Innovative Food Science and Emerging Technologies 112 (2026) 104681

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Impact of pH on microbial behaviour in watermelon juice preservation by hyperbaric storage at room temperature

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ARTICLE INFO

Keywords:
Food preservation
Food safety
Shelf-life extension
Microbial growth control and inactivation
Escherichia coli
Saccharomyces cerevisiae
Inactivation kinetics

ABSTRACT

The systematic effect of pH (4.00, 5.00 and 6.50) on hyperbaric storage (HS, 25-100 MPa) at uncontrolled room temperature (RT, 18-23 °C) for up to 28 days was studied for the first time, using watermelon juice (WJ) as a case-study, due to its high perishability, focusing on *Escherichia coli* and *Saccharomyces cerevisiae*'s behaviour, along with RT and refrigerated controls at atmospheric pressure for comparison.

Results showed that HS at RT could control *E. coli* and *S. cerevisiae*'s growth and that, during storage, inactivation also occurred, often reaching reductions over 5 log CFU/mL. HS' effect varied according to the microorganism, the juice's pH and storage pressure, and inactivation was observed regardless the pH and pressure level tested for both microorganisms. Higher pressures increased the inactivation rates of both microorganisms, while pH's impact depended on the microorganism. *E. coli* showed greater inactivation at pH 4.0 whereas *S. cerevisiae* exhibited comparable or slightly higher reductions at pH 6.50. Microbial inactivation was well described by both first-order and Weibull kinetic models, with the latter generally providing a better fit. At pH 4.00, *S. cerevisiae* obtained lower z_p-values than *E. coli*.

This work showed HS' potential for food preservation regardless of the pH (despite being influenced by it), without needing temperature control, which may lower energy costs and greenhouse gas emissions, being potentiality environmentally friendlier.

1. Introduction

Recently, the application of hyperbaric storage (HS) for food preservation has been gaining interest, as numerous publications evaluate its potential as an alternative to conventional refrigeration (RF). HS, a novel preservation technique based on food storage under moderate pressure levels, has been tested for perishable foods, often at room temperature (RT), and was shown to enhance microbial stability and safety and increased shelf-life (Basso et al., 2022; Santos et al., 2021). Using pressure as a hurdle to microbial growth allows for low energy usage since energy is only needed for pressurization, as the vessel sealing can maintain the required pressure throughout storage without additional energy consumption. This contrasts with RF, which requires an almost constant power supply to keep the low temperatures (Basso et al., 2022).

Microbial growth control and inactivation during HS have been demonstrated in several foods, including milk and related products, meat, fish, fruit juices, and ready-to-eat foods. HS studies report considerable inactivation results, frequently reaching levels below the detection limit (DL), with reductions over 6 log CFU/mL. Microbial behaviour during HS is strongly influenced by storage conditions (pressure, temperature, and duration), type of microorganism (Gram-positive, Gram-negative, moulds, yeasts, etc.), food composition, and food parameters like pH and water activity (a_W), among other factors (Lima & Saraiva, 2023). A low pH (below 4.6) is associated with increased microbial safety, generally providing a hurdle effect against microbial growth and enhancing microorganisms' inactivation, as shown in high pressure processing (HPP) studies (Lima et al., 2023). Despite not having been systematically studied yet, a pH-microbial inactivation effect has arised as a possibility in previous HS studies, with indigenous microorganisms showing increased pressure sensitivity in strawberry juice compared with melon and watermelon juices (WJ) (Lima & Saraiva, 2023).

Previous HS/RT studies with WJ have demonstrated a greater ability

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https://doi.org/10.1016/j.ifset.2026.104681
Received 19 January 2026; Received in revised form 15 May 2026; Accepted 22 June 2026
Available online 25 June 2026

than RF to control microbial growth and even promote the inactivation of total aerobic mesophiles (TAM), total aerobic psychrophiles, Enterobacteriaceae, yeasts and moulds (YM), as well as inoculated microorganisms such as *Escherichia coli* and *Listeria innocua*. Quality attributes such as colour and cloudiness were not consistently preserved during storage, whereas pH and total soluble solids remained relatively stable (Fidalgo et al., 2014; Lemos et al., 2017; Lemos et al., 2020; Pinto et al., 2016; Pinto et al., 2017; Santos et al., 2015). Comparable effects on microbial growth control and inactivation have been reported for melon (Queirós et al., 2014) and strawberry (Bermejo-Prada et al., 2016; Segovia-Bravo et al., 2012) juices. However, the influence of intrinsic factors such as pH on microbial behaviour under HS conditions has not been systematically addressed, which is the focus of the present study.

WJ is popular among consumers due to its appealing colour, flavour, and fresh aroma (Milczarek & Sedej, 2021). It has a high water content, sugars, vitamins (A, B, C, and E), minerals (K, Mg, Ca, and Fe), specific amino acids (citrulline and arginine), and bioactive compounds like carotenoids and phenolic compounds (Liu et al., 2018; Tlili et al., 2011). The high a_W (0.97–0.99) and low acidity (pH 5.2-6.7) make freshly blended WJ a suitable medium for microbial development and enzymatic deterioration (Lee et al., 2021).

The available research on pH's impact during food preservation by HS is quite scarce and nonexistent in a systematic manner, this way, it is important to provide additional insights into microbial development control by HS. WJ was chosen as a case study since it is highly perishable (due to its high pH and a_W) with no hurdles to delay or stop microbial growth, resulting in rapid microbial development and a short shelf-life, even when refrigerated. Thus, WJ allows for shorter experiments to study microbial behaviour and with minimum matrix interacting effects, due to its low nutritional complexity. Therefore, this work aimed at understanding the impact of pH variation in the behaviour of *Escherichia coli* and *Saccharomyces cerevisiae* inoculated in WJ at different pH levels (4.00, 5.00 and 6.50) stored under HS (25-100 MPa) at uncontrolled RT (18-23 °C) up to 28 days. *E. coli* was selected since it is a surrogate of the pathogenic *E. coli* O157:H7, which can be found in watermelon (Bhattacharjee et al., 2019) and is ubiquitous in nature, while *S. cerevisiae* is a relevant yeast in the food industry for its fermentative properties and its spoilage activity can cause problems resulting in considerable economic losses and was used as a model for yeasts overall. Additionally, yeasts can generally grow at lower pH values than *E. coli*, so its study is relevant, especially at lower pH values. The pH levels were selected as follows: pH 4.00 was selected to represent a value below 4.60, which is relevant in food safety, since pH is the borderline in the regulatory distinction between low acid and acid foods and values above 4.60 allow the growth and toxin production of pathogenic microorganisms like *Clostridium botulinum* under appropriate conditions (like anaerobiosis) (Park et al., 2014); pH 5.00 was chosen for still having some hurdle effect on microbial growth; and pH 6.50 was selected for resulting in more perishability, having basically no effect on microbial development and for being more similar to WJ's typical pH.

2. Materials and methods

2.1. Watermelon juice preparation

Mature seeded red watermelons (*Citrullus lanatus*) purchased from a local supermarket (Aveiro, Portugal) were kept at 4 °C until washing, peeling, crushing, and filtration with a sterilized cotton filter in sterile conditions, using a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) to avoid contaminations. The WJ was immediately used or frozen in 100 mL aliquots and stored at 20 °C until use.

Before each experiment, the juice was thawed at 4 °C overnight and equilibrated at 25 °C for 3 h. The fresh WJ pH values varied between 5.53 and 6.47 (differences related to the watermelons' maturity state). The pH was then adjusted to 4.00, 5.00, or 6.50 using HCl or NaOH solutions using a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S.A., Barcelona, Spain) at 25 °C. The WJ was sterilized at 121 °C for 15 min, inoculated with the microorganism and 0.4 mL were divided into 0.4 mL micro test tubes with cap type Beckman® (Kartell Labware, Noviglio, Italy).

2.2. Hyperbaric storage experiments

The experiments were conducted at RT (18-23 °C) in HS equipment (Stansted Fluid Power FPG13900, Stansted, UK) equipped with three pressure vessels, each with 30 mm in inner diameter and 500 mm in height (≈400 mL capacity each). A mixture of propylene glycol and water (40:60, *v*/v) was used as pressurization fluid.

