

Immunological Response of Bovine Mammary Cell Lines in Mastitis and Milk Hygiene

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Summary

Mammary adherent cells (MAC-T) were infected with six isolates of *Streptococcus uberis* (*S. uberis*). Three isolates were cases of mastitis in dairy cows and belonged to clonal complex 5, 143, which is associated with virulence and three were from cows with no clinical or laboratory evidence of mastitis. All these isolates belonged to clonal complex 86 which contains strains of low virulence. After incubation at 37°C for 24 h, there were no significant differences in the number of adherent or internalized *S. uberis* between mastitis (M) and non- mastitis (NM) isolates ($p > 0.05$).

The levels of tumour necrosis factor (TNF- α), measured in treated MAC-T cells supernatant with *S. uberis* by ELISA, were significantly elevated in cultures infected with NM isolates compared with M isolates, after 10h ($p > 0.05$) and 24h ($p > 0.001$) respectively. Expression of TNF- α , TLR2, TLR4 and NFkB genes were examined by Real-Time PCR. There are highly significant differences in the timing of expression. The levels of TNF- α mRNA increased 36 fold after 6 hour of infecting cells with M strains, but not in NM strains of *S. uberis*. These results suggest a vital role for TNF- α , in the defence against *S. uberis* in the bovine mammary glands.

Key words: Mastitis, *S.uberis*, Cytokines, TNF, TLR, NFkB, PCR, Real Time PCR.
This study was performed within Department of Biotechnology, School of Applied Science at RMIT University, Melbourne, Victoria, Australia

الاستجابة المناعية لخلايا الضرع المختبرية في التهاب الضرع بالابقار وصحة الحليب

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الخلاصة

اصيبت خلايا الضرع بستة عزلات من المكورات السبحية المسببة لالتهاب الضرع. ثلاثة عزلات من حالات التهاب الضرع وتعود الى الاستنساخ المعقدة 5 و 143 التي لها علاقة بالضراوة في التهاب الضرع. ثلاثة عزلات من حالات التهاب الضرع وتعود الى الاستنساخ المعقدة 86 التي لها علاقة بانخفاض الضراوة او التي لاتظهر علامات سريرية لالتهاب الضرع. لاتوجد اختلافات معنوية في اعداد البكتريا اللاصقة او الداخلة الى خلايا الضرع سواء كانت ضارية او غير ضارية. كان قياس معدل عامل نخر الورم بواسطة ELISA في مصل خلايا الضرع المختبرية المعرضة للمكورات السبحية مرتفع بصورة معنوية للبكتريا منخفضة الضراوة بعد 10 و 24 ساعة من التعرض الى الاصابة. التعابير الوراثية عامل نخر الورم والمستقبلات الشبيهة بعدد القتلى و العامل النووي للخلايا كآب ب فحصت بواسطة Real-Time PCR. ان معدل عامل نخر الورم مرتفع معنويا في البكتريا ذات الضراوة ليزيد على 36 مرة على البقية بعد 6 ساعات من التعرض للجرثومة ذات الضراوة المعنوية. اشارت النتائج الى وجود دور مهم يلعبه عامل نخر الورم في مناعة الجسم ضد البكتريا في الغدد اللبنية للابقار.

كلمات مفتاحية: التهاب الضرع، المكورات السبحية، عامل نخر الورم، المستقبلات الشبيهة، العامل النووي، البلمرة المتسلسل، الوقت الحقيقي للبلمرة المتسلسل.

اجريت هذه الدراسة في قسم تقنيات الحياة ، كلية العلوم التطبيقية ، جامعة ارمائتي، ملبورن، فكتوريا ، استراليا

Introduction

Bovine mastitis is an inflammation of one or more quarters of the udder. This disease is still the most costly infection of dairy cattle for the milk industry (1). *S. uberis* is one of 'environmental pathogen' which is responsible for a significant proportion of clinical mastitis (2). It is considered as an 'effective pathogen' because cows are likely to develop intramammary infections if their udders are exposed to contaminated material, especially if they have damaged teat skin or open teat ends. *S. uberis* is passed in the faeces of cattle (and other ruminants) and can survive for up to 2 weeks in fresh dung or faecal-contaminated mud or straw (3). Infections due to *S. uberis* are predominantly in subclinical mastitis (95%) and up to 33% of clinical cases per year in the United States and 30% of clinical cases in UK (4 and 5). The clinical and subclinical mastitis caused by *S. uberis* ranged from 75% in Australia and New Zealand (6 and 7).

Analysis of *S. uberis* by pulsed-field gel electrophoresis (PFGE) from the same and different farms has shown that the species is highly diverse (8). Evidence is emerging from multi-locus sequence typing, however, that some clonal complexes (CCs) are highly associated with clinical and subclinical mastitis, while others are found in the environment or are isolated mainly from cows with low somatic cell counts (9). Strains belonging to global clonal complex (GCC) sequence type (ST) 5 and GCC ST143 are associated with clinical and subclinical mastitis, whereas GCC ST 83 are considered to have less capacity to cause mastitis (10, 11, 12). This laboratory has previously speculated that strains belonging to GCC ST5 and GCC ST143 possess factors promoting survival in the environment, invasion of host tissue, internalization of mammary epithelial cells or evasion of host immune responses (12).

