Impact of different hyperbaric storage conditions on microbial, physicochemical and enzymatic parameters of watermelon juice

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Hyperbaric storage (HS) of raw watermelon juice, up to 10 days at 50, 75, and 100 MPa at variable/uncontrolled room temperature (18-23 °C, RT) was studied and compared with storage at atmospheric pressure (AP) under refrigeration (4 °C, RF) and RT, being evaluated microbiological (endogenous and inoculated), physicochemical parameters, and enzymatic activities.

Ten days of storage at 50 MPa resulted in a microbial growth evolution similar to RF, while at 75/100 MPa were observed microbial load reductions on endogenous and inoculated microorganisms (Escherichia coli and Listeria innocua, whose counts were reduced to below the detection limit of 1.00 log CFU/mL), resulting in a shelf-life extension compared to RF.

The physicochemical parameters remained stable at 75 MPa when compared to the initial raw juice, except for browning degree that increased 1.72-fold, whilst at 100 MPa were observed higher colour variations, attributed to a lycopene content decrease (25%), as well as reductions on peroxidase residual activity (16.8%) after 10 days, while both polyphenol oxidase and pectin methylesterase residual activities were similar to RF.

These outcomes hint HS as a reliable alternative to RF as a new food preservation methodology, allowing energy savings and shelf-life extension of food products. This is the first paper studying the effect of HS on inoculated microorganisms and on a broad number of physicochemical parameters and on endogenous enzymatic activities, for a preservation length surpassing the shelf-life by RF.

1. Introduction

Refrigeration (RF) is responsible for 35-50% of the total energy consumption in super and hypermarkets, contributing approximately 1% on the total CO_2 emissions worldwide (James & James, 2010), being also the third major source of CO_2 emissions in food industry (with 490 megatons of CO_2 released to the atmosphere in 2008) (Gilbert, 2012). Thus, food preservation methodologies capable of reducing the carbon footprint, while allowing energy savings and without compromising food safety and quality, are of great interest.

Hyperbaric storage (HS) is raising increasing interest as a new food preservation procedure, capable to preserve food products under pressure (between 25 and 220 MPa) at room temperature (RT) (Fernandes et al., 2015; Ko & Hsu, 2006; Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, & Otero, 2012). Some studies pointed out HS as an alternative to RF when performed at RT, since it does not require temperature control (Fidalgo et al., 2014; Moreira, Duarte, et al., 2015; Pinto et al., 2016; Santos et al., 2015), being only needed energy for the compression and decompression phases, differently than RF. In fact, Bermejo-Prada, Colmant, Otero, and Guignon (2017) estimated that the energetic costs to store 800 kg of strawberry juice under pressure (25 MPa) over 15 days was 0.002$, against 0.034$ for RF. HS has another major advantage over RF, since it can inactivate the endogen- ous microflora, particularly at 100 MPa or higher, additionally to the microbial growth slowdown as occurs for RF (Fidalgo et al., 2014; Pinto et al., 2016; Santos et al., 2015).

To assess HS feasibility, viz. its impact on the microbial and physicochemical parameters at and above RT, several studies were performed in the last three years with different food products, namely on strawberry juice (Bermejo-Prada, Segovia-Bravo, Guignon, & Otero, 2015; Bermejo-Prada, Vega, Pérez-Mateos, & Otero, 2015; Segovia- Bravo, Guignon, Bermejo-Prada, Sanz and Otero, 2012), watermelon juice (Fidalgo et al., 2014; Pinto et al., 2016; Santos et al., 2015), melon juice (Queirós et al., 2014), sliced cooked ham (Fernandes et al., 2015), requeijão (whey cheese) (Duarte et al., 2014), carrot soup (Moreira, Fernandes, et al., 2015), two ready-to-eat meals (Moreira, Duarte, et al., 2015), and raw bovine meat (Freitas et al., 2016). Cape hake loins were also preserved under pressure (50 MPa) but combined with RF (5 °C) over 7 days, allowing a shelf-life extension when compared to conven- tional AP/RF (Otero, Pérez-Mateos, & López-Caballero, 2017). The outcomes of these studies proved the feasibility of HS as a preservation methodology with potential to substitute RF.

More recently, two studies with highly perishable foods reported that HS/RT could extend the shelf-life compared to RF: for up to at least 7 and 10 days for raw watermelon juice at 100 MPa (Pinto et al., 2016) and raw bovine meat at 50 MPa (Freitas et al., 2016), respectively.

Thus, the aim of this work was to study HS feasibility for shelf-life extension of raw watermelon juice, at naturally variable/uncontrolled RT (18-23 °C) compared to RF. Raw watermelon juice was selected since it is a highly perishable food product (high water activity (a_w > 0.95) and pH-value close to neutral (5.20-5.60) (Bridges- & Mattice, 1939)). Three pressure levels were tested (50, 75, and 100 MPa) and microbial analyses: total aerobic mesophiles (TAM), total aerobic psychrophiles (TAP), Enterobacteriaceae (ENT), and yeasts and moulds (YM) and physicochemical parameters: pH, titratable acidity, total soluble solids (°brix), browning degree, cloudiness, colour, total phenolic compounds and lycopene content were studied. Due to the scarcity of data in the literature regarding the effect of HS on endogenous enzymes (the only data available are for strawberry juice (Bermejo-Prada, Segovia-Bravo, et al., 2015; Bermejo-Prada & Otero, 2016)), enzymatic activities of polyphenol oxidase (PPO), peroxidase (POD), and pectin methylesterase (PME) were also determined along storage. Additionally, HS effect on two specific microorganisms, L. innocua (ATCC 33090) and E. coli (ATCC 25992), as surrogates for pathogens (respectively for L. monocytogenes and pathogenic E. coli), was also studied for the first time (as the authors are aware), by inoculating these microorganisms on juice samples, to give a first insight of the possible HS effects on pathogenic microorganisms. A comparison between the juice stored at AP/RT (0.1 MPa/18-23 °C) and AP/RF (0.1 MPa/4 °C) conditions was established for all the performed analyses. As far as the authors are aware, this is the first work in literature, where such a broad range of microbial behaviour, physico- chemical, and enzymatic activities are studied, for a storage period that surpass the shelf-life achievable by RF.

2. Materials and methods

2.1. Reagents and solutions

Folin-Ciocalteu reagent, gallic acid, sodium carbonate, butylated hydroxytoluene (BHT), 2,2'-azinobis(3-ethylbenzthiazolm-6-sulfonate) (ABTS), and catechol were obtained from Sigma-Aldrich (Seelze, Germany). Sodium hydroxide was purchased from Fluka (St. Louis, Missouri), acetic acid was purchased from ChemLab (Zedelgem, Belgium), sodium acetate from Panreac (Barcelona, Spain), citric acid from Acros Organic (New Jersey, USA), sodium citrate from VWR- International (Carnaxide, Portugal), and citrus pectin was purchased from Riedel-de haen (Hanover, Germany). Plate count agar (PCA), violet red bile dextrose agar (VRBDA), rose-bengal chloramphenicol agar (RBCA), coliform count agar (CCA), Listeria identification agar base (PALCAM) (with a selective supplement PALCAM FD061), ringer tablets and ethanol (United States Pharmacopeia-grade, USP-grade) were acquired from Merck (Darmstadt, Germany).

