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## Keywords

Antimicrobial peptides, Innate immunity, Interleukin-8, *Mannheimia haemolytica*, Pneumonia, Sheep beta-defensin

## Disciplines

Large or Food Animal and Equine Medicine | Veterinary Pathology and Pathobiology

## Comments

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## Differential expression of sheep beta-defensin-1 and -2 and interleukin 8 during acute *Mannheimia haemolytica* pneumonia

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### Abstract

Beta-defensins are antimicrobial peptides produced by several cell types, including respiratory epithelia and leukocytes. Expression of some beta-defensins is increased by bacterial-induced inflammatory responses whereas expression of other beta-defensins is constitutive. Two beta-defensins are expressed in lungs of sheep (sheep beta-defensin-1 and -2; SBD-1/-2) and expression of SBD-1 is increased during parainfluenza virus type 3 (PI-3) infection. The effect of *Mannheimia haemolytica*, a Gram-negative bacteria known to induce expression of bovine beta-defensins and NF-kappa B in lung, has not been determined for SBD-1/-2. In this study, different concentrations of *M. haemolytica* were inoculated into pulmonary bronchi of lambs. SBD-1 and SBD-2 mRNA levels detected by real time reverse transcriptase polymerase chain reaction in lung homogenates did not increase. In fact, SBD-1 mRNA levels were significantly decreased with the highest administered inoculum concentration ( $10^9$ ). In contrast, mRNA levels of interleukin-8 (IL-8) were significantly increased over controls and progressively increased with *M. haemolytica* concentrations. Co-inoculation of *M. haemolytica* with xylitol, an osmotic agent, did not alter mRNA levels of SBD-1, SBD-2 or IL-8. SBD-1 mRNA expression was detected in lung epithelia, but not in leukocytes. This study suggests that SBD-1 expression occurs in epithelia and decreases during severe bacterial pneumonia, which is in contrast to the increase that occurs with PI-3 infection.

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**Keywords:** Antimicrobial peptides; Innate immunity; Interleukin-8; *Mannheimia haemolytica*; Pneumonia; Sheep beta-defensin

### 1. Introduction

Antimicrobial peptides are an integral component of the innate immune system. Defensins are cationic antimicrobial peptides produced by a wide range of species and have microbicidal activity against bacteria, fungi and enveloped viruses [1–5], including HIV-1 [6]. Alpha-defensins are produced by paneth cells and leukocytes and beta-defensins are produced by epithelia and leukocytes [3,5]. Antimicrobial activity of beta-defensins can be decreased by high salt concentrations [4,7–9]; however, recent studies indicate

that osmotic agents such as xylitol may enhance defensin activity, even in high salt conditions [10]. Over 26 human beta-defensins have been identified [11,12]. Expression of some beta-defensins, such as human beta-defensin-1 (HBD-1), is constitutive, whereas expression of other beta-defensins, such as HBD-2, 3, and 4, [4,8,13], mouse beta-defensin-2 [14], tracheal antimicrobial peptide (TAP) and lingual antimicrobial peptide (LAP) of cattle, is inducible [15–18]. Induction often occurs secondary to activation of Toll-like receptor 4 (TLR 4) and NF-kappa B signaling [4,5]. Sheep beta-defensin-1 and -2 (SBD-1 and -2) are also members of the beta-defensin family. SBD-1 is developmentally regulated in late gestation through the neonatal period with maximal expression in trachea, lung and rumen reached shortly after birth [19–21]. Although SBD-1 appears to lack NF-kappa B responsive elements,

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SBD-1 expression is increased during infection with ovine parainfluenza virus type 3 (PI-3) [22]. SBD-2 is also developmentally regulated and expressed primarily in the alimentary tract [20,23]; however, only low levels of SBD-2 mRNA are present in lung relative to SBD-1 [20,23]. Expression of SBD-1 and SBD-2 in response to bacterial infection has not been determined.

