Medical Microbiology

Bacterial Infections and Immunity

at the UMC Utrecht

Group Jos A.G. van Strijp

Therapeutic antibodies as alternative to antibiotics in the treatment of superficial skin infections with Staphylococcus

-Thesis-

for attainment of the academic degree of

Master of Science (M.Sc.)

Department of Biotechnology

University of Natural Resources and Life Sciences, Vienna

Curriculum: Biotechnology

presented by

Laura Wagner, B.Sc.

Utrecht, November 2018

Period of work:	01/05 – 30/11/2018
Supervisors:	Univ. Prof. Dr. Jos A.G. van Strijp
	Univ. Prof. Dipl.-Ing. Dr. Reingard Grabherr
	Dr. Beatrix Förster

Eidesstattliche Erklärung

Ich erkläre eidesstattlich, dass ich die Arbeit selbständig angefertigt habe. Es wurden keine anderen als die angegebenen Hilfsmittel benutzt. Die aus fremden Quellen direkt oder indirekt übernommenen Formulierungen und Gedanken sind als solche kenntlich gemacht. Diese schriftliche Arbeit wurde noch an keiner Stelle vorgelegt.

_____ _____

Datum, Ort Unterschrift der Verfasserin

Acknowledgements

I would like to thank those, who supported me throughout my studies, especially during the pursuit of my master project.

First, I would like to thank Univ. Prof. Dr. Jos van Strijp for giving me the opportunity to perform the practical part of my master thesis at the Medical Microbiology Department at the University Medical Center Utrecht (UMCU). I would also like to thank Univ. Prof. Dipl.-Ing. Dr. Reingard Grabherr for the supervision of my external master project at the University of Natural Resources and Life Sciences (BOKU).

In particular, I would like to acknowledge Dr. Beatrix Förster for the excellent daily supervision and constructive collaboration. I greatly appreciate the commitment and the support during my research and the writing process.

Last but not least, I would like to thank my family and friends for their unwavering encouragement and motivation during my studies.

Zusammenfassung

Staphylococcus pseudintermedius ist ein kommensales opportunistisch pathogenes Bakterium auf hündischer Haut und Schleimhaut. In Gegenwart von Hautbarriereschädigungen und Immunsuppremierung können sich bakterielle Hautentzündungen wie zum Beispiel Pyodermien entwickeln. Das Aufkommen von antibiotika-resistenten, zoonotischen Keimen erschwert die Behandlung von *S. pseudintermedius*. Der Druck zur Entwicklung neuer Therapieansätze für die effektive Behandlung von bakteriellen Infektionen, scheint immerwährend zu zunehmen.

In dieser Masterarbeit wurde die pharmakologische Testung polyklonaler boviner IgG Antikörper zur Behandlung kutaner *Staphylococcus pseudintermedius* Infektionen thematisiert. Mittels *ex-vivo* Experimenten konnte ein inhibitorischer Effekt auf die Kolonisierung der Hautoberfläche und auf die infektionsbedingte Hautschädigung nachgewiesen werden. Die Resultate legen somit das Fundament für zukünftige *in-vivo* Studien.

Abstract

Staphylococcus pseudintermedius is a commensal resident on canine skin and mucous membranes. *S. pseudintermedius* can become an opportunistic pathogen as skin develops susceptibility to bacterial infections due to skin barrier disruption and immune suppression. Zoonotic *Staphylococcus pseudintermedius* has recently been recognized as a growing threat for humans. Antimicrobial resistance to methicillin and other antibiotics has narrowed down treatment options in veterinary settings. In order to preserve antimicrobial efficacy, a one-health approach in the usage of antibiotics has to be implemented.

In this thesis the effect of therapeutic bovine IgG antibodies as alternatives to antibiotics in the treatment of superficial skin infections with *Staphylococcus* was tested. Treatment with bovine IgG from colostrum in *ex-vivo* experiments with canine skin not only reduced colonization, but also skin damage. Furthermore, the cytokine inflammation signal could be reduced. The results can provide guidance for *in-vivo* experiments in the future.

Table of contents

1. Introduction 9

1.1. Antibody therapy against infectious diseases 9

1.2. Antibiotic resistance in veterinary settings 13

1.3. Biotechnological recovery of bovine IgG from Colostrum milk 14

1.3.1. Composition of bovine milk 14

1.3.2. Immune components in bovine milk and colostrum 16

1.3.3. Immunoglobulin G (IgG) 18

1.3.4. Bioseparation of bovine IgG from Colostrum 20

1.3.5. Preparative chromatography for the isolation of bovine IgG from Ultrafiltration retentate 23

1.3.6. Formulation of bovine IgG in hydrogel 24

1.4. Canine skin infections 26

1.4.1. Canine skin and its immune system 26

1.4.2. Staphylococcus pseudintermedius 28

1.4.3. Canine pyoderma 31

1.4.1. Atopic dermatitis in dogs 33

2. Objectives 35

3. Materials and Methods 36

3.1. Pilot microfiltration experiment 36

3.2. Enzyme-linked immunosorbent assay (ELISA) 37

3.3. SDS-PAGE and Western Blot 37

3.4. Antibodies from bovine Colostrum 38

3.5. Staphylococcus pseudintermedius library 38

3.6. Rabbit red blood cells (RBC) haemolytic assay 40

3.7. Minimal inhibitory concentration assay 40

3.8. Biofilm formation assay 41
3.9. Formulation of bovine IgG 41
3.10. Canine *ex-vivo* model 41
3.11. Histology 42
3.12. Immunofluorescence microscopy 42
3.13. Cell culture 43
3.14. Bacterial adhesion and cell viability assay with MSCEK cells 43
3.15. Bacterial adhesion and cell viability assay with canine skin tissue 44
3.16. Scanning electron microscopy 44
3.17. Isolation of RNA from skin tissue 45
3.18. Isolation of RNA from MSCEK 45
3.19. cDNA synthesis 45
3.20. Quantitative polymerase chain reaction (qPCR) 46
4. Results 48
4.1. Pilot microfiltration experiment 48
4.2. Formulation of bovine IgG in hydrogel 51
4.3. Toxigenicity of *S. pseudintermedius* on rabbit erythrocytes 52
4.4. Antibiotic resistance of *S. pseudintermedius* in correlation with biofilm formation 54
4.5. The effect of bovine IgG on growth of antibiotic resistant *S. pseudintermedius* 56
4.6. The effect of bovine IgG on biofilm formation by antibiotic resistant *S. pseudintermedius* 58
4.7. Interface of colonization and infection of S. pseudintermedius in canine epidermal keratinocytes 61
4.8. Expression of pro-inflammatory cytokines in canine keratinocytes in presence of *S. pseudintermedius* 63
4.9. Interface of colonization and infection of *S. pseudintermedius* in a canine *ex-vivo* skin model 64

4.10. Investigating the bacterial infections in a canine *ex-vivo* model 68

4.11. Effect of bacterial infections and treatments on the expression level of pro-inflammatory cytokines in canine skin tissue.....77

5. Discussion.....79

Bibliography.....90

Annex.....98

I. Raw data of microfiltration pilot experiment 98

II. Primers for canine keratinocytes for qPCR 98

II.I. Housekeeping-genes.....98

II.II. Cytokine-genes.....99

Table of figures

Figure 1 The virulence factors of Staphylococcus aureus as possible targets for antibody therapy 10

Figure 2 Composition of milk. 14

Figure 3 Structure of Immunoglobulins 19

Figure 4 Comparison of mechanism of dead-end (A.) and cross-flow (B.) filtration. 21

Figure 5 Bioseparation of milk components with filtration membranes. 22

Figure 6 Combinational bioseparation process for the isolation of bovine IgG from Colostrum consisting of micro- and ultrafiltration in diafiltration mode. 23

Figure 7 Hematoxylin and eosin staining (HE stain) of canine skin. 27

Figure 8 Colonization of Staphylococcus pseudintermedius on dogs. 28

Figure 9 Examples of recurrent (>3 months) canine pyoderma with MRSP. 32

Figure 10 Influences on the pathogenesis of canine atopic dermatitis. 34

Figure 11 ELISA results of microfiltration permeate and retentate samples 48

Figure 12 A. Scan of SDS-PAGE with table summarizing the molecular weights (MW) of important milk proteins. 49

Figure 13 Western Blot of microfiltration samples. 50

Figure 14 Process monitoring of microfiltration pilot experiment. 51

Figure 15 Comparison of anti-hemolytic activity of AT AB and bovine IgG. 53

Figure 16 Hemolytic decrease by anti AT AB for A. 69687 and B. GL151A 54

Figure 17 Determination of antibiotic resistance profiles of S. pseudintermedius library and correlation of antibiotic resistance and biofilm formation. 56

Figure 18 Effect of bovine IgG on the growth of antibiotic resistant S. pseudintermedius in a solo and combinational approach. 57

Figure 19 Testing of anti-biofilm capability of bovine IgG for 69687 and GL151A 59

Figure 20 Combinational treatment approach with clindamycin and bovine IgG for A. 69687 and B. GL151A 60

Figure 21 Canine epidermal keratinocytes (MSCEK) image at 40x magnification.....61

Figure 22 Viability of canine epidermal keratinocytes after incubation with 10^4 GL151A (A.) and 10^6 GL151A (B.).....62

Figure 23 Colony forming units (CFU) of adherent S. pseudintermedius on canine epidermal keratinocytes.....63

Figure 24 Regulation of cytokine production (A. TNFα and B. IL-12p40) during bacterial infection in MSCEK cells. 64

Figure 25 Immunofluorescence microscopy of 3 µm skin cryosection. 65

Figure 26 Scanning electron microscopy images of skin biopsies cultivated with S. pseudintermedius.....66

Figure 27 Scanning electron microscopy images of skin biopsies cultivated with S. pseudintermedius and treated with 10 mg/mL clindamycin (A.) or 10 mg/mL bovine IgG (B.) ...68

Figure 28 Hematoxylin-Eosin staining of canine skin. 69

Figure 29 Challenges in the development of an ex-vivo skin model.....70

Figure 30 MRSA Colorex™ Chromogenic Media (bioTRADING Benelux B.V., The Netherlands) plate showing the multi-resistant S. pseudintermedius as pink colonies and methicillin-resistant S. lentus as blue colonies. 70

Figure 31 Ex-vivo skin model with clinical isolate 69687.....71

Figure 32 Quantification results for normal dog skin number 17 (NDS17) with 69687. 72

Figure 33 Quantification results for normal dog skin number 18 (NDS18) with 69687. 73

Figure 34 Ex-vivo skin model with clinical isolate GL151A.....74

Figure 35 Quantification results for normal dog skin number 19 (NDS19) with GL151A. 75

Figure 36 Quantification results for normal dog skin number 20 (NDS20) with GL151A. 76

Figure 37 Colony forming unit (CFU) of adherent bacteria on canine skin.....77

Figure 38 Expression of Th1 cytokine TNFα (A.) and Th2 cytokine IL-13 (B.) in NDS17 and NDS18 with bacterial strain 69687. 78

Table of tables

Table 1 Biological antibacterial agents in clinical development 12

Table 2 Summary of important milk proteins according to their molecular weight. 16

Table 3 Comparison of immunoglobulin content in bovine and human colostrum and mature milk. 17

Table 4 Antibiotic resistance profiles of sequenced Staphylococcus pseudintermedius library. 39

Table 5 cDNA reaction mixture 46

Table 6 Protocol for the transcription of RNA to cDNA 46

Table 7 qPCR reaction mixture 47

Table 8 qPCR protocol 47

Table 9 Cytokines produced by canine keratinocytes 63

Table 10 Cytokines produced by canine skin 78

List of abbreviations

Abs	Antibodies
agr	Accessory gene regulator
AIP	Autoinducing peptide
AMR	Antimicrobial resistance
antiDNP	Anti Dinitrophenyl
AT	Alpha-toxin
AT AB	Anti-alpha toxin IgG
bovIgG	Bovine IgG
BSA	Bovine serum albumin
CAD	Canine atopic dermatitis
CBP	Convalescent blood products
cDNA	Copy Deoxyribonucleic acid
CDR	Complementary determining region
CFU	Colony forming units
CMC	Carboxymethylcellulose
C_t	Cycle threshold
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent Assay
EPS	Extracellular polymer substances
FAO	Food and Agriculture Organization
FITC	Fluorescin isothiocyanate
GMP	Good manufacturing practice
ICD	Implantation of a cardioverter-defibrillator device
Ig	Immunoglobulin
mAb	Monoclonal antibody
MDR MSSP	Multidrug resistant Methicillin susceptible *S. pseudintermedius*
MDR MRSP	Multidrug resistant Methicillin resistant *S. pseudintermedius*
MHC I & II	Major histocompatibility complex I & II

MIC	Minimal inhibiting concentration
MRSA	Methicillin-resistant *S. aureus*
MRSP	Methicillin resistant *S. pseudintermedius*
MSCEK	Canine epidermal keratinocyte cell line
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSP	Methicillin susceptible *S. pseudintermedius*
NDS	Normal dog skin
OD	Optical density
OIE	World Organization for Animal Health
PAMPs	Pathogen associated molecular patterns
PAS	Periodic acid Schiff
PBP2a	Penicillin-binding protein
PMN	Polymorphonuclear neutrophil cells
qPCR	Quantitative Polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species enzymes
SC	Somatic cells
SIET	Staphylococcus pseudintermedius exfoliative toxins
SIG	*Staphylococcus intermedius* group
Sps	*Staphylococcus pseudintermedius* surface proteins
ROS	Reactive oxygen species enzymes
WHO	The World Health Organization

1. Introduction

1.1. Antibody therapy against infectious diseases

The discovery of antibiotics has been one of the biggest milestones in medical history. Effective antimicrobials have saved numerous lives and enabled fast progression of modern medicine. Antibiotic therapy has become an indispensable part of medicine due to availability, efficacy and low-cost[1]. Unfortunately, broad-spectrum antibiotics have been falsely and excessively used and as a result antimicrobial resistance (AMR) emerged. AMR is a global threat to not only veterinary, but also human health. Zoonotic diseases, which involve antibiotic resistant bacteria, are a growing problem and effective therapy from a one-health perspective is desirable[2]. The fight against antimicrobial resistance includes not only the reduction in general antibiotic prescription, but also a narrow treatment, the correct drug regimen and education. Although the medical need for new antibiotics is more relevant than ever, the development pipeline has been stagnating over the past years. Despite there has been a global effort of scientists to evolve new therapy strategies including antibodies, probiotics, lysins, bacteriophages and vaccines[3].

Especially the antibody-based approach has been widely acknowledged for treatment in infectious diseases. Antibodies (Abs) are single-pathogen antibacterial agents, which not only prevent infection, but also fight infectious diseases alone and in adjunction with classical antibiotics[1]. Abs are protein components of the adaptive immune response that are produced by B-cells. Antibodies specifically bind antigens of microbes to inhibit their host cell recognition[4]. Passive immunization with Abs can simultaneously neutralize bacteria and support the immune response in its activity. The benefits of antibody therapies include their specificity in pathogen recognition without impairing the microbiome, the preservation of antibiotics and the high safety levels, as anti-bacterial antibodies have no effect on the host cells[5]. The first attempts in antibody therapy date back to the 1880s and the serum therapy experiments against diphtheria by Emil van Behring[6]. Serum therapy can be defined as the transfer of specific humoral antibodies from an animal known to have a high titer of antibodies in its blood to another animal for immediate short-term immunization[7]. This therapy approach was neglected by the scientific community due the lack of standardized high-quality blood serum, serum sickness and high costs and the discovery of penicillin by Sir Alexander Fleming in 1928[8]. However, in rapid emerging infection outbreaks with no treatment options, such as the Ebola outbreak in West-Africa in 2014, passive immunizations with convalescent blood products (CBP) from patients that survived Ebola were

the only treatment options and thus, the science community once again gained interest in this technique[9].

The global emergence of methicillin-resistant *S. aureus* (MRSA) infections has become a major public health issue that has to be combated. *Staphylococcus aureus (S. aureus)* is a commensal cutaneous and mucosal gram-positive bacterium carried by 30% of the human population. In cases of disruption of skin or mucous membrane via chronic skin conditions, wound or surgical intervention, *S. aureus* can migrate to the blood stream and cause mild diseases such as impetigo or severe, life-threatening infections including pneumonia, endocarditis and sepsis. Humans with invasive medical devices or compromised immune systems[10]. The pathogenesis of the bacterium includes diverse virulence factors (see **Fehler! Verweisquelle konnte nicht gefunden werden.**) such as surface-associated proteins, carbohydrate structures and secreted factors for pinning down the complement system, suppressing antibody activity and killing of host cells. Virulence factors are distinct neutralization targets in antibody therapy. Numerous potential antibodies have failed in clinical trials until this day and proved that successful suppression of staphylococcal infections can only be the result of recognition of multiple *S. aureus* antigens and triggering of phagocytosis by neutrophils and opsonization for activation of the complement system[11].

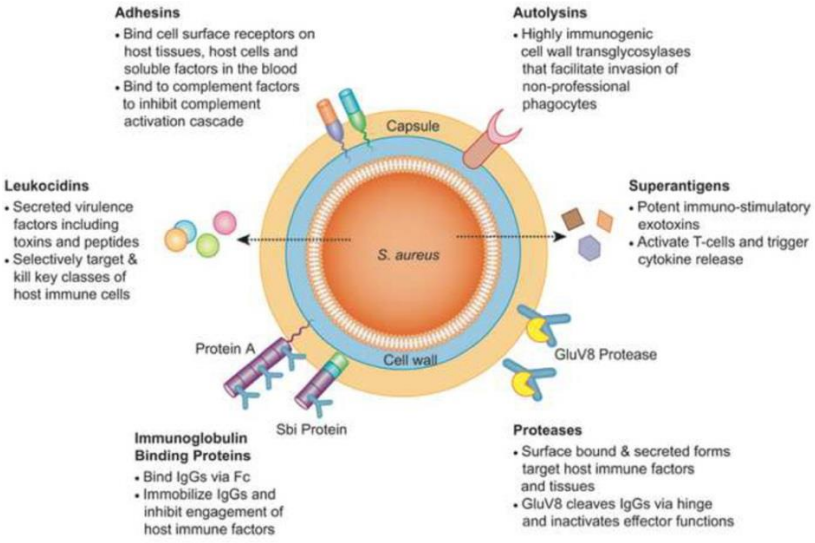


Figure 1 The virulence factors of Staphylococcus aureus as possible targets for antibody therapy [12].

The human polyclonal IgG antibody Altastaph was the first candidate in a Phase II clinical trial. Altastaph was extracted from the plasma of donors which received vaccine immunization with two

types of capsular polysaccharides (CP5 and CP8). The antibody proved to protect mice with *S. aureus* infections, but failed the proof of inhibiting bacteremia. Genetic mutations in the polysaccharide capsule and single antigen recognition limit the application. The biggest challenge was the large-scale production of polysaccharides in adequate amounts and high quality[13]. However, CP5 and CP8 remain interesting targets for future treatments.

Second generation monoclonal antibodies (mAb) against *S. aureus* such as ASN-1 and ASN-2 (Arsanis Biosciences GmbH, Biocenter Vienna, Austria), include multiple targets and combinational therapy approaches. ASN-1 effects α-toxin, which lyses endothelial and epithelial cells, and four leukocidins (LukSF-PV, LukED, HIgAB, HIgCB), which are responsible for the disruption of bacterial-killing phagocytes[14]. ASN-2 neutralizes another leukocidin (LukGH). The combinational therapy approach (ASN-100) can be seen a polyclonal antibody variant as multiple targets are being recognized. ASN-100 was under investigation in the clinical Phase II trial for the prevention of staphylococcal pneumonia in mechanically ventilated patients. Both mAbs proved *in-vitro* to prevent lysis of human neutrophils in presence of recombinant cytotoxins and native toxins. ASN-100 restored granulocytes, monocytes, natural-killer cells and T-lymphocytes derived from human blood[15]. However, the Phase II clinical trial failed in proving its effectiveness in mechanically ventilated patients[16]. Evaluation and publication of the data accumulated during the study on behalf of Arsanis Biosciences GmbH is up-to-date owed. The failure of Altastaph and ASN-100 stress the degree of difficulty in developing antibody therapy against *S. aureus*. **Fehler! Verweisquelle konnte nicht gefunden werden.** summarizes up-to-date biological antibacterial agents in clinical development (November 2018).

Table 1 Biological antibacterial agents in clinical development adapted from Theuretzbacher et al. [17]. C. difficile = Clostridum difficile, S. aureus= Staphylococcus aureus, P. aeruginosa= Pseudomonas aeruginosa; A= active, NA= non applicable

	Clinical Phase	**Antibody type**	**Route of administration**	**Developer**	**Expected activity against priority pathogens**		
					P. aeruginosa	*S. aureus*	*C. difficile*
DSTA-4637S	1	Anti *S. aureus* IgG monoclonal antibody/rifamycin	IV	Genentech/Roche	NA	A	NA
PolyCab	1	*C. difficile* polyclonal antibody	IV	MicroPharm	NA	NA	A
IMM-529	1/2	*C. difficile* polyclonal antibody	Oral	Immuron	NA	NA	A
AR-301 (tosatoxumab)	1/2	Anti *S. aureus* IgM monoclonal antibody	IV	Aridis	NA	A	NA
514G3	1/2	Anti *S. aureus* IgG monoclonal antibody	IV	XBiotech	NA	A	NA
SAL-200	2	Phage endolysin	IV	Intron	NA	A	NA
CF-301 (exebacase)	2	Phage endolysin	IV	Contrafect	NA	A	NA
Suvratoxumab	2	Anti *S. aureus* IgG monoclonal antibody	IV	MedImmune	NA	A	NA
MEDI-3902	2	Anti *P. aeruginosa* IgG monoclonal antibody	IV	MedImmune	A	NA	NA
AR-105 (Aerucin)	2	Anti *P. aeruginosa* IgG monoclonal	IV	Aridis	A	NA	NA

1.2. Antibiotic resistance in veterinary settings

AMR in companion animals, such as dogs, cats and horses, is an emerging threat in a one-health perspective. As microbes do not only affect humans, but also the entire eco-system containing animals and plants, a generalized effort including the environment as a whole, is the only choice in securing public health. The World Health Organization (WHO) has defined the most important aspects in the one-health approach to be food safety, control and surveillance of zoonotic diseases and combatting antimicrobial-resistance. Cooperation between the WHO, the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) promote the one-health approach[18].

Close contact to pets can lead to increasing rates in zoonotic transmissions of antibiotic resistant bacteria. Not only the interspecies transmission, but also the transfer of resistance genes has to be acknowledged. In veterinary settings, antimicrobials are used in therapy but also as prophylaxis. Data of antimicrobial prescription in veterinary medicine usually originate from drug manufacturer sales[19]. The effort in surveillance of AMR in companion animals has been widely neglected and therefore represents an important prospective research field[20]. Some antimicrobials authorized for human medicine are also used in companion animals, leading to challenges in the treatment of antibiotic resistant bacteria. Reserving antimicrobials for human mankind is of course always associated with an ethical question regarding animal welfare.

Different species of bacteria, such as *Staphylococci*, *Enterococci*, *Streptococci*, *Escherichia coli*, *Salmonella, Pseudomonas* and *Acinetobacter*, have been associated with AMR[20]. *Staphylococcus pseudintermedius* and *Staphylococcus aureus* are very significant bacteria in terms of human and veterinary medicine. Reports of human-to-dog and dog-to-human transmission of *S. aureus*[21] and *S. pseudintermedius*[22] show the significance of fighting antimicrobial resistance. In this thesis, a new anti-infective therapy against multi-resistant *S. pseudintermedius* was subject for pharmacological testing. The therapeutic effect of bovine immunoglobulins against skin infections for topical treatment was assessed. Furthermore, the biotechnological production and formulation of bovine IgG as a drug was broached.

1.3. Biotechnological recovery of bovine IgG from Colostrum milk

1.3.1. Composition of bovine milk[23]

Milk can be referred as a *post partum* lacteal secretion produced by the mammary gland containing heterogeneous components, which perform a wide variety of chemical and biological activities[24]. The biochemical composition of the milk depends on multiple endogenous and exogenous factors such as race, genetic background, animal feed, number of lactation-cycles, time point within the lactation cycle and health status. Bovine milk contains 88% water; the dry mass can be amounted to 12%.

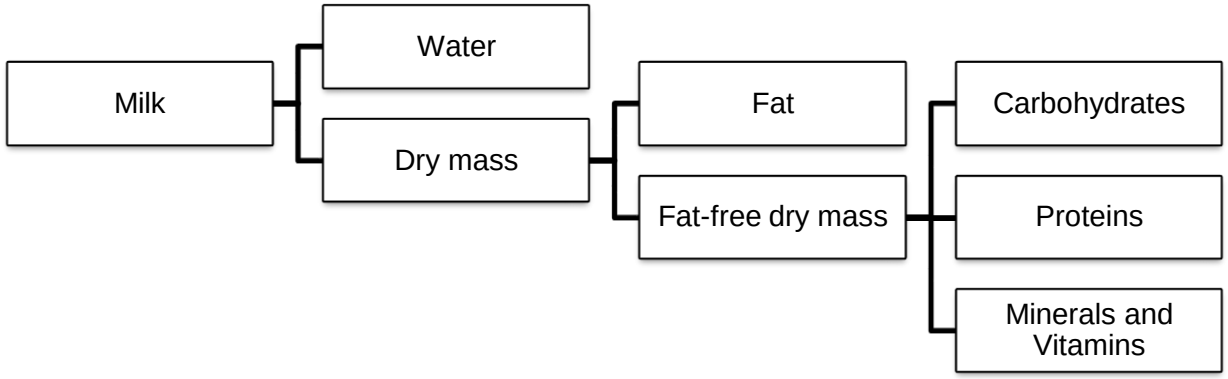


Figure 2 Composition of milk.

Milk contains between 3,5 and 6,0% fat, which is composed of lipids and acylglycerides and lipoids. Triglycerides are produced in the endoplasmatic reticulum by lactocytes via de-novo synthesis from acetate and β-hydroxybutyrat. Triglycerides contain glycerin and three fatty acids. Fat molecules are available as two to four µm big fat globules, which are surrounded by a membrane. Milk proteins constitute casein, whey proteins and amino compounds. Caseins and whey proteins are produced in the lactocytes of the mammary gland. Amino acids and low-molecular peptides are specifically transported from the blood into the lactocytes. The synthesis of proteins takes place at the ribosomes, segregation and aggregation is performed by the golgi-apparatus. The casein group can be divided into four subtypes (α_{s1}, α_{s2}, β, κ) and β-casein derivates (γ_1, γ_2, γ_3). Caseins can be defined as phosphoproteins, with exception to γ_1 and γ_2. The hydroxygroup of the amino acid serine is bound to organic phosphomolecules within characteristic patterns (Pse-X-glutamic acid or Pse-X-Pse). Caseins do not have a tertiary structure and are

therefore heat stable. In milk, caseins are present in form of micelles, which contain up to 94% proteins and 6% minerals (calcium, sodium, potassium, phosphate, magnesium and citrate ions). The production of cheese requires the degradation of caseins micelles by the enzyme chymosin at pH 6,7. The residual protein fractions, which are present at pH 4,6 and 20°C after degradation of caseins, are called whey proteins. Lactocytes produce α-lactalbumin, β-lactoglobulin, IgA, IgM, IgG2, whereas albumin and IgG1 are transported from the blood to the mammary gland. The functions of immunoglobulins in milk are described in section 1.3.2.

The physiological functions of β-Lactoglobulin are the binding of hydrophobic molecules and the transport of retinol (Vitamin A_1). α-Lactalbumin plays a major role in the production of lactose. Bovine serum albumin (BSA) is a globular molecule, which controls the osmotic pressure in the blood and transports zinc ions. Transferrin is transported from blood serum into the milk and is responsible for the transport of iron. Lactoferrin is specifically conveyed from the serum, but also produced in the mammary gland. It has an antimicrobial function as it binds iron which is thereby non-available for bacterial growth. Another enzyme to be found in milk is the lactoperoxidase. Lactoperoxidases can oxidize thiocyanate to hypothiocyanite, which has a bactericidal effect on gram-negative bacteria.

Table 2 Summary of important milk proteins according to their molecular weight.

Protein	Molecular weight [Dalton]
Casein	25 000 – 11 600
β-Lactoglobulin	18 300
α-Lactalbumin	14 200
BSA	66 000
IgG1	163 000
IgG2	150 000
IgA	390 000
IgM	950 000
Transferrin	75 000- 77 000
Lactoferrin	77 000 – 93 000
Lactoperoxidase	77 500

The major carbohydrate source in milk is lactose with a concentration of 4,5%. Lactose is a disaccharide of galactose and glucose connected with a glycosidic 1,4-β-O- bond between the C1 atom of D-galactose and the C4 atom of D-glucose. The production takes place in the golgi-apparatus of the mammary alveolus. Other carbohydrate components in milk are glucose, galactose and amino sugars. Milk is also a resource for minerals such as sodium, potassium, calcium, magnesium, iron or phosphor. Important vitamins as liposoluble vitamin A, D, E and K and watersoluble B_1, B_2, B_6, B_5, B_{12}, niacin, C, biotin and folic acid.

