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.

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 a strain belonging to Clonal Complex (CC)151 caused clinical mastitis, while a strain 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 from cows infected with each strain of *S. aureus* at 0, 24, 48, 72 and 168 hours post-infection (hpi) were analysed for differentially expressed (DE) genes in response to each strain.

Results: In response to MOK023 (CC97), 1278, 2278, 1986 and 1750 significant differentially expressed (DE) genes were found at 24, 48, 72 and 168 hpi, respectively, while 2293, 1979, 1428 and 1544 significant DE genes were found in response to MOK124 (CC151) 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.

48 ## Introduction

49 Bovine mastitis is a disease of the udder which affects milk yield and quality and causes significant
50 economic losses to the dairy industry (1-3). Mastitis can present as clinical or subclinical. Clinical
51 mastitis presents with overt signs such as clots in milk or swelling of the quarter. Clots in milk are a
52 result of the influx of substantial numbers of immune cells into the udder and are formed by
53 aggregates of leukocytes and blood clotting factors (4). Subclinical mastitis presents with no obvious
54 signs but typically causes a moderate influx of immune cells into the udder, leading to an increase in
55 somatic cell count (SCC) in milk (5). Individual cow SCC of < 100,000 cells/ml is typically considered to
56 be reflective of a healthy mammary gland, while an SCC > 200,000 cells/ml may be indicative of
57 bacterial infection (6).
58
59 *Staphylococcus aureus* is a major cause of bovine mastitis in Ireland (7, 8) and worldwide (9-12) and is
60 also one of the aetiological agents of human mastitis (13, 14). *S. aureus* is a clonal pathogen which
61 evolves slowly compared to many other bacteria (15). Nevertheless, substantial genetic diversity exists
62 among *S. aureus* strains, with many lineages identified to date, often adapted to different hosts (16).
63 Four main bovine-adapted lineages have been identified among Irish clinical mastitis isolates: Clonal
64 Complex (CC)71, CC97, ST136 and CC151 (17). CC97 is a predominantly bovine-associated lineage
65 although host jumps into humans with subsequent global spread have been demonstrated (16), while
66 CC151 appears to be a uniquely bovine lineage which has not been found in other ruminants, avian
67 species or in humans (16, 18-20).
68
69 During bovine IMI, pathogens enter the mammary gland through the teat canal. The immune response
70 in the mammary gland is presumably initiated by bovine mammary epithelial cells (bMEC) in the alveoli
71 of the udder and by resident macrophages. Pathogen-Associated Molecular Patterns such as
72 peptidoglycan and lipoteichoic acid on the surface of Gram-positive bacteria are recognised by Toll-
73 like receptor 2 (TLR2), which stimulates activation of the NF-κB signalling pathway in bMEC as well as

74 macrophages. NF-κB and other immune pathways stimulate the production of cytokines and
75 chemokines, which cause inflammation in the udder and stimulate neutrophil recruitment.
76 Neutrophils are the first cell type recruited into the udder to engulf the bacteria and attempt to clear
77 the infection (21). Previous studies have reported that *S. aureus* IMI induces a weak inflammatory
78 response with inhibition of the TLR2 and NF-κB pathways and low levels of pro-inflammatory cytokine
79 production (22-24). However, these studies used a variety of *S. aureus* strains and the magnitude of
80 the immune response to *S. aureus* has been reported to be strain-dependent (25). Therefore, the
81 general supposition that *S. aureus* causes a low immune response in bovine IMI (26) may only be true
82 for specific strains of *S. aureus* and there is a need to further study the immune response to different
83 strains of *S. aureus* during IMI.
84
85 The transcriptomic response to intra-mammary challenge with *S. aureus* has been previously studied
86 in bovine gland cistern tissue using microarrays (27, 28) and RNA sequencing (23); however, studies
87 were often short in duration (23, 27) or involved only one time point (28). Characterising the
88 transcriptomic response to *S. aureus* over several time points would be desirable, since it was
89 previously demonstrated *in vitro* and *in vivo* that the response to *S. aureus* IMI changes over time (25,
90 29). Furthermore, previous transcriptomic studies utilised only single strains of *S. aureus* for intra-
91 mammary challenge, and none used a strain belonging to CC151, a globally distributed bovine-
92 adapted lineage. Immune gene expression in bMEC in response to *S. aureus* differs according to
93 bacterial strain or lineage (29-31). Intramammary challenge of Holstein-Friesian cows with strains
94 MOK023 (CC97) or MOK124 (CC151) demonstrated that *S. aureus* strain MOK023 caused mild or
95 subclinical mastitis, while strain MOK124 caused severe clinical mastitis (25). Differences were
96 observed in secretion of cytokines IL-8 and IL-1β into milk between the MOK023 and the MOK124
97 infected cows, which further suggested that the immune response is strain-specific. Due to the
98 differences observed between the immune response to MOK023 and MOK124 in bMEC *in vitro* and
99 also *in vivo*, a further study to elucidate the molecular mechanisms of the host immune response *in*

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

105 # Results

106 ## Summary of *in vivo* disease presentation

107 Milk somatic cells collected during a previously described *S. aureus* IMI challenge trial were used. The
108 influence of bacterial strain on clinical signs, bacterial load, SCC and the concentration of select
109 cytokines and anti-*S. aureus* antibodies in milk were previously reported (25). Briefly, cows infected
110 with MOK124 generally developed clinical mastitis, while cows in the MOK023 group developed mild
111 or subclinical mastitis. There was a greater drop in milk yield in the MOK124 group and SCC was
112 significantly higher between 24 and 216 hpi in the MOK124 group compared to the MOK023 group.
113 The MOK124 bacterial load was significantly higher than that of MOK023 at 24 and 31 hpi and
114 subsequently decreased, while MOK023 bacterial load slowly increased and became significantly
115 higher than MOK124 later in the infection (216 to 504 hpi). The levels of IL-8 and IL-1β were also
116 significantly higher in milk of cows challenged with MOK124 compared to cows challenged with
117 MOK023 during the first week of infection.
118

119 ## LukM'

120 The severity of bovine IMI has been previously associated with secretion of the bi-component
121 leukocidin LukMF' by *S. aureus (32, 33)*. As the genes encoding this toxin are found in MOK124, but
122 not MOK023, LukM' in milk from infected animals was quantified. No LukM' was found in either
123 MOK023 or MOK124 group milk samples at any time point.
124

125 ## Sequence and mapping quality

126 Somatic cells collected pre-infection and at 24, 48, 72 and 168 hpi were subjected to 100 bp stranded
127 paired-end sequencing. On average, 95 % of the bases across all samples had a phred score of ≥ 30
128 and 98 % had a score of ≥ 20. The mean number of reads per sample was 83.5 M; however, less than
129 60 M reads were generated from 3 samples from time 0 due to the low number of somatic cells in the
130 samples. The distribution of read counts, phred scores and GC content per sample is available in
131 Supplementary File 1. Quality control, as assessed by FastQC, yielded satisfactory results for all
132 samples. On average, 93 % of reads mapped uniquely to the bovine genome UMD 3.1.93.

133

134 ## Data exploration

135 Raw read counts were used for DE gene analysis using DESeq2. After filtering for low counts (sum of
136 reads from all samples < 1) and non-protein coding genes, 17,334 genes were included in further
137 analysis. The expression of the 500 most variable genes was used for principal component analysis
138 (PCA) within the DESeq2 software package. Time 0 (pre-infection) samples clustered separately from
139 infected samples (Fig. 1A). Expression patterns from cows infected with either strain did not separate
140 on the PCA plot at 24 hpi. However, at 48 and 72 hpi, separation between the response to the 2 strains
141 was evident. At 48 hpi MOK124 infected animals' response had progressed to a state that was
142 maintained until 168 hpi, while the response in MOK023 infected animals progressed more slowly to
143 the same state and samples clustered in distinct groups at 48, 72 and 168 hpi. The difference in
144 progression of gene expression in response to each strain could also be seen in the expression profile
145 of a number of core immune genes across the experiment time course. This was illustrated by the NF-
146 κB subunit 1 (*NFKB1)* gene, which is a key gene involved in switching on production of immune
147 mediators through the NF-κB signalling pathway. A rapid increase in expression of this gene at 24 hpi
148 was followed by a rapid decrease in MOK124 infected animals. On the other hand, infection with
149 MOK023 caused a more sustained *NFKB1* induction (Fig 1B).

150

151 ## Differentially expressed genes

152 To identify highly up and downregulated genes, a $\log_2$ fold change threshold of 1 (indicating a 2 fold
153 increase or decrease in expression) was applied to the DE gene results in DESeq2 and genes with an
154 adjusted P value < 0.05 were subsequently selected as significant. In response to MOK023, 1278, 2278,
155 1986 and 1750 significant DE genes were found at 24, 48, 72 and 168 hpi, respectively, while 2293,
156 1979, 1428 and 1544 significant DE genes were found in response to MOK124 at those time points
157 (Fig. 2A). At 24 hpi the majority of genes with altered expression were downregulated in response to
158 both strains, which could indicate a suppression of normal cell function; while at 72 and 168 hpi,
159 upregulated DE genes predominated in response to both strains. Details of all significant DE genes are
160 available in Supplementary File 2.

