Universidade de Aveiro Departamento de Química

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo de Biotecnologia Alimentar, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, investigador auxiliar do Departamento de Química da Universidade de Aveiro

Dedico este trabalho aos meus pais, irmã e namorado pelo apoio incondicional ao longo da minha vida.

o júri

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palavras-chave Armazenamento Hiperbárico, Sangue, Hemólise, Preservação

resumo

Com este trabalho, pretendeu-se avaliar o efeito de uma nova metodologia de conservação, conhecida como armazenamento hiperbárico (AH) em combinação com diferentes temperaturas como possível alternativa ao método convencional de conservação (refrigeração) de sangue.

Para isso, duas soluções foram adicionadas ao sangue de suíno: um anticoagulante (ácido etilenodiamino tetra-acético, EDTA); ou uma solução aditiva (citrato fosfato dextrose adenina, CPDA-1). As amostras foram armazenadas à pressão atmosférica e à temperatura ambiente (0,1 MPa/RT), a temperaturas de refrigeração (0,1 MPa/RF), a 10 °C (0,1 MPa/10 °C) e sob pressão a diferentes temperaturas (50 MPa/RT, 50 MPa/10 °C, 25 MPa/RT, 25 MPa/10 °C) durante 35 dias. Além disso, as amostras com CPDA-1 foram também armazenadas a 25 MPa/-5 °C por um período de 7 dias, sendo este um ensaio preliminar. Todas as amostras foram analisadas relativamente ao crescimento microbiano, hemólise e variação de pH durante o período de armazenamento.

A adição de qualquer uma das soluções ao sangue mostrou ter impacto na hemólise e no crescimento microbiano, independentemente da combinação pressão-temperatura durante o armazenamento. Verificou-se que a hemólise foi superior para as amostras com solução anticoagulante apenas na sua composição, indicando a necessidade de uso de uma solução aditiva. O sangue armazenado sob pressão apresentou um aumento da hemólise para as pressões mais elevadas, tendo-se, contudo, verificado inativação microbiana. As temperaturas mais baixas tiveram um efeito positivo no sangue comparativamente com a mesma pressão de armazenamento, mas com temperatura ambiente. A 25 MPa/-5 °C, verificou-se que o pH não variou ao longo do armazenamento, abrindo a possibilidade de armazenamento de sangue sem a produção de ácido láctico resultante da glicólise. Todas as condições de armazenamento foram comparadas com a metodologia convencional de refrigeração e em nenhum dos ensaios se conseguiu aumentar o tempo de conservação mantendo uma baixa hemólise.

No entanto, este é o primeiro trabalho relativo ao uso do AH na preservação de sangue e mais estudos com outras combinações de pressão-temperatura devem ser testados.

keywords Hyperbaric Storage, Blood, Haemolysis, Preservation

abstract This work aimed to evaluate the effect of a new preservation methodology, known as hyperbaric storage (HS) in combination with different temperatures as a possible alternative to the conventional method of storage (refrigeration) of whole blood.

Two different solutions were added to the blood, an anticoagulant (Ethylenediaminetetraacetic Acid - EDTA) or an additive solution (Citrate Phosphate Dextrose Adenine - CPDA-1) and the samples were stored at atmospheric pressure at RT (0.1 MPa/RT), refrigerated temperatures (0.1 MPa/RF), 10 °C (0.1 MPa/10 °C) and under pressure (50 MPa/RT, 50 MPa/10 °C, 25 MPa/RT, 25 MPa/10 °C) up to 35 days. Samples with additive solution were also stored at 25 MPa/-5 °C for a period of 7 days. All the samples were analysed for microbial growth, haemolysis and pH variation during the storage period.

The solution added to the blood showed to have impact in the haemolysis and microbial growth regardless the pressure-temperature combination during storage. The haemolysis was higher for the samples with only anticoagulant in the composition indicating the necessity of an additive solution in the blood.

The samples stored under pressure revealed an increased haemolysis in the higher pressures (50 MPa/RT, 50 MPa/10 °C), but an inactivation effect regarding the microbial growth was verified. Lower temperatures had a positive impact in the blood when compared with the blood under the same pressure but at RT. At 25 MPa/-5 °C the lack of pH variation might open the possibility to storage blood without the production of lactic acid resulting from glycolysis.

All the conditions were compared with the standard method of storage (refrigeration) and in none of the assays it was possible to increase the preservation time and keep a low haemolysis. Nevertheless, this thesis is the first work regarding the use of HS to preserve blood and more studies with other combinations of pressure-temperature must be tested.

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List of abbreviations

Abbreviation	Designation
AS	Additive Solution
ATP	Adenine triphosphate
CFU	Colony Forming Unit
CP	Cryoprecipitate Plasma
CPD	Citrate Phosphate Dextrose
CPDA-1	Citrate Phosphate Dextrose Adenine
EDTA	Ethylenediaminetetraacetic Acid
FDA	Food and Drugs Administration
FFP	Fresh Frozen Plasma
fV	Factor V
fVIII	Factor VIII
HIV	Human Immunodeficiency Virus
HPP	High Pressure Processing
HS	Hyperbaric Storage
HS/RF	Hyperbaric Storage at Refrigeration Temperature
HS/RT	Hyperbaric Storage at Room Temperature
HS/SZ	Hyperbaric Storage at Sub-Zero Temperature
MPa	Mega Pascal
RBCs	Red Blood Cells
PATS	Pressure-Assisted Thermal Sterilization
PF24	Plasma Frozen within 24h
PF24RT24	Plasma Frozen within 24h held at Room Temperature
PME	Pectin-methylesterase
PRP	Platelet Rich Plasma
RF	Refrigeration Temperature
RT	Room Temperature
SZ	Sub-Zero
TP	Thawed Plasma
USA	United States of America
UV	Ultraviolet

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CHAPTER I – STATE OF ART

THIS SECTION COMPRISES AN EXTENSIVE, BRIEFLY COMPILED LITERATURE REVIEW REGARDING BLOOD PRESERVATION AND ITS CHARACTERISTICS

1. Blood

1.1. Introduction

The preservation and improvement of human life is of major importance, being large efforts made in order to improve technology that is necessary to fulfil this demand. Between the different branches of health, the preservation of biological materials has been increasingly important.

Stem cells, organs and blood are some examples of biological materials that has been used in a variety of life-threatening situations, being this possible due to the scientific and technological developments that allowed the arose of new techniques and methodologies for the preservation and storage of biological materials.

Regarding stem cells, application are already made in situations when exists a chronic disorder, a hereditary damage or a major disease (Hanna and Hubel, 2009), however, blood is needed in large quantities. For instance, every two seconds, someone in the United States of America (USA) needs a blood transfusion for situations such as surgeries, cancer treatment, chronic illnesses, and traumatic injuries making blood a high value product whose storage and preservation is of major importance (American Red Cross, 2018).

1.2. Blood collection

Blood is the body fluid responsible for transportation of oxygen and nutrients to the cells and transportation of carbon dioxide and waste products to the excretory organs. It is constituted by plasma, red blood cells or erythrocytes, white blood cells or lymphocytes, and platelets or thrombocytes (European Committee on Blood Transfusion, 2015; Castillo et al., 2018). All these components can be collected and used for transfusions, however, the time and conditions for the storage of each component is widely variable (European Committee on Blood Transfusion, 2015; Wong and Luban, 2016).

The collection of blood is made after the selection of the donor. All the donations are made by volunteers that are previously checked for infectious diseases that can be transferred to the blood receptor. Each volunteer can donate up to three times per year, donating 450 mL each time (Booth and Allard, 2017).

In **Figure A1 – Appendix A** is presented an example of a blood collection bag.

The tests made to evaluate the admissibility of a donor varies according with the country were the collection is being made. for example, in the United Kingdom, all donations are tested for hepatitis B, hepatitis C, syphilis, human T-lymphotropic virus type 1 and 2 and human immunodeficiency virus (HIV). In countries where other infection diseases are a threat, like malaria or West Nile virus, the tests for these diseases are also made, being this the main reason why the travel history for all donors must be known (European Committee on Blood Transfusion, 2015; Booth and Allard, 2017).

The collection of blood is made by venepuncture and all the procedure is made in aseptic conditions, using sterilized materials, in order to avoid contamination of the collected blood. The blood is collected for a bag with an anticoagulant solution, which is mixed with the blood to avoid coagulation. The anticoagulant solution is normally also a preservative solution (constitution and types of preservative solutions are mentioned further below) when the blood is collected for transfusion. When blood is collected for analysis, the anticoagulants used are heparin, sodium citrate or ethylenediaminetetraacetic acid (EDTA). The mechanism of each anticoagulant is different and act in different factors inhibiting the progression of the coagulation cascade (DeWald et al., 2018). The anticoagulant agent used varies accordingly with the final purpose of use (as for example for transfusion or analysis) (European Committee on Blood Transfusion, 2015).

The coagulation process begins with one of two pathways, or by an intrinsic pathway when factor XII contacts with a negatively charge surface such as glass or the surface of an activated platelet, or by an extrinsic pathway when an injury connects blood and tissue creating a connection between factor VII and tissue cells (Riddel et al., 2007). Both pathways trigger the coagulation cascade, where are involved a variety of factors that are activated and lead to the formation of a protein named fibrin by agglomeration of fibrinogen. Cross-linked fibrins aggregate platelets, leucocytes and red blood cells (RBC's) creating clots in blood. The trap of blood cells in the cross-linked fibrin eliminates the circulation capacity and normal functioning of cells. When coagulation extension is big, the blood is no longer suitable for transfusion (Riddel et al., 2007). **Figure 1** outlines the coagulation cascade process.

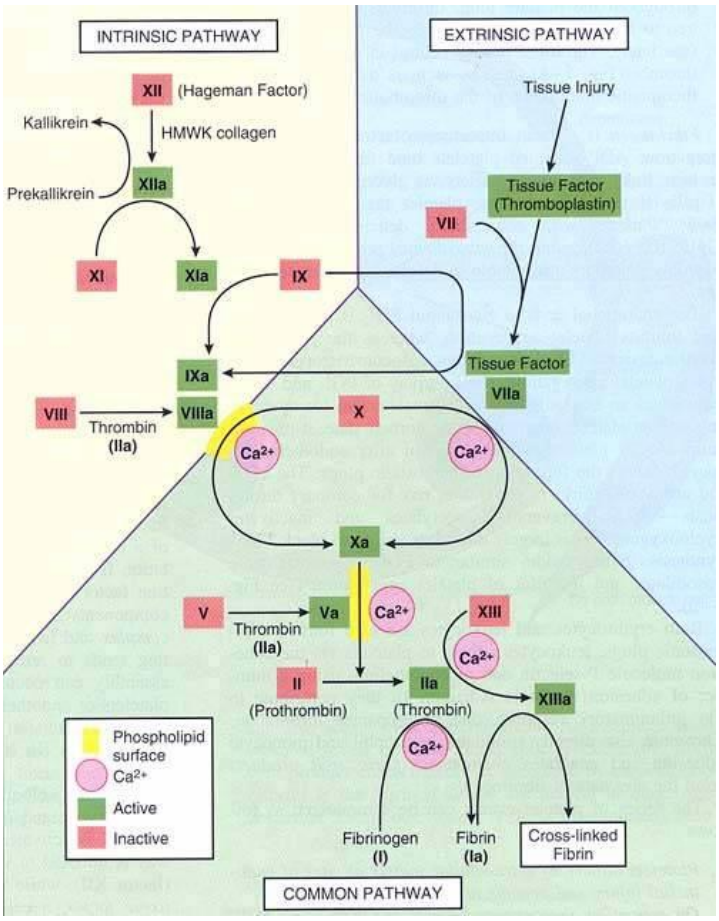


Figure 1 - Coagulation cascade. The initials XII, XI, VIII, IX, VII, X, V, II, and XIII represent factors of coagulation. The *a* letter present next to the factor means that these factors are activated. Adapted from Riddel et al. (2007).

When collecting blood, the ABO group and Rhesus group are identified and the bag of blood is marked with this information (Booth and Allard, 2017). The blood donated can be used in a variety of situations and can be separated by components for application in different situations as well (Booth and Allard, 2017; Castillo et al., 2018).

In the following sections, it will be described the different characteristics of blood components and the storage conditions used for each one of them with the end goal to using it for further transfusion.

1.3. Whole Blood

After collection, whole blood can be used without any treatment or change in its composition, however, nowadays, whole blood is rarely transfused and in the majority of cases it is processed and the several components of blood are separated and stored for further use in a variety of situations (Castillo et al., 2018; Turner, 2018).

When whole blood is used for transfusion without any change in is composition prior to storage, a preservative solution is also added. This solution is also an

anticoagulant solution, being already in the bag at the time of collection. In cases where the blood is separated in the several components the preservative solution is added after (Castillo et al., 2018).

A preservative solution is added to the blood in order to increase its shelf-life. Being a product with metabolism, the preservative solution will supply the blood with the necessary compounds, so the blood cells can be kept with normal metabolism, thus avoiding haemolysis (red cells rupture). There are a variety of preservatives solutions with different characteristics. For whole blood the preservative solution is typically citrate-phosphate-dextrose (CPD) or citrate-phosphate-dextrose-adenine 1 (CPDA-1) (Wong and Luban, 2016; Castillo et al., 2018).

Regarding the storage of whole blood, when CPD is added, the shelf-life is 21 days and when CPDA-1 is added it is 35 days. The shelf-life increase due to the addition of CPDA-1 is because of the presence of adenine that improves the production of adenine triphosphate (ATP). The presence of ATP increases the red cells survival and consequently the storage (Castillo et al., 2018). Once haemolysis reaches 0.8 % of the red cells mass, the blood is discarded, and so, in these cases, this limits the real shelf-life of the blood. (European Committee on Blood Transfusion, 2015).

Whole blood is typically kept at 1-6 ºC (refrigeration (RF) temperatures). All the storage times mentioned above for the preservatives solutions are at these temperatures (European Committee on Blood Transfusion, 2015).

1.4. Red Blood Cells

When whole blood is collected for separation into the different components, the first step after collection is centrifugation, so the components can be separated based on their densities.

The RBC's are obtained after a soft spin with a low *g*-force centrifugation that separates de RBC's from the platelet rich plasma (PRP). The RBC's product is a viscous solution with a haematocrit of 65-85 %. The haematocrit level is the volume ratio of packed RBC's per total volume of blood and for whole blood the haematocrit level varies between 40-48 % in humans. Since after centrifugation the PRP is separated the haematocrit level increase (Wennecke, 2004; Castillo et al., 2018).

In **Figure 2** is schematized a capillary tube with blood before and after centrifugation with the goal of measuring the haematocrit level.

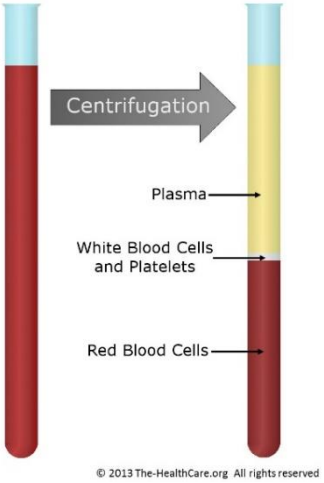


Figure 2 - Scheme of measurement of haematocrit level. Adapted from The Health Care (2018).

