Bovine milk somatic cell transcriptomic response to *Staphylococcus aureus* is dependent on strain genotype

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Abbreviations

bMEC – bovine mammary epithelial cells, CC – clonal complex, DE – differentially expressed, ECM – extracellular matrix, hpi – hours post-infection, GO – Gene Ontology, IMI – intramammary infection, KEGG - Kyoto Encyclopedia of Genes and Genomes, PCA – principal component analysis, RT- room temperature, ST – sequence type. Gene symbol abbreviations – Supplementary Table S2.

Abstract

Background: Mastitis is an economically important disease of dairy cows with *Staphylococcus aureus* a major cause worldwide. Challenge of Holstein-Friesian cows demonstrated that *S. aureus* strain MOK124, which belongs to Clonal Complex (CC)151, caused clinical mastitis, while strain MOK023, belonging to CC97, caused mild or subclinical mastitis. The aim of this study was to elucidate the

molecular mechanisms of the host immune response utilising a transcriptomic approach. Milk somatic cells were collected from cows infected with either *S. aureus* MOK023 or MOK124 at 0, 24, 48, 72 and 168 hours post-infection (hpi) and analysed for differentially expressed (DE) genes in response to each strain.

Results: In response to MOK023, 1278, 2278, 1986 and 1750 DE genes were found at 24, 48, 72 and 168 hpi, respectively, while 2293, 1979, 1428 and 1544 DE genes were found in response to MOK124 at those time points. Genes involved in milk production (*CSN1, CSN10, CSN1S2, CSN2, α-LACTA* and *PRLR)* were downregulated in response to both strains, with a more pronounced decrease in the MOK124 group. Immune response pathways such as NF-κB and TNF signalling were overrepresented in response to both strains at 24 hpi. These immune pathways continued to be overrepresented in the MOK023 group at 48 and 72 hpi, while the Hippo signalling, extracellular matrix interaction (ECM) and tight junction pathways were overrepresented in the MOK124 group between 48 and 168 hpi. Cellular composition analysis demonstrated that a neutrophil response was predominant in response to MOK124, while M1 macrophages were the main milk cell type post-infection in the MOK023 group.

Conclusions: A switch from immune response pathways to pathways involved in maintaining the integrity of the epithelial cell layer was observed in the MOK124 group from 48 hpi, which coincided with the occurrence of clinical signs in the infected animals. The higher proportion of M1 macrophages in the MOK023 group and lack of substantial neutrophil recruitment in response to MOK023 may indicate immune evasion by this strain. The results of this study highlight that the somatic cell transcriptomic response to *S. aureus* is dependent on the genotype of the infecting strain.

50 ## Introduction

51 Bovine mastitis is a disease of the udder which affects milk yield and quality and causes significant
52 economic losses to the dairy industry (1-3). Mastitis can present as clinical or subclinical. Clinical
53 mastitis presents with overt signs such as clots in milk or swelling of the quarter. Clots in milk are a
54 result of the influx of substantial numbers of immune cells into the udder and are formed by
55 aggregates of leukocytes and blood clotting factors (4). Subclinical mastitis presents with no obvious
56 signs but typically causes a moderate influx of immune cells into the udder, leading to an increase in
57 somatic cell count (SCC) in milk (5). Individual cow SCC of < 100,000 cells/ml is typically considered to
58 be reflective of a healthy mammary gland, while an SCC > 200,000 cells/ml may be indicative of
59 bacterial infection (6).

60

61 *Staphylococcus aureus* is a major cause of bovine mastitis in Ireland (7, 8) and worldwide (9-12) and
62 is also one of the aetiological agents of human mastitis (13, 14). *S. aureus* is a clonal pathogen which
63 evolves slowly compared to many other bacteria (15). Nevertheless, substantial genetic diversity
64 exists among *S. aureus* strains, with many lineages identified to date, often adapted to different
65 hosts (16). Four main bovine-adapted lineages have been identified among Irish clinical mastitis
66 isolates: Clonal Complex (CC)71, CC97, ST136 and CC151 (17). CC97 and CC151 are also the most
67 common mastitis-associated *S. aureus* lineages in Europe (18). CC97 is a predominantly bovine-
68 associated lineage although host jumps into humans with subsequent global spread have been
69 demonstrated (16), while CC151 appears to be a uniquely bovine lineage which has not been found
70 in other ruminants, avian species or in humans (16, 19-21).

71

72 During bovine IMI, pathogens enter the mammary gland through the teat canal. The immune
73 response in the mammary gland is presumably initiated by bovine mammary epithelial cells (bMEC)
74 in the alveoli of the udder and by resident macrophages (22). Pathogen-Associated Molecular
75 Patterns such as peptidoglycan and lipoteichoic acid on the surface of Gram-positive bacteria are

76 recognised by Toll-like receptor 2 (TLR2), which stimulates activation of the NF-κB signalling pathway
77 in bMEC as well as macrophages (23, 24). NF-κB and other immune pathways stimulate the
78 production of cytokines and chemokines, which cause inflammation in the udder and stimulate
79 neutrophil recruitment (25). Neutrophils are the first cell type recruited into the udder to engulf the
80 bacteria and attempt to clear the infection (26). Previous studies have reported that *S. aureus* IMI
81 induces a weak inflammatory response with inhibition of the TLR2 and NF-κB pathways and low
82 levels of pro-inflammatory cytokine production (27-29). However, these studies used a variety of *S.*
83 *aureus* strains and the magnitude of the immune response to *S. aureus* has been reported to be
84 strain-dependent (30). Therefore, the general supposition that *S. aureus* causes a low immune
85 response in bovine IMI (31) may only be true for specific strains of *S. aureus* and there is a need to
86 further study the immune response to different strains of *S. aureus* during IMI.
87
88 The transcriptomic response to intra-mammary challenge with *S. aureus* has been previously studied
89 in bovine gland cistern tissue using microarrays (32, 33) and RNA sequencing (28); however, studies
90 were often short in duration (28, 32) or involved only one time point (33). Characterising the
91 transcriptomic response to *S. aureus* over several time points would be desirable, since it was
92 previously demonstrated *in vitro* and *in vivo* that the response to *S. aureus* IMI changes over time
93 (30, 34). Furthermore, previous transcriptomic studies utilised only single strains of *S. aureus* for
94 intra-mammary challenge, and none used a strain belonging to CC151, a globally distributed bovine-
95 adapted lineage. Immune gene expression in bMEC in response to *S. aureus* differs according to
96 bacterial strain or lineage (25, 34, 35). Intramammary challenge of Holstein-Friesian cows with
97 strains MOK023 (CC97) or MOK124 (CC151) demonstrated that *S. aureus* strain MOK023 caused mild
98 or subclinical mastitis, while strain MOK124 caused severe clinical mastitis (30). Differences were
99 observed in secretion of cytokines IL-8 and IL-1β into milk between the MOK023 and the MOK124
100 infected cows, which further suggested that the immune response is strain-specific. Due to the
101 differences observed between the immune response to MOK023 and MOK124 in bMEC *in vitro* and

102 also *in vivo*, a further study to elucidate the molecular mechanisms of the host immune response *in*
103 *vivo* by utilising a transcriptomic approach was performed. The objective of this study was to identify
104 patterns of gene expression associated with the response to infection with two strains of *S. aureus*.
105 Expression of cell markers was also examined in order to explore the proportions of immune cells in
106 milk somatic cell samples in response to each strain.
107

108 # Results

109 ## Summary of *in vivo* disease presentation

110 Milk somatic cells collected during a previously described *S. aureus* IMI challenge trial were used.
111 The influence of bacterial strain on clinical signs, bacterial load, SCC and the concentration of select
112 cytokines and anti-*S. aureus* antibodies in milk were previously reported (30). Briefly, cows infected
113 with MOK124 generally developed clinical mastitis, while cows in the MOK023 group developed mild
114 or subclinical mastitis. There was a greater drop in milk yield in the MOK124 group and SCC was
115 significantly higher between 24 and 216 hpi in the MOK124 group compared to the MOK023 group.
116 The MOK124 bacterial load was significantly higher than that of MOK023 at 24 and 31 hpi and
117 subsequently decreased, while MOK023 bacterial load slowly increased and became significantly
118 higher than MOK124 later in the infection (216 to 504 hpi). The levels of interleukin 8 (IL-8) and IL-1β
119 were also significantly higher in milk of cows challenged with MOK124 compared to cows challenged
120 with MOK023 during the first week of infection. Post-infection milk somatic cells were primarily
121 neutrophils, which comprised 80-89 % of somatic cells in the MOK124 group and 41-69 % of somatic
122 cells in the MOK023 group. Infection with MOK124 resulted in a significantly higher proportion of
123 neutrophils in milk than infection with MOK023 (30).
124

125 ## LukM’

126 The severity of bovine IMI has been previously associated with secretion of the bi-component
127 leukocidin LukMF' by *S. aureus (36, 37)*. As the genes encoding this toxin are found in MOK124, but
128 not MOK023, LukM in milk from infected animals was quantified. No LukM’ was found in either
129 MOK023 or MOK124 group milk samples at any time point.

130

131 ## Sequence and mapping quality

132 Somatic cells collected pre-infection and at 24, 48, 72 and 168 hpi were subjected to 100 bp
133 stranded paired-end sequencing. On average, 95 % of the bases across all samples had a phred score
134 of ≥ 30 and 98 % had a score of ≥ 20. The mean number of reads per sample was 83.5 M; however,
135 less than 60 M reads were generated from 3 samples from time 0 due to the low number of somatic
136 cells in the samples. The distribution of read counts, phred scores and GC content per sample is
137 available in Supplementary File 1. Quality control, as assessed by FastQC, yielded satisfactory results
138 for all samples. On average, 93 % of reads mapped uniquely to the bovine genome UMD 3.1.93.