161

162 Among the most significant upregulated DE genes in response to MOK023, the gene for oncostatin M
163 (*OSM*) was in the top 5 at all time points post-infection (Fig. 2B). *OSM* encodes a cytokine involved in
164 inflammation and regulation of production of other cytokines. Oncostatin is also secreted by
165 neutrophils during their rolling in blood vessels in order to enhance neutrophil adhesion to endothelial
166 cells (34). In response to MOK124, the top 5 upregulated genes included those involved in the immune
167 response such as interleukin genes (*IL17F, IL18*) and interleukin-2 receptor (*CD25*) gene (Fig. 2C). The
168 top 5 downregulated genes in response to MOK124 included genes encoding milk proteins lactalbumin
169 alpha (*α-LACTA*), casein alpha subunit 2 (*CSN1S2*), casein alpha subunit 1 (*CSN1*) and casein kappa
170 (*CSN10*).

171

172 In order to examine the effect of strain on transcription of major genes involved in milk production,
173 $\log_2$ fold changes of relevant significantly DE genes were examined (Table 1). The main genes involved
174 in milk production were reviewed by Strucken, Laurenson (35) and include leptin, lactalbumin, caseins,
175 prolactin and several genes involved in fat transport, energy production and growth. No significant

176 change in expression of the selected milk production genes was observed in MOK023 infected animals
177 at 24 hpi; however, all examined genes with the exception of *AGPAT6* were significantly
178 downregulated at all other time points in this group. In the MOK124 group, downregulation of the
179 expression of milk production genes was already evident by 24 hpi and continued until 168 hpi. Log_2
180 fold changes in expression of genes encoding milk-related proteins (*α-LACTA, CSN1, CSN10, CSN1S2,*
181 *CSN2*) were 2-3 log lower in the MOK124 group than in the MOK023 group, indicating a more severe
182 reduction of protein synthesis in the first group.
183
184 **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

185 Log_2 fold changes of significant DE genes only are included (adjusted P-value < 0.05, log_2 fold change
186 threshold > 1).
187 N/A – gene not significantly DE. Gene names: *ABCG2* – ATP binding cassette sub-family G member 2,
188 *AGPAT6* - 1-acylglycerol-3-phosphate O-acyltransferase 6, *α-LACTA* – alpha lactalbumin, *CSN1* - casein
189 alpha subunit 1, *CSN10* – casein kappa, *CSN1S2* - casein alpha subunit 2, *CSN2* - casein beta, *GHR* -
190 growth hormone receptor, *PPARGC1A* - peroxisome proliferator activated receptor gamma
191 coactivator 1 alpha, *PRLR* - prolactin receptor. hpi - hours post-infection.
192
193 A total of 402 genes in the MOK023 group were DE at all time points post-infection, while 362 DE
194 genes were common in the response to MOK124 at all time points (Fig. 3 A-B). Of these, 130 were
195 shared between MOK023 and MOK124 at all time points and are proposed as strain-independent
196 biomarkers of *S. aureus* IMI during the first week of infection (Fig. 3C). Among the common genes, 42
197 were involved in immune response processes.
198

199 ## Pathway analysis

200 ### KEGG pathways

201 Pathway analysis of KEGG metabolic processes revealed 67 unique significantly overrepresented
202 pathways in response to MOK023 and 74 significantly overrepresented pathways in response to
203 MOK124. The pathways were enriched at one or more time points. Overall, 84 unique pathways were
204 enriched in response to either strain. The top 10 most significant KEGG pathways at each time point
205 are presented in Fig. 4, while a complete list of pathways at all time points is in Supplementary File 3.
206
207 KEGG pathway analysis illustrated that there was a sustained somatic cell immune response to
208 MOK023. At 24 hpi, the top 2 most significant pathways in response to both MOK023 and MOK124
209 were NF-κB and TNF signalling pathways, both of which are involved in the immune response (Fig. 4).
210 Genes involved in these two pathways displayed similar patterns of expression change in response to
211 both strains at 24 hpi (Fig. 5). Immune response pathways continued to be significantly upregulated
212 in the MOK023 group at 48 and 72 hpi, but not in the MOK124 group, even though mastitis was more
213 severe in the MOK124 group (Fig. 5). Instead, a specific response to MOK124 was evident at later time
214 points, characterised by overrepresentation of the Hippo signalling, extracellular matrix receptor
215 interaction (ECM) and tight junction pathways. The Hippo signalling pathway is involved in regulating
216 cell apoptosis or proliferation (36), while ECM receptor and tight junction pathways both represent
217 groups of genes that influence communication between epithelial cells and maintaining the integrity
218 of the epithelial cell layer (37, 38). When examining genes involved in the Hippo signalling pathway,
219 the majority of genes were downregulated relative to time 0 in response to MOK124 (Fig. 6A).
220
221 The ECM receptor interaction pathway was enriched in the MOK124 group at 48 hpi. Among ECM
222 receptor interaction genes, all collagen DE genes were downregulated in response to MOK124
223 between 48 and 168 hpi and included *COL4A1, COL4A2, COL6A1, COL6A2, COL8A2, COL12A1, COL14A1*

224 and *COL18A1* (Fig. 6B). The collagen genes encode proteins that are components of basement
225 membranes. Several integrin genes were upregulated in response to MOK124 at 48 hpi (*ITGA5, ITGA9,*
226 *ITGA2B, ITGB7*), while some were also downregulated between 48 and 168 hpi (*ITGA2, ITGA3, ITGB4,*
227 *ITGB6*).
228
229 For the tight junction pathway, in response to MOK124, most paracellular space genes were
230 downregulated, including genes encoding claudin (*CCLN*), occludin (*OCLN*), Marvel domain-containing
231 protein 3 (*MARVELD3*) and immunoglobulin superfamily member (*IGSF5*) (Fig. 6C). Several genes from
232 this pathway were upregulated at 24 hpi; however, the pathway was not significantly enriched in the
233 MOK124 group at that time point. Only two genes were upregulated at later time points: *ROCK1* was
234 upregulated at 48 hpi and *MYH1* was upregulated at 72 hpi, both of which are involved in regulation
235 of actin cytoskeleton. All other genes, involved in cell polarity (*PARD6B, TJP3, PATJ, AMOT, PRKCZ,*
236 *LLG2, TJP1, DLG3*), decreased paracellular permeability (*TJP1, CGN, CGNL1, CLDN3*), reduced cell
237 proliferation (*YBX3, CCND1*), cell differentiation (*YBX3, ERBB2*) and tight junction assembly (*RAB13,*
238 *TJP1, CGN*) were downregulated between 48 and 168 hpi.
239
240 While pathways specific to MOK124 emerged from 48 hpi, the tuberculosis pathway was solely
241 overrepresented in response to MOK023, at 24, 48 and 72 hpi. Notably, among the upregulated genes
242 in this pathway were *CTSD*, *ATP6A1* and *LAMP1*, which encode cathepsin D, ATPase H+ transporting
243 accessory protein 1 and lysosomal associated membrane protein 1, respectively. All of these proteins
244 are involved in internalisation into macrophages and arrest of phagosome maturation. Other
245 pathways specific to MOK023 were inflammation related pathways, such as inflammatory bowel
246 disease (IBD) pathway and rheumatoid arthritis pathway.
247

248 ### Gene ontology

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

254

255 Immune system processes were overrepresented in the MOK023 group at 24 and 48 hpi, while they
256 were only significant in MOK124 group at 24 hpi. While the GO terms in the MOK023 group varied
257 across time points, a small number were consistently overrepresented in the MOK124 group between
258 48 and 168 hpi. These included extracellular matrix organisation, wound healing and cell junction
259 organisation. Additionally, cell death was an enriched process in both groups at 168 hpi.

260

261 ## Immune cell populations

262 A significantly higher proportion of neutrophils was observed in milk from cows in the MOK124 group
263 than in the MOK023 group (25). In order to assess whether immune cell proportions in the milk
264 somatic cell samples could be inferred using transcriptomic data, a cell population analysis based on
265 gene expression of cell markers was performed using quanTIseq (39). The cell proportions at 0 hpi
266 were similar between the two groups, with most cells classified as "other" likely representing
267 mammary epithelial cells (Fig. 8A). Genes for epithelial cell markers such as cytokeratins 14, 18 and 19
268 (*KRT14, KRT18, KRT19*), E-cadherin (*CDH1*) and epithelial cell adhesion molecule (*EPCAM*) (40, 41)
269 showed the highest expression at 0 hpi and decreased post-infection (Fig. 8B). Immune cell
270 populations predominated at later time points and immune cell proportions differed between the two
271 groups (Fig. 8A). At 24 hpi, neutrophils represented the largest proportion of milk somatic cells in both
272 MOK023 and MOK124 infected animals; however, in the MOK124 group neutrophils represented a
273 greater proportion of somatic cells than in the MOK023 group. A major difference in cell proportions

274 was observed between 48 and 168 hpi. In the MOK023 group, the largest proportion of cells between
275 48 and 168 hpi were M1 macrophages, whereas in the MOK124 group neutrophils were the
276 predominant cell type. A slightly larger proportion of M2 macrophages could also be observed at each
277 time point post-infection in the MOK124 group when compared to the MOK023 group. M2
278 macrophages seemed to increase in proportion in both groups over time.
279

280 Milk secretion of chemokines and cytokines

281 Cytokine concentration was measured in milk at 0, 7, 24, 48, 72, 168, 336 and 504 hpi in order to
282 reflect both the acute and chronic response to infection. There were no differences in the
283 concentration of any of the examined cytokines or chemokines between the MOK023 and MOK124
284 groups prior to intramammary challenge or after 168 hpi. Protein concentrations increased post-
285 infection in both groups with a peak expression at 168 hpi, except for TNFα and IL-8 which
286 demonstrated a sharp increase at 24 hpi. A significant group x time interaction ($P < 0.05$) was observed
287 for IL-1α, IL-1β, IL-1RA, and IL-6, which are typical pro-inflammatory cytokines, but also IL-2, IL-4, IL-
288 10 and CCL3 (Fig. 9). In the case of all the above cytokines, MOK124 caused significantly higher protein
289 secretion at 168 hpi, which is also a time point at which the difference in milk yield was low.

