Gene 585 (2016) 247–255

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Streptococcus uberis ST439 and *ST475* induce differential inflammatory responses in a mouse intramammary infection model

Susweta Das Mitra [a,c], Bibek Ranjan Shome [a,*], Bhuvana Mani [a], D. Velu [a], Apala Banerjee [a], Kiran Bankar [b], Sankar Kumar Ghosh [c], Sandip Santra [a], K.P. Suresh [a], Habibur Rahman [a]

[a] *ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Ramagondanahalli, Yelahanka, Bengaluru, Karnataka, India*
[b] *BionividPvt Ltd, Kasturinagar, Bengaluru, Karnataka, India*
[c] *Department of Biotechnology, Assam University, Silchar, Assam, India*

ARTICLE INFO

Article history:
Received 11 January 2016
Received in revised form 4 March 2016
Accepted 28 March 2016
Available online 30 March 2016

Keywords:
Mastitis
Mice model
S. uberis
Immune response

ABSTRACT

Streptococcus uberis causing mastitis is a growing challenge to the dairy industry. Molecular, epidemiological and population structure studies have revealed clonal diversity among the infecting strains. In this study, mouse intramammary infection model was used to uncover the host immune response to two epidemiologically important live strains of *S. uberis* (*SU1*and *SU2*) obtained from subclinical case of mastitis possessing specific and unique multi locus sequence types (ST), pulsed field gel electrophoresis (PFGE) pulsotypes and virulence profiles. Temporal (2 h, 4 h, 8 h, 12 h, 24 h and 48 h) expression of key inflammatory mediators (IL2, IL4, IL6, IL12, TNFα, IFNγ, GMCSF, TLR2, TLR4, TLR9, TLR11, TLR12, CD14, IL1β, RANTES, Lactoferrin, and CXCl1) by reverse transcription and probe-based quantitative real-time PCR showed relative mRNA levels higher ($p < 0.05$) in response to *SU2* compared with *SU1* with 24 h PI serving as a critical point for the deviating behavior (*SU1 versus SU2*). Further employing the predicted biological processes under the influence of this pool of tested genes, the delineation of gene regulatory networks suggested *SU1* _favoring its persistence in the host environment; in contrast, *SU2* _which elevated gene expression indicating towards pathogen clearance or immune surveillance. This study suggested how these unique strains could manipulate the host immune response to influence the severity of mastitis; our results expand the available information on host pathogen interaction and provide a firm foundation needing further investigations to gain control over this pathogen.

1. Introduction

Bovine mastitis is the most serious economically significant disease affecting the dairy industry. Several factors complicate the disease condition, including the multiple causative agents, poor understanding of the early immune response and the complexities associated with mammary epithelial cell damage by both the agent and host factors (Awale et al., 2012).

Bacteria are the main etiological agents, with *Staphylococcus aureus*, *Coagulase Negative Staphylococci* (*CoNS*), *Escherichia coli* and *Streptococcus uberis* being the most common pathogens (Schukken et al., 2011; Kromker et al., 2014). Recently, *S. uberis* has been detected more frequently in a growing number of dairy herds and has caused both subclinical and clinical infections of the udder (Kromker et al., 2014; Swanson et al., 2009; Smolenski et al., 2014). Due to the importance of Streptococcal mastitis, several epidemiological studies have been performed across the globe to investigate its virulence potential and clonal diversity and to elucidate the pathogen biology (Wang et al., 2013; Shome et al., 2012; Rato et al., 2008; Zadoks, 2007). *S. uberis* possesses different virulence factors (i.e., CAMP factor (cfu), R-plasminogen activator (*pau*A/*skc*), and the adhesion molecule gene (*sua*)) that empower its adherence, internalization and persistence in the host environment and enable it to establish infection (Shome et al., 2012; Patel et al., 2009; Chen et al., 2010). The protein encoded by *sua* gene is immunogenic and plays a key role in adherence, thereby aiding persistent infection (Chen et al., 2010).

Over time, pathogens have evolved to be more ingenious than ever anticipated. Due to the existing difficulty in eradication and the increasing concern over antibiotic usage, alternative therapeutics are needed for mastitis control programs. In our previous study, we encountered novel clones of *S. uberis* that were prevalent in the southern province and caused subclinical mastitis (Shome et al., 2012) that required attention from the mastitis control program. These clones were unique and were reported for the first time in mastitis surveillance. This finding augmented the need to unravel the bacteria's strategic interactions

Abbreviations: IMI, Intramammary Infection; PI, Post inoculation; ST, Sequence Type; TLR, Toll Like Receptor; IF, Interferon; TNF, Tumor Necrosis Factor; IL, Interleukin; CXCl, Chemokine.

* Corresponding author at: ICAR-NIVEDI (formerly PD_ADMAS), Ramagondanahalli, Yelahanka, Bengaluru 560064, Karnataka, India.
E-mail address: brshome@gmail.com (B.R. Shome).

http://dx.doi.org/10.1016/j.gene.2016.03.054
0378-1119/

and behavior with the host immune defense system. Underpinning the immune response of the host is crucial to gain an insight into the host microbe interaction and intervene in the disease progress.

During intramammary infection (IMI), pathogen associated molecular patterns (PAMPs), or more precisely microbial associated molecular patterns (MAMPs), are recognized by pathogen recognition receptors (PRR),which are the known initiators of all immune responses (Kawasaki and Kawai, 2014; Akira et al., 2006). PRRs additionally recognize endogenous mediators that are released during infection by stressed tissues (danger associated molecular patterns or DAMPs) to warn the host of danger (Di Gioia and Zanoni, 2015; Vander Poll and Opal, 2008). Members of the toll-like receptor (TLR) family play a major role in sensing the invading organism and tailoring the activation of signaling cascades. These cascades lead to the activation of transcription factors such asAP-1 and NF-κB and interferon regulatory factors (IRFs) and eventually trigger a network of immune factors that can lead to pathogen clearance or the restoration of tissue homeostasis; however, these factors can be detrimental and fatal if left uncontrolled (Di Gioia and Zanoni, 2015). Additionally, TLR co receptors such as CD14 have been found to serve as specific accessory proteins that interact with the TLR, thereby facilitating the TLR-mediated immune response in addition to transducing their own signals (Di Gioia and Zanoni, 2015; Janot et al., 2008).Cytokines are key immune factors in addition to interferons and chemokines. IL6, TNFα and IL1β are the master pro-inflammatory cytokines that induce the production of other immune factors. Granulocyte macrophage colony stimulating factor (GM-CSF), the antibacterial protein Lactoferrin (Lf), interferons (IFNγ), interleukins (IL-2, IL-4, and IL-12) and chemokines (RANTES/CCl5 and CXCl1) direct the recruitment and activation of neutrophils into the infected mammary gland and are a few of the candidate immune factors that play a vital role in immune defense (Wellnitz et al., 2006; Bannerman, 2009; Zbinden et al., 2014). These wide spectra of immune genes have been shown to orchestrate both the local and systemic immune responses that decide the fate of the infection.

Notably, microbial pathogens have proven to be cleverer in manipulating and escaping host defenses than we ever assumed. bMECs (bovine Mammary Epithelial Cells) have been widely used as a model system in several studies related to mastitis (Wellnitz et al., 2006; Zbinden et al., 2014; Gunther et al., 2009; Griesbeck-Zilch et al., 2008), but recent reports have discussed their poor capacity to mimic whole animal data (Gunther et al., 2009). Our previous study successfully used a mouse model to study mastitis, thereby providing a suitable *in vivo* model system (Modak et al., 2012, 2014).

