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Investigation of the bovine immune response to repeated vaccinations against *Clostridium difficile*

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Table of contents

Table of contents ..... **i**

List of tables ..... **iv**

List of figures ..... **vi**

Abbreviations ..... **vii**

Summary **1**

Zusammenfassung **4**

Introduction **7**

Clostridium difficile 7

The pathogenic agent of Clostridium difficile infections 7

Treatment of Clostridium difficile infections 7

Active and passive immunizations against C. diff. 8

Immune milk 9

The milk antibodies IgG versus IgA 10

The (bovine) immune system exemplified by the immune response to vaccinations 10

Aim of the study **13**

Materials and Methods **14**

Test animals 14

Control animals 15

Vaccination 15

Notes to the investigation period 16

Milk yield recording and milk sampling 16

Treated Cows 16

Control cows 17

Blood sampling 17

Clostridium difficile specific IgA 18

Total IgA 19

Total and differential cell counting 19

Total RNA extraction from peripheral blood leukocytes and somatic cells obtained from milk 20

Reverse transcription PCR 20

Primer 21

Gene expression measurements 25

RT-qPCR data processing 25

Tissue Sampling and Preparation 26

Antibodies 26

Immunohistochemical staining 27

Quantitative Evaluation of Data 28

Statistical Analysis 28

Results and Discussion 30

Physical condition of the test animals and the vaccine tolerability 30

Milk yield and the gross milk composition 30

Clostridium difficile specific IgA in milk and blood 31

Total IgA in milk and blood 35

Total and differential cell counts in milk and blood 37

Gene expressions related to the innate immune system 44

In peripheral blood leukocytes 44

In bovine mammary epithelial cells of milk 50

In immune cells of milk 58

Gene expressions related to the adaptive immune system 63

In blood cells 63

In milk cells 70

Correlations 76

Immunohistological detection of lymphocytes in tissues 78

Distribution of T-lymphocyte subsets and IgA+ antibody secreting cells in the bovine lactating mammary gland 78

Distribution of T-lymphocyte subsets and IgA+ antibody secreting cells in the supramammary lymph nodes 82

Conclusions 84

References 85

Acknowledgments 96

Scientific communications 97

Peer-reviewed scientific publications 97

Abstracts and posters presented at scientific conferences 97

Appendix 98

Appendix I 98

Appendix II 98

Appendix III 98

List of tables

Table 1: Classification of the treated cows into LR and HR 15

Table 2: Application of the vaccines *MucoCD-I* batch B and *MucoCD-N* 16

Table 3: Primer for the RT-qPCR 22

Table 4: Classification of the gene regulation expressed as ΔΔCq range 26

Table 5: Antibodies for the IHC staining 27

Table 6: Milk yield and the components, protein, fat and SCC.....31

Table 7: Anti-*C. diff.* IgA contents in milk and blood of treated cows before the treatment (TW 0) and prior to the first use of *MucoCD-I* vaccine batch B (TW 16).....34

Table 8: Total IgA contents in milk and blood of treated cows before the treatment (TW 0) and prior to the first use of *MucoCD-I* vaccine batch B (TW 16) 37

Table 9: Total PBL counts and the relative distribution of the different leukocyte populations 40

Table 10: Total SCC in milk and relative distribution of the different leukocyte populations out of milk.....42

Table 11: ΔΔCq changes in gene expression of peripheral blood phagocytes' receptors relative to TW 0 or rather TW 16 46

Table 12: ΔΔCq changes in gene expression of blood phagocytes' chemokines / cytokines relative to TW 0 or rather TW 16 48

Table 13: ΔΔCq changes in gene expression of bMEC related Ig receptors and complement proteins relative to TW 1 or rather TW 15.....52

Table 14: ΔΔCq changes in gene expression of bMEC related chemokines relative to TW 1 or rather TW 15.....54

Table 15: ΔΔCq changes in gene expression of bMEC related antimicrobial peptides relative to TW 1 or rather TW 15.....56

Table 16: ΔΔCq changes in gene expression of milk phagocytes' receptors relative to TW 1 or rather TW 15.....59

Table 17: ΔΔCq changes in gene expression of milk phagocytes' cytokines relative to TW 1 or rather TW 15.....61

Table 18: ΔΔCq changes in gene expression of blood lymphocytes' receptors relative to TW 0 or rather TW 16 66

Table 19: ΔΔCq changes in gene expression of blood lymphocytes' cytokines relative to TW 0 or rather TW 16 68

Table 20: ΔΔCq changes in gene expression of milk lymphocytes' receptors relative to TW 1 or rather TW 15 72

Table 21: ΔΔCq changes in gene expression of milk lymphocytes' receptors and cytokines relative to TW 1 or rather TW 15 74

Table 22: Correlations between anti-*C. diff.*, total IgA and the main production factors in milk of treated cows (n = 9) 77

Table 23: Correlations between DCC and the belonging gene expression data. 78

Table 24: Incidence of lymphocytes resident in the MG parenchyma 78

Table 25: Incidence of lymphocytes resident in the SLN of the vaccinated groups 82

List of figures

Figure 1: Anti-*C. diff.* milk IgA contents at different lactation stages.....33

Figure 2: Anti-*C. diff.* IgA concentrations in milk and in blood during the 31-week treatment period.....34

Figure 3: Total milk IgA contents at different lactation stages.....36

Figure 4: Total IgA concentrations in milk and in blood during 31-weeks treatment period...37

Figure 5: Lymphocyte counts in the MG of the anti-*C. diff.* vaccinated groups (LR, HR) compared to the untreated group (Ctr).....79

Figure 6: Cell nuclei and IgA stained in the MG parenchyma and the spleen.....81

Figure 7: Lymphocyte counts in the SLN of the anti-*C. diff.* vaccinated groups.....83

Abbreviations

Ab	antibody(s)
ACTG1	actin gamma 1
APC	antigen-presenting cell(s)
ASC	antibody secreting cell(s)
BCR	B-cell receptor
BTC	before treatment control value
bMEC	bovine mammary epithelial cell(s)
bp	base pair(s)
C	complement
C3AR1	Complement C3a receptor 1
C5AR1	Complement C5a receptor 1
CCL	(C-C) motif chemokine ligand
CCR	(C-C) motif chemokine receptor
CD	cluster of differentiation
C. diff.	*Clostridium difficile*
CDI	*C. diff.* infection
cDNA	complementary deoxyribonucleic acid
Cq	cycle number
Ctr	control group
CXCL	(C-X-C) motif chemokine ligand
CXCR	(C-X-C) motif chemokine receptor
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DCC	differential cell count(s)
ΔCq	delta cycle number; difference between the target gene Cq and reference gene Cq
DiL	days in lactation
DNA	deoxyribonucleic acid
ECM	energy corrected milk
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (lat.), for example
ELISA	enzyme-linked immunosorbent assay
FA	formaldehyde
FcRN	neonatal Fc-receptor
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H3F3A	H3 histone, family 3A
HC	humid chamber
HR	high responder
i.a.	*inter alia* (lat.), among other things
IC	intracutaneous
iFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin(s)
IHC	immunohistochemistry
IL	interleukin
IM	immunization(s)
IR	immune response
IS	immune system
kg	kilogram(s)
KRT8	cytokeratin 8
L	liter(s)
LF	lactoferrin
LN	lymph node(s)
LR	low responder
LPO	lactoperoxidase
LPS	lipopolysaccharide
LSM	least square mean
Lym	lymphocytes
LYZ	lysozyme

MALT	mucosa associated lymphatic tissue
mc	monoclonal
MEC	mammary epithelial cell
mg	milligram(s)
MG	mammary gland
MHC	major histocompatibility complex
min	minute(s)
mL	milliliter(s)
MO	microorganism(s)
N	nasal
NALT	nasopharynx-associated lympoid tissue
ng	nanogram(s)
n.n.	*numerius negidius* (lat.); unavailable data
NTC	no-template control
PAMP	pathogen associated molecular patterns
PBL	peripheral blood leukocytes
PBS	phosphate buffered saline
PC	percutaneous
pc	polyclonal
PCR	polymerase chain reaction
Phag	phagocytes
PIGR	polymeric immunoglobulin receptor
PMN	polymorphnucleated neutrophilic leukocytes
PRR	pathogen recognition receptor
PS	plate shaker
RNA	ribonucleic acid
RT	room temperature
RT-qPCR	real time quantitative polymerase chain reaction
SC	subcutaneous
SD	standard error
sIgA	secretory IgA
SLN	supramammary lymph node(s)
SUZ12	SUZ12 polycomb repressive complex 2 subunit homolog
TAP	tracheal antimicrobial peptide
TBS	Tris-buffered saline
T_C-cell	cytoxic T-cell
TcdA	*C. diff.* toxin A
TcdB	*C. diff.* toxin B
TGFβ1	transforming growth factor beta 1
T_H-cell	T helper-cell
TLR	toll-like-receptor
TNFα	tumor necrosis factor alpha (cachectin)
TW	treatment week
UB3	polyubiquitin
µg	microgram(s)
µL	microliter(s)
YWHAZ	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase-activation protein zeta

Summary

The pathogen *Clostridium difficile* (*C. diff.*) causes severe diarrhoea in humans. Almost one of three patients suffered from *C. diff.* infection undergoes recurrences due to failed antibiotic treatment. The supplementary administration of a bovine whey protein concentrate enriched with anti-*C. diff.* IgA may improve the treatment success of antibiosis by diminishing the rate of recurrences as proven previously by v. Dissel et al. (2005). The production of bovine milk containing anti-*C. diff.* IgA requires the immunization of dairy cows against the virulent factors of *C. diff.*. The object of this doctoral thesis is to investigate the bovine immune response to repeated vaccinations against *C. diff.*.

Nine primiparous *Brown Swiss* cows were vaccinated several times against the pathogen within the 18-month treatment period. Different types of vaccines, containing inactivated *C. diff.* and the toxoids / toxins A and B, were administered nasally (*MucoCD-N*) and parenterally via injection (*MucoCD-I* batches A, B). The treated cows were divided into *low responder* (LR) and *high responder* (HR) cows measured by the level of anti-*C. diff.* milk IgA. The associated threshold was set at 8.00 µg / mL anti-*C. diff.* milk IgA. Untreated *Brown Swiss* cows were used as controls. The following investigations were performed to determine treatment effects: veterinary monitoring of the treated cows' health; milk yield recording by use of the TRU-Test milk meter; testing of the milk composition by means of the Fourier-transform infrared spectroscopy for milk fats and proteins plus fluorescence flow-cytometry for somatic cell counts (SCC); total cell counting of peripheral blood leukocytes (PBL); differential cell counting (DCC) of SCC and PBL by aid of light microscopy; IgA quantification in milk and blood with the enzyme-linked immunosorbent assay (ELISA); gene expression analysis of receptors and mediators associated with the immune system in SCC and PBL with the real-time quantitative polymerase chain reaction (RT-qPCR); correlation analysis of milk IgA and the main dairy production factors or rather DCC and associated gene expression data using the statistic software, SigmaPlot 11.0; immunohistochemically examination of different lymphocytes in mammary gland (MG) and supramammary lymph node (SLN) tissues.

The health of the treated cows was impaired after injecting *MucoCD-I* batch A, which contained an imbalanced mixture of *C. diff.* toxoids / toxins A and B. As a result, milk yield and quantities of milk fats and proteins ($P < 0.05$) were significantly diminished in early lactation of the treated cows compared to the control. The anti-*C. diff.* and total IgA concentrations in HR's milk were significantly higher up to 80% related to the "before treatment control" values (BTC) and the measurement values in LR's milk at any lactation

stage. Only in the late lactation, the LR's total milk IgA differed significantly from the associated BTC by roughly 47%. Total and anti-*C. diff.* IgA contents were affected more in milk than in blood due to the vaccinations. The total SCC of milk were untouched by the treatment. In blood, a short-term leukopenia followed by a leukocytosis were induced by application of *MucoCD-I* batch A. Significant changes in DCC of SCC and PBL among LR and HR were only partially treatment-related. Generally, the treatment influenced stronger the gene regulations in PBL than in SCC. The significantly different gene regulations in PBL and SCC among the treated groups were primarily triggered in response to *MucoCD-I* batch A. Especially the gene expressions of the chemokines / cytokines like *CXCL8*, *IL1*β, *IL12*β and *IFN*γ in PBL as well as *lactoferrin* and also *IFN*γ in SCC were significantly different between LR and HR. During the immunization period with *MucoCD-I* batch B, the treated groups' gene expressions were predominantly synchronized. They differed significantly from the control in case of *CXCL8*, *IL6*, *IL12*β, *IFN*γ, *IL2*, *TLR2*, *CD3*δ, *CD4* and *CD126* in PBL and for *lactoferrin*, *TNF*α, *IL6*, *IL12*β, *CCL20* and *CXCL3* in SCC. At no time of the treatment, the gene expressions of the epithelial IgA cell receptor *PIGR* were altered. The correlations among total or anti-*C. diff.* milk IgA and the main dairy production factors were determined as weak ($r < 0.5$). A significant linear dependency existed between anti-*C. diff.* and total milk IgA ($r = 0.69$). The correlation analysis of DCC and associated gene expression data revealed strong interrelationships between the SCC percentages of neutrophilic granulocytes and the related cell surface determinants *C5AR1* and *CXCR2* ($r > 0.8$) and a middle interrelationship between the SCC percentages of lymphocytes and the plasma cell marker *CD126* ($r = 0.75$). The median population sizes of CD4+ and CD8+ T-cells as well as IgA+ antibody secreting cells in MG and SLN tissues of HR were predominantly larger than in those of LR and control.

In conclusion, the persistent production of *C. diff.* specific IgA in milk depends on two important factors: a **potent and well-tolerated *C. diff.* vaccine** capable to induce a primarily mucosa-related immune response, and **sensitive dairy cows as recipients**. In advance, HR cows might be preselectable by *in vitro* testing of their PBL when challenged with *C. diff.* antigens and followed by gene expression analysis of *CXCL8*, *IL1*β, *IL12*β and *IFN*γ to check the induction of these genes. Overall, the following assumptions were deduced from the presented results and recommended as topics of subsequent investigations:

- In response to repeated immunizations against *C. diff.*, HR cows activated the humoral response to antigen directed by T_H2-lymphocytes, whereas LR cows emphasized rather the cellular immune response controlled by T_H1-lymphocytes, as indicated by the gene expression profiles of the chemokines / cytokines.
- Because the *PIGR* gene expressions were unaffected by the immunization, the epithelial transport capacity would not be a limiting factor for the secretion of milk IgA.
- The intensive homing of lymphocytes to the MG and SLN - as determined in HR's tissues - is crucial prerequisite for the persistent production of anti-*C. diff.* milk IgA.

Zusammenfassung

Der Krankheitserreger *Clostridium difficile* (*C. diff.*) verursacht gravierende Durchfälle beim Menschen. Annähernd ein Drittel an *C. diff.* erkrankten Patienten erfahren multiple Reinfektionen wegen fehlgeschlagener antibiotischer Behandlung. Die zur Antibiose supplementäre Verabreichung eines mit anti-*C. diff.* IgA angereicherten bovinen Molkenprotein-Konzentrats kann den Behandlungserfolg in Form geringerer Rückfallraten verbessern (van Dissel et al., 2005). Die Herstellung von bovinem anti-*C. diff.* Milch-IgA erfordert die Immunisierung von Milchkühen gegen die *C. diff.* typischen Virulenzfaktoren. In der vorliegenden Dissertation ist die bovine Immunantwort auf wiederholte Impfungen gegen C. diff. untersucht worden.

In einem Behandlungszeitraum von 18 Monate wurden neun erstkalbige *Brown Swiss* Kühe wiederholt gegen *C. diff.* geimpft. Verschiedene Impfstoffvarianten wurden nasal (*MucoCD-N*) und parenteral per Injektion (*MucoCD-I* Chargen A und B) verabreicht. Die gegen *C. diff.* geimpften Kühe wurden abhängig vom anti-*C. diff.* IgA Gehalt in der Milch in "Low Responder" (LR) und "High Responder" (HR) eingeteilt. Der zugehörige Grenzwert lag bei 8,00 µg / mL anti-*C. diff.* Milch-IgA. Unbehandelte *Brown Swiss* Kühe wurden als Kontrolltiere verwendet. Zur Feststellung von Behandlungseffekten wurden folgende Untersuchungen durchgeführt: tierärztliche Beurteilung der Tiergesundheit; Erfassung der Milchleistungsdaten inklusive des somatischen Milchzellgehaltes (SCC); Feststellung der Gesamtzahl peripherer Blutleukozyten (PBL); lichtmikroskopische Untersuchung der Differentialzellbilder (DCC) von SCC und PBL; Messung der IgA-Konzentrationen in Milch und Blut mit dem Enzyme-linked Immunosorbent Assay (ELISA); Genexpressionsanalysen von immunsystemrelevanten Zellrezeptoren und Botenstoffen in Milch- und Blutzellen mit der quantitativen Echtzeit-Polymerase-Kettenreaktion (RT-qPCR); Korrelationsanalyse von den Milch-IgA-Messwerten und Milchproduktionsfaktoren sowie von DCC und zughörigen Genexpressionsdaten mit SigmaPlot 11.0; immunhistochemischer Nachweis definierter Lymphozytentpyen in Eutergewebe (MG) und supramammären Lymphknotengewebe (SLN).

Die Impfung mit der *MucoCD-I* Charge A beeinträchtigte den Gesundheitszustand der behandelten Kühe aufgrund der unausgewogenen Toxoid/Toxin-Mixtur in dieser Impfstoffcharge. In der Folge waren in der Frühlaktation sowohl die durchschnittlichen Milchmengen als auch die Milchfette und -proteine der beiden behandelten Tiergruppen gegenüber der Kontrollgruppe signifikant reduziert ($P < 0.05$). Die Konzentrationen von anti-*C. diff.* und Gesamt-IgA in der HR-Milch überstiegen unabhängig vom Laktationsstadium signifikant um bis zu 80% die entsprechenden Kontrollwerte vor Behandlungsbeginn (BTC) und die Messwerte in der LR-Milch. Lediglich in der Spätlaktation war das Gesamt-IgA in der

LR-Milch um etwa 47% signifikant erhöht gegenüber dem BTC. Die Konzentrationen von anti-*C. diff.* und Gesamt-IgA in der Milch waren von den *C. diff.* Impfungen stärker beeinflusst als die im Blut. Der SCC blieb von den *C. diff.* Impfungen unberührt. Im Blut wurden durch die injizierte *MucoCD-I* Charge A eine kurzweilige Leukopenie und eine nachfolgende Leukozytose festgestellt. Die in den Milch- und Blut-DCC von LR und HR festgestellten signifikanten Änderungen wurden nicht ausschließlich vom Impfprogramm verursacht. Generell beeinflusste die Behandlung die Genregulationen in den PBL deutlicher als die in den SCC. Unterschiedliche Genregulationen in PBL und SCC von LR und HR traten vornehmlich nach der Verabreichung von *MucoCD-I* Charge A auf. Insbesondere die Gene für die Chemokine / Zytokine *CXCL8*, *IL1*β, *IL12*β und *IFN*γ in PBL sowie *Lactoferrin* und auch *IFN*γ in SCC waren von LR und HR signifikant unterschiedlich exprimiert. Während der Immunisierung mit *MucoCD-I* Charge B entwickelten sich die Genexpressionen der behandelten Gruppen überwiegend gleichförmig. Sie unterschieden sich signifikant von denen der Kontrollgruppe für *CXCL8*, *IL6*, *IL12*β, *IFN*γ, *IL2*, *TLR2*, *CD3*δ, *CD4* und *CD126* in den PBL sowie für *Lactoferrin*, *TNF*α, *IL6*, *IL12*β, *CCL20* und *CXCL3* in den SCC. Zu keinem Behandlungszeitpunkt waren die Genexpressionen für den epithelialen IgA-Zellrezeptor *PIGR* verändert. Für die Konzentrationen von anti-*C. diff.* und Gesamt-IgA in der Milch behandelter Tiere wurde eine deutliche lineare Abhängigkeit nachgewiesen ($r = 0.69$). Die Korrelationen beider IgA-Messwerte mit den Milchproduktionsfaktoren wurde als schwach ($r < 0.5$) bewertet. Der prozentuale Anteil von neutrophilen Granulozyten im SCC und die Genexpressionsdaten für die zugehörigen Zelloberflächendeterminanten *C5AR1* und *CXCR2* korrelierten stark ($r > 0.8$). Eine durchschnittliche lineare Abhängigkeit wurde für den prozentualen Anteil von Lymphozyten im SCC und dem Plasmazellmarker *CD126* festgestellt ($r = 0.75$). In den MG- und SLN-Geweben der HR waren die mittleren Populationsgrößen von CD4+ und CD8+ T-Zellen sowie von IgA+ Antikörper produzierenden Zellen (ASC) überwiegend größer als die von LR und Kontrolltieren.

Abschließend ist festzustellen, dass eine anhaltende Produktion von anti-*C. diff.* Milch-IgA von zwei wesentlichen Faktoren abhängt: einem **wirksamen und gut verträglichen *C. diff.* Impfstoff**, der es vermag eine primär Mukosa-assoziierte Immunantwort hervorzurufen, und von **sensitiv auf den Impfstoff reagierenden Milchkühen**. Solche HR-Kühe wären zukünftig im Voraus selektierbar, indem deren PBL mit *C. diff.* Antigenen *in vitro* getestet und nachfolgend mittels Genexpressionsanalysen die Induktion von *CXCL8*, *IL1*β, *IL12*β und *IFN*γ beurteilt werden. Folgende Annahmen, deren Überprüfung Folgeuntersuchungen erfordern, lassen sich anhand der vorliegenden Ergebnisse formulieren:

- Gemessen an den Chemokin- / Zytokin-Genexpressionen aktivieren HR-Kühe eine von T_H2-Lymphozyten kontrollierte humorale Immunantwort auf die *C. diff.* Impfungen und LR-Kühe eine von T_H1-Lymphozyten regulierte zelluläre Immunantwort.
- Die vom Impfprogramm unbeeinflussten *PIGR* Genexpressionen deuten auf eine nicht von der epithelialen IgA-Transportkapazität limitierten Milch-IgA Sekretion hin.
- Die intensive Besiedelung von MG- und SLN-Geweben mit T-Lymphozyten und IgA+ ASC - wie in diesen Geweben der HR nachgewiesen - ist eine entscheidende Voraussetzung für die anhaltend anhaltende Produktion von anti-*C. diff.* Milch-IgA.

Introduction

Clostridium difficile

The pathogenic agent of Clostridium difficile infections

The rod-shaped bacillus, *Clostridium difficile* (*C. diff.*), was discovered by Hall and O'Toole in 1935 [1]. It is an anaerobic and spore-forming bacterium with a markedly environmental resistance. Proven habitats of *C. diff.* are soil and sand beyond its high occurrence in humans and different animals as part of their intestinal flora [2] [3] [4] [5]. Therefore, the feces of these beings contained *C. diff.*, and animal products, such as retail meat, were found to be contaminated with the bacterium due to poor hygiene during their fabrication or storage [3] [5]. The *C. diff.* carriage rate in humans depends on their age and health status. The gut of up to 60% healthy neonates is physiologically loaded with *C. diff.*, whereas *C. diff.* carrier among adults are only 2-3%. In case of antibiotic usage their carriage rate increases to round about 20% because their normal intestinal flora is disturbed [1] [4] [6]. Besides the association to antibiotic treatment of an underlying disease, the accommodation of persons is also a predisposal factor for infections with *C. diff.* (CDI). To be resident in healthcare and nursing homes increases the risk for CDI. The severity of this disease is determined by the causing *C. diff.* strain. Some of them are hypervirulent, e. g. the BI/NAP1/027 *C. diff.* strain. These are characterized by production of a third binary toxin in addition to the both exotoxins, toxin A (TcdA) and toxin B (TcdB) [7]. They are crucial for the pathogenesis of CDI. Following infection with *C. diff.* spores on the fecal-oral route and their intestinal germination, the settled and matured bacteria emit the dangerous metabolites in the colon [4] [6]. Then, TcdA and TcdB complement each other with their effects because TcdA affects initially the epithelial integrity of the intestine and allowing the entry of the more potent cytotoxic TcdB, which is especially proinflammatory active [8]. Depending on the progression of CDI, its clinical presentations can be diarrhea with or without colitis and the colitis can be associated with pseudomembrane formation. Approximately 3% of CDI patients suffer life-threatening complications, such as chronic ileus, megacolon and intestinal perforation [9].

Treatment of Clostridium difficile infections

In the last decades, the global incidence of CDI increased, and hypervirulent strains emerged causing epidemics worldwide that are difficult to treat and responsible for huge health care costs [10] [11] [12] [13] [14]. The growing antibiotic resistance of *C. diff.*, *i.a.* because of their ability to form withstanding spores, reveal the high rate of relapses ranging between 15 to 35% after the first course of antibiotics and rising with every further episode [15] [16].

Apart from that, the changed disease severity is a further important root of the growing morbidity and mortality of CDI [7] [14] [17]. The 30-day case fatality rate for *C. diff.* related hospitalizations may range between 6 to 38%, and, as has been proved, it increases with the age of the patients [18] [19]. Additionally, the short-term doubling of the age-adjusted CDI-related mortality rate within the period of 2000 to 2004 in the United States was demonstrably influenced by the emergence of hypervirulent *C. diff.* strains [20]. To meet the challenges described above, the longstanding treatment by the exclusive use of antibiotics need to be improved. Therefore, the particular attention of management guidelines for CDI like the thoroughly observance of hygiene regulations is indispensably. Generally, the recommended antibiotics for CDI treatment, vancomycin and metronidazole, should be used restricted [1] [21] [22] [23] [24]. Different approaches accompanying the antibiotic therapy have been practically tested to deal with relapses more successful. Thereby, two main intentions were pursued, for one thing, the replenishment and care of the physiologically intestinal microbiome, and on the other hand, the remove of the *C. diff.* toxins. In this vein, the oral intake of probiotics (*e.g. Lactobacillus spp.* and *Saccharomyces boulardii*) or the transplantation of feces from healthy humans should provide assistance to the reconstitution of a normal flora with symbiotic microorganisms (MO) [4] [22] [25] [26] [27]. Whereas, the oral administration of adsorbents, such as ion-exchange resins and polymers, or of specific immunoglobulins (Ig) concerned the elimination of the *C. diff.* toxins [1] [28] [29] [30].

Active and passive immunizations against C. diff.

The active immunization (IM) elicits the cellular and humoral immune response (IR) of the vaccinates to the antigen. Important requirements to the vaccination are not only an effective vaccine containing the crucially virulent factors of the antigen, additionally, the vaccination routes need to adjust to the main points of the pathogens' attack in the body of the host. That means in case of *C. diff.*, the vaccine induced immunity is locally required in the gut to neutralize the enterotoxicity, and further, it should also be systemically present regarding the effect of TcdB. Therefore, inoculations with pure toxoid preparations, toxin/toxoid-mixtures with or without formaldehyde (FA)-inactivated *C. diff.* culture filtrate and partly with adjuvants were carried out mainly in animal models by combination of different and largely parenteral administration routes like nasal (N), intraperitoneal and intramuscular [6] [31] [32] [33]. To prevent and control CDI epidemics effective, compulsory vaccinations for risk groups would be need to implement. But individuals of the main risk groups have an impaired health, so an active IM would be a further burden of there IS. Passive IM are suitable for prophylactic and supplementary treatment of CDI. Different kinds of *C. diff.*-specific Ig were proven in animal models and humans. Exemplarily, anti-toxin IgG was intravenously administered and

mediated protection to mice and hamsters. Human infants suffering from CDI were treated successfully with human sera of donors with severe or recurrent CDI [4] [6] [32] [34]. Also different anti-*C. diff.* antibody (Ab)-preparations for oral application, such as specific egg IgY from immunized hens, bovine colostral IgG or rather milk secretory (s)IgA concentrates against *C. diff.*, were tested in hamsters, pigs and humans to be effective in neutralization the pathogen's toxins [6] [29] [33] [35]-[41].

Immune milk

Bovine milk meets the nutritional needs of the calf and is also a valuable foodstuff for humans. Apart from supplying essential nutrients, milk contains naturally a variety of immune factors. Along with the protection of the MG against invasive pathogens, these milk-derived bioactive compounds provide immunological protection of the consumers, especially of the offspring [42]. For instance, whey proteins with antimicrobial activity are lactoferrin (LF), lactoperoxidase (LPO) and lysozyme (LYZ). As part of the innate IS, they are produced by the bovine mammary epithelium itself. Other immune-effective ingredients of milk such as complement factors, acute phase proteins or cytokines like the interleukins IL1β and IL6 originated from immune cells resident in the MG, *e.g.* macrophages [43] [44] [45]. Of particular importance are milk Ig, because their production or rather their addition to milk are attuned to the pathogens prevalent in the immediate environment of the dairy cow. For this purpose, the bronchomammary link and, in case of humans, also the enteromammary link provide the necessary information transfer by assumption of the common mucosal IS [43] [46].

In retrospect, the specifically heterologous transfer of bovine milk Ab for human health is applied since the 1950s and was commercialized as *Stollait™ Immune Milk* first [47] [48] [49]. Scopes of application are the passive immunotherapy for immune-compromised humans like AIDS suffering patients and the prevention or rather the treatment of numerous gastrointestinal pathogens, such as *Helicobacter pylori*, rotavirus, *Shigella flexneri*, enterotoxigenic *Escherichia coli*, *C. diff.* and *Cryptosporidium parvum*. The production of specific milk Ig concentrates requires the hyperimmunization of cows against the particular pathogen of interest [40] [42] [47] [48]. Withal, the amount of the intended Ab collected per milking and the available Ig classes can be influenced by the time of vaccination and the route of vaccine administration. Hyperimmune bovine colostrum, and consequently very high antigen specific IgG contents, necessitates the repeated vaccination of drying-off cows at the end of their pregnancy. Preferential sIgA enrichment in milk against certain antigens is

achievable following continuous inoculations of the dairy cow during the course of lactation [45] [49] [50].

The milk antibodies IgG versus IgA

The most abundant Ab class in bovine lacteal secretions is IgG. In relation to the total Ab content, colostrum contains 86% and mature milk 75.5% IgG [45]. After its entry into the MG by the blood flow, this serum Ig is selectively transferred to milk mediated by the neonatal Fc-receptor (FcRN). Along with the end of parturition, the FcRN overexpression and the higher affinity of FcRN to bind IgG are the important prerequisites for the colostrum formation [51]. As a result, the IgG amount of colostrum may surpass the serum content at least eight times by reaching 200 mg/mL. By contrast, mature milk contains overall only < 1 mg/mL of all appearing Ab classes [47]. Besides IgG, the second most Ab class in mature milk is the sIgA with 18% of the total Ab content [45]. It is locally produced in the MG by plasma cells, and the polymeric Ig receptor (PIGR) ensures its transcytosis to the apical side of the mammary epithelial cells (MEC). Finally, after the endoproteolytic cleavage of PIGR, the secretory component remains tied to the J chain of the IgA dimer forming the sIgA complex [52] [53]. The secretory component protects IgA from enzymatic degradation in the digestive tract and preserves its biological efficacy [54]. Additionally, free secretory component, included in milk as well as in other body excretions, is also antimicrobial active [55]. The sIgA has a pivotal role in the defense of the mucosal surfaces of the body by neutralization of pathogens or of their harmful metabolites [46] [52]. In contrast to IgG, sIgA does neither act as an opsonin for phagocytes nor pin complement down. Thus, sIgA averts the induction of locally inflammatory reactions and maintains the integrity of the mucosa sheltering the epithelium [42] [52] [56].

The (bovine) immune system exemplified by the immune response to vaccinations

Active vaccinations are the induced exposure of living organism to a defined antigen. Besides the type of antigen (*e.g.* live attenuated or inactivated bacteria; partly inactivated, virulent metabolites of pathogens like toxins), the route of administration or rather the site of the primary contact with it direct the recipient's immune response. As a result, the vaccinate will acquire a local and systemic immunity against the antigen [57] [58]. The general process of the primary immune response is exemplarily described below for the mucosal IS.

Following the enteric, N or intramammary intake of the target antigen in a suitable dosage form, the mucosal epithelium is the first barrier for it. Immediately, innate defense

mechanisms act against the foreign particle. In addition to the epithelium itself as a mechanical barrier at the inductive site, the epithelial cells secret different bactericides (*e.g.* defensins) and opsonins like acute phase proteins and complement factors in contribution to the plenty of further antimicrobial peptides that are present in the mucus at the epithelial surface or within the lacteal secret of the MG [59] [60]. The coating of the antigen effects its rapid recognition by phagocytes, which are summoned by epithelial chemokines [61]. For instance, typical chemokines to call for neutrophilic granulocytes are CXCL3 and CXCL5, and they can also be attracted by CCL20 [62] [63]. With the aid of pattern recognition receptors (PRRs), especially toll-like receptors (TLRs) that are expressed by antigen-presenting cells (APCs) and also by epithelial cells among the non-immune cells, defined molecular patterns of a pathogen (PAMPs) are identified and may be decisive for the exclusion of harmless commensal microbiota [64] [65]. Apart from the "crosstalk" between epithelial cells and immune cells in the underlying connective tissue (e.g. small intestine lamina propria), APCs like dendritic cells (DCs) transmit the captured and processed antigen to lymphatic tissues. These may be the mucosa associated lymphatic tissue (MALT) including lymphoid follicles. Exemplarily, aggregated lymphoid follicles form the Peyer's patches related to the gut. A further way to induce the mucosal immunity by the APCs is their migration to the next lymph node (LN, *e.g.* the SLN next to the MG) by lymphatic transport [66]. In the lymphoid organ, the interface between innate and aquired IS, naïve T- and B-lymphocytes are activated by the cargo of the APCs expressed as major histocompatiblity (MHC)-II / peptide complex. Firstly, within the T-cell zone, CD4+ T-cells receive the antigen signal by complex interactions between different surface receptors, especially, with participation of the T-cell receptor (TCR). These are accompanied by the secretion of defined cytokines like IL12 and IL4 that influence the nature of the T-cell differentiation resulting in effector cells of the T_H1- or the T_H2-type. While T_H1-cells emphasize a stronger cellular immune response, the T_H2-cells support a stronger humoral immunity. A smaller part of the T helper (T_H)-cells differentiate to memory cells that are important for a rapid immune response to the same antigen repeatedly encountered following multiple vaccinations [67]. The B-cells, which shape the lymphoid follicles, take the antigen presented by the APCs up with their B-cell receptor (BCR). Generally, the promptly strong proliferation of the B-cells is stimulated with the aid of the activated T_H-cells. Besides the interplay of important surface receptors, the cytokines secreted by the T_H-cells coordinate the further differentiation and class switch of the B-cells [68] [69] [70]. As a result, activated T- and B-lymphocytes leave the lymphatic tissue and circulate in the body to home preferentially to the effector site. This is the tissue, wherein the induction of the immune response started by the first contact to the antigen. Furthermore, the activated lymphocytes (Lym) colonize other sites of the body

sharing the same conditions of entrance, such as the expression of defined surface receptors [63] [69] [71]. Exemplarily, the nasopharynx-associated lympoid tissue (NALT) derived effector cells bear homing molecules like CCR7 and CD62L, which ensure their more systemic admission to different body sites, *i.a.* to the MG, too [66]. After arriving there, the B-cells develop to Ab producing plasma cells, mainly of antigen specific sIgA because "the adaptive humoral immune defense at mucosal surfaces is to a large extent mediated by sIgA antibodies" [69].

The general process of an immune response, that is described above, illustrates that the active IM due to an antigen (vaccine) will elicit characteristic immune cell profiles and it will effect also a distinctive cell signaling.

Aim of the study

Dairy cows were repeatedly vaccinated against *C. diff.* for the long-term production of milk enriched with anti-*C. diff.* specific sIgA. The feasible output of the pathogen specific Ab in milk and the treated cows' immune responses to the numerous vaccinations were under examination.

The vaccination study was accompanied by a close monitoring of the treated cows' health and of the major components in their milk. The immune reactions of the vaccinated cows were firstly measured on their levels of milk sIgA specific for *C. diff.* using the ELISA. The primary assessment of the treated cows' anti-*C. diff.* specific sIgA production in milk spawned their different susceptibility against the vaccinations. The roots of their different immune responsiveness was subsequently identified. Therefore, besides of the measurement of anti-*C. diff.* specific sIgA in milk, this parameter was also quantified in blood. In addition, the total IgA contents in both body fluids were analyzed by use of the ELISA. To compare the IS of low and high responding cows, the cells out of milk and blood were counted differentially with aid of the light microscopy. On the molecular biological level, the gene expressions of selected immune cell markers and cell products, being relevant to perform the innate and acquired immune response to an antigen, were analyzed via RT-qPCR. Furthermore, following the sacrifice of the vaccinated cows, the colonization of the tissues, MG and SLN, with defined Lym was examined IHC.

It was expected that the continuous challenge of the test cows with *C. diff.* would initially trigger the increased production of the pathogen specific sIgA in the milk of all treated cows. Further, the ongoing treatment would guarantee the maintenance of the presumably heightened levels of production for this Ab. As reason for the different immune reactivity of the vaccinated cows classified as *low responder* (LR) and *high responder* (HR) was assumed that the LR would have more emphasized innate defense mechanisms than the HR. The exposure of the biological facts being responsible for an increased susceptibility to *C. diff.* vaccines could be of interest to select the appropriate dairy cows in case of intended commercialization of the milk Ab production.

Materials and Methods

Test animals

The government of Upper Bavaria approved the implementation of the animal trail (AZ 55.2-1-54-2532.6-17-2012). Nine primiparous *Brown Swiss* cows were selected as test animals for the anti-*C. diff.* vaccination study. At the outset of the treatment, they produced 21.3 $\pm$ 1.5 L / day being 28 $\pm$ 4 days in lactation (DiL). These cows were tethered in a separate byre. Their diet comprised a daily basic ration consisting of 22 kg of corn silage (with 33% dry matter), 10 kg of grass silage (with 40% dry matter), 2 kg of hay and 2 kg of high-protein crushing rape and soya (deuka Kompopur 404; Deutsche Tiernahrung Cremer, Düsseldorf, Germany) for energy equilibration. Additionally, 125 g of mineral mix (Josera, Kleinheubach, Germany) were supplemented. The feeding of 0.5 kg concentrate (deuka MK 194-UDP; Deutsche Tiernahrung Cremer) per delivered Liter milk covered the needs of their milk performance. All cows had water access *ad libitum*. After exit the trial, the treated cows were immediately slaughtered and, excepted from MG and SLN tissue samples of six cows, the carcasses were entirely disposed without touching the food chain. The dates of their passing depended on their total milk performance and, especially, on their anti-*C. diff.* sIgA production in milk.

The test animals were subdivided into *low responder* (LR, n = 4) and *high responder* (HR, n = 5) depending on their immune responsiveness to the vaccination against *C. diff.* and its toxins, TcdA and TcdB. The immune responsiveness was exclusively measured by the production of anti-*C. diff*. IgA in milk (µg / mL) on average not later than passing or to the end of the study. The threshold was set at 8.00 µg / mL anti-*C. diff*. IgA in milk. The classification of each single test animal are listed in Table 1.

Table 1: Classification of the treated cows into LR and HR

cow number	DiL up to the date of classification*)	average anti-*C. diff.* IgA (µg / mL)	assigned group
2	330	0.52	LR
6	341	1.99	LR
7	337	2.69	LR
3	501	5.19	LR
10	502	8.71	HR
5	510	10.65	HR
4	526	10.77	HR
1	501	10.94	HR
8	492	12.48	HR

**) The test cows were categorized into LR and HR following 505 DiL on average excepted from the cows 2, 6 and 7, that were early sacrified due to their very low anti-C. diff. specific sIgA contents in milk.*

Control animals

First lactating *Brown Swiss* cows out of the dairy herd of the research station Veitshof of the Technical University of Munich (Freising, Germany) formed the reference groups for different aims of analysis. Thirty cows were selected for the examination of the milk components, five of those served additionally as donors for milk and blood cells. The comparison group for the IHC staining of MG tissue consisted of three untreated cows of the breed *Simmental*, which were sacrificed in connection with a former study of our chair [72]. The feeding and watering of all control animals complied with the conditions of the test animals.

Vaccination

Every vaccination was performed by a veterinarian, who controlled also the health of the vaccinates immediately prior to the vaccination and on the following day. Generally, only healthy cows were vaccinated. The different *C. diff.* vaccines were produced by IDT Biologika (Dessau-Roßlau, Germany). Their components were formaldehyde-inactivated whole *C. diff.* cells (strain VPI 10463) and the partly disabled toxins, TcdA and TcdB, prepared from the *C. diff*. culture filtrate. The vaccine *MucoCD-N* (2 ml) was nasally given to each nostril biweekly. The two vaccine batches (A and B) of *MucoCD-I* (2 ml) were applied for injection either on the percutaneous (PC) route close to both SLN or on the subcutaneous (SC) and intracutaneous (IC) routes in the area of the scapula. *MucoCD-I* batch A was used only once in the treatment week (TW) 3 due to its inbalanced toxin / toxoid content resulting in poor toleration by the treated cows. The different application routes of *MucoCD-I* batch B

available from TW 17 in combination with *MucoCD-N* and the additional use of incomplete Freund's adjuvant (iFA) are summarized in Table 2.

Table 2: Application of the vaccines *MucoCD-I* batch B and *MucoCD-N*

TW		17	19	21	23	25	27	29	31	33	35	37	39	41	…
Application route	N	x	x	x	x	x	x	x	x	x	x	x	x	x	…
	PC	x		x		x		x		x		x		x	…
	SC			x											
	IC						x		x*		x*		x*		…

TW = treatment week; PC = percutaneous; SC = subcutaneous; IC = intracutaneous; "x" indicates vaccine application route used per TW; "" labels the additional use of incomplete Freund's adjuvant*

Notes to the investigation period

The investigation period differed between the aims of analysis. The most examinations presented in the study at hand, such as the differential counting of milk and blood cells, the gene expression analysis and, in part, the measurements of total and anti-*C. diff.* sIgA in the body fluids, were predominantly focus on the treatment period up to TW 31. Whereas the analysis of milk yield and components was extended to cover the 305-days lactation period of the treated cows. The anti-*C. diff.* sIgA analysis in milk were continued for the vaccination period until the sacrifice of the test cows. Overall, the IM period against *C. diff.* lasted 18 months (19 / 09 / 2012 - 06 / 02 / 2014). Thus the data of the anti-*C. diff.* sIgA contents in milk were available to assess the immune reactivity of the most test animals after one year of treatment. Generally, only samples of healthy cows were taken into account for study.

Milk yield recording and milk sampling

Treated Cows

The TRU-Test milk meter (Lemmer-Fullwood, Lohmar, Germany) was used to deposit a half liter of milk into a wide-mouth polyethylene plastic bottle weekly during the morning milking for milk sample collection. The milk yield was recorded bi-weekly. The measured quantities of milk were calculated as energy corrected milk (ECM) using the formula:

ECM = milk yield-kg[(0.38*milk fat-%) + (0.21*milk protein-%) + 1.05] / 3.28* [73].

Every milk sample was divided into three portions, which were immediately refrigerated. The first portion, 50 mL of milk supplemented with the preservative (acidiol), was used to analyze the major milk components. The Milchprüfring Bayern e.V. (Wolnzach, Germany) was commissioned with the milk testing encompassing the SCC with optical fluorescent technique

(*Fossomatic-FC* device, FOSS, Hamburg, Germany) and the analysis of milk fat and protein with the MilkoScan-FT-6000 device (FOSS, Hamburg, Germany) followed by the data transformation by the method of Fourier. The second portion, 20 mL of milk splitted into 10 mL aliquots, was sampled for the measurement of the Ig concentrations. These aliquots were defatted by centrifugation (4,000 xg, 4 °C, 15 min), aliquoted in 2-mL tubes afterwards and, finally, stored at -20 °C. The third portion, 250 mL of milk, was used to extract the included somatic cells. After degreasing the milk by centrifugation (1,800 xg, 4 °C, 15 min) and removal of the supernatant, the gained cell pellet was resolved in 25 mL phosphate buffered saline (PBS, pH 7.4). Following twice washing with PBS again, but with lesser rotation speed during the centrifugation (400 xg, 4 °C, 10 min), the cell pellet was resolved in 10 mL PBS. This cell suspension was filtrated by use of a cell strainer (mesh size: 100 µm pores, Corning Inc., Corning, NY, USA). Next, the filtrate was centrifuged (400 xg, 4 °C, 10 min), the supernatant was cleared away and the remaining cells were aspirated with 250 µL PBS. The gained cell extract was stored on ice until the milk smears and the RNA extraction were carried out.

Control cows

Unlike the test animals, the procedure differed to determine the milk quantities of the 30 control cows and to sample their milk. The data concerning milk yield and components of these control cows were recorded in the course of the regular analysis for the Bavarian Dairy Herd Improvement Association (LKV Bayern e. V., Munich, Germany). Furthermore, the milk of this control group (Ctr) was once only collected to measure the content of total and anti-*C. diff.*-sIgA.

Blood sampling

Blood of the treated cows was sampled weekly up to TW 8 and then biweekly for the remaining treatment period. Blood samples of the Ctr were taken only every two weeks. The jugular vein was punctured to collect the blood in two 9 mL vacuettes pre-coated with EDTA (Greiner Bio-One GmbH, Frickenhausen, Germany). The blood samples were instantly placed on ice. The admixture of 100 µL of 0.3 M EDTA [33.5 g of Titriplex III (Merck KgA, Darmstadt, Germany) solved in 300 mL bi-distillated water and supplemented with 1% acetylsalicylic acid (Merck KgA, Darmstadt, Germany)] ensured the anticoagulation of the blood taken. The plasma out of 9 mL blood used for the sIgA measurements was separated by centrifugation (2,000 xg, 4 °C, 15 min), split into 2 mL aliquots that were stored at -20 °C afterwards. To extract the peripheral blood leukocytes (PBL), 7 mL of the EDTA-blood were transferred into a 50-mL tube first and then 14 mL lysis buffer [8.3 g of NH_4Cl (Carl Roth

GmbH & CoKG, Karlsruhe, Germany), 0.37 g of Titriplex III (Merck) and 1.0 g of KCl (J.T. Baker Chemical Co. Phillipsburg, NJ, USA) solved in 1 L bi-distillated water, pH 7.4] were added. The tube was carefully inverted three times and placed on ice. The lysis of the erythrocytes was visible by the dark red coloring of the suspension. The PBL precipitated after the centrifugation (400 xg, 4 °C, 10 min). The supernatant was decanted, and thereafter, the cell pellet was repeatedly washed with 14 mL lysis buffer in each case. The PBL resuspended in 2 mL PBS were finally equally portioned into two 1.5 mL tubes. Both tubes were stored on ice until further processing to count the PBL and to extract the RNA.

Clostridium difficile specific IgA

A sandwich ELISA was used to measure the *C. diff.* specific IgA contained in the cows' milk and blood. For this, the following reagents and buffers were used: coating buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3 x $10H_2O$, pH 9.6), wash buffer (137 mM NaCl, 8 mM Na_2HPO_4 x $2H_2O$, 1.5 mM KH_2PO_4, 2.7 mM g/L KCl, 0.1% Tween 20), PBST (0.2 mM NaH_2PO_4 x H_2O, 1.2 mM Na_2HPO_4 x $2H_2O$, 0.05% Tween 20, pH 7.4), blocking buffer (2% gelatin in PBST), dilution buffer (0.2% gelatin in PBST), 3,3',5,5'-Tetramethylbenzidine (TMB) enzyme substrate mix and stop solution (2 M H_2SO_4).

A 96-well plate (Nunc MaxiSorp™, Sigma-Aldrich Chemie GmbH, Munich, Germany) was pretreated with *C. diff.* cells by application of 100 µL coating buffer (2.0×10^8 *C. diff.* cells/mL, IDT Biologika, Dessau-Roßlau, Germany) per well. The coating lasted 2 h at 70 °C first, then overnight at 4 °C, and it was ceased with 200 µL blocking buffer that incubated for 1 h at 37 °C. Four cycles of rinsing with wash buffer followed. *C. diff.* specific IgA (1.76 mg/mL, MucoVax b. v., Leiden, Netherlands) was used as standard stepwise diluted from 62.5 to 4,000 ng/µL with dilution buffer. The samples, skimmed milk or blood plasma, were diluted 1:10 to fall into the range of the standard dilutions. The control whey containing 2,998.98 ng/mL of anti-C. diff. sIgA and used as inter-assay control was diluted 1:1,000. The wells of every pre-coated plate were reserved as follows: the different standard dilutions to seven wells, pure dilution buffer used as blank to one well, duplicates of the diluted samples and the control whey to the wells remaining, and 100 µL of the liquids named before were poured into the wells. After an incubation period of 1.5 h at 37 °C, the antigen-Ab reactions were finished by repeated washing. The processed wells were covered with 100 µL of secondary Ab (1:70,000 diluted HRP conjugated sheep anti-bovine IgA (Bethyl Laboratories, Inc.; Montgomery, TX 77356, USA) protected from light for 1.5 h at 37 °C. This overlay was removed in the course of four washing cycles. To visualize the reaction products, 150 µL TMB substrate mix per well were added. The enzymatic reaction induced was running in the

dark and on a wave platform shaker for 40 min at RT. Its termination was induced by the stop solution applied (50 µL/well). The optical absorption per processed well was read off with the photometric instrument (microplate reader Sunrise™, Tecan Group Ltd., Männedorf, Switzerland) at 450 nm not later than 30 min after stopping the reaction. The anti-*C. diff.* sIgA contained in the samples were quantified in relation to the standard curve aided by the software Magellan™ V6.6 (Tecan Group Ltd., Männedorf, Switzerland).

Total IgA

The bovine IgA ELISA kit (Cat. No. E10-121) provided by Bethyl Laboratories, Inc. was used to analyze the total IgA quantities being present in the cows' milk and blood. Further materials used but unavailable in the kit were 96-well plates and the following chemicals: coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6), solution applied to washing, blocking and diluting (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0), enzyme substrate (TMB) and stop solution (0.18 M H_2SO_4). The ELISA was performed according to the manufacturer's recommendations. By using the plate reader (Sunrise™), the optical absorbance of the samples was measured at 450 nm within 30 min after pouring the stop solution. For the inter-plate calibration, the positive control as accessory component of the ELISA kit was used.

Total and differential cell counting

The total SCC in milk was part of the data received by the Milchprüfring Bayern e.V.. The total PBL counts were measured with the TC10 automated cell counter device (Bio-Rad Laboratories GmbH, Munich, Germany). For this, the PBL extracted and continuously cooled were diluted 1:10 with PBS to fall into the measuring range of the cell counter. The detailed procedure met the manufacturer's information.

In preparation of the differential counting of milk and blood cells, cell smears were produced as follows: 20 µL of the somatic cell-extract gained from milk were pipetted meandering on a standard microscope slide (76 x 26 mm, partly with frosted edge), and one droplet (3 µL) of the EDTA-blood was spread out on a microscope slide with the narrow edge of a second one. The milk and blood smears were used air dried for staining with the color solutions of the Haema-Quick-Stain Kit (Diff-Quick; Labor + Technik, Eberhard Lehmann GmbH, Berlin, Germany) considering the handling instructions of the manufacturer. Two droplets of the mounting media Eukitt® (Sigma-Aldrich) were applied to every slide with smears stained and completely air dried to fix permanently the cover slip (60 x 24 mm). The microscopic differentiation of the cells was performed with the light microscope (Axioskop 2 plus, Carl

Zeiss Microscopy GmbH, Göttingen, Germany). For this, 200 up to 300 cells were counted at 1,000-fold magnification and with oil immersion or at 400-fold magnification for the milk smears or for the blood smears, respectively, using the method of a "battlement track". The relative parts of the cell types differentiated in milk and blood were calculated in relation to the particular total cell counts of the samples.

Total RNA extraction from peripheral blood leukocytes and somatic cells obtained from milk

The RNA was extracted from milk and blood cells by use of the miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The cell pellet obtained by centrifuging (400 xg, RT, 5 min) 230 µL of somatic cell-extract or 1 mL of PBL-suspension, respectively, was lysed with 700 µl Qiazol lysis reagent (Qiagen) after disposing of the supernatant. Then the samples were homogenized using the vortex device for 5 min at RT. Either the homogenates were immediately processed or they were temporarily frozen at -80 °C until the RNA extraction was continued. The further procedure was in accordance with the manufacturer's instructions for the miRNeasy Mini Kit excepting the final volume of 25 µL of RNase-free water to solve the total RNA. Afterwards, the RNA-samples were directly cooled on ice or frozen at -80 °C until the RNA concentration was measured. The total RNA content per sample and its purity were determined at 260 nm using the NanoDrop ND-1000 (Peqlab, Erlangen, Germany). Furthermore, the RNA integrity was analyzed by aid of the 2100 Electrophoresis Bioanalyzer Instrument and the RNA 6000 Nano Assay Kit (Agilent Technologies, Waldbronn, Germany). The RNA samples were stored at -80 °C until the reverse transcription of RNA to complementary DNA (cDNA).

Reverse transcription PCR

The following samples and controls were part of every reverse transcription PCR performed: 300 ng or 1,000 ng of RNA gained from milk or blood cells used as template, respectively, and RNA extracted from the bovine tissues, MG and spleen, to control the efficiency of the RT-qPCR performed at a later time. A no-template control (NTC) was carried along replacing the template by RNase free water. Furthermore, RNA pooled from all samples to be analyzed was processed without the Reverse Transcriptase (RNase H Minus, Point Mutant; Promega, Mannheim, Germany) to check for unwanted genomic DNA contaminating the isolated RNA. 20 µL of the templates and the controls in duplicates were poured into at most 72 wells of a 96-well plate (4titude® Ltd., Berlin, Germany) and added with the master mix by the twofold volume. The master mix contained 5x buffer, 50 µM Random Hexamers

(Invitrogen Life Technologies, Darmstadt, Germany), 40 mM dNTP Mix and 200 units M-MLV Reverse Transcriptase. Normally, 60 µL of cDNA were produced per sample. Therefore, the covered multiwell plate was placed into the TPersonal Thermocycler (Biometra GmbH, Göttingen, Germany), wherein the reverse transcription PCR took place, after preheating the cover of the Thermocycler up to 104 °C. Then the following thermal cycling program was running: the annealing for 10 min at 21 °C, the transcription for 50 min at 48 °C and the final degradation of the Reverse Transcriptase for 2 min at 90 °C. Following the cooling down of the processed multiwell plate to 8 °C, it was sealed and stored at -20 °C.

Primer

By aid of the Primer-BLAST of the National Center for Biotechnology Information (NCBI, National Library of Medicine, Bethesda MD, USA. https://www.ncbi.nlm.nih.gov/tools/primer-blast/. Accessed 07 Mar 2017.), primer pairs suitable for the specific adhesion to the bovine sequences of seven reference genes and 35 target genes were designed (Table 3). Thereby, the length of the primer pairs drafted was limited to the range of 100 to 300 base pairs (bp) and their annealing temperature was adjusted to 60 °C. Desalted DNA oligonucleotides were synthesized by metabion international AG (Planegg, Germany) on the basis of the sequences of the designed primers. Their functionality were tested with cDNA of the control tissues, MG and spleen, by use of the RT-qPCR. The PCR products were examined via separation by gel electrophoresis to check the bp length and specificity of their adhesion (exclusion of undesired by-products). The optimization of the primer annealing resulted in the following annealing temperatures deviating from the intended 60 °C: 58°C for *CCR6*; *IL10*, 62°C *for CD126*, *CD163, PIGR*; and 64°C for *SUZ12*.

Table 3: Primer for the RT-qPCR

Gene name (alternative names) (*acronym*)	**NCBI[1] reference sequence number**	**Primer sequences (5' → 3'), forward[2] and reverse[3]**	**Primer length [bp][4]**
Reference genes			
Actin gamma 1 (*ACTG1*)	NM_001033618	AACTCCATCATGAAGTGTGAC GATCCACATCTGCTGGAAGG	234
Polyubiquitin (*UB3*)	Z18245	AGATCCAGGATAAGGGAAGGCAT GCTCCACCTCCAGGGTGAT	198
SUZ12 polycomb repressive complex 2 subunit homolog (*SUZ12*)	NM_001205587.2	AGCCATGCAGGAAATGGAAG GCAAGAGGTTTGGCTATAGG	183
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	NM_001034034.2	GTCTTCACTACCATGGAGAAGG TCATGGATGACCTTGGCCAG	197
H3 histone, family 3A (*H3F3A*)	NM_001014389.2	ACTTGCTACAAAAGCCGCTC ACTTGCCTCCTGCAAAGCAC	232
Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase-activation protein zeta (*YWHAZ*)	NM_174814.2	CAGGCTGAGCGATATGATGA GACCCTCCAAGATGACCTAC	141
Cytokeratin 8 (*KRT8*)	NM_001033610.1	TGGTGGAGGACTTCAAGACC CGTGTCAGAAATCTGAGACTGC	215
Cell surface receptors			
T-cell surface glycoprotein CD3 delta chain (*CD3δ*)	NM_001034033.2	CTGCCCTTCTCTCCCAAGTG ACAGTGGCAATGATGTCGGT	301
T-cell surface glycoprotein CD4 (*CD4*)	NM_001103225.1	TCCCAATCCTGGGTCACCT ATCCAGGGTCAGGGTTCCA	159
T-cell surface glycoprotein CD8 beta chain (*CD8β*)	NM_001105344.2	TTCTGGTGTCCTTGGGTGTG ATTTCCACGGCTGAGTGTGT	189
B cell surface marker CD19 (*CD19*)	NM_001245998.1	CTGTGCTCCACCTAACAGCA TGACCAGGGAGGTATGGGAG	110
B- and T-cells related ADP-ribosyl cyclase 1 (*CD38*)	NM_175798.3	TCGTTGGAGAACGCCTTTGA GAGGAGCCAGAGCATGAGTC	137
Activated B- and plasma cells associated interleukin 6 receptor subunit alpha (*CD126*)	NM_001110785.1	GGGATCAGATGACAGGCTCG GTAACACGGCCTTGGTGGTA	258
Monocytes and macrophages assigned scavenger receptor cysteine-rich type 1 protein M130 (*CD163*)	NM_001163413.1	CGAGTCCCATCTTTCACTCTG AGTGAGAGTTGCAGAGAGGTCC	285

Gene name (alternative names) (*acronym*)	**NCBI[1] reference sequence number**	**Primer sequences (5' → 3'), forward[2] and reverse[3]**	**Primer length [bp][4]**
Toll like receptor 2 (*TLR2*)	NM_174197.2	CATTCCTGGCAAGTGGATTATC	201
		GGAATGGCCTTCTTGTCAATGG	
IgG Fc receptor (*FcRN*)	AF141017.1	GAGCTGGCTCCTTGGATCTC	194
		ATACCAGGATTCCCGGAGGT	
Polymeric immunoglobulin receptor (*PIGR*)	NM_174143.1a	TGCGACCAGAACAGCCAG	278
		CCAGCAGCGTCCTTCACA	
Cytokines			
Interferon gamma (*IFNγ*)	NM_174086.1	TGGCAGCTCTGAGAAACTGG	199
		CAGGCAGGAGGACCATTACG	
Interleukin 1 beta (*IL1β*)	NM_174093.1	CAGTGCCTACGCACATGTCT	209
		AGAGGAGGTGGAGAGCCTTC	
Interleukin 2 (T-cell growth factor) (*IL2*)	NM_180997.2	TCAAGCTCTACGGGGAACAC	146
		GTAGCGTTAACCTTGGGCAC	
Interleukin 6 (Interferon beta 2) (*IL6*)	NM_173923.2	TGGTGATGACTTCTGCTTTCC	109
		AGAGCTTCGGTTTTCTCTGG	
Interleukin 10 (Cytokine synthesis inhibitory factor) (*IL10*)	NM_174088.1	AGCTGTATCCACTTGCCAACC	119
		TGGGTCAACAGTAAGCTGTGC	
Interleukin 12 subunit beta (Natural killer cell stimulatory factor 2, Cytotoxic lymphocyte maturation factor 2, p40) (*IL12β*)	NM_174356.1	GGTTTTCCCTGGTTTTGCTGG	175
		ACCTCACTGCTCTGGTCTGA	
Tumor necrosis factor alpha (Cachectin) (*TNFα*)	NM_173966.3	CCACGTTGTAGCCGACATC	108
		ACCACCAGCTGGTTGTCTTC	
Transforming growth factor beta 1 (*TGFβ1*)	NM_001166068.1	CCTGGACACCAACTACTGCT	185
		CCAGGACCTTGCTGTACTGT	
Chemokines and their receptors			
Chemokine (C-X-C motif) ligand 3 (Epithelial cell inflammatory protein) (*CXCL3*)	NM_001046513.2	TACAGAGCGTGAAGGTGACG	164
		CCCTCTAGGTCAGTTGGTGC	
Chemokine (C-X-C motif) ligand 5 (Chemokine (C-X-C motif) ligand 6, Granulocyte chemotactic protein 2) (*CXCL5*)	NM_174300.2	TTGTGAGAGAGCTGCGTTGT	112
		ACTTCCACCTTGGAGCACTG	
C-X-C motif chemokine receptor 2 (Interleukin 8 receptor beta) (*CXCR2*)	NM_001101285.1	CAACACTGACCTGCCCTCTA	197
		CCAGGTTCAGCAGGTAGACA	

Gene name (alternative names) (*acronym*)	**NCBI[1] reference sequence number**	**Primer sequences (5' → 3'), forward[2] and reverse[3]**	**Primer length [bp][4]**
C-X-C motif chemokine ligand 8 (Interleukin-8, Neutrophil activating peptide 1) (*CXCL8*)	NM_173925.2	AAGAATGAGTACAGAACTTCGATGC	160
		GTTTAGGCAGACCTCGTTTCC	
C-C motif chemokine ligand 5 (T-cell-specific protein RANTES) (*CCL5*)	NM_175827.2	TCCATGGCAGCAGTTGTCTT	129
		TTCAGGTTCAAGGCGTCCTC	
C-C motif chemokine ligand 20 (Macrophage inflammatory protein 3 alpha, MIP-3-alpha) (*CCL20*)	NM_174263.2	CTTGTGGGCTTCACACAGC	115
		GTTTCACCCACTTCTTCTTTGG	
C-C motif chemokine receptor 6 (*CCR6*)	NM_001194961.1	TCATGAAGGACCTGTGGTGC	132
		TGAAGGAAGACGGGTTGTCG	
C-C motif chemokine ligand 28 (*CCL28*)	NM_001101163.1	TGACGGGGATTGTGACTTGG	184
		CCGATGTGCCCCTTTACTGT	
Complement proteins and their receptors			
Complement C1q A chain (*C1QA*)	NM_001014945.2	CGTTGGACCGAATTCTGTCTC	224
		TGCTGTTGAAGTCACAGAAGCC	
Complement C3 (*C3*)	NM_001040469.2	AAGTTCATCACCCACATCAAG	191
		CACTGTTTCTGGTTCTCCTC	
Complement C3a receptor 1 (C3a anaphylatoxin chemotactic receptor 1) (*C3AR1*)	NM_001083752.1	CCCTCCATCATCATCCTCAAC	167
		CACATTACCAAAGCCACCACC	
Complement C5a receptor 1 (C5a anaphylatoxin chemotactic receptor 1) (*C5AR1*)	NM_001007810.3	ATACCGTCCTTTGTGTTCCG	158
		ATTGTAAGCGTGACCAGCG	
Antimicrobial peptides			
Lactotransferrin (Lactoferrin) (*LF*)	NM_180998.2	CGAAGTGTGGATGGCAAGGAA	215
		TTCAAGGTGGTCAAGTAGCGG	
Lactoperoxidase (*LPO*)	NM_173933.2	TGGCTGTCAACCAAGAAGC	134
		TGAGGCTCGAAAATCTCCC	
Lysozyme 1 (Lysozyme C) (*LYZ1*)	NM_001077829.1	AAGAAACTTGGATTGGATGGC	185
		ACTGCTTTTGGGGTTTTGC	
Tracheal antimicrobial peptide (*TAP*)	NM_174776.1	AGGAGTAGGAAATCCTGTAAGCTGTGT	113
		AGCATTTTACTGCCCGCCCGA	

1) NCBI = National Center for Biotechnology Information as source of the used gene database; 2) forward primer sequence in the upper line; 3) reverse primer sequence in the lower line; 4) bp = base pairs

Gene expression measurements

The thermal cycling of the RT-qPCR was done with the Rotor-Gene Q cycler (Rotor-Disc 72; Qiagen) controlled by the accessory software version 6.0.38 (Corbett Research 2004, aquired by Qiagen). In general, cDNA of samples and controls produced within one multiwell-plate were used within the same PCR-assay to analyze the gene expression. At most 72 strip tubes (0.1 mL, Qiagen) suitable for the Rotor-Disc were set into a cool plate and then filled with 1 µL of the cDNA template and 9 µL of the reaction mixture including 5 µL of the SsoFast EvaGreen Supermix (Bio-Rad Laboratories GmbH) plus 400 nM forward and reverse primers filled up with DNase-free water to the final volume of the mix. The strip tubes filled and capped were put in the cycler and the RT-qPCR was started being subject to the following temperature profile: heating-up for 30 sec at 98 °C, 40 cycles alternating between denaturation for 5 sec at 95 °C and primer annealing for 20 sec at the primer specific temperature for adhesion. The RT-qPCR finished with generating the melting curve up to 95 °C in steps of one degree. The PCR products were preserved frigidly at 8 °C.