290 ## Discussion

291 In this study, the transcriptional response of milk somatic cells to two genotypically distinct strains,
292 representing two important globally distributed bovine-adapted lineages of *S. aureus,* was assessed.
293 Differences in the response to the 2 strains were found in gene expression patterns and in
294 overrepresented pathways. An initial immune response to both strains was evident but the
295 transcriptional immune response was sustained in the MOK023 group between 24 and 72 hpi, while
296 immune genes were only significantly upregulated in the MOK124 group at 24 hpi. This may indicate
297 a difference in disease progression or a response to different virulence strategies employed by the
298 two strains. The expression of genetic markers of various immune cells also differed in response to
299 each strain, indicating differing immune cell proportions among somatic cells recruited in response to
300 each strain, a result that was confirmed by microscopy (25). More neutrophils were recruited in
301 response to MOK124 from 48 hpi and there were proportionally more M1 macrophages in the
302 MOK023 group.

303

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

316 point could represent the end of the acute innate immune response in both groups, since immune
317 pathways were not significantly enriched at this time point and few genes involved in the NF-kB and
318 TNF signalling pathways were upregulated in either group.
319
320 The transcriptional response observed in this study supported several findings of the corresponding
321 *in vivo* infection, such as the larger reduction in milk yield, higher inflammatory response at 24 hpi and
322 higher SCC in the MOK124 group, as well as the increase in the proportion of neutrophils in milk
323 observed in the MOK124 group. The downregulation of expression of milk production genes in the
324 MOK124 group coincided with a significant reduction in milk yield, which was observed in response to
325 MOK124 between 24 and 144 hpi when compared to MOK023 (25). This downregulation also indicates
326 that infection with MOK124 caused a greater disruption to the epithelial layer of the mammary gland,
327 possibly due to epithelial cell dysfunction and sloughing, when compared to MOK023 infection.
328 Overall, the cellular composition analysis indicated that the strain-specific response was mediated by
329 different cell populations. M1 macrophages were mainly involved in the response to MOK023, while
330 the response to MOK124 led to a higher neutrophil recruitment. There was no significant difference
331 between the MOK023 and MOK124 groups in the proportion of large mononuclear cells by
332 microscopy; however, large mononuclear cells would have been comprised of M1 and M2
333 macrophages as well as monocytes, and shifts in those respective populations could be observed when
334 looking at expression of cellular markers. The transcriptomic data therefore has the potential to
335 provide more detail on the proportions of different cell populations in milk, especially in species where
336 antibodies to cell markers are not available for the fine characterization of these cell subpopulations.
337
338 A larger proportion of M2 macrophages in somatic cells from the MOK124 group may indicate a trend
339 towards tissue repair. M2 macrophages synthesise polyamines and proline which stimulate cell
340 growth, collagen formation and tissue repair (42). M2 macrophages are also capable of producing ECM
341 components (43) and may have contributed to the ECM related pathways which were

342 overrepresented in response to MOK124. Therefore, it is possible that MOK124 creates high epithelial
343 dysfunction, which exposes the stroma to bacteria and bacterial products and induces a strong
344 inflammation that the organism attempts to repair. This leads to a higher recruitment of neutrophils
345 and more plasma leakage due to failure of the epithelium to function as a barrier.
346
347 Pathway analysis revealed that MOK124 elicited a strain-specific response indicative of tissue injury,
348 as illustrated by the downregulation of Hippo signalling, ECM receptor interaction and tight junction
349 pathways between 48 and 168 hpi. Moreover, MOK124-specific GO terms identified at those time
350 points, such as wound healing and ECM organisation, corresponded to the KEGG pathways identified.
351 These pathways are well studied in humans. The key genes in the Hippo signalling pathway are *YAP1*
352 and *WWTR* which encode transcription factors YAP and TAZ. A successful activation of this pathway
353 depends on translocation of the YAP/TAZ proteins to the nucleus, followed by binding of the
354 transcription factors to a TEAD protein. The YAP/TAZ-TEAD complex causes activation of
355 transcriptional responses which result in cell proliferation by controlling the expression of genes
356 directly involved in the cell cycle and by upregulation of anti-apoptotic genes (36). The genes involved
357 in the Hippo signalling pathway were downregulated, which could indicate a decrease in cell
358 proliferation. However, protein interactions and localisation in the cell would need to be studied in
359 order to confirm this hypothesis.
360
361 The ECM receptor interaction pathway is involved in maintenance of cell shape and the interaction of
362 epithelial cells with the basement membrane. Within the ECM receptor interaction pathway, several
363 integrin genes were upregulated in response to MOK124 at 48 hpi (*ITGA5, ITGA9, ITGB7, ITGA2B*). The
364 integrin proteins mediate cell to matrix and cell to cell adhesion (44). *ITGA5* encodes the light and
365 heavy chains that comprise the α5 subunit. This subunit associates with the β1 subunit to form a
366 fibronectin and fibrinogen receptor. Both fibronectin and fibrinogen can mediate adhesion of *S.*
367 *aureus* to host cells (45). The integrin α9 subunit encoded by the *ITGA9* gene, when bound to the β1

368 chain, forms an integrin that is a receptor for vascular cell adhesion molecule 1 (VCAM1), cytotactin
369 and osteopontin, all of which are involved in immune response. *ITGB7* encodes integrin subunit β7
370 which forms dimers with an α4 chain or an αE chain and plays a role in leukocyte adhesion (44). These
371 two upregulated integrin genes could indicate leukocyte adhesion and migration was taking place in
372 MOK124 infected animals at 48 hpi. Integrins can also act with cytoskeleton proteins from the tight
373 junction pathway and clustering of integrins increases cellular tension, which can lead to the activation
374 of the Hippo signalling pathway (44).
375
376 Tight junctions form barriers between cells to the flux of ions and molecules. Pore and leak pathways
377 can be differentiated within the action of the tight junction, and while claudins are involved in the
378 pore pathway, zonula occludens 1 (ZO-1) and occludins are involved in the leak pathway (46). In the
379 MOK124 group, claudin (*CCLN*), occludin (*OCLN*), Marvel (*MARVELD3*) and immunoglobulin
380 superfamily member (*IGSF5*) genes were downregulated as part of the tight junction pathway, which
381 suggests a decrease in cell to cell interaction and cell detachment. TNF-α has been shown to induce
382 barrier loss in epithelial cells during Crohn's disease *in vitro* and in mice. The loss of integrity of
383 intestinal epithelium in those studies was mediated by TNF-induced occludin internalisation (46).
384 Therefore, upregulation of the TNF pathway in the MOK124 group at 24 hpi could have contributed
385 to loss of cell integrity at later time points. On the other hand, dysregulation of cytoskeleton genes in
386 the tight junction pathway could be indicative of *S. aureus* internalisation into host cells. Modification
387 of the actin cytoskeleton in cells infected by *S. aureus* has previously been described (23) and was
388 attributed to host cell internalisation. A CC151 strain, RF122, has been hypothesised to be adapted to
389 an intracellular niche (18). *In vitro,* strain MOK023 was a significantly higher internaliser in bMEC than
390 strain MOK124 (29). The cytoskeleton reorganisation in response to MOK124 is therefore more likely
391 to be related to cell damage, loss of cellular structure and an attempt to repair the tissue rather than
392 due to host cell internalisation. The presence of clinical signs in MOK124 challenged animals (25) also
393 confirms that cell damage likely took place and a healing response would be necessary to rebuild the

394 damaged tissue. The ability of MOK124 to cause tissue damage may be associated with the toxin
395 encoding genes present in this lineage. MOK124 encodes enterotoxin genes *seg, sei, selm, seln, seln,*
396 *selo, egc, selu* and *ORFCM14* as well as the leukocidins *lukF-PV* and *lukM*, none of which are present
397 in MOK023 (17). While LukM' was not found in milk of MOK124 infected cows in this study, the other
398 toxins encoded by this strain could play a role in the virulence of this strain. Other CC151 strains have
399 been found to produce low amounts of LukM' in previous studies (47), therefore the mechanism of
400 neutrophil killing by CC151 strains remains to be determined. Since immune genes tended to be
401 upregulated for longer in response to MOK023 than in response to MOK124, the greater influx of
402 neutrophils in response to MOK124 is unlikely entirely due to immune signalling by milk somatic cells.
403 Instead, a larger influx of neutrophils could be related to cellular and tissue damage.
404
405 Although MOK023 caused immune gene induction both in bMEC *in vitro* and in milk somatic cells *in*
406 *vivo* (25, 29), immune signalling from bMEC was followed by a temporary recruitment of neutrophils
407 at 24 hpi, and M1 macrophages became the predominant cell type from 48 hpi. Since macrophages
408 are normally present in a healthy mammary gland, while neutrophils tend to be recruited to a site of
409 intramammary infection (48), the difference in cellular composition in milk post-infection in response
410 to the two strains may indicate that MOK023 subverted the immune response which failed to recruit
411 neutrophils to a significant extent. This is also supported by the fact that even though immune gene
412 expression was sustained in response to MOK023, bacterial load increased in the MOK023 infected
413 animals, to reach its maximum at 168 hpi. Lack of inflammation and relatively low neutrophil numbers
414 in response to MOK023 could be due to host cell internalisation. It was demonstrated that MOK023
415 can internalise into bMEC *in vitro* (29). It has also been reported that human-associated *S. aureus* can
416 internalise and survive within macrophages (49) and internalisation of MOK023 into MEC and/or
417 macrophages could aid in the evasion of immune response. Interestingly, among significantly DE
418 genes, *LAMP1* was found to be overexpressed solely in response to MOK023 (Supplementary File 2).
419 This gene has previously been associated with *S. aureus* survival inside a mature phagosome in

