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Preservação de leite com chocolate por armazenamento hiperbárico, uma nova tecnologia de preservação amiga do ambiente

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Chocolate milk preservation by hyperbaric storage, a new and environmentally friendlier preservation technology

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em 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 e ao meu irmão por sempre acreditarem em mim e sempre tornarem possíveis os meus sonhos.

o júri

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Palavras-chave Armazenamento hiperbárico, Leite com chocolate, Avaliação microbiológica, Avaliação físico-química, Conservação de alimentos.

Resumo

O principal objetivo deste trabalho centrou-se no estudodo efeito do armazenamento hiperbárico (AH) à temperatura ambiente, em leite achocolatado, de modo a evitar a sua deterioração a nível microbiológico e físico-químico, tentando assim aumentar o prazo de validade do produto comparativamente ao método de armazenamento mais tradicional, a refrigeração.

O AH foi estudado e comparado com o armazenamento refrigerado e armazenamento à temperatura ambiente (0.1 MPa). Assim, diferentes temperaturas (4 e15-25 °C) e níveis de pressão (0.1, 30, 50 e 100 MPa) foram utilizados durante um período de 30 dias. Algumas amostras foram inoculadas com *Listeria innocua* e *Escherichia coli* de forma a aferir o efeito do AH para nestes microrganismos.Um segundoestudo foi também realizado com amostras embaladas de duas diferentes formas, com ar e sem ar no seu interior, numa tentativa de concluirsobre possíveis diferenças a nível microbiológico e físico-químicopotenciadas pela presença de ar na embalagem quando armazenadas sob pressão.Em ambos estudos realizaram-se análises microbiológicas (mesófilos totais, *Enterobacteriaceae*, coliformes epsicrófilos) e físico-químicas (pH, A_w, cor e conteúdo em lactose e açúcares redutores), para além de visualização do produto emmicroscopia eletrónica.

Foram obtidosmelhores resultados no AH comparativamente à refrigeração, ocorrendo inibição de crescimento microbiano a 50 MPa e inativação de microrganismos a 100 MPa, sendo os mesofilos totais e os psicrófilos os microrganismos menos suscetíveis à pressão. No que respeita a parâmetros físico-químicos, o AHrevelou resultados semelhantes ou até melhores que a refrigeração. Assim, o AHà temperatura ambiente demonstrou ser uma possível alternativa à refrigeração para leite achocolatado, sendo esta uma metodologia amiga do ambiente uma vez que só é necessária energia na fase de compressão e descompressão, não sendo requerida para controlo de temperatura.

Keywords Hyperbaric storage, chocolate milk, microbiological evaluation, physicochemical evaluation, food preservation.

Abstract

The main goal of this work was to study the effect of hyperbaric storage (HS) at room temperature (RT)in a chocolate milk beverage, in order to avoid its deterioration at the microbiological and physicochemical level, trying at the same time to increase its shelf life without refrigeration.

HS was studied and compared with refrigeration (RF) and RT storage (0.1 MPa). The chocolate milk was stored at different temperatures (4 and 15-25 ºC) and pressure levels (0.1,30,50,100 MPa) over 30 days. Some samples were inoculated with *Listeria innocua* and *Escherichia coli* in order to study the HS effect on those microorganisms.

In addition, a second study was carried out with samples packed with and without air to observe possible microbiological and physicochemical differences empowered by the presence of air. For both studies,microbiological (total aerobic mesophiles, *Enterobacteriaceae*, coliforms, psychrophiles), physicochemical (pH, A_w, colour, lactose and reducing sugars content) and SEM analyses were performed.

HSallowed to obtain better results than RF, with a microbial growth inhibition at 50 MPa and a microbial inactivation at 100 MPa. The total aerobic mesophiles and psychrophileswere the microorganisms less susceptible to pressure. Regarding the physicochemical parameters analysed, HS showed similar or even better results than RF.

Therefore, food storage under pressure (HS) at RT, demonstrated to be a possible alternative to RF for chocolate milk, being this preservation methodology environmentally friendly, since energy is only required during compression/decompression phases, not being needed for temperature control.

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III. List of abbreviations

AP	Atmospheric pressure
CCA	Coliforms count agar
COL	Coliforms
DRI	Dietary recommended intake
ENT	*Enterobacteriaceae*
HPP	High pressure processing
HS	Hyperbaric storage
IU	International unit
MPa	Megapascal
PALCAM	Listeria identification agar base
PCA	Plate count agar
PSY	Psychrophiles
RF	Refrigeration
RT	Room temperature (15-25 ºC)
SFA	Saturated fatty acids
TAG	Triacylglycerol
TAM	Total aerobic mesophiles
UHT	Ultra-high temperature
USFA	Unsaturated fatty acids
VRBDA	Violet red bile dextrose agar
YM	Yeasts and moulds

1. INTRODUCTION

1.1 Overview

The progress and use of new technologies in food processing targets specific consumer requirements for safer, healthier, and minimally processed food products. These ground-breaking processes also include environmentally friendly and sustainable food manufacturing techniques, with low energy consumption and reduced water use that overwhelmed some restrictions given by present food processing performs (Knorr et al., 2011).

Gradually, consumers have been growing their knowledge about health benefits and risks associated with the intake of each food product. To come across consumers' perspectives, the food industry is committed to spending considerable capitals and know-how in the production of healthier and safer food products. This includes inspecting materials arriving from the food chain, suppressing microbial growth, and decreasing or eliminating the microbial load by processing and preventing post-processing contamination maintaining product quality (Lado & Yousef, 2002).

Diet and health have a relationship that is now well known to be one of the keys to avoiding disease and promoting wellbeing. Functional foods exert a positive influence on human health above their nutritive value, such as dairy products. Milk is a well-known food for being a rich source of bioactive components that have positively influence in health (Mills et al., 2011). Milk is a valuable source of important nutrients such as proteins, fats and carbohydrates and it is widely consumed. This food provides several physiologically functional compounds including bioactive peptides, antioxidants, essential vitamins/minerals, and nutritionally required fatty acids (Chung et al., 2016).

1.2 High pressure application on food

Fresh, healthy, minimal-processed food products with natural flavor and taste, as well as prolonged shelf life, are the characteristics that consumers showed preference for food products (Aymerich et al., 2008).

Temperature is an important thermodynamic parameter, but so is the pressure.High pressure processing (HPP) is a promising "non-thermal" techniquethat can affect molecular systems (Rastogi et al., 2007). This method isextensively used for food products preservation, allowing the inactivation of vegetative microorganisms most frequently related to food-borne diseases. HPP uses intense pressure, in the range

of 100-1000 MPa (**Figure 1**), at cold or even at elevated temperatures, in order to reach safety food products, with the most foods not being considerable affected on taste, texture or nutritional characteristics. The main advantage of HPP compared to thermal treatments is the maintenance of the sensorial and nutritional properties of food products (Balasubramaniam et al., 2008).

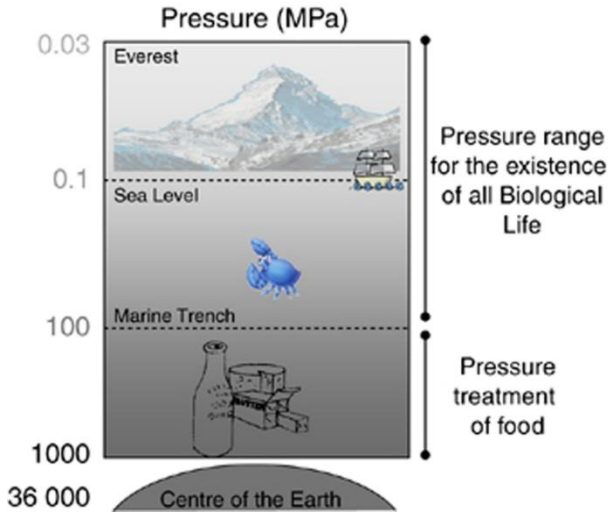


Figure 1. Representation of the pressures used in food processing. Adapted from (Considine et al., 2008).

1.2.1 HPP history

HPP technology has been discovered for more than a century as a preservation technique. Hite, 1899 was the first to publish that milk and fruit can be preserved by HPP at 680 MPa. Hite, 1899 showed that the shelf life of raw milk could be extended for 4 more days after pressure treatment at 600 MPa for 1 hour at RT. Years later Hite & Giddings, 1914 found that fruits treated with pressure remained commercially sterile for at least 5 years after processing at pressures ranging from 400 to 820 MPa. The HPP study forfood preservation started in the early 1990s, and can be considered the launch of industrial HPP biotechnology (Rastogi et al., 2007).

1.2.2 HPP process description and equipment

Inthe HPP, the packaged food is placed in a transporter and automatically loaded into the HPP vessel, being at the endthe vessel plugs locked. The pressure media, typically water, is driven to the vessel from one or both sides. After reaching the desired pressure the pumping is stopped, and no further energy effort is necessary to hold pressure during time. On the other hand, contrarily to thermal processing in which

temperature gradients occur, all molecules in the HPP vessel are exposed to the same amount of pressure at exactly the same time due to the isostatic principle of pressure diffusion (Rastogi et al., 2007; Heinz et al., 2009). The adiabatic heat of compression during processing estimated that temperature increases 3-9 ºC for each 100 MPa,beingneeded a few minutes to achieve the desired pressure (Knorr et al., 2011; Butz & Tauscher, 2002).

Through the years, the HPP equipment's development was considerable in gain of volume and increase of pressure. The volume of the vessel may vary from less than 100 mL under pressures above 1000 MPa in laboratory scales to an industrial scale equipment that can support more than 500 L with pressures beyond 600 MPa due to its size and material resistance (Hendrickx et al., 2001).

The basic principles that determine the behaviour of foods under pressure are Le Chatelier's principle and Isostatic principle. Le Chatelier's principle states that whenever an equilibrium system is disturbed, it tends to change to another equilibrium. Therefore, any reaction, conformational change, phase transition, attended by a decrease/increase in volume is improved/worse by pressure (Smelt, 1998). The isostatic principle states food products are compressed by uniform pressure from every direction and then reverted to their original shape when the pressure is released. The samples are compressed independently of the product size and geometry because transmission of pressure to the core is not mass/time dependant thus the process is minimized. If a food product contains sufficient moisture, pressure will not damage the product at the macroscopic levels as long as the pressure is applied uniformly in all directions (Smelt, 1998).

1.2.3 HPP impact on microorganisms

It can be predictable that the mode of action of pressure on whole organisms is not necessarily the same, but dependent on the pressure level applied. Hydrostatic pressures between 30 and 50 MPa can affect gene expression and protein synthesis. Usually, at pressures of ~100 MPa the nuclear membrane of yeasts isaffected and at >400-600 MPa more changesoccur in the mitochondria and cytoplasm (Smelt, 1998).

In literature it is proved that 50 MPa of pressure can obstruct protein synthesis in microorganisms and decreases the number of ribosomes. Pressure under 100 MPa can make partial protein desaturations, and 200 MPa causes damage to the cell membrane and internal cell structure (Huang et al., 2014).

1.2.3.1 Bacteria and bacterial spores

Bacteria are responsible for food poisoning and Gram-positive are considered more resistant to heat and pressure than Gram-negative bacteria (Smelt, 1998). This resistance is due to the rigidity of teichoic acids in thick peptidoglycan layer of the gram positive cell wall (Lado & Yousef, 2002).

The mode of action of pressure on bacterial spores is quite a matter of conjecture. Bacterial spores are killed directly by pressures higher than 1000 MPa. However, spores are sensitive to pressures between 50 and 300 MPa. It is commonly established that at such pressures, spores germinate, followed by death of the germinated spore. Activation of spores under atmospheric conditions is frequently needed prior to germination. Low pH or heat can be carried by activation of spores, but it seems to be a reversible event, but normally it is frequently tracked by germination (Griffiths & Walkling-Ribeiro, 2012).

1.2.3.2 Yeasts and moulds

Yeasts and moulds (YM) are a main problem because they can produce toxins. Under pressure, YM are sensitive and can be inactivated using relatively low pressures within 100 MPa which cause damage in cell wall of yeasts and at pressures of 200-300 MPa occur total inactivation of YM by damage of nuclear membrane, cytoplasm and mitochondria (Smelt, 1998).

1.2.3.3 *Escherichia coli*and*Listeria monocytogenes*

It is common that heat-resistant microorganisms are also more resistant to pressure, but there are numerous exceptions. Gram-positive bacteria are more resistant to heat and pressure than Gram-negative bacteria (Cheftel, 1995). *L. monocytogenes* is a Gram-positive bacteria and for that it is more resistant to pressure and temperature (Smelt, 1998). This bacteria can grow under RF without presence of oxygen, but under pressure, for instancein milk, inactivation can occur at 300, 400, 600 MPa, with decimal reduction times (D) of 14.03, 9.00,and 3.04,respectively (Dogan & Erkmen, 2004).

E. coli is a Gram-negative bacteria that is facultative anaerobic and does not produce spores. In milk between 0 fat (skimmed); 3.25 (whole) and 5% (high fat) it is shown that fat content has no significant influence on the HPP destruction of *E.coli*. Furthermore, it was found that the major contributors for baroprotection of *E.coli* in

milk during HPP treatment appear to be casein and lactose, rather than the fat content (Ramaswamy et al., 2009; Georget et al., 2015).Concerning pressure sensitive processes on microorganismsit is described that *E.coli*at low pressures, between 50 MPa and 60 MPa, shows some interference in DNA replication and growth, and at 77 MPa problems in the transcription process can occur (Mota et al., 2013).

1.3 Hyperbaric storage

Infood industry, RFis responsible for 50% of the total energy consumed, and thisenergy consumption contributes largely to the increase of carbon dioxide (CO_2) emission footprint(Bermejo-Prada, 2014).Recently and regarding this problem, more efforts have been made in the agro food industry to increase the efficiency of the food preservation technologies, finding new ways/technologies, better for the environment, with low energy costs(James & James, 2010; Tassou et al., 2010).

An accident that occurred 40 years ago revealed the possibility to preserve food products under pressure, since afterthe sinking of the research Submarine Alvin over 10 monthsat a depth of ~1540m (15MPa and 4ºC), well preserved food such as bouillon, apples and sandwiches were discovered. This new finding brought a new possibility to store food and other biomaterials under pressure, and it was named of Hyperbaric Storage(HS) methodology (Jannasch et al., 1971).

HS meaning food storage under pressure that preserves the quality of the product by microbial growth inhibition, brings a new way to preserve products without need of RF to keep their characteristics.At the beginning, studies concerningHSweretestedat very low temperatures with pressure, but over the years the tendency has been increasing it in order to useHS at RT. This approach brings the possibility to store food products at variable RT, reducing energy costs sinceenergy is only requiredin the compression/decompression phases to reach the desired pressure level, and no other energy is necessary to sustain food products under pressure during storage period(Fernandes et al., 2014).

In Japan, studies regardingHS at sub-zero temperatures showed promising resultson microbial growth inhibition, however, on the other hand the energy consumption is similar as the freezing method (Kalichevsky et al., 1995; Hendrickx & Knorr, 2001). Recent studies show the possibility to use low positive temperature and HPP to maintain food quality parameters for substantial periods of time(Kalichevsky, 1995; Charm et al., 1977). Nonetheless, otherHS studies carried out at RT and up to

37ºC provided higher energy savings(Queirós et al., 2014; Segovia-Bravo et al., 2012; Romanazzi et al., 2008). The state of the art will be addressed inmore detailin this work.

This new technology is important for the food industry as an alternative to the traditional frozen and refrigerated storage of foods. More importance is given to HS under naturally variable RT, since the energy costs are much lower than the conventional methodology (Fernandes et al., 2014). With this new technology it is possible not only to preserve the original characteristics ofthe product over storage but also to increase itsshelflife(Jannasch et al., 1971).

There are already several studies related to the influence of pressure on microorganisms in food processing at high pressures and during short periods. However, there are few studies exploring the microorganism's behaviour over long storage periodsatlow pressures.

1.3.1 Hyperbaric storage at sub-zero temperatures

The purpose of using HS at sub-zero temperatures allows to decrease the water freezing point, being possible to have liquid water at -22 ºC and 209 MPa by modification of low temperature and high pressure (Liplap et al., 2014). Thus, it is possible to have food products not subjugated to freezing and thawing processes, maintaining the nutritional properties and particularly texture characteristics (Charm et al., 1977).

Concerning HS at sub-zero until today, four studies were done in cod fish fillets, Pollock, chicken and beef (22,8 MPa for 36days at -3 ºC)(Charm et al., 1977), beef (200 MPa at -20 ºC)(Deuchi & Hayashi, 1990), strawberries and tomatoes (50 to 200 MPa at -5 to 20 ºC)(Deuchi & Hayashi, 1992) and chicken and carp (170 MPa for 50 days at -8 and -15 ºC) (Ooide et al., 1994)(see **Table 1**).

