DISAFA

FONDAZIONE Cassa di Risparmio di Asti

UniASTISS Polo Universitario Rita Levi-Montalcini

UNIVERSITÀ DI TORINO

PROCEEDINGS OF THE

26TH WORKSHOP ON THE DEVELOPMENTS IN THE ITALIAN PHD RESEARCH ON FOOD SCIENCE TECHNOLOGY AND BIOTECHNOLOGY

ASTI

19TH - 20TH - 21ST SEPT 2022

UniASTISS

Polo Universitario Asti Studi Superiori

"Rita Levi Montalcini"

UNIVERSITÀ DEGLI STUDI DI TORINO

SISTAL Società Italiana di Scienze e Tecnologie Alimentari

SIMTREA

SISS

Proceedings of the 26th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology

Università degli Studi di Torino – Asti (Italy), 19th-21st September 2022

Abstract: This book collects the conference proceedings of the *26th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology*, held at the *UniASTISS Polo Universitario Asti Studi Superiori "Rita Levi Montalcini"* from 19th to 21st September 2022. The goal of the conference is to gather PhD students from all Italian universities of whom projects deal with food-related topics to define the state of the art of the Italian academic research in this area of study.

Keywords: Food science, Food technology, Microbiology, Biotechnology, Italian PhD Research, PhDFood 2022.

Cover: Daniela Ghirardello

Cover background photo: beysim - stock.adobe.com

UNIVERSITÀ
DI TORINO

Collane@unito.it

Università degli Studi di Torino

ISBN: 9788875902278

Hyperbaric storage: An innovative and sustainable technology to extend stability and improve functionality of food

Federico Basso (basso.federico.1@spes.uniud.it)
Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Via Sondrio 2/A, 33100 Udine, Italy
Tutors: Prof. Lara Manzocco, Prof. Maria Cristina Nicoli

This PhD thesis consisted of a multi-aspect investigation on hyperbaric storage at room temperature, which is an innovative approach to sustainable food preservation. In particular, the following aspects of the technology were considered: i) the capability of guaranteeing food hygiene and microbiological safety; ii) the possibility to steer the functionality of industrially relevant food proteins; iii) the effect on the kinetics and mechanism of food alterative phenomena (enzymatic and non-enzymatic browning); iv) the effect on food packaging materials.

Hyperbaric storage: Una tecnologia innovativa e sostenibile per estendere la stabilità e migliorare la funzionalità degli alimenti

Questa tesi di dottorato consiste in una ricerca multi-aspetto sull'*hyperbaric storage* a temperatura ambiente. Tale tecnologia rappresenta un approccio innovativo per la conservazione sostenibile degli alimenti. In particolare, sono stati considerati i seguenti aspetti della tecnologia: i) la capacità di garantire l'igiene e la sicurezza microbiologica degli alimenti; ii) la possibilità di orientare la funzionalità tecnologica di proteine oggetto di interesse nell'industria alimentare; iii) l'effetto sulla cinetica e sul meccanismo di sviluppo di fenomeni alterativi (*i.e.*, imbrunimento enzimatico e non-enzimatico); iv) l'effetto sui materiali per il confezionamento.

Key words: Hyperbaric storage, food hygiene and safety, protein structure and functionality, enzymatic activity

1. Introduction

Food storage under pressure has been studied during the last decade as a sustainable substitute for refrigeration. The technology, also known as hyperbaric storage (HS), consists of applying moderate hydrostatic pressure ($P < 250$ MPa) to foods packaged into plastic pouches and inserted inside steel autoclaves (Santos *et al.*, 2020). As compared to refrigeration, the main advantage of HS is the capability to inhibit microbial growth in foods at a fraction of the energetic cost of cold storage (Bermejo-Prada *et al.*, 2017; Jannasch *et al.*, 1971). Recently, an increasing amount of evidence has surfaced, indicating that HS not only prevents growth of bacteria, but also induces substantial microbial inactivation (3-5 log reductions of Gram -, Gram + and sporogenic bacteria) and changes in structural and functional properties of food biomolecules (*i.e.*, proteins, lipids, enzymes, polysaccharides) (Santos *et al.*, 2020). Although limited to safety and quality aspects solely, this basis of evidence allows to expand the scope of HS beyond that of sustainable food storage. HS could represent a multi-tasking technology, capable to concomitantly guarantee: (i) food pasteurization *via* microbial inactivation, (ii) functionality improvement *via* biomolecules structure modification, and (iii) food preservation *via* pressure-induced control of the kinetics of chemical and enzymatic alterations. Nevertheless, the technology has not been investigated from such standpoints yet. Moreover, the technical aspects of HS (*e.g.*, equipment design, feasibility of plastic packaging materials) still require adequate assessment before the technology can bridge the gap between research laboratories and the industrial context.

