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Research Article

Metagenomic Profiling of Bovine Milk from Mastitis Infected Udder of the Cows before and after Treatment with Ethno-Veterinary Practice (EVP)

Santhosha Hegde[1], Pavithra Narendran[1], Malali Gowda[2] and M N Balakrishnan Nair[1]*

[1]*University of Trans-Disciplinary Health Sciences and Technology (TDU), Yelahanka, Bengaluru, India*

[2]*DNA Life, Yelahanka, Bengaluru, India*

***Corresponding Author:** M N Balakrishnan Nair, Professor, Centre for Ethno-Veterinary Science and Practice, University of Trans-Disciplinary Health Sciences and Technology (TDU), Yelahanka, Bengaluru, India.

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Abstract

This study presents the changes in the microbiome of milk from the cows with clinical mastitis before and after treatment with Ethno-veterinary herbal formulations. The mastitis was confirmed with CMT (California Mastitis Test). Besides presence of several species of bacteria, the mastitis causing bacteria were abundant before treatment. After 6 days of treatment with herbal formulations, the average abundance of *Staphylococcus* was reduced from 40.59% to 2.03% *(*20 times)*, Streptococcus* from 25.8% to 2.06% (12.52 times), *Pseudomonas*, *Pseudomonaceae* family 20.28% to 1.9% (10.67 times), *Klebsiella* from 8.4% to 0.26% (32.31 times) and *Enterobacteriaceae family* from 24% to 1.69% (14.37 times) indicating the cure of mastitis.

Keywords: *Mastitis; Metagenomic DNA; Ethno-Veterinary Practice*

Introduction

Bovine mastitis affects a large number of dairy cattle throughout the world. Prevalence of mastitis continues to remain as the most challenging disease and it is increasing among the cattle with higher production of milk. Mastitis is an inflammation of the udder generally caused by various microbes. Mastitis is the most common disease which affects dairy production world-wide [1]. Clinical mastitis infection is relatively easy to diagnose with the naked eye. However, subclinical mastitis cannot be as easily detected due to the absence of any visible indications. Although different varieties of bacteria were identified as causative agents of mastitis, only a few species of *Staphylococci, Streptococci* and Coliforms can create serious problems [2]. Even though bacteria are the main cause of mastitis, other organisms like *Mycoplasma californicum* and *Mycoplasma canadense* [3], viruses, and fungi might also be associated with the disease process [4,5]. Mastitis therapy protocol includes antibiotics. However, different treatment regimens and control strategy is necessary for different mastitis organism. The misuse of antibiotics led to development of the Antimicrobial resistance (AMR). Drug resistance bacteria in different regions of the world are creating threat to public health [6]. Antibiotic also find their way into the food chain as residue in the animal products such as milk and meat.

It is reported that the Ethno-veterinary herbal formulation can be used to combat mastitis in livestock and reduces the use of antibiotics [7-9]. *In-Vitro* antimicrobial activity of the extracts of the herbal formulation against mastitis had inhibitory activity against *Escherichia coli* and *Staphylococcus aureus* [9]. Clinical study using traditional formulation for Mastitis showed that somatic cell counts (SCC),

electrical conductivity (EC) and pH of the milk become normal within 6 days indicating cure of mastitis [7]. *In silico* approach bioactive compounds were tested for its effect against the target proteins of *S. aureus* using molecular docking studies [8]. It has been shown that traditional medicine can be used during dry period to reduce the incidence of mastitis [10]. The objective of this study is to compare the milk microbiome of cows with clinical mastitis before and after treatment with the ethno-veterinary practice based on herbal formulation. The genus level abundance of bacteria which causes mastitis before and after EVP treatment can indicate the effectiveness of the herbal formulation for cure of mastitis.

Methods such as real-time PCR [11-13], multiplex PCR (mPCR) [2] and denaturing gradient gel electrophoresis (DGGE) PCR [14] have been used to identify bacterial DNA in milk samples and which does not requires the culturing of individual bacteria. Currently with the advent of next generation sequencing technology, the sequencing of the 16S rRNA gene would help us to identify the entire commensal and pathogenic bacterial community which can overcome the limitations of the culture-based bacterial detection method [15].

