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ESCOLA SUPERIOR DE BIOTECNOLOGIA
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Terminal Sterilization Using Supercritical Carbon Dioxide ($_{SC}CO_2$): Influence of Several Processing Parameters

by

Fábio André Teixeira Pinheiro

Porto, 2018

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Mestrado em Engenharia Biomédica

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Resumo

Dada a expansão do mercado de biomateriais e sofisticação dos dispositivos médicos, os processos de esterilização terão necessariamente de ser reformulados e melhorados para responderem eficazmente. Algumas das técnicas de esterilização utilizadas atualmente não conseguem executar a sua função de uma forma eficaz. Em muitos casos o material é danificado durante o processo ou a esterilização não é realizada eficientemente. Assim sendo, a esterilização por dióxido de carbono em estado supercrítico ($_{SC}CO_2$) responde eficazmente ao esterilizar diversos materiais biológicos e polímeros sensíveis, que antes não eram esterilizados por outras técnicas. A par desta vertente, o custo de operação, a sua segurança e rapidez processual são características positivas na esterilização por $_{SC}CO_2$.

O trabalho realizado no âmbito da presente tese teve como objetivo estudar e analisar as variáveis de esterilização em tiras de esporos bacterianos de três estirpes diferentes: *Bacillus atrophaeus, Bacillus pumilus* e *Bacillus stearothermophilus*. Com este estudo, as variáveis como o tempo de esterilização efetiva, pressão e "Shelf life" foram aprimorados, aumentando assim a eficiência do processo. Em paralelo a este estudo, foram também estudadas amostras de placenta humana antes e depois do processo de esterilização de forma a verificar se a esterilização por $_{SC}CO_2$ poderá danificar ou modificar as propriedades físicas ou químicas deste material biológico.

Conclui-se que para esterilizar eficazmente as tiras de esporos bacterianos é recomendável um tempo de esterilização efetiva no mínimo de 3 horas com um *shelf life* mínimo de 1 semana. De forma a aumentar a viabilidade do processo, demonstrou-se que é possível a utilização dos materiais esterilizados logo após o processo sem reativação dos esporos, quando estes estão em contacto com o $_{SC}CO_2$ no mínimo 3 horas. No reator os valores recomendados são: pressão a 140 bar, 600 rpm, 40ºC e com a adição de 300 ppm H_2O_2. As análises de *High-performance liquid chromatography* (HPLC) e *Fourier-transform infrared spectroscopy* (FTIR) demonstraram que as amostras de placenta após esterilização não sofrem alterações químicas significativas. Contrariamente, os testes mecânicos e análises de *Differential scanning calorimetry* (DSC) revelaram mudanças físicas e químicas significativas nas amostras. A esterilização por $_{SC}CO_2$ conferiu um aumento de rigidez nas amostras biológicas estudadas, assim como um aumento na sua resistência térmica. O estudo da eficácia desta técnica de esterilização ($_{SC}CO_2$) em amostras biológicas deve ser continuado para que se

possa reunir mais informação que permita validar a sua aplicabilidade na área dos materiais médicos e biomateriais.

Palavras-chave: Esterilização, CO_2 supercrítico, Esporos bacterianos, placenta humana descelularizada.

Abstract

Due the expanding market for biomaterials and the sophistication of medical devices, sterilization processes need to be refined and reinvented to respond effectively to this demand. Nowadays the current sterilization techniques, cannot perform their function effectively without damaging the material during the process. Thus, supercritical carbon dioxide ($_{SC}CO_2$) sterilization can be an alternative solution for sterilizing various biological materials and complex/sensitive polymers. The great advantages of $_{SC}CO_2$ sterilization englobes the price of operation, its safety and procedural speed among others.

The main goal of this thesis project was to study and analyse different variables, like effective sterilization time or pressure in the effective sterilization process of three different bacterial spore strains: *Bacillus atrophaeus*, *Bacillus pumilus* and *Bacillus stearothermophilus*. In this study, the effect of the time interval between $_{SC}CO_2$ sterilization and the microbiological validation was also evaluated, despite of being a parameter that is not taken into account in the works described in literature to date. Parallel to this work, human placenta samples were analysed before and after the sterilization process to verify whether $_{SC}CO_2$ sterilization could damage or modify the physical or chemical properties of this biological material.

Regarding the results obtained of the sterilization of the bacterial spore strips, the mildest conditions optimized were: 140 bar, 600 rpm, 40ºC, 300 ppm H_2O_2 and 4 hours of effective sterilization. Interestingly, the effectiveness of terminal sterilization of these biological indicators was dependent on the time period after $scCO_2$ treatment. In addition, the minimum "shelf life" required is at least 1 week.

In the second part of the work, the placenta samples sterilized by $scCO_2$ did not undergo significant chemical changes as demonstrated by high-performance liquid chromatography (HPLC) and Fourier-transform infrared spectroscopy (FTIR) analyses that. In contrast, the mechanical tests and analyses of Differential scanning calorimetry (DSC) demonstrated significant physical and chemical changes in the samples. $_{SC}CO_2$ sterilization conferred rigidity to the biological sample and a different behaviour of the samples were detected when exposed to a constant increase of temperatures.

The study of the innovative sterilization technique ($_{SC}CO_2$) should be continued to increase its applicability in the area of medical materials and biomaterials and their efficiency.

Key words: Sterilization, supercritical CO_2, Bacterial spores, decellularized human placenta.

Contents

List of Figures

List of Tables

List of Abreviations

$_{SC}CO_2$	Supercritical carbon dioxide
60Co	Cobalt-60
Ar	Argon
AU	Maximum absorbance
BI	Biological indicators
BPR	Back-pressure regulator
$C_2H_4O_2$	Acetic acid
CFC-12	Dichlorodifluoromethane
CF_3COOH	Trifluoroacetic acid
CH_3CN	Acetonitrile
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate ion
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
ECM	Extracellular matrix
EtO	Ethylene oxide
FSR	Force-sensing resistor
FTIR	Fourier-transform infrared spectroscopy
H+	Hydron
H_2CO_3	Carbonic acid
H_2O	Water
H_2O_2	Hidrogen peroxide
HCO_3^-	Bicarbonate ion
HPLC	High-performance liquid chromatography
ISO	International Organization for Standardization
K	Kelvin
Kpa	Kilopascal
L	Liters
M	Molar
mg	Milligrams
min	Minute
ml	Milliliters

mm	Millimeters
MPa	Millipascal
MΩ	Megaohm
N_2	Nitrogen
N_2O	Nitrous oxide
nm	Nanometers
PAA	Paracetic Acid
PC	Computer
PDA	Photodiode array
pH	Potential of hydrogen
RNA	Ribonucleic acid
rpm	Rotations per minute
SAL	Sterilization Assurance Level
SFC	Supercritical Fluid Chromatography
SFR	Supercritical Fluid Reaction
TSB	Trypto-casein soy broth
wt %	Weight percent

1. Introduction

1.1 Sterilization History in Medicine

The enormous evolution and innovation of surgical techniques and medical devices has lead to the development of more and more complex and sophisticated sterilization procedures and machinery. In fact, despite the advances in medical surgery, the lack of knowledge in sterilization and asepsis procedures, has made of medical rooms and hospital materials lethal weapons of contamination with infectious diseases during surgical interventions.

During the nineteenth century, the first attempts to effectively sterilize operating rooms and surgical materials were taken by scientists and doctors dealing with high rates of postpartum mortality and infectious diseases. The father of modern microbiology, Louis Pasteur proved that microorganisms, such as bacteria and parasites, were a principal cause of disease in humans (Dubos, 1951). Besides that, he also demonstrated the high potential of bacterial propagation. These microorganisms could live in dead tissues and spread through the air as spores. The British surgeon Joseph Lister pioneered modern antisepsis by introducing the washing of exposed wounds with liquid solutions and aerosols with carbolic acid (phenol)(Lister, 1867). This invention, considered one of the great evolutions in medicine, proved to be quite efficient, since there was a reduction in deaths from postoperative infections. Besides, Lister suggested the use of phenol for cleaning medical instruments, surgical sites, among others, since this agent acts by destroying and preventing the growth of microorganisms in living tissue. Prior to his studies, the Hungarian Ignaz Semmelweis demonstrated the importance of the simple act of hand washing of doctors and nurses before contact with the patient (Semmelweis, 1861). The correct hand washing, by a chlorinated solution, could prevent infections and deaths in childbirths and operations (Mcdonnell et al., 1999). These procedures attempted to create a germ-free environment in the operation room and became a frequent practice before medical observations, after child-births and autopsies.

Furthermore, Earle Spaulding sought to find a rational approach to disinfection and sterilization of medical devices and surgical tools. Accordingly, “Spaulding classification”, presented to the medical community in 1939, the materials were divided into three distinct categories (Spaulding, 1939). Briefly, the noncritical materials where those contacting only with the outside of the skin, the semicritical, contacting with the mucous membranes, and the critical materials where the tools that contacted the inside of the body. Spaulding further

improved his classification with the type of treatment that should be implemented, based in the use of each material/tool. The disinfectant´s strength should be related to the type of decontamination requirements of a medical device or area (*From Ignaz Semmelweis to the present: Crucial problems of hospital hygiene*, n.d).

The first machines of sterilization began to be produced by Charles Chamberland in 1879. These pioneering machines, called autoclaves, were capable of sterilizing surgical materials with the use of water vapor at a temperature of 140 ºC, similar to nowadays' autoclaves. Over the years, these sterilization machines have undergone improvements and began to be implemented in hospitals with the purpose of eliminating all forms of live and other biological agents in the materials (Harvey, 2011).

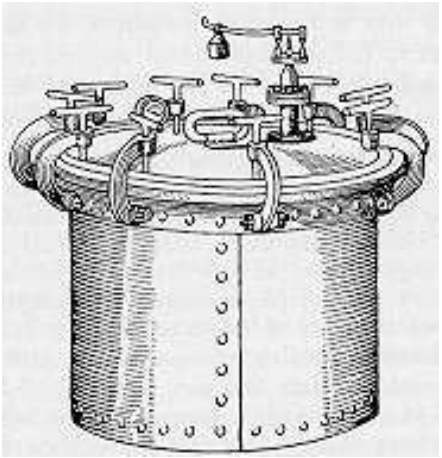


Figure 1.1–Pressure steam sterilizer built in 1884 by Charles Chamberland (Skellie, 2010).

The sterilization process has been following the advances of medicine and new methods using radiation and chemicals have been implemented. Therefore, the concept of sterilization has evolved, being defined by the destruction of all forms of life by heat, irradiation, gas or chemical treatment. Considering the several techniques and innovations in sterilization equipment, this field should contribute to a higher safety with obvious consequences in the quality care to the patients.

1.2 Main Sterilization Techniques in Medicine

There are several ways to perform sterilization efficiently. Figure1.2 lists the various sterilization techniques performed in various industrial sectors (National Institute of Open Schooling (NIOS), 1998).

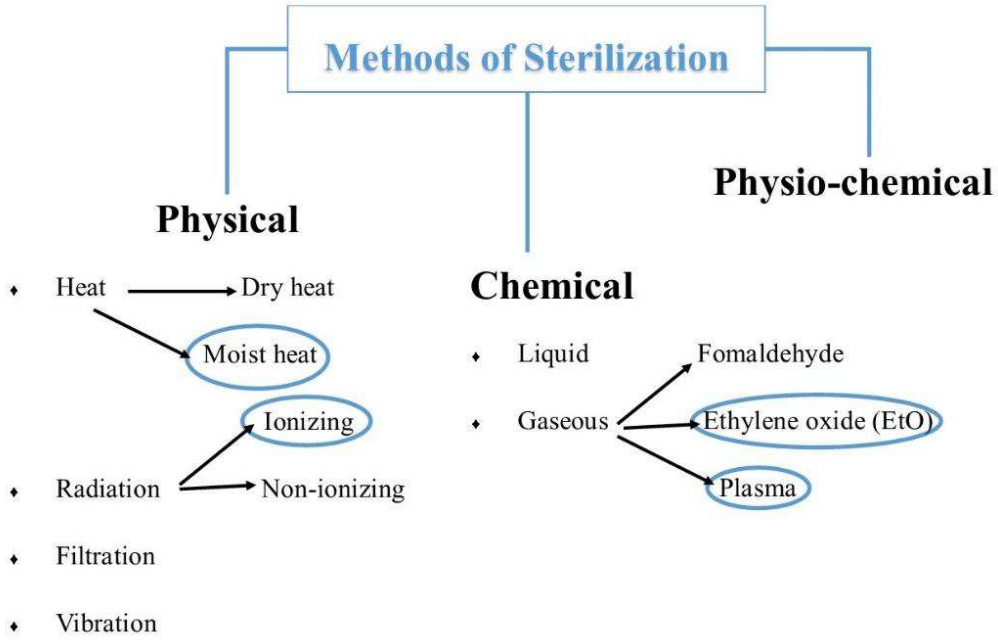


Figure 1.2– Scheme representing the main methods used for sterilization nowadays. Adapted from (National Institute of Open Schooling (NIOS), 1998)

The techniques most commonly found in hospital facilities, which are more suitable for medical devices, are physical sterilization using heat and ionizing radiation and chemical sterilization using gas plasma and ethylene oxide (EtO) (Hurrell, 1998). Autoclave is a physical method which uses moist heat, and the most used in any hospital for many years. Other methods are rarely adopted, due to the lack of studies demonstrating its efficiency or to the low benefits of implementing a new technique in a hospital.

Along with the evolution of medicine and material's industry, medical materials and devices have diversified and undergone changes in their composition. The need to keep the products treated implied the creation of appropriate methods for each type of product. On the other hand, the sterilization procedures for these devices has evolved and presently it must be carried out in accordance with an established Sterilization Assurance Level (SAL) which has

been standardized under ISO 14937-2009, by the directive EN 556-1. SAL is used to determine the effectiveness of a sterilization process, and expresses the likelihood that a specific product, from a batch of products submitted to this process, has not been sterilized. The value accepted for medical applications corresponds to SAL6, meaning the likelihood of 1 product being contaminated within a 1,000,000 batch of sterilized products (ISO, 2014).