420 macrophage cell lines (50, 51). However, no studies to date have examined the internalisation of *S.*
421 *aureus* into bovine macrophages. Upregulation of the tuberculosis pathway in the MOK023 group
422 further supports the hypothesis that MOK023 may internalise into host cell macrophages and be
423 immune evasive. The tuberculosis pathway represents a process where tubercle bacilli enter
424 macrophages in the lung, and following host cell internalisation they interfere with phagosomal
425 maturation, antigen presentation, apoptosis and host bactericidal responses to establish persistent or
426 latent infection (52). Persistence of infection of MOK023 was illustrated by a consistent bacterial load
427 in infected animals over 30 days; however, more research is needed to determine the precise
428 mechanisms by which this strain persists within the host. Notably, a recent study associated high
429 internalisation into bMEC, biofilm formation and low cytotoxicity with high within-herd prevalence of
430 a *S. aureus* CC9 strain (53).
431
432 The transcriptomic response to *S. aureus* mastitis in dairy cows has been studied previously with
433 samples taken at 3 hpi (23, 27) and 24 hpi (28). Udder biopsies were used in those studies to analyse
434 gene expression in response to a *S. aureus* IMI. This study characterises later events occurring in IMI,
435 and represents a response of different cell populations, as it would be expected that an udder biopsy
436 would be mostly comprised of bMEC. This is the first transcriptomics study of *S. aureus* mediated IMI
437 where the response to two different strains of *S. aureus* are compared, with full strain genotype
438 information available. Genotyping of bacterial strains used, use of multiple strains of *S. aureus* as well
439 as examination of the cellular populations of studied samples should be considered in future studies.
440 All of the above can ensure better understanding of strain-specific responses and an improved ability
441 to compare different studies of IMI.
442
443 The concentration of selected cytokines and chemokines in milk throughout the course of infection
444 confirmed and extended the finding that MOK124 induced higher IL-1β and IL-8 concentrations in
445 milk. A discrepancy was found between the gene expression results and the cytokine profiles since

446 cytokine and chemokine protein levels remained elevated in response to MOK124 at later time points,
447 while most of the cytokine genes were not DE post 24 hours in this group. While correlation between
448 transcriptomic and proteomic data is often poor (54-56) due to protein turnover, RNA degradation or
449 post-translational modifications, there could be other reasons for the discrepancy in this study. Firstly,
450 the protein levels were measured in milk, while the transcript levels were measured in milk somatic
451 cells. The mammary epithelial cells lining the bovine mammary gland could be contributing to cytokine
452 secretion into milk, and would not be captured by the RNA sequencing. Therefore, mammary
453 epithelial cells and other cells of the epithelial lining such as resident macrophages and lymphocytes
454 could be major contributors to cytokine and chemokine levels in the milk. Secondly, the number and
455 the type of milk cells likely influenced absolute protein concentration with higher neutrophil counts
456 in MOK124 infected cows. Finally, a systemic response could have contributed to increased milk
457 cytokine levels as exudation of plasma was likely in the MOK124 group with higher milk Ig
458 concentrations, but not evident in the MOK023 group (25). The milk cytokine data highlights the need
459 to implement proteomic studies alongside transcriptomic studies to better understand the molecular
460 mechanisms of host immune response. A study where each milk somatic cell type was assessed
461 separately during an *in vivo* trial, along with udder biopsies or post-mortem examinations at one pre-
462 defined time point, would be beneficial to further understanding of bovine immune response to *S.*
463 *aureus*. Furthermore, the pattern of cytokine expression measured *in vitro* on MEC culture infected
464 with either strain (29) does not correlate with the secretion profile detected in milk upon mammary
465 gland infection of dairy cows, showing that bacteria-MEC interactions do not summarize the complex
466 mechanisms occurring during mastitis and may not be predictive of the pathogenicity of *S. aureus*
467 strains.
468

469 ## Conclusions

470 This study further confirms the ability of bovine *S. aureus* strains to cause a strain-specific immune
471 response during IMI, with each strain resulting in a unique signature of infection in terms of the

472 number and type of immune cells recruited, the biological function of the recruited cells and the
473 resulting cytokine and chemokine concentration in milk. MOK124 caused more inflammation and
474 udder damage, as illustrated by the disruption of tight junction and Hippo signalling pathways
475 between 48 and 168 hpi, coupled with clinical signs observed in the infected animals. On the other
476 hand, MOK023 caused less inflammation and may be able to limit neutrophil recruitment and hence
477 persist in the mammary gland, as suggested by the sustained immune gene expression but low
478 increase in SCC, sustained bacterial load and mild clinical signs in MOK023 infected cows. Since
479 differences in early transcriptional response between the two strains were observed, followed by
480 either a severe or a mild, persistent infection, it is possible that the magnitude of this early response
481 may be crucial in determination of the course of staphylococcal IMI. A very strong response may be
482 detrimental to the host, and virulence or inflammatory factors like toxins may be the cause of
483 pathogen persistence due to the disruption of the epithelial barrier and tissue lesions.
484
485 The response to each strain also involved recruitment of specific immune cells, with M1 macrophages
486 primarily recruited in response to MOK023, while neutrophils were the main immune cell type
487 recruited in response to MOK124. This indicates that different cell types as well as the transcriptional
488 activity of each cell type may influence gene expression differences between groups when analysing
489 mixed cell population tissues such as blood or milk. Further studies are necessary to further
490 understand the molecular mechanisms utilised by these strains to cause inflammation or evade the
491 immune response. These studies could include examination of toxin secretion by the strains under
492 specific conditions or analysis of transcriptomic data from the bacterial strains under infection
493 conditions. The effect of other strains on the host immune response and gene expression *in vivo*
494 should also be studied, both to confirm whether the type of response is related to a specific strain, ST
495 or CC, and to establish whether biomarkers common to all strains of *S. aureus* can be determined.
496

497 # Methods

498 ## Study design

499 The aim of this study was to elucidate the molecular mechanisms of the host immune response *in vivo*
500 by utilising a transcriptomic approach. Fourteen first lactation Holstein-Friesian cows were purchased
501 from two commercial farms which were leptospirosis, salmonellosis and Johne's disease free. Animals
502 were selected based on composite milk SCC < 100,000 cells/ml. Milk bacteriology was performed prior
503 to challenge to confirm lack of contaminating organisms or mastitis pathogens. Cows were infected
504 with approximately 500 cfu of *S. aureus* strain MOK023 (ST3170, CC97) or MOK124 (ST151, CC151) in
505 400 µl of PBS as described previously (25). Animals in which a *S. aureus* infection was not established
506 (two cows challenged with MOK023 and one cow challenged with MOK124) were excluded from the
507 study. Therefore, the MOK023 infected group included 5 animals, while the MOK124 infected group
508 included 6 animals. Due to severe clinical mastitis developed by 2 cows in the MOK124 group, these
509 animals were treated with antibiotic and milk samples for transcriptomic analysis were not taken after
510 antibiotic treatment: cow 1776 was removed from the trial at 31 hpi, while cow 1920 was removed at
511 120 hpi. A further sample from the MOK124 group at 48 hpi was removed from the analysis for quality
512 control purposes as a sample from an uninfected quarter was mistakenly sequenced instead of the
513 sample from the infected quarter. The number of animals in each experimental group at each time
514 point is presented in Table 2.

515

516 **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

517

518 ## RNA extraction from milk somatic cells

519 Milk from the infected quarter was collected into a sterile universal tube (Sarstedt). In order to obtain
520 a sufficient quantity of RNA, 100 ml of milk was used for somatic cell isolation. Milk was centrifuged
521 at 800 x g, at 15 °C for 10 min. The fat layer and supernatant were removed and tubes were placed
522 upside down on tissue paper to drain for 10 min. Cell pellets were subsequently resuspended in 900
523 µl of Qiazol (Qiagen) and vortexed for 20 s to lyse the cells. Lysed samples were stored in Qiazol at -
524 20 °C until RNA extraction. Samples were rapidly thawed at 37 °C and maintained at room temperature
525 (RT) for the duration of the extraction. RNA was extracted using an RNeasy Plus Universal kit (Qiagen)
526 as per the manufacturer's instructions. RNA was eluted in 30 µl of RNase-free water (Sigma).
527
528 RNA quality was assessed using 2100 Bioanalyser RNA Nano chips (Agilent) as well as with a
529 NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA quantity
530 was determined with a Qubit RNA broad range assay (Invitrogen). Sample RIN values ranged between
531 5.9 and 9.5 (mean RIN value 7.7) and concentrations ranged from 4.1 – 930 ng/µl (mean concentration
532 of 127 ng/µl).
533