Materials and Methods

Sample collection: Enrollment criteria and treatments

Three cows from Aluva, Kerala state were selected for this study. CMT tests were conducted to confirm mastitis milk. Summary of collection of samples including the date of collection is given in table 1. Milk samples were collected from each quarter separately and numbered as follows; F1- Front left, F2 - Front right, B3 - Hind left, B4 - Hind right (Figure 1). Samples were collected before the starting of treatment, after 3 and 6 days of treatment (4 quarters of 3 Mastitis affected udder - 12 samples, After 3 days of EVP treatment 3 cows 4 quarters each - 12 samples, after 6 days of EVP treatment - 3 cows 4 quarter each - 12 samples. A total of 36 milk samples were collected (Table 1) from these treatments. The collected samples were placed in liquid nitrogen/dried ice and then transported to the lab.

No	Item	Date of collection	F1	F2	B3	B4	Total samples
1	Mastitis (CMT positive) before starting the treatment (3 cows)	15/02/2019	3	3	3	3	12
2	After 3 days of EVP treatment (3 cows)	19/02/2019	3	3	3	3	12
3	After 6 days of EVP treatment (3 cows)	21/02/2019	3	3	3	3	12

Table 1: *Summary of collection of Samples including the date of collection.*

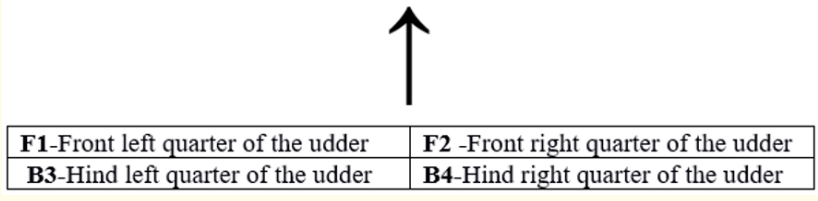

Figure 1: *Numbering of milk samples collected from each quarter of the udder.*

EVP treatment

Three cows were treated with an oil based emulsified herbal formulations. The formulation is sprayed on the udder after milking for 3 times a day for 5 days. Two lemons were also fed to the animal 3 times per day for 5 days.

DNA Isolation and PCR amplification of V3 and V4 region

Metagenomic DNA was extracted from milk samples and EVP formulation using Qiagen DNeasy Powersoil Metagenomic DNA extraction kit. The quality of Metagenomic DNA was checked on 0.8% agarose gel and quantified using Nanodrop. 25 ng of metagenomic DNA was used to amplify 16S rRNA hyper variable region V3-V4. The reaction includes KAPA HiFi Hot Start Ready Mix and 100 nm final concentrations of modified 341F and 785R primers. The PCR involved an initial denaturation of 95°C for 5 min followed by 30 cycles of 95°C for 30s, 55°C for 45s and 72°C for 30s and a final extension at 72°C for 7 min. The amplicons were purified using Ampure beads to remove unused primers.

Library preparation and sequencing

The purified 16S rRNA amplicons were used in library preparation. Eight cycles of PCR was performed using Illumina barcoded adapters to prepare the sequencing libraries using Nextera® XT DNA Index Kit. The libraries were quantified using Qubit DNA HS quantification assay. The sequence data was generated in fastq format using Illumina MiSeq. Data quality was checked using FastQC. The data was checked for basecall quality distribution, % bases above Q20, Q30, %GC, and sequencing adapter contamination.

Availability of data: Raw data of all 36 samples in fastq format has been deposited in NCBI Sequence Read Archive (SRA). Bio-project accession number is PRJNA480376.