*Mannheimia haemolytica* is a common respiratory pathogen of sheep and cattle [24–27]. *M. haemolytica* can cause serious outbreaks of acute pneumonia in neonates, weaned and growing animals as well as adults [24]. Vaccination against *M. haemolytica* can enhance resistance to infection; however, these vaccines are not completely effective [25,26]. Experimentally, *M. haemolytica* activates NF-kappa B signaling [15,28] and enhances expression of two inducible bovine defensins, TAP and LAP in cattle [16–18].

The purpose of this study was to: (1) determine the extent to which *M. haemolytica* infection in sheep alters SBD-1 and -2 gene expression in vivo by real time reverse transcriptase polymerase chain reaction (real time RT-PCR), (2) determine if xylitol treatment alters SBD-1 and -2 expression, (3) compare SBD-1 and -2 expression to interleukin 8 (IL-8), a cytokine known to have increased gene expression during *M. haemolytica* pneumonia in the bovine [29,30].

## 2. Results

### 2.1. Gross lesions

Animals inoculated with *M. haemolytica* developed characteristic, gross lesions for this agent in sheep at the site of bacterial deposition. These areas were consolidated and deep red/blue in color.

### 2.2. Histopathology

Animals inoculated with *M. haemolytica* developed characteristic microscopic lesions for this agent in sheep at the site of inoculation. Lesions included dense infiltrates of neutrophils, seroproteinaceous fluid, accumulation of polymerized fibrin and small amounts of cell debris within the airways, interlobular septae and lymphatic vessels. Non-inoculated areas and lungs from control animals lacked lesions or contained minimal multifocal peribronchial and peribronchiolar infiltrates of lymphocytes.

### 2.3. Quantitative plate counts in lung tissue and bronchoalveolar lavage fluid (BALF)

*M. haemolytica* was cultured from BALF and lung tissue from all animals inoculated with *M. haemolytica* and not from control animals (Table 1). Numbers of *M. haemolytica* in BALF (cfu/ml) increased progressively with increased

Table 1

Number of bacteria (*Mannheimia haemolytica*) isolated from bronchoalveolar lavage fluid (BALF) at 20 min and 24 h post-inoculation (PI) and number of bacteria isolated from lung tissue at 24 h PI

Group number <sup>a</sup>	Inoculum <sup>b</sup>	Log <sub>10</sub> bacteria (cfu/ml + sem) in BALF		Log <sub>10</sub> bacteria (cfu/g + sem) in lung 24 h PI
		20 min PI	24 h PI	
1	0	None	None	None
2	1.9 × 10 <sup>6</sup>	1.5 ± 0.9	0.9 ± 0.9	3.6 ± 0.5
3	2.3 × 10 <sup>7</sup>	2.9 ± 1.0	2.4 ± 1.4	2.8 ± 1.0
4	2.4 × 10 <sup>8</sup>	5.1 ± 0.2	3.5 ± 1.1	7.4 ± 0.9
5	3.2 × 10 <sup>9</sup>	6.7 ± 0.1	6.4 ± 0.5	9.6 ± 0.3

No *M. haemolytica* was cultured from control animals, and numbers of isolated bacteria increased as inoculation concentration of *M. haemolytica* increased.

<sup>a</sup> Three animals in Group 1, four animals in Groups 2, five animals in Group 4 and six animals in Group 5; xylitol and non-xylitol-treated animals are combined for this table.

<sup>b</sup> Number of viable *M. haemolytica*/ml in inoculum (5 cc/animal).

concentration of inoculated bacteria and were present at both 20 min and 24 h post-inoculation (PI). Numbers of bacteria in lung tissue at 24 h PI (cfu/g tissue) also increased progressively with increased concentration of inoculated bacteria.

### 2.4. IL-8 mRNA levels

IL-8 expression is known to increase during *M. haemolytica* pneumonia [29–31]. As expected, mRNA levels of IL-8 increased with increasing concentrations of *M. haemolytica*, and significantly so ( $P = 0.03$ ) when compared to controls (Fig. 1). Xylitol treatment did not alter IL-8 mRNA levels.

