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Adherent/invasive capacities of bovine-associated *Aerococcus viridans* contribute to pathogenesis of acute mastitis in a murine model

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ABSTRACT

Aerococcus viridans, a firmicutes bacteria widespread in the environment, is increasingly isolated from humans and animals, especially cows with mastitis. However, its pathogenicity in the bovine mammary gland is unclear. The objective was to explore pathogenic potential of putative virulent and avirulent *A. viridans* in murine systemic and intramammary infection and mechanistically in cultured bovine mammary epithelial cells (bMECs). Virulence of 9 strains of *A. viridans*, isolated from subclinical cases of mastitis, was tested for their ability to kill mice when systemically inoculated. Two *A. viridans* strains, causing highest and lowest survival rate in mice, were selected further as putative avirulent and virulent strains, respectively. *Staphylococcus aureus* N305 was used as a positive control. After intramammary inoculation, the virulent strain survived and replicated in the murine mammary gland for 9 d, whereas the avirulent strain was eliminated within 3 d. The virulent strain induced a robust inflammatory reaction in the mammary gland, characterized by acute histopathological changes, increased myeloperoxidase activity and higher expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) compared to the avirulent strain. The virulent strain produced CAMP factor and exhibited strong cytotoxic effects (LDH release) and adhering and invasive abilities in contact with bMECs. Adhesion and invasion of virulent strain to bMECs was further confirmed by scanning and transmission electron microscopy; there was severe damage, including cytomembrane disruption, swollen mitochondria and loss of organelles. In conclusion, the putative virulent strain of *A. viridans* activated a strong neutrophil-based inflammatory response in the mammary gland, attributed to its ability to adhere to and invade mammary epithelium.

1. Introduction

Mastitis is the most common and important disease of the dairy industry, causing huge economic losses (Barkema et al., 2009). During the last 40 y, intramammary infection (IMI) and mastitis caused by major pathogens have been reduced by implementation of specific mastitis preventive and control programs (Neave et al., 1969; Olde Riekerink et al., 2006). However, prevalence of IMI and the incidence of mastitis caused by minor pathogens has increased (Schukken et al., 2009). One of the increasingly commonly isolated bacteria from milk of dairy cows is *Aerococcus viridans* (Devriese et al., 1999; Liu et al., 2015; Zadoks et al., 2004). Although this bacterium was not traditionally considered a mastitis pathogen (Wyder et al., 2011), it has been associated with bovine mastitis (Liu et al., 2015; Saishu et al., 2015; Špaková et al., 2012; Sun et al., 2017).

Aerococcus viridans is a Gram-positive microaerophilic coccus, widely spread in the environment, including hospitals, seawater and animal farms (Vos et al., 2009). As an opportunistic pathogen, *A. viridans* has been clinically associated with endocarditis, arthritis and urinary infections in humans and pigs (Martin et al., 2007; Moreno et al., 2016; Rasmussen, 2016), septicemia and fatal infection in crustaceans and fish (Clark and Greenwood, 2011). Biochemically, *A. viridans* is catalase-negative or weakly-positive, oxidase-negative and causes green alpha hemolysis on blood agar (Liu et al., 2015). That this biochemical profile is similar to staphylococci or streptococci may have caused underestimation of the incidence and prevalence of *A. viridans* IMI (Raemy et al., 2013).

Questions regarding pathogenicity of *A. viridans* arose early in lobsters, with considerable variation in virulence among strains (Clark and Greenwood, 2011; Stewart et al., 2004). In cattle, *A. viridans* has been increasingly isolated from milk samples from cows with subclinical or clinical mastitis (Liu et al., 2015; Raemy et al., 2013; Špaková et al.,

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Table 1
Criteria of clinical scoring.

Clinical Score*	Criteria
1	Active, responsive and no signs of any illness
2	Slower in reaction to stimuli, but otherwise active and healthy
3	Slow and lethargic, but still active
4	Inactive but still responsive to stimuli, albeit slowly
5	Inactive and non-responsive to any stimuli
Additional score of 0.5 was added cumulatively with the presence of:	• Intestinal disturbances (diarrhea or loose fecal pellets) • Shivering • Dyspnea

* The maximal score is 6.5 (main clinical scores + additional scores of 0.5).

2012; Sun et al., 2017), although its pathogenic potential remains elusive. Of interest is to determine whether *A. viridans* is capable of inducing innate immune and inflammatory responses during IMI and characterize the type of mastitis produced. Therefore, the objective was to explore pathogenicity of *A. viridans* isolates from subclinical mastitis cases (Liu et al., 2015), based on capacity to produce systemic and mammary infections using murine models (systemic and mammary) and bovine mammary epithelial cells (bMECs).

2. Materials and methods

2.1. Statement of ethics

The present study was conducted in compliance with guidelines of the Beijing Municipality on the Review of Welfare and Ethics of Laboratory Animals, approved by the Beijing Municipality Administration Office of Laboratory Animals (BAOLA) and approved by the China Agricultural University Animal Ethics Committee (protocol CAU-AEC-2010-0603).

2.2. Bacterial strains

Nine genetically characterized (based on an RAPD subtyping assay) *A. viridans* strains (1 strain of each subtype) were selected from 60 strains isolated from subclinical mastitis milk samples (SCC > 500,000 cells/mL) collected from 10 dairy herds in Beijing, Tianjin and Hebei province in China (Liu et al., 2015). Isolates were identified by a commercially available identification system API Rapid 20 Strep system (bioMérieux, SA, Marcy l'Etoile, France), followed by the confirmation with sequence of 16S rRNA gene and comparison between sequencing data and the GenBank database. All isolates were stored at the mastitis diagnostic laboratory at the College of Veterinary Medicine, China Agricultural University (Beijing, China). *Staphylococcus aureus* N305 (ATCC 29740) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Prior to each experiment, fresh bacterial suspensions were prepared from frozen stocks by culture on tryptose soya agar (DifcoTM, Becton Dickison, Sparks, MD, USA) supplemented with 5% defibrinated sheep blood (SBA) and incubated at 37 °C for 24 h. Thereafter, strains were sub-cultured on brain heart infusion broth (DifcoTM) for 18 h to mid-log phase. The Colony forming units (CFUs)/mL were determined from the 1/100 diluted bacterial preculture by spectrophotometric measurements at 600 nm (OD600) and were confirmed a *posteriori* by plating on tryptic soy agar (DifcoTM) plates. The pathogens were suspended in PBS to achieve the required concentration.

2.3. Survival rate of mice systemically challenged by A. viridans

Virulence of *A. viridans* strains was initially investigated by systemically challenging mice. The 9 *A. viridans* strains were washed in triplicate in sterile saline and bacterial concentration adjusted to 1.0×10^8 CFUs/mL. Each strain (total of 0.5×10^8 CFUs in 0.5 mL), was injected intraperitoneally into female, 4-6-week-old Kunming (KM) mice (Merial-Vital Laboratory Animal Technology, Beijing, China). Mice injected with the same volume of sterile saline were used as a negative control. Mouse survival for 10 d was determined as described (Zhao et al., 2013). Base on survival rate, 1 putative virulent strain (caused highest mouse mortality) and 1 avirulent strain (caused lowest mouse mortality) were studied to determine potential to cause mastitis.