139

140 ## Data exploration

141 Raw read counts were used for DE gene analysis using DESeq2. After filtering for low counts (sum of
142 reads from all samples < 1) and non-protein coding genes, 17,334 genes were included in further
143 analysis. The expression of the 500 most variable genes was used for principal component analysis
144 (PCA) within the DESeq2 software package. Time 0 (pre-infection) samples clustered separately
145 from infected samples (Fig. 1A). Expression patterns from cows infected with either strain did not
146 separate on the PCA plot at 24 hpi. However, at 48 and 72 hpi, separation between the response to
147 the 2 strains was evident. At 48 hpi MOK124 infected animals’ response had progressed to a state
148 that was maintained until 168 hpi, while the response in MOK023 infected animals progressed more
149 slowly to the same state and samples clustered in distinct groups at 48, 72 and 168 hpi. The

150 difference in progression of gene expression in response to each strain could also be seen in the
151 expression profile of a number of core immune genes across the experiment time course. This was
152 illustrated by the NF-κB subunit 1 (*NFKB1)* gene, which is a key gene involved in switching on
153 production of immune mediators through the NF-κB signalling pathway. A rapid increase in
154 expression of this gene at 24 hpi was followed by a rapid decrease in MOK124 infected animals. On
155 the other hand, infection with MOK023 caused a more sustained *NFKB1* induction (Fig 1B).
156

157 ## Differentially expressed genes

158 To identify highly up and downregulated genes, a $\log_2$ fold change threshold of 1 (indicating a 2 fold
159 increase or decrease in expression) was applied to the DE gene results in DESeq2 and genes with an
160 adjusted P value < 0.05 were subsequently selected as significant. In response to MOK023, 1278,
161 2278, 1986 and 1750 significant DE genes were found at 24, 48, 72 and 168 hpi, respectively, while
162 2293, 1979, 1428 and 1544 significant DE genes were found in response to MOK124 at those time
163 points (Fig. 2A). At 24 hpi the majority of genes with altered expression were downregulated in
164 response to both strains, which could indicate a suppression of normal cell function; while at 72 and
165 168 hpi, upregulated DE genes predominated in response to both strains. Details of all significant DE
166 genes are available in Supplementary File 2.
167

168 Among the most significant upregulated DE genes in response to MOK023, the gene for oncostatin
169 M (*OSM*) was in the top 5 at all time points post-infection (Fig. 2B). *OSM* encodes a cytokine involved
170 in inflammation and regulation of production of other cytokines. Oncostatin is also secreted by
171 neutrophils during their rolling in blood vessels in order to enhance neutrophil adhesion to
172 endothelial cells (38). In response to MOK124, the top 5 upregulated genes included those involved
173 in the immune response such as interleukin genes (*IL17F, IL18*) and interleukin-2 receptor (*CD25*)
174 gene (Fig. 2C). The top 5 downregulated genes in response to MOK124 included genes encoding milk

175 proteins lactalbumin alpha (*α-LACTA*), casein alpha subunit 2 (*CSN1S2*), casein alpha subunit 1 (*CSN1*)
176 and casein kappa (*CSN10*).
177
178 In order to examine the effect of strain on transcription of major genes involved in milk production,
179 log_2 fold changes of relevant significantly DE genes were examined (Table 1). The main genes
180 involved in milk production were reviewed by Strucken et al. (2015) (39) and include leptin,
181 lactalbumin, caseins, prolactin and several genes involved in fat transport, energy production and
182 growth. No significant change in expression of the selected milk production genes was observed in
183 MOK023 infected animals at 24 hpi; however, all examined genes with the exception of the gene
184 coding for 1-acylglycerol-3-phosphate O-acyltransferase 6 (*AGPAT6)* were significantly
185 downregulated at all other time points in this group. In the MOK124 group, downregulation of the
186 expression of milk production genes was already evident by 24 hpi and continued until 168 hpi. Log_2
187 fold changes in expression of genes encoding milk-related proteins (*α-LACTA, CSN1, CSN10, CSN1S2,*
188 *CSN2*) were 2-3 log lower in the MOK124 group than in the MOK023 group, indicating a more severe
189 reduction of protein synthesis in the first group.
190
191 **Table 1.** Expression of genes involved in milk production.

Gene	MOK023				MOK124			
	24 hpi	48 hpi	72 hpi	168 hpi	24 hpi	48 hpi	72 hpi	168 hpi
ABCG2	N/A	-4.09	-4.12	-4.85	-2.48	-6.92	-6.04	-5.96
AGPAT6	N/A	N/A	N/A	N/A	3.29	N/A	N/A	N/A
α-LACTA	N/A	-4.17	-4.49	-5.53	-2.97	-8.44	-7.48	-7.35
CSN1	N/A	-4.09	-4.29	-5.27	-2.80	-6.72	-7.31	-7.04
CSN10	N/A	-4.25	-4.53	-5.34	-2.78	-6.39	-6.89	-6.84
CSN1S2	N/A	-4.29	-4.37	-5.21	-2.90	-6.75	-7.42	-6.81
CSN2	N/A	-4.03	-4.16	-5.34	N/A	-6.71	-7.22	-7.13
GHR	N/A	-3.18	-3.47	-4.16	N/A	-4.80	-5.17	-4.45
PPARGC1A	N/A	-4.12	-3.85	-4.30	N/A	-6.93	-7.06	-6.93
PRLR	N/A	-2.70	-3.04	-3.65	N/A	-4.35	-3.19	N/A

192 Log_2 fold changes of significant DE genes only are included (adjusted P-value < 0.05, log_2 fold change
193 threshold > 1).
194 N/A – gene not significantly DE. Gene names: *ABCG2* – ATP binding cassette sub-family G member 2,
195 *AGPAT6* - 1-acylglycerol-3-phosphate O-acyltransferase 6, *α-LACTA* – alpha lactalbumin, *CSN1* -

196 casein alpha subunit 1, *CSN10* – casein kappa, *CSN1S2* - casein alpha subunit 2, *CSN2* - casein beta,
197 *GHR* - growth hormone receptor, *PPARGC1A* - peroxisome proliferator activated receptor gamma
198 coactivator 1 alpha, *PRLR* - prolactin receptor. hpi - hours post-infection.
199

200 A total of 402 genes in the MOK023 group were DE at all time points post-infection, while 362 DE
201 genes were common in the response to MOK124 at all time points (Fig. 3 A-B). Of these, 130 were
202 shared between MOK023 and MOK124 at all time points and are proposed as strain-independent
203 biomarkers of *S. aureus* IMI during the first week of infection (Fig. 3C). Among the common genes,
204 42 were involved in immune response processes.
205

206 Pathway analysis

207 KEGG pathways

208 Pathway analysis of KEGG metabolic processes revealed 67 unique significantly overrepresented
209 pathways in response to MOK023 and 74 significantly overrepresented pathways in response to
210 MOK124. The pathways were enriched at one or more time points. Overall, 84 unique pathways
211 were enriched in response to either strain. The top 10 most significant KEGG pathways at each time
212 point are presented in Fig. 4, while a complete list of pathways at all time points is in Supplementary
213 File 3.
214

215 KEGG pathway analysis illustrated that there was a sustained somatic cell immune response to
216 MOK023. At 24 hpi, the top 2 most significant pathways in response to both MOK023 and MOK124
217 were NF-κB and TNF signalling pathways, both of which are involved in the immune response (Fig.
218 4). Genes involved in these two pathways displayed similar patterns of expression change in
219 response to both strains at 24 hpi (Fig. 5). Immune response pathways continued to be significantly
220 upregulated in the MOK023 group at 48 and 72 hpi, but not in the MOK124 group, even though
221 mastitis was more severe in the MOK124 group (Fig. 5). Instead, a specific response to MOK124 was
222 evident at later time points, characterised by overrepresentation of the Hippo signalling,

223 extracellular matrix receptor interaction (ECM) and tight junction pathways. The Hippo signalling
224 pathway is involved in regulating cell apoptosis or proliferation (40), while ECM receptor and tight
225 junction pathways both represent groups of genes that influence communication between epithelial
226 cells and maintaining the integrity of the epithelial cell layer (41, 42). When examining genes
227 involved in the Hippo signalling pathway, the majority of genes were downregulated relative to time
228 0 in response to MOK124 (Fig. 6A).
229
230 The ECM receptor interaction pathway was enriched in the MOK124 group at 48 hpi. Among ECM
231 receptor interaction genes, collagen genes *COL4A1, COL4A2, COL6A1, COL6A2, COL8A2, COL12A1,*
232 *COL14A1* and *COL18A1* were downregulated in response to MOK124 between 48 and 168 hpi (Fig.
233 6B). The collagen genes encode proteins that are components of basement membranes. Several
234 integrin genes were upregulated in response to MOK124 at 48 hpi (*ITGA5, ITGA9, ITGA2B, ITGB7*),
235 while some were also downregulated between 48 and 168 hpi (*ITGA2, ITGA3, ITGB4, ITGB6*).
236
237 For the tight junction pathway, in response to MOK124, most paracellular space genes were
238 downregulated, including genes encoding claudin (*CCLN*), occludin (*OCLN*), Marvel domain-
239 containing protein 3 (*MARVELD3*) and immunoglobulin superfamily member (*IGSF5*) (Fig. 6C).
240 Several genes from this pathway were upregulated at 24 hpi; however, the pathway was not
241 significantly enriched in the MOK124 group at that time point. Only two genes were upregulated at
242 later time points: Rho associated coiled-coil containing protein kinase 1 gene *ROCK1* was
243 upregulated at 48 hpi and myosin heavy chain 1 gene *MYH1* was upregulated at 72 hpi, both of
244 which are involved in regulation of actin cytoskeleton. All other genes, involved in cell polarity
245 (*PARD6B, TJP3, PATJ, AMOT, PRKCZ, LLG2, TJP1, DLG3*), decreased paracellular permeability (*TJP1,*
246 *CGN, CGNL1, CLDN3*), reduced cell proliferation (*YBX3, CCND1*), cell differentiation (*YBX3, ERBB2*)
247 and tight junction assembly (*RAB13, TJP1, CGN*) were downregulated between 48 and 168 hpi.
248

249 While pathways specific to MOK124 emerged from 48 hpi, the tuberculosis pathway was solely
250 overrepresented in response to MOK023, at 24, 48 and 72 hpi. Notably, among the upregulated
251 genes in this pathway were *CTSD*, *ATP6A1* and *LAMP1*, which encode cathepsin D, ATPase H+
252 transporting accessory protein 1 and lysosomal associated membrane protein 1, respectively. All of
253 these proteins are involved in internalisation into macrophages and arrest of phagosome
254 maturation. Other pathways specific to MOK023 were inflammation related pathways, such as
255 inflammatory bowel disease (IBD) pathway and rheumatoid arthritis pathway.
256

257 ### Gene ontology

258 Enrichment analysis of GO biological processes revealed 36 different significantly overrepresented
259 terms in response to MOK023 and 36 significantly overrepresented terms in response to MOK124.
260 Overall, 66 different GO terms were enriched in response to either strain. The top 10 enriched terms
261 in response to each strain at each time point are presented in Fig. 7, while a complete list at each
262 time point is attached in Supplementary File 3.
263

