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Comparison of the immune responses associated with experimental bovine mastitis caused by different strains of *Escherichia coli*

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We studied the mammary immune response to different mammary pathogenic *Escherichia coli* (MPEC) strains in cows, hypothesising that the dynamics of response would differ. *E. coli* is a major aetiologic agent of acute clinical bovine mastitis of various degrees of severity with specific strains being associated with persistent infections. We compared challenge with three distinct pathogenic MPEC strains (VL2874, VL2732 and P4), isolated from different forms of mastitis (per-acute, persistent and acute, respectively). A secondary objective was to verify the lack of mammary pathogenicity of an environmental isolate (K71) that is used for comparison against MPEC in genomic and phenotypic studies. Twelve cows were challenged by intra-mammary infusion with one of the strains. Cellular and chemokine responses and bacterial culture follow-up were performed for 35 d. All cows challenged by any of the MPEC strains developed clinical mastitis. Differences were found in the intensity and duration of response, in somatic cell count, secreted cytokines (TNF-α, IL-6 and IL-17) and levels of milk leucocyte membrane Toll-like receptor 4 (TLR4). A sharp decrease of TLR4 on leucocytes was observed concomitantly to peak bacterial counts in milk. Intra-mammary infusion of strain K71 did not elicit inflammation and bacteria were not recovered from milk. Results suggest some differences in the mammary immune response to distinct MPEC strains that could be correlated to their previously observed pathogenic traits. This is also the first report of an *E. coli* strain that is non-pathogenic to the bovine mammary gland.

Keywords: Cytokine, *Escherichia coli*, leucocyte, mastitis, TLR4.

Mastitis is a leading cause of economic losses in dairy production, with *Escherichia coli* being a major aetiologic agent of mastitis in cows worldwide. Economic losses are due to decreased milk yield, reduced price owing to increased somatic cell count (SCC), treatment expenses, early dry-off and, in severe cases, culling (Seegers et al. 2003; Halasa et al. 2007; Hogeveen et al. 2011). Although mastitis caused by *E. coli* is acute, udder health and milk quality may remain impaired for a long period after clinical healing. Increased SCC, decreased fat and lactose levels and altered coagulation properties can be observed up to 63 d after an event of *E. coli* mastitis, even in the absence of detectable infection (Blum et al. 2014). At the industry level, delivery of low quality milk due to mastitis affects the production of dairy products (Le Maréchal et al. 2011; Leitner et al. 2011).

E. coli causes clinical mastitis with clear signs of inflammation in the affected gland ranging from mild to severe (Burvenich et al. 2003). Experimentally, inflammation is evident in just a few hours after *E. coli* intra-mammary injection. Bacterial inoculum as low as 50 colony forming units (CFU) leads to increased SCC in just 9 h and clinical symptoms in 18 h (Oliver et al. 2012). *E. coli* is promptly detected by the innate immune system in the mammary gland (Schukken et al. 2011; Wellnitz & Bruckmaier 2012). Binding of bacterial lipopolysaccharide (LPS) to Toll-like receptor 4 (TLR4) and CD14 triggers the release of immune-modulating cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, leading to rapid and massive polymorphnuclear cell (neutrophils, PMN) infiltration into the gland. In some cases, *E. coli* causes persistent infection of the mammary gland, resulting in recurrent or chronic mastitis (Döpfer

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et al. 1999; Bradley & Green 2001; Blum et al. 2014). Persistent infections are associated with *E. coli* strains adapted to invade and survive in mammary epithelial cells (Dogan et al. 2006; Almeida et al. 2011). Severity of *E. coli*-mastitis has been attributed mainly to host factors (Burvenich et al. 2003). We hypothesise that factors pertaining to the pathogen also influence the severity of mastitis.

The objectives of the present work were to compare the mammary immune response and course of disease elicited by different *E. coli* strains isolated from distinct types of mastitis, namely from per-acute (VL2874) and persistent (VL2732) mastitis. Strain P4 (Bramley 1976) was included for comparison, as it is widely used as a model strain in mastitis research. A secondary objective of this work was to verify the lack of pathogenicity in the bovine mammary gland of an environmental isolate (K71), which is used for comparison against MPEC in genomic and phenotypic studies aiming to elucidate MPEC virulence mechanisms.