RT-qPCR data processing

The output of the Rotor-Gene Q software (Qiagen) displayed on completion of the RT-qPCR and the MIQE Guidelines [74] were taken into account for data evaluation. The calculation basis for the relative quantification of the PCR products was the cycle number (Cq) defined as the take-off point for the detection of the specific fluorescence signal. In the next step, the inter-"plate" or rather inter-PCR-assay calibration was performed. Therefore, the average Cq of the positive controls per every single PCR-assay was subtracted from their overall mean per analyzed gene expression based on all Cq values of the positive controls. The difference resulting was transmitted to the Cq values of the examined samples per well to balance possible differences between the PCR-assays conducted per gene. Following the calibration of the Cq values, a set of reference genes for normalization was determined by aid of the GenEx 'Normfinder' tool (GenEx software version 6.1; MultiD Analyses AB, Gothenburg, Sweden). As a result, seven reference genes were ascertained among the raw data of all examined genes. Among them, the reference gene *KRT8* was solely used for the normalization of the bovine MEC (bMEC) related genes to be found in milk samples. The normalization was implemented by subtraction of the average Cq value of all reference genes considered from the target gene specific Cq. Its result, the ΔCq value, was the basis to calculate the relative changes in gene expression to designated dates ($\Delta\Delta$Cq values) according to the method described elsewhere [75]. The following assessment of the gene regulations based on the scale of the $\Delta\Delta$Cq range during the 31 TWs. The applied grading is

summarized in Table 4. The TW with the maximal significant up- or down-regulation of each analyzed gene expression towards the belonging baseline, decided about its grading (0, +, ++, +++) regardless of the group (HR, LR, Ctr), in which the maximal change of gene expression was determined.

Table 4: Classification of the gene regulation expressed as ΔΔCq range

Defined grades of the gene regulation		ΔΔCq range
0	non-regulated	$\|x\|<1$
+	weak	$\|x\|\geq 1$ up to $\|x\|<3$
++	strong	$\|x\|\geq 3$ up to $\|x\|<5$
+++	very strong	$\|x\|\geq 5$

The grades of gene regulation are translated in words as follows: "0" for non-regulated, "+" for weak regulated, "++" for strong regulated, "+++" for very strong regulated [76].

Tissue Sampling and Preparation

The test cows were slaughtered in the abattoir of the Bavarian State Research Center for Agriculture location Grub (Poing, Germany). The tissues, MG (parenchyma of the upper body) and SLN (cortex and paracortex), were collected within half an hour *post mortem*. Then tissue pieces of less than 1 cm^3 were prepared, snap frozen in liquid nitrogen and placed on dry ice within an aluminium sachet. Finally, the tissue samples were stored in the deep freeze (-80 °C). In preparation of the cryosections, the tissue was mount with the Tissue-Tek O.C.T. compound (Sakura Finetek, Staufen, Germany) on the bracket accessory to the microtome cryostat (HM505 E, Microm, Walldorf, Germany). The embedded tissue was sectioned to the size of 5 µm by use of the microtome cryostat and the cryosections were subsequently transferred to poly-L-lysine (0.1% (w/v) in H_2O, Sigma-Aldrich, Taufkirchen, Germany) coated glass slides (76 x 26 mm). After drying them at room temperature (RT), they were fixed in -20 °C cold acetone-methanol (1:1, J. T Baker, Deventer, Netherlands) for 15 min. Following a further dry period (RT), the processed glass slides were wrapped in aluminium foil and stored in a 50 mL tube at -20 °C until use.

Antibodies

The set of Ab used for the IHC were purchased from Bio-Rad AbD Serotec GmbH (Puchheim, Germany). The monoclonal mouse Ab, anti-bovine CD4 (clone CC30, isotype IgG1) and anti-bovine CD8 (clone CC63, isotype IgG2a), were applied to the primary marking of T_H-cells and cytotoxic T (T_C)-cells, respectively. To visualize these antigen-Ab binding, the polyclonal goat anti-mouse IgG:FITC (isotype IgG) was utilized as secondary Ab.

The tracing of the IgA+ antibody secreting cells (ASC) was performed by direct IHC using the polyclonal sheep Ab anti-bovine IgA:FITC (isotype IgG). For the titration of the Ab, PBS was used as diluent. The Ab dilutions applied to the IHC of MG and SLN tissues are specified in Table 5.

Table 5: Antibodies for the IHC staining

primary Ab / secondary Ab	used Ab dilutions (in PBS)	
	for MG tissue	for SLN tissue
anti-bovine CD4 / anti-mouse IgG:FITC	1:150 / 1:50	1:150 / 1:40
anti-bovine CD8 / anti-mouse IgG:FITC	1:200 / 1:100	1:200 / 1:100
anti-bovine IgA:FITC / -	1:400	1:500

Ab = antibody; IHC = immunohistochemical; MG = mammary gland; SLN = supramammary lymph nodes

Immunohistochemical staining

The glass slides loaded with cryosections of the bovine spleen used as positive control for each Ab to analyze and those of the MG and SLN were defrosted at RT. After their framing with liquid blocker (Super Pap Pen, Daido Sangyo, Tokyo, Japan), they were rehydrated by triple washing with Tris-buffered saline (TBS, for 1, 5 and 9 min at RT) on a plate shaker (PS). Unspecific binding sites within the tissues were blocked with 10% (v/v) goat serum (Sigma-Aldrich, Taufkirchen, Germany) in PBS for 1 h at RT in a humid chamber (HC). Then the blocking solution was removed and the primary Ab dilution was spread on each section (Table 5) except for two covered with PBS to control the auto-fluorescence of the tissues. The incubation lasted over night at 4 °C (in HC) and the Ab solution was washed away by three TBS baths (shaded, on PS). To accomplish the indirect IHC, the fluorescein-labeled Ab dilution was applied to the tissue sections (Table 5). After its incubation for 45 min at RT (shaded, in HC), the surplus Ab solution was removed by triple washing with TBS. DAPI (1:1000 in PBS, Sigma-Aldrich, Taufkirchen, Germany) was used to stain the cell nuclei within 15 min at RT (shaded, in HC). Residues of the DAPI solution were cleared away in the course of four TBS baths (shaded, on PS, for 1, 5, 5 and 9 min at RT). To fix the Ab bindings, 10% (w/v) paraformaldehyde (Merck, Darmstadt, Germany) in PBS was spread on each section for 15 min at RT (shaded, in HC). Excessive fixation buffer was removed afterwards using TBS for 5 min at RT (shaded, on PS). Finally, two drops of fluoromount aqueous mounting medium (Sigma-Aldrich, Taufkirchen, Germany) were put on the glass slides loaded with the IHC stained tissue slices and topped with coverslips (60 x 24 mm). The preservation was completed by sealing the edges of the coverslips with clear nail polish.

Thus the stained tissue slices were protected against desiccation during their storage (shaded, 4 °C) until their microscopic examination.

Quantitative Evaluation of Data

The microscopic analysis of the IHC stained tissue slices was performed by aid of the Axioskop 2 Plus fluorescence microscope, the digital camera AxioCam MRc and the Axiovision 3.1 imaging software (Carl Zeiss Microscopy, Göttingen, Germany). At first the negative and positive controls were evaluated and, afterwards, ten slices of the MG and SLN tissues, each at 200 x magnification. To quantify the immune cells of interest, 15 photographs per slice were taken during the meandering screening of the slice. Thereby, the image areas were randomly selected and photographed twice within the relevant fluorescence channels, the FITC channel (green color, 530 nm) for the Ab specific staining and the DAPI channel (blue color, 460 nm) for the cell nuclei marking. Both photographs of the same image area were put on top of each other using the free photo editing software PhotoScape 3.7 (created by Mooi Tech; http://www.photoscape.org). Thus the FITC and the DAPI signals could be shown at once. Cells appearing with a blue shining nucleus surrounded by a bright green circle were evaluated as Ab specific stained cell and counted. The total count of positive events per picture (0.1462 mm^2) was converted to the cell count per 1 cm^2 image area used as data basis for the calculation of the average cell count per slice.

Statistical Analysis

The measurement results of the anti-*C. diff.* specific and total sIgA contents in milk and blood as well as the quantified relative changes of gene expressions ($\Delta\Delta$Cq values) were marked and sorted according to the date of treatment and their affiliation to the animal groups (HR, LR and Ctr). To find treatment effects and differences among groups during the treatment period, the statistics were performed by use of the MIXED procedure model in SAS/STAT® 9.22 (2010 SAS Institute Inc., Cary, NC, USA). Prerequisites of the statistical computations were that the data read in passed the log-likelihood calculation and met the convergence criteria. The estimated least square means (LSM) with belonging standard errors (SD) were the data output and basis of the following evaluation considering the significance level of $P < 0.05$. The software SigmaPlot 11.0 (2008 Systat Software, Inc., San Jose, CA 95131, USA) was applied to calculate the Pearson Product Moment Correlation coefficients (*r*) and to analyze the averaged Lym counts within the IHC stained tissues. Because the Lym counts and the ratio (CD4+ : CD8+) T-cells determined within the MG tissue were missing normality,

the Kruskal-Wallis One Way ANOVA on Ranks was applied as pairwise multiple comparison procedure for the statistics. The CD4+ and CD8+ T-cell counts and the belonging ratio, only examined within the SLN tissue of the treated groups, were tested by use of the Mann-Whitney Rank Sum Test following the failed normality tests. The IgA+ ASC counts of the SLN passed the testing of normality and were analyzed applying the t-test. The results outputted by SigmaPlot 11.0 were the median with the belonging lower and upper quartiles or the mean and standard deviation (SD) depending on the statistical method of testing. For all statistics performed, the significance level was set at $P < 0.05$ and results with $P < 0.01$ were defined as highly significant.

Results and Discussion

Physical condition of the test animals and the vaccine tolerability

The health of some test animals was impaired at the outset of the treatment without any link to it. One third of the treated cows (LR-2, HR-4, HR-10) were attested mastitis immediately prior to the treatment start or rather within the TW 1. In addition, the cow HR-10 came down with mastitis at two further dates (TW-16, TW-26). Four cases with diseases of the limbs like bursitis and lameness emerged up to TW 3. In context to the treatment, the vaccine batch A of *MucoCD-I* injected at TW 3 caused adverse reactions [77]. The majority of the vaccinated cows suffered subsequently from fever, reduced feed intake and rumen turnover. The injection sites on their udder showed clearly the cardinal signs of inflammation [78]. The milk of three treated cows was hemolytic. The cow HR-5 developed additionally a ketosis in TW 4. These local and systemic inflammatory reactions of the vaccinated cows were provoken by the insufficient detoxification of the vaccine batch A due to a production fault of the vaccine manufacturer, IDT Biologika. All other vaccines applied were well tolerated by the test animals [79].

Milk yield and the gross milk composition

The milk production was assessed in relation to the lactation stages "early", "mid" and "late", each with 100 DiL. The average values for the ECM yield and for selected milk components (protein, fat and SCC) per lactation stage are summarized in Table 6. Significant differences between the groups existed within the early lactation period. The milk production of the treated cows was inferior to the control animals by approximately 20% concerning ECM yield, milk protein and fat. These reduced production outputs resulted probably from the health disorders of the test animals at start of treatment and following the application of *MucoCD-I* batch A in TW 3. Mastitis, lameness and, especially, ketosis were associated with high milk production losses in dairy cows [80]. During the ongoing lactation, the SCC in HR's milk stuck out with more than 126,000 cells/mL to the mid lactation period exceeding the LR's SCC sixfold. Few individual SCC above 200,000 cells/mL were responsible for the elevated HR's SCC on average. Within each treated group, the milk production increased significantly towards the first 100 DiL as measured on the LR's ECM yield and the milk protein contents of LR and HR in mid lactation. Compared to the mid lactation, the last third of lactation revealed an excellent persistence of the treated cows' milk production. To this late lactation period, the HR could enhance their milk quantity towards the early lactation by 14% and, additionally,

their milk was significantly more concentrated with protein and fat by 36% and 27%, respectively. In sum, the treatment did not influence the macronutrient composition of milk except for the use of *MucoCD-I* batch A [79]. A comparative analysis of milk components from cows hyperimmunized with a multivalent bacterin (S100) and non-immunized cows led to the same outcome [81].

Table 6: Milk yield and the components, protein, fat and SCC

lactation stage	group	ECM yield (kg/d)			milk protein (kg/d)			milk fat (kg/d)			SCC (x1,000/mL)		
		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
early	Ctr	30.6	*1.3*	a	1.00	*0.05*	a	1.29	*0.10*	a	98.1	*28.9*	
	LR	23.7	*0.9*	b	0.76	*0.03*	b	0.96	*0.07*	b	60.4	*21.6*	
	HR	25.1	*0.9*	b	0.78	*0.03*	b	0.96	*0.07*	b	35.3	*20.1*	
mid	Ctr	31.9	*2.8*		1.07	*0.10*		1.43	*0.21*		33.0	*64.7*	ab
	LR	28.9	*1.0*	**	1.03	*0.03*	**	1.08	*0.07*		21.0	*22.0*	a
	HR	26.6	*0.9*		0.96	*0.03*	**	1.15	*0.07*		126.6	*21.2*	b**
late	Ctr	27.7	*1.4*		1.04	*0.05*		1.21	*0.11*		81.7	*32.3*	
	LR	27.3	*1.0*	*	1.00	*0.04*	**	1.04	*0.08*		22.9	*23.9*	
	HR	28.7	*0.9*	**	1.06	*0.03*	**	1.22	*0.07*	**	62.6	*21.2*	

The lactation stages are defined as follows: "early" for < 100 days in lactation (DiL), "mid" for 101 up to 200 DiL, "late" for > 201 DiL. The compared groups are the control group (Ctr, n = 30), low responder (LR, n = 4) and high responder (HR, n = 5). Milk yield is specified as energy corrected (ECM). The mapped values, including the SCC, are presented as LSM ± SD at the respective lactation stage. The lowercase letters (a, b) characterize significant differences (P < 0.05) between groups within the same lactation stage. Asterisks mark significant differences with P < 0.05 (*) or P < 0.01 (**) over time with regard to the early lactation stage within the same group [79].

Clostridium difficile specific IgA in milk and blood

The analysis of anti-*C. diff.* IgA in milk and blood of cows, being still untreated or rather selected as controls, spawned the natural antibody titers against *C. diff.* due to the omnipresence of *C. diff.* in the environment of dairy farms, too [82]. The before treatment control value (BTC) in milk of all test cows was 1.7 ± 1.3 µg/mL of anti-*C. diff.* IgA (

Figure 1). For comparison, control cows produced milk containing 5.0 ± 1.0 µg/mL of anti-*C. diff.* IgA in the early lactation stage (data not shown). The success of vaccination against *C. diff.* was bipolar concerning the intended concentration of specific sIgA in milk as illustrated by

Figure 1. During the ongoing lactation, the LR did not produce more anti-*C. diff.* IgA than before the vaccination start, whereas the HR significantly enriched this milk antibody compared to the LR and the BTC. The antibody titer in HR's milk increased by more than 80% towards the BTC at any lactation stage and reached already 8.3 ± 0.7 µg/mL in the

early lactation. A further concentration of anti-*C. diff.* IgA by approximately 20% to 10.6 ± 0.7 µg/mL in milk was achieved by the HR in the late lactation stage.

The early and mid lactation stages comprised the treatment period up to TW 31. At this, the early lactation was mainly influenced by the vaccination with *MucoCD-I* batch A once given and then the sole application of *MucoCD-N*. The mid lactation was characterized by the use of *MucoCD-I* batch B. Figure 2 depicts the direct effects of the administered vaccines and their routes of application on the anti-*C. diff.* IgA levels in milk and blood of the treated groups. The initial anti-*C. diff.* IgA concentrations of both body fluids in TW 0 were significantly exceeded after the PC injection of *MucoCD-I* batch A (Figure 2, Table 7) in TW 3. Compared with the specific antibody contents in milk of the treated groups at TW 0, the HR cows produced promptly more than triple anti-*C. diff.* IgA in milk (TWs 3 - 5), whereas the LR's antibody production in response to *MucoCD-I* batch A was only extended moderate to the before treatment level in TW 4. Following the boost of the HR's anti-*C. diff.* IgA production in TW 3, the specific antibody contents in milk were persistently different between the treated groups up to TW 31. During the pure nasal (N) vaccination period, the anti-*C. diff.* IgA production in milk declined to each initial levels of the LR and HR groups, but the antibody titer in HR's milk increased again after the first application of *MucoCD-I* batch B. The measured peak value in HR's milk was 15.1 ± 1.5 µg/mL anti-*C. diff.* IgA at TW 18. From TW 21, the HR maintained the specific antibody production in the range of 8.0 to 11.0 µg/mL milk for the remaining treatment period. In contrast to the HR's immune reactivity, the *MucoCD-I* batch B vaccinations stimulated the specific antibody production by the LR neither in milk nor in blood.

The anti-*C. diff.* IgA titers in blood were not found as being different between LR and HR during the first IM period up to TW 15. In contrast to each base levels in TW 0, only the LR's anti-*C. diff.* IgA concentrations in blood were significantly increased in the TWs 6 and 7 as delayed response to *MucoCD-I* batch A. The use of *MucoCD-I* batch B triggered the significantly enhanced anti-*C. diff.* IgA titer in HR's blood (15.2 ± 2.1 µg/mL) in TW 18 relating to the belonging reference titer in TW 16 and towards the LR's titer to the same time point, too (Figure 2, Table 7).

Regarding the anti-*C. diff.* IgA production in milk and blood, the used anti-*C. diff.* vaccines for injection were more effective than *MucoCD-N.* Thus the activation of the NALT did not evoke the intended humoral response in the MG considering the concept of the common mucosal IS [83]. The quantities of specific antibody produced in both body fluids seemed not to be affected by the used administration routes for *MucoCD-I* batch B (Figure 2). Questioning achievable anti-*C. diff.* IgA amounts in milk, 16 µg of anti-*C. diff.* sIgA/mL raw milk on

average were found as a possible reference value [84]. The HR's peak value in TW 18 underlay slightly the antibody concentration in milk quoted above. For this, the following influencing factors should be taken into account, which were not revealed by Young et al. [84]: the cow breed, the age of the test cows, the lactation number, the lactation stage at milk sampling and the base levels of anti-*C. diff.* sIgA in milk before the treatment. Additionally, it should be considered that the averaged measuring data of the specific antibody based on only five collected milk batches [84]. In general, it could be established that the individual susceptibility and immune responsiveness of treated animals are crucial factors for the antigen specific antibody production.

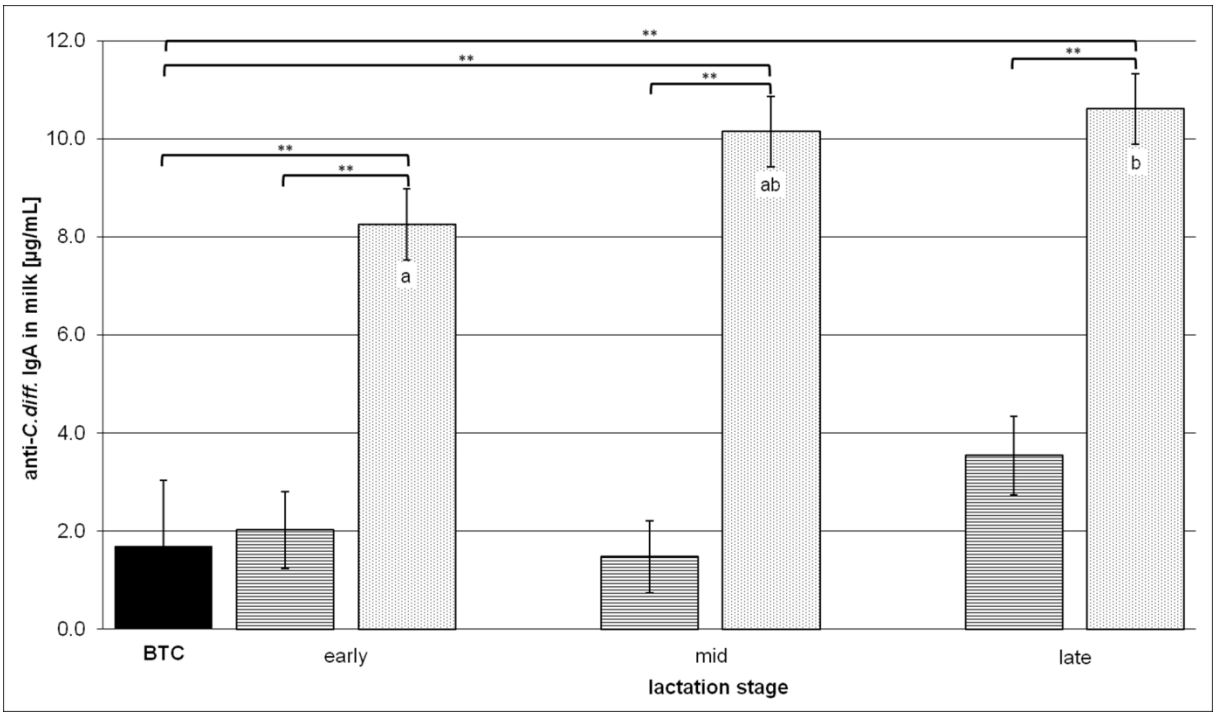


Figure 1: Anti-*C. diff.* milk IgA contents at different lactation stages

BTC signifies the "before treatment control" value of all cows in frame for vaccination. The lactation stages are defined as "early" for < 100 DiL, "mid" for 101-200 DiL, "late" for > 201 DiL. On the ordinate, C. diff. specific milk IgA concentrations are depicted as LSM + SD and presented comparatively for the BTC (n = 9, black bar ■), low responder (LR, n = 4, cross-striped bars ▤) and high responder (HR, n = 5, dotted bars ⚄) groups. Substantial or highly significant differences versus BTC and between LR and HR at the same lactation stage are marked by brackets with one (P < 0.05) or two asterisks (P < 0.01). Substantial or highly significant differences within the same group related to the early lactation stage are shown with lowercase letters (a, b) for P < 0.05 [79].

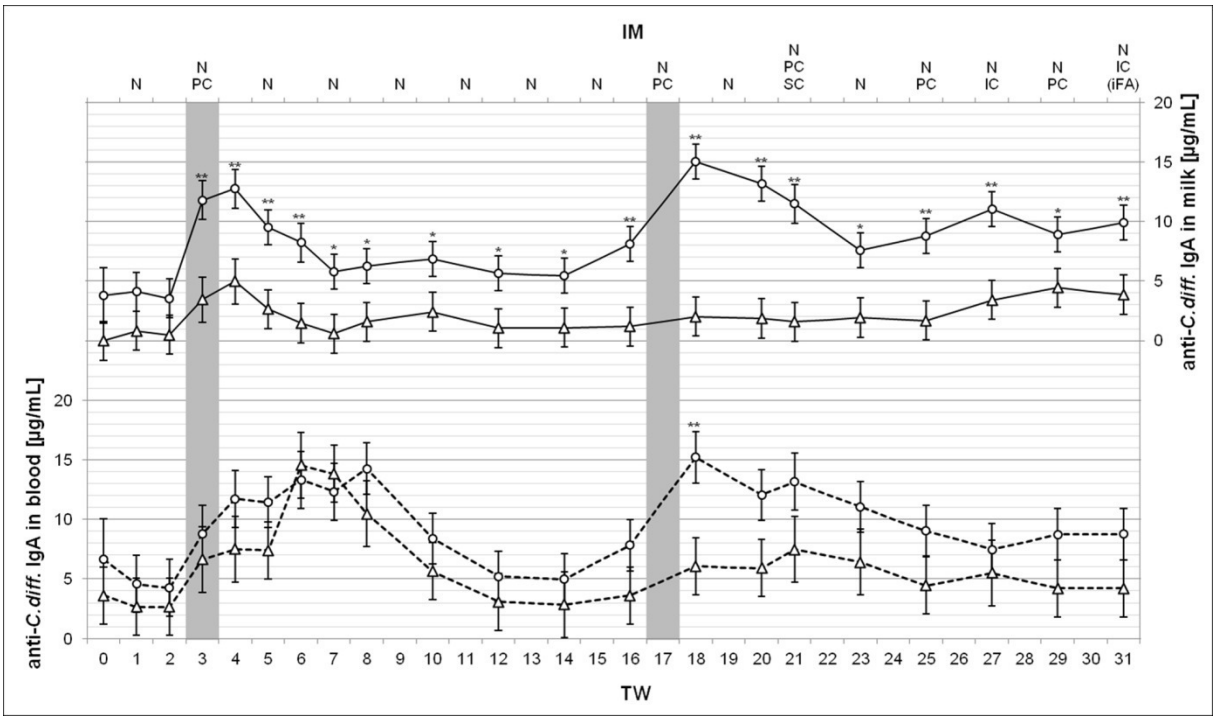


Figure 2: Anti-*C. diff.* IgA concentrations in milk and in blood during the 31-week treatment period

The ordinate values in milk (solid lines ▬, top) and in blood (broken lines ▬ ▬, bottom) are shown as LSM ± SD per treated groups, the low responder (LR, n = 4, triangles △) and the high responder (HR, n = 5, circle ○). Substantial or highly significant differences between both groups at the same point in time are marked by one (P < 0.05) or two asterisks (P < 0.01). The gray bars indicate the one-time application of MucoCD-I vaccine batch A in TW 3 and the first use of vaccine batch B for injection in TW 17. Utilized vaccination routes were nasal (N), percutaneous (PC), subcutaneous (SC) and intracutaneous (IC). Uniquely, the injected MucoCD-I vaccine was supplemented with incomplete Freund's adjuvant (iFA) [79].

Table 7: Anti-*C. diff.* IgA contents in milk and blood of treated cows before the treatment (TW 0) and prior to the first use of *MucoCD-I* vaccine batch B (TW 16)

group	TW	anti-*C. diff.* IgA (µg/mL)					
		in milk			in blood		
		LSM	*SD*		LSM	*SD*	
LR	**0**	0.0	*1.6*	* (TW 4)	3.6	*2.4*	**(TW 6,7)
HR	**0**	3.8	*2.3*	* (TW 3, 4) **(TW 5)	6.7	*3.4*	
LR	**16**	1.2	*1.6*	a	3.6	*2.4*	
HR	**16**	8.1	*1.5*	b, **(TW 18) * (TW 20)	7.8	*2.1*	* (TW 18)

The anti-C. diff. IgA is displayed as LSM ± SD group-related to LR (low responder, n = 4) and HR (high responder, n = 5) at different treatment weeks (TW). The lowercase letters (a, b) indicate significant differences (P < 0.05) between HR and LR within the same TW. Asterisks indicate significant differences between defined points in time towards TW 0 or TW 16 with P < 0.05 (*) or P < 0.01 (**) [79].

Total IgA in milk and blood

The BTC of the test cows amounted to 101.5 ± 35.9 µg/mL (Figure 3). The evaluation of the total milk IgA contents depending on the lactation stage showed that the concentration of the antibody class in the HR's milk proceeded stepwise up to 375.3 ± 19.2 µg/mL in the late lactation stage, exceeding the LR level by more than 55% to this period. The HR's total IgA production was significantly higher towards the BTC and the LR at any lactation stage. An enhanced total IgA amount in LR's milk was only determined in the late lactation stage by roughly 50% compared to the BTC (Figure 3).

At the BTC belonging measuring date, the test cows (with 28 DiL on average) had passed the first quarter of the early lactation stage. In relation to the treated groups total IgA production in milk of the fist 100 DiL, whether the average antibody amounts in LR's nor in HR's milk differed from the total milk IgA content of the Ctr group amounting to 168.4 ± 26.2 µg/mL (data not shown). Furthermore, another IM study with *C. diff.* ascertained that raw milk from immunized versus non-immunized cows did not differ significantly in total IgA content [84]. Generally, all milk Igs inclusive of sIgA are concentrated in the last third of lactation mainly due to the reduction in milk yield [85].

The total IgA levels in milk and blood closely examined during the 31-weeks treatment period displayed that the vaccination with *MucoCD-I batch* A in TW 3 affected the total contents of this antibody class in both body fluids (Figure 4). Immediately afterwards, the HR's milk contained significantly more total IgA than the LR's milk by almost three times. The top value of total milk IgA during the first treatment period up to TW 15 amounted to 357.1 ± 46.2 µg/ml and was achieved by the HR group in TW 4. This antibody concentration exceeded significantly the initial one in HR's milk at TW 0 besides that in LR's milk in TW 4, too (Figure 4, Table 8). Apart from an outlier value of total IgA in LR's milk at TW 10, of which cause was unknown, no further total IgA quantities measured in the first (TWs 0 - 15) and second (TWs 16 - 31) treatment period were significantly different to the belonging base levels in the TWs 0 and 16, respectively. However, in case of the HR, the use of *MucoCD-I* batch B promoted the increase of total IgA in milk twice in the TWs 20 and 27 by surpassing 400.0 µg/mL. As the secreted total IgA in the LR's milk seemed to be unimpaired by the treatment, its amounts produced by the HR were significantly higher at the most TWs during the second treatment period. However, neither the vaccinations with *MucoCD-I* batch B nor with *MucoCD-N* did change the total IgA concentrations in blood of both treated groups, which were stable at approximately 180 µg/mL from TW 5 up to the end of the treatment period (Figure 4, Table 8).

The development of the total IgA contents in blood and milk corroborate the common observation that under physiological conditions normal serum IgA levels of cattle fluctuate only within a narrow range, whereas IgA concentrations in milk vary clearly during the lactation period [85]. Furthermore, the accelerated total IgA response of HR after injecting the vaccine batches A and B for the first time could be indicate an response of the immunological memory [86]. In addition, cross reactions to memory B-cells specific for antigens structurally related to *C. diff*. and toxins might be responsible for the large differences in total milk IgA contents between HR and LR at the different lactation stages. Thus the vaccinations could have operated as polyclonal stimuli in favor of boosting IgA of various specificity [87].

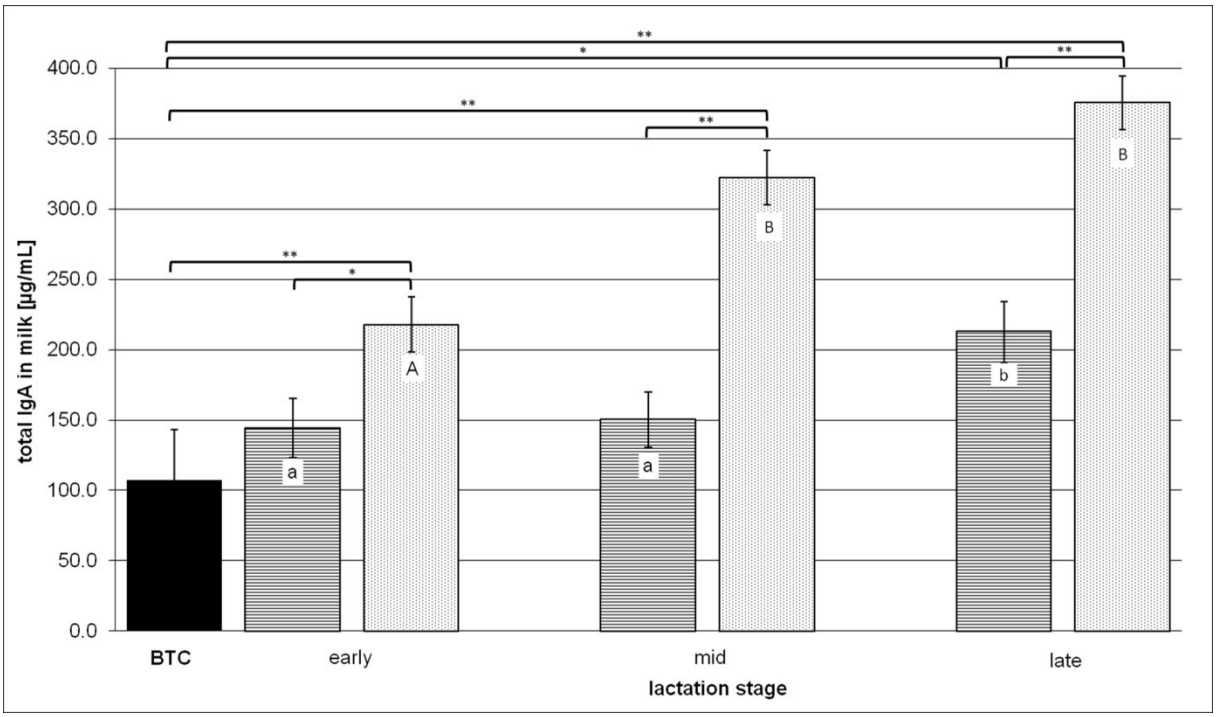


Figure 3: Total milk IgA contents at different lactation stages

BTC means the "before treatment control" value of all cows in frame for vaccination. The lactation stages are defined as "early" for < 100 DiL, "mid" for 101-200 DiL, "late" for > 201 DiL. The ordinate values represent comparatively LSM ± SD of the BTC (n = 9, black bar ■), low responder (LR, n = 4, cross-striped bars ▤) and high responder (HR, n = 5, dotted bars ⚄) groups. Brackets equipped with asterisks link treated groups with BTC and at the same lactation stage, if significant differences between them are given with P < 0.05 (*) or P < 0.01 (**). Substantial or highly significant differences within the same group related to the early lactation stage are illustrated with lowercase letters (a, b) in case of P < 0.05 and uppercase letters (A, B) for P < 0.01 [79].

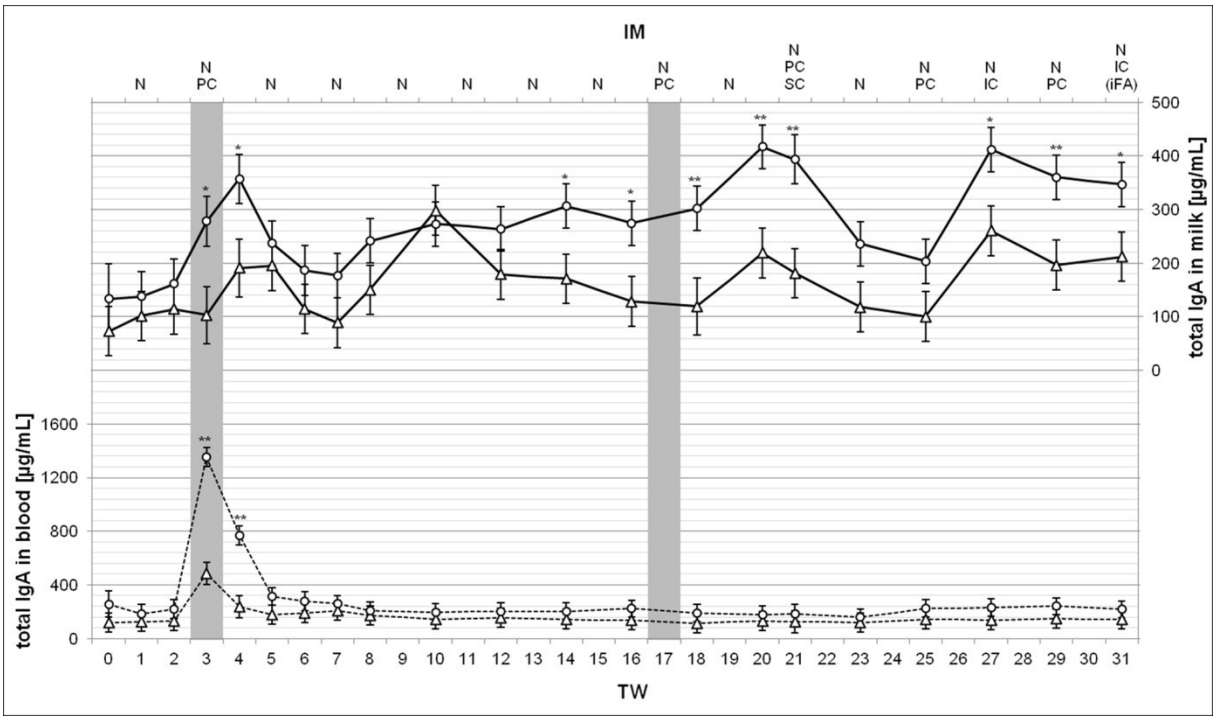


Figure 4: Total IgA concentrations in milk and in blood during 31-weeks treatment period

Measured total IgA values in milk (solid lines ▬, top) and in blood (broken lines ▬ ▬, bottom) are shown as LSM ± SD per treated groups, the low responder (LR, n = 4, triangle △) and the high responder (HR, n = 5, circle ○), on the ordinate. Substantial or highly significant differences between both groups to the same point in time are marked by one (P < 0.05) or two asterisks (P < 0.01). The gray bars indicate the one-time application of MucoCD-I vaccine batch A in TW 3 and the first use of vaccine batch B for injection in TW 17. Utilized vaccination routes were nasal (N), PC, SC and IC. Uniquely, the injected MucoCD-I vaccine was supplemented with iFA [79].

Table 8: Total IgA contents in milk and blood of treated cows before the treatment (TW 0) and prior to the first use of *MucoCD-I* vaccine batch B (TW 16)

group	TW	total IgA (µg/mL)					
		in milk			in blood		
		LSM	*SD*		LSM	*SD*	
LR	**0**	73.1	*46.2*	**(TW 10)	119.2	*70.8*	**(TW 3)
HR	**0**	133.6	*65.3*	**(TW 4) * (TW 14)	257.8	*100.2*	**(TW 3, 4)
LR	**16**	128.6	*46.2*	a	138.0	*70.8*	
HR	**16**	274.7	*41.3*	b **(TW 20, 27)	223.2	*63.4*	

The total IgA is displayed as LSM ± SD group-related to LR (low responder, n = 4) and HR (high responder, n = 5) at different treatment weeks (TW). The lower letters (a, b) indicate significant differences (P < 0.05) between HR and LR within the same TW. Asterisks indicate significant differences of defined points in time towards TW 0 or TW 16 with P < 0.05 (*) or P < 0.01 (**) [79].

Total and differential cell counts in milk and blood

The total counts of bovine PBL range physiologically between five to ten million cells per mL blood [88]. The treatment with *MucoCD-I* batch A effected promptly at first a significant

leukocytopenia in TW 3 followed by a leukocytosis in TW 5 each to similar extension for both treated groups (Table 9) matching the course of the Schilling's biological leukocyte curve as linked with physical burdens induced by infectious agents or toxins [88]. Additionally, the numbers of PBL of the LR and HR were below the threshold in the TWs 6 and 8, respectively. At the start of the second treatment period with *MucoCD-I* batch B, the PBL content in HR's blood touched the upper margin of the normal range. After the vaccine batch B was given the first time, the PBL counts of both treated groups developed similarly, and primarily, they fluctuated within the normal range for the remaining IM period. Apart from slight PBL shortfalls of the LR in TW 25 and of the HR in TW 31, the PBL numbers of the Ctr cows were out of range twice for unknown cause (TWs 25 and 27), too (Table 9).

The main PBL populations are Lym and polymorphnucleated neutrophilic granulocytes (PNM) with 45% to 65% and 25% to 45%, respectively. By respecting the monocytes share in PBL beyond 6%, the proportion of phagocytes (Phag) are normally lower than that of the Lym resulting in the ratio [Phag : Lym] < 1 [88]. The analysis of the differential cell counts (DCC) in bovine blood confirmed the Lym constituting the main share of PBL (Table 9). The normal range of Lym was exceeded up to TW 6 by the treated groups at several time points. Following the treatment with *MucoCD-I* batch A, the maximum of counted Lym was determined for the HR in TW 3 with the share of approximately 84%, which was significant higher than the Lym parts of the LR and the Ctr. Afterwards, the Lym percentages of the treated groups decreased clearly towards the Ctr (TWs 10 and 12). The treatment with the vaccine batch B did not effect to drop out of the normal range of Lym contents, but their percentages of the treated groups were enlarged to that of the Ctr in the TWs 25 and 27. The PMN numbers of all analyzed groups developed inversely to the Lym numbers during the examined treatment period (Table 9). Accordingly, the 8.6 $\pm$ 4.0% PMN of HR's PBL in TW 3 were clearly lower than that one of the other groups and below the upper threshold for PMN in bovine blood, too. Overall, the PMN counts of all groups seemed to be often underestimated in favor of the monocyte counts (Table 9). This finding was assumed as a shortcoming of the microscopic analysis of the DCC. This possible technical deficiency was balanced by the evaluation of the phagocytes' share of the PBL in relation to that of the Lym. The HR's ratio [Phag : Lym] was particularly low with 0.1 $\pm$ 0.1 following the treatment with *MucoCD-I* batch A corresponding to the extended Lym counts in TW 3 (Table 9). That one of the LR group overran plus 0.1 the upper margin of 1.0 due to an unusual high monocytes' count influencing the amount of phagocytes in TW 10. Throughout the second treatment period, the ratios [Phag : Lym] of the treated groups were within the normal range, but they were significantly different one-time, in TW 21, due to the many phagocytes counted in the HR's blood. A modest monocytosis can be a sign of physiological stress [90], and possibly,

the IS of the HR had reacted more sensitively than the LR's IS by additionally SC confrontation with the antigen for the first time. The Ctr group broke through the physiological range of Phag to Lym in TW 25. The ratio [Phag : Lym] of the Ctr was with 1.1 ± 0.1 significantly higher than that of the treated groups to this date.

The total SCC in milk was evaluated according to the reference value of 200,000 cells/mL, which is suggestive of bacterial infections [91]. The treated cows' SCCs remained constantly beneath this threshold within the complete IM period excepting of the outlier measured in LR's milk at TW 8 (Table 10). A single LR cow dominated the group related SCC to this date, but its general condition was attested as healthy. Likewise, the SCCs of the Ctr group stood out in the TWs 8 and 25. Thus, the single overrun of the SCCs threshold by the LR was not interpreted as being related to the vaccination program. Similarly, another IM study, testing the efficacy of a *Staphylococcus aureus* bacterin against mastitis in a lactating cow model, did not effected changed total SCCs in milk following the administration of the bacterin on the SC route. Therefore, it was suggested that vaccinations outside of the MG did not interfere the cellular homeostasis of its lacteal secretions, provided that the blood-udder barrier is intact [93].

The DCC in milk revealed atypical ratios [Phag : Lym] < 1 independent of the animal group, especially, up to TW 6 (Table 10). In contrast to the DCC in bovine blood, the macrophages are normally the dominant cell population in milk [91]. The Lym proportions of LRs' SCCs were approximately more than 80% in the TWs 5 and 6, which were significantly increased compared to the Ctr group. Nevertheless, these Lym percentages did not differ to the baseline in TW 1. Inversely to the Lym, the Phag proportions in LR's milk belonging to the TWs 5-7 were significantly low towards that detected in the milk of the Ctr group, and partly, of the HR group, too. Referring to the baseline values for the second treatment period in TW 15, the Lym proportion of LR's SCC was exceptionally low causing the high ratio [Phag : Lym] amounting to 4 ± 0.7. Likewise, the HR's ratio [Phag : Lym] was 3.8 ± 0.5, which was mainly influenced by enlarged PMN numbers. The following vaccinations with *MucoCD-I* batch B effected repeatedly heightened ratios [Phag : Lym] of the HR's SCCs compared to the Ctr and the LR groups, respectively, up to TW 25. Finally, assessing the DCC in milk (Table 10), two things stood out. Firstly, the proportions of the phagocytes seemed to be underrated in all. This could have been resulted by the polyethylene plastic bottles used for the milk sampling, which were proven to adhere macrophages [94]. Secondly, the DCCs in milk of all investigated groups indicated to be shaped by the physiological development of the milk cell populations during the ongoing lactation: growing numbers of phagocytes along with dropping lymphocyte counts [95].

Table 9: Total PBL counts and the relative distribution of the different leukocyte populations

IM	TW	group	PBL (x10^6/ml)			PMN (%)			Basophils (%)			Eosinophils (%)			Monocytes (%)			Lymphocytes (%)			ratio [Phag:Lym]		
			LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
	0	Ctr	9.6	*1.3*		23.8	*4.6*		0.2	*0.2*		6.1	*2.3*		7.7	*2.9*		62.2	*5.7*		0.6	*0.2*	
		LR	9.8	*1.3*		22.6	*4.0*		0.0	*0.1*		2.9	*2.0*		13.2	*2.5*		61.3	*4.9*		0.7	*0.1*	
		HR	9.2	*1.3*		17.8	*4.6*		0.2	*0.2*		1.1	*2.3*		6.3	*2.9*		74.5	*5.7*		0.4	*0.2*	
N	1	Ctr	7.3	*1.3*		25.5	*4.6*		0.1	*0.2*		4.0	*2.3*		10.7	*2.9*		59.7	*5.7*		0.6	*0.2*	
		LR	8.2	*1.2*		19.1	*4.0*		0.0	*0.1*		2.1	*2.0*		10.9	*2.5*		67.8	*4.9*		0.5	*0.1*	
		HR	7.2	*1.2*		19.3	*4.0*		0.2	*0.1*		2.3	*2.0*		7.2	*2.5*		71.1	*4.9*		0.4	*0.1*	
	2	Ctr	8.4	*1.3*		16.0	*4.6*		0.0	*0.2*		2.7	*2.3*		8.4	*2.9*		72.9	*5.7*		0.3	*0.2*	
		LR	7.7	*1.2*		27.6	*4.0*		0.1	*0.1*		3.6	*2.0*		8.4	*2.5*		60.3	*4.9*		0.6	*0.1*	
		HR	10.1	*1.2*		20.0	*4.0*		0.3	*0.1*		3.2	*2.0*		9.6	*2.5*		66.8	*4.9*		0.5	*0.1*	
N	3	Ctr	6.7	*1.3*		36.9	*4.6*	a*	0.1	*0.2*		5.7	*2.3*		4.9	*2.9*	ab	52.4	*5.7*	a	0.8	*0.2*	a
PC		LR	3.4	*1.3*	*	22.1	*4.6*	b	0.2	*0.2*		4.5	*2.3*		11.7	*2.9*	a	61.4	*5.7*	a	0.6	*0.2*	a
		HR	3.6	*1.2*	*	8.6	*4.0*	c	0.0	*0.1*		5.0	*2.0*		3.0	*2.5*	b	83.6	*4.9*	b	0.1	*0.1*	b
	4	Ctr	6.8	*1.3*		28.1	*4.6*		0.1	*0.2*		3.7	*2.3*		8.2	*2.9*		60.0	*5.7*		0.6	*0.2*	
		LR	8.3	*1.3*		27.1	*4.6*		0.0	*0.2*		2.0	*2.3*		2.1	*2.9*	*	68.8	*5.7*		0.4	*0.2*	
		HR	9.6	*1.2*		30.1	*4.0*	*	0.0	*0.1*		2.2	*2.0*		2.0	*2.5*		65.5	*4.9*		0.5	*0.1*	
N	5	Ctr	*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		LR	12.3	*1.2*		20.1	*4.0*		0.0	*0.1*		3.5	*2.0*		5.2	*2.5*	*	71.2	*4.9*		0.4	*0.1*	
		HR	11.9	*1.0*		20.1	*3.6*		0.3	*0.1*		2.3	*1.7*		4.6	*2.2*		72.8	*4.4*		0.3	*0.1*	
	6	Ctr	8.9	*1.6*		26.0	*4.6*		0.1	*0.2*		4.1	*2.3*		6.5	*2.9*		63.3	*5.7*		0.5	*0.2*	
		LR	3.5	*1.2*	*	20.7	*4.0*		0.1	*0.1*		5.6	*2.0*		9.0	*2.5*		64.5	*4.9*		0.5	*0.1*	
		HR	5.8	*1.0*	*	23.8	*3.6*		0.2	*0.1*		4.2	*1.7*		6.0	*2.2*		65.8	*4.4*		0.5	*0.1*	
N	7	Ctr	*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		LR	6.1	*1.2*	*	35.8	*4.0*	*	0.0	*0.1*		3.8	*2.0*		9.3	*2.5*		51.2	*4.9*		0.9	*0.1*	
		HR	5.4	*1.0*	*	29.0	*3.6*		0.0	*0.1*		2.7	*1.7*		8.0	*2.2*		60.1	*4.4*	*	0.6	*0.1*	
	8	Ctr	6.4	*1.3*		21.7	*4.6*		0.1	*0.2*		6.5	*2.3*		6.1	*2.9*		65.6	*5.7*		0.4	*0.2*	
		LR	4.7	*1.2*	*	29.5	*4.0*		0.3	*0.1*		4.3	*2.0*		6.9	*2.5*		59.0	*4.9*		0.6	*0.1*	
		HR	3.9	*1.0*	*	23.1	*3.6*		0.0	*0.1*		5.8	*1.7*		5.9	*2.2*		65.2	*4.4*		0.5	*0.1*	
N	10	Ctr	6.4	*1.3*		21.3	*4.6*		0.3	*0.2*		4.1	*2.3*	a	8.6	*2.9*	a	65.8	*5.7*	a	0.5	*0.2*	a
		LR	6.1	*1.2*	*	26.0	*4.0*		0.1	*0.1*		10.8	*2.0*	b*	19.8	*2.5*	b	43.3	*4.9*	b*	1.1	*0.1*	b*
		HR	7.0	*1.0*		28.5	*3.6*		0.0	*0.1*		4.0	*1.7*	a	11.0	*2.2*	a	56.5	*4.4*	a*	0.7	*0.1*	a
N	12	Ctr	7.1	*1.0*		24.1	*3.6*		0.3	*0.1*		4.2	*1.7*	a	6.9	*2.2*		64.5	*4.4*	a	0.5	*0.1*	a
		LR	8.4	*1.2*		25.9	*4.0*		0.0	*0.1*		10.1	*2.0*	b*	12.5	*2.5*		51.4	*4.9*	b	0.8	*0.1*	ab
		HR	7.7	*1.0*		32.6	*3.6*	*	0.0	*0.1*		6.0	*1.7*	ab	12.0	*2.2*		49.4	*4.4*	b*	0.9	*0.1*	b*

IM	TW	group	PBL (x10^6/ml)			PMN (%)			Basophils (%)			Eosinophils (%)			Monocytes (%)			Lymphocytes (%)			ratio [Phag:Lym]		
			LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	14	Ctr	6.3	*1.3*	a	28.0	*4.6*		0.3	*0.2*		4.5	*2.3*		4.9	*2.9*	a	62.3	*5.7*		0.6	*0.2*	
		LR	6.5	*1.2*	a	23.2	*4.0*		0.0	*0.1*		3.2	*2.0*		18.9	*2.5*	b	54.8	*4.9*		0.8	*0.1*	
		HR	9.8	*1.0*	b	25.8	*3.6*		0.0	*0.1*		7.7	*1.7*	*	16.9	*2.2*	b*	49.6	*4.4*	*	0.9	*0.1*	*
N	**16**	Ctr	7.3	*1.0*	a	30.5	*3.6*		0.5	*0.1*	a	4.5	*1.7*		9.2	*2.2*	a	55.3	*4.4*		0.8	*0.1*	
		LR	8.9	*1.2*	ab	28.2	*4.0*		0.0	*0.1*	b	4.1	*2.0*		14.8	*2.5*	ab	52.9	*4.9*		0.9	*0.1*	
		HR	10.3	*1.0*	b	24.4	*3.6*		0.0	*0.1*	b	6.8	*1.7*		17.4	*2.2*	b	51.4	*4.4*		0.9	*0.1*	
N	18	Ctr	6.6	*1.0*		23.5	*3.6*		0.2	*0.1*		3.1	*1.7*		9.0	*2.2*		64.1	*4.4*		0.5	*0.1*	
PC		LR	6.0	*1.2*		27.0	*4.0*		0.0	*0.1*		3.9	*2.0*		7.5	*2.5*	*	61.5	*4.9*		0.6	*0.1*	
		HR	7.6	*1.0*		23.6	*3.6*		0.0	*0.1*		5.0	*1.7*		10.8	*2.2*	*	60.4	*4.4*		0.6	*0.1*	
N	20	Ctr	6.6	*1.0*	a	29.4	*3.6*		0.3	*0.1*		5.3	*1.7*		7.7	*2.2*		57.3	*4.4*		0.7	*0.1*	
		LR	9.6	*1.2*	b	20.1	*4.0*		0.0	*0.1*		2.1	*2.0*		12.4	*2.5*		65.3	*4.9*		0.5	*0.1*	
		HR	9.0	*1.0*	ab	27.7	*3.6*		0.1	*0.1*		5.3	*1.7*		13.4	*2.2*		53.5	*4.4*		0.9	*0.1*	
N	21	Ctr	6.8	*1.0*		21.3	*3.6*		0.3	*0.1*		4.6	*1.7*		9.0	*2.2*	a	64.8	*4.4*		0.5	*0.1*	a
PC		LR	8.5	*1.2*		18.2	*4.0*		0.1	*0.1*		7.8	*2.0*		9.8	*2.5*	a	64.1	*4.9*		0.4	*0.1*	a*
SC		HR	8.2	*1.0*		24.0	*3.6*		0.1	*0.1*		6.1	*1.7*		16.9	*2.2*	b	52.8	*4.4*		0.9	*0.1*	b
N	23	Ctr	6.7	*1.0*		38.6	*3.6*	a	0.3	*0.1*		5.5	*1.7*		8.3	*2.2*	a	47.3	*4.4*		1.0	*0.1*	
		LR	5.9	*1.2*		23.0	*4.0*	b	0.3	*0.1*		2.9	*2.0*		18.0	*2.5*	b	55.8	*4.9*		0.7	*0.1*	
		HR	6.9	*1.0*	*	24.3	*3.6*	b	0.4	*0.1*	*	4.2	*1.7*		21.0	*2.2*	b	50.1	*4.4*		0.9	*0.1*	
N	25	Ctr	4.1	*1.0*	*	36.6	*3.6*	a	0.1	*0.1*	a*	5.8	*1.7*		10.2	*2.2*		47.3	*4.4*	a	1.1	*0.1*	a
PC		LR	4.5	*1.2*	*	23.8	*4.0*	b	0.4	*0.1*	ab*	3.5	*2.0*		13.8	*2.5*		58.5	*4.9*	ab	0.7	*0.1*	b
		HR	5.4	*1.0*	*	25.6	*3.6*	b	0.5	*0.1*	b*	2.8	*1.7*		9.4	*2.2*	*	61.7	*4.4*	b	0.6	*0.1*	b
N	27	Ctr	4.5	*1.2*	a	31.1	*4.0*	a	0.3	*0.1*		6.1	*2.0*		8.8	*2.5*		53.8	*4.9*	a	0.8	*0.1*	
IC		LR	8.6	*1.2*	b	13.8	*4.0*	b*	0.1	*0.1*		5.1	*2.0*		12.2	*2.5*		68.8	*4.9*	b*	0.4	*0.1*	*
		HR	7.1	*1.0*	ab*	20.1	*3.6*	b	0.2	*0.1*		3.4	*1.7*		13.4	*2.2*		65.0	*4.4*	ab*	0.6	*0.1*	
N	29	Ctr	5.6	*1.0*		35.5	*3.6*	a	0.2	*0.1*		3.7	*1.7*		6.9	*2.2*	a	53.9	*4.4*		0.8	*0.1*	
PC		LR	6.5	*1.2*		22.9	*4.0*	b	0.1	*0.1*		3.7	*2.0*		13.8	*2.5*	b	59.6	*4.9*		0.6	*0.1*	
		HR	7.0	*1.0*	*	17.0	*3.6*	b	0.2	*0.1*		4.9	*1.7*		12.6	*2.2*	ab	65.5	*4.4*	*	0.5	*0.1*	*
N	31	Ctr	6.3	*1.0*		28.1	*3.6*		0.3	*0.1*		6.2	*1.7*		6.7	*2.2*		58.7	*4.4*		0.6	*0.1*	
IC		LR	5.3	*1.2*	*	21.7	*4.0*		0.2	*0.1*		4.9	*2.0*		10.8	*2.5*		62.6	*4.9*		0.5	*0.1*	
(iFA)		HR	4.8	*1.0*	*	28.0	*3.6*		0.1	*0.1*		3.5	*1.7*		5.8	*2.2*	*	63.9	*4.4*	*	0.5	*0.1*	

The total and differential cell counts of the peripheral blood leukocytes (PBL) are listed as LSM ± SD per group (Ctr = control cows, n = 5; LR = low responder cows, n = 4; HR = high responder cows, n = 5), per treatment week and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N = nasal, PC = percutaneous, SC = subcutaneous, IC = intracutaneous (iFA = incomplete Freund's adjuvant)). TWs 0 and 16 in bold mark in each case the time point before the first use of the vaccine batch A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging PBL values related to the first uses of the vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 0 in the following time period until and including TW 14, and relating to TW 16 in the remaining treatment period. Lower case letters label significant differences (P < 0.05) between groups to the same time point. PMN = polymorphnucleated neutrophilic leukocytes; Phag = phagocytes (counts of PMN and monocytes); Lym = lymphocytes; n.n. = numerius negidius (unavailable data) [76].