2.4. Murine mammary infection model for A. viridans

A murine model of intrammamary challenge with bovine mastitis pathogens has been successfully used to assess bacterial infection and tissue damage (Pereyra et al., 2017). To determine the pathogenic role of *A. viridans* during IMI, female 6–8 wk old specific-pathogen-free pregnant (20 d) BALB/c mice were purchased from Merial-Vital Laboratory Animal Technology (Beijing, China). Mice were kept in germ-free isolators and fed *ad libitum* in a controlled environment with light and dark cycles (12 h light: 12 h darkness). The pups were removed 2 h before of the intramammary inoculation. At 3 d after parturition, mice were anesthetized (intramuscular injection with 50 mg/kg Zoletil 50 (Virbac, Carros, France)). The mammary gland ducts of the fourth pair of mammary gland were exposed by cutting the teat tip and the sample was slowly intraductally injected at a volume of 50 µL with a 33 gauge blunted needle. Four groups (n = 20 per group) of mice were allocated: two groups with either a virulent or an avirulent strain (10^6 CFUs), compared to a negative control group (sterile phosphate-buffered saline, PBS) and a positive control group (10^6 CFUs of *Staphylococcus aureus* N305 in PBS). Mice were sedated and euthanized by cervical dislocation and mammary glands aseptically collected at 1, 3, 5, and 9 d post inoculation (pi) (5 mice in each group).

2.5. Bacterial burden and clinical and histopathological evaluation

The general condition of each mouse was assessed daily by clinical scoring (Table 1; Johnzon et al., 2016). At necropsy, inoculated mammary glands were excised, weighed, placed in 5 mL of PBS, minced (scalpel) and homogenized (vortex with grinding beads). For bacterial burden, 50 µL of various dilutions of each tissue were plated onto SBA and incubated overnight at 37 °C. Numbers of viable colonies were counted and expressed as CFUs/g (Tuchscherr et al., 2005).

Mammary gland tissue was fixed in 10% formalin buffer, embedded, sectioned and stained with hematoxylin-eosin (H&E). Histological scoring was performed according to tissue necrosis, polymorphonuclear neutrophilic granulocyte inflammation (i.e., neutrophilic inflammation), lymphocytic inflammation (Breyne et al., 2017). Each feature was graded semi-quantitatively in ten different high power fields at 200× magnification and an average score for each category was calculated. The semi-quantitative scoring was performed using the criteria listed in Table 2. All scoring was evaluated by an experienced veterinary pathologist (blinded to inoculation status).

Table 2
Criteria of histological scoring.

Histological Score*	The histologic feature (necrosis, neutrophils, lymphocytes)
1	Absent
2	Minimal (the feature was present in scant or very small amount)
3	Mild (the feature was consistently present in low numbers)
4	Moderate (the feature was prominent and distinctive)
5	Severe (the feature was overwhelming and normal architecture of the gland was obscured)

2.6. Measurement of myeloperoxidase (MPO) activity and pro-inflammatory cytokines

MPO activity was analyzed with the test kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Briefly, the mammary gland tissues were homogenized and then fluidized in extraction buffer to obtain 5% of homogenate, heated to 37 °C in water for 15 min with 100 μL reaction buffer and 900 μL homogenate. The activity was evaluated by measuring the change in absorbance at 460 nm using a 96-well plate reader. The concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were determined using mouse specific enzyme-linked immunosorbent assays (eBioscience, San Diego, CA, USA).

2.7. Mammary epithelial cell culture

bMECs (MAC-T) (Shanghai Jingma Biological Technology Co., Ltd. China) were cultured in DMEM/F-12 (HyClone, Logan, UT, USA) supplemented with 10% (v/v) Gibco® Fetal Bovine Serum (FBS; HyClone, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C with 5% CO_2.

2.8. Cytotoxic LDH release assay

A lactate dehydrogenase (LDH) assay was used to evaluate cytotoxic effects of *A. viridans* on bMECs (Cytotoxicity LDH Assay Kit-WST®; Dojingdo Laboratories, Kumamoto, Japan). Cells cultured at 37 °C with 5% CO_2 in 96-well plates (Corning, New York, NY, USA) were challenged with either the virulent or avirulent strain, at a multiplicity of infection (MOI, ratio of *A. viridans* to cells) of 5:1 for 3, 6, 12 and 18 h. Non-infected cells were similarly incubated as control. After incubation, supernatants were collected and centrifuged at 18,000 g for 15 min. Supernatants were collected and LDH release quantified by absorbance at 490 nm (ELISA reader, Thermo Fisher Scientific, Waltham, MA, USA). Cytotoxicity (%) was calculated using Triton X-100 treatment cells as positive control (100% cytotoxicity), while cells only treated with cell culture medium were used as negative control (0% cytotoxicity), according to Eqation:

$$\%\,\text{cytotoxicity} = \frac{Abs_{experiment} - Abs_{\text{negative conctrol}}}{Abs_{\text{positive conctrol}} - Abs_{\text{negative conctrol}}} \times 100$$

2.9. Aerococcus viridans adhesion and invasion assays

Adherence and invasion are vital virulence mechanisms in bacterial IMI (Dogan et al., 2006). In our preliminary trials, 100 μg/mL of penicillin/gentamycin (Sigma Aldrich; St. Louis, MO, US) killed *A. viridans* (1×10^7 CFUs/mL) within 2 h (we tested penicillin/gentamycin 50, 100 and 150 μg/mL for 1, 2, and 3 h). Therefore, 100 μg/mL of penicillin/gentamycin for 2 h was used for the invasion assay of *A. viridans,* as described (Chen et al., 2017; Pereyra et al., 2016), with minor modifications. The bMECs cultured in 6-well plates (Corning; New York, NY, USA) and infected with either *A. viridans* or *S. aureus* N305 at an MOI of 50:1 for 30 min and for 1, 2, and 3 h. Following incubation, cells were washed with sterile PBS (pH 7.4) in triplicate and treated with 100 μg/mL penicillin/gentamycin or gentamicin (Sigma Aldrich) for 2 h to kill extracellular *A. viridans* or *S. aureus*, respectively. Supernatants were collected and plated on SBA to verify killing. Infected cells without antibiotic treatment were used as a control group. Cells were washed with sterile PBS in triplicate and lysed with 0.5% (v/v) triton X-100. Cell lysates were diluted 10-fold, cultured onto SBA and incubated at 37 °C for 24 h to count CFUs. Invasion rate of adhered bacteria was expressed as:

$$\text{Invasion rate of bacteria} = \frac{\text{Lysate of infected cells with antibiotic treatment (CFUs/mL)}}{\text{Cell lysates and treated bacterial supernatants in control group (CFUs/mL)}} \times 100$$

To assess adhesive capacity of *A. viridans* to bMECs, an adhesion assay was performed as described (Chen et al., 2017) with slight modifications. bMECs cultured in 6-well plates (Corning) for 3 d in antibiotic-free medium to reach confluency were infected with *A. viridans* or *S. aureus* (MOI 50:1) for 30 min and for 1, 2, and 3 h at 37 °C with 5% CO_2. After incubation, cells were washed in duplicate with sterile PBS (pH 7.4) to remove non-associated bacteria. Cells were lysed by adding 1 mL PBS and 1 mL 1% triton X-100 (0.5%v/v) to release associated bacteria (adhering and invasive). In the control group, both bacterial suspensions (1 mL) and cells were also treated with 1 mL triton X-100. Cell lysates and treated bacterial supernatants were diluted 10-fold, cultured onto SBA and incubated at 37 °C for 24 h to count CFUs. The adhesion rate was determined as follows:

$$\text{Adhesion rate of bacteria} = \frac{\text{Cell lysate of infected group (CFUs/mL)}}{\text{Cell lysates and treated bacterial supernatants in control group (CFUs/mL)}} \times 100 - \text{Invasion rate}$$

Invasion and adhesion assays were repeated 3 times and each experiment was performed in triplicate.

