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Culture-independent exploration of the teat apex microbiota of dairy cows reveals a wide bacterial species diversity

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ABSTRACT

Due to their close proximity to the mammary gland tissue, the bacterial communities lining the teat apex of the udders from lactating cows influence udder health. Denaturing gradient gel electrophoresis of the amplified V3 variable region of the 16S rRNA gene was used as a culture-independent method to reveal the bacterial composition of 48 samples originating from the teat apices of twelve Friesian-Holstein dairy cows suffering from clinical mastitis in one quarter. The microbiota belonged to four bacterial phyla: the Actinobacteria (32% of all genera), the Bacteroidetes (1%), the Firmicutes (42%), and the Proteobacteria (25%), encompassing 17 bacterial genera. Some differences in occurrence of these genera were seen when comparing quarters that were non-infected ($n = 22$), subclinically infected ($n = 14$), or clinically infected ($n = 12$). Besides commensal skin-associated bacteria, opportunistic pathogenic bacteria, and mastitis-causing pathogens were found as well. The species diversity varied considerably among the most prevalent bacterial genera. While *Corynebacterium* and *Staphylococcus* displayed a large diversity among the recovered sequences, indicating the possible presence of a variety of different species, only a single bacterial species (represented by one sequence) was obtained for the genera *Aerococcus*, *Acinetobacter*, and *Psychrobacter*.

In conclusion, introducing culture-independent analysis of teat apical skin swabs in mastitis research revealed an unexpected wide bacterial diversity, with variations between quarters with a different clinical status. In addition to potential mastitis-causing pathogens, it exposed the yet poorly mapped presence of skin-associated and other bacteria residing in close proximity to the mammary gland tissue. PCR-DGGE may thus be considered as a useful tool for the entanglement of animal skin microbiota, *in casu* the teat apices of dairy cows.

1. Introduction

A major constituent of the immune system of all mammals is the skin which harbors a vast microbial species diversity that is conserved over time (Costello et al., 2009). A disruption of either the bacterial communities or the skin can result in skin disorders and/or infections, sometimes involving bacteria previously identified as harmless commensals (Ziebuhr et al., 2006; Grice and Segre, 2011). In the bovine mammary glands, infections occur mostly upon transgression of bacteria past the teat canal (Paulrud, 2005). Mastitis, an intramammary infection (IMI) as a result of

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bacterial invasion, exists in two forms: the clinical form is characterized by local or systemic symptoms, whereas subclinically infected quarters show no symptoms, although the somatic cell count (SCC) of their milk is increased (Barkema et al., 2006).

The entrance of mastitis-causing bacteria through the teat orifice is mediated by the sphincter muscles. As those remain dilated after milking, bacteria colonizing the teat apex are among the most likely to enter the mammary gland (Sieber and Farnsworth, 1981; Fox and Norell, 1994). Hence, the type of colonizing bacteria and the time of colonization can influence the development of IMI (Krömker and Friedrich, 2009). Certain udder skin-associated bacteria, such as coagulase-negative staphylococci (CNS), have a contradictive behavior that either results in the establishment of IMI (Pyörälä and Taponen, 2009) or protects against new IMI by other pathogens, either when colonizing the teat apex (Piepers et al., 2011) or when causing IMI (Piepers et al., 2010). Therefore, a better understanding of the microbiota present on the teat apex as point of entrance is required.

In the present study, denaturating gradient gel electrophoresis (DGGE) of PCR amplicons targeting the V3 region of the 16S rRNA gene was used as a culture-independent methodology to analyze the microbiota colonizing teat apices from lactating Holstein-Friesian cows with quarters of different infection statuses (non-infected, subclinically infected, clinically infected), to assess the overall bacterial species diversity, and to uncover the presence of teat skin-associated, and/or mastitis-causing bacteria.

