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Identification of human monoclonal antibodies targeting lipoteichoic acid of *S. aureus* and whole cell *E. faecalis*

Dissertation

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Für meinen Vater

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Zusammenfassung

In dieser Arbeit wurden monoklonale Antikörper, die sich gegen Lipoteichonsäure (LTA) auf *Enterococcus faecalis* richten, auf ihre funktionellen Eigenschaften und die zugrunde liegende genetische Struktur untersucht. Da bei diesem Erreger vermehrt Antibiotika-Resistenzen auftreten, werden dringend alternative Behandlungsoptionen wie zum Beispiel eine Antikörpertherapie benötigt. Infektionen durch Enterokokken treten besonders bei immungeschwächten Patienten auf, beispielsweise bei älteren Menschen und Personen mit malignen Erkrankungen oder nach Transplantationen. In unseren Experimenten nutzten wir den Bakterienstamm *E. faecalis* 12030, dessen LTA von Huebner *et al*. isoliert und charakterisiert worden war. Dieses Zellwandpolymer ist als Zielstruktur von opsonophagozytierenden Antikörpern bekannt. LTA-bindende Antikörper schützen Mäuse vor Infektionen mit *E. faecalis* und zeigen *in vitro* und *in vivo* Kreuzreaktivität gegen *Staphylococcus. aureus* und *Staphylococcus epidermidis.* Die Grundstruktur von LTA ist bei Gram-positiven Bakterien hoch konserviert. Um monoklonale Antikörper zu identifizieren, die sich gegen LTA richten und außerdem zur Opsonophagozytose fähig sind, verglichen wir Seren verschiedener menschlicher Blutspender und wählten das Serum mit der höchsten „Killing"-Rate von *E. faecalis* 12030 im opsonophagozytischen Assay (OPA) aus. B-Zellen dieses Spenders wurden isoliert und kultiviert. Die von ihnen produzierten Antikörper wurden mittels „enzyme-linked immunosorbent assay" (ELISA) hinsichtlich ihrer Immunreaktivität gegen LTA von *S. aureus* analysiert. Die Antikörper mit der höchsten Immunreaktivität wurden daraufhin in OPA getestet, um diejenigen mit der höchsten „Killing" Aktivität gegen *E. faecalis* 12030 zu identifizieren. Um annährend Monoklonalität (Oligoklonalität, 1-9 Klone) herzustellen, wurden die B-Zell-Suspensionen mehrfach in Subkulturen aufgeteilt. Nun wurde der B-Zell-Pool ausgewählt, dessen Antikörper die höchste Immunreaktivität im ELISA und effektives Killing im OPA zeigte. Die Analyse der cDNA dieses Pools ergab 25 mögliche Kombinationen von variablen leichten und schweren Ketten des Antikörpers mit der höchsten gemessenen Aktivität. Die cDNA der variablen leichten oder schweren Kette wurde in den Klonierungsvektor pFUSE, ein Plasmid, welches bereits die konstante Region humaner Antikörper trägt, inseriert. Schließlich wurden Chinese Hamster Ovary (CHO $DHRF^-$) Zellen und Human Embryonal Kidney (HEK-293T) Zellen transient mit dem Vektor transfiziert. Anschließend verglichen wir die

Antikörperproduktion in den zwei Säugerzellreihen. Aufgrund des konsistenten und hohen Ertrags bei HEK-293T Zellen wurden diese für die weitere Produktion verwendet. Die Aktivität der rekombinanten monoklonalen Antikörper wurde im ELISA separat gegen LTA und gegen die ganze Zelle *E. faecalis* 12030 getestet. Dabei zeigte sich bei einigen Antikörpern hohe Immunreaktivität, die teilweise gegenüber LTA und der ganzen Zelle *E. faecalis* 12030 unterschiedlich ausgeprägt war. Im OPA zeigte der Antikörper A9 eine hohe „Killing" Aktivität. Eine genaue Analyse des Reaktionsmusters der Antikörper und der beinhalteten Ketten identifizierte zwei schwere Ketten VHC19 und VHA38, die die stärksten Reaktionen hervorriefen.

Mit unseren Untersuchungen konnten wir spezifische variable Domänen nachweisen, die Bestandteil immunreaktiver Antikörper gegen *E. faecalis* 12030 sind. Sie scheinen darüber hinaus nicht nur gegen LTA aktiv zu sein, sondern auch gegen weitere Antigene dieses Erregers.

Summary

In this study, monoclonal antibodies targeting lipoteichoic acid (LTA) from *Enterococcus faecalis* were investigated for functional properties and their underlying genetic structure. Due to the frequently observed antibiotic resistance of this emerging nosocomial pathogen, alternative treatment options such as antibody therapy are urgently needed. Enterococci cause infections especially in immunocompromised patient populations including elderly patients, solid organ and bone marrow transplant recipients, and cancer patients. The targeted strain used in our experiments was *E. faecalis* 12030, whose lipoteichoic acid (LTA) had been isolated and characterized by Huebner *et al*.. This cell wall polymer LTA has been described as a target of opsonophagocytic antibodies. Antibodies targeting this molecule confer protection against *E. faecalis* infections to mice and are cross-reactive *in vivo* and *in vitro* against *Staphylococcus aureus* and *Staphylococcus epidermidis*. The backbone structure of LTA is conserved among Gram-positive bacteria. In order to identify monoclonal antibodies targeting LTA with opsonophagocytic killing activity against *E. faecalis* 12030, we compared the sera from several human blood donors and selected the serum eliciting the highest killing activity of *E. faecalis* 12030 in the opsonophagocytic assay (OPA). B-cells from this donor were isolated and cultivated. Antibodies produced by them were analyzed by enzyme-linked immunosorbent assay (ELISA) regarding their immunoreactivity towards LTA from *S. aureus*. Those with the highest immunoreactivity were subsequently tested by OPA to identify the antibodies with the highest killing activity against *E. faecalis* 12030. In order to approximate monoclonality (oligoclonality, in theoretical calculations 1-9 clones) the B-cell suspension was repeatedly split into subcultures. Now, the B-cell pool whose antibodies elicited the highest immunoreactivity in ELISA and effective Killing in OPA was selected. Analysis of its cDNA resulted in 25 potential combinations of variable light and heavy chains of the antibody with the highest observed activity. The cDNA of their variable light or heavy chain was inserted into pFUSE, a plasmid already containing the constant region. Then, Chinese Hamster Ovary (CHO DHRF$^-$) cells and Human Embryonal Kidney cells (HEK-293T) were transiently transfected with this vector and antibody production was compared in the two mammalian cell lines. In HEK-293T cells a high yield of antibodies was achieved consistently and therefore they were used in the following. Activity of the recombinant monoclonal antibodies was assessed by ELISA testing separately

against LTA and whole cell *E. faecalis* 12030. Here, we found high reactivity in several antibodies. However, there were small differences between immunoreactivity for LTA and the whole cell *E. faecalis* 12030. Antibody A9 showed high killing activity in OPA. Close analysis of the reaction pattern of antibodies and their constitutive heavy and light chains showed two heavy chains VHC19 and VHA38 present in the most reactive antibodies.

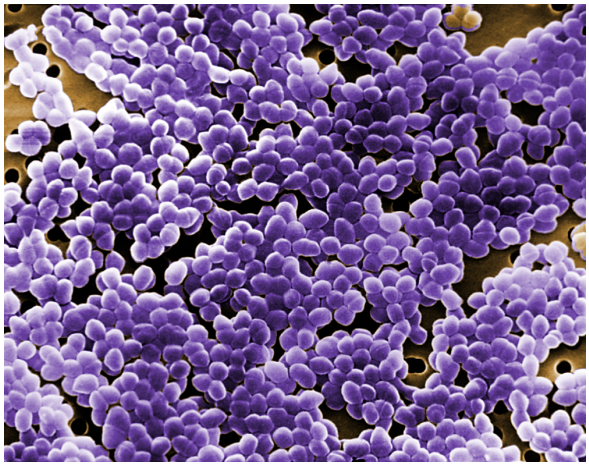
Thus, in our investigation, we were able to identify specific variable domains of antibodies immunoreactive against *E. faecalis* 12030. They appear to be directed not only against LTA but also against further antigens of this organism.

1. Introduction

1.1. Generalities of Enterococci

1.1.1. Classification and physiology

Enterococci were very first described by Thiercelin *et al.* in 1899. They introduced the name for a group of Gram-positive bacteria which were at that time commonly described as part of the genus streptococci [1] [2]. In 1984 the investigations of Schleifer and Kilpper-Bälz using DNA–DNA and DNA–RNA hybridization defined the genus of Enterococci. Their results confirmed that *Streptococcus faecalis* and *Streptococcus faecium* show only remote similarities with other strains of streptococci of serogroup D in contrast to the previous classification by Rebecca Lancefield [3] [4][5]. The non-spore-forming, facultative anaerobic Enterococci, which appear as single cocci, in pairs or short chains (see Fig.1.1.1a), do not show phenotypic differences from other Gram-positive, catalase-negative cocci. However, fermentation patterns, enzyme activities and other physiological characteristics can be used to distinguish them from other cocci [6]. Enterococci possess different properties enabling them to survive under rather harsh conditions and grow in aerobic conditions as well as in anaerobic atmospheres [7]. They tolerate a broad pH range (pH 4.6–9.9) and 6.5 % sodium chloride at 10° C. Moreover, they are able to survive heating at 60 °C for 30 minutes [6] [8].



1 Figure 1.1.1a Digitally colorized scanning electron microscopic image of *enterococci taken from the Centers for Disease Control and Prevention's (CDC) Public Health Image Library (PHIL).*

1.1.2. Occurrence of enterococci

In nature, enterococci occur in fruits and soil. They can also appear as commensals in the gastrointestinal and female genital tract of humans or other mammals, birds and many insects [9] [10]. Furthermore, enterococci which belong to the group of lactic acid bacteria are also an important ingredient as starter cultures in the preparation of cheese in Mediterranean countries [11]. Some Enterococci, like *E. faecium* and *E. faecalis,* have been used as probiotics – microorganisms prepared for a regular intake in order to stabilize the balance of the intestinal microbiome, thus contributing beneficially to human health [11][8][12]. For example, probiotics are reported to contribute to better control of rotavirus and *Clostridium difficile*-induced colitis, help the prevention of ulcers related to *Helicobacter pylori* and antagonize candidiasis and urinary tract infections and are also able to lower serum cholesterol [12]. Further studies have shown other positive effects like reduced severity of diarrhea in adults and children and shorter hospital stay particularly for children suffering from acute diarrhea [13] [14]. Some *E. faecalis* strains have been administered to humans, but they are more commonly used in animal food supplements [15]. However, the usage of Enterococci as probiotics is criticized since these bacteria may pose risks to human health and only few enterococcal strains are well characterized regarding safety for their use as probiotics, such as the *E. faecium* SF68 (NCIMB 10415 produced by Cerbios Pharma SA, Barbengo, Switzerland) and the *E. faecalis* Symbioflor 1 (produced by Symbiopharm, Herborn, Germany)[11]. For other preparations, it is still unknown whether the virulence traits as well as the mobile genetic elements of enterococci in particular might be transferred through conjugation to other bacteria colonizing the intestine [16] [11]. Especially immunocompromised and elderly patients would be exposed to the risks of growing antibiotic resistant bacteria that could trigger serious nosocomial infections [11].

1.1.3. Colonization of the GI tract in humans

In the human gastrointestinal (GI) tract enteroccci represent less than 1% of all commensal bacteria. Most of the other bacteria are anaerobic [17]. In order to maintain a balance in the human microbiota, the flora regulates itself through the so-called “colonization resistance” by several different mechanisms including secretion of bacteriocins [18]. Bacteriocins are small antimicrobial peptides used in different defense mechanisms to eliminate exogenous enterococcal competitors from the gut [19]. The majority of the bacteriocins identified so far and isolated from *E. faecalis* or *E. faecium* primarily target the cytoplasmatic cell wall [8]. If the

balance of the GI tract is disturbed – for example after antibiotic treatment – enterococci can turn from commensal bacteria into a serious threat for human health [18][20]. Colonization of the GI tract is the first step for these bacteria to turn into a pathogen in the human body [18]. For example, overgrowth of vancomycin-resistant enterococci (VRE) in the GI tract often precedes VRE bloodstream infection (Ubeda JCI). The lipopolysaccharide and flagellin of Gram-negative bacteria enhance the production of REGIIIγ. This is a C-type lectin having antimicrobial activity against Gram-positive bacteria and deprives growth of VRE by Paneth cells in mice. If the Gram-negative microbiota is reduced by antibiotics, the production of REGIIIγ and consequently the inhibition of VRE decreases [21] [22]. Different mechanisms enable Enterococci to survive the passage of the stomach where they are exposed to an acid environment. An H^+ -ATPase activated at lower pH levels may play a role [17]. Bacteria living in the GI tract regularly permeate the epithelial barrier to reach extra-intestinal organs such as lymph nodes, liver and spleen in a process known as translocation [23] [24]. Enterococci express various adherence factors in order to bind to the intestinal host cells and consequently translocate to the submucosa [24]. Normally, enterococci are phagocytosed by tissue macrophages and transported across the intestinal wall into the lymphatic system. However, some strains are able to survive within the phagocyte using them as a mean to translocate across the intestinal wall [20]. Furthermore, if the enterocytes' tight junctions are damaged after endotoxic or hemorrhagic shock or thermal injury enterococci can translocate directly through the epithelial cells and may reach the bloodstream [23].

1.1.4. Source and transmission of enterococcal infections

Enterococcal pathogenic potential was already noted in early research. Thiercelin isolated Enterococci from a patient with diarrhea and later septicemia caused by the transition of the bacteria from the intestine to the blood [1]. Patients, who are exposed to nosocomial pathogens, typically suffer from an altered gut flora or are immunosuppressed individuals. In those circumstances, an enterococcal infection often is believed to originate from the GIT [21]. Fecal contamination with antibiotic-resistant enterococcal strains in hospital settings and their spread via the hands of health care workers increase the risk of infection [19]. The capability of Enterococci to survive under harsh conditions also enables them to survive for long periods on surfaces. Therefore, appropriate hygiene practices have to be implemented to avoid nosocomial infections [21]. Moreover, antibiotic treatment of hospitalized patients has shown

to be associated with overgrowth of VRE in the GI tract prior to blood stream invasion [25]. Especially third-generation cephalosporins are likely to promote the growth of multiple resistant enterococci like VRE [19]. In immunocompetent hosts the natural gut flora limits the growth of Enterococci but when the balance of the gastrointestinal flora is disrupted, this regulation is disturbed. In debilitated patients with certain risk factors (e.g. antibiotic treatment, mucosa damage, shock or immunodeficiency) the consequence can be a systemic, life-threating infection [17][26][24][23].

1.1.5. Clinical importance

Before the introduction of third generation cephalosporins in the late 1970's, 90–95 % of clinical enterococcal infections were caused by *E. faecalis* [21]. *E. faecium,* which is more frequently resistant to vancomycin (VRE) and ampicillin than *E. faecalis,* has today almost caught up with *E. faecalis* as a cause of nosocomial infections [21]. Enterococci are the third most frequently isolated bacterial species in clinical practice and their prevalence was found to be rising [27]. A study conducted in Tasmania, Australia showed that the prevalence of new Vancomycin-resistant enterococcal strains has increased, especially since 2015, while the number of infections caused by *S. aureus* remained almost constant [28]. *E. faecalis* infections increased by 8,7% while *E. faecium* infections rose more than as fast, at 19,3% per year [29]. Surgical wound infections, endocarditis, endophthalmitis, hepatobiliary sepsis, urinary tract infections, bacteremia and neonatal sepsis are frequent forms of infection with these organisms. Almost half of all infections are community acquired [26]. More than 10% of infective endocarditis cases and catheter-associated urinary tract infections are caused by enterococci. Furthermore, hospital associated bacteremia caused by enterococci is ranked second place accounting for an average mortality of 33% [19].

1.2. Pathogenicity of Enterococci

Enterococci possess various virulence factors that enable them to spread systemically through adhesion to host cell surfaces and later evasion of the host immune response. [17]. During the actual infection, the host's body suffers tissue damages from toxins produced by the enterococci themselves or by inflammation processes [30][17]. In the following subsections various properties of Enterococci contributing to pathogenicity and respective virulence factors are outlined.

1.2.1. The enterococcal cell wall

The cell wall of Enterococci, like in other Gram-positive bacteria, contains multiple peptidoglycan layers, proteins, polysaccharides, lipids, lipoproteins, and glycopolymers [31] [32]. Glycopolymers are attached either to a peptidoglycan or inserted into membrane lipids. Teichoic acid (TA) and lipoteichoic acid (LTA) are the most common ones [32]. TAs consist of a glycosylated and D-alanine-substituted alditol phosphate repeating unit. Both of them are adherent to the peptidoglycan layers [31]. Cell-wall-glycopolymers can differ regarding the type of linked sugar, net charge or decoration of the repeating units. These characteristics can be strain- or species-specific. The charge of teichoic acids plays an important role in the affinity to antimicrobial peptides [32]. For instance, modification of the teichoic acids with positively charged d-alanine, such as naturally occurring for LTA of *E. faecalis*, contributes partially to resistance against cationic antimicrobial molecules [33].

1.2.2. Capsular polysaccharides of *E. faecalis* and serotyping

The first attempts to serotype *E. faecalis* were based on precipitin reactions and reciprocal absorption tests by Sharpe and later Maekawa *et al.*. However, this approach did not include defined antigen structures [34]. Later, Hufnagel et al. raised antisera against prototyped strains from Maekawa *et al.* and developed the Capsular Polysaccharide Serotyping System (CPS) analyzing immunoreactivity in ELISA and OPA [34] [35]. At that time the capsular polysaccharide lipoteichoic acid had been identified in *E. faecalis* and in a vancomycin-resistant *E. faecium*. Its chemical structure had been elucidated before [36]. Antibodies raised against this polysaccharide were demonstrated to opsonize, cross-react and provide protection from systemic enterococcal infection in a mouse model [37] [38]. Moreover, a gene locus *cps* that encodes the genes for the synthesis of a polysaccharide on the cell-wall surface of *E. faecalis* was described [39]. Restriction fragment length polymorphisms (RFLP) were used to determine how this *cps* locus and its genes were distributed among analyzed *E. faecalis* strains [35]. Four serotypes – CPS-A, -B, -C and -D – were postulated. From the investigated *E. faecalis* strains, 66% could be classified into one of these defined serotypes [35]. A cluster of 11 open reading frames determining the polysaccharide biosynthesis locus of *E. faecalis* type 2 capsular polysaccharides could be identified [40]. Two genes (*cpsA* and *cpsB)* were always observed in the RLFP pattern of the analyzed strains. This indicates that they are probably essential in *E. faecalis* [35] [41]. Only strains of serogroup CPS-C and CPS-D possess an additional eight to

nine genes. Those genes (from *cpsC* to *cpsK)* were shown to be important for capsule production suggesting that strains of CPS-A and CPS-B don't have a capsule [42] [35] [41]. These findings were confirmed by the observation that strains serotyped in CPS-A and CPS-B serovars were opsonized by antibodies raised against LTA from *E. faecalis* 12030, whereas CPS-C and CPS-D showed immunoreactivity in ELISA but were not opsonized by these antibodies [42]. However, serum raised against *E. faecalis* Type 2 strain opsonized strains of CPS-C and CPS-D. Theilacker *et al.* isolated this capsular polysaccharide named diheteroglycan (DHG) from *E. faecalis* strain Type 2 and elucidated its the chemical structure [43] [34] . Capsular polysaccharides of *E. faecalis* also play an important role in evasion of phagocytosis. One of them is the enterococcal polysaccharide antigen Epa [44]. The *epa* locus influences biofilm formation as well as enterocyte translocation, lower resistance to killing by polymorphonuclear neutrophils (PMNs), higher bacterial susceptibility to infection by phages and reduced virulence in mouse peritonitis and urinary tract infection [45] [32].

1.2.3. Lipoteichoic acid

Teichoic acids are found in most Gram-positive organisms including streptococci, staphylococci, enterococci and others [46]. There are two major types of teichoic acids: wall teichoic acids linked to peptidoglycans and LTA. LTAs are negatively charged polymers anchored in the cell membrane with a glycolipid anchor [46] [47].

When a capsular polysaccharide isolated from *E. faecalis* strain was compared with lipoteichoic acid (LTA) it was shown that it was structurally and immunologically identical to de-alanylated LTA to which opsonic antibodies against *E. faecalis* 12030 are mainly directed [37]. Antibodies directed against LTA from *E. faecalis* 12030 have been shown not only to bind to type I LTA of other Gram-positive bacteria but also to opsonize *S. aureus* and *S. epidermidis* [48]. This cross-reactivity in vitro by opsonophagocytic assays corresponds to *in vivo* protection through passive immunization against *E. faecalis* and *S. epidermidis* bacteremia in mice and reduced mortality in a mouse peritonitis model with *S. aureus* [48]. It has been suggested that these cross-reactive antibodies bind to the poly-1,3-(glycerolphosphate) backbone of the LTA molecule [48] [49]. It has also been demonstrated that LTA is part of the cell surface receptor interacting with aggregation substance (AS) [31]. Furthermore, decorations in the LTA structure may also contribute to the functional properties of this polysaccharide. For instance, D-alanylation of lipoteichoic acid effectuates several functions such as maintaining the cationic homeostasis and

modulating autolytic activities. An *E. faecalis* mutant lacking the *dlta* gene (encoding D-alanine esters in the LTA) produced less biofilm, was less adherent to eukaryotic cells and could better colonize the urothelium [50] [51]. Also, D-alanine esters in LTA provide bacterial cells with resistance to antimicrobial peptides and killing by neutrophils in opsonophagocytic assays [50].

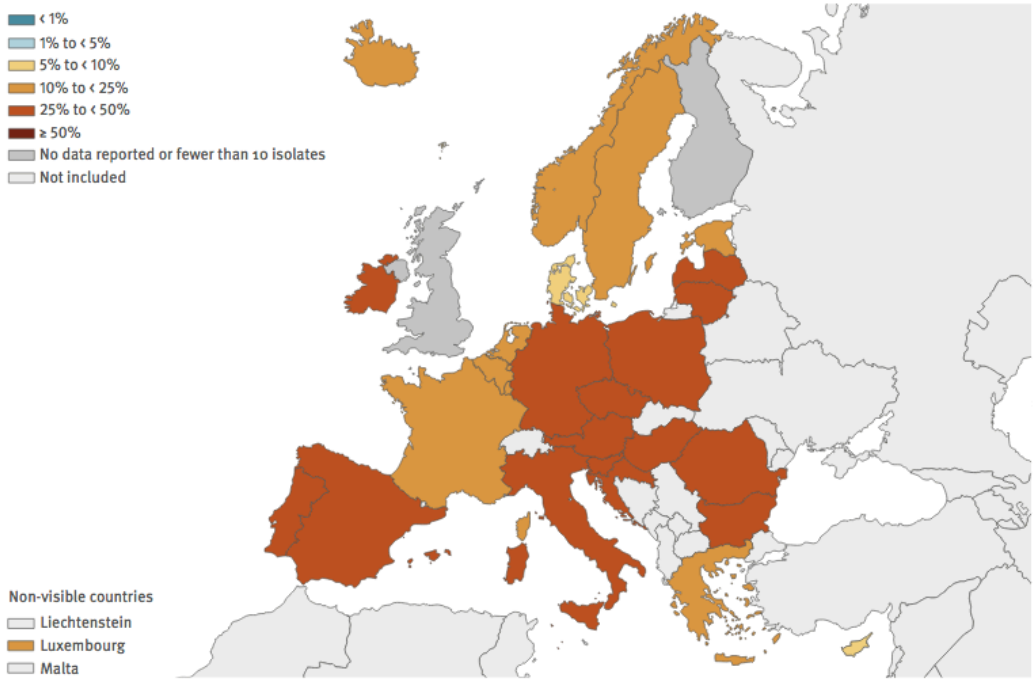
1.2.4. Diheteroglycan

The polysaccharide diheteroglycan (DHG), which is composed of glucose and galactose, was first described to be part of the cell wall of the then so-called *S. faecalis* (now *E. faecalis*) [52]. Later investigations showed that DHG occurs on the surface of *E. faecalis* strains of serotypes C and D. Additionally, it was observed that DHG is targeted by opsonic antibodies that mediate killing of these strains. Mice infected with *E. faecalis* of serotype C and D strains showed reduced bacteremia after immunization with serum raised against DHG. On the contrary, antibodies raised against serogroup A and B strains – targeting lipoteichoic acid – showed no mediation of opsonic killing in serogroups C and D [43]. Although these antibodies bound to LTA on the surface of *E. faecalis* groups C and D strains, they failed to mediate opsonic killing of these strains. This indicates that diheteroglycan may mask LTA in these serogroups [43] [42].

1.2.5. Antibiotic resistance

According to the EU Surveillance Report 2017 the majority of clinical enterococcal infections in humans are caused by *E. faecalis* and *E. faecium* [53]. Both of these strains harbor intrinsic and acquired resistances to antibiotic treatment [54]. Intrinsic resistance includes penicillins, cephalosporins, low levels of aminoglycosides or clindamycin. Acquired resistances range from chloramphenicol, erythromycin, high levels of clindamycin, tetracycline, high levels of aminoglycosides, penicillin, fluoroquinolones to vancomycin [21][54]. Mechanisms to acquire resistances include the exchange of genetic material via conjugative plasmids, conjugative transposons or by sporadic mutation of intrinsic genes [54]. Treatment therefore often involves a combination of penicillins and aminoglycosides or glycopeptides [53]. Still, even this combination therapy can be insufficient, since enterococci possess resistances against all these three classes of antibiotics. The expression of low-affinity Penicillin binding proteins (PBPs) leads to a low-level resistance to β-lactam antibiotics [21], [54]. PBPs (PBP4 in *E. faecalis*) are enzymes which link pentapeptide precursor molecules within the peptidoglycan cell wall. β-lactams are structural analogs of pentapeptide precursors and bind covalently to the cell wall

causing programmed cell death through reactive oxygen species [54] [21]. Additionally, enterococci can produce aminoglycoside-modifying enzymes changing amino- and hydroxyl-groups within the aminoglycoside molecule and thus decreasing the binding affinity between the antibiotic and the bacterial ribosome [21], [54]. *E. faecium* strains are intrinsically resistant to the synergistic treatment with aminoglycosides and penicillins when expressing an acetyltransferase. This makes treatments with tobramycin, kanamycin and dibekacin ineffective [54] [21]. Remarkably, there are no big differences, for example high-level gentamicin resistance occurs with similar frequency in northern and southern parts of the EU as figure 1.2.5a shows [53].



2 Figure 1.2.5a Percentage (%) of invasive isolates of E. faecalis with high-level resistance to gentamicin in the EU/EEA countries, 2017.

Data from the European Centre for Disease Prevention and Control [53] (page 58)

Today, resistance against VRE is of high relevance. In resistant enterococcal strains, the cell wall biosynthesis is reprogrammed using different peptidoglycan percursors like D-Ala-D-lactate, to which Glycopeptides usually bind (D-Lac producing high level resistance or D-Ala-D-Serine (D-Ser). This results in low-level resistance [54] [21]. Vancomycin resistance (by *Van* genes) is inducible and expressed only in the presence of extracellular glycopeptides. This occurs when signal transduction is mediated by a two-component system consisting of a sensor kinase (*VanS*) and a response regulator (*VanR*) [55]. Thereby, the high fitness cost during induced or

constitutive expression are reduced. Thus, vancomycin resistance spreads rapidly among many subspecies of enterococci [56].

1.2.6. Virulence factors promoting biofilm

Another virulent trait of enterococci is the formation of biofilm. In this structure, the bacteria transform from solitarily free-living unicellular organisms into a compound-structure living attached to the surface in organized groups [45]. The adhesion mechanisms allow communication with other bacteria, exchange of genetic material and attachment to host cells [45]. Enterococci also communicate through pheromones. A plasmid recipient *E. faecalis* cell secretes peptide pheromones recognized by *E. faecalis* cells harboring pheromone-responsive plasmids such as Aggregation Substance (AS) [6]. The AS protein enables enterococci to mate by binding to surface structures of other enterococci. Furthermore, the transfer of genetic material from the donor to a recipient cell(s) is possible in the aggregated bacterial cell clumps [57] [44]. Also, AS promotes adherence between enterococci and epithelial cells or renal tubular cells. Its expression has been shown to be associated with higher resistance to phagocytosis by inhibition of the respiratory burst, better surface attachment and enhanced biofilm formation *in vitro* [58] [45] [59]. Another factor associated with biofilm formation is the gene locus *Esp* contributing to *E. faecium* persistence within the host. Moreover, its gene product is the target of protective antibodies [32][60][44]. *Esp* has similarities with *Bap* (biofilm associated protein) which contributes to the formation of biofilms in *S. aureus* [17] [6]. Virulence factors, which are grouped in the complex of microbial surface components recognizing adhesive matrix molecules (MSCRAMM), contribute to the adhesion to extracellular matrix proteins [45]. So far, Ace (Adhesion of collagen from *E. faecalis*), Fss1 (*E. faecalis* surface protein), Fss2 as well as Fss3 all binding fibrinogen, have been found in *E. faecalis* and in *E. faecium* [61] [32]. *Ace,* is a collagen type I and IV laminin and dentin binding protein which recognizes adhesive matrix molecules playing a role in the pathogenesis of endocarditis [6] [62][63].

1.3. The host immune response

1.3.1. Phagocytosis

The host immune response to enterococcal infections requires efficient phagocytosis involving opsonic antibodies, the complement system, and granulocytes [64]. Generally, phagocytosis can be triggered in different ways. In the case of an enterococcal infection, monocytes and/or PMNs play a central role. These cells are activated either when their toll-like-Receptor 2 binds to a pathogen-associated-molecular-pattern (PAMP), such as lipoteichoid acid and peptidoglycan, or through complement or antibody mediated binding [65]. Phagocytes – including monocytes, macrophages, neutrophils, dendritic cells, osteoclasts, and eosinophils – display opsonic and not-opsonic receptors [66]. Many bacterial surface molecules can be recognized directly by phagocytes through not-opsonic receptors and consequently be destroyed. However, when bacteria express a capsule or other structures on their surface they can escape this immediate recognition. In this case encapsulated bacteria can be bound by opsonins, soluble particles like antibodies and complement factors [66]. Opsonic receptors of the phagocyte will then bind to those opsonins and trigger internalization of the pathogen into the phagocyte [66].

1.3.2. Innate immune response: The complement system

Among the different ways to neutralize bacteria, the complement system has an important role in the early stage of infection. It comes into play before B-lymphocytes could produce isotype-switched, specific antibodies [67]. The complement system involves between 35 and 40 (glyco-)proteins present in the blood plasma or on eukaryotic cell surfaces [68]. Their main function is to eliminate foreign particles through lysis or opsonization. The proteins are circulating as zymogenes or inactive forms. They are activated via three different pathways (classical, lectin and alternative pathway). Each can then be activated in the absence of antibodies but also be enhanced by antibody-antigen complexes [68]. These pathways lead eventually to the formation of the membrane-attack-complex (MAC) [68]. MAC formation then causes cell death by disrupting the proton gradient across the membrane [68] [69]. Some of the proteins are strong chemo-attractants involved in the recruitment of inflammatory cells such as neutrophils, eosinophils, monocytes, and T-lymphocytes [70].

1.3.3. Adaptive immune response: The role of immunoglobulins (antibodies) in phagocytosis

Pathogen-specific antibodies trigger the cascade of the classical complement activation pathway, stimulating opsonization. Hence, the Fc-fragment of IgG molecules (and IgAs) binds to the Fc-receptor on phagocytes, while IgMs lead to efficient complement activation [66] [67]. The Fc-receptors are crosslinked by multivalent antigen-antibody complexes and induce a signal cascade leading to phagocytosis [66]. Free immunoglobulin molecules show a very low affinity to Fc-receptors. Furthermore, only when bound to an antigen they form oligomers and bind with high affinity to phagocytes [67]. It was assumed that killing of *Enterococcus spp.* is mainly mediated through the complement system. This was demonstrated by a study comparing opsonophagocytic killing activity using sera from healthy adults, newborn infants and hypogammaglobulinemic serum [71]. Here, absorption of hypogammaglobulinemic serum with enterococcal cells inhibited opsonophagocytic killing. However, adding human immunoglobulins to the reaction led to opsonophagocytosis again. Therefore, antibodies along with complement appear to be required for opsonic killing of enterococci [72]. For encapsulated pathogens, a protective immune response is only achieved in the presence of all three components: specific antibodies directed against surface antigens (e.g. capsular polysaccharides), complement, and granulocytes [73] [74]. In enterococcal infections only few antigens have been identified to induce opsonic and protective antibodies. Antibodies targeting Ace, Esp and Gelatinase have shown no protection. AS can raise high antibody titers and an ABC transporter homologue was also found to be a potential target for antibody therapy as antibodies tested in a mouse model reduced colonization in kidney, liver and spleen [75]. Passive immunization with rabbit antibodies raised against different surface proteins reduced significantly the colony counts of an *E. faecium* strain in mice (i.e. SCP-like extracellular protein, low affinity penicillin-binding protein 5, basic membrane lipoprotein, peptidoglycan-binding protein LysM, D-alanyl-D-alanine carboxypeptidase and peptidyl-prolyl cis-trans isomerase) [76]. Antibodies against the purified secreted antigen SagA showed opsonic killing activity. In a mouse bacteraemia model, a significant reduction of the colony counts in blood was shown with rabbit immune serum [77]. Other important surface antigens also mediated opsonophagocytic killing, such as cell wall carbohydrate antigens like LTA and DHG [43] [37][78]. Moreover, vaccination with EbpA – an adhesin mediating attachment of bacteria to host fibrinogen – showed protection from catheter associated urinary tract infections in a

mouse model [79].

