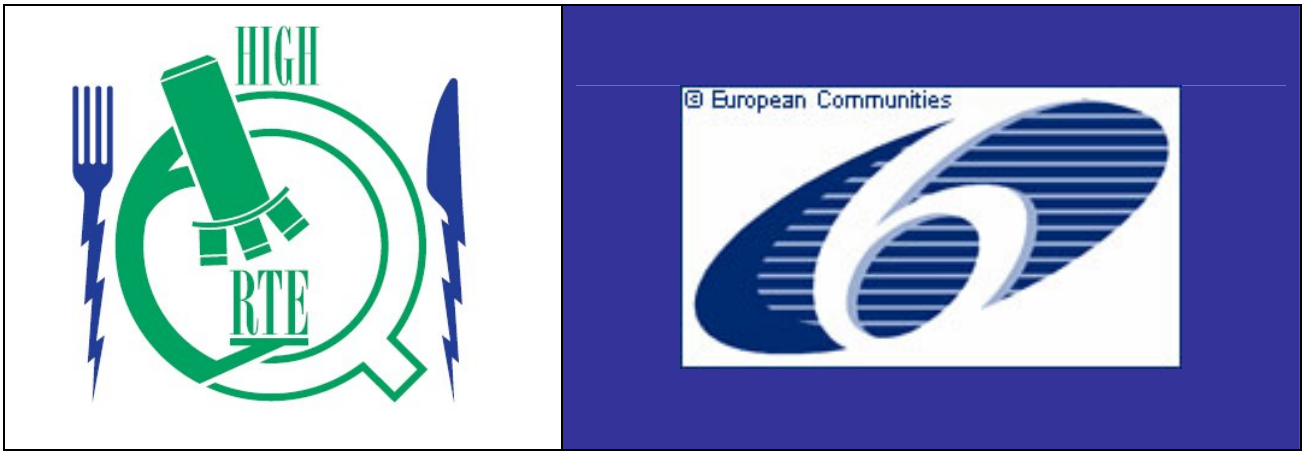

Project No. ***023140***
Project acronym: ***HighQ RTE***
Project full name: ***INNOVATIVE NON THERMAL PROCESSING TECHNOLOGIES TO IMPROVE THE QUALITY AND SAFETY OF READY-TO-EAT (RTE) MEALS***

Instrument type: Specific Targeted Research Projects

Priority name: Food Quality and Safety

Publishable Final Activity Report

Period covered: *01/10/2006* to *30/09/2009* **Date of preparation**: 14/11/2009

Start date of project: *01/10/2006* **Duration**: *36 months*

Project coordinator organisation name: *University of Bologna – Dipartimento di Scienze degli Alimenti*

.

Project coordinator name: Prof. Elisabetta Guerzoni **Revision** [1]

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HighQ RTE
"INNOVATIVE NON THERMAL PROCESSING TECHNOLOGIES TO IMPROVE THE QUALITY AND SAFETY OF READY-TO-EAT (RTE) MEALS"

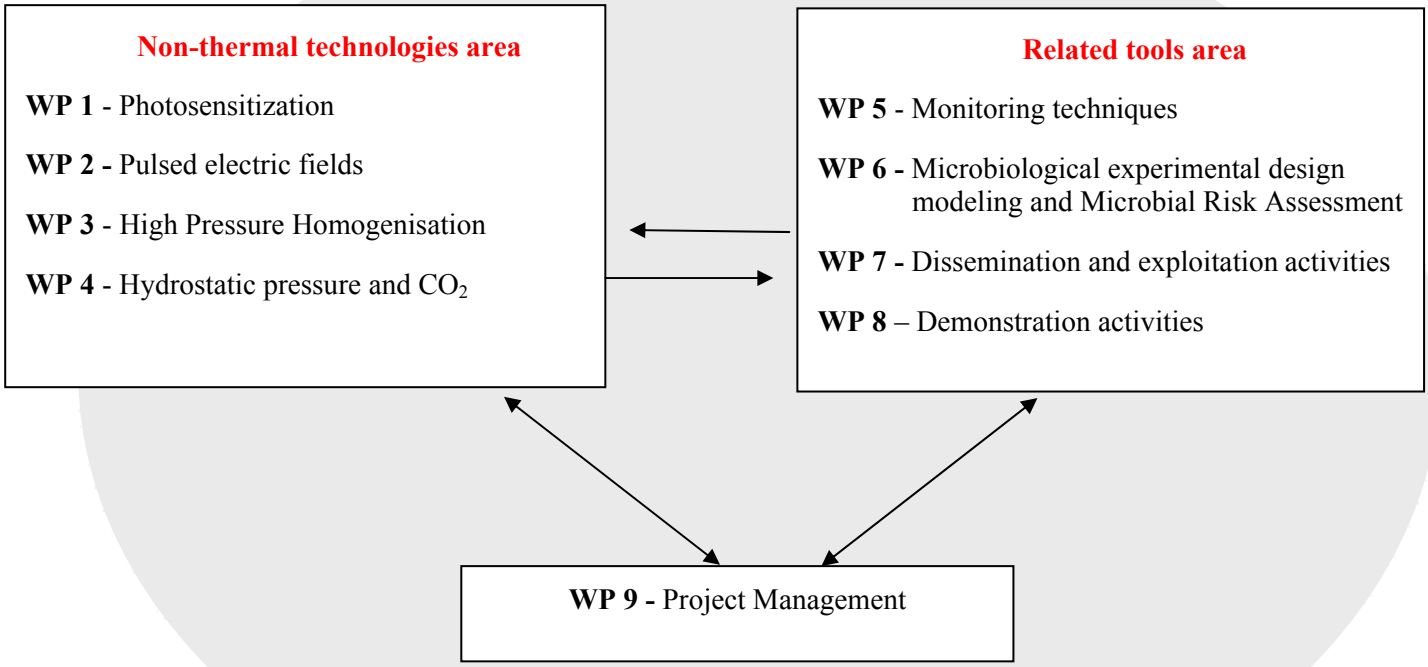
Project objectives

The project **HighQ RTE** aims to improve the safety and quality of three representative categories of European ready-to-eat foods: ready-to-eat salads, fluid foods, and ready-to-eat vegetable based meals.
In fact several papers reported that European ready-to-eat foods, and particularly Refrigerated Processed Foods of Extended Durability (REPFEDs), are frequently contaminated with pathogenic species. In order to enhance the safety of the three categories of foods, and at the same time prevent the heat-induced physicochemical and nutritional changes, non-thermal novel processes will be developed and applied.
For **ready-to-eat vegetable and fruit salads**, procedures based on Photosensitization (**PHOTO**) will be set up in WP1 in order to drastically reduce the contamination level of naturally occurring and inoculated pathogenic species in vegetable raw materials and packaging.
For **fluid foods,** Pulsed Electric Fields technologies (**PEF**) and semi-continuous High Pressure Homogenization (**HPH**) will be compared in WP2 and WP3, respectively both in terms of pathogenic species inactivation, microbiological quality and changes of the properties of proteins as well as food microstructure and rheology. The ability of the latter technology to activate naturally occurring or exogenous enzymes will be exploited in order to generate new bioactive natural food components.
For **ready-to-eat meals** of vegetable origin High Hydrostatic Pressure will be applied to meals packaged under CO_2 atmosphere (**HHPCO**) in comparison and in addition to traditional mild heat processes (WP4).

The optimisation of the formulation of the fluid or solid food systems to be subjected to the various technologies will be performed taking into consideration nutritional aspects (salt, lipid contents), the possible physicochemical and structural changes induced by the processes and the specific requirements of the various process technologies (PHOTO, PEF, HPH, HHPCO) in terms of physical state and rheology (appropriate viscosity, particles presence and size etc). For the various combinations products-processes also the optimisation of the formulation of the meals will be performed in relation to chemical, nutritional, microstructural and functional changes induced by the exposure to the various processes.

For each technology, (i) inactivation due to the different treatments; (ii) re-growth of inoculated pathogens during storage; and (iii) growth of indigenous flora will be modelled. The developed deterministic and probabilistic models will be integrated into quantitative risk assessment procedures in order to determine the parameters of the different treatments necessary to obtain the desired shelf-life and quality level of food with the various technologies.

The activities carried out in the HighQ RTE project, illustrated in the Figure below, are described in the following paragraphs together with a brief outline of the most significant results obtained.



Flowchart of the activities of the HighQ RTE project.

Contractors involved

Organisation name	Short name	Country	Person in charge
University of Bologna - Dipartimento di Scienze degli Alimenti	UNIBO	Italy	M.Elisabetta Guerzoni
CAMST	CAMST	Italy	Pasquale Saracino
Vilnius University - Institute of Material Sciences and Applied Research	VU	Lithuania	ZivileLuksiene
Universitat Politècnica de València	UPV	Spain	Isabel Pérez Munuera
SIK - Institutet for Livsmedel och Bioteknik AB	SIK	Sweden	Elisabeth Borch
Institute of Food Research	IFR	United Kingdom	Jozsef Baranyi
Laboratoire National de Métrologie et d'Essais	LNE	France	Catherine Loriot
Consejo Superior de Investigaciones Científicas	CSIC	Spain	M. Pilar Cano
UAB "Palink"	UAB	Lithuania	Modestas Keblys
Tecnoalimenti S.C.p.A.	TCA	Italy	Marco Gerevini
Ortoreale S.r.l.	ORT	Italy	Paola Massari
Cooperativa Sociale "Il Bettolino" S.C	CSB	Italy	Katia Verzellesi
Rubinetteria Webert S.r.l.	WEB	Italy	Emanuele Zanetta
Acetum s.r.l.	ACE	Italy	Cesare Mazzetti
Centro Tecnico Regionale di Ricerca sul Consumo Europeo	CTRRCE	Italy	Marino Melissano

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Project web-site

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Section 1 - Project Execution

Photosensitization (VU)

1. Objectives

The main objectives were to evaluate suitability of photosensitization as antimicrobial tool to decontaminate surface of raw material (fruits, vegetables) and packaging as well as ready-to-eat salads. It will allow identifying conditions, where PHOTO is useful and suitable for increase of food safety and quality. Specific objectives are:

1. To investigate sensitivity of several food-borne pathogens to photosensitization (*Salmonella* spp., *Escherichia coli*, *Listeria* spp., *Bacillus* spp).
2. To select the most suitable photoactive compounds (photosensitizers, such as 5-aminolevulinic acid, chlorophyll) *in vitro*, which afterwards will be used for decontamination of raw material (fruits, vegetables), packaging surfaces and ready-to-eat salads. To evaluate all experimental conditions (concentration of photosensitizer, incubation time, irradiation conditions etc.)
3. To develop a new technique based on light emitting diodes (LED's), suitable for decontamination of raw material from pathogenic microorganisms throughout the interdisciplinary approach of photobiologists, microbiologists, physicists and engineers.
4. To use photosensitization for decontamination of raw material (fruits, vegetables) and packaging surfaces.

2. Approach

Photosensitization is novel approach to decontaminate food or food related surfaces from food pathogens. For this purpose we had to construct 3 LED-based prototypes. We evaluated the antimicrobial efficiency of photosensitization, starting from the experiments *in vitro*, on the surface of packaging material and on the surface of fruits and vegetables. Antimicrobial efficiency of photosensitization was compared with conventional treatments. Nutritional, organoleptic properties of treated fruits and vegetables were estimated, activity of physiologically important enzymes in treated fruits and vegetables was measured. Experiments were performed in UAB using their facilities and evaluating organoleptic properties of treated by photosensitization ready to eat salads.

3. Materials and Methods

Photosensitizers and Chemicals

For this task we selected pro-drug of endogenous porphyrin-5-aminolevulinic acid (ALA) and Chlorophyll-Na-salt. Stock solutions of photosensitizers were prepared by dissolving them in phosphate-buffered saline (PBS, pH 7.2) to a concentration of 200 mM and NaOH was used to adjust pH of the solution to 7.2. Stock solutions were made instantly before use and sterilized by filtration through 0, 22 μm filter (Roth, Germany). ALA and all other reagents used were of analytical grade.

Pathogens

We investigated viability of *Salmonella enterica Serovar Typhimurium strain DS88, Listeria monocytogenes ATCL3C 7644* and *Bacillus cereus, Listeria monocytogenes 56Ly, Bacillus cereus SV90* after different photosensitization treatments.

Photosensitization procedure. 10 ml aliquots of pathogen suspension in PBS buffer at cell concentration of ~1x10^7 cfu/ml were incubated in dark with an appropriate concentration of ALA in 50 ml volume plastic tubes at 37^{o}C with aeration (175 rpm/min) for different periods (0.5 - 25 h). After incubation, 70 µl aliquots of bacterial suspension were withdrawn, placed into sterile wells of flat-bottomed 96-well plates and exposed to light (λ=400nm) for 30 min at a light dose of 37.5 mW/cm^2. To avoid overheating of the samples during irradiation the plates were kept on ice. In parallel, 70 µl aliquots of the ALA-incubated cell suspensions were kept on ice in dark and used as control samples.

Fluorescence measurements. After incubation with appropriate concentrations of ALA for the indicated periods of time in dark at 37°C 1 ml aliquots of bacterial suspensions (1x10^7 cfu/ml in PBS) were withdrawn, ice-cooled and used for fluorescence measurements. The fluorescence was measured by Hitachi 850 fluorescence spectrophotometer (Japan). The excitation wavelength was 395 nm, the emission was recorded in the range between 590 nm and 700 nm.

Photoinactivation of bacteria after adhesion to the surface of packaging

Packing yellow trays (polyolefine, a mixture of polyethylene/ polypropylene) were provided by LINPAC (West Yorks, United Kingdom). In order to simplify experiments and minimize illumination square, the packaging coupons for photosensitization experiments were cut into 2.5 cm × 4 cm pieces. To adhere the bacteria on the surface, each packaging sample was soaked in 25 mL *L. monocytogenes* suspension (~1x10^7 CFU/mL). The soaked samples were kept in a laminar for 30 min for better attachment of the bacterial cells. Then appropriate packing coupons with the adhered bacteria were incubated in the dark with the 7.5 - 10 mM ALA concentration for 15 min. The control samples were incubated with PBS (7.2 pH) buffer. After incubation with ALA, all packaging samples were dried in a laminar flow hood at room temperature for 20 min. The dried samples were placed in the treatment chamber and exposed to light for 15 min. The control samples were not illuminated.

Photoinactivation of bacterial biofilms on the surface of packaging material

The packaging samples (2.5 cm x 4 cm) were placed in sterile plastic tubes to keep separated from each other. *L. monocytogenes* cell suspension (~1x10^7 CFU/mL) was added until all samples were completely submerged for 3 h at 37°C. Afterwards the cell suspension was removed by aspiration and samples were separately washed three times with sterile PBS to exclude weakly adhered cells. Then the plastic coupons were transferred into plastic tubes containing 25 mL of TSYE medium, with each tube containing one sample. These tubes were incubated at 22.5°C for 48 h for biofilm development. Then samples were washed with sterile PBS three-times positioned in plastic tubes containing 25 mL of 7.5-10 mM ALA solutions, and incubated in the dark for 30 min. The control coupons were incubated with sterile PBS. After incubation with ALA all packaging samples were dried in a laminar at room temperature for 20 min. The dried samples were placed in the treatment chamber and exposed to light for 15 min up to the dose 18 J/cm^2. The control samples were not illuminated.

Photoinactivation of B. cereus spores in vitro and on the surface.

For preparation of inocula of *B. cereus* ATCC 12826 spores, culture was grown for 3 days at 370C in brain heart infusion broth (Liofilchem, Roseto degli Abruzzi, Italy) containing (per liter) 0.05 mg manganese until 80-90% sporulation was obtained (sporulation intensity was determined by Wirtz-Conklin staining method). Spore suspension was then washed three times by suspending the spores in 5 ml of sterile distilled water, centrifuging at 6000 x g for 20 min, and removing the supernatant. After washing, the spores were resuspended in 1 ml of distilled water, transferred to a sterile, plastic test tube, and heated to 800C for approximately

15 min to inactivate any remaining vegetative cells. Aliquots (10 ml) of spore suspension (~1x108 CFU ml-1 in 100 mmol l-1 PBS buffer (pH 7.2); initial spore count was established by spread plate method) with appropriate concentration of ALA (3mmol l-1 and 7.5 mmol l-1) were incubated in the dark in a plastic 50 ml bottle for cell culture cultivation at 37˚C for 30 min. After incubation, 150 µl aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and exposed to light for 15 min. Afterwards the purity of spores was tested by Wirtz-Conklin staining method again to assure any germination.

Photoinactivation of bacteria inoculated on food matrix

Photoinactivation of bacteria inoculated on food matrix.

The grains (as example of food matrix, 100 seeds, 3. 7 g) were rinsed with 100-mM PBS (pH 7.2) and soaked in 3.7 mL suspension of pathogen (~1x107 cfu mL-1) for 1 min with gentle agitation for inoculation. After the inoculum was decanted, fruits (seeds) were placed on sterile Petri dishes and dried in a laminar at room temperature (21˚C) for 24 h.

Dried fruit/seeds containing 105 to 106 CFU of pathogen per gram were soaked in 7.5-mM concentration of ALA, the control samples – in 100-mM PBS (pH 7.2). The inoculums were kept in the dark for 30 min. Then the inoculum was decanted and all samples were dried in the thermostat at 37˚C. Dried samples were placed in the treatment chamber in a sterile Petri dish without cover where they were exposed to light (λ=400nm, 20mW/cm2) for 5 min. The control sample was not illuminated.

Viability assay. The cell viability was defined as capability of the cell to form colonies on solid nutrient broth. After incubation with ALA and/or light exposure, the cell suspensions were serially diluted in 0.9 % NaCl and triplicate 100 µl aliquots were spread over the surfaces of solid nutrient broth (1,8% agar in LB nutrient broth) in Petri dishes. Colonies appearing on the plates after overnight incubation at 37°C in dark were counted. Survival fraction was determined as N/N_0, where N_0 is the number of CFU/ml in the sample of the untreated culture at time "zero", and N is the number of CFU/ml in the sample of culture after the indicated treatment.

Light source prototypes were constructed for photosensitization experiments in vitro and in vivo. LED-based light source emitting light at λ_1=405 nm, F_{1max}=810 lm, for photoactivation of Chlorophyll Na-salt and endogenous porphyrins was constructed. LED-based light source emitting light at λ_1=589 nm for hypericin activation was renovated and prepared for work.

Pectinesterase (PE) assay

Enzyme extract was prepared by homogenizing food samples (1g) with 10 ml 0.2 M NaCl for 1 min (Minimix, Interscience, France). For the spectrophotometric method, 2 ml of 0.5 % (w/v) citrus pectin was mixed with 0.15 ml of 0.01 % bromothymol blue (in 3 mM K phosphate buffer, pH 7.5) and 0.88 ml of distilled water. Reaction was initiated by addition of enzyme sample (0.5 ml) and the decrease of optical density was read at 620 nm.

Polyphenoloxidase (catechol oxidases) assay

Crude enzyme extract was prepared by homogenizing food samples (1g) with 10 ml of distilled water for 1 min (Minimix, Interscience, France). 50 mM potassium phosphate buffer (pH 6.5), 5 mM L-3,4-dihydroxyphenylalanine solution (L-DOPA), 2.1 mM L-ascorbic acid solution and enzyme extract were mixed by inversion in a 10 mm quartz cuvette and the decrease of optical density (OD) recorded in A_{265} nm for approximately 5 minutes.

Total antioxidants activity

Total antioxidant capacity was measured by FRAP (ferric reducing ability of plasma) method. Extracts for measurement was prepared by homogenizing 1 g of food sample with 50 ml 96% alcohol (Minimix). FRAP working solution consisted from 0.3 M acetate buffer (pH 3.6), 0.01 M 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 0.04 M HCl and 0.02 M $FeCl_3$ x $6H_2O$ in distilled water. For antioxidants measurement 1.5 ml FRAP reagent and 50 µl sample solution

were mixed and reading was performed up to 5 min at 593 nm, 1 cm light path. Fe (II) standard solution tested in parallel.

Total phenols assay

Food samples (3-10g) were homogenized for 1 min at maximum speed in a Minimix (Interscience, France) with 30-100 ml of a mixture containing acetone, distilled water and acetic acid (70:29.5:0.5). Samples were mixed and allowed to stand for 1 hour at room temperature. Extracts were centrifuged at 1640g for 15 min and supernatant was used for total phenols assay.

Total phenols concentration was measured using the Folin-Ciocalteu assay. In brief, 5 ml of distilled water, 0.5 ml of sample and 1 ml of Folin-Ciocalteu reagent were mixed and left at room temperature for 5 min. Afterwards 10 ml of 7% sodium carbonate solution was added and solution was filled to 25 ml final volume by the addition of distilled water. Solution was mixed well and left at room temperature for 2 hours. Then mixture was filtered through 8-layers cheesecloth prior to the determination of total phenols concentration using a spectrophotometer monitoring 750 nm. TP content was standardized against gallic acid and expressed as milligrams per liter of gallic acid equivalents (GAE).

Total flavonoid assay

Samples for total flavonoids assay were prepared by homogenizing fruits or vegetables (1g) with 100 ml 80% aqueous methanol for 1 min at maximum speed in a Minimix (Interscience, France). The extracts were centrifuged for 5 min at 14000 rpm.

Total flavonoid content was measured by the aluminum chloride colorimetric assay. 1 ml of food sample extract was mixed with 0.3 ml 5% $NaNO_2$ and incubated at room temperature for 5 min. Then 0.3 ml 10% $AlCl_3$ was added. After 1 min 2 ml 1 M NaOH was added and total volume up to 10 ml was filed by adding distilled water. The mixture was vortexed and the absorbance was read at 510 nm. Total flavonoid content was expressed as mg catechin equivalents (CE)/100 g fresh weight.

Total anthocyanins assay

Samples weighing 10 g of fresh fruits and vegetables were blended in a food processor for 1minute with 75 ml of a mixture of methanol, acetic acid, and distilled water (M:A:W) at a ratio of 25:1:24. Mixture was centrifuged at 12 000 rpm for 20 min. The remaining residue from centrifugation after the supernatant was removed and mixed with 75mL M: A: W, then centrifuged again, and the supernatant was separated. Each sample was extracted 3 times. Optical density was measured using 1 cm path length quartz cuvettes.

Measurement of colour

Changes of strawberry colour after photosensitization with Na-Chl salt was evaluated from absorption spectrum measuring optical density (OD) in visible region of spectrum. Samples weighting 10 g of fresh berry were blended in a food processor for 1minute with 75 ml of a mixture of methanol, acetic acid, and distilled water (M:A:W) at a ratio of 25:1:24. Afterwards the mixture was centrifuged at 12 000 rpm for 20 min. and repeated 3 times. All three collected supernatants were used for following colour measurements. Optical density (310 – 650 nm) was measured using 1 cm path length quartz cuvettes. Each sample was extracted 3 times.

Temperature measurement

Precision Celsius temperature sensors („Deltha Ohm“, Italy) were used for temperature measurements on the surface of fruits and vegetables.

4. Results

Construction of light sources (prototypes) for photosensitization (VU)

Light source prototypes were constructed for photosensitization experiments *in vitro* and *in vivo*. As different photosensitizers absorb different light we had to construct prototype for aminolevulinic acid and Chlorophyll-Na-salt (400nm) and for hypericin (589nm). LED-based light source emitting light at λ_1=405 nm, for photoactivation of Chlorophyll Na-salt and endogenous porphyrins is presented in Fig.1. LED-based light source emitting light at λ_1=589 nm for hypericin activation was renovated, prepared for work and is depicted in Fig.2.

Fig.1 Photo of pilot-scale LED-based light source prototype with LED's matrix emitting light from bottom and from top.

Fig.2 Photo of lab-scale LED-based light source emitting light at 589 nm.

LED-based light and high power pulsed light were combined in device prototype to decontaminate fruits and vegetables with higher efficiency and shorter exposition time. This prototype was emitting light of 400 nm and 20 mW/cm^2 power and broad spectrum (UV, visible, IR) high power pulsed light. These technical light illumination characteristics were extrapolated from the data *in vitro*.

Fig.3. Photo of combined LED-based and high power pulsed light prototype.

Microbial response in model system: Inactivation of food pathogens by photosensitization in vitro (VU)

Three food pathogens-*Listeria monocytogenes, Bacillus cereus* and *Salmonella enterica* might be inactivated *in vitro* by ALA-based photosensitization by 4-6 log. Chlorophyll sodium salt exhibits much more promising properties in photosensitized inactivation of all these pathogens: it works at extremely low concentrations (10^{-7}M) and does not require additional incubation time to produce endogenous photosensitizers i*n vitro*.

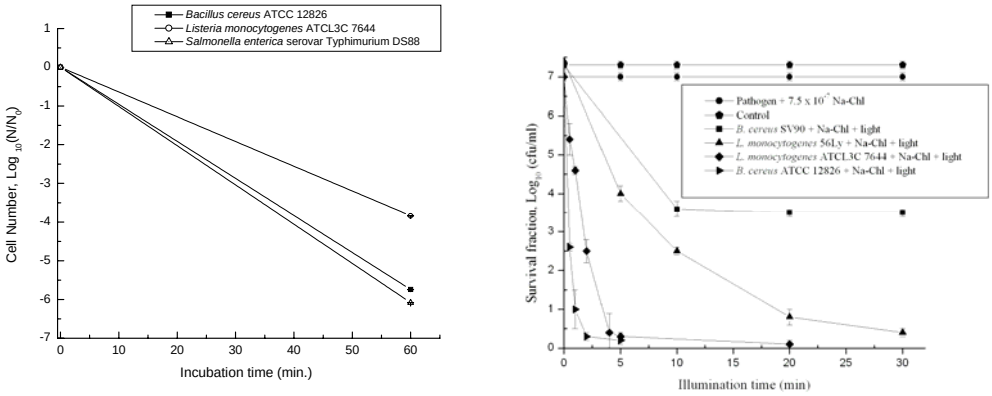

Fig.4ab. 7.5mM ALA photosensitization (a) and Na-Chl-photosensitization based inactivation of food pathogens *in vitro*.

ALA-based photosensitization was effective against investigated pathogens, just required longer exposures. For experiments *in vitro* even 10^{-7}M Na-Chl salt was effective photosensitizer (7 log inactivation) for *Bacillus* and *Listeria*, but not *Salmonella* (2 log inactivation) or *Pseudomonas* (2 log inactivation) in vitro (Fig.4ab).

Effects on packaging decontamination (VU, LNE)

Decontamination of packaging from selected pathogens, inoculated on the surface of packaging material seems real and effective. No physical or chemical changes in packaging material were detected after photosensitization treatment. Chlorophyll sodium salt showed higher efficiency and is much cheaper photosensitizer than ALA. Decontamination of packaging material by ALA-based photosensitization from these pathogens was effective and reached 4-6 log, decontamination with Na-Chl salt reached the same efficiency within shorter incubation and illumination times (Table 1-3).

Table 1. Surface decontamination from several *L. monocytogenes* strains.

Treatment type	***L. monocytogenes* ATCL3C 7644**
Control	4.5 log ± 0.13
Washing with water	3.8 log ± 0.03
100 ppm hypochlorite solution, 1 min treatment	3.3 log ± 0.03
200 ppm hypochlorite solution, 1 min treatment	2.8 log ± 0.02
7.5 x 10^{-7} M Chlorophyll-Na salt + 5 min light	0 log

Treatment type	***L. monocytogenes* 56Ly**
Control	4 log ± 0.04
Washing with water	3.2 log ± 0.04
100 ppm hypochlorite solution, 1 min treatment	2.9 log ± 0.04
200 ppm hypochlorite solution, 1 min treatment	2.5 log ± 0.02
7.5 x 10^{-4} M chlorophyll-Na salt + 15 min light	0 log

Table 2. Inactivation of *L. monocytogenes* ATCL3C 7644 biofilms by Na-Chlorophyllin-based photosensitization onto packaging material polyolefine.

Treatment type	***L. monocytogenes* ATCL3C 7644**
Control	4.5 log ± 0.02
Chlorophyll-Na salt without light	4.3 log ± 0.03
1.5 x 10^{-5} M Chlorophyll-Na salt + 5 min light	3 log ± 0.03
1.5 x 10^{-4} M Chlorophyll-Na salt + 5 min light	0 log

Table 3. Inactivation of *B. cereus* ATCC 12826 spores on the surface of polyolefine by Na-Chlorophyllin-based photosensitization.

Treatment type	***B. cereus* ATCC 12826**
Control	6.1 log ± 0.03
Chlorophyll-Na salt without light	6 log ± 0.01
1.5 x 10^{-5} M Chlorophyll-Na salt + 5 min light	3.9 log ± 0.03
1.5 x 10^{-4} M Chlorophyll-Na salt + 5 min light	1.2 log ± 0.02

Microbial response in food systems

Pathogens inoculated on different raw material (beans, wheat grains, nectarines) were inactivated by ALA and Chlorophyll-Na salt-based photosensitization in less extent if compared with inactivation *in vitro*. Decontamination of food matrix (surface of fruits) from these pathogens by ALA-based photosensitization was less effective and reached just 1-1.5 log.

Decontamination of fruits and vegetables using 10^{-5} M Chl-Na salt reached 1-1.5 log inactivation, using 10-^{4} M Na-Chl salt-2-3 log inactivation. More flat surface fruits were decontaminated better, that for instance salad. Total microbial count on the surface of different fruits decreased as well by 2-3 log. Shelf-life of treated fruits was 10-25% longer, depending on fruit (Fig. 5-7).

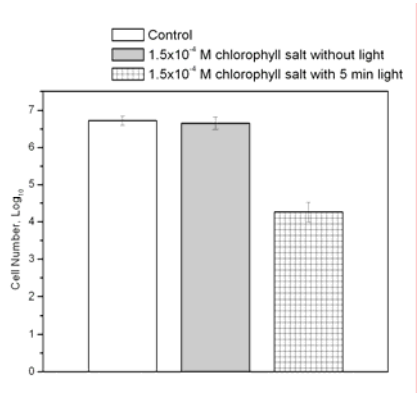

Fig. 5 Inactivation of *Bacillus cereus* ATCC 12826 by 1.5 x 10^{-4} M chlorophyll sodium salt based photosensitization onto cocktail tomatoes. Results – 2, 5 log.

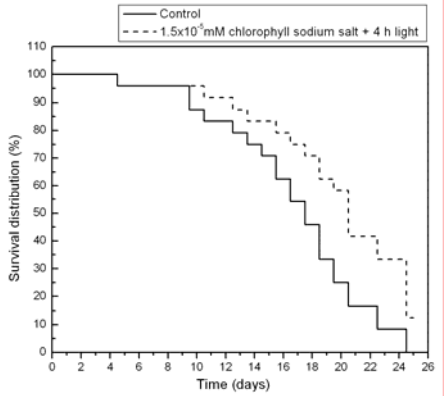

Fig. 6 Cocktail tomatoes, producer Holland, (2008.06.06), soaked in 1.5×10^{-5} Na-Chl salt 10 min, irrad. 20 min., kept 23 ˚C.

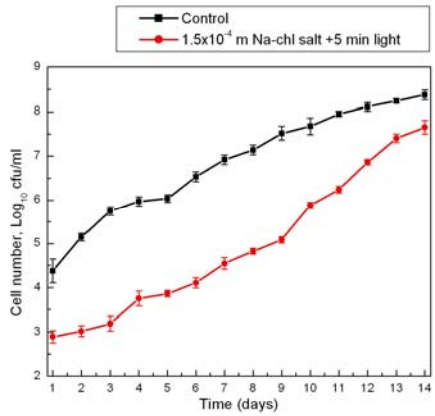

Fig. 7. *Listeria monocytogenes* 56Ly regrowth onto cherry tomatoes after photosensitization with Na-Chl salt.

Hypericin was very effective photosensitizer *in vitro* (6 log inactivation), but using fruit matrix 10^{-5}M concentration reduced *Bacillus* just 0.75 log. Thus, we did not continue these investigations.

Chl-Na- salt –based PHOTO treatment can specifically inactivate vegetative cells *B. cereus*, *S. enteritidis, L. monocytogenes, Pseudomonas fluorescence*, their spores and biofilms *in vitro* and attached to the surface of packaging.

Combined PHOTO- high power pulsed light treatment can increase pathogen inactivation efficiency up to 3-4 log on the food matrix (Fig.7).

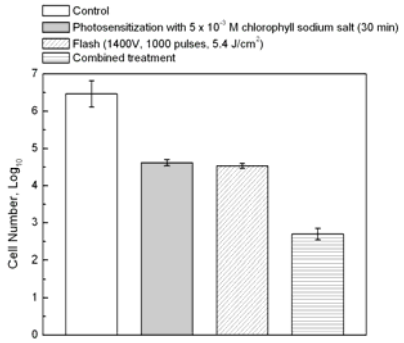

Fig. 7 Inactivation of *Bacillus cereus* ATCC 12826 by photosensitization with chlorophyll sodium salt (5x10-3 M), pulsed light (0.162 J/cm2) treatment and combined treatment onto red pepper.

Comparison of different treatments with PHOTO (VU)

Decontamination of vegetables or packaging from inoculated pathogens or naturally distributed mesophyls by PHOTO treatment was more effective than washing with water or hypochlorite (Fig.8-9).

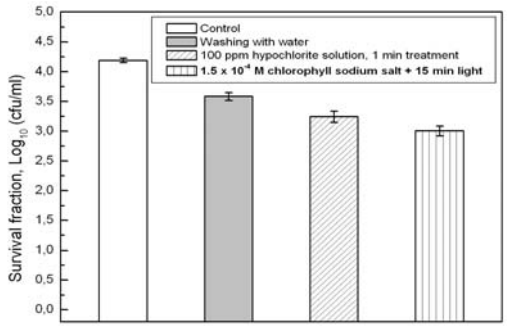

Fig.8. Comparative analysis: inactivation of termoresistant *Bacillus cereus* SV60 inoculated on tomatoes

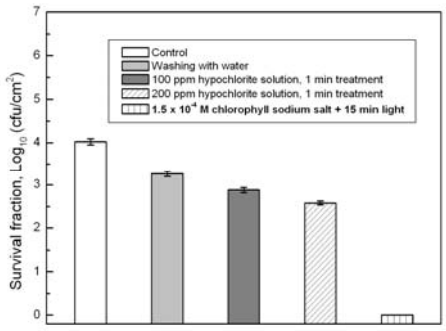

Fig.9. Comparative analysis: inactivation of *Listeria monocytogenes* 56Ly on packaging.

Effects on organoleptic properties, enzymes and colour in food systems after PHOTO (VU, UNIBO, UAB)

No sensorial changes or decrease of nutrition were observed in treated intact or freshly cut fruits and vegetables. We found: about 2 log pathogen inactivation on food matrix without significant impact on enzymatic activity (polyphenoloxidase, pectinesterase, antioxidant enzymes) or amount of total flavonoids, anthocyanines, or phenols (Fig.10-12).

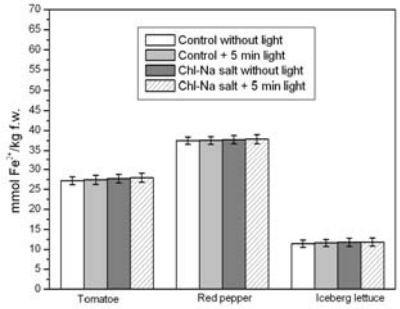

Fig.10. Total antioxidant activity of tomatoes, red pepper and Iceberg lettuce (1, 5 x 10^{-4} M Na-chl salt, 5 min illumination)

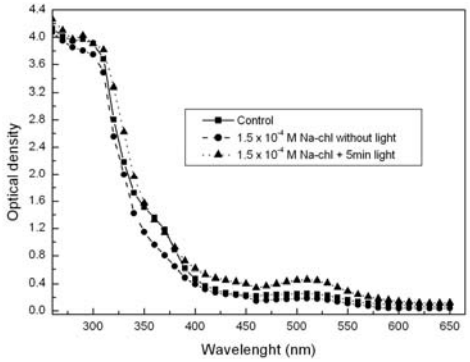

Fig.11. Strawberries colour changes after Chl-Na-based PHOTO treatment (1.5 x 10^{-4} M Na-chl salt, 5 min illumination)

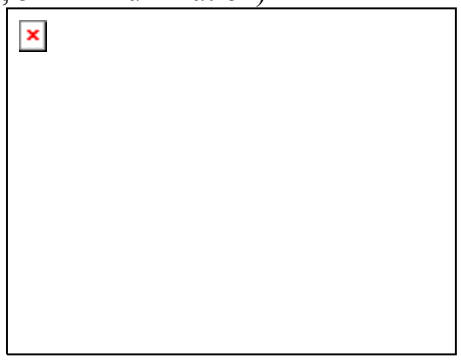

Fig.12. Temperature changes in strawberries during photosensitization.

We examinated organoleptic properties of treated by PHOTO strawberries as well as raw material for salad after PHOTO treatment. As presented in Table 4 no impact on organoleptic properties of strawberries was found after PHOTO treatment.

Table. 4.Treated by Photo strawberries were tested by 20 volunteers.

Nr. of voluntiers	Taste	
	No detected changes of sweetness after treatment of strawberries	No detected changes of firmness after treatment of strawberries
20	20	20

Effects of PHOTO on organoleptic properties of ready to eat salads

We performed pilot scale experiments in UAB "Palink" and found first results on decontamination of ready to eat salads by PHOTO in industrial environment. 12 hours prolongation of salad shelf life is possible to achieve when salad is treated by PHOTO. Thus, preliminary results obtained with ready-to-eat salads look promising when raw material or salad were treated by PHOTO before dressing (Fig.13).

According to the data obtained in UAB "Palink", photosensitization can delay total microbial counts when "raw material for salad", and "salad before dressing" were used for experiments. But data obtained on the decontamination of "raw material for salad" and "salad before dressing" in laboratory are much better than those in UAB "Palink". It is likely that possible cross contamination will be significantly reduced when automatic work will replace the huge amount of hand work (cutting of vegetables, mixing them, delivering to the packs etc.) that has been actually performed by UAB personnel during these activities caried out within the project in their industrial sites.

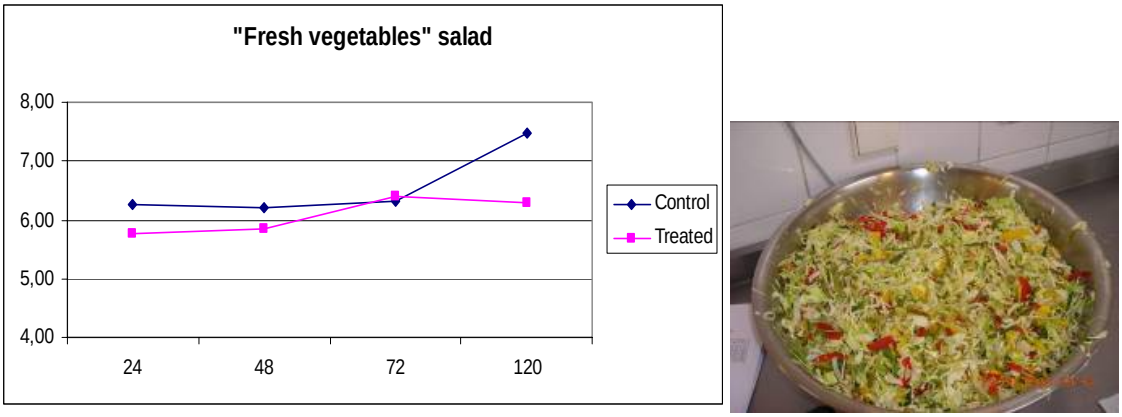

Fig.13 Microbial regrowth in treated by PHOTO "Fresh vegetable salad".

Some organoleptic changes were observed in treated salads before dressing as salad before dressing was not completely dried after the treatment (Table 5-6).

Results of the "Fresh vegetable" salads tests performed at Lithuanian National risk evaluation institute (credited method)

Table 5. Organoleptic testing of the PHOTO treated"Fresh vegetable salad" - treated raw materials.

	Question	Answer/conclusion (Number of positive answers of 10 degustators)	
		Number of positive answers	Conclusion, (Risk margin)
1.	If there is difference on appearance	0	No effect (Risk margin 0, 1%)
2.	If there is difference on smell	1	No effect (Risk margin 0, 1%)
3.	If there is difference on taste	0	No effect (Risk margin 0, 1%)

Table 6. Organoleptic testing of the PHOTO treated"Fresh vegetable salads" (treated finished product before dressing).

	Question	Answer/conclusion (Number of positive answers of 10 degustators)	
		Number of positive answers	Conclusion, (Risk margin)
1.	If there is difference on appearance	2	No effect (Risk margin 0, 1%)
2.	If there is difference on smell	4	No effect (Risk margin 0, 1%)

Thus we can conclude that decontamination of the raw materials with followed full processing cycle is an effective way for increasing the quality of the product when less hand work will be used for processing of salad. Decontamination of the prepared salads before dressing indicates reduction of the initial bacterial decontamination. But without following drying it does not fit as influences the organoleptic properties of them.

5. Conclusions

1. LED-based light source for pilot-scale food decontamination by photosensitization was developed. It emits light of 400 nm and 20 mW/cm^2 power. These technical light illumination characteristics were extrapolated from the data *in vitro*.
2. LED-based light source emitting light at λ_1=589 nm for hypericin activation was prepared for work.
3. LED-based light and high power pulsed light were combined in device prototype to decontaminate fruits and vegetables with higher efficiency and shorter exposition time.
4. Three food pathogens-*Listeria monocytogenes, Bacillus cereus and Salmonella typhimurium* might be inactivated *in vitro* by ALA-based photosensitization by 4-6 log. *Pseudomonas fluorescence* is the most resistant one to this treatment and can be inactivated by no more than 2 log at certain experimental conditions.
5. Chlorophyll sodium salt (Na-Chl salt) exhibits much more promising properties in photosensitized inactivation of all these pathogens: it works at extremely low concentrations (10^{-7}M, 7 log inactivation) and does not require additional incubation time to produce endogenous photosensitizers *in vitro.*
6. Decontamination of packaging from selected pathogens, inoculated on the surface of packaging material seems real and effective as can clean the surface totally by ALA- as well as Na-Chl salt based PHOTO treatment. Chlorophyll sodium salt showed higher efficiency and is a much cheaper photosensitizer than ALA. Biofilms and spores were susceptible to ALA- as well as Na-Chl salt based photosensitization.
7. PHOTO-Na-Chl salt treatment can specifically inactivate *B. cereus, S. enteritidis, L. monocytogenes,* as well as their termoresistant strains on the surface of fruits and reaches 1.4-2.9 log depending on surface structure when 10^{-4}M Na-Chl salt was used as photosensitizer. Inactivation efficiency strongly depends on fruit surface structure: flat surfaces are decontaminated better than irregular, round etc.
8. PHOTO-hyp treatment at 10^{-5}M photosensitizer concentration effective as Na-Chl salt. Its main disadvantage is that at this concentration it colours freshly-cut fruits and vegetables. Thus it was rejected from the further experiments.

9. Shelf-life of fruits and vegetables treated by PHOTO-Na-Chl salt (when Na-Chl salt concentration was 10^{-4}M) was prolonged by 10-25 % in comparison with control (untreated ones).
10. Combined PHOTO- high power pulsed light treatment can increase pathogen inactivation efficiency up to 3-4 log on the surface of fruits and vegetables.
11. Comparative analysis indicate that decontamination of vegetables or packaging material from pathogens and mesophyls by PHOTO was more effective than such conventional treatments as washing with water or hypochlorite.
12. No sensory or nutritional changes were observed in treated intact or freshly cut fruits and vegetables after PHOTO based on Na-Chl salt.

6. Future perspectives

Photosensitization induces lethal effects in all investigated pathogens, their vegetative cells, spores and biofilms. No microbial resistance to this treatment was observed. Moreover, photosensitization does not require high intensities (can be used as hurdle), thus the preservation of sensory, nutritional and functional properties of foods is higher.

Chlorophyll sodium salt exhibited high photosensitizing activity against food pathogens and can be used for the decontamination of fruits and vegetables, food related surfaces as belongs to food additives in EU classification (E100-E199, it is E140(color)). Its concentration for food, cosmetics and drugs is not limited as it is based on natural product (reglamentated by directives 88/343 EEB, 97/60 EB ir 94/36 EB).
Light power of LED-based light sources developed during this project is not maximal, as more powerful LEDs exist, but their usage needs additional financial support. Meanwhile the possibility exists to increase the power of LED-based prototype and in the same time to reduce technology duration and costs.

In our opinion, this phenomenon opens a new and prospective avenue for the development of effective, human and environmentally friendly, low-cost and easy to maintain antimicrobial treatment. Its proper application for the treatment of food, packaging and processing equipment might be really useful to increase microbial food control. Nevertheless, this is just beginning. Taking into account the low budget for this activity, the complexity of food matrixes, microbial ecology of food, interaction of food matrix with treatment, shelf-life and quality of foods after treatment further deep research is needed. Whilst it is not suggested that photosensitization will solve all problems of antimicrobial issues, improvements may be obtained using this new approach in special cases or combining photosensitization with accepted technologies for microbial control.

7. Impact of the results on industry or the research

1. The main results obtained during running of HighQRTE project are presented in more than 100 figures and 20 tables. Data are published in 16 ISI Web of Science journals and per-reviewing journals, 1 patent and presented in more than 10 international conferences.
2. This study significantly contributes to the fundamental knowledge related to photoinactivation of food pathogens as it is the first and successful attempt.
3. A lot of scientific and technological publishable results have been obtained related to the effect of PHOTO on the inactivation of foodborne pathogens like *Listeria monocytogenes*, *Bacillus cereus* and *Salmonella enteritidis.* These results show the effectiveness of PHOTO to

eliminate these pathogens and to reduce the risk of re-contamination of the ready-to-eat vegetable food after the treatment and during the storage and distribution.
4. The results provided important information about parameters which are necessary for LED-based light sources in order to have high pathogen inactivation.
5. These results provided the industry with valuable information about new emerging technology showing that PHOTO can be a viable alternative to conventional treatments, such as washing with sterile water or hypochlorite in terms of microbiological safety.
6. Moreover, PHOTO could significantly increase the commercial shelf-life of fresh fruits and vegetables avoiding the use of additives and with any loss of the nutritional and sensory properties.

The initial expected potential impact related to food processing innovation and competitive value has been achieved. To apply photosensitization for food safety purposes is pioneering approach. This project is the first attempt. Despite it this study has generated a lot of information about this new technology from a scientific and technological standpoint. By no means, this study is useful for the industry as suggests a novel and effective tool which may in the future be developed into food safety technology and will enable to change the conventional technologies such as washing with water of hypochlorite.

Pulsed Electric Fields (SIK)

1. Introduction

Pulsed Electric Field (PEF) treatment involves the application of high voltage pulses to a product between two electrodes. The process has been applied to fluid food products such as fruit or vegetable juices, milk and liquid egg (Ohlsson and Bengtsson, 2002). When evaluating microbial and quality effects of products it is important to identify conditions enabling satisfactory microbial inactivation not having any negative effect on the total quality. It is well known that the temperature will increase due to ohmic heating where the pulsed electric field energy is transformed into heat. Therefore for heat sensitive products, the PEF process needs to be designed to give a limited temperature increase during treatment together with a satisfactory bacterial inactivation.

Many factors affect the microbial inactivation, and needs to be accounted for (Anon. 2000). Important process factors are electric field strength, pulse length, energy input, pulse wave shape and treatment temperature. The electric field strength is dependent on the geometric design of the treatment chamber. Most treatment chambers have uneven flow and many have uneven field. Product characteristics such as pH-vale and conductivity also have a high influence (Garcia *et al.* 2005). A synergistic effect on bacterial inactivation is is reported in some studies when PEF treatment is combined with a subsequent mild heat treatment (Li *et al* 2005; Jin *et al*. 2009).

In order to produce safe food, PEF processing must inactivate pathogenic bacteria. In RTE products, *L. monocytogenes* are of particular importance since it is of a major concern in this type of product. *Salmonella* is of importance in several products, but in particular in heat sensitive products containing liquid whole egg. The inactivation of spoilage bacteria by PEF will prolong the product shelf life. The objective can also be to reduce spoilage of a high value product such as balsamic vinegar by the inactivation of specific bacteria causing unwanted fermentation.

L. monocytogenes is very resistant towards PEF at neutral pH value. The inactivation of *L. monocytogenes* by PEF in non acid RTE food/ RTE food ingredients constitutes a challenge when the product quality is negatively affected by high temperatures. By using a high energy input (3490kJ/kg), *L. monocytogenes* is reported to be reduced by 4.8 log CFU at pH 7 (Àlvarez *et al.*, 2003). However, high energy input might result in too high temperature depending on the conductivity in the product. The inactivation of *Salmonella* by PEF in liquid whole egg is reported to be about 1 log CFU reduction using PEF (Hermawan *et al.*, 2004). No information is found in the literature on the inactivation of bacteria associated with spoilage of vinegar.

PEF is reported to exert a small impact, if any, on most of the quality parameters such as protein stability, vitamin content, colour, flavour of milk, juices and egg products (Manas and Vercet, 2006). The resistance of enzymes is higher than that showed by microorganisms. The effect of PEF on food microstructure and functional properties is not well known. Some studies have been carried out on solid foods; Bazhal *et al.* (2003) studied the effect of PEF on apple tissue and the results showed that both plasma lemma and cell wall were affected.

Gudmundsson and Hofsteinsson (2001) investigated the microstructure of muscle foods and roes after PEF treatment; a visible effect was observed on the microstructure of salmon and chicken but lumpfish roes were affected at a minor level.

2. Objectives

The aim of the work performed using PEF technology was to evaluate and improve the efficiency of treatment, in terms of quality and safety for RTE fluid ingredients and foods including milk, liquid whole egg, vinegar and heat sensitive sauces. This treatment should enable a good inactivation of *L. monocytogenes, S. enteritidis* and spoilage bacteria while at the same time avoiding the negative effects obtained by conventional heat pasteurization on the over all quality such as physical and nutritional properties.

3. Materials and Methods

PEF processing was performed using a pilot plant consisting of a treatment chamber with a novel design, heat exchangers for the heating and cooling of samples during processing, and a holding cell for a post PEF treatment. The pulse generator (Scandinova; Uppsala, Sweden) produced mono-polar rectangular pulses. The pulses were square pulses and the top value of the voltage was used for defining the electric field strength of the treatment.

Studies were performed on: phosphate buffer (pH 7.0; conductivity 5.0 mS/cm); UHT milk 3.2% fat (pH 6.6; conductivity 4.6 mS/cm), liquid, non pasteurized, homogenized whole egg ((pH 7.7-8.2; conductivity 5.8 to 7.0 mS/ cm); mayog dressing (pH 5.4-5.5; conductivity 6.4-7.9 mS/ cm); béchamel sauce (pH 6.8-7.2; conductivity 5.9 mS/cm, 6.9 mS/cm) and balsamic vinegar (pH 3.1; conductivity 3.9 mS/cm). The mayog dressing was an emulsion of sunflower oil (22.3%), yoghurt with 3% fat (30,4 %), whole egg (25,4%), water (19.2%), salt (0.5%) and Starch (2.0%; Swelly gel 700, Lyckeby Culinar, Sweden).

The effect of PEF regarding microbial response was evaluated studying i) basic PEF factors such as electric field strength, energy input, pulse length and number of pulses; ii) PEF combined with subsequent mild heat treatment and iii) PEF combined with sorbate. The combinations of electric field strength, pulse width and number of pulses were selected in order to achieve a treatment where the energy input resulted in a maximum product temperature of 50°C. A mild heat treatment post PEF processing was performed by maintaining the product temperature at 53°C for 10 minutes.

Inactivation and re-growth of inoculated *Listeria monocytogenes, Salmonella* spp. *Glucanoacetobacter* sp. and of natural flora was studied. The bacterial strains used for inoculation of samples were *L. monocytogenes* SIK 564 (=*L. monocytogenes* Scott A), *L. monocytogenes* UNIBO 56Ly (received from the University of Bologna), and *S. enteritidis* UNIBO155 (received from the University of Bologna). The *Glucanoacetobacter* sp. that was used for inoculation of vinegar was isolated from a spoiled sample of balsamic vinegar received from ACE. The isolate was identified to *Glucanoacetobacter* sp. regarding 16S rRNA sequencing. Analyses of aerobic total count, *L. monocytogenes*, *S. enteritidis*, *Glucanoacetobacter* sp., *Pseudomonas* spp., lactic acid bacteria, yeast and mold were made as colony forming units on non-selective and selective agars. Colonies from aerobic count plates were picked for identification of the dominating total aerobic microflora based on phenotypic characterization using API. Colonies with different appearance were picked in order to

represent the diversity of strains in the sample. Genetic responses after PEF of three genes involved in protein synthesis (*rpoB)*, stress response (*gadA*) and acid adaptation (*opuCA*) in *L. monocytogenes* was measured using RT-q-PCR.

The effect of microstructure, functionality, enzymatic activities, macroscopic properties, sensorial properties, and nutritional properties in i) liquid whole egg was evaluated concerning basic PEF factors and PEF combined with subsequent mild heat treatment and ii) mayog dressing concerning PEF with and without subsequent mild heat treatment.

Microstructure and functionality were analyzed by microscopic techniques and physical properties. The microscopic techniques used were low temperature Scanning Electron Microscopy, Transmission electron Microscopy, Light Microscopy, and Confocal Laser Scanning Microscopy. Foaming capacity, foam stability, pH-value, viscosity, textural profile analysis, water holding capacity and colour were the physical properties analyzed.

Enzymatic responses were analysed by extraction and quantification of water-soluble proteins, study of total and water-soluble protein fractions by electrophoresis. Lipids were extracted and acidity grade, hydroperoxides and secondary oxidation products were analyzed.

Macroscopic properties were tested in a standard size back extrusion container.

A sensory panel consisting of 42 persons was used for sensory tests. Each tester described texture, taste/flavour, smell/flavour, colour/appearance and ddisposed to re-eat the product. Each indicator was described between ranges of values from 1 to 5. The value 1 corresponds to a bad evaluation while the value 5 corresponded to excellent product.

To evidence possible adverse effects on the nutritional quality due to the treatment, three different analyses have been performed on samples: triacylglycerol hydrolysis, oxidative damage, and vitamin E content. The entity of triacylglycerol hydrolysis was determined by evaluating the free acidity in the samples. Free acidity is expressed as percentage by weight of linoleic acid in mayog samples and oleic acid in liquid whole egg samples, since linoleic acid and oleic acid were the most abundant fatty acid in the mayog and liquid whole egg samples respectively. Oxidative damage was assayed in by quantifying the content of conjugated dienes. Vitamin E was determined on samples extracted by cold saponification and n-hexane by direct phase NP-HPLC. Each extraction method was replicated three times. Vitamin E content was calculated by comparison of the respective peak areas to standards. Chromatograms were recorded and processed using HP Chemstation software.

Vinegar was analysed for pH, relative density, total acidity, total sulphur dioxide, total dried extract sugar, deduced died extract and turbidity in order to evaluate against the Commission regulation (EEC) No 2676/90 for the analysis of wines.

4. Results and Discussion

Pilot plant

The PEF pilot plant consisted of a pulse generator (Scandinova (Uppsala, Sweden), heat exchangers, PEF treatment chamber and a holding cell (Fig.14). The product temperature was

adjusted using heat exchangers. A holding cell was used for maintaining the product temperature for a defined time after the PEF treatment.

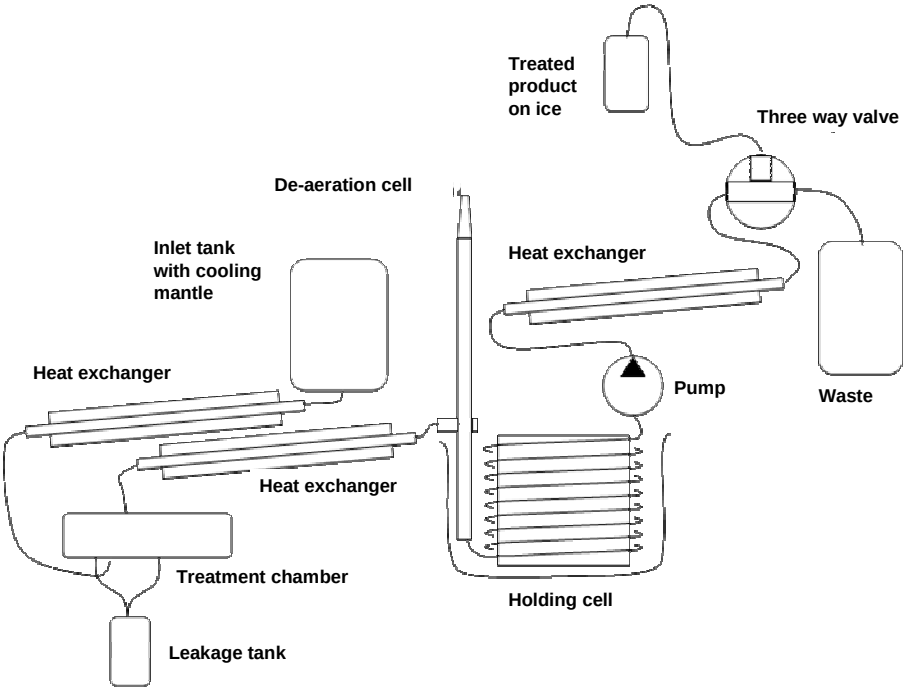

Figure 14. Schematic figure over the PEF pilot plant

The treatment chamber had a cylindrical shape where the two circular ends were the electrodes. The chamber was closed during PEF treatment. After treatment the chamber was completely emptied and refilled; this was continuously executed. The shape of the chamber and the arrangement with two parallel electrode surfaces made the electrical field in the chamber homogeneous. The enclosement of the chamber and the homogenous electric field ascertained that the entire product volume got exactly the same treatment even if there were some circulation in the chamber during the treatment. This feature made the treatment chamber very suitable for evaluating the influence of PEF treatment on microorganisms.

The product was transported continuously through the pilot plant via plastic tubes. The system volume and flow capacity chosen provided a product volume sufficient for the setting of treatment levels and laboratory analysis. The plastic tubes were disconnected and autoclaved between experiments. The treatment chamber was disinfected with 70% ethanol. All other equipment was autoclaved.

Microbial response

PEF conditions that increased the product temperature to maximum 50ºC were not efficient for the inactivation of *L. monocytogenes* or *Salmonella* spp. in phosphate buffer, milk, and liquid whole egg. The reduction of *L. monocytogenes* that could be achieved was ~1.5 log CFU. However, in phosphate buffer almost 3 log CFU reductions of *S. enteritidis* were achieved.

PEF treatment of balsamic vinegar decreased the number of *Glucanoacetobacter* sp. with more than 3.5 log CFU/ml. After 2 months of storage at room temperature, no bacteria were

detected in vinegar naturally contaminated or inoculated with *Glucanoacetobacter* sp. when the applied electric field strength was 35 kV/cm.
The PEF treatments parameters (electric field strength, pulse width and number of pulses) applied varied from product to product in order to reach the criteria of product temperature ≤50°C. One important reason was the sample conductivity. In addition, minimal air bubbles, invisible for the eye in the Béchamel sauce is believed to have limited the maximum applied energy input.

Three different approaches were used in order to increase the bacterial inactivation by PEF treatment:

1. Application of consecutive PEF treatments with cooling in between.
2. Use of sorbate.
3. Application of a mild heat treatment subsequent to the PEF treatment.

Different PEF treatments were investigated for the effect of consecutive PEF treatments (Fig. 15). The highest reduction of *L. monocytogenes* was found for the condition 32kV/cm, 10µs, 5 pulses; 3 log units were inactivated in phosphate buffer during three PEF cycles. In each cycle, the inactivation was about 1 log unit. The reduction was less for conditions (i) 27kV/cm, 6µs, 5 pulses; (ii) 35kV/cm, 6µs, 3 pulses and (iii) 35kV/cm, 6µs, 5 pulses. During these conditions about one log reduction was found after three consecutive PEF treatments.

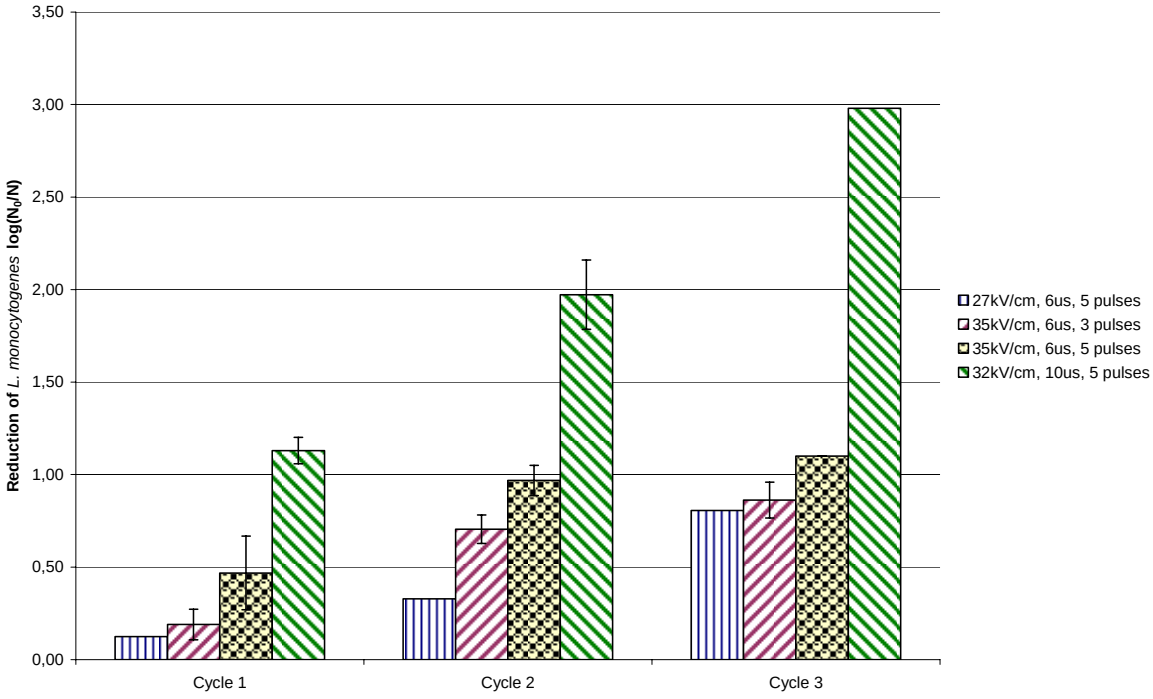

Figure 15. Reduction of *L. monocytogenes* SIK 564 during consecutive PEF treatment in phosphate buffer (pH 7.0, 5.0 mS/cm). The treatments performed were (i) 27kV/cm, 6µs, 5 pulses; (ii) 35kV/cm, 6µs, 3 pulses; (iii) 35kV/cm, 6µs, 5 pulses and (iv) 32kV/cm, 10µs, 5 pulses. The inlet temperature was 5°C and the temperature increase during the treatments was 15, 15, 32 and 45°C, respectively.

