2 | 4.4 |
| M38 | >10,000,000 | | 4.7 | | 4.4 | | 4.5 | 4.3 |
| M39 | >10,000,000 | 5.6 | 4.5 | | 6.3 | 5.4 | 6.3 | 4.1 |
| M40 | >10,000,000 | 7.2 | 6.2 | | 7.5 | 5.3 | 6.8 | 4.6 |
| M41 | >10,000,000 | 6.1 | | 8.2 | 7.7 | 5.9 | 6.9 | 5.2 |
| M42 | >10,000,000 | 7.1 | 7.0 | | 7.3 | 7.3 | 7.0 | 5.1 |
| M43 | >10,000,000 | | | | 5.8 | 6.7 | 7.5 | 6.4 |
| M44 | >10,000,000 | 6.4 | 5.7 | | 7.5 | 2.0 | 7.1 | 3.7 |
| M45 | >10,000,000 | 6.4 | 6.5 | | 5.8 | 2.2 | | 5.0 |
| M46 | >10,000,000 | | 8.1 | | 2.6 | | 7.3 | 5.5 |
| M47 | >10,000,000 | 4.6 | 3.3 | | 4.8 | 3.9 | 5.6 | |
| M48 | >10,000,000 | 6.2 | | | 5.6 | 2.2 | 6.8 | 3.8 |
| M49 | >10,000,000 | | 3.8 | | 4.0 | 4.2 | 3.9 | |
| M50 | >10,000,000 | 5.6 | 6.8 | | | 2.0 | 7.1 | 6.2 |

[a] SCC are also shown. Colour intensity corresponds to the microbial load on various media. Red represents bacterial numbers approaching 10 log cfu $mL^{-1}$ and white represents absence of microbial growth in the tested media.

*Staphylococcus* whereas in samples M10, M15 and M25, the main genus detected was *Streptococcus*. Sample M22 and M40 were dominated by *Pseudomonas*, sample M34 was dominated by *Trueperella*, and sample M30 was dominated by *Escherichia*. However, this was not the case for the other samples. Group 2 consisted of 17 samples (M1, M11, M14, M23, M24, M26, M27, M31, M32, M38, M39, M44–M46 and M48–M50) displaying few similarities between the two data sets while the 18 samples in Group 3 displayed no similarities (M2–M6, M8, M16, M18–M21, M28, M29, M33, M36, M41, M42 and M47). Finally, Group 4 (M12, M17, M34, M35 and M37) comprised of 5 samples that did not give rise to colonies on blood agar plates.

Of 36 genera detected in the bovine mastitic milk samples by 16S rRNA analysis, only 8 were found in the culture-dependent analysis; namely *Staphylococcus*, *Bacillus*, *Carnobacterium*, *Escherichia/Shigella*, *Enterococcus*, *Streptococcus*, *Trueperella* and *Pseudomonas*. Moreover, from the culture-based approach we detected *Barnesiella* sp., *Kocuria*, *Microbacterium and Raoultella* sp., which were not detected in the 16S rRNA analysis. Aerococci were detected in higher percentages in Blood agar plates than via