Autoclave

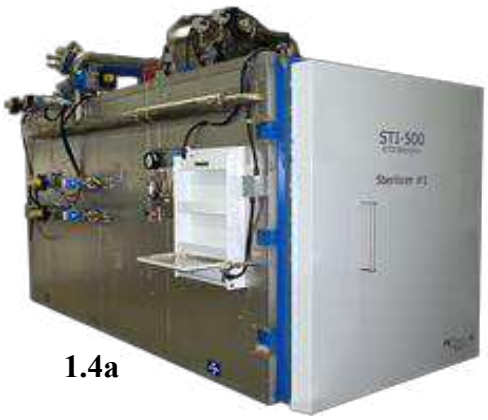
As mentioned before, autoclave is widely used to sterilize medical devices. Created in the nineteenth century, the autoclave machine provides a physical method for disinfection and sterilization (Sultana, 2007). It uses a combination of heat with humidity and pressure during a stipulated time (Figure 1.3). Basically, the high-temperature steam is forced in under high pressure, thereby displacing air. Inside of the autoclave´s chamber, moist heat destroys all microorganisms by denaturation of enzymes and/or structural proteins (Ravikrishna, 2013). The two variables, time and temperature, may vary according to the type of microorganisms to be inactivated, packaging material and the material nature. There are two basic types of steam sterilization, which are gravity displacement autoclave and high-speed pre-vacuum sterilizer. In the first type, steam displaces air in the chamber by gravity without mechanical procedures. The second, the air is mechanically removed and load through a series of vacuum and pressure pulses. By this procedure, the steam penetrates the porous areas of the material which couldn't otherwise be reached with other types of steam sterilization. Heat sterilization processes are indicated to heat resistant materials, like metallic surgical instruments and stainless-steel sutures ("The Chamber Blog," 2016).

Figure 1.3 –Recent model of an autoclave machine (STE Class B, IcanClave).

This sterilization procedure presents a low-cost machinery, a fast sterilization cycle, it is easy to control and non-toxic to the staff and the environment. However, the autoclaving process can damage materials which are sensitive to high temperatures. On the other hand, when considering metallic based devices and instruments, there is the possibility of corrosion due to the humid environment inside of the chamber (Tankeshwar, 2013).

Ethylene Oxide (EtO)

Sterilization can also be performed using chemical substances like EtO commonly used for sterilization of medical devices (Figure 1.4a, b).



1.4a

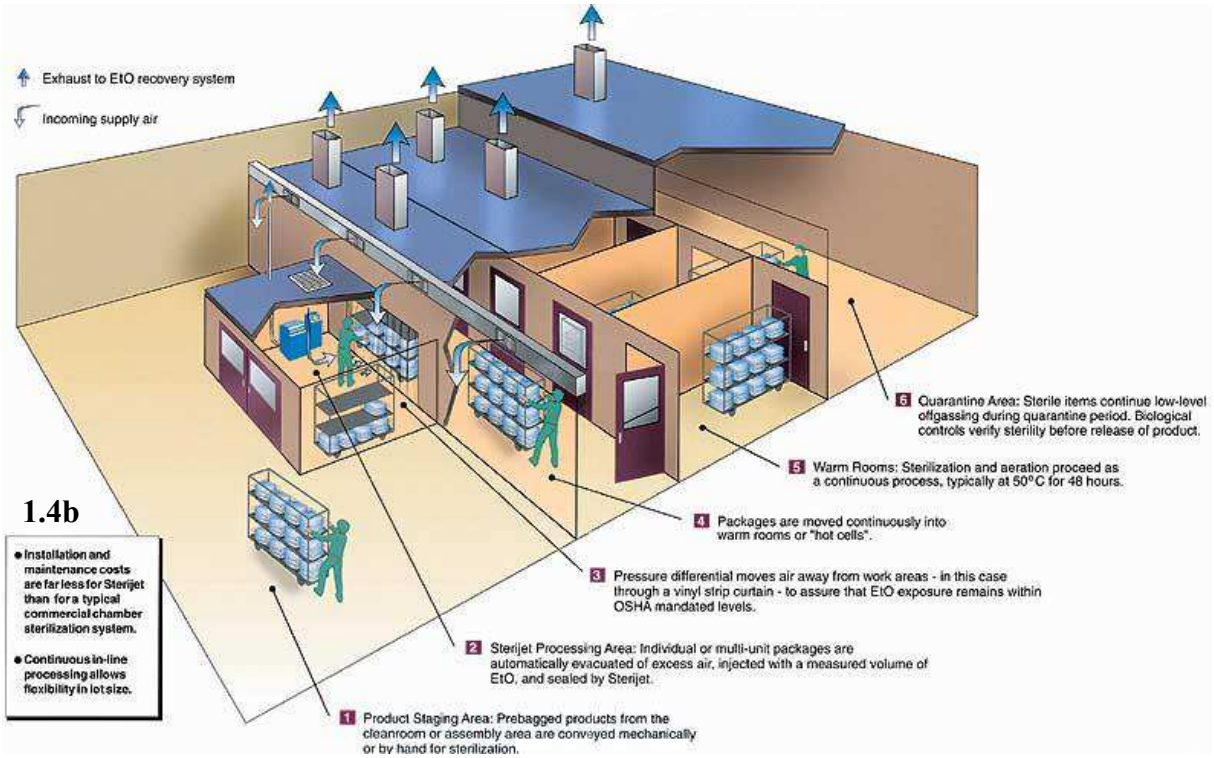


Figure 1.4a –Example of a recent model of EtO sterilizer machine (STI-500 Sterilizer, Treated Technologies).
1.4b – Sterilization facility floor layout (Sterijet, North Carolina, USA).

EtO was developed in 1859 but only implemented in the industry and medicine in 1900s (Wurtz, 1859). This method can work at low temperatures and so, it can be applied to a vast variety of compatible materials, as compared to autoclave. The EtO sterilization cycle is divided in three stages: preconditioning, sterilization and aeration. This cycle begins with the preparation of the chamber environment to meet the ideal sterilization conditions for temperature, pressure and humidity. Then the EtO enters in the chamber via evaporation and sterilizes the material. When the sterilization process is finished, the material is exposed to an excessive aeration stage to remove any remaining EtO gas and to allow absorbed gas to

evaporate again from the sterilized items (according to Figure 1.4b, performed, typically at 50 ºC for 2 hours, in room 5 “warm rooms”). If the material does not pass through this stage, materials, like plastics and rubbers, absorbed the gas and when applied to patients, the toxic gas could damage their body tissue (Henk & Finkiel, 2013). It is an alternative to medical materials that cannot support conventional steam sterilization with high temperatures. Yet, it is necessary to ensure the item to be sterilized is compatible with the sterilant. EtO gas is considered toxic, carcinogenic, flammable and explosive. Hence, pure EtO should be handled in explosion proof equipment. In addition, it is common to use EtO mixed with other components, like Chlorofluorocarbon (CFC-12) to become non-flammable sterilant. Efficiency depends heavily on the concentration of the gas, time of exposure, temperature, humidity and the nature of the material. EtO sterilization is applied to a wide range of medical material, like heart valves and stents coated with bioactive compounds (Govindaraj & Muthuraman, 2015)(Rutala & Weber, 1999).

The evident weakness of this technology is the time it takes for the sterilized material to become operational. After the sterilization process, the sterilized material is required to be placed in aeration for a minimum of 6 hours. In its entirety, the cycle has a minimum duration of 12 hours. Also, it requires special room conditions, safety equipment and separate ventilation system. EtO entails safety concerns due to its carcinogenic and flammable characteristics. Another obvious disadvantage, but less likely to occur, is the possibility of the presence of toxic EtO residues in surgical instruments and medical devices after sterilization. Ventilation may not always be sufficient to remove all residues of sterilized materials. This gas is not able to be recovered, thus it is not recyclable. Nevertheless, EtO operates under low temperature, which allows its use in thermo-sensitive materials while it is non-corrosive to plastic, rubber and metal (Henk & Finkiel, 2013).

Ionizing Radiation

E-beam, x-ray and gamma radiation are the three common ionizing radiation types used in sterilization procedures (Silindir & Özer, 2009). The first gamma irradiator was created in 1973 to sterilize medical material. One of the first industrial sterilization facilities was raised to sterilize surgical sutures with cobalt-60 (60Co) (Nahm, 2010). The innovation of this method has never ceased, and currently gamma radiation with 60Co can sterilize a larger range of materials and medical devices than EtO and autoclave. 60Co, which emits gamma rays during

radioactive decay, destroys microorganisms by breaking the covalent bonds of bacterial DNA. Despite that, the process presents a large amount of wasted energy, because rays are emitted in all directions beyond the desired one. Therefore, it must be carried out with safety concerns as ionizing radiation is harmful to human. In the case of 60Co, microorganisms are indirectly inactivated by the reaction of the free radicals produced in the cellular fluid. Radiation is considered one of the most effective methods for sterilization of medical devices and hospital material, despite one of the most expensive current methods (Govindaraj & Muthuraman, 2015). This technology is often found in hospitals, Figure 1.5, and in radiation sterilization plants.

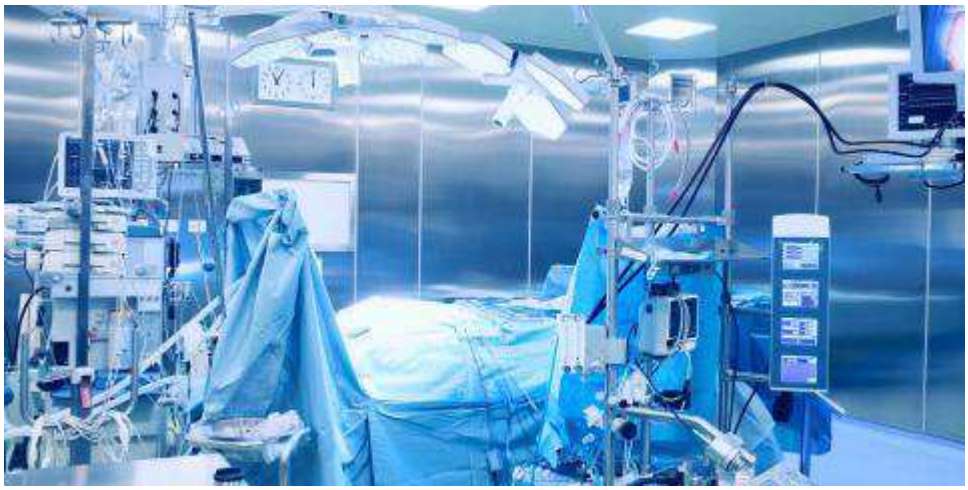


Figure 1.5 –Gamma radiation used during surgical procedures in hospitals to maintain patient and practitioner safety in the healthcare industry (Pacific Integrated Manufacturing, 2018).

Despite the high sterilization efficiency rate of the technology, gamma radiation can change structural characteristics in medical devices that have been sterilized several times. These structural changes are more frequent in tissue allografts and polymer medical devices and negatively affect their clinical use. Moreover, according to studies, gamma radiation generates free hydroxyl radicals and other radiotoxins that induces toxigenic and mutagenic effects which in turn to potentially promotes cancer. Withal, sterilization by radiation demands machinery of high monetary and maintenance cost (Harrell et al., 2018).

Gas Plasma: Hydrogen Peroxide (H_2O_2)

In the late 1980's, the first sterilization system using hydrogen peroxide gas plasma was developed (Centers for Disease Control and Prevention, 2008). Ten years later, the company

Sterrad (ASP) (Irvine, USA) began the commercialization of gas plasma sterilization (Jacobs & Lin, 1996). In Figure 1.6 is represented one of the most recent models of this company.

Figure 1.6 –Hydrogen peroxide sterilizer (Sterrad 100S, Advanced Sterilization Products (ASP)).

The sterilization process begins when a strictly calculated dose of an aqueous solution of H_2O_2 is inserted in the plasma machine vacuum chamber where its evaporation and dispersion occur. Once the pressure inside the chamber drops, the particles become excited enough to ionize and the H_2O_2 turns into plasma. In this stage, the plasma breaks the genetic material of the microorganism into smaller molecules. The interaction between the free radicals generated by plasma and the cellular components such as enzymes, phospholipids, DNA or RNA, preventing normal cellular metabolism, eliminates the possibility of harmful reactions in the materials (Boiano & Steege, 2015) (Thierry Corporation, 2017).

This method presents profitable values in sterilization efficiency rates, fast sterilization cycles and a high compatibility with a lot of different medical devices and biological materials. Like EtO and radiation technologies, this entails a high price of equipment and specialized personnel (Govindaraj & Muthuraman, 2015). Compared with EtO, the H_2O_2 sterilization presents a better cost-efficiency ratio and begins to gain a more solid and attractive position in the market (TSO3, 2016).

1.3 Supercritical Carbon Dioxide ($_{SC}CO_2$) as a Sterilization Method

1.3.1 $_{SC}CO_2$ Technology

Charles Cagniard de La Tour is the man behind the discover of the critical point and, in consequence, supercritical fluids (de la Tour, 1822). The French used Papin´s digester (Figure 1.7) filled with liquid and with a silex ball inside, to study experiments in acoustic. When rotating the equipment, a noise was produced whenever the ball penetrated the liquid-vapor interface. However, when the system reaches a temperature way beyond the boiling point of the liquid, the expected noise of the stone falling into the liquid disappeared. This experiment marks the discover of the supercritical fluid phase (Berche et al ., 2009) (de la Tour, 1822).

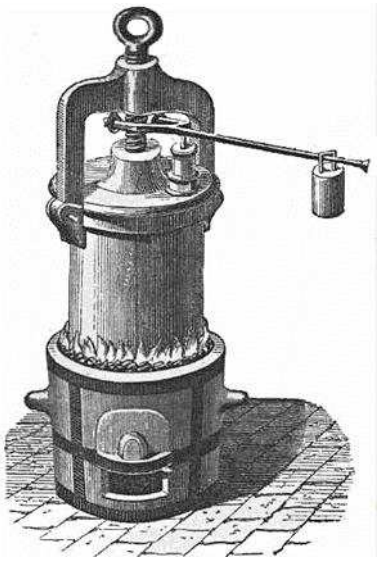


Figure 1.7 –A 1680 version of Papin's steam digester. Adapt from Thurston (1878).

A supercritical fluid consists of any substance whose temperature and pressure values exceed its critical values. The physicochemical properties of these fluids assume intermediate values relative to those presented in liquids and gases. So, these cannot be classified as being liquids, nor gases, and can be described as an intermediate between these two states. Under these conditions, they will have the solvability and density comparable to that of a liquid, equivalent values of viscosity compared to a gas, and values of diffusion coefficients intermediate to a liquid and a gas, thus presenting a considerable capacity of penetration into a

solid matrix facilitated by its carrying capacity. Furthermore, the ability to change the dissolving power of a supercritical fluid, in a continuous manner without the occurrence of a phase transition, only by adjusting the pressure and temperature values, represents an advantage which must be emphasized (Nakayma & Boucher, 1999). This combination of properties allows the application of these fluids in several areas of interest. In case of CO_2, when in the supercritical state, it can be used in several applications as described in Figure 1.8.

