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Analysis of raw goat milk microbiota: Impact of stage of lactation and lysozyme on microbial diversity

Elizabeth A. McInnis [a], Karen M. Kalanetra [b], David A. Mills [b, c], Elizabeth A. Maga [a, *]

[a] Department of Animal Science, University of California, Davis, One Shields Ave, Davis, CA 95616, USA
[b] Department of Viticulture and Enology, University of California, Davis, One Shields Ave, Davis, CA 95616, USA
[c] Department of Food Science & Technology, University of California, Davis, One Shields Ave, Davis, CA 95616, USA

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ABSTRACT

To protect infants from infection, human milk contains high levels of the enzyme lysozyme, unlike the milk of dairy animals. We have genetically engineered goats to express human lysozyme (hLZ milk) in their milk at 68% the amount found in human milk to help extend this protection. This study looked at the effect of hLZ on bacteria in raw milk over time. As the microbial diversity of goats' milk has yet to be investigated in depth using next-generation sequencing (NGS) technologies, we applied NGS and clone library sequencing (CLS) to determine the microbiota of raw goat milk (WT milk) and hLZ milk at early, mid and late lactation. Overall, in WT milk, the bacterial populations in milk at early and mid lactation were similar to each other with a shift occurring at late lactation. Both methods found *Proteobacteria* as the dominant bacteria at early and mid lactation, while *Actinobacteria* surged at late lactation. These changes were related to decreases in *Pseudomonas* and increases in *Micrococcus*. The bacterial populations in hLZ milk were similar to WT milk at early and mid lactation with the only significant differences occurring at late lactation with the elevation of Bacillaceae, Alicyclobacillaceae, Clostridiaceae and Halomonadaceae.

1. Introduction

The types of bacteria present in milk can influence cheese-making, shelf-life and can promote health or cause disease in consumers of the milk and milk products. The microbial profile of raw milk can also provide insight into the health status of the lactating dam since it changes during the course of lactation (D'Amico and Donnelly, 2010) and in response to infections such as mastitis (Alawa et al., 2000). However, the complex nature of milk and milk products makes determining what bacteria are present and what influence they exert a challenge.

Culture-independent methods of microbial population analysis have grown more sophisticated in recent years. Next-generation sequencing (NGS) is able to generate far more reads than traditional clone library sequencing (CLS) (Hamady and Knight, 2009). While older studies relied on culturing bacteria for identification (Foschino et al., 2002; Holm et al., 2004; D'Amico and Donnelly, 2010), NGS does not rely on selective media and can provide greater depth and breadth to the study of milk. These new technologies have not been widely applied to the bacteria in goat milk, as yet. Studies such as Callon et al. (2007) which used molecular techniques including single-stranded conformation polymorphism (SSCP) analysis and restriction fragment length polymorphism (RFLP) typing, have found bacteria in raw milk of small ruminants not found in previous culture-based studies which focused on specific groups such as staphylococci (Blagitz et al., 2011) or coliforms (Araya et al., 2008). In light of this, this study used NGS and traditional CLS to determine the microbial diversity in raw goat milk throughout the course of lactation and compare it to that of milk from genetically engineered goats producing the antimicrobial human lysozyme (hLZ) in their milk.

Lysozyme is a muramidase found in tears, saliva and milk of all mammals that specifically cleaves the 1,4-β-D-linkage between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of bacterial cell walls, resulting in cell lysis (Masschalck and Michiels, 2003). Lysozyme is present in human milk at much higher levels than the milk of dairy animals (400 μg/ml compared to 0.130 μg/ml in cow milk and 0.250 μg/ml in goat milk (Chandan et al., 1968)) to help protect infants against pathogenic bacteria and promote the formation of a healthy gut microbiota (Lonnerdal, 2003). Goats were genetically engineered to express increased levels of lysozyme in the mammary gland with the intent of

* Corresponding author. Tel.: +1 530 752 5930.
E-mail address: eamaga@ucdavis.edu (E.A. Maga).

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improving human health upon consumption of the milk (Maga et al., 2003). These transgenic goats produce active hLZ in their milk at levels of 270 μg/ml, 68% of the level of human milk (Maga et al., 2006a). Expression of hLZ did not disrupt yield or the gross composition (fat and protein content) of milk (Maga et al., 2006a) and finer analysis demonstrated that the presence of hLZ was the only difference in protein composition between the milk of transgenic does and their non-transgenic herd mates (Maga et al., 2012). The milk from hLZ goats has been shown to have a longer shelf-life and *in vitro* slowed the growth of bacterial isolates responsible for causing the spoilage of milk (*Pseudomonas fragi*) and mastitis (*Escherichia coli* and *Staphylococcus aureus*) but not *Lactococcus lactis* (Maga et al., 2006b) as the milk can still be used to produce cheese (Scharfen et al., 2007). When consumed by animal models, pasteurized hLZ milk beneficially modulates gut microbiota (Maga et al., 2012), improves gut morphology and circulating metabolites in young pigs (Brundige et al., 2010; Cooper et al., 2011) and helps resolve the symptoms of diarrhea (Cooper et al., 2013), all indicating potential human health benefits. One important question to answer is if lysozyme itself is causing these changes or if by-products of lysozyme presence in milk (different types of bacteria or metabolites) are influencing the antimicrobial action of the milk. In addition, the production of lysozyme in the udder of transgenic goats has the potential to alter the bacterial population of the raw milk, alterations which could have effects on the doe, milk processing and any consumers of the milk. In this study we used CLS and NGS approaches for an in depth characterization of the microbial diversity of raw goat milk and how these populations change in response to the presence of hLZ.

2. Materials and methods

2.1. Animals

All goats used in this study were housed in adjacent dry lots at the University of California, Davis (UCD) under Association for Assessment and Accreditation of Lab Animal Care (AALAC)-approved conditions. All animals were fed the same diet consisting of alfalfa provided daily and 3.3 kg of a corn, oat, barley and cottonseed concentrate at each milking, once in the morning and once in the evening. None of the study goats required antibiotics throughout the sample collection period and all udder halves appeared healthy throughout lactation. All does kidded within one month of one another and were milked twice daily in a milking parlor throughout the course of lactation (late February–October). Four does of the UCD herd, 2 each in their first and fourth parity, representing an Alpine, Toggenburg, Saanen and LaMancha were used for the analysis of raw goat milk to represent the milk of a standard dairy herd (wild-type (WT) milk). Analysis of the milk from the hLZ transgenic line (Alpine and Toggenburg in origin) used milk from four hLZ transgenic does, 2 in their fourth parity and 1 each in their second and first parity (hLZ milk).

