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Molecular and proteomic identification of *Arthrobacter gandavensis* isolated from cows with subclinical mastitis in a dairy farm

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ABSTRACT

Aims: The genus *Arthrobacter* is a pleomorphic and heterogeneous Gram-positive bacteria mainly isolated from the soil, only two species of *Arthrobacter* have been reported worldwide as pathogens of veterinary importance. This paper aims to report the isolation and identification of the *Arthrobacter gandavensis* from cows with subclinical mastitis at a dairy farm in the savanna of Bogotá, Colombia.
Methodology and results: A total of 209 milk and skin samples were taken from cows with and without subclinical mastitis, nasal swabs from workers and the environment. All samples were cultured in blood and MacConkey agar and identified by 16S rRNA gene sequencing and mass spectrometry MALDI TOF-MS. From the isolates identified, 33 corresponded to *Staphylococcus* spp., nine to the Enterobacteriaceae family and seven from *Arthrobacter* spp. (only identified by MALDI-ToF MS). The *A. gandavensis* isolates were obtained from six different positive cows for the California mastitis test, all with a matching pattern corresponding to *Arthrobacter gandavensis* strain DSM N: 15046, isolated from milk from cows with subclinical mastitis in Belgium. Analysis of the 16S rRNA gene showed 100% genetic similarity with sequences of *A. gandavensis* previously reported in the NCBI databases.
Conclusion, significance and impact of study: The identification by MALDI-ToF-MS and molecular, as shown in this report, is important to provide data that allow us to approach the actual ecology of the opportunistic pathogens of subclinical mastitis, especially in regions where the infection is endemic.

Keywords: Arthrobacter gandavensis, MALDI-TOF MS, 16S rRNA, subclinical mastitis bovine

INTRODUCTION

Mastitis is the inflammation of the udder in dairy cows (Su *et al*., 2016). In Latin America, the most prevalent etiological agents of bovine mastitis are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis*, non-aureus staphylococci (NAS) and *Streptococcus uberis*. Mastitis is a polymicrobial disease (Angelopoulou *et al*., 2019), most microorganisms form part of the microbiota of the skin of the apex and the canal of the teats and they are also classified as minor pathogens (Cobirka *et al*., 2020), principal NAS and others like *Aerococcus viridans* (Liu *et al*., 2015), *Lactococcus lactis*, *Lactococcus garvieae* (Rodrigues *et al*., 2017) and *Arthrobacter gandavensis* (Storms *et al*., 2003). Only two species of the genus *Anthrobacter* have been identified as pathogens of domestic animals (Storms *et al*., 2003; Yassin *et al*., 2011). The characterization of new bovine mastitis pathogens using molecular and proteomic techniques leads us to reflect on the importance of correctly identifying the microorganisms that circulate in dairy herds. This purpose is relevant in countries where this disease is endemic in order to improve prevention and control strategies. The objective of this paper was to report the isolation identification of the species *Arthrobacter gandavensis* in *Holstein* cows with subclinical mastitis from a dairy farm in the Bogotá savanna in Colombia by using molecular and proteomic tools.

MATERIALS AND METHODS

Description of the study area

The study was carried out in a dairy herd of the geographical subregion corresponding to the Bogotá savanna. More than 95% of the farms are small, mainly subsistence farming. The farm had 107 Holstein cattle of

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which 65 were lactating cows with a high percentage (>50%) of subclinical mastitis in recent years. The milking system was a portable milking machine with good water availability. Raw milk was stored in traditional drums until it was sold. This research was endorsed by the ethics committee of the Faculty of Health Sciences of La Salle University, through act number 040 dated September 22, 2017.

Sampling

Milk samples were collected according to standard procedures recommended by the National Mastitis Council (Hogan *et al*., 1999). Before milking, the teats were disinfected with 70% ethyl alcohol, then the teat apices of a cow were swabbed with a single sterile swab. Before and after milking, the surfaces of stalls and milking equipment were sampled, and the inside of all liners was sampled by rubbing and turning the sterile swab. Nasal samples from milkers were taken from both nares using sterile swabs. The swabs were placed into Stuart medium (Innovation® - Italy) and transported to the laboratory, where they were immediately cultured.