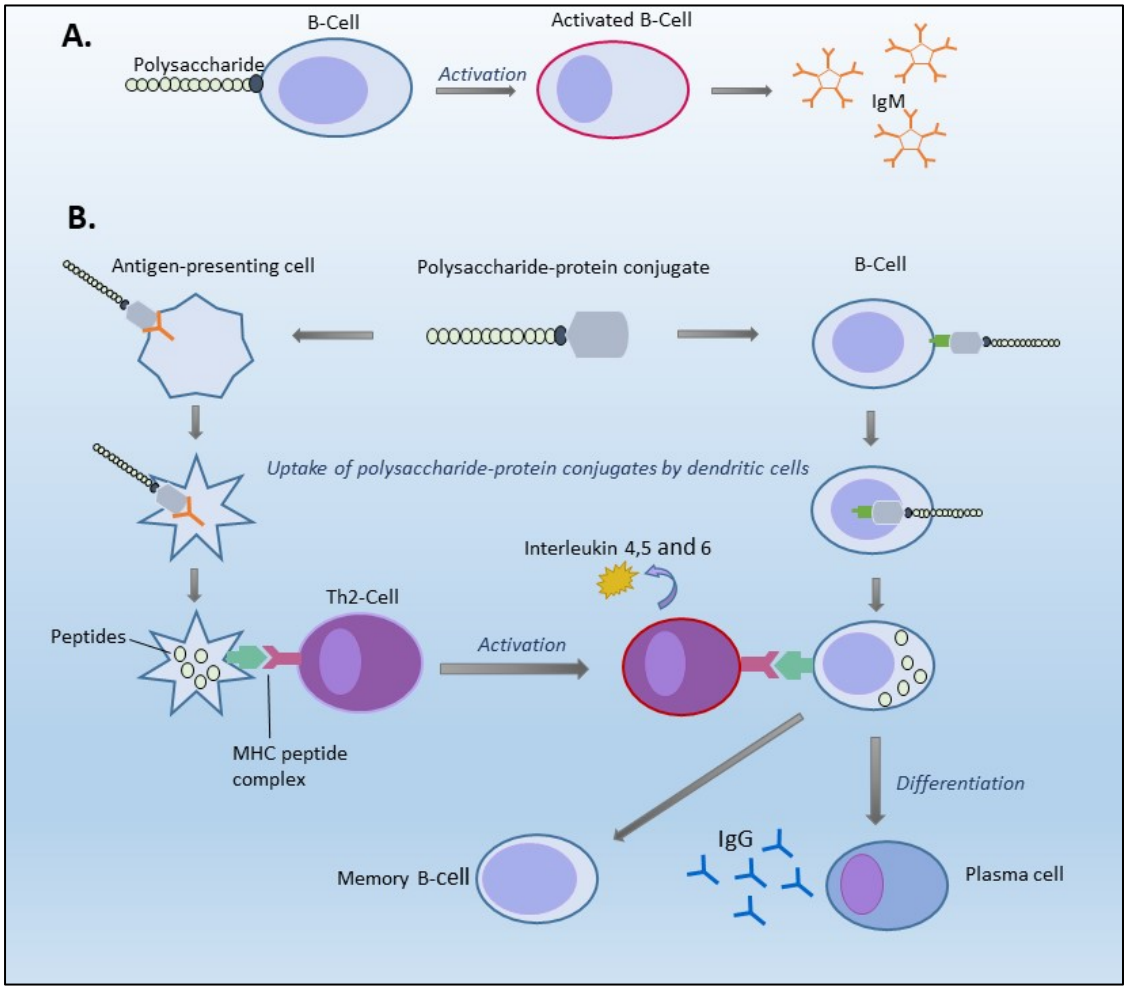
1.4. New treatment options for enterococcal infection

Different approaches to prevent or treat enterococcal infections are currently under research. Among them are antibodies, probiotics, phages, vaccines, antimicrobial and anti-biofilm peptides as well as other treatments [80]. However, as described in a review published in The Lancet in 2016 most of the alternatives under investigation are still in preclinical phase [80].

1.4.1. Vaccine therapy

Stimulation of the immune system and generation of antibodies display a broad variety of effector mechanisms to fight bacterial pathogens. This includes altered motility and metabolism of pathogens as well as effective humoral immune responses (see Fig. 1.4.4a) [81]. Various aspects play a role in the development of a vaccine, such as anti-bacterial immunogenicity, bacterial pathogenicity (for vaccine antigen selection) and the patient population to whom a vaccine would eventually be given [82] [83]. For full immunity a response of T- and B-cells is required as T-helper cells are needed for the generation of functional antibodies and Th17 cells support neutrophils' activity [83]. Since pathogens exhibit different virulence factors that are strain or species dependent, immunization with multiple antigens may lead to a higher level of protection than immunization with individual antigens [84]. Regarding the target group of patients, vaccination against enterococci could be administered to patients who suffer from defined risk factors like exposure to broad-spectrum antibiotics [82]. However, most patients with enterococcal infection suffer from a suppressed immune system which leads to a weak immune response to active immunization. Nevertheless, passive immunotherapy could be an option in this case [82]. There have been different approaches which are conducted to investigate vaccination against Gram-positive pathogens after the emergence of antibiotic resistance has increased in the last decades [83][76][82]. Since Staphylococci and Enterococci share some antigenic structures, vaccines targeting these structures from *S. aureus* also hold potential in fighting infections with this bacteria [85]. Still, so far there are no vaccines in clinical use for the treatment of Gram-positive pathogens such as *S. aureus* or enterococci [83] [86]. Different bacterial antigens have been proposed as vaccine candidates. Vaccine therapy against enterococci has been investigated especially concerning urinary tract infections, but in various animal models passive immunization protected from

bacteremia and reduced bacterial counts in endocarditis, sepsis and peritonitis models [87] [78][88] [38] [48] [89] [90] [82] [91]. Cell surface bacterial proteins and polysaccharides have been used as vaccine antigens often conjugated to a protein carrier [92] [93]. Vaccinations were tested in several trials studying "whole cell" vaccines, including whole bacteria (either live attenuated or inactivated) and bacterial lysates, and "specific-antigen" vaccines including one or more antigens (subunit, toxoid, or conjugate vaccines) [87]. Polysaccharides used in antibacterial vaccines include antigens from *H. influenzae*, diverse capsular polysaccharides from *S. pneumoniae,* and capsular polysaccharides from the various serotypes of *Neisseria meningitides,* usually conjugated to carrier proteins such as tetanus or diphtheria toxoid [94] [92] [93]. Polysaccharides can shield the bacterial surface to avoid complement deposition and activation as well as phagocyte activation [44]. Therefore, they are good target antigens for vaccine development since antibodies against these antigens could mediate immune responses such as activation of complement and/or phagocytes [95]. However, capsular polysaccharides are T-cell independent antigens inducing the production of antibodies without generating memory B-cells nor eliciting an isotype switch [94]. Instead, memory B-cells dedifferentiate to plasma cells and the pool of memory cells is diminished, lowering future immune response [94]. To trigger a T-cell dependent response, polysaccharides can be conjugated with a protein inducing the production of IgG antibodies [94][96]. Antigen-presenting cells (i.e. dendritic cells or B-cells) recognize proteins and polysaccharide-protein complex by type 2 helper T-cells (Th2 cells) [94], [96]. B-cells finally differentiate to memory and plasma cells and produce IgGs directed against the polysaccharide. This process is illustrated in Figure 1.4.1a. Thus, a long-lasting immune response is triggered making a vaccine a useful tool for immunization susceptible populations, such as infants and elderly people [96] [94].



3 Figure 1.4.1a Antibody Responses

A. B cells possess IgM receptors on their surface which bind to a polysaccharide antigen in lymphoid tissues. Activated B cells produce and then secrete IgM antibody molecules. **B.** Polysaccharide –protein conjugates are taken up by dendritic cells (B cells and other antigen-presenting cells). The peptides from the protein portion of the conjugate are presented to type 2 helper T (Th2) cells. B cells that have IgM receptors specific to the carbohydrate moiety bind the conjugates. Through endocytosis the conjugates are processed by the B cell. Class II MHC molecules on the surface of the B cell express the resulting peptides. The activated Th2 cells recognize this complex and consequently secrete interleukin-4, interleukin-5, and interleukin-6 and cause B cells to differentiate. B cells then express IgG molecules with polysaccharide specificity. They mature in lymphoid follicles and only cells expressing very-high-affinity IgG molecules become plasma cells. These antibodies bind strongly to encapsulated bacteria and mediate opsonic activity and complement-mediated bactericidal activities. Adapted from Ada [96].

In order to produce polysaccharide-based vaccines, the synthesis of oligosaccharides is an attractive alternative because purification of carbohydrates is complicated and often only yields small amounts of antigen. The produced synthetic antigens show a well-defined structure and reproducible physical, chemical and biological properties [92]. The synthetic oligosaccharide vaccine against *H. influenzae* type b was the first successful example of large-scale synthesis of a glycoconjugate [97]. Since then, several synthetic vaccine candidates against various bacteria like Shigella, *V. cholerae* and *S. pneumoniae* have been described [98]

[88], [99]. Laverde et al. conjugated a synthetic LTA fragment with Bovine Serum Albumin (BSA). They raised rabbit polyclonal antibodies which mediated opsonic killing of *E. faecalis 12030*, *E. faecium* and a community-acquired methicillin-resistant *S. aureus.* Prophylactic immunization with rabbit sera raised against these antigens led to a reduction of colony counts in blood/internal organs in animal models of enterococcal infections [88]. For enterococci, only a limited number of serotypes seem to exist, and therefore polysaccharides from the different serotypes seem to be good targets to develop a multi-component vaccine. Also, surface proteins of enterococci have been suggested potential vaccine candidates that could be used as carrier proteins to produce enterococcal glycoconjugate vaccines [85] [76].

1.4.2. Antibody therapy in the past and today

Long before the discovery of antibiotics revolutionized the treatment infectious diseases, Behring and Kitasato found immunization with serum to be effective in animals against infection with diphtheria [100]. This was the first passive immunization or preventive antibody therapy on record [100]. Later, serum therapy was used to treat several infectious disease outbreaks, such as the influenza pandemic in 1918 and an Ebola outbreak in 1976 [101]. However, this therapeutic approach had a limited use in the battle against infectious diseases. In contrast, in recent times monoclonal antibodies (mAbs) have become an important element in the treatment of non-communicable diseases such as cancer and autoimmune disorders [101]. Furthermore, in the last decades, the interest in antibody therapy as an alternative treatment to fight infectious diseases has tremendously increased [20][101]. Monoclonal antibody therapy offers safe use and consistent target-specificity. This therapy is considered mainly for three groups of patients: As treatment for infected individuals (passive immunotherapy), as prophylaxis to protect high-risk individuals (passive immunoprophylaxis), and as transmission prophylaxis in average-risk populations [101]. Modern techniques have enabled the identification of monoclonal antibodies by expressing the variable heavy and light chains in eukaryotic cells as well as their *in vitro* immunological evaluation [101]. The development of hybridoma technology where B-cells are fused with myeloma cells in order to produce monoclonal antibodies was awarded with the Nobel prize [102]. Immortalization of human B cells for antibody development provides the opportunity to isolate rare antibodies [17]. Later, antibody production in immortalized B-cells as well as recombinant DNA technology led to the development of the first clinically licensed recombinant mAbs for infectious diseases.

They were used in prevention of respiratory syncytial virus (RSV) in newborns [103]. Therapeutic antibodies are considered a low-risk approach with strong scientific basis, a history of safe use and a high degree of technical feasibility. According to a recent Lancet review on all alternative treatments to antibiotics, therapeutic antibodies seem very likely to be accepted and established successfully within the next decade [80].

1.4.3. Production of monoclonal antibodies

Different techniques have been developed to produce antibodies since the times of Behring and Kitasato. The "{…} ability of the cell-free blood fluid to render harmless the toxic substance which the tetanus bacillus produces" was later determined to rely on the presence of antibodies – a term which was coined by Paul Ehrlich in 1891 [100] (p.138) [104]. An antibody is composed of two heavy chains (VH) consisting of a joining (J), a diversity (D), and a constant (C) region and two light chains consisting of a variable (VL), a joining (J), and a constant (C) region that are both linked covalently with disulfide bonds [105]. Epitopes are recognized and bound by complementary determining regions (CDR3) [106]. The process of artificially constructing an antibody includes defining its characteristics like the isotype (class, subclass and light chain composition) [106].

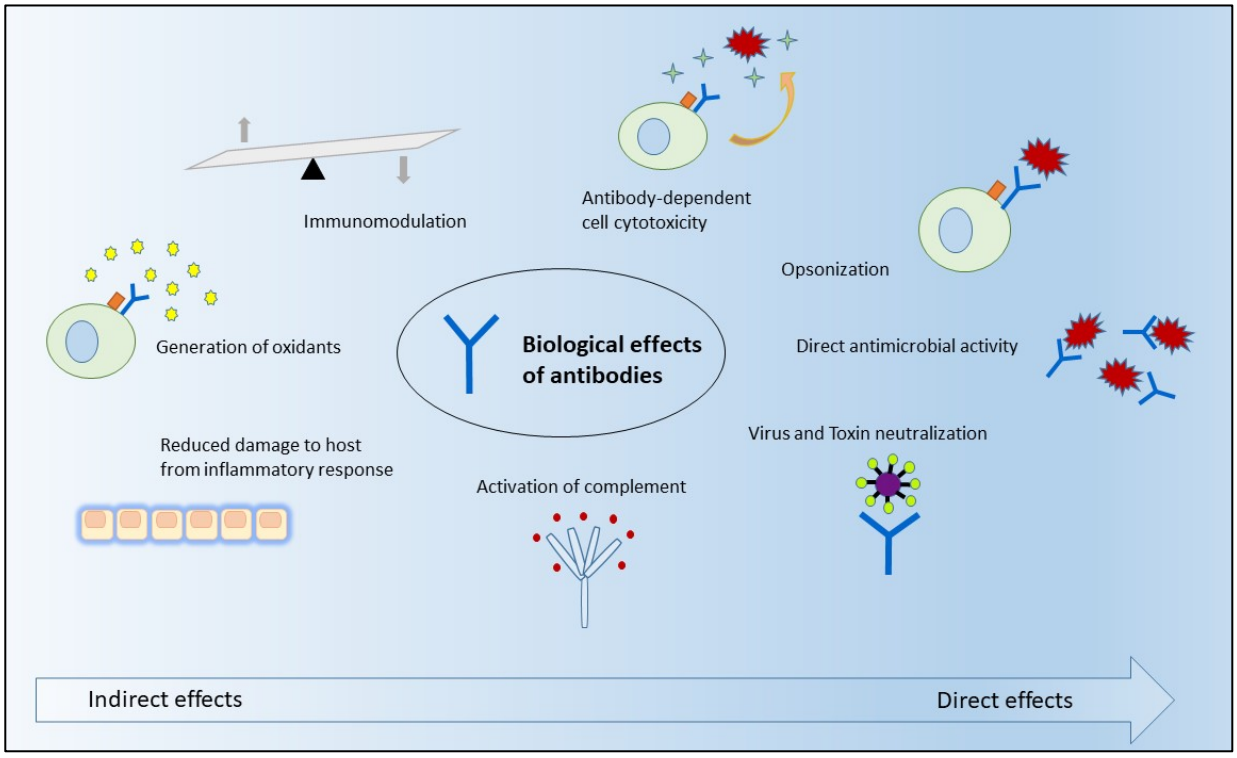
Immunological analysis encompasses the assessment of immunoreactivity, cross-reactivity, analysis of binding properties as well as characterization of the antigen and determination of effector cell function [106]. IgG production, correct folding and precise post-translational modifications require complex conditions such as an oxidizing environment for disulfide bonds [107].

Antibody engineering aims to optimize the biological properties of the effector domains of mAbs to improve clinical efficacy [106]. For instance, an antibody fragment, the single chain fragment variable (scFv) is formed when a variable light chain and a variable heavy chain are linked by a short peptide and stabilized, enabling the fragment to properly bind the antigen without loss of affinity compared to the whole antibody molecule. VL and VH are constructed and can be later modified. In the antigen binding fragment (Fab), VH and CH (variable and constant heavy chain) and VL and CL (variable and constant light chain) are combined [106]. Expression of these fragments is possible in phage display libraries, where bacteriophages are modified to express antibody fragments on their surface [108][106]. Usually, heavy chains and light chains are randomly paired to create a library of potential antibody combinations [109].

The expression of antibodies or antibody fragments is usually performed in prokaryotic, mammalian or myeloma cell lines, whereas phage display libraries are expressed in *E. coli* [107]. The latter holds several advantages for the investigated antibody fragments such as easier handling, faster culture procedures and the possibly higher yields of antibody protein [106]. *E. coli* is one of the most suitable organisms for the expression of antibody fragments as its genome has been thoroughly investigated [107]. However, in prokaryotic cells it is not possible to generate properly glycosylated antibodies. This may reduce immunoreactivity, increase immunogenicity and accelerate elimination [110][107]. For the large scale production of monoclonal antibodies, expression in transgenic plants or animals could play an important role [106]. Today, mammalian cell lines are still the major source (95%) of antibodies produced for therapeutic application. Here, folding, secretion and post-translational modifications are ensured [107]. CHO cells – possibly in a DHFR expression system – are most commonly used in commercial production [107][110]. For transient transfections used during screening of antibodies, production in HEK-293T is preferred. They are efficiently transfected with plasmid DNA [107].

1.4.4. Therapeutic application of antibodies

Monoclonal antibodies are today used in cancer, bone marrow and solid organ transplantation as well as in autoimmune, cardiovascular and infectious diseases [106]. The development of monoclonal antibodies to prevent enterococcal infections, is an appropriate approach for patients at high risk of infection (prolonged stay in the hospital, especially in intensive care unit)[21]. For this group of patients, active vaccination is not an attractive alternative. Especially for polysaccharide antigens successful induction of immunity in an immunocompromised patient is less likely and slow [92] [20]. Therefore, passive immunization with immunoglobulins would be the treatment of choice [20].



4 Figure 1.4.4a Different biological effects of antibodies.

Adapted from Casadevall *et al.* [103]

Monoclonal antibodies allow the induction of different effector functions either by directly interacting with the pathogen or through indirect effects (see Figure 1.4.4a). If first activated by antibodies, leucocytes kill infected host cells that present microbial antigens on their surface. This process is known as antibody-dependent cellular cytotoxicity, which involves antibody binding to the pathogen and to the Fc receptor of the effector cells. Effector leucocytes can be monocytes, neutrophils, and natural killer cells. [111] [112] [113]. By binding to the surface of the pathogen, antibodies can cause agglutination of bacteria thus reducing the amount of viable pathogens, inhibit the microbial motility and make bacteria more susceptible to phagocytosis [111]. Also, by targeting a transport molecule on the bacterial cell surface and thus preventing the uptake of nutrients antibodies can inhibit growth of pathogens [111]. The major clinical impact is expected from antibodies binding to the virulence factors or toxins of a pathogen like the toxins of diphtheria, tetanus, and anthrax all of which have been an important subject of research [80][113]. A recent study found a single intravenous dose of Bezlotoxumab (Merck, Darmstadt, Germany) effective against *C. difficile* toxin B. It was - given with standard-of-care antibiotics - protective against recurrent *C. difficile* infection and found to

be superior to antibiotic prophylaxis alone [114]. Antibody therapy against different antigens of *S. aureus* and *P. aeruginosa* is currently being investigated in phase 1 and 2 clinical trials [80]. A good example of passive immunization against viral pathogens is the application of RS-Virus antibody in the preventive care for premature infants and infants with bronchopulmonary dysplasia. This antibody has been used for more than a decade successfully [115]. Despite the advantages of antibody therapy its widespread use may lead to antibody resistance by changes in the antigen structure or protease production [111]. Antibody-resistant mutants can be selected *in vitro,* as it was observed for mAbs against *Borrelia burgdorferi* [111] [116].

1.4.5. Antibody therapy for enterococcal infections

In order to combat or prevent enterococcal infections, different target molecules have been studied. Passive immunization with anti-EbpA^{NTD} antisera binding the N-terminal domain of the EbpA pilus is protective in a murine catheter associated urinary tract infection model. This procedure was shown to be protective against different enterococcal clinical strains (i.e. *E. faecalis*, *E. faecium*, and VRE) [117]. Two therapeutic monoclonal antibodies have been published to be in preclinical trials: an enterococcal antibody produced by Nabi Biopharmaceuticals and a fully human mAb against Enterococcus targeting MSCRAMM proteins (Inhibitex/Dyxan) [95]. Rossmann et al. isolated two human monoclonal antibodies binding to polysaccharide structures, possibly LTA. Their mAbs showed strong opsonizing activity against enterococcal and staphylococcal strains, among these a multidrug-resistant *S. aureus* (MRSA) and *E. faecium* [78]. Protection against infection was also observed *in vivo* in a rat and a mouse model for infection with MRSA, *E. faecium* as well as *E. faecalis* 12030 [78]. Kalfopoulou et al. recently developed two mouse antibodies directed against DHG and the secreted antigen (SagA) with confirmed opsonophagocytic activity in vitro [118]. Yet another monoclonal antibody directed against LTA (Pagibaximab) was produced by Biosnyexus (Gaithersburg, MD) [119]. Pagibaximab is a human chimeric monoclonal antibody which was safe and well tolerated in phase 1 studies and effective against staphylococci preclinically [119]. However, a recent study did not show reduction of septicaemia in preterm infants, and no effective killing of enterococcal strains in vitro. This indicates that it may bind another epitope than the mAbs developed by Rossmann et al. [78]. In any case, in this investigation the administration of the mAb was safe in neonates [120]. To our knowledge, there are no mAbs used in clinical management of enterococcal infections currently.

2. Objective

Enterococci pose an increasing threat to patients by causing severe nosocomial infections which can be challenging or sometimes impossible to treat [21] [121]. Due to various resistance mechanisms, enterococci often do not respond to conventional treatment and thus it is of high clinical relevance to develop alternative treatment options and/or preventive measures [122] [54]. It has been previously shown that the cell-wall-associated polymer LTA, isolated from the *E. faecalis* 12030, is present on the surface of many enterococci and that antibodies targeting this molecule (anti-LTA) mediate *in vitro* phagocytosis and confer *in vivo* protection [43] [37]. In addition, anti-LTA have been proven to be cross-reactive against *E. faecalis*, *S. aureus* and *S. epidermidis,* demonstrating that LTA is a potential vaccine candidate [48][88]. Based on these findings, the present study aims to develop a pipeline which enables the identification and characterization of human monoclonal antibodies that target LTA and are able to mediate phagocytosis of *E. faecalis* 12030. More specifically, the following objectives are pursued:

- Immortalize B-cells from a healthy human donor for the identification and selection of cells that produce antibodies with the desired immunological properties.
- Identify the immortalized B-cells from a healthy human donor which produce antibodies that target LTA by ELISA using LTA from *S. aureus* (which has been shown to be cross-reactive with enterococcal LTA)
- Use the selected the B-cell pools to identify and characterize monoclonal antibodies with opsonophagocytic activity against *E. faecalis* 12030 by *in vitro* opsonophagocytosis
- Analyze the constant and variable chains of antibodies by producing the cDNA from the selected B-cell clone(s)
- Recombinantly produce the identified monoclonal antibodies by transfecting eukaryotic cell lines
- Evaluate the activity of the recombinant monoclonal antibodies by *in vitro* opsonophagocytosis and ELISA
- Study the impact of the different combinations of variable heavy and light chains on the immunoreactivity and production of the identified monoclonal antibodies targeting LTA

3. Material and Methods

3.1. Mammalian cell culture

3.1.1. Isolation of human B-cells and Infection with Epstein-Barr-Virus (EBV)

Immortalization of B-cells with EBV was performed as described by Tosato *et al.* [123] with the following modifications. In brief, 8mL of blood were drawn by venipuncture from a healthy donor and a volume of sterile Dubelco's phosphate buffered saline (PBS) was mixed up to 10mL. The Blood-PBS mixture was carefully added to 4 mL of Ficoll Plaque medium (GE Healthcare, Uppsala, Sweden) without disturbing the Ficoll phase. The tube was centrifuged at 2200rpm for 20 minutes (min) at 22°C and the cloud of peripheral blood mononuclear cells (PBMC) was aspirated and poured into a 15mL Falcon tube. Sterile PBS was added to fill the volume up to 15mL and once more the tube was centrifuged at 1500 rpm for 5 min at 22°C. Cells were washed with 10mL of sterile PBS and centrifuged again under the same conditions. Meanwhile, 1.5 mL of medium A (see Table 3.1.1a) was added to an equal volume of an EBV suspension (kindly provided by Prof. Christoph Klein, Dr. von Hauner Children Hospital, Ludwig-Maximilians-University, Munich, Germany). PBMCs were resuspended with the EBV/Medium mixture and incubated for 2hours at 37°C. Finally, cyclosporine A was added to a final concentration of 0,01% which functionally inactivates T-cells present in the sera of adult blood donors [124]. Cells were then incubated at 37°C with 5% CO_2 for one week. Periodically, medium A was added and the growth of cells was checked regularly during six weeks. After, EBV immortalized cells were aliquoted in three batches and frozen at -80°C.

1 Table 3.1.1a. Medium with antibiotics for immortalized B-cells.

Medium (A) with antibiotics for immortalized B-cells	Manufacturer
450mL RPMI 1640 Medium GlutaMAX™ Supplement	Gibco
10% Fetal Bovine Serum FBS	Gibco
50U/mL Penicillin-Streptomycin	Gibco
10mM HEPES	Gibco
1mM Sodium Pyruvate	Gibco
0.5µg/mL Plasmocin	Invivogen

2 Table 3.1.1b. Medium without antibiotics for immortalized B-cells.

Medium (B) without antibiotics for immortalized B-cells	Manufacturer
450mL RPMI + GlutaMax	Gibco
10% FBS	Gibco
10mM HEPES	Gibco
1mM Sodium Pyruvate	Gibco
0.5µg/mL Plasmocin	Invivogen

3.1.2. Freezing and thawing of B-cells

To assess the number of cells per mL, a 100µL aliquot of EBV immortalized B-cells was mixed with three volumes of Tryptan blue (Gibco) and counted in a Neubauer chamber with the following formula:

$$\frac{cells}{mL} = \frac{Counted\ cells\ in\ Neubauer\ Chamber}{4} \times Dilution\ Factor\ (4) \times 10^4$$

Before freezing, the cells were adjusted to a final concentration of $5x10^6$ cells/mL or more in FBS supplemented with 10% DMSO. Tubes were frozen at -80°C using a CoolCell freezing container (Biocision, USA). To thaw the cells an equal volume of pre-warmed medium at 37°C (see Table 3.1.1a) was added and mixed with the cells by pipetting in and out until thawing. The mixture was added to a 15mL falcon tube containing 10mL of warm medium and centrifuged at 1500rpm for 5min at 22°C. Afterwards, the cell pellet was resuspended in fresh medium and transferred either to a T25 flask containing 10mL of fresh medium or split into a 96 well plate containing 150µL/well of fresh medium and 50µL/well of cell suspension. Cells were incubated at 37°C for 5 days with 5% CO_2 and monitored for growth.

3.1.3. Splitting and Culturing of B-cells during immunological screening period

After thawing the EBV immortalized cells, cells were split in a 96 well plate using medium with antibiotics (Medium A). After immunological evaluation by ELISA and OPA of cells' supernatants (SPNs), cells in a specific well were counted and resuspended in the volume needed to split them either in 48 or 96 wells. To avoid a high dilution of cells in the well, 96 well plates were used in both cases. To this purpose, 200µL of cells' suspension were resuspended in 14.4mL or in 19mL to split the suspension into either 48 or 96 wells, respectively. Prior to OPA, the medium of the wells was exchanged at least twice for medium without antibiotics (Medium B

see Table 3.1.1b). For maintenance of split B-cells, the medium was changed every 2-3 days while cells were sitting in 48 or 96-well plates. Culturing of the B-cells was performed in the absence of stimulating factors.

3.1.4. Culture of CHO Cells

CHO-DHFR$^-$ cells were kindly provided by Prof. Lawrence Chasin (Fairchild Center, Columbia University, NY, USA) [78]. For culturing CHO-DHFR$^-$ we used Alpha Medium (Mod. MEM) with Nucleosides (Merck, Berlin, Germany) supplemented with Glutamine, FBS and Pen/Strep (αCHO-DHFR$^-$ Start medium see table 3.1.4a). For calibration of selection pressure of antibiotics Alpha Medium (Modified MEM) without Nucleosides by Merck (Berlin, Germany) was used as control medium. For thawing, cells were warmed at 37°C in a water bath, then centrifuged at 200xg for 5 min and resuspended in fresh media. Cells were washed once more with medium to remove remaining Dimethyl sulfoxide (DMSO). Later, cells were resuspended in 10mL of αCHO medium, poured into a T25 flask and incubated at 37°C with 5% CO_2. Cells were checked daily for growth. After cells reached confluency, the supernatant was removed and cells were washed twice with 10mL of 1xPBS before adding 1 mL of Trypsin solution (EDTA free) at 0.05% in PBS (Gibco). After 2.5 min of incubation at 37°C the flask was hit manually to detach cells. Complete trypsination of the cells was checked by microscopy. Then, 4mL of medium were added and cells resuspended by pipetting. The mixture was transferred to a 15mL falcon tube and centrifuged at 200xg for 5 min. A second wash of the cells was performed with 10mL of fresh medium. After removing the supernatant, cells were resuspended in 10mL of fresh medium and counted in a Neubauer chamber (100µL of cells suspension+ 300µL trypan blue). Then, cells were split in a T25 flasks by doing a 1:20 dilution of initial cell suspension in a final volume of 10mL. Cells were incubated for 3 days at 37°C with 5% CO_2.

3 Table 3.1.4a. Medium for CHO-DHFR$^-$ cells: αCHO Start-, αCHO Growth -, αCHO Transfection- medium.

	αCHO Start medium	**αCHO Growth medium**	**αCHO Transfection medium**
Alpha MEM Medium	to start CHO-DHFR- cells	To growth CHO-DHFR- cells	After transfection of CHO-DHFR- cells
Medium with Nucleosides	440mL	445mL	445mL
Glutamine	5mL	5mL	5mL
FBS	10%	50mL	50mL
Penicillin-Streptomycin	5mL		
Zeocin			300µg/mL
Blasticidin			7,5 µg/mL

After four to six passages CHO-DHFR$^-$ cells were subcultured before transfection in a T175 flask. First two passages were done using αCHO Start medium and from third passage cells αCHO Growth medium was used (see table 3.1.4a). For transfection, after trypsination cells were washed twice, counted and seeded at $3x10^5$cells/well in a six well plate and incubated at 37°C and 5%CO_2. The amount of antibiotics needed to select transfected CHO-DHFR$^-$ cells was standardized by growing cells in αCHO Growth medium with different concentrations of antibiotic (Zeocin and Blasticidin).

3.1.5. Culture of HEK-293T

HEK-293T cells (kindly provided by Prof. Christoph Klein, Dr. von Hauner Children Hospital, Ludwig-Maximilians-University, Munich, Germany) were cultured in DMEM Glutamax medium (Gibco) supplemented with 10% FBS Fetal Bovine Serum, qualified, US origin. The amount of FBS was reduced slowly by regular medium changes in order to adapt the cells to a final concentration of 2% FBS. Thus, cells were trypsinized as described above for CHO-DHFR$^-$ cells but using 0.05% Trypsin EDTA, Gibco. Cells were adapted first to DMEM Glutamax medium containing 5% FBS and grown under this condition periodically for two weeks. This procedure was repeated for two more weeks using DMEM Glutamax medium with 2% FBS. One day prior to transfection, cells were seeded at $3x10^5$ cells/well in 1mL DMEM-Glutamax 2%FBS in a 12-well plate and incubated at 37°C and 5% CO_2. In order to reach a higher yield of produced antibodies, transfection was performed in 100mm dishes at a seeding density of approximately $8.8x10^6$ cells per dish. One day prior to transfection, $4.8x10^6$ cells per dish were seeded in DMEM-Glutamax media without antibiotics.

3.1.6. Control for *Mycoplasma* infection

All the cultured cells were screened for infection with *Mycoplasma* by using the colorimetric PlasmoTest (Invivogen, San Diego, USA). All steps were performed following the manufacturer's instructions. Cells were heated at 100° for 15 min and mixed with suspended HEK-Blue cells included in the Kit to be incubated for 16 hours. On the next day wells were observed and controlled for color development (from pink to purple/blue in case of infection). Positive and negative controls were always included.

3.2. Bacterial strains and culture conditions

3.2.1. Bacterial strains

The reference strain in this study was *Enterococcus faecalis* 12030 isolated from a patient in the USA [36]. For plasmid constructions, *Escherischa coli* Top 10 was used (Invitrogen). The detailed information of the strains used in this study is listed in Table 3.2.1a.

4 Table 3.2.1a. Bacterial strains used in this study.

Strains	***Description***	***Reference***
***E.faecalis* strain**		
E.faecalis 12030	Isolated from a patient in US 1999	Huebner *et al*.[36]
E. coli strains		
E.coli Top 10	Gram negative cloning host	Invitrogen
pFUSE_AL_34	harbouring sequence of light chain AL34 on pFUSE plasmid	This study
pFUSE_AL_35	harbouring sequence of light chain AL35 on pFUSE plasmid	This study
pFUSE_CL_33	harbouring sequence of light chain CL33 on pFUSE plasmid	This study
pFUSE_CL_56	harbouring sequence of light chain CL56 on pFUSE plasmid	This study
pFUSE_CL_510	harbouring sequence of light chain CL510 on pFUSE plasmid	This study
pFUSE_CL_75	harbouring sequence of light chain CL75 on pFUSE plasmid	This study
pFUSE_CH_12	harbouring sequence of heavy chain CH12 on pFUSE plasmid	This study
pFUSE_CH_19	harbouring sequence of heavy chain CH19 on pFUSE plasmid	This study
pFUSE_AH_38	harbouring sequence of heavy chain AH38 on pFUSE plasmid	This study
pFUSE_AH_51	harbouring sequence of heavy chain AH51 on pFUSE plasmid	This study

3.2.2. Culture conditions for the *E. coli* strains

E. coli strains were cultured under vigorous shaking at 37°C in LB (Luria Bertani) medium. When necessary, ampicillin (100µg/mL) or kanamycin (50µg/mL) were added to the medium. Recombinant colonies were selected by blue-white screening by adding 50µg/mL of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to the LB agar plates. Solid LB cultures were

stored at 4°C For short term cultivation. For long term storage, permanent stocks of bacterial cells were done by growing the bacteria overnight an then supplementing the media with 20% glycerol before storage at -80°C. The exact composition of the different media is shown in Table 3.2.2a.

5 Table 3.2.2a. Medium (LB broth and LB Agar) used for culture of *E.coli*.

LB Broth	LB Agar
Tryptone 10 g/L	Tryptone 10 g/L
Yeast extract 5 g/L	Yeast extract 5 g/L
Sodium chloride 10 g/L	Sodium chloride 10 g/L
	Agar 15 g/L
pH 7.0 ± 0.2	pH 7.0 ± 0.2

3.2.3. Preparation of electrocompetent *E. coli* cells

In order to prepare *E. coli* cells for electroporation, 20mL of LB broth were inoculated with 5µL of *E. coli* Top10 from a frozen stock and incubated overnight at 37°C with agitation. The next day, the culture was used to re-inoculate 500mL of fresh LB media and bacterial cells were grown until OD reached 0.8 at 600nm. The cells were centrifuged for 30 min at 4000rpm and the pellet was resuspended in 500mL of ice cold sterile ultrapure water. Then, cells were centrifuged under the same conditions as mentioned above and washed twice with 350 and 125mL of ice cold water. After, cells were washed twice with decreasing volumes of ice cold sterile 10% glycerol. Finally, cell pellet was resuspended in 1mL of ice cold sterile 10% glycerol and aliquoted in 60µL portions before storing them at -80°C.

