The immune response of bovine mammary epithelial cells to live or heat-inactivated *Mycoplasma bovis*

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Abstract

Mycoplasma bovis is an emerging bacterial agent causing bovine mastitis. Although these cell wall-free bacteria lack classical virulence factors, they are able to activate the immune system of the host. However, effects on the bovine mammary immune system are not yet well characterized and detailed knowledge would improve the prevention and therapy of mycoplasmal mastitis. The aim of this study was to investigate the immunogenic effects of *M. bovis* on the mammary gland in an established primary bovine mammary epithelial cell (bMEC) culture system. Primary bMEC of four different cows were challenged with live and heat-inactivated *M. bovis* strain JF4278 isolated from acute bovine mastitis, as well as with the type strain PG45. The immune response was evaluated 6 and 24 h after mycoplasmal challenge by measuring the relative mRNA expression of selected immune factors by quantitative PCR. *M. bovis* triggered an immune response in bMEC, reflected by the upregulation of tumor necrosis factor-α, interleukin(IL)-1β, IL-6, IL-8, lactoferrin, Toll-like receptor-2, RANTES, and serum amyloid A mRNA. Interestingly, this cellular reaction was only observed in response to live, but not to heat-inactivated *M. bovis*, in contrast to other bacterial pathogens of mastitis such as *Staphylococcus aureus*. This study provides evidence that bMEC exhibit a strong inflammatory reaction in response to live *M. bovis*. The lack of a cellular response to heat-inactivated *M. bovis* supports the current hypothesis that mycoplasmas activate the immune system through secreted secondary metabolites.

Keywords: Bovine mammary epithelial cells, mastitis, *Mycoplasma bovis*, innate immune response

Introduction

Mycoplasmas represent the smallest self-replicating microorganisms characterized by a minimal genome, and by the lack of a cell wall (Razin et al., 1998). In dairy cattle, *Mycoplasma bovis* belongs to the most prevalent contagious mastitis pathogens (Olde Riekerink et al., 2006) that is often unresponsive to antibiotics and tends to cause chronic infections (Kauf et al., 2007).

In general, bacterial species and strain specific severities of mastitis are characterized by differences in their induction of the mammary immune system, i.e. the regulation of several immune factors (i.e. cytokines) and recognition receptors (i.e. toll-like receptors; TLR) (Wellnitz and Bruckmaier, 2012; Zbinden et al., 2014). *M. bovis* displays a broad range of immunogenic and immunosuppressive characteristics that determine its pathogenicity. The extensive variation of cell surface antigens, also known as variable surface proteins (Vsps), enables the pathogen to evade the host immunity which contributes to the persistent nature of *M. bovis* infections (Razin et al., 1998; Buchenau et al., 2010). Among these Vsps, lipoproteins represent the most abundant fraction (Razin et al., 1998). Besides their antigenic properties, lipoproteins play a central role in adhesion and are expected to trigger the production of pro-inflammatory cytokines (Pilo et al., 2007). Furthermore, *Mycoplasma*-derived secondary metabolites have been demonstrated to exert cytotoxic effects in infected host cells (Pilo et al., 2005).

As *M. bovis* resists antibiotic therapies, it is of high importance to understand the bovine mammary immune response to this pathogen to optimize prevention and therapy strategies of mycoplasmal mastitis in dairy cows. Therefore, the objective of the present study was to investigate the innate immune response of bovine mammary epithelial cells (bMEC) to *M. bovis,* characterized by the mRNA expression of selected immune factors. Primary cultures of bMEC challenged with live and heat-inactivated *M. bovis* were used to investigate host-pathogen interactions that trigger the immune reaction in the mammary epithelium.

Materials and Methods

M. bovis strains

The field strain JF4278, isolated from mastitic milk in 2008 in Switzerland (Aebi et al., 2012), and the type strain PG45 (Hale et al., 1962) were used in this study. *M. bovis* cultures were grown in SP4 broth medium supplemented with 50 µg/ml cefoxitin sodium salt (Sigma-Aldrich, Buchs, Switzerland) for 18 hours (Freundt, 1983).