The first HS study at sub-zero temperatures, which was made by Charm et al., 1977 in codfish fillets showed that samples maintained its initial microbial load. In other experimental studies, done in raw pork, beef, strawberries and tomatoes stored under 50-200 MPa, at -5 to -20 ºC, the colour and fresh flavour were preserved, and the microbial counts of most microorganisms decreased (Deuchi & Hayashi, 1990; Deuchi & Hayashi, 1991). The enzymatic activity assessment in food is important since the enzymatic activity is related tochanges in the food product characteristics.Thus,Deuchi & Hayashi, 1992showed that some enzymes activity (catalase, β-amylase, cathepsin and lactate dehydrogenase) werereduced under sub-zero HS conditions (200 MPa and -20

ºC), but not inactivated as observed in the freezing process, preserving the characteristics of the product.

In the close combination of temperature and pressure at sub-zero temperature, food products shelf-lives can be extended as a result of dropping the enzyme and microbial activities. This method had demonstrated to be similar to better than freezing on the reduction of the microbial loads, avoiding damages caused by freezing/thawing (Charm et al., 1977).

Table 1:Studies regarding HS at sub-zero (adapted from Fernandes et al., 2014).

Product	Conditions	Results	Reference
Sub-Zero Temperature			
Cod fish fillets, pollock, chicken and beef	22.8 MPa for 36 days at -3 °C	Stable for at least 36 days. Classified with only 7 days of shelf life at 0.1 MPa.	Charm et al., 1977
Beef	200 MPa at -20 °C	Microbial load reduction and inactivation of yeasts and some bacteria.	Deuchi & Hayashi, 1990
Strawberries and tomatos	50 to 200 MPa at -5 to -20 °C	Stable for a few days/weeks. Fresh flavour and colour were preserved.	Deuchi & Hayashi, 1992
Chicken and carp	170 MPa for 50 days at -8 and -15 °C	Stable for 50 days. Enzymatic activity was reduced.	Ooide et al., 1994

1.3.2 Hyperbaric storage at refrigeration temperatures

As mentioned before, HS at RF temperatures was discovered at the time of the research submarine Alvin sinking(Jannasch et al., 1971). Some years later a similar studied showed the effect of 24 MPa at 1 ºC in cod fish fillets and Pollock (Charm et al., 1977). The results obtained showed that under pressure the microbial load remains stable over storage. The samples subjected to HS were considered acceptable for consumption after 12 and 21 days of storage for Pollock and codfish, respectively.

Studies were done concerning enzymes and physicochemical properties under different conditions, including pressure and temperature (see **Table 2**). Trypsin and peroxidase were studied at temperatures of -3, 0, 4 and 23 ºC and pressures of 0, 27.6,

34.5 and 41.3 MPa. The authors concluded that the increase in temperature and pressure caused a decrease in the enzyme activity at constant pressure and temperature, respectively. Nonetheless, trypsin activity increased and decreased when the pressure was increased at temperatures near 23 ºC or below 4 ºC, respectively. An hypothesis was made and consisted that an enzyme has a critical temperature value under which the pressure reduces the reaction rate and beyond it the reaction rate increases (Charm et al., 1977).Several authors express that high pressure at low temperatures may have a higher inhibition in the biochemical activity of microbial cells, when compared to low temperature alone based on the studies made (Jannasch et al., 1971).

Table 2: Studies regarding HS at low temperature (adapted from Fernandes et al. 2014).

Products	Conditions	Results	Reference
Low Temperature			
Bouillon, sandwiches and apples	15 MPa for 10 months at 3-4 °C	All the products were stable for the 10 months and for a few weeks at RF and 0.1 MPa.	Jannasch et al., 1971
Rice, wheat and soy beans	3.5 MPa for 1 year at 1 °C	Stable for 1 year. Biochemical changes less pronounced.	Mitsuda, 1972
Cod fish fillets	24.12 MPa for 21 days at 1 °C	Stable for 21 days. Classified with only 8.2 days of shelf life at 0.1 MPa.	Charm et al., 1977
Pollock	24.12 MPa for 21 days at 1 °C	Stable for 12 days. Classified with only 6.7 days of shelf life at 0.1 MPa.	Charm et al., 1977
Mume fruit	0.5 MPa for 5 days at 5 °C	Stable for at least 5 days. Acceptable colour quality, decrease ethylene and CO_2 production.	Baba & Ikeda, 2003
Mume fruit, sweet basil	0.5 MPa for 10 days at 4 °C	Stable for 10 days. Inhibition of discoloration and chilling injuries for mume fruit. Sweet basil exhibited browning injuries.	Baba et al., 2008
Rocket salad	0.025 MPa for 2 months at 4 °C	Prevention against fungal growth.	Baba et al., 2008
Peach	0.414 MPa for 4 weeks at 4.4 °C	Decrease of total volatiles production.	Yang et al., 2009
Tomato	0.1, 0.3, 0.5, 0.7 and 0.9 MPa for 5, 10 and 15 days at 13 ºC	Respiration rate decrease, weight loss and delay in ripening process.	Goyette et al., 2012

1.3.3 Hyperbaric storage at room temperature

HS of food at RT is a good opportunity to reduce energy costs sinceHSonly requires energy during the compression and decompression phases, and do not need energy consumption to maintain the pressure level throughout time (Bermejo-Prada, Segovia-Bravo, et al., 2015). This technique can be very important since it could allow to have domestic storage at consumers' homes or restaurants, using equipment's named as hyperstorers or simply,storers (Fidalgo et al., 2013).

In the last few years, an increasingly number of researchers have published scientific papers about HS at RT, and have explored the possibilities of this technology to extend the shelf life of fresh fruits and vegetables or processed food products (see **Table 3** for HS at RT regarding studies from 1981 to 2014).

The first study on HS at RT was achieved by compressing air at 35 atm (~3.6 MPa) and 20 ºC with an atmosphere composed by O_2, N_2, and CO. These authors tested the moisture loss, respiration rate and quality of mushrooms during 96 to 393 h at the same conditions described above (Robitaille & Badenhop, 1981). This study showed a lower moisture loss and browning extent when compared to those stored at 0.1 MPa. On the other hand, pressure demonstrated no effect on respiration rate. Only after 1 week occurred larval growth in samples under pressure after depressurization and storage at 0.1 MPa, however in control samples larval growth appeared during storage (Robitaille & Badenhop, 1981). Although, in this case air was used to create the pressure level desired,the most common is to use a liquid medium to create pressure, like water, for instance in Romanazzi et al., 2008; Liplap, Charlebois, et al., 2013 studies.

In processed food, normally pressure is transmitted by a liquid medium and it can be increased considerably (25-220 MPa), especially in homogenized products (Bermejo-Prada, Vega, et al., 2015).Tilapia fillets were studied under 101 MPa for 12h at 25 ºC with pressure transmitted by a liquid medium showing similar inhibitory effect on rotting agent growth as mentioned above. In this experiment was observed an inhibitory effect in the growth of total plate counts, a reduction of 2.0 log CFU/g was detected as well in fillets stored under 203 MPa for 12 h at 25 ºC. After this study, the authors also analyzed the post-HS (the preservationof the product over hours todays at atmospheric pressure (AP) after a storage period under pressure) and have seen some enzymes and microbes reactivation (Ko & Hsu, 2001).

Recent studies are more focused on fruit juices preservation, the first one was developed by Segovia-Bravo et al., 2012 where raw strawberry juice preserved by HS was studied at pressure levels of 25, 100 and 250 MPa, at 5 and 20 ºC, for 15 days, as well as pasteurized juice. The results showed that control sample (juice preserved at 20 ºC and 0.1 MPa) presented higher microbial counts (total aerobic mesophiles (TAM), and YM) than pasteurized and pressurized juice which were below detection limits. In the samples stored at 20 ºC pressure was effective onviscosity and color lossesattenuation. Similar results were obtained by Fidalgo et al., 2013 for watermelon juice stored under 100 MPa, at variable RT (18-21 ºC) and above 30 ºC, for 60 and 8h, respectively. The pressurized watermelon juice preserved under pressure showed a TAM, *Enterobacteriaceae* (ENT), and YM growth reduction while control juice at 0.1 MPa presented an unpleasant odour and strong off-flavors, which was relatedto the higher microbial load. Also, HS reduced the increase oftitrable acidity parameter verified at AP, but caused higher color changes, speciallya higher lightness. A similar study was made also on watermelon juice for 8h at pressure of 25-150 MPa with temperatures ranging 20-37 ºC showing that 75 MPa had an inhibitory effect on microbial growth, with a performance similar to RF, and at higher pressures, 100 and 150 MPa, the reduction of the initial microbial counts to ≤ 1.00 Log CFU/mL for ENTand YM was observed (Santos et al., 2015).

In 2016 a study was published also on watermelon juice preserved under pressure for a longer period of time (Pinto et al. 2016). In this short communication watermelon juice was preserved over 7 days at 100 MPa and at variable RTbeing then compared with RF. At the end, there was an increase of the microbial counts (TAM, YM and psychrophiles (PSY)) above 6 Log_{10} CFU/mL for samples stored at AP/RT. HS/RT showed maximum values of about 2 Log_{10} CFU/mL for TAM and PSY and below the detection limit for YM. Also watermelon juice stored at 100 MPa presented physicochemical parameters similar to the initial ones.

Another study carried outon melon juice stored under 25-150 MPa at variable RT (25, 30 and 37 ºC), for 8h, detected a microbial growth inhibition only above 25 MPa, while sample stored under pressures of 100-150 MPa resulted ina decrease of the initial microbial load. Physicochemical parameters of all samples stored under pressure (pH, titratable acidity, total soluble solids, browning degree and cloudiness) did not show a clear variation tendency with pressure, being the results generally similar to RF storage (Queirós et al., 2014).

From 2015 and 2016 were published several studies regarding the effect of HS onfoods(see **Table 4**) (Fernandes et al., 2015; Santos et al., 2015; Moreira et al., 2015; Bermejo-Prada & Otero, 2016; Bermejo-Prada, Vega, et al., 2015; Duarte et al., 2014; Moreira et al., 2015) concerning sliced cooked ham, watermelon juice, *Bacalhau com nata*s, *caldo verde*, strawberries juice, carrot soup,*requeijão* and raw bovine meat.

Sliced cooked ham was preserved at temperatures ranging from 25 to 37 ºC under25-150 MPa over4 and 8h. This study demonstrated that HS at naturally variable RT is a promising alternative to RF showing an inactivation of the microorganisms at 100 and 150 MPa, and a microbial growth inhibition similar to RFat 50 MPa/30 ºC, of about 3.8 Log_{10} CFU/g for TAM and lactic acid bacteria. In general HS of sliced cooked ham showed physicochemical parameters (lipid oxidation, pH and colour) similar to the refrigerated samples(Fernandes et al., 2015).

A similar study in two ready-to-eat pre-cooked food: *Bacalhau com nata*s and *caldo verde* stored for 12h at 0.1, 50,100 and 150 MPa at naturally uncontrolled RT (~21 ºC). The resultswere similar to the ones found in the latter study described, with a microbial growth inhibition at 100 MPa for all microorganisms, moreover an additional inactivation effect at 150 MPa was verified, resulting in values below the detection limit for ENT and YM. These results showedan equal to better behaviour when compared to RF. In all HS conditions studied, the physicochemical parameters were similar to the samples stored under RF.

Another two studies concerning carrot soup and *requeijão* were made atsimilar conditions. The carrot soup was tested with 100 and 150 MPa for 4 and 8h at 25 and 30 ºC and *requeijão* with 100 and 150 MPa for 4 and 8h at 25 ºC, 30 ºC and 37 ºC. The carrot soup presented amicrobial growth inactivation at 150 MPa during 8h and a microbial growth inhibition at 100 MPa during 4h, being the results better than the ones obtained under RF. TAM showed less susceptibility to HS compared to ENT and YM. The physicochemical parameters were maintained generally at values similar to RF(Moreira et al., 2015). In *requeijão*, the microbial analyses showed that storage for 4h at 100 MPa was capable to maintain the microbial counts similarto RF and the initial load, ≈3 Log_{10} CFU/g, at all tested temperatures. At higher pressure (150 MPa), during 8h, the microbial loads were reduced to undetectable counts, except for TAM that were reduced to about ≈1 Log unit. The pH, water activity and lipid oxidation in HS samples presented similar values to RF(Duarte et al., 2014).

Bermejo-Prada & Otero, 2016 and Bermejo-Prada, Veja et al, 2015 made two studies on the same food, strawberry juice evaluating the viscosity, pectin methylesterase (PME) activity and colour degradation. The effect of HS during 1, 2, 5, 7, 10, and 15 days at50 and 200 MPa and 20 ºC was tested showing that HS affects some mechanisms of colour degradation, displaying a significant peroxidase inactivation and lower percent polymeric colour during 5, 7 and 15 days of storage at 200 MPa compared with those maintained at AP(Bermejo-Prada & Otero, 2016). The study made in strawberry juice evaluated the viscosity and PME activity atthe same conditions of Bermejo-Prada & Otero 2016and showed that HS enhanced viscosity decay particularly at the beginning of storage although no increase in PME activity was observed (Bermejo-Prada, Segovia-Bravo, et al., 2015)

A more recent study concerning raw bovine meat preserved under pressure was studied and compared to AP storage. Samples were stored initially for 12h at 50, 100 and 150 MPa at RT (without temperature control) and in a second experiment for a longer period of days (10 days) at 50 MPa. For the 12h storage, RF and 50 MPa presented a similar microbial growth inhibition and 100 and 150 MPa storage conditionsrevealed a microbial inactivation effect. Over 10 days at 50 MPa,a longer shelf life was obtained when compared to samples stored under RF. For both storages (12h and 10 days) samples preserved under pressure showed no detrimental effect on physicochemical parameters comparatively to the initial and refrigerated samples.

Table 3: Studies regarding HS at RT from 1981 to 2014 (adapted from Fernandes et al. 2014).

Products	Conditions	Results	References
Room Temperature			
Mushroom	3.5 MPa for 4 days at 20 °C	Reduction of moisture loss and browning.	Robitaille & Badenhop, 1981
Tilapia fillets	203 MPa for 12h at 25 ºC	K value under 40%. Inhibition of deterioration only under pressure.	Ko & Hsu, 2001
Sweet cherries	0.15 MPa for 4 hours at 20 °C	Decrease of mould contamination (brow and total rots, grey and blue moulds).	Romanazzi et al., 2008
Table grapes	0.15 MPa for 1 day at 20 °C	Reduction of infected berry and percentage of lesion diameter.	Romanazzi et al., 2008
Strawberries juice	25,100 and 220 MPa for 15 days at 20 ºC	Stable for 15 days under pressure + 15 days at 0.1 MPa at 5 °C. Microbial load below the detection limits. Attenuation of viscosity decay. No significant colour degradation.	Segovia-Bravo et al., 2012
Tomato	0.1, 0.3, 0.5, 0.7 and 0.9 MPa for 4 days at 20 ºC	Lycopene synthesis inhibition during HS. No influence in total phenolics and ascorbic acid content.	Liplap, Charlebois, et al., 2013
Tomato	0.1, 0.3, 0.5, 0.7 and 0.9 MPa for 4 days at 20 ºC	Effective reduction of weight loss. Firmness conservation and delay in ripening colour development.	Liplap, Vigneault, Toivonen, et al., 2013
Lettuce	0.1, 0.2, 0.4, 0.6 and 0.85 MPa for 3, 5 and 7 days at 20 °C	Product marketable for 5 days. Sensory and visual quality similar to RF.	Liplap, Vigneault, Rennie, et al., 2013
Melon juice	25, 50, 75, 100 and 150 MPa at 20, 30 and 37 ºC for 8h	Stable at all temperatures. Microbial inhibition at 50/75 MPa and reduction at 100/150 MPa.	Fidalgo et al., 2013
Watermelon Juice	100 MPa for 60h at 18-21 ºC	Avoid microbial growth up to 60 h. Decrease the initial loads. Extended shelf life.	Queirós et al., 2014
Requeijão	100 and 150 MPa for 4 and 8h at 25 ºC, 30 ºC and 37 ºC	Storage for 4h at 100 MPa was able to maintain microbial counts similar to RF and initial load at all temperatures. At 150 MPa and the storage time to 8h, microbial loads were reduced to undetectable counts, except TAM. HS maintained pH, $A_w$and lipid oxidation values, similar to RF.	(Duarte et al., 2014)

Table 4: Studies regarding HS at RT from 2015 to 2016(adapted from Fernandes et al. 2014).