After centrifugation, the RBC's can undergo through a variety of processes such as reduction of leukocytes, irradiation, wash, or frozen. The reduction of leukocytes has become a standard procedure in Europe and countries like USA and Canada, this can be made pre-storage or pre-transfusion and the goal of this procedure is to minimize possible reactions of leukocytes from the donor with different tissues in the receptor that is recognize as foreign. This procedure can also be made in whole blood. Irradiation has the same goal as leukocytes reduction. By submitting the RBC's to irradiation, the leukocytes present will became unable to proliferate leading to a leukocyte reduction as well (European Committee on Blood Transfusion, 2015; Wong and Luban, 2016; Castillo et al., 2018). Regarding RBC's washing, this procedure is made for cases where the blood receptor has clinical diseases like immunoglobulin A deficiency that, when in contact with plasma proteins remained in the RBC's solution, can cause an anaphylactic reaction. By washing the cells and remove all the remaining plasma in the product, the RBC's can be transfused for the patient (Trompeter et al., 2014). In **Figure 3** is presented an image of RBC's obtained with scanning electron microscopy.

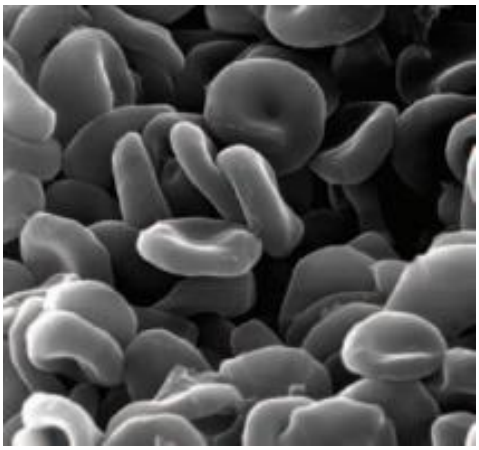


Figure 3 - RBC's visualized in scanning electron microscopy. Adapted from Toumey (2011)

According with the treatment/process and the preservative solution used, the shelf-life of the RBC's varies. For units of RBC's with no other treatment after centrifugation and RBC's leukoreduced, the storage time is dependent on the preservative solution, and, as said before for CPD, it is of 21 days and for CPDA-1 is 35 days. RBC's can also be preserved with additive solution (AS) (constitution not disclosure by the manufacturers) that extend the shelf-life of the product to 42 days (Wong and Luban, 2016; Castillo et al., 2018) under refrigeration conditions.

In **Table 1** is presented the shelf-life and storage temperatures for the different RBC's according with the treatments made.

Table 1- RBC's shelf-life and storage temperatures accordingly with the treatments made. Adapted from Castillo et al. (2018).

Product	**Shelf-life**	**Storage temperature (ºC)**
Whole blood/RBC's	CPD – 21 days; CPDA-1 – 35 days AS – 42 days	1–6
Leukoreduced RBC's	CPD – 21 days; CPDA-1 – 35 days AS – 42 days	1–6
Irradiated RBC's	28 days	1–6
Frozen RBC's	10 years	≤ -65 in 40 % glycerol
Deglycerolized RBC's	24 hours*	1–6
Washed RBC's	24 hours*	1–6

* in open system (without sterile conditions)

As mentioned above if haemolysis reach 0.8 % of red blood cells mass, RBC's units are discarded despite the time of storage at that point (European Committee on Blood Transfusion, 2015).

1.5. Leukocytes

Leukocytes represent about 1 % of blood and are constituted by five different types with different characteristics and functions. From all the types of leukocytes, only granulocytes are used in transfusions (American society of hematology, 2018). In **Figure 4** are identified the different types of leukocytes present in blood (Sarrafzadeh et al., 2014; Med-Health, 2019).

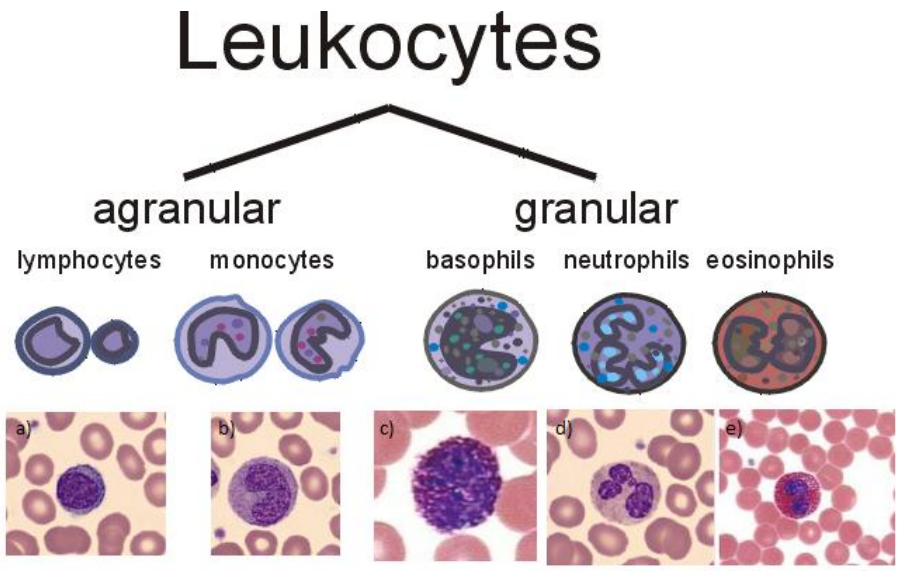


Figure 4 - Different types of leukocytes in the blood. a) lymphocytes b) monocytes c) basophils d) neutrophils and e) eosinophils. Adapted from Sarrafzadeh et al. (2014) and Med-Health (2019).

The collection of granulocytes is exclusively made by apheresis. This methodology is made by using a medical device where the blood passes through an equipment that separates a component of the blood and returns the remaining components to the donor's body (Castillo et al., 2018).

In **Figure A2 – Appendix A** is displayed an example of an apheresis device.

Since granulocytes represent a small percentage of the blood, the donors are stimulated with corticosteroids so the number of granulocytes in the blood increases and in order to be collect higher amounts. The use of granulocytes is not licensed by the Food and Drugs Administration (FDA) and, when used, it is necessary a specialized consent

and approval by the institutional review board since it is necessary the evaluation of risk-benefit in each case (Castillo et al., 2018).

After collection granulocytes can be kept at temperatures between 20-24ºC for up to 24 hours (Castillo et al., 2018).

1.6. Platelets

The transfusion of platelets as a standard procedure has increase in the last five decades and has been used in patients with thrombocytopenia (low platelet counts), with bone marrow failure or before invasive procedures like major surgeries, eyes surgeries or neurosurgeries (Goodnough et al., 2013; Booth and Allard, 2017).

Platelets can be obtained from PRP with a second centrifugation or through apheresis directly from the donor. In both cases the shelf-life of platelets is usually 5 days. However, FDA has recently approved the use of additive solutions, thus allowing the extension platelets' shelf-life for up to 7 days if all quality parameters (that will be further mentioned) are kept (European Committee on Blood Transfusion, 2015; Wong and Luban, 2016).

The storage of platelets is made at temperatures between 20-24ºC in bags with high permeability that allows gas exchange. Being platelets metabolically active, oxygen is necessary to maintain the metabolism since oxidative phosphorylation occurs during all the storage period. Also, platelets units need to be in continuous agitation, so aggregation of platelets does not occur and the gas exchange across the suspended platelets can be maintained (Castillo et al., 2018).

Regarding quality parameters, it is necessary that platelets units have a minimum number of platelets per unity. This value was defined as 5.5×10^{10} platelets if obtained from whole blood, and 3.0×10^{11} platelets if obtained from apheresis. The other quality parameter is the pH value at the end of the shelf-life. For platelets, pH cannot be lower than 6.2-6.4, as they are metabolically active, glycolysis occur along storage and lactic acid is produced, which is controlled by the additive solution, however, the pH can decrease despite the buffering solution and if the pH goes lower than 6.2, the recovery of platelets function poorer (European Committee on Blood Transfusion, 2015; Castillo et al., 2018).

1.7. Plasma

After centrifugation and RBC's, leukocytes and platelets separation, the remaining part is named plasma, the major component of blood (Castillo et al., 2018).

Plasma contains a mixture of water, proteins, sugars, fats, salts and chemical messengers (American society of hematology, 2018). Besides the regular proteins, plasma also contains the proteins called coagulation factors, and that's why plasma is collected and transfused (Castillo et al., 2018). It is used essentially in cases of active bleeding before surgery, in patients with acquired deficiencies on one or more coagulation factors, when disseminated intravascular coagulation with active bleeding and in patients with a congenital coagulation factor deficiency for which no alternative treatments exists (Goodnough et al., 2013).

There are a variety of plasma products available for transfusion, accordingly with the methodology of freezing and thawing and the time lapse between the collection and freezing and the thawing and transfusion of the plasma. The temperature of storage and processing is also an important factor for the type of plasma product. In **Table 2** is presented the different plasma products and definition for each of them.

Table 2- Plasma characteristics accordingly with the methodology of freezing and thawing. Adapted from European Committee on Blood Transfusion (2015), Wong and Luban (2016) and Castillo et al. (2018).

Product	Definition	Shelf-life	Storage temperature (ºC)
Fresh Frozen Plasma (FFP)	Plasma frozen within the first 8 hours after collection.	1 year 7 years	- 18 - 65
Plasma Frozen within 24 hours after venepuncture (PF24)	Plasma frozen within the first 24 hours after collection.	1 year	- 18
Plasma Frozen within 24 hours after venepuncture held at room temperature (PF24RT24)	Plasma frozen within the first 24 hours after collection, held at room temperature for that period.	1 year	- 18
Thawed Plasma (TP)	Plasma product (FFP, PF24, PF24RT24) that has been thawed and stored longer than 24 hours.	4 days*	1 to 6
Cryoprecipitate Plasma (CP)	Plasma concentrated after collection containing an high amount of cryoglobulins.	1 year	- 18

*After the initial 24 hours of being thawed.

As mentioned in **Table 2**, FFP is plasma frozen within the first 8 hours of collection. This product contains all the coagulation and anticoagulation factors present in normal blood (Erickson 2018). PF24 is an equivalent product to FFP since all the factors present in plasma are in comparable levels, has showed by Nifong et al. (2002). However, PF24 has a higher time window for freezing (24 hours) than FFP, making it logistically more feasible, especially for the blood collected in drives (Castillo et al., 2018).

The PF24RT24 is another plasma product that can be obtained. This type of plasma can only be collected from apheresis, unlike FFP and PF24, that can be obtained also from whole blood (Castillo et al., 2018). Regarding the coagulation factors present in PF24RT24, there are a reduction in some coagulation factors, being fVIII the most affected one, having a reduction up to 20 % when compared with FFP and PF24 (Alhumaidan et al., 2010). This difference in the values of fVIII and other factors is due to the time of hold and the temperature at which this happens (Castillo et al., 2018).

When FFP, PF24 and PF24RT24 are thawed and stored longer than 24 hours it is considered thawed plasma. As mentioned above, TP has a shelf-life of 5 days (the first 24 hours after being thawed plus 4 days after being renamed as thawed plasma) at temperatures of 1-6 ºC. The major difference of TP when compared with FFP and PF24 is the decreased amounts of cofactors, factor V (fV) and fVIII (Wong and Luban, 2016; Castillo et al., 2018).

All plasma products mentioned until now do not suffer any procedure after collection (and centrifugation in case of blood collected through venepuncture) having been only frozen or kept stored under RF. However, after collection, plasma can be furtherly processed and other plasma products can be obtained, like CP and cryoprecipitate-reduced plasma.

CP can be obtained by re-centrifugation at refrigeration temperatures. This centrifugation allows the sedimentation of plasma proteins like cofactors and anticoagulation factors, that by being at these temperatures aggregate and sediment (cryoglobulins) (European Committee on Blood Transfusion, 2015; Osterman and Arora, 2017). CP have high amounts of fibrinogen, fVIII and Von Willebrand Factor being used specially in patients with acquired hypofibrinogenemia or in patients with active bleedings (Goodnough et al., 2013; European Committee on Blood Transfusion, 2015). Cryoprecipitate-reduced plasma, the supernatant obtained after centrifugation, can also

be stored and used for transfusion, however it is rarely used and the evidences of its effectiveness are controversial (Castillo et al., 2018).

1.7. Bacterial and Viral Contamination of Blood

The contamination of blood or blood components used for transfusions can be endogenous or exogenous. The endogenous contamination is related with the donor of the blood, while the exogenous contamination causes are mostly attributed to the process of blood collection (introduction of bacteria from the skin). It can also be due to inappropriate handling of the sterile materials during the collection or management of the blood (Vasconcelos and Seghatchian, 2004).

Regarding the endogenous contamination and as mentioned before, the use of whole blood or blood components for transfusion requires the analysis of blood at the time of collection to avoid the transmission of bacteria and virus from the donor to the receptor of the blood (European Committee on Blood Transfusion, 2015). These procedures have decreased the transfusion of blood with bacteria and/or virus coming from the donor, however, the existence of new infectious threats and emerging diseases require a continuous analysis of threat potential (Booth and Allard, 2017).

When a blood collection is made, sterility of the materials and asepsis in the procedure is required, however, bacterial contamination from the donor skin or surrounding environment can occur. The rate of contamination in whole blood is between 0.3 to 0.6 % when tested in the first 12 hours after collection (Vasconcelos and Seghatchian, 2004).

Depending on the blood component, the contamination and posterior multiplication can be more feasible than others. Certain conditions such as the size of inoculum, storage temperature, preparation methodology, type of bacteria and component have a great impact on the capability of bacterial contamination and replication (Vasconcelos and Seghatchian, 2004). For example, since platelets are stored at RT the capability of bacterial replication in platelets is ideal, that's why platelets has been identified as the main blood component responsible for bacterial transmission in blood transfusions (Osterman and Arora, 2017). *Staphylococcus aureus*, *Escherichia coli*, *Bacillus* spp., *Salmonella* spp., *Streptococcus* spp., and *Klebsiella* spp. are some of the bacteria that can be find in contaminated platelets bags (Osterman and Arora, 2017; Bah et al., 2018).

Regarding RBC's the most common bacteria is *Yersinia enterocolitica* since it grows well in an environment rich in citrate and iron (Osterman and Arora, 2017). For plasma products, the main source of contamination is from water baths during the thawing of plasma products like FFP and CP (Vasconcelos and Seghatchian, 2004).

In the past 20 years, techniques to inactivate and reduce the level of infection agents, has been used in plasma derivatives such as immunoglobulins and cofactors, more recently, these techniques have been also applied in blood components like RBC's, platelets and FFP. There are two approaches for bacterial and viral inactivation. One of the techniques is a method that disrupt lipid membranes like solvent detergent treatment and visible light exposure and the other one is a method that target RNA and DNA like riboflavin plus ultraviolet (UV), light exposure or UV light exposure alone (Coté et al., 2018).

However, these techniques are limited. In cases when the microbial loads are high, these techniques may not inactivate all microorganisms, and for nonlipid virus the use of methods to disrupt lipid membranes is not effective. Another disadvantage of these techniques is the damages caused on plasma proteins and platelets, affecting their effectiveness (Coté et al., 2018).

2. High Pressure Processing (HPP)

HPP is an emergent processing technology that relies on the use of high hydrostatic pressure to inactivate both deteriorative and pathogenic vegetative microorganisms (Lado and Yousef, 2002). This technology has been used in a variety of industries, however, in food industry this technology as has grown in the last decades being already a well-established technology applied in a wide range of products such as fruit juices, ready-to-eat meals, meat products (such as ham) and vegetables (Lado and Yousef, 2002).

This technology relies, essentially, on two principles, namely on the *Le Chatelier's* principle and on the isostatic principle. The *Le Chatelier's* principle states that when an equilibrium system is perturbed, it tends to respond in order to counteract that perturbation. So, in an equilibrium process, any phenomenon that promotes a pressure increase is compensated by a volume decrease, and vice-versa (Knorr et al., 2011; Muntean et al., 2016) and, because of that, the packages used in HPP need to be flexible to support these variations (Balasubramaniam et al., 2015), while the isostatic principle states that pressure is instantaneously and uniformly applied through products, regardless of their shape, size and composition (Smelt, 1998; Knorr et al., 2011). During compression/decompression phases, there is a temperature increase/decrease due to adiabatic heating/cooling, respectively. The increase/decrease of temperature varies between 3-9 ºC for each 100 MPa, depending on the product matrix and pressurization fluid (Knorr et al., 2011).