264 Immune system processes were overrepresented in the MOK023 group at 24 and 48 hpi, while they
265 were only significant in MOK124 group at 24 hpi. While the GO terms in the MOK023 group varied
266 across time points, a small number were consistently overrepresented in the MOK124 group
267 between 48 and 168 hpi. These included extracellular matrix organisation, wound healing and cell
268 junction organisation. Additionally, cell death was an enriched process in both groups at 168 hpi.
269

270 ## Immune cell populations

271 A significantly higher proportion of neutrophils was observed in milk from cows in the MOK124
272 group than in the MOK023 group (30). In order to assess whether immune cell proportions in the
273 milk somatic cell samples could be inferred using transcriptomic data, a cell population analysis
274 based on gene expression of cell markers was performed using quanTIseq (43). The cell proportions

275 at 0 hpi were similar between the two groups, with most cells classified as "other" likely
276 representing mammary epithelial cells (Fig. 8A). Genes for epithelial cell markers such as
277 cytokeratins 14, 18 and 19 (*KRT14, KRT18, KRT19*), E-cadherin (*CDH1*) and epithelial cell adhesion
278 molecule (*EPCAM*) (44, 45) showed the highest expression at 0 hpi and decreased post-infection (Fig.
279 8B). Immune cell populations predominated at later time points and immune cell proportions
280 differed between the two groups (Fig. 8A). At 24 hpi, neutrophils represented the largest proportion
281 of milk somatic cells in both MOK023 and MOK124 infected animals; however, in the MOK124 group
282 neutrophils represented a greater proportion of somatic cells than in the MOK023 group. A major
283 difference in cell proportions was observed between 48 and 168 hpi. In the MOK023 group, the
284 largest proportion of cells between 48 and 168 hpi were M1 macrophages, whereas in the MOK124
285 group neutrophils were the predominant cell type. A slightly larger proportion of M2 macrophages
286 could also be observed at each time point post-infection in the MOK124 group when compared to
287 the MOK023 group. M2 macrophages seemed to increase in proportion in both groups over time.
288

289 Milk secretion of chemokines and cytokines

290 Cytokine concentration was measured in milk at 0, 7, 24, 48, 72, 168, 336 and 504 hpi in order to
291 reflect both the acute and chronic response to infection. There were no differences in the
292 concentration of any of the examined cytokines or chemokines between the MOK023 and MOK124
293 groups prior to intramammary challenge or after 168 hpi. Protein concentrations increased post-
294 infection in both groups with a peak expression at 168 hpi, except for TNFα and IL-8 which
295 demonstrated a sharp increase at 24 hpi. A significant group x time interaction ($P < 0.05$) was
296 observed for IL-1α, IL-1β, IL-1RA, and IL-6, which are typical pro-inflammatory cytokines, but also IL-
297 2, IL-4, IL-10 and CCL3 (Fig. 9). In the case of all the above cytokines, MOK124 caused significantly
298 higher protein secretion at 168 hpi, which is also a time point at which the difference in milk yield
299 was low.

300 ## Discussion

301 In this study, the transcriptional response of milk somatic cells to two genotypically distinct strains,
302 representing two important globally distributed bovine-adapted lineages of *S. aureus,* was assessed.
303 Differences in the response to the 2 strains were found in gene expression patterns and in
304 overrepresented pathways. An initial immune response to both strains was evident but the
305 transcriptional immune response was sustained in the MOK023 group between 24 and 72 hpi, while
306 immune genes were only significantly upregulated in the MOK124 group at 24 hpi. This may indicate
307 a difference in disease progression or a response to different virulence strategies employed by the
308 two strains. The expression of genetic markers of various immune cells also differed in response to
309 each strain, indicating differing immune cell proportions among somatic cells recruited in response
310 to each strain, a result that was confirmed by a differential milk somatic cell count using microscopy
311 (30). More neutrophils were recruited in response to MOK124 from 48 hpi and there were
312 proportionally more M1 macrophages in the MOK023 group.

313

314 Exploration of normalised gene expression data yielded an interesting insight into the dynamics of
315 the transcriptional response to the two infecting strains, with a strain-specific transcriptional
316 response evident by 48 hpi. At this time point, gene expression in the MOK023 group was similar to
317 that at 24 hpi while in the MOK124 group gene expression was more similar to that at 72 and 168
318 hpi. Examination of normalised counts of *NFKB1* and other key genes suggested a difference in the
319 pro-inflammatory response, with a more rapid and higher response to MOK124, which later
320 subsided; in contrast, more sustained upregulation of immune gene expression was evident in
321 response to MOK023. The sustained transcriptional upregulation of immune genes in the MOK023
322 group was supported by pathway analysis – the overrepresentation of both the NF-κB and TNF
323 signalling pathways was sustained for longer after infection with MOK023. This difference in the
324 transcriptional response may be related to a rapid switch from activation of the immune response to
325 a disruption in cellular proliferation and the integrity of the epithelial cell layer in the MOK124

326 group. The 168 h time point could represent the end of the acute innate immune response in both
327 groups, since immune pathways were not significantly enriched at this time point and few genes
328 involved in the NF-kB and TNF signalling pathways were upregulated in either group.
329
330 The transcriptional response observed in this study supported several findings of the corresponding
331 *in vivo* infection, such as the larger reduction in milk yield, higher inflammatory response at 24 hpi
332 and higher SCC in the MOK124 group, as well as the increase in the proportion of neutrophils in milk
333 observed in the MOK124 group. The downregulation of expression of milk production genes in the
334 MOK124 group coincided with a significant reduction in milk yield, which was observed in response
335 to MOK124 between 24 and 144 hpi when compared to MOK023 (30). This downregulation also
336 indicates that infection with MOK124 caused a greater disruption to the epithelial layer of the
337 mammary gland, possibly due to epithelial cell dysfunction and sloughing, when compared to
338 MOK023 infection. Overall, the cellular composition analysis indicated that the strain-specific
339 response was mediated by different cell populations. M1 macrophages were mainly involved in the
340 response to MOK023, while the response to MOK124 led to a higher neutrophil recruitment. There
341 was no significant difference between the MOK023 and MOK124 groups in the proportion of large
342 mononuclear cells by microscopy; however, large mononuclear cells would have been comprised of
343 M1 and M2 macrophages as well as monocytes, and shifts in those respective populations could be
344 observed when looking at expression of cellular markers. The transcriptomic data therefore has the
345 potential to provide more detail on the proportions of different cell populations in milk, especially in
346 species where antibodies to cell markers are not available for the fine characterization of these cell
347 subpopulations.
348
349 A larger proportion of M2 macrophages in somatic cells from the MOK124 group may indicate a
350 trend towards tissue repair. M2 macrophages synthesise polyamines and proline which stimulate
351 cell growth, collagen formation and tissue repair (46). M2 macrophages are also capable of

352 producing ECM components (47) and may have contributed to the ECM related pathways which
353 were overrepresented in response to MOK124. Therefore, it is possible that MOK124 creates high
354 epithelial dysfunction, which exposes the stroma to bacteria and bacterial products and induces a
355 strong inflammation that the organism attempts to repair. This leads to a higher recruitment of
356 neutrophils and more plasma leakage due to failure of the epithelium to function as a barrier.
357
358 Pathway analysis revealed that MOK124 elicited a strain-specific response indicative of tissue injury,
359 as illustrated by the downregulation of Hippo signalling, ECM receptor interaction and tight junction
360 pathways between 48 and 168 hpi. Moreover, MOK124-specific GO terms identified at those time
361 points, such as wound healing and ECM organisation, corresponded to the KEGG pathways
362 identified. These pathways are well studied in humans. The key genes in the Hippo signalling
363 pathway are *YAP1* and *WWTR* which encode transcription factors YAP and TAZ. A successful
364 activation of this pathway depends on translocation of the YAP/TAZ proteins to the nucleus,
365 followed by binding of the transcription factors to a TEAD protein. The YAP/TAZ-TEAD complex
366 causes activation of transcriptional responses which result in cell proliferation by controlling the
367 expression of genes directly involved in the cell cycle and by upregulation of anti-apoptotic genes
368 (40). The genes involved in the Hippo signalling pathway were downregulated, which could indicate
369 a decrease in cell proliferation. However, protein interactions and localisation in the cell would need
370 to be studied in order to confirm this hypothesis.
371
372 The ECM receptor interaction pathway is involved in maintenance of cell shape and the interaction
373 of epithelial cells with the basement membrane. Within the ECM receptor interaction pathway,
374 several integrin genes were upregulated in response to MOK124 at 48 hpi (*ITGA5, ITGA9, ITGB7,*
375 *ITGA2B*). The integrin proteins mediate cell to matrix and cell to cell adhesion (48). *ITGA5* encodes
376 the light and heavy chains that comprise the α5 subunit. This subunit associates with the β1 subunit
377 to form a fibronectin and fibrinogen receptor. Both fibronectin and fibrinogen can mediate adhesion

378 of *S. aureus* to host cells (49), and increase of fibronectin levels in whey of Holstein-Friesian cows is
379 associated with mastitis progression (50). The integrin α9 subunit encoded by the *ITGA9* gene, when
380 bound to the β1 chain, forms an integrin that is a receptor for vascular cell adhesion molecule 1
381 (VCAM1), cytotactin and osteopontin, all of which are involved in immune response. *ITGB7* encodes
382 integrin subunit β7 which forms dimers with an α4 chain or an αE chain and plays a role in leukocyte
383 adhesion (48). These two upregulated integrin genes could indicate leukocyte adhesion and
384 migration was taking place in MOK124 infected animals at 48 hpi. Integrins can also act with
385 cytoskeleton proteins from the tight junction pathway and clustering of integrins increases cellular
386 tension, which can lead to the activation of the Hippo signalling pathway (48).
387
388 Tight junctions form barriers between cells to the flux of ions and molecules. Pore and leak pathways
389 can be differentiated within the action of the tight junction, and while claudins are involved in the
390 pore pathway, zonula occludens 1 (ZO-1) and occludins are involved in the leak pathway (51). In the
391 MOK124 group, claudin (*CCLN*), occludin (*OCLN*), Marvel (*MARVELD3*) and immunoglobulin
392 superfamily member (*IGSF5*) genes were downregulated as part of the tight junction pathway, which
393 suggests a decrease in cell to cell interaction and cell detachment. TNF-α has been shown to induce
394 barrier loss in epithelial cells during Crohn's disease *in vitro* and in mice. The loss of integrity of
395 intestinal epithelium in those studies was mediated by TNF-induced occludin internalisation (51).
396 Therefore, upregulation of the TNF pathway in the MOK124 group at 24 hpi could have contributed
397 to loss of cell integrity at later time points. On the other hand, dysregulation of cytoskeleton genes in
398 the tight junction pathway could be indicative of *S. aureus* internalisation into host cells.
399 Modification of the actin cytoskeleton in cells infected by *S. aureus* has previously been described
400 (28) and was attributed to host cell internalisation. A CC151 strain, RF122, has been hypothesised to
401 be adapted to an intracellular niche (19). *In vitro,* strain MOK023 was a significantly higher
402 internaliser in bMEC than strain MOK124 (34). The cytoskeleton reorganisation in response to
403 MOK124 is therefore more likely to be related to cell damage, loss of cellular structure and an