To date, *S. aureus* and *E. coli* have comparatively gained more attention in mastitis research (Petzl et al., 2008) than *S. uberis*; hence, the available information on *S. uberis* pathogenesis and the associated host immune response is meager. Encountering the unique and epidemiologically important clones of *S. uberis* in our previous study emphasized the need to understand the interaction between these two strains and the host defense system.

Here, we expanded the study by using an *in vivo* mouse model to delineate how the two epidemiologically important *S. uberis* strains could manipulate the host immune response over the course of infection. Importantly, these two selected strains had specific multi locus sequence typing (MLST), Pulsed Field Gel Electrophoresis (PFGE) and virulence profiles. Notably, one strain was positive for the virulence factor *sua* gene, while the other strain was not.

2. Materials and methods

2.1. Ethics statement

The Institutional Animal Ethics Committee (IAEC) of ICAR-NIVEDI (formerly PD_ADMAS) approved the animal experiments. The animal experiments were performed under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) registration no. 881/03/ac/05/CPCSEA. The animal experiments were performed as per the guidelines of CPCSEA, Government of India, New Delhi, as previously described (Modak et al., 2012; Modak et al., 2014; Krishnamoorthy et al., 2014).

2.2. Bacterial strains and growth conditions

Two representative field isolates of *S. uberis* (designated *SU1*_ and *SU2*_ with specific characteristics (Table 1) were selected in the present study for intramammary infection (IMI) in an established mouse model. These *S. uberis* field isolates were grown in brain heart infusion (BHI) broth overnight at 37 °C. The bacterial cells were centrifuged at 3000 ×*g* for 5 min at room temperature to pellet the cells. The *S. uberis* pellet was washed with sterile PBS (HiMedia) twice to remove all of the media components and finally suspended in PBS. The standard plate count (SPC) method was used to decide the inoculum dose and final bacterial load. For intramammary inoculation 50 μL (5×10^3 cfu) (50) was used.

2.3. In vivo intramammary infection (Swiss albino mice)

Swiss albino mice (timed pregnant) were procured from the National Centre for Laboratory Animal Science (NCLAS), National Institute of Nutrition, Hyderabad, India, and acclimatized under controlled conditions in individually ventilated cages (IVC). The *S. uberis* intramammary infection (IMI) experiments were performed following the protocol described by Chandler, 1970 with modifications as previously described (Modak et al., 2012, 2014; Krishnamoorthy et al., 2014) for our *S. aureus* and *E. coli* challenge studies. A total of 18 mice comprising a group of three mice for each time point (2, 4, 8, 12, 24, and 48 h) and twelve mice for the PBS control were simultaneously inoculated. The mice were sacrificed, and the samples were collected in RNA-later. The RNAlater was discarded, and the RNA stabilized tissues were flash frozen in liquid nitrogen and stored at −80 °C prior to use.

2.4. Total RNA extraction and reverse transcription

Total RNA was extracted from mouse mammary tissues using the RNeasy mini kit (Qiagen) as per the manufacturer's instructions. The total RNA yield and purity were determined with a NanoDrop 2000c (Denmark). Finally, 300 ng of total RNA was subjected to cDNA synthesis using the Revertaid H-minus cDNA Synthesis kit (Invitrogen) under the following conditions: denaturationat 95 °C for 10 s and amplification for 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The concentration of the cDNA was determined using the NanoDrop 2000c (Denmark).

2.5. Quantitative real-time PCR

The relative mRNA concentrations were quantified via quantitative real-time PCR (qRT-PCR) using gene-specific primers and compatible probes from the Roche Universal Probe Library (Table 2). The assay was performed with the Roche Light Cycler® 480 real-time PCR system with the LC480 Probe master kit (Roche).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene for normalization. Sense and antisense primers for the genes are shown in Table 2. Each reaction volume was 20 μl, and

Table 1
Characteristics of the *Streptococcus uberis* (SU) isolates used for IMI in the mouse model.

Isolate ID	Gene bank Accession no.	MLST Sequence type	PFGE pulsotype	Virulence		
				cfu	*Skc*	*Sua*
SU1	HM-355974	ST439	A1	−	+	+
SU2	HM355979	ST475	B	+	+	−

Reference: Shome et al., 2012.

Table 2
Sequences and annealing temperatures of primers and probes and amplicon size (Modak et al., 2014).

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe Cat No (Universal Probe Library)	Annealing temperature	AMP size (bp)
IL2	GCTGTTGATGGACCTACAGGA	ATCCTGGGGAGTTTCAGGTT	#15, cat. no. 04685148001	60	69
IL 4	CATCGGCATTTTGAACGAG	GACGTTTGGCACATCCATCT	#2, cat. no. 04684982001	60	67
IL 6	ATCAGGAAATTTGCCTATTGAAA	CCAGGTAGCTATGGTACTCCAGA	#6, cat. no. 04685032001	58	60
IL 12	CTTAGCCAGTCCCGAAACCT	TTTTCTCTGGCCGTCTTCAC	#114, cat. no. 04693485001	60	62
TNFα	AAGGGGGACCAACTCAGC	CGGACTCCGCAAAGTCTAAG	#113, cat. no. 04693477001	58	61
IFNγ	CGCTACACACTGCATCTTGG	GACTGTGCCGTGGCAGTA	#129, cat. no. 04693655001	60	73
GM CSF	TGTAGAGGCCATCAAAGAAGC	ACCTCTTCATTCAACGTGACAG	#79, cat. no. 04689020001	60	66
TLR 2	CTGCACTGGTGTCTGGAGTC	GGGCACCTACGAGCAAGAT	#2, cat. no. 04684982001	60	101
TLR 4	GGACTCTGATCATGGCACTG	CTGATCCATGCATTGGTAGGT	#2, cat. no. 04684982001	60	101
TLR 9	CTCGGAACAACCTGGTGACT	ACTGGAGGCGTGAGAGATTG	#106, cat. no. 04692250001	60	60
TLR 11	ATGGGGCTTTATCCCTTTTG	AGATGTTATTGCCACTCAACCA	#1, cat.no. 04684974001	60	60
TLR 12	TTTCAAGCACTGGCCTAACC	GAAGCCTAGGCATGGCAGT	#31, cat.no. 04687647001	60	60
CD 14	AAAGAAACTGAAGCCTTTCTCG	AGCAACAAGCCAAGCACAC	#26, cat.no. 04687574001	60	89
IL 1ß	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG	#78, cat. no. 04689011001	60	68
RANTES	CCTACTCCCACTCGGTCCT	GTTTCTTGGGTTTGCTGTGC	#105, cat. no. 04692241001	60	73
Lactoferrin	CGGACAGACAAGGTGGAAGT	CCATTTCTCCCAAACTGAGC	#62, cat. no. 04688619001	60	68
CXCL1	AGACTCCAGCCACACTCCAA	TGACAGCGCAGCTCATTG	#83, cat. no. 04689062001	60	130
GAPDH	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG	#9, cat. no. 04685075001	60	62

the reaction conditions were as follows: 95 °C for 5 min, followed by 40 cycles at 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 30 s. The relative expression levels were normalized to GAPDH against the tested *versus* PBS control using the ΔΔCT inbuilt algorithm in the LC480 system.

2.6. Predicted gene ontology and pathway analysis

Biological analysis of the tested genes (n = 17) was assessed using the predicted Gene Ontology and Pathways with DAVID (http://david.abcc.ncifcrf.gov/).

2.7. Biological network analysis of tested genes and predicted enriched biological processes

The list of the tested genes (n = 17) and their predicted biological process clusters were provided as an input to the Bridge-Island software to elucidate the gene: process connections with enrichment scores. Then, the connections were imported into Cytoscape V 2.8.3 to visualize the network. Nodes/tested genes were colored based on their fold changes in expression upon infection separately for each time point to elucidate the regulatory dynamics upon infection.