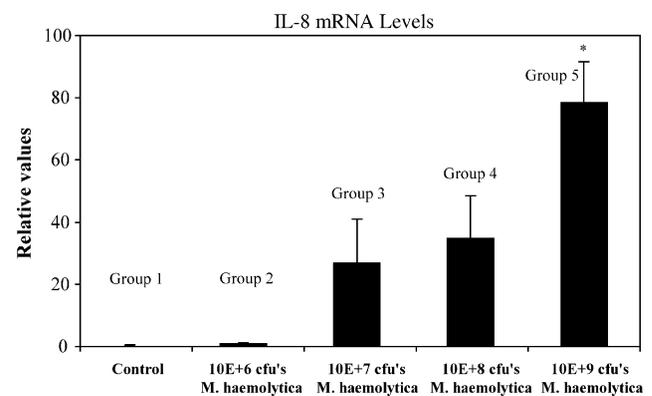


Fig. 1. Interleukin-8 (IL-8) mRNA levels (normalized to Ribo 18S) in lungs of sheep determined by real time RT-PCR. Group 1, controls; Group 2, 10<sup>6</sup> *M. haemolytica*; Group 3, 10<sup>7</sup> *M. haemolytica*; Group 4, 10<sup>8</sup> *M. haemolytica*; Group 5, 10<sup>9</sup> *M. haemolytica* ( $n = 3$ /group). IL-8 mRNA levels progressively increase with increased bacterial concentrations and are significantly increased in Group 5 versus controls (Group 1).

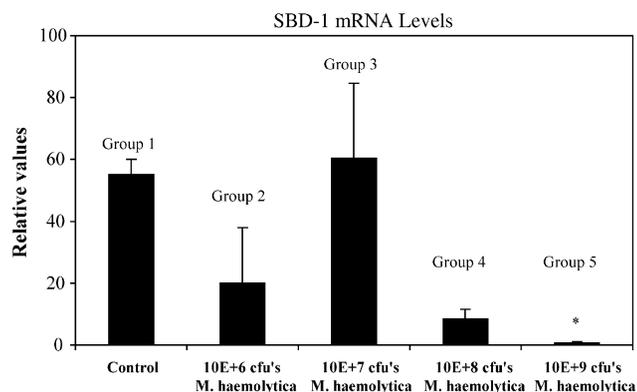


Fig. 2. SBD-1 mRNA levels (normalized to Ribo 18S) in lungs of sheep determined by real time RT-PCR. Group 1, controls; Group 2,  $10^6$  *M. haemolytica*; Group 3,  $10^7$  *M. haemolytica*; Group 4,  $10^8$  *M. haemolytica*; Group 5,  $10^9$  *M. haemolytica* ( $n = 3$ /group). SBD-1 mRNA levels are significantly decreased in Group 5 versus controls (Group 1).

### 2.5. SBD-1 and SBD-2 mRNA levels

In contrast to the increase in IL-8 mRNA expression, mRNA levels of SBD-1 decreased significantly ( $P = 0.01$ ) in lambs that received the highest amounts of *M. haemolytica* (Fig. 2). Xylitol treatment did not alter SBD-1 mRNA levels. SBD-2 mRNA levels were not significantly altered (Fig. 3) in animals inoculated with *M. haemolytica* compared to controls. mRNA levels of SBD-1 and, to a greater extent, SBD-2, were somewhat variable as seen previously.

### 2.6. Levels of SBD-1 mRNA in ovine epithelial cells retrieved by laser capture microdissection (LCM)

SBD-1 mRNA levels in respiratory epithelia were determined in epithelial cells retrieved by LCM in order to define the contribution of respiratory epithelia (versus leukocytes) in SBD-1 expression. By real time RT-PCR assessment of mRNA isolated from lung epithelial cells