2.2. Watermelon juice samples

Mature, seeded red watermelons (Citrullus lanatus) were purchased at a local supermarket and kept at 4 °C until washing, peeling, crushing, and filtration with a sterilized cotton filter. In sterile conditions, the filtered juice was separated in different aliquots (10 mL), and was aseptically placed in low permeability polyamide-polyethylene bags (PA/PE-90, Albipack - Packaging Solutions, Águeda, Portugal), pre- viously sterilized by UV light irradiation

for 15 min, using a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) to avoid contaminations. The bags were manually heat sealed with care to avoid as much as possible to leave air inside. All samples were immediately stored at -20 °C, being thawed and kept at 4 °C (for as much as 5 h) before each experiment.

2.3. Storage conditions

Storage experiments were performed in a 2 L high pressure equip- ment (FPG7100, Stanstead Fluid Power, Stansted, UK), with a pressure vessel of100 mm inner diameter and 250 mm height, using a mixture of propylene glycol and water (40:60) as pressurization fluid. HS was performed at three pressure levels (50, 75 and 100 MPa) up to 10 days, at variable RT (18-23 °C). At the same time, two control samples were kept at AP, one at RT (18-23 °C) and the other under RF (4 °C), immersed in the same fluid (used in HS) and kept in the dark to avoid differences between samples.

2.4. Microbial inoculations

To evaluate the effect of HS/RT on the development of pathogenic surrogated microorganisms, juice samples were previously inoculated with two non-pathogenic surrogate strains: L. innocua ATCC 33090 and E. coli ATCC 25922. The inoculations were carried out by suspending plated pure cultures of these microorganisms in Ringer's solution, being adjusted each suspension to 0.5 McFarland (Jenway 6405 UV/Vis spectrophotometer, Stone, Staffordshire, UK). Decimal dilutions were prepared to obtain a final microbial load between 3.00 and 4.00 log CFU/mL for E. coli and L. innocua, respectively.

2.5. Microbial analyses

Microbial analyses were carried out by adding 1.0 mL of water- melon juice to 9.0 mL of Ringer's buffer solution. Then, decimal dilutions were made and plated in specific culture media, according to the microorganisms analysed: (1) TAM and TAP were enumerated in PCA culture media and the plates were stored at 30 ± 1 °C and 20 ± 1 °C during 72 h and 5 days, respectively (ISO 4833: 2003); (2) ENT were plated and counted in VRBDA after incubation at 37 ± 1 °C for 24 h (ISO 8523: 1991); (3) YM were counted in RBCA culture medium, after incubation at 25 ± 1 °C for 5 days (ISO 7984: 1987); (4) Listeria innocua ATCC 33090 was plated in PALCAM Listeria agar base with the selective supplement PALCAM (FD061), and incubated at 37 ± 1 °C for 48 h (ISO 11290: 1997); (5) Escherichia coli ATCC 25922 was plated and counted in CCA after incubation at 37 ± 1 °C for 24 h (ISO 9308-1).

The results were expressed as decimal logarithm of colony forming units per millilitre of watermelon juice (log CFU/mL). The maximum load considered in this study was 6.00 log CFU/mL, while the detection and quantification limits considered were 1.00 log CFU/mL and 2.00 log CFU/mL, respectively.

2.6. Physicochemical analyses

2.6.1. pH and titratable acidity

The pH-value was obtained at 25 °C by using a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S.A., Spain). The titratable acidity of each sample was determined with an automatic titrator (Titromatic 1S, Crison Instruments, S.A., Spain) by titrating 10 mL of diluted watermelon juice (3 mL of watermelon juice and 7 mL of distilled water) with a standardized solution of 0.02 M sodium hydroxide until reaching a final pH of 8.1. The results were expressed as milligrams of citric acid per liter of watermelon juice (mg citric acid/ L) (Liu, Hu, Zhao, & Song, 2012).

2.6.2. Total soluble solids content

Total soluble solids content was determined by measuring the °brix (Handheld Refractometer Atago ATC-IE, Tokyo, Japan) at 20 °C (Wang et al., 2005) based on the official AOAC Official Method 932.12 (AOAC International, 1932) and the results were expressed as °brix.

2.6.3. Browning degree

Juice samples were centrifuged at 9000 g at 4 °C for 20 min and the absorbance of the supernatant was measured at 420 nm in a UV-Vis spectrophotometer (Microplate Spectrophotometer Multiskan Go, Thermo Scientific, USA) (Zhang et al., 2011). The results of the browning degree were expressed as optical density at 420 nm (OD_{420nm}).

2.6.4. Cloudiness

Cloudiness was quantified by direct measurement of juice absor- bance at 700 nm, using a UV-Vis spectrophotometer (Microplate Spectrophotometer Multiskan GO, Thermo Scientific) (Okoth, Kaahwa, & Imungi, 2000). The results were expressed as optical density at 700 nm ($OD_{700 nm}$).

2.6.5. Total phenolic compounds content

Determination of total phenolics content was carried out using the Folin-Ciocalteu spectrophotometric assay (Singleton & Rossi, 1965), by mixing 0.125 mL of watermelon juice with 0.500 mL of distilled water in a test tube, followed by the addition of 0.125 mL of Folin- Ciocalteu solution. After vigorously stirring, the mixture was kept in the dark for 6 min. Then, 1.25 mL of sodium carbonate solution (7%, w/v) was added to each tube, followed by vigorously stirring. After 60 min, the absorbance at 760 nm was measured using a UV-Vis spectro- photometer (Microplate Spectrophotometer Multiskan Go, Thermo Scientific, USA). Gallic acid (0-200 pg/mL) was used as standard, being the total phenolic content expressed as micrograms of gallic acid per millilitre of juice (pg gallic acid/mL).

2.6.6. Total lycopene content

The total lycopene content was measured as proposed by Davis, Fish, and Perkins-Veazie (2003), where 0.6 g of watermelon juice were weighted and added to 5 mL of a solution of BHT in acetone (0.05%, w/v), 5 mL of USP-grade ethanol and 10 mL of hexane. After shaking, 3 mL of distilled water were added and the mixture was stirred and left to rest to allow phase separation. After phase separation, the absorbance of the upper phase (hexane layer) was measured at 503 nm (Jenway 6405 UV/Vis spectrophotometer, Stone, Staffordshire, UK). Eq. (1) was used to quantify the lycopene content in each sample:

$$Lycopene = \frac{A_{503} \times MW \times DF \times 1000}{} \qquad (1)$$

where A_{503} is the absorbance (at 503 nm) of the sample, MW is the molecular weight of lycopene (536.9 g/mol), DF is the dilution factor, L is the path length (cm) and e is the molar extinction coefficient for lycopene (172,000 L/mol.cm). Lycopene content was expressed in milligram of lycopene per liter of watermelon juice (mg/L).