2.8. Statistical analyses

The mRNA data are presented as the means ± SEM of three biological replicates for each time point. Statistical analysis of the inflammatory response data was performed with ANOVA using SAS (Release 9.3; SAS Institute Inc., Cary, NC, USA) with Proc GLM and the Tukey post hoc-test to assess the pairwise significance. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Relative mRNA expression levels of immune determinants

Evaluation of three test mice corresponding to each time point (2 h, 4 h, 8 h, 12 h, 24 h and 48 h) against PBS-inoculated control mice allowed us to record the relative fold-changes in the expression of these three biological replicates under each condition for both SU1 and SU2 IMI.

The measured relative fold changes in expression showed significant over-expression of the majority of the pro-inflammatory cytokines in the infected tissues compared to the controls. In contrast, few of the TLRs were over-expressed. Temporal observation revealed that challenge of the mammary glands with *S. uberis* for only 2 h had no significant effect on the relative mRNA expression of the measured factors, with the exception of TLR2, TLR9, TLR11, TLR12 and RANTES (Table 3).

3.1.1. TLR expression

Among the five TLRs tested (TLR 2, 4, 9,11 and 12), strain *SU1*_induced increased mRNA levels of TLR2 in the mammary gland at 2 h PI, TLR4 at 8 h PI and TLR9 at 12 h PI. In contrast, *SU2* significantly increased the mRNA levels of TLR2, TLR4, TLR9, TLR11 and TLR12 at 24 h PI (Fig 1).

3.1.2. Pro-inflammatory response

The relative mRNA expression of the master pro-inflammatory cytokines was analyzed. TNFα was significantly increased after 12 h of infection with *SU1*. However, a high elevation in its mRNA expression level was observed following infection with *SU2* but not *SU1*, at 24 h post-inoculation. In contrast, IFNγ mRNA expression was significantly increased at 8 h PI and achieved a high elevation at 24 h PI in response to *SU2* Similarly, IL1β mRNA expression was increased at 12 hand significantly peaked at 48 h PI in response to *SU2*.

3.1.3. Interleukins

SU2 strongly induced high levels of IL2 and IL4 mRNA expression that significantly peaked at 24 h PI in comparison to *SU1*. There was no significant difference in the mRNA level of IL12 between the two test conditions.

3.1.4. Chemokines

The mRNA expression levels of RANTES and CXCl1 were affected at 2 h PI with *SU1*, buta significant elevation was noted at 24 PI in response to *SU2*IMI. At 48 h, the expression of these chemokines dropped drastically (Fig 2 a & b).

Interestingly, at 48 h PI most of these genes showed a decrease in their mRNA levels, which was essential for sustained *S. uberis* infection. Lactoferrin was found to be high at 12 h PI in *SU2* IMI and was moderately expressed in SU1 IMI; however, there was no significant difference between SU1 and SU2.

In response to *SU2* infection, GMCSF mRNA expression was significantly increased after 24 h PI and then drastically decreased at 48 h PI. The mRNA level of GMCSF in *SU1* IMI was significantly high at 8 h PI and then decreased over time.

The co-stimulatory molecule CD14 was found to be elevated in both *SU2* and *SU1*IMI; however, there was no significant difference between the two conditions.

Table 3
Fold changes of mRNA levels (mean ± SEM of the ΔΔCT[1]) of immune factors (n = 17) in mouse mammary gland stimulated with two live *S. uberis* strains for 2 h, 4 h, 8 h, 12 h, 24 h and 48 h.

Genes[2]	Time	SU1[3]	SU2[3]	p-Value		Time	SU1[3]	SU2[3]	p-Value
IL2	2 h	3.2 ± 1.3	0.37817 ± 0.06	0.112	TNFα	2 h	2.3 ± 0.8	0.86 ± 0.4	0.191
	4 h	5.7 ± 3	12.3860 ± 5	0.375		4 h	3.0 ± 2	7.8 ± 4	0.340
	8 h	2.0 ± 0.6	9.9 ± 2.2	**0.028***		8 h	2.04 ± 0.5	2.8 ± 1	0.602
	12 h	1.9 ± 0.2	2.2 ± 0.8	0.724		12 h	3.3 ± 0.6	0.9 ± 0.5	**0.041***
	24 h	0.4 ± 0.1	8.5 ± 2.6	**0.037***		**24 h**	0.6 ± 0.2	28.3 ± 12	**0.089#**
	48 h	1.3 ± 0.	4.1 ± 1.5	0.160		48 h	1.2 ± 0.4	6.2 ± 2	**0.076#**
IL4	2 h	4.3 ± 2	0.49 ± 0.14	0.153	IFNγ	2 h	3.0 ± 1.2	0.38 ± 0.06	0.107
	4 h	4.2 ± 2	9.2 ± 4.4	0.387		4 h	5.2 ± 2.6	26.7 ± 15	0.238
	8 h	2.2 ± 0.6	2.1 ± 0.5	0.872		8 h	1.9 ± 0.5	10.6 ± 2	**0.024***
	12 h	7.6 ± 1[a]	0.3 ± 0.2	**0.003****		12 h	4.5 ± 0.4	2.4 ± 1	0.149
	24 h	0.4 ± 0.06[b]	10.2 ± 2.5	**0.016***		**24 h**	0.6 ± 0.1	20.0 ± 6	**0.049***
	48 h	1. ± 0.3	2.2 ± 1	0.339		48 h	1.4 ± 0.6	10.9 ± 4	0.112
IL6	2 h	3.1 ± 1	4.6 ± 4	0.736	CD14	2 h	1.3 ± 0.1	2.9 ± 1.5	0.329
	4 h	4.6 ± 2	9.3 ± 4	0.408		4 h	0.9 ± 0.1	1.3 ± 0.3	0.319
	8 h	3. ± 2	6.6 ± 1	0.196		8 h	4.5 ± 2	1.4 ± 0.3	0.219
	12 h	6.1 ± 2	2.6 ± 1	0.223		12 h	2.4 ± 1.3	6.5 ± 3.2	0.308
	24 h	0.5 ± 0.1	50.1 ± 39	0.273		24 h	1.5 ± 0.5	28.4 ± 21	0.285
	48 h	1.3 ± 0.5	3.2 ± 1.1	0.212		48 h	1.0 ± 0.1	1.2 ± 0.3	0.812
IL12	2 h	2.5 ± 0.8	2.1 ± 0.4	0.595	IL1β	2 h	1.2 ± 0.1	3.5 ± 2.6	0.435
	4 h	3.8 ± 1	3.0 ± 0.6	0.676		4 h	4.2 ± 2.6	1.2 ± 0.5	0.324
	8 h	2.6 ± 1	0.32 ± 0.08	0.182		8 h	6.1 ± 2	2.6 ± 1.3	0.270
	12 h	4.7 ± 1	1.7 ± 1.5	0.275		12 h	0.9 ± 0.4	27.4 ± 24	0.348
	24 h	0.2 ± 0.05	22.7 ± 16	0.242		24 h	1.7 ± 0.6	20.4 ± 9.7	0.130
	48 h	1.3 ± 0.3	2.1 ± 0.15	0.160		48 h	1.3 ± 0.09	7.1 ± 0.7	**0.002****
GMCSF	2 h	1.4 ± 0.5 [a]	1.3 ± 1.2	0.955	TLR2	2	1.5 ± 0.1	0.83 ± 0.07 [a]	0.017*
	4 h	0.8 ± 0.5 [a]	2.0 ± 1.4	0.498		4	1.3 ± 0.1	0.78 ± 0.1 [a]	**0.046***
	8 h	7.4 ± 1 [a]	2.5 ± 0.7	**0.037***		8	1.3 ± 0.2	0.68 ± 0.3 [a]	0.207
	12 h	1.2 ± 0.4 [b]	2.4 ± 1.1	0.364		12	0.7 ± 0.1	2.8 ± 0.7 [b]	**0.045***
	24 h	0.6 ± 0.3 [a]	38.7 ± 19	0.124		24	0.5 ± 0.02	0.84 ± 0.2 [a]	0.129
	48 h	3.6 ± 1	1.4 ± 0.2	0.299		48	1.1 ± 0.03	1.085 ± 0.06	0.418
RANTES	2 h	2.2 ± 0.2	0.05 ± 0.04	**0.001****	TLR4	2	0.6 ± 0.1[a]	2.0 ± 0.7	0.129
	4 h	14.1 ± 7	4.1 ± 1.7	0.263		4	0.3 ± 0.1[a]	0.4 ± 0.1	0.769
	8 h	2.4 ± 0.1	1.0 ± 0.08	**0.002****		8	1.7 ± 0.3[b]	0.6 ± 0.1	0.048*
	12 h	3.5 ± 2.7	3.9 ± 3	0.922		12	0.6 ± 0.1[a]	1.5 ± 0.6	0.233
	24 h	0.1 ± 0.02	83.71 ± 51	0.183		**24**	0.5 ± 0.03[a]	2.7 ± 0.8	0.070#
	48 h	2.5 ± 1	2.5 ± 0.7	0.968		48	0.5 ± 0.1[a]	0.9 ± 0.3	0.338
LTF	2 h	1.7 ± 0.4	0.7 ± 0.2	0.124	TLR9	2	2.4 ± 0.8	0.3 ± 0.06 [ab]	0.075#
	4 h	2.8 ± 1	2.6 ± 1.6	0.930		4	2.9 ± 1.2	3.9 ± 1.7 [ab]	0.661
	8 h	1.5 ± 0.5	0.9 ± 0.6	0.496		8	2.4 ± 0.3	3.9 ± 1.8 [ab]	0.472
	12 h	2.7 ± 0.19	7.3 ± 5.5	0.457		12	1.7 ± 0.16	0.6 ± 0.2 [a b]	**0.018***
	24 h	1.2 ± 0.43	1.8 ± 1.6	0.739		24	0.2 ± 0.02	14.9 ± 6.6 [c]	0.092
	48 h	1.1 ± 0.1	0.8 ± 0.4	0.514		48	1.1 ± 0.1	2.4 ± 0.6	0.115
CXCL1	2 h	2.7 ± 0.3	2.8 ± 1.4	0.949	TLR11	2	2.8 ± 1	0.2 ± 0.03 [a]	0.090#
	4 h	4.5 ± 1	1.7 ± 0.5	0.232		4	4.7 ± 2.2	9.3 ± 4.4	0.400
	8 h	3.5 ± 0.4	1.6 ± 0.4	**0.037***		8	1.7 ± 0.5	48.0 ± 18.9 [b]	**0.071#**
	12 h	13.3 ± 11	5.2 ± 1.4	0.516		12	4.9 ± 0.3	0.8 ± 0.3 [c]	**0.001****
	24 h	0.98 ± 0.1	70.3 ± 24	**0.049***		**24**	0.4 ± 0.1	21.3 ± 8.9	**0.080#**
	48 h	1.1 ± 0.1	2.1 ± 0.2	**0.037***		48	1.2 ± 0.4	3.0 ± 0.3 [d]	**0.033***
TLR12	2 h	2.6 ± 0.9	0.3 ± 0.05	0.076#					
	4 h	5.5 ± 2.5	2.6 ± .1.1	0.371					
	8 h	1.7 ± 0.4	2.1 ± 0.2	0.513					
	12 h	4.1 ± 0.4	1.2 ± 0.4 [a]	**0.009****					
	24 h	0.5 ± 0.1	4.5 ± 0.7 [b]	**0.008****					
	48 h	1.3 ± 0.09	1.4 ± 0.2 [a c]	0.686					