MAC-T cells, an immortalized epithelial cell line isolated from bovine mammary tissue, were routinely cultured according to the recommended conditions (13). MAC-T cells were used widely in the experimental design instead of live animals. Bovine mammary epithelial cells (bMEC or MAC-T cells) are capable to produce neutrophil-mobilizing chemokines and pro-inflammatory cytokines such as Interleukin (IL)-6 and TNF- α upon bacterial stimulation (14, 15 and 16). MAC-T cells are crucial to delay the attacking bacteria while sending chemoattractant signals to circulate neutrophils and lymphocytes therapy facilitating generation of rapid stronger local innate immune defences mediated by infiltrating immune cells and ultimately antigen-specific protective immune responses (17, 18).

The innate immune system is the major contributor to acute inflammation induced by microbial infection or tissue damage. Innate immune cells including macrophages and dendritic cells (DCs) play important roles, nonprofessional cells such as epithelial cells, endothelial cells, and fibroblasts also contribute to innate immunity (19). The endothelial tissue and their pro-inflammatory cytokines play an important role during inflammation and caused reduction in the intracellular of *S. epidermidis* with bovine endothelial cells (20).

Toll-like receptors (TLRs) function is to distinguish antigens and to initiate an appropriate immune response (21). Toll-like receptors are key sensors of pathogen-associated molecular patterns (PAMPs) (22). Bovine mammary epithelial cells contribute to the innate immune response to intramammary infections by recognizing pathogens through specialized pattern recognition receptors. Toll-like receptor 4 (TLR4) is activated by lipopolysaccharide (LPS), a component of the outer envelope of Gram-negative bacteria (23).

The level of both tumour necrosis factor (TNF)- α and IL-12 were increased and played a role in regulation of the immune responses of bovine mammary gland in *S. aureus* infection (24). Real-time reverse transcriptase-polymerase chain reaction (RT-

PCR) was used to quantify interleukin-1 (IL-1), IL-1 receptor antagonist (IL-1Ra), tumour necrosis factor (TNF)- α , toll-like receptor 2 (TLR2), and toll-like receptor 4 (TLR4) (25). These various cytokines (TNF- α , IL-1 β , IL-6, IL-8, IL-4, IL-10 and IL-13) have synergistic and antagonistic effects in the immuno-inflammatory response of infections (26). TNF- α has ability to trigger directly or indirectly by inducing the production of secondary mediators. Recent results strongly suggest that the classical and alternative pathways to NF- κ B activation have distinct regulatory functions, one that is mostly involved in innate immunity and the other in adaptive immunity. Recently, only a single NF- κ B signaling pathway was known, whereby NF- κ B activity is stimulated by pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1), as well as by pathogen associated molecular patterns (PAMPs)(27).

The goal of this study was to compare strains of GCC ST3/ 143 with GCC ST 83 and investigate the mRNA expression of TNF- α , TLR2, TLR4 and NF κ B immune components during the acute phase of mammary inflammation caused by *S. uberis*, and establish a new model design of vaccination against bovine mastitis.

Materials and Methods

Bacterial strains: Six *S. uberis* isolates from the milk of cows were used in this study. Three isolates 5851, 2520.1 and 2893.1 were from cows with clinical mastitis had previously been placed in GCC ST5 and 143 respectively. The other three isolates from cows without mastitis (milk somatic cell counts <250,000 /ml belonged to GCC St83 (12). One to two separate colonies were streaked on Columbia agar plates (CAB) incubated at 37°C for 18 h and then separate colonies were inoculated into a media contains beads. The cultures were stored at -80°C as stock cultures. One bead from the stock culture was streaked onto CAB and incubated overnight at 37 °C. Bacteria was harvested in sterile 20 ml Todd–Hewitt broth (THB), mixed in 150 rpm orbital shaker for 2 h at 37 °C. Bacterial suspension was washed three times with PBS, then estimated the number of bacterial counts by OD₆₀₀ spectrophotometer (Invitrogen). Bacterial suspension was diluted with DMEM to a concentration of $\sim 1.3 \times 10^7$ colony-forming units (CFU).

Invasion of MAC-T cells with *S. uberis*: Dulbecco Modified Edward medium (DMEM) with 10% foetal bovine serum (FBS) plus antibiotics was removed from 24-well plates containing monolayer MAC-T cell. 1 ml of fresh DMEM medium only contains *S. uberis* (1.3×10^7 CFU/ml) per well in triplicate for each strain. Co-culture MAC-T medium were incubated for 60 minutes at 37°C and 5% CO₂. Mammary epithelial cell monolayers were washed three times with PBS, then added 1 ml/well fresh DMEM medium with 10% foetal bovine serum (FBS), 50 μ g/mL of streptomycin, and 50 IU/mL (Invitrogen) 24-well plate was incubated for 24 h at 37°C, 5% CO₂. The MAC-T cells were detached after 24 h of incubation with 0.25% trypsin, checked the viability with counter (Invitrogen™ countess, Automated Cell Counter) of cells and lyses with 0.25% Triton X-100 at a final concentration of 0.025% (v/v) in sterile distilled water. Serial dilutions of Lyses cells were streaked into CAB then incubated for 18 h at 37°C. The colonies were counted and multiplied by dilution factor. These experiments were repeated three times.