534 ## Library preparation and sequencing

535 RNA was sent on dry ice to Macrogen (South Korea) for library preparation and sequencing. RNA
536 quality was confirmed on arrival using a TapeStation HSRNA Screen Tape and analysed on a 2200
537 TapeStation (Agilent). Library preparation was performed with 100 ng of RNA using a TruSeq Stranded
538 mRNA LT Sample Prep Kit (Illumina) according to the manufacturer's instructions (Part #15031047 Rev.
539 E). Library size was verified with a 2100 Bioanalyzer using a DNA 1000 chip (Agilent) and library
540 quantity was determined with qPCR according to the Illumina qPCR Quantification Protocol Guide. The
541 mean library fragment size was 348 bp while the mean library concentration was 92.63 nM. Libraries

542 were diluted and pooled with 300 pM of each library and sequenced (100 bp, paired-end) on a
543 NovaSeq (Illumina). Samples were divided across two sequencing runs.
544

545 Quality control, assembly and DE gene analysis

546 All the bioinformatics pipeline bash, Perl and R scripts used for computational analyses were deposited
547 in a GitHub repository at https://github.com/DagmaraNiedziela/RNAseq_Saureus_cattle_infection.
548 RStudio v1.1.463 and R v3.5.2 were used for the analyses. Sequence quality was assessed using FastQC
549 v11.5 (57). Trimming was performed using fastp v12.1 (58) with default settings as well as enabling
550 base correction for paired end data and enabling overrepresented sequence analysis; on average 0.5
551 % of the reads were trimmed per sample. Trimmed fastq files were re-checked with FastQC to confirm
552 sequence read quality. Trimmed sequences were mapped to the bovine genome (UMD3.1) using the
553 STAR aligner v2.5.2 (59), with gene counts generated simultaneously with the STAR software using the
554 UMD3.1 v93 of the *B. taurus* reference genome and annotation. Genes with zero read counts across
555 all samples as well as non-protein coding genes were removed in a filtering step (60). DeSeq2 v1.18.1
556 (61) was then used to visualise gene expression data and perform differentially expressed (DE) gene
557 analysis. Data was normalised with a vst function and visualised with a principal component analysis
558 (PCA) plot of the samples based on the 500 genes with the highest inter-sample variation. DE gene
559 lists were generated using a negative binomial generalized linear model. Wald tests were performed
560 to obtain DE genes separately for each group at each time point using time 0 as a reference, with P
561 values adjusted for multiple comparisons using a Benjamini and Hochberg (B-H) method. DE genes
562 with an adjusted P value < 0.05 and a $\log_2$ fold change threshold of 1 were used for further DE gene
563 data exploration and pathway analysis.
564

565 ## Pathway and gene correlation analysis

566 Significant (P adj < 0.05) and highly overexpressed (|$\log_2$ fold change|> 1) DE genes were converted
567 to their 1 to 1 human orthologs using BioMart, ordered by adjusted P value and analysed for
568 enrichment of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG)
569 pathways using gProfiler R package v0.6.6 (62). When a group of DE genes matched to multiple
570 pathways, the pathway that was represented by the highest number of DE genes was selected in a
571 filtering step.

572

573 ## Cell composition analysis

574 Transcripts per million (TPMs) were generated from raw gene counts. Bovine Gene IDs converted to
575 1 to 1 human orthologs were used for the analysis. To estimate the proportion of the different immune
576 cell populations in each somatic cell sample, a quanTIseq algorithm which is based on expression of
577 cell specific surface marker genes was used (39).

578

579 ## Quantification of a panel of cytokines and chemokines in milk

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

586

587 ## LukM ELISA

588 ELISA to detect the LukM subunit of the LukMF' Staphylococcal toxin in milk samples was performed
589 as described previously (33). Briefly, anti-LukM polyclonal bovine IgG (32 µg/ml) was used to coat the

590 wells of an ELISA plate at 4 °C overnight. The plate was emptied and washed twice with 300 µl of PBS-
591 T, blocked with 200 µl of 4 % skimmed milk powder in PBS-T for 30 min with shaking and washed once
592 with 300 µl PBS-T. A 100 µl aliquot of each sample (skimmed milk heated to 95 °C for 10 minutes to
593 inactivate any existing antibodies against LukM) or standard (recombinant LukM) was added to the
594 wells and incubated for 60 min at room temperature (RT) with shaking, followed by 2 washes with 300
595 µl PBS-T. Mouse anti-LukM monoclonal antibody (KoMa43, Podiceps, The Netherlands) was diluted to
596 3 µg/ml in 1 % skimmed milk-PBS-T and 50 µl was added to each well. The plate was incubated for 60
597 min at RT, with shaking, and washed 3 times. Polyclonal HRP conjugated goat anti-mouse IgG
598 (BioLegend) was diluted 1 in 1000 and 100 µl was added to each well. The plate was incubated for 60
599 min at RT, with shaking, and washed 3 times. TMB (100 µl) was subsequently added and incubated in
600 the dark at RT for 15 min, followed by addition of 100 µl of the stop solution (0.05 M H_2SO_4).
601 Absorbance was read at 450 nm using a microplate reader.
602

603 ## Statistical analysis

604 Data generated from cytokine and LukM' protein quantification was analysed with a repeated
605 measures ANOVA using a MIXED® procedure in SAS (SAS Version 9.4, SAS Institute, Cary NC, USA) to
606 compare strain-dependent differences. Strain, time and the strain x time interaction were included in
607 the model, with time as a repeated measure. Time 0 measurements for each cytokine were included
608 as a covariate, and all variable interactions were explored. Log_{10} transformations were used for all
609 cytokine variables to satisfy the distributional requirements of ANOVA. Correlations among repeated
610 measurements across time between strains were modelled using the compound symmetry covariance
611 structure for each parameter analysed. Tukey's post-hoc multiple comparison test was applied as
612 appropriate.
613

Declarations

Ethics approval

All procedures involving animals received approval from the University College Dublin Animal Research Ethics Committee (protocol number Leonard AREC 16 44), and a project authorisation from the Health Product Regulatory Authority (HPRA) (project authorisation number AE18982/P108).

Consent for publication

Not applicable.

Availability of data and materials

Raw fastq files used during the current study are available in the European Nucleotide Archive (ENA) repository with a project number [to be added].

Code used to analyse the data is available at: https://github.com/DagmaraNiedziela/RNAseq_Saureus_cattle_infection

Any other data from the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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638 ## Author contributions

639 DAN optimised and conducted the laboratory-based work, performed bioinformatics analysis of the
640 RNAseq data and drafted the manuscript.
641 OMK conceived the initial idea of the study and participated in its design and coordination.
642 FCL conceived the initial idea of the study and participated in its design and coordination.
643 PC provided mentoring and supervision during the analysis of RNAseq data and performed the cell
644 composition analysis.
645 GF conducted/supervised protein laboratory-based work and statistical analysis of protein data.
646 All authors helped to draft the manuscript and read and approved the final manuscript.
647

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655
656

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814 # Figures

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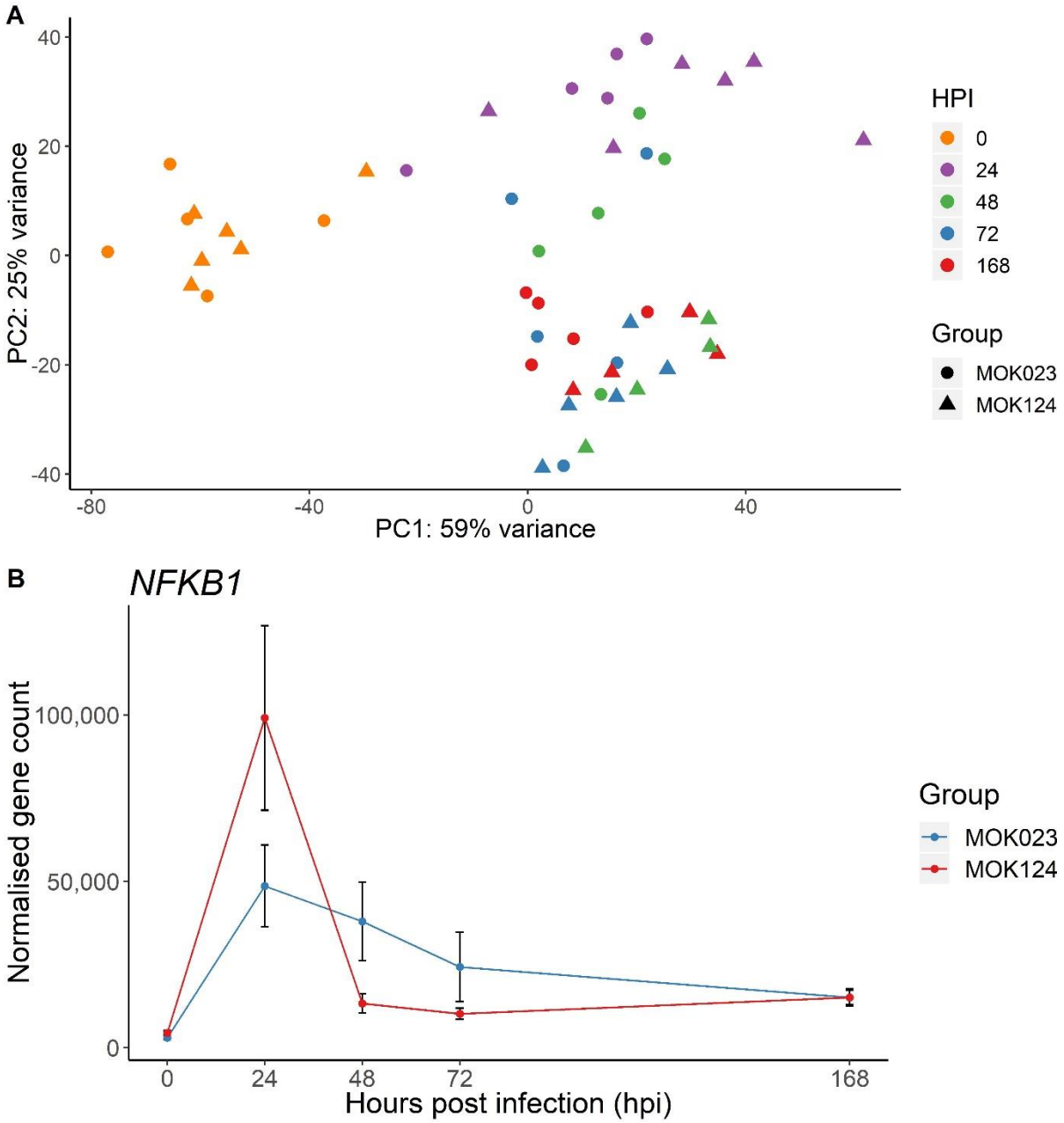

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817 **Fig. 1**. Results of exploration of raw counts data.