a-e Means within a column without common superscript letters differ (P < 0.05).
*Means differ significantly between SU1 *vs* SU2.
** Highly significant difference between SU1 and SU2.
# Suggested significant difference between SU1 and SU2.
[1]ΔΔCT values are normalized to the PBS control and referenced to the GAPDH gene.
[2]IL2, IL4, IL6, IL12 and IL1β: Interleukins; TLR2, 4, 9, 11 and 12: Toll-Like Receptors (TLR); TNFα: Tumor Necrosis Factor-α; IFNγ: Interferon-γ; CD14-cluster of differentiation 14; GMCSF: Granulocyte-macrophage colony-stimulating factor; RANTES: Regulated on activation, normal T cell expressed and secreted, also known as CCl5; LTF: Lactoferrin.

Collectively, we observed markedly higher expression of pro-inflammatory genes in *SU2*-infected samples compared to *SU1*-infected tissues. This finding highlights the differential host tissue responses to the different strains. Notably, the strain positive for the *sua* gene induced moderate expression of all tested genes, whereas strain SU2 (which was negative for the *sua* gene) showed elevated expression that peaked at 24 hPI (Fig 2).

3.2. Dynamics of the predicted regulatory network of biological processes associated with the tested genes

Further analysis of the predicted biological processes in the public database dysregulated by the pool of these tested gene showed clustering of several biological processes that were relevant to immune defense. The lists of the biological processes are available in Table S1.

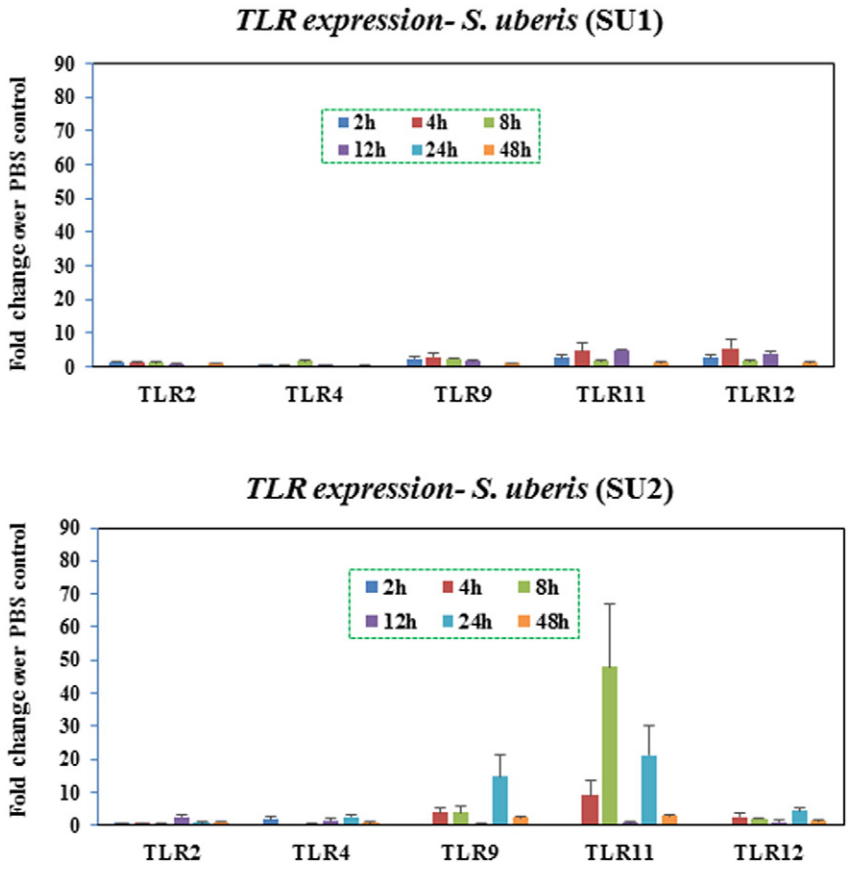

Fig. 1. *S. uberis* infection induces typical pathogen recognition receptor (PRR) expression. A. Temporal expression of pathogen recognition receptors of the TLR family (TLR2, TLR4, TLR9, TLR11 and TLR12) in infected tissue compared to the PBS control was analyzed by qRT-PCR. The fold over expression of genes in the SU1- infected tissue compared to the control tissue was calculated and plotted as the average of three biological replicates. B. Temporal expression of pathogen recognition receptors of the TLR family (TLR2, TLR4, TLR9, TLR11 and TLR12) in infected tissue compared to the PBS control was analyzed by qRT-PCR. The fold over expression of genes in the SU2- infected tissue compared to the control tissue was calculated and plotted as the average of three biological replicates.