2.6.7. Colour

Colour measurements were performed using a spectrophotometer Konica Minolta CM 2300d (Osaka, Japan). The colour parameters were recorded according to the Commission internationale de l'éclairage (CIE) system and the data was processed with the original SpectraMagic™ NX software (Konica Minolta, Osaka, Japan) in accordance to the International Commission on Illumination regulations: red/green col- our (a·), yellow/blue colour (b·), and luminosity (L·) parameters. The values of each parameter were obtained by doing six measurements for each sample, in duplicate, in order to

calculate the total colour change variation (4E∗), as shown in Eq. (2):

$$\Delta E^* = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \tag{2}$$

where 4E∗ is the total colour change variation between a sample and the control (initial values identified with the subscript "0").

2.7. Enzymatic activities

2.7.1. PPO, POD, and PME

PPO activity was assayed according to the method described by Duangmal and Owusu Apenten (1999) with minor modifications. Aliquots of watermelon juice (0.6 mL) were mixed with 2.4 mL of pre- incubated substrate solution at 30 °C, containing 100 mM catechol in 100 mM citrate buffer (pH 5.4), and the absorbance was measured at 420 nm (Lambda 35 UV/Vis spectrometer, PerkinElmer Instruments Inc., MA, USA).

POD activity assays were performed as reported by Childs and Bardsley (1975). Aliquots of watermelon juice (10 pL) were mixed with 0.36 mM ABTS, and 0.1 M sodium acetate buffer (pH 6.0) to a final volume of 1.9 mL. After a pre-incubation at 20 °C, 100 pL of 0.5 M hydrogen peroxide was added to initiate the reaction, being monitored the formation of the ABTS radical cation at 414 nm (Lambda 35 UV/Vis spectrometer, PerkinElmer Instruments Inc., MA, USA) during 5 min.

PME activity was measured based on the method described by Hagerman and Austin (1986). Before each analysis, all solutions were adjusted to a pH of 7.5 using 2.0 M sodium hydroxide, and the enzymatic extracts were adjusted to the same pH with 0.5 M sodium hydroxide. Juice aliquots were mixed with 1% polyvinylpolypyrroli- done and 1% Triton X-100 at 4 °C, for 1 h, before centrifugation (10,000 g, 4 °C, 20 min). To the supernatant were added 4 mL of citrus pectin solution (0.5%, w/v), 300 pL of bromothymol blue (0.01%, w/v), and distilled water up to a final volume of 6.0 mL. The absorbance was measured at 620 nm (Lambda 35 UV/Vis spectrometer, PerkinElmer Instruments Inc., MA, USA) during 1 min.

The enzymatic activities were obtained from the linear portion of the absorbance-time curves, and expressed as AAbs/min/mL at 420, 414 and 620 nm, respectively, for PPO, POD and PME.

2.8. Statistical analyses

All the studied microorganisms, physicochemical parameters, and enzymatic activities were evaluated in triplicate, each one from duplicated samples, for all storage conditions, except for samples stored at AP/RT for 10 days due to their severe spoilage stage. The results were statistically analysed using one-way Analysis of Variance (ANOVA), followed by Turkey's HSD test at 5% of significance and were expressed as mean ± standard deviation.

3. Results and discussion

3.1. Microbial analyses

The initial microbial loads of raw watermelon juice were 4.10 ± 0.14, 4.22 ± 0.09, 3.36 ± 0.16 and 3.55 ± 0.07 log CFU/ mL for total aerobic mesophiles (TAM), total aerobic psychrophiles (TAP), Enterobacteriaceae (ENT) and yeasts and moulds (YM), and its evolution throughout storage over 10 days is displayed on Fig. 1 (A-D).

Generally, after 4 days of storage at AP/RT, microbial loads above 6.00 log CFU/mL (maximum load considered) were observed for all the analysed microorganisms, a clear sign of spoilage, while HS/RT resulted in a general reduction (p < 0.05) of all microbial loads at 75 and 100 MPa throughout storage time. HS/RT at 50 MPa yielded loads similar (p > 0.05) to AP/RF for both TAM, TAP and ENT (4.57 ± 0.22, 4.67 ± 0.17 and 4.44 ± 0.01 log CFU/mL, respectively) at the 4th day of storage, as seen in Fig. 1 (A-B), respectively, but values above 6.00 log CFU/mL were observed after 10 days for both 50 MPa/RT and AP/RF conditions. Regarding YM counts, after 4 days at HS/RT, a decrease

Total aerobic mesophiles (A)

Total aerobic psychrophiles (B)

Enterobacteriaceae (C)
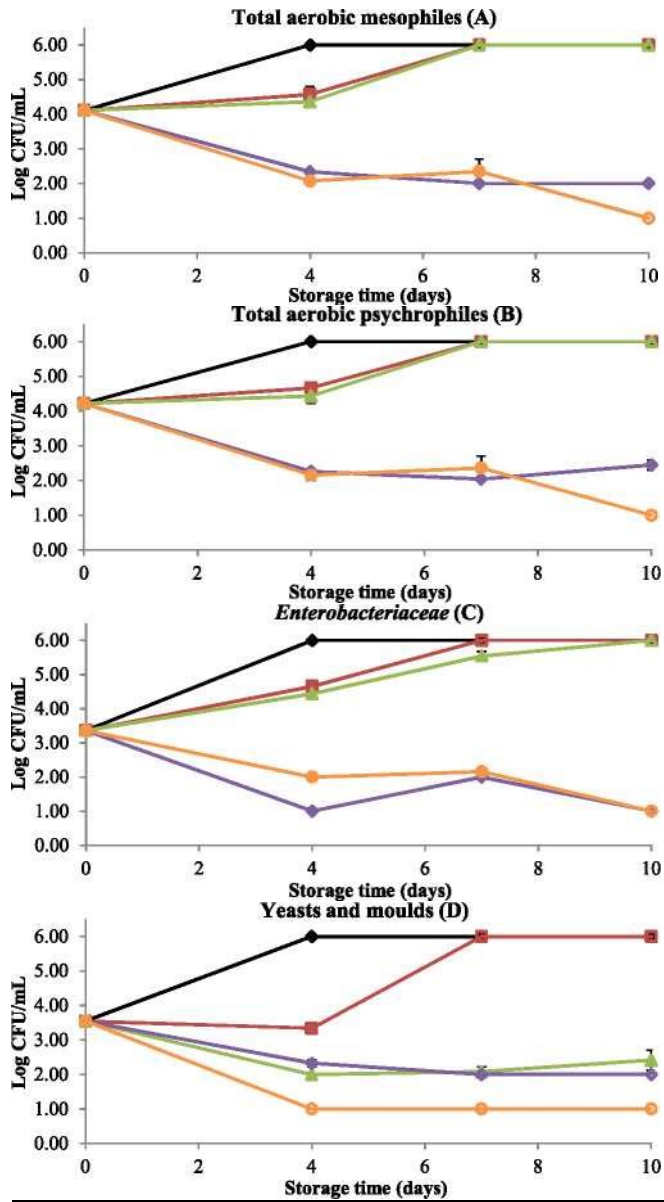
Yeasts and moulds (D)