Inoculated WJ samples in the micro test tubes were kept inside small low permeability polyamide/polyethylene bags with 90 μm of thickness (PA/PE-90, Ideiapack - Comércio de embalagens Lda, Abraveses, Viseu, Portugal) and stored for up to 28 days at different pressure levels (25-100 MPa), while control samples were kept in the dark at atmospheric pressure (AP, 0.1 MPa) at RT (AP/RT, 18-23 °C) or under refrigerated conditions (AP/RF, ~4 °C).

2.3. Microbiological analyses

2.3.1. Inoculated microorganisms' preparation

A non-pathogenic surrogate strain *E. coli* ATCC 25922 and *S. cerevisiae* were used in this study. A pure culture of *E. coli* was previously grown in Tryptic Soy Broth (Liofilchem, Roseto degli Abruzzi, Italy) at 37 °C overnight in an incubating orbital shaker (VWR, Carnaxide, Portugal) at 150 rpm. *S. cerevisiae* was isolated from baker's yeast (Condi, Camarate, Portugal) purchased from a local supermarket: baker's yeast was plated in Yeast Malt Agar medium (YMA, HiMedia, Maharashtra, India) and incubated at 30 °C for 3 days; then, a single colony was inoculated in 200 mL of Yeast Malt Broth (HiMedia, Maharashtra, India), followed by incubation at 30 °C for 3 days under orbital agitation (150 rpm) in the orbital shaker. Glycerol (10% *v*/v) (Biochem Chemopharma, Cosne-Cours-sur-Loire, France) was added to the grown microorganisms' stocks for cryoprotection and divided in aliquots in 2 mL microtubes (Deltalab, S. L., Barcelona, Spain) and frozen at -80 °C until use. After thawing at RT, the necessary micro tubes were centrifuged at 6000 *g* for 10 min and the supernatant was discarded, while the remaining mass of cells (pellet) was inoculated in the WJ. The initial microbial counts of the inoculums ranged between 8 and 9 log CFU/mL. The inoculation of microorganisms in WJ and samples' packaging process did not take >1 h in order to minimise the effects of pH only (especially low pH) on the viability of microorganisms.

2.3.2. Quantification of microorganisms

WJ samples were serially diluted in Ringer's solution after each experiment and plated on the appropriate media using the Miles and Misra technique (Miles et al., 1938). *E. coli* was enumerated in Tryptic Soy Agar medium (VWR International, Radnor, Pennsylvania, United States of America (USA)) after incubation at 37 °C for 24 h and *S. cerevisiae* was enumerated in YMA after incubation at 30 °C for 36 h. Petri dishes containing 10–100 colony forming units (CFU) were selected for counting. The results were expressed as log CFU/mL ± standard deviation of WJ and presented as microbial log load variation ($\log (N/N_0)$), calculated by the log load difference between the microbial load at each storage day (N), and the initial microbial load (N_0). The detection limit was 1.7 log CFU/mL according to the Miles and Misra technique (Miles et al., 1938), when no counts were found in the lowest dilution (i.e. when the WJ was directly placed on the plates), while when only 1-10 colonies were counted in the lowest dilution, the quantification limit was 2.7 log CFU/mL (Lopes et al., 2024).

2.3.3. Microbial inactivation kinetic modelling

Microbial inactivation kinetic parameters were calculated using two

mathematical models (first-order and Weibull), based on the kinetics profiles observed and when microbial inactivation was observed with enough data points to apply the models. The first-order model is frequently used in the food industry, the Weibull model was chosen for being one of the most applied nonlinear models and for having shown good fits for HPP microbial inactivation data (Serment-Moreno, 2020). The data fitting was performed using Matlab R2023a (MathWorks Inc., Natick, Massachusetts, USA) using the Trust-region nonlinear regression method with only quantifiable experimental values (i.e., values above the QL). To evaluate the quality of the fit for each model, the root mean square error (*RMSE*), the coefficient of determination (R^2) and the adjusted R^2 ($Adj\text{-}R^2$) were determined and the adequacy of the model to describe the data indicated by a *RMSE* value close to 0 and an $Adj\text{-}R^2$ close to 1. The kinetic parameter values estimated for each model were presented as value ±95% confidence interval.

The application of first-order linear model (Eq. 1) for the microbial inactivation by pressure assumes that the N_0 of microbial population is reduced to N after some storage time t at a constant pressure. The decimal reduction time (D_p, in days) is the time necessary to achieve a decimal reduction in microbial load and was directly calculated using Eq. 1 (Erkmen & Barazi, 2012). -valuesAdj-$R^2 > 0.900$ were considered.

$$log\left(\frac{N}{N0}\right) = -\frac{t}{Dp} \quad (1)$$

The z_p, expressed in MPa, represents the necessary change in the pressure level to change the D_p by a decimal factor and was obtained through Eq. 2 (Erkmen & Barazi, 2012), where D_{ref} is the D_p-value at the reference pressure (p_{ref}, MPa), and p is the pressure. Only z_p-values estimated with an $Adj\text{-}R^2 > 0.850$ were considered.

$$log\left(\frac{Dp}{D_{ref}}\right) = -\frac{p_{ref} - p}{z_p} \quad (2)$$

In the Weibull model (Eq. 3) the parameter b represents the microbial inactivation rate constant, and n regards the shaping factor. When $n < 1$ an upward concave curve is displayed, indicating an adaptation of the microbial population that shows moderate or high resistance to the applied stress through gradual count reductions as time goes by. In the case of $n > 1$, a downward concave curve appears, meaning that inactivation occurs faster due to cumulate damage caused by the applied stress, while the less frequent case of $n = 1$ results in a first-order kinetics (Serment-Moreno, 2020).

$$log\left(\frac{N}{N0}\right) = -b^{*}t^{n} \quad (3)$$

2.4. Statistical analysis

The microbiological analyses were performed with three different replicates (samples), each one plated in duplicate. Then, a one-way Analysis of Variance (ANOVA) followed by the post hoc Tukey's test were used to evaluate the differences at different storage times for each curve. For microbial inactivation, kinetic parameters were used to identify differences, based on the 95% confidence interval of each one, between storage pressure at different pH or between different pressures at the same pH. The level of significance was set in all cases at 5%.

