J. Dairy Sci. 96:1499–1510
http://dx.doi.org/10.3168/jds.2012-5493

Unraveling the microbiota of teat apices of clinically healthy lactating dairy cows, with special emphasis on coagulase-negative staphylococci

G. Braem,* S. De Vliegher,† B. Verbist,‡ V. Piessens,§ E. Van Coillie,§ L. De Vuyst,* and F. Leroy*[1]
*Research Group of Industrial Microbiology and Food Biotechnology, Faculty of Sciences and Bio-engineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium
†M-team and Mastitis and Milk Quality Research Unit, Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium
‡Ministry of the Flemish Community, Institute for Agriculture and Fisheries Research, Technology and Food Science Unit, Agricultural Engineering Research Area, Van Gansberghelaan 115 bus 1, B-9820 Merelbeke, Belgium
§Ministry of the Flemish Community, Institute for Agriculture and Fisheries Research, Technology and Food Science Unit, Food Safety Research Area, Brusselsesteenweg 370, B-9090 Melle, Belgium

ABSTRACT

Swab samples (n = 72) obtained from the teat apex of lactating dairy cows without visual signs of inflammation (n = 18) were gathered on 2 well-managed Flemish dairy herds (herds 1 and 2) during the same month to assess the bacterial diversity of teat apices before milking. A combination of both culture-dependent [plating and $(GTG)_5$-PCR fingerprinting of the colonies] and culture-independent [denaturing gradient gel electrophoresis (PCR-DGGE)] techniques indicated that the teat apices contain a wide diversity of bacterial genera. Despite a low bacterial load, 20 bacterial genera of 3 phyla (*Actinobacteria*, *Firmicutes*, and *Proteobacteria*) were present. The most prevalent bacteria were the coagulase-negative staphylococci (CNS), encompassing a total of 15 species, which were identified to the species level using a combination of $(GTG)_5$-PCR fingerprinting, gene sequencing (16S ribosomal RNA and *rpoB* genes), and a novel PCR-DGGE technique based on the *tuf*-PCR amplicon. Overall bacterial diversity did not differ significantly between the herds or between noninfected and subclinically infected quarters in herd 1. In herd 1, borderline significant lower CNS species diversity was found on teat apices of noninfected quarters compared with subclinically infected quarters. The most prevalent CNS species were *Staphylococcus haemolyticus* and *Staphylococcus equorum* in both herds and *Staphylococcus carnosus* in herd 2.

Key words: bacterial species diversity, coagulase-negative staphylococci, teat apex, udder health

INTRODUCTION

Mastitis, or inflammation of the mammary gland, remains one of the most costly diseases for the dairy farmer (Huijps et al., 2009). An IMI, potentially leading to mastitis, occurs upon passage of bacteria from the teat canal into the udder. Although the keratin plug in the teat canal offers a primary defense against invading bacteria, 60% of teat canals of heifers are already open 60 d before parturition (Krömker and Friedrich, 2009). Six weeks into the dry period, up to 20% of the teats have not closed (Dingwell et al., 2002). In lactating cows, the sphincter muscles of the teat canal remain dilated for 1 to 2 h after milking, imposing a risk of bacterial infection by skin-colonizing, contagious, and environmental bacteria (Sieber and Farnsworth, 1981; Fox and Norell, 1994; Neijenhuis et al., 2000). Bacteria that have accumulated on the teat apex between milking rounds are the most likely to enter the mammary gland. Such bacteria may also end up in the milk and influence both its safety and processing (Verdier-Metz et al., 2012). Hence, a better understanding of the composition of the teat apex microbiota is needed.

The microbiology of the teat apex has previously been analyzed mostly with culture-dependent methods and the teat apex is known to carry a microbiota that includes both skin-associated bacteria (e.g., CNS) and mastitis-causing pathogens (e.g., *Streptococcus agalactiae* and *Staphylococcus aureus*; Woodward et al., 1987). However, *Staph. aureus* strains located on the teat apex may differ from strains found in the milk (Zadoks et al., 2002). Although the precise consequence of a continuous presence of CNS on the teat apex is under discussion, studies indicate that this type of colonizing bacteria and the time of colonization are relevant for udder health (Roberson et al., 1994; De Vliegher et al., 2003; Krömker and Friedrich, 2009; Piepers et al., 2011). Recently, a culture-independent analysis of

Received March 1, 2012.
Accepted November 11, 2012.
[1]Corresponding author: fleroy@vub.ac.be

the teat apices of clinically infected cows revealed wide bacterial species diversity (Braem et al., 2012).

