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Serum resistance of *Mycoplasma agalactiae* and phase variable lipoproteins

Bachelor Thesis

University of Veterinary Medicine Vienna

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submitted by

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DIE ÖSTERREICHISCHE GESELLSCHAFT FÜR HYGIENE, MIKROBIOLOGIE UND PRÄVENTIVMEDIZIN

hat beschlossen

Frau

Katja Sommer

für den eingereichten Abstract

Serum resistance of different *Mycoplasma agalactiae* strains and mutants bearing different lipoprotein profiles

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mit einem Tagungsstipendium für die 37. ÖGHMP Jahrestagung

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Summary

Mycoplasma agalactiae causes contagious agalactia in small ruminants. Symptoms of this disease include mastitis, arthritis, conjunctivitis, septicaemia and sporadic genital infections. The molecular basis for pathogenicity has yet to be elucidated, however, the variable proteins of *M. agalactiae* (Vpma) multigene family seem to play a role in adhesion and immune evasion via antigenic variation. Vpmas are surface-lipoproteins, similar to the variable surface proteins (Vsa) of *Mycoplasma pulmonis*, where longer Vsa proteins have been shown to protect against complement. Since the ability to evade complement in serum is an important factor in systemic spread, the role of Vpmas in the differential pathogenicity of strains and mutants needs to be investigated.

Bactericidal assays in sera were carried out with (i) *M. agalactiae* type strain PG2, which exhibits high-frequency phase variation of all six Vpma lipoproteins, (ii) strain GM139, which shows a unique Vpma profile expressing just VpmaV and (iii) six different Vpma phase-locked mutants (PLMs) expressing single stable Vpma products. After incubation with active or heat-inactivated PG2-sensitized sheep serum, serial dilutions and plating, the resulting number of colony-forming units was assessed to calculate the percentage survival. Guinea pig complement was tested using the same methodology. Additionally, western blot analyses of Triton X-114 phase extracts of PG2 and GM139 were carried out using VpmaV-specific polyclonal rabbit antibodies (α-VpmaV) and PG2-sensitized sheep serum, revealing stark differences in the profiles of the two strains.

Overall data demonstrate the PG2 strain to be more susceptible to sheep serum compared to the GM139 strain bearing a different Vpma profile. Significant differences were also observed between the different PLMs, with PLMU and PLMX showing the highest serum susceptibility in serum. The results are in good correlation with previous studies where shorter lipoprotein variants were more susceptible to complement. Since none of the strains and PLMs showed susceptibility in non-sensitized sheep serum in previous studies, antibodies seem to play an important role in serum killing. Proteins of GM139 in the gel band identified by polyclonal α-VpmaV antibodies were digested with trypsin or trypsin/Lys-C mix and peptides analysed by nano-LC-Orbitrap-MS/MS. Database search revealed that the GM139 proteins displayed high sequence coverages of different Vpmas of various *M. agalactiae* strains. The originally expected VpmaV however, could not be found. In conclusion, Vpmas might play an important factor in *M. agalactiae*'s serum sensitivity and their antigenic variation likely allows the pathogen to select for variants that increase its serum resistance during systemic infections.

Zusammenfassung

Mycoplasma agalactiae verursacht bei kleinen Wiederkäuern die ansteckende Agalaktie. Symptome dieser Krankheit sind Mastitis, Arthritis und Konjunktivitis. Die molekularen Grundlagen für die Pathogenität müssen noch aufgeklärt werden, jedoch scheinen die Variablen Proteine der Multigenfamilie von *M. agalactiae* (Vpma) eine Rolle bei der Adhäsion und Immunevasion durch Antigenvariation zu spielen. Vpmas sind Oberflächenlipoproteine, ähnlich den Variablen Oberflächenproteinen (Vsa) von *Mycoplasma pulmonis*, bei denen längere Vsa-Proteine nachweislich vor Komplement schützen. Da die Fähigkeit, Komplement im Serum zu umgehen, ein wichtiger Faktor bei der systemischen Ausbreitung ist, wird die Rolle von Vpmas bei der differentiellen Pathogenität von Stämmen und Mutanten untersucht.
Bakterizide Assays in Seren wurden mit (i) dem *M. agalactiae*-Stamm PG2 durchgeführt, der eine hochfrequente Phasenvariation aller sechs Vpma-Lipoproteine aufweist, (ii) dem Stamm GM139, der nur VpmaV exprimiert, und (iii) sechs verschiedenen Vpma-Phase-Locked-Mutanten (PLMs), die einzelne Vpma-Produkte stabil exprimieren. Nach der Inkubation mit PG2-sensibilisiertem Schafserum wurden serielle Verdünnungen plattiert und die resultierende Anzahl koloniebildender Einheiten im Vergleich zu den hitzeinaktivierten Serumkontrollen bewertet, um das prozentuelle Überleben zu berechnen. Meerschweinchen-Komplement wurde ebenfalls mit der gleichen Methodik getestet. Zusätzlich wurden Western-Blot-Analysen von Triton X-114-Extrakten aus PG2 und GM139 unter Verwendung von polyklonalen Kaninchen Antikörpern α-VpmaV und PG2-sensibilisiertem Schafserum durchgeführt, wodurch starke Unterschiede in den Profilen der Stämme aufgezeigt wurden.
Die Gesamtdaten zeigen, dass der PG2-Stamm im Vergleich zum GM139-Stamm, der ein anderes Vpma-Profil trägt, anfälliger für Schafserum ist. Signifikante Unterschiede wurden auch zwischen den PLMs beobachtet, wobei PLMU und PLMX die höchste Serumempfindlichkeit aufwiesen. Da die Stämme und PLMs in früheren Experimenten keine Anfälligkeit in nicht sensibilisiertem Schafserum zeigten, scheinen Antikörper eine wichtige Rolle bei der Serumabtötung zu spielen. Proteine von GM139 in der mit polyklonalen α-VpmaV Antikörpern identifizierten Gelbande wurden mit Nano-LC-Orbitrap-MS/MS analysiert. Die Datenbanksuche ergab hohe Sequenzabdeckungen von Vpmas verschiedener *M. agalactiae* Stämme. Das ursprünglich erwartete VpmaV konnte jedoch nicht gefunden werden. Zusammenfassend könnten Vpmas eine wichtige Rolle bei der Serumempfindlichkeit von *M. agalactiae* spielen, und die Antigen-Variation ermöglicht es dem Erreger wahrscheinlich, nach Varianten zu selektieren, die die Serumresistenz während systemischer Infektionen erhöhen.

List of abbreviations

4-CN	4-chloro-1-naphthol
A	Adenine
ABC	Ammonium bicarbonate
ADCC	Antibody-dependent cellular cytotoxicity
APS	Ammonium persulfate
AQ-phase	Aqueous-phase
ATP	Adenosine triphosphate
Avg	Agalactiae variable gene
C	Cytosine
C1-9	Complement component 1-9
CA	Contagious agalactia
CFU	Colony-forming unit
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EF-Tu	Elongation factor thermo-unstable
ELISA	Enzyme-linked immunosorbent assay
G	Guanine
GlcNAc	N-Acetylglucosamine
GPS	Guinea pig serum
H. influenzae	*Haemophilus influenzae*
HRP	Horseradish peroxidase
hsp70	Heat-shock protein 70
IgA	Immunglobulin A
IgG	Immunglobulin G
IgM	Immunglobulin M
kb	Kilobases
kDa	Kilodalton
LEB	Laemmli Electrode Buffer
LPS	Lipopolysaccharide
M. agalactiae	*Mycoplasma agalactiae*
M. gallisepticum	*Mycoplasma gallisepticum*

M. genitalium	*Mycoplasma genitalium*
M. hypopneumoniae	*Mycoplasma hypopneumoniae*
M. pneumoniae	*Mycoplasma pneumoniae*
M. pulmonis	*Mycoplasma pulmonis*
M. bovis	*Mycoplasma bovis*
MAC	Membrane attack complex
mol%	Molar percent
OmpP2	Outer membrane protein P2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLM	Phase-locked mutants
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
RT	Room temperature
rpoA/rpoB/rpoC	RNA polymerase subunit alpha/beta/beta'
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
Sp.	Species
Staphylococcus aureus	*S. aureus*
T	Thymine
T_0	Sample taken directly after addition of serum
T_1	Sample taken after addition with serum and 1 h of incubation
TBS	Tris-buffered saline
Temed	N,N,N',N'-Tetramethylethylendiamin
Th cells	T helper cells
tRNA	Transfer ribonucleic acid
TX-phase	Triton-phase
U	Uracil
V	Volt
Vlp	Variable lipoproteins
Vpma	Variable proteins of *M. agalactiae*
Vsa	Variable surface antigens
Vsp	Variable surface proteins
Xer1	Integrase-recombinase, Xer1

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1. Introduction

1.1 Mycoplasma

Mycoplasma are procaryotic bacteria that belong to the class of Mollicutes (derived from the Latin *mollis*, soft and *cutis*, skin). They are the smallest self-replicating organisms and their shape can be polymorphic. The genome size of *Mycoplasma* spp. is very small with 580-1840 kb and an extraordinarily low content of guanine (G) and cytosine (C) from 8-40 mol%. Their optimal growth-temperature is 37 °C and they are pathogens and commensals in both humans and animals[2–4]. They are difficult to detect and often persistent in the host. Moreover, they can cause problems when contaminating cell culture and thus altering research results[5].

1.1.1 Taxonomy

The class of Mollicutes consists of four different orders, namely the Mycoplasmatales, the Entomoplasmatales, the Acholeplasmatales and the Anaeroplasmatales. The Mycoplasmatales comprise the family of Mycoplasmataceae with the geni of *Mycoplasma* and *Ureaplasma*. This class is highly variable in both phenotypes and genotypes.

To determine the taxonomy, in addition to 16s rRNA sequencing, ribosomal proteins, the elongation factor thermo-unstable (EF-Tu), the heat shock protein gene *hsp70*, as well as intergenic sequences were analysed. Ribosomal proteins provide the advantage of varying in size and sequence more than the 16s rRNA genes, facilitating the differentiation between closely related strains. Data from an evaluation of housekeeping genes support the hypothesis, that Mollicutes are likely derived from gram-positive bacteria which went through degenerative evolution[2,6]. They are most closely related to some clostridia[5].

1.1.2 Cell structure

Mycoplasma are the smallest free-living organisms, with diameters of 0.2 to 0.3 nm. Their exceptionally small size initially even led to the misconception, that they were viruses. Intracellularly, they contain a double-stranded circular DNA molecule and ribosomes which are enclosed by a plasma membrane (Figure 1)[3,5,7].

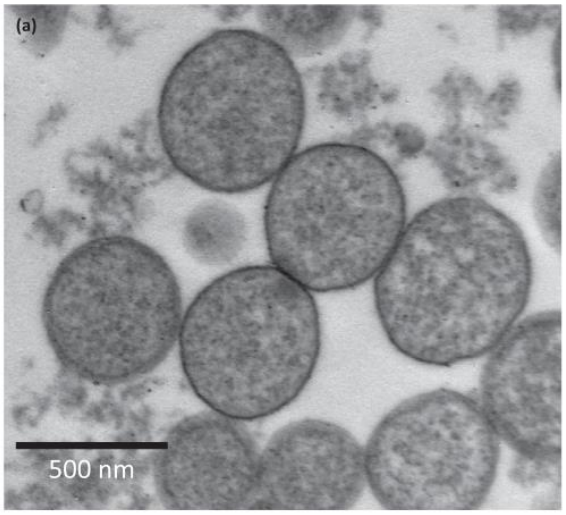

Figure 1: Transmission electron microscopy of *M. agalactiae*
The cells appear in the typical signet-ring shape with only one phospholipid membrane. The chromosomes inside are visible as threads, while the ribosomes appear as dense spots (image from Citti & Blanchard 2013[8]).

1.1.2.1 Cell membrane

Mycoplasma lack a cell wall, which means that their outer border consists solely of their cell membrane, which is about 10 nm wide. Thus, they often appear polymorphic, but still keep cell polarity and different shapes. These are for example pear-, flask-shapes, or helical filaments, although the favoured form is a sphere[3]. Hence, a cytoskeleton is likely in place to hold the cell stable. By usage of so-called terminal tip structures, some mycoplasma, like *M. gallisepticum* or *M. pneumoniae,* can glide on surfaces[9]. These structures connect the cytoskeleton with proteins like the bacterial actin, MreB (murein formation gene cluster B) and adhesins, which contain a motor activity.

The cytoplasmic membrane of mycoplasma consists of lipids, especially so sterols, glycolipids, lipoglycans and up to 50 % of proteins, for example cytadhesins. Many of these (lipo-)proteins play an important role as virulence factors and can undergo antigenic phase variation[5,10,11].

1.1.2.2 Glycocalyx

Similar to other bacteria, Mollicutes also possess a glycocalyx in the form of a polysaccharide capsule or a slime layer, with lipoglycans, glycolipids and adhesive polysaccharides, which are associated with biofilms.

Bacteria can be surrounded by either a capsule or a slime layer, which differ in the sense that a capsule is directly attached to the bacteria's surface and more complex, while a slime layer is anchored more loosely and simpler. Polysaccharides can either be fixed upon the cell surface or exported as exopolysaccharides. In general, they serve as a defence against the host's immune system by inhibiting bacteriolytic activity, phagocytosis, protecting against complement and in evading the immune response. Moreover, they play a role in adhesion, biofilm formation and even host mimicry.

The presence of a capsule in different *Mycoplasma* spp. has been suggested by different studies with dyes reacting to saccharides. Polysaccharides containing N-Acetylglucosamine (GlcNAc) form especially robust biofilms. Additionally, glycoconjugates which are adsorbed from the bacteria's surroundings can be included in forming the glycocalyx. Filamentous polysaccharides form the adhesive matrix in which the biofilm can assemble. The enclosing in such a biofilm can thus protect mycoplasma from the innate immune system and play a role in adhesion to epithelial cells. Moreover, the susceptibility to complement and macrophages is lessened in comparison to bacteria that are not encased in a biofilm[12,13].

1.1.3 Cultivation

Mycoplasma are difficult to grow in culture since they lack many genes for biosynthetic pathways due to their minimal genome. Therefore, they are dependent on the host, respectively their surroundings to provide nutrients. Components like cholesterol and fatty acids are especially important for growth and are hence supplemented through serum in the medium. Peptone, yeast extract and glucose are added as nutrients, while penicillin serves as a preventative measure against overgrowth with other, faster-growing bacteria. Mycoplasma grow only very slowly in liquid culture medium and enriched agar, taking several days to produce colonies visible in the light-microscope[2,3,5]. Difficulties in cultivating mycoplasma have led to problems in diagnosis as well as when identifying the bacteria as possible causes of different diseases[8].