1.3.2. Immune components in bovine milk and colostrum

Colostrum, also referred as beast or first milk, includes a complete diet for newborn cows. Next to the nutritional aspect, it also provides neonates with passive immunization for primary protection against microbes until the immune system of the calf has developed. In cows the immunoglobulins (Ig) are transported via Fcγ receptors from the blood stream through the epithelium into the mammary gland and accumulate to be transferred postnatally through suckling to the calf[25]. The *in-utero* transport through the placenta as characteristic in humans, is not

present in cows. The antibody concentration in the mammary gland decreases significantly within the first 12-36 hours after birth. In the calf's gut the molecules will be transported into the blood stream via intestinal cells. The composition of the Igs represent the immune recognition of the mother against pathogens. In bovine colostrum the concentration of Igs is significantly higher than in mature milk. Immunoglobulins constitute 70-80% of the total protein content, whereas in milk they make up 1-2% of the proteins Marnila[26].The most abundant immunoglobulin class is IgG, whereas in human colostrum IgA constitutes the largest amount of immunoglobulins[27].

Table 3 Comparison of immunoglobulin content in bovine and human colostrum and mature milk. [27]

Species	Immunoglobulin	Concentration [g/L]	
		Colostrum	Mature milk
Bovine	IgG_1	46,40	0,58
	IgG_2	2,87	0,06
	IgA	5,36	0,08
	IgM	6,77	0,09
Human	IgG	0,43	0,04
	IgA	17,35	1,00
	IgM	1,59	0,10

Beast milk includes somatic cells (SCs) such as epithelial cells and leucocytes (macrophages, polymorphonuclear neutrophil cells (PMNs) and lymphocytes)[28]. Epithelial cells are responsible for the milk production and can shed from the mammary gland during lactation. The leucocytes are part of the innate immune system, which is activated as first-line defense mechanism upon infection by bacteria. As bacteria migrate through the physical and chemical skin barrier, the immune cells can detect pathogen-associated molecular patterns (PAMPS) such as cell-wall components. The primary cellular defense mechanism against pathogens is phagocytosis. Macrophages, neutrophils and dendritic cells perform phagocytosis in tissue, whereas monocytes engulf bacteria in the blood. The bacteria are engulfed by pseudopodia and ingested into a phagosome. In the next step the phagosome fuses with a lysosome. Lysosomal enzymes can degrade the microbes. The dead end-products are finally released from the cell. Furthermore, the

first-line defense can trigger activation of multiple other cellular innate immune responses by expression of antimicrobial peptides, interferons, chemokines and cytokines[29].

The main function of SCs in colostrum is to protect the mammary gland from infections. Macrophages can prevent microbial infections by engulfment. If infection occurs, macrophages release chemical messengers and chemoattractants to guide PMNs to the site of infection. Both cell types can phagocytose bacteria and play an important role in innate immunity. After ingestion of bacteria by phagocytosis, PMNs kill the invaders by oxidative reactions with reactive oxygen species enzymes (ROS) and by non-oxidative reactions conducted by granular enzymes. Lymphocytes can react specifically to microbes by detection of antigens via membrane receptors. Furthermore they are responsible for the production of regulatory cytokines[30].

1.3.3. Immunoglobulin G (IgG)[31]

Immunoglobulins are biologically active proteins produced by plasma B-cells, which selectively bind epitopes on pathogens, such as bacteria or viruses, to induce immune responses. Antibody activated immune responses are so-called humoral responses. The basic polypeptide composition of all immunoglobulins are two identical light chains (25 kDa) and heavy chains (50 kDa), which form a 150 kDa Y shaped structure via disulfide bonds.

Antibodies can be separated into five different classes (IgA, IgG, IgE, IgD, IgM) depending on their heavy chain structures. Each heavy chain consists of one variable region (V_H) and depending on the antibody subclass three to four constant regions (C_H1, C_H2, C_H3, C_H4). IgG contain γ heavy chains, IgM have μ heavy chains, IgA have α heavy chains, IgD have δ heavy chains and IgE have ε heavy chains. The light chain can be differentiated between κ or λ according to the amino acid sequences. Selfsame contain a constant (C_L) and (V_L) region. The variable regions of the heavy and the light chain contain three so-called complementary-determining regions (CDRs), which constitute the immunological specificity and response of the antibody. In total six CDRs arise from three hypervariable regions from the heavy (CDR H1, CDR H2, CDR H3) and the light (CDR L1, CDR L2, CDR L3) chain. Variation in the amino acid sequences provide diversity in antigen recognition. 10% of the bovine antibodies are unique in their exceptionally long CDR H3 region, which consists of up to 69 amino acids. The residual 90% contain approximately 23 residues. The composition of the bovine ultralong CDR H3 includes large amounts of cysteins[32]. CDR H3 forms a knob-like structure, sticking out of the variable regions. It is probable, that the ultralong CDR H3 alone binds epitopes of the antigen, whereas the other CDRs contribute to the

structure. Furthermore, the knob-like structure could be beneficial for binding of antigen-sites that are not accessible for smaller antibodies[33].

The light chains are bound to the heavy chains by disulfide-bonds, whereas the heavy chains are held together by disulfide-bonds in the hinge region. This region compromises the flexibility of the molecule, which is needed for the antigen binding. The functionally-folded and connected polypeptide chain constitutes two antigen-binding fragments (Fab) formed by the N-terminus of the variable domains of the heavy and light chain and one constant region (Fc) formed by the C-terminus of the two heavy chains. The Fc region can bind to receptors on immune cells and activate the cellular immune system.

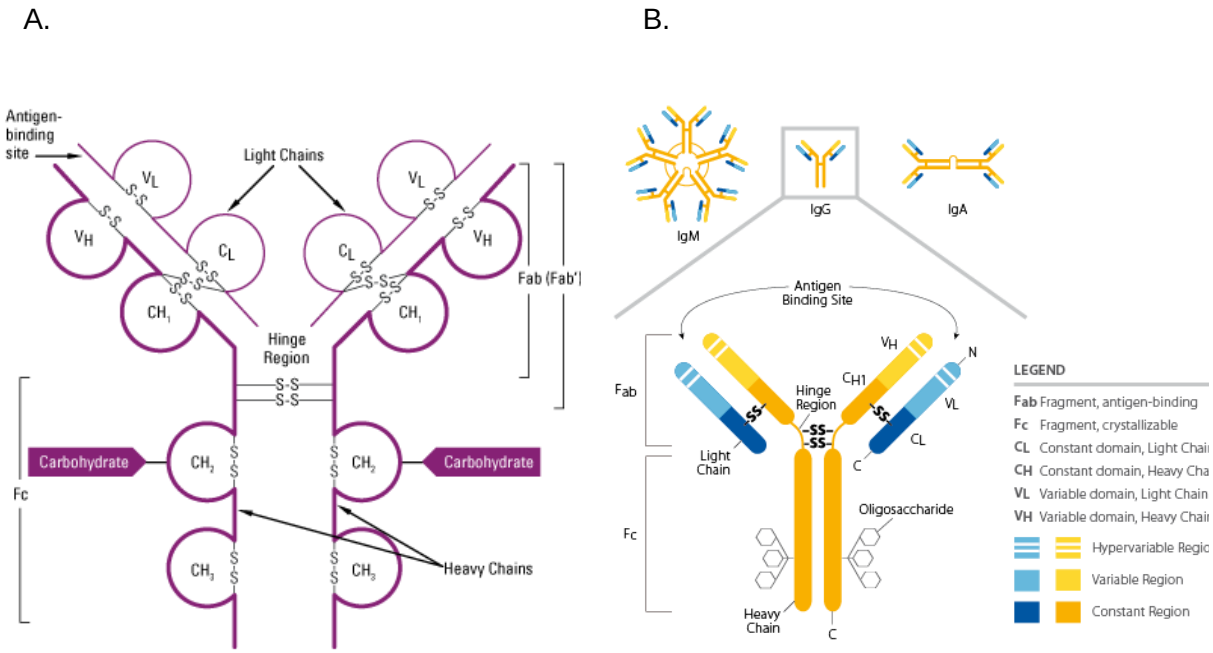


Figure 3 Structure of Immunoglobulins : A. Generalized structure of immunoglobulins (example IgG), B. Structures of immunoglobulin subclasses IgM and IgA in comparison to IgG. [34])

The most important antibody classes in bovine colostrum and milk are IgG, IgA and IgM. IgG occurs as a monomer, whereas IgA can form either monomers or dimers, which are connected via a polypeptide chain (J-chain) and an epithelial glycoprotein. So-called secretory IgA has a molecular weight of 380 kDa. IgM consists of five antibodies bound together circularly with a J-chain and disulfide bonds (see Figure 3)[26].

The IgG class can be differentiated into four subclasses: IgG1, IgG2, IgG3 and IgG4. The amino acid chains of all four types share 90% homology, resulting in different properties in antigen-binding, effector functions and half-life. The most variants in the amino acid sequences can be found in the hinge region and the N-terminus of the C_H2 domain. IgG1 has the highest abundance and mainly binds soluble protein antigens and membrane proteins. The function of IgG2 compromises the binding of capsular polysaccharide antigens. IgG3 activates effector functions and IgG4 is often associated with long-term exposure to certain antigens [35]. The most abundant IgG subclass in cows is IgG1 and IgG2[25]

1.3.4. Bioseparation of bovine IgG from Colostrum

Beast milk is a heterogeneous biofluid with high concentrations of nutrients and bioactive components. From a technological point of view, it can be defined as a polydisperse system of emulsions of fat in water and colloidal dissolved proteins and dissolved ions[23]. For the preparation of antibodies from bovine colostrum for application on human or companion animals as pharmaceutical agent, processes according to the good manufacturing guidelines (GMP) have to be used. The chosen bioseparation methods should not only extract the desired component, but also ensure removal of hazardous pathogens.

Filtration is defined as the separation of two or more solid components from a fluid with the help of a selective membrane. The particles are separated from each other due to size-dependent permeability through the filter membrane. Depending on the size of the separated molecules different filtration methods can be defined. Filtrations can be performed in three different modes: dead end, cross-flow or diafiltration mode. Dead end filtrations are characterized by a conventional feed flow, which causes development of a stable filter cake proportional to the feed stream. The porosity of the membrane decreases as the formation of the filter cake plugs the membrane steadily throughout the process time. The filtrate flow through the membrane decreases according to the progression of filter cake formation. Cross flow filtrations have a tangential feed flow, which results in the formation of a dynamic gel layer instead of a stable filter cake. After a certain process time a steady state in dynamic gel-layer formation is reached and the flow can be held constant. Cross-flow filtrations can be performed in a continuous mode, whereas dead-end filtrations can only be applied for batch processes[36]. Cross-flow filtrations performed by pressure difference are number one choice in dairy food membrane separations[37]. Diafiltration is used to remove membrane-passing components or to change the buffer in the feed. In this mode diafiltration medium is either added step-wise or continuous to the

feed tank according to the removed amount of permeate. Diafiltration media are usually water of buffer solutions[38].

A. B.

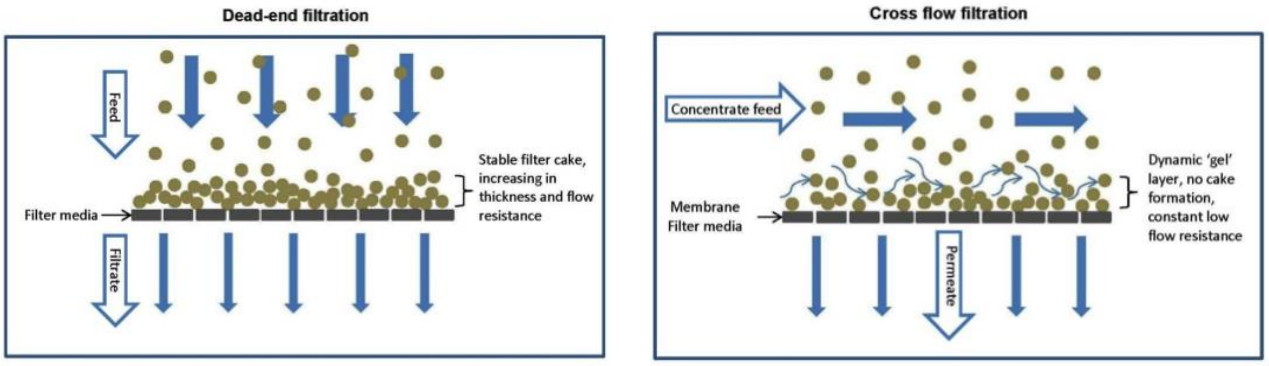


Figure 4 Comparison of mechanism of dead-end (A.) and cross-flow (B.) filtration. [39]

As milk contains bioactive, heat-sensitive components such as proteins, a gentle separation technology, which can provide fractions in their native state as well as high product quality, has to be chosen. Furthermore, the separation of different fractions from milk is a challenging undertaking, as the particle sizes of different components vary between 10 µm to 0,1 nm, the concentration of different components vary with high extend and natural variability have to be taken into consideration[40]. Figure 5 provides an overview of possible membrane separation technologies for certain milk components according to their size.

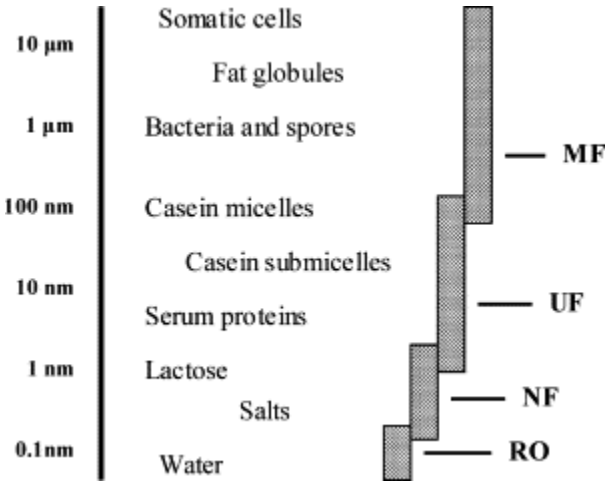


Figure 5 Bioseparation of milk components with filtration membranes. MF= microfiltration, UF= ultrafiltration, NF= nanofiltration, RO= reverse osmosis. [40]

Microfiltration can be performed with membranes of 0,1 to 0,2 µm pore size. Casein micelles, fat globules, microorganisms and somatic cells can be removed. The resulting product is native whey free of hazardous bacteria[41]. With ultrafiltration molecules in the range of 100 to 1 nm can be removed. Via ultrafiltration the casein micelles, salts and sugars such as lactose can be removed, while immunoglobulins are being enriched.

At the Chair of Food and Bioprocess Engineering TUM School of Life Sciences Weihenstephan a biotechnological process for the recovery of bovine IgG from colostrum milk was developed. Within this process raw colostrum is collected within day one to seven after birth. The milk is heated to a temperature of 50°C with a plate heat exchanger and the fat is removed to <0,1% by centrifugation in a milk separator. The defatted milk is cooled down with the plate heat exchanger and stored at 4°C. For the removal of casein micelles and bacteria a combination of cross-flow micro- and ultrafiltration in diafiltration mode is used.

Because the milk contains high amounts of proteins, which results in high viscosity and possible membrane fouling, the milk is firstly diluted with water to a dry mass of 10%. Before the start of the bioseparation, the milk is again heated to 50°C and poured into the sample tank. The microfiltration is performed by seven ceramic ISOFLUX™-membranes (TAMI Industries, France) with 0,14 µm pore size and a filter area of 0,35 m^2 per membrane. The transmembrane pressure (Δp_{TM}) is kept constant at 2 bar. The retentate is lead back to the sample vessel, whereas the permeate is lead into the ultrafiltration plant. At this stage the fluid is considered to be immunoglobulin-rich whey.

The ultrafiltration process is performed by a polypropylene spiral wound membrane with a molecular weight cut off of 10 kDa (DSS Silkeborg AS, Denmark). The retained IgG (retentate) is collected and lead back to the sample vessel of the ultrafiltration plant. Meanwhile the permeate is transported into the sample tank of the microfiltration for diafiltration. Thereby a reduction in viscosity rising and a continuous filtration process with constant flux can be ensured. After seven diafiltration steps the bioprocess is stopped. Process monitoring includes documentation of the temperature, inlet and outlet pressure, Δp_{TM}, flux of permeate and retentate and regular sampling of permeate in retentate at defined time points.

For the recovery of bovine IgG from the whey, the ultrafiltration is separated from the microfiltration. To remove the lactose and salt contents, demineralized water is used as a diafiltration medium. The retentate is collected in the tank of the plant and the permeate is removed. In total seven washing steps are performed before the retentate is concentrated.

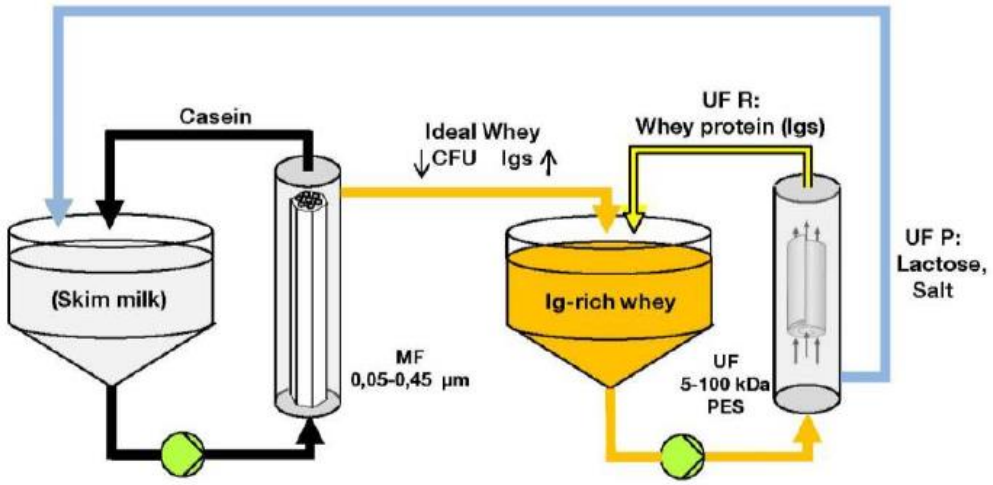


Figure 6 Combinational bioseparation process for the isolation of bovine IgG from Colostrum consisting of micro- and ultrafiltration in diafiltration mode. [42]

1.3.5. Preparative chromatography for the isolation of bovine IgG from Ultrafiltration retentate[43]

For the purification of bovine antibodies from the IgG-enriched whey, a large-scale chromatography process was developed at the Chair of Food and Bioprocess Engineering at the

Technical University of Munich. Chromatography allows the time-resolved separation based on physical or chemical properties of components in mixtures between a stationary and mobile phase. The preparative chromatography process has to provide high yields, a product in its native form and cost-effectiveness.

Multiple chromatographic methods for the sufficient isolation of bovine IgG from whey have been reported. Immunoaffinity chromatography with egg-yolk derived IgY antibodies, protein G, thiophilic chromatography, size-exclusion or metal-chelate interaction chromatography with Cu or Zn ions have been proven to be suitable for high purity capture. Nevertheless, many difficulties in up-scaling and high costs as well as leaching of Cu or Zn ions into the product summarize the downsides of these methods. Mixed-mode chromatography (MMC) represents a chromatography technique, suitable for the purification of components from complex biofluids. The stationary phase in MMC columns contain multi-functional ligands, which increase efficiency and purity within the process. Within one separation column multiple chromatographic separation processes can take place simultaneously. The interaction between the ligand and the samples can be dependent or independent from each other. The strength of the binding between ligand and protein is the result from the physicochemical properties of the sample and the process parameters.

For the isolation of bovine IgG from the micro- and ultrafiltrated IgG enriched whey (see section 1.3.4) the 1 mL Mercapto-Ethyl-Pyridine-Hypercel™ (Pall Corpotation, United States of America) was chosen. IgG binds to the column with hydrophobic interactions, whereas the residual proteins with more hydrophilic characters are flushed through the column. Lactoperoxidase has a similar hydrophobic character as IgG and therefore impurified the extract. To increase the purity of the chromatography, a second chromatography step with the 1 mL Capto™-MMC with N-benzoyl-homocysteine ligands (GE Healthcare, Sweden) was incorporated into the bioseparation. In this column the ligands are negatively charged at pH 7,5, which causes the positively charged lactoperoxidase to selectively bind. The combinational process resulted in a >96,1% purity for IgG and a yield between 60-80%. Upscaling the process to 8800 mL Mercapto-Ethyl-Pyridine-Hypercel™ and 3000 mL Capto™-MMC columns resulted in 130-150 g IgG/3 liter colostrum.

1.3.6. Formulation of bovine IgG in hydrogel[44, 45]

The formulation of therapeutic proteins is a challenging endeavor in which different aspects have to be considered. The protein has to be provided as an effective applicable product and product

stability has to be ensured. Furthermore, the formulation vehicle should ensure a long product life, high loading capabilities and GMP production possibilities. Parameters influencing the formulation development arise from the protein type, the administration form and the therapeutic target. Protein characteristics such as size, charge and solubility summarize only a small spectrum of challenges. Hydrogels are a promising type of vehicle for formulating proteins for topical cutaneous application according to GMP guidelines.

Hydrogels are elastic, cross-linked networks of hydrophilic monomers of natural or synthetic production that bind large amounts of water. The production of hydrogels is relatively easy by mixing of biocompatible materials in liquid solution at room temperature. Immunoglobulins with sensitive stability, can be formulated with a high degree in stability and functionality. The linking of substrate monomers takes place by chemical reaction, physical interaction or ionizing radiation. Often the therapeutic protein is already involved in the reaction mixture during cross-linking. The high water content, the softness and the porosity levels make hydrogels interesting for therapeutic applications. One of the most critical parameter in choosing a hydrogel type is the mesh size. The hydrogel structure determines the diffusion kinetics of the cargo protein through the gel to the target. Depending on the mesh size the local drug release can be influenced towards a therapeutically relevant drug concentration. Disadvantages of hydrogel formulations are limitations in the loading capacities, interaction of the proteins with the hydrogel components and fast release of hydrophilic proteins within hours or days.

For the formulation of bovine IgG for topical application Sodium Carboxymethylcellulose (CMC) was chosen. CMC is an anionic, water-soluble, purified and dried polymer. The hydrogel is the result of sodium monochloroacetate reacting with alkalicellulose. As a container the Ursatec 3K® horizontal spray system was chosen. This device provides an airless- and preservative-free spraying without any risk of contamination. In order to test the capability of the formulation of the antibody, different testing formats were performed at the Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics (Ludwig Maximilians-Universität München)[46].

In different tests the sprayability and rheological characteristics at different CMC concentration were tested. 1,5% CMC proofed to ensure a viscosity of the fluid with maximal adhesiveness on the skin and constant dosing. The possible formation of antibody aggregates in hydrogel was tested by size exclusion chromatography. Within eleven weeks of storage at 2-8°C approximately

15% of the antibodies formed dimers and 5% aggregated. The secondary structure of the antibody was tested by far-UV Circular dichroism at 180-240 nm. A formulation in 1,5% CMC did not show an altered spectrum compared to antibody in PBS. Furthermore a time-dependent change in structure within seven weeks of storage could not be determined, leading to the assumption that the secondary structure is not affected by preparation in CMC and storage at 2-8°C. To compare the functional activity of the bovine IgG in hydrogel and in the PBS control, binding to α-toxin from *Staphylococcus aureus* was tested in an ELISA assay format. As the curves were identical, the activity of the antibody could be ensured. To test the power of neutralization of toxins by the antibody, a hemolysis assay with rabbit erythrocytes was performed. Both, the antibody in PBS as well as the antibody in hydrogel could neutralize hemolysis to the same degree.

1.4. Canine skin infections

1.4.1. Canine skin and its immune system

The skin is the largest organ of mammals and has diverse functions. It serves as a barrier to protect the body from environmental influences, facilitates communication with the surrounding matter and establishes homeostasis. Canine skin represents a complex microenvironment with high bacterial diversity in the microbiome[47]. Dog skin varies in thickness between 0,5 and 5 mm depending on the location of the body. The thickest skin can be found on the back and the dorsal neck. The thinnest skin covers the inguinal and axillary parts of the body[48].

Canine skin consists of two associated layers: the epidermis and the dermis. The epidermis is composed of dead (*stratum corneum*) and living (*stratum granulosum, stratum lucidum, stratum spinosum and stratum germinativum)* cells. Due to the haircoat the epidermal layer is much thinner than in humans. Below the epidermis lies the dermis, which constitutes a papillary and reticular layer.

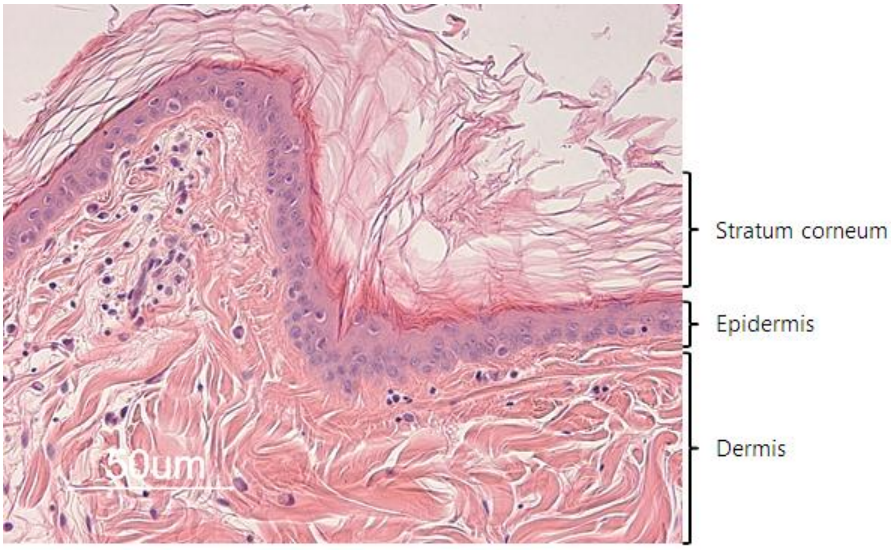


Figure 7 Hematoxylin and eosin staining (HE stain) of canine skin. Hematoxylin stains basophilic structures such as cell nuclei with their DNA and the rough endoplasmatic reticulum (ER) blue. Eosin is an acidic stain, which colors acidophilic components such as cell plasma protein, mitochondria, smooth ER, collagen and keratin red.

The *stratum corneum* is derived from multiple layers of dead cells and contains macromolecules such as lipids and first-line defense immunoglobulins (IgG, IgA, IgM), complement factor C3 and serum albumin[49]. The *stratum corneum* sheds over time and is replaced by cells originating from the *stratum granulosum* or *spinosum*. The *stratum lucidum* is only present in the paws and comprised of keratinocytes without a nucleus. The *stratum granulosum* is formed by the *stratum spinosum* and shows a characteristic shrunken and flattened shape. The *stratum spinosum* contains prickly looking cells, which are connected to each other by different cell junctions. The deepest layer of the epidermis, the *stratum germinativum*, appears as a single keratinocyte and melanocyte cell layer and is embedded in the basal membrane, which can be visualized with Periodic acid–Schiff (PAS) staining. Keratinocytes produce keratin for protection against environmental influences. Langerhans cells, a specific type of dendritic cells, are present in both the *stratum germinativum* and *spinosum*. If microbes pass the *stratum corneum* Langerhans cells as antigen-presenting cells bind and process them for presentation at their major histocompatibility complex class I and II (MHC I and II). In the next step migration to the lymph nodes takes place where T-cells are activated[50].

Underneath the epidermis lies the connective-tissue dermis. The function of this layer is the protection against physical trauma, supporting function and water storage. The uppermost layer is defined by a network of fine collagen fibers in loose arrangement. The layer below is composed of thicker collagen fibers and fewer elastic macromolecules. The dermis contains different cell

types including fibroblasts for the production of collagen, mast cells for production of histamine and histiocytes for phagocytosis of microbes. In presence of infections, a number of other cells such as lymphocytes, eosinophils, neutrophils and plasma cells can infiltrate the dermis from the blood. Below the dermis lies the subcutis, which contains connective tissue and large amounts of fat cells[51].

Dogs are covered by fur, which increase the physical and mechanical protection to the environment and attribute thermoregulation, sensory functions, immunological protection and camouflage. Hair follicles are keratin filaments which are self-renewing for every hair cycle throughout life-time[52]. The canine skin contains multiple appendages with distinct functions. Sebaceous glands regulate the hydration of the skin by producing fatty acids, cholesterol and waxes[53]. Apocrine glands produce sweat during active movement and regulate the body temperature of the dog.