TNF- α test by ELISA: TNF- α tested by an ELISA procedure was followed (R&D research manufacture). MAC-T cells were infected with *S. uberis* for one hour. TNF- α protein level were measured in infected MAC-T supernatants harvested after incubation at 37°C and 5% CO₂ for 0 h, 3 h, 10 h and 24 h and stored at -20°C until use. TNF- α was measured with ELISA followed (R&D research manufacture). Briefly, coated a 96-well

microplate with 100 μ L per well of the diluted Capture Antibody. The plate was sealed and incubated overnight in refrigeration. Aspirated each well and washed with Wash Buffer for three times. The plate was inverted and blotted against clean paper towels. The plate was blocked Block Buffer. Then the plate was incubated at room temperature for 1 hour. The plate was washed again. The sample or standard was added to the well. An adhesive strip covered the plate and incubated 2 hours at room temperature. The plate was washed again. The Detecting Antibody was added to each well. The plate was covered with a new adhesive strip and incubated 2 hours at room temperature. Repeating the aspiration/wash was applied. Streptavidin-HRP was added to each well. The plate was covered and incubated for 20 minutes at room temperature in dark place. The plate was washed again. Substrate Solution was added to each well. The plate was incubated for 20 minutes at room temperature in a dark place. Stop Solution was added to each well, and thorough mixing. The plate was determined by optical density of each well immediately, using a microplate reader set to 450 nm.

RNA Isolation and Quantitative: *S. uberis* strains cultures of (5851 and 3327.3) were prepared as above-mentioned method in invasion of MAC-T cells with *S. uberis*. Stimulate MAC-T with DMEM only containing *S. uberis* $\sim 1.3 \times 10^7$ for 1 h at 37°C 5% CO₂, wash MAC-T with PBS three times. Fresh DMEM only was added and incubate for 0, 6, 12 and 24 h, MAC-T after which total RNA was determined and Gel electrophoresis.

RNA isolation from MAC-T cells: The cell culture medium was removed completely by aspiration. The cells were washed once with PBS pH 7.2. The RNA extraction was followed (Bioline instructions). Briefly, Added 1 ml of TRIsure (BIOLINE) to T75 ml tissue culture flask, scraped quickly by scraper. The cells were collected in eppendorf tube by aspiration, and then added 0.2 ml chloroform, secure cap tube and shake it vigorously by hand for 15 seconds. The tube was incubated at room temperature for 3-5 min, then centrifuge the sample at 12000 xg for 15 min. at 2-8°C. The upper layer was collected into a new collection tube, and then added 0.5 ml Isopropyl alcohol to the collection tube incubated at room temperature for 15 minutes. Centrifuge the mixture 12000 x g for 15 minutes at 2-8°C. The supernatant was removed and the pellet was washed once with 1 ml 70% ethanol. The sample was vortex and centrifuge at 7500 xg for 5 min. 2-8°C. RNA was stored at -80°C until used it further.

The complementary DNA: The complementary DNA (cDNA) was prepared by the following on ice: Mixing the reagents in PCR tube 1 μ g RNA (3 μ l), Oligo (dt)₁₈ (1 μ l) 10mM dNTP (1 μ l) and diethyl pyrocarbonate-treated water (DEPC-H₂O) up to (10 μ l). The tubes were put in PCR machine, and then the mixture was incubated at 65°C for 10 min. All tubes were placed in ice for 2 minutes. In the meantime these reagents 5x RT Buffer 4 μ l, RNase inhibitor 1 μ l, Reverse transcriptase (200u/ μ l) 0.25 μ l and DEPC-H₂O up to 10 μ l. The last 10 μ l reagents were added to 10 μ l of the above reaction mix to a tube containing the primed RNA. Samples were incubated at 45°C for 60 min and the reaction was terminated by incubating at 70°C for 15 min. The samples were stored at -20°C until next step real time PCR was be ready.

Real-Time PCR: 2 X SensiMix 25 μ l final dilutions was 1X was added to 5 μ l Template, then added 2 μ l of each 10 μ M forward TLR2, TLR4, NF κ B and TNF- α Primers final concentration 200nM. 2 μ l of 10 μ M reverse Primers final concentration 200nM was added. RNase free water was added up to 50 μ l. The mixture was placed in Real-Time PCR special tubes or 48 wells and sealed. The tubes or plate were put in Real-Time PCR machine which was programmed as follows. Cycle 1 was programmed at 95°C for 10 min. The 40th cycle was planned at temperature 95°C for 10 seconds and 60°C for 60 seconds acquire at end of step.

Statistical Analysis: Statistic for data was performed by student *t-test*, ANOVAs two way statistics.

Results and Discussion

Adherence, Invasion and Viability of MAC-T cells: There were no significant differences between M and NM isolates in their ability to adhere to (Figure 1) invade (Figure2), or kill (Figure 3), MAC-T cells after a 24 h incubation period at 37°C.

MAC-T was infected with six M and NM strains of *S. uberis*. The viability of MAC-T cells in both M and NM of *S. uberis* strains were compared. Neither the adherent of M strain *S. uberis* counts to MAC-T cells or NM *S. uberis* counts were different in numbers after MAC-T cells infected with both strains of *S. uberis*. These results agreed with the results accomplished by (28, 29 and 30), these authors summarised that M and NM of *S. uberis* strains were adherent to MAC-T cells, but equivalent in the adherence counts between M and NM of *S. uberis* strains. The surviving of both M and NM of *S. uberis* strains were estimated by SPC after 24 h incubation in MAC-T cells, no significant differences between two strains. These results are supported by (1) who also mentioned that *S. uberis* survived intracellular for 120 h without loss of viability in MAC-T cells.