2.10. Ultrastructural morphology of bovine mammary epithelial cells challenged by Aerococcus viridans

For scanning electron microscopy (SEM), bMECs were cultured on coverslips and inoculated with both strains of *A. viridans* (MOI 5:1) for 3, 6, 12 and 18 h. Treatment with the same volume of sterile PBS was used as negative control. After treatment, coverslips were washed with sterile PBS in duplicate and fixed with 2.5% glutaraldehyde (Sigma Aldrich; St. Louis, MO, USA) at 4 °C for 1.5 h. After washing, cells were dehydrated through a graded series of ethanol (30, 50, 70, 80, 90 and 100% ethanol) for 15 min for each step at room temperature. Cells were dried (critical point method) before being sputter coated with gold in aE-1010 Ion Sputter Coater (TESCAN 5136, Brno, Czech Republic) and then examined with SEM (Hitachi H-7650, Tokyo, Japan).

For transmission electron microscopy (TEM), cells were harvested and treated as SEM before dehydration. After dehydration by graded ethanol and acetone (three changes, 10 min each), cells were sequentially embedded in epoxy resin acetone mixtures (2:1) for 2 h and in pure resin overnight at 37 °C. After resin had polymerized, ultra-thin sections were cut by an ultramicrotome (Leica EM, Germany), stained with 1% uranyl acetate followed by lead citrate and examined with TEM (Hitachi H-7650, Japan).

2.11. Gene expression of pro-inflammatory cytokines and Toll-like receptors in mammary epithelial cells challenged by Aerococcus viridans

bMECs were challenged with either *A. viridans* or *S. aureus* N305 (MOI of 5:1) for 1, 3, 6 and 12 h. Total RNA was isolated from bMECs using Trizol Reagent (Invitrogen; Carlsbad, CA, USA) and reverse-transcribed using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). A quantitative real-time polymerase chain reaction was performed at 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 60 s, 72 °C for 60 s. Primer sequences are shown (Table 3). Gene

Table 3
Primers used in this study.

Gene	Primer	Sequence 5′ > 3′	Product size (bp)
Tumor necrosis factor-α	Forward	GCCTCCCTCTCATCAGTTCTA	246
	Reverse	GGCAGCCTTGTCCCTTG	
Interleukin-1β	Forward	ACCTGTGTCTTTCCCGTGG	162
	Reverse	TCATCTCGGAGCCTGTAGTG	
Interleukin-6	Forward	AGTTGTGCAATGGCAATTCTGA	223
	Reverse	CCCCAGCATCGAAGGTAGA	
TLR2	Forward	CGATGACTACCGCTGTGACTC	224
	Reverse	CCTTCCTGGGCTTCCTCTT	
TLR4	Forward	TGCCTTCACTACAGGGACTTT	101
	Reverse	TGGGACACCACGACAATAAC	
GAPDH	Forward	TGCTGTCCCTGTATGCCTCT	224
	Reverse	TTTGATGTCACGCACGATTT	
β-actin	Forward	TCACCAACTGGGACGACA	206
	Reverse	GCATACAGGGACAGCACA	

expression levels were standardized to the corresponding GAPDH threshold cycle (Ct) values using the $2^{-\Delta\Delta Ct}$ comparative method.

2.12. . Activity of CAMP factor in Aerococcus viridans

The standard CAMP test was performed as described (Brown et al., 1974). Briefly, *A. viridians* were streaked on SBA perpendicular to, but not touching, a β-hemolysin-producing *S. aureus* (ATCC25923). After 20 h of incubation at 37 °C, *A. viridians* were inspected for CAMP hemolysis.

2.13. Statistical analyses

Data were presented as means ± standard deviation (SD) and each experiment was repeated three times. The differences between groups were assessed using one-way ANOVA, followed by the least significant difference (LSD) multiple comparisons of treatment means. Statistical analyses were performed using Origin version 8.0 (OriginLab; Northampton, MA, USA). A P-value < 0.05 was considered significant.

3. Results

3.1. Certain strains of A. viridans had lethal effects on mice when challenged systemically

Mouse survival after intraperitoneal challenge was determined using 9 strains of *A. viridans* isolated from bovine mastitis (Fig. 1). Highest mortality occurred in mice challenged with *A. viridans* isolate AV40, with only 10% of mice surviving after 4 d pi (Fig. 1). Lowest mortality occurred in mice challenged by *A. viridans* isolate AV36, with 90% of mice surviving after 10 days pi (Fig. 1). Mortality rates of other *A. viridans* isolates ranged from 10 to 20%. Hence, *A. viridans* strains AV40 and AV36 were selected as putative virulent and avirulent strains, respectively.

3.2. Virulent Aerococcus viridans caused severe clinical and histopathological mastitis in mice

Pathogenicity of two *A. viridans* strains was assessed in a mouse IMI model, compared to *S. aureus* N305. Clinical mastitis was scored from 1 to 5, with 1 representing normal activity and 5 representing inactive, unresponsive animals (Table 1), with an additional score of 0.5 added if other clinical symptoms (intestinal disturbances, shivering and dyspnea) were present. Both virulent *A. viridans* strain and *S. aureus* N305 provoked a significantly higher clinical score than the avirulent strain from days 1 to 9 pi (Fig. 2A), including manifestations such as exacerbated dyspnea, anorexia, sluggishness and an irregular hair coat.

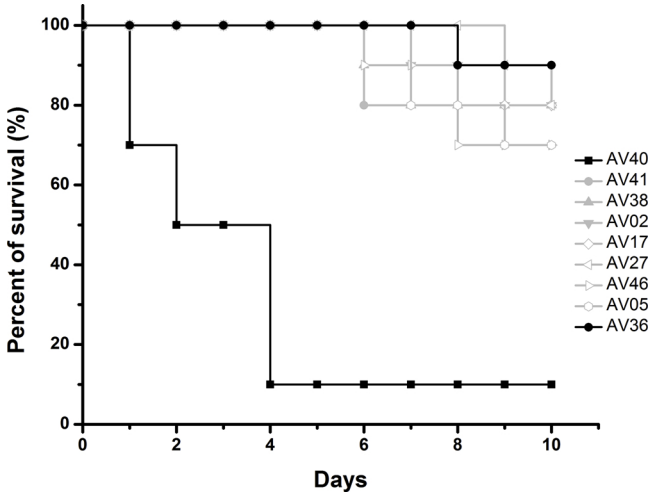

Fig. 1. Survival rate of mice intraperitoneally challenged by 9 strains of *Aerococcus viridans*.

Macroscopically, mice challenged with virulent *A. viridans* had severe edema and hyperaemia in mammary glands at days 1 and 5 pi. Mice challenged with the virulent strain suffered from dyspnea from days 5 to 9 pi, with histological evidence of congestion and edema in lungs detected, with *A. viridans* isolated from these tissues (data not shown).