Materials and methods

Bacteria

Three MPEC and one environmental strain were studied. Strain VL2874 was isolated from severe, per-acute mastitis and strain VL2732 was isolated from recurrent, persistent mastitis, which lasted for about 6 months (Blum et al. 2014). P4 was obtained from NCIMB (No. 702070) and propagated once before storage. Environmental strain K71, isolated from a cow shed is diverged from MPEC in phenotypes and genomic features that are putatively associated with pathogenicity in the mammary gland. This strain is non-pathogenic in murine mammary glands (Blum et al. 2008; 2015). K71 was used here to confirm that it is non-pathogenic in bovine mammary glands. All strains were previously genotyped and analysed by whole genome sequencing and phenotyping (Blum & Leitner 2013; Blum et al. 2015; Kempf et al. 2016). Bacteria were recovered from stock (−80 °C in brain heart infusion, 25% glycerol) on blood agar (Tryptose Blood Agar Base; Becton-Dickinson, Sparks, MD, USA, with 5% washed sheep erythrocytes) incubated aerobically at 37 °C overnight. Bacteria were harvested and washed in pyrogenic-free saline (PFS). Bacterial concentration was measured by plate counting. Bacteria were then suspended in PFS and stored at 4 °C for 10 h. Bacterial concentration was adjusted with PFS just before challenge, aiming at an inoculum of about 50 CFU per 3 ml. Final bacterial concentrations assessed from inoculum aliquots separated just prior to the challenge ranged between 10–30 CFU per 3 ml PFS.

Animals

Twelve Israeli Holstein cows entered the study. Cows were milked thrice daily in a dairy parlour equipped with a computerised AfiFarm Herd Management system and AfiLab milk analyser (Afimilk Ltd., Afikim, Israel), which provided on-line data on gross milk composition and conductivity as a measure for mastitis. Herd average milk yield was >11 000 L/305 lactation days. Animals were fed typical Israeli mixed ration (65% concentrate and 35% forage, 17% protein) *ad lib* in mangers located in the sheds. All mammary glands of the 12 cows were free of infection, based on three consecutive bacteriological examinations and with milk SCC <100 000 cells/ml. Lactation number ranged from 1–6 d in milk from 133–442, and daily milk yield 22·0–38·4 L/day. Cows were randomly assigned to four groups for intra-mammary challenge, each with a different *E. coli* strain. Groups were checked for balance according to lactation number, days in milk and milk yield and reviewed to avoid bias. Animal experiments were approved by the ARO Committee of Animal Experimentation and followed its ethical guidelines.

One gland per cow was challenged, while the other glands were combined (pooled into one sample from three glands) as a control. Strains VL2874 and VL2732 were challenged in three cows each. Strain P4 was used as a control, since it is widely used as a model strain for MPEC research in vivo and was therefore challenged in two cows. Because no previous information on the pathogenicity of strain K71 in cows was available, this strain was challenged in four cows. Before challenge, milk yield and composition, SCC and leucocytes distribution were evaluated at −2 d and day 0, separately for challenge and control glands. Intramammary inoculation was performed aseptically after morning milking. Teats were thoroughly cleaned, dried, disinfected with iodine and wiped with antiseptic cloths. Development of clinical symptoms was recorded during the first 24 h, including rectal temperature measurement every 4 h. Milk and blood samples were taken on challenge day before bacterial infusion (0), after 4, 8, 12, and 16 h and 1, 2, 4, 7, 10, 14, 21, 28 and 35 d post-challenge (DPC).

Sample collection and analyses

Samples were taken from challenged glands and pooled samples were taken from the control glands. For bacteriological tests, teats were cleaned, disinfected, foremilk was discarded and 3 ml milk was collected aseptically into sterile tubes. For the other tests, the challenged gland was milked into a separate container and milk volume was recorded. Milk was gently mixed and a sample of 0·5–1·0 L was collected for SCC, leucocytes differentiation and cytokines measurement (see below). Milk samples were kept refrigerated and transferred to the laboratory within 1 h. Blood was collected by venipuncture of the tail vein. Serum was separated by centrifugation and stored at −20 °C. Control glands were milked normally and milk yield was recorded.

One hundred µl of milk and serial 1:10 dilutions were streaked onto MacConkey agar plates for enumeration of *E. coli* colonies. Ten µl of milk was streaked onto blood agar for detection of other bacteria. Plates were incubated aerobically at 37 °C overnight. Two mL of milk collected

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aseptically were stored frozen for PCR detection of bacteria. SCC was performed using the Fossomatic 360 (Foss Electric, Hillerød, Denmark) at the Israel Cattle Breeders Association Laboratory (Caesarea, Israel). Leucocytes differentiation was performed by flow cytometry (FACs Calibur, Becton-Dickinson, San Jose, CA, USA) as described by Leitner et al. (2003) using the following anti-bovine monoclonal antibodies: anti-CD18/11a – BAT 75A (IgG-1) (total leucocytes), anti-CD4 – GC 50A1 138A (IgM) and anti-CD8 – CACT 80C (IgG-1) (T lymphocytes), anti-CD21 – BAQ 15A (IgM) (B lymphocytes), anti-CD14 – CAM 36A (IgG-1) (monocytes), anti-polymorphonuclear (PMN) (G1) (IgM) (VMRD Inc., Pullman, WA, USA), anti-TNF alexa-conjugated – MCA2334A488 (UgG_{2a}) (Serotec, Oxford, UK) and anti-TLR4 CD284 FITC-conjugated (IMG5031) (Ingenex, CA, USA). Secondary polyclonal antibodies were: TRI-COLOR (TC) labelled goat anti-mouse IgG-1 and FITC labelled goat anti-mouse IgM (CALTAG Laboratories, Burlingame, CA, USA). Tests were performed within 24 h after sample collection with milk stored at 4 °C.

