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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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# Innovative Food Science and Emerging Technologies

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

--Manuscript Draft--

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<b>Abstract:</b>	<p>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. <i>S. aureus</i> and <i>S. enterica</i> 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.</p>

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 sustainable 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 particularly interesting since they indicate that hyperbaric storage could be applied not only to preserve hygienic 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,

Prof. Lara Manzocco, PhD

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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

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

3  
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14

15 **ABSTRACT**

16 Egg white was submitted to hyperbaric storage at 200 MPa at room temperature for up to 28 days.

17 Control samples were stored at 4 °C and 0.1 MPa. Storage conditions were compared for

18 antimicrobial capacity and changes in physical, structural and functional properties of egg white

19 proteins. *S. aureus* and *S. enterica* were completely inactivated within 3 hours of hyperbaric

20 storage. Prolonged hyperbaric storage promoted slight egg white yellowing, probably due to non-

21 enzymatic browning or riboflavin-protein decomplexation, and induced minor changes in egg

22 white proteins structure. Partial conversion of ovalbumin into S-ovalbumin lead to slightly

23 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

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

28

## 29 **Industrial relevance**

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

35

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

38

## 39 **1 Introduction**

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

47 has the undoubted advantage of much lower energy cost. Energy is only required for  
48 pressurization, while pressure maintenance during storage is guaranteed by vessel sealing solely  
49 (Bermejo-Prada, Colmant, Otero, & Guignon, 2017; Freitas et al., 2016; Santos et al., 2020).  
50 Additional energy might be required only when, depending on the desired application, storage  
51 temperature needs to be controlled. HS units can actually work in a wide temperature range (-  
52 20/40°C) by implementing thermal insulation of the vessel. To this regard, the technology is  
53 mentioned as HS-RT if pressure is applied at room temperature with no specific control, or HS-  
54 LT when pressure is combined with low temperature to assist food refrigeration or freezing.  
55 During the last few years, HS has sparked substantial interest to maintain safety and hygienic  
56 properties during storage of many fresh foods, such as meat, fish and fruit juices (Fidalgo et al.,  
57 2019; Lemos, Ribeiro, Delgadillo, & Saraiva, 2020; Santos, Castro, Delgadillo, & Saraiva, 2019).  
58 For instance, strawberry juice stored at room temperature at 100 MPa for 15 days presented 5 and  
59 3.8 log reductions in total bacteria count and yeasts and molds, respectively. In the case of fresh  
60 beef meat, the application of 75 MPa at 25 °C for 14 days promoted a 3 log unit-reduction in  
61 inoculated *L. innocua* and *E. coli* (Santos et al., 2019). Interestingly, HS performed at 100 MPa  
62 for 20 days at room temperature was also successful at inactivating endospores (4.5 log reductions)  
63 of *B. subtilis* in carrot juice (Pinto, Santos, Fidalgo, Delgadillo, & Saraiva, 2018). Despite  
64 promoting extensive microbial inactivation, with no recovery in microbial activity even after  
65 months under pressure, HS did not promote significant changes in food physical and sensory  
66 properties. To this regard, Lemos et al., (2020) actually reported that watermelon juice stored for  
67 one year at 75 MPa only presented a slightly faded color. In the case of protein rich foods, such as  
68 meat and fish, denaturation of myofibrillar and sarcoplasmic proteins was detected, resulting in  
69 minor changes of techno-functional properties such as water-holding capacity and texture.

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

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

84

## 85 **2 Materials and Methods**

86

### 87 **2.2 Sample preparation**

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



92 <sup>2</sup> · 24 h<sup>-1</sup>; Niederwieser Group S.p.A., Campogalliano, Italy), which were heat-sealed with  
93 headspace not exceeding 5 % of samples volume (Orved, VM-16, Musile di Piave, Italy).  
94 Egg white samples for microbiological analyses were prepared separately. Egg shells were cleaned  
95 with hydroalcoholic solution (ethanol 70%) and allowed to air dry for a few minutes before aseptic  
96 breaking. The egg white was manually separated from the yolk and chalazae under sterile  
97 conditions, and collected in a sterilized beaker. For the inoculum, a bacteria suspension was  
98 prepared using strains of *Salmonella enterica* subsp. *enterica* 9898 DSMZ and *Staphylococcus*  
99 *aureus*, obtained from the bacterial culture collection of the Department of Agricultural, Food,  
100 Animal and Environmental Sciences of the University of Udine (Italy). Strains were maintained at  
101 -80 °C in Brain Heart Infusion broth (BHI, Oxoid, Milan, Italy) with 30% sterile glycerol as  
102 cryoprotectant until use. Strains were incubated in BHI at 37 °C for 24 h, subsequently cultured in  
103 5 mL of BHI at 37 °C for 24 h, and finally collected by centrifugation at 14,170 × g for 10 min at  
104 4 °C (Beckman, Avanti TM J-25, Palo Alto, CA, USA) and washed three times with Maximum  
105 Recovery Diluent (MRD, Oxoid, Milan, Italy). The final pellets were suspended in MRD. An  
106 adequate aliquot of the bacteria suspension was added to the egg white to obtain a final  
107 concentration of 10<sup>3-4</sup> CFU g<sup>-1</sup>. The inoculated egg white was distributed in 50 g aliquots and  
108 packaged as for the other samples.