3.2.4. Culture of *Enterococcus faecalis* 12030

The *E. faecalis* 12030 strain was grown in Tryptic Soy Broth (TSB, CASO broth, Carl Roth) or Tryptic Soy Agar (TSA, CASO agar, Carl Roth) at 37°C without agitation. For preparing bacterial stocks, one colony was inoculated in freshly prepared TSB and bacterial cells were grown until OD reached 0.4 at 600nm. Then, bacterial suspension was supplemented with glycerol at a final concentration of 20% and stored at -80°C. The exact composition of the media is shown in Table 3.2.4a.

6 Table 3.2.4a. Medium used for culture of E. faecalis 12030: TSA und TSB.

TSB (pH 7.3 ± 0.2)	TSA (pH 7.3 ± 0.2)
Casein peptone (pancreatic digest.) 17 g/L	Casein peptone (pancreatic digest.) 15 g/L
Soya peptone (papain digest.) 3 g/L	Soya peptone (papain digest.) 5 g/L
Sodium chloride 5 g/L	Sodium chloride 5 g/L
Dipotassium hydrogen phosphate 2.5 g/L	Agar 15 g/L
Glucose 2.5 g/L	

3.3. Biological and immunological methods

3.3.1. LTA as screening target

For immune-screening assays LTA of *Staphylococcus aureus* (Sigma-Aldrich, St. Louis, Mo) was used. As positive controls in immunological assays, rabbit polyclonal serum raised against LTA from *E. faecalis* (anti-LTA) was used [48].

3.3.2. Indirect ELISA for screening of LTA-targeting antibodies

To test the affinity of antibodies towards LTA and DHG, an indirect ELISA was performed as described elsewhere [125]. DHG is a capsular polysaccharide isolated from the enterococcal cell wall and was purified as previously described [43]. For detailed composition of the buffers used see Table 3.3.2a.

7 Table 3.3.2a. Buffer used in indirect ELISA.

Buffer for indirect ELISA	Preparation
Coating Buffer	0.2M Sodium carbonate / bicarbonate buffer, pH 9.4
Washing Buffer	1xPBS+0.05%Tween
Blocking Buffer	1xPBS + 3%BSA (Bovine Albumin Serum)
Antibody diluting Buffer	1xPBS + 1%BSA
First antibody	Supernatant of B-cells / transfected eukaryotic cells
Secondary antibody	AP-conjugated anti-Human IgG / polyvalent immunoglobulin anti α, ϒ, μ
Detection	p-nitrophenyl phosphate
Detection Buffer	0.1M glycine buffer with 1mM $MgCl_2$ and 1 mM $ZnCl_2$, pH 10,4
Stop Solution	3M NaOH

Nunc-immuno Maxisorp 96 well plates were coated with 2µg/well of LTA from *Staphylococcus aureus* (Sigma-Aldrich, St. Lois, Mo) in Coating buffer and incubated overnight at 4°C. After incubation, wells were washed three times with 200µL of washing buffer and blocked for 1 hour with 100µL of blocking buffer at 37°C. Later, wells were washed twice with 200µL of washing buffer. After the washing, 100µL of either B-cell culture supernatants or supernatants from transfected cells were plated in the corresponding wells. Immune rabbit serum raised against LTA from *E. faecalis* 12030 [36] at 1:500 dilution was used as positive control. When cross-reactivity of cell supernatants was assessed against DHG, rabbit immune serum raised against DHG [43] was used as positive control. Samples were incubated for 1 hour at room temperature. Then, wells were washed three times with 200µL of washing buffer prior to incubation with secondary antibody. After, 100µL of alkaline phosphatase conjugated secondary antibody at 1:1000 dilution were added to each well (anti-Human IgG was used to evaluate supernatants after transfection or anti-human polyvalent immunoglobulin – α, ϒ and µ chain specific to detect antibodies during immunological screening period, both purchased from Sigma-Aldrich, St. Lois, Mo-). For positive controls Alkaline-phosphatase-conjugated anti-rabbit IgG produced in goat (Sigma-Aldrich, St. Lois, Mo) was used. After 1 hour of incubation at room temperature, plates were washed four to five times prior to addition of the p-nitrophenyl phosphate substrate (Sigma-Aldrich, St. Lois, Mo) prepared in detection buffer following manufacturer's instructions. After 15 to 30 min of incubation in the dark at room temperature the reaction was stopped by addition of 50µL 3M NaOH. Absorbance was measured at 405nm in an ELISA reader (Synergy H1 Hybrid reader, Bio-Tek).

3.3.3. Whole cell ELISA

E. faecalis 12030 stored at -80°C in a stock was streak out on a TSA plate and grown overnight at 37°C. Next day, the bacteria on the plate was used to inoculate 50 mL of TSB at OD650nm of 0.1 and culture was grown at 37°C until OD650nm reached approximately 0.38. The culture was split equally into two 50mL falcon tubes and centrifuged at 8000rpm for 10 min at 4°C. After two washes with one volume of 1xPBS, cells were resuspended and pooled together with 25mL of 8% paraformaldehyde in PBS. Paraformaldehyde was prepared by adding 4g of paraformaldehyde to 50mL of pre-warmed PBS. Sodium hydroxide 3M was added under the chemical hood until the solution cleared and pH was neutralized to 7 by titration with 5M hydrochloric acid. Then, bacterial cells were incubated in a rotor rack at 4°C for 30 min and

afterwards centrifuged as described above. After two more washes with 25mL of PBS cells were resuspended in 10mL of Coating Buffer and 100µL of cell suspension per well were used to coat a Nunc-immuno Maxisorp 96 well plate. Plates were coated at 4°C overnight and the next day ELISA was performed as described in section 3.3.2.

3.3.4. IgG and IgM quantification

To differentiate between IgG and IgM isotypes and quantify antibodies in the different cell culture supernatant, a sandwich ELISA was performed as previously described [89]. For detailed composition of the buffers used see Table 3.3.4a.

8 Table 3.3.4a. Buffers used in IgG quantification.

Buffer for IgG quantification	Preparation
Coating Buffer	0,2M Sodium carbonate / bicarbonate buffer, pH 9.4
Washing Buffer	0,9% NaCl + 0,1%Tween 20
Blocking Buffer	1x PBS + 2%BSA (Bovine Serum Albumin)
Antibody diluting Buffer	1x PBS + 1% BSA
First antibody	Standard IgG, Supernatant of B-cells or transfected eukaryotic cells
Secondary antibody	AP-conjugated anti-Human IgG
Detection	p-nitrophenyl phosphate
Detection Buffer	0.1M glycine buffer with 1mM $MgCl_2$ and 1 mM $ZnCl_2$, pH 10,4
Stop Solution	3M NaOH

Nunc-immuno Maxisorp MicroWell 96 well plates were coated with 0,1µg/well of unlabeled anti-human IgG (Southern Biotech, Birmingham, AL) or unlabeled anti-human IgM (Sigma-Aldrich, St. Lois, Mo) diluted in Coating buffer and incubated overnight at 4°C. After incubation, wells were washed three times with 200µL of washing buffer and incubated for one hour at room temperature with 200µL of blocking buffer. Dilutions of the standard IgG (Invitrogen, Carlsbad, US) were prepared in blocking buffer as follows, standard IgG was prepared at a concentration of 100µg/µL and 13 serial dilutions (1:2) were performed up to 0.12 ng/µL. Plain antibody diluting buffer was included as negative control. After, the wells were washed three times with 200µL of washing buffer and 100µL of dilutions of standard ranging from 31.2ng/mL to 0.0ng/mL were plated in triplicate as well as 100µL of the supernatants to be tested and incubated for two hours at room temperature. Then, wells were washed three times with 200µL of washing buffer and 100µL of alkaline-phosphatase conjugated anti-human IgG

produced in goat (Sigma-Aldrich, St. Lois, Mo) at 1:1000 dilution in blocking buffer was added to each well and incubated for two hours at room temperature. Four additional washes were performed to each well with 200µL of washing buffer. Afterwards, the p-nitrophenyl phosphate substrate at 1mg/mL (Sigma-Aldrich, St. Lois, Mo) in detection buffer was added to each well. After 30 min of incubation in the dark at room temperature, the reaction was stopped by addition of 50µL of 3M NaOH per well. Finally, absorbance was measured at 405 nm in an ELISA reader (Synergy H1 Hybrid reader, Bio-Tek).

3.3.5. Opsonophagocytic Assay

An in vitro OPA was performed as described by Kropec *et al.* [77] using the supernatant of either B-cells or the transfected eukaryotic cells, the target bacterial strain *E. faecalis* 12030, white blood cells and baby rabbit serum as complement source. The composition of buffers and media used in the OPA are shown in Table 3.3.5a.

9 Table 3.3.5a. Buffers used in OPA.

Buffer for OPA	Preparation
RPMI + 15% FBS	Rosewell Park Memorial Institute Medium (RPMI) with 15% Fetal Bovine Serum heat inactivated for 30 min at 56° Filtered sterile
Heparin-Dextran Buffer	4,5gNaCl 32,5mg Heparin-Sulfate 10g dextran 500 Fill up to 500mL with dH2O Filtered sterile
Lysis Solution	*(NH_4Cl) 1%* 1 g NH_4Cl dissolve in 100mL dH2O Filtered sterile

E. faecalis 12030 strain was plated on tryptic soy agar and incubated overnight at 37°C. Colonies were taken with a cotton swab from the plate and used to inoculate fresh TSB to an OD of ~0.100 at 650nm. Bacterial culture was grown until OD reached ~0.400 at 650nm. Then, cells were centrifuged at 13000 rpm for 5 min, washed and diluted with RPMI + 15 % FBS to yield a final concentration of 2×10^7 CFU/ mL. Freshly drawn blood from a healthy donor was mixed with an equal volume of heparin-dextran buffer and incubated for 45 min at 37°C. Then, the upper layer containing the leukocytes was transferred to a fresh Falcon tube and centrifuged at 2700 rpm for 10 minutes at 10°C. The pellet was resuspended in 10mL of RPMI + 15%FBS and centrifuged again under the same conditions. Afterwards, the pellet was resuspended in 10mL of lysis buffer and incubated for 10-20 min at room temperature for a

complete hypotonic lysis of the remaining erythrocytes. Cells were centrifuged again under the same conditions and washed once more with 10mL RPMI+ 15%FBS. Finally, the cell pellet was resuspended in 3mL of RPMI+15%FBS at a final concentration of 2×10^7 cells/mL. Lyophilized baby rabbit complement (Cerdalane, Burlington, NC) was diluted in sterile medium (RPMI + 15% FBS) to a final concentration of 6.7 % and absorbed for 60 min at 4°C on a rotor rack with bacterial cells of the target strain *E. faecalis* 12030 collected from a plate with a cotton swab. After 1 hour of incubation, the complement was centrifuged at 2700 rpm for 10 min at 10°C and filtered sterile. As positive control, immune rabbit serum raised against LTA from *E. faecalis* 12030 [36] was used at 1:100 dilution. For the assay 100µL of each reagent (neutrophils, complement, antibody sample or positive control and bacteria) were mixed in 2mL microcentrifuge and incubated for 90 min at 37°C on a rotor rack. After incubation, samples were diluted 1:100 in TSB and 10µL of each dilution were plated in quadruplicate on a TSA plates. Plates were incubated at 37°C overnight and colonies were counted the next day. By comparing the colony forming units (CFUs) surviving in the tubes with bacteria, white blood cells (WBC), complement and antibody (WBC^{pos})to the CFUs surviving in the tubes with all these components but lacking neutrophils, percentage of killing was calculated as follows:

$$\%\ \text{Killing} = 100 - \left(100 \times \frac{(mean\ CFU\ WBCpos\ at\ t90)}{(mean\ CFU\ WBCneg\ at\ t90)}\right)$$

Effective opsonophagocytic killing activity of the antibodies or supernatant of B-cells was only considered when percentages of killing were 35% or higher.

3.3.6. Concentration and purification of antibodies

Prior to test the antibodies produced by the transient transfections of either CHO-$DHFR^-$ or HEK-293T cells the cell culture supernatants were concentrated by diafiltration with Amicon Ultra - 0.5 mL Centrifugal Filters and later purified with Protein A HP Spin Trap columns according to manufacturer's instructions (GE healthcare Europe, Germany). During and after IgG purification the samples were stored at 4°C.

3.4. Genetic and molecular biology methods

3.4.1. Extraction of nucleic acids

The RNA was extracted from B-cells using the RNeasy Minikit by Quiagen according to the manufacturer's instructions. Briefly, the immortalized B-cells were harvested by centrifugation at 300*xg* for 5 min at 22°C and the supernatants were carefully collected for further analysis in ELISA and OPA. To lyse the cells (≤ $5x10^6$ cells), 350µL of RLT buffer supplemented with 10µL of beta-mercaptoethanol was added and the mixture was homogenized quickly with a syringe gauge of 0.9mm. After, 1 volume of 70% Ethanol was added to the sample and the mixture was transferred to a RNeasy column and centrifuged for 15 seconds at 8000*xg*. The flow-through was discarded and the column was washed with 700µL of RW1 buffer. Afterwards, two more washes with 500µL of RPE buffer were performed and the column was dried by centrifugation for 2 min at 8000*xg*. In a new collection tube 35µL of RNase free water was added to the column and incubated for 2 min at room temperature. Then, RNA was eluted by centrifugation for 1 minute at 8000*xg*.

3.4.2. Synthesis of cDNA

To synthesize cDNA, the SuperscriptIII First Strand Synthesis System (Invitrogen, Carlsbad, CA) for RT-PCR was used following the manufacturer's instructions. Right after RNA extraction, 4µL of RNAse OUT was added to 30-35µL of RNA. Then, random hexamers and dNTPs were added and a denaturation step was performed at 65°C for 5 min. After cooling down the reaction mixture for one min, RT Buffer, MgCl2, DTT and the superscript III reverse transciptase were added (see Table 3.4.2a.). Then, cDNA synthesis was performed using the temperature program shown in Table 3.4.2b. Subsequently, RNAseH was added to the mixture and the samples were incubated for 20 min at 37°C to remove remaining RNA. Quality and quantity of cDNA were measured by NanoDrop (Thermoscientific Nanodrop2000). Finally, cDNA samples were aliquoted and stored at -20°C for further analysis.

10 Table 3.4.2a. Reagents for cDNA synthesis.

Reagents used in cDNA synthesis	Volume
eluted RNA	35 µL
RNAseOUT	4 µL
random primers	4 µL
dNTP	4 µL
RT buffer	8 µL
DTT	8 µL
$MgCl_2$	16 µL
SuperScriptIII Reverse Transcriptase	4 µL

11 Table 3.4.2b. PCR program for cDNA synthesis.

	Time	Temperature
Denature conditions:	5 min	65°C
cDNA synthesis conditions:	10 min	25°C
	50 min	50°C
	5 min	85°C
	∞	4°C
RNA elimination:	20 min	37°C

3.4.3. Design of primers for amplification of variable domains

To amplify the sequence of cDNA that codifies for the variable domain of the antibodies produced by our immortalized B-cells, primers were designed according to the published Kabat database [126]. Most of the primers used were previously described by Kelly-Quintos *et al.* [127] and used previously in our research group by Rossmann *et al*. [78]. The sequences of the primers were reviewed and analyzed again to ensure the correct annealing of the chosen oligomers. The primers used in this study along with their more relevant characteristics are summarized in Table 3.4.3a and Table 3.4.3b.

12 Table 3.4.3a. Primer sequence of light chains.

Light Chains	Primer Sequence
L1	5'AGATCTCTCACC ATG GCC RGC TTC CCT CTC CTC
L2	5'AGATCTCTCACC ATG ACC TGC TTC CCT CTC CTC
L3	5'AGATCTCTCACC ATG GCC TGG GCT CTG CT
L4	5'AGATCTCTCACC ATG ACT TGG ATC CCT CTC TTC
L5	5'AGATCTCTCACC ATG GCA TGG ATC CCT CTC TTC
L6	5'AGATCTCTCACC ATG GCA TGG ATC CCT CTC TTC
L7	5'AGATCTCTCACC ATG GCC TGG ATG ATG CTT CTC
Light constant	5'GACCGAGGGGGCAGCCTTGGGCTGACCTAGG

13 Table 3.4.3b. Primer sequence of heavy chains.

Heavy Chains	Primer Sequence
H1	5'GTCGAC ATG GAC TGG ACC TGG A
H2	5'GTCGAC ATG GAC ATA CTT TGT TCC AC
H3	5'GTCGAC ATG GAG YYK GGG CTG AGC
H5	5'GTCGAC ATG GGG TCA ACC GCC ATC CT
H6	5'GTCGAC ATG TCT GTC TCC TTC CTC AT
H7	5'GTCGAC ATG AAA CAT CTG TGG TTC TTC
Heavy constant	5'TGGGCCCTTGGTGCTAGCTGAGGAGAC

3.4.4. Polymerase chain reaction (PCR) experimental conditions

In all cases the PCRs were performed following the manufacturer's instructions. For routine PCR analyses, i.e. colony screening, plasmid verification and gene identification the reactions were carried out using the GoTaq Master Mix (Promega, Mannheim, Germany). To optimize some PCR applications the reactions were performed using OneTaq 2X MasterMix with GC Buffer (New England Biolab, Ipswich, MA). For PCR experiments that require further DNA manipulations (enzymatic digestion, ligation and cloning) the reactions were performed using the highfidelity DNA polymerase Q5, High-Fidelity 2X Master Mix (New England BioLabs, Ipswich, MA). Exact composition of the reaction mixtures and temperature protocols used are shown in Tables 3.4.4.a.-c. The Bio Rad T 100 Thermal Cycler (Biorad, Hercules, CA) was used for the PCR reaction and Biorad Chemidoc MP Imaging System (Biorad, Hercules CA) to detect

bands in agarose gels. The samples were then run in 1% agarose gel according to Sambrook and Russel [128]. To make a 1% agarose gel, 1 g of agarose was dissolved in 100 mL of boiling TBE buffer (Tris acetate 40 mM, EDTA 2 mM pH 8.2). Subsequently, 1µL/100mL of Midori Green (NIPPON Genetics Europe, Germany) was added to the boiling agarose solution to enable visualization of the DNA. After cooling down, the gels were placed in a Mini-Sub® Cell GT Cell (Bio-Rad, Hercules, CA) electrophoresis chamber and covered completely with TBE buffer. The electrophoresis was run for 35 min at 120 mV and 400 A using the PowerPac™ (Bio-Rad, Hercules, CA) as power supply. The size of the DNA fragments was estimated by comparison with linear molecular weight marker O' GeneRuler 1kb DNA ladder (Thermo Scientific, USA).

14 Table 3.4.4a. Reagents and their volume for PCR reaction with GoTaq and OneTaq

Reagents for GoTaq and OneTaq	Volume
Master Mix	25 µl
Constant Primer	1 µl
Variable Primer	1 µl
Template DNA	1 - 3 µl
H_2O	up to 25 µl
Final volume	25 µl

15 Table 3.4.4b. Reagents and their volume for PCR reaction with Q5 High Fidelity Enzyme

Reagents for Q5 High Fidelity	Volume
Q5 High-Fidelity 2X Master Mix	12,5 µl
Constant Primer	1,25 µl
Variable Primer	1,25 µl
Template DNA	1 µl
H_2O	up to 25 µl
Final volume	50 µl

16 Table 3.4.4c. PCR program A. GoTaq B. OneTaq C. Q5 High Fidelity Enzyme

	A GoTaq		B One Taq		C Q5	
Cycle Step ***(Number of Cycles)***	**Time**	**Temperature**	**Time**	**Temperature**	**Time**	**Temperature**
Initial Denaturation *(1x)*	2 min	95°C	0:30 min	94°C	0:30 min	98°C
Denaturation *(30x)*	0:30 min	95°C	0:30 min	94°C	0:08 min	98°C
Annealing *(30x)*	0:30 min	60°C	0:30 min	45-68°C	0:20 min	50-72°C
Extension *(30x)*	1 min	73°C	1 min	68°C	0:15 min	72°C
Final Extension *(1x)*	5 min	73°C	5 min	68°C	2 min	72°C
Hold	∞	4°	∞	4°	∞	4°

3.4.5. Analysis of cDNA through sequencing

Amplified cDNA was purified using the Wizard SVGel and PCR Cleanup System (Promega, Mannheim, Germany) according to manufacturer's instructions. After eluting 35µL of DNA in DNAse free water, the DNA fragment was cloned into a plasmid using the pCR™2.1-TOPO® vector and the TOPO™ TA Cloning™ Kit for Subcloning (Invitrogen, Carlsbad, CA). Reagents are listed in Table 3.4.5a.

17 Table 3.4.5a. Reagents for cloning into TOPO® vector

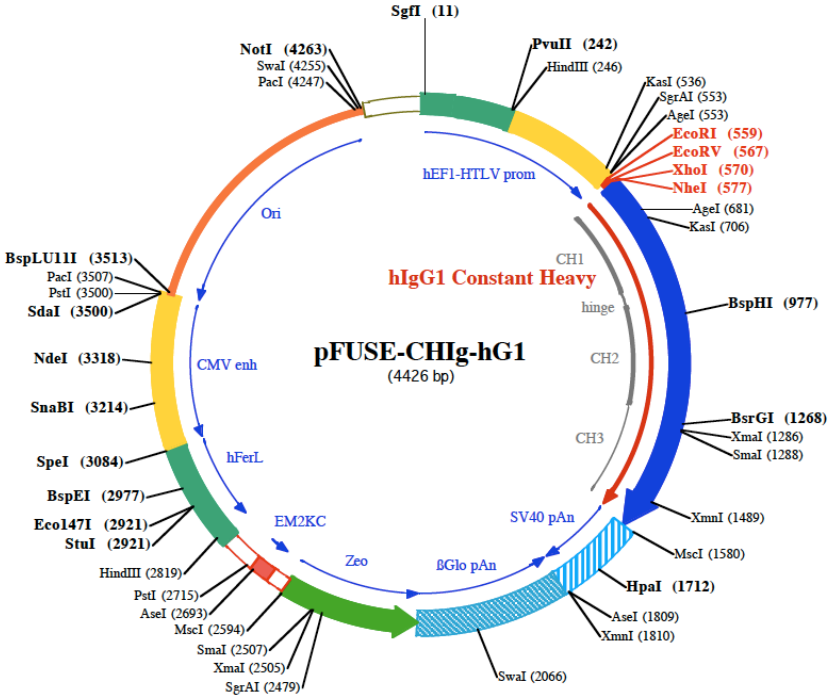
Reagent	Volume
PCR product	0.5–4 µL
Salt Solution	1 µL
Water	add to a total volume of 5 µL
TOPO® vector	1 µL
Final Volume	6 µL

The reaction was mixed gently and incubated for 20 minutes at room temperature. Electrocompetent *E. coli* cells (100µL prepared as described above) were transformed with 2µL of TOPO-Reaction using the Electro Cell Manipulator® (BTX®, USA) with a voltage of 2.5kV, 200Ω and a capacitance of 25F. Warm LB media was immediately added after transformation and the transformed cells were incubated for 40 min shaking at 37°C. Bacterial cells were spread on LB agar plates supplemented with X-gal at 50µg/mL and ampicillin at 100µg/mL using different volumes of bacterial suspension per plate (10µL, 30µL and 100µL) and incubated at 37°C overnight. Next day white colonies were transferred to a fresh plate and incubated overnight at 37°C again. On the following day 10 colonies were picked to verify the content of the insert by PCR using the GoTaq Maxter Mix (Promega, Mannheim, Germany) as described above. Bacteria were disrupted using MilliQ H_2O and 1µL of disrupted cells were used as DNA template. Colonies that contained the plasmid with inserts of around 450bp were grown in 5mL of LB supplemented with ampicillin at 100µg/mL at 37°C overnight with shaking. The Pure Yield Plasmid Miniprep System (Promega, Mannheim, Germany) was used to purify the plasmid and a small aliquot was sent to Eurofins Genomics (Germany) for sequencing. All the nucleotide sequences were translated to amino acid sequences using the ExPASy translate tool available online (https://web.expasy.org/translate/). Afterwards, the sequences were reviewed for the presence of all main characteristics of a variable domain in a functional antibody (i.e. three

complementary determining regions (CDR), three Framework Regions (FR) without stop codons in between and a leader sequence). The sequences were compared to each other and reviewed for correspondence to a known antibody sequence using the online tool IG-BLAST. They were checked for the signal peptide sequence with the SignalP 4.1 online tool server (http://www.cbs.dtu.dk/services/SignalP/). The variation between protein sequences was measured by Restricted Damerau-Levenshtein distance [129], [130].

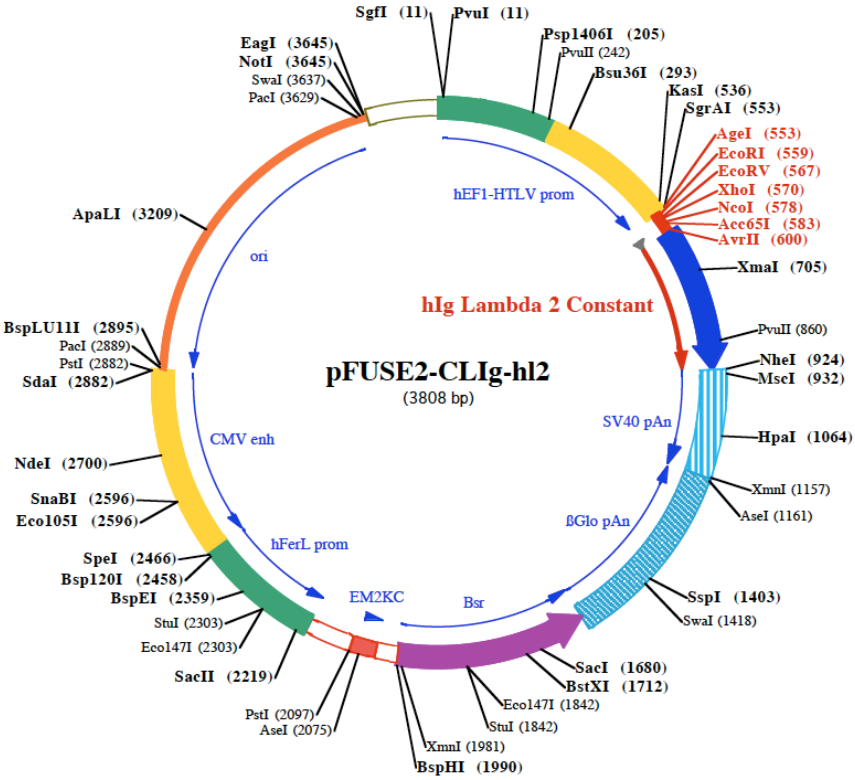
3.4.6. pFUSE System as Cloning vector

For transfection of mammalian cells the pFUSE System (InvivoGen, San Diego, USA) was used as a cloning system. pFUSE-CHIg and pFUSE2-CLIg vectors codify the constant region of the heavy or the light chain, respectively. In this case pFUSE-CHIg-hG1 expressing human IgG1 and pFUSE2-CLIg-hl2 expressing human lambda 2 chain were used. In this vector, upstream the constant region there is a multiple cloning site where inserting the variable domain is possible. The generated plasmids are listed in Table 3.4.6a. By transfecting mammalian cells with pFUSE-CHIg-hG1 and pFUSE2-CLIg-hl2 a full IgG antibody was generated that could be purified from the supernatant. Transfected clones were selected by blasticidin for pFUSE2-CLIg and zeocin for pFUSE-CHIg in both – prokaryotic and eukaryotic cells (see figure 3.4.6a/b).



5 Figure 3.4.6a pFUSE-CHIg-hG1.

Vector for the constant heavy chain. Illustration by Invivogen (San Diego, USA)



6 Figure 3.4.6b pFUSE2-VLIg-hl2.

Vector for the light chain. Illustration by Invivogen (San Diego, USA)

18 Table 3.4.6a. Plasmids generated in this study and the description of the respective insert.

Plasmid	Description	Reference
pF_AH38	pFUSE plasmid carrying heavy chain AH38 with peptide signal	This study
pF_AH51	pFUSE plasmid carrying heavy chain AH51 with peptide signal	This study
pF_CH12	pFUSE plasmid carrying heavy chain CH12 with peptide signal	This study
pF_CH19	pFUSE plasmid carrying heavy chain CH19 with peptide signal	This study
pF_AL34	pFUSE plasmid carrying light chain AH34 with peptide signal	This study
pF_AL35	pFUSE plasmid carrying light chain AH35 with peptide signal	This study
pF_CL33	pFUSE plasmid carrying light chain CH33 with peptide signal	This study
pF_CL56	pFUSE plasmid carrying light chain CH56 with peptide signal	This study
pF_CL510	pFUSE plasmid carrying light chain CH510 with peptide signal	This study
pF_CL75	pFUSE plasmid carrying light chain CH75 with peptide signal	This study

To clone the variable regions into the pFUSE System new forward primers were designed including a site for the respective enzyme to digest the insert (see Table 3.4.6c). Restriction enzymes were purchased from New England Biolabs (Frankfurt a.M., Germany). A Kozak sequence was added upstream the starting ATG to ensure the correct expression of the protein [131]. The reverse primer located in the constant primers remained the same since it already encodes the sequence for the desired restriction site (see Tables 3.4.3a and 3.4.3b). Before digestion and ligation of the plasmid all samples were controlled to contain the insert by PCR using primers designed for pFUSE. For the digestion two different reactions containing either the insert or the pFUSE plasmid were prepared. Restriction Enzymes are listed in Table 3.4.6b. Since all restriction enzymes of New England BioLabs (Ipswich, MA) use the same CutSmart Buffer for digestion only one reaction was carried out together with other components (see Table 3.4.6d.). Reactions were incubated for 1h at 37°C and the enzymes were heat inactivated at 80°C for 20min.

19 Table 3.4.6b. Digestion enzymes used for cloning of the heavy and light chain in pFUSE vector.

Variable region	3‘ end	5‘ end
Heavy chains	Nhe I	Eco RI
Light chains	Avr II	Age I

20 Table 3.4.6c. Modified primer sequences amplifying variable region including restriction site of restriction enzyme (underlined sequence).

Name of primer for pFUSE	Primer Sequence
pFU_VH1_EcoRI_Fw	5’ GAT C GAATTC GAC ATG GAC TGG ACC TGG A
pFU_VH3_EcoRI_Fw	5‘ GATC GAATTC GAC ATG GAG YYK GGG CTG AGC
pFU_VH5_EcoRI_Fw	5‘ GATC GAATTC GAC ATG GGG TCA ACC GCC ATC CT
pFu_Lam3_AgeI_Fw	5’ GAT C ACCGGT ACC ATG GCC TGG GCT CTG CT
pFu_Lam5_AgeI_Fw	5‘ GATC ACCGGT ACC ATG GCA TGG ATC CCT CTC TTC
pFu_Lam7_AgeI_Fw	5‘ GATC ACCGGT ACC ATG GCC TGG ATG ATG CTT CTC

21 Table 3.4.6d. Reagents for digestion in pFUSE plasmid preparation

Reagent	Volume
For Reaction 1: DNA (insert)	1 µg
For Reaction 2: pFUSE plasmid	4 µl
Both Reactions: Restriction Enzymes	Each 1 µl
Both Reactions: CutSmart Buffer	5 µl
Both Reactions: H_2O	Up to 50 µl

After purification of the DNA with the Wizard SVGel and PCR Cleanup System (Promega, Mannheim, Germany), the concentration was quantified by Nanodrop. The NEBio calculator available online was used to calculate the molar ratio 7:1 (insert: plasmid) needed for ligation in pFUSE plasmid according to their mass and length. Reagents used for ligation are listed in Table 3.4.6e.

22 Table 3.4.6e. Reagents for ligation of the insert into the pFUSE plasmid

Reagent	Volume
T4 DNA ligase (NEB)	1 µl
Buffer	2 µl
Cutted plasmid	40 ng
Insert	2,8 ng
H_2O	Up to 20 µl

After incubation of 1 hour at room temperature and heat inactivation at 65°C in a water bath, electro-competent *E. coli* were transformed with the plasmid and grown overnight on plates as explained previously. To select transformed clones Agar plates were prepared with low salt LB

agar medium (pH 8) and supplemented with 100 µg/ml of Blasticidin for pFUSE2-CLIg or 25 µg/ml of Zeocin for pFUSE-CHIg. After identifying transformed clones through selection and control PCR for the insert, Minipreps of selected colonies were prepared to send to sequence. In preparation for the transfection Midiprep and Maxiprep Pure Yield Plasmid System by Promega were used according to manufacturer's instructions to optimize the yield of DNA in order to transfect eukaryotic cells successfully. The final volumes of some samples were reduced using a SpeedVac concentrator system to achieve the concentrations of DNA recommended for the transfection of eukaryotic cells (i.e. 1-5µg/µL).

3.4.7. Transfection of mammalian cells

Cells were plated in a 12-well plate as described above one day prior to transfection. To transfect the mammalian cells 3µg of plasmid was needed (in this case 1.5 µg of each pFUSE plasmid containing the heavy and light chains, respectively). The quantity of DNA was measured before by NanoDrop. As recommended a ratio of 2:3 (1.2 : 1.8 µg) was used for plasmids containing the heavy and light chains, respectively. OptiMEM + Glutamaxx Opti-MEM™ Reduced Serum Medium, GlutaMAX™ Supplement (Gibco) was used as well as Lipofectamine™ 2000 Transfection Reagent by Invitrogen. 1 hour prior to transfection the medium was changed to 1 mL of OptiMEM Medium and cells were incubated at 37°C. Plasmid DNA and lipofectamine were diluted to required concentrations in OptiMEM medium and 37.5 µl of each plasmid was mixed. After adding an equal volume (75 µL) of lipofectamine to the DNA the reaction mixture was incubated at room temperature for 15min. Drop by drop the mixture (125 µL) was added to the wells and the plate was hit slightly to guarantee homogenization of the solution. Transfected cells were incubated at 37°C for six hours before changing the medium to Alpha MEM with Nucleosides for CHO-DHFR$^-$ cells or DMEM + Glutamax for HEK-293T cells. Media were always supplemented with Ultra Low IgG Fetal Bovine Serum (Gibco). On every plate one well containing Pmax (green fluorescent protein - GFP 1.0 µg/µL) by Lonza (Basel, Switzerland) was included in order to estimate the amount of transfected cells. In addition, one well was transfected with the empty pFUSE plasmid, supernatant that would be used as negative control in subsequent antibody testing assays. After 24 hours (supernatant day 0) of transfection the medium was changed to medium with antibiotics. After 72 hours supernatant was collected (day 2) and medium was changed to medium with antibiotics. Every two days medium was collected and changed for medium with

antibiotics for 4 days more (day 4 and 6, respectively).