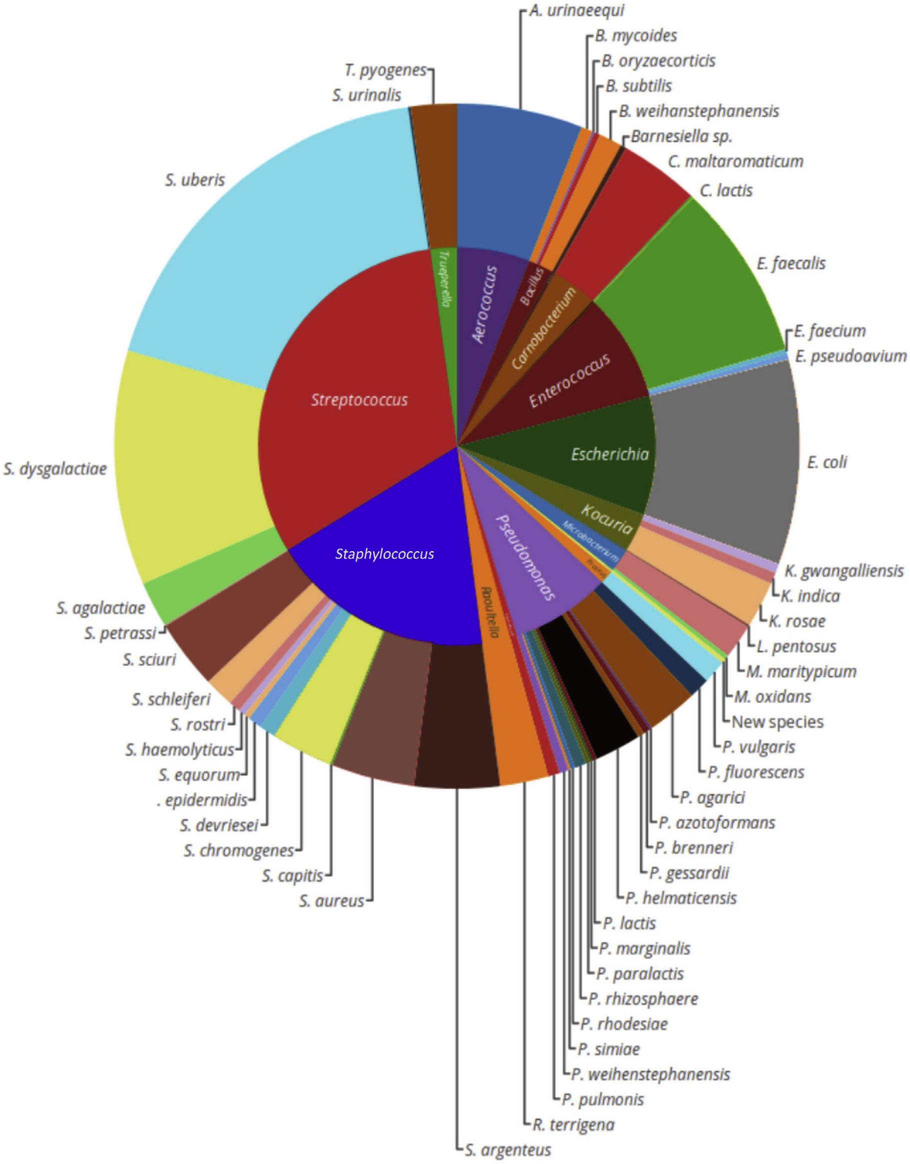

**Fig. 1.** Average species identification on blood agar from fifty mastitic milk samples. Inner circle depicts genus and outer circle indicates species. Results depicting *S. argenteus* were inconclusive as to whether it was *S. argenteus* or *S. aureus.*

sequencing. It should be emphasised that 16S rRNA profiling only provides relative abundances and is not quantitative, albeit that *Corynebacterium* was found in similar relative abundances using the two methods. Furthermore, no colonies were obtained from samples M7, M11 and M24 on Baird Parker plates, even though staphylococci were found on blood agar plates for M7 and M11. This may demonstrate differences in nutritional requirements. 16S rRNA profiling indicated relative abundances for *Staphylococcus* at 83.81% (M7), 0.01% (M11) and 5.27% (M24).