TNF- α Measurement by ELISA: In supernatant of MAC-T cells incubated with *S. uberis*, there was a steady increase in levels of TNF- α over the incubation period of 24 h. The levels TNF- α were elevated significantly ($P \leq 0.05$) in MAC-T cell's supernatant which was stimulated by NM strains of *S. uberis* more than M strains of *S. uberis* in 10 h incubation ($P \leq 0.05$). The difference was that TNF- α levels (yellow columns) were highly significant after 24 h incubation. The Level of TNF- α in stimulated MAC-T cells with M strains were increased after 10 h and 24 h incubation but less extent than NM strain. The negative control (blue columns) and positive control (red columns) were conducted in each experiment (Figure 4). The up-regulation of TNF- α in M *S. uberis* was converted from nanograms to picograms. TNF- α was 213.8 pg after 24 h incubation time of supernatant of MAC-T cells, in 10 h incubation time, the measurement of TNF- α was 15.1 pg, while in 3 h time was nearly close to 0 time measurement.

Activated macrophages release mediators, such as interleukin-1 (IL-1) and tumour necrosis factor (TNF) families appear to be uniquely important in initiation the next series of reaction. These cytokines have pleiotropic activity and act both locally and distally (31). In this study we found that TNF- α elevated after 10 h and 24 h of infected MAC-T with M and NM *S. uberis*. TNF- α was increased significantly in NM strains over M strains.

These results indicated that one or two of these 3 strains possessed influential antigenic activity to stimulate TNF- α more than the M strain. These results disagreed with the findings by (32) who designed their experiment in the macrophages of milk. The level of TNF- α in NM *S. uberis* strains up-regulated 10 folds in time 10 h and 24 h higher than in NM strains of *S. uberis*. The mRNAs tend to have abundant AU-rich elements in their 3'UTRs compared with mRNAs expressed at later time points (33). Therefore, control of mRNA decay may be as important as control of transcription in terms of the regulation of innate immune responses.

Real-Time PCR: Extraction RNA was measured by gel electrophoresis as shown in (Figure 5). The primer genes have been analysed by gel electrophoresis as revealed in (Figure 6). Normalise data from real time PCR were showed that TNF- α measurement has been much higher than TLR2, TLR4, and NFkB genes. Folds of TLR2, TLR4, and TNF- α treated with M of *S. uberis* strains was high. TNF- α level was 3.59 in 6 h and 0.209 in 12 h of incubation, while TNF- α Treated with NM 0.02 in 6 h and 0.002 in 12 h of incubation. TLR2 level treated with M of *S. uberis* was 0.15 in 6 h and 0.02 in 12 h of

incubation while TLR2 level treated with NM of *S. uberis* was 0.003 in 6 h and 0.02 in 12 h of incubation. TLR4 level treated with M of *S. uberis* was 0.06 in 6 h and 0.08 in 12 h of incubation while TLR4 level treated with NM of *S. uberis* was 0.02 in 6 h and 0.02 in 12 h of incubation. NFkB results in different time of incubation were below the TNF- α level so, in (Figure 7) was used sigmaplot graph to display lower columns in the graph for matching with TNF- α .

The inflammatory response is orchestrated by pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6. These cytokines are pleiotropic proteins that regulate the cell death of inflammatory tissues, modify vascular endothelial permeability, recruit blood cells to inflamed tissues, and induce the production of acute-phase proteins. Although TNF and IL-6 are mainly regulated at the transcriptional and translational levels, the production of IL-1b is regulated by a two-step mechanism. The first step is the expression of an IL-1 β zymogen, pro-IL-1 β , which is regulated by the synthesis of its mRNA in a TLR signal-dependent manner. However, IL-1b maturation requires cleavage of pro-IL-1b by a protease, caspase-1, which is activated independently of TLR signaling. The complex that activates caspase-1, called the inflammasome, is composed of Nod-like receptor (NLRs), Apoptosis associated speck-like protein containing a caspase recruitment (ASC), and caspase-1 (34).

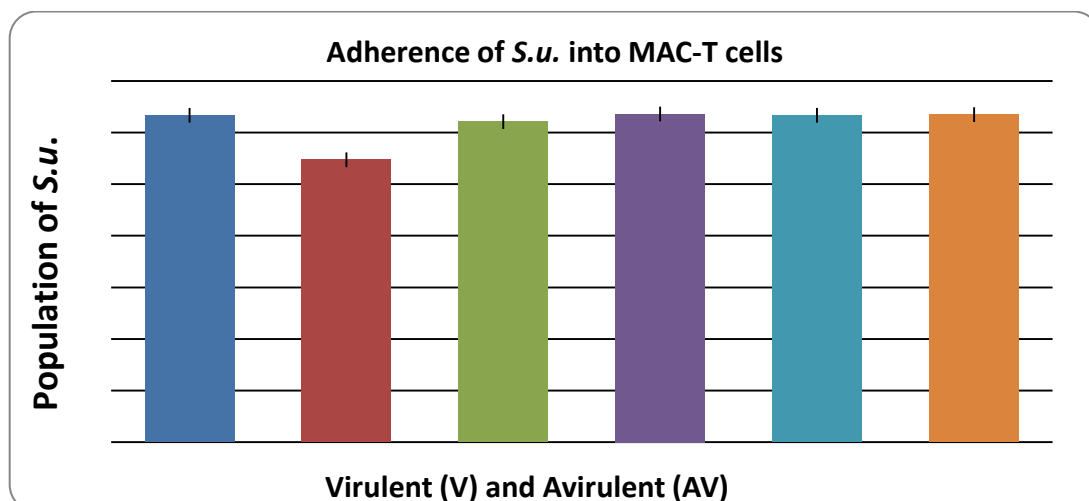


Figure1. Mean with SE number of Virulence and Non-Virulence of *S. uberis* adherent to MAC-T cells. Each point was the result of adhesion assays performed on these isolates of *S. uberis* either Virulence or Non-Virulence in triplicate.