Table 10: Total SCC in milk and relative distribution of the different leukocyte populations out of milk

IM	TW	group	SCC (x10^3/ml)			PMN (%)			Basophils (%)			Macrophages (%)			Lymphocytes (%)			ratio [Phag:Lym]		
			LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	**1**	Ctr	27.3	*139.8*		24.8	*8.8*		3.7	*1.5*		9.9	*2.9*		61.6	*7.6*		0.7	*0.6*	
		LR	128.0	*161.5*		34.0	*10.1*		0.6	*1.7*		3.4	*3.3*		62.0	*8.7*		0.6	*0.7*	
		HR	16.5	*197.8*		20.4	*12.4*		0.7	*2.1*		8.8	*4.1*		70.1	*10.7*		0.4	*0.8*	
N PC	3	Ctr	48.7	*161.5*		23.5	*10.1*		1.0	*1.7*		9.0	*3.3*		66.5	*8.7*		0.5	*0.7*	
		LR	26.3	*161.5*		15.5	*10.1*		2.3	*1.7*		7.9	*3.3*		75.1	*8.7*		0.3	*0.7*	
		HR	51.5	*139.8*		19.9	*8.8*		2.7	*1.5*		6.4	*2.9*		71.0	*7.6*		0.4	*0.6*	
	4	Ctr	33.8	*125.1*		23.0	*7.9*		1.9	*1.3*		16.5	*2.6*		66.2	*6.8*		0.7	*0.5*	
		LR	*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		HR	36.7	*161.5*		19.1	*10.1*		4.7	*1.7*		8.3	*3.3*		67.9	*8.7*		0.4	*0.7*	
N	5	Ctr	50.3	*139.8*		30.7	*8.8*		2.9	*1.5*		7.8	*2.9*		58.6	*7.6*	a	0.7	*0.6*	
		LR	8.0	*139.8*		7.0	*8.8*	*	4.9	*1.5*		7.3	*2.9*		80.8	*7.6*	b	0.2	*0.6*	
		HR	19.3	*139.8*		23.2	*8.8*		3.1	*1.5*		5.8	*2.9*		67.6	*7.6*	ab	0.4	*0.6*	
	6	Ctr	88.3	*139.8*		38.9	*8.8*	a	2.2	*1.5*		3.8	*2.9*		55.1	*7.6*	a	0.9	*0.6*	
		LR	7.0	*197.8*		6.7	*12.4*	b	1.7	*2.1*		5.8	*4.1*		85.8	*10.7*	b	0.1	*0.8*	
		HR	31.0	*197.8*		45.6	*12.4*	a	0.9	*2.1*		5.5	*4.1*		48.1	*10.7*	ab	1.1	*0.8*	
N	7	Ctr	178.5	*139.8*		50.2	*8.8*	a*	1.4	*1.5*	a	1.9	*2.9*		46.3	*7.6*		1.3	*0.6*	
		LR	7.0	*161.5*		13.5	*10.1*	b	8.6	*1.7*	b*	9.0	*3.3*		68.9	*8.7*		0.3	*0.7*	
		HR	27.8	*139.8*		30.4	*8.8*	ab	1.7	*1.5*	a	5.6	*2.9*		62.0	*7.6*		0.7	*0.6*	
	8	Ctr	479.3	*139.8*	a*	44.8	*8.8*		5.0	*1.5*		7.6	*2.9*		42.6	*7.6*		1.5	*0.6*	
		LR	910.1	*139.8*	b*	26.5	*8.8*		4.5	*1.5*		7.2	*2.9*		61.9	*7.6*		1.1	*0.6*	
		HR	83.6	*125.1*	c	43.5	*7.9*		1.9	*1.3*		3.6	*2.6*		51.0	*6.8*		1.1	*0.5*	
N	9	Ctr	78.3	*139.8*		36.9	*8.8*		3.8	*1.5*		7.3	*2.9*		52.0	*7.6*		0.9	*0.6*	
		LR	29.0	*139.8*		27.6	*8.8*		1.4	*1.5*		6.2	*2.9*		64.9	*7.6*		0.5	*0.6*	
		HR	42.6	*125.1*		43.3	*7.9*		1.5	*1.3*		3.0	*2.6*		52.1	*6.8*		1.1	*0.5*	
N	11	Ctr	91.8	*139.8*		49.8	*8.8*	*	5.3	*1.5*	a	4.3	*2.9*		40.6	*7.6*		1.6	*0.6*	
		LR	28.0	*161.5*		33.3	*10.1*		1.1	*1.7*	ab	4.6	*3.3*		61.0	*8.7*		0.8	*0.7*	
		HR	81.0	*125.1*		53.3	*7.9*	*	0.9	*1.3*	b	3.7	*2.6*		42.4	*6.8*	*	1.7	*0.5*	
N	13	Ctr	73.6	*125.1*		34.7	*7.9*		4.6	*1.3*	a	7.5	*2.6*		53.2	*6.8*		0.8	*0.5*	
		LR	19.8	*139.8*		33.0	*8.8*		1.4	*1.5*	ab	6.4	*2.9*		59.2	*7.6*		0.7	*0.6*	
		HR	58.8	*139.8*		49.2	*8.8*		0.5	*1.5*	b	1.3	*2.9*		49.1	*7.6*		1.3	*0.6*	
N	**15**	Ctr	62.8	*125.1*		40.5	*7.9*	a	5.0	*1.3*	ab	7.8	*2.6*		46.8	*6.8*	a	1.1	*0.5*	a
		LR	41.1	*161.5*		65.2	*10.1*	ab	8.9	*1.7*	a	7.4	*3.3*		18.5	*8.7*	b	4.0	*0.7*	b
		HR	138.6	*125.1*		64.4	*7.9*	b	3.8	*1.3*	b	1.5	*2.6*		30.3	*6.8*	ab	3.8	*0.5*	b

IM	TW	group	SCC (x10³/ml)		PMN (%)			Basophils (%)			Macrophages (%)			Lymphocytes (%)			ratio [Phag:Lym]		
			LSM	*SD*	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N PC	17	Ctr	66.2	*125.1*	46.6	*7.9*	a	3.8	*1.3*		6.0	*2.6*		43.7	*6.8*		1.5	*0.5*	ab
		LR	23.0	*139.8*	42.5	*8.8*	ab	0.8	*1.5*	*	5.3	*2.9*		51.4	*7.6*	*	1.1	*0.6*	a*
		HR	175.6	*125.1*	62.1	*7.9*	b	1.3	*1.3*		2.7	*2.6*		33.9	*6.8*		2.8	*0.5*	b
N	19	Ctr	162.6	*125.1*	45.2	*7.9*	ab	3.6	*1.3*		6.0	*2.6*		45.3	*6.8*	ab	1.3	*0.5*	ab
		LR	45.8	*139.8*	32.6	*8.8*	a*	1.5	*1.5*	*	5.8	*2.9*		59.8	*7.6*	a*	0.7	*0.6*	a*
		HR	164.2	*125.1*	60.7	*7.9*	b	1.1	*1.3*		1.8	*2.6*		36.5	*6.8*	b	2.6	*0.5*	b
N PC SC	21	Ctr	56.5	*139.8*	34.9	*8.8*		2.5	*1.5*		6.0	*2.9*	ab	56.7	*7.6*	a	0.8	*0.6*	a
		LR	24.3	*139.8*	38.4	*8.8*	*	1.0	*1.5*	*	10.8	*2.9*	a	49.9	*7.6*	ab*	1.1	*0.6*	ab*
		HR	140.0	*125.1*	59.3	*7.9*		2.0	*1.3*		2.7	*2.6*	b	36.1	*6.8*	b	2.5	*0.5*	b
N	23	Ctr	73.6	*125.1*	55.8	*7.9*		1.8	*1.3*		4.3	*2.6*		38.1	*6.8*		1.7	*0.5*	
		LR	28.3	*139.8*	38.5	*8.8*	*	0.8	*1.5*	*	7.0	*2.9*		53.7	*7.6*	*	1.1	*0.6*	*
		HR	143.8	*125.1*	54.2	*7.9*		1.9	*1.3*		2.1	*2.6*		41.8	*6.8*		2.2	*0.5*	*
N PC	25	Ctr	267.8	*139.8*	51.5	*8.8*		1.8	*1.5*		3.8	*2.9*		42.9	*7.6*		1.5	*0.6*	a
		LR	28.0	*197.8*	70.9	*12.4*		0.0	*2.1*	*	0.0	*4.1*		28.5	*10.7*		5.7	*0.8*	b
		HR	83.0	*139.8*	55.2	*8.8*		1.9	*1.5*		1.2	*2.9*		41.8	*7.6*		1.5	*0.6*	a*
N IC	27	Ctr	95.5	*139.8*	54.9	*8.8*		2.3	*1.5*		5.8	*2.9*		37.0	*7.6*		1.8	*0.6*	
		LR	24.3	*161.5*	42.0	*10.1*		2.4	*1.7*	*	8.4	*3.3*		47.2	*8.7*	*	1.1	*0.7*	*
		HR	73.3	*139.8*	46.1	*8.8*		4.7	*1.5*		2.0	*2.9*		47.1	*7.6*		1.1	*0.6*	*
N PC	29	Ctr	72.8	*139.8*	47.6	*8.8*		1.8	*1.5*		6.3	*2.9*		44.3	*7.6*		1.3	*0.6*	
		LR	23.3	*139.8*	54.7	*8.8*		2.4	*1.5*	*	7.8	*2.9*		35.0	*7.6*		2.2	*0.6*	
		HR	65.6	*125.1*	57.6	*7.9*		1.9	*1.3*		2.6	*2.6*		37.9	*6.8*		1.8	*0.5*	*
N IC (iFA)	31	Ctr	70.5	*139.8*	55.7	*8.8*		3.0	*1.5*		3.9	*2.9*		37.4	*7.6*		1.7	*0.6*	
		LR	25.8	*139.8*	49.0	*8.8*		1.1	*1.5*	*	7.3	*2.9*		42.7	*7.6*	*	1.5	*0.6*	*
		HR	60.8	*125.1*	64.2	*7.9*		2.3	*1.3*		1.5	*2.6*		32.0	*6.8*		2.3	*0.5*	*

The total and differential cell counts of the somatic cells (SCC) in milk are listed as LSM ± SD per group (Ctr = control cows, n = 5; LR = low responder cows, n = 4; HR = high responder cows, n = 5), per treatment week and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N = nasal, PC = percutaneous, SC = subcutaneous, IC = intracutaneous (iFA = incomplete Freund's adjuvant)). TWs 1 and 15 in bold mark in each case the time point before the first use of the vaccine batch A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging SCC values related to the first uses of the vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 1 in the following time period until and including TW 13, and relating to TW 15 in the remaining treatment period. Lower case letters label significant differences (P < 0.05) between groups to the same time point. PMN = polymorphnucleated neutrophilic leukocytes; Phag = phagocytes (counts of PMN and macrophages); Lym = lymphocytes; n.n. = numerius negidius (unavailable data) [76].

Gene expressions related to the innate immune system

The expression of genes indicating surface determinants / receptors and immune mediators of the innate IS were analyzed in blood and milk cells. Whose evaluation based on their regulation per group within the first and second treatment periods starting at TWs 0/ 1 and TWs 16/ 17, respectively. Additionally, gene regulations were considered regarding significant differences between the groups at the same time of treatment. The results of the gene expression analysis in PBL and milk cells encompass the Table 11-Table 17. The detailed description of every gene regulation investigated can be found in the original paper of Schmautz et al. (2018b) presented in the Appendix II [76].

In peripheral blood leukocytes

The gene expressions of all phagocytes' related surface determinants examined in the blood cells were weakly influenced by the treatment (Table 11). Among the mediators emitted by phagocytes in blood, the genes of the examined interleukins were (very) strongly regulated during the observation period, whereas that of the chemokines, *TNFα* and *CXCL8*, were weakly affected (Table 12).

The initial down-regulation of several receptors of phagocytes, like *C5AR1* and *CXCR2*, got started subsequent to the application of *MucoCD-I batch A* in TW 3 (Table 11). One week later, it was significantly given for *TLR2* and *CD163*, too. Likewise, noticeable reduced PBL were counted following injecting *MucoCD-I batch A* (Table 9). Additionally, the low PMN counts of the HR towards the other groups correspond to the belonging expressions of *CXCR2* and *C5AR1* in TW 3. Further corresponding results were not determined between gene expression of phagocytes' specific surface determinants and the DCC in PBL. The down-regulations of *CXCR2* and *CD163*, marking PMN and monocytes / macrophages, respectively, lasted in PBL of HR and, partly, LR up to the end of the first IM period. The innate immune response acts immediately following antigen contact, within few minutes. Then the adaptive IS obtains the leading role to eliminate the threat originating from the antigen [59]. An ongoing confrontation with the same antigen activates the immunological memory [86]. Thus cells of the innate IS could lose their relevance attacking the injecting antigens during the solely N treatment. Noticeable differences between the treated groups existed regarding their chemokine profiles in the first IM period (Table 12). The HR's one was characterized by more pronounced down-regulations of *IL1β* and *IL6* and a partly stronger up-regulation of *CXCL8* in contrast to the LR. In combination, these regulation of chemokines could have encouraged vasodilatation for recall of defined leukocyte populations but without activating more lymphocytes [59] [96]. Possibly, the decreased transcription of *IL1β* and *IL6*

prevented an overflowing response by the specific IS of the HR, especially, of their specific Ab production (Figure 2). In contrast, the LR showed a strong up-regulation of *IL12β*. Adjuvants effects of *IL12β* were proven in cattle promoting enhanced type 1 cytokine responses [67] [97]. This cytokine is *ex vivo* a potent inducer of *IFNγ* in T-cells stimulating their differentiation memory / effector cells and effecting increased IgG1 responses, *i.a.* [97] [98]. Correspondingly, *IFNγ* was up-regulated by the LR in TW 8 (Table 19).

The second IM period was indicated by chemokines similarly regulated when comparing the treated groups. Uniformly, *IL1β* was down-regulated once short-term, *IL12β* was lastingly down-regulated, and *IL6* was permanently up-regulated in PBL of HR and LR following the triple-vaccination in TW 21. *IL6* contributes to the maturation of T_H2 cells and also of B-cells into APC [67] [96]. The promotion of the T_H2 immune response indicated by antigen specific IgA-formation was only recognizable in case of the HR (Figure 2), despite stronger up-regulations of *IL6* by the LR in the TWs 18 and 27.

Table 11: ΔΔCq changes in gene expression of peripheral blood phagocytes' receptors relative to TW 0 or rather TW 16

		gene	C3AR1			C5AR1			CXCR2			TLR2			CD163		
		regulation	+			+			+			+			+		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
	0	Ctr	0.00	*0.64*		0.00	*0.63*		0.00	*0.51*		0.00	*0.43*		0.00	*0.57*	
		LR	0.00	*0.55*		0.00	*0.55*		0.00	*0.44*		0.00	*0.37*		0.00	*0.50*	
		HR	0.00	*0.64*		0.00	*0.63*		0.00	*0.51*		0.00	*0.43*		0.00	*0.57*	
N	1	Ctr	-0.41	*0.64*		0.49	*0.63*		-0.11	*0.51*		-0.02	*0.43*		-0.04	*0.57*	
		LR	0.53	*0.55*		0.86	*0.55*		-0.62	*0.44*		0.18	*0.37*		1.21	*0.50*	
		HR	0.39	*0.55*		-0.43	*0.55*		-0.84	*0.44*		-0.25	*0.37*		0.26	*0.50*	
	2	Ctr	-0.65	*0.64*		0.25	*0.63*		-0.25	*0.51*		-0.03	*0.43*		0.48	*0.57*	ab
		LR	-0.02	*0.55*		0.91	*0.55*		-0.29	*0.44*		-0.02	*0.37*		0.96	*0.50*	a
		HR	0.09	*0.55*		0.29	*0.55*		-0.52	*0.44*		-0.38	*0.37*		-0.57	*0.50*	b
N PC	3	Ctr	-0.20	*0.64*		1.43	*0.63*	a	0.73	*0.51*	a	0.04	*0.43*		-0.23	*0.57*	
		LR	0.41	*0.64*		-0.15	*0.63*	ab	-1.22	*0.51*	b	0.08	*0.43*		0.15	*0.57*	
		HR	0.20	*0.55*		-0.75	*0.55*	b	-2.03	*0.44*	b*	-0.31	*0.37*		-0.13	*0.50*	
	4	Ctr	0.06	*0.64*		0.36	*0.63*	a	-0.17	*0.51*	a	0.23	*0.43*	a	0.13	*0.57*	a
		LR	0.64	*0.64*		-1.56	*0.63*	b	-2.26	*0.51*	b*	-1.16	*0.43*	b*	-2.29	*0.57*	b*
		HR	0.12	*0.55*		-2.63	*0.55*	b*	-2.36	*0.44*	b*	-1.20	*0.37*	b*	-2.01	*0.50*	b*
N	5	Ctr	*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		LR	0.31	*0.55*		-0.57	*0.55*		-1.97	*0.51*	*	-0.92	*0.37*		-0.44	*0.50*	
		HR	0.27	*0.49*		-0.63	*0.49*		-0.79	*0.51*		-0.71	*0.33*		-0.50	*0.44*	
	6	Ctr	-0.16	*0.64*		0.37	*0.63*		0.16	*0.51*	a	-0.21	*0.43*		0.63	*0.57*	
		LR	0.30	*0.55*		-0.98	*0.55*		-1.61	*0.63*	b*	-0.73	*0.37*		-0.07	*0.50*	
		HR	0.99	*0.49*		-0.99	*0.49*		-1.10	*0.44*	ab	-0.03	*0.33*		-0.76	*0.44*	
N	7	Ctr	*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		LR	-0.13	*0.55*		-0.10	*0.55*		-1.08	*0.44*		-0.50	*0.37*		-0.64	*0.50*	
		HR	0.69	*0.49*		-1.19	*0.49*		-2.07	*0.40*	**	-0.98	*0.33*		-1.87	*0.44*	*
	8	Ctr	0.15	*0.64*	a	-0.02	*0.63*		-0.34	*0.51*	a	-0.58	*0.43*	ab	-0.16	*0.57*	a
		LR	1.82	*0.55*	b*	-0.63	*0.55*		-2.56	*0.44*	b*	-0.21	*0.37*	a	0.24	*0.50*	a
		HR	0.90	*0.49*	ab	-0.88	*0.49*		-2.85	*0.40*	b*	-1.32	*0.33*	b*	-2.16	*0.44*	b*
N	10	Ctr	0.12	*0.64*		0.19	*0.63*	ab	-0.18	*0.51*	a	-1.15	*0.43*		0.40	*0.57*	ab
		LR	0.11	*0.55*		1.74	*0.55*	a*	-1.24	*0.44*	ab	-0.44	*0.37*		0.61	*0.50*	a
		HR	-0.20	*0.49*		-0.26	*0.49*	b	-1.69	*0.40*	b*	-1.36	*0.33*	*	-0.86	*0.44*	b
N	12	Ctr	1.10	*0.49*		-0.04	*0.49*		-0.17	*0.40*	a	-1.40	*0.33*	*	0.42	*0.44*	a
		LR	0.09	*0.55*		-0.86	*0.55*		-2.29	*0.44*	b*	-1.97	*0.37*	*	-0.97	*0.50*	b
		HR	0.68	*0.49*		-0.58	*0.49*		-1.51	*0.40*	b*	-1.77	*0.33*	*	-1.36	*0.44*	b

		gene	C3AR1			C5AR1			CXCR2			TLR2			CD163		
		regulation	+			+			+			+			+		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	14	Ctr	1.12	*0.64*		-0.45	*0.63*	ab	-0.20	*0.51*	a	-1.32	*0.43*	*	-0.10	*0.57*	a
		LR	0.39	*0.55*		0.34	*0.55*	a	-1.51	*0.44*	ab*	-1.91	*0.37*	*	-0.78	*0.50*	a
		HR	-0.20	*0.49*		-1.16	*0.49*	b	-2.41	*0.40*	b*	-2.16	*0.33*	*	-2.12	*0.44*	b*
N	**16**	Ctr	0.00	*0.49*		0.00	*0.49*		0.00	*0.40*		0.00	*0.33*		0.00	*0.44*	
		LR	0.00	*0.55*		0.00	*0.55*		0.00	*0.44*		0.00	*0.37*		0.00	*0.50*	
		HR	0.00	*0.49*		0.00	*0.49*		0.00	*0.40*		0.00	*0.33*		0.00	*0.44*	
N	18	Ctr	0.25	*0.49*	a	-0.03	*0.49*		-0.35	*0.40*	ab	-1.01	*0.33*	a	0.03	*0.44*	
PC		LR	1.83	*0.55*	b*	-0.25	*0.55*		-0.84	*0.44*	a	0.56	*0.37*	b	-0.55	*0.50*	
		HR	1.97	*0.49*	b*	1.15	*0.49*		0.53	*0.40*	b	0.49	*0.33*	b	-0.57	*0.44*	
N	20	Ctr	0.19	*0.49*		0.71	*0.49*		0.13	*0.40*	a	-0.91	*0.33*		-0.39	*0.44*	
		LR	0.05	*0.55*		1.17	*0.55*		0.92	*0.44*	ab	-0.30	*0.37*		-0.18	*0.50*	
		HR	1.06	*0.49*		2.04	*0.49*	*	1.68	*0.40*	b*	-0.24	*0.33*		-0.46	*0.44*	
N	21	Ctr	0.58	*0.49*	a	0.36	*0.49*		-0.04	*0.40*		-1.06	*0.33*	a*	-0.20	*0.44*	
PC		LR	0.65	*0.55*	ab	-0.97	*0.55*		-0.98	*0.44*		1.98	*0.37*	b*	0.01	*0.50*	
SC		HR	2.02	*0.49*	b*	-0.86	*0.49*		-0.62	*0.40*		2.08	*0.33*	b*	0.48	*0.44*	
N	23	Ctr	0.66	*0.49*	ab	0.76	*0.49*	a	0.16	*0.40*		-0.38	*0.33*	a	0.00	*0.44*	
		LR	-0.51	*0.55*	a	-0.46	*0.63*	ab	-0.51	*0.44*		1.91	*0.37*	b*	-0.16	*0.50*	
		HR	1.22	*0.49*	b	-1.03	*0.49*	b	-0.50	*0.40*		2.09	*0.33*	b*	0.11	*0.44*	
N	25	Ctr	0.15	*0.49*		0.41	*0.49*		0.03	*0.40*		-1.15	*0.33*	a*	-0.17	*0.44*	
PC		LR	0.04	*0.55*		-0.14	*0.55*		0.44	*0.44*		1.54	*0.37*	b*	-0.66	*0.50*	
		HR	1.24	*0.49*		-0.05	*0.49*		0.58	*0.40*		1.35	*0.33*	b*	-0.39	*0.44*	
N	27	Ctr	-0.57	*0.55*	a	1.14	*0.55*		0.61	*0.44*		-0.61	*0.37*	a	0.11	*0.50*	
IC		LR	1.60	*0.55*	b*	-0.27	*0.55*		0.15	*0.44*		1.38	*0.37*	b*	0.68	*0.50*	
		HR	1.85	*0.49*	b*	0.06	*0.49*		1.24	*0.44*	*	1.00	*0.33*	b*	-0.11	*0.44*	
N	29	Ctr	0.23	*0.49*		1.03	*0.49*		1.07	*0.40*		-0.73	*0.33*	a	0.17	*0.44*	
PC		LR	0.57	*0.55*		-0.13	*0.55*		0.70	*0.44*		1.07	*0.37*	b*	-0.15	*0.50*	
		HR	1.37	*0.49*		0.30	*0.49*		0.95	*0.40*		1.04	*0.33*	b*	-0.36	*0.44*	
N	31	Ctr	0.01	*0.49*	a	0.71	*0.49*		0.59	*0.40*		-0.73	*0.33*	a	-0.19	*0.44*	
IC		LR	0.35	*0.55*	ab	0.08	*0.55*		0.90	*0.44*		1.03	*0.37*	b	-0.27	*0.50*	
(iFA)		HR	1.71	*0.49*	b*	0.04	*0.49*		1.21	*0.40*	*	1.08	*0.33*	b*	0.00	*0.44*	

The gene regulation is graded as "+" for weak regulated. The ΔΔCq data for the gene expression of the blood phagocytes' receptors (C3AR1, C5AR1, CXCR2, TLR2, CD163) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 0 and 16 in bold mark in each case the time point before the first use of the vaccine batch A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 0 in the time period until and including TW 14, and relating to TW 16 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point. Unavailable data are described as n.n.(numerius negidius) [76].

Table 12: ΔΔCq changes in gene expression of blood phagocytes' chemokines / cytokines relative to TW 0 or rather TW 16

		gene	CXCL8			TNFα			IL1β			IL6			IL12β		
		regulation	+			+			++			+++			++		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
	0	Ctr	0.00	*0.58*		0.00	*0.42*		0.00	*0.57*		0.00	*0.71*		0.00	*0.52*	
		LR	0.00	*0.50*		0.00	*0.36*		0.00	*0.49*		0.00	*0.61*		0.00	*0.45*	
		HR	0.00	*0.58*		0.00	*0.42*		0.00	*0.57*		0.00	*0.71*		0.00	*0.52*	
N	1	Ctr	0.07	*0.58*	a	-0.44	*0.42*		0.23	*0.57*	a	0.01	*0.71*		-0.63	*0.52*	a
		LR	-2.29	*0.50*	b*	0.18	*0.36*		0.26	*0.49*	a	0.76	*0.61*		1.38	*0.45*	b*
		HR	-1.09	*0.50*	ab	0.60	*0.36*		-1.54	*0.49*	b*	0.43	*0.61*		-0.12	*0.45*	a
	2	Ctr	-0.31	*0.58*		-0.05	*0.42*		0.53	*0.57*	a	0.57	*0.71*		-0.51	*0.52*	a
		LR	-1.07	*0.50*		-0.32	*0.36*		0.01	*0.49*	ab	-0.69	*0.61*		1.21	*0.45*	b
		HR	-1.06	*0.50*		0.32	*0.36*		-0.98	*0.49*	b	-0.77	*0.61*		-0.09	*0.45*	a
N PC	3	Ctr	-0.55	*0.58*	a	0.20	*0.42*		0.39	*0.57*	a	1.01	*0.71*		-0.75	*0.52*	a
		LR	-2.79	*0.58*	b*	0.05	*0.42*		-0.62	*0.57*	a	0.21	*0.71*		1.81	*0.52*	b*
		HR	-1.37	*0.50*	ab	0.59	*0.36*		-2.60	*0.49*	b*	0.22	*0.61*		0.42	*0.45*	a
	4	Ctr	0.00	*0.58*	a	-0.34	*0.42*		0.56	*0.57*	a	0.13	*0.71*		-0.37	*0.52*	a
		LR	1.58	*0.58*	ab*	-0.26	*0.42*		-0.96	*0.57*	ab	0.41	*0.71*		-1.02	*0.52*	ab
		HR	2.90	*0.50*	b*	0.11	*0.36*		-2.35	*0.49*	b*	0.29	*0.61*		-1.96	*0.45*	b*
N	5	Ctr	*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		LR	-0.42	*0.50*	a	-0.04	*0.36*		-0.64	*0.49*		-0.92	*0.61*		0.48	*0.45*	
		HR	1.39	*0.45*	b	0.13	*0.32*		-0.91	*0.44*		-0.36	*0.55*		-0.02	*0.40*	
	6	Ctr	0.54	*0.58*	ab	0.11	*0.42*		1.33	*0.57*	a	0.93	*0.71*		-0.77	*0.52*	a
		LR	0.00	*0.50*	a	-0.20	*0.36*		-0.32	*0.57*	b	0.23	*0.61*		0.88	*0.45*	b
		HR	1.53	*0.45*	b*	0.27	*0.32*		-1.31	*0.44*	b	0.56	*0.55*		-0.30	*0.40*	ab
N	7	Ctr	*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		LR	-0.28	*0.50*	a	0.18	*0.36*		-2.12	*0.49*	*	-0.49	*0.61*		0.22	*0.45*	
		HR	1.61	*0.45*	b*	0.22	*0.32*		-3.19	*0.49*	*	-0.45	*0.55*		-0.67	*0.40*	
	8	Ctr	0.29	*0.58*	a	-0.29	*0.42*		0.88	*0.57*	a	0.66	*0.71*	ab	-0.39	*0.52*	a
		LR	1.49	*0.50*	ab*	-0.18	*0.36*		-2.08	*0.49*	b*	1.02	*0.61*	a	3.09	*0.45*	b*
		HR	2.34	*0.45*	b*	-0.07	*0.32*		-3.97	*0.44*	c*	-1.02	*0.55*	b	1.41	*0.40*	c*
N	10	Ctr	1.93	*0.58*	a*	0.25	*0.42*		1.57	*0.57*	a	1.03	*0.71*	a	-0.09	*0.52*	a
		LR	-1.24	*0.50*	b	0.96	*0.36*		-1.19	*0.49*	b	-0.31	*0.61*	a	3.51	*0.45*	b*
		HR	0.84	*0.45*	a	0.05	*0.32*		-2.29	*0.44*	b*	-2.00	*0.55*	b*	2.07	*0.40*	c*
N	12	Ctr	1.90	*0.45*	a*	-0.20	*0.32*		2.06	*0.44*	a*	1.10	*0.55*	a	-0.36	*0.40*	a
		LR	0.51	*0.50*	b	-0.59	*0.36*		-1.49	*0.49*	b*	-0.77	*0.61*	b	2.44	*0.45*	b*
		HR	2.18	*0.45*	a*	0.14	*0.32*		-1.43	*0.44*	b*	-0.54	*0.55*	b	1.73	*0.40*	b*

		gene	CXCL8			TNFα			IL1β			IL6			IL12β		
		regulation	+			+			++			+++			++		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	14	Ctr	0.45	*0.58*	a	-0.31	*0.42*		1.49	*0.57*	a	0.37	*0.71*	a	-0.41	*0.52*	a
		LR	-1.33	*0.50*	b	-0.28	*0.36*		-0.81	*0.49*	b	-2.91	*0.61*	b*	2.67	*0.45*	b*
		HR	0.74	*0.45*	a	-0.19	*0.32*		-2.07	*0.44*	b*	-2.60	*0.55*	b*	0.83	*0.40*	a
N	**16**	Ctr	0.00	*0.45*		0.00	*0.32*		0.00	*0.44*		0.00	*0.55*		0.00	*0.40*	
		LR	0.00	*0.50*		0.00	*0.36*		0.00	*0.49*		0.00	*0.61*		0.00	*0.45*	
		HR	0.00	*0.45*		0.00	*0.32*		0.00	*0.44*		0.00	*0.55*		0.00	*0.40*	
N	18	Ctr	-1.03	*0.45*	a	0.69	*0.32*		-0.09	*0.44*		0.77	*0.55*	a	-0.05	*0.40*	
PC		LR	0.70	*0.50*	b	0.61	*0.36*		0.28	*0.49*		2.76	*0.61*	b*	0.56	*0.45*	
		HR	0.85	*0.45*	b	1.19	*0.32*	*	0.87	*0.44*		0.87	*0.55*	a	0.81	*0.40*	
N	20	Ctr	-1.31	*0.45*	a*	0.57	*0.32*		-0.13	*0.44*	a	0.01	*0.55*		-0.07	*0.40*	
		LR	-0.18	*0.50*	ab	0.65	*0.36*		-0.16	*0.57*	a	0.42	*0.61*		0.47	*0.52*	
		HR	0.08	*0.45*	b	1.06	*0.32*	*	-2.20	*0.49*	b*	0.30	*0.55*		0.06	*0.40*	
N	21	Ctr	-1.54	*0.45*	a*	1.04	*0.32*	*	-0.22	*0.44*	a	-0.18	*0.55*	a	0.16	*0.40*	a
PC		LR	-0.31	*0.50*	ab	1.46	*0.36*	*	-1.78	*0.49*	b	3.70	*0.61*	b*	-2.41	*0.52*	b*
SC		HR	0.12	*0.45*	b	1.86	*0.32*	*	-0.24	*0.49*	a	3.80	*0.55*	b*	-2.85	*0.63*	b*
N	23	Ctr	-2.56	*0.45*	a*	0.94	*0.32*	*	0.40	*0.44*		-0.32	*0.55*	a	0.18	*0.40*	a
		LR	1.01	*0.50*	b	0.61	*0.36*		0.39	*0.49*		3.39	*0.61*	b*	-3.46	*0.45*	b*
		HR	0.86	*0.45*	b	0.91	*0.32*	*	0.54	*0.44*		4.06	*0.55*	b*	-2.98	*0.63*	b*
N	25	Ctr	-1.69	*0.45*	a*	0.83	*0.32*		-0.31	*0.44*		-0.63	*0.55*	a	-0.15	*0.40*	a
PC		LR	1.17	*0.50*	b	0.69	*0.36*		0.61	*0.49*		1.82	*0.61*	b*	-2.86	*0.45*	b*
		HR	1.18	*0.45*	b	0.93	*0.32*	*	0.46	*0.44*		2.10	*0.55*	b*	-3.16	*0.40*	b*
N	27	Ctr	-2.37	*0.50*	a*	-0.02	*0.36*	a	-0.02	*0.49*		-0.47	*0.61*	a	-0.27	*0.45*	a
IC		LR	0.60	*0.50*	b	1.93	*0.36*	b*	-0.20	*0.49*		5.80	*0.61*	b*	-2.52	*0.89*	b*
		HR	1.05	*0.45*	b	1.82	*0.32*	b*	0.37	*0.44*		4.07	*0.55*	c*	-3.08	*0.52*	b*
N	29	Ctr	-2.41	*0.45*	a*	0.53	*0.32*		0.35	*0.44*		-1.01	*0.55*	a	-0.51	*0.40*	a
PC		LR	0.72	*0.50*	b	0.90	*0.36*		0.27	*0.49*		3.37	*0.61*	b*	-2.94	*0.45*	b*
		HR	0.97	*0.45*	b	1.12	*0.32*	*	0.99	*0.44*		2.07	*0.55*	b*	-2.13	*0.40*	b*
N	31	Ctr	-1.33	*0.45*	a*	1.75	*0.32*	*	-0.29	*0.44*		0.93	*0.55*	a	-1.25	*0.40*	a*
IC		LR	0.33	*0.50*	b	0.90	*0.36*		0.33	*0.49*		2.10	*0.61*	ab*	-2.47	*0.45*	b*
(iFA)		HR	0.43	*0.45*	b	1.00	*0.32*	*	0.79	*0.44*		2.69	*0.55*	b*	-2.30	*0.45*	ab*

The gene regulation is graded as "+" for weak regulated, "++" for strong regulated and "+++" for very strong regulated. The ΔΔCq data for the gene expression of the blood phagocytes' chemokines and cytokines (CXCL8, TNFα, IL1β, IL6 and IL12β) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 0 and 16 in bold mark in each case the time point before the first use of the vaccine batch A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of the vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 0 in the time period until and including TW 14, and relating to TW 16 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point. Unavailable data are described as n.n.(numerius negidius) [76].

In bovine mammary epithelial cells of milk

The genes for the epithelial IgG-receptor (*FcRN*), the complement protein *C1QA* and the chemokine *CXCL5* expressed by bMEC were proven as to be unregulated. The gene expressions of *CCL5*, *CCL28*, *CXCL3, CXCL8*, the complement protein *C3* and the antimicrobial peptides, *LF*, *LYZ1* and *TAP*, were weakly changed due to the treatment. The chemokine *CCL20*, the bactericidal enzyme *LPO* and the epithelial IgA-receptor *PIGR* were classified as strongly regulated (Table 13-Table 15).

The application of *MucoCD-I* batch A effected increased *CCL28* expressions of the treated groups towards the Ctr (Table 14), possibly, fulfilling its essential role in the accumulation of IgA-ASC in the MG [99]. Among antimicrobial factors produced by bMEC, *LF* was differently expressed between LR and HR several times up to TW 13 (Table 15). This result was in line with the *in vitro* study profiling the gene expression in primary bMEC [100]. But in contrast to the *in vitro* study referred [100], the relative expression of *LF* by the LR increased while that of the HR declined as proven several times in the second half of the first IM period. *LPO* was stronger expressed by the treated groups compared with the Ctr in the TWs 5 and 6 (Table 15). But unlike to the outcome of the *in vitro* study referred [100], no different regulations of this bactericidal enzyme were determined between LR and HR. The frequent triggering with antigen PC next to the MG did not cause any indications of self-arming as measured on the expression of genes related to the bMEC.

Quite the contrary, the repeated PC inoculations with *MucoCD-I* batch B in the area of the SLN seemed to result in a calming effect on the IS of the MG respecting the down-regulations of *CXCL3* and *CCL20* by the HR, especially (Table 14). Both cytokines aim at the activation of lymphocytes and phagocytes [62] [63] [101]. But at this stage of IM, the injected antigen was well-known by the treated cows' IS, wherefore the immunological memory was in the frontline combating *C. diff.* and its toxins. Thus the treated cows' IS habituates on the repeated stimulations with the same antigens, being under control of the immunological memory. As any direct injuries of the MG, causing defensive forces of this organ, were absent, a beneficial effect of a physiological stress response in the MG is conceivable [102]. Accordingly, many milk phagocytes' related genes were reduced transcribed for several times during the second IM period (Table 16 and Table 17).

The crucial receptor for the epithelial IgA transfer into milk, *PIGR,* was not ascertained as expressed significantly different between HR and LR at any time of the treatment (Table 13). In response to the vaccination with *MucoCD-I* batch A, the LR's *PIGR* was solely up-regulated compared to the Ctr in TW 6, downstream of the triggered anti-*C. diff.* IgA production by the vaccine batch A (Figure 2). Nevertheless, this up-regulation of the receptor

in bMEC of LR corresponds to their also enhanced *IFNγ* expression to this date (Table 21). Some cytokines are also regulators of the PIGR expression beside hormones, dietary and microbial factors, and *IFNγ* induces the transcription of the human *PIGR* gene, *i.e.* [53] [103]. This relation was not demonstrable again, when *PIGR* was up-regulated twice by the HR during the second treatment period.

Table 13: ΔΔCq changes in gene expression of bMEC related Ig receptors and complement proteins relative to TW 1 or rather TW 15

		gene	PIGR		FcRN		C1QA		C3		
		regulation	++		0		0		+		
IM	**TW**	**group**	LSM	*SD*	LSM	*SD*	LSM	*SD*	LSM	*SD*	
N	**1**	Ctr	0.00	*1.48*	0.00	*0.76*	0.00	*0.86*	0.00	*0.50*	
		LR	0.00	*1.48*	0.00	*0.76*	0.00	*0.99*	0.00	*0.57*	
		HR	0.00	*1.29*	0.00	*0.66*	0.00	*0.86*	0.00	*0.50*	
N PC	3	Ctr	-1.74	*1.29*	-0.74	*0.66*	0.32	*0.86*	-0.10	*0.50*	
		LR	0.47	*1.48*	-0.57	*0.76*	-0.59	*0.99*	-0.16	*0.57*	
		HR	0.61	*1.29*	0.18	*0.66*	0.31	*0.86*	0.64	*0.57*	
	4	Ctr	-0.17	*1.15*	-0.75	*0.66*	0.17	*0.99*	0.07	*0.50*	
		LR	-0.13	*2.57*	-0.68	*1.32*	-0.77	*1.72*	0.33	*1.00*	
		HR	0.23	*1.48*	0.40	*0.76*	0.57	*0.99*	0.20	*0.57*	
N	5	Ctr	-1.11	*1.15*	-0.74	*0.59*	-0.89	*0.86*	-0.12	*0.50*	a
		LR	1.54	*1.29*	-1.16	*0.66*	-0.92	*0.86*	1.61	*0.50*	b*
		HR	1.55	*1.29*	-1.09	*0.66*	-1.90	*0.86*	1.12	*0.50*	ab
	6	Ctr	-2.44	*1.29*	a	-1.31	*0.66*	0.80	*0.86*	-0.31	*0.50*
		LR	2.91	*1.82*	b	-1.24	*0.93*	-1.14	*1.22*	1.16	*0.70*
		HR	0.43	*1.82*	ab	-0.07	*0.93*	-1.10	*1.22*	0.85	*0.70*
N	7	Ctr	0.62	*1.29*		-0.81	*0.66*	0.53	*0.86*	-0.02	*0.50*
		LR	2.72	*1.48*		-1.20	*0.76*	-0.01	*0.99*	1.26	*0.57*
		HR	0.67	*1.29*		-1.05	*0.66*	0.13	*0.86*	0.11	*0.50*
	8	Ctr	1.69	*1.29*		-0.40	*0.66*	0.48	*0.86*	0.60	*0.50* a
		LR	0.31	*1.29*		-0.83	*0.66*	0.59	*0.99*	0.60	*0.50* a
		HR	-0.07	*1.15*		-0.64	*0.59*	0.35	*0.77*	-0.84	*0.45* b
N	9	Ctr	0.22	*1.29*		0.15	*0.66*	0.97	*0.86*	0.00	*0.50*
		LR	1.10	*1.29*		-0.22	*0.66*	1.16	*0.86*	-0.14	*0.50*
		HR	-1.80	*1.15*		-0.23	*0.59*	0.91	*0.77*	-0.64	*0.45*
N	11	Ctr	1.14	*1.15*		-0.06	*0.59*	0.23	*0.77*	0.38	*0.45*
		LR	1.22	*1.48*		0.23	*0.76*	0.41	*0.86*	0.13	*0.50*
		HR	-0.43	*1.15*		0.66	*0.59*	0.14	*0.99*	0.10	*0.57*
N	13	Ctr	0.19	*1.15*		0.27	*0.66*	0.23	*0.86*	0.99	*0.50*
		LR	-0.99	*1.29*		0.00	*0.66*	-0.62	*0.86*	0.10	*0.50*
		HR	-1.96	*1.29*		0.45	*0.66*	-0.15	*0.86*	-0.31	*0.50*

		gene	PIGR			FcRN		C1QA		C3		
		regulation	++			0		0		+		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*	LSM	*SD*	LSM	*SD*	
N	**15**	Ctr	0.00	*1.15*		0.00	*0.59*	0.00	*0.77*	0.00	*0.45*	
		LR	0.00	*1.29*		0.00	*0.66*	0.00	*0.86*	0.00	*0.50*	
		HR	0.00	*1.15*		0.00	*0.59*	0.00	*0.77*	0.00	*0.45*	
N PC	17	Ctr	-1.66	*1.15*	a	0.17	*0.66*	-0.13	*0.86*	0.55	*0.45*	
		LR	0.65	*1.29*	ab	1.40	*0.66*	0.48	*0.86*	-0.05	*0.50*	
		HR	2.21	*1.15*	b	0.23	*0.59*	-0.39	*0.77*	0.15	*0.45*	
N	19	Ctr	0.15	*1.15*		0.09	*0.59*	-0.11	*0.77*	0.19	*0.45*	
		LR	1.58	*1.29*		1.00	*0.66*	-0.06	*0.86*	-0.51	*0.50*	
		HR	0.89	*1.15*		0.65	*0.59*	0.18	*0.77*	0.16	*0.45*	
N PC SC	21	Ctr	1.57	*1.29*		0.56	*0.66*	-1.49	*0.99*	0.99	*0.57*	
		LR	2.87	*1.29*		1.10	*0.66*	-0.29	*0.86*	0.31	*0.50*	
		HR	3.34	*1.15*	*	0.14	*0.59*	0.47	*0.77*	1.09	*0.45*	
N	23	Ctr	0.60	*1.15*		-0.05	*0.59*	-0.60	*0.77*	0.68	*0.45*	
		LR	1.98	*1.29*		0.98	*0.66*	0.30	*0.86*	0.70	*0.50*	
		HR	2.81	*1.15*		0.19	*0.59*	0.20	*0.77*	1.06	*0.45*	
N PC	25	Ctr	0.00	*1.29*		-0.15	*0.66*	-0.65	*0.86*	0.46	*0.50*	
		LR	2.05	*1.82*		0.70	*0.93*	-0.03	*1.22*	-0.23	*0.70*	
		HR	1.52	*1.29*		0.02	*0.66*	0.25	*0.86*	0.05	*0.50*	
N IC	27	Ctr	0.64	*1.29*		-0.02	*0.66*	-0.24	*0.86*	0.87	*0.50*	a
		LR	1.59	*1.48*		0.29	*0.76*	-0.01	*0.99*	-0.76	*0.57*	b
		HR	0.13	*1.29*		-0.09	*0.66*	-0.16	*0.99*	-0.46	*0.50*	ab
N PC	29	Ctr	1.17	*1.29*		-0.07	*0.66*	-0.09	*0.86*	0.80	*0.50*	
		LR	2.15	*1.29*		-0.13	*0.66*	0.68	*0.86*	-0.53	*0.50*	
		HR	1.92	*1.15*		-0.34	*0.59*	1.00	*0.86*	0.00	*0.50*	
N IC (iFA)	31	Ctr	0.38	*1.29*		1.04	*0.66*	0.81	*0.99*	0.61	*0.50*	
		LR	2.03	*1.29*		0.07	*0.66*	0.93	*0.86*	-0.20	*0.50*	
		HR	1.49	*1.15*		-0.57	*0.59*	1.00	*0.77*	-0.22	*0.45*	

The gene regulation is graded as "0" for non regulated, "+" for weak regulated and "++" for strong regulated. The ΔΔCq data for the gene expression of the bovine mammary epithelial cells' (bMEC) receptors (PIGR, FcRN) and complement proteins (C1QA, C3) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 1 and 15 in bold mark in each case the time point before the first use of the vaccine batches A (TW 3) and B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 1 in the following time period until and including TW 13, and relating to TW 15 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point [76].

Table 14: ΔΔCq changes in gene expression of bMEC related chemokines relative to TW 1 or rather TW 15

		gene	CCL5			CCL20			CCL28			CXCL3			CXCL5			CXCL8		
		regulation	+			++			+			+			0			+		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	**1**	Ctr	0.00	*0.81*		0.00	*1.05*		0.00	*1.07*		0.00	*0.83*		0.00	*1.15*		0.00	*1.29*	
		LR	0.00	*0.81*		0.00	*1.05*		0.00	*1.07*		0.00	*0.83*		0.00	*1.15*		0.00	*1.29*	
		HR	0.00	*0.70*		0.00	*0.91*		0.00	*0.93*		0.00	*0.72*		0.00	*0.99*		0.00	*1.12*	
N PC	3	Ctr	-0.10	*0.70*		2.37	*0.91*	a	-0.51	*0.93*		-0.71	*0.72*		-0.25	*0.99*		-0.41	*1.12*	
		LR	-0.24	*0.81*		-0.18	*1.05*	ab	0.85	*1.07*		0.28	*0.83*		1.27	*1.15*		0.92	*1.29*	
		HR	-1.59	*0.70*		-0.31	*0.91*	b	0.57	*0.93*		0.78	*0.72*		-0.59	*0.99*		0.77	*1.12*	
	4	Ctr	0.24	*0.63*		-0.91	*0.91*		-1.08	*0.83*		-1.24	*0.64*		-0.39	*0.89*		-0.70	*1.00*	
		LR	1.57	*1.40*		1.47	*1.82*		-0.23	*1.86*		-0.73	*1.43*		0.72	*1.98*		-0.68	*2.23*	
		HR	-0.97	*0.81*		0.02	*1.05*		0.78	*1.07*		-0.98	*0.83*		-0.23	*1.15*		-0.97	*1.29*	
N	5	Ctr	1.22	*0.70*	a	-0.24	*0.81*		-1.63	*0.83*	a	-0.71	*0.64*		-0.11	*0.89*		-0.09	*1.00*	
		LR	0.38	*0.70*	ab	-0.52	*0.91*		2.47	*0.93*	b	-0.48	*0.72*		-1.01	*0.99*		-2.16	*1.12*	
		HR	-1.00	*0.70*	b	-1.38	*0.91*		1.49	*0.93*	b	-1.01	*0.72*		-1.53	*0.99*		-0.76	*1.12*	
	6	Ctr	1.12	*0.70*		-0.07	*0.91*		-0.94	*0.93*	a	-0.73	*0.72*		0.86	*0.99*		1.26	*1.12*	
		LR	1.10	*0.99*		-1.67	*1.29*		2.49	*1.31*	b	-0.77	*1.01*		-0.59	*1.40*		-0.82	*1.58*	
		HR	-0.17	*0.99*		1.36	*1.29*		1.86	*1.31*	ab	1.06	*1.01*		0.63	*1.40*		2.28	*1.58*	
N	7	Ctr	-0.60	*0.81*		-2.40	*0.91*		-1.96	*0.93*	a	-1.39	*0.72*		0.02	*0.99*		0.92	*1.12*	
		LR	0.46	*0.81*		-3.12	*1.05*	*	2.40	*1.07*	b	-1.18	*0.83*		-0.13	*1.15*		-1.57	*1.29*	
		HR	-0.23	*0.70*		-1.29	*0.91*		0.38	*0.93*	ab	-0.89	*0.72*		-0.56	*0.99*		0.08	*1.12*	
	8	Ctr	-0.37	*0.70*		-1.25	*0.91*		-1.56	*0.93*		-0.27	*0.72*		-0.66	*0.99*		-0.03	*1.12*	
		LR	0.50	*0.70*		-1.50	*0.91*		0.81	*0.93*		-0.95	*0.72*		0.29	*0.99*		-0.17	*1.12*	
		HR	0.33	*0.63*		-0.76	*0.81*		-0.61	*0.83*		0.04	*0.64*		0.30	*0.89*		1.42	*1.00*	
N	9	Ctr	0.50	*0.70*		-2.96	*0.91*	a*	-2.45	*0.93*	a	-1.17	*0.72*		-0.43	*0.99*		-0.52	*1.12*	
		LR	1.08	*0.70*		-0.51	*0.91*	ab	1.31	*0.93*	b	0.05	*0.72*		1.39	*0.99*		1.21	*1.12*	
		HR	0.66	*0.63*		-0.46	*0.81*	b	-1.39	*0.83*	a	0.21	*0.64*		0.94	*0.89*		2.23	*1.00*	
N	11	Ctr	0.02	*0.63*		-1.85	*0.81*		-1.61	*0.83*	a	-0.68	*0.64*		0.36	*0.89*		0.26	*1.00*	
		LR	1.64	*0.81*		-2.02	*1.05*		1.57	*1.07*	b	0.44	*0.83*		1.53	*1.15*		0.65	*1.29*	
		HR	0.15	*0.63*		-1.18	*0.81*		-0.13	*0.83*	ab	0.68	*0.64*		1.16	*0.89*		2.48	*1.00*	
N	13	Ctr	0.61	*0.63*		-2.21	*0.91*		-1.74	*0.83*	a	-0.34	*0.64*		0.52	*0.89*		0.25	*1.00*	
		LR	1.25	*0.70*		-0.97	*0.91*		1.08	*0.93*	b	0.91	*0.72*		2.18	*0.99*		1.95	*1.12*	
		HR	0.97	*0.70*		-1.68	*0.91*		-0.79	*0.93*	ab	-0.20	*0.72*		1.04	*0.99*		2.40	*1.12*	

		gene	CCL5		CCL20			CCL28			CXCL3			CXCL5			CXCL8		
		regulation	+		++			+			+			0			+		
IM	**TW**	**group**	LSM	*SD*	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	**15**	Ctr	0.00	*0.63*	0.00	*0.81*		0.00	*0.83*		0.00	*0.64*		0.00	*0.89*		0.00	*1.00*	
		LR	0.00	*0.70*	0.00	*0.91*		0.00	*0.93*		0.00	*0.72*		0.00	*0.99*		0.00	*1.12*	
		HR	0.00	*0.63*	0.00	*0.81*		0.00	*0.83*		0.00	*0.64*		0.00	*0.89*		0.00	*1.00*	
N PC	17	Ctr	-0.03	*0.63*	0.62	*0.91*	a	0.59	*0.83*		0.70	*0.64*		-0.40	*0.89*		0.07	*1.00*	
		LR	0.85	*0.70*	-0.03	*0.91*	ab	-0.10	*0.93*		-0.33	*0.72*		0.55	*0.99*		-0.15	*1.12*	
		HR	-0.31	*0.63*	-2.39	*0.81*	b*	0.95	*0.83*		-1.93	*0.64*	*	-0.55	*0.89*		-1.25	*1.00*	
N	19	Ctr	-0.14	*0.63*	1.25	*0.81*	a	-0.61	*0.83*		0.55	*0.64*	a	-0.64	*0.89*		-0.01	*1.00*	
		LR	0.55	*0.70*	-0.30	*0.91*	ab	0.75	*0.93*		-1.50	*0.72*	b	-0.35	*0.99*		-1.40	*1.12*	
		HR	-0.31	*0.63*	-1.67	*0.81*	b	-0.17	*0.83*		-1.99	*0.64*	b*	-0.93	*0.89*		-1.35	*1.00*	
N PC SC	21	Ctr	-0.87	*0.70*	2.09	*0.91*	a	0.94	*0.93*		0.86	*0.72*	a	-1.30	*0.99*		-0.91	*1.12*	
		LR	-0.48	*0.70*	-0.65	*0.91*	b	1.80	*0.93*		-0.65	*0.72*	ab	-0.65	*0.99*		-1.15	*1.12*	
		HR	-1.66	*0.63*	-2.28	*0.81*	b*	2.10	*0.83*		-1.92	*0.64*	b*	-1.32	*0.89*		-1.66	*1.00*	
N	23	Ctr	-0.20	*0.63*	2.32	*0.81*	a*	-0.01	*0.83*		1.16	*0.64*	a	0.08	*0.89*		1.15	*1.00*	a
		LR	-0.43	*0.70*	-2.48	*0.91*	b	0.90	*0.93*		-1.28	*0.72*	b	-0.75	*0.99*		-2.13	*1.12*	b
		HR	-1.23	*0.63*	-2.27	*0.81*	b*	1.59	*0.83*		-1.55	*0.64*	b	-1.57	*0.89*		-1.89	*1.00*	b
N PC	25	Ctr	-0.92	*0.81*	2.38	*1.05*	a	-0.32	*0.93*		1.66	*0.72*	a	-0.93	*0.99*		0.30	*1.12*	
		LR	0.40	*0.99*	-1.30	*1.29*	b	0.15	*1.31*		-1.75	*1.01*	b	0.75	*1.40*		-0.95	*1.58*	
		HR	0.17	*0.70*	-1.38	*0.91*	b	0.49	*0.93*		-1.86	*0.72*	b	-0.56	*0.99*		-1.28	*1.12*	
N IC	27	Ctr	-0.85	*0.70*	3.46	*0.91*	a*	-0.07	*0.93*		2.09	*0.72*	a	-0.08	*0.99*		1.56	*1.12*	
		LR	0.70	*0.81*	1.87	*1.05*	a	1.09	*1.07*		-0.13	*0.83*	b	1.54	*1.15*		2.02	*1.29*	
		HR	-0.77	*0.70*	-0.99	*0.91*	b	0.28	*0.93*		-1.89	*0.72*	b	-0.24	*0.99*		-1.02	*1.12*	
N PC	29	Ctr	-0.23	*0.70*	2.59	*0.91*	a*	0.35	*0.93*		1.75	*0.72*	a	-0.05	*0.99*		0.80	*1.12*	
		LR	0.47	*0.70*	0.90	*0.91*	ab	1.35	*0.93*		-1.33	*0.72*	b	0.90	*0.99*		1.00	*1.12*	
		HR	-0.43	*0.63*	-0.53	*0.81*	b	0.68	*0.83*		-2.15	*0.64*	b*	-0.31	*0.89*		-0.86	*1.00*	
N IC (iFA)	31	Ctr	0.41	*0.70*	3.69	*0.91*	a*	-0.22	*0.93*		1.76	*0.72*	a	-0.09	*0.99*		1.51	*1.12*	
		LR	1.13	*0.70*	1.13	*0.91*	b	1.30	*0.93*		-0.77	*0.72*	b	1.23	*0.99*		0.77	*1.12*	
		HR	-0.42	*0.63*	-1.02	*0.81*	b	0.63	*0.83*		-1.76	*0.64*	b	0.52	*0.89*		-0.15	*1.00*	

The gene regulation is graded as "0" for non regulated, "+" for weak regulated and "++" for strong regulated. The ΔΔCq data for the gene expression of the bovine mammary epithelial cells' (bMEC) chemokines (CCL5, CCL20, CCL28, CXCL3, CXCL5, CXCL8) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 1 and 15 in bold mark in each case the time point before the first use of the vaccine batches A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of the vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 1 in the following time period until and including TW 13, and relating to TW 15 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point [76].

Table 15: ΔΔCq changes in gene expression of bMEC related antimicrobial peptides relative to TW 1 or rather TW 15

		gene	LPO			LF			LYZ1			TAP		
		regulation	++			+			+			+		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	**1**	Ctr	0.00	*1.37*		0.00	*0.79*		0.00	*0.90*		0.00	*0.79*	
		LR	0.00	*1.37*		0.00	*0.91*		0.00	*0.90*		0.00	*0.91*	
		HR	0.00	*1.19*		0.00	*0.91*		0.00	*0.64*		0.00	*0.79*	
N PC	3	Ctr	-0.51	*1.19*		-0.83	*0.79*		-1.66	*0.74*		1.65	*0.79*	a
		LR	1.67	*1.37*		1.21	*0.91*		0.11	*0.90*		-1.52	*0.91*	b
		HR	2.39	*1.19*		0.59	*0.91*		-0.72	*0.90*		-2.08	*0.79*	b
	4	Ctr	0.66	*1.06*		-0.84	*0.79*		-2.86	*0.74*	*	0.71	*0.79*	
		LR	0.22	*2.37*		0.73	*1.58*		*n.n.*			-1.67	*1.58*	
		HR	0.87	*1.37*		0.58	*0.91*		-0.68	*0.90*		-0.95	*0.91*	
N	5	Ctr	-1.44	*1.06*	a	-0.92	*0.79*	a	-2.89	*0.90*	*	1.96	*0.91*	a
		LR	2.23	*1.19*	b	1.97	*0.79*	b	-0.93	*0.74*		-0.99	*0.79*	b
		HR	1.82	*1.19*	b	0.11	*0.79*	ab	-1.90	*0.90*		-2.18	*0.79*	b
	6	Ctr	-1.62	*1.19*	a	-1.25	*0.79*	a	-2.03	*0.64*		0.88	*0.79*	a
		LR	3.41	*1.68*	b	2.11	*1.12*	b	-2.32	*0.90*		-0.84	*1.12*	ab
		HR	1.53	*1.68*	ab	-0.09	*1.12*	ab	-0.32	*0.90*		-2.32	*1.12*	b
N	7	Ctr	-0.91	*1.19*		-0.50	*0.79*	a	-2.87	*0.64*	*	0.25	*0.79*	
		LR	2.26	*1.37*		2.05	*0.91*	b	-1.18	*1.28*		-0.14	*0.91*	
		HR	0.48	*1.19*		-0.84	*0.79*	a	-1.09	*0.74*		-0.93	*0.79*	
	8	Ctr	-0.63	*1.19*		-0.27	*0.79*	ab	-2.94	*0.64*	*	0.43	*0.79*	
		LR	0.83	*1.19*		1.48	*0.91*	a	-2.35	*0.90*		-0.70	*0.79*	
		HR	-0.24	*1.06*		-1.69	*0.71*	b	-1.68	*0.57*		-0.78	*0.71*	
N	9	Ctr	-1.35	*1.19*		-0.58	*0.79*	ab	-1.22	*0.90*		1.90	*0.79*	
		LR	1.13	*1.19*		1.08	*0.79*	a	-0.91	*0.90*		0.21	*0.79*	
		HR	-1.51	*1.06*		-1.43	*0.71*	b	-1.24	*0.64*		0.34	*0.71*	
N	11	Ctr	-0.64	*1.06*		-0.05	*0.71*		-2.00	*0.64*		1.09	*0.71*	
		LR	1.33	*1.37*		1.74	*0.91*		-1.13	*0.90*		-0.04	*0.79*	
		HR	0.00	*1.06*		-0.71	*0.91*		-2.27	*0.90*	*	-0.89	*0.91*	
N	13	Ctr	-0.87	*1.06*		-0.34	*0.79*	a	-1.05	*0.64*		1.50	*0.79*	
		LR	0.55	*1.19*		-0.11	*0.79*	a	-1.41	*0.74*		1.41	*0.91*	
		HR	-0.87	*1.19*		-2.60	*0.79*	b*	-1.52	*0.74*		0.30	*0.91*	

		gene	LPO		LF			LYZ1		TAP		
		regulation	++		+			+		+		
IM	**TW**	**group**	LSM	*SD*	LSM	*SD*		LSM	*SD*	LSM	*SD*	
N	**15**	Ctr	0.00	*1.19*	0.00	*0.71*		0.00	*0.64*	0.00	*0.71*	
		LR	0.00	*1.19*	0.00	*0.79*		0.00	*0.64*	0.00	*0.79*	
		HR	0.00	*1.06*	0.00	*0.71*		0.00	*0.64*	0.00	*0.71*	
N PC	17	Ctr	-0.85	*1.37*	-0.50	*0.71*	a	-1.12	*1.28*	-0.30	*0.79*	
		LR	0.35	*1.19*	1.03	*0.79*	ab	1.09	*0.90*	1.15	*0.79*	
		HR	0.79	*1.06*	1.53	*0.71*	b	-0.65	*0.57*	-0.45	*0.71*	
N	19	Ctr	-1.69	*1.19*	-1.02	*0.71*		-1.03	*0.64*	-0.23	*0.71*	
		LR	0.90	*1.19*	1.14	*0.79*		-0.07	*0.74*	0.34	*0.79*	
		HR	-0.41	*1.06*	0.54	*0.71*		-0.56	*0.64*	0.10	*0.71*	
N PC SC	21	Ctr	-0.66	*1.37*	0.27	*0.91*	a	-0.54	*0.74*	-0.19	*0.91*	
		LR	2.32	*1.19*	2.81	*0.79*	b*	-1.12	*0.64*	-0.42	*0.79*	
		HR	1.98	*1.06*	2.81	*0.71*	b*	-1.06	*0.57*	-0.79	*0.71*	
N	23	Ctr	-1.04	*1.06*	-0.21	*0.71*	a	-0.26	*0.64*	-0.48	*0.71*	
		LR	1.50	*1.19*	2.40	*0.79*	b*	0.73	*0.90*	1.13	*0.91*	
		HR	1.69	*1.06*	1.72	*0.71*	ab	-0.22	*0.64*	0.26	*0.71*	
N PC	25	Ctr	-1.75	*1.19*	-0.44	*0.79*		-1.36	*0.64*	-0.25	*0.79*	
		LR	1.60	*1.68*	1.33	*1.58*		-0.48	*0.90*	0.28	*1.12*	
		HR	0.32	*1.19*	0.78	*0.79*		-0.04	*0.64*	-0.20	*0.79*	
N IC	27	Ctr	-1.17	*1.19*	0.33	*0.79*		-1.57	*0.74*	-1.26	*0.79*	
		LR	0.90	*1.37*	1.09	*0.91*		-0.04	*0.74*	0.61	*0.91*	
		HR	-0.74	*1.19*	0.75	*0.79*		-0.44	*0.64*	-0.38	*0.79*	
N PC	29	Ctr	0.02	*1.19*	0.98	*0.79*		-0.43	*0.64*	-1.09	*0.79*	
		LR	1.90	*1.19*	2.18	*0.79*		0.71	*0.74*	0.02	*0.79*	
		HR	0.49	*1.06*	0.28	*0.79*		0.29	*0.64*	0.22	*0.79*	
N IC (iFA)	31	Ctr	-1.58	*1.37*	0.56	*0.79*		-0.37	*0.64*	-1.10	*0.79*	a
		LR	1.40	*1.19*	2.03	*0.79*		0.95	*0.90*	1.35	*0.79*	b
		HR	0.20	*1.06*	0.54	*0.71*		0.74	*0.57*	0.27	*0.71*	ab

The gene regulation is graded as "+" for weak regulated and "++" for strong regulated. The ΔΔCq data for the gene expression of the bovine mammary epithelial cells' (bMEC) antimicrobial peptides (LPO, LF, LYZ1, TAP) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 1 and 15 in bold mark in each case the time point before the first use of the vaccine batches A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of the vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 1 in the following time period until and including TW 13, and relating to TW 15 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point. Unavailable data are described as n.n.(numerius negidius) [76].