For high yield transfection HEK-293T cells were seeded on 100mm dishes and one hour prior to transfection the medium was changed to 12.5 ml of OptiMEM + Glutamax (Gibco) as described above. Meanwhile DNA samples were prepared as described above and as transfection agent Polyethylenimine (PEI25K) by Polysciences Inc. (Warrington, PA) was used. 600 µL of the plasmid preparation and 600 µL of PEI were added drop by drop. Afterwards the samples were incubated for 15 min and then 1.2 mL of reaction mixture was added to each well. After six hours the medium was changed to 20 mL of DMEM + Glutamax. After 24 hours the medium was changed to DMEM + GLUTAMAX + Zeocin (300 µg/mL) and Blasticidin (7.5 µg/mL) and supernatant was collected. Supernatant was collected three more times every second day.

3.4.8. Dialysis of supernatants after transfection of HEK-293T cells

Supernatants of transfected HEK-293T cells were collected and stored at 4°C. After day six collection of the supernatants was no longer carried out. Samples were centrifuged at 3000rpm for 10min at 4°C and filtered using 0.45µm CME syringe filters (Carl Roth, GmbH, Germany). Supernatants from day four were dialyzed using regenerated cellular tubular membrane of nominal molecular weight cut of 3500 Da (ZelluTrans, Carl Roth, GmbH, Germany). The dialysis was done against 10mM Tris Buffer, pH 7.4 filtered sterile. The buffer was exchanged three times during 32 hours and procedure was performed at 4°C.

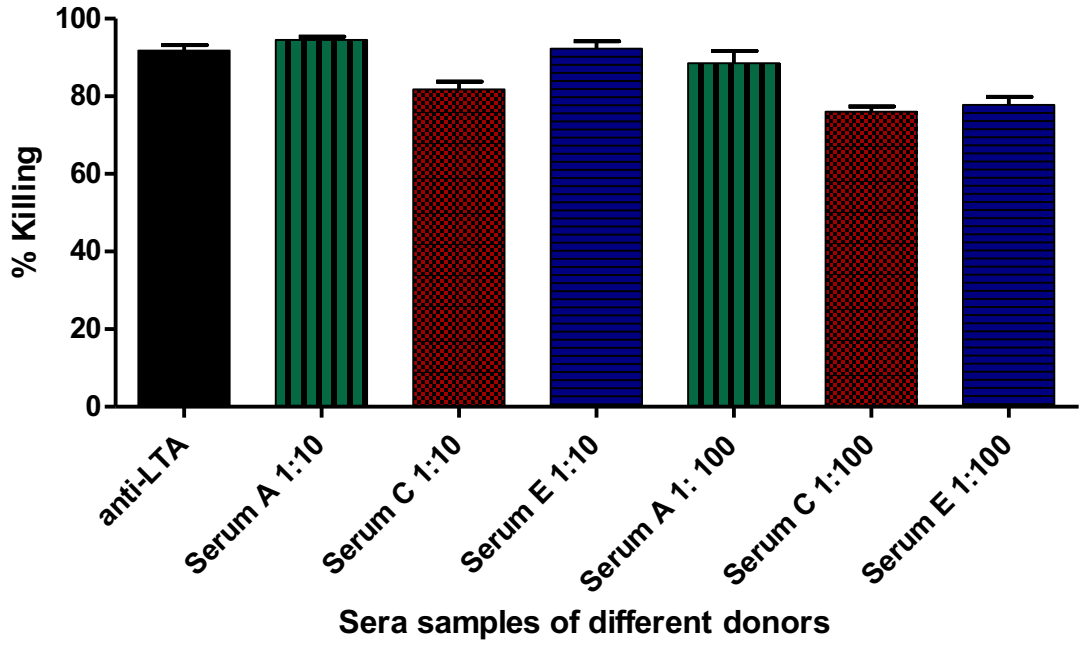
3.5. Statistical Analysis

Results were analyzed using the software program GraphPad PRISM version 5.00. Mean and 95% cut off were calculated and used to identify outstanding samples. The percentage of opsonophagocytic killing was expressed as the geometrical mean ± the standard errors of the means. Statistical significance (P values ≤0.05) was determined using Dunnett Test for multiple comparisons to one control or Tukey test for the comparison of values between each other. Two-way-analysis of variance (ANOVA) with Bonferroni-Post-Test was used to identify significantly reactive antibodies. Fisher-Exact Test was used to examine the significance of the contingency between the two kinds of classification. F-Test was applied to analyze variances in two groups.

4. Results

4.1. Selection of blood donor

Blood sera from different healthy donors were screened in an opsonophagocytic assay. The serum exhibiting the highest opsonophagocytic killing activity against *E. faecalis* 12030 was selected for isolation and immortalization of blood PBMCs and investigation in this project. All sera tested showed high opsonophagocytic killing activity (Fig.4.1a). Hence, all sera samples obtained could be used in our study. However, since Serum A exhibited the highest opsonophagocytic killing activity against the target strain at the two tested dilutions, it was selected. The immortalized B-cells were later split and screened for their content of immunoreactive and opsonic antibodies towards the target bacterial strain and LTA.



7 Figure 4.1a. Opsonophagocytic killing activity of sera for selection of best blood donor for PBMC isolation and immortalization.

Opsonophagocytic assay was performed using human sera from different donors: Serum A (green bars), Serum C (red bars) and Serum E (blue bars) at two different dilutions (1:10 and 1:100). Anti-LTA serum (black bar) was used as positive control at final dilution of 1:100 and the strain tested was E. faecalis 12030. Bars represent the mean of data (n=4) and error bars represent the standard error of the mean.

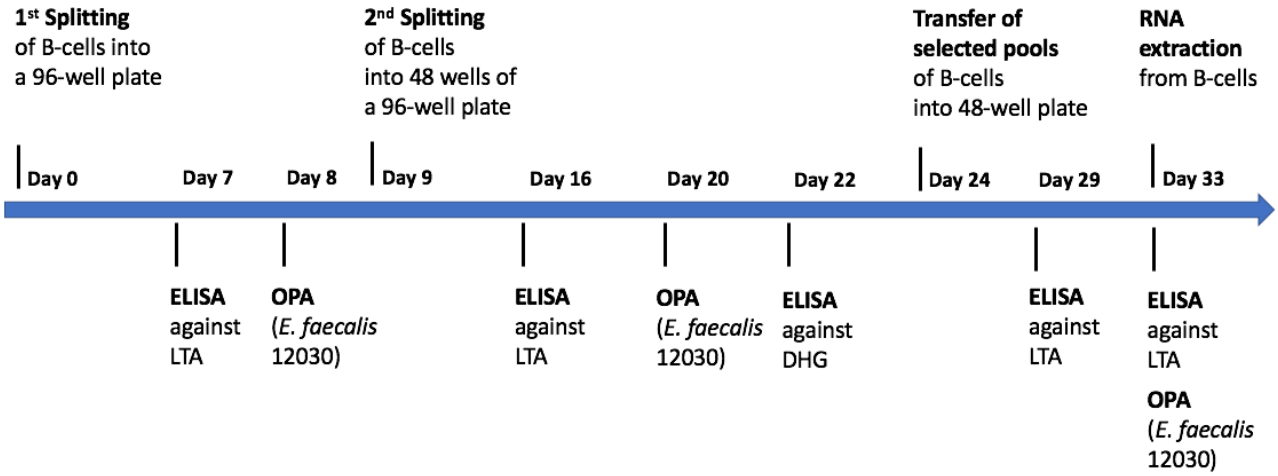
4.2. Pipeline development for immunological screening and selection of immunoreactive and opsonic antibodies

EBV-immortalized B-cells were split into a 96 well plate. A theoretical approximation was made based on the estimated number of initially isolated B-cells to ensure reaching the single-cell level of the cultured B-cells through the splitting steps, (from 8mL of blood). The yields of the different performed procedures were also considered to calculate the remaining cells after each step (i.e. EBV immortalization, successive splitting). Table 4.2a shows the minimal and maximal possible amount of B-cells present in blood and the corresponding number of cells in a blood volume of 8mL. In order to obtain a single clone of the B-cell, only two successive splits of the cells are needed (first into 96 wells plate and then into 48 wells plate) as only about 1% of isolated B-cells are immortalized with EBV (see theoretical calculation in Table 4.2a) [132].

23 Table 4.2a. Theoretical number of B-cells after PBMC isolation, EBV immortalization and B-cell splitting.

	cells/mL in Blood	cells in 8mL of Blood	1% EBV immortalized cells	1st Split in 96 wells Cells/well	2nd split in 48 wells Cells/well
B-cells	0.7 - 5.3 x 10^5	5.6- 42.4 x 10^5	5.6- 42.4 x 10^3	58-442	1-9

Figure 4.2a represents the time scale used during the screening of the B-cell repertoire of the healthy donor of Serum A. On “Day 0”, thawed cells were split into a 96-well plate and grown for the seven following days. The supernatants of immortalized B-cells were then screened for antibodies with high immunoreactivity towards LTA and opsonophagocytic killing activity against *E. faecalis* 12030. On “Day 7”, immunoreactivity of the supernatants was assessed in ELISA and the samples with high immunoreactivity were tested on “Day 8” in OPA. To increase the concentration of antibodies in the culture, a second split of three pools into 48 wells (using a 96 well plate) was performed on “Day 9”. Cells were then left to grow for another seven days before the next ELISA was performed on “Day 16”. On “Day 20” OPA was performed and cells were left to grow for nine days. Furthermore, the supernatants of the B-cells were simultaneously screened in ELISA against DHG, another known surface antigen of *E. faecalis* Type 2 [43]. This was performed on “Day 22”. On “Day 24” selected pools were transferred to a 48-well plate for further culturing. On “Day 29” immunoreactivity in ELISA was assessed again. Subsequently, RNA was extracted from the cells on “DAY 33” and the supernatant was used to confirm previously obtained results in ELISA and OPA.

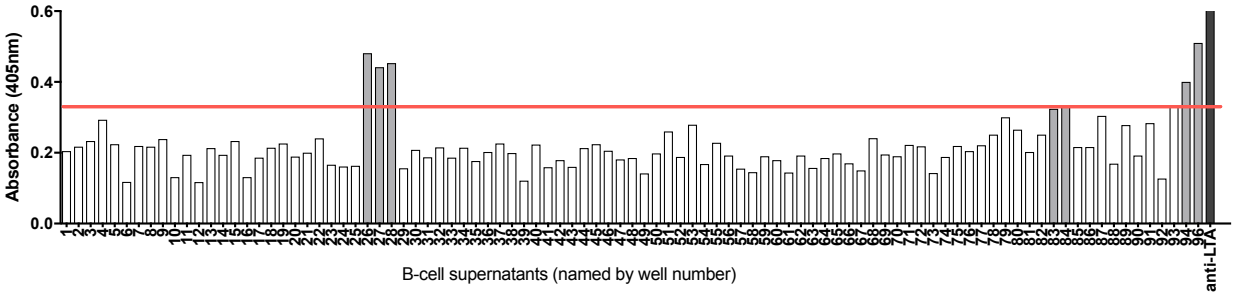


8 Figure 4.2a Time scale used in this study.

All experiments performed during the selection process are shown in the time scale on the day when they were carried out. The "Day n" indicates the nth day after the first split of transfected B-cells into 96 wells.

4.3. Identification of pools of B-cells producing immunoreactive and opsonic antibodies

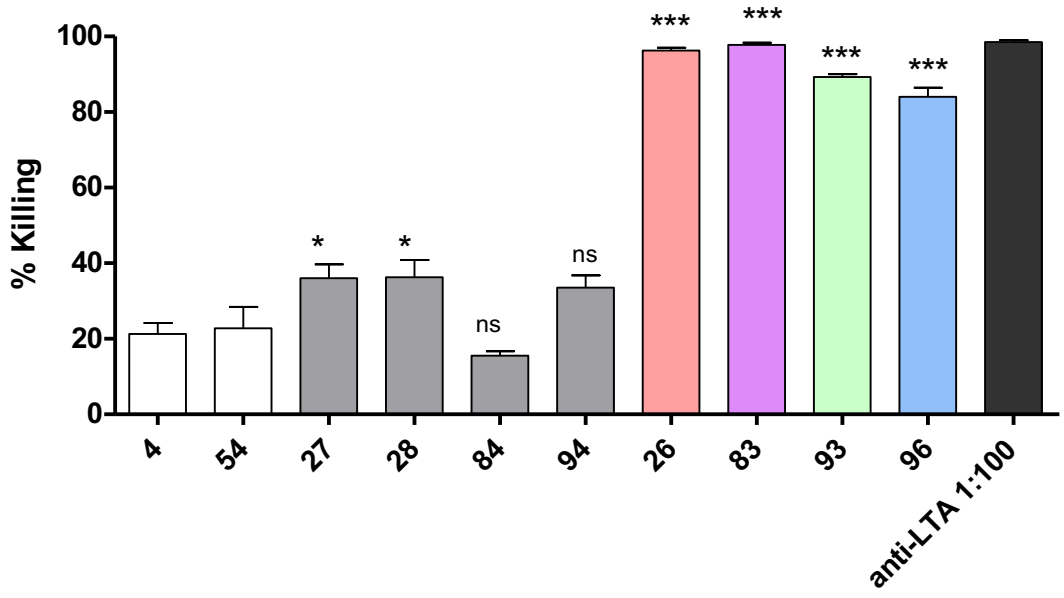
Cells were incubated for seven days after the first splitting and the immunoreactivity towards LTA of the supernatants of pools 1 to 96 was tested in ELISA (see Figure 4.3a). In order to have a reasonable number of samples to consequently test in OPA, we established the number of a maximum of 10, arbitrarily. Thus, seven out of the 96 pools were selected for further analysis: Pools 26, 27, 28, 83, 84, 93 and 96. Pools 4 and 54 showed lower immunoreactivity against LTA and therefore were selected as negative controls in OPA.



9 Figure 4.3a. Immunoreactivity of B-cell SPNs of 1st split against LTA on "Day 7".

Indirect ELISA was performed to test the 96 B-cells pools after the 1st splitting. Anti-LTA served as a positive control (black bar). Pools that showed high immunoreactivity (above red line) were selected for further analysis in OPA: 26, 27, 28, 83, 84, 93, 94 and 96. Pools 4 and 54, with low immunoreactivity towards LTA served as negative controls.

On “Day 8”, the supernatants of the pools mentioned above were taken and tested in OPA. As observed in Figure 4.3b only some pools with high immunoreactivity in ELISA showed high opsonophagocytic killing activity (pools 96, 93, 26, 96); whereas pools 27, 28 and 84 showed lower killing activity. In ELISA control pools with low immunoreactivity against LTA (4, 54) did not exhibit effective opsonophagocytic killing activity against the target bacterial strain.

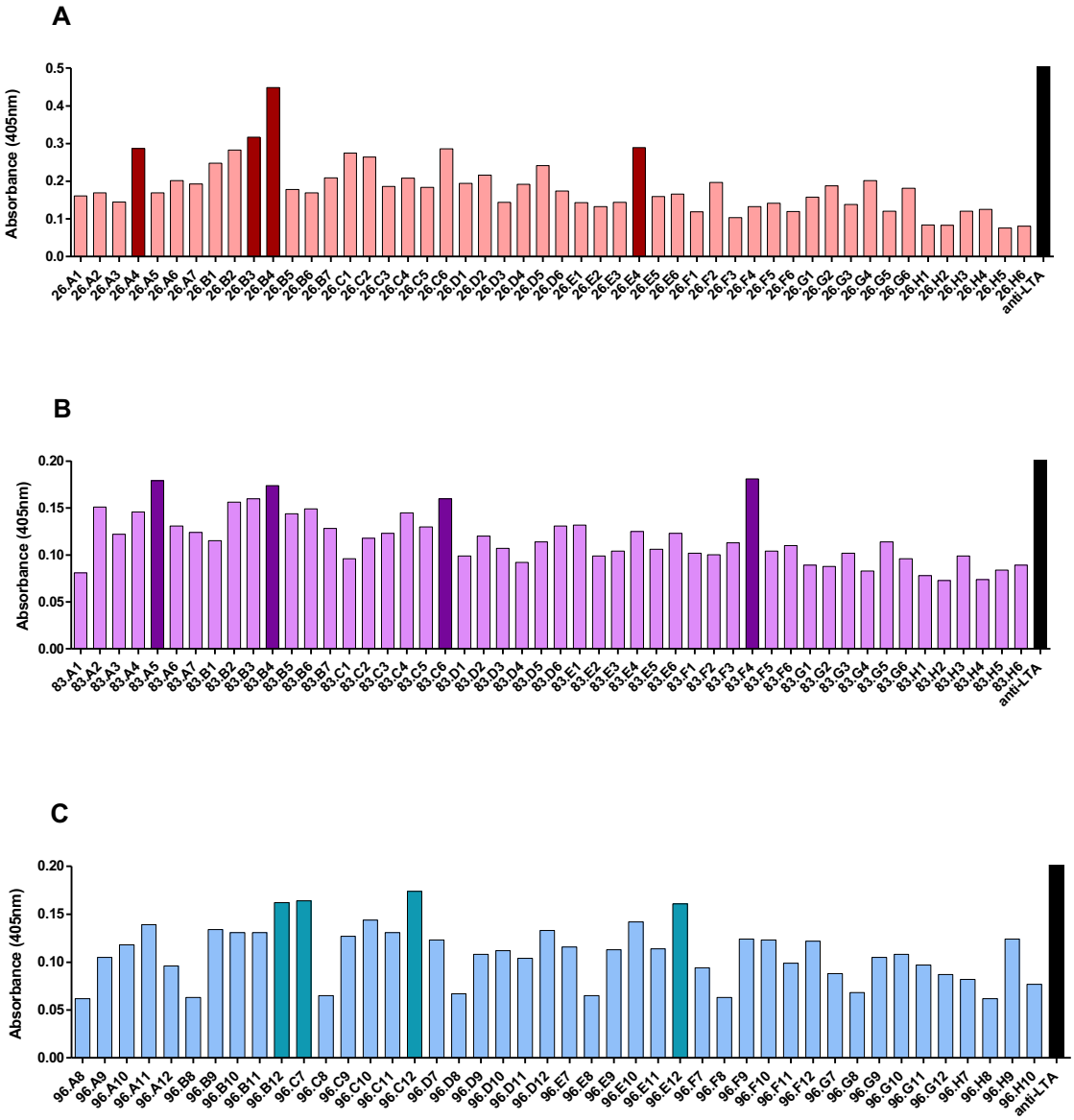


10 Figure 4.3b. Opsonophagocytic killing activity of selected B-cell SPNs after the 1st split against E. faecalis 12030 on “Day 8”.

Anti-LTA serum was used as positive control at final dilution of 1:100 (black bar). The tested strain was E. faecalis 12030. Pools 4 and 54 showing low immunoreactivity towards LTA in ELISA were chosen as negative controls in the assay (white bars). Pools 26 (red bar), 83 (purple bar), 96 (blue bar) and 93 (green bar) showed the highest opsonophagocytic killing activity against the target strain. Dunnett Test for multiple comparisons showed that pools 26, 27, 28, 83, 93, and 96 exhibited statistically significant higher killing, whereas pools 84 and 94 were not significantly different from both negative controls (pools 4 and 54). Bars represent the mean of data and the error bars represent the standard error of the mean. Asterisks denote significance (ns, not significant, * P≤0.05, ** P ≤ 0.01, *** P ≤ 0.001).

Only pools showing positive results in both assays – ELISA and OPA – were selected for further splitting into 48 wells on “Day 9”. Therefore, pools 26, 83 and 96 were further analyzed in this study. Pool 93 was not included. B-cells were not split in a 48 well plate but into 48 wells of a 96-well plate since using a higher volume-per-well plate would over-dilute the culture and thus lead to cell death [123]. The cells were left to grow for seven days after the splitting and

immunoreactivity was assessed in ELISA (see Figure 4.3c). Of every group (e.g. all pools of 26 or 83 or 96) four pools with the highest immunoreactivity were selected and were further tested for their opsonophagocytic killing activity (i.e. pools 26. A4, 26.B3, 26.B4, 26.E4, 83.A5, 83.B4, 83.C6, 83.F4, 96.B12, 96.C7, 96.C12 and 96.E12).



11 Figure 4.3c. Immunoreactivity of B-cell SPNs after the second splitting in ELISA against LTA on "Day 16".

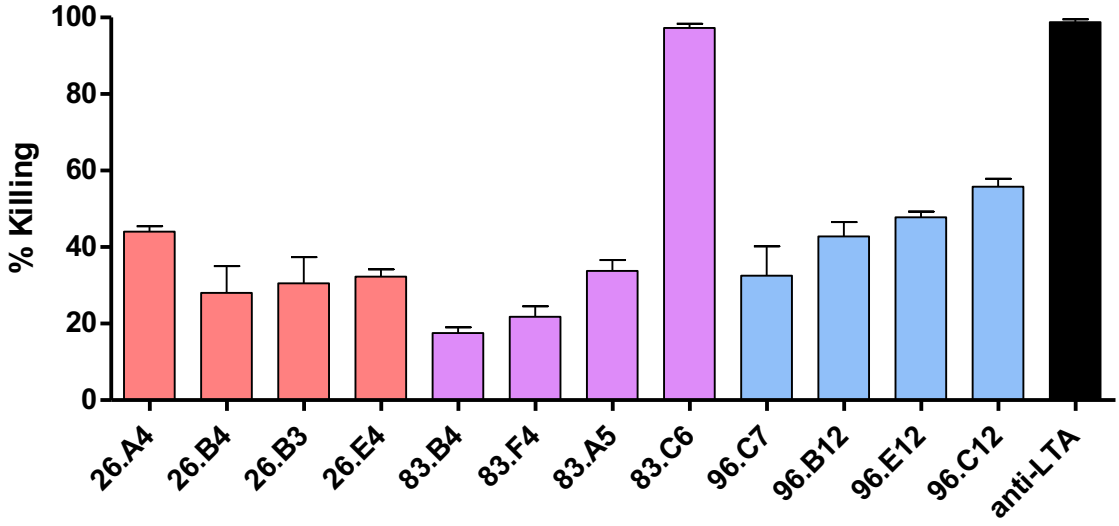
Indirect ELISA was performed to test the B-cell pools after the second splitting. Anti-LTA was included as a positive control (black bars).

A. Pools 26. A4, B3, 26.B4, 26.E4 (dark red) were selected for analysis in OPA.

B. Pools 83.A5, 83.B4, 83.C6 and 83.F4 (dark purple) were selected for analysis in OPA

C. Pools 96.B12, 96.C7, 96.C12, 96.E12 (dark blue) were selected for analysis in OPA.

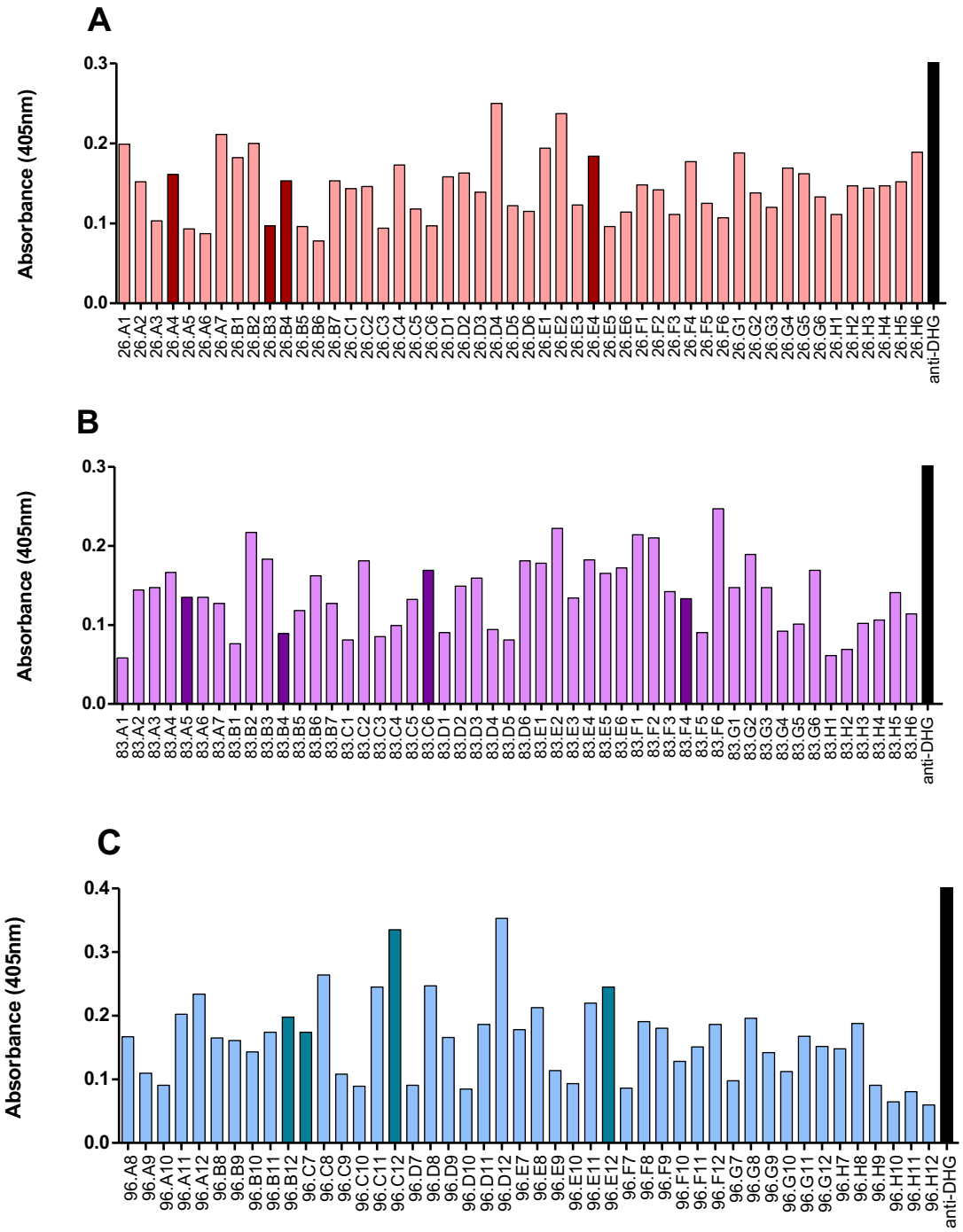
The opsonophagocytic activity of the selected pools of B-cells is shown in Figure 4.3d. Although, some pools (26.A4, 26.B4, 26.B3, 26.E4, 83.B4, 83.F4, 83.A5, 96.C7) showed high immunoreactivity towards LTA in ELISA, they exhibited less than 50% of opsonophagocytic killing activity. Only two pools, 83.C6 and 96.C12, showed killing activity higher than 50% on "Day 20".



12 Figure 4.3d. Opsonophagocytic killing activity of selected B-cell SPNs after second splitting against E. faecalis 12030 on "Day 20".

Opsonophagocytic assay of SPNs from B-cells after the second splitting in 48 wells. Anti-LTA serum was used as positive control at a final dilution of 1:100 (black bar) and the strain tested was E. faecalis 12030. Pools derived from the splitting of Pool 26, 83 and 96 are shown in red, purple and blue bars, respectively. Bars represent the mean of data and the error bars represent the standard error of the mean.

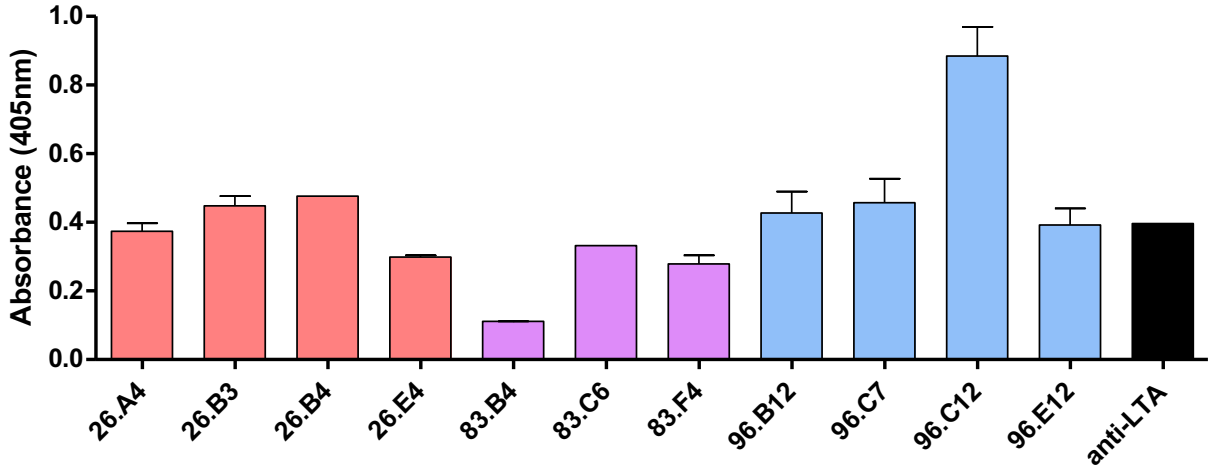
In order to identify antibodies with cross-reactivity towards other bacterial antigens, ELISA against DHG was performed to the supernatants of the selected pools on "Day 22". DHG is a known surface antigen from *E. faecalis* T2. Overall, absorbance values in the assay against DHG were higher than in the assay against LTA (see Figure 4.3c and Figure 4.3e). Pool 26.E4, 83.C6 and pool 96.C12 showed the highest immunoreactivity towards both antigens, DHG and LTA, in comparison with other pools on the same plate. The purpose of this assay was to assess cross-reactivity potential but these results were not included in the screening process.



13 Figure 4.3e. Immunoreactivity of B-cell SPNs of second splitting in indirect ELISA against DHG on "Day 22".

Anti-DHG was included as a positive control (black bar). The pools selected due to high immunoreactivity against LTA are marked in darker shades. **A.** Split pools of pool 26. Supernatant of pool 26. E4 showed highest overlap of immunoreactivity against LTA and DHG. **B.** Split pools of pool 83. Supernatant of pool 83.C6 showed highest overlapped immunoreactivity against LTA and DHG. **C.** Split pools of pool 96. Supernatant of pool 96.C12 showed highest overlapped immunoreactivity against LTA and DHG.

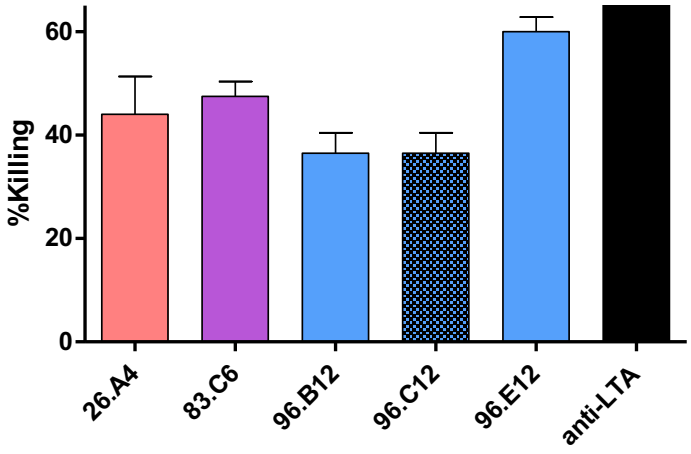
The pools of immortalized B-cells selected by ELISA and OPA were cultured in a 48-well plate until “Day 33”. During this time the activity the supernatant was tested once more in ELISA on “Day 29”. Here, pool 96.C12 showed the highest immunoreactivity towards LTA (see Figure 4.3f).



14 Figure 4.3f. Immunoreactivity of B-cell SPNs of second split in indirect ELISA against LTA on “Day 29”.

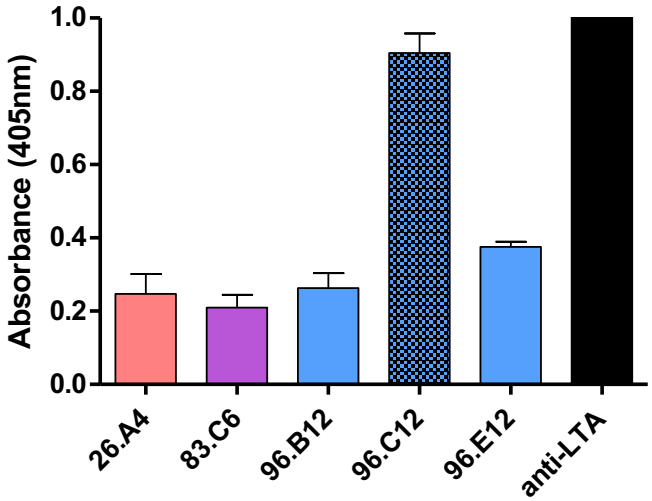
Indirect ELISA was performed to monitor the activity of SPNs of B-cell pools after the second splitting. Anti-LTA was included as a positive control (black bar). Pools derived from the split Pool 26 are shown in red bars, while those derived from pool 83 and 96 are shown in purple and blue bars, respectively. Bars represent the mean of data and the error bars represent the standard error of the mean.

On “Day 33”, B-cells were taken to perform the RNA extraction. At this time each pool should have contained between one and nine clones according to our theoretical calculations of the number of clones per well. This was considered a reasonable number of clones and no further splitting of the B-cells was performed. Before the RNA extraction, supernatants of pools 96.C12, 83.C6, 96.B12 and 96.E12 were kept for further immunological assays. All of them were tested in ELISA and OPA. In Figure 4.3g the opsonophagocytic killing activities of all pools are shown. Although the percentages of killing were lower than in previous tests, effective neutralization of the bacterial target was still observed. However, when tested again in ELISA, only pool 96.C12 showed very high immunoreactivity towards LTA (see Figure 4.3h) and therefore, its cDNA was selected for further analysis by molecular biology techniques. Figure 4.3.i summarizes experiments conducted during the selection period.