Since the center of the colony grows deeper into the agar, the typical fried egg shape appears, as visible in Figure 2 (Razin 1996).

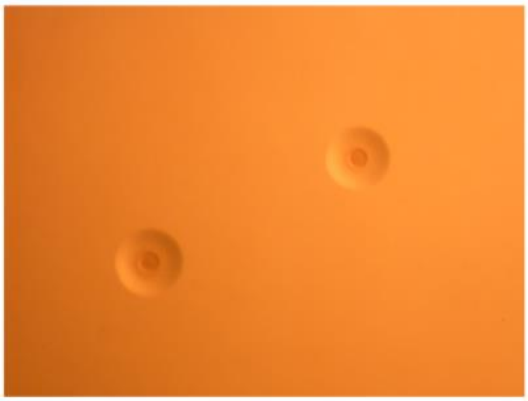

Figure 2: Colonies of *Mycoplasma agalactiae* strain PG2
The fried-egg morphology of the colonies is visible, the centre of the colony grows deeper into the agar, leading to a darker appearance (image from Hegde et al. 2015[14]).

1.1.4 Genome

A characteristic feature of mycoplasma is their small genome of about 580-1840 kb. They have preserved enough of their synthesizing capabilities for DNA, RNA and proteins to enable them to live a parasitic lifestyle. Because they can be grown in rich media in the laboratory setting, they are said to be the smallest self-replicating organisms[4]. Their guanine and cytosine content is very low, ranging from 8-40 mol%[7]. As mentioned, they have evolved from gram-positive bacteria and lost their cell wall and many biosynthetic pathways for macromolecules, making them dependent on the host or the media[5]. Mycoplasma's ribosomal genes are extremely conserved, making them a useful tool to determine phylogeny. Especially the 16S rRNA genes have been closely studied[2].

As already mentioned, mycoplasma have a high content of adenine (A) and thymine (T) due to directional mutation pressure during their development. Because of the preferred use of A and T, these bases are favoured over C or G in the 3' wobble position of codons in the case of synonymous codons. Amino acids encoded by C- or G-rich codons, for example glycine, proline, alanine, or arginine, are used more seldomly[2,15]. Nevertheless, the use of C and G across the genome is not uniform. Especially in regions that code for rRNA, tRNA, ribosome-genes, ATP-synthase genes, the RNA polymerase, as well as in sequences for variable surface proteins, the use of G and C is heightened in comparison to the rest of the genome. Furthermore, due to the increased use of A and T, UGA, where U stands for uracil, encodes tryptophan instead the typical translation as a stop-codon, in some Mollicutes. This codon is preferred over UGG which also encodes tryptophan, but features more G[15]. This can in turn

lead to problems when expressing mycoplasma genes in *E. coli* where UGA is used as a stop-codon[2].

1.1.5 Disease

Mycoplasma live parasitically, often causing persistent chronic infections. This means that they are highly adapted to the host[2]. Many different species can cause different endemic diseases in cattle, sheep and goats, which are of economic significance since, for example, milk production is curbed[16,17]. The main illness caused by *M. agalactiae* is contagious agalactia (CA), which affects small ruminants like sheep or goats all over the world, although the prevalence is highest in the Mediterranean Basin and certain Asian and African regions. It is a syndrome, composed of mammary symptoms like mastitis and decreased milk production, ocular infections like conjunctivitis or keratitis, joint symptoms like arthritis and sometimes respiratory illnesses including coughing and lesions. If a pregnant ewe is infected, mycoplasma can cause abortions as well. The infection can be spread orally or mammary and thus infect kids and lambs. Diseases similar to the CA caused by *M. agalactiae* can also be triggered by *Mycoplasma mycoides* subsp. *mycoides*, *Mycoplasma capricolum* subsp. *capricolum* and *Mycoplasma putrefaciens*. An outbreak of CA either occurs slowly with gradually increasing cases or spreads fast throughout the flock. Defining factors in prevalence are the immune status of the animals and the transhumance, which can cause extensive stress and contact between flocks.

As already mentioned, *M. agalactiae* can persist for months to years in an animal and relapses are possible even for healthy-appearing individuals. In this case, clinical periods can alternate with symptom-free periods, making infection control difficult. Antibiotic treatment is often ineffective and serves only to reduce the symptoms. This situation is even more intensified by the breeding of animals on increasingly large-scale farms. Mycoplasma are difficult and slow in cultivation, impeding diagnosis. Hence, growth inhibition tests, enzyme-linked immunosorbent assays (ELISA), complement fixation tests, polymerase chain reaction (PCR) and immunohistochemical techniques serve better to detect mycoplasma. The pathogenic mechanisms behind infection have not been identified completely, due to difficulties in genetic manipulation and lack of animal models[17–19].

1.2 Serum resistance & Pathogenicity

After infection of the host, microorganisms utilize various pathogenic mechanisms to cause disease. To prevent this, the immune system has developed two interconnected branches of defence, the adaptive and innate immune system.

1.2.1 Immune system components in serum

Out of the subsets of the immune system, there are different components present in serum, for example the complement system of the innate part and antibodies of the adaptive immune system[20,21].

1.2.1.1 The complement system

The innate immune system is the first line of defence against pathogens, with one of its major players being the complement system. It consists of plasma and membrane-associated serum proteins, which can induce inflammatory and cell-lytic pathways against different pathogens. The identified microorganisms are opsonized, proinflammatory mediators are created and the target cell is lysed through the membrane attack complex (MAC). The activation can happen in three different ways, via the classical, lectin and alternative pathway.

In the classical pathway, the activation happens through the C1 complex, which binds to antibodies attached to a pathogen's surface. This leads to cleavage of C4 and C2, which associate to form C4bC2a, called C3 convertase, which then attaches to the pathogen's surface. C3 can thus be cleaved into C3a and C3b, an anaphylatoxin, and an opsonin, respectively. The lectin pathway functions similarly, though the activation is due to pattern-recognition receptors like mannose-binding lectin. These receptors recognize pathogen-associated molecular patterns (PAMPs), like endotoxins. Through the incorporation of serine proteases, the complement proteins C4 and C2 are once again cleaved and the C3 convertase is made. When C3b associates with the C3 convertase, the C5 convertase is formed (C4bC2aC3b). In the alternative pathway, C3 cleaves spontaneously and C3b, together with Factor B, Factor D and Properdin, forms the C3 and C5 convertase. In the end, the pathways all form convertases, which in turn generate pro-inflammatory molecules, called anaphylatoxins (C4a, C3a, C5a), opsonins (C3b, C4b and others) and the membrane attack complex. The MAC relies on the further incorporation of C5b in the membrane which

opsonizes the pathogens and then assembles C6-9, which form a pore in the target cell, leading to its lysis (Figure 3).

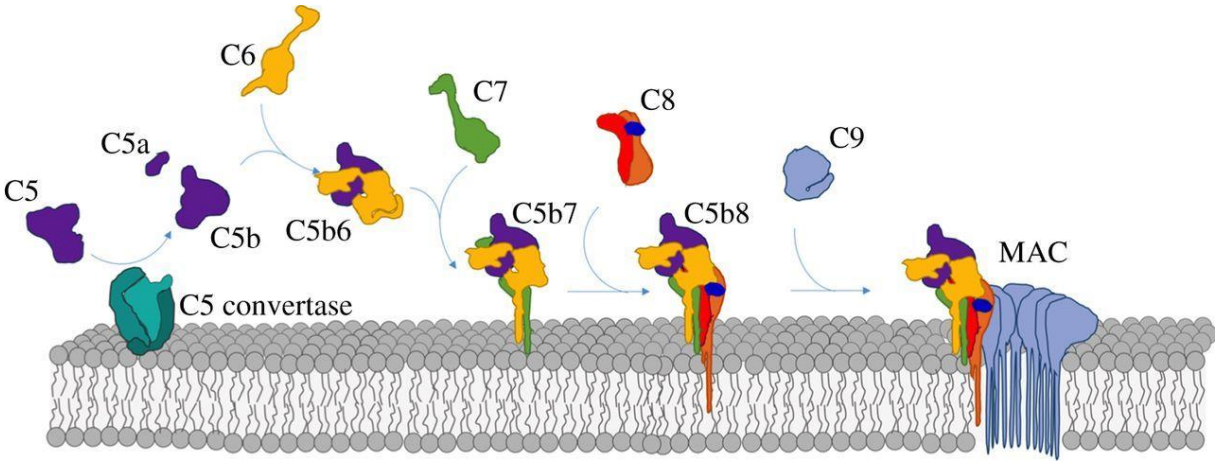

Figure 3: The membrane attack complex

The membrane attack complex is assembled after cleavage of C5 into C5a and C5b. C5b then associates with C6, C7, C8 and multiple monomers of C9. The C9 monomers insert into the membrane and form a pore. Thus, unregulated ion diffusion through the membrane ultimately leads to lysis of the attacked cell (image from Bayly-Jones et al. 2017[22]).

Different anaphylatoxins can then induce downstream signalling after binding to a receptor and thus activate different cells of the innate immune system like macrophages, neutrophils, basophils, eosinophils and mast cells. Out of these, neutrophils and macrophages can perform phagocytosis. While neutrophils kill pathogens through an oxidative burst, macrophages can process protein antigens and present these them T-cells. Eosinophils, basophils and mast cells degranulate and thereby secrete cytokines, which can attract other immune cells and mediate inflammatory reactions. Moreover, the complement system is connected to parts of the adaptive immune system. For example, the complement system plays an important role in trapping antigens and helps lower the B-cell activation threshold. Complement-opsonized pathogens can activate B-cells and lead to their expansion and differentiation. C5aR and other different complement inhibitory proteins also modulate T-cell immunity[20,21,23].

1.2.1.2 Antibodies

As already mentioned, the innate immune system interacts with the acquired immune system, for example via antibodies. Antibodies are produced by B-cells and are specific to

certain antigen-epitopes. They can have different effects. After the binding of antibodies to a pathogen's surface for example, the complement system can be activated through the classical pathway. Thus, more complement components can be attracted for opsonization and signalling. Antibodies can further help with phagocytosis and have complement-independent functions as well. They neutralize bacterial toxins like tetanus, diphtheria and pertussis toxins and play a role in antibody-dependent cellular cytotoxicity (ADCC)[24,25]. One of the main defence mechanisms against mycoplasma are IgM, IgG and IgA antibodies[5].

1.2.1.3 Phagocytes

Phagocytes (neutrophils and macrophages) recognize pathogens through different receptors, for example lipopolysaccharide (LPS), mannose, or glucan receptors. Opsonisation through complement or antibodies further facilitates the recognition of said pathogen by the phagocytes. The microbe is internalized by the phagocyte and killed through different enzymes, acidification and toxic products, for example hydrogen peroxide, the superoxide anion and nitric oxide. Fragments of the pathogen can be presented to T helper (Th) cells via the major histocompatibility class II molecules, which then secrete cytokines. The process of phagocytosis is especially important because for one, gram-positive bacteria cannot be lysed by the MAC due to their thick cell wall and some pathogens have evolved to become resistant to the MAC[23,24].

1.2.2 Virulence factors involved in serum resistance

Virulence genes in mycoplasma are still not fully identified. They comprise genes for adhesion, invasion, toxins, immune evasion and immunostimulation – or suppression. Besides that, certain sequences code for procurement pathways of nutrients necessary for toxic products or for persisting in fastidious environments. Another important factor in pathogenesis are intense immunopathological reactions of the host, inflammation, as well as cell damage due to adhesion and invasion of mycoplasma into host cells. To further investigate disease pathogenesis and to allow for better diagnosis, the virulence factors need to be better characterized. Obstacles in this field are, however, lacking research interest and difficulties in genetic manipulations[26,27].

The first step in colonizing host tissue is adhesion to host cells. Some mycoplasma, *M. pneumoniae* and *M. genitalium* for example, even have specialized terminal structures, called tip, for adhesion[27]. Some mycoplasma are also able to invade host cells. This allows

them to evade the host's immune system, antibiotic therapy and pass into the bloodstream, leading to systemic infections. This is the basis for mycoplasma to become persistent and cause chronic diseases[19,28].

Various elements can account for differences in serum resistance, for example, biofilm formation. Other factors are the complement cleaving, polysaccharide secretion, phagocytosis resistance and phase variation, leading to the evasion of the immune system. While serum resistance of *M. agalactiae* has not yet been studied, there are many examples from other bacteria[27].

1.2.2.1 Serum resistance of different bacteria

For *Klebsiella pneumoniae*, it has been described that the polysaccharide of the capsule protects against killing via the MAC and against C3b opsonization. Differences in the sugar composition of the capsule can affect complement activation via the lectin pathway. Longer LPS on the surface inhibit the deposition of C3b and the MAC, either through sterically hindering the components or through recognition of certain epitopes[29].

In the case of *Mycoplasma pulmonis*, since it lacks a cell wall, its outer most layer is the cell membrane, including surface proteins, for example the variable surface antigen family (Vsa). These are size- and high-frequency phase-variable lipoproteins and can aid in avoiding lysis through complement. Bacteria bearing Vsa proteins with an increased length of 40-60 tandem repeats have an enhanced resistance against killing, while cells with repeats of five or less are more susceptible. Hence, Vsa proteins can sterically hinder complement interactions with the bacteria's surface. However, biofilm formation is more efficient when the Vsa is short. Biofilms then once again aid in conferring complement resistance, even for mycoplasma with short Vsa proteins[13,30,31]. Adherence is also dependent on the number of Vsa repeats, with shorter Vsa proteins being more adherent to red blood cells and polystyrene[32]. While different Vsa types exhibit differential adherence and susceptibility, these variations were solely dependent on the overall length and not the Vsa type[33]. Additionally, cells with a high number of Vsa repeats are rather resistant to phagocytosis by macrophages, possibly due to Vsa proteins shielding surface proteins from receptor recognition of the macrophage[34].

Complement resistance is also enhanced in *Mycoplasma pneumoniae* when encased in biofilms[35]. For *Mycoplasma bovis*, it was demonstrated that antibody-dependent complement

killing is significantly stronger than regular complement killing[36]. *Mycoplasma hypopneumoniae* binds Factor H – an inhibitor of the complement system – over EF-Tu to reduce C3 deposition and block complement activation. Factor H helps with adhesion to epithelium as well[37]. *Haemophilus influenzae* can delay C3b deposition and thus evade killing by the MAC[38]. For *H. parasuis*, the outer membrane protein P2 (OmpP2) plays an important role in conferring serum resistance[39]. *Staphylococcus aureus* and *Pseudomonas aeruginosa* have developed proteins inhibiting the C3 convertase or C3b respectively. *S. aureus* can bind immunoglobulins to prevent phagocytosis and complement activation and it can cleave surface-bound C3b and immunoglobulins. *Borrelia burgdorferi* uses a certain surface protein to block MAC assembly. Similar to *M. hypopneumoniae*, *Neisseria meningitidis and Neisseria gonorrhoeae* use Factor H to inhibit complement activation[20].

