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ORIGINAL RESEARCH PAPER

ASSESSMENT OF L. MONOCYTOGENES CELLS' CAPACITY TO RECOVER IN CUCUMBER JUICE AFTER HIGH PRESSURE TREATMENT

FLORENTINA IONELA BUCUR, DANIELA BORDA, ANCA IOANA NICOLAU, LEONTINA GRIGORE-GURGU*

Dunarea de Jos University, 111 Domneasca Street, 800201 Galati, Romania
*corresponding author: leontina.gurgu@ugal.ro

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Abstract

This study aimed at assessing the ability of the foodborne pathogenic bacterium *Listeria monocytogenes* to resist high pressure processing when present in cucumber juice and evaluating its capacity to recover during post-processing storage at 8°C for 72 hours. Two *L. monocytogenes* strains were selected, Scott A and RO15, a reference and a food isolated strains, respectively. The cucumber juice was inoculated with either Scott A or RO15 strain at a concentration of approximately 7 log CFU/mL and treated at 300 and 400 MPa, 8°C, for 8 minutes. The behavior of *L. monocytogenes* populations was studied by employing both quantitative and qualitative approaches: enumeration of *L. monocytogenes* viable cells by plate counting on a selective growth medium and measuring the fluorescence intensity emitted by the cells when stained with propidium iodide (PI). The treatment at 300 MPa, 8°C, for 8 minutes determined a reduction rate of less than 1 log CFU/mL for both *L. monocytogenes* strains, while treatment at 400 MPa led to a significantly higher reduction in cells number, 6 and 5 log CFU/mL for Scott A and RO15 strain, respectively. *L. monocytogenes* viable cells counting at each time point (0, 24, 48 and 72 hours) revealed no multiplication of survivors during storage at 8°C. However, the fluorescence method suggested a possible cellular membrane recovery process, since fluorescence intensity emitted by the HP treated *L. monocytogenes* cells decreased continuously over the storage period compared to time 0. The results of this experiment emphasize the necessity of employing other barriers against *L. monocytogenes* in combination with HPP, to ensure its consumption safety during storage for periods longer than 72 hours.

Keywords: *Listeria monocytogenes*, high pressure processing, vegetable juice, food safety, fluorescence

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Introduction

Consumers' tendency towards a diet based on raw and minimally processed vegetables can expose them to the risk of contracting food-borne diseases, especially due to the wide distribution of the pathogenic bacterium *Listeria monocytogenes* on plants (Beuchat, 1996). Vegetables are considered the main vehicle of *L. monocytogenes* transmission because they can get in direct contact with decaying plants, soil, and sewage waters. Additional causes of contamination with *L. monocytogenes* may be represented by harvesting, handling procedures, and transportation of vegetables in improper conditions (Ajayeoba *et al.*, 2015; Donnelly, 2001). Fresh vegetables with increased incidence of *L. monocytogenes* are lettuce, cabbage, celery, cucumber, corn, tomato, and melon, some of these being involved in serious listeriosis outbreaks over time (Beuchat, 2002).

L. monocytogenes prevalence in naturally contaminated cucumbers is 6%, the contamination level ranging from 0.04 to 0.170 CFU/25 g sample (Hadjilouka *et al*., 2015). Although stored under refrigeration conditions, cucumbers can provide optimal conditions for *L. mononocytogenes* proliferation. Moreover, this bacterium has been shown to resist freezing temperatures, being capable of surviving on whole and sliced cucumbers for at least 4 months (Bardsley *et al*., 2019). Besides cold survival, *L. monocytogenes* has been shown to tolerate salt concentrations up to 10% (McClure *et al*., 1989) and to grow at a water activity value of 0.92 (de Daza *et al*., 1991; Farber *et al.,* 1992). The ability of this pathogen to cope with low temperatures is due to several molecular mechanisms, among which should be mentioned the alteration in the membrane composition in order to maintain its fluidity and to prevent the formation of a gel-like state that may result in the loss of the cytoplasmic content (Beales, 2004; Neunlist *et al*., 2005), the synthesis of the cold-shock proteins, as result of *csp* genes increased expression, that aim at facilitating replication, transcription and translation processes (Schmid *et al*., 2009; Lee *et al*., 2012), and the accumulation of osmolytes such as glycine betaine and carnitine through transport systems encoded by *gbu*ABC and *opu*CABCD operons, respectively (Miladi *et al*., 2006; Chan *et al*., 2007; Bucur *et al*., 2018). The latest mechanism is also involved in the resistance to osmotic pressure that is generated by high salt concentrations' presence in the growth medium (Cacace *et al*., 2010; Bae *et al*., 2012). This variety of features renders *L. monocytogenes* difficult to control in food products by traditional processing techniques, the microorganism posing, consequently, a particular challenge to food industry (Donnelly, 2001).