Products	Conditions	Results	References
Room Temperature			
Watermelon Juice	25-150 MPa for 8h at 20-37 ºC	At 75ºC show an inhibitory effect on microbial growth and additional inactivation effect at 100 and 150 MPa for ENT and YM.	Santos et al., 2015
Sliced cooked ham	25-150 MPa for 4 and 8h at 25-37 ºC	At 25ºC, 30ºC and 37ºC no effect on microbial growth at 25 MPa. The storage at 50 MPa and 30ºC result on microbial growth inhibition. At 100 and 150 MPa result in microbial inactivation.	Fernandes et al., 2015
two ready-to-eat pre-cooked food: *Bacalhau com nata*s and *caldo verde*	50, 100 and 150 MPa for 12h at 21 ºC	Microbial growth inhibition at 100 MPa and inactivation effect at 150 MPa in values below the detection limit for ENT and YM.	Moreira et al., 2015
Carrot soup	100 and 150 MPa for 4 and 8h at 25 and 30 ºC	Similar microbial growth inhibition to better microbial inactivation results compared to RF. HS maintained the physicochemical parameters at values similar to RF.	Moreira et al., 2015
Strawberries juice	50 and 200 MPa for 1, 2, 5, 7, 10, 15 days at 20 ºC	Viscosity decay and at the beginning storage and no increase in PME (pectin methilesterase) activity.	Bermejo-Prada, Segovia-Bravo, et al., 2015
Strawberries juice	50 and 200 MPa for 1, 2, 5, 7, 10, 15 days at 20 ºC	Storage affect some mechanism of colour degradation, peroxidase inactivation and lower percent polymeric colour during 5, 7 and 10 days.	Bermejo-Prada & Otero, 2016
Watermelon juice	100 MPa for 7 days at RT	Increase of microbial counts for samples at AP to $\geq$6 Log_{10} CFU/mL, while samples under HS/RT showed a maximum value of $\approx$2 Log_{10} CFU/mL for TAM. HS/RT juice showed physicochemical parameters similar to the initial juice.	Pinto et al., 2016
Raw bovine meat	50, 100 and 150 MPa for 12h and 50 MPa for 10 days at RT	For the 12h storage, RF and 50 MPa presented a similar microbial growth inhibition, and 100/150 MPa revealed a microbial inactivation. For the longer experiment, samples show shelf life increase compared to RF.	Freitas et al., 2016

1.4 Milk

Milk is the secretion of the mammary gland of females of mammalian species, and contains the required nutrients to sustain the life of infants and helps the rapid growth and gain of weight that new-borns need, having compounds that offer critical nutritive elements, biologically active substances and immunological protection (Séverin & Wenshui, 2005). Milk is considered to be a dairy food and known for its balanced profile, being an important key on a healthy diet (Pereira, 2014).

In Europe, milk is the main dairy product of the dairy sector and the source for the production of other dairy products (cream, cheese, butter, yogurt, and whey) (González-García et al., 2013). The milk of sheep and goats is significant to the manufacture of dairy products although bovine milk is the most generally consumed in the world (Early, 2012).In the Portuguese industries, milk in its different forms (whole, semi-skimmed and skimmed milk) is the leading product (~890.000 tonnes), tracked by yogurts and cheese reflected by the consumption trends(González-García et al., 2013).

Milk composition can be influenced by several aspects, depending normally on animal species and genetics, environmental conditions, lactation stage, and animal nutritional status (Caroli et al., 2009). When comparing cow's milk, the most consumed, with sheep, goat and human some differences can be found. Comparing these types of milk, some studies revealed that sheep milk has higher protein and fat content while goat milk presents higher amounts of A, B1 and B12 vitamins, as also calcium and phosphorus concentration when compared to cow and sheep milk (Gartner et al., 2005; Jandal, 1996; Park et al., 2007).

Milk is composed by more than 100 components as fat, proteins, lactose, vitamins, minerals and water. The major component is water at 87 %, and the typical composition of cows' milk is 3.6 % of fat, 3.2 % of protein, 4.7 % of lactose, 122 mg/100g of calcium, 119 mg/100g of phosphorus, 126 IU of vitamin A and 2.0 IU of vitamin D (Månsson, 2008). The energy value of cow's milk is about 70 Kcal per 100 ml and the pH level ranges between 6.65 and 6.71 (Park et al., 2007). An example of the composition of milk from different mammals is exposed in **Table 5**(Pereira, 2014).

Table 5: Typical composition of milk in goat, sheep, cow and human milk (Pereira, 2014).

	Milk source			
	Goat	**Sheep**	**Cow**	**Human**
Fat (%)	3.8	7.9	3.6	4.0
Water	88.7	81.0	88.5	88.8
Lactose (%)	4.1	4.9	4.7	6.0
Protein (%)	3.4	6.2	3.2	1.2
Energy (Kcal/100 mL)	70	105	69	68
Calcium (mg/100 g)	134	193	122	33
Phosphorus (mg/100 g)	121	158	119	43
Vitamin A (IU)	185	146	126	190
Vitamin D (IU)	2.3	0.18 (µg)	2.0	1.4

1.4.1 Milk proteins

Milk is an important protein source in human diet having a value of 32g protein/L. Proteins in this food can be divided into soluble and insoluble fractions, where the soluble (whey proteins) represent 20% and insoluble (caseins) proteins represent 80% of the total protein in milk, respectively as represented in**Table 6**. The two fractions are categorised as high quality proteins considering human amino acid necessities, digestibility and bioavailability (Séverin & Wenshui, 2005; Haug et al., 2007). These are considered to be the best protein source taking into account the essential amino acid score and protein-digestibility corrected amino acid score (Boye et al., 2012). The amino acids content are different between the two fractions, on the one hand casein has a higher proportion of histidine, methionine and phenylalanine while whey is rich in leucine, isoleucine, lysine and valine (Tang et al., 2009).

Table 6: Typical composition of proteins in cow milk found in a study of Pereira, 2014.

Protein	**Concentration (g/L)**

TOTAL CASEINS	26.0
α-Casein	13.0
β-Casein	9.3
κ-Casein	3.3
TOTAL WHEY PROTEINS	6.3
β-Lactoglobulin	3.2
α-Lactoalbumin	1.2
Immunoglobulin (IgA, IgM, IgE, IgG)	0.7
Serum albumin	0.4
Lactoferrin	0.1
Lactoperoxidase	0.03
Lysozyme	0.0004
Others	0.8
Proteose-peptone	1.2
Glycomacropeptides	1.2

The soluble protein fraction includes β-lactoglobulin, α-lactoglobulin, immunoglobulins (Ig), serum albumin, lactoferrin, lactoperoxidase, lysozyme, proteose-peptone and transferrin (Séverin & Wenshui, 2005). Soluble proteins that are important antimicrobial agents are lactoferrin, lactoperoxidase and lysozyme (Jenssen & Hancock, 2009), on the other hand β-lactoglobulin is a retinol carrier and has antioxidant capacities, and lactoferrin is a crucial element in iron absorption and in exerting antioxidant and anticarcinogenic effects (Mills et al., 2011; González-Chávez et al., 2009).

Regarding β-lactoglobulin, it is a polypeptide chain comprising 162 amino acid residues. This protein has several functions in milk such as retinol carrier, binds Cu^{2+} and Fe^{2+} ions and inhibits auto oxidation of fats (Micinskia et al., 2013).

α-lactoglobulin constitutes 22% of total whey protein. It is an albumin well soluble in water, and has a chain with 123 amino acid residues with high concentrations of cysteine lysine and particularly tryptophan, which is a precursor of serotonin. This protein participates in the formation of lactose and also binds metals such as cobalt, magnesium and zinc (Micinskia et al., 2013). Serum albumin is similar to α-Casein, β-

Casein, and α-lactoglobulin and it is inactivated at 70 ºC – 80 ºC (Micinskia et al., 2013).

Concerning lactoferrin, it is a protein synthesized by the secretory epithelial cells of the mammary gland and the main function of this protein is binding iron and transporting it to the intestinal vascular system (Micinskia et al., 2013).

Lactoperoxidase and lysozyme are biologically active enzymes. Lactoperoxidase is an oxidoreductase with antibacterial function, viral growth inhibitor and antineoplastic agent on the other hand lysozyme lyses bacteria in milk, in special Gram-positive bacteria, and has antiviral and anti-inflammatory properties (Micinskia et al., 2013).

Immunoglobulins are antibodies produced in response to viruses, bacteria and animal antigens. These antibodies take place in five types: A, G, M, E and D at the intestinal lumen and intestinal wall. Transferrin is a glycoprotein similar to lactoglobulin and presents an iron-binding capacity, transporting iron form. Iron in muscles is used to biosynthesis of cytochromes and myoglobin to tissues, and in bone narrow is used in the manufacture of hemoglobin. Proteose-peptones are present in minor amounts compared to the other proteins, being the products of enzymatic degradation of casein (Micinskia et al., 2013).

Total caseins can be separated in α-, β-, and κ-Caseins. These proteins transport calcium and phosphorus, forming a coagulum and refining their digestibility in the stomach. Casein fractions differ in concentration, contents of phosphorus, amino acid composition, molecular weight and isoelectric point. Caseins from bovine milk may induce inflammatory reaction in mucous membrane of patients with celiac disease (Holt et al., 2013).

1.4.2 Milk lipids

The lipid fraction in milk is mainly composed by triacylglycerols (TAGs) forming 98% of the fraction, where other lipids like diacylglycerol (2%), cholesterol (<0.5%), phospholipids (~1%) and free fatty acids (0.1%) can also be found. Additionally, there are trace amounts of hydrocarbons, fat-soluble vitamins, flavour compounds, and other ingredients introduced through the animal feed. Lipid matrix in milk is the most complex of all natural fats, having more than 400 fatty acids, forming different TAGs (Månsson, 2008).

Lipid fraction in milk is essentially present in globules that are unaffected to pancreatic lipolysis except if prior submitted to gastric digestion (Ye et al., 2010). The quantity and composition of milk fatty acids depend on the animal origin, stage of lactation, ruminal fermentation, or feed related factors (see **Table 7**).

Table 7: Fatty acids composition in cow milk (adapted from (Månsson, 2008; Lindmark-Månsson et al., 2003)).

Fatty acids	%
Unsaturated fatty acids	30
α-linoleic	0.7
Linoleic	1.6
Oleic	25
Vaccenic	2.7
Saturated fatty acids	70
Palmitic	30
Myristic	11
Stearic	12
Butyric	4.4
Caproic	2.4
Others	10.2

1.4.3 Milk vitamins and minerals

Raw milk, as a dairy product, has an especially particular micronutrient composition. Milk has an elevated concentration of calcium but other minerals can be found too, like phosphorus, magnesium, zinc and selenium (see**Table 8**)(Gaucheron, 2011).

Table 8: Mineral composition in cow milk (Pereira, 2014).

Mineral	mg/100g	Amount in 1cup (244g)	% DRI
Calcium	119-124	297.50-310	37-40
Phosphorus	93-101	232.50-252.5	16-32
Magnesium	11-14	27.5-35	8-10
Potassium	151-166	377.5-415	8-9
Zinc	0.4-0.6	1-1.5	9-14

%DRI- Dietary recommended intake

Calcium is a macro element present in higher concentrations in milk, 120 mg/L on average, being this content distributed between the micellar and aqueous phases. In the aqueous phase it is linked to whey proteins or inorganic forms of phosphate-forming salts, while in the micellar phase it is associated with the phosphoseryl residues of caseins. These two phases are in thermodynamic equilibrium but at different physicochemical conditions, such as pH and temperature, calcium can pass from one phase to another (Pereira, 2014).

In milk, phosphorus is present in organic and inorganic forms. Inorganic phosphate is located in the aqueous phase and depending on the pH level, just like calcium, exhibits equilibrium between the two phases that can be changed by different pHvalues. The organic phosphate is linked to proteins, phospholipids, organic acids and nucleotides, being present in the micellar phase (Gaucheron, 2011).

Magnesium content is not so high in milk but has the same function of calcium and phosphorus to keep the dynamic equilibrium between the two phases. In even lower concentrations are zinc and selenium, which are present commonly in the micellar phase associated with casein (Pereira, 2014).

The vitamins fraction present in milk are liposoluble (vitamin A, D and E) and hidrosoluble (B complex, thiamine and riboflavin) (**Table 9)**. The concentrations of fat-soluble vitamins in milk depend on milk fat content, therefore low-fat and skim milk varieties have lower amounts of A, D, and E vitamins (Pereira, 2014). Vitamin A is very important in growth, immunity and eye health and the concentration in milk ranges largely on fat amount, but also on aspects like animal feed and season (Gaucheron, 2011). In milk, vitamin D is not present on considerable amounts, except when milk is improved and fortified. Previous studies have reported values within 5 and 35 IU/L (Leerbeck & Søndergaard, 1980).

Table 9: Vitamin composition in milk (Pereira, 2014).

Vitamin	/100g
B1 (thiamin)	0.04-0.05 mg
B2 (riboflavin)	0.16-0.17 mg
B3 (niacin)	0.08-0.09 mg
B6 (piridoxin)	0.04-0.04 mg
Folate	5-5.2 µg
B12 (cobalamin)	0.357-0.500 µg

1.4.4 Lactose

In milkthe main carbohydrate present is lactose, and it can be found at values around 4.8%. This level of sugar does not turn milk an excessively sweet food product because lactose is less sweet than sucrose as well as less sweet than an equimolar mixture of its components, galactose and glucose. Lactose has a major influence in the colligative properties of milk (osmotic pressure, freezing point depression, boiling point elevation). Associated with many other sugars, lactose ismoderately less soluble in water, being its solubility at 25 °C of 17.8 g/100 g solution. The low solubility of lactose can provoke some production problems because lactose crystals are gritty in texture. Lactose crystallization is also responsible for caking and lumping of dried milk during storage. Like other reducing sugars, lactose can react with free amino groups of proteins to give products that are brown in colour(Singh & Bennett, 2002).

1.5 Health benefits

Since 1980, the dairy industry has been adapting and improving milk products to fulfil consumers requests, and for that reason industries had committed themselves to change the fat content according to milk classification, finding new strategies for less-caloric products ensuring nutritional richness, proper flavour, texture, and odour, with lower fat and lower energy (Pereira, 2014).

Milk is the first food for mammals and gives the nutrients and energy required to ensure proper growth and development in mammals' growth. This product is a complex food with many components presenting benefits for human health (such as cardiovascular well-being) and others with negative effect. The main problem with milk

is related with heart disease due to the saturated fat content, representing 70% of total milk fat. High consumption of saturated fat was earlier associated with improved risk of cardiovascular disease. Additionally, others milk elements, such as minerals like calcium, magnesium and potassium, can take a defensive part in cardiovascular disease due to the antihypertensive effect (Pereira, 2014).

Milk as several minerals, but calcium has a higher concentration compared to other minerals and it is very important in bone density. Elevated bone mass is very important to prevent osteoporosis, and it is very significant the consumption of food products with calcium in early life in order to prevent health problems latter. In Pereira 2014 it was demonstrated that several types of minerals and other constituents like peptides, can play a positive part in bone mass, such as osteoporosis prevention and inferior occurrence of fractures.This can be reinforced by information about nutrients that are supposed to influence production and maintenance of bone matrix such as protein, vitamin C, D and K, as well as minerals like copper manganese and zinc. For all these aims milk consumption is worldwide suggested as a supporter of good bone health(Pereira, 2014).

1.6 Chocolate milk

Chocolate milk formulation comprises milk, cocoa powder, sugar and hydrocolloids. The final composition of chocolate milk, as well as the physical and sensory properties, principally depend on the levels of the components including fat, the type of cocoa and the type of hydrocolloid present (Yanes et al., 2002).

Usually hydrocolloids are added to UHT flavoured milk to increase the creaminess of the final product and to give a more lasting taste.The largest dairy application for kappa-carrageenan (κ-car) is in hot-processed chocolate milk as a favourable mouth-feel to the milk and to permit long-term suspension of the cocoa particles (Bixler et al., 2001). The enhanced apparent viscosity of the carrageenan–casein network results in a favourable mouth-feel (Bixler et al., 2001). The heat treatment increases the shelf life and also the hydration of the hydrocolloid, on the other hand heat treatment promotes the increase of the carrageenan gels strength, and helps in the stability of the product at long-term. The displacement of carrageenan complexed to κ-casein by the denatured β-lactoglobulin increases the stability of the product, therefore increasing the carrageenan availability for carrageenan–carrageenan

connections which are mainly in charge for the formation of the weak gel network (Sedlmeyer and Kulozik, 2007; Tijssen et al., 2007).

Nevertheless, during sterilization, problems may appear due to the increased viscosity accomplished by the hydrocolloids and also due to the interactions between the flavourcomponents and milk that can result in flocculation, coagulation and sediments formation (Tziboula & Horne, 2000). If the quantity and type of stabilizer are not acceptable, the final product can disclose a number of unwanted characteristics such as flocculation and coagulation.

κ-carrageenan has been used as an additive to stabilize protein systems (Langendorff, 2000). Chocolate milk studies had mainly been focused in its sensory (Folkenberg et al., 1999), rheological and optical properties but few in microbiology and physical chemical characteristics (Yanes et al., 2002)

The chocolate milk processed with heat treatment can show three types of instability: sedimentation of cocoa particles, large flocs formation and formation of light- and dark-coloured layers (Yanes et al., 2002) that ascend due to interactions between the chocolate ingredients and milk components (Boomgaard et al., 2007). In industry it is an every-day concern the fouling or deposit formation being very common the occurrence of problems during UHT processing of chocolate milk.Thus, the application of new technologies for chocolate milk processing and/or preservationare welcome (Harwood et al., 2012).