Cytokines

Cytokines were measured in skim milk prepared by centrifugation at 1000 ***g*** for 20 min at 4 °C with the following ELISA assays, according to the manufacturer's instructions: IL-1β (ESS0027), IL-4 (ESS0031), IL-6 (ESS0029) (Thermo Scientific, Rockford, IL, USA), TNFα (VS0285B-002) and IL-17A (VS0284B-002) (VetSet, Kingfisher Biotech., St. Paul, MN, USA). TNFα was also measured in blood serum.

Molecular detection and identification of bacteria

Presence of bacteria in milk samples without positive bacterial cultures was assessed by PCR. DNA was extracted with Milk Bacterial DNA Isolation Kit (Norgen Biotek Corp., Ontario, Canada) following the manufacturers' protocol. Bacterial DNA was tested by PCR with universal 16S primers 4F and 801R as described by Blum et al. (2010). *E. coli* isolates in positive cultures were confirmed as being identical to the respective challenged strain by genotyping as described by Blum & Leitner (2013).

Statistical analysis

Statistical analyses were performed with JMP software (SAS Institute, 2000). The effects of strain, time and interaction between strain and time were evaluated by two-way ANOVA in a repeated measurements (split-plot) design. Experimental units were 'cow' (major) and 'time within cow' (minor). The statistical model was:

$$Y_{ijk} = \mu + \alpha_i + e^1 + \beta_j + \alpha\beta_{ij} + e^2_{ijk}$$

where: μ = mean of all data, α_i = difference between the mean of strain i from the trial mean, e^1 = variance between cows (Random Error), β_j = difference between the mean of time j from the trial mean, $\alpha\beta_{ij}$ = strain × time interaction, e^2_{ijk} = residual variance between measurements (Random Error). The strain effect was tested against variance between cows, and time and interaction effects were tested against residual variance. The same model was used to study changes within 24 h post-challenge. Statistical significance considered was $\alpha \leq 0{\cdot}05$.

Results

Clinical symptoms and milk yield

Rectal temperatures rose after 8 h (38·2 °C), peaked at 12 h (39·2 °C) and decreased after 16 h (38·2 °C) from bacterial infusion in cows challenged with strain VL2784. In cows challenged with strain VL2732, peak rectal temperature was observed 12 h from challenge (40·1 °C), remained high at 16 h (39·5 °C) and decreased after 24 h (38 °C). No significant changes in body temperature were noted in cows challenged with P4 or K71. Clinical symptoms, when observed, were limited to the challenged glands in all the cows. Of the cows challenged with VL2874, one showed swelling from 12 h to 7 DPC, and in two cows swelling remained until the end of the study. Of the cows challenged with VL2732, two showed swelling from 16 h to 2 DPC, and in one cow swelling of the gland persisted until the end of the study. In cows challenged with P4, one showed swelling from 16 h to 2 DPC. No clinical symptoms were observed in the four cows challenged with K71.

Milk yield was analysed as percentage from yield at day 0 (100%). Milk yield of the challenged glands was compared to the control glands combined in each cow (Fig. 1). All the glands challenged with any of the three MPEC strains showed decreased milk yield. The most accentuated decrease was observed in glands challenged with VL2874, followed by VL2732 and P4. Decreased milk yield in control glands was observed to a lesser extent and for a shorter period than those of the challenged glands. None of the glands challenged with VL2874 fully recovered during the study period, with approximately 60% loss in milk yield, compared to 20% loss in control glands. Milk yield loss in glands challenged with VL2732 and P4 was similar, about 40%, but control glands in cows challenged with VL2732 lost about 20% of its milk yield throughout the study. However, milk yield of control glands in cows challenged with P4 fully recovered within 10 DPC. Milk yield was unaffected in glands challenged with strain K71, with only one cow showing a mild decrease in milk yield until 2 DPC. No effect was observed in control glands in these cows.

Bacteriology

Bacteria of strains VL2874 and VL2732 were detected in milk after 4 h and of P4, after 8 h. Peak bacterial counts were observed between 16 and 24 h in glands challenged with MPEC. In glands challenged with VL2874, bacteria