California mastitis test (CMT)

A sample of milk from each quarter was collected into a plastic paddle, an equal amount of CMT reagent (sodium lauryl sulfate, bromocresol purple and bromothymol blue) was added to the milk and the contents were mixed. The CMT result was interpreted as negative (0), trace (T), weakly positive (1+), distinct positive (2+) and strongly positive (3+). Cows were considered positive when at least one quarter turned out to be trace or more.

Culture and microbiological identification

Each sample was cultured on blood agar and MacConkey agar and incubated at 37 °C for 24 h. The milk samples were considered positive if a single sample had ≥1 Colony Forming Unit (CFU)/10 µL (Hogan *et al*., 1999). Isolated colonies were identified by Gram's stain and subculture on trypticase soy agar under the same conditions for subsequent identification by the automated Vitek® 2 compact system (Biomerieux, Marcy l'Etoile, France) according to the manufacturer´s recommendations.

Proteomic identification by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

A colony incubated for 18-24 h at 37 °C in trypticase soy agar was placed on a 96-point steel plate (Bruker Daltonik, Bremen, Germany). A solution of 0.8 µL of 100% formic acid was applied (Sigma Aldrich, St. Louis, MO-Germany) along with 0.8 µL of the matrix solution of α-cyano-4-hydroxycinnamic acid (HCCA) in acetonitrile at 50% and 2.5% trifluoroacetic acid (Sigma Aldrich, St. Louis, MO Germany) and allowed to dry at 25 °C after adding each reagent. All samples were processed in duplicate. Protein mass spectra were analyzed using Flex Control® software and MALDI Biotyper version 3.1 7311 reference spectra (main spectra) (Bruker Daltonics, Bremen, Germany). The MALDI-ToF MS results were analyzed according to the manufacturer's technical specifications as follows: the correct identification of genus and species (≥2.0), the correct identification of the genus (1.7-2.0), or no reliable identification (<1.7). The MALDI-TOF Biotyper 3.1® software was used to analyze the protein profiles of the isolates that obtained a score of ≥2.0, with which a dendrogram was generated using a distance level corresponding to 1.0 as the arbitrary cut-off point.

Molecular identification by 16S rRNA analysis

The DNA (deoxyribonucleic acid) of 2 × 10^9 CFU obtained from the culture plate was extracted with PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA), after washing with ultra-pure water and following the recommendations of the kit. The 16S rRNA gene PCR (polymerase chain reaction) was performed according to the procedures reported in previous studies (Geha *et al*., 1994). The primers used to amplify of the 16S rRNA gene were: primer X 5'-GGA ATTCAA A[T/G, 1:1]G AAT TGA CGG GGG C and primer Y 5'-CGG GAT CCC AGG CCC GGG AAC GTA TTC AC, with a product size of 479 bp. A mixture of 200 µM dNTP, Tris 10 mM (pH 8.5), 50 mM KCl, 1.5 µM $MgCl_2$ and 1 µM of each primer (16S rRNA) was used for amplification in a final reaction volume of 50 µL. The PCR conditions were: 94 °C for 4 min, 30 cycles of 94 °C for 45 sec, 50 °C for 45 sec, 72 °C for 1 min, with a final extension of 72 °C for 2 min. Bacteria of the *Staphylococcus* genus (ATCC 43300) were used as a positive control to corroborate the amplification of gene 16S rRNA. The reaction control of all PCR reagents without template DNA was used to verify that there is no contamination with bacterial DNA. The products were purified by alcohol precipitation and sequenced with BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) using the ABI3730XL sequencer (Applied Biosystems). Sequence analysis was performed with MEGA 7 software (DNAStar, USA). The phylogenetic tree was built using the neighbor-joining method with the same software. Reference sequences from different species of *Arthrobacter* and specifically of *A. gandavensis* were included in the analysis, obtained from National Center for Biotechnology Information (NCBI).