Data analysis

The quality of raw reads of Illumina sequencing was checked for the ambiguous bases, Phred score > Q30, read length, nucleotide base content and other parameters by using FASTQC toolkit [16]. The quality checked raw data was uploaded into the open-source pipeline Quantitative Insights into Microbial Ecology (QIIME) version 1.9.1 [17]. Sequences were filtered based on quality (Phred score > 20) and merged using fastq-join [18]. The merged reads were assigned to operational taxonomic units (OTUs) with 97% identity using UCLUST to identify microbial community [19]. These OTU's were further used for taxonomic assignment using Greengenes database 13.8 [20] as a reference. The constructed OTUs were used to get bacterial abundance in each sample.

Results

Diagnosis of mastitis and sample collection results

The milk from all quarters of the 3 selected cows was CMT positive. The CMT showed positive results after 3 days of EVP treatment in B3 quarter in (EVP-1), F2, B3 quarters in EVP2 and, F2 and B4 quarters in (EVP3). However, after 6 days of treatment all the quarters showed negative result (Supplementary 1).

Microbial abundance estimation

Total of 6,920,244 paired end reads with an average read length of 300 bases were generated for 36 samples. These reads were considered for further analysis in QIIME. The most abundant microbial phyla (average abundance > 0.1%) are shown in figure 2.

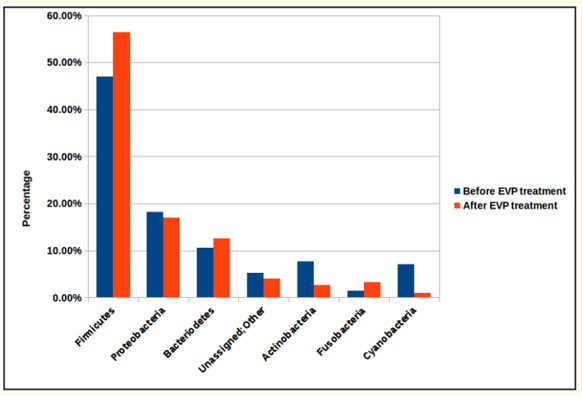

Figure 2: *Phylum level bacterial abundance in mastitis milk before and after Ethno-veterinary practice treatment.*

Genus level bacterial abundance before EVP and after treatment

The bacterial abundance in the milk from the mastitis affected cows before treating with herbal formulation, after 3 and 6 days of treatment is given in table 2-4. In the milk samples has prevalent mastitis causing most abundant genera including *Staphylococcus, Streptococcus, Enterobacteriaceae family, Pseudomonaceae family and Enterococcaceae family* before treatment with EVP. There is an increase in the abundance of *Enterobacteriaceae* after 3 days of treatment with herbal formulation in F1, F2, B3 quarters of the cow No 1 and F1 quarter in cow No 3 (Table 2 and 4). *Pseudomonas* also showed an increased abundance in F1 quarter in cow No 1 (Table 2) and cow No3 (Table 4). *Streptococcus* increased in B4 quarter of cow No 3 (Table 4) after 3rd day of treatment with herbal formulation. However, the abundance mastitis causing *Staphylococcus, Streptococcus, Pseudomonaceae, Pseudomonas and Enterobacteriaceae (includes E. coli)* have reduced after 6 days (Table 2-4) indicating the cure of mastitis. The abundance of *Staphylococcus, Streptococcus* before and after treatment is shown in figure 3.