109

### 110 **2.3 Hyperbaric storage**

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

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

126

#### 127 **2.4 Microbial analyses**

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

132 Preliminary trials were carried out on non-inoculated egg white samples to check the *S. enterica*  
133 or *S. aureus* presence. For *S. aureus*, 20 g of egg white was diluted 1:5 v/v in MRD and 0.1 mL  
134 aliquots of appropriate dilutions were plated onto Baird Parker agar (BP, Oxoid, Milan, Italy),  
135 incubated at 37 °C for 24 h. For *S. enterica*, 25 g of non-inoculated egg white were diluted with  
136 225 mL of Buffered Peptone Water (BPW, Oxoid, Milan, Italy), homogenized in a Stomacher for  
137 2 min at 37 °C for 24 h. A volume of 0.1 mL of BPW was added with 9.9 mL Rappaport Vassiliadis

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

141

## 142 **2.5 Colour**

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

147

## 148 **2.6 Absorbance**

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

153

## 154 **2.7 Free sulfhydryl groups**

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

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

$$165 \quad SH = \frac{73.53 \cdot A \cdot D}{C} \quad (\text{Eq.1})$$

166  
167 where  $A$  is the absorbance;  $C$  is egg white concentration ( $\text{mg mL}^{-1}$ );  $D$  is the dilution factor; and  
168 73.53 is derived from  $\frac{10^6}{1.36 \cdot 10^4}$ ;  $1.36 \cdot 10^4$  is the molar absorptivity (Ellman, 1959).

169

## 170 **2.8 Differential scanning calorimetry**

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

179

## 180 **2.9 Fourier transform infrared spectroscopy**

181 Fourier transform infrared spectroscopy (FT-IR) analysis was performed at  $25 \pm 1$   $^{\circ}\text{C}$  on freeze-  
182 dried samples by using an Alpha-P (Bruker Optics, Milan, Italy) instrument equipped with an  
183 attenuated total reflection accessory and a Zn-Se crystal, as previously described by Melchior,

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

191

## 192 **2.10 Particle size and Z-potential**

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

201

## 202 **2.11 Apparent viscosity**

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

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

208

## 209 **2.12 Gelling properties**

210 Aliquots of 50 mL of sample were heated at  $90 \text{ }^\circ\text{C}$  for 15 min in 50 mL-capacity sealed plastic  
211 Falcon tubes. Samples were then rapidly cooled in ice and stored at  $4 \text{ }^\circ\text{C}$  for 12 h. The gelled  
212 samples were extracted from the Falcon tubes and manually cut by a sharp knife to obtain  $1.5 \pm$   
213  $0.1 \text{ mm}$  thick slices. Mechanical spectra of the heat-set gels were obtained using a RS6000  
214 Rheometer equipped with a parallel plates geometry having 40 mm diameter and 1 mm gap. To  
215 determine samples linear visco-elastic stress domain, stress sweep analysis was performed by  
216 increasing the applied stress from 1 to 200 Pa at 1 Hz frequency. Frequency sweep analysis was  
217 performed by increasing oscillatory frequency from 0.1 to 16 Hz, applying a stress within the linear  
218 visco-elastic stress domain. The gelling capacity was expressed as the elastic modulus of the gelled  
219 sample at a frequency of 1 Hz.