The combination of PEF and sorbate was evaluated. The time to growth (TTG) after PEF treatment during storage was, however, increased in the presence of 0.1 % potassium sorbate

(Fig. 16). The TTG of *L. monocytogenes* UNIBO 56 Ly was about 80 hours at 10 °C after PEF treatment or in the presence of sorbate. When combining PEF treatment and the addition of sorbate, the TTG was 200 hours.

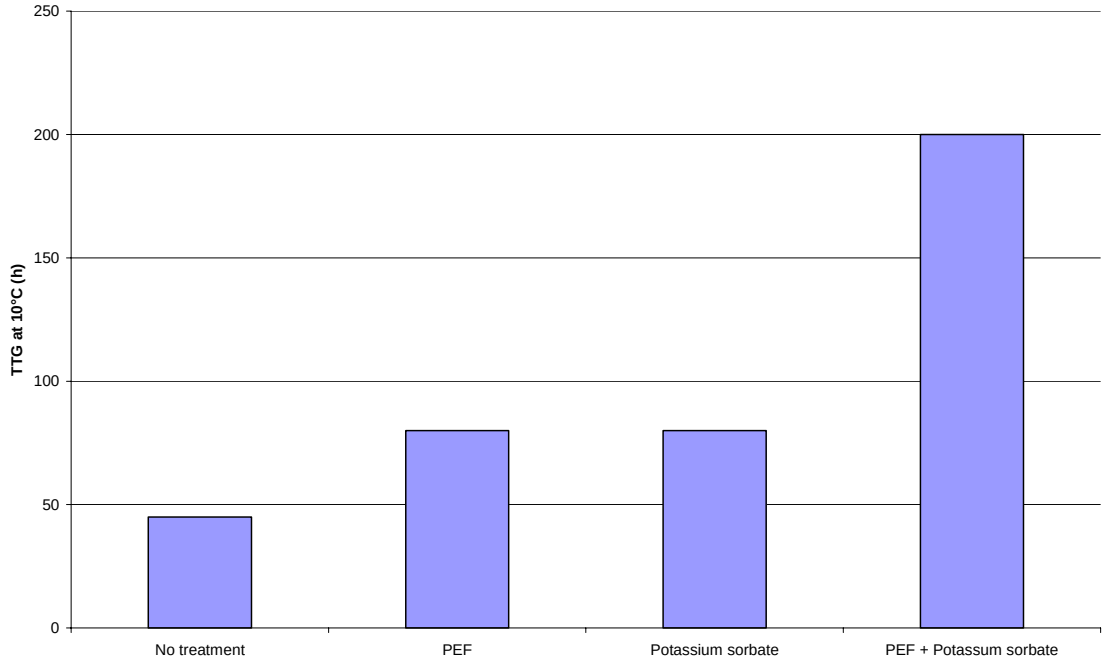

Figure 16. Time to growth (TTG) of *L. monocytogenes* UNIBO 56 Ly at 10°C after PEF treatment, addition of 0.1 % potassium sorbate and their combination. PEF treatment was performed at 32 kV/cm, 10 µs and 5 pulses. TTG corresponds to an increase of 0.5 log CFU/ml.

When combining PEF with heat treatment, both the PEF conditions and the time temperature conditions applied were important. A higher energy input, and higher electric field strength during the PEF treatment increased the sensitivity of *L. monocytogenes* to heat treatment at 55°C in phosphate buffer pH 7.0. Gene expression analysis of *rpoB* indicated a disturbance in the bacterial protein synthesis due to PEF treatment which may have influenced the heat sensitivity of *L. monocytogenes*. The gene *rpoB* behaved differently depending on high or low energy input during the PEF treatment; a high energy input down regulated the protein synthesis and a low energy input up regulated the protein synthesis. These results were obtained for a non continuous process where the sample was resuscitated at 25°C for 10 minutes between the PEF and subsequent heat treatment. Using continuous combined PEF and heat treatment on real products without resuscitation at 25°C, the level of energy input did not affect the reduction of *L. monocytogenes* in liquid whole egg and mayog dressing.

An increased inactivation of *L. monocytogenes* and *S. enteritidis* was obtained in real products by combining PEF treatment and mild heat treatment. As opposed, the growth rate of these two bacteria was not affected by PEF treatment. The inactivation of *S. enteritidis* UNIBO 155 in egg and mayog dressing was >6 log CFU using PEF treatment and subsequent mild heat treatment at 53°C for 10 minutes. During the same conditions, *L. monocytogenes* was inactivated with 2.6 – 3.1 log CFU/ml (Table 7); the effect varied with the type of product treated. By carefully selecting PEF and heat treatment parameters the reduction may be increased and become equivalent to heat pasteurisation (target value 6 log reduction) even for

the PEF resistant *L. monocytogenes.* For example, by increasing the temperature during the heat treatment from 53°C to 55°C an additional 2 log CFU reduction was obtained in phosphate buffer.

The shelf life at chill temperatures of the tested products was increased by PEF and mild heat treatment. This was due to the inactivation of psychotropic bacteria able to grow during the storage of products. A selection of the types of microorganisms able to grow in the product was also seen. In untreated liquid whole egg a heterogeneous microflora including *Enterococcus* spp., *Stenotrophomonas* spp., *Pseudomonas* spp., *Yersinia* spp. and lactic acid bacteria, was growing during the cold storage. In the PEF and mild heat treated samples, *Yersinia* spp. and *Enterococcus* spp. were identified.

Table 7. Inactivation of *L. monocytogenes* UNIBO 56 Ly and *S. enteritidis* UNIBO 155 in liquid whole egg, milk, mayog dressing and béchamel sauce. Product inlet temperature was 5°C.

Product	PEF treatment			Heat treatment		Bacterial inactivation (log CFU)	
	Electric field strength (kV/cm)	Pulse width (µs)	Number of pulses	Temperature (°C)	Time (minutes)	*L. monocytogenes* UNIBO 56 Ly	*S. enteritidis* UNIBO 155
Liquid whole egg	34	6	5	53	10	3.6±0,3	6.3±0.7
	35	6	3	53	10	3.3±0,5	6.3±0.7
			0	53	10	1.8±0.2	3.7±0.2
Milk	34	6	6	53	10	3.1±0.4	nt[1)]
	35	6	4	53	10	1.8±0.2	nt
			0	53	10	0.5±0.0	
Mayog dressing	32	6	4	53	10	2.7±1.4	>7.4; >6.6
	32	6	3	53	10	2.9±1.6	>7.4; >6.6
			0	53	10	1.7±1.4	>7.4; 6.4
Béchamel sauce	30	6	5	53	10	2.6±0.0	nt
	27	6	5	53	10	2.3	nt
			0	53	10	0.6±0.1	nt

1) nt, not tested

Response of the microstructure and functionality

When the effect of field strength was investigated, the microstructural results showed a highly affected lipoprotein matrix in whole liquid egg treated by PEF. The continuity of the protein network was broken and some protein granules from the yolk were degraded, especially when high field strength was applied (Figure 17). In the pasteurised samples, the microstructure shows the aggregation of the protein network due to the temperature.

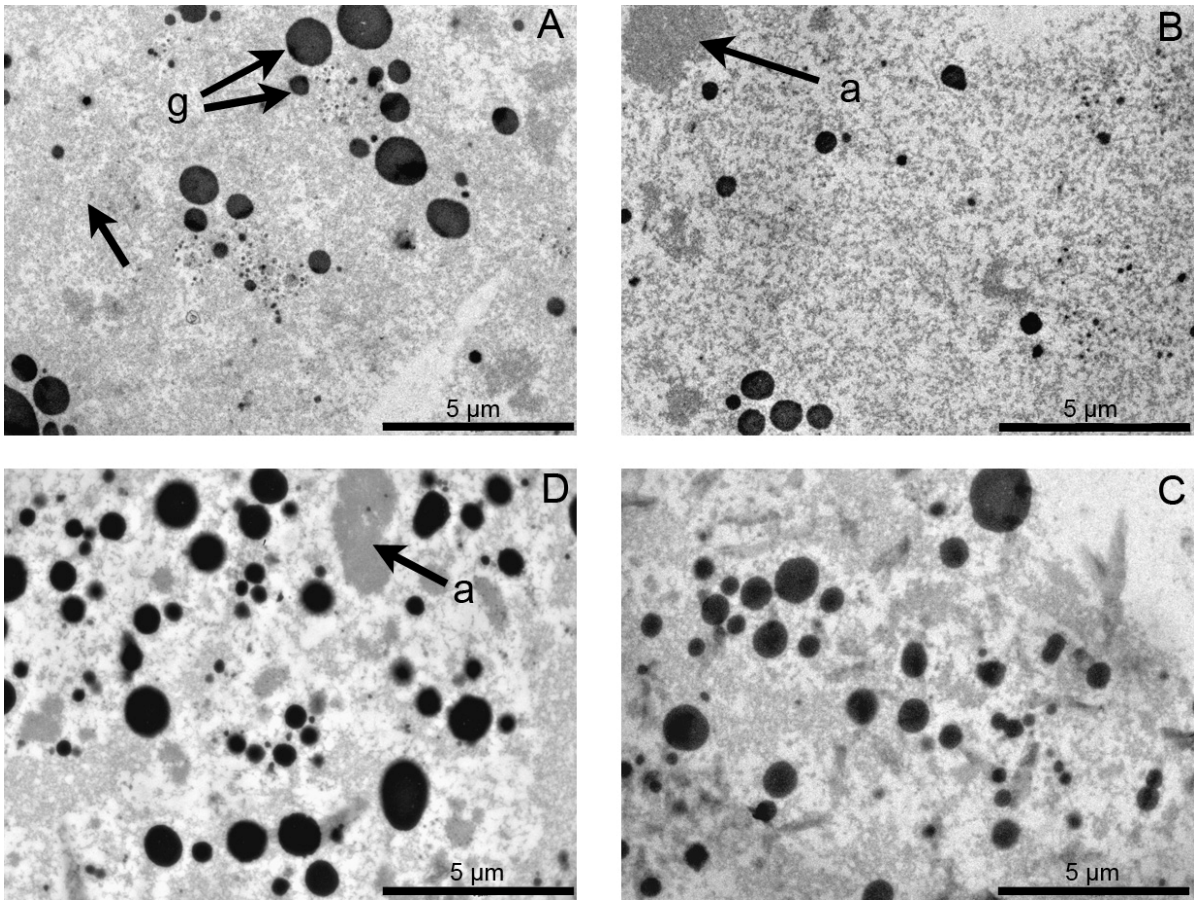

Figure 17. TEM. A: Control whole liquid egg; B: liquid whole egg treated by PEF (19 kV; 8kV/cm-6µs-5 pulses); C: liquid whole egg treated by PEF (32 kV; 29kV/cm-6µs-5 pulses); D: pasteurised liquid whole egg. 1650x (arrow: lipoprotein matrix; g: protein granules; a: aggregated compounds).

Viscosity, colour and textural profiles were unaffected by the PEF treatments in whole egg samples, but the foam stability was higher in PEF treated samples than after pasteurization. However, a lower stability of food systems based on a gel structure, as liquid whole egg, would be predictable after PEF treatments, especially when high field strength are used (32 or 37kV/cm).

When PEF in combination with mild temperature was applied in liquid whole egg, microstructure and some physical properties as pH and viscosity were not greatly affected.

The structure of the mayog dressing samples, heat sensitive sauces, treated only by PEF was found to be similar to that of the control, but when the sample was treated with PEF

in combination with mild temperature, the lipoprotein network was aggregated (Figure 18). pH and colour were not affected by PEF in combination with mild temperature. A protein insolubilisation was detected what can be related to the increase of viscosity observed.

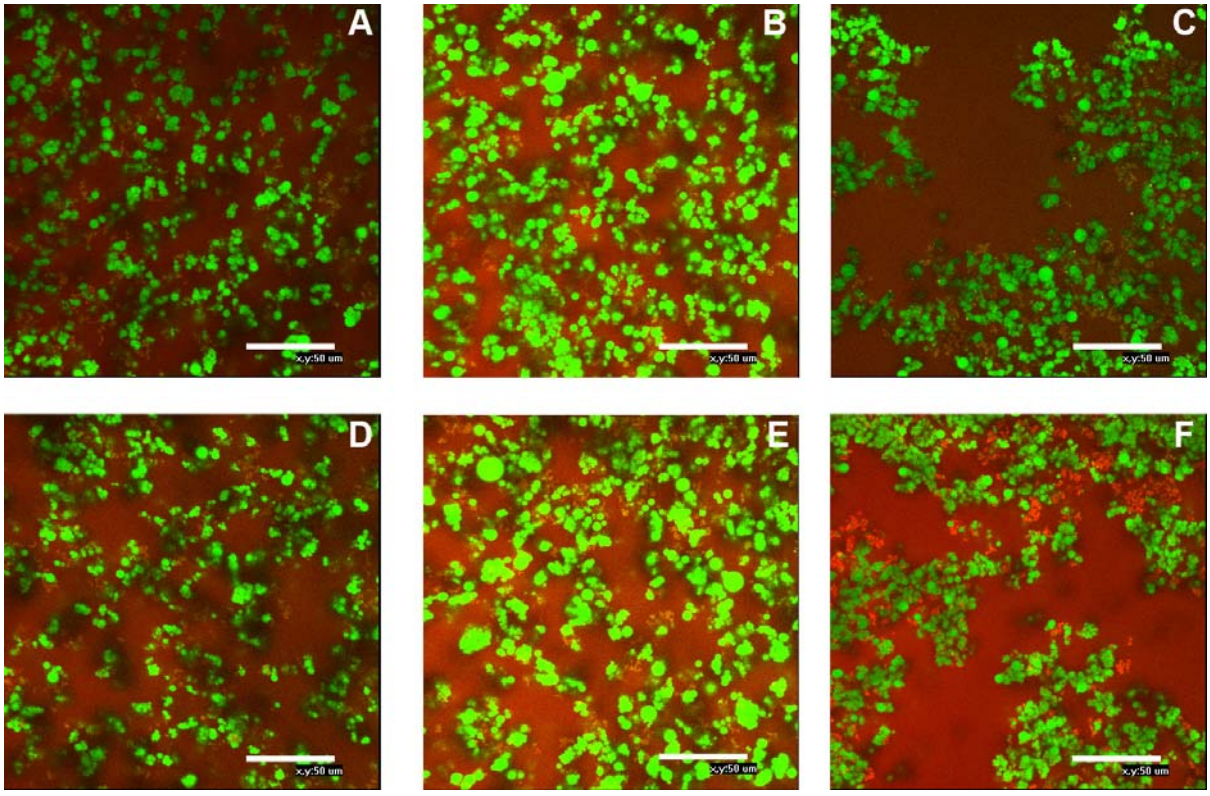

Figure 18. CLSM. A: Control: not PEF treated mayog dressing; B: mayog dressing treated by PEF; C: mayog dressing treated by (PEF+T^a); D: Control, after 1 week at 4 ºC; E: mayog dressing treated by PEF, after 1 week at 4 ºC; F: mayog dressing treated by (PEF+T^a), after 1 week at 4 ºC. 60x (arrow: oil droplets)

Enzymatic response

Proteolytic activity was not detected in PEF treated samples (19 to 37 kV; Figure 19). Only a slight hydrolytic activity of the lipid fraction was observed in PEF treated samples, after one week at 4ºC.

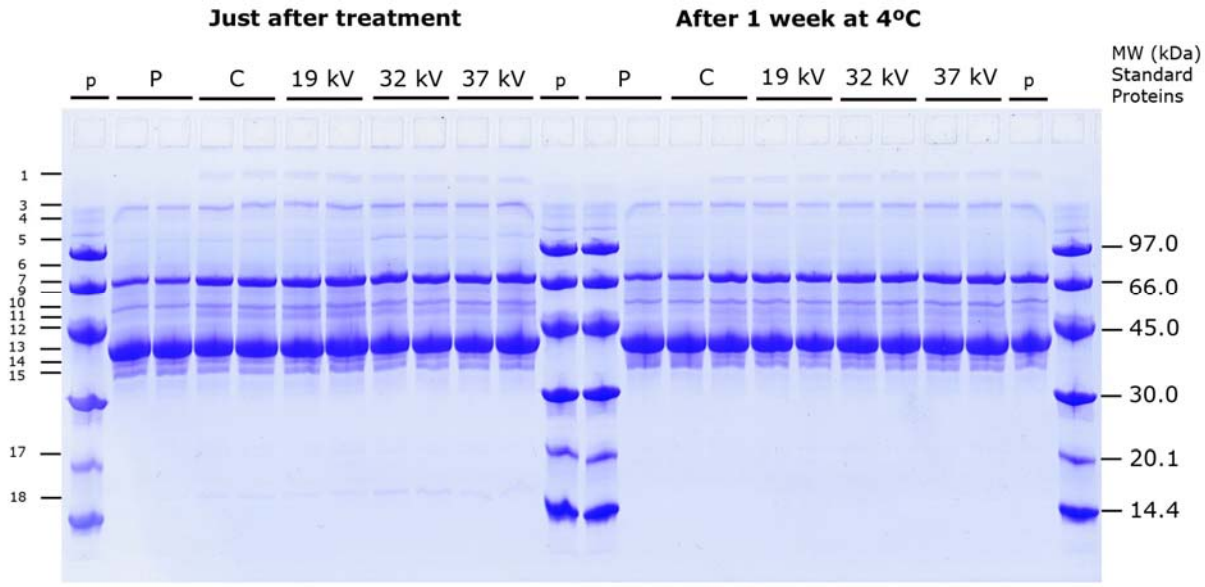

Figure 19. Water soluble protein electrophoregram of whole liquid egg **P**: pasteurised liquid whole egg; **C**: liquid whole egg control; **19 kV**: low intensity PEF ((18kV/cm-6µs-5 pulses)) treated liquid whole egg; **32 kV**: medium intensity PEF (29kV/cm-6µs-5 pulses) treated liquid whole egg; **37 kV**: high intensity PEF (34kV/cm-6µs-3 pulses) treated liquid whole egg; **p**: standard protein.

When PEF in combination with mild temperature was applied in liquid whole egg, proteolytic activity was neither detected. However, hydrolysis and a slight oxidation of the lipid fraction were observed in PEF in combination with mild temperature treated samples.

A slight hydrolysis was produced in the lipid fraction of mayog dressing samples if compared with non-treated ones.

Nutritional response

The effect of PEF treatment on the nutritional value was evaluated in liquid whole egg and mayog dressing. Both are lipid-rich matrices, and since lipids are one of the most chemically unstable food components and readily undergo free-radical chain reactions the attention was focused on three different analysis, i.e triacylglycerol hydrolysis, oxidative damage, and vitamin E content. In fact the above mentioned free-radical chain reactions not only deteriorate the lipids but also a) produce oxidative fragments, some of which are volatile and are perceived as the off-flavors of rancidity; b) degrade proteins, vitamins and pigments and (c) cross-link lipids and other macromolecules into non-nutritive polymers.

Lipid oxidation was evaluated by measuring the level of conjugated diene containing lipids, a well known index of lipid peroxidation. In both matrices no differences were detected among PEF treated and untreated samples, indicating that PEF treatment does not cause lipid oxidation, at least in our experimental condition. Regarding lipolysis, in both liquid whole egg and mayog dressing samples, PEF treatment did not cause any modification in triacylglycerol content (measured as free acidity). Our data on triacylglycerol hydrolisis are in agreement with data on lipid peroxidation. In food the increase of free fatty acid content due to triacylglycerol hydrolysis is undesirable not

only because they are more prone to oxidation but also because they lead to off flavour in the product. Finally, no differences in vitamin E content were detected in PEF treated samples.

Sensory response

All of the indicators selected to describe sensory characteristics of the custard samples produced by PEF treated liquid whole egg reveal that the sample corresponding to a PEF treatment of 34kV/cm-6µs-5pulses was the most appreciated one. In particular, this sample showed similar trend if compared with the sample corresponding to the custard obtained from untreated eggs. These preliminary results suggest that a high level of PEF treatment seems not to affect the sensory or technological properties of the treated products.

No clear differences have been described by the panelists (42 people) on the samples of mayog dressing treated with only PEF (30kV/cm, 6µs 4 pulses), PEF + mild heat (53°C, 10 minutes) and not treated. The heat step after PEF treatment resulted in positive differences in the consistence and flavor on the final products. The absolute value of the parameters was not high because the mayog dressing formulation was not added with flavorings to avoid interferences.

The PEF treatment did not affect the mayog in comparison with the untreated samples or samples subjected to a mild heat treatment after PEF treatment. In this case, as well as in the case of mayog obtained from treated eggs, a tiny decrease of the consistence has been revealed when the sample was mildly heat treated.

Response on the macrostructure

Texture properties of a fluid food are essential characteristic to be investigated for industrial products when a scale up of production is required. Indeed in order to understand the "workability" of a fluid food parameters such as firmness, consistency, cohesiveness and viscosity must be evaluated. In our case the values of the final products have to reach the texture parameters needed for a proper working of the filling equipment of an industrial plant.

Test results obtained from custard produced by PEF treated liquid whole eggs regarding firmness, consistency, cohesiveness and viscosity showed that one of the samples, corresponding to a PEF treatment of 34kV/cm-6µs-3 pulses, had a significant difference from the sample containing untreated eggs. Probably such an electric field intensity induces structural modification at the level of the protein or fat of the treated eggs. On the contrary a higher level of treatment, corresponding to sample with 34kV/cm-6µs-5 pulses, did not apparently affect the technological properties of the eggs yielding a final product (custard) similar to that produced by using untreated eggs.

The samples of heat sensitive sauce (*mayog*) produced from PEF + mild heat (53°C for 10 minutes) treated liquid whole eggs showed slight differences in terms of consistency and cohesiveness if compared to the controls. On the contrary the sample obtained with untreated eggs showed slight differences from those obtained from PEF treated eggs or pasteurized eggs in term of firmness and viscosity. In all cases, the range of workability for the mayog filling equipment showed conformity to the starndards.

In the second phase of the work the texture properties of the samples of mayog dressing produced by SIK, having a modified formulation and a reduced fat content, have been investigated. The PEF treatments on the modified mayog resulted in a reduction of the firmness, consistency and cohesiveness if compared the control (no PEF treatment). In this case the PEF treatment seems to slightly weaken the emulsion of the investigated sauce. On the contrary, the viscosity seem not be affected by the treatment. The technological range corresponds to those required for a proper workability.

Quality of Balsamic vinegar

According to the analytical data obtained during the present research, PEF reveals to be a non invasive method for the treatment of vinegars, and in particular of balsamic vinegar of Modena, because it did not alter the chemico-physical features of the product. The content in acetic acid, which is a legal parameter, was not affected by the treatment. It can be supposed that also nutritional properties like the percentage of carbohydrates (mostly sugars) remain unaltered.

5. Conclusions

Pilot plant

A PEF pilot plant consisting of a pulse generator, heat exchangers, a PEF treatment chamber and a holding cell was developed. A new type of treatment chamber was designed for achieving a controlled homogeneous electric field.

Microbial response

The safety and shelf life of products will be increased in PEF treated products due to a reduction of the initial level of important hazardous bacteria such as *L. monocytogenes* and *Salmonella*, and spoilage bacteria. The PEF and mild treatment conditions to be applied should be optimized in a product specific way, accounting for product characteristics, specific pathogens, spoilage microorganisms and important quality attributes. Important factors to take into consideration are the initial bacterial load (numbers and type of microorganisms) and the inactivation kinetics. The effect of the injury from the PEF treatment on first order heat inactivation kinetics could be modelled as a function of the electric field strength and treatment time.
PEF treatment of balsamic vinegar PEF treatment inactivated *Glucanoacetobacter* sp. when the applied electric field strength was higher than 35 kV/cm.
PEF conditions that increased the product temperature to maximum 50ºC was not sufficient for the inactivation of *L. monocytogenes* or *Salmonella* spp..
Three different approaches were used in order to increase the bacterial inactivation by PEF treatment:

- Application of consecutive PEF treatments with cooling in between

  A satisfying reduction of *L. monocytogenes* and *Salmonella* is possible using equipment that cools down the product during treatment and increases the total energy input by increasing the number of pulses.

- Use of sorbate

The use of PEF treatment and addition of potassium sorbate give an additive effect delaying the time for *L. monocytogenes* to grow to unacceptable numbers during storage.

- Application of a mild heat treatment subsequent to the PEF treatment

By combining PEF and mild heat treatment (53ºC, 10 minutes) more than 6 log CFU reductions of *S. enteritidis* was obtained in whole liquid egg and mayog dressing.

By combining PEF and mild heat treatment (53ºC, 10 minutes) up to 4 log CFU reductions of *L. monocytogenes* was obtained depending on the product (liquid whole egg, milk, mayog dressing and béchamel sauce). By increasing the heat treatment temperature from 53 to 55°C an additional 2 log CFU reduction was obtained in phosphate buffer.

PEF and heat treatment resulted in a longer shelf life of RTE ingredients and products. This was due to a reduction of initial levels of *L. monocytogenes* and *S. enteritidis* prior to storage, but not to a change in growth rate. In un-inoculated samples the time to reach high numbers during storage was increased as a result of inactivation of the number of bacteria (potentially spoilage bacteria) able to grow in the products. A change in the dominating microbial flora was noted due to PEF in combination with heat treatment.

The study of gene expression showed that the heat shock protein *groES* in *L. monocytogenes* was highly up-regulated when exposed to heat between 40-50°C up to 15 minutes. As opposed, PEF treatment alone caused a down regulation, suggesting that the PEF treatment is to brief to cause any damage to the cell that are comparable to heat stress.

Response of the microstructure and functionality

In whole liquid egg treated by high intensity PEF (18kV/cm, 6µs, 5 pulses; 34kV/cm, 6µs, 3 pulses), the continuity of the protein network was broken. However, when PEF in combination with mild temperature was applied the microstructure was not greatly affected. In mayog dressing, the lipoprotein network was observed aggregated only when the sample was treated with PEF in combination with mild temperature.
Viscosity, colour and textural profiles were unaffected by the PEF or PEF in combination with mild temperature treatments in whole egg samples. The foam stability was higher in PEF treated samples than in pasteurised ones. In mayog the main functional properties were not affected, only an increase of viscosity was observed.

Enzymatic response
Proteolytic activity was not detected in PEF treated liquid whole egg (18kV/cm, 6µs, 5 pulses; 34kV/cm, 6µs, 3 pulses), even when mild temperature was applied. However, hydrolysis and a slight oxidation of the lipid fraction were observed in PEF in combination with mild temperature treated samples. A slight hydrolysis was produced in the lipid fraction of PEF treated mayog samples.

Nutritional response

PEF treatment, in the conditions and on the matrices considered in the project, does not cause trygliceride hydrolysis or an increased lipid oxidation. Furthermore, vitamin E content is preserved.

Response on the macrostructure

High level of intensity of PEF seems to positively affect the properties of eggs when utilized as an ingredient. Moreover, the combination of PEF+heat on eggs showed slight differencess from un-treated eggs in terms of consistency and flavour when mayog were produced. The workability (value ranges for filling machinery) has been respected.

Balsamic vinegar

PEF treatments did not cause any detectable variations in density, turbidity and viscosity at normal laboratory control stage.

6. Impact on industry or research sector

It is demonstrated that PEF is an innovative non thermal processing technology that when combined with mild heating improves the quality, safety and shelf life of fluid ready-to-eat (RTE) foods and ingredients used in RTE foods. The results provided new information about PEF processing of heat sensitive and non acid RTE foods/ingredients. The results obtained demonstrated that some quality aspects may be affected; for examples are reduced O/W emulsion stability and increased foam stability of egg. Of particular importance for food safety is the inactivation of *L. monocytogenes*. This technology could significantly improve the shelf life of heat sensitive products. Interesting results were obtained for total bacterial counts and specific spoilage bacteria such as *Glucanoacetobacter* sp..

PEF combined with mild heat treatment is an alternative to commercial pasteurisation in terms of microbiological safety, but with the added value of an improved product quality. A great number of scientific and technological publishable results have been obtained related to the effect on the microbial response such as inactivation and post treatment growth of the foodborne pathogens *Listeria monocytogenes* and *Salmonella enteritidis*.

During PEF processing, the temperature increases due to ohmic heating where the pulsed electric field energy is transformed into heat. However, results have been obtained demonstrating that PEF enhances heat inactivation of bacteria. By combining PEF and mild heat treatment it is shown that even if the treatment is designed in order to keep the maximum temperature below 55°C, a satisfactory bacterial inactivation and a high overall quality is obtained.

The potential to apply PEF technology in combination with mild heat treatment on several types of ingredients/products such as liquid whole egg, heat sensitive sauces and vinegar is of high relevance to catering industry and food industry producing raw egg products/ingredients and RTE dressings. An important obstacle for the introduction of PEF technology on an industrial scale is the lack of commercial PEF equipment. A

SME is not likely to start developing a product produced by PEF technology until this aspect is solved.

The novel design of treatment chamber developed in the project enabled a homogenous treatment in a continuous flow. This was essential from a scientific point of view providing a precisely applied electric field. For production purpose a system with high flow is needed. The treatment chamber used is well suited for a high flow design. To give a good opportunity for commercialization a patent protection of the design would be advantageous. There are many features both in the single chamber and the production design that could be patented.

Research studies evaluating the effect of PEF treatment on bacteria uses bacterial counts as the normative. Developments in DNA technology will in near future give additional methods for investigation and evaluation of how bacteria respond at a physiological level by electric fields. Quantitative RT-PCR can provide novel information to the food processing industry both regarding bacterial counts, including stressed bacteria cells and as have been applied in the project, genetic responses for the bacterium's metabolic activity and stress. This is an important issue, which needs more focus in future research to secure the food safety in conjunction to PEF technology.

7. Measure of the realised impact against the potential impact described in part 5 of the DoW

- *The initial expected potential impact related to food processing innovation and competitive value has been achieved.*

The project has contributed with new knowledge about PEF technology as an innovative non thermal processing technology that improves the quality and safety of fluid ready-to-eat (RTE) foods and ingredients used in RTE foods. The potential to apply PEF technology in combination with mild heat treatment on several types of ingredients/products such as liquid whole egg, heat sensitive sauces and vinegar is of high relevance to catering industry and food industry producing raw egg products or ingredients and RTE fluid products such as dressings, sauces and beverages. PEF in combination with mild heat treatment is an alternative to commercial pasteurisation in terms of microbiological safety, but with the added value of an improved product quality. The technology could significantly improve the shelf life of heat sensitive products. It will also enable development of new products, unable to be produced using conventional heat processing.

- *The initial expected potential impact to generate more information to define microbial standards for new process has been achieved.*

The results generated about PEF have produced increasing knowledge about the lethality level for several pathogens and overall microbial flora on the basis of the inactivation kinetics and recovery during storage. This information allows industries to evaluate commercial shelf-life accounting for microbial safety and quality, and overall quality aspects.

- *The initial expected potential impact related to contribution to policy developments has been achieved*.

The integration of research and industrial area in order to develop new process to improve the safety and quality of food systems has been addressed by the collaboration of two SME`s Italian company (CAMST and Acetum), dedicated to manufacture of RTE foods and vinegar, in the validation of this new technology and in the organization of the PEF Open testing day. Also the PEF technology results were exposed in the two meetings (Gothenburg-Sweden and Bertinoro-Italy) held in conjunction with NOVEL-Q project.

- *The initial expected potential impact related to risk assessment has been achieved*.

The results generated about PEF in the project have produced increasing knowledge about the lethality level for pathogens *Listeria monocytogenes* and *Salmonella enteritidis* on basis of the inactivation kinetics and recovery during storage and contribute to improved risk assessment.

High Pressure Homogenisation (UNIBO)

1.Introduction

Homogenization is a fluid mechanical process that involves the subdivision of particles or droplets into micron sizes to create a stable dispersion or emulsion for further processing. The process occurs in a special homogenizing valve, the design of which is the heart of the homogenizing equipment. The fluid passes through a minute gap in the homogenizing valve. This creates conditions of high turbulence and shear, combined with compression, acceleration, pressure drop, and impact causing the disintegration of particles and dispersion throughout the product.

Low-pressure homogenization (<20 MPa) has been widely used by the dairy industry to avoid fat separation (creaming) in milk since its invention by Auguste Gaulin in 1900 (Diels and Michiels, 2006). In the early 1900s, homogenizers able to reach ~150 MPa were developed, while current technology can achieve 400 MPa of homogenizing pressure (Paquin, 1999; Diels et al., 2004 and 2005; Diels and Michiels, 2006). Several physical phenomena including, shear stress, turbulence, cavitation, impingement, and temperature increase are imposed on a fluid food subjected to high-pressure homogenization (HPH). HPH is a continuous process with relatively lower pressure (<400 MPa) and shorter exposure time (milliseconds) than HHP, which is a batch process requiring longer exposure times (minutes).

The HPH technology has proven to be effective for the inactivation of bacteria and enzymes (Diels et al., 2004; Picart et al., 2006; Taylor et al., 2007), stabilization of emulsions (Dybowska, 2005), and polysaccharide molecular weight reduction (Corredig and Wicker, 2001). HPH causes bacterial inactivation and cell disruption probably due to sudden pressure drop, torsion, and shear stresses, but mostly by cavitation shock waves from imploding gas bubbles (Brinez et al., 2006; Taylor et al., 2007). HPH has been studied for the inactivation of *Escherichia coli* K-12 in apple juice and apple cider (Kumar et al., 2009), for the inactivation of *E. coli* O58:H21 and *E. coli* O157:H7 in orange juice (Brinez et al., 2006), and for treatment of milk containing *Staphylococcus aureus* before cheesemaking (Lo´pez-Pedemonte et al., 2006), showing promising utilization by the food industry.

Concerning the dairy industry, Guerzoni and coworkers deeply investigated the resistance of different microorganisms to homogenisation up to 100 MPa in milk (Lanciotti et al., 1994 and 1996). In particular, the Aauthors focused on the HPH treatment of milk for the production of yoghurt (Lanciotti et al., 2004b) and of cheese (Guerzoni et al., 1999; Lanciotti et al., 2004a and 2006), highlighting that HPH had both direct and indirect effects on cheese characteristics and their evolution during ripening, in addition to a significant contribution to the non-thermal pasteurisation of the raw milk. Similarly, Vachon and coworkers investigated the effect of HPH treatment of milk (up to 200 MPa for five cycles) in the production of Cheddar cheese, showing a three to four logcycle reduction of *L. innocua* and reductions in size of casein micelles and fat globules (Kheadr et al., 2002). Guerzoni et al. (2002) demonstrated the efficacy of HPH in the decontamination and stabilization of mayonnaise based products deliberately inoculated with *Salmonella enteritidis*. Moreover, the treatment resulted in the enhancement of the antimicrobial activity of naturally occurring or exogenously added enzymes (Vannini et al. 2004).

In the pharmaceutical, cosmetic, chemical and food industries, high-pressure homogenisation is used for the preparation or stabilization of emulsions and

suspensions, or for creating physical changes, such as viscosity changes, in products. For some applications that require a high degree of disperion, pressures up to 200 MPa or higher are currently being investigated. Another application is cell disruption of yeasts or bacteria in order to release intracellular products such as recombinant proteins.
Although the range of products that undergo high pressure homogenisation is quite diverse, their microbiological quality is invariably an important parameter. Many pharmaceutical products need to be sterile, but also for most products in the food industry a partial or complete inactivation of the microbial population is desired.
Notwithstanding the number of works on the subject, uncertainty exists in the literature about the exact cause of cell disruption in high-pressure homogenisers. Experimental evidence was reported that Gram-negative bacteria are more sensitive to highpressure homogenisation than Gram-positive bacteria (Wuytack et al., 2002; Vachon et al., 2002), suggesting a correlation between cell wall structure and high-pressure resistance, which indicates that high-pressure homogenisation kills vegetative bacteria mainly through mechanical destruction of the cell integrity. Cells are indeed disrupted by high-pressure homogenisation without causing any sublethal injury (Vachon et al., 2002).

2. Objectives

The main objective of these activities was to evaluate, in comparison to the traditional heat treatments (HT), the efficacy of High Pressure Homogenisation (HPH) on the decontamination of fluid food products and its effects on antimicrobial enzymes naturally occurring or deliberately added.

Specific objectives were:

- To determine how the microbial response is affected by the technological (pressure, inlet temperature), physico-chemical (viscosity, aw, pH), compositional and biological (bacterial species and strains) variables.
- To determine how naturally occurring or deliberately added enzymes are affected by HPH doses applied and physico-chemical conditions.
- To evaluate the effects of the treatment and of the interaction process/composition on the water binding capacity and other functional properties of the proteins and on the consequent effects on food rheological properties and microstructural differentiation.
- To verify the possible changes in the health promoting characteristics of the foods (i.e. antioxidant potential) and possible changes in food ingredients and sensory properties

The effect of HPH on microbial response was evaluated by modulating; i) process variables, such as pressure, inlet temperature, number of repeated passe; ii) system variables, such as intrinsic characteristic (plant or animal origin, complexity), pH and Aw.
Inactivation and re-grow ability of some spoilage and pathogenic bacteria (*Saccharomyces cerevisiae, Zygosaccharomyces bailii, Pseudomonas fluorescens, Listeria monocytogenes, Salmonella enteritidis*, *Escherichia coli, B. cereus*) was evaluated. Studies were performed both in simple systems (model systems, milk, liquid whole eggs, apricot and carrot juices) and complex foods, i.e. milk-egg emulsions with different NaCl contents, mixed fruit juices and vegetable soups.

Also the the possible enhancement or loss of enzyme activity as a consequence of HPH treatments have been assessed for 3 antimicrobial enzymes, i.e. lactoferrin, lactoperoxidase system and lysozyme, both in model and in real systems.

Finally, the effects of HPH treatments on microstructure, protein functionality, macroscopic properties, sensory and nutritional properties were also evaluated.

3. Materials and Methods

The experiments to evaluate the effects of HPH or heat treatments on the various microorganisms inoculated in simple or complex foods have been performed according to the following procedures and equipments:

HPH treatments - A continuous high pressure homogenizer PANDA (Niro Soavi, Parma, Italy) was used for all the homogenizing treatments with the lab scale equipment (maximum pressure = 160 MPa). The inlet temperature of samples ranged between 19 and 23°C.
Treatments with higher pressure (up to 400 MPa) were performed by using a UHP unit (GEA) with a flux of 200 liter /hour and equipped with a thermal exchanger immediately after the valve. The inlet temperature of samples ranged between 19 and 75°C according the experimental plan.

Food matrices – Tomato, apricot, and carrot juices, raw whole eggs and raw milk, which were used as simple food matrices, were supplied by CAMST (Italy).
Concerning complex systems, i.e. egg/milk emulsions whose pH and Aw values had been modified with respect to the basic formulation, vegetable soups and mixed fruit juices, were produced by using rw materials supplied by CAMST.

Microorganisms – The following strains were used for the test performed during the project life: *S. cerevisiae* strain 635, *Zygosaccharomyces bailii* strain 45, *L. monocytogenes* 56Ly, *E. coli* 555, *Pseudomonas fluorescens, S. enteritidis* strain 155, *Bacillus cereus* SV90. All the strains belong to the collection of the Dipartimento di Scienze degli Alimenti – UNIBO. Yeasts were cultured in Sabouraud media at 28°C for 48 hour, while bacteria in BHI at 37°C for 24 hours before being used. Cell viability of the target organisms before and after the treatments and during the storage of treated foods was evaluated by plate counts onto Sabouraud agar plates (yeasts), BHI (bacteria) and selective media, i.e. Listeria Selective Agar for *L. monocytogenes*, Bacillus Cereus Selective Agar for *B. cereus*, Brilliant Gren Agar for *S. enteritidis*, Violet Red Bile Glucose Agar with Mug added for *E. coli*, Pseudomonas Agar Base with supplement added for *Ps. fluorescens*. All the media were from Oxoid.
For each microbiological analysis 3 or 5 repetitions of the same sample were made.

Heat treatments - For each product different thermal treatments have been performed by using different inculum levels. Treatments have been evaluated by monitoring the temperature in the core of the product. Following the treatments, samples have been immediately cooled before being used for the microbiological analyses.

Enzyme activation or deactivation – The effects of HPH treatments up to 100 MPa on the antimicrobial activity of lysozyme, lactoferrin or lactoperoxidase system on *Listeria monocytogenes* 56Ly inoculated in milk/egg emulsions have been studeied. In particular, lysozyme (3.3 mg/100 ml) and lactoferrin (2.0 mg/ml) were added directly to

the emulsions which were simultaneously inoculated with *L. monocytogenes* strain 56 Ly. As far as the lactoperoxidase systems, also hydrogen peroxide (9 ml/l) and thyocianate (11 mg/l) were added to the system to activate the enzyme (30 μg/ml).
The food systems were then subjected to HPH treatments at 75 and 100 MPa (inlet temperature: 20°C; outlet temperature: 38°C). The viable cells were detected by plate counts immediately after the HPH treatments and during refrigerated storage at 10°C.

Microstructure and functionality – Such aspects were studied by microscopic techniques and physical proterties.

Microscopic techniques

Low Temperature Scanning Electron Microscopy (Cryo-SEM) -The samples were immersed in slush Nitrogen (–210 ºC) and transferred to a cryo-trans (CT 15000 C from Oxford Instruments, Oxford, England) linked to a scanning electron microscope JEOL JSM 5410 (Jeol Tokio, Japan), operating at a temperature below –130 ºC. Samples were cryo-fractured at –180 ºC and etched at –90 ºC. The observations in the microscope were carried out at 15 kV and at a working distance of 15 mm.

Transmission Electron Microscopy (TEM) - The samples were cut into 1-mm^3 cubes, fixed (primary fixation with 2.5% glutaraldehyde and secondary fixation with 2% osmium tetroxide) and dehydrated with 30, 50 and 70% ethanol for 10 minutes. They were then embedded in epoxy resin (durcupan) (FLUKA) and the blocks thus obtained were cut in an Ultracut ultramicrotome from Reichter-Jung. The sections obtained (≅100 Å) were collected on copper grills and stained with 4% lead citrate to allow for observation in the Philips EM 400 Transmission Microscope at 100 kV.

Light Microscopy (LM) - Semithin sections were obtained as for TEM and they were observed in a Nikkon Eclipse E800 light microscope

Confocal laser scanning microscopy (CLSM) - A drop of the sample was put on a slide and stained with Rodhamine B solution (2 g/l) and Nile Red (1 g/l). Then, the mixture was covered with a cover glass. The samples were observed in a CSLM (Nikon confocal microscope C1 fitted to a Nikon Eclipse E800 microscope, Nikon Co., Ltd., Tokyo, Japan) in single photon mode equipped with an Ar-Kr laser. The excitation wavelength and emission maxima of the applied fluorescent dyes were 568/625 nm for Rhodamine B and 647/675 nm for Nile Red. Images were stored using EZ-C1 software (Nikon Co., Ltd., Tokyo, Japan).

Physical properties

Properties of foams (Foaming capacity and foam stability) - Foaming properties were evaluated by foaming capacity (FC) and foam stability (FS) according with the method of Ferreira et al., (1995). The volumes of foam and of the liquid phase are measured in stoppered graduated cylinders. For the determination of FC and FS the following formulae were used:

FC (%) = (FV/ILV) x 100%

FS (%) = [(ILV – DV) / ILV] x 100%

Drainage (ml): LVM – LVS,

where: FV: volume of foam; ILV: volume of the initial liquid phase; DV: volume of drainage; LVM: volume of the liquid phase at t = 60min after foaming was finished; LVS: volume of the liquid phase at t = 30s after foaming was finished

pH and viscosity - The pH was measured with a Crison pHmeter (Crison Instruments, Spain), and the viscosity was measured using a Brookfield rotating type viscometer (Brookfield Engineering labs. INC., Milddleboro, USA) with the nº 61 spindle at 60 rpm. Viscosity measurements were carried out at different temperatures (10 – 55 ºC).

Textural profile analysis - Egg gels were cut into cylindrical samples (20mm dia x 20mm ht). Texture profile analysis (TPA) was carried out with a TA-XT2 Texture Analyzer. A double compression test was performed using a 2.54 cm diameter rod to 50% of their original height. Textural variables obtained from force and area measurements were: Hardness, elasticity, cohesiveness, chewiness and adhesivity.

Water-holding capacity - The water-holding capacity (WHC) of gels was analysed by centrifugation at 1000g for 30min of gel cylinders in 1,5 ml centrifuge tubes. The WHC is given by the relative weight: WHC (%) = (W gel after centrifugation/ W gel before centrifugation) x 100%

Color - The gel and liquid egg color was measured using a Minolta Chroma Meter CR-400 (Minolta Co. Ltd., Osaka, Japan). The parameters measured were: L*, a* and b* (lightness, redness and yelloness).

Enzymatic response was analysed by extraction and quantification of water-soluble proteins followed by SDS-PAGE eletrophoresis. Lipids were extracted and acidity grade, hydroperoxides and secondary oxidation products were analysed.

Extraction and quantification of the water-soluble proteins - Water-soluble nitrogen was extracted according to AOAC 932.08 (AOAC, 2000) and quantified by Kjeldahl, using a mixture of K_2SO_4, $CuSO_4$ and Se (10:1:0.1) as catalyser. All the results were expressed as protein using a conversion factor of 6.25.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) - The electrophoretic study of the total protein fraction was carried out using directly an aliquot of each whole liquid egg sample. Protein concentration in these samples was determined by N-Kjeldahl, using a mixture of K_2SO_4, $CuSO_4$ and Se (10:1:0.1) as a catalyser. The water-soluble proteins were extracted as follows. First, samples were freeze-dried (TELSTAR Lioalfa-6, Terrassa, Spain) for 24 h at 10^3 Pa and –45ºC. After freeze-drying, they were defatted in a continuous extraction method (soxhlet) for 16 h with n-hexane-isopropanol (77:23) as solvent (Boselli et al., 2001; Chung et al., 1991). The extraction of the water-soluble protein fraction was carried out weighting 2.5 g of the defatted samples and adding 15 mL of distilled water. Next, these mixtures were centrifuged at 3500 rpm for 20 min in a Sorvall Super T_{21} centrifuge. Protein concentration of these water-soluble extracts was determined by the Bradford method (1976) using standard BSA for the preparation of the standard curve. The preparation of the samples for their electrophoretic study was carried out adjusting the protein concentration to 1.25 mg/mL with Laemmli buffer.

Electrophoresis was performed using the method of Laemmli (1970) using 12.5% polyacrylamide gels ExcelGel SDS Homogeneous at 600 V, 38 mA, 23 W and 15 ºC for 1h 30 min, and 8 µl of each sample were loaded in the gel in duplicate.

The standard was an Amersham low molecular weight calibration kit (GE Healthcare, UK). Protein bands were stained with Coomassie Brilliant Blue tablets (PhastGel Blue R., Pharmacia Biotech). Destaining was performed in an aqueous solution of 25% ethanol and 8% acetic acid. Samples were conserved in a solution of 10% glycerol and 7.2% acetic acid. The molecular weight of each band was determined by a densitometer intelligent Dark Box II Fujifilm LAS 1000 (Fujifilm, USA) with the programme Image Gauge (Fujifilm, USA).

Analysis of lipids - Lipids were extracted from the whole liquid egg samples according to Boselli et al. (2001). The acidity grade was determined in accordance with AOAC (1990) regulations. Hydroperoxides and secondary oxidation products were detected by spectrophotometric methods (UNE 55-047-73).

Nutritional quality – The effect of HPH treatments up to 400 MPa on the nutritional value of vegetable soups and mixed fruit juices was evaluated by measuring the total antioxidant activity (TAA) and the vitamin C content of the treated samples.
TAA was measured using the method of Re *et al*. (Re, 1999), on the basis of the ability of the antioxidant molecules in the sample to reduce the radical cation of ABTS, while vitamin C concentration was determined by titration with iodine using starch as indicator (Ciancaglini 2001).
Lipid peroxidation was evaluated by measuring the level of conjugated diene containing lipids, lipolysis measuring the free acidity in the sample, the content of Vitamin E by HPLC, and the fatty acid composition was determined by gas-chromatography.

Quality assessment – The evaluation of the effects of HPH treatments performed with the industrial-scale equipment on the quality of the complex foods has been performed through a panel test. The treated samples have been stored immediately after HPH processing at 4°C for 1 day and then served at the most suitable temperature, i.e. 60°C for the vegetable soup, 6 °C for the fruit juice mixture and milk-egg emulsions, to a group composed of 42 untrained panellist. Each panellist had to assess four indicators, i.e the consistence/texture, the taste/flavour, the smell/flavour and the colour/appearance, by attributing a score ranging from 1 to 5. The lowest value corresponded to a negative evaluation, while the maximum one to an excellent product. The indicators chosen describe the main features encountered by consumers of self-service restaurants and canteens.

4. Results and Discussion

Microbial response in vegetable and fruit based foods
A continuous lab-scale high pressure homogenizer PANDA (Niro Soavi, Parma, Italy) was preliminary used. This equipment was generally employed with repeated passes of the fluid systems at 100 MPa. The use of such an equipment allowed reductions generally not exceeding 1-1.5 log CFU/ml of the target microorganismis (*L monocytogenes* 56Ly, *S. enteritidis* 155, *S. cerevisiae* 635, *Z. bailii* 45, vegetative cells of *B. cereus* SV90) when a range of 100-150 MPa (1 pass) was applied. Repeated passes at 100 MPa allowed cell viability reductions of *L. monocytogenes* 56Ly and *S. enteritidis* 155 in raw milk and eggs up to 5 log CFU/ml. Concerning *S. cerevisiae* strain 635, 8 repeated passes at 100 MPa were able to reduce its viability in carrot juice by 5 log CFU/ml.
In order to enhance the effectiveness of the process without repeated treatments, an industrial homogenizer, able to work up to 400 MPa, was used. Significant cell reductions were obtained for all the microbial species when the industrial scale equipment was employed. Treatments higher than 200 MPa resulted in cell reductions down to the detection limit for *B. cereus* SV90, *S. enteritidis* 155, *S. cerevisiae* 635 in vegetable soups (Table 8). On the contrary, *Listeria monocytogenes* 56Ly resulted to be more resistant: in fact, only an HPH treatment higher than 300 MPa gave rise to a reduction down to the detection limit. The most severe treatments prevented the cell re-growth over a 20-day storage period at both 4°C and 10°C (abuse condition). Similar results were obtained immediately after the HPH treatments for all the microbial species inoculated in fruit juices, with the exception of *L. monocytogenes* 56 Ly which showed a higher sensitivity in the fruit juices than in the vegetable soup. In fact, probably due to

the lower pH of the fruit juice (3.8 *vs* 4.5), an inactivation of 5 log CFU/ml was obtained at 200 MPa and no recovery was observed over refrigerated storage.
Although the fluid temperature was upstream reduced immediately after the treatment, an ultra-rapid temperature increase during the treatment contributed, as combined action, to the cell inactivation. In order to better evaluate the effect of the temperature on microbial inactivation, also the inlet temperature was modulated up to 75°C. The comparison of the results relative to spores of *B. cereus* SV90 inoculated in tomato soup and treated at 32 and 75°C showed that spore inactivation was improved by increasing the inlet temperature (Figures 20 and 21). In particular, when 400 MPa was used in combination with an inlet temperature of 75°C, an inactivation down to the detection limit was observed. However, the comparison of the data obtained after a 1-week storage at 25°C showed that the initial inactivation of this sporeformer strain was apparent and that a cell load of about 3 log CFU/ml was detected also in the most severely treated samples (inlet temperature: 75°C; pressure: 400 MPa). However, no recovery was observed when the samples were stored at 4°C over a period exceeding one week.
Also the sensitivity of *S. cerevisiae* 635 and *Zygosaccharomyces bailii* 45 and the target pathogens to pressure was improved by the increase of the inlet temperature.
The importance of the combined effect pressure/high inlet temperature on microbial inactivation was confirmed also in fruit juices.
The inlet temperature had strong effects also on soup microstructure and some physico-chemical properties. It is interesting to note that the increase of the inlet temperature resulted in reduced mean sizes of particles, and the consequent product lower viscosity induced a lower rise in temperature during HPH treatments. As far as the colour, the most severe treatment resulted in an increase of the "b" value of the vegetable soup, while no significant differences in relation to the HPH severity were observed for the fruit juice probably due to the lower pH values. No significant changes were observed for the pH in both fruit juices and vegetable soups.

Microbial response in emulsions
Concerning milk-egg emulsions having pH 4.5 and characterised by different NaCl contents, the effect of the pressure level on the inactivation of *Listeria monocytogenes* 56 Ly increased with the pressure up to a threshold level of pressure after which the effect did not increase due to a phase separation, which favours the microbial growth with respect to a compartimentalised system. The pressure at which a phase separation was observed was >170 or >150 when NaCl was 1% and 2%, respectively. The Figures 22 and 23 show the effects of pressures up to the threshold level and NaCl addition on the inactivation level and viability recovery overtime of *L. monocytogenes* 56Ly. An inactivation level of about 0.5-0.7 log CFU/ml was achieved when the pressure ranged between 70 and 100 MPa. A drastic dose/effect discontinuity was observed when 150 MPa was used with a consequent temperature increase up to 55°C. In such systems without NaCl added a pressure higher than 170 MPa induced a phase separation. In Table 9 the effects of pressure and inlet temperature on emulsions outlet temperatures are reported.
When the NaCl content increased up to 1% and 2% the effect of pressures higher than 150-170 MPa was lower than that observed in salt-free emulsions. Moreover, while no recovery and growth over storage was observed in the NaCl free emulsions, a major recovery rate was observed in the presence of NaCl.

A possible explanation of these results can be found in the microstructural results. In fact when no salt was added, oil droplets of the emulsion were surrounded by a barrier of natural emulsifiers. When pressure increased, a coalescence phenomenon progressively occurred due to the loss of the emulsifiers barrier. At a pressure level higher than 150-170MPa a separation phase occurred.

Data relative to the D values and the equivalent processes (thermal or HPH) able to kill a specific microbial level have been obtained (Table 10). Such values have been calculated by using the linear part of the deactivation curves. In fact, as reported by many Authors the inactivation curves relative to physical and particularly thermal treatments are generally non linear and show a tail of survivors.

Microstructure and functionality of emulsions

The effects of HPH treatments up to 400 MPa on the microstructure, protein functionality and chemico-physical properties of egg/milk emulsions have been evaluated.

The emulsions were oil in water emulsions in which oil was trapped in a protein matrix.

The sample treated at 200 MPa showed a disruption immediately after the treatment due to a too high pressure. Pressures higher than 190 MPa (the pressure level at which the disruption occurred) are not suitable for this kind of product although they have a good efficiency in killing microorganisms.

The control sample (Figure 24A) showed a continuous water phase composed by the water soluble components of the sample mainly soluble proteins from egg and milk. In addition, also a continuous phase, made up with caseins and other components from the yoghurt and the egg (fat globules from milk surrounded by a protein membrane, protein granules from egg,...), was also observed; it can be described as a lipoprotein phase. Oil droplets (1 to 15 μm) from the sunflower oil were observed closely interacting with this lipoprotein network.

The structure of the samples treated at 70 MPa (Figure 24B) was found to be similar to that of the control sample (0.1 MPa). However, the lipoprotein phase was thicker and the oil droplets were larger than in the control sample. This was probably due to a homogenisation effect during the treatment.

When the sample was treated at 100 MPa, the lipoprotein matrix was observed clearly aggregated. The oil droplets from the sunflower oil appear bigger and deformed due to a coalescence phenomenon (Figure 24C). When higher pressure was applied (150 MPa) very big oil droplets appeared (Figure 24D). A new fat phase was forming and the other components (mainly proteins) were now closely interacting with each other (this could be related to the N-Soluble study, which showed a progressively insolubilisation of proteins). This effect indicated that a progressive separation of phases was going on as the pressure increased, with a complete disruption of the emulsion at 250 MPa (Figure 24E).

The oil droplets trapped in the protein matrix can be observed in details at an ultrastructural level in Figure 25A, in which the samples were stained with Nile Red, showing the fat phase in green. The oil droplets are surrounded by a barrier of natural emulsifiers (caseins from the yoghurt and phospholipids from the egg) that stabilise the oil of the O/W emulsion. In the samples treated by HPH ≥ 100 MPa (Figures 25C, D, E and F) a coalescence phenomenon progressively occured when pressure increased. This phenomenon was due to the loss of the emulsifiers barrier that surrounds the oil

droplets. An accumulation of tensoactives was produced on the new interface water-oil, with phase separation due to the HPH effect after a certain pressure threshold.
These results can provide a possible explanation of the dose/effect discontinuity of the pressure on *L. monocytogenes* 56Ly above reported. In fact the stability of the emulsions is regarded as the limiting factor of the microbial growth in the emulsions.

According to the SDS-PAGE analysis of the N-soluble protein fraction of the various HPH-treated emulsions, the electrophoretic pattern changed when the pressure applied was higher than 150 MPa; a progressively insolubilisation of proteins was produced mainly in band 4 (Figure 26). Furthermore, unsolubilisation of proteins occurred also when the sample was treated at 250 MPa and band corresponding to the ovalbumin was lost. It could be attributed to the increase of the T^a during the process. Ovalbumin from the egg is the most sensitive protein to heat in this system.

The chemical analysis of the fat extracted from the samples showed neither changes in the acidity index as the applied pressure increased, nor during storage. This result could indicate that lipolysis was not produced. Furthermore, any changes in the oxidation parameters were observed neither with the HPH treatment nor after 1 week of storage at 4 º C.

Generally, no significant changes in the pH of the HPH-treated samples were noticeable. Moreover, pH-values appear to be stable for a week of storage.

Enzyme activation or deactivation in real system
The effects of HPH treatments up to 100 MPa on the antimicrobial activity of lysozyme, lactoferrin or lactoperoxidase system on *Listeria monocytogenes* 56Ly inoculated in milk/egg emulsions have been studied.
Data reported in Table 11, detected immediately after the treatments, indicate that lactoperoxidase is the most effective antimicrobial enzyme under the adopted experimental conditions. Moreover, the antimicrobial activity of this enzyme increased by increasing the pressure level up to 100 MPa. Also the effectiveness of lactoferrin and lysozyme were maximized by a treatment at 100 MPa. During the refrigerated storage the cell load of the target organisms remained constant in the control sample, while it decreased by 1.5 and 2 log CFU/ml within 24 hours in the samples treated at 75 or 100 MPa, respectively (no added enzyme). The presence of the antimicrobials fastened the death kinetics of *Listeria monocytogens* 56Ly particularly in the samples treated at 75 and 100 MPa. In fact after 24 hours of refrigerated storage *L. monocytogenes* 56Ly was under the detection limit in all the samples with the enzymes and HPH treated at the highest pressures.
A refrigerated storage up to 5 days did not affect the antimicrobial activity of the 3 enzymes previously sucbjected to HPH treatments at 100 MPa, thus evidencing that no significant reduction of the antimicrobial activity, due to the storage, occurred.

Rheological behaviour
The emulsions behaved as a non-newtonian fluid. Data obtained showed substantial differences in the rheological behavior of the fluid as a consequence of High Pressure processing (Figure 27). Namely, pressurization appeared to induce a substantial increase in the shear stress of the emulsion at any temperature. It is remarkable that the shear

strength at a shear rate of zero was also close to zero for the non-treated samples, thus resembling the behavior of a pseudoplastic fluid. Differently, the treated samples behaved as a Herschel-Bulkley fluid, which means that shear stresses took non-zero values when shear rates were close to zero.

Effects of HPH treatment on nutritional quality

The effect of HPH treatments up to 400 MPa on the nutritional value of vegetable soups and mixed fruit juices was evaluated by measuring the total antioxidant activity (TAA) and the vitamin C content of the treated samples.

The HPH treatment overall increased the TAA of vegetable soups; in particular, a treatment at 300 MPa resulted in a significant increase of TAA compared to that of untreated samples (Figure 28). The increase of TAA could be explained by the break of intact vegetable cell walls after HPH processing with a consequent extraction of intracellular antioxidant, particularly polyphenols, into extracellular matrix. Although oxidation has been proposed to cause a progressive decrease in polyphenol antioxidant properties, polyphenols with an intermediate oxidation state can exhibit higher radical scavenging efficiency.

No significant decrease in vitamin C content was observed following HPH treatments of both vegetable soups and fruit juices.

The effects of different HPH pressure levels on nutritional quality of milk-egg emulsions with different NaCl concentrations was evaluated by measuring lipid peroxidation and lipolysis.

In the milk-egg emulsions, the fatty acids present in the highest amounts were linoleic acid>oleic acid>palmitic acid>stearic acid. Myristic, palmitoleic, and docosanoic acid were also found in traces. The fatty acid composition of the emulsions reflects the fatty acid composition of the single constituents. In fact the fatty acids mainly present in sunflower oil, eggs, yogurt are linoleic acid, oleic and palmitic acid, respectively.

No significant modifications were observed in the fatty acid composition and lipid peroxidation of the samples in relation to their NaCl contents or of the pressure level used for the treatments(Figures 29 and 30). Lipid peroxidation is a major cause of quality deterioration of many food products, impairing both flavor and nutritional value. In addition, an increase in peroxidizing lipids may pose health risks.

In this study, at the tested conditions, an increase in lipid oxidation, in spite of the high content of linoleic acid in the sample, was observed. This could also be explained by the inactivation of lipoxygenase, the enzyme that catalyses the oxygenation of fatty acids into the corresponding lipid hydroperoxides. According to Hendrickx (1998), soybean lipoxygenase is inactivated by several combinations of high pressure (up to 750 MPa) and temperature (0–75°C) for a period of 5 min.

Regarding vitamin E content, no differences were detected in relation either to the NaCl content or the pressure used for treatments. The preservation of the Vitamin E in the HPH treated samples contributed to the stability of lipids, in agreement with our data on lipid peroxidation.

Grouping samples on the basis of their NaCl content, free acidity appeared significantly higher in samples without NaCl, while no differences in free acidity were detected grouping the samples on the basis of the pressure used for their treatments.

The lower acidity observed samples added with NaCl could be attributed to changes in sample pH and to modification of the environment leading to a different activity of lipases, i.e. the enzymes responsible for hydrolysis of animal and vegetable fats and

oils. Also HPH treatments could affect the functionality of the enzymes due to changes in their protein conformation.

Quality assessment

The suitability of the HPH treatments to be used at industrial scale for 3 complex foods, i.e. fruit juices, vegetable soups and milk/egg emulsions, has been assessed by evaluating the shelf-life and the sensory properties of the HPH-treated products.