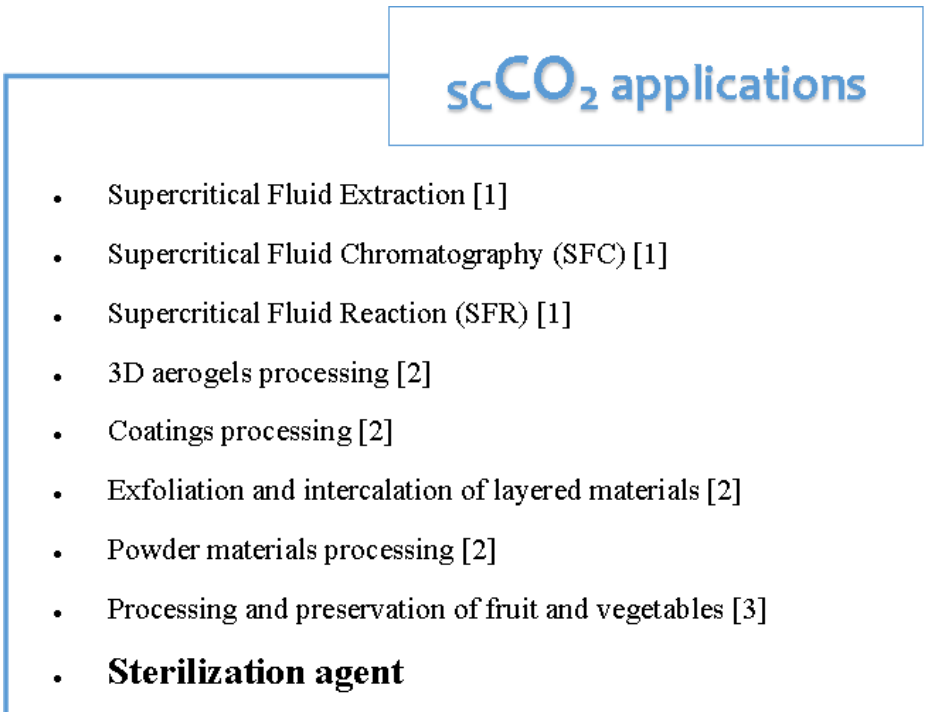


Figure 1.8 –Examples of several applications of $_{sc}CO_2$. Adapted from:[1] (Lewa, 2017) ; [2](Zhang et al., 2014) and [3](Rawson et al., 2012).

Supercritical Carbon Dioxide ($_{sc}CO_2$)

According to pressure-temperature (p-T) phase diagram of CO_2 presented in Figure 1.9, CO_2 transits to supercritical phase at the critical points of 7.38 MPa, and temperature of 31.1°C (304 K). This supercritical fluid is chemically inert, non-toxic, non-inflammable and non-polluting. It has desirable properties such as high density, low viscosity and high diffusivity that make it suiTable for use as a solvent in supercritical fluid extraction method (Lozowski, 2010).

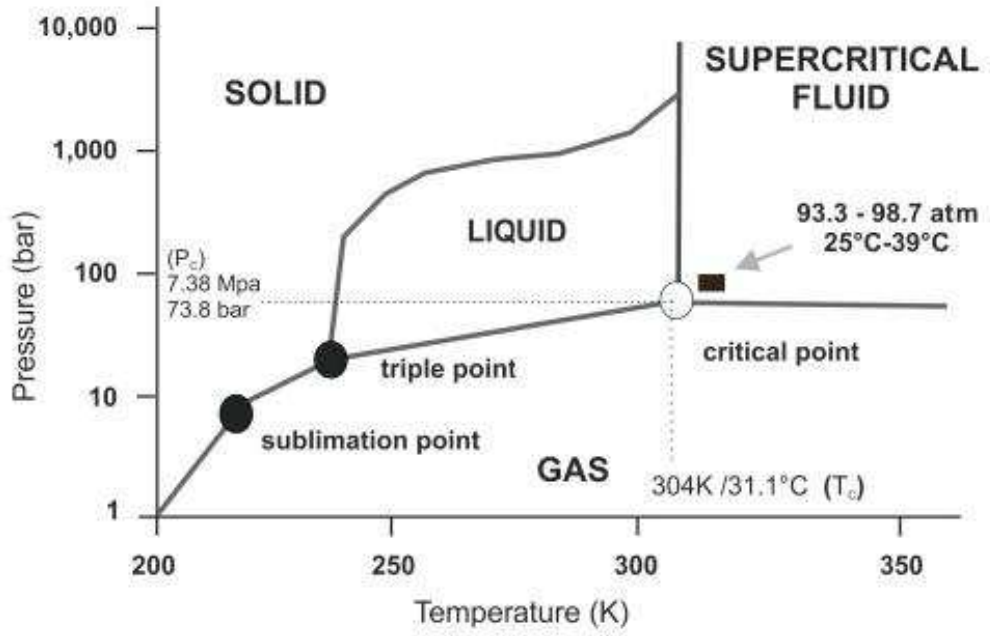


Figure 1.9 –Schematic p-T phase diagram of CO_2. From Budisa & Schulze-Makuch (2014).

1.3.2 Principles of $_{SC}CO_2$ Sterilization Technique

The results presented by the scientist Fraser in the middle of the 20th century (Fraser, 1951), triggered the interest of using carbon dioxide (CO_2) in sterilization processes. The experiment was based on blasting off *Escherichia coli* bacteria with the use of pressurized gases (Ar, N_2, N_2O, and CO_2). It was noted that the rates of deactivation of *E. coli* between 95 and 99% were achieved with 3.4 MPa of CO_2 (Enomoto et al., 1997). Some years later, in response to the strict regulations implemented on EtO and radiation sterilization by the Japanese Government, experiments were initiated using high pressured gases to treat various materials. Based on previous knowledge about the potentialities of CO_2, the efficiency of gas with residual amounts of additives such as 0.5 wt.% of acetic acid or 2 wt.% of ethanol was tested in endospores of *Bacillus subtilis* and *Geobacillus stearothermophilus*. The conclusive results demonstrated that bacterial endospores were resistant to this type of treatment (Takahashi, 2004).

Kamihira *et al.* (1987) stated that the use of compressed CO_2 caused the death of microbial cells by inactivation of important enzymes, pH decreases and/or by the extraction of intracellular substances. It was also demonstrated that CO_2 is a safe sterilizing reagent to be applied to heat-sensitive substances. There is a high interest in understanding the mechanism

behind the bactericidal effect of $_{SC}CO_2$. There are two proposed mechanisms which aim to explain how this inactivation occurs. Mechanical, cell rupture is one of the accepted explanations. It is assumed that fast depressurising of CO_2 at elevated pressures leads to an uncontrolled increase in cell volume, and consequently the cell lysis (Fraser, 1951).

On the other hand, it is suggested that the sudden pressure drop may not cause a significant role in microbial inactivation (Enomoto et al., 1997). Published work by Haas et al (1989) and Wei *et al* (1991) demonstrated that during pressurization with CO_2, the gas causes the inactivation of key enzymes essential to the metabolic process of microorganisms most likely caused by the decreased pH value inside the cells and/or the solubilization of intracellular substances. According to experimental studies focused on the inactivation of microorganisms in food, the physiological deactivation was described as a complex mechanism, divided into several stages occurring simultaneously(Garcia-Gonzalez et al., 2007). In an initial phase a pressurized CO_2 solubilization occurs in the external liquid phase. It occurs the formation and dissociation of carbonic acid (H_2CO_3), triggered by the contact of pressurized CO_2 with water. This dissociation results in hydrogen cations (H^+), responsible for the acidification of the medium. The use of a more acidic medium may decrease microbial resistance to inactivation due to an increase in energy consumption to achieve stable conditions that allow cell survival (Hutkins & Nannen, 1993) (Hong & Pyun, 1999).

The acid medium may also facilitate the penetration of CO_2 into microbial cells, due to an increase in cellular permeability (Ho-mu et al., 1993) (Lin et al., 1994). The next step sums up to cell membrane modifications, due to the high affinity of CO_2 with the plasma membrane. The CO_2 in the aqueous state may have an ability to diffuse into the cell membrane and accumulate in its inner layer, causing functional and structural damages. The increase in permeability of the membrane to the passage of pressurized CO_2 will cause its accumulation in the cytoplasm, causing a decrease in intracellular pH, inactivating essential enzymes for cellular metabolism. The presence of CO_2 and HCO_3^-, in the cytoplasm, will interfere and deregulate the carboxylation and decarboxylation reactions, translating into an inhibitory effect on microbial metabolism. This accumulation of CO_2 also causes an increase in the pressure which, when released, converts HCO_3^- into CO_3^{2-}, interfering with the electrolyte balance and damaging the cell's biological system. The high solvability of $_{SC}CO_2$ can also cause disturbances, with the removal of intracellular constituents (Garcia-Gonzalez et al., 2007).

The $_{SC}CO_2$ sterilization must also ensure efficient elimination/inactivation of bacterial spores. Bacterial spores (endospores) are structures created by bacteria themselves when subjected to unfavourable environmental conditions. They are quite resistant to chemical and heat sterilization and are also responsible for bacterial multiplication and propagation. Nevertheless, some experimental studies revealed that CO_2 at high pressure induced the inactivation of these forms by affecting the membrane's structure of the spores as well as inhibiting the role of important proteins associated with spore germination (Rao et al., 2016). To increase the effectiveness of sterilization, several studies demonstrated the potential of $_{SC}CO_2$ sterilization combined with low amounts of additives. The following table lists several publications where different combinations of additives have been used to achieve the required SAL of 10^{-6} for the tested bacterial spores.

Table 1.1– Overview on publications describing the potential of $_{SC}CO_2$ sterilization with effective additives. Adapted from Bernhardt *et al* (2015).

Author (year)	Bacterial spores tested	Effective additives (log reduction >6)
White (2006)	*G. stearothermophilus* *B. subtilis*	PAA (0.002%), H_2O (0.15%); TFA
Zhang (2006)	*B. pumilus*	H_2O_2 (0.0002%)
Hemmer (2007)	*G. stearothermophilus* *B. atrophaeus*	H_2O_2 (0.6%)
Shieh (2009)	*B. pumilus*	H_2O/methanol/formic acid (3.3% / 0.33 % / 0.033%), H_2O/methanol/formic acid/H_2O_2 (3.3% / 0.33 % / 0.033% / 0.11%) (log reduction 5)
Checinska (2011)	*B. pumilus*	H_2O / H_2O_2 (3.3% / 0.1%)
Donati (2012)	*G. stearothermophilus*	H_2O_2 (0.0002% - 0.0006%)
Park (2013)	*B. cereus*	Ethanol (2%)

More recent work has established that $_{SC}CO_2$ sterilization should occur generally under addition of 0.25% water, 0.15% hydrogen peroxide and 0.5% acetic anhydride. This

combination was successfully tested for the inactivation of a wide range of microorganisms including endospores of different bacterial species (Bernhardt et al., 2015).

1.3.3 Current Sterilization Technology *vs* $_{SC}CO_2$

$_{SC}CO_2$ technology compared to the sterilization methods referred to in section 1.2 "Main Sterilization Techniques in Medicine", stands out because it uses very small amounts of non-toxic sterilant, has a high penetration ability in complex structures, enables short cycle of sterilization, maintaining the intrinsic properties of materials. Figure 1.10 shows a brief comparison between technologies.




Sterilization methods	$scCO_2$	Autoclave	EtO	Ionizing Radiation	H_2O_2
Price	−	+	+ +	−	−
Toxicity	Non-toxic	Non-toxic	Toxic	Mostly Non-toxic	Non-toxic
Temperature	< 40 °C	< 121 °C	30 to 80 °C	Room temperature	6 to 60°C
Penetration in material	High	Mainly at surface	High	High	High
Time for cycle sterilization	6 hours	2 to 3 hours	24 to 48 hours	Material dependent	+/- 1 hour
Maintenance of Material properties	+ +	−	+	−	+

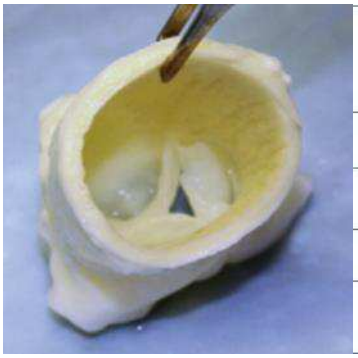
Figure 1.10 – Comparison of the standard sterilization techniques with the $_{SC}CO_2$ process. ++: High probability that the sterilization method does not interfere with the properties of the material; –: Low: probability that the sterilization method does not interfere with the properties of the material.

1.3.4 Sterilization of Biological Material by using $_{SC}CO_2$

New emerging biomaterials, based on natural polymers, require the development of advanced methods of sterilization. During the procedures of collection and processing of the

biomaterials and tissue grafts, these can be easily contaminated with multiple organisms. This explains the importance of mild sterilization technologies such as $_{SC}CO_2$.

Several works have demonstrated the effectiveness of $_{SC}CO_2$ in the sterilization of various biological materials. Examples are tissue grafts and engineered tissues which have been extensively studied and tested for valve replacement in humans inflicted with vascular failure or disease. In case of the sterilization of decellularized heart valves, $_{SC}CO_2$ sterilization technology has been demonstrating to be superior to other tested techniques, according to histology, microbiological culture and electron microscopy results (Figure 1.11) (Hennessy et al., 2017). In fact, the standard technologies do not sterilize efficiently without impacting the structure of the biological material reflecting in several ways such as a cross-linking effect, molecular fragmentation or protein denaturation. In particular, gamma radiation provides an amount of radiation that could destroy and damage the tissue matrix of the valve cusps (Somers et al., 2009). Others, such as sterilization techniques using electrolyzed water and H_2O_2, proved to be inefficient to sterilize heart valves due to microbial remnants (Hennessy et al., 2017).



Sterilization methods	Sterilized ?
Electrolyzed Water	No
Gamma Radiation	No
H_2O_2	No
sc CO_2	Yes

Figure 1.11 – Decellularized heart valve and the results of sterilization for the different methods. Adapted from Hennessy *et al* (2017).