To standardize the assays, standard growth curves relating turbidity (OD_{600}) of liquid cultures to colony forming units (CFU) of *M. bovis* were obtained by determining colony counts on SP4 agar supplemented with 50 µg/ml cefoxitin sodium salt incubated for 3-4 days at 37 °C in a humidified atmosphere. Growth of *M. bovis* was tested to assess variations among each individual SP4 batch. Each culture was centrifuged for 30 min at 400 g and at 4 °C. The pellet was suspended in challenge medium consisting of DMEM/F12 supplemented with 5 % FBS, and ITS containing 0.5 mg/ml insulin, 0.5 mg/ml apo-transferrin, 0.5 µg/ml sodium selenite (all components were received from Sigma-Aldrich, Buchs, Switzerland) to reach the appropriate concentration. To evaluate the effective mycoplasmal concentrations, serial 10-fold dilutions of *M. bovis* cultures were simultaneously plated on SP4 agar for colony enumeration. For heat-inactivation, *M. bovis* suspensions were incubated for 30 min at 95 °C. The efficiency of this method was previously assessed by plating serial 10-fold dilutions of the *M. bovis* suspension on SP4 agar plates.

Treatment of bMEC with live or heat-inactivated M. bovis

Primary cultures of mammary gland epithelial cells of four lactating Holstein dairy cows with clinically healthy udders (somatic cell count $<10^5$ cells/ml) were performed as previously described (Wellnitz and Kerr, 2004). Mammary tissue was removed directly after slaughter with permission of the slaughterhouse Marmy SA, Estavayer-le-Lac, Switzerland. Cells were passaged twice and

cryopreserved in DMEM/F12 containing 20 % FBS and 10 % DMSO (Sigma-Aldrich, Buchs, Switzerland) and stored in aliquots at –80 °C until the experiment.

Exogenous *Mycoplasma* sp. contamination of cell cultures from all four cows was excluded using the Venor™GeM Mycoplasma Detection Kit (Minerva Biolabs, Berlin, Germany) following the manufacturer's instructions.

Cells from all cows were thawed and separately cultured in growth medium (DMEM/F12 supplemented with 10 % FBS, penicillin G [500 units, Sigma-Aldrich], streptomycin [100 µg/ml, Sigma-Aldrich], and ITS). After two passages, cells were seeded at a concentration of 3 x 10^5 cells/well on BD Falcon™ 6-well cell culture plates (BD Biosciences, San Jose, CA, USA). On the following day, growth medium was replaced with medium without antibiotics.

Assuming that 3 x 10^5 cells/well after 24 h of incubation and a confluence of about 70 % represent approximately 1 x 10^6 cells/well, bMEC were challenged with an expected multiplicity of infection (MOI) of 150 of live, or heat-inactivated *M. bovis* suspensions. As a positive control, cells were challenged with 150 MOI of heat-inactivated *S. aureus* strain 1904 from bovine mastitis (Zbinden et al., 2014). Cells incubated in growth medium only served as negative controls.

Cells were grown and incubated at 37 °C with 5 % CO_2.

Total RNA extraction and quantitative real-time PCR

After 6 and 24 h of incubation cells were harvested with 1 ml peqGOLD Trifast™ (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Total RNA was extracted according to the manufacturer’s protocol. Reverse transcription with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega Corp., Madison, WI, USA) and quantitative real-time PCR analysis using the Sensimix DNA Kit (Quantace, Biolabo, Châtel St. Denis, Switzerland) on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) was described before (Zbinden et al., 2014). Primer sequences and annealing temperatures are shown in table 1.

Cycle threshold (Ct) values were determined by the Rotor-Gene software version 1.7.75, and the relative mRNA expression was calculated using the comparative Ct method using the following equation (Schmittgen and Livak, 2008): ΔCt = Ct target gene – Ct endogenous control (arithmetic mean of housekeeping genes).

To visualize the impact of *M. bovis*, or *S. aureus* on the immune response of bMEC, data are presented as ΔΔCt ± SEM, where: ΔΔCt = ΔCt sample (6, or 24 h; treated) – ΔCt negative control (6, or 24 h; untreated).

Statistical Analysis

Data from the cells of four cows were expressed as means ± SEM from ΔΔCt values of the selected factors. Statistical analysis of the inflammatory response data was tested for significance ($P < 0.05$) by ANOVA using PROC MIXED of SAS (Release 9.4; SAS Institute Inc., Cary, NC, USA). The model included time, strain, and their interaction as fixed effects, and the cow as repeated subject. A Bonferroni correction was applied.

