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Partial permeabilisation and depolarization of *Salmonella enterica Typhimurium* cells after treatment with pulsed electric fields and high pressure carbon dioxide

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ABSTRACT

The aim of the present study is to investigate the efficiency of the combined pulsed electric fields and high pressure carbon dioxide (PEF + HPCD) treatment on the Gram-negative pathogen *Salmonella Typhimurium* in a liquid medium, by means of both plate count technique and flow cytometry (FCM). PEF was applied at two conditions: (1) 1 single pulse of 1 ms length at 30 kV/cm and (2) 12 pulses of 4 ms length at 30 kV/cm, while HPCD at 12 MPa, 22 °C and 35 °C for different treating times (0–45 min). At both temperatures, the application of PEF as HPCD pre-treatment was demonstrated to enhance the inactivation kinetics and to decrease the treatment time to inactivate *S. Typhimurium* if compared to HPCD alone. Further, the approach based on FCM permitted to investigate the functional status of bacterial cells after PEF and HPCD treatments distinguishing among viable bacteria (considered as intact cells), permeabilised cells and depolarised cells simultaneously. It has been demonstrated that the synergistic effect is due to the electroporation effect of PEF which lead to changes in the cell membrane potential but also in a partial structural damage, favoring the subsequent CO_2 penetration into the cells and increasing the inactivation kinetics, thus improving the efficiency of the entire process.

1. Introduction

Foodborne illness is a public health challenge that, according to a World Health Organization report [1], caused almost 1.8 million human deaths in 2005 [2]. Such illness is mostly caused by eating food contaminated with pathogenic bacteria, which enter the food supply through cross-contamination events or food handlers' poor hygiene.

The most desirable objective of a novel food pasteurization technique would be the capability of destroying high levels of microorganisms at low temperature in short time. In this regard, alternative techniques have recently been studied to inactivate microorganisms, such as high-intensity pulse electrical fields [3], high pressure carbon dioxide [4], high hydrostatic pressure [5], and ultrasound treatments [6].

High pressure carbon dioxide (HPCD) has been increasingly investigated as a technique able to induce the inactivation of the natural microbial flora but also pathogens occurring in solid and liquid matrices [7–10]. Theories explaining the inactivation mechanism of HPCD involve the diffusion and solubility of CO_2 in the culture medium, the decrease of the pH in the medium, the increase of the membrane fluidity and permeability, the diffusion of CO_2 into the cells, the cell membrane rupture caused by the increase of the internal pressure, and the resultant changes in the cellular environment, such as a decrease in pH, inactivation of key enzymes, and extraction of critical intracellular materials [11,12]. Nevertheless, in some cases quite a high temperature and long time are required to achieve a satisfactory inactivation level, limiting the feasibility of HPCD inactivation processes [13,14]. That is the reason why there is increasing scientific interest in combining HPCD processes with synergistic techniques to enhance its inactivation efficiency.

Pulsed electric fields (PEF) is becoming a promising alternative technique in liquid food processing as it avoids, or at least greatly reduces, the detrimental changes induced by the thermal treatment to the sensory and physical properties of foods [3]. PEF processing

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involves the application of pulses of high electric strength (typically 15–80 kV/cm) to food flowing through or placed between two electrodes at ambient, sub-ambient or slightly above ambient temperature, for less than 1 s [15]. The application of an external electric field to biological cells induces an alteration of the electrical potential across the cell membrane (normally at −60/−70 mV) which, when exceeding critical levels (about 1 V), leads to an electrical breakdown and local structural changes of the cell membrane, causing the formation of pores, i.e. electroporation, which can be reversible or irreversible, depending on the degree of the structural changes [16]. The electric field strength, the length, the shape and the number of pulses and the initial temperature are considered as the main process parameters which affect microbial inactivation by PEF. In general, an increase of each parameter enhances the microbial inactivation. Moreover, the bacterial inactivation by PEF has been demonstrated to depend on the physical and chemical characteristics of the product and on the type of microorganisms: bacterial cells are more resistant than yeasts and Gram-positive are more resistant than Gram-negative species, because of the composition of the cell wall [17–20].

Recently, the effect of coupling PEF with HPCD to achieve a high microbial inactivation of a Gram negative *E. coli*, a Gram positive *Staphylococcus aureus* and a spore forming bacteria *Bacillus cereus*, at temperatures not higher than 40 °C has been successfully investigated [16], but the ultrastructural effect of this treatment on the physiological status of microorganisms has never been elucidated. To the best of our knowledge, no studies regarding the effect of combined PEF + HPCD process on the inactivation mechanisms and particularly on permeabilization and depolarization of bacterial cell membranes are present in literature.