404 attempt to repair the tissue rather than due to host cell internalisation. The presence of clinical signs
405 in MOK124 challenged animals (30) also confirms that cell damage likely took place and a healing
406 response would be necessary to rebuild the damaged tissue. The ability of MOK124 to cause tissue
407 damage may be associated with the toxin encoding genes present in this lineage. MOK124 encodes
408 enterotoxin genes *seg, sei, selm, seln, seln, selo, egc, selu* and *ORFCM14* as well as the leukocidins
409 *lukF-PV* and *lukM*, none of which are present in MOK023 (17). Staphylococcal enterotoxin genes *seg,*
410 *sei, selm* and *sei* as well as *agrII* (which is the agr type of MOK124) have recently been found to be
411 associated with a higher likelihood of causing clinical mastitis in a collection of European *S. aureus*
412 strains isolated from natural infections (18). While LukM' was not found in milk of MOK124 infected
413 cows in this study, the other toxins encoded by this strain could play a role in the virulence of this
414 strain. Other CC151 strains have been found to produce low amounts of LukM' in previous studies
415 (52), therefore the mechanism of neutrophil killing by CC151 strains remains to be determined. Since
416 immune genes tended to be upregulated for longer in response to MOK023 than in response to
417 MOK124, the greater influx of neutrophils in response to MOK124 is unlikely entirely due to immune
418 signalling by milk somatic cells. Instead, a larger influx of neutrophils could be related to cellular and
419 tissue damage.
420
421 Although MOK023 caused immune gene induction both in bMEC *in vitro* and in milk somatic cells *in*
422 *vivo* (30, 34), immune signalling from bMEC was followed by a temporary recruitment of neutrophils
423 at 24 hpi, and M1 macrophages became the predominant cell type from 48 hpi. Since macrophages
424 are normally present in a healthy mammary gland, while neutrophils tend to be recruited to a site of
425 intramammary infection (53), the difference in cellular composition in milk post-infection in
426 response to the two strains may indicate that MOK023 subverted the immune response which failed
427 to recruit neutrophils to a significant extent. This is also supported by the fact that even though
428 immune gene expression was sustained in response to MOK023, bacterial load increased in the
429 MOK023 infected animals, to reach its maximum at 168 hpi. Lack of inflammation and relatively low

430 neutrophil numbers in response to MOK023 could be due to host cell internalisation. It was
431 demonstrated that MOK023 can internalise into bMEC *in vitro* (34). It has also been reported that
432 human-associated *S. aureus* can internalise and survive within human (54) and bovine macrophages
433 (55). *S. aureus* SH1000 (CC8) internalised within murine or human macrophages can exhibit a
434 persister phenotype and evade antimicrobial treatment (56), and strain ATCC 29213 (CC5; (57)) was
435 found to promote its survival in bovine macrophages by blocking autophagic flux (55). Internalisation
436 of MOK023 into MEC and/or macrophages could aid in the evasion of immune response.
437 Interestingly, among significantly DE genes, *LAMP1* was found to be overexpressed solely in
438 response to MOK023 (Supplementary File 2). This gene has previously been associated with *S.*
439 *aureus* survival inside a mature phagosome in macrophage cell lines (58, 59). Upregulation of the
440 tuberculosis pathway in the MOK023 group further supports the hypothesis that MOK023 may
441 internalise into host cell macrophages and be immune evasive. The tuberculosis pathway represents
442 a process where tubercle bacilli enter macrophages in the lung, and following host cell
443 internalisation they interfere with phagosomal maturation, antigen presentation, apoptosis and host
444 bactericidal responses to establish persistent or latent infection (60). Persistence of infection of
445 MOK023 was illustrated by a consistent bacterial load in infected animals over 30 days; however,
446 more research is needed to determine the precise mechanisms by which this strain persists within
447 the host. Notably, a recent study associated high internalisation into bMEC, biofilm formation and
448 low cytotoxicity with high within-herd prevalence of a *S. aureus* CC9 strain (61).
449
450 The transcriptomic response to *S. aureus* mastitis in dairy cows has been studied previously with
451 samples taken at single time points: 3 hpi (28, 32), 24 hpi (33) and 48 hpi (62). Udder biopsies were
452 used in those studies to analyse gene expression in response to a *S. aureus* IMI, and single *S. aureus*
453 strains were used. This study characterises later events occurring in IMI, and represents a response
454 of different cell populations, as it would be expected that an udder biopsy would be mostly
455 comprised of bMEC. Transcriptomic analysis of *S. aureus* mastitis in naturally infected dairy cows has

456 also been performed on post-mortem mammary tissue and peripheral blood leukocytes (63, 64),
457 with the extent of time post-infection being unknown, and the infecting strains not identified. This is
458 the first transcriptomics study of *S. aureus* mediated IMI where the response to two different strains
459 of *S. aureus* are compared, with full strain genotype information available. Genotyping of bacterial
460 strains used, use of multiple strains of *S. aureus* as well as examination of the cellular populations of
461 studied samples should be considered in future studies. All of the above can ensure better
462 understanding of strain-specific responses and an improved ability to compare different studies of
463 IMI.
464
465 The concentration of selected cytokines and chemokines in milk throughout the course of infection
466 confirmed and extended the finding that MOK124 induced higher IL-1β and IL-8 concentrations in
467 milk. A discrepancy was found between the gene expression results and the cytokine profiles since
468 cytokine and chemokine protein levels remained elevated in response to MOK124 at later time
469 points, while most of the cytokine genes were not DE post 24 hours in this group. While correlation
470 between transcriptomic and proteomic data is often poor (65-67) due to protein turnover, RNA
471 degradation or post-translational modifications, there could be other reasons for the discrepancy in
472 this study. Firstly, the protein levels were measured in milk, while the transcript levels were
473 measured in milk somatic cells. The mammary epithelial cells lining the bovine mammary gland
474 could be contributing to cytokine secretion into milk, and would not be captured by the RNA
475 sequencing. Therefore, mammary epithelial cells and other cells of the epithelial lining such as
476 resident macrophages and lymphocytes could be major contributors to cytokine and chemokine
477 levels in the milk. Secondly, the number and the type of milk cells likely influenced absolute protein
478 concentration with higher neutrophil counts in MOK124 infected cows. Finally, a systemic response
479 could have contributed to increased milk cytokine levels as exudation of plasma was likely in the
480 MOK124 group with higher milk Ig concentrations, but not evident in the MOK023 group (30). The
481 milk cytokine data highlights the need to implement proteomic studies alongside transcriptomic

482 studies to better understand the molecular mechanisms of host immune response. A study where
483 each milk somatic cell type was assessed separately during an *in vivo* trial, along with udder biopsies
484 or post-mortem examinations at one pre-defined time point, would be beneficial to further
485 understanding of bovine immune response to *S. aureus*. Furthermore, the pattern of cytokine
486 expression measured *in vitro* on MEC culture infected with either strain (34) does not correlate with
487 the secretion profile detected in milk upon mammary gland infection of dairy cows, showing that
488 bacteria-MEC interactions do not summarize the complex mechanisms occurring during mastitis and
489 may not be predictive of the pathogenicity of *S. aureus* strains.
490

491 ## Conclusions

492 This study confirms the ability of bovine *S. aureus* strains to cause a strain-specific immune response
493 during IMI, with each strain resulting in a unique signature of infection in terms of the number and
494 type of immune cells recruited, the biological function of the recruited cells and the resulting
495 cytokine and chemokine concentration in milk. MOK124 caused more inflammation and udder
496 damage, as illustrated by the disruption of tight junction and Hippo signalling pathways between 48
497 and 168 hpi, coupled with clinical signs observed in the infected animals. On the other hand,
498 MOK023 caused less inflammation and may be able to limit neutrophil recruitment and hence
499 persist in the mammary gland, as suggested by the sustained immune gene expression but low
500 increase in SCC, sustained bacterial load and mild clinical signs in MOK023 infected cows. Since
501 differences in early transcriptional response between the two strains were observed, followed by
502 either a severe or a mild, persistent infection, it is possible that the magnitude of this early response
503 may be crucial in determination of the course of staphylococcal IMI. The response to each strain also
504 involved recruitment of specific immune cells, with M1 macrophages primarily recruited in response
505 to MOK023, while neutrophils were the main immune cell type recruited in response to MOK124.
506 This indicates that different cell types as well as the transcriptional activity of each cell type may
507 influence gene expression differences between groups when analysing mixed cell population tissues

508 such as blood or milk. Further studies are necessary to further understand the molecular
509 mechanisms utilised by these strains to cause inflammation or evade the immune response.
510

511 # Methods

512 ## Study design

513 The aim of this study was to elucidate the molecular mechanisms of the host immune response *in*
514 *vivo* by utilising a transcriptomic approach. Fourteen first lactation Holstein-Friesian cows were
515 purchased from two commercial farms which were leptospirosis, salmonellosis and Johne's disease
516 free. Animals were selected based on composite milk SCC < 100,000 cells/ml. Milk bacteriology was
517 performed prior to challenge to confirm lack of contaminating organisms or mastitis pathogens.
518 Cows were infected with approximately 500 cfu of *S. aureus* strain MOK023 (ST3170, CC97) or
519 MOK124 (ST151, CC151) in 400 µl of PBS as described previously (30). Animals in which a *S. aureus*
520 infection was not established (two cows challenged with MOK023 and one cow challenged with
521 MOK124) were excluded from the study. Therefore, the MOK023 infected group included 5 animals,
522 while the MOK124 infected group included 6 animals. Due to severe clinical mastitis developed by 2
523 cows in the MOK124 group, these animals were treated with antibiotic and milk samples for
524 transcriptomic analysis were not taken after antibiotic treatment: cow 1776 was removed from the
525 trial at 31 hpi, while cow 1920 was removed at 120 hpi. A further sample from the MOK124 group at
526 48 hpi was removed from the analysis for quality control purposes as a sample from an uninfected
527 quarter was mistakenly sequenced instead of the sample from the infected quarter. The number of
528 animals in each experimental group at each time point is presented in Table 2.
529

530 **Table 2.** The numbers of animals (N) included in the study at each time point.