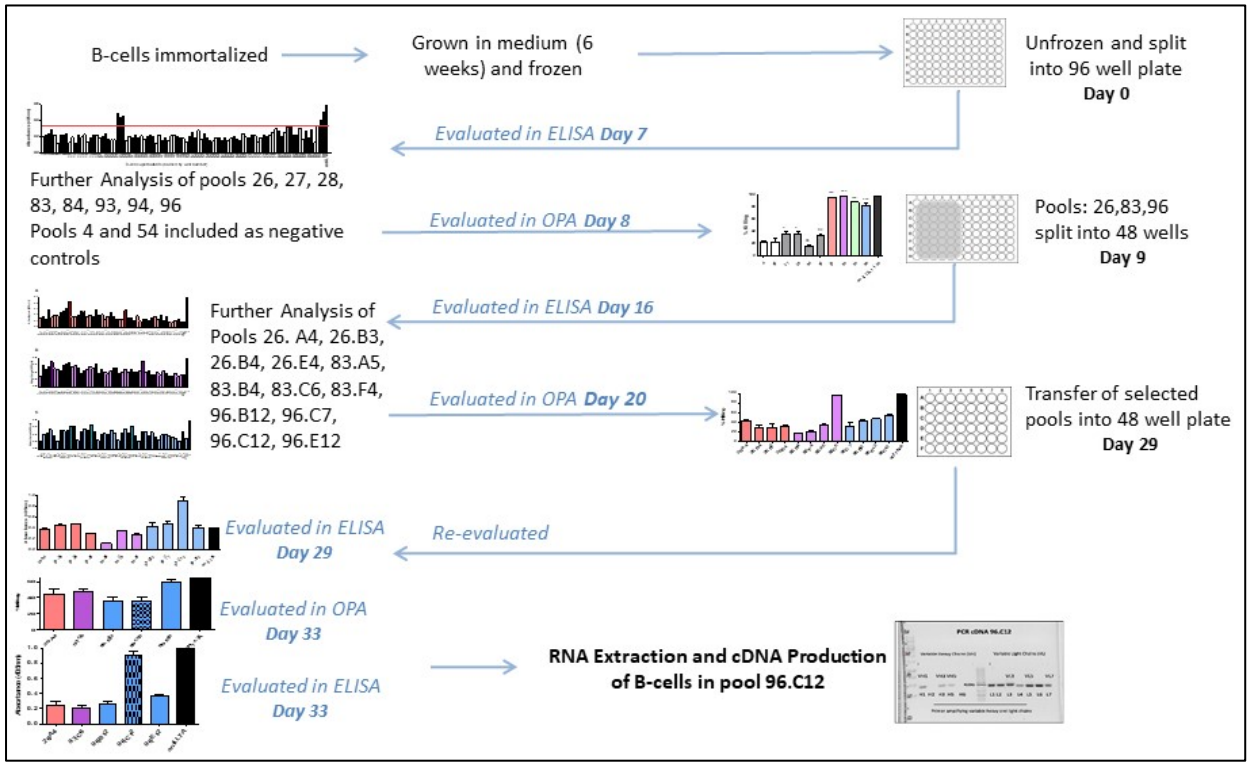
15 Figure 4.3g. Opsonophagocytic assay of B-cell SPNs after the second splitting on ”Day 33”.

Opsonophagocytic assay was performed using SPNs of B-cells cultured in a 48-well-plate after the second splitting. Anti-LTA serum was used as positive control at a final dilution of 1:100 (black bar) and the strain tested was E. faecalis 12030. Pool 26.A4 and pool 83.C6 are represented by red and purple bars, respectively. Pools derived from pool 96 are shown in blue bars. Pool 96.C12 (black and blue squares) was selected for further analysis. Bars represent the mean of data and the error bars represent the standard error of the mean.



16 Figure 4.3h. Immunoreactivity of B-cell supernatant of second splitting against LTA on “Day 33”.

Indirect ELISA was performed in order to monitor the immunoreactivity of SPNs of B-cell pools taken on the same day as RNA extraction was performed. Anti-LTA was included as a positive control (black bar). Pool 26.A4 and pool 83.C6 are represented by red and purple bars, respectively. Pools derived from pool 96 are shown in blue bars. Pool 96.C12 (black and blue squares) was selected for further analysis. Bars represent the mean of data and the error bars represent the standard error of the mean.



17 Figure 4.3i. Summary of selecting process

B-cells were immortalized by EBV-infection and cultured. Immortalized B-cells were further split into 96 wells and supernatants of pools of cells were selected according to their immunoreactivity towards LTA from S. aureus. Immunoreactive antibodies produced by pools of B-cells were later tested by OPA to determine those with highest killing activity against E. faecalis 12030. Further splitting of the immunoreactive and opsonic pools of B-cells was performed. Finally, pool 96.C12 was selected since it produced antibodies showing the highest immunoreactivity in ELISA and effective killing in OPA. RNA was extracted and cDNA was subsequently produced.

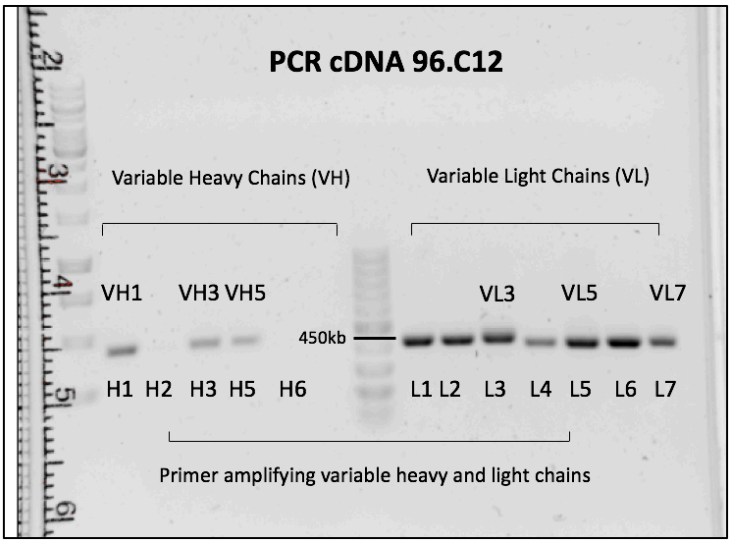
4.4. Identification of encoding sequences of variable heavy and light chains of the antibody by cDNA analysis

The cDNA of B-cells from pool 96.C12 encoding the antibody/ies that mediated opsonophagocytic killing of *E. faecalis* 12030 and immunoreactivity against LTA was analyzed. Later the coding sequences of the variable domains of light and heavy chains were determined and compared to each other. Finally, plasmids containing an insert with the encoding variable domains were generated to transfect eukaryotic cells and overexpress the respective antibodies.

4.4.1. Heavy and Light chains expressed in pool 96.C12

The cDNA of pool 96.C12 was synthetized after RNA extraction, and a concentration of 1511,2 ng/µL was obtained. According to manufacturer's standards the 260/280 ratio of 1,77

indicated a good quality of the cDNA which is considered to be pure at a 260/280 ratio of ~1,8 [133]. The cDNA was then amplified by PCR using GoTaq enzyme and primers as listed in Tables 3.4.2a and 3.4.2b. Figure 4.4.1a shows a gel electrophorogram of the PCR products. Bands at approximately 450bp were expected, since the variable heavy and light chain encoded a full variable region of the antibody. Only PCR products that yielded intense bands were selected for further analysis, as faint bands normally indicate unspecific binding of the primer (see Fig. 4.4.1a). Not all sets of primers amplified the variable domains of the heavy chains, only primers H1, H3, H5 yielded a product and were named accordingly VH1, VH3 and VH5 (VH representing variable heavy chain) for further analysis. These PCR products contain sequences for all theoretically possible heavy chains present in antibody/ies that mediated opsonophagocytic killing activity and were immunoreactive against LTA in pool 96.C12. Regarding the analysis of light chains, all sets of used primers yielded products. This was expected since these primer sets include degenerated nucleotide sequences binding unspecifically and amplify a broad range of variable region sequences (see Figure 4.4.1a.). The products of primers L3, L5 and L7, were named VL3, VL5 and VL7 respectively, and chosen for further analysis.



18 Figure 4.4.1a. Gel electrophoresis of amplified variable domain sequences of mAbs present B-cell pool 96.C12.

PCR was performed according to manufacturer's instructions for **GoTaq** (Promega, Mannheim, Germany). The band expected to correspond to the size of the amplified variable heavy and light chain sequence is indicated by the black line (around 450kb). By using the set of primers **H1**, **H2**, **H3**, **H5** and **H6**, variable heavy chains (**VH**) **VH1**, **VH3** and **VH5** were amplified. Sets of primers **L1**, **L2**, **L3**, **L4**, **L5**, **L6** and **L7** yielded a product but only amplified variable light chains (**VL**) **VL3**, **VL5** and **VL7** were further analyzed.

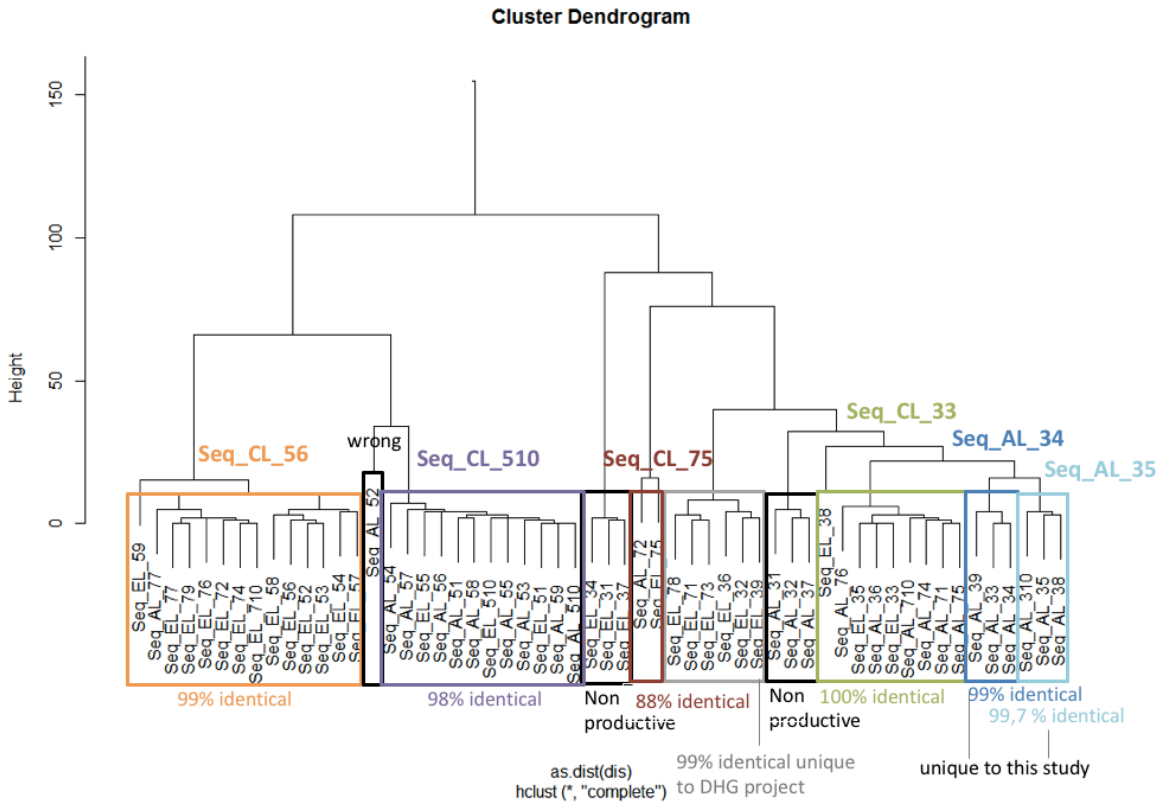
In order to sequence the variable domains, PCR products were purified and inserted into the 2.1-TOPO vector. Up to ten successfully transfected colonies were chosen to be sequenced. Therefore, the number of amplified inserts in *E. coli* varies from sample to sample (see Table 4.4.1a). All samples were controlled by PCR for the presence of an insert of the desired variable chain before they were sent to be sequenced.

24 Table 4.4.1a. *E. coli* generated for sequencing of the variable light and heavy chains.

Primer amplifying the inserts	Insert in electrocompetent *E.coli* Top 10
H1	AH11, AH12, AH13, AH14, AH15, AH16, AH17, AH18, AH19, AH10
H3	AH31, AH32, AH33, AH34, AH35, AH36, AH37, AH38
H5	AH51, AH52, AH53
L3	AL31, AL32, AL33, AL34, AL35, AL36, AL37, AL38, AL39, AL310
L5	AL51, AL52, AL53, AL54, AL55, AL56, AL57, AL58, AL59, AL510
L7	AL71, AL72, AL73, AL74, AL75, AL76, AL77, AL78, AL79, AL710

4.4.2. Analysis of variable light chain sequences

All samples were sequenced by Eurofins genomics. The sequences were then translated into amino acid sequences with the online available Expasy translate tool. They were compared to known antibody sequences by IgBlast and were finally checked for containing a signal peptide. Also, amino acid sequences were compared to each other by protein blast. Figure 4.4.2a shows the Optimal String Alignment (according to Restricted Damerau-Levenshtein distance) clustering the amino acid sequences based on their similarities [130]. Since in our group antibodies targeting DHG were analyzed as well, all sequences resulting from the DHG work were named Seq_**EL**_X while the ones from this study (targeting LTA and *E. faecalis* 12030) are named Seq_**AL**_X, with X indicating the insert in electrocompetent *E. coli* Top 10 described in Table 4.5.1b. (see Figure 4.4.2a.). Some sequences of variable light chains overlap between the two projects and were named **CL**_X (common light chain). The most complete sequence analyzed, showing no mutations according to IgBlast, being very similar to the other chains and encoding a full IgG, was chosen to perform the corresponding construction in the pFUSE plasmid (i.e. Sequences CL_56, CL_510, CL_75, CL_33, AL_34 and AL_35). Seq_AL_34 and Seq_AL_35 were unique to this study, while Seq_EL_39 was unique for the DHG project. Sequences failing to be analyzed (i.e. Seq_AL_52 wrong, black) or to codify for an IgG molecule (i.e. non-productive, black) were excluded from this study.



19 Figure 4.4.2a. Cluster Dendrogram of optimal string alignment according to Restricted Damerau-Levenshtein distance for the sequenced variable light chains of pool 96.C12.

Identical sequences (overlapping amino acid sequences by the dendrogram) were grouped and one of them used to insert the variable domain in the plasmid containing the constant domain's light chains. Grouped sequences Seq_CL_56 were 99% identical to each other (orange). Grouped sequences Seq_CL_510 were 98% identical to each other (purple). Sequence Seq_CL_75 was 88% identical to Seq_CL_72 (both red). Grouped sequences Seq_CL_33 were 100% identical to each other (green). Grouped sequences Seq_AL_34 were 99% identical with each other (dark blue). Grouped sequences Seq_AL_34 were 99% identical with each other (light blue). Sequences that failed to be analyzed (i.e. Seq_AL_52 wrong, black) or to codify for an IgG molecule (i.e. non-productive, black) were excluded from this study.

In Table 4.4.2a the sequences that were chosen to be cloned into the pFUSE2-CLIg-hl2 vector as well as the identical sequences within their group (identity ≥87%, see Figure 4.4.2a) are listed. All of the chosen sequences were analyzed using IgBlast and SignalP4.1 online servers. They were controlled for the Top V and J gene match, the chain type, the presence of a stop codon, for being in-frame and for encoding a complete IgG (see Table 4.4.2b).

25 Table 4.4.2a. Inserts chosen to be cloned in the pFUSE plasmid and the respective identical inserts

Insert to be cloned in pFUSE	Identical Insert
AL34	AL33, AL39
AL35	AL38, AL310
CL33	AL 71, AL 74, AL75, AL 76, AL 710, AL36
CL56	AL77
CL510	AL51, AL53, AL54, AL55, AL56, AL57, AL58, AL59, AL510
CL75	AL72

26 Table 4.4.2b. Selected variable light chain sequences analyzed by IgBlast and SignalP4.1

	Seq_AL_34	Seq_AL_35	Seq_CL_33	Seq_CL_56	Seq_CL_510	Seq_CL_75
Top V gene match	IGLV2-14*01	IGLV2-11*01	IGLV2-18*02	IGLV3-25*02	IGLV3-1*01	IGLV8-61*01
Top J gene match	IGLJ2*01, IGLJ3*01	IGLJ3*02	IGLJ2*01, IGLJ3*01	IGLJ3*02	IGLJ1*01	IGLJ3*02
Chain type	VL	VL	VL	VL	VL	VL
Stop codon	No	No	No	No	No	No
V-J frame	In-frame	In-frame	In-frame	In-frame	In-frame	In-frame
Productive	Yes	Yes	Yes	Yes	Yes	Yes
Signal Peptide	Yes	Yes	Yes	No	Yes	Yes

4.4.3. Analysis of variable heavy chain sequences

Multiple identical sequences of variable heavy chains were chosen to perform the cloning in the pFUSE-CHIg-hG1 vector (identity ≥ 90%). The Analysis in IgBlast showed that sequences Seq_CH_12 and Seq_CH_19 overlap with sequences in the DHG project (data not shown), while Seq_AH_38 and Seq_AH_51 were unique to this study. Table 4.4.3a lists all inserts that were selected to be cloned into the pFUSE system and the inserts identical with them. Inserts not listed in this table did not encode a complete IgG and therefore were not used for further analysis. The sequences of all selected inserts were analyzed through IgBlast and SignalP4.1 online servers. They were controlled for the Top V and J gene match, chain type, the presence of a stop codon, for being in-frame and for encoding a complete IgG (Table 4.4.3b).

27 Table 4.4.3a Inserts chosen to be inserted in the pFUSE plasmid and their respective identical sequences

Inserts to be cloned in pFUSE	Identical inserts
CH12	AH11, AH13, AH17
CH19	AH19
AH38	AH32, AH34, AH35, AH36, AH37
AH51	AH51, AH52, AH53

28 Table 4.4.3b. Selected variable heavy chain sequences analyzed by IgBlast and SignalP4.1

	CH19	CH12	AH38	AH51
Top V gene match	IGHV1-3*01	IGHV1-18*01, IGHV1-18*03	IGHV3-33*01, IGHV3-33*05	IGHV5-51*01
Top D gene match	IGHD3-3*01	IGHD3-10*01	IGHD1-26*01	IGHD2-21*02
Top J gene match	IGHJ6*02	IGHJ5*02	IGHJ6*02	IGHJ5*02
Chain type	VH	VH	VH	VH
Stop codon	No	No	No	No
V-J frame	In-frame	In-frame	In-frame	In-frame
Productive	Yes	Yes	Yes	Yes
Signal Peptide	Yes	Yes	Yes	Yes

4.4.4. Amino acid sequences variable chains used for antibody production

The amino acid sequences of the fragments cloned into the pFUSE system to be overexpressed in CHO-DHFR$^-$ and HEK-293T cells are listed in Table 4.4.4a. All sequences encode for the variable domains of an antibody according to the IGBLAST analysis. They contain three complementary determining regions (CDRs) and four framework regions (FRs) which are crucial for correct antigen binding [134] [112]. All inserts encode a whole protein without any stop codons according to Expasy Translate tool. Except from AL56 chain, the variable chains include a signal peptide, ensuring the secretion of the protein.

The DNA insert of variable and heavy chains using primers designed for pFUSE was ligated into the pFUSE plasmid before the plasmid was electroporated into electrocompetent *E.coli* Top 10. Then, the resulting bacterial colonies were analyzed by PCR to identify the correctly transfected clones. A plasmid preparation from these clones was performed for subsequent analysis of the insert's sequence. Once the sequence of the variable domains was verified, a plasmid preparation was carried out for transfection of the pFUSE plasmids in HEK-293T cells. Plasmid DNA concentrations are shown in Table 4.4.4b.

29 Table 4.4.4a. Amino acid sequences of variable heavy and light chains used in this study

Name	Aminoacid Sequence
CH12	MDWTWSILFLVAAATGAHSQDHLVQSGAVVRKPGASVKVSCKANGYSFSDYGITWVRQAPGQGLEWM GWISAYNGNTVYAQKFHDRLTMTTETSTSTAYLELRSLRFDDRAVYYCARAPYNSNYGDWFDPWGQGTL VTVSSASTKGP
CH19	MDWTWRIPFLVAAATGAHSQVQLVQSGAEVKKPGASVKVSCKASGYTFTSYAMHWVRQAPGQRLEW MGWIDADDGNTKYSQKFQGRVTISRGTSATTAYMELSSLRSEDTAVYYCARDNHWIFGVERYHYFGMDV W GQGTTVTVSSASTKGP
AH38	MESGLSWVFLVALLRGVQCQVHLVESGGGVVQPGRSLRLSCAASGFTFRSSGMHWVRQAPGKGLEWVA VIYSDGSNKYYADSVKGRFTISRDNSKSTLYLQMNSLRAEDTAVYYCARGVGATVSVRYYTMDVWGQGTT VTVSSAS
AH51	MGSTAILALLLAVLQGVCAEVQLVQSGAEVKKPGESLKISCKTSGYTFTSYWIGWVRQMPGKGLEWMGIIY PGDFESRYSPSFQGQVTISVDKSINTAYLQWSSLKASDTAMYYCARLIAYCSGDCYSRFDPWGQGTLVTVSS AS
AL34	MAWALLFLTLLTQGTGSWAQSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIY DVSNRPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTPYVVFGGGTKLTVLGQPKAAPSV
AL35	MAWALLLLSLLTQGTGSWAQSALTQPRSVSGSPGQSVTISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIY DVSKRPSGVPDRFSGSKSGSTASLTISGLQAEDEADYYCCSYAGSYTFWVFGGGTKLTVLGQPKAAPSV
CL33	MAWALLLLTLLTQGTGSWAQSALTQPPSVSGSPGQSVTISCTGTSSDVGSYNRVSWYQQPPGTAPKLMIY EVSNRPSGVPDRFSGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTLVFGGGTKLTVLGQPKAAPSV
CL56	MAWMMLLPLLTLCTGSEASYELTQPPSVSVSPGQTARITCSADALPKQYAYWYQQKPGQAPVLVIYKDSE RPSGIPERFSGSSSGTTVTLTISGVQAEDEADYYCQSADSSGAYQVFGGGTKLTVLGQPKAAPSV
CL510	MAWIPLFLGVLAYCTGSVASYELTQPPSVSVSPGQTASITCSGDKLGDKYACWYQQKPGQSPVLVIYQDSK RPSGIPERFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTAVFGTGTKVTVLGQPKAAPSV
CL75	MAWMMLLLGLLAYGSGVGSQTVVTQEPSFSVSPGGTVTLTCGLGSGSVSTSYYPSWYQQTPGQAPRTLIY STNTRSSGVPDRFSGSILGNKAALTITGAQADDESDYYCVLYMGSGIWVFGGGTKLTVLGQPKAAPSV

30 Table 4.4.4b. Concentration of plasmid DNA used for transfection procedures

Plasmid	Concentration ng/µL
pF_CH12	759,6
pF_CH19	890,3
pF_AH38	578,4
pF_AH51	703,9
pF_AL34*	765
pF_AL35	506
pF_CL33*	1311,9
pF_CL56*	1331,8
pF_CL510*	1269
pF_CL75*	1379

* Samples were concentrated using the SpeedVac

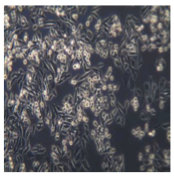
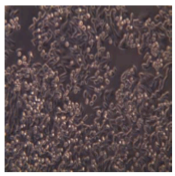
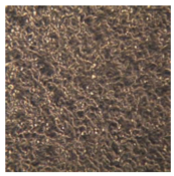
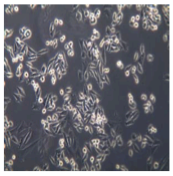
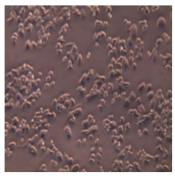
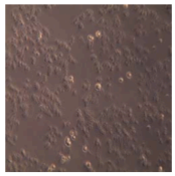
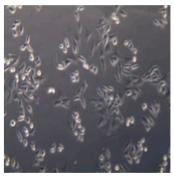
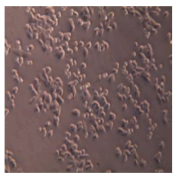
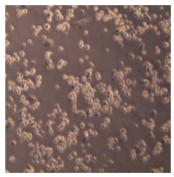
4.5. Transfection of eukaryotic cells

4.5.1. Standardization of antibiotic concentration for transfection of CHO-DHFR$^-$ cell line with pFUSE plasmids

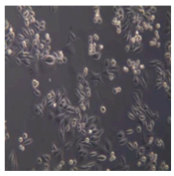
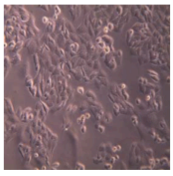
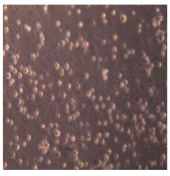
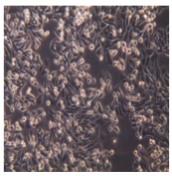
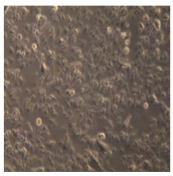
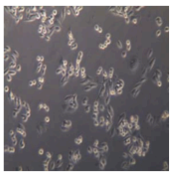
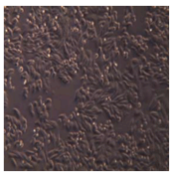
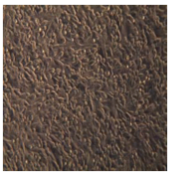
The CHO-DHFR$^-$ cell line does not carry the genetic information conferring resistance against the antibiotics used to overexpress the antibodies with the pFUSE vector system. The pFUSE-plasmids carry resistance genes against Blasticidin (pFUSE2-CLIg-hl2) and Zeocin (pFUSE-CHIg-hG1), respectively. To establish the antibiotic concentration (i.e. of Zeocin and Blasticidin) to be used with our CHO-DHFR$^-$ cell line after transfection, cells were seeded on a six-well plate and one day later, Blasticidin was added in a concentration of 10 µg/mL and 7 µg/mL while Zeocin was added in a concentration of 200 µg/mL and 300 µg/mL. Cells were periodically observed under the microscope for changes in morphology, adherence and survival. Medium without nucleosides was used as positive control causing rapid cell death since our cell line was DHFR deficient and requires glycine, hypoxanthine, and thymidine for growth [135]. For Blasticidin, no marked differences between the two concentrations were observed, both leading to cell death from day one to two. For Zeocin, 300 µg/mL lead to cell death after one to two days, while 200 µg/mL did not affect cell survival even after two days in medium with antibiotics (see Figure 4.5.1a.). Thus, Blasticidin was used in a concentration of 7.5 µg/mL (sufficient to select cells containing pFUSE2-CLIg-hG1 vector) and 300 µg/mL of Zeocin (to select pFUSE-CHIg-hl2 vector).

According to the manufacturer's instructions, the concentrations of Zeocin and Blasticidin used for CHO-cell lines can also be used for HEK-293T-cell lines. Therefore, identical antibiotic concentrations were used for transfection of HEK-293T cells (Invivogen, San Diego, USA).

A.

Medium	Day 1	Day 2	Day 3
	with nucleosides	without nucleosides	without nucleosides
Control αCHO Growth Medium			
	without antibiotics	with antibiotics	with antibiotics
αCHO Growth Medium with Nucleosides supplemented with **10 µg/mL Blasticidin** from Day 2			
αCHO Growth Medium with Nucleosides supplemented with **7,5 µg/mL Blasticdin** from Day 2			

B.

Medium	Day 1	Day 2	Day 3
	with nucleosides	without nucleosides	without nucleosides
Control αCHO Growth Medium			
	without antibiotics	with antibiotics	with antibiotics
αCHO Growth Medium with Nucleosides supplemented with **300 µg/mL Zeocin** from Day 2			
αCHO Growth Medium with Nucleosides supplemented with **200 µg/mL Zeocin** from Day 2			

20 Figure 4.5.1a. Standardization of antibiotic concentration for transfection of CHO-DHFR$^-$cell line with pFUSE vector system.

CHO-DHFR$^-$ cells were cultured for three days. After day one, antibiotics were added to the medium at different concentrations. **A.** Blasticidin was added for final concentrations of 7,5 µg/mL and 10 µg/mL. **B**. Zeocin was added for final concentrations of 200 µg/mL and 300 µg/mL. After 24 hours, cells were observed under the microscope and changes in cell shape and survival were detected. A second check was done after 48 hours of culturing. For both analyses a positive control was included in which cells were cultured in medium without nucleosides.

4.5.2. Production of antibodies in mammalian cell lines

The transient production of antibodies was performed in two different mammalian cell lines: CHO-DHFR$^-$ and HEK-293T cells. Transfection of pFUSE plasmid was performed both on CHO-DHFR$^-$ and HEK-293T cell lines to compare the production levels of each antibody of interest. Based on our results, the HEK-293T cell line was chosen to perform transfections at a larger scale (see below). Absence of mycoplasma infection during culturing of both cell lines was periodically confirmed.

pFUSE2-CHIg-hG1 and pFUSE2-CLIg-hl2 vectors encoded the constant region of either the heavy or the light chain, respectively. As the original combination of light and heavy chains in the original B-cell clone could not be determined, we generated 25 antibodies from all possible combinations of heavy and light chains as shown in Table 4.5.2a. Antibodies A4-A15, A17-A20, A23-A26 and A28-A31 were produced in mammalian cells with our constructs in pFUSE-CHIg-hG1 and pFUSE2-CLIg-hl2 vectors. As a negative control, antibody A22 containing the "empty" plasmids of pFUSE-CHIg-hG1 and pFUSE2-CLIg-hl2 (containing no insert) was produced. Variable chains produced were named VLX or VHX for light and heavy chains respectively. X refers to the number of the variable chain: "A" denotes chains that were unique to this study whereas "C" (common) indicates the chains shared with the DHG project.

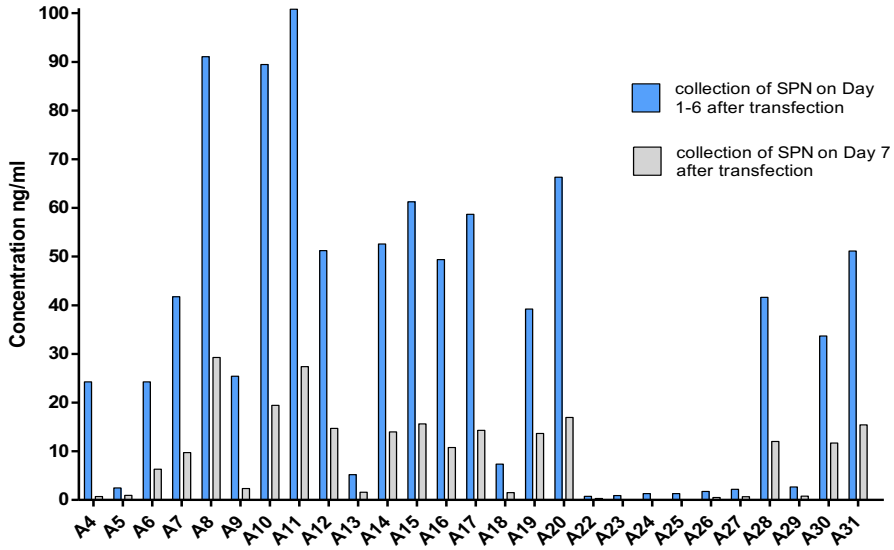
31 Table 4.5.2a. Name of antibodies containing the inserts of variable domains of light and heavy chains produced in mammalian cell lines

	VL Variable light chain	VH Variable heavy chain
A4	VLA34	VHC12
A5	VLA34	VHC19
A6	VLA34	VHA38
A7	VLA34	VHA51
A8	VLA35	VHC12
A9	VLA35	VHC19
A10	VLA35	VHA38
A11	VLA35	VHA51
A12	VLC33	VHC12
A13	VLC33	VHC19
A14	VLC33	VHA38
A15	VLC33	VHA51
A17	VLC510	VHC12
A18	VLC510	VHC19
A19	VLC510	VHA38
A20	VLC510	VHA51
A22	pFUSE VLCLIg	pFUSE VHIg
A23	VLC56	VHC12
A24	VLC56	VHC19
A25	VLC56	VHA38
A26	VLC56	VHA51
A28	VLC75	VHC12
A29	VLC75	VHC19
A30	VLC75	VHA38
A31	VLC75	VHA51

4.5.3. Transfection of CHO-DHFR$^-$cells

CHO-DHFR$^-$ cells were cultured in a 12-well plate and transfected as described above. The transfection rate was 50% for Pmax control plasmid (data not shown). The supernatant of the cells was collected seven times in 10 days. Supernatants from days one to six were pooled and supernatant from day seven was collected separately. Antibodies in these two pools were quantified as described above. On the seventh day after transfection, a pronounced decrease in antibody production was observed and therefore supernatants were no longer collected and cells discarded. The quantity of produced antibodies assessed in ELISA showed no detectable antibodies for the negative control A22 (< 1 ng/mL) and a maximum of 100,8 ng/mL for antibody A11, whereas antibodies A23, A24, A25 and A26 were produced in very small amounts only (< 1.7 ng/mL) (see Figure 4.5.3a).

A.



21 Figure 4.5.3a. Quantification of antibodies produced in CHO-DHFR- cells after transfection (total concentration).

A. Antibodies in the supernatant of CHO-DHFR$^-$ cells were quantified through ELISA and concentration of the antibodies are shown either after pooling the supernatants from day one to sixth after transfection (blue bars) and on the seventh after transfection (grey bars).

B.

Antibody	Concentration of SPN Day 1-6 ng/mL	Concentration of SPN Day 7 ng/mL
A4	24,241	0,711
A5	2,486	0,943
A6	24,241	6,338
A7	41,746	9,729
A8	91,070	29,273
A9	25,404	2,330
A10	89,463	19,425
A11	100,816	27,370
A12	51,218	14,719
A13	5,192	1,601
A14	52,571	13,980
A15	61,239	15,619
A17	58,681	14,314
A18	7,370	1,500
A19	39,209	13,656
A20	66,292	16,945
A22	0,732	0,296
A23	0,922	-0,069
A24	1,323	-0,069
A25	1,323	0,032
A26	1,746	0,498
A28	41,641	12,006
A29	2,655	0,771
A30	33,691	11,682
A31	51,133	15,447

21 Figure 4.5.3a. Quantification of antibodies produced in CHO-DHFR- cells after transfection (total concentration).

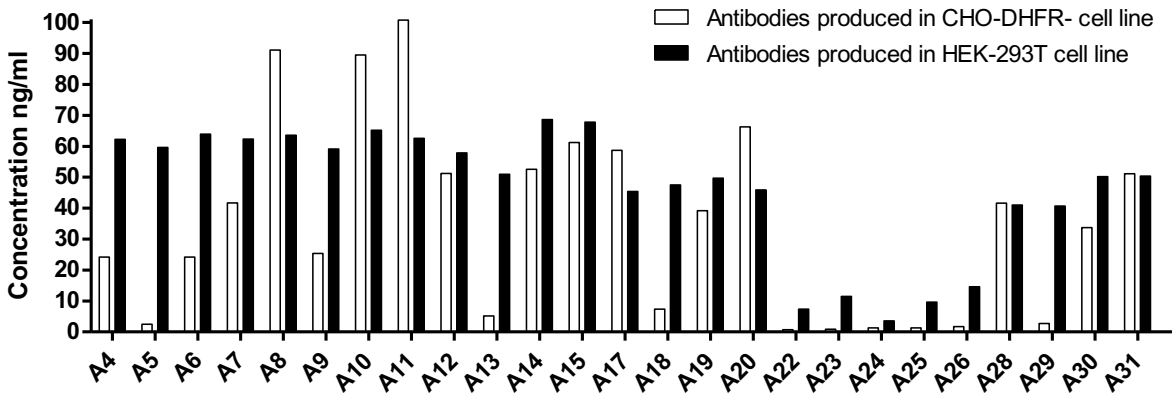
B. Values of concentrations calculated from the human IgG standard calibration curve.

