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Hyperbaric storage of egg white at room temperature: effects on hygienic properties, protein structure and technological functionality

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Abstract:	Egg white was submitted to hyperbaric storage at 200 MPa at room temperature for up to 28 days. Control samples were stored at 4 °C and 0.1 MPa. Storage conditions were compared for antimicrobial capacity and changes in physical, structural and functional properties of egg white proteins. S. aureus and S. enterica were completely inactivated within 3 hours of hyperbaric storage. Prolonged hyperbaric storage promoted slight egg white yellowing, probably due to non-enzymatic browning or riboflavin-protein decomplexation, and induced minor changes in egg white proteins structure. Partial conversion of ovalbumin into S-ovalbumin lead to slightly decreased gelling capacity. Pressurized egg white proteins also resulted slightly compressed and electrically stabilized, becoming more prone to solvent interactions. Based on these effects, viscosity and foaming capacity of egg white increased. Our work demonstrates for the first time that hyperbaric storage guarantees safety and hygiene of egg white without detriment to its technological functionality.

Dear Editor,

We send to your attention the research article "**Hyperbaric storage of egg white at room temperature: effects on hygienic properties, protein structure and technological functionality**" by Federico Basso, Lara Manzocco, Michela Maifreni, and Maria Cristina Nicoli. All the authors have read and approved the manuscript.

Hyperbaric storage was investigated as a stustainable alternative to refrigeration for protein rich food ingredients. To the best of our knowledge, this technology has never been studied with reference to these highly industrially relevant food matrices. To this aim, the case of egg white was considered as an example of perishable, protein-rich food ingredient. The effects of hyperbaric storage and conventional refrigeration on egg white hygienic properties (*i.e.* counts of inoculated *Staphylococcus aureus* and *Salmonella enterica*), protein structure and technological functionality (*i.e.* foaming and gelling properties) were compared. Results appear particulatly interesting since they indicate that hyperbaric storage could be applied not only to preserve hygenic quality but also to enhance technological functionality of egg white.

We hope that this article could satisfy the requirements of Innovative Food Science and Emerging Technologies, so that you might consider it for publication in this Journal.

Best regards,

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1 HS at 200 MPa for 1 day inactivates *S. aureus* and *S. enterica* in egg white

2 HS slightly decreases egg white protein diameter and Z-potential

3 HS allows S-ovalbumin formation and decreases egg white gelling

4 Hyperbarically stored egg white is more viscous and better foamin

Hyperbaric storage of egg white at room temperature: effects on hygienic properties, protein structure and technological functionality

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ABSTRACT

Egg white was submitted to hyperbaric storage at 200 MPa at room temperature for up to 28 days. Control samples were stored at 4 °C and 0.1 MPa. Storage conditions were compared for antimicrobial capacity and changes in physical, structural and functional properties of egg white proteins. *S. aureus* and *S. enterica* were completely inactivated within 3 hours of hyperbaric storage. Prolonged hyperbaric storage promoted slight egg white yellowing, probably due to non-enzymatic browning or riboflavin-protein decomplexation, and induced minor changes in egg white proteins structure. Partial conversion of ovalbumin into S-ovalbumin lead to slightly decreased gelling capacity. Pressurized egg white proteins also resulted slightly compressed and

Abbreviations: HS: Hyperbaric storage; HS-RT: Hyperbaric storage at room temperature; HS-LT: Hyperbaric storage at low temperature; HHP: High hydrostatic pressure; BHI: Brain heart infusion; MRD: Maximum recovery diluent; BP: Baird Parker agar; BPW: Buffered peptone water; DTNB: 5′,5-dithiobis (2-nitrobenzoic acid): EDTA: Ethylenediaminetetraacetic acid

electrically stabilized, becoming more prone to solvent interactions. Based on these effects, viscosity and foaming capacity of egg white increased. Our work demonstrates for the first time that hyperbaric storage guarantees safety and hygiene of egg white without detriment to its technological functionality.

Industrial relevance

Hyperbaric storage could represent an interesting alternative to refrigeration due to its capability to preserve food hygienic properties. Concomitantly, it could be used to pasteurize and even enhance technological functionality of protein-rich food ingredients. These goals could be achieved at *quasi*-zero energetic consumption if working units were made viable for industrial application.

Keywords: hyperbaric storage, egg white, microbiological safety, protein structure, technological functionality

1 Introduction

Hyperbaric storage (HS) is a novel technology, based on the application of moderate hydrostatic pressure (up to 250 MPa) to extend food stability over time. Similarly to high hydrostatic pressure (HHP), HS working units consist in pressure-holding steel tanks where hydrostatic pressure is applied by means of a pressurizing fluid, which is often water (Fernandes et al., 2019). Nevertheless, HS equipment is less expensive and easier to operate than HHP due to lower pressure levels. On the other hand, HS is also similar to refrigeration, since they are both based on the control of a thermodynamic variable (*i.e.* pressure or temperature) during storage. However, HS

has the undoubted advantage of much lower energy cost. Energy is only required for pressurization, while pressure maintenance during storage is guaranteed by vessel sealing solely (Bermejo-Prada, Colmant, Otero, & Guignon, 2017; Freitas et al., 2016; Santos et al., 2020). Additional energy might be required only when, depending on the desired application, storage temperature needs to be controlled. HS units can actually work in a wide temperature range (-20/40°C) by implementing thermal insulation of the vessel. To this regard, the technology is mentioned as HS-RT if pressure is applied at room temperature with no specific control, or HS-LT when pressure is combined with low temperature to assist food refrigeration or freezing.