Lately, food research has focused on the development of new processing approaches that aim at ensuring food safety, retaining, at the same time, the nutritional value and fresh-like sensorial properties of food products (Patrignani and Lanciotti, 2016). Under the umbrella of alternative food processing technologies the high pressure processing (HPP) is included; this is a relatively new non-thermal technology capable of inactivating spoilage and pathogenic microorganisms. HPP can be an alternative to the classical thermal pasteurization of liquid, semi-solid and solid food products, such as fruit and vegetable juices and smoothies, fruit purees, jams and jellies, shellfish, cured hams, cooked meats, and

salad dressings (Muntean *et al.,* 2016). This preservation technique enhances food safety and extends food products' shelf-life (Considine *et al*., 2008).

This article focusses on the efficiency of HPP in inactivating *L. monocytogenes* in cucumber juice and evaluates the ability of *L. monocytogenes* survivors to recover during juice storage under refrigeration conditions for 72 hours.

Materials and methods

Cucumber juice preparation

The cucumbers (*Cucumis sativus*) used to obtain the juice were purchased at a local supermarket in Galati, Romania, one day before experiments and stored at 4°C until use. Prior processing, the cucumbers were thoroughly washed with potable tap water and dried by tapping with clean paper towels. The cucumber juice was obtained in a regular fruit and vegetable juicer (Severin, ES 3562, Germany), being, afterwards, clarified by centrifugation at 6000 rpm, 4°C for 15 minutes (Hermile, Z 326K, Germany). The clarified juice was collected in sterile glass vessels and stored overnight at 8°C.

Determination of cucumber juice's dry matter content

The dry matter content of the cucumber juice was determined by gravimetric method according to EN 12145:1996 standard.

Determination of cucumber juice's pH and water activity

The pH of the cucumber juice was determined with the pH meter Lab 865 (SI Analytics, Mainz, Germany) and the water activity of it was measured with the water activity meter FA-st lab (GBX Scientific Ltd, Germany). Both determinations were performed in triplicate.

L. monocytogenes strains, growth conditions and inoculum preparation

L. monocytogenes strains used in this work, Scott A and RO15 (both being serovar 4b strains), were provided by the culture collection of the Faculty of Food Science and Engineering from Dunarea de Jos University of Galati, Romania. Scott A is a reference strain, while RO15 is a food-isolated one, which is described in Ciolacu *et al*. (2016). The bacterial strains were stored at -20°C, in tryptic soy broth supplemented with 0.6% yeast extract (TSB-YE; Oxoid, Basingstoke, Hampshire, England) containing 30% glycerol. Prior use, stock cultures of *L monocytogenes* strains were activated by streaking onto tryptic soy agar supplemented with 0.6% yeast extract (TSA-YE, Oxoid, Basingstoke, Hampshire, England) and incubation at 37°C for 48 h. To obtain the inoculum, a single colony of either Scott A or RO15 strain was inoculated in 10 mL of TSB-YE and grown at 30°C for 18 h in order to reach the stationary phase. The resulting cultures, used further as inocula, yielded approximately 4 x 10^9 CFU/mL for both *L. monocytogenes* strains.