1.7 Milk deterioration and food safety

1.7.1 Food safety in milk

Generally, milk is collected from a lactating animal at least twice a day and is considered as a highly perishable food easily exposed to microbial contamination. This contamination can diverge widely due to milk-handling performs. Normally, milk is refrigerated straightaway and stored in tanks until picked up to process (Singh & Bennett, 2002).

During production, collection and handling of milk several types of microorganisms can grow. After milk storage at ≤ 4 ºC the bacterial growth delay is feasible for at least 24h. A valuable indicator for checking the sanitary conditions present through production, collection and handling of raw milk is total bacterial count for a preliminary assessment. Followed by microbial assessments for psychrotrophs,

spore-forming bacteria, streptococci and coliforms (COL) to determining the sanitary deficiencies (Singh & Bennett, 2002).Total bacterial can range from <1000 mL^{-1}, where contamination during manufacture is minimal, to >$1x10^6$ mL^{-1}. In many countries raw milk with a total bacterial load of <$1x10^5$ mL^{-1} is used for heat treatment before consumption. For raw milk consumption more severe rules are mandatory for the reason that consumers of raw milk are more subject to salmonellosis(Singh & Bennett, 2002).

1.7.2 Milk deterioration

The effects caused by spoilage bacteria in food are sour, off-flavours and structural defects produced by proteolytic, lipolytic and phospholipase enzymes. Off-flavours can manifest by bitter, putrid, stale, rancid, fruity, yeasty or sour. The bitter flavour is caused by protease activity on the proteins, although rancid and fruity flavours are caused by lipases (Delhi, 2010).

One of the major causes of deterioration of milk is the light incidence. Off-flavours can be developed and are due to oxidative processes, mainly linking changes in proteins and lipids. Lipid oxidation in milk conduct to the creation of carbonyl compounds, and protein degradation creating off-flavours in milk (Delhi, 2010).

Ultra high temperature (UHT) milk is bacteriologically stable for months at ambient temperatures, but its shelf life is frequently compromised by age gelation. This singularity process rises milk viscosity through storage and eventually results in a loss of fluidity with the development of a gel. The gel is a three dimensional protein network shaped by the whey proteins, particularly β-lactoglubulin, interaction with κ-casein of the casein micelle. Age gelation is started by proteolytic activity initiating from either native milk protease (plasmin) or bacterial proteases that survive the UHT treatment (Delhi, 2010).Hydrolysis of milk caseins by proteases leads to a weakening of the casein micelle. After heat treatment, interactions between κ-casein and β-lactoglobulin occur, resulting in the release of the β-lactoglobulin-κ-casein complex (βκ-complex) from the micelle. The released complex then aggregates and forms the typical network of cross-linked proteins, which causes the milk to gel (Delhi, 2010).

1.8 UHT milk production process and other processing technologies

Milk is a nutritive medium that allows the growth of spoilage and pathogenic microorganisms. As a result, raw milk has a short shelf

life and requires to be processed in order to increase that (short) period. Typically, commercial milk undergoes several processes including standardization of fat content according to the type of milk required, homogenisation, and pasteurisation. In conventional homogenisation, milk is passed through two homogenisation valves (2-stage homogenisation) under moderate pressures (~18 to 20 MPa), avoiding creaming during storage due to the decrease of fat globule size (Andersson et al., 1995; Pereda et al., 2007).

Heat treatment has been by far the most important technology to improve milk shelf life, as it inactivates pathogen microorganisms, enzymes and most of the spoilage bacteria. The temperature increase causes cooked off-flavour by denaturation of the whey protein during the processing, being this fact the biggest disadvantage of heat treatments, along with the loss of nutritional and physicochemical quality (Andersson et al., 1995; Pereda et al., 2007).

The production line of milk is separated in two main subsystems (**Figure 2**): the dairy farm (subsystem 1) and the dairy factory (subsystem 2) (González-García et al., 2013).

• Subsystem 1—dairy farm: a representative farm in order to produce the raw milk essential in the milk factory (González-García et al., 2013).

• Subsystem 2—dairy factory: all the activities that take place in the dairy factory are in this subsystem contains, starting in the raw milk reception to packaging and storingthe product. The delivery of raw milk from the dairy farm is made in tankers, and they are washed in the delivery. Raw milk is kept under cold conditions in storage tanks for the coming processes. The first step is the pre-warming shadow by the skimming in order to isolate the cream from the milk by a centrifugation process. Afterwards, it is homogenised for the uniform distribution of fat in the milk and also to avoid cream formation. The pasteurisation step is an obligatory process and involves a thermal treatment to inactivate microorganisms and preserve milk quality. The temperature and time conditions are factors associated to the quality and characteristics of the final milk product. Lastly, the whole, semi-skimmed and skimmed milk are placed under cold environments (González-García et al., 2013).

The following thermal milk treatment is the UHT treatment which permits shelf life extension up to 6 or 9 months until opened. The UHT treatment involves the heating of milk for a short period of time, around 2-3s at a high temperature (145 ºC) and trailed by a rapid cooling at 20 ºC (RT). For the UHT cocoa milk production, the milk is

previous mixed with other ingredients (i.e., chocolate), and following to thermal treatment the milk product is packaged. This method takes place automatically in filling machines and under sterilised conditions at RT. The widely used Tetra Brik® is a package established by a number of layers of polyethylene, cardboard and aluminium, avoiding the contact of the packaged milk with oxygen and light (González-García et al., 2013).

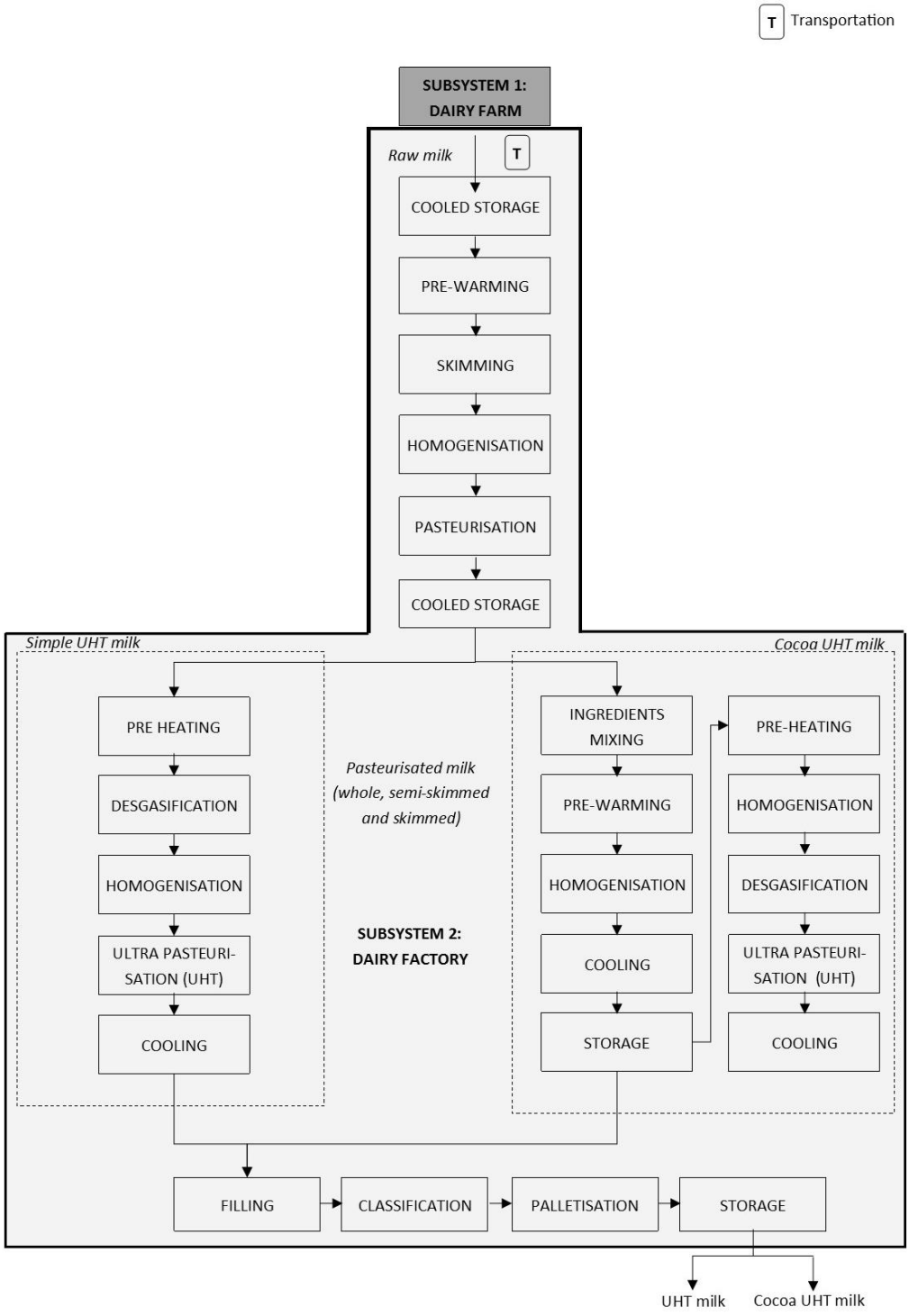


Figure 2. Flow chart of the UHT milk production process (González-García et al., 2013).

1.8.1 Pasteurisation

Pasteurisation is a thermal processing technology frequently applied to liquid milk in which milk can be heated quickly to 72°C for 15 to 20 s (high-temperature short-time pasteurisation) or heated to 80 to 90 °C for 15 s (high-pasteurisation)(Pereda et al., 2007). The main objective of this treatment is to eliminate any potential vegetativemicroorganisms that might be present and to reduce spoilage bacteria, which can generate negative sensory attributes decreasing processed milk shelf life (Walstra et al., 2005). However, thermal processes can cause changes in nutritional and organoleptic properties of milk (Fox & McSweeney, 1998; Walstra et al., 2005; Datta & Deeth, 2003).

1.8.2 Ultra-high temperature (UHT)

When heat treatment is applied for the production of longer shelf life products is named "sterilization." The treated product in this process is open to such powerful heat treatment that microorganisms and most of the enzymes are inactivated, being stored after the process for months under ambient conditions. UHT processing uses continuous flow of milk, which lead to less chemical changes in contrast to retort processing. pH, water activity, viscosity, composition, and dissolved oxygen, point out the necessary processing conditions to achieve commercial sterility. The selection criteria of UHT and aseptic packaging systems are designed to ensure commercial sterility and acceptable sensory attributes throughout shelf life (Chavan et al., 2011).

Studies regarding thermal treatments applied to milk, show that UHT reveal problems of cooked off-favour, protein gelation and nutritional compound destruction. Thus, food industries are looking for new processing technologies to treat milk, with the purpose of reducing the use of heat, by substituting it with non-thermal technologies (Amador-Espejo et al., 2014).

A company in Mexico developed a milk pasteurized by HPP at cold temperatures and is available in the Mexican market. This company was the first to sell milk processed by HPP to the consumers (Villa de Patos, 2014).

2. OBJECTIVES

Usually food products are handled at refrigerated temperatures to retard their spoilage, involving this procedure high energy costs, raising at the same timesustainability concerns, as well as high carbon footprint issues.As there is no commercial alternative to this technology,the HS emerged as an alternative to RF, capable to extend food products shelf-livesat RT requiring for that a low energy consumption since it is only necessary in the compression and decompression phases (Fernandes et al., 2014).

The main goal of this work was to study the HS effect on chocolate milk in order to assure its microbiological safety and shelf life extension,evaluating at the same time possible changes on physicochemical parameters and organoleptic characteristics.For this experience the following microorganisms were evaluated: TAM, PSY, ENT, COL.*E.coli* and *L.innocua*were also inoculated in some samples to ensure the microbiological safety of milk under HS. For the physicochemical parameters, the pH, A_w, colour, reducing sugars, lactose and SEM images were evaluated.

Chocolate milk stored under RF and at RT (15-25ºC) at AP (0.1 MPa)was studied and compared to milk stored under pressure. For that, different combinations of pressure levels and temperatureswere consideredover different storage times. In addition,it was tested possible differences at the microbial and physicochemical levels empowered by the presence of air inside the packages, being prepared for that samples with air and without air(**Table 10**).

Table 10: Pressure, temperature and storage period of HS experiments performed.

HS experiments		
Pressure (MPa)	**Temperature (°C)**	**Storage period (days)**
30 MPa	15-25°C	0, 2, 7
50 MPa	15-25°C	0, 2, 7, 14, 30
50 MPa	30°C	0, 2, 7
100 MPa	15-25°C	0, 2, 7, 14, 30
Control samples		
0.1 MPa	15-25°C	0, 2, 7
0.1 MPa	30°C	0, 2, 7
0.1 MPa	4°C	0, 2, 7, 14, 30

3. MATERIALS AND METHODS

3.1 Chemicals

Ringer's solution were purchased from Merck (Darmstadt, Germany) and 5-dinitrosalicylic (DNS) acid was purchased from Acros (New Jersey,USA).

3.2 Preparation of sample

Chocolate milk was purchased from a local supermarket. At each experiment new packages were purchased to start a new study.

As thepurchased chocolate milk showed microbial loads below the detection limit, astrategic experiment to increase it above the detection limit was always performed. Thus, firstly, two packages of chocolate milk were opened, mixed (total of 400 mL) and transferred to a cup of glass, being left opened at RT (~20 °C) for 48h (mixed occasionally). To verify themicrobial load (TAM and ENT) present in the chocolate milk,serial dilutionswere made and plated on the proper media. It was obtained for TAMand ENT values of$8.62\pm0.01 Log_{10}$ CFU/mL and $8.25\pm0.08 Log_{10}$ CFU/mL respectively. (see **Appendix CTable** 12and**Table 13**). After that, chocolate milk was frozen and stored at -80 °C (under sterile conditions to avoid microbial load changes) in aliquots of 2 mL. After that, itwas calculated the amount of spoiled chocolate milk (stored at -80 °C) requiredto add to the purchased/pasteurized chocolate milk (with microbial loads below detection limits) to have an initial load in each experimentof≈2.5 Log_{10} CFU/mL for TAM and ENT. These inoculations were made in sterile conditions.

3.2.1 Inoculation of *Listeria innocua* and *Escherichia coli* on chocolate milk samples

To study the HS effect on the growth of two non-pathogens microorganisms, specifically *Listeria innocua* ATCC 33090 and *Escherichia coli* ATCC 25922, chocolate milk was inoculated with these microorganisms with a final microbial load of 2.51 ± 0.19 and 2.58 ± 0.10, respectively, using for that the McFarland scale (600 nm) (jenway 6405 UV/Vis. Spectrophotometer, Stone, Staffordshire, UK).

3.3 HS experiments

HS experiments were carried out in a hydrostatic press (FPG7100, Stansted Fluid Power, Stanstead, United Kingdom). This equipment has a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to control the temperature, and another equipment with a vessel of 37 mm inner diameter and 500 mm

height without temperature control (FPG13900, Stansted Fluid Power, Stanstead, United Kingdom).

A mixture of propylene glycol and water (40:60) was used as pressurising fluid. The chocolate milk samples were aseptically placed in low permeability polyamide-polyethylene bags (PA/PE-90, Albipack – Packaging Solutions, Águeda, Portugal), using a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) to avoid contaminations. The bags were heat sealed manually with care to avoid as much as possible to leave air inside the bags.In the experimentswhere samples packed with air inside were used, the same amount of air(58.13±5.5%) in each bag was attested.Each bag was afterwards inserted into a second bag that was heat sealed under vacuum. The packaging film was previously sterilized by irradiation with UV light for 15 min (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain).

3.4 Microbial analyses

3.4.1 Sample preparation and dilution

In the same day of the HS, decimal dilutions with 1 mL of chocolate milk were prepared, allowing a maximum microbiological quantification of 6.00 Log_{10} CFU/mL and a minimum of 1.00 Log_{10} CFU/mL. Triplicates of each sample and duplicates of each dilution were plated on the appropriate media. All samples were analysed for counts of TAM, ENT, COL, PSY.

3.4.2 Count of microorganisms

TAM counts were determinate in PCA after aerobic incubation at 30 ± 1 ºC for 72 ± 3 h (ISO 4833 2003). ENT counts were quantified in VRBDA, being incubated aerobically at 37 ± 1 ºC for 24 h(ISO 8523 1991). COL counts were quantified in CCA, being incubated aerobically at 37 ± 1 ºC for 24 h (ISO 4831 2006). PSYcounts were determinate in PCA after aerobic incubation at 20 ± 1 ºC for 5 days (ISO 4833 2003). *L.innocua* counts were quantified in PALCAM, being incubated aerobically at 37 ± 1 ºC for 48 h (ISO 11290 1996). *E.coli*counts were quantified in CCA, being incubated aerobically at 37 ± 1 ºC for 24 h (ISO 4831 2006). The method used for all culture medium was the pour-plated, using 1.0 mL of diluted solution sample. All culture mediums were purchased from Merck (Darmstadt, Germany).