In immune cells of milk

Concerning the regulation of surface determinants specific for phagocytes (Table 16), *CD163* expressed by monocytes / macrophages' was unaffected by the treatment. The genes coding *C3AR1*, CXCR2, TLR2, *IL1β*, *IL6* and *IL12β* were weakly influenced by vaccinations against *C. diff.*, whereas *C5AR1* and *TNFα* were strongly regulated (Table 16 and Table 17).

In consequence of the vaccination with *MucoCD-I* batch A and the ongoing triggering with antigen on the N route, up-regulations of the complement receptors in both treated groups and also of *CXCR2* and *TLR2* in case of the HR resulted (Table 16), possibly indicating an increased attentiveness of the innate IS. Concerning the maintenance of these chemotactic receptors, *C5AR1* recognizes the antigens opsonized by complement for phagocytosis [104]. *CXCR2*, primarily on PMN surfaces, ensures to restore chemokine homeostasis by binding to the CXC-ligands 1-8, when these chemokines accumulate within the tissue contaminated by antigen designated for elimination [104]. Among the PAMPs recognizing Toll-like receptors, *TLR2* has a strong affinity to bind lipoteichoic acids of Gram-positive bacteria like *C. diff.*. In response to microbial components, the Toll-like receptors trigger the induction of inflammatory cytokines [64]. Even though the receptors' counterparts, being related to the bMEC in milk (Table 13 andTable 14), were not regulated correspondingly. But the induction of the TLR pathway and the increased expression of several chemokines attracting phagocytes were proven *in vitro* following the immune stimulation of primary bMEC [100].

As noticed in the bMEC related gene expressions described above, the innate IS should be possibly calmed overall in the course of the second treatment period. Because the treatment with *MucoCD-I* batch B resulted in the partial down-regulation of several receptors and all chemokines /cytokines, analyzed and related to phagocytes in milk, of the treated groups towards the Ctr group (Table 16 and Table 17).

Table 16: ΔΔCq changes in gene expression of milk phagocytes' receptors relative to TW 1 or rather TW 15

		gene	C3AR1			C5AR1			CXCR2			TLR2			CD163		
		regulation	+			++			+			+			0		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	**1**	Ctr	0.00	*0.99*		0.00	*1.14*		0.00	*1.12*		0.00	*0.78*		0.00	*1.13*	
		LR	0.00	*0.99*		0.00	*1.14*		0.00	*1.12*		0.00	*0.78*		0.00	*1.13*	
		HR	0.00	*0.86*		0.00	*0.98*		0.00	*0.97*		0.00	*0.68*		0.00	*0.97*	
N PC	3	Ctr	-0.52	*0.86*		-0.02	*0.98*		-0.45	*0.97*		-0.70	*0.68*		-0.44	*0.97*	
		LR	0.31	*0.99*		1.77	*1.14*		1.35	*1.12*		-0.54	*0.78*		1.40	*1.13*	
		HR	0.46	*0.86*		0.89	*0.98*		-0.39	*0.97*		1.14	*0.68*		0.68	*0.97*	
	4	Ctr	-0.50	*0.77*		0.44	*0.88*		-0.03	*0.86*		-0.60	*0.61*		-0.24	*0.87*	
		LR	0.22	*1.72*		1.97	*1.97*		0.02	*1.93*		1.12	*1.35*		2.67	*1.95*	
		HR	0.46	*0.99*		0.42	*1.14*		-1.26	*1.12*		0.71	*0.78*		-0.51	*1.13*	
N	5	Ctr	-0.14	*0.77*		0.10	*0.88*		-0.09	*0.86*		-0.18	*0.61*		-0.43	*0.87*	
		LR	-0.56	*0.86*		-0.64	*0.98*		-1.76	*0.97*		-0.94	*0.68*		-0.92	*0.97*	
		HR	-0.86	*0.86*		-0.14	*0.98*		-0.95	*0.97*		0.13	*0.68*		-2.12	*0.97*	
	6	Ctr	0.16	*0.86*		1.54	*0.98*		1.34	*0.97*		-0.65	*0.78*		0.51	*0.97*	
		LR	-0.30	*1.21*		0.95	*1.39*		-0.54	*1.37*		-0.48	*0.96*		-0.82	*1.38*	
		HR	0.68	*1.21*		1.44	*1.39*		1.31	*1.37*		1.11	*0.96*		-2.82	*1.38*	
N	7	Ctr	-0.74	*0.86*		1.70	*0.98*		1.64	*0.97*		-0.18	*0.68*		-0.28	*0.97*	
		LR	0.39	*0.99*		0.84	*1.14*		-0.40	*1.12*		-0.43	*0.78*		0.44	*1.13*	
		HR	0.39	*0.86*		1.02	*0.98*		0.75	*0.97*		0.79	*0.68*		-1.66	*0.97*	
	8	Ctr	-0.94	*0.86*		0.65	*0.98*		0.57	*0.97*		0.15	*0.68*		-0.16	*0.97*	
		LR	0.65	*0.86*		1.53	*0.98*		1.44	*0.97*		0.22	*0.68*		0.54	*0.97*	
		HR	1.16	*0.77*		2.47	*0.88*		2.09	*0.86*		1.86	*0.61*	*	-0.44	*0.87*	
N	9	Ctr	-1.40	*0.86*	a	0.34	*0.98*		0.15	*0.97*		-0.25	*0.68*	a	0.11	*0.97*	
		LR	0.91	*0.86*	ab	2.37	*0.98*		2.11	*0.97*		0.52	*0.68*	ab	0.72	*0.97*	
		HR	1.28	*0.77*	b	2.35	*0.88*		2.61	*0.86*	*	1.56	*0.61*	b	-0.16	*0.87*	
N	11	Ctr	-1.51	*0.77*	a	0.84	*0.88*		0.65	*0.86*		-0.77	*0.68*	a	-0.25	*0.87*	
		LR	0.06	*0.99*	ab	2.38	*1.14*		1.63	*1.12*		-0.70	*0.78*	a	-0.78	*1.13*	
		HR	1.47	*0.77*	b	3.25	*0.88*	*	2.72	*0.86*	*	1.56	*0.61*	b	0.35	*0.87*	
N	13	Ctr	-1.49	*0.77*	a	0.31	*0.88*	a	0.21	*0.86*	a	0.30	*0.61*		0.33	*0.87*	
		LR	2.04	*0.86*	b	2.97	*0.98*	b*	1.69	*0.97*	ab	0.72	*0.68*		0.70	*0.97*	
		HR	2.03	*0.86*	b	3.61	*0.98*	b*	2.88	*0.97*	b*	1.63	*0.68*		0.05	*0.97*	

		gene	C3AR1			C5AR1			CXCR2			TLR2			CD163		
		regulation	+			++			+			+			0		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	**15**	Ctr	0.00	*0.77*		0.00	*0.88*		0.00	*0.86*		0.00	*0.61*		0.00	*0.87*	
		LR	0.00	*0.86*		0.00	*0.98*		0.00	*0.97*		0.00	*0.68*		0.00	*0.97*	
		HR	0.00	*0.77*		0.00	*0.88*		0.00	*0.86*		0.00	*0.61*		0.00	*0.87*	
N PC	17	Ctr	0.51	*0.77*		-0.02	*0.88*		0.14	*0.86*		1.00	*0.61*		0.01	*0.87*	
		LR	0.22	*0.86*		-0.75	*0.98*		0.20	*0.97*		0.15	*0.68*		0.34	*0.97*	
		HR	-0.78	*0.77*		-0.80	*0.88*		-0.02	*0.86*		-0.62	*0.61*		-0.34	*0.87*	
N	19	Ctr	0.94	*0.77*		0.33	*0.88*		0.67	*0.86*		0.58	*0.61*		0.31	*0.87*	
		LR	-0.25	*0.86*		-1.37	*0.98*		-0.47	*0.97*		-0.42	*0.68*		-0.52	*0.97*	
		HR	0.05	*0.77*		-0.65	*0.88*		-0.13	*0.86*		0.03	*0.61*		0.21	*0.87*	
N PC SC	21	Ctr	1.00	*0.86*		-0.61	*0.98*		-0.66	*0.97*		0.68	*0.68*		0.02	*0.97*	
		LR	-0.73	*0.86*		-1.19	*0.98*		-0.71	*0.97*		-0.56	*0.68*		0.07	*0.97*	
		HR	-0.63	*0.77*		-0.91	*0.88*		-0.95	*0.86*		-0.41	*0.61*		0.09	*0.87*	
N	23	Ctr	1.09	*0.77*		0.84	*0.88*		1.54	*0.86*	a	1.56	*0.61*	a	0.23	*0.87*	
		LR	-0.25	*0.86*		-1.28	*0.98*		-1.35	*0.97*	b	-0.55	*0.68*	b	0.27	*0.97*	
		HR	-0.89	*0.77*		-1.55	*0.88*		-1.69	*0.86*	b	-0.57	*0.61*	b	-0.17	*0.87*	
N PC	25	Ctr	0.86	*0.86*		-0.36	*0.98*		-0.08	*0.97*		0.43	*0.68*		-0.46	*0.97*	
		LR	-0.90	*1.21*		-1.00	*1.39*		0.30	*1.37*		-0.75	*0.96*		-0.85	*1.38*	
		HR	-0.59	*0.86*		-1.34	*0.98*		-0.34	*0.97*		-0.02	*0.68*		0.16	*0.97*	
N IC	27	Ctr	1.30	*0.86*		1.11	*0.98*	a	1.61	*0.97*		2.15	*0.68*	*	0.54	*0.97*	
		LR	-0.87	*0.99*		-0.05	*1.14*	ab	1.42	*1.12*		1.20	*0.78*		1.03	*1.13*	
		HR	-0.28	*0.86*		-1.78	*0.98*	b	-0.83	*0.97*		1.02	*0.68*		1.07	*0.97*	
N PC	29	Ctr	1.58	*0.86*	a	1.12	*0.98*	a	0.94	*0.97*		1.24	*0.68*		0.01	*0.97*	
		LR	-1.79	*0.86*	b	-1.14	*0.98*	ab	0.59	*0.97*		-0.11	*0.68*		-0.34	*0.97*	
		HR	-1.49	*0.77*	b	-2.02	*0.88*	b	-0.50	*0.86*		0.17	*0.61*		0.53	*0.87*	
N IC (iFA)	31	Ctr	1.91	*0.86*	a	1.04	*0.98*	a	0.86	*0.97*		1.51	*0.68*		0.63	*0.97*	
		LR	-1.30	*0.86*	b	-1.37	*0.98*	ab	0.23	*0.97*		0.43	*0.68*		0.11	*0.97*	
		HR	-1.13	*0.77*	b	-1.62	*0.88*	b	-0.20	*0.86*		0.67	*0.61*		0.45	*0.87*	

The gene regulation is graded as "0" for non regulated, "+" for weak regulated and "++" for strong regulated. The ΔΔCq data for the gene expression of the milk phagocytes' receptors (C3AR1, C5AR1, CXCR2, TLR2, CD163) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 1 and 15 in bold mark in each case the time point before the first use of the vaccine batches A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 1 in the following time period until and including TW 13, and relating to TW 15 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point [76].

Table 17: ΔΔCq changes in gene expression of milk phagocytes' cytokines relative to TW 1 or rather TW 15

		gene	TNFα		IL1β		IL6		IL12β	
		regulation	++		+		+		+	
IM	**TW**	**group**	LSM	*SD*	LSM	*SD*	LSM	*SD*	LSM	*SD*
N	**1**	Ctr	0.00	*0.90*	0.00	*1.20*	0.00	*0.99*	0.00	*0.88*
		LR	0.00	*0.90*	0.00	*1.20*	0.00	*0.99*	0.00	*0.88*
		HR	0.00	*0.78*	0.00	*1.04*	0.00	*0.86*	0.00	*0.76*
N PC	3	Ctr	0.45	*0.78*	-0.04	*1.04*	0.61	*0.86*	1.03	*0.76*
		LR	-0.15	*0.90*	0.62	*1.20*	1.29	*0.99*	0.87	*0.88*
		HR	-0.32	*0.78*	0.01	*1.04*	0.73	*0.86*	-0.55	*0.76*
	4	Ctr	-0.10	*0.69*	-0.02	*0.93*	-0.20	*0.86*	-1.14	*0.76*
		LR	0.97	*1.55*	2.05	*2.08*	-1.42	*1.71*	-1.07	*1.53*
		HR	-2.29	*0.90*	-2.48	*1.20*	-0.59	*0.99*	-0.23	*0.88*
N	5	Ctr	-0.17	*0.69* a	0.37	*0.93* a	-0.57	*0.86* ab	0.34	*0.76*
		LR	-1.45	*0.78* ab	-2.43	*1.04* b	0.32	*0.86* a	0.16	*0.76*
		HR	-2.45	*0.78* b*	-1.17	*1.04* ab	-2.47	*0.86* b*	-1.52	*0.76*
	6	Ctr	0.77	*0.78*	1.64	*1.04*	1.84	*0.99*	0.55	*0.88*
		LR	-0.49	*1.10*	0.32	*1.47*	0.52	*1.21*	-0.31	*1.08*
		HR	-1.54	*1.10*	0.39	*1.47*	0.51	*1.21*	0.06	*1.08*
N	7	Ctr	-0.75	*0.78*	0.91	*1.04*	-1.94	*0.86*	-0.95	*0.76*
		LR	-1.32	*0.90*	-1.09	*1.20*	0.05	*0.99*	-0.30	*0.88*
		HR	-2.05	*0.78*	-0.76	*1.04*	-1.31	*0.86*	0.06	*0.76*
	8	Ctr	-1.11	*0.78*	0.25	*1.04*	-1.61	*0.86*	-0.14	*0.76*
		LR	-1.62	*0.78*	0.03	*1.04*	-1.69	*0.86*	-0.05	*0.76*
		HR	-0.97	*0.69*	0.46	*0.93*	-0.30	*0.77*	0.22	*0.68*
N	9	Ctr	-1.71	*0.78*	0.27	*1.04*	0.00	*0.86*	-1.50	*0.76* a
		LR	0.33	*0.78*	1.72	*1.04*	0.58	*0.86*	1.06	*0.76* b
		HR	0.19	*0.69*	1.32	*0.93*	0.58	*0.77*	0.66	*0.68* b
N	11	Ctr	-2.02	*0.69*	0.67	*0.93*	-1.69	*0.77*	-1.02	*0.68*
		LR	-1.75	*0.90*	1.53	*1.20*	-0.07	*0.99*	0.24	*0.88*
		HR	-1.54	*0.69*	1.75	*0.93*	-0.59	*0.77*	0.19	*0.68*
N	13	Ctr	-1.65	*0.69*	0.71	*0.93*	-2.61	*0.86* a*	-1.05	*0.76*
		LR	-1.85	*0.78*	2.22	*1.04*	-0.09	*0.86* b	0.97	*0.76*
		HR	-2.65	*0.78* *	1.95	*1.04*	-1.54	*0.86* ab	0.91	*0.76*

		gene	TNFα			IL1β			IL6			IL12β		
		regulation	++			+			+			+		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	**15**	Ctr	0.00	*0.69*		0.00	*0.93*		0.00	*0.77*		0.00	*0.68*	
		LR	0.00	*0.78*		0.00	*1.04*		0.00	*0.86*		0.00	*0.76*	
		HR	0.00	*0.69*		0.00	*0.93*		0.00	*0.77*		0.00	*0.68*	
N PC	17	Ctr	1.04	*0.69*	a	-0.28	*0.93*		-0.16	*0.77*		0.97	*0.68*	
		LR	-0.05	*0.78*	ab	0.09	*1.04*		-0.25	*0.86*		-0.50	*0.76*	
		HR	-1.46	*0.69*	b	-1.22	*0.93*		-1.26	*0.77*		-0.78	*0.68*	
N	19	Ctr	0.55	*0.69*	a	-0.15	*0.93*		0.02	*0.77*		1.34	*0.68*	a
		LR	-1.32	*0.78*	ab	-0.62	*1.04*		-1.45	*0.86*		-1.02	*0.76*	b
		HR	-1.65	*0.69*	b	-0.61	*0.93*		-1.85	*0.77*		-0.53	*0.68*	ab
N PC SC	21	Ctr	1.77	*0.78*	a	-0.48	*1.04*		0.53	*0.86*	a	1.58	*0.76*	a
		LR	-2.06	*0.78*	b	-0.93	*1.04*		-2.21	*0.86*	b	-1.39	*0.76*	b
		HR	-2.43	*0.69*	b*	-1.49	*0.93*		-1.87	*0.77*	b	-1.77	*0.68*	b
N	23	Ctr	2.34	*0.69*	a*	1.44	*0.93*	a	0.90	*0.77*	a	2.66	*0.68*	a*
		LR	-1.53	*0.78*	b	-1.50	*1.04*	b	-1.88	*0.86*	b	-1.43	*0.76*	b
		HR	-2.03	*0.69*	b*	-1.97	*0.93*	b	-2.15	*0.77*	b*	-1.37	*0.68*	b
N PC	25	Ctr	2.04	*0.78*	a	0.04	*1.04*		0.13	*0.86*		1.91	*0.76*	a
		LR	-1.50	*1.10*	b	-0.30	*1.47*		-0.90	*1.21*		-0.95	*1.08*	b
		HR	-1.22	*0.78*	b	-1.07	*1.04*		-0.89	*0.86*		0.34	*0.76*	ab
N IC	27	Ctr	2.99	*0.78*	a*	2.19	*1.04*	a	1.80	*0.86*	a	2.75	*0.76*	a*
		LR	-0.33	*0.90*	b	1.35	*1.20*	a	0.00	*0.99*	ab	1.40	*0.88*	ab
		HR	-1.88	*0.78*	b	-2.01	*1.04*	b	-0.81	*0.86*	b	-0.86	*0.76*	b
N PC	29	Ctr	2.67	*0.78*	a*	1.09	*1.04*		1.69	*0.86*	a	2.29	*0.76*	a*
		LR	-0.69	*0.78*	b	-0.19	*1.04*		-1.16	*0.86*	b	0.36	*0.76*	ab
		HR	-0.23	*0.69*	b	-1.14	*0.93*		-0.81	*0.77*	b	-0.43	*0.68*	b
N IC (iFA)	31	Ctr	3.26	*0.78*	a*	1.19	*1.04*		1.51	*0.86*		1.94	*0.76*	a
		LR	0.41	*0.78*	b	0.03	*1.04*		0.43	*0.86*		0.20	*0.76*	ab
		HR	0.31	*0.69*	b	-0.98	*0.93*		0.53	*0.77*		-0.57	*0.68*	b

The gene regulation is graded as "+" for weak regulated and "++" for strong regulated. The ΔΔCq data for the gene expression of the milk phagocytes' cytokines (TNFα, IL1β, IL6 and IL12β) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 1 and 15 in bold mark in each case the time point before the first use of the vaccine batches A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 1 in the following time period until and including TW 13, and relating to TW 15 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point [76].

Gene expressions related to the adaptive immune system

Concerning the adaptive IS, the results of gene expressions analyzed in PBL and milk cells were evaluated by the criteria introductorily described for the gene expressions related to the innate IS. The belonging data are summarized in the Table 18-Table 21, and being also described in words as traceable in the original paper of Schmautz et al. (2018b) presented in the Appendix II [76].

In blood cells

The Table 18 and Table 19 included the gene expressions of T- and B-cell markers as well as cytokines investigated in PBL. The genes for *CD3δ*, the surface determinant associated with T-cells, and *CD126*, used as plasma cell marker, were very strongly influenced by the *C. diff.* vaccinations. The transcriptions of the genes *CD4* and *CD8β*, coding T-cell subpopulations, were proven as strongly affected by the treatment, whereas the expression of the B-cell marker *CD19* was only weakly regulated. Among the investigated cytokines of blood lymphocytes, only the expression of *IL2* was very strongly influenced by the vaccinations. All other cytokines analyzed in PBL were weakly regulated.

The first two months of treatment was characterized by the frequent up-regulation of *IFNγ* in PBL of HR in contrast to the other groups. This cytokine are co-responsible in shaping the type of the T-cell response [59]. The promotion of phagocytosis and the T_H1-cell response is attributed to *IFNγ* [98]. Interestingly, significant down-regulations of HR's PBL, marked by *CD163* or rather *CD4*, were noticed towards the LR and Ctr groups at the time when *IFNγ* was no further differently regulated between HR and the other groups from TW 8. *In vitro* studies, analyzing the role of *IFNγ* in the regulation of bovine antibody responses, showed *IFNγ* as stimulator of IgG2 production when it was added to BCR or pokeweed mitogen-activated bovine B-cells [105]. Thus it could have also affected the (activated) B-cell populations in blood. But no increased *CD126* expressions in blood of HR, indicating a possibly enhanced presence of activated B- and plasma cells towards the other groups, was determined following the vaccination with *MucoCD-I* batch A. In contrast, the relative *CD126* transcriptions of the LR showed the tendency to be larger than those of the HR. But the drastic decrease of *CD126* expressions in PBL of both treated groups from TW 7, followed by the sustained *CD126* down-regulations, corresponded with the down-regulations of *IFNγ* from TW 10. Likewise, *CD4*+ and *CD8*+ T-cells of HR were reduced expressed from TW 7 and TW 8, respectively, concomitant with the flattening production of anti-*C. diff.* blood IgA (Figure 2) and the normalization of the total PBL counts (Table 9). Accordingly, the regulator

of the mucosal immunity by stimulating B-cells to secrete IgA, *TGFβ1*, was also down-regulated by both treated groups in the TW 8 [59] [98]. As exceptional case during the first IM period, the *CD19*+ B-cells were up-regulated among the LR's blood cells in TW 10, whereas those of the HR were unchanged. Regarding the DCC, the ratio [Phag:Lym] in LR's blood was >1, for once during the first IM period (Table 9). There could be an interdependence to the noticeably high SCC of LR's milk in TW 8 (Table 10) indicative for inflammation, and resulting systemic effects cannot be excluded. The T-cells as marked by *CD3δ* were also up-regulated by the LR in TW 10, presumably, following their stimulation by *IL2*. This cytokine, known as T-cell growth factor, was beforehand up-regulated by the LR and could have induced the proliferation of the T-cells or, more precisely, of T_H1-cells [98]. The T-cell subpopulations, *CD4*+ and *CD8*+ cells, were proven to be more transcribed by the LR than by the HR in TW 10, too. Overall, the immune responses to the once administered *MucoCD-I* batch A and to the inflammation conceivably present in the LR group seemed not to be suppressed by *IL10* which was especially down-regulated by the HR for several times.

In accordance with the anti-*C. diff*. IgA production by the HR (Figure 2), initially stimulated by injection of *MucoCD-I* batch B for the first time, their *TGFβ1* expression was relatively increased. *IL10* was up-regulated by both treated groups towards the Ctr when the vaccine batch B was administered SC and IC, presumably to control the immune response. *IL2* was up-regulated significantly more by the LR than by the HR in the TWs 18 and 27. Corresponding *CD3δ* gene up-regulations in LR's PBL, indicating different T-cell proliferations between the treated groups, were not determined. On the contrary, *CD3δ* was significantly down-regulated by LR and HR, simultaneously, subsequent to the vaccination combining three different routes of application (TW 21). Otherwise, the increased transcription of *CD4* could be co-induced by *IL2*, as both genes were up-regulated in LR and HR compared to the Ctr to this time. In the following treatment period, the *CD4* expressions remained equally enhanced by both treated groups. Several up-regulations of *IL2* and *IFNγ*, mainly related to the SC and IC vaccinations, could have contributed to the maintenance of the increased *CD4* transcription levels. Both cytokines are produced by T_H1-cells to stimulate autocrine the own proliferation [98]. Nevertheless, a further promotion of T_H1-cell differentiation was presumably stopped, considering the significant down-regulation of *IL12β*, the key cytokine to polarization into the T_H1 phenotype [98], in both treated groups from TW 21 (Table 12). It could be a hint for activation of the immunological memory being featured by *CD4*+ T_H-cell dependent antibody-responses [86]. The sustained *CD4* up-regulations indicated possibly memory T-cells increased circulating in blood and ready to migrate into tissues, especially affected by SC and IC vaccinations. A contemporaneous up-regulation of *CD4* in milk cells was not determined (Table 20). The *CD8β* expressions were

slightly increased in HR's blood several times from TW 21, maybe ensuring enough T_C-cells patrolling in blood and being on the alert against *C. diff*. repeatedly administered. The *CD126*+ PBL were proven as weakly up-regulated by the treated groups towards the Ctr from TW 21, too, being in concert with the strong *IL6* up-regulations in the same period. Also the weak up-regulations of *IL10* by the treated groups within the same period could have triggered the *CD126* up-regulations, as *IL10* contribute to proliferation of activated B-cells, *i.a.* [98]. In contrast, the humoral response to *C. diff*. was adjusted inversely because the anti-*C. diff*. blood IgA of the HR decreased from TW 21 (Figure 2). But it should be kept in mind that the IgA-response to antigens is primarily a mucosal one, and the antibodies of the class IgG dominate in blood [106]. The latter were not analyzed in this study due to the missing appropriate standard.

Table 18: ΔΔCq changes in gene expression of blood lymphocytes' receptors relative to TW 0 or rather TW 16

		gene	CD3δ			CD4			CD8β			CD19			CD126		
		regulation	+++			++			++			+			+++		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
	0	Ctr	0.00	*0.63*		0.00	*0.43*		0.00	*0.47*		0.00	*0.50*		0.00	*0.79*	
		LR	0.00	*0.55*		0.00	*0.38*		0.00	*0.41*		0.00	*0.43*		0.00	*0.69*	
		HR	0.00	*0.63*		0.00	*0.43*		0.00	*0.47*		0.00	*0.50*		0.00	*0.79*	
N	1	Ctr	-0.89	*0.63*		-1.10	*0.43*	a	-0.20	*0.47*		-0.11	*0.50*		-0.54	*0.79*	
		LR	0.08	*0.55*		0.78	*0.38*	b	0.38	*0.41*		0.91	*0.43*		-0.44	*0.69*	
		HR	0.19	*0.55*		0.20	*0.38*	b	1.03	*0.41*		0.60	*0.43*		1.32	*0.79*	
	2	Ctr	0.10	*0.63*		-0.14	*0.43*		0.23	*0.47*		-0.19	*0.50*		-0.12	*0.79*	
		LR	-0.49	*0.55*		0.38	*0.38*		0.08	*0.41*		1.11	*0.43*		0.18	*0.69*	
		HR	0.09	*0.55*		0.55	*0.38*		0.62	*0.41*		0.82	*0.43*		0.18	*0.69*	
N PC	3	Ctr	-0.82	*0.63*		-0.45	*0.43*		0.25	*0.47*		-0.30	*0.50*	a	-0.13	*0.79*	a
		LR	-0.25	*0.63*		0.05	*0.43*		0.08	*0.47*		1.05	*0.50*	ab	2.98	*0.79*	b*
		HR	-0.35	*0.55*		-0.46	*0.38*		0.81	*0.41*		1.01	*0.43*	b	1.30	*0.69*	ab
	4	Ctr	-0.37	*0.63*		-0.54	*0.43*		0.16	*0.47*		-0.14	*0.50*		-0.37	*0.79*	a
		LR	-0.96	*0.63*		-0.16	*0.43*		-0.46	*0.47*		-0.29	*0.50*		2.04	*0.79*	b
		HR	-0.85	*0.55*		-0.57	*0.38*		-0.20	*0.41*		0.16	*0.43*		1.06	*0.69*	ab
N	5	Ctr	*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		LR	-0.42	*0.55*		0.21	*0.38*		-0.39	*0.41*		0.66	*0.43*		1.91	*0.69*	
		HR	-0.45	*0.49*		-0.55	*0.34*		-0.47	*0.36*		0.35	*0.39*		1.56	*0.61*	
	6	Ctr	-0.12	*0.63*		0.04	*0.43*		-0.46	*0.47*		-0.27	*0.50*		-0.51	*0.79*	a
		LR	-0.95	*0.55*		-0.27	*0.38*		-0.68	*0.41*		0.48	*0.43*		2.08	*0.69*	b*
		HR	-0.80	*0.63*		-0.39	*0.34*		-0.23	*0.36*		0.55	*0.39*		0.91	*0.69*	ab
N	7	Ctr	*n.n.*			*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		LR	-0.19	*0.55*		0.16	*0.38*	a	-0.16	*0.41*		0.16	*0.43*		-1.40	*0.97*	a
		HR	-0.82	*0.55*		-1.40	*0.34*	b*	-0.76	*0.36*		-0.66	*0.39*		-5.33	*0.69*	b*
	8	Ctr	-0.19	*0.63*		-0.21	*0.43*	a	-0.84	*0.47*	a	0.01	*0.50*		-0.60	*0.79*	a
		LR	-1.48	*0.55*		-2.31	*0.38*	b*	-3.18	*0.41*	b*	-1.03	*0.43*		-4.16	*0.97*	b*
		HR	-0.33	*0.49*		-2.55	*0.34*	b*	-2.19	*0.36*	b*	-0.81	*0.39*		-5.71	*0.61*	b*
N	10	Ctr	0.36	*0.63*	a	0.09	*0.43*	a	-1.46	*0.47*	ab*	-0.06	*0.50*	a	-0.95	*0.79*	a
		LR	2.09	*0.55*	b*	0.29	*0.38*	a	-0.64	*0.41*	a	2.14	*0.43*	b*	-3.21	*0.69*	b*
		HR	0.67	*0.49*	ab	-1.85	*0.34*	b*	-2.19	*0.36*	b*	0.73	*0.39*	a	-4.51	*0.61*	b*
N	12	Ctr	0.19	*0.49*		-0.28	*0.34*	a	-2.33	*0.36*	*	-0.14	*0.39*		-0.97	*0.61*	a
		LR	-0.14	*0.55*		-2.84	*0.38*	b*	-2.24	*0.41*	*	-0.34	*0.43*		-4.52	*0.69*	b*
		HR	-0.35	*0.49*		-3.30	*0.34*	b*	-2.56	*0.36*	*	-0.78	*0.39*		-5.01	*0.61*	b*

		gene	CD3δ			CD4			CD8β			CD19			CD126		
		regulation	+++			++			++			+			+++		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	14	Ctr	-0.01	*0.63*		-0.29	*0.43*	a	-2.23	*0.47*	*	-0.30	*0.50*		-1.20	*0.79*	a
		LR	1.34	*0.55*		-0.78	*0.38*	a	-2.16	*0.41*	*	-0.06	*0.43*		-4.06	*0.69*	b*
		HR	0.53	*0.49*		-2.21	*0.34*	b*	-2.25	*0.36*	*	-0.47	*0.39*		-5.29	*0.61*	b*
N	**16**	Ctr	0.00	*0.49*		0.00	*0.34*		0.00	*0.36*		0.00	*0.39*		0.00	*0.61*	
		LR	0.00	*0.55*		0.00	*0.38*		0.00	*0.41*		0.00	*0.43*		0.00	*0.69*	
		HR	0.00	*0.49*		0.00	*0.34*		0.00	*0.36*		0.00	*0.39*		0.00	*0.61*	
N	18	Ctr	0.07	*0.49*		-0.13	*0.34*		-0.35	*0.36*	ab	0.05	*0.39*		0.21	*0.61*	
PC		LR	-1.87	*0.55*	*	-0.37	*0.38*		-1.00	*0.41*	a	-0.87	*0.43*		-0.72	*0.69*	
		HR	-0.45	*0.49*		0.11	*0.34*		0.15	*0.36*	b	-0.11	*0.39*		0.33	*0.61*	
N	20	Ctr	0.39	*0.49*		-0.05	*0.34*	a	0.17	*0.36*		0.25	*0.39*		0.07	*0.61*	
		LR	0.52	*0.55*		0.60	*0.38*	ab	0.02	*0.41*		0.65	*0.43*		0.62	*0.69*	
		HR	0.64	*0.49*		1.26	*0.34*	b*	0.22	*0.36*		0.64	*0.39*		0.68	*0.61*	
N	21	Ctr	0.60	*0.49*	a	0.02	*0.34*	a	0.14	*0.36*	a	0.74	*0.39*		0.18	*0.61*	a
PC		LR	-4.01	*0.63*	b*	2.90	*0.38*	b*	0.59	*0.41*	ab	-0.16	*0.43*		1.58	*0.69*	ab
SC		HR	-5.36	*0.49*	b*	3.10	*0.34*	b*	1.30	*0.36*	b*	-0.44	*0.39*		1.90	*0.61*	b*
N	23	Ctr	0.32	*0.49*	a	-0.08	*0.34*	a	-0.02	*0.36*		0.32	*0.39*		-0.04	*0.61*	a
		LR	-4.71	*0.55*	b*	1.76	*0.38*	b*	0.09	*0.41*		-0.14	*0.43*		1.49	*0.69*	ab
		HR	-5.52	*0.49*	b*	1.82	*0.34*	b*	0.54	*0.36*		-0.74	*0.39*		1.87	*0.61*	b*
N	25	Ctr	0.29	*0.49*	a	0.09	*0.34*	a	-0.17	*0.36*	a	0.61	*0.39*		-0.52	*0.69*	a
PC		LR	-2.74	*0.55*	b*	2.14	*0.38*	b*	0.41	*0.41*	ab	0.84	*0.43*		1.59	*0.69*	b
		HR	-2.70	*0.49*	b*	2.34	*0.34*	b*	1.00	*0.36*	b	0.34	*0.39*		1.97	*0.61*	b*
N	27	Ctr	-0.59	*0.55*	a	-1.19	*0.38*	a*	-0.66	*0.41*	a	0.54	*0.43*		-0.27	*0.69*	a
IC		LR	-2.55	*0.55*	b*	3.18	*0.38*	b*	1.13	*0.41*	b	0.55	*0.43*		1.58	*0.69*	ab
		HR	-2.33	*0.49*	b*	3.07	*0.34*	b*	1.19	*0.36*	b*	0.43	*0.39*		1.90	*0.61*	b*
N	29	Ctr	-0.61	*0.49*		-0.81	*0.34*	a	-0.31	*0.36*	a	0.25	*0.39*		0.23	*0.61*	a
PC		LR	-1.85	*0.55*	*	2.47	*0.38*	b*	0.27	*0.41*	ab	1.02	*0.43*		1.77	*0.69*	ab
		HR	-1.65	*0.49*	*	2.53	*0.34*	b*	0.91	*0.36*	b	0.87	*0.39*		2.02	*0.61*	b*
N	31	Ctr	0.39	*0.49*	a	-0.29	*0.34*	a	-0.27	*0.36*	a	0.61	*0.39*		0.36	*0.61*	
IC		LR	-1.42	*0.55*	b	2.38	*0.38*	b*	0.63	*0.41*	ab	1.23	*0.43*	*	1.88	*0.69*	
(iFA)		HR	-1.29	*0.49*	b	2.13	*0.34*	b*	1.15	*0.36*	b*	0.89	*0.39*		1.34	*0.61*	

The gene regulation is graded as "+" for weak regulated, "++" for strong regulated, "+++" for very strong regulated. The ΔΔCq data for the gene expression of the lymphocytes' receptors (CD3δ, CD4, CD8, CD19, CD126) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 0 and 16 in bold mark in each case the time point before the first use of the vaccine batch A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 0 in the time period until and including TW 14, and relating to TW 16 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point. "n.n." means unavailable data [76].

Table 19: ΔΔCq changes in gene expression of blood lymphocytes' cytokines relative to TW 0 or rather TW 16

		gene	IFNγ			TGFβ1			IL2			IL10		
		regulation	+			+			+++			+		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
	0	Ctr	0.00	*0.70*		0.00	*0.63*		0.00	*0.93*		0.00	*0.58*	
		LR	0.00	*0.61*		0.00	*0.54*		0.00	*0.81*		0.00	*0.50*	
		HR	0.00	*0.70*		0.00	*0.63*		0.00	*0.93*		0.00	*0.58*	
N	1	Ctr	-0.68	*0.70*		-0.70	*0.63*		-0.99	*0.93*		0.16	*0.58*	
		LR	-0.17	*0.61*		0.06	*0.54*		-0.22	*0.81*		0.38	*0.50*	
		HR	0.91	*0.61*		-0.17	*0.54*		0.25	*0.81*		-0.29	*0.50*	
	2	Ctr	-0.35	*0.70*	ab	-0.77	*0.63*		-0.73	*0.93*		0.35	*0.58*	a
		LR	-0.94	*0.61*	a	-0.09	*0.54*		-1.12	*0.81*		0.06	*0.50*	a
		HR	1.03	*0.61*	b	0.56	*0.54*		-0.20	*0.81*		-1.35	*0.50*	b
N PC	3	Ctr	-0.67	*0.70*		-0.51	*0.63*		0.28	*0.93*		-0.50	*0.58*	
		LR	-0.62	*0.70*		0.01	*0.63*		-1.15	*0.93*		0.41	*0.58*	
		HR	-0.33	*0.61*		0.07	*0.54*		-0.54	*0.81*		-0.38	*0.50*	
	4	Ctr	-0.40	*0.70*	ab	-0.84	*0.63*		-0.90	*0.93*		0.03	*0.58*	
		LR	-0.59	*0.70*	a	-0.29	*0.63*		-0.32	*0.93*		-1.16	*0.58*	
		HR	1.36	*0.61*	b	-0.29	*0.54*		0.31	*0.81*		-1.39	*0.50*	
N	5	Ctr	*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		LR	-0.59	*0.61*		0.18	*0.54*		-0.69	*0.81*		-0.29	*0.50*	
		HR	0.82	*0.55*		0.22	*0.49*		0.07	*0.72*		-1.28	*0.45*	
	6	Ctr	-0.07	*0.70*	ab	-1.50	*0.63*	a	-0.53	*0.93*		0.26	*0.58*	
		LR	-0.55	*0.61*	a	-0.13	*0.63*	ab	-0.75	*0.81*		0.13	*0.50*	
		HR	1.08	*0.55*	b	0.18	*0.49*	b	0.33	*0.72*		-0.36	*0.45*	
N	7	Ctr	*n.n.*			*n.n.*			*n.n.*			*n.n.*		
		LR	-0.13	*0.61*	a	0.38	*0.63*		-0.52	*0.81*		-0.15	*0.50*	
		HR	2.09	*0.55*	b*	0.37	*0.49*		0.55	*0.81*		-0.77	*0.45*	
	8	Ctr	-0.76	*0.70*	a	-0.98	*0.63*		-1.17	*0.93*	a	0.50	*0.58*	a
		LR	2.19	*0.61*	b*	-1.96	*0.54*	*	5.29	*0.81*	b*	-0.36	*0.58*	ab
		HR	1.55	*0.55*	b	-1.96	*0.49*	*	2.60	*0.81*	c*	-1.65	*0.50*	b*
N	10	Ctr	0.16	*0.70*	a	-1.37	*0.63*		-1.44	*0.93*	a	0.59	*0.58*	a
		LR	-1.84	*0.61*	b*	-2.01	*0.77*	*	1.21	*0.81*	b	1.14	*0.50*	a
		HR	-0.97	*0.55*	ab	-1.01	*0.63*		0.59	*0.72*	ab	-1.55	*0.45*	b*
N	12	Ctr	0.55	*0.55*		-2.06	*0.49*	a*	-0.74	*0.72*	a	0.47	*0.45*	
		LR	-0.72	*0.61*		0.44	*0.63*	b	2.69	*0.81*	b*	-0.61	*0.58*	
		HR	-0.31	*0.70*		1.00	*0.54*	b	3.72	*0.72*	b*	0.01	*0.45*	

		gene	IFNγ			TGFβ1			IL2			IL10		
		regulation	+			+			+++			+		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	14	Ctr	0.15	*0.70*		-2.45	*0.63*	a*	-2.00	*0.93*	a	0.27	*0.58*	a
		LR	-0.51	*0.61*		1.22	*0.54*	b	0.97	*0.81*	b	-1.51	*0.50*	b*
		HR	0.29	*0.55*		0.90	*0.49*	b	2.13	*0.72*	b	-2.45	*0.45*	b*
N	**16**	Ctr	0.00	*0.55*		0.00	*0.49*		0.00	*0.72*		0.00	*0.45*	
		LR	0.00	*0.61*		0.00	*0.54*		0.00	*0.81*		0.00	*0.50*	
		HR	0.00	*0.55*		0.00	*0.49*		0.00	*0.72*		0.00	*0.45*	
N	18	Ctr	0.01	*0.55*	a	0.17	*0.49*	a	0.65	*0.72*	a	0.17	*0.45*	
PC		LR	2.71	*0.61*	b*	0.95	*0.54*	ab	4.36	*0.81*	b*	1.00	*0.58*	
		HR	1.67	*0.55*	b*	1.53	*0.49*	b*	1.89	*0.72*	a	1.13	*0.45*	
N	20	Ctr	-0.11	*0.55*		0.23	*0.49*		0.31	*0.72*		-0.13	*0.45*	
		LR	0.40	*0.61*		0.60	*0.54*		-0.10	*0.81*		-0.10	*0.50*	
		HR	0.86	*0.55*		1.02	*0.49*		1.48	*0.72*		-0.10	*0.45*	
N	21	Ctr	-0.14	*0.55*	a	0.72	*0.49*		-0.50	*0.72*	a	-0.60	*0.45*	a
PC		LR	2.28	*0.61*	b*	0.75	*0.54*		3.43	*0.81*	b*	1.65	*0.50*	b*
SC		HR	2.99	*0.55*	b*	0.86	*0.49*		3.94	*0.72*	b*	1.81	*0.50*	b*
N	23	Ctr	-1.26	*0.55*	a	0.48	*0.49*		-0.64	*0.72*	a	0.10	*0.45*	a
		LR	0.11	*0.61*	a	0.14	*0.54*		1.19	*0.81*	ab	1.11	*0.50*	ab
		HR	2.49	*0.55*	b*	0.14	*0.49*		3.15	*0.72*	b*	1.86	*0.45*	b*
N	25	Ctr	-1.36	*0.55*		0.53	*0.49*		-0.11	*0.72*		-0.87	*0.45*	a
PC		LR	-0.16	*0.61*		0.79	*0.54*		0.22	*0.81*		0.34	*0.50*	ab
		HR	0.11	*0.61*		0.96	*0.49*		0.49	*0.72*		0.76	*0.45*	b
N	27	Ctr	-1.04	*0.61*	a	-0.47	*0.54*	a	-1.12	*0.81*	a	-0.02	*0.50*	a
IC		LR	2.29	*0.61*	b*	1.98	*0.54*	b*	5.18	*0.81*	b*	1.98	*0.50*	b*
		HR	2.80	*0.55*	b*	1.57	*0.49*	b*	2.98	*0.72*	c*	1.25	*0.45*	ab*
N	29	Ctr	-0.49	*0.55*	a	0.31	*0.49*	a	-0.29	*0.72*	a	0.01	*0.45*	
PC		LR	0.72	*0.61*	ab	1.00	*0.54*	ab	2.00	*0.81*	b	0.92	*0.50*	
		HR	1.29	*0.55*	b	1.69	*0.49*	b	1.90	*0.72*	b	0.81	*0.45*	
N	31	Ctr	-0.20	*0.55*		0.41	*0.49*		-0.09	*0.72*		0.19	*0.45*	
IC		LR	-0.06	*0.70*		1.10	*0.54*		0.68	*0.81*		1.33	*0.50*	
(iFA)		HR	0.07	*0.70*		1.01	*0.49*		1.18	*0.72*		0.51	*0.58*	

The gene regulation is graded as "+" for weak regulated and "+++" for very strong regulated. The ΔΔCq data for the gene expression of the lymphocytes' cytokines (IFNγ, TGFβ1, IL2, IL10) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 0 and 16 in bold mark in each case the time point before the first use of the vaccine batch A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 0 in the time period until and including TW 14, and relating to TW 16 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point. "n.n." means unavailable data [76].

In milk cells

The genes of all cell markers examined in milk were determined as weakly regulated due to the treatment, excepting the T-cell marker *CD4* and the immune suppressive cytokine *IL10*, which were strongly regulated (Table 20 and Table 21).

IL10 has moderating effects on the IS [59]. It was not proven as differently regulated between LR and HR, but it was down-regulated by both treated groups towards the Ctr after the use of vaccine batch A. Additionally, the decline of the HR's *IFNγ* expressions could have kept the HR's anti-*C. diff.* production safe from inhibitory effects by *IFNγ*, known as suppressor of the humoral immune response of the T_H2-type [59] [67] [107]. Thus the opposite regulation of *IFNγ* in milk lymphocytes by the treated groups corresponds to their different *C. diff.* specific Ab production in milk (Figure 2). Furthermore, *IFNγ* acts as activator of macrophages [59] and stimulation of phagocytosis [98]. Therefore, it could have also promoted the up-regulation of several phagocytes' receptors in milk during the first IM period, although those were determined as no differing between the treated groups (Table 16). Among the examined surface determinants, the expressions of *CD4* and *CD38* were triggered in the lymphocytes of the LR directly after the injection of *MucoCD-I* batch A and, additionally, *CD3δ* was up-regulated by the LR towards the Ctr. *CD4*+ T-cells are one part of the entirety of *CD3δ*+ T-cells, and they are important for the activation of B-cells [59]. *CD38* is expressed by activated T- and B-lymphocytes assessed in mice and humans, and it was identified in human breast milk-derived B-cells, *i.a.* [108] [109]. The up-regulations of *CD4* and *CD38* could indicate the promotion of the LR's humoral response to *C. diff.*, but that was proven as weakly accentuated (Figure 2). ASC were marked by *CD126*, also known as IL6-receptor [110]. This plasma cell marker was lower expressed by the LR than by the other groups, being consistent with the expression levels of the counterpart *IL6* by the LR (Table 17) and the weaker Ab production of the LR, respectively, towards the HR (Figure 2).

In the course of the second treatment period, *CD3δ*, *CD4* and *CD8β* were expressed slightly reduced few times by the treated groups compared to the Ctr from TW 21. Following the singular up-regulation of *IFNγ* in milk cells of LR in TW 23, no changes of other gene expressions analyzed in SCC of LR were obviously induced. Solely, the *CCR6* regulation was conspicuous during the treatment with *MucoCD-I* batch B (Table 21). This receptor is the counterpart of the chemoattractant *CCL20* and mainly expressed by "almost all mucosa-homing and most skin-homing effector / memory T-cells". Among them, *CCR6* is more present on CD4+ than on CD8+ T-cells. But this receptor is also expressed by other leukocytes, depending on their subtype, maturation and / or differentiation stage. Being

linked with *CCL20*, *CCR6* is important for the endothelial T-cell arrest prior to extravasal migration [63]. Although *CCL20* was not proven to be stronger expressed by the treated groups (Table 14), the transcript amounts of *CCR6* were increased in milk cells of the HR from TW 25. The *CCR6* expression of LR showed initially the trend to be enhanced, and to the end of the treatment, it was also proven to be significantly increased.

Referring to a list compiled by Ogra et al. (2001) comprising events of immunological activity associated with mucosal immune response, the sites of antigen exposure are characterized by activation of *CD4*+ and *CD8*+ T-cells and B-cell activation inducted by *TGFβ* and *IL6*, *i.a.* [68]. Whereas the effector sites are marked by homing of the activated and antigen-reactive lymphocytes, the induction of *PIGR,* partly depending on *IFNγ*, and furthermore, the activation, proliferation and terminal differentiation of antigen-reactive IgA B-cells to plasma cells partly promoted by *IL6* and *IL10*. Beside these typical T_H2-cytokines, T_H1 cytokines like *IL2* and *IFNγ* are able to induce sIgA responses, too [68]. Interestingly, these characteristics of mucosal immune responses compared to the outcomes of the gene expression analysis were mainly confirmed for both treated groups during the second treatment period. The different responsiveness of the treated cows, as measured by their *C. diff.* specific milk IgA, was showed by the gene expression analysis being primarily present in PBL following the first PC vaccination with *MucoCD-I* batch A. For this, the different patterns of chemokine / cytokines like *CXCL8*, *IL1β*, *IL12β* and, in particular, *IFNγ* (also in SCC), seemed to be crucial influence factors.

Table 20: ΔΔCq changes in gene expression of milk lymphocytes' receptors relative to TW 1 or rather TW 15

		gene	CD3δ			CD4			CD8β			CD19			CD38		
		regulation	+			++			+			+			+		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	**1**	Ctr	0.00	*0.83*		0.00	*0.78*		0.00	*0.80*		0.00	*1.08*		0.00	*0.78*	
		LR	0.00	*0.83*		0.00	*0.78*		0.00	*0.80*		0.00	*0.88*		0.00	*0.78*	
		HR	0.00	*0.72*		0.00	*0.68*		0.00	*0.69*		0.00	*0.76*		0.00	*0.68*	
N PC	3	Ctr	-0.54	*0.72*	a	-0.61	*0.78*	a	-0.25	*0.69*		2.55	*0.76*	a	-0.15	*0.68*	ab
		LR	1.79	*0.83*	b	3.05	*0.78*	b*	1.48	*0.80*		0.17	*0.88*	b	1.02	*0.78*	a
		HR	0.02	*0.72*	ab	0.96	*0.68*	a	-0.44	*0.69*		0.95	*0.76*	ab	-1.14	*0.68*	b
	4	Ctr	0.35	*0.65*		0.77	*0.60*		-0.53	*0.69*		0.76	*0.88*		-0.16	*0.61*	
		LR	1.02	*1.45*		0.77	*1.35*		1.32	*1.38*		-1.68	*1.52*		0.47	*1.35*	
		HR	-0.14	*0.83*		0.31	*0.78*		-0.51	*0.80*		0.71	*0.88*		-0.54	*0.78*	
N	5	Ctr	0.13	*0.65*		0.58	*0.60*		0.05	*0.62*		2.59	*0.68*	a*	-0.13	*0.61*	
		LR	0.84	*0.72*		0.38	*0.68*		0.92	*0.69*		0.72	*0.76*	ab	-0.69	*0.68*	
		HR	0.26	*0.72*		-0.67	*0.68*		-0.77	*0.69*		0.20	*0.76*	b	-1.54	*0.68*	
	6	Ctr	-0.27	*0.72*	a	-0.23	*0.68*		-0.30	*0.69*		1.70	*0.88*		0.46	*0.68*	
		LR	2.52	*1.02*	b	1.42	*0.96*		2.06	*0.98*		1.30	*1.08*		0.17	*0.96*	
		HR	0.09	*1.02*	ab	0.16	*0.96*		-0.36	*0.98*		-0.64	*1.08*		-0.02	*0.96*	
N	7	Ctr	0.40	*0.72*		0.86	*0.68*		-0.82	*0.69*		0.36	*0.76*		-1.39	*0.78*	
		LR	0.75	*0.83*		0.24	*0.78*		-0.11	*0.80*		0.18	*0.88*		-0.44	*0.78*	
		HR	0.12	*0.72*		0.84	*0.68*		-0.73	*0.69*		0.18	*0.76*		-0.34	*0.68*	
	8	Ctr	-1.12	*0.72*		-0.09	*0.68*		-1.93	*0.69*		0.48	*0.76*		-1.29	*0.68*	
		LR	-0.01	*0.72*		-0.82	*0.68*		-0.40	*0.69*		-0.34	*0.76*		-1.05	*0.68*	
		HR	-0.14	*0.65*		0.34	*0.60*		-0.88	*0.62*		-0.03	*0.68*		-0.04	*0.61*	
N	9	Ctr	-0.11	*0.72*		0.72	*0.68*		-1.09	*0.69*	a	0.95	*0.76*		-1.62	*0.68*	a
		LR	1.65	*0.72*		1.77	*0.68*		1.14	*0.69*	b	1.15	*0.76*		0.21	*0.68*	ab
		HR	0.74	*0.65*		1.16	*0.60*		0.56	*0.62*	ab	0.81	*0.68*		0.44	*0.61*	b
N	11	Ctr	-0.22	*0.65*		0.08	*0.60*		-1.39	*0.62*	a	0.64	*0.68*		-1.47	*0.61*	
		LR	1.41	*0.83*		0.73	*0.78*		1.16	*0.80*	b	0.99	*0.88*		0.09	*0.78*	
		HR	0.59	*0.65*		0.40	*0.60*		0.53	*0.62*	b	1.54	*0.68*		-0.15	*0.61*	
N	13	Ctr	0.58	*0.65*		0.65	*0.60*		-0.10	*0.62*		1.14	*0.68*		-0.72	*0.61*	
		LR	1.11	*0.72*		0.70	*0.68*		1.66	*0.69*		1.33	*0.76*		0.99	*0.68*	
		HR	0.08	*0.72*		0.06	*0.68*		0.10	*0.69*		0.66	*0.76*		0.43	*0.68*	

		gene	CD3δ			CD4			CD8β			CD19			CD38		
		regulation	+			++			+			+			+		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	**15**	Ctr	0.00	*0.65*		0.00	*0.60*		0.00	*0.62*		0.00	*0.68*		0.00	*0.61*	
		LR	0.00	*0.72*		0.00	*0.68*		0.00	*0.69*		0.00	*0.76*		0.00	*0.68*	
		HR	0.00	*0.65*		0.00	*0.60*		0.00	*0.62*		0.00	*0.68*		0.00	*0.61*	
N PC	17	Ctr	0.73	*0.65*		0.82	*0.60*		0.77	*0.62*		-0.14	*0.68*		0.70	*0.61*	
		LR	0.05	*0.72*		0.09	*0.68*		0.25	*0.69*		0.09	*0.76*		1.15	*0.68*	
		HR	0.22	*0.65*		0.14	*0.60*		0.10	*0.62*		-0.82	*0.68*		-0.42	*0.61*	
N	19	Ctr	0.90	*0.65*		1.20	*0.60*		0.71	*0.62*		-0.04	*0.68*		0.19	*0.61*	
		LR	-0.17	*0.72*		0.13	*0.68*		0.20	*0.69*		0.08	*0.76*		0.75	*0.68*	
		HR	-0.23	*0.65*		0.15	*0.60*		-0.07	*0.62*		-0.65	*0.68*		-0.07	*0.61*	
N PC SC	21	Ctr	1.18	*0.72*	a	1.25	*0.68*		0.29	*0.69*		0.50	*0.76*		0.34	*0.68*	
		LR	-0.96	*0.72*	b	-0.11	*0.68*		-0.54	*0.69*		-0.61	*0.76*		-0.48	*0.68*	
		HR	-0.63	*0.65*	ab	-0.51	*0.60*		-0.69	*0.62*		-0.61	*0.68*		-0.75	*0.61*	
N	23	Ctr	1.36	*0.65*	a	1.38	*0.60*	a	0.89	*0.62*	a	0.39	*0.68*		0.56	*0.61*	
		LR	-0.63	*0.72*	b	-0.15	*0.68*	ab	-0.45	*0.69*	ab	-0.15	*0.76*		0.47	*0.68*	
		HR	-0.95	*0.65*	b	-0.51	*0.60*	b*	-1.01	*0.62*	b	-0.95	*0.68*		-0.65	*0.61*	
N PC	25	Ctr	0.18	*0.72*		0.26	*0.68*		0.19	*0.69*		0.31	*0.76*		-0.60	*0.78*	
		LR	-1.15	*1.02*		-0.45	*0.96*		-1.00	*0.98*		-1.65	*1.08*		0.35	*0.96*	
		HR	0.09	*0.72*		0.71	*0.68*		-0.22	*0.69*		-0.64	*0.76*		0.48	*0.68*	
N IC	27	Ctr	0.23	*0.72*		0.48	*0.68*		-0.04	*0.69*		0.15	*0.76*		0.16	*0.68*	
		LR	-0.73	*0.83*		0.22	*0.78*		-0.27	*0.80*		1.13	*0.88*		-0.77	*0.78*	
		HR	-1.51	*0.72*		-0.16	*0.68*		-1.48	*0.69*		-0.25	*0.76*		-0.38	*0.68*	
N PC	29	Ctr	0.89	*0.72*		1.45	*0.68*	a	0.56	*0.69*		0.18	*0.76*		0.32	*0.68*	
		LR	-0.89	*0.72*		-0.79	*0.68*	b	-0.59	*0.69*		0.01	*0.76*		0.24	*0.68*	
		HR	-0.27	*0.65*		-0.08	*0.60*	ab	-0.47	*0.62*		0.45	*0.68*		-0.13	*0.61*	
N IC (iFA)	31	Ctr	0.98	*0.72*	a	0.84	*0.78*		0.86	*0.69*		-0.30	*0.76*		0.54	*0.68*	
		LR	-0.72	*0.72*	ab	-0.12	*0.68*		-0.17	*0.69*		0.33	*0.76*		0.58	*0.68*	
		HR	-1.31	*0.65*	b	0.08	*0.60*		-0.87	*0.62*		0.13	*0.68*		0.19	*0.61*	

The gene regulation is graded as "+" for weak regulated and "++" for strong regulated. The ΔΔCq data for the gene expression of the milk lymphocytes' receptors (CD3δ, CD4, CD8, CD19, CD38) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 1 and 15 in bold mark in each case the time point before the first use of the vaccine batches A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 1 in the following time period until and including TW 13, and relating to TW 15 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point [76].

Table 21: ΔΔCq changes in gene expression of milk lymphocytes' receptors and cytokines relative to TW 1 or rather TW 15

		gene	CD126			CCR6			IFNγ			IL10		
		regulation	+			+			+			++		
IM	**TW**	**group**	LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
N	**1**	Ctr	0.00	*0.75*		0.00	*0.87*		0.00	*0.95*		0.00	*1.08*	
		LR	0.00	*0.75*		0.00	*0.87*		0.00	*0.78*		0.00	*1.33*	
		HR	0.00	*0.75*		0.00	*0.75*		0.00	*0.78*		0.00	*0.94*	
N PC	3	Ctr	0.88	*0.65*		0.01	*0.75*		-0.46	*0.78*	ab	0.21	*1.08*	
		LR	0.89	*0.75*		0.83	*0.87*		0.86	*0.78*	a	-1.63	*1.08*	
		HR	0.27	*0.65*		0.40	*0.75*		-1.78	*0.67*	b	-0.39	*0.94*	
	4	Ctr	0.72	*0.65*		0.63	*0.67*		1.36	*0.78*	a	1.28	*1.08*	a
		LR	-1.12	*1.29*		0.38	*1.50*		*n.n.*			-0.22	*1.87*	ab
		HR	-0.22	*0.75*		0.52	*0.87*		-2.15	*0.78*	b	-2.17	*1.08*	b
N	5	Ctr	0.40	*0.65*		1.48	*0.67*	a	-0.29	*0.67*	ab	2.25	*1.08*	a
		LR	-0.93	*0.65*		-0.15	*0.75*	ab	1.54	*0.78*	a	-2.73	*0.94*	b
		HR	-0.65	*0.65*		-0.78	*0.75*	b	-1.34	*0.67*	b	-2.53	*0.94*	b
	6	Ctr	1.70	*0.65*	a	0.92	*0.75*		0.93	*0.78*	ab	2.14	*0.94*	a
		LR	-0.54	*0.92*	b	-0.38	*1.06*		2.41	*0.95*	a	-0.14	*1.33*	ab
		HR	0.65	*0.92*	ab	-0.02	*1.06*		-0.71	*0.95*	b	-1.38	*1.33*	b
N	7	Ctr	1.55	*0.65*	a	0.34	*0.75*		-0.19	*0.67*	a	1.49	*0.94*	a
		LR	-0.70	*0.75*	b	1.01	*0.87*		2.69	*0.95*	b*	-0.56	*1.08*	ab
		HR	0.24	*0.65*	ab	-0.13	*0.75*		-0.43	*0.67*	a	-1.15	*0.94*	b
	8	Ctr	0.86	*0.65*	a	0.61	*0.75*		1.29	*0.78*	a	0.78	*1.08*	a
		LR	-1.10	*0.75*	b	0.07	*0.75*		0.73	*0.67*	a	-2.36	*0.94*	b
		HR	0.85	*0.58*	a	0.81	*0.67*		-1.42	*0.60*	b	-0.08	*0.84*	ab
N	9	Ctr	0.81	*0.65*		1.05	*0.75*		0.02	*0.67*		0.62	*1.08*	
		LR	0.40	*0.65*		1.47	*0.75*		1.89	*0.67*		-0.71	*0.94*	
		HR	1.25	*0.58*		0.67	*0.67*		0.36	*0.60*		0.70	*0.84*	
N	11	Ctr	1.04	*0.65*		0.50	*0.67*		0.10	*0.60*	a	0.75	*0.84*	
		LR	0.74	*0.75*		0.00	*0.87*		2.11	*0.67*	b*	-0.11	*1.08*	
		HR	1.10	*0.65*		-0.06	*0.67*		0.15	*0.67*	a	-0.54	*1.08*	
N	13	Ctr	0.86	*0.65*		0.62	*0.67*		1.04	*0.67*	ab	-0.57	*1.08*	
		LR	0.07	*0.65*		-0.35	*0.75*		2.30	*0.67*	a*	-4.10	*1.08*	*
		HR	0.52	*0.75*		-0.22	*0.75*		-0.76	*0.67*	b	-2.28	*0.94*	

		gene	CD126		CCR6			IFNγ			IL10	
		regulation	+		+			+			++	
IM	**TW**	**group**	LSM	*SD*	LSM	*SD*		LSM	*SD*		LSM	*SD*
N	**15**	Ctr	0.00	*0.58*	0.00	*0.67*		0.00	*0.67*		0.00	*0.94*
		LR	0.00	*0.65*	0.00	*0.75*		0.00	*0.67*		0.00	*0.94*
		HR	0.00	*0.65*	0.00	*0.67*		0.00	*0.60*		0.00	*0.84*
N PC	17	Ctr	-0.05	*0.58*	-0.19	*0.67*		-0.43	*0.67*		0.22	*0.94*
		LR	0.24	*0.65*	0.32	*0.75*		0.84	*0.67*		-0.11	*0.94*
		HR	0.05	*0.58*	0.74	*0.67*		0.68	*0.60*		-0.98	*0.84*
N	19	Ctr	-0.35	*0.58*	-0.10	*0.67*		0.79	*0.60*		0.01	*0.94*
		LR	-0.09	*0.65*	-0.25	*0.75*		0.96	*0.67*		-0.84	*0.94*
		HR	-0.13	*0.58*	0.41	*0.67*		0.86	*0.60*		-1.24	*0.84*
N PC SC	21	Ctr	-0.38	*0.75*	-0.72	*0.75*		-0.56	*0.78*		-0.49	*1.08*
		LR	-0.36	*0.65*	0.27	*0.75*		0.30	*0.67*		-1.68	*0.94*
		HR	-0.15	*0.58*	0.47	*0.67*		-0.10	*0.60*		-1.32	*0.84*
N	23	Ctr	0.51	*0.58*	-0.20	*0.67*		-0.03	*0.60*	ab	0.73	*0.84*
		LR	-0.28	*0.65*	0.35	*0.75*		1.63	*0.78*	a	-0.81	*0.94*
		HR	-0.66	*0.58*	0.25	*0.67*		-0.50	*0.60*	b	-1.50	*0.84*
N PC	25	Ctr	-0.27	*0.65*	-1.10	*0.75*	a	-0.05	*0.78*		1.02	*0.94*
		LR	-0.05	*0.92*	0.55	*1.06*	ab	0.12	*1.35*		-0.50	*1.33*
		HR	-0.15	*0.65*	1.26	*0.75*	b	-0.19	*0.67*		-0.59	*0.94*
N IC	27	Ctr	0.58	*0.65*	-0.18	*0.75*	a	-1.18	*0.67*		1.67	*0.94*
		LR	1.43	*0.75*	1.52	*0.87*	ab	0.20	*0.78*		1.76	*1.08*
		HR	-0.33	*0.65*	1.95	*0.75*	b	-1.77	*0.67*		-0.71	*0.94*
N PC	29	Ctr	0.19	*0.65*	-0.89	*0.75*	a	-0.24	*0.67*		0.48	*0.94*
		LR	1.12	*0.65*	0.96	*0.75*	ab	-0.18	*0.67*		1.22	*0.94*
		HR	0.99	*0.65*	1.34	*0.67*	b	0.05	*0.67*		1.70	*0.94*
N IC (iFA)	31	Ctr	0.39	*0.65*	-0.82	*0.75*	a	0.33	*0.67*		1.11	*0.94*
		LR	0.93	*0.65*	1.43	*0.75*	b	0.56	*0.67*		2.23	*0.94*
		HR	0.70	*0.58*	1.90	*0.67*	b*	-0.81	*0.60*		1.16	*0.84*

The gene regulation is graded as "+" for weak regulated, "++" for strong regulated. The ΔΔCq data for the gene expression of the milk lymphocytes' receptors (CD126, CCR6) and cytokines (IFNγ, IL10) are listed as LSM ± SD per group (Ctr, LR, HR), per treatment week (TW) and per immunization (IM) taking effect in this TW. The different IMs are indicated by the vaccines' administration routes (N, PC, SC, IC (iFA = incomplete Freund's adjuvant)). TWs 1 and 15 in bold mark in each case the time point before the first use of the vaccine batches A (TW 3) or rather B (TW 17). Grey background: Highlighting of IM, TW and the belonging ΔΔCq data related to the first uses of vaccine batches A and B, respectively. An asterisk (*) indicates significant differences (P < 0.05) within a group relating to TW 1 in the following time period until and including TW 13, and relating to TW 15 in the remaining treatment period. Lowercase letters label significant differences (P < 0.05) between groups to the same time point. "n.n." means unavailable data [76].