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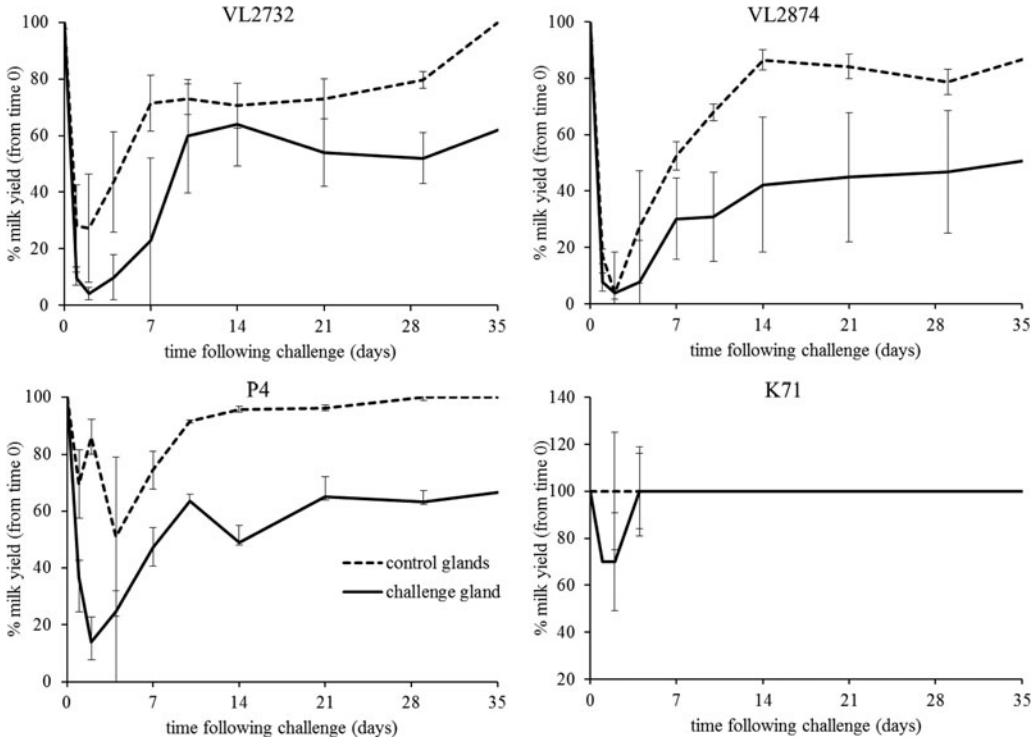

Fig. 1. Milk yield of cows challenged with different *E. coli* MPEC strains (VL2874, VL2732 and P4) and non-mammary pathogenic strain K71measured separately for challenged glands and pooled control glands, showing mean and SE. Milk yield is presented as percentage of milk yield from day 0 (100%).

were detected in milk up to 28 DPC and up to 7 DPC in those challenged with P4. VL2732 bacteria were detected in milk up to 28 DPC but were undetected at 10 and 14 DPC (Fig. 2a). Overall, bacteria were secreted in milk of glands challenged with VL2874 or VL2732 in higher numbers and for a longer time than P4, although differences were not statistically significant. Bacterial isolates from all cows at different points of time were genotypically identical to inoculum strains (data not shown). No bacteria were detected in milk from glands challenged with strain K71. No bacterial DNA was identified in samples without bacterial growth in the culture.

Immune response

No significant changes were observed in any of the parameters studied in the milk of all control glands in the MPEC challenged cows and in the glands challenged with strain K71. Therefore, the following refers only to glands challenged with the three MPEC.

Analysis of immune parameters that differed according to strain are presented in Table 1 and a full analysis of all parameters is given in Supplementary Material, Table S1. All the evaluated parameters changed significantly throughout the study in glands challenged with one of the three MPEC, except for IL-4 and blood TNF levels (Supplementary Material, Table S1). Differences in response to challenge by distinct MPEC strains were evaluated using the interaction of strain × time effects. Significant differences were found in $\log_{10}$ SCC, intra-cellular TNFα in PMN and IL-17. Significant differences were found in IL-6 and TLR4 levels within 24 h post challenge (Table 1 and Supplementary Material, Table S2).

Based on the sharp increase in SCC (Fig. 2b), onset of mastitis was already observed at 12 h from infusion in glands challenged with strain VL2732, and at 16 h in glands challenged with the other two MPEC strains. SCC decreased slowly in glands challenged with VL2874 and VL2732. At 10 DPC and thereafter, SCC was significantly lower in glands challenged with P4 and became comparable to SCC before challenge at 35 DPC. No significant increase in SCC in glands challenged with K71 was observed.

An acute immune response in glands challenged with VL2874 (PMN above 80%) was detected at 12 h after infusion and remained until the end of the study (Fig. 2c). In glands challenged with VL2732, the response was detected at 16 h, and P4 between 16 and 24 h. At 14 DPC, PMN decreased to less than 50% with VL2732 and to 20% with P4. The dynamics of leucocytes change is shown in details in Supplementary Material (Figs S1–S3). In glands challenged with strain K71, leucocytes levels did not increase following bacterial infusion and somatic cell types remained unchanged, comprised mainly of epithelial cells.