The list of the tested genes and their biological process clusters elucidated the gene: process connections with enrichment scores. Nodes/genes were colored by their fold changes (green–low to red-high), and the gene size variation was based on the number of processes associated with the gene. The analyses showed that more than 200 processes (Table S1) were under the influence of this pool of tested genes. Of these processes, a total of 138 processes were associated with the regulation of different processes, such as the regulation of cytokine production, positive regulation of lymphocyte activation, positive regulation of signal transduction, and positive regulation of TNF production. The gene nodes for TLR4, TNFα, IL4, IL6 and IFNγ were found to comparatively possess maximum process connections, suggesting their crucial roles in immune defense to *S. uberis* IMI (Figs. 3 and 4). When compared across the time points, a mixed response was observed at the beginning (2 hPI) (Fig 3). However, a change in the response pattern was evident over time. Notably, the mRNA expression levels of all tested genes at 24 h PI with *SU2* were on the higher side (RED) in comparison to the mRNA levels induced by *SU1* (GREEN), revealing a strain-directed immune response (Fig 4).

Genes that are over expressed compared to PBS are shown in RED, and genes with moderate expression or no changes are marked in GREEN. The size of the genes is shown on the base of the number of biochemical processes to which they connect in the immune response. The lines represent the connections between the expressed node levels shown in the red to green range.

4. Discussion

Mastitis is a disease condition that is complicated by the influence of several pathogen and host factors. One of the key reasons for the failure to control mastitis is attributed to the limited knowledge concerning the epidemiology of the causative pathogen (Wang et al., 2013). A stepwise understanding of the population genetics of the causative pathogens will enable future studies to uncover the dominant types causing mastitis and delineate its interaction with the host defense system. Completing this process within a systematic framework is a prerequisite to achieving alternative therapeutic interventions for mastitis. Our previous study reported the population diversity of *S. uberis* in an Indian province and revealed that three novel clones (ST439, ST474 and ST475) of *S. uberis* predominantly caused subclinical mastitis; these clones were found to have evolved under Indian dairy farming conditions (Shome et al., 2012). To contribute to the understanding of the pathogenesis of these unique strains in mastitis, we used an *in vivo* mouse model to elucidate the host immune response to intramammary challenge with two representative *S. uberis* strains designated *SU1* and *SU2*. Real-time quantitation of mRNA levels of 17 key genes (IL2, IL4, IL6, IL12, TNFα, IFNγ, GMCSF, TLR2, TLR4, TLR9, TLR11, TLR12, CD14, IL1β, RANTES, Lactoferrin, and CXCl1) involved in different aspects of the active immune system (from sensing the pathogen to inflammation and immunity) allowed a comprehensive insight into the host immune response in a temporal manner (2 h, 4 h, 8 h, 12 h, 24 h and 48 h). Furthermore, the biological reproducibility was strengthened by the use of three biological replicates under each test condition (*i.e.*, 3 mice/time point for a total of 18 mice covering all time points and three mice separately for the PBS reference control). The use of a probe-based assay further strengthened the accuracy of the measurement of mRNA abundance. However, a few exceptions were noted in the observations that could be attributed to the unavoidable biological variations in mice.

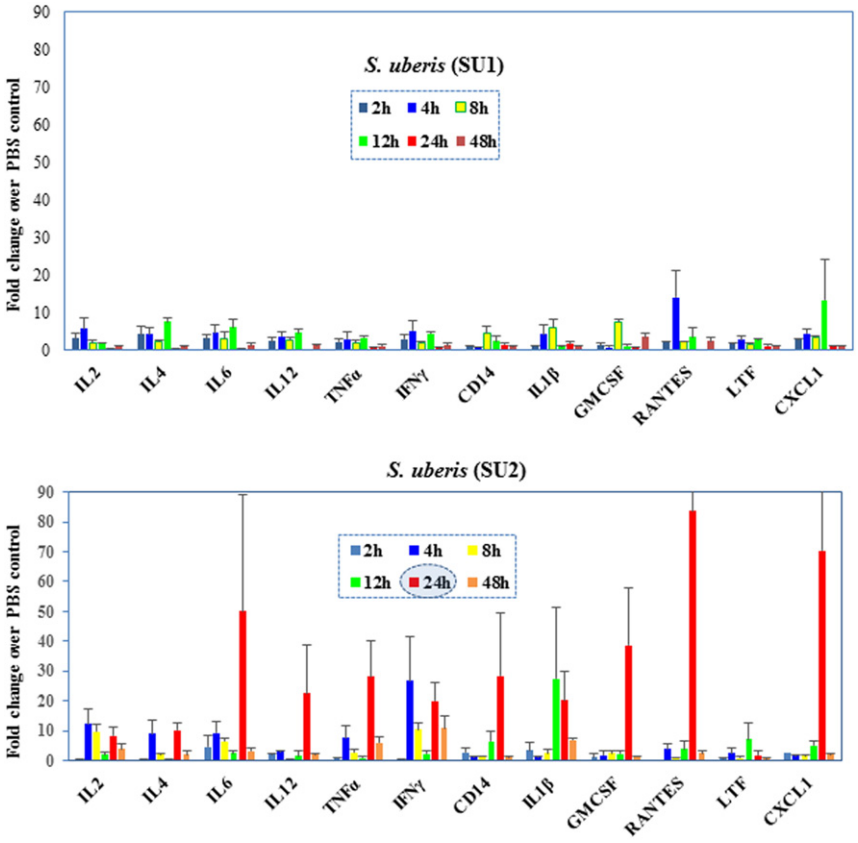

Fig. 2. *S. uberis* infection induces an inflammatory response in mouse mammary glands. A. The temporal expression of 12 key immune genes involved in diverse immune response pathways in infected tissue compared to the PBS control was analyzed by qRT-PCR. The fold over expression of genes in the SU1-infected tissue compared to the control tissue was calculated and plotted as the average of three biological replicates. B. The temporal expression of various genes in infected tissue compared to the PBS control was analyzed by qRT-PCR. The fold over expression of genes in the SU2-infected tissue compared to the control tissue was calculated and plotted as the average of three biological replicates SU2 infection consistently resulted in a higher inflammatory response compared to SU1 at all-time points. The 24 h PI was a critical point showing a typical deviating response.

The temporal comparative kinetics revealed that the two strains induced differential immune responses. The SU1 strain suggested towards favoring its persistence in the host environment by limiting immune gene expression. In contrast, SU2 indicated its possible elimination by significantly elevating the immune response.