Histological characteristics of the *A. viridans* and *S. aureus* N305 infected mammary glands were assessed by H&E staining (Fig. 2B and C). Histologically, both virulent *A. viridans* and *S. aureus* N305 caused more severe mastitis compared to both control and avirulent strain groups until day 9 pi (Fig. 2B). Specifically, at 1 d pi, infiltrating inflammatory cells (mainly lymphocytes and neutrophils) were identified in the interstitium of the mammary gland in mice infected with virulent *A. viridans* and some mammary epithelial cells were present in the lumen of mammary acini, due to cell degradation and necrosis. Some infiltrating inflammatory cells were noticed in the lumen of *S. aureus* N305 chanllenged mammary gland, without degraded epithelial cells (Fig. 2C). Massive infiltration of inflammatory cells and discontinuous linings of epithelial cells were observed in mice infected with both virulent *A. viridans* and *S. aureus* N305 at 5 d pi, whereas at 9 d pi, countless inflammatory cells were present in interstitium and acini, with total disruption of normal acinar structure (Fig. 2C). In contrast, there was only mild infiltration of inflammatory cells in the mammary gland interstitium in mice infected with avirulent *A. viridans* (Fig. 2C). There was no evidence of inflammation in uninfected control mice (Fig. 2C).

3.3. Virulent Aerococcus viridans increased neutrophil myeloperoxidase activity and production of pro-inflammatory cytokines

Mice intrammarily challenged with virulent *A. viridans* had significantly higher MPO activity in mammary tissues compared to the other three treatment groups from 1 to 9 d pi (Fig. 3A). However, there was no MPO increase in mice challenged with avirulent *A. viridans* (Fig. 3A). Challenge with virulent *A. viridans* (but not avirulent *A. viridans*) and *S. aureus* N305 increased protein expression of TNF-α, IL-1β and IL-6, from 1 to 9 d pi in mammary tissues (Fig. 3B-D).

3.4. Virulent Aerococcus viridans persisted in murine mammary gland

Bacteria were isolated from mammary glands of mice challenged with *S. aureus* N305, virulent or avirulent strains (Fig. 4), whereas no bacteria were isolated from the non-infected control group. The highest number of CFUs was recorded on day 1 pi for both virulent (6.3×10^6 CFUs/g) and avirulent (7.9×10^6 CFUs/g) *A. viridans*

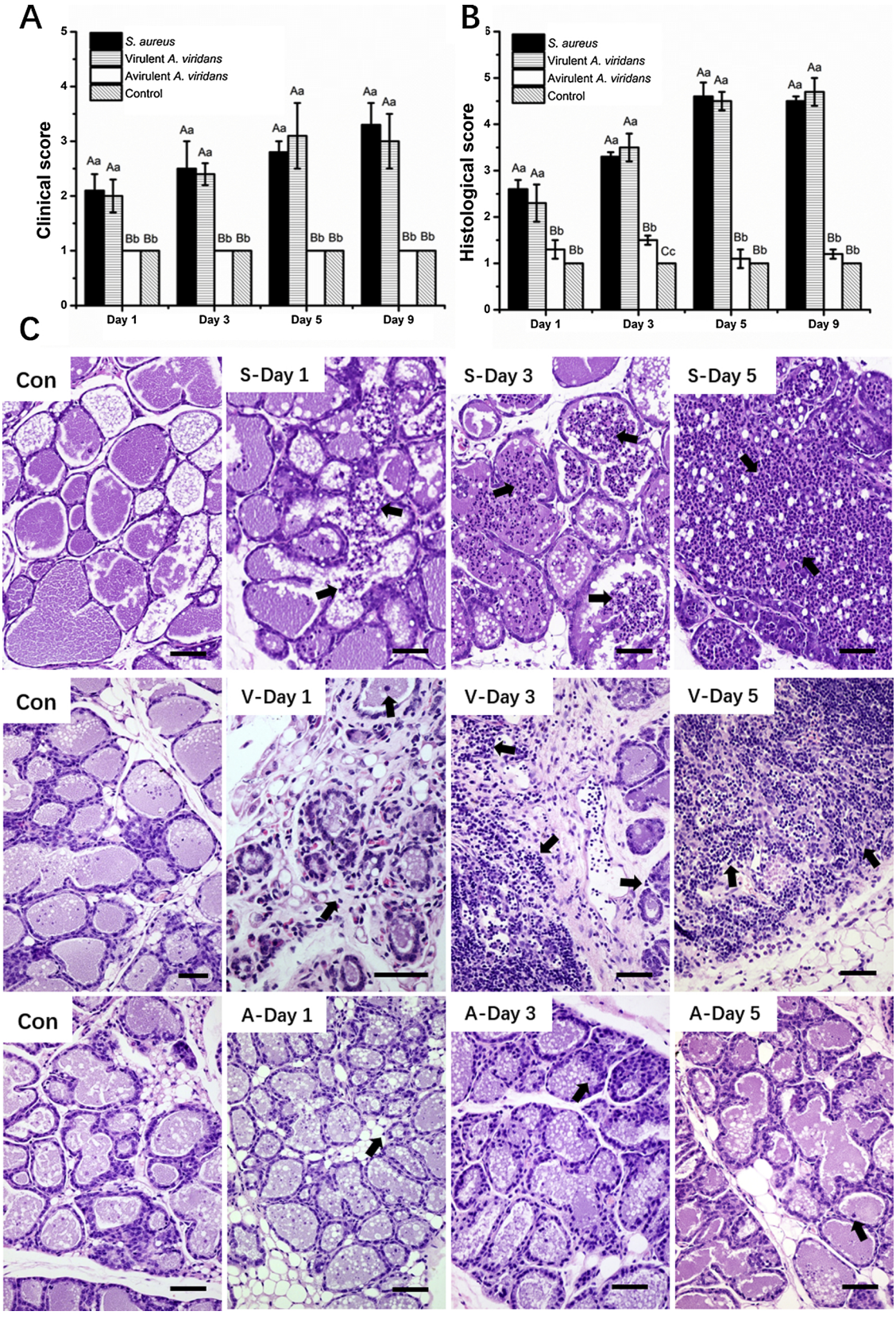

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Fig. 2. The clinical outcome and damage to murine mammary tissue caused by intramammary infection of *Aerococcus viridans* and *Staphylococcus aureus*. (A) The clinical score was evaluated for the challenged mice. (B–C) Mammary tissue was processed for histological evaluation (H&E staining 200×) and histopathological scores. Both *S. aureus* N305 and virulent *A. viridans* provoked acute mastitis; an increasing number of inflammatory cells (mainly lymphocytes and PMNs) infiltrated the interstitium of the mammary gland **(black** arrow) at day 1 pi. Note the massive infiltration of inflammatory cells **(black** arrow) on day 3 pi. At day 5 pi, both *S. aureus* N305 and virulent *A. viridans* had caused massive infiltration of inflammatory cells in interstitium and acini **(black** arrow), and the normal structure of mammary gland was disrupted. Only mild infiltration of inflammatory cells **(black** arrow) in interstitium of the mammary gland was observed in mice challenged with avirulent *A. viridans*. Bar = 25 μm. Results are presented as mean ± SD of 3 independent experiments. At each sampling time, different capitalized and lowercase letters correspond to statistically significant differences ($P < 0.01$) and ($P < 0.05$), respectively. The same lowercase letters correspond to no statistically significant differences. S-*S. aureus* N305, V-virulent strain, A-avirulent strain.

strains, compared to *S. aureus* N305 (1.6×10^7 CFUs/g) (Fig. 4). Whereas the infection burden rapidly decreased in mice infected with avirulent *A. viridans* (16 CFUs/g on day 3 pi and < 10 CFUs/gland afterward), mice infected with virulent *A. viridans* (2.0×10^3 CFUs/g on day 9) and *S. aureus* N305 (4.0×10^4 CFUs/g on day 9) had persistent infection (Fig. 4). Number of CFUs/g in mice infected with virulent *A. viridans* and *S. aureus* N305 was higher than avirulent *A. viridans* at all time point except day 1 ($P < 0.01$). Thus, virulent *A. viridans* isolated from the udder of dairy cows with mastitis persistently infected and provoked severe acute mastitis in a murine model of intramammary challenge.