1.2.2.2 Virulence factors and serum resistance of *Mycoplasma agalactiae*

It has recently been described that *M. agalactiae* can secrete polysaccharides and attach these to the cell as a β-(1→6)-glucopyranose structure. The responsible synthase gene and, therefore, the glucan-secretion undergo high-frequency phase variation. In *M. mycoides* subsp. *mycoides*, the complement susceptibility was reduced when galactan was attached to the cell. For *M. agalactiae* producing β-(1→6)-glucans, however, the survival both in goat serum and in guinea pig complement was greatly reduced. This stands somewhat in contrast to the shielding hypothesis of longer Vsa proteins attached to the *M. pulmonis* surface[33]. However, this polysaccharide is abundantly expressed in fungal cell walls, and fungi are common pathogens of goats. Therefore, antibodies against this structure could be present in goat serum. Another possible explanation is the direct binding of complement components to the glucans, which explains the reduced resistance to guinea pig complement. Switching off the secretion of β-(1→6)-glucan can therefore promote systemic dissemination[40,41].

A prominent mechanism that *M. agalactiae* employs to evade the host immune system is the high-frequency antigenic phase variations of the family of surface lipoproteins called Vpmas (**V**ariable **P**roteins of ***M. a****galactiae*)[42]. This allows them to adapt to different host environments. Phase variation is present in other mycoplasma as well, for example, *Mycoplasma hyorhinis* with the variable lipoproteins (Vlp), the Vsas of *M. pulmonis*[6] and the variable surface proteins (Vsp) of *M. bovis.* Antigenic variation is driven by different genetic systems, through DNA slippage, site-specific recombination, gene conversion, or reciprocal recombination. In the case of *M. agalactiae* the recombinase Xer1 exhibits site-specific

recombination events in the *vpma* gene locus leading to switches in Vpma expression. The Vpma size can be varied through DNA slippage and domains shuffled via DNA recombination and gene duplication[42]. Under selection pressure *in vivo* and the presence of corresponding Vpma-antibodies *in vitro,* however, *Xer1* independent Vpma switching has been observed via the formation of chimeras, duplications and deletions. This proves the role of Vpmas in immune evasion[43,44]. The type strain of *M. agalactiae*, PG2, has six different Vpma genes (VpmaU-Z), as visible in Figure 4, which range in size from about 23 kDa (VpmaU and VpmaX) to about 34 kDa (VpmaV, VpmaW, VpmaY, VpmaZ)[42]. In other *M. agalactiae* strains, like 5632 and 627, other Vpmas are also present. Vpmas first identified in the 627 strain, however, were called Agalactiae variable gene (Avg) proteins[45].

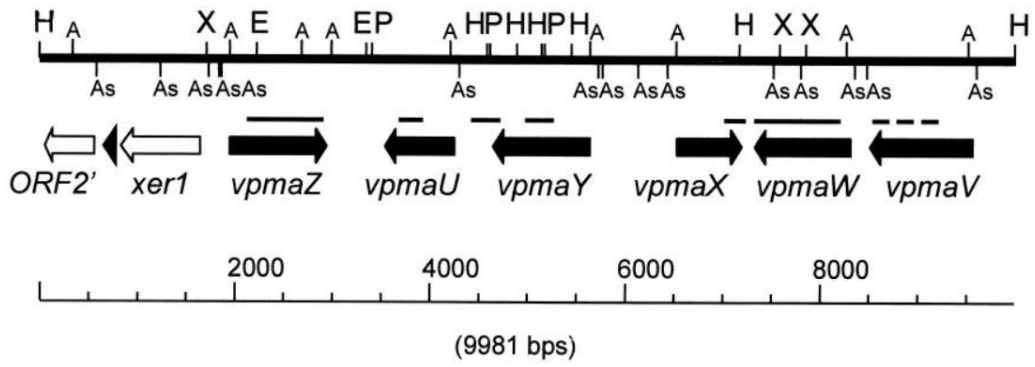

Figure 4: Vpmas of *M. agalactiae* type strain PG2

The six different Vpmas (VpmaU-Z) of *M. agalactiae* type strain PG2, the different ORFs and the recombinase Xer1 are illustrated (image from Glew et al. 2002[46]).

To study the role of different Vpma proteins during *M. agalactiae* infections and in vitro pathogenicity related assays, Vpma phase-locked mutants (PLMs) were generated through the targeted disruption of Xer1 recombinase, so that only one of the respective Vpmas is constitutively expressed without further switching[47]. *In vitro* studies with PLMs have demonstrated, that PLMV, followed by PLMW is highly adhesive to HeLa, sheep mammary epithelial and stromal cells, while PLMU was least adhesive. The invasion of cells by the PLMs seems to be closely correlated to their adhesion rate. This highlights the decreased fitness of PLMU during host colonization. VpmaU could nevertheless be advantageous when switching from local infection to a systemic spread, where lower adherence to the host cells is necessary for detachment[48]. Strains capable of Vpma phase variation, for example PG2, have been suggested to be more invasive and therefore better at spreading systemically *in vivo* than PLMU and PLMY in a sheep trial[49]. The dominance of PLMY over PLMU at local

infection sites during experimental co-challenge studies indicates a better *in vivo* fitness for PLMY, possibly due to better shielding against phagocytes, better adherence, or nutrient acquisition. To become systemic or to evade the immune system at later infection stages, Xer1 independent switching could lead to phase variation. This suggests that the various Vpmas have different properties concerning different stages of colonization and are most likely beneficial for a successful infection, as already seen with the increased invasiveness of PG2[43]. In another sheep infection study, PLMU initially exhibited less colonization and multiplication after infection, which is in line with the previous results demonstrating less adhesion and lower *in vivo* fitness compared to PLMY during intramammary infections. PLMV and PLMX infected tissue displayed hyperplasia of the left udder lymph nodes, inflammatory cells, as well as purulent galactophoritis. This indicates an increased virulence of these PLMs. PLMV and PLMW also showed the highest numbers of re-isolations from sheep. For PLMV, a significantly higher spread between the udder halves than for other PLMs could be confirmed via immunohistochemistry, whereas PLMW and PLMU were not detected, possibly due to clearance of the pathogen. The results from the re-isolations and the immunohistochemistry highlight the high adhesion and invasion potential of PLMV, as already shown *in vitro*. In addition, all PLMs, regardless of their inability to perform phase-variation, were able to infect and colonize host tissue[50].

In addition to Vpmas, the proteins P40 and MAG_6130 have a role as cytadhesins, binding to lactoferrin, fibronectin, fibrinogen and/or plasminogen. The adhesion of plasminogen to the pathogen's surface can inhibit complement activation through degradation of C3b and C5. Therefore, this process might enhance immune evasion. The other molecules can aid in binding to host cells and therefore lead to cell invasion and biofilm formation. P40 is also able to trigger pro-apoptotic pathways. Taken together, different factors aid in adhesion, invasion, systemic spread and immune evasion of *M. agalactiae*[51].

Analysing the *M. agalactiae* strain GM139[52] with colony immunoblots and western blots, it became evident that only the VpmaV phenotype is expressed. In the type strain PG2 all Vpmas are visible in colony immunoblots and western blots with the respective Vpma-specific antibodies. The underlying Vpma sequence for VpmaV in GM139, however, is different from the one in PG2. Therefore, this alteration in Vpma profile might play a role in differential serum susceptibility between the two strains.

1.3 Project Hypothesis

As already mentioned, the serum resistance of *M. agalactiae* has not yet been studied, except for one case[40]. Nevertheless, serum resistance has been proven to be important for the systemic spread of pathogens. In the case of the Vsa protein family of *M. pulmonis*, which are homologous to the Vpmas of *M. agalactiae*, the Vsa profile plays a role in serum resistance. Vsa proteins with more and longer tandem repeats sterically hinder complement deposition and activation, inhibit phagocytosis and display less biofilm formation in comparison to shorter Vsas[13,30–34]. While it has been proven that the secretion of a certain glucan of *M. agalactiae* can affect serum resistance, the role of Vpmas has not yet been investigated in complement or serum resistance.

To examine differences in the serum susceptibility of different strains/mutants expressing different Vpma profiles, for instance, the type strain PG2[19,43,49], the recently characterized GM139[52] – a wild-type strain exhibiting sole expression of VpmaV, and the six PLMs (PLMU-Z; [43,47,48]) will be analysed in serum bactericidal assays. Different sera will be tested, including sheep serum obtained four days before infection (D-4) and on Day 17 after infection with PG2 (D+17), as well as commercially obtained guinea pig serum complement, to assess the killing characteristics of these. Different standardisation parameters were already briefly studied previously, although the project had to be abruptly stopped due to the first Covid-19 lockdown[53]. From the collected data, we seek to derive, whether the Vpma-profiles influence susceptibility to serum components[39,54,55].

To further assess the presence, and differential reactivity of the *M. agalactiae*- specific antibodies present in the serum, western blots with the Triton-X114 phase-extractions of the PG2 and the GM139 would be performed. Additionally, analysis of the Vpma protein being expressed in strain GM139 would be carried out by mass spectrometry (LC-MS). The amino acid sequence and structural differences of GM139's altered Vpma profile could be a cause for differential serum resistance.

2. Materials and Methods

2.1 Bacterial Strains, Culture Conditions

M. agalactiae type strain PG2 (Madrid, Spain)[19,43,49], strain GM139 (CA, USA)[52], which had previously demonstrated sole expression of VpmaV, as well as the different Vpma phase-locked-mutants of *M. agalactiae* (PLMs)[48] were tested in serum bactericidal assays.

Table 1: Composition of SP-4 Broth

SP-4 Broth, components for 1 l	
BBL™ Mycoplasma Broth Base (Gibco Life Technologies, USA)	3.5 g
Bacto™ Tryptone (Gibco Life Technologies, USA)	10 g
Bacto™ Peptone (BD, USA)	5.325 g
675 ml of double distilled water added	
pH set to 7.8 with NaOH	
Sterile components added after autoclaving:	
Fetal Bovine Serum, qualified (heat-inactivated at 56 °C, 40 min; Gibco Life Technologies, USA)	170 ml
CMRL 1066 10x (Gibco Life Technologies, USA)	50 ml
4 % Aqueous Yeastolate Solution	50 ml
15 % Yeast Extract Solution (Gibco Life Technologies, USA))	25 ml
50 % Glucose Solution (Sigma-Aldrich, USA)	10 ml
Penicillin G 10 E5 (Sandoz GmbH, Austria)	5 ml
0.5 % Phenol Red Dye (Sigma-Aldrich, USA)	10 ml
25 % Sodium Pyruvate (Sigma-Aldrich, USA)	20 ml

For the first assay, equivalent amounts of culture were scraped off from the frozen -80 °C stocks of the different mycoplasma cultures. The strains were grown in SP-4 broth (prepared as described in Table 1) for 16 h until the appropriate colour change to orange-yellow due to the phenol red indicator was visible[56]. After the first assay, the respective remaining cultures were aliquoted and stored at -80 °C, to reduce the freeze-thaw cycles and to ensure that the amount and condition of the inoculated culture are homogenous between experiments.

2.2 Complement/Serum Killing Assay

In this assay, the cell survival after treatment with different types of active and heat-inactivated serum was assessed after making serial dilutions and plating the solution on SP-4 agar[33,39,55]. After incubation at 37 °C for three to five days, the resulting CFU was counted. The experiment is visualized in detail in Figure 5.

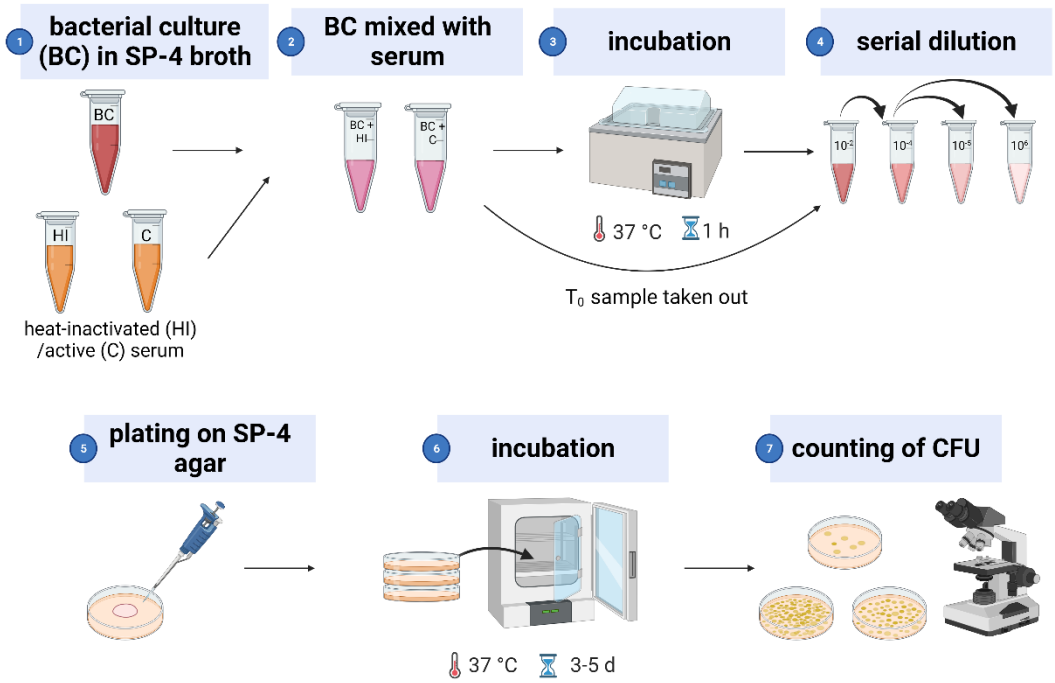

Figure 5: Bactericidal assay

Bacterial cultures of the different strains/PLMs were individually mixed with equal amounts of heat-inactivated/active sera from sheep or guinea pig. For the first experiments, an aliquot was taken out (T_0 sample), before incubating the sample for 1 h at 37 °C. Afterwards, the sample was serially diluted to 10^{-4}, 10^{-5}, 10^{-6} and plated onto SP-4 agar plates. These were incubated for three to five days at 37 °C and the resulting CFU was counted under a stereomicroscope. Figure created with BioRender.com

2.2.1 Sera in complement/serum killing assays

The sheep serum used in the assays was obtained from a sheep (No. 1) both four days before inoculation (Day 4 p.i., D-4) and 17 days after inoculation (Day 17 p.i., D+17) with *M. agalactiae* type strain PG2 during previous intramammary infection experiments in 2012. Other than that guinea pig serum complement (Sigma-Aldrich, USA) and fetal bovine serum (Gibco Life Technologies, USA) was used.