Comprehensively, the study showed how the novel clones of *Streptococcus uberis* may favor their sustainment in the host environment, as observed in the case *SU1*_ against immune surveillance compared to *SU2*_. Our results collectively enrich the available information on host pathogen interplay and establish a firm basis for future studies to finally achieve the ultimate goal of gaining control over this pathogen.

The measurement of the fold changes in mRNA expression levels was performed between each of the challenged animals and the PBS inoculated control (SUI/SU2 *versus* PBS) as well as between the two infected conditions (SU1 *versus* SU2).

The study revealed that the two *S. uberis* IMI models induced immune responses in a strain-dependent manner. Differential expression was observed for the pool of 17 genes tested, suggesting that the strains had a deviating impact on the course of mastitis that influenced the outcome of infection. PRRs sense invading microbes; in the case of Gram-positive bacteria, the microbes are reported to be detected by TLR 2 (Goldammer et al., 2004). In this study, TLR 4, TLR 9, TLR 11 and TLR 12 were found to be upregulated together with TLR2 under both the conditions. A significant increase in the mRNA levels of all of the TLRs was noted in the mice infected with *SU2* compared to the mice infected with *SU1*. Farhat et al. (2008) similarly observed that *S. uberis* and *S. agalactiae* surprisingly failed to activate TLR2. However, Modak et al. (2012) showed higher induction of TLR2 and TLR4 expression in *E. coli* challenge studies. The diminished TLR2 expression in the *SU2* condition at 2 h PI indicated that *S. uberis* might have used alternative pathways through other PRRs, such as the nod-like receptors (NLR) and C-type lectin receptors (CLR), to mount the immune response that led to the pronounced mRNA expression of the other inflammatory mediators over time. This finding also suggests that there may be cross talk between the TLRs and different PRRs to induce an effective immune response (Kawai and Akira, 2011). Intracellular TLR9 can recognize CpG motifs in pathogen DNA and can enhance IL12 production due to positive feedback. A recent report showed that TLR9 activated two signaling pathways within different intracellular compartments as well as sensors of apoptosis (Kawai and Akira, 2011; Krysko et al., 2011). This result suggests that *SU2* IMI induced stronger recognition, leading to its internalization in mouse mammary gland.

The activation of TLR-induced signaling pathways leads to the production of the major proinflammatory cytokines TNFα and IL1β, which are locally produced by several cell types and trigger an inflammatory cascade (Zbinden et al., 2014). In the present study a strong and significant induction of TNFα mRNA levels was observed at 12 h PI that peaked at 24 hPI following infection with *SU2*, which was in agreement with previous studies (Swanson et al., 2009; Gunther et al., 2009). This type of initial delayed response followed by a very strong response was also observed by Bannerman (2009). Rambeaud et al. (2003) in their study found experimental *S. uberis* IMI induced local production of TNF-alpha, IL-1beta and IL-8, which may play a role in the pathogenesis of *S. uberis* mastitis. There was no significant differential expression was observed for IL6 expression, which is an important pro-inflammatory cytokine which mediate the passage from innate to adaptive immune response (Bannerman, 2009; Zbinden et al., 2014; Gunther et al., 2009). IL2 and IL4 were significantly increased during the initial course of infection, but their mRNA levels dropped at 24 h PI in the *SU1* condition. However, IL2 showed a significant elevation in

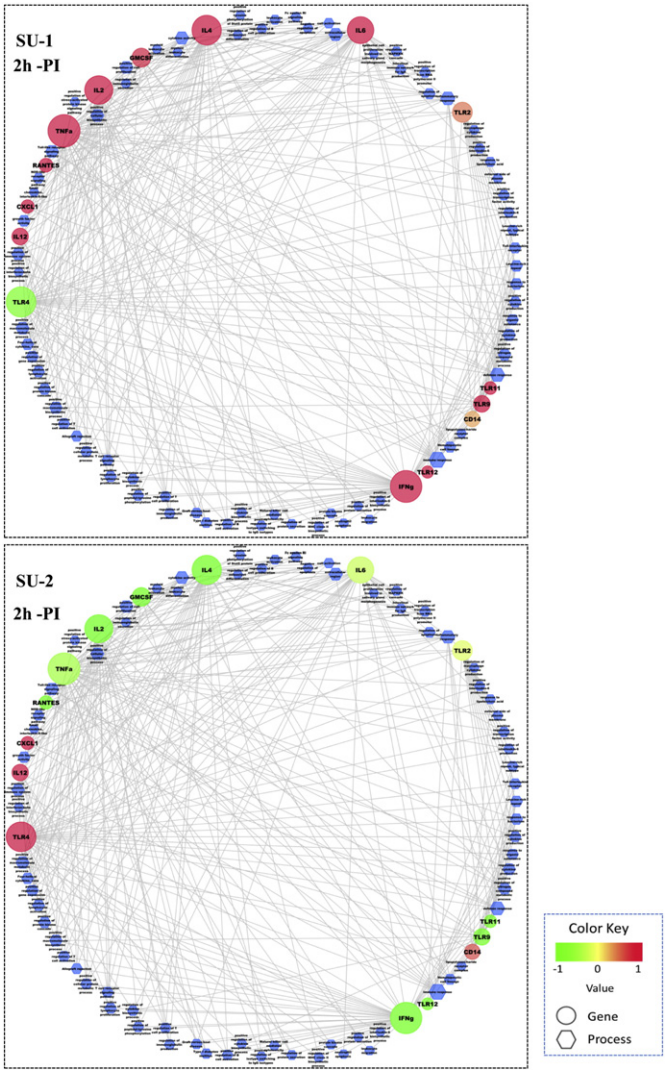

Fig. 3. The different biochemical process controlled by the activated inflammatory genes expressed 2 h post-*S. uberis* infection. A. Biological network analyses showing the differential expression of the tested genes involved in the immune response and the predicted biochemical pathways in the SU1-infected mouse mammary tissue 2 h post-inoculation. B. Biological network analyses showing the differential expression of the tested genes involved in the immune response and the predicted biochemical pathways in the SU2-infected mouse mammary tissue 2 h post-inoculation. Genes that are over expressed compared to PBS are shown in RED, and genes with moderate expression or no changes are marked in GREEN. The size of the genes is shown on the base of the number of biochemical processes to which they connect in the immune response. The lines represent the connections between the expressed node levels shown in the red to green range.

