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

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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

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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