Results

Cell cultures

Primary bMEC cultures of all four cows grew to an approximate confluence of 70% within ~20 h after seeding into 6-well plates. According to Venor™GeM Mycoplasma Detection Kit, the cultures of all cows were free of *Mycoplasma sp.* contamination. After 24 h of incubation with live or heat-inactivated mycoplasmas, cells were nearly confluent, and no morphological changes were visible.

Growth of M. bovis strains

For both *M. bovis* strains a MOI of 150 was expected based on the assumption/observation that an OD_{600} value of 0.1 corresponded to 4.2×10^8 CFU/ml and 1.65×10^9 CFU/ml, for JF4278 and

PG45, respectively. The plating of serial dilutions indicated that the growth kinetics differed between *M. bovis* strains, resulting in effective MOIs of 108 (JF4278), and 30 (PG45), respectively. Plating of heat-inactivated *M. bovis* did not result in any bacterial growth.

Relative mRNA expression of immune factors

The mRNA expression of measured immune factors was not increased in negative controls.

In cells challenged with *S. aureus* 1904 as positive control, an expected induction of mRNA expression of interleukin (IL)-1β, tumor-necrosis factor (TNF)-α, IL-8, serum amyloid A (SAA), lactoferrin (Lf), and TLR2 after 6 h and 24 h of stimulation was detectable. The chemokine RANTES (regulated on activation, normal T-cell expressed and secreted) was induced at 6 h, but not at 24 h after challenge. IL-6, IL-10, cyclooxygenase (COX)-2, TLR1, and TLR6 were not expressed at the mRNA level at either of the time points in response to *S. aureus*.

Induction of mRNA expression after challenge and differences between challenges with live and heat-inactivated *M. bovis* are shown in Table 2 and Figure 1. Transcription of IL-1β, TNF-α, and IL-8 significantly increased ($P<0.05$) in bMEC infected with live *M. bovis* at both, 6 and 24 h post infection. The abundance of IL-6 mRNA was increased in response to JF4278 after 24 h. RANTES was only induced at 6 h post infection in bMEC infected by live JF4278 whereas SAA was upregulated in bMEC at 24 h post infection with both live *M. bovis* strains. Lactoferrin mRNA expression was upregulated in bMEC 6 h after infection with live PG45, whereas transcription of Lf was increased 24 h after infection with both live *M. bovis* strains. Expression of TLR2 mRNA was transiently induced after 6 h in response to live JF4278, and at 24 h post infection in response to live PG45. Interleukin-10, COX-2, TLR1, and TLR6 were not induced in bMEC by either live or heat-inactivated *M. bovis*. With heat-inactivated *M. bovis*, only IL-8 mRNA was upregulated after 6 h of challenge by JF4278. A further increase of mRNA expression from 6 to 24 h of challenge was only detectable for SAA and Lf with live mycoplasmas.

Discussion

Cryopreserved primary bMEC cultures in a low passage were previously shown to immunologically respond to different bacteria that play a role in mastitis development (Wellnitz and Kerr, 2004). The cells responded to the stimulation with *M. bovis* by increased transcription of genes relevant in mammary gland innate immunity, which confirms the suitability of this model to investigate the immune response to mycoplasmas. As all cell cultures were free of *Mycoplasma* sp. contamination, a cellular response induced by other *Mycoplasma* species than *M. bovis* was excluded. The biological reproducibility was provided by using cells from four different cows. Different periods of challenge (6 and 24 h) were used to investigate immune factor expression in earlier and later phases of the response. The *S. aureus strain* 1904 was used as a positive control (Zbinden et al., 2014) and proved responsiveness of the cultures used.

M. bovis strain JF4278 used in the present study represents a Swiss field strain that was isolated from severe mastitis in 2008, when *M. bovis* mastitis outbreaks started to emerge in Switzerland (Aebi et al., 2012). In parallel, bMEC were challenged with the type strain PG45 that was isolated in 1961 in the US (Hale et al., 1962). Both *M. bovis* strains induced an immune response in bMEC in the present study; however, a direct quantitative comparison is not possible since effective MOIs were not identical due to differential growth. Although Wellnitz et al. (2006) found that an increasing MOIs of *E. coli* or *S. aureus* did not necessarily result in a stronger immune response in bMEC, an effect of the bacterial cell concentration used for infection/challenge can be possible.