Hours post-infection (hpi)	N, MOK023 group	N, MOK124 group
0	5	6
24	5	6
48	5	4
72	5	5

168	5	4

RNA extraction from milk somatic cells

Milk from the infected quarter was collected into a sterile universal tube (Sarstedt, Nümbrecht, Germany). In order to obtain a sufficient quantity of RNA, 100 ml of milk was used for somatic cell isolation. Milk was centrifuged at 800 x g, at 15 °C for 10 min. The fat layer and supernatant were removed and tubes were placed upside down on tissue paper to drain for 10 min. Cell pellets were subsequently resuspended in 900 µl of Qiazol (Qiagen, Manchester, UK) and vortexed for 20 s to lyse the cells. Lysed samples were stored in Qiazol at -20 °C until RNA extraction. Samples were rapidly thawed at 37 °C and maintained at room temperature (RT) for the duration of the extraction. RNA was extracted using an RNeasy Plus Universal kit (Qiagen, Manchester, UK) as per the manufacturer's instructions. RNA was eluted in 30 µl of RNase-free water (Sigma Aldrich Ireland Ltd., Wicklow, Ireland).

RNA quality was assessed using 2100 Bioanalyser RNA Nano chips (Agilent Technologies Ireland Limited, Cork, Ireland) as well as with a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA quantity was determined with a Qubit RNA broad range assay (Invitrogen: Bio-Sciences, Dun Laoghaire, Ireland). Sample RIN values ranged between 5.9 and 9.5 with a mean RIN value of 7.82 (Supplementary Table S1), and concentrations ranged from 4.1 – 930 ng/µl (mean concentration of 154 ng/µl).

Library preparation and sequencing

RNA was sent on dry ice to Macrogen (South Korea) for library preparation and sequencing. RNA quality was confirmed on arrival using a TapeStation HSRNA Screen Tape and analysed on a 2200 TapeStation (Agilent Technologies Korea Ltd, Seoul, Korea). Library preparation was performed with 100 ng of RNA using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, USA)

556 according to the manufacturer's instructions (Part #15031047 Rev. E). Library size was verified with a
557 2100 Bioanalyzer using a DNA 1000 chip (Agilent, Korea) and library quantity was determined with
558 qPCR according to the Illumina qPCR Quantification Protocol Guide. The mean library fragment size
559 was 348 bp while the mean library concentration was 92.63 nM. Libraries were diluted and pooled
560 with 300 pM of each library and sequenced (100 bp, paired-end) on a NovaSeq (Illumina, USA).
561 Samples were divided across two sequencing runs.
562

563 Quality control, assembly and DE gene analysis

564 All the bioinformatics pipeline bash, Perl and R scripts used for computational analyses were
565 deposited in a GitHub repository at
566 https://github.com/DagmaraNiedziela/RNAseq_Saureus_cattle_infection. RStudio v1.1.463 and R
567 v3.5.2 were used for the analyses. Sequence quality was assessed using FastQC v11.5 (68). Trimming
568 was performed using fastp v12.1 (69) with default settings as well as enabling base correction for
569 paired end data and enabling overrepresented sequence analysis; on average 0.5 % of the reads
570 were trimmed per sample. Trimmed fastq files were re-checked with FastQC to confirm sequence
571 read quality. Trimmed sequences were mapped to the bovine genome (UMD3.1) using the STAR
572 aligner v2.5.2 (70), with gene counts generated simultaneously with the STAR software using the
573 UMD3.1 v93 of the *B. taurus* reference genome and annotation. Genes with zero read counts across
574 all samples as well as non-protein coding genes were removed in a filtering step (71). DeSeq2
575 v1.18.1 (72) was then used to visualise gene expression data and perform differentially expressed
576 (DE) gene analysis. Data was normalised with a vst function and visualised with a principal
577 component analysis (PCA) plot of the samples based on the 500 genes with the highest inter-sample
578 variation. DE gene lists were generated using a negative binomial generalized linear model. Wald
579 tests were performed to obtain DE genes separately for each group at each time point using time 0
580 as a reference, with P values adjusted for multiple comparisons using a Benjamini and Hochberg (B-

581 H) method. DE genes with an adjusted P value < 0.05 and a $\log_2$ fold change threshold of 1 were used
582 for further DE gene data exploration and pathway analysis.
583

584 Pathway and gene correlation analysis

585 Significant (P adj < 0.05) and highly overexpressed ($|\log_2$ fold change$|$ > 1) DE genes were converted
586 to their 1 to 1 human orthologs using BioMart, ordered by adjusted P value and analysed for
587 enrichment of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG)
588 pathways using gProfiler R package v0.6.6 (73). When a group of DE genes matched to multiple
589 pathways, the pathway that was represented by the highest number of DE genes was selected in a
590 filtering step.
591

592 Cell composition analysis

593 Transcripts per million (TPMs) were generated from raw gene counts. Bovine Gene IDs converted to
594 1 to 1 human orthologs were used for the analysis. To estimate the proportion of the different
595 immune cell populations in each somatic cell sample, a quanTIseq algorithm which is based on
596 expression of cell specific surface marker genes was used (43).
597

598 Quantification of a panel of cytokines and chemokines in milk

599 Milk was defatted by centrifugation at 800 x g, 15 °C for 10 min. The top fat layer was discarded and
600 the skim milk supernatant was stored in 1 ml aliquots at -20 °C until use. Prior to protein
601 quantification milk was diluted 1:2 in assay buffer (Merck-Millipore, Burlington, USA).
602 Concentrations for 15 cytokines (IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-17a, IFNγ, CCL-2, CCL-3, CCL-4,
603 CXCL8, CXCL10, and TNFα) were determined using a custom bovine cytokines MilliPlex xMAP assay
604 (Merck Millipore, USA). Data were recorded on a MAGPIX flow cytometer using Xponent software
605 (Luminex, Austin, USA).

606

607 ## LukM ELISA

608 ELISA to detect the LukM subunit of the LukMF' Staphylococcal toxin in milk samples was performed
609 as described previously (37). Briefly, anti-LukM polyclonal bovine IgG (32 µg/ml) was used to coat
610 the wells of an ELISA plate at 4 °C overnight. The plate was emptied and washed twice with 300 µl of
611 PBS-T (DBPS: Gibco, UK; 0.05% Tween-20: Sigma-Aldrich, St. Louis, MO), blocked with 200 µl of 4 %
612 skimmed milk powder (Marvel, DSDelta Ltd) in PBS-T for 30 min with shaking and washed once with
613 300 µl PBS-T. A 100 µl aliquot of each sample (skimmed milk heated to 95 °C for 10 minutes to
614 inactivate any existing antibodies against LukM) or standard (recombinant LukM; kindly provided by
615 Utrecht University) was added to the wells and incubated for 60 min at room temperature (RT) with
616 shaking, followed by 2 washes with 300 µl PBS-T. Mouse anti-LukM monoclonal antibody (KoMa43,
617 Podiceps, The Netherlands) was diluted to 3 µg/ml in 1 % skimmed milk-PBS-T and 50 µl was added
618 to each well. The plate was incubated for 60 min at RT, with shaking, and washed 3 times. Polyclonal
619 HRP conjugated goat anti-mouse IgG (BioLegend, London, UK) was diluted 1 in 1000 and 100 µl was
620 added to each well. The plate was incubated for 60 min at RT, with shaking, and washed 3 times.
621 TMB (100 µl; Thermo Fisher Scientific, Waltham, USA) was subsequently added and incubated in the
622 dark at RT for 15 min, followed by addition of 100 µl of the stop solution (0.05 M H_2SO_4; Thermo
623 Fisher Scientific, Waltham, USA). Absorbance was read at 450 nm using a microplate reader (xMark,
624 Bio-Rad, Watford, UK).

625

626 ## Statistical analysis

627 Data generated from cytokine and LukM' protein quantification was analysed with a repeated
628 measures ANOVA using a MIXED® procedure in SAS (SAS Version 9.4, SAS Institute, Cary NC, USA) to
629 compare strain-dependent differences. Strain, time and the strain x time interaction were included
630 in the model, with time as a repeated measure. Time 0 measurements for each cytokine were
631 included as a covariate, and all variable interactions were explored. Log_{10} transformations were used

632 for all cytokine variables to satisfy the distributional requirements of ANOVA. Correlations among
633 repeated measurements across time between strains were modelled using the compound symmetry
634 covariance structure for each parameter analysed. Tukey's post-hoc multiple comparison test was
635 applied as appropriate.
636

637 # Declarations

638 ## Ethics approval

639 All procedures involving animals were performed in accordance with the relevant guidelines and
640 regulations of the European Directive 2010/63/EU and received approval from the University College
641 Dublin Animal Research Ethics Committee (protocol number Leonard AREC 16 44), and a project
642 authorisation from the Health Product Regulatory Authority (HPRA) (project authorisation number
643 AE18982/P108). The study was carried out in compliance with the ARRIVE guidelines and has been
644 described in detail previously (Niedziela DA, *et al.* 2020. *J Dairy Sci*. 103(9):8453-66).
645

646 ## Consent for publication

647 Not applicable.
648

649 ## Availability of data and materials

650 Raw fastq files used during the current study are available in the European Nucleotide Archive (ENA)
651 repository with a project number PRJEB43443 .
652 Code used to analyse the data is available at:
653 https://github.com/DagmaraNiedziela/RNAseq_Saureus_cattle_infection
654 Any other data from the current study are available from the corresponding author on reasonable
655 request.
656

657 ## Competing interests

658 The authors declare that they have no competing interests.

659

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663

664 ## Author contributions

665 DAN optimised and conducted the laboratory-based work, performed bioinformatics analysis of the

666 RNAseq data and drafted the manuscript.

667 OMK conceived the initial idea of the study and participated in its design and coordination.

668 FCL conceived the initial idea of the study and participated in its design and coordination.

669 PC provided mentoring and supervision during the analysis of RNAseq data and performed the cell

670 composition analysis.