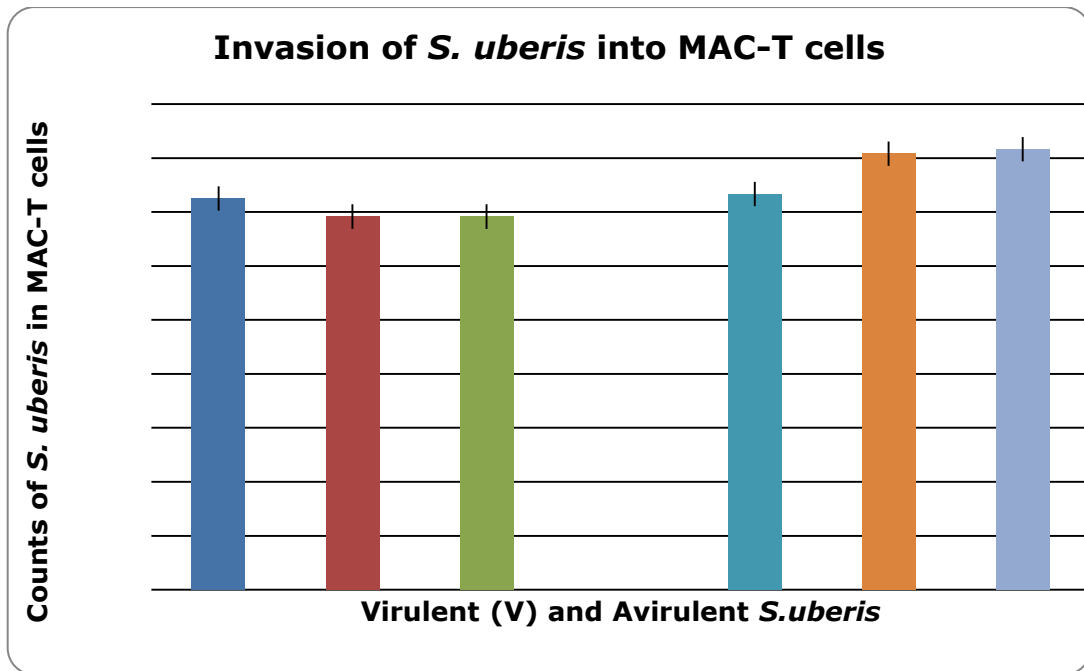


Figure 2. Mean with SE number of Virulence and Non-Virulence *S. uberis* invasion to MAC-T cells. Each point was the result of invasion assays performed on these isolates of *S. uberis* (either mastitis or non-mastitis associated) in triplicate.

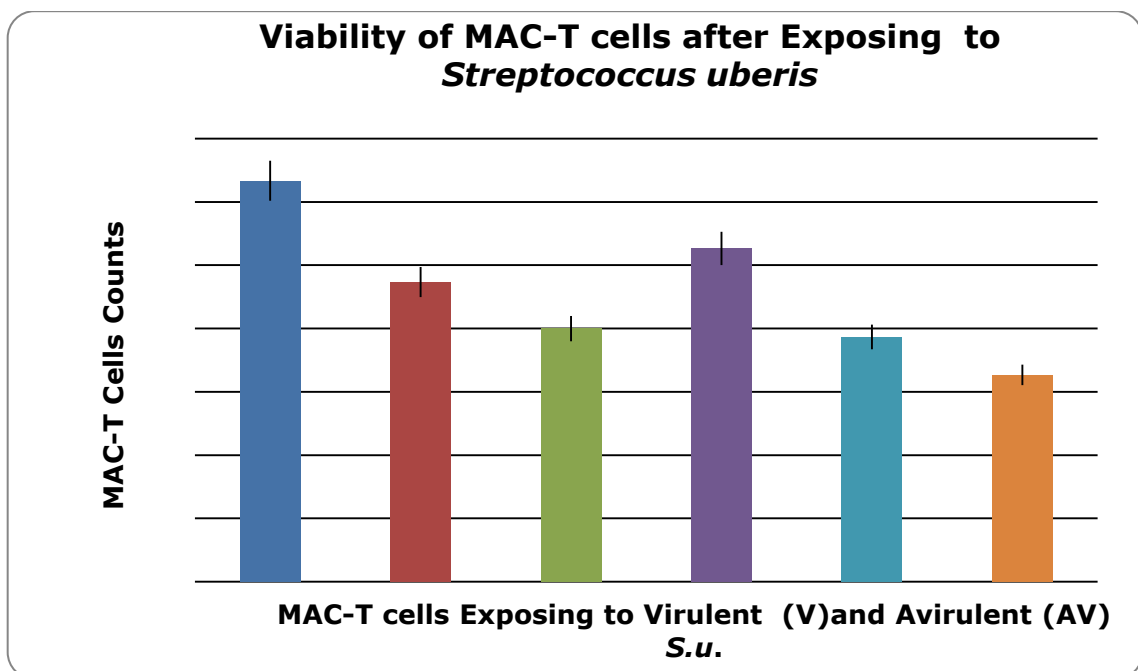


Figure 3. Mean with SE number of Virulence and Non-Virulence *S. uberis* to MAC-T cells viability. Each point was the result of killing performance of these isolates (either mastitis or non-mastitis associated) to MAC-T cells in triplicate.