*Pseudomonas* was detected in 6 samples (M1, M4, M5, M22, M41 and M47) by both cultivation and Sanger Sequencing, while it was predominant in the 16S profiling in 9 other samples (M18, M22, M31, M39, M40, M43, M44, M46 and M48). The detected differences could be due to the fact that *Pseudomonas* either could not grow in the cultivation conditions used or that it was there in different amounts, albeit 16S rRNA profiling only provides relative abundances and does not differentiate between viable and non-viable bacteria. *Pseudomonas* has been found in previous studies in raw milk (von Neubeck et al., 2015), bulk tank milk (Rodrigues, Lima, Canniatti-Brazaca, & Bicalho, 2017) and is a member of the healthy core microbiome in human milk (Murphy et al., 2017). Nevertheless, individual cases or sporadic outbreaks of mastitis may be caused by *Pseudomonas* sp., *T. pyogenes, Serratia* sp., or other unusual pathogens (Harmon, 1994). *Pseudomonas* has also been associated with water contamination, including purified water systems (Kuehn et al., 2013; Ryan, Pembroke, & Adley, 2011). Water could be a significant source of microbial contamination considering that modern milking practices depend heavily on water for cleaning milking units. Indeed, mastitis caused by *P. aeruginosa* has been previously linked with contamination of water systems and teat disinfectants in the milking parlour (Kirk & Bartlett, 1984).

Eighteen out of fifty samples were dominated by *Rhodococcus* according to 16S rRNA profiling. In particular, 8 samples (M5, M6, M16, M21, M23, M29, M36 and M37) had over 80% *Rhodococcus* and were accompanied by low total mesophilic counts (up to 5 log cfu mL$^{-1}$). *Rhodococcus* was not detected from the colonies grown on Blood agar plates and it is likely that the species detected by the 16S

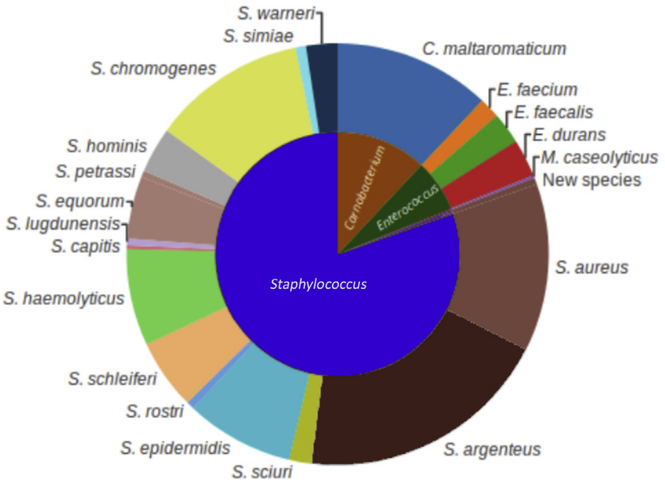

**Fig. 2.** Average species identification on Baird Parker agar plates from fifty mastitic milk samples. Inner circle depicts genus and outer circle indicates species. Results depicting *S. argenteus* were inconclusive as to whether it was *S. argenteus* or *S. aureus.*

rRNA sequencing were either anaerobic or could not grow under the conditions used in this study. *Rhodococcus* was previously misidentified in bovine mastitis milk samples as *Corynebacterium bovis* (Watts, Lowery, Teel, & Rossbach, 2000) while *Rhodococcus equi* was identified as the causative agent in an immunocompromised woman with granulomatous mastitis (Nath, Mathew, Mohan, & Anila, 2013). One study has identified *Rhodococcus* sp. as a causative agent in 4 out of 65 paired milk samples, collected from mastitic and healthy quarters of diseased dairy cows (Oultram, Ganda, Boulding, Bicalho, & Oikonomou, 2017).

Based on 16S rRNA profiling, 5 samples (M4, M10, M14, M20 and M47) were dominated by streptococci (ranging from 79.93 to 99.68%), however, three of those samples (M4, M20 and M47) were negative for streptococci on blood agar plates, possibly due to their anaerobicity. In other studies, streptococci not only have been linked with high SCC milk samples (Park et al., 2007; Rodrigues et al., 2017; Zanardi et al., 2014), but they were also found in the healthy core microbiome of bovine (Quigley et al., 2013) and human milk (Murphy et al., 2017).