1.4.2. Staphylococcus pseudintermedius

The Staphylococcus *intermedius* group (SIG) contains three bacterial strains: Staphylococcus *intermedius*, Staphylococcus *pseudintermedius* and Staphylococcus *delphini.* Out of the three, S. *pseudintermedius* is the most relevant strain with respect to clinical impact. S. *pseudintermedius* is a commensal gram- and coagulase-positive bacterium found on skin and mucous membranes of healthy dogs. As part of the dermal microflora the bacterium colonizes the skin, hair follicles and the mucosal membranes of anus, nose and mouth[54].

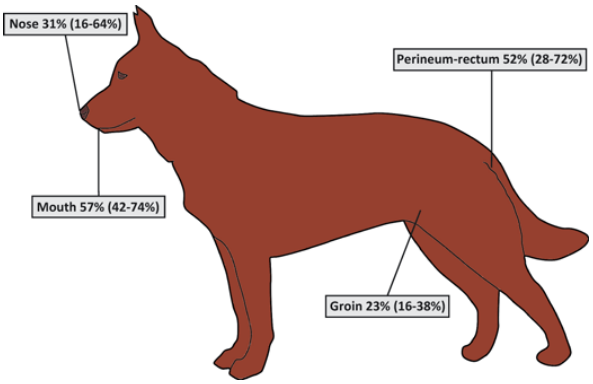


Figure 8 Colonization of Staphylococcus pseudintermedius on dogs. Depending on the localization on the body, different *S. pseudintermedius* levels were identified. [55]

An important factor for predisposing of carriage of S. *pseudintermedius* is the canine health status. The bacterial strain can be characterized as an opportunistic pathogen, causing infections in

immunosuppressive susceptible hosts or dogs suffering from dysfunctional skin barrier diseases such as atopic dermatitis[56].

The pathogenicity of S. *pseudintermedius* depends on several expressed virulence factors such as exoenzymes, cell wall anchored proteins and toxins. Virulence factors have distinct functions such as facilitation of colonization, nutritional supply and dissemination[56]. Part of produced virulence factors are functionally identical to *S. aureus* pendants.

S. *pseudintermedius* surface proteins (Sps) facilitate adherence to epithelial cells for colonization and infection through their cell wall anchoring presence and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Sps can bind to fibrinogen, fibronectin, cytokeratin 10, elastin, collagen type I, vitronectin and laminin[57]. Pietrocola et al.[58] reported that the cell wall-anchored fibronectin-binding proteins SpsD and SpsL are fundamental for bacterial infection in epidermal keratinocytes. Additionally, *S. pseudintermedius* has been reported to produce protein A (spa) for binding of the Fc region of IgG and evasion from phagocytosis[59]. However, protein A binds bovine IgG only weakly[60]. Regarding exoenzymes, *S. pseudintermedius* is known to produce coagulase to activate prothrombin to convert fibrinogen to fibrin. The formed staphylothrombin protects the bacteria from the immune system[56].

S. *pseudintermedius* produces different so-called cytotoxins, which lyse different cell types in the host. α-haemolysin is a pore-forming unit, which causes damages in the cell membrane of erythrocytes and thereby escapes from the phagosome. β-haemolysin attacks the cell membrane component sphingomyelin[56]. In addition, they may express the leukotoxin Luk-I to kill PMNs for evasion from the immune system[61]. *Staphylococcus pseudintermedius* exfoliative toxins (SIET) cause rounding deformation of epithelium and induce the development of pyoderma[62]. Via producing superantigens, such as the enterotoxins SEA, SEB, SEC, SED and toxic-shock syndrome toxin-1 immune evasion from recognizing antibodies can be facilitated[63]. Superantigens activate an uncontrolled recruitment of T-cells and can lead to fever, sepsis and death[56]. It has been shown that the accessory gene regulator (agr) system known from Staphylococcus a*ureus* is also present in S. *pseudintermedius* strains. The effect of this communication system ensures colonization by expression of virulence factors in a cell-density and growth dependent manner[55]. The autoinducing peptide (AIP), which is encoded by the *agrD* gene in S. *aureus,* has been identified to be encoded by different alleles and expressed in different variations in S. *pseudintermedius*.

In addition, *Staphylococcus pseudintermedius* has been associated with biofilm formation. Biofilms are complex matrices composed of extracellular polymer substances (EPS) containing various polysaccharides, proteins and extracellular DNA. Biofilms can shield bacteria from antibiotics and disinfectants as well as from the host immune system, enabling continuous growth and chronic infections. The external DNA forms a network, which selectively allows diffusion of nutrients for growth[64]. Pompilio et al.[65] described that an S. *pseudintermedius* isolate from a human skin wound is capable of forming an antibiotic-resistant biofilm consisting of multiple microcolonies embedded in EPS. The minimal inhibiting concentrations of last-resort antibiotics (e.g. linezolid, tigecyclin and vancomycin) observed in this study were higher than in serum.

The transmission of *S. pseudintermedius* between dogs can be either of vertical or horizontal origin. Vertical transfer from the bitch to the newborns occurs perinatal and after birth. Horizontally transmitted bacteria takes place between adult dogs upon contact. Interspecies transmission between dogs and humans is a growing problem which challenges veterinarians and medical doctors. *S. pseudintermedius* is not part of the skin microbiome in healthy humans. However, humans can be colonized and become transient carriers when in close contact to dogs. Especially pet owners and veterinary personnel are at risk for infections. The most prominent colonizer of human skin is *S. aureus.* As the veterinary *S. pseudintermedius* encodes similar virulence factors as *S. aureus*, zoonotic infections can develop. Various human pathologies have been reported in association with canine *S. pseudintermedius* including animal bite wound infections, endocarditis, bacteremia, pneumonia, otitis externa, mastoiditis and brain abscess. The first report of human *S. pseudintermedius* infection was published in 2006. A patient suffered from endocarditis after the implantation of a cardioverter-debrifillator device (ICD). The origin of transmission was very likely community-acquired- the patient did not own pets[66].

The significance of animals as reservoirs for antibiotic resistant zoonotic bacteria increases rapidly. Methicillin-resistant *S. pseudintermedius* (MRSP) encode the *mecA* gene, which expresses the low affinity penicillin-binding protein (PBP2a). PBP2a is a transpeptidase, which is responsible for the crosslinking of the major cell wall component peptidoglycan. Peptidoglycan is produced by transglycosylases and comprised of multiple repeats of the disaccharide *N*-acetylglucosamine (NAG)-*N*- acetylmuramic acid (NAM). NAM contains strain-dependent peptide-structures, which are crosslinked by PBP2a during maturation of the cell wall. As integrity of the cell wall is necessary for survival of bacteria, interference during the synthesis process is an auspicious drug target. β-lactam antibiotics inhibit the cell wall synthesis by binding D-alanin-

transpeptidases and preventing the crosslinking of the carbohydrate backbone. PBP2a is weakly affected by the antibiotics, allowing progression in activity of the transpeptidase and ensuring survival of the bacterial cells in presence of penicillins, cephalosporins and carbapenems[67].

1.4.3. Canine pyoderma

Canine pyoderma is one of the most common reasons for presentation at veterinarians and for prescription of antibiotics in small animal practice. The bacterium most often isolated from pyoderma skin infection is the commensal *S. pseudintermedius*. In rare cases pyoderma can become a life-threatening disease with systemic inflammation. With the emergence of MRSP the necessity of new treatment options has to be stressed. Reasons for alleviation of infection in canine skins are the thin epidermal layer, the loose *stratum corneum* and the structure of hair follicle. Chronic and recurrent pyoderma can be often be found in dogs with underlying skin or systemic diseases such as atopic dermatitis or dogs with immunological defects[68].

In general three types of superficial pyoderma can be differentiated: surface, superficial and deep pyoderma[69]. The syndrome in surface pyoderma varies from acute moist dermatitis inflammation, intertrigo and erythrema. Cytology of superficial pyoderma inflamed skin is characterized by overgrowth of bacteria. Superficial pyoderma is the most common type of clinical manifested pyoderma. In this type bacteria can be identified in the epidermis, leading to development of papules, pustules and collarettes. As bacteria migrate through the dermis to the blood vessel system in deep pyoderma, life-threatening conditions can develop in dogs.

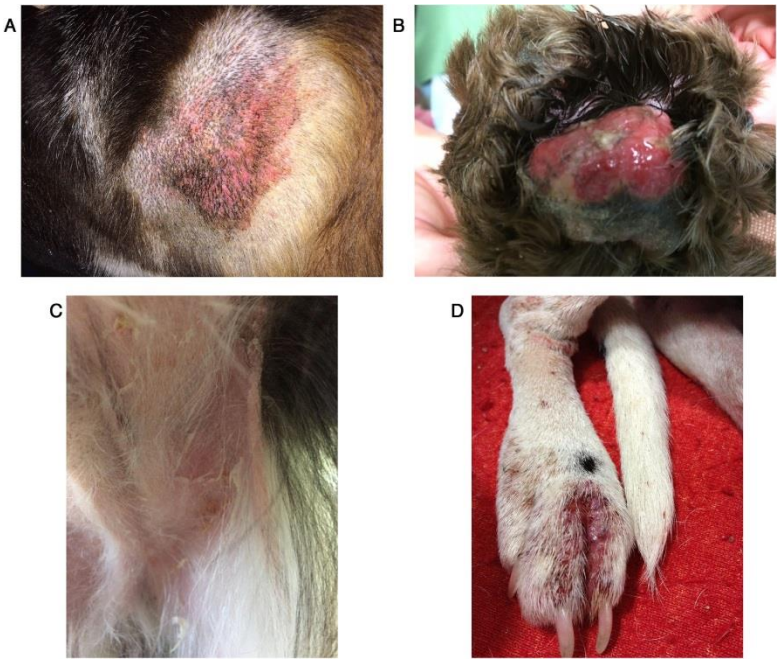


Figure 9 Examples of recurrent (>3 months) canine pyoderma with MRSP. by Loeffler et al.[69]. A. Acute moist dermatitis by MRSP at the neck region, B. Purulent *Klebsielle spp.* Infection, C. Recurrent superficial pyoderma with expanding collarettes caused by MRSA, D. Deep pyoderma due to *Pseudomonas aeruginosa* infection.

The treatment of canine pyoderma should be carefully chosen in terms of MRSP increase and according to bacteriology results with correct administration of antimicrobial substances. To reduce the prescription of systemic antibiotic therapy, topical therapy should be considered. Topical treatment may be efficient for surface pyoderma and in combination with systemic therapy for superficial and deep pyoderma. Different type of formulations are available for topical treatment such as shampoos, creams, gels, ointments and dips[70]. Shampoos containing 0,5-4% chlorhexidine, 10% ethyl lactate or 2,5-3% benzoyl peroxide are effective in killing *S. pseudintermedius*. Bathing should be repeated daily and contact should last 10 to 15 minutes. Ointments, creams, gels or liquids are effective vehicles in the antimicrobial treatment against local MRSP infections. The antibiotics used in topical treatment include mupirocin, fusidic acid and amikacin. In the Netherlands gold standard topical antibiotic treatment is Clindacutin® cream, which contains 10 mg of clindamycinehydrochloride. The treatments should be applied twice daily with a minimum contact time of ten minutes[70].

If deep or superficial pyoderma are diagnosed, systemic therapy should be chosen as a treatment[69]. As the effectiveness of this therapy option depends on the susceptibility of the bacterial strain, correct administration, doses, status of disease and owner compliance. Any prescription of antimicrobials should precede antibiogram testing. Surface pyoderma should be

treated for three weeks (or one week beyond cure), superficial and deep require treatment for four to eight weeks (or two weeks beyond cure)[69].

Clindamycin, first generation cephalosporins, amoxicillin-clavulanate or sulphonamides include possible prescript antimicrobials. First choice is usually clindamycin due to its narrow antimicrobial spectrum against gram-positive aerobe, gram-negative anaerobe bacteria and bacteria of the genus *chlamydia*. Second tier agents (e.g. fluoroquinolones) should only be used after susceptibility testing. Morris et al.[71] manifested in the Clinical Consensus Guidelines following rules for the treatment or MRSP in skin infections: β-lactam antibiotics should in general not be used for treatment. Before applying clindamycin, susceptibility should be tested. Switching of treatment within the tetracycline class should not be undertaken as resistance can be traced back to multiple genes. Lastly, resistance to one type fluoroquinolone indicates a high likelihood of resistance to other fluoroquinolones.

1.4.1. Atopic dermatitis in dogs

Canine atopic dermatitis (CAD) is an inflammatory and pruritic allergic skin disease with a strong genetical predisposition. IgE antibodies directed against environmental allergens are associated with this type of disease[72]. CAD is the most common skin disease diagnosed in dogs. The prevalence in the general dog population is estimated between 3 to15%. When skin diseased dogs are presented at veterinarians, between 3 to 58% of the patients are diagnosed with atopic dermatitis[73]. Atopic dermatitis is a life-long manageable disease without the possibility of cure. Clinical features develop within the first three years after birth and result in skin barrier dysfunction. The most characteristic symptom is pruritus in combination with skin lesions around the mouth, eyes, ears, elbows, carpal and tarsal joints abdomen, perineum and the proximal tail. Pathogenesis is influenced by genetic background, cutaneous condition, infections by bacteria or yeasts, psychogenic factors and reaction to the surrounding environment. Environmental influences include allergens such as food, microbes and insects. Upon exposure the skin reacts by infiltration of immune cells, activation of resident cells and production of inflammation factors[74].

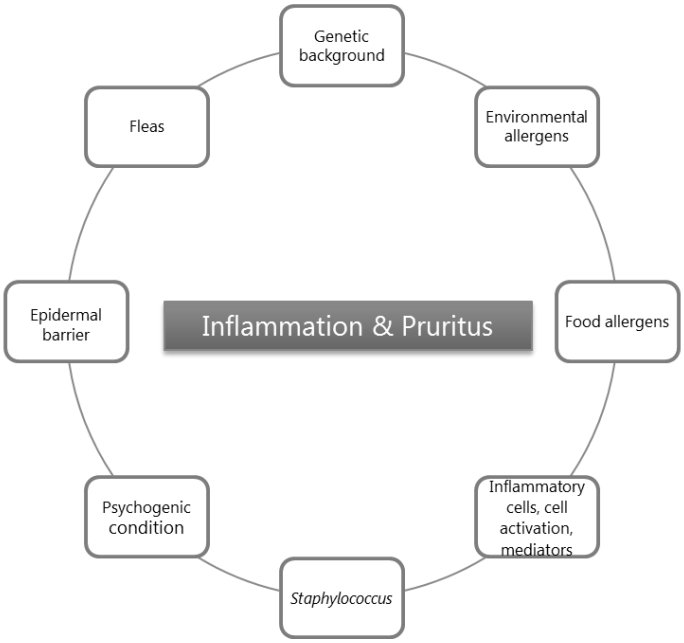


Figure 10 Influences on the pathogenesis of canine atopic dermatitis. Single influences interact with the skin, but also interact and depend on each other. Adapted from Saridomichelakis et al.[74]

Patients with atopic dermatitis are susceptible to microbial infection. Fazakerley et al.[75] have reported in a study including 48 healthy and 24 CAD dogs that 87,5% of the atopic dogs were colonized by *S. pseudintermedius* compared to only 32,7% in the healthy group. Reasons for enhanced bacterial colonization include increased expression of skin adhesion molecules, reduced or impaired antimicrobial peptide deposition, skin barrier dysfunction, chronic inflammation and self-trauma[74]. Bacteria colonize cornified, lesional and to a lower extend non-lesional skin. Superficial pyoderma underlying atopic dermatitis has been associated with highly increased antistaphylococcal IgE levels in serum. Hypersensitivity of IgE can lead to recurrent skin inflammation via activating cutaneous mast cells to produce inflammation mediators such as histamine. Histamine causes inflammation in the skin with symptoms such as pruritus and erythema. Histamine may also affect the chemotaxis of bacteria-recognizing neutrophils and thereby increases persistence of *Staphylococci* on the skin[76].

The immune-response in chronic canine AD is compromised by different cytokines produced by keratinocytes. Cytokines are protein compounds, which enable cell-to-cell communication and interaction in immune cells. Cytokines control activation, proliferation and differentiation of target cells by binding their receptors and influencing the pathways. As a consequence enzyme activity and gene expression can be altered[77]. The most abundant cytokine producing cells are T cells

and macrophages[78]. Canine atopic dermatitis is associated with characteristic cytokine production such as Th1 specific cytokines (IL-12p35, IL-12p40, IL-2, IFNγ, TNFα) for the promotion of cell-mediated immunity, Th2-type cytokines (IL-4, IL-6, IL-13) for activation of humoral immunity and regulatory T-cells cytokines (IL-10, TGFβ) for immune suppression[79]. Schlotter et al.[80] reported different cytokine expression levels depending on the lesional events on the atopic skin. The Th1 specific cytokine IL-12p40 was downregulated in expression level in lesional skin. IL-13 (TH2-type cytokine) was upregulated in lesional and non-lesional atopic skin compared to healthy skin. Not only Th1 and Th2 cytokines could be identified, but also increased levels of regulatory T-cell cytokine (IL-10) was detected in lesional and non-lesional skin samples. In summary, canine atopic skin indicated immune responses from Th1, Th2 and regulatory T-cells.

The treatment for atopic dogs has to be individualized according to the symptoms in atopic dermatitis. Flea prevention and avoiding of food responsible for allergic skin reaction can be undertaking rather easily. Treatments against underlying bacteria and yeast infections, include etiologic therapy (allergen-specific immunotherapy), highly effective systemic and topical symptomatic treatment with Glucocorticoids and low effective systemic and topical symptomatic treatment with e.g. antihistamine[74]. Dupilumab, which is a monoclonal IgG4 antibody inhibiting the signaling pathway of IL-4 and IL-13 and thereby the Th2 immune response, is the newest achievement in treating atopic dermatitis in humans[81]. In two Phase III clinical studies dupilumab proved to reduce symptoms of atopic dermatitis such as pruritus and the overall life-quality could be improved[82]. Since September 26th, 2017 Dupixent is authorized for the European market by the European Union as the first therapeutic antibody for the treatment of atopic dermatitis.

2. Objectives

The aim of this master project was the determination of the therapeutic effect of bovine IgG as a novel anti-infective antibody therapy against antibiotic resistant *S. pseudintermedius* in canine skin infections. The operated experiments involved following aspects:

- Purification of IgG antibodies from bovine colostrum with a GMP certified microfiltration membrane (Membralox® Ceramic Membrane)
- Formulation of bovine IgG in a hydrogel for topical, cutaneous application
- Characterization of virulence factors of clinical *S. pseudintermedius* isolates

- Testing of functionality of bovine IgG in *in-vitro* assays with canine keratocytes
- Development of a canine *ex-vivo* skin model for the determination of the therapeutic effect of bovine IgG in skin infections caused by *S. pseudintermedius*
- Determination of effect of bovine IgG on expression levels of pro-inflammatory cytokines during infection with *S. pseudintermedius*

3. Materials and Methods

3.1. Pilot microfiltration experiment

At the Chair of Food and Bioprocess Engineering TUM School of Life Sciences Weihenstephan a biotechnological process including micro- und ultrafiltration for the recovery of bovine IgG from colostrum milk was developed. The microfiltration process consists of seven ISOFLUX™-membranes (TAMI Industries, France) with 0,14 µm pore size and a filter area of 0,35 m^2 per membrane. This membrane system is accredited for food technological processes, but not applicable for pharmaceutical GMP-conform production. Therefore, a pilot study was performed with a 0,1 µm Membralox® Ceramic Membrane (PALL Corporation, United States of America). In the first step the fat was separated via centrifugation at 4000xg for ten minutes at 10°C (Sorvall LYNX 4000 Superspeed centrifuge, Thermo Fisher Scientific, United States of America).

Before the bioseparation run was started, the plant was cleaned and flushed through with 0,5 M NaOH for 30 minutes. To remove the NaOH out of the system, the plant was flushed with demineralized water for 30 minutes. In total 30 liter of defatted colostrum were filtrated in diafiltration mode. The permeate was lead into a jerrycan, which was placed on a scale to measure the increase in weight throughout the process. The retentate was lead back into the vessel of the plant for diafiltration. The process was run for 170 min. Process monitoring included documentation of $p_{retentate}$, $p_{permeate}$, retentate flux, volume of the permeate, temperature and continuous sampling of the permeate and retentate at defined time points throughout the process.

After the filtration the plant was cleaned by flushing with 2-3% Ecolab P3-ultrasil 115 (Ecolab Inc., United Stated of America) for 30 minutes and impacted overnight. The next day the system was flushed with water until no foam formation could be detected. Subsequently cleaning with 0,5 M NaOH for 70 minutes while heating the system slowly to 40°C occurred. Lastly the system was flushed with 0,8% acetic acid for 70 minutes. After cleaning the performance of the membrane was checked.

3.2. Enzyme-linked immunosorbent assay (ELISA)

To analyze the amount of isolated bovine IgG over time during the filtration process, the CellTrend IgG bovine ELISA kit (CellTrend GmbH, Germany) was used. The IgG detection occurred via a direct Sandwich-ELISA. In this test system the anti-bovine IgG coated microtiter plate was incubated with the filtration sampled for one hour on a shaker at room temperature. The wells in the microtiter plate were washed three times with washing buffer and subsequently peroxidase-marked IgG was pipetted into the wells for detection of bovine IgG. Once again, the plate was incubated for one hour on a shaker at room temperature. The wells were washed with buffer three times and 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution were added. This reaction mixture was incubated for 15 minutes in the dark. The optical density was measured at 450 nm with the VersaMax Microplate Reader (Molecular Devices, LLC, United States of America).

3.3. SDS-PAGE and Western Blot

The protein content of the filtration samples was determined with the NanoDrop One (Thermo Fisher Scientific, United States of America) UV-Vis spectrophotometer. For the separation with a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-Page) the samples with concentrations above 100 mg/mL were diluted 1:50 and below 100 mg/mL were diluted 1:5. The samples were mixed with 4x Laemmli SDS Sample buffer (Bio-Rad Laboratories Inc., United States of America) and heated to 96°C. 15 µL of the denatured samples were applied to a pre-cast Bolt 4-12% Bis-tris Plus gel (Thermo Fisher Scientific, United States of America). As a marker Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories Inc., United States of America) was chosen. The gels were run at 130 V in SDS-PAGE running buffer (20X Bolt™ MES SDS Running Buffer, Thermo Fisher Scientific, United States of America) until the blue front was near the end of the gel (approximately one hour). Subsequently the gels were blotted with the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories Inc., United States of America) on 0,2 µm Trans-Blot® Turbo™ Mini PVDF Transfer Packs (Bio-Rad Laboratories Inc., United States of America) at 25V for ten minutes. The membranes were blocked at 4°C with 5% (v/v) milk powder on a roller bank. Anti-bovine IgG labelled with horse-radish peroxidase (Jackson ImmunoResearch Laboratories, Inc., United Kingdom) was incubated on the membrane overnight at 4°C on a roller bank. The membrane was washed five times with cold TBS and incubated for five minutes in developing solution (Pierce™ ECL Western Blotting Substrate, Thermo Fisher

Scientific, United States of America) . The membrane was developed with ImageQuant LAS 4000 (GE Healthcare, United States of America).

3.4. Antibodies from bovine Colostrum

Two different types of polyclonal bovine antibodies were used to assess their pharmacological impact:

- Anti-alpha toxin IgG (AT AB)
- Natural IgG (bovIgG).

Antibodies specific against the pore-forming *Staphylococcus* alpha-toxin were extracted from colostrum from cows vaccinated with the non-toxic alpha-toxin mutant H35L. The mutant protein expresses all alpha toxin subunits, but cannot assemble into a pore. The vaccine was recombinantly expressed as a his-tagged protein in *Escherichia coli*. Prime immunization was administered 6 weeks before calving. Boostering occurred on day 7 and day 21. The vaccination occurred subcutaneously in use of Saponin Quil-A adjuvant. The bovine Placebo IgG was obtained from colostrum of unvaccinated cows. These antibodies have a natural activity against various factors of the bacterial species *Staphylococcus*. The antibodies were purified via a combinational filtration process including a microfiltration and diafiltration step in diafiltration mode and chromatography (see 1.3.4, 1.3.5).

3.5. Staphylococcus pseudintermedius library

Eight clinical isolates of *Staphylococcus pseudintermedius* strains from dogs was obtained from the Royal Veterinary College, University of London. All bacterial strains were sequenced and the antibiotic resistance profiles were identified[83]. The clinical isolates were stored as glycerol stocks. 600 µL of bacterial overnight cultures were transferred into cryo tubes and 200 µL of 85% glycerol were added for a final concentration of 20% glycerol. The samples were stored at -80°C.

Table 4 Antibiotic resistance profiles of sequenced Staphylococcus pseudintermedius library. OXA-oxacillin, PEN-penicillin, AMP-ampicillin, AMC-amoxicillin/clavulanate, LEX-cefalexin, GEN- gentamicin, KAN-kanamycin, ERY-erythromycin, CLI-clindamycin, SXT- trimethoprim/sulfamethoxazole, CIP-ciprofloxacin, TET-tetracycline

Strain	Phenotypic group	Origin			Phenotypic resistance profile	Resistance to antibiotic classes
		Country	Year	Isolation site		
69687	MDR MRSP	UK	2012	Skin	OXA-PEN-AMP-AMC-LEX-GEN-KAN-ERY-CLI-SXT-CIP	7
HH15	MDR MRSP	Germany	2012	Skin	OXA-PEN-AMP-AMC-LEX-GEN-KAN-ERY-CLI-TET-SXT-CIP	7
GL151A	MDR MRSP	Germany	2012	Wound	OXA-PEN-AMP-AMC-LEX-GEN-KAN- ERY-CLI-TET-SXT-CIP	7
23929	MDR MRSP	Ireland	2008	Skin	OXA-PEN-AMP-LEX-KAN-ERY-CLI-TET-SXT-CIP	7
BNG1	MRSP	UK	2011	Skin	OXA-PEN-AMP-LEX-TET	2
GL117B	MDR MSSP	Germany	2011	Ear	PEN-AMP-KAN-ERY	3
GL118B	MDR MSSP	Germany	2011	Skin	PEN-AMP-KAN-TET	3
463949	MSSP	USA	2012	Skin	PEN-AMP-TET	2
MSSP	Methicillin susceptible *S. pseudintermedius*				Phenotypic susceptible to oxacillin and *mecA* negative, resistant to fewer than three antimicrobial classes	
MRSP	Methicillin resistant *S. pseudintermedius*				Phenotypic resistant to oxacillin and *mecA* positive, resistant to fewer than three antimicrobial classes	
MDR MSSP	Multidrug resistant Methicillin susceptible *S. pseudintermedius*				Phenotypic susceptible to oxacillin and *mecA* negative, resistant to at least three antimicrobial classes	
MDR MRSP	Multidrug resistant Methicillin resistant *S. pseudintermedius*				Phenotypic resistant to oxacillin and *mecA* positive, resistant to at least three antimicrobial classes	

3.6. Rabbit red blood cells (RBC) haemolytic assay

To characterize the clinical *Staphylococcus pseudintermedius* strains, the haemolytic activity was measured. 0,5 mL of rabbit blood in 50% Alsever solution were washed 3x with 10 mL PBS, centrifuged for five minutes at 500xg at room temperature and the supernatant was removed. The washed red blood cells were gently resuspended in 5 mL sterile PBS and counted in the TC20 Automated cell counter (Bio-Rad Laboratories Inc., United States of America). The cell number was adjusted to $1x10^8$ cells/mL. In one half of the microtiter plate serial dilutions (1:2) of supernatant containing α-toxin from overnight bacterial cultures in combination of a constant antibody concentration (1 mg/mL) were prepared. The second half of the plate contained serial dilutions (1:2) of bovine IgG with a starting concentration of 10 mg/mL and bacterial supernatant from overnight cultures. The 96-well plate was incubated for two hours on a shaker (450 rpm) at room temperature. The plate was centrifuged at room temperature for five minutes at 500xg (Rotanta 460 RS, Hettich Benelux B.V., United States of America). 100 µL of supernatant were transferred into a new 96-well plate and the optical density was measured at 415 nm in the VersaMax Microplate Reader (Molecular Devices, LLC, United States of America).