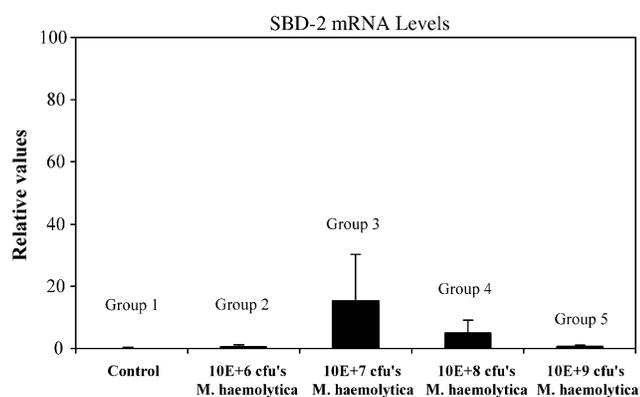


Fig. 3. SBD-2 mRNA levels (normalized to Ribo 18S) in lungs of sheep determined by real time RT-PCR. Group 1, controls; Group 2,  $10^6$  *M. haemolytica*; Group 3,  $10^7$  *M. haemolytica*; Group 4,  $10^8$  *M. haemolytica*; Group 5,  $10^9$  *M. haemolytica* ( $n = 3$ /group). SBD-2 mRNA levels are not significantly altered between groups.

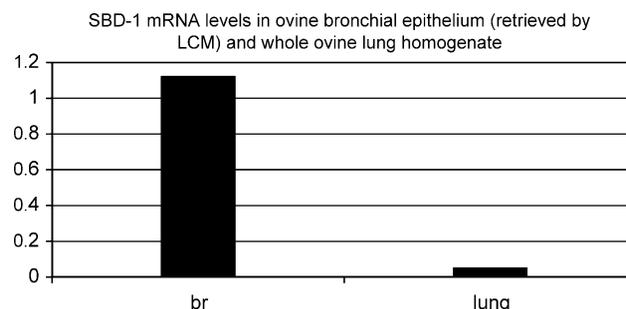


Fig. 4. SBD-1 mRNA levels (normalized to Ribo 18S) in ovine bronchial epithelium retrieved by laser capture microdissection are detectable and higher than whole lung homogenate. In contrast, ovine buffy coat cells lacked SBD-1 mRNA real time signal even after 50 PCR cycles (Fig. 5).

(bronchi) retrieved by LCM, SBD-1 PCR amplification resulted in linear amplification that was well within the suggested threshold levels. SBD-1 mRNA levels were significantly higher than levels observed in lung homogenates when normalized to the reference signal (Fig. 4).

### 2.7. Levels of SBD-1 and IL-8 mRNA in LPS-stimulated ovine buffy coat cells

In order to determine if circulating leukocytes, which infiltrate the lung during pneumonia, contribute to SBD-1 and IL-8 expression, mRNA levels were determined in LPS-stimulated and control buffy coat cells. LPS-treated cells had roughly three fold increases in raw total RNA content at 3 and 24 h incubation with LPS as compared to control cells (which lacked LPS treatment). SBD-1 mRNA levels were not detectable at 3 and 24 h in buffy coat cells with or without LPS-treatment. In contrast, IL-8 had robust expression in both LPS-treated and control buffy coat cells. IL-8 expression was slightly increased at 3 h in LPS-treated

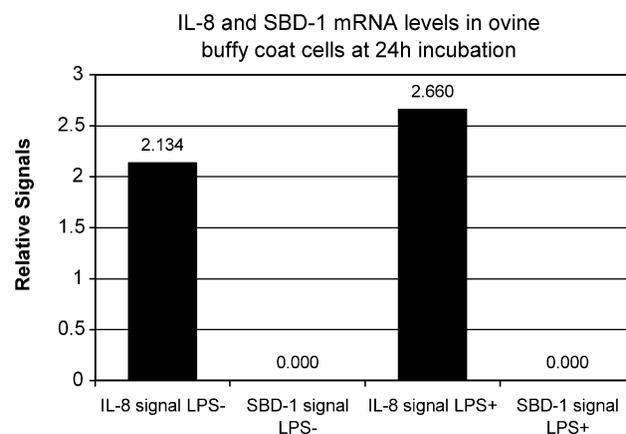


Fig. 5. IL-8 mRNA levels (normalized to Ribo 18S) determined by real time RT-PCR in ovine buffy coat cells incubated 24 h with lipopolysaccharide (LPS +) or incubated without LPS (LPS -). IL-8 mRNA levels are mildly increased with LPS incubation. In contrast, ovine buffy coat cells lacked SBD-1 mRNA levels after 50 PCR cycles with or without LPS stimulation.

