Inactivation of pathogenic bacteria in food matrices: high pressure processing, photodynamic inactivation and pressure-assisted photodynamic inactivation

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Abstract. Traditional food processing methods frequently depend on the application of high temperature. However, heat may cause undesirable changes in food properties and often has a negative impact on nutritional value and organoleptic characteristics. Therefore, reducing the microbial load without compromising the desirable properties of food products is still a technological challenge. High-pressure processing (HPP) can be classified as a cold pasteurization technique, since it is a non-thermal food preservation method that uses hydrostatic pressure to inactivate spoilage microorganisms. At the same time, it increases shelf life and retains the original features of food. Photodynamic inactivation (PDI) is also regarded as promising approach for the decontamination of food matrices. In this case, the inactivation of bacterial cells is achieved by the cytotoxic effects of reactive oxygens species (ROS) produced from the combined interaction of a photosensitizer molecule, light and oxygen. This short review examines some recent developments on the application of HPP and PDI with food-grade photosensitizers for the inactivation of listeriae, taken as a food pathogen model. The results of a proof-of-concept trial of the use of high-pressure as a coadjutant to increase the efficiency of photodynamic inactivation of bacterial endospores is also addressed.

1. Introduction

Over time and starting from the moment of harvest, fresh foodstuff loses quality by physical, chemical, and biological processes. Microorganisms and enzymes are key agents of deterioration and consequently priority targets of preservation techniques.

Traditional food processing methods frequently depend on the application of high temperature. However, heat may cause undesirable changes in food properties and often has a negative impact on nutritional value and organoleptic characteristics. Therefore, reducing the microbial load without compromising the desirable properties of food products is still a technological challenge.

An ideal method of food preservation should be inexpensive and convenient to apply to ensure the inactivation of microorganisms and enzymes and/or inhibition of microbial activity and growth [1] while extending shelf life, preserving organoleptic and nutritional attributes, without leaving chemical residues and not raising objections from consumers and legislators [1, 2]. By using temperatures lower than those typically used in thermal processing, non-thermal food processing technologies are expected to exert a minimal impact on the nutritional, physical, chemical and sensory properties of
food [3]. High pressure processing (HPP) and photodynamic inactivation (PDI) are examples of non-thermal techniques that inactivate/eliminate microorganisms at ambient temperatures [3, 4].

HPP uses hydrostatic pressure - force per unit area applied in a direction perpendicular to the surface - to inactivate pathogens and vegetative forms of spoilage microorganisms [5]. Additionally, it increases shelf life and retains the original features of food. This technique can be used in different types of solid or liquid food matrices, at pressure values between 100 and 1000 MPa (1 MPa = 0.101 atm = 0.1 bar = 6.89 × 10^3 psi) in a range of temperature between -20 and 80 °C, during periods that can range from seconds to minutes [6, 7]. During the pressurization, a decrease in food volume proportional to the pressure applied occurs but food material returns to its initial volume during decompression [8]. Pressure is applied in an isostatic mode, i.e. the transmission of pressure occurs uniformly and almost instantly through the food material regardless of its shape and size, making this technique suitable for the inactivation of pathogens present at the surface or imbed in the food matrix [9]. The level of bacterial inactivation depends on the type of microorganism, but also on the composition and pH of the food matrix and therefore, it is necessary to carefully choose the appropriate processing protocol [2, 10].

PDI is also regarded as a promising approach for the decontamination of food matrices. In this case, the inactivation of bacterial cells is achieved by the cytotoxic effects of reactive oxygen species (ROS) produced from the combined interaction of a photosensitizer (PS) molecule, light and oxygen. The PS is excited by light and changes to a long-lived triplet state. Molecular oxygen is regarded as a key factor in PDI. In the type I photochemical mechanism, the PS in the excited state interacts with molecular oxygen by electron or hydrogen transfer, generating radical species including the superoxide anion, which can further originate other ROS like hydroxyl radicals. Type II mechanism is associated to the interaction of the excited state of the PS with molecular oxygen, in this case by energy transfer, which leads to the production of singlet oxygen [11]. PDI has been demonstrated as an efficient alternative for the inactivation of virus, bacteria and fungi [12]. However, the application of this technique to the inactivation of microbes in food products is dependent on the availability of food-compatible PSs that do not compromise food composition, appearance, taste and flavour. Some natural compounds with photosensitizing potential, such as hypericin, chlorophyll, riboflavin and curcumin, are common additives in foods and drinks that have been proposed to overcome this problem [13].