2.2.2 Treatment of bacteria with serum

Heat inactivation of 1 ml sera aliquots was carried out at 56 °C in a water bath for 40 min for sheep serum and fetal bovine serum, and 1 h for guinea pig serum. For the bactericidal assay, 50-100 µl aliquots of the mycoplasma culture were mixed with an equal amount of active or heat-inactivated serum, respectively. To keep a check on the equivalence of the starting bacterial counts, 5 µl were occasionally removed as controls at time point zero (T_0). The remaining mixture was then incubated for 1 h at 37 °C in a water bath (T_1)[33,39,40,55,57,58].

2.2.3 Serial dilutions

Because the bacterial cell suspensions would have a colony-forming unit (CFU) count too high to enable counting after plating, the samples were diluted. Hundred-fold serial dilutions of the T_0 and the T_1 samples were made by mixing 5 µl of sample with 495 µl of SP-4 broth, thereby, achieving a 10^4-fold and a 10^6-fold dilution. To prepare a 10^5-fold dilution 50 µl were taken out of the10^{-4} dilution and mixed with 450 µl of SP-4 broth.

2.2.4 Cultivation

2.2.4.1 Preparation of plates

SP-4 agar was prepared with the same components as SP-4 broth (Table 1). After mixing Mycoplasma Broth Base, Tryptone and Peptone and setting the pH to 7.8, Difco Agar Noble (BD, USA) was added in a concentration of 1 % of the total volume to the solution. After autoclaving, this was cooled slowly to about 54 °C and kept warm in the water bath at the same temperature. The sterile components were prepared as stated in Table 1. However, both phenol red and pyruvate were substituted with water as they are not needed in the agar as growth indicator. These components were warmed to about 37 °C and after mixing everything, small plates were poured with about 7 ml of SP-4 agar medium each.

2.2.4.2 Plating of dilutions

Depending on the dryness of the plates, 60-100 µl aliquots of the appropriate serial dilutions were spread per plate. For the T_0 samples, only the 10^6-fold dilutions were plated, whereas 10^4-, 10^5- and 10^6-fold dilutions were used to evaluate optimal dilutions for counting. After drying for a few minutes in the laminar flow hood, the plates were incubated at 37 °C for three to five days, depending on the size of the colonies. The CFU were counted under the light stereomicroscope.

2.2.4.3 Spot-plating assay

To assess differences in serum-killing and resistances between the active and heat-inactivated sera side-by-side in a preliminary manner, larger plates were poured and 3- and 4-fold serial dilutions of T_1 samples were prepared in microtiter-plates with SP-4 medium as visualized in Figure 6[59]. This assay was performed only for the T_1 samples of PG2 and GM139 with active and heat-inactivated sheep serum, respectively. In the beginning, 2 µl of 3^1- to 3^{17}-fold dilutions of culture with active and heat-inactivated serum were plated below each other. After evaluating optimal dilutions for counting CFUs, 10^{-3}- to 10^{-9}-dilutions were plated. The microtiter plates and the spot-plating-assay plates were incubated at 37 °C and the colour change in the microtiter plate and the number/density of colonies appearing on the agar plate were assessed daily to see changes. The CFUs on the plate were counted after three to five days of incubation.

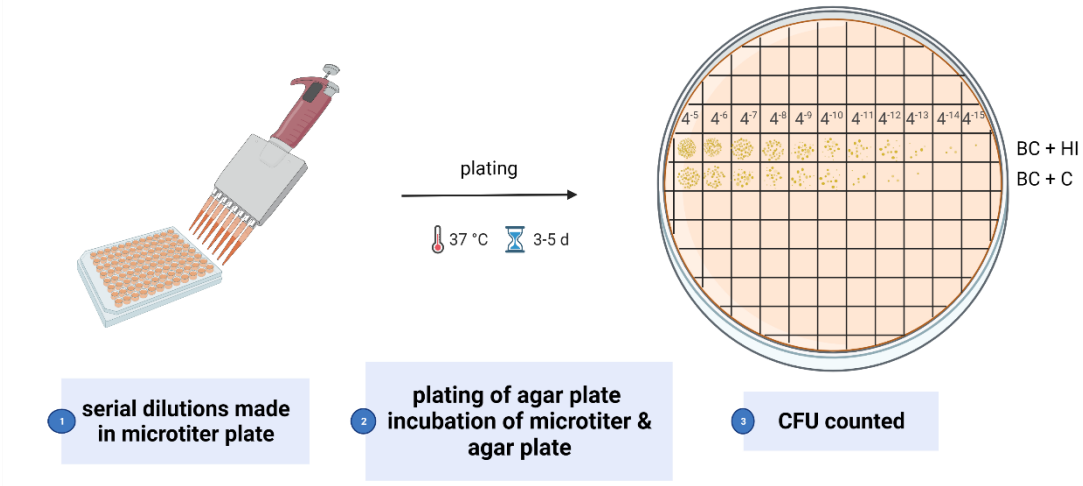

Figure 6: Spot plating assay

The samples with active and heat-inactivated serum were diluted in a microtiter plate with SP-4 medium up to 10^{-9} and 2 µl of each dilution plated onto a large SP-4 agar plate. The CFU were counted after three to five days of incubation. Figure created with BioRender.com

2.2.5 Calculation of serum/complement susceptibility

To calculate the serum resistance the CFU/ml was determined via this formula:

$$\frac{CFU}{ml} = \frac{number\ of\ counted\ colonies * dilution\ factor * 1000}{\mu l\ plated}$$

This was done both for the T_0 and the T_1 samples with active or heat-inactivated sera. The CFU/ml for the complement sample was divided by CFU/ml for the heat-inactivated one to obtain the % survival. Alternatively, fold-killing was calculated by dividing the heat-inactivated CFU/ml by the CFU/ml of the active complement sample.

$$\%\ survival = \frac{\frac{CFU}{ml} of\ sample\ treated\ with\ active\ complement}{\frac{CFU}{ml} of\ sample\ treated\ with\ heat-inactivated\ complement} * 100$$

$$fold-killing = \frac{\frac{CFU}{ml} of\ sample\ treated\ with\ heat-inactivated\ complement}{\frac{CFU}{ml} of\ sample\ treated\ with\ active\ complement}$$

To assess whether there may have already been a difference in cell count before the assay, which could, in turn, falsify the calculation of the killing ratio between the active and heat-inactivated complement, the T_0 counts were incorporated as follows:

$$\%\ survival = \frac{\frac{\frac{CFU}{ml} of\ sample\ treated\ with\ active\ complement\ of\ T_1}{\frac{CFU}{ml} of\ sample\ treated\ with\ active\ complement\ of\ T_0}}{\frac{\frac{CFU}{ml} of\ sample\ treated\ with\ heat-inactivated\ complement\ of\ T_1}{\frac{CFU}{ml} of\ sample\ treated\ with\ heat-inactivated\ complement\ of\ T_0}} * 100$$

$$fold-killing = \frac{\frac{\frac{CFU}{ml} of\ sample\ treated\ with\ heat-inactivated\ complement\ of\ T_1}{\frac{CFU}{ml} of\ sample\ treated\ with\ heat-inactivated\ complement\ of\ T_0}}{\frac{\frac{CFU}{ml} of\ sample\ treated\ with\ active\ complement\ of\ T_1}{\frac{CFU}{ml} of\ sample\ treated\ with\ active\ complement\ of\ T_0}}$$

2.2.6 Statistical analysis

The differences in serum resistance were analysed in GraphPad Prism. Firstly, normality and the distribution of residuals were examined. Depending on the sample size, a Student's t-test and ANOVA or the respective non-parametric test were performed. Results with a p-value of 0.05 were considered statistically significant. Graphs were made in GraphPad Prism.

2.3 Triton-X114 Phase Extraction & Blotting

As mentioned before, the sensitized sheep serum was obtained at Day 17 p.i. from a sheep inoculated with *M. agalactiae* PG2. Therefore, the serum killing during bactericidal assays, could as well be due to the presence of *M. agalactiae*-specific antibodies and not just because of complement.

To analyse this, the membrane proteins from the different *M. agalactiae* strains and mutants were extracted with Triton-X114, a detergent that incorporates said proteins into micelles[52]. Through this, the proteins are divided into three fractions: the insoluble, aqueous and detergent part. The insoluble fraction usually comprises cytoskeleton proteins, the aqueous part cytoplasmic proteins and the detergent fraction contains membrane proteins. Then the samples were put through gel electrophoresis and blotted onto a nitrocellulose membrane. The sensitized sheep serum used in the bactericidal assays served as the primary antibody and rabbit anti-sheep IgG, conjugated to horseradish peroxidase (DakoCytomation, Denmark) as secondary antibodies. Only if antibodies against the fractionated mycoplasma proteins of the different strains are present in the serum, they would bind the secondary antibody and would be visualized as distinct bands on the blot.

Similarly, Triton-X114 phase extracts of GM139 strain were tested with α-VpmaV polyclonal antibodies as previously demonstrated by Barbosa et al. 2022[52]. This was done to identify and excise the corresponding bands from the Coomassie gel run in parallel to define the exact Vpma protein expressed by the mass spectrometry method described ahead.

2.3.1 Triton-X114 phase extraction

Firstly, a 1 ml preculture of the strain was prepared with 25 µl of the -80 °C stock in SP4-broth. Subsequently, 100 µl of GM139 and PG2 precultures were respectively inoculated into 10 ml SP4 in 15 ml Falcon tubes. The cultures were grown for the next 2 days at 37 °C.

To start the extraction, each culture was centrifuged for 5 min, at 4 °C at 12,000 × g. The supernatant was discarded and the resulting pellet was resuspended into the original volume in PBS (phosphate buffered saline; Gibco Life Technologies, USA) containing 10 % PMSF (Phenylmethylsulfonyl fluoride; PBS/PMSF), which served as a protease inhibitor. This was washed three times to remove any residues of the serum components in the SP-4 broth which could later interact with the blotting antibodies. After centrifuging, the supernatant was

discarded again and the resulting pellet was resuspended in 500 µl of PBS/PMSF in between washing steps. After the final wash, the pellet was resuspended in about 800 µl PBS/PMSF.

Next, 10 % of Triton-X114 (Sigma-Aldrich, USA) were added. The optimal concentration of Triton-X114 had to be previously evaluated so that after the later steps, the proportion of the Triton phase is close to 10 % of the total volume. After the addition of Triton-X114, the tubes were rotated for 2 h at 4 °C. The solution was centrifuged and the supernatant transferred to a new tube. The resulting pellet contained the insoluble proteins. If needed for further analyses, it was resuspended in 500 µl of PBS/PMSF with 10 % Triton-X114, rotated again for 2 h at 4 °C, centrifuged, the supernatant discarded and the pellet dissolved in PBS/PMSF.

The supernatant from the earlier step was incubated for 5 min at 37 °C and centrifuged for 3 min at 12,000 × g at room temperature (RT). The Triton-phase (TX-phase) which is indicated by a cloudy phase at the bottom of the tube, was separated from the aqueous-phase (AQ-phase) above into different tubes. The TX-phase was washed three times by resuspending into 800 µl of PBS/PMSF, vortexed and incubated for 5 min on ice and 5 min at 37 °C. After centrifugation for 3 min at 12,000 × g, the AQ-phase was discarded every time. After the final wash, the solution was diluted 1:10 in cold methanol and stored at -80 °C overnight, to allow the sample to precipitate. Afterward, the sample was centrifuged for 5 min at 4 °C at 12,000 × g. The methanol was discarded, and the pellet was resuspended in 120 µl of PBS/PMSF. The AQ-phase was also washed three times, and 80 µl of Triton-X114 added to the solution each time. After 5 min of incubation on ice, 5 min at 37 °C and centrifugation for 3 min at 12,000 × g the AQ-phase was transferred to a new tube and the TX-phase discarded.

The resulting protein concentration was measured in NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Different standards were prepared out of Albumin Standard (2 mg/ml; Thermo Fisher Scientific, USA) in PBS/PMSF, ranging from 0 ng/µl to 1000 ng/µl. 2 µl of sample were mixed with 15 µl of Pierce™ 600 nm Protein Assay Reagent (Thermo Fisher Scientific, USA). After blanking with H_2O and measuring the standards, the samples were measured at a wavelength of 660 nm. The resulting samples were stored at -80 °C or used directly for further analysis[47,60].

2.3.2 Gel electrophoresis

The Triton-X114 phase extraction samples were thawed on ice and prepared for separation according to their size in an SDS-PAGE, consisting of a 12 % resolving gel and a 5 % stacking gel[47,52]. Before loading, the samples were mixed 1:5 with 5 x SDS Loading Dye (5 % β-Mercaptoethanol, 0.02 % Bromophenol blue, 30 % Glycerol, 10 % sodium dodecyl sulphate (SDS) and 250 mM Tris-HCl (pH 6.8)). The solution was heated for 5 min at 95 °C and immediately cooled on ice thereafter. The apparatus for pouring the gels was prepared by wiping the glass plates with 70 % alcohol and one glass plate was aligned with a back This set-up was tested with water for any leaks for about 15 min before casting the acrylamide gels. Small 12 % resolving gels were prepared with the following components for two gels: 6.4 ml of deionized water, 4.5 ml of 40 % Acrylamide mix (Rotiphorese® Gel 40 (29:1), Carl Roth GmbH + Co. KG, DE), 3.8 ml of 1.5 M Tris (pH 8.8), 150 µl of 10 % SDS, 150 µl of ammonium persulfate (APS) and 6 µl of N,N,N′,N′-Tetramethylethylendiamin (TEMED, Carl Roth GmbH + Co. KG, DE). TEMED splits APS, thereby forming free radicals to start the polymerisation of the acrylamide and bisacrylamide. Therefore, the gel must be poured instantly. 500 µl of 70 % ethanol was poured on top of the gel to prevent bubbles from forming. After about one and a half hours of polymerization, the alcohol was removed and the gel top washed with water. The 5 % stacking gel was prepared similarly, with 5.8 ml of deionized water, 1 ml of 40 % Acrylamide mix, 1 ml of 0.5 M Tris (pH 6.8), 80 µl of SDS, 80 µl of APS and 8 µl of Temed. A comb was put in and the gel was left to polymerize for half an hour. The gels were then put into the apparatus for running in SDS-PAGE Running Buffer (1:10 dilution of 10X Laemmli Electrode Buffer (LEB) stock (250 mM Tris, 1.92 M Glycine) plus 1 % SDS). The samples and the Protein marker Roti®-Mark TRICOLOR (Carl Roth, Germany) or Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Fisher Scientific, USA) were loaded with a syringe. The gels were run in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad, USA) at 150 V and 2 mA for about one and a half hours until the loading dye reached the bottom of the gel. The gels were subsequently stained or blotted onto a nitrocellulose membrane. For Coomassie staining, the gel was put in Coomassie Brilliant Blue R-250 for half an hour on a shaker. For preparing the Coomassie solution, (2.5 g of Coomassie were dissolved in 450 ml of methanol (Honeywell, USA), 450 ml of deionized water and 100 ml of glacial acetic acid (Carl Roth GmbH + Co. KG, DE). The gel was destained with deionized water or using destaining solution (40 % methanol, 10 % glacial acetic acid) and imaged with ChemiDoc MP (Biorad, USA).