RESULTS

On the study farm, 76.3% had subclinical mastitis with positive CMT. A total of 209 samples were analyzed, of which 199 (154 from milk and 45 from teats) were from animals, two from humans and eight from the environment. In 80 milk samples from cows with negative or trace CMT, there was no microbiological growth. Seventy of the isolated bacteria were not identified by any

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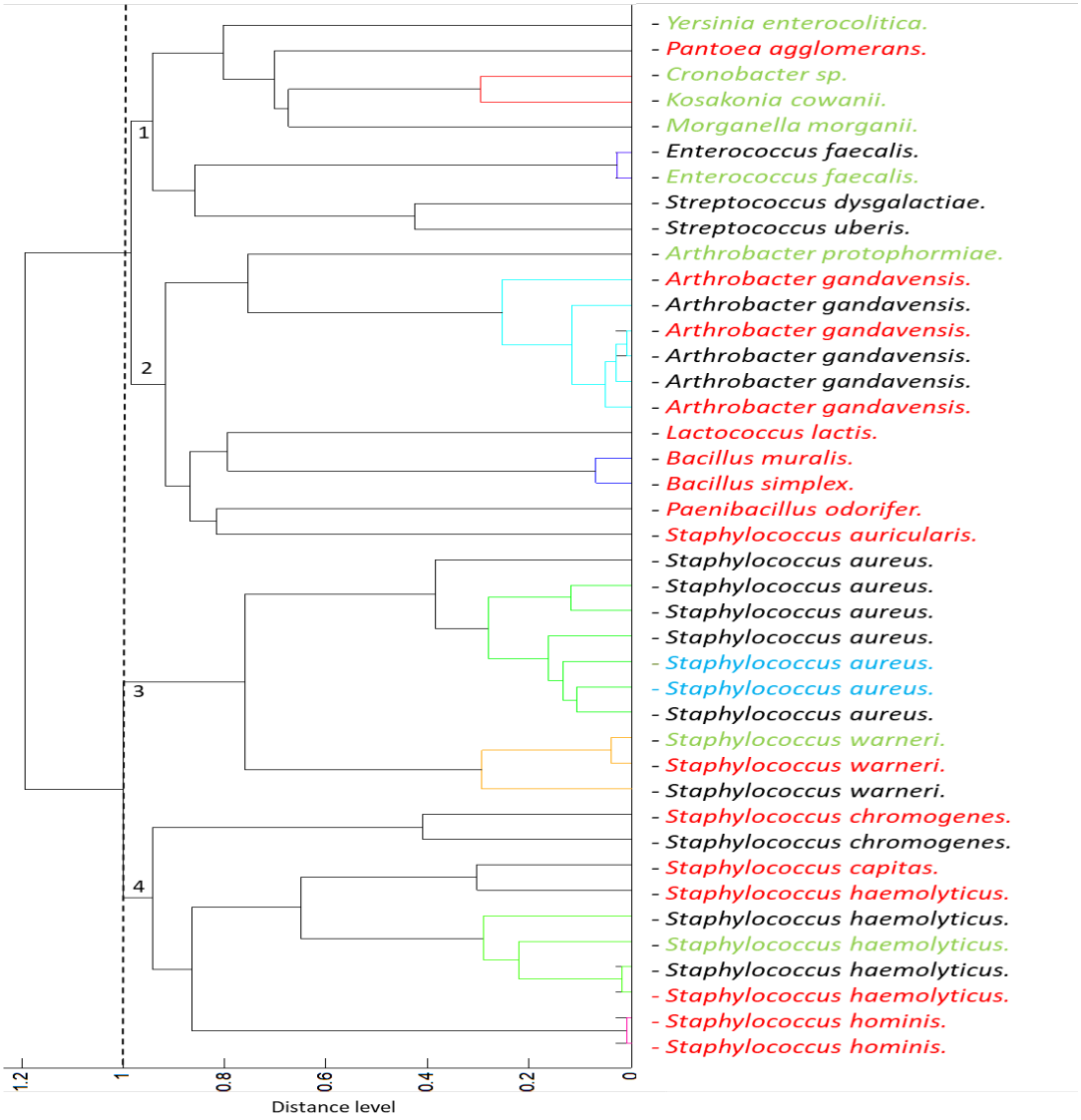

Figure 1: Dendrogram of the bacterial species identified by MALDI-TOF from cultures of milk (black) and teat apices (red) samples from cows with subclinical mastitis, nasal swabs from workers (blue) and from the environment (green) of the study farm, obtained by analyzing main components (PCA). Two clades and four sub-groups were evident, taking into account the cut-off point at a distance level of 1.0. The dendrogram was performed with the MALDI-TOF Biotyper 3.1 software.

methodology; nine were identified only by MALDI-TOF. From the isolates identified by Vitek® or MALDI-TOF, 33 corresponded to *Staphylococcus* spp., four isolates to the *Enterococcus* spp. and *Streptococcus* spp., seven from *Arthrobacter* (identified only by MALDI-TOF), nine to the Enterobacteriaceae family, one *Pseudomona* sp. and seven belonged to other Gram-positive rods.