Storage time (days)	Storage conditions	Total aerobic mesophiles	Total aerobic psychrophiles	*Enterobacteriaceae*	Yeasts and moulds
	AP/RT	#	#	#	#
	AP/RF	aB	aB	aB	aB E
4	50 MPa	aB e	aB E	aA	+
	75 MPa	aA	abA	*	aA
	100 MPa	aA	aA	+	*
	AP/RT	#	#	#	#
	AP/RF	#	#	#	#
7	50 MPa	#	#	bB	aA
	75 MPa	+	aA	+	+
	100 MPa	aA	aA	aA	♦
	AP/RT	#	#	#	#
	AP/RF	#	#	#	#
10	50 MPa	#	#	#	bA
	75 MPa	aA	bA	*	+
	100 MPa	*	*	*	*

(caption on next page)

Fig. 1. Total aerobic mesophiles (A), total aerobic psychrophiles (B), Enterobacteriaceae (C), and yeasts and moulds (D) counts (expressed as log CFU/mL) in watermelon juice after 4, 7 and 10 days of storage at different conditions: AP/RT (»), AP/RF (■), and HS/RT at 50 MPa (△—), 75 MPa (△—) and 100 MPa (•). In the table, different upper/lower case letters (A-B)/(a-b) indicate significant differences (p < 0.05) between different storage conditions/storage times, while # and * represent the maximum load considered on this study (> 6.00 log CFU/mL) and the detection limit (< 1.00 log CFU/mL) respectively, being shown as empty markers in the graphs and + represents the quantification limit (2.00 log CFU/ mL). The Greek letter £ indicates values that are not statistically different (p > 0.05) from the initial value.

(p < 0.05) of about 2.00 log CFU/mL was verified, being observed a slight increase (p < 0.05) to 2.42 ± 0.29 log CFU/mL by the 10th day of storage. Nonetheless, this increase of YM on HS/RT samples was lower than the one verified for AP/RF samples (Fig. 1D).

A significant decrease (p < 0.05) of microbial loads was observed for HS/RT at 75 MPa, since after 4 days of storage both TAM and TAP counts reached values of 2.34 ± 0.02 and 2.26 ± 0.03 log CFU/mL, respec- tively, which were maintained (p > 0.05) until the 10th day. The ENT counts were reduced to below the detection limit (< 1.00 log CFU/mL) after 10 days of HS/RT and YM counts were below the quantification limit (2.00 log CFU/mL), as seen in the Fig. 1 (C-D). These results seem very promising, since a low-pressure level as 75 MPa caused a microbial inactivation for all studied microorganisms, thus allowing surpassing the juice shelf-life obtained under AP/RF for at least more three days.

HS/RT at 100 MPa yielded a significant reduction (p < 0.05) of both TAM and TAP counts to below the quantification limit after 10 days of storage. Concerning ENT and YM counts, it was observed a gradual decrease (p < 0.05) throughout storage, reaching values below the detection limit at the 10th day.

3.2. Inoculated microorganisms

Regarding the inoculated samples, the initial counts were 4.39 ± 0.27 and 3.10 ± 0.08 log CFU/mL for L. innocua and E. coli, respectively. Three days at AP/RT resulted in microbial loads incre- ments to values above 6.00 log CFU/mL for both microorganisms, being these values maintained (p > 0.05) until the end of storage experi- ments, as seen in Fig. 2 (A-B). After 10 days at AP/RF storage
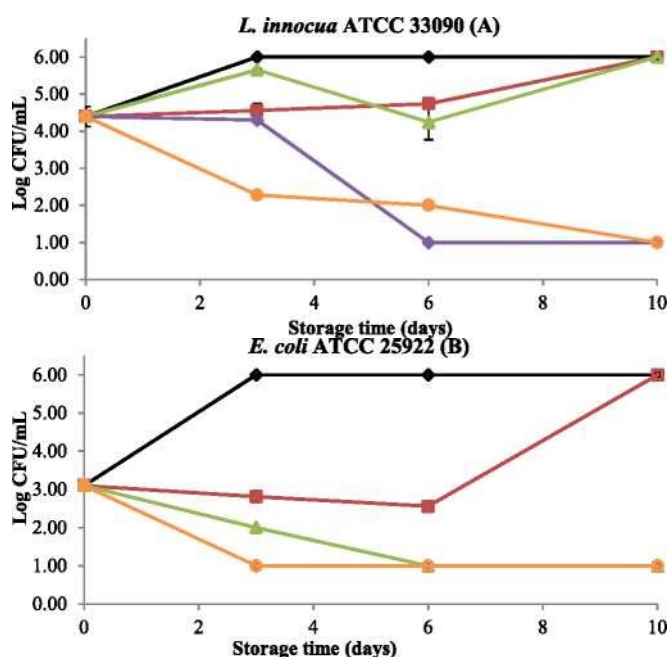
Storage time (days)	Storage condition	Escheríchia coli	Listeria innocua
	AP/RT	#	#
	AP/RF	aA	aB E
	50 MPa	+	bC
	75 MPa	*	aB E
	100 MPa	*	aA
	AP/RT	#	#
	AP/RF	bA	aB E
6	50 MPa	«	aAe
	75 MPa	*	*
	100 MPa	*	+
	AP/RT	#	#
	AP/RF	#	#
10	50 MPa	*	#
	75 MPa	*	*
	100 MPa	*	*

Fig. 2. Inoculated L. innocua ATCC 33090 (A) and E. coli ATCC 25922 (B) loads (expressed as log CFU/mL) in watermelon juice after 4, 7 and 10 days of storage at different conditions: AP/RT (»), AP/RF, (■), and HS/RT at 50 MPa (△—), 75MPa (△—) and 100MPa (△). In the table, different upper/lower case letters (A-C)/(a-b) indicate significant differences (p < 0.05) between different storage conditions/ storage times, while # and * represent the maximum load considered on this study (> 6.00 log CFU/mL) and the detection limit (< 1.00 log CFU/mL) respectively, being shown as empty markers in the graphs and + represents the quantification limit (2.00 log CFU/mL). The Greek letter £ indicates values that are not statistically different (p > 0.05) from the initial value.

conditions, both E. coli and L. innocua loads presented values above 6.00 log CFU/mL.

HS/RT at 50 MPa reduced ($p < 0.05$) E. coli loads in about 1.00 log CFU/mL after 3 days, and to below the detection limit at the 6th day of storage and onwards ($p > 0.05$). This indicates that HS/RT at 50 MPa was more effective to inhibit E. coli growth than AP/RF, contrarily to L. innocua, whose counts increased ($p < 0.05$) to above 6.00 log CFU/mL after 10 days of storage, similarly to the juice stored at AP/RF. This behaviour might be related with the fact that gram-positive bacteria (as L. innocua) are more pressure-resistant than gram-negative bacteria (as E. coli) due to the presence of a peptidoglycan layer on gram-positive bacteria (Wuytack, Diels, & Michiels, 2002).