2.3.3 Western blot

For blotting[47,52], the gel was put into blotting buffer, prepared by mixing 100 ml of 1 X LEB, 200 ml of methanol (Honeywell, USA) and 700 ml of deionized water. Whatman sheets were prepared as filters. These, as well as the fiber pads and the GE Healthcare Amersham™ Protran™ 0.2 µm NC (Thermo Fisher Scientific, USA) nitrocellulose membrane were soaked in blotting buffer. This was arranged in the blotting apparatus Mini Trans-Blot® Cell (Bio-Rad, USA) as illustrated in Figure 7. After filling this with blotting buffer to the top, the gel was run at constant voltage of 100 V for one hour.

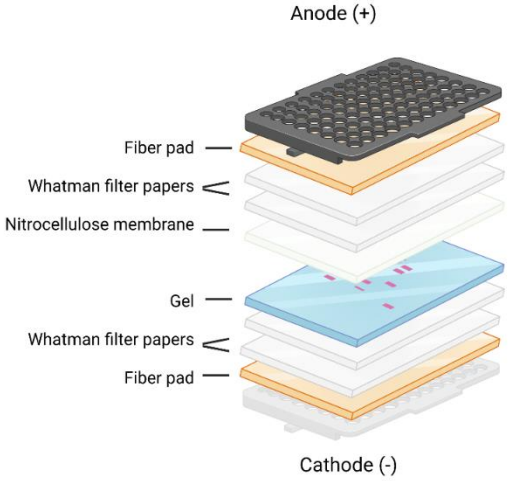

Figure 7: Blotting arrangement

After soaking in blotting buffer, from cathode to anode, a fiber pad, two Whatman filter papers, the gel, the nitrocellulose membrane, again two Whatman filter papers and a fiber pad were arranged. This was placed into the blotting apparatus. Figure created with BioRender.com

The bound proteins were probed with different antibodies (Figure 8). The first step was to rinse the membrane in tris buffered saline (TBS) 2-3 times for 5 min each. TBS was prepared with 10 mM Tris-HCl, 154 mM NaCl and pH set to 7.4. Afterwards, proper blotting was verified by staining the membrane with Ponceau S stain (0.5 % Ponceau S; Sigma-Aldrich, USA). The membrane was washed with deionized water to remove the Ponceau stain and put into blocking buffer, which consisted of 3 % milk powder (Carl Roth GmbH + Co. KG, DE) in TBS-Tween (0.05% Tween® 20; Carl Roth GmbH + Co. KG, DE). After incubation for 1 h at RT the membrane was washed with TBS for 2-3 min. The sensitized sheep serum, as also used in the bactericidal assays, was applied here as a primary antibody after 1:100 dilution in TBS and incubated overnight at 4 °C on a shaker. After washing in TBS-Tween three times for 10-15 min each, the blots were treated with 1:2000 diluted secondary antibody (rabbit

anti-sheep IgG, conjugated to horseradish peroxidase; DakoCytomation, Denmark) in TBS. The membranes were incubated for 1-1.5 h at RT and washed again three times in TBS for 10-15 min each. To prepare the color development solution 20 ml of TBS were mixed with 20 µl of H_2O_2 and 4 ml of 4-CN stock solution. The 4-CN stock consists of 3 mg of HRP Color Development Reagent, DAB (BioRad, USA) per ml of methanol (Honeywell, USA). The horseradish-peroxidase (HRP) catalyzes H_2O_2 and 4-CN to H_2O and benzo-4-chloro cyclohexadienone, leading to the appearance of darkened spots on the membrane where the antibodies were bound. The membrane was incubated in this solution for 15-40 min at RT on a gentle shaker until the desired grade of coloring was reached. The membrane was rinsed in deionized water 2-3 times or left in water for 10-15 min to intensify the staining and was subsequently dried and imaged with ChemiDoc MP (Biorad, USA).

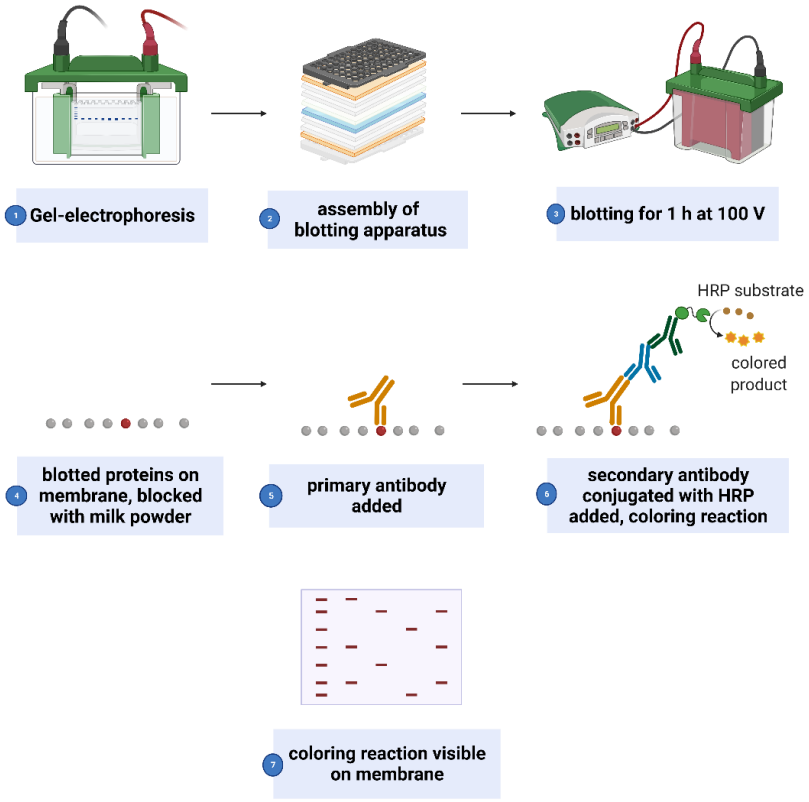

Figure 8: Blotting and color reaction

After gel electrophoresis, the blotting apparatus was assembled as visible in Figure 7. The blotting proceeds for 1 h at 100 V. The membrane was blocked with 3 % milk powder for 1 h and the membrane was treated with the primary antibody and a secondary antibody which is conjugated with horseradish peroxidase. After the addition of the color development solution (4-CN stock with H_2O_2), the bands where the primary antibodies had bound were visible as purplish black bands on the membrane. Figure created with BioRender.com

2.3.4 Analysis of GM139 Vpma

To identify the Vpma protein expressed in GM139, an SDS-PAGE was run as described above. The gel was cut in half. One half was stained with Coomassie and the other half blotted according to the protocol. The primary antibody was a polyclonal antibody α-VpmaV, raised in rabbits, used in a 1:100 dilution and the secondary antibody was a polyclonal swine anti-rabbit Immunoglobulins/HRP (DakoCytomation, Denmark) in a dilution of 1:2000. Afterwards, different gel bands corresponding to the bands recognized in the blot were cut out of the gel and prepared for an in-gel digest of the proteins.

The band was cut into small cubes. 100 µl H_2O were added and removed after vortexing. After this, the bands were washed and destained. 100 µl of aqueous ammonium bicarbonate (ABC; 100 mM; Fluka Analytical, USA) were added, followed by ultrasonication at RT for 5 min and removal of the supernatant. Then, 100 µl ABC and ethanol, absolute, >= 99.8 % HPLC grade (Thermo Fisher Scientific, USA) in equal amounts were added to the gel cubes (followed by ultrasonication, removal). This was repeated until the blue color of the Coomassie staining had disappeared. After further washing with H_2O, ABC and acetonitrile hypergrade for LC/MS (Merck, DE), the gel cubes were dehydrated in the vacuum concentrator (Eppendorf, DE) for 10 min at RT. For the reduction of disulphide bonds, the dried gel cubes were rehydrated in 50 µl of DL-dithiothreitol (DTT, 10 mM; Sigma-Aldrich, USA) and incubated at 56 °C for 1 h at 550 rpm on the thermomixer (Eppendorf, DE). After DTT removal, iodoacetamide (55 mM; Sigma-Aldrich, USA) was added for alkylation for 45 min at RT in the dark[61]. The sample was washed with ABC twice and once with acetonitrile, each step followed by ultrasonication and removal of solution. After drying for 10 min in the vacuum concentrator, the proteins were digested. Two different types of enzymatic digest were applied: Either Trypsin Gold: trypsin working solution composed of 120 µl of H_2O, 120 µl of ABC and 10 µl aqueous $CaCl_2$ (120 mM; Sigma-Aldrich, USA) added to 5 µl of trypsin stock (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI) or Trypsin/Lys-C Mix (Mass Spectrometry Grade, Promega, Madison, WI). The dried gel cubes were rehydrated in an appropriate amount of working solution in order to be covered with liquid. After a rehydration time of 20 min at 4 °C in both cases, the supernatant was discarded and 30 µl ABC (50 mM, pH 8.5) were added to cover the gel cubes. Next, the proteins were digested for 8 h at 37 °C on the thermomixer (550 rpm) and cooled to 4 °C[62]. For the peptide extraction, the supernatant was transferred to a new tube and 30 µl of acetonitrile:H_2O:trifluoroacetic acid Optima LC/MS (Thermo Fisher Scientific, USA) (50:45:5

v/v) were added, after 10 min in the ultrasonic bath, the supernatant was collected in the same peptide collection tube. This step was repeated two times. The sample was vacuum concentrated for 2.5 h at 45 °C and peptides dissolved in 8 µl of 0.1 % trifluoroacetic acid before analysis.

The following steps were performed by the staff at VetCore Facility (Proteomics) of the University of Veterinary Medicine. A nano- HPLC Ultimate 3000 RSLC system (Dionex) was used for high-performance liquid chromatography. The sample was desalted and pre-concentrated with a 5 mm Acclaim PepMap µ-Precolumn (300 µm inner diameter, 5 µm particle size and 100 Å pore size; Dionex). The solution was loaded with 2 % acetonitrile in ultra pure H_2O (Water, Optima LC/MS; Thermo Fisher Scientific, USA) with 0.05 % trifluoroacetic acid as a mobile phase at a flow rate of 5 µl/min. The separation was performed on a 25 cm Acclaim PepMap C18 column (75 µm inner diameter, 2 µm particle size and 100 Å pore size) with a flow rate of 300 nl/min. For elution from the column a gradient of 4 % solution B (80 % acetonitrile with 0.08 % formic acid Optima LC/MS (Thermo Fisher Scientific, USA)) was used for the first 7 min, which increased from 4 % to 31 % in the following 30 min and then to 44 % in the last 5 min. Solution A consisted of ultra pure H_2O with 0.1 % formic acid. Lastly, the column was washed with 95 % solution B. The HPLC system was coupled to a high-resolution Q Exactive HF Orbitrap mass spectrometer via a heated nano-electrospray ion source. The mass spectrometric data acquisition was done in the mass range of m/z of 350-2000 Da, with a resolution of 60000, a maximum injection time of 50 ms and the automatic gain control at 3×10^6. The ten most intense ions were further fragmented by Orbitrap via high energy collision dissociation activation over an m/z range of 200-2000, with a resolution of 15000 (intensity threshold at 4×10^3). Ions charged +1, +7, +8 and greater than +8 were omitted. The collision energy was set to 28. The automatic gain control was set to 5×10^4 for each scan and the maximum injection time to 50 ms. To inhibit repeated peak fragmentation the precursor ion masses were dynamically excluded over a time range of 30 s. The resulting spectra were analyzed with Proteome Discoverer Software 2.4.0.305 (Thermo Fisher Scientific, USA) and compared to the UniProt database and to the gene sequence of GM139 (after transformation to an amino acid sequence via BLAST, kindly provided by Dr.med.vet. Priv.-Doz. Joachim Spergser) to identify the expressed Vpma. Furthermore, a database with common contaminants was included (https://www.thegpm.org/crap/).

3. Results

3.1 Bactericidal Assays

3.1.1 Sheep serum

3.1.1.1 Sensitized sheep serum

After treatment with the *M. agalactiae* sensitized sheep serum, the strains and PLMs displayed differential survival. The use of the T_0 sample to take into account different CFU/ml counts at the start of the experiments did not lead to any prominent change in the results as already demonstrated in previous studies[53], and was therefore only performed once in the beginning to confirm this (data not shown). For the GM139 strain, there was no significant difference between the CFU/ml counts of the complement (C) and heat-inactivated (HI) serum treated cultures, whereas PG2 type strain showed a significant difference with a p-value of 0.006. Treatment with sensitized sheep serum led to a significantly higher killing of the PG2 strain compared to the GM139 strain (p-value <0.0001) as demonstrated in Figure 9. PG2 exhibited about 50 % survival in contrast to 95 % of GM139.

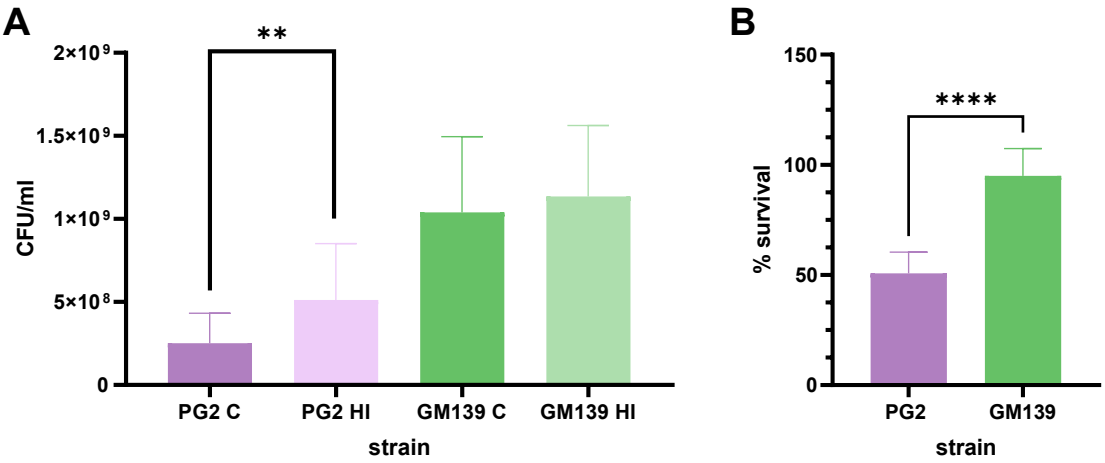

Figure 9: CFU/ml (A) and percentage survival (B) of PG2 and GM139 strains treated with heat-inactivated (HI) and complement intact (C) *M. agalactiae*- sensitized sheep serum obtained at Day 17 p.i. (**p < 0.01, ****p <0.0001).