HP treatments

The cucumber juice samples were inoculated in duplicate with either *L. monocytogenes* ScottA or RO15 at a final concentration of approximately 2 x 10^7

CFU/mL. The artificially contaminated cucumber juice samples were then loaded into 30 mL Teflon cylinders/containers and subjected to the HPP carried out in high pressure equipment (Resato, Roden, Netherlands) composed of four vessels with the capacity of 100 mL. A mixture of water and propylene glycol (TR15, Resato) was used as pressure transmitting fluid. Treatments were performed at two pressure values, namely 300 and 400 MPa, 8ºC, with a maintaining time of 8 minutes. A 1 min. equilibration time was considered after the come-up time to allow temperature stabilization inside the samples. The compression rate was 100 MPa/min. and the vessels' decompression took place in approximately 5 seconds.

Determination of L. monocytogenes viable cells number

To determine the viable *L. monocytogenes* cells number, both untreated (used as control) and HP treated artificially contaminated cucumber juice samples were diluted 1:10 in phosphate saline buffer (PBS; Sigma Aldrich, St. Louis, SUA), pH 7.4, and the appropriate dilutions were plated on the selective growth medium Agar Listeria according to Ottaviani and Agosti (ALOA; Scharlau, Spain). The plates were incubated at 37ºC for 48 hours and the typical colonies (green-blue colonies surrounded by an opaque halo) were counted. The cell viability was expressed as CFU/mL cucumber juice.

Assessment of L. monocytogenes cells` capacity to recover in the cucumber juice after HP treatments

After HP treatments, at each analysis time point, *L. monocytogenes* cells from the HP treated cucumber juice samples were sedimented by centrifugation (13.000 rpm/min, 4°C, 10 min.), washed three times with 0.9% saline solution and finally resuspended in an equal volume of washing solution. *L. monocytogenes* cells harvested from the untreated cucumber juice samples represented the control samples. In a 96-well plate (Nunc™ Microwell™), 100 µL cells suspension portions were distributed in triplicate, stained with 50 µL of 15 µM propidium iodide (S-34854, Molecular probes) and incubated in the dark for 15 minutes at the room temperature. Fluorescence intensity was measured by excitation at 488 nm (with the emission at 617 nm) in a microplate reader (Tecan Infinite Pro 200).

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 8 (GraphPad Software Inc., San Diego, CA, USA) by analysis of variance (ANOVA) with post-hoc multiple comparison t-test. Differences were considered significant at $p<0.05$

Results and discussion

Characterization of cucumber juice

Some properties of the cucumber juice (Table 1) were analyzed in order to check if this food matrix can sustain the growth of *L. monocytogenes*.

As shown in Table 1, the cucumber juice provides optimal conditions for bacteria growth, including *L. monocytogenes*, especially due to its almost neutral pH (6.58±0.04) and water activity value (a_w=0.995±0.0005).

Table 1. Cucumber juice properties.

Property	Value
pH	6.58±0.04
a_w	0.995±0.0005
% dry matter	2.26±0.14

As cucumber was found to be one of the most contaminated vegetables, carrying pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus and L. monocytogenes* (Meldrum *et al*., 2009; Ajayeoba *et al*., 2015), the unprocessed cucumber-based food products may pose a serious threat to people's health.

Effect of HPP on L. monocytogenes populations in cucumber juice

The results of the HP treatments of the cucumber juice inoculated with *L. monocytogenes* Scott A and RO15 are presented in figures 1 and 2, respectively. HP treatments efficiency varied according to the pressure intensity for both *L. monocytogenes* Scott A and RO15.

As food products' shelf life depends primary on the storage temperature, storage of the HP treated cucumber at 8°C was performed for a short period of time (72 h), since fresh juices were shown to be stable at such temperature for only 5 days (Fellers, 1988).