3.7. Minimal inhibitory concentration assay

In order to test the antibiotic resistance of *Staphylococcus pseudintermedius* against Clindamycin, which is the standard antibiotic used in clinics, the minimal inhibiting concentration was assessed. Bacteria were grown overnight in 12 mL tubes (Greiner Bio-One International GmbH, Germany) with sterile T-Hewitt medium at 37°C (Elbanton LT650 incubator, Gembini BV, The Netherlands) and 600 rpm (VWR Mini Shaker, VWR International, United States of America). The OD_{600} was measured and the bacterial concentration adjusted to 10^6 bacteria/mL. 20 µg/mL clindamycin was weighed in and dissolved in T-Hewitt medium. The triple assay was performed in 96-well u-bottom microplates (Greiner Bio-One International GmbH, Germany). The clindamycin was serially diluted (1:2) with a Pipet-Lite XLS+ (Mettler-Toledo International Inc., United States of America) multichannel pipet and the bacteria were added. The positive control contained no antibiotic and the negative control contained neither antibiotics nor bacteria. The plates were incubated for 24 h at 37°C without shaking. After the incubation the microplates were scanned with the Epson Perfection V700 Photo scanner (Seiko Epson K.K, Japan) and the minimal inhibitory concentration was calculated according to the dilution step of clindamycin.

3.8. Biofilm formation assay

As bacteria embedded in their secreted biofilm structures increase the resistance against antibiotics, the biofilm forming capabilities of different *Staphylococcus pseudintermedius* were tested. In the next step the activity of bovine IgG against biofilm formation was studied. Bacteria are plated on BD™ Trypticase™ Soy Agar II with 5% Sheep Blood (Becton, Dickinson and Company, United States of America) and grown overnight at 37°C. One colony was picked from the plate and transferred into 100 µL T-Hewitt medium in a Corning™ Clear Polystyrene 96-Well Microplate (Corning Inc., United States of America) with flat bottoms. The plate was incubated for 24h at 37°C. Consecutively 100 µL of 1% glucose (Sigma-Aldrich Corporation, United States of America) enriched T-Hewitt medium were pipetted into respective wells for six replicates. 3 µL of the overnight bacteria cultures were transferred into the wells and incubated for five hours at 37°C. For the negative control no bacteria were added. After the incubation, the medium was carefully removed and 170 µL of 0,2% crystal violet (Sigma-Aldrich Corporation, United States of America) in phosphate-buffered saline (PBS) were added. The plate was incubated for 15 minutes at room temperature. The wells were washed 2x with 195 µL sterile PBS and remaining liquid was carefully removed by pipetting. The plates were scanned with the Epson Perfection V700 Photo scanner (Seiko Epson K.K, Japan) for visual examination of biofilm formation. Lastly 170 µL of 96% ethanol (Merck KGaA, Germany) were pipetted into the wells and incubated for one hour. The absorbance of the eluted crystal violet was measured at 595 nm (VersaMax Microplate Reader, Molecular Devices, LLC, United States of America).

3.9. Formulation of bovine IgG

The hydrogel was prepared by adding 1,5% of Sodium Carboxymethylcellulose (CMC) to phosphate buffered saline (PBS) under stirring at 150 rpm (IKA RW20, IKA-Werke GmbH & Co. KG, Germany). To enhance the dissolvation-process of CMC, the mixture was heated to 40°C with a heating plate. The sterilization was performed under standard conditions (121°C, 2 bar, 15 min). After autoclaving 1,5 mL aliquots of the sterile CMC hydrogel stock were prepared and antibody was added to the gel for a final concentration of 10 mg/mL.

3.10. Canine *ex-vivo* model

Thoracic normal dog skin (NDS) was provided as waste material from sacrificed beagle-dogs, which were used for a terminal animal experiment (approval by the Committee for Experiments on Animals of the University of Utrecht, The Netherlands). To remove commensal bacteria, the skin

was incubated in Williams' E Medium (Sigma-Aldrich Corporation, United States of America) supplemented with 100 units/mL Penicillin and 100 µg/mL Streptomycin for 20 minutes and subsequently washed with medium without antibiotics 3x. The skin was dried with sterile compresses and 8 mm biopsies were cut out with skin biopsy punches (Stiefel, GlaxoSmithKline plc, United Kingdom). For cultivation Williams' E Medium (Sigma-Aldrich Corporation, United States of America) enriched with 100 units/mL Penicillin and 100 µg/mL Streptomycin (Gibson Laboratories LLC, United States of America), 0,1% Insulin Sigma-Aldrich Corporation, United States of America), 0,02% Hydrocortisone (Sigma-Aldrich Corporation, United States of America) and 1% L-Glutamine (Lonza Group, Switzerland) was prepared. The biopsies were placed into 0,4 µm pore size liquid-air trans-well systems (Greiner Bio-One, Germany) with 500 µL of medium below the filter and 50 µL of medium on top of the filter and either $1x10^8$ CFU bacteria or bacteria plus hydrogel or bacteria plus hydrogel containing 10 mg/mL antibody were applied on the epidermal side. The skin was incubated for 30h at 37°C and 5% CO_2. Storage occurred for cryo-conservation at -150°C and for paraffin-sections in tubes containing 4% formalin at room temperature.

3.11. Histology

Hematoxylin and eosin (H&E), Periodic acid–Schiff (PAS) and Gram staining was executed on paraffin slides from canine skin stored in 4% formalin by the Department of Pathology of the University Medical Centre Utrecht. The slides were microscoped at 40x-magnification with the Nikon Eclipse E800M light microscope (Nikon Corporation, Japan). For each slide ten randomly chosen epidermal sections were photographed and viable, pyknotic and dead cells were counted. The analysis was done with GraphPad Prism 7.04 software

3.12. Immunofluorescence microscopy

Immunofluorescence staining was executed from 3 µm cryosections. The skin biopsies were treated with Fluorescin isothiocyanate (FITC, Sigma-Aldrich Corporation, United States of America) labelled *S. pseudintermedius* and 10 mg/mL antibody in hydrogel. The cultivation occurred in accordance to the canine *ex-vivo* model. The cryosections were incubated in Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific, United States of America) enriched with 1% BSA (Sigma-Aldrich Corporation, United States of America) for ten minutes. To counterstain the bovine IgG, the sections were incubated in the dark with Alexa Fluor® 647-conjugated goat anti bovine IgG (Jackson ImmunoResearch Laboratories, Inc., United Kingdom) at room

temperature for one hour. In the next step the slides were washed with buffer, coverslips were placed on top and fixation with ProLong™ Gold Antifade with DAPI reagent (Thermo Fisher Scientific, United States of America) was performed. The images were photographed with the Leica Confocal Microscope CTR6500 (Leica Camera AG, Germany) using the Leica Application Suite Advanced Fluorescence (LAS AF) software.

3.13. Cell culture

The canine epidermal keratinocyte cell line (MSCEK) was obtained from the Department of Infectious Diseases and Immunology at the University of Utrecht. The cells were grown in tissue culture flasks (TC Flasks T75, Sarstedt AG & Co., Germany). As a medium Williams' E Medium (Sigma-Aldrich Corporation, United States of America) enriched with 1% L-Glutamine (Lonza Group, Switzerland), 10% Newborn Calf Serum (Corning Inc., United States of America), 100 units/mL Penicillin, 100 µg/mL Streptomycin, 0,01 mg/mL Epidermal Growth Factor (EGF, Thermo Fisher Scientific, United States of America) and 0,05 mg/mL Choleratoxin (Thermo Fisher Scientific, United States of America) was freshly prepared. The incubation took place at 37°C and 5% CO_2.

3.14. Bacterial adhesion and cell viability assay with MSCEK cells

The MSCEK cells were seeded in 12-well plates (Corning Inc., United States of America) with $1x10^5$ cells per well. The plates were incubated until confluency at 37°C and 5% CO_2. To remove the remaining Penicillin and Streptomycin from the plate, the cells were washed with sterile PBS (Lonza Group, Switzerland). Bacterial overnight cultures were centrifuged and resuspended in 1 mL of Williams' E Medium supplemented with 10% Newborn Calf Serum (Corning Inc., United States of America). The bacteria were diluted to a final concentration of $1x10^4$ cells/mL in medium and pipetted onto the MSCEK cells. As a treatment 10 mg/mL antibody in PBS were added. The cells were incubated for four hours at 37°C and 5% CO_2. To remove non-adherent bacteria, the cells were washed 3x with PBS and trypsinized with 150 µL 10x Trypsin. The trypsinization was stopped with 500 µL medium (without Pen/Strep). To check the viability of the mammalian cells, the cells were counted with trypan blue in the TC20 Automated cell counter (Bio-Rad Laboratories Inc., United States of America). Afterwards the cells were spinned down at 2000 rpm for ten minutes (Hettich® MIKRO 120 centrifuge, Hettich Benelux B.V., United States of America). The supernatant was removed and the bacterial pellet was resuspended in 1 mL MQ- water to lyse remaining mammalian cells by osmotic shock. To assess the number of adherent bacteria 100 µL

of 1:10 and 1:100 dilutions were prepared and plated on MRSA Colorex™ Chromogenic Media (bioTRADING Benelux B.V., The Netherlands). The plates were incubated for 24h at 37°C and the formed colonies were counted. With respect to the prepared dilutions the colony forming units adherent to the keratinocytes were measured.

3.15. Bacterial adhesion and cell viability assay with canine skin tissue

The canine skin was obtained as waste material from terminal animal experiments from the Veterinary Clinic at the University of Utrecht and biopsies were cut out as previously described. The tissue was incubated with $1x10^6$ bacteria for four hours at 37°C and 5% CO_2. After cultivation the biopsies were washed 3x with sterile PBS to remove non-adherent bacteria. Subsequently, the samples were placed in cryotubes containing twelve sterile bead beater beads and 1 mL of sterile physiological salt solution was added. The cells were lysed for 45 seconds at full speed with the help of the Bead Bug Microtube Homogenizer (Benchmark Scientific, United States of America). After the homogenization the lysed tissue was diluted 1:10 and 1:100 in sterile MQ-water. 100 µL of these dilutions were plated on MRSA Colorex™ Chromogenic Media (bioTRADING Benelux B.V., The Netherlands). The plates were incubated overnight at 37°C. The colony forming units were counted and according to the dilutions the amount of bacteria adherent to the mammalian cells were calculated.

3.16. Scanning electron microscopy

The skin tissue was fixed in 1% (v/v) glutaraldehyde (Sigma-Aldrich Corporation, United States of America) in PBS at 4°C. In the next step the samples were consecutively dehydrated in 30 minutes incubations with 10% (v/v), 25% (v/v) and 50% (v/v) ethanol (Merck KGaA, Germany) diluted in PBS. Subsequently incubation with 75% (v/v) and 90% (v/v) ethanol-water dilutions occurred, followed by two incubations with 96% ethanol. Lastly the tissue was treated with 50% (v/v) ethanol-hexamethyldisilazane (HMDS, Sigma-Aldrich Corporation, United States of America) and 100% HMDS. The samples were air-dried at room temperature overnight and mounted onto 12 mm specimen stubs (Agar Scientific Ltd., United Kingdom). The coating with 5 nm gold was performed using a Quorum Q150R sputter coater (Quorum Technologies Ltd., United Kingdom). The images were taken with the scanning electron microscope (SEM) Scios™ DualBeam™ (Thermo Fisher Scientific, United States of America) and colored using the Adobe Photoshop CS6 software.

3.17. Isolation of RNA from skin tissue

RNA was isolated from 50-60 slices of 20 µm canine skin sections using the RNeasy® Micro Kit (QUIAGEN, Germany). Before starting with the isolation, the working space and used non-sterile materials were cleaned with RNAseZAP to reduce the presence of RNases. Prior following the kit-protocol, the samples were thawed on ice, vortexed and syringed up and down six times through a 1 mL syringe with 0,6 mm needle to homogenize the sample. In the next step 580 µL of RNAse free water were pipetted to the sample and 20 µL of a 10 mg/mL proteinase K were added. The sections were incubated for ten minutes at 55°C on a heating block and spinned down for eight minutes at 12000 rpm (Hettich® MIKRO 120 centrifuge, Hettich Benelux B.V., United States of America). The supernatant was transferred into new 2 mL tubes and the standard protocol for the RNeasy® Micro Kit was executed. The RNA was stored at -150°C.

3.18. Isolation of RNA from MSCEK

For the isolation of RNA from canine epidermal keratinocytes, $1x10^5$ cells were seeded in 1 mL medium (composition previously described) in 12 well-plates (Corning Inc., United States of America). The cells were grown until 80% confluency at 37°C and 5% CO_2. To remove the Penicillin and Streptomycin the cells were washed with PBS (Lonza Group, Switzerland). The keratinocytes were incubated in media without Penicillin or Streptomycin with $1x10^6$ bacteria with or without addition of 10 mg/mL Clindamycin hydrochloride (Bio-Connect Life Sciences, The Netherlands) or bovine IgG in hydrogel for four hours at 37°C. To remove non-adherent bacteria, the wells were washed with two times with PBS. From this point on, the RNA isolation was started according to the RNeasy® Micro Kit protocol with the lysis of the cells with 350 µL RTL buffer. In the last step, the RNA was eluted in 14 µL RNAse-free water and the concentration was determined with the NanoDrop One (Thermo Fisher Scientific, United States of America). Storage occurred at -150°C.

3.19. cDNA synthesis

The isolated RNA, which originated from either canine skin tissue or MSCEK cells, was transcribed into cDNA with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., United States of America). The RNA of all samples was diluted to the concentration of the samples with the lowest RNA content. Table 5 summarizes the reaction mix for a total volume 20 µL.

Table 5 cDNA reaction mixture

Component	**Volume per reaction [µL]**
5x iScript Reaction mix	4
iScript Reverse Transcriptase	1
Nuclease-free water	Variable
RNA template 9100 fg-1µg total RNA	Variable
Total Volume	20

The transcription reaction occurred in the Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Unites States of America) according the protocol in Table 6:

Table 6 Protocol for the transcription of RNA to cDNA

	Temperature [°C]	**Time [minutes]**
Priming	25	5
Reverse Transcription (RT)	46	20
RT inactivation	95	1
Optional step	4	Holding

The cDNA was stored at -20°C.

3.20. Quantitative polymerase chain reaction (qPCR)

Real-time quantitative PCR was performed with the Step One Plus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Unites States of America), using iQ SYBR Green Supermix kit (Bio-Rad Laboratories Inc., United States of America). The sequences of the primers can be found in the Annex (see page 98). The genomic DNA was diluted to a concentration of 10 pg/µL. Following Table 7 summarizes the reaction mixture:

Table 7 qPCR reaction mixture

	1x Preparation [µL]
SybR MMX	12,5
Forward and Reverse Primer [150 nM]	2x0,5
Template	11,5
Total	25

The reaction was programmed according Table 8:

Table 8 qPCR protocol

			Time [min]	**Temperature [°C]**
Holding Stage			10:00	95
Cycling Stage	**Number of cycles: 40**	Step 1	00:15	95
		Step 2	01:00	60
Melt Curve Stage		Step 1	00:15	95
		Step 2	01:00	60
		Step 3	00:15	95

For the analysis, the cycle threshold (C_t) values of the samples were referred to a housekeeping gene (RPS19). To understand the effect of the bovine IgG or clindamycin treatment, the calculated ΔC_t values of treatment samples were compared to non-treatment values ($\Delta\Delta C_t$). The fold-expression compared to the untreated samples, could be determined by calculating $2^{-\Delta\Delta Ct}$.

$$\Delta C_t = C_{t\,Target} - C_{t\,Housekeeping\ gene}$$

$$\Delta\Delta C_t = C_{t\,Treated} - C_{t\,Untreated}$$

$$Fold\ increase = 2^{-\Delta\Delta C_t}$$

4. Results

4.1. Pilot microfiltration experiment

To analyze the efficiency of the microfiltration with a good manufacturing process (GMP) conform membrane module three distinct methods were chosen. Since the OD-measurements of the ELISA-samples showed higher signals than the standard curve, the experiment had to be repeated three times to find the correct sample dilutions. The third ELISA (indicated in green in Figure 11 A.) showed an exponential increase in bovine IgG isolation over time for the permeate. However, the retentate samples did not show the expected decrease in concentration. The second ELISA (indicated in red in Figure 11 B.) showed that the IgG content in the retentate increased during the first 120 minutes and dramatically dropped until the end of the pilot experiment. This could be explained due to an overall temperature increase within the first 120 minutes from 13,3°C to 31,1°C up to 42,5°C at minute 170. Accompanied with the temperature increase, an increase in flux, which might have a positive impact on the crossflow filtration, was detected. Unfortunately the ELISA analysis could not be repeated, because the samples showed degradation after two weeks of storage at 4°C.

A. B.

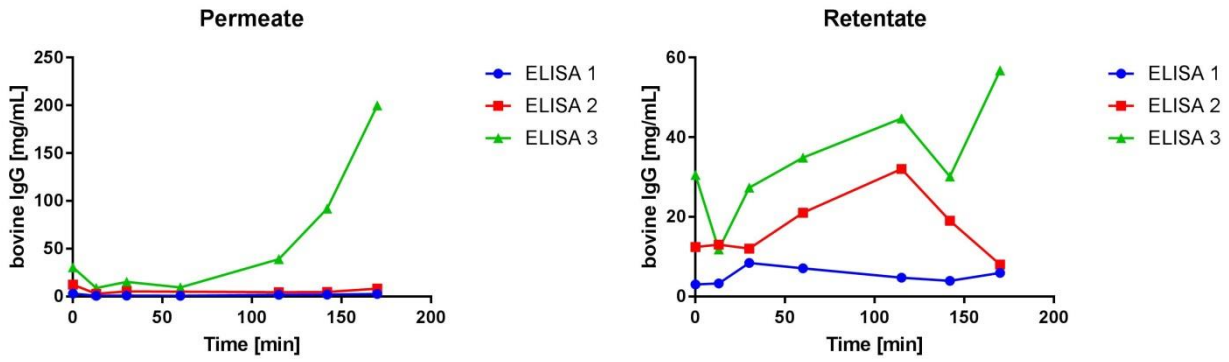
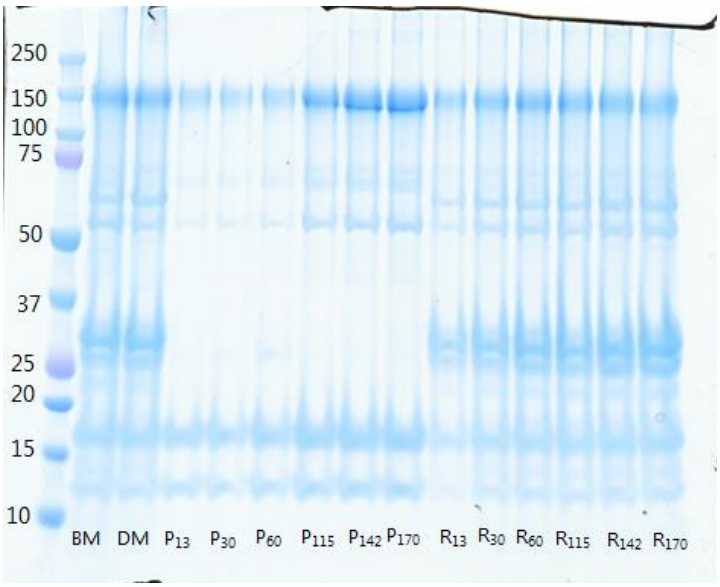


Figure 11 ELISA results of microfiltration permeate and retentate samples

Figure 12 shows a scan of the SDS-PAGE Gel from the samples drawn during the microfiltration process. Colostrum as a biofluid contains heterogeneous components including proteins, sugars, fats, salt and water. The protein fraction compromises molecules of different structures and physicochemical characteristics. Therefore the interpretation of the gel is rather difficult. IgG has

a molecular weight of 150 kDa and the band running at this height increased in intensity over the time (p_{13}-p_{170}). Over time IgG was enriched in the permeate. The retentate samples did not show the expected decrease in intensity throughout the process, indicating that IgG was present after the bioseparation process. Another purpose of the microfiltration is the removal of the casein fraction forming a micelle. Caseins have a molecular weight between 11,6 to 25 kDa. For the permeate samples, the SDS-PAGE revealed no band at this height. Thus, the MF depleted the caseins. In comparison, the retentate samples showed a band at this height. In addition other whey proteins could pass the membrane and were present in the permeate. At approximately 65 kDa bovine serum albumin (BSA) showed a band. Concerning the actual production process, these impurities can be removed by ultrafiltration and chromatography (see section 1.3.4 and 1.3.5).



Protein	MW [Dalton]
Casein	25 000 – 11 600
β-Lactoglobulin	18 300
α-Lactalbumin	14 200
BSA	66 000
IgG1	163 000
IgG2	150 000
IgA	390 000
IgM	950 000
Transferrin	75 000- 77 000
Lactoferrin	77 000 – 93 000
Lactoperoxidase	77 500

Figure 12 A. Scan of SDS-PAGE with table summarizing the molecular weights (MW) of important milk proteins. [23] BM=beast milk, DM=defatted milk, P=permeate at defined time point, R=retentate at defined time point. B. Summary of molecular weight of milk proteins.

To specifically identify bovine IgG a Western Blot analysis was performed. At time points 115, 142 and 170 minutes the IgG band increased in intensity confirming the data obtained by SDS-PAGE (see Figure 13).

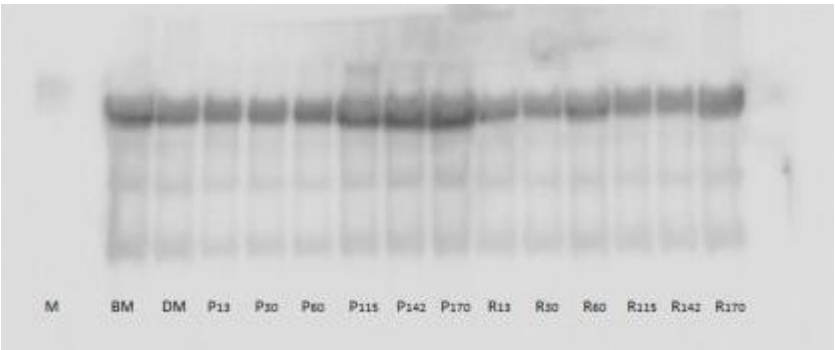
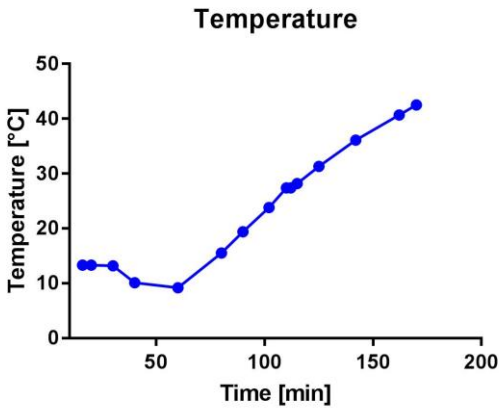


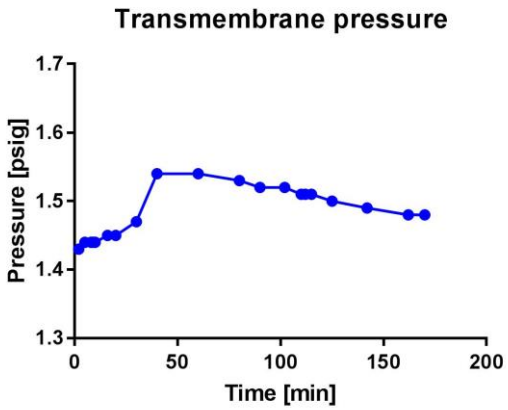
Figure 13 Western Blot of microfiltration samples. BM=beast milk, DM=defatted milk, P=permeate at defined time point, R=retentate at defined time point

Figure 14 summarizes the monitored process parameters throughout the process time of 170 minutes. After the experiment start the permeate flow ($Q_{Permeate}$) declined throughout the first 80 minutes. From this time point on the flow increased up to 88 mL/min. The TMP stayed within an acceptable range and was rather stable. After 50 minutes the temperature increased from 10°C to 45°C. Simultaneous with the increase of temperature, also $Q_{Permeate}$ increased. It is likely that the increasing temperature had a beneficial effect on the physicochemical properties of the milk and thereby enhanced the flow within the filtration system.

A.



B.



C.

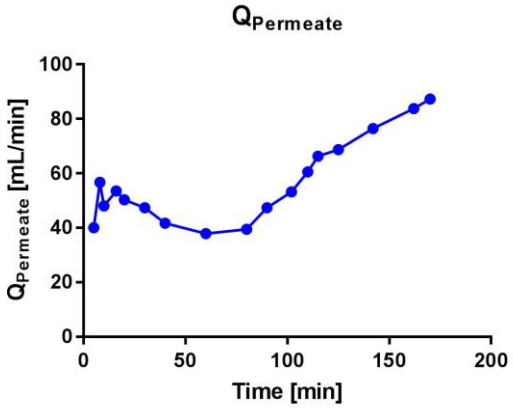


Figure 14 Process monitoring of microfiltration pilot experiment. A. Temperature, B. Transmembrane pressure, C. $Q_{Permeate}$

According to its performance, the GMP conform membrane is suited for the process. IgG is enriched while simultaneously casein is removed. The process parameters are in an acceptable range.

4.2. Formulation of bovine IgG in hydrogel

For the formulation of bovine IgG in Sodium Carboxymethylcellulose (CMC) two different CMC concentrations were tested. A concentration of 3% CMC resulted in a viscosity that could no longer

be mixed in a sufficient way. The powder aggregated in the bottom of the beaker glass. The attempt to decrease the viscosity by heating the hydrogel to a higher temperature of approximately 50°C was interrupted due to possible degradation of the hydrogel components. Thus, a second CMC gel was prepared with a concentration of 1,5%. In the second attempt, CMC power was successively poured into the beaker glass under continuous stirring to prevent clump formation of the gel. These adjustments lowered the viscosity and improved the performance of the IKA RW20 stirrer. After autoclaving the gel was plated on sheep blood agar plates to test the sterility of the product. No bacterial growth could be detected after 24h of incubation. The antibody was added to the hydrogel under sterile conditions in the flow cabinet. The gel was stored at room temperature and for every *ex-vivo* dog experiment a new aliquot containing 10 mg/mL bovine IgG was prepared. The storage of hydrogel with bovine IgG at 4°C did not result in precipitation of protein.

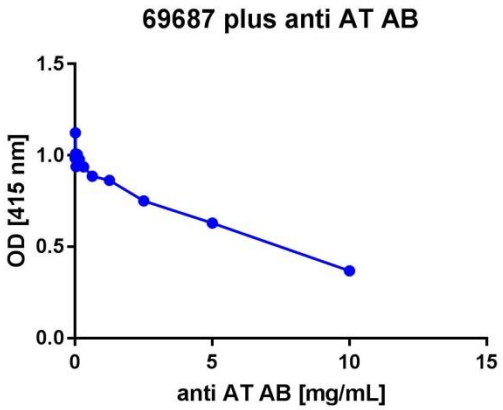
4.3. Toxigenicity of *S. pseudintermedius* on rabbit erythrocytes

Toxic damage of erythrocytes by α-toxin (AT) can be assessed by incubation of culture supernatant of *S. pseudintermedius* with rabbit blood cells and determination of hemolysis extend. The experiments were designed to cover two different aspects: on one hand the effect of bovine IgG on the hemolysis potential in a concentration dependent manner was studied and on the other hand the AT containing bacterial supernatant was titrated and a constant antibody concentration added to see a AT concentration dependent binding by the IgG. Furthermore, the possible beneficial effect of vaccinating cows with the non-toxic alpha-toxin mutant H35L was tested.

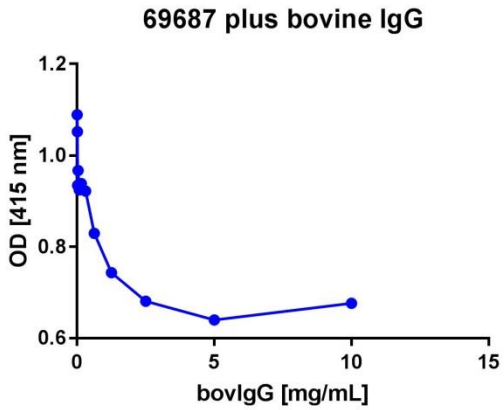
Figure 15 shows the anti-hemolytic potency of the anti AT AB versus the naturally occurring bovine IgG. The hemolysis caused by culture supernatant of 69687 can be reduced by the AT AB in a linear concentration dependent manner. At the highest concentration (10 mg/mL) an OD_{600} of 0,4 could be reached. The trend of the curve suggests, that at higher antibody concentrations the hemolysis could have been reduced even more. The naturally occurring bovine IgG shows at its highest concentration a weaker reduction (OD_{600} = 0,65) in hemolysis, leading to the assumption that vaccination increases the recognition level of α-toxin. At concentrations above 5 mg/mL the antibody reaches its plateau and cannot decrease hemolysis to a higher extend.

The bacterial strain GL151A was tested with the anti AT antibody and showed an overall lower hemolysis. The hemolysis could be reduced with 5 mg/mL anti AT AB to OD_{600} = 0,4. The trend of the curve suggests, that concentrations above 5 mg/mL anti AT AB would not result in a higher hemolysis decrease.

A.



B.



C.

