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# COMPARING *EX VIVO* HUMAN BLOOD MODEL WITH *IN VITRO* MAMMALS BLOOD MODELS FOR PHENOTYPIC CHARACTERIZATION OF *STAPHYLOCOCCUS EPIDERMIDIS*

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# COMPARING *EX VIVO* HUMAN BLOOD MODEL WITH *IN VITRO* MAMMALS BLOOD MODELS FOR PHENOTYPIC CHARACTERIZATION OF *STAPHYLOCOCCUS EPIDERMIDIS*

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by
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## Resumo

*Staphylococcus epidermidis* é uma bactéria comensal presente na pele e nas membranas mucosas humanas. Através da implantação de dispositivos médicos esta pode entrar na corrente sanguínea e provocar infeções graves, em particular, em recém-nascidos e em pacientes imunocomprometidos. Essas infeções são difíceis de diagnosticar e de tratar, resultando num aumento das taxas de morbilidade e mortalidade. Assim, devido à relevância clínica das infeções provocadas por *S. epidermidis* e devido à necessidade de encontrar novas opções de tratamento, o estudo dos fatores de virulência desta bactéria é de grande importância. Para tal, vários modelos biológicos têm vindo a ser utilizados, tendo o modelo *ex vivo* de sangue humano despertado especial interesse, uma vez que permite o estudo da bactéria no contexto das infeções da corrente sanguínea sem necessitar de instalações ou técnicos especializados. No entanto, apesar dessas vantagens, este modelo também apresenta limitações, especialmente no que diz respeito à disponibilidade de dadores, dificultando a sua implementação no contexto académico.

Assim, o objetivo desta dissertação foi superar essa limitação propondo a substituição do sangue humano fresco por sangue comercial de outros mamíferos. Para testar esta hipótese, a sobrevivência de várias estirpes de *S. epidermidis*, a secreção de proteases e o nível de transcrição dos genes *sepA* e *hld* foram estudados após a interação com sangue humano fresco e sangue comercial de cavalo e de carneiro. Os resultados obtidos mostraram que embora em sangue humano se tenha verificado uma diminuição no número bactérias (entre 4 e 6×) durante o período de incubação (4 horas), em ambas as concentrações de inóculo testadas ($10^8$ e $10^5$ CFU/mL), nos sangues comerciais de carneiro e cavalo foi observado um aumento do número de bactérias (entre 2 e 4×). Relativamente à secreção de proteases, nas duas concentrações de bactéria estudadas, a substituição de sangue humano pelos sangues de cavalo ou carneiro parece não ter causado alterações significativas. Finalmente, a expressão genética foi variável nas diferentes condições, com o gene *sepA* a ser transcrito ao mesmo nível nos 3 sangues, mas com a transcrição do gene *hld* significativamente mais elevada em sangue humano (entre 5 e 50×).

De uma forma geral, os resultados obtidos indicam que dependendo dos parâmetros sob estudo, o sangue humano fresco poderá ou não ser substituído pelos sangues comerciais de cavalo ou carneiro, implicando, assim, uma análise prévia à sua substituição.



**Palavras-chave:** *Staphylococcus epidermidis*; Infeções da corrente sanguínea; Modelos *ex vivo;* Sangue humano; Sangues comerciais de carneiro e cavalo.

## Abstract

*Staphylococcus epidermidis* is a commensal bacterium of healthy human skin and mucous membranes. Through the implantation of medical devices, *S. epidermidis* can enter the bloodstream and cause serious infections, in particular in neonates and in immunocompromised patients. These infections are difficult to diagnose and to treat, resulting in increased morbidity and mortality rates. Thus, due to the clinical relevance of the infections caused by *S. epidermidis* and the urgent need to find new treatment options, the study of its virulence factors is of paramount importance. To do so, several biological models have been used, having *ex vivo* human blood model gained special interest since it does not require neither specialized facilities nor personnel, and because it enables the study of *S. epidermidis* in the context of a bloodstream infection. However, despite these advantages, this model also has important limitations, mainly related to availability of donors, complicating its implementation in the academic context.

Hence, the aim of this dissertation was to overcome this limitation by proposing the replacement of human blood by commercial bloods from other mammals. To test this hypothesis, the survival of several *S. epidermidi*s strains, the secretion of proteases and the level of transcription of the genes *sepA* and *hld* were determined after interaction with fresh human blood and commercial horse and sheep bloods. The results obtained showed that although in human blood the number of bacteria tended to decrease (4 to 6-fold) during the period of incubation (4 hours), in the two inocula used ($10^8$ and $10^5$ CFU/mL), in both horse and sheep bloods a significant increase in the number of bacteria was observed (2 to 4-fold). To what concerns the secretion of proteases, in both inocula tested, our results suggested that the replacement of human blood by horse or sheep did not cause significant alterations. Finally, gene expression under the different conditions was variable, with *sepA* being similarly expressed in the 3 tested bloods, but with *hld* being significantly higher expressed in human blood (5 to 50-fold).

Overall, the results obtained show that depending on the parameters under analysis, fresh human blood may or not be replaced by commercial horse or sheep bloods implicating, thus, a previous evaluation of its substitution.



**Keywords:** *Staphylococcus epidermidis*; Bloodstream infections; *ex vivo* models; Human blood; Commercial sheep and horse bloods.

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# Introduction

## 1. Nosocomial infections

Since birth, our immune system has a key role in our survival, protecting us from bacteria, viruses, parasites, etc. However, in some situations, our immune system fails and serious infections can emerge.

Nosocomial or healthcare-associated infections are defined as "infections acquired in the hospital, or other healthcare institutions, by a patient who was admitted for a reason other than that infection" (Ducel *et al*., 2002; Castro-Sánchez & Holmes, 2015). Patients submitted to invasive techniques or with compromised immune system (due to age, illness or invasive treatments), neonates, the presence of other infections and poor sanitary conditions are the major factors contributing to the development of nosocomial infections, which lead to increased morbidity and mortality rates (Jenkins, 2017). In addition, these infections have an important economic impact due to the high costs associated with prolonged hospitalization and additional diagnostics and treatments (Ducel *et al*., 2002; Breathnach *et al*., 2013; Khan *et al.*, 2017). These infections occur worldwide affecting both industrialized and non-industrialized countries (Dong *et al.,* 2018). A study carried out by the World Health Organization has determined that, in 2002, the prevalence of nosocomial infections in Europe was around 7.7% (Ducel *et al*., 2002), having increased over the years reaching, in 2011, 15% (Sydnor and Perl, 2011). In regards to non-industrialized countries, the analysis of the literature revealed a clear fragmented picture of the endemic burden of nosocomial infections, resulting in the absence of reliable estimates. This is mainly because the surveillance of healthcare-associated infections implicates time, resources and personnel with experience on study design, data collection, analysis and interpretation. Thus, very few low- and middle-income countries can mount such surveillance systems (Allegranzi *et al.*, 2011). However, some studies report that the prevalence of nosocomial infections in non-industrialized countries are higher than in industrialized countries (Allegranzi *et al.*, 2011; Nejad *et al.*, 2011; Mbim *et al.*, 2016). So, considering these high values, preventive measures are necessary to control and reduce the incidence of these infections. For that reason, hospitals and healthcare facilities must follow strict prevention programmes (Zingg *et al.,* 2015).

Nosocomial infections can be caused by exogenous microorganisms, which are those transmitted among patients or by the medical staff, visitors or contaminated objects (Breathnach

*et al*., 2013). In addition, they can also be caused by endogenous microorganisms, i.e., those present in patients' natural flora (Ducel *et al*., 2002). Indeed, the majority of the nosocomial infections diagnosed nowadays are caused by endogenous microorganisms like the gram-negative bacteria *Escherichia coli, Proteus mirabilis* and *Klebsiella pneumonia* and by the Gram-positive *Staphylococcus aureus* and coagulase-negative Staphylococci (Breathnach *et al*., 2013; Khan *et al.,* 2015).

## 2. Genus *Staphylococcus*

The genus *Staphylococcus* consists of non-motile, non-spore forming and spherical cocci, with sizes between 0.5-1.5 μm in diameter. They are facultative anaerobes that grow by aerobic respiration or by fermentation (Pfaller and Herwaldt, 1988; Harris *et al.*, 2002). The term *Staphylococcus* was proposed in 1880 by Ogston, who linked this microorganism to wound infections (Ogton, 1880). A few years later, in 1891, a pathologist named Welch defined *Staphylococcus epidermidis albus* as a colonizing microorganism of the human skin, which was also found in aseptic wounds (Becker *et al*., 2014). In the 1940s, after being suggested that *Staphylococcus* pathogenic strains were the ones producing the enzyme coagulase (Chapman *et al* 1934; Cruickshank 1937), Fairbrother proposed the production of this enzyme as the main differentiating factor among staphylococcal species (Fairbrother, 1940), classifying the species as coagulase-positive or coagulase-negative Staphylococci (CoNS) (Becker *et al*., 2014). The genus *Staphylococcus* has suffered several changes over the years but since 2014 it consists in 47 species, 38 of which fulfil the categorization of CoNS (Becker *et al*., 2014). These are the most prevalent and frequent colonizers found in healthy human skin and mucous membranes (Bierowiec *et al.,* 2019). However, previously regarded as innocuous commensals, CoNS are now recognized as important opportunistic microorganisms being *Staphylococcus epidermidis* the most prevalent and important species (Cogen *et al.,* 2008).