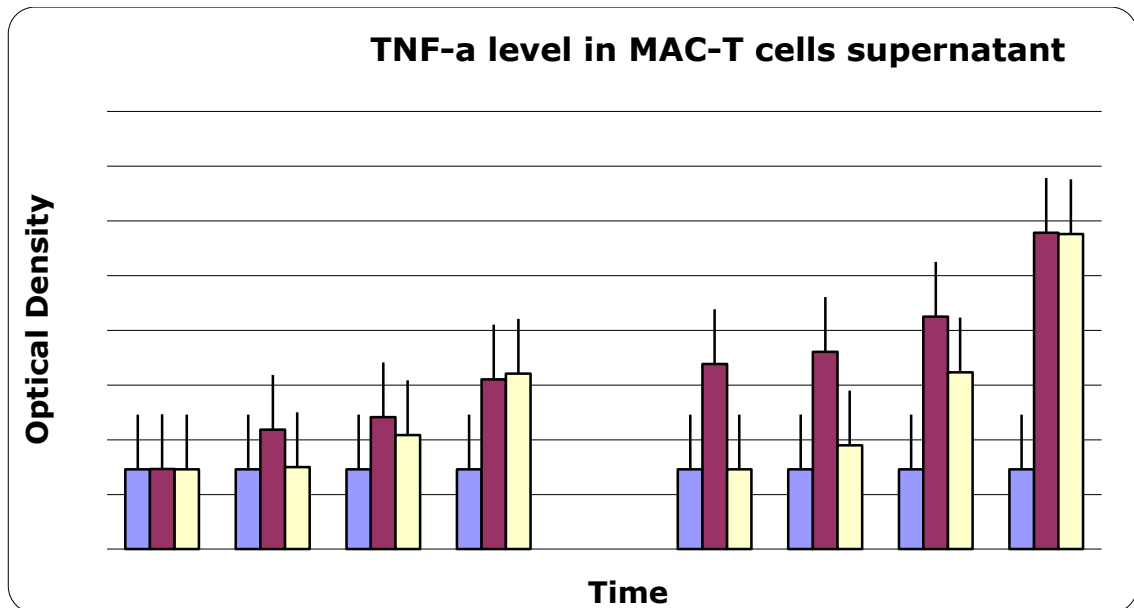


Figure 4. The level of TNF- α measurement level in Pictograms at time 0h, 3h, 10 h, and 24 h incubation period in both M and NM strains of *S. uberis*. Blue Columns are Negative control, red Columns are positive control, yellow columns are Mastitis left and non-mastitis right.

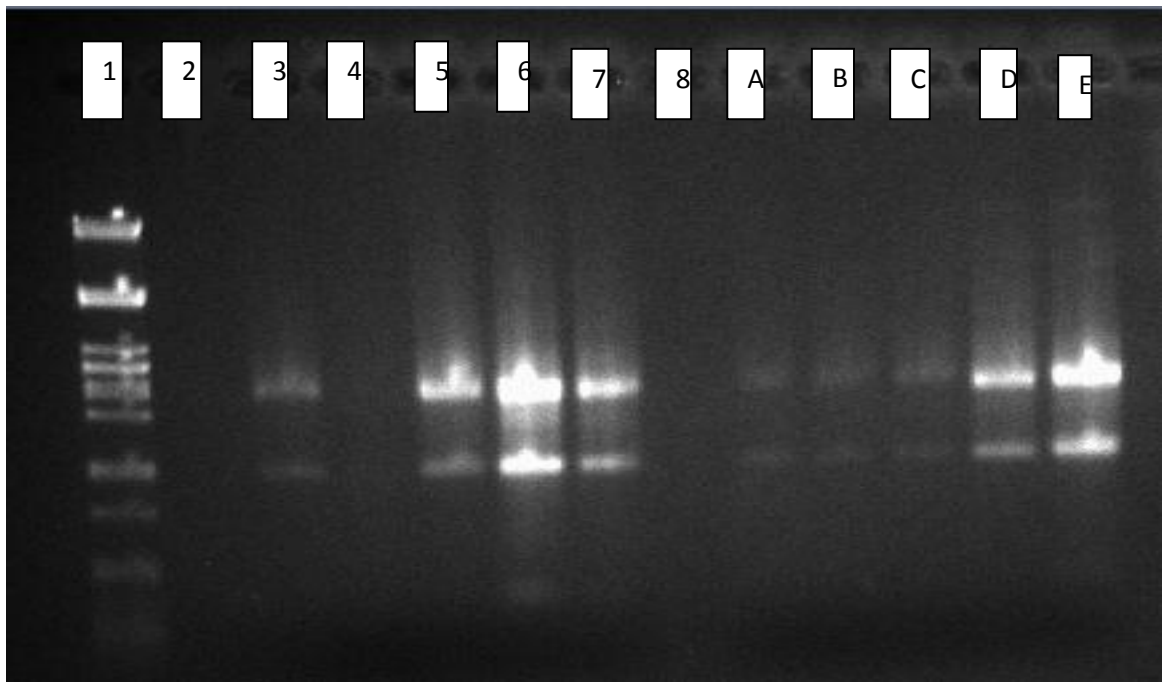


Figure 5. RNA extraction from MAC-T cells after stimulation with *S. uberis*. 1= Marker, 2= Empty, 3=M 0h, 4= M 3h, 5= M 6h, 6= M 12h, 7= M 24h, 8 = Empty, A= NM 0h, B= NM 3h, C= NM 6h, D= NM12h, E= NM24h.