3.5. Virulent Aerococcus viridans adhered to and invaded cultured bovine mammary epithelial cells

To determine pathogenic mechanisms used by virulent *A. viridans* to induce mastitis, the interaction between *A. viridans* and mammary epithelial cells was studied using bMECs. The virulent *A. viridans* (10.2%) and *S. aureus* N305 (12.5%) rapidly (30 min) adhered to bMEC, greater than avirulent *A. viridans* ($P < 0.01$) (Fig. 5A). Indeed, virulent *A. viridans* (54.2%) and *S. aureus* N305 (69.1%) had their peak adherence at 3 h, compared to only 3% of avirulent *A. viridans* attached to bMECs (Fig. 5A).

The ability of virulent *A. viridans* to adhere to bMECs in comparison to avirulent *A. viridans* was further confirmed by SEM (Fig. 6). Virulent *A. viridans* provoked epithelial cell membrane disruption with patches of bacteria adhered to the disrupted cell membrane where microvilli had disappeared at 3 h pi (Fig. 6B), followed by prominent structural changes such as cell shrinkage, disappearance of microvilli, cell membrane rupture and desquamation with abundant adhered cells at 12 h pi (Fig. 6D). Only a few avirulent *A. viridans* attached to cells and no cell membrane disruption was apparent (Fig. 6C). There were no morphological changes in the negative control group (Fig. 6A).

A high variability in percentages of internalization into bMECs between *S. aureus* N305 and *A. viridans* strains was observed. Percentage of bacterial invasion was the highest in bMECs exposed to *S. aureus* N305, followed by the virulent *A. viridans* ($P < 0.01$). In contrast, avirulent *A. viridans* possessed a significantly lower invasive ability ($P < 0.01$) (Fig. 5B). TEM studies at 6 h pi confirmed invasion of virulent *A. viridans* into cytoplasma of bMECs, with swelling of

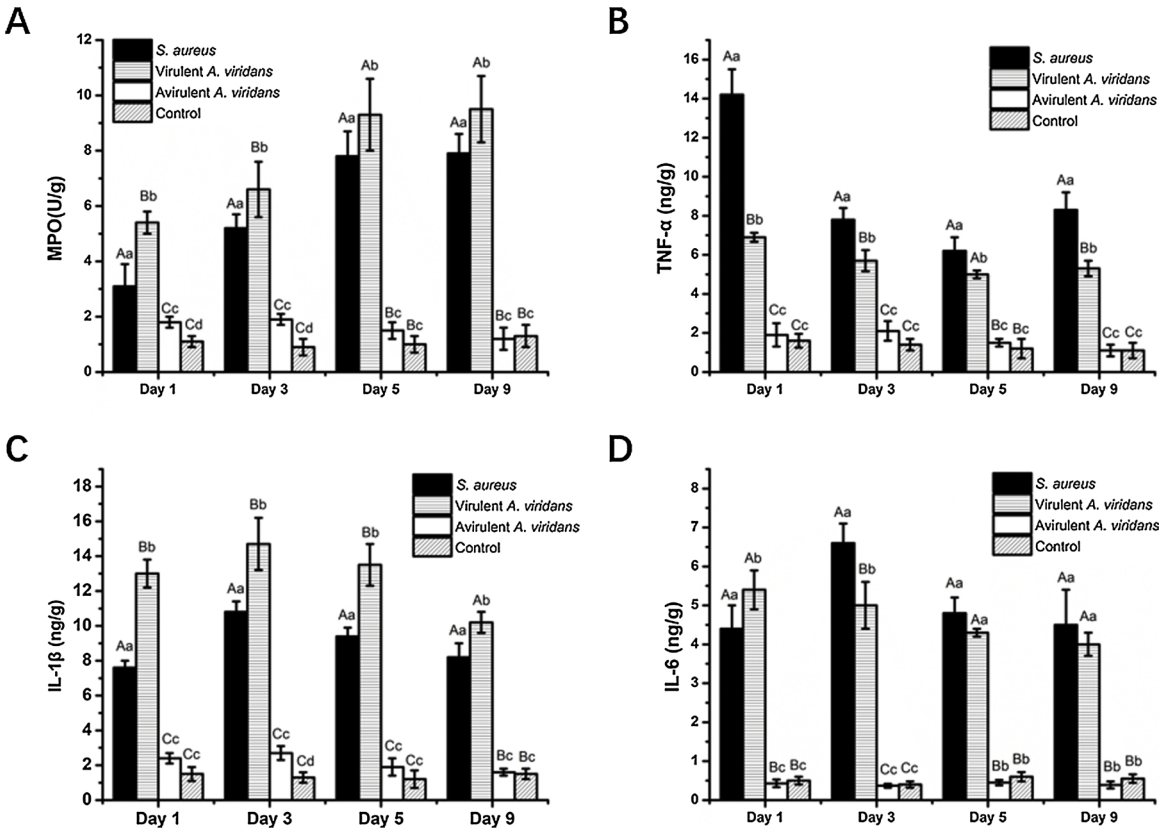

Fig. 3. Neutrophil myeloperoxidase activity and protein levels of proinflammatory cytokines in mammary glands challenged by *Aerococcus viridans* and *Staphylococcus aureus*. Levels of MPO activity (A), tumor necrosis factor-α (TNF-α; B), interleukin-1β (IL-1β; C), and interleukin-6 (IL-6; D) in murine mammary gland tissue were measured with an enzyme-linked immunosorbent assay. Results are presented as mean ± SD. At each sampling time, different capitalized and lowercase letters correspond to statistically significant differences ($P < 0.01$) and ($P < 0.05$), respectively. The same lowercase letters correspond to no statistically significant differences.