cells and moderately increased again at 24 h post-incubation with LPS as compared to controls (Fig. 5). Because SBD-2 levels were nearly undetectable in control and *M. haemolytic*-infected lung, and bronchiolar epithelial cells, SBD-2 was not assessed in buffy coat cells. The lack of SBD-1 expression in buffy coat cells along with the detection of SBD-1 in LCM retrieved epithelia, suggest that respiratory epithelia cells, and not leukocytes, are primarily responsible for SBD-1 mRNA expression in lung.

### 3. Discussion

Our observed increase in the expression of IL-8 during *M. haemolytica* infection in lambs is consistent with similar, previous findings in cattle [29–31]. *M. haemolytica* is a Gram-negative bacteria that causes an intense inflammatory reaction characterized in the acute stages (first 24 h) by vascular leakage of protein, polymerization of fibrin, and dense infiltrates of neutrophils [24]. Virulence factors released by *M. haemolytica* include lipopolysaccharide and capsular polysaccharide (both of which can incite inflammatory responses) and a leukotoxin that binds the CD18 subunit of the beta-2 integrins of leukocytes, resulting in apoptosis/necrosis [24]. The severity of pneumonia can be reduced with dexamethasone, suggesting that the inflammatory response induced by *M. haemolytica* can be considered excessive [32]. Indeed, several studies have demonstrated a marked increase in IL-8 and other inflammatory cytokines including IL-1 and TNF-alpha during *M. haemolytica* pneumonia [29–31]. The inability of xylitol to alter IL-8 mRNA levels was not altogether surprising since the high numbers of bacteria in inoculate likely overwhelm any potentially observable localized effect(s) of xylitol. The expression of IL-8 mRNA in buffy coat cells suggests that infiltrating leukocytes may contribute significantly to IL-8 levels detected in whole lung homogenates. The lack of SBD-1 mRNA expression in buffy coat cells in combination with the proportionally high SBD-1 mRNA expression in respiratory epithelial cells retrieved by LCM suggests that SBD-1 is expressed primarily by pulmonary epithelia of the lung.

The lack of increase in mRNA levels for SBD-1 and SBD-2 suggests that these beta-defensins are not responsive or regulated by the acute inflammatory conditions induced by *M. haemolytica*. Additional work is needed to determine if SBD-1 and SBD-2 expression is affected similarly by other microbial pathogens and their products. As with IL-8, xylitol did not alter SBD-1 or SBD-2 mRNA levels. Although SBD-2 expression was slightly elevated in Groups 3 and 4, SBD-2 expression in these animals was highly variable, not statistically significant, and occurred at much higher cycle points during real time RT-PCR (>45 cycles and often reaches the allowed maximum [50]) than observed for SBD-1 (<40 cycles) or IL-8 (<32 cycles); suggesting that SBD-2 mRNA levels are very low in comparison to

SBD-1 or IL-8. In our experience, rare mRNA signals (cDNA signals which generally appear above the threshold of detection at 40 cycles and above), when detected by real time RT-PCR, seem prone to generate variable target amplification data. Such has been the case with SBD-2 signal in lung—and the reason it cannot be fully interpreted to any acceptable degree of statistical confidence. However, in sheep gastrointestinal tract tissues (e.g. ileum, jejunum, cecum, spiral colon and rectum), SBD-2 expression is much more abundant [20,23].