As regards to HS/RT at 75 MPa, E. coli was reduced ($p < 0.05$) to below the detection limit in the first 3 days of storage, being this value maintained ($p > 0.05$) until the 10th day. Concerning L. innocua, storage at 75 MPa led to an inhibitory effect, keeping the microbial load similar to the initial one ($p > 0.05$), being verified that after 3 days of storage, HS/RT was equally efficient as AP/RF ($p > 0.05$) to control L. innocua. However, at the 6th day, the microbial load of AP/RF samples grew above 6.00 log CFU/mL, contrarily to juice stored at 75 MPa that showed a microbial load below the detection limit, indicating that pressure was effective for L. innocua microbial inactivation during storage, as occurred for HS/RT at 100 MPa, for both microbial strains (Fig. 2A-B).

These results indicate that HS/RT at 75 and 100 MPa allows a better performance than AP/RF on microbial growth inhibition and inactiva- tion for both L. innocua and E. coli on watermelon juice, considering that in both cases the microbial loads reached values below the detection limit.

4. Physicochemical parameters
4.1. pH and titratable acidity

The initial pH of watermelon juice was 6.23 ± 0.02 and, as shown in Table 1, no considerable changes in pH values were detected for samples kept at HS/RT and AP/RF conditions. Nevertheless, for samples stored at AP/RT, a significant pH decrease ($p > 0.05$) was observed at the 7th day of storage (4.78 ± 0.01). After the 10 days of storage, AP/ RT samples were not studied due to its heavy spoilage state, presenting an unpleasant odour and gas formation. Regarding samples under HS/ RT at 50 MPa, they showed a slight pH decrease to 5.82 ± 0.03, with a value similar to RF samples ($p < 0.05$) at the 10th day of storage (5.78 ± 0.05), while HS/RT at 75 and 100 MPa were able to maintain ($p < 0.05$) the sample's pH stable after 10 days of storage.

The initial juice titratable acidity was 411.7 ± 18.5 mg citric acid/L and this value increased sharply ($p < 0.05$), about 3.8-fold after 4 days at AP/RT remaining thereafter constant ($p > 0.05$) after 7 days), probably due to the microbial development observed for these samples. The AP/RF samples presented a titratable acidity increase ($p < 0.05$) of about 1.9- fold after 10 days, while for HS/RT the samples titratable acidity showed a smaller increase (maximum of 0.37-fold for 50 MPa at the 10th day, $p < 0.05$) compared to the samples kept at AP/RF, since after 10 days at 50, 75 and 100 MPa, the titratable acidity reached 566.3 ± 2.8, 529.2 ± 25.8 and 547.3 ± 7.3 mg citric acid/L, respectively, showing no significant differences ($p > 0.05$) between these conditions.

4.2. Total soluble solids (°brix)

Total soluble solids decreased ($p < 0.05$, Table 1) for samples stored at AP/RT conditions, from the initial value (8.1 ± 0.1°brix) reaching a minimum of 6.9 ± 0.1°brix. Concerning juice stored at AP/ RF, no significant changes ($p > 0.05$) were detected on °brix along storage, remaining similar to the initial value. Regarding HS/RT at 50 MPa, total soluble solids faced some fluctuations along storage, reaching a value of 7.6 ± 0.1°brix after 10 days, which is lower ($p < 0.05$) than the initial value. Concerning samples stored at 75 MPa, a value of 8.3 ± 0.1°brix was reached after 10 days of storage, a value slightly higher ($p < 0.05$) than that obtained for the initial juice, while at 100 MPa this parameter remained stable ($p > 0.05$) during the 10 days of storage (8.0 ± 0.1°brix).

4.3. Browning degree

A decrease of about 2.4-fold ($p < 0.05$) occurred for the browning degree (initial value of 0.257 ± 0.008 OD$_{420\,nm}$) of AP/RT stored samples, reaching a minimum of 0.105 ± 0.015 OD$_{420\,nm}$ after 7 days of storage, while AP/RF samples showed a browning degree similar ($p > 0.05$) to the initial juice until the OD$_{420\,nm}$) (Table 1). 10th day (0.247 ± 0.014

Table 1

Physicochemical properties of watermelon juice for each storage condition: at atmospheric pressure at naturally variable/uncontrolled room temperature (AP/RT) and refrigeration (AP/ RF), and under pressure at 50, 75, and 100 MPa at RT. Different upper/lower case letters (A-D)/(a-c) indicate significant differences ($p < 0.05$) between different storage conditions/ storage times. The Greek letter e indicates values that are not statistically different ($p > 0.05$) from the initial value.

Storage time (days)	Storage conditions	pH	Titratable acidity (mg citric acid/L)	Total soluble solids (°brix)	Browning degree (OD$_{420nm}$)	Cloudiness (OD$_{600nm}$)	Total phenolics (Rg gallic acid/mL)	Lycopene content (mg/L)
Initial	6.23 ± 0.02		411.7 ± 18.5	8.1 ± 0.1	0.257 ± 0.008	0.534 ± 0.004	265.8 ± 0.4	1.169 ± 0.111
4	AP/RT	5.15 ± 0.03 bA	1541.7 ± 0.4 aC	7.1 ± 0.1 bA	0.147 ± 0.053 bA	1.037 ± 0.010 aC	197.9 ± 2.1 aC	1.548 ± 0.121 aC
	AP/RF	6.12 ± 0.02 bC	441.5 ± 1.7 aA e	8.0 ± 0.1 aC e	0.242 ± 0.007 aB e	0.631 ± 0.004 aB	242.2 ± 1.0 bD	1.605 ± 0.082 aC
	50 MPa/RT	6.01 ± 0.02 bB	447.4 ± 1.7 aA e	7.4 ± 0.1 bB	0.193 ± 0.013 aAB a	0.500 ± 0.006 aA	176.6 ± 1.2 bA	1.203 ± 0.044 bB e
	75 MPa/RT	6.10 ± 0.02 bC	548.4 ± 28.0 cB	7.5 ± 0.1 aB	0.349 ± 0.041 aC	0.621 ± 0.004 bB	189.8 ± 4.2 aB	1.640 ± 0.050 aC
	100 MPa/RT	6.05 ± 0.02 bBC	563.8 ± 38.6 aB	8.0 ± 0.1 aC e	0.231 ± 0.016 aB e	0.513 ± 0.006 bA e	179.7 ± 0.3 bA	0.711 ± 0.044 aA
7	AP/RT	4.78 ± 0.01 aA	1543.2 ± 0.2 aD	6.9 ± 0.1 aA	0.105 ± 0.015 aA	1.209 ± 0.017 bD	200.0 ± 1.0 aBC	1.447 ± 0.015 aB
	AP/RF	5.79 ± 0.05 aB	621.0 ± 10.5 bC	8.0 ± 0.1 aC e	0.219 ± 0.006 aB e	0.667 ± 0.007 bC	203.8 ± 0.6 aC	1.539 ± 0.183 aB
	50 MPa/RT	5.94 ± 0.01 bC	493.5 ± 12.8 aA	7.2 ± 0.1 aB	0.289 ± 0.043 bC	0.516 ± 0.009 bA	170.9 ± 0.6 aA e	0.819 ± 0.094 aA
	75 MPa/RT	5.98 ± 0.02 aCD	469.4 ± 23.3 aA	8.0 ± 0.1 bC e	0.486 ± 0.068 bD	0.581 ± 0.002 aB	195.5 ± 4.1 bB	1.546 ± 0.179 aB
	100 MPa/RT	6.04 ± 0.01 bD	538.2 ± 8.8 aB	8.0 ± 0.1 aC e	0.215 ± 0.032 aB e	0.505 ± 0.003 bA	175.9 ± 0.7 bA	0.935 ± 0.035 aA e
10	AP/RT	-	-	-	-	-	-	-
	AP/RF	5.78 ± 0.05 bA	776.0 ± 8.3 cB	8.0 ± 0.1 aB e	0.247 ± 0.014 aB	0.678 ± 0.023 bD	277.7 ± 0.4 cD	1.012 ± 0.049 bA e
	50 MPa/RT	5.82 ± 0.03 aB	566.3 ± 2.8 bA	7.6 ± 0.1 cA	0.349 ± 0.041 cC e	0.515 ± 0.007 bB	181.1 ± 0.9 bB	0.920 ± 0.033 aA e
	75 MPa/RT	6.05 ± 0.02 abD	529.2 ± 25.8 bA	8.3 ± 0.1 cC	0.444 ± 0.023 bD	0.577 ± 0.006 aC	192.6 ± 1.0 abC	1.446 ± 0.033 aB
	100 MPa/RT	5.91 ± 0.01 aC	547.3 ± 7.3 aA	8.0 ± 0.1 aB e	0.172 ± 0.005 aA e	0.414 ± 0.008 aA	150.9 ± 0.8 aA	0.885 ± 0.058 aA