2. Materials and methods

2.1. Sampling

Teat apices ($n = 48$) from twelve lactating Holstein-Friesian cows from four farms were sampled cross-sectionally. The twelve cows included in this study were selected out of an accessible pool of 60 cows, divided over six herds. Selection of the cows was based on the presence of a clinical infection in one quarter, while the other three quarters were either non-infected or subclinically infected. Diagnosis of clinical mastitis was based on local signs of inflammation (abnormal milk, swelling of quarter, etc.) and/or general symptoms of the cow. Depending on the SCC and presence/absence of clinical symptoms, quarters were divided in three categories: non-infected (no clinical symptoms and an SCC $<$ 200,000 cells/ml), subclinically infected (no clinical symptoms and an SCC $\geq$ 200,000 cells/ml), and clinically infected (presence of clinical symptoms).

Sampling was carried out using dry sterile swabs (Copan, Brescia, Italy), covering the area around the apices of the teat canals and prior to milking, as described by De Vliegher et al. (2003). All samples were transported under refrigerated conditions to and processed within 30 h in the laboratory. Under sterile working conditions, the tip of the swabs was suspended in plastic tubes containing 5 ml of sterile saline [0.85% (w/v) NaCl] and 0.01% (w/v) Tween 80 (VWR International, Darmstadt, Germany). The tubes were vortexed vigorously (5 min) and the cell suspensions were allocated into sterile plastic vials (Eppendorf AG, Hamburg, Germany). Centrifugation was performed at 21,000 $\times$ *g* for 30 min at 4 °C, followed by a careful removal of the supernatants. The cell pellets were stored at −20 °C.

2.2. DNA extraction

DNA extraction and purification were carried out using a DNA extraction kit (NucleoSpin® Tissue XS; Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's guidelines with an adjusted lysis step. Therefore, thawed cell pellets were resuspended in 80 μl of lysis solution [Tris–HCl (VWR International), 20 mM; EDTA, 2 mM; Triton X-100 (Sigma–Aldrich Chemie, Steinheim, Germany), 1 g/l; lysozyme (VWR International), 50 g/l; mutanolysin (Sigma–Aldrich Chemie), 500,000 U/l; pH 8.0] and incubated at 37 °C for 1 h. Subsequently, 8 μl of a 28.9 g/l proteinase K solution (VWR International) was added, followed by 1 h of incubation at 56 °C. After purification, DNA was obtained in a final dilution of 20 μl. DNA concentrations were measured using a NanoDrop ND-2000 (Thermo Scientific, Wilmington, DE, USA).

2.3. PCR conditions

DNA was subjected to PCR analysis using 16S rRNA V3-targeting primer combinations [forward primer (primer-F; F357): 5′-TAC GGG AGG CAG CAG-3′; reverse primer (primer-R; 518R): 5′-ATT ACC GCG GCT GCT GG-3′]. The forward primer was supplemented with a GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG-3′) to counter complete denaturation of the PCR amplicons inside the gels. For PCR amplification, a T3000 Thermocycler (Biometra GmbH, Goettingen, Germany) was applied. The mastermix contained 5 μl of 10× buffer (Roche, Mannheim, Germany), 2.5 μl of 0.1 mg/ml bovine serum albumin (BSA; Acros Organics, Geel, Belgium), 2.5 μM of deoxyribonucleoside triphosphates (dNTPs; Fermentas GmbH, St. Leon-Rot, Germany), 2 U of *Taq* polymerase (Roche), 3 μl of the DNA template, and 2 μl of 5 μM primer-F and primer-R each (Sigma–Aldrich Chemie). The PCR assay consisted of an initial denaturation at 95 °C for 5 min; a 38-cycle reaction of denaturation at 94 °C for 1 min, annealing at 55 °C for 75 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min. All PCR amplicons were checked on agarose gels (1.4%, w/v) for purity and the presence of DNA bands of the expected size.

2.4. DGGE and DNA band identification

A 1-mm thick 8% polyacrylamide gel (160 mm × 160 mm), using a denaturing gradient from 35 to 70% consisting of urea and formamide [100% denaturation corresponded to 7 M urea and 40% (w/v) formamide], was casted inside a DGGE apparatus (CBS Scientific, San Diego, CA, USA). For electrophoresis, a constant temperature of 60 °C and a constant voltage of 70 V were applied for 16 h in a tank filled with 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Each gel was loaded with PCR-amplified samples as described above and with a reference ladder