In order to generate the representative dendrogram of the associations of protein profiles of the different isolates, mass spectra with scores ≥2.0 were used. Two clades and four subgroups are observed. Isolates of environmental origin with or without pathogenic potential were grouped in the first clade and in the second clade were grouped the different species of the *Staphylococcus*, *S. aureus* and NAS, classified as contagious and opportunistic agents of bovine mastitis, respectively (Figure 1). In the first clade of the dendrogram in subgroup 2, six isolates of *A. gandavensis* were identified, all with a score >2.0 and with a matching pattern corresponding to *Arthrobacter gandavensis* strain DSM N: 15046 (NBCI Identifier 169960), isolated from milk from cows with subclinical mastitis in Belgium (Storms *et al.*, 2003). In this subgroup, one environmental isolation of the same genus was also found, obtained from the stalls in the portable milking machine (Figure 1). The *A. gandavensis* isolates were obtained from six different

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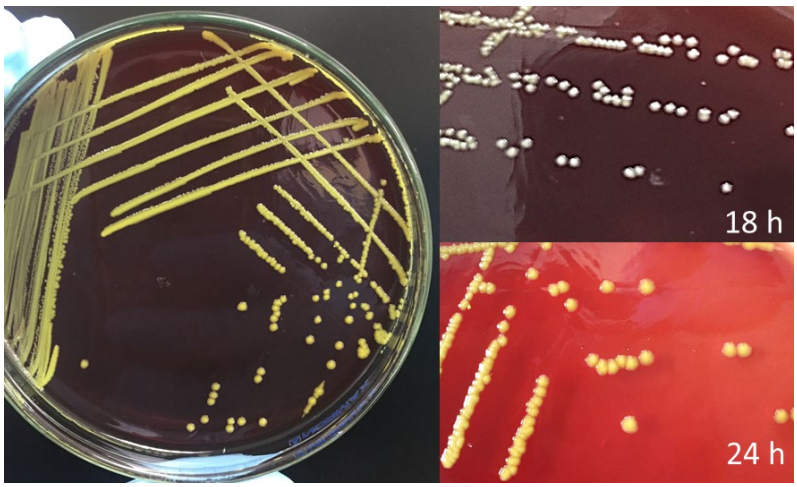

Figure 2: Culture of *Arthrobacter gandavensis* on blood agar at 37 °C isolated from milk and teat apices of cows with a diagnosis of subclinical mastitis. Bright cream-colored round colonies were observed at 18 h and yellow colonies at 24 h.

cows, three from the teat apex and three isolates from milk from positive cows for CMT (score 2+, 1+ and traces).

The microbiological culture of *A. gandavensis* on blood agar and trypticase soy agar was characterized by bright yellow colonies in which their intensity increased over time (Figure 2). The six isolates were observed with Gram staining as dotted Gram-positive coccobacilli; however, these isolates were not identified by Vitek®. They reacted positively for the following biochemicals: N-acetyl-d-glucosamine, methyl-bd-glucopyranoside, leucine arylamidase, l-proline-arylamidase, d-galactose, d-ribose, d-xylose d-mannose, d-maltose, d-trehalose, lactose and sucrose. Analysis of the 16S rRNA gene showed 100% genetic similarity with sequences of *A. gandavensis* previously reported in the NCBI databases (Figure 3). The bootstrap resampling value to infer the phylogenetic tree was 99% (1000 replicates).