## 3. *Staphylococcus epidermidis* and its clinical implications

*S. epidermidis* is normally found in the axillae, head, arms and legs and in the mucous membranes of the nasopharynx (Zhou and Li, 2015). This bacterium plays an important role in the maintenance of the normal flora of the skin and in the protection against pathogenic

microorganisms through mechanisms of competition (Cogen *et al*., 2008). To survive the stressful conditions existing in human skin, such as high concentration of salt and osmotic pressure, *S. epidermidis* is equipped with several genes that encode proteins that enable its survival in such environment (Otto, 2008, 2013). Although *S. epidermidis* has a benign relationship with the host, in the last decades it has become one of the most important opportunist nosocomial agents. This bacterium primarily affects immunocompromised adults as well as preterm, low weight (less than 1500 g) and low gestational age neonates (Cheung and Otto, 2010) that have indwelling medical devices such as central line catheters, which are frequently required for feeding and administration of fluids or drugs (Cheung and Otto, 2010). This association with the use of indwelling medical devices has to do with *S. epidermidis* tenacious capacity to attach to the surfaces of such devices and form biofilms (Otto, 2013; Becker *et al*., 2014).

Biofilms are agglomerates of cells attached to a surface and covered by a self-produced extracellular matrix, which is mainly composed by polysaccharides, proteins, lipids and extracellular DNA (Costerton *et al.*, 1978; Kristian *et al.*, 2008; López *et al.*, 2010; Le *et al.*, 2018). The development of a biofilm occurs in 3 steps: attachment, maturation and detachment (Figure 1.1) (O'Toole *et al.*, 2000; Otto, 2013). During attachment, *S. epidermidis* produces dozens of microbial surface components that recognize and bind to human matrix proteins, which readily cover the implanted medical device (Otto, 2008). After the attachment stage, maturation begins with the aggregation of cells that, in this bacterium, is mainly mediated by the polysaccharide intercellular adhesin (PIA), also called poly-N-acetylglucosamine (PNAG) (Mack *et al*., 1996; Otto, 2008). This molecule is the largest constituent of the extracellular matrix of *S. epidermidis* biofilms (Heilmann *et al*., 1996; Otto, 2012). However, several strains of *S. epidermidis* can form biofilms in the absence of PNAG (Mack *et al.*, 2006). In this case, the formation of biofilms is mediated by proteins such as the extracellular matrix binding protein (Embp) (Christner *et al.*, 2010), accumulation-associated protein (Aap) (Schaeffer *et al.*, 2015; Alabdullatif and Ramirez-Arcos, 2018) and the biofilm homolog protein (Bhp) (Chokr *et al.*, 2006; Geoghegan *et al.*, 2010; Speziale *et al.*, 2014). PNAG-independent biofilms are, though, normally weaker than the ones formed in the presence of PNAG (Schommer *et al.*, 2011; Cue *et al.*, 2012; Le *et al.*, 2018). Finally, the life cycle of the biofilm ends with the dispersion of cells to the involving environment, a process primarily intermediated by phenol-soluble modulins (PSMs) (Otto, 2013). The cells released from the biofilm can cause serious

biofilm secondary infections such as bacteraemia and sepsis (O'Gara & Humphreys, 2001; Wang *et al*., 2011).

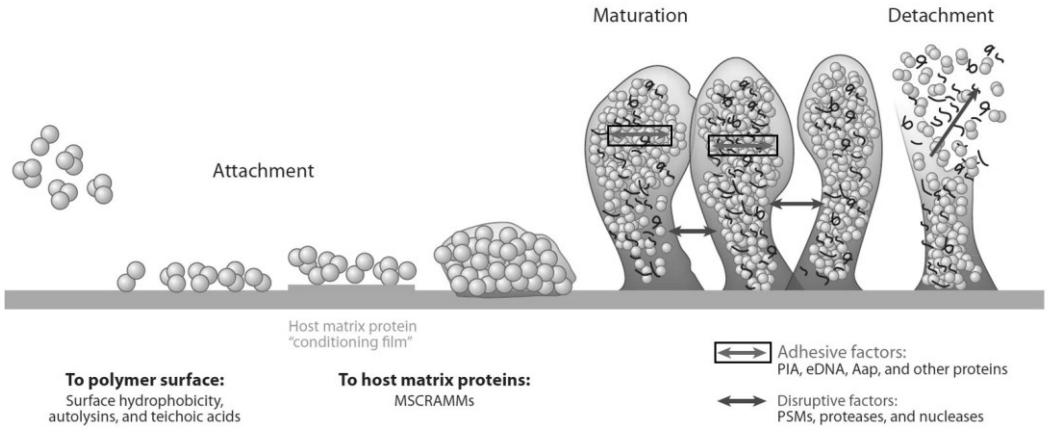


**Figure 1.1. Model of *Staphylococcus* biofilm development.** Aap-accumulation-associated protein; eDNA-extracellular DNA; MSCRAMMs-microbial surface components recognizing adhesive matrix molecules; PIA-polysaccharide intercellular adhesin and PSMs-phenol-soluble modulins (adapted from Otto, 2013).

Due to the gradients of nutrients, waste products and signalling factors formed throughout the biofilm, depending on its location, bacterial cells will experience a slightly different environment within the same biofilm. As a consequence, biofilms are very heterogeneous populations of cells, including cells with different biological activities and in different metabolic states (Stewart and Franklin, 2008). Dormant cells, for instance, are a subpopulation of cells that have a reduced metabolic rate (Otto, 2012; Carvalhais *et al*., 2014). This characteristic enables them to evade antibiotics targeting cell wall, protein and nucleic acids biosynthesis (Lewis, 2012; Wood *et al.*, 2013; Cerca *et al.*, 2014). Furthermore, due to their reduced metabolic activity, dormant cells are often undetected by culture-based detection methods (Cerca *et al.,* 2011), complicating the diagnosis of *S. epidermidis* biofilm-related infections. In addition, biofilms also harbour persister cells, which are a variant of dormant cells that are highly tolerant to antibiotics (Lewis, 2010; Cerca *et al.*, 2011, 2014; Wood *et al.*, 2013; Roberts *et al.*, 2015). Also protecting biofilm cells from the action of antibiotics as well as from

the host immune system attack is the biofilm matrix (Figure 1.2) (Otto, 2012; Kleinschmidt *et al.*, 2015; Le *et al.*, 2018).

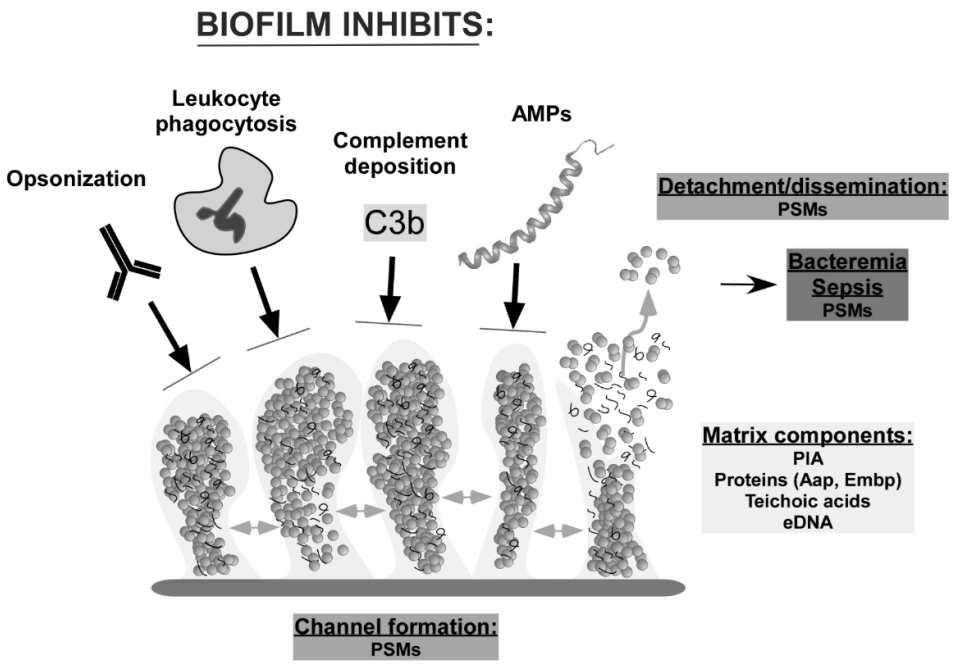


**Figure 1.2. Mechanisms employed by *S. epidermidis* biofilms to evade the host immune system**. Aap-accumulation-associated protein; Embp-extracellular matrix-binding protein; eDNA-extracellular DNA; PIA-polysaccharide intercellular adhesin; PSMs-phenol-soluble modulins (Le *et al.*, 2018).

Thus, *S. epidermidis* biofilm-associated infections present a recalcitrant and relapsing nature often implicating the removal and replacement of the colonized device (Mack *et al.*, 2013; Galac *et al.*, 2017). This not only leads to increased morbidity and mortality rates but also implicates extra costs associated with additional surgeries, hospitalization and treatment (Nguyen *et al.*, 2017; Le *et al.*, 2018).

