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Effects of bovine subclinical mastitis caused by *Corynebacterium* spp. on somatic cell count, milk yield and composition by comparing contralateral quarters

Juliano Leonel Gonçalves [a], Tiago Tomazi [a], Juliana Regina Barreiro [a], Daniele Cristine Beuron [a], Marcos André Arcari [a], Sarah Hwa In Lee [b], Cristian Marlon de Magalhães Rodrigues Martins [a], João Pessoa Araújo Junior [c], Marcos Veiga dos Santos [a,*]

[a] Department of Animal Sciences, School of Veterinary Medicine and Animal Sciences, University of São Paulo (USP), Pirassununga, SP, Brazil
[b] Department of Food Engineering, School of Animal Sciences and Food Engineering, University of São Paulo (USP), Pirassununga, SP, Brazil
[c] Institute of Biosciences, State University of São Paulo Júlio de Mesquita (UNESP), Botucatu, SP, Brazil

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ABSTRACT

Subclinical mastitis caused by *Corynebacterium* spp. (as a group and at the species level) was investigated by evaluating contralateral (healthy and infected) mammary quarters for somatic cell count (SCC), milk yield and composition. Selection of cows with subclinical mastitis caused by *Corynebacterium* spp. was performed by microbiological culture of composite samples collected from 1242 dairy cows from 21 dairy herds. For each of the selected cows, milk yield was measured and milk samples were collected at the mammary quarter level (i.e., 1140 mammary samples collected from 285 cows) for analysis of milk composition and SCC. The identification of *Corynebacterium* spp. isolates was performed by 16S rRNA gene sequencing.

One hundred and eighty *Corynebacterium* spp. isolates were identified, of which 167 (92.77%) were *C. bovis* and eight (4.44%) non-*C. bovis*; for five of the *Corynebacterium* spp. isolates (2.77%), sequencing of 16S rRNA genes did not allow identification at the species level. Mammary quarters infected with *Corynebacterium* spp. as a group had a higher geometric mean SCC (197,900 cells/mL) than healthy contralateral mammary quarters (85,800 cells/mL). Species of *Corynebacterium* non-*C. bovis* were infrequently isolated and did not change SCC, milk yield or milk solid contents when evaluated at the contralateral quarter level. Although *C. bovis* infection showed no effect on milk yield, fat, protein, casein or total solids in milk, it increased SCC and decreased lactose and milk solids non-fat content.

Introduction

Mastitis is the most costly disease affecting dairy cattle (Djabri et al., 2007; Halasa et al., 2007) and it presents most commonly in the subclinical form caused by bacteria (Djabri et al., 2002; Andersen et al., 2010). Subclinical mastitis increases the somatic cell count (SCC) and reduces the milk yield of dairy cows (Forsbäck et al., 2009). Losses occur due to damage caused by microorganisms to the secretory tissues of the mammary gland and the breakage of cell junctions, which may result in the permanent loss of milk synthesis capacity (Auldist et al., 1995).

Adoption of various strategies to control mastitis during the last few decades has resulted in a decreased frequency of clinical and subclinical mastitis caused by major pathogens. However, the frequency of subclinical mastitis caused by minor pathogens is still a challenge for dairy farmers (Haltia et al., 2006; Souto et al., 2008; Taponen and Pyorala, 2009). *Corynebacterium bovis* is a contagious microorganism frequently isolated from cases of subclinical mastitis. Despite the high frequency of isolation, *Corynebacterium* spp. are considered minor pathogens of mastitis (Bradley and Green, 2005; Schukken et al., 2009). The effects of *Corynebacterium* spp. on milk yield and composition remain largely unknown.

C. bovis has been described as a commensal of bovine mammary glands (Brooks and Barnum, 1984) and quarters infected with this bacterium may be less susceptible to intramammary infections caused by other mastitis pathogens (Rainard and Poutrel, 1982; Sordillo et al., 1989; Lam et al., 1997; Schukken et al., 1999; Blagitz et al., 2013). It has been reported that *C. bovis* only colonises the teat canal of dairy cows, and as such, it has been used as an indicator of milking hygiene (Watts et al., 2000). *C. bovis* has however

* Corresponding author. Tel.: +55 19 3545 4240.
E-mail address: mveiga@usp.br (M.V. dos Santos).