During the last few years, HS has sparked substantial interest to maintain safety and hygienic properties during storage of many fresh foods, such as meat, fish and fruit juices (Fidalgo et al., 2019; Lemos, Ribeiro, Delgadillo, & Saraiva, 2020; Santos, Castro, Delgadillo, & Saraiva, 2019). For instance, strawberry juice stored at room temperature at 100 MPa for 15 days presented 5 and 3.8 log reductions in total bacteria count and yeasts and molds, respectively. In the case of fresh beef meat, the application of 75 MPa at 25 °C for 14 days promoted a 3 log unit-reduction in inoculated *L. innocua* and *E. coli* (Santos et al., 2019). Interestingly, HS performed at 100 MPa for 20 days at room temperature was also successful at inactivating endospores (4.5 log reductions) of *B. subtilis* in carrot juice (Pinto, Santos, Fidalgo, Delgadillo, & Saraiva, 2018). Despite promoting extensive microbial inactivation, with no recovery in microbial activity even after months under pressure, HS did not promote significant changes in food physical and sensory properties. To this regard, Lemos et al., (2020) actually reported that watermelon juice stored for one year at 75 MPa only presented a slightly faded color. In the case of protein rich foods, such as meat and fish, denaturation of myofibrillar and sarcoplasmic proteins was detected, resulting in minor changes of techno-functional properties such as water-holding capacity and texture.

Although these changes are negligible, modifications in protein structure might become particularly critical for food ingredients (*e.g.* milk, soy and egg, and derivatives), which are used to produce and stabilize food structures, including emulsions, gels and foams. Nevertheless, to our knowledge, no information is available in the literature about this topic.

The aim of the present study was to investigate the effects of hyperbaric storage at room temperature (HS-RT) on microbial inactivation, physical and structural properties, and techno-functionality of egg white. The latter was taken as an example of a highly perishable protein rich food ingredient. To this aim, egg white was inoculated with *Staphylococcus aureus* and *Salmonella enterica*, and subjected to hyperbaric storage at 200 MPa at 20 °C. During storage up to 28 days, samples were analyzed for microbial counts, physical and structural properties (colour, sulfhydryl groups, absorbance at 280, 380 and 680 nm, denaturation temperature, secondary structure, particle size and Z-potential) and techno-functionality (viscosity, gelling and foaming properties). The intention was to evaluate the possibility to use HS to guarantee food safety and hygienic properties of protein-rich food ingredients without impairing their functionality.

2 Materials and Methods

2.2 Sample preparation

Fresh eggs were purchased from a local retailer. Egg white was obtained by manually separating the yolk and the chalazae and by gently stirring for 2 min, in order to mix the naturally occurring egg white fractions (*i.e.* thick and thin). Egg white was poured in polyethylene/ethylene vinyl alcohol/polypropylene pouches (15 x 30 cm; 80 μm thickness, water vapor permeability < 1 g · m^{-}

^{2} · 24 h^{-1}; Niederwieser Group S.p.A., Campogalliano, Italy), which were heat-sealed with headspace not exceeding 5 % of samples volume (Orved, VM-16, Musile di Piave, Italy).

Egg white samples for microbiological analyses were prepared separately. Egg shells were cleaned with hydroalcoholic solution (ethanol 70%) and allowed to air dry for a few minutes before aseptic breaking. The egg white was manually separated from the yolk and chalazae under sterile conditions, and collected in a sterilized beaker. For the inoculum, a bacteria suspension was prepared using strains of *Salmonella enterica* subsp. *enterica* 9898 DSMZ and *Staphylococcus aureus*, obtained from the bacterial culture collection of the Department of Agricultural, Food, Animal and Environmental Sciences of the University of Udine (Italy). Strains were maintained at -80 °C in Brain Heart Infusion broth (BHI, Oxoid, Milan, Italy) with 30% sterile glycerol as cryoprotectant until use. Strains were incubated in BHI at 37 °C for 24 h, subsequently cultured in 5 mL of BHI at 37 °C for 24 h, and finally collected by centrifugation at 14,170 × g for 10 min at 4 °C (Beckman, Avanti TM J-25, Palo Alto, CA, USA) and washed three times with Maximum Recovery Diluent (MRD, Oxoid, Milan, Italy). The final pellets were suspended in MRD. An adequate aliquot of the bacteria suspension was added to the egg white to obtain a final concentration of 10^{3-4} CFU g^{-1}. The inoculated egg white was distributed in 50 g aliquots and packaged as for the other samples.

2.3 Hyperbaric storage

A HS-RT working unit assembled by Comer Srl (Bologna, Italy) was used. It consisted of a water-tight steel vessel (Hystat, Slaithwaite, Huddersfield, United Kingdom) pressurized by a Haskel International high pressure pump (Burbank, CA, USA). The pressure-mediating fluid was an aqueous solution containing 0.2 % (w/w) potassium sorbate and 0.2 % (w/w) sodium benzoate

(Carlo Erba Reagents Srl, Milan, Italy) to prevent mold growth in the fluid reservoir. Samples were introduced in the vessel and pressurized at 200 MPa at room temperature (20 ± 2 °C). Control samples were stored under refrigerated conditions (4 °C, 0.1 MPa). In-shell eggs stored at room conditions (20 ± 2 °C, 0.1 MPa) were also used as additional control. At increasing time during storage for up to 28 days, samples for microbial analyses were removed from the HS vessel or from the refrigerator and analyzed. Other samples were divided in two aliquots. The first one was submitted to analysis within 24 h from depressurization. The second aliquot was removed from the pouches, frozen in thin layer at -30 °C in a shock freezer ("air-o-chill", Electrolux Professional S.p.A., Pordenone, Italy) and freeze-dried (Mini-Fast Edwards, mod. 1700, Edwards Alto Vuoto, Milan, Italy). Freeze dried samples were stored in desiccators at room temperature under dark until further analyses.

2.4 Microbial analyses

From each pouch, 20 g of egg white sample, inoculated with *S. enterica* or *S. aureus*, was diluted in 80 mL of MRD (1:5 v/v) (Oxoid, Milan, Italy). 0.1 mL aliquots of appropriate dilutions were plated onto Plate Count Agar (Oxoid, Milan, Italy) and incubated at 37 °C for 24 h or 36 - 48 h respectively for *S. enterica* and *S. aureus*.