## 4. Bloodstream infections caused by *S. epidermidis*

Bloodstream infections caused by *S. epidermidis* have lower frequency compared to other nosocomial infections, like urinary tract infections, but the associated mortality rate is higher (Ducel *et al*., 2002; Mack *et al*., 2013; Kleinschmidt *et al.*, 2015). Aggravating this situation is the emergence of strains resistant to several antibiotics used for the treatment of

staphylococcal infections such as methicillin (methicillin-resistant *S. epidermidis*) and vancomycin (vancomycin-intermediate *S. epidermidis*) (Struelens *et al*., 1998; Otto, 2012; Howden *et al.,* 2014). As already referred, bloodstream infections are mainly caused by the cells released from the biofilms formed by *S. epidermidis,* in particular those formed on central line catheters (Donegan & Cheung, 2009; Nguyen *et al*., 2017) (Figure 1.3).

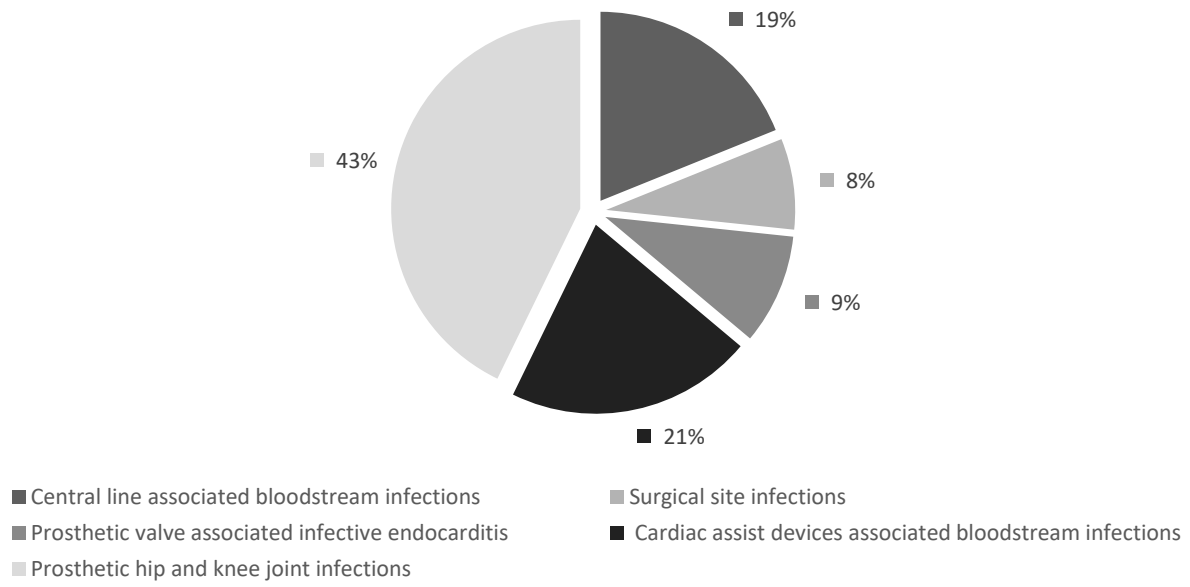


**Figure 1.3. Medical device-associated infections caused by CoNS in the USA.** (adapted from Mack *et al.*, 2013).

Patients can develop bacteraemia, which means that the bacterium is present in the bloodstream, but without necessarily showing signs of infection. These infections are, thus, very difficult to detect at an early stage being, consequently, hard to treat and may progress to sepsis (Kleinschmidt *et al*., 2015; Otto, 2017). Sepsis develops when bacteria in the bloodstream start multiplying. To protect the host, the immune system initiates an overwhelming defensive response (Qin *et al.*, 2017). This reaction is activated by several bacterial factors such as the molecular patterns on the surface of the bacterium (Otto, 2012). Additionally, *S. epidermidis* produce toxins such as PSMs that also exhibit proinflammatory activity (Namvar *et al.*, 2014). These toxins attack and eliminate important cells of the immune system making it difficult to combat the infection (Donegan *et al*., 2009; Otto, 2012). Complicating the treatment of these infections is the difficulty to diagnose them. When there is a clinical suspicion of infection, blood samples are routinely taken to try to detect the infectious agent. Since *S. epidermidis* is a commensal bacterium of human skin and mucous membranes

(Kleinschmidt *et al*., 2015), it can be transferred into the sample during the collection procedure. Hence, the presence of *S. epidermidis* in a blood culture is frequently considered a contamination (Loonen *et al.*, 2014) and, therefore, treatment is not administered immediately, allowing the progression of the infection (Kirn & Weinstein, 2013; Dargère *et al.*, 2014). Thus, the study of *S. epidermidis* bloodstream infections is of paramount importance to help design and develop new and more effective treatment options. This can be pursued by identifying specific mechanisms employed by the bacteria to survive inside the host.

## 5. Models to study *S. epidermidis* virulence in bloodstream infections

*In vitro* models have been used for a long time to study bacterial infections due to its easiness and have provided invaluable information. However, these biological models present several limitations as the laboratory environment is far from being close to the one encountered during infection (Coenye & Nelis, 2010; Roberts *et al*., 2015). The best way to mimic the conditions found in the host is to use *in vivo* vertebrate models, in particular, mouse models. Nevertheless, beside the clear advantages, *in vivo* mouse models present several ethical considerations and implicate higher costs, as well as specialized facilities and personnel. Furthermore, although mouse and humans have several similarities, there are also important differences that may have an impact on the outcome. Moreover, bearing in mind the 3R policy: Reduce, Refine and Reuse, *in vivo* mouse models shall be kept to a minimum and must be replaced when possible (Coenye & Nelis, 2010; Fröhlich & Salar-behzadi, 2014; Moleiro *et al*., 2017). Hence, *ex vivo* models, which can integrate characteristics of both *in vitro* and *in vivo* models, have gained special interest. Evidently, *ex vivo* models cannot stand alone but are useful to screen for the most promising conditions to be tested in *in vivo* mouse models. The *ex vivo* human blood model is particularly interesting to study *S. epidermidis* and has been widely used in the last years (França *et al.*, 2014; Alves *et al.*, 2016; França & Cerca, 2016; Bras *et al.*, 2017; Rosman *et al.,* 2018). Notwithstanding, the *ex vivo* human blood model also has limitations, namely the fact that this is a closed system, making impossible the constant renewal of cells, as it occurs in the host. In addition, human blood donors are necessary, also raising ethical considerations. Furthermore, in the academia context, is not easy to find donors, complicating the implementation of this model.

## 6. Aim of the dissertation

Having into consideration the clinical relevance of the bloodstream infections caused by *S. epidermidis* and the importance of the *ex vivo* human blood model to study the virulence of this bacterium, this Master dissertation had as primary objective to overcome the limitations of this model by investigating the possibility of replacing fresh human blood by commercial bloods of other mammals. To do so, the behavior of the bacterium in fresh human blood and in commercially available horse and sheep bloods was evaluated in regards to:

1. Survival, determined by CFU counting;
2. Proteases secretion, determined by macroscopic observation of the colonies on skim milk plates;
3. Transcription of genes involved in the evasion from the host immune system, determined through quantitative PCR.

It is important to refer that this dissertation was developed as a part of an ongoing project, which aims to explore the role of biofilm cells dormancy and iron uptake in the virulence of *S. epidermidis*, by studying the fitness of strains lacking genes of interest in an *ex vivo* human blood model.

# Materials and Methods

## 1. Human blood collection and ethics statement

Human blood was collected from healthy adult volunteers, by venipuncture, into Vacuette-Greiner Bio-One® tubes spray-coated with lithium heparin (Kremsmünster, Austria), under a protocol approved by the Institutional Review Board of the University of Minho (SECVS002/2014 (ADENDA)). This procedure was performed in agreement with Helsinki declaration and Oviedo convention. A written consent was signed by all donors before blood collection.

## 2. Commercial bloods

Horse and sheep bloods were obtained commercially. Defibrinated horse blood was bought from Fisher (Thermo Fisher Scientific, MA, USA) and defibrinated sheep blood was bought from Liofilchem (Teramo, Italy). The same lot of sheep blood was used for all the experiments. In the case of horse blood, two different lots were used: one for the evaluation of bacterial survival and for proteases secretion and another lot for gene expression quantification.

## 3. Bacterial strains, growth conditions and inoculum preparation

*S. epidermidis* strain 1457, isolated from a patient with an infected central venous catheter (Mack *et al*., 1992), and isogenic mutant strains were used in this dissertation (Table 2.1). All strains, kept in Tryptic Soy Broth (TSB) (BD, Le Pont de Claix, France) supplemented with 30% glycerol (v/v) (Invitrogen, CA, USA), were streaked directly from -80 ºC to Tryptic Soy Agar (TSA) plates, which were prepared using TSB (VWR, Leuven, Belgium) plus 1.5% agar (w/v) (Liofilchem), and incubated overnight at 37 ºC. After that, a single colony of each strain was inoculated into 3 mL of TSB (BD), in a 10 mL Erlenmeyer flask, and incubated for 16 hours at 37 °C and 120 rpm, in an orbital shaker with 10 mm orbit (Biosan, Riga, Latvia).