Other studies were conducted on decellularized human tendons and cortical bones using different sterilization methods and compared to $_{SC}CO_2$ sterilization. For example the biomechanical properties of these two grafts sterilized with $_{SC}CO_2$ were better reinforced and maintained when compared to those sterilized by gamma irradiation (Nichols et al., 2009). Russell *et al.* (2013) has also demonstrated identical results in terms of mechanical properties for cortical bones from rabbits when compared to gamma radiation. Baldini *et al.* (2016) studied the strength and stiffness of soft tissue allografts comparing unprocessed grafts, irradiated grafts and grafts treated (sterilized) with $_{SC}CO_2$. As a result, the two tested methods of sterilization do

not affect allograft strength. The stiffness of the treated allografts was significantly lower when used $_{SC}CO_2$ sterilization compared to gamma radiation.

Currently, in pulmonary tissue engineering peracetic acid (PAA) is being used at high concentrations to sterilize lung tissue which negatively interferes with the extracellular matrix (ECM) (Balestrini et al., 2016). $_{SC}CO_2$ sterilization, compared to PAA treatment, does not significantly alter the physical or chemical properties of the lung. According to Balestrini et al (2016), PAA and $_{SC}CO_2$ sterilization strategies were effective in removing bioburden in decellularized lungs. Nevertheless, the histological, biochemical and mechanical assays revealed different results for both sterilization techniques. Namely, the acellular lung tissue treated with $_{SC}CO_2$ retained its structural and mechanical integrity and protein content like collagen, or elastin molecules, in contrast, with PAA sterilization treatment.

Qiu *et al* (2009) studied the effect of $_{SC}CO_2$ sterilization on acellular dermal matrices using PAA as additive. The mechanical parameters like maximum load, maximum stress, elasticity, and tear strength were assessed and compared between $_{SC}CO_2$ sterilized samples and non-sterilized samples (control group). Only differences in terms of elasticity were found, in which sterilized samples presented lower elasticity values than the control group. Regarding the biochemical and biomechanical properties of the acellular dermal matrix the changes were almost minimal. These results were consistent with other analyses which demonstrated that sterilization did not cause significant denaturation or cross-linking. However, this change can be indicative of a modification in the structure of the collagen which may affect the response in vivo.

In another work carried out by Meyer *et al* (2015) a set of collagen-based materials was sterilized using $_{SC}CO_2$ with H_2O_2 as an additive while other was sterilized by gamma-irradiation sterilization. Once again, the measured parameters, as structure, tensile strength and tear resistance of the $_{SC}CO_2$ treated samples were improved, in this case the mechanical properties, or kept as desired when compared with the standard treatment (gamma irradiation).

There is a need to reinvent and create new sterilization techniques to respond to the modernization and complexity of areas such as biomaterials, tissue engineering and tissue grafting. So far, $_{SC}CO_2$ sterilization has proven to be effective on a large diversity of biological materials. However, there is still a long way to go when considering the regulatory barriers of launching a new sterilization method to the market. In this sense, the present work aims at

investigating the ideal process parameters of $scCO_2$ sterilization and further analysis of the impact on the physical and chemical properties of a biological samples of placenta.

2. Material and Methods

2.1 Biological Samples

Placenta

Placenta samples were obtained from 4 women with normal pregnancies undergoing a term (38–40 weeks of gestation) scheduled cesarean section. Samples were obtained from Hospital São João (Porto, Portugal), with the informed consent from patients and after ethical approval from Ethics Committee for Health from the referred hospital. Decellularization was performed at the Biomaterials for Multistage Drug and Cell Delivery group at i3s (Instituto de Investigação e Inovação em Saúde, Porto, Portugal) by adapting the protocol of Choi *et al* (2013), followed by lyophilisation performed according to the parameters of i3s. After obtaining the decellularized and lyophilized tissue, a set of samples were submitted to sterilization. Two of them of 60 mg and 135 mg were selected for structural/chemical analysis.

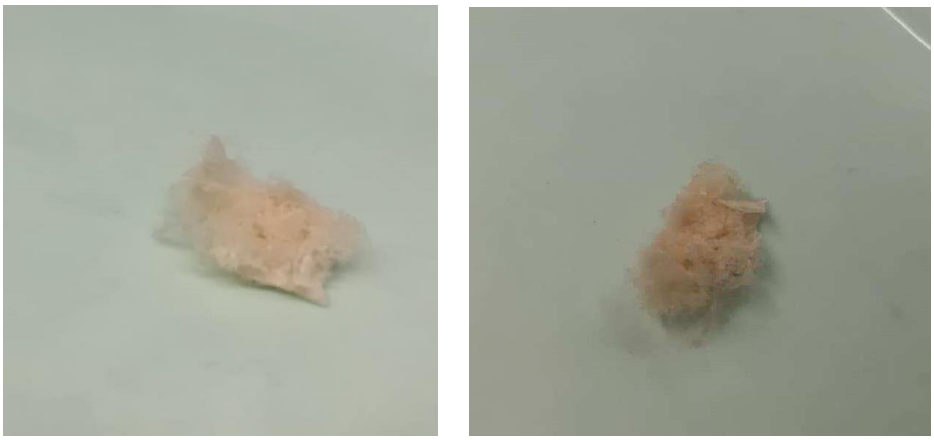


Figure 2.1– Decellularized human placenta after lyophilization.

Biological Indicators

Spore strips of the species *B. stearothermophilus*, *B. atrophaeus* and *B. pumilus* containing 10^6 spores each one, were used as biological indicators (BI) to test the efficiency of the sterilization process (control samples). Namely, *B. stearothermophilus* is a BI for steam sterilization, *B. atrophaeus* for ethylene oxide and dry heat sterilization and *B. pumilus* for sterilization by gamma radiation (Figure 2.2).

Figure 2.2– *B. stearothermophilus*, *B. atrophaeus* and *B. pumilus* spore strips.

2.2 $_{SC}CO_2$ Sterilization

The $_{SC}CO_2$ equipment used for the sterilization of the previously described biological samples is illustrated in Figure 2.3.

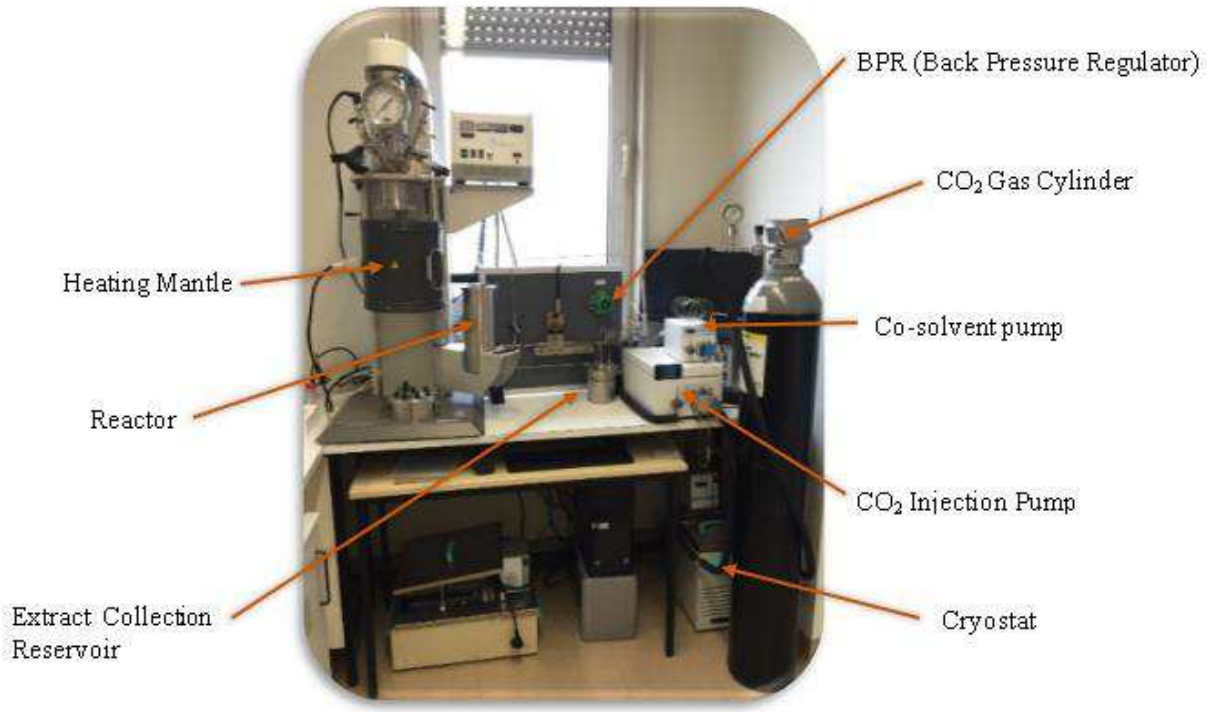


Figure 2.3– $_{SC}CO_2$ sterilization equipment.

Herein, the sterilization system is composed of several connected components. The CO_2 supplied to the system is stored and ready for use in a cylinder with a capacity of 50 L of premium CO_2 Liquid Premier with 99.995 % of purity provided by Gasin Air Products (Porto, Portugal). The CO_2 will pass through a cryostat, where it liquefies before it reaches the injection pump. The CO_2 will be transported to the reactor through a system of adapted pipes and valves. The correct heating and pressurizing parameters allow CO_2 to reach the supercritical phase. The reactor present in the system is a Parr Instruments series 4540 high pressure reactor (Parr Instrument Company, Illinois, USA) with a capacity of 1.2 L, with a workable volume estimated in 1 L, due to the existence of the agitator and the refrigeration circuit inside it. The samples and materials that are intended to be sterilized are placed inside the reactor before closing. After processing, the $_{SC}CO_2$ is transported from the reactor to the atmosphere, passing through a back-pressure regulator (BPR) valve, allowing the continuous flow of $_{SC}CO_2$, and a trap, intended for

the precipitation of relevant compounds dissolved in the $_{SC}CO_2$, although this item is not important for sterilization. The system is composed of several resistors before and after the reactor to prevent CO_2 from freezing and clogging the pipes during the depressurization. All the remaining equipment was supplied and assembled by Paralab (Porto, Portugal).

To control the conditions under which the process will take place, a customized computer program (Universidade Católica Portuguesa SC) from Paralab is used (Figure 2.4). This operating system controls the flow rate of CO_2, inlet/outlet temperature (in this case 29 and 32 ºC, respectively), BPR and Trap temperatures (84 ºC and 82 ºC, respectively) and the pressure and temperature inside of the chamber (31.9 ºC and 51.5 bar). These parameters are controlled in real time.

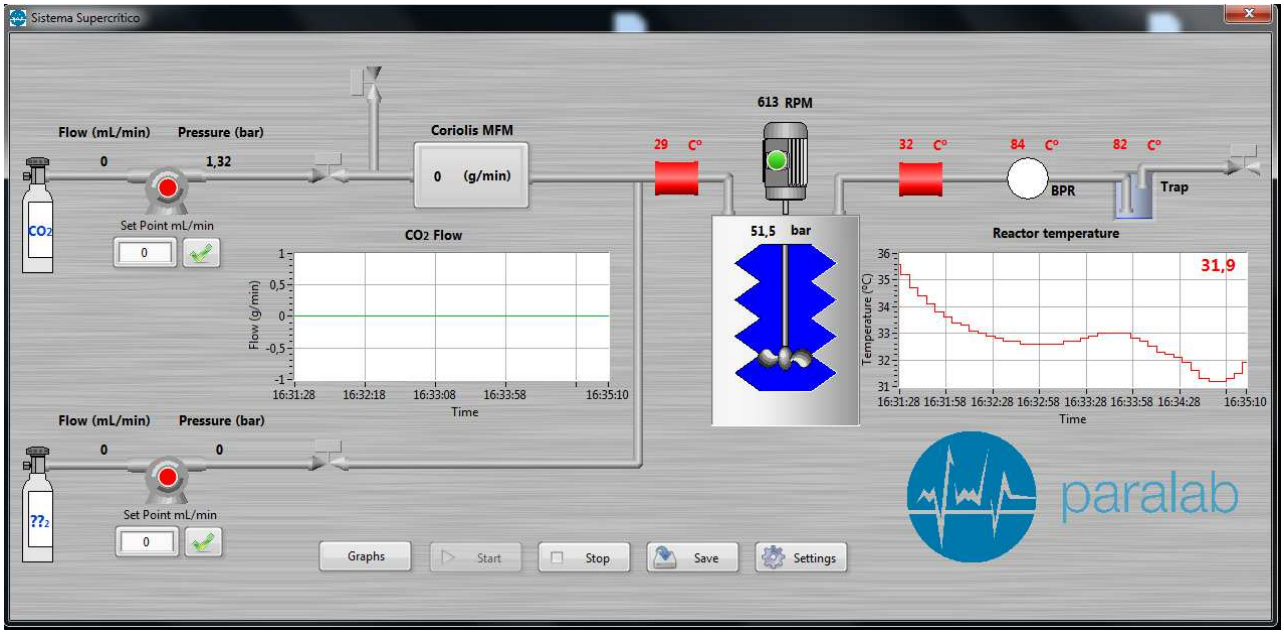


Figure 2.4– Operating system created to the sterilization system.

Placenta samples and biological indicators were individually packaged in Tyvek pouches (Palex, Barcelona, Spain), as shown in Figure 2.5a,b , and heat-sealed before exposure to the $scCO_2$ sterilization treatment.

Figure 2.5a – Biological indicators packed and sealed in Tyvek pouches. 2.5b– Decellularized and lyophilized placenta packaged in Tyvek pouches. 2.5c – placed in the reactor for the sterilization process.

2.2.1 Optimization of the Mildest Conditions for Effective $_{SC}CO_2$ Sterilization

Different approaches of the $_{SC}CO_2$ sterilization methodology were tested in order to determine the ideal pressure and time for terminal sterilization of *B. pumilus*, *B. atrophaeus* and *B. stearothermophilus* spores. Namely, 12 different sterilization conditions were performed at 2 distinct pressures (140 bar and 245 bar) and 6 different cycle periods (1, 2, 3, 4, 5 and 6 hours of effective sterilization), all of them at 40 °C, 600 rpm and with 300 ppm H_2O_2 30% (Merck, Darmstadt, Germany), as presented in Table 1.1.

Table 2.1– Sterilization parameters for optimizing of the mildest conditions for effective $_{SC}CO_2$ sterilization.

Sterilization			
#	B.I. Quantity (1)	Conditions	
		Pressure (bar)	Time (h)
1	6 x strips	140	1
2	6 x strips	140	2
3	6 x strips	140	3
4	6 x strips	140	4
5	6 x strips	140	5
6	6 x strips	140	6
7	6 x strips	245	1
8	6 x strips	245	2
9	6 x strips	245	3
10	6 x strips	245	4
11	6 x strips	245	5
12	6 x strips	245	6

(1) Quantity of Biological indicator (B.I.): 2 strips of spore of each species, namely *B. pumilus*, *B. atrophaeus* and *B. stearothermophilus.*

Each sterilization cycle presents one pressurizing stage, one depressurising phase and the effective sterilization phase, also called plateau phase (Figure 2.6). The pressuring and depressurizing intervals are not considered for the effective sterilization time but are both monitored in all sterilizations.