An experimental mammary *M. bovis* infection increased the pro-inflammatory cytokines TNF-α and IL-1β in milk (Kauf et al., 2007). This was reflected by the transcriptional upregulation of IL-1β and TNF-α in bMEC in the present study. The cytokine IL-6 seems to be involved at later stages of the inflammatory response of bMEC to *M. bovis*, since live *M. bovis* JF4278 induced IL-6 transcription not until 24 h after stimulation.

Chemokines like IL-8 or RANTES mediate the influx of neutrophils from the blood to the site of infection, reflected by an increase of milk somatic cell count during mastitis. In bMEC IL-8 mRNA expression was increased by *M. bovis* which is in agreement with increased milk IL-8 concentrations *M. bovis* mastitis (Kauf et al., 2007).

Acute phase protein synthesis also reflects an immune response. In this study, SAA expression significantly raised 24 h after infection with live *M. bovis* but not with heat inactivated mycoplasmas. Since SAA is known to act as a chemoattractant, pathogens that upregulate SAA transcription are likely to be associated with an increase of somatic cell count in the mammary gland, which could influence the progress of the mastitis (Badolato et al., 1994).

The enzyme COX-2 is involved in prostaglandin synthesis that is upregulated in pulmonary epithelial cells from pigs by *Mycoplasma hyopneumoniae* (Andrada et al., 2014). However, in the present study COX-2 was not upregulated in bMEC in response to either of the *M. bovis* strains.

Lactoferrin is an antibacterial protein that is produced by mammary epithelial cells during clinical bovine mastitis (Wellnitz and Bruckmaier, 2012). Live *M. bovis* induced Lf transcription in the present study. The earlier induction of Lf transcription of strain PG45 compared to JF4278 despite the lower MOI could indicate that Lf expression follows a strain-dependent mechanism. Although it was reported that Lf production increases over time in bMEC cultures, likely due to the increasing cell density (Wellnitz and Kerr, 2004), the increased transcription of Lf was not due to this effect since control cells did not express increased levels of Lf mRNA during the time of the experiment.

Toll-like receptors, i.e. TLR2 in combination with TLR1 or TLR6, on the surface of host cells recognize lipopeptides within the cell membranes of mycoplasmas (Omueti et al., 2005). The slight increase of TLR2 transcription in bMEC after infection with *M. bovis* shows that TLR2 is involved in *M. bovis* recognition by mammary epithelial cells. In contrast, transcription of TLR1, and TLR6 was not affected.

Mycoplasmal infections are not necessarily associated with a strong inflammatory response and can proceed without apparent clinical symptoms (Chambaud et al., 1999). In the present study the immune suppressive cytokine IL-10 was not upregulated in bMEC by *M. bovis*, which is consistent with the results of Lahouassa et al. (2007). An increase of IL-10 in milk after experimental infection of cows with *M. bovis* shown by Kauf et al. (2007) could be derived from other immune cells.

Most interestingly, in contrast to other mastitis pathogens like *E. coli* or *S. aureus* (Griesbeck-Zilch et al., 2008; Zbinden et al., 2014), bMEC responded only to live, but not to heat-inactivated *M. bovis*. For *Mycoplasma mycoides* subsp. *mycoides*, it was previously shown that heat-inactivation results in impaired cytotoxicity (Dedieu et al., 2005). The main reason for this difference is that cell wall containing bacteria have components like lipoteichoic acid, peptidoglycans, or lipopolysaccharide that are recognized by the host immune system, whereas wall-less mycoplasmas do not have these classical virulence factors. Therefore, mycoplasmas exhibit alternative mechanisms that induce an immune response in bMEC, which are apparently antagonized by heat treatment. Putative *M. bovis*-specific immunogenic factors include cell surface molecules such as lipoproteins that are recognized by bMEC via TLRs. However, the most important role in the activation of an immune response seem to play secreted metabolites that are associated with cell damage and, as a consequence, inflammation and disease (Pilo et al., 2007).

In conclusion, the present study indicates that an infection with *M. bovis* induces a considerable immune response in bMEC shown by increased transcription of several immune factors. Remarkably, a substantial immune response only occurs if bMEC are infected with live but not with heat-inactivated *M. bovis*, indicating a role of released metabolites in the activation of the mammary immune response.

Acknowledgments

We thank Sibylle Bürki, of the Institute for Veterinary Bacteriology, Vetsuisse Faculty University of Bern, Switzerland, for mycoplasma preparation. The *Staphylococcus aureus* strain 1904 was kindly provided by Prof. R. Stephan, Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Switzerland.

Conflict of interests

The authors have declared that no competing interests exist.