This technology was firstly applied at the ceramic industry to produce different microstructures and avoid small defects that exists when other methodologies are used to produce ceramic pieces (Pfeifer et al., 1999). In the food industry, the first application of elevated pressures for non-thermal processing of food products was attributed to Bert Hite, in 1899, who proved that it was possible to pasteurize raw milk by HPP, promoting its shelf-life extension (Hite, 1899).

In medicine, HPP can be applied in a variety of situation like production of modified proteins of interest in pharmaceutical fields, stabilization of enzymes and modulation of their activity and specificity and sterilization of fragile biopharmaceuticals or medical material (Masson et al., 2001).

2.1. HPP applied in Blood

Regarding the use of HPP in blood the studies are scarce. Matser et al. (2005) applied pressures of 200-500 MPa at different temperatures and verified several changes in the different components of blood. RBC's were processed at 6 ºC and at 500 MPa, with treatment leading to a gel formation and completely loss of functional properties. For the RBC's treated at lower pressures (200 and 350 MPa) no gel formation was verified however the treatment darkened the colour of the samples at 350 MPa. In all the pressures applied the fragmentation of cells and consequently the haemolysis was high indicating the loss of functionality of RBC's. Regarding plasma, the treatment at 500 MPa and 6 ºC result in a completely loos of fVIII activity. For the samples whose treatment was made while frozen and with a start temperature of -10 ºC, significantly better results were obtained. At 300 MPa the fVIII activity reduced about 30-35 %. Changes in the shape of blood platelets wer also verified.

In another study, Toldrà et al. (2008) study the use of HPP in porcine blood with the goal to preserve animal blood, having analysed several parameters such as colour, protein solubility, foaming and emulsifying properties, texture and water holding capacity. The impact in the microbial loads was also tested. The results in the microbial loads was promising since the HPP treatment lead to a reduction in all the microbial counts for the bacteria analysed. Regarding colour, changes were verified for whole blood and RBC's concentrate solution, and for protein solubility and emulsifying properties changes were only verified for RBC's concentrate solution.

2.2. Effect of HPP in Microorganisms

As mentioned before, HPP inactivate vegetative microorganisms by destroying cell membranes, but several others changes occur when high pressure is applied, namely changes in the proteins structures, inactivation of enzymes like ATPase and rupture of nucleic acids and ribosomes involved in the protein synthesis (Georget et al., 2015; Dattaa and Deeth, 2018). The gram-positive bacteria normally require more intensive pressure treatments than gram-negative bacteria. This difference in resistance is attributed to the lack of the teichoic acids in the cell wall of gram-negative bacteria. Regarding to the resistance of yeasts, it takes place between these two bacterial groups (Dattaa and Deeth, 2018).

The inactivation of bacterial endospores by HPP cannot be achieved. The resistance mechanisms allow the survival of endospores through high pressure treatments up to 1200 MPa at room temperature, however high pressure can stimulate germination of bacterial endospores that can subsequently be inactivated (Knorr et al., 2011). With PATS, it is possible to inactivate bacterial endospores by combining high pressures and temperatures to obtain sterile products. Due to the adiabatic heating, the necessary temperatures are more easily achieved and uniformly applied through the food, reducing the thermal damage that occur in traditional sterilization (Martinez-Monteagudo and Saldaña, 2014; Dattaa and Deeth, 2018).

2.3. HPP in food industry

At an industrial level, the sales of HPP equipment are increasing over the years, showing the potential of this non-thermal technology. In **Figure 5** is presented the number of equipment operating worldwide, in the last years, and the percentage of machines used for each food category.

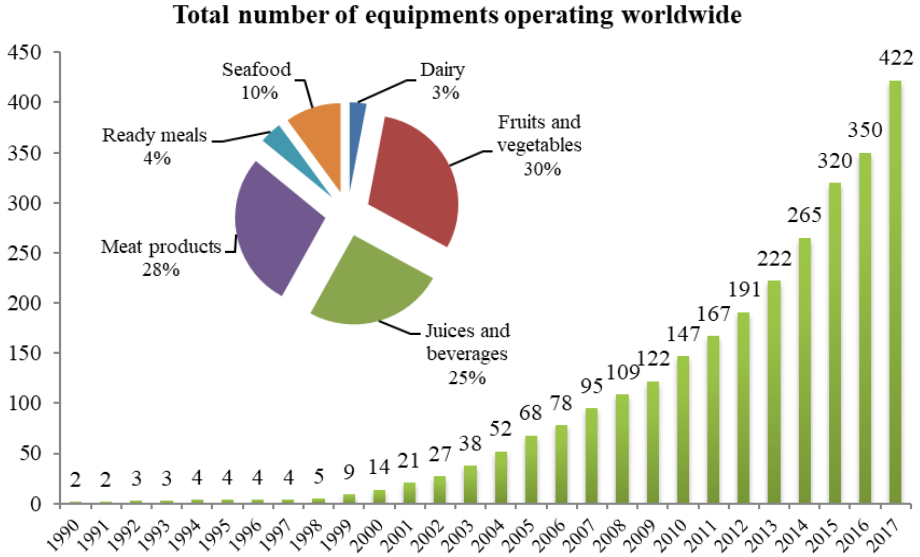


Figure 5 - Number of equipment operating worldwide, and percentage of food categories using the equipment. Courtesy of Hiperbaric S.A. (Burgos, Spain).

The continuous increase in the HPP equipment sales that is being verified in the last few years show the potential of this technology. The greatest advantages of HPP are the preservation of nutrients and flavours, the reduced processing times, no evidences of toxicity, the possibility of processing products in-package and, as mentioned before, the

ability of processing products despite the size or shape, being the treatment uniformly applied (Penchalaraju and Shireesha, 2013), which leads to the needless scale-up phase. However, despite the advantages of HPP, there are some disadvantages that have been hurdling an even bigger application of this methodology worldwide. The expensive equipment, especially for small companies with low budgets and the batch processing, are the major disadvantages (Penchalaraju and Shireesha, 2013; Muntean et al., 2016).

Regarding the costs of HPP equipment, this disadvantage can be overcome in a near future, since the number of companies applying this technology and the arose of new manufacturers in the market may lead to an equipment's costs decrease.

In **Figure 6** is schematized the HPP food market forecast between 2014 and 2025 and the share in 2015 through the different sectors of food products commercialization (Huang et al., 2017), demonstrating that the HPP food market will continuously grow over the next years.

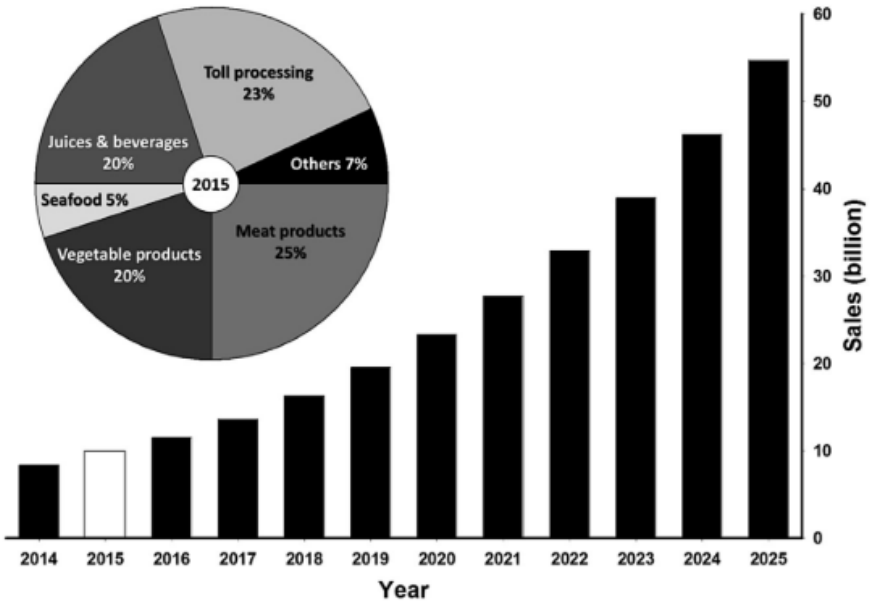


Figure 6 - The HPP food market forecast from 2014 to 2025 and the share in 2015 thru the sectors of food products. Adapted from Huang et al. (2017).

2.4. Hyperbaric Storage

Hyperbaric storage (HS) is a new food preservation methodology that consist in storage food under pressure (up to 220 MPa) for a certain period of time (from a few hours to months) (Fernandes et al., 2014).

This methodology was discovered by accident when the submarine Alvin (**Figure 7 (a**)) sank in October of 1968. After almost a year at 1540 meters (≈ 15 MPa) deep-sea

and temperatures of 3-4 ºC, the submarine was recovered, and with it some of the crew's foodstuff, namely apples, sandwiches and bouillon, despite of being soggy, seemed well preserved and edible (**Figure 7 (b)**). The recovered food products were stored at RF conditions and, a few days later, spoiled as expected (Jannasch et al., 1971). This event opened the possibility to store food products under pressure at RF temperatures to extend their shelf-lives.

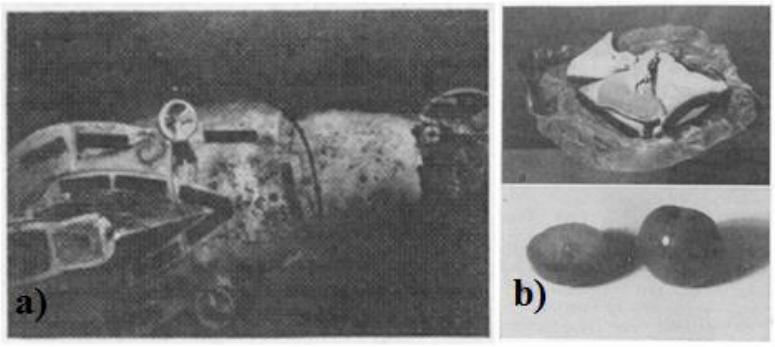


Figure 7 - (a) Alvin submarine on deep sea a few months before is recovery. (b) Food products that were inside of the submarine for almost a year in deep sea. Adapted from Jannasch et al. (1971).

2.4.1. Hyperbaric Storage at Low Temperatures

Conventional RF is the most used methodology (mainly, between 1 and 7 ºC) to preserve a wide range of products, not only in the food industries but also in the medical and pharmaceutical industries. This methodology is effective since it retards microbial growth, allowing a microbiological shelf-life extension (since the majority of microorganisms grow above 12 ºC, as well the majority of the deteriorative biochemical reactions occurs at that temperature) (Ashie et al., 1996; Gould, 2000).

However, there are many foods that are highly perishable, such as fish, where deterioration begins immediately after the harvesting (Otero et al., 2017) and other products like meat, fresh fruit juices and milk. Therefore, in order to maintain high quality and microbiological safety, a combination of preservation techniques, like the combination of HS and RF, can be a solution.

To test this, several studies have been conducted in recent years to evaluate the effect of HS/RF on various food products at RF temperatures (as an improvement to RF). Some of the results obtained are shown in **Table 3**.

Table 3 - Studies concerning HS/RF above 1.0 MPa (and up to 220 MPa) found in the literature. Adapted from Fernandes et al. (2014).

Product	Conditions	Results	Reference
Apples, bouillon and sandwiches	15 MPa at 3-4ºC for 10 months	Stable for 10 months under sea (15 MPa). After recovery, at RF temperatures, rapidly spoiled	Jannasch et al., (1971)
Rice, wheat and soy	3.5 MPa at 1ºC for a year	Stable for a year. Minor changes in seeds moisture, fatty acids and reducing sugars	Mitsuda et al., (1972)
Dressed cod	24 MPa at 1ºC for 21 days	Stable for 21 days with better quality than the samples storage at 0.1 MPa	Charm et al., (1977)
Pollock	24 MPa at 1ºC for 12 days	Stable and edible for 12 days with better quality than the samples storage at 0.1 MPa	Charm et al., (1977)
Hake loins	50 MPa at 5ºC for 7 days	Samples storage at 50 MPa were stable after 7 days, while at 0.1 MPa the microorganisms counts exceed the limits	Otero et al., (2017)
Atlantic mackerel fillets	50 MPa at 5ºC for 12 days	Microbial growth inhibition after 12 days at 50 MPa. No significant lipid degradation was observed	Otero, (2018)

2.4.2. Hyperbaric Storage at Sub-Zero Temperatures

Hyperbaric storage at sub-zero temperatures (HS/SZ) allows storage of products at temperatures below 0 °C without freezing, since pressure decreases the freezing point of the water. As for HS/RF, also in HS/SZ, the storage can vary between a few days to a few months (Kalichevsky et al., 1995; Fidalgo et al., 2014). In **Figure 8** is presented the diagram of the high-pressure shift freezing process.

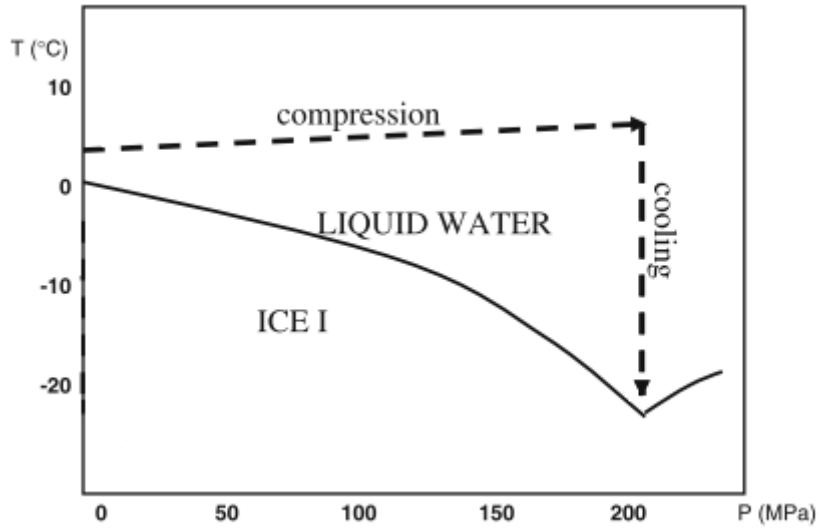


Figure 8 -Schematic diagram of the high-pressure shift freezing process. Adapted from Fernández et al., (2008).

This method of preservation is particularly important for solid foods, where the freezing and thawing process can cause damage to cells and tissues resulting in modifications in the texture of foods such as meat and fish (Fidalgo et al., 2014). Therefore, it is important to ensure the microbial safety and stability of the physicochemical parameters of the different types of food when stored in HS/SZ.

This methodology can also be applied in medicine. The storage at sub-zero temperatures without freezing can be used for cells, animal tissues, blood cells and even in organs for transplant (Masson et al., 2001).

In **Table 4** are presented the studies concerning HS/SZ.

Table 4 - Studies concerning HS/SZ above 1.0 MPa (and up to 220 MPa) found in the literature. Adapted from Fernandes et al. (2014).

Product	Conditions	Results	Reference
Cod fillets	22.8 MPa at -3ºC for 36 days	Stable and consumable for at least 36 days. Similar in quality to samples at 0.1 MPa	Charm et al. (1977)
Beef	200 MPa at -20ºC for a few days or weeks	Microbial load reduction and inactivation of yeasts and some bacteria	Hayashi and Deuchi, (1992)
Strawberry and tomatoes	50 - 200 MPa at -5 to -20ºC for a few days or weeks	Stable for a few more days/weeks. Fresh flavour and colour preserved	Hayashi and Deuchi, (1992)
Chicken and carp	17 MPa at -8 to -15ºC for 50 days	Stable for 50 days. Enzymatic activity reduced	Ooide et al. (1994)

Like HS/RF, in order to apply this methodology, is necessary to control and keep low temperatures and because of that, high energy costs are associated (Fernandes et al. 2014).