220

## 221 **2.13 Foaming properties**

222 Foaming properties were determined by adapting the method from Melchior et al. (2020). Briefly,  
223 10 mL of sample was diluted 1:10 (w/w) with MilliQ water and homogenized (Polytron DI 25  
224 basic, IKA Werke GmbH & Co., Germany) for 3 min at 9,500 rpm in a graduated cylinder. The  
225 total volume of the foamed samples was measured after 0 and 15 min. Foaming capacity and foam  
226 stability were calculated as follows:

227

$$228 \text{ Foaming capacity (\%)} = \frac{V_0 - V_i}{V_i} \cdot 100 \quad (\text{Eq.2})$$

229 
$$\text{Foaming stability (\%)} = \frac{V_{15} - V_0}{V_0} \cdot 100 \quad (\text{Eq.3})$$

230

231 Where  $V_0$  (mL) is the sample volume after homogenization,  $V_i$  (mL) is the initial sample volume  
232 (10 mL) and  $V_{15}$  (mL) is the sample volume after 15 min from homogenization.

233

## 234 **2.14 Statistical analysis**

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

242

## 243 **3 Results and Discussion**

244

### 245 **3.1 Hygienic properties**

246 Preliminary microbial analyses were carried out on non-inoculated egg white to ensure the absence  
247 of *Salmonella* and to quantify the naturally occurring *S. aureus* load, which resulted to be always  
248 below the detection limit ( $1.7 \log \text{CFU g}^{-1}$ ). Egg white was then inoculated with *S. enterica* ( $4.05$   
249  $\pm 0.35 \log \text{CFU g}^{-1}$ ) and *S. aureus* ( $3.96 \pm 0.20 \log \text{CFU g}^{-1}$ ) and the evolution of the counts of  
250 these bacteria were followed throughout storage under hyperbaric and refrigerated conditions over  
251 28 days (Table 1).

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

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



275 (as *Salmonella* spp.). This is known to be due to the presence of a thick peptidoglycan layer in the  
276 cell wall of gram-positive bacteria (Wuytack, Diels, & Michiels, 2002).  
277 These results indicate that hyperbaric storage at 200 MPa allows an efficient performance on  
278 microbial growth inhibition and inactivation for both *S. enterica* and *S. aureus* in egg white. In  
279 particular, 1 day of storage seems sufficient to achieve a satisfactory level of inactivation of these  
280 microorganisms.

281

### 282 **3.2 Physical and structural properties**

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

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

294 Although being mainly constituted by proteins, egg white also contains reducing sugars, which  
295 could make it particularly prone to non-enzymatic browning reactions (Sisak, Csanádi, Rónay, &  
296 Szajáni, 2006). Literature actually reports the early steps of non-enzymatic browning reactions to  
297 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<sup>-1</sup>,  
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<sup>-1</sup>) clearly showed  
311 the presence of three protein components. Peaks identified at 1630, 1654 and 1684 cm<sup>-1</sup> were  
312 associated to low-frequency  $\beta$ -sheet,  $\alpha$ -helix highly overlapped to random coil and high-frequency  
313  $\beta$ -sheet structures, respectively (Uygun-Sarıbay, Ergun, Kalaycı, & Köseoğlu, 2017).

314 Data relevant to refrigerated egg white showed the occurrence of minor fluctuations in the  $\alpha$ -  
315 Helix/random coil domain (Table 3). In the pressurized samples, only a slight increase in the  
316 average value of the percentage of  $\alpha$ -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 than 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.,

321 2013; Smith, Fiebig, Schwalbe, & Dobson, 1996). To better study structural changes leading to  
322 protein denaturation, egg white samples were also analyzed for absorbance at 280, particle size  
323 and Z-potential (Table 4).

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

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

346

### 347 **3.3 Techno-functional properties**

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

351 No changes in these properties were detected in egg white stored under refrigeration. By contrast,  
352 pressurized egg white presented a remarkable increase in apparent viscosity after 14 days storage.

353 The higher viscosity of pressurized egg white is consistent with protein structural changes  
354 previously described (Table 4). Even if more compact, water swollen proteins with higher surface  
355 charge would better interact with the solvent, preventing free flowing of the aqueous media in a  
356 more efficient way as compared to native ones. Actually, a good correlation ( $r = 0.93$ ;  $p < 0.05$ )  
357 was found between particle size and apparent viscosity. Based on the better interaction with water,  
358 pressurized proteins would be less prone to interparticle interactions. To this regard, it is  
359 noteworthy that a slight decrease in gelling capacity of egg white was observed. In order to better  
360 investigate the mechanism at the basis of this change, specific information was obtained by  
361 differential scanning calorimetry analysis. The thermograms relevant to egg white stored for  
362 increasing time under hyperbaric condition are shown as examples in Figure 2.