Composite milk samples from individual does underwent monthly analysis through the California Dairy Herd Improvement Association (DHIA) testing program for weight percent fat and protein, somatic cell count and daily milk production. DHI analysis was carried out within 1 week of all sample collections. Means from individual does of each type at each time point were compared using the Student's *t*-test ($\alpha = 0.05$). Values are reported as mean ± standard deviation. Pooled milk samples from WT and hLZ does was subjected to a spot-on-lawn activity assay by incubating 30 μl milk in a punched hole of an agarose plate with 10% *Micrococcus lysodeikticus* incorporated. Plates were incubated at 37 °C overnight. *M. lysodeikticus* is a test substrate for lysozyme and clearing of the lawn indicates lysozyme activity. In addition, after kidding, milk from each doe was screened for the presence of mastitis pathogens using bovine blood agar plates.

Raw milk from each animal was collected 3 times during lactation representing early, mid and late lactation. The first time-point was 2 weeks after parturition (early lactation), the second time-point was collected during the third month of lactation (mid lactation) and the last time-point was taken 1 month before cessation of lactation (late lactation, approximately the seventh month of lactation). For the early lactation time-point, all does except 2 were sampled the same day in mid-March with the remaining 2 sampled on the same day in early April. For the mid and late lactation time-points, all does were sampled on the same day in early July and late October, respectively. Milk was collected from each half of the udder separately during the morning milking after the primary teat dip, stripping and wiping with an alcohol wipe by a milker who wore gloves. Milk was collected into a sterile 50 mL tube and kept on ice until processing, less than 2 h later.

Genomic DNA was extracted from the raw milk of each udder half using a commercially available kit specifically designed for milk (Norgen Milk Bacterial DNA Isolation Kit, Norgen Biotek, Thorold, ON, CAN) using the protocol for 'Gram-Positive or Unknown bacteria' and minor modifications including extending incubations from 45 min to 1 h in a shaking incubator. The concentration of recovered DNA was quantified using a NanoDrop and the DNA from each udder half was subjected to bacterial 16S rRNA gene sequence analysis by both CLS and NGS.

2.2. Preparation of clone libraries

The generation of the clone library for CLS was carried out as using the conditions as described in Maga et al. (2012). Briefly, bacterial 16S ribosomal DNA was PCR amplified using the primers 27F and 1392R (Lane, 1991), resulting PCR products were ligated into a vector and transformed into competent *E. coli* using the StrataClone PCR cloning kit (Agilent Technologies, Santa Clara, CA) and DNA templates prepared from the resulting colonies with rolling circle amplification followed by Sanger sequencing with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosciences, Foster City, CA). A total of 96 colonies per udder half were prepared for sequencing to give 192 sequences per animal. Resulting 16S rRNA sequences were identified and compared using the Ribosomal Database Project (RDP, Release 10 http://rdp.cme.msu.edu/). Similarity scores ≥0.8 were considered a significant match. Data from the transgenic goats at each stage of lactation were combined and compared as a library to the combination of bacterial species from the control goats at each time point and statistically analyzed using the RDP 10 LibCompare pipeline (Cole et al., 2009). Differences were considered statistically significant if $P < 0.001$ (Wang et al., 2007).

2.3. Next generation amplicon sequencing preparation

For NGS amplicon preparation, the V4 region of the 16S rRNA gene was amplified from bacterial DNA using a barcoded forward primer and an unbarcoded reverse V4 primer (Bokulich et al., 2012). Bacterial DNA from each udder half was amplified in triplicate using the GoTaq 2X PCR kit (Promega, Madison, WI) and then combined. The PCR products were purified using a Qiagen 96 PCR purification kit (Qiagen, Valencia, CA) and DNA concentration was fluorescently quantified using the Quant-iT™ PicoGreen® kit (Invitrogen, Grand Island, NY) per the manufacturers instructions. All the samples were then combined to an equimolar concentration into one volume, run on a gel and extracted using the QiaQuick Gel Extraction kit (Qiagen, Valencia, CA). Cluster generation and sequencing was carried out at the UCD Genome Center DNA

Technologies Core on the Illumina GAIIx platform to produce 160 bp pair-end reads.

2.4. NGS data analysis

Samples were demultiplexed, quality filtered and further analyzed using QIIME v1.7.0 (Quantitative Insights Into Microbial Ecology, Caporaso et al., 2010). Quality filtering was carried out with the default settings. Reads with ambiguous base calls or a high quality region of less than 75 percent of the total read length were discarded. Operational taxonomic units (OTUs) were assigned using the pick open reference otu command (Edgar, 2010) with 97% pairwise identity and the Greengenes bacterial 16S rRNA database 13_8 release (DeSantis et al., 2006). Taxonomy was assigned and sequences aligned using the QIIME-based RDP classifier reference library (Greengenes 13_8 release) at the 97% confidence level. Illumina FASTA files were rarefied to 10,000 sequences per animal (5000 per udder half), combined to 40,000 sequences per library using the program MOTHUR (Schloss et al., 2009) and analyzed using the RDP 10 LibCompare pipeline (Cole et al., 2009) to directly compare to CLS data. Samples were combined into libraries by both stage of lactation and herd (control or transgenic) for comparisons. QIIME was also used to generate rarefaction curves as well as estimates of beta-diversity using weighted UniFrac (Lozupone and Knight, 2005) and even sampling of 8000 reads per sample (16,000 reads per animal) to create principal coordinate analysis (PCoA) plots to visualize differences in the data.

The linear discriminate effect size (LefSe) program was used with the relative abundance data generated from the QIIME analysis to ascertain any significant differences in taxonomic abundance between both stage of lactation and herd (WT or transgenic) (Segata et al., 2011). The LefSe program uses the Kruska-Wallis sum-rank test to detect taxa with significant differential abundance in relation to class and then biological significance is determined by pairwise tests between subclasses using the Wilcoxon rank-sum test. Finally, linear discriminate analysis is used to estimate effect size of each differentially abundant taxa and taxonomic cladograms generated to highlight significant differences in taxa due to herd and time point ($P < 0.01$).

3. Results

Compositional analysis of milk samples revealed no significant differences between WT and hLZ does at each time-point (Table 1). Changes characteristic of the end of lactation (decreased production, increased fat and protein content and somatic cells) were evident in both groups of animals at the late lactation time-point. At late lactation, hLZ does produced significantly less milk ($P = 0.002$) and significantly more protein ($P < 0.001$) and tended to have more fat ($P = 0.065$) than at mid lactation. WT does followed same trend but the differences were not significant. Spot-on-lawn assay demonstrated that the milk from hLZ does was active against *M. lysodeikticus* (Fig. 1). Milk from each doe was cultured at early lactation to detect the presence of mastitis pathogens and the growth of *Staphylococcus* species (<100 colonies) was detected in the milk of 1 WT and 1 hLZ doe (data not shown).

A total of 2,377,734 reads were generated using NGS after quality filtering. Alpha-diversity analysis (richness within samples) indicated that the lowest number of reads for a sample (udder half) was 8,711, thus all further QIIME analysis was rarefied to 8000 reads per sample for a total of 16,000 reads per animal. CLS generated libraries ranging from 328 to 714 sequences (Fig. 2).