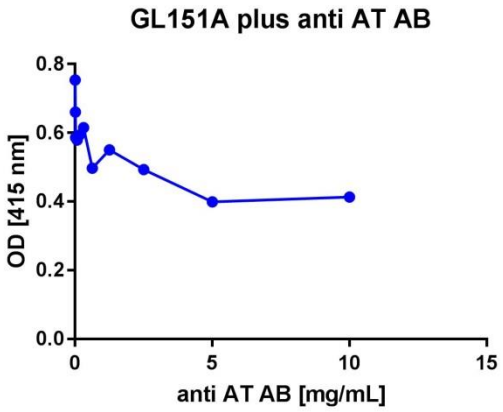
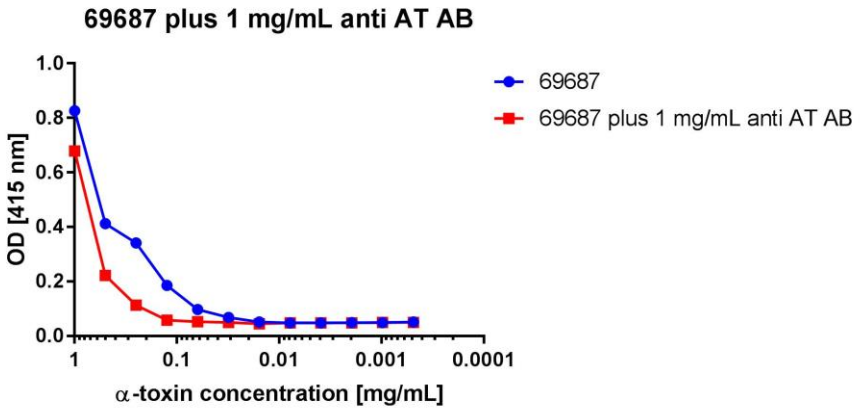


Figure 15 Comparison of anti-hemolytic activity of AT AB and bovine IgG. A. anti AT AB titration for 69687, B. bovine IgG titration for 69687, C. anti AT AB titration for GL151A

In order to compare the rate of hemolysis in the absence or presence of the toxin neutralizing antibody, supernatants of the bacterial strains GL151A and 69687 were titrated and a constant concentration of 1 mg/mL anti AT AB or buffer as control was added (see Figure 16). The hemolysis by 69687 and GL151A supernatants could both be reduced and proof hemolytic activity of both canine bacterial strains which can be inhibited by the bovine anti AT antibodies.

A.



B.

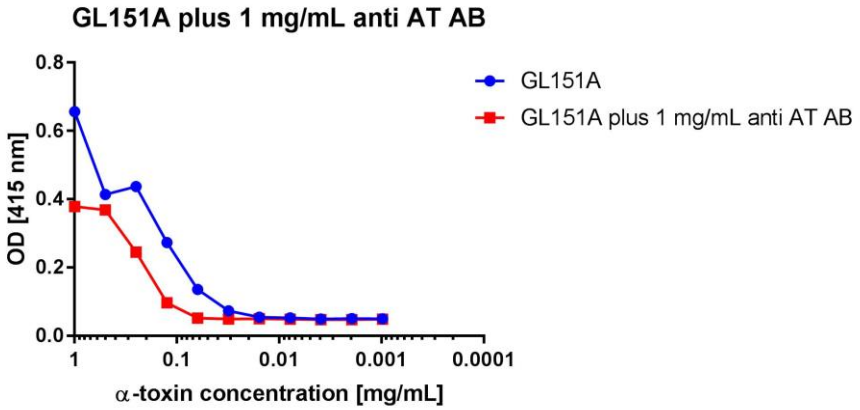


Figure 16 Hemolytic decrease by anti AT AB for A. 69687 and B. GL151A

4.4. Antibiotic resistance of *S. pseudintermedius* in correlation with biofilm formation

As antibiotic susceptibility testing is an important feature for determination of correct treatment option, the *S. pseudintermedius* library was screened for resistance to the gold-standard antimicrobial therapy clindamycin (see Figure 17). The bacteria were incubated with a serial dilution of clindamycin to test for the minimal inhibiting concentration (MIC), which is the lowest antibiotic concentration that shows growth inhibition of bacteria. The higher the MIC, the higher the antibiotic resistance to a certain drug. According to the British Society of Antimicrobial

Chemotherapy, *Staphylococci* are considered to be clindamycin susceptible at MIC breakpoints less than or equal to 0,00025 mg/mL. The MIC for clindamycin resistant *Staphylococci* lies above concentrations of 0,0005 mg/mL.

The clinical isolates 69687, HH15, GL151A and 23929 were described to be MDR MRSP (multidrug resistant methicillin resistant *S. pseudintermedius*) and phenotypically resistant to clindamycin by McCarthy et al. [83]. The resistance to clindamycin could be confirmed for all four strains. Within the MDR MRSP group, GL151A showed growth at the highest clindamycin concentration (2,5 mg/mL), followed by 69687 and HH15 at 1,25 mg/mL and 23929 at 0,16 mg/mL. The colonies of 69687 showed a precise shape, whereas HH15 and 23929 appeared to grow in form of weak colonies and GL151A showed diffuse colonies.

BNG1 is defined to be *mecA* positive, phenotypically resistant to oxacillin and methicillin resistant *S. pseudintermedius* (MRSP) with resistance to two antimicrobial classes. McCarthy et al. [83] did not define clindamycin resistance for this isolate. In presence of clindamycin, BNG1 was inhibited in its growth up to a clindamycin concentration of 0,16 mg/mL. The MDR MSSP (multidrug resistant methicillin susceptible *S. pseudintermedius*) group, including GL117B and GL118B, did not grow at any clindamycin concentration, which is in compliance to McCarthy et al. [83]. Although MSSP (methicillin susceptible *S. pseudintermedius*) 463949 was not defined to be resistant to clindamycin, a resistance profile with a MIC of 0,625 mg/mL could be determined.

As biofilm formation is an important virulence factor for successful and persistent infection by bacteria, the biofilm forming potential of the *S. pseudintermedius* library was tested. The MDR MRSP group showed excessive biofilm growth. Interestingly, this shows the compliance to antibiotic resistance and suggests the hypothesis that antibiotic resistance is associated with biofilm formation.

A. B.

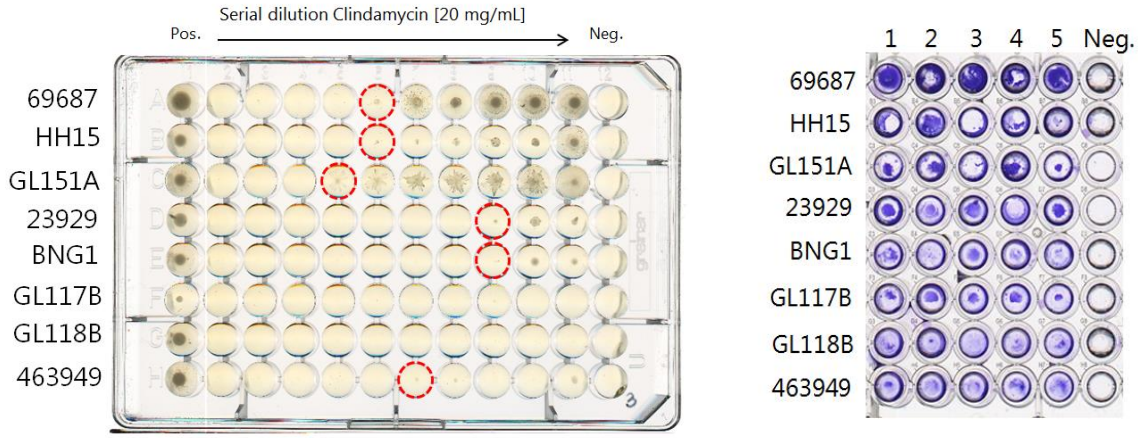


Figure 17 Determination of antibiotic resistance profiles of *S. pseudintermedius* library and correlation of antibiotic resistance and biofilm formation. A. Minimal inhibiting concentration of S. pseudintermedius library against clindamycin. Indicated in red are the breakthrough points at which bacteria show their resistance. B. Biofilm screening for *S. pseudintermedius* library

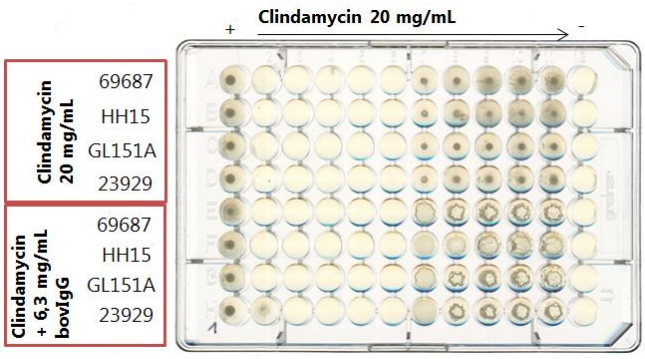
4.5. The effect of bovine IgG on growth of antibiotic resistant *S. pseudintermedius*

To assess the effect of bovine IgG against the canine *S. pseudintermedius* a combination of MIC assay and pharmacological testing was developed. Within this assay the growth-inhibiting effect of clindamycin, the combinational therapy of antibiotics and bovine IgG and the solo effect of bovine IgG in a concentration dependent manner could be determined. As a control bovine serum albumin (BSA) was added to the assay. The experiment was repeated two times.

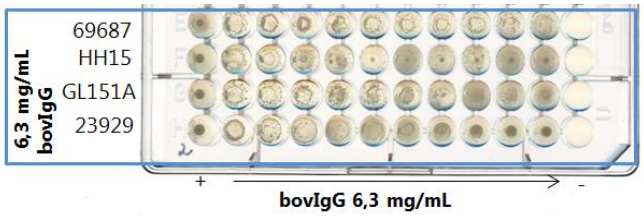
In Figure 18 A. the MDR MRSP minimal inhibiting concentrations against clindamycin can be determined. 69687, HH15, GL151A and 23929 show their breakthrough points at 0,625 mg/ml clindamycin. When a constant concentration of 6,3 mg/mL bovine IgG was added, the colonies showed deformation and a different morphology. This suggests, that the antibodies interfered with the bacterial growth. A combinational treatment approach would have the advantage of attacking the bacteria with antimicrobial substances and simultaneously working with antibodies binding virulence factors. Figure 18 B. shows the concentration dependent effect of bovine IgG on the bacterial growth. The growth of 69687 was changed throughout the entire dilution series of bovine IgG. HH15, GL151A and 23929 showed altered and reduced colony formation at certain antibody

dilutions. The combination of clindamycin and BSA and the dilution of BSA showed no alteration and leads to the assumption, that the growth inhibiting effect of bovine IgG is specific.

A.



B.



C.

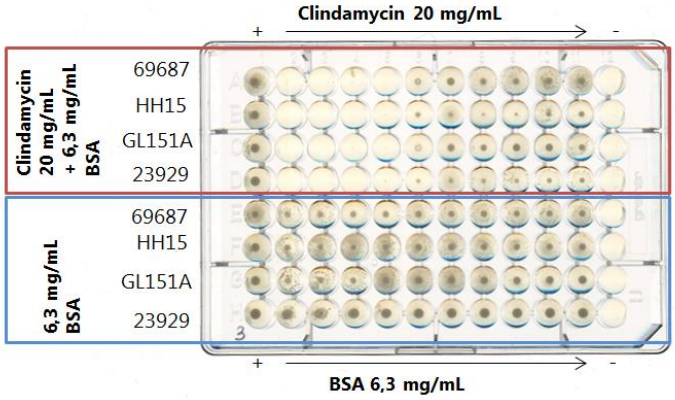


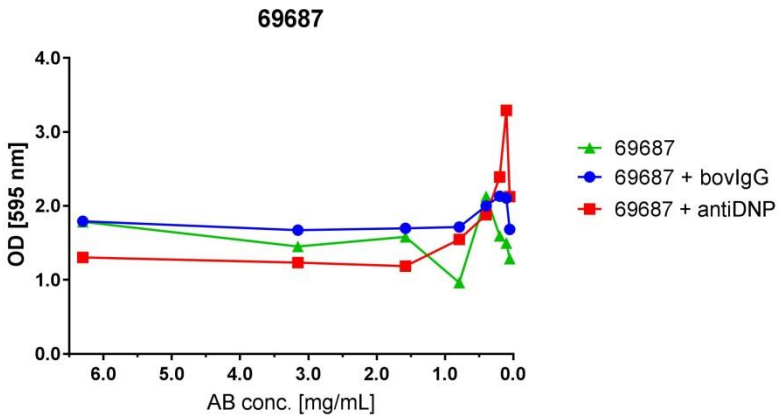
Figure 18 Effect of bovine IgG on the growth of antibiotic resistant *S. pseudintermedius* in a solo and combinational approach.

4.6. The effect of bovine IgG on biofilm formation by antibiotic resistant *S. pseudintermedius*

Biofilm formation causes challenges in treatment of bacterial infections. *S. pseudintermedius* has been associated with growth in form of biofilms. The *S. pseudintermedius* library was tested for biofilm information (see 4.4). In order to test the possible anti-biofilm effect of bovine IgG a new biofilm assay was developed. In the first attempts the antibody was titrated to determine the concentration-dependent effect against biofilms. As a control the anti DNP antibody was included into the assay. This monoclonal antibody binding to a chemical compound should not have an effect on the biofilm formation.

69687 and GL151A were both identified to build biofilms. An addition of bovine IgG did not reduce the biofilm formation for 69687. Anti DNP showed a higher reduction in biofilm formation than bovine IgG. This effect has to be unspecific as the target of anti-DNP Abs should not influence biofilm formation. GL151A showed higher biofilm formation in presence of both antibodies. This result cannot be explained rationally with antibody-binding. In general, high variabilities within the quantification of the biofilm assays remained a challenge.

A.



B.

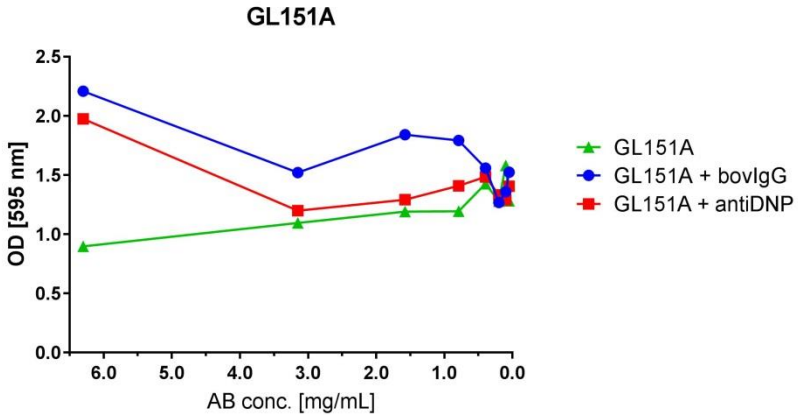
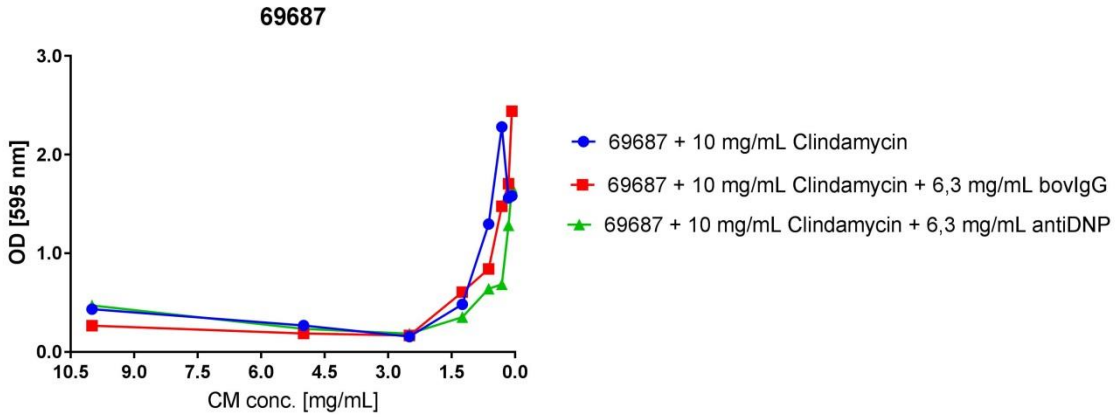


Figure 19 Testing of anti-biofilm capability of bovine IgG for 69687 and GL151A

In advanced experiments, the combinational therapy including clindamycin and bovine IgG, was tested. For both 69687 and GL151A, no biofilm inhibiting effect could be identified. The quantification graphs for 69687 and GL151A show the same curve trend for bacteria without and with treatment.

A.



B.

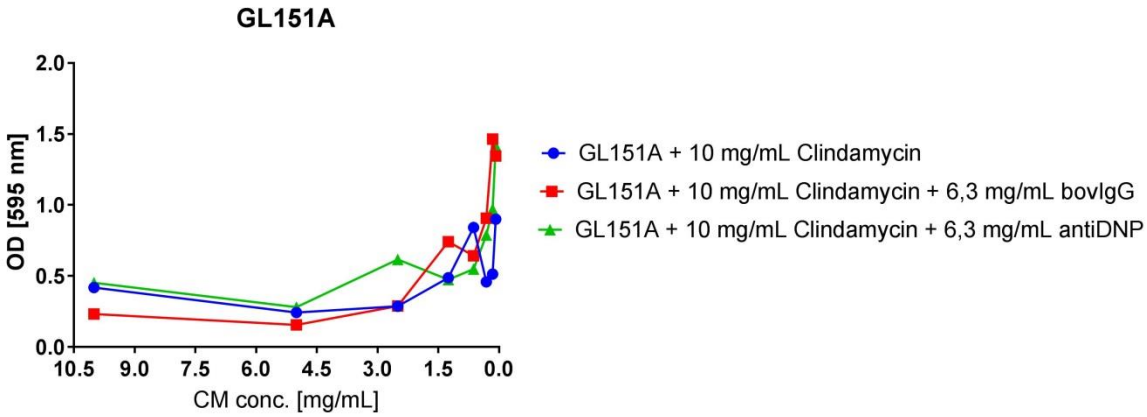


Figure 20 Combinational treatment approach with clindamycin and bovine IgG for A. 69687 and B. GL151A

These results lead to the assumption that clindamycin has little to no chance of effecting growth in biofilm forming bacteria, because EPS shields the colonies effectively against antimicrobials. Furthermore, bovine IgG does not reduce the degree of biofilm formation as single treatment or in combination with clindamycin. In general it has to be recognized that biofilm assays have the downside of error proneness. In the execution of the experiments several washing steps are required. With each washing step the chances of unintentional removal of the visible biofilm increases. Furthermore, minimal amounts of residual crystal violet after washing, led to intense

coloring during excavation with ethanol. Subsequently, the quantification showed high levels of fluctuation in several assay replications and validation of results was problematic.

4.7. Interface of colonization and infection of S. pseudintermedius in canine epidermal keratinocytes

To study the interface of *S. pseudintermedius* colonization and infection on the skin *in-vitro* experiments with canine keratinocytes were developed. The assays were performed with a canine epidermal keratinocyte cell line (MSCEK) originating from skin-biopsy from healthy mixed-breed dogs[84]. The cells are adherent and show distinct cell nuclei with precise nucleoli (see Figure 21).

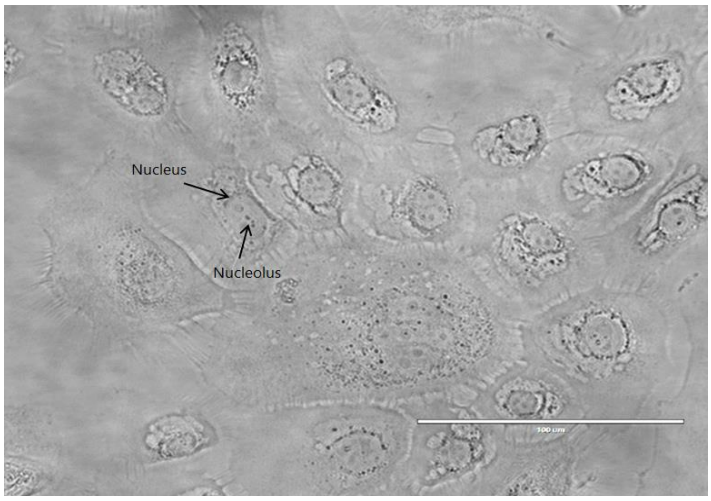
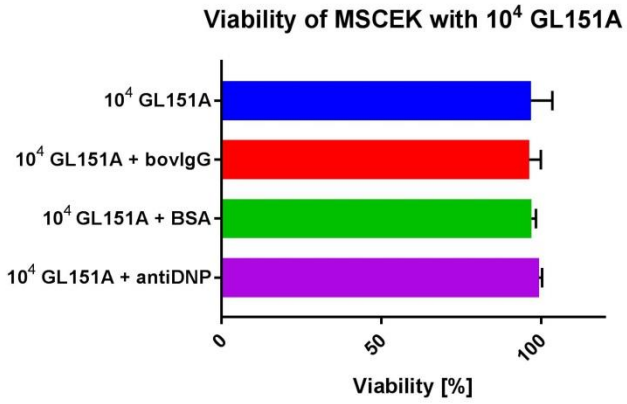


Figure 21 Canine epidermal keratinocytes (MSCEK) image at 40x magnification

Within the first experiments, the effect of bacteria on the viability of the MSCEK cell line was tested. Confluent cells were incubated with 10^4 or 10^6 *S. pseudintermedius* for four hours. Afterwards, the cells were trypsinized and the viability was checked with a cell counter. In preliminary experiments bovine serum albumin was used as a control. Subsequently, monoclonal anti dinitrophenyl antibodies were used as an antibody control. Figure 22 shows the viability percentage at the two different bacterial concentrations (10^4 and 10^6). Within multiple experiment repetitions the cell viability was proven to be stable between 95-100% with and without bovine IgG. The BSA and anti-DNP controls also showed high amount of viable cells.

A.



B.

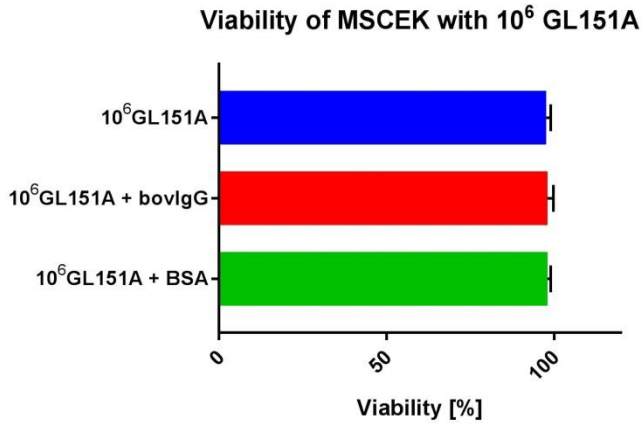


Figure 22 Viability of canine epidermal keratinocytes after incubation with 10^4 GL151A (A.) and 10^6 GL151A (B.). Bovine IgG was added at a concentration of 6,3 mg/mL and antiDNP at a concentration of 5,2 mg/mL. bovIgG= bovine IgG, BSA= bovine serum albumin, antiDNP= anti dinitrophenyl antibody

For determination of adherence of *S. pseudintermedius* to canine keratinocytes, adhesion assays were performed. The confluent cells were incubated with bacteria for four hours and washed multiple times to remove non-adherent bacteria. The bacteria, adherent to the MSCEK cells, were plated on MRSA Colorex™ Chromogenic Media (bioTRADING Benelux B.V., The Netherlands) plates, which are commonly used in routine diagnostics for *S. aureus* identification. In presence of 10^4 *S. pseudintermedius* approximately 20 000 CFU could be found, a reduction to 3 600 could be obtained with bovine IgG (see Figure 23). Anti-DNP also had a reductive effect on colony formation. However, this effect has to be unspecific. The bovine IgG antibody seems to have a beneficial outcome on reduction in bacterial adhesion. In general, it has to be stated, that determination of quantities of adherent bacteria is a rather difficult undertaking, as the numbers of bacteria on the MRSA selective plates can vary strongly between experiments.

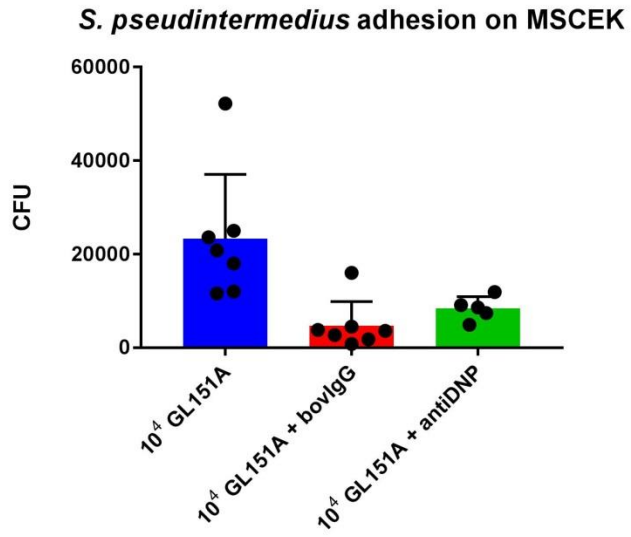


Figure 23 Colony forming units (CFU) of adherent S. pseudintermedius on canine epidermal keratinocytes

4.8. Expression of pro-inflammatory cytokines in canine keratinocytes in presence of *S. pseudintermedius*

Bacterial infections can alter the expression profile and level of cytokines. Th1 cytokines activate the cellular immunity, while Th2 cytokines support the humoral immune system[85]. Immunosuppressive cytokines produced by regulatory T-cells play a major role in regulation and control of pro-inflammatory cytokines. To gain more insight into the cytokine production by the MSCEK cell line during bacterial infection, qPCR analysis was performed. Table 9 summarizes the screened cytokines.

Table 9 Cytokines produced by canine keratinocytes

Th1	IL-12p35, IL-12p40, IFNγ, TNFα
Th2	IL-4, IL-13
Regulatory T-cells	IL-10, TGFβ

The pro-inflammatory cytokine TNFα showed alteration in expression levels in presence of the bacterial strains 69687 and GL151A. It was increased to a 10-fold expression with GL151A (see

Figure 24 A). The antibody treatment reduced this production to a 3-fold expression. In comparison 69687 showed a lower induction of TNFα production. The bovine IgG did not have a beneficial effect in this setting. Both bacterial strains, GL151A and 69687, TNFα expression could not be determined with clindamycin treatment. For IL-12p40 (see Figure 24 B) increased cytokine production could be determined for clindamycin treated samples. Compared to the cytokine production in the control, cytokine levels for GL151A and GL151A plus bovine IgG as well as 69687 were downregulated. The expression level of IL-12p40 for the combination of 69687 and bovine IgG could not be determined.

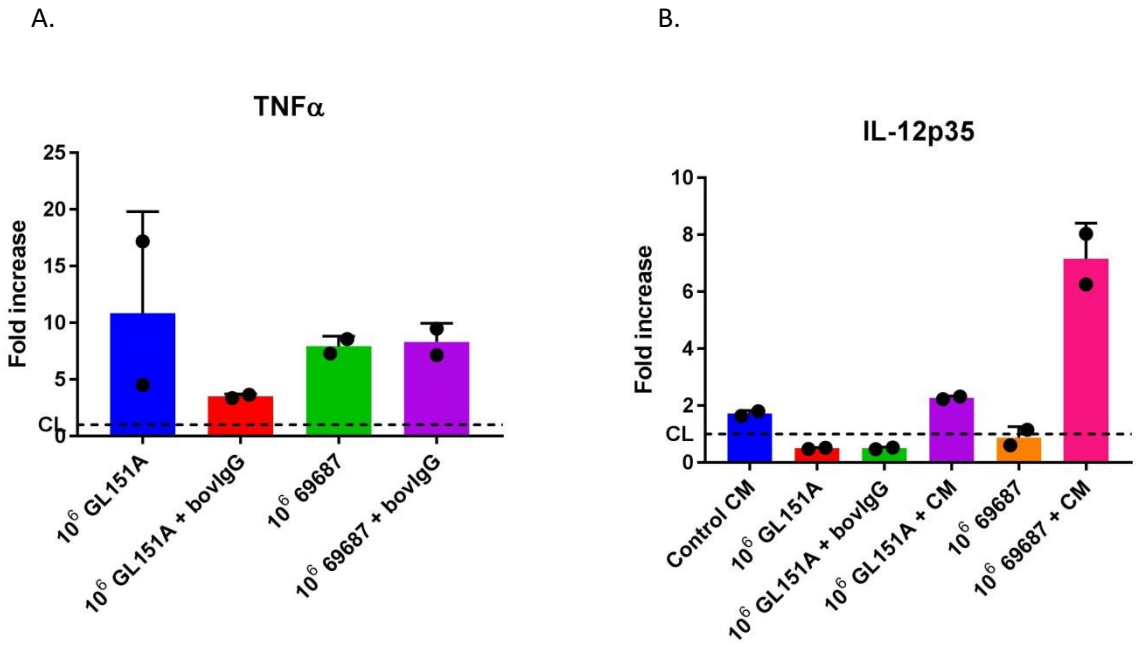


Figure 24 Regulation of cytokine production (A. TNFα and B. IL-12p40) during bacterial infection in MSCEK cells. BovIgG= bovine IgG, CM= clindamycin

4.9. Interface of colonization and infection of *S. pseudintermedius* in a canine *ex-vivo* skin model

In order to investigate the interface between bacterial colonization and infection of *S. pseudintermedius* on canine skin microscope techniques were used. Bacterial infections in skin are often found in combination with skin barrier dysfunctions and immunosuppremised individuals. As canine skin is thinner than human skin, it is likely to be more sensitive to microbal colonization.