The inability for *M. haemolytica* infection to induce SBD-1 expression stands in sharp contrast to bovine defensins, TAP and LAP, both of which are highly expressed during *M. haemolytica* pneumonia [15–19]. The lack of SBD-1 expression suggests that signaling pathways such as NF-kappa B, which is induced by *M. haemolytica* pneumonia [15,28], do not induce or regulate expression; however, the mechanistic basis underlying the regulation of SBD-1 and SBD-2 requires further characterization. It is also reasonable that *M. haemolytica* may suppresses SBD-1 expression, since expression of HBD-1 and the cathelicidin LL37 are both inhibited/reduced during *Shigella* ssp.-induced bacillary dysentery and with *Shigella* spp. infection of cultured epithelial cells and monocytes [33].

The lack of observable increase in lung SBD-1 mRNA levels during experimental pneumonia caused by *M. haemolytica* is contrary to our findings that demonstrate increased SBD-1 and surfactant protein A and D mRNA expression during viral (PI-3) infection [22]. The different levels of SBD-1 and surfactant protein A and D expression that occurs in *M. haemolytica* and PI-3 infections may influence the degree of clearance/persistence of each of these pathogens during pneumonia and may also influence susceptibility/resistance to secondary pathogens. The divergent response of SBD-1 expression as demonstrated by a decrease with *M. haemolytica* infection and increase with PI-3 infection presents an interesting regulatory process biologically. Other constitutively expressed beta-defensins include HBD-1 and mouse beta-defensin-1 (MBD-1) [7,9, 34]. Although HBD-1 expression is constitutive, HBD-1 production in some tissues, such as breast, is relatively high [35] and HBD-1 mRNA levels can increase with certain infections, which is somewhat analogous to the increased SBD-1 expression seen with PI-3 infection.

HBD-1 expression occurs in macrophages and the lack of SBD-1 mRNA expression in buffy coat cells was somewhat unexpected. The majority of buffy coat cells were neutrophils and lymphocytes, however, a small number of monocytes were also present. It is possible that the lack of SBD-1 mRNA expression by buffy coat cells could be due to several reasons: (1) the number of monocytes in the buffy coat samples was too low for significant SBD-1 mRNA detection, (2) monocytes in the buffy coat may have different expression characteristics of SBD-1 than fully differentiated macrophages, even under LPS

stimulation, and (3) SBD-1 expression may simply not occur or is limited in resting monocytes and LPS-stimulated monocytes, but may be increased by other stimuli (such as viral infection or interferons). Also, because lung tissues in this study were collected at one time point (24 h), it cannot be ruled out that defensin expression may have risen prior to or after this time point.

Levels of SBD-1 and SBD-2 mRNA expression in lung homogenates were quite variable. We have noted variability of SBD-1 expression among similar control animals before, and variable expression is a feature of other beta-defensins as well. Such inter-animal variability in defensin expression is not currently understood, but may be simply due in part to potentially varying amounts of bronchi, bronchiolar and alveolar epithelium in each whole lung tissue homogenate. Exposure to small airborne particles from feed grains, airborne LPS, or subclinical infections with agents such as PI-3, or slight differences in lung development or age between animals could also result in alterations in SBD-1 expression.

This work demonstrates differential expression of IL-8 and SBD-1 and SBD-2 during acute *M. haemolytica* pneumonia. The decreased SBD-1 expression that occurs during *M. haemolytica* pneumonia contrasts sharply with the increased SBD-1 expression, that occurs with PI-3 infection. This contrast identifies an interesting and potentially unique biological regulatory pathway in defensin gene expression.

## 4. Materials and methods

### 4.1. Bacterial inoculum

*M. haemolytica* serotype 1 strain 82-25, was originally isolated from a sheep with pneumonia. It was grown overnight on blood agar containing trypticase soy agar with 5% defibrinated sheep blood (Lab Supply Co., Des Moines, IA) as described [27]. The overnight growth was transferred to 35 ml of tryptose broth (Difco Laboratories, Detroit, MI) and incubated at 37 °C for 3 h on a magnetic stirrer. The culture was centrifuged at 5860g (SS-34 rotor) for 10 min at 4 °C, suspended in 140 mM NaCl, and adjusted to 0.108 OD at 600 nm in a spectrophotometer (Coleman model 35, Bacharach Instrument Co.) to contain approximately  $1.0 \times 10^8$  CFU/ml. The bacterial cells in 150 ml of suspension were split and then pelleted by centrifugation at 10,000g for 10 min at 4 °C and re-suspended in 300 mOsm saline or 300 mOsm xylitol. An exact viable bacterial concentration was determined by standard plate count on blood agar. The inoculum was kept on ice throughout the procedure.