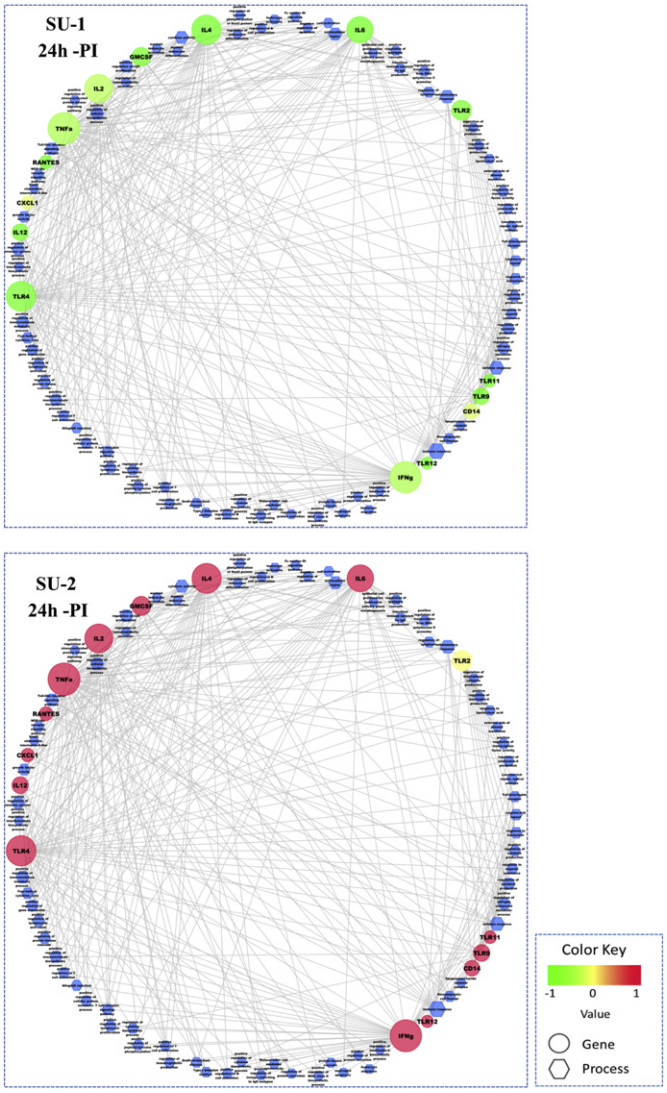

Fig. 4. The different biochemical processes controlled by the activated inflammatory genes expressed 24 h post-*S. uberis* infection. A. Biological network analyses showing the differential expression of the tested genes involved in the immune response and the predicted biochemical pathways in the SU1-infected mouse mammary tissue 24 h post-inoculation. B. Biological network analyses showing the differential expression of the tested genes involved in the immune response and the predicted biochemical pathways in the SU2-infected mouse mammary tissue 24 h post-inoculation. Genes that are over expressed compared to PBS are shown in RED, and genes with moderate expression or no changes are marked in GREEN. The size of the genes is shown on the base of the number of biochemical processes to which they connect in the immune response. The lines represent the connections between the expressed node levels shown in the red to green range.

mRNA abundance at 24 hPI in comparison to SU1. Tassi et al. (2013) in their study on *S. uberis* challenge found the cytokine response followed a specific order, with an increase in IL-1β, IL-6, and IL-8 levels at the time of first SCC elevation, followed by an increase in IL-10, IL-12p40, and TNFα levels approximately 6 h later.

Interferon-γ (IFNγ) is a linker of the innate and adaptive immune arms and enhances microbicidal activity via receptor-mediated phagocytosis (Bannerman et al., 2004). Kauf et al. (2007) and Bannerman et al. (2004) observed high levels of IFNγ in persistent IMI. Similarly, a higher induction was also noted for *SU2* IMI at 4 h PI, with a significant elevation in mRNA abundance at 24 h PI.

At the site of infection, chemokines recruit cellular factors involved in the immune defense to direct effective inflammatory responses that lead to pathogen clearance. The mRNA expression of the chemokines CXCl1 and CCL5/RANTES in the *SU2* condition gradually increased and peaked at 24 h PI. However, in the $SU1_{ST439,\ sua+}$ condition the highly induced expression of chemokines during the initial hours PI eventually diminished over time. This result suggests that *SU1* must have used a certain strategy to disturb and subvert the host defenses. Although the mRNA abundance of Lactoferrin, IL12 and CD14 were high in each condition, there was no significant change in the mRNA levels between the two conditions. Zbinden et al. (2014) and Modak et al. (2012, 2014) observed an increase in the mRNA abundance of IL12, Lactoferrin and CD14 in *E. coli* and *S. aureus* IMI. Bannerman (2009) noted high expression of the CD14 protein in the milk throughout the study. Another study (Swanson et al., 2009) noted the increase in mRNA expression of immune-related genes complement component 3, clusterin, IL-8, calgranulin C, IFN-gamma, IL-10, IL-1beta, IL-6, toll-like receptor-2, tumor necrosis factor-alpha, serum amyloid A3, lactoferrin, LPS-

bonding protein, and oxidative stress-related genes metallothionein 1A and superoxide dimutase 2 to *S. uberis* challenge.

Collectively, *SU2* strongly induced the expression of most of the immune factors tested in the study throughout the course of infection. Conversely, the SU1-induced elevation was eventually followed by a reduction in the mRNA abundance, which could be connected to its virulence profile. SU1 was positive for the *sua* gene, which may enhance the adhesive ability of SU1 and allowed its persistence by strategically manipulating the host environment in its favor over time. Tassi et al. (2013) observed in his study a strain-specific pathogenicity to be consistent across animals, implying that it is determined by pathogen factors rather than host factors.

In this study, the predicted biological processes were assessed for the tested genes (n = 17), which showed more than 200 biochemical processes comprising several regulatory processes of the immune pathways (i.e., the regulation of cytokine production, positive regulation of signal transduction, and positive regulation of TNFα production) connected to this pool of genes. The predicted biological networks showed an association between diverse biological processes involved in immune defense with these master inflammatory mediators. TNFα, IL6, IFNγ and TLR4 were found to be the most crucial based on their connections to the maximum number of processes. This result indicated the master role of the tested genes in ruling the immune defense system.

The most interesting observation was made at 24 h PI, when *SU1* and *SU2* showed a complete deviation in the expression of all of the tested genes, with significant differences noted for their IL2, IL4, TNFα, IFNγ, CXCl1, TLR4, TLR9, TLR11, and TLR12 levels (Table 3, Fig 4). *SU2* infection elevated most of the mRNA expression levels compared with *SU1* infection, indicating the possible capacity of *SU1* to manipulate conditions in the host environment in its favor to result in its sustainment/persistence. However, *SU2* induced very high expression levels that were followed by a gradual reduction at 48 h PI; notably, the level of expression did not drop drastically at 48 h PI, which suggested that it did not inhibit further proliferation of the immune response. At 48 h PI, the levels were still high enough to probably mount an effective immune defense. This result suggested that the host may be able to eliminate this strain (*SU2* over time, leading to pathogen clearance. Overall, our results suggest that 24 h PI may be a critical time point in the course of infection for directing the probable outcome of infection in *S. uberis* IMI. Similarly, Modak et al. (2012) found a sharp peak increase in immune gene expression at 48 h PI in *E. coli* IMI, indicating progress towards acute mastitis. In contrast, Modak et al. (2014) observed a fall in the expression level at 48 h PI in *S. aureus* IMI. These observations in mouse intramammary infection mouse model evoke the need for further studies.

5. Conclusion

In conclusion, the emergence of diverse and novel clones of *S. uberis* that cause mastitis evokes the need to understand the mechanisms of their pathogenesis to aid in the successful development of alternative therapeutics for mastitis control programs. Using an *in vivo* mouse model, we unraveled the host response to two epidemiologically important novel clones of *S. uberis* with known molecular characteristics and virulence profiles. Comprehensive and comparative analyses revealed that *SU1* and *SU2* induced inflammatory responses in a deviating manner in the mouse mammary gland. The pattern of expression over time indicated a capacity of *SU1* towards persistence and sustenance in the host tissue; in contrast, *SU2* augmented the immune response, thereby suggesting its contribution to its clearance. Thus, there is a complex interaction between *S. uberis* and an array of host factors that manipulate and determine the differences in the severity of mastitis. The study establishes a firm basis evoking the need for further research. The observations as well provide an impetus for future studies with more diverse clones and challenge studies in cattle to delineate and reaffirm the regulatory mechanisms associated with the deviating response. The results of this study warrant the generation of additional important information for alternative therapeutic interventions in mastitis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2016.03.054.

Acknowledgement

The research was supported by grants from National Agricultural Innovative Project (NAIP), Indian Council of Agricultural Research, New Delhi, Govt. of India under component 4: Basic and Strategic Research vide NAIP/Comp-4/C-30017/2008-09 Dt. 23.12.2008 to B. R. Shome. Thankful to Manu Kumar and Sreedevi G. C for their assistance.