2. Non-thermal inactivation of listeriae

The genus *Listeria* is composed of short Gram positive rods with 0.4 – 0.5 μm of diameter and 1.2 μm of length, appearing singly or in short chains. This genus is closely related to other food pathogens like *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus* and *Staphylococcus*. *Listeria* spp. are facultative anaerobes, do not produce spores and grow at temperatures between 0 and 45 °C, although their optimum temperature for growth is between 30 and 37 °C. Listeriae easily form biofilms and grow in high concentrations of salt (10% NaCl) and at pH values between 4.5 and 9. This genus entails six species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. grayi*, *L. welshimeri* and *L. seeligeri*. Only the first two species are pathogenic [14, 16]. *L. monocytogenes* is a food pathogen responsible for an opportunistic infection called listeriosis, most often transmitted by raw food like soft cheese, fruits and vegetables but also by cooked meat [17, 18]. Although listeriosis is not very frequent, it can be lethal among adults and neonates [19]. *L. innocua*, is a non-pathogenic species that has been often used as a surrogate for the pathogenic *L. monocytogenes* in biological studies, since it presents similar responses to chemical or thermal treatments [20].

2.1. Inactivation of listeriae by HPP

The exposure of bacterial cells to high pressure causes damage to the cell membrane and denaturation of proteins, affects the function of enzymes and ribosomes, deregulates homeostasis and may ultimately cause cell disruption [6, 21]. With low pressure values (<50 MPa), processes like gene
expression and protein synthesis are affected but at pressure values above 400 MPa significant structural damage occurs [22]. Although not all the previously mentioned effects are demonstrated for listeriae, morphological, structural, physiological and genetic changes have been reported [23].

The inactivation efficiency is strongly affected by intrinsic (related with the bacterial cell) and/or extrinsic (related with the extracellular medium) factors. L. innocua cells in exponential growth phase are more susceptible that stationary phase cells probably because of the stress response proteins expressed by the latter [24, 25]. The composition of the food matrix, particularly in terms of pH and \( a_w \) has also a significant effect on the efficiency of inactivation of listeriae. In general, lower pH like in fruit juices, enhances inactivation since the survival of pressure-damaged cells is reduced in acid media [22]. Lower \( a_w \) decreases susceptibility to HPP, although the magnitude of the effect seems to be dependent on the type of solute [26, 27]. As an example, literature data indicate that milk significantly protects listeriae from HPP but this effect may not be observed in milk derivatives such as cheese, because of the particular properties of this product in terms of pH and \( a_w \) [23]. Cells that have been previously exposed to low temperatures (10 - 25 °C) are more susceptible to HPP than cells grown at temperatures near 43 °C [28]. This may implicate that contamination originating from warm-blood animals may be more difficult to control than contaminants from other environmental sources.

Pressurization parameters are of major importance in the inactivation of listeriae by HPP. In general, L. innocua is completely inactivated in food matrices at 400-600 MPa [29]. There is a direct relation between holding time (the time during which pressure is applied) and the efficiency of inactivation. At moderate pressure values, extending the holding time from 5 to 15 minutes may increase the factor of inactivation of L. innocua by as much as 5 log but at very high pressure values (>500 MPa), holding time losses relative importance as an operational parameter [30]. A study conducted with stationary phase cells of L. innocua demonstrated that lower compression/decompression rates increased inactivation efficiency [29]. A large set of data obtained in different food matrices and with different combinations of pressure and temperature indicates that inactivation efficiency seems to be the highest at refrigeration temperatures (4-10 °C) or when high pressure is combined with heat (50 °C) [23, 29]. All this further stresses the importance of a careful design of HPP protocols.

2.2. Photodynamic inactivation of listeriae

Literature reports of successful photodynamic inactivation of listeriae are strikingly scarce. Tests conducted with Escherichia coli O157:H7 and L monocytogenes revealed that the latter was more easily inactivated than the former, which was attributed to the differences in the composition of the cell wall. Also, photosensitization was more efficient with the dyes toluidine blue O (TBO) and methylene blue trihydrate (MB), than with a porphyrin, the tetrasalicylate salt of 5,10,15,20-tetrais(1-methylpyridinium-4-yl)porphyrin (Tetra-Py\(^{4+}\)-Me) [31]. However, TBO and MB cannot be used in foodstuff. The results of experiments in which a colourless and odourless solution of 5-aminolevulinic acid (ALA) was used to induce the synthesis of endogenous porphyrins demonstrated that plankton cells and biofilms of L. monocytogenes could be efficiently inactivated [32]. But again, the use of natural compounds, already approved as food additives, is preferable and more easily accepted by consumers.