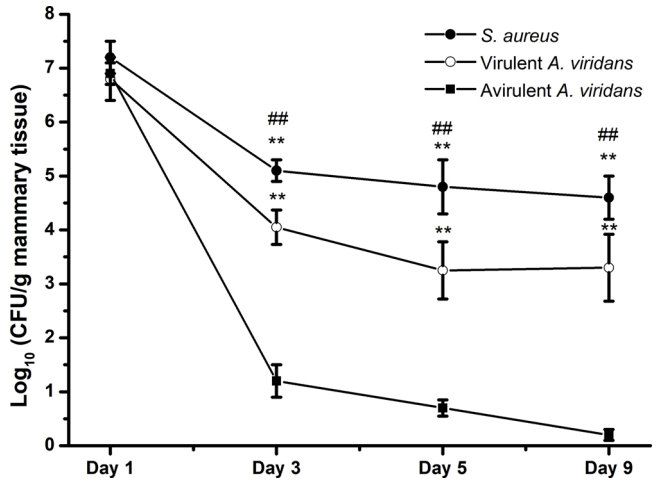

Fig. 4. Bacterial burden in mammary glands of mice after intramammary inoculation with *Aerococcus viridans* and *Staphylococcus aureus*. Each time point represents 5 mice in each of the 2 groups. ** = $P < 0.01$ versus the avirulent strain; ## = $P < 0.01$ versus the virulent strain.

mitochondria and disrupted microvilli, whereas no *A. viridans* was detected in cells infected by avirulent *A. viridans* (Fig. 7B and C). At 18 h pi, there was severe disruption of cells and loss of organelles, with *A. viridans* invading cytoplasm (Fig. 7D). There were no morphological changes in the negative control group (Fig. 7A).

Pathogenicity of virulent *A. viridans* included cytotoxic effects on bMECs as this strain disrupted cell membranes and increased LDH release from cells, reaching 71.0% of LDH released by virulent *A. viridans* compared to 78.1% by *S. aureus* N305 at 18 h pi (Fig. 8).

3.6. Virulent Aerococcus viridans increased gene expression of inflammatory cytokines and toll-like receptors in mammary epithelial cells

Gene mRNA expression of TNF-α, IL-6, IL-1β determined by qRT-PCR, were increased in bMECs exposed to virulent *A. viridans* (Fig. 9A-C). Expression of TLR-2 increased from 1 to 12 h pi, whereas expression of TLR-4 increased after 6 h pi in bMECs infected with virulent *A. viridans* (Fig. 9D-E). A similar pattern of cytokines and TLRs expression was observed in *S. aureus* N305 treated bMECs.

3.7. Virulent Aerococcus viridans produced active CAMP factor

Virulent *A. viridans* had positive CAMP factor activity, whereas avirulent *A. viridans* was CAMP-negative. CAMP tests were also performed with the remaining 7 *A. viridans* strains, although no CAMP factor was detected among these strains.

4. Discussion

Aerococcus viridans has been isolated from bovine milk (Devriese et al., 1999; Zadoks et al., 2004) and was recently associated with clinical and subclinical bovine mastitis (Liu et al., 2015; Saishu et al., 2015; Špaková et al., 2012). Although *A. viridans* is mentioned as an emerging pathogen associated with bovine subclinical mastitis (Sun et al., 2017), pathogenicity of this bacterium in bovine mastitis is unclear. In the present study, various strains of *A. viridans* isolated from milk of cows with subclinical mastitis hade variable virulence, with some of them capable of inducing strong experimental murine mastitis by establishment of IMI, due to bacterial adhesion and invasion to mammary epithelial cells.

Among all the *A. viridans* strains, AV40 and AV36 had the highest and lowest virulence, respectively, as determined by a murine intraperitoneal challenge survival test. Such variation of virulence among *A. viridans* strains was reported in lobsters, related to the expression of chaperonin 60 or production of capsules (Clark and Greenwood, 2011; Stewart et al., 2004). We reported substantial genetic diversity of *A. viridans* strains isolated from bovine mastitis based on RAPD and PFGE, which might account for variations in virulence between virulent and avirulent strains (Liu et al., 2015). We demonstrated that other *A. viridans* strains tested had relatively low virulence, with similar survival results compared to the avirulent strain (AV36). A limited virulence for most of *A. viridans* strains agrees with isolation of *A. viridans* from milk samples with minor or no pathogenicity (Wyder et al., 2011). However, particular virulent *A. viridans* strains (e.g., AV40) can persist and infect the murine mammary gland for up to 9 d pi. This persistent infection in a mouse model coincides with experimentally induced mastitis studies using standard chronic bovine mastitis pathogens, such as *Staphylococcus aureus* and *Streptococcus uberis* (Almeida et al., 2015; Hensen et al., 2000).

A mouse mastitis model was developed and characterized to study the specific host immune response to IMI with mastitis pathogens, such as *S. aureus* (Chandler, 1970). Therefore, *S. aureus* N305 was used as a positive control in the present study. Mastitis caused by virulent *A. viridans* in a mouse model was similar to that by *S. aureus* N305, evidenced by massive infiltrations of inflammatory cells and progressive mammary alveolar damage with discontinuous linings of epithelial and luminal cells and ultimately complete disruption of alveolar structure. Mammary alveolar damage is the main feature in mastitis and

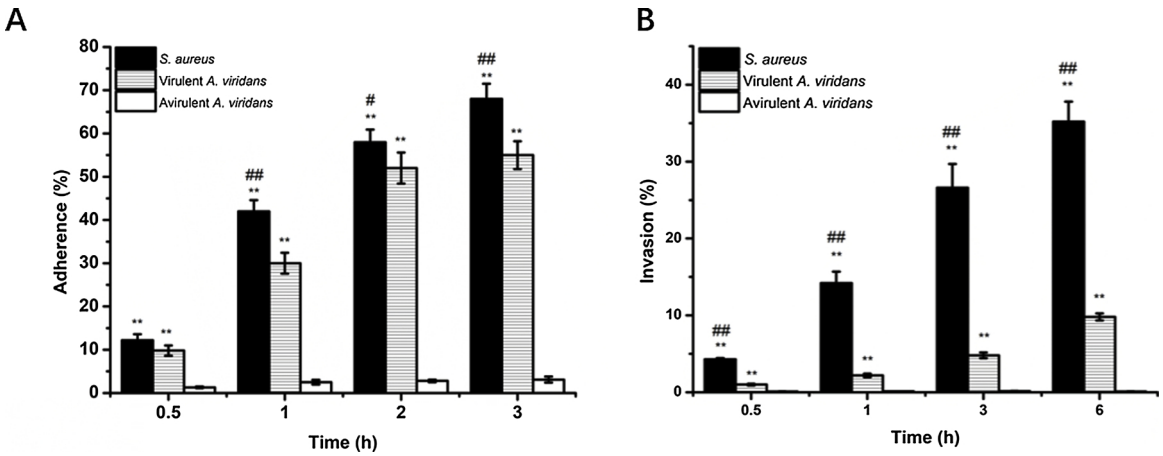

Fig. 5. Adhesion and invasion of *Aerococcus viridans* and *Staphylococcus aureus* to cultured bovine mammary epithelial cells. (A) Adhesion rate of *A. viridans* to bMECs. Results were presented as mean ± SD of 3 independent experiments. ** = $P < 0.01$ versus the avirulent strain; ## = $P < 0.01$ versus the virulent strain; # = $P < 0.05$ versus the virulent strain.