This Ph.D. thesis was intended as a multi-aspect investigation on hyperbaric storage. In particular, the technology was assessed for its multi-tasking character based on its capability to inactivate microorganisms, enhance food functionality, and control enzymatic and non-enzymatic browning. Attention was particularly paid to the influence of the native structural organization of proteins on the outcome of HS application, and on the relationship between protein structural changes and functionality. The same research approach was also adopted to study the effect of HS on food packaging materials.

In this presentation, the main results inherent to the effect of HS on food microbiological safety, protein functionality and enzymatic browning are critically overviewed.

2. Materials and Methods

2.1 Samples preparation and hyperbaric storage

Fresh hen (*Gallus gallus domesticus*) eggs and "Golden delicious" apples were purchased at a local retailer. Raw skim milk was obtained at a local dairy processing plant. Egg white and egg yolk were manually separated and homogenized by gentle stirring to obtain the samples. Apple juice samples were prepared according to Manzocco *et al.* (2013) with minimal modifications. Polyphenoloxidase (PPO) model solutions were prepared by dissolving mushroom tyrosinase (5771 U/mg, Sigma Aldrich, Milan, Italy) in 0.01, 0.05 and 0.1 M pH 4.5 sodium acetate and pH 7 potassium phosphate buffers. Samples were heat-sealed into polypropylene/ethylene-vinyl-alcohol/polyethylene pouches (Niederwieser Group S.p.A., Campogalliano, Italy) and subjected to HS in a pilot-scale working unit assembled by Comer Srl. (Bologna, Italy). The latter consisted of a water-tight steel autoclave (Hystat, Slaithwaite, Huddersfield, United Kingdom), filled with an aqueous solution containing 0.2% (w/w) potassium sorbate and 0.2% (w/w) sodium benzoate (Carlo Erba Reagents Srl, Milan, Italy), and pressurized by a Haskel International high-pressure pump (Burbank, CA, USA). Control samples were maintained under refrigerated (4 °C) or room temperature conditions (20 ± 1 °C) at atmospheric pressure (0.1 MPa).

2.2 Microbial counts

Total bacteria count (TBC) was evaluated by enumeration on Plate Count Agar (Oxoid, Milan, Italy) after incubation at 30 ± 1 °C for 48-72 h. *Salmonella enterica* subsp. *enterica* 9898 DSMZ, *S. aureus* 226 and *E. coli* 8048 were inoculated in egg white, egg yolk and raw skim milk and counted during HS. Counts of *Salmonella enterica* and *S. aureus* were performed after incubation at 37 ± 1 °C for 24-48 h onto Plate Count Agar (Oxoid). *E. coli* was counted on ColiID (bio-Merieux, Grassina, Italia) after incubation at 37 ± 1 °C for 24 h.

2.3 Physical properties, protein structure and technological functionality

Samples physical properties and protein structure was evaluated by determination of colour (tristimulus colorimeter), thermal properties (differential scanning calorimetry), infrared absorbance spectrum (FT-IR), particle size and zeta potential (dynamic light scattering). Samples protein fractions were further analysed for their structure by quantification of free SH groups, determination of absorbance at 280, 380 and 680 nm, and reverse phase-high performance liquid chromatography (RP-HPLC). Samples technological functionality was assessed by evaluating viscosity, solubility, and foaming, gelling, and emulsifying properties. Analyses were performed as described by Basso *et al.* (2021, 2022).

2.4 Enzymatic activity

PPO activity was assessed as described by Manzocco *et al.* (2013). The enzymatic unit (U) was defined as the amount of enzyme leading to 0.001/min increase in absorbance (420 nm) by adding 20 µL sample in 1980 µL of $1.5 \cdot 10^{-3}$ M L-Dopa (Sigma Aldrich).