3.1. Bacterial profile of raw goat milk

The bacteria in raw goat milk belonged predominantly to 4 phyla: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*. The relative proportions of each of these groups changed over the course of lactation and both sequencing methods showed similar trends over time (Fig. 2). Both sequencing methods found that *Proteobacteria* was the predominant phylum at early and mid lactation, with the predominance more pronounced with NGS, followed by *Actinobacteria*. However, at late lactation, *Actinobacteria* increased to be the dominant phylum as *Proteobacteria* decreased from mid to late lactation in both NGS and CLS. The increase in *Actinobacteria* could be traced to the family Micrococcaceae in both NGS and CLS. *Firmicutes* increased between all three stages of lactation in the CLS with a further increase at late lactation seen using NGS. NGS detected a higher number of phylum *Bacteroidetes* at early lactation. This could be traced to a single udder half in one doe. CLS found very low levels (<1.0%) of *Bacteroidetes* at every time-point. Other phyla were found in the NGS at lower levels.

Within the phyla, QIIME analysis of the NGS data (Fig. 3a) found the largest family in the early and mid stages to be family Pseudomonadaceae within phylum *Proteobacteria* with 47.5% and 54.4%, respectively of the identified bacterial sequences. This group fell to 2% at late lactation. The genus *Pseudomonas* accounted for 97% of the Pseudomonadaceae at all time points. The largest group at late lactation using NGS (Fig. 3a) was phylum *Actinobacteria* family Micrococcaceae, with 68.0%. Prior to late lactation, this group held only 2.9% and 2.1% of the identified bacterial sequences in early and mid lactation, respectively. All the Micrococcaceae detected was *Micrococcus*. The *Actinobacteria* family Nocardiaceae (Fig. 3a) showed the opposite trend, dipping from 15.2% and 11.4% at early and mid lactation, respectively, to 0.2% at late lactation and the genus *Rhodococcus* represented all the Nocardiaceae. Similar results were obtained using RDP to assign taxonomy to the NGS data (data not shown).

Using CLS, the same trends were observed but with the order of the most prevalent families being switched (Fig. 3b). The largest group detected with CLS at early and mid lactation was phylum *Actinobacteria* family Nocardiaceae containing 35.2% and 36.4% of

Table 1
Compositional analysis of milk samples from WT and hLZ does at early, mid and late lactation.

	WT ($n = 4$)			hLZ ($n = 4$)		
	Early	Mid	Late	Early	Mid	Late
Milk (kg)	5.5 ± 2.4	5.3 ± 2.7	4.2 ± 4.1	5.0 ± 0.7	5.3 ± 1.4a	2.8 ± 1.6b
% Fat	2.9 ± 0.8	2.7 ± 0.4	3.1 ± 0.7	3.0 ± 0.1	2.6 ± 0.3	3.4 ± 0.6
% Protein	2.7 ± 0.2	2.5 ± 0.4	3.1 ± 0.5	2.8 ± 0.3	2.3 ± 0.1a	3.1 ± 0.1b
SCS[a]	3.5 ± 1.9	4.7 ± 1.7	5.9 ± 3.3	3.7 ± 1.5	5.7 ± 0.9	6.2 ± 1.5

a, b: Means with different online letters significantly differ, $P < 0.05$.
[a] Somatic cell score.

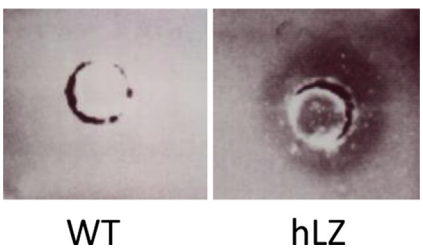

Fig. 1. Antimicrobial activity of WT and hLZ milk. Representative spot-on-lawn assay for milk from WT (left panel) and hLZ (right panel) goats at mid lactation. Clearing (dark ring) indicates lysozyme activity.

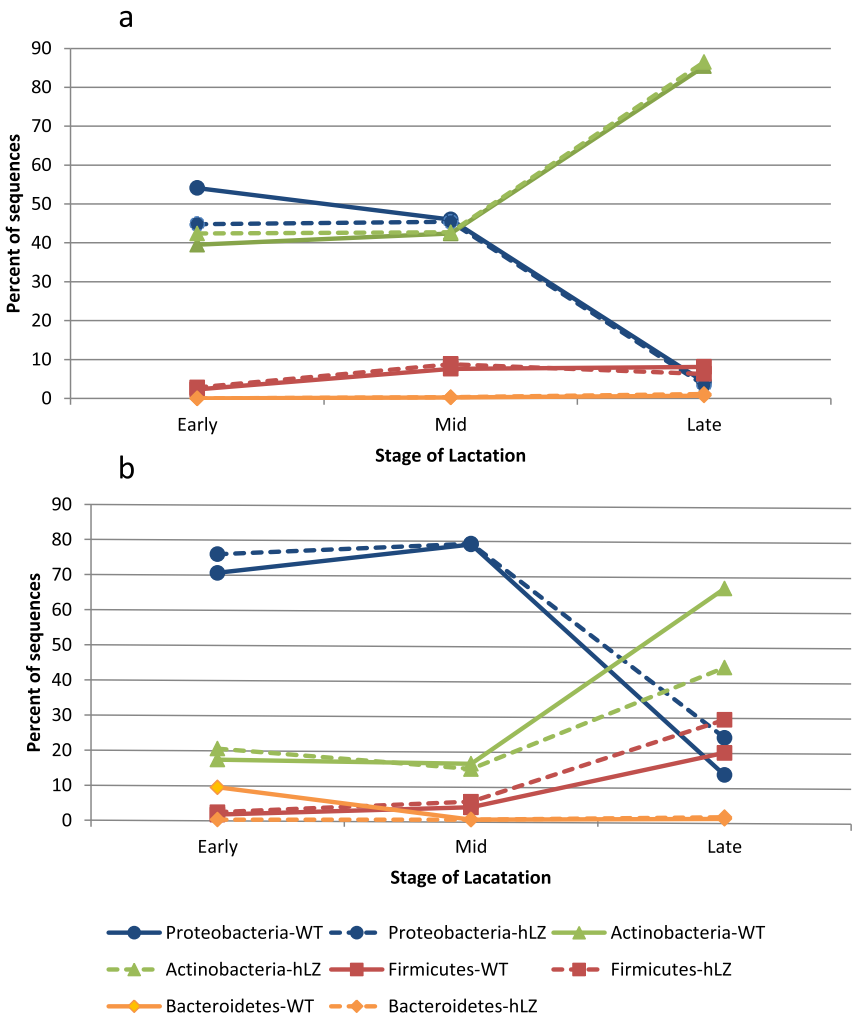

Fig. 2. Changes in the major phyla of goat milk over time. Percentages of sequences assigned to a phylum using RDP for CLS (a) and NGS (b) of milk from WT ($n = 4$) and hLZ transgenic ($n = 4$) goats. The number of sequences of in the CLS libraries of WT milk were 529, 420, and 714 at early, mid and late lactation, respectively and there were 422, 328 and 650 sequences in the hLZ milk CLS libraries at early, mid and late lactation, respectively. For NGS, the libraries were 40,000 sequences each.