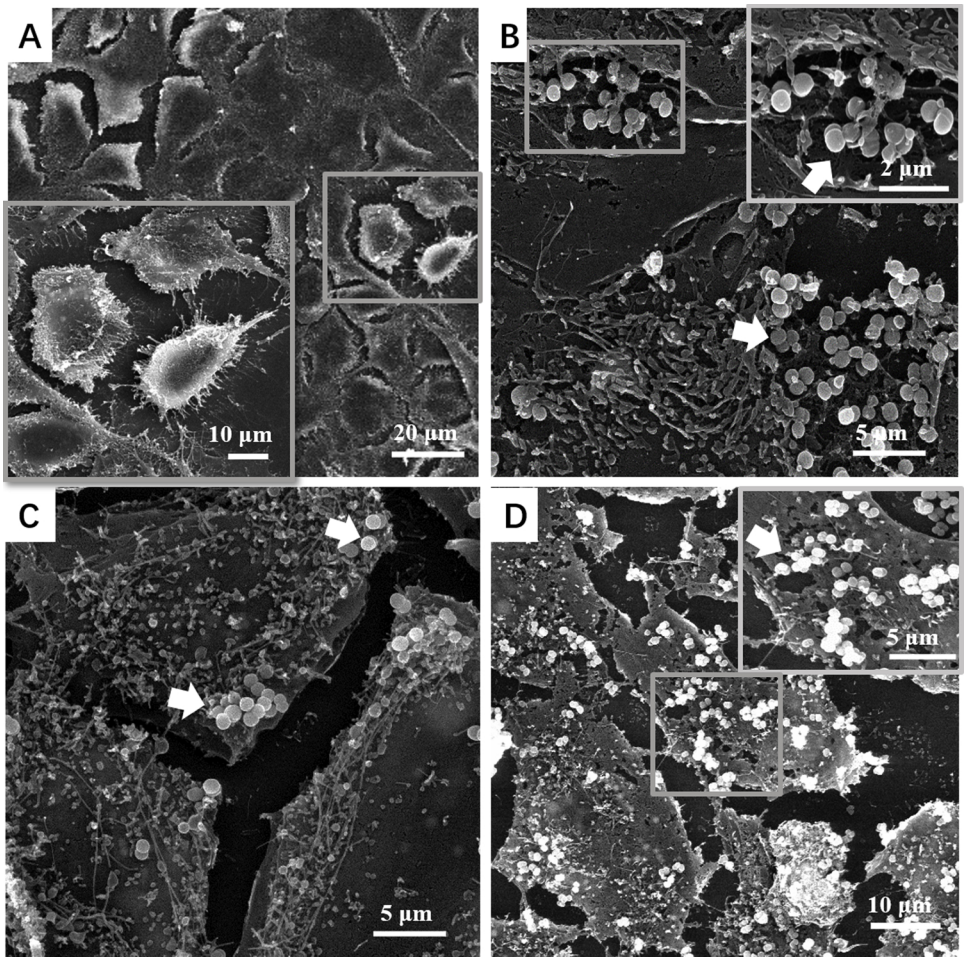

Fig. 6. Representatives of scanning electron microscope (SEM) image of bMECs challenged by *Aerococcus viridans*. (A) SEM image of bMECSs treated with sterile PBS (negative control). (B) SEM image of bMECs at 3 h pi challenged by virulent *A. viridans*. Adhered bacteria disrupted cell membrane and microvilli. (C) SEM image of bMECs at 3 h pi challenged by avirulent *A. viridians*, with few bacteria attached to cells and no disrupted cell membranes. (D) SEM image of bMECs at 6 h pi with virulent *A. viridans*. There were prominent cell structural alterations, including cell shrinkage, disappearance of microvilli, cell membrane rupture and desquamation with abundant adhered cells. The arrow points at *A. viridans* adhered to bMECs.

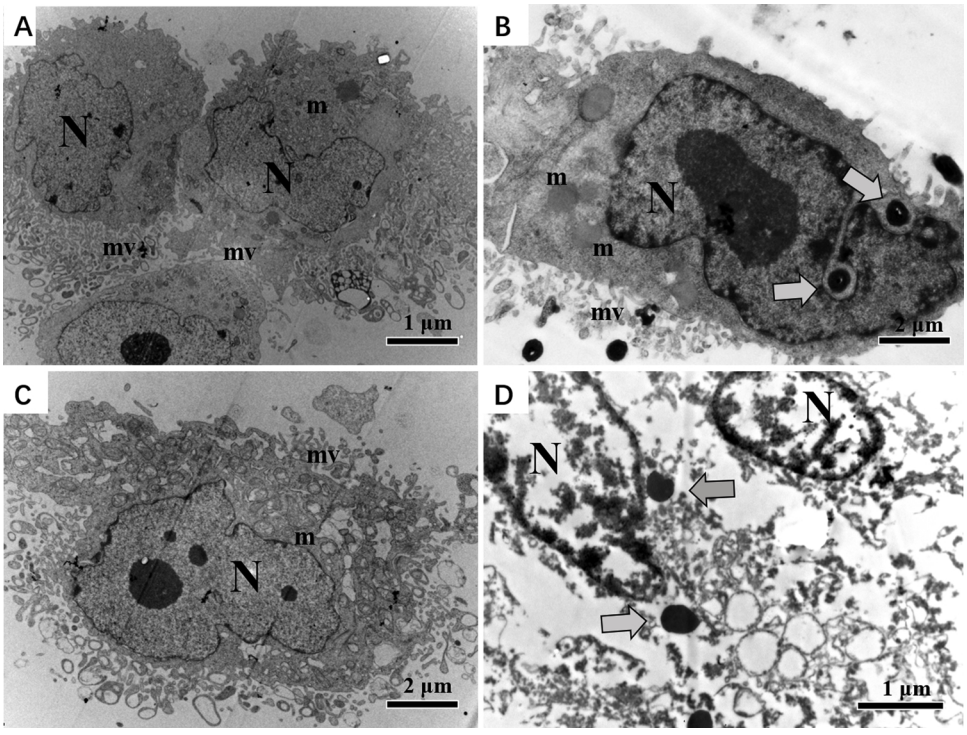

Fig. 7. Representatives of Transmission electron microscope (TEM) image of bMECs challenged by *Aerococcus viridans*. (A) TEM image of bMECSs treated with sterile PBS (negative control). (B) TEM image of bMECs at 6 h pi challenged by virulent *A. viridans*; these bacteria invaded cytoplasm, causing swelling of mitochondria and disrupted microvilli. (C) TEM image of bMECs at 6 h pi challenged by avirulent *A. viridans*. No bacteria were detected in bMECs. (D) TEM image of bMECs at 18 h pi challenged by virulent *A. viridans*. Severe disruption of cells and loss of organelles were observed with invaded *A. viridans*. The arrow points at the *A. viridans* invaded into bMECs. *n*-nucleus, *m*-mitochondrion, *mv*-microvillus.

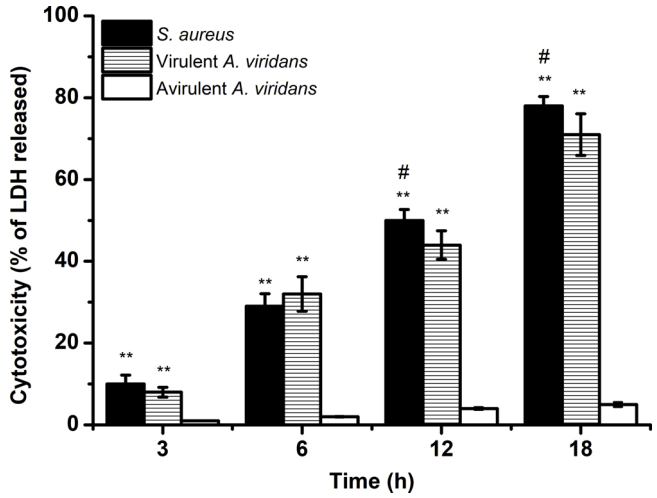

Fig. 8. LDH release of cultured bovine mammary epithelial cells caused by *Aerococcus viridans* and *Staphylococcus aureus*. Results are presented as mean ± SD of 3 independent experiments. ** = $P < 0.01$ versus the avirulent strain; # = $P < 0.05$ versus the virulent strain.

associated with permanent decreased milk production (Barkema et al., 2009). Such mammary epithelial damage may be due to cell apoptosis and necrosis caused by migration of inflammatory cells into the mammary gland tissue (Johnzon et al., 2016). In the present study, experimental IMI with virulent *A. viridans* eventually spread systemically and provoked lung infection, evidenced by recovery of these bacteria from murine lung tissue. Indeed, severe respiratory infection in cattle has been associated with *A. viridans* (Guccione et al., 2013).