The treatment of the PLMs with heat-inactivated (HI) and complement active (C) sensitized sheep serum led to significant differences in the CFU/ml of PLMU, PLMV, PLMX and PLMZ.

For PLMW and PLMY no significant killing could observed (Figure 10). PLMW grew to a significantly higher CFU/ml than all the other PLMs when cultured on agar plates.

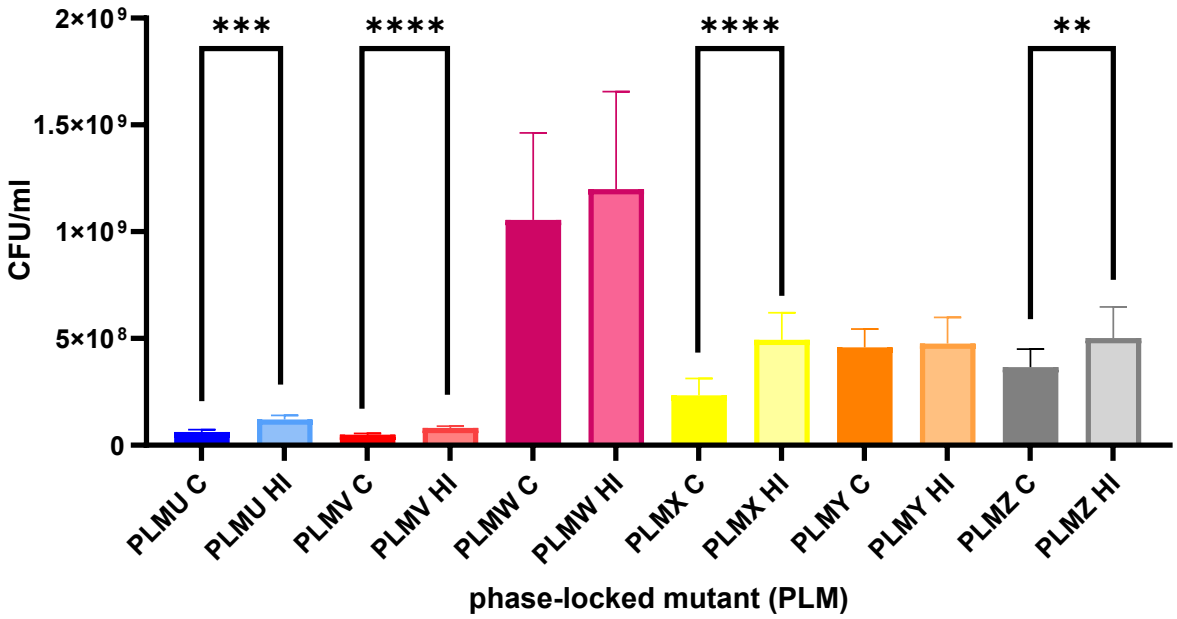

Figure 10: CFU/ml of PLMs treated with heat-inactivated (HI) and complement intact (C) *M. agalactiae*- sensitized sheep serum obtained at Day 17 p.i.

Significant differences were observed for PLMV and PLMX (****p <0.0001), PLMU (***p <0.001), and PLMZ (**p <0.01). For PLMW and PLMY no significant differences were observed pointing towards insignificant serum-killing.

For the different PLMs, the survival averages were as follows: PLMU with a survival of 52.5 %, PLMV 65.8 %, PLMW 87.3 %, PLMX 46.7 %, PLMY 97.1 %, PLMZ 72.6 %. Through a two-tailed ANOVA, differences in the mean survival between the PLMs was assessed. The difference between these is significant for almost all PLMs except for PLMU vs. PLMX, PLMV vs. PLMZ and PLMW vs. PLMZ. As visible in Figure 11, PLMU and PLMX were the most susceptible to serum and PLMY was the least affected.

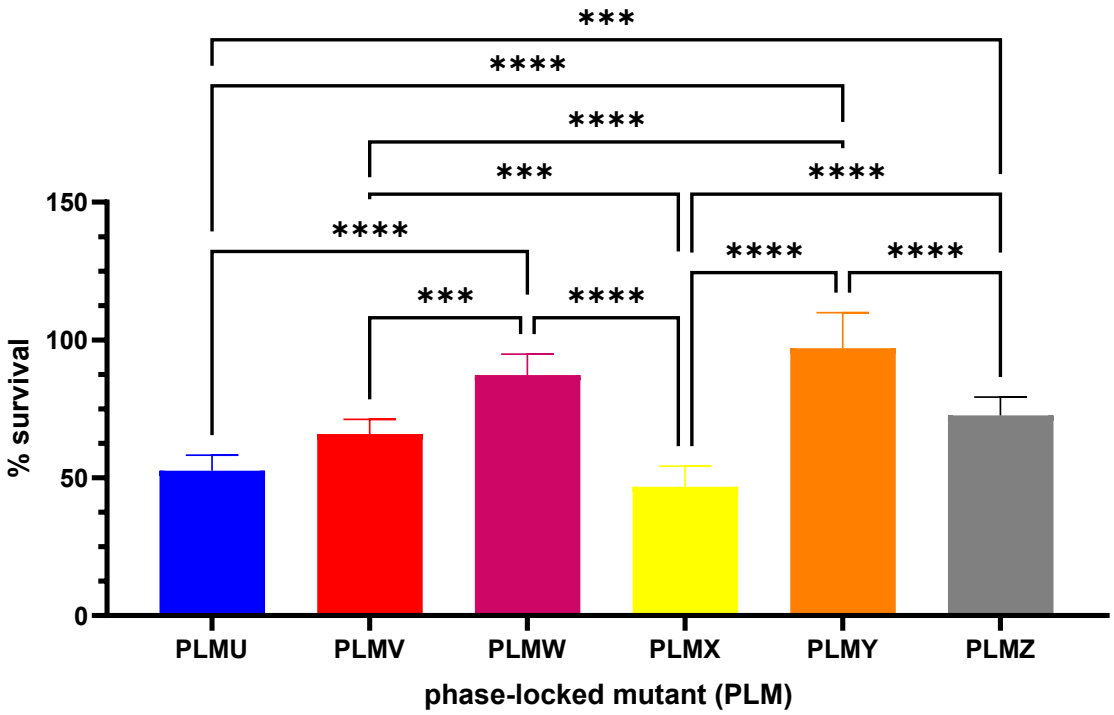

Figure 11: Percentage survival of PLMs treated with *M. agalactiae*- sensitized sheep serum obtained at Day 17 p.i.

The six PLMs varied in their rates of survival with the sensitized sheep serum, with PLMU and PLMX being the least resistant and PLMY the most.

To summarize, PG2, PLMU and PLMX were the most susceptible showing about 50% killing, whereas GM139 and PLMY were the most resistant (Table 2).

Table 2: Mean percentage survival of strains and mutants treated with sheep serum D+17

	PG2	**GM139**	**PLMU**	**PLMV**	**PLMW**	**PLMX**	**PLMY**	**PLMZ**
% survival	50.2 %	94.9 %	52.5 %	65.9 %	87.3 %	46.7 %	97.1 %	72.6 %

For PG2 and GM139 treated with sensitized sheep serum the spot-plating assay was performed as described by Schieck et al. 2016[59]. Contrary to the results visible in this paper, this method was rather inconsistent and did not reveal any stark differences in the CFU

counts during our experimentation. Hence, the CFU were counted as with the normal method of plating on small plates to calculate percentage survival. The reason for this difference is, that in the referenced paper, the killing effect was extremely strong over a few log-phases, while there was only 2-fold killing at the most in the experiments with PG2 and GM139.

3.1.1.2 Comparison of unsensitized and sensitized sheep serum

In previous experiments from the lab, serum obtained 4 days before infection (D-4) from the same sheep No. 1 (from whom the currently tested D+17 sensitized serum was obtained) was tested in bactericidal assays on some strains and PLMs using the same methodology as for the sensitized serum. For PG2 and PLMU the susceptibility was significantly higher in sensitized serum, while GM139 and PLMY did not differ significantly between the pre-immune serum and the sensitized serum (Figure 12).

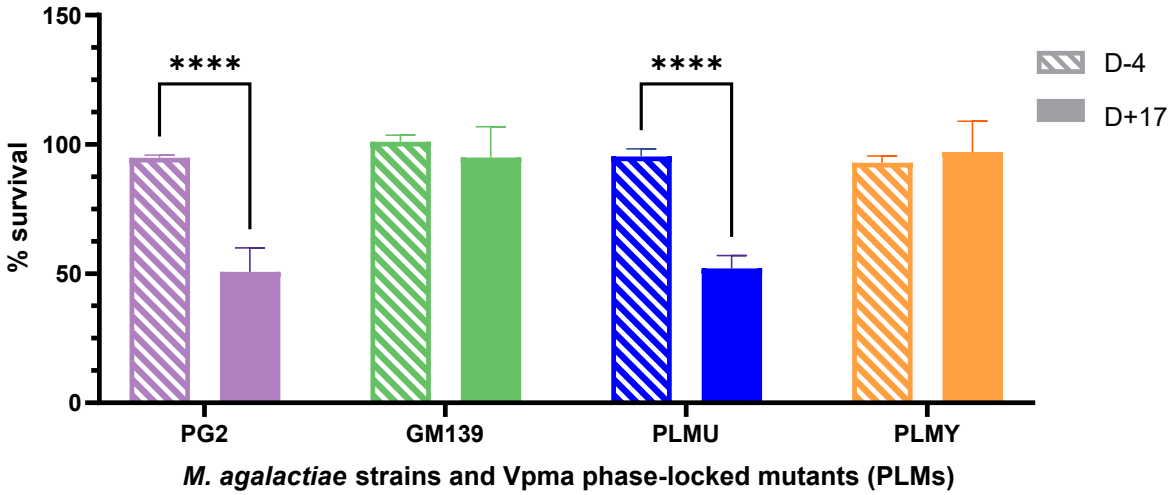

Figure 12: Percentage survival of PG2, GM139, PLMU and PLMY treated with non-sensitized (D-4) and *M. agalactiae*-sensitized (D+17) sheep serum.

PG2 and PLMU showed significantly lower survival (****p <0.0001) in the sensitized serum as compared to non-sensitized (D-4) serum.

3.1.2 Guinea pig serum complement

After treatment with guinea pig serum complement, the CFU/ml of neither PG2 nor GM139 differed significantly compared to the corresponding heat inactivated (HI) serum controls (Figure 13 A). PG2 had a mean survival of 92 % and GM139 had a mean survival of 110 %. Although these two strains demonstrate a significantly different survival rate relative to each other ($p < 0.0001$), there is no significant complement-killing observed for both.

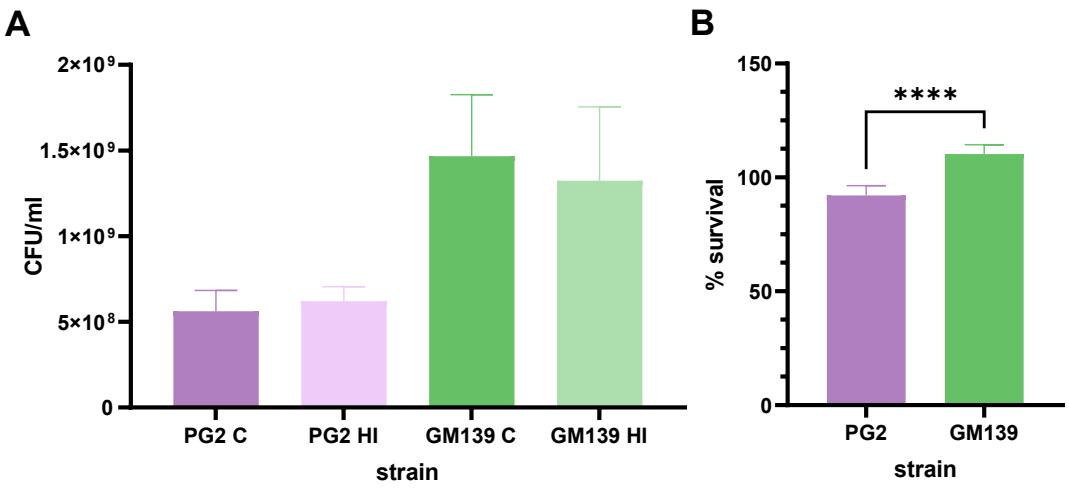

Figure 13: CFU/ml (A) and percentage survival of (B) *M. agalactiae* strains PG2 and GM139 treated with heat-inactivated (HI) and complement intact (C) guinea pig serum complement

For the *M. agalactiae* PLMs, treatment with guinea pig complement (C) and corresponding heat-inactivated (HI) controls, did not lead to any significant differences in the two sets of CFU/ml except for PLMX (Figure 14). When treating the PLMs with guinea pig complement, the mean survival was somehow greater than 100 % for all strains except for PLMX. PLMX displayed only 50 % survival, which is significantly less compared to all other PLMs (Figure 15).