4.5.4. Transfection of HEK-293T cells

The transfection of HEK-293T cells was performed exactly under the same conditions as for CHO-DHFR$^-$ cells in order to enable comparison of the two different cell lines and selection of the one which yielded the higher concentration of antibodies. 24 hours after transfection the GFP control vector showed that around 60% of HEK-293T cells were successfully transfected (data not shown). The supernatant was collected four times within the following six days. On day seven, cells were over confluency and were not further cultured. IgG quantification showed a similar antibody production for almost all produced antibodies (~50ng/mL). When produced in HEK-293T cells, antibodies A23, A24, A25, and A26 increased their low yield in comparison with CHO-DHFR$^-$ cells production, markedly by a 13, 3, 7 and 8 fold, respectively. Also, production of antibodies A5, A13, A18, and A29 increased in HEK-293T cells by a factor of 24, 10, 6 and 15 respectively in comparison to CHO-DHFR$^-$ cells. Interestingly, A22 also increased its production by a factor of 10 when produced in HEK-293T cells.

Figure 4.5.4a shows the difference in antibody production between CHO-DHFR$^-$ and HEK-293T cells. A consistent production of almost all antibodies could be observed in HEK-293T cells, including antibodies produced in very low yield in CHO-DHFR$^-$ cells (i.e. A5, A9, A13). Despite the increased production rates of antibodies A23-A26 by transfected HEK-293T cells, concentrations were still quite low when compared to other produced antibodies. All of those antibodies shared the variable light chain VLC56 lacking the signal peptide. Additionally, their activity could not be fairly compared to that of the other antibodies due to different concentrations. Therefore, antibodies A23 to A26 poorly produced in either CHO-DHFR$^-$ or HEK-293T cells were no longer produced or studied. Since we wanted to identify the cell line permitting the best and most consistent production of the studied antibodies, we selected the HEK-293T cell-line for further production of the antibodies of interest at higher scale.

A.



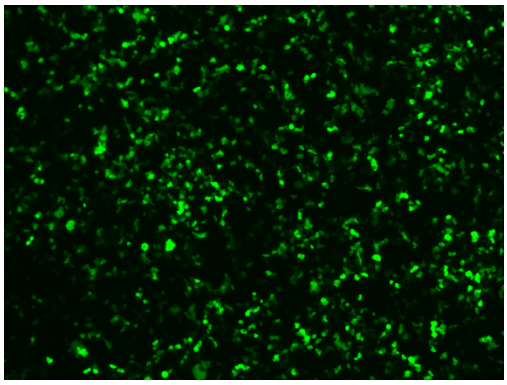
B.

Antibody	Production in HEK-293T cell line ng/mL	Production in CHO-DHFR- cell line ng/ml
A4	62,23	24,24
A5	59,56	2,49
A6	63,93	24,24
A7	62,35	41,75
A8	63,65	91,07
A9	59,06	25,40
A10	65,16	89,46
A11	62,56	100,82
A12	57,93	51,22
A13	51,05	5,19
A14	68,55	52,571
A15	67,83	61,24
A17	45,41	58,68
A18	47,52	7,37
A19	49,66	39,21
A20	45,87	66,29
A22	7,43	0,73
A23	11,54	0,92
A24	3,60	1,32
A25	9,55	1,32
A26	14,61	1,75
A28	41,00	41,64
A29	40,70	2,66
A30	50,21	33,69
A31	50,41	51,13

22 Figure 4.5.4a. Comparison of antibody concentration when produced either in CHO-DHFR$^-$ or HEK-293T cells.
A. Concentrations of antibodies produced in CHO-DHFR$^-$cells from day 1 to day 6 after transfection are represented as white bars, and those of antibodies produced in HEK-293T cells as black bars. F-test showed that variances of the groups are significant. **B.** Antibodies produced in the HEK-293T and CHO-DHFR$^-$ cells were quantified in triplicates and concentrations were calculated according to a standard calibration curve.

4.5.5. High scale production of antibodies in HEK-293T cells

In order to achieve higher yields of antibodies, HEK-293T cells were seeded close to confluency in 10mm plates and transfected one day later. Supernatant was collected on day 0, 2, 4 and 6. On day 2, the transfection rate was evaluated in the cells transfected with GFP vector by fluorescence microscopy and approximately 60% of the cells appeared to be transfected (see Figure 4.5.5a.).

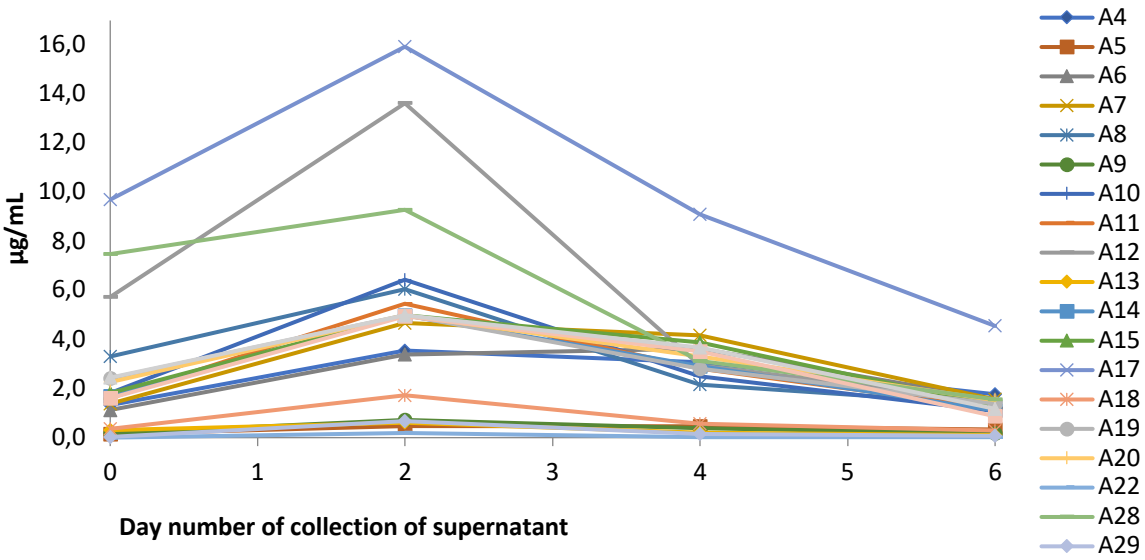


23 Figure 4.5.5a. Evaluation of transfected cells by GFP.

Green fluorescent protein vector (GFP) marks successfully transfected cells (approximately 60%).

In order to analyze the production of the antibodies in HEK-293T cells after transfection, the supernatant was taken on different days, separately quantified and compared to each other. As shown in Figure 4.5.5b, cells produced the highest amount of antibody on day 2 and antibody production was lowest on day 6. Collection of supernatants beyond this day was not carried out. As mentioned before, antibodies A23, A24, A25 and A26 were not transfected as these combinations had not given a reasonable yield in the pilot assessment (see section 4.5.3.). In general, most antibodies were produced by the 15 to 216-fold compared to the previous production in HEK-293T cells at lower scale. However, antibodies A5, A9, A13 and A29 only increased by a factor of 6 (see Figure 4.5.5b)

A.



B.

Antibody	µg/mL	Factor of increased production for higher scale transfection
A4	2,43	39
A5	0,36	6
A6	2,43	38
A7	2,94	47
A8	3,22	51
A9	0,37	6
A10	2,96	45
A11	2,77	44
A12	5,94	103
A13	0,30	6
A14	2,68	39
A15	2,99	44
A17	9,82	216
A18	0,74	15
A19	2,90	58
A20	3,02	66
A22	0,06	8
A28	5,36	131
A29	0,23	6
A30	2,73	54
A31	3,07	61

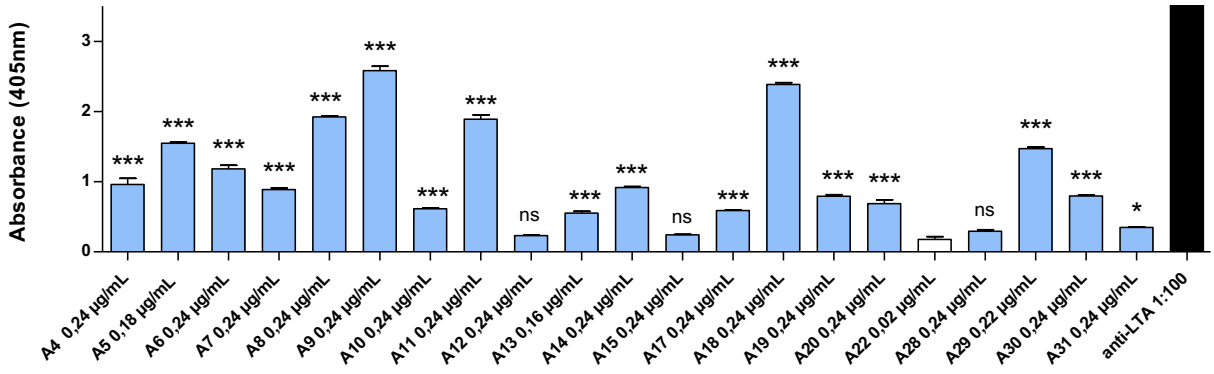
24 Figure 4.5.5b. Production of antibodies in HEK-293T cells during six days after transfection.

A. The concentration of antibodies (in µg/mL and colours according to the legend) in the supernatant was measured for each day of collection. Days 2 and 4 after transfection showed the highest antibody production **B.** Antibodies produced in the HEK-293T cells were quantified and values of concentrations were calculated according to a standard calibration curve. The increase factor was calculated comparing the concentration of antibodies produced in the high yield transfection and the concentration of antibodies when produced at small scale in HEK-293T cells.

4.6. Activity of monoclonal antibodies

4.6.1. Immunoreactivity towards LTA and *E. faecalis* 12030 in ELISA

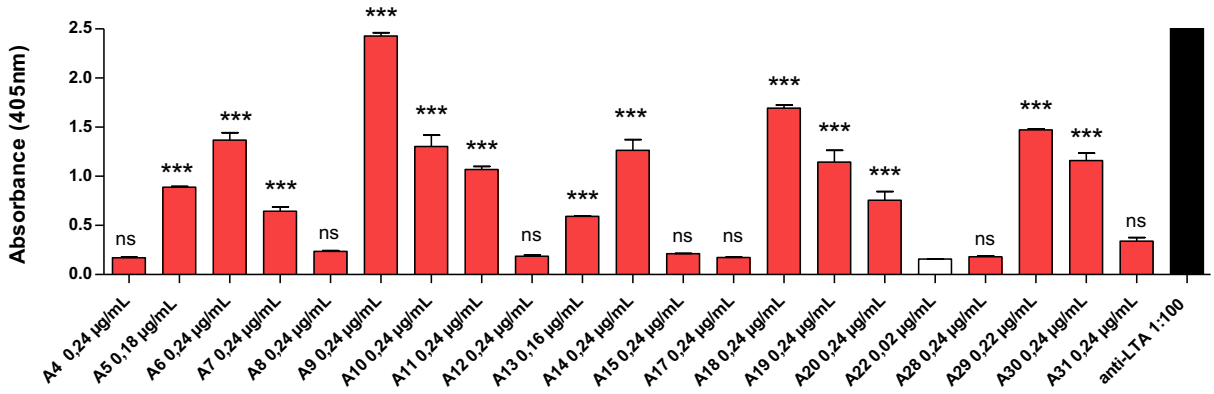
The immunoreactivity of *in vitro* produced monoclonal antibodies was assessed in ELISA against LTA and in a whole cell ELISA against target strain *E. faecalis* 12030 (See Figures 4.6.1a and 4.6.1b respectively). Tests were performed using the dialyzed supernatant collected on day 4 as described above. The antibody concentration of the samples was adjusted to 0.24 µg/mL to compare the immunoreactivity of all antibodies. Some samples did not reach this concentration and were therefore evaluated at lower concentrations (the concentration of each sample tested is indicated in the respective figures). A22, dialyzed from the supernatant of HEK-293T cells transfected with pFUSE plasmids containing no insert for variable light and heavy chains, was included as negative control although its concentration was not in the same range as the rest of the produced antibodies. Monoclonal antibodies A4, A5, A6, A7, A8, A9, A10, A11, A13, A14, A17, A18, A19, A20, A29, A30 amd A31 showed significant immunoreactivity towards LTA assessed in ELISA (see Figure 4.6.1a.), while antibodies A12, A15 and A28 did not. All samples tested differ significantly from the negative control A22 (Dunnett test for multiple comparisons).



25 Figure 4.6.1a. Immunoreactivity of monoclonal antibodies towards LTA.

Indirect ELISA was performed to test antibodies in the SPN of transfected HEK-293T cells. Anti-LTA was included as a positive control (black bar). Dunnett Test for multiple comparisons showed that the antibodies A4, A5, A6, A7, A8, A9, A10, A11, A13, A14, A17, A18, A19, A20, A29, A30, A31 (asterisk) were statistically significant from negative control A22 (white bar). Asterisks denote significance (ns, not significant, * P≤0.05, ** P ≤ 0.01, *** P ≤ 0.001).

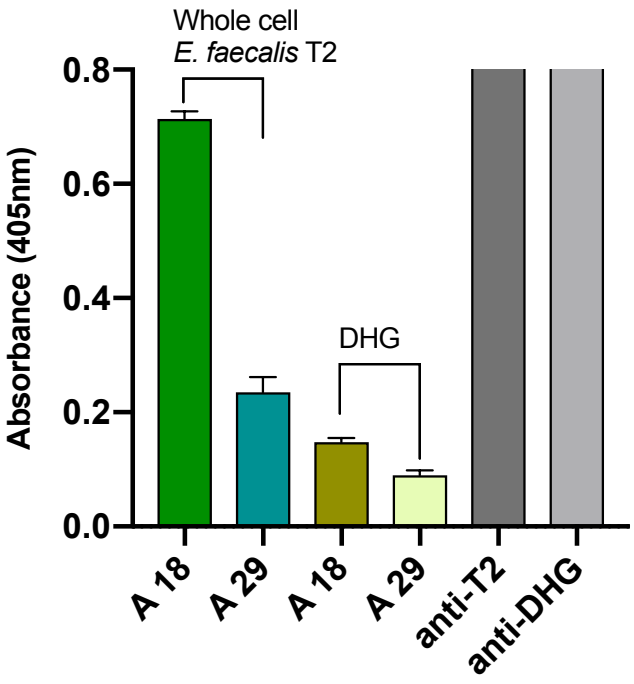
Immunoreactivity towards *E. faecalis* 12030 was assessed by whole cell ELISA. Monoclonal antibodies A5, A6, A7, A9, A10, A11, A13, A14, A18, A19, A20, A29 and A30 exhibited significant immunoreactivity (see Figure 4.6.1b.); while antibodies A4, A8, A12, A15, A17, A28 and A31 failed to do so.



26 Figure 4.6.1b. Immunoreactivity of monoclonal antibodies towards E. faecalis 12030.

Whole cell ELISA was performed to test antibodies in the SPN of HEK-293T cells. Anti-LTA was included as a positive control (black bar). Antibodies A5, A6, A7, A9, A10, A11, A13, A14, A18, A19, A20, A29, A30 (asterisk) were significantly from negative controls A22 (white bar) (Dunnett Test for multiple comparisons). Asterisks denote significance (ns, not significant, * P≤0.05, ** P ≤ 0.01, *** P ≤ 0.001).

Simultaneously, immunoreactivity in ELISA towards DHG, a polysaccharide masking LTA in encapsulating strains such as *E. faecalis* Type 2, was performed to all antibodies. In Figure 4.6.1c, the immunoreactivity of the two antibodies (i.e. A18 and A29) that exhibited the highest immunoreactivity against DHG and the whole cell of *E. faecalis* Type 2 is shown.

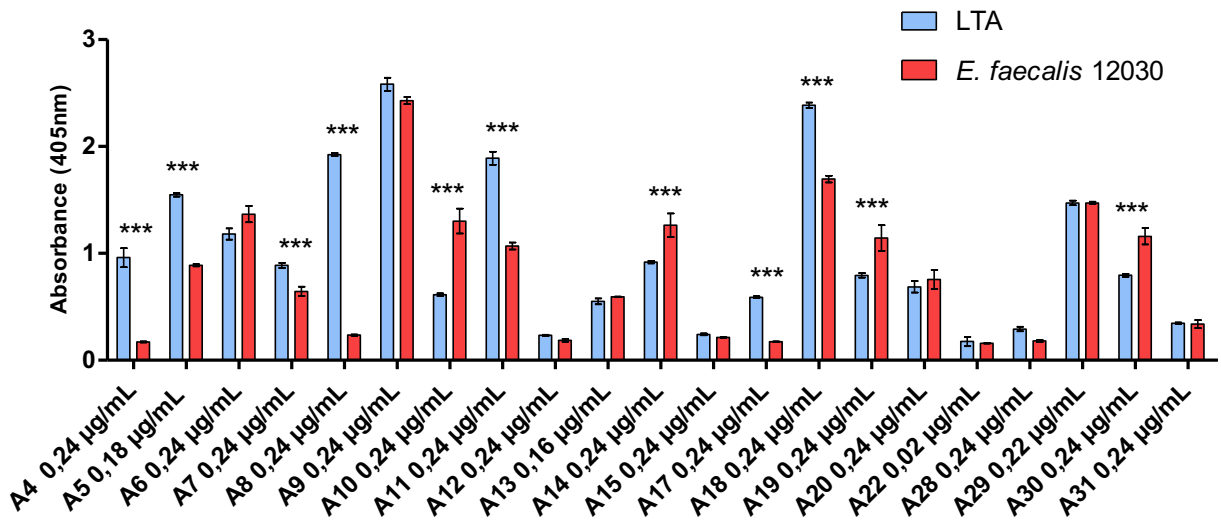


27 Figure 4.6.1c. Immunoreactivity of monoclonal antibodies towards E. faecalis T2 and DHG.

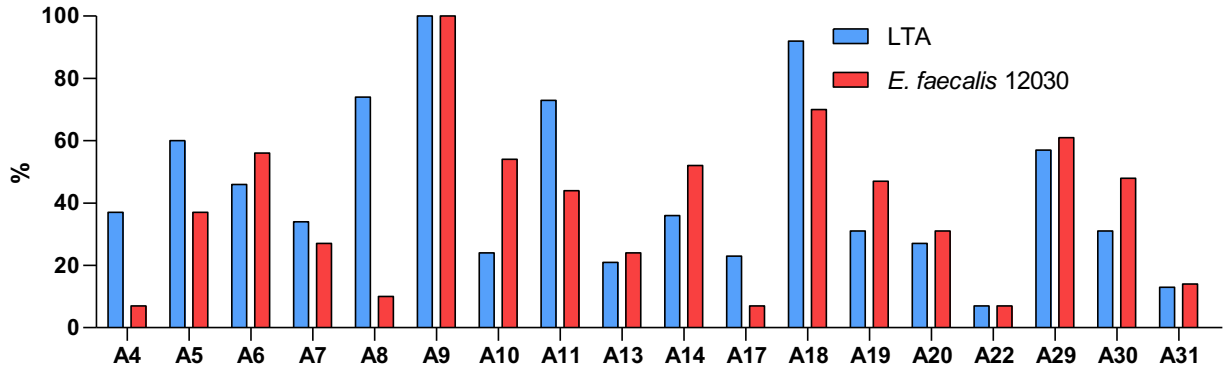
Whole cell ELISA was performed to test antibodies in the supernatant of HEK-293T cells. Anti-T2 and anti DHG were included as a positive controls (dark grey and light grey bar, respectively).

As shown in Figure 4.6.1d A., immunoreactivity towards *E. faecalis* 12030 and LTA were compared among all antibodies tested. Two-way ANOVA and Bonferroni post-test were used to determinate significance of immunoreactivity towards LTA or *E. faecalis* 12030. Most samples exhibiting a significant value of immunoreactivity against LTA also showed significance when tested against *E. faecalis* 12030. Only some antibodies showed differences in immunoreactivity against LTA from *S. aureus* and whole bug of *E. faecalis* 12030. To compare the two ELISA assays in a valid way we also showed this correlation when values were normalized to the highest value of each data set (ELISA against *E. faecalis* 12030 and against LTA, respectively).

A.



B.

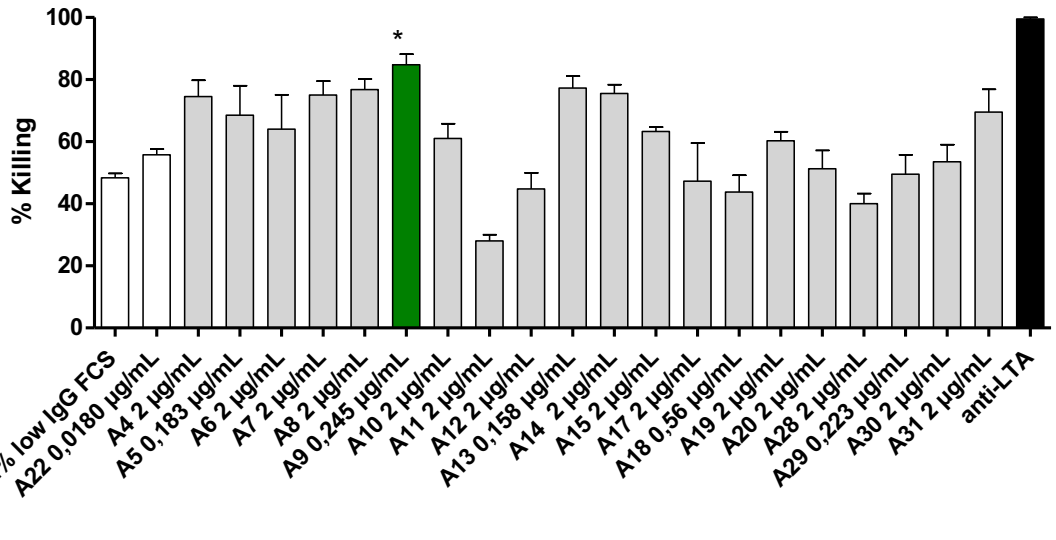


28 Figure 4.6.1d A. Immunoreactivity towards LTA and E. faecalis 12030 in ELISA.

Immunoreactivity towards LTA and E. faecalis 12030 represented by blue and red bars, respectively. Two way ANOVA and Bonferroni Post were used to assess whether monoclonal antibodies showed significant differences between their immunoreactivity towards LTA and that to E. faecalis 12030. Antibodies A4, A5, A7, A8, A10, A11, A14, A17, A18, A19 and A30 immunoreact significantly differently towards LTA than to whole bug of E. faecalis 12030. Asterisks denote significance (ns, not significant, * P≤0.05, ** P ≤ 0.01, *** P ≤ 0.001). **B. Correlation of immunoreactivity towards LTA and *E. faecalis* 12030 in ELISA.** Values of Figures 4.6.1a and 4.6.1b have been normalized to the highest value of the data set. Normalized values of immunoreactivity towards LTA and E. faecalis 12030 are represented by blue and red bars, respectively.

4.6.2. Opsonophagocytic killing activity of monoclonal antibodies

The opsonophagocytic killing activity of antibodies produced in HEK-293T cells was assessed using samples at a concentration adjusted to 2 µg/mL. This was the highest concentration possible to use, that most of the samples could be adjusted to. Only A5, A9, A13, A18, A22 and A29 were tested at lower concentrations as indicated in Figure 4.6.2a. All antibodies tested in the assay exhibited killing activities higher than 20%. Statistical analysis revealed that killing activity of antibody A9 (>80%), which was tested at a concentration of 0.245 µg/mL, was significantly higher than that of the negative controls and the rest of the antibodies tested. At 2µg/mL antibody A11 showed the lowest opsonophagocytic activity from all antibodies tested confirming that medium has an impact in OPA, since this highly produced antibody was diluted to 2µg/mL in OPA medium for the assay (diluted 1:4 for the assay). As the negative controls used in the assay resulted to be unreliable, we cannot conclude which antibody(ies) possesses opsonophagocytic killing activity. Still, A9 showed high opsonophagocytic killing activity in OPA and high immunoreactivity in ELISA against whole cell *E. faecalis* 12030 and LTA of *S. aureus* even if tested at a lower concentration of (0.245µg/mL).

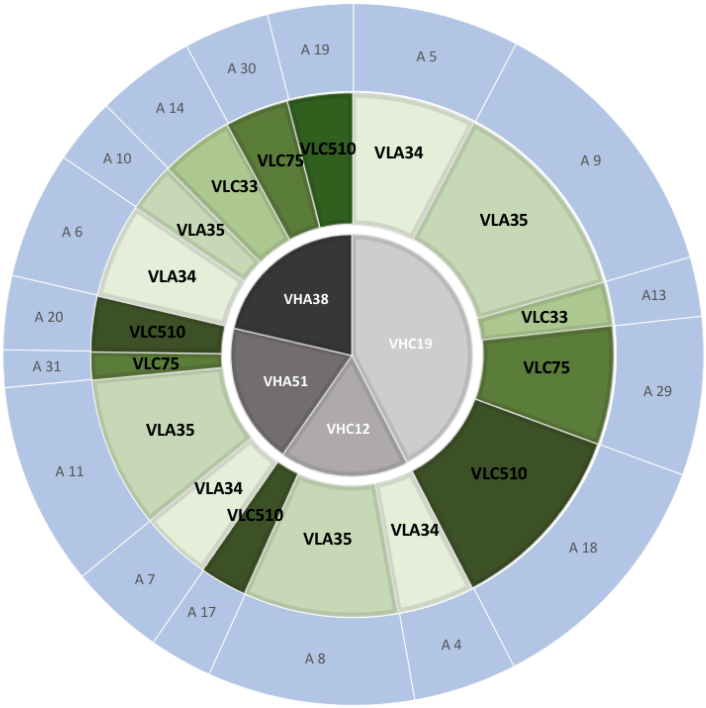


29 Figure 4.6.2a. Opsonophagocytic killing activity of antibodies produced in HEK-293T cells against E. faecalis 12030

Anti-LTA serum was used as positive control at the final dilution of 1:100 (black bar) and the strain tested was E. faecalis 12030. Negative controls are A22 as well as fresh medium (white bars). Dunnett Test for multiple comparisons showed that only A9 (green bar) was significantly different from A22. Bars represent the mean of data and the error bars represent the standard error of the mean. Asterisks denote significance (* P≤0.05, ** P ≤ 0.01, *** P ≤ 0.001).

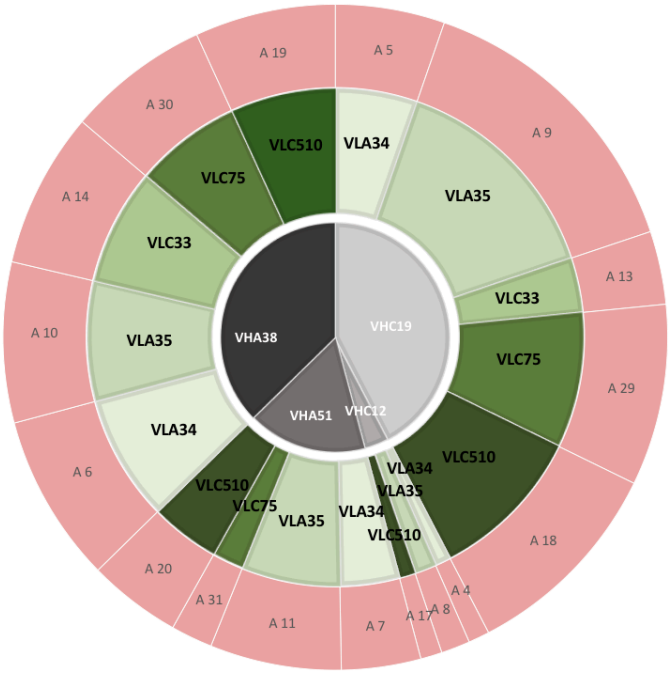
4.6.3. Immunoreactivity of specific variable light and heavy chains

Each of the antibodies produced is unique in its combination of variable heavy and light chains but in this study variable heavy or light chains are shared between some antibodies. In order to understand if a certain variable chain influences the binding activity to LTA of *S. aureus* or whole cell *E. faecalis 12030*, the antibodies – their variable chains and reaction pattern – are displayed in Figure 4.6.3a/b. These figures show that antibodies which exhibited the highest immunoreactivity towards LTA and/or *E. faecalis* 12030 (absorbance $\geq$ 0.8 U.A., units of absorbance) contained the heavy chain VHC19 (i.e. A5, A9, A18 and A29), besides antibody A13. Antibodies showing high immunoreactivity in both assays (absorbance $\geq$ 0.5 U.A.) contained the heavy chain VHA38 (i.e. A6, A10, A14, A19 and A30). Higher immunoreactivity towards *E. faecalis* 12030 than to LTA from *S. aureus* was only shown by antibodies sharing VHA38 where four out of five antibodies (i.e. A10, A14, A19 and A30) showed this pattern of reaction while the fifth one (i.e. A6) exhibited similar immunoreactivity in both assays. Finally, antibodies harboring the variable heavy chain VHA51 showed immunoreactivity towards both evaluated samples – i.e. A7 and A11 showed to be more reactive towards whole cell *E. faecalis* 12030, while A20 and A31 showed same immunoreactivity for LTA as well – but were not substantially better than other evaluated antibodies. Antibodies A4, A8, A17 sharing the heavy chain VHC12 showed generally low immunoreactivity but appeared to be more reactive against purified LTA than against the whole cell *E. faecalis* 12030.



30 Figure 4.6.3a Reaction pattern of monoclonal antibodies (mAbs) against LTA depending of contained variable heavy and light chains.

The outer circle is partitioned by percentages depending on the reactivity against LTA. The inner circle (grey to black) displays the heavy chains and the middle circle (shades of green) the light chains.



31 Figure 4.6.3b Reaction pattern against E. faecalis 12030 of monoclonal antibodies depending of contained variable heavy and light chains.

The outer circle is partitioned by percentages depending on the reactivity against E. faecalis 12030. The inner circle (grey to black) displays the heavy chains and the middle circle (shades of green) the light chains.

5. Discussion

Today, alternatives to antibiotic treatment especially for nosocomial infections are urgently needed. Immunotherapy by monoclonal antibodies exclusively or combined with conventional antibiotic treatment is one of the most promising options for the future [90] [80]. They are used in cancer therapy as well as to treat autoimmune cardiovascular and infectious diseases [106]. Antibodies offer several advantages such as low toxicity and high specificity (avoiding alteration of the host flora) as well as a low risk of resistance development [103][136]. Highly specific monoclonal antibodies can be a therapeutic approach or used as a supportive measure in other management regimens [103]. For patients at risk of enterococcal infections active vaccination is, due to the little success in immunocompromised patients, a less favorable option, whereas passive antibody therapy offers good possibilities of treatment [21] [92] [20]. Possible effects include neutralization of microbial toxins, promotion of phagocytosis at the site of infection, activation of complement as well as microbial lysis among others as shown in Fig. 1.4.4a [111] [103]. On the other hand, high specificity makes the development of antibodies for a broad market less attractive. Moreover, secondary effects and administration of the drug have to be taken into account when antibodies are considered for treatment and it is known that early treatment is crucial to fight infections effectively. Therefore, detailed knowledge of the impact on the host immune system and rapid diagnosis is needed [103]. Despite the recent progress of monoclonal antibody technology many procedures to identify suitable antibodies are timeconsuming, labour intensive, expensive and of low efficiency [101]. In the present work, a pipeline for the identification and production of immunoreactive monoclonal antibodies directed against the well-known cell surface antigen LTA present in *E. faecalis* and other Gram-positive bacteria is described.

Here, we propose an outline for the rapid and efficient isolation and identification of antibodies from B-cells, their cloning and production in mammalian cell lines and their immunological evaluation. In the following we will discuss the advantages and disadvantages of the selection and production as well as future perspectives of the monoclonal antibodies studied in this work.

5.1. Isolation of immunoreactive antibodies

Different methods to isolate monoclonal antibodies have been investigated so far. In the 1970'ies antibodies were mostly isolated from infected mice, nowadays they can be derived directly from patients and most recently cloning of variable heavy and light chains is a frequently used technique in antibody production (Marston, Paul 2018). A very common method to isolate monoclonal antibodies is the production of a hybridoma cell line from the stable fusion of immortalized myeloma cells with B-cells from immunized mice [136]. This implies the construction of chimeric antibodies combining human constant heavy and mouse variable chains [136]. This has been carried out successfully by our group to produce two murine antibodies against DHG and SagA [118]. However, mouse antibodies being foreign proteins in humans are highly immunogenic leading to lower efficacy or even neutralization of the monoclonal antibody by the human immune system [136]. Interactions of mouse antibodies with human complement and FcγRs are weak, causing an insufficient immune response [109] [136]. Moreover, an anaphylactic or human-anti-mouse-antibody (HAMA) response is more likely [113]. Therefore approaches to humanize mouse antibodies have been developed inserting the murine CDR regions into a human IgG [136] [108]. Another option poses the production in transfected eukaryotic cell lines like CHO cells which are commonly used in clinical development as they often produce higher antibody titers [136]. There is also the possibility of phage or ribosomal display [136]. Here, heavy and light chains are paired randomly and there is no negative-selection for self-reactivity [109]. Furthermore, it is unlikely that the combinations are the same as by natural selection in B-cells of a healthy donor undergoing regular exposure to the antigen [136] [109]. In this approach the immunogenic parts of the antibody are expressed in *E.coli* which may lead to suboptimal expression in mammalian cell lines later [109].