the identified sequences at early and mid lactation, respectively, and dropped to 0.1% of identified sequences at late lactation. Again, the genus *Rhodococcus* accounted for all the Nocardiaceae. The second largest family group was Pseudomonadaceae within the phylum *Proteobacteria* at 17.4% and 11.7% of identified sequences at early and mid lactation, respectively and was comprised of *Pseudomonas*. As with NGS, Micrococcaceae within the *Actinobacteria* phylum was the most abundant family detected by CLS at late lactation increasing from early and mid lactation with 3.4% and 2.9% respectively of the identified sequences to 83.2% at late lactation. The Micrococcaceae were composed of genus *Arthrobacter* at early and mid lactation and both *Arthrobacter* and *Micrococcus* at late lactation.

Overall, the genera detected in raw WT goat milk using NGS and CLS were similar (Table 2). As a percent of total sequences, *Pseudomonas*, *Micrococcus*, *Rhodococcus*, *Stenotrophomonas*, *Phyllobacterium*, *Streptococcus* and *Agrobacterium* were the most prevalent genera identified by NGS regardless of time point. Using CLS, the same main genera were found, with minor differences in the order of prevalence and lack of *Streptococcus* and *Agrobacterium*, with the addition of *Arthrobacter*, *Rhizobium* and *Staphylococcus* also being present at greater than 1% of total sequences.

3.2. Bacterial profile of raw hLZ milk

The same four phyla were represented in hLZ milk in similar proportions and the changes over time closely mirrored that of WT milk (Fig. 2). At early and mid lactation, *Proteobacteria* was the main phylum detected with both methods followed by *Actinobacteria* and as with WT milk, NGS detected higher proportions of *Proteobacteria* and lower proportions of *Actinobacteria* than CLS (Fig. 2). At late lactation, the shift to *Actinobacteria* at the expense of *Proteobacteria* was also observed. As with WT milk, increases in family Micrococcaceae accounted for the increase in *Actinobacteria* in hLZ milk at late lactation. NGS found *Firmicutes* increasing to a greater extent at late lactation than CLS and with both methods, *Bacteroidetes* remained constantly low (<1%) at all time points. Like the WT milk, the hLZ milk had only minor changes between early and mid lactation.

At the family level, NGS found Pseudomonadaceae to be the most prevalent family at early and mid lactation (40.7% and 56.9%, respectively), falling to 2.5% of identified sequences at late lactation (Fig. 3a) with 98% of the Pseudomonadaceae being *Pseudomonas*. Phylum *Actinobacteria* family Micrococcaceae was the largest family represented at late lactation, comprising 44.3% of identified reads (Fig. 3a). Micrococcaceae was present as 4.5% of reads at early

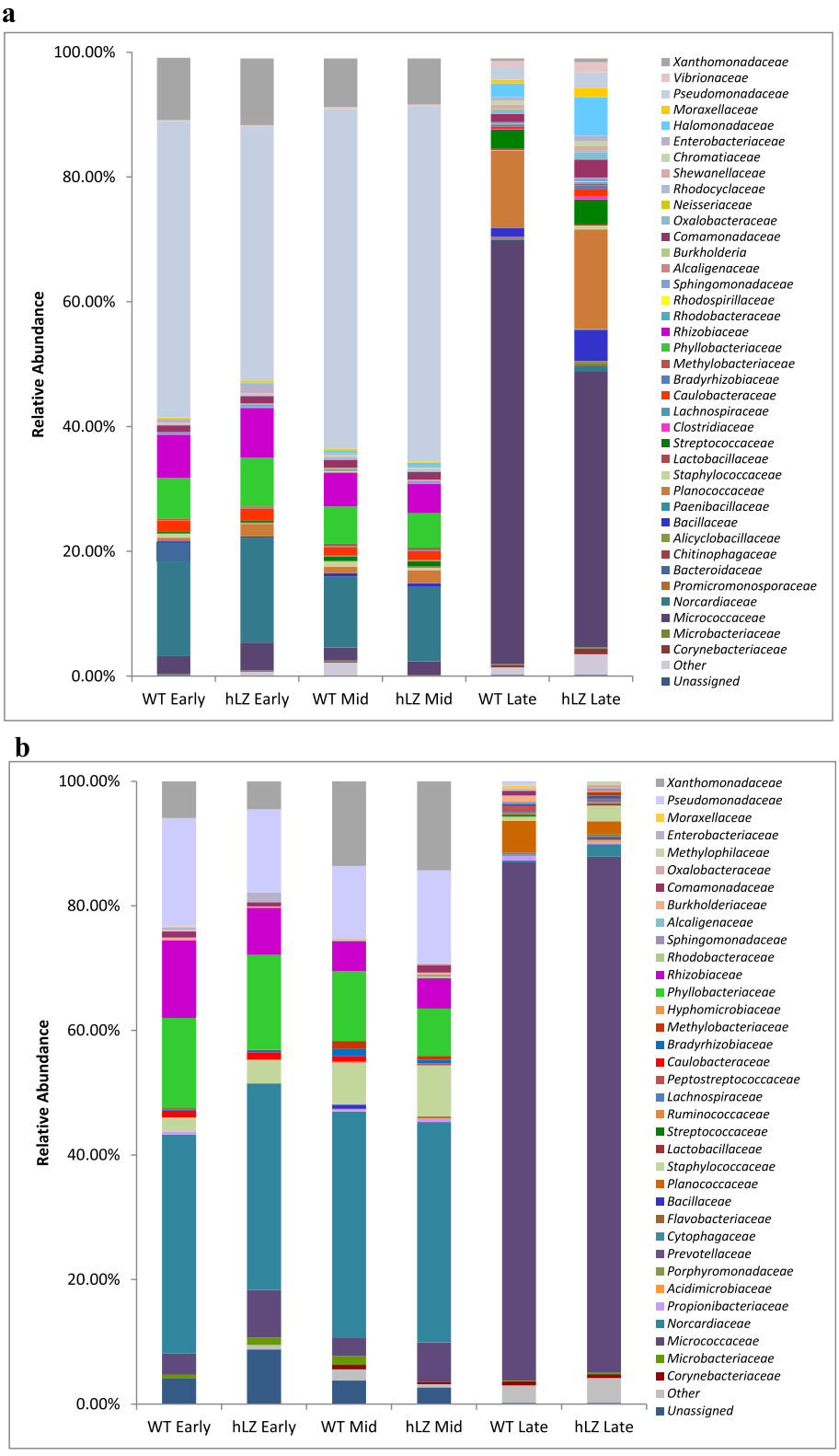

Fig. 3. Bacterial community structure at the family level of the milk from WT ($n = 4$) and hLZ transgenic ($n = 4$) animals at early, mid and late lactation using NGS (a) and CLS (b).

lactation and 2.2% at mid lactation and was entirely *Micrococcus*. As with WT milk, the *Actinobacteria* family Nocardiaceae (Fig. 3a) dropped from 16.8% and 12% at early and mid lactation, respectively, to 0.9% at late lactation and was comprised of the genus *Rhodococcus*. Planococcaceae from phylum *Firmicutes* class *Bacilli* and order *Bacillales* was the second largest family at late lactation representing 16% of reads (Fig. 3a). As with WT milk, these results with QIIME were similar results to RDP (data not shown).