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also been reported to colonize the teat cistern, gland cistern, and mammary parenchyma (Benites et al., 2003).

Different methods have been used to estimate production losses due to subclinical mastitis in dairy cows. The most commonly used technique is based on the SCC for estimating production losses between herds, among cows, between mammary quarters, or even between identical twin cows (Petrovski et al., 2006; Pearson et al., 2013). The majority of studies have evaluated milk yield and milk composition between healthy and infected mammary quarters based on SCC (Barkema et al., 1997; Wilson et al., 1997; Forsbäck et al., 2010a, 2010b). However, these studies compared the mammary quarters of different cows and this may represent a bias due to heterogeneity between different animals and herds. To the best of our knowledge, no study has reported the effect of subclinical mastitis caused by *Corynebacterium* spp. on SCC, milk yield and composition by comparing healthy and infected contralateral mammary quarters. This approach could minimize confounding factors at both cow and herd level (such as the cow's immune status at the time of infection, management systems or environmental challenge). Such an experimental design may prove to be more reliable in evaluating the effect of *Corynebacterium* spp. on SCC, milk quality and yield.

The objectives of the present study were: (1) to determine the effect of subclinical *Corynebacterium* spp. mastitis as a group and at a species level on milk yield and SCC by evaluating the contralateral (healthy and infected) mammary quarters, and (2) to determine the effect of subclinical mastitis caused by *Corynebacterium* spp. on concentrations of milk fat, protein, lactose, casein, total solids and solids non fat.

Materials and methods

Herds and cow selection and sample collection

Twenty-one dairy herds located in the Mid-west area of São Paulo State, Brazil, were enrolled in this study over a 14-month sample collection period. To be enrolled in the study, herds needed to meet the following requirements: (1) good cow identification and recording systems and (2) proper milking management and mastitis control practices (including disposal of first streams of milk, disinfection of teats prior to and after milking using disinfectant solutions, drying teats with disposable towels, and treatment of clinical mastitis cases).

Cows were enrolled in the study based on microbiological cultures performed after the collection of two milk samples. First, composite milk samples (milk from all mammary quarters) were collected aseptically from each cow for screening for *Corynebacterium* spp. subclinical mastitis following National Mastitis Council guidelines (Oliver et al., 2004). After microbiological culture of the first milk sample, cows with *Corynebacterium* spp. were individually sampled at the quarter level within 15 days. At the second sample collection, milk yield was measured at the quarter level, and quarter milk samples were collected for microbiological culture and for analyses of composition and SCC.

To determine quarter milk yield and for analyses of composition and SCC, mammary quarters were milked individually using a bucket milking system (Intermaq Milking Systems), which was connected to the milking machine vacuum line. The equipment included a pulsator and a cluster of four liners connected to individual silicone tubing equipped with valves for vacuum release. The system allowed the milk to flow from each mammary quarter to a four-compartment stainless steel bucket. Quarter milk was stirred and weighed, and milk samples (40 mL) were collected into plastic tubes containing the antimicrobial Bronopol (2-bromo-2-nitropropane-1,3-diol) as preservative (0.05 g/100 mL milk) according to the International Dairy Federation guidelines (IDF (International Dairy Federation), 1995). Samples were kept refrigerated (4–7 °C) until analysis of composition and SCC.

This study (protocol number 2231/2011) was in conformity with the Ethical Principles in Animal Research adopted by the Ethical Committee on the Use of Animals of the School of Veterinary Medicine and Animal Science, at the University of São Paulo.

Microbiological culture procedures

Microbiological cultures of milk samples were performed according to the National Mastitis Council guidelines (Oliver et al., 2004). Briefly, 10 μL of milk was inoculated on blood agar with 5% defibrinated bovine blood. Inverted plates were incubated aerobically at 37 °C for 72 h and observed every 24 h for colonial characteristics (shape, size, number, and colour), haemolytic ability (presence and type), and possible contamination. Smears were stained by Gram and a catalase test was performed to determine the morphology and differentiation between *Corynebacterium* spp. genera.