Preliminary trials were carried out on non-inoculated egg white samples to check the *S. enterica* or *S. aureus* presence. For *S. aureus*, 20 g of egg white was diluted 1:5 v/v in MRD and 0.1 mL aliquots of appropriate dilutions were plated onto Baird Parker agar (BP, Oxoid, Milan, Italy), incubated at 37 °C for 24 h. For *S. enterica*, 25 g of non-inoculated egg white were diluted with 225 mL of Buffered Peptone Water (BPW, Oxoid, Milan, Italy), homogenized in a Stomacher for 2 min at 37 °C for 24 h. A volume of 0.1 mL of BPW was added with 9.9 mL Rappaport Vassiliadis

(RV, Oxoid, Milan, Italy) and incubated at 42 – 43 °C for 18 - 24 h. Presence/absence of *S. enterica* was checked by spreading onto Xylose-Lysine-Desoxycholate agar (Oxoid, Milan, Italy) and incubated at 37 °C for 24 h.

2.5 Colour

Samples were placed into Petri dishes positioned over a white surface and analyzed for colour by using a tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-300 measuring head. The instrument was standardized against a white tile before analysis. Colour was expressed in L*, a* and b* Hunter scale parameters.

2.6 Absorbance

Freeze dried samples were diluted 1:1000 (w/v) in 0.05 M Tris-HCl buffer pH 9.0 containing 0.04 M NaCl. Samples were very gently stirred at 4 °C overnight to ensure solubilization. Absorbance at 280, 380 and 680 nm was detected at 4 °C by a UV-VIS spectrophotometer (UV-2501 PC, Shimadzu Kyoto, Japan) in 1 cm path-length quartz cuvettes.

2.7 Free sulfhydryl groups

Free sulfhydryl groups content was determined using Ellman's reagent (5′,5-dithiobis (2-nitrobenzoic acid), DTNB) (Sigma Aldrich. Milan, Italy), adapting the method of Manzocco, Panozzo, & Nicoli (2013). Briefly, freeze dried samples were diluted 1:1000 (w/v) in Tris–glycine buffer (10.4 g Tris, 6.9 g glycine, 1.2 g EDTA per liter, pH 8.0) containing 1% (w/v) NaCl (Sigma Aldrich, Milan, Italy) by very gentle stirring overnight. 1.93 mL of 0.5% SDS in Tris–glycine buffer was added to 0.067 mL of diluted sample and 0.013 mL of Ellman's reagent (4 mg mL^{-1}

DTNB in Tris–glycine buffer) to develop colour. After 15 min, absorbance was measured at 412 nm by a UV–VIS spectrophotometer (UV-2501 PC, Shimadzu Kyoto, Japan). Concentration of free sulfhydryl groups (μM g^{-1}) was calculated using the following equation:

$$SH = \frac{73.53 \cdot A \cdot D}{C} \quad \text{(Eq.1)}$$

where *A* is the absorbance; *C* is egg white concentration (mg mL^{-1}); *D* is the dilution factor; and 73.53 is derived from $\frac{10^6}{1.36 \cdot 10^4}$; $1.36 \cdot 10^4$ is the molar absorptivity (Ellman, 1959).

2.8 Differential scanning calorimetry

Approximately 20 mg of egg white was weighed into 40 μL aluminum pans, which were hermetically pressure-sealed and heated from 45 to 95 °C at 5 °C min^{-1} in a DSC 3 Stare System differential scanning calorimeter (Mettler-Toledo, Greifensee, Swiss). An empty pan was used as a reference. Transitions peak temperatures were extrapolated from the thermograms and total peak enthalpies were calculated by peak integration using the program STARe ver. 16.10 (Mettler-Toledo, Greifensee, Switzerland). The transition peak associated to ovalbumin unfolding was deconvoluted using Origin Pro 9 (OriginLab, Northampton, MA, USA). Multiple peak fitting was applied adopting $R^2_{adj} > 0.997$ as goodness of fit threshold.

2.9 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) analysis was performed at 25 ± 1 °C on freeze-dried samples by using an Alpha-P (Bruker Optics, Milan, Italy) instrument equipped with an attenuated total reflection accessory and a Zn-Se crystal, as previously described by Melchior,

Calligaris, Bisson, & Manzocco, (2020). Spectra were acquired by performing 32 scans per measurement in the 4000 - 400 cm^{-1} wavelength range, with a resolution of 4 cm^{-1}. Amide I band of every spectra (1700 - 1600 cm-1) was extrapolated, smoothed, baselined and normalized using the OPUS software (version 7.0 for Microsoft Windows, Bruker Optics, Milan, Italy). Amide I band Fourier self-deconvolution and Gaussian multiple peak fitting were performed using Origin Pro 9 (OriginLab, Northampton, MA, USA). $R^2_{adj} > 0.997$ was adopted as goodness of fit threshold.

2.10 Particle size and Z-potential

Freeze-dried samples were diluted 1:100 (w/v) in 0.05 M Tris-HCl buffer pH 9.0 containing 0.04 M NaCl, as previously described for absorbance spectroscopy analysis samples. Samples were then filtered through Whatman n °1 paper and, subsequently, through 25 mm PVDF syringe filters (cutoff 0.45 μm; Lab Logistics Group GmbH, Meckenheim, Germany). Filtered samples were further diluted 1:100 (v/v) with Tris-HCl buffer at 4 °C. Particle size and Z-potential were determined at 4 °C by using a dynamic light scattering system (NanoSizer 3000, Malvern Instruments, Malvern, UK) equipped with a Peltier temperature control system. The refractive index was set at 1.333 and the viscosity was approximated to that of pure water at 4 °C.

2.11 Apparent viscosity

Apparent viscosity at 20 °C was determined using a RS6000 Rheometer (ThermoScientific Rheo Stress, Haake, Germany) equipped with a Peltier temperature control system. Flow curves were obtained in the 0.1 - 200 s^{-1} shear rate range by using a bob-cup geometry with a gap of 27.2 mm

(bob: CC25 DIN Ti; cup: CCB25 DIN/SS; ThermoScientific, Haake, Germany). Apparent viscosity at 21.79 s^{-1} was considered for sample comparison.