Note: Watermelon juice stored at AP/RT conditions for 10 days presented severe spoilage, for so, the physicochemical parameters regarding those samples were not analysed (-).

HS/RT at 50 MPa caused a decrease ($p > 0.05$) of the browning degree value after 4 days of storage (0.193 ± 0.013 $OD_{420\,nm}$), being then verified an increase in the subsequent days, reaching a value of 0.349 ± 0.041 $OD_{420\,nm}$ at the 10th day, which is significantly different ($p < 0.05$) from the initial value. Juice samples stored at 75 MPa presented a gradual increase ($p < 0.05$) of the browning degree, since after 10 days it was reached a value of 0.444 ± 0.023 $OD_{420\,nm}$, while at 100 MPa it was detected a significant decrease ($p < 0.05$) of this parameter for all storage period, reaching a minimum of 0.172 ± 0.005 $OD_{420\,nm}$ by the 10th day.

4.4. Cloudiness

There was a statistically significant increase ($p < 0.05$) of about 2.3-fold for AP/RT samples, reaching values of 1.209 ± 0.017 $OD_{700\,nm}$ after 7 days of storage, compared to the initial value (0.534 ± 0.004 $OD_{700\,nm}$, Table 1). Concerning AP/RF samples, sig- nificant differences ($p < 0.05$) were observed when compared to the initial value, (1.27-fold increase), although, by the 10th day, the cloudiness value was similar ($p > 0.05$) to the initial one.

Regarding HS/RT, it was verified a global decrease at 50 and 100 MPa, since after 10 days of storage, cloudiness values of 0.515 ± 0.007 $OD_{700\,nm}$ ($p > 0.05$) and 0.414 ± 0.008 $OD_{700\,nm}$ ($p < 0.05$), respec- tively, were observed, except at 75 MPa, wherein the cloudiness value increased ($p < 0.05$), showing values between 0.577 ± 0.006 and 0.621 ± 0.004 $OD_{700\,nm}$.

4.5. Total phenolic compounds content

Globally, there was a decrease ($p < 0.05$) of total phenolics compounds content during storage, independently of storage conditions (initial value of 265.8 ± 0.4 pg gallic acid/mL, Table 1).

Storage at AP/RT caused a decrease ($p < 0.05$) of the total phenolics content (0.74-fold) after 4 days of storage reaching 197.9 ± 2.1 pg gallic acid/mL, remaining thereafter similar ($p > 0.05$) throughout storage. Similar results were observed for AP/ RF storage, where a decrease ($p < 0.05$) of this parameter was verified (0.91-fold) at the 4th day (to 242.2 ± 1.0 pg gallic acid/mL), followed by an increase ($p < 0.05$) after 10 days to 277.7 ± 0.4 pg gallic acid/ mL.

HS/RT at 75 MPa over 4 days induced a decrease ($p < 0.05$) of the total phenolics content to 189.8 ± 4.2 pg gallic acid/mL (0.71-fold), and no further variations ($p > 0.05$) were observed. Moreover, HS/RT at 50 and 100 MPa enhanced the decay ($p < 0.05$) of this parameter until the 7th day (contrarily to that reported by Fidalgo et al. (2014), who found a slight increase of the total phenolics content after 60 h of HS/RT at 100 MPa, what might be related with the time extension under pressure in the present study). By the 10th day of HS/RT at 100 MPa, a reduction ($p < 0.05$) to about half the initial value was noticed, while at 50 MPa it was detected a slight increase ($p < 0.05$) to 181.1 ± 0.9 pg gallic acid/mL.

4.6. Lycopene content

The effect of HS/RT on the lycopene content of watermelon juice was studied for the first time (as the authors are aware), being the initial value of 1.169 ± 0.111 mg/L, higher than the concentration obtained by Zhang et al. (2011) (0.0175 mg/L). The major discrepan- cies between lycopene concentrations reported on the literature are known to vary with cultivar, seasons and production sources (Perkins- Veazie, Collins, Pair, & Roberts, 2001).

As seen in Table 1, the juice stored at AP/RT, AP/RF and HS/RT at 75 MPa showed a lycopene content similar ($p > 0.05$) to the initial value (1.169 ± 0.111 mg/L) at the 4th day of storage (1.548 ± 0.121, 1.605 ± 0.082 and 1.640 ± 0.050 mg/L, respectively). Ten days at AP/RF caused a lycopene content decrease ($p > 0.05$) to 1.012 ± 0.049 mg/L, while HS/RT at 75 MPa caused an increment ($p < 0.05$) of about 1.2-fold to 1.446 ± 0.033 mg/L. HS/RT at 50 MPa caused no changes for 4 days of storage ($p > 0.05$), although it was observed a decrease ($p < 0.05$) throughout storage, showing no significant differences ($p > 0.05$) between the 7th and 10th days of storage (0.819 ± 0.094 and 0.920 ± 0.033 mg/L, respectively).

At 100 MPa a significant lycopene concentration decay ($p < 0.05$) was observed after 4 days of storage (0.711 ± 0.044 mg/L), followed by an increase ($p < 0.05$) to a value of 0.885 ± 0.058 mg/L at the 10th day.