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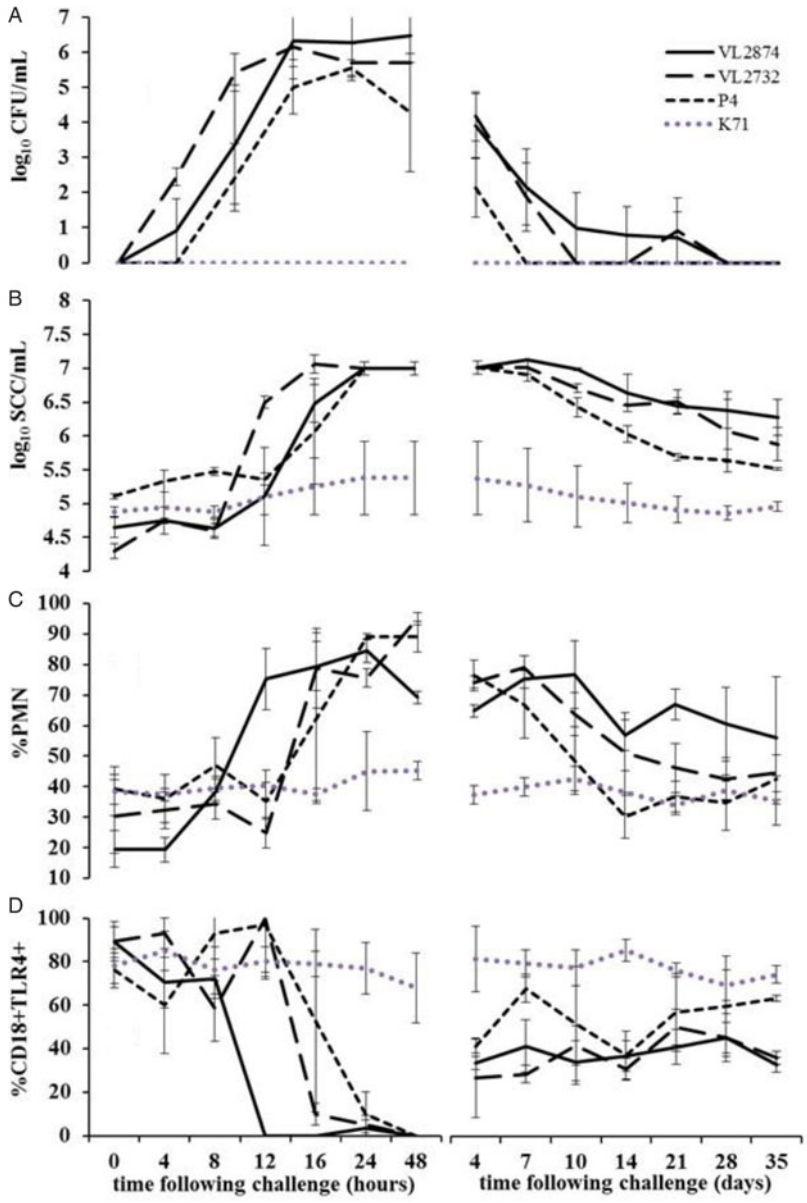

Fig. 2. Evolution of various immune function parameters in milk of glands challenged with different *E. coli* MPEC strains (VL2874, VL2732 and P4) and non-pathogenic strain K71 showing mean and SE within 48 h (left panel) and 35 d (right panel) following challenge. (a) bacterial secretion (Log_{10} CFU/ml: non-mammary pathogenic strain K71 was not recovered). (b) Somatic cell counts (Log_{10} SCC/ml). (c) Neutrophils (PMN) percentage. (d) TLR4-positive leucocytes ($CD18^{+}$).

The levels of secreted cytokines in milk followed an overall expected pattern. The dynamics of cytokines secretion in milk is depicted in Supplementary Material (Fig. S4). Peak TNFα was observed at 16 h, followed by IL-1β at 24 h and IL-6 at 48 h. A peak of IL-4 was recorded 8 h after bacterial infusion in glands challenged with VL2874 and VL2732, but not with P4. Specifically, in glands challenged with P4, the cytokine response was either lower (IL-4, IL1β, IL6) or delayed (TNFα) compared to glands challenged with the other MPEC strains, except for IL-17, for which significant higher levels in milk were recorded in glands challenged with P4. Peak IL-17 was recorded at 7 DPC, coinciding with peak CD4:CD8 ratio (Supplementary Material Fig. S3), and followed by a decrease in PMN percentage (Fig. 2c) and SCC (Fig. 2b).

TNFα was measured also as intra-cellular TNF by FACS. Intra-cellular TNF was detected in all leucocytes types but not in epithelial cells ($CD18^{-}$). Data presented for the total $CD18^{+}TNF^{+}$ cells is depicted in Supplementary Material (Fig. S5). Before challenge, intra-cellular TNF was detected on the average in approximately 70% of the leucocytes. In all the challenged glands, above 95% of the leucocytes

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Table 1. ANOVA analysis of parameters that were affected by *E. coli* MPEC strain and/or strain/time interaction at 24 h and during the whole study