Correlations

The correlation analysis was performed to trace interdependencies between the following variables:

- Anti-C. diff IgA, total IgA and the main production factors in milk of the treated cows (Table 22) and
- Percentages of immune cell types (DCC) and gene expression data of belonging cell surface markers in blood and milk of treated and Ctr cows (Table 23).

The Pearson correlation coefficients (*r*) calculated for the different pairs of variables were evaluated as follows: $|r| < 0.5$, $0.5 \leq |r| < 0.8$ and $|r| \geq 0.8$ indicate "weak", "middle" and "strong" relations between the variables, respectively.

Concerning the correlations among the different variables of milk production, they were primarily determined being positive apart from the negative interdependency between the ECM yield and both variables of IgA (Table 22). This outcome was in concordance with a former study, ascertaining phenotypic correlations among milk constituents to be positive among them but negative towards the milk yield [111]. The root of the inverse development of milk Ig concentrations and milk yield during lactation is a major reduction in milk yield within late-lactation [85]. Excepting the *middle* correlation ($r = 0.69$) between *C. diff.* specific and total IgA, the relations between all other pairs of variables were determined to be weak (Table 22). In contrast to anti-*C. diff.* IgA, the correlations between total IgA and the variables, DiL, milk proteins and fats, showed the tendency to be stronger linked, presumably, because of the differently relative shares of the specific and total IgA in milk. As shown in Figure 3, the total IgA concentrations are subject to the natural dynamic of milk constituents during lactation. However, *C. diff.* specific IgA was influenced by the vaccination when comparing its percentages in total milk IgA before the treatment and at the late-lactation stage. On average, 1.6% anti-*C. diff.* specific in total IgA contained the BTC, and at late-lactation, remaining unchanged in case of the LR and achieving roughly 3% in case of the HR.

Table 22: Correlations between anti-*C. diff.*, total IgA and the main production factors in milk of treated cows (n = 9).

Variables	anti-*C. diff.* IgA (µg/mL)		total IgA (µg/mL)	
	r	P	*r*	P
DiL	*0.19*	< 0.05	*0.37*	< 0.01
ECM yield (kg/d)	*-0.25*	< 0.01	*-0.30*	< 0.01
milk protein (kg/d)	*0.27*	< 0.01	*0.47*	< 0.01
milk fat (kg/d)	*0.30*	< 0.01	*0.44*	< 0.01
SCC (x1,000/mL)	*0.19*	< 0.05	*0.21*	< 0.01
total IgA (µg/mL)	*0.69*	< 0.01	-	-

"r" indicates the Pearson product moment correlation coefficient. Significant correlations between the investigated variables must be proven by P < 0.05 and highly significant correlations are assessed with P < 0.01. Positive "r" displays pairs of variables being linearly intererdependent. For the pairs with negative "r", their interdependence is inverse. Abbreviations used for some variables are: DiL for days in lactation, ECM yield for energy corrected milk yield, and SCC for somatic cell counts. The evaluated number of samples was 165 [79].

The correlations analyzed between the relative parts of immune cells and the normalized gene expressions of corresponding surface determinants in PBL and SCC, respectively, are summarized in Table 23. Strong interdependencies were proven between the milk PMN and the gene of receptors for various chemokines (*CXCR2*) and the complement C5a (*C5AR1*) expressed by SCC. Both receptors are necessary to sense the respective chemoattractants, which are deposed in milk following antigenic stimulation [60]. Activated PMN, bearing these receptors *i.e.*, migrate more often into mammary tissue providing the first immunological line of defense against bacterial invasion. But their residence within the MG is short. Continuously, they were removed by milking and replaced by freshly migrated PMN [112]. Thus activated PMN could be more present in milk than in blood resulting in the strong correlations between PMN in milk and the gene expressions of the receptors, *CXCR2* and *C5AR1*, in SCC. The weakly negative correlation between macrophages in milk and *CD163* might be caused by the absolute underrepresentation of this cell type, being typically predominant in milk, within the DCC of SCC due to methodical shortcomings. The lymphocytes in blood and the gene expressions of *CD4* and *CD8β* of PBL, marking the T-cell subpopulations, were weakly correlated, considering that T_H- and T_C-cells shaping lymphocytes in blood only to smaller proportions. No correlations were proven between lymphocytes in milk and the expression of these T-cell receptors. A middle interdependency was proven for lymphocytes and the plasma cell marker *CD126* in milk and blood, showing the tendency to be stronger in milk. Although *CD126* is also weakly expressed by the most leukocytes [59], possibly, the proportional development of lymphocytes in SCC towards PBL could be stronger stamped by activated B-cells / plasma cells strongly expressing *CD126*. The MG recruits primarily plasmablasts / plasma cells for homing [113]. In particular, IgA producing ASC are settled

next to the alveolar epithelium of the MG [114], owning the potential to be removed by milking. In contrast, the lymphocyte traffic in peripheral blood is characterized by a variety of B-cell maturation states [115].

Table 23: Correlations between DCC and the belonging gene expression data.

Pair of Variables [immune cell type (%) - specific marker of cell surface (ΔCq)]	Source of Cells	*r*	P
PMN - C5AR1	milk	*0.85*	< 0.01
	blood	*0.28*	< 0.05
PMN - CXCR2	milk	*0.89*	< 0.01
	blood	*0.31*	< 0.05
Monocytes / Macrophages - CD163	milk	*-0.30*	< 0.01
	blood	*-0.09*	0.50
Lymphocytes - CD4	milk	*-0.17*	0.23
	blood	*0.29*	< 0.05
Lymphocytes - CD8β	milk	*-0.20*	0.14
	blood	*0.42*	< 0.01
Lymphocytes - CD126	milk	*0.75*	< 0.01
	blood	*0.53*	< 0.01

PMN = polymorph-nucleated neutrophils; "r" indicates the Pearson Product Moment Correlation coefficient. The correlations between the investigated variables were substantiated as significant with P < 0.05 and as highly significant with P < 0.01. The LSMs of all examined groups were used as data basis (n = 54) for each variable.

Immunohistological detection of lymphocytes in tissues

Distribution of T-lymphocyte subsets and IgA+ antibody secreting cells in the bovine lactating mammary gland

The averaged numbers of the lymphocyte populations counted per square centimeter of MG parenchyma are outlined in Table 24. Differences between the groups could only determined by comparison of the median values plotted in Figure 5.

Table 24: Incidence of lymphocytes resident in the MG parenchyma

group	CD4+ T-cells / cm^2 tissue		CD8+ T-cells / cm^2 tissue		IgA+ ASC / cm^2 tissue		ratio [CD4+ : CD8+] T-cells	
	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*
Ctr	378.5	*173.6*	4212.8	*3150.6*	61.1	*36.5*	0.22	*0.29*
LR	1668.9	*957.9*	3038.3	*1414.9*	64.0	*54.4*	0.80	*0.78*
HR	2246.5	*850.6*	7152.5	*4412.3*	238.0	*150.7*	0.51	*0.44*

Abbreviations used are: MG for mammary gland, ASC for antibody secreting cells. The evaluated number of samples was 30 per each group. No significant differences (P < 0.05) between the means of the groups exist.

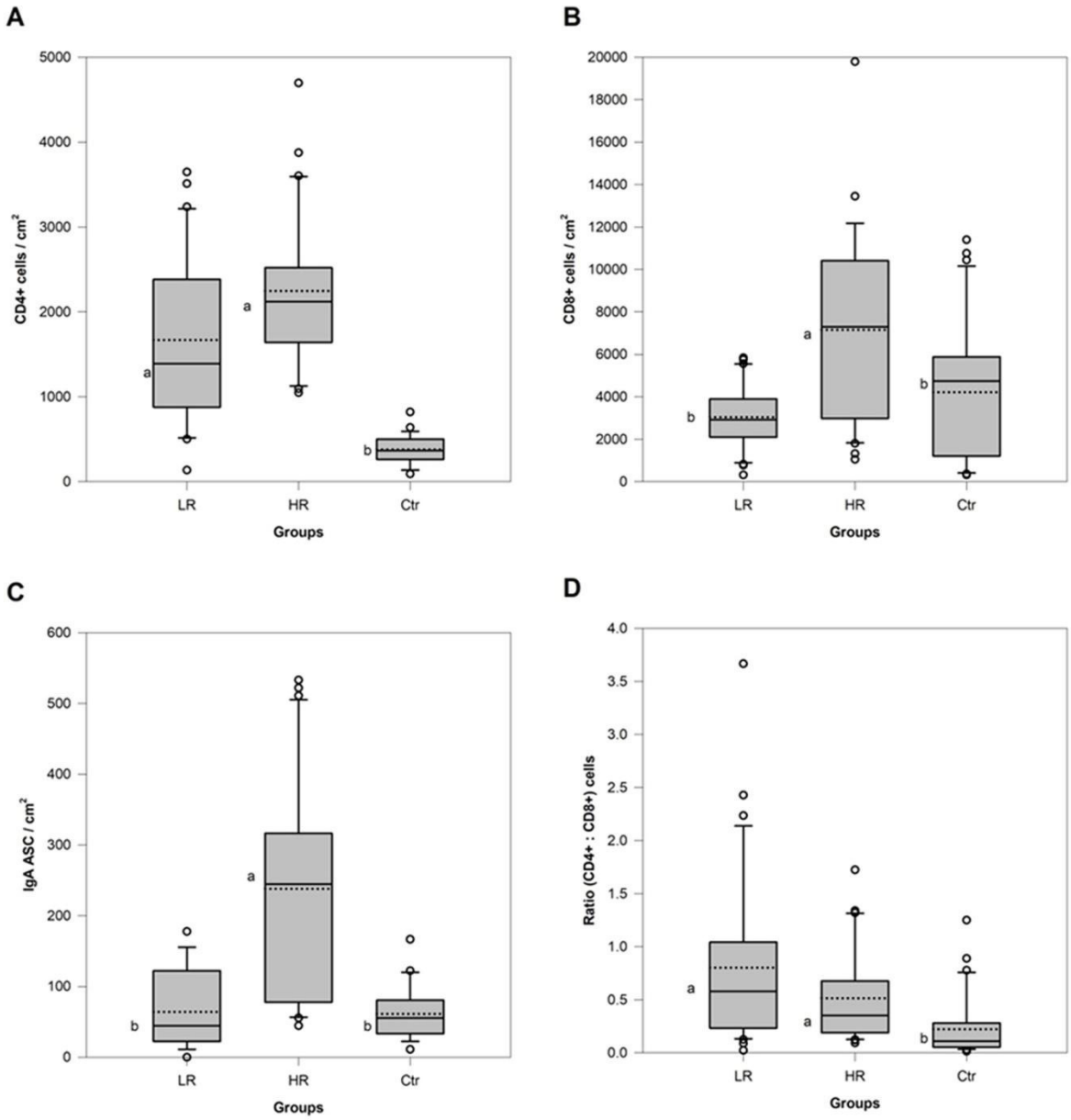


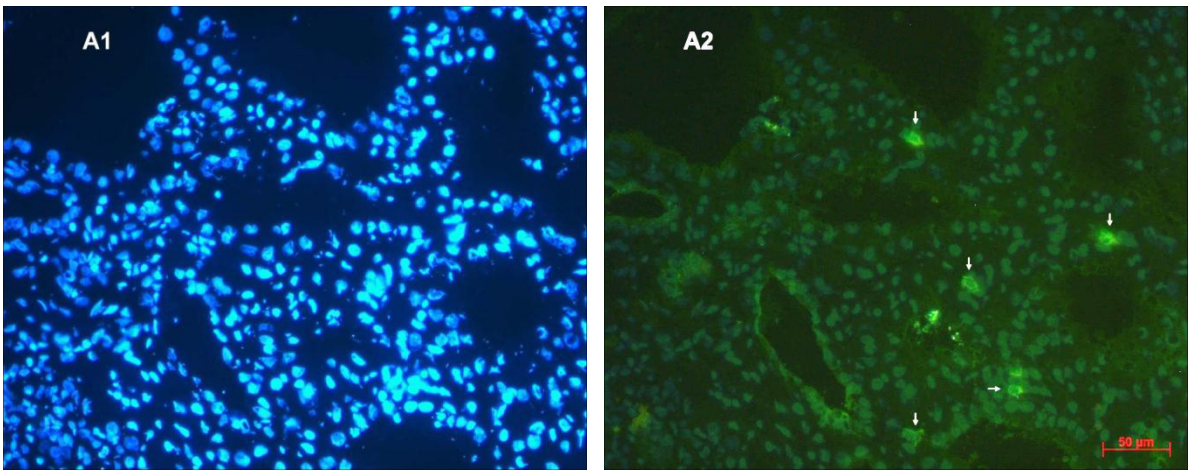
Figure 5: Lymphocyte counts in the MG of the anti-*C. diff.* vaccinated groups (LR, HR) compared to the untreated group (Ctr)

The plotted lymphocyte counts per square centimeter of MG tissue are CD4+ T-cells (A), CD8+ T-cells (B) and IgA+ antibody secreting cells (ASC) (C). The figure part D presents the ratio of (CD4+ : CD8+) T-cells. Within each box plot, the solid line labels the median and the dotted line labels the mean of the lymphocyte count or rather the ratio of the T-cell subpopulations. The lower and upper quartiles margin (25% and 75%) the box plots, and the belonging tails (5% and 95%) are shown by the lower and upper whiskers. The data points out of the box plot range are marked by circles. Substantial differences ($P < 0.05$) of ranks among the groups are illustrated with lowercase letters (a, b, c) [116].

The median numbers of IgA+ ASC, CD4+ and CD8+ T-cells in MG parenchyma of HR were significantly elevated towards the Ctr group. Leaving the CD4+ T-cells aside, the median of lymphocytes counted in MG tissue of HR and LR were significantly different, too (Figure 1, parts A-C). Shifts in lymphocyte trafficking and colonization of peripheral tissues were

triggered by various factors like hormonal and inflammatory stimuli [113]. Exemplarily, local inoculation of the MG resulted in generally higher numbers of IgA producing plasma cells settled in the immunized glands in contrast to the control glands [117]. Thus the repeated anti-*C. diff.* IM might have encouraged the lymphocyte homing to the MG, too. T_H-cells seemed to be attracted in the MG of all treated cows. But the type of T_H-cell emerging in the MG was presumably different between HR and LR concerning their differently regulated chemokines in PBL and SCC [67] [76]. As a result, more activated B-cells could have entered the MG of the HR, forming IgA+ ASC and, finally, producing more anti-*C. diff*. milk IgA than LR in response to the treatment. CD8+ T-cells are the prevalent lymphocyte subpopulation in the MG and the examined ratio of [CD4+ : CD8+] T-cells is typically < 1 [118] [119] [120]. Likewise, this was determined in the present study. CD8+ T-cells are either of the cytotoxic or suppressor type, probably, owning an immunoregulatory function in the MG [118]. Because T_C cells seemed to be more present in the HR's MG compared to the other groups, presumably, ensuring the orderly resolution of the immune response of the more antigen-sensitive HR cows.

The MG parenchyma of HR and LR IHC stained with DAPI and anti-bovine IgA:FITC in comparison to the negative and positive controls are exemplarily shown in Figure 6.



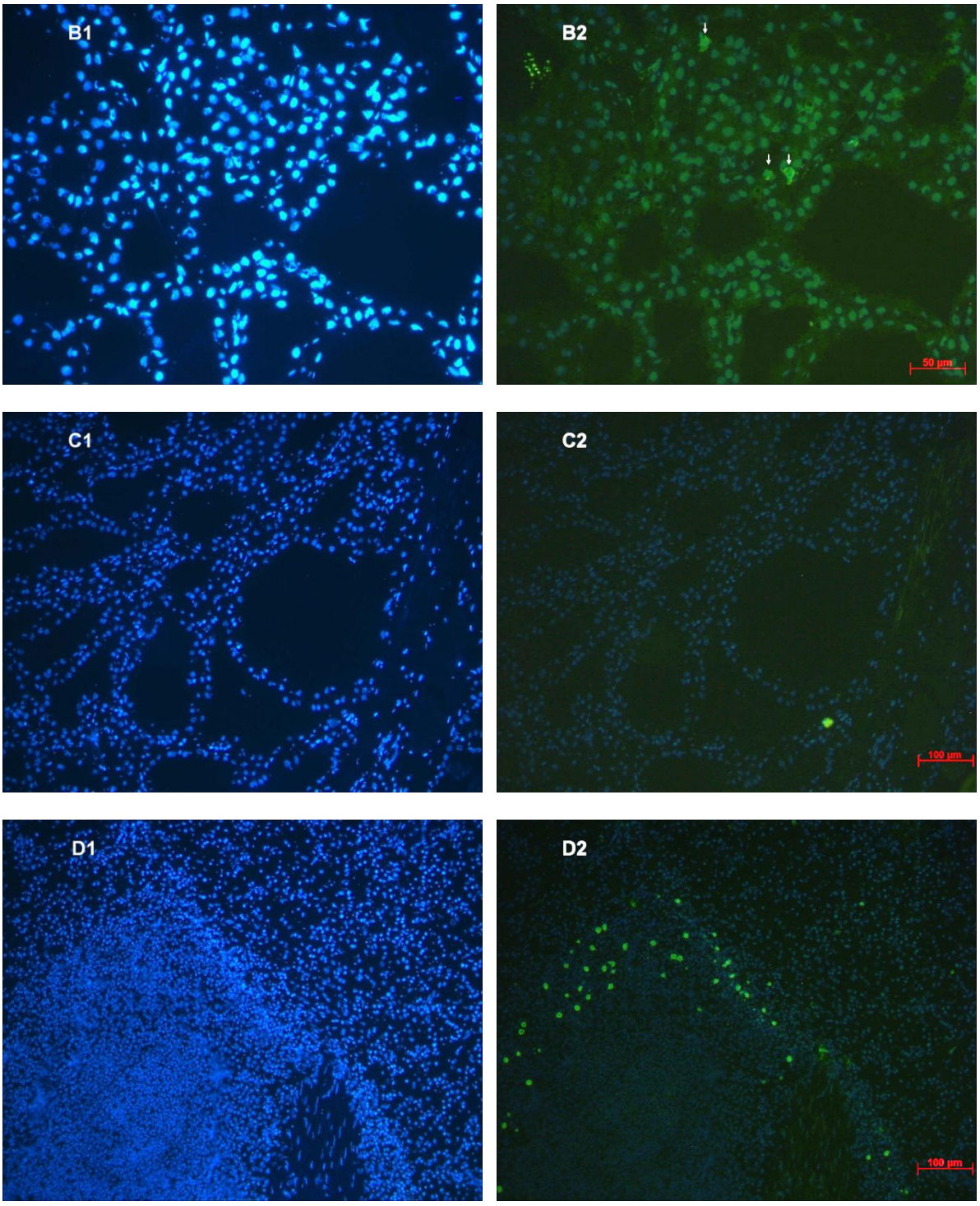


Figure 6: Cell nuclei and IgA stained in the MG parenchyma and the spleen

IgA+ antibody secreting cells (ASC) stained in MG parenchyma of a HR cow (A1, A2) and a LR cow (B1, B2) are marked by white arrows (IHC, x200, scale bar represents 50 µm). C1 and C2 present the negative control (IHC, x100, scale bar represents 100 µm). C2 includes an auto-fluorescent debris. The IgA+ ASC are the bright green circles in the spleen used as positive control (D2) (IHC, x100, scale bar represents 100 µm) [116].

Distribution of T-lymphocyte subsets and IgA+ antibody secreting cells in the supramammary lymph nodes

The density of lymphocytes resident in the SLN was exclusively analyzed for the treated groups, because no untreated bovine SLN tissue of known origin was available. The SLN of HR were proven to host more IgA+ ASC than those of the LR respecting the averaged counts of IgA+ ASC per square centimeter of SLN tissue (Table 25, Figure 7, part C). Counts of CD4+ and CD8+ T-cells seemed to be increased within the SLN of HR evaluating the belonging median values presented in Figure 7 (parts A and B), which differed significantly compared to the LR group. LN are the body sites of professional antigen recognition. The probability of encounter the cognate antigen increases by accumulation of recirculating lymphocytes within the peripheral lymphoid organs [121]. Multiplications of T- and / or B-cells in LN post infection or vaccination of the adjacent tissues occur but need not to be caused in any case [122] - [124]. The possibly reinforced presence of these lymphocyte populations, particularly, of CD4+ T-cells and IgA+ ASC, in the HR's SLN might have improved their acquired immune responsiveness towards the PC *C. diff.* vaccinations in contrast to the LR. Atypically, the averaged ratio of (CD4+ : CD8+) T-cells was < 1 in the SLN of LR (Table 25). It is generally proven to be > 1 in bovine SLN, just like in peripheral blood [119]. The significantly differing median values of both treated groups were also below the base level (Figure 7, part D). Apart from the wide scattering of data, the atypical T-cell ratios of both treated groups could have been caused by a possibly imbalanced selection of tissue pieces by respecting the preferential localization of CD4+ and CD8+ T-cells in different compartments of LN [123].

Table 25: Incidence of lymphocytes resident in the SLN of the vaccinated groups

group	**CD4+ T-cells / cm^2 tissue**			**CD8+ T-cells / cm^2 tissue**			**IgA+ ASC / cm^2 tissue**			**ratio [CD4+ : CD8+] T-cells**		
	mean	*SD*		mean	*SD*		mean	*SD*		mean	*SD*	
LR	1055.1	*959.6*	a	3881.4	*3661.0*	a	1875.5	*571.7*	a	0.43	*0.43*	a
HR	5031.2	*5501.3*	a	7799.1	*6798.2*	a	2421.4	*766.2*	b	1.11	*1.34*	a

Abbreviations used are: SLN for supramammary lymph nodes, ASC for antibody secreting cells. The evaluated number of samples was 30 per each group. The lowercase letters (a, b) characterize significant differences (P < 0.05) between the groups.

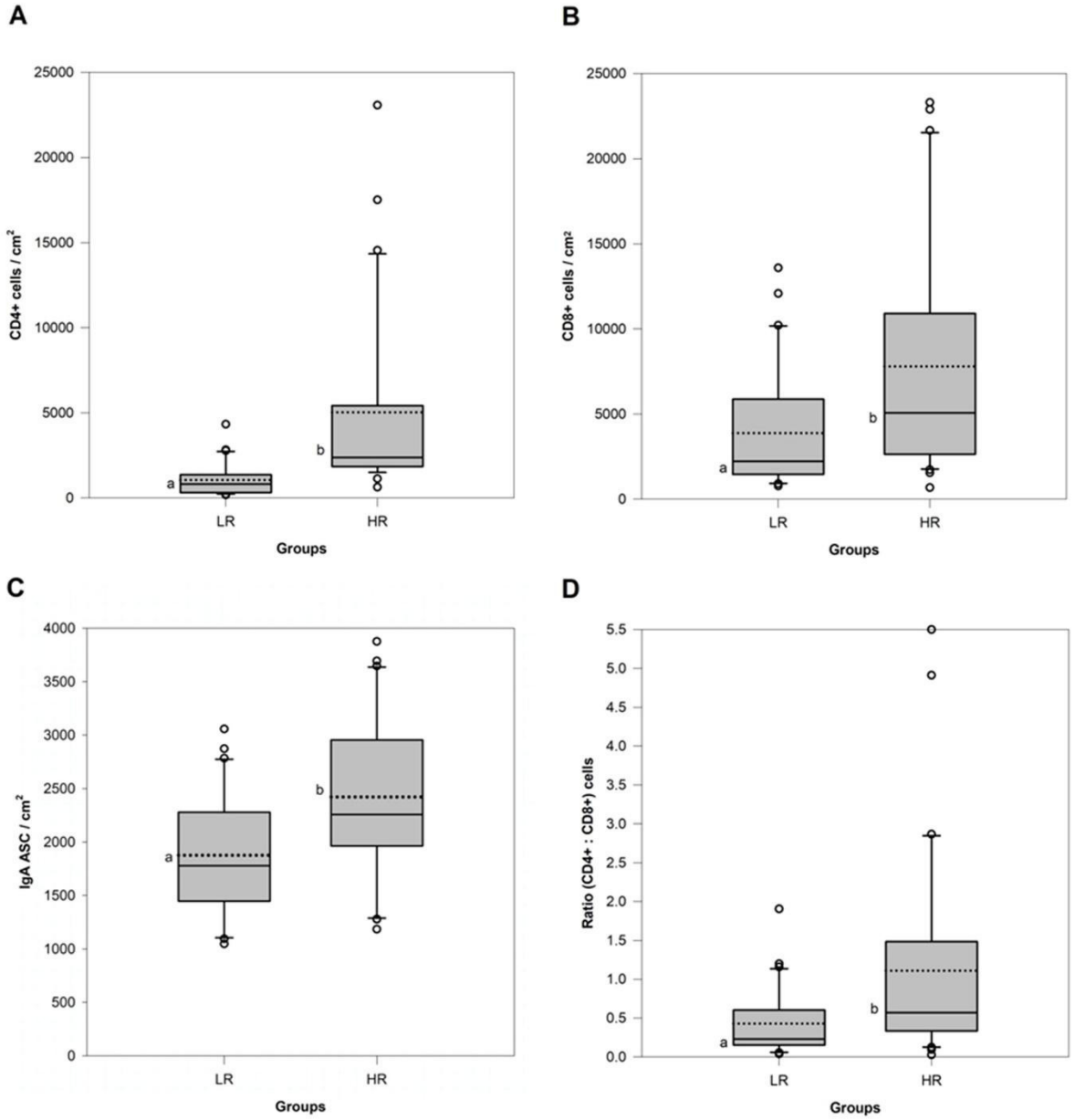


Figure 7: Lymphocyte counts in the SLN of the anti-*C. diff.* vaccinated groups

CD4+ T-cells (A), CD8+ T-cells (B) and IgA+ antibody secreting cells (ASC) (C) are specified per square centimeter of SLN tissue. The ratios of (CD4+ : CD8+) T-cells are illustrated in the figure part D. Each box plot indicates the median (solid line), the mean (dotted line), the lower and upper quartiles of the lymphocyte count or rather the T-cells' ratio. The lower and upper whiskers extend to the 5% and 95% quantiles of the depicted data. Circles characterize outlying data points. Lowercase letters (a, b) mark significant differences (P < 0.05) in the median values among the treatment groups (figure parts A, B, C) and the mean values of IgA+ ASC (figure part C), respectively [116].

Conclusions

In sum, the persistent concentration of *C. diff.* specific IgA in milk is achievable by frequent IM of dairy cows. For this purpose, crucial preconditions are the use of a potent anti-*C. diff.* vaccine capable to induce a primarily mucosa-related immune response and preselected dairy cows as recipients showing particular sensitivity towards *C. diff.* and its toxins.

No impacts on the physical conditions of the vaccinated cows, the main composition of their milk and the DCC of milk and blood shaped up when a well tolerated vaccine is used. The gene expression analysis of selected chemokines / cytokines in PBL after stimulation with the relevant *C. diff.* antigens might be a suitable tool to identify dairy cows especially sensitive to *C. diff.*. Beside *CXCL8*, *IL1*β and *IL12*β, particularly, *IFN*γ are eligible for testing, as these mediators were regulated significantly different among HR and LR in response to the vaccine *MucoCD-I batch A*.

Concerning the different immune responsiveness of LR and HR, the following assumptions were deduced:

- The results of the gene expression analysis indicated the activation of different T_H-cell types after the first treatment with *C. diff.*.The IS of the HR was driven by T_H2-lymphocytes supporting the humoral response to antigen, whereas the LR promoted the T_H1-cell response, which aims at a strong cellular immunity.
- Regarding the similar PIGR expression patterns of the treated groups, the epithelial transport of IgA was no reason for the low mucosal immune response towards *C. diff.* in the MG of LR.
- The intense lymphocytes colonization of the MG and SLN, as revealed in the HR's tissues for IgA+ ASC, CD4+ and CD8+ T-cells, is a the crucial prerequisite for the persistent production of anti-*C. diff.* IgA.

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Appendix

Appendix I

Schmautz C, Hillreiner M, Ballweg I, Pfaffl MW, Kliem H. Stimulated enrichment of *Clostridium difficile* specific IgA in mature cow's milk. PLoS One. 2018 Apr 25;13(4):e0195275. doi: 10.1371/journal.pone.0195275.

Appendix II

Schmautz C, Mueller N, Auer M, Ballweg I, Pfaffl MW, Kliem H. Immune cell counts and signaling in body fluids of cows vaccinated against *Clostridium difficile*. J Biol Res (Thessalon). 2018 Dec 10;25:20. doi: 10.1186/s40709-018-0092-4

Appendix III

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RESEARCH ARTICLE

Stimulated enrichment of *Clostridium difficile* specific IgA in mature cow's milk

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Abstract

Cow milk products enriched with *Clostridium difficile* (*C. diff.*) specific IgA are possible alternative therapeutics against *C. diff.* associated diarrhea. A persistently high level of *C. diff.* specific IgA in mature milk triggered by continuous immunizations of dairy cows against *C. diff.* was hypothesized. Nine *Brown Swiss* cows were repeatedly vaccinated against *C. diff.* and divided into low responder (LR) and high responder (HR) cows, as measured by their production of anti-*C. diff.* specific IgA in milk (threshold: 8.0 μg anti-*C. diff.* specific IgA/mL on average). Total and *C. diff.* specific IgA were quantified in bovine milk and blood using a sandwich ELISA. Important milk production factors were analyzed per lactation stage. Milk yield, milk fats and proteins were significantly different ($P < 0.05$) in the early lactation stage when the treated with the untreated cows ($n = 30$) were compared. In contrast to the "before treatment control" values, the HR's milk anti-*C. diff.* IgA was approximately 80% higher at any lactation stage, and the HR's total milk IgA increased up to 72% in the late lactation stage. The LR's total milk IgA differed from the baseline by roughly 47% only in the late lactation stage. The total and specific IgA contents in milk were more influenced by the anti-*C. diff.* immunizations than in blood. The correlations between anti-*C. diff.* specific IgA, total IgA and the main production factors in milk were classified as weak ($I\,r\,I < 0.5$), except for the close relation of anti-*C. diff.* specific IgA and total IgA ($r = 0.69$). To conclude, a sustainable *C. diff.* specific IgA enrichment in milk can be achieved by continuous immunization of dairy cows, provided a potent and well-formulated anti-*C. diff.* vaccine is given to dairy cows preselected due to their proven anti-*C. diff.* receptivity.

Introduction

Milk is a foodstuff of high biological value in mammalian nutrition. In addition to the species-adapted proportions of the macronutrients, lactose, milk fat and protein, milk delivers various health-promoting constituents. The main immune-relevant components in milk, and especially in colostrum, are immunoglobulins (Igs). Depending on species, breed, age, stage of lactation and health status, varying proportions of the different immunoglobulin (Ig) classes are inherent in mammary secretions [1]. In humans, the predominant Ig class is IgA, whereas IgG has the largest share of colostral and milk antibodies in cattle [2]. Concentrations of 20–200

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mg IgG/mL milk, being dominated by the principal subclass IgG1, are found in bovine colostrum. To ensure the supply of calves with essential antibodies, colostrum of the dam contains remarkably higher amounts of IgG, which comprise 70–80% of total protein in contrast to its lower remaining level of 1–2% in mature milk [3].

In line with passive immunization (IM) of bovine neonates, the concept of immune milk for human health was developed as early as in the mid-20th century. Petersen & Campbell (1955) pointed out "*the use of protective principles in milk and colostrum in prevention of disease in man and animals*" [4]. They had laid the cornerstone for the utilization of minor milk components in the health care and dairy industries, first commercialized by Ralph Stolle (http://www.smbimilk.com/) [5]. IM procedures with polyvalent vaccines showed that the specificity of lacteal antibodies can be influenced [6]. The volume of antigen-specific lacteal antibodies would grow if pregnant cows were vaccinated with certain antigens at the end of their gestation so they could benefit from the natural accumulation of Igs in bovine colostrum. A hyperimmune bovine colostrum (HBC) was generated in this way, in retrospect mainly for its application in the prevention or treatment of animal and/or human enteric diseases [7, 8]. For instance, cryptosporidiosis, shigellosis, infantile rotavirus gastroenteritis, enterotoxigenic *E. coli* caused diarrhea and *Clostridium difficile* (*C. diff.*) associated diarrhea (CDAD) were successfully treated with nutraceuticals like HBC or purified derivate products [9]. The therapeutic activity of such an orally administered bovine Ig concentrate depends directly on the survival of the included antigen-specific IgG during its passage through the recipient's intestinal tract. In this context, Kelly et al. (1997) found that the consumed bovine colostral IgG undergoes partial degradation while retaining antigen-specific neutralization capacity [10]. More than 10% of IgG was recovered in stools from infants treated with an Ig concentrate [11]. Resistance to proteolytic digestion depends on the isotype of antibody. The secretory IgA (sIgA), naturally occurring in mucosal sites, resists proteolytic digestion better than IgG due to the protection of sIgA by the linked secretory component [12]. Bovine colostrum and milk comprise sIgA in concentrations of 1–6 mg/mL and 0.05–0.1 mg/mL, respectively [3]. However, the average 18% sIgA of total milk Igs provide a powerful antigen-specific neutralization capacity [1]. Exemplarily, polymeric IgA was revealed as superior to monomeric IgA and IgG in preventing the damaging effects of *C. diff.* toxin A *in vitro* [13]. Furthermore, sIgA does not encourage inflammatory processes as it is neither able to opsonize nor to activate complement, unlike IgG [14]. Overall, the features of sIgA described above are advantageous for the complementary immunotherapy of CDAD, as demonstrated by using a particular sIgA-enriched bovine whey protein concentrate in the hamster model and in a clinical trial performed with human beings [15, 16].

C. diff. is a gram-positive, anaerobic, spore-forming and rod-shaped bacterium ubiquitously present in the environment. It is present as a normal, but minor inhabitant of the intestinal microflora of healthy human adults and animals [17]. Toxigenic strains of *C. diff.* produce two exotoxins, named toxin A (TcdA) and B (TcdB), which affect the intestinal integrity by destroying the cytoskeleton of the enterocytes. Essential prerequisites for *C. diff.* infections (CDI) in humans are a weakened immune system (IS) due to advanced age, co-morbidity, antibiotic therapy and residence in nursing homes and hospitals with increased *C. diff.* spore load. Antibiosis disturbs the indigenous flora of the gastrointestinal tract and offers *C. diff.* a niche for adherence and vegetation. The clinical picture of CDI varies from asymptomatic carriage to life-threatening pseudomembranous colitis or toxic megacolon [18, 19]. 20–35% of patients experience a relapse after standard antibiotic treatment with the commonly used antibiotics vancomycin or metronidazole [20]. Furthermore, hypervirulent *C. diff.* strains like BI/NAP1/027, which produce a third, binary toxin, have become more prevalent worldwide since the CDI epidemics in North America and Europe (2003 & 2004) [21]. The increased incidence

and severity of this disease invite alternative treatment approaches. Oral immunotherapy with *C. diff*. specific sIgA from bovine immune milk would not eradicate *C. diff*. right now, but could serve as a possible supplementary approach to antibiotic therapy to prevent CDAD.

As the history of immune milk production has shown, repeated exposure of dairy cows to the selected pathogen would be necessary to produce of cow's milk enriched with *C. diff*. specific antibodies. The present study builds on the promising approach regarding the IM procedure utilized by van Dissel et al. in 2005 [15]. In contrast to the previously tested efficacy of the immune whey protein concentrate on CDAD prevention [15], the aim of this study was to focus on the quantities of anti-*C. diff*. sIgA that can be steadily produced in milk. It was hypothesized that a persistently high level of *C. diff*. specific IgA in mature milk would be ensured by continuous triggering of the dairy cows with the virulent factors of *C. diff*. until completion of lactation. Therefore, specific IM procedures were carried out and the immune response of the vaccinated cows was closely supervised.

Materials and methods

Animals

The government of Upper Bavaria gave its approval to conduct the animal trial (AZ 55.2-1-54-2532.6-17-2012). Nine healthy and primiparous *Brown Swiss* cows were used for the IM study. They were allocated to a separate byre with tethering for easier operation. The early lactating cows with 28 ± 4 milking days (DiL) on average produced 21.3 ± 1.5 liters milk per day. After the lactation time ended, they were slaughtered and their carcasses fully disposed of without touching the food chain. The reference dairy cattle group was provided by the Veitshof research station of the Technical University of Munich at Freising, Germany, where they were housed in a freestall barn. Thirty *Brown Swiss* cows in the first or second lactation were selected and assigned to an early, mid- or late lactation group depending on their lactation state. Throughout the entire study period, all cows had water access *ad libitum* and were fed a daily basic ration that consisted of 22 kg of corn silage (33% dry matter), 10 kg of grass silage (40% dry matter) and 2 kg of hay. For energy balance, they received 2 kg of high-protein crushing rape and soy (deuka Kompopur 404; Deutsche Tiernahrung Cremer, Düsseldorf, Germany) in their diet. Their mineral balance was achieved by supplementation of 125 g of mineral mix (Josera, Kleinheubach, Germany). Dairy cow performance requirements were met by giving them 0.5 kg of concentrate (deuka MK 194-UDP; Deutsche Tiernahrung Cremer) per liter of milk delivered.

Vaccination plan

Before launching the experiment, the animals assigned for treatment were allowed a five-day acclimatization period in the cowshed environment. After a health check by a veterinarian, only those cows showing no signs of illness were vaccinated. The same procedure was carried out prior to every further vaccination. Additionally, the health status of the animals was always audited on the first day after the vaccination. Samples associated with sick animals were excluded from the analysis. The *vaccine* consisted of whole *C. diff*. cells (strain VPI 10463) formaldehyde-inactivated, and the partly disabled TcdA and TcdB prepared from the *C. diff*. culture filtrate. Two *MucoCD-I* vaccine batches (A and B) for injection, including different toxoid A and B proportions in relation to the respective toxins, and the vaccine *MucoCD-N* for nasal (N) application were provided by IDT Biologika (Dessau-Roßlau, Germany). The *vaccination schedule* implemented is summarized in Table 1. The cows were immunized on the N route biweekly. The periodically administered N vaccinations were complemented with *MucoCD-I* given percutaneously (PC) close to both supramammary lymph nodes (SLN) every

Table 1. Immunization schedule.

TW		1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31
MucoCD-N vaccine		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
MucoCD-I vaccine batch			A							B		B		B	B	B	B
+ iFA																	X
vaccine application	*PC*		X							X		X		X		X	
	SC											X					
	IC														X		X

During the 31-week treatment period, anti-*C. diff*. vaccinations were conducted only in the indicated TWs. The types of vaccine application were marked by crosses. The N vaccination with 2 mL *MucoCD-N* vaccine to each nostril was regularly given bi-weekly. PC close to the SLN, 2 mL of *MucoCD-I* vaccine (A or B) were injected in TWs 3, 17, 21, 25 and 29. On the SC route, 4 mL of *MucoCD-I* vaccine (B) were administered only once in TW 21 and on the IC route in TW 27. Additionally, 2 mL of iFA combined with 2 mL *MucoCD-I* vaccine (B) were IC applied in TW 31.

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four weeks and subcutaneously (SC) or rather intracutaneously (IC) on the lateral torso (by several punctures into a 20 x 15 cm shorn area caudal of the scapula) at different points in time from treatment week (TW) 17. Because of incompatible concentrations of intact toxins TcdA and TcdB existing in vaccine batch A, the immune stimulation was restricted to the application of *MucoCD-N* from TW 3 up to TW 15, the end of the first IM period. The second treatment period started with the application of vaccine batch B. Its administration was modified by replacing the SC injection with the intracutaneous (IC) one due the improved efficacy of this administration route. To stimulate a long-lasting antibody response, incomplete Freund's adjuvant (iFA) was additionally injected in TW 31. The 31-week vaccination period was accompanied by intensive surveillance of its physiological effects.

Rationale of vaccination sites

The *N IM* was administered by injecting 2 mL of *MucoCD-N* vaccine in each nostril with a needle-free syringe. The direct contact of antigen with the N mucous membrane should stimulate the NALT (nasal associated tissue), a lymphoid tissue located in the olfactory organ and part of the mucosal IS, being closely connected with other mucosal tissues of the body. The local administration of the anti-*C. diff*. vaccine close to both SLN on the *PC route* should lead the antigen to the SLN draining the udder by way of the afferent lymphatics. Ensuring the encounter of antigen with naïve lymphocytes, an augmented egress and settlement of specific antibody producing cells (APCs) in the mammary gland were expected. *SC or IC administrations* of antigen caudal of both scapulae should trigger the systemic humoral immunity against *C. diff*. and its toxins by increasing the specific serum antibodies. Naïve lymphocytes of peripheral lymph nodes are steadily on an inter-nodal journey in search for their target antigen, thus increasing the probability of an encounter. After lymphocyte activation by antigen contact, their homing is followed by specific antibody production, and the blood becomes enriched with them [22, 23].

Milk yield and sampling

Milk yield and its composition were monitored in all cows with at least 300 DiL until lactation ended. The quantities of milk gathered were calculated as energy corrected milk (ECM) using the formula: ECM = milk yield-kg*[(0.38*milk fat %) + (0.21*milk protein %) + 1.05] / 3.28 [24].

Treated cows. Milk yield was recorded every two weeks with a TRU-Test milk meter (Lemmer-Fullwood, Lohmar, Germany). Once a week, half a liter of milk per cow was deposited during the morning milking. Of this quantity, a 50 mL aliquot containing a preservative (acidiol) was taken to analyze the major milk components, including somatic cell counts (SCC). This milk testing was carried out by the Milchprüfring Bayern Association (Wolnzach, Germany). The milk fats and proteins were measured based on their infrared absorption behavior with the MilkoScan-FT-6000 device (FOSS, Hamburg, Germany). Afterwards, the data were evaluated after transformation using the Fourier method. SCC in milk were analyzed with an optical fluorescent technique (*Fossomatic-FC* device, FOSS, Hamburg, Germany). Milk for measurement of the Ig concentrations was collected weekly in the first two months of treatment and every two weeks subsequently. Two 10 mL samples of the residual 450 mL milk sample per cow were defatted by centrifugation (4,000 xg, 4˚C, 15 min). Skimmed milk was aliquoted in 2-mL tubes and stored at -20˚C until further processing. Milk samples of cows suspected of mastitis were bacteriologically examined by the Central Bavarian Animal Health Service Association (TGD Bayern, Grub, Germany).

Control cows. Milk yield and proximates were captured during the course of the regular analysis for the Bavarian Dairy Herd Improvement Association (LKV Bayern e. V., Munich, Germany). Milk of the selected control cows was collected only once to analyze the IgA content.

Blood sampling

Blood of the treated cows to measure IgA concentration was collected simultaneously to the milk samples. After morning milking, blood of the jugular vein was collected with a 9 mL EDTA pre-coated vacuette (Greiner Bio-One GmbH, Frickenhausen, Germany) and subsequently stored in ice. Additional 100 μL of 0.3 M EDTA [33.5 g Titriplex III (Merck KgA, Darmstadt, Germany) dissolved in 300 mL bi-distillated water and supplemented with 1% acetylsalicylic acid (Merck KgA, Darmstadt, Germany)] were added to guarantee the stabilization of the bovine blood. After centrifugation (2,000 xg, 4˚C, 15 min), the plasma was divided into 2 mL aliquots and stored at -20˚C.

C. diff. specific IgA

C. diff. specific IgA in cow's milk and blood was quantified with a sandwich ELISA. The reagents and buffers used were: Coating buffer (35 mM $NaHCO_3$, 15 mM $Na_2CO_3x10H_2O$, pH 9.6), wash buffer (137 mM NaCl, 8 mM $Na_2HPO_4x2H_2O$, 1.5 mM KH_2PO_4, 2.7 mM g/l KCl, 0.1% Tween 20), PBST (0.2 mM $NaH_2PO_4xH_2O$, 1.2 mM $Na_2HPO_4x2H_2O$, 0.05% Tween 20, pH 7.4), blocking buffer (2% gelatin in PBST), dilution buffer (0.2% gelatin in PBST), 3,3',5,5'-tetramethylbenzidine (TMB) enzyme substrate mix and stop solution (2 M H_2SO_4). First of all, 96-well plates (Nunc MaxiSorp™, Sigma-Aldrich Chemie GmbH, Munich, Germany) were pretreated with 100 μL/well of *C. diff.* cells included in coating buffer ($2.0x10^8$ cells/mL, IDT Biologika, Dessau, Germany). The coating lasted 2 h at 70˚C and then overnight at 4˚C. The addition of 200 μL of blocking buffer for 1 h at 37˚C blocked the overlay. After its decantation, the wells were rinsed four times with wash buffer. *C. diff.* specific IgA (1.76 mg/mL, MucoVax b. v., Leiden, Netherlands) used as standard was diluted within the range of 62.5 to 4,000 ng/μL with dilution buffer. The samples for testing, skimmed milk or blood serum were diluted 1:10 so they would fall within the range of the standard. Seven dilution stages of the standard, a blank (pure dilution buffer), the samples and intra-assay controls additionally used were applied to the pre-coated plate (100 μL/well) in duplicates for incubation lasting 1.5 h at 37˚C. The incubation was completed by repeated washing and the

subsequent addition of 100 μL of secondary antibody (1:70,000 diluted HRP conjugated sheep anti-bovine IgA (Bethyl Laboratories, Inc.; Montgomery, TX 77356, USA) to the processed wells. After 1.5 h of exposure at 37˚C and protected from light, the wells were rinsed four times and then filled with 150 μL of the TMB substrate mix. The enzymatic reaction took place on a wave platform shaker and in the dark within 40 min at RT. It was terminated by pouring 50 μL of stop solution per well. During the subsequent 30 min, absorbance was evaluated at 450 nm with the photometric instrument (microplate reader Sunrise™, Tecan Group Ltd., Männedorf, Switzerland). The calculation of *C. diff*. specific IgA quantities in unknown samples is based on the standard curve generated by the Magellan™ V6.6 reader software (Tecan Group Ltd., Männedorf, Switzerland).

Total IgA

The bovine IgA ELISA kit (Cat. No. E10-121) provided by Bethyl Laboratories, Inc. was used for the quantitation of total IgA in cow's milk and blood. Other test components used and separately supplied were 96-well plates and the ensuing buffer preparations: Coating buffer (0.05 M carbonate-bicarbonate, pH 9.6), solution for washing, blocking and as diluent (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0), enzyme substrate (TMB) and stop solution (0.18 M H_2SO_4). The ELISA test was carried out according to the manufacture's recommendations. Finally, the absorbance of each sample was measured within 30 min at 450 nm using the plate reader (Sunrise™).

Statistics

The MIXED procedure model in SAS/STAT® 9.22 (2010 SAS Institute Inc., Cary, NC, USA) was used to find treatment effects and differences among groups during the treatment period. The computational procedure could only work when the convergence criteria were met, which were evaluated following the log-likelihood calculation. The results were expressed as estimated least square means (LSM) with associated standard errors (SD). The Pearson product moment correlation coefficients (*r*) were calculated by means of SigmaPlot 11.0 (2008 Systat Software, Inc., San Jose, CA 95131, USA). The significance level was set at $P < 0.05$. Results with $P < 0.01$ were defined as highly significant.

Results

Note

As a preliminary remark, it has to be pointed out that all the data surveyed for the treated cows were evaluated and stringently divided into 'low responder' (LR) and 'high responder' (HR) cows. The enrichment of *C. diff*. specific IgA in milk was done by repeated IM. In particular, the overall view of this parameter had shown that cows were differently sensitive towards the given antigen. Based on their immunological reactivity, the treated cows were assessed as LR or HR by the fixed threshold of 8.0 μg/mL *C. diff*. specific IgA in milk. For this purpose, the total average of *C. diff*. specific IgA in milk, relating to all measurement points of the entire treatment period, was calculated per cow.

Animal health

Table 2 summarizes all diseases of the vaccinated cows occurring at the start and during the treatment period. The TW, in which the different diseases emerged, are quoted therein. No health impairments of the control cows to the different dates of sampling were noticed.

Bursitis, and sometimes lameness and mastitis, were diagnosed mainly when treatment began. These diseases conceivably arose due to local transition of the animals in question to

Table 2. Diseases of treated cows and the TW per vaccinated cow in which diseases emerged.

Diseases	Treated cows								
	LR-2	LR-3	LR-6	LR-7	HR-1	HR-4	HR-5	HR-8	HR-10
Mastitis	1, 8					0			0, 16, 26
Bursitis				1			1		
Lameness			2, 3		0, 1				
Fever				1, **3**	1	**3**	**3, 4**	**3**	**3**, 23
Udder swelling on IM side	**3, 4**	**3, 4**		**3, 4**		**3, 4**	**3, 4**	**3, 4**	**3**
Hemolysis		**4**		**3, 4**			**4**		
Ketosis							**4**		

TWs with side effects associated with the one-time PC administered vaccine batch A are in bold.
LR = low responder cow numbers 2, 3, 6, 7; HR = high responder cow numbers 1, 4, 5, 8, 10.

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the new byre environment. Because of its impaired health, cow HR-1 did not receive the first N anti-*C. diff.* vaccination in TW 1. Clinical signs like fever, udder swelling on the IM side, hemolysis and ketosis, but also reduced feed intake and rumen turnover were deemed to be the cause of the high dosage of the *C. diff.* toxins delivered with *MucoCD-I* batch A in TW 3. Cow LR-6 was not immunized in TW 3 owing to its persisting lameness. Therefore, no treatment-related signs could develop hereinafter.

Milk yield & proximates

With the onset of lactation, control cows produced 30.6 ± 1.3 kg milk per day on average, at least 5 kg/d more milk than the treated cows (Table 3). In association with the adverse health effects caused by the first PC vaccination, a reduction in emitted milk volume was noted for the treated cows in early lactation. When lactation proceeded, significant differences in dairy production no longer existed among the groups, and all cows had persistent milk production

Table 3. Milk yield and proximates related to different groups and lactation stages.

lactation stage	group	ECM yield (kg/d)			milk proteins (kg/d)			milk proteins (%)			milk fats (kg/d)			milk fats (%)		SCC (x1,000/mL)		
		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	LSM	*SD*	
early	control	30.6	*1.3*	a	1.00	*0.05*	a	3.3	*0.2*		1.29	*0.10*	a	4.2	*0.3*	98.1	*28.9*	
	LR	23.7	*0.9*	b	0.76	*0.03*	b	3.2	*0.3*		0.96	*0.07*	b	3.8	*1.3*	60.4	*21.6*	
	HR	25.1	*0.9*	b	0.78	*0.03*	b	3.1	*0.2*		0.96	*0.07*	b	3.8	*0.6*	35.3	*20.1*	
mid	control	31.9	*2.8*		1.07	*0.10*		3.4	*0.5*		1.43	*0.21*		4.6	*0.5*	33.0	*64.7*	a b
	LR	28.9	*1.0*	**	1.03	*0.03*	**	3.6	*0.3*		1.08	*0.07*		3.7	*0.7*	21.0	*22.0*	a
	HR	26.6	*0.9*		0.96	*0.03*	**	3.6	*0.2*	*	1.15	*0.07*		4.3	*0.4*	126.6	*21.2*	b**
late	control	27.7	*1.4*		1.04	*0.05*		3.7	*0.2*		1.21	*0.11*		4.3	*0.5*	81.7	*32.3*	
	LR	27.3	*1.0*	*	1.00	*0.04*	**	3.6	*0.3*		1.04	*0.08*		3.8	*0.6*	22.9	*23.9*	
	HR	28.7	*0.9*	**	1.06	*0.03*	**	3.7	*0.1*	*	1.22	*0.07*	**	4.3	*0.4*	62.6	*21.2*	

The lactation stages are defined as follows: "early" for ≤ 100 days in lactation (DiL), "mid" for 101 up to 200 DiL, "late" for ≥ 201 DiL. The groups compared are the control group (n = 30), LR (low responder, n = 4) and HR (high responder, n = 5). Milk yield is specified as energy corrected (ECM). The mapped values, including the SCC, are presented as LSM ± SD in the respective lactation stage. The superscripted letters ($^{a, b}$) characterize significant differences ($P < 0.05$) between groups within the same lactation stage. Asterisks indicate significant differences (*; $P < 0.05$) or highly significant differences (**; $P < 0.01$) over time with regard to the early lactation stage within the same group.

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within the range of 27 kg/d to 32 kg/d. The daily averages of the treated cows' milk production increased significantly by 22% in the mid-lactation of the LR group and by 14% in the late lactation of the HR group, both with regard to the initial 100 DiL. The concentrations of milk fats and proteins of the control group were 1.29 ± 0.1 kg/d and 1.00 ± 0.1 kg/d, respectively, in early lactation. In this lactation stage, these milk components were significantly higher than those of the treated groups by at least 26% for milk fats and 22% for milk proteins. These group differences ceased in the following lactation stages due to the increased ECM yields of both treated groups. Moreover, the percentages of the major milk components did not differ among all examined groups at any time. Regarding the SCC in milk, the threshold of 10^5 SCC/mL was exceeded only by the HR group in the mid-lactation stage (Table 3). Several times during this lactation period, mainly between TW 16 and 20, milk of cow HR-8 contained up to 240,000 SCC/mL, and a peak value of 540,000 SCC/mL was determined in the milk of cow HR-10. In sharp contrast, cows of the LR group had produced milk poor in SCC after the first 100 DiL. Control cows approached the threshold with a mean number of 98,100 SCC/mL in early lactation, because about 40% of the control cows had more than 150,000 SCC/mL during this period.

C. diff. specific and total IgA

The cows in frame for vaccination showed a baseline value (BTC) of 1.7 ± 1.3 μg/mL anti-*C. diff.* IgA in milk at TW 0 (Fig 1), which indicated a natural confrontation with the omnipresent germ *C. diff.*. At this initial point in time, milk samples included *C. diff.* specific IgA up to 5.1 μg/mL, and the HR cows tended to have more milk anti-*C. diff.* IgA than the LR cows (Table 4). *C. diff.* specific as well as total IgA in milk were analyzed to compare them with the three lactation stages of the LR and HR groups (early, mid- and late lactation) in order to assess a possible concentration of the milk IgA during the course of lactation. At this point, the anti-*C. diff.* IgA milk contents were examined with regard to the BTC, among LR and HR in the same lactation stage, and within the same group compared with the early lactation stage (Fig 1). Compared to BTC, the HR cows had approximately 80% more anti-*C. diff.* IgA in milk during any lactation stage, whereas no enrichment of this Ig was found in the milk of the LR group. Accordingly, the amounts of anti-*C. diff.* milk IgA produced by the HR group in each lactation stage were significantly higher than those of the LR group. With a view to the continuing lactation and treatment, an increase of 2.4 ± 1.0 μg/mL anti-*C. diff.* IgA in HR milk was calculated by the SAS/STAT software starting from 8.3 ± 0.7 μg/mL in early lactation and achieving 10.6 ± 0.7 μg/mL in late lactation.

In accordance with the anti-*C. diff.* IgA contents of milk, the same aspects were reconsidered when the total milk IgA contents were evaluated during the three lactation stages (Fig 2). The BTC of total milk IgA was 107.5 ± 35.9 μg/mL, but the total IgA values related to the treated groups differed by nearly the factor of two in TW 0 as indicated in Table 4. This imbalance of the total IgA amounts among milk of HR and LR at the outset of treatment increased by the treatment in the further course of lactation. The HR cows produced an average of 217.8 ± 19.5 μg of total IgA per mL milk in early lactation, two times more than the BTC and roughly one third more than the LR cows secreted during this early lactation period. The total IgA in HR milk increased further by 48% and 72%, respectively, in the subsequent mid- and late lactation stages. An increase of roughly 47% was determined in LR milk only between the early and late lactation periods, peaking at 212.7 ± 21.6 μg/mL. Consequently, this total IgA level in LR cows' milk also deviated significantly compared to the BTC. The calculation of the relative shares of *C. diff.* specific IgA in total milk IgA resulted in 1.6% for the BTC and 1.6% for the LR group in late lactation. The proportion of *C. diff.* specific IgA in total milk IgA more

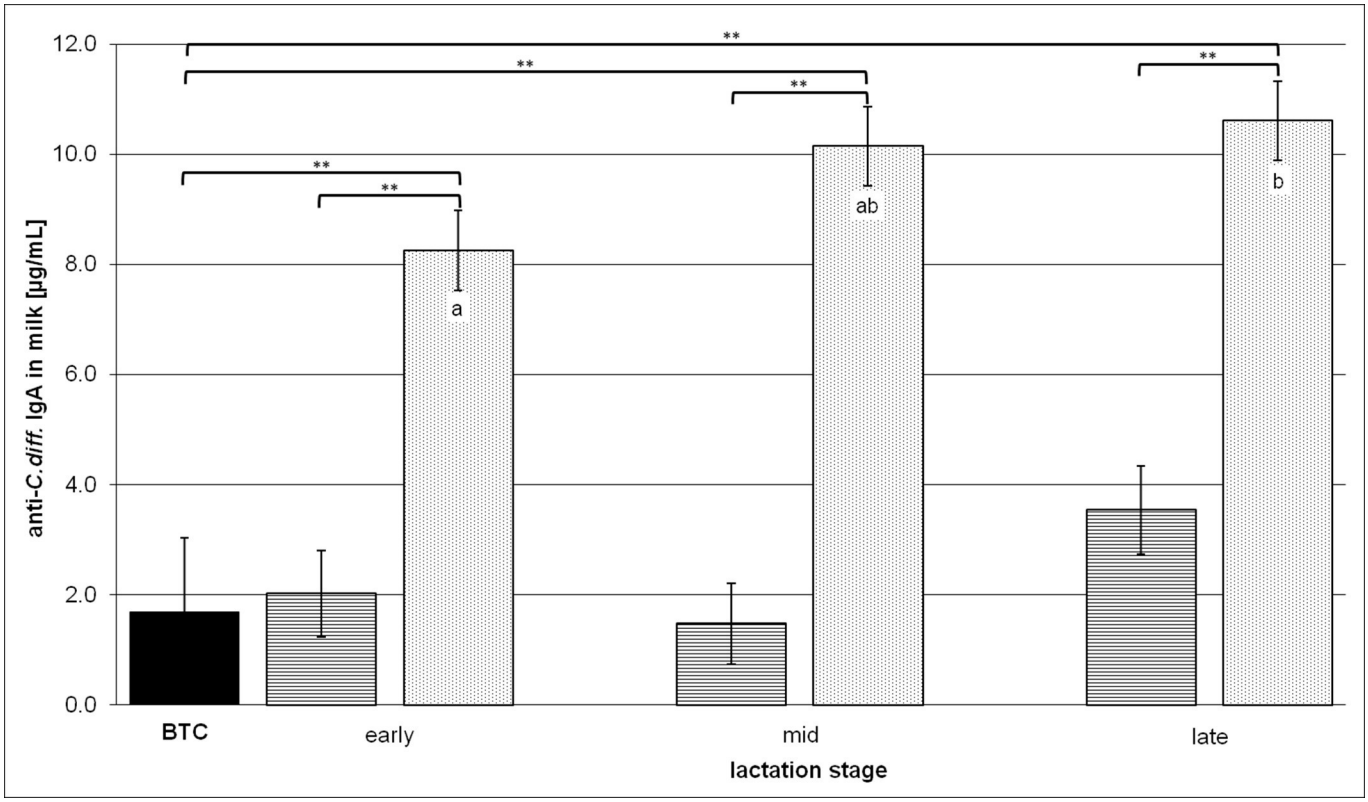

Fig 1. Anti-*C. diff*. milk IgA contents in the different lactation stages. BTC means the "before treatment control" value of all cows in frame for vaccination. The lactation stages are defined as "early" for ≤ 100 DiL, "mid" for 101–200 DiL and "late" for ≥ 201 DiL. On the ordinate, *C. diff*. specific milk IgA concentrations are depicted as LSM ± SD and presented comparatively for the BTC (n = 9, black bar), low responder (LR, n = 4, cross-striped bars) and high responder (HR, n = 5, dotted bars) groups. Substantial or highly significant differences versus BTC and between LR and HR in the same lactation stage are marked by brackets with one ($P < 0.05$) or two asterisks ($P < 0.01$). Significant differences within the same group related to the early lactation stage are shown in lower case letters (a, b) for $P < 0.05$.