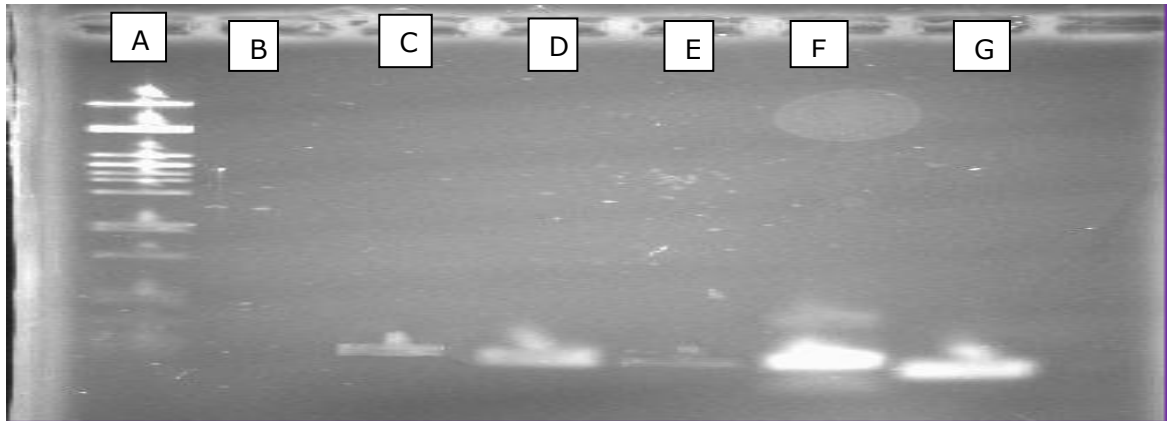


Figure 6. cDNA of RNA extraction from MAC-T cells after stimulation with *S. uberis*. A=Marker, B=NA, C=TLR2, D=TLR4, E=NFkB, F=GAPDH, F=TNF- α .

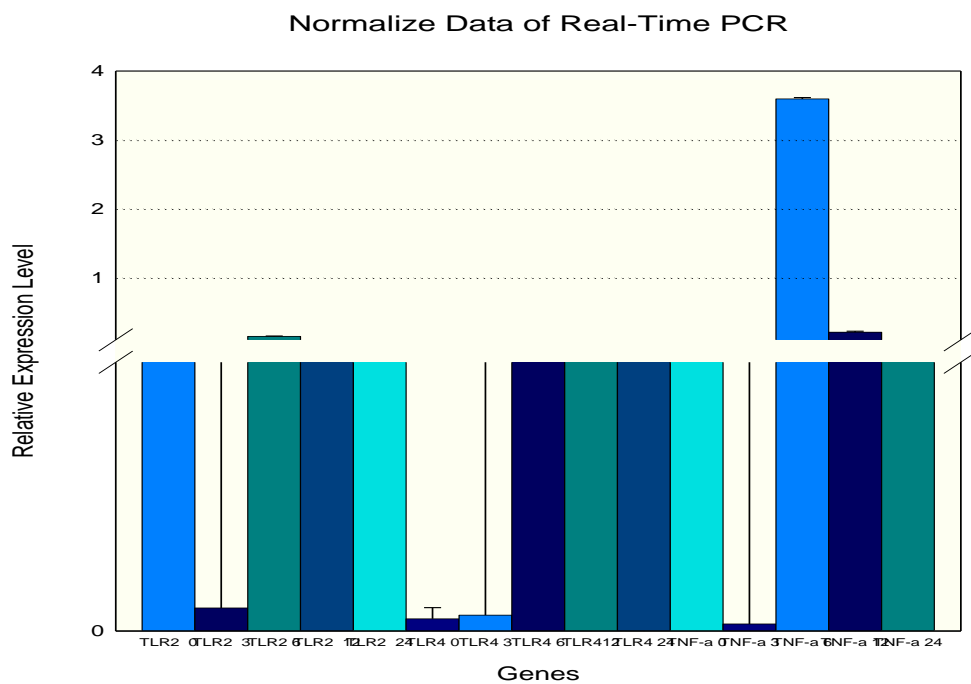


Figure7. Normalise data of mRNA, TLR2 0, 3,6, 12, 24 h - TLR4 0, 3,6, 12, 24 h, TNF- α 0, 3,6, 12, 24 h.

Table 1. Percentage of immune components after stimulation with Mastitis and Non-Mastitis of *S. uberis* strains.

Immune components	0h	3h	6h	12h	24h
TLR2 (M)	3%	3%	80%	10%	4%
TLR2 (NM)	2%	10%	6%	80%	2%
TLR4 (M)	1%	2%	40%	55%	2%
TLR4(NM)	2%	30%	33%	33%	2%
TNF-a (M)	1%	1%	90%	6%	2%
TNF-a(NM)	1%	1%	50%	45%	3%