The petri dishes presenting15-300 colony forming units (CFU) were considered and the results were expressed as logarithmic of CFU per mL of chocolate milk(Log_{10} CFU/mL). The microbial counts were calculated following **Equation 1.**

$$N = \frac{\sum characteristic\ colonies}{V\ [\ (n1 + 0.1 \times n2) \times d]}$$

Equation 1

Being:

N - Colony forming units per mL of chocolate milk (CFU/mL)

V - Sample volume (mL) – 1.0 mL for all microorganisms

n1 - Number of plates countable in the first dilution

n2 - Number of plates countable in the second dilution

d - First countable dilution

3.5 Physicochemical analyses

3.5.1 Water activity (A_w) determination

The sample was placed in the cuvette of the Novasina – LabSwift-A_w analyser (Novasina, Zurich, Switzerland). Direct reading was performed in the equipment at ≈25 °C.

3.5.2 pH determination

Samples (12 mL) were mixed in themagnetic stirrer. The pH value of the samples was measured at RT (≈ 20 °C) with a properly calibrated glass electrode (Crison, Barcelona, Spain) which was calibrated with 4.0 and 7.0 buffer. The pH resulted from duplicate of sample and quadruplicate of analysis.

3.5.3 Sample clarification

Approximately 1g of chocolate milk was weighed and transferred into a 100 mL volumetric flask and 60 mL of distilled water was added. The flask was mixed and placed in a bath at 50 ºC for 15 min with occasional swirling. Then the following

solutions were added and mixed after each addition:2 mL of Carrez I solution (3.60 g of potassium hexacyanoferrate (II) [$K_4(Fe(CN)_6).3H_2O$] in 100 mL of distilled water), 2 mL of Carrez II solution (7.20 g of zinc sulphate ($ZnSO_47H_2O$) in 100 mL of distilled water) and 4 mL of NaOH solution (100 mM). Finally, the volume was adjusted to 100 mL with distilled water, mixed and filtered through Whatman No. 1 filter paper.This procedure wasrequired for the reducing sugars and lactose analyses.

3.5.4 Reducing sugars

To determinate the reducing sugars content in chocolate milk samples it was applied a colorimetric method using 3,5-dinitrosalicylic acid reagent (DNS). 1.0 mL of chocolate milk after clarification and 1.0 mL of DNS reagent were mixed. The mixture was heated to 100 ºC for 5 minutes and then placed inice for a quickcooling(to stop the reaction). After that the mixture was diluted with 10 mL of distilled water. Absorbance was measured using a Multiskan Go microplate spectrophotometer (Thermo Scientific, USA) with microplate of 96 wells (Brand, Wertheim, Germany). 300 µL of the mixture were added, being the blank prepared by adding 1.0 mL of distilled water. Then the plate was shaken for 10 seconds and the absorbance at 540 nm. The reducing sugars value resulted from the difference between the absorbance of the blank and the absorbance of samples. Six quantifications (duplicate of sample and triplicate of analysis) were performed and the value determined using the calibration curve(see**APPENDIX B**) represented in **Equation 2**and expressed in milligram of reducing sugars per gram of sample (mg of reducing sugars/g chocolate milk).

$$Abs\ (540\ nm) = 0.5162x - 0.0218 \qquad R^2 = 0.9955$$

Equation 2

3.5.5 Lactose & D-Galactose concentration

In this work, lactose and D-galactose concentrations were determined with an enzymatic test kit Lactose & D-galactose (K-LACGAR MPF 06/11) (Megazyme, International Wicklow, Ireland) according to the manufacturer's instructions and adapted to use in 96-well microplates.With this enzymatic kit only lactose was tested. The determination of lactose is based on the following three coupled reactions (Megazyme 2011).

$$lactose + H_2O \xrightarrow{\beta-\text{galactosidase}} D-galactose + D-glucose$$

$$\propto -D-galactose \xleftrightarrow{GalM} \beta-D-galactose$$

$$\beta-D-galactose + NAD^+ \xrightarrow{\beta-GalDH} D-galactonic\ acid + NADH + H^+$$

In this enzymatic kit the amount of NADH andlactoseobtained wasin a ratio of 1:1, being the NADH measured at 340 nm.

3.5.6 Colour analysis

Colour was measured two times per sampleand six random measures of each sample at RT were performed, using the CIE*Lab* system by a single operator. It was determined using the Minolta Konica CM 2300d (Minolta Konica, Osaka, Japan). The CIE*Lab* parameters were determined using the original SpectraMagic^{TM} NX software, Konica Minolta, USA, according to regulations of the International Comission on Illumination. The colour parameters were determined taking into account the parameters L^* (luminosity), a^*(red/green colour) and b^* (yellow/blue colour).

In addition, the difference in colour (ΔE) was calculated usingthe **Equation** 3 in which the control sample was always the day 0.

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

Equation 3

3.5.7 Scanning electron microscopy

Chocolate milk samples at different pressures, temperatures and days were observed by scanning electron microscope, HITACHIS-4100 (Tokyo, Japan) provided with an electron emission system with a maximum resolution of 15Å and an accelerating potential of 10 kV. The samples subjected to this test were prepared in advance. To this end, fixed to double-sided carbon tape on a disk of aluminium on which dispersed a minimum amount of chocolate milk and glued small pieces of coverglass. In order to increase the electrical conductivity of the samples, was elaborated a thin film of carbon, using the Emitech K950 equipment (Quorum Technologies, East Sussex, England).

3.6 Statistical analyses

All results obtained for each treatment were carried out in duplicate and all analyses were done in triplicate (quadruplicate and sextuplicate for pH and colour respectively). Treatment groups were compared using Analyses of variance (ANOVA), followed by a multiple comparison post hoc test, Tukey's HSD Test, at a 5% level of significance, and the results were expressed as mean ± standard deviation

4. RESULTS AND DISCUSSION

4.1 Microbial analyses

The initial microbial load of TAM, ENT, COL, PSY in the chocolate milk were 2.67±0.13, 2.69±0.14, 2.73±0.14, 2.74±0.08 Log_{10} CFU/mL, respectively. For *L. innocua* and *E. coli* the initial average counts were 2.51±0.19 and 2.58±0.10Log_{10} CFU/mL respectively (**Figure 3, 5, 7, 9, 11, 12**).

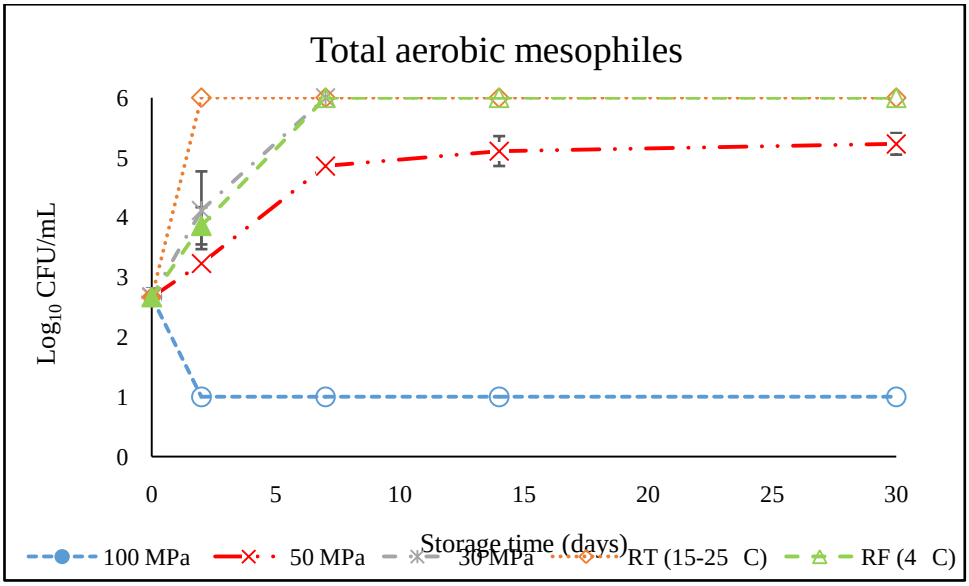
4.1.1 Effect of hyperbaric storage on total aerobic mesophiles

Concerning TAM counts, the initial value was 2.67±0.13 (see **Figure 3**). At 0.1 MPa/RT the microbial load increased about 3.5 Log_{10} CFU/mL after 2 days of storage, and were above 6 Log_{10}CFU/mL over the next 28 days. The counts obtained for storage at 0.1 MPa were higher ($p<0.05$) than those obtained for storage under 50 and 100 MPa.

Storage at 30 MPa/RT and 0.1 MPa/RF were statistically similar ($p>0.05$) in the 2^{nd} day. Until day 7, for the 30 MPa storage condition, the behaviour was similar to 0.1 MPa/RF, with counts above 6 Log_{10} CFU/mL.

When the pressure level was increased to 50 MPa, it was observed that after 2 days the counts obtained were 3.23±0.08 Log_{10} CFU/mL, lower ($p<0.05$) than those obtained at 30 MPa, 0.1 MPa/RF, and 0.1 MPa/RT. On the 7^{th} day, the microbial load at 50 MPa increased to 4.86±0.01 Log_{10} CFU/mL remaining lower ($p<0.05$) than in the 30 MPa storage condition, 0.1 MPa/RF, and 0.1 MPa/RT until the end of the experiment (day 30^{th}).

Moreover, when chocolate milk was stored under 100 MPa, it was observed a microbial inactivation effect, causing a microbial load reduction from 2.67±0.13 Log_{10} CFU/mL to $\leq$1Log_{10} CFU/mL, up to the 2^{nd} day. After that, the results remained lower than 1Log_{10} CFU/mL for more 28 days of storage. These results for TAM at 100 MPa are in accordance with Fidalgo et al. (2013) where a general growth inhibition/inactivation was found for more than 56 hours in watermelon juice under HS. In a recent published paper (Pinto et al.,2016), a microbial growth inhibition was obtained for TAM over a storage period of 7 days at 100MPa/RT, being presented a decrease of $\approx$2 Log_{10} CFU/mL from the initial value.



	Storage time (days)				
	0	2	7	14	30
100 MPa	a	*	*	*	*
50 MPa	a	b	d	d	d
30 MPa	a	c			
RT (15-25 °C)	a	#	#	#	#
RF (4 °C)	a	c	#	#	#

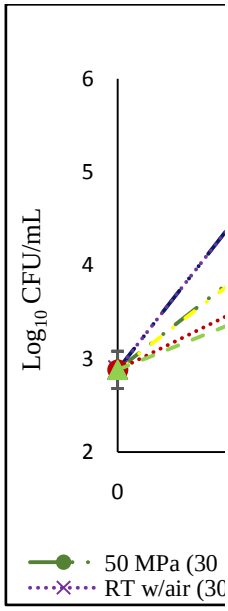
Figure 3: Microbial counts (Log_{10} CFU/mL chocolate milk) ofTAM in chocolate milk during 30 days of storage at 30 MPa, 50 MPa and 100 MPa at uncontrolled RT (15-25 °C). Two control conditions were also stored at 0.1 MPa/RT, and 0.1 MPa/RF. In the table different letters indicate significant differences ($p<0.05$) between conditions. Values shown as 6 (#) and 1 (*) log units, mean values higher than 6 and lower than 1 log units, respectively.

In this work, it was also studied the influence of air inside chocolate milk packages. Thus, chocolate milk packages, some of them with air and others without air inside, were stored at 50 MPa/30 °C (see **Figure 4**). In this HS experiment performed over 7 days, the initial microbial load was 2.88±0.2 Log_{10} CFU/mL. A storage condition of 0.1 MPa/30 ºC led to similar results in all samples (with and without air) when compared to 0.1 MPa/RT storage (**Figure 3**). After 2 days, the microbial counts for samples stored at 0.1 MPa/30 ºC, with and without air, were above 6 Log_{10} CFU/mL until day 7.

On the other hand, the microbial load of the samples stored at 50 MPa/30 ºC, with and without air, were above the counts obtained under 0.1 MPa/RF and below the value obtained at 0.1 MPa/30 ºC (with statistical significance, $p<0.05$). After 7 days, samples stored at all these conditions were above 6 Log_{10} CFU/mL. As expected, the

comparison between 50 MPa/RT and 50 MPa/30 ºC (samples without air inside) after 2 days of storage revealed a faster microbial growth for the latter condition due to high temperature storage.

Queirós et al. (2014) showed for the same storage condition (50 MPa/30 ºC without air), using melon juice, that microbial load increased when compared to the initial value just after 8 hours.



	Storage time (days)		
	0	2	7
RF (4 °C)	a	b	#
RF w/air (4 °C)	a	b	#
RT (30 °C)	a	#	#
RT w/air (30 °C)	a	#	#
50 MPa (30 °C)	a	c	#
50 MPa w/air (30 °C)	a	c	#

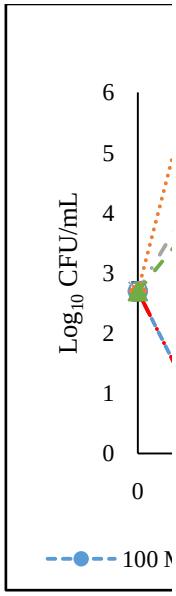
Figure 4:Microbial counts (Log_{10} CFU/mL chocolate milk) ofTAM in chocolate milk during 7 days of storage at 50 MPa without and 50 MPa with air at 30 °C. Four control conditions were also stored at 0.1 MPa/30 °C without air, 0.1 MPa/30 °C with air, 0.1 MPa/RF without air and 0.1 MPa/RF with air. In the table different letters indicate significant differences ($p<0.05$) between conditions. Values shown as 6 (#) and 1 (*) log units, mean values higher than 6 and lower than 1 log units, respectively.

4.1.2 Effect of hyperbaric storage on *Enterobacteriaceae*

The ENT counts obtained are expressed in **Figure 5** and**Figure 6**for the different HS experiments. A storage of 30 days at 0.1 MPa/RT led to a microbial growth increase to ≥6 Log_{10} CFU/mL only after 2 days when compared to the initial value (2.69±0.14

Log_{10} CFU/mL), and on the 0.1 MPa/RF storage, a microbial growth was also detected after 2 days, being close to 6 Log_{10} CFU/mL on the 7th day (5.88±0.21Log_{10} CFU/mL).

Concerning HS, although 30 MPa was not capable to decrease the initial microbial load, samples stored at that condition showed an important decrease between the 2nd and the 7th day, being these results better than the ones obtained at 0.1 MPa. However, when HS was performed at 50 MPa and 100 MPa, ENT presented similar results since after day 0, the initial value decreased to ≤1 Log_{10} CFU/mL and remained stable for more 28 days. Fidalgo et al. (2014) showed similar results for ENT in watermelon juice stored at 100 MPa and 18-21 ºC over 60 hours.



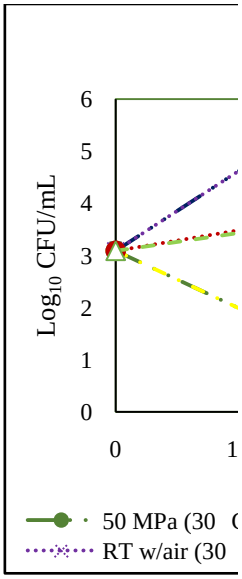
	Storage time (days)				
	0	2	7	14	30
100 MPa	a	*	*	*	*
50 MPa	a	*	*	*	*
30 MPa	a	c			
RT (15-25 °C)	a	#	#	#	#
RF (4 °C)	a	b	d	#	#

Figure 5:Microbial counts (Log_{10} CFU/mL chocolate milk) ofENT in chocolate milk during 30 days of storage at 30 MPa, 50 MPa and 100 MPa at uncontrolled RT (15-25 °C). Two control conditions were also stored at 0.1 MPa/RT, and at 0.1 MPa/RF. In the table different letters indicate significant differences (p<0.05) between conditions. Values shown as 6 (#) and 1 (*) log units, mean values higher than 6 and lower than 1 log units, respectively.

When chocolate milk samples were preserved at 30 ºC, the initial value for ENT was 3.09±0.15 Log_{10}CFU/mL (**Figure 6**),being detected a microbial growth when those samples, packaged with and without air, were stored at 0.1 MPa/30 ºC (≥6

Log_{10}CFU/mL).0.1 MPa/4 ºC led to a microbial growth after 2 days of storage with no significant differences ($p>0.05$) from the initial value, being the microbial counts above 6 Log_{10}CFU/mL on the 7th day.

In all samples (with and without air) stored at 50 MPa/30 ºC a decrease of the microbial load to ≤1 Log_{10} CFU/mL was observed. This storage condition was capable not only to inhibit the microbial growth but also to inactivate the microorganisms present on the samples, showing better results compared to 0.1 MPa/RT and 0.1 MPa/RF. As verified for TAM counts, it was not detected considerable differences between samples stored in packages with and without air.