With CLS, the same trends as seen with WT milk were also seen with hLZ milk (Fig. 3b). Instead of Pseudomonadaceae from phylum

Table 2
Genera detected in WT and hLZ milk using NGS and CLS. Genera present at ≥1% of the total sequences at any time point are listed in order of prevalence. Genera below the bold line in each column are present at <1%.

WT		hLZ	
NGS	CLS	NGS	CLS
Pseudomonas	*Micrococcus*	*Pseudomonas*	*Micrococcus*
Micrococcus	*Rhodococcus*	*Micrococcus*	*Rhodococcus*
Rhodococcus	*Pseudomonas*	*Rhodococcus*	*Arthrobacter*
Stenotrophomonas	*Arthrobacter*	*Stenotrophomonas*	*Pseudomonas*
Phyllobacterium	*Phyllobacterium*	*Phyllobacterium*	*Phyllobacterium*
Streptococcus	*Stenotrophomonas*	*Halomonas*	*Stenotrophomonas*
Agrobacterium	*Rhizobium*	*Streptococcus*	*Staphylococcus*
Halomonas	*Staphylococcus*	*Agrobacterium*	*Rhizobium*
Vibrio	*Brevundimonas*	*Acinetobacter*	*Brevundimonas*
Acinetobacter	*Aminobacter*	*Staphylococcus*	*Escherichia*
Ralstonia	*Ralstonia*	*Vibrio*	*Methylobacterium*
Aminobacter	*Corynebacterium*	*Escherichia*	*Microbacterium*
Methylobacterium	*Microbacterium*	*Ralstonia*	*Corynebacterium*
Shewanella	*Propionibacterium*	*Corynebacterium*	*Propionibacterium*
Corynebacterium	*Methylobacterium*	*Shewanella*	*Bradyrhizobium*
Staphylococcus	*Bradyrhizobium*	*Methylobacterium*	*Ralstonia*
Escherichia	*Jeotgalicoccus*	*Aminobacter*	*Lactobacillus*
Lactococcus	*Acinetobacter*	*Sphingomonas*	*Sphingomonas*
Sphingobium	*Lactococcus*	*Alicyclobacillus*	
Sphingomonas	*Sphingomonas*	*Sphingobium*	
	Escherichia	*Thermoanaerobacterium*	
	Kocuria	*Lactobacillus*	

Proteobacteria being the predominant family at early and mid lactation, Nocardiaceae from phylum *Actinobacteria* was found to be most prevalent representing 33.2% and 35.4% of the clones at early and mid lactation, respectively and comprised 2% at late lactation and consisted of the genus *Rhodococcus*. In contrast to WT milk, Phyllobacteriaceae comprised the second most prevalent family at early lactation (15.2%) with Pseudomonadaceae accounting for 13.3% of reads. At mid lactation, Pseudomonadaceae was the second most predominant family (14.9%) and was also not detected at late lactation. All of the Pseudomonadaceae detected were *Pseudomonas*. As seen with NGS, Micrococcaceae within the *Actinobacteria* phylum was predominant at late lactation rising to 82.8% of clones from 7.6% at early lactation and 6.4% at mid lactation. Of the Micrococcaceae, the genera *Arthrobacter* and *Micrococcus* were present at both early and late lactation with *Micrococcus* accounting for 19% of the Micrococcaceae at early lactation and 86% at late lactation. Only *Micrococcus* was present at mid lactation.

The most prevalent genera found in hLZ milk at any time point using NGS were *Pseudomonas*, *Micrococcus*, *Rhodococcus*, *Stenotrophomonas*, *Phyllobacterium*, *Halomonas*, *Streptococcus* and *Agrobacterium* (Table 2). CLS indentified the same main genera with the addition of *Arthrobacter*, *Staphylococcus* and *Rhizobium* and did not detect *Agrobacterium*. *Streptococcus* was detected but at less than 0.1% (0.07%) of total sequences. The genera of bacteria found in hLZ milk and their relative prevalence closely mirrored those found in WT milk with *Lactobacillus* (0.1%), *Alicyclobacillus* (0.13%) and *Thermoanaerobacterium* (0.13%) being the only genera found in hLZ milk at greater than 0.1% of the total reads and not in WT milk.

3.3. Comparison of WT and hLZ milk

Analysis of the CLS data using the LibCompare function in RDP to determine the likelihood that the frequency of membership in a given taxon was the same for the WT and hLZ libraries at each time point found only one significant difference in phyla between WT and hLZ milk. *Proteobacteria* tended to be significantly higher in the WT than the hLZ milk at early lactation ($P = 0.0011$). However, NGS found *Proteobacteria* present at a greater proportion in hLZ milk compared to WT milk. Due to the larger size of the NGS data set, different types of statistical analysis were carried out.

Principal component analysis of the NGS data clearly showed the difference between late lactation and the other time-points in both groups (Fig. 4). Early and mid lactation cluster very closely with one outlier with no discernible difference at any time point between WT and hLZ milk. Late lactation (circled in red) does not appear to have a distinction between hLZ or WT milk, but does show a change from earlier samples.

Further analysis of the NGS data to the family level with LEfSe, found multiple significant differences in bacterial abundance due to stage of lactation (Fig. 5a) but only 4 significant differences due to herd (Fig. 5b). At late lactation, Bacillaceae, Alicyclobacillaceae, Clostridiaceae and Halomonadaceae were significantly more abundant in hLZ milk ($P < 0.01$) with some variation between individual udder halves (Fig. 6a–d). With less stringent analysis, Micrococcaceae were significantly less abundant in hLZ milk at late lactation ($P < 0.05$, Fig. 6e). At the family level, the WT and hLZ milk had many similarities at both early and mid lactation (Fig. 3a). No significant differences were found due to herd at these time points.