3. Results and discussion

3.1. Microbial analyses

3.1.1. Escherichia coli

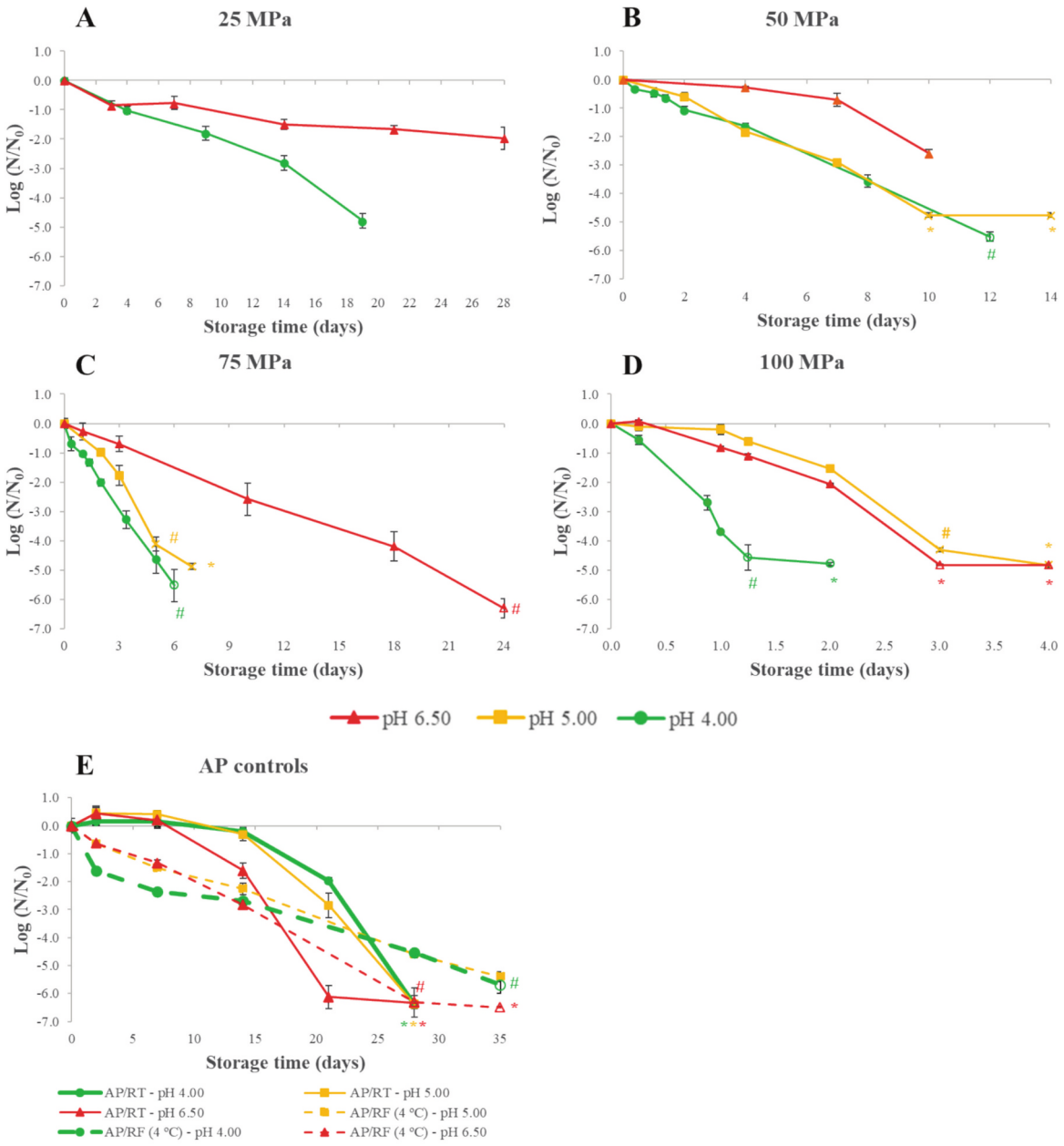
The impact of WJ's pH on *E. coli*'s behaviour during HS at RT was studied for pH 4.00, 5.00, and 6.50, at pressure levels from 25 MPa (for pH 4.00 and 6.50) or 50 MPa (for pH 5.00) to 100 MPa, and the results compared to conventional RF. HS/RT at 25 MPa was tested after seeing that higher pressures succeed in controlling *E. coli*'s growth, to assess whether a lower pressure would still achieve this goal. Table S1 details the initial *E. coli* loads (N_0) for the different experiments, which varied between 6.5 and 8.2 log CFU/mL (average of 7.5 ± 0.7 log CFU/mL). Since considerable inactivating effects were detected during the first experiments, it was decided to increase the inoculated *E. coli* load to test HS' inactivating potential deeply. This is the reason for the differences in initial loads seen in Table S1, with the initial loads of subsequent experiments being higher.

Fig. 1 depicts the impact of the different pH levels for each HS condition, including the AP controls at refrigerated (4 °C) and RT conditions (Fig. 1E) and the statistical analysis for each curve (Fig. 1F). The results show that, during HS at all pressures and, regardless the pH, *E. coli* growth was controlled, including the control at RT (AP/RT) and AP/RF (for AP/RT, a very slight growth for the first 7 days was observed, followed by decay). Additionally, albeit at varied rates, *E. coli*'s inactivation was also seen during storage for all cases studied, with overall inactivation being faster under HS at pH 4.00 and 5.00 at all pressures studied and at pH 6.50 at 100 MPa. The impact of pH was tested first at 50, 75, and 100 MPa and, generally, inactivation was faster from pH 6.50 to 4.00 (with pH 4.00 showing similar results to pH 5.00 at 50 and 75 MPa and faster inactivation at 100 MPa) (Fig. 1), with the loads often being reduced to values below the quantification or detection limits (QL and DL, respectively). One example of this trend was the reduction of, at least, 4. log CFU/mL after 2 days at 100 MPa for pH 4.00, whereas a comparable reduction (4.8 log CFU/mL) in pH 6.50 stored at 100 MPa took one extra day. Further, below in section 3.2, kinetic modelling results are presented with a quantitative comparison of inactivation rates.

Afterwards, since the results revealed that pH 4.00 obtained the fastest inactivation and pH 6.50 presented the slowest, these two pH levels were chosen to test HS at 25 MPa, to understand whether a lower storage pressure would suffice to control *E. coli* growth, as well as causing its inactivation. The results confirmed these expectations since slower inactivation was observed at 25 MPa, especially at pH 6.50, where after 28 days the 2.0 log units' reduction was smaller compared to the 4.8 log units' reduction at pH 4.00 after 19 days. In fact, the lower inactivation at pH 6.50 was observed for pressures of 25 and 50 MPa compared to controls at RT and RF indicates that pressure at these levels may have conferred protection against inactivation.

In Fig. 1, especially for pH 4.00 and pH 5.00, it is noteworthy that higher pressure levels gradually increase the inactivation rates (with at least, 4.8 log CFU/mL inactivated after 4, 7, and 10 days for pH 5.00 stored at 100, 75 and 50 MPa, respectively, for example). This way, for pH 6.50 the most evident benefit occurred at 100 MPa, considering that lower pressure levels (75 MPa) obtained results comparable to AP controls and lower pressure (25 and 50 MPa) resulted in lower inactivation rates, thus indicating a protective effect against inactivation at these pressure levels at pH 6.5, as above. It should be noted that even at pH 6.50, the most perishable level, HS at 100 MPa not only effectively controlled *E. coli* development in the WJ but also caused its inactivation of, at least, about 5 log CFU/mL in about 3 days. The inactivation levels of *E. coli* measured at the last sampling day in each case ranged from a minimum of 1.8 log CFU/mL (pH 6.50 after 28 days at 25 MPa) to, a maximum of at least, 6.3 log CFU/mL (pH 6.50 after 24 days at 75 MPa).

The major trends seen in Fig. 1 are consistent with previous HS works since higher inactivation rates have been associated with higher storage pressures and, although no direct comparison has been reported yet, some HS works also have pointed at higher inactivating effect of an acidic pH (Lima & Saraiva, 2023). The inactivation effects of HS are likely related in the cumulative disruption of essential cellular processes, such as nutrient uptake, DNA replication, and protein synthesis, where the prolonged inhibition of these vital functions under HS conditions (working as a stress source for microorganisms) eventually surpassing the ability of cells to be able to recover, leading to an irreversible loss of viability and cell death. Nevertheless, the exact mechanisms behind the combined effects of pH and HS on microorganisms' inactivation should



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HS/RT	pH 4.00								pH 5.00							pH 6.50						
(days)	0	4	9	14	19											0	3	7	14			
25 MPa	a	b	c	d	e											a	b	b	c			
(days)	0	0.38	1	1.38	2	4	8	12	0	2	4	7	10	14		0	4	7	10	18		
50 MPa	a	b	bc	c	d	e	f	#	a	b	c	d	*	*		a	b	c	d	e		
(days)	0	0.38	1	1.38	2	3.38	5	6	0	2	3	5	7			0	1	3	10	24		
75 MPa	a	b	bc	c	d	e	f	#	a	b	c	#	*			a	ab	b	c	#		
(days)	0	0.25	0.88	1	1.25	2			0	0.25	1	1.25	2	3	4	0	0.25	1	1.25	2	3	4
100 MPa	a	b	c	d	#	*			a	ab	bc	c	d	#	*	a	a	b	b	c	*	*
(days)	0	2	7	14	21	28			0	2	7	14	21	28		0	2	7	14	21	28	
AP/RT	b	a	a	c	d	*			bc	a	ab	c	d	*		c	a	b	d	#	*	
(days)	0	2	7	14	28	35			0	2	7	14	28	35		0	2	7	14	28	35	
AP/RF (4 °C)	a	b	c	d	e	#			a	b	c	d	e	f		a	b	c	d	#	*	