Concerning natural microflora, HPH treatments at 200 MPa of fruit juices and vegetable soups resulted in a dramatic cell viability reduction of total aerobic microflora, yeasts, lactobacilli and lactococci immediately after the treatment. Data relative to the evolution of the surviving cells have been monitored during the storage over a 30 day period at 4°C and 10°C (abuse condition) and then used to calculate the shelf-life of the untreated and HPH treated samples (Table 12). The shelf-lives have been calculated as the time necessary to reach a cell load of 7 log CFU/ml and 6 log CFU/ml for bacteria and yeasts, respectively. HPH treatments at pressure levels of 300 and 400 MPa allowed to obtain shelf-lives > 30 days, also for vegetable soups, having a pH of about 6, stored also at 10°C.

As far as the milk/egg emulsions, no pressure levels higher than 150 MPa was adopted due to the emulsion rupture. However, the treatment at 150 MPa assured a shelf-life of 14 and > 30 days for samples stored at 10 and 4°C, respectively.

Concerning the panel test, it was performed by a group of 42 untrained panellists who had to describe the main features encountered by consumers of self-service restaurants and canteens. Significant differences were observed for the palatability which received higher rates for the all the products treated by HPH, particularly at the highest pressures (Table 13). On the contrary no significant differences were observed for taste/flavour, smell/flavour and the colour of both vegetable soups and fruit juices. Concerning the milk-egg emulsions, the HPH treatment allowed the maintenance of the natural colour with a positive response by the panellists also for the taste.

5. Conclusions

The results above described, related to the inactivation levels and growth of the pathogenic species in vegetable soups, fruit juice mixtures and milk-egg emulsions processed with the industrial-scale equipment, allowed to draw some conclusions on the safety of the processed foods. It is important to point out that the inoculum levels were > 5.5 log CFU/ml, while the most frequent concentration (mode) of the major part of the pathogens in raw materials of plant and animal origin does not exceed -2 log CFU/ml. In Table 14 data relative to the pressure levels at which inactivations ≥ 5 log CFU/ml were achieved for the target pathogens and no recovery was observed during refrigerated storage, for 30-50 days, are summarised. The safety level in terms of pathogen inactivation in fruit juices and vegetable soups increased with the pressure up to the maximum level tested (400 MPa). However, a continuous linear relationships between the pressure applied and inactivation levels were not observed. In fact for the pathogenic and spoilage species taken into consideration a discontinuity in the inactivation rate was detected regardless the food system: the inactivation level of the various strains did not exceed 1.5-1.7 log units per treatment up to 150 MPa. On the

other hand, a treatment at 200 MPawas able to inactivate more than 5 log CFU/ml for the major part of the species particularly when inoculated in vegetable and fruit juices
Concerning egg/milk emulsions, the exposure to pressures > 150-170 MPa gave rise to a phase separation which made unfeasible the use of higher pressure levels particularly in the presence of NaCl which enhanced the coalescence phenomenon.
The physical factor that most significantly affected the pressure efficacy was the inlet temperature. In fact an inlet temperature of 75°C enabled the inactivation of 4 log CFU/ml of *Bacillus cereus* spores at 400 MPa in tomato soup. Under these conditions no recovery was observed over storage at room temperature.
Therefore an appropriate combination of sublethal temperatures and pressures >200 MPa can assure the safety and stability also of food systems whose pH allow the survival and growth of sporeforming and dangerous bacteria.
A promising opportunity to be developed at industrial level, to enhance the food stability, is the addition of antimicrobial enzymes such as lysozyme, lactoferrin or lactoperoxidase system pretreated at 100 MPa.
The HPH can induce significant changes in the emulsion microstructure at pressure > 100 MPa. Oil droplets appeared bigger and deformed due to a coalescence phenomenon and a visible phase separation was generated at pressures > 150 MPa particularly in the presence of NaCl.
Under the adopted conditions HPH treatments did not induce a significant loss of vitamins C and E in the considered food systems, while it induced an increase in the antioxidant activity (TAA) of raw vegetable soups, which can be attributed to a generation of polyphenol derivatives having higher oxygen scavenging properties with respect to their precursors.The HPH treatment of the emulsions did not enhance the lipid peroxidation with respect to the untreated controls.
In general the results obtained demonstrate that HPH treatments can be used as a safe alternative technique to thermal treatments. In addition it can give rise to products that maintain their original colour as well as nutritional and antioxidant properties, while having an improved palatability.

6. Impact on industry or research sector

The results obtained during the project life evidenced the relevant potential impact of HPH as an alternative process to thermal treatments. In fact the HPH process, particularly when applied by using already available state of the art equipments able to work up to 400 MPa, is able to: i) inactivate, in simple and complex food, up to 5 log units of pathogenic species such as *Listeria monocytogenes, Salmonella enteritidis,* toxinogenic sporeformers such as *B. cereus*, and spoilage bacteria and yeasts; ii) to prevent the regrowth of the inoculated species in HPH-treated vegetable soups, fruit juices and appropriately formulated emulsions.
Additional factors, such as the use of inlet temperatures of 32-75°C resulted in a significant inactivation of spores of *B. cereus*. On the other hand the instantaneous temperature increase recorded during the process did not negatively affect the quality of the products due the extremely rapid process (a few milliseconds) and the subsequent immediate cooling of the products.
On the basis of these results and of the existence of already available industrial equipments this technology can be successfully applied in the various industrial sectors such as vegetable, egg and egg-derived products, catering, fruit juices, dairy products,

sauces, in order to totally inactivate pathogens and improve the shelf-life in terms of microbial, nutritional and organoleptic property stability.
Although specific experiments have not been developed, an important application could be the cold decontamination of grape must which can allow the desired elimination of the use of SO_2 with relevant advantages for the consumer safety. In fact the HPH treatment can reduce both the contamination of unwanted microorganisms and oxidative enxymes such as polyphenoloxidase.
A side effect of the HPH process regarded the changes on the fluid viscosity and particularly fruit juices. The possibility to modulate this aspect in addition to texture modifications can be exploited by the industry for product differentiation/innovation. In fact previous published data of this research group showed that an HPH pre-treatment of milk results in dairy products (cheeses, yoghurts, probiotic fermented milks..) having different ripening dynamics and profiles, microstructural and organileptic properties, taste and shelf-life.
Moreover it has been demonstrated and published that HPH is able to inactivate several phages specific for the most diffused lactic acid bacteria species used as starters for the dairy industry. These results suggest the possibility of an important exploitable use in the decontamination of fluid foods from human pathogenic viruses. This aspect opens a new research perspective for food virus decontamination, which is currently performed by using chemicals or pasteurization.
Another aspect related to the research sector is the need to better understand the mechanisms and the thermodynamic aspects of HPH for microbial or virus inactivation. Such a knowledge is essential to identify and optimize additional factors for increasing the process efficacy.
In conclusion the results obtained can be easily exploited by the food industry and realistically applied at industrial level in alternative to heat btreatments also because the existing equipments are suitable to be used to achieve the quality and safety objectives.

7. Measure of the realised impact against the potential impact described in part 5 of the DoW

- *The initial expected potential impact related to food processing innovation and competitive value has been achieved.*

The results obtained provided new knowledge on the advantages and disadvantages of HPH technology as an innovative non thermal technology. The technological potential of HPH can be successfully exploited for the production of ingredients/RTE foods like stable whole eggs, refrigerated milk, dressings, emulsions, fruit juices and vegetable soups.
The quantitative dose/effect data obtained for the various microorganisms and systems can allow the development of safe and high quality food products having an extended shelf-life also in comparison with the heat-treated ones.
Innovative vegetable and fruit based foods having the colour, flavour, nutritional properties of untreated fresh products can also be obtained, while reducing the side effects of heat treatments.
In terms of possible limits of this technology it has to be mentioned that while no pressure limits have been identified for vegetable and fruit fluid foods, the use of pressures > 200 MPa in food emulsions results in a phase separation.

- *The initial expected potential impact to generate more knowledge and strategies to minimize the public health impact of food borne pathogens has been achieved*.

The results obtained on HPH use alone or in combination with other factors provided new information regarding the sensitiveness of selected pathogens or toxinogenic species such as *Listeria monocytogenes, Salmonella enteritidis, Bacillus cereus* in the varios food systems and conditions. The data concerning the inactivation rate, dose response, regrowth ability under the various conditions allow the identification of appropriate measures and strategies to generate innovative products minimizing the public health impact of the most diffused in Europe foodborne pathogens.

- *The initial expected potential impact to improve the knowledge on the effect of this technology on chemico-physical, microbiological and structural modification has been achieved.*

The results obtained within this project allowed the identification of compositive and process conditions suitable to maintain the desired qualitative and nutritional food properties and in some cases to improve organoleptic properties such as the palatability.

- *The initial expected potential impact to enhance the application of this technology in the food sector has been achieved*.

The dissemination activities of the data produced within this project during the various meetings and events attracted the interest of the food industry, and industrial projects based on the use of HPH have been submitted (Italian project: Industria 2015) and will be co-founded by the involved industries.

- *The initial expected potential impact related to contribution to policy developments has been achieved*.

The integration of research and industrial partners allowed the development of new processes and products. These products have been validated at industrial level in collaboration with Gea Niro Soavi (Parma, Italy), which produces HPH equipments, and CAMST producing emulsions, fruit juices and vegetable soups. In particular the integrated approach involving mechanical and food industry resulted in 1) a deeper knowledge of the performances and limits of the equipment for the mechanical industry and an instrument for its improvement; 2) an innovation tool for the food industry in general in terms of products and process. During the meetings in Gothenburg (Sweden), Madrid (Spain) and Bertinoro (Italy) the results on HPH technology have been critically exposed and discussed with some partners of the NovelQ project.

- *The initial expected potential impact related to risk assessment has been achieved*.

The generated results on the inactivation kinetics and regrowth ability of foodborne pathogens (*L. monocytogenes, S. enteritidis, B. cereus*) by HPH treatments allowed to develop survival models and implement them in a user-friendly software tool aiding to validate this new technology. With a laboratory equipment where pressures were limited to 160 MPa, a satisfactory level of inactivation was achieved by repeating the treatments. On the other hand, the safety level in terms of pathogens inactivation can be improved with an industrial scale homogenizer able to reach pressures up to 400 MPa: with this equipment inactivations ≥ 5 log CFU/ml were achieved for the target pathogens with only one treatment and no recovery was observed during refrigerated storage for 30-50 days.
The new concept of Food Safety Objective for selected pathogenic species like *Listeria monocytogenes*, related to risk assessment, has been taken into consideration for the fluid vegetable products in relation to the heat treatment. By considering the use of repeated treatments at pressures <150 MPa as unrealistic for the food industry, pressures >200 MPa have been used with an industrial scale equipment. The modulation of pressures between 200-300 MPa allowed not only the shelf-life improvement, but also the eradication of the inoculated *Listeria monocytogenes* and *Bacillus cereus* strains and the prevention of their regrowth also under abuse conditions. The efficacy of the treatment, at least for fruit and vegetable juices, allowed the achievement of the Food Safety Objectives for *Listeria monocytogenes, Salmonella enteritidis* and *Bacillus cereus* also when products with an extended shelf-life were considered.

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Table 8. Effects of HPH treatments on *Escherichia coli* strain 555, *Pseudomonas fluorescens, B. cereus* SV 90, *L. monocytogenes* 56 Ly, *Saccharomyces cerevisiae* 635 and *Zygosaccharomyces bailii* 45 inactivation in vegetable soup in relation to the inoculum level and pressure applied

Microbial species	**Inoculum level (log CFU/ml)**	**Cell Load immediately after HPH treatment**		
		Pressure level (MPa)		
		100	**160**	**300**
S. cerevisiae 635	5.5	4.6	3.2	-
	1.8	-*	-	-
Ps. fluorescens	5.9	3.0	1.8	-
	2.3	-	-	-
B. cereus SV90	6.0	5.1	3.9	-
	2.3	1.4	-	-
L. monocytogenes 56Ly	6.8	6.4	5.9	-
	2.5	2.0	1.1	-
Salmonella enteritidis 155	6.5	4.9	3.5	-
	2.1	-	-	-
Zygosaccharomyces bailii 45	5.5	4.3	4.0	-
	1.9	-	-	-
				-

*Under the detection limit, i.e. 1 log CFU/ml

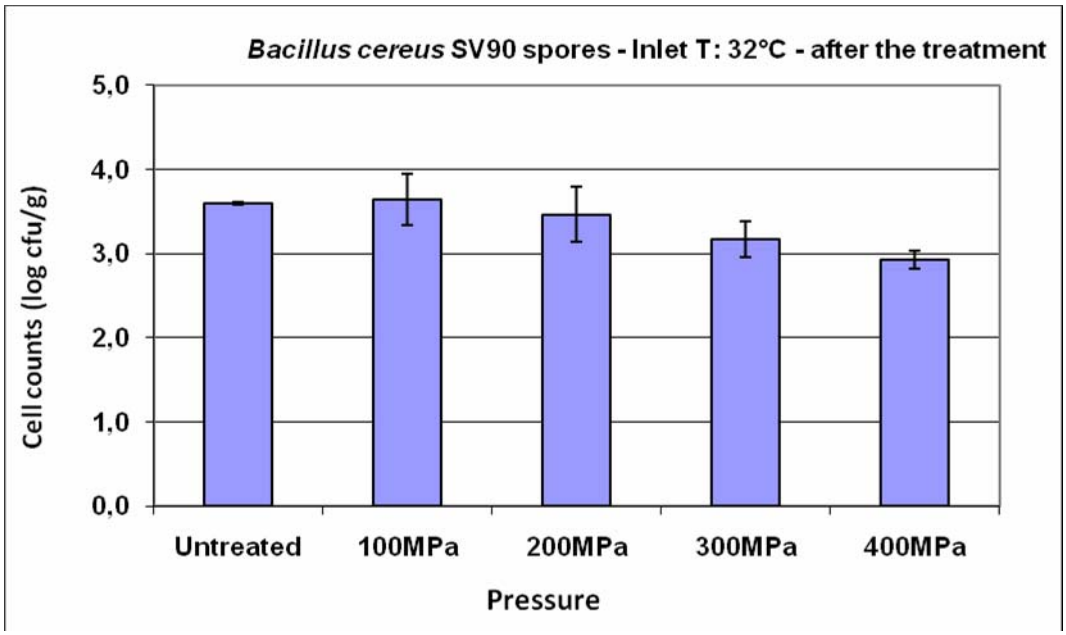

Figure 20- Cell counts of *Bacillus cereus* SV90 spores inoculated in tomato soup detected immediately after HPH treatments at increasing pressure levels (Industrial-scale equipment; Inlet temperature: 32°C).

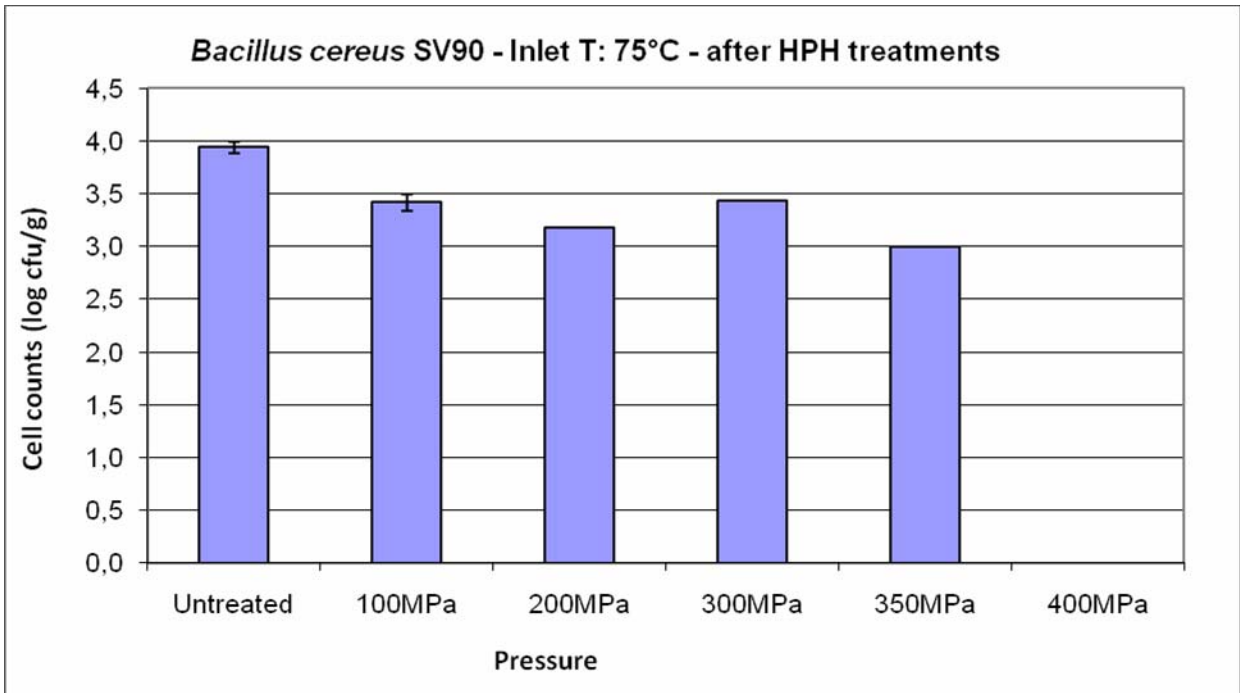

Figure 21- Cell counts of *Bacillus cereus* SV90 spores inoculated in tomato soup detected immediately after HPH treatments at increasing pressure levels (Industrial-scale equipment; Inlet temperature: 75°C).
* Under the detection limit

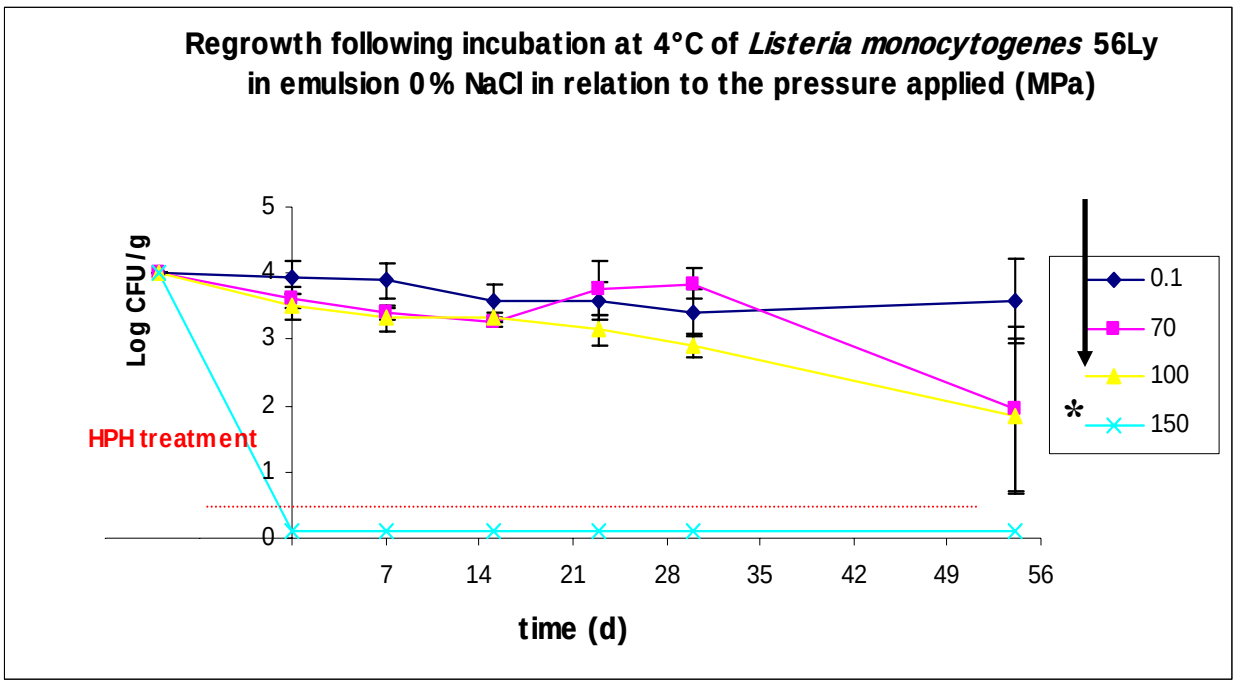

Figure 22- Effect of HPH treatments at different pressures (industrial-scale equipment) on viability loss and cell recovery of *Listeria monocytogenes* 56Ly inoculated in milk-egg emulsions with no NaCl added and stored at 4°C.

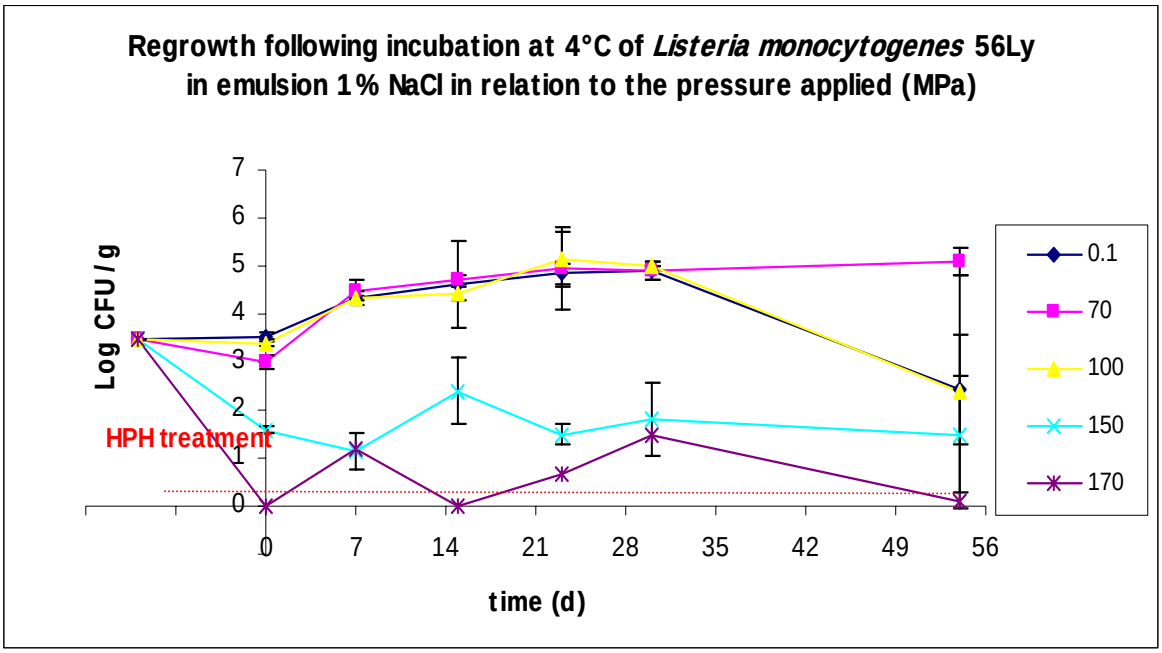

Figure 23- Effect of HPH treatments at different pressures (industrial-scale equipment) on viability loss and cell recovery of *Listeria monocytogenes* 56Ly inoculated in milk-egg emulsions with 1% NaCl added and stored at 4°C.

Table 9 – Data relative to the temperature increase following HPH treatments at different pressures of milk-egg emulsions (industrial-scale equipment).

NaCl content	Pressure (MPa)	Inlet temperature (°C)	Outlet Temperature (°C)
0%	0.1	15.5	15.7
	70	15.3	32.5
	100	15.3	41.2
	150	15.3	55.2
1%	0.1	14.2	17.4
	70	14.0	30.5
	100	13.9	40.2
	150	13.6	57.3
	170	13.5	62.0
2%	0.1	14.7	19.4
	70	14.7	33.7
	100	14.7	42.1
	130	14.7	54.9
	150	14.7	56.0

Table 10 - HPH and heat (HT) treatments equivalent in terms of inactivation efficacy. The equivalence was defined on the basis of the ability to reduce a defined extent (log CFU/ml) of the target organism in specific food systems. The last column reports the D values at 70°C for the various species in the different food systems taken into consideration.

Microorganism	System	pH	log CFU/ml reduction	HPH Pressure (MPa)	HEAT Treatment time (minutes)	TREATMENTS D_{70} (minutes)
Listeria monocytogenes 56Ly	Vegetable soup	5.6	6	350	0.48	2.88
	Fruit juice	3.5	6	200	0.19	1.14
	Egg-dairy emulsion 0% NaCl 1% NaCl	5.6	 4 4	 150 170	 0.30 0.20	 1.2 0.8
Salmonella Enteritidis	Vegetable soup	5.6	6	300	0.35	2.1
	Fruit juice	3.5	5.5	200	0.11	0.605
Saccharomyces cerevisiae 635	Vegetable soup	5.6	6	300	0.15 – 0.20	0.9 – 1.2
	Fruit juice	3.5	5.5	200	0.15 – 0.20	0.15 – 0.20
Bacillus cereus SV90 (vegetative cells)	Vegetable soup	5.6	6	350	1.25	7.5
	Fruit juice	3.5	6	200	1.05	16.3

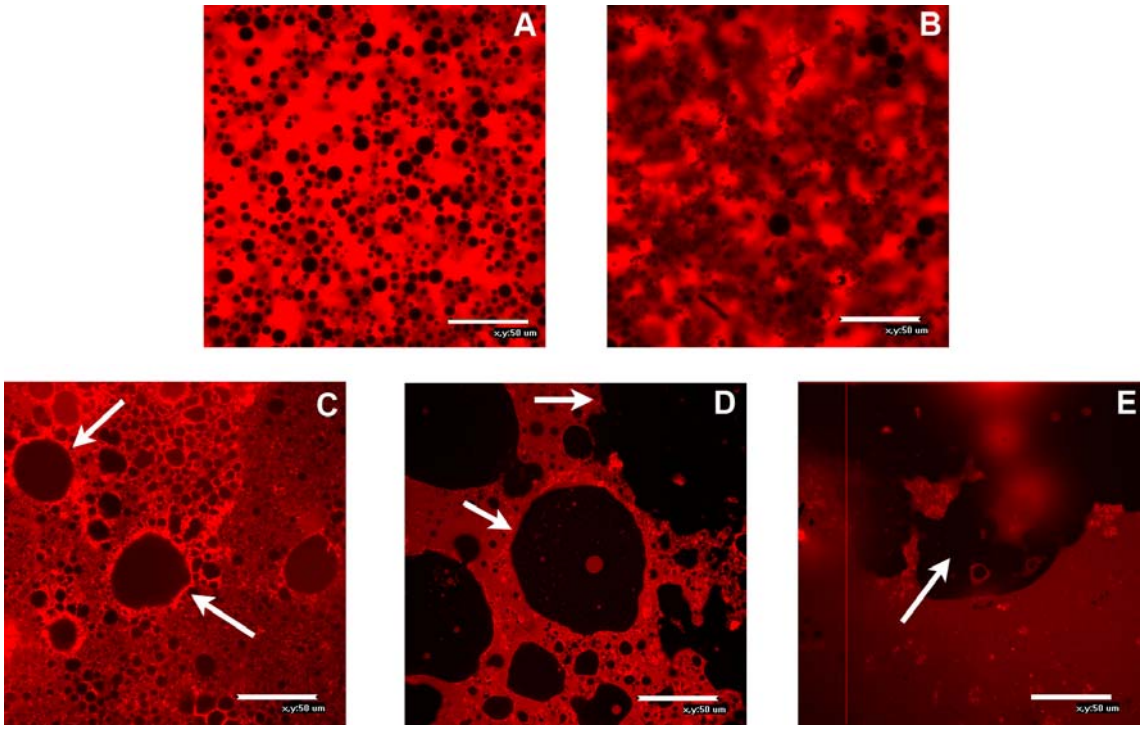

Figure 24. CLSM (Excitation wavelength and emission maxima were 568/625 nm for Rhodamine B). A: Control: Samples treated by HPH at 0.1 MPa; B: Samples treated by HPH at 70 MPa; C: Samples treated by HPH at 100 MPa; D: Samples treated by HPH at 150 MPa; E: Samples treated by HPH at 250 MPa (E). 60x (arrow: oil droplets)

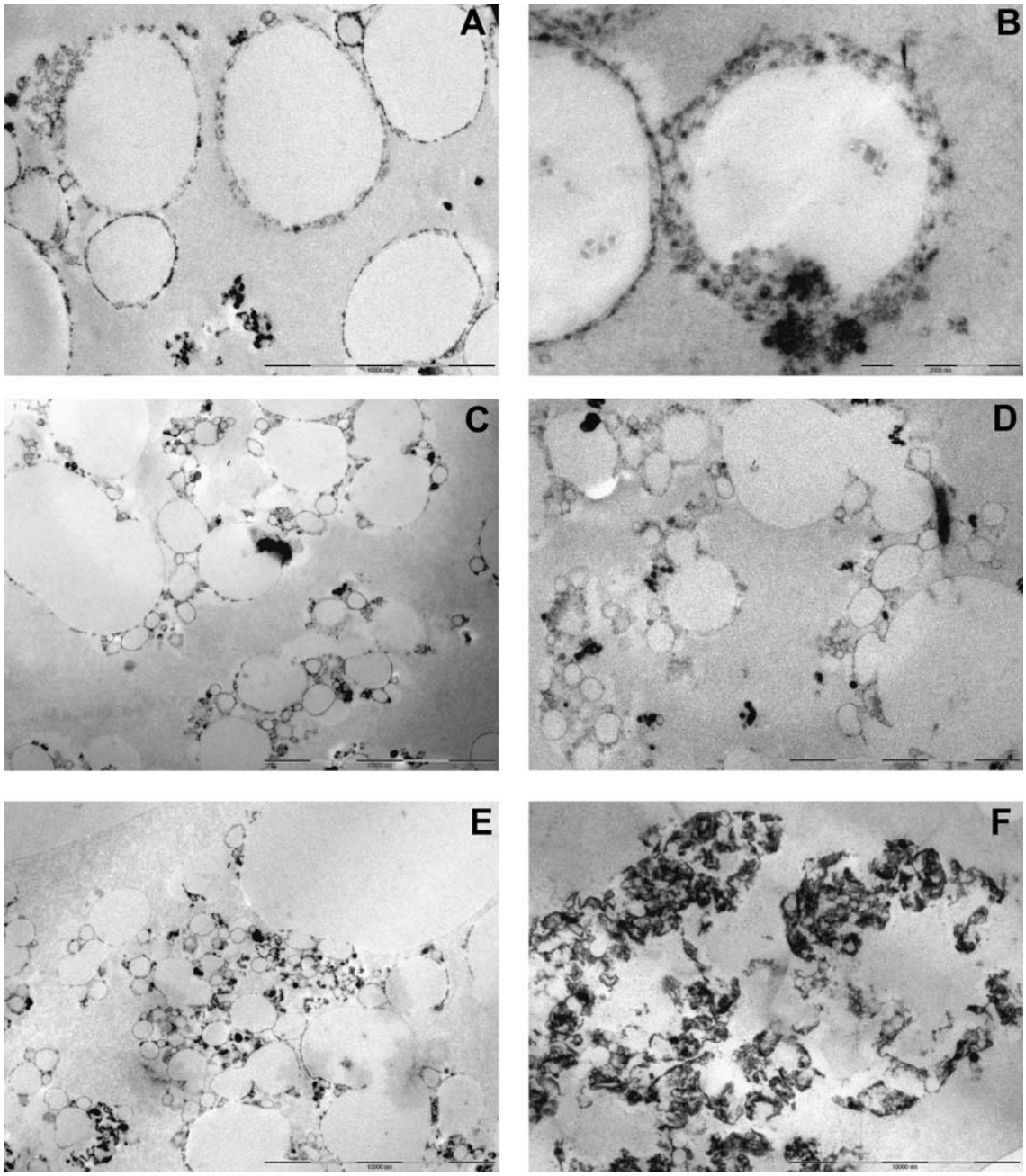

Figure 25. TEM. Control: Samples treated by HPH at 0.1 MPa (A); Detail of control samples (B); Samples treated by HPH at 70 MPa (C); Samples treated by HPH at 100 MPa (D); Samples treated by HPH at 150 MPa (E); Samples treated by HPH at 250 MPa (F). 1200x

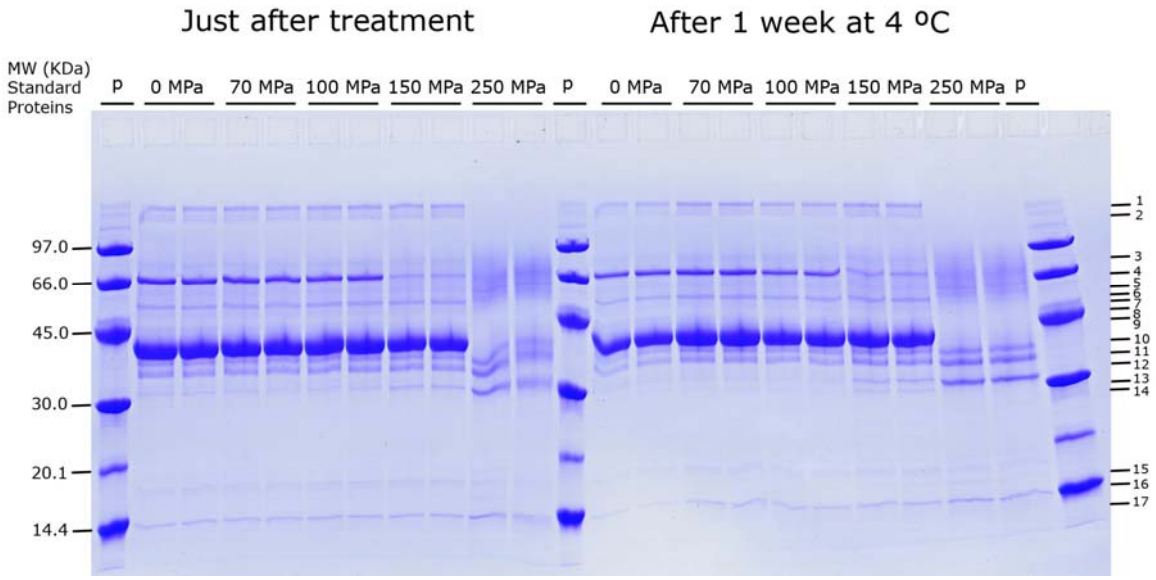

Figure 26. Electrophoregram of N-soluble of HPH treated Mayog samples, just after treatment and after 1 week at 4 ºC

Table 11. Effects HPH treatments on the antimicrobial activity of lysozyme, lactoperoxidase system and lactoferrin evaluated towards *Listeria monocytogenes* 56Ly inoculated in a milk/egg emulsion (Lab scale equipment). The inoculum level was 6.5 log CFU/ml.

Enzyme	**Cell counts (log CFU/ml) detected immediately after HPH treatment**		
		Pressure level (MPa)	
	0.1	**75**	**100**
Control*	6.45	6.00	5.30
Lysozyme	5.90	5.50	5.00
Lactoferrin	5.80	5.40	4.50
Lactoperoxidase system	5.70	4.80	3.60

*No enzyme added

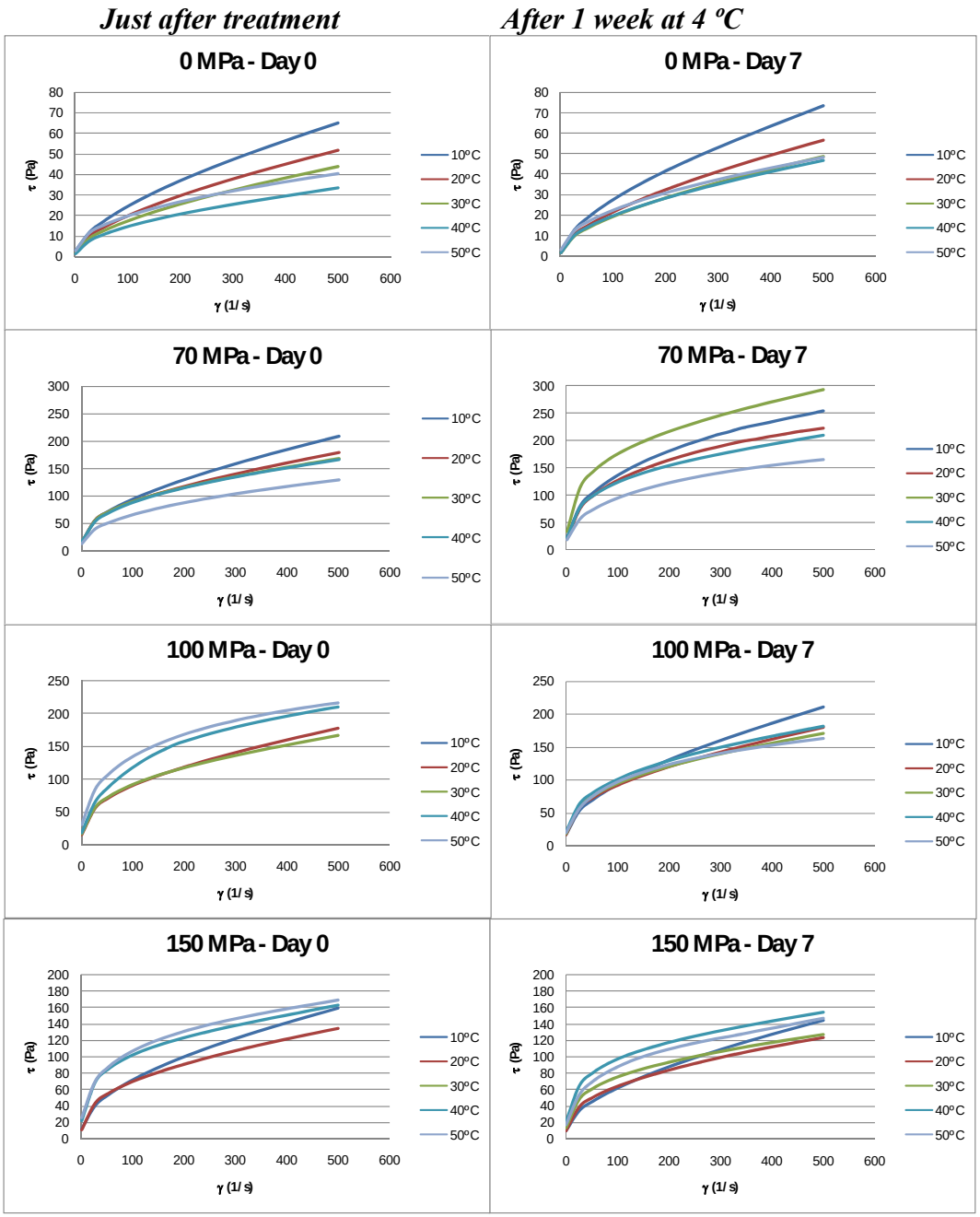

Figure 27. Rheological behaviour of HPH treated samples, just treated and after 1 week at 4 ºC

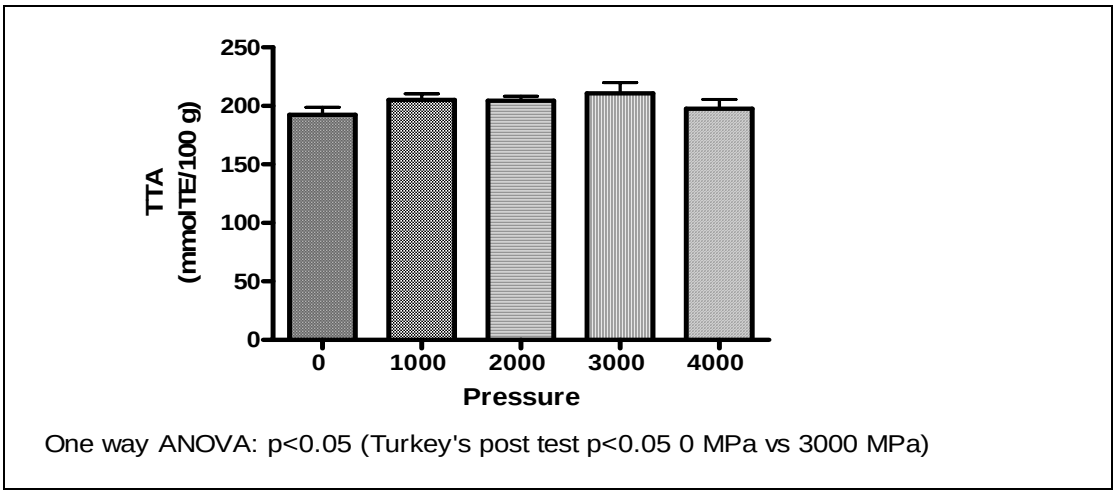

Figure 28. Total antioxidant activity in the vegetable soups subjected to HPH processing. Statistical analysis was by one way ANOVA, using Tukey's as post tes : $p<0.05$ (0.1MPa vs 300MPa $p<0.05$).

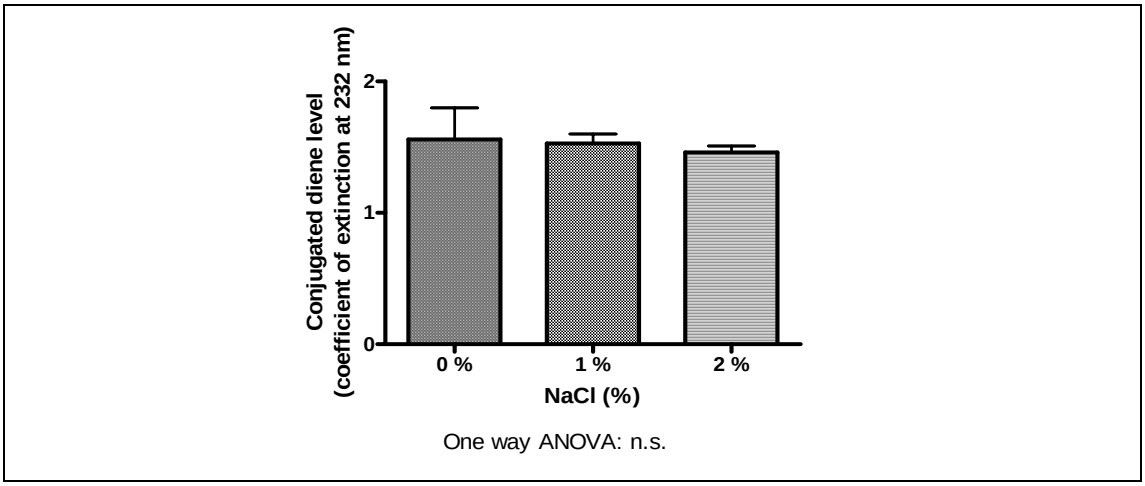

Figure 29. Level of conjugated diene (expressed as coefficient of extinction at 232 nm) on the basis of NaCl content in milk-egg emulsions. Statistical analysis was by one way ANOVA, using Tukey's as post tes : n.s.

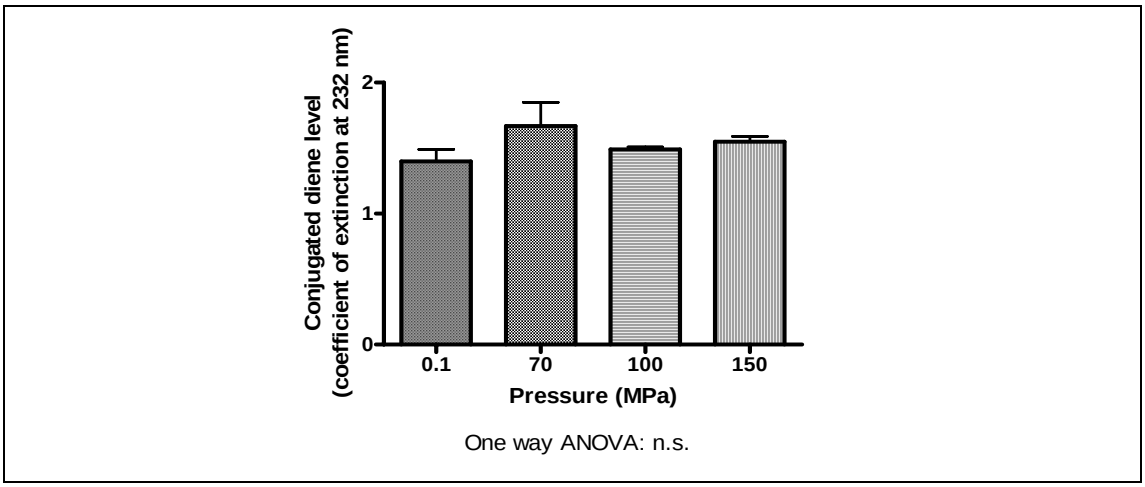

Figure 30. Level of conjugated diene (expressed as coefficient of extinction at 232 nm) on the basis of high pressure treatment in milk-egg emulsions. Statistical analysis was by one way ANOVA, using Tukey's as post tes : n.s.

Table 12. Data relative to the shelf-live values of vegetable soups, fruit juices and milk-egg emulsions subjected to HPH treatments (industrial-scale equipment) at different pressure levels and stored at 4°C.

Product	Pressure level (MPa)	Shelf-life (days)
Vegetable soup	0.1	7
	100	9
	200	15
	300	>30
	400	>30
Fruit juices	0.1	30
	100	>30
	200	>30
	300	>30
	400	>30
Milk-egg emulsion	0.1	6
	70	12
	100	13
	150	>30

Table 13- Score attributed by the panelist to the various parameters in relation to the food system and the treatment performed.

Food	Treatment	Consistence /texture	Taste/ flavour	Appearance/ colour	Smell/ flavour
Vegetable soup	**Heat**	3.0 ±0.2	4.0±0.3	3.8±0.2	3.5±0.5
	HPH	4.2±0.3	4.3±0.1	4.5±0.3	3.4±0.1
Fruit juice	**Heat**	3.2±0.5	4.3±0.4	3.5±0.3	3.8±0.5
	HPH	4.3±0.4	4.5±0.2	4.1±0.1	3.5±0.3
Milk-egg emulsions	**Heat**	2.9±0.3	4.5±0.3	4.0±0.3	3.1±0.2
	HPH	4.5±0.4	4.2±0.5	4.3±0.5	3.3±0.4

Table 14- Pressure levels at which inactivations ≥ 5 log CFU/ml was achieved for the target pathogens and no recovery was observed during refrigerated storage for 30-50 days.

Product	Microorganims	Pressure level (MPa)
Vegetable soup	*Listeria monocytogenes* 56 Ly	400
	Salmonella enteritidis 155	≥300
	Bacillus cereus SV90	≥ 200
Fruit juices	*Listeria monocytogenes* 56 Ly	>200
	Salmonella enteritidis 155	≥ 200
	Bacillus cereus SV90	≥ 200
Milk-egg emulsion	*Listeria monocytogenes* 56 Ly	
0% NaCl		150
1% NaCl		170

*Inoculum levels were > 5 log CFU/ml

High Hydrostatic pressure and CO_2 (CSIC)

1. Introduction

Hydrostatic Pressure Processing is less detrimental than thermal processes to low molecular weight food compounds such as flavouring agents, pigments, vitamins. Vegetative cells, including yeasts and moulds can be inactivated by pressures between 300 MPa and 600 MPa, while bacterial spores are highly pressure resistant.
High-pressure processing (HPP) can be applied to inactivate the microbials and food quality related enzymes (PPO, POD, PME) of plant foods, and thus enhance safety, quality and extend shelf-life. Modified atmosphere packaging technology (MAP) is largely used for minimally processed fruits and vegetables to control microbial growth and food enzyme activity. HPP should be used in combination with other processes to assure microbial safety.
Carbon dioxide (CO_2) is a candidate for such a combination because of its ability to inactivate microorganisms. Either modified atmosphere packaging (MAP) or high-pressure processing (HPP) applied alone is not effective enough to avoid the adverse effects of enzymes and to control the human pathogens growing during the refrigerated storage. In fact, *Listeria monocytogenes* has been isolated from refrigerated pre-packaged salads. Therefore, combined HPP of MAP products could be a promising alternative to preserve the quality of plant-derived products during processing and storage.
Lethal effect of HPP on vegetative microorganism is thought to be the result of a number of changes taking place simultaneously in the microbial cell. High pressure disrupts the thermodynamics of the chemical reactions hence disturbing the "normal" metabolic activity of the cells. It is thought hat the main cause of inactivation is related to the disruption or changes in the permeability of membrane cell, other mechanisms as the inactivation of key enzymes as that involved in DNA replication and transcription, decrease of intracellular pH (died by acidification) and protein denaturalization or aggregation can occur.
The carbon dioxide (95%) inside of the package of vegetable products treated with pressure greater than 100 MPa reach the properties of supercritical carbon dioxide because the conditions are above the critical point of this gas (Pressure 7.38 MPa and Temperature 31 ºC). With carbon dioxide in a supercritical state, the process of inhibition is potentially enhanced by further disruption to the physiology of the cell caused by the dissolution of carbon dioxide in the liquid biolayer or solid matrix of foods and penetration in the cell. Indeed the supercritical state of carbon dioxide makes it behave like a gas in term of transport properties (diffusivity, solubility) and like a liquid in term of density and so is more efficient than carbon dioxide in other states. Supercritical carbon dioxide ($scCO_2$) is an excellent non-polar solvent for many organic compounds.
The combination of High Hydrostatic Pressure and packaging under CO_2 (HHPCO) as novel processing technique to enhance the safety and shelf life of several foods can be regarded as clear progress beyond the state of the art.

2. Objectives

The main objective was to evaluate the efficiency of High Pressure Processing (HPP) combined with CO_2 packaging (HHPCO), in comparison with traditional heat treatments (HT), in terms of quality and safety to obtain ready-to eat vegetable foods.

The specific objectives were:

- **Objective 1**. Development and validation of a new approach to perform a ready to-eat vegetable product by the combination of high hydrostatic pressure with CO_2 (HHPCO) and subsequent refrigerated storage.
- **Objective 2**. Apply this approach to systematically investigate the effects of high hydrostatic pressure combined with CO_2 and subsequent refrigerated storage on the inactivation of spoilage and pathogenic microorganisms.
- **Objective 3**. To establish the effect of combined treatment HHPCO and subsequent refrigerated storage on the inactivation of plant food quality related enzymes.
- **Objective 4.** To study the effect of combined treatment HHPCO and subsequent refrigerated storage on nutritional and health-promoting compounds.
- **Objective 5**. To compare the results obtained in objectives 1-4 with those obtained with a thermal treatment in terms of microbiological and enzymatic inactivation and retention of nutritional and health-promoting compounds.

To achieve the stated objectives different activities were carried out in the framework of the next tasks:

1. Standard procedures (CSIC, CAMST, UNIBO, LNE)
2. Packaging selection (LNE, CSIC)
3. Microbial response in food systems (CSIC)
4. Enzymatic activity in food systems (CSIC)
5. Nutritional quality (UNIBO, CSIC)
6. Sensory quality (CSIC)
7 Performance in Pilot-scale (CSIC, CAMST)

3. Materials and Methods (See Annex I, II and III)

4. Results and Discussion

4.1 Protocols for the formulation of vegetable dishes to be processed with HHP treatments (alone or combined with temperature) and different packaging (CO_2 and vacuum) (CSIC, CAMST).

Standard procedures for sample preparation of vegetable dishes to be processed by HHPCO were defined by CSIC in collaboration with CAMST (ORMA) in order to know the influence of the process on the composition and quality of raw plant material and the effects of boiling, grilling and dressing.

Two different **ready to-eat vegetable based dishes** has been chosen as product targets and the procedures to prepare each dish have been defined:

- For a serving size of 250 g, a mix of boiled spinach (120 g) and carrot (120 g) dressing with salt (3 g) and virgin olive oil (7 g).

- For a serving size of 250 g, a mix of grilled vegetables as zucchini (85 g), eggplant (90 g) and red pepper (65 g) dressing with salt (3 g) and virgin olive oil (7 g).

The studies have been carried out with target components of the vegetable dishes selected. Therefore, the studies have been done with raw carrots slices as one of the ingredients of the dish of mix of boiled vegetables and raw and grilled red pepper cut in stripes as one of the main component of the dish of grilled vegetables.

4.2 Protocols for standardized HHPCO processing parameters (CSIC)

Determination of the protocols for the inoculation and enumeration of microorganisms no-inoculated (aerobic mesophilic microorganisms, yeast and moulds) and inoculated (*Listeria innocua*) in real plant foods as carrot slices and red bell pepper stripes were achieved (Annex I).

Determination of the protocols for the enzymatic activity of different vegetable products. Standard procedures to determine the activity of quality-related plant food enzymes such as Polyphenoloxidase (PPO), Peroxidase (POD) and Pectinmethylesterase (PME) in carrot, zucchini, red pepper, spinach and eggplant were established. In all cases, a basic method of enzyme activity determination was adjusted for each vegetable in order to optimize the methods of enzyme extraction and measurement. (Annex I).

Studies to identify optimal conditions for inactivation of plant food microorganism by HHPCO. In order to select the more convenient treatment parameters [pressure, time of treatment, temperature, and type of packaging (air, 95%CO_2 or vacuum packaging)] to obtain a ready-to-eat vegetable dish, it was studied the effect of HP processing (HPP) on an inoculated pathogenic microorganism ***(Listeria innocua as surrogate of Listeria monocytogenes)*** in raw carrots slices and in raw and grilled red bell pepper strips.

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Results: Listeria innocua inactivation

In general, from the point of view of *Listeria innocua* inactivation in raw and grilled pre-cut red bell pepper and raw carrot, the more convenient HPP treatment could be the combination of Pressure at **400 MPa, temperature at 42.5 ºC (or 60 ºC) and time of treatment 3-5 minutes,** independently of the type of packaging employed. In this conditions, it was achieved a *L. innocua* reduction > 5 logs CFU/g.

Also it is important to consider that the type of packaging (air, vacuum and 95%CO_2) seems not to be a determining factor affecting the inactivation of *Listeria innocua* by HPP, meanwhile the temperature was a determining factor affecting but only at higher temperatures than 42.5°C.

Related with the type of food, inactivation by HPP of *Listeria innocua* in carrot was the most efficient, and the inactivation by HPP in grilled red bell pepper was more efficient than in raw red bell pepper.

The parameters of treatment assayed for *Listeria innocua* inactivation are showed in **Table 15**.

Table 15. Pressure and temperature conditions (at fixed time of treatment of 3 min) employed for the inactivation of *Listeria innocua* inoculated in raw and grilled red bell pepper and raw carrot packaging under different packaging (air, vacuum, 95% CO2)

Condition	Pressure (MPa)	Temperature (ºC)
1	100	25
2	100	42,5
3	100	60
4	250	25
5	250	42,5
6	250	60
7	400	25
8	400	42,5
9	*400*	*60*

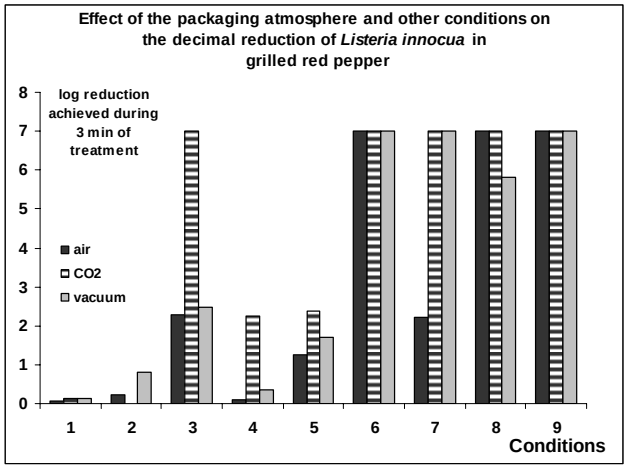


Figure 31. Effects of pressure, temperature and packaging atmosphere at a fixed time of treatment (3 min) on the inactivation of *Listeria innocua* inoculated in grilled red pepper. The pressure/temperature combinations are given for each condition number in Table 15.

4.3. Specifications of packaging materials to be used with HHPCO technology (CSIC, LNE).

LNE had contacts with packaging suppliers such as Breger, Tetrapak or Klöckner Pentaplast. CSIC partner also provides us contact with EDV and AMCOR:
At last, after intensive contacts with these packaging material suppliers to collect information about the potentiality of different materials to be used in HPPCO processing of foods and between CSIC and LNE to exchange information about the data provided, the selected packaging were:

- a transparent film and a transparent doypack provided by AMCOR,
- a transparent cup and a transparent tray provided by EDV.

The tests on untreated and treated packaging have been performed and the results are given in (Annex III).

Moreover, to assess the chemical safety and the mechanical resistance of the packaging after treatment and so, to check if the selected packaging fulfill HPPCO conditions, some analyses have to be performed to:

- Follow the structure modification of the packaging by IR analysis of both side of the materials;
- Scanning electronic microscope experiments to characterize the surface state;
- Verify the migration of additives by establishment of a fingerprint by extraction and specific migration (water, 95 % ethanol, and 3% acetic acid at 40°C for 10 days and isooctane at 20°C for 2 days) of the packaging;
- Check by overall migration (water, 95 % ethanol, and 3% acetic acid at 40°C for 10 days and isooctane at 20°C for 2 days) that the treated packaging complies with rules;
- Assess the physical and physico-chemical properties by performing mechanical tests, permeability to CO_2, O_2, and water vapor.

There are no significant chemical and physical modifications in the 2 packaging by the HHPCO treatment (400 MPa/60 ºC/5 min).

4.4. Report on standardized minimum enzyme (PPO, POD, PME) activity in real plant foods prcessed with HHP treatments combined with temperature and different packaging (CO_2 or vacuum) (CSIC).

A. Standard Procedures to determine food related enzyme activity (PPO, POD, PME) in real plant foods (CSIC)

In order to identify optimal conditions for inactivation of plant food enzymes by HHPCO, sliced raw carrots and pre-cut raw red bell peppers were selected as indicators (one for each ready to-eat vegetable based meal, mix of boiled vegetables and mix of grilled vegetables, respectively). The impact of high-pressure processing (HPP) combined with CO_2 on POD, PPO and PME activities were studied. In order to determine optimal conditions for enzyme inactivation, the influence of high-pressure parameters (pressure, temperature, treatment time and compression speed) combined with different headspace gas compositions (95% CO_2, air and vacuum) was evaluated

Results: Enzyme inactivation

Identification of the optimal conditions to inactivate plant food enzymes by HHPCO and set up of the related activities

1. *Carrot slices*: Treatments at 300 MPa/5 min or 400 MPa/5 min (at any assayed temperature) were sufficient to obtain POD and PPO inactivation levels higher than 50 and 85%, respectively, for any atmosphere in raw carrot slices.

2. *Eggplant slices*: The combination of 400 MPa/5min/60 ºC and CO_2 packaging was an efficient treatment to reduce 86% the initial PPO activity in eggplant products. However, assayed combined treatments of high-pressure and temperature were not able to inactivate POD in eggplant slices. Higher enzymatic inactivation was observed at lower compression speed or when the treatment was applied in two cycles.

3. *Zucchini slices*: Samples treated at 400 MPa/60 ºC/5 min presented the highest enzymatic inactivation for POD (55 and 45% in vacuum and CO_2, respectively) and PPO (85 and 82% in vacuum and CO_2, respectively), being similar for both types of packaging. The highest POD and PPO inactivation (38% and 69%, respectively) was observed when the compression speed was 0, 5 MPa/s and packaging under CO_2 was employed.
4. *Red bell pepper strips*: The compression speed and the high-pressure treatment in one or two cycles did not influence POD activity for any type of packaging.

B. Identification of the optimal conditions to inactivate plant food enzymes by HHP combined treatments and set up the related activities (CSIC)

1. Raw Carrot Slices: In general, PME and POD inactivation was influenced by the atmosphere and the combination of pressure and temperature. For PME, higher inactivation levels were achieved at lower compression speed and when treatment was applied in two cycles. Treatments at 300 MPa/5min/60 ºC or 400 MPa/5min/25 ºC were sufficient to obtain PPO inactivation levels higher than 75% for any atmosphere in raw carrot slices.

PME of sliced raw carrots:
The effect of different combined treatments on Pectinmethylesterase (PME) activity of raw carrot slices was shown in **Figure 32**.

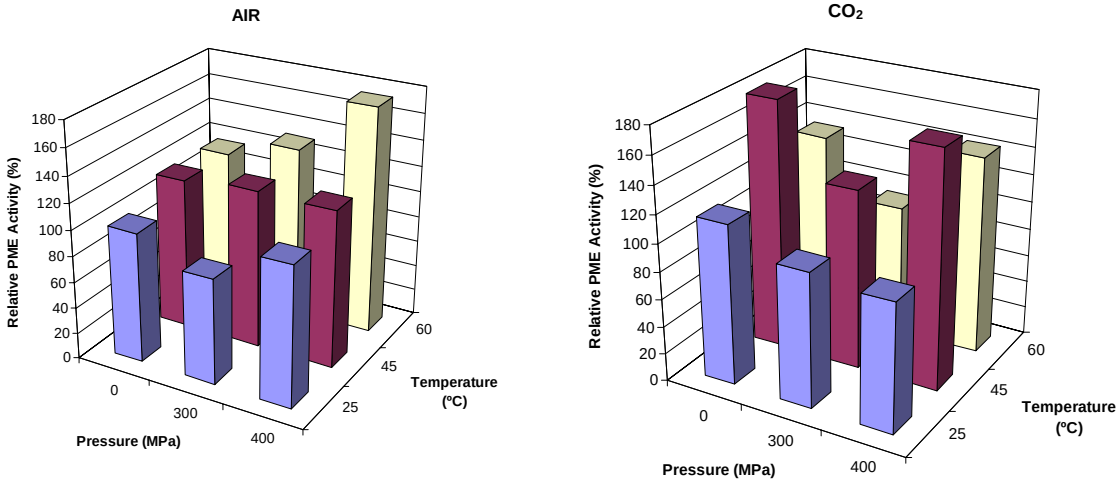


Figure 32. Effects of pressure, temperature and headspace atmosphere (CO_2 or air) on PME activity of sliced carrot (5 min/1.6 MPa s^{-1})

Results:. The maximum PME inactivation was observed at 300 MPa/25 ºC (19.03 %) for air and at 400 MPa/25 ºC (7.15 %) for CO_2.

POD and PPO of sliced raw carrots:
The effect of different combined treatments on Polyphenoloxidase (PPO) and Peroxidase (POD) activities of raw carrot slices was shown in **Table 16.**

Table 16. POD and PPO activities in carrot slices. Effects of treatment.