Milk samples with more than two morphologically different colonies were considered contaminated. Briefly, after growth on blood agar, a single small, circular colony (approximately 1 mm in diameter) with a white-grey or yellowish colour and a slightly raised, dry and/or flaky, non-haemolytic appearance and Gram-positive rods was considered as *Corynebacterium* spp. Each isolate of *Corynebacterium* spp. was inoculated in a tube containing 1 mL of trypticase soy broth (TSB, Becton Dickinson). Tubes were incubated at 37 °C for 48 h, and centrifuged at 10,000 *g* for 10 min. Pellets were washed with 1 mL of sterile Milli-Q water, centrifuged again under the same conditions, and 1 mL of TSB containing 10% glycerol was added, followed by homogenization. A loop from each microtube was streaked on a plate containing tryptic soy agar (TSA, Becton, Dickinson) supplemented with 1% Tween 80 (Sigma Chemical Company) for confirmation (Watts et al., 2000). Only microtubes containing *Corynebacterium* spp. were cryopreserved at –20 °C.

Milk composition and SCC

Concentrations of milk fat, protein, lactose, casein, total solids and solids non-fat were determined by infrared absorption system using a milk analyser (MilkoScan FT+, Foss Electric). The SCC was determined by flow cytometry using a high-capacity somatic cell counter (Fossomatic FC, Foss Electric).

Corynebacterium *spp. subclinical mastitis*

Mammary quarters were considered to have subclinical mastitis when milk samples showed isolation of >10 colonies (1000 cfu/mL) of *Corynebacterium* spp. (Andersen et al., 2010; Dohoo et al., 2011a, 2011b). Mammary quarters were considered healthy when they had no growth of bacteria within 72 h incubation of milk from either sampling and an SCC of <200,000 cells/mL (Bradley and Green, 2005).

Gene sequencing for identification of Corynebacterium *spp.*

The isolates were identified genotypically by 16S rRNA sequencing analysis as described by Watts et al. (2000). A DNA extraction protocol was performed by adding lysozyme buffer solution, lysozyme (10 mg/mL; Merck) and resin solution (10 mg/mL, Chelex100 resin, Bio-Rad Laboratories), heated in a thermocycler at 99 °C for 10 min (Vaneechoutte et al., 1995). All isolates of *Corynebacterium* spp. were submitted to amplification with a pair of primers (F – 5′GCGAACGGGTGAGTAACACG3′ and R – 5′TCTGCGATTACTAGCGACTCCG3′) as described by Huxley et al. (2004). Isolates with no amplification were submitted to a second round of PCR using pairs of primers (F – 5′AGAGTTTGATCCTGGCTCAG3′ and R – 5′AAGGAGGTGATCCAGCCGCA3′) (Watts et al., 2000).

The second protocol amplification was used to amplify non-*C. bovis* species. All PCR reactions targeted the 16S rRNA gene. After electrophoresis analysis, the purified PCR products were sequenced unidirectionally using the reverse primers. All sequences obtained from the 16S rRNA gene sequences were analysed with GenBank[1] Library Reference online data. Isolates were identified at the species level when their similarities to reference sequences were ≥98% (Watts et al., 2000).

Statistical analysis

The effects of subclinical mastitis caused by *Corynebacterium* spp. as a group and at species level on quarter milk yield, composition and SCC were evaluated. The milk yield, composition and SCC of infected quarters were compared with the same variables from healthy contralateral quarters by a strip-plot design by splitting the anterior and posterior mammary quarters in halves. Thus, the left and right contralateral mammary quarters were compared within the half and cow using the following mixed model:

$$Y = \mu + IMI + Q + (IMI \times Q) + \{[C + C(H) + (C \times Q) + [IMI \times C(Q)]\} + e$$

where Y is the dependent variable, μ is the overall mean, IMI and Q are the fixed effects of variables in that IMI is the presence or absence of subclinical mastitis caused by *Corynebacterium* spp., and Q is the contralateral quarters (right or left) within cow; IMI × Q is the interaction between the fixed effects; C is the random effect of cow, C(H) is the random effect of cows nested within herd, and *e* is the random error term.

Somatic cell count at quarter level was converted to linear scores (LS) by the formula (Schukken et al., 2003) and was presented as geometric mean:

$$LS\ SCC = \log_2 (SCC/100) + 3$$

Statistical models were analysed using the MIXED procedure of SAS version 9.2. Statistical significance was defined at $P < 0.05$.

[1] See: http://www.ncbi.nlm.nih.gov/nuccore/AF311433.1 (accessed 2 August 2015).