In the present work we aimed at the development of monoclonal antibodies that could be applied in clinical therapy. Since we generated fully human IgGs our antibodies do not have the disadvantages of the aforementioned technologies. Production of fully human antibodies can be carried out in single B-cell culture or analysis of a whole pool of B-cells. Antibodies produced by human B-cells generally lead to a powerful immune response due to long human CDR3 regions and a very low risk of cross-reactivity with self-antigens [137]. The strategy of single B-cell-culture is mostly applied to plasma cells collected after antigenic boost [109]. However, a

high frequency of somatic mutations limits the significance of this approach in a pipeline developed for a fast, inexpensive screening method. The mutated sequences may not align to the designed primers and therefore variable chains cannot be amplified [109]. In our approach, the pool of B-cells of a healthy donor was analyzed after cells had been immortalized using EBV. This method offers the advantage of receiving a high yield of antibodies secreted by the immortalized clones. Additionally, there is no description of ongoing somatic mutations in EBV-immortalized clones [109]. It has been shown that healthy adults possess antibodies against enterococcal polysaccharide antigens regardless of a previous infection [138]. This may be due to the ongoing small-scale exposition to Enterococci which translocate from the intestine as described above [138]. Since our investigation aimed to develop an effective screening method for the identification of opsonic antibodies, we chose to evaluate the B-cell repertoire of the donor whose sera would achieve highest killing in the opsonophagocytic assay. Previously, we had successfully followed this approach when looking for antibodies with opsonic and protective activity against *S. aureus* LAC and *E. faecalis* 12030 [78]. The results obtained indicate that the opsonic antibodies found are directed against LTA [78]. Yet, we realized that our platform for the identification of B-cell clones had some shortcomings. Cell counting of the initial B-cell population was not performed in order to avoid loss of B-cells. Therefore, we could not determinate the B cell population that should be split to reach monoclonality and thus had to rely on theoretical calculations. Furthermore, it was difficult to test the B-cell pools repeatedly. Repeated testing of supernatant harvested at another point of time was not feasible since B-cells tend to become monoclonal and the predominance of clones in each well could change [139]. We also could not analyze the whole repertoire as only a part of B-cells are immortalized and sub-culturing them for a longer time can impair B-cell function resulting in diminished reactivity of the antibodies [108].

In the present study we screened the antibodies produced by B-cells not only for phagocytic activity but also for affinity towards LTA in ELISA in order to quickly limit the number of pools of clones to analyze and thus fasten and simplify the selection procedure. The target structure was LTA from *S. aureus* which is expressed in human disease, accessible for functional antibodies and has been demonstrated to have a conserved chemical structure among Gram positive pathogens [95] [48]. LTA was chosen as a marker of immunoreactivity during the screening of the pools due to its immunogenic properties. As described above, it is an

important virulence factor present in many clinically relevant Gram-positive bacteria such as staphylococci and streptococci and has been demonstrated to induce opsonic and protective antibodies against Gram-positive infections [37]. Antibodies raised against LTA from *E. faecalis* 12030 have been shown to be cross-reactive against *S. aureus* and *E. faecalis* since they primarily bind to the teichoic acid backbone of LTA [48]. Therefore, LTA was considered a suitable target antigen for the selection and identification of opsonic antibodies that neutralize and bind LTA in Enterococci and/or other Gram-positive bacterial pathogens.

We used a common affinity method to identify antibodies by pre-screening the antibodies in ELISA and select the immunoreactive ones to be tested in OPA. OPA has been shown to reliably identify functional antibodies against bacterial antigens [140]. Both assays were necessary in order to select not only immunoreactive but also opsonic antibodies. This approach was pursued before in our team by Kalfopoulou et al. selecting hybridoma cells to produce murine antibodies [118]. *In vivo* models could not be used with the small amount of samples collected (from 150µL to 400µL) in the short period of time available for screening and would be difficult to handle with the number of samples evaluated (96 to 180 samples) in this study. In contrast, ELISA could easily be used to process the numerous samples and to limit the number of pools to be analyzed in OPA. Different studies have shown good correlation between ELISA and/or OPA with in vivo activity for several bacterial pathogens, such as *S. aureus*, *E. coli*, *S. pyogenes, E. faecalis and S. pneumoniae* [141] [142] [143] [88]. In our study not all immunoreactive antibodies showed proof of opsonophagocytic activity. One reason may be that the antibody concentrations reached after transfections were low (below 1µg/mL) and not enough to observe opsonophagocytic killing of the target strain [142] [127]. Nevertheless, there are more advantages of testing the antibody in ELISA: It requires only a low amount of antibodies meaning that immunoreactive antibodies can be detected at any time of the screening process. Since the aim of this study was to develop a cross-reactive antibody directed against LTA (Enterococci and *S. aureus*) constant proven affinity to LTA was crucial in the selection process. However, although the immunoreactivity of an antibody indicates its ability to interact with an antigen, opsonophagocytosis represents its potential to mediate bacterial killing under physiological conditions. Therefore, additional assessment in OPA was needed to evaluate the functional activity of the antibody. On some occasions, we observed small differences in opsonophagocytic killing for some pools at independent time points, which were probably

related to the respective antibody concentration. We think that isolation of phagocytes from different blood donors may affect opsonophagocytic killing as well. A macrophage cell line culture in order to standardize the OPA and reduce donor-donor variability could be feasible [144]. Yet, using these cell lines may not solve this problem completely, since some of the antibodies do not express the appropriate Fc-receptor and may therefore fail to mimic the *in vivo* conditions in the experiment. For this reason other studies as well as this one preferred freshly prepared PMNs [145] [146].

Another aim of the study was to detect antibodies with (cross-)immunoreactivity against other serotypes of enterococci or Gram positive bacteria. Cross-reactivity was considered interesting as it indicates that targets of the antibody could be different enterococcal cell-wall polysaccharides. In Enterococci, the previously described serotype CPS-C (serotype CPS-C or *E. faecalis* Type 2) expresses DHG on the surface probably masking the lipoteichoic acid [43] [42]. In ELISA, the absorbance values in the assay against DHG were higher than in that against LTA (see Figure 4.3c and Figure 4.3e). This observation could be explained by differences in B-cell population and antibody concentration within the pool on the day that the supernatants were taken. When the supernatants were collected to test immunoreactivity towards DHG, B-cells had proliferated for 13 days after splitting vs seven days for testing against LTA. Therefore, a lower amount of cells and antibody concentration on “Day 16” would yield lower immunoreactivity towards LTA than towards DHG on “Day 22”. Pool 26.E4, 83.C6 and pool 96.C12 showed the highest immunoreactivity towards both antigens, DHG and LTA, in comparison with other pools on the same plate. The antibodies A18 and A29 were immunoreactive by whole cell ELISA against *E. faecalis* Type 2 and also towards DHG. This confirms the findings of the screening period, when pool 96.C12 showed cross-reactivity against DHG. These antibodies could be responsible for the observed cross-reactivity of pool 96.C12.

After completing the screening procedure on “Day 33” one pool of B-cells was selected to perform RNA extraction. As shown in Figures 4.3f and 4.3h the immunoreactivity values towards LTA observed for pools 96.C12, 96.E12, 83.C6 and 26.A4 showed only minimal variation. However, phagocytic killing activities for these selected pools on “Day20” and “Day33” showed pronounced differences. For instance, phagocytic activities of pools 96.C12 and 83.C3 dropped between the two dates by 20% and 55% respectively. In contrast, activities

for pools 96.E12 and 26.A4 increased during this period by 15% and 55%, respectively. In our research group we have observed effective opsonophagocytic killing activity when antibody concentration in supernatants of EBV transfected B-cells ranged between 2 to 20 µg/mL (data not shown). We observed that absorbance values (i.e. in ELISA) are dependent on antibody concentration. Therefore, reduced immunoreactivity and killing activity are not surprising after the splitting of B-cells (i.e. by splitting either 1/96 or 1/48). The differences in phagocytic killing over time can also be explained by the tendency of EBV-infected B-cells to become monoclonal with time and the uncertain persistence of the clone(s) producing opsonophagocytic antibodies to the end of the process [139]. For this reason, we recommend that projects using this platform for the identification of active antibodies perform the RNA extraction as soon as the pool is screened and selected. Otherwise activity could be lost completely with further manipulations and/or culturing. For the identification of pools producing the antibodies with the highest immunoreactivity and opsonic killing activity results were always compared between the different pools of one plate at a certain date. They were then analyzed for consistency of the obtained immunoreactivity during the whole screening period. Pool 96.C12 was selected because of its high immunoreactivity in ELISA on “Day 33” suggesting the predominance of a B-cell clone producing a highly immunoeactive antibody.

5.2. Production and analysis of monoclonal antibodies

Different vectors have been used to transfect variable light and heavy chains into mammalian cells for antibody expression. The TCAE system, for example, contains the human IgG1 heavy chain and the kappa light chain constant region [78]. It allows the use of only one selection marker for the transfection of the eukaryotic cell with a single plasmid [78]. Yet, this single plasmid has to be generated for every possible combination of variable and heavy chains [147] [148] [127] [78]. In contrast, the commercially available pFUSE system used in this study allows the cloning of the variable heavy and light chains independently to transfect them in diverse combinations. This minimizes the number of cloning procedures, complications and time compared to the use of other plasmids, such as TCAE. pFUSE has been used mainly for both transient and stable expression of phage display libraries in CHO as well as in HEK-293T cells [118] [149] [150] [151]. For expression of the protein of interest in a mammalian cell line, CHO-cells are in wide-spread use and still play an important role in the production of biotherapeutics [152]. However, in the industry setting stable CHO cell lines producing antibodies are also difficult to establish. The feasible culture format for CHO cells would be in suspension, in contrast to the adherent cell-line used in our project [153]. Adaption of our CHO-DHFR$^-$ cell line would take about nine months and was therefore not attempted in this project [154]. On the other hand, HEK-cells are commonly used for transient transfection as they usually yield large amounts of protein within a few days [153] [155] [156]. The HEK-293T cell line carries the Simian Virus 40 large T leading to episomal retention of the expression vector and thus to enhanced transcription and translation [153]. Jäger *et al*. effectively expressed antibodies from a phage display library in HEK-293T cells [157]. Jain *et al*. compared antibodies produced in CHO and HEK-293T cells and found no differences in antibody affinity, but observed a more variable protein secretion in CHO cells, which is comparable to our findings [158]. However, it has been shown that protein titers produced in stable CHO cell lines and transient HEK cell lines do indeed correlate [159]. In that study the highest production in HEK cells was observed between 12-72 hours after transfection which is the same in our data. As shown in Figure 4.5.5b cells produced the highest amount of antibodies on day 2, while antibody production was lowest on day 6 and would continue diminishing over time. Over the last ten years usage of HEK-293T cells has increased and also therapeutic compounds have been produced in this system, even if glycosylation differences are present between proteins produced in CHO and in HEK cells [153]

[160]. These discrepancies can lead to different aggregation behavior influencing the functional results of the antibody [153] [160] [161]. Under our experimental conditions HEK-293T cells were feasible for transient production regarding yield and consistency of production as compared to CHO-cells. After antibodies had been harvested from HEK 293T cells, their immunoreactivity was assessed. The activity of antibodies produced in HEK-293T was comparable to those secreted by B-cells. The highest immunoreactivity among the produced monoclonal antibodies was observed for A9. It also showed the highest opsonophagocytic killing activity (more than 80%), exhibited at a very low concentration (0.245 µg/mL) in comparison to other evaluated antibodies. However, killing activity of the constructed antibodies in OPA was not comparable to the ones observed during the selection period. Differences between recombinant antibodies' activity and the original activity have been described also for monoclonal mouse antibodies [118]. The discrepancy between concentration and functional activity in OPA has been noted before for IgGs against *Streptococcus pneumoniae* and it was shown to depend on their avidity towards the pneumococcal polysaccharide [162]. This may explain the high activity of the A9 antibody at very low concentration. Another possible explanation for the antibodies' lack of functional activity in OPA could be an impairment in the Fc fragment. It is important for the activity in OPA but not for immunoreactivity in ELISA, as we used secondary antibodies targeting the whole IgG molecule in ELISA. As mentioned before, the discrepancy between activity in ELISA and the missing opsonophagocytic activity of antibodies produced in HEK-293T cells could be due to low concentration. The antibody concentration in the supernatant during the selection process was probably higher than the one achieved after cloning and expression in HEK cells. Lanzavecchia and Corti reported that antibodies collected from EBV- immortalized B-cell clones are at concentrations between 5 - 50 µg/ml [109]. It has been described beforehand that monoclonal antibodies against *S. aureus* exhibited opsonophagocytic activity at a range of concentration of 3 µg/mL [127] [163]. In other studies antibodies produced in HEK-T293 cells were tested at 2µg/ml [164]. For *N. meningitidis* opsonophagocytic killing activity was present at a concentration of 0,11 µg/ml [146]. Thus, we would expect to observe similar opsonophagocytic activity at a similar concentration (i.e. 2µg/mL) against *E. faecalis* 12030. Opsonophagocytic killing activity of our antibodies may therefore increase at higher antibody concentrations. Additionally, the DMEM media used yielded a background opsonophagocytic

activity in the OPA, questioning the activities observed for the tested monoclonal antibodies. The medium used for culturing HEK-293T cells had very low IgG content and samples had been dialyzed before being tested in OPA. However, A22 (at 0,018 µg/mL) and control medium which were included as negative controls in the assay showed killing rates higher than 50%. This indicates that the cell culture medium interferes in the OPA. Since the negative controls used in the assay turned out unreliable, we cannot conclude which antibody(/ies) possesses opsonophagocytic killing activity. Nevertheless, A9, taking into account its low concentration (0.245µg/mL), its high opsonophagocytic killing activity and its high immunoreactivity in ELISA against whole cell *E. faecalis* 12030 and LTA of *S. aureus*, could correspond to the antibody present in pool 96.C12 during the screening period of the study (see figure 4.6.2a). However, further analysis should be performed on A9 to confirm these findings. As a future recommendation for testing activity of the antibodies in OPA, we suggest that after transfection the medium is changed to the same used for OPA (with 2% low IgG) or to a medium with very low interference in the assay like serum-free media.

The question remains which kind of antibody was responsible for the high killing percentages observed during the selection process. This may be partly due to non-specific uptake of bacteria by IgMs [146] [165]. The pentameric IgM molecule can lead to complement activation and may contribute to formation of antigen-antibody complexes [166] [167]. These in turn can be recognized by other components of the immune system [166] [167]. Different immunoglobulin classes vary in their opsonophagocytic activity [90]. A study of commercially available immunoglobulin preparations for clinical usage demonstrated that IgG only preparations yielded higher opsonophagocytic killing of *E. faecalis* in comparison to preparations containing IgG, but also IgM and IgA [90]. However, in protection studies of *in vivo* models, IgM yielded better results, probably by targeting carbohydrate structures of the cell wall more broadly [90].

In this study we constructed antibodies of the IgG1 subclass, since the IgG1 and IgG3 subclasses have been shown to be most effective in activation of complement and to bind to phagocytic cells through Fc receptors [95] [166]. IgGs, in contrast to IgMs, trigger antibody-dependent cellular toxicity [113]. Furthermore, they are the most stable antibody class with a serum half-life of 20 days in peripheral blood, rather than less than 10 days for other classes [113] [136]. In IgGs there are two subclasses of light chains, lambda or kappa [168]. Here, the lambda light

chains were analyzed representing roughly half of all serum antibodies. In comparable earlier studies, the lambda domain was also analyzed and cloned [78] [127] [169]. The exact mechanisms how kappa or lambda light chains influence the functional activity of the antibody are not well explored. When B-cells start producing the lambda light chain isotype they may broaden the repertoire leading to a more effective immune response [169]. Lambda light chains are only expressed if multiple rearrangement of kappa chains fails and they contain a more varied structure [169]. However, there seem to be only slight differences in affinity and activity between monoclonal antibodies differing in lambda or kappa chains, apart from a shorter half-life *in vivo* [168]. Molecular stability in the kappa-bearing antibodies was higher, but no other significant differences were observed [170]. Recently, Smith *et al.* have analyzed the ratio of kappa to lambda antibodies against various immunogens. They have demonstrated that the immune response as well as the light chain subtype differ according to the type of the microorganism and the polysaccharide [169]. Half of the antibodies produced during the immune response possess the lambda light chain. They display an entirely different spectrum of specificities as well as cross-reactivities compared to kappa antibodies. The lambda light chain therefore seems to be crucial for a successful immune response against a broad variety of pathogens [169]. Antibodies expressing only the kappa light chain are produced on exposure to a simple antigen, but both (kappa and lambda) are produced if the immune system is exposed to more complex or variable structures such as complex multimers of the Influenza virus [169]. A lower number of potential B-cells recognizing the epitope probably accounts for this. Thus, only one light chain is produced [169]. E.g. a simple epitope of the antigen EBNA-1 of EB-Virus elicited a “kappa only” response. However, a more complex structure like the whole protein lead to antibodies having both light chains. Similar results were obtained for *S. pneumoniae* [169]. Regarding monoclonal antibodies against polysaccharides, either lambda, kappa or both are expressed depending on the serotype. The switch to lambda light chain antibody production requires NF-κB signaling [171]. Moreover, Smith *et al.* observed that individuals would predominantly express either lambda or kappa to the same antigen/pathogen even if some antigens showed a distinct ratio either to kappa or to lambda antibodies [169]. The lack of opsonphagocytic potential in our monoclonal antibodies may be due to the fact that kappa antibodies have been responsible for opsonic killing, as observed in pool 96. C12. Further analysis of the kappa light chains is needed to fully understand the activity which was observed

in pool 96.C12 during selection period.

When analyzing the immunogenic properties of the produced antibodies, similar immunoreactivities towards LTA from *S. aureus* and whole cell *E. faecalis* 12030 could be observed. This could be explained by similar structural epitopes that the antibody recognizes in the LTA from *S. aureus* and in the whole cell of *E. faecalis* 12030. On the other hand, different immunoreactivities may be due to the presence of polysaccharide(s) or other cell-wall-associated antigens that could hinder the LTA epitopes in the whole bacterium of *E. faecalis* 12030. Thus, immunorecognition is only possible if these are completely exposed, e.g. when purified LTA is being tested. Differences in immunoreactivity could as well be explained by antibodies binding epitopes present in LTA from *S. aureus,* but lacking in the LTA from *E. faecalis 12030*, or vice versa. Antibodies with higher immunoreactivity to *E. faecalis* 12030 may be directed against a basic structure of teichoic acids, like the common backbone, or may bind to other antigens on the surface of *E. faecalis* 12030. Antibodies showing higher immunoreactivity to LTA (A4, A5, A7, A8, A11, A17, A18) than to *E. faecalis* 12030 may target a LTA decoration or a contaminant present in the LTA from *S. aureus* preparation, missing in the whole bacterium of *E. faecalis* 12030. Antibodies showing no immunoreactivity either towards LTA or to the whole cell *E. faecalis* 12030 may target an epitope which is not present in our preparations, or simply not bind any specific epitope.

In a closer analysis we looked at the variable heavy and light chains that the immunoreactive antibodies contained. In Figure 4.6.3a and Figure 4.6.3b it can be observed that antibodies that exhibited the highest immunoreactivity towards LTA and/or *E. faecalis* 12030 (absorbance $\geq$ 0.8 U.A., units of absorbance) contained the heavy chain VHC19 (i.e. A5, A9, A18 and A29), with exception of antibody A13. Their target epitope could be a structure present in LTA of *S. aureus* and *E. faecalis* 12030 which is accessible and affine to the antibody. Another chain shared by antibodies (i.e. A6, A10, A14, A19 and A30) showing high immunoreactivity in both assays (absorbance $\geq$ 0.5 U.A.) is the variable heavy chain VHA38. The same chain forms part of antibodies that exhibit higher immunoreactivity towards *E. faecalis* 12030 than to LTA from *S. aureus*: four out of five antibodies (i.e. A10, A14, A19 and A30) showed this pattern of reaction while the fifth one (i.e. A6) exhibited similar immunoreactivity in both assays. This may indicate preferential immunoreactivity to epitopes present in LTA from *E. faecalis* 12030 than to those in LTA from *S. aureus*. Decorations in the LTA from *S. aureus* may hinder the target epitope of

these antibodies. Another observed trend was that those antibodies containing variable heavy chain VHC12 showed preferential binding to LTA from *S. aureus* and not to the whole cell of *E. faecalis* 12030 (i.e. significant results for A4, A8 and A17). As mentioned above, this could be due to immunoreactivity towards either an epitope present in the LTA of *S. aureus* and absent from the *E. faecalis* 12030 LTA or a contaminant in the LTA preparation from *S. aureus*. Finally, antibodies harboring the variable heavy chain VHA51 showed immunoreactivity towards both evaluated samples. However, taking small differences of reactivity into account, A7 and A11 showed to be more reactive towards whole cell *E. faecalis* 12030, while A20 and A31 showed the same immunoreactivity as for LTA. Despite the fact that antibodies A13 and A14 contain the variable heavy chains VHC19 or VHA38, their immunoreactivity was low within their group (i.e. of antibodies sharing the same heavy chain). Interestingly, A15 and A12 harboring the same variable light chain as A13 and A14 (i.e. VLC33), yielded very low immunoreactivity (absorbance $\geq$ 0.2 U.A.). Most of the antibodies harboring variable light chain VLC75 (i.e. A28, A30 and A31) yielded antibodies with very low or no immunoreactivity against the molecules tested, especially if not in combination with the heavy chains appearing in antibodies with the highest immunoreactivity (i.e. VHC12 or VHA38). It can be observed that all antibodies with VLA35 (i.e. A8, A9, A10, A11) show high immunoreactivity towards the antigens tested (especially against LTA), indicating that it may be responsible for good binding against the targeted molecules. To sum up, certain variable heavy chains (i.e. VHC19, and VHA38) are part of antibodies exhibiting particularly high immunoreactivity. VHC19 occurs in the two most affine antibodies A9 and A18. In fact, antibodies possessing VHC19 account for more than a third of all immunoreactive antibodies against LTA or whole cell *E. faecalis* 12030. Furthermore, VHA38 was present in antibodies immunoreactive against *E. faecalis* 12030, while the other VH chains seem to favor the binding of antibodies to the LTA of *S. aureus.*

This observation raises the following question: can a single variable chain be responsible for a specific binding of the antibody? If true, this would suggest a direct influence of the interaction between the variable light and heavy chains on binding capacity. The variable light and heavy chains of an antibody form the antigen binding site at the Fab-region consisting of three CDR loops embedded in the framework regions [172] [136] [166]. The rearrangement of Variable (V), Diversity (D) and Joining (J) immunoglobulin genes accounts for diversity of B-cells [166]. CDRs 1 and 2 are encoded within the *IGHV/IGKV/IGLV* genes and the repertoire of their

diversity corresponds to *IGV* gene usage. In contrast, the CDR3 regions encoded by various gene segments, are the most variable part of the antibody [166]. Light chain rearrangement involves only V and J regions, thus the CDR3 of light chains (CDRL3) is less diverse than that of heavy chains (CDRH3). CDRH3 includes two joining sites, one between IGHV-IGHD and another between IGHD-IGHJ. The CDRH3 - generated through recombination of V, D and J genes - is engaged in one third of the antigen-antibody contacts [173]. In humans, long CDRH3 regions have been reported to influence the potential of an antibody in virus neutralization [174]. Since exonucleases may remove nucleotides and nucleotides are randomly added by the terminal deoxynucleotidyl transferase, gene rearrangement is imprecise. As a result diversity at these joining sites increases [166]. Since the CDRH3 shows such great variability, it is considered the most important component of the antibody regarding binding specificity and activity [175]. However, antigen recognition and affinity may correspond to VH and VL pairing as well as individual characteristics of variable heavy and light chains [176] [177] [173].

Recently, the significance of CDRH3 has been reviewed and put into a new perspective. Despite the critical role of CDRH3 in antigen binding (even peptides derived from this region show themselves binding to the antigens) antibody specificity is largely defined by the VL and VH germline genes [175]. In a group of antibodies selected for target-specificity, antibodies with the same CDRH3 show different binding affinity [175]. In contrast, if not selected for binding capacity, most antibodies from a library sharing the same CDRH3 do not bind to the antigen [175]. They also report that in antibodies binding to the target the same single VDJ arrangement was found [175]. This confirms results of another study demonstrating that CDRH3 possesses the same amino acid sequence [172]. Still, variability was seen in its conformation which can be influenced by heavy and light chain pairing. Additionally, conformation can change while binding the antigen [172]. Consequently, affinity of different antibodies containing the identical CDRH3 differs up to 100-fold [175]. In summary, the binding site of an antibody (especially the CDRH3 region) is not only defined by its amino acid sequence but to a great extent by the pairing of variable heavy and light chains [175] [172].

This fits with our data. The presence of a variable (heavy or light) chain associated with high immunoreactivity, leads to high activity of the antibody, even if its paired variable (light or heavy) chain itself is not associated with high immunoreactivity. In our investigation we were able to identify specific variable domains associated with high immunoreactivity against whole

cell *E. faecalis* 12030 and/or LTA of *S. aureus.* Precisely, we defined two heavy chains (i.e. VHC19, VHA38) and one light chain (i.e. VLA35) present in most immunoreactive recombinant antibodies.

5.3. Future perspective

In this study 25 recombinant antibodies were identified, 16 of which showed immunoreactivity against LTA from *S. aureus* and/or whole cell *E. faecalis* 12030. To specify opsonophagocytic activity of the antibodies, future investigation should include the use of chemically defined media, production at higher scale and purification of the antibodies. For further development of a therapeutic human monoclonal antibody there are various approaches currently being investigated. Phage display and ribosomal display offer the possibility of *in vitro* antibody affinity maturation. Several methods are being pursued to optimize the Fc region [106]. Other options include randomization of CDR3 regions, light chain shuffling by changing the variable light chain, but keeping the variable heavy chain, or generation of an antibody library with mutations in the variable genes [113] [106].

These methods may permit an effective monoclonal antibody to be constructed. In the era of increasing antibiotic resistance immunotherapy is a promising advancement in the treatment of enterococcal infections. We hope our work represents one of the useful steps in this direction.

6. References

[1] Thiercelin M. E., “Morphologie et modes de reproduction de l’enterocoque,” *Comptes Rendus des Seances de la Societe de Biologie et des ses Filiales*, vol. 11, no. 11, pp. 551–553, 1899.

[2] W. Köhler, “The present state of species within the genera Streptococcus and Enterococcus,” *International Journal of Medical Microbiology*, vol. 297, no. 3. pp. 133–150, Jun. 11, 2007.

[3] R. C. Lancefield, “A serological differentiation of human and other groups of hemolytic streptococci,” *Journal of Experimental Medicine*, vol. 57, no. 4, pp. 571–595, Apr. 1933.

[4] A. P. Kalina, “The taxonomy and nomenclature of enterococci,” *International Journal of Systematic Bacteriology*, vol. 20, no. 2, pp. 185–189, Apr. 1970.

[5] K. H. Schleifer and R. Kilpper-Balz, “Transfer of Streptococcus faecalis and Streptococcus faecium to the Genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov.,” *International Journal of Systematic Bacteriology*, vol. 34, no. 1, pp. 31–34, 1984.

[6] K. Fisher and C. Phillips, “The ecology, epidemiology and virulence of Enterococcus,” *Microbiology*, vol. 155, no. 6. pp. 1749–1757, Jun. 01, 2009.

[7] K. J. Domig, H. K. Mayer, and W. Kneifel, “Methods used for the isolation, enumeration, characterisation and identification of Enterococcus spp. - 2. Pheno- and genotypic criteria,” *International Journal of Food Microbiology*, vol. 88, no. 2–3, pp. 165–188, Dec. 2003.

[8] M. R. Foulquié Moreno, P. Sarantinopoulos, E. Tsakalidou, and L. De Vuyst, “The role and application of enterococci in food and health,” *International Journal of Food Microbiology*, vol. 106, no. 1. pp. 1–24, Jan. 2006.

[9] J. O. Mundt, “Occurrence of enterococci on plants in a wild environment.,” *Applied microbiology*, vol. 11, no. 2, pp. 141–4, Mar. 1963.

[10] J. O. Mundt, “Occurrence of Enterococci: Bud, Blossom, and Soil Studies.,” *Applied microbiology*, vol. 9, no. 6, pp. 541–4, Nov. 1961.

[11] C. M. A. P. Franz, M. Huch, H. Abriouel, W. Holzapfel, and A. Gálvez, “Enterococci as probiotics and their implications in food safety,” *International Journal of Food Microbiology*, vol. 151, no. 2. pp. 125–140, Dec. 2011.

[12] J. M. Saavedra, “Clinical applications of probiotic agents.,” *The American journal of clinical nutrition*, vol. 73, no. 6, pp. 1147S-1151S, Jun. 2001.

[13] C.-C. Chen *et al.*, “Probiotics have clinical, microbiologic, and immunologic efficacy in acute infectious diarrhea.,” *The Pediatric infectious disease journal*, vol. 29, no. 2, pp. 135–8, Feb. 2010.

[14] S. J. Allen, E. G. Martinez, G. V Gregorio, and L. F. Dans, “Probiotics for treating acute infectious diarrhoea,” *Cochrane Database of Systematic Reviews*, no. 11, p. CD003048, Nov. 2010.

[15] I. F. Nes, D. B. Diep, and Y. Ike, “Enterococcal Bacteriocins and Antimicrobial Proteins that Contribute to Niche Control,” *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, pp. 1–29, Feb. 2014.

[16] M. López *et al.*, “Detection of vanA and vanB2-containing enterococci from food samples in Spain, including Enterococcus faecium strains of CC17 and the new singleton ST425,” *International Journal of Food Microbiology*, vol. 133, no. 1–2, pp. 172–178, Jul. 2009.

[17] L. E. Hancock and M. S. Gilmore, “Pathogenicity of Enterococci,” *Gram-Positive Pathogens, Second Edition*, pp. 37–41, Jan. 2006.

[18] T. D. Lawley and A. W. Walker, “Intestinal colonization resistance.,” *Immunology*, vol. 138, no. 1, pp. 1–11, Jan. 2013.

[19] K. Dubin and E. G. Pamer, “Enterococci and Their Interactions with the Intestinal Microbiome,” *Microbiology spectrum*, vol. 5, no. 6, pp. 1–16, Nov. 2017.

[20] S. Koch, M. Hufnagel, C. Theilacker, and J. Huebner, “Enterococcal infections: host response, therapeutic, and prophylactic possibilities,” *Vaccine*, vol. 22, no. 7, pp. 822–830, Feb. 2004.

[21] C. A. Arias and B. E. Murray, “The rise of the Enterococcus: Beyond vancomycin resistance,” *Nature Reviews Microbiology*, vol. 10, no. 4. pp. 266–278, Mar. 16, 2012.

[22] K. Brandl *et al.*, "Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits," *Nature*, vol. 455, no. 7214, pp. 804–807, Oct. 2008.

[23] R. Nagpal and H. Yadav, "Bacterial translocation from the gut to the distant organs: An overview," *Annals of Nutrition and Metabolism*, vol. 71, no. 1. pp. 11–16, 2017.

[24] R. D. Berg, "Bacterial translocation from the gastrointestinal tract.," *Advances in experimental medicine and biology*, vol. 473, no. 4, pp. 11–30, 1999.

[25] C. Ubeda *et al.*, "Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans," *Journal of Clinical Investigation*, vol. 120, no. 12, pp. 4332–4341, Dec. 2010.

[26] C. J. Donskey, "The Role of the Intestinal Tract as a Reservoir and Source for Transmission of Nosocomial Pathogens," *Clinical Infectious Diseases*, vol. 39, no. 2, pp. 219–226, Jul. 2004.

[27] M. E. A. de Kraker, V. Jarlier, J. C. M. Monen, O. E. Heuer, N. van de Sande, and H. Grundmann, "The changing epidemiology of bacteraemias in Europe: Trends from the European antimicrobial resistance surveillance system," *Clinical Microbiology and Infection*, vol. 19, no. 9, pp. 860–868, Sep. 2013.

[28] K. W. C. Leong *et al.*, "Emergence of Vancomycin-Resistant Enterococcus faecium at an Australian Hospital: A Whole Genome Sequencing Analysis," *Scientific Reports*, vol. 8, no. 1, p. 6274, Dec. 2018.

[29] C. H. Poh, H. M. L. Oh, and A. L. Tan, "Epidemiology and clinical outcome of enterococcal bacteraemia in an acute care hospital," *Journal of Infection*, vol. 52, no. 5, pp. 383–386, May 2006.

[30] D. A. Garsin *et al.*, "Pathogenesis and Models of Enterococcal Infection," *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, pp. 1–57, Feb. 2014.

[31] F. C. Neuhaus and J. Baddiley, "A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria," *Microbiology and molecular biology reviews : MMBR*, vol. 67, no. 4, pp. 686–723, Dec. 2003.

[32] I. G. Sava, E. Heikens, and J. Huebner, "Pathogenesis and immunity in enterococcal infections," *Clinical Microbiology and Infection*, vol. 16, no. 6. Elsevier, pp. 533–540, Jun.

01, 2010.

[33] C. Weidenmaier and A. Peschel, “Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions,” *Nature Reviews Microbiology*, vol. 6, no. 4. pp. 276–287, Apr. 10, 2008.

[34] S. Maekawa, M. Yoshioka, and Y. Kumamoto, “Proposal of a New Scheme for the Serological Typing of Enterococcus faecalis Strains,” *Microbiology and Immunology*, vol. 36, no. 7, pp. 671–681, 1992.

[35] M. Hufnagel, L. E. Hancock, S. Koch, C. Theilacker, M. S. Gilmore, and J. Huebner, “Serological and genetic diversity of capsular polysaccharides in Enterococcus faecalis,” *Journal of Clinical Microbiology*, vol. 42, no. 6, pp. 2548–2557, Jun. 2004.

[36] J. Huebner *et al.*, “Isolation and chemical characterization of a capsular polysaccharide antigen shared by clinical isolates of Enterococcus faecalis and vancomycin-resistant Enterococcus faecium.,” *Infection and immunity*, vol. 67, no. 3, pp. 1213–9, Mar. 1999.

[37] C. Theilacker *et al.*, “Opsonic antibodies to Enterococcus faecalis strain 12030 are directed against lipoteichoic acid,” *Infection and Immunity*, vol. 74, no. 10, pp. 5703–5712, 2006.

[38] J. Huebner, A. Quaas, W. A. Krueger, D. A. Goldmann, and G. B. Pier, “Prophylactic and therapeutic efficacy of antibodies to a capsular polysaccharide shared among vancomycin-sensitive and -resistant enterococci,” *Infection and Immunity*, vol. 68, no. 8, pp. 4631–4636, Aug. 2000.

[39] L. E. Hancock and M. S. Gilmore, “The capsular polysaccharide of Enterococcus faecalis and its relationship to other polysaccharides in the cell wall,” *Proceedings of the National Academy of Sciences*, vol. 99, no. 3, pp. 1574–1579, Feb. 2002.

[40] L. E. Hancock, B. D. Shepard, and M. S. Gilmore, “Molecular analysis of the Enterococcus faecalis serotype 2 polysaccharide determinant,” *Journal of Bacteriology*, vol. 185, no. 15, pp. 4393–4401, Aug. 2003.