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than doubled in the HR cows' early lactation milk by 3.8% towards the BTC, dropping off to roughly 3% during the later lactation stages.

During the treatment, two different *MucoCD-I* vaccine batches, named batch A and B, were tested. Additionally, the well-tolerated vaccine batch B was administered on various routes. To

Table 4. Anti-*C. diff*. and total IgA contents in milk and blood of treated cows when the initial period (TW 0) and prior to the *MucoCD-I* vaccine batch B was used for the first time (TW 16).

Group	TW	anti-*C. diff*. IgA (µg/mL)						total IgA (µg/mL)					
		in milk			in blood			in milk			in blood		
		LSM	*SD*		LSM	*SD*		LSM	*SD*		LSM	*SD*	
LR	**0**	0.0	*1.6*	* (TW 4)	3.6	*2.4*	** (TW 6,7)	73.1	*46.2*	** (TW 10)	119.2	*70.8*	** (TW 3)
HR	**0**	3.8	*2.3*	* (TW 3, 4) ** (TW 5)	6.7	*3.4*		133.6	*65.3*	** (TW 4) * (TW 14)	257.8	*100.2*	** (TW 3, 4)
LR	**16**	1.2	*1.6*	a	3.6	*2.4*		128.6	*46.2*		138.0	*70.8*	
HR	**16**	8.1	*1.5*	b ** (TW 18) * (TW 20)	7.8	*2.1*	* (TW 18)	274.7	*41.3*	** (TW 20, 27)	223.2	*63.4*	

All IgA data are displayed as LSM ± SD group-related to LR (low responder, n = 4) and HR (high responder, n = 5) in different treatment weeks (TW). The superscripted letters ($^{a, b}$) indicate significant differences ($P < 0.05$) between HR and LR group within the same TW. Asterisks indicate significant differences between defined points in time towards TW 0 or TW 16 with $P < 0.05$ (*) or $P < 0.01$ (**).

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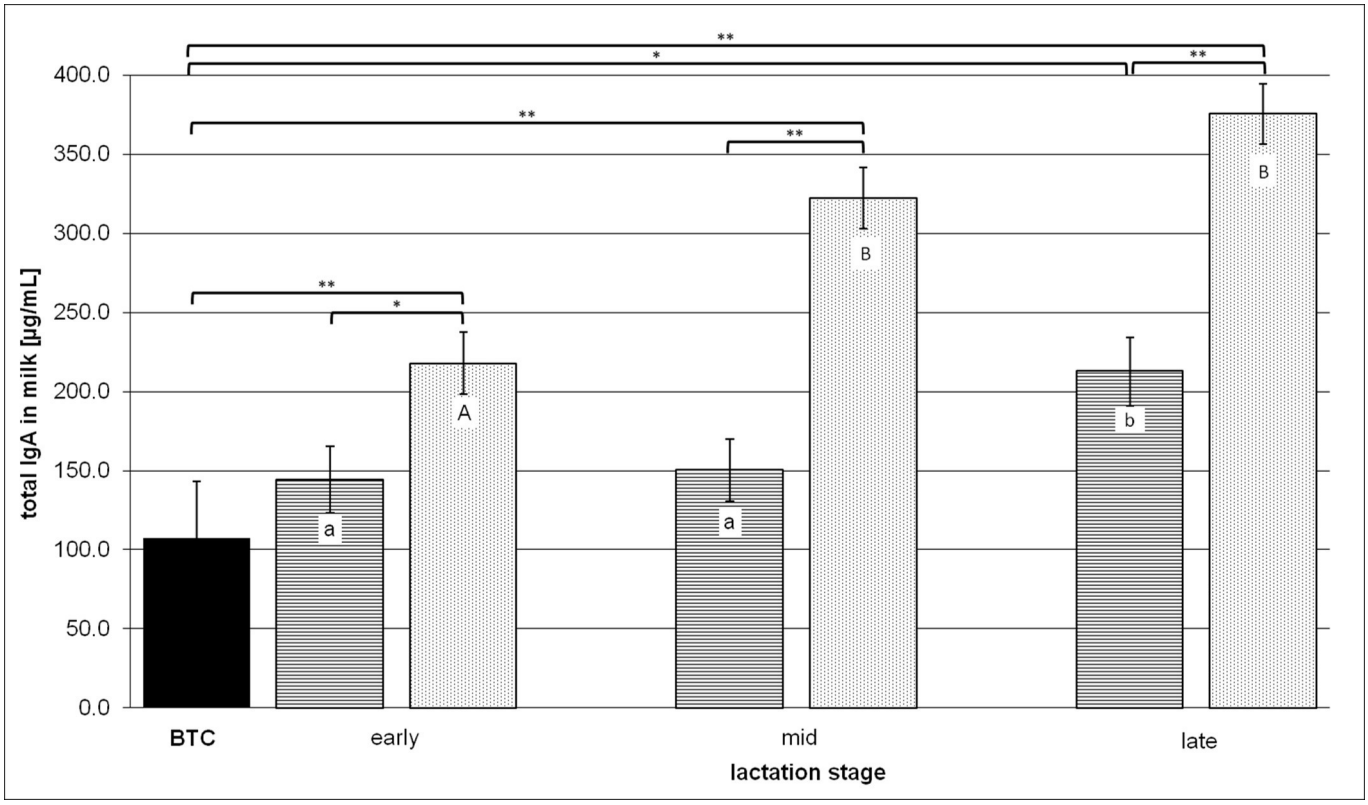

Fig 2. Total milk IgA contents in the different lactation stages. BTC means the "before treatment control" value of all cows in frame for vaccination. The lactation stages are defined as "early" for ≤ 100 DiL, "mid" for 101–200 DiL, "late" for ≥ 201 DiL. The ordinate values represent comparatively LSM ± SD of the BTC (n = 9, black bar), low responder (LR, n = 4, cross-striped bars) and high responder (HR, n = 5, dotted bars) groups. Brackets with asterisks link treated groups with BTC and during the same lactation stage, if significant differences between them are indicated by $P < 0.05$ (*) or $P < 0.01$ (**). Significant differences within the same group related to the early lactation stage are indicated by lower case letters (a, b) in case of $P < 0.05$ and upper case letters (A, B) for $P < 0.01$.

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evaluate in detail the *C. diff.* specific and total IgA concentrations in milk and blood possibly changed by the treatment, both parameters were analyzed close-meshed in these body fluids of the treated animals (Figs 3 and 4). Table 4 includes the initial amounts of the measured Igs before the treatment started. Obviously, regarding the investigated antibodies, no significant differences were determined in each body fluid between LR and HR cows at this point in time (TW 0). In terms of *C. diff.* specific IgA production in milk, a radical increase resulted from the vaccine batch A given once PC for both treated groups. The value for the HR group initially measured more than tripled in TWs 3 and 4. With approximately 5.0 µg/mL *C. diff.* specific IgA in milk, the LR group achieved a substantially different level against TW 0 in TW 4. Following the vaccination in TW 3, all subsequent analysis of the *C. diff.* specific IgA in milk showed significant differences between HR and LR. They flattened during the pure N application of vaccine, but increased again after IM with vaccine batch B in TW 16 due to the strong responsiveness of the HR cows. Their *C. diff.* specific IgA production peaked at 15.1 ± 1.5 µg/mL in milk in TW 18. This top value and the following in TW 20 were substantially higher than the measured values that were found during the previously N IM period. The *C. diff.* specific IgA level in HR milk caused by the first PC injection of vaccine batch B could not be maintained. No later than in TW 23, the level reached was at least 30% lower, and then maintained until the end of treatment, unaffected by other administration routes for the vaccine. The *C. diff.* specific IgA contents of the HR group monitored in the blood showed a similar development. Compared with the measured values in HR milk, the specific antibody content

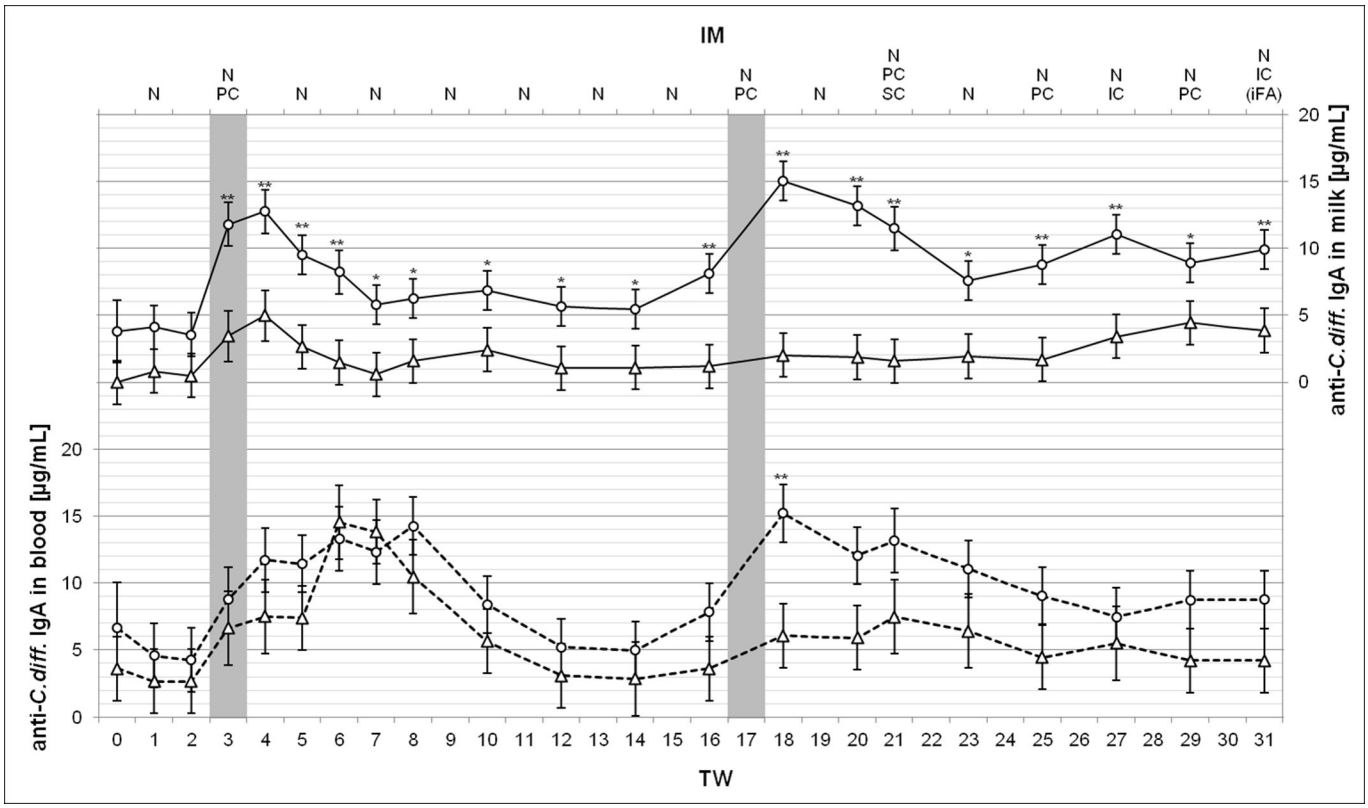

Fig 3. Anti-*C. diff*. IgA concentrations in milk and in blood during the 31-week treatment period. The ordinate values in milk (solid lines, top) and in blood (broken lines, bottom) are shown as LSM ± SD per treated groups, the low responder (LR, n = 4, triangles) and the high responder (HR, n = 5, circles). Substantial or highly significant differences between both groups at the same point in time are marked by one ($P < 0.05$) or two asterisks ($P < 0.01$). The gray bars indicate the one-time application of *MucoCD-I* vaccine batch A in TW 3 and the first use of vaccine batch B for injection in TW 17. The vaccination routes were nasal (N), percutaneous (PC), subcutaneous (SC) and intracutaneous (IC). The injected *MucoCD-I* vaccine was uniquely supplemented with incomplete Freund's adjuvant (iFA).

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of blood increased more slowly after the PC administered vaccine batch A. However, the time period with more than 10 μg/mL *C. diff*. specific IgA in blood was prolonged and ended with the highest value of 14.3 ± 2.1 μg/mL in TW 8. The vaccine batch B given helped to boost this antibody once again to 15.2 ± 2.1 μg/mL in TW 18. Only then did the *C. diff*. specific IgA level in HR blood differ from that in LR blood. During the remaining treatment period, a slight decrease to the primary level in HR blood was noticed. The *C. diff*. specific IgA level in LR blood tended to be lower than that of its counterpart, apart from TWs 6 and 7. Only in these TWs, the measured values of approximately 15 μg/mL *C. diff*. specific IgA in LR blood were significantly higher than the initial values, reaching 3.6 ± 2.4 μg/mL (Table 4). The use of vaccine batch B showed no similar effects.

The analysis of total IgA concentrations in milk (Fig 4) revealed results similar to *C. diff*. specific IgA concentrations regarding their development over the complete treatment period. The HR group data showed once again a rapid increase after injecting vaccine batch A in TW 3, a slowdown during the pure N IM, and a renewed increase after vaccine batch B was administered. The first IC injection of vaccine batch B in TW 27 induced increased total IgA in milk, which was on the same level, as measured in TWs 20 and 21. With about 400 μg/mL, the HR cows produced three times more than when treatment started. Regarding the dates of the top values determined in HR milk, the differences with regard to the LR group were significant when comparing the total IgA milk contents. The range of total IgA in LR milk was between

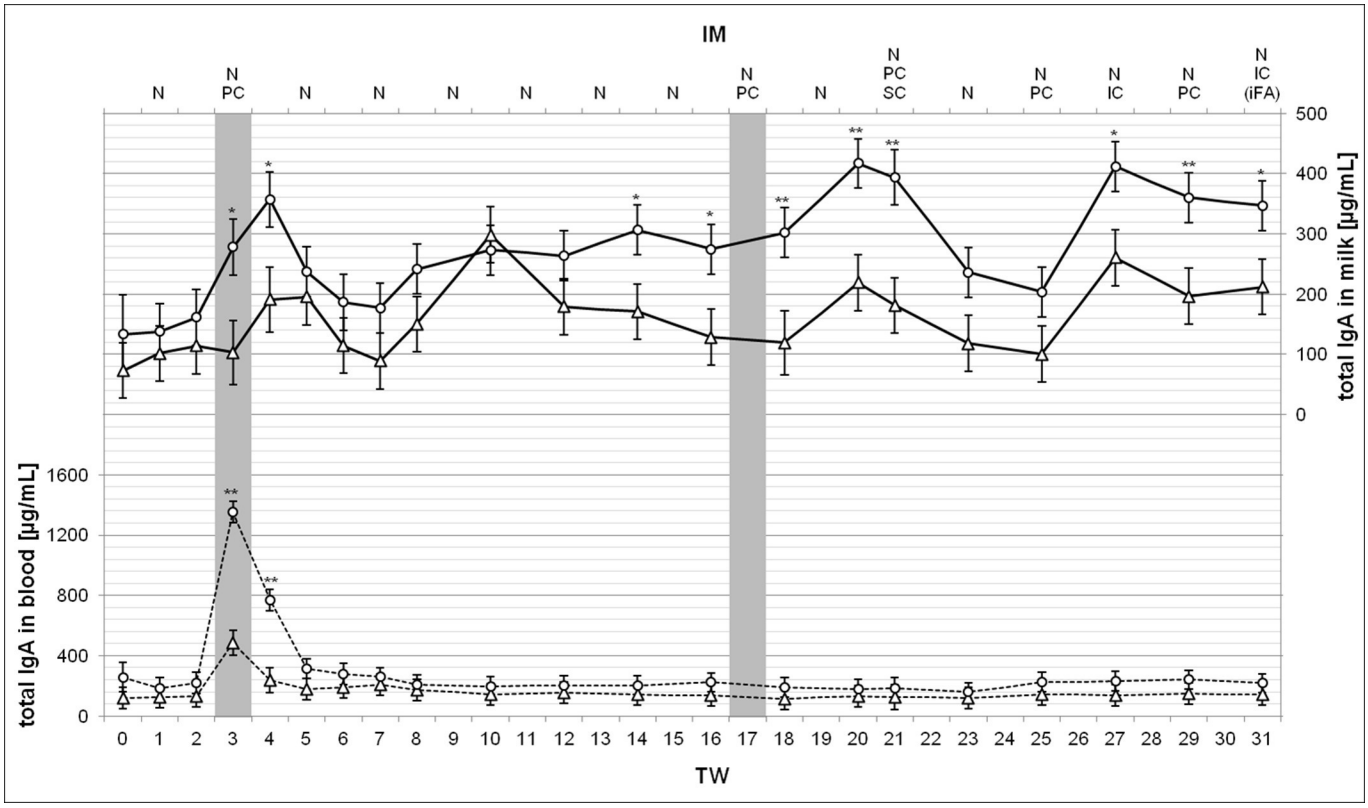

Fig 4. Total IgA concentrations in milk and in blood during 31-weeks treatment period. Measured total IgA values in milk (solid lines, top) and in blood (broken lines, bottom) are shown as LSM ± SD per treated groups, the low responder (LR, n = 4, triangles) and the high responder (HR, n = 5, circles), on the ordinate. Substantial or highly significant differences between both groups compared to the same point in time are marked by one ($P < 0.05$) or two asterisks ($P < 0.01$). The gray bars indicate the one-time application of *MucoCD-I* vaccine batch A in TW 3 and the first use of vaccine batch B for injection in TW 17. The vaccination routes were nasal (N), PC, SC and IC. The injected *MucoCD-I* vaccine was uniquely supplemented with iFA.

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70 and 360 µg/mL. Overall, total IgA secretion by LR seemed to be unaffected by the treatment. The cause of the outlier value recorded in TW 10 remained unknown. Except for the effect of vaccine batch A on HR cows, the total IgA concentrations of HR and LR did not differ in the blood. Consequently, the blood serum contents of both groups for total IgA clearly peaked only in TWs 3 and 4 even though the LR blood contained four times more total IgA, whereas the HR cows reached a fivefold increase.

Correlation analysis

The calculated Pearson correlation coefficients were classified as follows: $I\,r\,I < 0.5$ as "weak", $0.5 \leq I\,r\,I < 0.8$ as "middle" and $I\,r\,I \geq 0.8$ as "strong" relations between the examined variable pairs. Apart from the milk yield, all other variables summarized in Table 5 showed positive linear dependency in milk referring to *C. diff.* specific and total IgA. Primarily, weak relations were determined between the pairs of variables that were the subject of the research. Nevertheless, total IgA concentrations tended to be stronger depending on some factors like duration of lactation (marked by DiL) and major milk components, milk proteins and fats, than the *C. diff.* specific IgA content of milk. This difference in the specific and total Igs dependency on the above-mentioned dairy production suggests that these factors could be caused by the vaccination schedule, which aimed at the enrichment of *C. diff.* specific IgA in milk. Additionally, total IgA content was more affected by the natural concentration of milk components

Table 5. Correlations between anti-*C. diff*., total IgA and the main production factors in milk of treated cows (n = 9).

(n = 165)	anti-*C. diff*. IgA (μg/mL)		total IgA (μg/mL)	
	r	**P**	***r***	**P**
DiL	*0.19*	< 0.05	*0.37*	< 0.01
ECM yield (kg/d)	*-0.25*	< 0.01	*-0.30*	< 0.01
milk protein (kg/d)	*0.27*	< 0.01	*0.47*	< 0.01
milk fat (kg/d)	*0.30*	< 0.01	*0.44*	< 0.01
SCC (x1,000/mL)	*0.19*	< 0.05	*0.21*	< 0.01
total IgA (μg/mL)	*0.69*	< 0.01	-	-

"*r*" indicates the Pearson product moment correlation coefficient. Significant correlations between the investigated variables must be proven by $P < 0.05$ and highly significant correlations are assessed with $P < 0.01$. Positive *r* displays pairs of variables tending to increase linearly together. For the pairs with negative *r*, one variable tends to develop in an inversely proportional way. Abbreviations used for some variables are: *DiL* for days in lactation, *ECM yield* for energy corrected milk yield, and *SCC* for somatic cell counts. The evaluated number of samples (n) was 165.

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in the late lactation stage. Due to the correlation analysis, the relationship between *C. diff*. specific IgA and total IgA contents could only be classified as moderate. Compared with the weaker dependencies on the other variables, this result was not surprising because *C. diff*. specific IgA presented some of the total IgA as well.

Discussion

The present study aimed to achieve the enrichment of *C. diff*. specific antibodies, preferably sIgA in mature cow's milk, based on the similar vaccination schedule that has proven to be effective earlier [15]. The different vaccines used for IM against *C. diff*. were well tolerated by the treated cows apart from the *MucoCD-I* vaccine batch A, when their monitored health was taken into account. *MucoCD-I* vaccine batch A contained excessive amounts of TcdA and TcdB as a vaccine production-related fault. Its one-time administration implicated pro-inflammatory effects in the treated cows due to the remaining biological activity of these potent exotoxins. Consequently, several vaccinated animals fell ill in TW 3, and the administration of antigen via injection had to be discontinued until a newly composed *MucoCD-I* vaccine was available. To maintain the stimulation of the treated cows' IS with *C. diff*., the bi-weekly N IM was steadily carried out assuming the concept of the "common mucosal IS" [25]. Thus, the IM of the NALT against *C. diff*. should also evoke a humoral immune response in the mammary gland. Nevertheless, the systemic IM on parenteral routes was pursued with *MucoCD-I* vaccine batch B to trigger a cumulative antibody response in peripheral lymphoid tissues. Following treatment with *MucoCD-I* batch B when PC was given for the first time, a new dynamic in production of *C. diff*. specific IgA was achieved as measured, especially, in the milk and blood of the HR group (Fig 3). However, the specific antibody levels within the range of 15 μg per mL milk were unachievable, neither on the SC nor on the IC route. The humoral immune response of the treated animals was also evaluated depending on the indirect measurement of serum IgA. This approach is known to be problematic because it does "not necessarily reflect the effector response at the mucosal surface" [26]. Even though the treatment influenced the development of the anti-*C. diff*. IgA concentrations in blood serum, the mucosal immune response should not assessed solely with the specific antibodies, considering the LR immune response during the pure N treatment period.

Regarding treatment effects on the milk yield and its composition, the *MucoCD-I* vaccine batch A applied in TW 3 caused a reduced daily milk output, including the total quantities of milk fats and proteins within early lactation (Table 3). Due to the harmful effect of this vaccine, the treated cows' general condition was impaired and their milk performance reduced. Health and performance are closely interrelated throughout lactation [27]. Apart from *MucoCD-I* vaccine batch A given in TW 3, all other anti-*C. diff.* vaccinations in this study had no impact on any cow health parameter, milk production and macronutrient composition. These findings were in accordance with study results of Gingerich et al. (2008), exploring the safety of milk ingredients from hyperimmunized cows with a multivalent bacterin (S100) [28]. In the study referred to, the specificity but not the total amount of the evaluated milk antibodies was affected by the S100 treatment [28]. Another *C. diff.* IM study showed significantly increased antibody concentrations against *C. diff.* and its exotoxins in raw milk [29]. In contrast to the present study, Young et al. (2007) did not release the development of the antigen-specific sIgA production during the IM period. Their mean value disclosed for anti-*C. diff.* sIgA in raw milk was 16 μg/mL on average [29]. Hereinafter, it was used as reference for discussing the outcomes, as shown in Figs 1 and 3, because the IM procedure applied against *C. diff.* was similar, and the ELISA was also used as measuring method to ascertain the specific Ig milk contents. The reference value for *C. diff.* specific sIgA revealed by Young et al. (2007) was comparatively higher than 10 to 11 μg/mL of the specific milk antibody produced by the HR group during mid- and late lactation (Fig 1). The interrupted vaccination on parenteral routes following TW 3 may have led to this minor amount. However, the following facts should be respected when making that assessment. Firstly, the individual immune responsiveness of the treated animals varied dramatically as seen, for example, in the two extremes in the last two thirds of lactation: HR-cow 8 produced on average 15 μg/mL of *C. diff.* specific sIgA, whereas LR-cow 2 did not even reach 1 μg/mL. Secondly, the dairy cow breed *Brown Swiss* used for immune milk production could basically be less sensitive to immune challenges than other breeds due to stronger innate immune defense mechanisms. For instance, the *Holstein Frisian* with a history of selection for milk yield at the expense of disease resistance [30] may trigger a more powerful humoral immune response. In principle, colostral Ig concentrations were proven to be as variable between dairy breeds [31]. Likewise, such variations may be present in mature milk due to the genetic differences of the investigated breeds. Thirdly, the age or the number of elapsed lactations shape the immunological status of mammalians, as measured exemplarily by amounts of bovine colostral Igs [32]. Finally, the vaccine used for triggering the immune response probably had a different composition, e.g. included adjuvants. Neither van Dissel et al. (2005) nor Young et al. (2007) disclosed the breed of the immunized dairy cattle and the vaccine composition that was used [15, 29]. In contrast to these previous IM studies, the study at hand highlights the achievable quantities of anti-*C. diff.* IgA in bovine milk during the progressing lactation following sustained immune stimulation of dairy cows. The potential output is important knowledge for every dairy producer intending to commercialize the anti-*C. diff.* IgA production in bovine milk as a dietary supplement for human health. The necessarily qualitative assessment of the anti-*C. diff.* IgA produced in milk is still pending.

No direct comparison could be made between the total milk IgA measurements presented and the same ones obtained by Young et al. (2007) due to different analytical approaches [29]. As depicted in Fig 2, the vaccinated animals classified by their different immune responsiveness showed a sharp contrast between the group-related total IgA contents. That was not expected as resulting from the *C. diff.* specific IM, considering that the secreted *C. diff.* specific IgA was only slightly less than 5% of total IgA in milk. Cross-reactions in antibody production of various sizes may have contributed to different increases of total IgA when LR and HR are compared. In addition, fundamental differences between the number of APCs in mammary

tissues or the mammary epithelial placements with the IgA transport mediating specific receptors (pIgR) might be responsible for the varying immune responsiveness of the treated cows. The concentrations of all milk Igs were shown to increase during late lactation, which coincides with a major reduction in milk yield [33]. This result parallels the weak inverse relation between anti-*C. diff*. or total IgA and milk yield as calculated in the present study (Table 5), whereas the correlation analysis of all other production factors investigated and anti-*C. diff*. or total milk IgA revealed a slightly positive interdependency. For example, the SCC was found to be a factor with strong significant correlation to total milk IgA concentration [34]. An elevated SCC is often accompanied by increased proportions of a few whey proteins, including Igs [35]. Furthermore, milk IgA concentrations are obviously associated with the stage of lactation [34] that was reproduced by the examined correlation to DiL. The dependency could only be described as weak, presumably due to the sample size that was considerably smaller in the available study as opposed to the comparative study [34]. Given the fact that total IgA also covers anti-*C. diff*. IgA and that this whey protein is, in turn, part of total milk proteins, the closely commutated linkages of these variables could be verified by the results of the correlation analysis (Table 5). Phenotypic correlations among milk constituents are known to be strongly positive [36], regardless of the significant differences between their proportions among breeds and parities [37]. Accordingly, anti-*C. diff*. and total milk IgA as elements of total milk protein content are also related to the daily milk fat amount. Notwithstanding the close knitted relation of anti-*C. diff*. to total milk IgA ($r = 0.69$), the correlations between the *C. diff*. specific IgA and the production factors were generally weaker than the findings for total IgA.

Conclusion

To conclude, a lasting *C. diff*. specific IgA enrichment in milk can be achieved by continuous IM of dairy cows based on two conditions. Firstly, a potent vaccine including the pivotal virulent factors of *C. diff*. is crucial. However, a poorly effective vaccine cannot be compensated by the IM procedure, which is carried out to induce a more mucosa-related immune response. Secondly, dairy cows sensitive towards *C. diff*. should be preselected as measured by their *C. diff*. specific milk IgA. Factors possibly causing different immune responsiveness might be intramammary variations given the prevalence of immune cells like APCs, differences between intercellular signaling following the IM or differences between the epithelial IgA transport capacities depending on the pIgR. These topics will be addressed in a further publication.

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RESEARCH

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Immune cell counts and signaling in body fluids of cows vaccinated against *Clostridium difficile*

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Abstract

Background: New treatment options are needed to prevent relapses following failed antibiotic therapies of *Clostridium difficile* infections (CDI) in humans. The concomitant therapy with an anti-*C. difficile* IgA containing whey protein concentrate can support the sustainable recovery of CDI patients. For 31 weeks, nine dairy cows were continuously vaccinated with several anti-*C. difficile* vaccines by certain routes of administration to produce anti-*C. difficile* IgA enriched milk. The study aimed at finding decisive differences between low responder (LR) and high responder (HR) cows (> 8.0 µg ml^{-1} total milk *C. difficile* specific IgA) concerning their immune response to vaccination on cellular and molecular biological levels.

Results: The results of total and differential cell counting (DCC) in blood and milk and the outcomes of the gene expression analysis of selected immune factors were assessed relating to the usage of two vaccine batches for injection (*MucoCD-I* batch A and B), marking two immunization (IM) periods, and compared to a control group (Ctr). The *MucoCD-I* batch A caused short-term leukopenia followed by leukocytosis in the blood of LR and HR. The total somatic cell counts in milk were not altered by the treatment. The DCC revealed that the leukocytes of the treated groups were partly impaired by the treatment. The gene expression analysis exposed cumulative and sustainable differences ($p < 0.05$) between LR and HR for the genes encoding for *lactoferrin*, *CXCL8*, *IL1β*, *IL2*, *IL6*, *IL12β*, *IFNγ*, *CD4* and *CD163*. The regulation of the epithelial IgA cell receptor *PIGR* was not impaired by the IM. In contrast to the vaccination with *MucoCD-I* batch A, the second IM period with *MucoCD-I* batch B resulted in mitigation and synchronization of the treated groups' immune responses.

Conclusions: The inversely regulated cytokines in the blood and milk cells of the treated groups led to a variously directed, local T cell response resulting in their different production intensities of *C. difficile* specific IgA in milk.

Keywords: Gene expression, Cytokines, Dairy cattle, Immunization, *Clostridium difficile*

Background

Clostridium difficile is a gram-positive, endospore-forming and fastidious anaerobe bacterium [1]. The majority of *C. difficile* isolates is toxigenic and produces two large exotoxins, known as *C. difficile* toxin A (TcdA) and toxin B (TcdB), with enterotoxic and cytotoxic activities. In some epidemic strains (such as the ribotypes 078 and 027) a third toxin, the binary toxin *C. difficile* transferase, is related to germ virulence [2, 3]. *Clostridium difficile* endospores are widely disseminated in the environment, although the primary habitat of the vegetative form is the gastrointestinal tract of humans and animals [4]. An interference of the microbiome in the gut, for instance due to the intake of antibiotics, an impaired mucosal barrier, as symptomatic of inflammatory bowel disease (IBD), and, generally, a weakened immune system (IS) are the prominent risk factors of susceptibility to *C. difficile* infection (CDI) [2, 5]. In 2015, the U.S. Centers for Disease Control and Prevention (CDC) classified *C. difficile* as an urgent antibiotic resistance threat [6]. CDI is deemed to be the leading cause of health care-associated

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diarrhea, but according to the latest whole genome sequencing studies, CDI is mainly community-acquired and may have a foodborne or zoonotic etiology [1, 7, 8]. Serious and fatal progressions of this disease have been increasing for the last two decades [9]. The risk to relapse or to re-infect after the first finalized episode of CDI is in the range of 20 to 25% and continues to grow with every further episode [10, 11]. The best practice advices for managing the CDI, particularly in IBD, comprise treatment with vancomycin or fidaxomicin instead of metronidazole. To recover the colonization resistance, fecal microbiota transplantation should be recommended to patients with recurrent CDI [12, 13]. Another way to reduce recurrences is additional passive immunization of CDI patients. Anti-*C. difficile* specific antibodies directed against *C. difficile* main virulence factors, TcdA and TcdB, were concomitantly applied to antibiotic therapy and proven effective to prevent renewed *C. difficile* associated disease [1, 14, 15]. Therefore, a whey protein concentrate enriched with anti-*C. difficile* specific antibodies is an appropriate dosage form [16–18]. The targeted concentration of anti-*C. difficile* specific antibodies in bovine milk requires a continuous vaccination of dairy cows against the characteristic features of the microbe. Following vaccination, a complex set of interacting factors governs the development of systemic and secretory immune responses in vivo [19]. Exogenous factors include the characteristics of the vaccine, the route of administration and the possibly utilized immunologic vehicles for vaccine delivery such as adjuvants. Furthermore, environmental factors, psychological stress, nutrition and infectious diseases may have an impact on the vaccination response, whereas genetic factors, age and sex determine the intrinsic capacity of the recipients to respond to a vaccine [20]. Hence, the outcome of their immune reaction to the same immunological stimulus may vary fundamentally. Given this fact, high responder (HR) and low responder (LR) cows were identified, as measured by their production of anti-*C. difficile* specific antibodies in milk [21]. In the study at hand, the source of different immunological reactivity of the treated cows was examined within selected endogenous factors, as the exogenous factors influencing an active immunization were standardized by the experimental setup. In any case, vaccines are antigens and the in vivo challenge with an antigen causes a cellular reaction and leads ideally to a humoral response of the IS. The first encounter with the antigen provokes the innate IS, which includes components like the complement system or antigen-presenting cells (APCs). The latter activate the second arm of the IS, the adaptive IS, which is associated with lymphocytes as effector cells, antibody production and immunological memory [22]. The repeated exposure to the same antigen heightens these lymphocyte functions [23]. In the current study, the activation of the cellular stimulation by vaccination was assessed in blood and milk by the use of total and differential cell counting (DCC) as diagnostic tool for progress monitoring [24, 25]. A more fine-tuned cause analysis for the determined different responsiveness of the treated cows was exemplarily achieved by molecular genetic analysis to verify the degree of cellular activity by the expression of genes coding for specific surface determinants and for typical chemokines [26–30].

Results

Clostridium difficile specific IgA in milk

The full description of the *C. difficile* specific IgA contents in milk and their development over the first 31 treatment weeks (TWs) was disclosed by Schmautz et al. [21]. In summary, all cows designated for immunization (IM) showed a basic level of less than 2 µg of anti-*C. difficile* IgA per milliliter milk on average owing to the natural dissemination of *C. difficile*. The repeated IM challenged the IS of the treated cows, thereby triggering the production and release of specific antibodies in milk, *inter alia*. Since the extent of anti-*C. difficile* IgA enrichment in milk differed significantly between the immunized cows, they were classified into "low responder" (LR) and "high responder" (HR) by a threshold of 8.0 µg ml^{-1} *C. difficile* specific IgA in milk, as published by Schmautz et al. [21]. On completion of their above-mentioned 31-week treatment period, LR cows produced ordinarily 3.1 ± 0.3 µg and HR cows 9.6 ± 0.3 µg of anti-*C. difficile* IgA per milliliter milk, respectively. The initial applications of the two tested vaccines for injection, *MucoCD-I* batches A and B, HR accomplished peak values of roughly 15 µg anti-*C. difficile* IgA per milliliter milk, whereas LR never exceeded 5 µg ml^{-1}.

Gene expression data

In order to assess possible gene regulatory effects by IM in PBL and SCC, the gene expressions of different cell type marking receptors as well as immunologically relevant factors like chemokines were analyzed. A more detailed description of all analyzed genes is shown in Additional file 1: Table S1. The relative changes in gene expression were determined in relation to the baselines before the first-time application of the *MucoCD-I* vaccine batches [TW 0 (for PBL and SCC) and TW 15 (only for SCC) or TW 16 (only for PBL)]. The delta values, representing the gene expression changes measured in PBL and SCC, are accessible in Additional file 2: Table S2 and Additional file 3: Table S3, respectively. Table 1 shows the grading system being applied for the gene expression data.

Table 1 Classification of the gene regulation expressed as ΔΔCq range

Defined grades of gene regulation		ΔΔCq range
0	Non-regulated	$\lvert < 1 \rvert$
+	Weak	$\lvert \geq 1 \rvert$ up to $\lvert < 3 \rvert$
++	Strong	$\lvert \geq 3 \rvert$ up to $\lvert < 5 \rvert$
+++	Very strong	$\lvert \geq 5 \rvert$

The grades of gene regulation are given as follows: "0" for non-regulated, "+" for weakly regulated, "++" for strongly regulated, "+++" for very strongly regulated

In favor of a structured compilation, the determined gene regulations were classified depending on their scale of the ΔΔCq range during the 31 TWs. The TW, marking the maximal significant up- or down-regulation of the investigated gene expression in relation to the belonging baseline, decided about the affiliation to one of the four grades of gene regulation (0, +, ++, +++). The group (HR, LR, control), in which the maximal change of gene expression was determined, did not matter for the classification. Table 2 summarizes the analyzed genes in PBL and SCC plus the information described above according to their cell type related affiliation.

Phagocyte-related genes in PBL

The investigated receptors of phagocytes (*C3AR1*, *C5AR1*, *CXCR2*, *TLR2* and *CD163*) were regulated on a weak level in PBL. During the first IM period up to TW 14, only the complement receptor *C3AR1* in PBL of the LR was found up-regulated within the group and to the control group (Ctr) in TW 8. Following treatment with vaccine batch B, the HR showed a steadily increased *C3AR1* expression in PBL with significant differences to the Ctr on four dates, and additionally, to the LR in TW 23. *C5AR1*, the counterpart of the complement fragment C5a, was down-regulated in PBL of all treated cows directly after the injection of vaccine batch A. After the LR group *C5AR1* expression turned upward, it exceeded that of the HR group by achieving the ΔΔCq value of 1.7 in TW 10. Furthermore, the *C5AR1* expression differed between the treated groups in TW 14. At the second IM period, the expressions of *C5AR1* developed similarly toward its down-regulation by all treated cows from TW 21, and therefore the *C5AR1* transcript amount of HR was significantly lower than that of the Ctr in TW 23. The expression of the PMN specific chemokine receptor *CXCR2* was lastingly diminished in the treated groups after the application of *MucoCD-I* vaccine batch A. The treatment with vaccine batch B led to the enhanced transcription of *CXCR2* in HR compared to the LR in TW 18 and to the Ctr group in TW 20. In association with the vaccine batch A, the toll-like receptor 2 (*TLR2*) was down-regulated by half in the PBL of the treated groups in TW 4. Comparing HR and LR, *TLR2* was significantly down-regulated in the HR group in TW 8. During the second IM period, both treated groups exhibited an identical up-regulation of the *TLR2* transcription compared to the Ctr. The monocytes/macrophages specific surface determinant *CD163* was found significantly up-regulated in the LR compared to the HR (TW 2). Similar declines of *CD163* transcripts were determined in PBL of HR and LR in TW 4, the result of the previously given vaccine batch A on the PC route. Afterwards, the *CD163* expression by the HR PBL was mainly reduced up to TW 14 and it differed markedly from that in the LR PBL in TWs 8, 10 and 14. The second treatment period did not cause any alterations of the *CD163* expressions. The genes coding for the phagocyte-related cytokines, *CXCL8* and *TNFα*, were graded as weakly regulated in PBL. Following vaccination with the *MucoCD-I* batch A, *CXCL8* was more up-regulated in the PBL of HR than of LR, and its expression varied significantly between both treated groups at the TWs 5–7, 10, 12 and 14. The increased transcription of *CXCL8* by the Ctr, contrary to the lower one by the LR, led to clear differences between both groups in the TWs 10 and 12. As from TW 16, the second IM period was marked by reduced amounts of *CXCL8* transcripts, up to one-fifth compared to the baseline value in PBL of the Ctr group. Consequently, considering the scarcely altered *CXCL8* expression levels of the treated groups in the same time period, many times of investigation had significant discrepancies between them and the Ctr group. The amounts of *TNFα* transcripts were not impaired in any group during the first vaccination period, whereas *TNFα* was slightly up-regulated within all groups at various TWs during the use of vaccine batch B. Different *TNFα* expressions between HR and LR were not measured, but those of the treated groups were significantly up-regulated toward the Ctr group *TNFα* expression in TW 27. The investigated interleukins, *IL1β* and *IL12 β,* were proven as strongly regulated, and especially, the expression of *IL6* was very strongly affected by the IM. Within the HR group, a down-regulation of *IL1β* in PBL proceeded during the first 2 months of treatment. In relation to the baseline, the lowest point of *IL1β* expression was reached with remaining six percent of *IL1β* transcripts in TW 8. In contrast to the HR, the LR group's *IL1β* expression was depressed to a lesser extent in PBL during the same time period. Therefore, differently pronounced down-regulations of *IL1β* were proven between them in TWs 1, 3 and 8. Comparing the *IL1β* gene regulations between the treated cows and the Ctr cows, considerable differences were revealed at numerous measurement points up to TW 14. In the ensuing second treatment period, the *IL1β* transcriptions of HR

Table 2 Spectrum of genes analyzed in PBL and in SCC out of milk and the classification of their regulation

Genes	Performed gene expression analysis		Classification of the gene regulation	
	In PBL	In SCC	In PBL	In SCC
bMEC′-related genes				
KRT8	No	Yes	/	+
PIGR[a]	No	Yes	/	++
FcRN[a]	No	Yes	/	0
C1QA[a]	No	Yes	/	0
C3[a]	No	Yes	/	+
CCL5[a]	No	Yes	/	+
CCL20[a]	No	yes	/	++
CCL28[a]	No	Yes	/	+
CXCL3[a]	No	Yes	/	+
CXCL5[a]	No	Yes	/	0
CXCL8[a]	No	Yes	/	+
LPO[a]	No	Yes	/	++
LF[a]	No	Yes	/	+
LYZ1[a]	No	Yes	/	+
TAP[a]	No	Yes	/	+
Phagocyte-related genes				
C3AR1	Yes	Yes	+	+
C5AR1	Yes	Yes	+	++
CXCR2	Yes	Yes	+	+
TLR2	Yes	Yes	+	+
CD163	Yes	Yes	+	0
CXCL8	Yes	See above	+	See above
TNFα	Yes	Yes	+	++
IL1β	Yes	Yes	++	+
IL6	Yes	Yes	+++	+
IL12β	Yes	Yes	++	+
Lymphocyte-related genes				
CD3δ	Yes	Yes	+++	+
CD4	Yes	Yes	++	++
CD8β	Yes	Yes	++	+
CD19	Yes	Yes	+	+
CD38	No	Yes	/	+
CD126	Yes	Yes	+++	+
CCR6	No	Yes	/	+
IFNγ	Yes	Yes	+	+
TGFβ1	Yes	No	+	/
IL2	Yes	No	+++	/
IL10	Yes	Yes	+	++

PBL peripheral blood leukocytes, *SCC* somatic cell count, *bMEC* bovine epithelial cells

[a] characterizes the bMEC-related genes, which were additionally normalized with KRT8 as the epithelial cell marker; the sign / means that the gene expression was not analyzed in the study objects, PBL or SCC

and LR were down-regulated only once in TW 20 and TW 21, respectively. On these dates, the measurement data of both treated groups deviated from each other and from the Ctr group, too. The transcription of *IL6* varied between the HR and LR in PBL in TWs 8 and 10 due to a partly weak *IL6* down-regulation in HR PBL. At the end of this IM period, the relative quantity of *IL6* transcripts of both treated groups undercut that of the Ctr group. In contrast to the first IM period, treatment with vaccine batch B evoked at least strong up-regulations of *IL6* in all treated cows, which were very strongly pronounced in the LR in TW 27. To this date and subsequent to the first PC injection of vaccine batch B, the *IL6* expression by the LR PBL surpassed clearly that of the HR. The courses of *IL12β* expressions were generally similar to the treated groups during both IM periods. Nevertheless, the HR and LR expressed *IL12β* in PBL at distinguishable levels during the first IM period, where the relative amounts of *IL12β* transcripts in LR PBL were larger than that in HR PBL at the TWs 1–3, 8 and 10. The LR showed a weak up-regulation of *IL12β* up to the application of *MucoCD-I* batch A, which was followed by a slight down-regulation only in TW 4, and a strong up-regulation of *IL12β* developed after TW 7. From TW 21, the expressions of *IL12β* in PBL of all treated cows were reduced to more than a quarter in the remaining second IM period.

Lymphocyte-related genes in PBL

By focusing on the acquired IS, *CD3δ*, a part of the T cell antigen receptor (TCR), was mainly uniformly regulated in HR and LR. During the first IM period, *CD3δ* was slightly more expressed in PBL of the LR group compared to the other groups in TW 10 only. From TW 20, *CD3δ* was found very strongly down-regulated in HR and LR PBL compared to Ctr in relation with the combined PC/SC injection of *MucoCD-I* batch B. The expression of *CD4*, the T-helper (T_H) cell-specific co-receptor for major histocompatibility complex (MHC) class II molecules, was graded as strongly regulated in the PBL of the treated groups. From TW 7, four TWs after the vaccination with *MucoCD-I* batch A, the *CD4* expression was lastingly depressed in PBL of the HR. Consequently, the HR relative level of *CD4* transcripts was distinct towards the LR in TWs 7, 10 and 14. In contrast to the first IM period, increased *CD4* transcriptions were determined in all cows treated with *MucoCD-I* batch B from TW 20, which mainly amounted to > 2 ΔΔCq. *CD8β*, a part of the co-receptor for MHC I molecules, is a characteristic feature of cytotoxic T (T_C) cells. The *CD8β* expression was unaltered in all groups up to TW 7. During the remaining first IM period, *CD8β* was down-regulated in the treated groups from TW 8, except for the LR in TW 10, and in the Ctr from TW 10. The one-time increase of LR

CD8β transcripts with the simultaneously depressed HR relative amount of *CD8β* transcripts led to the significant different *CD8β* expressions between them in TW 10. In terms of the treatment with vaccine batch B, the *CD8β* transcription was weakly enhanced in the HR PBL in TW 21 and after TW 25 for the remaining treatment period. Between HR and LR, only one significant variance was found in TW 18. Regarding the low regulated gene *CD19*, coding the B cell-specific co-receptor, the LR stuck out solely with a significant up-regulation of *CD19* in TW 10 compared to HR and Ctr. Contemporaneously with the once injected vaccine batch A, the relative increased transcription of *CD19* in HR PBL was higher than that in PBL of the Ctr group (TW 3). Only one substantially altered regulation of *CD19* was noticed in the second IM period. In TW 31, the transcription of *CD19* in the LR PBL was more than double compared to the associated baseline value. The regulation of the plasma cell typical *CD126*, also known as the cytokine receptor *IL6Rα*, was very strongly changed in the HR group from TW 7 up to TW 14. During this pure nasal IM period, the relative transcription of *CD126* in HR PBL was lowered to roughly three percent of the initial value as measured at the start of the treatment. After the determined higher relative transcript amount of *CD126* in LR PBL towards that of the Ctr up to TW 6, the *CD126* expression of the LR was also reduced for the remaining nasal IM period. However, the extent of this depression in LR PBL was lower than that in HR PBL causing the significant difference between their *CD126* expressions in TW 8. In the Ctr group, the transcription of *CD126* remained unchanged during both treatment periods. With regard to the second IM period, *CD126* was up-regulated in the HR PBLs from TW 21 to TW 29; meanwhile, its relative expressions clearly differed from those of the Ctr group. Additionally, the relative expression of *CD126* in LR PBL exceeded that of the Ctr in TW 25. Among the lymphocyte-related cytokines, *IFNγ*, *TGFβ1*, *IL2* and *IL10* were investigated to assess the T cell activation state. The expression of the gene coding for *IFNγ*, a cytokine relevant to the activation of macrophages and the Ig class switching, was only slightly impaired by vaccination against *C. difficile* (Fig. 1). This gene was clearly up-regulated in the HR PBL and its relative expressions differed compared to that of the LR at the TWs 2, 4, 6 and 7 in proximity to the once given *MucoCD-I* vaccine batch A. At the mid-term of nasal IM, the *IFNγ* transcription peaked once only in the LR PBL. Apart from TW 23, conform developments of the LR and HR relative *IFNγ* expressions in PBL were marked by three simultaneous peaks in the second treatment period with the *MucoCD-I* vaccine batch B. The Ctr cows expressed *IFNγ* in PBL primarily steadily. *TGFβ1*, which is known to induce the antibody class switch to IgA production in plasma cells, was found down-regulated in PBL of the Ctr group in TWs 12 and 14. The expressions of *TGFβ1* by the treated groups were not changed for the first 7 weeks of treatment. After depressions up to a quarter of the initial *TGFβ1* levels in PBL of LR and HR in TWs 8 to 10, they were lifted up to the baselines again. Furthermore, the *TGFβ1* starting values of the treated groups in TW 16, marking the second IM period, were significantly raised in TW 27. Immediately after the first treatment with vaccine batch B and additionally in TW 29, the relative *TGFβ1* levels of the HR were significantly superior to that of the Ctr. From TW 8, the IM caused a very strong regulation of the T cell growth factor *IL2*. On this date, undulated expressions of *IL2* in PBL of both treated groups started and lasted during the second vaccination period, too. The Ctr expressed *IL2* in a relatively stable way, but among the treated cows, strong up-regulations by >4 ΔΔCq of *IL2* in PBL were partly presented by the LR in TWs 8, 18 and 27. On these dates, the relative *IL2* expression levels of the LR differed considerably from those of the HR. The *IL10* expression was analyzed with respect to this cytokine role as an active suppressor of macrophage functions, *inter alia*. The Ctr group showed unchanged *IL10* expressions in PBL over time. Among the treated cows, the transcriptions of *IL10* were weakly depressed several times during the first IM period, mainly by the HR. Consequently, significant differences between the relative *IL10* expression levels in HR and LR PBL existed at the TWs 2 and 10. Within the second IM period, *IL10* was slightly up-regulated in the PBL of both treated groups in TW 21. In addition, the relative *IL10* transcript amounts in PBL varied between Ctr and HR in TWs 23 and 25, and in TW 27 in case of the Ctr and the LR.

Total and differential cell counts in blood

The total and differential cell numbers were assessed referring to the physiological ranges of bovine PBL [25]. Typically, bovine blood contains five to ten million PBL per milliliter. Comparing within the groups, control (Ctr), LR and HR, the average PBL amounts per milliliter blood were similar with 6.8 ± 1.3 (Ctr), 7.1 ± 2.6 (LR) or 7.5 ± 2.4 (HR) million cells for the examined treatment period of 31 weeks. During the course of treatment, the graphs for the PBL concentrations of both treated groups developed predominantly in a similar manner (Fig. 2). After a singular IM with *MucoCD-I* batch A, the LR and the HR cows blood included the significantly lowest and highest PBL concentrations in TW 3 and TW 5, respectively, and furthermore beyond the normal range. One more time, the LR PBL count fell considerably below the minimum threshold in TW 6. With some delay, the PBL number of the HR group achieved a second low with

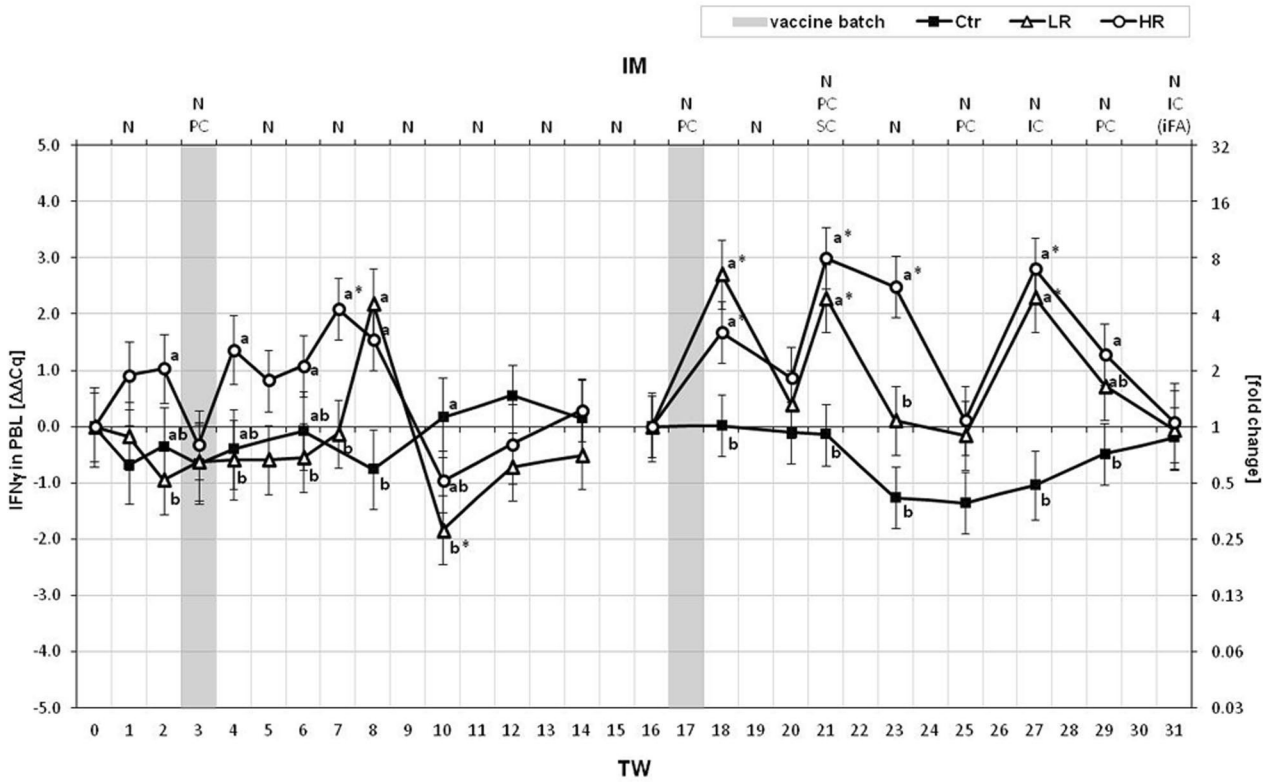

Fig. 1 IFNγ gene expression in PBL. The ΔΔCq values of IFNγ as LSM ± SD and the corresponding fold changes are mapped on the ordinates for the control ("Ctr", n = 5) and the treated animal groups ("LR", low responder cows, n = 4; "HR", high responder cows n = 5). The lower X-axis shows the 31-week treatment period (TW). The upper X-axis depicts the immunization (IM) specified by the routes of vaccine application [nasal (N), percutaneous (PC), subcutaneous (SC) and intracutaneous (IC)] per TW. Incomplete Freund's adjuvant (iFA) was solely part of the *MucoCD-I* vaccine in TW 31. The gray bars in TWs 3 and 17 highlight the times of the first uses of vaccine batches A and B, respectively. During the treatment periods with both vaccine batches and the particular TW before the first use of vaccine batch A or B, substantial differences within the same group are marked by asterisks ($p < 0.05$). Lower case (a, b, c) indicate evident differences ($p < 0.05$) between the groups on the same dates

3.9 ± 1.0 million cells ml^{-1} in TW 8. During the following exclusively nasal IM, the continuous increase of the HR PBL contents led to a distinctively higher value, amounting to more than nine million cells in TW 14. In the second vaccination period starting in TW 17, the treated cows showed a trend to reduce PBL concentrations to slightly below the physiological minimum level for the LR in TW 25 and for the HR in TW 31. The Ctr group stood out with considerable few PBL concentrations of unknown cause in TW 25 and TW 27. The relative distributions of the single leukocyte populations depending on different times of measurement are summarized in Additional file 4: Table S4. In principle, the proportion of lymphocytes in bovine blood varies between 45 and 65%, and the subjacent range of PMN is between 25 and 45% [25]. These are the dominant cell types and, by taking also the monocytes into account for up to 6%, the normal ratio of phagocytes to lymphocytes is faintly < 1 in bovine blood. Evaluating significantly positive divergences of this ratio during the IM period, 1.2 ± 0.2 was determined for the LR group in TW 10 (Fig. 2). Additionally, out of the standard range, the same level of this ratio was found for the Ctr group in TW 25. Focusing on the development of the blood lymphocytes (Fig. 3), the first IM period with the once injected *MucoCD-I* batch A followed by the solely nasal IM, a clear reduction of the HR lymphocyte concentrations in blood emerged in TW 12 and TW 14. However, the delta values of lymphocytes in blood of LR and Ctr progressed steadily further on. During the treatment period with *MucoCD-I* batch B, there were no noticeable alterations of the lymphocyte contents in blood.

bMEC-related genes in SCC

In terms of the systematic triggering of the cows IS by vaccination against *C. difficile*, the adjustments of the bMEC' related *PIGR* and *FcRN* were analyzed as the key receptors for epithelial transports of IgA and IgG,

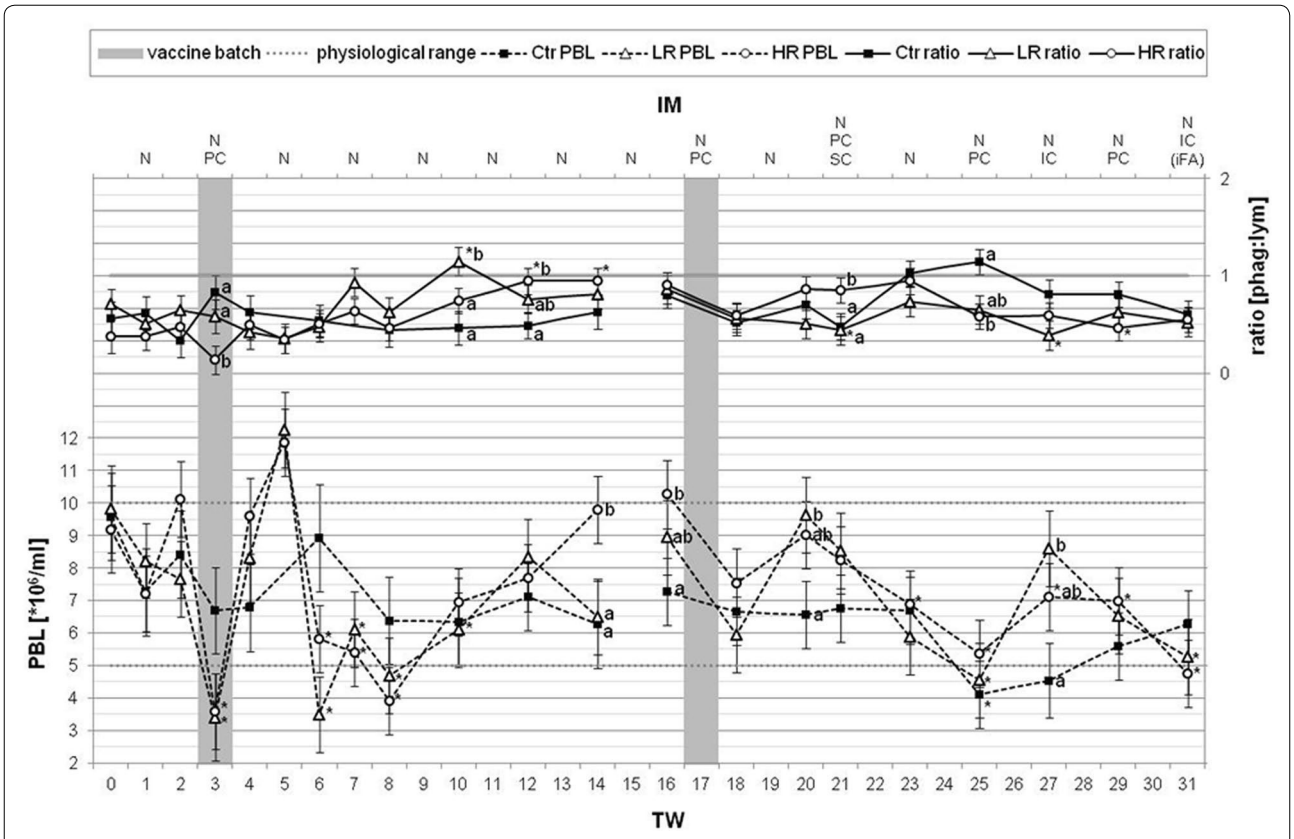

Fig. 2 Total PBL contents and the ratios of phagocytes to lymphocytes in blood. On the ordinates, the total PBL contents and the ratios of phagocytes to lymphocytes in blood are depicted as LSM ± SD for the three examined animal groups [("Ctr" control animals (n = 5), "LR" treated, low responder animals (n = 4), "HR" treated, high responder animals (n = 5)]. The physiological range of PBL (5 up to 10 million leukocytes per milliliter blood) was accented by two gray dotted lines. The 31-week treatment period (TW) is depicted on the lower abscissa. The upper abscissa indicates the different application routes [(nasal (N), percutaneous (PC), subcutaneous (SC) and intracutaneous (IC)] of the vaccines to the TWs with immunization (IM). Only once, incomplete Freund's adjuvant (iFA) was supplemented to the *MucoCD-I* vaccine. The gray bars in TWs 3 and 17 highlight the times of the first uses of the vaccine batches A and B, respectively. During the treatment periods with both vaccine batches and the particular TW before the first use of vaccine batch A or B, substantial differences within the same group are marked by asterisks ($p < 0.05$). Lower case (a, b, c) label evident differences ($p < 0.05$) between the groups at the same dates

respectively. *PIGR* was graded as strong regulated due to its increased expression by >3 ΔΔCq in HR SCC in TW 21. Differently altered expression levels of *PIGR* existed only once in each IM period, between LR and Ctr in TW 6 and between HR and Ctr in TW 17. The expression of *FcRN* was not affected by the treatment in any group. The complement system, represented by *C1QA* and *C3*, was barely impaired by the vaccination procedure. No relative expression changes of *C1QA* were determined during the treatment. The gene *C1QA* codes for the recognition protein C1q, a part of the complement component C1 that initiates the classical pathway of complement activation by antigens. Regarding the alternative pathway that can be initiated by cleavage of the complement component C3, the gene *C3* was weakly regulated. During the first IM period, the relative *C3* expression in SCC of LR more than doubled, evoking also the significant difference to that of the Ctr in TW 5. Three TWs later, in TW 8, the same relative *C3* transcript amounts of LR and Ctr were, on average, significantly higher than those of the HR. Following treatment with *MucoCD-I* batch B, the LR *C3* was expressed less compared to the other groups in TW 27. A selection of chemokines classified as bMEC' related was analyzed to verify the relevance of bMEC for the recruitment of leukocytes to the mammary gland in response to the repeated vaccination against *C. difficile*. Among the genes that encode the chemokines, the expression of *CCL5* was slightly interfered by the vaccination, which was significantly down-regulated in HR compared to Ctr in TW 5. *CCL20* was found strongly regulated during treatment against *C. difficile*. In the course of the first IM period, the relative expression levels of *CCL20* differed