### 4.2. Lambs and inoculation

Twenty-seven, 8-month old lambs (approximately 60 kg each), were housed in isolation rooms and treated by

methods approved by the National Animal Disease Center Animal Care and Use Committee. In order to assess the ability of xylitol to reduce microbial viability in situ, lambs were first randomly assigned to eight groups of three. Just prior to inoculation, lambs were lightly sedated with 20 mg xylazine (Rompun, Bayer Corp., Shawnee Mission, KS). A small plastic tube was inserted into the oral cavity and served as a speculum for inoculation as performed and described previously [27,36]. A bronchoscope (Model administered to ease the scope past the larynx. The tip of the bronchoscope was moved to the dorsum of the caudal portion of the cranial lobe of the right lung (pulmonary deposition site) in each animal for deposition of inocula. After inoculation, the effect of xylazine was reversed by intravenous injection of 100 mg tolazoline HCl (Tolazine, Lloyd Laboratories, Shenandoah, IA).

Lambs in Group 1 were saline-inoculated controls ( $n = 3$ ). Lambs in Group 2 received  $10^6$  CFU *M. haemolytica* ( $n = 3$ ) in 1 ml 300 mM NaCl and 300 mM xylitol ( $n = 3$ ), respectively. Lambs in Group 3 received  $10^7$  CFU *M. haemolytica* in 1 ml 300 mM NaCl ( $n = 3$ ) and 300 mM xylitol ( $n = 3$ ), respectively. Lambs in Group 4 received  $10^8$  CFU *M. haemolytica* in 1 ml 300 mM NaCl ( $n = 3$ ) and 300 mM xylitol ( $n = 3$ ), respectively. Lambs in Group 5 received  $10^9$  CFU *M. haemolytica* in 1 ml 300 mM NaCl ( $n = 3$ ) and 300 mM xylitol ( $n = 3$ ), respectively.

### 4.3. Bronchoalveolar lavage (BAL) and necropsy

At 20 min PI, the lungs of all lambs were lavaged five times with 20 ml saline per lavage, then pooled (100 ml total). At 24 h PI, all lambs were euthanized with pentobarbital and exsanguinated. At necropsy, lungs were evaluated grossly, and affected lobes were lavaged with 100 ml saline. Pieces of tissue were taken from pulmonary deposition sites, fixed in 10% neutral buffered formalin solution and processed for histopathologic examination. Pieces of lung were also collected for quantitative bacterial culture. Lung sections were scored according to the type of infiltrate present (lymphocyte, neutrophil, and macrophage), and according to the extent of necrosis, hemorrhage, collapse, and fibrosis. Scores ranged from 0 (no changes) to 4 (most severe changes) in each category. The final score was a total of the individual scores for each animal (less the score for lymphocyte infiltrates and degree of collapse). Lung tissue and diluted BAL fluid were used for quantitative plate counts of *M. haemolytica* (Table 1).

### 4.4. Real time RT-PCR

SBD-1 and IL-8 gene expression were determined by two-step fluorogenic real time RT-PCR using primers and probe sequences (Table 2) listed, and according procedures previously described in our laboratory [15,22]. Briefly, real time RT-PCR was carried out according to manufacturer's specifications using the GeneAmp 5700

Table 2

Primer and TaqMan fluorescent probe sequences used in real time reverse transcriptase-PCR for relative quantification of mRNA expression of sheep beta-defensin-1 and -2 (SBD-1 and SBD-2) and ovine interleukin-8 (IL-8)