2.4.3. Hyperbaric Storage at Room Temperature

Some years after HS/RF and HS/SZ studies began, the possibility of use HS without the need for temperature control (HS/RT) arose. This methodology has opened a new area of study and, in recent years, several studies have been published on the effect of HS/RT on different food matrices (microbiological safety and organoleptic characteristics) (Fidalgo et al., 2014).

In **Table 5** are presented the studies concerning HS/RT.

Table 5 - Studies concerning HS/RT above 1.0 MPa (and up to 220 MPa) found in the literature. Adapted from Fernandes et al. (2014).

Product	Conditions	Results	Reference
Tilapia filets	200 MPa at 25 ºC for 12 hours	Improved freshness than in those storage at 0.1 MPa	Ko et al. (2006)
Sea cucumber guts	60 MPa at 30 ºC for 24 hours	Reduction of the psychotrophic counts	Okazaki et al. (2007)
Melon juice	25-150 MPa at 20-37 ºC for 8 hours	Microbial growth inhibition achieved at 50/75 MPa and inhibition plus reduction at 100/150 MPa	Queirós et al. (2014)
***Requeijão* (Portuguese whey cheese)**	100 and 150 MPa at 25-37 ºC for 8 hours	Microbial loads reduction after HS at 100 and 150 MPa. No significant changes on lipid oxidation levels when compared to RF at 0.1 MPa	Duarte et al. (2014)
	100 MPa at ≈17-21 ºC for 24 hours and 10 days	Microbial loads reduction after HS at 100 MPa for 24 hours and 10 days of storage. No change in the physicochemical parameters.	Duarte et al. (2017)
Sliced cooked ham	25-150 MPa at 23-37 ºC for 8 hours	Microbial growth inhibition at 50 MPa and microbial inactivation at 100 and 150 MPa. No changes on physicochemical parameters	Fernandes et al. (2015)
Carrot soup	100-150 MPa at 25-30 ºC for 8 hours	Microbial growth inhibition at 100 MPa and inactivation at 150 MPa	Moreira et al., (2015)
Caldo verde* and *bacalhau com natas	50-150 MPa at ≈21 ºC for 12 hours	Microbial growth inhibition at 100 MPa and inactivation at 150 MPa. No significant changes in physicochemical parameters	Moreira et al. (2015)
Raw bovine meat	100 MPa at ≈21 ºC for 10 days	The raw bovine meat shelf-life was extended over RF at 0.1 MPa	Freitas et al. (2016)

Table 5 - Studies concerning HS/RT above 1.0 MPa (and up to 220 MPa) found in the literature. Adapted from Fernandes et al. (2014) (continued).

Product	Conditions	Results	Reference
Strawberry juice	25-220 MPa at 20 ºC for 15 days	Stable for 15 days during HS and more 15 days at RF conditions. Microbial counts at 100 and 200 MPa below detection	Segovia-Bravo et al., (2012)
		HS kept stable the samples stored for 15 days and the volatile profile of the strawberry juice was similar to the initial samples	Bermejo-Prada et al. (2015)
	50-200 MPa at 20 ºC up to 15 days	The catalytic activity of pectin-methylesterase (PME) was not affected by the pressure in the strawberry extract	Bermejo-Prada et al. (2015)
	25-200 MPa at 20 ºC up to 15 days	Microbial growth inhibition at 50 MPa. At higher pressures, further microbial loads reductions were observed	Bermejo-Prada et al. (2016)
Watermelon juice	100 MPa at 18-21 ºC for 60 hours	Inhibition of microbial growth up to 60 hours	Fidalgo et al. (2014)
	25-150 MPa at 20-37 ºC for 8 hours	Microbial growth inhibition at 75 MPa and inactivation at 100 and 150 MPa	Santos et al. (2015)
	100 MPa at 18-21 ºC for 7 days	Shelf-life extension when compared to the juice kept at 4 ºC and 0.1 MPa	Pinto et al. (2016)
	50-75 MPa at 10-25 ºC up to 58 days	Microbial loads reduction after HS. No significant changes on physicochemical parameters. Shelf-life extension up to 58 days	Lemos et al. (2017)
	50-100 MPa at 18-23 ºC for up to 10 days	Microbial load reductions at 75 and 100 MPa were observed on endogenous and inoculated microorganisms.	Pinto et al. (2017)
Fresh Salmon	50-75 MPa at 25-37 ºC for 10 and 28 days	Microbial growth inhibition at 60 and 75 MPa. Shelf-life extension for at least 25 days at 75 MPa	Fidalgo et al., (2018)
Carrot juice	25-100 MPa at 18-23 ºC for up to 60 days	*Bacillus subtilis* endospore loads reductions in carrot juice (and Brain-Heart Infusion broth) at 50 and 100 MPa.	Pinto et al. (2018)
Apple juice	25-100 MPa at 18-23 ºC for up to 30 days	*Alicyclobacillus acidoterrestris* endospores inactivation at rapid rates reaching levels below the detection limit.	Pinto et al. (2019)

The major advantage of HS/RT over RF, HS/RF and HS/SZ is the reduced associated energetic costs, since energy is just necessary on the compression and decompression phases of the pressure vessel, and, once the desired pressure is reached, energy is not required to keep it along storage. This way, HS/RT could be a possible alternative to RF, since it keeps the majority of food characteristics and attributes, sometimes for longer periods than RF, with lower energetic costs. A recent study has showed the possibility to extend the watermelon juice shelf-life after HS/RT for up to 58 days (Lemos et al., 2017). Lemos et al. (2017) studied the effect of HS in watermelon juice at different pressures (50, 62.5 and 75 MPa) and verified that at 50 MPa the microbial loads of total aerobic mesophiles, total aerobic psychrophiles, *Enterobacteriaceae* and yeasts and moulds increased during storage reaching 6.0 CFU/mL after 7 days. However, for 62.5 and 75 MPa the microbial loads decreased, and the self-life of watermelon juice was increased. In this study the colour and pH were also analysed, and the values obtained for the samples at higher pressures support the extension of shelf-life.

2.4.4. Energy Costs of Hyperbaric Storage

HS is a new preservation methodology that is not yet applied in the food industry, being scarce data related to the energy costs. In fact, until now, only high pressure equipment applied for non-thermal pasteurization (reaching 600 MPa in a few minutes) has been commercialized. The energy costs related to HPP equipment does not relate with HS energy costs, since the pressure levels and pressurization rates required for HS are much lower (Bermejo-Prada et al., 2017).

For HS/RF and HS/SZ the energy costs are obviously higher than those associated to the conventional RF since it is necessary to control temperature and pressure (Fernandes et al., 2014). However, these methodologies can be used in products with high value where the preservation of organoleptic characteristics may compensate the increase in the energy costs.

Regarding HS/RT, the energy costs related are much lower than the energy costs of conventional RF since, as mentioned before, it only necessary energy on the compression and decompression phases of the pressure vessel, and, once the desired pressure is reached, energy is not required to keep it along storage (Lemos et al., 2017). However, at the moment this methodology HS/RT is more expensive than the conventional RF due to equipment costs. Thus, it is expected that in the future the

equipment costs will tend to be lower, which might open the possibility of HS/RT to become an affordable alternative to conventional RF (Bermejo-Prada et al., 2017).

In a study carried out by Bermejo-Prada et al. (2017) it was concluded that the energetic costs associated with HS were much lower for HS/RT compared to conventional RF, being the values 0.001 € and 0.026 € respectively per kg of juice, for 15 days. This difference in energy costs in the CO_2 release (carbon footprint) into the environment by conventional RF is an important factor to consider in a world where environmentally friendly technologies are becoming increasingly valued (Bermejo-Prada et al., 2017).

In **Figure 9** are schematized and indicated the costs related with HS/RT and conventional RF.

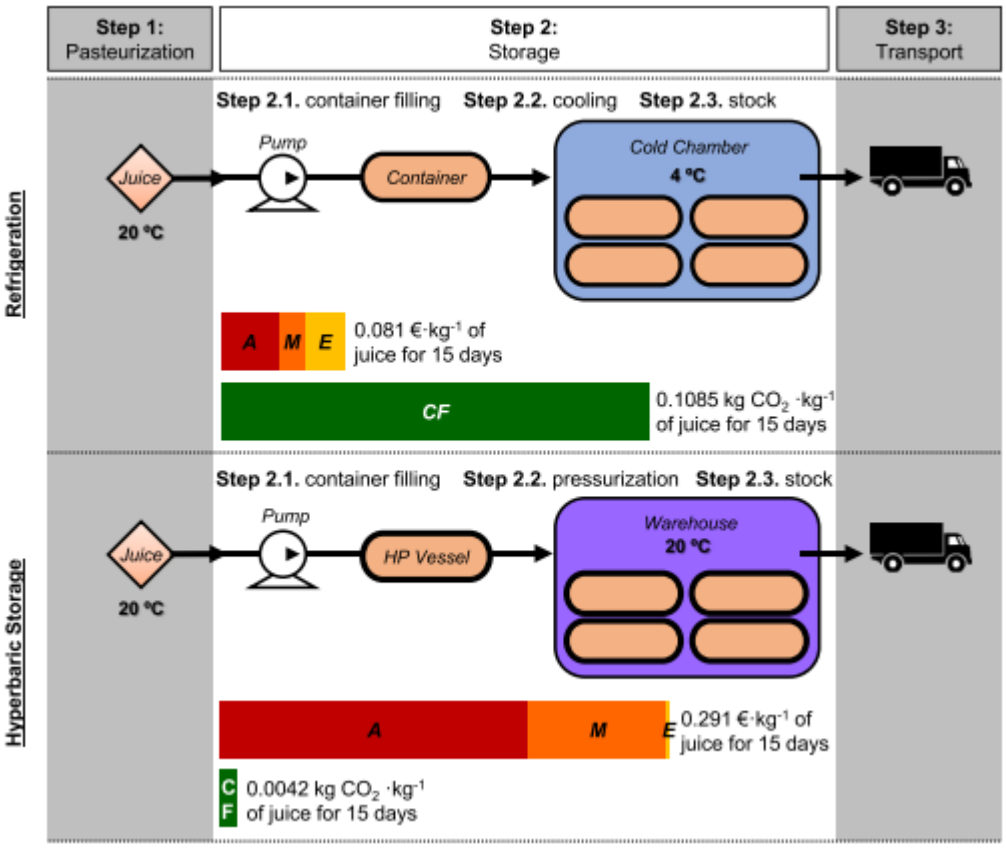


Figure 9 - Scenarios for storage according to each methodology. The stacked bar charts allow the comparison of the results for amortization (A), maintenance (M) and energy (E) costs and for carbon footprints (CF). Adapted from Bermejo-Prada et al. (2017).

3. Objectives

Nowadays blood and blood components are stored at RF temperatures or frozen. However, being blood a high value product with an high demand, the use of new methodologies, to store and keep the characteristics of blood, can be a solution to improve not only its quality but also to possibly extend the shelf-life. So, the main goal of this thesis is to study the effect of hyperbaric storage in blood, in terms of shelf-life, by means of microbiology and quality parameters.

To do so, porcine whole blood was used, and some parameters such as haemolysis, pH and microbial development was evaluated. Also, the storage conditions evaluated varied in a range of pressure between 0.1 to 50 MPa and temperatures between -5 to 25 ºC. These conditions were combined to evaluate the best pressure and temperature to preserve blood under pressure.

CHAPTER II – MATERIALS AND METHODS

1. Culture media and reagents

The EDTA was acquired from Biochem Chemopharma (Cosne-Cours-sur-Loire, France). Citric acid and monobasic sodium phosphate were purchased from Applichem Panreac (Darmstadt, Germany), adenine, dextrose, ringer, plate count agar (PCA), violet red bile dextrose agar (VRBDA) and rose bengal chloramphenicol agar (RBCA) were bought from Merck (Seelze, Germany) and trisodium citrate was acquired from VWR (Barcelona, Spain).

2. Solutions preparation

2.1. Ethylenediaminetetraacetic Acid

The anticoagulant EDTA (0.4M with pH of 8.0) was prepared by dissolution on distilled water. Potassium hydroxide (1M) was added until complete dissolution (pH = 8).

2.2. Citrate-phosphate-dextrose-adenine 1

The additive solution was prepared by adding citric acid, monobasic sodium phosphate, adenine, dextrose and trisodium phosphate to distilled water, according the indications provided by American Association of Blood Banks (AABB Technical Manual 2017).

3. Sample collection

All porcine blood used in this work was kindly provided by a local slaughterhouse (Taboeira, Aveiro, Portugal). Before the collection of the porcine blood samples, EDTA or CPDA-1 solutions were prepared and sterilized. EDTA was heat-sterilized and CPDA-1 was filtered through a 0.22 µm pore size membrane filter due to the heat sensitivity of the components. All the samples were collected in a laboratory glass bottle of 500 mL with the respective solution (anticoagulant or additive solution) and transported/kept in ice until storage (not longer than 2 hours).

For the samples with anticoagulant, 5.5 mL of EDTA were added to 500 mL and for the samples with additive solution, 70 mL were added to 500 mL when the collection of the blood.

4. Hyperbaric storage

HS experiments were carried out in two hydrostatic pressure equipment (High pressure system U33 (Institute of High Pressure Physics, Warsaw, Poland) and the multivessel system SFP 13900 (Stansted, United Kingdom). A mixture of propylene glycol and water (40:60) was used as a pressurisation fluid. Blood samples were aseptically packed in UV-light sterilized low permeability polyamide–polyethylene bags PA/PE-90 (Plásticos Macar - Indústria de Plásticos Lda, Palmeira, Portugal), using a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) to avoid contaminations. The samples were stored for 35 days at different pressures (25 and 50 MPa) and temperatures (10 ºC and uncontrolled room temperature, RT). Also, an experimental assay was done at 25 MPa and -5 ºC for 7 days. This assay was done for the first time in the research group so, the procedures and the vessel behaviour during the storage were carefully monitored.

Simultaneously, three control samples were kept at atmospheric pressure (0.1 MPa) and RT (0.1 MPa/RT), 10 ºC (0.1 MPa/10 ºC) and at RF (0.1 MPa/4 ºC), submersed in the same pressurization fluid and kept in the dark, to mimic the samples stored under hyperbaric conditions.

5. Microbial analyses

5.1. Sample preparation and dilution

In each microbial analysis, decimal dilutions were performed (1.0 mL of each sample for 9.0 mL of ringer solution) up to dilution of 10^{-4}, allowing a maximum microbiological quantification of 6.48 log CFU/mL. All the samples were analysed for counts of total aerobic mesophiles, *Enterobacteriaceae* and yeast and moulds. The preparation of culture media is summarized in the **Table C1 (Appendix C).**

5.2. Total aerobic mesophiles

Total aerobic mesophiles (TAM) counts were quantified by pour-plating 1.0 mL of each dilution in PCA after aerobic incubation at 30 ± 1 ºC for 72 ± 3 h (ISO 4833:2013).

5.3. Enterobacteriaceae

Enterobacteriaceae (ENT) counts were quantified by pour-plating 1.0 mL of each dilution in VRBDA, being incubated aerobically at 37 ± 1 ºC for 24 ± 1 h (ISO 21528:2017).

5.4. Yeasts and moulds

Yeasts and moulds (YM) were enumerated by spread plating 200 µL of each dilution in RBCA after incubation at 25 ± 1 ºC for 120 ± 3 h (ISO 21527:2008).