		P		
Parameter	Period	Strain	Time	Interaction
Log_{10} SCC	24 h	n.s.	<0·001	<0·05
	35 d	n.s.	<0·001	<0·01
$CD14^+$ PMN	24 h	n.s.	<0·001	n.s.
	35 d	<0·05	<0·001	n.s.
$TLR4^+CD18^+$	24 h	n.s.	<0·001	<0·01
	35 d	n.s.	<0·001	n.s.
TNF^+ PMN	24 h	n.s.	<0·001	<0·05
	35 d	n.s.	<0·001	n.s.
TNF in blood	24 h	0·01	<0·001	n.s.
	35 d	0·01	n.s.	n.s.
IL-6	24 h	<0·05	<0·001	<0·01
	35 d	n.s.	<0·001	n.s.
IL-17	24 h	n.s.	<0·001	<0·05
	35 d	<0·01	<0·001	<0·05

Results restricted to individual glands challenged with MPEC strains (VL2732, VL2874 and P4). Parameters that were not affected by strain or interaction: $CD18^+G1^+$; $CD4^+$; $CD8^+$; $CD14^+$ macrophages; TNF^+ macrophages, lymphocytes and total; TNF in milk; IgG; IL-1β; log_{10}CFU. Parameters that did not change during the study: IL-4. n.s.: not significant.

were positive to intra-cellular TNF within 16 h, corresponding to peak secreted TNFα in milk. However, contrarily to secreted TNFα, the per cent of TNF-positive leucocytes declined in mammary glands infected with P4 from 4 DPC, whereas in glands challenged with VL2732 or VL2874 a decline was recorded only after 28 DPC.

TLR4

TLR4 were detected on 70–80% of all leucocytes types, but not on epithelial cells. No significant differences were observed between leucocytes cell types; data is exhibited for total leucocytes ($CD18^+$: Fig. 2d). A sharp decrease in milk $TLR4^+$ leucocytes was observed within 12–24 h post-challenge. At 12 h post-challenge, TLR4 was not detected on leucocytes in milk from glands challenged with VL2874, at 16 h with VL2732 h and at 24 h with P4. The percentage of $TLR4^+$ leucocytes increased at 4 DPC, with a higher increase observed in glands challenged with P4, but in none of the cows $TLR4^+$ leucocytes returned to the pre-challenge levels until the end of the study. No such changes were recorded in milk of glands challenged with K71.

Discussion

E. coli is often considered an opportunistic pathogen of bovine mastitis. Factors that relate to the infecting strain have been considered to be of little or no effect on mastitis severity (Burvenich et al. 2003). Yet, there are increasing evidences that MPEC comprise selected strains and that MPEC isolated from distinct types of mastitis differ in pathogenic properties (Dogan et al. 2006, 2012; Blum et al. 2008, 2015; Kerro Dego et al. 2012).

The overall pattern of the immune response observed in the present study is comparable to similar studies (Bannerman et al. 2004; Oliver et al. 2012). Some differences could be observed between MPEC strains, although statistical results should be analyzed with proper care due to the low number of cows used. The response to P4 tended to be delayed, lower or shorter in various parameters and with milder clinical signs. VL2874 and VL2732 caused a higher decrease in milk yield and increase in SCC compared to P4, and bacteria were detected in milk for a longer time than in P4. These observations correlate with the original patterns of mastitis from which these strains were isolated (Blum et al. 2014) and with results of experimentation in mice (Blum et al. 2015). It is possible that VL2874 and VL2732 cause more extensive tissue damage or colonise the gland epithelium, thus triggering a longer PMN response compared to P4. Genomic properties that could be associated with increased tissue damage or with intra-cellular survival, colonisation and biofilm formation were found in strains VL2874 and VL2732, respectively, by whole genome analysis (Blum et al. 2015). P4 lacks these features. Therefore, our findings suggest that P4 does not possess the whole array of pathogenic potentials found in MPEC, although it is usually used as a 'universal model' strain for *E. coli*-mastitis research.

CD8 lymphocytes infiltration into milk was shown to be influenced by *E. coli* counts (Mehrzad et al. 2008). Here, increased bacterial counts in milk were inversely correlated to CD4:CD8 ratio at 16 h post-challenge. P4 lead to increased CD4:CD8 ratio of above 1·5 at 7 DPC, whereas this ratio decreased with VL2874 and VL2732. The increased CD4:CD8 ratio with P4 coincided with significant increase in IL-17 secretion. IL-17 secretion in milk during mastitis is associated with CD4 lymphocytes activity (Rainard et al. 2013). Although up-regulation of IL-17 genes is detected in the mammary tissue after intra-mammary *E. coli* challenge (Roussel et al. 2015), measurable IL-17 secretion in milk was not detected before.