DISCUSSION

The use of molecular and proteomic techniques such as 16S rRNA gene sequencing, pulsed-field gel electrophoresis, random amplified polymorphic DNA (RAPD) and MALDI-TOF MS have revealed a great diversity of the opportunistic pathogen, which are part of three phyla mainly: Firmicutes (*Staphylococcus*, *Aerococcus*, *Streptococcus* and *Lactococcus*), Actinobacteria (*Corynebacterium*, *Kocuria*, *Propionibacterium* and *Arthrobacter*) and Proteobacteria (*Acinetobacter*, *Pseudomonas* and *Enterobacter*) (Piessens *et al*., 2012; Verdier-Metz *et al*., 2012; Derakhshani *et al*., 2018).

The genus *Arthrobacter* belongs to the phylum Actinobacteria and the Micrococcaceae family. It is characterized by being pleomorphic and heterogeneous bacteria, Gram-positive, aerobic cocobacilli, with one pointed end and not spore-forming. This genus can to survive under stressful conditions; therefore, it is widely distributed in the environment, mainly in the soil (Mongodin *et al*., 2006; Wang and Xie, 2012; Liu *et al*., 2019; Viegas *et al*., 2019). Two species of the genus *Anthrobacter* have been identified as pathogens of domestic animals, *A. equi*, isolated from a mare's vaginal discharge and identified as a new species within the genus by sequencing the 16SrRNA gene showed in phylogenetic analysis a high similarity to other environmental species such as *A. chlorophenolicus*, *A. defluvii* and *A. niigatensis*. *A. equi* has a wide variety of enzymes that allow it to degrade almost all carbohydrates, using nitro-based compounds and the ability to degrade oxygen reagents (Yassin *et al*., 2011). The second species, *A. gandavensis* was first identified in milk from cows with subclinical mastitis on dairy farms in Ghent, Belgium. Its genome has been fully sequenced and classified as an animal pathogen within the risk group that requires biosafety level 2. Up to now, 3,360 proteins of the bacterium have been identified, including enzymes such as galactosidases, glucosidases, mannosidases and others (Storms *et al*., 2003). The isolates of *Arthrobacter* in this study also had positive ten carbohydrate assimilation tests, indicating that genus *Arthrobacter* can degrade almost all these compounds of bovine milk.

The isolates from different cows in this study were identified by mass spectrometry with a score >2.0 and the matching pattern corresponding to the DSM N: 15046 strain isolated from subclinical mastitis in Belgium (Storms *et al*., 2003). These results were also confirmed through the analysis of the 16S rRNA gene. In the USA on a farm at the University of Illinois, the genus *Arthorbacter* was also identified as an etiological agent of subclinical mastitis in *Holstein* cows, but without being able to identify the species (Dhoble *et al*., 2019). Other researchers in France using 16S rRNA sequencing also identified this species as skin commensals of the teats of *Holstein* and *Montbéliarde* cows (Verdier-Metz *et al*.,