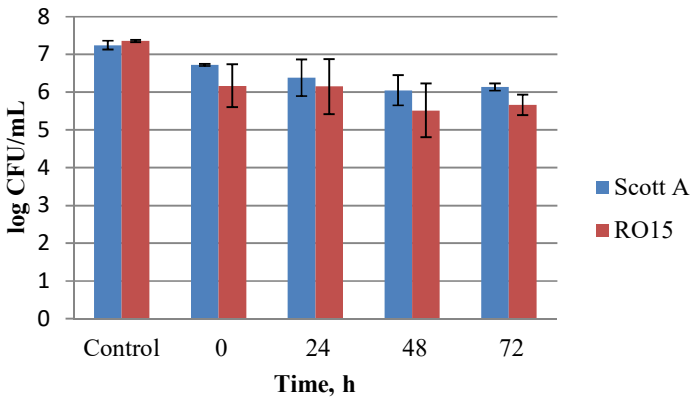

Figure 1. Surviving populations of *L. monocytogenes* Scott A and RO15 in cucumber juice after HP treatment at 300 MPa, 8°C, 8 min. and their behavior during storage at 8°C for 72 hours.

The initial *L. monocytogenes* population residing in the cucumber juice (~7 log CFU/mL) was reduced, following treatment at 300 MPa, 8°C for 8 min., with 0.51±0.08 log CFU/ mL and 1.18±0.54 log CFU/mL for Scott A and RO15 strains,

respectively. A similar reduction (1.6±0.5 log CFU/g) was observed after HPP of cantaloupe puree, artificially contaminated with *L. monocytogenes* at a concentration of ~6.7 log CFU/g, at 300 MPa, 8°C for 5 min. (Mukhopadhyay *et al*., 2016).

It is generally thought that stationary-phase *L. monocytogenes* cells exhibit a higher resistance to HPP, an observation that is supported by many studies (Mackey *et al*., 1995; Smiddy *et al*., 2005; Morales *et al.,* 2006). This phenomenon may be due to the cells' size, which is smaller compared to that of the exponential phase cells, and to their more spherical shape (Hayman *et al*., 2007).Changes in cells morphology result in cytoplasmic condensation and intracellular water activity reduction that are also thought to contribute to HPP resistance (Wen *et al*., 2009).

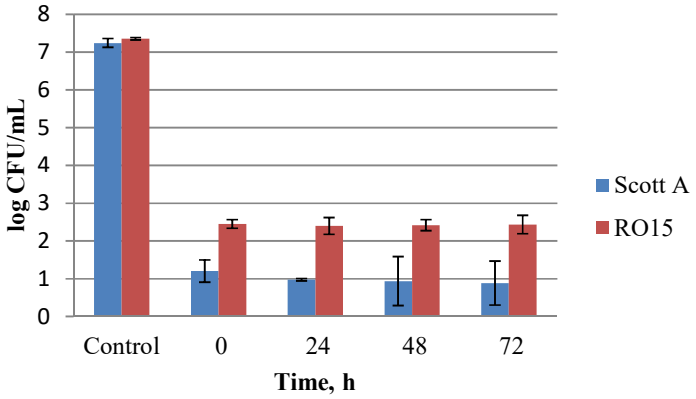

Figure 2. Surviving populations of *L. monocytogenes* Scott A and RO15 in cucumber juice after HP treatment at 400 MPa, 8°C, 8 min. and their behavior during storage at 8° C for 72 hours.

As expected, after HPP at 400 MPa, a higher reduction in *L. monocytogenes* cells number was obtained (Figure 2). RO15 strain proved to be more resistant to this treatment (p=0.03), the cells number reduction being with ~1.25 log CFU/mL lower compared to Scott A strain (Figure 2). The treatment at 400 MPa, 8°C for 8 min. determined a reduction in the *L. monocytogenes* Scott A cells number to a value lower than the safety limit of 100 CFU/mL, which is in accordance with the EC 1441/2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. For RO15 strain, the reduction rate after 400 MPa treatment was close to this limit as well.