With the help of an immunofluoresence experiment the colonization of *S. pseudintermedius* on skin and the interaction of bovine IgG with the canine bacteria could be examined. In Figure 25

the successful DAPI staining of s*tratum corneum*, epidermis and dermis can be seen in blue. The FITC labelled *S. pseudintermedius* are visible as green patches and individual spots. Bovine IgG could be detected with Alexa Fluor® 647-conjugated goat anti bovine IgG in red. The overlay of the individual staining images detects green bacteria on the surface of the skin, but also invading into the s*tratum corneum*. The bovine IgG could be identified on the surface of the skin sample but also in the s*tratum corneum.* The interaction between green bacteria and red bovine IgG results in a yellow coloration and proves that bovine IgG is co-localized with *S. pseudintermedius*.

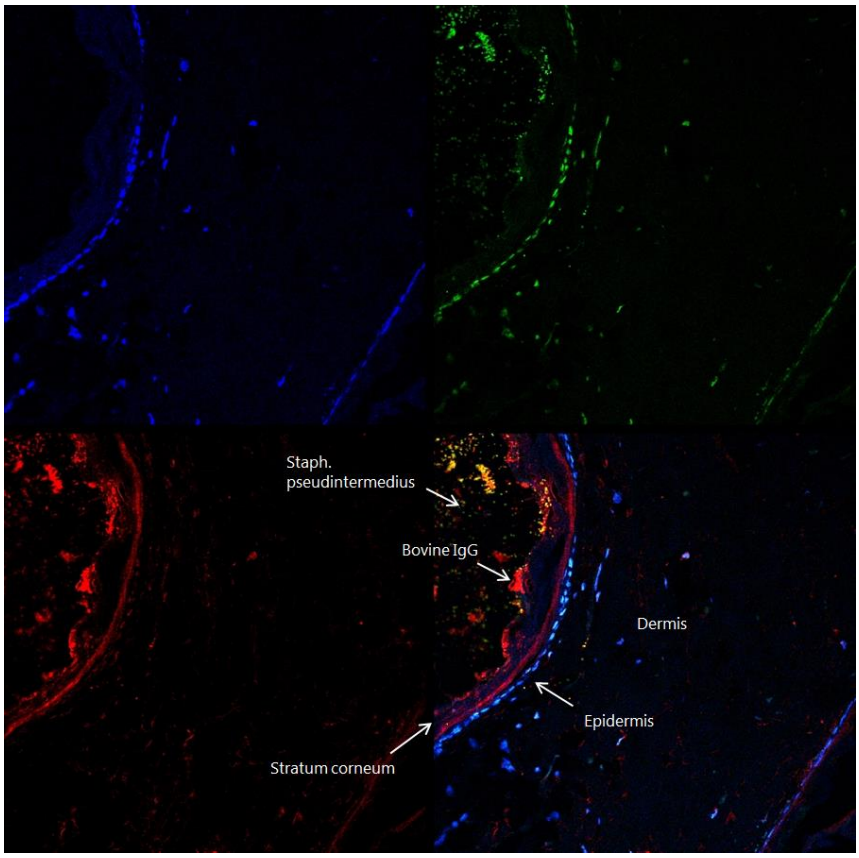
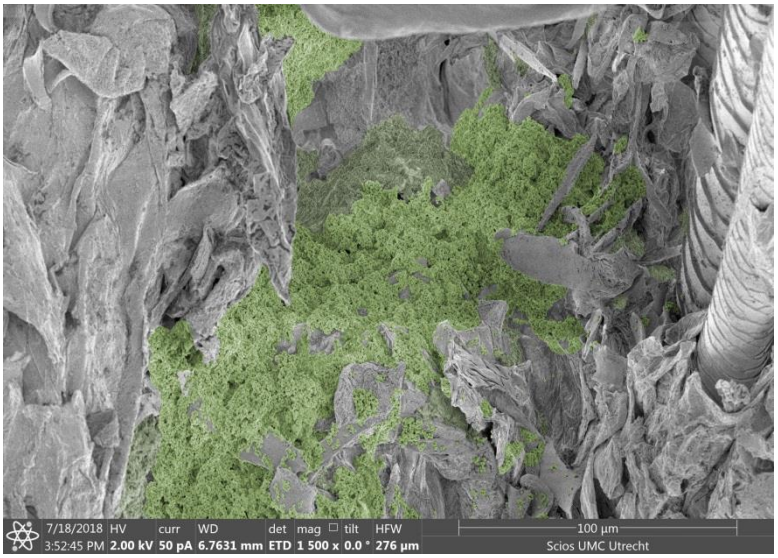


Figure 25 Immunofluorescence microscopy of 3 µm skin cryosection. The canine *S. pseudintermedius* were FITC labelled. Bovine IgG was detected with Alexa Fluor® 647-conjugated goat anti bovine IgG. The nuclei of the skin cells were stained with DAPI.

For understanding of the localization and colony formation of *Staphylococci* on canine skin scanning electron microscopy was utilized. After a 24h colonization with bacteria with or without antibody or antibiotic treatment the skin samples were serially dehydrated and coated with gold. The skin samples without treatment (see Figure 26) showed massive bacterial colonies. *S. pseudintermedius* was rarely found in form of individual cells but rather as comprehensive

patches. Furthermore, they appeared close to hair follicles where migration into the skin is likely to be facilitated.

A.



B.

Figure 26 Scanning electron microscopy images of skin biopsies cultivated with S. pseudintermedius. A. Bacterial colonies could often be found close to hair follicles where migration into the skin is facilitated; Scale= 100 µm. B. Colonization of bacteria on s*tratum corneum*; Scale= 30 µm.

In presence of 10 mg/mL clindamycin or bovine IgG, the bacteria showed an altered behavior. Comprehensive bacterial colonization could not be identified. Contrariwise, small patches of bacteria very often hidden underneath dead cells of the *stratum corneum*. In these spots it is probably more difficult for topical treatment to deploy complete therapy spectrum.

A.

B.

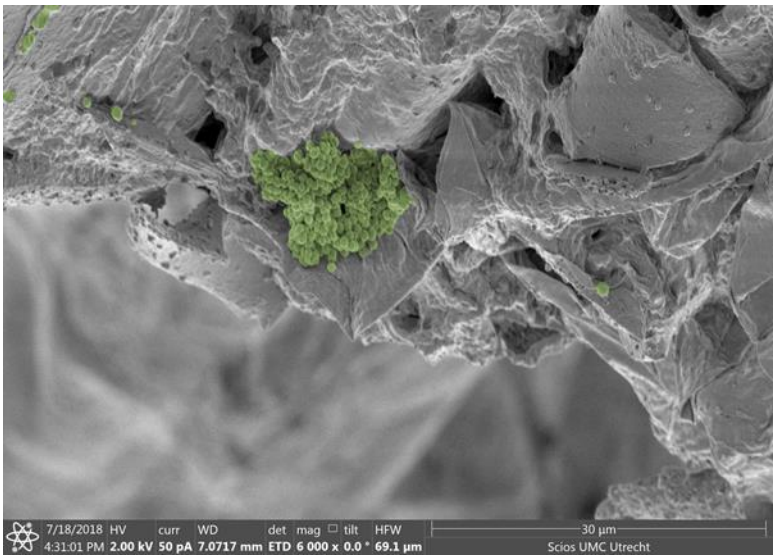


Figure 27 Scanning electron microscopy images of skin biopsies cultivated with *S. pseudintermedius* and treated with 10 mg/mL clindamycin (A.) or 10 mg/mL bovine IgG (B.)

4.10. Investigating the bacterial infections in a canine *ex-vivo* model

For histological examination of canine skin three different types of staining were used. Hematoxylin-eosin staining was used to identify morphological changes in the tissue and

determine three different conditions of epidermal cells (see **Fehler! Verweisquelle konnte nicht gefunden werden.**). Viable cells could be seen as purple dots. Cells that currently undergo apoptosis (pyknotic cells) showed a condensed cell content. Dead cells, also referred to as ghost cells, appeared as white spots without any content.

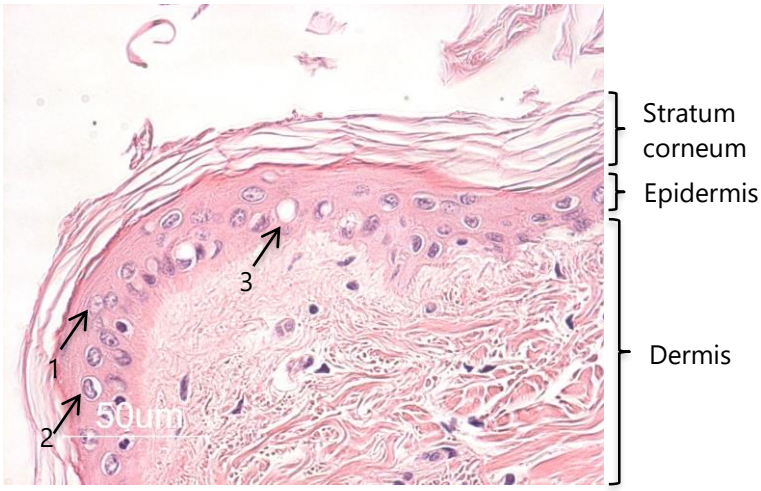


Figure 28 Hematoxylin-Eosin staining of canine skin. Viable cells= 1; Pyknotic cells= 2; Dead cells= 3.

In the development of the canine *ex-vivo* model multiple challenges had to be tackled. Preliminary experiments failed as bacteria massively invaded into the skin and destroyed the integrity of the skin structures. Figure 29 shows an example of immense bacterial infection by *S. pseudintermedius* strain 69687. At time point 0 the skin had full integrity. After 30h of cultivation (T1) the biological viability had already lowered and more pyknotic as well as ghost cells could be seen. Furthermore, the epidermal layer started to separate from the underlying dermis. The addition of bacteria showed heavy migration of bacteria into the skin and lead to disruption of the integrity. The epidermal layer was torn apart and appeared as single keratinocytes. *stratum corneum*, epidermis and dermis separated from each other and a conclusion about the outcome of the experiment was difficult to draw. In presence of bovine IgG the severity of the infection could be reduced and less damage in the viability of the cells was seen. Nevertheless, bacteria were regardless present between epidermis and dermis. Clindamycin also reduced the infection, but skin integrity could not be restored. In order to increase the mimicry of *in-vivo* bacterial infections, the skin biopsies were washed with Williams' E Medium containing 100 units/mL Penicillin and 100 µg/mL Streptomycin to remove commensal bacteria. Furthermore, Penicillin and Streptomycin was added into the cultivation medium. Subsequent experiments showed less biological damage after the adjustment in the protocol.

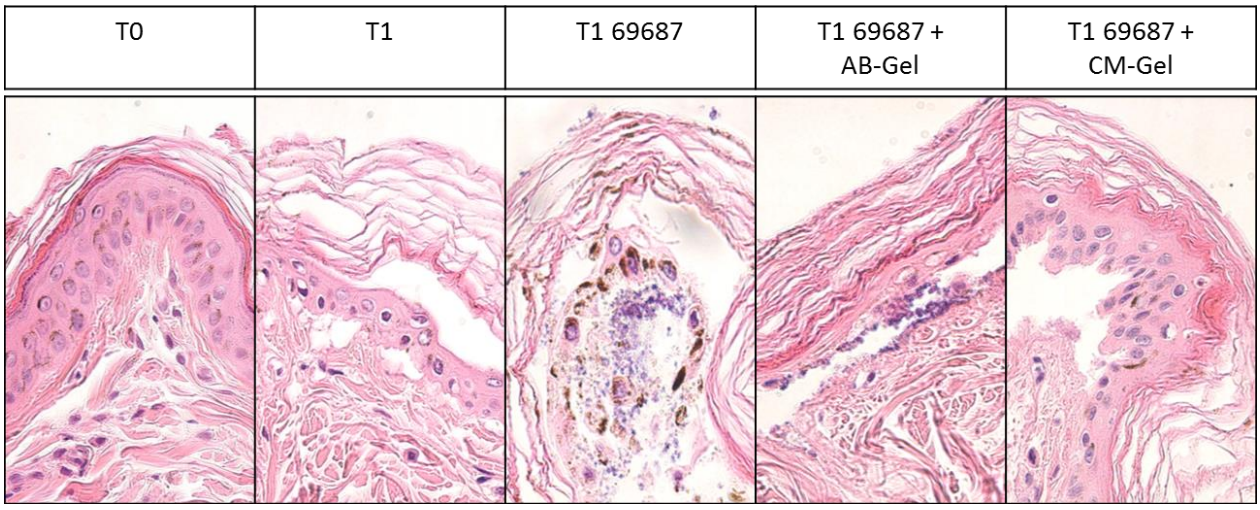


Figure 29 Challenges in the development of an *ex-vivo* skin model. The biopsy at time point 0 (T0) was taken immediately after obtaining the tissue. Time point 1 represents the skin samples after 30 h of cultivation. AB-Gel= bovine IgG antibody in hydrogel, CM-Gel= clindamycin in hydrogel

To gain deeper insight into commensal colonizing antibiotic-resistant bacteria, a skin biopsy was incubated with *S. pseudintermedius* for four hours and subsequently the tissue was lysed with bead beating. The supernatant was plated on MRSA Colorex™ Chromogenic Media (bioTRADING Benelux B.V., The Netherlands). These plates are frequently used in microbiological diagnostics for identification of MRSA. Colonies formed on the plates had either MRSA specific pink or blue coloration. Blue colonies are according to the manufacturer´s description non-inhibited, methicillin resistant *Staphylococci*. Analysis by MALDI-TOF identified pink colonies as *S. pseudintermedius* and blue colonies as *S. lentus*. This finding indicates the significance of antibiotic-resistant commensal bacteria in dogs.

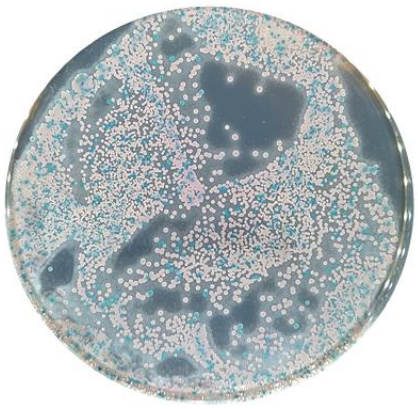


Figure 30 MRSA Colorex™ Chromogenic Media (bioTRADING Benelux B.V., The Netherlands) plate showing the multi-resistant *S. pseudintermedius* as pink colonies and methicillin-resistant *S. lentus* as blue colonies.

The differences in the infection profiles of the clinical isolates 69687 and GL151A were tested in the *ex-vivo* model. **Fehler! Verweisquelle konnte nicht gefunden werden.** shows that at time point T1 after 30h of cultivation the cells show some signs of degradation, compared to T0. However, compared to previous experiments without washing and cultivation with antibiotics (see Figure 29), the cultivation seemed to have less effect on the viability of the cells. In presence of 69687 more dead and pyknotic cells could be identified. The bacterial infection furthermore caused dissemination of epidermis and dermis. Addition of bovine IgG as a treatment, reduced the infectious effect of 69687. The addition of clindamycin also led to a reduction in bacterial damage in the skin. The combinational therapy of clindamycin and antibodies could restore the natural integrity of the skin and showed high amounts of viable cells.

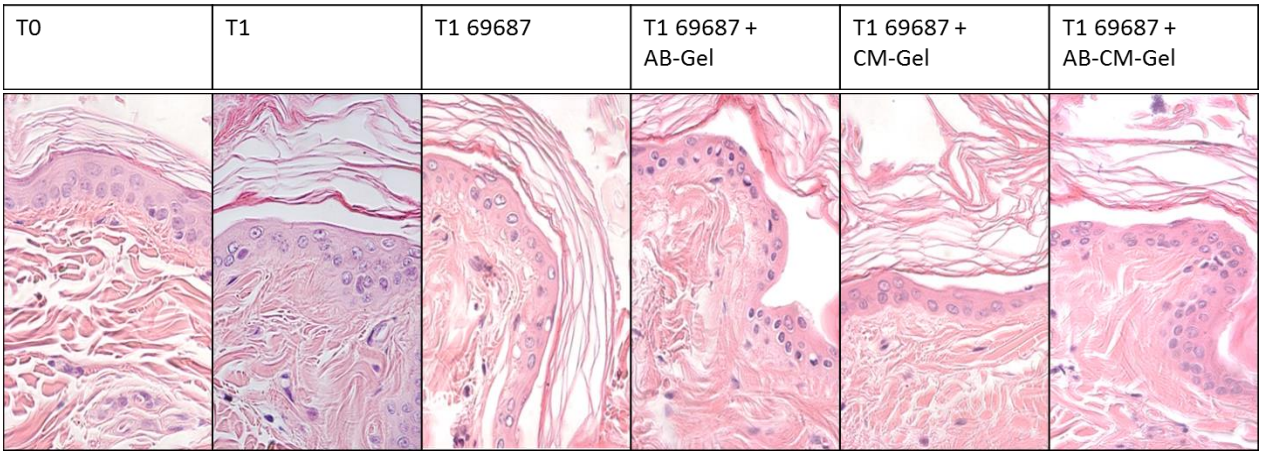


Figure 31 Ex-vivo skin model with clinical isolate 69687. The biopsy at time point 0 (T0) was taken immediately after obtaining the tissue. Time point 1 represents the skin samples after 30 h of cultivation. AB-Gel= bovine IgG antibody in hydrogel, CM-Gel= clindamycin in hydrogel, AB-CM-Gel= bovine IgG antibody and clindamycin in hydrogel

To quantify the effect of bacteria on the skin under conditions with and without treatment, the viable, pyknotic and dead cells were counted in a blinded fashion and relativized. The quantification for bacterial strain 69687 in skin obtained from dog number 17 (NDS17= normal dog skin number 17) can be found in Figure 32. The control showed approximately 97% viability in the cells. This leads to the assumption that the *ex-vivo* cultivation was successfully performed and had little effect on the epidermal cells. The addition of 69687 resulted in an increase of 9% pyknotic cells and 1% ghost cells. If hydrogel was added to the skin, the same effect as with just 69687 could be observed, leading to the assumption that the gel did not have a beneficial effect on the infection outcome. Bovine IgG slightly improved the amount of viable cells. However, clindamycin

had an even stronger positive effect on the skin. The combinational therapy showed higher amounts of pyknotic cells compared to just clindamycin.

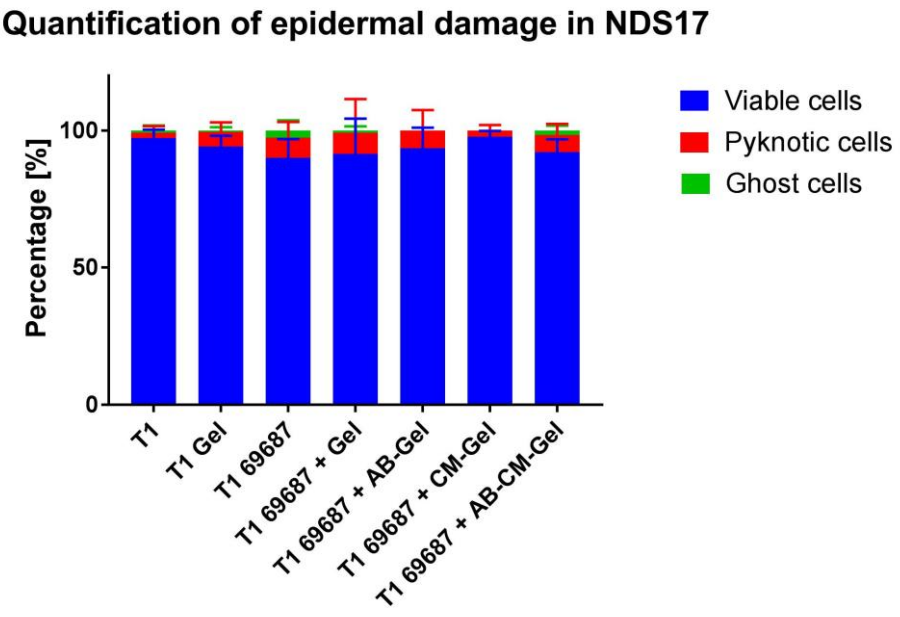


Figure 32 Quantification results for normal dog skin number 17 (NDS17) with 69687. Time point 1 represents the skin samples after 30 h of cultivation. AB-Gel= bovine IgG antibody in hydrogel, CM-Gel= clindamycin in hydrogel, AB-CM-Gel=bovine IgG and clindamycin in hydrogel

Dog number 18 (NDS18) was infected with 69687 and showed a different quantification pattern (see Figure 33). After 30h of cultivation (T1) the viability of the cells was 100%. The same applied for application of antibody in hydrogel without addition of bacteria. The antibody in hydrogel did not show an effect on the viability of the cells. The presence of bacterial strain 69687 reduced the amount of viable cells to 88%. The addition of hydrogel to the bacteria, reduced resulted in approximately 2% of pyknotic cells and reduced the negative effect of bacteria on keratinocytes. The addition of antibody or clindamycin treatment could restore almost 100% viability. The combinational therapy approach of antibody and antibiotics could not be tested in NDS18 due to lack of material.

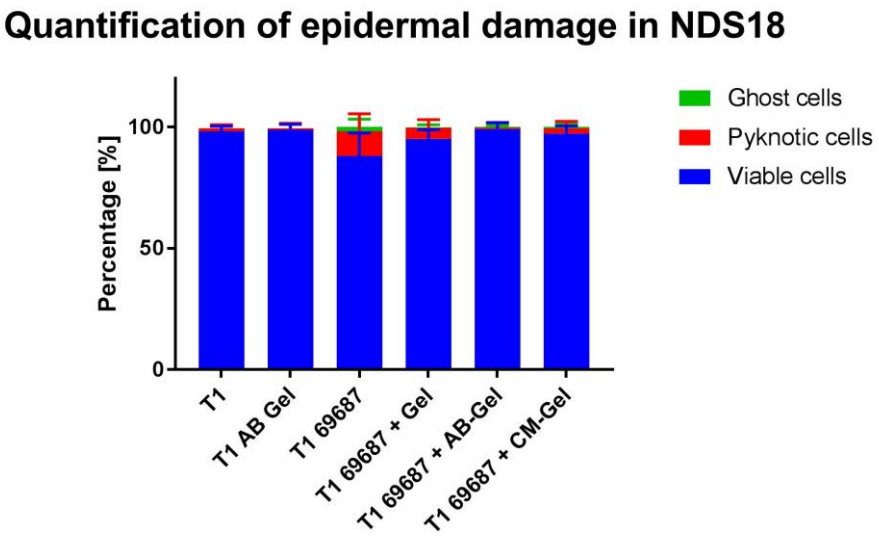


Figure 33 Quantification results for normal dog skin number 18 (NDS18) with 69687. Time point 1 represents the skin samples after 30 h of cultivation. AB-Gel= bovine IgG antibody in hydrogel, Gel= hydrogel, CM-Gel= clindamycin in hydrogel, AB-CM-Gel=bovine IgG and clindamycin

The difference in viability in keratinocytes in NDS17 and NDS18 during infection with *S. pseudintermedius* 69687 stresses the biological variability in the dog skin. Compared with 69687, GL151A showed a different infection profile (see Figure 34). The histological staining of T1 proved, that the cultivation per se worked without a high impact on the viability of the epidermal keratinocytes. GL151A migrated into the dermis and caused dissemination of epidermis and dermis. A plausible explanation for this separation could be the production of exotoxins. The viability of the cells was subsequently strongly affected by the bacteria. By adding bovine IgG, the destructive effect of the bacteria could be prevented. Nevertheless, pyknotic and dead cells could be identified. Clindamycin showed a beneficial effect on the skin integrity, but large amounts of apoptotic and ghost cells were present. The combinational therapy approach consisting of bovine IgG and clindamycin could however restore the integrity and viability of the skin.

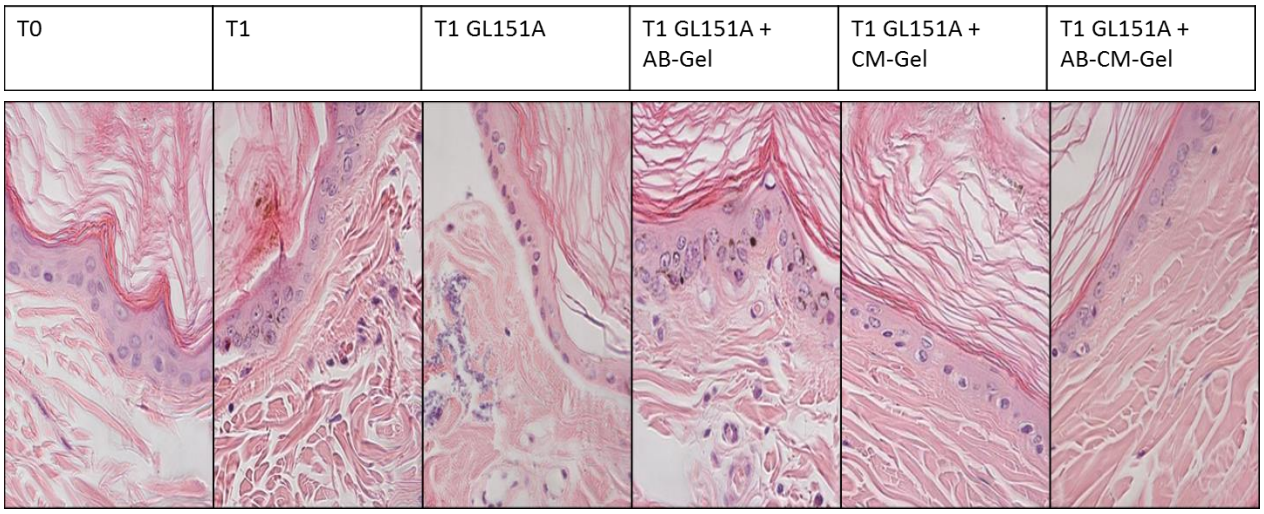


Figure 34 *Ex-vivo* skin model with clinical isolate GL151A. The biopsy at time point 0 (T0) was taken immediately after obtaining the tissue. Time point 1 represents the skin samples after 30 h of cultivation. AB-Gel= bovine IgG antibody in hydrogel, CM-Gel= clindamycin in hydrogel, AB-CM-Gel=bovine IgG and clindamycin in hydrogel

The quantification of the three cell types were reflective for the overall outcome of the characteristics visible in the HE stainings. The cultivation itself had small effect on the cell function. For NDS19 the T1 control with clindamycin in gel was added. Compared to T1, the clindamycin showed a small reduction in viability. GL151A infection resulted in a massive reduction in cell viability to approximately 65%. Hence, GL151A had a bigger toxic effect on the skin in comparison to 69687. By addition of hydrogel without any therapeutic components, the viability could slightly be increased. The therapeutic bovine IgG resulted in a beneficial outcome for the canine skin. Surprisingly, adding clindamycin as an antimicrobial showed a larger amount of pyknotic cells compared to cultivation with just bacteria. This finding was unique for dog number 19. In addition it has to be recognized, that the clindamycin control and the adjunctive therapy with bovine IgG and clindamycin did not reflect the same effect. For rational explanation of this result, additional *ex-vivo* models with GL151A are necessary.