5.5. Microbial counts

For all the microorganisms analysed, the petri dishes containing 15-300 colony forming units (CFU) were counted and the results were expressed as logarithmic of CFU per mL (log CFU/mL) of blood. For yeasts and moulds (RBCA media), there were considered plates with 1-150 colonies. The microbial counts were calculated following **Equation 1** (ISO 4833:2003).

$$N = \frac{\Sigma\, characteristic\ colonies}{V[(n_1 + 0.1 \times n_2) \times d]} \qquad \textbf{(Equation 1)}$$

being:

N – Colony forming units per mL of blood (CFU/mL)

V – Sample volume (mL)

n_1 – Number of plates countable in the first dilution

n_2 – Number of plates countable in the second dilution

d – First countable dilution

All the results presented were obtained from triplicated samples and duplicate of analysis for TAM and ENT and quintuplicate of analyses for YM. The results were expressed as the decimal logarithm variation (log (N/N_0)), obtained by the difference between the microbial load at each storage day (N) and the initial microbial load (N_0). The quantification limit of 2.00 log CFU/mL was established (ten colonies or below per plate at dilution 10^{-1}). Values above 6.48 log CFU/mL were indicated as above the quantification limit. When no CFU were detected in the plates, the values were presented as below the detection limit and considered 1.00 log CFU/mL. The detention limit for YM was established at 2.00 log CFU/mL (no CFU detected in the plates).

6. Physicochemical analyses

6.1. pH

The pH value of the samples was measured at 25 ºC with a properly calibrated glass electrode (Crison Instruments, S.A., Spain) which was calibrated with pH 4.0 and 7.0 buffer solutions.

6.2. Haemolysis

The haemolysis was measured with the Harboe direct spectrophotometric method (Wians et al. 1988; Cookson et al. 2004). The samples of blood were first centrifuged (8,000 ×g for 10 min at 25 ºC) to separate blood cells from plasma. The plasma obtained was then diluted (when necessary to be within the linear spectrophotometric range) and measured at 380, 415 and 450 nm. The spectrophotometric measurements at 380 and 450 nm were made in order to obtain a real haemoglobin concentration in plasma, since some plasma impurities and bilirubin/albumin complexes can also be detected at 415 nm (Cookson et al. 2004). The **Equation 2** was used to determine the haemolysis in mg/dL (Cookson et al. 2004; Han et al. 2010).

$$Hb\ (mg/dL) = \frac{(167.2A_{415} - 83.6A_{380} - 83.6A_{450})}{dil H_2O} \qquad \textbf{(Equation 2)}$$

To be able to make a comparison between the haemolysis values obtained and the limit value (which the blood can be used for transfusion), a conversion to percentage of haemolysis was made using the **Equation 3** presented below (Han et al. 2010).

$$Haemolysis\ (\%) = \frac{Supernatant\ Hb\ (mg/dL) \times [100 - haematocrit\ (\%)]}{Total\ Hb\ (mg/dL)} \qquad \textbf{(Equation 3)}$$

7. Statistical analyses

The microbial samples were performed in duplicate, each one from triplicated samples. The pH and haemolysis values were measured in triplicate. The results were statistically analysed using main effects analysis of variance (ANOVA), followed by Turkey's honest significand differences (HSD) test at 5 % of significance and were expressed as mean ± standard deviation.

CHAPTER III – RESULTS AND DISCUTION

THIS SECTION REPORTS ALL THE OBTAINED RESULTS REGARDING BLOOD STORAGE WITH TWO DIFFERENT SOLUTIONS

1. Ethylenediamine tetraacetic acid experiments

As the author of this thesis is aware, there are no studies in the literature regarding the effect of HS (or HS-like conditions) in blood. So, to understand the effect of pressure (with and without temperature control) in blood without additive solution (normally added) only an anticoagulant was added to the blood to prevent it to clot.

1.1. Microbial Analysis

As mentioned before, all the porcine blood used in this thesis was provided by a local slaughterhouse and, despite all the hygiene procedures used, the presence of microorganisms in the blood was possible. So, the microbial analyses were made regarding TAM and ENT. The presence of YM was also tested but were not detected.

Regarding TAM, an initial microbial load of 2.10 ± 0.05 log CFU/mL (2.57 ± 0.16 log CFU/mL for the assay at 50 MPa/RT) was quantified, while for ENT, the initial microbial load was below the detection limit (initial value below 1.00 log CFU/mL) (**Figure 10 (a and b)**).

At 4th day, at all storage conditions (50 MPa/RT, 50 MPa/10 °C, 25 MPa/RT, 25 MPa/10 °C, 0.1 MPa/RT, 0.1 MPa/10 °C and 0.1 MPa/RF) the microbial load for TAM was below the quantification limit (2.00 log CFU/mL). At the 7th day of storage experiments, the microbial load for TAM reached the detection limit (1.00 log CFU/mL) for the samples stored at 25 MPa/RT and 0.1 MPa/RT. For all other conditions, the microbial load was below 2.00 log CFU/mL. The detection limit was also reached for the samples at 50 MPa/10 °C and at 0.1 MPa/10 °C by the 12th day and for samples at 25 MPa/RT by the 21th day. Additionally, only the refrigerated samples (0.1MPa/RF) were below 2.00 log CFU/mL, having reached the detection limit by the 35th day.

Throughout all the assays the presence of ENT was evaluated since it is a good indicator of faecal contamination and the blood was not collected in sterile conditions. However, no microbial presence of ENT was verified in all samples tested

All the values regarding the microbial load for TAM and ENT are presented in **Table D1 – Appendix D**.

According with the results presented by Fidalgo et al. (2018), Lemos et al. (2017) and several other authors, at 50 MPa/RT was expected an increase in the microbial loads since this pressure does not have, normally, an impact in the microorganisms. For lower storage pressures, it was expected a similar behaviour. However, in all storage conditions,

a decrease in microbial loads was verified, being even reached the detection limit (1.00 log CFU/mL). This might have happened due to the lack of nutrients in the blood since only EDTA was added. Without nutrients, the microorganisms present in the blood were unable to growth and proliferate, despite the pressure and temperature hurdle for it. This evidence is supported by the evolution of haemolysis during the 35 days of storage and it will be discussed in the next topic.

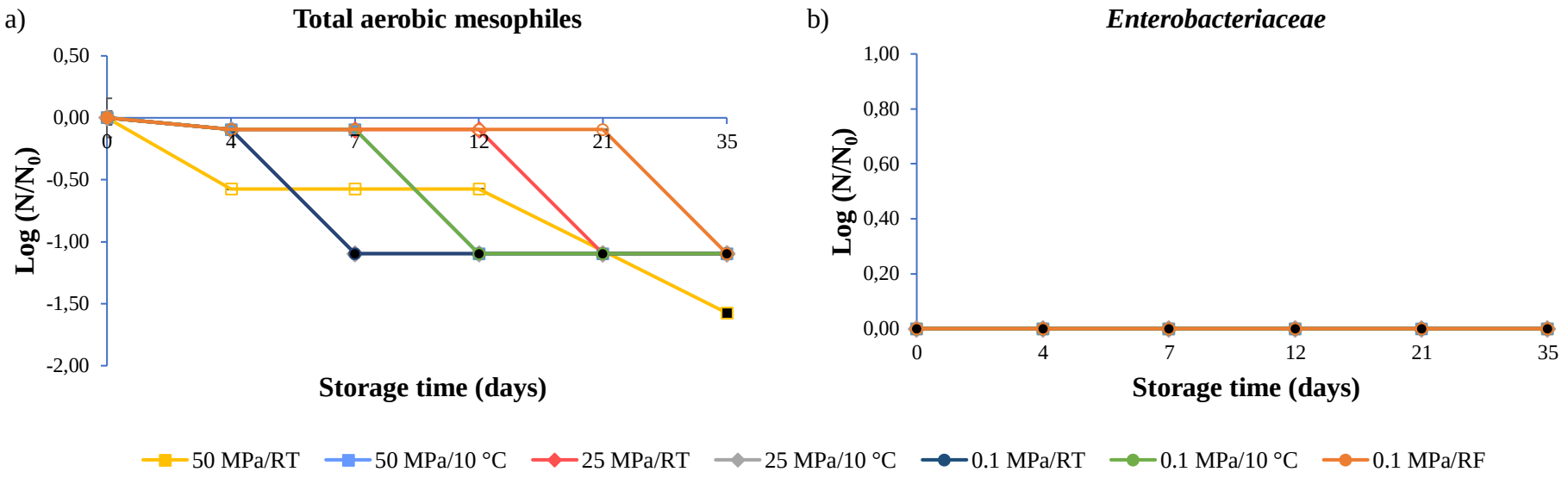


Figure 10 - a) Total aerobic mesophiles and b) *Enterobacteriaceae* loads evolution on porcine blood with anticoagulant during 35 days of storage at: 50 MPa/RT, 50 MPa/10 °C, 25 MPa/RT, 25 MPa/10 °C, 0.1 MPa/RT, 0.1 MPa/10 °C and 0.1 MPa/RF. Black filled/empty symbols mean that the detection/quantification limit (1.00 and 2.00 log CFU/mL, respectively) was reached. At 50 MPa/RT, the 21th day is not presented since it was not possible to analyse due to the holidays break.

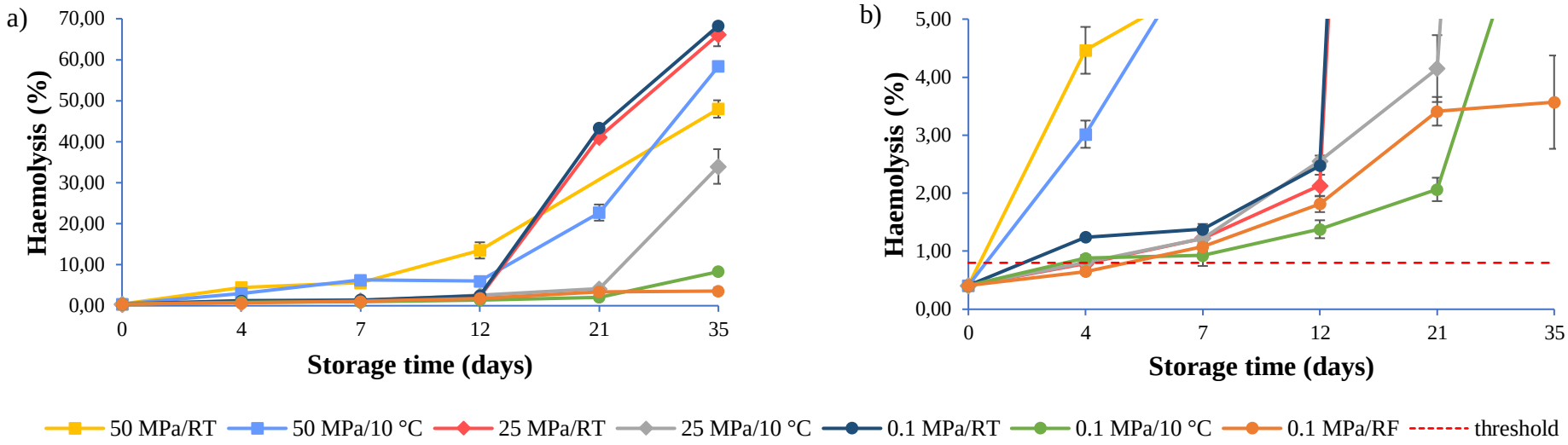
1.2. Haemolysis

Haemolysis is an important parameter for blood transfusion since the main goal for a transfusion is to increase the red blood cells to allow a correct and enough oxygenation of the body cells and tissues. Thus, the haemolysis percentage was measured for each storage condition at each time and the evolution was presented in a graphic (**Figure 11**).

According with the European Committee on Blood Transfusion (2015) the maximum percentage of haemolysis allowed for the transfusion of blood is 0.8 %. All the blood collected that reaches this value before the 35 days of shelf-life is discarded. So, in **Figure 11b)** (an amplified section of **Figure 11a)**) a dashed line is presented and indicate this value for an easier comparation with the different storage conditions. All values obtained for all conditions and days are presents in **Table E1 – Appendix E**.

The initial haemolysis was 0.41 ± 0.03 % (0.44 ± 0.03 % for the assay at 50 MPa/RT). At the 4th day, all the storage conditions (with exception of 50 MPa/RT) were similar ($p>0.05$) between each other and the initial value, however only the samples kept at 25 MPa/RT, at 25 MPa/10 °C and at 0.1 MPa/RF were below the limit of 0.8 % of haemolysis. By the 7th day, the haemolysis percentage of all samples under different storage conditions were above the limit of 0.8 %. This is in agreement with the microbial analyses, since without the presence of nutrients, no microbial growth will happen, neither the RBC will be kept alive and consequently haemolysis will occur. At this point, no significant differences ($p>0.05$) at 50 MPa, both at RT and 10 ºC were observed, showing that despite the temperature difference, no changes between the samples were verified. All the other conditions were statistically different ($p<0.05$) of the samples at 50 MPa (RT and 10 °C) but similar ($p>0.05$) when compared between each other (**Table E1 – Appendix E**). The differences between the first ones and the second ones is mainly due to the impact that pressure have in the RBC's promoting cell lysis, and consequently the increase of haemolysis.

All the samples that at the 7th day did not have significant differences ($p<0.05$) kept this way by the 12th day, however at 50 MPa/RT and 50 MPa/10 °C the similarity did not hold, with a higher increase of haemolysis at 50 MPa/RT.



Condition/Storage time (days)	4	7	12	21	35
50 MPa/RT	aB	aB	bC	-	cD
50 MPa/10 °C	aABε	aB	aB	bB	cE
25 MPa/RT	aAε	aAε	aAε	bC	cF
25 MPa/10 °C	aAε	aAε	aAε	aA	bC
0.1 MPa/RT	aABε	aAε	aAε	bC	cF
0.1 MPa/10 °C	aAε	aAε	aAε	aA	bB
0.1 MPa/RF	aAε	aAε	aAε	aAε	aAε

Figure 11 - Haemolysis percentage evolution in blood with anticoagulant during 35 days of storage at: 50 MPa/RT, 50 MPa/10 °C, 25 MPa/RT, 25 MPa/10 °C, 0.1 MPa/RT, 0.1 MPa/10 °C and 0.1 MPa/RF. The Figure b) in an amplified section of Figure a) where is indicated the maximum percentage of haemolysis allowed for blood transfusion with a red dashed line denominated "threshold". In the table, different upper/lower case letters (A-F)/(a-c) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. The Greek letter ε indicates values that are statistically similar ($p>0.05$) to the initial value. At 50 MPa/RT, the 21th day is not presented since it was not possible to analyse due to the holidays break.

At the 21st day, with exception of 25 MPa/10 °C, 0.1 MPa/10 °C and 0.1 MPa/RF that slightly increase (not statistically different ($p>0.05$)), all other condition showed a significant increase in the haemolysis ($p<0.05$) when compared with the 12th day.

Despite having exceeded the limit of haemolysis stipulated, by the 35th day only the samples storage at 0.1MPa/RF were similar ($p>0.05$) with all the prior days of storage and with the initial value of haemolysis (0.41 ± 0.03 %).

Regarding the values of haemolysis obtained for all the conditions after the 35 days of storage is possible to conclude that higher pressures have a higher impact in the haemolysis as expected, however the lack of nutrients in the blood had also an important role in the increasing of haemolysis since RBC's cannot be able to keep the metabolism running and cellular lysis occur.