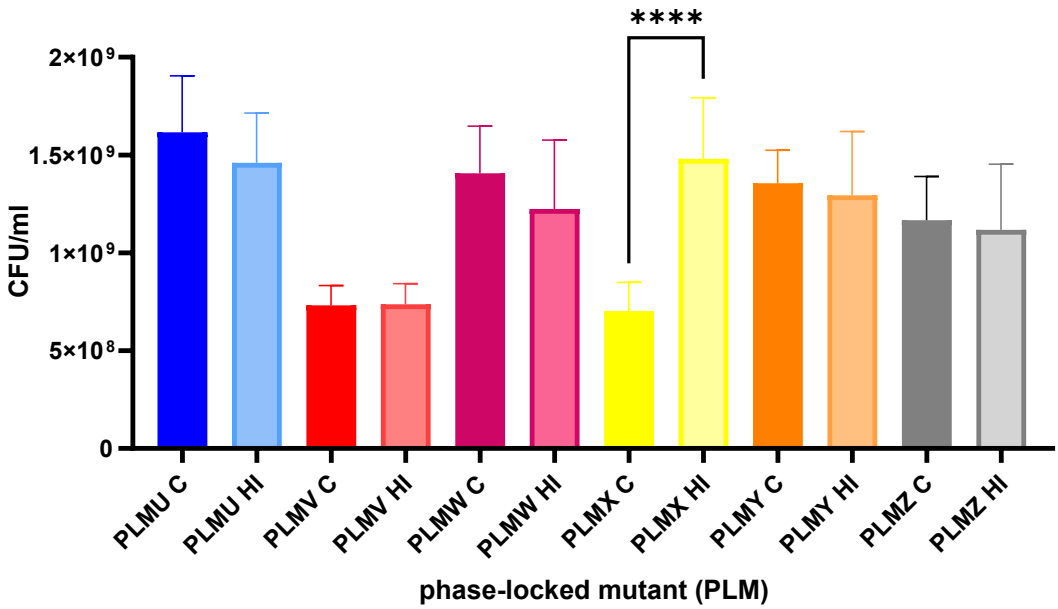

Figure 14: CFU/ml of PLMs treated with heat-inactivated (HI) and complement intact (C) guinea pig serum complement

The CFU/ml did not differ significantly for the cultures treated with active or inactivated complement for any PLM except for PLMX (****p <0.0001).

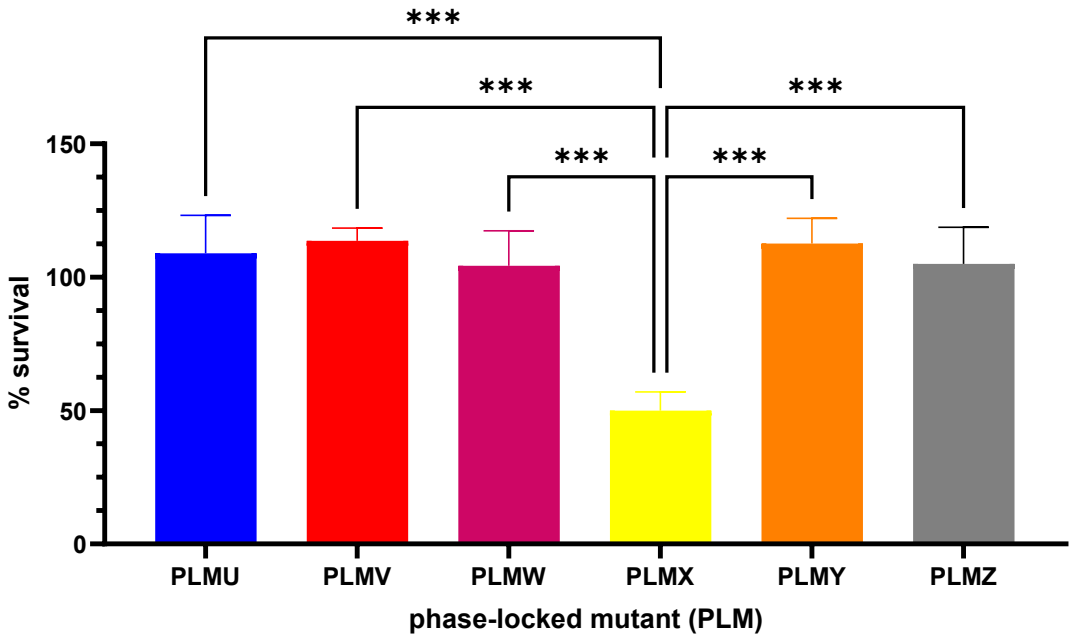

Figure 15: Percentage survival of *M. agalactiae* PLMs treated with guinea pig serum complement.

PLMX shows significantly lower survival compared to all other PLMs (***p <0.001).

For all the tested strains and mutants, only PLMX demonstrated significant killing in presence of guinea pig complement. Noticeably, all the other PLMs and GM139 exhibited more than a 100 % survival, meaning that the CFU/ml of the complement fraction exceeds the one of the heat-inactivated fraction.

Table 3: Mean percentage survival of strains and mutants treated with guinea pig serum complement

	PG2	GM139	PLMU	PLMV	PLMW	PLMX	PLMY	PLMZ
% survival	92.1 %	110.2 %	109 %	113.7 %	104.3 %	50 %	112.7 %	105 %

3.1.3 Fetal bovine serum

Since for both PG2 and GM139 the percentage survival was close to 100 % (data not shown) in the first preliminary experiments, the use of this serum was not pursued further in serum killing assays.

3.2 Western Blot with Sensitized Sheep Serum

To analyse the comparative reactivity of the sensitized sheep serum to GM139 and PG2 strains, triton and aqueous phase extracts of both strains were run on an SDS-PAGE, blotted onto a nitrocellulose membrane and probed with the D+17 sheep serum to check the antibody reactivity with the respective protein extracts. Firstly, a vast range of the proteins visible in the gel could also be seen in the western blot. This means that antibodies are present against many different PG2 and GM139 antigens (Figure 16). While only a few bands are visible in the aqueous phases (cytoplasmic proteins), as expected the membrane proteins (in the triton phase) are recognized extensively. This is especially true for the PG2 strain, where several bands, including the ones at around 43 kDa and 34 kDa, are extremely strong. These likely correspond with the abundantly expressed Vpma proteins that usually run at similar positions in SDS-PAGE, which is a little higher than their actual molecular weight[47]. For the GM139 strain, most proteins are comparatively less strongly recognized except for one band at around 25 kDa.

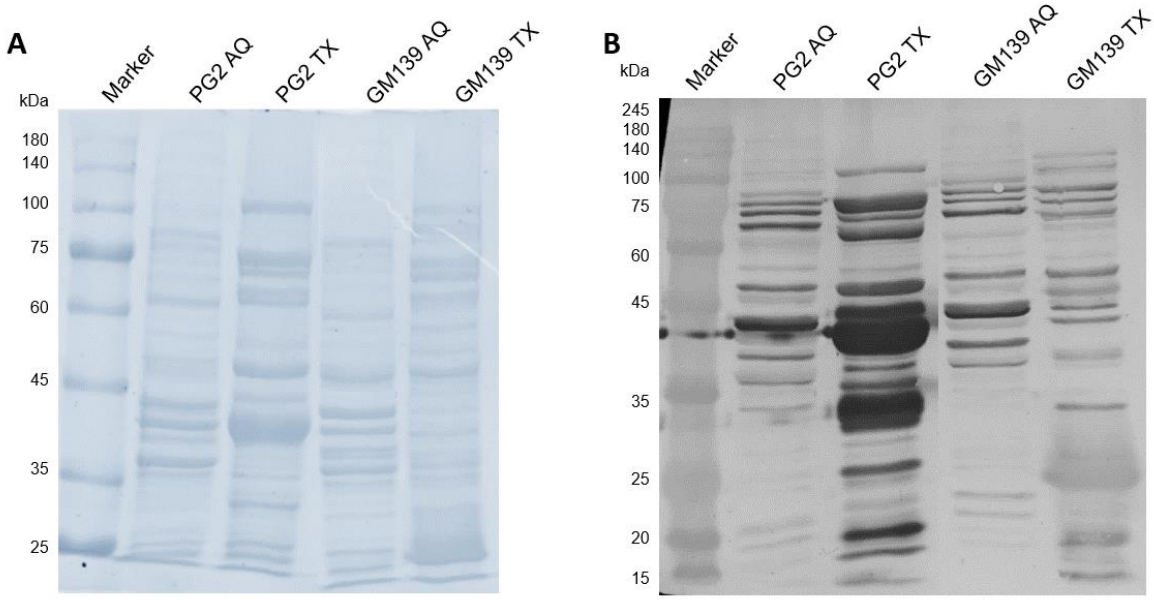

Figure 16: SDS-PAGE (A) and western blot analysis (B) of triton (TX) and aqueous (AQ) phases of *M. agalactiae* strains PG2 and GM139 treated with *M. agalactiae* PG2-sensitized sheep serum (D+17).

3.3 Mass Spectrometry Analysis of GM139 Vpma

In a previous study GM139 was shown to express only VpmaV (on colony-immunoblots and western blots) and did not recognize any of the other five Vpma–specific antibodies[52]. However, GM139 genome sequence analysis by Priv.-Doz. Dr.med.vet. Joachim Spergser demonstrated the absence of the typical *vpmaV* gene of PG2 type strain. In order to identify the Vpma protein being expressed in GM139, western blotting of the PG2 and GM139 triton phases was carried out with α-VpmaV polyclonal antibodies. This revealed a strong band for PG2 at around 43 kDa and three different bands at 85 kDa, 43 kDa and 14 kDa for GM139, which also showed a strongly reacting running front. The Vpmas in PG2 have a theoretical size ranging from 23 kDa (VpmaU and VpmaX) to about 33-35 kDa (VpmaW, VpmaZ, VpmaY, VpmaV) and run about 5-10 kDa higher on SDS-PAGE, with VpmaV running at around 43 kDa and VpmaX at around 27 kDa[47]. The Coomassie stained gel band corresponding to the Western blot band of 43 kDa was excised from the GM139 blot and analysed by LC-MS. Protein digestion was performed with trypsin. However, after mass spectrometric analysis and database comparisons, a hint of a large 136 Da fragment was found, with 33 % sequence coverage to the sequence found directly next to the promotor (data not shown). This protein featured a lot of proline directly after lysine – where the

enzyme trypsin is rather unable to cut. Therefore, the lowest band, which ran at around 14 kDa, and the running front were digested again with a Trypsin/Lys-C Mix for a new analysis round and adjusted settings regarding enzyme specificity and missed cleavage sites were applied. Additionally, the database search for the peptides of the original 43 kDa fragment was repeated.

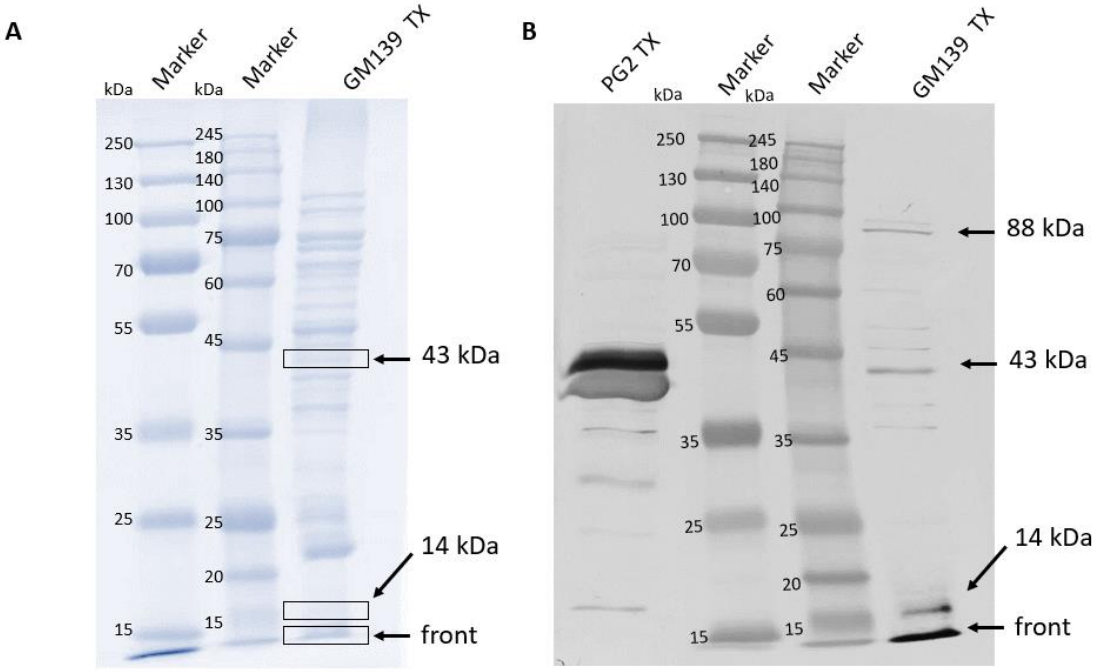

Figure 17: SDS-PAGE (A) and western blot (B) of triton phase (TX) of *M. agalactiae* strains PG2 and GM139 treated with α-VpmaV antibody

After treatment of the PG2 and GM139 triton phase with polyclonal α-VpmaV antibodies, a strong band was visible for PG2 and three different strong bands for GM139 at around 85 kDa, 43 kDa and 14 kDa. In addition, there seems to be a lot of protein in the running front.

The chromatogram of the 43 kDa band was unusual with a lot of hills that are out of order and usually appear due to the presence of polymers in the sample. For the other gel bands, at 14 kDa and the running front, digested with Trypsin/Lys-C, the chromatograms appeared normal, with distinct peaks (Figure 18).

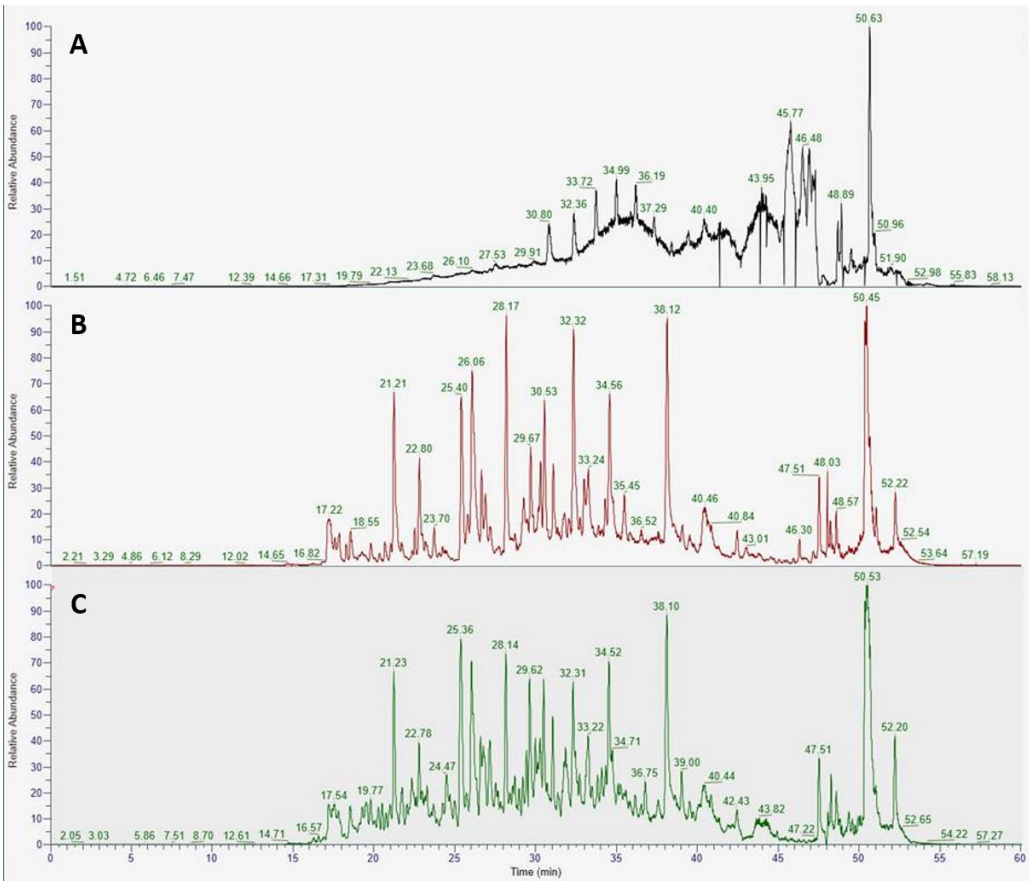

Figure 18: Chromatograms of GM139 43 kDa (A), 14 kDa (B) and running front (C) protein samples.