It was expected that recurrent mastitis could be reproduced with strain VL2732, but recurrence was observed only at 21 DPC. Using a similar experimental protocol, but a different persistent strain, Oliver et al. (2012) reported intermittent bacterial excretion in milk over 1 week only in three out of seven cows. The persistent strain was not detected in milk after 10–11 d post-challenge, however, it was isolated again at about 21 d, which is the same pattern found with strain VL2732 here. The next isolation of the persistent strain by Oliver et al. was 43 d post-challenge, which is beyond the follow-up time performed in the present study. One difference between both studies is that Oliver et al. used heifers close to parturition, whereas mid-late-lactation cows were used here. It is possible that lactation number and stage play a role on predisposition of potentially persistent strains to actually become

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persistent. Long-term SCC and PMN infiltration into milk regardless of detectable viable bacteria in milk could be reproduced with VL2732 and VL2874, similarly to original mastitis (Blum et al. 2014).

TLR4 is a main receptor contributing to *E. coli* detection in the mammary gland (Schukken et al. 2011), being upregulated in all regions in the gland following *E. coli* infusion (Rinaldi et al. 2010). Although early bacterial detection is critical for an effective response and pathogen clearing, uncontrolled TLR4 signalling could lead to excessive damage to tissues owing to exacerbated inflammation and possibly even exhaustion and death. Here, a sharp decrease of $TLR4^{+}$ was observed in all glands challenged with MPEC. Because membrane TLR4 was measured and not mRNA, it is suggested that TLR4 down-regulation resulted due to mechanisms associated with release or degradation of the receptor. The time at which TLR4 was not detected coincides with the time at which TLR4 mRNA was previously found to be up-regulated in other studies (Petzl et al. 2008; Rinaldi et al. 2010), suggesting that post-transcriptional regulatory mechanisms could exist. With all three MPEC strains, decrease in TLR4 followed peak bacterial counts in milk. This could, therefore, be an essential mechanism of preventing an over-reaction to the bacteria after the initial triggering of the immune response and its maintenance by further pathways. The different times of decrease in TLR4 expression correlate with overall onset of inflammation; VL2874 elicited the earliest inflammatory response and decrease in TLR4 and P4 were the latest.

Finally, K71 did not elicit any detectable immune or inflammatory response. This strain was isolated from a cow shed and showed phenotypes suggesting that it would not be adapted to cause mastitis (Blum et al. 2008). It does not cause mastitis in mice (Blum et al. 2015) and we have now confirmed that K71 is non-mammary-pathogenic to cows. This finding contradicts the widely accepted notion that all *E. coli* bacteria are equally pathogenic in the mammary gland. Moreover, K71 may be used in comparative studies of MPEC, aiming to identify virulence factors of *E. coli* associated with mastitis pathogenicity. Apart from the present work, very few such strains were reported previously (Anderson et al. 1977).

In conclusion, our results suggest that some differences can be found between the immune response elicited by distinct MPEC strains. These differences may correspond to different pathogenic properties of these strains. In addition, an environmental strain was shown to be non-pathogenic in the bovine mammary gland and is therefore suitable for comparative studies aiming to characterise the virulence factors of MPEC. Novel results include also the induction of IL-17 secretion in milk by strain P4 and significant decrease of TLR4 expression on milk leucocytes at peak stages of infection, possibly as a protective or a tolerance mechanism.

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Supplementary Material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029917000206

References

Almeida RA, Dogan B, Klaessing S, Schukken YH & Oliver SP 2011 Intracellular fate of strains of Escherichia coli isolated from dairy cows with acute or chronic mastitis. *Veterinary Research Communications* **35** 89–101

Anderson JC, Burrows MR & Bramley AJ 1977 Bacterial adherence in mastitis caused by *Escherichia coli*. *Veterinary Pathology* **14** 618–628

Bannerman DD, Paape MJ, Lee J-W, Zhao X, Hope JC & Rainard P 2004 Escherichia coli and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clinical and Diagnostic Laboratory Immunology* **11** 463–472

Blum SE & Leitner G 2013 Genotyping and virulence factors assessment of bovine mastitis *Escherichia coli*. *Veterinary Microbiology* **163** 305–312

Blum SE, Heller ED, Krifucks O, Sela S, Hammer-Muntz O & Leitner G 2008 Identification of a bovine mastitis *Escherichia coli* subset. *Veterinary Microbiology* **132** 135–148

Blum SE, Freed M, Zukin N, Shwimmer A, Weissblit L, Khatib N, Bojesen AM, Larsen J, Friedman S & Elad D 2010 Bovine subclinical mastitis caused by *Mannheimia granulomatis*. *Journal of Veterinary Diagnostic Investigation* **22** 995–997

Blum SE, Heller ED & Leitner G 2014 Long term effects of *Escherichia coli* mastitis. *Veterinary Journal* **201** 72–77

Blum SE, Heller ED, Sela S, Elad D, Edery N & Leitner G 2015 Genomic and phenomic study of mammary pathogenic *Escherichia coli*. *PLoS ONE* **10** e0136387

Bradley AJ & Green MJ 2001 Adaptation of *Escherichia coli* to the bovine mammary gland. *Journal of Clinical Microbiology* **39** 1845–1849

Bramley AJ 1976 Variations in the susceptibility of lactating and non-lactating bovine udders to infection when infused with *Escherichia coli*. *Journal of Dairy Science* **43** 205–211