2.5 Data analysis

Microbiological and RP-HPLC analyses were performed in single on two independent experiments and in duplicate, respectively. Other data were obtained by at least triplicate measurements. Data were reported as mean ± standard deviation and subjected to one-way analysis of variance (ANOVA) and Tukey's Honest Significant Differences test ($p<0.05$) using R for Windows (The R foundation for statistical computing).

3. Results and Discussion

3.1 Antimicrobial efficacy of hyperbaric storage

The efficacy of HS in guaranteeing food microbiological quality and safety was evaluated in egg white, egg yolk, apple juice, and raw skim milk (Table 1).

Table 1 *Time required for HS inactivation (t_i) below the detection limit (L.o.D.) of total bacteria count (TBC) and inoculated microorganisms in egg white, egg yolk, apple juice, and raw skim milk.*

Sample	Hyperbaric storage P (MPa)	TBC* Initial load	t_i (h)	*E. coli*** Initial load	t_i (h)	*S. aureus** Initial load	t_i (h)	*S. enterica** Initial load	t_i (h)
Egg white	200	N.a.	N.a.	N.a.	N.a.	3.96 ± 0.20	24	3.50 ± 0.07	3
Egg yolk	200	N.a.	N.a.	N.a.	N.a.	2.78 ± 0.19	48	3.35 ± 0.12	24
Apple juice	200	1.70 ± 0.05	24	N.a.	N.a.	N.a.	N.a.	N.a.	N.a.
Skim milk	150	3.89 ± 0.16	144	5.13 ± 0.33	48	5.66 ± 0.93	144	N.a.	N.a.

P: pressure; Initial load expressed as logCFU mL^{-1}; *L.o.D. = 1 logCFU g^{-1}; **L.o.D. = 0 logCFU g^{-1}; N.a. Not analyzed.

Egg white and yolk, which are sterile in normal conditions, were inoculated with *Salmonella enterica* and *S. aureus* (3-4 logCFU mL^{-1}) to simulate a process-related contamination. HS promoted the inactivation of both bacteria below the detection limit within few days (Table 1). In particular, *Salmonella enterica* was inactivated after just 3 hours at 200 MPa in egg white, whereas it took up to 1 day to reach the same effect in egg yolk. The higher

microbial resistance to pressurized conditions in egg yolk as compared to egg white was probably due to its richer composition, which probably exerted a protective effect on bacterial cells. As compared to *Salmonella enterica* (Gram -), *S. aureus* (Gram +) showed a far higher pressure resistance, which was most definitely due to its thick peptidoglycan layer.
The naturally present microflora of both apple juice and raw skim milk (*i.e.*, TBC) was completely inactivated upon HS, indicating the capability of the technology to guarantee optimal hygienic conditions regardless of food pH and microbial susceptibility (Table 1). The possibility of using HS as a novel approach for food pasteurization was also investigated in raw skim milk inoculated with *E. coli* and *S. aureus* (5-6 logCFU mL^{-1}) (Basso *et al.*, 2022). In this case, 150 MPa-HS completely inactivated *E. coli* and *S. aureus* below the detection limit after 2 and 6 days, respectively. This inactivation resulted to be irreversible, as demonstrated by no microbial recovery during 12 days of milk storage under refrigerated conditions (data not shown).
Results indicated that HS could be efficaciously used not only as a sustainable alternative to refrigeration, but also as an innovative and *quasi*-energy-free approach to achieve food decontamination and pasteurization.

3.2 Effect of hyperbaric storage on proteins

Based on the promising antimicrobial efficacy of HS, the possibility to apply the technology to steer the functional properties of egg white, egg yolk and raw skim milk was further investigated. Results allowed to summarize the main effects of HS on both protein structure and technological functionality of the tested foods (Table 2).