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

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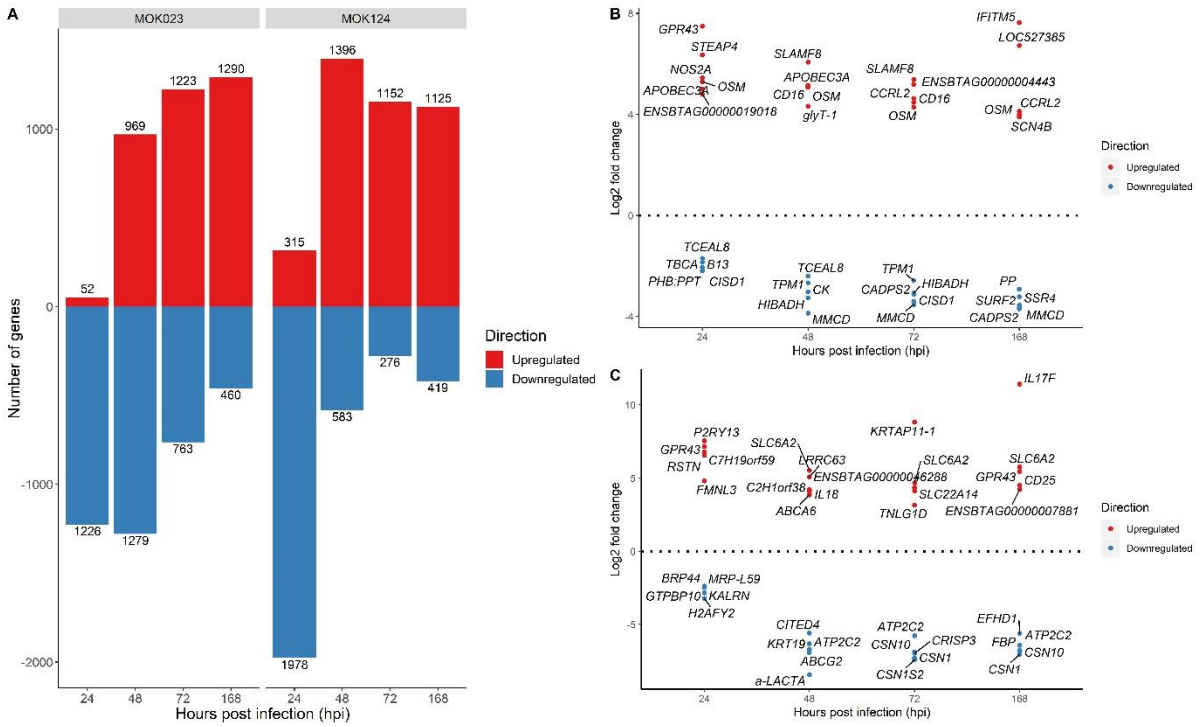

Fig. 2. Differentially expressed genes in response to *S. aureus* MOK023 and MOK124. **a** The number of significant DE genes (Benjamini-Hochberg adjusted P-value < 0.05, $\log_2$ fold change threshold > 1) which were up- (red) and downregulated (blue) in milk somatic cells in response to strains MOK023 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.

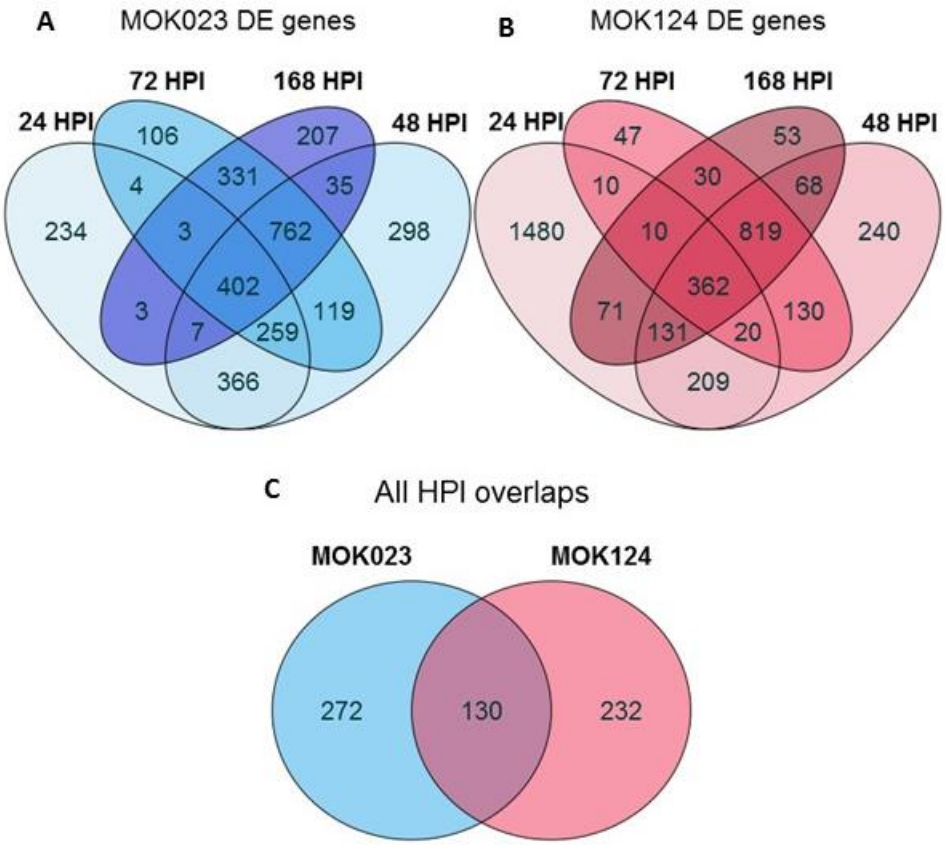

Fig. 3. Common DE genes in milk somatic cells across time points.
a Common DE genes in response to MOK023. **b** Common DE genes in response to MOK124. **c** Overlap of the DE genes common at all time points between the two strains. HPI - hours post infection.

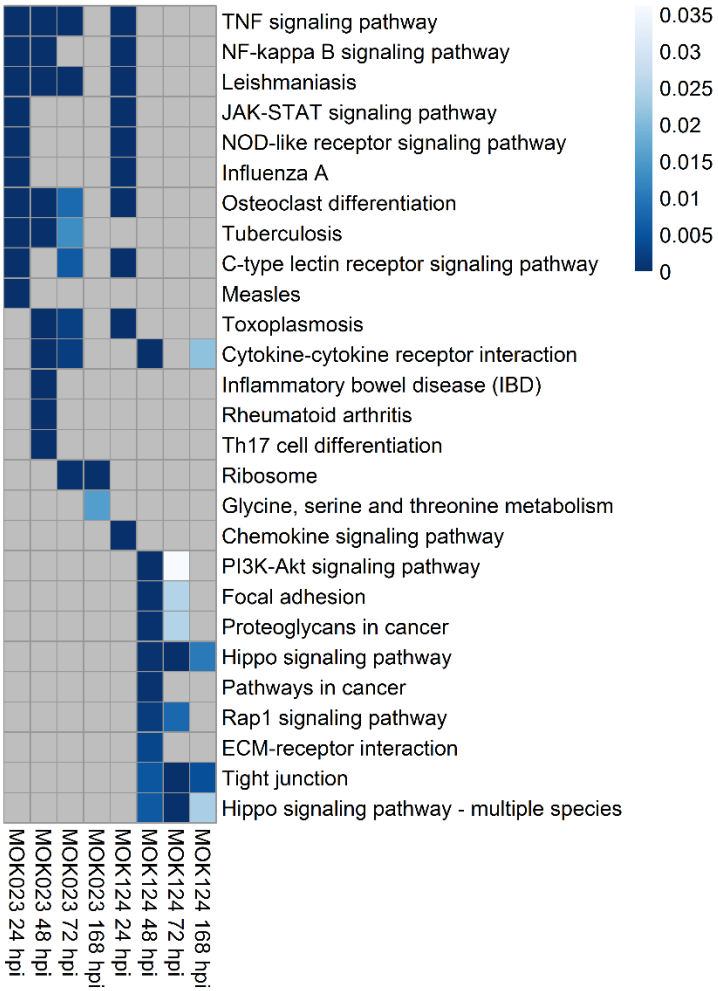

Fig. 4. KEGG pathways in response to infection with *S. aureus* MOK023 or MOK124 over time. Significant ($P < 0.05$) and unique (filtered for shared genes) are shown. Colour scale represents P value, grey squares represent non-significance. Up to 10 of the most significant pathways at each time point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