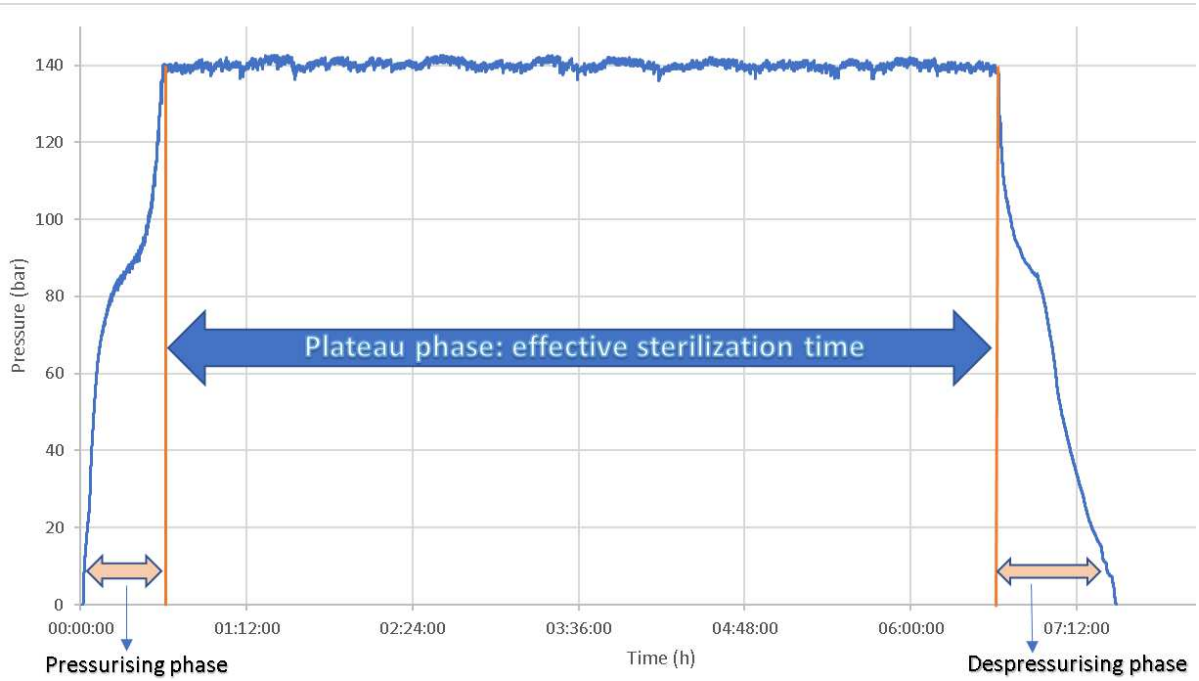


Figure 2.6– An example of a complete sterilization process.

The effectiveness of the sterilization process was verified by turbidity tests carried out on liquid suspensions of trypto-casein soy broth (TSB) (Biokar, Pantin, France) containing the spores strips after $_{SC}CO_2$ treatment. The analysis was performed as shown in "Effective Validation Sterilization", Figures 2.7 and 2.8. The growth was verified 15 days after the incubation start.

Effective Validation of Sterilization

Turbid medium	Clear medium
	
Existance of microorganism growth. • **Note:** Unefficient sterilization process.	No microorganism growth. • **Note:** Efficient sterilization process.

Figure 2.7–Interpretation of results in a broth medium inoculation.

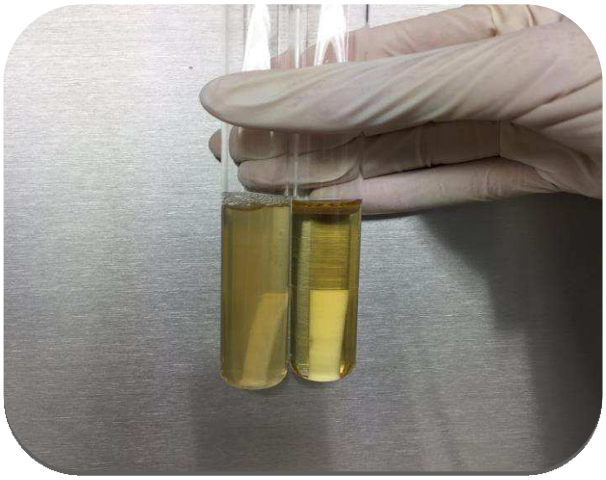


Figure 2.8–Comparation between a turbid medium (left test tube) and clear medium (right test tube) containing spores strips before and after the $_{SC}CO_2$ treatment, respectively.

2.2.2 Influence of the Time Interval between the $_{SC}CO_2$ Sterilization and Microbiological Validation

The terminal sterilization of *Bacillus pumilus* and *Bacillus atrophaeus* spores using the supercritical carbon dioxide as function of the time interval between the sterilization cycle and the start of the turbidity tests was studied. Herein it was considered one $scCO_2$ effective sterilization time of 2 hours and another of 3 hours, both at 140 bar, 40 °C and 600 rpm, with 300 ppm H_2O_2 30 % (Merck, Darmstadt, Germany), as shown in Table 2.2. 3 independent sterilization processes were performed for each condition and BI. Turbidity tests were carried out on the liquid suspensions containing the spore strips selected after $scCO_2$ treatment. The incubation of the 3 replicates of each spore species was performed at specific time points after $scCO_2$ sterilization: same day (right after sterilization), 24 hours, 7 days and 1 month after $scCO_2$ treatment. The growth was verified 15 days after the incubation start.

Table 2.2 – Defined sterilization conditions to evaluate the effectiveness of terminal sterilization.

#	**B.I. Quantity**	**t**	Incubation Start Date
13	10 x strips of *B. atrophaeus*	3	0 Day
			1 Day
			7 Days
			30 Days
14	10 x strips of *B.atrophaeus*	3	0 Day
			1 Day
			7 Days
			30 Days
15	12 x strips of *B.atrophaeus*	3	0 Day
			1 Day
			7 Days
			30 Days
16	12 x strips of *B.atrophaeus*	2	0 Day
			1 Day
			15 Days
			30 Days
17	12 x strips of *B.atrophaeus*	2	0 Day
			1 Day
			7 Days
			30 Days
18	12 x strips of *B.atrophaeus*	2	0 Day
			1 Day
			7 Days
			30 Days

#	**B.I. Quantity**	**t**	Incubation Start Date
19	12 x strips of *B.pumilus*	3	0 Day
			1 Day
			7 Days
			30 Days
20	12 x strips of *B.pumilus*	3	0 Day
			1 Day
			7 Days
			30 Days
21	12 x strips of *B.pumilus*	3	0 Day
			1 Day
			7 Days
			30 Days
22	12 x strips of *B.pumilus*	2	0 Day
			1 Day
			7 Days
			30 Days
23	12 x strips of *B.pumilus*	2	0 Day
			1 Day
			7 Days
			30 Days
24	12 x strips of *B.pumilus*	2	0 Day
			1 Day
			7 Days
			30 Days

t: Time of effective sterilization;

2.3 Sterilization of Decellularized Placenta and further Evaluation of its Physicochemical Properties

Two human decellularized and lyophilized placenta samples of 60 mg and 135 mg were sterilized with the following conditions: 140 bar, 40 ºC, 600 rpm, with 300 ppm of H_2O_2 (Durox, Rio Claro, Brazil) for 6 hours. The samples of each condition before and after sterilization were analyzed by Fourier transform infrared) spectroscopy (FTIR), High-performance Liquid Chromatography (HPLC), Differential Scanning Calorimetry (DSC), and mechanical tests using a texture analyser. For the sample's treatment glacial Acetic Acid ($C_2H_4O_2$) 1M (Merck, Darmstadt, Germany) was used as received.

Fourier Transform Infrared (FTIR)

The characterization of the sample's chemical structure was performed using a Perkin Elmer (Wellesley, U.S.A.) Spectrum 100 FT-IR spectrometer. The analysis was performed before and after sterilization. The characterization was done at a spectral resolution of 4 cm^{-1} on a frequency region to 450 to 4000 cm^{-1}. One hundred scans were accumulated per sample.

High-Performance Liquid Chromatography (HPLC)

HPLC analysis was performed using a Waters 2690 separations module connected to a Waters 2998 (PDA) photodiode array detector (Waters, Milford, USA). Absorption spectra were recorded between 270 and 550 nm. The system is connected to a PC for data acquisition and analysis using the software package (ChromQuest). The system is also composed by a Beckman 126 Pump (Analytical Instruments) and a Beckman 168 DAD Detector. The HPLC column used was C_{18} 250 x 4.6 mm I.D. (Phenomenex, California, USA) and was packed with 5µm Kromasil. For HPLC detection, the mobile phase A consisted of Acetonitrile gradient grade 0.2% (CH_3CN) (Merck), Trifluoroacetic acid (TFA) (CF_3COOH) (Sigma-Aldrich, St. Louis, MO, EUA) and the mobile phase B Acetonitrile 0.2% TFA and ultra-pure H_2O with 18.2 MΩ.cm resistivity (5:95). The flow rate was set at 2.0 $mL \cdot min^{-1}$ and the signal was monitored at 220 nm. Two solutions were made to dissolve placental samples for further HPLC analysis, as follows: 8.0 mg of treated placenta in 10 ml of acetic acid solution 1.5 M and 17.1 mg of untreated placenta in 40 ml of acetic acid solution 1.5 M. There were some difficulties in

dissolving the untreated sample, so a larger amount of solution was added. The injections were executed as mentioned in the programmed flow (Figure 2.9). The HPLC tests served to compare the chemical composition of treated and untreated human placenta.

General | Degas | Events | Flow | Temperature | Solvents | Channel

Pressure Limits

High Limit (psi) 4000.0 Low Limit (psi) 0.0

Programmed Flow

Accelerate to 10.0 mL/min in:

Pump Mode Gradient 2.00 min (5.00 mL/min/min)

	Time	Flow	%A	%B	%C	%D	Curve
1		1.00	100.0	0.0	0.0	0.0	
2	1.00	1.00	100.0	0.0	0.0	0.0	6
3	30.00	1.00	79.0	21.0	0.0	0.0	6
4	42.00	1.00	73.0	27.0	0.0	0.0	6
5	55.00	1.00	42.0	58.0	0.0	0.0	6
6	60.00	1.00	100.0	0.0	0.0	0.0	6
7	62.00	1.00	100.0	0.0	0.0	0.0	6

Figure 2.9– Pressure limits and programmed flow from the HPLC system.

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) was employed for thermal analysis of human decellularized placenta. It was used a Micro DSC III Analyzer from Setaram instrumentation (Caluire, France). The placenta samples were closed inside aluminium pans and was used a flow rate of 40 ml/min of nitrogen to keep an inert atmosphere. The samples were heated from 10ºC to 150ºC with a scan rate of 1ºC/min to detect the thermal properties. The system is connected to a PC for data acquisition and analysis using the software Thermal Analysis Software Calisto.

Mechanical Tests

The mechanical properties of the placenta samples were assessed by uniaxial tensile testing using the texture analyser TA. XT.Plus® (Stable Micro Systems, Surrey, UK), based on ASTMD882-02 methods. The thickness of the decellularized and lyophilized matrices analysed per condition, before and after $_{SC}CO_2$ sterilization, was measured with a digital micrometer (MI20, Adamel Lhomargy, Ivry sur Seine Cedex, France) being 1.733 ± 0.026 mm and 1.982 ± 0.003 mm. In brief, placenta samples were mounted on a support rig (HDP/FSR) and punctured by a 5 mm spherical probe (P5/S) at a speed of 1 mm/s. The most relevant parameters were obtained from the stress/strain curves as follows: Young's Modulus, stress at burst and strain at burst. The Young's modulus refers to the slope of stress-strain curves. Stress at burst was calculated as the ratio between the required force to break the matrices and their cross-sectional area, while strain percentage at burst was evaluated as the deformation of matrices at the point of rupture. The results represent the average of three samples obtained from the lyophilized placenta after $_{SC}CO_2$ treatment and four samples from untreated lyophilized placenta.

3. Results

3.1 Optimization of the Mildest Conditions for Effective $_{SC}CO_2$ Sterilization

In this first phase of the work, 12 sterilization cycles with different conditions were performed on biological indicators, to optimize the mildest conditions required for an effective $_{SC}CO_2$ sterilization. In a typical sterilization cycle (Figure 2.6) the pressurising and depressurising stages were around 10 to 40 minutes and 10 to 50 minutes, respectively. The effective sterilization times ranged from 1 to 6 hours. There are some doubts about pressurization interfering with sterility of the spore strips. It is estimated that a pressurising interval less than 10 minutes may adversely affect the process of sterilization due to the pressure shock generated. The same happens with the depressurising stage, the final sterilization step. The results obtained for all the $_{SC}CO_2$ cycles taken place are shown in the following Table 3.1.

Table 3.1– Microbiological validation: results of microbiological growth in TBS medium

#	Conditions		Growth in TBS medium (15 days)					
	Pressure (bar)	Time (h)	Sample*					
			B. stearothermophilus		*B. atrophaeus*		*B. pumilus*	
1	140	1	+	+	+	+	+	+
2		2	+	+	+	–	+	+
3		3	–	–	–	–	–	–
4		4	–	–	–	–	–	–
5		5	–	–	–	–	–	–
6		6	–	–	–	–	–	+
7	245	1	–	–	–	–	–	+
8		2	–	+	–	+	–	+
9		3	+	+	–	+	+	+
10		4	–	–	–	+	+	+
11		5	–	–	–	–	–	–
12		6	–	–	–	–	–	–

* quantity: 2x strips of each spore strain;

+ refers to growth of the spore species; – refers to a nonexistence of growth.

According to the carried out turbidity tests all the spore strains selected had grown in TBS medium after the sterilization cycles at 140 bar and with 2 hours or 1 hour of effective sterilization. For cycles at 140 bar, with periods of effective sterilization equal or higher than 3 hours the different spore strains, were inactivated, except one strip of *B. pumilus* with 6 hours of effective sterilization. In this isolated case, growth may have been caused by contamination during lab incubation procedures.