2.12 Gelling properties

Aliquots of 50 mL of sample were heated at 90 °C for 15 min in 50 mL-capacity sealed plastic Falcon tubes. Samples were then rapidly cooled in ice and stored at 4 °C for 12 h. The gelled samples were extracted from the Falcon tubes and manually cut by a sharp knife to obtain 1.5 ± 0.1 mm thick slices. Mechanical spectra of the heat-set gels were obtained using a RS6000 Rheometer equipped with a parallel plates geometry having 40 mm diameter and 1 mm gap. To determine samples linear visco-elastic stress domain, stress sweep analysis was performed by increasing the applied stress from 1 to 200 Pa at 1 Hz frequency. Frequency sweep analysis was performed by increasing oscillatory frequency from 0.1 to 16 Hz, applying a stress within the linear visco-elastic stress domain. The gelling capacity was expressed as the elastic modulus of the gelled sample at a frequency of 1 Hz.

2.13 Foaming properties

Foaming properties were determined by adapting the method from Melchior et al. (2020). Briefly, 10 mL of sample was diluted 1:10 (w/w) with MilliQ water and homogenized (Polytron DI 25 basic, IKA Werke GmbH & Co., Germany) for 3 min at 9,500 rpm in a graduated cylinder. The total volume of the foamed samples was measured after 0 and 15 min. Foaming capacity and foam stability were calculated as follows:

$$Foaming\ capacity\ (\%) = \frac{V_0 - V_i}{V_i} \cdot 100 \qquad \text{(Eq.2)}$$

$$Foaming\ stability\ (\%) = \frac{V_{15} - V_0}{V_0} \cdot 100 \quad \text{(Eq.3)}$$

Where V_0 (mL) is the sample volume after homogenization, V_i (mL) is the initial sample volume (10 mL) and V_{15} (mL) is the sample volume after 15 min from homogenization.

2.14 Statistical analysis

Microbiological analyses were performed in single on samples from two independent experiments. Data of colour, absorbance spectroscopy, free sulfhydryl groups content, FT-IR, particle size, Z-potential and foaming properties were obtained by triplicate measurements. Data of differential scanning calorimetry, apparent viscosity and gelling properties were obtained in duplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's Honest Significant Differences test ($p < 0.05$) using R v. 3.6.1 for Windows (The R foundation for statistical computing).

3 Results and Discussion

3.1 Hygienic properties

Preliminary microbial analyses were carried out on non-inoculated egg white to ensure the absence of *Salmonella* and to quantify the naturally occurring *S. aureus* load, which resulted to be always below the detection limit (1.7 log CFU g^{-1}). Egg white was then inoculated with *S. enterica* (4.05 ± 0.35 log CFU g^{-1}) and *S. aureus* (3.96 ± 0.20 log CFU g^{-1}) and the evolution of the counts of these bacteria were followed throughout storage under hyperbaric and refrigerated conditions over 28 days (Table 1).

After just 3 h under hyperbaric conditions, values below the detection limit were reached for the counts of both *S. enterica* and *S. aureus*. Interestingly, these values were maintained throughout the 28 days storage, suggesting the capability of hyperbaric storage to maintain egg white microbiological stability as long as pressure is applied. Such findings are coherent with the literature on hyperbaric storage applied to fresh meat, fresh fish and fruit juices (Fidalgo, Lemos, Delgadillo, & Saraiva, 2018; Pinto et al., 2017; Santos et al., 2020). Conversely, in the refrigerated samples, values of *S. aureus* remained almost the same as the initial concentrations, and these values remained similar until the 14^{th} day of storage. Prolonging the storage period up to 28 days, the *S. aureus* concentration decreased to reach a concentration of about 2.43 log CFU g^{-1}. Regarding *S. enterica* under refrigerated condition, a behavior similar to *S. aureus* was observed, even though significant count reduction occurred only after 21 days. By comparing the results observed under hyperbaric and refrigerated conditions, it appears that pressure actively induces microbial inactivation, while, on the other hand, low temperature only slows down microbial growth. Such behavior has been repeatedly observed in other food matrices subjected to hyperbaric storage (Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, & Otero, 2012). Based on these interesting results, the effects of hyperbaric storage on *S. enterica* and *S. aureus* counts were investigated within 24 hours under hyperbaric conditions to evaluate the differences in their inactivation under hyperbaric conditions (Table 2).

As a result, a reduction of *Salmonella* of about 1.3 log was observed after just 30 min of storage. Subsequently, the count value reached the detection limit after only 3 hours. On the other hand, *S. aureus* was more resistant to pressure. In fact, after 3 hours, only a slightly decrease of the microorganism was observed. This behavior might be explained by the fact that gram-positive bacteria (as *Staphylococcus* spp.) are inherently more pressure-resistant than gram-negative ones

(as *Salmonella* spp.). This is known to be due to the presence of a thick peptidoglycan layer in the cell wall of gram-positive bacteria (Wuytack, Diels, & Michiels, 2002).

These results indicate that hyperbaric storage at 200 MPa allows an efficient performance on microbial growth inhibition and inactivation for both *S. enterica* and *S. aureus* in egg white. In particular, 1 day of storage seems sufficient to achieve a satisfactory level of inactivation of these microorganisms.

3.2 Physical and structural properties

In the light of the encouraging results relevant to the effect of hyperbaric storage on the hygienic properties of egg white, further analyses were performed. In particular, the attention was focused on the physical and structural properties of egg white proteins, given their importance for the techno-functionality of this food ingredient.

Egg white samples were initially analyzed for colour changes. Figure 1 compares the evolution of luminosity and yellowness of egg white during storage under hyperbaric and refrigerated conditions. A progressive decrease in egg white luminosity and a significant increase in yellowness were detected during hyperbaric storage whereas much less pronounced colour changes were observed under refrigeration. These changes were also confirmed by measurements of absorbance at 380 nm. The latter remained almost constant (0.057 ± 0.004) during refrigeration for 28 days, while almost triplicated (0.150 ± 0.007) during pressurized storage.