Currently, CNS are the most frequently isolated group of bacteria in almost all mastitis-related surveys (Pitkälä et al., 2004; Piepers et al., 2007; Schukken et al., 2009). In the past, these bacteria were considered of limited importance for udder health and were thus classified as minor pathogens (Taponen et al., 2006). Although the effect of CNS on the SCC of milk is smaller than the effect of major pathogens (Supré et al., 2011), the prevalence among cows of IMI caused by CNS may vary between 6 and 27% (Djabri et al., 2002). Further, CNS have been reported to cause cases of (mild) clinical mastitis (Schukken et al., 2009). Finally, reports indicate the presence of penicillin resistance in CNS in older cows (Rajala-Schultz et al., 2004). Although these findings suggest undesired properties associated with CNS, heifers with a CNS infection at calving have been reported to out-produce noninfected herd mates during first lactation and to have a lower incidence of clinical mastitis (Piepers et al., 2010). These seemingly conflicting results indicate that more research is needed to study the diverse group of CNS in more detail.

As a follow-up of a previous study that investigated the total microbiota of clinically infected cows (Braem et al., 2012), the aim of the present paper was to characterize the diversity of bacterial species present on the teat apices of clinically healthy lactating dairy cows, with a focus on CNS because of their suggested role in udder health. A culture-dependent method, with molecular identification of the isolates through $(GTG)_5$-PCR fingerprinting, and a culture-independent method—denaturing gradient gel electrophoresis (**DGGE**) of PCR amplicons 16S rRNA (V6-V8)-PCR and *tuf*-PCR—were used in a complementary way to maximize bacterial coverage.

MATERIALS AND METHODS

Sampling and Infection Status of Quarters

Samples were taken from 18 clinically healthy lactating Holstein-Friesian cows in 2 Flemish dairy herds (herds 1 and 2) during the same month (April 2009). Herd 1 consisted of 87 cows characterized by an average milk production of 9,955 kg of milk per cow per year and an average bulk milk SCC of 202,000 cells/mL, whereas herd 2 consisted of 63 cows characterized by an average milk production of 10,372 kg of milk per cow per year and an average bulk milk SCC of 175,000 cells/mL. Lactating cows from both herds were milked twice a day (approximately every 12 h) and subjected to postmilking teat dipping, using an iodine disinfectant (Blockade; DeLaval, Drongen, Belgium), implying an average of 12 h between last dip and sampling.

Immediately before milking, the teat apices (n = 72) from 8 (herd 1) and 10 (herd 2) cows were sampled using a sterile swab (Copan, Brescia, Italy), covering the area (± 0.5 cm^2) around the teat orifice, as previously described (De Vliegher et al., 2003). One sterile swab was used for each teat, and contamination of the swab with milk was avoided. After sampling of the teat apex and removing some streams of milk, quarter milk samples were taken for bacteriological analysis, as previously described (Piepers et al., 2007; Dohoo et al., 2011). Briefly, 0.01 mL of each quarter milk sample was spread on a quadrant of a blood-esculin agar plate and incubated aerobically at 37°C for 36 h ± 12 h. A quarter was classified as infected when growth of at least one colony was detected (Dohoo et al., 2011). For all infected quarters included in this study, no clinical signs (e.g., redness and abnormal milk) were observed by the veterinarian performing the teat apex and milk samplings. Therefore, these quarters were labeled as "subclinically infected," whereas quarters that were not infected were designated "noninfected." The 8 sampled cows of herd 1 had 15 noninfected and 17 infected quarters; the 10 cows of herd 2 had 38 noninfected and 2 infected quarters. Swabs were transported to the laboratory under refrigeration and processed within 30 h. Briefly, the tips of the swabs were suspended in 5 mL of sterile saline solution [0.85% (wt/vol) NaCl and 0.01% (wt/vol) Tween 80; VWR International, Darmstadt, Germany] and vortexed vigorously for 5 min, yielding 5 mL of cell suspensions containing the bacteria under investigation (further referred to as bacterial swab samples). Each bacterial swab sample was analyzed in a culture-dependent and a culture-independent manner.

Growth Media

Bacterial cultivation was performed by plating on agar-containing growth media, including brain heart infusion (**BHI**; Oxoid, Basingstoke, UK) as a nonselective growth medium; de Man, Rogosa, Sharpe medium (**MRS**; Oxoid) for the growth of lactobacilli; MacConkey medium (**McC**; Oxoid) for the growth of gram-negative bacteria; medium 17 (**M17**; Oxoid) for the growth of streptococci; and mannitol salt agar (**MSA**; VWR International) for the growth of staphylococci. All media contained 1.5% (wt/vol) agar (Oxoid), were prepared according to the manufacturers' guidelines, and were autoclaved at 121°C for 20 min. Media were inoculated with 100 μL of the bacterial swab samples and incubated at 37°C for 24 to 48 h. Colony-forming units were enumerated and expressed per swabbed sur-