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Table 1. Sequences, accession numbers, annealing temperature of the PCR primers, and length of the PCR products.

Gene[a]		Sequence 5'→3'	GenBank accession no.	Annealing temperature (°C)	Length (bp)
TNF-α	for	CCA CGT TGT AGC CGA CAT C[b]	NM173966	60	155
	rev	CCC TGA AGA GGA CCT GTG AG[b]			
IL-1β	for	AGT GCC TAC GCA CAT GTC TTC[b]	M37211	60	114
	rev	TGC GTC ACA CAG AAA CTC GTC[b]			
IL-6	for	CTT CAC AAG CGC CTT CAC TC[b]	NM173923.2	62	132
	rev	GTC AGA AGT AGT CTG CCT GG[c]			
IL-8	for	ATG ACT TCC AAG CTG GCT GTT G[b]	AF232704	60	149
	rev	TTG ATA AAT TTG GGG TGG AAA G[b]			
RANTES	for	GCC AAC CCA GAG AAG AAG TG[b]	BC102064	60	119
	rev	CTG CTT AGG ACA AGA GCG AGA[b]			
SAA	for	GGG CAT CAT TTT CTG CTT CCT[d]	AF540564	60	106
	rev	TTG GTA AGC TCT CCA CAT GTC TTT AG[d]			
COX-2	for	TCC TGA AAC CCA CTC CCA ACA[e]	NM174445	62	242
	rev	TGG GCA GTC ATC AGG CAC AG[e]			
Lf	for	GGC CTT TGC CTT GGA ATG TAT[e]	L08604	62	338
	rev	ATT TAG CCA CAG CTC CCT GGA G[e]			
TLR1	for	ACT TGG AAT TCC TTC TTC ACG A[f]	NM001046504	60	176
	rev	GGA AGA CTG AAC ACA TCA TGG A[f]			
TLR2	for	GGT TTT AAG GCA GAA TCG TTT G[f]	NM174197	60	190
	rev	AAG GCA CTG GGT TAA ACT GTG T[f]			
TLR6	for	CCT TGT TTT TCA CCC AAA TAG C[f]	NM001001159	60	154
	rev	TAA GGT TGG TCC TCC AGT GAG T[f]			

IL-10	for	TGT TGA CCC AGT CTC TGC TG[g]	NM174088.1	60	94
	rev	GGC ATC ACC TCT TCC AGG TA[g]			
GAPDH	for	GTC TTC ACT ACC ATG GAG AAG G[b]	NM001034034	60	197
	rev	TCA TGG ATG ACC TTG GCC AG[b]			
Ubiquitin	for	AGA TCC AGG ATA AGG AAG GCA T[b]	NM174133	62	198
	rev	GCT CCA CCT CCA GGG TGA T[b]			

for = forward, rev = reverse.

[a]RANTES = regulated on activation, normal T cell expressed and secreted; SAA = serum amyloid A; COX-2 = cyclooxygenase-2; Lf = lactoferrin.

Primer sequence references : [b](Griesbeck-Zilch et al., 2008); [c](Zarrin et al., 2014), [d](Mukesh et al., 2010), [e](Zbinden et al., 2014), [f](Davies et al., 2008), [g](Dreesen et al., 2012).

Table 2. Changes of mRNA abundance (Mean ± SEM ΔΔCt[c]) of immune factors in bMEC challenged with 2 strains of live or heat-inactivated (h.i.) *M. bovis* for 6 or 24 h.