**Table 2.1**. **List of strains used and the function of the genes deleted in the mutant strains.** NA-not applicable.

| Strains | Function of the genes deleted | Source of the mutants |
|---|---|---|
| **1457 WT Germany** | NA | Fernando Oliveira PhD thesis (unpublished work) |
| **1457 Germany *ΔSERP1775-77*** | Siderophore ABC transporter (Oliveira *et al.,* 2017) | |
| **1457 Germany *ΔSERP1778-81*** | Siderophore biosynthesis (Oliveira *et al.,* 2017) | |
| **1457 WT USA** | NA | Vânia Gaio PhD thesis (unpublished work) |
| **1457 USA *ΔmazEF*** | Toxin-antitoxin type II complex (Carvalhais *et al.*, 2014) | |

For all the experiments described below, bacterial inocula were prepared as follows. Overnight bacterial cultures were transferred into 1.5 mL tubes and harvested by centrifugation at 16,000 *g* for 7 minutes at 4 °C (ScanSpeed 1730R, Lynge, Denmark). Next, cell pellets were washed twice with 0.9% (w/v) sodium chloride (VWR) solution (NaCl), suspended in 600 µL of the same solution, and then submitted to 10 seconds pulse of sonication at 33% amplitude (Ultrasonic Processor, IL, USA). This sonication step was performed to break down cell clusters without interfering with cells viability (Freitas *et al.*, 2013). Bacterial suspensions with different concentrations were prepared by measuring the initial optical density at 640 nm ($OD_{640nm}$) in a spectrophotometer (UV/VIS 3100 PC, VWR) and by adjusting, in 0.9% NaCl, to the pretended OD.

## 4. Assessment of optical density correlation with bacteria concentration

In order to confirm the correlation between optical density and bacterial concentration (CFU/mL) among the strains used, after an overnight growth, the $OD_{640nm}$ of all strains was measured and adjusted, in NaCl, (VWR) to 0.150 (± 0.025). Thereafter, for CFU counting, a 20 µL aliquot was taken and serially diluted (10-fold) in 180 µL of 0.9% NaCl. Ten µL of different dilutions were plated, in duplicate, on TSA plates. The plates were then incubated for 18 hours at 37°C.

## 5. Assessment of bacteria concentration and incubation time for co-incubation assays

For the determination of the concentration of bacteria and time points for co-incubation assays, the $OD_{640nm}$ of 1457 WT (Germany) bacterial suspension was adjusted to 2.5, which corresponds to $2\text{-}3\times10^9$ CFU/mL, and then serially diluted (10-fold) until a concentration of $10^6$ CFU/mL was obtained. In 2 mL tubes, 900 µL of human blood and 100 µL of bacterial suspension, with different concentrations, were mixed together in order to obtain $10^8$, $10^7$, $10^6$ and $10^5$ CFU/mL. The tubes were then incubated at 37 ºC and 80 rpm, in a 10 mm orbit shaker (Biosan). At time points 0.5, 1, 2 and 4 hours, an aliquot of 20 µL was taken for CFU counting and processed as described in section 4. This experiment was repeated 5 times, using blood of 5 different female donors. The number of CFU/mL before interacting with blood was used as control to calculate the percentage of survival (2.1.1):

$$Percentage\ of\ survival = \left(\frac{number\ of\ CFU/mLTxh}{number\ of\ CFU/mLT0h}\right) \times 100 \qquad (2.1.1)$$

**Equation 2.1.1.** Percentage of survival after interaction with blood.

$Txh$ is the number of CFU/mL obtained in the specific time point being analyzed.

## 6. Skim milk medium preparation for protease secretion analysis

Skim milk medium was used to assess the secretion of proteases by *S. epidermidis* after interaction with the three different bloods. The preparation of this medium had to be optimized and was prepared as follows. First, 2% (w/v) of tryptone (Liofilchem), 1% (w/v) of yeast extract (Liofilchem), 1% (w/v) of NaCl (VWR) and 3% (w/v) of agar (Liofilchem), were mixed together and autoclaved for 15 minutes at 121 ºC. In a separate flask, a solution of 2% of skim milk (Oxoid, Hampshire, UK) was prepared, stirred for 1 hour and then autoclaved at 121 ºC for 5 minutes. Both media were cooled down to 55 ºC, in a water bath, and mixed together, aseptically, while stirring. Hence, at the end, the concentration of each component is 2-fold diluted. Finally, 13 mL of medium were transferred into 90 mm petri dishes and the plates kept at 4 ºC. It is important to emphasize that the skim milk medium needs to be fresh (not older than 10 days), otherwise it is difficult to detect the proteolysis around the colonies.

## 7. Assessment of *S. epidermidis* survival and protease secretion in fresh human blood and in commercial horse and sheep bloods

The number of surviving bacteria and its proteolytic activity after interaction with fresh human blood and commercial sheep and horse bloods was assessed after 0.5, 2 and 4 hours of incubation. In brief, the bacterial inoculum was prepared as described in section 3 and by adjusting the concentration of the suspension to $10^9$ CFU/mL, being then serially diluted (10-fold) until a concentration of $10^6$ CFU/mL was obtained. Thereafter, 50 µL of bacteria at $10^9$ and $10^6$ CFU/mL were mixed, in a 2 mL tube, with 450 µL of each blood. At each time point, 20 µL aliquots were removed and CFU counting performed as described in section 4 but using skim milk plates instead. In the case of sheep and horse bloods, the aliquot taken was sonicated for 5 seconds at 33% amplitude before diluting down. Clear patches on skim milk plates indicate regions where proteins have been broken down and, thus, these colonies were considered positive for proteolysis. The percentage of positive colonies was calculated in relation to the total number of colonies obtained in each time point. This experiment was repeated 4 times with human blood (1 male and 3 female donors) and 3 times with either sheep or horse bloods (using the same lot).

## 8. Gene expression quantification by quantitative PCR

After incubating bacteria for 4 hours with human, sheep or horse bloods, the transcription of the genes *sepA,* an extracellular serine protease that degrades antimicrobial peptides (Le *et al.,* 2018) and *hld*, a δ-hemolysin with cytolytic activity (Otto, 2009), was assessed by quantitative PCR (qPCR), as previously optimized (França *et al*., 2012; França *et al*., 2016).

### 8.1. RNA extraction

Total RNA was isolated using the E.Z.N.A. total RNA Kit (Omega Bio-tek, GA, USA) with minor modifications. Briefly, 4 hours after the co-incubation of 1457 WT USA and human, horse or sheep bloods, samples were sonicated for 5 seconds at 33% amplitude, in order to lyse eukaryotic cells and allow the collection of bacteria. Then, bacteria were harvested by centrifugation for 7 minutes at 16,000 *g* and 4 ºC. From then on, all the procedure was performed at room temperature unless otherwise stated. Bacterial pellets were suspended in 500

µL of TRK lysis buffer supplemented with β-mercaptoethanol (National Diagnostics, GA, USA) and then transferred into a 2 mL safe lock tube containing 0.4 g of acid washed 150–212 µm glass beads (Sigma, MO, USA). Cells were lysed in a cell disruptor (FastPrep®-24, MP Bio, CA, USA) using the following settings: 6.5 meters/second for 35 seconds. Samples were then cooled on ice for 5 minutes. The lysis process was repeated two times more, always including the 5 minutes incubation on ice. After that, the samples were centrifuged at 12,000 *g* for 1 minute, the supernatant transferred into a new tube and mixed with equal volume of 70% ethanol (Thermo Fisher Scientific), which was prepared in RNase free water (NZYTech, Lisbon, Portugal). Then, up to 700 µL of the lysate were transferred to the RNA isolation columns and centrifuged at 12,000 *g* for 1 minute. To wash the columns, 500 µL of RYBW1 were added to the columns and centrifuged under the same conditions described above. The flow-through was rejected and the column inserted into the same collection tube. Next, 500 µL of RYBW2 were added to the columns and the columns centrifuged. The flow-through was discarded and the columns reinsert into the same collection tube. A second wash with 500 µL of RYBW2 was performed. The flow-though was discarded, the column inserted into a new collection tube. To dry the membrane the columns were centrifuged for 2 more minutes. Finally, each column was inserted into a 1.5 mL tube and total RNA was eluted by adding 40 µL of RNase free water into the center of the membrane and centrifuging for 2 minutes. The columns were discarded and total RNA was immediately transferred to ice or stored at -80 ºC until further use.

### 8.2. DNase treatment

Genomic DNA co-purified with total RNA was digested using DNase I RNase free kit (Thermo Fisher Scientific). In brief, 2 µL of DNase I and 4 µL of 10 × reaction buffer were added to the RNA sample and incubated at 37 °C for 30 minutes. Next, to inactivate the enzyme, 4 µL of 25 mM EDTA were added to the mixture and incubated at 65ºC for 10 minutes.

### 8.3. RNA quality determination

The concentration and purity of total RNA was determined using a NanoDrop™ One (Thermo Fisher Scientific). Before measuring the concentration of total RNA, the NanoDrop was activated and the light source was allowed to warm up and stabilize. Two measurements of the same sample were performed. The absorbance ratio $A_{260}/A_{280}$ was used as an indicator of

protein contamination and $A_{260}/A_{230}$ as an indicator of polysaccharide and/or phenol contamination (Yamaguchi *et al.*, 1992).

### 8.4.Complementary DNA synthesis

Complementary DNA (cDNA) was synthesized, in a 10 µL reaction volume, using 0.4 µg of total RNA in the presence of the enzyme RevertAid H minus reverse transcriptase (Thermo Fisher Scientific) and using random primers as priming strategy (NZYTech). The following cycle parameter was used: 25ºC for 10 minutes, 42 ºC for 1 hour and 70 ºC for 10 minutes. To determine genomic DNA carry-over a control reaction lacking the reverse transcriptase enzyme (NRT) was prepared.