Table 2 *HS effects on the physical, structural and functional properties of egg white, egg yolk and raw skim milk.*

Matrix	Protein	Structure	Hyperbaric storage Pressure (MPa)	Time (days)	Structural effects	Functional effec	References
Egg white	Ovalbumin	Globular	200	28	Compression and electrical stabilization	Viscosity and foaming increase	Basso *et al.*, 2021
Egg Yolk	Apolipoprotein	Lipoproteins	200	28	Unfolding and swelling	Pressure-induced gelling	Basso *et al.*, *under review*
Skim milk	Casein and whey protein	Micellar and globular	150, 200	6	Casein-globulin complexation	Enhanced foaming capacity	Basso *et al.*, 2022

Egg white globular proteins (*e.g.*, conalbumin, ovalbumin) showed slight structural modification during pressurized storage, leading to an increase in viscosity and foaming properties. On the other hand, egg yolk proteins, which are primarily found embedded in lipoproteins membranes, were severely unfolded under the same pressure conditions, resulting in a fully gelled matrix after 28 days (Table 2) (Basso, 2021).
In the case of milk proteins, which occur either in highly organized micellar structure or as globular solvated proteins, the effects of HS were more complex (Table 3).

Table 3 *Size of micelles and submicelles, β-lactoglobulin concentration, proteose peptones RP-HPLC peak area and foaming capacity of raw skim milk during storage under refrigerated (0.1 MPa, 4 ± 1 °C) and hyperbaric (150 MPa, 20 ± 1 °C) conditions.*

Storage	Time (days)	Micelle size (nm)	Submicelle size (nm)	β-lactoglobulin concentration (g L^{-1})	Proteose peptones peak area (Arbitrary units · 10^7)	Foaming capacity (%)
Fresh	0	169.1 ± 2.6^{d}	N.d.	2.38 ± 0.28	1.40 ± 0.01	72.5 ± 4.4^{d}
Refrigerated	6	N.a.	N.a.	2.22 ± 0.08	2.27 ± 0.03	92.8 ± 5.1^{c}
Hyperbaric	1	237.0 ± 4.0^{c}	N.d.	0.44 ± 0.10	1.70 ± 0.21	119.5 ± 7.9^{b}
	2	275.8 ± 7.9^{b}	52.1 ± 8.0^{a}	0.56 ± 0.00	3.43 ± 0.20	123.4 ± 8.5^{b}
	5	377.9 ± 11.0^{a}	51.1 ± 2.8^{a}	N.a.	N.a.	N.a.
	6	371.1 ± 8.1^{a}	N.d.	0.11 ± 0.08	4.97 ± 0.08	267.3 ± 15.7^{a}

N.d. Not detected; N.a. Not analyzed; * Different letters in the same column indicate statistically different means ($p<0.05$; ANOVA).

Upon HS at 150 MPa for up to 6 days, milk casein micelles and globular whey proteins were highly destabilized. Dynamic light scattering and RP-HPLC analyses actually highlighted the release of sub-micellar particles (~ 50 nm) and a decrease in β-lactoglobulin content during 150 MPa-HS (Table 3). Consequently, destabilized casein micelles served as local aggregation points for unfolded β-lactoglobulin molecules, leading to a progressive increase in micelles size (Table 3). HS-destabilized casein micelles were also more susceptible to the attack of proteolytic enzymes (*i.e.*, plasmin), as clearly indicated by the enhancement of proteose-peptones, which typically derive from casein enzymatic hydrolysis (Table 3). Due to the extensive unfolding of β-lactoglobulin and enhanced formation of proteose peptones, HS increased the foaming capacity of milk to almost 4-fold that of fresh milk (Table 3). To this regard, it is reasonable that the formation of proteose-peptones was the primary driver of this

improvement, due to their exceptional bubble-stabilizing activity. Actually, a strong positive correlation (r=0.9085) was found between milk foaming capacity and proteose-peptones RP-HPLC peak area.
Results indicate that HS could be regarded as a novel approach to steer functional properties of protein-rich foods, with effects substantially influenced by the native organization of proteins.

3.3 Effect of hyperbaric storage on polyphenoloxidase activity

Based on the capacity of HS to modify protein structure and functionality, HS was further evaluated for its ability to inactivate food-spoiling enzymes. To this aim, PPO was specifically considered based on its well-known browning effect in fresh fruit derivatives viable for HS (*e.g.*, fresh-cut vegetables, fruit juices). The effect of HS at 100 and 200 MPa was firstly studied on PPO activity and color of apple juice, using samples kept under room conditions as a reference (Table 4).