16S rRNA profiling identified *Trueperella* in 5 samples (M27, M31, M33, M34, M48 and M49 at 0.1%, 4.1%, 21.4%, 49.5% and 0.7%, respectively). Sample M34 was dominated by *Trueperella,* and *T. pyogenes* was the only member of this genus cultured. *T. pyogenes* has been shown to act synergistically with anaerobic bacteria, namely *Fusobacterium necrophorum*, *Bacteroides* sp, *Porphyromonas levii* in summer mastitis (Pyörälä et al., 1992). Oikonomou et al. (2012) found that milk samples which were diagnosed as *T. pyogenes* mastitis, had a high prevalence of *F. necrophorum* subsp. *funduliforne*. We were unable to confirm this finding.

Samples M2, M8 and M19 were dominated by *Acinetobacter*. *Brochothrix* and *Pseudomonas* were detected in all three samples by 16S rRNA profiling. In sample M8, clostridia were detected while in sample M19 *Bacillus* was identified. Patel et al. (2017) demonstrated that 18 healthy mothers were rich in *Acinetobacter* compared with women with mastitis. Moreover, Kable et al. (2016) showed that *Acinetobacter* belongs to the core milk microbiota while Quigley et al. (2013) reported that *Acinetobacter* is often found in raw milk. *Acinetobacter* has the ability to adapt to various environmental conditions and several emerging pathogens have been described (Gurung et al., 2013). *Acinetobacter* is also known to cause spoilage (Hantsis-Zacharov & Halpern, 2007) and is rarely a primary cause of mastitis (Oliver & Murinda, 2012).

Five samples contained *Escherichia/Shigella* at genus level (M15, M26, M30, M38 and M48 at 40.6%, 20.4%, 50.2%, 10.8% and 29.6% respectively) which agreed with higher abundances of Enterobacteriaceae at family level and high abundances of Proteobacteria (Madigan, Bender, Buckley, Sattley, & Stahl, 2018). These findings are in agreement with previously published studies (Ganda et al., 2016; Lima, Bicalho, & Bicalho, 2018; Vasquez et al., 2019) which also found the same pattern. *E. coli* was not detected in aerobic culture in sample M15, while for sample M26 only 25% of the colonies were identified *as E. coli*. Nonetheless, *E. coli* was the only bacterium recovered from samples M30 and M38 on blood agar plates.

16S rRNA profiling of samples M30 and M38 revealed a large diversity of taxonomic families, which is in accordance with previous characterisation of the microbiota of mastitic and healthy human and bovine milk, a finding that supports the possibility of an entero-mammary pathway (Angelopoulou et al., 2018; Perez et al., 2007). This is a pathway in which bacteria from the gastrointestinal