Fig. 1. *Escherichia coli* load variation (log (N/N_0)) for watermelon juice (pH 4.00, 5.00, and 6.50) preserved by hyperbaric storage (HS) at uncontrolled room temperature (RT, 18-23 °C) at different pressure levels of 25 MPa (A), 50 MPa (B), 75 MPa (C), 100 MPa (D) and atmospheric pressure controls (E) at RT (AP/RT) or refrigerated (AP/RF, 4 °C) conditions. Results are expressed in log CFU/mL and initial loads are available in Table S1. Different letters in Table (F) denote significant differences ($p < 0.05$) between storage days for each storage condition at a given pH level (a-f). Unfilled symbols on the graphs represent counts below the quantification or the detection limits (2.7 and 1.7 log CFU/mL, respectively), indicated in the graphs and the Table (F) by # and *, respectively.

be further explored (Oger & Jebbar, 2010). Although no systematic work is reported in literature on the effect of pH on food storage by HS, the influence of pH on the current commercial HPP is widely reported, where, in most cases, an acidic pH boosted inactivation, being this attributed to a synergistic effect between the stress induced by low pH and the physical damages caused by pressure (Lima et al., 2023).

The behaviour of *E. coli* inoculated in foods preserved by HS was studied by different authors, including in WJ (pH 6.23) preserved up to 10 days at 50, 75, and 100 MPa at RT (18-23 °C), demonstrated the ability of HS to gradually inactivate microorganisms, especially at higher pressure levels. After 3 days at 75-100 MPa, *E. coli* counts reached the DL, with an inactivation of, at least, 2.1 log CFU/mL, while at 50 MPa, it was observed a decrease of 1 log CFU/mL after the first 3 days and, by the 6th day, the DL was reached (Pinto et al., 2017). This is one example of faster and/or higher inactivation results achieved by higher storage pressure levels, like in Fig. 1. The same trend was noted for other foods stored at varied storage pressure levels, like raw milk (pH 6.68) preserved by HS/RT for up to 31 days at 50-100 MPa that reduced *E. coli* counts by, at least, 4.1 log CFU/mL after 21 days at 50 MPa, 10 days at 75 MPa or 3 days at 100 MPa (Duarte, Pinto, et al., 2022). Another example was milk (pH 6.73) pasteurised by HPP (two consecutive cycles at 600 MPa for 90 and 120 s, respectively) and subsequently preserved for up to 120 days at 50-100 MPa/RT (20 °C), revealing that, at least, 6.7 log units' reduction was observed after 10 days at 100 MPa or 21 days at 75 MPa but, at 50 MPa, a reduction of only 2-3 log units was observed after 21 days (Lemos, Lopes-da-Silva, et al., 2023). Comparisons between WJ and milk results should consider that, for milk, the matrix may be exerting a protective effect, for example, due to the amount of casein or lactose, as reported in the literature for HPP (Ramaswamy et al., 2009).

The changes in the WJ's pH affected how suitable the environment was for *E. coli*: since its growth can occur between pH 4.4-10.0 being the optimal pH range 6.0-7.0 (McAuley & Fegan, 2022), lowering to pH 4.00 was expected to reduce counts, as well as providing faster and/or higher inactivation results when combined with pressure. Furthermore, *E. coli* has an optimal growth temperature of 35-40 °C but can grow from 7 to 8 °C to 46 °C (McAuley & Fegan, 2022) and the RT range studied (18-23 °C) is below the optimal range of growth temperature, whereas AP/RF control (4 °C) is even below the temperature growth range. This way, in face of the results, no straightforward conclusion can be reached concerning the effect of temperature on the behaviour of *E. coli* under HS. This is supported by the results obtained for AP/RT and AP/RF, since at AP/RF inactivation occurs faster in the beginning and, at AP/RT, a very slight growth occurs at the beginning, but, for longer storage times, AP/RT results in equal to higher inactivation level.

When viewing the above findings as possible indications of *E. coli* behaviour and comparing them with the results displayed in Fig. 1 for HS/RT, the most notable difference is that not only no growth was observed, but inactivation occurred during HS/RT. This can be attributed to the impact on HS on *E. coli*'s behaviour, while inactivation observed for AP controls was slower, and for 25 and 50 MPa (particularly for the former and particularly for pH 6.50) a protective effect of pressure was observed, this being interesting for further research.

Fig. 1 shows the HS' potential of HS for food preservation, since not only microbial growth was controlled for all pH levels, but considerable (fast) inactivation also occurred (in some cases) to pasteurisation-like values, ≥5 log CFU/mL, with reduction of the most resistant microorganism that poses a threat to public health, as defined by the U.S. Food and Drug Administration (U.S. Food and Drug Administration, 2004; Lee et al., 2023).

3.1.2. *Saccharomyces cerevisiae*

S. cerevisiae was used as model for yeasts overall, due to the facility to carry out experiments with it, to systematically study, for the first time, the effect of pH on yeasts during HS. Food spoilage by *S. cerevisiae* is associated with fermentation, leading to gassiness, cloudiness, and off-flavours linked with acetic acid and hydrogen sulphide (Paniagua-Martínez et al., 2018). Additionally, YM can grow in a broader pH range (2.0-10.0) than bacteria (4.5-10.0) (Park et al., 2014), thus favouring competition for YM compared to bacteria to grow at lower pH levels.

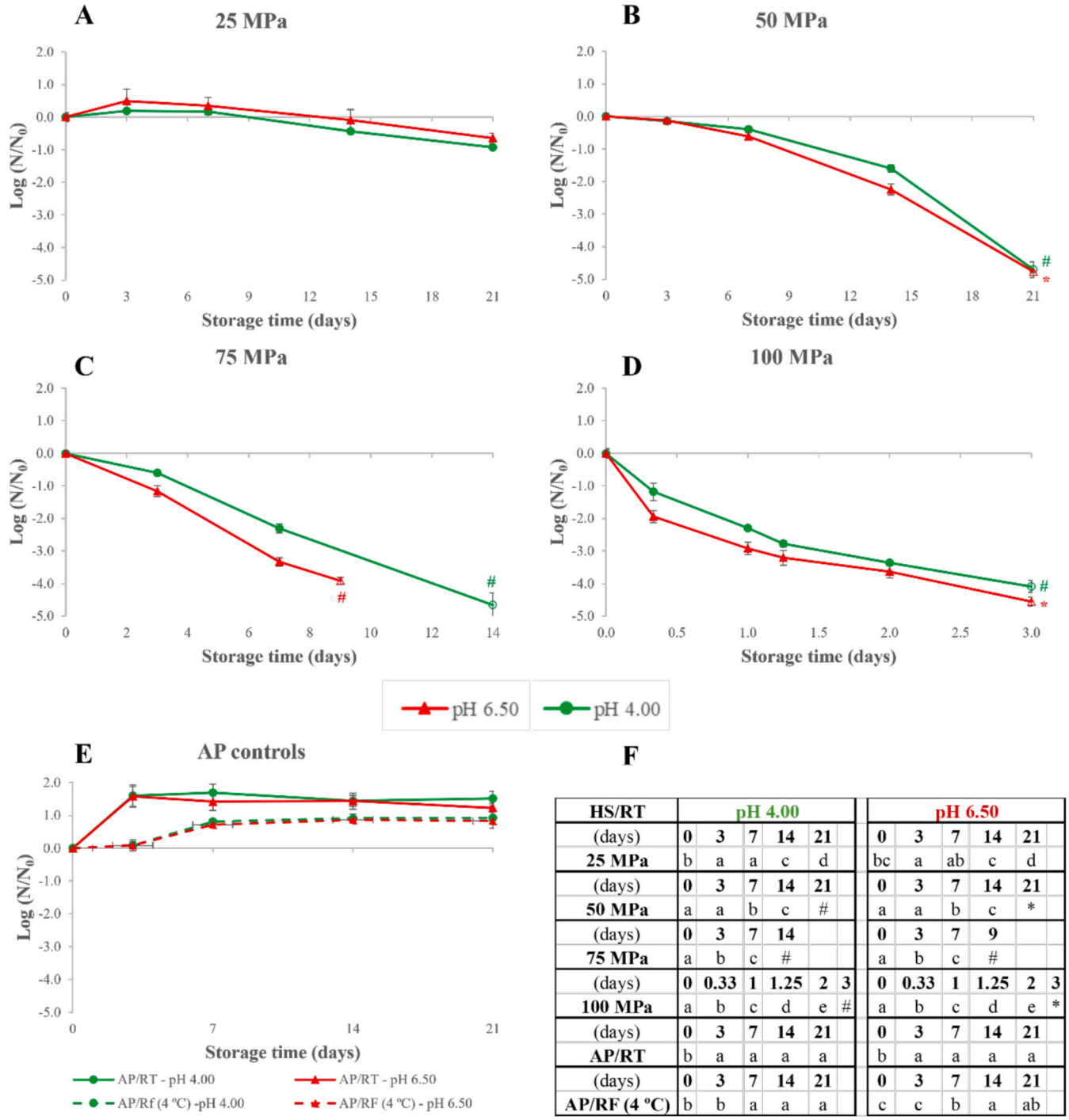
The influence of pH on *S. cerevisiae*'s behaviour during the HS/RT (25-100 MPa) of WJ was studied for pH 4.00 and 6.50. Table S2 presents the inoculated *S. cerevisiae* loads, which ranged from 6.3 to 6.6 log CFU/mL (average of 6.4 ± 0.1 log CFU/mL). Fig. 2 shows these HS/RT curves, along with the AP controls under RF (4 °C) and RT conditions (Fig. 2E) and the statistical analyses for each curve (Fig. 2F).