4.7. Colour

Storage at AP/RT conditions resulted in a total colour variation ($\hat{A}E^*$) ($p < 0.05$) of about 2.714 ± 0.184, when compared to the initial juice, remaining unchanged ($p > 0.05$) after 7 days (Table 2). AP/RF and HS/RT at 75 MPa conditions resulted in a similar ($p > 0.05$) evolution of $\hat{A}E^*$ until the 4th day of storage, being detected a $\hat{A}E^*$increase ($p < 0.05$) for AP/RF samples after 7 days of storage, while at 75 MPa it was verified a decrease ($p < 0.05$) of this

Table 2

Colour parameters of the watermelon juice for each storage condition: at atmospheric pressure at naturally variable/uncontrolled room temperature (AP/RT) and refrigeration (AP/RF), and under pressure at 75, and 100 MPa at RT. Different upper/lower case letters (A-D)/(a-c) indicate significant differences (p < 0.05) between different storage conditions/storage times. The Greek letter e indicates values that are not statistically different (p > 0.05) from the initial value.

Storage time	Storage conditions	L*		a*		b*		ÁE*	
Initial		33.774	± 0.247	11.941	± 0.230	6.723	± 0.164	-	
4	AP/RT	34.558	± 0.383 aB	9.609 ±	1 0.237 aA	5.468	± 0.180 aA	2.714	± 0.184 aB
	AP/RF	34.446	± 0.257 aAB	12.640	± 0.168 cC	5.874	± 0.102 bB	1.611	± 0.149 aA
	75 MPa/RT	34.178	± 0.335 aA e	11.649	± 0.394 aB e	5.547	± 0.249 aA	1.450	± 0.194 bA
	100 MPa/RT	38.042	± 0.308 cC	9.620 ±	1 0.174 aA	6.930	± 0.207 cC e	5.056	± 0.216 cC
7	AP/RT	35.050	± 0.113 bB	10.393	± 0.361 bA	5.334	± 0.136 aA	2.492	± 0.239 aC
	AP/RF	35.124	± 0.238 bB	11.382	± 0.197 aB	5.385	± 0.174 aA	2.170	± 0.065 bB
	75 MPa/RT	34.049	± 0.357 aA e	11.908	± 0.295 aC e	7.090	± 0.336 cC	0.889	± 0.248 aA
	100 MPa/RT	36.384	± 0.206 bC	10.457	± 0.198 bA	5.978	± 0.179 bB	3.276	± 0.156 bD
10	AP/RT	-		-		-		-	
	AP/RF	35.248	± 0.176 bB	11.937	± 0.252 bA e	5.534	± 0.162 aB	2.170	± 0.094 bB
	75 MPa/RT	35.670	± 0.335 bC	11.655	± 0.221 aA e	6.186	± 0.185 bC	2.295	± 0.217 cB
	100 MPa/RT	33.292	± 0.260 aA	11.790	± 0.212 cA e	4.946	± 0.184 aA	1.787	± 0.135 aA

Note: Colour parameters of juice stored at 50 MPa were not studied, due to the reduced amount of sample, since the pressure vessel has a small volume and taking into consideration that under this condition samples presented a behaviour similar to those at AP/RF for the same storage period. Watermelon juice stored at AP/RT conditions for 10 days presented severe spoilage, for so, the physicochemical parameters regarding those samples were not analysed (-).

parameter. This decrease on 4E* was caused by the fact that b* parameter was less affected at 75 MPa when compared to other storage conditions. After 10 days of HS/RT at 75 MPa and AP/RF, juice samples reached similar (p > 0.05) ÁE* values (2.295 ± 0.217 and 2.170 ± 0.094, respectively) showing that HS was as effective as AP/RF in maintaining colour parameters. Regarding juice stored at 100 MPa, it was verified a ÁE* of 5.056 ± 0.216 after 4 days of storage due to a pronounced increase (p < 0.05) of L*, nonetheless, a ÁE* of 1.787 ± 0.135 was found at the 10th day of storage.
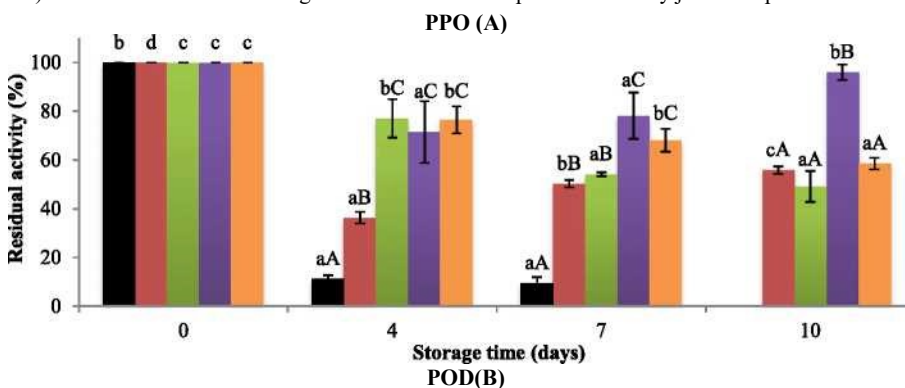
5. Enzymatic activities
5.1. PPO

Fig. 3(A) shows the evolution of PPO residual activity in juice stored at different pressure levels and RT during the 10 days of storage as well as the juice samples stored at both AP/RT and AP/RF (the initial value of PPO activity in watermelon juice was 0.015 ± 0.003 AAbs$_{420}$/min/ mL). The results revealed that PPO activity was statistically (p < 0.05) reduced after 4 and 7 days of storage at AP/RT conditions, showing a residual activity of 9.5% after 7 days. Storage under AP/RF resulted in a reduction to 36.3% (p < 0.05) of initial activity after 4 days of storage, and presented thereafter a linear residual activity increment ($r^2 = 0.9423$, (%) = 3.3 x storage time (days) + 24.7) until the 10th day to a value of 55.9%.

PPO activity of the juice preserved by HS was less affected along storage time compared to AP/RF, showing a less pronounced reduction on activity. After 4 days, PPO activity was reduced to a residual activity between 71.5 and 77.1%, without statistic differences (p > 0.05) between the different pressure levels. However, for 75 MPa was observed an increment of activity from the 4th to the 10th day of storage (p < 0.05) that showed a linear trend ($r^2 = 0.9356$, residual activity (%) = 4.1 x storage time (days) + 53.3), reaching a value of 96.1%. At 50 and 100 MPa, it was observed an activity decrease (p < 0.05) along the 10 days of storage showing residual activities of 49.2 and 58.7%, respectively. The browning degree decrease along the 10 days of storage at 100 MPa reported above correlated well ($r^2 = 0.9962$) with the decrease of PPO activity in the same samples, thus pointing to an effect of PPO on the browning degree.