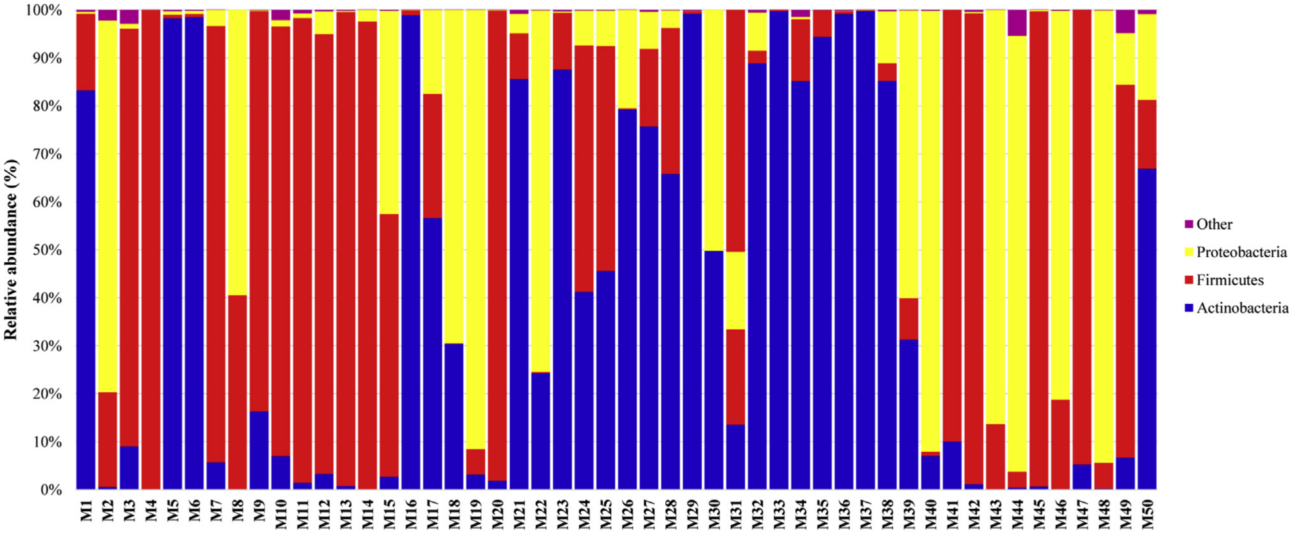

**Fig. 3.** Phylum level assignments of average relative abundances of the microbiota in fifty bovine mastitic milk samples. Phyla with abundances below 1% are grouped as "Other".

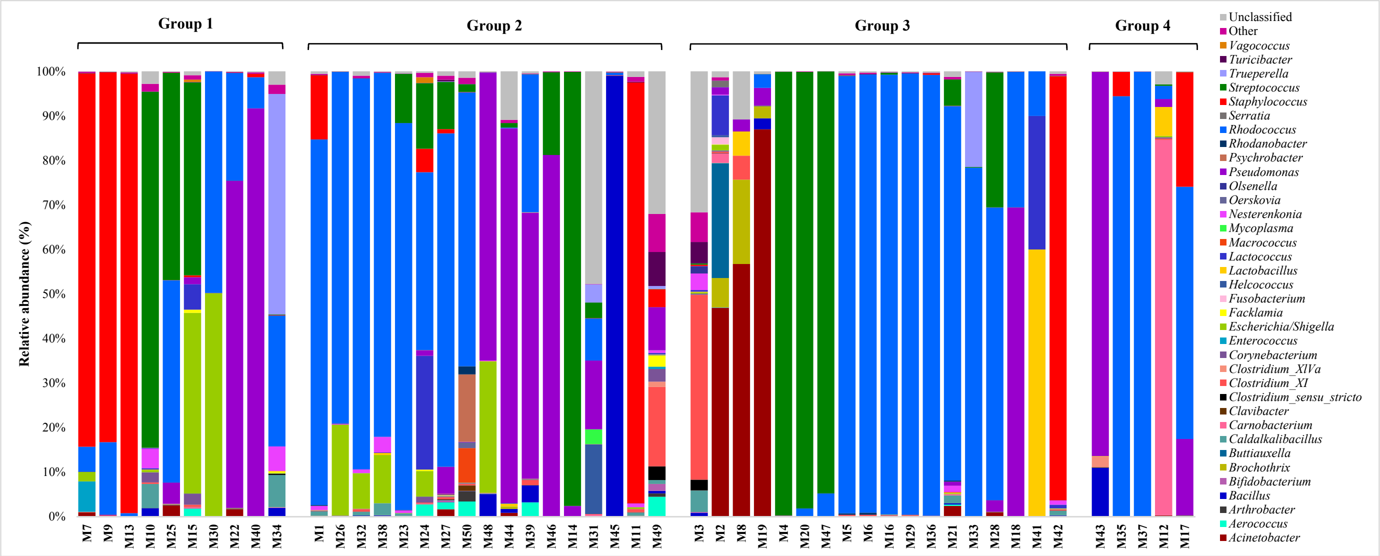

**Fig. 4.** Genus level assignments of average relative abundances of the microbiota in bovine mastitic milk samples. Bacterial genera under 1% are grouped as "Other". The samples are represented in 4 groups based on level of similarity between genus level assignments of relative abundances and results from 16S Sanger sequencing on blood agar plates. Group 1 is comprised of samples where in both cases the dominant genus is identified. Group 2 displays some similarities, group 3 has no similarities between the 2 data sets and group 4 did not show any growth on blood agar plates.