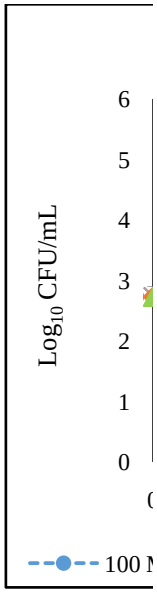
	Storage time (days)		
	0	2	7
RF (4 °C)	a	a	#
RF w/air (4 °C)	a	a	#
RT (30 °C)	a	#	#
RT w/air (30 °C)	a	#	#
50 MPa (30 °C)	a	*	*
50 MPa w/air (30 °C)	a	*	*

Figure 6:Microbial counts (Log_{10} CFU/mL chocolate milk) ofENT in chocolate milk during 7 days of storage at 50 MPa and 50 MPa with air at 30 °C. Four control conditions were also stored at 0.1 MPa/30 °C, 0.1 MPa/30 °C with air, 0.1 MPa/RF and 0.1 MPa/RF with air. In the table different letters indicate significant differences ($p<0.05$) between conditions. Values shown as 6 (#) and 1 (*) log units, mean values higher than 6 and lower than 1 log units, respectively.

4.1.3 Effect of hyperbaric storage on coliforms

On 0.1 MPa/RT storage a microbial load increase was detected after 2 days to values above 6 Log_{10} CFU/mL. For 0.1 MPa/RF storage, the results exhibited a slower microbial load increase, reaching values above 6 Log_{10} CFU/mL only after 7 days of

storage. A storage for 7 days at 30 MPa led to an increase on COL of 1 Log_{10} CFU/mL in the 2nd day of storage from an initial value of 2.73±0.14 Log_{10} CFU/mL, similarly to 0.1 MPa/RF ($p>0.05$) (**Figure 7**). In the 7th day, the microbial load was maintained with no significant changes ($p>0.05$). When the pressure level of the HS was increased to 50 and 100 MPa, it was observed a decrease of almost 2Log_{10} CFU/mL, reaching values of ≤1Log_{10}CFU/mL over the remain storage period.

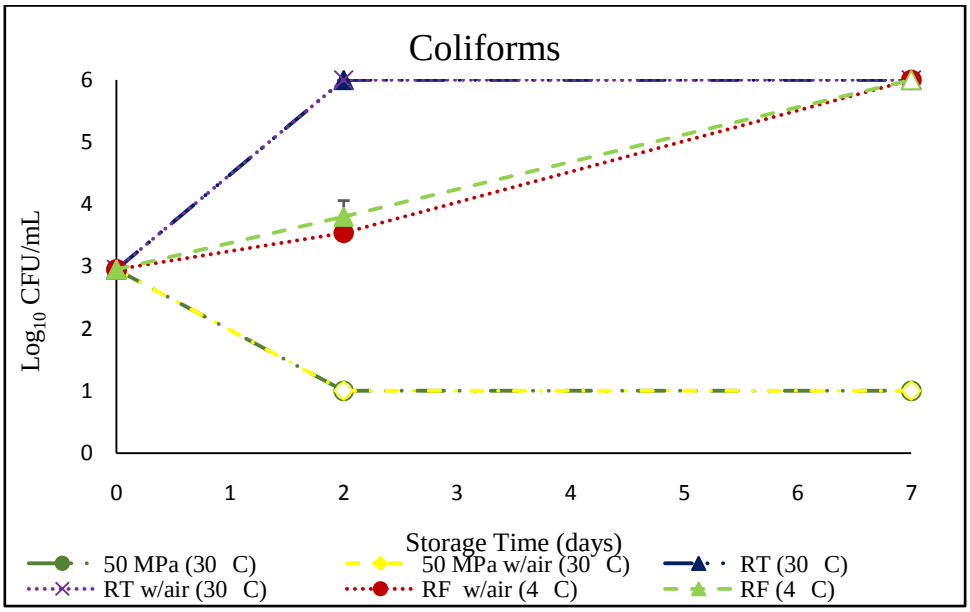


	Storage time (days)				
	0	2	7	14	30
100 MPa	a	*	*	*	*
50 MPa	a	*	*	*	*
30 MPa	a	b	b		
RT (15-25 °C)	a	#	#	#	#
RF (4 °C)	a	b	#	#	#

Figure 7:Microbial counts (Log_{10} CFU/mL chocolate milk) of COL in chocolate milk during 30 days of storage at 30 MPa, 50 MPa and 100 MPa atuncontrolledRT (15-25 °C). Two control conditions were also stored at 0.1 MPa/RT and 0.1 MPa/RF.In the table different letters indicate significant differences ($p<0.05$) between conditions. Values shown as 6 (#) and 1 (*) log units, mean values higher than 6 and lower than 1 log units, respectively.

Regarding COL, chocolate milk preservation at 50 MPa/30 ºC led to similar results when compared to 50 MPa/RT (**Figure 8**). As it is possible to observe in **Figure 8,** HS experiments of 50 MPa/30 ºC, on samples with and without air, led to values of ≤1 Log_{10} CFU/mL after 2 days of storage, being the initial value of 2.95±0.08 Log_{10}CFU/mL.

Regarding packaging conditions, no significate differences ($p<0.05$) were detected between samples with and without air on coliforms analyses.



	Storage time (days)		
	0	2	7
RF (4 °C)	b	c	#
RF w/air (4 °C)	b	b	#
RT (30 °C)	b	#	#
RT w/air (30 °C)	b	#	#
50 MPa (30 °C)	b	*	*
50 MPa w/air (30 °C)	b	*	*

Figure 8: Microbial counts (Log10 CFU/mL chocolate milk) of COL in chocolate milk during 7 days of storage at 50 MPa without air and 50 MPa with air at 30 °C. Four control conditions were also stored at 0.1 MPa/ 30 °C without air, 0.1 MPa/ 30 °C with air, 0.1 MPa/RF without air and0.1 MPa/RF with air. In the table different letters indicate significant differences ($p<0.05$) between conditions. Values shown as 6 (#) and 1 (*) log units, mean values higher than 6 and lower than 1 log units, respectively.

4.1.4 Effect of hyperbaric storage in psychrophiles

The results obtained for PSY were similar to the ones verified for TAM in **figure 3**. The initial value for PSY was 2.73±0.14Log_{10} CFU/mL (**Figure 9**). Regarding samples stored under pressure, 30 MPa was not capable to inhibit the microbial growth, being found values above 6 Log_{10} CFU/mL on the 7th day of storage. For 50 MPa the microbial growth was similar to 0.1 MPa/RF in the 2nd day ($p>0.05$), however, being present an increase tendency over storage time, reaching values close to 6 Log_{10}CFU/mL only at the 30th day. At 100 MPa the microbial load decreased to values

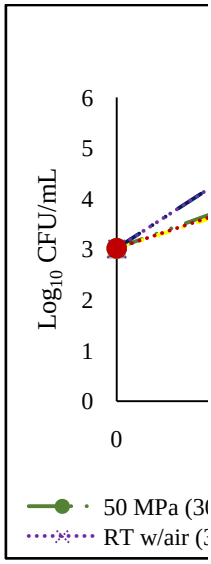
≤1 Log_{10} CFU/mL after 2 days, remaining below 1 Log_{10}CFU/mL for more 28 days. As expected, 0.1 MPa/RT and 0.1 MPa/RF allowed a microbial load increase to values above 6 Log_{10} CFU/mL.

	Storage time (days)				
	0	2	7	14	30
100 MPa	a	*	*	*	*
50 MPa	a	b	d	d	e
30 MPa	a	c			
RT (15-25 °C)	a	#	#	#	#
RF (4 °C)	a	b	#	#	#

Figure 9:Microbial counts (Log_{10} CFU/mL chocolate milk) ofPSY in chocolate milk during 30 days of storage at 30 MPa, 50 MPa and 100 MPa atuncontrolled RT (15-25 °C). Two control conditions were also stored at 0.1 MPa/RT and 0.1 MPa/RF. In the table different letters indicate significant differences (p<0.05) between conditions. Values shown as 6 (#) and 1 (*) log units, mean values higher than 6 and lower than 1 log units, respectively.

For the experiments carried out at 30 ºC, the initial value of PSY was 3.02±0.04 Log_{10} CFU/mL. (**Figure 10**). All samples, with and without air, at 50 MPa/30 ºC showed an increase of 1 Log_{10} CFU/mL after 2 days of storage with no significant differences between them. At the 7th day, the microbial load was above 6 Log_{10} CFU/mL for all conditions. Under RF, differences (p<0.05) between packages (with and without air) were detected in the 2nd day, still, presenting both packages at the end of storage values above 6 Log_{10} CFU/mL.

Storage time (days)



	0	2	7
RF (4 °C)	a	b	#
RF w/air (4 °C)	a	c	#
RT (30 °C)	a	#	#
RT w/air (30 °C)	a	#	#
50 MPa (30 °C)	a	c	#
50 MPa w/air (30 °C)	a	c	#

Figure 10:Microbial counts (Log10 CFU/mL chocolate milk) of PSY in chocolate milk during 7 days of storage at 50 MPa without and 50 MPa with air at 30 °C. Four control conditions were also stored at 0.1 MPa/30 °C without air, 0.1 MPa/30 °C with air, 0.1 MPa/RF without air, and 0.1 MPa/RF with air.In the table different letters indicate significant differences (p<0.05) between conditions. Values shown as 6 (#) and 1 (*) log units, mean values higher than 6 and lower than 1 log units, respectively.

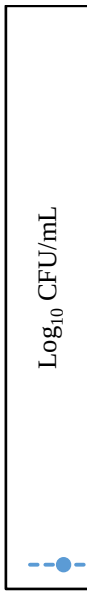
4.1.5 Effect of hyperbaric storage in *E.coli*

The initial value for samples inoculated with *E. coli* was 2.58±0.10 Log_{10} CFU/mL (**Figure 11**).

The storage conditions of 30 MPa, 0.1 MPa/RF and 0.1 MPa/RTallowed the microbial growth increase. In the 30 MPa experiment, the microbial growth observed in the 2nd day (≈2 Log_{10} CFU/mL) presented significant differences ($p<0.05$) compared with the other storage conditions at the same day. Still, at the 7th day, all these three storage conditions presented values of *E. coli* above 6 Log_{10} CFU/mL.

For 50 MPa storage, *E. coli*were capable to growth after 2 days but with no significant differences from the initial value of the experience. At the 7th and 14th day of storage, the results were statistically similar ($p>0.05$) without significant differences between these days. From the 14th day of storage, *E. coli* decreased more than 2 Log_{10}CFU/mL to value below 1 Log_{10}CFU/mL.

For 100 MPa storage, a significant ($p<0.05$) increase of microbial growth in the initial 2 days of storage was detected, but after that, the microbial load decreased to values below 1 Log_{10}CFU/mL up to the 30th day of storage.



	Storage time (days)				
	0	2	7	14	30
100 MPa	a	cd	*	*	*
50 MPa	a	abc	d	cd	*
30 MPa	a	e	#		
RT (15-25 °C)	a	#	#	#	#
RF (4 °C)	a	ab	bcd	#	#

Figure 11: Microbial counts (Log_{10} CFU/mL chocolate milk) of*E.coli* in chocolate milk during 30 days of storage at 30 MPa, 50 MPa and 100 MPa at uncontrolledRT (15-25 °C). Two control conditions were also stored at 0.1 MPa/RT and 0.1 MPa/RF. In the table different letters indicate significant differences ($p<0.05$) between conditions. Values shown as 6 (#) and 1 (*) log units, mean values higher than 6 and lower than 1 log units, respectively.

4.1.6 Effect of hyperbaric storage in *L.innocua*

Concerning the inoculated samples with *L. innocua*, after 2 days of HS at 100 MPa at RT, the microbial load was reduced from 2.51±0.19Log_{10} CFU/mL to ≤1.00 Log_{10} CFU/mL, being this value kept up to the end of the storage (**Figure 12**). In the HS experiments at 50 MPa, similar results were obtained ($p>0.05$) during the first two days. After the 2nd day at 50 MPa the microbial load increased about 1.00 Log_{10} CFU/mL up to the 7th day, decreasing afterwards up to the end of the experiment.

The storage at 30 MPa, 0.1 MPa/RT and 0.1 MPa/RF presented similar results after the 7th day of storage up to the end of the experiment.

	Storage time (days)				
	0	2	7	14	30
100 MPa	a	a	*	*	*
50 MPa	a	a	b	a	*
30 MPa	a	c			
RT (15-25 °C)	a		#	#	#
RF (4 °C)	a	b	c	#	#

Figure 12:Microbial counts (Log_{10} CFU/mL chocolate milk) of*L. innocua* in chocolate milk during 30 days of storage at 30 MPa, 50 MPa and 100 MPa at uncontrolled RT (15-25 °C). Two control conditions were also stored at 0.1 MPa/RT and 0.1 MPa/RF. In the table different letters indicate significant differences ($p<0.05$) between conditions. Values shown as 6 (#) and 1 (*) log units, mean values higher than 6 and lower than 1 log units, respectively.

4.1.7 Microbial analyses – overview

Generally, a storage condition of 0.1 MPa/RT over 30 days led to a microbial load increase ($p<0.05$) when compared to the initial value, reaching a maximum of ≥6.00 Log_{10} CFU/mL for all microorganisms just after 2 days. These results were expected due to high water activity of chocolate milk and its low acidity.When chocolate milk was stored at 0.1 MPa/RF the microbial loads increased ($p<0.05$) comparatively to the initial value, reaching ≥6.00 Log_{10} CFU/mL after 7 days of storage.

When chocolate milk was stored under higher pressures (30, 50 and 100 MPa) the microbial counts obtained were lower ($p<0.05$) than those verified at 0.1 MPa/RT for the same period of time. HS at 30 MPa, revealed a preservation effect similar to RF,

since the limit of 6.00 Log_{10} CFU/mL was reached at the same time for TAM and PSY, after 7 days of storage.

In addition, for 50 MPa storage condition, similar results were obtained between TAM and PSY (**Figure 3** and **Figure 9**) with a slow microbial growth rate being present. In this case, for ENT and COL (**Figure 5** and **Figure 7**) a microbial inactivation was detected (≤1.00 Log_{10} CFU/mL). The pressure level increase to 100 MPa, led to a microbial inactivation of all microorganismsobserved at the 2nd day of storage, being these values stable until the end (30th day).

To test HS at higher temperatures, chocolate milk was stored at 50 MPa/30 ºC (**Figure 4, 6, 8, 10**), in which samples were packed with and without air, in order to compare at the same time possible microbiological differences empowered by the presence of air. These experiments did not show significant differences between samples packed with and without air, but demonstrated that samples at 50 MPa/30 ºC without air have a faster microbial growth when compared to 50 MPa/RT (samples packed also without air). These results revealed a better performance of HS at RT and 30 ºC, when compared to 0.1 MPa/RF and 0.1 MPa/RT.

4.2 Physicochemical analysis

4.2.1 Water activity

As pH, water activity (A_w) is one of the major factors influencing microbial growth. As expected, the initial water activity value fluctuates between 0.971±0.001 and 0.989±0.001, allowing the microbial spoilage by the majority of microorganisms. For all storage conditions, water activity did not significantly change throughout storage period compared to the initial value (**Table 11**). This behaviour is in accordance with one published study regarding *requeijão* preserved under HS, where the water activity was also very high and at the end of storage the changes on this parameter were not significant (Duarte et al., 2014).

Table 11: Water activity for chocolate milk stored for 30 days at 30 ºC and 15-25 ºCand pressure (MPa) conditions.

Water activity					
	Days				
Conditions	0	2	7	14	30
30 MPa	0.989±0.001	0.988±0.001	0.986±0.001		
50 MPa	0.989±0.001	0.985±0.001	0.986±0.001	0.987±0.001	0.985±10.001
100 MPa	0.971±0.001	0.960±0.001	0.966±0.001	0.989±0.001	0.964±0.001
RT(15-25ºC)	0.989±0.001	*	*	0.988±0.001	0.985±0.001
RF(4ºC)	0.989±0.001	*	*	0.986±0.001	0.985±0.001
RF (4 ºC) w/air	*	*	*	*	*
50 MPa 30ºC	0.975±0.001	0.975±0.001	0.971±0.001		
50 MPa w/air 30ºC	0.975±0.001	0.971±0.001	0.968±0.001		
RT 30 ºC	*	*	*	*	*
RT 30 ºC w/air	*	*	*	*	

*- Experiments were not carried out in these conditions

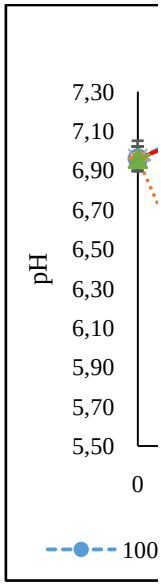
4.2.2 pH

The initial pH of chocolate milk was 6.96±0.08 for all studies (0.1 MPa/RT, 0.1 MPa/RF, 30 MPa, 50 MPa, and 100 MPa). In general,the values varied for 0.1 MPa/RT, 0.1 MPa/RF, 30 MPa, 50 MPa, but remained stable, without significant changes,for 100 MPa ($p>0.05$) (see**Figure 13**).