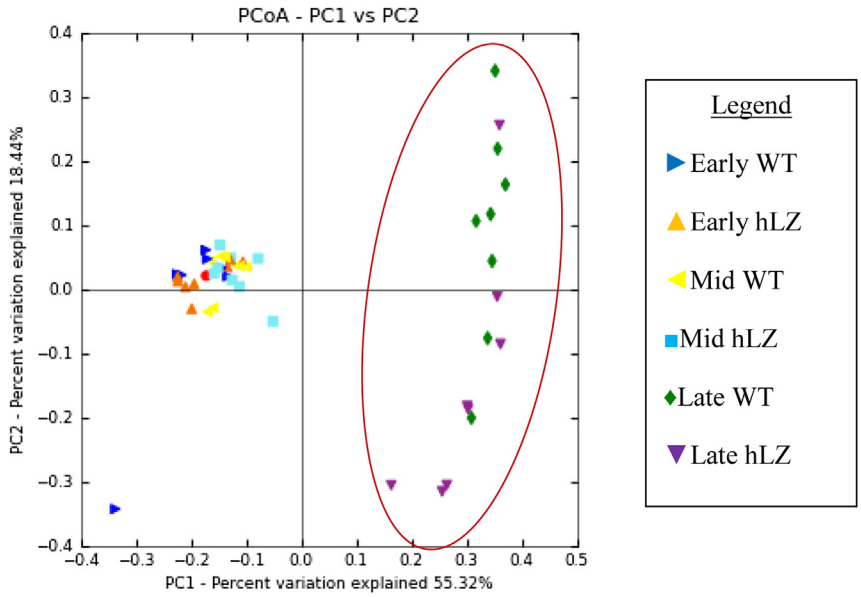

Fig. 4. PCoA plot of the milk from WT ($n = 4$) and hLZ transgenic ($n = 4$) goats grouped by udder half at early, mid and late lactation. The red oval encircles all of the late lactation samples from both herds and no samples from any other time-point. The first coordinate (PC1) explained 55.32% of the variation and the second coordinate (PC2) explained 18.44% of the variation between samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Stage of lactation significantly influenced bacterial abundance in both WT and hLZ milk (Fig. 5a). At early lactation, Nocardiaceae was elevated as were *Alphaproteobacteria* including Phyllobacteriaceae, Rhizobiaceae and Caulobacteraceae as well as Xanthomonadaceae and Enterobacteriaceae compared to the other time points. At mid lactation, Staphylococcaceae and the phylum *Proteobacteria* including the *Gammaproteobacteria* and Pseudomonadaceae were significantly elevated as were several families at late lactation including the phylum *Actinobacteria*, Corynebacteriaceae, the *Firmicutes*, Bacillaceae, Streptococcaceae, and Planococcaceae and several *Alphaproteobacteria* including Halomonadaceae, Chromatiaceae and Vibrionaceae. All these changes occurred in both WT and hLZ milk with no differences between the two.

4. Discussion

Direct amplicon and clone library sequencing of ribosomal RNA genes provided a largely unbiased and proportional picture of the bacterial species present in goat milk. Similar to studies of gut microbiota that found the characteristic coliforms to be a small percentage of the total bacteria load (Hamady and Knight, 2009), this work demonstrated that *Firmicutes*, especially lactic acid bacteria and other classic milk bacteria form only a small part of the total bacterial profile of goat milk. The ubiquitous *Actinobacteria* and *Proteobacteria* form a larger part of the microbiota.

The dramatic shift toward *Actinobacteria* at late lactation was caused by a growth of Micrococcaceae species. Preponderance of *Micrococcus* has been seen in goat milk previously, associated with an increase in hygiene. Verdier-Metz et al. (2009) found bacterial populations with up to 67–77% *Micrococcus* on some farms which cleaned teats before and after milking, had aerated housing, and correct turbulence and temperature in the machines during milking. These researchers associated the high numbers of specific species with management practices aimed at decreasing bacterial contamination. While the same hygienic techniques were maintained throughout this study, by late lactation they have effectively altered the bacterial populations of the raw milk toward certain groups (*Actinobacteria*) and farther away from others (*Proteobacteria*). It is likely that environmental conditions (temperature, humidity) played a role in this shift seen at late lactation as the families changing represented environmental bacteria.

Both CLS and NGS were in general agreement with overall trends, despite the fact that NGS had more than 50 times as many reads per library. At early lactation, NGS identified more *Proteobacteria* than CLS at the expense of *Actinobacteria* with *Actinobacteria* accounting for 20% of the NGS reads while with CLS, *Actinobacteria* represented 40% of the population. This could either be a consequence of the shorter sequence reads generated with NGS, a reflection of larger sample size or more likely the use of different primers for each method resulted in the differential amplification of different populations. The increase in the number of sequences led to greater statistical power and more comprehensive view of the bacteria in goat milk. Both CLS and NGS identified the same four major phyla and thus the CLS data validated the identities of the short amplicons generated with NGS. The dramatic drop in *Proteobacteria* at late lactation and corresponding increase in *Actinobacteria* was seen using both methods. The NGS detected a higher level of *Bacteroidetes* at early lactation which was not observed in the CLS. While CLS may be useful to identify dominant bacteria down to the genus level, NGS likely provides a more accurate view of the overall bacterial population by providing more reads.

Previous studies using both culture-dependent and culture-independent molecular methods (SSCP) have reported that the most prevalent genera detected in raw goat milk are *Enterococcus* spp., *Kocuria* spp., *Lactococcus* spp., *Pseudomonas* spp. and *Staphylococcus* spp. with *Acinetobacter* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Microbacterium* spp., *Pantoea* spp. and *Stenotrophomonas* spp. also being commonly found but less prevalent and genera such as *Arthrobacter* spp., *Corynebacterium* spp., *Micrococcus* spp., *Streptococcus* spp., *Jeotgalicoccus* spp. and several others being detected only occasionally (Alonso-Calleja et al., 2002; Callon et al., 2007; Quigley et al., 2013). In this study, NGS and CLS detected all these genera albeit at different frequencies. With the exception of *Pseudomonas* and *Staphylococcus*, the other prevalently reported genera