671 GF conducted/supervised protein laboratory-based work and statistical analysis of protein data.

672 All authors helped to draft the manuscript and read and approved the final manuscript.

673

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681

682

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875 ## Figures

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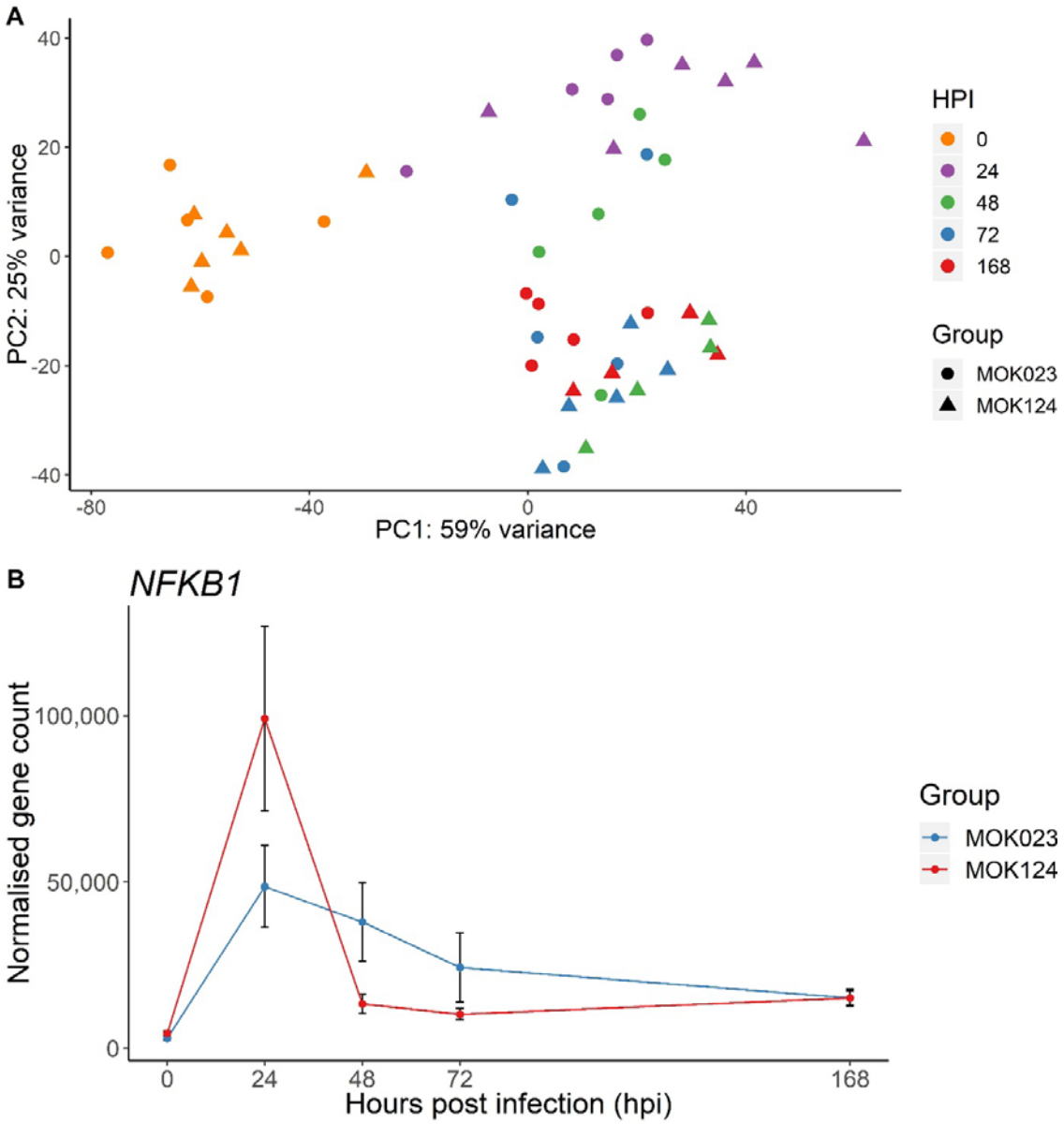

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878 **Fig. 1**. Results of exploration of raw counts data.

879 **a** PCA plot generated from milk somatic cell gene expression data of the 500 most variable genes
880 following infection with MOK023 (circles) or MOK124 (triangles). Samples from each time point are
881 represented by a different colour: 0 hpi - orange, 24 hpi - purple, 48 hpi - green, 72 hpi - blue, 168
882 hpi - red. HPI - hours post infection. **b** Normalised expression (mean ± SEM) of the NF-κB subunit 1
883 (*NFKB1*) gene in milk somatic cells in response to infection with MOK023 (blue) or MOK124 (red).

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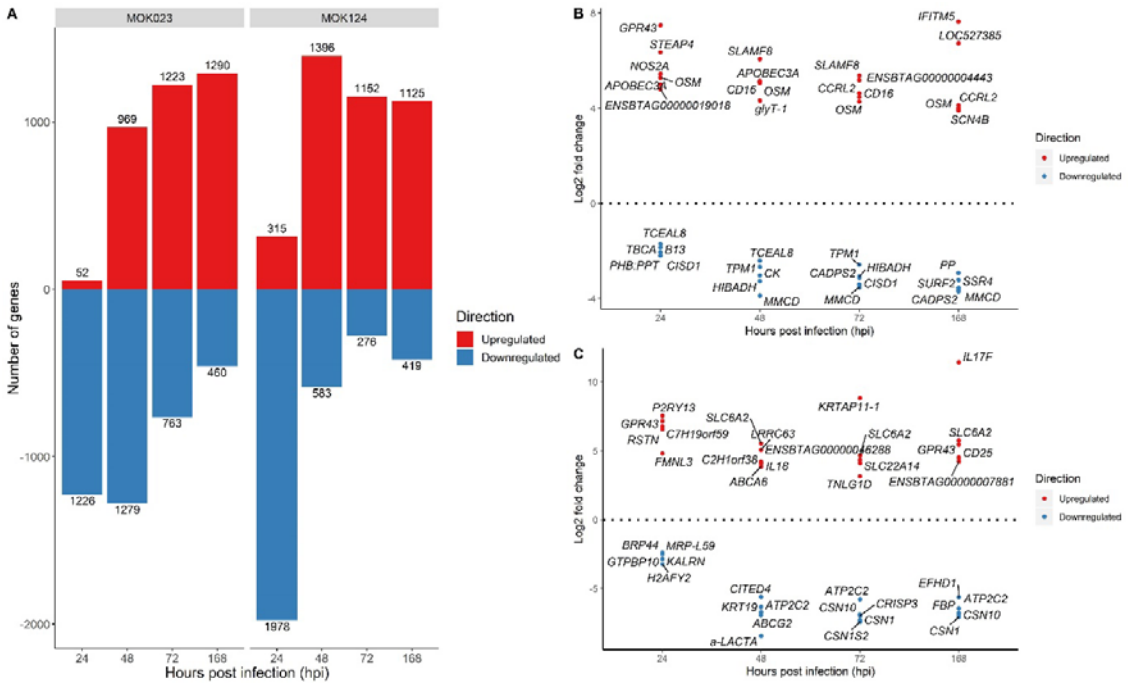

Fig. 2. Differentially expressed genes in response to *S. aureus* MOK023 and MOK124. **a** The number of significant DE genes (Benjamini-Hochberg adjusted P-value < 0.05, $\log_2$ fold change threshold > 1) which were up- (red) and downregulated (blue) in milk somatic cells in response to strains MOK023 and MOK124 relative to pre-infection. **b** Top 5 most significant up- and downregulated DE genes in response to infection with MOK023. **c** Top 5 most significant up- and downregulated DE genes in response to infection with MOK124. Genes with lowest adjusted P value were selected at each time point. In the case of genes upregulated at 24 hpi in response to infection with MOK023 two genes had the same adjusted P value and so both are included.

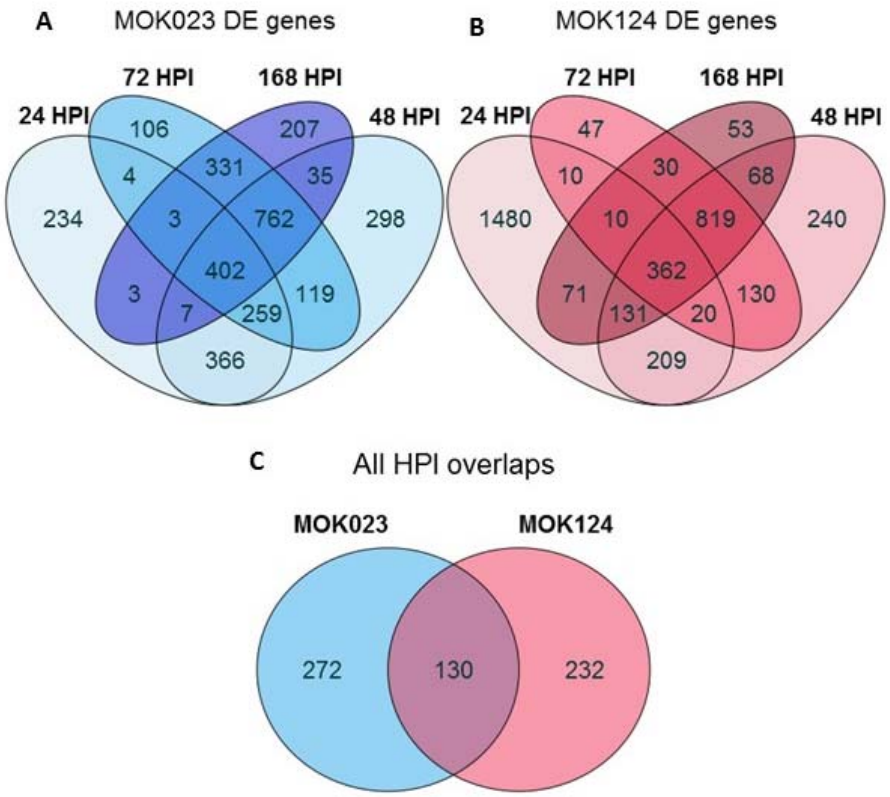

Fig. 3. Common DE genes in milk somatic cells across time points.
a Common DE genes in response to MOK023. **b** Common DE genes in response to MOK124. **c** Overlap of the DE genes common at all time points between the two strains. HPI - hours post infection.