Fig. 2 demonstrates that HS could control the growth of *S. cerevisiae* during storage, as well as inactive it. Compared to the *E. coli* results, the pH showed a much lower effect at all pressures tested for *S. cerevisiae*. Considerable inactivation was observed for pressures equal or above 50 MPa, with the effect increasing with the pressure increment (for 25 MPa a slight growth was observed at beginning (maximum of 0.5 log CFU/mL after 3 days for pH 6.50 WJ), followed by a small reduction in counts until day 21 (0.9 and 0.7 log CFU/mL for pH 4.00 and 6.50, respectively). HS/RT's success in inactivating *S. cerevisiae* contrasts with the growth measured in the AP controls: around 1.6 log CFU/mL for AP/RT after 3 days (with minor variation throughout storage) and AP/RF, in which growth was delayed for longer (7 days) and only a slight growth occurred (maximum of 0.9 log CFU/mL). In accordance with these results, unlike the WJ samples preserved by HS, the samples stored at AP showed evidence of gas formation (Fig. 3) at both pH levels, especially at RT.

Differently than *E. coli*, *S. cerevisiae* was inactivated slightly quicker in pH 6.50 compared to pH 4.00 WJ stored at pressure levels of 50-100 MPa (Fig. 2), although the difference between the curves of the two pH levels was smaller, thus indicating a lower effect of pH. The most noticeable example occurred after 7 days at 75 MPa, with a difference in inactivation of only around 1 log CFU/mL. One possible explanation for the smaller pH effect (compared to *E. coli*) is related to most *S. cerevisiae* strains growing at pH values of 2.5-8.5 and being acidophilic organisms with an optimum pH growth range between 4.0 and 6.0 (Liu et al., 2015) and thus being above the optimal pH growth range when inoculated at pH 6.50.

Increasing the storage pressure accelerated *S. cerevisiae*'s inactivation in WJ at both pH levels as, for example, for pH 4.00 WJ: it took 14 days to achieve a 1.6 log CFU/mL reduction at 50 MPa, but during this period, at 75 MPa, an additional 3 log CFU/mL inactivation was measured; or, for example, the 2.3 log CFU/mL reduction observed after 7 days at 75 MPa took only 1 day at 100 MPa. At the end of storage (21 days), when the highest reduction for each curve was observed, the inactivation was as low as 0.7 log CFU/mL (for pH 6.50 WJ stored at 25 MPa) and as high as, at least, 4.8 log CFU/mL (for pH 6.50 WJ stored at 50 MPa). Apart from the results for WJ stored at 25 MPa, the inactivation levels at 50, 75 and 100 MPa were over 4 log CFU/mL (often closer to 5 log units), regardless of the pH.

These results are consistent with the expectation of increased inactivation rates for higher pressures since, despite no HS works with *S. cerevisiae* having been published, results for YM (as a group) are available. In this context, the loss of yeast viability under HS may be happening by the cumulative disruption of cellular homeostasis and structural integrity that occur at low/moderate hydrostatic pressures (50–100 MPa, like those used for HS studies) is known to inhibit the plasma membrane H^+ATPase, which compromises the cell's ability to maintain an internal pH gradient and leads to cytoplasmic pH shifts that can compromise yeasts viability (Abe, 2007), being this one possible reason for the observed faster inactivation at pH 6.50 and that at 4.00 (which is within to the optimal growth pH range). Several studies have demonstrated that HS/RT can inactivate YM in WJ to levels below the DL (Fidalgo et al., 2014; Lemos et al., 2017; Lemos et al., 2020; Pinto et al., 2016; Pinto et al., 2017; Santos et al., 2015) and some revealed that the maximum inactivation occurred at the highest storage pressure



F

HS/RT	pH 4.00						pH 6.50					
(days)	0	3	7	14	21		0	3	7	14	21	
25 MPa	b	a	a	c	d		bc	a	ab	c	d	
(days)	0	3	7	14	21		0	3	7	14	21	
50 MPa	a	a	b	c	#		a	a	b	c	*	
(days)	0	3	7	14			0	3	7	9		
75 MPa	a	b	c	#			a	b	c	#		
(days)	0	0.33	1	1.25	2	3	0	0.33	1	1.25	2	3
100 MPa	a	b	c	d	e	#	a	b	c	d	e	*
(days)	0	3	7	14	21		0	3	7	14	21	
AP/RT	b	a	a	a	a		b	a	a	a	a	
(days)	0	3	7	14	21		0	3	7	14	21	
AP/RF (4 ºC)	b	b	a	a	a		c	c	b	a	ab	

Fig. 2. *Saccharomyces cerevisiae* load variation (log (N/N_0)) for watermelon juice at pH 4.00 or 6.50 preserved by hyperbaric storage (HS) at uncontrolled room temperature (RT, 18-23 °C) at different pressure levels of 25 MPa (A), 50 MPa (B), 75 MPa (C), 100 MPa (D) and the atmospheric pressure controls (E) at RT (AP/RT) and refrigerated (AP/RF, 4 °C) conditions. Results are expressed in log CFU/mL and initial loads are available in Table S2. Different letters in Table (F) denote significant differences ($p < 0.05$) between storage days for each storage condition at a given pH level (a-e). Unfilled symbols on the graphs represent counts below the quantification or the detection limits (2.7 and 1.7 log CFU/mL, respectively), indicated in the graphs and in Table (F) by # and *, respectively.

level tested. This occurred for WJ (pH 6.23) stored for up to 10 days at 50-100 MPa/RT (18-23 °C): inactivation to levels below the DL (at least 2.6 log CFU/mL) were observed after 4 days at 100 MPa and 7 days at 75 MPa whereas, at 50 MPa, inactivation did not occur (Pinto et al., 2017). Similar results were also described for WJ (pH 6.50) stored at a lower temperature (15 °C) at 50-75 MPa: a reduction of, at least, 2.3 log CFU/mL to levels below the DL was noticed after 14 days at 75 MPa, 58 days at 62.5 MPa while, at 50 MPa, growth was observed at the 7th day (the last sampling point) (Lemos et al., 2017). The inactivating effect of increasing storage pressure was also found in raw milk (pH 6.68) (Duarte, Pinto, et al., 2022), cow and goat fresh cheese (pH 6.45 and 6.58, respectively) (Duarte et al., 2023), ready-to-eat fish soup (pH 6.0) (Lemos, Martins, et al., 2023) and pork meat in pieces (pH 6.39) (Santos et al., 2020). These works also reported that YM are more susceptible to HS than bacteria and more susceptible to preservation by HS than by RF, which is in accordance with Fig. 2.