While the chromatograms of the 14 kDa and running front protein samples appear normal, with distinct peaks, the 43 kDa features a lot of unusual hills.

After analysis of the database search results, various proteins could be identified in the different bands. In the front 181 proteins with more than one peptide, in the 14 kDa band 137 proteins and 99 proteins in the 43 kDa band were found. Filtered for the terms “vpma”, “avg” or “variable” up to eight protein IDs were left in each band (see Table 4-6). The putative proteins obtained from sequencing are signified by *fig|6666666* at the start of the description, while the other proteins were derived from the Uniprot database. Interestingly, the 745vpmaON protein, which corresponds to the gene sequence found directly next to the promoter in the sequenced GM139 strain, was present in all bands. The Sequest HT score sums the scores of individual peptides. Thus, the higher the Sequest HT score, the better the identification of the protein.

Table 4: Proteins identified in the 43 kDa gel band of GM139 strain after database search (filtered list)

Description	Score Sequest HT	Coverage [%]	MW [kDa]	# of Peptides
fig|6666666.901569.peg.754vpma	443.98	67.5	43	31
fig|6666666.901569.peg.745vpmaON	43.48	72.4	14.2	11
VpmaY OS=Mycoplasma agalactiae OX=2110 GN=vpmaY PE=4 SV=1	37.52	44.2	37.5	11
Variable surface lipoprotein OS=Mycoplasma agalactiae OX=2110 GN=E5287_02600 PE=4 SV=1	35.87	31.6	36.2	9

Table 5: Proteins identified in the 14 kDa gel band after database search (filtered list)

Description	Score Sequest HT	Coverage [%]	MW [kDa]	# of Peptides
fig|6666666.901569.peg.754vpma	15.47	19.0	43	7
fig|6666666.901569.peg.755vpmaG	11.51	30.4	34.4	5
fig|6666666.901569.peg.745vpmaON	8.66	47.8	14.2	4
AvgA OS=Mycoplasma agalactiae OX=2110 GN=avgA PE=4 SV=1	5.83	22.6	24.6	3
Variable surface lipoprotein B OS=Mycoplasma agalactiae OX=2110 GN=avgB PE=4 SV=1	4.82	17.5	26.5	3
Variable surface lipoprotein B (VpmaB) OS=Mycoplasma agalactiae OX=2110 GN=vpmaB PE=4 SV=1	4.02	17.8	26.1	3
fig|6666666.901569.peg.744vpmaL	2.77	6.5	33.8	2
fig|6666666.901569.peg.756vpma	0	10.5	23.6	2

Table 6: Proteins identified in the running front gel band after database search (filtered list)

Description	Score Sequest HT	Coverage [%]	MW [kDa]	# of Peptides
fig|6666666.901569.peg.745vpmaON	10.96	18.7	14.2	5
Variable surface lipoprotein B OS=Mycoplasma agalactiae OX=2110 GN=avgB PE=4 SV=1	8.66	23.2	26.5	7
fig|6666666.901569.peg.754vpma	5.77	5.1	43	2
fig|6666666.901569.peg.755vpmaG	5.73	30.4	34.4	3
fig|6666666.901569.peg.744vpmaL	2.86	6.5	33.8	1
AvgA OS=Mycoplasma agalactiae OX=2110 GN=avgA PE=4 SV=1	0	19.0	24.6	3

In the 43 kDa gel band, the 754vpma protein with 43 kDa had a high coverage of 67.5 %. Nevertheless, the 745vpmaOn protein, with a size of 14.2 kDa was found with an even higher coverage of 72.4 %, but lower number of identified peptides as well as peptide spectrum matches (peptide spectrum matches not shown). Additionally, VpmaY seems to be present.

In the 14 kDa band, different proteins from sequencing were found, the 754vpma, 755vpmaG and 745vpmaOn with the highest Sequest HT scores. Here, once again, 745vpmaOn had the highest coverage, but it is also the smallest protein with only 14.2 kDa. The agalactiae variable gene A (AvgA) protein, of the *M. agalactiae* strain 627, which is closely related to VpmaX of PG2, VpmaB, of the strain 5632 and AvgB of the strain 627 were also present, however, with less sequence coverage. The AvgB protein also features a lot of similarity to VpmaY, while parts of VpmaB can also be found in VpmaW of the strain PG2[42].

In the running front, the 755vpmaG revealed the highest coverage of the sample proteins. Moreover, peptides of 745vpmaOn, 755vpmaG, 744vpmaL, AvgB and AvgA were detected.

4. Discussion

To spread systemically and to colonize tissue, resistance to complement and evasion of the cellular and humoral immune system of the blood is necessary[39,57].

Neither PG2 nor GM139 were susceptible to pre-immune serum and guinea pig complement, which means that complement and non-sensitized immune factors likely do not suffice to elicit a significant bactericidal response. In the PG2-sensitized serum, however, PG2 showed only about 50 % survival, meaning that antibodies or other factors only present in sensitized serum likely play an essential role in the serum-killing of the PG2 strain. For *M. bovis,* complement killing was strongest in the presence of antibodies against surface proteins which activates the classical complement pathway and promotes phagocytosis and C3b deposition[36]. For *E. coli* too, only sensitized serum led to killing[63]. As can be seen in the blot treated with D+17 sheep serum, there is extensive reactivity for the PG2-bands at expected Vpma sizes and also for GM139 bands, though fewer and fainter than PG2. This revealed that *M. agalactiae*-specific antibodies are present in the sensitized sheep serum and highlights the immunogenicity of Vpmas. Apparently, there is stronger and more frequent binding to proteins in the PG2-triton-phase. This could be due to a higher immunogenicity of the PG2 proteins when compared to GM139, or simply because the serum was obtained from a PG2-sensitized sheep and therefore more PG2-specific antibodies are present in it.

In the case of the PLMs, PLMU and PLMX were the most susceptible in sensitized serum and PLMX was the only strain susceptible to guinea pig complement, which fits the hypothesis that isogenic variants expressing smaller Vpmas are more susceptible to serum killing, as also seen for *M. pulmonis* expressing shorter variants of Vsa lipoproteins[33]. PLMU however was not susceptible to pre-immune serum, which contrasts with this theory. Another possible explanation is that PLMU is only sensitive in the presence of antibodies. Depending on which Vpmas were expressed during the experimental infection with PG2, different proportions of antibodies against the Vpmas could be present in the D+17 serum, which could be an explanation for the differential susceptibility. All the longer Vpma PLMs (PLMV, PLMW, PLMY, PLMZ) were less sensitive to sheep serum, which could be either due to fewer antibodies present in the serum against these Vpma variants, or an increased resistance due to a better shielding of the important antigenic epitopes from antibodies and/or complement by longer Vpmas as compared to the shorter ones. PLMY had a higher survival rate, both in the sensitized and the non-sensitized serum, which is in line with

previous results where PLMY was dominant over PLMU in co-challenge infection studies in sheep via the intramammary and conjunctival routes, and in vitro adhesion assays[19,43,48]. Therefore, PLMY seems to be better equipped to withstand the host immune factors, including those in host serum, as compared to PLMU.

For the variable surface antigens (Vsa) of *M. pulmonis*, a strain closely related to *M. agalactiae,* strains producing Vsa proteins with more and longer tandem repeats are less susceptible to complement. While the complement cascade was activated both in strains with shorter and longer tandem repeats, the serum susceptibility differed, which indicates an inhibition of the MAC-formation conferring the complement resistance. Longer Vsa proteins prevented access of larger molecules to the mycoplasmal membrane. However, the size variation for Vsa is between 200 kDa to 32 kDa, a much bigger range as compared to the range of Vpmas (from 43 kDa to 25 kDa). Therefore, size variation might not be playing such an important role in differential PLM survival[33,34].

In previous experiments[53] with different sheep sera, GM139 was more resistant than PG2, while both were susceptible to the sensitized serum obtained 17 days after infection with PLMZ. PG2 exhibited similar survival with both sera at around 40 % while PLMU was resistant to both sera. PLMY displayed about 55 % survival for sensitized serum. So even though the PG2 results were rather similar to the current results, here the pre-immune serum caused stronger killing. This might be due to the presence of better or more active immune factors in this serum. The PLM survival in sensitized serum, with PLMU being more resistant than PLMY, was opposite for our tested serum, which might indicate that different antibodies were present here than in the case of the PG2-sensitized D+17 serum. Nevertheless, these experiments were not repeated often enough to be reliable (due to sudden complete lockdown due to Covid pandemic). Therefore, this could be a reason for discrepancies.

Additionally, the sheep serum had already been stored at -80 °C for a few years in all cases and had undergone multiple freeze-thaw cycles, which probably led to degradation or inactivation of the humoral and cellular components. Therefore, for optimal comparability of results, non-sensitized and sensitized sera should be obtained from several different individuals and used directly when still fresh.

Interestingly, PLMW grew to a significantly higher CFU/ml in both HI and C sheep serum as compared to all the other PLMs, whereas there were no stark differences in CFU/ml for the

cultures in guinea pig complement, where all PLMs reached a CFU/ml similar to the one of PLMW in sheep serum. As already mentioned, the sheep serum had already been stored at -80 °C for multiple years before this experiment. Therefore, the growth-promoting factors in the serum were probably not as pronounced in sheep serum as in the guinea pig serum. This means that either PLMW has a growth advantage over the other PLMs even in the presence of the older serum or that even the HI fractions of the other PLMs in sheep serum were somewhat active and decimated the PLM CFU count. In guinea pig serum complement, GM139 and all PLMs except for PLMX showed a percentage survival greater than 100 %. Therefore, the CFU in the C-sample was higher in number than for the HI-part. The reason for this could be growth-promoting factors present in the serum which were inactivated during heat-inactivation.

An additional variable in such experiments is the heat-inactivation of serum, because complement is not the only protein component inactivated in this process. Other antimicrobial factors like phospholipases, lysozyme and amidases, which aid in bacterial killing are inactivated as well. This means that several other elements, besides complement, affect killing even in unsensitized serum[29]. Therefore, specific complement inhibitors are necessary to determine the role of complement-killing in unsensitized serum. This could for example be a C3 or C5 inhibitor or C1s inhibitor to impede the activation of the antibody-dependent complement cascade. This would offer the opportunity to discriminate between antibody-dependent complement killing and complement killing through other activation pathways.

For future experiments, the biofilm-forming ability of strains with different Vpma-profiles could be tested. In the case of the Vsa proteins, shorter proteins around the size of the Vpmas formed biofilms more readily, which then once again conferred protection from complement. Because there is negligible size variation in Vpmas, biofilm formation is probably very similar for all Vpmas. Interestingly, *M. pulmonis* strains expressing longer Vsa proteins and a certain polysaccharide on their surface were also able to form biofilms and thus be protected from complement[13,34]. Given these results, comparing the biofilm-formation of the different strains and mutants and how this impairs complement susceptibility would be especially interesting to consider when doing infectious trials in the future.

By LC-MS analyses a mixture of different Vpmas was found in the GM139 strain. While some proteins of the GM139 sequencing database were found, high sequence coverages of proteins from other *M. agalactiae* strains like 5632 and 627 were also detected. The

sequence of the protein 745vpmaOn was observed to be directly next to the promoter and therefore most likely to be expressed. Consistent with this, the 745vpmaOn protein was found to be present in all three analysed bands. Interestingly, even though many different Vpmas and Avg proteins were detected, the PG2 VpmaV protein did not appear in the sequence coverage analyses of any of the bands, even though GM139 reacted exclusively with the polyclonal α-VpmaV antibodies during blotting[52]. Nevertheless, this changed Vpma profile, in comparison to PG2, could explain differences in the serum susceptibility since the shielding against complement could be altered. Moreover, the different amino-acid sequence of the expressed Vpma could promote immune evasion because antibodies already present in the host or different receptors are unable to recognize the altered epitopes.

5. Conclusion and Outlook

Strains with different Vpma profiles exhibit different serum and complement susceptibilities, with PG2 being less resistant than GM139 in all cases. PLMX, the mutant expressing the smallest Vpma, was the only one sensitive to guinea pig complement. Both in previous experiments[53] and in the current study it became obvious that strains are more susceptible to sensitized serum. Therefore, antibodies likely play a significant role in serum-killing of *M. agalactiae*. This is emphasized since there was almost no bactericidal effect of the guinea pig serum complement. In summary, the PLMs with the smallest Vpmas (PLMU and PLMX) and PG2 were the most susceptible. Moreover, the increased fitness of PLMY in comparison to PLMU was once again proven, with PLMY being resistant to sensitized serum to a much greater extent. When cultured in the presence of sheep serum, PLMW grew to significantly higher CFU counts than all other PLMs, meaning this strain likely has an *in vitro* growth advantage over the others.

The GM139 strain, although reactive with the α-VpmaV rabbit polyclonal antibodies, was found to have a quite different Vpma-profile than the type strain PG2, with distinct Vpmas, also bearing epitopes similar to the *M. agalactiae* strain 5632 and strain 627. This is a possible reason for differential pathogenicity and serum resistance as compared to the type strain PG2.

In future experiments, using fresh serum from different sheep would provide a better reflection of the *in vivo* serum-killing. Additionally, more studies need to be performed to discriminate between the role of complement versus other factors like antibodies in serum. For this, C3 inhibitors would allow evaluating how much of the killing is due to the complement system, while C1s inhibitors would allow the specific inhibition of the antibody-activated classical complement pathway. Furthermore, it would be interesting to compare the strains' biofilm-forming-ability to explain different survival *in vivo* and *in vitro* and to assess the killing of bacteria in a biofilm.

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