At 245 bar microbial growth was observed for sterilization cycles with 4 or less hours of effective sterilization. The growth was verified, at 1 hour of effective sterilization, in a strip of *B. pumilus*. With 2 hours, on a strip of *B. stearothermophilus*, *B. atrophaeus* and *B. pumilus*. At 3 hours, growth occurred on the two strips of *B. stearothermophilus* and *B. pumilus* and one of *B. atrophaeus*. With 4 hours of effective sterilization growth occurred on the two strips of *B. pumilus* and one of *B. atrophaeus*. Over 4 hours of effective sterilization, bacterial growth was not observed in the spore strips of the 3 species under study.

3.2 Influence of the Time between $_{SC}CO_2$ Sterilization and Microbiological Validation: Shelf Life

The influence of the time between the $_{SC}CO_2$ treatment and the turbidity assay on the effectiveness of sterilization of *B. pumilus* and *B. atrophaeus* spores, is presented in Table 3.2.

Table 3.2– Microbiological validation: results of microbiological growth in TBS medium.

Sterilization			Growth in TBS medium (15 days)											
#	Sample*	Conditions Time (h)	Incubation Start Date											
			0 Day			1 Day			7 Days			30 Days		
13	*Bacillus atrophaeus*	3	–	–		–	–		–	–		–	–	–
14			–	–	–	–	–	–	–	–	–	–	–	–
15			–	–	–	–	–	–	–	–	–	–	–	–
16	*Bacillus atrophaeus*	2	+	+	+	–	–	–	–	–	–	–	–	–
17			+	+	+	–	–	–	–	–	–	–	–	+
18			–	–	+	–	–	–	–	–	–	–	–	–
19	*Bacillus pumilus*	3	–	–	–	–	–	–	–	–	+	–	–	–
20			–	–	–	–	–	+	–	+	+	–	–	–
21			–	–	–	–	–	–	–	–	–	–	–	+
22		2	+	+	+	+	+	+	–	–	+	–	–	–
23			+	+	+	–	–	+	–	–	–	–	–	–
24			+	+	+	+	+	+	–	–	+	–	–	+

* quantity: 12x strips of the strain for each sterilization; 10x strips of the strain for each sterilization

+ refers to growth of the spore species; – refers to a nonexistence of growth.

The turbidity test results revealed that *B. atrophaeus* spores were inactivated with a 3 hours sterilization cycle at 140 bar. All the strips incubated on the same day of sterilization or in subsequent 1, 7 or 30 days did not show microbial growth. Thus, the shelf life for this strain has no significant influence on growth, since terminal sterilization occurred just after the $_{SC}CO_2$ process. The same did not happen when the time for effective sterilization was reduced to 2 hours. For the strips sterilized and incubated on the same day, the results presented microbial growth in 7 out of 9 strips under study. For time intervals greater than or equal to 1 day, no growth was observed, except in one spore strip maybe contaminated during lab procedures.

Regarding sterilization of *B. pumilus* spores, for 3 hours of sterilization, microbial growth was evidenced in a spore strip incubated 1 day after sterilization and in three strips incubated strips 7 days after sterilization. There was no microbial growth for the strips incubated 30 days after sterilization. For 2 hours of effective sterilization, microbial growth occurred in all strips incubated in the same day as sterilization. For the strips incubated 1 day after the sterilization, 7 of them presented microbial growth. The strips incubated 7 days after sterilization, only 2 strips showed microbial growth. When incubation was done 30 days after sterilization, only 1 spore strip showed growth.

These results demonstrate that the effect of $_{SC}CO_2$ on spore inactivation goes beyond the process itself and is time-dependent.

3.3 Physical, Morphological and Chemical Characterization of the Decellularized Placentas

3.3.1 Fourier Transform Infrared (FTIR)

FTIR analysis was performed on placenta samples before and after the sterilization in order to study and identify changes in the chemical structure caused by the process, as presented in Figure 3.1.

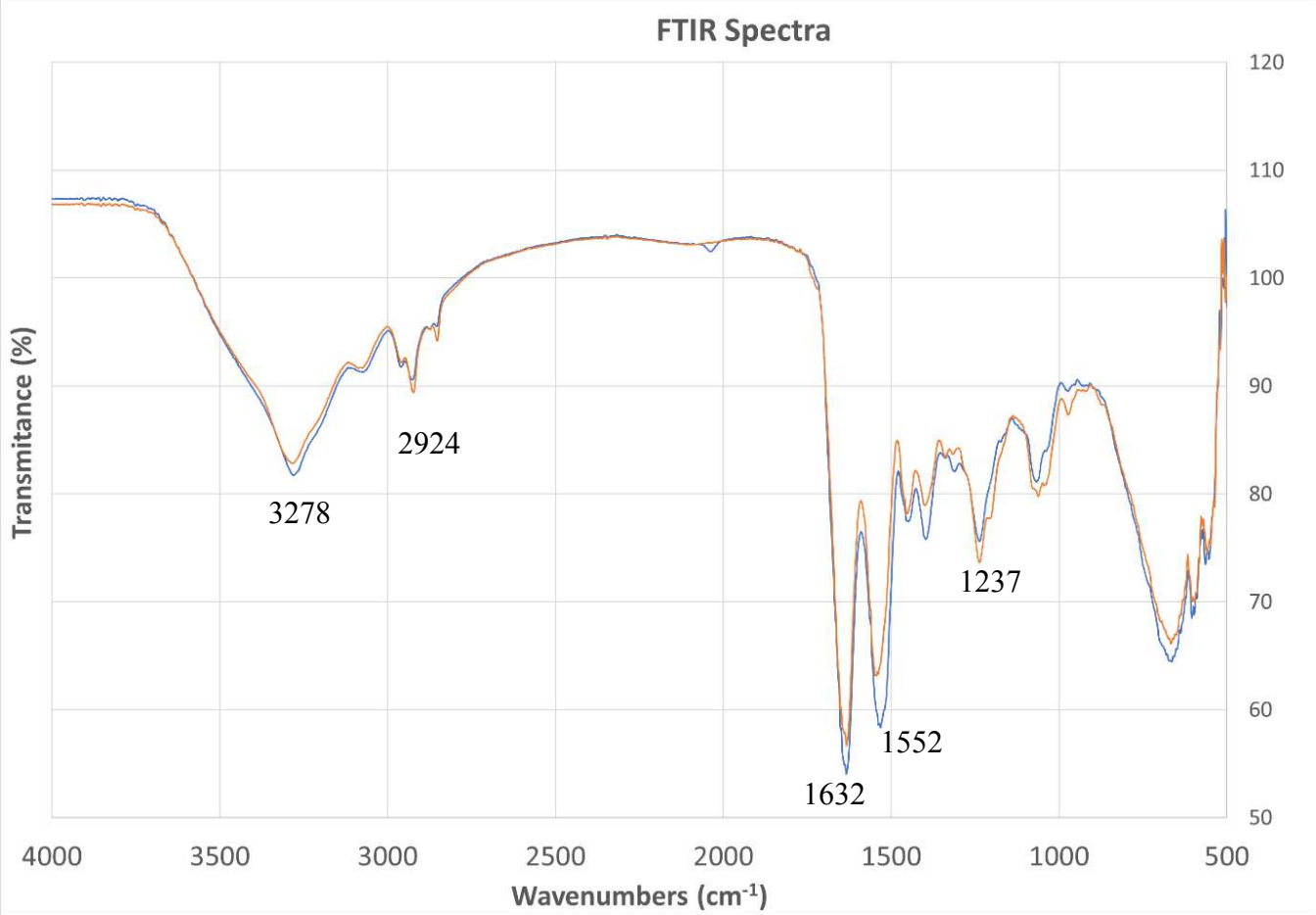


Figure 3.1– Superimposed FTIR spectra of the lyophilized and decellularized placenta samples. **Blue**: Before sterilization; **Orange**: After sterilization.

Comparing the FTIR spectra of the treated and untreated samples, with respect to the identified bands and their transmittance values, a clear similarity between both is visible. The bands identified in the spectra of the placenta before and after sterilization process, corresponding to the functional groups of placental tissue (Figure 3.1), are very

similar which indicates that the sterilization process does not affect the chemical structure. Treated samples exhibited amide absorption bands at 1632 cm^{-1}, 1552 cm^{-1} and 1237 cm^{-1}, for amide I, II and III respectively, similar to those observed in untreated placenta samples. The peaks of the treated placenta with absorption bands at 2924 cm^{-1} and 3278 cm^{-1}, corresponding to C-H (alkane) and N-H (amine) stretching vibrations are also found in both sample spectra. The bands corresponding to amides I, II and III are associated with collagen molecules present in the placenta tissue and the bands corresponding to the alkane and amine stretching vibrations are associated with lipid alkyl chains present in the placenta structure (Wehmeyer et al., 2015).

3.3.2 High-performance Liquid Chromatography (HPLC)

The HPLC technique was applied in treated and untreated placenta samples in order to compare their chemical content. At 220 nm, the chromatograms of the untreated and treated samples are represented in Figure 3.2a and 3.2c, respectively. Figure 3.2b shows 4 peaks, from the untreated sample, with the retention times: 5.408, 5.666, 6.053 and 56.883 minutes. Figure 3.2d shows 3 peaks from the treated samples with the retention times: 5.981, 6.478 and 56.762 minutes. The 3 peaks detected in the treated placenta sample are clearly found in the chromatogram of the untreated placenta sample. Thus, it is possible to correlate the peaks of the untreated sample 1, 3 and 4 with the peaks of the treated sample 1, 2 and 3.

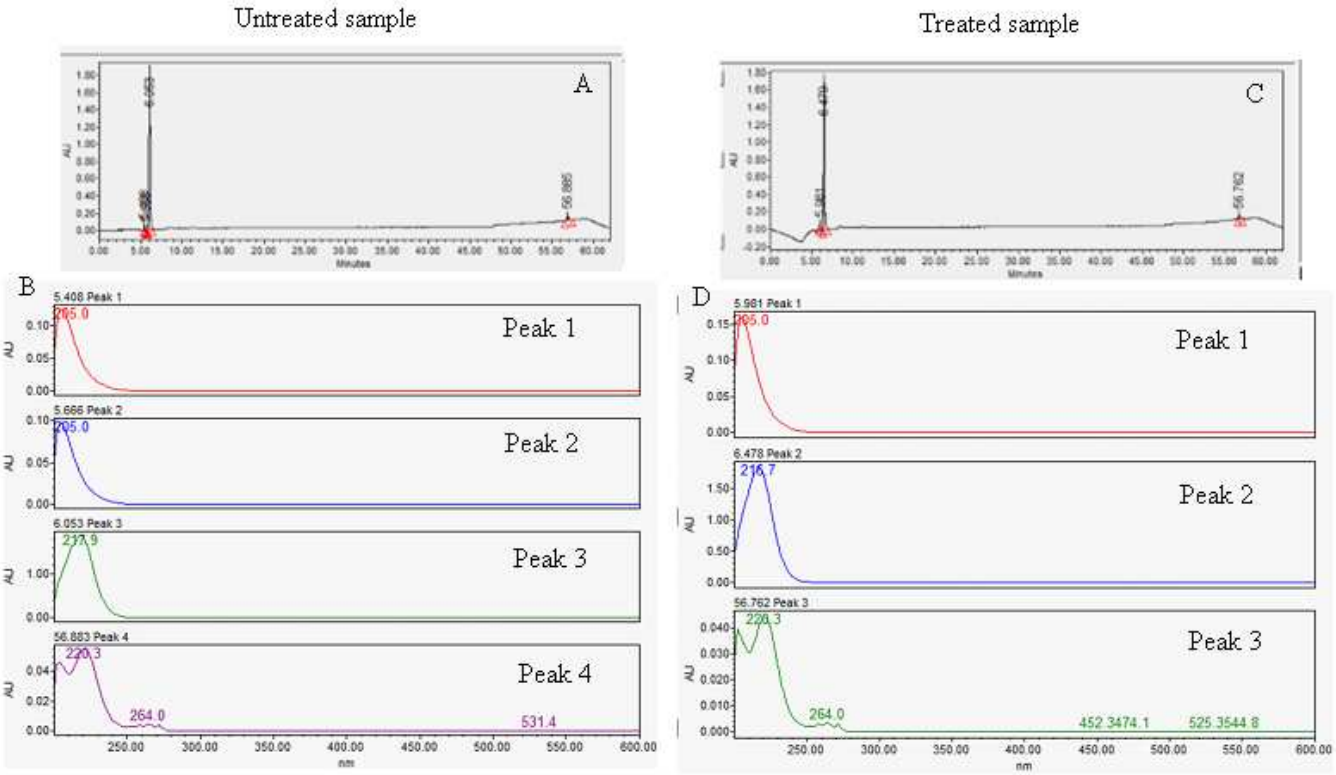


Figure 3.2a – HPLC chromatogram of untreated placenta sample. 3.2b – Chromatograms of the 4 separated peaks from the untreated placenta. 3.2c – HPLC chromatogram of treated placenta sample. 3.2d – Chromatograms of the 3 separated peaks from the treated placenta sample.

In Table 3.3 the correlated peaks are compared in their values of retention time and maximum absorbance.

Table 3.3 – Comparison between the calculated retention time values, in minutes, and the maximum absorbance, in absorbance units, of the peaks of untreated and treated placenta.

Sample	Peak	Retention time (min)	Maximum absorbance (AU)
Untreated	1	5.408	0.12481
Treated	1	5.981	0.15967
Untreated	3	6.053	1.67913
Treated	2	6.478	1.84527
Untreated	4	56.883	1.67913
Treated	3	56.762	0.04409
Untreated	2	5.666	0.09780

The HPLC technique was repeated, as described earlier, but this time with a temperature increase in the separation column from room temperature (+/- 25 ºC) to 45 °C thereby increasing the separation efficiency of the compounds. The chromatogram of the untreated and treated placenta is represented in Figure 3.3a and 3.3c. The peaks highlighted in the chromatogram, from the untreated sample, have the retention time of: 1.678, 2.200, 3.062, 3.442, 4.037 and 54.319 minutes, represented in Figure 3.3b. The peaks highlighted in the chromatogram, from the treated sample, have the retention time of: 2.195, 3.420, 3.635, 3.967 and 54.331 minutes, represented 3.3d. The 6 peaks detected in the treated placenta sample are clearly found in the chromatogram of the untreated placenta sample. Thus, it is possible to correlate the peaks of the untreated sample 2, 4, 3, 5 and 6 with the peaks of the treated sample 1, 2, 3, 4 and 5.