		JF4278		PG45	
Gene[d]	Time (h)	live	h.i.	live	h.i.
TNF-α	6	4.5 ± 0.9 [a*]	1.8 ± 0.8 [b]	4.9 ± 0.4 [A*]	0.4 ± 1.0 [B]
	24	4.9 ± 0.4 [a*]	-0.3 ± 1.0 [b]	4.4 ± 0.5 [A*]	0.5 ± 0.4 [B]
IL-1β	6	7.9 ± 1.0 [a*]	1.6 ± 1.1 [b]	7.9 ± 1.1 [A*]	-0.2 ± 0.2 [B]
	24	8.1 ± 0.7 [a*]	0.2 ± 0.5 [b]	9.2 ± 0.5 [A*]	-0.1 ± 0.4 [B]
IL-6	6	0.9 ± 2.2 [a]	0.5 ± 0.3 [a]	1.8 ± 0.6 [A]	0.0 ± 0.6 [A]
	24	2.1 ± 0.4 [a*]	-0.1 ± 0.1 [a]	2.2 ± 1.0 [A]	-0.2 ± 0.6 [A]
IL-8	6	5.6 ± 0.9 [a*]	1.1 ± 0.3 [b*]	5.6 ± 0.3 [A*]	0.3 ± 0.3 [B]
	24	7.1 ± 1.1 [a*]	1.3 ± 1.1 [b]	7.6 ± 1.0 [A*]	0.1 ± 1.3 [B]
RANTES	6	1.3 ± 0.4 [a*]	1.0 ± 0.8 [a]	0.3 ± 0.4 [A]	-1.2 ± 0.9 [A]
	24	1.3 ± 0.6 [a]	-0.7 ± 0.9 [a]	1.6 ± 0.8 [A]	-0.5 ± 0.6 [A]
SAA	6	3.1 ± 1.8 [a]	0.4 ± 1.2 [a]	2.9 ± 1.6 [A]	-0.3 ± 1.8 [A]
	24	8.5 ± 0.7 [a*#]	-0.6 ± 0.4 [b]	7.7 ± 0.1 [A*#]	-1.1 ± 0.7 [B]
COX-2	6	0.6 ± 0.8 [a]	0.7 ± 1.2 [a]	1.4 ± 1.0 [A]	0.1 ± 0.9 [A]
	24	0.5 ± 1.5 [a]	0.1 ± 0.6 [a]	3.0 ± 1.2 [A]	2.2 ± 1.1 [A]
Lf	6	0.1 ± 0.3 [a]	-0.4 ± 0.3 [a]	1.1 ± 0.2 [A*]	-1.4 ± 0.3 [B]
	24	4.4 ± 1.0 [a*#]	0.3 ± 0.7 [b]	4.0 ± 1.4 [A*#]	-1.3 ± 0.1 [B]
TLR1	6	0.6 ± 0.5 [a]	0.0 ± 1.0 [a]	0.7 ± 0.2 [A]	0.3 ± 2.0 [A]
	24	-1.6 ± 0.7 [a]	-0.4 ± 0.3 [a]	-0.8 ± 0.4 [A]	-0.6 ± 0.8 [A]

TLR2	6	1.9 ± 0.3 [a*]	0.2 ± 0.7 [b]	1.1 ± 1.0 [A]	-0.4 ± 0.8 [A]
	24	1.2 ± 0.9 [a]	0.4 ± 0.6 [a]	2.3 ± 0.7 [A*]	1.0 ± 0.5 [A]
TLR6	6	0.1 ± 0.5 [a]	-0.3 ± 0.2 [a]	0.0 ± 0.7 [A]	0.0 ± 0.9 [A]
	24	-1.3 ± 0.6 [a]	-0.9 ± 0.3 [a]	-0.5 ± 0.5 [A]	0.2 ± 0.4 [A]
IL-10	6	1.4 ± 1.6 [a]	0.7 ± 1.3 [a]	0.9 ± 1.0 [A]	0.4 ± 0.4 [A]
	24	-2.8 ± 0.6 [a]	0.0 ± 0.4 [a]	-2.3 ± 0.8 [A]	0.3 ± 0.5 [A]

*Means differ significantly from negative control.

[#]Means between 6 h and 24 h treatment differ significantly.

[a, b]Means between live and heat-inactivated JF4278 without common superscript letters differ ($P<0.05$).

[A,B]Means between live and heat-inactivated PG45 without common superscript letters differ ($P<0.05$).

[c]ΔΔCt values are normalized to negative controls and corrected for the two reference genes, GAPDH and ubiquitin.

[d]RANTES = regulated on activation, normal T cell expressed and secreted; SAA = serum amyloid A; COX-2 = cyclooxygenase-2; Lf = lactoferrin.

Figure. 1. Changes of TNF-*α* relative mRNA abundance as a representative for several other immune factors in bMEC challenged with *M. bovis* JF4278.

Changes of relative mRNA abundance (ΔΔCt) of TNF-α in bMEC challenged for 6 h, or 24 h with live or heat-inactivated (h.i.) *M. bovis* strain JF4278 (108 MOI). Data are presented as Means ± SEM.

[a,b,A,B]Means within time point between live and heat-inactivated JF4278 without common superscript letters differ ($P<0.05$).

*Means differ from negative control ($P<0.05$).