Pressure (MPa)	**Time of treatment** (Min)	**Temperatura** (ºC)	**Compression speed** (MPa/s)	**Atmosphere**	**POD** RA (%)	**PPO** RA (%)
300	5	60	1.6	CO_2	37.58 ± 2.96	9.99 ± 1.22
300	5	60	1.6	Air	35.10 ± 5.62	1.65 ± 0.76
300	2.5 + 2.5	60	1.6	CO_2	43.19 ± 3.99	8.26 ± 1.45
300	2.5 + 2.5	60	1.6	Air	32.83 ± 4.50	1.43 ± 1.56
300	5	60	0.5	CO_2	24.85 ± 3.68	2.27 ± 1.16
300	5	60	0.5	Air	31.31 ± 2.85	1.60 ± 1.18
300	5	60	1.0	CO_2	33.44 ± 1.36	2.42 ± 0.95
300	5	60	1.0	Air	32.95 ± 2.47	1.65 ± 0.97
300	5	10	1.6	CO_2	36.82 ± 2.55	2.61 ± 1.59
300	5	10	1.6	Air	46.71 ± 2.46	14.76 ± 5.54
300	5	25	1.6	CO_2	39.39 ± 3.85	4.45 ± 1.62
300	5	25	1.6	Air	22.96 ± 1.98	5.96 ± 2.08
400	5	25	1.6	CO_2	38.45 ± 2.33	3.67 ± 1.28
400	5	25	1.6	Air	25.61 ± 1.99	3.02 ± 1.58
0	0	25	0	CO_2	81.57 ± 17.50	60.24 ± 20.69

Conclusion: Treatments at 300 MPa/5min/60 ºC or 400 MPa/5min/25 ºC were sufficient to obtain PPO inactivation levels higher than 75% for any atmosphere in raw carrot slices. POD inactivation was influenced by the atmosphere and the combination of pressure and temperature (**Table 16**).

2. Raw Eggplant Slices: Maximum PME inactivation in eggplants was achieved at 400 MPa/60 ºC/5 (29.63 and 9.42% for vacuum and CO_2 samples, respectively). The combination of 400 MPa/5min/60 ºC and CO_2 packaging was an efficient treatment to reduce 86% the initial PPO activity in eggplant products. The higher POD and PPO inactivation in CO_2 packaging of 30% and 53%, respectively, and 57% in vacuum was observed when the compression speed was 5 bar/s, being significantly higher than the results obtained at 16 bar/s.

PME of sliced raw eggplant. The effect of different HHPCO combined treatments on Pectinmethylesterase (PME) activity of raw eggplant slices was shown in **Table 17**.

Table 17. PME activity in sliced raw eggplants. Effect of treatment.

Treatment	Packaging	PME (mmol galacturonic acid min^{-1} g^{-1})
Control		73.47 ± 11.76
25 ºC / 1min	Vacuum	90.89 ± 5.27
100 MPa / 25 ºC / 1min	Vacuum	90.94 ± 7.58
42.5 ºC / 3min	Vacuum	81.64 ± 1.62
250 MPa / 42.5 ºC / 3min	Vacuum	68.82 ± 3.42
60 ºC / 5min	Vacuum	100.31 ± 2.55
400 MPa / 60 º C/ 5min	Vacuum	51.70 ± 8.11
25 ºC / 1min	CO_2	87.33 ± 13.38
100 MPa / 25 ºC / 1min	CO_2	92.65 ± 4.75
42.5 ºC / 3min	CO_2	82.39 ± 15.98
250 MPa / 42.5ºC / 3min	CO_2	88.79 ± 4.41
60 ºC / 5min	CO_2	68.41 ± 4.58
400 MPa / 60ºC / 5min	CO_2	66.55 ± 5.99

***Conclusion*:** Maximum PME inactivation in eggplants was achieved at 400 MPa / 60 ºC /5 (29.63 and 9.42 % for vacuum and CO_2 samples, respectively).

POD and PPO of sliced raw eggplants: The effect of different combined treatments on Polyphenoloxidase (PPO) and Peroxidase (POD) activities of raw eggplant slices was shown in **Table 18**.

Table 18. POD and PPO activities in sliced raw eggplants. Effect of treatment.

Treatment	Packaging	POD (ΔDO/min)/g fw	PPO (ΔDO/min)/g fw
Control		11.92 ± 0.73	13.91 ± 0.76
25 ºC / 1min	Vacuum	8.92 ± 1.58	19.00 ± 3.56
100 MPa / 25 ºC / 1min	Vacuum	13.04 ± 0.19	19.29 ± 1.47
42.5 ºC / 3min	Vacuum	6.67 ± 1.05	17.41 ± 2.36
250 MPa / 42.5 ºC / 3min	Vacuum	10.91 ± 1.95	7.35 ± 1.56

60 ºC / 5min	Vacuum	6.91 ± 1.40	4.66 ± 0.31
400 MPa / 60 º C/ 5min	Vacuum	11.65 ± 1.35	4.87 ± 1.51
25 ºC / 1min	CO_2	10.74 ± 1.48	16.78 ± 1.28
100 MPa / 25 ºC / 1min	CO_2	11.34 ± 1.45	21.57 ± 2.26
42.5 ºC / 3min	CO_2	11.38 ± 1.84	17.21 ± 2.64
250 MPa / 42.5ºC / 3min	CO_2	13.08 ± 3.17	15.79 ± 1.18
60 ºC / 5min	CO_2	15.00 ±1.43	20.92 ± 0.67
400 MPa / 60ºC / 5min	CO_2	10.68 ± 2.75	1.87 ± 0.61

Conclusion: *The combination of 400 MPa/5min/60 ºC and CO_2 packaging was an efficient treatment to reduce 86% the initial PPO activity in eggplant products.*

3. Raw Zucchini Slices: POD and PPO activities decreased significantly when the pressure increased. Samples treated at 400 MPa/60 ºC/5 min presented the highest enzymatic inactivation for both enzymes. Higher enzymatic inactivation was achieved when the compression speed decreased and when the treatment was applied in two cycles.

POD and PPO of sliced raw zucchinis: The effect of different combined treatments on Polyphenoloxidase (PPO) and Peroxidase (POD) activities of raw zucchini slices was shown in **Table 19.**

Table 19. POD and PPO activities in sliced raw zucchinis. Effect of treatment.

Treatment	**Packaging**	**POD** (ΔDO/min)/g fw	**PPO** (ΔDO/min)/g fw
Control		40.41 ± 3.50	12.58 ± 0.37
25 ºC / 1min	Vacuum	48.69 ± 3.61	13.47 ± 0.78
100 MPa / 25 ºC / 1min	Vacuum	35.48 ± 0.51	12.70 ± 0.96
42.5 ºC / 3min	Vacuum	48.47 ± 5.39	14.99 ± 0.77
250 MPa / 42.5 ºC / 3min	Vacuum	35.22 ± 0.41	15.72 ± 0.47
60 ºC / 5min	Vacuum	31.53 ± 10.70	3.83 ± 1.38
400 MPa / 60 º C/ 5min	Vacuum	17.96 ± 0.66	1.93 ± 0.16
25 ºC / 1min	CO_2	46.41 ± 5.95	13.72 ± 0.46
100 MPa / 25 ºC / 1min	CO_2	34.61 ± 5.11	10.98 ± 0.38
42.5 ºC / 3min	CO_2	54.14 ± 3.54	15.62 ± 0.81

250 MPa / 42.5ºC / 3min	CO_2	24.06 ± 2.51	4.01 ± 0.46
60 ºC / 5min	CO_2	55.91 ± 3.11	16.70 ± 1.47
400 MPa / 60ºC / 5min	CO_2	22.06 ± 1.64	2.22 ± 0.41

Conclusion: *Samples treated at 400 MPa/60 ºC/5 min presented the highest enzymatic inactivation for POD (55 and 45% in vacuum and CO2, respectively) and PPO (85 and 82% in vacuum and CO2, respectively).*

4. <u>Raw Red Bell Pepper Strips</u>: The highest POD inactivation was observed when pressure increased up to 400 MPa at 25 ºC for any atmosphere. The compresssion speed and the application of treatment in one or two cycles did not influence POD activity for any atmosphere.

POD of pre-cut raw red bell peppers: Response surface methodology was employed to investigate the combined effect of high pressure (100-400 MPa), temperature (25-60 ºC) and treatment time (1-5 min), with different headspace gas composition (95% CO_2, air and vacuum), on POD activity. Response surfaces for POD activity of raw red bell peppers packaging under 95% CO_2 were shown in **Figure 33.**

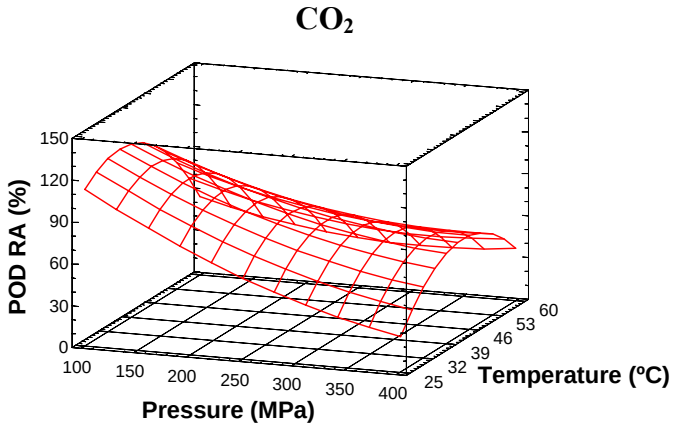


Figure 33. Response surface plot of POD relative activity (RA) in raw red bell pepper packaging under 95% CO_2 atmosphere. Treatment time was constant at 3 min.

***Conclusion*:** The highest POD inactivation was observed when pressure increased up to 400 MPa at low temperatures for any atmosphere.

5. <u>Raw and Grilled Red Bell Pepper Strips</u>: The highest POD inactivation was found in HP treated samples (400 MPa/45 ºC/3 min) under 95% CO_2 atmosphere for raw red bell peppers. PPO was almost completely inactivated after HP treatment of raw samples. Grilled red bell peppers HP treated did not show any POD or PPO activity.

4.5. Report on standarized minimum lethality levels for microorganism (endogenous and inoculated) in real plant foods treated by HHP treatments (alone or combined with temperature) and different packaging (CO_2 or vacuum)(CSIC)

A.-Identification of the optimal conditions to inactivate microbial population (endogenous and inoculated) by HHP combined treatments in real plant foods

-.Effecst of HHPCO treatment on endogenous (non-inoculated) microorganisms of raw red bell pepper. HP processing (400 MPa/45 ºC/3min) completely reduced (6 logs units) the total aerobic mesophilic counts and psychrophilic bacteria counts (under the detection limit) in raw pre-cut pepper in both types of packaging (vacuum and 95% CO_2).

-.*Effect of HHPCO on inoculated microorganism (Listeria innocua) of grilled red bell pepper*. High-pressure treatment combined with medium temperature (400 MPa/45 ºC/3min) combined with packaging under 95%CO_2, produced a significant reduction of 6 Logs in the counts of *Listeria innocua* inoculated in grilled pre-cut red bell pepper.

-.Effecst of HHPCO treatment on endogenous (non-inoculated) microorganisms of grilled red bell pepper. High-pressure treatment combined with medium temperature (400 MPa/45 ºC/3min) reduced 2-3 logs units the total aerobic mesophilic counts and 3.8 logs units the psychrophilic bacteria counts in the both types of packaging assayed (vacuum and 95%CO_2).

Differences between vacuum and CO_2 packaging in raw and grilled red bell pepper were not detected just after HHP treatment.

B.- Inactivation kinetics of inoculated microorganisms (Salmonella enteritidis, Bacillus cereus, Listeria monocytogenes) in real plant food treated with HHP treatments (combined with temperature and different packaging (CO_2 or vacuum) (CSIC, IFR)

Inactivation kinetics of Listeria innocua inoculated in real food

The inactivation of Listeria innocua CECT910 as a surrogate for *Listeria monocytogenes* was studied. More than 200 data of *Listeria innocua* inactivation in raw carrot and in raw and grilled red bell pepper, in different packaging (vacuum, air and 95% CO2) treated with pressure (100-400 MPa), temperature (25-60°C) and time of treatment (1-5 minutes), were obtained. This data was formatting according to the ComBase database by the researchers of Dr Baranyi´s group (Institute of Food Research) in order to obtain inactivation kinetics for *Listeria innocua* inoculated in real food.

The inoculum of *Listeria innocua* 910 (CECT, Spanish Type Culture Collection) was prepared to allow a viable cell count of 10^8 cells per g of vegetable product quantified in a selective medium (Palcam)(Protocol described in Annex I).

Results:

Primary model:
At the lower pressure (100 and 250 MPa), the inactivation of *Listeria innocua* in raw and grilled red bell pepper and raw carrot seems to be linear. The inactivation curves of grilled red bell pepper are showed in **Figure 34**.

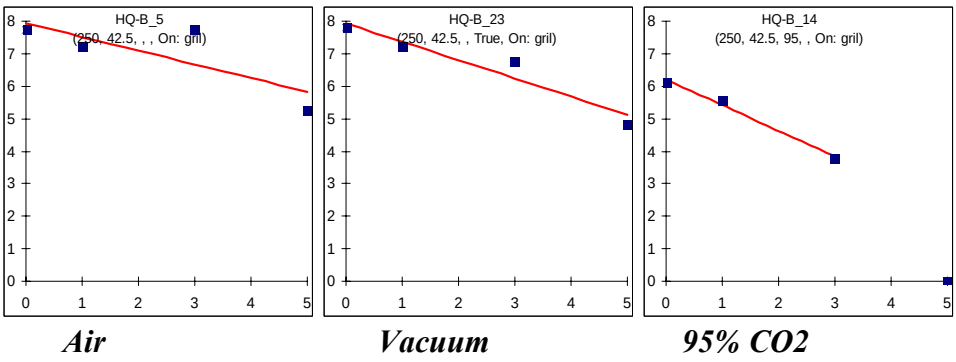


Figure 34. Inactivation of *Listeria innocua* as a function of the treatment time (in min.) inoculated on grilled red pepper treated at 250 MPa / 42.5°C packaged with air, vacuum and atmosphere of 95% CO_2.

When higher pressure was employed up to 400 MPa, the inactivation of *Listeria innocua* in raw and grilled red bell pepper and raw carrot showed a rate decrease or tailing which corresponds to the convex shape most usually observed with high pressure treatment. The inactivation curves of grilled red bell pepper are showed in **Figure 35.**

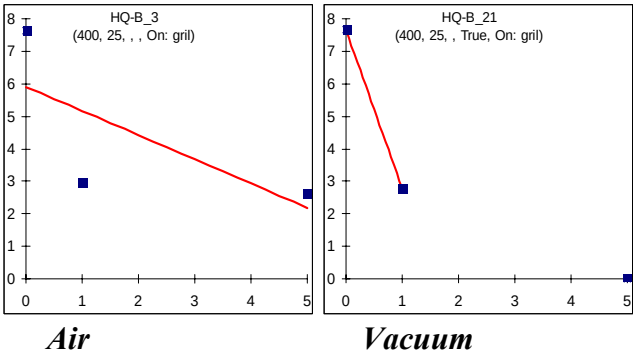


Figure 35. Inactivation of *Listeria innocua* as a function of the treatment time (in min.) inoculated on grilled red pepper treated at 400MPa/25°C packaged with air and vacuum.

The primary model of *Listeria innocua* inactivation suggest a linear trend at the lower pressures (100 and 250 MPa) for treatments up to 5 minutes, but at higher pressure up to 400 MPa the primary model could be convex. A non linear model log-logistic model was employed.

Inactivation of Listeria monocytogenes 56Y, Salmonella enteritidis 155 and Bacillus cereus SV90 inoculated in real food

Listeria monocytogene 56Y, *Salmonella enteritidis 155* and *Bacillus cereus SV90* (strains provided by UNIBO) were inoculated on grilled red pepper to be treated by high pressure with supercritical carbon dioxide (packaging under 95%CO_2). Combinations of pressure and temperature were applied with pressure ranging between 250 and 600 MPa, temperature between 10 and 45°C for up to 5 min. **I**nitial pathogen counts in grilled red bell pepper were 10^7 CFU/g. The results obtained were presented below:

Bacillus cereus
Survival curves of *Bacillus cereus* inoculated in grilled red bell pepper packaging under 95%CO_2 and HHPCO treated at 250 and 400 MPa were showed in **Figures 36 and 37**, respectively.

In general, the inactivation of *B. cereus* in grilled red bell pepper HHPCO treated did not follow a first-order kinetic and exhibit non-linear behaviour. The tailing phenomenon occurred in practically all survival curves. The curves that seemed fit a first-order linear kinetics could be convex if more time of treatments should be assayed.

The non-linear model log-logistic function has been employed by Dr Baranyi´s group (IFR) for modeling the inactivation data obtained by the application of HHPCO (See Quantitative Tools and Methods Section).

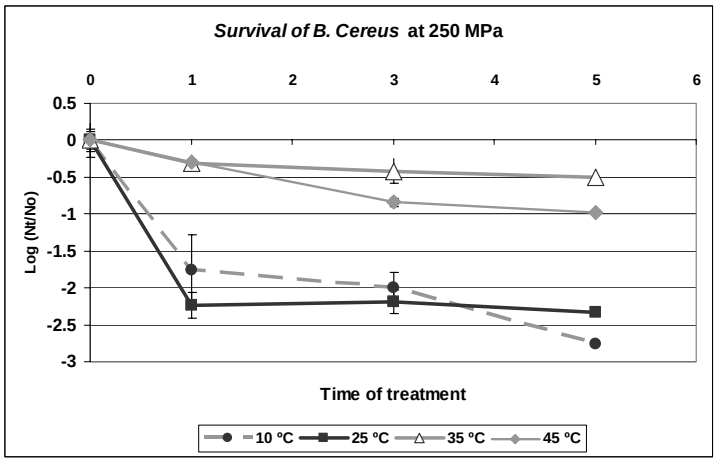


Figure 36. Survival curves of *Bacillus cereus* inoculated in grilled red bell pepper packaging under 95%CO_2, treated at 250 MPa with different temperatures and time of treatment. (Data are the mean value of two different determinations).

Taking into consideration that the curves are not linear in all the phases of the inactivation, an aproximate D-values (t/log(Nt/N0) have been calculated in order to compare the treatments (**Table 20**)

Table 20. Aproximate D-value for *B. cereus* in grilled red bell pepper packaging under 95% CO_2 (supercritical carbon dioxide) by high pressure combined with temperature.

Treatments (MPa/ ºC)	D-value (min)
250 / 10	1.29
250 / 25	1.32
250 / 35	6,81
250 / 45	4.00
400 / 25	0.48
400 / 35	0.50
400 / 45	0.46

The D-value was similar for the treatments at 400 MPa assayed. Temperature seemed not be an important factor for the inactivation of *B. cereus* at this pressure.

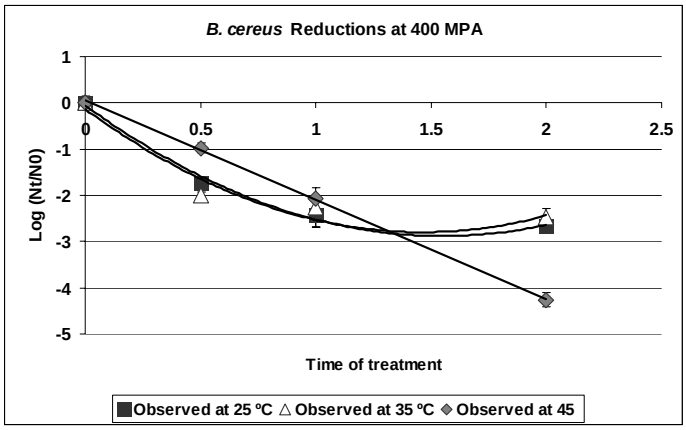


Figure 37: Survival curves of *Bacillus cereus* inoculated in grilled red bell pepper packaging under 95%CO_2, treated at 400 MPa and different temperatures and time of treatment (Data are the mean value of two different determinations).

Salmonella enteritidis

Survival curves of *Salmonella enteritidis* inoculated in grilled red bell pepper packaging under 95%CO2 and HHPCO treated at 250 MPa and 400 MPa have been shown in **Figure 38** and **Figure 39.**

Non-linear curves represented the inactivation of *Salmonella enteritidis* in grilled red bell pepper treated at 250 MPa at differente temperatures (10, 25, 35 and 45 ºC) (**Figure 38**).

Survival curves of *Salmonella enteritidis* at 400 MPa also showed a non-linear curves with tailing for representing the inactivation at different temperatures (25, 35 and 45 ºC) Only the survival curve of Salmonella enteritidis at 400 MPa with 35 ºC fitted with a linear inactivation equation (inactivation rate= -3.51, R^2 =0,996) (**Figure 39**).

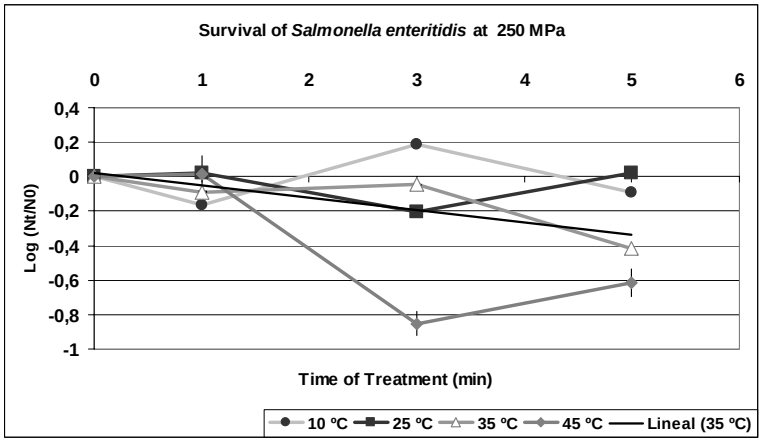


Figure 38. Survival curves of *Salmonella enteritidis* inoculated in grilled red bell pepper packaging under 95%CO_2, treated at 250 MPa and different temperatures and time of treatment. (data are the mean value of two different determinations).

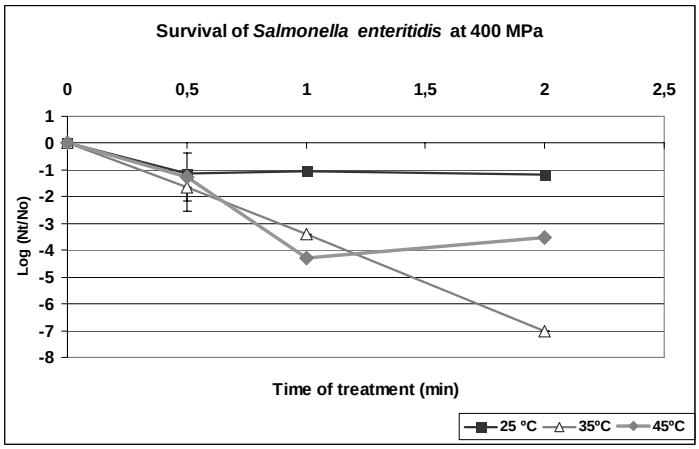


Figure 39. Survival curves of *Salmonella enteritidis* inoculated in grilled red bell pepper packaging under 95%CO_2, treated at 400 MPa and different temperatures and time of treatment. (data are the mean value of two different determinations).

Table 21. Aproximate D-value for *Salmonella enteritidis* in grilled red bell pepper packaging under 95% CO2 (supercritical carbon dioxide) and treated by High pressure and temperature.

Treatments (MPa/ ºC)	D-value (min)
250 / 10	25.74
250 / 25	88.79
250 / 35	29.51
250 / 45	22.39
400 / 25	1.045
400 / 35	0.24
400 / 45	0.40

Salmonella enteritidis at 250 MPa seems to have higher D-value than at 400 MPa, independently of temperature (**Table 21).**

The non-linear model log-logistic function has been employed by Dr Baranyi´s group (IFR) for modeling the inactivation data obtained by the application of HHPCO (See Quantitative Tools and Methods Section).

Listeria monocytogenes

Survival curves of *Listeria monocytogenes* inoculated in grilled red bell pepper packaging under 95%CO_2 and HHPCO treated at 250 MPa and 400 MPa have been presented in **Figure 40 and Figure 41.**

Treatments at 250 MPa combined with temperature (10, 25, 35 and 45 ºC) and time of treatment between 1-5 min, produced very low log reductions of *Listeria monocytogenes* (< 0.2 log CFU/g) (**Figure 40).**

When treatments at 400 MPa were combined with 25 ºC and 35 ºC, the inactivation of *Listeria monocytogenes* increased systematically when the time of treatment increase and the maximum reduction of 7 log CFU/g of *Listeria monocytogenes* was achieved after 5 minutes at 400 MPa/25 ºC and after 3 minutes at 400 MPa/35 ºC. Treatment at 400 MPa and 45 ºC reduced completely *Listeria monocytogenes* (7 log CFU/g reduction) after 0.5, 1 and 2 minutes of treatment (**Figure 41**).

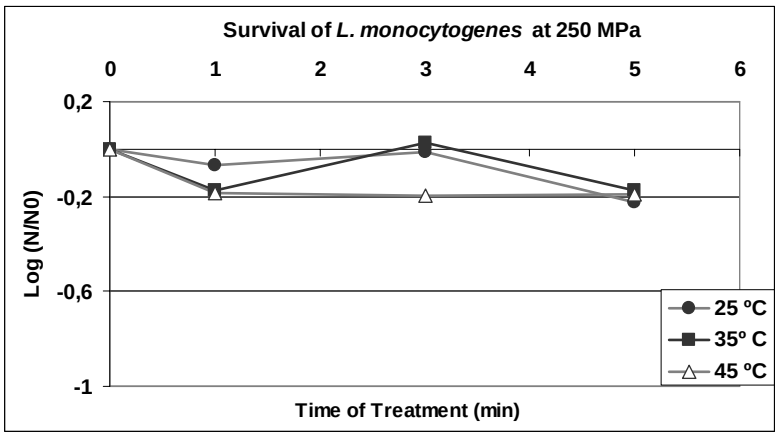


Figure 40. Survival curves of *Listeria monocytogenes* inoculated in grilled red bell pepper packaging under 95%CO_2, treated at 250 MPa and different temperatures and time of treatment. (data are the mean value of two different determinations)

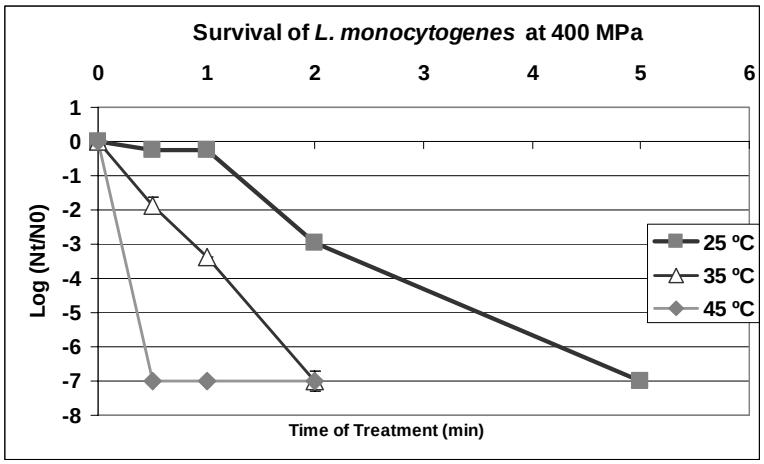


Figure 41. Survival curves of *Listeria monocytogenes* inoculated in grilled red bell pepper packaging under 95%CO_2, treated at 400 MPa and different temperatures and time of treatment. (Data are the mean value of two different determinations)

Inactivation curves at 600 MPa was imposible to obtain taking into account that with a time of treatment of 0.5 min and 25 ºC, no survival cells were detected. The inactivation assays at 35 ºC and 45 ºC were not done.

Table 22 showed the aproximate D-value for *Listeria monocytogenes* in grilled red bell pepper HPPCO trated. D-value for 400 MPa at 35 ºC (0.23 min) and 45 ºC (0.21 min) was very similar.
Comparing the aproximate D-values for *B. cereus* (0, 46 min) (**Table 20**), *Salmonella enteritidis* (0,40 min) (**Table 21**), and *Listeria monocytogenes* (0,21 min) (**Table 22**) *Salmonella enteritidis*) obtained for the treament 400 MPa/45 ºC, it it is posible to conclude that *Listeria monocytogens* was easier to inactivate than *Salmonella enteritidis* and *B.cereus*.

Table 22. Aproximate D-value for *Listeria moncytogenes* in grilled red bell pepper packaging under 95% CO_2 (supercritical carbon dioxide) treatd by high pressure combined with temperature.

Treatments (MPa/ ºC)	D-value (min)
250 / 45	20,73
400 / 25	1.23
400 / 35	0.23
400 / 45	0.21
600/25	0,07

The non-linear model log-logistic function has been employed by Dr Baranyi´s group (IFR) for modeling the inactivation data obtained by the application of HHPCO (See Quantitative Tools and Methods Section).

Comparision with Thermal Treatment (Commercial Pasteurization)
In general, survival curves of *Listeria innocua*, *Salmonella enteritidis* and *B. cereus* at 250 MPa and 400 MPa are nonlinear curves with tailing phenomena represented the inactivation. For that D-value can not be calculated. To obtain information about the traditional thermal pasteurizarion equivalent to one of the HHPCO treatments that better

results yield (400 MPa/45ºC/2 min), approximate D-values have been calculated (t/(Log Nt/N0) for each combined treatment of pressure /temperature, with the aim to compare the behaviour of the different microorganisms.

For that the equivalence of HHPCO treatment at 400MPa/45 ºC/2 min with a traditional thermal treatment have been done taking into account the log reduction obtained and calculating the time need to obtain similar reduction by thermal treatment at 70 ºC.

Treatment at 400 MPa/45 ºC/2 min	**Time (min) for Pasteurization at 70 ºC**
B.cereus (4,24 log reduction)	8,5
Salmonella enteritidis (3,5 log reduction)	7 min
Listeria monocytogenes (8,6 log reduction)	17,2 min

Effects of the different factors on the inactivation of inoculated pathogens:
To compare the effect of the different factors, the decimal reduction in cell number obtained in 3 minutes were estimated based on the linear kinetics for the vegetative cells and biphasic kinetics for *Bacillus cereus.* The initial counts of the pathogens were 10^7 log CFU/g.
Regarding the effect of the treatment parameters, pressure had the biggest effect on the inactivation while higher temperatures than 42,5 ºC tend to favour the inactivation.
Similar conclusions have been achieved with *Listeria innocua.* The results for *Listeria monocytogeens* are shown in **Figure 42.**

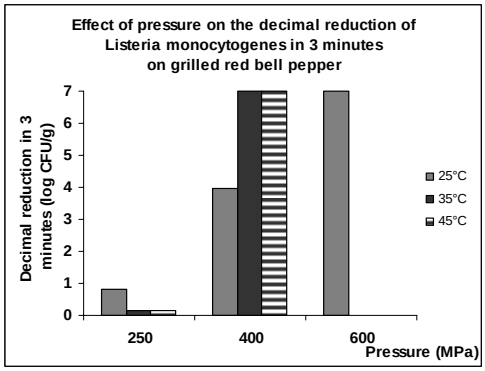


Figure 42. Effect of inactivation of *Listeria monocytogenes* in grilled red pepper by HHPCO at 3 minutes of treatment time.

Inactivation kinetics of Listeria monocytogenes 56Y, Salmonella enteritidis 155 and Bacillus cereus SV90 inoculated in real food (CSIC, IFR)

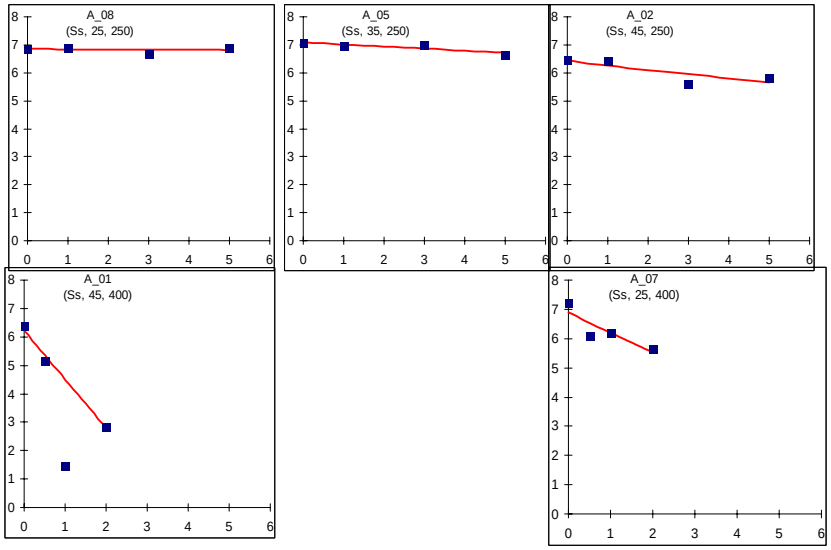
Inactivation data of *Listeria monocytogenes 56Y*, *Salmonella enteritidis155* and *Bacillus cereus90V* inoculated on grilled red pepper packaging under 95%CO_2 and treated by high pressure between 250 and 600 MPa and temperature between 10 and 45°C for up to 5 min have been formated according to the ComBase database by Dr Baranyi´s group IFR) in order to obtain inactivation kinetics for the three pathogens inoculated in real

food. The inactivation of the vegetative cells of *Listeria monocytogenes* and *Salmonella enteritidis* looked linear on the log scale but again considering longer experimental times, it would be convex (**Figure 43 and 44**).
In contrast, for *Bacillus cereus*, the inactivation curves were convex even for short treatment times and they were fitted with biphasic models (**Figure 45**).

Salmonella enteritidis

250 MPa

400 MPa



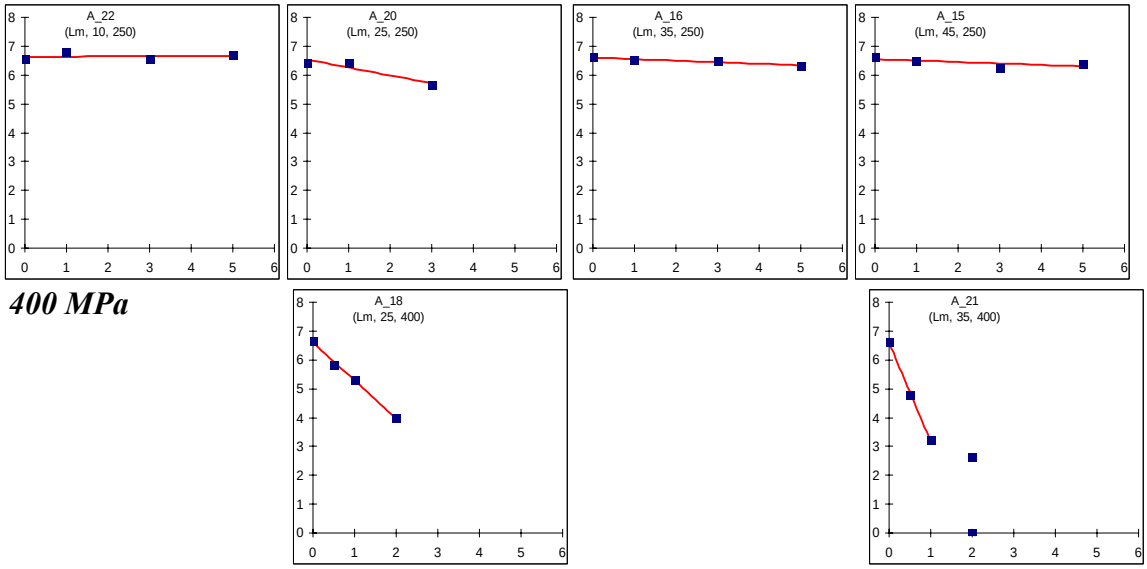
10ºC ***25 ºC*** ***35 ºC*** ***45ºC***

Figure 43. Inactivation of *Salmonella enteritidis* as a function of the treatment time (in min.) inoculated on grilled red pepper packaging with 95% CO2 and treated at 250 and 400 MPa and different temperatures (10, 25, 35 and 45 ºC)

Listeria monocytogenes

250 MPa

400 MPa



10ºC ***25 ºC*** ***35 ºC*** ***45ºC***

Figure 44. Inactivation of *Listeria monocytogenes* as a function of the treatment time (in min.) inoculated on grilled red pepper packaging with 95% CO2 and treated at 250 and 400 MPa and different temperatures (10, 25, 35 and 45 ºC)

Bacillus cereus
250 MPa

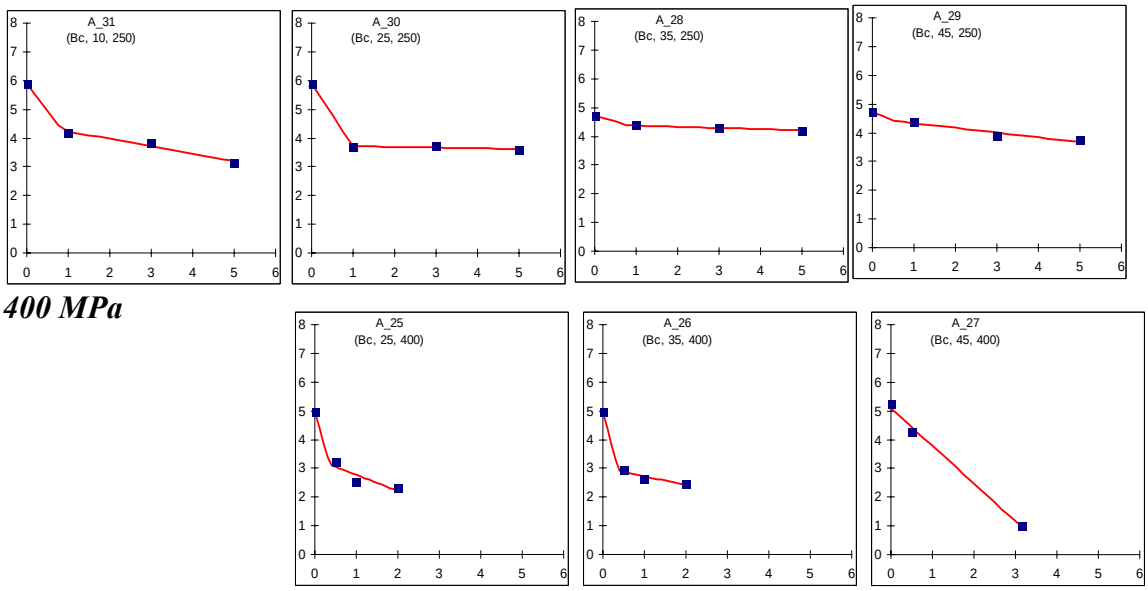


Figure 45. Inactivation of *Bacillus cereus* as a function of the treatment time (in min.) inoculated on grilled red pepper packaging with 95% CO2 and treated at 250 and 400 MPa and different temperatures (10, 25, 35 and 45 ºC)

The non-linear model log-logistic function has been employed by Dr Baranyi´s group (IFR) for modeling the inactivation data obtained by the application of HHPCO (See Quantitative Tools and Methods Section).

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Re-growth of inoculated pathogens in grilled red bell pepper HHPCO treated during refrigerated storage.

Listeria innocua

The behavior of *Listeria innocua* inoculated in grilled red bell pepper, cut in strips, packed under vacuum or 95% CO2, treated by HHP (400 MPa/45 ºC/3 min) and storage at 4 ºC and 20 ºC during one week, have been studied. Therefore it has been evaluated the possibility of the re-growth of the cells during storage at 4 ºC and 20 ºC.

The inoculum of *Listeria innocua* 910 (CECT, Spanish Type Culture Collection) was prepared to allow a viable cell count of 10^8 cells per g quantified in a selective medium (Palcam). Sublethal injury of *Listeria innocua* exposed to combined treatment HP/temperature and packaging under vacuum or 95% CO2 in grilled red bell pepper was assessed by the difference between the counts on the non-selective agar medium (PCA) and the selective agar medium (Palcam)(Protocol in Annex I).

Results:
The combined treatment at 400 MPa/45 ºC during 3 minutes produced 5.11 and 5.24 Log CFU/g reduction of *Listeria innocua* in 95%CO_2 and vacuum packaging, respectively. No significant differences were found between the two type of packaging (vacuum and 95%CO_2) (**Figure 46**).

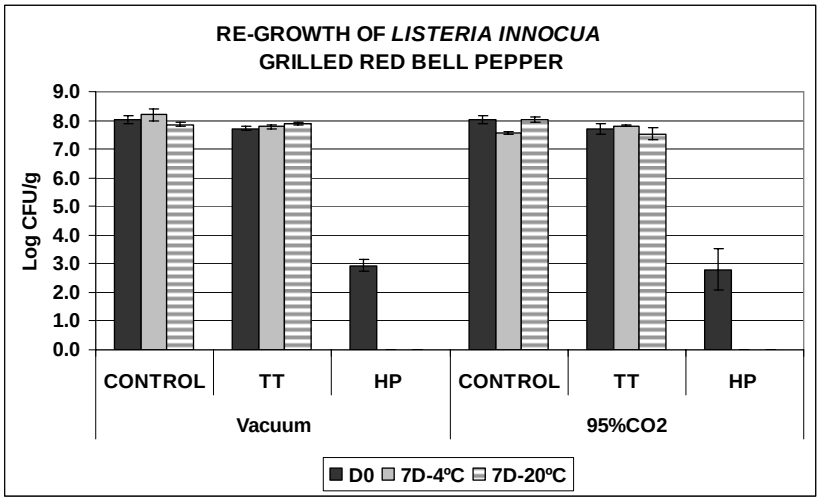


Figure 46. Re-growth study: behavior of *Listeria innocua* inoculated in grilled red bell pepper packaging under vacuum and 95 %CO_2 and treated by HPP (400 MPa/45 ºC/3 min) and thermal treatment (45 ºC/3 min) during storage at 4 ºC and 20 ºC.

After 7 days or storage at 4 ºC and 20 ºC, viable cells of *Listeria innocua* in red bell pepper treated at 400 MPa/45 ºC/3 min were not detected. No re-growth of *Listeria innocua* was observed, even more, surviving cells were inactivated during storage. This result was similar in both types of packaging (vacuum and 95%CO_2).

Listeria monocytogenes
The behavior of *Listeria monocytogenes* inoculated in grilled red bell pepper, cut in strips, packed under 95% CO2 (supercritical carbon dioxide) and treated by HPP (400 MPa/35 ºC/1 min) and thermal treatment (70 ºC/15 min) have been studied. The samples were stored at 4ºC and 10°C for 30 days to monitor the growth following the treatments.
The inoculum of *Listeria monocytogenes* 56LY (UNIBO Type Culture Collection) was prepared to allow a viable cell count of 10^6-10^7 cells per g quantified in Brain Heart Infusion (BHI) agar by spread plating. Plates were incubated at 37 ºC± 1ºC during 48 ± 3 hours (Protocols in Annex I).

***Results*:**
The HPP (400 MPa/35 ºC/1 min) and thermal treatement (70ºC/15 min) produced a reduction in concentration *Listeria monocytogeenes* of 3.5 and 4.5 logs of CFU/g, respectively. No subsequent growth was observed in any case. The results are shown in **Figure 47**. There was no subsequent growth of *Listeria monocytogenes* in red grilled pepper- even with a relatively mild inactivation treatment- contrary to what has been observed in phosphate buffered saline solution.

These results confirm again that microorganism inactivation was very dependent on the pH and food composition and the results obtained in a buffer solution are not extrapolated for real food.

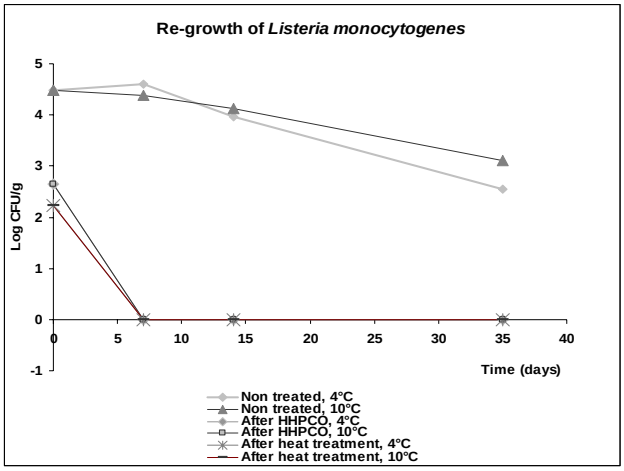


Figure 47: Growth of *Listeria monocytogenes* inoculated in grilled red bell pepper packaging under 95%CO_2, in no treated, and in HHPCO treated (400 MPa/35ºC/1 min) and in heat treated (70 ºC/15 min) products, during storage at 4 ºC and 10 ºC.

In additon to the cell reduction due to the treatments assayed in grilled red bell pepper, the remaining cells damaged by HHPCO treatment are inactivated faster (inactivation rate= 0.348 log c/day) than if they had not been treated (inactivation rate= 0.048 log c/day) during refrigerated storage (**Figure 48**).

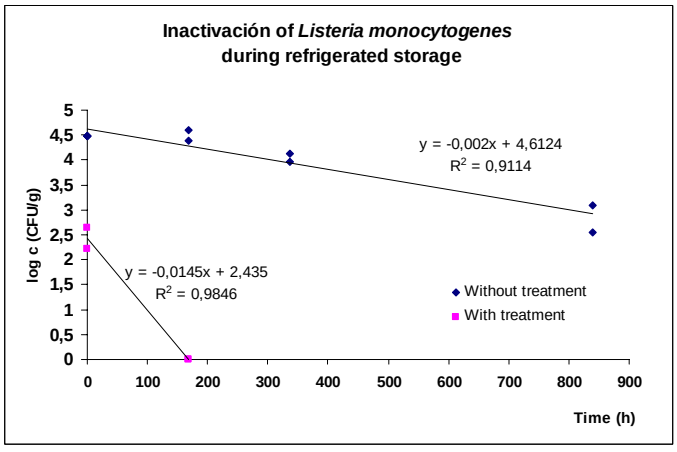


Figure 48. Inactivation of *Listeria monocytogenes* inoculated in grilled red bell pepper packaging under 95%CO_2, in non treated products and in HHPCO treated (400 MPa/35ºC/1 min), assuming no differences between the two temperatures assayed (4 ºC and 10ºC).

4.6. Specifications of treatment parameters (pressure, temperature, time of treatment, vacuum or CO_2 packaging) to apply the most convenient treatment in terms of microorganism (endogenous and inoculated) and enzyme inactivation.

After reviewing the main results found in previous sections regarding the treatment parameters: pressure, temperature, time of treatment, vacuum or CO_2 packaging in order to found the most adequate and convenient treatment in terms of microorganisms and enzyme inactivation, we found that, among the parameters studied, pressure had the biggest effect on the microorganism inactivation, while high temperatures compared with low temperature tend to favour the microorganism inactivation.
For enzyme inactivation, low pressures combined with high temperatures, as well as high pressures combined with low temperatures achieved good inactivation levels for the enzymes studied.
In addition, the type of packaging (air, 95% CO_2 and vacuum) did not significantly affect the microorganism and enzyme inactivation, although the packaging under 95%CO_2 better controlled microbiological growth than vacuum packaging during refrigerated storage up to 75 days at 4 ºC.

In conclusion, **HP treatment (400 MPa/45 ºC/ 3 min) combined with packaging under 95% CO_2 or vacuum** was chosen as a good alternative to guarantee the microbial safety and to preserve the enzymatic and sensory quality, as well as the nutritional and health related compounds in real plant foods during refrigerated storage.

4.7. Report of the shelf-life of a plant food treated with a combined HHP treatement selected in terms of enzymatic and microbological quality

Studies about the behaviour of endogenous microorganisms (total mesophilic bacteria, yeast and moulds and psycrophilic bacteria counts) and enzymatic activity during refrigerated storage (4 ºC) of HHPCO-treated products have been carried out. The red bell pepper products employed in this study have been provided by CAMST (ORMA). In this experimental design we have included the study of the effect of HP combined with different packaging systems (MAP with 95% CO_2 and vacuum) and vegetables in different physiological states (raw and grilled). Also we have analyzed the commercial product of CAMST with the aim to study the beneficial effects of the application of combined treatment HHP/MAP to increase the shelf-life of the product. All the products were storage at 4 ºC during 75 days. Treatments employed were 400 MPa/3 min/45 ºC). Some results have been shown below:

Refrigerated storage effect on POD and PPO of pre-cut grilled red bell peppers HHPCO treated:

Effects of cold storage on POD and PPO activities of HHPCO treated and commercial grilled red bell peppers have been studied.

In general, enzymatic activity in grilled red bell peppers was very low [POD activity 0.13 (ΔDO/min)/g fw]. Grilled samples treated by HHP and packaged under CO_2 showed higher POD inactivation levels during refrigerated storage than those HHP

treated and packaged under vacuum. Commercial samples did not show any POD activity. Also, no PPO activity was detected for grilled and commercial grilled red bell peppers.

Refrigerated storage on endogenous microorganisms of HHPCO grilled and commercial pasteurized red bell peppers:

High-pressure treatment combined with medium temperature (400 MPa/45 ºC/3min) reduced 2-3 logs units the total aerobic mesophilic counts and 3.8 logs units the psychrophilic bacteria counts in the both types of packaging assayed (vacuum and 95%CO_2)(**Figure 49**)
After 75 days at 4 ºC, the product packaging under 95%CO_2 presented a aerobic mesophilic total counts (1.97 log CFU/g) that was 2.5 log units lower than vacuum packaging and 3.58 logs units lower than the commercial thermal product after 75 days at 4 ºC. Also yeast and moulds were total inactivated and no counts were observed during the 75 days of refrigerated storage at 4ºC.

Differences between vacuum and CO_2 packaging in grilled pepper were not detected just after treatment, although the packaging under 95%CO_2 better control microbiological growth than HHP comibined with vacuum packaging or pasteurized thermal treatment combined with MAP packaging during refrigerated storage.

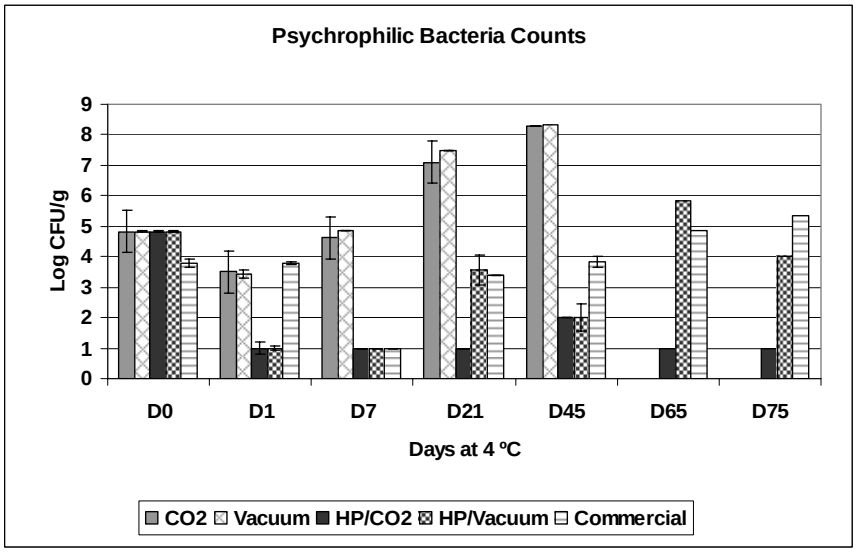


Figure 49. Storage effects on Total Psychrophilic Bacteria (TPB) counts in grilled red bell pepper packed under vacuum and CO_2. Comparison between HP treated products and untreated ones.

HHP (400MPa/45 ºC/3 min) in combination with packaging under CO_2 atmosphere could be a good alternative to preserve the quality of pre-cut red bell peppers during refrigerated storage.

4.8 Report on the effects of selected HHP combined treatment on nutritional and health promoting compounds (CSIC, UNIBO).

Study the effects of a combined HHP treatment selected (400 MPa/45 ºC/3 min) according to the results of packaging section (CO_2 and vacuum), microbial response, and enzymatic activity, and subsequent refrigerated storage on nutritional and health-promoting compounds (vitamin C, vitamin A, carotenoids, and antioxidant activity) of red bell peppers. Comparison with the results obtained with a thermal treatment. The plant material had been provided by CAMST.

Conclusions

Effect of treatment in raw red bell peppers (CSIC, UNIBO):
HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under vacuum increased the vitamin E content in *raw red bell peppers* compared with peppers vacuum-packaged without HHP treatment.
HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under vacuum increased the vitamin A content in *raw red bell peppers* compared with HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under 95% CO_2.
HHP treatment (400 MPa / 45 ºC / 3 min) combined with different packaging systems (95% CO_2 or vacuum) did not modified the antioxidant activity in *raw red bell peppers* compared with peppers without HHP treatment and packaged in these atmospheres.

Effect of treatment in grilled red bell peppers (CSIC, UNIBO)(Table 9):
HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under vacuum increased the vitamin E content in *grilled red bell peppers* compared with HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under 95% CO_2.
HHP treatment (400 MPa / 45 ºC / 3 min) combined with different packaging systems (95% CO_2 or vacuum) did not modified the vitamin A content and antioxidant activity in *grilled red bell peppers* compared with peppers without HHP treatment and packaged in these atmospheres.

Table 23. Vitamin E, vitamin A, and antioxidant activity in grilled red bell peppers. Effect of treatments.

	Vitamin E (mg/100 g)	**Vitamin A (β-carotene) (μg /100 g)**	**AA (mmol TE/100 g)**
95% CO_2	3.21 ± 0.57	295.00 ± 80.29	385.79 ± 35.85
Vacuum	3.47 ± 0.24	177.60 ± 48.81	352.59 ± 47.83
95% CO_2 + 400 MPa / 45 ºC / 3 min	1.52 ± 1.32	183.67 ± 61.50	295.09 ± 40.30
vacuum + 400 MPa / 45 ºC / 3 min	4.76 ± 0.56	297.00 ± 81.61	277.31 ± 46.02
ORMA (CAMST)	5.49 ± 0.17	432.33 ± 40.50	373.84 ± 0.91

Effect of treatment and refrigerated storage in raw red bell peppers (CSIC):

HHP treatment (400 MPa / 45 ºC / 3 min) combined with different packaging systems (95% CO_2 or vacuum) maintained the vitamin C content stable for *raw red bell peppers* during refrigerated storage (45 days).

HHP treatment (400 MPa / 45 ºC / 3 min) combined with different packaging systems (95% CO_2 or vacuum) maintained the total carotenoid content stable for *raw red bell peppers* until day 21 of refrigerated storage.

Regarding the antioxidant activity determined by the FRAP and DPPH$^{\bullet}$ assays, raw red bell peppers packaged with different packaging systems (95% CO_2 and vacuum) and those treated combining HHP with different packaging systems (95% CO_2 and vacuum) tended to increase the antioxidant activity from day 1 to day 45 compared with the untreated raw red bell peppers. In addition, this antioxidant activity was maintained stable for these days, and FRAP and DPPH$^{\bullet}$ values were significantly correlated.

Effect of treatment and refrigerated storage in grilled red bell peppers (CSIC):

Vitamin C significantly increased in pasteurized samples compared with untreated samples, being this increase stable during 21 days of refrigerated storage, and increasing from day 21 to days 45 and 65. HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under 95% CO_2 maintained the vitamin C content stable for *grilled red bell peppers* during 45 days of refrigerated storage, decreasing in days 65 and 75 (**Figure 50**).

The content of total carotenoids was maintained stable from day 1 to day 75 of refrigerated storage without significant differences among *grilled red bell peppers* with different treatments.

The antioxidant activity, determined by the three methods, significantly increased in pasteurized samples compared with untreated samples. Different packaging systems (95% CO_2 and vacuum) and HHP treatment (400 MPa / 45 ºC / 3 min) combined with these packaging systems (95% CO_2 or vacuum) increased in the antioxidant activity determined by FRAP and DPPH$^{\bullet}$ assays in day 1 compared with the untreated samples (**Figures 51 and 52**). However, this increase was not maintained during the storage period, decreasing in day 7. In addition, a significant correlations were found between vitamin C values and antioxidant activity values determined by FRAP and DPPH$^{\bullet}$, along with a significant correlation between FRAP and DPPH$^{\bullet}$ values (**Figures 53 and 54**).

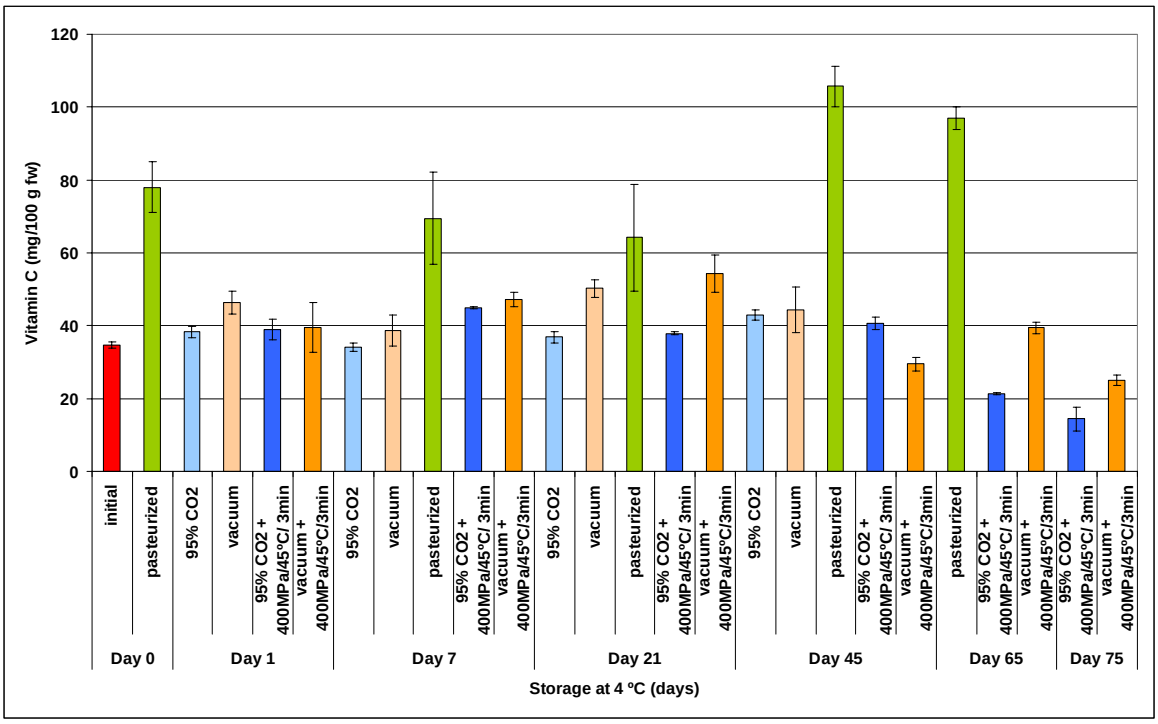


Figure 50. Vitamin C content in grilled red bell peppers stored at 4 ºC for up to 75 days.

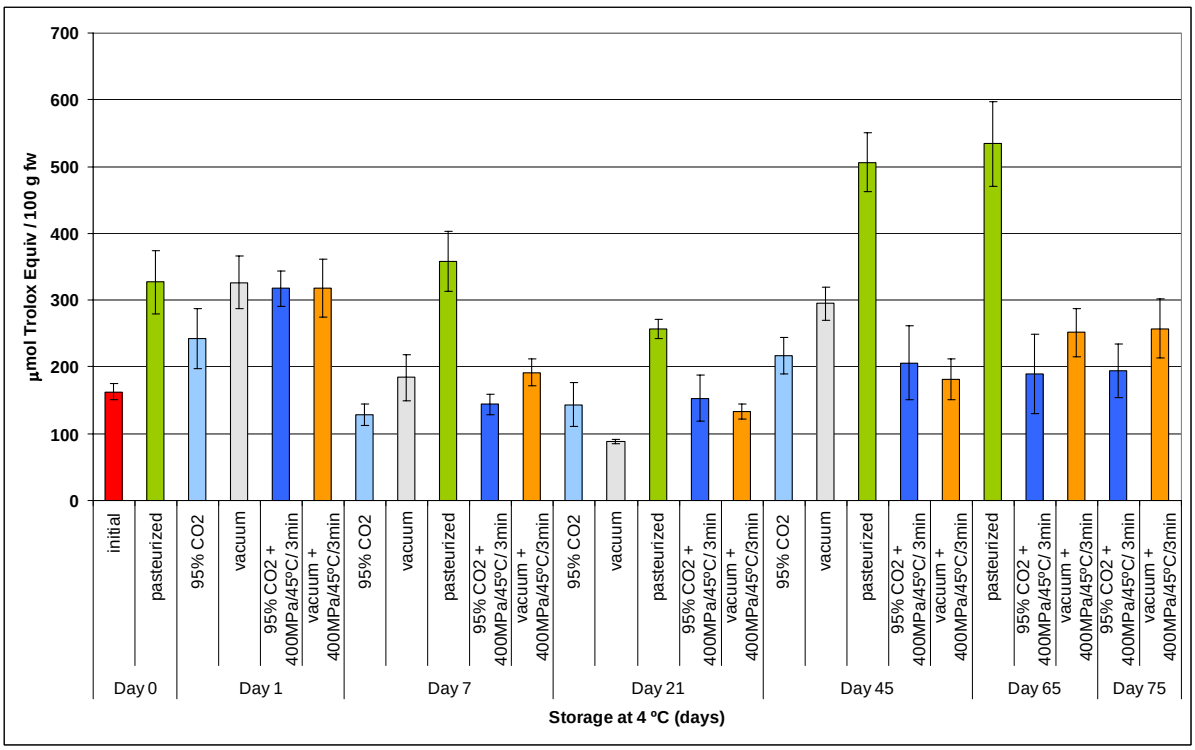


Figure 51. Antioxidant activity determined by the FRAP assay of grilled red bell peppers stored at 4 ºC for up to 75 days.