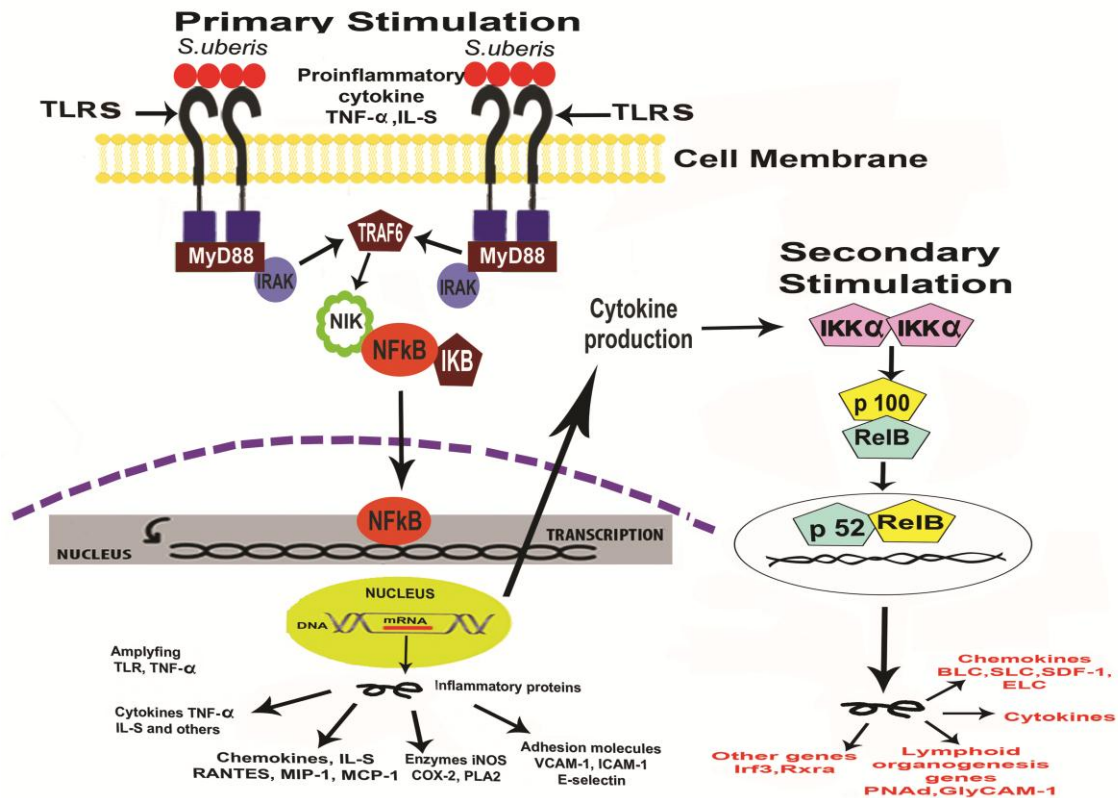


Diagram 1. Primary and secondary stimulation of innate immunity induced by mastitis and non-mastitis strains of *S. uberis*.

TNF- α , TLR2, TLR4, NF κ B were determined and quantitative by Real-time PCR (Table 1) in this method we found that TNF- α was highly shifted in M strains of *S. uberis* than NM strains of *S. uberis*. TNF- α level was elevated 35.9 times, than others (Figure 7). Transcripts that increased in both cell lines by at least 20 fold included IL-8, CXCL6 and TNF- α , but in each case there was a much greater fold increase in the bMEC. IL-1b and b-defensin were markedly up-regulated by greater than 500 fold in bMEC but only 4.26 and 2.75 fold, respectively, in the MAC-T cells (17). Gram positive and gram negative bacteria fluctuate in their dose-dependent patterns of induction of TLR2 and TLR4 (35). Nearly all of the genes directly involved in the Toll-Like Receptor activation pathway (i.e. TLR2, TLR4, CD14, IRAK-1, IRAK-4, IRAK-M and TRAF-6) were expressed in both cell lines but in each case was largely unaffected by LPS treatment (17). In case of TLR2 and TLR4 were elevated in the M strains more than NM strains. The number of TLR2 copies correlated well with those of TLR4, indicating coordinated regulation of these two PRRs during infection of the udder (36). For that reason it thought that M strains were more reliable to induce TNF- α than NM strains of *S. uberis*. TLR4 is the major receptor for LPS and causes intracellular signal transduction (37). The initial stimulation of *S. uberis* induced innate immunity, while secondary stimulation induced cytokines which take part in pro-inflammatory responses or cure by production of anti-inflammatory immune components (Diagram 1). The function of TNF- α activates, as a synergistic in normal level and as an antagonistic or adverse reaction in high level.

In conclusion, the function of TNF- α activates as a synergistic in normal level and an antagonistic or adverse reaction in high level. TNF- α in the mRNA of MAC-T cells is induced by mastitis *S. uberis* strains after 6 h due to: Firstly, the cells being able to

produce TNF- α in the first 6 hours after infected with *S. uberis* due to stimulation of TNFR4 receptor which lead to inducing TNF- α . Secondly, mastitis strains of *S. uberis* have the ability to attack mammary tissue in the first 6 h and inhibit the secretion of cytokines to make the tissue medium safe for multiplication. Thirdly, MAC-T cells are defending themselves against bacteria by secreting cytokines to stop their action and sending a signal to other immunity cells and components to participate in the body defence. Fourthly, TLR2, TLR4 are involved indirectly in this process by activation of NFkB, for that reason low level of NFkB, TLR2 and TLR4 were detected in this process. Fifthly, NM strains of *S. uberis* have induced the TNF- α from MAC-T in supernatant, for that reason TNF- α in ELISA was detected in 12 and 24 hours. Sixthly, NFkB have appeared only during time 3 h after stimulation with M and NM. In M case NFkB was very low during this early stage while TNF- α level was the highest 36 folds and higher than TNF- α in NM stimulation. In NM case NFkB was considerably high while TNF- α was lower than M stimulation. Our results showed TNF- α had a negative correlation with NFkB. Seventhly, it recommond in future studies to design synthetic peptides of MAC-T cells TNF- α to use it as a vaccination against bovine mastitis as a trial proposal.

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