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SBD-1 Fwd primer-5'-CCA TAG GAA TAA AGG CGT CTG TGT-3'
SBD-1 Rev primer-5'-CGC GAC AGG TGC CAA TCT-3'
SBD-1 Probe-5'-6FAM-CCG AGC AGG TGC CCT AGA CAC ATG A-TAMRA-3'
IL-8 Fwd primer-5'-TTC CAA GCT GGC TGT TGC T-3'
IL-8 Rev primer-5'-TTG ACA GAA CTG CAG CTT CAC A-3'
IL-8 Probe-5'-6FAM-CCG CTT TCC TGC TCT CTG CAG CTC-TAMRA-3'
SBD-2 Fwd primer-5'-AAG CTG CCG TTG GAA GAA AG-3'
SBD-2 Rev primer-5'-CCC GAA ACA GGT GCC AAT C-3'
SBD-2 Probe-5'-6FAM-TGT GTG CTG ACC AAG TGC CCT GGA ACC ATG AG-TAMRA-3'

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6FAM, Fluorescent reporter dye; TAMRA, Fluorescent quencher dye.

Sequence Detection System (Applied Biosystems). In this procedure, total RNA was isolated by Trizol following manufacturer's directions (Invitrogen) and cDNA was generated by reverse transcriptase (following Promega and Applied Biosystems procedural specifications) in a separate step preceding the fluorogenic real time RT-PCR procedure (during which ribosomal 18S RNA was used as the endogenous reference gene).

#### 4.5. Assessment of SBD-1 and SBD-2 expression in bronchiolar epithelial cells retrieved by laser capture microdissection

In order to determine if SBD-1 expression occurred in respiratory epithelia cells or infiltrating leukocytes, bronchiolar epithelial cells were retrieved by LCM. Briefly, lung tissue from normal lambs frozen in OCT media (Sakura Finetek Inc., Torrance, CA) were sectioned at 8  $\mu$ m in a cryostat (Leica) and placed onto a glass slide. The sections were processed as indicated by a commercial kit (Arcturus, Inc.). Bronchiolar epithelial cells were retrieved under adequate power and duration settings with a PixCell II LCM system (Arcturus, Inc.) and placed in a microfuge tube. Captured cells were lysed and total RNA was isolated with a commercial kit (PicoPure RNA isolation kit; Arcturus, Inc.). SBD-1 and SBD-2 mRNA levels were determined by real time RT-PCR as described previously [15,22] and above.

#### 4.6. Assessment of SBD-1 and IL-8 mRNA levels in ovine leukocytes (buffy coat)

In order to determine if SBD-1 expression is present in leukocytes that infiltrate into the lung during pneumonia, SBD-1 and IL-8 levels were determined in buffy coat cells isolated from normal lambs. Briefly, blood was collected in EDTA, complete blood counts were determined, and buffy coats were obtained by centrifugation and separation using Histopaque-1083 (Sigma, St Louis, MO). Buffy coat cells

were maintained in RPMI-1640 media with 10% fetal calf serum, additional (2 mM) L-glutamine and trace amounts of  $\beta$ -mercaptoethanol. Cells were incubated with 3.6  $\mu$ g/ml purified *M. haemolytica* lipopolysaccharide (LPS) for 3 and 24 h. Control cells lacked LPS treatment. Total RNA was isolated from floating and trypsin-EDTA harvested cells using Trizol according to manufacturer's recommendations except our modification of using a 16,100g spin for the final RNA pellet collection (instead of 7500g). SBD-1 and IL-8 mRNA levels were determined by real time RT-PCR as described previously [15,22] and outlined above.

#### 4.7. Statistical analysis

Statistical analysis was performed using the means of the three replicate wells for each cDNA sample being analyzed for the presence of SBD-1, -2 and IL-8 levels normalized to their respective ribosomal 18S RNA reference ('house-keeping') signals. To determine if there were significant treatment and time effects, a two-factor analysis of variance (ANOVA) (SPSS, SPSS Inc., Version 9.0, Chicago, Ill.) was used on all samples. Frozen lung tissues from all animals (controls and infected) from both groups were assessed. Real time RT-PCR mRNA levels were compared for significance with the Kruskal–Wallis ANOVA.

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