Fig. 3. Gas formation (blue arrow) in the test tubes containing watermelon juice inoculated with *Saccharomyces cerevisiae* at pH 4.00, in a polyamide/polyethylene bag stored at atmospheric pressure under room temperature (AP/RT, 18-23 °C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The storage temperature observed in these experiments (18-23 °C), fits well within *S. cerevisiae*'s growth range, as growth was observed (on average for 10 strains) between 2.9 °C and 45.4 °C, with an optimal temperature of 32.3 °C (Salvadó et al., 2011). The smaller growth observed in AP/RF samples, in comparison to AP/RT (at both pH levels), may be related to the AP/RF temperature (4 °C) being closer to the minimum growth temperature.

The impact of cold storage of WJ at AP on YM has been studied by some authors, for example: citrulline-enriched acidified WJ (pH 3.80) was thermally pasteurised (80 °C for 40-90 s) and stored for 30 days at 4 °C and considerable growth of 4.9 and 4.3 log CFU/mL was measured during this period, from the initial 1.3 and 1.6 log CFU/mL after the 40 and the 90 s pasteurisation, respectively (Aguayo et al., 2017). YM counts in WJ (pH 5.34-5.35) treated with UV-C at different intensities and stored at 5 °C decreased by around 1 log CFU/mL from the initial 2.9 and 2.5 log CFU/mL (for intensities 2.7 and 37.5 J/mL, respectively) until day 13 (for 2.7 J/mL) and day 17 (for 37.5 J/mL), before increasing by around 5 and 3 log CFU/mL for 2.7 and 37.5 J/mL, respectively, at the end of storage (day 37) (Feng et al., 2013). Acidified WJ (pH 3.80) was pasteurised for 20 s at 87.7 °C and stored for 30 days at 4 or 8 °C, showing a decrease in YM counts to levels below the DL after 10 days (from the initial 2.2 log CFU/mL), a subsequent increase to 1.4 log CFU/mL after 20 days at 8 °C, and values of 1.8 and 1.9 log CFU/mL after 30 days for WJ stored at 4 and 8 °C, respectively (Tarazona-Díaz & Aguayo, 2013). Using the same storage and pasteurisation conditions of the last study, a different paper by the same research group presented results for the yeasts and filamentous fungi group in acidified WJ (pH 3.80) during cold storage: after a small reduction caused by the pasteurisation, counts grew gradually from 1 to 2 log CFU/mL (depending on the duration) until reaching around 6 log CFU/mL at day 30 (Tarazona-Díaz et al., 2017). Unlike all these last studies, a work focusing on the cold storage (4 °C, 14 days) of pasteurised (80 °C for 1-15 min) WJ (pH 5.40) reduced YM counts to levels below the DL and, during storage, counts remained below detection, regardless of the pasteurisation duration) (Mandha et al., 2023). The aforementioned results are aligned with the ones obtained in the present study, as it was observed an overall increase of about 1 log unit for *S. cerevisiae* over the 21 days of refrigerated storage, at pH 4.00 and 6.50.

The comparison between the *S. cerevisiae* data of the present work and the abovementioned YM (in general) results do not allow for straightforward extrapolations, but it provides interesting points like the growth observed in WJ at both pH levels stored at AP/RF conditions matching the YM behaviour described by most authors cited. Considering this comparison, it is worth noting that, unlike the growth observed during cold storage by most of these YM works, HS could control *S. cerevisiae*'s growth during WJ storage at uncontrolled RT, as well as cause inactivation to values up to, at least, 5 log CFU/mL.

3.2. Effect of hyperbaric storage on the inactivation kinetic parameters

Figs. 1 and 2 showed that inactivation kinetics did not always follow a linear inactivation kinetics. This is not unusual considering that nonlinear kinetics models are reported for HPP inactivation curves and, in these cases, the Weibull model is one of the most used nonlinear models showing good fits for inactivation of microorganisms by HPP (Serment-Moreno, 2020).

The impact of the different HS conditions and pH levels was mathematically modelled by the first-order kinetic model (D_p- and z_p-values) and the Weibull model (b and n values) (Table 1 for *E. coli* and Table 2 for *S. cerevisiae*). Their graphical representation and the fitted vs experimental values plots are available as supplementary material (for fits with an $Adj\text{-}R^2 > 0.90$, Figs. S1-S7 and Figs. S8-S14, respectively). The z_p-values andwere estimated with a minimum of 3 pressure levels and had an $Adj\text{-}R^2 > 0.850$ and should be cautiously interpreted due to the low number of pressures used. Additional research is required to obtain a higher number of pressures to better calculate z_p-values, but they are worth discussing here due to the *quasi* absence of z_p-values under HS available in the literature.

Generally, for *E. coli* (Table 1) the Weibull model performed better than the first-order kinetics model, presenting overall higher $Adj\text{-}R^2$ and lower RMSE values, despite two cases where the first-order kinetics model was more adequate. The D_p-values decreased as pressure increased and for example, at pH 4.00, there was a gradual decrease of the D_p-values from 4.21 ± 0.48 to 0.28 ± 0.03 days when the pressure increased from 25 to 100 MPa, respectively (Table 1). An increase in the D_p-values was observed when the WJ's pH increased, as seen in Table 1 at 75 MPa, for example. The results show a comparable D_p for pH 6.50 WJ/100 MPa and pH 4.00 WJ/25 MPa, clearly indicating a relevant effect of the pH on *E. coli's* inactivation. The highest D_p was observed for pH 4.00 WJ at 100 MPa and the lowest was seen for pH 6.50 WJ kept at 75 MPa, but it was still faster than pH 5.00 WJ stored by AP/RF (4 °C). The z_p was only calculated for pH 4.00 WJ (64.75 ± 39.99), since the other pH levels had insufficient experimental data points.

Limited published information exists regarding inactivation kinetic data for microorganisms in foods preserved by HS. A work on raw milk (pH 6.68) stored by HS/RT (50-100 MPa, 18-22 °C) reported a D_p of 3.7 days at 50 MPa for *E. coli*, which is higher than the D_p-values estimated in the present work for the same pressure level (2.32-2.34 days), as seen in Table 1. The authors also estimated the D_p-values for TAM, reporting values of 25.6 and 12.8 days for 75 and 100 MPa, respectively (Duarte, Pinto, et al., 2022). Other work reported the D_p of the inactivation of TAM and Enterobacteriaceae in raw milk (pH 6.68) stored at 75 MPa (18-22 °C), obtaining very different results: 18.81-19.81 days and 3.50-3.56 days, respectively (Duarte, Gomes, et al., 2022). A third HS/RT

Table 1
Estimated first-order kinetic (D_p and z_p) and Weibull (b and n) parameters (± 95% confidence interval) of the inactivation of *Escherichia coli* in watermelon juice (WJ) at different pH levels preserved by hyperbaric storage (HS, 25-100 MPa) at uncontrolled room temperature (RT, 18-23 °C) and the atmospheric pressure controls at RT (AP/RT) and refrigerated conditions (AP/RF, 4 °C). *Adj-R^2* values in bold represent the best fit between both models.*