Although the two strains belong to the same serotype - 4b, the higher resistance to HPP of *Listeria monocytogenes* RO15 compared to Scott A may be due to an increased expression of the genes involved in DNA repair mechanisms, proteins complexes synthesis, cells division, or peptidoglycan biosynthesis networks. Juices are heat-sensitive food products that, when treated at high temperatures, undergo negative changes in sensorial, nutritional, and functional properties (Gonzalez *et*

al., 2010). Therefore, thermal treatments such as low temperature long time (LTLT) pasteurization need to be employed in fruit or vegetables juices' processing in order to ensure high quality products as consumers demand. A research conducted in this regard showed that cabbage juice (pH 5.6) should be treated at 56°C for 30 minutes in order to reduce *L. monocytogenes* Scott A residing population by 5 log CFU/mL. The study suggests the need of other hurdles employment, such as salt and acidic pH, to lower the processing time and to prevent *Listeria* cells recovery during storage under refrigeration conditions (Beuchat *et al*., 1986). With all these aspects reviewed, it can be stated that HPP is more effective in juices' processing compared to pasteurization from cost, quality and safety points of view.

The cucumber juice was stored at 8°C for 72 h with the purpose to assess the behavior of the surviving *L. monocytogenes* population. During storage at 8°C for 72 hours, no increase or decrease in cells number was observed for both Scott A and RO15 strains subjected to both HP treatments (p>0.05) (300MPa and 400 MPa).

The ability of L. monocytogenes cells to recover in the cucumber juice after HPP

The main effects of HPP on bacterial cells are damages occurred on the cell membrane level (Pagan and Mackey, 2000). Research studies have shown that bacterial cells which survived the HP treatment are able to recover and even proliferate during storage of food products as function of food matrix type and storage temperature (Bull *et al*., 2005; Bozoglu *et al*., 2004; Chilton *et al*., 2001). It seems that the cellular recovery process implies a structural reorganization and pores sealing rather than biosynthesis o new compounds (Chilton *et al*., 2001).

The recovery capacity of *L. monocytogenes* cells after high pressure treatments was evaluated by staining with propidium iodide and measuring the relative intensity of the fluorescence emitted by the cells. Propidium iodide is a red fluorescent dye used in microscopy techniques, cytometry and fluorometry to identify and count the damaged or dead cells in a population. The fluorochrome enters the bacterial cells only when their membrane is permeabilized, staining the nucleic acids by intercalating between nitrogenous bases (Stiefel *et al*., 2015).

The *L. monocytogenes* Scott A and RO15 cells ability to recover in the cucumber juice after HPP is presented in the figures 3 and 4, respectively. Immediately after the HP treatment at 300 MPa (t_0), the fluorescence intensity of *L. monocytogenes* Scott A cells increased by 47.5 % compared to the control, indicating a higher capacity of this dye to enter the cells and bind the nucleic material, as consequence of cellular membrane damage. Moreover, in the case of 400 MPa treatment, at t_0, the fluorescence intensity emitted by the bacterial cells increased by 56.75% compared to the control, indicating a higher degree of cell membrane damage caused by HPP (Figure 3).

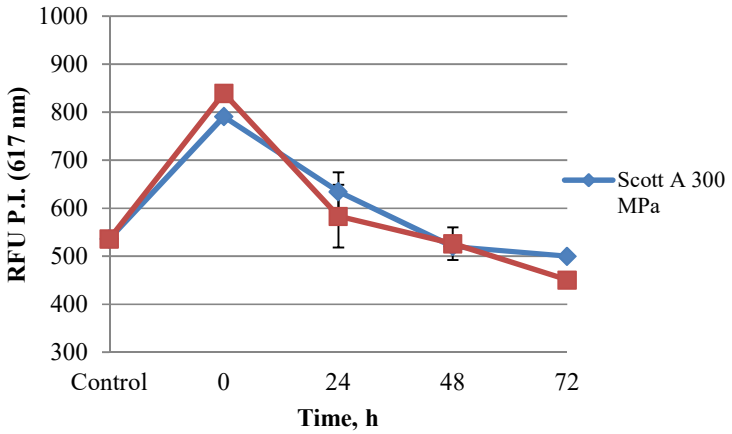

Figure. 3. Fluorescence intensity emitted by the propidium iodide stained *L. monocytogenes* Scott A cells at the analysis time points following HPP.