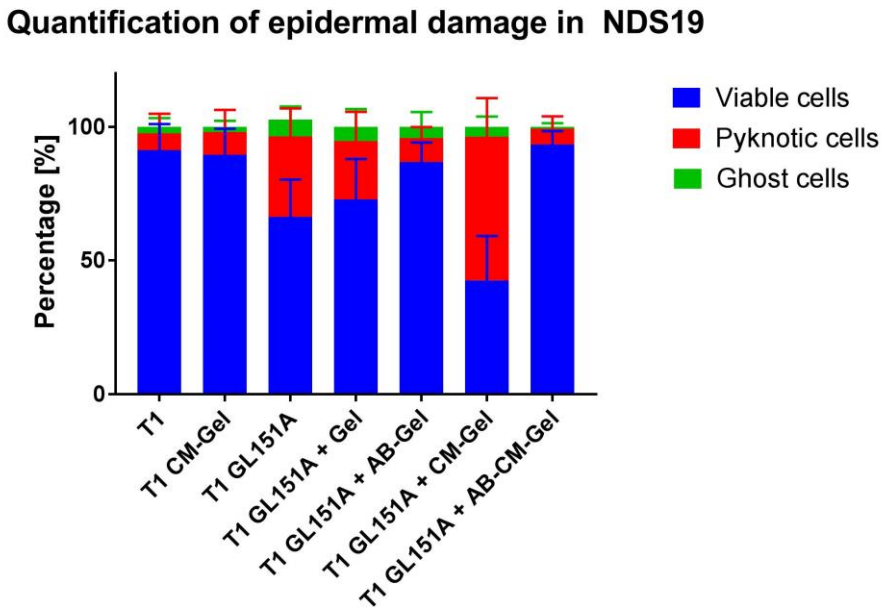


Figure 35 Quantification results for normal dog skin number 19 (NDS19) with GL151A. Time point 1 represents the skin samples after 30 h of cultivation. CM-Gel = clindamycin in gel, AB-Gel= bovine IgG antibody in hydrogel, CM-Gel= clindamycin in hydrogel, AB-CM-Gel=bovine IgG and clindamycin in hydrogel

Dog Number 20 (NDS20) was likewise infected with GL151A, but showed a different quantification pattern (see Figure 36). The 30h cultivation and the addition of bovine IgG did not affect the viability of the keratinocytes. The addition of GL151A reduced the amount of viable cells to 70%. In this setting approximately 10% of dead cells were found. The addition of bovine IgG, clindamycin or the combination could beneficially result in 100% viability. NDS20 showed in comparison to NDS19 a less severe infection. The massive viability loss with clindamycin in NDS19 could not be seen in NDS20. Contrariwise clindamycin treatment resulted in 100% viability. Biological viability in the tissue samples from different dogs could explain this finding and represents the downside of the *ex-vivo* model.

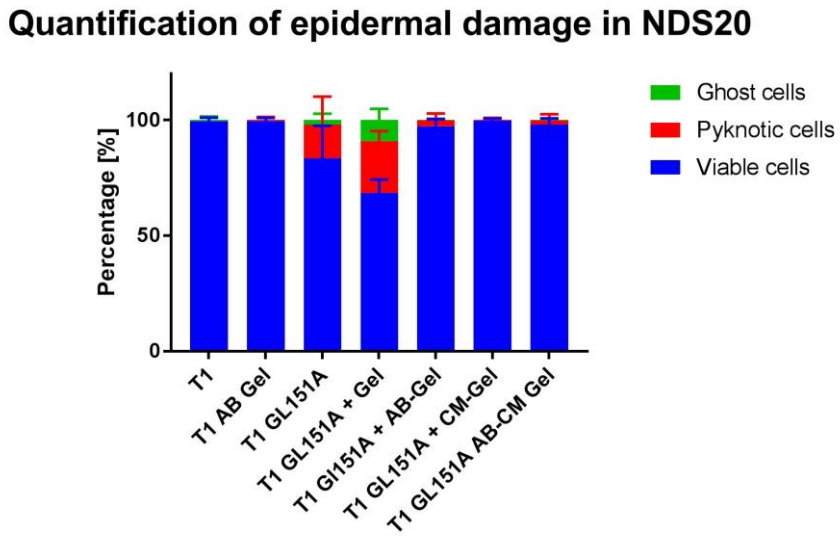


Figure 36 Quantification results for normal dog skin number 20 (NDS20) with GL151A. Time point 1 represents the skin samples after 30 h of cultivation. AB-Gel= bovine IgG antibody in hydrogel, CM-Gel= clindamycin in hydrogel, Gel= hydrogel, AB-CM-Gel=bovine IgG and clindamycin

For the attempt to quantify the adherent bacteria on canine skin, adhesion assays were performed. The graph in Figure 37 shows a reduction in colony forming units in presence of bovine IgG. The anti-DNP control antibody also showed a decrease in bacterial colonies. However, this result has to be unspecific. The findings with canine skin can be equalized with the adhesion assays from the MSCEK *in-vitro* results (see Figure 23). Both experiments show a therapeutic effect of antibody therapy.

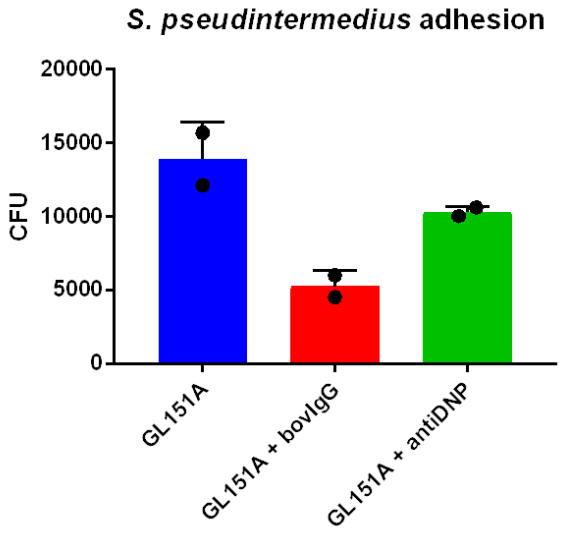


Figure 37 Colony forming unit (CFU) of adherent bacteria on canine skin

4.11. Effect of bacterial infections and treatments on the expression level of pro-inflammatory cytokines in canine skin tissue

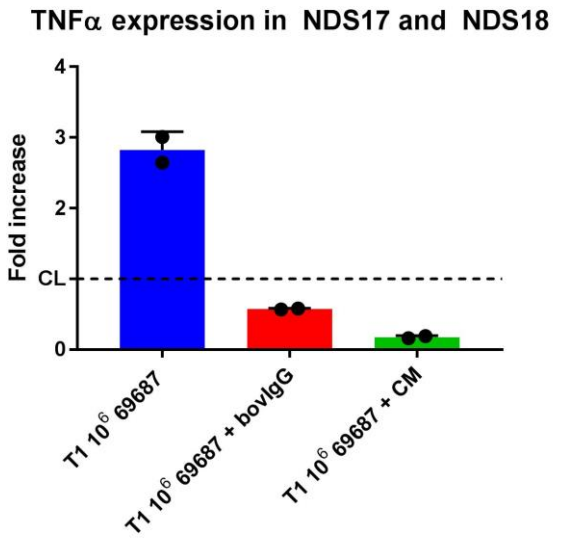
Bacterial infections can induce and effect the production of pro-inflammatory cytokines. Th1 cytokines are known to activate cellular immunity, whilst Th2 cytokines induce the humoral immune response. In general allergies are associated with an imbalance of Th1 and Th2 lymphocytes towards the Th2 cells. Consequently, atopic dermatitis is characterized by higher Th2 cytokine production levels[86]. Regulatory T-cells produce immunosuppressive cytokines to control the overall immune response[85]. With the help of qPCR the expression of cytokines, produced by canine explant skin during 30h cultivation with *S. pseudintermedius*, were studied. Table 10 summarizes the screened cytokines. The experiments were performed in correlation to the *in-vitro* qPCR experiments with the MSCEK cell line which displays skin keratinocytes (see section 4.8). Quantification of the fold expression compared to the reference sample was calculated according to the equations in section 3.20.

Table 10 Cytokines produced by canine skin

Th1	IL-12p35, IL-12p40, IFNγ, TNFα
Th2	IL-4, IL-13
Regulatory T-cells	IL-10, TGFβ

NDS17 and NDS 18 were both infected with *S. pseudintermedius* strain 69687. The expression of the pro-inflammatory cytokine TNFα was elevated approximately 3x in comparison to the uninfected and untreated reference sample. Treatment with bovine IgG could downregulate the expression level, clindamycin lowered the expression to a higher extend. The Th2 cytokine IL-13 showed a different expression pattern. Infection with 69687 upregulated its expression 4x. Bovine IgG strongly downregulated the cytokine quantities, whereas clindamycin treatment upregulated the production. Thus, TNFα was produced in a lower quantity than IL-13 during infection with 69687. Clindamycin had a downregulating effect for TNFα, whereas IL-13 was upregulated during infection with 69687.

A.



B.

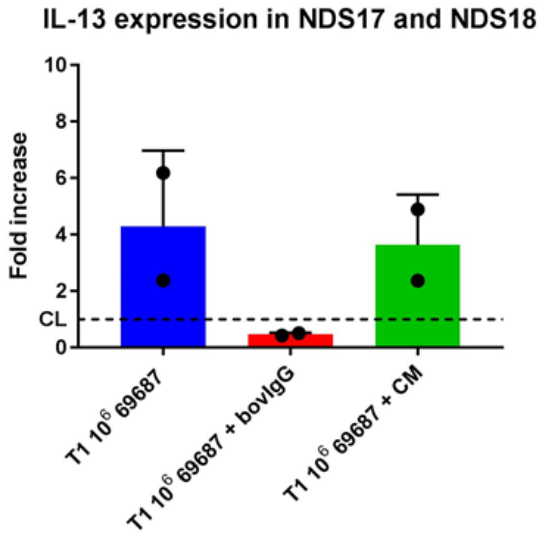


Figure 38 Expression of Th1 cytokine TNFα (A.) and Th2 cytokine IL-13 (B.) in NDS17 and NDS18 with bacterial strain 69687. BovIgG= bovine IgG, CM= clindamycin

For NDS19 and NDS20, which were infected with bacterial strain GL151A, cytokine production could not be measured. The fold increases in expression were very low and no distinct pattern could be deducted from the measured results. The H&E staining of NDS19 and NDS20 revealed high impact of GL151A on the viability of the epidermis. The low cytokine expression levels could be explained by loss in activity of the epidermal skin layer.

5. Discussion

The aim of this master thesis was the determination of pharmacological efficacy of bovine antibodies against skin infections caused by *Staphylococci* in dogs. Bovine antibodies might be a prospective effective alternative treatment against pathogenic bacteria. According to Ulfman et al.[87] bovine antibodies can bind human pathogens and allergens, render *in-vitro* infection in human cells and reduce gut inflammation. In addition bovine IgG can promote phagocytosis, neutralization of bacteria and presentation of pathogens on antigen-presenting cells. With regard to emerging antimicrobial resistance in *Staphylococcus*, the natural activity of bovine IgG against these pathogens can be beneficially utilized. Bovine immunoglobulins can impair antibiotic resistant bacteria without effecting their antibiotic resistance profile. Furthermore, bovine IgG can neutralize exotoxins and interfere with tissue damage and immune evasion. Established antibody therapy could lead to a reduction in antibiotic prescription and thus minimize the emergence of antibiotic resistant bacteria[5].

Applicability of the GMP certified 0,1 µm Membralox® Ceramic Membrane for isolating bovine IgG from colostrum

From a production point of view, bovine IgG can be isolated from colostrum at low cost and in large-scale at high concentrations according to GMP. The benefits of colostrum as an antibody source not only include the high natural titer, but also high availability. More and more companies are investigating the possibility of bovine antibody therapy. For instance, Immuron Ltd.(Blackburn North, Australia) investigates the development and commercialization of gut immunotherapy with bovine colostrum against *Clostridium difficile* infections.

The large-scale extraction of bovine IgG from colostrum according to good manufacturing guidelines, is one of the most critical steps in biotechnological production of therapeutics. The biotechnological isolation process developed at the Chair of Food and Bioprocess Engineering TUM School of Life Sciences Weihenstephan included microfiltration membranes (0,14 µm

ISOFLUX™- ceramic membrane; TAMI Industries, France) applicable for GMP food technology. In order to facilitate GMP certified production, a GMP qualified microfiltration module (0,1 µm Membralox® Ceramic Membrane; PALL Corporation, United States of America) was tested. A pilot microfiltration experiment was performed to determine whether the exchanged membrane could meet the requirements. Its demands include the depletion of the impurities such as caseins and hazardous pathogens whilst simultaneously concentrating IgG. In addition the filtration module should process high volumes in an acceptable time range and with constant and reasonable process parameters (temperature, TMP, Q). The isolation process should not be affect or imperil the natural integrity of the desired extraction compound.

Heidebrecht et al.[41] investigated different microfiltration membranes (ceramic standard and gradient), pore sizes (0,14-0,8 µm), TMP (0,5-2,5 bar) and temperatures (10, 50°C) for the concentration of IgG and simultaneous reduction of caseins and lactose from skim milk. The experiments showed the inapplicability of ceramic as well as gradient membranes with pore sizes above 0,2 µm at 1 or 2 bar TMP and 10°C and 50°C due to casein transmission. However, ceramic gradient membranes with 0,14 µm pore sizes proved to isolate bovine IgG (45 to 65%), whilst simultaneously depleting the casein content to 1% in the permeate. Furthermore, reduced TMP improved the Abs isolation. The performed microfiltration experiment with the 0,1 µm Membralox® Ceramic Membrane module was reviewed on the basis of these experiments.

The pilot microfiltration experiment with a GMP certified membrane, stressed the applicability of the tested equipment. IgG could be extracted, whereas caseins were depleted within the time span of 170 minutes. The $Q_{Permeate}$ strongly depended on the temperature within the system. After running the filtration for 60 minutes, the temperature increased steadily from 10°C to 45°C. The permeate flow showed a likewise behavior. After the first 60 minutes the transmembrane pressure could be kept constant around 1,5 psig. Within 170 minutes 30 liters of colostrum could be filtrated- on a eight hour workday 480 liter could be processed. As the antibody concentration in the retentate did not result in an exponential depletion, process improvements are required. Adjusting process parameters such as temperature or dry mass concentration of the biofluid can have a beneficial effect on the process output. In addition increasing the number of membrane modules within the process could succeed in a more efficient isolation. it has to be stated that the pilot experiment was executed a single time and adjustments to improve the process parameters could not be taken into consideration

For large-scale production with the Membralox® Ceramic Membrane more validation experiments have to be performed. The membrane has to be integrated into the established bioseparation process including micro- and ultrafiltration to verify the effectiveness and efficiency. Including this membrane into the bioprocess enables new GMP conform microfiltration production possibilities.

Formulation of bovine IgG in CMC hydrogel for topical, cutaneous application

State-of-the-art bovine IgG therapy are developed for oral application. Various studies have been performed to assess the beneficial effect of bovine Abs on the gut health[87]. The novelty of formulating bovine IgG in a hydrogel is the topical, cutaneous application. The formulation has to meet its requirements such as stability of the antibody, good sprayability for topical application by sprays on canine skin. At this point it has to be recognized that treatment application on canine skin is a challenging undertaking as dogs are covered with fur and thus the hydrogel has to enable efficient accessibility of the cutaneous infection site.

Poelstra et al.[88] reported a beneficial effect on the outcome of MRSA infection by applying polyclonal human IgG formulated in aqueous CMC gel locally at the infection site in a murine surgical implant model. Within these experiments the human IgG was rapidly released from the hydrogel via first order kinetics and thus lead to the assumption that CMC is a suitable vehicle for antibody delivery. The formulation of bovine antibodies in Sodium Carboxymethylcellulose (CMC) proved to be an appropriate medium. Integrity and stability of the protein content in hydrogels could be confirmed and points out its suitability. In addition, the physicochemical properties of hydrogels facilitate adequacy for packaging in sterile pump sprays. Hydrogel with 3% CMC proved to be inapplicable as the high viscosity impaired efficient mixing. Adjusting the protocol by reducing the CMC concentration to 1,5% and progressive addition of the powder into PBS, led to a lower viscosity and sufficient mixing. By autoclaving the formulation depletion of microorganisms could be performed.

Suitability of bovine IgG isolated from milk to target clinical canine *S. pseudintermedius* strains

S. pseudintermedius is a natural colonizing gram-positive bacteria on canine mucous membranes and skin[55]. In case of skin-barrier dysfunction or immunosuppressive dogs, *S. pseudintermedius*

can become opportunistic pathogenic and induce mild to life-threatening infections. Furthermore, *S. pseudintermedius* has been increasingly associated with zoonotic transmission. *S. pseudintermedius* has high phenotypic similarities with *S. aureus* and can thus be easily misdiagnosed[89].

Cows are colonized by *S. aureus* and are known to suffer from its pathogenicity in form of e.g. mastitis[90]. Thus, IgG occurring naturally in colostrum have activity against *Staphylococcus aureus* which could be used to target *S. pseudintermedius* due to various virulence factors that are shared by both[91]. As antibiotic-resistant *S. pseudintermedius* infections in veterinary but also human settings are an emerging one-health problem and the veterinary antibiotic pipeline is stagnating, the medical need in alternative treatment options is ranked as a priority[19]. Bovine Abs could be one novel treatment approach in bacterial skin infections in dogs. Toxins play a major role in virulence of *S. pseudintermedius* [92] and bovine milk derived antibodies are known to bind exotoxins and thus neutralizing them as well as adhesion molecules [93]. In previous, unpublished experiments, the IgG response profile against 122 *S. aureus* antigens was assessed. Bovine IgG form cows immunized with H35L as well as IgG from non-vaccinated cows was proven to bind to 77 different targets including toxins and adhesion molecules being the major recognized epitopes supporting the findings published by Tempelmans et al.[93].

The clinical *S. pseudintermedius* isolates available in the laboratory were tested for the expression of hemolytic toxins and therefore for similarities to important virulence factors known to be expressed by *S. aureus*. The clinical isolates GL151A and 69687 proved to lyse rabbit erythrocytes. Anti AT AB from vaccinated cows could significantly reduce the hemolytic effect. which led to the assumption that α-toxin can be neutralized. Natural bovine IgG also reduced the hemolysis, but to a lower extend. Presumably, vaccination of cows with the H35L mutant may be beneficial for the efficiency in neutralization of α-toxin.

Determination of the resistance profiles of the clinical isolates against the gold-standard therapy clindamycin, was rather challenging as experiment repetitions revealed variability in the MICs measured in different experiments. However, overall the described MDR MRSP strains 69687, HH15, GL151A and 23929 with a resistance to clindamycin based on a genomic analysis (McCarthy et al.[83]) revealed a MIC between 2,5 and 0,16 mg/mL, which indicates a clindamycin resistance with regard to the British Society of Antimicrobial Chemotherapy. The other strains tested, showed no clindamycin resistance based on genomic data published by McCarthy et

al.[83], the MIC test however revealed MICs between 0,16 and 0,625 mg/mL. These results show the known difficulty in correlating geno- and phenotype in resistance. Gow et al.[94] hypothesized in their publication that AMR is likely to be caused by an interplay of multiple resistance genes, after failing in confirming genotypic antibiotic resistance by phenotypic MIC determination of fecal *Escherichia coli* isolates from cow calves.

As antibodies could exhibit efficacy against antibiotic resistant bacteria, a MIC assay combining antibiotic and antibody treatment, was developed. Bovine IgG could negatively affect the bacterial growth and colony formation in presence of antibiotics, but also solely. This proves the efficacy of bovine antibodies against antibiotic resistant bacteria and could underline the therapeutic effect.

Formation of a bacterial biofilm is implicated as a survival strategy, enhances the virulence of the pathogen and is effected by antibiotic pressure[95]. Concerning the biofilm formation of the clinical *S. pseudintermedius* isolates, the bacteria with the highest MICs (69687, HH15, GL151A and 23929) produced the most excessive biofilms. This underlines the observation that biofilm production is associated with antibiotic resistance[96]. Bovine IgG although binding to molecules involved in biofilm formation as well as clindamycin could not inhibit biofilm formation.

Development of a novel canine *ex-vivo* model

To test the pharmacological efficiency of bovine IgG in canine skin, a canine *ex-vivo* model in a trans-well system with an air-liquid interface was established. State-of-the-art models such as cell-culture mimic bacterial infections insufficient as the interaction of *Staphylococci* and skin is more complex. The developed *ex-vivo* model could overcome current limitations and allowed research of interface between infection and immunity. Olanivi et al.[97] recently published data of the establishment of a new human *ex-vivo* skin model to assess the role of α-toxin and Panton-Valentine Leukocidin in *S. aureus* skin infections. This publication identified that the two toxins are important mediators in skin toxicity during infection.

During establishment of the skin explant model, numerous challenges had to be tackled. Preliminary experiments failed as commensal and applied bacteria massively invaded the skin and subsequently destroyed skin integrity. Conclusions about the infection mechanism, immune response and pathology could not be drawn. Inclusion of a washing step with medium containing antibiotics and addition of antibiotics in the cultivation medium significantly improved the outcome of the experiments. In addition the duration of cultivation reflected the thin line between mimicking

bacterial infection and cultivating in favor of bacterial growth. Cultivations longer than 30 hours resulted in high amounts of dead and pyknotic cells and loss of skin integrity. Previously, Abramo et al.[98] described 5 day long canine skin cultivation in 6-well plates. This long cultivation might have been possible due to the different plate system used. The skin biopsies are likely to swim in the medium and nutrient supply might be facilitated. However, this 6-well system does not allow the mimicking of the liquid-air interface present on skin.

Progressive *ex-vivo* models reflected the natural events during bacterial infection. *S. pseudintermedius* migrated through the *stratum corneum* into the epidermis and dermis and resulted in reduction of viability and loss in skin integrity. Separation of dermis and epidermis in progressive infection is likely to be caused by exotoxins (e.g. α-toxins) produced by *S. pseudintermedius*. The limitations in explant skin cultivation arise from loss in cell viability over cultivation time and migration of bacteria to the bottom of the tissue towards the medium, reducing the topical amount of bacteria. Within the master thesis project this problem could not be solved.

In addition it has to be mentioned that the skin samples lack blood supply and thereby leukocytes cannot infiltrate the infection site. Consequently, the immune response depends on the immune cells present in the explant tissue and infiltration of the site of infection by neutrophils as shown by Baeumer et al.[99] in an experimental model of canine superficial pyoderma on living dogs could not be displayed . Another downside of the established method is the limitation in the amount of donor samples in combination with the natural diversity in skin characteristics of different donors. In conclusion the developed canine *ex-vivo* skin model can be clinically important for understanding primary tissue-specific pathophysiology. Optimization of the cultivation time and bacterial infection could increase the significance of the model.

The finding of methicillin resistant *Staphylococcus lentus* as a commensal bacterium on canine skin from healthy laboratory dogs stresses the medical need in novel antimicrobial therapies in veterinary settings. As companion animals are in close contact to owners, interspecies transfer is very likely to occur. Perretten et al.[100] reported occurrence of *S. pseudintermedius* with resistance to the most important orally administered antibiotics used in veterinary medicine in Europe and North America. The susceptible antibiotics included for human treatment authorized drugs, making the treatment decision more difficult.

A canine *ex-vivo* skin model of superficial pyoderma could be established which displays adhesion and infiltration of *S. pseudintermedius,* epidermal damage by bacterial toxins. This models allow

to estimate the therapeutic effect of topically applied bovine IgG in buffer or gel in comparison to clindamycin as antibiotic treatment.

Tolerability of bovine IgG in gel by canine skin or keratinocytes

Confocal microscopy of canine *ex vivo* skin infected with *S. pseudintermedius* and treated with fluorescence labelled bovine IgG revealed that the antibody is localized within the *stratum corneum* and on the surface of the epidermis and does not penetrate into deeper skin layers. This finding is in compliance with the 500 Dalton rule of drugs for skin penetration since the antibody has a molecular weight of about 150 kDa[101].

To analyze adverse effect of the hydrogel or bovine IgG in comparison with clindamycin on canine skin, samples derived from the *ex vivo* model underwent a histological staining with H&E to investigate morphological changes. Neither hydrogel alone, nor bovine IgG in hydrogel or clindamycin induced any morphological changes in the skin. To investigate the effect of bovine IgG directly on canine keratinocytes IgG was applied on MSCEK cells and cell viability was measured. After treatment the cell viability remained at almost 100 %.

Bovine IgG in gel is localized on the skin surface and highly tolerated by canine skin.

Therapeutic effect of bovine IgG in the canine superficial pyoderma model

Confocal microscopy of canine *ex vivo* skin infected with *S. pseudintermedius* and treated with bovine IgG revealed co-localization. The green FITC labelled bacteria and the bovine IgG detected in red with Alexa Fluor® 647-conjugated goat anti bovine IgG showed a yellow overlay and thus bioavailability of the antibody on the site of infection can be assumed.

In the canine *ex-vivo* skin model bovine IgG could deploy its function shown *in-vitro* and proved to reduce the toxic effect of *S. pseudintermedius* on the skin. In comparison clindamycin effectively inhibited bacteria in exploiting skin damage. The combinational therapy proved to be sufficient in restoring the natural integrity of the skin.

In the canine superficial pyoderma could be shown that bovine IgG is co-localized with *S. pseudintermedius* and due to neutralization of toxins protects the skin from epidermal damage. These results are in accordance with *in vitro* data.

Effect of bovine IgG on bacterial adhesion and colonization on canine skin or keratinocytes

Testing of the therapeutic effect of bovine IgG on canine skin colonized by *S. pseudintermedius* involved development of bacterial adhesion assays *in-vitro* with a canine epidermal keratinocyte cell line and *ex-vivo* skin. Both assays resulted in a reduction of colony forming units in the presence of bovine IgG and thus can be used to prove reduced bacterial adherence. However, assessing bacterial counts from adherent bacteria is a challenging undertaking as the practical execution strongly impacts the experiment outcome. Optimizing the protocol of the assay by including washing steps and improving the time-management led to higher consistency in the results of repetitive experiments.

The visualization of bacterial colonization by scanning electron microscopy revealed patch colonies in close vicinity to hair follicles where migration into the skin might be facilitated. In presence of bovine IgG or clindamycin, the overall colonization was reduced and smaller colonies of bacteria could be detected mainly under skin flakes. This leads to the assumption that topical treatment with bovine antibody or clindamycin leads to a reduced number and colony size of bacteria. However infection sites with limited accessibility cannot always be reached by both treatment options. In general, application of topical treatment in dogs is rather challenging as fur covers the body and might reduce the accessibility of treatment. Veterinarians can seek information on contact time for different therapy types from published guidelines for canine bacterial skin infections[70, 102].

In the canine *ex vivo* model for superficial pyoderma as well as on canine keratinocytes could be shown that bovine IgG leads to a reduced adhesion and colonization of *S. pseudintermedius*, but seems to have no direct bactericidal effect.

Effect of bovine IgG on expression levels of pro-inflammatory cytokines of canine skin or keratinocytes infected with *S. pseudintermedius*

Previously, Schlotter et al.[80] described mixed Th1, Th2 and regulatory T-cell cytokine expressions in lesional atopic skin in dogs. For this publication the expression levels of the Th1 cytokines IL-12p35, IL-12p40 and INFγ, the Th2 cytokines IL-4 and IL-13 and the regulatory cytokines IL-10 and TGF-β were measured by quantitative real-time PCR of healthy, non-lesional and lesional skin. IL-12p40 production was down-regulated in lesional skin, whereas IL-13 and IL-10 was expressed in higher amounts in non-lesional and lesional skin. In comparison Nuttall et

al.[103] published different cytokine expression patterns. In this study canine atopic dermatitis was associated with increased IL-4 and decreased TGF-β expression. Lesional skin was identified with increased production levels of IFN-γ, TNFα and IL-2. Both publications indicate, that cytokine production in the skin induces Th1 and simultaneously Th2 and regulatory T-cell immune responses.

The production of pro-inflammatory cytokines by canine keratinocytes *in-vivo* and *in-vitro* displayed different expression levels after co-cultivation with *S. pseudintermedius* in the absence or presence of treatment over four hours. The MSCEK cells expressed the pro-inflammatory Th1 cytokines TNFα and IL-12p35 at higher levels. Expression changes in Th2 and regulatory T-cell cytokines could not be detected. TNFα production differed between infection with 69687 and GL151A. GL151A induced a ten-fold increase in TNFα production, whereas 69687 induced an eight-fold increase. Bovine IgG treatment could reduce the cytokine production with GL151A to a 2,5- fold increase. However, the antibodies could not reduce the TNFα expression during infection with 69687. For IL-12p35 GL151A as well as 69687 had a downregulating effect on the production. Surprisingly, clindamycin increased the expression levels up to an eight-fold increase. These qPCR results were deducted from two experiments, leading to the assumption that validation of the data will need to be performed in the future. The results could be explained with regard to Del Rosso et al.[104], who reported that the overall inflammatory response can be increased with clindamycin as phagocytosis and opsonization is facilitated at enhanced levels.

The overall cytokine expression in *ex-vivo* canine skin after 30h of cultivation was significantly lower in comparison to the keratinocytes cell line and distinct expression patterns were difficult to deduct. The isolation of RNA from the skin explants was a rather difficult undertaking and resulted in low concentrations and high impurity. From the four dogs tested (NDS17-20), only two (NDS17 and NDS18) allowed measurement of reasonable quantities in cytokine production. The skin cells from these dogs produced increased levels of TNFα and IL-13 during skin colonizing with *S. pseudintermedius*. Bovine IgG and clindamycin treatment could downregulate the expression of TNFα and thus resulted in an antagonistic effect. IL-13 was expressed in a slightly higher amount, however clindamycin treatment did not result in a reduction in production. NDS19 and NDS20, which were cultivated with GL151A expressed cytokines at very low to indeterminable levels. Quantification of the skin damage revealed, that GL151A had a high impact on the viability of the keratinocytes. This leads to the assumption that after 30h of cultivation, the skin cells could have

been apoptotic or dead due to severe bacterial infection and thus did not produce cytokines any longer.