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

367 upon high hydrostatic pressure (Rivalain, Roquain, & Demazeau, 2010; Singh & Ramaswamy,  
368 2015; Van der Plancken, Van Loey, & Hendrickx, 2005). Accordingly, the intensity of this  
369 phenomenon progressively decreased during hyperbaric storage. A second complex transition in  
370 the temperature range 75-87 °C was attributed to ovalbumin, whose native form is characterised  
371 by a denaturation temperature of *circa* 80 °C. The ovalbumin double peak shape revealed the  
372 presence of an intermediate ovalbumin form showing peak temperature at about 85 °C (De Groot  
373 & De Jongh, 2003). During hyperbaric storage, the thermal phenomena associated to the  
374 denaturation of ovalbumin native fraction progressively decreased with the increase of the  
375 intermediate form of ovalbumin and the appearance of a novel shoulder at temperatures above 89  
376 °C. The latter was attributed to S-ovalbumin. Spontaneous ovalbumin conversion into S-  
377 ovalbumin is due to an irreversible multi-step process, involving L-D isomerization of Ser-164,  
378 Ser-236 and Ser-320, as well as distancing motion of 1A and 2A strands and burying of residues  
379 surrounding Phe-99 (Yamasaki, Takahashi, & Hirose, 2003). To get a quantitative information  
380 about the effect of storage conditions on the shift of ovalbumin to S-ovalbumin, enthalpy values  
381 of this thermal phenomenon were computed (Figure 3). Analogous data were also acquired for egg  
382 white stored under refrigerated conditions or maintained in shell at room temperature.