1.3. pH

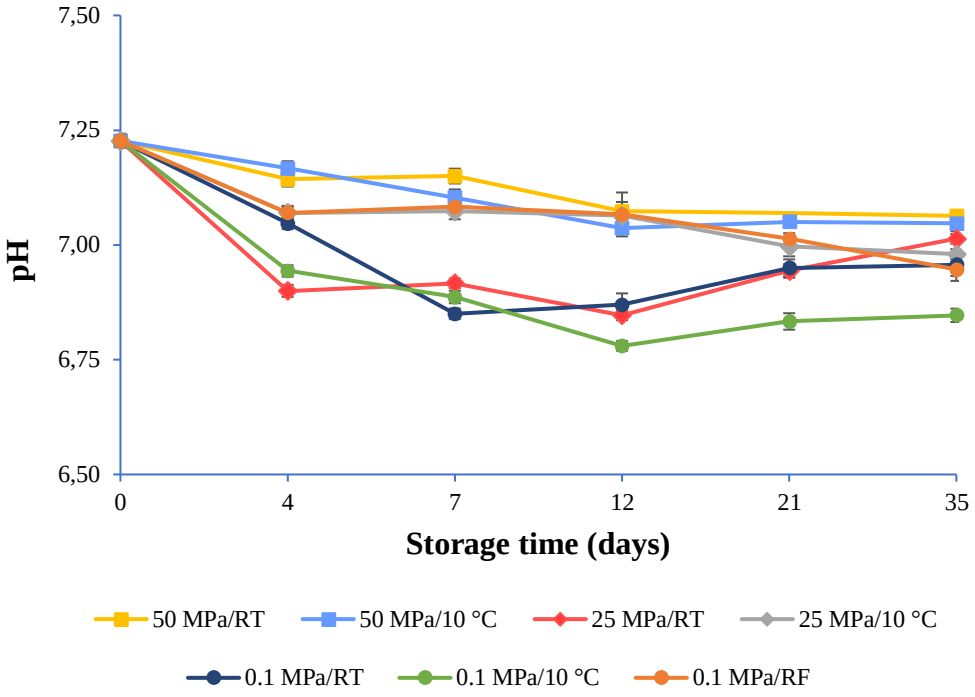
The blood pH on the body is normally around 7.35 and 7.45 (Donaldson and Lamont 2013), but when collected, due to the anticoagulant or the additive solution added, it might drop slightly. However, these values are for blood in the bloodstream (at 37 °C). All values presented next were measured at 25 °C so, the values are lower than those if measured at 37 °C. Nevertheless, the changes (variation) provoked in the pH during the storage period by the different conditions is the same independent of the temperature at which the pH was measured.

The samples collected had an initial pH of 7.23 ± 0.02 (7.23 ± 0.03 for the assay at 50 MPa/RT).

According with Donaldson and Lamont (2013) it was expected a decrease in the pH values during the 35 days of storage due to the production of lactic acid by glycolysis. In fact, this was verified as it is showed in **Figure 12**.

However, the drop in the pH values was different according the storage conditions. At 50 MPa/RT and 50 MPa/10 °C the variation between the initial day and the 35th day was smaller than for the other conditions. This might be related to the haemolysis percentage at these conditions since the increased haemolysis implies a smaller amount of RBC's that are producing lactic acid and consequently a smaller drop in the pH is verified. It may also be related to the release of non-acid metabolites from the RBC's at the time of haemolysis. The pH values by the 35th day for the samples at 25 MPa/RT and 0.1 MPa/RT support this theory since at the 21st day is verified a significant increase ($p<0.05$) in the haemolysis percentage and the pH values for the same conditions at the same day increased.

The blood stored under RF conditions (0.1 MPa/RF) showed a different behaviour along storage, since the pH decreased, showing no significant differences ($p>0.05$) compared with the samples stored at 25 MPa/10 °C and 0.1 MPa/RT by the 4th day, but also showing no significant differences ($p>0.05$) with the samples stored at 25 MPa/10 °C, 50 MPa/RT and 50 MPa/10 °C by the 12th day instead of keep the decrease tendency has the other conditions without pressure applied. This cannot be justified with the haemolysis since the values for the blood at 0.1 MPa/RF are the smallest. But can be



Condition/Storage time (days)	4	7	12	21	35
50 MPa/RT	bC	bD	aC	-	aCD
50 MPa/10 °C	cC	bCD	aC	abC	aCD
25 MPa/RT	abA	bB	aB	bB	cC
25 MPa/10 °C	bB	bC	bC	aBC	aBC
0.1 MPa/RT	cB	aA	aB	bB	bB
0.1 MPa/10 °C	cA	bAB	aA	abA	bA
0.1 MPa/RF	cB	cC	bcC	bC	aB

Figure 12 - pH evolution in blood with anticoagulant during 35 days of storage at: 50 MPa/RT, 50 MPa/10 °C, 25 MPa/RT, 25 MPa/10 °C, 0.1 MPa/RT, 0.1 MPa/10 °C and 0.1 MPa/RF. In the table, different upper/lower case letters (A-D)/(a-c) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. At 50 MPa/RT, the 21th day is not presented since it was not possible to analyse due to the holidays break.

justified with the metabolism deceleration due to the low temperature and consequently lower glycolysis rate and lower lactic acid production.

Regarding the samples kept at 25 MPa/10 ℃, it was expected a similar behaviour to 25 MPa/RT since for those at 50 MPa (RT and 10 ℃) the temperature did not have an impact in the pH. However, instead of it, no significant differences ($p>0.05$) in the behaviour was verified when compared with the results obtained for the blood stored at 0.1 MPa/RF. These results might be due to a combination of pressure-temperature that have a similar impact in the RBC's which is supported by the haemolysis results that also show no significant differences ($p>0.05$) between each other for the first 21 days.

All values obtained for all conditions and days are presents in **Table F1 – Appendix F**.

2. Citrate phosphate dextrose adenine-1 experiments

To evaluate the effect of HS in blood with the same composition of the one normally stored for transfusion, an additive solution was added to the blood at the time of collection and stored at different pressures and combined with different temperatures. The additive solution added was CPDA-1, typically used for the storage of whole blood to provide the necessary nutrients to maintaining the RBC's in good conditions and preventing the cell lysis.

The assay at 25 MPa/-5 °C had only the duration of 7 days since no prior tests were ever made at SZ temperatures in the research group and there were no guaranties about the behaviour and capability to maintaining the low temperature and pressure of the vessel for long periods of time. **Figure B1 – Appendix B** shows the pressure vessel at 25 MPa/-5 °C and it is possible to see the ice on top of the vessel.

In this section, the results regarding the blood samples with this additive solution will be presented and discussed.

2.1. Microbial Analysis

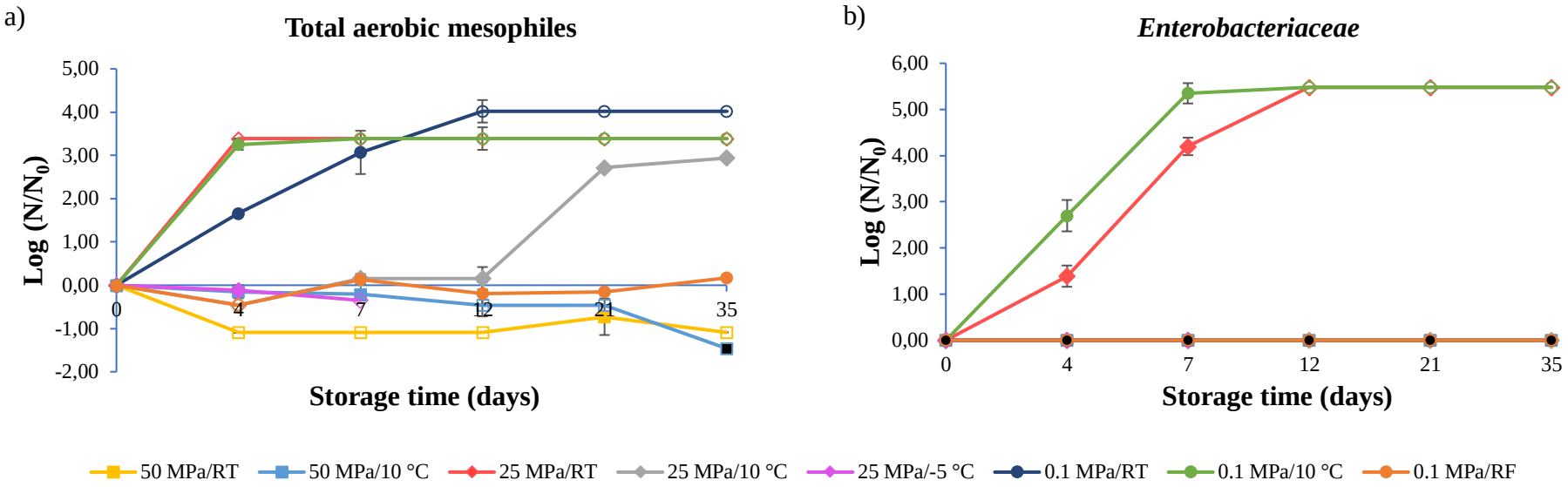
As for the blood samples with EDTA, for the samples with CPDA-1 was also carried out microbial analysis regarding TAM and ENT as showed in **Figure 13**.

The additive solution added have a large amount of dextrose, giving the nutrients necessary for the growth of microorganisms, allowing this way the microbial growth when possible (when the conditions used for storage were not enough to prevent it) what did not happen with the blood with EDTA.

Regarding TAM, an initial microbial load of 2.46 ± 0.04 log CFU/mL (3.09 ± 0.06 log CFU/mL for the assay at 50 MPa/RT, 25 MPa/RT and 0.1 MPa/10 °C and 2.35 ± 0.10 log CFU/mL for the assay at 25 MPa/-5 °C) was quantified. No microbial load was detected for ENT at day zero (initial value below 1.00 log CFU/mL for all the assays).

The samples kept at 50 MPa (RT and 10 °C) showed a decrease in the microbial load for TAM reaching the quantification limit (2.00 log CFU/mL) by the 4^{th} and 12^{th} days, respectively. Throughout the first 21 days in both cases, no significant differences ($p>0.05$) were verified between the different days of each condition. By the 35^{th} day the samples at 50 MPa/10 °C reached the detection limit (1.00 log CFU/mL) and the samples

at 50 MPa/RT kept below the quantification limit. These results show the possibility of reducing the microbial load of blood with pressure.



Condition/Storage time (days)	4	7	12	21	35
50 MPa/RT	aA	aA	aA	aA	bA
50 MPa/10 °C	bBε	bBε	bBε	bAε	aA
25 MPa/RT	aD	aC	aD	aC	aC
25 MPa/10 °C	aBε	bBε	bCε	cB	cC
25 MPa/-5 °C	aBε	aBε	-	-	-
0.1 MPa/RT	aC	bC	dC	cD	cD
0.1 MPa/10 °C	aD	aC	aD	aC	aC
0.1 MPa/RF	aBε	bBε	abBε	abAε	abBε

Condition/Storage time (days)	4	7	12	21	35
50 MPa/RT	aAε	aAε	aAε	aAε	aAε
50 MPa/10 °C	aAε	aAε	aAε	aAε	aAε
25 MPa/RT	aB	bB	cB	cB	cB
25 MPa/10 °C	aAε	aAε	aAε	aAε	aAε
25 MPa/-5 °C	aAε	aAε	-	-	-
0.1 MPa/RT	aAε	aAε	aAε	aAε	aAε
0.1 MPa/10 °C	aC	bC	bB	bB	bB
0.1 MPa/RF	aAε	aAε	aAε	aAε	aAε

Figure 13 - Total aerobic mesophiles and *Enterobacteriaceae* loads evolution in blood with additive solution during 35 days of storage at: 50 MPa/RT, 50 MPa/10 °C, 25 MPa/RT, 25 MPa/10 °C, 25 MPa/-5 °C, 0.1 MPa/RT, 0.1 MPa/10 °C and 0.1 MPa/RF. Black filled/empty symbols mean that the detection/quantification limit (1.00 and 2.00 or 6.48 log CFU/mL, respectively) was reached. In the table, different upper/lower case letters (A-D)/(a-c) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. The Greek letter ε indicates values that are statistically similar ($p>0.05$) to the initial value. The assay at 25 MPa/-5 °C had only a duration of 7 days.

Contrary to the samples at 50 MPa (RT and 10 °C), for the blood samples kept at 25 MPa/RT and 25 MPa/10 °C, an increase in the microbial loads of TAM was verified and, by the 4th day, the value was above 6.48 log CFU/mL for those kept at 25 MPa/RT, showing no significant differences ($p>0.05$) between the different days of storage. For 25 MPa/10 °C the lower temperature prevented the fast growth as verified for 25 MPa/RT. However, by the 21st day, an increase of more than 2.5 log CFU/mL occurred, reaching 5.40 ± 0.43 log CFU/mL by the 35th day. It is worth to note that, until the 7th day of storage, the microbial loads for the samples at 25 MPa/10 °C and 0.1 MPa/RF were not statistically different ($p>0.05$) indicating a possible pressure-temperature effect similar to the one induced by 0.1 MPa/RF in the TAM. For the samples at 25 MPa/-5 °C during the 7 days of storage a slightly decrease was verified reaching the quantification limit (2.00 log CFU/mL) by the 7th day and showing also no differences ($p>0.05$) compared to 0.1 MPa/RF.

The samples at 0.1 MPa/RT and 0.1 MPa/10 °C showed an increase throughout the 35 days of storage as expected having both reached values above 6.48 log CFU/mL. For 0.1 MPa/10 °C the increase was similar ($p>0.05$) to 25 MPa/RT.

Regarding samples under 0.1 MPa/RF, the microbial load for TAM showed small oscillations during the 35 days of storage but having not reached 3.00 log CFU/mL and the values were always not statistically different ($p>0.05$) from the initial value (2.46 ± 0.04 log CFU/mL).

As mentioned above, all the samples were tested for the presence (and quantification) of ENT and only the samples kept at 50 MPa/RT, 25 MPa/RT and 0.1 MPa/10 °C revealed the presence of ENT. The initial microbial load for ENT was below the detection limit (1.00 log CFU/mL), however, for the remaining days of storage the presence of ENT was verified.

Despite no detection of ENT for the samples at 50 MPa/RT, all samples stored under these conditions were from the same batch of blood, showing that at 50 MPa/RT ENT were inactivated/unable to grow. For the remaining conditions by the 21st day the microbial load was above 6.48 log CFU/mL. However, at 25 MPa/RT the growth rate was lower than at 0.1 MPa/10 °C, being statistically different ($p<0.05$) by the 4th and 7th day of storage, indicating an impact of pressure in the microbial growth.

The values obtained for all conditions and days are displayed in **Table G1 – Appendix G**.

2.2. Haemolysis

As mentioned before, haemolysis is an important parameter when the blood is collected for further transfusion, so for the blood stored with CPDA-1 the haemolysis was also measured.