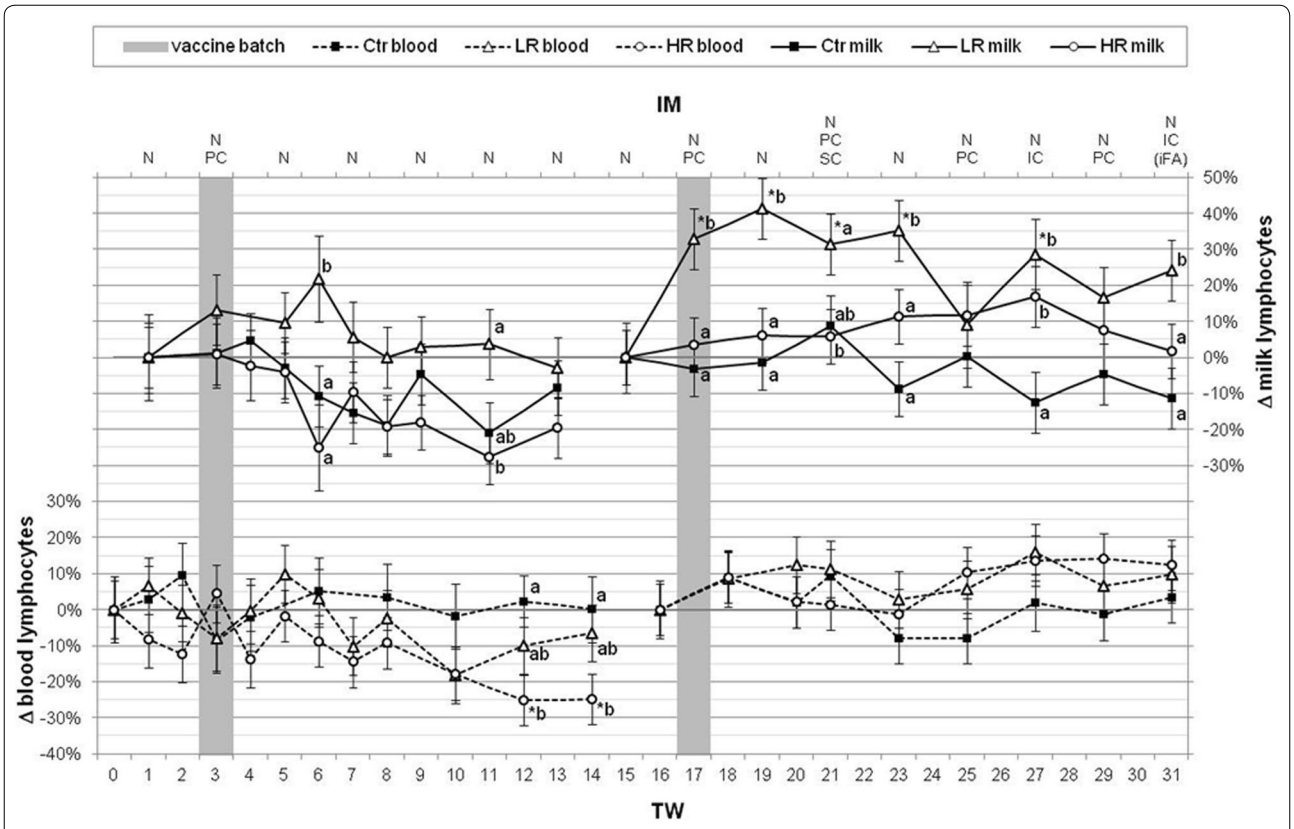

Fig. 3 Relative changes of lymphocytes out of milk and blood related to the used *MucoCD-I* vaccine batches. The percentage variations of lymphocyte contents per group [("Ctr" control animals (n = 5), "LR" treated, low responder animals (n = 4), "HR" treated, high responder animals (n = 5)] and per milk and blood are represented as LSM ± SD on the vertical axes. The comparative indices are the treatment weeks (TW) before the first-time application of the vaccine batches A or B, respectively. The upper abscissa shows the used routes for administration of the vaccine [nasal (N), percutaneous (PC), subcutaneous (SC) and intracutaneous (IC)] marking the dates of immunization (IM), too. The *MucoCD-I* was given only once plus incomplete Freund's adjuvant (iFA) in TW 31. In TWs 3 and TW 17, the primal injections of the vaccine batches A or B are accented by the gray bars. Deviated by group, the asterisks ($p < 0.05$) indicate considerable differences relating to the dates immediately before the first use of each vaccine batch. The significant variations ($p < 0.05$) between the groups at the same times are characterized by small letters (a, b, c)

to their baselines for the LR in TW 7 and for the Ctr in TW 9. Concerning heterogeneous regulations of *CCL20* between the groups, two gaps were determined between HR and Ctr in TWs 3 and 9, whereas during the second IM period, the down-regulated transcriptions of *CCL20* in SCC of HR compared to the up-regulated ones of the Ctr varied on each date of analysis. Without developing divergent expression levels of *CCL20* in SCCs to the belonging initial value in TW 15, the LR produced lower relative transcript amounts of this chemokine than the Ctr on four dates. In TW 27, the date of the first IC application of the vaccine batch B stood out with only one-time different expression levels of LR and HR. At no time of treatment, *CCL28* was altered to its baselines in SCCs of any group. Nevertheless, the regulation of CCL28 was assessed to be weak due to the proven differences between groups. Variations between the transcription levels of *CCL28* in SCCs per group existed only during the pure nasal IM period. Apart from TW 8, the calculated transcript amounts for the LR cows were significantly higher to that for the Ctr cows on every date of measurement beginning with TW 5. The HR *CCL28* expression was markedly down-regulated compared to the LR in TW 9. Likewise, *CXCL3* was graded as slightly regulated. While no alterations of the gene expression could be identified in any group during the first IM period, the down-regulation of *CXCL3*, as found in SCCs of the HR group by vaccination with *MucoCD-I* batch B, was decisive for the classification of this gene. Significant variations between the relative expression profiles of *CXCL3* in SCCs of the treated groups and the Ctr group existed predominantly on the same dates. No changed regulations of *CXCL5* were noticed as induced by the vaccination against *C. difficile*. Regarding the chemokine *CXCL8*, a weak regulation was shown because of a single significant down-regulation between the treated groups

and the Ctr group in TW 23. Within the analyzed antimicrobial factors as produced by bMEC, the gene coded for lactoperoxidase, *LPO*, was solely found as strongly regulated. The *LPO* expression patterns of all groups had not been changed towards the associated baselines in TWs 1 and 16, but two dates with discrepancies between treated and Ctr cows were found. At the beginning of the pure nasal IM, the relative *LPO* transcript amounts of the treated groups exceeded that of the Ctr group in TW 5. One TW later, the ΔΔCq value of 3.4 for the LR *LPO* transcription was significantly above that for the Ctr group. The treatment interfered only slightly with additional genes belonging to the division of antimicrobials like lactoferrin (*LF*), lysozyme (*LYZ1*) and tracheal antimicrobial peptide (*TAP*). Following vaccination with *MucoCD-I* batch A, *LF* was inversely regulated in the SCCs of the treated groups. The up-regulation of *LF* in the LR group and its simultaneous down-regulation in the HR group resulted in significant differences between them in TWs 7–9 and 13. The LR *LF* transcription was also intensified towards the Ctr in TWs 5–7. At the first application of *MucoCD-I* batch B, the HR *LF* expression level exceeded significantly compared to that of the Ctr (TW 17). In context with the triple-vaccination on the nasal, PC and SC route, *LF* was clearly expressed more in the treated cows compared to the baselines and to the Ctr in the TWs 21 and 23. In mid-term of the first IM period, sharply reduced transcriptions of *LYZ1* were noticed for the Ctr cows on several dates and for the HR cows only in TW 11. Different regulations of *LYZ1* between the groups were not determined during the period to be analyzed. Related to the application of *MucoCD-I* batch A, the apparently diminished *TAP* expressions in the SCCs of both treated groups led to the proven variations towards the *TAP* expression levels in Ctr SCCs in TWs 3 and 5. Nevertheless, those of the treated groups were neither different to the respective initial values on these dates nor to other measurement points during the complete treatment period. Finally, at the end of treatment with vaccine batch B, a higher relative transcript amount of *TAP* was found in LR than in Ctr cows.

Phagocyte-related genes in SCC

Among both analyzed genes, which encode complement receptors, *C3AR1* was assessed to be regulated on a minor level in SCCs than in *C5AR1*. In the course of the complete treatment period, no group showed considerable alterations of *C3AR1* expressions towards the belonging initial values. But always towards the end of both vaccination periods, the expression profiles of the treated groups differed clearly from those of the Ctr group. For the HR and LR, the first IM period finished with expression levels of *C3AR1* showing an upward tendency. Conversely, their expression levels of *C3AR1* tended to be reduced at the end of the second IM period. During both IM periods, the developments of the *C5AR1* transcriptions in SCCs of the treated groups towards the Ctr group were similar to those of the *C3AR1* transcriptions. However, the relative transcript amounts of *C5AR1* in the SCCs of the treated groups exceeded considerably towards the baselines when finishing the first IM period. That was also the case for the development of *CXCR2* transcripts in the HR SCCs. Generally, this chemokine receptor was regulated on a low level. In contrast to the Ctr cows, CXCR2 was significantly higher expressed by the HR in TW 13 and significantly lower expressed by HR and LR in TW 23. *TLR2* belonged to the category of weakly regulated genes by the treatment. During the first IM period, only in the HR group, a single up-regulation of *TLR2* towards the baseline was determined in TW 8. Comparing the relative expression levels of *TLR2* in SCCs between the three groups, those of the HR were markedly higher than those of the Ctr in TWs 9 and 11. Furthermore, the relative HR transcript amounts of *TLR2* also topped those of the LR in TW 11. At mid-term of the vaccination with *MucoCD-I* batch B, the *TLR2* transcriptions in SCCs of both treated groups were significantly reduced compared to the Ctr group. The gene for the specific surface determinant of monocytes and macrophages, *CD163*, was graded as non-regulated by the vaccination against *C. difficile*. Concerning the investigated gene regulations of phagocyte-specific cytokines, *TNFα* was only determined to be strongly interfered by the treatment. The steadily increasing *TNFα* transcription in SCCs of the Ctr group, as proven during the second IM period, was crucial for this classification. Two weeks after PC injection of vaccine batch A, the reduced transcription of *TNFα* by more than 80% in the SCC of HR was considerable towards the baseline, and, in addition, by comparing it with the *TNFα* transcription of the Ctr group (TW 5). Similarly, the *TNFα* expression in the HR SCC was depressed in TW 13. During the second IM period, the *TNFα* expression profiles of the treated groups developed similarly. They were shaped by a reduction up to TW 21 followed by an increase that reached their initial values at the last point of measurement in TW 31. Continuously, *TNFα* transcript amounts in the treated groups were significantly lower than those of the Ctr cows. The interleukins, *IL1β*, *IL6* and *IL12β*, were graded as slightly regulated in SCCs during 31 TWs. After the *MucoCD-I* vaccine batch A was administered, the LR *IL1β* was exclusively markedly lower expressed than the Ctr one in TW 5. Regarding the second period of vaccination, both *IL1β* transcriptions in the SCCs of the treated groups were significantly diminished as compared with that of the Ctr in TW 23. At the following points to

be analyzed, the *IL1β* transcriptions in SCCs of LR were steadily multiplied and those of HR were constantly reduced resulting in significant differences between HR and LR as well as between HR and Ctr in TW 27. In context with the once given vaccine batch A, *IL6* was clearly down-regulated in the HR SCC in TW 5. On the same date, this gene was also differently expressed between the HR and LR cows. In TW 13, the *IL6* expression in the Ctr SCC was noticed by its significant decrease towards the associated baseline value. During the second IM period, the development of the treated cows *IL6* expression profiles was characterized by initially reduced transcriptions of *IL6* compared with the Ctr cows. These were significant in TWs 21, 23, 27 (only HR) and 29. Then, the *IL6* expressions in SCCs of HR and LR returned to the baseline levels in the last TW. In case of the examined *IL12β* expressions in SCCs of the treated and the Ctr groups, a significant gap existed between them during the first IM period in TW 9. Within the treatment period that used vaccine batch B, the increasing transcription of *IL12β* in SCCs of the Ctr resulted in their clearly higher transcript amounts as measured by those of the treated groups. The significant variances between the groups were supported by a similar trend towards reduced *IL12β* transcriptions of all treated cows up to TW 23.

Lymphocyte-related genes in SCC

Considering the adaptive IS, the T cell population marker *CD3δ* was only weakly impaired in SCCs by the repeated vaccinations against *C. difficile*. In context with the once injected vaccine batch A, the relative expression of *CD3δ* of the LR was found changed by roughly 2 ΔΔCq compared to the other groups in TWs 3 and 6. Within the second vaccination period, the differences in *CD3δ* expression levels between the treated and the Ctr groups were mainly attributed to their inverse courses of *CD3δ* regulation. *CD4*, tagging the T_H cell populations, was very strongly up-regulated in the SCC of LR in TW 3. On this date, the LR transcription of *CD4* amounted to 3 ΔΔCq, which was considerably different from HR and Ctr. The extent of this peak led to the assessment of *CD4* being strongly influenced by the treatment. From mid-term of treatment with *MucoCD-I* batch B, two gaps between the developments of the *CD4* expressions by the treated cows and the Ctr cows became obvious. The relative *CD4* transcript amount in the SCC of the HR group was significantly lower than the Ctr one in TW 23, and this was also the case between the LR and the Ctr in TW 29. Regarding *CD8β*, marking the T_C cells, this gene was graded as slightly interfered by the vaccination on numerous dates to be analyzed. In SCCs of LR and Ctr, their *CD8β* expressions developed contrarily in TWs 9 and 11 within the solely nasal IM period. Additionally, the HR and Ctr expression levels of *CD8β* differed in the SCCs in TW 11. During the second IM period, *CD8β* was markedly lower expressed in the HR compared to the Ctr group in TW 23. The transcription of the B cell receptor *CD19* was slightly influenced by the vaccination as measured in SCCs. The inconsistent development of the Ctr *CD19* expression for the first TWs was decisive for the variations from the treated groups. Following the treatment, *CD38* marking activated B and T cells within the SCCs, was found to be weakly regulated. Within the groups, no different *CD38* expressions in opposition to the baselines were noticed. Between the groups, two gaps were determined in the course of the first IM period. The first one was present between the relative *CD38* transcriptions of the HR and LR in TW 3, and the second one was determined between those of the HR and Ctr in TW 9. The surface determinant of activated B cells and plasma cells *CD126* was characterized by a commonly tenuous regulation related to its expression by SCCs of all groups. Only the LR group showed the trend to a decreasing expression of *CD126* between TW 6 and TW 8, which was sufficient to achieve distinct differences towards the Ctr group. Additionally, the course of the LR *CD126* expression in SCCs led also to reduced transcript amounts of *CD126* versus the HR in TW 8. The generated transcript profiles of *CCR6*, the counterpart to *CCL28,* were similar to one another for both treated groups. Apart from the single disparity between the *CCR6* regulations of the HR and the Ctr in TW 5, no further alterations of the generally classified as weak *CCR6* regulation were found within and between the groups during the first IM period. In the course of the second IM period, the LR differed from the Ctr more hesitantly than the HR from the Ctr and the HR *CCR6* was significantly up-regulated from TW 25 up to TW 31. *IFNγ* and *IL10*, which, *inter alia*, are produced by T lymphocytes, were dissimilarly regulated due to the vaccination procedures. A weak altered expression characterized *IFNγ*, whereas the expression of *IL10* was classified as strongly changed. Comparing the treated groups, inversely progressed *IFNγ* expressions in the SCCs were detected from the beginning of treatment (Fig. 4). In these first TWs, the LR's transcription of *IFNγ* was increased by partly > 2 ΔΔCq in TWs 3, 5–13, while that of the HR was noticeably depressed towards the LR all the time except for TW 9. In the course of treatment with the *MucoCD-I* vaccine batch B and, in contrast to the first IM period, the relative expressions of *IFNγ* in SCCs of the LR and the HR varied only once in TW 23. The Ctr cows expressed *IFNγ* in SCCs primarily steady during the 31 TWs. Differently

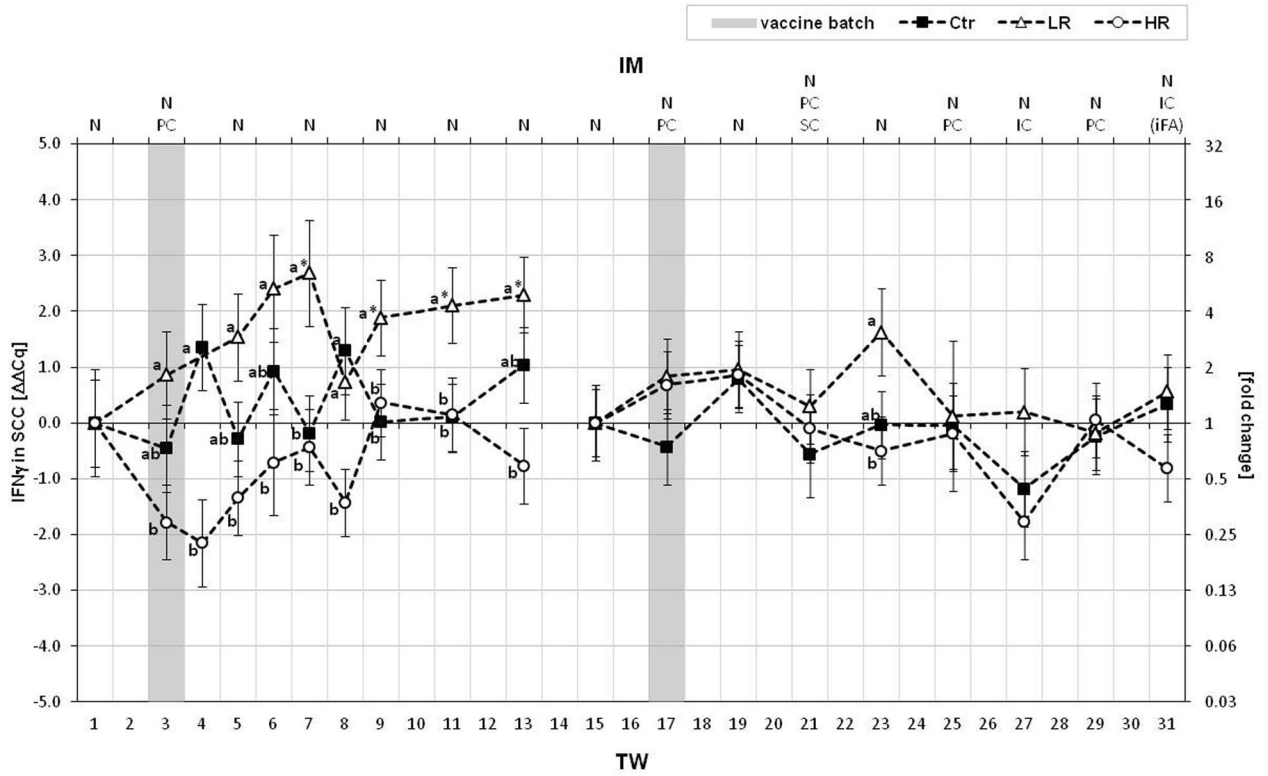

Fig. 4 IFNγ gene expression in SCC. The Y-axis shows the ΔΔCq values of IFNγ as LSM ± SD and the corresponding fold changes for the control ("Ctr", n = 5) and the treated animal groups ("LR", low responder cows, n = 4; "HR", high responder cows n = 5). The 31-week treatment period (TW) is depicted on the lower X-axis. The immunization (IM) specified by the routes of vaccine application [nasal (N), percutaneous (PC), subcutaneous (SC) and intracutaneous (IC)] per TW is illustrated by the upper X-axis. Incomplete Freund's adjuvant (iFA) was only once part of the *MucoCD-I* vaccine in TW 31. The gray bars in TWs 3 and 17 highlight the times of the first uses of vaccine batches A and B, respectively. During the treatment periods with both vaccine batches and the particular TW before the first use of vaccine batch A or B, substantial differences within the same group are marked by asterisks ($p < 0.05$). Lower case (a, b, c) indicate evident differences ($p < 0.05$) between the groups on the same dates

developed *IFNγ* expressions between the Ctr and the treated groups existed only in TWs 4, 7, 8 and 11 during the first IM period. Unlike the Ctr, the inhibitor of important immune functions of the innate IS, *IL10*, showed to be more down-regulated in the SCCs of the treated cows following the IM with *MucoCD-I* vaccine batch A on several dates. However, only in TW 13 was the depressed expression of *IL10* in the SCC of the LR heavy enough to differ clearly from the baseline. The levels of *IL10* expressions in the SCCs varied from TW 15 neither within nor between the groups.

Total and differential cell counts in milk

Generally, dairy farming accepts the cut-off value of 200 thousand cells per milliliter bovine milk for assessing udder health [31, 32]. When monitoring the SCC in milk (Fig. 5) over the IM period, this practice-related upper limit was exceeded by a multiple only at one time. In TW 8, the LR and Ctr groups protruded with high SCCs amounting to 910.1 ± 139.8 and 479.3 ± 139.8 thousand cells per milliliter milk, respectively. Those group-related SCC levels were dominated by data of single cows. An acute mastitis was demonstrated for cow LR-2 causing more than 3.5 million cells per milliliter milk, whereas the SCCs of the other LR cows accounted for around 15 thousand cells per milliliter milk. The source of the striking Ctr cow SCC with nearly 2 million cells per milliliter milk was not found. Its udder health seemed to be unimpaired. All other cows of the Ctr group showed a SCC of 70 thousand cells per milliliter milk, on average, in TW 8. The results of the DCC in milk are compiled in detail in Additional file 5: Table S5. The development of the lymphocyte proportions in milk was investigated in relation to the date before the first use of each *MucoCD*-I vaccine batch (Fig. 3). The untreated Ctr showed a clear decrease of these immune cells unlike the homeostatically balanced lymphocyte contents in blood during both IM periods. In LR milk, the level of the lymphocytes grew by 20% up to TW 6 and declined towards the baseline in TW 13. Similarly to the Ctr, the lymphocyte contents in

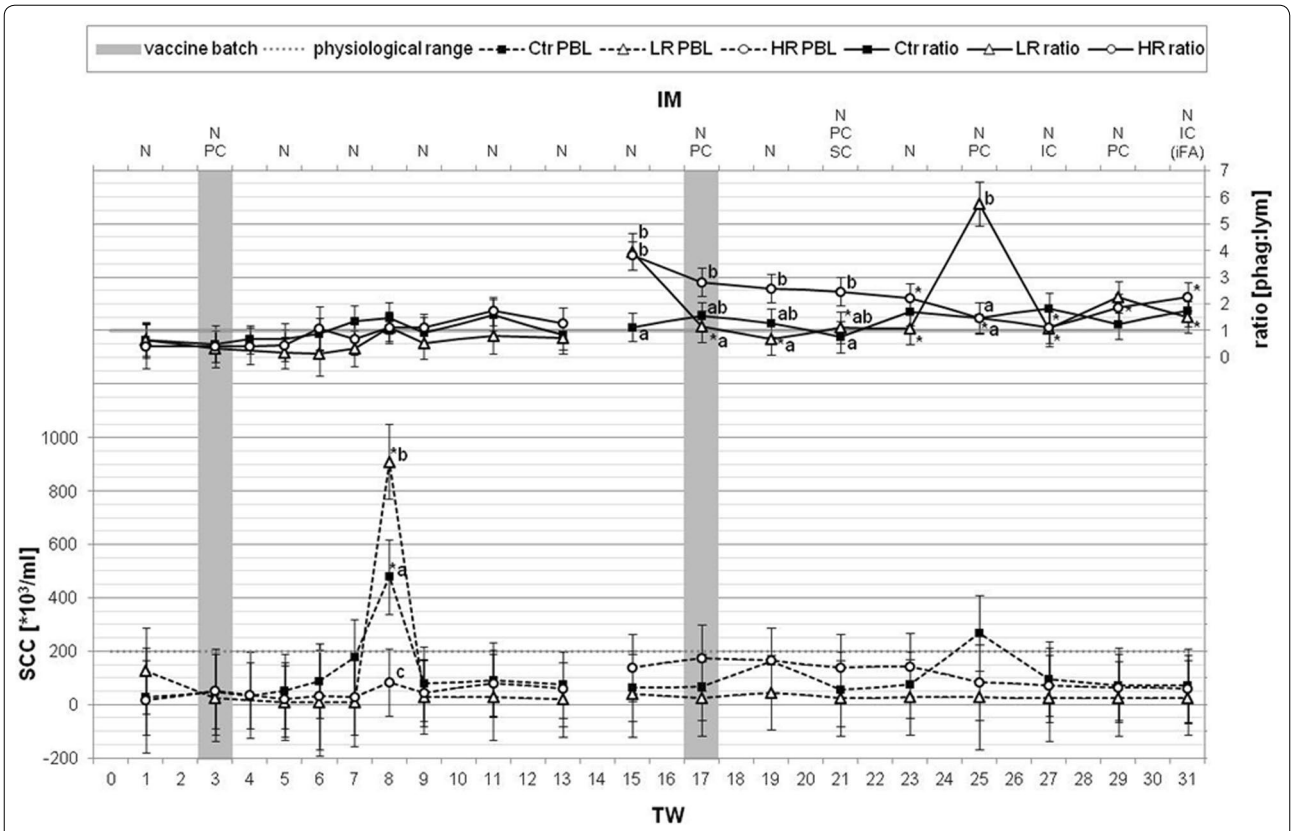

Fig. 5 Total SCC in milk and the associated ratio of phagocytes to lymphocytes. Within 31 treatment weeks (TW), the recorded total SCC in milk are shown in groups ["Ctr" control animals (n = 5), "LR" treated, low responder animals (n = 4), "HR" treated, high responder animals (n = 5)] as LSM ± SD on the lower vertical axis. The gray dotted line at 200 thousand cells per milliliter marks the commonly used cut-off value for normal SCC. The upper line chart depicts the ratio of phagocytes to lymphocytes in milk. The routes of vaccination [nasal (N), percutaneous (PC), subcutaneous (SC) and intracutaneous (IC), incomplete Freund's adjuvant (iFA)] are presented on the upper abscissa on the immunization (IM) dates. Two gray bars represent the IM times using the *MucoCD-I* vaccine batches A or B. The asterisks ($p < 0.05$) indicate the inner-group deviations in relation to the dates before the initial uses of both *MucoCD-I* vaccine batches. The significant differences ($p < 0.05$) between groups on the same dates are marked by lower case letters (a, b, c)

milk of HR declined reaching a low of −30% in TW 12. After the application of vaccine batch B in TW 17, the development of the lymphocyte proportions in milk of LR was strongly influenced by the extraordinarily low baseline value in TW 15. Ruling out this effect, the lymphocyte levels in the milk of the LR group also showed a declining trend based on TW 19. In comparison, the lymphocyte contents of the HR milk appeared slightly volatile by maximal +17% to the date of the firstly IC given *MucoCD-I* and returned to the initial level in the last TW. Emphasizing the calculated ratios of phagocytes to lymphocytes per group (Fig. 5), they developed predominantly uniformly within TW 1 up to TW 13. During this first IM period, a slight increase of this ratio from 0.6 up to 0.9 was equally noticed for all three groups. This development continued in case of the Ctr, even in the remaining TWs. General phagocyte growth was mainly characterized by the increasing percentages of the PMNs in proportion to the lymphocytes. Notwithstanding the varying development of these cell populations in the treated groups within TW 15 and TW 25, the ratios of phagocytes to lymphocytes of all groups averaged 1.8. The extraordinarily high values of this ratio of about 3.9 in each treated group were due to an encounter of maximal PMN and low lymphocyte concentrations in milk in TW 15. Likewise, the LR milk contained a large share of phagocytes due to an individual PMN count of around 90% in TW 25. The physical check of the responsible LR cow revealed an unremarkable status of health. The described extreme levels of the phagocytes to lymphocytes ratios in milk were not assessed as to be induced by the vaccination regime, whereas the quotients formed by phagocytes and lymphocytes differed distinctively

between the LR and HR after the first injection of vaccine batch B for the following 4 weeks.

Discussion

Clostridium difficile specific IgA in milk

The specific antibody response against *C. difficile*, more precisely the level of anti-*C. difficile* IgA in milk, was the decisive criterion for assessing the immune reactivity of the treated cows [21]. In search of indications that primarily explained their different responsiveness to the repeated challenging with *C. difficile*, the outcomes of this study were appraised hereinafter. For this, in particular, the first three TWs were of interest upon receiving the *MucoCD-I* batches A and B for the first time. For these dates, the main gaps between the specific antibody responses of both groups were ascertained amounting to a threefold (batch A) or sevenfold (batch B) varying anti-*C. difficile* IgA content in milk [21]. In case of the LR, the amount of anti-*C. difficile* specific IgA in milk exceeded only once the associated initial value in TW 5. But the lastingly increased production level of *C. difficile* specific IgA in HR milk of about at least a triple upon TW 3 remained unaffected by this single peak value in LR milk [21].

Gene expression profiling in PBL

Within the scope of the gene expression analysis, an impression of alterations on the molecular biological level induced by the repeatedly administered inoculation was received. Commencing with the genes investigated in PBL and related to the innate IS, the two-part vaccination schedule had scarcely interfered with the expression of the phagocytes characterizing surface determinants. Even though, in response to the poorly tolerated vaccine batch A, the first phase of the biological leukocyte curve, as shown by clearly reduced PBL counts (Fig. 2), were also determined by the depressed expression of the blood phagocyte receptors (*C5aR1, CXCR2, TLR2, CD163*) in TW 4. In part, the down-regulation persisted for these genes throughout the remaining first IM period like for HR and LR *CXCR2* and for the HR *CD163*. Although this might hint at a possibly induced migration of phagocytes leaving the circulation, the phenotypic confirmation of this assumption by DCC was not given. In response to the first application of *MucoCD-I* batch B (TW 18), the HR cows responded more sensitively than the LR, as measured by increased *CXCR2* expression in HR PBL. Simultaneously increased PMN numbers in HR blood were not determined, even though *CXCR2* is predominantly expressed by PMN [33]. Possibly, the *CXCR2* up-regulation in HR PBL was necessary in order to clear the corresponding ligands that were released at the vaccination site, and to restore their homeostasis [34]. Tracing the injected *C. difficile* antigens by complement during the second IM period seemed to be of subordinate importance assessed by means of the phagocyte-related complement receptors. Likewise, the *C. difficile* antigen detection by the *TLR2*, which is of prime importance for the recognition of gram-positive cell wand components, appeared to be on a low level [35]. Nonetheless, *TLR2* was equally up-regulated within the PBL of the treated groups following the triple vaccination in TW 21. Conceivably, the dendritic cells were involved predominantly in the sensing of the *C. difficile* antigens subsequent to its inoculations. These APCs are located within the peripheral tissue out of the blood circulation and they are the main initiator and modulator of the immune response [36]. The examined chemokines, which are known to be primarily emitted by monocytes/macrophages, were differently expressed in the HR and LR PBL, especially in relation to the application of *MucoCD-I* batch A. During the first IM period, the chemokine profile of the HR was characterized by more pronounced down-regulations of *IL1β* and *IL6* and a partly stronger up-regulation of *CXCL8* in contrast to the LR. For the HR, this might mean that vasodilation for recall of defined leukocyte populations, like PMNs and effector *CD8* positive T cells, was promoted, but that no further pro-inflammatory signaling was supported [28, 37], whereas the LR showed a strong up-regulation of *IL12β*, the prerequisite for the T_H1 cell response [38]. The second IM period indicated more consistent expressions of the chemokines by both treated groups. Uniformly, following the triple vaccination in TW 21, *IL1β* was down-regulated once in the short term, *IL12β* was down-regulated in the long term, and *IL6* was permanently up-regulated in PBL of HR and LR. Thus, the triple vaccination could have caused the switch to a T_H2 immune response driving the antibody production by plasma cells [38]. Owing to the chemokines importance to orchestrate the lymphocytes, their different expression patterns might have been one of the crucial determinants for the different reactions of the treated groups to the vaccinations against *C. difficile.* Based on the partly strong regulation of lymphocyte specific receptors, like *CD3δ* and *CD126,* it can be assumed that the associated cell proportions of T cells and activated B cells were equally influenced by the treatment. While *CD3δ* was not differently expressed between HR and LR in the course of the first IM period, the T cell subpopulations marking surface determinants, *CD4* and *CD8β*, were regulated in a clearly different way between them. Following three and four TWs after the treatment with *MucoCD-I* batch A, *CD4* and *CD8β* positive T cells were down-regulated more sustainably in HR than in LR. This could be caused by their differing profiles of chemokines on this date. Exemplarily, the previously

enhanced *CXCL8* expression levels could have activated the withdrawal of *CD8β* positive cells out of the blood stream [37]. Additionally, *IL6* was contemporaneously down-regulated with the *CD4* positive T cell subset. The primarily lower depressed expression of *CD4* and *CD8β* positive T cells in LR than in HR could be associated with the more intense increase of *IL12β* transcripts, as *IL12β* supports cellular immunity [38]. The pronounced up-regulation of the T cell growth factor *IL2* in LR PBL in TW 8 as well as the missing up-regulation of the inhibitory active *IL10* could also have contributed to that [39]. *IL10* was partly down-regulated in the HR PBL, whereby the chemotactic effect of possibly secreted *CXCL8* would not have been hampered. Concerning the B cells, the extent of the drastic down-regulation of the *IL6* recipient, *CD126*, in LR and HR did not reflect the corresponding *IL6* expression patterns. With a view on the second IM period, similarly to the interleukins, the expressions of the different lymphocyte markers were mainly uniformly developed by HR and LR during this treatment period, too. Obviously, the triple vaccination (TW 21) could have caused a very strong decrease of *CD3δ* positive T cells paired with the growth of the associated subpopulation, the *CD4* positive T cells, whereas the level of *CD8β* positive T cells seemed not to be affected by this treatment. The TCR down-modulation upon ligation with MHC:peptide complexes has been demonstrated to be important in T cell activation [40]. The T cell activation through the TCR lead to the subsequently de novo synthesis of *IL2* [41]. The presumed proliferation of *CD4* positive T cells in TW 21 might be triggered by the simultaneously increased release of *IL2* and *IL6*. The likely withdrawal of *IL12* could indicate the switch of *CD4* T cell differentiation into T_H2-like cells. Their maturation is probably activated by *IL6* [38]. Finally, the extent of the possible T_H2 immune response was regulated by probably secreted *IL10*, because the associated transcripts were multiplied in the treated groups from TW 21, too. *IL10* participates in inhibiting *IL12* and *IFNγ* secretions [39]. Although *IFNγ* and *IL2* are generally classified as typical T_H1 cytokines, both were proven to be up-regulated at the same time during treatment with vaccine batch B. This is remarkable considering the clear down-regulation of *IL12* at these points in time, which is deemed to be a potent inducer of *IFNγ* in T cells [42]. On the other hand, a positive feedback mechanism interconnects both cytokines in inflammation [39], 42]. However, the causal link between *IL12* and *IFNγ* could not even be fully established for the first treatment period, as *IL12* and *IFNγ* were predominantly inverse regulated following the anti-*C. difficile* vaccinations during this period. It is known that discrepancies in the T_H1/T_H2 hypothesis exist [38, 39]. The paradigm of type 1 and type 2 immune responses was established with laboratory rodents and it is not entirely applicable to large ruminants [38, 43]. Considering the effect of *IFNγ* to promote the antigen recognition and the Ig class switching in favor of IgG, this cytokine seemed to play an important role in the two-part responsiveness of the treated cows [43, 44]. As opposed to the LR, the HR showed stronger induced *IFNγ* expressions by the treatment on four dates during the first IM period and to a further date following treatment with vaccine batch B (Fig. 1). Generally, *IFNγ* supports intense phagocytic activity and only a weak humoral antibody response [39]. However, the DCC did not confirm enhanced concentrations of phagocytes in the TWs with up-regulation of *IFNγ* in the treated groups. In addition, the measurement of IgG concentrations in blood and milk were not part of the study at hand, so the possible effects of *IFNγ* were not verifiable. *TGFβ1* is referred to as an important regulator of mucosal immunity and as the co-responsible cytokine for induction of the antibody class switch to IgA [39, 44]. Regarding the examined anti-*C. difficile* IgA response, *TGFβ1* was not differently regulated between the treated groups. Also, in the crucial TWs of significantly increased IgA concentrations in HR milk, the *TGFβ1* expression did not differ between HR and Ctr in PBL. The examination of the local IS of the mammary gland as measured by gene regulations in SCC revealed only few differences between the treated groups in response to the vaccination. The different anti-*C. difficile* IgA production in milk of LR and HR did not seem to be influenced by different expression levels of the epithelial IgA receptor. The *PIGR* expression in the lactating mammary gland is assumed to be primarily controllable by steroid hormones rather than by immunoregulatory factors [45, 46]. Concerning the complement system, the complement activation product, C3a, is described as an inflammatory modulator [47]. The differently regulated complement component *C3* between HR and the other groups, LR and Ctr, in TW 8 has to be seen within the context of the excessive SCC values of LR and Ctr to this date (Fig. 5). Inflated SCC in milk usually indicates an inflammation, and for example, intramammary infections might cause increased *C3* expressions by bMECs [48]. In contrast to the HR and partly to the Ctr, the antimicrobial peptide *LF* and the chemokine *CCL28* were up-regulated in the LR group around TW 8, too. Possibly, both immunological factors had contributed to overcome an impending udder infection of the LR. Considering their up-regulation from TW 5 towards unchanged expressions of *LF* and *CCL28* in the Ctr, it is also likely that the vaccination increased their transcription in LR. Besides the direct antibacterial activity of *LF*, it operates cytokine-like as an "alarm", summoning assistance by various leukocytes [49]. *CCL28*

possesses also antimicrobial activities, but the typical feature attributed to this chemokine is to accumulate lymphocytes, especially IgA plasma cells, in mucosal tissues [50]. Thus, *CCL28* is designated as key regulator of B lymphocyte migration and retention in the mammary gland [51]. In all treated cows inoculated with *MucoCD-I* batch A, *CCL28* was up-regulated between TWs 5 and 7, supposedly to utilize the *CCL28* functions. The increased expression of *LPO* in the treated cows within the same period could have been precautionary, as this host defense peptide should participate to resolve the potentially microbial threat for the mammary gland by vaccination due to its bacteriostatic and bactericidal properties [52].

Total and differential cell counts in blood

Concerning the immune cell counts, their total numbers in blood revealed no disparities between LR and HR (Fig. 2) related to the vaccination program. Nonetheless, the PBL counts of all treated cows were definitely impaired by the continuous vaccinations, and they exceed uniformly their physiological range following the once PC administered *MucoCD-I* batch A (Fig. 2). In connection with the injection of this poorly tolerated vaccine, the PBL counts of the treated cows developed similarly to Schilling's biological leukocyte curve as being characteristic of bodily injuries caused by infections or toxins [25]. The PBL falls noticeably for all groups in TW 25 and cannot be exclusively explained by the vaccination schedule because the Ctr group was affected more particularly. An error in the PBL measurements up to this date was excluded, as the Ctr group was also below the physiological range of PBL in TW 27. The blood sampling date was in the early spring and it was assumed that health monitoring did not perceive the effect of a seasonal viral infection.

Regarding the DCC, the development of the lymphocyte parts in blood was primarily investigated in relation to the associated baselines for each IM period and compared to the phagocytes contained therein. Lymphocytes perform the specific immune response to an antigen, and in the narrower sense, they control antibody production [53]. Mucosal immunization, in particular, boosts lymphocyte migration following their activation by an antigen in the draining LNs next to the vaccination site. Afterwards, the activated lymphocytes colonize the related mucosal tissues via the lymph, and finally, via the blood circulation [54]. Thus, the apparent differences between the *C. difficile* specific IgA levels in the treated groups could be caused by crucial variations between lymphocyte numbers. Additionally, because every vaccine itself and the vaccination procedure act as immune stressors, the phagocytes to lymphocytes ratio was evaluated, which is frequently reported as an indicator of physiological stress in farm animals [55]. After the first vaccination with *MucoCD-I* batch A, the DCC in the blood of the HR group was immediately dominated by especially low-diameter lymphocytes in contrast to the LR and control groups, as demonstrated by the ratio of phagocytes to lymphocytes (Fig. 2, Additional file 4: Table S4). Nevertheless, the lymphocyte percentage of the HR remained unchanged compared to the baseline in TW 3 (Fig. 3). However, the apparently increased young blood lymphocytes, together with the diminished total PBL content, led to the assumption that a higher proportion of these effector cells of the adaptive IS might have been transported to the vaccination site of the HR cows on this date. The atypical ratio of phagocytes to lymphocytes >1 in LR blood in TW 10 could not be explained to be caused by any confrontation of the body with the *C. difficile* antigens. This appraisal was assured by noticing a similar event in the Ctr group in TW 25. The discrepancy between the ratios of phagocytes to lymphocytes within the PBL of the treated groups in TW 21 was evoked by the significant increased percentage of phagocytes in the HR DCC, which depended mainly on the enhanced proportion of monocytes in contrast to the LR (Fig. 2, Additional file 4: Table S4). A modest monocytosis can be a sign of physiological stress [56], and possibly, the IS of the HR had reacted more sensitively than the LR IS by an additional SC confrontation with the antigen for the first time.

Contrary to the one-time administered vaccine batch A, the systemically re-exposure to *C. difficile* by vaccine batch B did not initially lead to increased lymphocyte percentages in the blood of HR cows, whereas their specific antibody production against *C. difficile* was clearly enhanced between TWs 17 and 21 (Fig. 3, Additional file 4: Table S4) [21]. Therefore, a boost of the existing *C. difficile* specific T and B memory cells can be assumed, causing shifts of subtypes within the relative proportion of the blood lymphocytes but without impacting the size of the relative proportion itself. The continual re-stimulation of B cells to proliferate and differentiate into antibody producing cells by intermittent re-exposure to the pathogen, as is the case in vaccination, promotes long-term antibody production, and usually the magnitude of antibody production depends on the lifespan of plasma cells [57]. Under steady-state conditions in vivo, one-time vaccination triggers initially the rise of antigen specific serum antibody levels, circulating plasma cells and activated memory B cells, but this correlation can be lost after a boost, which causes only a pronounced increase in antibody levels [53].

bMEC-related genes in SCC

No further indications for the self-arming of bMEC or their call for the assistance by professional immune cells could be detected during the first IM period. During the second IM period, the chemokines *CCL20* and *CXCL3* stuck out with a persistent down-regulation in the treated cows towards the Ctr. These chemokines released by bMEC would be aimed at the activation of lymphocytes and phagocytes, respectively [58–60]. However, frequent antigen challenging next to the mammary gland, as was implemented by the repeated PC inoculations in the area of the supramammary LNs, could have caused the bMEC' habituation on these stimuli. Since many of the investigated phagocyte-related genes, like the complement receptors and the analyzed phagocyte cytokines, were also reduced transcribed in HR and LR, the innate IS should presumably be calmed overall during the second IM period.

Gene expressions of phagocytes and lymphocytes in SCC

Following treatment with *MucoCD-I* batch A, the phagocyte-related genes were expressed rarely different between the groups, although the prevalent up-regulation of phagocyte receptors was conspicuous in HR and LR until the end of the first IM period. This could indicate a regeneration of phagocyte numbers, mainly of PMNs, in the mammary gland tissue after their previous consumption due to the removal of *C. difficile* antigens inoculated in TW 3, as is especially visible for the LR (Additional file 5: Table S5). This assumption could not be depicted by a significant down-regulation of these genes after this vaccination date but by corresponding data of the DCC (Additional file 5: Table S5) in TW 13. Although *IL1β*, *IL6* and *TLR2* were differently expressed by HR and LR each once and on separate dates during the entire treatment period, none of the examined phagocyte-related genes in the SCC were found to explain the basically different responsiveness in the treated groups. For this, the reactions within the specific IS challenged by the administered *C. difficile* vaccines seemed to be rather decisive. At the beginning of the treatment, the treated cows responded differently to vaccine batch A, as determined for the lymphocyte surface determinants *CD4* and *CD38*. Both were clearly up-regulated in the SCC of the LR towards the HR. *CD4* positive T cells can become the predominant phenotype in milk following their increased recruitment in the mammary gland during inflammatory reactions. One of their central functions is the activation of B cells in response to recognized antigens [61]. Within human breast milk cells, *CD38* displays the phenotypic hallmark of activated B lymphocytes [51]. The presence of both lymphocyte populations might have encouraged a further differentiation of B cells to generate antibody secreting cells. However, the humoral response in terms of increased anti-*C. difficile* IgA production failed to appear, and accordingly, the plasma cell marker *CD126* was finally lower expressed by the LR than by the other groups. Additionally, *IL10*, known to promote the humoral response, was repeatedly down-regulated in the treated groups during the first IM period [62]. Presumably, in favor of the cellular immunity, the *CD8β* positive lymphocytes were activated, which had shown the tendency towards their up-regulation in LR SCC from TW 3, and this alteration was verified from TW 8. *CD8β* positive T cells are the main lymphocyte subpopulation in the mammary gland and its secretions. They are able to act in a cytotoxic as well as suppressive way. *CD8β* positive T cells of the suppressive type are frequently responsible for the hypo-responsiveness of the IS of the mammary gland confronted with intramammary pathogens, like *Staphylococcus aureus* [48]. The TW 8 was also marked by high SCC levels (Fig. 5) of LR, which are generally a crucial clinical symptom of intramammary infections. The increase of SCC resulted possibly in the course of a counter-attack by *CD8β* T cells. Conflictingly, one TW later, *CD8β* of the HR was also increased, expressed towards the Ctr. The persistently inverse *IFNγ* regulations by the treated groups within the first treatment period could affirm the theory of pronounced cell-mediated immune responses developed by the LR (Fig. 4). *IFNγ* is a crucial intermediator between the innate and the adaptive IS by promoting the anti-microbicidal activity of phagocytes and the up-regulations of MHC-I and MHC-II molecules each for antigen presentation [62]. In contrast to its expression in PBL by the treated cows, *IFNγ* was sustainably over-expressed by the LR compared to its reduction in the HR. One more time, this *IFNγ* expression pattern of the treated groups emerged also during the second treatment period following the triple vaccination. However, further different alterations of lymphocyte-related genes between the treated groups could not be measured in the course of the treatment with *MucoCD-I* batch B.

Total and differential cell counts in milk

The development of the total SCCs in milk seemed not to be impaired by the treatment and remained constantly beneath the upper threshold except for the outlier measured in LR milk in TW 8 (Fig. 5). By SC vaccination unaffected concentrations of milk SCCs were also found in a study evaluating the efficacy of a *Staphylococcus aureus* bacterin against mastitis in a lactating cow model [63]. Therefore, it can be assumed that vaccinations beyond the mammary gland generally do not interfere with the cellular homeostasis of the lacteal secretions, provided that the blood-udder barrier is intact [64]. The ratios of phagocytes to lymphocytes in milk did not vary between

groups, as proven for the first IM period (Fig. 5). However, in terms of the normal level of this cell ratio in milk > 1, it should be noted that this was not applicable either for any group up to TW 6 and the LR group showed consistently cell ratios < 1 on any date of the first IM period. The examination of the differential SCCs revealed strikingly low proportions of macrophages, being usually below 10%, whereas this cell population out of the milk of healthy udders is known as the dominating share of the DCC (Additional file 5: Table S5) [31, 65]. Technical factors could have influenced the DCC in milk. Exemplarily, polyethylene plastic bottles were used for milk sampling, which might have minimized the macrophage population by adherence [65]. Furthermore, the microscopic evaluation of the different somatic cell populations could have understated the PMN fraction [66]. The outset of the second IM period was characterized by physiologically positive charged values for the ratio of phagocytes to lymphocytes in the milk of all treated cows (Fig. 5), but following the application of *MucoCD-I* batch B, this milk cell ratio diverged between HR and LR on numerous dates. While the HR could maintain the cell ratio within the normal range, the LR lost proportionally more lymphocytes than phagocytes in their lacteal secretions up to TW 23 (Fig. 5, Additional file 5: Table S5). Regarding the development of the milk lymphocyte concentrations in relation to their initial values, the delta values for the LR lymphocyte parts tended to increase while that of the HR showed an inverse drift subsequent to the PC injection of vaccine batch A (Fig. 3). Their contrary development led to clear differences between the treated groups in TWs 6 and 11. After treatment with vaccine batch B, considerably enhanced lymphocyte proportions were determined within the LR SCCs, which resulted in significant discrepancies towards the predominantly constant HR lymphocyte parts up to the end of the treatment. However, a unilateral attribution of the growth of the LR lymphocyte percentages to the vaccination program of the second IM period has to be doubted for two reasons. Firstly, the reference value used for the LR lymphocyte percentage in TW 16 was extraordinary low, which had a critical impact on the calculated delta values (Additional file 5: Table S5). Secondly, the extent of their increase seemed to be curious when taking into account that the likewise PC administered vaccine batch A, containing the imbalanced and poorer tolerated mixture of the *C. difficile* toxins and toxoids, triggered no significant altered lymphocyte concentrations in LR milk related to the baseline (TW 1). In general, the DCCs in the milk of all investigated groups indicated to be shaped by the physiological development of the milk cell populations in the course of lactation. As commonly specified for healthy cows with low SCC milk and as is particularly obvious in the Ctr group, the phagocyte portion grows, whereas the lymphocyte content declines as lactation progresses (Additional file 5: Table S5) [66].

Conclusions

To sum up, the total immune cell counts in blood but not in milk of HR and LR were altered by the vaccination with *MucoCD-I* batch A. The variations of the DCCs in blood and milk between the treated groups were not unequivocally attributable to the IM against *C. difficile*. To evaluate the DCC, shortcomings of the selected examination method, such as light microscopy, could not be excluded. The gene expression analysis of PBL spawned sustainable differences between HR and LR. In particular, their expression patterns of the examined cytokines differed significantly during the first IM period. This outcome might have been crucial for the different response of the treated groups to the vaccinations against *C. difficile* because the intercellular mediators are important to orchestrate the immune cells for thwarting a pathogen attack. In doing so, *IFNγ* seemed to be a key player, as it stood out as a potent influencing factor of the local response in the mammary gland, too. The reason for the inversely expressed *IFNγ* in the SCC towards the PBL of the treated groups remained open. By confrontation with the *C. difficile* antigens, the LR seemed to be targeted at a stronger cellular immunity of the mammary gland in contrast to the HR. This will be proven by immunohistological examinations of different lymphocyte populations settled in the udder parenchyma. As analyzed in the SCC, the low anti-*C. difficile* IgA level in LR milk seemed not to be dependent on the limited transport capacity of the associated epithelial receptor *PIGR*. This was not found as differently regulated between the treated groups. Unlike the LR, more anti-*C. difficile* IgA secreting plasma cells had possibly been attracted for homing in the HR mammary gland due to the vaccination schedule. A subsequent immunohistological analysis will verify this assumption.

Methods

Animals and vaccination

The government of Upper Bavaria permitted the animal trial (AZ 55.2-1-54-2532.6-17-2012), as described below. During a 31-week treatment period, nine early lactating *Brown Swiss* cows were repeatedly vaccinated against *C. difficile*. The *MucoCD* vaccines (IDT Biologika, Dessau-Roßlau, Germany) comprised the crucial virulence factors of *C. difficile,* formaldehyde-inactivated whole cells (strain VPI 10463) and the exotoxins, TcdA and TcdB, being partly available as toxoids. More detailed information about their composition is subject to the company secret. For stimulating the mucosal IM system,

the *MucoCD-N* vaccine was used for the biweekly nasal inoculation. With purpose of systemic IM, the *MucoCD-I* vaccine was injected via PC, IC or SC routes. The PC application was close to both supramammary LN. Two batches of *MucoCD-I* were administered. In TW 3, a single PC injection of vaccine batch A caused undesirable side effects and was consequently discharged. After the receipt of vaccine batch B, the IM via injection was administered in TW 17. The PC and the SC or IC vaccinations were scheduled at 4 weekly intervals. The detailed description of the IM routines was released by Schmautz et al. [21]. The treated cows were divided into two groups due to their immune responsiveness. The anti-*C. difficile* specific IgA content in milk was the distinctive feature and, at the end of the treatment, its total average value in cow's milk determined the group membership. Treated cows with more than 8.0 µg ml^{-1} anti-*C. difficile* specific IgA in milk belonged to the high responder (HR) group (n = 5), those with lower anti-*C. difficile* specific IgA in milk formed the low responder (LR) group (n = 4). The control group (Ctr) consisted of five early lactating *Brown Swiss* cows supplied by the research station Veitshof of the Technical University of Munich (TUM, Freising, Germany).

Milk sampling and preparation

Milk samples were taken once a week up to TW 8. Afterwards, they were collected biweekly. During the morning milking process, 500 ml of milk per cow were branched off with a TRU-Test milk meter (Lemmer-Fullwood, Lohmar, Germany), and then transferred to a wide-mouth polyethylene plastic bottle. A tenth of the milk volume taken was used for the SCC determination using optical fluorescent technique (*Fossomatic-FC* device, FOSS, Hamburg, Germany) by the contracted Milchprüfring Bayern association of Wolnzach, Germany. Out of 250 ml refrigerated raw milk the included somatic cells were extracted according to the following procedure: after centrifugation (1800×*g*, 4 °C, 15 min) the milk sample was defatted, the supernatant discarded, and the gained cell pellet re-suspended in 25 ml phosphate buffered saline (PBS, pH 7.4). Once again, the cell suspension was centrifuged with lower rotation speed (400×*g*, 4 °C, 10 min). This washing was repeated one more time. Afterwards, for the filtration step, only 10 ml of PBS were admitted to the cell pellet and then the cell suspension was percolated through a cell strainer (mesh size: 100 µm pores, Corning Inc., Corning, NY, USA). The filtrate was centrifuged (400×*g*, 4 °C, 10 min) and, after removal of the supernatant, the cells were aspirated with 250 µl of PBS. This cell extract was stored on ice until the preparation of the milk smears and the RNA extraction. For the IgA analysis, 20 ml of the raw milk sample were defatted (4000×*g*, 4 °C, 15 min), apportioned to 2-ml tubes and refrigerated at − 20 °C until further processing.

Blood sampling and preparation

In the first 8 treatment weeks, 9 ml of blood of the cow jugular vein were sampled weekly and, in the remaining treatment period, their collection was performed in a regular 2-week cycles. EDTA pre-coated vacuettes (Greiner Bio-One GmbH, Frickenhausen, Germany) were used and put immediately on ice after the sampling. The blood stabilization was belatedly upgraded by addition of 100 µl of 0.3 M EDTA [33.5 g Titriplex III (Merck KgA, Darmstadt, Germany) dissolved in 300 ml of double distilled water and supplemented with 1% acetylsalicylic acid (Merck)]. PBL were extracted out of the blood as follows: after transferring 7 ml of EDTA blood into a 50-ml tube, 14 ml of lysis buffer [8.3 g NH_4Cl (Carl Roth GmbH & CoKG, Karlsruhe, Germany), 0.37 g of Titriplex III (Merck) and 1.0 g of KCl (J.T. Baker Chemical Co. Phillipsburg, NJ, USA) dissolved in 1 l of double distilled water, pH 7.4] were added, intended for the lysis of the erythrocytes. The tube was carefully inverted three times and placed on ice until the dark red coloring of the suspension was visible. The PBL were gained after the centrifugation (400×*g*, 4 °C, 10 min) and the decantation of the supernatant. The following washing routine of the cells was done twice with 14 ml of lysis buffer in each case. Finally, the cell pellet was re-suspended in 2 ml of PBS, then the cell suspension was equally splitted into two 1.5 ml tubes and both tubes were placed on ice, one tube retained for RNA extraction and the other one for total PBL counting by using the TC10 automated cell counter (Bio-Rad Laboratories GmbH, Munich, Germany).

Clostridium difficile specific IgA in milk

The particular procedure of the used sandwich ELISA determining the *C. difficile* specific IgA in cow milk can be looked up in the previous publication by Schmautz et al. [21]. In brief, a dilution series of the standard (1.76 mg ml^{-1} of *C. difficile* specific IgA, MucoVax b. v., Leiden, Netherlands) and a thinning of the milk samples at a ratio of one to ten were prepared with PBS. In duplicates, 100 µl standard dilutions or milk samples per well were applied to 96-well plates (Nunc MaxiSorp™, Sigma-Aldrich Chemie GmbH, Munich, Germany) precoated with *C. difficile* cells (2.0×10^8 cells ml^{-1}, IDT Biologika, Dessau, Germany). The incubation lasted for 1.5 h at 37 °C and was followed by multiple rinses with wash buffer. HRP conjugated sheep anti-bovine IgA (diluted 1:70,000, Bethyl Laboratories, Inc.; Montgomery, TX 77356, USA) was used as secondary antibody

(100 µl well^{-1}) and allowed to take effect under shelter for 1.5 h at 37 °C. Multiple washings were done before the TMB substrate mix was added (150 µl well^{-1}) visualizing the antibody reactivity. After 40 min at RT, the chromogenic reaction was finished by adding the stop solution (50 µl well^{-1}). The absorbance of the colored products were measured at 450 nm with a microplate reader (Sunrise™, Tecan Group Ltd., Männedorf, Switzerland). The concentration of *C. difficile* specific IgA in every milk sample was quantified relative to the standard curve by the software Magellan™ V6.6 (Tecan) belonging to the photometer.

Differential cell counting (DCC) of milk and blood smears

For the milk smears, 20 µl of the prepared somatic cell-extract out of milk were pipetted on a standard microscope slide (76 × 26 mm, partly with frosted edge) in a meandering shape. For the blood smears, one droplet (3 µl) of the EDTA blood sample was spread out on a microscope slide with the narrow edge of a second one. Both, the milk and blood smears, were air dried and then processed with the Haema-Quick-Stain Kit (Diff-Quick; Labor + Technik, Eberhard Lehmann GmbH, Berlin, Germany) according to the manufacturer's recommendations, producing staining results equivalent to the Pappenheim method. On completely dried smears, coverglasses (60 × 24 mm) were stuck with two droplets of the mounting media Eukitt® (Sigma-Aldrich Chemie GmbH, Munich, Germany) per slide conserving the smear up to the microscopic differentiation. The evaluation was performed by light microscopy (Axioskop 2 plus, Carl Zeiss Microscopy GmbH, Göttingen, Germany). Using a "battlement track" method, between 200 and 300 cells were differentiated at 400-fold magnification for the blood smears, or at 1000-fold magnification and with oil immersion for the milk smears. The percentages of the various cell types in both body fluids were calculated in relation to the particular total cell counts of the samples.

Total RNA extraction out of SC and PBL and transcription to cDNA

The miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) was used to reap the RNA out of milk cells and PBL. For this purpose, 230 µl of somatic cell-extract or 1 ml of PBL suspension, were centrifuged for 5 min at 400×*g* and RT, respectively. After disposal of the supernatants, every gained cell pellet was lysed with 700 µl of Qiazol lysis reagent (Qiagen). Following cell disruption and homogenization for 5 min at RT, the samples could be stored interim at −80 °C. The frozen homogenates were thawed at RT, continuing the RNA extraction in accordance with the manufacturer's instructions. The total RNA was collected in 25 µl of RNase-free water departing from the manufacturer's recommendation. The yielded RNA samples were immediately placed on ice. The total RNA concentration and its purity were determined at 260 nm with the NanoDrop ND-1000 (Peqlab, Erlangen, Germany). By using the 2100 Electrophoresis Bioanalyzer Instrument and the RNA 6000 Nano Assay Kit (Agilent Technologies, Waldbronn, Germany), the RNA integrity was examined. Until further processing, the RNA samples were stored at −80 °C. For the reverse transcription of RNA to complementary DNA (cDNA), 300 ng or 1000 ng of RNA from each sample containing milk cells or PBL were used, respectively. The master mix included 5× buffer, 50 µM of random hexamers (Invitrogen Life Technologies, Darmstadt, Germany), 40 mM of dNTP Mix and 200 units of M-MLV reverse transcriptase (RNase H Minus, Point Mutant; Promega, Mannheim, Germany). At the ratio of one to three, the template and the master mix were mixed in 96-well plates (4titude® Ltd., Berlin, Germany) to achieve a total volume of 60 µl of cDNA per sample. The thermal cycling of the reverse transcription was conducted by using the TPersonal Thermocycler (Biometra GmbH, Göttingen, Deutschland). After preheating the cover of the Thermocycler up to 104 °C, the single heating steps of the thermal cycling program were annealing for 10 min at 21 °C, transcription for 50 min at 48 °C and degradation of the reverse transcriptase for 2 min at 90 °C. The entrained positive controls were RNA extracts out of bovine mammary gland tissue and bovine spleen tissue to check the effective conducting of the following RT-qPCR. Excluding of non-inherent DNase in the RNA samples, a pooled sample with RNA out of all samples for examination was carried along without adding the reverse transcriptase. RNase-free water was used as a non-template control assuring a reverse transcription of the RNA without contaminations. The sealed cDNA containing multi-well plates were stored at −20 °C.