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

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

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

408

#### 409 **4 Conclusions**

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

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

422

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426

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428

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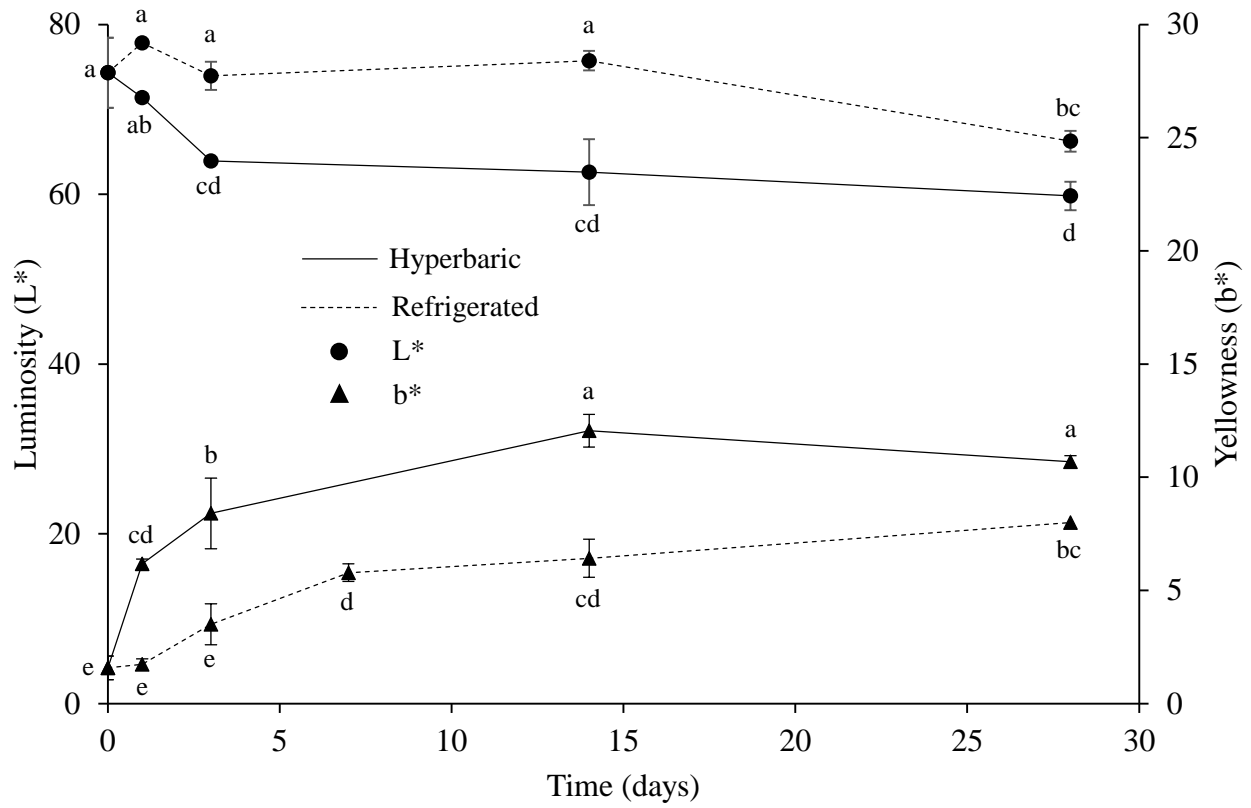
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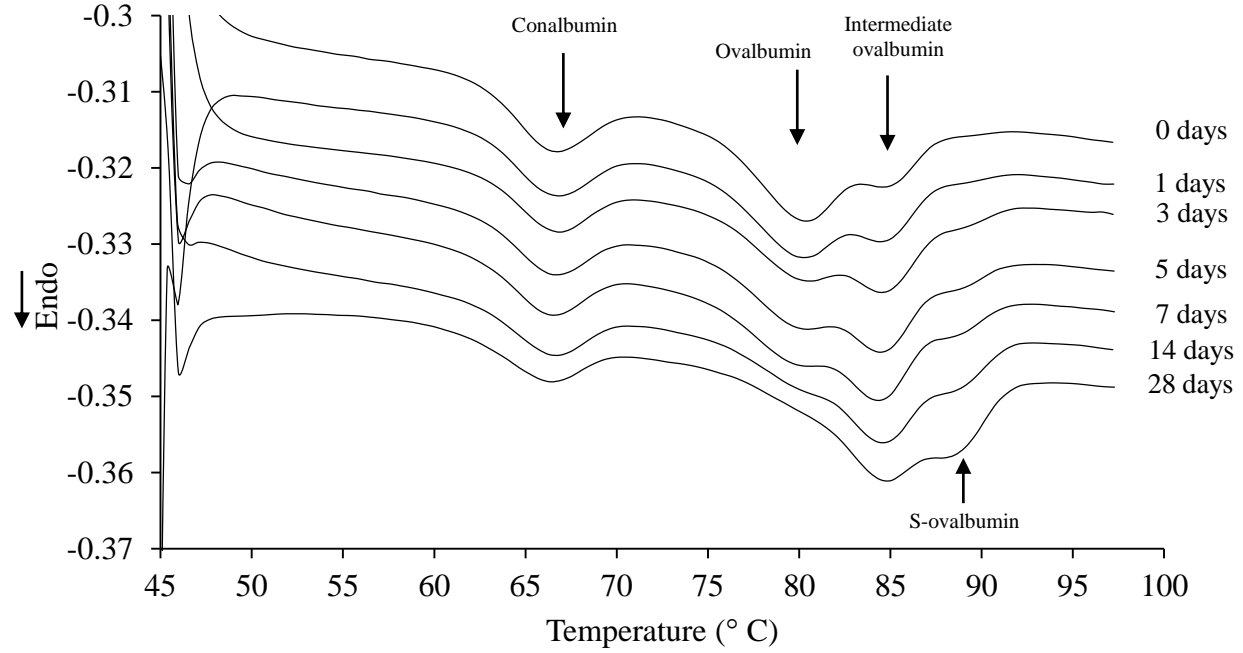
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1

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

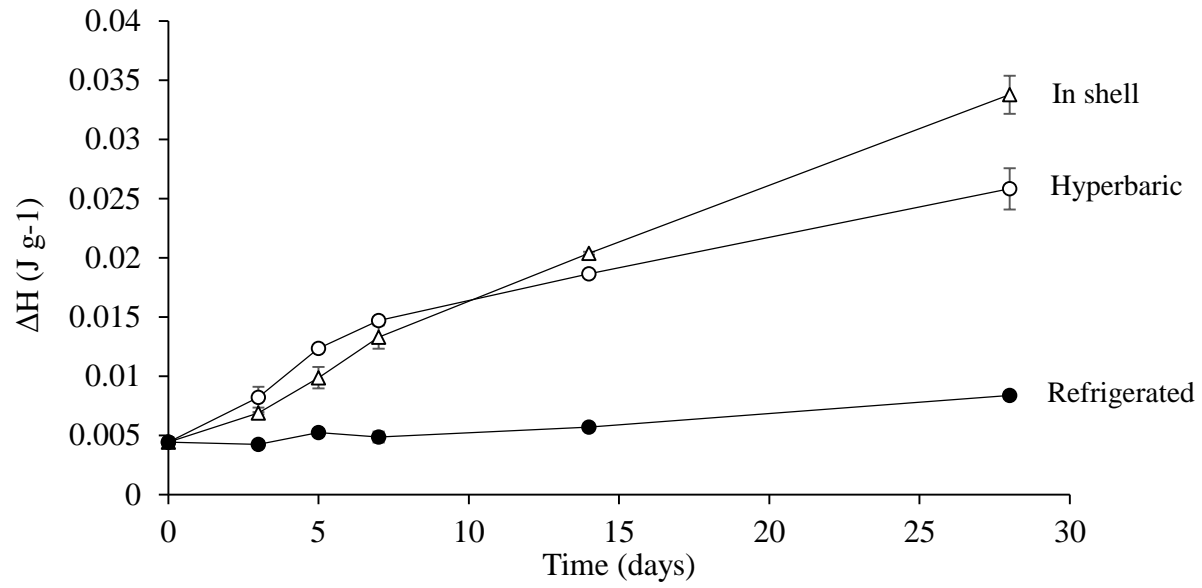
3 hyperbaric conditions.