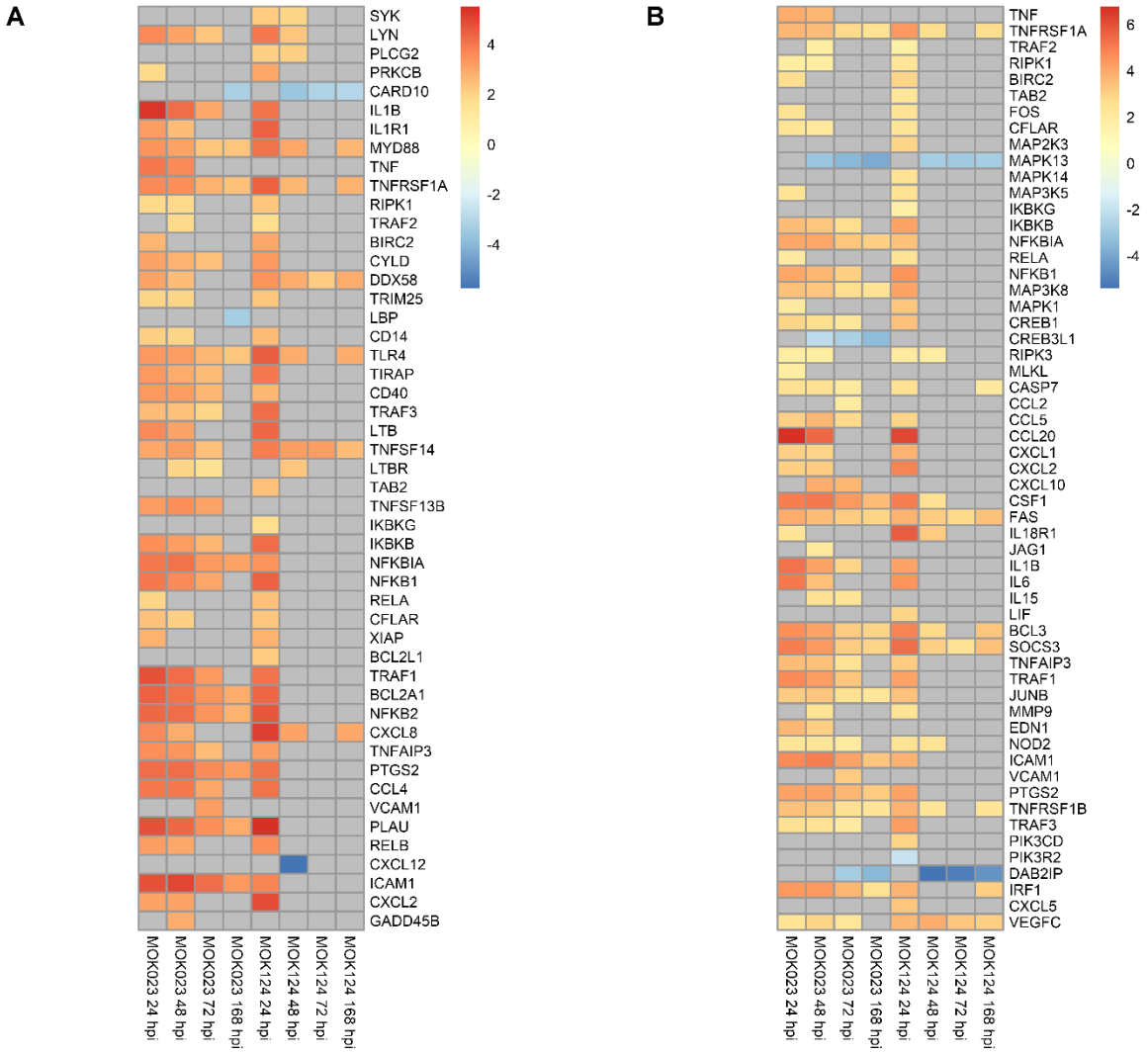

Fig. 5. Log_2fold change of genes involved in selected KEGG pathways in response to MOK023 and MOK124.

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

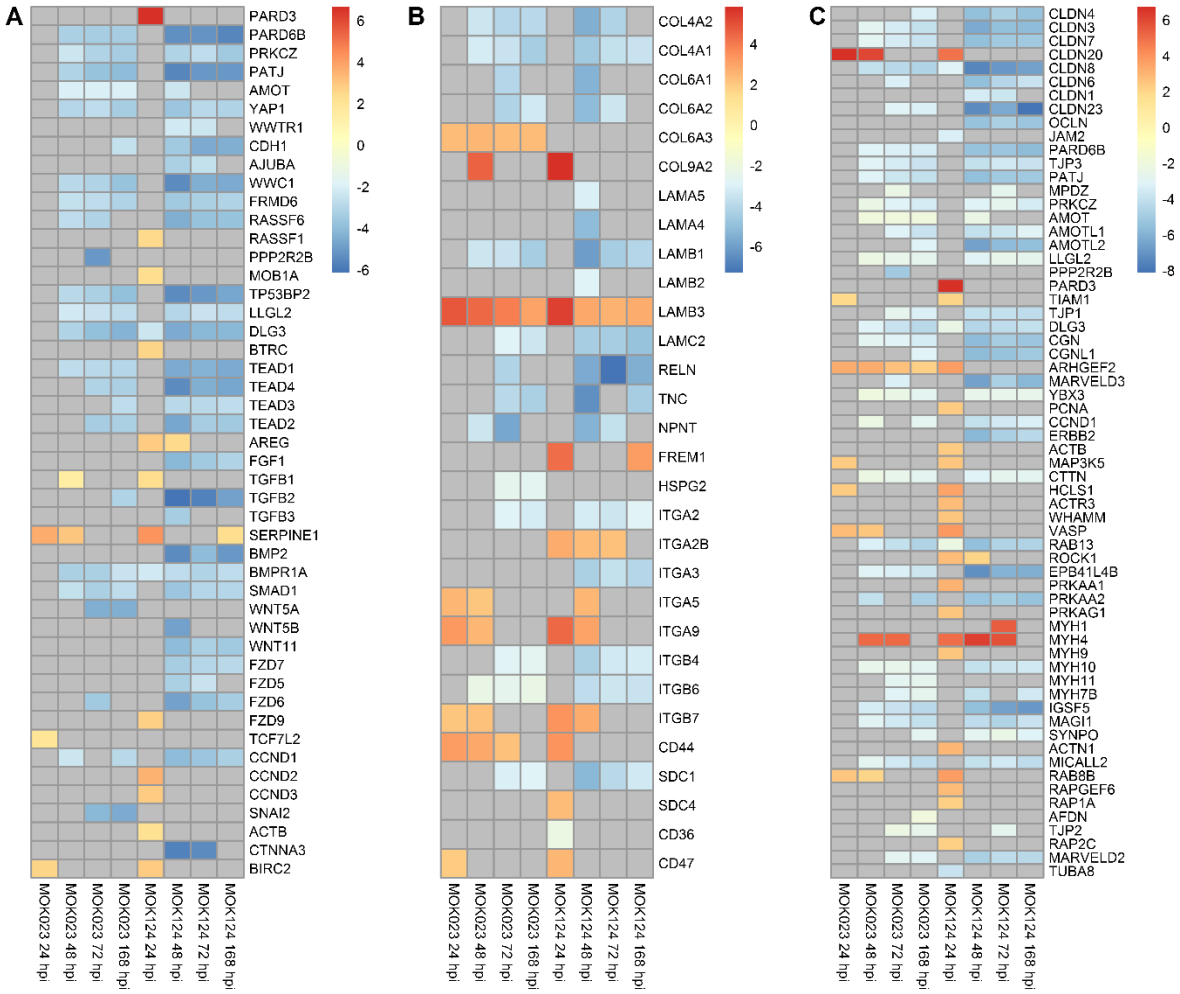

Fig. 6. Log_2fold change of genes involved in selected KEGG pathways in response to MOK023 and MOK124. **a** Hippo signalling pathway. **b** ECM receptor interaction pathway. **c** tight junction pathway. Grey tiles show genes that were not significantly DE.