Table 4 *PPO activity, luminosity and redness of apple juice during storage under room (0.1 MPa, 20 ± 1 °C) and hyperbaric (100, 200 MPa, 20 ± 1 °C) conditions.*

Storage	Time (h)	PPO residual activity (%)	Luminosity (L*)	Red point (a*)
Fresh	0	100.00 ± 0.00^{a}	67.12 ± 0.84^{a}	1.13 ± 0.14^{c}
0.1 MPa	24	71.43 ± 4.04^{b}	53.87 ± 1.86^{cd}	2.78 ± 0.53^{bc}
	48	57.14 ± 8.08^{c}	46.14 ± 2.95^{d}	7.00 ± 0.79^{a}
	144	19.05 ± 0.00^{e}	39.29 ± 1.12^{e}	6.39 ± 0.06^{a}
100 MPa	24	73.87 ± 3.12^{b}	N.a.	N.a.
	48	40.54 ± 3.82^{d}	N.a.	N.a.
	144	0.54 ± 2.36^{f}	N.a.	N.a.
200 MPa	5	51.43 ± 0.00^{cd}	62.11 ± 1.38^{b}	2.93 ± 0.35^{b}
	48	5.71 ± 0.00^{f}	53.76 ± 1.45^{cd}	2.49 ± 0.35^{bc}
	144	1.05 ± 1.98^{f}	53.82 ± 0.61^{c}	2.63 ± 0.08^{bc}

N.a. Not analyzed; [a] Different letters in the same column indicate statistically different means ($p<0.05$; ANOVA).

HS allowed for a much faster enzyme inactivation than room pressure storage. In addition, PPO inactivation was pressure-dependent, being significantly faster at 200 than at 100 MPa. As a result, HS allowed to slow down the browning of apple juice, whose change in luminosity (L*) and redness (a*) was significantly lower when stored under pressure (Table 4). Based on these results, HS would not only limit food microbial spoilage, but also prevent the quality decay deriving from enzymatic activity.
The effect of HS was further tested on the activity of mushroom tyrosinase (*i.e.*, PPO) in buffered solutions to understand the mechanism of PPO inactivation under pressure and the influence of environmental conditions. Firstly, the influence of enzyme crowding was tested by storing at 200 MPa solutions containing different amount of PPO (2, 6, 14, 26 U). The effect of HS on PPO activity resulted significant only in the 2 U samples (data not shown). In accordance with the literature, these results indicate that enzyme inactivation probably occurs following a structural unfolding mechanism, which takes place only if enzyme molecules have sufficient solvent volume around them. The influence of the environmental composition was then assessed by pressurizing 2 U PPO solutions at different pH and ionic strength (Figure 1). Results clearly indicate that the effect of pressure was particularly significant at neutral pH (Figure 1 A, B, C) and that the enzyme was substantially more stable at higher ionic strength. These results might be due to the stabilizing effect of acidic pH and salt ions on the molecular structure of PPO, which would make enzyme unfolding more difficult.