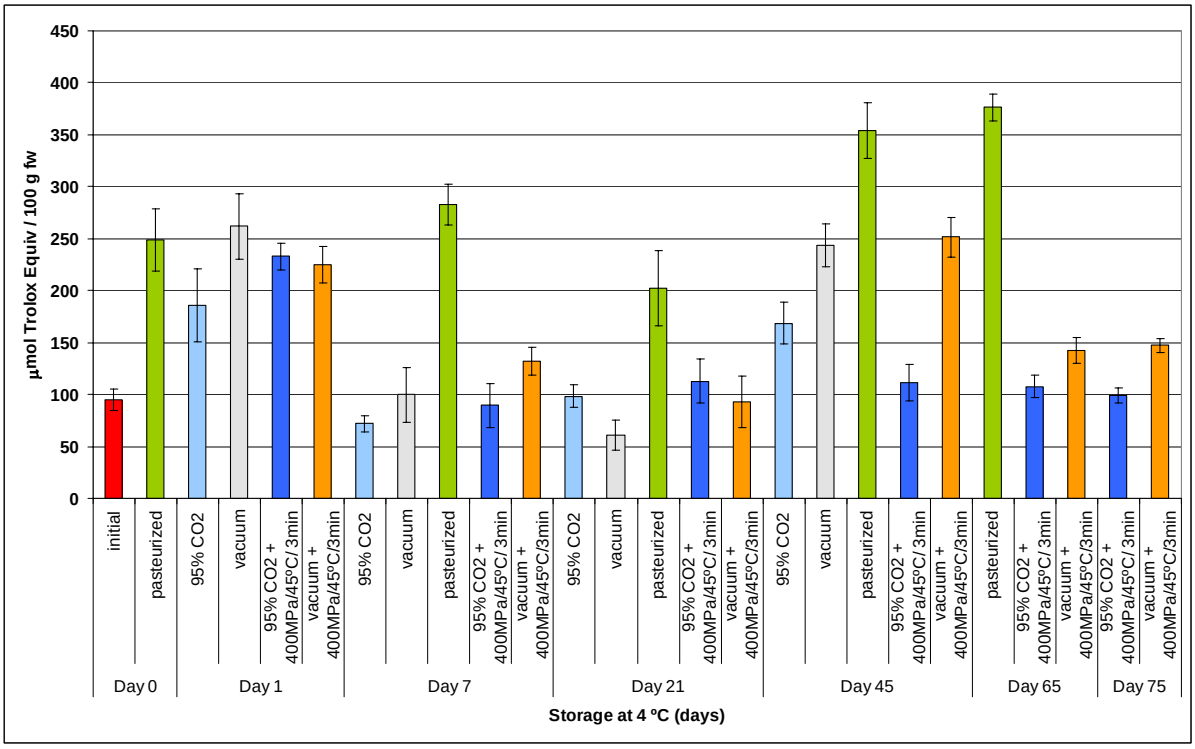


Figure 52. Antioxidant activity determined by the DPPH• assay of grilled red bell peppers stored at 4 ºC for up to 75 days.

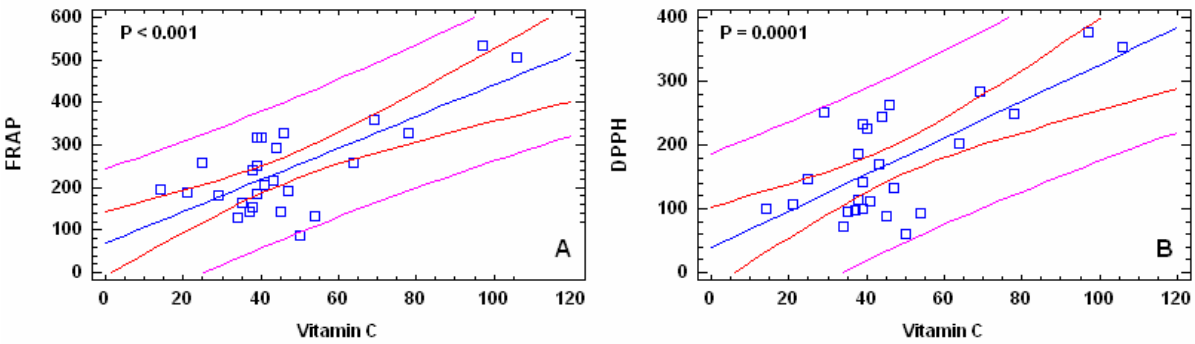


Figure 53. Correlation between vitamin C and FRAP values (r = 0.7261, P < 0.001) (A) and vitamin C and DPPH• values (r = 0.6937, P = 0.0001) (B) for grilled red bell peppers stored at 4 ºC for up to 75 days.

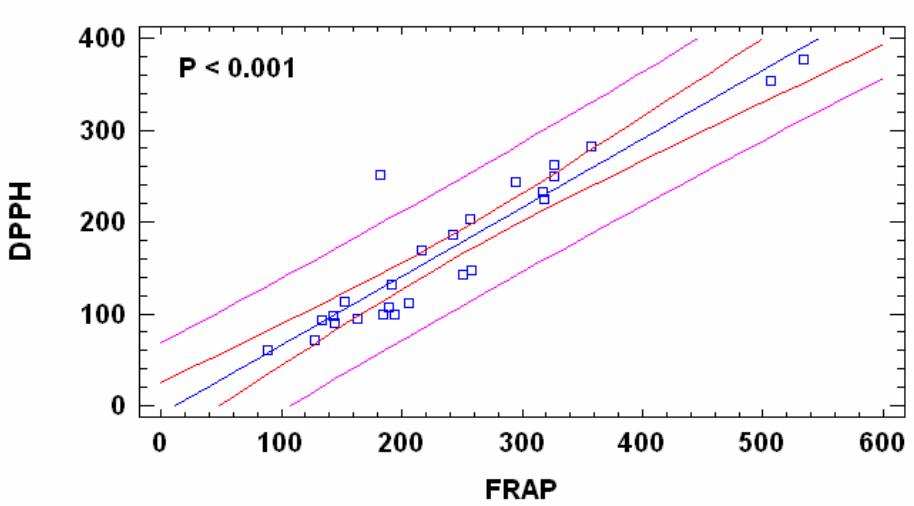


Figure 54. Correlation between FRAP and DPPH˙ values for grilled red bell peppers stored at 4 ºC for up to 75 days (r = 0.9282, P < 0.001).

4.9. Report on the effects of HHP selected combined treatment on sensory quality

Grilled red bell peppers were selected as products for the sensory evaluation. The effect of HPP combined with different packaging systems (95% CO_2 and vacuum) on sensory quality of grilled pre-cut red bell pepper during refrigerated storage was studied. Raw red bell peppers were minimally processed (washed, drained and cut into strips). Grilled samples were obtained grilling peppers at 180 ºC for 45 min. Then, grilled samples were packaged under carbon dioxide atmosphere (95% CO_2) or vacuum and were treated at 400 MPa and 45 ºC for 3 min (treatment selected in terms of microbiological and enzymatic quality). Treated samples were stored at 4 ºC for up to 60 days. Sensory quality (colour, texture, flavour and general acceptability) of treated grilled red bell peppers was evaluated.

Sensory evaluation: A panel of 15 members was selected on their ability to perceive differences between test products and to describe these perceptions. Samples were evaluated by panellists, throughout a 60 days period of storage at 4 ºC. They filled in a questionnaire using an acceptability test (colour, texture, flavour and general acceptability) on a scale of 1-10 and descriptive test (colour, texture and flavour). The questionnaire is shown in Annex III.

Conclusion Sensory analysis showed that HP treatment (400 MPa/45 ºC/3 min) combined with packaging under CO_2 atmosphere (95 % CO_2) or vacuum did not result in significant changes on the sensory quality (colour, texture, flavour and general acceptability) of grilled red bell peppers during 60 days of refrigerated storage at 4 ºC. In addition, panelists gave high scores to these kinds of products, indicating that they would buy any of them.

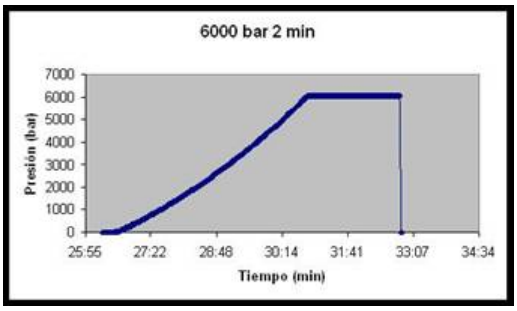
4.10. Report on the effects of technology based on HHP treatment combined with temperature and CO_2 packaging on microbial safety, shelf-life, enzymatic activity, sensory quality, nutritional and health promoting compounds in real plant foods, containing statistical information

HHPCO treatments performed at lab-scale have been validated at industrial pilot-scale using the industrial equipment located in NC-Hyperbaric (Burgos, Spain). NC-Hyperbaric is a Spanish industry that designs, manufactures and markets high pressure equipment for food processing since 1999.
The validation of HHPCO treatment on Ortoreale (ORT) products (grilled yellow and red bell peppers, and grilled vegetable mix) with regard to the traditional process was studied. Traditional treatment was pasteurized product (86-89 ºC/48-54 min) and packaged under MAP.
No pasteurized ORT products were prepared for HHPCO treatment in the IF-CSIC pilot experimental plant. They were placed in small cups (100 g) and, then, cups were thermosealed under 95% CO_2 atmosphere. The cups (EDV) employed were analyzed by LNE and the chemical safety and the mechanical resistance of the packaging after HHPCO have been demostrated.
Thermosealing machine and cups with a mix of yellow and red bell pepper packaged under 95% CO_2.

HP treatments: HP treatments were carried out in the industrial equipment mod. Wave 6000/55 located in NC-Hyperbaric (Burgos). This equipment has a horizontal vessel and it is equipped with an automatic loading and unloading system. The maximum work pressure was 600 MPa and temperature between 16-30 ºC. Pressure and time were controlled by a computer program, being constantly monitored and recorded during the process.

HP treatments assayed at industrial scale in NC-Hyperbaric was 600 MPa/25 ºC/2 min and 400MPa/25ºC/5 min. These treatments were equivalent to the lab-scale treatment (400 MPa/45 ºC/3 min) selected in IF-CSIC in terms of enzymatic inactivation (results of section 4.4), microbial reduction (>5 logs reduction of inoculated *Listeria monocytogenes, B.cereus and Salmonella enteritidis* and reduction of total aerobic mesophilic microoganisms)(results of section 4.5) and sensorial analysis (results of section 4.9).

NC-Hyperbaric equipment Wave 6000/55



HP parameters recorded in NC-Hyperbaric equipment (Wave 6000/55)

Conclusions related to Performance in Pilot-scale (Validation Task)

a.- *Effects of HHPCO treatment and refrigerated storage on the microbiological quality*: Microbiological quality was excellent for commercial pasteurized (ORT products) and HHPCO treated (600 MPa/25 ºC/2 min) samples (grilled yellow and red bell peppers and mix of grilled vegetables) during 105 days of refrigerated storage at 4 ºC.
b.- *Effects of HHPCO treatment and refrigerated storage on physicochemical parameters*: After 105 days of refrigerated storage at 4 ºC, commercial pasteurized samples reached the reject threshold of ORT (pH > 4.35), corresponding to highest acidity values, whereas HHPCO samples remained under this limit. Soluble solids showed similar values for pasteurized and HHPCO samples.
c.- *Effects of HHPCO treatment on sensory quality*: Sensory analysis showed that sensory quality (colour, texture, flavour and general acceptability) for grilled yellow and red bell peppers treated by HHPCO (600 MPa/25 ºC/2 min or 400 MPa/25 ºC/5 min) or pasteurized (commercial) was acceptable. However, sensory quality for the mix of grilled vegetables was low acceptable or even unsatisfactory. In general, acceptability of HHPCO products was higher with regard the commercial ones.
d.- *Effects of HHPCO treatment on POD activity*: No activity was detected for any of treatments assayed for grilled yellow and red peppers and for the mix of grilled vegetables.
e.- *Effects of HHPCO treatment on vitamin C content*: There were not significant differences in the vitamin C content between pasteurized samples (Commercial ORT) and samples treated by HHPCO (600 MPa/25 ºC/2 min or 400 MPa/25 ºC/5 min).

5. Conclusions

Packaging Conclusions

Chemical safety and mechanical resistance of two types of packaging (cups and Doypack bags) after HHPCO treatment (400 MPa/60ºC/5min) have been proved that HHPCO technology slightly affected the packaging selected (EDV-cups and Doypacks bags) treated at 400 MPa, 60°C for 5 min under air atmosphere (LNE).

There are no significant chemical and physical modifications in the 2 packaging by the HHPCO treatment.

Enzymatic Inactivation Conclusions

Enzyme Inactivation: Identification of the optimal conditions to inactivate plant food enzymes by HHPCO and set up of the related activities.
1. *Carrot slices*: Treatments at 300 MPa/5 min or 400 MPa/5 min (at any assayed temperature) were sufficient to obtain POD and PPO inactivation levels higher than 50 and 85%, respectively, for any atmosphere in raw carrot slices.
2. *Eggplant slices*: The combination of 400 MPa/5min/60 ºC and CO_2 packaging was an efficient treatment to reduce 86% the initial PPO activity in eggplant products. However, assayed combined treatments of high-pressure and temperature were not able to inactivate POD in eggplant slices. Higher enzymatic inactivation was observed at lower compression speed or when the treatment was applied in two cycles.
3. *Zucchini slices*: Samples treated at 400 MPa/60 ºC/5 min presented the highest enzymatic inactivation for POD (55 and 45% in vacuum and CO_2, respectively) and PPO (85 and 82% in vacuum and CO_2, respectively), being similar for both types of packaging. The highest POD and PPO inactivation (38% and 69%, respectively) was observed when the compression speed was 0, 5 MPa/s and packaging under CO_2 was employed.
4. *Red bell pepper strips*: The compression speed and the high-pressure treatment in one or two cycles did not influence POD activity for any type of packaging.

Microbial Inactivation Conclusions

Listeria innocua inactivation by HHPCO
In general, from the point of view of *Listeria innocua* inactivation in raw and grilled pre-cut red bell pepper and raw carrot, the more convenient HPP treatment could be the combination of Pressure at **400 MPa, temperature at 42.5 ºC (or 60 ºC) and time of treatment 3-5 minutes,** independently of the type of packaging employed. In this conditions was achieved a *L. innocua* reduction > 5 logs CFU/g.
Also it is important to considerer that the type of packaging (air, vacuum and 95%CO_2) seemed not to be determined factors in the inactivation of *Listeria innocua* by HPP and the temperatue also was a determined factor in the inactivation but only at higher temperatures than 42.5 or 60°C.

Inoculated microorganisms (Listeria innocua) of grilled red bell peppers. Effecst of HHPCO treatment and subsequent chilled storage. High-pressure treatment combined with medium temperature (400 MPa/45 ºC/3min) combined with packaging under 95%CO_2, produced a significant reduction of 6 Logs in the counts of *Listeria innocua* inoculated in grilled pre-cut red bell pepper. During 75 days of storage at 4 ºC it was not observed sublethal injury and subsequent recovery of the injury cells. No differences were found between vacuum and CO_2 packaging.

Non-inoculated microorganisms of raw red bell peppers. Effecst of HHPCO treatment and subsequent chilled storage. HP processing (400 MPa/45 ºC/3min) completely reduced (6 logs units) the total aerobic mesophilic counts and psychrophilic bacteria counts (under the detection limit) in raw pre-cut pepper in both types of

packaging (vacuum and 95% CO_2). The counts increased in both types of packaging during refrigerated storage, but after 45 days at 4 ºC the packaging under 95%CO_2 (6.9 log CFU/g) was 1 log unit lower than in vacuum packaging (8 log CFU/g). Also yeast and moulds counts were total inactivated and no counts were observed during the 45 days of refrigerated storage at 4ºC. Packaging under 95%CO_2 increase the shelf-life of raw red bell pepper up to 45 days.
Differences between vacuum and CO_2 packaging in raw pepper were not detected just after treatment, although the packaging under 95%CO_2 better control microbiological growth than vacuum packaging during refrigerated storage.

Non-inoculated microorganisms of grilled and commercial red bell peppers. Effect of HHPCO treatment and subsequent chilled storage. High-pressure treatment combined with medium temperature (400 MPa/45 ºC/3min) reduced 2-3 logs units the total aerobic mesophilic counts and 3.8 logs units the psychrophilic bacteria counts in the both types of packaging assayed (vacuum and 95%CO_2).
After 75 days at 4 ºC, the product packaging under 95%CO_2 presented a total mesophilic total count (1.97 log CFU/g) that was 2.5 log units lower than vacuum packaging and 3.58 logs units lower than the commercial thermal product after 75 days at 4 ºC. Also yeast and moulds were total inactivated and no counts were observed during the 75 days of refrigerated storage at 4ºC.

Differences between vacuum and CO_2 packaging in grilled pepper were not detected just after treatment, although the packaging under 95%CO_2 better control microbiological growth than vacuum packaging or pasteurized thermal treatment during refrigerated storage.

Inactivation kinetics of inoculated microorganisms (Listeria innocua CECT910, Salmonella enteritidis 155, Bacillus cereus SV90, Listeria monocytogene 56LY) in real plant food treated with HHP treatments (combined with temperature and different packaging (CO_2 or vacuum)(CSIC,IFR)

1. *Listeria innocua CECT 910.* The primary model of *Listeria innocua* inactivation suggest a linear trend at the lower pressures (100 and 250 MPa) for treatments up to 5 minutes, but at higher pressure up to 400 MPa the primary model could be convex. A non linear model log-logistic model was employed by IFR.

2. *Listeria monocytogenes*: When treatment at 400 MPa was combined with 25 ºC and 35 ºC, the inactivation of *Listeria monocytogenes* in grilled red bell pepper increased systematically when the time of treatment increase and the maximum reduction of 7 log CFU/g of *Listeria monocytogenes* was achieved after 5 minutes at 400 MPa/25 ºC and after 3 minutes at 400 MPa/35 ºC. Treatment at 400 MPa and 45 ºC reduced completely *Listeria monocytogenes* (7 log CFU/g reduction) just after 0.5 minutes of treatment.

3. *Bacillus cereus.* The inactivation of *Bacillus cereus* inoculated in grilled red bell pepper increased when pressure, time of treatment (holding pressure time) and temperature increase. The maximum reductions of 4.2 log cycles was achieved when 400 MPa was combined with 45 ºC during 2 minutes

4. *Salmonella enteritidis.* Treatments at 400 MPa / 25 ºC produced only 1 log reduction independently of the time of treatment (0.5, 1 and 2 min). When treatment at 400 MPa was combined with 35 ºC, the inactivation of *Salmonella* increased systematically when the time of treatment increase. Treatment of 2 minutes was enough to produce 7 log reductions of *Salmonella enteritidis* at 400 MPa combined with 35 ºC.

5. *Listeria monocytogenes* was the pathogen most easily eliminated at 400 MPa while *Bacillus cereus* is the most difficult to eliminate.
Regarding the effect of the treatment parameters, pressure had the biggest effect on the inactivation while higher temperatures tend to favour the inactivation.

Re-growth of Listeria monocytogenes 56Y during refrigerated storage at 4 and 10 ºC.
The HPP (400 MPa/35 ºC/1 min) and thermal treatement (70ºC/15 min) produced a reduction in concentration of *Listeria monocytogenes* of 3.5 and 4.5 logs of CFU/g, respectively. No subsequent re-growth was observed in any case during refrigerated storage at 4 ºC and 10ºC, moreover the remaining cells damaged by the thermal and HHPCO treatment was inactivated faster than if they had not been treated.

Nutritional Quality Conclusions

1.-Effect of treatment in raw red bell peppers:
HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under vacuum increased the vitamin E content in *raw red bell peppers* compared with peppers vacuum-packaged without HHP treatment.
HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under vacuum increased the vitamin A content in *raw red bell peppers* compared with HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under 95% CO_2.
HHP treatment (400 MPa / 45 ºC / 3 min) combined with different packaging systems (95% CO_2 or vacuum) did not modified the antioxidant activity in *raw red bell peppers* compared with peppers without HHP treatment and packaged in these atmospheres.

2.-Effect of treatment in grilled red bell peppers:
HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under vacuum increased the vitamin E content in *grilled red bell peppers* compared with HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under 95% CO_2.
HHP treatment (400 MPa / 45 ºC / 3 min) combined with different packaging systems (95% CO_2 or vacuum) did not modified the vitamin A content and antioxidant activity in *grilled red bell peppers* compared with peppers without HHP treatment and packaged in these atmospheres.

3.- Effect of treatment and refrigerated storage in raw red bell peppers:
HHP treatment (400 MPa / 45 ºC / 3 min) combined with different packaging systems (95% CO_2 or vacuum) maintained the vitamin C content stable for *raw red bell peppers* during refrigerated storage (45 days).

HHP treatment (400 MPa / 45 ºC / 3 min) combined with different packaging systems (95% CO_2 or vacuum) maintained the total carotenoid content stable for *raw red bell peppers* until day 21 of refrigerated storage.

Regarding the antioxidant activity determined by the FRAP and DPPH• assays, raw red bell peppers packaged with different packaging systems (95% CO_2 and vacuum) and those treated combining HHP with different packaging systems (95% CO_2 and vacuum) tended to increase the antioxidant activity from day 1 to day 45 compared with the untreated raw red bell peppers. In addition, this antioxidant activity was maintained stable for these days, and FRAP and DPPH• values were significantly correlated.

4.-Effect of treatment and refrigerated storage in grilled red bell peppers:

Vitamin C significantly increased in pasteurized samples compared with untreated samples, being this increase stable during 21 days of refrigerated storage, and increasing from day 21 to days 45 and 65. HHP treatment (400 MPa / 45 ºC / 3 min) combined with packaging under 95% CO_2 maintained the vitamin C content stable for *grilled red bell peppers* during 45 days of refrigerated storage, decreasing in days 65 and 75.

The content of total carotenoids was maintained stable from day 1 to day 75 of refrigerated storage without significant differences among *grilled red bell peppers* with different treatments.

The antioxidant activity, determined by the three methods, significantly increased in pasteurized samples compared with untreated samples. Different packaging systems (95% CO_2 and vacuum) and HHP treatment (400 MPa / 45 ºC / 3 min) combined with these packaging systems (95% CO_2 or vacuum) increased in the antioxidant activity determined by FRAP and DPPH• assays in day 1 compared with the untreated samples. However, this increase was not maintained during the storage period, decreasing in day 7. In addition, a significant correlations were found between vitamin C values and antioxidant activity values determined by FRAP and DPPH•, along with a significant correlation between FRAP and DPPH• values.

Performance in Pilot-scale (Validation Task) Conclusions

a.- *Effects of HHPCO treatment and refrigerated storage on the microbiological quality*: Microbiological quality was excellent for commercial pasteurized (ORT products) and HHPCO treated (600 MPa/25 ºC/2 min) samples (grilled yellow and red bell peppers and mix of grilled vegetables) during 105 days of refrigerated storage at 4 ºC.
b.- *Effects of HHPCO treatment and refrigerated storage on physicochemical parameters*: After 105 days of refrigerated storage at 4 ºC, commercial pasteurized samples reached the reject threshold of ORT (pH > 4.35), corresponding to highest acidity values, whereas HHPCO samples remained under this limit. Soluble solids showed similar values for pasteurized and HHPCO samples.
c.- *Effects of HHPCO treatment on sensory quality*: Sensory analysis showed that sensory quality (colour, texture, flavour and general acceptability) for grilled yellow and red bell peppers treated by HHPCO (600 MPa/25 ºC/2 min or 400 MPa/25 ºC/5 min) or pasteurized (commercial) was acceptable. However, sensory quality for the mix of grilled vegetables was low acceptable or even unsatisfactory. In general, acceptability of HHPCO products was higher with regard the commercial ones.

d.- *Effects of HHPCO treatment on POD activity*: No activity was detected for any of treatments assayed for grilled yellow and red peppers and for the mix of grilled vegetables.
e.- *Effects of HHPCO treatment on vitamin C content*: There were not significant differences in the vitamin C content between pasteurized samples (Commercial ORT) and samples treated by HHPCO (600 MPa/25 ºC/2 min or 400 MPa/25 ºC/5 min).

6. Impact on industry or research sector

The impact of the results obtained applying the combination of high pressure processing with a modified atmosphere packaging with 95% of CO_2 (HHPCO) to improve the shelf-life of ready-to-eat vegetable dishes has been summarized in the next paragraphs.

1.- This study has deeply contributed to improve the scientific knowledge related to chemical, physical and microbiological effects of HHPCO on vegetables, because is the first time that this combined technologies have been studied in depth for ready-to-eat (RTE) vegetable products.

2.-A great number of scientific and technological publishable results have been obtained related to the effect of this combined technology, HHPCO, on the inactivation of foodborne pathogens like *Listeria monocytogenes*, *Bacillus cereus* and *Salmonella enteritidis.* These results show the effectiveness of HHPCO to eliminate these pathogens and to reduce the risk of re-contamination of the ready-to-eat vegetable food after the treatment and during the storage and distribution of the manufactured product.

3.- The great data generated related to the inactivation of foodborne pathogens (*Listeria monocytogenes*, *Bacillus cereus* and *Salmonella enteritidis)* by HHPCO have allowed to develop survival models and implement them in a user-friendly software tool aiding to validate this new technology (HHPCO) and comparing it with other technologies by using Predictive Microbiology and Quantitative Microbial Risk Assessment (PM and QMRA) methods.

4.- The results provided new information about the duality of HHPCO treatment parameters (doses) and pathogen lethality level. The definition of these two factors could provide useful information for the selection of the manufacturing process parameters in order to design safety processes without detrimental sensorial and nutritional quality.

5.-These results provided to the industry a valuable information about this emerging technology showing that HPPCO can be a viable alternative to commercial pasteurisation in terms of microbiological safety, but with the added value that enhances the sensory characteristics of the product. Moreover, HHPCO could significantly increase the commercial shelf-life of the ready-to-eat vegetable dishes avoiding the use of additives and with minimum loss of the nutritional properties and sensorial characteristics.

6.- These results were reported to several of the major Spanish SMEs agri-food industries by the communication and dissemiantion activities planned in the Open-testing day in collaboratin with an Italian SME`s industry, i.e. Ortoreale.

7. Measure of the realised impact against the potential impact described in part 5 of the DoW

- *The initial expected potential impact related to food processing innovation and competitive value has been achieved*. This project has generated enough information about this new technology, HHPCO, from a scientific and technological standpoint, and for the industries to change the traditional thermal technology. These results have demonstrated that HHPCO could be a competitive food processing technology that significant increase the shelf-life of the RTE vegetable dishes avoiding the use of additives and with minimal nutritional and sensorial loss.

- *The initial expected potential impact related to generate more information to define microbial standards for new process has been achieved*. The results generated in the project have allowed increasing the knowledge about the lethality level for several pathogens on basis of the HHPCO death kinetics and recovery during storage and of the risk assessment. This information allows industries to predict commercial shelf-life and to define consumption patters.

- *The initial expected potential impact related to contribution to policy developments has been achieved*. The integration of research and industrial area in order to develop new process to improve the safety and quality of food systems have been addressed by the collaboration of one SME`s Italian company (ORT), dedicated to manufacture RTE vetable foods, in the validation of this new technology and in the organization and development of the HHPCO Open testing day, with the participation of several major Spanish agri-food industries. Also the HHPCO technology results were exposed in the two Join meeting (Gothemburg-Sweden and Bertinoro-Italy) held in conjunction with NOVEL-Q project.

- *The initial expected potential impact related to risk assessment has been achieved*. The great data generated related to the inactivation of foodborne pathogens (*Listeria monocytogenes*, *Bacillus cereus* and *Salmonella enteritidis)* by HHPCO have allowed to develop survival models and implement them in a user-friendly software tool aiding to validate this new technology (HHPCO) and comparing it with other technologies by using Predictive Microbiology and Quantitative Microbial Risk Assessment (PM and QMRA) methods.

Monitoring Techniques (UNIBO)

1. Introduction

Traditional methods for the detection of *L. monocytogenes* in food, as for other bacterial food-borne pathogens, are time-consuming. The methods include enrichment in selective media, subsequent plating on agar plates, and various tests for species identification (Anonymous, 1996; 2004). Rapid detection methods are required for microbiological quality control programs, which have to be applied throughout the food production chain. Various tests have been developed for this purpose. Immunological methods such as enzyme immunoassays, although relatively rapid, need a high number of target organisms for detection because assay sensitivity is low (Hara-Kudo *et al.,* 2005). Molecular assays such as PCR can obtain results within 1 day and are specific and sensitive. However, traditional PCR requires gel electrophoresis to detect and identify the target amplicons, followed by confirmation. Real-time PCR (rt-PCR) with fluorescent dyes (e.g., SYBR Green I) or fluorescent probes eliminates the need for agarose gel electrophoresis and subsequent confirmation. Detection is based on the increase in fluorescence as the DNA is amplified, reducing assay time by at least 2 h. Moreover, compared to conventional PCR, real-time PCR involves a lower risk of cross-contamination because the presence of the target in the sample is indicated by an increase in fluorescence, and no post-PCR processing of the samples is required (Heid *et al,* 1996). In addition, the fluorescent signal is proportional to the amount of target present in the sample and the method has the potential for automation.
Although these molecular assays have good sensitivity, DNA-based PCR methods cannot typically differentiate between dead and live or viable bacterial cells. Reverse Transcriptase (RT) PCR methods that target mRNA instead of DNA have a greater potential for detecting viable infectious cells (Maurer, 2006) or recent contamination because the half-life of mRNA is shorter than that of DNA. Moreover, RNA-based techniques, such as (RT) PCR, can be used to study genes and systems which play important roles in adaptation.
Adaptation to (sudden) adverse conditions in the environment of a bacterium requires the ability to respond rapidly. Such a bacterial response to environmental changes involves activation of existing enzymes and enhanced rates of transcription of genes, resulting in enhanced levels of proteins.
Understanding how bacteria sense and respond to stresses is essential in order to design optimal minimal processing regimes, combining maximum safety along the food chain with consumer demands for freshness and wholesomeness. The application of quantitative gene expression analysis techniques such as real-time RT-PCR (qRT-PCR) contributes immensely to the understanding of the complex biological processes behind stress adaptation. As a result, these molecular techniques are now increasingly being applied for rapid and sensitive quantification of the desired mRNA targets in cells (Bustin, 2000; Bustin *et al*., 2005). However, various challenges remain due to variations associated with RNA isolation, enzymatic efficiencies and standardization of the quantification results (Bustin, 2000; Vandesompele *et al*., 2002; Bustin *et al*., 2005).
Extensive investigation focused on the effects of environmental factors on the bacterial heat resistance has been performed as heat treatment is one of the principal methods used in food industry to eliminate pathogens from food products. As was shown, microorganisms show modifications in their heat resistance after exposure to certain

environmental and preservation stresses, such as acid and cold stresses (Annous and Kozempel, 1998; Rowan and Anderson, 1998; Martı´nez *et al*., 2003; A´ lvarez-Ordo´n˜ez *et al*., 2008).
It is well known that the heat shock response has the potential to enhance bacterial survival in processed foods. *L.monocytogenes* has the capacity to elicit a heat shock response and also clearly induces synthesis of the conserved heat-shock proteins, DnaK and GroEL, following sub-lethal heat shock (Hill *et al.,* 2002). These heat shock proteins act as molecular chaperonins, protecting essential bacterial proteins from heat denaturation. In addition to their role in heat stress, DnaK and GroEL are induced following exposure to other environmental stresses such as low pH, elevated salt and ethanol indicating a protective role in the general stress response (Hartke *et al.,* 1997; Kilstrup *et al.*, 1997; Gahan *et al.,* 2001).
Wemekamp-Kamphuis *et al*. (2004) described the role of σ^B in acid stress adaptation of exponentially growing *L. monocytogenes* cells. Additionally, the role of σ^B in two industrially important processes, high-hydrostatic-pressure (HHP) treatment and freezing, was assessed in combination with pretreatment at a low pH in order to assess the protective effect of adaptation to these food-processing methods. Up to now, no research has been made on the effects of high pressure homogenization treatments on gene expression in *Listeria monocytogees.*

2. Objectives

The main objective was to assess, with molecular methods, the survival, recovery and response of subletally damaged cells of selected species following the treatment with PEF, HPH and thermal treatments in model systems.
In particular, protocols for PCR-DGGE, FISH, quantitative-PCR, quantitative reverse transcriptase PCR have been developed and compared in terms of efficiency, sensitivity, specificity and feasibility to detect viable *Listeria monocytogenes* in model systems.
Moreover protocols based on the quantification of mRNA have been developed and used to determine the expression of some selected stress induced genes in *L. monocytogenes* following the exposure to selected PEF, HPH or heat treatments.

3. Materials and Methods

L. monocytogenes strains and culture conditions

Bacterial strains used for these studies included *Listeriamonocytogenes* strains SIK 564/Scott A and UNIBO 56Ly belonging to the collections of Institutet for Livsmedel och Bioteknik AB (SIK) and Department of Food Science of University of Bologna, respectively.
The bacteria were maintained at −80 ◦C and routinely cultured in Brain-Heart Infusion (BHI) (Oxoid) at 37°C.

Detection of viable *L. monocytogenes* by Quantitative reverse Transcription PCR

As far as the detection through the RT-q-PCR method of RNA expression from low concentrations of cells, extraction of total RNA from cell cultures from *L. monocytogenes* 56Ly was made using Ambion RNAqueous, Small Scale Phenol-Free Total RNA Isolation Kit. Contaminating DNA was removed by adding DNase and thereafter the RNA was translated into cDNA by using with random primers. Finally

PCR amplification of the cDNA was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) as described in the manufacturer's instructions.

Gene expression following Technological treatments

The influence of different technological treatments on target genes expression was assessed in model system (BHI medium or phosphate buffer) by using exponential-phase cultures.

In particular *L. monocytogenes* cells (~10^7 CFU/ml) were exposed to the following treatment conditions:

1) PEF – 35kV/cm, 6µs, 3 pulses; 35kV/cm, 6µs, 5 pulses; 32 kV/cm, 10µs, 5 pulses. All the treatments were performed in phosphate buffer with a conductivity of 5 mS/cm. After each PEF treatment samples were maintained at 25°C for 9, 6, 10, 15, 20 and 45 minutes followed by RNA isolation.
2) HPH – 0.1, 20, 40, 60, 80 and 100 MPa with a lab-scale equipment.
3) HT - 70°C for 30, 60 or 120 seconds.

Immediately after HPH and HT treatments and after 15 min, 30 min, 1 hour, 3, 6 and 24 hours of incubaton at 37°C samples were withdrawn, added with RNAprotect™ Bacteria Reagent (Quiagen) and processed for total RNA template isolation.

The various total RNA templates prepared from the different samples were subsequently applied for the analysis of target genes expression in gene-specific real-time RT-PCR assays.

In particular the genes taken into consideration have been: *rpoB*, *opuCA*, *gadA, groEl, groES, clpP*, *clpC* and *16SrRNA*, which was used as the housekeeping one.

Total RNA isolation

Total RNA was isolated from each sample using the Trizol® Reagent (Sigma) and following the instructions of the manufacturer.

RNA purification and cDNA synthesis

RNAs were treated with DNase I according to the manufacturer's directions (Promega) prior to cDNA synthesis. The RNA concentration was measured by absorbance at 260 nm, while purity was monitored by inspection of the 260/280nm and 230/260nm ratios to assess protein and organic substances contamination, respectively.

The reverse transcription step was performed using the-MLV Reverse transcriptase (Promega). This procedure incorporated a blend of dNTPs and random primers for cDNA synthesis. The cDNA template generated from each sample was subsequently used to assess the various transcript levels in gene-specific real-time PCR assays.

Real-time PCR

The real-time PCR reactions were performed in a Rotor Gene 6000 instrument (Corbett) in a total reaction volume of 20 µl. This reaction contained 5 ng cDNA template, 0.4 mM of each primer and 1 x Sybr® Premix ExTaq (Takara).

The target genes and their primer sets are shown in Table 24.

Genomic DNA-based standard curves were used to determine the efficiencies of the genes target amplification by real-time PCR

$$E = 10^{-1/slope}$$

Data on the expression level of the various genes were obtained in the form of crossing points (CP) values; further data analysis was performed to calculate the Relative Gene Expression (RGE) according to Pfaffl (2001).

$$RGE = \frac{\left(E_{\text{target gene}}\right)^{\Delta Ct_{\text{target}}(control - sample)}}{\left(E_{\text{housekeeping gene}}\right)^{\Delta Ct_{\text{housekeeping}}(control - sample)}}$$

The *16S rRNA* has been used as the housekeeping gene.

Table 24. Details of primers for each gene of *Listeria monocytogenes*.

Target Gene	Primer sequence (5'→3')	Rerefence
16SrRNA F	CTTCCGCAATGGACGAAAGT	Frazer *et al*., 2003
16SrRNA R	ACGATCCGAAAACCTTCTTCATAC	
gadA F	TGGCGGTTTGGCAATGA	Kazmierczak *et al.*,2006
gadA R	TGCCTGTATATCCAGACCTCGTT	
opuCA F	ACATCGATAAAGGAGAATTTGTTTGTT	Sue *et al.*,2004
opuCA R	GCCGGTTAATCATCTTCATTGTT	
rpoB F	TGTAAAATATGGACGGCATCGT	Sue *et al.*,2004
rpoB R	GCTGTTTGAATCTCACTTCCGTTTGG	
groES F	GCCAAAGAAAAACCGCAATC	Pang *et al*., 2007
groES R	TGTGTCACCTTCTGCAACTTC	
groEL F	GTAGTAGCCGTGAAAGC	Pang *et al*., 2007
groEL R	GTAGAGCGGAACGTGTTA	
clpP F	AGCGGACGTACAAACAATCG	Gaillot *et al*., 2000
clpP R	AATTTCAGCGTTTGGCAAGG	
clpC F	AGTCGATGTTTGGCGATGAG	Rouquette *et al*., 1998
clpC R	TGGAGGAGCCCCAACTAAAC	

4. Results

Development of viable *L. monocytogenes*

Protocols for different molecular techniques based on DNA and RNA extraction have been developed and compared. In particular, protocols for PCR-DGGE, FISH, q-RT-PCR have been developed for *Listeria monocytogenes*. The developed PCR DGGE protocol, aimed to be used to study the possible shift and the microbial community of foods following processing, showed a good specificity for *Listeria*; however, PCR-DGGE method is not sensitive enough to detect low amounts of bacteria. Also an attempt to develop a method based on q-PCR and PMA able to differentiate between viable and dead cells was not successful. Despite all the experiments made in order to optimise several factors (PMA concentration, light exposure and light distance, light source, shaking), inconsistent and not always significant results have been obtained.

As far as the detection through the RT-q-PCR method of RNA expression from low concentrations of cells, the results obtained evidenced that at cell concentrations $<10^5$ CFU/ml it was difficult to extract RNA as the RNA concentration did no decrease with decreasing CFU (Figure 55). This may result in inconsistent gene expression analysis.

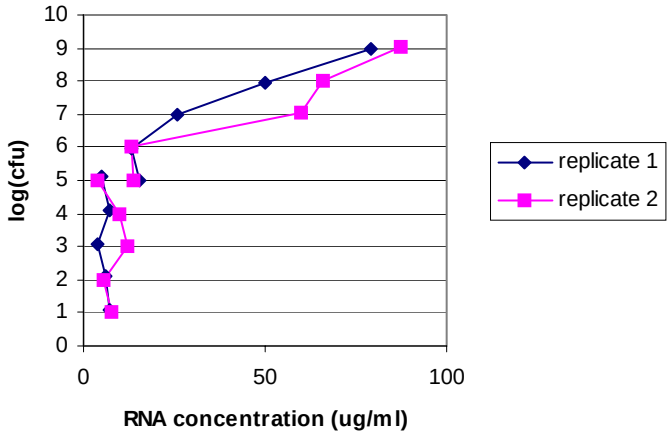


Figure 55. log CFU versus RNA concentration.

The total RNA dilutions were analysed with the RT-q-PCR methods for the *16SrRNA* and *rpoB* and plotted versus log CFU (Figure 56).

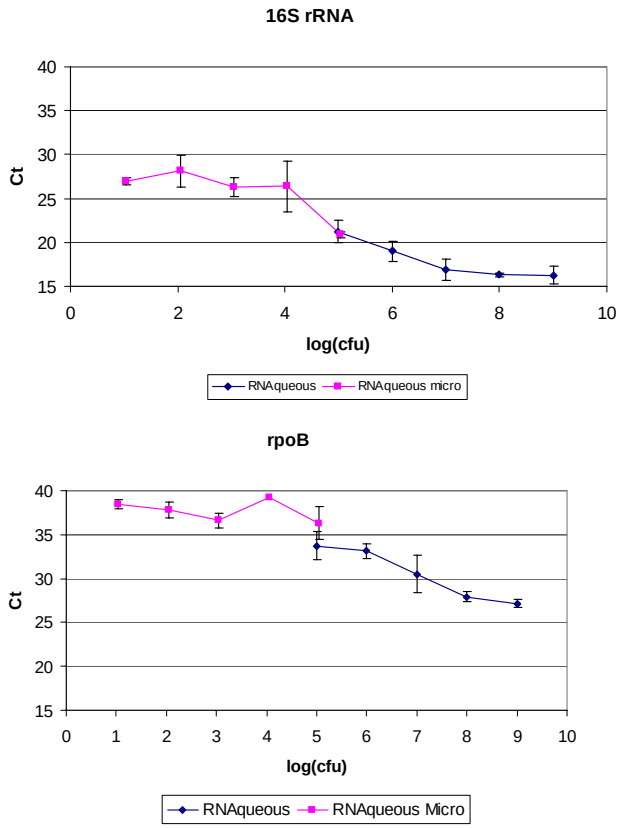


Figure 56. Standard curves for *16S rRNA* (upper) and *rpoB* (lower).

In Figure 57 the linear ranges of the curves are reported. For the *16S rRNA* the amplification efficiency in this range was determined to be E=1.16 and for the *rpoB* the E-value was determined to E=1.29. Although good amplification efficiencies were obtained for both the genes, the range of linearity was quite narrow (from 4 to 7 log CFU), thus not allowing the use of the RT-q-PCR methods for the detection of low concentrations of viable cells.

On the other hand, RT-q-PCR protocols to determine the expression of 7 stress induced genes in *L. monocytogenes* have been developed. Such protocols have been used to evaluate the effects of different PEF (by SIK), HPH and heat (by UNIBO) treatments on gene expression profiles of the selected genes, i.e.*gadA*, *rpoB, opuCA. groEl, groES*, *clpP*, *clpC* and *16SrRNA*, which was used as the housekeeping one. All the studies have been performed in a model system by using *L. monocytogenes* 56 Ly as target organism.

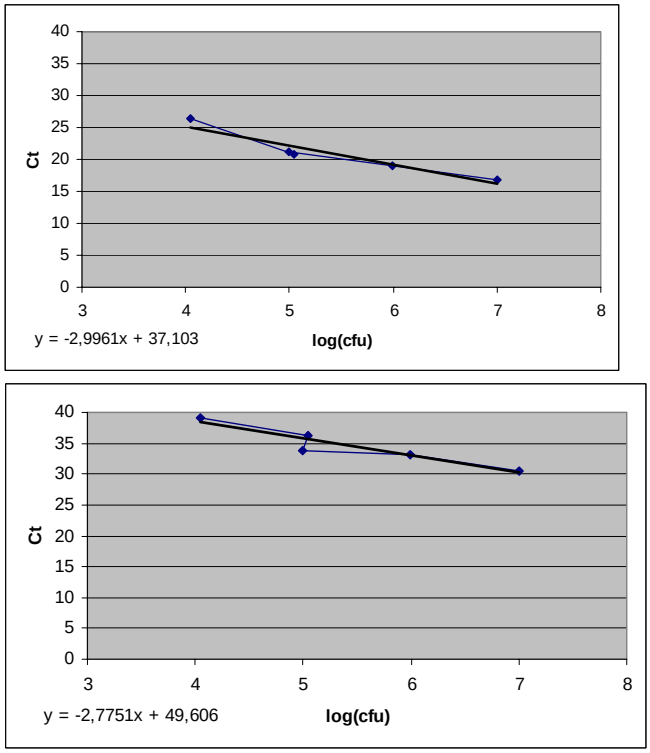


Figure 57. Linear range of detection for the *16S rRNA* gene (upper) and *rpoB* gene (lower).

Gene expression profiles after PEF treatments

The effect of PEF treatments on *L. monocytogenes* SIK 564/Scott A was evaluated by measuring the activity of three genes involved in protein synthesis (*rpoB)*, stress response (*gadA*) and acid adaptation (*opuCA*) during a time span of 0 to 45 min following the different treatments.

All three PEF treatments had an effect on the genetic activity of *opuCA* and *rpoB* (Figures 58 and 59). For *opuCA* the highest expression (70-85-fold higher than untreated cells) was observed after 10 minutes in 25 °C (Figure 58). This result is consistent for both PEF treatments with the field strength of 35kV/cm, but not for PEF treatment with field strength 32 kV/cm. Also at 15-20 minutes after PEF treatment with 35kV/cm, 6µs, 3 pulses, the *opuCA* was still induced (14-fold higher than untreated cells). On the other hand when increasing the pulse length to 10 µs and decreasing the

field strength to 32 kV/cm the *opuCA* had the highest induction after 45 minutes (11-fold higher than untreated cells).

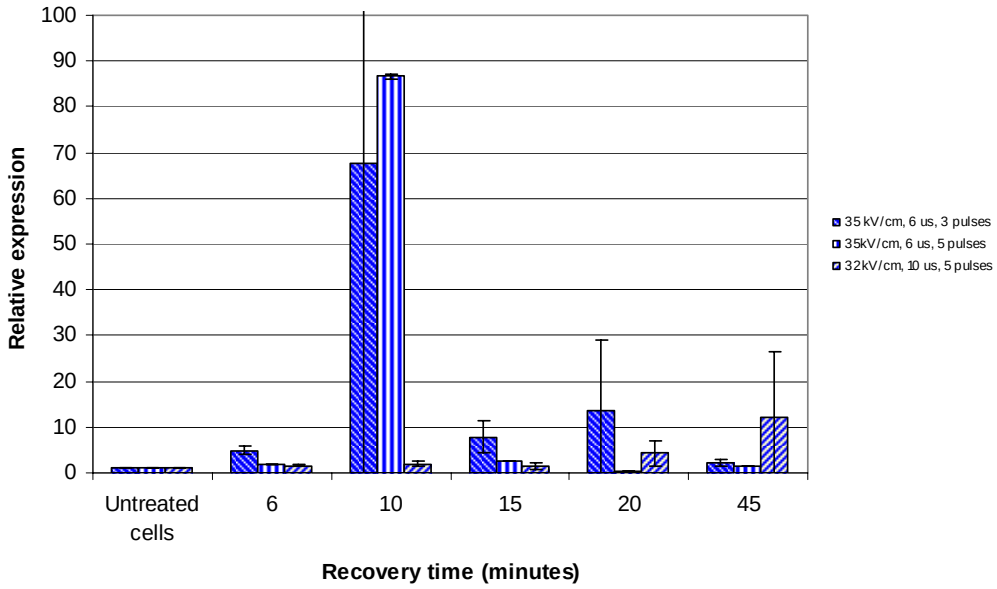


Figure 58. The relative expression of *opuCA* in *L. monocytogenes* SIK 654/Scott A before and up to 45 minutes at 25°C after PEF treatment. The reference sample is untreated cells (relative expression of 1.00).

The same trend was observed for the *rpoB* gene, with the highest expression after 10 minutes at 25°C after PEF treatments 35kV/cm, 6µs, 3 pulses and 35kV/cm, 6µs, 5 pulses. After PEF treatment 35kV/cm, 6µs, 3 pulses, the *rpoB* was still induced after 45 minutes in 25°C (8 times higher compared to untreated cells).

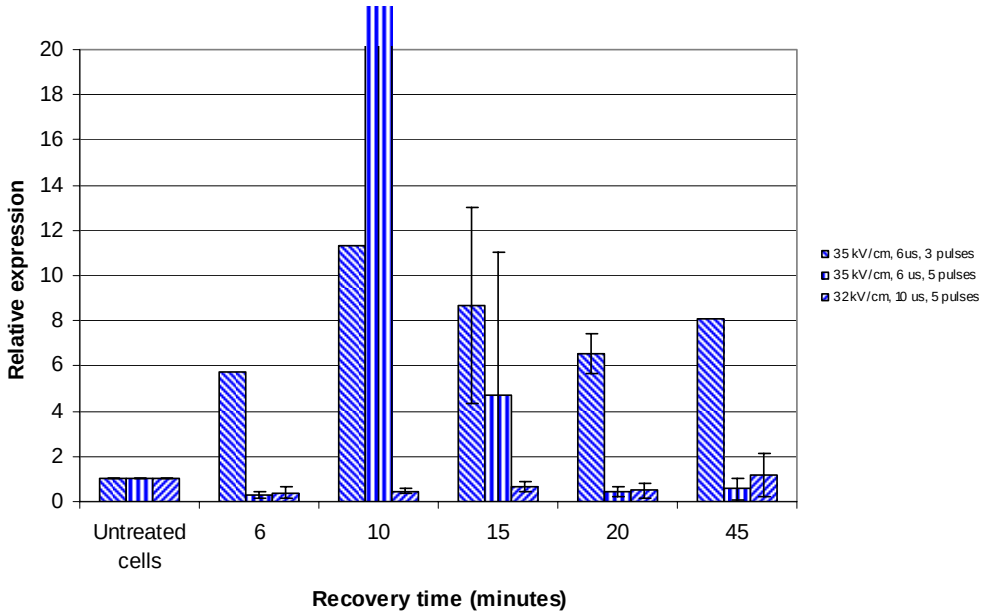


Figure 59. The relative expression of *rpoB* in *L. monocytogenes* SIK 654/Scott A before and up to 45 minutes at 25°C after PEF treatment. The reference sample is ★

untreated cells (relative expression of 1.00). A high variation in relative expression ratio between the two individual replicates was observed.

The expression of the heat shock gene, *groES*, was also evaluated after PEF treatment on *L. monocytogenes* SIK 564/Scott A and *L. monocytogenes* UNIBO 56 Ly by measuring its activity both after: (i) mild heat treatment for 5 or 15 minutes at 40, 45 and 50°C; (ii) PEF treatment 32kV/cm, 10µs, 5 pulses. After the PEF treatment, samples were maintained at 25°C for 6, 10, 15 minutes until sampled for RT-q-PCR analysis. All treatments were performed in phosphate buffer with a conductivity of 5 mS/cm.

Figure 60 shows the expression of *L. monocytogenes* SIK 564/Scott A after mild heat treatment for 5 and 15 minutes, with no PEF treatment. The *groES* was highly induced after all treatments with the highest increase in expression after 5 minutes in 50°C.
After the PEF treatment, which resulted in a maximum temperature increase in the chamber of 45°C, the *groES* activity was down regulated. This was the same after 6, 10 or 15 minutes post PEF treatment. The relative expression level was between 20-30% of the expression of untreated cells.

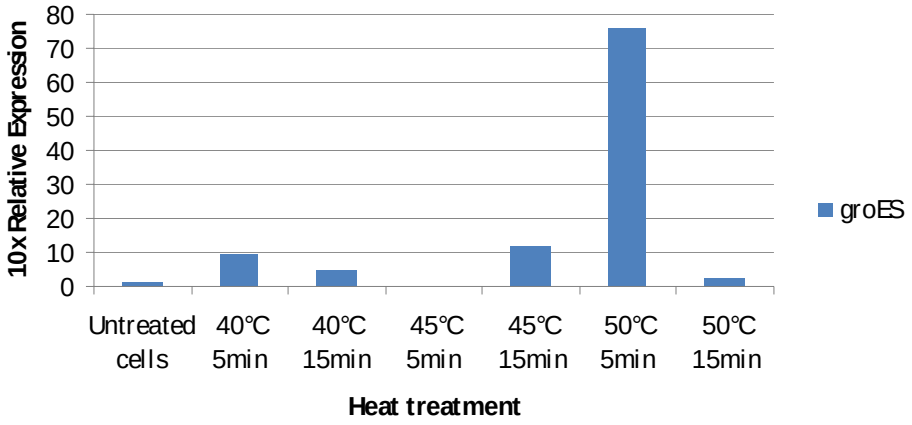


Figure 60. Expression of *groES* in *L. monocytogenes* ScottA after different mild heat treatments in phosphate buffer. No data was obtained after 5 minutes in 45°C. The reference samples are untreated *L. monocytogenes* cells.

Gene expression profiles after HPH and thermal treatments
The evaluation of the effects of technological treatments on the gene expression was performed by subjecting cell suspensions (~$10^{7\text{-}8}$CFU/ml) of *L. monocytogenes* 56Ly to:

1) a single HPH treatment at 0.1, 20, 40, 60, 80 or 100 MPa
2) 1, 3 or 5 repeated HPH treatments at 80 MPa
3) thermal treatments at 70 °C for 0, 30, 60 and 120 seconds.

Concerning the *opuCa* and *clpP* genes, their expression was increased up to 8 times by treating the cells at increasing pressures up 60MPa; however, a further increase in the pressure resulted in a reduction in both their relative gene expressions. Also the genes *rpoB*, *gadA* and *clpP* were over-expressed immediately after the HPH treatments at

pressures up to 60MPa; on the other hand a repression of all these genes was observed from 15 minutes to 24 hours following the HPH treatments (Figure 61). No significant changes were observed for the *groES* gene regardless the pressure used and the sampling time, while the *groEL* gene was never expressed in any of the experimental conditions taken into consideration.
Repeated HPH treatments performed at 80MPa resulted in a down-regulation of all the genes regardless the number of repeated cycles applied and the sampling time following the treatment.

When the cells were subjected to thermal treatments at 70°C for different times up to 2 minutes, an immediate increase in the gene expression was observed for *opuCA*, *rpoB*, *gadA*, *clpC*, *groES* and *groEL* (Figure 62). The extent of their over-expression was dependent on the treatment time. Moreover, such over-expressions were almost stable for at least 30 minutes following the thermal treatment, after which their values decreased.

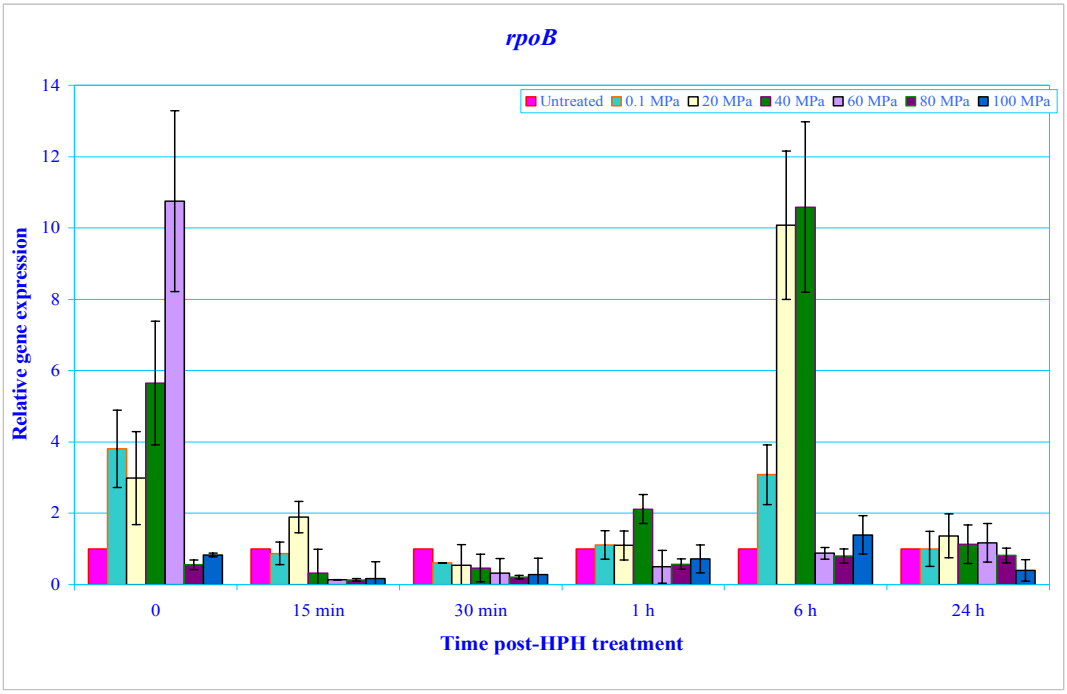


Figure 61 – Evolution of relative gene expression values of the gene *rpoB* in *Listeria monocytogenes* 56Ly in model system (BHI) following HPH treatments at different pressure levels.

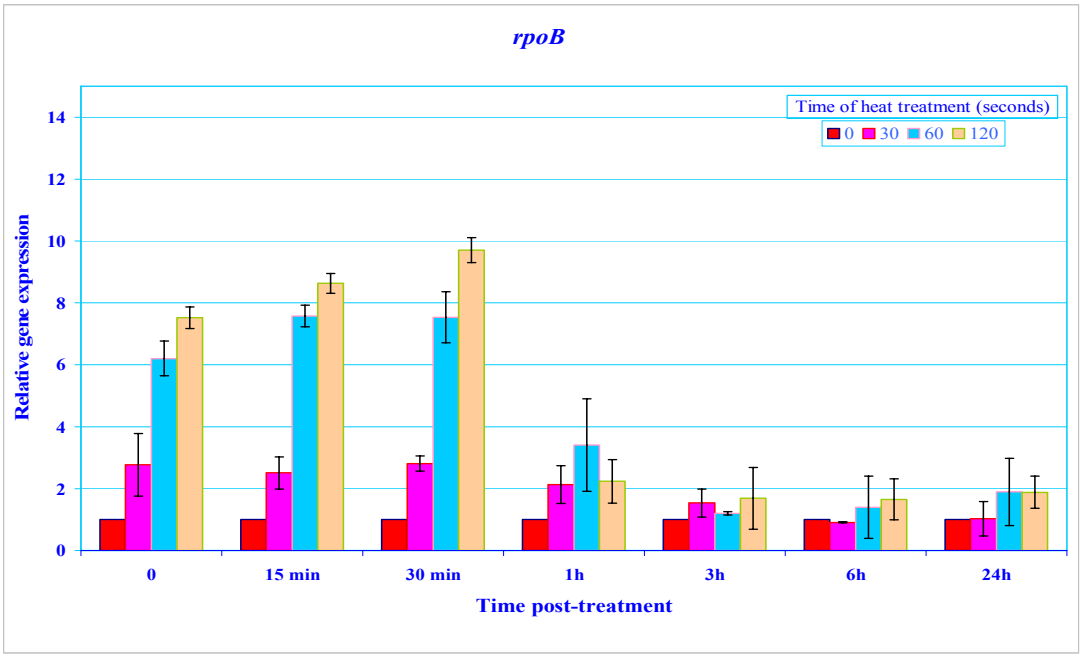


Figure 62 – Evolution of relative gene expression values of the gene *rpoB* in *Listeria monocytogenes* 56Ly in model system (BHI) following thermal treatments at 70°C for different times.

5.Conclusions

Protocols for different molecular techniques based both on DNA and RNA extraction have been developed and compared. In particular, protocols for PCR-DGGE, FISH, q-RT-PCR have been developed for *Listeria monocytogenes* in model systems and tested in real fluid foods. Although the DNA-methods were charcaterised by good specificity for the target microorganism, they failed to be sensitive enough to detect low levels of bacteria. Also the RT-q-PCR method based on mRNA, targeting the *16S rRNA* could not be used for cell concentrations $<10^5$ CFU/m as below this threshold level the RNA concentration did no decrease with decreasing CFU.

On the other hand the developed q-RT-PCR protocols were successfully used to monitor the effects of different technologies on the expression of some selected genes in *Listeria monocytogenes* in model system.
When *L. monocytogenes* was exposed to heat between 40-50°C for 5-15 minutes it was found that the *groES* was highly upregulated. GroES is a heat shock protein whose gene expression is upregulated when bacterial cells are exposed to stress factors such as heat. However, after PEF treatment the expression of *groES* was down regulated to approximately 20-30% of the expression in untreated cells. This suggests that the PEF treatment is to brief to cause any damage to the cell that are comparable to heat stress.
Concerning HPH treatments, a threshold pressure value corresponding to the maximum relative gene expression value was found. In fact several genes were over-expressed immediately after the exposure to a HPH single treatment at 60 MPa; however, a further increase in the pressure level resulted in a reduction in their relative gene expression. On the other hand, repeated HPH treatments performed at 80MPa resulted in a down-regulation of all the genes regardless the number of repeated cycles applied and the sampling time following the treatment.

Another interesting aspect related to the experimental results obtained was the time-dependant relationship between the relative gene expression and the specific processing conditions adopted. In fact all the PEF treatments taken into consideration had a significant effect on the *gadA*, *rpoB* and *opuCA* genes, particularly 10 minutes after the treatment. While the maximum over-expression due to heat treatments at 70°C was observed 30 minutes after the treatments, HPH treatments resulted in an up- or down-regulation of most of the gens immediately after the treatments when pressure levels of 60 MPa or >60 MPa were used, respectively.

These results have important implications for the food industry as the application of quantitative gene expression analysis techniques such as qRT-PCR can contribute to the understanding of the complex biological processes behind stress adaptation. It is well known that the food-borne pathogen *Listeria monocytogenes* continues to be involved in both sporadic and epidemic food-borne illnesses around the world. This organism must frequently adapt and overcome various forms of hostile conditions during transmission in foods and related environments.
The versatile adaptation capacity of *L. monocytogenes* to the stress challenges (e.g. low or high temperatures, low pH, high NaCl content, technological processes) stems from a well-coordinated appropriate induction of multiple molecular response mechanisms by stressed cells. Understanding how bacteria sense and respond to mild stresses may provide targets for inactivation strategies, and therefore it will be essential to design optimal processing regimes, assuring maximum safety with consumer demands for freshness and wholesomeness.

6. Impact on industry or research sector

This study has deeply contributed to improve the scientific knowledge on the effects of different processing technologies (PEF, HPH and heat treatments) on gene expression in *Listeria monocytogenes*.
L.monocytogenes is a versatile organism, capable of growth under many stressing conditions, such as refrigeration temperatures, high salt foods, acid foods and within the human immune system, or survival/recovery following exposure to different processing technologies. Such versatility requires that the organism has the ability to assimilate information about its environment, and process this information quickly in order to adapt and/or respond to changing conditions.
The identification of response mechanisms to technological/environmental stressing conditions may provide targets to optimize inactivation strategies.
Although the resistance of *L. monocytogenes* to several processing /stressing conditions, particularly heat shock exposure, has been widely studied and a number of genes involved in heat stresses have been identified, no paper on the effects of the studied technologies, i.e. PEF and HPH, on response mechanisms in *L. monocytogenes* has been published yet.
The preliminary results obtained within the HighQ RTE project can provide useful information for both the research sector aiding the identification and further understanding of stress sensing and food industry in order to set up and optimize inactivation strategies to assure safe foods.