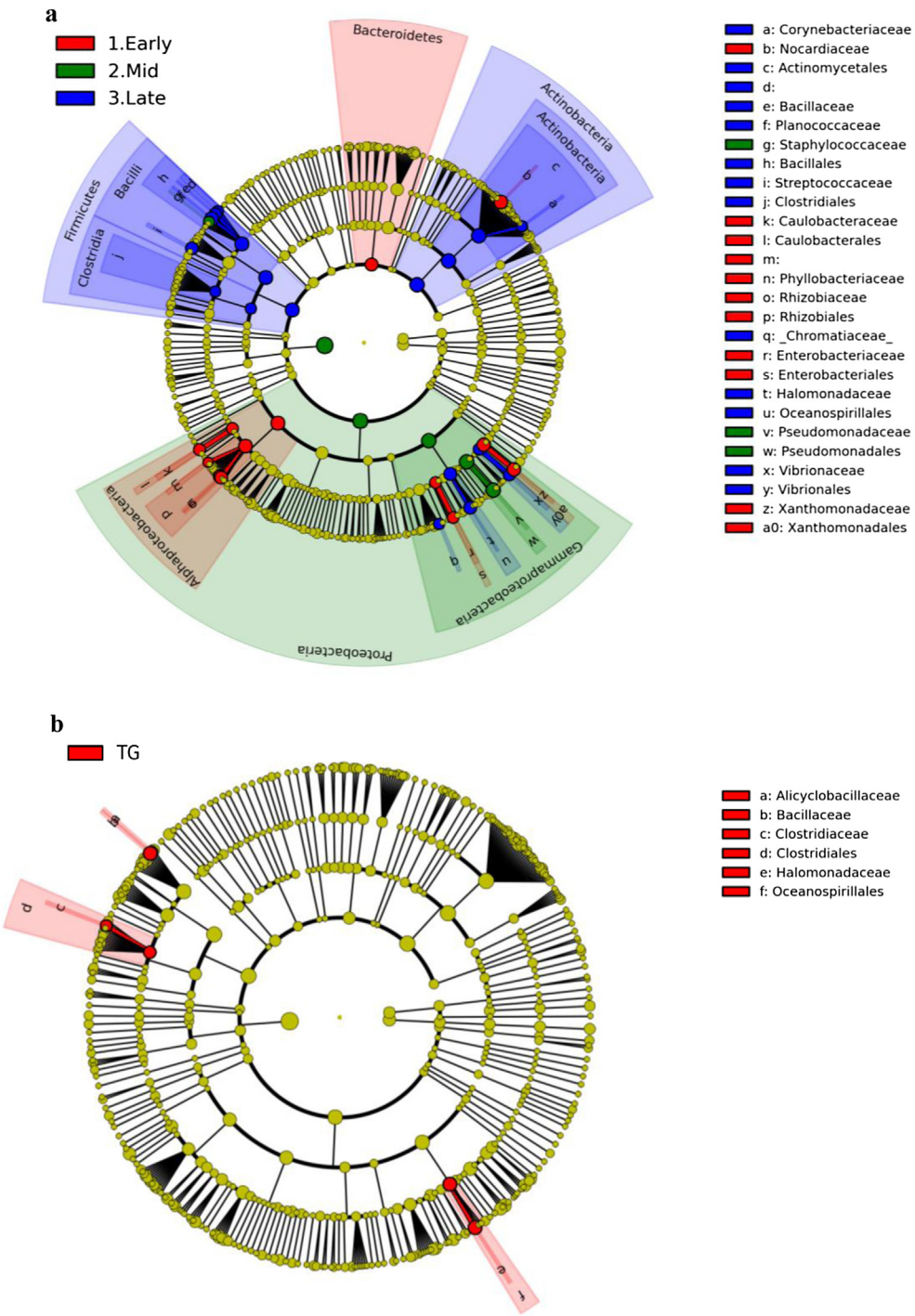

Fig. 5. LEfSe cladogram (family level) demonstrating taxonomic differences between the stage of lactation in all goats (a) and the milk of hLZ transgenic (TG) and WT goats at late lactation (b). (a) Taxa and nodes highlighted in red, green and blue were significantly elevated at early, mid and late lactation, respectively in both WT and hLZ does. (b) Taxa and nodes highlighted in red were significantly elevated in the milk of hLZ (TG) does compared to WT. Nodes remaining yellow indicate taxa that were not significantly differentially represented ($P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were found in raw WT milk at low frequency. *Enterococcus* spp., one of the reported prevalent genera, was detected with NGS but only at 0.005% of the total sequences and was not detected with CLS. The genera that were reported to be found frequently but less prevalent were generally in agreement with the exception that *Stenotrophomonas* was found at a relatively high prevalence in WT milk and *Lactobacillus*, *Leuconostoc* and *Pantoea* were all detected at less than 0.06% of total reads. Several of the genera reported to be found only occasionally were found here quite frequently, including *Micrococcus*, *Streptococcus* and *Arthrobacter*. The presence of *Rhodococcus*, *Phyllobacterium*, *Halomonas*, *Agrobacterium* and *Rhizobium*, the other prevalent genera found here, were not reported in these previous studies. These findings suggest that while similar types of bacteria may be present in raw goat milk, their presence may be predicated by many factors such as diet and environment and determining how their relative abundance is related to animal