So far, bacterial inactivation was commonly evalutated by conventional standard plate count methods, which measure the bacterial ability to replicate and form colonies on rich seletive agar media. On the other hand, it is well known that, under environmental stress conditions (e.g. nutrient limitation, pressure, temperature), a number of bacterial cells enter into a socalled Viable But Not Cultivable state (VBNC), becoming even more resistant to stress [21]. Thus, plate counts may overestimate pasteurization efficiency, by not detecting as viable reversibly damaged bacterial cells [22]. Innovative viability test methods may be more suited for studying the efficiency of pasteurization treatment on bacterial cells, including e.g. flow cytometry [23], wich employs cell-membrane permeability as viability parameter.

The aim of the present study is to investigate the efficiency of the combined PEF + HPCD treatment on the Gram-negative pathogen *Salmonella Typhimurium* in a liquid medium, by means of both plate count technique and flow cytometry (FCM). Untreated and HPCD treated samples were also investigated, at the same process conditions, in order to confirm the synergistic effect of PEF and HPCD treatments in series. The approach based on FCM permitted to investigate the physiological status of bacterial cells after PEF and HPCD treatments distinguishing among viable cells (considered as intact cells), permeabilised cells and depolarised cells simultaneously.

2. Materials and methods

2.1. PEF apparatus

The electroporation apparatus is the Micropulser™ BIO-RAD® and consists of a pulse generator module, a shocking chamber and a cuvette with incorporated 2 parallel stainless steel electrodes, separated with a gap of 0.1 cm. The apparatus produces electric strengths up to 30 kV/cm. The MicroPulser module contains a capacitor charged to high electric strength that discharged the current in the capacitor to the sample in the cuvette. The capacitance discharge circuit generates an electrical pulse with an exponential decay wave form. The ranges of operative parameters tested in the experimental runs were the following: electric strength from 10 to 30 kV/cm, pulse length from 1 to 4 ms and number of pulses from 1 to 12. The choice was based on recent publications focusing on the inactivation of *Salmonella* strain with PEF [24–27]. The maximum number of 12 pulses was chosen to avoid electrical arcing in the cuvette and the overheating of the samples: the temperature of the samples was measured after the treatment and it was about 25 ± 2 °C, even after 12 pulses of 4 ms.

2.2. HPCD multibatch apparatus

The apparatus consists of ten 15 ml cylinders, provided with a magnetic system for stirring (Velp, about 300 rpm). The cylinders operate in parallel, so that each experimental run provides a set of experimental data taken at identical process conditions but different treatment times. Each reactor is connected to an on-off valve that can be used to depressurize it independently from the others. The reactors are submerged in a single temperature-controlled water bath. Liquid CO_2 (Messer, Carbon dioxide 4.0, purity 99.990%) is fed into the reactors by a volumetric pump (LEWA, mod. LCD1/M910s) that increases the pressure to the desired processing levels with a rate of about 6 MPa/min. The apparatus is provided with a transducer (Endress + Hauser GmbH, Maulburg, Germany) to control the pressure values while one cover lid of the 10 reactors is equipped with a fixed thermocouple (Pt 100Ω) to control the product temperature. At the end of the process, two micrometric valves and one on-off valve are used to depressurize and release CO_2 from the apparatus occurred over approximately 1 min. The processed samples are collected in sterile containers, cooled down immediately at 4 °C until further use [9]. The operating parameters (temperature, pressure and time) are continuously recorded by a real time acquisition data system (National Instruments, FieldPoint FP-1000) and monitored by a specific software (LabVIEW™ 5.0).

3. Experimental procedure and operative conditions

3.1. Microbial strain and plate counts

Salmonella enterica subsp. *enterica* serovar *Typhimurium* (ATCC® 14023™; DSMZ, Braunschweig, Germany) was used in this study. A fresh colony of *S. Typhimurium* was inoculated into 10 ml of Luria Bertani (LB, Sigma–Aldrich) medium and incubated at 37 °C, with constant shaking (200 rpm) to stationary phase (16 h). For plate counts, untreated and treated samples were serially diluted in sterile phosphate buffer solution (PBS) and plated in duplicate onto *Plate Count Agar* (Liofilchem, TE, Italy), a non-selective medium. The plate were incubated at 37 °C for 24 h according to ISO 6579:2002 [28]. The degree of inactivation was determined by evaluating the $\log(N/N_0)$, where N_0 was the number of CFUs (colonies forming unit) initially present in the untreated sample and N was the number of CFUs after the treatment. Three independent experiments were carried out for each experimental condition and the results were calculated as the mean value of three replications. Standard deviations were shown by error bars in the graphs. The detection limit was 10 CFU/ml.

3.2. PEF treatment

The cells were centrifuged at 6000 rpm for 10 min and resuspended in an equal volume of a cold solution of 10% glycerol. Subsequently, other three centrifugations at 5000 rpm for 15 min were performed to remove any trace of the culture medium from