### 8.5.Quantitative PCR

Quantitative PCR (qPCR) run was performed by mixing 2 µL of 1:200 diluted cDNA with 5 µL of Xpert Fast SYBR Green (GRISP, Porto, Portugal), 0.5 µL of each forward and reverse primers at 10 µM and 2 µL of nuclease-free water. The run was completed in a CFX96$^{TM}$ thermal cycler (Bio-Rad) with the following parameters: 95°C for 2 minutes and 39 cycles of 95°C for 5 seconds and at 60°C for 30 seconds. The primers utilized were previously designed using Primer3 software (Koressaar & Remm, 2007; Untergasse *et al.,* 2012) and synthesized by Metabion (Steinkirchen, Germany) (Table 2.2). Primer efficiencies were determined by the dilution method (Pfaffl, 2004b, 2004a) at 60 °C (Table 2.2). RNA samples were considered free from significant genomic DNA contamination if the quantification cycle (Cq) difference between the specific signal and the respective NRT control was greater than 10. A control reaction lacking cDNA template (normally referred as NTC) was prepared to evaluate reagents contamination. Neither unanticipated products nor primer dimers were detected by melting curve analysis. The abundance of transcripts was determined by applying a variation of the Livak equation (Livak and Schmittgen, 2001), $E^{\Delta Cq}$, where $\Delta Cq$ = Cq reference gene - Cq target gene and E is the experimentally determined reaction efficiency. 16S ribosomal RNA was used as reference gene. Data analysis was based on 4 independent experiments using 4 different donors (1 male and 3 female donors).

**Table 2.2. List of primers used for qPCR analysis.** F-forward, R-reverse.

| Genes | Function | Primer sequences 5' to 3' | Amplicon (bp) | Efficiency (%) |
|---|---|---|---|---|
| ***16S rRNA*** | Reference gene | F- GGGCTACACACGTGCTACAA<br>R- GTACAAGACCCGGGAACGTA | 176 | 93 |
| ***sepA*** | Extracellular serine protease | F-TCTTAAGGCATCTCCGCCTA<br>R- GTCTGGTGCGAATGATGTTG | 196 | 90 |
| ***hld*** | δ-hemolysin | F- ATGGCAGCAGATATCATTTCTAC<br>R-AACAAAGTTACAAAAGTTACAATAGACTC | 116 | 105 |

## 9. Statistical analysis

The statistical significance of the results obtained was determined by the application of the unpaired two tailed t-test with equal variances, using the Microsoft Excel 2016 software, or by one-way ANOVA with Holm-Sidak's multiple comparisons test or by two-way ANOVA with Dunnett's multiple comparisons test, using GraphPad Prism 6 software (CA, USA). More information regarding the tests used are detailed in each figure caption. A confidence level of 95% was considered.

## Results and Discussion

The *ex vivo* human blood model is an important tool to help us identify the mechanisms used by *S. epidermidis* to survive in the host and establish infection. However, the ethical considerations raised by the use of human subjects and the difficulty to find donors in the academia context, complicates the implementation of this model. Therefore, the primary goal of this dissertation was to investigate the possibility to replace fresh human blood by commercial horse or sheep bloods for the study of *S. epidermidi*s virulence.

Since this dissertation was integrated in a project that aims to evaluate the role of iron uptake and dormancy emergence in *S. epidermidis* virulence, by using an *ex vivo* human blood model, three strains lacking genes putatively involved in these mechanisms, which were constructed in the ambit of that project, were used. A mutant for the set of genes *SERP1775 to SERP1777* (*ΔSERP1775-77*), which constitute the operon responsible for the codification of a siderophore ABC transporter, a mutant for the genes *SERP1778 to 1781* (*ΔSERP1778-81*), which are responsible for the codification of a siderophore biosynthesis (Oliveira *et al.*, 2017), and a mutant that lacks the genes *mazE* and *mazF* (*ΔmazEF*), which are responsible for the codification of a toxin-antitoxin type II system that has been associated with the emergence of dormant cells (Lewis, 2007; Chandra *et al.*, 2013; Carvalhais *et al.*, 2014). In addition, the wild type (WT) strain used to construct these mutants was also included. However, considering that the three mutants were constructed using WT strains from two different laboratories (one in Germany and another in the USA), and that the different techniques carried out to handle bacteria differ from one laboratory to another and that can alter the genetic makeup of the same strain, the two WT strains were included in this dissertation. The WT from Germany (1457 WT Germany) was used to construct the mutants deficient in genes involved in iron uptake, and the WT from the USA (1457 WT USA) was used to construct the mutant deficient in the *mazEF* operon.

Taking into consideration that the deletion of particular genes may have important consequences on strains phenotype, a preliminary experiment was performed to assess if the mutations constructed have impaired the relationship between cells optical density (OD) and CFU counting. This is important as this relationship is frequently used to adjust cells concentration and prepare inocula. Hence, the OD at 640 mn ($OD_{640\ nm}$) of all strains was adjusted to 0.150 and the number of CFU/mL in the suspensions was determined (Figure 3.1).

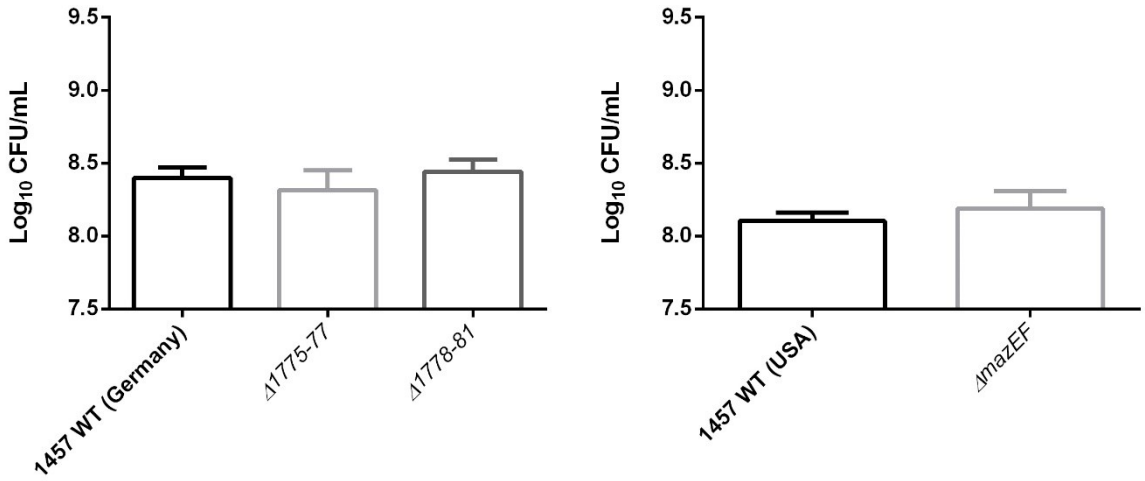


**Figure 3.1. $Log_{10}$ CFU/mL of *S. epidermidis* strains after adjusting the optical density ($OD_{640nm}$) of all strains to 0.150.** Bars correspond to average plus standard deviation of 3 independent assays. Statistical analysis was performed using unpaired two tailed t-test with equal variances.

As can be seen in Figure 3.1, the mutations constructed did not impact the relationship between $OD_{640nm}$ and CFU/mL, as no significant differences were found when comparing with the respective WT. This way, the same $OD_{640nm}$ was used in all strains to obtain the same initial concentration of bacteria for all the experiments presented.

### 1. Survival of *S. epidermidis* cells in fresh human blood and in commercial horse and sheep bloods

In order to select the time points and the concentration of bacteria to be used in this dissertation, before moving forward to the characterization of the behavior of *S. epidermidis* strains in different bloods, different time-points (up to 4 hours) and concentrations of bacteria ($10^4$ to $10^8$ CFU/mL) were tested using the 1457 WT (Germany) strain incubated in human blood (Figure 3.2).

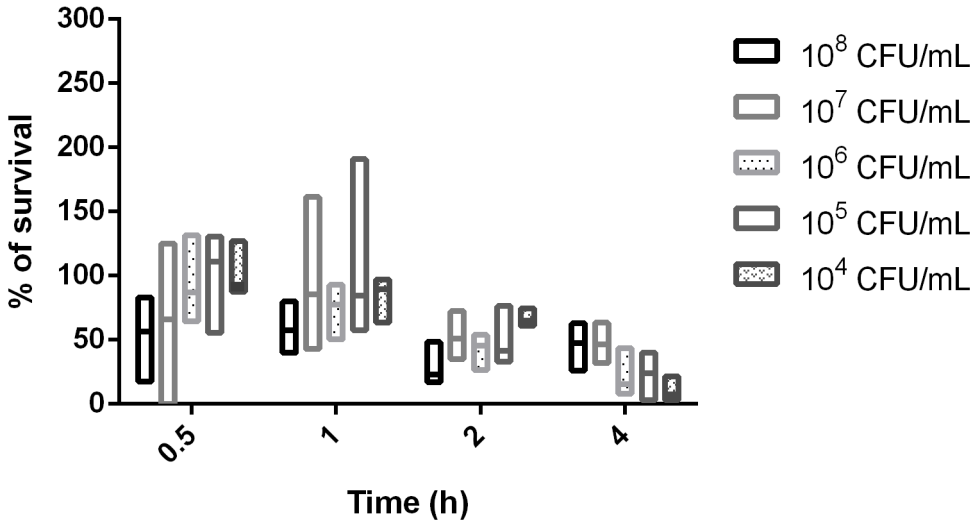


**Figure 3.2. Evaluation of different concentrations and time points using *S. epidermidis* 1457 WT (Germany) incubated in human blood.** Boxes correspond to interquartile range values of 5 independent assays and the horizontal lines represent the median. Human blood was collected from 5 different female donors.