The stability of PPO activity on strawberry juice was also studied under HS conditions (50 and 200 MPa, 20 °C, 15 days) by Bermejo- Prada, Segovia-Bravo, et al. (2015), being verified that PPO activity significantly increased, showing an evolution similar to the juice stored at AP (both RT and RF). This different behaviour might be due to the acidic pH of strawberry juice compared to watermelon.
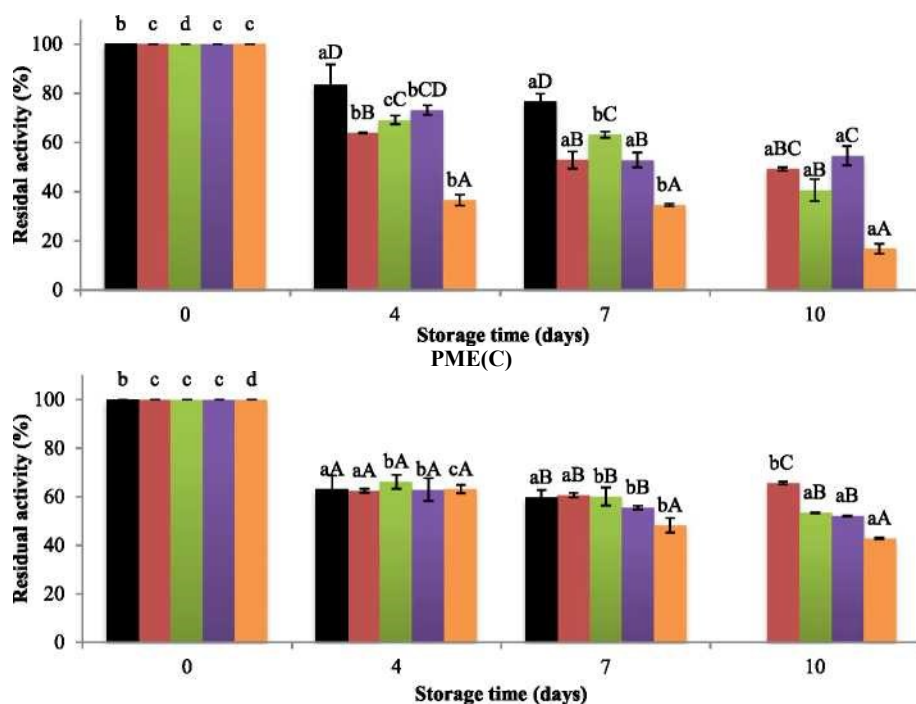

PPO (A)

POD(B)

PME(C)

Fig. 3. Enzymatic activities of polyphenol oxidase (PPO), peroxidase (POD), and pectin methylesterase (PME) of watermelon juice, expressed as residual activity (%), after 4, 7 and 10 days of storage at different conditions: AP/RT ( ), AP/RF ( ), and HS/RT at 50MPa ( ), 75 MPa ( ), and 100 MPa ( ). Different upper/lower case letters (A-C)/(a-d) indicate significant differences (p < 0.05) between different storage conditions/ storage times.

5.2. POD

POD activity was statistically (p < 0.05) affected by storage time at AP (Fig. 3 (B)). After 4 and 7 days of storage at AP/RT, it was observed a residual activity of 83.5 and 76.5%, respectively (the initial value of

POD activity was 0.58 ± 0.05 AAbs$_{414}$/min/mL). Under AP/RF, it was verified a higher activity reduction (p < 0.05) with a residual activity of 49.2% after 10 days ($r^2 = 0.9231$, residual activity (%) = — 2.5 x storage time (days) + 72.5). POD activity on samples stored at 100 MPa was significantly affected, showing a decrease (p < 0.05) to a residual activity of 16.8% after 10 days. However, HS/RT at 50 and 75 MPa showed an activity decrease (p < 0.05) similar to AP/RF, presenting residual activities of 40.6% (50 MPa) and 54.6% (75 MPa) at the end of storage. Another study on strawberry juice storage by HS reported that after 15 days at 200 MPa and 20 °C POD showed lower reduction of activity (15%) (Bermejo-Prada & Otero, 2016), compared to the values just described for watermelon, what might be due to the acidic pH of strawberry juice compared to watermelon.

5.3. PME

Regardless of storage condition (AP or HS), a reduction of PME activity (p < 0.05) was observed after 4 days of storage to a residual activity around 64% for all samples (Fig. 3 C) (the initial value of PME activity in watermelon juice was 0.043 ± 0.006 AAbs$_{620}$/min/mL). For samples stored at AP (RT and RF), this residual activity was maintained (p > 0.05) after 7 and 10 days, respectively. A similar behaviour was found for samples stored at 50 MPa, showing a PME residual activity of 60.1% after 7 days, and a slightly reduction (p < 0.05) after 10 days to a value of 53.5% ($r^2 = 0.9994$, residual activity (%) = — 2.1 x storage time (days) + 74.7). In this case, when the pressure level increased, the PME activity tended to decrease further, since after 10 days of HS/RT at 100 MPa it was verified a residual activity of 42.8%.

PME activity was already evaluated in HS/RT of strawberry juice by Bermejo-Prada, Segovia-Bravo, et al. (2015). In this study, PME activity decreased throughout the 15 days of storage in all strawberry juice samples, and pressure up to 200 MPa did not affect PME activity compared to the remaining storage procedures, whereas an approx- imate reduction of 43% of PME residual activity was observed. These results are overall similar to the results observed in the present study for watermelon juice.

6. Conclusions

HS/RT at 50 MPa showed to be as efficient as AP/RF for microbial growth inhibition in raw watermelon juice. Moreover, HS/RT at 75 and 100 MPa showed to be a more efficient storage procedure when compared to AP/RF, allowing a shelf-life extension of at least more three days over RF. Concerning the inoculated E. coli and L. innocua, storage under pressure enhanced microbial load reductions to below the detection limit for both microorganisms, except for L. innocua at 50 MPa that grew above 6.00 log CFU/mL after 10 days at HS/RT, similarly to AP/RF.

Regarding physicochemical analyses, HS/RT did not affect the overall studied parameters when compared to AP/RF along storage, except at 100 MPa over 4 days wherein higher colour variations (AE*), along with a lycopene content decrease were observed. Moreover, it was faced an increase of browning degree for samples stored at 50 and 75 MPa, while a cloudiness reduction was observed at 100 MPa, as well as a bigger impact on the residual activity of POD for this pressure.

The outcomes of this work are very promising and open the possibility to replace the traditional RF by HS/RT, since it allowed a longer shelf-life at 75 and 100 MPa. At the same time, this occurs with lower energetic costs, since energy is not necessary throughout storage, because the storage take place at room temperature. The results obtained in this work for a highly perishable food (watermelon juice), clearly show the potential of HS to achieve longer shelf-life of food products, compared to RF, with lower energetic cost and CO_2 emissions and so with a lower carbon footprint. Further studies are of interest to be carried out, such as, different food products, longer storage periods at lower/higher storage temperatures, other microbial and physico-chemical parameters as well as biochemical parameters, like enzymatic activities, in order to deepen the knowledge about this new food preservation procedure.

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