Although being mainly constituted by proteins, egg white also contains reducing sugars, which could make it particularly prone to non-enzymatic browning reactions (Sisak, Csanádi, Rónay, & Szajáni, 2006). Literature actually reports the early steps of non-enzymatic browning reactions to be characterized by a negative activation volume (Isaacs & Coulson, 1996). It can be thus

298 hypothesized that pressurization could favor condensation reactions between aminoacids and
299 reducing sugars, leading to melanoidin formation during prolonged hyperbaric storage of egg
300 white. To this regard, Hill, Ledward, & Ames, (1996) reported the development of non-enzymatic
301 browning to be triggered under hyperbaric conditions at temperatures as low as 40 °C.
302 Nevertheless, a further mechanism of egg white yellowing upon hyperbaric storage could involve
303 the increase in free riboflavin. Literature actually reports that riboflavin occurs in egg white as
304 complexed with a riboflavin-binding protein. Pressure-induced dissociation of this complex would
305 thus increase the amount of free riboflavin, which has a higher absorption capacity at 380 nm
306 (Shiga et al., 1979; Thomas, Weber, Hook, & Drickamer, 1976).
307 Protein structural changes were investigated by FT-IR analysis of freeze-dried samples. Spectra
308 (not shown) exhibited the typical peaks of amide I and amide II within the range 1500 - 1700 cm^{-1}
309 , associated to C=O and N–H stretching, and bending of the peptide bonds, respectively (Ami,
310 Mereghetti, & Maria, 2013). Deconvolution of Amide I peak (1600 – 1700 cm^{-1}) clearly showed
311 the presence of three protein components. Peaks identified at 1630, 1654 and 1684 cm^{-1} were
312 associated to low-frequency β-sheet, α-helix highly overlapped to random coil and high-frequency
313 β-sheet structures, respectively (Uygun-Sarıbay, Ergun, Kalaycı, & Köseoğlu, 2017).
314 Data relevant to refrigerated egg white showed the occurrence of minor fluctuations in the α-
315 Helix/random coil domain (Table 3). In the pressurized samples, only a slight increase in the
316 average value of the percentage of α-Helix/random coil was noticed (Ngarize, Herman, Adams, &
317 Howell, 2004), suggesting that the secondary structure of egg white proteins was largely retained
318 during hyperbaric storage. Nevertheless, pressurized egg white appeared significantly more turbid
319 then the refrigerated one, as indicated by the increase in absorbance at 680 nm (Table 4). This
320 effect typically indicates the occurrence of protein denaturation phenomena (Manzocco et al.,

2013; Smith, Fiebig, Schwalbe, & Dobson, 1996). To better study structural changes leading to protein denaturation, egg white samples were also analyzed for absorbance at 280, particle size and Z-potential (Table 4).

Under refrigerated conditions, a minor increase in absorbance at 280 nm was observed, suggesting a marginally higher exposure of tyrosine, tryptophan and cysteine residues. Under hyperbaric conditions, no significant changes in absorbance at 280 nm were observed. The lack of changes in cysteine groups exposure was also confirmed by data relevant to sulfhydryl group, which remained almost constant (about 51 $\mu M\ g^{-1}$), independently on storage condition and time. This confirms that S-S/SH exchange plays a negligible role during egg white storage under both refrigerated and hyperbaric conditions. By contrast, dynamic light scattering analysis indicated that the size of pressurized egg white proteins was significantly lower than that of proteins in fresh and refrigerated-stored samples (Table 4). A concomitant increase in the absolute value of the Z-potential also indicated a slightly higher stability of hyperbarically stored proteins towards inter-particle interactions. Similar Z-potential changes were reported for proteins other than those of egg white, and attributed to an increased exposure of carboxyl groups upon pressurization (Kurpiewska et al., 2018; Wang et al., 2019; Zhao, Mu, Zhang, & Richel, 2018). Data shown in Table 4 suggest pressurized storage to favor the formation of protein structures with reduced excluded volume and higher exposure of negatively charged groups, which are typically associated to a more efficient interaction with surrounding water molecules. These effects are in agreement with those reported in the literature for proteins submitted to HHP (Harano, Yoshidome, & Kinoshita, 2008). The latter would turn protein into moderately less compact structures with much larger water-accessible surface. According to this mechanistic interpretation, water would penetrate into the protein interior, leading to a swollen structure stabilized by water molecules with

limited translational and rotational mobility (Harano et al., 2008). Reversely, translational restriction for water molecules outside the protein would be greatly reduced.

3.3 Techno-functional properties

To understand whether the changes in egg white protein structure observed during hyperbaric storage could be associated to modifications in their functional properties, samples were also analyzed for apparent viscosity as well as for gelling and foaming properties (Table 5).

No changes in these properties were detected in egg white stored under refrigeration. By contrast, pressurized egg white presented a remarkable increase in apparent viscosity after 14 days storage. The higher viscosity of pressurized egg white is consistent with protein structural changes previously described (Table 4). Even if more compact, water swollen proteins with higher surface charge would better interact with the solvent, preventing free flowing of the aqueous media in a more efficient way as compared to native ones. Actually, a good correlation ($r = 0.93$; $p < 0.05$) was found between particle size and apparent viscosity. Based on the better interaction with water, pressurized proteins would be less prone to interparticle interactions. To this regard, it is noteworthy that a slight decrease in gelling capacity of egg white was observed. In order to better investigate the mechanism at the basis of this change, specific information was obtained by differential scanning calorimetry analysis. The thermograms relevant to egg white stored for increasing time under hyperbaric condition are shown as examples in Figure 2.

Fresh egg white showed the presence of two phenomena, which were associated to the denaturation of the main protein fractions in egg white. In particular, the endothermal phenomenon between 62 and 70 °C was attributed to the denaturation of conalbumin (Singh & Ramaswamy, 2015). The latter is a highly pressure-sensitive protein that easily undergoes consistent tertiary structure loss

upon high hydrostatic pressure (Rivalain, Roquain, & Demazeau, 2010; Singh & Ramaswamy, 2015; Van der Plancken, Van Loey, & Hendrickx, 2005). Accordingly, the intensity of this phenomenon progressively decreased during hyperbaric storage. A second complex transition in the temperature range 75-87 °C was attributed to ovalbumin, whose native form is characterised by a denaturation temperature of *circa* 80 °C. The ovalbumin double peak shape revealed the presence of an intermediate ovalbumin form showing peak temperature at about 85 °C (De Groot & De Jongh, 2003). During hyperbaric storage, the thermal phenomena associated to the denaturation of ovalbumin native fraction progressively decreased with the increase of the intermediate form of ovalbumin and the appearance of a novel shoulder at temperatures above 89 °C. The latter was attributed to S-ovalbumin. Spontaneous ovalbumin conversion into S-ovalbumin is due to an irreversible multi-step process, involving L-D isomerization of Ser-164, Ser-236 and Ser-320, as well as distancing motion of 1A and 2A strands and burying of residues surrounding Phe-99 (Yamasaki, Takahashi, & Hirose, 2003). To get a quantitative information about the effect of storage conditions on the shift of ovalbumin to S-ovalbumin, enthalpy values of this thermal phenomenon were computed (Figure 3). Analogous data were also acquired for egg white stored under refrigerated conditions or maintained in shell at room temperature.