[41] M. Hufnagel, V. J. Carey, L. Baldassarri, R. R. Reinert, and J. Huebner, “Distribution of four capsular serotypes of Enterococcus faecalis among clinical isolates from different geographical origins and infection sites,” *Infection*, vol. 34, no. 1, pp. 22–25, Feb. 2006.

[42] L. R. Thurlow, V. C. Thomas, and L. E. Hancock, “Capsular polysaccharide production in Enterococcus faecalis and contribution of CpsF to capsule serospecificity,” *Journal of Bacteriology*, vol. 191, no. 20, pp. 6203–6210, Oct. 2009.

[43] C. Theilacker *et al.*, “Serodiversity of opsonic antibodies against Enterococcus faecalis - glycans of the cell wall revisited,” *PLoS ONE*, vol. 6, no. 3, p. e17839, Mar. 2011.

[44] D. Van Tyne and M. S. Gilmore, “Friend Turned Foe: Evolution of Enterococcal Virulence and Antibiotic Resistance,” *Annual Review of Microbiology*, vol. 68, no. 1, pp. 337–356, 2014.

[45] G. M. Dunny, L. E. Hancock, and N. Shankar, “Enterococcal Biofilm Structure and Role in Colonization and Disease,” in *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, 2014, pp. 1–17.

[46] T. J. Silhavy, D. Kahne, and S. Walker, “The bacterial cell envelope.,” *Cold Spring Harbor perspectives in biology*, vol. 2, no. 5. p. a000414, May 2010.

[47] Y. Wang, J. Huebner, A. O. Tzianabos, G. Martirosian, D. L. Kasper, and G. B. Pier, “Structure of an antigenic teichoic acid shared by clinical isolates of Enterococcus faecalis and vancomycin-resistant Enterococcus faecium,” *Carbohydrate Research*, vol. 316, no. 1–4, pp. 155–160, Mar. 1999.

[48] C. Theilacker *et al.*, “Protection against Staphylococcus aureus by antibody to the polyglycerolphosphate backbone of heterologous lipoteichoic acid,” *Journal of Infectious Diseases*, vol. 205, no. 7, pp. 1076–1085, Apr. 2012.

[49] W. F. J. Hogendorf *et al.*, “Automated solid phase synthesis of teichoic acids,” *Chemical Communications*, vol. 47, no. 31, p. 8961, Jul. 2011.

[50] F. Fabretti *et al.*, “Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides,” *Infection and Immunity*, vol. 74, no. 7, pp. 4164–4171, 2006.

[51] D. Wobser, L. Ali, E. Grohmann, J. Huebner, and T. Sakinc, “A Novel Role for D-Alanylation of Lipoteichoic Acid of Enterococcus faecalis in Urinary Tract Infection,” *PLoS ONE*, vol. 9, no. 10, p. e107827, 2014.

[52] J. H. Pazur, J. S. Anderson, and W. W. Karakawa, “Glycans from Streptococcal Cell Walls

immunological and chemical properties of a new diheteroglycan from Streptococcus faecalis," *The Journal of Biological Chemnistry*, vol. 246, no. 6, pp. 1793–1798, 1971.

[53] "European Centre for Disease Prevention and Control. Surveillance of antimicrobial resistance in Europe – Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2017. Stockholm: ECDC;," pp. 57–60, 2018.

[54] B. L. Hollenbeck and L. B. Rice, "Intrinsic and acquired resistance mechanisms in enterococcus," *Virulence*, vol. 3, no. 5. pp. 421–433, 2012.

[55] H. J. Hong, M. I. Hutchings, and M. J. Buttner, "Vancomycin resistance VanS/VanR two-component systems," *Advances in Experimental Medicine and Biology*, vol. 631. pp. 200–213, 2008.

[56] M.-L. Foucault, F. Depardieu, P. Courvalin, and C. Grillot-Courvalin, "Inducible expression eliminates the fitness cost of vancomycin resistance in enterococci," *Proceedings of the National Academy of Sciences*, vol. 107, no. 39, pp. 16964–16969, Sep. 2010.

[57] G. M. Dunny, B. A. B. Leonard, and P. J. Hedberg, "Pheromone-inducible conjugation in Enterococcus faecalis: Interbacterial and host-parasite chemical communication," *Journal of Bacteriology*, vol. 177, no. 4. pp. 871–876, 1995.

[58] S. D. Sussmuth, A. Muscholl-Silberhorn, R. Wirth, M. Susa, R. Marre, and E. Rozdzinski, "Aggregation substance promotes adherence, phagocytosis, and intracellular survival of Enterococcus faecalis within human macrophages and suppresses respiratory burst," *Infection and Immunity*, vol. 68, no. 9, pp. 4900–4906, Sep. 2000.

[59] P. M. Tendolkar, A. S. Baghdayan, and N. Shankar, "Pathogenic enterococci: New developments in the 21st century," *Cellular and Molecular Life Sciences*, vol. 60, no. 12. pp. 2622–2636, Dec. 01, 2003.

[60] A. Toledo-Arana *et al.*, "The Enterococcal Surface Protein, Esp, Is Involved in Enterococcus faecalis Biofilm Formation," *Applied and Environmental Microbiology*, vol. 67, no. 10, pp. 4538–4545, Oct. 2001.

[61] J. Sillanpää *et al.*, "A family of fibrinogen-binding MSCRAMMs from Enterococcus faecalis.," *Microbiology (Reading, England)*, vol. 155, no. Pt 7, pp. 2390–400, Jul. 2009.

[62] J. Sillanpää, Y. Xu, S. R. Nallapareddy, B. E. Murray, and M. Höök, "A family of putative

MSCRAMMs from Enterococcus faecalis," *Microbiology*, vol. 150, no. 7, pp. 2069–2078, 2004.

[63] K. V Singh, S. R. Nallapareddy, J. Sillanpää, and B. E. Murray, "Importance of the collagen adhesin ace in pathogenesis and protection against Enterococcus faecalis experimental endocarditis," *PLoS Pathogens*, vol. 6, no. 1, 2010.

[64] A. Kropec, M. Hufnagel, K. Zimmermann, and J. Huebner, "In vitro assessment of the host response against Enterococcus faecalis used in probiotic preparations," *Infection*, vol. 33, no. 5–6, pp. 377–379, Oct. 2005.

[65] T. H. Mogensen, "Pathogen recognition and inflammatory signaling in innate immune defenses," *Clinical Microbiology Reviews*, vol. 22, no. 2. pp. 240–273, Apr. 2009.

[66] C. Rosales and E. Uribe-Querol, "Phagocytosis: A Fundamental Process in Immunity," *BioMed Research International*, pp. 1–18, Jun. 2017.

[67] C. A. J. Janeway, P. Travers, and M. Walport, "The destruction of antibody-coated pathogens via Fc receptors," *Janeway's Immunobiology The Immune System in Health and Disease*, 2001.

[68] M. V. Carroll and R. B. Sim, "Complement in health and disease," *Advanced Drug Delivery Reviews*, vol. 63, no. 12, pp. 965–975, Sep. 2011.

[69] J. V. Sarma and P. A. Ward, "The complement system.," *Cell and Tissue Research*, vol. 343, no. 1, pp. 227–35, Jan. 2011.

[70] M. Chen, M. R. Daha, and C. G. M. Kallenberg, "The complement system in systemic autoimmune disease," *Journal of Autoimmunity*, vol. 34, no. 3, pp. J276–J286, May 2010.

[71] B. S. Harvey, C. J. Baker, and M. S. Edwards, "Contributions of complement and immunoglobulin to neutrophil-mediated killing of enterococci," *Infection and Immunity*, vol. 60, no. 9, pp. 3635–3640, 1992.

[72] M. J. Gaglani, C. J. Baker, and M. S. Edwards, "Contribution of antibody to neutrophil-mediated killing of Enterococcus faecalis," *Journal of Clinical Immunology*, vol. 17, no. 6, pp. 478–484, Nov. 1997.

[73] J. Huebner, "Antibody-Antigen Interactions and Measurements of Immunologic

Reactions," in *Immunology, Infection, and Immunity*, 2004, pp. 224–225.

[74] R. C. Arduino, B. E. Murray, and R. M. Rakita, "Roles of antibodies and complement in phagocytic killing of enterococci," *Infection and Immunity*, vol. 62, no. 3, pp. 987–993, Mar. 1994.

[75] J. Burnie, T. Carter, G. Rigg, S. Hodgetts, M. Donohoe, and R. Matthews, "Identification of ABC transporters in vancomycin-resistant Enterococcus faecium as potential targets for antibody therapy," *FEMS Immunology & Medical Microbiology*, vol. 33, no. 3, pp. 179–189, Jul. 2002.

[76] F. Romero-Saavedra *et al.*, "Identification of peptidoglycan-Associated proteins as vaccine candidates for enterococcal infections," *PLoS ONE*, vol. 9, no. 11, p. e111880, 2014.

[77] A. Kropec, I. G. Sava, C. Vonend, T. Sakinc, E. Grohmann, and J. Huebner, "Identification of SagA as a novel vaccine target for the prevention of Enterococcus faecium infections," *Microbiology*, vol. 157, no. 12, pp. 3429–3434, Dec. 2011.

[78] F. S. Rossmann, D. Laverde, A. Kropec, F. Romero-Saavedra, M. Meyer-Buehn, and J. Huebner, "Isolation of highly active monoclonal antibodies against multiresistant Gram-positive bacteria," *PLoS ONE*, vol. 10, no. 2, p. e0118405, 2015.

[79] A. L. Flores-Mireles, J. S. Pinkner, M. G. Caparon, and S. J. Hultgren, "EbpA vaccine antibodies block binding of Enterococcus faecalis to fibrinogen to prevent catheter-associated bladder infection in mice," *Science Translational Medicine*, vol. 6, no. 254, p. 254ra127, Sep. 2014.

[80] L. Czaplewski *et al.*, "Alternatives to antibiotics—a pipeline portfolio review," *The Lancet Infectious Diseases*, vol. 16, no. 2, pp. 239–251, Feb. 2016.

[81] L. R. Berghman, D. Abi-Ghanem, S. D. Waghela, and S. C. Ricke, "Antibodies: An Alternative for Antibiotics?," *Poultry Science*, vol. 84, no. 4, pp. 660–666, 2005.

[82] C. Theilacker, W. A. Krueger, A. Kropec, and J. Huebner, "Rationale for the development of immunotherapy regimens against enterococcal infections," *Vaccine*, vol. 22, pp. S31–S38, Dec. 2004.

[83] K. U. Jansen, D. Q. Girgenti, I. L. Scully, and A. S. Anderson, "Vaccine review:

‘Staphyloccocus aureus vaccines: Problems and prospects,’” *Vaccine*, vol. 31, no. 25, pp. 2723–2730, Jun. 2013.

[84] Y. K. Stranger-Jones, T. Bae, and O. Schneewind, “Vaccine assembly from surface proteins of Staphylococcus aureus,” *Proceedings of the National Academy of Sciences*, vol. 103, no. 45, pp. 16942–16947, Nov. 2006.

[85] S. Koch, M. Hufnagel, and J. Huebner, “Treatment and prevention of enterococcal infections - alternative and experimental approaches.,” *Expert opinion on biological therapy*, vol. 4, no. 9, pp. 1519–31, Sep. 2004.

[86] B. K. Giersing, S. S. Dastgheyb, K. Modjarrad, and V. Moorthy, “Status of vaccine research and development of vaccines for Staphylococcus aureus,” *Vaccine*, vol. 34, no. 26, pp. 2962–2966, Jun. 2016.

[87] V. P. O’Brien, T. J. Hannan, H. V Nielsen, and S. J. Hultgren, “Drug and Vaccine Development for the Treatment and Prevention of Urinary Tract Infections,” *Microbiology Spectrum*, vol. 4, no. 1, Feb. 2016.

[88] D. Laverde *et al.*, “Synthetic teichoic acid conjugate vaccine against nosocomial Gram-positive bacteria,” *PLoS ONE*, vol. 9, no. 10, p. e110953, 2014.

[89] D. Laverde, Z. Kaczynski, J. Huebner, and A. Hartke, “Effector-Function-Directed Identification and Purification of Putative Vaccine Antigens from Enterococcus spp,” Thèse de doctorat Université de Caen Basse-Normandie, 2015.

[90] F. S. Rossmann, A. Kropec, D. Laverde, F. R. Saaverda, D. Wobser, and J. Huebner, “In vitro and in vivo activity of hyperimmune globulin preparations against multiresistant nosocomial pathogens,” *Infection*, vol. 43, no. 2, pp. 169–175, Apr. 2015.

[91] J. Huebner, A. Quaas, W. A. Krueger, D. A. Goldmann, and G. B. Pier, “Prophylactic and therapeutic efficacy of antibodies to a capsular polysaccharide shared among vancomycin-sensitive and -resistant enterococci,” *Infection and Immunity*, vol. 68, no. 8, pp. 4631–4636, Aug. 2000.

[92] R. D. Astronomo and D. R. Burton, “Carbohydrate vaccines: Developing sweet solutions to sticky situations?,” *Nature Reviews Drug Discovery*, vol. 9, no. 4, pp. 308–324, Apr. 2010.

[93] R. S. Daum and B. Spellberg, “Progress toward a staphylococcus aureus vaccine,” *Clinical Infectious Diseases*, vol. 54, no. 4, pp. 560–567, Feb. 2012.

[94] A. J. Pollard, K. P. Perrett, and P. C. Beverley, “Maintaining protection against invasive bacteria with protein–polysaccharide conjugate vaccines,” *Nature Reviews Immunology*, vol. 9, no. 3, pp. 213–220, Mar. 2009.

[95] E. Nagy, C. Giefing, and A. von Gabain, “Anti-infective antibodies: a novel tool to prevent and treat nosocomial diseases,” *Expert Review of Anti-infective Therapy*, vol. 6, no. 1, pp. 21–30, Feb. 2008.

[96] G. Ada, “Vaccines and Vaccination,” *New England Journal of Medicine*, vol. 345, no. 14, pp. 1042–1053, Oct. 2001.

[97] V. Verez-Bencomo *et al.*, “A synthetic conjugate polysaccharide vaccine against Haemophilus influenzae type b,” *Science*, vol. 305, no. 5683, pp. 522–5, Jul. 2004.

[98] A. Phalipon *et al.*, “A Synthetic Carbohydrate-Protein Conjugate Vaccine Candidate against Shigella flexneri 2a Infection,” *The Journal of Immunology*, vol. 182, no. 4, pp. 2241–2247, Feb. 2009.

[99] C. Grandjean, A. Boutonnier, B. Dassy, J. M. Fournier, and L. A. Mulard, “Investigation towards bivalent chemically defined glycoconjugate immunogens prepared from acid-detoxified lipopolysaccharide of Vibrio cholerae O1, serotype Inaba,” *Glycoconjugate Journal*, vol. 26, no. 1, pp. 41–55, Jan. 2009.

[100] E. von Behring and S. Kitasato, “The mechanism of immunity in animals to diphtheria and tetanus,” *Deutsche Medizinische Wochenschrift*, vol. 16, pp. 1113–1114, 1890.

[101] H. D. Marston, C. I. Paules, and A. S. Fauci, “Monoclonal Antibodies for Emerging Infectious Diseases — Borrowing from History,” *New England Journal of Medicine*, p. p1802256, 2018.

[102] G. Köhler and C. Milstein, “Continuous cultures of fused cells secreting antibody of predefined specificity,” *Nature*, vol. 256, no. 5517, pp. 495–497, Aug. 1975.

[103] A. Casadevall, E. Dadachova, and L. A. Pirofski, “Passive antibody therapy for infectious diseases,” *Nature Reviews Microbiology*, vol. 2, no. 9. pp. 695–703, Sep. 01, 2004.

[104] P. Ehrlich, “Experimentelle Untersuchungen über Immunität,” *Deutsche Medizinische Wochenschrift*, vol. 32, p. 976, 1891.

[105] C. Hamers-Casterman *et al.*, “Naturally occurring antibodies devoid of light chains,” *Nature*, vol. 363, no. 6428, pp. 446–448, Jun. 1993.

[106] A. F. U. H. Saeed, R. Wang, S. Ling, and S. Wang, “Antibody engineering for pursuing a healthier future,” *Frontiers in Microbiology*, vol. 8, no. MAR, 2017.

[107] A. Frenzel, M. Hust, and T. Schirrmann, “Expression of recombinant antibodies,” *Frontiers in Immunology*, vol. 4, no. JUL. Frontiers, p. 217, Jul. 29, 2013.

[108] M. J. Kwakkenbos, P. M. van Helden, T. Beaumont, and H. Spits, “Stable long-term cultures of self-renewing B cells and their applications.,” *Immunological reviews*, vol. 270, no. 1, pp. 65–77, Mar. 2016.

[109] A. Lanzavecchia and D. Corti, “Efficient Methods To Isolate Human Monoclonal Antibodies from Memory B Cells and Plasma Cells,” *Microbiology Spectrum*, vol. 2, no. 5, Oct. 2014.

[110] J. Zhang, “Mammalian Cell Culture for Biopharmaceutical Production,” *Manual of Industrial Microbiology and Biotechnology, Third Edition*, pp. 157–178, 2010.

[111] H. B. Oral, C. A. Akdiş, and C. Özakin, “Back to the future: Antibody-based strategies for the treatment of infectious diseases,” *Applied Biochemistry and Biotechnology - Part B Molecular Biotechnology*, vol. 21, no. 3, pp. 225–239, 2002.

[112] H. W. Schroeder, L. Cavacini, H. W. Schroeder Jr., L. Cavacini, H. W. Schroeder, and L. Cavacini, “Structure and function of immunoglobulins,” *The Journal of allergy and clinical immunology*, vol. 125, no. 2 Suppl, pp. S41-52, Feb. 2010.

[113] O. H. Brekke and I. Sandlie, “Therapeutic antibodies for human diseases at the dawn of the twenty-first century,” *Nature Reviews Drug Discovery*, vol. 2, no. 1, pp. 52–62, Jan. 2003.

[114] M. H. Wilcox *et al.*, “Bezlotoxumab for Prevention of Recurrent Clostridium difficile Infection,” *New England Journal of Medicine*, vol. 376, no. 4, pp. 305–317, Jan. 2017.

[115] X. Saez-Llorens *et al.*, “Safety and pharmacokinetics of an intramuscular humanized

monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia," *Pediatric Infectious Disease Journal*, vol. 17, no. 9, pp. 787–791, Feb. 1998.

[116] A. Sădziene, P. A. Rosa, P. A. Thompson, D. M. Hogan, and A. G. Barbour, "Antibody-resistant mutants of Borrelia burgdorferi: in vitro selection and characterization.," *The Journal of experimental medicine*, vol. 176, no. 3, pp. 799–809, Sep. 1992.

[117] A. L. Flores-Mireles *et al.*, "Antibody-based therapy for enterococcal catheter-associated urinary tract infections," *mBio*, vol. 7, no. 5, pp. e01653-16, Nov. 2016.

[118] E. Kalfopoulou *et al.*, "Development of opsonic mouse monoclonal antibodies against multidrug-resistant enterococci," *Infection and Immunity*, vol. 87, no. 9, Sep. 2019.

[119] L. E. Weisman *et al.*, "A Randomized Study of a Monoclonal Antibody (Pagibaximab) to Prevent Staphylococcal Sepsis," *PEDIATRICS*, vol. 128, no. 2, pp. 271–279, Aug. 2011.

[120] M. Patel and D. A. Kaufman, "Anti-lipoteichoic acid monoclonal antibody (pagibaximab) studies for the prevention of staphylococcal bloodstream infections in preterm infants," *Expert Opinion on Biological Therapy*, vol. 15, no. 4, pp. 595–600, Apr. 2015.

[121] A. M. Guzman Prieto *et al.*, "Global emergence and dissemination of enterococci as nosocomial pathogens: Attack of the clones?," *Frontiers in Microbiology*, vol. 7, no. MAY. Frontiers Media SA, p. 788, 2016.

[122] M. Nigo, J. M. Munita, C. A. Arias, and B. E. Murray, "What's New in the Treatment of Enterococcal Endocarditis?," *Current Infectious Disease Reports*, vol. 16, no. 10. p. 431, Oct. 2014.

[123] G. Tosato and J. I. Cohen, "Generation of Epstein-Barr Virus (EBV)-immortalized B cell lines.," *Current protocols in immunology*, p. Unit 7.22, 2007.

[124] A. B. Rickinson, D. J. Moss, and J. H. Pope, "Long-term T-cell-mediated immunity to Epstein-Barr virus in man. II. Components necessary for regression in virus-infected leukocyte cultures," *International Journal of Cancer*, vol. 23, no. 5, pp. 610–617, May 1979.

[125] E. Engvall, "Enzyme immunoassay ELISA and EMIT," *Methods in Enzymology*, vol. 70, no. C, pp. 419–439, 1980.

[126] E. A. Kabat, T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Koeler, “Sequences of proteins of immunological interest,” *Analytical Biochemistry*, vol. 138, no. 1. p. 265, 1984.

[127] C. Kelly-Quintos, L. A. Cavacini, M. R. Posner, D. Goldmann, and G. B. Pier, “Characterization of the opsonic and protective activity against Staphylococcus aureus of fully human monoclonal antibodies specific for the bacterial surface polysaccharide poly-N-acetylglucosamine,” *Infection and Immunity*, vol. 74, no. 5, pp. 2742–2750, 2006.

[128] J. Sambrook and D. W. Russell, “Molecular Cloning: A laboratory manual 3th edition,” *CSHL Press*, 2001.

[129] V. Levenshtein, “Binary codes capable of correcting deletions, insertions, and reversals,” *Soviet Physics Doklady*, vol. 10, no. 8, pp. 707–710, 1966.

[130] F. J. Damerau, “A technique for computer detection and correction of spelling errors,” *Communications of the ACM*, vol. 7, no. 3, pp. 171–176, Mar. 1964.

[131] M. Kozak, “An analysis of 5’-noncoding sequences from 699 vertebrate messenger rNAS,” *Nucleic Acids Research*, vol. 15, no. 20, pp. 8125–8148, Oct. 1987.

[132] B. Sugden and W. Mark, “Clonal transformation of adult human leukocytes by Epstein-Barr virus.,” *Journal of virology*, vol. 23, no. 3, pp. 503–8, Sep. 1977.

[133] W. W. Wilfinger, K. Mackey, and P. Chomczynski, “Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity,” *BioTechniques*, vol. 22, no. 3, pp. 474–481, Mar. 1997.

[134] I. Sela-Culang, V. Kunik, and Y. Ofran, “The structural basis of antibody-antigen recognition,” *Frontiers in Immunology*, vol. 4, no. OCT, p. 302, Oct. 2013.

[135] A. S. Lubiniecki, “Large-Scale Mammalian Cell Culture Technology,” pp. 21–22, 1990.

[136] P. J. Carter, “Potent antibody therapeutics by design,” *Nature Reviews Immunology*, vol. 6, no. 5, pp. 343–357, 2006.

[137] A. Lanzavecchia, N. Bernasconi, E. Traggiai, C. R. Ruprecht, D. Corti, and F. Sallusto, “Understanding and making use of human memory B cells,” *Immunological Reviews*, vol. 211, no. 1, pp. 303–309, Jun. 2006.

[138] M. Hufnagel and J. Huebner, “Analysis of the specific immune response against capsular

polysaccharides of two patients with systemic enterococcal infections," *Infection*, vol. 33, no. 5–6, pp. 373–376, Oct. 2005.

[139] G. Nilsson, Kenneth; Klein, "Phenotypic and Cytogenetic Characteristics of Human B-Lymphoid Cell Lines and Their Relevance for the Etiology of Burkitt'S Lymphoma," *Advances in Cancer Research*, vol. 37, pp. 319–380, 1982.

[140] C. E. Rose *et al.*, "Multilaboratory comparison of Streptococcus pneumoniae opsonophagocytic killing assays and their level of agreement for the determination of functional antibody activity in human reference sera.," *Clinical and vaccine immunology : CVI*, vol. 18, no. 1, pp. 135–42, Jan. 2011.

[141] C. Cordonnier *et al.*, "Relationship between IgG titers and opsonocytophagocytic activity of anti-pneumococcal antibodies after immunization with the 7-valent conjugate vaccine in allogeneic stem cell transplant," *Bone Marrow Transplantation*, vol. 45, no. 9, pp. 1423–1426, Sep. 2010.

[142] A. Soininen, M. Karpala, S.-L. Wahlman, H. Lehtonen, and H. Käyhty, "Specificities and opsonophagocytic activities of antibodies to pneumococcal capsular polysaccharides in sera of unimmunized young children.," *Clinical and diagnostic laboratory immunology*, vol. 9, no. 5, pp. 1032–8, Sep. 2002.

[143] P. S. Hiemstra, J. Brands-Tajouiti, and R. van Furth, "Comparison of antibody activity against various microorganisms in intravenous immunoglobulin preparations determined by ELISA and opsonic assay.," *The Journal of laboratory and clinical medicine*, vol. 123, no. 2, pp. 241–6, Feb. 1994.

[144] S. Romero-Steiner *et al.*, "Standardization of an Opsonophagocytic Assay for the Measurement of Functional Antibody Activity against Streptococcus pneumoniae Using Differentiated HL-60 Cells," *Clinical and diagnostic laboratory immunology*, vol. 4, no. 4, pp. 415–422, 1997.

[145] F. Lamari, E. D. Anastassiou, G. Dimitracopoulos, and N. K. Karamanos, "Immunochemical-based assays in the bioanalysis of immunoglobulins," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 23, no. 6, pp. 939–946, Nov. 2000.

[146] J. S. Plested *et al.*, "Functional Opsonic Activity of Human Serum Antibodies to Inner Core

Lipopolysaccharide (galE) of Serogroup B Meningococci Measured by Flow Cytometry," *Infection and Immunity*, vol. 69, no. 5, pp. 3203–3213, 2001.

[147] G. B. Pier *et al.*, "Human Monoclonal Antibodies to Pseudomonas aeruginosa Alginate That Protect against Infection by Both Mucoid and Nonmucoid Strains," *The Journal of Immunology*, vol. 173, no. 9, pp. 5671–5678, 2004.

[148] M. et al. Preston, "Production and Characterization of a Set of Mouse-Human Chimeric Immunoglobulin G (IgG) Subclass and IgA Monoclonal Antibodies with Identical Variable Regions Specific for Pseudomonas aeruginosa Serogroup O6 Lipopolysaccharide," *Infection and Immunity*, vol. 66, no. 9, pp. 4137–4142, 1998.

[149] T. Keller *et al.*, "Selection of scFv antibody fragments binding to human blood versus lymphatic endothelial surface antigens by direct cell phage display," *PLoS ONE*, vol. 10, no. 5, p. e0127169, 2015.

[150] S. Moutel *et al.*, "A multi-Fc-species system for recombinant antibody production," *BMC Biotechnology*, vol. 9, p. 14, Feb. 2009.

[151] L. Xu *et al.*, "Design and Characterization of a Human Monoclonal Antibody that Modulates Mutant Connexin 26 Hemichannels Implicated in Deafness and Skin Disorders," *Frontiers in Molecular Neuroscience*, vol. 10, p. 298, 2017.

[152] J. Zhu, "Mammalian cell protein expression for biopharmaceutical production," *Biotechnology Advances*, vol. 30, no. 5, pp. 1158–1170, Sep. 2012.

[153] J. Dumont, D. Euwart, B. Mei, S. Estes, and R. Kshirsagar, "Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives," *Critical Reviews in Biotechnology*, vol. 36, no. 6. pp. 1110–1122, 2016.

[154] J. R. Birch and A. J. Racher, "Antibody production," *Advanced Drug Delivery Reviews*, vol. 58, no. 5–6, pp. 671–685, Aug. 2006.

[155] H. C. Chiou, S. Vasu, C. Y. Liu, I. Cisneros, M. B. Jones, and J. F. Zmuda, "Scalable transient protein expression," *Methods in Molecular Biology*, vol. 1104, pp. 35–55, 2014.

[156] S. Geisse and B. Voedisch, "Transient expression technologies: Past, present, and future," *Methods in Molecular Biology*, vol. 899, pp. 203–219, 2012.

[157] V. Jäger *et al.*, "High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells," *BMC Biotechnology*, vol. 13, p. 1, 2013.

[158] N. K. Jain *et al.*, "A high density CHO-S transient transfection system: Comparison of ExpiCHO and Expi293," *Protein Expression and Purification*, vol. 134, pp. 38–46, Jun. 2017.

[159] C. Diepenbruck, M. Klinger, T. Urbig, P. Baeuerle, and R. Neef, "Productivity and quality of recombinant proteins produced by stable CHO cell clones can be predicted by transient expression in HEK cells," *Molecular Biotechnology*, vol. 54, no. 2, pp. 497–503, Jun. 2013.

[160] A. Croset *et al.*, "Differences in the glycosylation of recombinant proteins expressed in HEK and CHO cells," *Journal of Biotechnology*, vol. 161, no. 3, pp. 336–348, Oct. 2012.

[161] K. Zheng, C. Bantog, and R. Bayer, "The impact of glycosylation on monoclonal antibody conformation and stability," *mAbs*, vol. 3, no. 6, pp. 568–76, 2011.

[162] S. Romero-Steiner, D. M. Musher, M. S. Cetron, L. B. Pais, J. E. Groover, and G. M. Carlone, "Reduction in Functional Antibody Activity Against Streptococcus pneumoniae in Vaccinated Elderly Individuals Highly Correlates with Decreased IgG Antibody Avidity," *Clinical Infectious Diseases*, vol. 29, no. 2, pp. 281–288, Aug. 1999.

[163] T. K. Held, N. R. M. Jendrike, T. Rukavina, R. Podschun, and M. Trautmann, "Binding to and Opsonophagocytic Activity of O-Antigen-Specific Monoclonal Antibodies against Encapsulated and Nonencapsulated Klebsiella pneumoniae Serotype O1 Strains," *Infection and Immunity*, vol. 68, no. 5, pp. 2402–2409, 2000.

[164] M. Väkeväinen *et al.*, "Are the opsonophagocytic activities of antibodies in infant sera measured by different pneumococcal phagocytosis assays comparable?," *Clinical and diagnostic laboratory immunology*, vol. 8, no. 2, pp. 363–9, Mar. 2001.

[165] B. Simell, A. Nurkka, N. Ekström, N. Givon-Lavi, H. Käyhty, and R. Dagan, "Serum IgM antibodies contribute to high levels of opsonophagocytic activities in toddlers immunized with a single dose of the 9-valent pneumococcal conjugate vaccine.," *Clinical and vaccine immunology : CVI*, vol. 19, no. 10, pp. 1618–23, Oct. 2012.

[166] D. Dunn-Walters, C. Townsend, E. Sinclair, and A. Stewart, "Immunoglobulin gene

analysis as a tool for investigating human immune responses.," *Immunological reviews*, vol. 284, no. 1, pp. 132–147, 2018.

[167] J. Charles A Janeway, P. Travers, M. Walport, and M. J. Shlomchik, "The distribution and functions of immunoglobulin isotypes," 2001.

[168] R. F. Montano and S. L. Morrison, "Influence of the Isotype of the Light Chain on the Properties of IgG," *The Journal of Immunology*, vol. 168, no. 1, pp. 224–231, Jan. 2002.

[169] K. Smith, H. Shah, J. J. Muther, A. L. Duke, K. Haley, and J. A. James, "Antigen Nature and Complexity Influence Human Antibody Light Chain Usage and Specificity HHS Public Access," *Vaccine*, vol. 34, no. 25, pp. 2813–2820, 2016.

[170] R. Toughiri *et al.*, "Comparing domain interactions within antibody Fabs with kappa and lambda light chains," *mAbs*, vol. 8, 2016.

[171] E. Derudder *et al.*, "Development of immunoglobulin λ-chain-positive B cells, but not editing of immunoglobulin κ-chain, depends on NF-κB signals," *Nature Immunology*, vol. 10, no. 6, pp. 647–654, Jun. 2009.

[172] A. Teplyakov *et al.*, "Structural diversity in a human antibody germline library.," *mAbs*, vol. 8, no. 6, pp. 1045–63, 2016.

[173] B. D. Weitzner, R. L. Dunbrack, and J. J. Gray, "The Origin of CDR H3 Structural Diversity," *Structure/Folding and Design*, vol. 23, pp. 302–311, 2015.

[174] F. Wang *et al.*, "Reshaping Antibody Diversity," *Cell*, vol. 153, no. 6, pp. 1379–1393, 2013.

[175] S. D'Angelo, F. Ferrara, L. Naranjo, M. F. Erasmus, P. Hraber, and A. R. M. Bradbury, "Many routes to an antibody heavy-chain CDR3: Necessary, yet insufficient, for specific binding," *Frontiers in Immunology*, vol. 9, no. MAR, pp. 3953389–395, 2018.

[176] V. Kunik and Y. Ofran, "The indistinguishability of epitopes from protein surface is explained by the distinct binding preferences of each of the six antigen-binding loops," *Protein Engineering, Design and Selection*, vol. 26, no. 10, pp. 599–609, Oct. 2013.

[177] Y. Pasman and A. K. Kaushik, "VHand VLDomains of Polyspecific IgM and Monospecific IgG Antibodies Contribute Differentially to Antigen Recognition and Virus Neutralization Functions," *Scandinavian Journal of Immunology*, vol. 84, no. 1, pp. 28–38, Jul. 2016.

7. List of Figures

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9. Abbreviations

ADCC	Antibody-dependent cell mediated cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
AS	Aggregation substance
BSA	Bovine Serum Albumin
CDC	Complement dependent cytotoxicity
(c)DNA	(Complementary) deoxyribonucleic acid
CDR	Complementary determining regions
CHO	Chinese Hamster Ovary
CPS	Capsular Polysaccharide Serotyping System
DHG	Diheteroglycan
DHRF$^-$	Dihydrofolate reductase deficient
DMSO	Dimethylsulfoxide
EBV	Eppstein- Barr-Virus
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen binding fragment
FBS	Fetal bovine serum
Fc	Fragment crystallizable region
FR	Framework region
GFP	Green fluorescent protein
GI	Gastrointestinal
HEK	Human Embryonal Kidney
HEPES	Hydroxyethyl)-1-piperazinyl)-ethansulfon acid
IgG/IgA/IgM	Immunoglobuline G/A/M
LB	Luria Bertani
(L)TA	(Lipo-)teichoic acid
mAbs	Monoclonal antibodies
MAC	Membrane-attack-complex
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
OPA	Opsonophagocytic assay
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBPs	Penicillin binding proteins
PBS	Phosphate buffers saline
PCR	Polymerase chain reaction
PMNs	Polymorphonuclear neutrophils
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute (culture medium)
RSV	Respiratory syncytial virus
scFv	Single chain fragment variable
SPN	Supernatant
TBE	Tris borate EDTA
TSA /TSB	Tryptic soy agar/broth
VH	Variable heavy
VL	Variable light
VRE	Vancomycin resistant Enterococci

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