For the concentrations $10^8$ and $10^7$ CFU/mL, a higher percentage of survival was found in the 4-hour time point, when comparing with the other concentrations tested. This may have to do with neutrophils incapacity to kill bacteria due to the high bacterial load present. Considering that normally human blood has $10^6$ neutrophils/mL (Li *et al.*, 2002), the ratio between phagocytes and bacteria was, approximately, 1:100 (in the $10^8$ CFU/mL inoculum) and 1:10 (in the $10^7$ CFU/mL inoculum). Also, this observation may be related to the inability of neutrophils to kill bacteria as fast as bacteria are replicating. In addition, since this model is a closed system, where blood circulation is not possible, there is no cells renewal overtime and, thus, neutrophils may start dying and bacterial cells can start proliferating freely (Alberts *et al*., 2002; Venezia *et al.*, 2004). Finally, the toxic factors that may be released by the bacterium may also impact neutrophils viability, allowing bacteria to grow in the host (Li *et al.*, 2002). Interestingly, it was observed that in the first 2 hours of incubation, there was a higher decrease in the number of CFU/mL in the concentration $10^8$ CFU/mL than in the other concentrations tested. A possible explanation for this observation is that the high initial concentration of bacteria promptly activated neutrophils (Li *et al.,* 2004) and so, bacteria were more readily phagocytosed. Hence, due to this interesting difference between concentrations, a high ($10^8$ CFU/mL) and low ($10^5$ CFU/mL) concentration of bacteria were selected for further studies. In addition, these concentrations were chosen as they have been previously used in other studies

of the group (França *et al.,* 2016) enabling the comparison of the results obtained. Moreover, the selection of the concentration of $10^8$ CFU/mL was also made since for gene transcription studies a higher concentration of bacteria is often necessary.

Regarding time points, an early (30 minutes), middle (2 hours) and late (4 hours) time points of incubation were selected. Incubations superior to 4 hours were not considered, as it was shown by members of the group that blood cells start losing their viability after 4 hours of incubation, having an important impact on the results obtained (unpublished data). Additionally, the use of biological tissues such as blood has to be kept to a minimum. Bearing that in mind, it was previously tested by members of the group if the use of small volumes of blood may impair bacterium survival and gene expression levels (Bras *et al*., 2017). Three different volumes (0.2, 0.5, 0.6 mL) were tested and compared with the results obtained when using 1 mL of blood (França & Cerca, 2016; França *et al.,* 2016). The results obtained showed that the use of smaller volumes did not affect neither the transcription levels of the genes tested nor the culturability of bacterial cells. Hence, 0.5 mL of blood were used for all the experiments performed afterwards.

Having all the conditions defined, the survival of bacteria in the three different bloods was then assessed. As could be expected, with the highest concentration tested ($10^8$ CFU/mL) (Figure 3.3), when incubated with horse and sheep bloods, the number of bacteria tend to increase overtime. Interestingly, in the case of the WT from Germany, in sheep blood (Figure 3.3), there was also an increase in the number of CFU/mL until 2 hours of interaction but, after that, the number of CFU/mL decreased.

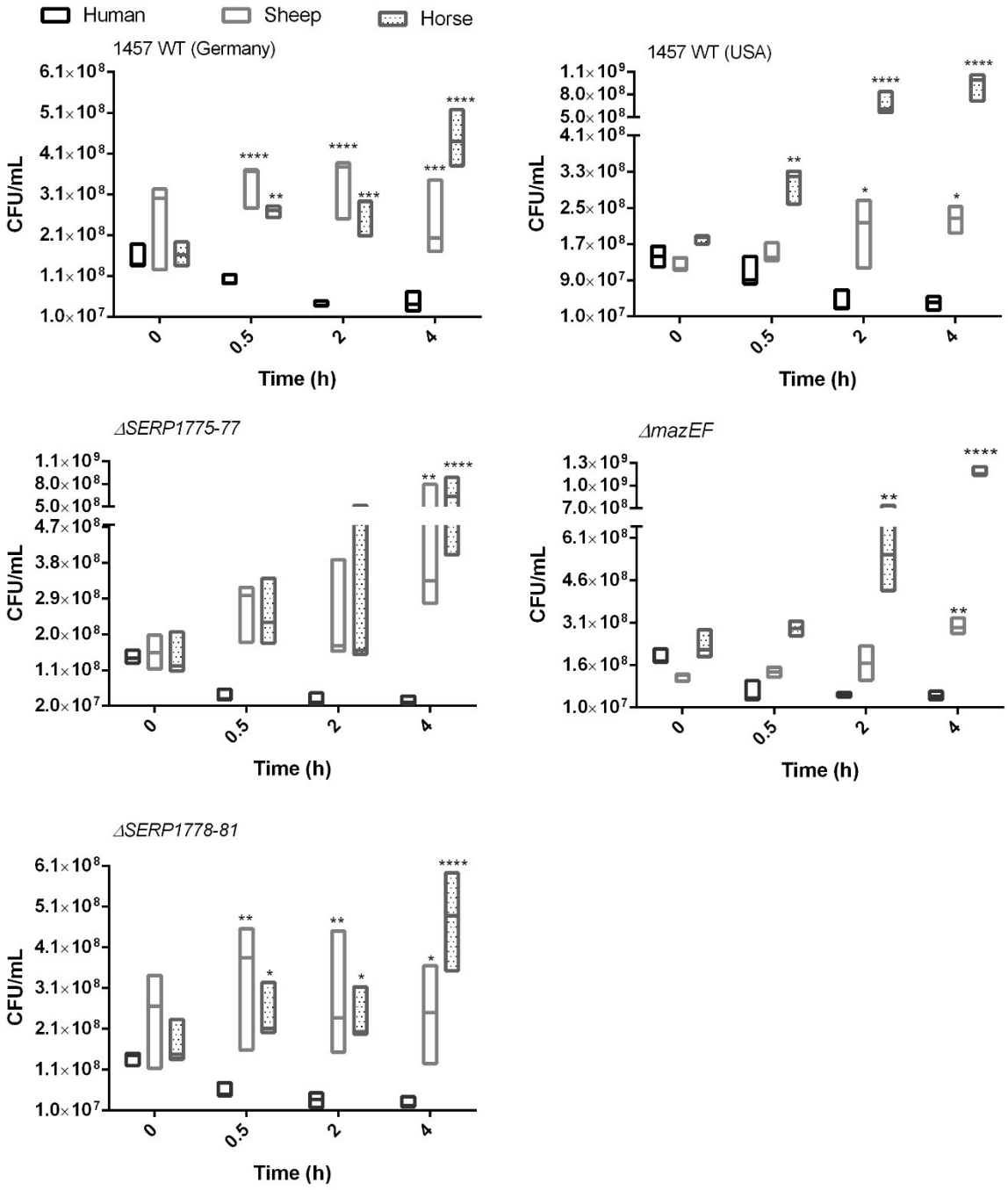


**Figure 3.3. *S. epidermidis* strains survival in the three different bloods using $10^8$ CFU/mL of initial inoculum.** Boxes correspond to interquartile range values of 3 (horse and sheep bloods) to 4 (human blood) independent assays and the horizontal lines represent the median of the values. Human blood was collected from 4 different donors (1 male and 3 female donors). Statistical differences among groups were evaluated with two-way ANOVA and posthoc Dunnett's multiple comparisons test using the values obtained with human blood as control. * $P< 0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