In summary, the *in-vitro* MSCEK qPCR results showed sole induction of Th1 cytokine (IL-12p35 and TNFα) production. The mixed Th1 and Th2 immune responsiveness, identified by Schlotter et al.[80] and Nuttall et al.[79], could not be proven. Depending on the bacterial strain, bovine IgG could reduce the TNFα production levels. The Th1 cytokine IL-12p35 was downregulated during bacterial infection, however, in presence of clindamycin an upregulation could be determined. The cytokine determination with the help of the *ex-vivo* skin model proved to be challenging as the RNA isolation resulted in low concentrations and high impurities. As TNFα and IL-13 were upregulated, a mixed Type-1 and Type-2 immune response was identified. TNFα production was reduced by bovine IgG and clindamycin, whereas IL-13 was solely downregulated in presence of antibody treatment. With regard to the published data by Schlotter et al.[80] the four-fold expression level of IL-13 in the *ex-vivo* skin used in the experiments was comparable to the three-fold cytokine production in non-lesional skin. The three-fold increase in TNFα can be compared to the two-fold expression reported by Nuttall et al.[103] in lesional atopic dog skin.

Concluding remarks

The data collected within this master thesis not only comprise valuable insights about the biotechnological large-scale isolation of bovine IgG, but also about the applicability of cow antibodies for topical treatment in canine skin infections by *S. pseudintermedius*. The Membralox® Ceramic Membrane proved to be suitable for the microfiltration of bovine colostrum. The formulation of bovine IgG in CMC hydrogel verified to be useful for topical treatment without affecting the viability of the skin. The therapeutic spectrum of bovine IgG included neutralization of exotoxins to impair skin damage whilst simultaneously reducing the adhesion and colonization on skin. Furthermore, the antibodies proved to limit skin inflammation by down-regulation of cytokines.

In conclusion it has to be stated that for full assessment of the spectrum of therapeutic effect exploited by bovine IgG in bacterial skin infections, *in-vivo* studies have to be performed. The established *ex-vivo* model can be a useful tool in gaining insight in pathophysiology. However, imitating *in-vivo* events in assessing pharmacological effects of prospective new treatments with the help of models remains a challenging undertaking. In particular the *in-vitro* simulation of immune and inflammatory responses in course of infections is up till now problematic. The

experiments performed and the data generated in this master project can be used as guidance for planning a clinical trial. Bovine IgG could be a novel treatment option in the fight against infectious diseases.

Bibliography

[1] DiGiandomenico A, Sellman B. Antibacterial monoclonal antibodies: the next generation? Current Opinion in Microbiology 2015;27:78-85.

[2] The World Health Organization. Antimicrobial resistance 2018 Available from: http://www.who.int/en/news-room/fact-sheets/detail/antimicrobial-resistance.

[3] Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti V, et al. Alternatives to antibiotics- a pipeline portfolio review. The Lancet Infectious Diseases 2016;16(2):239-51.

[4] Alberts B, Johnson A, Lewis J. The Adaptive Immune System. Molecular Biology of the Cell. New York: Garland Science 2002.

[5] Francois B. Antibody-based therapy to combat Staphylococcus aureus infections *European Congress of Clinical Microbiology and Infectious Diseases (ECCMID).* Vienna; 2017.

[6] Winau F, Winau R. Emil von Behring and serum therapy. Microbes and Infections 2002;4(2):185-8.

[7] Dolman C. Serum therapy. Canadian Medical Aassociation Journal 1936;35(6):628-35.

[8] Ligon B. Penicillin: its discovery and early development. Seminars in Pediatric Infectious Diseases 2004;15(1):52-7.

[9] Marano G, Vaglio S, Pupella S, Facco G, Catalano L, Liumbruno G, et al. Convalescent plasma: new evidence for an old therapeutic tool? Blood Transfusion 2016;14(5):152-7.

[10] Lee A, de Lencastre H, Garau J, Kluytmans J, Malhotra-Kumar S, Peschel A, et al. Methicillin-resistant Staphylococcus aureus. Nature Reviews Disease Primers 2018;4:18033.

[11] Sause W, Buckley P, Strohl W, Lynch A, Torres V. Antibody-Based Biologics and Their Promise to Combat Staphylococcus aureus Infections. Trends in Pharmacological Sciences 2016;37(3):231-41.

[12] Sause W, Buckley P, Strohl W, Lynch A, Torres V. S. aureus Immune Evasion Factors Targeted by Experimental Biologic Agents. 494 KB. Trends in Pharmacological Sciences; 2016.

[13] Benjamin D, Schelonka R, White R, Holley H, Bifano E, Cummings J, et al. A blinded, randomized, multicenter study of an intravenous Staphylococcus aureus immune globulin. Journal of Perinatology 2006;26(5):290-5.

[14] Rouha H, Badarau A, Visram Z, Battles M, Prinz B, Magyarics Z, et al. Five birds, one stone: Neutralization of α-hemolysin and 4 bi-component leukocidins ofStaphylococcus aureuswith a single human monoclonal antibody. mAbs 2014;7(1):243-54.

[15] Rouha H, Weber S, Janesch P, Maierhofer B, Gross K, Dolezilkova I, et al. Disarming Staphylococcus aureus from destroying human cells by simultaneously neutralizing six cytotoxins with two human monoclonal antibodies. Virulence 2017;9(1):231-47.

[16] Genetic Engineering and Biotechnology News. Arsanis Halts Phase II Trial of Pneumonia Candidate ASN100; 2018. Available from: https://www.genengnews.com/topics/drug-

discovery/arsanis-halts-phase-ii-trial-of-pneumonia-candidate-asn100/. [Accessed 28. June 2018].

[17] Theuretzbacher U, Gottwalt S, Beyer P, Butler M, Czaplewski L, Lienhardt C, et al. Analysis of the clinical antibacterial and antituberculosis pipeline. https://www.thelancet.com/journals/laninf/article/PIIS1473-3099(18)30513-9/fulltext: The Lancet; 2018.

[18] The World Health Organization. One health 2017. Available from: http://www.who.int/features/qa/one-health/en/.

[19] Pomba C, Rantala M, Greko C, Baptiste K, Catry B, van Duijkeren E, et al. Public health risk of antimicrobial resistance transfer from companion animals. Journal of Antimicrobial Chemotherapy 2016:957-68.

[20] Scott Weese J. Antimicrobial resistance in companion animals. Animal Health Research Reviews 2008;9(02):169-76.

[21] Baptiste K, Williams K, Willams N. Methicillin-resistant staphylococci in companion animals Emerging infectious diseases 2005;11(12):1942-4.

[22] Kmieciak W, Szewczyk E. Are zoonotic Staphylococcus pseudintermedius strains a growing threat for humans? Folia Microbiologica 2018;63(6):743-7.

[23] Maertlbauer E, Becker H. Milchkunde und Milchhygiene. Verlag Eugen Ulmer; 2016.

[24] Park Y, Nam M. Bioactive Peptides in Milk and Dairy Products: A Review. Korean Journal for Food Science of Animal Resources 2015;35(6):831-40.

[25] Korhonen H, Marnila P, Gill H. Milk immunoglobulins and complement factors. British Journal of Nutrition 2007;84(S1).

[26] Marnila P. Milk proteins, Immunoglobulins. Encyclopedia of Dairy Science 2011;3:807-15.

[27] Wheeler T, Hodgkinson A, Prosser C, Davis S. Immune Components of Colostrum and Milk-A Historical Perspective. Journal of Mammary Gland Biology and Neoplasia 2007;12(4):237-47.

[28] Boutinaud M, Jammes H. Potential uses of milk epithelial cells: a review. Reproduction Nutrition Development 2002;42(2):133-47.

[29] Kindt T, Goldsby R, Osborne B, Kuby J. Innate Immunity Immunology United States of America; 2009, p. 141-92.

[30] Li N, Richoux R, Boutinaud M, Martin P, Gagnaire V. Role of somatic cells on dairy processes and products: a review. Dairy Science & Technology 2014;94(6):517-38.

[31] Janeway C, Travers P, Walport M. The structure of a typical antibody molecule. Immunobiology. Garland Science; 2001.

[32] Stanfield R, Wilson I, Smider V. Conservation and diversity in the ultralong third heavy-chain complementarity-determining region of bovine antibodies. Science Immunology 2016;1(1):aaf7962-aaf.

[33] Haakenson J, Huang R, Smider V. Diversity in the Cow Ultralong CDR H3 Antibody Repertoire. Frontiers in Immunology 2018;9.

[34] Thermo Fisher Scientific. Immunoglobulin Structure and Classes; Available from: https://www.thermofisher.com/nl/en/home/life-science/antibodies/antibodies-learning-center/antibodies-resource-library/antibody-methods/immunoglobulin-structure-classes.html. [Accessed 22.11. 2018].

[35] Vidarsson G, Dekkers G, Rispens T. IgG Subclasses and Allotypes: From Structure to Effector Functions. Frontiers in Immunology 2014;5.

[36] Hamatschek J. Die erste Verarbeitungsstufe: Wertstoffgewinnung aus agrarischen Rohstoffen. Lebensmitteltechnologie. utb/Ulmer; 2015, p. 186-201.

[37] Datta N, Tomasula P. Crossflow Microfiltration in the Dairy Industry. Emerging Dairy Processing Technologies: Opportunities for the Dairy Industry. Wiley-Blackwel; 2015, p. 1-30.

[38] Lipnizki F. Industrial Applications of Ultrafiltration in Pharmaceutical Biotechnology. Engineering in Life Sciences 2005;5(1):81-3.

[39] Porex Filtration Group. Cross Flow Filtration and Dead-End Filtration 2018. Available from: http://www.porexfiltration.com/learning-center/technology/what-is-cross-flow-filtration/. [Accessed 22.11. 2018].

[40] Brans G, Schroën C, van der Sman R, Boom R. Membrane fractionation of milk: state of the art and challenges. Journal of Membrane Science 2004;243(1-2):263-72.

[41] Heidebrecht H, Toro-Sierra J, Kulozik U. Concentration of Immunoglobulins in Microfiltration Permeates of Skim Milk: Impact of Transmembrane Pressure and Temperature on the IgG Transmission Using Different Ceramic Membrane Types and Pore Sizes. Foods 2018;7(7):101.

[42] Heidebrecht H, Toro Sierra J, Kulozik U. Separation of Immunoglobulins from Milk *Technology Seminar, Food Bioprocessing: New functionalities through production, concentration and stabilization of biologically active components.* . Freising-Weihenstephan; 2014.

[43] Heidebrecht H, Kainz B, Schopf R, Godl K, Karcier Z, Kulozik U, et al. Isolation of biofunctional bovine immunoglobulin G from milk- and colostral whey with mixed-mode chromatography at lab and pilot scale. Journal of Chromatography 2018;1562:59-68.

[44] Warne N, Mahler H. Formulation Development of Therapeutic Proteins Challenges in Protein Product Development Springer, p. 144-61.

[45] Ickenstein L, Garidel P. Hydrogel formultions for biologics: current spotlight from a commercial perspective Therapeutic Delivery 2018;9(3).

[46] Geh K, Stelzl A, Groene A, Wagner L, Foerster B, Winter G. Development of a drug carrier for the skin application of therapeutic antibodies 2018.

[47] Cuscó A, Belanger J, Gershony L, Islas-Trejo A, Levy K, Medrano J, et al. Individual signatures and environmental factors shape skin microbiota in healthy dogs. Microbiome 2017;5(1).

[48] Affolter V, Moore P. Histologic Features of Normal Canine and Feline Skin Clinics in Dermatology 1994;12(4):491-7.

[49] Garthwaite. Location of immunoglobulins and complement C3 at the surface and within the skin of dogs. Journal of Comparative Pathology 1983;93(2):185-93.

[50] Nakagawa S, Bos J. Role of Langerhans cells in the skin. What's new? Journal of The European Academy of Dermatology and Venereology 2008;15(5):399-402.

[51] Thomsett L. Structure of canine skin. British Veterinary Journal 1986;142(2):116-23.

[52] Welle M, Wiener D. The Hair Follicle. Toxicologic Pathology 2016;44(4):564-74.

[53] Thomsett LR. Structure of canine skin. British Veterinary Journal 1986;142(2):116-23.

[54] Bannoehr J, Ben Zakour N, Waller AS, Guardabassi L, Thoday K, van den Broek A, et al. Population Genetic Structure of the Staphylococcus intermedius Group: Insights into agr Diversification and the Emergence of Methicillin-Resistant Strains. Journal of Bacteriology 2007;189(23):8685-92.

[55] Bannoehr J, Guardabassi L. Staphylococcus pseudintermedius in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. Veterinary Dermatology 2012;23(4):253-e52.

[56] Chrobak-Chmiel D, Golke A, Dembele K, Ćwiek K, Kizerwetter-Świda M, Rzewuska M, et al. Staphylococcus pseudintermedius, both commensal and pathogen. Medycyna Weterynaryjna 2018;74(1):6042-2018.

[57] Geoghegan J, Smith E, Speziale P, Foster T. Staphylococcus pseudintermedius expresses surface proteins that closely resemble those from Staphylococcus aureus. Veterinary Microbiology 2009;138(3-4):345-52.

[58] Pietrocola G, Gianotti V, Richards A, Nobile G, Geoghegan J, Rindi S, et al. Fibronectin Binding Proteins SpsD and SpsL Both Support Invasion of Canine Epithelial Cells by Staphylococcus pseudintermedius. Infection and Immunity 2015;83(10):4093-102.

[59] Moodley A, Stegger M, Ben Zakour N, Fitzgerald J, Guardabassi L. Tandem repeat sequence analysis of staphylococcal protein A (spa) gene in methicillin-resistant Staphylococcus pseudintermedius. Veterinary Microbiology 2009;135(3-4):320-6.

[60] GE Healthcare Bio-Sciences A. Affinity Chromatography. 2016;1.

[61] Futagawa-Saito K, Sugiyama T, Karube S, Sakurai N, Ba-Thein W, Fukuyasu T. Prevalence and Characterization of Leukotoxin-Producing Staphylococcus intermedius in Isolates from Dogs and Pigeons. Journal of Clinical Microbiology 2004;42(11):5324-6.

[62] Terauchi R. Isolation of exfoliative toxin from Staphylococcus intermedius and its local toxicity in dogs. Veterinary Microbiology 2003;94(1):19-29.

[63] Hendricks A, Schuberth H, Schueler K, Lloyd D. Frequency of superantigen-producing Staphylococcus intermedius isolates from canine pyoderma and proliferation-inducing potential of superantigens in dogs. Research in Veterinary Science 2002;73(3):273-7.

[64] Yu X, Robbie G, Wu Y, Esser M, Jensen K, Schwartz H, et al. Safety, Tolerability, and Pharmacokinetics of MEDI4893, an Investigational, Extended-Half-Life, Anti-Staphylococcus aureus Alpha-Toxin Human Monoclonal Antibody, in Healthy Adults. Antimicrobial Agents and Chemotherapy 2017;61(1).

[65] Pompilio A, De Nicola S, Crocetta V, Guarnieri S, Savini V, Carretto E, et al. New insights in Staphylococcus pseudintermedius pathogenicity: antibiotic-resistant biofilm formation by a human wound-associated strain. BMC Microbiology 2015;15(1).

[66] Van Hoovels L, Vankeerberghen A, Boel A, Van Vaerenbergh K, De Beenhouwer H. First Case of Staphylococcus pseudintermedius Infection in a Human. Journal of Clinical Microbiology 2006;44(12):4609-12.

[67] Loeffler A, Linek M, Moodley A, Guardabassi L, Sung J, Winkler M, et al. First report of multiresistant, mecA-positive Staphylococcus intermedius in Europe: 12 cases from a veterinary dermatology referral clinic in Germany. Veterinary Dermatology 2007;18(6):412-21.

[68] Schmidt V. Canine pyoderma Part 1: Clinical features. Companion animal 2010;15(8):17-21.

[69] Loeffler A, Lloyd D. What has changed in canine pyoderma? A narrative review. The Veterinary Journal 2018;235:73-82.

[70] Jeffers J. Topical Therapy for Drug-Resistant Pyoderma in Small Animals. Veterinary Clinics of North America: Small Animal Practice 2013;43(1):41-50.

[71] Morris DO, Loeffler A, Davis MF, Guardabassi L, Weese JS. Recommendations for approaches to meticillin-resistant staphylococcal infections of small animals: diagnosis, therapeutic considerations and preventative measures. Veterinary Dermatology 2017;28(3):304-e69.

[72] Halliwell R. Revised nomenclature for veterinary allergy. Veterinary Immunology and Immunopathology 2006;114(3-4):207-8.

[73] Hillier A, Griffin C. The ACVD task force on canine atopic dermatitis (I): incidence and prevalence. Veterinary Immunology and Immunopathology 2001;2001(81):147-51.

[74] Saridomichelakis M, Olivry T. An update on the treatment of canine atopic dermatitis. The Veterinary Journal 2016;207:29-37.

[75] Fazakerley J, Nuttall T, Sales D, Schmidt V, Carter S, Hart C, et al. Staphylococcal colonization of mucosal and lesional skin sites in atopic and healthy dogs. Veterinary Dermatology 2009;20(3):179-84.

[76] Morales C, Schultz K, DeBoer D. Antistaphylococcal antibodies in dogs with recurrent staphylococcal pyoderma. Veterinary Immunology and Immunopathology 1994;42(2):137-47.

[77] Owen J, Punt J, Stranford S, Jones P. Receptors and Signaling: Cytokines and Chemokines. Immunology. W.H.Freeman & Co Ltd; 2013, p. 105-40.

[78] Zhang J, An J. Cytokines, Inflammation, and Pain. International Anesthesiology Clinics 2007;45(2):27-37.

[79] Nuttall T, Knight P, McAleese S, Lamb J, Hill P. Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis. Clinical and Experimental Allergy 2002;35(5):789-95.

[80] Schlotter Y, Rutten V, Riemers F, Knol E, Willemse T. Lesional skin in atopic dogs shows a mixed Type-1 and Type-2 immune responsiveness. Veterinary Immunology and Immunopathology 2011;143(1-2):20-6.

[81] Kraft M, Worm M. Dupilumab in the treatment of moderate-to-severe atopic dermatitis. Expert Review of Clinical Immunology 2017;13(4):301-10.

[82] Simpson E, Bieber T, Guttman-Yassky E, Beck L, Blauvelt A, Cork M, et al. Two Phase 3 Trials of Dupilumab versus Placebo in Atopic Dermatitis. New England Journal of Medicine 2016;375(24):2335-48.

[83] McCarthy A, Harrison E, Stanczak-Mrozek K, Leggett B, Waller A, Holmes M, et al. Genomic insights into the rapid emergence and evolution of MDR in Staphylococcus pseudintermedius. Journal of Antimicrobial Chemotherapy 2014.

[84] Ide K, Yamamoto W, Sato M, Yabuzoe A, Iwasaki T, Nishifuji K. Expression Analysis of Desmosomal Components of the Novel Canine Epidermal Keratinocyte Cell Line (MSCEK). Journal of Veterinary Medical Science 2010;72(11):1479-82.

[85] Turner M, Nedjai B, Hurst T, Pennington D. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 2014;1843(11):2563-82.

[86] Helmholtz Zentrum München AK, Allergieinformationsdienst. Forschungsgebiet T-Helferzellen bei Allergien; 2017. Available from: https://www.allergieinformationsdienst.de/forschung/immunmechanismen-und-entzuendung/t-helferzellen.html. [Accessed 28.11. 2018].

[87] Ulfman L, Leusen J, Savelkoul H, Warner J, van Neerven R. Effects of Bovine Immunoglobulins on Immune Function, Allergy, and Infection. Frontiers in Nutrition 2018;5.

[88] Poelstra KA, Barekzi NA, Rediske AM, Felts AG, Slunt JB, Grainger DW. Prophylactic treatment of gram-positive and gram-negative abdominal implant infections using locally delivered polyclonal antibodies. Journal of Biomedical Materials Research 2002;60(1):206-15.

[89] Börjesson S, Gómez-Sanz E, Ekström K, Torres C, Grönlund U. Staphylococcus pseudintermedius can be misdiagnosed as Staphylococcus aureus in humans with dog bite wounds. European Journal of Clinical Microbiology & Infectious Diseases 2015;34(4):839-44.

[90] Gomes F, Saavedra M, Henriques H. Bovine mastitis disease/pathogenicity: evidence of the potential role of microbial biofilms. Pathogens and Disease 2016;74(3).

[91] Suzuki H, Lefébure T, Pavinski Bitar P, Stanhope M. Comparative genomic analysis of the genus Staphylococcus including Staphylococcus aureus and its newly described sister species Staphylococcus simiae. BMC Genomics 2012;12(38).

[92] Maali Y, Badiou C, Martins-Simões P, Hodille E, Bes M, Vandenesch F, et al. Understanding the Virulence of Staphylococcus pseudintermedius: A Major Role of Pore-Forming Toxins. Frontiers in Cellular and Infection Microbiology 2018;8(221).

[93] Tempelmans Plat-Sinnige M, Verkaika N, van Wamel W, de Groot N, Actonb D, van Belkuma A. Induction of Staphylococcus aureus-specific IgA and agglutination potency in milk of cows by mucosal immunization. Vaccine 2009;27:4001-9.

[94] Gow S, Waldner C, Harel J, Boerlin P. Associations between Antimicrobial Resistance Genes in Fecal Generic Escherichia coli Isolates from Cow-Calf Herds in Western Canada. Applied and Environmental Microbiology 2008;74(12): 3658–66.

[95] Liu J, Yang L, Hou Y, Soteyome T, Zeng B, Su J, et al. Transcriptomics Study on Staphylococcus aureus Biofilm Under Low Concentration of Ampicillin. Frontiers in Microbiology 2018;9.

[96] Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofub O. Antibiotic resistance of bacterial biofilms. International Journal of Antimicrobial Agents 2009;32:322-32.

[97] Olaniyi R, Pancotto L, Grimaldi L, Bagnoli F. Deciphering the Pathological Role of Staphylococcal α-Toxin and Panton–Valentine Leukocidin Using a Novel Ex Vivo Human Skin Model. Frontiers in Immunology 2018;9.

[98] Abramo F, Lazzarini G, Pirone A, Lenzi C, Albertini S, della Valle M, et al. Ultramicronized palmitoylethanolamide counteracts the effects of compound 48/80 in a canine skin organ culture model. Veterinary Dermatology 2017;28(5):456-e104.

[99] Bäumer W, Bizikova P, Jacob M, Linder KE. Establishing a canine superficial pyoderma model. Journal of Applied Microbiology 2017;122(2):331-7.

[100] Perreten V, Kadlec K, Schwarz S, Gronlund Andersson U, Finn M, Greko C, et al. Clonal spread of methicillin-resistant Staphylococcus pseudintermedius in Europe and North America: an international multicentre study. Journal of Antimicrobial Chemotherapy 2010;65(6):1145-54.

[101] Bos J, Meinardi M. The 500 Dalton rule for the skin penetration of chemical compounds and drugs Experimental Dermatology 2000;9:165-9.

[102] Hillier A, Lloyd D, Weese J, Blondeau J, Boothe D, Breitschwerdt E, et al. Guidelines for the diagnosis and antimicrobial therapy of canine superficial bacterial folliculitis (Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases). Veterinary Dermatology 2014;25(3):163-43.

[103] Nuttall T, Knight P, McAleese S, Lamb J, Hill P. T-helper 1, T-helper 2 and immunosuppressive cytokines in canine atopic dermatitis Veterinary Immunology and Immunopathology 2002;87:379-84.

[104] Del Rosso J, Schmidt N. A Review of the Anti-inflammatory Properties of Clindamycin in the Treatment of Acne Vulgaris. Cutis 2010;5:15-24.

Annex

I. Raw data of microfiltration pilot experiment

Time	P_{inlet}	$P_{retentate}$	$P_{permeate}$	TMP	DP	Q_{outlet}	Q_{outlet}	$Q_{permeate}$	Q_{inlet}	$S_{retentate}$	$M_{permeate}$	$V_{permeate}$	Temp
min	psig	psig	psig	psig	psig	m3/H	mL/min	mL/min	mL/min	cm/sec	g	ml	°C
0	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	0	0.0	0	0.0	na
2	2.22	0.63	0.00	1.43	1.59	4.57	76167	120.00	76286.67	0.0	240	240.0	na
5	2.23	0.64	0.00	1.44	1.59	4.58	76333	40.00	76373	0.0	360	360.0	na
8	2.23	0.64	0.00	1.44	1.59	4.58	76333	56.67	76390	0.0	530	530.0	na
9	2.24	0.64	0.00	1.44	1.60	4.57	76167	-485.00	75682	0.0	45	45.0	12
10	2.24	0.64	0.00	1.44	1.60	4.57	76167	48.00	76215	0.0	93	93.0	na
16	2.26	0.64	0.00	1.45	1.62	4.57	76167	53.50	76220	0.0	414	414.0	13.3
20	2.26	0.64	0.00	1.45	1.62	4.54	75667	50.25	75717	0.0	615	615.0	13.3
30	2.29	0.64	0.00	1.47	1.65	4.52	75333	47.40	75381	0.0	1089	1089.0	13.2
40	2.42	0.65	0.00	1.54	1.77	4.45	74167	41.70	74208	0.0	1506	1506.0	10.1
60	2.42	0.65	0.00	1.54	1.77	4.35	72500	37.85	72538	0.0	2263	2263.0	9.2
80	2.40	0.65	0.00	1.53	1.75	4.41	73500	39.45	73539	0.0	3052	3052.0	15.5
90	2.39	0.65	0.00	1.52	1.74	4.49	74833	47.40	74881	0.0	3526	3526.0	19.4
102	2.37	0.67	0.00	1.52	1.70	4.56	76000	53.17	76053	0.0	4164	4164.0	23.8
110	2.35	0.67	0.00	1.51	1.68	4.60	76667	60.50	76727	0.0	4648	4648.0	27.4
112	2.35	0.67	0.00	1.51	1.68	4.60	76667	-2316.00	74351	0.0	16	16.0	27.4
115	2.34	0.67	0.00	1.51	1.67	4.62	77000	66.33	77066	0.0	215	215.0	28.2
125	2.33	0.67	0.00	1.50	1.66	4.66	77667	68.70	77735	0.0	902	902.0	31.3
142	2.31	0.67	0.00	1.49	1.64	4.71	78500	76.47	78576	0.0	2202	2202.0	36.1
162	2.29	0.67	0.00	1.48	1.62	4.75	79167	83.75	79250	0.0	3877	3877.0	40.7
170	2.28	0.67	0.00	1.48	1.61	4.76	79333	87.25	79421	0.0	4575	4575.0	42.5

II. Primers for canine keratinocytes for qPCR

II.I. Housekeeping-genes

GUSB	Forward	AGACGCTTCCAAGTACCCC
	Reverse	AGGTGTGGTGTAGAGGAGCAC
RPS19	Forward	CCTTCCTCAAAAAGTCTGGG
	Reverse	GTTCTCATCGTAGGGAGCAAG

RPL8	Forward	CCATGAATCCTGTGGAGC
	Reverse	GTAGAGGGTTTGCCGATG
RPS5	Forward	TCACTGGTGAGAACCCCCT
	Reverse	CCTGATTCACACGGCGTAG
HPRT	Forward	AGCTTGCTGGTGAAAAGGAC
	Reverse	TTATAGTCAAGGGCATATCC

II.II. Cytokine-genes

IL-4	Forward	CCAAAGAACACAAGCGATAAGGAA
	Reverse	GTTTGCCATGCTGCTGAGGTT
IL-10	Forward	CCTTCCTCAAAAAGTCTGGG
	Reverse	AAATGCGCTCTTCACCTGCTCCAC
IL-12p35	Forward	TAATGGATCCCAAGAGGCAG
	Reverse	TCAAGGGAGGATTTCTGTGG
IL-12p40	Forward	GGACGTTTCACATGCTGGT
	Reverse	CCACTCTGACCCTCTCTGCT
IL-13	Forward	GAGGAGCTGGTCAACATCA
	Reverse	TGCAGTCGGAGACATTGA
IFN-γ	Forward	AGCGCAAGGCGATAAATG
	Reverse	GCGGCCTCGAAACAGATT
TGFβ1	Forward	CAAGGATCTGGGCTGGAAGTGGA
	Reverse	CCAGGACCTTGCTGTACTGCGTGT
TNFα	Forward	CCCCGGGCTCCAGAAGGTG
	Reverse	GCAGCAGGCAGAAGAGTGTGGTG