References

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.
Awale, M.M., Dhudhatra, G.B., Avinash, K., et al., 2012. Bovine mastitis: a threat to economy. Open Access Sci. Rep. 1, 5.
Bannerman, D.D., 2009. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. J. Anim. Sci. 87, 10–25.
Bannerman, D.D., Paape, M.J., Lee, J.W., Zhao, X., Hope, J.C., et al., 2004. *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. Clin. Diagn. Lab. Immunol. 11, 463–472.
Chandler, R.L., 1970. Experimental bacterial mastitis in the mouse. J. Med. Microbiol. 3, 273–282.
Chen, X., Dego, O.K., Almeida, R.A., Fuller, T.E., Luther, D.A., et al., 2010. Deletion of suagene reduces the ability of *Streptococcus uberis* to adhere to and internalize into bovine mammary epithelial cells. Vet. Microbiol. 147, 426–434.
Di Gioia, M., Zanoni, I., 2015. Toll-like co-receptors as master regulators of the immune response. Mol. Immunol. 63, 143–152.
Farhat, K., Sauter, K.S., Brcic, M., et al., 2008. The response of HEK293 cells transfected with bovine TLR2 to established pathogen-associated molecular patterns and to bacteria causing mastitis in cattle. Vet. Immunol. Immunopathol. 125, 326–336.
Goldammer, T., Zerbe, H., Molenaar, A., Schuberth, H.J., Brunner, R.M., et al., 2004. Mastitis increases mammary mRNA abundance of beta-defensin 5, toll-likereceptor 2 (TLR2), and TLR4 but not TLR9 in cattle. Clin. Diagn. Lab. Immunol. 11, 174–185.
Griesbeck-Zilch, B., Meyer, H.H., Kuhn, C.H., Schwerin, M., Wellnitz, O., 2008. *Staphylococcus aureus* and *Escherichia coli* cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. J. Dairy Sci. 91, 2215–2224.
Gunther, J., Koczan, D., Yang, W., Nurnberg, G., Repsilber, D., et al., 2009. Assessment of the immune capacity of mammary epithelial cells: comparison with mammary tissue after challenge with *Escherichia coli*. Vet. Res. 40, 31.
Janot, L., Secher, T., Torres, D., Maillet, I., Pfeilschifter, J., Quesniaux, V.F., Landmann, R., Ryffel, B., Erard, F., 2008. CD14 works with toll-like receptor 2 to contribute to recognition and control of Listeria monocyto genes infection. J. Infect. Dis. 198, 115–124.
Kauf, A.C.W., Rosenbusch, R.F., Paape, M.J., et al., 2007. Innate immune response to intramammary *Mycoplasma bovis* infection. J. Dairy Sci. 90, 3336–3348.
Kawai, T., Akira, S., 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34, 637–650.
Kawasaki, T., Kawai, T., 2014. Toll-like receptor signaling pathways. Front. Immunol. 5, 461.
Krishnamoorthy, P., Satyanarayana, M.L., Shome, B.R., Rahman, H., 2014. Mouse milk somatic cell count in coagulase negative Staphylococcus species induced mastitis. Scand. J. Lab. Anim. Sci. 2, 16–19.
Kromker, V., Reinecke, F., Paduch, J.H., Grabowski, N., 2014. Bovine *Streptococcus uberis* intramammary infections and mastitis. Clin. Microbial 3, 157.
Krysko, D.V., Kaczmarek, A., Krysko, O., Heyndrickx, L., Woznicki, J., et al., 2011. TLR-2 and TLR-9 are sensors of apoptosis in a mouse model of doxorubicin-induced acute inflammation. Cell Death Differ. 18, 1316–1325.
Modak, R., Das Mitra, S., Krishnamoorthy, P., Bhat, A., Banerjee, A., Gowsica, B.R., Bhuvana, M., et al., 2012. Histone H3K14 and H4K8 hyperacetylation is associated with *Escherichia coli*-induced mastitis in mice. Epigenetics 7, 492–501.
Modak, R., Mitra, S.D., Vasudevan, M., Krishnamoorthy, K., Manoj, K., Bhuvana, M., et al., 2014. Epigenetic response in mice mastitis: role of histone H3 acetylation and micro RNA(s) in the regulation of host inflammatory gene expression during *Staphylococcus aureus* infection. Clin. Epigen. 6, 12.
Patel, D., Almeida, R.A., Dunlap, J.R., Oliver, S.P., Petti, C.A., et al., 2009. Bovine lactoferrin serves as a molecular bridge for internalization of *Streptococcus uberis* into bovine mammary epithelial cells. Vet. Microbiol. 137, 297–301.
Petzl, W., Zerbe, H., Günther, J., Yang, W., Seyfert, H.M., Nürnberg, G., Schuberth, H.J., 2008. *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. Vet. Res. 39, 18.
Rambeaud, M., Almeida, R.A., Pighetti, G.M., Oliver, S.P., 2003. Dynamics of leukocytes and cytokines during experimentally induced *Streptococcus uberis* mastitis. Vet. Immunol. Immunopathol. 96, 193–205.

Rato, M.G., Bexiga, R., Nunes, S.F., Cavaco, L.M., Vilela, C.L., et al., 2008. Molecular epidemiology and population structure of bovine *Streptococcus uberis*. J. Dairy Sci. 91, 4542–4551.

Schukken, Y.H., Gunther, J., Fitzpatrick, J., et al., 2011. Host-Response Patterns of Intramammary Infections in Dairy Cows. Vet Immunol and Immuno Pathol Vol. 144, pp. 270–289.

Shome, B.R., Bhuvana, M., Mitra, S.D., Krithiga, N., Shome, R., Velu, D., et al., 2012. Molecular characterization of *Streptococcus agalactiae* and *Streptococcus uberis* isolates from bovine milk. Trop. Anim. Health Prod. 44, 1981–1992.

Smolenski, G.A., Broadhurst, M.K., Stelwagen, K., et al., 2014. Host define related responses in bovine milk during an experimentally induced *Streptococcus uberis* infection. Proteome Sci. 12, 19.

Swanson, K.M., Stelwagen, K., Dobson, J., et al., 2009. Transcriptome profiling of *Streptococcus uberis*-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. J. Dairy Sci. 92, 117–129.

Tassi, R., McNeilly, T.N., Fitzpatrick, J.L., Fontaine, M.C., Reddick, D., et al., 2013. Strain-specific pathogenicity of putative host-adapted and nonadapted strains of Streptococcusuberis in dairy cattle. J. Dairy Sci. 96, 5129–5145.

Vander Poll, T., Opal, S.M., 2008. Host-pathogen interactions in sepsis. Lancet Infect. Dis. 8, 32–43.

Wang, L., Chen, W., Zhang, L., et al., 2013. Genetic diversity of *Streptococcus uberis* isolates from dairy cows with subclinical mastitis in Southern Xinjiang Province, China. J. Gen. Appl. Microbiol. 59, 287–293.

Wellnitz, O., Reith, P., Haas, S.C., Meyer, H.H.D., 2006. Immune relevant gene expression of mammary epithelial cells and their influence on leukocyte chemotaxis in response to different mastitis pathogens. Vet. Med. (Praha) 51, 125–132.

Zadoks, R.N., 2007. Sources and epidemiology of *Streptococcus uberis*, with special emphasis on mastitis in dairy cattle. CAB Rev: Perspect Agric. Vet. Sci. Nutr. Nat. Res. 2.

Zbinden, C., Stephan, R., Johler, S., Borel, N., Bunter, J., Bruckmaier, R.M., et al., 2014. The inflammatory response of primary bovine mammary epithelial cells to *Staphylococcus aureus* strains is linked to the bacterial phenotype. PLoS One 9 (1), e87374.