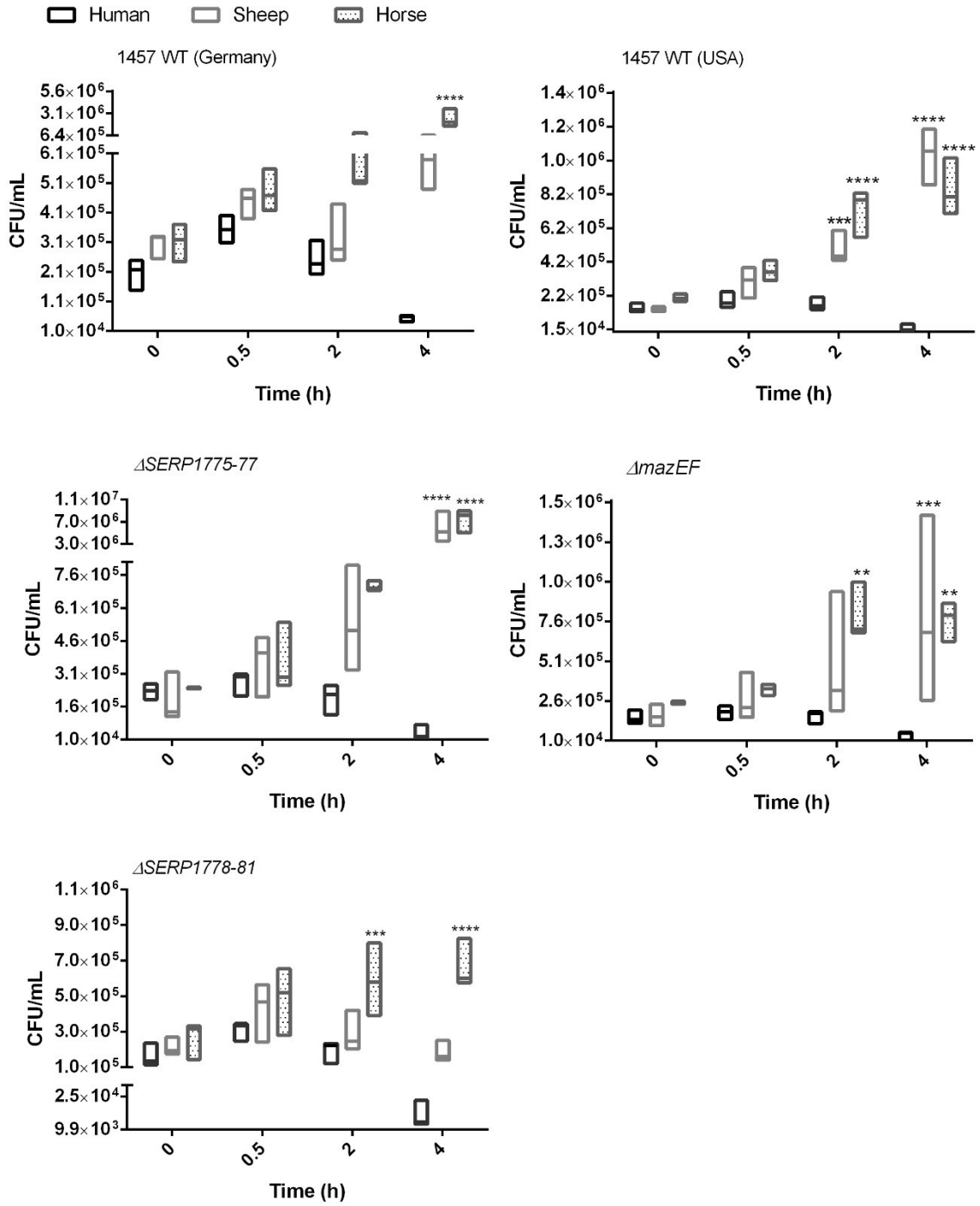


**Figure 3.4. *S. epidermidis* strains survival in the three different bloods using $10^5$ CFU/mL of initial inoculum.** Boxes correspond to interquartile range values of 3 (horse and sheep bloods) to 4 (human blood) independent assays and the horizontal lines represent the median. Human blood was collected from 4 different donors (1 male and 3 female donors). Statistical differences among groups were evaluated with two-way ANOVA and posthoc Dunnett's multiple comparisons test using the values obtained with human blood as control. * $P< 0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

Regarding the $10^5$ CFU/mL inoculum (Figure 3.4), in both sheep and horse bloods, bacteria concentration tends to increase during the 4 hours of incubation, with the exception of the strain *ΔSERP1778-81,* in sheep blood, that after 2 hours of interaction the number of CFU/mL decreased (Figure 3.4). This may have to do with the lack of genes responsible for the siderophore biosynthesis, which affects the ability of the bacterium to capture the amount of iron necessary for its growth. The same was not observed in horse blood, which may be due to the fact that horse blood has more erythrocytes (Adili *et al.*, 2016) and, thus, is likely to have a higher amount of iron available to keep the bacteria growing.

In human blood, however, a marked decrease in the number of bacteria was observed in all strains, over the 4-hour period, when using an initial bacterial load of $10^8$ CFU/mL. When $10^5$ CFU/mL was used, during the first 30 minutes of interaction with human blood the number of bacteria either maintained (*ΔSERP1775-77,* 1457 WT USA and *ΔmazEF*) or increased (1457 WT Germany and *ΔSERP1778-81*), starting to decrease, after that, to values below the initial load. This probably had to do with the fact that the low initial inoculum did not trigger phagocytic cells as quickly as the high initial inoculum, giving the opportunity to bacteria to replicate in the first hours.

It is important to stress that since *Staphylococcu*s species have a high propensity to agglomerate (Dastgheyb *et al.*, 2015; Ortiz-Gila *et al.*, 2015), during the incubation with human blood some aggregation may have occurred. To break down the cluster of cells possibly formed, a sonication step had to be done. However, contrarily to what was performed in the case of horse and sheep bloods, a sonication step was not executed in human blood samples before CFU plating. If performed, the sonication step would lyse phagocytic cells and release its contents, probably including alive bacteria (it is not known yet if *S. epidermidis* cells are capable of surviving inside phagocytic cells as *S. aureus* does). Therefore, since our goal was to count the number of non-phagocytized bacteria, the sonication step was not completed. Therefore, it is important to consider that the number of CFU/mL obtained after incubation with human blood may be higher than the ones presented. Nevertheless, independently of this limitation, the trend of CFU/mL in human blood is the opposite of what was observed in sheep or horse bloods.

Overall these results indicate that contrarily to what was observed in human blood, both sheep and horse bloods do not seem to present antimicrobial activity. Indeed, it seems to enhance bacterial growth, what could be expected as these are often used as supplement for

bacterial growth. Hence, for the study of *S. epidermidis* survival, fresh human blood cannot be replaced by neither sheep nor horse commercial bloods.

## 2. Protease secretion by *S. epidermidis* cells after interaction with fresh human blood and with commercial horse and sheep bloods

Bacterial proteases are enzymes that have an important role in bacterial survival. In one hand, by cleaving proteins, proteases provide peptides to the bacterium that function as nutrients. On the other hand, by targeting antimicrobial peptides, proteases contribute to the virulence of the bacterium (Martínez-García *et al.,* 2018). *S. epidermidis* secretes proteases that can target both immune cells and antimicrobial peptides, contributing to the evasion from the host immune system response (Rohde *et al.,* 2005). It was therefore interesting to evaluate if the interaction of the bacterium with different bloods altered the secretion of proteases. Interestingly, the strains 1457 WT Germany and *ΔSERP1775-77* did not originally show proteolytic activity and this phenotype did not change during the incubation with the three different bloods. Thus, the results obtained with the set of strains constructed to evaluate the importance of iron uptake are not presented. The absence of proteolytic activity in milk medium suggest a mutation in the *agr* system, the regulator of the majority of the staphylococcal proteases (Vuong *et al.,* 2000), that frequently and spontaneously occurs in *Staphylococcus* species, in particular in *S. epidermidis*, having an important impact on bacterium virulence profile (Villaruz *et al.*, 2009).

Despite the high variability and fluctuations detected, in both inocula, in the percentage of proteolytic activity of the WT (USA) strain, in the case of the mutant for the operon *mazEF*, independently of the origin of the blood, time point or the initial concentration of bacteria, the proteolytic activity was close to 100% (Figure 3.5 and 3.6). The operon *mazEF* codifies a toxin (MazF) and an antitoxin (MazE) (Chandra *et al.*, 2013; Schuster and Bertram, 2016). Whereas MazE is very labile protein, MazF is a long-lived protein. Hence, when MazE is degraded due to the presence of stress or due to the trigger of some particular environmental condition, the levels of MazF rise and exert its toxic activity by, for instance, inhibiting the synthesis of several proteins (Engelberg-kulka *et al.*, 2005; Schuster and Bertram, 2016). This may be one possible explanation for the high variability found in the percentage of proteolytic activity in the WT but not in the *ΔmazEF* strain, as the last does not has the toxin-antitoxin module and, thus, it is not impaired by the effect of the toxin MazF.

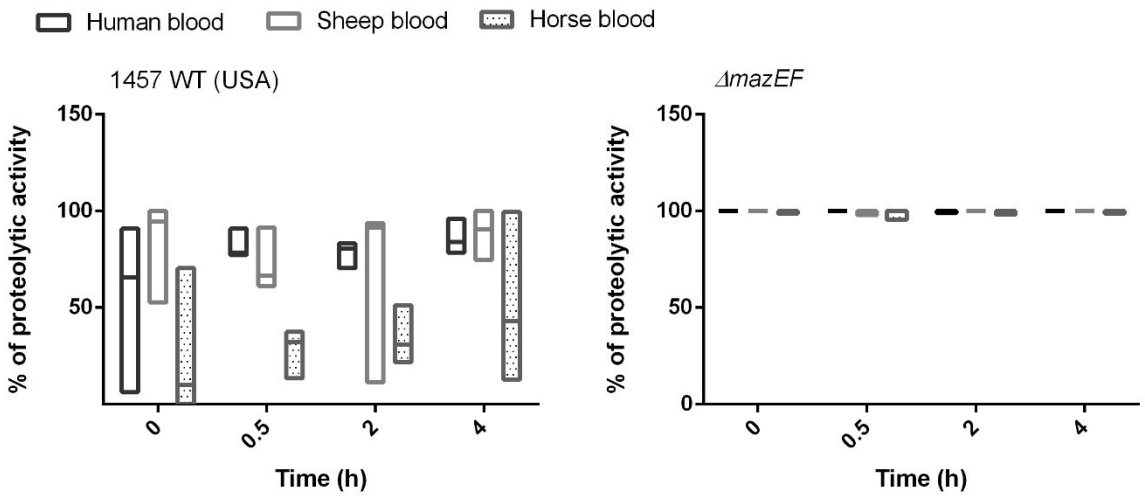


**Figure 3.5. Proteolytic activity of *S. epidermidis* strains after interaction with the three different bloods using $10^8$ CFU/mL of initial inoculum.** Boxes correspond to interquartile range of the values of 3 (horse and sheep bloods) to 4 (human blood) independent assays and horizontal lines represent the median. Human blood was collected from 4 different donors (1 male and 3 female donors). Statistical differences among groups were evaluated with two-way ANOVA and posthoc Dunnett's multiple comparisons test using the values obtained with the T0h of each blood as control. * $P< 0.05$.