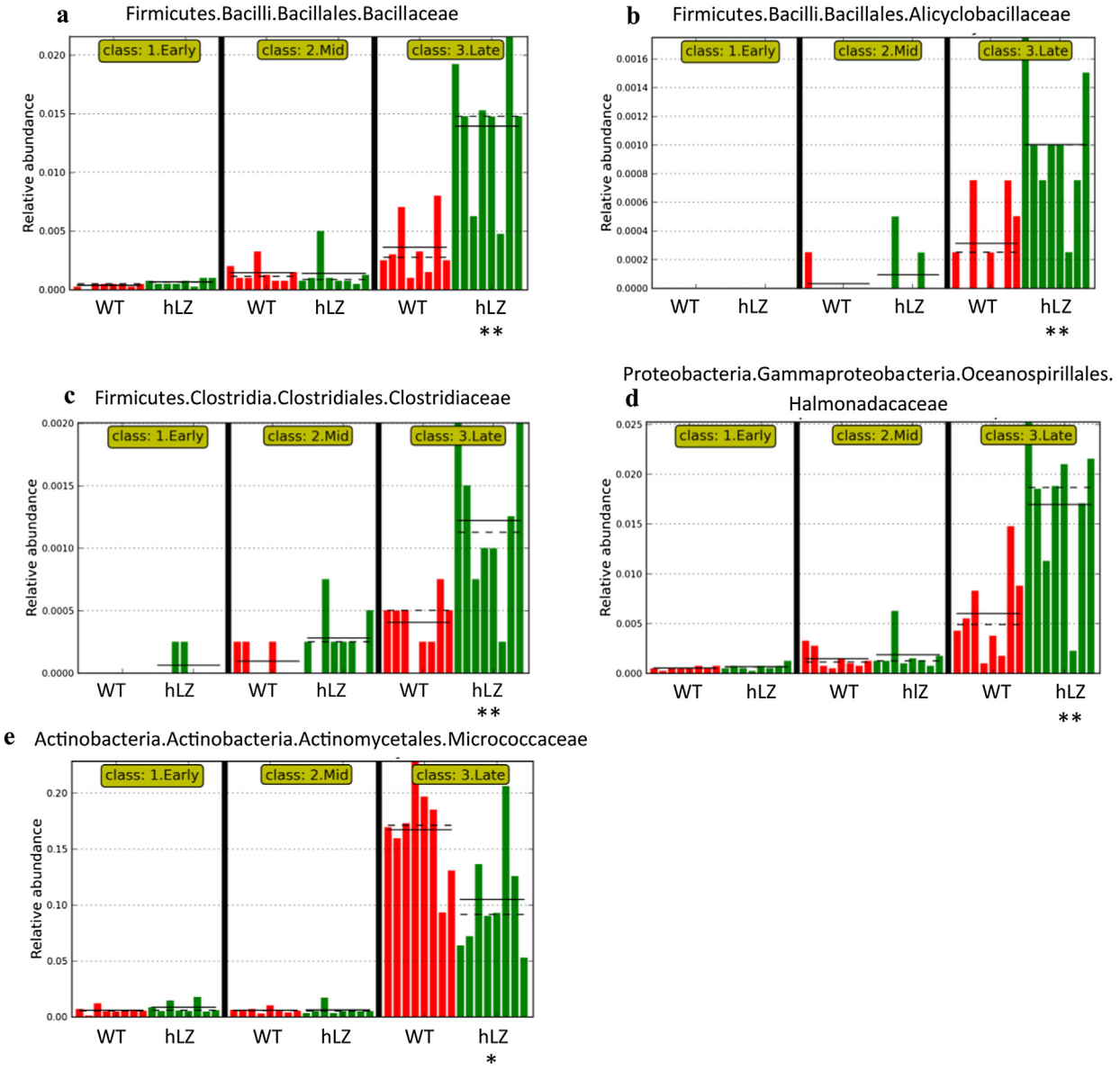

Fig. 6. LEfSe relative abundance in individual samples from families significantly different between WT and hLZ milk ($P < 0.01$). Relative abundance of Bacillaceae (a), Alicyclobacillaceae (b), Clostridiaceae (c) and Halomonadaceae (d) Micrococcaceae (e) in the milk of each udder half from WT and hLZ transgenic goats at early (left panel), mid (middle panel) and late (right panel) lactation. Each bar represents an udder half in the designated group. Solid and dashed horizontal lines indicate group mean and median, respectively. *Indicates a significant difference in abundance between hLZ and WT goats at a given time point (*$P < 0.05$ and **$P < 0.01$, respectively).

health and milk quality and manufacturing properties remains the challenge.

Expression of hLZ in the mammary gland had some effect on the bacterial population of milk, but not as much as stage of lactation since the milk from the hLZ goats followed the same pattern as the milk from the WT does. This is somewhat surprising due to the antimicrobial nature of lysozyme in general and previous work with hLZ milk which demonstrated that the only protein difference in WT and hLZ milk was the presence of hLZ, that the hLZ was active and that consumption of the milk by animal models influenced the relative abundance of gut microbiota populations (Maga et al., 2006a, 2006b; 2006c, 2012). With the samples used in this particular study, gross component composition of the milk was not significantly different between WT and hLZ does (Table 1), the presence of hLZ was documented throughout lactation at a consistent level by western blot (data not shown) and the milk from hLZ does possessed antimicrobial activity (Fig. 1). We therefore expected to observe differences in the types of bacteria in the milk of hLZ does. However, the quantity of bacteria in each sample was not determined hence the effectiveness of lysozyme in altering the total amount of bacteria present in milk is not known.

It is thought that milk from the udder proper contains very few bacteria and that the bacteria found in milk originate from the environment of the animal, its surroundings and milking equipment (Quigley et al., 2013). Therefore, the bacteria detected here were likely environmental bacteria and the lack of changes seen between raw hLZ and WT milk indicate that hLZ was either not active against these particular bacteria or the bacteria were present for too short time for hLZ to have a significant impact. For instance, at early and mid lactation, Gram-negative bacteria were predominant with Gram-positive species taking over at late lactation where the few differences between WT and hLZ milk were seen. Lysozyme is considered more effective against Gram-positive bacteria as it has direct access to its substrate, peptidoglycan (Masschalck and

Michiels, 2003). Therefore, at late lactation, the Gram-positive *Actinobacteria* and *Firmicutes* were present in greater proportions than at early and mid lactation making it easier for hLZ to impact milk bacterial populations. At late lactation, hLZ milk had fewer Micrococcaceae and three families of *Firmicutes* and one *Gammaproteobacteria* were found to be differentially abundant at a significant level. These families were all found in WT milk and were elevated in hLZ milk. We speculate that hLZ was able to act on the Micrococcaceae present to decrease their prevalence which then allowed a more favorable environment for these other families to proliferate. The impact of these changes on milk quality and safety remain to be determined.

The hLZ transgenic goats were developed to provide a source of milk with the protective properties of human milk to benefit the health of consumers of the milk. It is thought that lysozyme in human milk plays a role in establishing a beneficial gut microbiota (Lonnerdal, 2003) and when hLZ milk was fed to a human-relevant animal model (the pig), the fecal microbiota population was distinct from animals fed WT milk demonstrating that hLZ milk can indeed modulate gut microbial populations in a fashion similar to human milk (Maga et al., 2012). In these trials, pasteurized milk from mid lactation was fed to the animals, thus eliminating any role live bacteria would have had, and as these results show, there was no significant difference in the relative abundance of bacteria present at mid lactation between hLZ and WT milk nor was there any difference in milk components (Table 1) pointing to the direct activity of lysozyme in the intestinal tract or an indirect effect via some yet unidentified secondary metabolites present in the milk. As lysozyme is heat and acid stable and survives transit through the gastrointestinal tract (Masschalck and Michiels, 2003; Eschenburg et al., 1990), we speculate that lysozyme in milk can act at the level of the intestine to influence the relative abundance of microbial community members. It is interesting to note that hLZ influenced Clostridiaceae both in the milk at late lactation where the relative abundance was increased compared to WT milk and in the pig where Clostridiaceae were significantly underrepresented in the fecal microbiota of pigs consuming hLZ milk compared to those consuming WT milk (Maga et al., 2012). These opposite findings in these two different milieus points to the antimicrobial action of hLZ being impacted by its surroundings. This type of information is not only important for future applications with this milk, but also as part of a risk analysis of the safety of the milk derived from lysozyme transgenic dairy animals.

5. Conclusion

The use of NGS and CLS revealed the diversity of raw goat milk microbiota in greater detail than previously reported. Stage of lactation influenced bacterial populations with the microbiota at late lactation being distinct from that at early and mid lactation. As opposed to stage of lactation, the presence of lysozyme had a little effect on the microbiota of raw milk. The types of bacteria in hLZ milk and their changes over time closely mirrored that of WT milk. While the bacterial profile changed dramatically over the course of the study, the goats remained healthy, suggesting that these changes in largely non-pathogenic commensal and/or environmental bacteria in both the WT and transgenic goats are part of the normal lactational cycle. As the cost of NGS decreases, more studies should take advantage of these techniques to analyze the bacterial populations of goat milk and dairy products and their ramifications.

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