For RO15 strain, at t_0, the fluorescence intensity emitted by the HP treated cells increased by 64.28 and 84.16% in the case of 300 MPa and 400 MPa treatments, respectively (Figure 4).

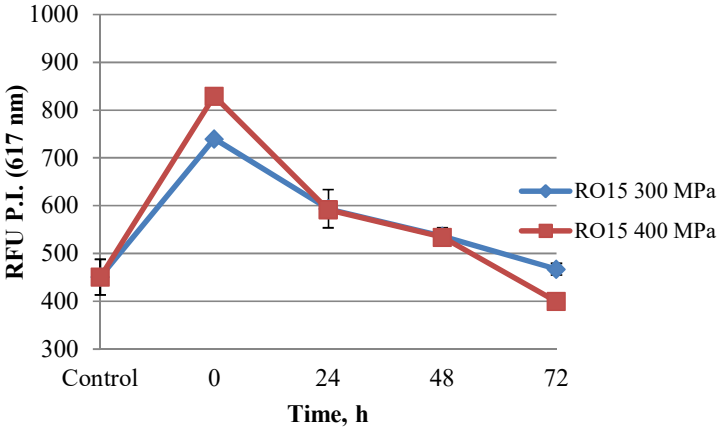

Figure 4. Fluorescence intensity emitted by the propidium iodide stained *L. monocytogenes* RO15 cells at the analysis time points following HPP.

Interestingly, after 24 h of storing at 8°C the cucumber juice treated at 300 MPa, the fluorescence intensity emitted by *L. monocytogenes* Scott A and RO15 cells decreased by 19.75 and 19.8%, respectively, compared to t_0. In the case of 400 MPa treated cucumber juice, at t_{24}, the fluorescence intensity decreased by 30.52 and 28.69% for Scott A and RO15 strain, respectively.

For both strains, at the end of the 72-hour storage period, the fluorescence intensity decreased, being approximately at the same level with the fluorescence intensity

emitted by the controls, suggesting, thus, a continuous process of cells regeneration.

From biological point of view, HPP injuries the cellular membrane and the cells can be inactivated or killed. In case of cells inactivation, the protein complexes dissociate, the enzymes are activated or blocked (depending on the protein flexibility), the nucleoids condense, the organelles become vulnerable and the membrane loses its integrity (Niven *et al.,* 1999; Manas and Mackey, 2004).

Actually, there is an opposing influence on proteins due to the action of two pressures, the hydrostatic and the osmotic one. It has been proven that, at 300 MPa, the *L. monocytogenes* cells were sublethal injured being slightly degraded, having the capacity to recover on cultivation media (Ritz *et al*., 2006; Bowman *et al*., 2008).

At the gene level, it may be activated the main stress response regulator, *sig*B, which can activate other protective genes like *tuf*A and *rpo*C, some DNA repair genes (Aertsen and Michiels, 2005), histone-like proteins genes (Ishii *et al*., 2005; Malone *et al*., 2006), cold-shock protein genes (Wemekamp-Kamphuis *et al*., 2004; Semrad *et al*., 2004), protein secretion and trafficking genes or other several genes associated with fatty acid biosynthesis (Schujman *et al*., 2003).

Conclusions

This study shows that the HPP at 300 MPa, 8°C, 8 min. is not effective in inactivating *L. monocytogenes* cells residing in the cucumber juice, but the HPP at 400 MPa, determines a reduction in *L. monocytogenes* cells number of 5 to 6 log CFU/mL, which is in accordance with the requirements of food safety regulations (EC 1441/2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs). The recovery process of the HP treated *Listeria monocytogenes* cells, at 8°C, might take place since the fluorescence emitted by cells stained with PI decreased over storage period, fact which emphasizes the necessity of employing other antimicrobial barriers in combination with HPP, for an effective processing technology.

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