Bacteria - Genus	Before EVP Treatment	After 3 days of Treatment	After 6 days of Treatment	Quarter
Enterobacteriaceae family	**4.8%**	**22.6%**	**1.5%**	**F1**
Lachnospiraceae family	3.8%	-	11%	F1
Lactobacillus	5.3%	-	4.3%	F1
Lactococcus	7.2%	24.5%	4.3%	F1
Neisseria	5.8%	0.02%	4.3%	F1
Pseudomonas	**2.3%**	**42.7%**	**1.9%**	**F1**
Ruminococcus	7.86%	-	6.6%	F1
Staphylococcus	**5.1%**	**0.18%**	**4.3%**	**F1**
Streptococcus	**2.17%**	**0.02%**	**2.8%**	**F1**
Acinetobacter	11.4%	1.1%	1.3%	F2
Bacteroidales family	4%	5.42%	12.13%	F2
Enterobacteriaceae family	**0.3%**	**39%**	**2.84%**	**F2**
Lactobacillus	0.14%	3%	5.5%	F2
Neisseria	0.2%	1.9%	4.4%	F2
Ruminococcus	0.3%	3%	4.4%	F2
Staphylococcus	**59.3%**	**5.8%**	**5.4%**	**F2**
Enterobacteriaceae family	**9.4%**	**36%**	**2.5%**	**B3**
Klebsiella	**-**	**3.9%**	**0.2**	**B3**
Lactococcus	3.3%	2.8%	4.6%	B3
Neisseria	2.6%	2.8%	4.6%	B3
Pseudomonaceae family	**17.6%**	**Nil**	**Nil**	**B3**
Ruminococcus	0.2%	2.1%	6.3%	B3
Staphylococcs	**8.8%**	**4.2%**	**5%**	**B3**
Streptococcus	**3.5%**	**1.9%**	**3.1%**	**B3**
Acinetobacter	1.5%	6%	0.3%	B4
Aeromonadaceae family	-	9.3%	-	B4
Bacteroidales order	15%	4.1%	10.2%	B4
Clostridiales family	4%	-	-	B4

Enterobacteriaceae family	**1.6%**	**37.7%**	**2.3%**	**B4**
Klebsiella	**-**	**4.5%**	**0.06%**	**B4**
Lactococcus	0.4%	2.4%	6%	B4
Neisseria	0.8%	1.7%	6.4%	B4
Pseudomonadaceae family	**12.16%**	**1.1%**	**3.9%**	**B4**
Pseudomonas	**0.9%**	**1.06%**	**3.7%**	**B4**
Ruminococcacieae family	18.6%	-	-	B4
Ruminococcus	0.16%	2.1%	4.5%	B4
Staphylococcus	**0.9%**	**5.3%**	**6.1%**	**B4**

Table 2: *Genus level bacterial abundance in the milk cow No 1, before and after from EVP treatment (F1, F2, B3 and B4 Quarters).*

Genus level bacteria	Before EVP treatment	After 3 days of treatment	After 6 days of treatment	Quarter
Bacteroides	2.2%	0.2%	0.1%	F1
Corynebacterium	0.03%	3.2%	3%	F1
Lactobcillus	0.2%	4.6%	2.7%	F1
Planococcaceae family	17%	0.3%	0.04%	F1
Pretvotella	2%	3.9%	2.1%	F1
Ruminococcus	0.01%	6.2%	3.8%	F1
Staphylococcus	**60.7%**	**1.6%**	**2.1%**	**F1**
Streptococcus	**0.1%**	**2.4%**	**5.7%**	**F1**
Streptophyta Family	0.03%	4.3%	3.8%	F1
Bacteroides	3%	2.1%	0.6%	F2
Enterococcaceae family	**27%**	**2%**	**0.1%**	**F2**
Enterococcus	3%	0.1%	0.1	F2
Kaistobacter	-	5%	0.09%	F2
Micrococcus	-	0.01%	4%	F2
Mitochondria	0.03%	0.3%	10%	F2
Planococcaceae	0.06%	4%	0.39%	F2
Prevotella	2.4%	3%	1.8%	F2
Staphylococcus	**45%**	**11%**	**1%**	**F2**
Streptophyta	0.5%	1.1%	!7%	F2
Synechococcus	-	0.02%	10%	F2
Corynebacterium	-	0.03%	6%	B3
Fusobacterium	4.5%	4.6%	1.8%	B3
Haemophilus	4.2%	2.2%	0.7%	B3
Micrococcus	-	0.03%	6%	B3
Neisseria	5.2%	4.2%	1.1%	B3
Prevotella	3.6%	4.5%	2.2%	B3
Pseudomonas	8.2 %	1.8%	1.8%	B3
Streptococcus	**31%**	**1.4%**	**2.2%**	**B3**