lumen reach the mammary gland with the help of dendritic cells and CD18$^+$ cells (Macpherson & Uhr, 2004; Rescigno et al., 2001). We detected many families in mastitic milk that are normally present in the gastrointestinal tract (GIT), such as Ruminococcaceae, Clostridiaceae, Peptostreptococcaceae and Lachnospiraceae. This is consistent with findings from normally sampled quarters and samples acquired via cannula (Ganda et al., 2016; Jost, Lacroix, Braegger, Rochat, & Chassard, 2014; Oikonomou et al., 2014; Pang et al., 2018; Vasquez et al., 2019; Young, Hine, Wallace, Callaghan, & Bibiloni, 2015). Members of these families have been previously detected in samples from different anatomical parts of the bovine GIT (Lima et al., 2015; Mao, Zhang, Liu, & Zhu, 2015). Ruminococcaceae, Clostridiaceae, Peptostreptococcaceae and Lachnospiraceae were also identified in faecal matter from cows; making it possible that their presence represents either contamination of samples or translocation into the udder (Young et al., 2015).

It is obvious that there are limitations to both culture-based and culture-independent diagnostics. Not all organisms causing infection can be cultivated and/or are recovered on culture while 16S rRNA compositional profiling does not provide sufficient resolution to pinpoint particular species and/or strains and furthermore, cannot differentiate between live and dead bacteria. Additionally, practical considerations such as price, time and labour intensity will influence the choice of method. On one hand, culturing bacteria is laborious, has a set price per sample (effect of sample number if limited) and can take up to a week to get results. On the other hand, 16S compositional sequencing is less laborious, its price per sample can be greatly affected by number of samples and can be very time consuming (outsourced sequencing usually takes 6 weeks, followed by data analysis). Nevertheless, metagenomic approaches are increasingly applied to acquire a detailed picture of the bacteria involved in the pathogenesis of mastitis.

Both strategies are almost certainly compromised by the low microbial biomass of most milk samples (and other types of samples). Salter et al. (2014) and Glassing, Dowd, Galandiuk, Davis, and Chiodini (2016) have both pointed out the potential for incorrect results in low biomass samples due to contamination or other artefacts that could lead to the discrepancies observed between the different studies examining the mastitic bovine milk microbiota (Kuehn et al., 2013; Lima et al., 2018; Oikonomou et al., 2012, 2014; Oultram et al., 2017; Pang et al., 2018; Rodrigues et al., 2017; Vasquez et al., 2019). However, we feel that this is less likely to be an issue in this study given that the majority of samples have a relatively high bacterial load. Storage conditions are another factor that should be taken into consideration as all samples were stored below 4 °C, overnight until they were further processed. However, psychrotrophs such as *Pseudomonas* sp. could proliferate at low temperature, complicating the interpretation of the finding that 16% of samples were dominated by *Pseudomonas*. However, it should be emphasised that *Pseudomonas* has been found in the core microbiome of healthy human milk samples (Murphy et al., 2017) and therefore it remains possible for samples to be dominated by *Pseudomonas* at the time of sampling. Moreover, *S. aureus*, the main causative agent of bovine mastitis in Ireland has a temperature range for growth of 7–48 °C and so numbers should not increase on refrigeration. As storage conditions could influence the results of both culture-dependent and culture-independent methods, it is essential to minimise effects of sample handling in particular collection method, time until sample processing and sample storage.

We identified the microbiota composition of fifty bovine mastitic milks using both culture-dependent and independent approaches with 20% (n = 10) of the tested samples giving similar outputs (Group 1). Group 2 displayed few similarities when 16S rRNA profiling was compared with culturing. Group 3 consisted of samples for which the two approaches were inconclusive and Group 4 samples gave no growth on Blood agar plates, indicating that the culture conditions used were not appropriate for the bacteria present in these samples. A high inter-sample diversity was noted in the 16S rRNA profiling, which was not always reflected in plating results. Thus, we suggest that the combination of the two methods sheds light into the microbial complexity of the disease and that symptoms might be driven or exacerbated by more than one insulting organism.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.104539.

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