HS at 100 MPa showed no significant differences ($p>0.05$) over all storage period (30 days). Concerning lower pressure levels, for 50 MPa storage, until the 14th day, it was not found significant changes ($p>0.05$) compared to the initial value, but after that, pH decreased to 6.73±0.05. For 30 MPa no significant changes ($p>0.05$) were noticed during the 7 days of storage.

On the other hand, samples stored under 0.1 MPa/RF presented a decrease after the 7th day of storage to values of 6.41±0.02 and 6.25±0.03 in days 14th and 30th, respectively, with statistical differences ($p<0.05$) between the 14th and 30th day. 0.1 MPa/RT led to a decrease after the 2nd day of storage to a value of 6.36±0.03, decreasing even more up to the end (5.87±0.04).

Storage time (days)

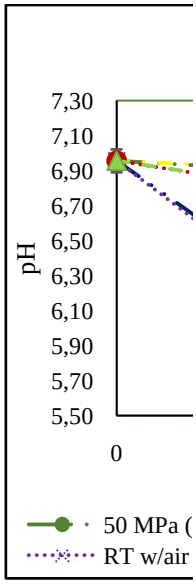


	0	2	7	14	30
100 MPa	gh	h	gh	gh	gh
50 MPa	gh	h	h	gh	e
30 MPa	gh	fg	ef		
RT (15-25 °C)	gh	cd	b	b	a
RF (4 °C)	gh	h	gh	d	c

Figure 13:Chocolate milk pH during 30 days of storage at 30 MPa, 50 MPa and 100 MPa at uncontrolled RT (15-25 °C). Control samples conditions were also stored at 0.1 MPa/RT and 0.1 MPa/RF. In the table different letters indicate significant differences (p<0.05) between conditions.

In HS experiments at 50 MPa/30 ºC the initial pH value of chocolate milk was 6.78±0.04 (**Figure 14**). Samples stored at 50 MPa/30 ºC, packaged with and without air, presented a similar behaviour (p>0.05) from day 0 up to the 2nd day. At the 7th day of storage, the pH of the samples packages without air decreased significantly (p<0.05) compared to 50 MPa/30 ºC with air for the same day of storage. No significant differences (p<0.05) were detected between samples packed with and without air on 0.1 MPa/RF storage at the 2nd day.

HS at 100 MPa, 50 MPa and 50 MPa/30ºC allowed to avoid the pH decrease when compared to 0.1 MPa/RT storage.



	0	2	7
RF (4 °C)	e	b	
RF w/air (4 °C)	e	bc	
RT (30 °C)	e	a	
RT w/air (30 °C)	e	a	
50 MPa (30 °C)	e	de	cd
50 MPa w/air (30 °C)	e	de	b

Figure 14:Chocolate milk pH during 7 days of storage at 50 MPa at 30 °C (samples packed with and without air). Control samples were also stored at 0.1 MPa/30 °C, 0.1 MPa/4°C. In the table different letters indicate significant differences (p<0.05) between conditions.

4.2.3 Colour

Currently the available data regarding colour preservation of chocolate milk over storage is scarce. The initial chocolate milk samples showed a bright brown colour (*L**= 49.24±1.69) (**Figure 15**), tending to red (*a**= 4.00±0.59) (**Figure 16**) and yellow (*b**=4.49±0.60) (**Figure 17**). The *L** value (luminosity) show, in general for all conditions,a decrease of luminosityat the end of storagecompared to the initial value. Regarding *a** parameter, all conditions presented an increase of this value over storage with two exceptions, 100 MPa and 0.1 MPa/RF. On the other hand, an increase of *b** was verified for all storage conditions with the exception of 0.1 MPa/RF at the end of storage. The total colour changes (ΔE) were significant ($p<0.05$) after the 7th day of storage up to the end.

The luminosity parameter (**Figure 15**)presented a decrease tendency at the end of storage. Regarding 30 MPa, 0.1 MPa/RT and 0.1 MPa/RF storage conditions, it was

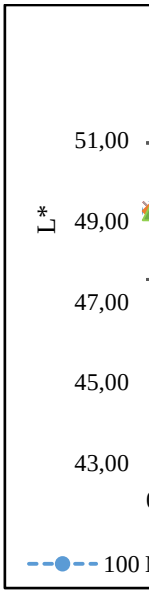
not found significant differences ($p>0.05$) over period of storage. After 7 days, forHS at 50 and100MPa, the values were below the initial value ($p<0.05$), but at the end of the storage the results presented no significant differences compared to the initial value for 50 MPa ($p>0.05$). For 100 MPa the values remained stable until the end of storage with significant differences compared to the initial value ($p<0.05$).

The redness (**Figure 16**) showed no significant differences ($p>0.05$) on the first 14 days of storage compared to the initial value, except for 0.1 MPa/RT.Storage at 50 MPa after the 14^{th} show an increase in redness with significant differences compared to the initial value and with the other conditions for the same day.

The yellowness (**Figure 17**) displayed no significant differences ($p>0.05$) on the first 14 days of storage compared to the initial value, except for 100 MPa a* decreased after the 7^{nd} day. Storage at 50 MPa after the 14^{th} show an increase in redness, to values compared to the day 0. For 100 MPa, after the 2^{nd} day, the values maintained below the initial one ($p<0.05$) for the remain storage period.

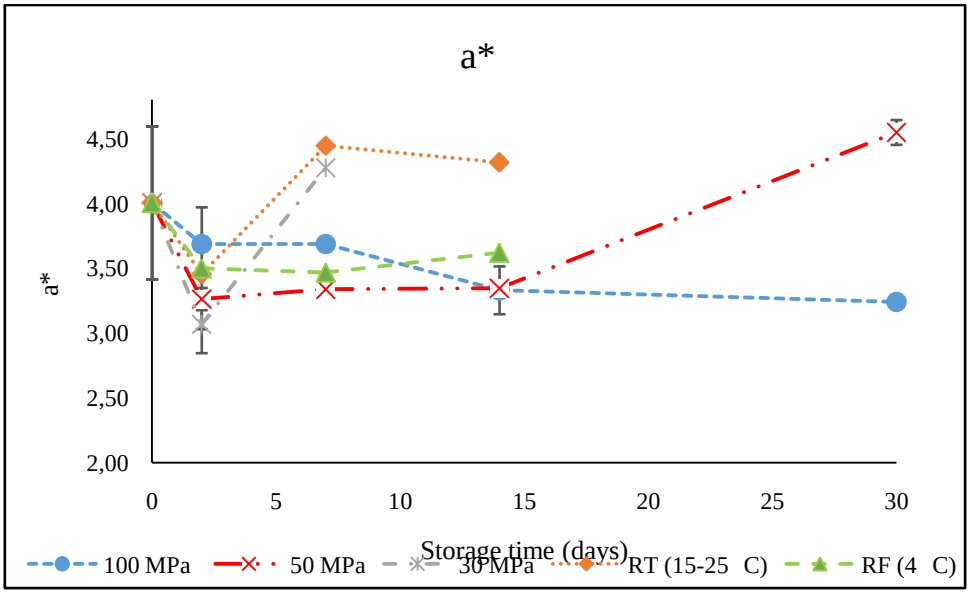
The colour preservation under HS conditions so far published, show that HS preserved the food colour when compared to AP at the same temperature for carrot soup and strawberry juice (Moreira et al., 2015; Bermejo-Prada & Otero, 2016). In this case (**Figure 18**) storage at 100 MPa exhibit significant changes compared to the initial value, but with similar results to 0.1 MPa/RT. For 50 MPa and 30 MPa no significant changes were detected during storage as well as for 0.1 MPa/RF. However, should be highlighted the relative high error associated with the ΔE calculation.

Storage time (days)



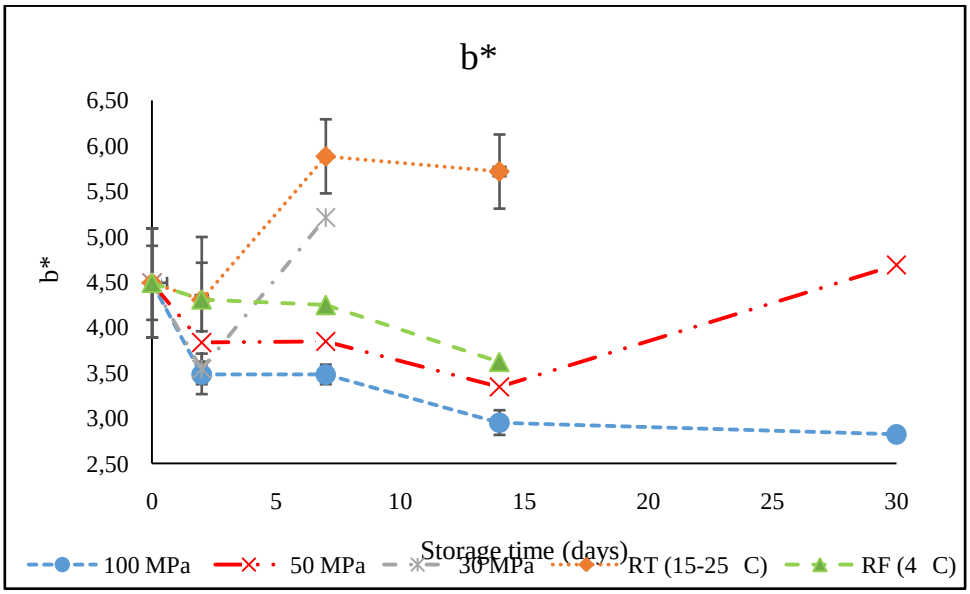
	0	2	7	14	30
100 MPa	ef	cde	ab	a	a
50 MPa	ef	ab	abcd	abcd	cde
30 MPa	ef	abc	def		
RT (15-25 °C)	ef	bcde	f	cdef	
RF (4 °C)	ef	cde	cde	abcd	

Figure 15: Chocolate milk colour (L*) during 30 days of storage at 30 MPa, 50 MPa and 100 MPa at uncontrolled RT (15-25 °C). Control samples were also stored at 0.1 MPa/RT and 0.1 MPa/RF. In the table different letters indicate significant differences ($p<0.05$) between conditions.



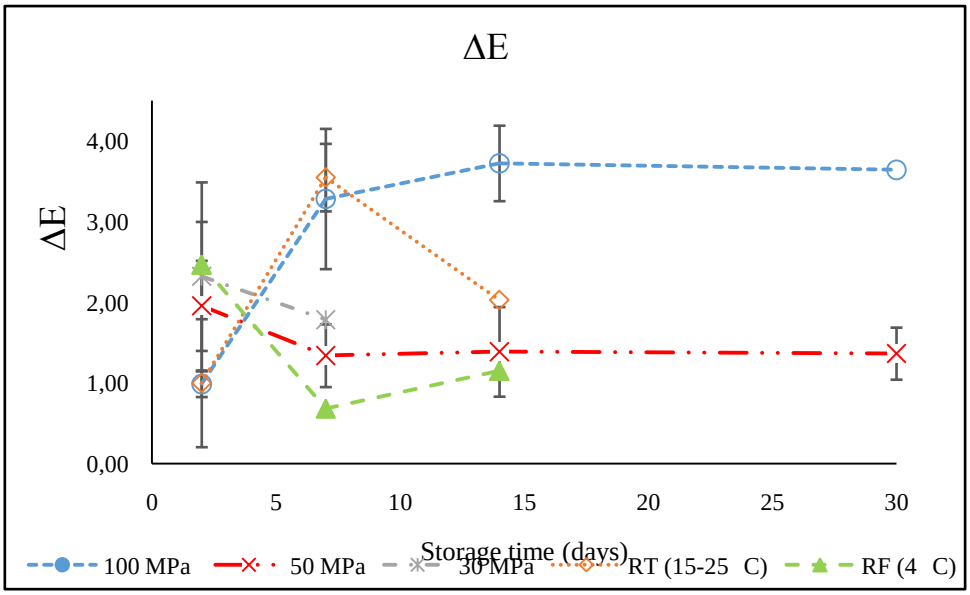
	Storage time (days)				
	0	2	7	14	30
100 MPa	cdef	bcdef	bcdef	abc	abc
50 MPa	cdef	a	abc	abc	f
30 MPa	cdef	ab	def		
RT (15-25 °C)	cdef	bcd	ef	def	
RF (4 °C)	cdef	bcd	bcd	bcde	

Figure 16: Chocolate milk colour (a*) during 30 days of storage at 30 MPa, 50 MPa and 100 MPa at RT uncontrolled (15-25 °C). Control samples conditions were also stored at 0.1 MPa/RT and 0.1 MPa/RF. In the table different letters indicate significant differences (p<0.05) between conditions.



	Storage time (days)				
	0	2	7	14	30
100 MPa	efg	def	abcd	abc	ab
50 MPa	efg	a	cdef	bcde	fg
30 MPa	efg	bcd	gh		
RT (15-25 °C)	efg	defg	h	h	
RF (4 °C)	efg	defg	def	fg	

Figure 17: Chocolate milk colour (b*) during 30 days of storage at 30 MPa, 50 MPa and 100 MPa atuncontrolledRT (15-25 °C). Control sampleswere also stored at 0.1 MPa/RT and 0.1 MPa/RF. Different letters indicate significantdifferences (p<0.05) between conditions.



	Storage time (days)			
	2	7	14	30
100 MPa	ab	de	e	e
50 MPa	abcd	abc	abc	abc
30 MPa	bcde	abc		
RT (15-25 °C)	abc	e	abcd	
RF (4 °C)	cde	a	abc	

Figure 18: Chocolate milk colour (ΔE)during 30 days of storage at 30 MPa, 50 MPa and 100 MPa at RT uncontrolled (15-25 °C). Control samples conditions were also stored at 0.1 MPa/RT and 0.1 MPa/RF. In the table different letters indicate significantdifferences (p<0.05) between conditions.

In **Figure 19** is shown the colour evolution of samples preserved at 50 MPa, 0.1 MPa/RF and 0.1 MPa/RT over 14 days. In image A, it is present the initial colour of the chocolate milk used in this study. After 2 days, (image B) shows some colour degradation in 0.1 MPa/RT storage (the microbial counts were above 6 Log_{10} CFU/mL), and the maintenance of the apparent colour for 0.1 MPa/RF and 50 MPa storages. At the 7th day (image C), 0.1 MPa/RT showed a continue colour degradation, as well as the size of the bag was increased due to gas production. At the 14th day (image D), samples preserved under 0.1 MPa/RT showed significant changes on its aspect, with a decrease of colour intensity, as well as 0.1 MPa/RF increased the size of the bag due to gas production. 50 MPa at 14th day showed the same darkening colour like on the7th day.