Burvenich C, Van Merris V, Mehrzad J, Diez-Fraile A & Duchateau L 2003 Severity of *E. coli* mastitis is mainly determined by cow factors. *Veterinary Research* **34** 521–564

Dogan B, Klaessig S, Rishniw M, Almeida RA, Oliver SP, Simpson K & Schukken YH 2006 Adherent and invasive *Escherichia coli* are associated with persistent bovine mastitis. *Veterinary Microbiology* **116** 270–282

Dogan B, Rishniw M, Bruant G, Harel J, Schukken YH & Simpson KW 2012 Phylogroup and lpfA influence epithelial invasion by mastitis associated *Escherichia coli*. *Veterinary Microbiology* **159** 163–170

Döpfer D, Barkema HW, Lam TJ, Schukken YH & Gaastra W 1999 Recurrent clinical mastitis caused by *Escherichia coli* in dairy cows. *Journal of Dairy Science* **82** 80–85

Halasa T, Huijps K, Østerås O & Hogeveen H 2007 Economic effects of bovine mastitis and mastitis management: a review. *The Veterinary Quarterly* **29** 18–31

Hogeveen H, Huijps K & Lam TJGM 2011 Economic aspects of mastitis: new developments. *New Zealand Veterinary Journal* **59** 16–23

Kempf F, Slugocki C, Blum SE, Leitner G & Germon P 2016 Genomic Comparative Study of Bovine Mastitis *Escherichia coli*. *PLoS ONE* **11** e0147954

Kerro Dego O, Oliver SP & Almeida RA 2012 Host-pathogen gene expression profiles during infection of primary bovine mammary epithelial cells with *Escherichia coli* strains associated with acute or persistent bovine mastitis. *Veterinary Microbiology* **155** 291–297

Leitner G, Eligulashvily R, Krifucks O, Perl S & Saran A 2003 Immune cell differentiation in mammary gland tissues and milk of cows chronically infected with *Staphylococcus aureus*. *Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health* **50** 45–52

Leitner G, Merin U & Silanikove N 2011 Effects of glandular bacterial infection and stage of lactation on milk clotting parameters: comparison among cows, goats and sheep. *International Dairy Journal* **21** 279–285

Le Maréchal C, Thiéry R, Vautor E & Le Loir Y 2011 Mastitis impact on technological properties of milk and quality of milk products - a review. *Dairy Science and Technology* **91** 247–282

Mehrzad J, Janssen D, Duchateau L & Burvenich C 2008 Increase in *Escherichia coli* inoculum dose accelerates $CD8^+$ T-cell trafficking in the primiparous bovine mammary gland. *Journal of Dairy Science* **91** 193–201

Oliver SP, Headrick SI, Lewis MJ, Gillespie BE, Johnson DL & Almeida RA 2012 Experimental intramammary infection with a strain of *Escherichia coli* isolated from a cow with persistent *E. coli* Mastitis. *Open Journal of Veterinary Medicine* **2** 186–190

Petzl W, Zerbe H, Günther J, Yang W, Seyfert HM, Nürnberg G, Schuberth HJ 2008 Escherichia coli, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. *Veterinary Research* **39** 18–00

Rainard P, Cunha P, Bougarn S, Fromageau A, Rossignol C, Gilbert FB & Berthon P 2013 T helper 17-associated cytokines are produced during antigen-specific inflammation in the mammary gland. *PLoS ONE* **8** e63471

Rinaldi M, Li RW, Bannerman DD, Daniels KM, Evock-Clover C, Silva MVB, Paape MJ, Van Ryssen B, Burvenich C & Capuco AV 2010 A sentinel function for teat tissues in dairy cows: dominant innate immune response elements define early response to *E.* coli *mastitis*. Functional & Integrative Genomics **10** 21–38

Roussel P, Cunha P, Porcherie A, Petzl W, Gilbert FB, Riollet C, Zerbe H, Rainard P & Germon P 2015 Investigating the contribution of IL-17A and IL-17F to the host response during *Escherichia coli* mastitis. *Veterinary Research* **46** 56

SAS Institute 2000 JMP®, Version 5. SAS Institute Inc., Cary, NC, 1989-2007.

Schukken YH, Günther J, Fitzpatrick J, Fontaine MC, Goetze L, Holst O, Leigh J, Petzl W, Schuberth HJ, Sipka A, Smith DGE, Quesnell R, Watts J, Yancey R, Zerbe H, Gurjar A, Zadoks RN & Seyfert HM 2011 Host-response patterns of intramammary infections in dairy cows. *Veterinary Immunology and Immunopathology* **144** 270–289

Seegers H, Fourichon C & Beaudeau F 2003 Production effects related to mastitis and mastitis economics in dairy cattle herds. *Veterinary Research* **34** 475–491

Wellnitz O & Bruckmaier RM 2012 The innate immune response of the bovine mammary gland to bacterial infection. *Veterinary Journal* **192** 148–152

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