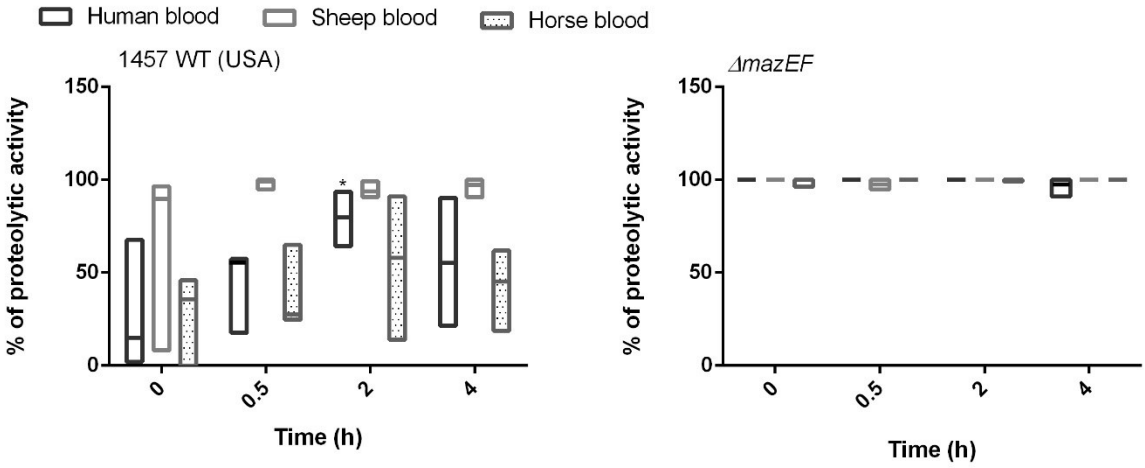


**Figure 3.6. Proteolytic activity of *S. epidermidis* strains after interaction with the three different bloods using $10^5$ CFU/mL of initial inoculum.** Boxes correspond to interquartile range of the values of 3 (horse and sheep bloods) to 4 (human blood) independent assays and horizontal lines represent the median. Human blood was collected from 4 different donors (1 male and 3 female donors). Statistical differences among groups were evaluated with two-way ANOVA and posthoc Dunnett's multiple comparisons test using the values obtained with the T0h of each blood as control. * $P< 0.05$.

So, in general, these results suggest that although the influence of changing bloods on the secretion of proteases may be strain-dependent, either sheep or horse bloods can be used to assess this bacterial phenotype. However, due to the high variability observed, another methodology such as high performance liquid chromatography or quantitative PCR, should be used to confirm these results. However, in that case, specific proteases need to be targeted as these methods are not as broad as the skim milk medium.

### 3. Transcription level of *sepA* and *hld* genes after *S. epidermidis* interaction with fresh human blood and with commercial horse and sheep bloods

The study of the bacterial gene expression profile in the presence of peripheral human blood cells is very important to help characterize the specific bacterial response elicited when inside the host (Whitney *et al*., 2003; Xu *et al*., 2011; Joehanes *et al*., 2012; França *et al*., 2014; França & Cerca, 2016; França *et al*., 2016). Therefore, it was also important to evaluate if using horse or sheep bloods bacteria gene expression would be similar to the one obtained in the presence of fresh human blood. To do so, the transcription level of two genes was analyzed: *sepA,* a gene that codifies the extracellular serine protease SepA that breaks down antimicrobial peptides (Lai *et al.*, 2007) and *hld,* a gene that codifies the protein δ-hemolysin that has cytolytic activity (Pinheiro *et al.*, 2015). Since time was limiting, it was not possible to perform gene expression assays in all of the five strains used so far. Thus, for the purpose of studying the influence of replacing human blood on gene expression profile, the 1457 WT USA strain was selected since one of the genes under study is a protease and this strain was one of the five used that have shown proteolytic activity. Although the mutant *ΔmazEF* have also shown proteolytic activity, it was not chosen as the absence of the operon could indirectly influence the outcome and lead to misleading conclusions. In addition, gene expression was only assessed 4 hours after the interaction with the three different bloods. A late time point was preferred as it is observed, in previous studies done by the group, that in the first 2 hours upon contact with human blood the bacterium's transcriptome changes in a non-specific manner, i.e., the transcriptomic changes detected in the bacterium after interaction with human blood were also detected after incubation with rich medium routinely used in the laboratory (França *et al.*, 2014, 2016). Also, for these assays only the $10^8$ CFU/mL inoculum was used. As explained before, for gene expression studies high concentrations of bacteria are normally necessary as during the procedure several losses may occur.

As can be seen in Figure 3.7 the transcription of the gene *hld* was markedly different among the three bloods, where a higher level of expression was found when in contact with human blood being, respectively, 5× and 50× higher than in horse and sheep bloods.

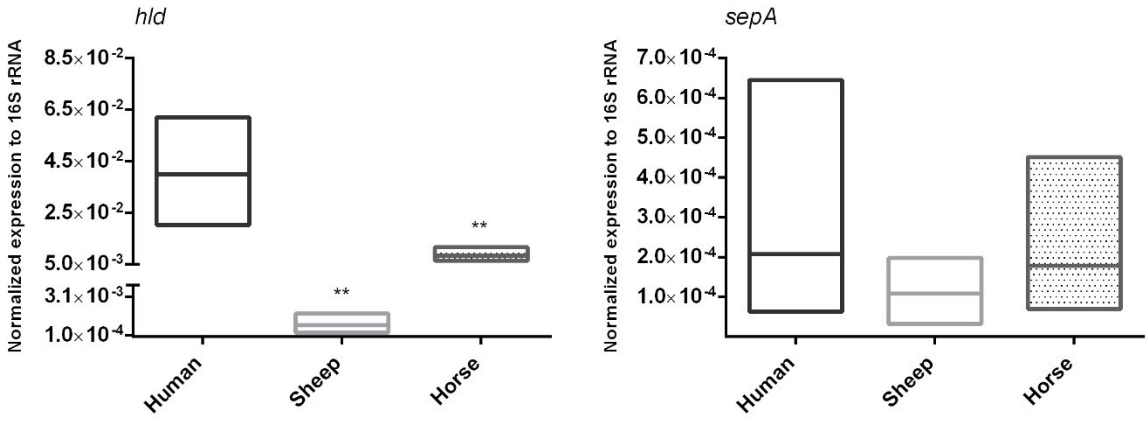


**Figure 3.7. Normalized expression of *hld* and *sepA* genes after *S. epidermidis* 1457 WT (USA) interaction with the three different bloods.** Boxes represent interquartile range of 4 independent assays and the horizontal lines the median. Human blood was collected from 4 different donors (1 male and 3 female donors). Statistical differences among groups were evaluated with one-way ANOVA and posthoc Holm-Sidak's multiple comparisons test using the values obtained with human blood as control. ** $P < 0.01$.

This probably has to do with the fact that δ-hemolysin is more necessary in the presence of human blood as more pressure is exerted by phagocytic cells, which seem not to be present in commercial bloods. However, in the case of the transcription levels of the gene *sepA* (Figure 3.7), although some variability was observed in human blood, as could be expected due to donor singularity, no significant differences were found among bloods. Since SepA targets soluble compounds, which are possibly still present and viable in the commercial bloods, the bacterium had the same pressure and, thus, the response was similar in the three cases. Furthermore, it was previously shown that the alterations occurring in the transcription of some particular genes, which included *sep*A, are mainly mediated by plasma components and not by cells (França and Cerca, 2016). Hence, still considering that the soluble factors are viable in the commercial bloods, it could be expected to detect similar levels of transcription of the gene *sepA* in the three bloods.

Overall, these results show that depending on the gene under study, human blood may be replaced by either sheep or horse bloods for gene transcription studies. However, it is

important to consider that only two genes, one strain and one time point were evaluated, being necessary to deepen this analysis to better understand the consequences of replacing human blood in gene expression profile.

## Conclusions

In conclusion, our results showed that:

1) Human blood cannot be replaced by commercial horse or sheep bloods for bacterial survival studies;
2) Human blood can be replaced by either horse or sheep bloods for the analysis of protease secretion, however, this seems to be strain-dependent. Hence, this shall be validated for the strain under study before replacing fresh human blood by commercial horse or sheep bloods;
3) Human blood can be replaced by either horse or sheep bloods for gene expression studies, however, this seems to be gene-dependent. Thus, it must be validated for the gene under study before replacing fresh human blood by commercial horse or sheep bloods.

## Further Work

Although this dissertation have answered some of the questions initially asked, several other important questions were raised. In addition, this work presents some limitations that should be addressed in the future. Thus, for future work, it would be important to consider:

1) Test more strains, as herein only *S. epidermidis* 1457 background was evaluated;
2) Test more mutants, as the absence of genes with particular function may have an important impact on how bacteria respond to the replacement of human blood;
3) Test other methods for the analysis of the secretion of proteases in order to confirm the results obtained;
4) Test more genes, including genes involved in metabolism and biofilm formation, in addition to more genes involved in evasion from the host immune system attack;
5) Test other lots of commercial blood to ensure that the results are not lot-dependent;
6) Test fresh human blood prepared in the same way (desfibrinated) as the commercial horse and sheep bloods used. Use sheep or horse bloods prepared in the same way as human blood was processed (with lithium heparin as anticoagulant). This would help understanding if the differences found have to do with the way bloods coagulation was prevented. In addition, it would be interesting to test outdated human blood to better understand the consequences of not having active phagocytic cells and the role of plasma components;
7) Analyze the immune components present and their viability in fresh human and commercial horse and sheep bloods.

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