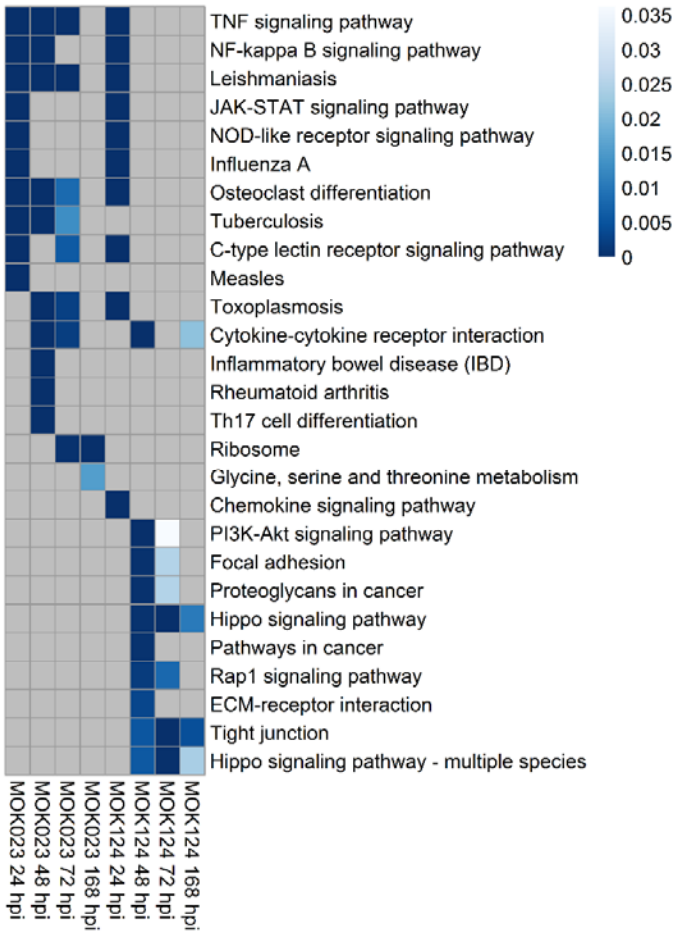

Fig. 4. KEGG pathways in response to infection with *S. aureus* MOK023 or MOK124 over time. Significant ($P < 0.05$) and unique (filtered for shared genes) are shown. Colour scale represents P value, grey squares represent non-significance. Up to 10 of the most significant pathways at each time point are presented. hpi - hours post infection.

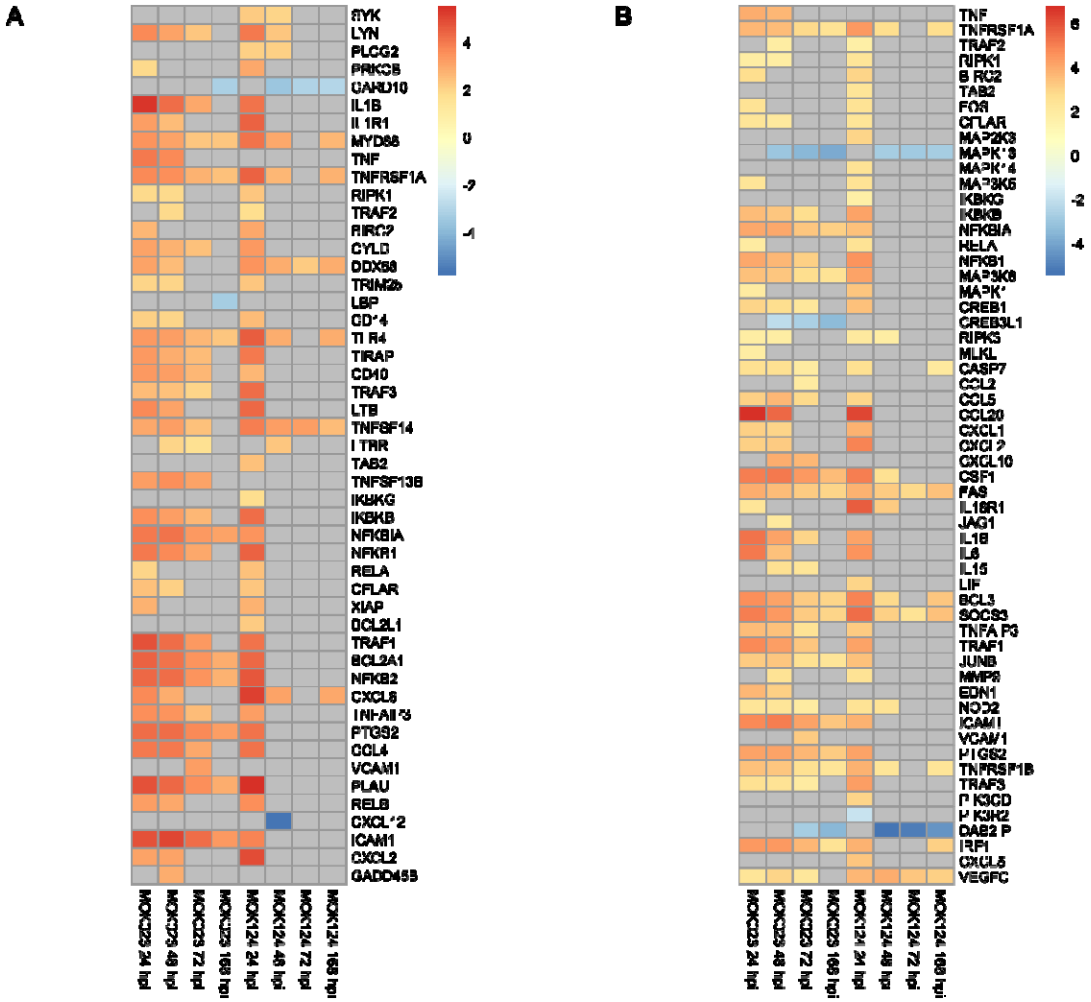

Fig. 5. Log_2fold change of genes involved in selected KEGG pathways in response to MOK023 and MOK124.

a NF-κB signalling pathway. **b** the TNF signalling pathway. Grey tiles show genes that were not significantly DE.

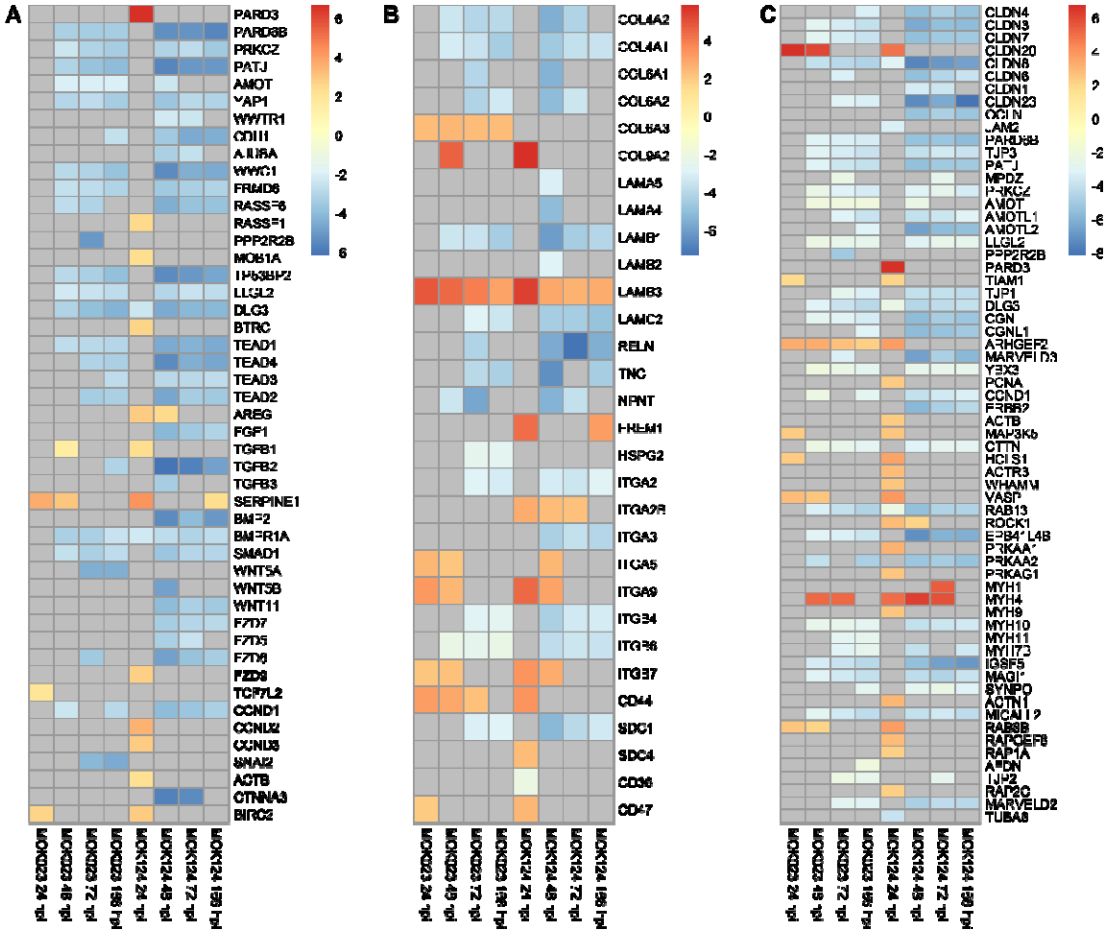

Fig. 6. Log_2fold change of genes involved in selected KEGG pathways in response to MOK023 and MOK124. **a** Hippo signalling pathway. **b** ECM receptor interaction pathway. **c** tight junction pathway. Grey tiles show genes that were not significantly DE.