Curcumin (food additive E100) is a yellow pigment and the active constituent of turmeric, which is obtained from the dried rhizome of Curcuma longa. Turmeric powder is an essential ingredient in curry, and it is often used in ready-to-eat meat dishes. Curcumin is a natural PS, able to generate cytotoxic ROS when activated with blue light [33] and photosensitization with curcumin has been successfully tested on yeasts and bacteria [34-36]. In recent experiments conducted by our group, irradiation of biofilms of L. innocua with blue light in presence of 10 \( \mu \)M of commercial curcumin caused an approximate 5 log reduction in the concentration of viable cells, whereas with an equivalent concentration of the reference cationic porphyrin, the tetrathosphate salt of Tetra-Py\(^{4+}\)-Me, the inactivation factor was only of ~1 log. The planktonic form was much more susceptible to PDI and in planktonic cells, photosensitization was more efficient with the porphyrin, which caused complete inactivation (unpublished). Bacterial biofilms represent a considerable challenge in terms of PDI because the extracellular matrix limits the penetration of the photosensitizer and the diffusion of oxygen to inner layers and cytotoxic ROS are efficiently captured by the extracellular polymeric substances.
Our results indicate that curcumin may be a particularly advantageous PS for the control of biofilm bacteria in packaged ready-to-eat meat meals.

3. Pressure-assisted photodynamic inactivation of bacterial endospores

Sporulation is a biological mechanism of resistance that enhances bacterial survival in harsh environmental conditions for considerably long periods of time. They represent a challenge in terms of inactivation since endospores resist pasteurization temperatures, desiccation, and chemical biocides like organic acids, alcohols and phenols, which easily destroy vegetative cells [38]. *Bacillus cereus*, *Clostridium perfringens* and *C. botulinum* are examples of foodborne endospore producing bacteria that due to their ubiquity in the environment and resistance to thermal processing represent a major concern in terms of the safety and stability of foods [39].

HPP inactivation of endospores is a two-step process in which an initial treatment with moderate to low temperature and pressure (100-200 MPa, T<50 °C) induces spore germination and higher pressure and temperature (400-600 MPa, T>60 °C) cause significant endospore inactivation [40]. In general, the process requires the application of very high pressure values and quite long holding times which makes it costly in terms equipment and energy. PDI of bacterial endospores has also received attention in the perspective of an alternative non-thermal inactivation approach. Although much less susceptible than vegetative cells, endospores of *Bacillus* spp. have been successfully inactivated with phenothiazine dyes and cationic porphyrins [41, 42]. However, complete inactivation is not easily achieved and the binding of the PS to the essentially inert spore coating has been identified as a limiting step in the photosensitzation process [42].

Using endospores of *B. cereus* as biological models, we tested the hypothesis that high pressure could be used as a physical coadjuvant to enhance PS binding and therefore to improve the PDI of bacterial endospores [pressure-assisted photodynamic inactivation (HP-PDI)]. In repeated independent trials, pressurized endospore suspensions (300 MPa for 30 minutes in the presence of 20 µM *Tetra-Py*'-Me) revealed up to 76-fold increases in PS binding, in relation to the controls in which the dark exposure to PS was conducted at atmospheric pressure, and a significant increase in photosensitization efficiency upon irradiation (unpublished). Considering that the proof-of-concept of HP-PDI was successful, the challenge is now to optimize the approach using food-grade photosensitizers for endospore inactivation in food products.

4. Future perspectives

HPP and PDI represent promising non-thermal alternatives for the control of bacterial pathogens and food-spoilage microorganisms, such as inferred from experiments with *Listeria* spp. Both approaches still face technical challenges. Future developments of HPP protocols must address the problem of protein denaturation, so that it can be used in a wider variety of food products, and also the cost effectiveness of the process, in terms of equipment and energy requirements. A major step forward in PDI of food pathogens will be the development of efficient food-grade photosensitzers and the combined application of physical co-adjutants, like high-pressure or heat, to overcome the problem of more resistant forms like microbial biofilms, protozoan cysts, fungal spores and bacterial endospores.

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