It can be noted that the increase in S-ovalbumin enthalpy was more pronounced in egg white stored under hyperbaric conditions as compared to refrigerated ones. This difference could further account for the lower gelling properties of pressurized egg white. In fact, the presence of even small amounts of ovalbumin forms undergoing denaturation at higher temperature, has been reported to almost halve the radius of the aggregates generated upon heat treatment. For this reason, S- and intermediate- ovalbumin are known to be characterized by impaired gel network formation as compared to native ovalbumin (De Groot & De Jongh, 2003; Deleu et al., 2015).

Nevertheless, data shown in Figure 3 clearly show that the intensity of conversion from native ovalbumin to thermally resistant ovalbumin forms in pressurized egg white was comparable to that observed in egg white maintained in shell at room temperature.

Despite the lower capacity of proteins to network, pressurized egg white presented a remarkable increase in foaming properties (Table 5). Being smaller and electrically more stable (Table 4), pressurized proteins would quickly set at the interface between water and gas phases, leading to more efficient air encapsulation. To this regard, it is noteworthy that changes in pH and ionic force are generally associated to better foaming capacity (Li et al., 2018). In addition, a good correlation ($r = 0.95$; $p < 0.05$) between foaming capacity and apparent viscosity was actually found, suggesting that the higher foaming capacity could also result from the lower mobility of protein particles in the aqueous interstices among air bubbles. This is also known to be associated to lower solvent drainage from the foams (Fameau & Salonen, 2014). Nevertheless, the stability of the foams obtained from hyperbarically stored egg white resulted comparable to that of refrigerated samples. Egg white foam stability also depends on the capacity of proteins to network upon air contact at the gas-water interfaces. This property would be impaired by the lower networking capacity of pressurized proteins. In other words, the stability of pressurized egg white foams would be the result of two counterbalancing effects: an increase in viscosity, which stabilizes the foam, and a decrease in networking capacity, which has an opposite effect.

4 Conclusions

This study demonstrates that hyperbaric storage at room temperature might represent an interesting sustainable technology to guarantee safety and hygienic properties of egg white. In particular, the capability to effectively inactivate *S. aureus* and *S. enterica* indicates that HS could be employed

to pasteurize egg white and to keep it under optimal hygienic conditions. However, the possibility to adopt HS as an alternative technological approach for pasteurization is bound to the availability of validation studies, which must provide clear evidence of its efficacy in achieving the required inactivation levels of specific target microorganisms. At the same time, due to minor changes in egg white proteins structure, HS could allow to boost the technological functionality of this matrix, with particular reference to foaming properties. The results achieved were relevant to the case of egg white, but analogous results are expected also for other matrices and, especially, for other protein-rich food ingredients. The spectrum of foods feasible for HS is wide and shall include not only fluid foods, but also solid ones, making HS an interesting alternative to refrigeration.

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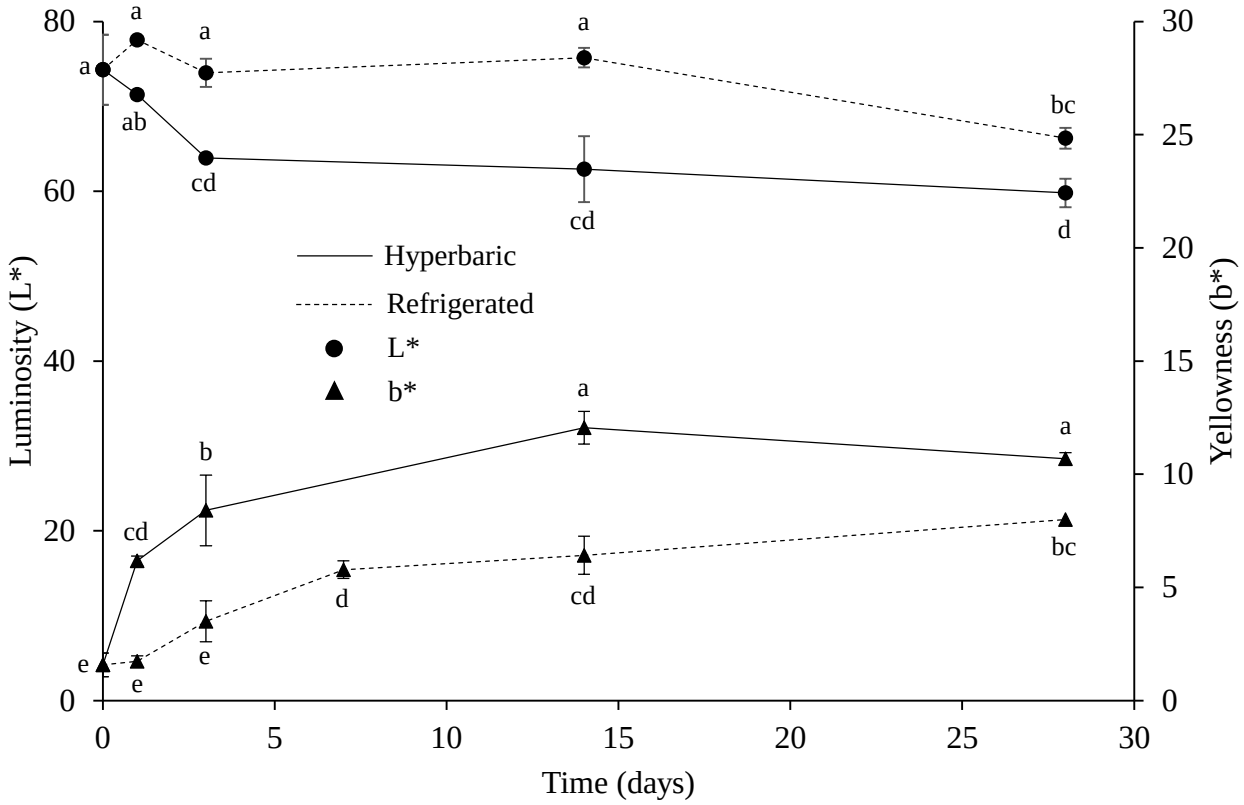