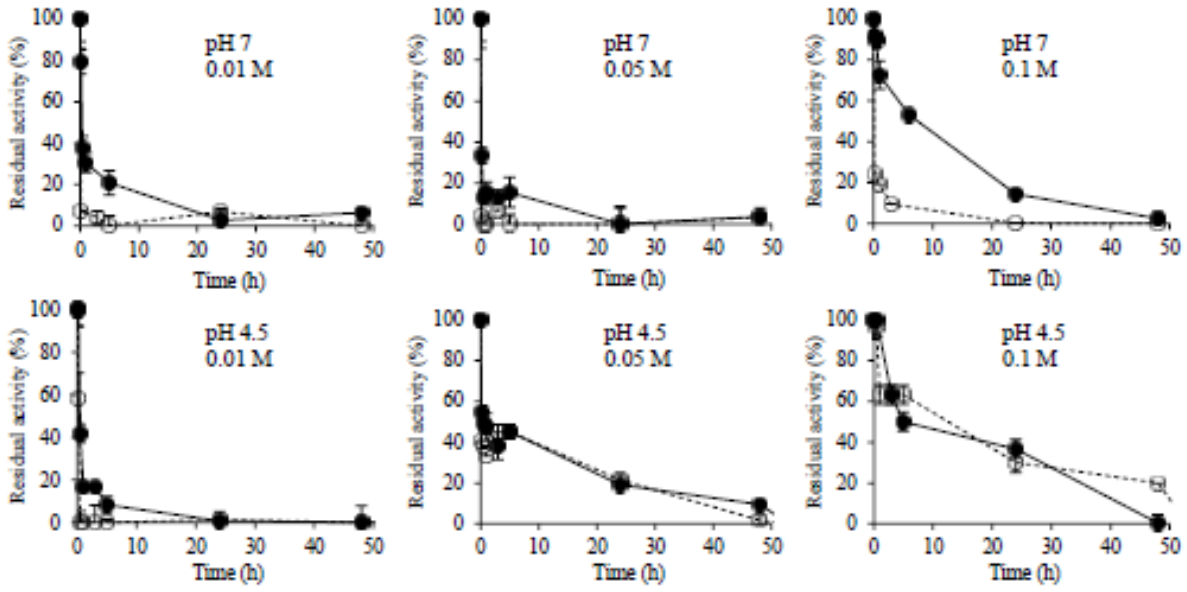

Figure 1 *Mushroom PPO activity at different pH (4.5 and 7) and ionic strength (0.01, 0.05 and 0.1 M) during storage under room (0.1 MPa, 20 ± 1 °C; ●) and hyperbaric (200 MPa, 20 ± 1 °C; ○) conditions.*

These results confirm the role of HS-induced unfolding in the loss of PPO activity during pressurized storage as well as the critical role of the electrical landscape of the enzyme.

4. Conclusions and future perspectives

The results acquired with this Ph.D. thesis clearly indicate that HS represents a promising innovative non-thermal food technology. Besides its sustainability, HS has a multi-purpose character, based on its concomitant capability to preserve, pasteurize, functionalize, and control alteration in food. This evidence strongly supports the urge to fulfill the research gaps that currently prevent the widespread application of the technology in an industrial context. To this aim, future research on HS should adopt a multi-disciplinary approach to boost TRL by aligning data achieved at laboratory level with those relevant to pilot and industrial scale. In particular, attention should be especially paid to the design of cost-effective steel or composite autoclaves, and to the development of mathematical models allowing to implement automated HS storage/treatment cycles.

5. References

Basso F (2021) Hyperbaric storage: An innovative and sustainable preservation technology for fresh food ingredients. In Proc.s of the 1st Virtual Workshop on the *Developments in the Italian PhD Research on Food Science, Technology and Biotechnology*, Palermo (Italy), 14-15th September, 2021, pp. 226–227.

Basso F, Maifreni M, Innocente N, Manzocco L, Nicoli MC (2022) Raw milk preservation by hyperbaric storage: Effect on microbial counts, protein structure and technological functionality. *Food Res Int* **156**: Article 111090.

Basso F, Manzocco L, Maifreni M, Alongi M, Nicoli MC (*Under review*) Changes in hygienic, chemical, physical and techno-functional properties of egg yolk during hyperbaric storage. *Food Chem*.

Basso F, Manzocco L, Maifreni M, Nicoli MC (2021) Hyperbaric storage of egg white at room temperature: Effects on hygienic properties, protein structure and technological functionality. *Inn Food Sci Emerg Technol* **74**: Article 102847.

Bermejo-Prada A, Colmant A, Otero L, Guignon B (2017) Industrial viability of the hyperbaric method to store perishable foods at room temperature. *J Food Eng* **193**: 76–85.

Jannasch HW, Eimhjellen K, Wirsen CO, Farmanfarmaian A (1971) Microbial degradation of organic matter in the deep sea. *Sci* **171**: 672–675.

Manzocco L, Panozzo A, Nicoli MC (2013) Inactivation of polyphenoloxidase by pulsed light. *J Food Sci* **78**: 1183-1187.

Santos MD, Fidalgo LG, Pinto CA, Duarte RV, Lemos AT, Delgadillo I, Saraiva JA (2020) Hyperbaric storage at room like temperatures as a possible alternative to refrigeration: Evolution and recent advances. *Crit Rev Food Sci Nut* **61**:1–12.