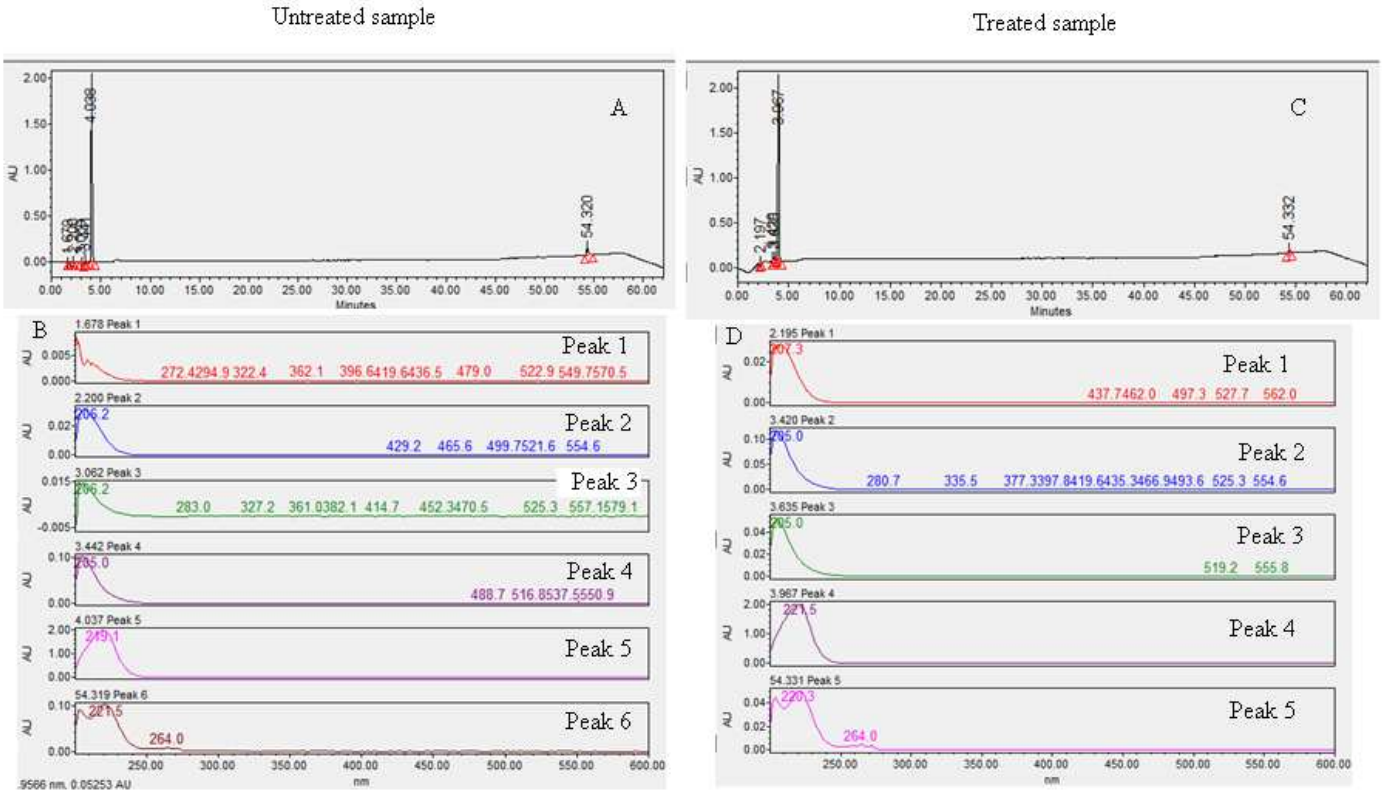


Figure 3.3a – HPLC chromatogram of untreated placenta sample. 3.2b – Chromatograms of the 6 separated peaks from the untreated placenta. 3.2c – HPLC chromatogram of treated placenta sample. 3.2d – Chromatograms of the 5 separated peaks from the treated placenta sample.

In Table 3.4 the correlated peaks are compared in their values of retention time and maximum absorbance.

Table 3.4– Comparison between the calculated retention time values, in minutes, and the maximum absorbance, in absorbance units, of the peaks of untreated and treated placenta.

Sample	Peak	Retention time (min)	Maximum absorbance (AU)
Untreated	2	2.200	0.03236
Treated	1	2.195	0.02909
Untreated	4	3.442	0.10196
Treated	2	3.420	0.11607
Untreated	3	3.062	0.01473
Treated	3	3.635	0.05394
Untreated	5	4.037	1.97526
Treated	4	3.967	1.99127
Untreated	6	54.319	0.10244
Treated	5	54.331	0.05044
Untreated	1	1.679	0.00912

3.3.3 Differential Scanning Calorimetry (DSC)

The DSC was performed to analyse the thermal transitions, of placenta and $_{SC}CO_2$ treated placenta samples (Figure 3.4). The enthalpies for each peak were presented in Table 3.5.

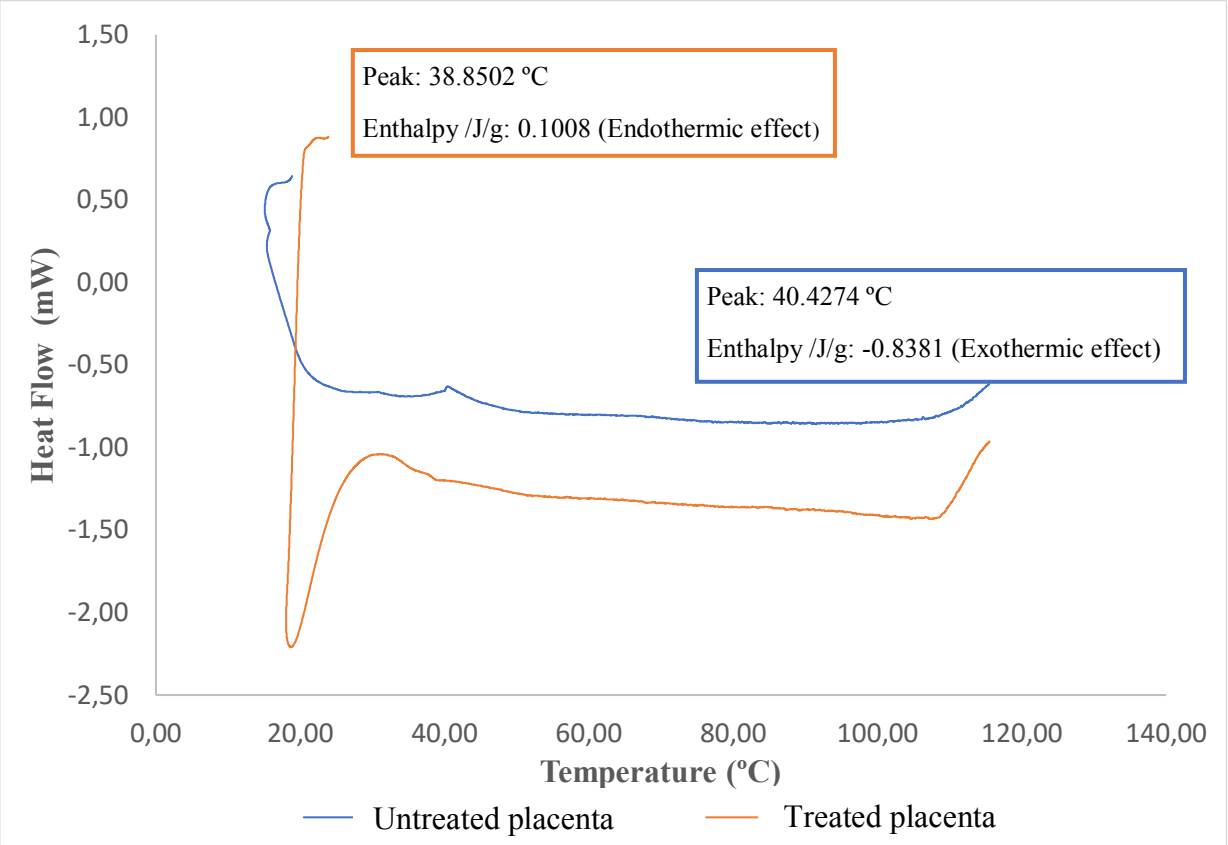


Figure 3.4– Comparison of DSC thermograms of untreated and treated placenta.

Table 3.5–DSC thermograms peaks and respective enthalpies.

Sample	Peak (ºC)	Enthalpy (J)
Untreated	40.4274	-0.8381
Treated	38.8502	0.1008

The first thermal transition occurred around at 40.4 ºC and 19.0 ºC for untreated and treated placenta, respectively. At 19.0 ºC, for the treated samples, the endothermal effect may be representative of denaturation of a protein collagen. Around 31.2 ºC a thermal transition was observed in the treated placenta sample that was not detected in

the untreated sample. The second thermal transition occurred around 96.8 ºC and 108.6 ºC for untreated and treated placenta, respectively. DSC presented different thermal profiles of the samples, untreated placenta exhibited exothermic effect, possibly representative of a physical state change, while treated placenta exhibited an endothermic effect. The increasing of enthalpy values maybe indicates a matrix alteration via cross-linking.

3.3.4 Mechanical Tests

In Figure 3.5, is represented the stress-strain curve of the placenta samples. The values of Young's modulus, stress and strain at burst obtained after the tensile test performed to the placenta samples were evaluated and compared as presented in Table 3.6.

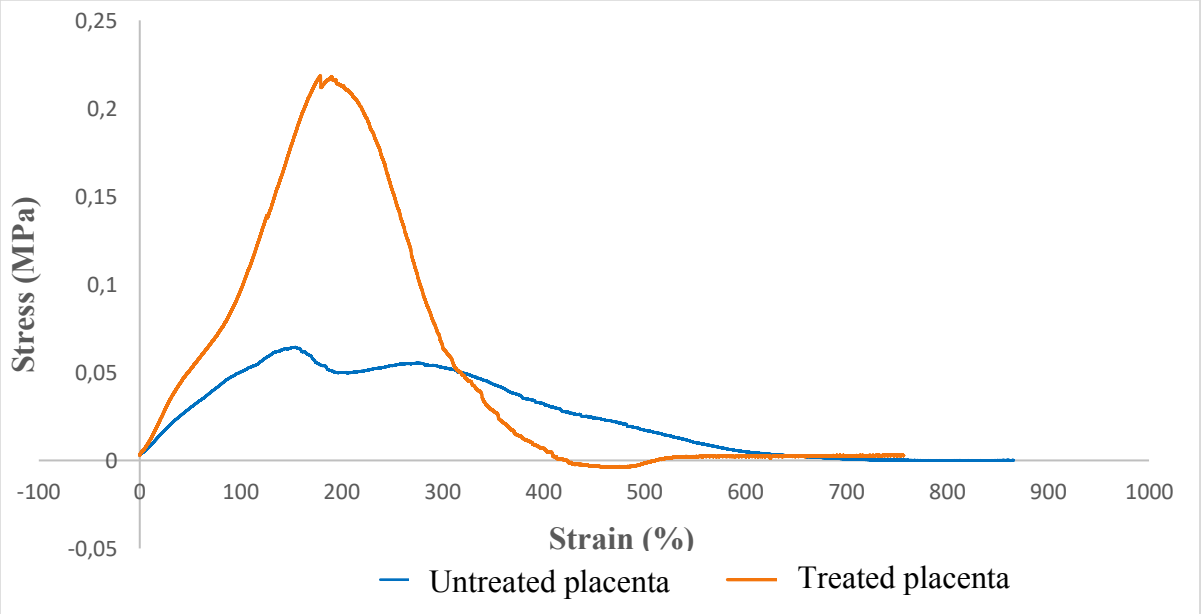


Figure 3.5– Representative stress-strain curves of untreated and treated placenta samples.

Table 3. 6 – Calculated values of Young´s modulus, stress and strain at burst.

Sample	Young's modulus (Kpa)	Stress at burst (Kpa)	Strain at burst (Kpa)
Untreated	0.535	116.542	43.722
Treated	1.345	189.635	149.011

By the analysis of the stress-strain curve and the values in Table 3.6, it can be stated that treated placenta samples undergo considerable changes in its physical properties. Treated placenta was significantly stiffer than untreated placenta sample. The values of Young´s modulus, stress and strain at burst are significantly higher for sterilized placenta.

4. Discussion

4.1. Different Processing Parameters on $_{SC}CO_2$ Sterilization Efficiency

Bacterial spores are microorganisms very resistant to adverse environmental conditions and, therefore, are used as biological indicators in sterilization processes (Booth, 1998) (Hemmer et al., 2007). In addition, the nutritious medium used in the validation tests, TSB, allows the microbiological growth as soon as there is the minimal prevalence of live bacteria. An absence of growth in these tests will translate in an efficient sterilization. The exclusive use of $_{SC}CO_2$ in the sterilization process does not ensure the complete elimination of all types of contaminating microorganisms, at mild conditions of temperature/pressure (Balestrini et al., 2016). In the work of J. Zhang *et al* (2006), the $_{SC}CO_2$ sterilization with 200 ppm of H_2O_2 presents better inactivation results than a sterilization without the use of additives. It was assumed that the permeability barrier of the spore, spore envelope, is destroyed by the $_{SC}CO_2$ allowing the penetration of H_2O_2 which oxidizes the internal structures, leading to the spore death, as hypothesized by Zhang *et al* (2006) using *B. pumilus* endospores. Hemmer *et al* (2007) also demonstrated that the use of H_2O_2 (<100 ppm) as an active additive to the process corresponds to a greater population reduction in *G. stearothermophilus* and *B. atrophaeus* spores. Compared to $_{SC}CO_2$ sterilization without additives, with distilled H_2O as a passive additive and with H_2O_2, as active additive, the H_2O_2 added allows more efficient sterilization with lower temperatures, lower pressures and shorter cycle times. In addition to H_2O_2, other additives have been used such as ethanol to inactivate *B. cereus* (Park et al., 2013) and PAA for *G. stearothermophilus* and *B. Subtilis* (White et al., 2006), and have demonstrated to be efficient in sterilization. In the present thesis, the use of H_2O_2 (300 ppm) has been adopted as a process additive, during all the sterilization cycles carried out.

There is a lack of information about the times indicated for the pressurising and depressurising stages, but it is assumed that abrupt injections/degassing of the carbon dioxide could damage more delicate materials. In a specific case using lung tissue, the slow depressurising rate (30 to 45 minutes) allowed it to maintain the temperature at 25 ºC, preventing tissue damage, through freezing and CO_2 expansion in the lung (Balestrini

et al., 2016). Therefore, depressurising and pressurising stages should be controlled and performed slowly.