Escherichia coli		First-order model parameters							
pH	**Storage**	**D_p (day)**	**R^2**	***Adj-R^2***	***RMSE***	**z_p (MPa)**	**R^2**	***Adj-R^2***	***RMSE***
4.00	25 MPa/RT	4.21 ± 0.48	0.966	0.963	0.3273	64.75 ± 39.99	0.960	0.941	0.1240
	50 MPa/RT	2.32 ± 0.11	0.990	**0.990**	0.1189				
	75 MPa/RT	1.10 ± 0.07	0.984	0.983	0.2026				
	100 MPa/RT	0.28 ± 0.03	0.975	0.972	0.2660				
	AP/RT	11.37 ± 4.41*	0.705	0.682	0.4759				
	AP/RF (4 °C)	7.29 ± 1.59	0.883	0.874	0.5431				
5.00	50 MPa/RT	2.34 ± 0.21	0.985	0.983	0.1512				
	75 MPa/RT	1.73 ± 0.32	0.960	0.954	0.1679				
	100 MPa/RT	1.38 ± 0.76	0.840	0.827	0.2442				
	AP/RT	7.55 ± 2.97*	0.699	0.675	0.7273				
	AP/RF (4 °C)	6.46 ± 0.34	0.990	0.990	0.2135				
6.50	25 MPa/RT	16.03 ± 3.50	0.855	0.846	0.2709				
	50 MPa/RT	4.32 ± 3.90	0.890	0.882	0.5309				
	75 MPa/RT	4.24 ± 0.33	0.983	0.982	0.2259				
	100 MPa/RT	0.91 ± 0.12	0.955	0.952	0.1831				
	AP/RT	7.98 ± 3.57*	0.713	0.684	0.2135				
	AP/RF (4 °C)	5.16 ± 0.39	0.989	**0.988**	0.1223				

Escherichia coli		Weibull model parameters				
pH	**Storage**	***b***	***n***	**R^2**	***Adj-R^2***	***RMSE***
4.00	25 MPa/RT	0.122 ± 0.070	1.233 ± 0.208	0.975	**0.973**	0.2774
	50 MPa/RT	0.474 ± 0.063	0.963 ± 0.071	0.989	0.988	0.1241
	75 MPa/RT	1.090 ± 0.123	0.900 ± 0.083	0.984	**0.983**	0.2023
	100 MPa/RT	3.556 ± 0.195	1.516 ± 1.400	0.989	**0.987**	0.1787
	AP/RT	$8.414 \times 10^{-8} \pm 2.859 \times 10^{-9}$ **	5.604 ± 1.823	0.981	**0.980**	0.1205
	AP/RF (4 °C)	0.982 ± 0.244	0.443 ± 0.086	0.967	**0.965**	0.2877
5.00	50 MPa/RT	0.365 ± 0.110	1.074 ± 0.168	0.985	**0.983**	0.1507
	75 MPa/RT	0.363 ± 0.190	1.447 ± 0.516	0.975	**0.971**	0.1325
	100 MPa/RT	0.316 ± 0.081	2.300 ± 0.396	0.974	**0.972**	0.0981
	AP/RT	$9.100^{-8} \pm 5.9225 \times 10^{-9}$ **	5.702 ± 3.625	0.938	**0.933**	0.3310
	AP/RF (4 °C)	0.250 ± 0.070	0.872 ± 0.084	0.990	**0.990**	0.2117
6.50	25 MPa/RT	0.400 ± 0.146	0.474 ± 0.125	0.932	**0.928**	0.1848
	50 MPa/RT	0.946 ± 0.176	0.536 ± 0.075	0.986	**0.984**	0.1931
	75 MPa/RT	0.292 ± 0.109	0.925 ± 0.137	0.984	**0.983**	0.2169
	100 MPa/RT	0.948 ± 0.163	1.145 ± 0.299	0.952	**0.948**	0.1909
	AP/RT	$3.774 \times 10^{-8} \pm 3.500 \times 10^{-9}$ **	8.327 ± 101.713	0.911	**0.902**	0.2629
	AP/RF (4 °C)	0.244 ± 0.081	0.922 ± 0.135	0.988	0.987	0.1274

* : microbial growth.
** : These values are presented in scientific notation considering that the *b*-values (from the Weibull model) were very close to zero due to the fact that microbial growth was observed in the first days at AP/RT before occurring inactivation.

work (15-22 °C), this time in cow and goat fresh cheese, reported a D_p of 17.8 and 13.4 days (cow cheese) and 9.9 and 3.4 days (goat cheese) for TAM when the foods were stored at 75 and 100 MPa, respectively; 14.7 days (cow cheese at 50 MPa), 11.2 and 4.2 days (goat cheese at 50 and 75 MPa, respectively) for coliforms; and, lastly 11.3 and 9.6 days (cow cheese) and 5.4 and 3.5 days (goat cheese) for Enterobacteriaceae for HS at 50 and 75 MPa, respectively (Duarte et al., 2023). These results reveal the underlying differences between microbial groups, as well as food matrixes, which is relevant since microbial inactivation depends on the nutritional composition, since some food components can provide a protective effect against HPP (Lima & Saraiva, 2023).

The application of the Weibull model to *E. coli*'s inactivation during HS showed an increase in the parameter b (related to the inactivation rate magnitude increment (Evelyn, and Silva, 2015) for pH 4.00 WJ when the storage pressure increased (Table 1). For instance, greater b values were observed when the pressure was increased from 25 to 100 MPa, from 0.122 ± 0.070 to 3.556 ± 0.195, respectively, revealing a similar trend to that observed for the D_p-values. Differently, for pH 5.00, the b values are quite similar at 50 and 100 MPa, respectively, whereas for pH 6.50, there is no clear trend. Measured n values include values below, above, and of 1, without clear variation trend. When the confidence interval at 95% for the n value includes 1 (pH 4.00 WJ/50 and 100 MPa, pH 5.00 WJ/50-75 MPa and pH 6.50 WJ/75-100 MPa), this means that the concavity of the curve changes from concave upwards/downwards to linear and a linear inactivation kinetics (first-order kinetics model) is possible (Serment-Moreno, 2020). Overall, a trend for n to increase with pressure was observed, particularly for pH 6.50. The high confidence interval of the n value for pH 4.00 WJ/100 MPa may be related to a lower number of experimental points used (3 as opposed to the 4 or 5 for the other cases). An $n > 1$ value suggests that the cumulative damage induced by the exposure to pressure progressively weakens the surviving microbial population causing a faster inactivation (Peleg, 2021; Serment-Moreno, 2020).

The increase in b related to the increased pressure level observed for pH 4.00 WJ was also reported by Moody and colleagues when apple juice (pH 3.52) was treated by HPP (300-600 MPa, 0-7 min, 21 °C) (Moody et al., 2014) but differs from the decrease in b registered in other work of apple juice (pH 4.515) treated by HPP (400-475 MPa, 5 °C) (Usaga et al., 2021). A $n < 1$ trend was noted for pH 6.50 WJ, except for the $n > 1$ value for 100 MPa, signalling a change in shape from an upward to a downward curve at 100 MPa (Table 1), possibly indicating that below 100 MPa a tailing effect has a higher prevalence (Fay et al., 2023). This behaviour is in accordance with results observed for most pressure used to treat (300-600 MPa, 0-7 min, 21 °C) apple juice (pH 3.52), as only at 300 MPa the n was over 1 (Moody et al., 2014). Differently, for apple juice (pH 4.52) processed by HPP (400-475 MPa, 5 °C), the calculated n was always above 1 (Usaga et al., 2021).

The same models were applied to *S. cerevisiae*'s inactivation data (Table 2), revealing higher *Adj-R^2* and lower *RMSE* values for the Weibull model, which showed a better fitting. Both models were unable to fit

Table 2
Estimated first-order kinetic (D_p and z_p) and Weibull (b and n) parameters (± 95% confidence interval) of the inactivation of *Saccharomyces cerevisiae* in watermelon juice (WJ) at different pH levels preserved by hyperbaric storage (HS, 25-100 MPa) at uncontrolled room temperature (RT, 18-23 °C) and the atmospheric pressure controls at RT (AP/RT) and refrigerated conditions (AP/RF, 4 °C). *Adj-R^2* values in bold represent the best fit between both models.