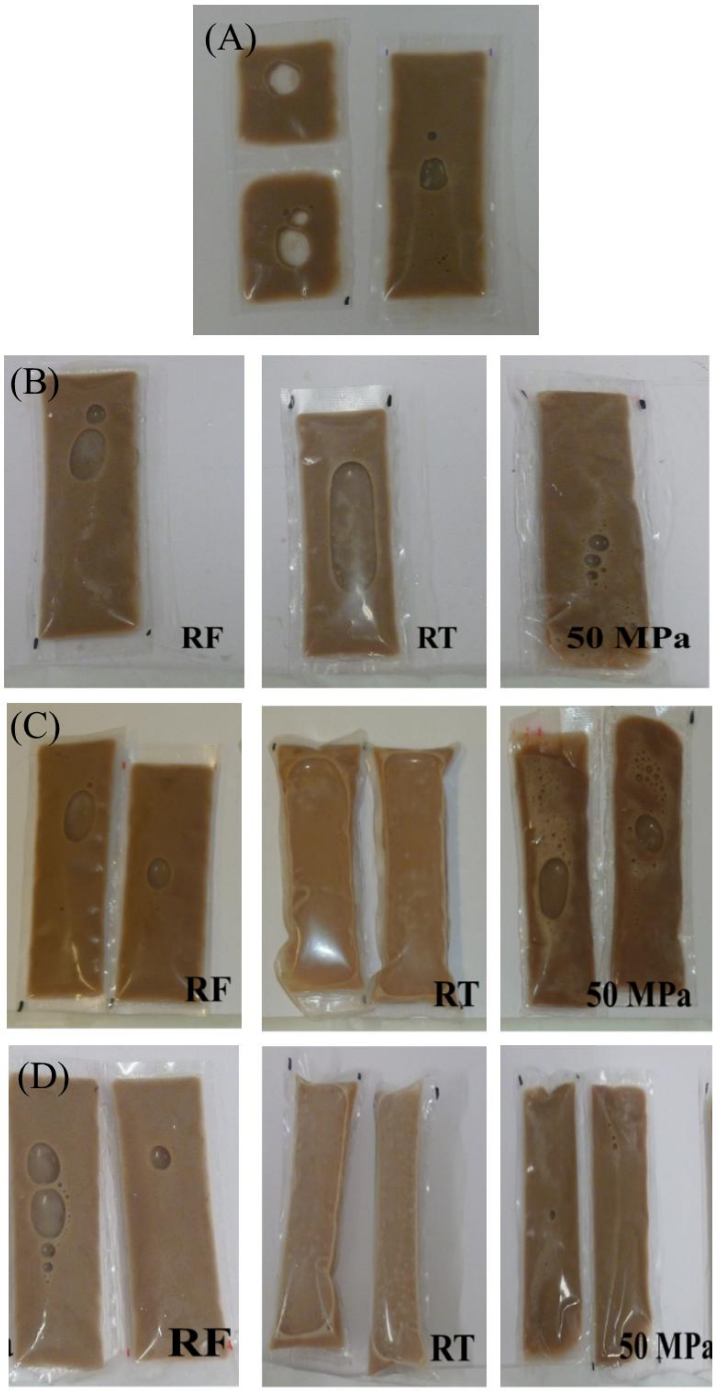
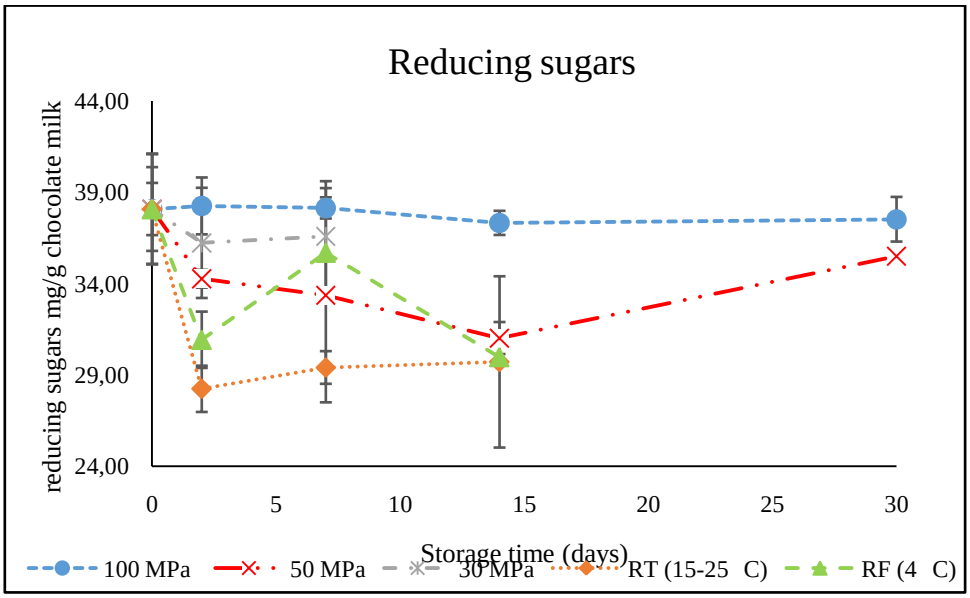


Figure 19: Samples of chocolate milk during the days of storage at 0.1 MPa/RT, 0.1 MPa/RF, 50 MPa (A) day 0, (B) day 2, (C) day 7 and (D) day 14.

4.2.4 Reducing sugars

The major sugars present in chocolate milk are lactose and glucose. In**Figure 20**is possible to observe the initial value of reducing sugars (38.082±3.02 mg reducing sugars/g chocolate milk). Storage at 100 MPa led to no significant changes ($p>0.05$)

during the 30 days of storage regarding this parameter. On the other hand, the pressure level decrease to 50 MPa led to a decrease of the reducing sugars content during the first 14 days, and an increase for the remain storage time with no significant changes ($p>0.05$). An even lower pressure level, 30 MPa, allowed to obtain a similar behaviour since a decrease was observed with no significant changes ($p>0.05$) over time. The control samples, 0.1 MPa/RF and 0.1 MPa/RT presented an even lower decrease tendency of reducing sugars content over storage time.



	Storage time (days)				
	0	2	7	14	30
100 MPa	defg	g	ef	cdefg	defg
50 MPa	defg	abcdef	abcdef	abcde	Bcdef
30 MPa	defg	bcdef	abcdef		
RT (15-25 °C)	defg	a	ab	abc	
RF (4 °C)	defg	abcd	abcdef	ab	

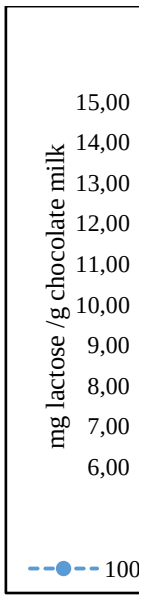
Figure 20: Reducing sugars in chocolate milk during 30 days of storage at 30 MPa, 50 MPa and 100 MPa atuncontrolledRT (15-25 °C). Control sampleswere also stored at 0.1 MPa/RT and 0.1 MPa/RF. In the table different letters indicate significantdifferences ($p<0.05$) between conditions.

4.2.5 Lactose

Lactose is a white crystalline disaccharide, formed in the mammary glands of all lactating animals, being present in their milk. Lactose yields D-galactose and D-glucose on hydrolysis by lactase (β-galactosidase). Enzymatic methods for the measurement of lactose are well known and are generally based on the hydrolysis of lactose to D-

galactose and D-glucose with β-galactosidase, followed by determination of either D-galactose or D-glucose. The lactose measurement as D-galactose is more reliable than the measurement as D-glucose since preparations generally contain more free D-glucose than free D-galactose.

In this assay, galactose was not detected, only lactose content was determined. During all storage time were not detected significant differences ($p>0.05$) between all the conditions (**Figure 21**).



	Storage time (days)				
	0	2	7	14	30
100 MPa	a	a	a	a	a
50 MPa	a	a	a	a	a
30 MPa	a	a	a		
RT (15-25 °C)	a	a	a	a	a
RF (4 °C)	a	a	a	a	a

Figure 21: Lactose concentration in chocolate milkduring 30 days of storage at 30 MPa, 50 MPa and 100 MPa at uncontrolledRT (15-25 °C). Two control conditions were also stored at 0.1 MPa/RT and 0.1 MPa/RF. In the table different letters indicate significantdifferences ($p<0.05$) between conditions.

4.2.6 SEM

SEM images were used as an attempt to determine possible differences on chocolate milk fat globules during different storage conditions. In **Figure 22** is shown the fat globules in chocolate milk in the initial sample and after 2 days for the different storage conditions. Figure (A) shows the fat globules before storage.Although figure A presents many clusters at day 0, during the remain storage period the clusters of fat globules decreased (Figure B, C, D). Storage at 0.1 MPa/RT (B) and 0.1 MPa/RF (C),

demonstrated less fat globules compared to the initial sample. For 100 MPa(D), SEM allowed to observea smaller amount of fat globules, being also visible an aggregation of these.

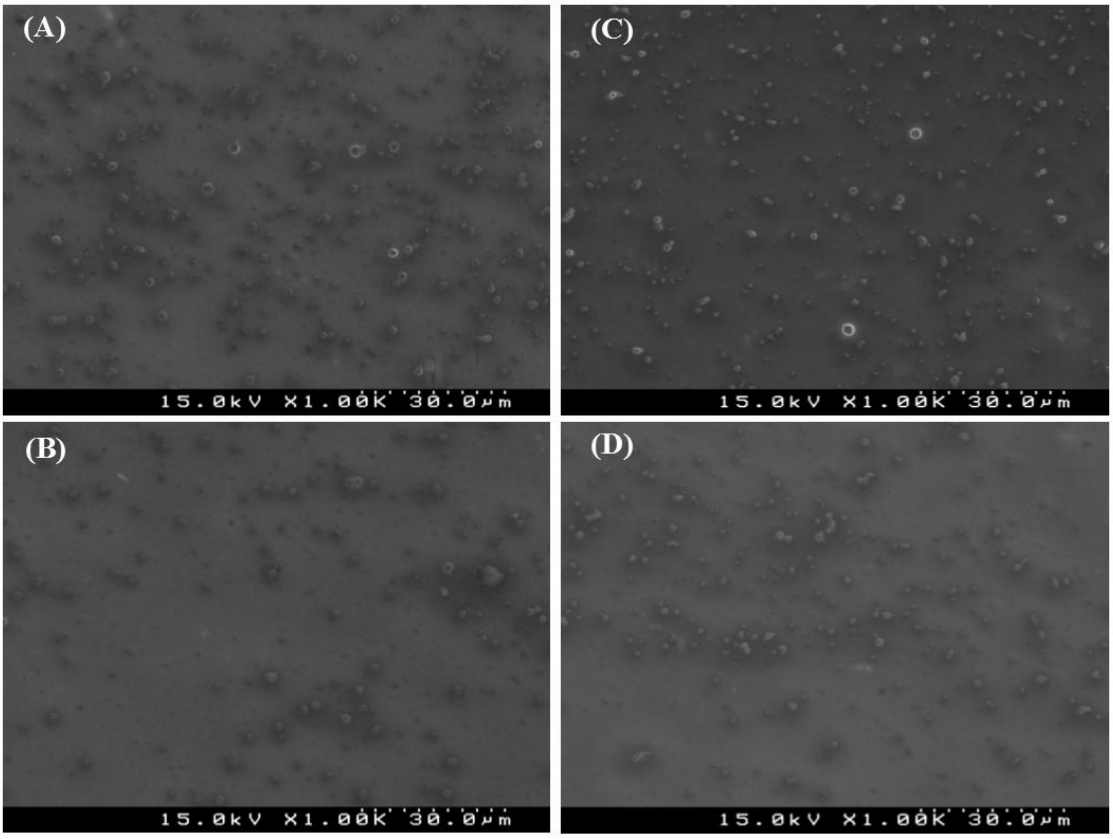


Figure 22: Fat globules in chocolate milk in the initial day of storage (A) and after 2 days of storage for 0.1 MPa/RT(B), 0.1 MPa /RF(C) and 100 MPa(D).

5. CONCLUSION AND FUTURE WORK

Currently, dairy products are preserved under RF, leading to high energetic costs. In this study, the HS feasibility at uncontrolled RT (15-25 ºC) or above it (30 ºC), was assessed as a possible alternative to RF for chocolate milk.

The HS at uncontrolled RT (15-25 ºC) of chocolate milk showed equal to better microbiological and physicochemical results compared to RF, since HS samples presented a microbial growth inhibition and for some pressure levels, a microbial load reduction (microorganisms inactivation). Storage at 0.1 MPa/RT led to microbial loads higher than the initial ones, being above 6.00 Log_{10} CFU/mL for all microorganisms after 2 days of storage. Microbial inactivation was more pronounced at 100 MPa, and microbial growth inhibition prevailed for the lower pressure levels 50 MPa and 30 MPa, being for the latter similar to 0.1 MPa/RF storage condition. In 50 MPa preservation, a microbial inactivation occurred for COL and ENT, but a slow growth inhibition was verified for TAM and PSY. After 2 days of HS at 100 MPa all microorganisms reduced about 1.50 Log_{10} CFU/mL to values below the detection limit remaining unchanged for the rest of the 28 days of storage. The most resistant microorganisms to pressure were TAM and PSY, instead, COL and ENT were much more easily inactivated by pressure. For 50 MPa/30 ºC, a microbial inactivation was observed for COL and ENT but TAM and PSY presented a similar behaviour compared to 0.1 MPa/RF. The temperature increase (30 ºC) led to a more pronounced growth of TAM and PSY, but no significant changes were detected between samples packaged with air and without air.

Physicochemical parameters revealed small changes, normally having no significant differences between the chocolate milk stored under pressure and under RF. HS was capable to maintain pH, reducing sugars and colour values compared to RF and RT.

Regardless of these results, more studies are needed under other pressure levels and temperatures. Other physicochemical parameters such as viscosity and fatty acids determination should be evaluated.

In order to apply this food preservation methodology at an industrial level, new studies must be done with different food products. It is relevant to understand how microorganisms behave under HS in different foods, as well as physicochemical and sensorial parameters are important to assess the HS feasibility as an alternative to the conventional RF.

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APPENDIX

APPENDIX A: Standards preparation and calibration curve construction for the determination of reducing sugars content in chocolate milk

A1. DNS reagent preparation:

10 g of DNS were weighted and dissolved in 200 mL of a 2N NaOH solution. The solution was then heated and stirred intensively. Simultaneously a solution of 300 g of potassium tartrate in 500 mL of distilled water was prepared and heated (with intense stirring). The two solutions were mixed and stirred. Distilled water was added to make up 1 L.

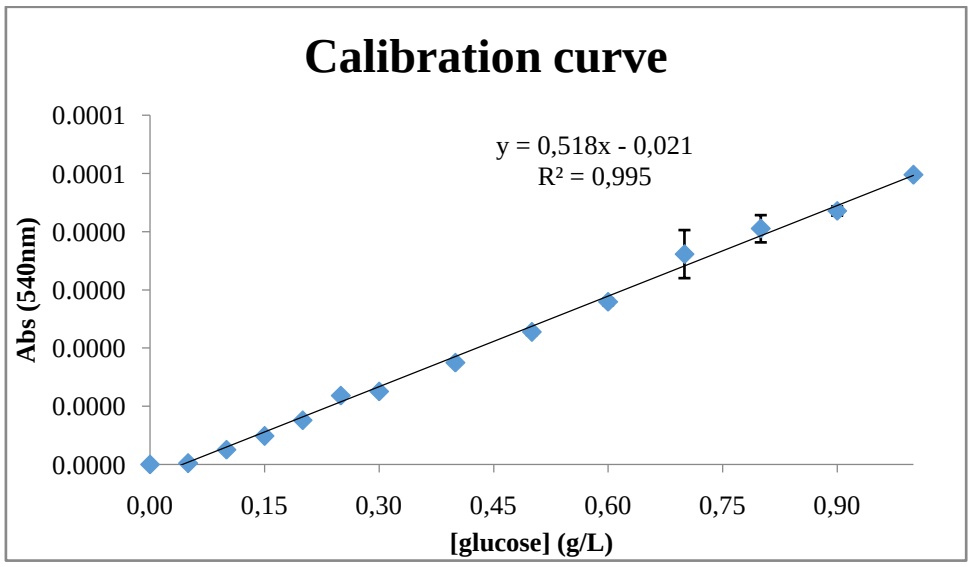

Figure A1: Standard curve used for determining reducing sugars content in chocolate milk.

APPENDIX B: Calibration curve of galactose to determine concentration of lactose in chocolate milk

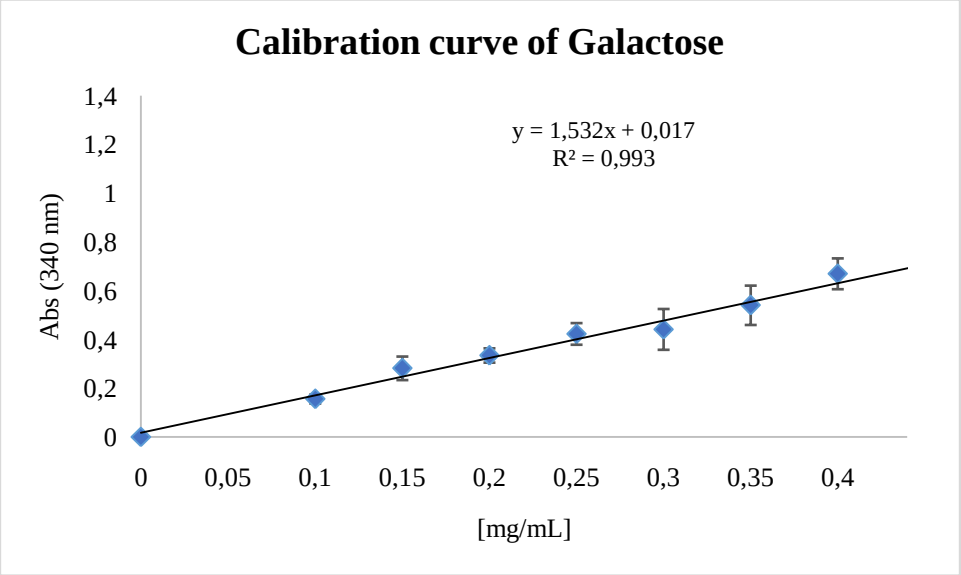

Figure B1: Standard curve used to determine lactose in chocolate milk.

APPENDIX C: TAMand ENT counts in chocolate milk after increasing the microbial loads

Table 12: TAM counts in chocolate milk after increasing the microbial loads.

	Total aerobic meshophiles						$Log_{10}(N)$	$Log_{10}(N)\pm ST$
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
1	-	-	-	Unc*	Unc*	424	8.61	8.62±0.01
	-	-	-	Unc*	Unc*	396		
2	-	-	-	Unc*	Unc*	440	8.63	
	-	-	-	Unc*	Unc*	440		

*Inc- uncountable

Table 13:ENT counts in chocolate milk after increasing the microbial loads.

	Enterobacteriaceae						$Log_{10}(N)$	$Log_{10}(N)\pm ST$
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
1	-	-	-	Unc*	Unc*	216	8.31	8.25±0.08
	-	-	-	Unc*	Unc*	190		
2	-	-	-	Unc*	Unc*	144	8.20	
	-	-	-	Unc*	Unc*	171		

*Inc- uncountable