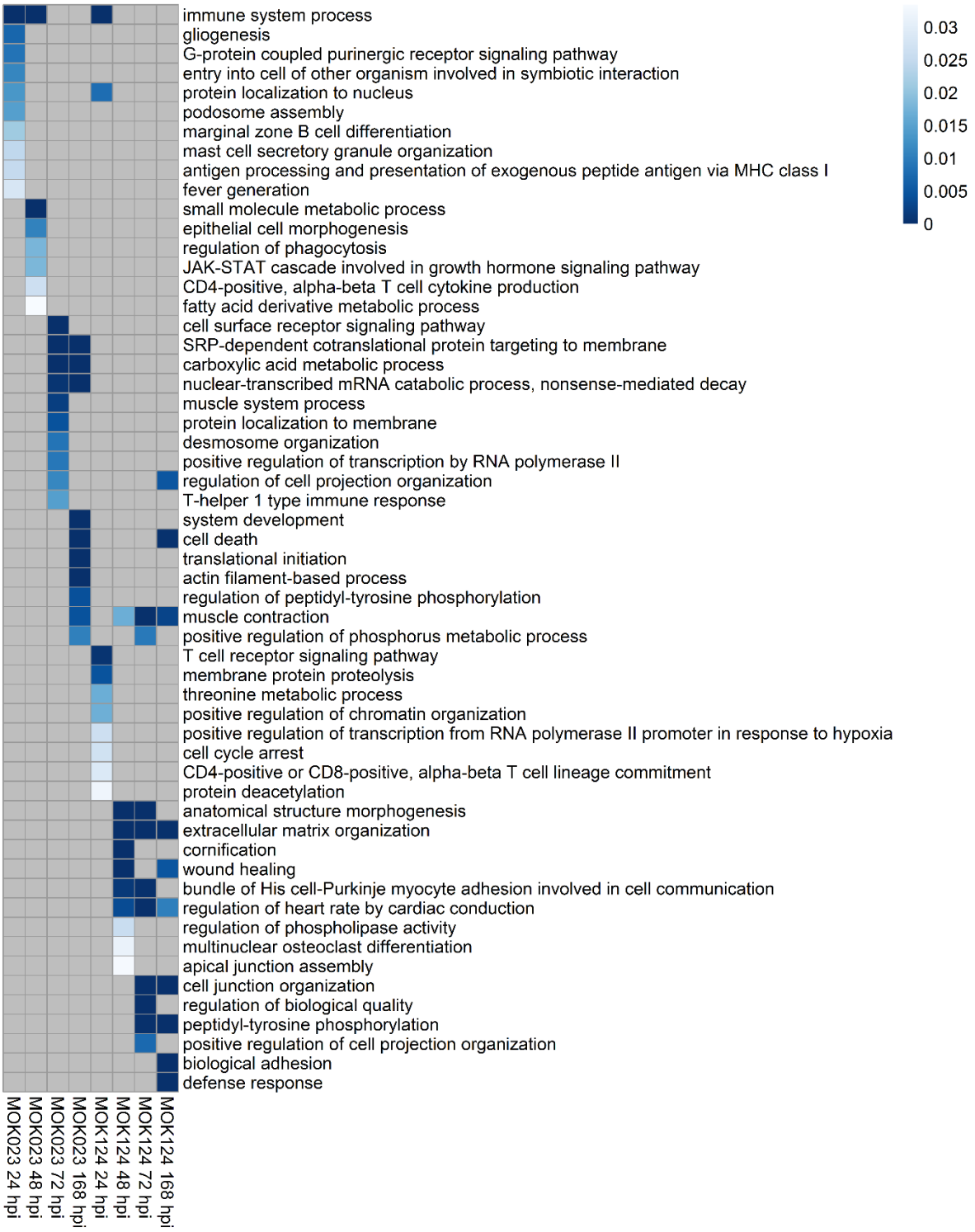

Fig. 7. GO biological processes in response to infection with *S. aureus* MOK023 or MOK124 over time. Top 10 most significant ($P < 0.05$) and unique (filtered for shared genes) are shown. Colour scale represents P value, grey squares represent non-significance. hpi - hours post infection.

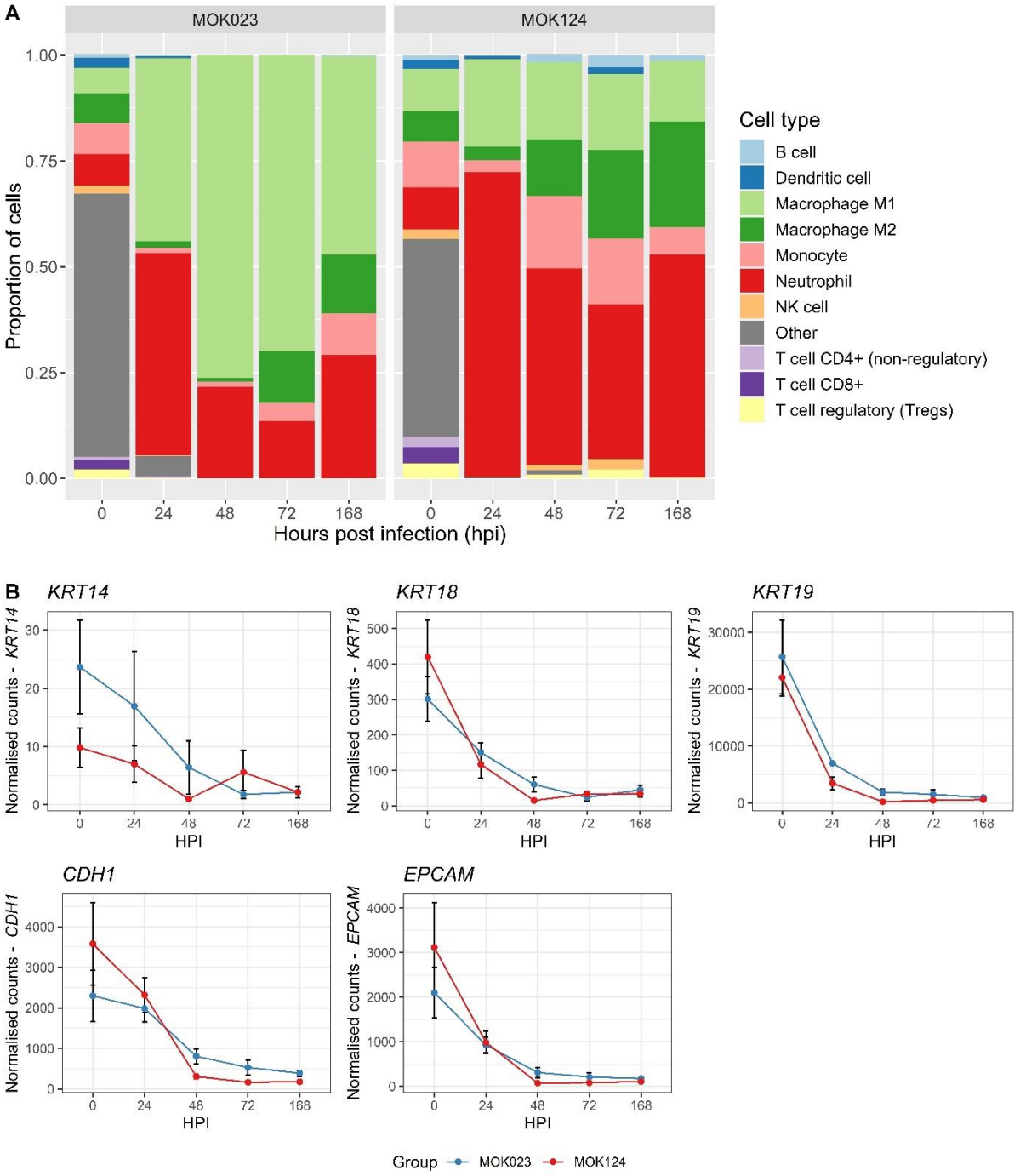

Fig. 8. Cell composition analysis.

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.

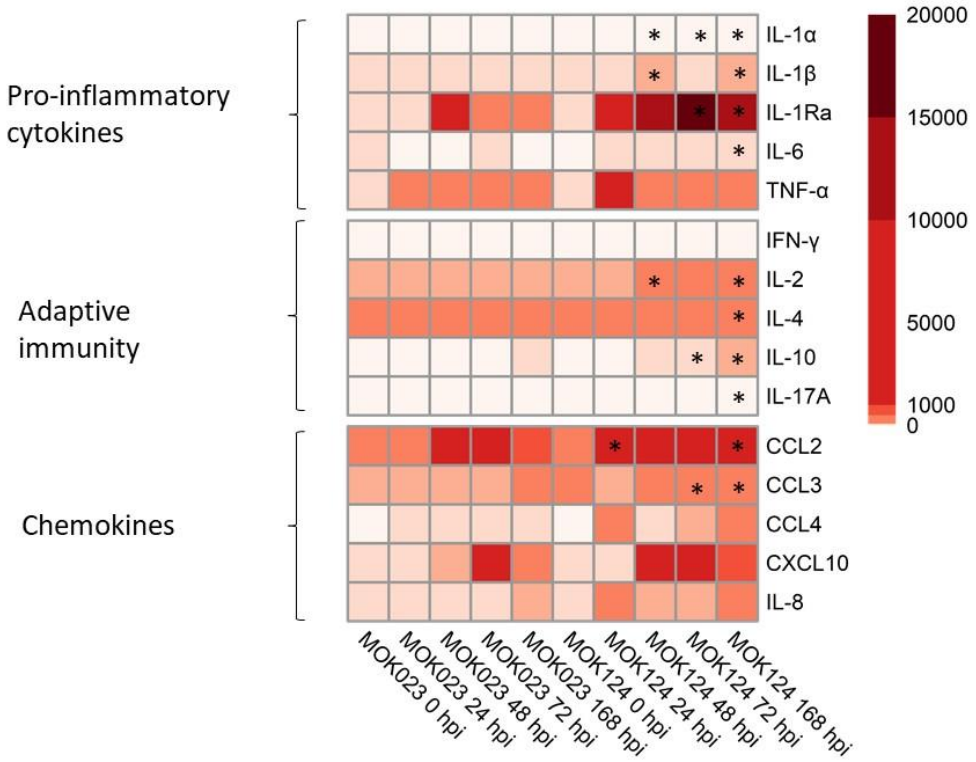

Fig. 9. Concentration of selected immune markers in milk.
The immune markers are divided into: pro-inflammatory cytokines: IL-1α, IL-1β, IL-1RA, IL-6, TNFa; cytokines involved in adaptive immunity: IFNγ, IL-2, IL-4, IL-10, IL-17A; and chemokines: CCL2, CCL3, CCL4, CXCL10, IL-8.
Colour scale represents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