1

2 Figure 1: Luminosity and yellowness of egg white stored for increasing time under refrigerated or

3 hyperbaric conditions.

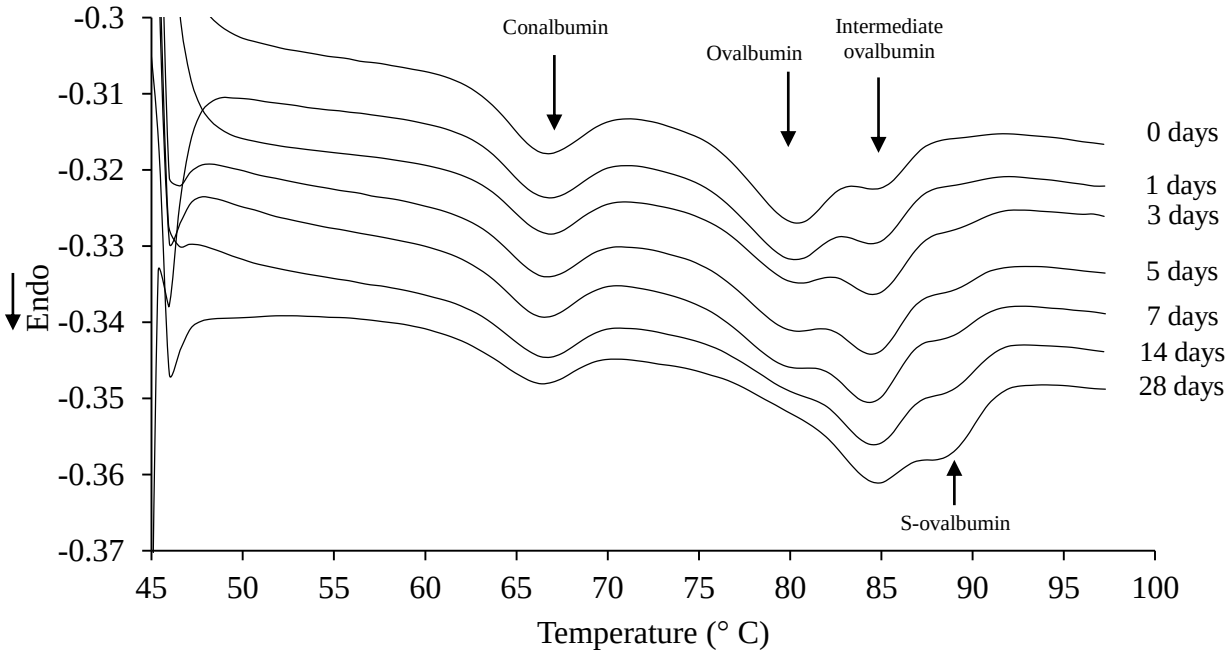
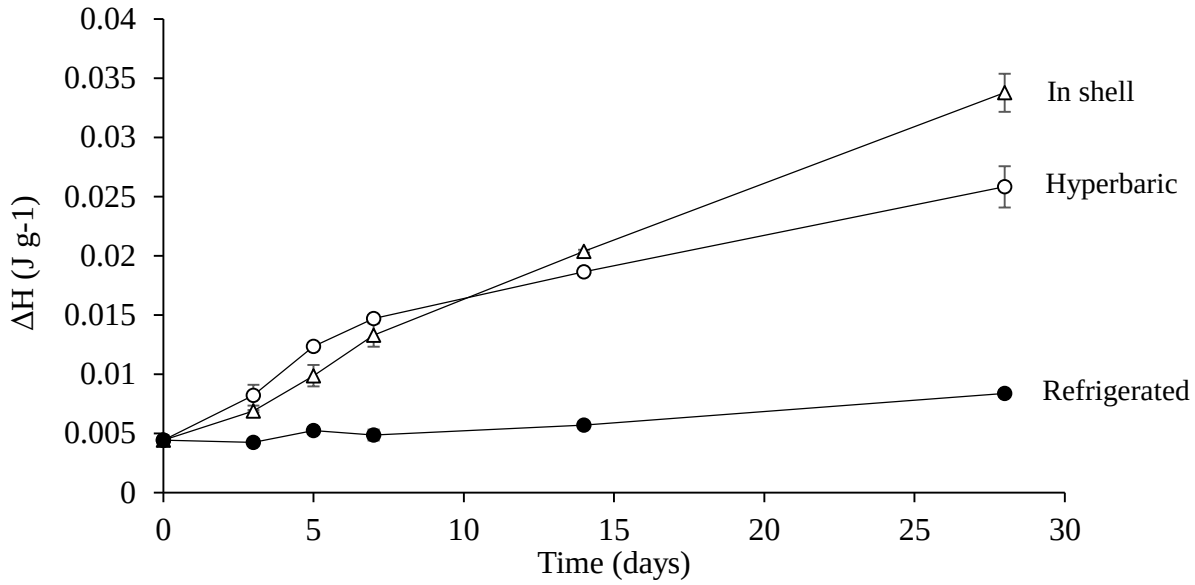


Figure 2: Differential scanning calorimetry thermograms of egg white stored for increasing time under hyperbaric conditions.



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2 Figure 3: Transition enthalpy of S-ovalbumin in egg white stored for increasing time under

3 refrigerated or hyperbaric conditions. Egg white from shell egg is shown as additional control.