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Quantitative Tools and Methods (IFR)

1.State of the art at the beginning of the project

The modelling of inactivation of pathogens by heat treatment is often recognised as the first example of predictive microbiology. Esty and Meyer (1922) demonstrated a linear relationship between the heating temperature and the logarithm of the time needed to inactivate suspensions containing 60 billion ($6*10^{10}$) spores of proteolytic *Clostridium botulinum*. This led to the standard for canned food sterilization being based on a reduction of the spore population by a factor of 10^{12}. Studies on the kinetics of thermal inactivation established that the concentration of viable vegetative cells or spores decreases exponentially with time during the heat treatment.
The model describing the logarithm of the viable cell concentration as a function of time is called the primary model. If it is linearly decreasing, we can define a `D' value, where D is the time needed for one decimal reduction in viable cell numbers. Deviations from this linearity have often been observed, particularly in the case of vegetative cells, with `shoulders' and/or `tails' on the semi-log scale of the survival curves (Peleg and Cole, 1988). For such cases, alternative models have been proposed including the log-logistic model, Weibull model, Baranyi model etc. (Geeraerd *et al.*, 2004).
The model describing the effect of environmental parameters on the inactivation parameters (the parameters of the primary model, such as the D-value) is called the secondary model. For heat treatment, it has been shown that the temperature has an exponential effect on the D-value, so the 'z' value has been introduced, representing the temperature change needed to bring about a tenfold change in D. The effect of temperature on the death of pathogens is often explained on the basis of thermodynamic principles whereby the change in temperature displaces the equilibrium of the chemical reactions taking place in the cells leading to protein denaturation and other alterations of the cells that may be lethal.

High hydrostatic pressure has been investigated as a mean of preservation of food for a long time (Hite, 1899), but was effectively applied to food products at the industrial scale only recently; e.g. for acidic food such as jam, fruit juices and now for vacuum-packed ham and "ready to eat" meals or oysters. It is very effective with vegetative cells but not so much with spores, where very high pressures or combinations of treatments such as pressure pasteurisation have to be applied (Gould, 2005). The shape of the primary model on the semi-log scale has been reported to be linear, concave or convex, sometimes with a shoulder (Klotz *et al.,* 2007). For primary models, various approaches have been used, e.g. Gompertz, Weibull (Burzul, 2008) and bi-phasic models (Klotz *et al.,* 2007).
The pressure and temperature have been shown to be two major factors for inactivation by high pressure. In general the sensitivity of bacteria to pressure treatment depends greatly on the species. Gram negative vegetative cells tend to be more resistant to pressure because of the thickness of their wall. Spores are actually encouraged to germinate with pressure treatments of 300 to 600 MPa (Gould, 2005) while vegetative cells cannot sustain these pressures. Treatments at pressures higher than 600 MPa are lethal for both vegetative cells and spores. The effect of pressure has been modelled following the thermodynamic laws of entropy, which (at constant temperature) suggests linearity between the logarithm of the specific death rate (the reciprocal of the D-value)

and the pressure (Klotz *et al*., 2007, Kim *et al.*, 2007). The secondary model of Ratkowsky has also been proposed where the square root of the inactivation rate depends linearly on the pressure (Koseki and Yamamoto, 2007).
Without carbon dioxide, the temperature has been shown to increase the efficiency of the treatment with different bacteria for medium range pressures (Alpas *et al*., 2000). By having carbon dioxide in the atmosphere, the process of inhibition is potentially enhanced by further disruption to the physiology of the cell caused by the dissolution of carbon dioxide in the liquid biolayer or solid matrix of foods and penetration in the cell. Indeed at high pressure, carbon dioxide is in a supercritical state which confers it gas properties in terms of transport (diffusivity, solubility) and liquid properties in terms of density and therefore it is more efficient than carbon dioxide in either gas, liquid or solid states (Garcia-Gonzales *et al*.,2007). The effect of pressure with carbon dioxide (HHPCO treatment) has been tested mostly for low pressures, that is under 50 MPa and for liquid food (Garcia-Gonzales *et al.*, 2007). In this range of pressure, higher pressure increased the rate of inactivation of vegetative cells and shortened the treatment time for vegetative cells. In the same way, higher temperatures generally increased the efficiency of the HHPCO treatment. The effect of temperature has been modelled following thermodynamic considerations with the logarithm of the rate as a function of the inverse temperature (in Kelvin), (Kim *et al*., 2007). Since pressure (P) and temperature (T) (shown by thermodynamic principles) are not independent from each other, P-T contour plots showing iso-inactivation levels have been used as an alternative representation (Diels *et al.,* 2007). Other factors affecting pressure HHPCO treatments have also been indentified, including agitation for liquid, low water activity and pH of the medium, depressurisation rate, physiological state of the bacteria and medium composition (additives, presence of Mg^{+} etc.); see Garcia-Gonzales *et al*., 2007.

High pressure homogenisation (HPH) is used widely in the industry to prepare and stabilise emulsions and suspensions. Inactivation of micro-organisms by high pressure homogenisation is different from high hydrostatic pressure because it involves mechanical stress rather than the disruption of chemical reactions. The most commonly recognised physical phenomena which cause the mechanical disruption of the cells stresses are cavitations, turbulence, impact with the solid surface and extensional stress (Diels *et al*., 2005).
Mechanistic modelling would involve complex mathematics and the values of physical variables that are not easily measured since this is a fluid dynamic problem. A traditional primary model of the decimal reduction of the concentration of pathogens cannot be defined as a function of time since time has not the same cumulative effect as for hydrostatic pressure treatment or heat treatment. However, the number of passes (pushes or cycles) does show a cumulative effect (Wuytack *et al.*, 2002) and could be used for primary models. The pressure has been identified as a main parameter which influences the inactivation of pathogens. There is no reason why the log-linear secondary model (linearity of the logarithm of the inactivation rate with pressure) observed with high hydrostatic pressure treatments should hold. Therefore full investigation is needed for this particular process with different foodborne pathogens. The viscosity of the food has also been shown to be an important factor in the inactivation of pathogens by HPH while the water activity did not influence the performance of the process (Diels et al., 2005).

Pulsed Electric Field (PEF) treatment consists of the application of short high-power electric field pulses to liquid food .The duration of the pulse is in the order of the □s,

using electric field strength between 20 and 80 kV/cm. If the voltage gradient is sufficient, the cell membrane of the bacteria become permeable and leakage of the cells contents leads to death (Dutreux et *al.*, 2000). The inactivation has been shown to depend on the bacteria, product parameters such as the pH, the conductivity of the fluid and on process parameters such as the electrical field strength, treatment time, pulse number, treatment temperature (Wouters *et al*., 2001). Since the process is exothermic, if the temperature is not controlled by a cooling process, the temperature increases with the treatment time. This increase has been modelled with the Arhenius equation and exploited to enhance the efficiency of PEF treatment (Amiali *et al.*, 2007). Alternatively it has been proposed to use the treatment in conjunction with bacteriocins or high pressure (Gould, 2005). The modelling of inactivation has sometimes followed the traditional two-steps modelling with a primary model as a function of the treatment time following log-linear kinetics, the Weibull model or lo-logistic models (Wouters *et al.,* 2001, San Martin *et al.*, 2007). One-step models have also been proposed based on a linear relationship between the logarithm of the survival fraction and the electric field strength and a linear relationship between the logarithm of the survival fraction and the logarithm of treatment time (Wouters *et al.*, 2001).

PHOTO has been used to inactivate mammalian cells but the application to food safety is a novelty so there is no background information available on the modelling of the inactivation of pathogens. A primary model can be defined as a function of the treatment time. The obvious parameters to consider for the secondary modelling are the nature and concentration of the photosensitizer as well as the incubation time.

2. Objectives

This workpackage consists of cross-package activities aiding all the four studied technology, mainly using Predictive Microbiology and Quantitative Microbial Risk Assessment (PM and QMRA) methods. The aims are:

(1) Provide help to partners with the quantitative aspects of experimental design.

(2) Assess the efficiency of the four new technologies in terms of microbial safety.

(3) Develop survival models and implement them in a user-friendly software tool aiding to validate the new technologies and comparing them with other technologies and with each other.

Data collection

The absence of available data has been identified as one of the problems to develop predictive modelling for the inactivation of foodborne pathogens by high pressure (Diels *et al.*, 2007). This is even more of a problem with the other emerging technologies that have been less studied. We have collected all the data produced during the project and recorded in a format compatible with the ComBase system (www.combase.cc). ComBase is a free database of responses of foodborne pathogens to environmental factors (temperature, pH, water activity, additives in the food etc.). It currently contains more than 40,000 entries and is on average visited by 200 “genuine” users a day (“genuine” means that automated searches from the likes of Google, Yahoo,

etc. are excluded). The database is supported by internationally renowned institutions and the European Union had also supported the initiative (see EU project QLAM-2001-00513)

The number of new records deposited in ComBase during this project and relevant data from the literature are given in Table 25.

	Inactivation		**Growth/Inactivation by heat**	**Total number of records**
Source of data	Provided by the partners	Literature	Partners	
PHOTO	213	0	2	215
PEF	116	138	0	254
PEF + heat	41	0	79	120
HPH	442	0	1003	1445
HHPCO	121	117	10	248

Table 25: Data recorded in the databases at the end of the project. One record is made of experiments in one set of conditions so it usually includes several experiments.

It is intended that as the data are published, they will be included in ComBase on the internet. This way they will be not only freely available but also easily accessible on a web site that is already known by many food scientists, contributing to the dissemination of the results to a wide audience.

Primary models

The primary models used in this project are: linear (HPH), Weibull (PHOTO), bi-phasic (PEF), with tailing or secondary effects (HHPCO, PEF, heat after PEF). Sometimes they were simplified to linear for a limited range of times (HHPCO, heat treatment after PEF).

The primary model used for PHOTO inactivation was the Weibull model:
$\mathrm{Ln}(N/N_0)=-kt^p$ (1)
with N the number of cell at time t; N_0 the initial number of cells, k and p constants depending on the photosensitizer (ALA) concentration and the incubation time.

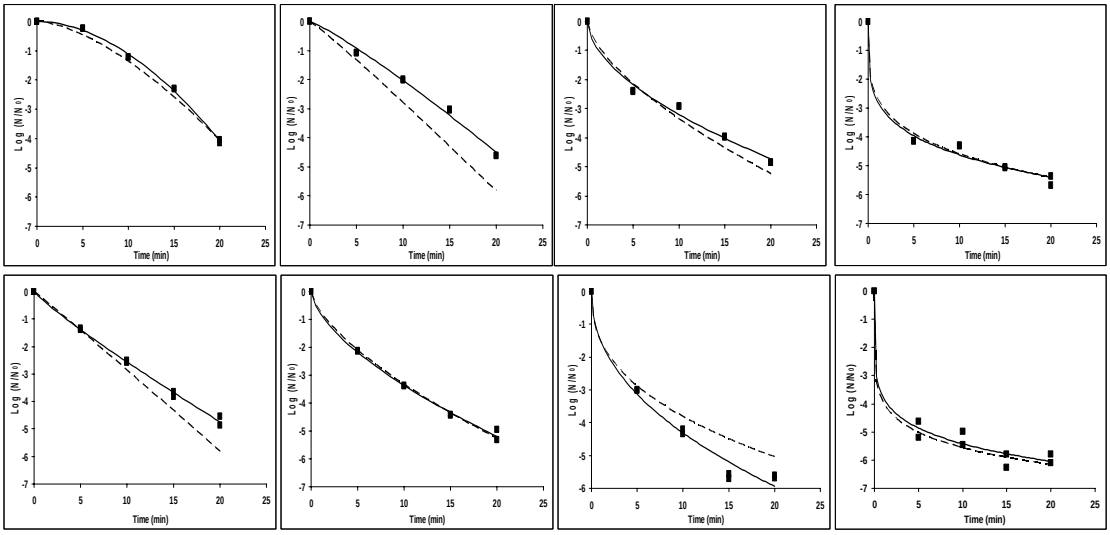

Figure 63: Inactivation of *Bacillus cereus* by PHOTO in broth after incubation in the presence of ALA for 2, 15, 30 and 60 min respectively (from left to right). Square, measured log counts; solid line, fit of the Weibull model; dotted line, predictions. In the first row the ALA concentration was 3mM and in the second row, it was 7.5 mM. Published in Le Marc *et al.*, 2009, Journal of Applied Microbiology, 107:1006-1011.

The primary model for inactivation by PEF in broth was developed from published data and was the result of collaboration with NovelQ partners. In June 2009, József Baranyi, the leader of Work Package 6 at IFR, visited partners of the NovelQ project at the University of Zaragoza and it was agreed that the original raw data of articles on PEF treatment published by the colleagues in Zaragoza would be included in the HighQ RTE database. In return, NovelQ will receive the ComBase-formatted version of the data together with the software developed at IFR that aids the data processing. A fitting program was used to create Fig.64. The primary model is bi-phasic linear.

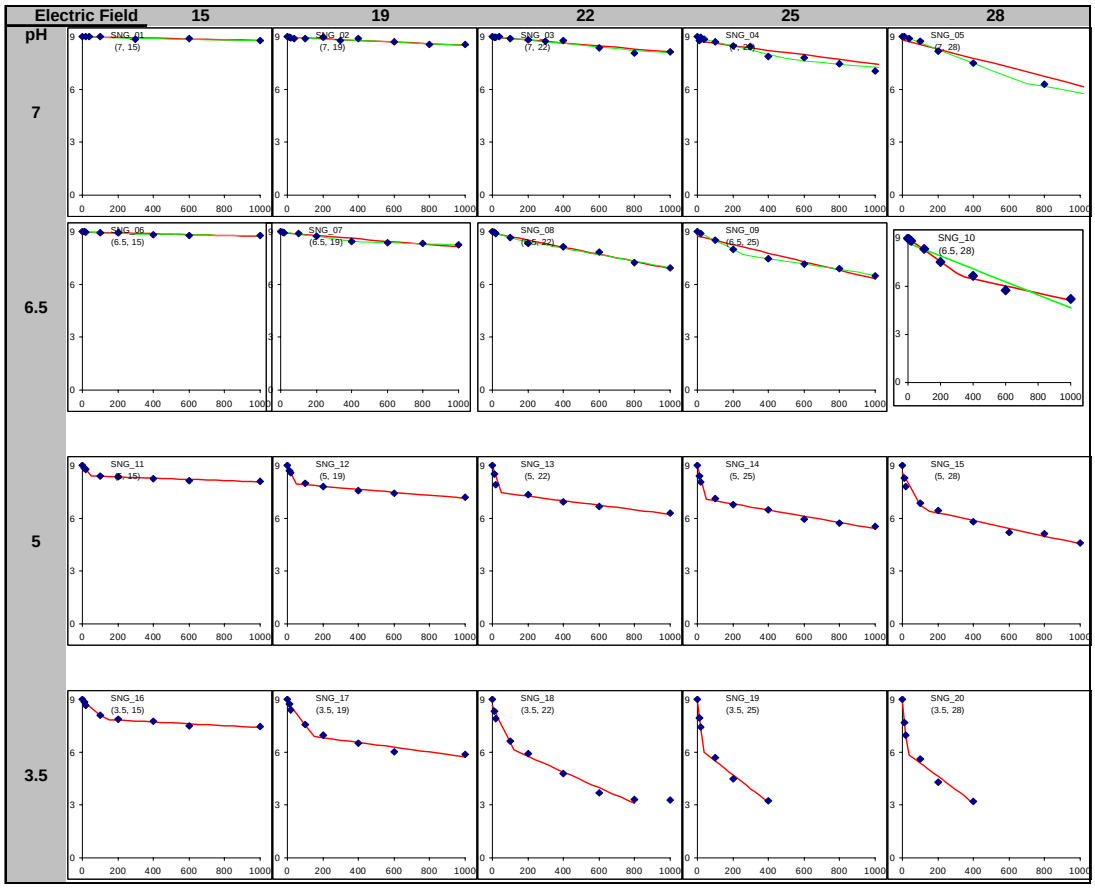

Figure 64. Inactivation of *Listeria monocytogenes* in Tryptone Soya Broth. The cell concentration (Log CFU) *v.* PEF treatment time (micro second) is represented as a function of pH and electric field (kV/cm). Data of Gomez *et al.* (2005). At pH 7 and 6.5, the inactivation is linear. At low pH it is convex, indicating that the initial low pH stress causes an abrupt initial death that later slows down.

To improve the efficiency of PEF it was proposed that PEF treated samples were held at a temperature of 53-55°C for up to 20 min. For heat treatments up to 10 min, the inactivation was linear with sometimes a tailing effect for longer treatment times.

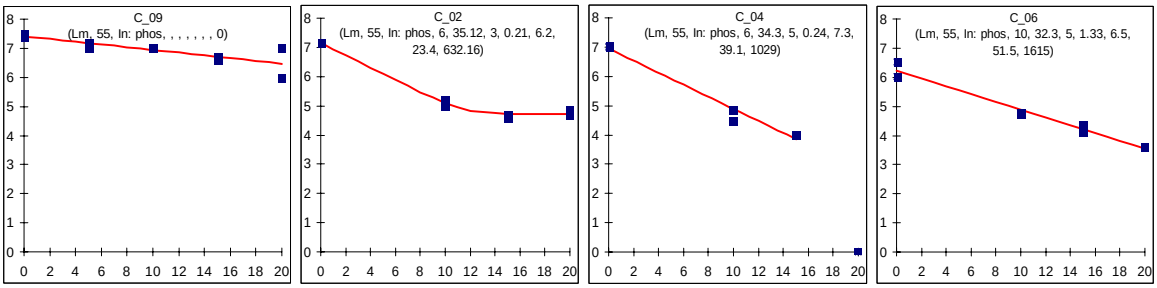

Figure 65: Log CFU/ml as a function of time (in min.) during the inactivation of *Listeria monocytogenes* by holding at 55°C in phosphate buffer after different PEF treatment (curve C_09: no PEF treatment, curve C_02 and C_04: the PEF treatments consisted of 3 and 6 pulses of 6 □s with electric fields of 35.12 and 34.3 kV/cm respectively, curve C_08: the PEF treatments consisted of 5 pulses of 10 □s with electric fields of 25kV/cm. The kinetics were assumed to be linear for the first 10 min of treatment.

As explained in the introduction, in the case of HPH, time could not be chosen as the explanatory variable for the primary model. Instead we considered the number of "pushes". The primary model was indeed linear.

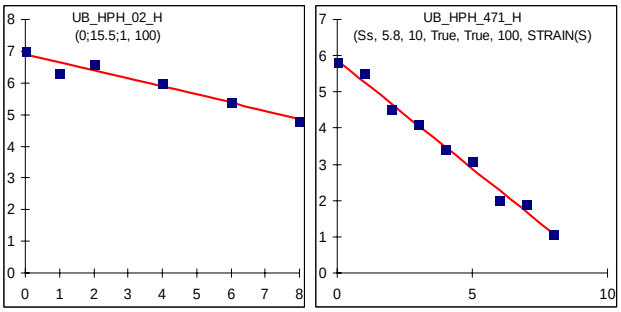

Figure 66: Inactivation of *Listeria monocytogenes* in milk and *Salmonella typhimurium* in egg as a function of the number of "pushes" by HPH. For primary models, the usual variable is the duration of treatment. In this case the number of pushes represents the cumulative effect of the treatment. The curves are linear.

For high hydrostatic pressure treatments, at the lower pressure (100 and 250MPa), the inactivation of *Listeria* appeared to be linear. However, the observation times are relatively short, and the data generally show less than 4 log reductions in cell numbers. For pressures higher than 400 MPa, where more than 2 points are present, we could observe a rate decrease, or tailing shape (convex from below), which corresponds to the convex shape most usually observed with high pressure treatments (figure 67a). Data on the effect of pressure on *Listeria monocytogenes* inoculated in TSA in the same range of temperature and pressure were found in Alpas *et al*., 2000. These data suggest a convex shape for the inactivation. These results are not in disagreement with the current data since they had longer treatment times which would explain why we did not observe the tailing at the lower pressures (see figure 67b).

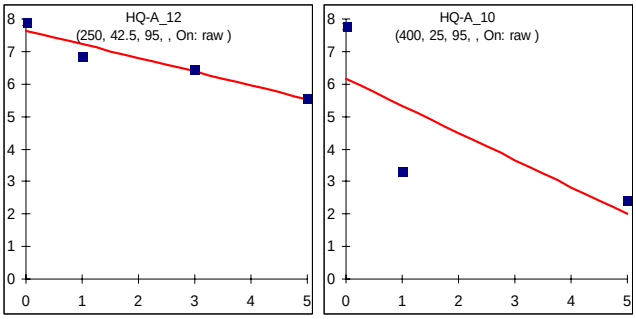

Figure 67a: Inactivation of *Listeria innocua* as a function of the treatment time (in min.) inoculated on raw red pepper at 250 MPa, 42.5°C and 400 MPa, 25°C, respectively, packaged with an atmosphere of 95% CO_2. The former is linear in this time scale while the latter indicates a convex shape typical of pressure treatments.

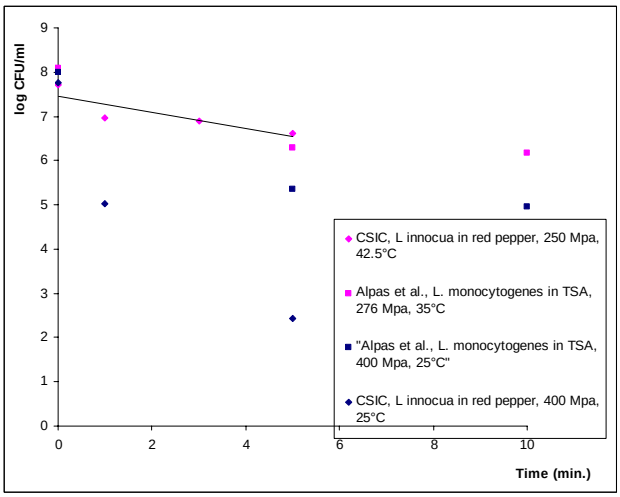

Figure 67b: Although a linear trend has been observed at the lower pressures (100 and 250 MPa) for treatments up to 5 minutes in this study, the actual primary model could be convex as suggested at higher pressure (400 MPa) in agreement with the results of Alpas *et al*., 2000.

In addition the analytic features of the primary model depended on the bacteria, in the same conditions (figure 68).

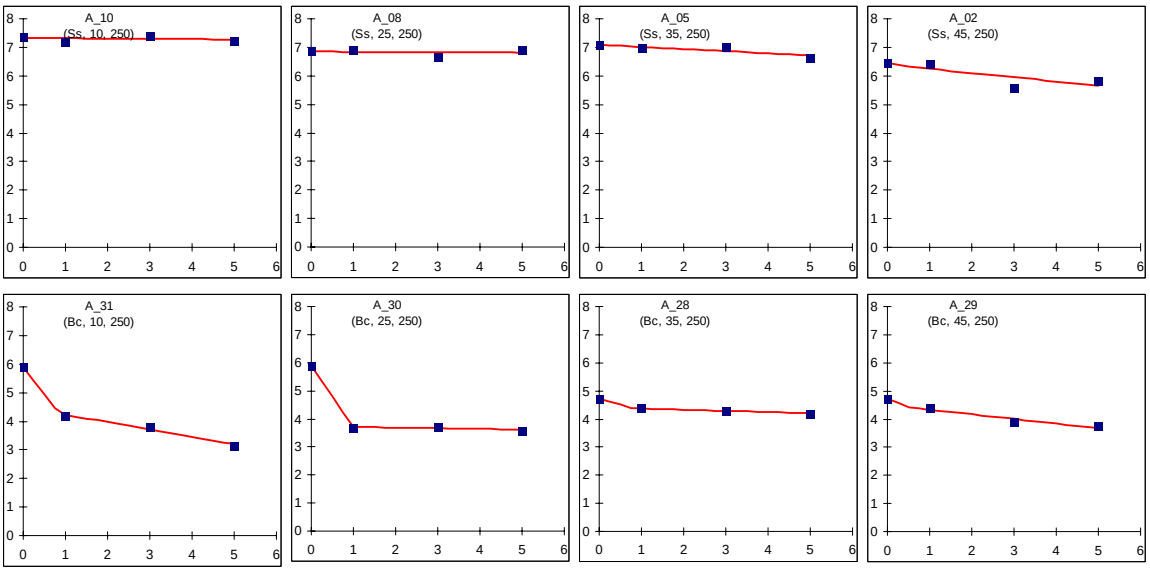

Figure 68: Inactivation of *Salmonella typhimurium* (first row) and *Bacillus cereus* (second row) in grilled red peppers by HHPCO at 250 MPa. The temperatures were (from left to right) 10, 25, 35 and 45°C. A straight line was fitted to the curves in the case of *Salmonellae* while biphasic models were fitted in the case of *Bacillus* to take into account the convexity of the curve.

Secondary models

Secondary models describe the effect of environmental factors on the parameters of the primary models.

The primary model used for PHOTO was the Weibull model, with two parameters, *k* and *p,* which depended on the concentration of ALA and the incubation time. It was shown that the two parameters are strongly correlated so only one of them needed to be modelled (figure 69).

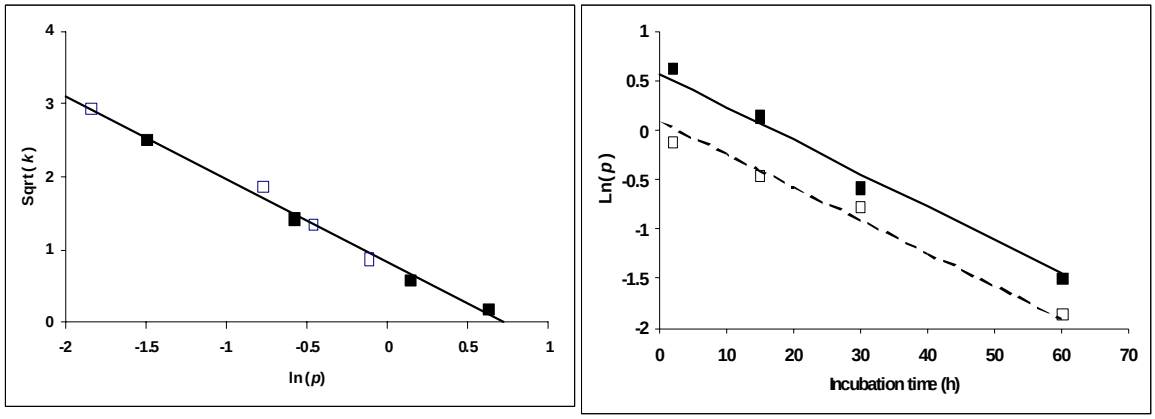

Figure 69: Left: The plain squares and empty squares are the Weibull parameters fitted by the primary model after 3 mM and 7.5 mM ALA treatment, respectively, at various treatment times. Since they are correlated, the power *p* was chosen as the variable to model. Right: ln(*p*) values fitted by the primary model at 3 mM and 7.5 mM ALA respectively. The predictions are represented by solid and dotted lines respectively.

For the effect of PEF treatment in broth, a secondary model equivalent of that of Ratkowsky, 1988, was chosen to describe the effect of *E* (kV/cm) on the square root of the *d* death rates (log10(CFU)/µS). This choice was based on the study of data from the literature and on an analogy to thermal inactivation. Indeed, if we assume that the fraction of bacteria inactivated in a unit time is proportional to the energy they are exposed to, then, since the energy of the electric field is proportional to the square of the electric field (E^2), the conductivity of the media and the exposure time, the square root of the death rate in terms of log(CFU)/µs is proportional to *E*:

$Sq(d) = b \cdot x = b \cdot (E-E_0)$

For instance, the minimum electric field strength reported was 7.5 kV/cm on lactic acid bacteria in apple cider (Geveke *et al.* 2009). It could be established from the data that the value of E_0 was between 5 and 10 kV/cm for all organisms tested, independently of the media, so E_0 was fixed independently of organisms and media to E_0 = 7.5 kV/cm . This modelling approach will be published (Baranyi *et al*., in preparation).

However, the data obtained in HighQ RTE for *Listeria* in milk were significantly different from these results (figure 70). This may be because the laboratory system was different or because the results in milk were based on the fitting of one straight line to two points as opposed to the bi-phasic models used in broth.

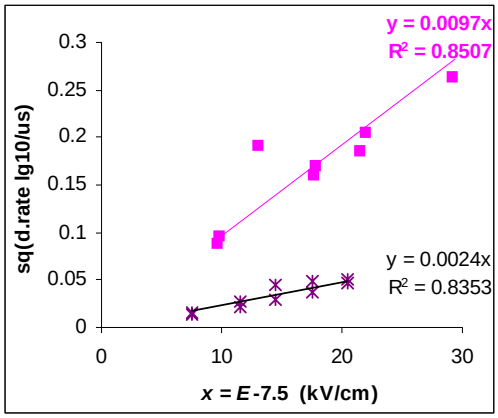

Figure 70: Crosses, *Listeria* model in broth (NovelQ data); and square, in milk (HighQ RTE data). The discrepancy may be due to the difference in the set-up of the process or to the fitting of one straight line to two points in milk as opposed to the bi-phasic models used in broth.

For heat treatment following PEF, the inactivation rate of the heat treatment consistently increased with the severity of the PEF treatment. We assumed that the death rate increased proportionally to the damage to the cells produced by the PEF treatment. That is $D_{heat/PEF}=D_{heat}*damage_{PEF}$. Applying the logarithmic link function, $\ln D_{heat/PEF} = \ln D_{heat}+\ln(damage_{PEF})$. We proposed that $\ln(damage_{PEF})=k*E*t$, k being a constant if all the other parameters of the PEF treatment are equal (i.e. inlet temperature, medium, frequency etc.). The plot of $\ln(D_{heat/PEF}) = f(E*t)$ is given in figure 71 for *Listeria monocytogenes* with 2 different pulse lengths.

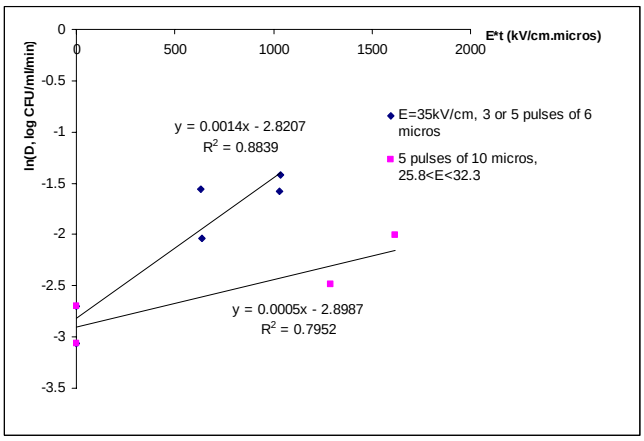

Figure 71: Logarithm of the death rate of heat treatment as a function of the damage of the cells induced by PEF treatments characterised by the electric field, E (in kV/cm) time the treatment times (in □s). The pulse length seems to be a significant factor in the way the cells are damaged.

In food, the concentration after the PEF treatment was not measured so at time t=0 the initial concentration is taken as the concentration of the inoculum before the PEF treatment. Because the decimal reduction in cell number due to the PEF treatment was small compared to the effect of heat treatment in the range of conditions tested (the highest that has been measured is 1.6 log CFU/min. in phosphate buffer), the rate is not only due to the heat treatment but this discrepancy was neglected and this rate was used in the following analysis.

The cells appeared to be damaged in milk, egg and phosphate buffer in a similar fashion. In fact it could be shown statistically that there were no significant differences between the three media. The overall fitting is: $\ln(D')=0.0012*E*t-2.52$ with a coefficient of correlation of 0.85.
In the mayog, *Listeria* seemed to be more sensitive to heat but less affected by the PEF treatment (higher intercept, lower slope, $\ln(D')=0.0005*E*t-1.25$

The effect of pressure on the rate of inactivation for treatment by HPH was plotted with a logarithmic link function. These curves were linear in cases when lab-scale equipment was used and the results were independent of the temperature for inlet temperature up to 15.5°C (figure 72).

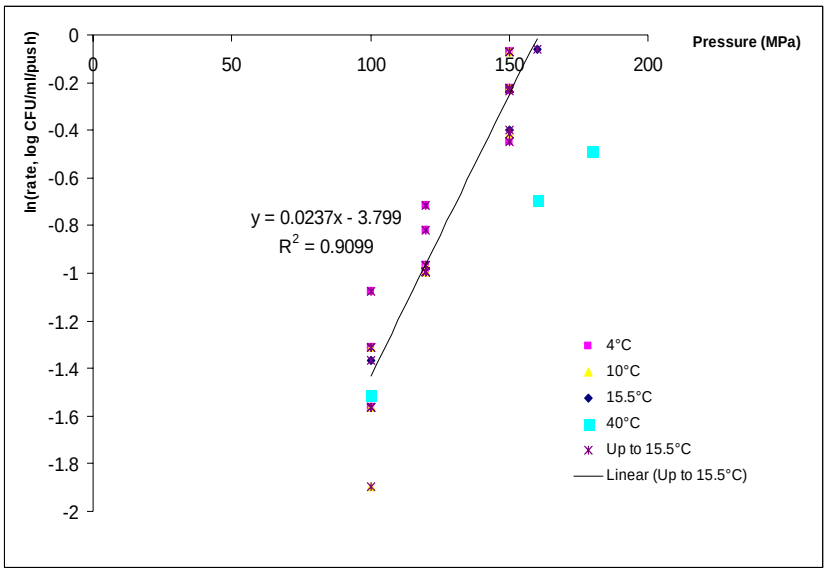

Figure 72: Logarithm of the inactivation rate as a function of the pressure for *Listeria* in milk treated by HHP.

For industrial scale equipment, only one "push" was applied. The effect of pressure depended on the organism (figure 73).

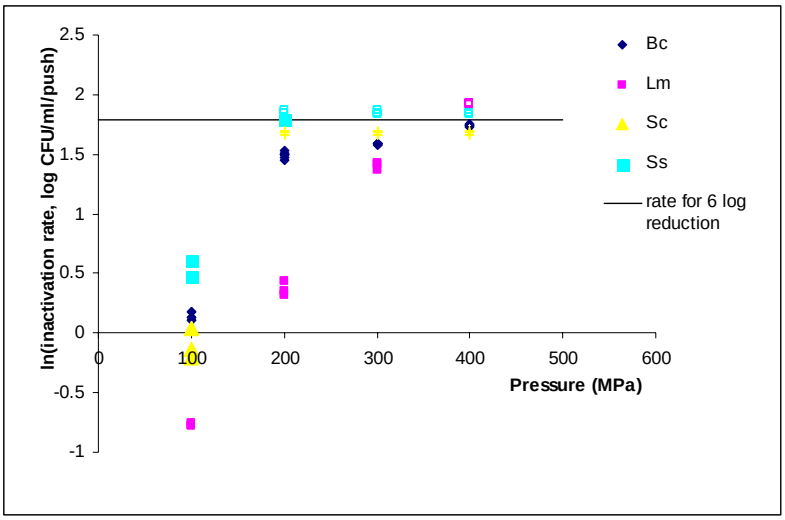

Figure 73: Logarithm of the inactivation rate in soup as a function of the pressure for different micro-organisms with industrial scale equipment. The straight line and empty symbols show data where the final concentration was under the detection levels so the rate was "more than" what is represented. The micro-organisms were *Bacillus cereus*, *Listeria monocytogenes*, *Saccharomyces cerevisiae* and *Salmonella typhymurium* respectively. *Listeria* was the most resistant to HPH while the yeasts were the least resistant, perhaps because of their size.

Since there were not enough observations to fit a sophisticated secondary model to the specific rates obtained with HHPCO inactivation of *Listeria* in pepper, the following was proposed.
To model the effect of the pressure, P (in MPa), we kept the traditional enthropy- based secondary model: $\ln(k)=a*P+b$ at constant temperature. The dependence of the temperature, T (in °C), was assumed to be so that both $a=a(T)$ and $b=b(T)$ are linear. This is equivalent to the assumption that $\ln(k)=f(P,T)$ is linear, possibly with an interaction term.
It can be observed that the increase of the rate with temperature at 250 MPa was not clear from the data. It was assumed to be independent of the temperature at this pressure so we took the average of the two rates we are more confident about, that is $\ln(k)=-2.95$.
The resulting equation, obtained by linear regression was:
$\ln(k) = -4.378 - 0.158*T + 0.00571*P + 0.000632*T*P$
The predicted and measured rates, as a function of pressure, are shown in figure 74.

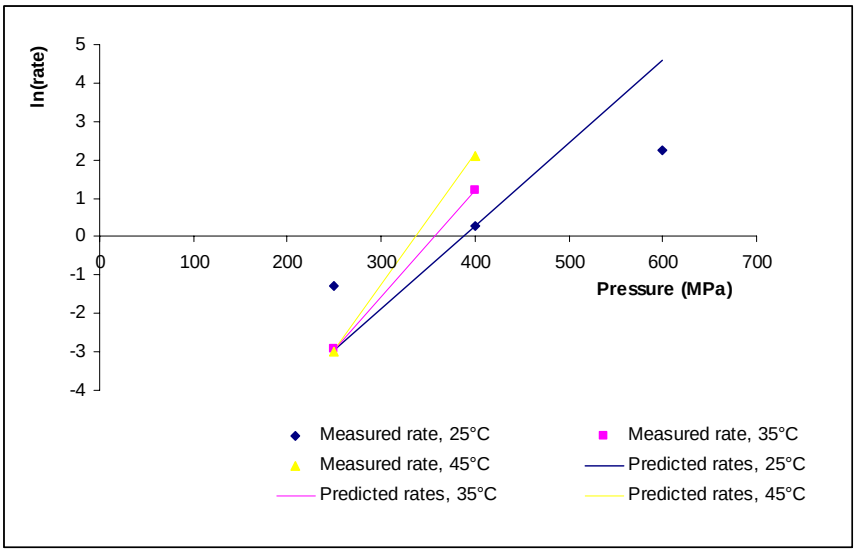

Figure 74: Prediction and measurements of the inactivation rates of *Listeria monocytogenes* in red grilled pepper by HHPCO.

Case studies:

PHOTO inactivation of *Listeria monocytogenes* on tomatoes:

The growth of *L. monocytogenes* was followed at 8°C on the surface of tomatoes after i) washing of tomatoes (control) and ii) after applying irradiation after sensitization.
The treatment induced a reduction of 1.48±0.40 $\log_{10}$ CFU/g. The bacterial growth curves were fitted with the model of Baranyi and Roberts (1994) in order to calculate the lag times and maximum growth rates. Although the growth of *L. monocytogenes* is reported not to occur in tomatoes, our data clearly show that *L. monocytogenes* is able to growth on their surface.
The treatment was found to induce a lag time of 74h at 8°C (no lag was observed for the control experiment). F-tests highlighted significant difference (at p=0.05) between the two specific growth rates. The specific rate estimated for the control is 0.033 h^{-1}, a lower value than the value of 0.045h^{-1} observed after treatment. We assume that the background flora was able to reduce the growth of *L. monocytogenes* in the control experiment. The treatment may have also reduced the population of the background flora, resulting in a higher growth rate of *L. monocytogenes*.

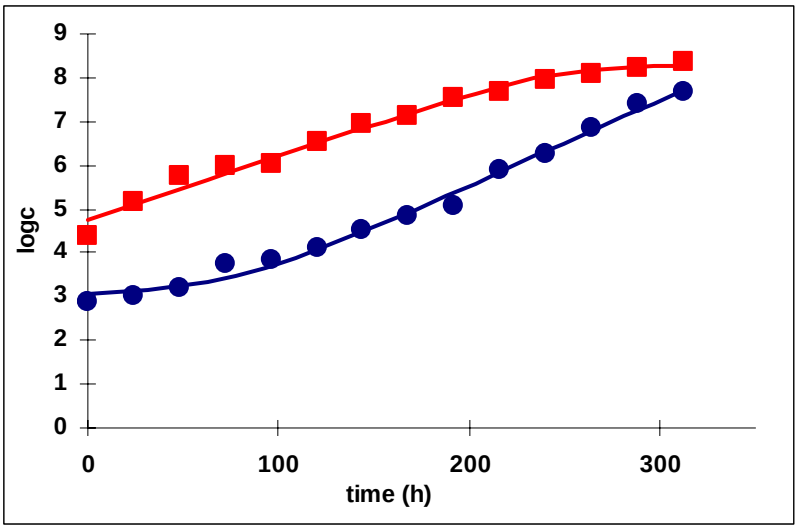

Figure 75: Growth of *L. monocytogenes* on the surface of whole tomatoes after treatment (circle) and control experiment (square).

A QRMA for predictions of the regrowth of *L. monocytogenes* after washing only and after treatment was implemented in an Excel workbook.
For the initial concentration of *L. monocytogenes*, we used a normal distribution proposed by Crepet *et al.* (2007) for *L. monocytogenes* on unprocessed or minimally processed vegetables. For the fridge temperature, we assumed a normal distribution (mean: 6.62°C, std=2.52). A Ratkowsky model, $sqrt(\mu_{max})=b(T-T_{min})$ was used for predicting the bacterial growth rate. T_{min} was obtained from Koseki *et al.* (2005). The parameter b was derived from the reference curves at 8°C. Lag time predictions are based on an h_0 value ("work to be done"; see Baranyi and Roberts, 1994) calculated at 8°C (h_0=3.82 after treatment, h_0=0 for washing). The prevalence of *Listeria* on the food product was not included in the calculations.
For short periods of storage (i.e 8-10 days), the final distribution for treated tomatoes is skewed to the left compared to the "only washed" tomatoes. For longer periods and high fridge temperatures, the final concentration of *Listeria* can be higher than for the control experiments (due to the fact that μ_{max} is higher).

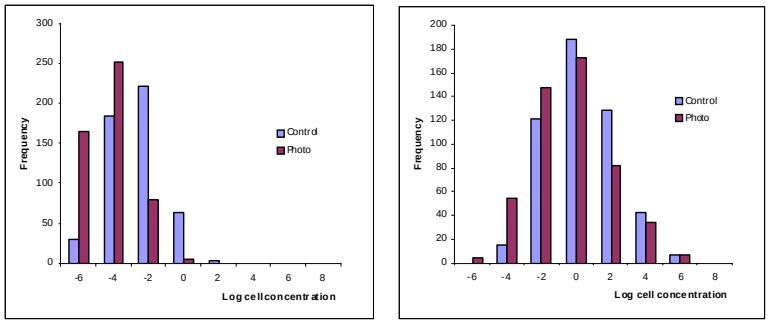

Figure 76: Predicted distribution of the concentration of *Listeria* at 0 and 10 days for untreated and after a PHOTO treatment. Initially the PHOTO treatment decreases the pathogen concentration further than the washing and because of the lag time, the treated samples are less contaminated. However in the long term the contamination level with treatment is higher perhaps because the indigenous flora has also been damaged and the pathogen has less competition.

It has to be emphasized that this study is based on insufficient data to draw robust conclusions. As this is a new technology which is aimed at solid foods (that show a great variability), more data are needed to validate the results.

Inactivation of *Listeria monocytogenes* in whole UHT milk by heat, HPH (with lab scale equipment) and PEF combined with heat and subsequent growth at fridge temperature.

This study takes into account different sources of variability and uncertainties to show their relative influence.

The inactivation by HPH and PEF combined with heat were modelled following the modelling principles explained above. The inactivation by heat treatment was modelled using data in ComBase (www.combase.cc) in the temperature range of 50 to 64°C (47 rates). The logarithm of the rate was a linear function of the temperature and the resulting model was: $\ln(k)=0.3977*T-18.952$ with k, the specific death rate in ln(CFU/ml/h) and T, the temperature, in °C ($R^2=0.83$).
For all inactivation models, the primary model (log concentration as a function of the treatment time or number of pushes for HPH) was assumed to be linear in the range of conditions studied. It was also assumed that a maximum of 4 decimal reductions could be achieved with all techniques because of the possible effect of tailing which was not investigated in this project. The uncertainty of the predictions were accounted for by generating random numbers around the predicted inactivation rates assuming the error is normally distributed with a standard deviation equal to the standard error of the fit.

The fridge temperature was assumed to follow a normal distribution with average 6.62 and standard deviation 2.31°C which was found to be the distribution of fridge temperatures in Europe.

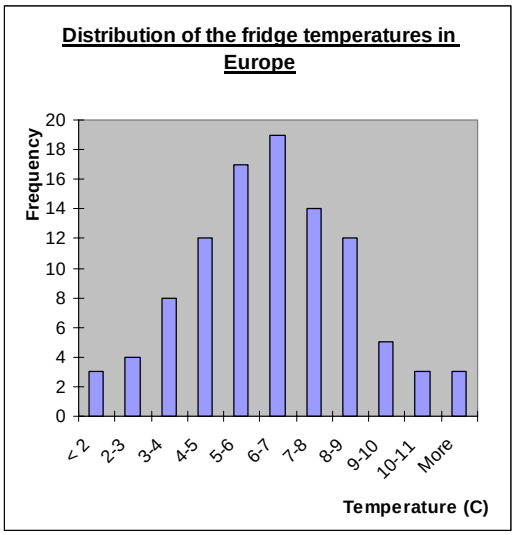

Figure 77: Histogram of the distribution of the temperature of fridges in Europe (Nauta *et al.*, 2003).

Data from ComBase and the data gathered during the project were pulled together to determine the growth rate, , as a function of the temperature, T, in milk. A Ratkowsky

model was used to describe the effect of temperature on the specific growth rate; that is sqrt( )=a*(T-T_0) with a as constant and T_0 a notional minimum temperature.
The growth rates obtained in the experiments of this project showed a great range of variability. Possible explanations include: rates from UNIBO are determined from a high inoculum so the growth rate is less certain; different samples of milk can be quite different in composition and flora; the treatments may affect the flora in different ways although this is in UHT milk where the flora should be greatly reduced.
The secondary model was built as follows: it was assumed that a is independent of the medium (note that a is from broth experiments and was determined from ComBase data (source=Bajard_97). Then the deviation around the model was assumed to follow a normal distribution and was estimated from the experimental data of UNIBO and SIK pooled together. Lastly T_0 was determined by assuming that 95% of the data are under the rates predicted in broth.
Validation: the resulting model was in agreement with the growth rates estimated by Bajard *et al.*, in whole UHT milk (source=Bajard_96), see Figure 78.

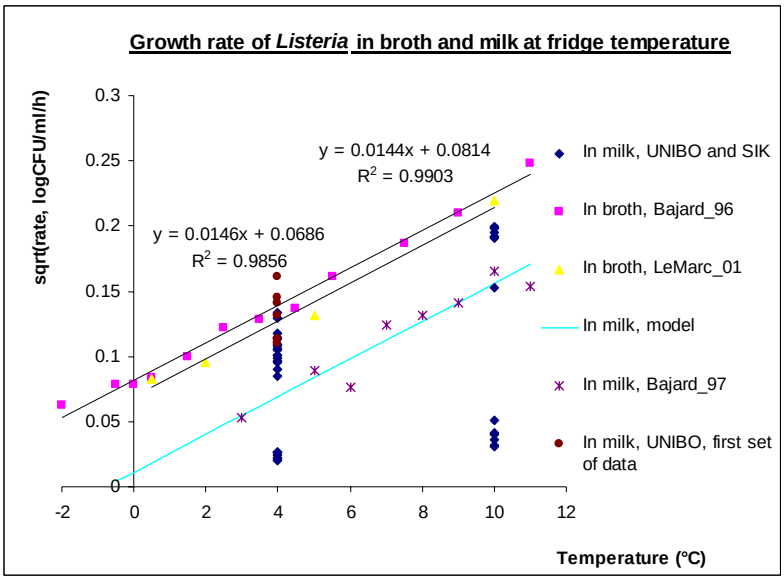

Figure 78: Growth rate of *Listeria* in broth and milk; data from HighQ RTE and ComBase (www.combase.cc). The resulting model was: a=0.0145h^{-1} and T_0=-0.79°C.

To compare the effect of sources of variability and uncertainty the following case is illustrated in figure 79. The initial inoculation level was assumed to be normally distributed with average and standard deviation of -2 and 1.5 [log CFU/ml], respectively.
The HPH treatment was 7 pushes at 120MPa, the heat treatment was 10 min at 53°C and the PEF treatment was 5 pulses of 6 □s with a field of 35mV/cm followed by a heat treatment of 10 min. at 53°C.

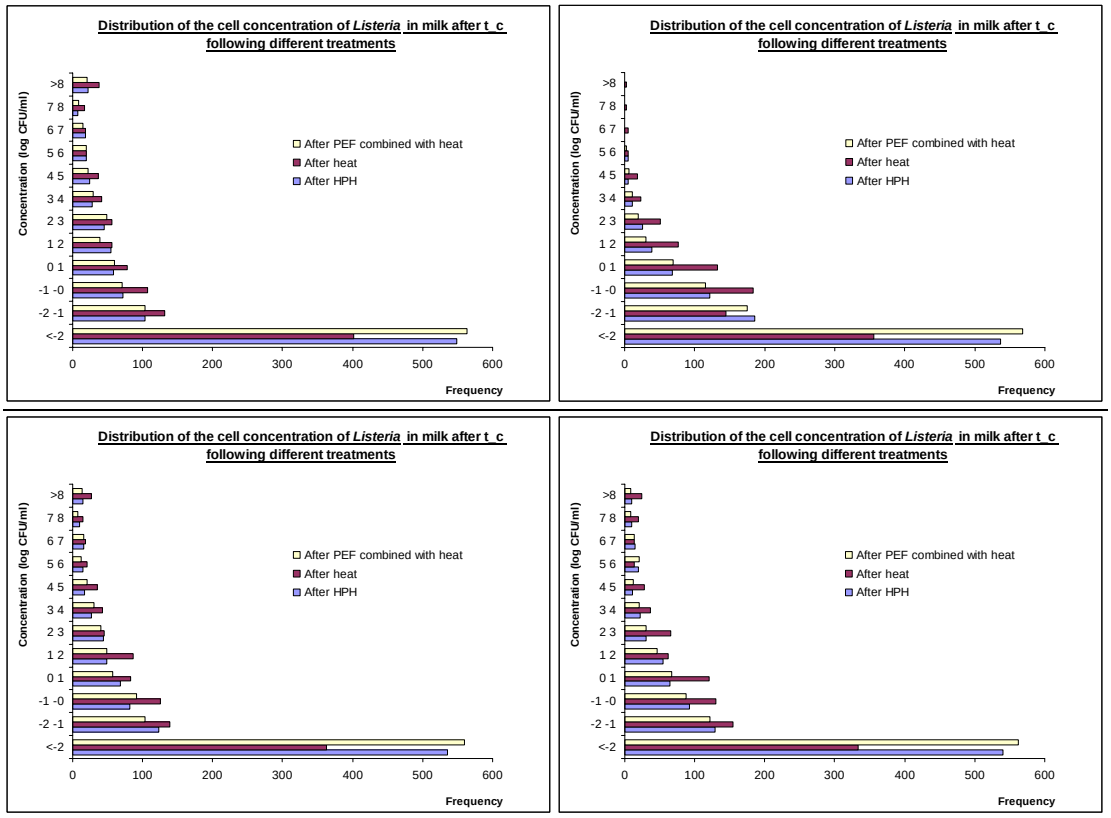

Figure 79: Predicted concentration of *Listeria* in whole milk after 10 days in the fridge after different types of inactivation. From left to right 1) taking account all the sources of variability and uncertainty described above; 2) not considering the growth rate variability in the different samples; 3) not considering the fridge temperature variability; 4) not considering the uncertainty of the modelling of inactivation. It can be seen that the highly contaminated samples come from the high growth rate sometimes observed in whole milk (case 2). While the model uncertainty may be reduced by carrying out additional experiments, the food variability and the fridge temperature variability cannot be easily decreased in practice.

The modelling of inactivation is adequate for QMRA, it is the fact that high growth rates of *Listeria* may occur in milk that make it possible to reaches high levels. As a consequence, the inactivation needs to be high enough so that no *Listeria* is able to recover.

Inactivation of *Salmonella typhimurium* in egg by HPH (with lab scale equipment) and PEF combined with heat and subsequent growth at 10°C.

The inactivation by HPH and PEF combined with heat were modelled following the modelling principles explained above.

The growth rates in eggs at 10°C were significantly different for eggs from Italy and eggs from Sweden. This is possibly because of the differences of the indigenous flora; chickens are fed differently and differences such as climate may also have an effect on the biota around the chicken as well.

Growth rates in Swedish eggs:

For non-treated eggs, no growth of *Salmonella* was observed.
For treated eggs, the growth rates were independent of the intensity of PEF & heat treatment. However, there was a cluster of higher growth rates and it was assumed that the growth rate was the average of this cluster since it is a worse case scenario study.

Growth rates in Italian eggs:
The growth rate after treatment by HPH was influenced by the number of pushes, see figure 80.

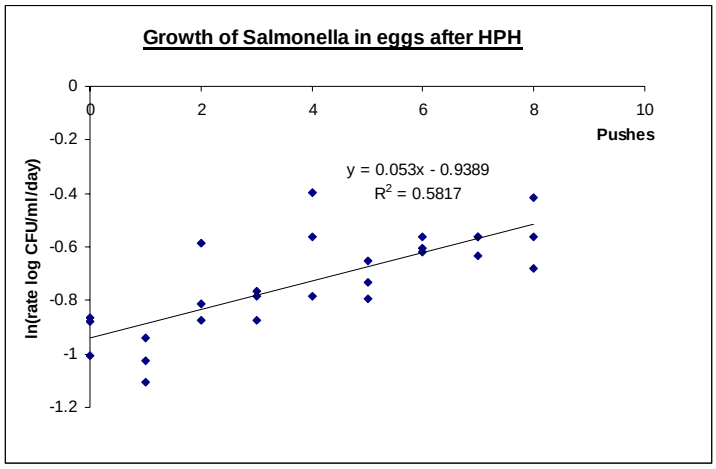

Figure 80: Growth rate of *Salmonella* in eggs at 10°C after different numbers of pushes. The HPH treatment also affects the flora and the pathogen is actually growing faster after more intense treatments.

As a consequence of the food variability, it was not possible to compare directly the effect of HPH and PEF combined with heat. An example of contamination after 3 days assuming the same initial contamination level, that is a normal distribution of average 5 log CFU/ml and standard deviation 1.5 log CFU/ml of egg, is given in figure 81.

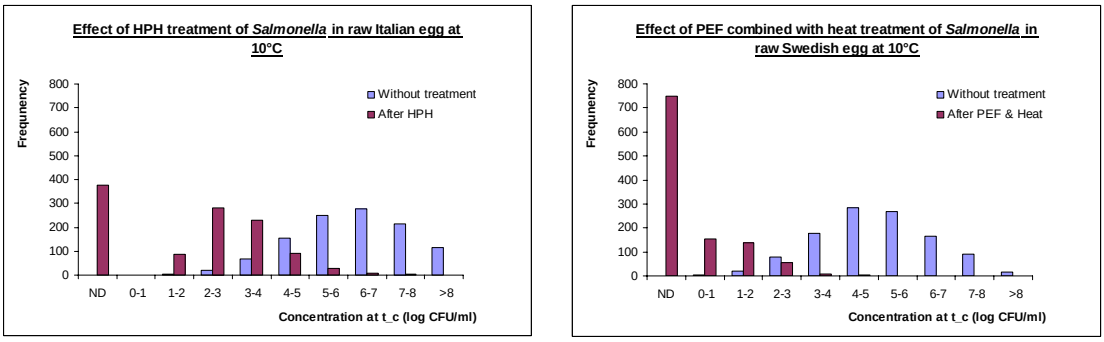

Figure 81: Predicted distribution of the concentration of *Salmonella* after 3 days storage at 10°C in Italian egg (left) and Swedish egg (right) both untreated (blue bars) or after treatment (red bars) after 7 pushes at 100MPa for HPH and 5 pulses of 6 □s at E=32 kV/cm for PEF followed by 10 min heat at 53°C. The initial contamination was the same in both cases, Normal(5,1.5) in log CFU/ml. Even without treatment, the contamination is different in the eggs presumably because of the difference in flora of the eggs from different origins.

Inactivation by HPH (at industrial scale) and heat treatment and subsequent growth of different pathogens in soup

With industrial equipment where only one push was applied, the rate of inactivation by HPH is the difference between the logarithm of the initial inoculum cell concentration and the logarithm of the count after the treatment. The effect of pressure on inactivation of different micro-organisms is shown in figure 73. *Listeria* and *Bacillus cereus* are the "limiting" organisms to determine the pressure to be applied for safe food since they are more resistant to the HPH treatments.

Growth was not observed for cases where all micro-organisms were inactivated by HPH treatment (growth is defined as two successive measured cell concentrations above the detection level). In cases where not all micro-organisms were inactivated, at 10°C, both *Listeria* and *Bacillus cereus* were able to grow before dying after HPH treatment.

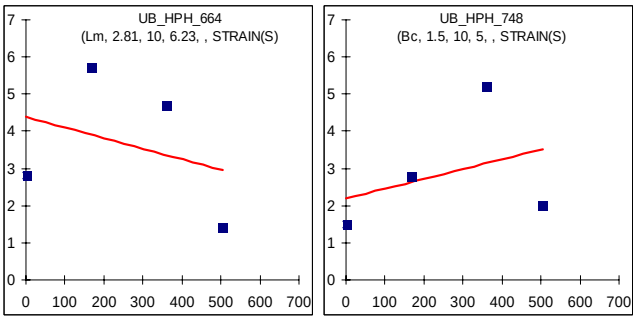

Figure 82: Left: Growth of *Listeria* in vegetable soup, at 10°C, pH 6.24 after HPH treatment at 300MPa. Right: Growth of *Bacillus cereus* in vegetable soup, at 10°C, pH 5 after HPH treatment at 200MPa. The time (x-axis) is given in hours. The survivors were able to grow before dying.

Comparison with heat treatment:
To compare HPH with heat treatment, samples were incubated in a bath at 80°C. The inactivation curve obtained for *Listeria* has a tail, see figure 46.

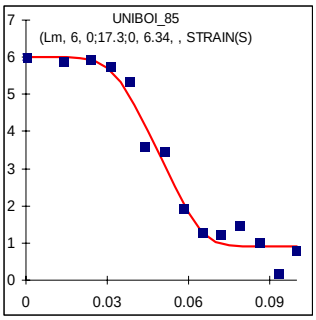

Figure 83: Inactivation of *Listeria* in a water bath for up to 6 minutes at 80°C. The tailing effect means that some cells are not inactivated and might recover after treatment. Usually a long lag time is observed but in the case of vegetable soup, low levels of *Listeria* have been shown to recover without lag time.

To ensure the safety of food, for HPH treatments as for the case of heat treatments in soup, the treatment has to be severe enough to inactivate all the bacteria.
In this particular case, it means pressure treatments of 400MPa to ensure that 10^6 cells of *Listeria*, the limiting micro-organisms, are inactivated.

Growth of *Listeria monocytogenes* at 10°C in grilled red pepper after inactivation by HHPCO and heat treatment.

Inactivation by HPH and PEF combined with heat was modelled following the modelling principles explained above.

Listeria did not grow on grilled red pepper, with or without treatment, so no growth was observed after HHPCO. This is in contradiction to what was observed in phosphate buffered saline solution for *E. coli* (Koseki and Yamamoto, 2006). In addition to the cell reduction due to the treatment, the survivors damaged by the treatment die faster than the untreated cells.

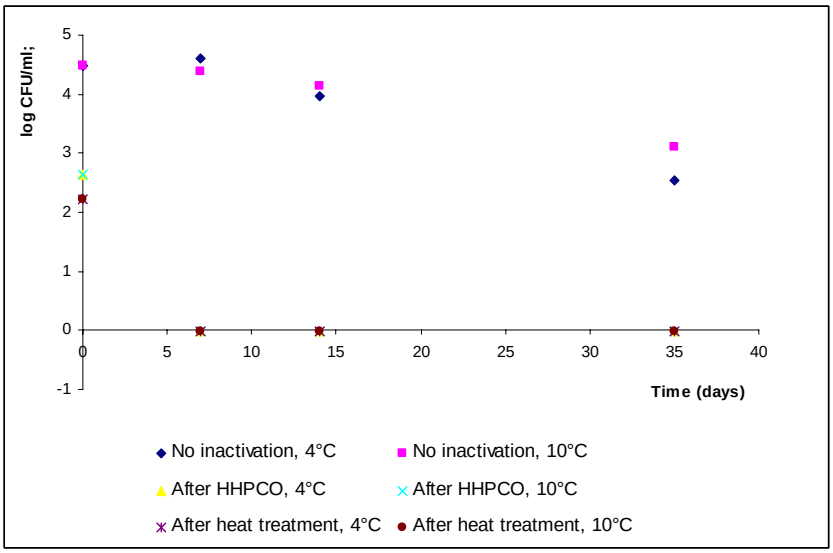

Figure 84: Growth of *Listeria monocytogenes* in the fridge at 4 and 10°C following different types of inactivation. The pressure treatment was 3 min at 400 MPa, 35°C and the heat treatment was 15 min at 70°C which both resulted in a decimal reduction of about 3 logs.

The study shows a worse-case scenario: the temperature was fixed at 10°C as an abuse temperature for the fridge and the inactivation rates during storage are underestimated. It does not reflect the actual contamination level after the chosen observation time (2 days in the example below).

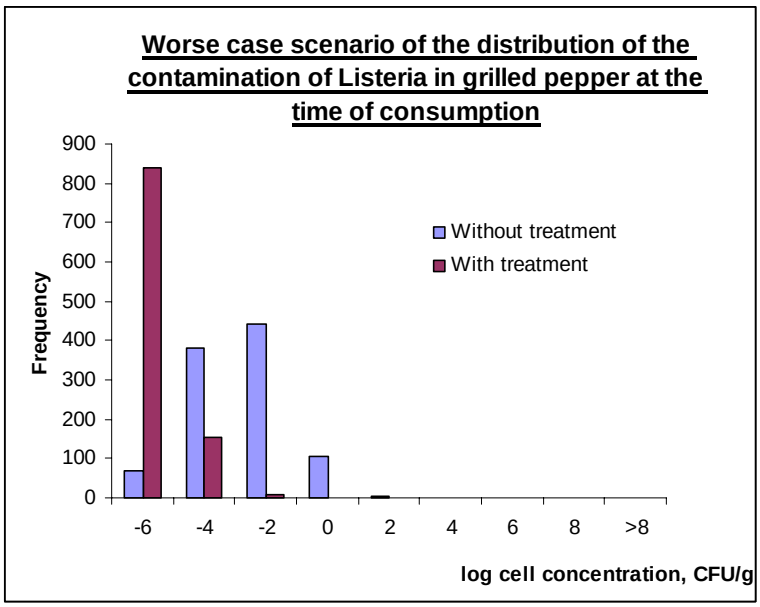

Figure 85: Contamination level of *Listeria* after treatment (350MPa at 30°C for 5 minutes) and without treatment after 2 days at 10°C. The initial contamination level was assumed to be on average -3.68 log CFU/g. Without treatment, 0.6% of the samples had more than the 100 cells/mg permitted by European regulation, whereas none of the 1000 samples had reached that level after the pressure treatment. For longer observation times, the contamination levels would even be lower as *Listeria* is inactivated in raw pepper.