1

2 Figure 2: Differential scanning calorimetry thermograms of egg white stored for increasing time

3 under hyperbaric conditions.



1

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.



- 1 Table 1: *S. enterica* and *S. aureus* counts (log CFU g<sup>-1</sup>) in egg white stored for up to 28 days under  
 2 refrigerated or hyperbaric conditions.

Time (days)	<i>S. enterica</i>		<i>S. aureus</i>	
	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.

3 N.D. Not determined

4 L.o.D. 1.7 log CFU g<sup>-1</sup>

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

Time (hours)	<i>S. enterica</i>	<i>S. aureus</i>
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.

3 L.o.D. 1.7 log CFU g<sup>-1</sup>

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

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

3 a, b, c: Different letters in the same column indicate significantly different means (ANOVA;  $p <$   
 4 0.05).

- 1 Table 4: Absorbance at 680 and 280 nm, particle size and Z-potential of egg white stored for  
 2 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 <sup>b</sup>	0.376 ± 0.005 <sup>b</sup>	224.65 ± 4.97 <sup>a</sup>	-12.25 ± 0.78 <sup>a</sup>
Refrigerated	14	0.018 ± 0.003 <sup>bc</sup>	0.391 ± 0.009 <sup>b</sup>	226.63 ± 11.71 <sup>a</sup>	-12.48 ± 1.02 <sup>a</sup>
	28	0.016 ± 0.001 <sup>c</sup>	0.410 ± 0.009 <sup>a</sup>	225.50 ± 11.47 <sup>a</sup>	-12.14 ± 0.24 <sup>a</sup>
Hyperbaric	14	0.050 ± 0.005 <sup>a</sup>	0.382 ± 0.010 <sup>b</sup>	198.29 ± 4.20 <sup>b</sup>	-15.95 ± 0.53 <sup>b</sup>
	28	0.046 ± 0.005 <sup>a</sup>	0.377 ± 0.006 <sup>b</sup>	192.78 ± 5.26 <sup>b</sup>	-15.15 ± 0.91 <sup>b</sup>

- 3 a, b, c: Different letters in the same column indicate significantly different means (ANOVA; p <  
 4 0.05).

- 1 Table 5: Apparent viscosity, gel elastic modulus ( $G'$ ), foaming capacity and foaming stability of  
 2 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	$0.078 \pm 0.038^b$	$5.95 \pm 0.63^{ab}$	$63.3 \pm 15.3^c$	$93.5 \pm 6.7^a$
Refrigerated	5	$0.050 \pm 0.023^b$	$7.20 \pm 0.22^a$	$90.0 \pm 10.0^{bc}$	$96.5 \pm 3.1^a$
	14	$0.058 \pm 0.032^b$	-	$86.7 \pm 14.1^{bc}$	$91.2 \pm 2.5^a$
	28	$0.014 \pm 0.002^b$	$6.57 \pm 0.35^a$	$66.7 \pm 15.3^c$	$93.9 \pm 5.9^a$
Hyperbaric	5	$0.120 \pm 0.071^b$	$5.80 \pm 0.13^{ab}$	$133.3 \pm 11.5^a$	$91.5 \pm 4.0^a$
	14	$0.421 \pm 0.029^a$	-	$113.3 \pm 5.8^{ab}$	$89.6 \pm 5.4^a$
	28	$0.318 \pm 0.042^a$	$4.41 \pm 0.32^b$	$100.0 \pm 10.0^{ab}$	$96.7 \pm 2.8^a$

- 3 a, b, c: Different letters in the same column indicate significantly different means (ANOVA;  $p <$   
 4 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.