Saccharomyces cerevisiae		First-order model parameters						
pH	**Storage**	**D_p (days)**	***R^2***	***Adj-R^2***	***RMSE***	**z_p (MPa)**	***R^2***	***Adj-R^2***	***RMSE***
4.00	25 MPa/RT	19.59 ± 5.55*	0.817	0.803	0.1974	43.36 ± 64.20	0.987	0.973	0.0950
	50 MPa/RT	8.63 ± 1.76	0.923	0.915	0.1929				
	75 MPa/RT	2.99 ± 0.52	0.963	0.958	0.2121				
	100 MPa/RT	0.61 ± 0.10	0.926	0.921	0.3510				
	AP/RT		*						
	AP/RF (4 °C)		*						
6.50	25 MPa/RT	24.30 ± 12.71*	0.568	0.534	0.2938				
	50 MPa/RT	6.05 ± 1.15	0.932	0.926	0.2556				
	75 MPa/RT	2.08 ± 0.20	0.989	0.987	0.1654				
	100 MPa/RT	0.60 ± 0.17	0.813	0.799	0.6009				
	AP/RT		*						
	AP/RF (4 °C)		*						

Saccharomyces cerevisiae		Weibull model parameters				
pH	**Storage**	***b***	***n***	***R^2***	***Adj-R^2***	***RMSE***
4.00	25 MPa/RT			*		
	50 MPa/RT	0.010 ± 0.007	1.938 ± 0.276	0.991	**0.990**	0.0652
	75 MPa/RT	0.106 ± 0.032	1.581 ± 0.156	0.997	**0.997**	0.0565
	100 MPa/RT	2.317 ± 0.096	0.569 ± 0.075	0.989	**0.988**	0.1383
	AP/RT			*		
	AP/RF (4 °C)			*		
6.50	25 MPa/RT			*		
	50 MPa/RT	0.016 ± 0.010	1.874 ± 0.234	0.993	**0.992**	0.0818
	75 MPa/RT	0.295 ± 0.068	1.246 ± 0.122	0.997	**0.997**	0.0830
	100 MPa/RT	2.901 ± 0.044	0.344 ± 0.027	0.998	**0.997**	0.0683
	AP/RT			*		
	AP/RF (4 °C)			*		

"*": microbial growth was observed

the curves for the AP controls, possibly due to the slight growth observed before the small (and slow) inactivation verified during the 21 days of storage (Fig. 2).

The observed reduction in D_p-values when storage pressure increased, regardless of the pH, confirms the expectation of higher inactivation rates at higher pressure. pH's effect was also visible: the biggest difference wasat 50 MPa with higher D_p-values for pH 4.00 than pH 6.50. The lowest D_p was quantified for pH 4.00 WJ/100 MPa and the highest was for pH 6.50 WJ/50 MPa. Additionally, the $z_p$for pH 4.00 was calculated but had a high 95% confidence interval, possibly due to the small number of pressure levels used.

The lack of published HS-related *S. cerevisiae* kinetic data does not allow for a direct comparison, however, when cow and goat fresh cheese (pH 6.45 and 6.48, respectively) were preserved by HS/RT (50-100 MPa), D_p-values of4.7 and 3.9 days for cow and goat, respectively at 50 MPa were calculated for YM (as a group) (Duarte et al., 2023), being lower than what was measured for pH 6.50 WJ stored at 50 MPa (6.05 ± 1.15 days).

Table 2 shows the increase in the b parameter when pressure increased for both pH levels,indicating a higher inactivation rate, especially from 75 to 100 MPa. The b values were also higher for pH 6.50, confirming Fig. 2's data. A descending trend of the n parameter was observed for increasing pressures: $n > 1$ values at 50-75 MPa and $n < 1$ at 100 MPa.

The increase in b detected for higher pressures and $n < 1$ values were also observed in the inactivation of *S. cerevisiae* ascospores in beer treated by HPP: in a first study, beer with different alcohol content (0.0-7.0%) was subjected to 200-400 MPa for up to 40 min (at temperatures not exceeding 36 °C) and small variations in n were observed between different beers or pressure levels applied (Milani & Silva, 2016); whereas a second study (200-400 MPa, up to 60 min, 23 °C) showed unchanged n values (Milani et al., 2016). A change from $n > 1$ to $n < 1$ values was detected for the inactivation of *S. cerevisiae* in grape juice (pH 5.92) by pulsed electric field treatment (40 °C, 34-92 μs) after the electric field strength was increased (9-27 kV/cm^2) (Huang et al., 2014).

3.3. Effect of hyperbaric storage on the inactivation kinetic parameters of *Escherichia coli* and *Saccharomyces cerevisiae*

Tables 1 and 2 also allow for a brief comparison of the inactivation kinetic parameters of both microorganisms studied for the same pH level at the same storage conditions and present four cases when D_p-values can be compared for both microorganisms, showing differences in the resistance to inactivation under HS: the higher D_p-values reveal that *S. cerevisiae* was more resistant at pH 4.00 but the difference decreased as pressure rose up to 100 MPa. However, at pH 6.50 at 75 MPa, a shift in HS resistance was observed since *E. coli*'s D_p-value was higher. The z_p-values obtained for pH 4.00 WJ indicate that *S. cerevisiae* was the most sensitive to pressure-value - 43.36 ± 64.20 days), followed by *E. coli* (64.75 ± 39.99 days), although this should be cautiously interpreted due to the high confidence interval of the z_p-values.

4. Conclusions

This work evaluated the impact of pH variation (4.00-6.50) on HS/RT's potential to ensure microbial growth control in WJ, and the results revealed that during storage at pressures up to 100 MPa, not only *E. coli* and *S. cerevisiae*'s growth could be controlled, but also microbial inactivation occurred and was frequently to levels below the DL and reaching 5 log CFU/mL (the pasteurisation level required for bacterial vegetative pathogenic microorganisms).

HS' effect in microbial counts varied depending on the microorganism, WJ's pH level and storage pressure. Generally, *E. coli* and *S. cerevisiae* inactivation occurred at the pH level and pressure tested (albeit at different levels). WJ's pH influenced *E. coli*'s results, since lowering the pH to 4.00 increased inactivation rates whereas, for *S. cerevisiae*, the pH effect was minor but pH 6.50 allowed for slightly higher reductions at 50-100 MPa. Furthermore, the increase in pressure resulted in higher inactivation rates for both microorganisms. The microbial inactivation kinetic data revealed differences in microbial resistance to HS/RT, depending on the pH: lower D_p-values (50-100

MPa) were estimated for *E. coli* at pH 4.00 but, at pH 6.50 at 75 MPa, the D_p-values were lower for *S. cerevisiae* and lower z_p-values were estimated for *S. cerevisiae*'s inactivation in pH 4.00 WJ (43.36 ± 64.20 vs 64.75 ± 39.99 MPa for *S. cerevisiae* and *E. coli*, respectively).

The microbial growth control and inactivation findings presented confirm HS's potential as a food preservation methodology regardless of pH, although influenced by it, with overall better results than RF and the ability to operate without temperature control, making HS, from a functional point of view, almost energetically costless and with lower greenhouse gas emissions, being environmentally friendlier. The inactivation levels reached 5 log CFU/mL for *E. coli* (a surrogate of a vegetative pathogenic bacteria) and *S. cerevisiae* (while growth was observed at RT and even at RF at 4 °C) during storage, indicating that HS also has great potential as a novel nonthermal pasteurisation technique at RT. This study focused on microbial parameters, but the impact of HS and pH variation should be further studied, namely on physicochemical, quality, and sensory attributes to evaluate whether WJ properties are also well preserved. Additionally, further research is required to understand the mechanisms behind the impact on microorganism caused by HS and pH variation.

CRediT authorship contribution statement

Vasco Lima: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Carlos A. Pinto:** Writing – review & editing, Validation, Software, Methodology, Investigation, Formal analysis. **Jorge A. Saraiva:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work received financial support from PT national funds (FCT/MCTES, Fundação para a Ciência e Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior) through the project UID/50006/2025, DOI 10.54499/UID/50006/2025-Laboratório Associado para a Química Verde - Tecnologias e Processos Limpos (LAQV-REQUIMTE). Thanks are also due to FCT/MCTES for financing the PhD grants of Vasco Lima (SFRH/BD/146080/2019) and Carlos A. Pinto (SFRH/BD/137036/2018 and COVID/BD/153220/2023).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2026.104681.

Data availability

Data will be made available on request.

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