3.Conclusions

In general the modelling of inactivation of "emerging" technology is still in its infancy. This may be in part because these technologies are expensive to implement and experiments are costly. As data are accumulated for instance via HighQ-RTE, progress has been made and new models and concepts are being published (Le Marc *et al*, 2009; inactivation by PHOTO in broth; Baranyi *et al*., inactivation by PEF in broth, in preparation; Metris *et al.*, inactivation by heat treatment following PEF in broth and other liquid food, in preparation). However, the data are still limited and it is of paramount importance to make them available to the community. It is also important that users are able to access the data that are already published. With this in mind, all datasets produced and published in the HighQ RTE project, have been made compatible with the ComBase system and will be added to the next version of ComBase. It will be available on-line (see www.combase.cc), which is an added value to the HighQ RTE project.

PEF treatment modelling was improved by collaboration with NovelQ partners and will result in the publication of a paper (Baranyi *et al*., in preparation; secondary modelling of PEF) whereby their (already published) data were used in our modelling and in return they received training to compile data in the ComBase format.
From the case studies, it emerged that, from the microbiological safety point of view, high pressure is suitable for the treatment of food. For high hydrostatic pressure treatments it was, however, not clear that CO_2 in the packaging atmosphere did improve the efficiency of the process to treat *Listeria*. For HPH, industrial scale equipment was

able to deliver pressure high enough to eradicate a whole range of micro-organisms in soup and fruit juice. With laboratory equipment where pressures were limited to 200MPa, a satisfactory level of inactivation could be achieved by repeating the experiments (applying successive "pushes"). PEF on its own was not sufficient to kill any significant numbers of *Listeria.* However, in combination with mild heat treatment it may be suitable to treat new products such as dressings where their properties might be adversely affected by treatment at high temperatures. PHOTO is a technique which has not been applied to food before and which results in a decimal reduction of pathogens on a solid surface comparable to washing.
The variability in the ability of the pathogens to grow in food was found to be mainly due to the food variability; e.g. the flora in eggs was so different for Swedish and Italian eggs that even without treatment the growth of *Salmonella* was significantly different. The effect of modelling uncertainty of the new technologies on the contamination of *Listeria* in milk after storage at refrigeration temperature was less than the effect caused by the variability of the food itself (composition of food and flora, although it was UHT milk). For PEF treatments, a significant difference of efficiency was found compared to the results obtained in the NovelQ project, a difference that may be due to the difference of equipment.

4.Impact of the research

New data were produced on the inactivation and subsequent recovery and growth of pathogens in food for which innovative technologies have been used during manufacturing/processing. These data are invaluable for industry, containing vital information regarding food safety issues. They help the industry to save money when designing experiments or optimising the applied processes. The data will be made publicly and readily available via ComBase (www.combase.cc), a world-leader of food safety databases. In addition, the models developed during the project gives a first insight about the practical use of the four new technologies and help to optimise the effort and cost that these technologies will need.

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Dissemination and Exploitation (TCA)

1. Objectives

The main objective of this activity was to share and disseminate among the stakeholders, food industrial community, consumers' organisations and policy makers the scientific knowledge generated by each HighQ RTE partner.

2.Approach

The consortium disseminated the results of the project by means of:

- a project web-site
- an easy-to-read project pamphlet
- papers published in international scientific journals
- final scientific workshop
- survey among consumer organisations
- contacts with catering stakeholders

3.Activities carried out

At the beginning of the project the web-site http://www.highqrte.eu has been set up and published. The web is composed of 4 sections that provide a brief description on:

- project and the basic information
- project partners
- project contacts
- events and publications

During the project life the web-site has periodically been updated and particularly the section "*events and publications*" in which all the events, conferences or other dissemination activities related to the project have been published. Moreover, a new section dedicated to the "*dissemination actives and results*" has been created and published. In such a session the project brochure and Consumers survey document have been published as downloadable documents. Also the pdf files of the published manuscripts or poster presentatis made at conferences on the HighQ RTE results have been uploaded.

Also an **easy-to-read brochure**, describing the scope of the project and the main activities that it covers, has been prepared and printed.

In order to investigate the consumers opinion on the non-thermal technologies studied within the HighQ RTE project, a questionnaire that was used for a survey among the consumers and which has been published on the web-site has been prepared. Such a questionnaire has been sent to all the project's partners, to national and European consumers' associations, institutions and universities. A total of 2100 questionnaires has been collected from all over Europe: Italy, Spain, Sweden, UK, Portugal, Greece, Ireland, France, Poland, the Netherlands. The survey revealed that consumers seemed to be very interested in new technologies aiming at the elimination or reduction of degradative and particularly pathogenic microorganisms that are frequently associated with milk, mayonnaise, cheese, vegetables, juice, and ready-to-eat meals. Most of them consider food safety as a very importantobjective, and appreciate the possibility of having it by using treatments that do not affect the quality and the consistency of food.

An interesting result emerged from two specific questions regarding organoleptic characteristics and the price of new non-thermally processed food: 38% of consumers consider consistency (feel) as the most important character; 62% of them stated they would prefer innovative organoleptic characteristics, but only 49% would be willing to pay a higher price if new (non thermally processed) products were launched on the market. And they would only accept 10% increase over the original price.

Many contacts with companies and catering stakeholders representatives (in particular with R&D Managers and production managers of SMEs) have been established in order to disseminate the results and achievements obtained during the project. For this purpose also a final workshop has been organized in Bertinoro (Italy) in September 2009 during which the benefits and promising applications of the non-thermal technologies studied in the HighQ RTE project have been presented to scientists and food companies (SMEs) representatives.
Finally, the results of the research activities carried out during the HighQ RTE have been presented at several national and international conferences and disseminated through the publication of reports or manuscript on international journals.

Demonstration activities (UNIBO)

1. Objectives

The main objective of these activities was to evaluate the efficiency of the 4 novel processes on pilot scale, validate products and improve dissemination.

2.Approach

The activities carried out included:

PHOTO demonstration task

23 tests of 4 different products at two different stages of production in 2 different production facilities of UAB "Palink" have been performed. During experiments the possibility of microbial inactivation in three types of salads and strawberries indented to pastry production was evaluated. In particular, samples were produced and subjected to Photo treatment under conventional production conditions in UAB "Palink" production unit to assess the commercial feasibility of photo technology. The relevance and applicability of photo sensitizer on the food matrix were examined and the commercial feasibility and the legal status evaluated at Lithuanian National Risk Evaluation Institute accredited by Deutscher Akkrediteirungs Rat laboratory (Kairukščio str. 10 Vilnius Lithuania). Effects on organoleptic properties of the selected products were also tested after photosensitization and treatment with the photo device developed by VU. Photo-treated products were compared to standard products. In particular; appearance/color, sensory profiles of fresh products and their shelf-life performances were compared in order to determine the performance of the novel process for RTE products at Lithuanian National Risk Evaluation Institute.

PEF demonstration task

PEF technology in combination with mild heat treatments was validated for mayog dressing, béchamel sauce and balsamic vinegar. Treatments were performed by using the pilot scale PEF equipment used for the RTD work, which was modified in order to adapt to the need of the demonstration activity. Technical feasibility, commercial feasibility and process criteria to obtain safe products were also evaluated.

Product treatments were performed on 16 occasions. During each occasion, one to four different treatments combinations were tested depending on the volume to be treated. In addition, equipment assembling and disassembling and cleaning were performed.

Variable treatment conditions were tested. Experiments were performed in order to treat the different products without getting a too high temperature increase in the product or electrical arcing. The pulse length was held at a constant value. The electric field strength and the number of pulses was either set constant or increased as much as possible. From these experiments, the setting of parameters for evaluation the product quality was selected.

Analyses of microbiology, structure, enzymes, technological properties and sensorial properties were performed by the Partners. Moreover, the obtained analytical data were used for an evaluation of technical feasibility, commercial feasibility and process criteria necessary to obtain safe products. The results of the evaluation were presented and discussed during a demonstration day.

An open demonstration day on Pulsed electric field processing was organized by SIK and took place on the 3rd of September 2009. Treatment of liquid whole egg was demonstrated and experimental results of the treatments of whole liquid egg, mayog dressing, béchamel sauce and balsamic vinegar, which had been obtained by the Partners, were presented and discussed.

HPH demonstration task
Three complex foods chosen on the basis of the results of the RTD activities were prepared by CAMST and treated at Niro Soavi (Sub-contractor of UNIBO) with an industrial-scale equipment at pressure levels up to 400 MPa. In particular the foods used for the demonstration were: mixed fruit juices, vegetable soups and milk/egg emulsions. The quality of the HPH-treated products has been evaluated by CAMST by a panel test, while the safety has been evaluated by UNIBO. Also the evolution of the surviving cells has been evaluated during the storage over a 30 day period at 4°C and 10°C in order to define the shelf-life of the treated products and determine the performances of the novel process and the new RTE products.

HHPCO Desmostration task
The demonstration activities were carried out by CSIC in collaboration with ORT by organizing several experiments. In particular, RTE dishes produced by ORT have been subjected to HHP treatments by using an industrial high-pressure equipment located in NC-Hyperbaric (Burgos). Treated samples were then analysed in order to evaluate the effects of the treatments on chemico-physical parameters, sensory features, flavour, colour, texture (CSIC, ORT).
In addition, a ***"technology open testing day"*** in which SME´s assayed their experiments with the objective to investigate if this new technology could be useful for them has been organised by CSIC.

3.Results

PHOTO demonstration task (UAB, VU, CSB)

The main objective of this activity was to assess the suitability of the Photosensistization technology to be used for the production of RTE-meals proposed by SMEs.
In particular the following activities have been carried out:

1. Presentation of the portable PHOTO device developed by VU at people working in 2 selected industrial sites.
2. Testing the portable PHOTO device in UAB "Palink" in two different sites in Vilnius and Alytus. In particular, 23 tests of 4 different products at two different stages of production were performed.
   2.1. Incorporation of the PHOTO technology by treatment of the raw vegetables before full production cycle (Vilnius delicatessen production unit).
   2.2. Incorporation of the PHOTO technology by treatment 3 different finished products (salads) before their dressing (Vilnius delicatessen production unit).
   2.3. Treatment of fruits (strawberries).
3. Evaluation of the performances of the portable Photo device on UAB products with respect to the traditional process/products by assessing:
   3.1. the effects of PHOTO treatment on the safety of the products
   3.2. the effects of PHOTO treatment on shelf-life of the products
   3.3. the effects of PHOTO treatment on the quality of the products

3.4. the possible changes in the production costs
3.5. a proposals for a possible adaptation of the technology to the industrial requirement

1.Presentation of the PHOTO device

The portable PHOTO device developed by VU was tested at real production; method and equipment were presented for 120 persons working in Vilnius and Alytus production sites. Photo device was introduced into real production cycle. Employees of UAB Palink get acquainted with PHOTO microbial deactivation method, procedures and methods of the work.
The implementation and introduction of this technology, which enables to increase the safety of the product, would be very important for UAB “Palink”. Currently the shelf-life of salads is 60 hours for products which are packed in ordinary atmosphere, while it corresponds to five days for the products packed under modified atmosphere. The extension of the shelf-life would have a great economic effect. Strawberries are used for the decoration of the pastry products and in general the shelf-life of the pastry products depends on the shelf-life of the fresh fruits used. The implementation of this technology is considered a target for UAB Palink which aims to extend the shelf-life of the products up to 5 days without using a modified atmosphere packaging.
UAB "Palink" is the second biggest retail network in Lithuania and operates also in Latvia. UAB "Palink" operates from 1993 and is specialized in retail and production of food products. UAB “Palink operates 282 shops in Lithuania, 92 of them having butchery units in the shops where fresh meat are produced, as well 31 units for pastry production and 7 units for production of fresh fruit and vegetable salads also ready-to-eat meat and fish product production. For the production of its products UAB "Palink" do not use chemical preservatives and this is the reason why there is a big interest in the development of any non thermal processing of fresh products.
After the presentation of this equipment, UAB “Palink” employees attending the meeting were interviewed. A general impression and conclusion of the interview was that this method slows very much the production cycle.

2. Verification of performances of the PHOTO on UAB products using PHOTO and conventional production cycle

The verification was performed by using a portable PHOTO device and this verification is valid only for production by this prototype. If PHOTO device would be upgraded, processes can be faster.

Salads containing most popular vegetable for preparation of salads such as paprika, broccoli, leek, cucumber, China cabbage, radish, dill, cabbage, carrots were tested. In particular three types of fresh vegetable salads, i.e. “Popular” salad (“Pupuliariosios”), “Spring “salad (“Pavasaris”) and “”Fresh vegetable” salad (“šviežių daržovių salotos”), were selected. The selected salads are mainly produced from fresh fruit and vegetables and added with a dressing containing oil according to the procedures reported in the Annex IV.
Also strawberries intended to be used for the decoration of pastry products have been used for the demonstration activities. The effects of photo treatment on the organoleptic

properties as well as microbiological quality and shelf-life of the treated products have been evaluated.

Materials and methods

Prepared raw materials were cut to fit in to the illumination equipment. Before final preparation they were photo photosensitized illuminated and then the preparation process continued according to the Annex IV.



After illumination the products were finally processed, dressed and packed. The same preparation and storage conditions were kept for control and treated salads. Prepared salads were then packed for shelf-life period testing. Packages were stored at +4 ºC and delivered to the Lithuanian National Risk Evaluation Institute. Some of them were kept in the production unit for determination of the sensory profiles.

Microbiological analysis

For this experiment *Salmonella* and *Listeria monocytogenes* were tested according to the annex of European Regulation (EC) Nr. 1441/2007. In accordance with the EC regulation, food products must be free from *Salmonella* and *Listeria monocytogenes* in 25 grams of the product.

Staphylococus aureus and *Bacillus cereus* were tested according to the Lithuanian legislation for microbiological contamination according to the Lithuanian Hygiene norm Nr 26-2006. The limits which are set for these microorganisms are the following:

Stapylocosus aureus: 1000 CFU/1g of product

Bacilus cereus: 10000 CFU/1g.

Salmonella and *Listeria monocytogenes* were tested only the first day of the experiments because these contaminants must be absent.

Total microorganisms counts are not regulated in Lithuania; however, they have been counted as they are generally considered one of the principal indicators for the evaluation of the of the shelf-life.

Microbiological analysis were carried out at the Laboratory of National Food and Veterinary Risk Assessment Institute. The conditions adopted for the analysis were the following:

1. For the detection of spoilage bacteria “Plate Count Agar” plates, which were incubated at 30°C, were used. Data are the mean of 5 repetitions.
2. For the detection of *Salmonella* “KLD” plates, incubated at 37ºC, were used. The umber of repetitions was 5.
3. For the detection of *Listeria monocytogenes* “Oxford” plates incubated at 37°C were employed. 5 repetitions were made.

Validation tests were performed in Lithuanian National risk evaluation institute accredited by Deutscher Akkrediteirungs Rat laboratory (Kairukščio str. 10 Vilnius Lithuania). Only accredited methods were used to perform tests.

Photo treatment

Products were incubated for 5minutes by using as photosensistizer chlorophyll sodium salt at a concentration of 1.5 x 10^{-4} mM. Products were submerged in chlorophyll Natrium water solution, while control vegetables were not exposed to any treatment, because it is not foreseen in the production technology.

Products were then illuminated with the PHOTO device for 5 minutes at 100 percent power.

Packaging

Products were packed into plastic package designed for contact with food products package. These packages (barcode 795.08993 and 795.63681 supplier UAB "Rotoma") are used for packaging of the products. Products were packed in ordinary atmosphere.

Organoleptic testings

10 panellists were involved for each experiment. The questionnaire used was based on the following attributes: global appearance, smell and taste. The statistical analysis of the results was performed at the Laboratory of National Food and Veterinary Risk Assessment Institute according to LST EN ISO 4120:2007.

Results

2.1 Incorporation of the PHOTO technology by treatment of the raw vegetables before full production cycle

None of the pathogens was found in any of the tested product regardless the treatment and the sampling time during refrigerated storage.

Raw materials for "Spring" salads treated by Photo

Table 26 Microbiological test results „Spring"salads produced 2009 06 09 – Control.

Bacteria tested	2009 06 10	2009 06 11	2009 06 12	2009 06 15
Salmonella	0			
Listeria monocytogenes	0			
Staphylococus	0	0	0	0
Bacillus cereus	45	790	220	150000
Total microbial count	1100000	1000000	2100000	30000000

Table 27 Microbiological test results „Spring“salads produced 2009 06 09 – Treated by photosensitization and illumination.

Bacteria tested	**2009 06 10**	**2009 06 11**	**2009 06 12**	**2009 06 15**
Salmonella	0			
Listeria monocytogenes	0			
Staphylococus	0	0	0	0
Bacillus cereus	40	990	1600	150000
Total microbial count	2300000	970000	4300000	30000000

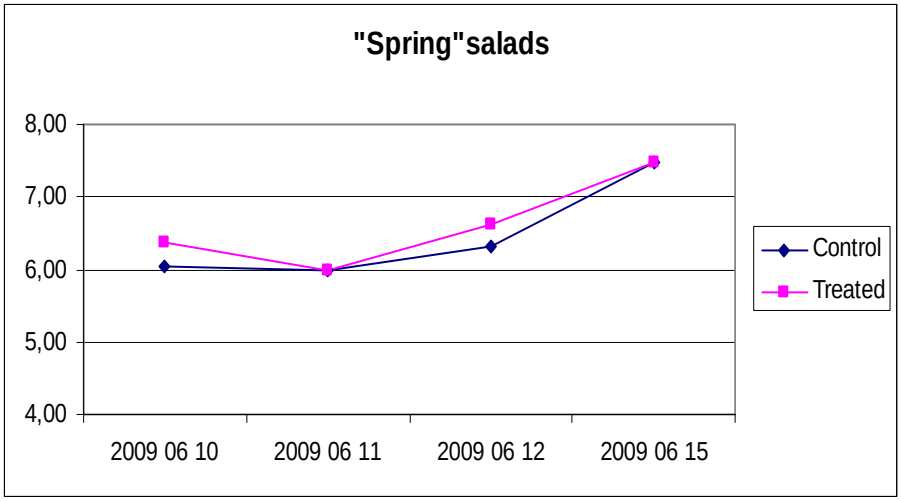


Figure 86. Comparison of total microbial counts in „Spring” salads. Raw materials treated by photosensitization and control.

No significant differences were detected between treated salads and the controls.

Raw materials for “Popular” salads treated by Photo

Popular salads were prepared in the same way as “Spring” salads; however different vegetables were used, paprika before photosensitization and illumination was cut and removed seeds and Cabage cut into straws according to the Annex IV – section 1.1.

Some fluctuations from normal salad production process procedures occurred. The photosensitization and illumination phases of the product have extends for 2 hours and experimental product were stored at production are at 15ºC. Normally the processing of one batch of product takes 15- 20 minutes.

Table 28. Microbiological test results „Popular“salads produced 2009 06 09 – Control.

Bacteria tested	**2009 06 10**	**2009 06 11**	**2009 06 12**	**2009 06 15**
Salmonella	0			
Listeria monocytogenes	0			
Staphylococus	0	0	0	0
Bacillus cereus	0	40	40	40
Total microbial count	59000	41000	26000	830000

Table 29 Microbiological test results „Popular“ salads produced 2009 06 09 – Treated by photosensitization and illumination.

Bacteria tested	**2009 06 10**	**2009 06 11**	**2009 06 12**	**2009 06 15**
Salmonella	0			
Listeria monocytogenes	0			
Staphylococus	0	0	0	0
Bacillus cereus	0	0	0	0
Total microbial count	110000	120000	40000	59000

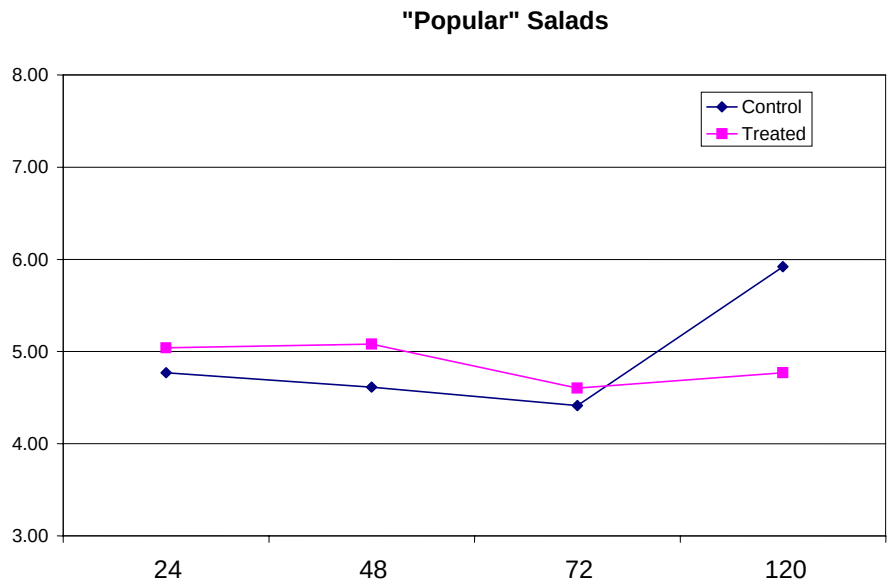


Figure 87. Comparison of total microbial counts in „Popular” salads. Raw materials treated by photosensitization and control.

For popular salads a higher total microbial count was observed in the untreated products after 5 days of refrigerated storage, while no growth was detected in the photo-treated products.

Raw materials for "Fresh vegetable" salads treated by Photo

Experiments were performed the same way as "Spring" and "Popular"salads.
Some fluctuations from normal salad production process procedures occurred. In fact photosensitization and illumination of the product have extended for 2 hours and the experimental products were stored in the production area at 15ºC. Usually the processing of a batch of productkes 15- 20 minutes.

From the comparison of data reported in the Tables 30-31 and figure 88 it is clear that a lower cell count was detected 120 hours following Photo treatment with respect to the controls.

Table 30 Microbiological test results „Fresh vegetable" salads produced 2009 06 09 – Control**.**

Bacteria tested	2009 06 10	2009 06 11	2009 06 12	2009 06 15
Salmonella	0			
Listeria monocytogenes	0			
Staphylococus	0	0	0	0
Bacillus cereus	40	4700	540	11000
Total microbial count	1800000	1600000	2100000	30000000

Table 31 Microbiological test results „ Fresh vegetable" salads produced 2009 06 09 – Treated by photosensitization and illumination.

Bacteria tested	2009 06 10	2009 06 11	2009 06 12	2009 06 15
Salmonella	0			
Listeria monocytogenes	0			
Staphylococus	0	0	0	
Bacillus cereus	40	4200	1300	12000
Total microbial count	590000	690000	2500000	1900000

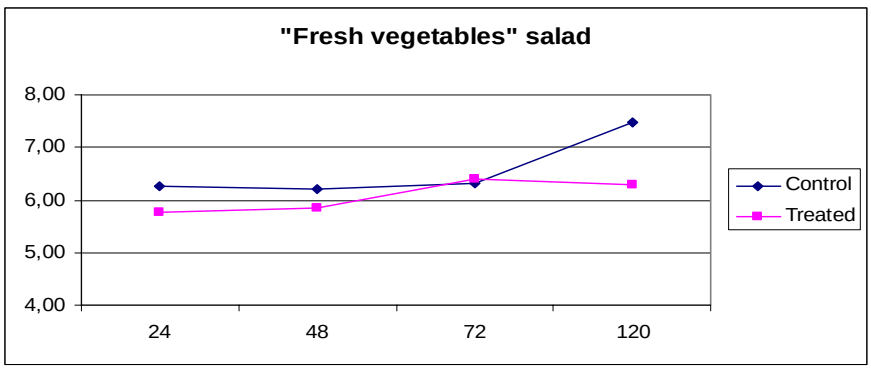


Figure 88 Comparison of total microbial counts for treated by photosensitization and illumination and control „Fresh vegetables" salads produced 2009 06 09.

2.2Incorporation of the PHOTO technology by treatment of the finished products before dressing

A second group of experiments was performed on 2009 06 15, 16 and 22 by using raw materials that were processed according to the procedures described at points 1.1; 2.1 and 3.1 of the Annex IV and used to prepare salads according to the procedures described at points 1.2; 2.2 and 3.2 of the same Annex. Afterwards, they were washed with the photo sensitizer solution and illuminated with light. Illuminated vegetables were then dried by removing the exceeding water. Organoleptic properties as well as microbial contamination and the effects on the shelf-life were tested. Salads which were made from photosensitized and photo-treated ingredients were compared with salads prepared in usual way of the production.

Finished "Popular" salads treated before dressing

"Popular" salads prepared according article 1.2 of Annex IV, photosensitized and irradiated.

After irradiation salads was packet and transported to Laboratory.

Table 32 Microbiological test results „Popular"salads produced 2009 06 15 – Control.

Bacteria tested	**2009 06 15**	**2009 06 17**	**2009 06 18**
Staphylococus aureus	0	0	0
Bacillus cereus	0	40	40
Total microbial counts	200000	71000	89000

Table 33 Microbiological test results „Popular“ salads produced 2009 06 15 – Treated by photosensitization and illumination.

Bacteria tested	**2009 06 15**	**2009 06 17**	**2009 06 18**
Staphylococcus aureus	0	0	0
Bacillus cereus	110	40	40
Total microbial counts	72000	74000	130000

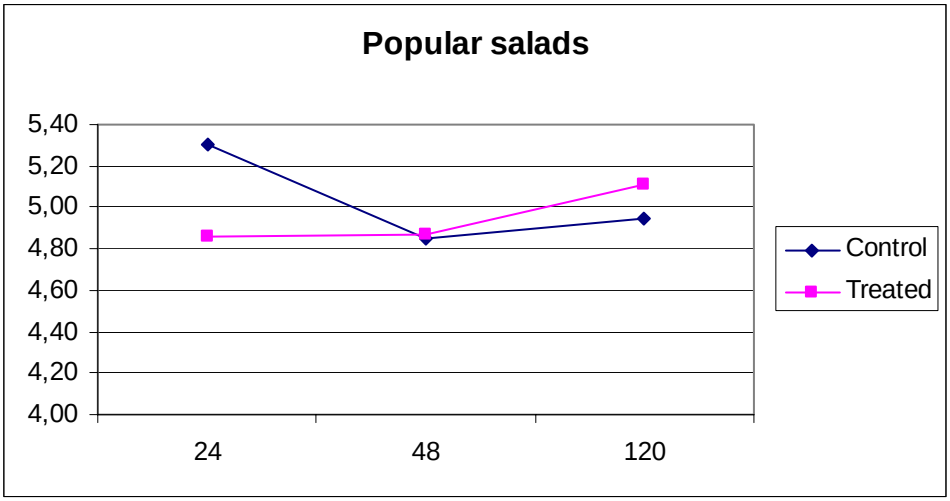


Figure 89 Comparison of total microbial counts for treated by photosensitization and illumination and control „Popular“salads produced 2009 06 15.

A slight decrease in the initial contamination of finished Popular salad was observed immediately after the photo treatment; however, such a difference tended to disappear after 2days of refrigerated storage (Figure 89).

Finished “Spring” salads treated before dressing

“Spring” salads prepared according to the article 2.2 of the Annex IV.

Photo sensitized salad

Irradiated. packed and transported to laboratory

Table 34 Microbiological test results „Spring"salads produced 2009 06 16 – Control.

Bacteria tested	**2009 06 16**	**2009 06 18**	**2009 06 23**
Staphylococus aureus	0	0	0
Bacillus cereus	40	40	40
Total microbial counts	1300000	2700000	980000

Table 35 Microbiological test results „Spring" salads produced 2009 06 16 – Treated by photosensitization and illumination.

Bacteria tested	**2009 06 16**	**2009 06 18**	**2009 06 23**
Staphylococus aureus	0	0	0
Bacillus cereus	40	40	40
Total microbial counts	900000	150000	290000

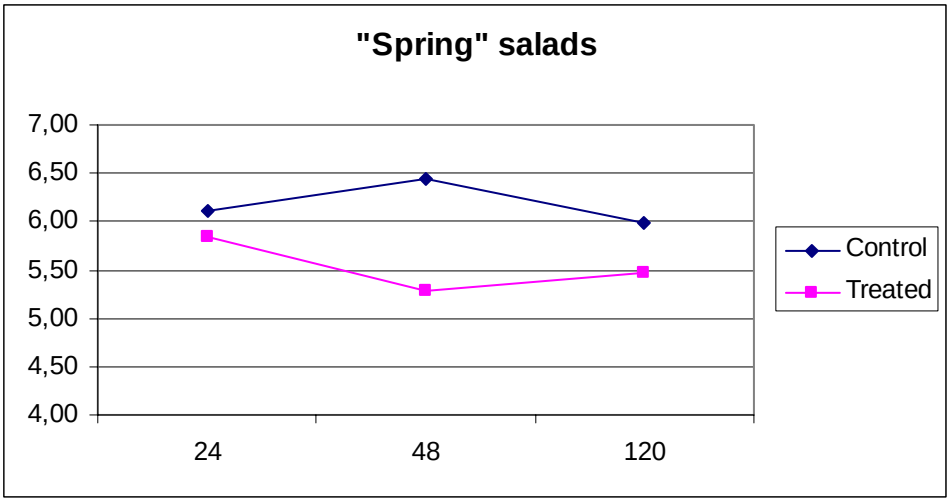


Figure 90 Comparison of total microbial counts for treated by photosensitization and illumination and control „Spring“salads produced 2009 06 16.

No significant differences were observed in cell counts of “Spring” salads immediately after Photo treatment with respect to the controls; on the other hand, no viability cell increase was detected during a 5 days refrigerated storage in Photo-treated samples (Figure 90).

Finished “Fresh vegetable” salads treated before dressing

“Fresh vegetable” salads were prepared according to the article 3.2 of the Annex IV.

Photosensitized and irradiated, and packed and transported to the laboratory.

Table 36 Microbiological test results „Fresh vegetable“salads produced 2009 06 22 – Control.

Bacteria tested	**2009 06 22**	**2009 06 23**	**2009 06 25**
Staphylococus aureus	0	0	0
Bacillus cereus	0	0	0
Total microbial counts	430000	130000	150000

Table37 Microbiological test results „Fresh vegetable“ salads produced 2009 06 22 – Treated by photosensitization and illumination.

Bacteria tested	**2009 06 22**	**2009 06 23**	**2009 06 25**
Staphylococus aureus	0	0	0
Bacillus cereus	0	40	40
Total microbial counts	54000	53000	75000

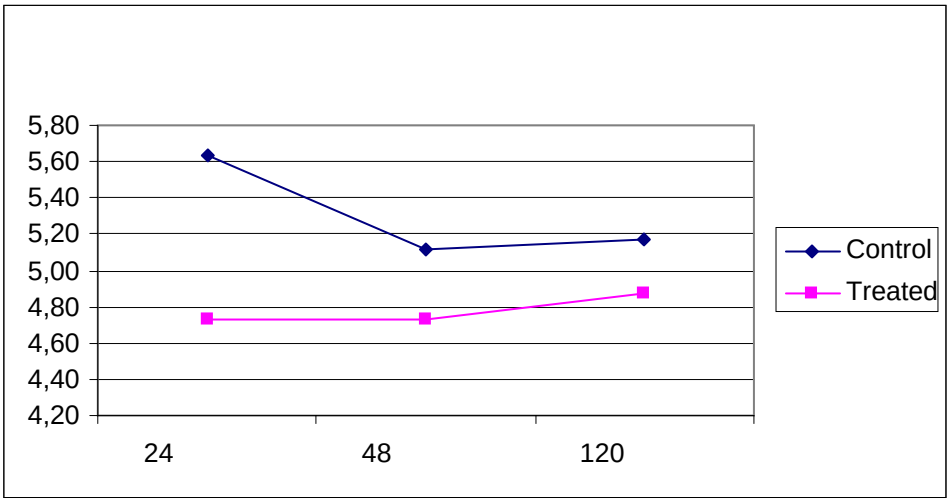


Figure 91 Comparison of total microbial counts for treated by photosensitization and illumination and control „Fresh vegetable“salads produced 2009 06 22.

Photo treatment of finished “Fresh vegetables” salads resulted in a 0.8 log reduction of the initial contamination (Figure 91); no cell counts increase was observed during refrigerated storage in photo-treated samples nor in the controls.

2.3 Treatment of the fresh strawberries to be used for future production

Fresh strawberries, were washed with photosensitizer solution and illuminated with light. Afterwards berries were dried and packed. Organoleptic properties as well as Microbial contamination and shelf life were evaluated.

Data reported in figure 92 evidenced that no significant effect of Photo on initial contamination occurred also taking into consideration that a high variability (20%) was observed among the 5 repetitions. This result may probably be due to rough surface of the strawberries which reduced the efficacy of the photosensitization.

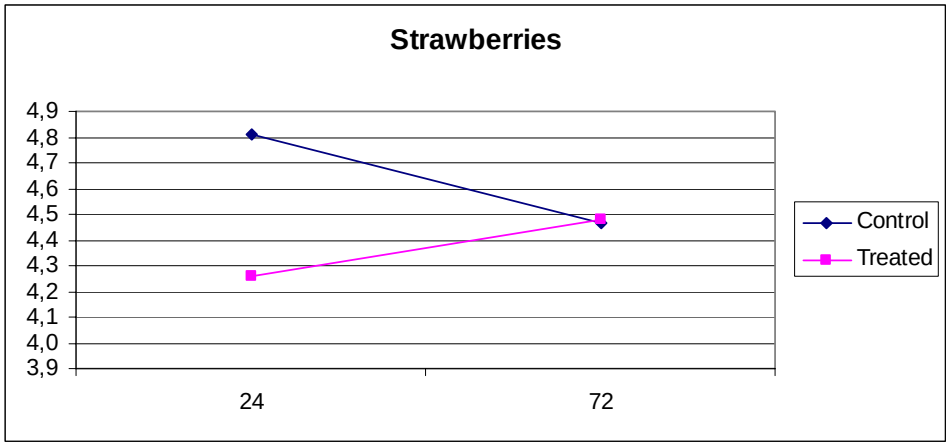


Figure 92. Total microbial counts in strawberries.

3. Evaluation of the performances of the PHOTO device

Verification of the effects of PHOTO treatment on the safety of the products

During laboratory tests, PHOTO method resulted to have very good performances for the reduction of both pathogenic microorganisms and spoilage bacteria.

During experiments performed under real industrial conditions, it was not possible to assess effect of the method on pathogenic microorganisms because it is impossible to use contaminated raw materials under real production conditions. The only way to evidence the possible effect of the PHOTO treatment is to analyze changes in total bacterial contamination. Significant reductions of the initial contamination by spoilage bacteria were observed only if the products were PHOTO treated after all the production steps; on the contrary, treatment of the raw materials did not result in any significant effect. The reason of this result could be that some of the vegetables that were used for the production of the salads (e.g. dill, leek, broccoli, china cabbage) makes many shadows and the decontamination is not effective. During the following process phases, spoilage bacteria were naturally spread ant this could be a reason that no significant effect was observed.

Another negative factor occurring during real conditions is that during the decontamination of the products by photo treatment it is practically impossible to control the product temperature. An implemented temperature control for this processing stage is possible only if the products are produced in a refrigerated area (0 + 5ºC). But this aspect would significantly increase the labor power costs for all the products produced in such area.

Verification of the effects of PHOTO treatment on the shelf life of the products

During the experimental activities also the possibility to extend the shelf-life of photo-treated products was evaluated. The shelf-life was defined as the time necessary to attain a threshold value of cell counts (i.e. 7 Log CFU/g of product) over which the products can be considered as unacceptable.

Table 38. Efficiency of photosensitization treatment of the raw vegetables before production cycle on the shelf life of the product

Product	Condition	Shelf-life (days)
Spring salad	Control	2
	Photo-treated	2
Fresh vegetable salad	Control	3.5
	Photo-treated	>5
Popular salad	Control	>5
	Photo-treated	>5

Table 39. Efficiency of photosensitization treatment of the prepared salads before dresing on the shelf life of the product

Product	Condition	Shelf-life (days)
Popular salad	Control	>5
	Photo-treated	>5
Spring salad	Control	>5
	Photo-treated	>5
Fresh vegetable salad	Control	>5
	Photo-treated	>5

Concerning strawberries, no significant differences were observed between photo-treated products and the control also considering that the variability of cell counts among the data of Log CFU/g obtained for 5 samples was 20% probably due to the rough surface of the products.

Verification of the effects of PHOTO treatment on the quality of the products

Organoleptic properties are very important for salads, because consumers' choice is mainly based on organoleptic properties of the products.

Photo treatment of the raw materials did not show significant effects on organoleptic parameters of the product; however, when products were treated on the final stages of the production cycle, the PHOTO treatment significantly reduced organoleptic features of the product if water in excess was not removed during the process. In fact residual water can activate spoilage enzymatic activities and namely oxidation inducing browning phenomena. This effect cannot actually be attributed to the Photo treatment, but to the processing.

3.4 Verification of the possible changes in the production costs

For the verification of the changes of the production costs for delicatessen product, calculations were made only in relation to labor power costs including all taxes paid by

company and employee. Changes for operational costs (equipment, building, water, electricity, package and etc) were not taken into consideration.
During a normal production cycle for production of one kilogram of the selected product 0,022 working hours are normally used, whereas treatment by photo of the raw materials significantly increases labor costs. Average labor power cost for one hour of production is 7,53 Eur.

Table 40. Demand for extra labor power cost of PHOTO treated salads.

Salads	**Treatment of the raw materials**		**Treatment of the finished salads**	
	Demand of extra labor power hours per 1 kg	**Extra labor power cost Euro per 1kg**	**Demand of extra labor power hours per 1 kg**	**Extra labor power cost Euro per 1kg**
"Spring" salads	3,00	22,59	3,73	28,24
"Popular" salads	3,73	28,24	5,25	39,53
"Fresh vegetable" salads	3,00	22,59	3,73	28,24

According to the data reported in Table 15 PHOTO treatment very significantly increased labor power costs for the production of salads. Cost has increased from original 0,17EUR up to 39,53 EUR. Labor cost for the PHOTO treatment of the raw materials is significantly lower than those of the treatment of the finished products.
This result indicates that the prototype must be upgraded and adjusted to real production lines to reduce labor power costs for production.

3.5 Identifications of proposals for a possible adaptation of the method

Treatment of the products by Photo device can be effective if it is possible to reduce Photo irradiation time from 5 minutes down to few seconds; in this way it would be possible to include the technology into current processing lines. In fact this solution would result in an increased food safety and would significantly reduce labor costs.

3.6 Verification of possibilities for the validation of this technology

It is possible to validate the PHOTO treatment, because Chlorophyll natrium salt is registered as the food additive E140 (colouring), and exposure by light do not requires any special validation procedures. However, due to low effectiveness of the Photo device actually available its industrial usage will be possible only when the prototype will be upgraded.

Conclusions of PHOTO demonstration activities

During the demonstration activities and research performed at UAB "Palink" food processing facilities it was established that for a possible use of this technology it is necessary to reduce the illumination time and adapt the method to the industrial conditions and equipment.

1. Photo treatment significantly increases labour costs for salad production.
2. Photo treatment is effective only on final stages of the product production, while decontamination of the raw materials is not enough effective.
3. In general a significant effect of this decontamination technology for the extension of the shelf-life of the products was not observed.
4. Photosensitization and irradiation method can be used at industrial level only if adapted to production lines.
5. It is possible to validate this method if effectiveness would be reached.
6. Photosensitization can reduce total microbial growth treating both raw materials for salads and salads before their dressing. Decontamination of the prepared salads before dressing results in a reduction of the initial bacterial contamination. However, for development of the method it is necessary to reduce time of photosensitization (application chlorophyll sodium salt solution).
7. For a possible future implementation of an industrial scale equipment it is necessary to reduce also the time of irradiation, because a slow production process increases the risk of contamination of the products and decreases the economic value of the technology due to an increase in the production costs.

PEF demonstration task (SIK, CAMST, ACE, UPV)

Modifications of equipment and product composition

The treatment equipment was modified in order to be able to treat the real RTE products (mayog dressing, béchamel sauce and balsamic vinegar). Additional pumping capacity was added due to a higher viscosity of some products. For each product, the maximal energy input was carefully evaluated by step wise changes in applied electrical field strength, number of pulses and pulse time. The composition of the heat sensitive sauces were modified in order to suit PEF treatment regarding conductivity.

Demonstration day

The application of PEF on liquid whole egg, béchamel, mayog and balsamic vinegar in pilot scale was demonstrated and discussed during a one-day demonstration day. The program is shown in Figure 93. Industries producing liquid food were invited to attend the workshop.

Figure 93. Program for PEF open technology testing day.

Commercial feasibility

The industry partners CAMST and ACETUM evaluated the commercial feasibility taking mayog dressing and balsamic vinegar as examples.

- ***Mayog dressing***

On the basis of all the experiments, previously made by the Partners at demonstration level, it emerged that the high level of intensity of PEF affected positively the properties of eggs when utilized as ingredients for the preparation of custards. Moreover, the combination of PEF and heat on eggs showed slight differencess from no treated eggs in terms of consistency and flavour for the preparation of mayog dressing. The workability specifications (value ranges for filling machinery) were achieved. The PEF treated samples complied the workability values. In fact the firmness was included in the range >1 <10 Kg, the consistency in the range >5 <10 Kg*sec, the cohesiveness in the range >-5 <-1 Kg and the viscosity in the range >-1.0 < -0.5 Kg*sec.
In conclusion, the application of PEF allowed the preparation of a heat sensitive mayog dressing which otherwise could not be produced due to the breakage of its structure.

- ***Balsamic vinegar***

On the basis of the experimental data it was concluded that PEF is a promising method for a non invasive treatment of vinegars and their related products. Improvement and maybe specific set ups are required before its implementation in the vinegar industry. It seems to be a good preventive method (like ultrafiltration and addition of sulphites), but not definitive against side fermentations.

Its transfer to an industrial scale requires investment costs that are likely to be too high for the majority of target companies. Maybe in the future, PEF will become a more widespread technology, whose diffusion will provide easier customized solutions and lower costs.
The quality parameters for the balsamic vinegar treated with different PEF conditions were in accordance to the quality norm. In particular, PEF-treated samples complied for the chemico-physical limits established for balsamic vinegars of good quality (Table 41).

Table 41. Physico-chemical properties of PEF treated balsamic vinegar of Modena.

	Mean value ± Standard Deviation	Normal Quality
Relative density	1,06636 ± 0,00010	1,06 – 1,07
Total Acidity (Acetic acid g/100mL)	6,202 ± 0,013	6,0 – 6,25
pH	3,214 ± 0,010	2,0 – 3,50
Total sulphur dioxide (ppm)	8,856 ± 1,215	< 60
Total dried extract (g/L)	150,3 ± 0,3	130 – 160
Deduced dried extract (g/L)	38,08 ± 3,25	30 – 40
Sugars (Fehling - g/L)	112,22 ± 3,16	110 – 120
Turbidity NTU	3,14 ± 0,073	2 – 5

Process criteria to achieve "safe" products
By combining PEF treatments and mild heat treatments it is most likely possible to give a product a treatment equivalent to traditional pasteurisation by carefully choosing the PEF and heat parameters. The reduction required for a specific product depends on the number of bacteria in the product before treatment and on the number of infected products that is accepted. According to the EU legislation, the producer is responsible for choosing an appropriate treatment. In the USA, pasteurization requirements for liquid whole egg is 60°C for 3.5 minutes giving a 8.75 log CFU reduction of Salmonella according to FSIS (National advisory committee on microbiological criteria for foods 2004). For RTE food often a 6 log CFU reduction is desired. The heat treatment required for achieving this is 2 minutes at 70°C for *L. monocytogenes* (Inventory report, FAIR CT96-1020).
The inactivation of *S. enteritidis* UNIBO 155 in egg and mayog dressing was >6 log CFU using PEF treatment and subsequent mild heat at 53°C for 10 minutes. *L. monocytogenes* was inactivated to a lesser extent; 3.6, 3.1, 2.7 and 2.6 log CFU reductions were obtained in liquid whole egg, milk, mayog dressing and béchamel sauce, respectively.

The obtained reduction of *S. enteritidis* using PEF and mild heat was more than 6 log CFU. If the level of the bacterium before treatment is assumed to be 1 CFU/ml, less than 4 CFU/100 000 liter product would be expected after the treatment. If PEF alone is used, a 2 log reduction was achieved. The predicted level after treatment would be 4 CFU/10 liter product.
The PEF treatment applied in the project on samples inoculated with *L. monocytogenes* did not achieve a 6 log CFU inactivation. However, a sufficient inactivation of *L. monocytogenes* using PEF and subsequent heat treatment is expected to be achieved by increasing the heat temperature and/or heating time slightly. The inactivation of *L. monocytogenes* in phosphate buffer increased from 1 log CFU to 3 log CFU when the temperature was increased from 50°C to 53°C. When the temperature was increased to 55°C, the inactivation was >5.5 log CFU.

Conclusions of PEF demonstration task

Liquid whole egg treated by PEF and subsequent mild heat treatment was successfully used for the production of custard. Mayog dressing was successfully produced using PEF and subsequent mild heat treatment. Unwanted side fermentation in balsamic vinegar was delayed due to PEF and subsequent heat treatment.
Conditions applied during PEF and subsequent mild heat treatment inactivated *S. enteritidis* by more then 6 log CFU. As opposed, the inactivation of *L. monocytogenes* was $\leq$ 3.6 log CFU applying similar conditions. By optimising the subsequent mild heat treatment, a further reduction is expected.

HPH demonstration task (UNIBO, CAMST, Niro-Soavi)

In order to evaluate the suitability of the HPH treatments at industrial scale level, mixed fruit juices, vegetable soups and milk/egg emulsions were prepared by CAMST and treated at Niro Soavi (Sub-contractor of UNIBO) with an industrial-scale equipment at pressure levels up to 400 MPa. The immediate microbial viability of the naturally occurring total aerobic microflora, yeasts, lactobacilli and lactococci in vegetable soup and fruit juice are reported in Table 42. The initial contamination levels of the total mesophilic bacteria in raw fruit juice and vegetable soup were ca 4.90 and 4.5 log CFU/ml, respectively, while cells counts of about 2 log CFU/ml were detected for yeasts. Moreover, the vegetable soup showed higher cell counts of lactobacilli.
HPH treatments at 200 MPa of fruit juices resulted in a dramatic cell viability reduction of all the microbial groups tested. By increasing the pressure up to 300 MPa, counts under the detection limit were observed for all the microbial groups considered, except for the total aerobic mesophiles in the fruit juice. Similar results were observed also for the vegetable soup.
The evolution of the surviving cells has been evaluated during the storage over a 30 day period at 4°C and 10°C (abuse condition). All the data obtained have been used to define the shelf-lives of the untreated and HPH treated samples, which have been calculated as the time necessary to reach a cell load of 7 log CFU/ml and 6 log CFU/ml for bacteria and yeasts, respectively. In Tables 44 and 45 the data relative to the shelf-lives are reported. HPH treatments at pressure levels of 300 and 400 MPa allowed to obtain shelf-lives > 30 days, also for vegetable soups, having a pH of about 6, stored at

10°C.
None of the following species, i.e. *Listeria monocytognes, Salmonella* spp., *Bacillus cereus, Escherichia coli*, were detected both in the raw materials and HPH treated samples by using the standard procedures for their detection.
As far as the milk/egg emulsions subjected to HPH treatments in an industrial scale equipment, no pressure levels higher than 150 MPa was adopted due to the emulsion rupture. In Table 43 the immediate cell reduction detected for the total aerobic bacteria are reported. Cell counts < 2 log CFU/ml were detected only in the most severely treated samples. An abuse temperature storage halfed the shelf-life of the products, regardless the pressure applied. However, the treatment at 150 MPa assured a shelf-life of 14 and > 30 days for samples stored at 4 and 10°C, respectively (Tables 44 and 45).

In order to evaluate the effects of HPH treatments performed with the industrial-scale equipment on the quality of the foods, a panel test has been organised. The treated samples have been stored immediately after HPH processing at 4°C for 1 day and then served at the most suitable temperature, i.e. 60°C for the vegetable soup, 6 °C for the fruit juice mixture and milk-egg emulsions, to a group composed of 42 untrained panellist (Tables 46 and 47).
Each panellist had to assess four indicators, i.e the consistence/texture, the taste/flavour, the smell/flavour and the colour/appearance, by attributing a score ranging from 1 to 5. The lowest value corresponded to a negative evaluation, while the maximum one to an excellent product. The indicators chosen describe the main features encountered by consumers of self-service restaurants and canteens.
Significant differences were observed for the palatability which received higher rates for the all the products treated by HPH, particularly at the highest pressures (Figures 94 and 98; Table 48). No significant differences were observed for taste/flavour, smell/flavour and the colour of both vegetable soups and fruit juices (Figures 95-97; Figures 99-101). Concerning the milk-egg emulsions, the HPH treatment allowed the maintenance of the natural colour with a positive response by the panellists also for the taste (Table 48).

Table 42. Effects HPH treatments on cell counts of mesophiles, yeasts and lactic acid bacteria detected in vegetable soups or fruit juices in relation to the pressure level adopted (industrial-scale equipment).

Product	**Microbial group**	**Cell load (log CFU/ml)**				
		Pressure level (MPa)				
		0.1	**100**	**200**	**300**	**400**
Vegetable soup	Mesophylic aerobic bacteria	4.51	4.41	2.66	1.00	0.63
	Yeasts	2.23	-*	-	-	-
	Lactobacilli	4.30	3.95	2.00	-	-
	Lactococci	4.23	3.90	2.30	0.70	-
Fruit juices	Mesophylic aerobic bacteria	4.92	4.31	1.97	1.5	1.05
	Yeasts	1.80	-	-	-	-
	Lactobacilli	2.30	1.80	0.81	-	-
	Lactococci	2.05	1.30	0.69	-	-

*Not detectable in a 1-ml sample

Table 43. Effects HPH treatments on cell counts of mesophiles in milk-egg emulsions with different NaCl contents in relation to the pressure level adopted (industrial-scale equipment).

NaCl (%)	Cell load (log CFU/ml)			
	Pressure level (MPa)			
	0.1	**70**	**100**	**150**
0	4.43	2.3	2.47	<2
1	3.51	3.06	2.0	1.8
2	2.3	2.3	2.01	<2

Table 44. Data relative to the shelf-live values of vegetable soups, fruit juices and milk-egg emulsions subjected to HPH treatments (industrial-scale equipment) at different pressure levels and stored at 4°C.

Product	Pressure level (MPa)	Shelf-life (days)
Vegetable soup	0.1	7
	100	9
	200	15
	300	>30
	400	>30
Fruit juices	0.1	30
	100	>30
	200	>30
	300	>30
	400	>30
Milk-egg emulsion	0.1	6
	70	12
	100	13
	150	>30

Table 45- Data relative to the shelf-live values of vegetable soups, fruit juices and milk-egg emulsions subjected to HPH treatments (industrial-scale equipment) at different pressure levels and stored at 10°C.

Product	Pressure level (MPa)	Shelf-life (days)
Vegetable soup	0.1	5
	100	6
	200	9
	300	15
	400	24
Fruit juices	0.1	10
	100	11
	200	14
	300	>30
	400	>30
Milk-egg emulsion	0.1	3
	70	5
	100	6
	150	14

Table 46 – Age of the 42 panelist involved in the panel test on complex foods subjected to HPH treatments.

20-30 years old	30-40 years old	40-50 years old
52%	43%	5%

Table 47 – Composition of the group of panellists involved in the panel test on complex foods subjected to HPH treatments.

female	male
52%	48%

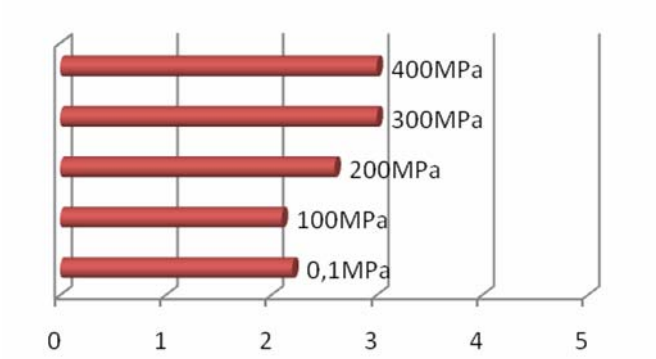


Figure 94 - Consistence/texture values obtained from a panel test on vegetable soups subjected to HPH treatments at different pressure levels.

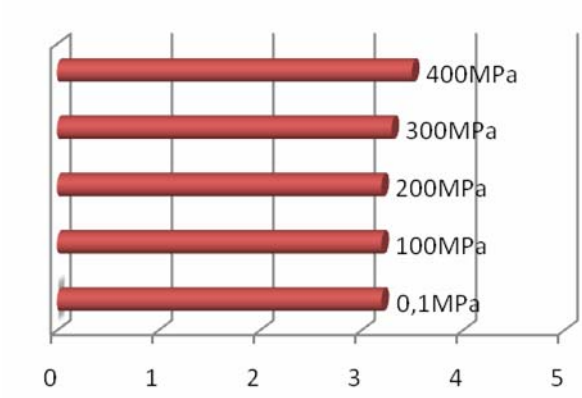


Figure 95 - Taste/flavour values obtained from a panel test on vegetable soups subjected to HPH treatments at different pressure levels.

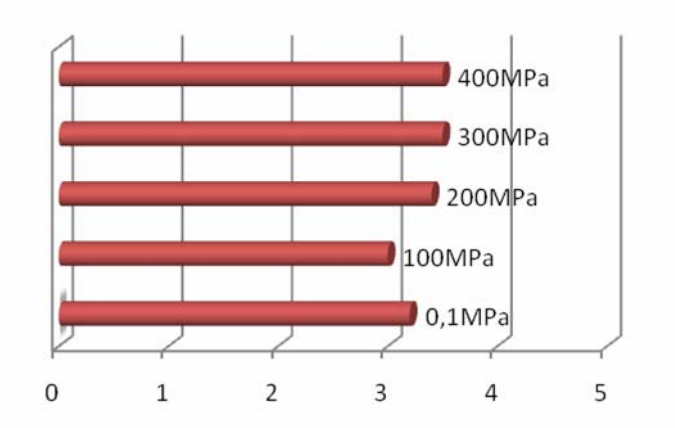


Figure 96 - Smell/flavour values obtained from a panel test on vegetable soups subjected to HPH treatments at different pressure levels.

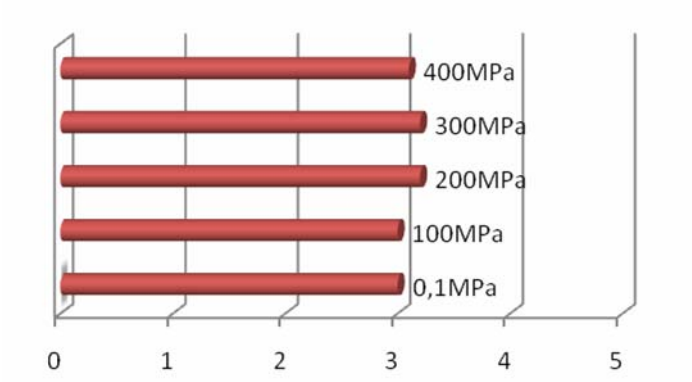


Figure 97 - Appearance/colour values obtained from a panel test on vegetable soups subjected to HPH treatments at different pressure levels.

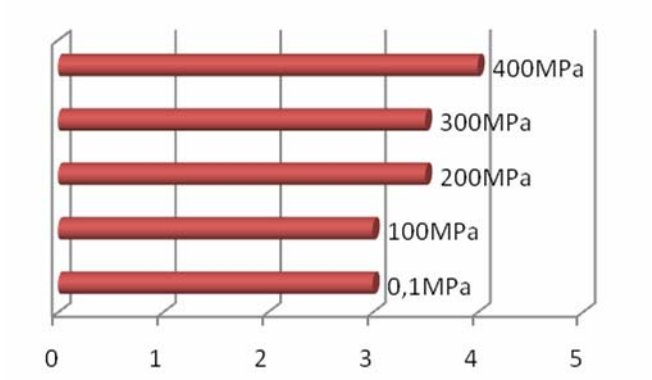


Figure 98 - Consistence/texture values obtained from a panel test on fruit juices subjected to HPH treatments at different pressure levels.

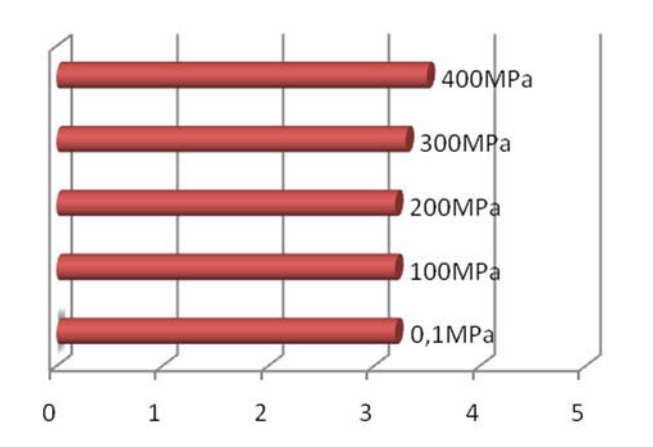


Figure 99 - Taste/flavour values obtained from a panel test on fruit juices subjected to HPH treatments at different pressure levels.

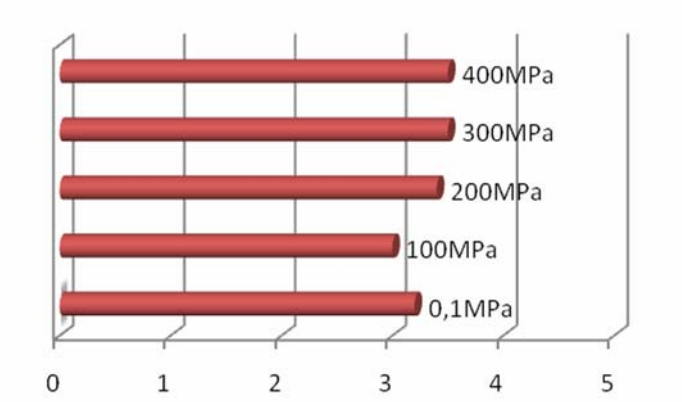


Figure 100 - Smell/flavour values obtained from a panel test on fruit juices subjected to HPH treatments at different pressure levels.

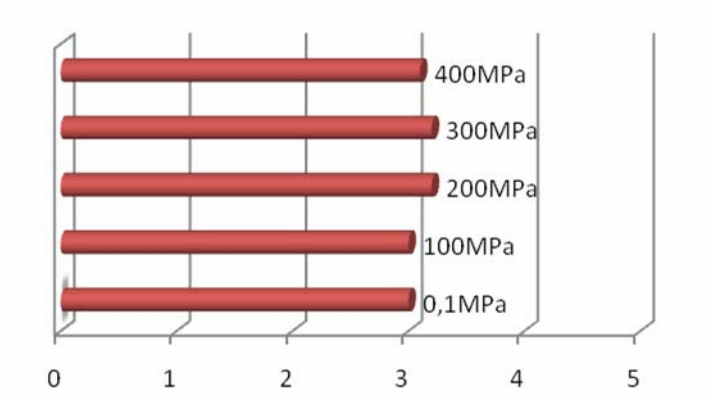


Figure 101 - Appearance/colour values obtained from a panel test on fruit juices subjected to HPH treatments at different pressure levels.

Table 48- Score attributed by the panelist to the various parameters in relation to the food system and the treatment performed.

Food	Treatment	Consistence /texture	Taste/ flavour	Appearance/ colour	Smell/ flavour
Vegetable soup	**Heat: 70°C; 3 min**	3.0 ±0.2	4.0±0.3	3.8±0.2	3.5±0.5
	HPH: 350 MPa	4.2±0.3	4.3±0.1	4.5±0.3	3.4±0.1
Fruit juice	**Heat: 70°C; 1.2 min**	3.2±0.5	4.3±0.4	3.5±0.3	3.8±0.5
	HPH: 200 MPa	4.3±0.4	4.5±0.2	4.1±0.1	3.5±0.3
Milk-egg emulsions	**Heat: 70°C; 3 min**	2.9±0.3	4.5±0.3	4.0±0.3	3.1±0.2
	HPH: 150 MPa	4.5±0.4	4.2±0.5	4.3±0.5	3.3±0.4

Conclusions of HPH demonstration task

In general the results obtained demonstrated that HPH treatments can be used as a safe alternative technique to thermal treatments. In fact cell reductions up to 2-3 log CFU/ml have been achieved for different spoilage microorganisms immediately after HPH treatments at 200 MPa. In particular, this technology resulted to be very appropriate for the decontamination and shelf-life extension of fruit juices and vegetable soups. Moreover, the sensory analysis performed on the HPH-treated samples of both vegetable soups and fruit juices evidenced that the consumers prefer the products subjected to HPH treatments with respect to the heat-treated ones.

Concerning the milk-egg emulsions, the HPH treatments gave rise to a satisfying inactivation level (abot 2 log units) of the target microorganisms also with a pressure level not exceeding 150 MPa; however, HPH treatments can not be performed at higher pressure values due to protein coalescence phenomena that affect the emulsion stability and generates phase separation with a consequent lower microbial stability and unaceptable quality parameters. Data relative to the panel test evidenced that HPH treatments at a pressure < 150 MPa give rise to products that maintain their original colour, while having an improved palatability.

HHPCO demonstration task (CSIC, ORT)

1. Description

A ***"technology open testing day"*** in which SME´s assayed their experiments with the objective to investigate if this new technology could be useful for them was organized and supervised by CSIC. In particular, the demonstration task was conduced on RTE dishes produced by ORT, using an industrial high-pressure equipment located in NC-Hyperbaric (Burgos).

2. Activities carried out

2.1. ***Open testing day agenda.*** On 29 May, an open testing day with the next agenda was organized:

- Welcome to NC-Hyperbaric facilities (Carolle Tonello, responsible of R&D).
- Introduction to HPP new applications and products (Francisco Purroy, NC-Hyperbaric).

- Brief introduction to HighQ-RTE project activities (M. Pilar Cano, Instituto del Frío-CSIC).
- Visit to NC-Hyperbaric facilities.
- Assays with industrial HPP equipments. HPPCO treatment in real RTD vegetable foods.
- Discussion of the validation assays.
- Sensorial analysis of HPPCO products.
- Discussion about interests of the SME´s for HPP.
- Final remarks.
- Lunch in Burgos.