Table 1: *S. enterica* and *S. aureus* counts (log CFU g^{-1}) in egg white stored for up to 28 days under refrigerated or hyperbaric conditions.

Time (days)	*S. enterica*		*S. aureus*	
	Refrigerated	Hyperbaric	Refrigerated	Hyperbaric
0	4.05 ± 0.35	4.05 ± 0.35	3.96 ± 0.20	3.96 ± 0.20
1	3.85 ± 0.35	< L.o.D.	3.95 ± 0.21	< L.o.D.
3	3.50 ± 0.07	< L.o.D.	3.75 ± 0.22	< L.o.D.
7	3.40 ± 0.57	< L.o.D.	3.70 ± 0.25	< L.o.D.
14	3.08 ± 0.11	< L.o.D.	3.38 ± 0.11	< L.o.D.
21	3.05 ± 0.07	< L.o.D.	2.62 ± 0.17	< L.o.D.
28	2.37 ± 0.05	< L.o.D.	2.43 ± 0.02	< L.o.D.

N.D. Not determined

L.o.D. 1.7 log CFU g^{-1}

Table 2: *S. enterica* and *S. aureus* counts (log CFU g^{-1}) in egg white stored for up to 3 hours under hyperbaric conditions.

Time (hours)	*S. enterica*	*S. aureus*
0	3.50 ± 0.07	3.96 ± 0.20
0.5	2.30 ± 0.36	3.52 ± 0.35
1	< L.o.D.	3.19 ± 0.06
3	< L.o.D.	< L.o.D.

L.o.D. 1.7 log CFU g^{-1}

Table 3: Percentage of secondary structures of egg white stored for increasing time under refrigerated or hyperbaric conditions.

Storage	Time (days)	α-Helix and random coil (%)	Low frequency β-Sheet (%)	High frequency β-Sheet (%)
Fresh	0	33.50 ± 5.82^{bc}	49.83 ± 3.59^{a}	16.67 ± 2.97^{a}
Refrigerated	14	28.86 ± 2.88^{c}	50.26 ± 3.23^{a}	17.62 ± 3.84^{a}
	28	34.30 ± 3.48^{ab}	49.95 ± 3.41^{a}	15.30 ± 2.35^{a}
Hyperbaric	5	37.17 ± 2.44^{ab}	47.11 ± 1.63^{ab}	15.72 ± 1.66^{a}
	7	38.45 ± 3.72^{ab}	46.42 ± 1.63^{ab}	15.92 ± 2.28^{a}
	14	37.82 ± 2.01^{ab}	46.54 ± 2.44^{ab}	15.64 ± 0.59^{a}
	28	39.88 ± 3.62^{a}	45.01 ± 3.22^{b}	15.69 ± 2.48^{a}

a, b, c: Different letters in the same column indicate significantly different means (ANOVA; $p < 0.05$).

Table 4: Absorbance at 680 and 280 nm, particle size and Z-potential of egg white stored for increasing time under refrigerated or hyperbaric conditions.

Storage	Time (days)	Absorbance		Particle size (nm)	Z-potential (mV)
		680 nm	280 nm		
Fresh	0	0.020 ± 0.001^{b}	0.376 ± 0.005^{b}	224.65 ± 4.97^{a}	-12.25 ± 0.78^{a}
Refrigerated	14	0.018 ± 0.003^{bc}	0.391 ± 0.009^{b}	226.63 ± 11.71^{a}	-12.48 ± 1.02^{a}
	28	0.016 ± 0.001^{c}	0.410 ± 0.009^{a}	225.50 ± 11.47^{a}	-12.14 ± 0.24^{a}
Hyperbaric	14	0.050 ± 0.005^{a}	0.382 ± 0.010^{b}	198.29 ± 4.20^{b}	-15.95 ± 0.53^{b}
	28	0.046 ± 0.005^{a}	0.377 ± 0.006^{b}	192.78 ± 5.26^{b}	-15.15 ± 0.91^{b}

a, b, c: Different letters in the same column indicate significantly different means (ANOVA; $p < 0.05$).

Table 5: Apparent viscosity, gel elastic modulus (G'), foaming capacity and foaming stability of egg white stored for increasing time under refrigerated or hyperbaric conditions.

Storage	Time (days)	Apparent viscosity (Pa s)	G' (Pa · 1000)	Foaming capacity (%)	Foaming stability (%)
Fresh	0	0.078 ± 0.038^{b}	5.95 ± 0.63^{ab}	63.3 ± 15.3^{c}	93.5 ± 6.7^{a}
Refrigerated	5	0.050 ± 0.023^{b}	7.20 ± 0.22^{a}	90.0 ± 10.0^{bc}	96.5 ± 3.1^{a}
	14	0.058 ± 0.032^{b}	-	86.7 ± 14.1^{bc}	91.2 ± 2.5^{a}
	28	0.014 ± 0.002^{b}	6.57 ± 0.35^{a}	66.7 ± 15.3^{c}	93.9 ± 5.9^{a}
Hyperbaric	5	0.120 ± 0.071^{b}	5.80 ± 0.13^{ab}	133.3 ± 11.5^{a}	91.5 ± 4.0^{a}
	14	0.421 ± 0.029^{a}	-	113.3 ± 5.8^{ab}	89.6 ± 5.4^{a}
	28	0.318 ± 0.042^{a}	4.41 ± 0.32^{b}	100.0 ± 10.0^{ab}	96.7 ± 2.8^{a}

a, b, c: Different letters in the same column indicate significantly different means (ANOVA; $p < 0.05$).

Conflict of Interest and Authorship Confirmation Form

The Authors declare that:

- ✓ All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

- ✓ This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

- ✓ The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

Federico Basso: Investigation, Formal analysis, Data curation, Writing - Original Draft, Visualization; **Lara Manzocco**: Conceptualization, Data curation, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision; **Michela Maifreni**: Investigation, Formal analysis, Data curation, Writing - Original Draft; **Maria Cristina Nicoli**: Conceptualization, Resources, Writing - Review & Editing, Supervision.