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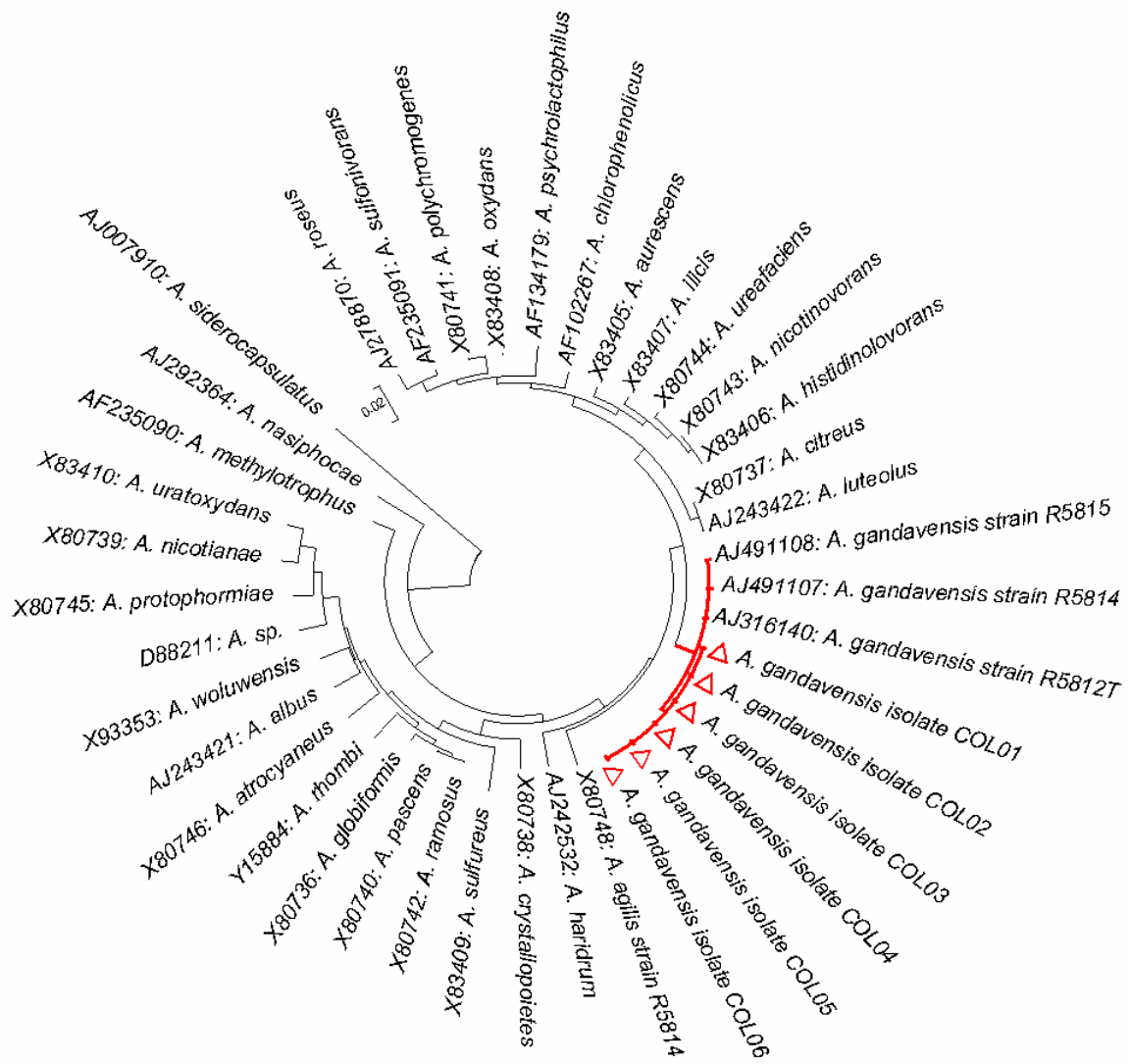

Figure 3: Phylogenetic relationships derived from analysis of 16S rRNA sequences of six isolates of *A. gandavensis* (red triangles) and type strains of other species within the genus *Arthrobacter*. The neighbor-joining method was used to construct the phylogenetic tree. The bootstrap resampling value was 99% (1000 bootstrap replicates). Bar: 2% sequence divergence.

2012). Likewise, we identified three isolates of *A. gandavensis* in the skin of cows' teats, confirming that this bacterium also forms part of the microbiota of the skin of the teats. *Arthorbacter* has also been identified in raw tank milk obtained from farms in central New York, along with the genera *Corynebacterium*, *Streptococcus*, *Lactobacillus*, *Coxiella* and *Lactococcus*. Although a strong association between the number of *Arthrobacter* observed taxonomic units (UTO) and high somatic cell count (RCS) (>200,000 cells/mL milk) could not be established, no association with UTOs in the microbiome was found of environmental origin (Rodrigues *et al.*, 2017). The precise identification of these agents, a minor causative agent of bovine mastitis, will help not only in the diagnostic confirmation but also to elucidate the epidemiology and ecology of these bacteria in order to guide the management and control plans for the eradication of the disease.

CONCLUSION

The genus *Arthrobacter* is a Gram-positive bacterium widely distributed in the environment characterized by its ability to survive in adverse conditions. The species *A. gandavensis* was identified by proteomics and 16S rRNA analysis in the milk and teat apex of cows with subclinical mastitis. Those isolates had the same phenotypic characteristics previously reported in cows with mastitis in Belgium, so we consider it is important to continue with the study of these and other opportunistic causative

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agents of bovine mastitis in order to know their epidemiology and ecology. These results also show the importance of using more sensitive and specific diagnostic methods to identify the great diversity of etiological agents of polymicrobial diseases such as subclinical mastitis.

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