2.2. ***Companies that attended the open testing day.***

- INDUSTRIAS ALIMENTARIAS DE NAVARRA (IAN) (Spain):
  Mr. Joaquín Aramendía and Mr. Francisco Javier Salcedo
- INSTITUTO TECNOLÓGICO DE EXTREMADURA (INTAEX)(Spain):
  Mr. Rosario Ramírez and Mr. David González
- FRUTIBON, Bonnysa, Alicante (Spain):
  Mr. Rafael Alberola
- AVOMIX, Málaga (Spain):
  Mr. Luis Bravo
- NC-HYPERBARIC, Burgos (Spain):
  Mr. Francisco Purroy, Mrs. M Jesús Alonso and Mr. Andrés Hernández
- INSTITUTO DEL FRÍO-CSIC
  Dr. M. Pilar Cano, Dr. Begoña de Ancos, Dr. Concepción Sánchez-Moreno and Dr. Lucía Plaza
- Dario Srl, Italy (Mr. Andrea Dario), Dr. Lucía Vannini (UNIBO) and Mrs. Paola Massari (ORT). At the last minute, they could not attend.

2.3. ***Collaboration between CSIC and ORT.*** In May 2009, ORT sent to the Instituto del Frío (IF) different vegetable products (selected for the demonstration study) under refrigerated conditions packaged in trays of 500 g.

These vegetable products were:

- Pasteurized (86-89 ºC/48-54 min) and packaged under MAP, mix of yellow and red bell peppers, grilled and spicy (ORT commercial product).
- Pasteurized (86-89 ºC/48-54 min) and packaged under MAP, mix of vegetables (zucchini, eggplant and red bell pepper), grilled and spicy (ORT commercial product).
- No pasteurized, mix of yellow and red bell peppers, grilled and spicy.
- No pasteurized, mix of vegetables (zucchini, eggplant and red bell pepper), grilled and spicy.

No pasteurized ORT products were prepared for HHPCO treatment in the IF. They were placed in small cups (100 g) and, then, cups were thermosealed under 95 % CO_2 atmosphere. HP treatments were carried out in an industrial equipment (Wave 6000/55) located in NC-Hyperbaric (Burgos). The HP treatment assayed was: 600 MPa/25 ºC/2 min (industrial treatment).

2.4. ***Preparation of IF-CSIC products.*** Red bell peppers were grilled in the pilot plant of the Instituto del Frío (180 ºC/45 min). They were placed in small cups (100 g) and, then, cups were thermosealed under 95 % CO_2 or air atmospheres. HP treatments were carried out in an industrial equipment (Wave 6000/55) located in NC-Hyperbaric (Burgos). The HP treatment assayed was: 600 MPa/25 ºC/2 min (industrial treatment).

Conclusions of HHPCO demonstration task

a.- *Effects of HHPCO treatment on physicochemical parameters (ORT and IF-CSIC products)*: Grilled peppers treated by HHPCO (IF-CSIC product) showed higher pH than HP-air samples, whereas there were no significant differences for acidity. Also, the mix of grilled vegetables treated by HHPCO (ORT product) showed higher pH than the commercial one, corresponding to a lower acidity. Total and soluble solids of grilled peppers (IF-CSIC and ORT products) were higher for HHPCO treated samples.

b.- *Effects of HHPCO treatment on sensory quality (ORT and IF-CSIC products)*: Sensory analysis showed that sensory quality (colour, texture, flavour and general acceptability) for ORT products was only acceptable for grilled peppers. HHPCO treated (600 MPa/25 ºC/2 min) grilled peppers obtained higher score than the commercial ones. With regard to IF-CSIC products, red bell peppers grilled in the IF (without additives) were better scored than ORT products. No significant differences were found between HHPCO and HP-air samples.

The SMEs invited to the "Technology Open testing day" were pleasantly surprised by the new technology, the combination of high pressure, temperature and packaging under 95%CO2. The results of sensory analysis confirmed that these HHPCO products have a flavour similar to that of "freshly" grilled product, significantly better than pasteurized commercial products that require the addition of additives such as citric acid, that disturb its natural sensorial characteristics, to prolong its shelf-life. Moreover, the HHPCO products have a shelf-life up to 105 days at 4ºC according with validation results.

Section 2 - Dissemination and Use

The results obtained in this project provided new scientific and technological knowledge on the advantages and disadvantages of different mild non-thermal technologies. Some of the results obtained have already been published in scientific refereed journals and can be downloaded also from the web-site (http://www.highqrte.eu). After the end of the project no future dissemination activities are foreseen by the Consortium. However, it is likely that some scientific papers, which are currently under evaluation, will be published on international journals. Also additional oral or poster presentations will be given during 2010-2011 at national or international conferences.

However, most of the results obtained are new and can be successfully be exploited in various industrial sectors. Therefore they are not available for public use because of commercial confidentiality reasons. Concerning PEF, HPH and HHP technologies, which have already been investigated in other projects and industrial equipments are available, the studies carried out in the HighQ RTE project by integrating microbiological, technical, nutritional, microstructural and sensory aspects resulted in many cases in process or RTE product/ingredient innovations, which have been, or are going to be, protected by patents. Moreover, a novel design of treatment chamber, enabling an homogenous treatment in a continuous flow, has been developed for PEF.

In general, the IPR belongs to the relevant Partners that have developed/improved the technology or that were significantly involved in the research activities. Most of the work carried out, which can be exploited or lead to further research, has been described in the documents submitted to the EC.

Annex I to *High Hydrostatic pressure and CO_2 Technology*

MATERIAL AND METHODS

A.-Raw material

Raw red bell peppers and carrot were purchased from Mercamadrid (Central Market of Fruits and Vegetables) from Madrid, Spain. The variety, stage of maturity and origin of the raw products were controlled by the supplier. Raw carrot roots (*Daucus carota*, var. Nantesa) and red bell pepper (*Capsicum annumm* L., cv. Lamuyo) were minimally processed: washed, drained, hand peeled and sliced (1 cm slices or 3 cm strips) with mechanical cutter Sammic CA-300). Red Pepper (*Capsicum annumm* L., cv. Lamuyo) also was minimally processed: washed, drained, and grilling at 180 ºC for 45 min. The physicochemical characteristics of raw carrots, raw red bell pepper and grilled red bell peppers were shown in **Table 1, 2 and 3**.

Table 1. Physicochemical characteristics of raw carrots (cv. Nantesa)

Characteristic	Value
Titratable acidity (g citric acid/100 g fw)	0.084 ± .005
pH	5.95 ± 0.09
Soluble solids ºBrix at 20 °C	9.76 ± 0.53
Total solids g/100 g fw	10.45 ± 0.21

Values are the mean of three independent determinations±standard deviations.

Table 2. Physicochemical characteristics of raw red peppers (cv. Lamuyo)

Characteristic	Value
Titratable acidity (g citric acid/100 g fw)	0.204 ± .018
pH	4.64 ± 0.01
Soluble solids ºBrix at 20 °C	7.12 ± 0.15
Total solids g/100 g fw	7.40 ± 0.05

Values are the mean of three independent determinations±standard deviations.

Table 3. Physicochemical characteristics of grilled red bell peppers (cv. Lamuyo)

Characteristic	Value
Titratable acidity (g citric acid/100 g fw)	0.301 ± 0.01
pH	4.76 ± 0.01
Soluble solids ºBrix at 20 °C	13,15±0,47
Total solids g/100 g fw	11,40±0,13

Values are the mean of three independent determinations±standard deviations.

B.-High-pressure Treatment:

High-pressure processing (HPP) were carried out in a high-pressure unit (Gec Alsthom ACB 900 HP, type ACIP 665, Nantes, France) using a hydrostatic pump and a fluid of low compressibility (water) sealed in a vessel (steel container 100 mm in diameter, 300 mm in height and 2.35 L in volume). Pressure was increased and released at 1.6 MPa/s. The maximum working pressure in this equipment is 400 MPa. Time of the treatments was the holding pressure time. The pressure chamber was heated to a desired level by means of a thermostat jacket connected to a water bath. Because of adiabatic compression, the temperature of treatment (temperature of treatment during holding time) was the maximum temperature at the pressure of work set. Pressure, time and temperature were controlled by a computer program, being constantly monitored and recorded during the process. After the instantaneous decompression, the samples were storage without light at 4 ºC and analyzed two hours after treatment.

Graphic 1: Graphic of treatment parameter (pressure, temperature, time of treatment) of combined process High Pressure / Temperature recorded in the Instituto del Frío HP equipment during all the phases of the process.

C.-Temperature of treatment:

Temperature of treatment was defined as the maximum temperature achieved when the system reach the pressure of treatment selected. The temperature and the pressure were recorded during all the phases of the treatment (initial, when the pressure is going up, when the pressure is holding during one minute or more and after depressurization) (**Graphic 1**).

The lowest temperature that could be combined with pressure (300-400 MPa) in the HHP equipment located at the Instituto del Frío (IF-CSIC) was 10 ºC due to mechanical problems.

Because of adiabatic compression, the temperature of treatment (temperature of treatment during holding time) was the maximum temperature at the pressure of work set and was controlled by a computer program, being constantly monitored and recorded during the process. In our equipment every 100 MPa of increasing pressure increases 4 ºC the starting temperature.

For example, a combined treatment Pressure/Temperature at 400 MPa/ 45 ºC means that we begin with a starting temperature at 33-34 ºC to reach 45 ºC at 400 MPa. This temperature is maintained during the pressure holding time (time of treatment). The temperature returns to its initial value when the pressure is released. **Table 4** shows the data of starting temperature, temperature during pressure holding and after treatments at 300 and 400 MPa:

Table 4. Starting temperature, temperature during pressure holding time and after treatment at 300 and 400 MPa:

Pressure(MPa)/ Temperature (ºC)	**Starting Temperature (ºC)**	**Temperture during HT (ºC)**	**Temperture After Treatment (ºC)**
300 / 60	48	60	48
300 / 45	33	45	33
400 /60	44	60	44
400 /45	29	45	29

HT= Holding Pressure Time or Time of Treatment (3 min, 1 min, etc..)
Temperature during Holding Time = Temperature of HP treatment

E.- Microbiological Analysis

Protocols for the analysis of total mesophilic bacteria, yeast and moulds and psycrophilic bacteria counts in pre-cut vegetables

a) Total Aerobic Mesophilic Bacteria (TAMB) count by plate method (Plate Count Agar-PCA). Detection limit is <10 CFU/g in solid foods. Plates were incubated at 30 ºC± 1ºC during 72 ± 3 hours (ISO 4833).
b) Yeast and Moulds count by plate method (YM)(Chloranphenicol Glucose Agar). Detection limit is <10 CFU/g in solid foods. Plates were incubated at 25 ºC during 3-5 days.

Psychrophilic Bacteria counts (PB) by plate method (Plate Count Agar-PCA). Plates were incubated at 6-6,5 ºC during 10 days.

Experimental Design and Protocol for the inoculation and count of Listeria innocua in raw carrot, and raw and grilled red bell pepper

a. Experimental Design. In order to study the effect of the combination of high pressure, temperature and time of treatment on the behaviour of *Listeria innocua* inoculated in pre-cut vegetable (carrot and red-bell pepper) in different physiological

state (raw and grilled) and packaged under modified atmosphere (95% CO_2), air or vacuum, a **Response Surface Methodology Experimental Design (RSM)** has been employed**.** A RSM was used for analyzing the effect of independent variables (pressure, temperature and time of treatment) on a dependent variable or response: the inactivation of *Listeria innocua* artificially inoculated on real food systems. Based on the results, response surface methodology was employed to determine the log-cycles reduction of the pathogen inoculated in the products.

The main advantage of RSM is its ability to decrease the experimental runs required to provide sufficient information for statistically acceptable results. The experiment was carried out according to a Face Centred-Central Composite Design (CCD). The coded and uncoded values of three independent parameters and their corresponding levels for each food are presented in Table 5. The experimental design used for this study consisted of three levels for each of the three factors and two replications for each experimental condition. Furthermore, four error degrees of freedom for carrot slices and red pepper stripes experimental design were included.

Table 5. Variables and levels used in response surface methodology experimental design

Independent Variables	**Levels**		
	-1	0	1
Pressure (MPa)	100	250	400
Temperature (ºC)	25	42.5	60
Time of treatment (min)	1	3	5

NOTE: The statistical information corresponding to Response Surface Plots concerning *Listeria Innocua* inactivation and produced after analyzed the data obtained with this experimental designed is showed in the Annex II – Appendix to High Hydrostatic pressure and CO_2

b) Bacterial strain and preparation of inoculum

Listeria innocua 910 from Colección Española de Cultivos Tipo (CECT)]. The strain was cultivated in TSB (tryptic soy broth) at 37 ºC for 24 h to prepare a stock culture. The stock culture was refreshed at two successive 24-h intervals. A solution of 0,1% of the stock culture was prepared by adding 1 ml of the stock culture to 1 litre of TSB (OD= 0.04). After 16 hours of incubation at 37 ºC, this solution reached a OD = 0.97 (at its early stationary phase) (approximatly 10^9 CFU/ml) (Inoculum solution).

c)Procedure for inoculation

1.-Preparation of vegetables products

The raw products were washed with tap water, and drained during several minutes in a domestic drain. Then, the product were peeled (if it is necessary) and cut (slices, strips, etc) with sharp stainless steel knives. After that the cut products were placed in special basket full of holes.

2.-Inoculation

Approximatly 400 g of products placed in the special basket and put in a special bowl with 2 L (25g/100 ml) of the overnight TSB culture solution (10^9 CFU/ml) for 5 min with slight agitation. It is important that the product was totally covered by the TSB culture solution. After this 5 minutes, the basket were took out from the bath and the artificially contaminated vegetables pieces were placed over a sterile test tube rack and dried at 22±2ºC under a laminar flow hood for 15 min.

d) Packaging and Processing

The artificially contaminated product were placed in Doypack bags (25 g), packaged under MAP (95% C02) or vacuum packaging and then introduced in the High Pressure vessel for high-pressure treatment. After each treatment, the products were placed at room temperature in the laminar flow hood for 1 hour before open the bags and begin with the microbial analysis.

e) Microbial analysis

Microbial analysis of untreated products was used as initial values for calculations of logarithmic reductions in microbial counts.

Ten-fold serial dilutions

10 g of samples were placed in a stomacher bags with 90 ml of buffered peptone water (BPW) (10^{-1} dilution) and homogenized in a stomacher for 1 minute (normal speed). After 1 hour at room temperature, ten-fold dilutions for each sample were prepared in buffered peptone (Example: 1 ml of the 10^{-1} dilution and 9 ml of buffered peptone water produce the 10^{-2} dilution; 1 ml of this dilution (-2) and 9 ml of buffered peptone water produce the 10^{-3} dilution, etc.

Counts of viable cells

Viable cells of *Listeria innocua* were determined by the pour plate technique in duplicate on PALCAM. 0.1 ml of each dilution was pour plated and scattered by a rake over the entire plate surface. After 15 min the plates were incubated at 37 ºC during 48±2 hours (inverse position).

The enumeration was made only in plates with less than 150 colonies. *L. innocua* colonies are very characteristics: they are grey with a black point in the middle and a black halo around them.

The reduction of *L. innocua* was evaluated as the difference in log CFU per gram between the colony of untreated and treated samples.

The sublethal injuries of cells of *L. innocua* exposed to high pressure combined treatments in red bell pepper were assessed by the difference between the counts on the nonselective agar medium (PCA) and the selective medium (Palcam).

Experimental Desig for the Inoculation of Listeria monocytogenes 56Y, Salmonella enteritidis 155 and Bacillus cereus SV90 in grilled red bell pepper

More data is needed to model and to obtain inactivation kinetics of target pathogens inoculated in real food. Experimental Design to complete the information has been shown in **Table 6**. One of the outputs of the project is a Quantitative Microbiological Risk Assessment (QMRA) to optimise food formulation with regards of food safety and food quality. As QMRA requires a considerable amount of work, **Table 6** shows the different combinations of pressure, temperature and time of treatment designed to study the inactivation of *Listeria monocytogenes*, *Salmonella typhimurium* and *Bacillus cereus* inoculated on grilled red pepper to be treated by high pressure with supercritical carbon dioxide (packaging under 95%CO2). Experimental design was provided by "Quantitatie tools and methods" (**Table 6**).

Table 6. Experimental design based in the combination of pressure, temperature and time of treatment for grilled red bell pepper packaging under 95% CO2

Pressure	Sampling time (min)			
	T=10˚C	T=25˚C	T=35°C	T=45˚C
250	1,3,5	1,3,5	1,3,5	1,3,5
400	---	0.5,1,2	0.5,1,2	0.5,1,2
600	---	0.5,1	---	---

Protocol for the inoculation and enumeration of Listeria monocytogenes 56Y, Salmonella enteritidis 155 and Bacillus cereus SV90 in grilled red bell pepper

Bacterial strain and preparation of inoculum

Strains of *Listeria monocytogenes 56Y, Salmonella enteritidis 155 and Bacillus cereus SV90* were obtained from the food microbiology culture collection of the University of Bologna (UNIBO). Upon arrival at Instituto del Frío (CSIC) strains were stored at -80 ºC in Brain Heart Infusion (BHI) Broth supplemented with 25% glycerol. Prior to experiments strains were cultivated overnight in BHI at 37 ± 1ºC (*B. cereus* 30 ºC) to prepare a stock culture (10^8-10^9 CFU/ml for *Listeria monocytogenes* and *Salmonella enteritidis* and 10^5-10^6 *Bacillus cereus*) Appropriate dilutions of the stock culture were prepared (50 ml in 600 ml in peptone water for *Listeria monocytogenes* and *Salmonella enteritidis* and 100 ml in 600 ml in peptone water for *Bacillus cereus*) to obtain a culture solution to inoculate the product and produce 10^6 -10^7 CFU/g for *Salmonella enteritidis* and *Listeria monocytogenes* and 10^5 -10^6 CFU/g for *Bacillus cereus*.

Procedure for inoculation

Approximatly 25 g of cut red bell pepper in small pieces were placed in cups with 40 ml of the grown and diluted BHI broth for 10 min It is important that the product was totally covered by the solution. After these 10 minutes, the artificially contaminated vegetables pieces were drained at 22±2ºC in a BioSafety Cabinet for 15 min.

Packaging and Processing

The artificially contaminated product weas placed in plastic cups (100 g) and thermoselled with 95% CO2. Then the selled cups were introduced in the high Pressure vessel for high-pressure treatment. After each treatment, the products were stored 24 hours at 4 ºC. After that the cups were opened in the BioSafety Cabinet and microbiological analysis was started.

Enumeration of viable cells

The number of suriving cells was determined after a proper dilution of the untreated and the treated products. Portions of 0.1 ml of selected dilutions were spread plated on BHI agar plates. The enumeration was made only in plates with less than 250 colonies.

Microbial analysis of untreated products was used as initial values for calculations of logarithmic reductions in microbial counts. Initially samples of heat treated uninoculated red bell pepper were analyzed in order to assess the effect of the heat treatment and to assure absence of interference of endogenous microorganisms in the experiments.

Counts of viable cells

Listeria monocytogenes were determined by duplicate spread plating on Brain Heart Infusion (BHI) agar. The plates were incubated at 37 ºC during 48±2 hours.

Salmonella enteritidis were determined by duplicate spread plating on Brain Heart Infusion (BHI) agar. The plates were incubated at 37 ºC during 48±2 hours.

Bacillus cereus were determined by duplicate spread plating on Brain Heart Infusion (BHI) agar. The plates were incubated at 30 ºC during 24±2 hours.

F. Enzimatic Analysis

Protocol for the determination of POD and PPO activities in pre-cut vegetables

Enzyme extraction

The same enzyme extraction procedure was used for POD and PPO activities determinations. Enzyme extracts were prepared as follows: 10 g of sample was homogenised with 25 ml of sodium phosphate buffer (pH 7.5 and 0.2 M for carrots; pH 7.5 and 0.05 M for eggplants; pH 7 and 0.1 M for red bell peppers; pH 6.5 and 0.1 M for zucchinis). The mixture was centrifuged at 15,000g for 15 min at 4 ºC and filtered through six cheesecloth layers.

PPO activity assay

The PPO activity was assayed using an aliquot (0.1 ml) of extract and 2.9 ml of a 0.11 M catechol solution in sodium phosphate buffer (pH 6.5 and 0.1 M for carrots; pH 6.5 and 0.025 M for eggplants; pH 6.5 and 0.05 M for red bell peppers; pH 6.5 and 0.05 M for zucchinis). The reaction was measured with a double beam spectrophotometer (Perkin Elmer, model Lambda 15, Bodenseewerk, Germany) at 420 nm and 25 ºC.

POD activity assay

The POD activity was determined spectrophotometrically at 485 nm. A reaction mixture consisting of 2.65 ml of 0.05 M sodium phosphate buffer (pH 6.5 and 0.1 M for carrots; pH 6.5 and 0.025 M for eggplants; pH 6.5 and 0.05 M for red bell peppers; pH 6.5 and 0.05 M for zucchinis), 0.1 ml hydrogen peroxide (1.5%, v/v) as oxidant, 0.2 ml of 1,4-phenylenediamine (1%, w/v) as H-donor and 0.05 ml extract was used. The oxidation of p-phenylenediamine was measured.

PPO and POD activities were determined by measuring the slope of reaction and the enzyme activity unit was defined as the change in absorbance per minute per gram of fresh weight of sample or as relative activity (%).

Protocol for the determination of PME activity in pre-cut vegetables

Enzyme extraction

The enzyme was extracted by homogenisation (4 min at 10,000g) of 10 g of sample with 20 ml of an extraction solution (1 M NaCl in 0.2 M sodium phosphate buffer pH 7.5; 0.01 M dithiothreitol for carrot and 1 % PVPP for eggplant). The resulting mixture was shaken for one hour at 4 _C, centrifuged at 16,000g for 30 min at 4 ºC, the supernatant filtered through six cheesecloth layers and then assayed for PME activity.

PME activity assay

PME activity was measured titrimetrically at 25 ºC. Enzyme extract (0.5 ml for eggplant and 1 ml for carrot) was mixed with 40 ml of a pectin-salt substrate solution (0.1 or 0.5 % pectin for eggplant and carrot, respectively, and 0.1 M NaCl). The solution was adjusted to pH 7 with 1 N sodium hydroxide, and then the pH of the solution was

readjusted to 7.5 with sodium hydroxide 0.05 N. After the pH reached 7.5, 0.10 ml of 0.05 N sodium hydroxide was added. The time until the pH of the solution regained pH 7.5 was measured. The PME activity unit (PEU) was defined as the amount of enzyme necessary to release 1 mmol galacturonic acid/min/g fresh weight.

G.- Nutritional Analysis

Protocol for the determination of VITAMIN E and β-CAROTENE in raw and grilled red bell peppers (UNIBO – A. Bordoni)

Ten grams of each sample were extracted by cold saponification. The saponification solution was composed of 5 ml KOH 50%, 100 mL ethanol and 200 mg of antioxidant mixture (butylated hydroxytoluene – BHT-, EDTA, vitamin C). Samples were shaken for 15 min, and incubated in the dark overnight. Samples were then extracted twice with n-hexane, the extraction solution combined, filtered and evaporated under vacuum using a Rotavapor. Then, 10 mL of n-hexane was used to dissolve the extracted, and transferred into a HPLC vial. Vitamin E and β-carotene content was determined by direct phase NP-HPLC.
The HPLC system consisted of Helwett Pakard- serie1100Agilent, UV detector, binPump, and column thermostat and rheodyne injector. Samples were injected into a 250 × 4.6 mm diameter of 5-μm Luna Si-60 - Phenomenex column. The mobile phase consisted of n-hexane/isopropanol (99.2/0.8). It was in isocratic at a flow rate of 1.5 mL/min for vitamin E and 0.8 ml/min for β-carotene. The UV detector was set at 290 nm and 450 nm for vitamin E and β-carotene detection, respectively.
Each extraction method was replicated three times. Vitamin E and β carotene content was calculated by comparison of the respective peak areas to standards. Chromatograms were recorded and processed using HP Chemstation software.

Protocol for the determination of ANTIOXIDANT ACTIVITY (AA) in raw and grilled red bell peppers (UNIBO – A. Bordoni)

Red bell peppers were homogenized in a food processor without addition of any solvent, and 10 g of the homogenized material were dissolved in 100 mL of 5 mM phosphate buffered saline, pH 7.4 (PBS), getting a dilution 1:10 (w/v). The samples were then filtered and the resulting filtrate was used to determine the antioxidant activity (AA). All phases of homogenization were done in ice and each extract immediately analyze.
AA was measured using the method of Re and others (1999) on the basis of the ability of the antioxidant molecules in the sample to reduce the radical cation of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), determined by the decolorization of ABTS+·, and measured as the quenching of the absorbance at 734 nm. Values obtained for each sample were compared to the concentration–response curve of the standard trolox solution and expressed as micromoles of Trolox equivalent (TE).

Protocol for the determination of VITAMIN C content in raw and grilled red bell peppers (CSIC)

Extraction
10 g of sample was homogenised triplicate in an ultrahomogeniser (Omni mixer, model ES-270, Omni International Inc., Gainesville, VA, USA) with 40 mL of an extraction solution (3% meta-phosphoric acid + 8% acetic acid). The resulting mixture was centrifuged at 10,000 g for 15 min at 4 ºC and adjusted up to 100 mL with distilled water. Samples were filtered through a 0.45-μm membrane filter and duplicates of 20 μL for each extract were analysed by HPLC. Results were expressed as mg ascorbic acid per 100 mg fresh weight.

HPLC procedure
Vitamin C was determined by high performance liquid chromatography (HPLC). A Hewlett-Packard Model 1100 quaternary solvent delivery controller pump was used for analyses. Samples were introduced onto the column via a manual injector (Rheodyne) equipped with a sample loop (20 μL). Separation of ascorbic acid was performed by HPLC using a reversed phase Mediterranean Sea C18 (5 μm) stainless steel column (250×4.6 mm i.d.) (Technochroma, Spain). The solvent system used was an isocratic gradient of a solution 0.01% H_2SO_4, adjusted to pH 2.5–2.6. The flow rate was fixed at 1 ml/min. The column is thermostatized at 25 °C. A Hewlett-Packard 1040A UV–vis photodiode array detector was set at 245 nm; chromatographic data and UV–vis spectra were collected, stored and integrated using a Hewlett-Packard Chem Station and related software. Identification of the ascorbic acid was carried out by HPLC by comparing the retention time and UV–vis absorption spectrum with those of the standard previously referred to.

Protocol for the determination of TOTAL CAROTENOIDS in raw and grilled red bell peppers (CSIC)

10 g of sample was used to measure the total carotenoids. First the samples were saponified to remove the chlorophylls. Subsequently, the carotenoids were extracted according to Rodríguez-Amaya (1999).
The total carotenoids content was measured spectrophotometrically at 450 nm using the extinction coefficient of 2500 and results were expressed as β-carotene equivalents (μg per 100 g fresh weight).

Protocol for the determination of ANTIOXIDANT ACTIVITY in raw and grilled red bell peppers (CSIC)

Extraction
Enzyme extracts were prepared as follows: 10 g of sample was homogenised with 20 ml of methanol/water (50:50, v/v) for 1 minute in triplicate in an ultrahomogeniser (Omni mixer, model ES-270, Omni International Inc., Gainesville, VA, USA). After 1 hour of shaking at 4 ºC, the mixture was centrifuged at 10,000 g for 15 min at 4 ºC and vacuum filtered. Extracts were made up to 50 mL with distilled water. Triplicates of 10 μL for each extract were measured by three different antioxidant capacity methods.

Ferric reducing antioxidant power (FRAP) assay
The total antioxidant potential of a sample was also determined using the ferric reducing antioxidant power (FRAP) assay by Benzie & Strain (1996). The method is based on

reducing power of an antioxidant compound. A potential antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) at low pH; the latter forms a blue complex (Fe^{2+}/TPTZ), measured at 593 nm. The FRAP reagent was freshly prepared by mixing together 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in a proportion 10:1:1 (v/v/v), respectively. The assay was carried out in 96-well microplate by adding 10 µL of each extract and then adding 290 µL of the FRAP reagent. After 20 min of incubation in the dark at 37 ºC and shaking, absorbance was read at 593 nm. All samples were run in triplicate. Results were compared with a standard curve prepared daily with different concentrations of Trolox and were expressed as µM trolox equivalents (TE).

2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging assay

DPPH$^{\bullet}$ is a stable radical widely used to monitor the free radical scavenging abilities (the ability of a compound to donate an electron) of various antioxidants. The DPPH$^{\bullet}$ radical has a deep violet colour due to its impaired electron, and radical scavenging can be followed spectrophotometrically by the loss of absorbance at 515 nm, as the pale yellow nonradical form is produced. To optimize the conditions used to run the DPPH$^{\bullet}$ assay in microplates we modified the DPPH$^{\bullet}$ concentration and the time of reaction. As a result, we finally set the DPPH$^{\bullet}$ concentration at 100 µM and the time of reaction at 1 h. Briefly, in a 96-well microplate, it was added 10 µL of each extract and then it was added 290 µL of 100 µM DPPH$^{\bullet}$ in methanol, mixed well, and after one hour of incubation in the dark, absorbance was measured at 515 nm in a microplate reader. All samples were run in triplicate. Results were compared with a standard curve prepared daily with different concentrations of Trolox and were expressed as µM trolox equivalents (TE).

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^{\bullet+}$) radical cation scavenging assay

The ABTS$^{\bullet+}$ assay is a decolorization assay applicable to both lipophilic and hydrophilic antioxidants. The pre-formed radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence hydrogen-donating antioxidants. Briefly, ABTS radical cation (ABTS$^{\bullet+}$) was produced by reacting ABTS with 2.45 mM potassium persulphate ($K_2S_2O_8$) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS$^{\bullet+}$ solution (two days stable) was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Then, in a 96-well microplate, it was added 10 µL of each extract and then it was added 290 µL of 7 mM ABTS$^{\bullet+}$, mixed well, and after 20 minutes of incubation in the dark at 30 ºC, absorbance was measured at 734 nm in a microplate reader. All samples were run in triplicate. Results were compared with a standard curve prepared daily with different concentrations of Trolox and were expressed as µM trolox equivalents (TE).

H. Protocols for Validation Task

Enumeration of endogenous microorganisms in pasteurized (commercial) and HHPCO treated products

The number of surviving cells was determined after a proper dilution of the untreated and the treated products. Portions of 0.1 ml of selected dilutions were spread plated on

nutrient agar plates (Brain Heart Infusion Agar). The enumeration was made only in plates with less than 250 colonies.
Microbial analysis of untreated products was used as initial values for calculations of logarithmic reductions in microbial counts.

1. Total Aerobic Mesophilic Bacteria count by pour plate method (Plate Count Agar-PCA). Detection limit is <10 CFU/g in solid foods. Plates were incubated at 30 ºC± 1ºC during 72 ± 3 hours (ISO 4833:2003)
2. Yeast and Moulds count by spread plate method (Chloranphenicol Glucose Agar). Detection limit is <100 CFU/g in solid foods. Plates were incubated at 25 ºC during 3-5 days.
3. Psychrophilic Bacteria counts by plate method (Plate Count Agar-PCA). Plates were incubated at 6-6,5 ºC during 10 days. (ISO 6730:2005.)
4. Total coliforms counts by pour plating two layer method (Violet Red Bile Agar with lactose-VRBL). Plates were incubate at 30 ºC± 1ºC during 24± 1 hours (ISO 4832:2006)
5. *Staphylococcus aureus* counts by plate method (Baird Parker Agar). Plates wer incubated at 37ºC± 1ºC during 48± 3 hours (ISO 6888-1:1999)

Presence/Absence of Listeria monocytogenes in 25 g of product (ISO 11295-1:1996)
25 g of product was suspended in 225 ml of half-concentrated Fraser Broth and incubated at 30ºC during 24 ± 3 hours. 0.1 ml of this solution was inoculated in 10 ml Fraser broth and incubated at 37 ºC during 48±3 hours. From this solution, ALOA and Oxford agar plates were inoculated and incubated at 37 ºC during 48±3 hours.

Presence/Absence of Salmonella spp in 25 g of product (ISO 6579:2002)
25 g of product was suspended in 225 ml buffered Peptone Water (BPW) and incubated at 37º ± 1C during 18 ± 2 hours. 0,1 ml of this BPW solution was inoculated in 10 ml of RVS broth and incubated at 41,5 ºC during 24±3 hours. 1 ml of the BPW solution was inoculated in 10 ml of MKTTn broth and incubated at 37 ºC during 24±3 hours. Both broths were inoculated on XLD and SM-ID2 plates.

Protocol for the determination of pH, titratable acidity and soluble solids
pH
10 g of sample was blended with 20ml deionized water. The mixture was heated to 100 ºC. 20 ml deionized water was added to this mixture, and the resulting mixture was cooled to 20 ºC. The pH was measured at this temperature with a Crison pH meter (Barcelona, Spain).
Titratable acidity
After determining pH, the solution was titrated with 0.1M NaOH to pH8.1, monitoring with an electrode pH meter (Barcelona, Spain). Results were expressed as grams of citric acid per kilogram fresh weight (fw).
Soluble solids
Soluble solids were measured using an Atago digital refractometer dbx-30 (Tokyo, Japan) at 20 ºC. Results are reported as ºBrix.

Annex II to High Hydrostatic pressure and CO_2 Technology
ANNEX II

QUESTIONNAIRE FOR SENSORY ANALYSIS OF GRILLED PEPPER

NAME……..... DATE.....

Please, consider samples for their quality as processed product. Do not communicate with other members of taster panel your interpretations while the analysis is carrying out.

Use numeric values according to the range shown in the first table. Put the value that you consider opportune for each codified sample in the corresponding box. If you have any observation, please, make a note of it.

Preliminary question:
Are you a regular consumer of grilled pepper?

YES NO

GRILLED PEPPER

MARKS

RANGE OF QUALITY	CLASSIFICATION	MARK
UNSATISFACTORY	VERY BAD	1-2
	BAD	3-4
LOW ACCEPTABLE	FAIR	5-6
ACCEPTABLE	GOOD	7-8
HIGH ACCEPTABLE	VERY GOOD	9-10

EVALUATION

CHARACTERISTIC		A	B
COLOUR	EVALUATION		
	OBSERVATIONS		
TEXTURE	EVALUATION		
	OBSERVATIONS		
FLAVOUR	EVALUATION		
	OBSERVATIONS		

NAME……..... DATE.....

Determinate the intensity of attributes of each sample marking with a cross (X) those that were in accordance with your perception.

GRILLED PEPPER

		A	B
COLOUR	**5. Normal**		
	4. Intense		
	3. Light		
	2. Brown		
	1. Dark Brown		
FLAVOUR	**5. Normal**		
	4. Slightly Sweet		
	3. Sweet		
	2. Acid		
	1. Strange		
TEXTURE	**5. Normal**		
	4. Fleshy		
	3. Firm		
	2. Soft		
	1. Very Soft		

OBSERVATIONS: (Strange smells, etc.)

NAME……..... DATE.....

Consider the samples according to their general quality. For this, employ the range of marks previously indicated.

GRILLED PEPPER

		A	B
GENERAL EVALUATION	**EVALUATION**		
	OBSERVATIONS		

Would you buy any of these products?

SI NO

In affirmative case, which one or which ones?

A B

Annex III to High Hydrostatic pressure and CO_2 Technology
PACKAGING SELECTION (LNE)

References, composition and dimensions

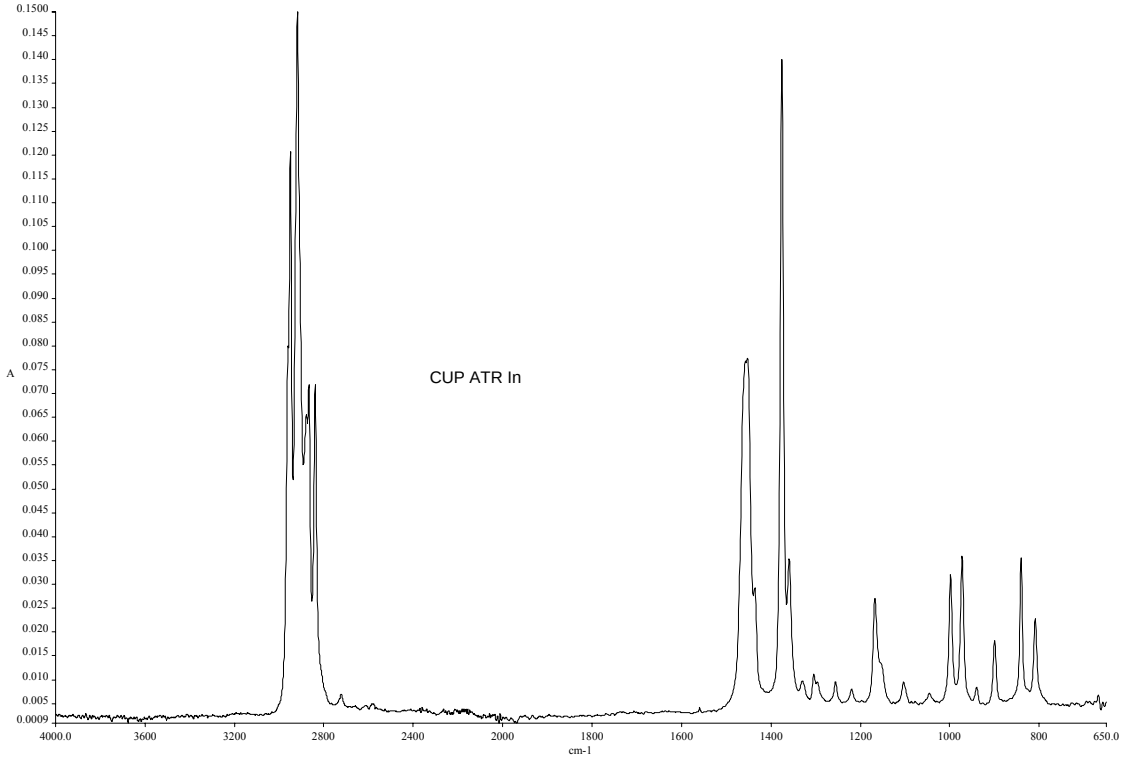
Reference	Supplier	Reference supplier	Type of packaging	Type of materials	Type of polymer and thickness	Dimension
Film AKNS	AMCOR Flexibles	SIDAMIL M Type AKNS	Film	Multilayer	o Oriented polyester film, metal oxide coated (12 µm) o Adhesive (Polyurethane based, ± 4 µm) o Polyamide film (20 µm) o Adhesive (Polyurethane based, ± 4 µm) o White polyethylene film with peelable blend seal layer (50 µm)	
Doypack AMCOR	AMCOR Flexibles	Polyskin XL	Doypack	Multilayer	o Polyester/Polyvinilidene chloride (13,3 µm) o Polyethylene (100 µm)	L : 200 mm W : 110 mm
FT Material EDV	EDV	X X684STDSL2 Standard 84MM 6IT H 510,0X1,4MM	Tray	Multilayer	o Polypropylene (145 µm) o Polypropylene + REC (495 µm) o Adhesive polymer (15 µm) o Ethylene vinyl alcohol (40 µm) o Adhesive polymer (15 µm) o Polypropylene + REC (495 µm) o Polypropylene (145 µm)	L : 153 mm W : 130 mm H : 31 mm
Cups EDV	EDV	E E692020001 EDV 84MM 4OZ Rigid barrier cups	Cup	Multilayer	o Polypropylene/Ethylene vinyl alcohol/Polypropylene (1200 µm)	Base diameter : 64 mm Top diameter : 91 mm H : 109 mm
Doypack BREGER	BREGER	Doypack Tramier vertes 400g FRANCAIS	Doypack	Multilayer	o Polyethylene terephthalate (12 µm) o Polyethylene (120 µm)	L: 495 mm W : 130 mm

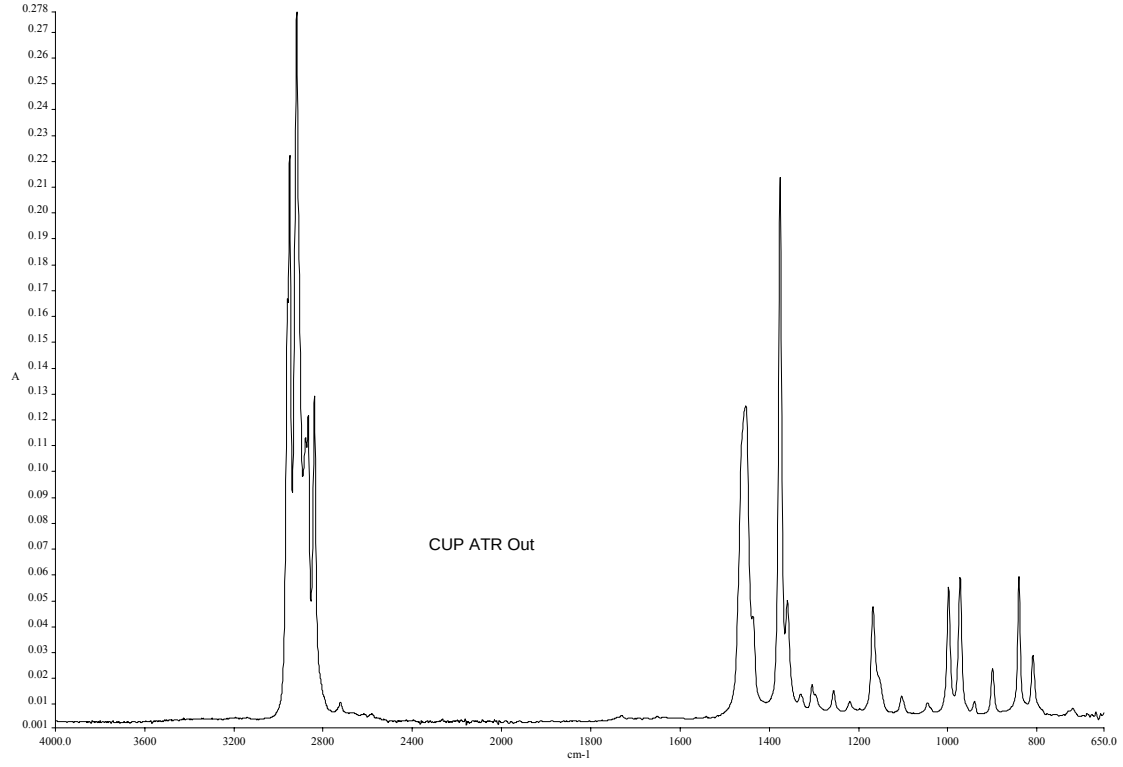
Physical characteristics

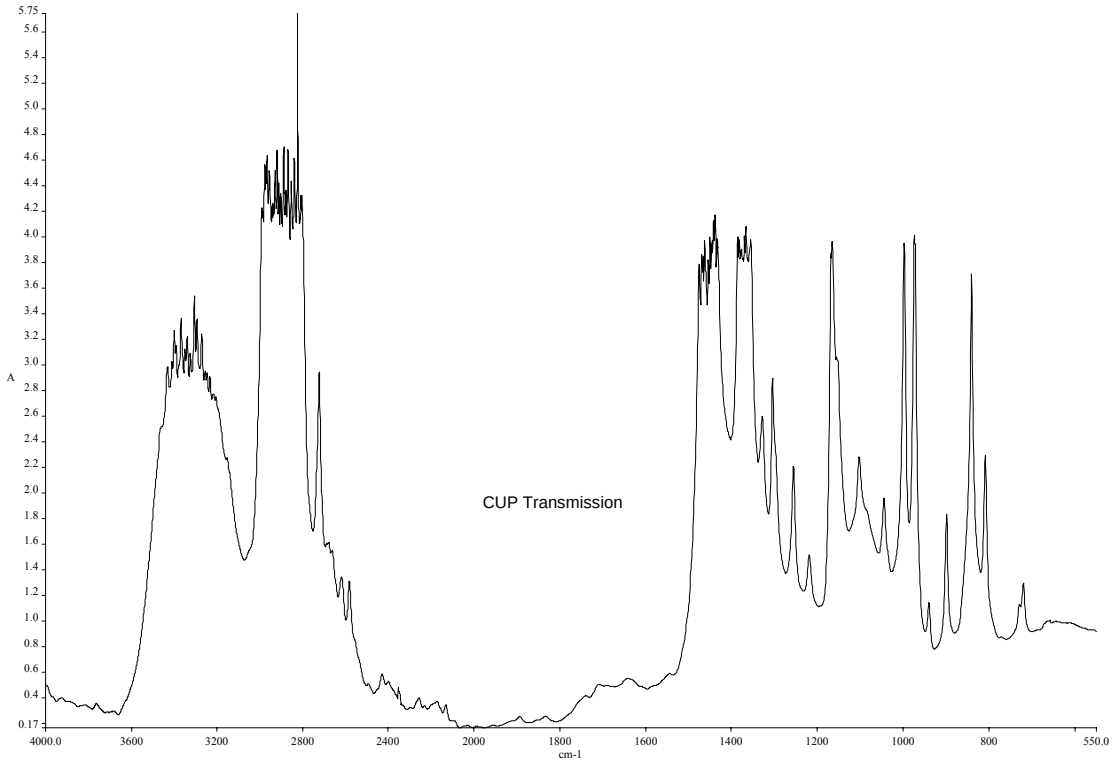
Reference	Density (g/cm^3)	Surface weight (g/dm^2)	Overall migration (10 days at 40°C except for isooctane 2 days at 20°C)	Permeability	Seal range	Seal strength	Maximal stress	Failure elongation	Maximal force
Film AKNS	0.87	0.78	o H2O : 2.7 mg/dm² o AcOH 3% : 8.2 mg/dm² o EtOH 95% : 0.6 mg/dm² o Isooctane : 1.9 mg/dm²	o Oxygen : 1.5 cm3/m2.d.atm o Water vapour : 1.5 g/m2.d	160-200 °C	4 N/15mm (90°C) 10 N/15mm (180°C)			
Doypack AMCOR	0.99	1.12	o H2O : 4.5 mg/dm² o AcOH 3% : 8.3 mg/dm² o EtOH 95% : 1.7 mg/dm² o Isooctane : 1.4 mg/dm²	o Transmission coefficient of oxygen : 5.0 cm3/m².d.bar o Transmission coefficient of water vapour : 2.7g/m².d	120-150 °C		27.7 MPa	63.4 %	59.1 N
FT Material EDV	0.94	12.7	o H2O : 9.8 mg/dm² o AcOH 3% : 13.9 mg/dm² o EtOH 95% : 1.4 mg/dm² o Isooctane : 4.0 mg/dm²						
Cups EDV	0.95	11.4	o H2O : 10.3 mg/dm² o AcOH 3% : 14.6 mg/dm² o EtOH 95% : 2.6 mg/dm² o Isooctane : 3.8 mg/dm²	o Global permeability of oxygen : 0.0015 cm3/d o Transmission coefficient of water vapour : 0.018 g/d			134.4 MPa	18.1 %	291 N
Doypack BREGER	0.94	1.24	o H2O : 4.9 mg/dm² o AcOH 3% : 3.5 mg/dm² o EtOH 95% : 1.3 mg/dm² o Isooctane : 1.6 mg/dm²						

IR Spectrum

Cup



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5.4
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4.6
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3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
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1600
1400
1200
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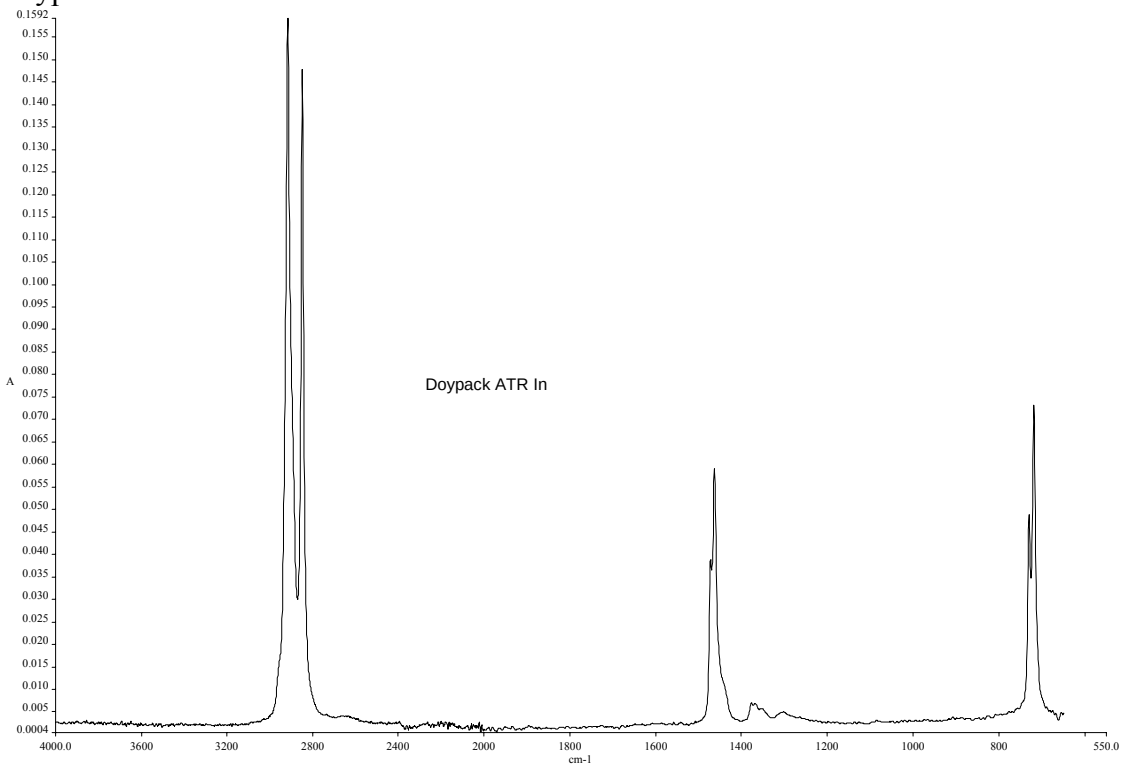
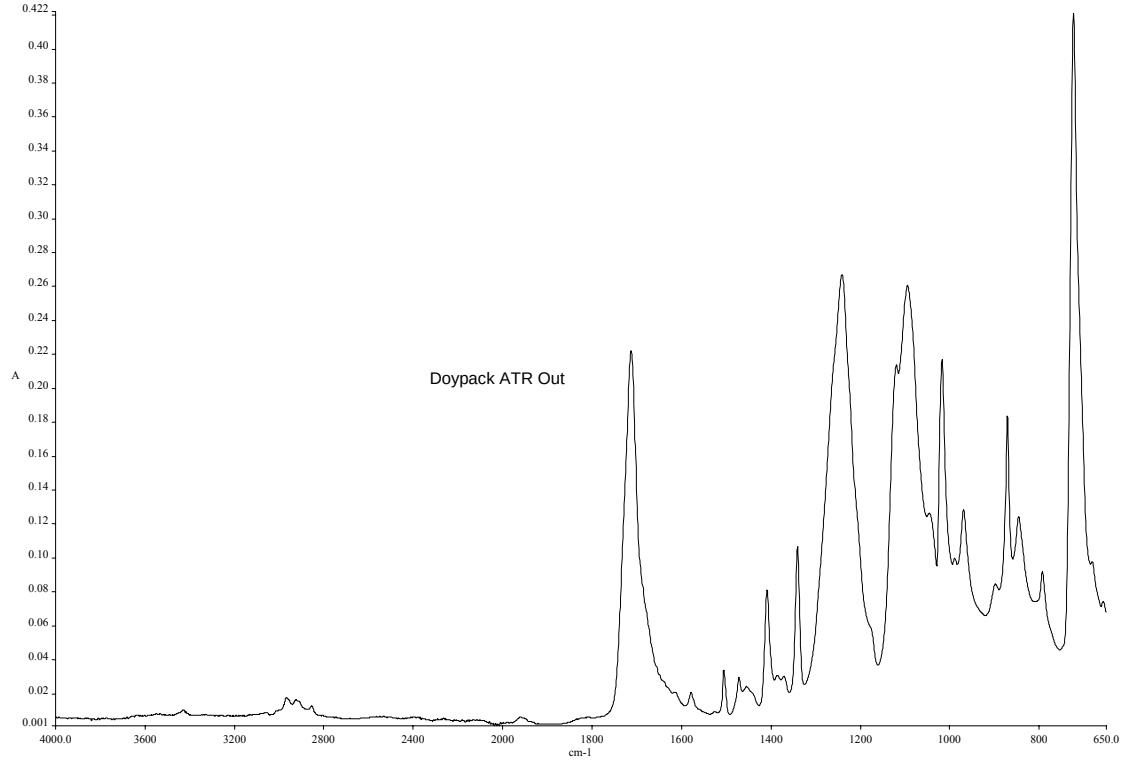
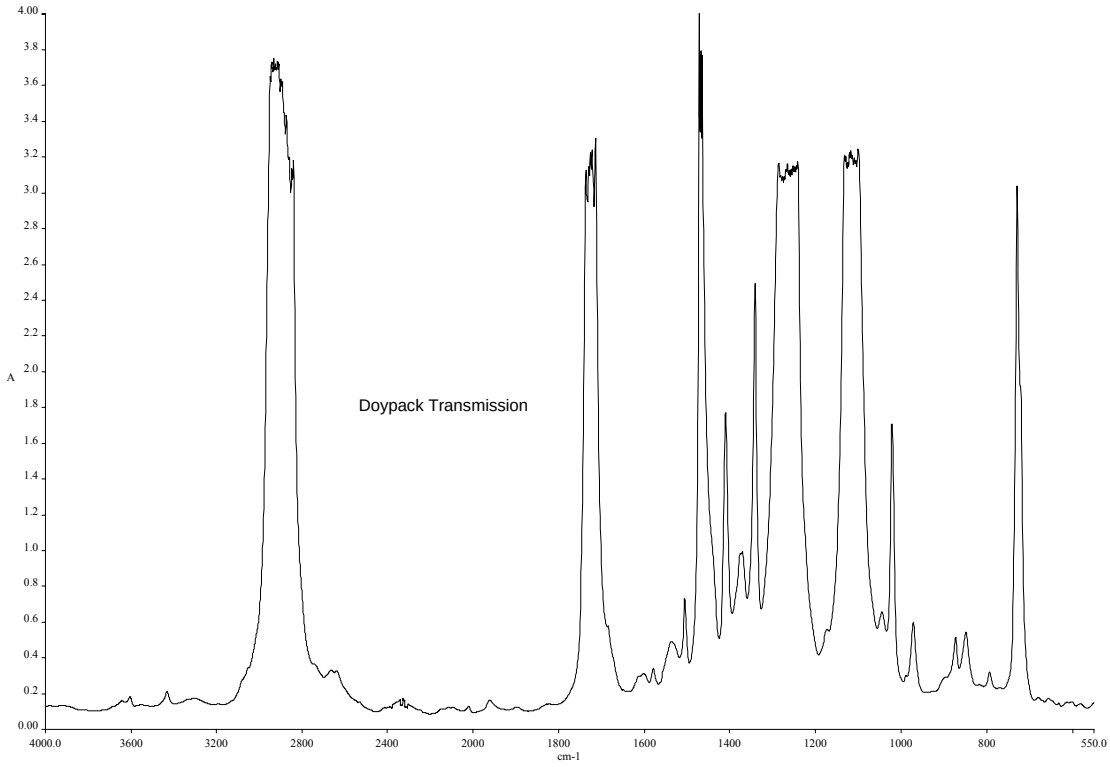
Doypack



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MEB pictures

Cup

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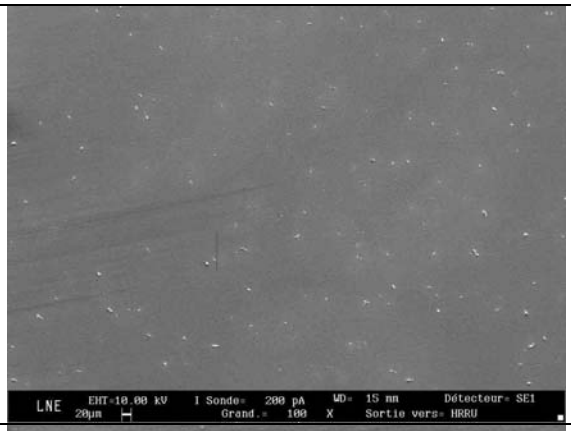
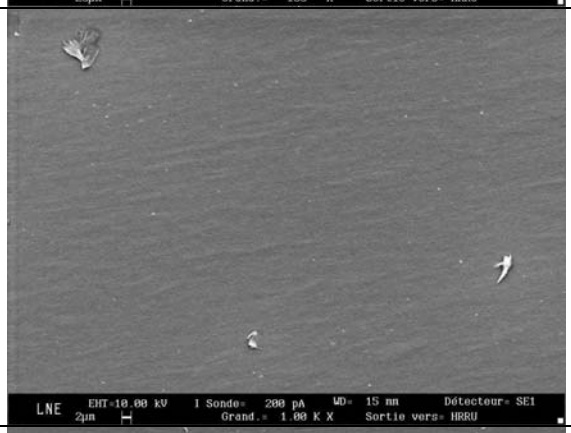
Face in X100	
Face in X1000	
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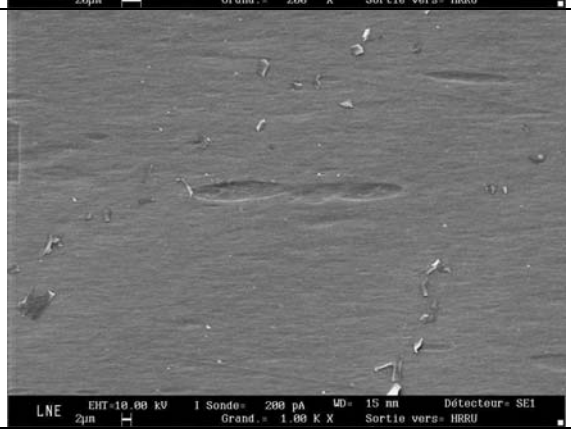
Face out X1000	

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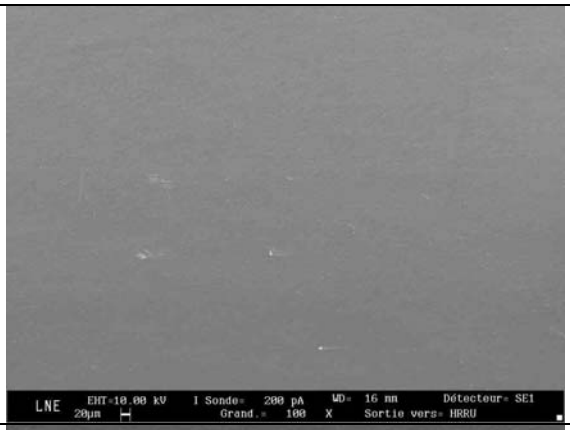
Face in X100	
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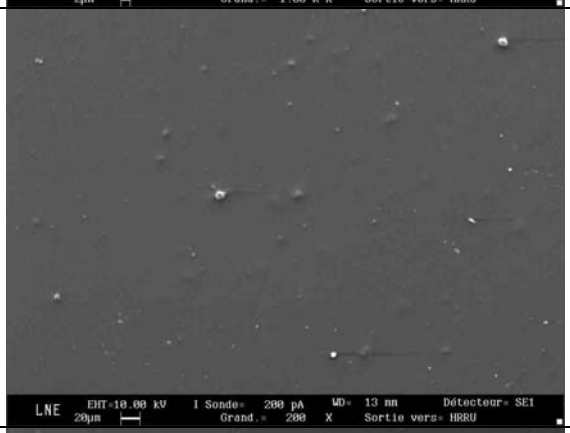
Face out X200	
Face out X1000	

Annex IV to Photosensitization Technology Demonstration Activity

Salad preparation steps

1. Populiariosios salotos (popular salads)

Composition: Cabbage, marinated cucumbers, paprika, “French dressing”(oil, lemon juice , mustard, vinegar, sugar, salt species) leeks, species, salt.

1.1. Preparation of the raw materials

1.1.1. Cabbage cleaned removed stem chopped by 1,5 – 2cm length straws and washed.
1.1.2. Leeks cleaned, removed roots and washed.
1.1.3. Paprika washed, cut and cleaned from seeds.
1.1.4. Marinated cucumbers drained.
1.1.5.

1.2. Preparation ingredients:

1.2.1. Cabbage there is no preparation.
1.2.2. Leeks chopped 5mm wide straws
1.2.3. Paprika chopped in to 2mm wide and 1,5 cm length straws.
1.2.4. Marinated cucumbers chopped in to 2mm wide and 1,5 cm length straws.

1.3. Preparation of Salads

1.3.1. Al ingredients put in to dish.
1.3.2. Added “French dressing”
1.3.3. Well mixed.

“Popular” salads general view

2. "Pavasaris" salotos “Spring” salads

Composition: fresh broccoli, radish, paprika, fresh cucumbers “salad dressing”(oil, lemon juice , vinegar, garlic, sugar, species, salt, pepper). , dill, vinegar, salt.

2.1. Preparation of the raw materials

2.1.1. Broccoli is cleaned divided in to separate trusses, washed.
2.1.2. Paprika washed, cut, cleaned from seeds.
2.1.3. Radish cleaned, removed residues of roots and leaves, washed.
2.1.4. Cucumbers cleaned, washed removed stumps.
2.1.5. Dill well washed and cut roots.

2.2. Preparation ingredients:

2.2.1. Broccoli and put in to, water with vinegar and salt and kept for 1,5 hour.
2.2.2. Paprika chopped in to 2mm wide and 1,5 cm length straws.
2.2.3. Radish sliced in to 5mm slices.
2.2.4. Cucumbers sliced in two peaces and chopped in to 5mm semi slices.
2.2.5. Dill chopped.

2.3. Preparation of Salads

2.3.1. Al ingredients put in to dish.
2.3.2. Added salt
2.3.3. Added “French dressing”
2.3.4. Carefully mixed.

“Spring” salads general view

3. Šviežių daržovių salotos (fresh vegetable salads)

Composition: China cabbage, fresh cucumbers, carrots, “French dressing”(oil, lemon juice , mustard, vinegar, sugar, salt species) salt.

3.1. Preparation of the raw materials

3.1.1. China cabbage is cleaned removed stem, well washed.
3.1.2. Carrots cleaned, washed.
3.1.3. Cucumbers cleaned, washed removed stumps.

3.2. Preparation ingredients:

3.2.1. China chopped by 5mm wide straws.
3.2.2. Carrots chopped by 2mm wide straws.
3.2.3. Cucumbers sliced in two peaces and chopped in to 5mm semi slices.

3.3. Preparation of Salads

3.3.1. Al ingredients put in to dish.
3.3.2. Added “French dressing”
3.3.3. Well mixed.

“Fresh vegatable” salads general view