In the mammary gland, epithelial cells recognize bacterial infection through activation of several pattern recognition receptors (PRRs), and subsequently initiate an immune response by producing inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Schukken et al., 2011). Recognition of virulent *A. viridans* seemed to involve TLR-2, an important receptor for detection of Gram-positive bacteria, as TLR-2 production increased 1 h pi. Such stimulation of TLR-2 by virulent *A. viridans* could enhance bacterial invasion into epithelial cells as TLR2 stimulation enhanced internalization of *Neisseriae* into human bronchial epithelial cells (Toussi et al., 2016). Notably, the exposure of both virulent *A. viridans* and *S. aureus* N305 increased the expression of TLR-4 in bMECs at 6 h and 12 h pi. TLR-4 has been identified as an important receptor for detection of Gram-negative bacteria, while recently study demonstrated that this receptor could also be induced either by detecting lipoteichoic acid (LTA) directly or through recognition of endogenous mediators induced by the interaction between LTA and TLR2 (Knapp et al., 2008). This was in accordance with our finding that the increasing expression of TLR4 (6 h and 12 h pi) followed the expression of TLR2 (3 h pi). Virulent *A. viridans* induced protein expression of all these pro-inflammatory cytokines in mammary tissues, TNF-α, IL-1β and IL-6. These cytokines seemed to characterize the local inflammatory response against *A. viridans* infection, although overproduction of these cytokines may cause systemic and destructive inflammation (Schukken et al., 2011).

Mechanistically, virulent *A. viridans* adhered to, invaded and eventually killed mammary epithelial cells, as confirmed by SEM, TEM and high LDH release. Adherence of pathogens to mammary gland epithelial is the first stage of pathogenicity to persist and proliferate, whereas invasion into host cells protects bacteria from humoral defenses and antibiotic treatments (Chen et al., 2017). Rapid adherence of virulent *A. viridans* may facilitate intracellular invasion, cell death and contribute to the chronic character of the disease. Perhaps bacteria adhered to the mammary epithelium or internalized into epithelial cells maintain infections within the udder and act as a reservoir.

The adherent/invasive ability of virulent *A. viridans* might be attributed to expression of CAMP factor, a pore-forming virulence factor in several Streptococcus species (Gase et al., 1999). *Streptococcus pyogenes* CAMP factor promoted bacterial adhesion and invasion in pharyngeal epithelial cells (Kurosawa et al., 2018) and *Propionibacterium acnes* CAMP factor contributed to bacterial invasion in skin keratinocytes (Lo et al., 2011). Our virulent strain had an active CAMP factor; to the best of our knowledge, this is the first report of CAMP factor in *A. viridans*, however an approach of targeted mutagenesis needs to be

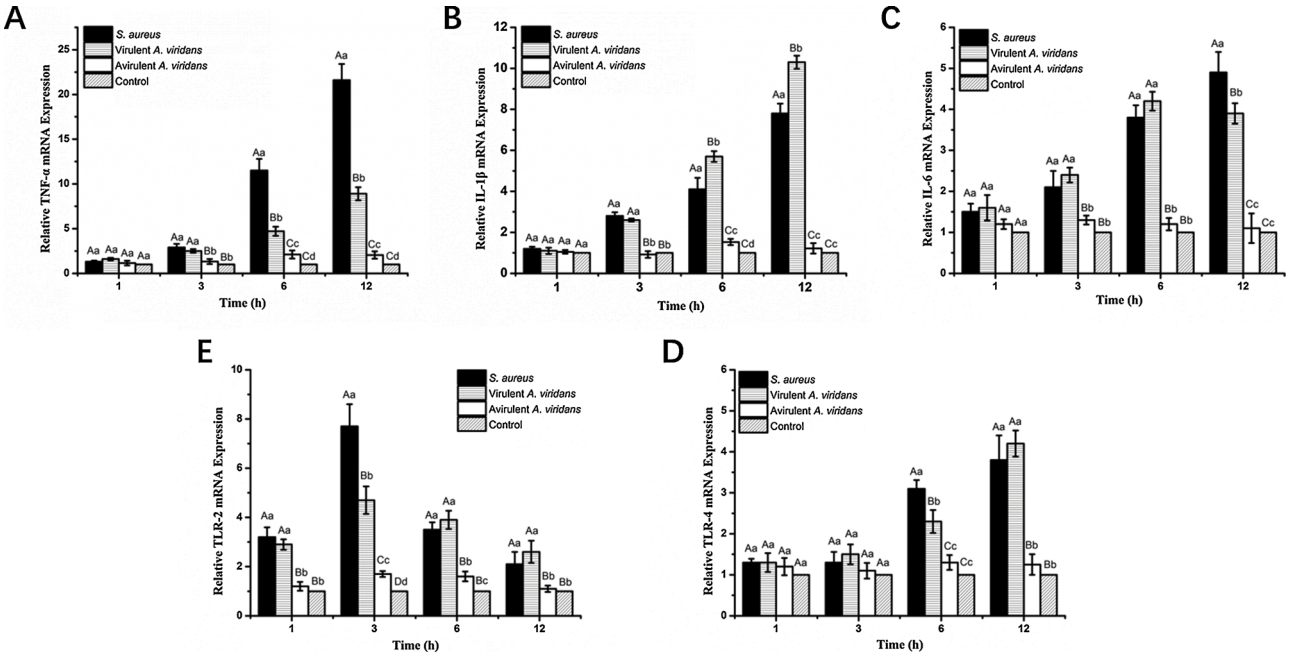

Fig. 9. mRNA levels of cytokines and toll like receptors in cultured mammary epithelial cells challenged by *Aerococcus viridans* and *Staphylococcus aureus*. Levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), Toll like receptor 2 (TLR-2) and Toll like receptor 4 (TLR-4) were measured by real-time PCR assay. Results are presented as mean ± SD. At each sampling time, different capitalized and lowercase letters correspond to statistically significant differences ($P < 0.01$) and ($P < 0.05$), respectively. The same lowercase letters correspond to no statistically significant differences.

adopted to verify the role of endogenous CAMP factor production in *A. viridans* pathogenicity.

5. Conclusion

This study demonstrated considerable pathogenic variation among *A. viridans* isolated from dairy cows with subclinical mastitis. The virulent *A. viridans* strain possessed strong adhering and invasive ability into bMECs which facilited their survival and replication in the mammary gland, inducing acute mastitis in a murine model. These findings are foundational for future studies in cattle models to elucidate the importance of *A. viridans* as a mastitis pathogen.

Competing interests

The authors have declared that no competing interests exist.

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