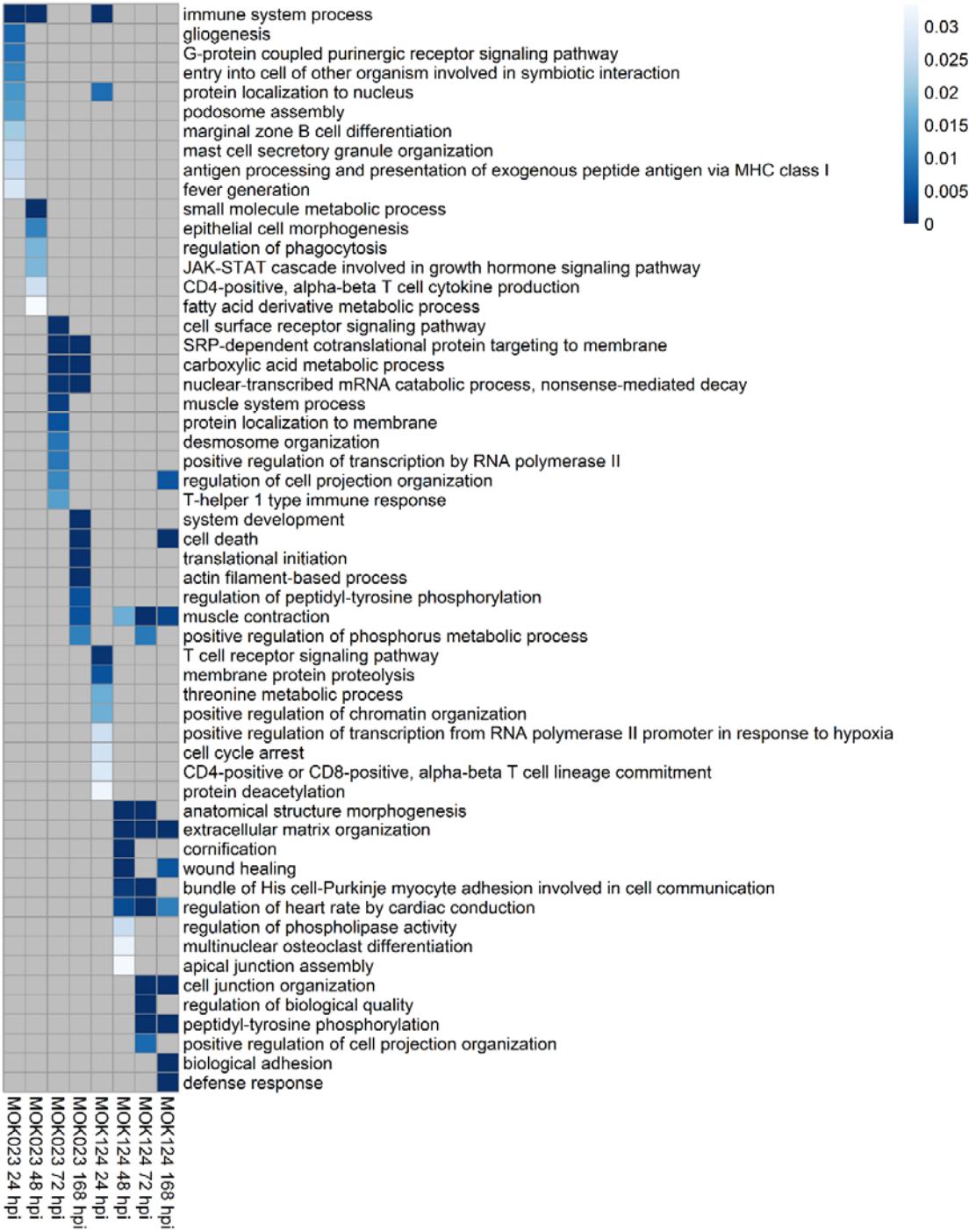

Fig. 7. GO biological processes in response to infection with *S. aureus* MOK023 or MOK124 over time.

Top 10 most significant ($P < 0.05$) and unique (filtered for shared genes) are shown. Colour scale represents P value, grey squares represent non-significance. hpi - hours post infection.

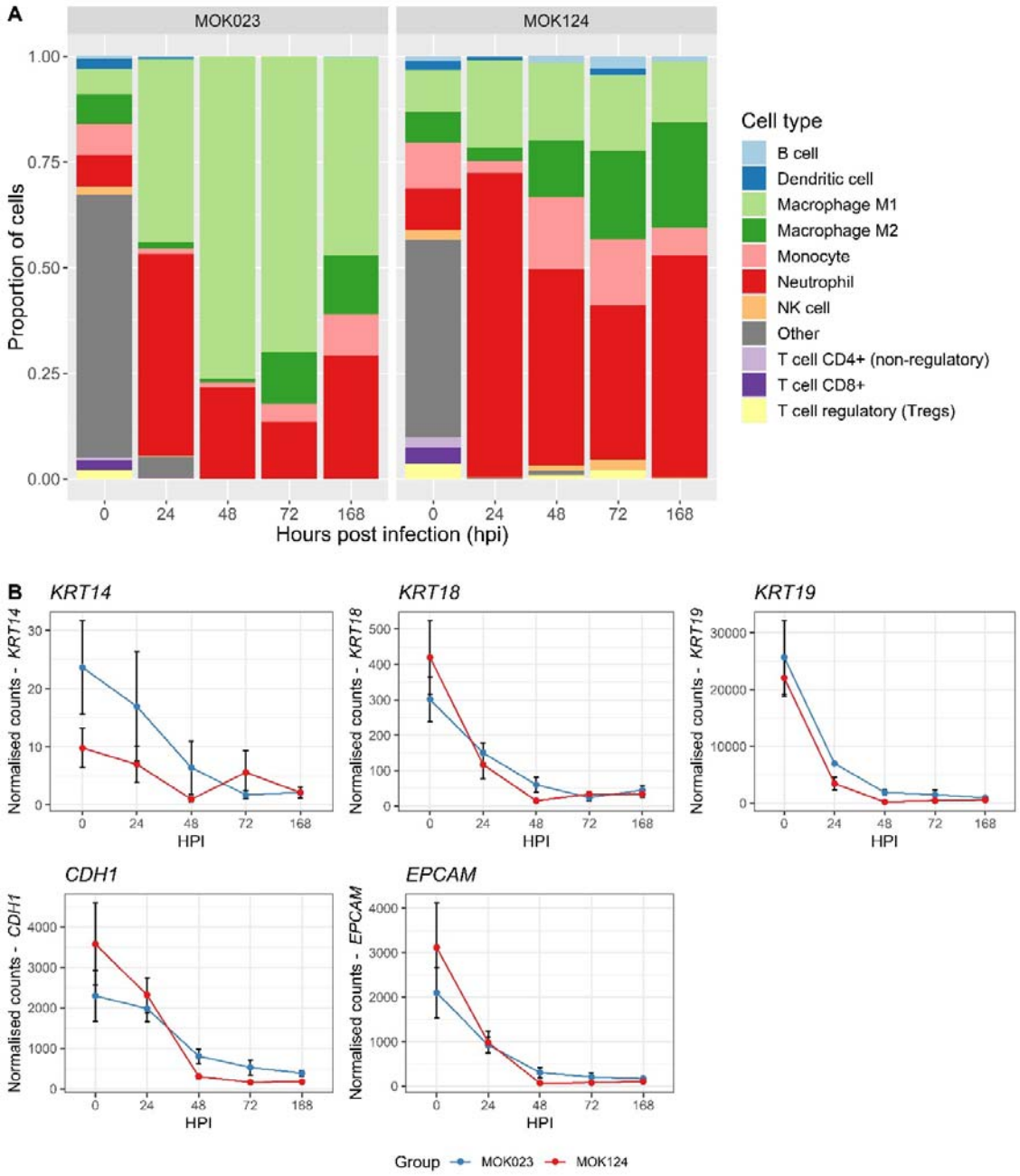

Fig. 8. Cell composition analysis.

a Changes in cellular composition in milk somatic cells over time illustrated by cell proportions. **b** Expression of epithelial cell marker genes in milk somatic cells over time in response to infection with MOK023 (blue) or MOK124 (red). *KRT14* - cytokeratin 14, *KRT18* - cytokeratin 18, *KRT19* - cytokeratin 19, *CDH1* - E-cadherin, *EPCAM* - epithelial cell adhesion molecule, HPI - hours post infection.

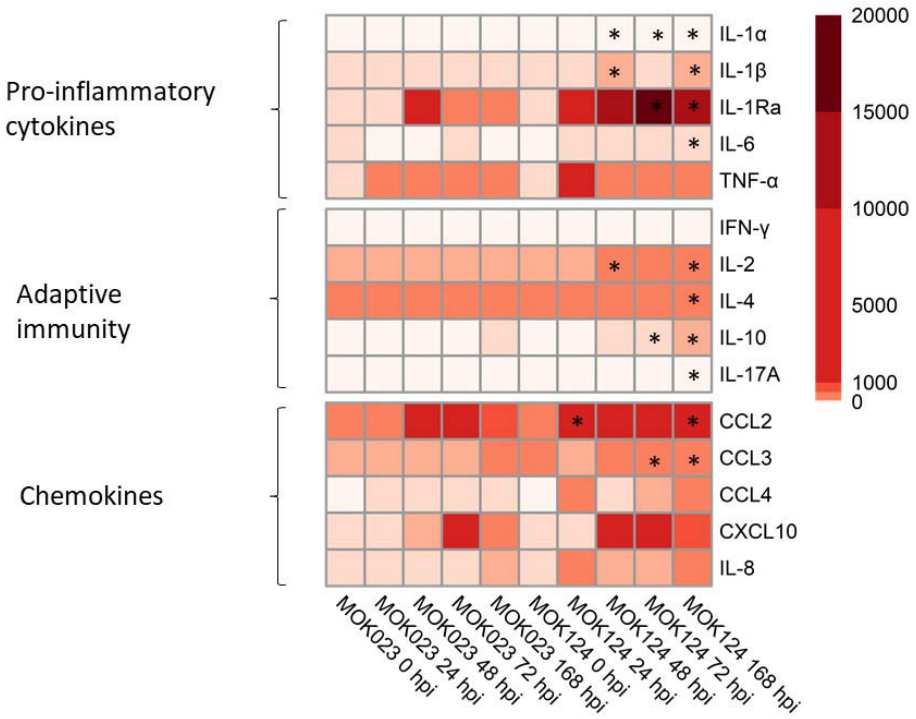

Fig. 9. Concentration of selected immune markers in milk.
The immune markers are divided into: pro-inflammatory cytokines: IL-1α, IL-1β, IL-1RA, IL-6, TNFa; cytokines involved in adaptive immunity: IFNγ, IL-2, IL-4, IL-10, IL-17A; and chemokines: CCL2, CCL3, CCL4, CXCL10, IL-8.
Colour scale represents mean fluorescence intensity (MFI). Significant time points where cytokine concentration in MOK124 group was higher than in the MOK023 group are marked with *.