Streptophyta	0.7%	2.9%	6.7%	B3
Synechococcus	-	0.2%	3.4%	B3
Veillonella	2.1%	4.5%	1.06%	B3
Acinetobacter	6.4%	0..8%	0.3%	B4
Delftia	6%	0.11%	0.06%	B4
Fusobacterium	0.65%	5.7%	1.1%	B4
Leptotrichia	0.08%	6%	0.2%	B4
Mitochondria	-	0.7%	13.5%	B4
Planococcace	11.6%	0.4%	0.08%	B4
Prevotella	1.05%	6.6%	2.3%	B4
Staphylococcus	**31.4%**	**1.6%**	**1.6%**	**B4**
Streptococcus	**30%**	**0.8%**	**2.3 %**	**B4**
Streptophyta	0.06%	1.69%	29.3%	B4
Synechococcus	-	0.25	3%	B4
Synechococcus	-	0.25%	3%	B4
Unassigned	2.6%	6.5%	4%	B4
Veillonella	0.09%	4%	0.6%	B4

Table 3: *Genus level bacterial abundance in the milk from EVP treated cow No.2.*

Genus level bacteria	Before EVP Treatment	After 3 days of Treatment	After 6 days of Treatment	Quarter
Bacillus	68%	0.5%	21%	F1
Bacteroides	3.3%	0.5	1.7%	F1
Cetobacterium	0.2%	9%	0.02%	F1
Enterobacteriaceae	**0.8%**	**10%**	**0.3%**	**F1**
Enterobacteriaceae	**10%**	**0.3%**	**0.8%**	**F1**
Paenibacillus	1.3%	-	-	F1
Planococcaceae	0.05%	0.07%	26%	F1
Prevotella	1.3%	0.3%	2%	F1
Staphylococcus	**30%**	**28%**	**1%**	**F1**
Streptococcus	**5%**	**0.04%**	**5%**	**F1**
Acetobactoer	0.03%	6%	1.5%	F2
Bacillaceae	9%	-	19%	F2
Bacillus	47%	-	20%	F2
Bacteroides	1.5%	0.3%	8.3%	F2
Elizabethkingia	30%	28%	0.8%	F2
Enterobacteriaceae	**23%**	**0.23%**	**3.1%**	**F2**
Lactococcus	-	2%	0.05%	F2
Neisseria	0.23%	0.06%	4.2%	F2
Staphylococcus	**34%**	**1.4%**	**0.7%**	**F2**

Streptococcus	**28%**	**9%**	**1.9%**	**F2**
Veillonella	0.05%	0.02%	4.4%	F2
Bacillus	33%	-	0.5%	B3
Bacteroides	1.4%	4%	11%	B3
Comamona	0.02%	0.01	19%	B3
Enterobacteriaceae family	**11%**	**0.7%**	**1.8%**	**B3**
Lactococcus	0.01%	4.4%	0.01%	B3
Planococcaceae	0.03%	2.2%	14%	B3
Staphylococcus	**49.3%**	**38%**	**0.13%**	**B3**
Streptococcus	**30%**	**29%**	**0.1%**	**B3**
Streptomyces	4.9%	-	-	B3
Unassigned	1.8%	1.5%	3.9%	B3
Bacillus	3.4%	0.06%	1.8%	B4
Bacteroides	2.2%	0.5.8%	20.9%	B4
Bifidobacterium	0.5%	4%	2.1%	B4
Enterobacteriaceae	**0.7%**	**5.2%**	**6.8%**	**B4**
Neisseria	1.07%	3.2%	4.7%	B4
Planococcaceae	**19.7%**	**0.0.1%**	**0.2%**	B4
Ruminococcaceae	1.1%	6.7%	5.3%	B4
Staphylococcus	**46.8%**	**1.65%**	**2.1%**	**B4**
Streptococcus	**2.1%**	**10%**	**3.8%**	**B4**
Streptomyces -	6.9%	-	-	B4