Primer design

For the detection of transcripts of 35 target genes and seven reference genes (*ACTG1, UB3, SUZ12, GAPDH, H3F3A, YWHAZ,* and *KRT8*), the corresponding bovine primer pairs were designed utilizing the Primer-BLAST of the National Center for Biotechnology Information (NCBI, National Library of Medicine, Bethesda, MD, USA, https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed 07 Mar 2017.). The applied primers and the belonging details are compiled in the Additional file 1: Table S1. In each case, the primers functionality and optimal annealing temperature of 60 °C were tested with

cDNA of the named positive control tissues using the RT-qPCR. The following primer deviated from the intended annealing temperature: *CCR6* (58 °C); *IL10*, *CD126*, *CD163* and *PIGR* (62 °C); *SUZ12* (64 °C). The amplification efficiency of each primer was tested pursuant to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. Only primers showing >85% amplification efficiency in the RT-qPCR were used for the following gene expression measurements [67]. Furthermore, the primer specificity verified with gel electrophoresis of the PCR products was part of the quality assessment prior to usage of the primers.

Gene expression measurements

The RT-qPCR was performed with the Rotor-Gene Q cycler (Rotor-Disc 72; Qiagen GmbH, Hilden, Germany). The reaction components, 1 µl of cDNA template, 5 µl of SsoFast EvaGreen Supermix (Bio-Rad Laboratories GmbH, Munich, Germany), 400 nM forward and reverse primers (metabion international AG, Planegg, Germany) filled up with DNase-free water to a final volume of 10 µl, were poured into the cycler accessory strip tubes (0.1 ml, Qiagen GmbH, Hilden, Germany). The cycler was operated by the related Rotor-Gene Q software version 6.0.38 (Corbett Research 2004, acquired by Qiagen GmbH, Hilden, Germany). The temperature profile of the RT-qPCR comprised first of all 30 s at 98 °C, followed by 40 cycles of two steps with 5 s at 95 °C and 20 s at the primer specific annealing temperature, and finally, generating the melting curve up to 95 °C in one-degree increments. The PCR products were preserved frigidly at 8 °C.

RT-qPCR data processing

Based on the assay information outputted by the Rotor-Gene Q software (Qiagen GmbH, Hilden, Germany), the PCR performance was evaluated considering the MIQE Guidelines [67]. The PCR raw data for analysis are the generated Cq, which define the cycle number as take-off point for the detection of the specific fluorescence signal. The relative quantification of the PCR signals required a set of reference genes for normalization. The GenEx 'Normfinder' tool (GenEx software version 6.1; MultiD Analyses AB, Gothenburg, Sweden) extracted seven possible reference genes out of the raw data of all examined genes. For the normalization, six of the suggested seven reference genes were regularly used. The seventh one, KRT8, was respected only for the bMEC-related genes to be found in milk samples. After the interplate calibration based on the entrained and sample source relevant positive controls, the difference between the mean Cq value of all considered reference genes and the target gene-specific Cq was calculated per sample (ΔCq). The relative changes in gene expression to designated dates was calculated with the $2^{-\Delta\Delta Cq}$ method [68].

Statistics

The data of each individual animal were assigned to the related group time-dependently. Variations within or between groups occurring during the treatment were figured using the MIXED procedure model in SAS/STAT ® 9.22 (2010 SAS Institute Inc., Cary, NC, USA). After checking the convergence criteria by the log-likelihood calculation, the estimated least square means (LSM) plus standard errors (SD) were released by the applied statistic software. For all statistics, the significance level at $p < 0.05$ was decisive.

Additional files

Additional file 1: Table S1. Primer used for the RT-qPCR examinations. The primer construction based on the nucleotide sequences provided by the gene database of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/nuccore/, accessed 07 Mar 2017). The associated NCBI reference sequence numbers are listed in the Additional file 1: Table S1.

Additional file 2: Table S2. ΔΔCq changes in gene expression of PBL relative to TW 0 or rather TW 16.

Additional file 3: Table S3. ΔΔCq changes in gene expression of SC relative to TW 1 or rather TW 15.

Additional file 4: Table S4. Total PBL counts and the relative distribution of the different leukocyte populations.

Additional file 5: Table S5. Total SC counts and the relative distribution of the different leukocyte populations in milk.

Abbreviations

APCs: antigen presenting cells; bMEC: bovine mammary epithelial cells; CDI: *C. difficile* infection; CDAD: *C. difficile* associated diarrhea; Cq: quantitation cycle; Ctr: control; DCC: differential cell counting; HR: high responder; IC: intracutaneous; iFA: incomplete Freund's adjuvant; IM: immunization; IS: immune system; LN: lymph node; LR: low responder; MHC: major histocompatibility complex; PAMPs: pathogen-associated molecular patterns; PBL: peripheral blood leukocytes; PC: percutaneous; PMN: polymorph nucleated neutrophil; PRR: pattern recognition receptor; RT: room temperature; RT-qPCR: real time quantitative PCR; SC: subcutaneous; SCC: somatic cell count; TcdA: *C. difficile* toxin A; TcdB: *C. difficile* toxin B; TCR: T cell antigen receptor; T_C cell: cytotoxic T cell; T_H cell: T helper cell; TW: treatment week.

Authors' contributions

CS was the main person responsible for the sample analysis, data evaluation and writing of the script. NM carried out the milk sample preparation steps and the gene expression measurements. MA evaluated the milk smears. IB and HK executed the immunizations of the cows designated for treatment. MWP and HK managed the project and revised all working drafts. All named writers checked and authorized the final manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Data availability statement
All data generated or analyzed during this study are included in this published article and its additional information files.

Ethics approval and consent to participate
The animal trial was approved by the government of Upper Bavaria (AZ 55.2-1-54-2532.6-17-2012).

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Differential colonization of the bovine mammary gland and supramammary lymph nodes with lymphocytes in response to the vaccination against *Clostridium difficile*

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Short title: Lymphocytes in bovine tissues after *C. difficile* vaccination

Abstract

The purpose of this study was to ascertain, if the bovine mammary gland (**MG**) and supramammary lymph nodes (**SLN**) would finally be populated differently with lymphocytes following vaccinations with *Clostridium difficile* (***C. difficile***). Beforehand, inoculations of dairy cows were necessary to pave the way for the production of anti-*C. difficile* IgA enriched milk with the intended use as nutraceutical against human *C. difficile* infections. Referring to the anti-*C. difficile* IgA output in milk of the vaccinated cows, their immune responsiveness were significantly different. MG and SLN tissues of high and low responder cows were obtained after slaughtering. Both tissues were immunohistochemically stained with specific antibodies for CD4+, CD8+ and IgA+ lymphocytes. The population sizes of CD4+ and CD8+ T-cells as well as IgA+ antibody secreting cells in MG and SLN of high responder topped widely

those of the low responding cows. The MG of all treated cows harboured more CD4+ T-cells, and also their CD4+ to CD8+ T-cell ratios were larger compared with the control cows. In conclusion, the predominantly higher population density by lymphocytes in the tissues of the high responding cows could be a crucial factor for their more intense *C. difficile* specific IgA production in milk. In preparation of the large-scale anti- *C. difficile* milk IgA production, the preselection of dairy cows, being sensitive against the related vaccine, is recommended.

Keywords

cattle, CD4+ T-cells, CD8+ T-cells, IgA+ antibody secreting cells, immunization

Implications

Bovine milk can be used as a nutraceutical in the treatment of *C. difficile* infections of humans. The production of anti-*C. difficile* IgA enriched milk for this intended use requires the vaccination of dairy cows against *C. difficile*. The output target is a high titre of anti-*C. difficile* milk IgA. But the intensity of anti-*C. difficile* milk IgA production by the immunized cows was proven to be significantly different. The study at hand is part of the causal analysis to identify the prerequisites of cows producing the intended milk antibody at high level.

Introduction

Bovine milk is a functional food. Besides the valuable nutrient supply by milk, some ingredients possess also a therapeutic value that could serve as a new source of income for the farmers. The successful application of bovine milk immunoglobulin (**Ig**) preparations as nutraceuticals is proven in numerous clinical studies in animals

and humans (Korhonen *et al*., 2000; Steijns, 2001). The *Clostridium difficile* (***C. difficile***) infection (**CDI**) is one of the human disorders, whose therapy can be aided by the administration of an anti-*C. difficile* antibody-rich milk protein concentrate (van Dissel *et al*., 2005). The ubiquitously occurring bacterium *Clostridium difficile* (***C. difficile***) is the causing agent of the serious *C. difficile* associated diarrhea. The main virulence factors of *C. difficile* are two exotoxins, toxin A (**TcdA**) and toxin B (**TcdB**), which take effect in the gut of the host. Hypervirulent strains, producing a third toxin, the binary actin-ADP-ribosylating toxin, have emerged in the last decades (Freeman *et al.*, 2010; Monaghan *et al.*, 2015). Their treatability by use of antibiotics was marked by restricted success considering the increased rate of relapses (McFarland, 2005). In support of a solid recovery of patients with CDI or to prevent CDI, various immunological approaches have been tested (Giannasca and Warny, 2004; Zhao *et al*., 2014). A considerable improvement of the long-lasting treatment success could be reached by the application of an anti-*C. difficile* antibody-rich milk protein concentrate concomitant to the standard CDI antibiotic therapy with vancomycin or metronidazole (van Dissel *et al*., 2005; Numan *et al*., 2007). The production of milk preparations enriched with antigen specific antibodies, like anti-*C. difficile* antibodies, requires the targeted immunization of dairy cows. For this, in retrospect, two strategies were pursued, that differ in regard to the timing of anti-*C. difficile* inoculation and the primarily produced antibody class in the lacteal secretions. If the vaccination is performed during the dry period of the cow prior to the parturition, then anti-*C. difficile* IgG will be abundant in the colostrum (Sponseller *et al*., 2015). Whereas *C. difficile* specific secretory IgA (sIgA) and IgG will be contained in milk of dairy cows vaccinated against *C. difficile postpartum* (van Dissel *et al*., 2005; Numan *et al*., 2007). The treatment of CDI patients with such

hyperimmune lacteal products were proven to be effective in diminishing the CDI recurrences (van Dissel *et al*., 2005; Numan *et al*., 2007; Mattila *et al*., 2009). With respect to the human digestive tract as the operation site of the dietary supplement, the therapeutic use of the milk protein concentrate with anti-*C. difficile* sIgA as main active ingredient has advantages over anti-*C. difficile* IgG (Simon *et al.*, 2014). Exemplarily, sIgA has an anti-inflammatory capacity in terms of antigen neutralization without provoking an influx of polymorphonuclear neutrophilic granulocytes (Snoeck *et al*., 2006). Additionally, sIgA possesses a natural self-protection by the secretory component withstanding the gastro-intestinal digestion (Korhonen *et al.*, 2000). Regarding the commercial viewpoint, a large-scale anti-*C. difficile* sIgA production of hyperimmunized dairy cows during their entire lactation is in the manufacturer's interest. Therefore, an efficient immunization protocol which increases the antigen specific levels of sIgA in ruminant milk is a crucial prerequisite as shown for the production of *Candida albicans* IgA antibodies (Hodgkinson *et al*., 2007). The internal trial to immunize dairy cows against *C. difficile* intended the production of milk including anti-*C. difficile* sIgA. The treated cows were classified as low responder (**LR**) or high responder (**HR**) depending on the threshold of 8 µg/ml anti-*C. difficile* sIgA in milk on average of their annual production. In case of the LR, the treatment spawned a restricted success of immunization as measured by the milk sIgA specific for *C. difficile* (Schmautz *et al*., 2018a). In spite of refinements of the used vaccines, the administration routes and, finally, the additional application of defined adjuvants, the anti-*C. difficile* sIgA contained in LR's milk could not be increased on average. Possible reasons for the different responsiveness of the treated cows have been stepwise analyzed. The molecular biological examination of crucial factors of the innate and adaptive immune system in peripheral blood cells and somatic cells out of

101 milk indicated differences regarding the T-helper-cell responses of the vaccinates
102 (Schmautz *et al*., 2018b).
103 The objective of the study at hand was to investigate the population of the bovine MG
104 and the SLN with the lymphocytes' subsets, CD4+ and CD8+ T-cells as well as IgA+
105 antibody secreting cells (**ASC**) in anti-*C. difficile* vaccinated cows and control cows.
106
107 **Material and methods**
108
109 *Animals*
110 The approval for the animal trail was obtained from the government of Upper Bavaria
111 (AZ. 55.2-1-54-2532.6-17-2012). Six of totally nine *Brown Swiss* cows, that were
112 destined for the treatment with different *C. difficile* vaccines, were the donors of MG
113 and SLN tissues for the immunohistochemical examinations in the present study. The
114 remaining three test cows were not included within the immunohistochemical
115 examinations due to their abundant anti-*C. difficile* IgA production. The cows were
116 kept in a stanchion barn. They had anytime water access and their feeding met the
117 requirements of their milk performance as formerly specified (Schmautz *et al*.,
118 2018a). The disease history of the cows was already published in Schmautz *et al*.
119 (2018a). At the time of treatment start, the primiparous cows (n = 6) were 40 ± 9 days
120 in lactation (**DiL**) and they were continuously vaccinated against *C. difficile* pursuant
121 to the immunization schedule until their slaughter in the slaughterhouse of the
122 Bavarian State Research Center for Agriculture location Grub (Poing, Germany)
123 (Table 1). The carcasses were completely discarded without touching the food chain
124 after sampling the tissue for analysis. For ethical reasons the trial did not include the
125 permission to sacrifice untreated *Brown Swiss* cows. Therefore MG control tissue

originated from three primiparous *Simmental* cows (**Ctr** group), which were needed in a former study of our chair (Kohlenberg, 2001) (Table 1). Thus the health history of the cows providing the control tissue was known. SLN tissues of untreated cows suitable for control purposes were unavailable.

Vaccines and the immunization schedule

The used vaccines and the immunization schedule were previously disclosed (Schmautz *et al*., 2018a). In brief, different anti-*C. difficile* vaccines were used depending on the route of their parenteral administration. Their principal constituents were formaldehyde-inactivated whole *C. difficile* cells (strain VPI 10463) and a toxin/toxoid mixture of TcdA and TcdB. The exotoxins were extracted from the *C. difficile* culture filtrate. All used anti-*C. difficile* vaccines, MucoCD-N and MucoCD-I, were supplied by IDT Biologika (Dessau-Roßlau, Germany). In a perpetually two-week interval, MucoCD-N was intra-nasally inoculated in each nostril. MucoCD-I was generally applied per injection on the percutaneous route in the area of both SLN on a regular cycle of four weeks. The subcutaneous and intracutaneous vaccinations in the caudal area of the scapulae were largely intermediary. The treatment with MucoCD-I was interrupted within the treatment weeks (**TWs**) 4 and 16, because the only one-time inoculated MucoCD-I batch A (TW 3) was poorly tolerated by the cows. The treatment with the improved MucoCD-I vaccine, batch B, was continued from TW 17. From the TW 31, this vaccine was injected in combination with the incomplete Freund's Adjuvant in case of the subcutaneous administration and, with the temporal distance of two weeks, in alternation with the percutaneous vaccination.

Tissue sampling and preparation

The tissues, MG (parenchyma of the upper body) and SLN (cortex and paracortex), were collected directly *post mortem* in the slaughterhouse. Subsequently, tissue pieces of less than 1 cm^3 were snap frozen in liquid nitrogen, placed on dry ice within an aluminum sachet for transport and stored then in the deep freeze (-80 °C). After embedding the tissue with the Tissue-Tek O.C.T. compound (Sakura Finetek, Staufen, Germany), sections were cut (5 µm) by use of the microtome cryostat (HM505 E, Microm, Walldorf, Germany). They were transferred to poly-L-lysine (0.1% (w/v) in H_2O, Sigma-Aldrich, Taufkirchen, Germany) coated glass slides (76x26 mm), dried at room temperature (**RT**), fixed in -20 °C cold acetone-methanol (1:1, J. T Baker, Deventer, Netherlands) for 15 min, dried again (RT), wrapped in aluminum foil and stored in a 50 ml tube at -20 °C until use.

Antibodies

All used antibodies (**Ab**) were purchased from Bio-Rad AbD Serotec GmbH (Puchheim, Germany). As primary monoclonal mouse Ab were used anti-bovine CD4 (clone CC30, isotype IgG1)) to detect T helper ($\mathbf{T_H}$)-cells and anti-bovine CD8 (clone CC63, isotype IgG2a) to trace cytotoxic T ($\mathbf{T_C}$)-cells. The applied secondary Ab was the polyclonal goat anti-mouse IgG/fluorescein isothiocyanate (**FITC**) (isotype IgG). IgA+ ASC were recognized with the polyclonal sheep Ab anti-bovine IgA/FITC (isotype IgG). The Ab were titrated with phosphate-buffered saline (**PBS**) and the applied Ab dilutions for each examined tissue are summarized in Table 2.

Immunohistochemical staining

Prepared cryosections of the bovine spleen were used as positive controls for each Ab to analyze. Two negative control sections were produced in addition to the tissue slices intended for cell counting to evaluate the background and to control the staining with the secondary antibody. The defrosted tissue sections (at RT) were framed with liquid blocker (Super Pap Pen, Daido Sangyo, Tokyo, Japan) and then rehydrated by triple washing with Tris-buffered saline (**TBS**, for 1, 5 and 9 min at RT) on a plate shaker (**PS**). For blocking unspecific binding sites, 10% (v/v) goat serum (Sigma-Aldrich, Taufkirchen, Germany) in PBS was applied for 1 h at RT in a humid chamber (**HC**). After removing the blocking solution, the primary Ab was spread on each section (Table 2) except for the negative control sections that were only covered with PBS. The incubation was over night at 4 °C (in HC). Surplus Ab solutions were cleared away by triple washing with TBS (shaded, on PS). In case of unlabeled primary Ab, the secondary Ab dilution was applied to the tissue sections (Table 2) except for one negative control section, which was treated with PBS once more. After incubation for 45 min at RT (shaded, in HC), the excess Ab solutions were removed with three TBS washes. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (**DAPI**; 1/1 000 in PBS, Sigma-Aldrich, Taufkirchen, Germany) for 15 min at RT (shaded, in HC). Following four TBS washes (shaded, on PS, for 1, 5, 5 and 9 min at RT) to get rid of DAPI solution residues, the Ab bindings were fixed by use of 10% (w/v) paraformaldehyde (Merck, Darmstadt, Germany) in PBS on each section for 15 min at RT (shaded, in HC). Remains of the fixation buffer were washed away with TBS for 5 min at RT (shaded, on PS). To preserve the immunohistochemical stained tissue slices, fluoromount aqueous mounting medium (Sigma-Aldrich, Taufkirchen, Germany) was dropped on the glass slides, and

198 subsequently, cover glasses (60x24 mm) were applied. The edges of the cover
199 glasses were sealed with clear nail polish to prevent the desiccation of the stained
200 tissue slices during their storage (shaded, 4 °C) until their microscopic examination.
201
202 *Quantitative evaluation of data*
203 The immunohistochemical stained tissue slices were analyzed by use of the
204 Axioskop 2 Plus fluorescence microscope, equipped with the digital camera
205 AxioCam MRc and the Axiovision 3.1 imaging software (Carl Zeiss Microscopy,
206 Göttingen, Germany). After microscopic evaluation (200x magnification) of the
207 negative and positive controls, the immune cells of interest were examined in 15
208 photographs per slice and overall ten slices of each tissue, MG and SLN. The
209 meandering course of the microscopic examination prevented multiple photographs.
210 The randomly selected image areas were photographed in the Ab labeling related
211 FITC channel (green color, 530 nm) and in the cell nuclei marking related DAPI
212 channel (blue color, 460 nm). By aid of the free photo editing software
213 PhotoScape 3.7 (created by Mooi Tech; http://www.photoscape.org), photographs of
214 the same image areas and both colors were put on top of each other. Fluorescent
215 signals that showed clearly a blue shining nucleus surrounded by a bright green
216 circle were considered as Ab specific stained cell for counting. The total count of
217 positive events per picture (0.1462 mm^2) was converted to the cell count per 1 cm^2
218 image area used as data basis for the average cell count per slice.
219
220 *Statistics*
221 The averaged lymphocyte counts per tissue slice were the data basis for the
222 following statistical analysis with SigmaPlot 11.0 (2008 Systat Software, Inc., San

223 Jose, CA 95131, USA). The statistic software operated with ten cell counts entered
224 per Ab staining and group (HR, LR and Ctr) owing to ten tissue slices evaluated for
225 each group. The significance level was set at $P<0.05$. The Kruskal-Wallis One Way
226 ANOVA on Ranks was executed for all analyzed lymphocyte counts in the MG
227 including the ratio of CD4+ to CD8+ T-cells due to the missing normality of the data
228 basis. The applied pairwise multiple comparison procedure based on the Tukey Test.
229 In case of the additionally examined lymphocytes in the SLN tissues of the treated
230 groups, the t-test was implemented for the IgA+ ASC and the Mann-Whitney Rank
231 Sum Test was used for the CD4+ and CD8+ T-cells after their failed normality tests.
232 Depending on the statistical method of testing, the mean and standard deviation or
233 the median with the belonging lower and upper quartiles were the outputs and used
234 for comparing the analyzed groups.
235
236 **Results**
237
238 *Distribution of T-lymphocyte subsets and IgA+ ASC in the bovine lactating mammary*
239 *gland*
240 The average numbers of CD4+ T-cells per cm^2 MG tissue of the groups were 2 247
241 $\pm$850.6 for the HR, 1 669 $\pm$957.9 for the LR and 379 $\pm$173.6 for the Ctr. The statistical
242 incidences of CD4+ T-cells in the treated groups towards the Ctr were proven as
243 significantly different only for the belonging median values (Figure 1, A). The stained
244 T_H-cells were predominantly located solitary in the connective tissue between the
245 alveoli of the udder. The CD8+ T-cells were spaced separately in close proximity to
246 the alveolar epithelium. Their counts per square centimeter averaged 7 153 $\pm$4 412.3
247 for the HR, 3 038 $\pm$1 414.9 for the LR and 4 213 $\pm$3 150.6 for the Ctr. The median of

248 the HR's T_C-cells amounted to 7 296/cm^2 differing significantly from those of the other
249 groups as depicted in part B of Figure 1. The ratios of CD4+ to CD8+ T-cells were <1
250 for each group. Especially, the Ctr differed significantly from the other groups with a
251 very low ratio of the T-cell subsets (Figure 1, D). Likewise to the investigated T-cell
252 populations, the IgA+ ASC appeared without clustering, but they were considerably
253 rarer distributed in the parenchyma of the MG. The mean values of the counted
254 plasma cells per square centimeter tissue were 238 $\pm$150.7 for the HR, 64 $\pm$54.4 for
255 the LR and 61 $\pm$36.5 for the Ctr. The median of the HR's IgA+ ASC was 244/cm^2 and
256 at least four times higher than those of the other groups (Figure 1, C). The MG
257 parenchyma of HR and LR stained with DAPI and anti-bovine IgA/FITC in
258 comparison to the negative and positive controls are exemplarily shown in Figure 2.
259 The mean values of counted lymphocytes in every MG slice are provided in the
260 Supplementary Tables S1-S3.
261
262 *Distribution of T-lymphocyte subsets and IgA+ ASC in the supramammary lymph*
263 *node*
264 The supplementary immunohistochemical analysis of the target lymphocytes in the
265 SLN of the treated cows revealed that the HR's SLN were more densely populated by
266 the T-cell subsets than the LR's by comparing on ranks. The averaged numbers of
267 CD4+ T-cells per square centimeter SLN tissue were 5 031 $\pm$5 501.3 for the HR and
268 1055 $\pm$959.6 for the LR. Their median values differed significantly by the factor of
269 about three as shown in the part A of Figure 3. The median belonging to the counted
270 CD8+ T-cells were more close to each other (Figure 3, B). The SLN of HR contained
271 with 5062 T_C-cells/cm^2 twice as much than the LR's. The averages of the T_C-cell
272 counts in SLN were 7 799 $\pm$6 798.2/cm^2 for the HR and 3 881 $\pm$3 661.0/cm^2 for the

LR. Likewise, the ratio of CD4+ to CD8+ T-cells differed by the factor two between both groups concerning the median values (Figure 3, D) amounting to 0.57 for the HR and 0.23 for the LR. The averaged T-cell ratio of the HR was 1.1, which was characterized by the massive scattering of the raw data leading to the SD of 1.3. Exclusively among all objects of analysis, the population sizes of IgA+ ASC in SLN of the treated groups were statistically comparable by their mean values. The LR's SLN were colonized lower by these plasma cells than that of the HR by approximately 20% (Figure 3, C). 2 421 $\pm$766.2 IgA+ ASC/cm^2 were counted in SLN of HR and 1 876 $\pm$571.7 IgA+ ASC/cm^2 in that of LR. The lymphocyte counts per each SLN slice are compiled in the Supplementary Tables S4-S6.

Discussion

The vaccination trial against *C. difficile* with dairy cows revealed the different immune responsiveness of the vaccinated cows as measured by the intended anti-*C. difficile* specific IgA contents in milk (Schmautz *et al*., 2018a). This finding gave reason to believe that the colonization of the MG and the SLN with lymphocytes may be different between the HR and LR as these immune cells are indispensable for the generation of an effective specific immunity (Sordillo *et al.*, 1997). Indisputably, the significance of the study at hand was limited by some factors listed below. Firstly, the HR cows were not slaughtered at the same physiological state of lactation compared to the LR and Ctr cows (Table 1). While the LR and Ctr cows produced milk on a medium level at date of sacrifice, the HR cows were going to drying-off. But mainly macrophages are attracted among the leukocytes populations with the incipient involution (Sinowatz *et al*., 2000). Thus the numbers of lymphocytes should not be influenced by the different dates of slaughter. Secondly, the MG control tissues

originated from cows of another breed. Among breeds milk yield and the gross milk composition may significantly vary (Schutz *et al*., 1990). But leukocyte numbers prevalent within the MG and also as part of the somatic milk cell count are primarily influenced by the health and physiological state of the cows (Bradley and Green, 2005). Thirdly, the nonexisting Ctr group for comparison with the analyzed lymphocytes in the SLN of treated cows curtails the informative value of the belonging findings. However, the analysis of SLN was valuable regarding the significantly different mean values of IgA+ ASC between HR and LR (Figure 3, C). Fourthly, and lastly, the overall IgA+ ASC were examined instead of *C. difficile* specific IgA+ ASC. The applicable antibody for verification of last-named ASC lacked. Generally, the overall IgA+ ASC include only a small portion *C. difficile* specific IgA+ ASC. But cross-reactions in response to the injected antigen are able to cause an increase of the total IgA+ ASC numbers as previously shown in milk (Schmautz *et al.*, 2018a). Despite all these limitations listed above, the research results in the present study are important for tracing the different immune responsiveness of the anti-*C. difficile* vaccinated cows.

The examined lymphocytes in the mammary gland

The CD4+ and CD8+ T-cell numbers as well as the IgA+ ASC counted in HR's MG tissue were clearly above those in the other groups apart from the LR's CD4+ T-cell count (Figure 1, A-C). Insofar, the repeated anti-*C. difficile* inoculations might have caused a stronger homing of these lymphocytes to the HR's MG, and consequently, the HR had an higher potential to produce more specific antibodies in milk. Shifts in the lymphocyte proportions can be initiated by various factors. Physiological states, like parturition and lactation, and also local pathological changes, such as mastitis,

323 influence the number and the functionality of lymphocyte subsets in the MG (Shaver-
324 Weaver *et al*., 1996; Tanneau *et al*., 1999; Leitner *et al*., 2003; Ezzat Alnakip *et al.*,
325 2014). Similarly, targeted intramammary immunizations with pathogenic
326 microorganisms, as practiced exemplarily with *Candida albicans*, can trigger a
327 multiplication of T-lymphocytes and plasma cells, especially of IgA+ ASC
328 (Hodgkinson *et al.*, 2009). The repeated vaccination has encouraged the settlement
329 of T_H cells in all treated cows to a similar extent, which was determined to be denser
330 compared to the Ctr cows. Nevertheless, the nature of the T_H cell response, that is
331 characterized by its specific cytokine profile, could have been different among the
332 treated groups. The results of the previous gene expression profiling in blood and
333 milk cells of the treated cows corroborate this assumption (Schmautz *et al*., 2018b).
334 Depending on the promoted T_H cell subtype, the antibody formation by B-cells is
335 differently emphasized (Kidd, 2003). Consequently, the IgA+ ASC colonization of the
336 HR's MG could have been more supported than of the LR's MG. Finally, this could
337 have been the prerequisite for the HR's more intensive anti-*C. difficile* IgA production
338 in milk. Concerning the CD8+ T-cells, the study at hand verified also the
339 predominance of this T-cell subpopulation in the MG as referred hitherto (Sordillo *et*
340 *al*., 1997; Ezzat Alnakip *et al*., 2014). Confirming their tissue location in our study,
341 they accumulate generally close to the mammary epithelium. Thereby, they may
342 contribute crucially to maintain the integrity of the mucosal epithelium in the MG
343 (Taylor *et al.*, 1994). CD8+ T-cells are either of the cytotoxic or suppressor type
344 (Sordillo *et al*., 1997; Ezzat Alnakip *et al*., 2014). Possibly, the vigorously local
345 immune response in the HR's MG to the anti-*C. difficile* inoculations has necessitated
346 a stronger influx of CD8+ T-cells for acting immunoregulatory (Figure 1, B). However,
347 different levels of the CD4+ to CD8+ T-cells' ratios did not result between HR and LR

but between them and the Ctr (Figure 1, D). Each examined T-cells' ratio was <1 as typical for the bovine MG (Shaver-Weaver *et al*., 1996; Yamaguchi *et al*., 1999).

Lymphocytes differentiated in the supramammary lymph nodes

The efficient recognition of antigens by immune cells is ensured in lymph nodes by control of the afferent and efferent lymph flow. Thus, in case of antigen stimulation, internodally recirculating lymphocytes can quickly accumulate within these peripheral lymphoid organs and they have a high chance to encounter the cognate antigen (Girard *et al*., 2012). Therefore, T- and B-cell numbers can be increased in the draining lymph node post infection of the adjacent tissue as it was demonstrated for SLN due to mastitis (Yang *et al*., 1988; Trigo *et al*., 2009). On the contrary, the relative parts of different lymphocyte subsets in SLN of chronically *Staphylococcus aureus* infected udders remained unchanged and no histological differences between SLN of healthy and control cows were found (Leitner *et al*., 2003). Likewise, following vaccinations with *Brucella abortus* in cattle, T-cell subsets in lymph nodes draining the injection sites were not depleted (Kunkle *et al*., 1995). In the present vaccination study with *C. difficile*, the distribution of lymphocytes in the examined SLN of HR and LR was significantly different for the median values of the T-cell subsets and for the averaged IgA+ ASC (Figure 3, A-C). The tissue slices of HR's SLN comprised more of each examined immune cell type than that of the LR. These results correspond also to those ascertained in the MG of the treated groups. The CD4+ to CD8+ T-cell ratio, which is generally expected to be >1 in bovine SLN, just like in peripheral blood (Shaver-Weaver *et al*., 1996), was found to be below the base level (Figure 3, D). To compare the HR's and the LR's T-cell ratios, that of the LR showed a strikingly low level of the median value with only 0.2 due to fewer CD4+ T-cell counts.

Consequently, the T-cell dependent activation of B-cells, resulting from the antigenic stimulation by the *C. difficile* inoculations, may be impaired in the LR's tissue. Additionally, the slight preponderance of HR's CD8+ T-cells could indicate the necessarily stronger regulation of the HR's specific immune response than in case of the LR.

Conclusions

In sum, MG and SLN of HR and LR cows were colonized with CD4+ and CD8+ T-cells as well as with IgA+ ASC significantly different. The population density by lymphocytes ascertained in the HR's tissues was predominantly higher than in the LR's ones, which may have been partly responsible for the more intense production of *C. difficile* specific IgA in HR's milk compared to LR. One crucial element on the path to commercialize the anti-*C. difficile* enriched milk protein as nutraceutical will be the selection of dairy cows being sensitive towards the related vaccine. For this, the *in vitro* analysis of molecular markers described formerly is recommended as possible tool to identify suitable candidates (Schmautz *et al*., 2018b).

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Declaration of interest

The authors declare that they have no competing interests.

Ethics approval

The animal trial was approved by the government of Upper Bavaria (AZ 55.2-1-54-2532.6-17-2012).

Software and data repository resources

All data are available upon request.

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509 **Table 1** *Background for the different dates of slaughter*

Cows	Vaccinated against *C. difficile*	DiL[1] to the date of slaughter	Statement to the date of slaughter
LR[2]-2	yes	330	minimal anti-*C. difficile* sIgA production in milk (<3 µg/ml) (Schmautz *et al*., 2018a)
LR-6	yes	341	
LR-7	yes	337	
HR[3]-4	yes	849	
HR-5	yes	720	natural drying-off
HR-10	yes	545	
Ctr[4]-*Coka*	no	252	PhD thesis of Kohlenberg (2001) investigating relevant transcriptional factors for different physiological states of lactation
Ctr-*Golda*	no	240	
Ctr-*Azalee*	no	360	

510 [1] DiL = days in lactation

511 [2] Low responder cow

512 [3] High responder cow

513 [4] Control cow

514

515 **Table 2** *Antibodies for the immunohistochemical staining*

primary Ab[1] & secondary Ab	Used Ab dilutions for MG[2] tissue	for SLN[3] tissue
anti-bovine CD4 & anti-mouse IgG/FITC	1/150 & 1/50	1/150 & 1/40
anti-bovine CD8 & anti-mouse IgG/FITC	1/200 & 1/100	1/200 & 1/100
anti-bovine IgA/FITC	1/400	1/500

516 [1] Antibody

517 [2] Mammary gland

518 [3] Supramammary lymph node

519

Figure captions

Figure 1 Lymphocyte counts of CD4+ T-cells (Figure part A), CD8+ T-cells (Figure part B), IgA+ antibody secreting cells (ASC) (Figure part C) and the ratio of CD4+ to CD8+ T-cells (Figure part D) in the mammary gland of the anti-*C. difficile* vaccinated cows (LR = low responder, HR = high responder) and control cows (Ctr). Significant differences ($P<0.05$) between the groups are marked by lower case letters (a, b).

Figure 2 Cell nuclei and IgA+ antibody secreting cells (ASC) immunohistochemically stained in mammary gland parenchyma of a high responder cow (A1, A2) and a low responder cow (B1, B2) are marked by white arrows (x200, scale bar represents 50 µm). C1 and C2 present the negative control (x100, scale bar represents 100 µm). C2 includes an auto-fluorescent debris. The IgA+ ASC are the bright green circles in the spleen used as positive control (D2) (x100, scale bar represents 100 µm).

Figure 3 Lymphocyte counts of CD4+ T-cells (Figure part A), CD8+ T-cells (Figure part B), IgA+ antibody secreting cells (ASC) (Figure part C) and the ratio of CD4+ to CD8+ T-cells (Figure part D) in the supramammary lymph nodes of the anti-*C. difficile* vaccinated groups (LR = low responder, HR = high responder). Significant differences ($P<0.05$) between the groups are marked by lower case letters (a, b).

Figure 1

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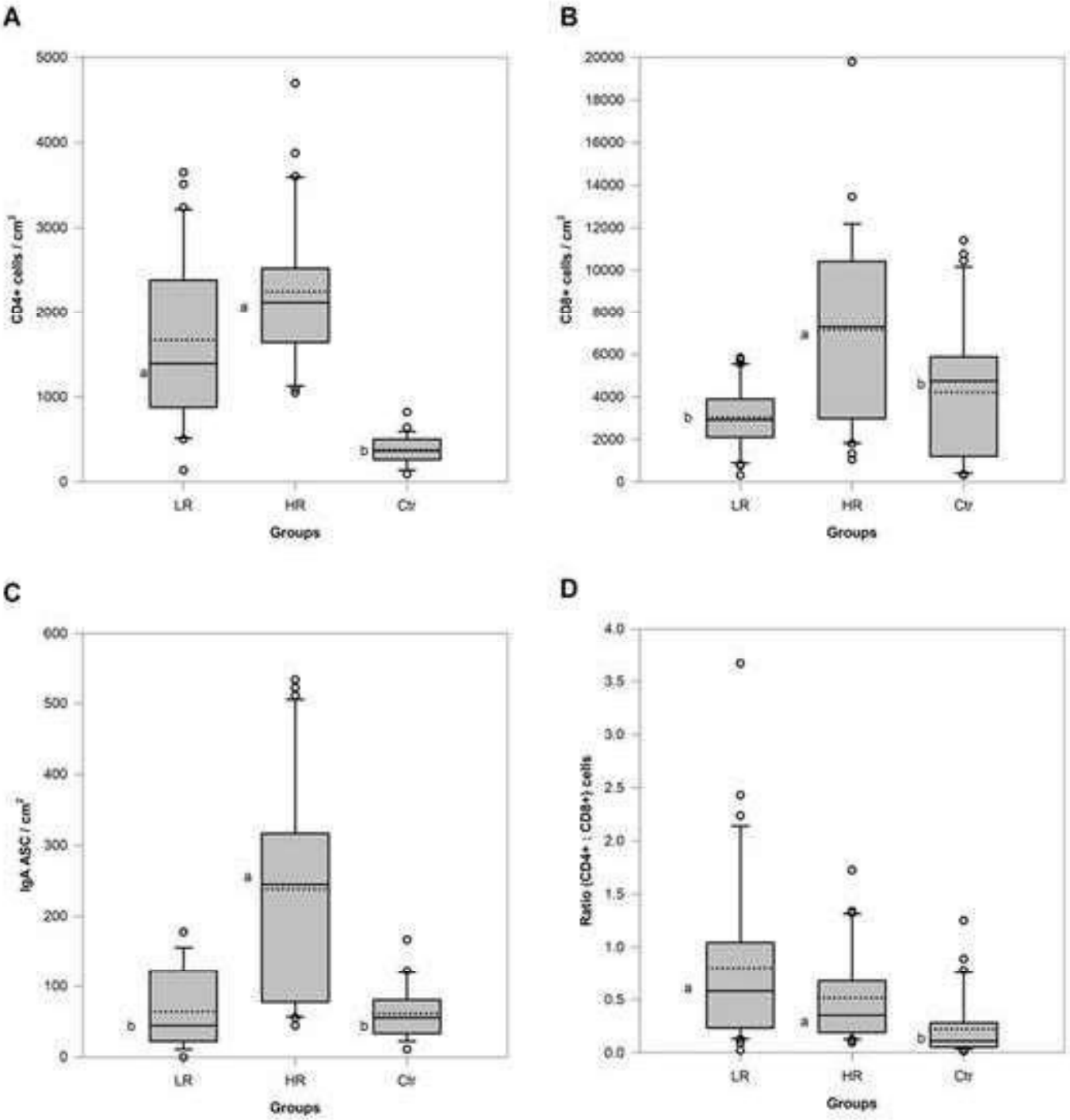

Figure 2

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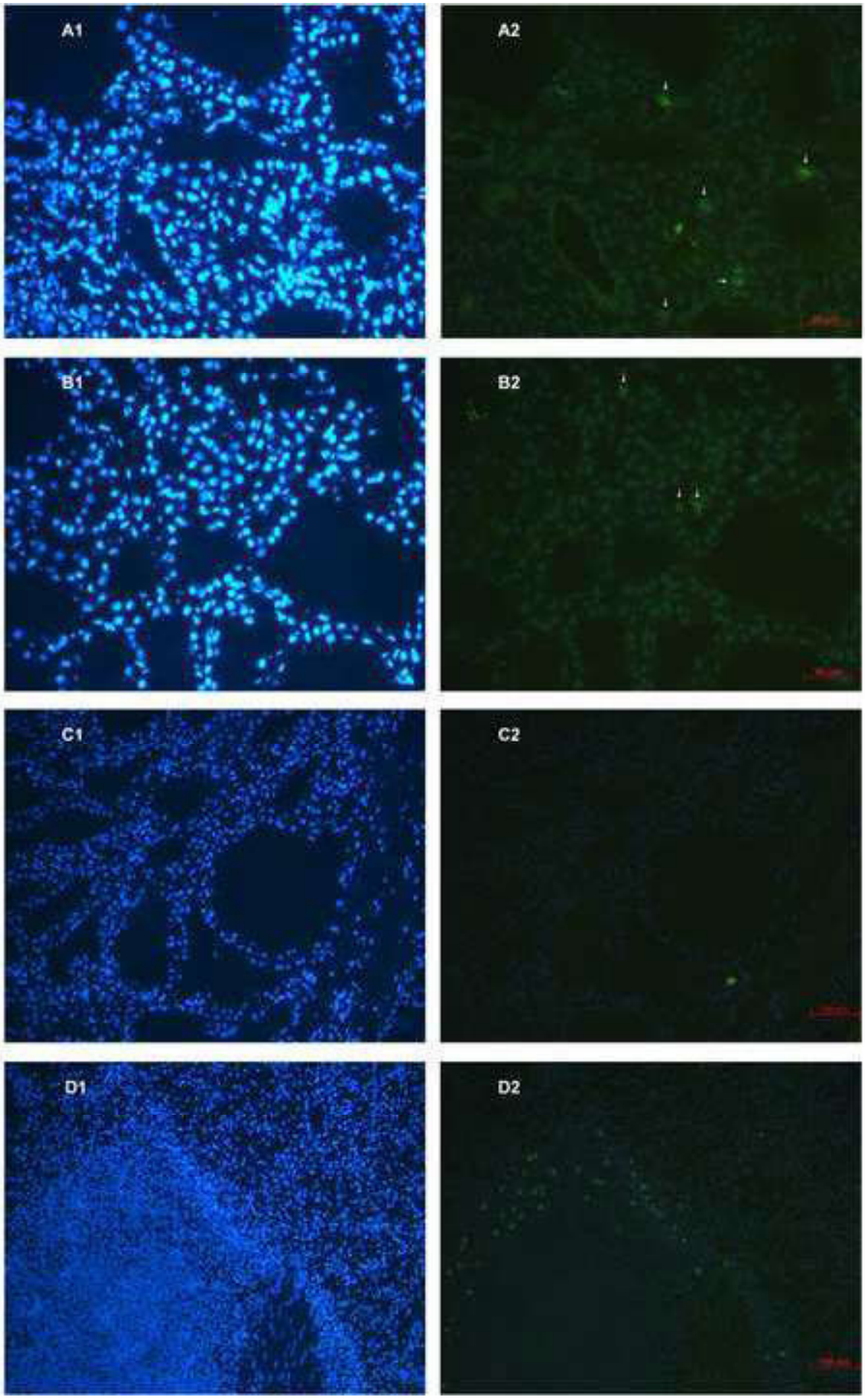

Figure 3

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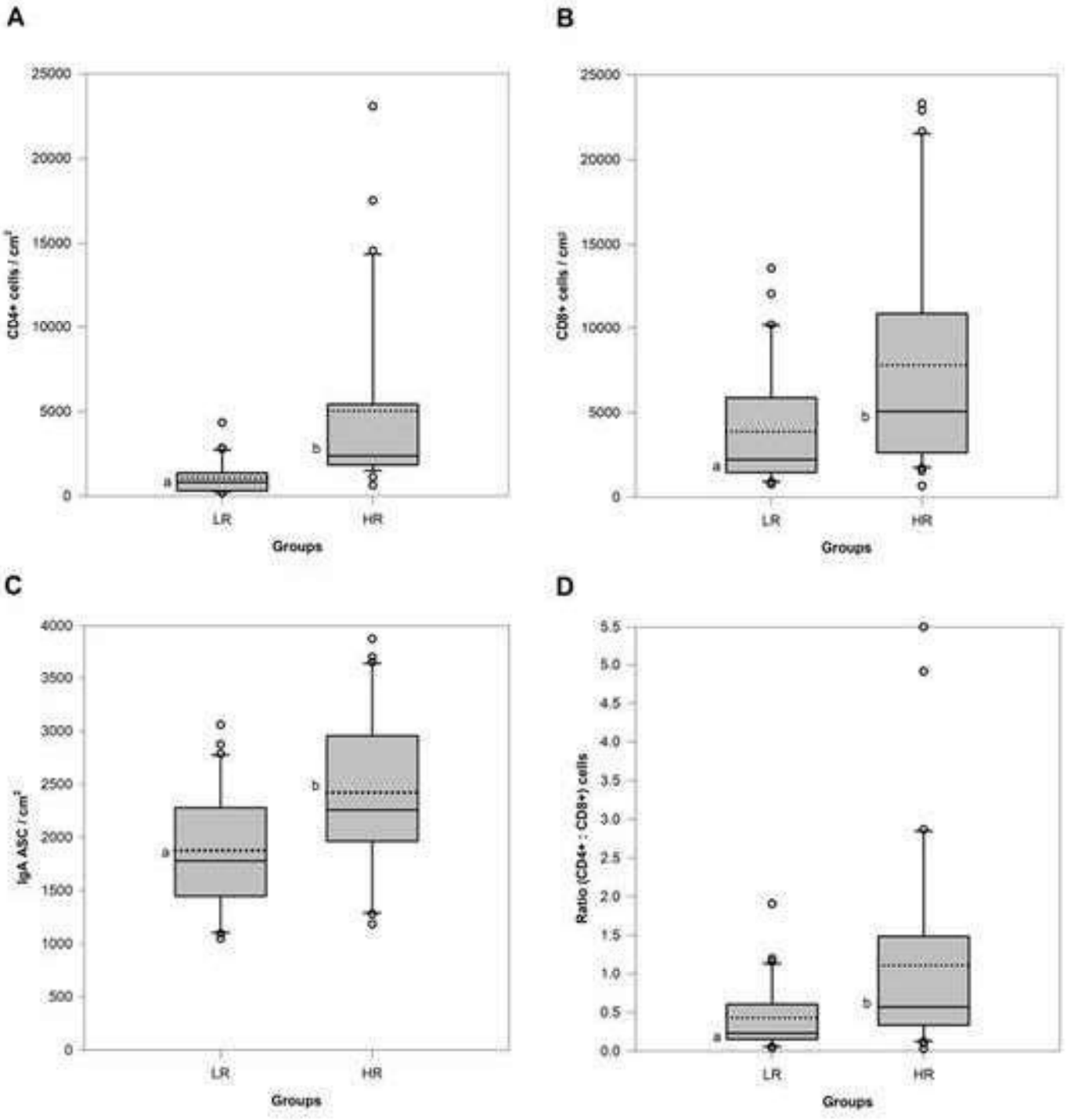

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Differential colonization of the bovine mammary gland and supramammary lymph nodes with lymphocytes in response to the vaccination against *Clostridium difficile*

C. Schmautz, S. Wißmiller, M. Auer, I. Ballweg, M. W. Pfaffl and H. Kliem

Supplementary Table S1 CD8+ T cell counts of MG tissue

Cow	slice-1		slice-2		slice-3		slice-4		slice-5		slice-6		slice-7		slice-8		slice-9		slice-10	
	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*
LR-2	3.9	*1.37*	3.6	*0.92*	8.4	*0.62*	8.5	*0.99*	4.4	*1.11*	4.2	*0.90*	3.1	*0.58*	2.5	*0.55*	1.1	*0.29*	n.a.	*n.a.*
LR-6	2.8	*0.58*	5.7	*1.11*	5.1	*0.84*	5.8	*0.88*	1.2	*0.38*	4.5	*0.83*	4.3	*0.71*	6.5	*1.35*	3.1	*0.68*	4.9	*0.81*
LR-7	2.3	*0.57*	5.0	*0.75*	3.1	*0.97*	3.7	*0.62*	5.1	*0.51*	5.9	*1.52*	7.8	*1.25*	4.3	*0.98*	4.0	*0.75*	8.1	*1.28*
HR-4	10.7	*1.43*	6.5	*1.09*	7.3	*1.27*	7.7	*1.39*	10.8	*1.24*	16.3	*0.93*	14.9	*1.61*	17.8	*2.07*	17.8	*1.74*	28.9	*1.81*
HR-5	19.7	*2.28*	16.8	*2.21*	8.7	*1.46*	10.7	*1.32*	5.9	*0.72*	12.5	*2.21*	13.5	*2.38*	13.3	*2.19*	13.4	*2.20*	16.5	*3.10*
HR-10	4.2	*1.27*	4.2	*0.96*	2.6	*0.79*	3.3	*1.53*	1.9	*0.37*	1.5	*0.31*	6.5	*1.06*	2.9	*0.42*	12.5	*2.16*	4.4	*0.70*
Ctr-*Coka*	15.3	*1.39*	6.9	*1.04*	8.6	*1.23*	7.9	*1.40*	7.8	*1.02*	15.7	*1.54*	7.7	*1.24*	5.5	*0.82*	3.1	*0.55*	8.9	*1.80*
Ctr-*Golda*	16.7	*1.44*	7.9	*1.08*	9.4	*1.22*	7.7	*0.94*	5.3	*1.19*	7.3	*1.20*	8.6	*1.20*	11.1	*1.60*	6.9	*1.30*	3.4	*0.42*
Ctr-*Azalee*	1.7	*0.27*	0.5	*0.24*	1.4	*0.36*	2.8	*0.77*	1.8	*0.39*	1.9	*0.50*	1.1	*0.26*	0.6	*0.19*	0.5	*0.24*	0.6	*0.27*

"MG" = mammary gland; "LR" = low responder cow; "HR" = high responder cow; "Ctr" = control cow; "mean" referred to averaged cell number of 15 photographs per tissue slice; "n.a." = not available

Supplementary Table S2

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Differential colonization of the bovine mammary gland and supramammary lymph nodes with lymphocytes in response to the vaccination against *Clostridium difficile*

C. Schmautz, S. Wißmiller, M. Auer, I. Ballweg, M. W. Pfaffl and H. Kliem

Supplementary Table S2 CD4+ T cell counts of MG tissue

Cow	slice-1		slice-2		slice-3		slice-4		slice-5		slice-6		slice-7		slice-8		slice-9		slice-10	
	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*
LR-2	3.2	*0.51*	1.9	*0.34*	0.2	*0.11*	2.0	*0.32*	3.3	*0.45*	1.6	*0.41*	3.5	*0.40*	2.3	*0.32*	2.5	*0.35*	1.1	*0.3*
LR-6	3.5	*0.40*	5.1	*0.47*	2.1	*0.40*	3.9	*0.38*	4.4	*0.57*	4.7	*0.53*	5.3	*0.61*	3.7	*0.40*	3.3	*0.53*	2.9	*0.43*
LR-7	1.9	*0.41*	1.1	*0.32*	1.5	*0.41*	1.6	*0.34*	1.0	*0.28*	0.7	*0.18*	1.6	*0.21*	0.9	*0.25*	1.3	*0.23*	0.7	*0.18*
HR-4	6.9	*0.83*	3.6	*0.39*	2.4	*0.49*	2.9	*0.39*	2.7	*0.61*	3.0	*0.43*	3.5	*0.93*	2.4	*0.48*	3.4	*0.51*	3.6	*0.51*
HR-5	4.3	*0.50*	2.6	*0.39*	3.3	*0.54*	3.9	*0.64*	3.3	*0.56*	5.1	*0.81*	2.1	*0.28*	1.6	*0.39*	3.2	*0.38*	1.5	*0.36*
HR-10	5.3	*0.75*	5.7	*0.80*	2.9	*0.42*	4.4	*0.49*	3.3	*0.29*	1.6	*0.38*	2.2	*0.35*	2.1	*0.40*	2.9	*0.68*	2.9	*0.44*
Ctr-*Coka*	0.9	*0.17*	0.6	*0.19*	0.9	*0.24*	0.7	*0.21*	0.5	*0.17*	0.6	*0.16*	0.8	*0.22*	0.9	*0.15*	0.7	*0.21*	1.2	*0.31*
Ctr-*Golda*	0.5	*0.19*	0.4	*0.16*	0.1	*0.09*	0.5	*0.22*	0.2	*0.14*	0.4	*0.16*	0.1	*0.09*	0.6	*0.13*	0.9	*0.19*	0.7	*0.13*
Ctr-*Azalee*	0.2	*0.11*	0.7	*0.19*	0.5	*0.17*	0.3	*0.13*	0.5	*0.17*	0.5	*0.17*	0.3	*0.13*	0.5	*0.13*	0.3	*0.15*	0.5	*0.17*

"MG" = mammary gland; "LR" = low responder cow; "HR" = high responder cow; "Ctr" = control cow; "mean" referred to averaged cell number of 15 photographs per tissue slice

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Differential colonization of the bovine mammary gland and supramammary lymph nodes with lymphocytes in response to the vaccination against *Clostridium difficile*

C. Schmautz, S. Wißmiller, M. Auer, I. Ballweg, M. W. Pfaffl and H. Kliem

Supplementary Table S3 IgA+ ASC counts of MG tissue

Cow	slice-1		slice-2		slice-3		slice-4		slice-5		slice-6		slice-7		slice-8		slice-9		slice-10	
	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*
LR-2	0.2	*0.11*	0.2	*0.14*	0.1	*0.09*	0.3	*0.12*	0.3	*0.12*	0.3	*0.12*	0.0	*0.00*	0.2	*0.14*	0.2	*0.11*	0.3	*0.1*
LR-6	1.1	*0.33*	0.7	*0.21*	0.6	*0.19*	0.9	*0.25*	0.3	*0.12*	0.7	*0.21*	0.9	*0.24*	0.9	*0.32*	0.8	*0.30*	0.9	*0.23*
LR-7	0.1	*0.07*	0.3	*0.16*	0.2	*0.11*	0.1	*0.07*	0.4	*0.19*	0.0	*0.00*	0.1	*0.09*	0.1	*0.09*	0.2	*0.11*	0.1	*0.09*
HR-4	3.1	*0.67*	2.7	*0.56*	1.7	*0.35*	2.3	*0.55*	2.4	*0.49*	3.1	*0.52*	1.1	*0.40*	3.2	*0.34*	1.2	*0.22*	1.9	*0.49*
HR-5	1.9	*0.35*	1.5	*0.32*	1.7	*0.25*	1.7	*0.45*	1.8	*0.28*	1.1	*0.31*	1.5	*0.29*	1.3	*0.32*	1.6	*0.31*	2.0	*0.37*
HR-10	0.4	*0.16*	0.5	*0.17*	0.5	*0.17*	0.3	*0.13*	0.3	*0.15*	0.4	*0.16*	0.5	*0.19*	0.7	*0.30*	0.4	*0.16*	0.3	*0.12*
Ctr-*Coka*	0.5	*0.22*	0.5	*0.19*	0.7	*0.21*	0.4	*0.13*	0.5	*0.17*	1.0	*0.28*	0.6	*0.16*	0.5	*0.40*	0.5	*0.22*	0.7	*0.23*
Ctr-*Golda*	0.3	*0.15*	0.5	*0.24*	0.4	*0.16*	0.3	*0.21*	0.3	*0.13*	0.4	*0.21*	0.5	*0.13*	0.1	*0.07*	0.1	*0.09*	0.3	*0.13*
Ctr-*Azalee*	0.1	*0.07*	0.2	*0.11*	0.2	*0.11*	0.3	*0.13*	0.2	*0.14*	0.1	*0.09*	0.3	*0.15*	0.1	*0.09*	0.1	*0.09*	0.2	*0.11*

"ASC" = antibody secreting cells; "MG" = mammary gland; "LR" = low responder cow; "HR" = high responder cow; "Ctr" = control cow; "mean" referred to averaged cell number of 15 photographs per tissue slice

Supplementary Table S4

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Differential colonization of the bovine mammary gland and supramammary lymph nodes with lymphocytes in response to the vaccination against *Clostridium difficile*

C. Schmautz, S. Wißmiller, M. Auer, I. Ballweg, M. W. Pfaffl and H. Kliem

Supplementary Table S4 CD8+ T cell counts of SLN tissue

Cow	slice-1		slice-2		slice-3		slice-4		slice-5		slice-6		slice-7		slice-8		slice-9		slice-10	
	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*
LR-2	2.1	*0.40*	2.1	*0.36*	1.3	*0.29*	3.5	*0.63*	1.9	*0.32*	1.7	*0.37*	2.6	*0.66*	2.4	*0.49*	3.1	*0.47*	2.1	*0.4*
LR-6	4.8	*0.67*	14.9	*5.05*	8.9	*2.94*	11.1	*2.98*	5.2	*1.10*	3.6	*1.09*	5.5	*1.22*	4.1	*1.21*	8.5	*2.57*	2.3	*0.55*
LR-7	19.9	*3.98*	14.3	*2.14*	13.5	*2.21*	17.7	*5.24*	1.7	*0.42*	1.3	*0.29*	2.5	*0.42*	2.4	*0.32*	4.3	*0.55*	1.1	*0.26*
HR-4	3.1	*0.98*	6.1	*1.27*	4.8	*0.89*	5.6	*1.02*	3.6	*1.09*	4.5	*1.04*	10.1	*1.47*	4.4	*1.03*	9.4	*1.55*	2.5	*0.43*
HR-5	34.1	*5.40*	31.7	*11.37*	13.2	*2.27*	29.7	*6.60*	8.1	*1.43*	20.2	*6.10*	9.1	*1.86*	18.4	*3.60*	15.1	*2.32*	13.2	*3.47*
HR-10	5.3	*1.33*	6.7	*2.09*	13.2	*2.73*	33.5	*6.43*	1.0	*0.34*	3.9	*0.70*	21.9	*4.27*	3.5	*0.52*	2.3	*0.73*	3.9	*1.21*

"SLN" = supramammary lymph node; "LR" = low responder cow; "HR" = high responder cow; "mean" referred to averaged cell number of 15 photographs per tissue slice

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Differential colonization of the bovine mammary gland and supramammary lymph nodes with lymphocytes in response to the vaccination against *Clostridium difficile*

C. Schmautz, S. Wißmiller, M. Auer, I. Ballweg, M. W. Pfaffl and H. Kliem

Supplementary Table S5 CD4+ T cell counts of SLN tissue

Cow	slice-1		slice-2		slice-3		slice-4		slice-5		slice-6		slice-7		slice-8		slice-9		slice-10	
	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*
LR-2	0.3	*0.19*	4.1	*1.33*	0.9	*0.27*	1.7	*0.53*	0.4	*0.16*	0.7	*0.19*	0.5	*0.17*	0.5	*0.19*	2.7	*0.90*	0.4	*0.19*
LR-6	4.1	*1.20*	2.0	*0.60*	1.5	*0.35*	1.6	*0.62*	1.3	*0.37*	3.3	*0.73*	6.3	*2.29*	2.0	*0.70*	2.2	*0.58*	0.5	*0.19*
LR-7	1.1	*0.31*	0.7	*0.27*	0.5	*0.17*	1.5	*0.40*	0.3	*0.15*	1.6	*0.79*	0.7	*0.21*	0.3	*0.15*	1.9	*0.49*	0.7	*0.29*
HR-4	6.9	*0.83*	3.6	*0.39*	2.4	*0.49*	2.9	*0.39*	2.7	*0.61*	3.0	*0.43*	3.5	*0.93*	2.4	*0.48*	3.4	*0.51*	3.6	*0.51*
HR-5	18.2	*2.63*	0.9	*0.36*	3.1	*0.95*	2.9	*1.19*	2.6	*0.70*	33.7	*5.20*	6.3	*1.76*	6.3	*1.24*	2.2	*0.54*	2.2	*0.87*
HR-10	6.1	*1.51*	17.8	*4.25*	1.7	*0.40*	25.6	*7.52*	2.9	*0.90*	21.3	*3.96*	11.4	*2.10*	3.4	*1.43*	11.1	*1.69*	6.7	*1.16*

"SLN" = supramammary lymph node; "LR" = low responder cow; "HR" = high responder cow; "mean" referred to averaged cell number of 15 photographs per tissue slice

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Differential colonization of the bovine mammary gland and supramammary lymph nodes with lymphocytes in response to the vaccination against *Clostridium difficile*

C. Schmautz, S. Wißmiller, M. Auer, I. Ballweg, M. W. Pfaffl and H. Kliem

Supplementary Table S6 IgA+ ASC counts of SLN tissue

Cow	slice-1		slice-2		slice-3		slice-4		slice-5		slice-6		slice-7		slice-8		slice-9		slice-10	
	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*	MS	*SD*
LR-2	2.5	*0.60*	2.1	*0.58*	3.2	*0.83*	2.9	*0.85*	3.0	*0.74*	4.5	*1.64*	3.1	*0.82*	3.6	*0.71*	4.1	*1.14*	3.3	*0.85*
LR-6	2.2	*0.47*	1.7	*0.42*	1.9	*0.49*	1.5	*0.43*	2.3	*0.53*	2.0	*0.40*	1.5	*0.36*	1.6	*0.49*	2.1	*0.51*	2.2	*0.55*
LR-7	2.4	*0.36*	3.1	*0.86*	4.2	*1.22*	3.3	*0.89*	3.9	*0.89*	3.7	*0.69*	2.1	*0.63*	2.3	*0.72*	2.7	*0.81*	3.2	*0.61*
HR-4	3.1	*0.62*	3.1	*0.56*	1.9	*0.50*	3.4	*0.65*	2.5	*0.80*	2.9	*1.13*	2.5	*0.45*	5.7	*1.55*	5.4	*0.95*	4.9	*0.81*
HR-5	3.1	*0.56*	3.9	*0.75*	3.5	*0.68*	4.5	*1.00*	3.1	*0.57*	1.7	*0.34*	3.3	*0.78*	4.3	*1.00*	5.3	*1.05*	1.9	*0.47*
HR-10	4.1	*0.92*	5.1	*1.23*	5.2	*1.99*	2.0	*0.81*	4.1	*0.90*	2.9	*0.98*	3.0	*0.94*	3.0	*0.65*	3.3	*1.02*	3.5	*0.66*

"ASC" = antibody secreting cells; "SLN" = supramammary lymph node; "LR" = low responder cow; "HR" = high responder cow; "mean" referred to averaged cell number of 15 photographs per tissue slice