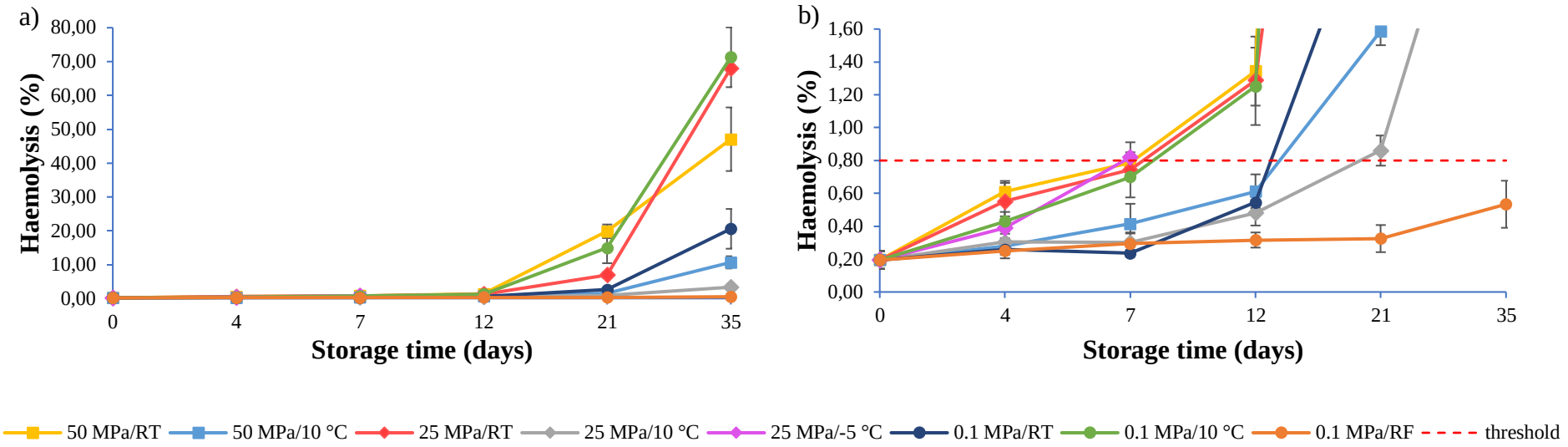
The blood with CPDA-1 showed a totally different behaviour regarding haemolysis (and microbial growth as seen above) when compared with the blood with EDTA, regardless the combination of pressure and temperature at what the blood was stored. This is most likely due to the additive solution added, since with this solution the necessary nutrients were added to prevent cell lysis and haemoglobin release. However, it is possible to identify differences between the different conditions at which the blood was kept during the storage period.

Figure 14 shows the haemolysis evolution during the 35 days of storage experiments of all the conditions. In **Figure 14b)** (an amplified section of **Figure 14a)**) a dashed line is presented and indicate the 0.8 % of haemolysis (the limit allowed for blood transfusion) to an easier comparation with the different storage conditions, as was made for the blood samples with EDTA. All haemolysis values obtained for all conditions and days are presents in **Table H1 – Appendix H**.

For the haemolysis values to the assays with CPDA-1 the statistical analysis was made only for the days of each condition at which the value was below or at the limit to allow a better comparison between the results, since if all the values were contemplated, the values below the limit would have been all identified as not statistically different ($p>0.05$) from each other, i.e. would lose significance, and, from a perspective of ability for transfusion, small differences such as those verified between the storage conditions are crucial and significant to discard or not the blood.

The initial value of haemolysis was 0.16 ± 0.05 % (0.19 ± 0.03 % for the assay at 50 MPa/RT, 25 MPa/RT and 0.1 MPa/10 °C and 0.21 ± 0.04 % for the assay at 25 MPa/-5 °C), lower values than those obtained for the blood with EDTA (0.41 ± 0.03 % and 0.44 ± 0.03 %), possibly due to the solution of CPDA-1 added.

At the 4^{th} and 7^{th} days of storage all the samples at all storage conditions where below or at the haemolysis limit while with EDTA the haemolysis for all conditions were already above the limit at this point, which is considerably better from the point of view of a future transfusion.



Condition/Storage time (days)	4	7	12	21	35
50 MPa/RT	Ab	aB	#	#	#
50 MPa/10 °C	aAε	abAε	bB	#	#
25 MPa/RT	aB	aB	#	#	#
25 MPa/10 °C	aAε	aAε	aAB	bB	#
25 MPa/-5 °C	aAε	bB	-	-	-
0.1 MPa/RT	aAε	aAε	bAB	#	#
0.1 MPa/10 °C	aABε	bB	#	#	#
0.1 MPa/RF	aAε	aAε	aAε	aAε	bA

Figure 14 - Haemolysis percentage evolution in blood with additive solution during 35 days of storage at: 50 MPa/RT, 50 MPa/10 °C, 25 MPa/RT, 25 MPa/10 °C, 25 MPa/-5 °C, 0.1 MPa/RT, 0.1 MPa/10 °C and 0.1 MPa/RF. The Figure b) in an amplified section of Figure a) where is indicated the maximum percentage of haemolysis allowed for blood transfusion with a red dashed line denominated "threshold". In the table, different upper/lower case letters (A-B)/(a-b) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. The Greek letter ε indicates values that are statistically similar ($p>0.05$) to the initial value. The symbol # indicate the haemolysis values that were not statistically analysed. The assay at 25 MPa/-5 °C had only a duration of 7 days.

The samples at 50 MPa/RT, 25 MPa/RT and 0.1 MPa/10 °C were above the limit by the 12th day. The sample kept at 50 MPa/RT showed a higher haemolysis percentage possibly due to the impact of pressure in the RBC's promoting cell lysis. At 25 MPa/RT and at 0.1 MPa/10 °C, the high haemolysis can be justified by the increasing of microbial loads observed to these conditions. At this point for both samples, the microbial loads for TAM and ENT were above 6.48 log CFU/mL each. The growth of these microorganisms in addition to the consumption of the nutrients necessary to keep the RBC's intact also had a negative impact in the cells promoting haemolysis (Ribault et al. 2005). At 25 and 50 MPa (at 10 °C) lower haemolysis was observed, as temperature possibly had a protective effect in the RBC's and slowed down the metabolism of the cells. For the samples at 25 MPa/10 °C the microbial load was much lower than at 25 MPa/RT which also had a positive impact in the haemolysis. When the microbial load increase by the 21st day the haemolysis percentage also increase reaching the limit allowed for transfusion. It would be interesting to make the same assay with blood collected in aseptic conditions (with the minimum microbial load possible) to understand, what is the real effect of the microbial development on RBC's and if without the negative impact of microorganisms in the RBC's the haemolysis by the 35th days would be below the limit and able to be used for transfusion. The samples kept at 0.1 MPa/RF (the standard condition at which the blood is stored) presented the best results of all storage conditions reaching the 35 days of storage with the haemolysis percentage below the limit. Moreover, it is noteworthy that by the 12th day no significant differences ($p>0.05$) between 25 MPa/10 °C and 0.1 MPa/RF samples were verified.

For the samples kept at 25 MPa/-5 °C it was expected better results regarding the haemolysis percentage. For this assay, it was necessary to lower the vessel temperature to 5 °C, then the samples were placed in the vessel and it was pressurized to 25 MPa. The temperature reached approximately 11 °C due to the adiabatic heating and then decreased to 5 °C in a couple of minutes. The temperature was then lowered to -5 °C. With the decrease in the temperature the pressure also decreased reaching 16 MPa and the vessel had to be recompressed to 25 MPa. As mentioned in the introduction it is possible to keep products at negative temperatures without freezing when a certain pressure level is applied since pressure shift the freezing point. In each day at which the samples were removed from the vessel, reverse procedure had to be made to preventing the samples from freezing. The temperature was changed to 5 °C and because of it the pressure reached the 34 MPa. At this point the samples were removed from the vessel. These

oscillations of pressures and temperatures during this procedure might be the cause for the haemolysis percentage by the 7th day, since the samples was subjected to two cycles of pressurization and depressurization as explained above. Another possible explanation might be the formation of very small ice crystals (it was not seen any type of freezing indicators at naked eye) since the shift in the freezing point of blood was never studied (the existent values are relative to pure water). Further studies with temperatures higher than -5 °C (but still below 0 °C) and in the 25 MPa range in order to find the best combination pressure-temperature to preserve blood. This is even more interesting of further studies when the pH evolution is analysed, which will be discussed in the next section.

2.3. pH

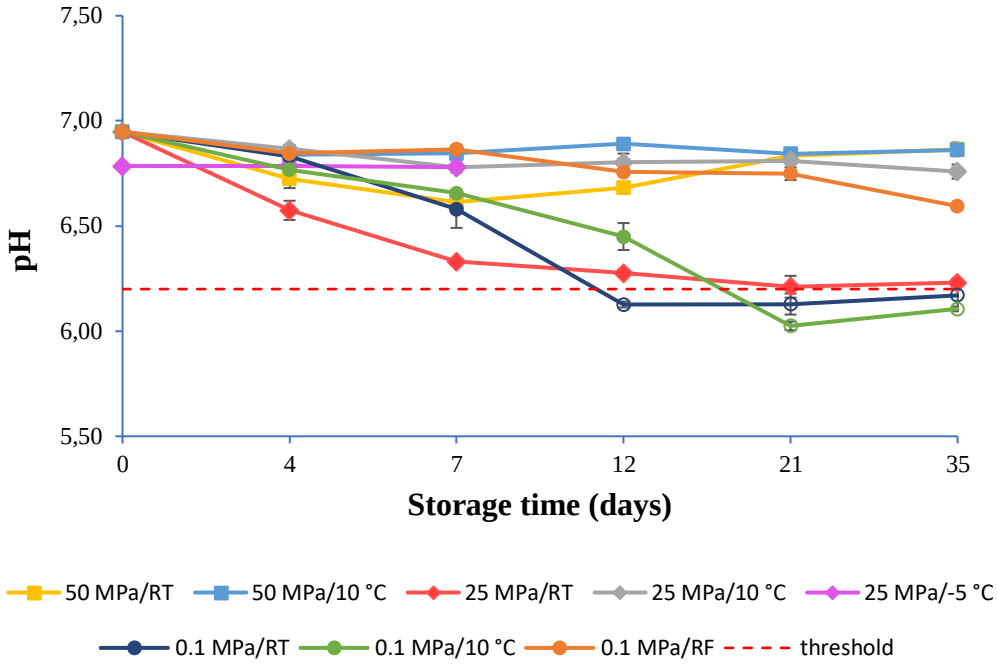
The initial pH for all the blood with additive solution was lower than the pH for the blood with EDTA and this is due to the additive solution added (Latham et al. 1982). The samples collected had an initial pH of 6.95 ± 0.02 (6.97 ± 0.02 for the assay at 50 MPa/RT, 25 MPa/RT and 0.1 MPa/10 °C and 6.78 ± 0.01 for the assay at 25 MPa/-5 °C).

In a global perspective, the behaviour of the pH was similar to the behaviour of pH for the blood with EDTA in all conditions. The pH evolution for all conditions and all the days of analysis is presented in **Figure 15**. All pH values obtained for all conditions and days are presents in **Table I1 – Appendix I**.

The samples kept at higher pressures (50 MPa/RT and 50 MPa/10 °C) had a smaller decrease in the pH during the 35 days, showing no significant differences ($p>0.05$) with the initial day of storage and this might be justified by the haemolysis percentage, as it was explained for the pH variation of the blood with EDTA at the same conditions.

The blood stored at 25 MPa/RT showed a different evolution of the pH at the same condition but with anticoagulant instead of the additive solution. With the increase in the haemolysis, and assuming what was indicated as possible reason, to the EDTA solution, it would be expected an increase in the pH, however, no increase in the 35 days of storage was verified. However, there was one critical difference between these two solutions added. The microbial load for samples with CPDA-1 was significantly higher and this might explain this difference, since microorganisms produce metabolites that usually lower the pH has is verified by Lemos et al. (2017). The samples at 0.1 MPa/RT and 0.1 MPa/10 °C also showed a decrease in the pH until the 35th day that can also be justified by what was said above. For these two conditions the pH reached and surpassed the minimum pH value for the blood since at 6.2 the platelets in the blood suffer changes and lose functionality. These values might be higher than 6.2 at 37 °C nevertheless the values will not be far from the limit and from 6.4 below a decrease in the recovery of functionality of platelets is verified (Castillo et al. 2018).

At 25 MPa/10 °C the pH variation is similar to 0.1 MPa/RF having no significant differences ($p>0.05$) until the 21st day. By the 35th day a decrease in the pH is verified for the samples at 0.1 MPa/RF, which is not seen for those at 25 MPa/10 °C. This might be explained by the increase in the haemolysis that counterbalance the effect of the lactic



Condition/Storage time (days)	4	7	12	21	35
50 MPa/RT	abB	aB	aD	bCε	bDε
50 MPa/10 °C	aBCε	aCε	aEε	aCε	aDε
25 MPa/RT	cA	bA	abB	aB	abB
25 MPa/10 °C	aCε	aC	aE	aC	aD
25 MPa/-5 °C	aBCε	aCε	-	-	-
0.1 MPa/RT	cBCε	bB	aA	aAB	aAB
0.1 MPa/10 °C	cBC	cB	bC	aAB	aA
0.1 MPa/RF	bCε	bCε	bDE	bC	aC

Figure 15 - pH evolution in blood with additive solution during 35 days of storage at: 50 MPa/RT, 50 MPa/10 °C, 25 MPa/RT, 25 MPa/10 °C, 25 MPa/-5 °C 0.1 MPa/RT, 0.1 MPa/10 °C and 0.1 MPa/RF. In the figure is indicated the minimum pH allowed for blood transfusion with a red dashed line denominated "threshold". In the table, different upper/lower case letters (A-E)/(a-c) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. The Greek letter ε indicates values that are not statistically different ($p>0.05$) from the initial value. The assay at 25 MPa/-5 °C had only a duration of 7 days.

acid in the pH that does not occur at 0.1 MPa/RF, that, despite having a slowdown effect in the lactic acid production, it is not capable of cease it.

The blood at 25 MPa/-5 °C showed no change in the pH during the 7 days of storage, having the exact same pH value of the initial day, opening the possibility of blood storage without pH variation, which is still a problem in the storage of blood for transfusion. The lower temperature at which the blood was subjected during storage might have decreased the metabolism to a point that no lactic acid was produced, or the amount was so low that no changes in the pH were detected (Donaldson and Lamont, 2013).

CHAPTER IV – CONCLUSIONS

This work aimed the evaluation of HS effect in blood preservation and, to do so, several combinations of pressure and temperature were tested in blood with anticoagulant and with additive solutions.

The solution added to the blood proved to have impact in the blood quality parameters regardless the pressure-temperature combination, since with the anticoagulant the haemolysis evolution along storage was higher than for the blood with additive solution. At the higher level of pressure used (50 MPa), either at RT and 10 °C, independently the solution used a positive effect in the inactivation of microorganisms was verified, nevertheless, the haemolysis was higher and by the first days the values were already superior than the limit allowed for transfusion.

Concerning the effect of HS in the blood with citrate phosphate dextrose adenine-1 (CPDA-1), all the remaining combinations of pressure-temperature were unable to control the microbial growth with exception of 0.1 MPa/RF and 25 MPa/-5 °C (assay with only 7 days of duration). In both cases the microbial growth inhibition was due to mainly the low temperature and not so much because the pressure applied.

For haemolysis, the results were better for the samples with additive solution and it was possible to conclude that with higher pressure, higher the haemolysis percentage evolution throughout the storage. The temperature had also an important impact in the haemolysis showing a lower haemolysis percentage when compared with the samples at the same pressure but with higher temperature (RT).

The pH was also influenced by the pressure and temperature applied during the storage, however the results in the pH might be related with the increased haemolysis. The pH values obtained for the blood at 25 MPa/-5 °C revealed the possibility to storage blood probably without the production of lactic acid during the storage. More pressure-temperature combinations (at sub-zero conditions) and longer storage periods should be tested to verify this possibility in future works.

With the results obtained from this thesis, it is possible to conclude that none of the pressure-temperature combinations tested showed better results in all the analysed parameters than those at 0.1 MPa/RF, the standard method used to storage whole blood for posterior transfusion. However, some combinations showed results that with some changes in the pressure or in the temperature might be capable of preserve blood with better quality parameters that the blood stored at 0.1 MPa/RF. Besides that, for short term storage (<7 days), at higher pressures (50 MPa) the haemolysis is under control and it is possible to reduce the microbial load of blood with the advantage of low energetic costs.

CHAPTER V – FUTURE WORK

Until now, no studies regarding the effect of HS in blood were made so far, as the author of this work is aware. The results obtained in this thesis have an important impact in the pressure and temperature ranges that should be tested for blood storage under pressure.

Further experiments, with lower pressures in combination with lower temperatures (refrigeration and sub-zero) should be performed to understand if it is possible to preserve blood under pressure with better results than those obtained under refrigeration temperatures and even extend the shelf-life of blood.