Table 4: *Genus level bacterial abundance for in case of EVP treatment-3.*

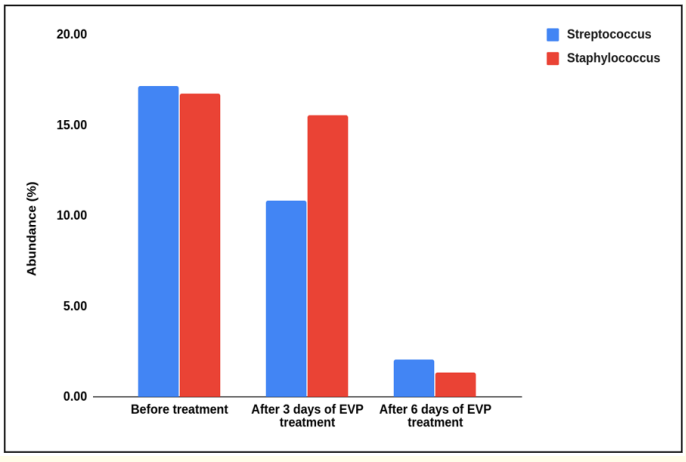

Figure 3: *Abundance of Streptococcus and Staphylococcus in the Mastitis affected cow milk before treatment, after 3days and 6 days of treatment with herbal formulation based on EVP.*

The microbial abundance of EVP formulations was given in table 5. *Fusobacterium* genera have more abundance followed by *Capnocytophaga, Prevotella, Veillonella, Leptotrichia* in case of EVP formulation.

No	EVP spray	Abundance in %
1	*Fusobacterium*	13
2	Other	8.4
3	*Capnocytophaga*	8.3
4	*Prevotella*	7.2
5	*Veillonella*	6.4

Table 5: *Microbial abundance of EVP formulations.*

Discussion

It is reported that microbiome of bovine milk constitutes normal microbiome along with certain contaminated genera [21]. The previous reports have identified *Staphylococcus aureus* and *Streptococcus agalactiae are most common pathogen related to mastitis* [22]. *Acinetobacter, Pseudomonas, Klebsiella, Escherichia, Enterobacter, Staphylococcus, Streptococcus, Bacillus, Pantoea, Shewanella, Ralstonia etc*. as the microbes associated with bovine mastitis. The present study we have also identified *Staphylococcus, Streptococcus, Pseudomonaceae family, Pseudomonas and Enterobacteriaceae family* (include *E. coli*) in the mastitis infected cattle.

Generally, antibiotics are administered for the treatment of mastitis in cows. However, the antibiotic therapy for prevention and cure of mastitis is not fully effective due to AMR resulting in chronic and recurrent mastitis. Therefore, the effective best alternative is EVP based on herbal formulation. Issues related to antimicrobial resistance may be avoided by using ethno-veterinary medicines successfully for the health care of livestock against several microbial diseases and also minimizes the residues in animal products like milk, meat and egg [6]. Present study indicates that abundance mastitis causing microbes like *Staphylococcus, Streptococcus, Pseudomonaceae, Pseudomonas and Enterobacteriaceae* (includes *E. coli*) have reduced systematically after 6 days of treatment with herbal formulation indicating the cure of mastitis. Gut bacteria like *Prevotella*, *Ruminococcus, Bacteroides* was one of the predominant microbial contamination in milk [23].

Conclusion

Bovine mastitis the most challenging disease which affects dairy production worldwide and the misuse of antibiotics led to residue in the animal products such as milk and antimicrobial resistance. On an average the abundance mastitis causing microbes have reduced systematically (*Staphylococcus* - 20 times, *Streptococcus* -12.52 times, *Pseudomonaceae family, Pseudomonas* - 10.67 times, *Enterobacteriaceae family* (includes *E. coli*) - 14.37 times and *Klebsiella* - 32.31 times) after 6 days of treatment with herbal formulation indicating the cure of mastitis.

Acknowledgement

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Author Contributions

Dr. Malali Gowda was instrumental in experimental design for microbiome sequencing. Dr. Pavithra Narendran and Mr. Santhosha Hegde carried out microbiome studies, data analysis, interpretation, Prof M N Balakrishnan Nair field experiments, sample collection and data interpretation and manuscript preparation.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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