The optimization of the mildest conditions for effective $_{SC}CO_2$ sterilization were conducted at 2 distinct pressure values (140 bar and 245 bar) and 6 different cycle periods (1 to 6 hours of effective sterilization). Regarding the results obtained from the validation of microbial growth, the 6 (#**1** to #**6**) sterilizations performed at 140 bar, indicated that sterilization cycles of 1 and 2 hours were insufficient for the sterilization of the tested spore species (Table 3.1). In the contrary, the following effective sterilization cycles equal or higher than 3 hours (#**3**, #**4, #5** and **#6**), at the same pressure (140 bar), allowing an inactivation of all bacteria spore strains. Except for a single replicate of *B. pumilus*, which growth was most probably caused by contamination during laboratory manipulation procedures. The sterilization processes carried out at 245 bar (#**7** to #**12**) revealed very different results from the ones obtained at 140 bar. In fact, 3 hours of effective sterilization process was not enough to prevent the microbial growth of all spore species. At this point the focus was to determine the minimum time for effective sterilization, which revealed to be 3 hours when the sterilization process is carried out at 140 bar and 5 hours when using 245 bar. Logically, at 245 bar less effective time was expected to perform sterilization efficiently. It would therefore be essential to repeat the sterilizations at this pressure value to see if the results prevail. These results are similar to what has been reported in the literature by Zhang & Burrows *et a*l (2006) efficiently sterilized *B. pumilus* within 4 hours at 60 °C, 275 bar and with 200 ppm H_2O_2; Donati *et al* (2012) sterilized *G. stearothermophilus* using a 4 hours process under the conditions: 40 °C, 270 bar and with 200 ppm H_2O_2; Balestrini *et al* (2016) stated that 3.5 hours of $_{SC}CO_2$ sterilization, including preconditioning and $_{SC}CO_2$ exposure, at 35 ºC and 100 bar it is sufficient to sterilize *B. atrophaeus* efficiently. According to Hemmer *et al* (2007), under similar conditions (40 ºC, additive: H_2O_2 and pressure at 203 and at 304 bar), complete deactivation of *G. stearothermophilus* and *B. atrophaeus* spores was observed after a 1 hour sterilization treatment. This referred work was carried out with an industrialized machinery composed by three separate systems which allows a better use of the CO_2.

The effectiveness of terminal sterilization of *B. pumilus* and *B. atrophaeus* spores using $_{SC}CO_2$ as function of the time interval between the sterilization cycle and the start of the turbidity tests was assessed by the following 12 sterilizations (#**13** to # **24**) and considering the previously results of microbial growth at 140 bar. Strain *B.*

stearothermophilus being used for sterilization tests by autoclaving, is of the 3 strains less resistant and thus was left aside in this study. Herein, the 12 sterilization cycles were executed at 140 bar and with an effective sterilization cycle of 2 or 3 hours. From this study, it was possible to analyse and compare the behaviour of both strains according to its shelf life, i.e. the time interval between the sterilization cycle and its application, in this case, the start of the turbidity tests. Namely, *B. pumilus* and *B. atrophaeus* strips were placed in growth media right after the sterilization treatment (day 0) or after 1 day, 7 days or 30 days.

For the *B. pumilus* spore strips, with 2 hours of effective sterilization, increasing the shelf life from 0 and 1 day to 7 and 30 days would prevent the microbial growth. Previously, it has been shown that it was not possible to efficiently sterilize *B. pumilus* strips with 2 hours of effective sterilization. However, by increasing the shelf life, the sterilization, under these conditions, may present non-growth results. For the same strain, with 3 hours of effective sterilization, it has been shown that the shelf life did not interfere with sterilization efficiency. On the other hand, for shelf life of 1 and 7 days microbial growth occurred probably due to manipulation errors causing contamination in the spore strips.

In the case of *B. atrophaeus* spores treated with a cycle of 2 hours of effective sterilization, the increase of the shelf life led to absence of microbial growth. For the cycle of 3 hours of effective sterilization, the same variable had no effect since no growth occurred in any of the sterilization treatments. Briefly, it was possible to sterilize spore strips of the *B. pumilus*, *B. stearothermophilus* and *B. atrophaeus* strains under the following conditions: 3 hours of effective sterilization, 40 °C, 140 bar, 600 rpm, with addition of 300 ppm H_2O_2 and a shelf life from 1 day to 1 month.

These results indicate that the effective sterilization of 3 hours is enough to kill all the spores of the strips. Probably for this period of effective sterilization the shelf life will not be an important variable. At 2 hours of effective sterilization, most of the spores are not killed but are damaged in some way. After the sterilization is completed, a delayed effect caused by CO_2 and/or H_2O_2 that is still inside the spores and which in the same way ends up inactivating them is evidenced for longer shelf life periods. Assuming this hypothesis, CO_2 and H_2O_2 are diluted in the culture medium if the strips are introduced into the culture medium immediately after sterilization is complete. Thus, the delayed effect is cancelled and the spores are able to maintain their viability. Thus, shelf life is an important variable

for reduced-period sterilizations. Differently, shelf life periods greater than 1 week may be inconvenient since they make the process too long and are not recommended for perishable materials. It is important to ensure that the various materials like some prostheses and polymers during the shelf life retain their physical and chemical intrinsic properties. These promising results can be the basis for other studies aimed at analysing the influence of shelf life on the sterility and durability of a panoply of biomaterials and on their successful implementation on a patient.

4.2. Effective Sterilization of placenta by $_{SC}CO_2$

Placenta tissue has a complex and heterogeneous composition and its structure is composed essential by collagen. Placenta extracts are used in the medical field, by the Japanese companies Melsmon (Tokyo, Japan) and Laennec (Tokyo, Japan), for menopause and chronic hepatitis treatment, respectively. Other Japanese company, Plantec Co (Osaka, Japan), created a drink with raw placenta which claims to help in the activity of human metabolism. According to the American company Americord (New York, USA), placental tissue can be applied, in a near future, on a wide range of medical conditions, including: heart and liver diseases, spinal cord injuries and many more. Lyophilized and decellularized human placenta samples were sterilized using $_{SC}CO_2$ and further analysed in terms of physical and/or chemical characteristics. FTIR and HPLC techniques allowed to compare the chemical composition of placenta samples before and after $scCO_2$ sterilization treatment. FTIR spectra identified similar absorption bands for the placenta samples before and after treatment by $_{SC}CO_2$, corresponding to the functional groups for amide I, II and III. In the same way, bands were identified for the functional group C-H (alkane) and N-H (amine) stretching vibrations in both spectra. The amide groups, associated with the collagen molecules present in the placenta and the alkane and amine stretching vibrations, associated with lipid alkyl chains, did not suffer changes in chemical composition caused by $_{SC}CO_2$ treatment. Regarding the $_{SC}CO_2$ sterilization of amniotic membrane tissue graft, Wehmeyer *et al* (2015) demonstrated, by FTIR and DSC analyses that the sterilization imposes minimal to no changes in molecular organization of extracellular matrix protein present in the graft. FTIR analysis showed no significant changes in the spectral frequencies corresponding to the amide absorption peaks typical to collagenous tissues.

Comparing the HPLC chromatograms of both unprocessed samples and samples that undergo $scCO_2$ sterilization treatment, the detected peaks revealed similar values of retention and maximum absorbance. Despite the increase in temperature to allow separation efficiency of the components, the maximum absorbance values were quite identical; however, the values of maximum absorbance differ greatly in the last peaks of both chromatograms of the untreated and treated samples. This may be correlated with decreased composition of a specific placental tissue compound caused by the sterilization process. Wehmeyer *et al* (2015) demonstrated by FTIR and DSC analyses that $_{SC}CO_2$ sterilization imposes minimal to no changes in molecular organization of extracellular matrix protein present amniotic membrane tissue grafts. FTIR analysis showed no significant changes in the spectral frequencies corresponding to the amide absorption peaks typical to collagenous tissues. The influence of $_{SC}CO_2$ sterilization on the physicochemical properties of natural and synthetic biomaterials has been little explored. An experiment by Dai *et al* (2016) reported that $_{SC}CO_2$ sterilization did not cause any damage in the structural properties of biodegradable scaffolds. The treatment did not create new radicals or reactive species. Dillow *et al* (1999) demonstrated that $_{SC}CO_2$ did not cause any changes in the physical and chemical properties of the biodegradable polymers poly(lactic-co-glycolic) acid (PLGA) and polylactic acid (PLA).

In contrast to the FTIR and HPLC tests, DSC and the mechanical analysis showed significant differences between the treated and untreated placenta samples. The thermograms obtained by DSC assays demonstrated that the untreated sample had an exothermic effect whereas another sample of treated placenta presented an endothermic effect, indicating changes in the composition of the placenta after the sterilization process. The denaturation of the collagen molecule, in solution, occurs around 39-40 ºC (Miles & Bailey, 1999). For the treated sample an endothermic effect occurred around 19.0 ºC indicating a substantial denaturation of the collagen (Hennessy et al., 2017). This value does not coincide with the denaturation temperatures of the collagen and with the detected peak of the untreated sample. The enthalpy values of the treated sample are greater than the untreated indicating that the sterilization altered the matrix via cross-linking.

Placenta samples presented a different mechanical behaviour before and after the sterilization treatment. Namely, the treated samples revealed a Young's modulus significantly higher than the untreated placenta samples. This means that the sterilization process conferred an increase of the strength to those structures. Sterilization have also

contributed to remove any residual unbounded water from the biological samples. These physical modifications can be reduced or even eliminated if certain sterilization parameters are controlled. Indeed, placental sterilization was performed with the addition of 300 ppm H_2O_2 and with 6 hours of effective sterilization cycle. Nevertheless, after gather knowledge about the best processing parameters it would be interesting to repeat the sterilization of placenta samples with the shorter cycles as the ones previously optimized (3 and 4 hours) in order to verify any change in the final properties. Also, it would be also interesting to carry out a histological evaluation, to compare the tissues before and after $_{SC}CO_2$ sterilization.

5. General Conclusions

Considering the results obtained, it is possible to effectively sterilize by $_{SC}CO_2$ the spore strains: *B. pumilus*, *B. atrophaeus* and *B. stearothermophilus* under the following procedural conditions: 40 ° C, 600 rpm, 3 to 6 h of effective sterilization, at 140 or 245 bar and with 300 ppm of H_2O_2. In order to sterilize *B. atrophaeus* in 2 hours of effective sterilization, it is suggested to add 1 week of shelf life to the strips for sterilization to be efficient. Sterilization of *B. pumilus* with 2 or less hours of sterilization is not advisable according to the results. It is clear that certain spores are more resistant than others, as it is possible to eliminate the action of these very resistant microorganisms, the results obtained are interesting but required the continuation of the research and accomplishment of future work. The sterilization at 245 bar should be tested for 2 and 3 hours of effective sterilization in a future work. The analysis of the action of H_2O_2 in the process should also be analysed, thus carrying out sterilizations with less quantity of the same.

In relation to placenta treatment by $_{SC}CO_2$ sterilization, HPLC and FTIR tests did not address any significant changes in the compositions of treated and untreated samples. Nevertheless, the reason for some values to differ may be due to the heterogeneity of the samples. DSC analysis demonstrated different thermal behaviours when exposed to a temperature rise. The placenta matrix suffered alterations via cross-linking. Mechanical tests reported an increase in stiffness of the treated sample when compared to the untreated sample. In the next work, tests will be carried out in order to know the ideal conditions to sterilize biological samples without damaging them.

Biomaterials are assuming an increasingly important role in many areas and performing a wide variety of functions. Today's biomaterials are more complex and sophisticated, which require sterilization under specific conditions so as not to alter their properties. Because $_{SC}CO_2$ sterilization operates under low pressure and temperature conditions, it can be adapted and used in various biomaterials and medical devices that are not sterilized by the procedures already known in the market. It has all the potential to be next emergent technique in the sterilization market. However, it is important to analyse and test other biological materials with this technology and new methods of chemical and physical analysis will be used.

6. Ongoing Projects

6.1 Human Tissue and Bone

There is an increase in the use of musculoskeletal allografts by the knee surgeons for various purposes such as: ligament reconstruction, meniscal transplantation, and articular surface reconstruction (Vangsness et al., 2003). Parallel to this, the doctors show some concern about the safety and the possibility of the sterilization process to negatively affect tissue biology and biomechanics. Nowadays, the methods used for sterilization of allografts are gamma sterilization and EtO, which in certain cases may decrease the mechanical properties (McAllister et al., 2007)(Mroz et al., 2006). Due to this, new methods need to be developed and tested to sterilize musculoskeletal allografts.

In this way, the work to be performed is based on the sterilization of human bone and tissue samples. The main objective is to evaluate the possible causes of $_{SC}CO_2$ sterilization in biological samples in relation to structure, mechanics, composition and histology. For such, several tests will be performed on samples before and after sterilization, which will serve as a comparison. In a first analysis, it is estimated that the sterilizations are carried out with 5 hours of effective sterilization, at 140 bar with, at least, 1 week of waiting time.

The work is done in collaboration with Dr. Paulo Amado and Hospital Lusíadas. Bone and tissue were obtained from 4 ankle and hip. Samples were obtained from Hospital Lusíadas (Porto, Portugal) with informed consent from patients and after ethical approval from Ethics Committee for Health from the referred hospital.

6.2 Human Platelet Lysate (hPL)

Human platelet lysate (hPL) motivated interest in the scientific community due to the possibility of its use as an appropriate substitute for fetal bovine serum (FBS) in culture. Platelet lysate is a human blood component which has in its constitution several growth factors (Delfina & Gonzalez, 2016). Several studies detailed the disadvantages of using FBS and the importance of and the potentialities of hPL. Platelet lysate can be used for human mesenchymal stem cells (hMSC) expand in flatbed cultures to maintain their multipotency and therapeutic properties (Delfina and Gonzalez, 2016). Lepperdinger et al (2008) concluded in their studies that using hPL instead of bovine serum in hMSC culture increases proliferation rates without changing morphology, immunophenotype or differentiation capacity.

Also, Food and Drug Administration (FDA), European Medicines Agency (EMA) and International Good Manufacturing Practice (GMP) proposed guidelines for minimizing the use of animal products due to related risks. It is considered that methods involving the processing of FBS are not adequate to guarantee the quality of the hMSCs for use in human therapeutic. FBS may allow the transmission of pathogens, prions and mycoplasmas when used for the expansion of hMSCs intended for clinical trials (Gstraunthaler, 2003) (Bieback et al., 2009).

The future is aimed at verifying the efficiency of sterilization in human platelet lysate. This project is carried out in collaboration with the company Stemmatters (Braga, Portugal).

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