More analysis should also be performed to verify the quality of the blood, such as lactic acid quantification, cell viability, microscopy analysis and factors activity quantification.

CHAPTER VI – REFERENCES

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APPENDICES – COMPLEMENTARY INFORMATION

THIS SECTION COMPRISES ALL THE COMPLEMENTARY INFORMATION MENTIONED ALONG THE VARIOUS THESIS CHAPTERS

Appendix A - Examples

Figure A1 - Blood bag used for collection of whole blood. Adapted from British Society for Immunology (2018).

Figure A2 - Example of an apheresis device. Adapted from News Medical Life Sciences (2018)

Appendix B – Sub-zero assay

Figure B1 – Frozen pressure vessel top during the assay at 25 MPa/-5 °C.

Appendix C – Culture media preparation

Table C1 - Preparation scheme of the culture media used.

Culture media	Amount of media powder* (g)
PCA	23.5
RBCA	31.7
VRBDA	39.5

(*) Amount of powder for each 1.0 L of distilled water.

Appendix D – Total aerobic mesophiles and *Enterobacteriaceae* loads for blood with EDTA

Table D1 - Total aerobic mesophiles and *Enterobacteriaceae* loads (expressed as value ± standard deviation, in log CFU/mL) evolution in blood with EDTA (anticoagulant) throughout each storage condition.

Storage time (days)	Storage condition	Total aerobic mesophiles (log CFU/mL)	*Enterobacteriaceae* (log CFU/mL)
0	Initial	2.10 ± 0.05	1.00
	Initial (50 MPa/RT)	2.57 ± 0.16	1.00
4	50 MPa/RT	2.00	1.00
	50 MPa/10 °C	2.00	1.00
	25 MPa/RT	2.00	1.00
	25 MPa/10 °C	2.00	1.00
	0.1 MPa/RT	2.00	1.00
	0.1 MPa/10 °C	2.00	1.00
	0.1 MPa/RF	2.00	1.00
7	50 MPa/RT	2.00	1.00
	50 MPa/10 °C	2.00	1.00
	25 MPa/RT	2.00	1.00
	25 MPa/10 °C	1.00	1.00
	0.1 MPa/RT	1.00	1.00
	0.1 MPa/10 °C	2.00	1.00
	0.1 MPa/RF	2.00	1.00
12	50 MPa/RT	2.00	1.00
	50 MPa/10 °C	1.00	1.00
	25 MPa/RT	2.00	1.00
	25 MPa/10 °C	1.00	1.00
	0.1 MPa/RT	1.00	1.00
	0.1 MPa/10 °C	1.00	1.00
	0.1 MPa/RF	2.00	1.00
21	50 MPa/RT	-	-
	50 MPa/10 °C	1.00	1.00
	25 MPa/RT	1.00	1.00
	25 MPa/10 °C	1.00	1.00
	0.1 MPa/RT	1.00	1.00
	0.1 MPa/10 °C	1.00	1.00
	0.1 MPa/RF	2.00	1.00
35	50 MPa/RT	1.00	1.00
	50 MPa/10 °C	1.00	1.00
	25 MPa/RT	1.00	1.00
	25 MPa/10 °C	1.00	1.00
	0.1 MPa/RT	1.00	1.00
	0.1 MPa/10 °C	1.00	1.00
	0.1 MPa/RF	1.00	1.00

Appendix E – Haemolysis values for blood with EDTA

Table E1 - Haemolysis evolution (expressed as value ± standard deviation, in percentage) in blood with EDTA (anticoagulant) throughout each storage condition.

Storage time (days)	Storage condition	Haemolysis (%)
0	Initial	0.41 ± 0.03
	Initial (50 MPa/RT)	0.44 ± 0.03
4	50 MPa/RT	4.46 ± 0.40
	50 MPa/10 °C	3.02 ± 0.23
	25 MPa/RT	0.79 ± 0.06
	25 MPa/10 °C	0.81 ± 0.12
	0.1 MPa/RT	1.24 ± 0.01
	0.1 MPa/10 °C	0.88 ± 0.02
	0.1 MPa/RF	0.65 ±0.04
7	50 MPa/RT	5.62 ± 0.58
	50 MPa/10 °C	6.30 ± 0.28
	25 MPa/RT	1.22 ± 0.04
	25 MPa/10 °C	1.22 ± 0.24
	0.1 MPa/RT	1.38 ± 0.12
	0.1 MPa/10 °C	0.93 ± 0.18
	0.1 MPa/RF	1.08 ± 0.07
12	50 MPa/RT	13.49 ± 1.98
	50 MPa/10 °C	6.02 ± 0.39
	25 MPa/RT	2.13 ± 0.19
	25 MPa/10 °C	2.55 ± 0.10
	0.1 MPa/RT	2.48 ± 0.06
	0.1 MPa/10 °C	1.38 ± 0.15
	0.1 MPa/RF	1.81 ± 0.14
21	50 MPa/RT	-
	50 MPa/10 °C	22.72 ± 1.97
	25 MPa/RT	41.12 ± 0.52
	25 MPa/10 °C	4.15 ± 0.58
	0.1 MPa/RT	43.31 ± 1.36
	0.1 MPa/10 °C	2.07 ± 0.20
	0.1 MPa/RF	3.41 ± 0.25
35	50 MPa/RT	47.98 ± 2.12
	50 MPa/10 °C	58.36 ± 0.63
	25 MPa/RT	66.14 ± 2.86
	25 MPa/10 °C	33.96 ± 4.22
	0.1 MPa/RT	68.31 ± 1.38
	0.1 MPa/10 °C	8.30 ± 0.45
	0.1 MPa/RF	3.57 ± 0.80

Appendix F – pH values for blood with EDTA

Table F1 - pH evolution (expressed as value ± standard deviation) in blood with EDTA (anticoagulant) throughout each storage condition.

Storage time (days)	Storage condition	pH
0	Initial	7.23 ± 0.02
	Initial (50 MPa/RT)	7.23 ± 0.03
4	50 MPa/RT	7.14 ± 0.02
	50 MPa/10 °C	7.17 ± 0.02
	25 MPa/RT	6.90 ± 0.01
	25 MPa/10 °C	7.07 ± 0.02
	0.1 MPa/RT	7.05 ± 0.01
	0.1 MPa/10 °C	6.94 ± 0.01
	0.1 MPa/RF	7.07 ± 0.01
7	50 MPa/RT	7.15 ± 0.02
	50 MPa/10 °C	7.10 ± 0.02
	25 MPa/RT	6.92 ± 0.01
	25 MPa/10 °C	7.07 ± 0.02
	0.1 MPa/RT	6.85 ± 0.01
	0.1 MPa/10 °C	6.89 ± 0.01
	0.1 MPa/RF	7.08 ± 0.01
12	50 MPa/RT	7.07 ± 0.04
	50 MPa/10 °C	7.04 ± 0.02
	25 MPa/RT	6.85 ± 0.01
	25 MPa/10 °C	7.06 ± 0.01
	0.1 MPa/RT	6.87 ± 0.02
	0.1 MPa/10 °C	6.78 ± 0.01
	0.1 MPa/RF	7.07 ± 0.03
21	50 MPa/RT	-
	50 MPa/10 °C	7.05 ± 0.01
	25 MPa/RT	6.94 ± 0.01
	25 MPa/10 °C	7.00 ± 0.02
	0.1 MPa/RT	6.95 ± 0.02
	0.1 MPa/10 °C	6.83 ± 0.02
	0.1 MPa/RF	7.01 ± 0.01
35	50 MPa/RT	7.06 ± 0.01
	50 MPa/10 °C	7.05 ± 0.02
	25 MPa/RT	7.01 ± 0.01
	25 MPa/10 °C	6.98 ± 0.01
	0.1 MPa/RT	6.96 ± 0.02
	0.1 MPa/10 °C	6.85 ± 0.01
	0.1 MPa/RF	6.95 ± 0.03

Appendix G – Total aerobic mesophiles and *Enterobacteriaceae* loads for blood with CPDA-1

Table G1 - Total aerobic mesophiles and Enterobacteriaceae loads (expressed as value ± standard deviation, in log CFU/mL) evolution in blood with CPDA-1 (additive solution) throughout each storage condition.

Storage time (days)	Storage condition	Total aerobic mesophiles (log CFU/mL)	*Enterobacteriaceae* (log CFU/mL)
0	Initial	2.46 ± 0.04	1.00
	Initial (50 MPa/RT, 25MPa/RT 0.1MPa/10 °C)	3.09 ± 0.06	1.00
	Initial (25 MPa/-5 °C)	2.35 ± 0.10	1.00
4	50 MPa/RT	2.00	1.00
	50 MPa/10 °C	2.31 ± 0.15	1.00
	25 MPa/RT	6.48	2.39 ± 0.23
	25 MPa/10 °C	2.00	1.00
	25 MPa/-5 °C	2.24 ± 0.01	1.00
	0.1 MPa/RT	4.12 ± 0.00	1.00
	0.1 MPa/10 °C	6.34 ± 0.12	3.70 ± 0.34
	0.1 MPa/RF	2.00	1.00
7	50 MPa/RT	2.00	1.00
	50 MPa/10 °C	2.25 ± 0.03	1.00
	25 MPa/RT	6.48	5.20 ± 0.19
	25 MPa/10 °C	2.62 ± 0.10	1.00
	25 MPa/-5 °C	2.00	1.00
	0.1 MPa/RT	5.53 ± 0.50	1.00
	0.1 MPa/10 °C	6.48	6.35 ± 0.22
	0.1 MPa/RF	2.59 ± 0.10	1.00
12	50 MPa/RT	2.00	1.00
	50 MPa/10 °C	2.00	1.00
	25 MPa/RT	6.48	6.48
	25 MPa/10 °C	2.62 ± 0.25	1.00
	0.1 MPa/RT	6.48	1.00
	0.1 MPa/10 °C	6.48	6.48
	0.1 MPa/RF	2.27 ± 0.05	1.00
21	50 MPa/RT	2.36 ± 0.42	1.00
	50 MPa/10 °C	2.00	1.00
	25 MPa/RT	6.48	6.48
	25 MPa/10 °C	5.18 ± 0.43	1.00
	0.1 MPa/RT	6.48	1.00
	0.1 MPa/10 °C	6.48	6.48
	0.1 MPa/RF	2.31 ± 0.01	1.00
35	50 MPa/RT	2.00	1.00
	50 MPa/10 °C	1.00	1.00
	25 MPa/RT	6.48	6.48

	25 MPa/10 °C	5.40 ± 0.43	1.00
	0.1 MPa/RT	6.48	1.00
	0.1 MPa/10 °C	6.48	6.48
	0.1 MPa/RF	2.63 ± 0.55	1.00

Appendix H – Haemolysis values for blood with CPDA-1

Table H1 - Haemolysis evolution (expressed as value ± standard deviation, in percentage) in blood with CPDA-1 (additive solution) throughout each storage condition.

Storage time (days)	Storage condition	Haemolysis (%)
0	Initial	0.16 ± 0.05
	Initial (50 MPa/RT, 25MPa/RT, 0.1MPa/10 °C)	0.19 ± 0.03
	Initial (25 MPa/-5 °C)	0.21 ± 0.04
4	50 MPa/RT	0.61 ± 0.07
	50 MPa/10 °C	0.28 ± 0.07
	25 MPa/RT	0.55 ± 0.11
	25 MPa/10 °C	0.30 ± 0.02
	25 MPa/-5 °C	0.39 ± 0.07
	0.1 MPa/RT	0.26 ± 0.01
	0.1 MPa/10 °C	0.43 ± 0.06
	0.1 MPa/RF	0.25 ± 0.02
7	50 MPa/RT	0.79 ± 0.01
	50 MPa/10 °C	0.41 ± 0.12
	25 MPa/RT	0.74 ± 0.17
	25 MPa/10 °C	0.30 ± 0.06
	25 MPa/-5 °C	0.82 ± 0.03
	0.1 MPa/RT	0.24 ± 0.02
	0.1 MPa/10 °C	0.70 ± 0.01
	0.1 MPa/RF	0.29 ± 0.06
12	50 MPa/RT	1.34 ± 0.21
	50 MPa/10 °C	0.61 ± 0.10
	25 MPa/RT	1.29 ± 0.02
	25 MPa/10 °C	0.48 ± 0.08
	0.1 MPa/RT	0.54 ± 0.03
	0.1 MPa/10 °C	1.25 ± 0.24
	0.1 MPa/RF	0.32 ± 0.05
21	50 MPa/RT	19.87 ± 2.01
	50 MPa/10 °C	1.59 ± 0.08
	25 MPa/RT	6.96 ± 0.87
	25 MPa/10 °C	0.86 ± 0.09
	0.1 MPa/RT	2.60 ± 0.62
	0.1 MPa/10 °C	14.93 ± 4.50
	0.1 MPa/RF	0.32 ± 0.08
35	50 MPa/RT	47.06 ± 9.39
	50 MPa/10 °C	10.69 ± 1.80
	25 MPa/RT	68.06 ± 1.61
	25 MPa/10 °C	3.35 ± 0.13
	0.1 MPa/RT	20.59 ± 5.87
	0.1 MPa/10 °C	71.27 ± 8.84
	0.1 MPa/RF	0.53 ± 0.14

Appendix I – pH values for blood with CPDA-1

Table I1 - pH evolution (expressed as value ± standard deviation) in blood with CPDA-1 (additive solution) throughout each storage condition.

Storage time (days)	Storage condition	pH
0	Initial	6.95 ± 0.02
	Initial (50 MPa/RT, 25MPa/RT, 0.1MPa/10 °C)	6.97 ± 0.02
	Initial (25 MPa/-5 °C)	6.78 ± 0.01
4	50 MPa/RT	6.72 ± 0.04
	50 MPa/10 °C	6.84 ± 0.02
	25 MPa/RT	6.57 ± 0.05
	25 MPa/10 °C	6.87 ± 0.02
	25 MPa/-5 °C	6.79 ± 0.01
	0.1 MPa/RT	6.83 ± 0.02
	0.1 MPa/10 °C	6.77 ± 0.03
	0.1 MPa/RF	6.85 ± 0.02
7	50 MPa/RT	6.61 ± 0.02
	50 MPa/10 °C	6.85 ± 0.01
	25 MPa/RT	6.33 ± 0.01
	25 MPa/10 °C	6.78 ± 0.02
	25 MPa/-5 °C	6.78 ± 0.01
	0.1 MPa/RT	6.58 ± 0.09
	0.1 MPa/10 °C	6.66 ± 0.01
	0.1 MPa/RF	6.87 ± 0.02
12	50 MPa/RT	6.68 ± 0.03
	50 MPa/10 °C	6.89 ± 0.01
	25 MPa/RT	6.28 ± 0.02
	25 MPa/10 °C	6.80 ± 0.04
	0.1 MPa/RT	6.13 ± 0.01
	0.1 MPa/10 °C	6.45 ± 0.06
	0.1 MPa/RF	6.76 ± 0.01
21	50 MPa/RT	6.83 ±0.02
	50 MPa/10 °C	6.84 ± 0.02
	25 MPa/RT	6.21 ± 0.05
	25 MPa/10 °C	6.81 ± 0.01
	0.1 MPa/RT	6.13 ± 0.05
	0.1 MPa/10 °C	6.03 ± 0.02
	0.1 MPa/RF	6.75 ± 0.03
35	50 MPa/RT	6.86 ± 0.03
	50 MPa/10 °C	6.86 ± 0.03
	25 MPa/RT	6.23 ± 0.02
	25 MPa/10 °C	6.76 ± 0.03
	0.1 MPa/RT	6.17 ± 0.07
	0.1 MPa/10 °C	6.11 ± 0.01
	0.1 MPa/RF	6.59 ± 0.01