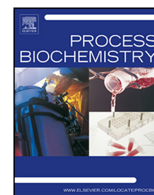
Process Biochemistry 50 (2015) 188–193

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio



Application of culture-independent methods for monitoring *Listeria monocytogenes* inactivation on food products

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ARTICLE INFO

Article history:
Received 15 July 2014
Received in revised form
19 November 2014
Accepted 11 December 2014
Available online 26 December 2014

Keywords:
Flow cytometry
Quantitative PCR
Microbial inactivation
Membrane integrity
Listeria monocytogenes
Supercritical carbon dioxide

ABSTRACT

When new food processing technologies are investigated as alternative to traditional thermal pasteurization processes, conventional cultivation-based methods are usually applied to evaluate microbial concentration before and after the treatment to determine the process efficiency. However, these standard methods lead to a typical underestimation of the microbes present in the sample, which may represent an issue when pathogenic strains have to be detected. Here, the efficiency of SC-CO_2 pasteurization treatment in the inactivation of *Listeria monocytogenes* spiked on cured ham skin surface was evaluated using plate counts, flow cytometry (FCM) coupled with SYBR-Green I (SYBR-I) and propidium iodide (PI), and propidium monoazide quantitative PCR (PMA-qPCR), at different process conditions. SC-CO_2 best performed at 12 MPa, 45 and 50 °C, resulting in a 7.5 log reduction of cultivable cells quantified by plate counts after 15 min of treatment, while FCM and PMA-qPCR revealed a 4 log and 2 log reduction of intact cells, respectively. This striking difference between culture-based and culture-independent quantification methods was independent from treatment time and indicated that a large fraction of the cells lost cultivability after treatment but maintained an intact membrane, likely entering in a so-called Viable But Not Culturable (VBNC) state. Our study highlights the usefulness of FCM and PMA-qPCR to assess the viability status of microbial populations and support their application in microbiological quality control in the food industry, in particular when mild pasteurization technologies are used.

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1. Introduction

Supercritical carbon dioxide (SC-CO_2) has been widely investigated as an innovative technology for the preservation of food products. It is a promising alternative to the traditional processes used to inactivate pathogens [1,2] and spoilage microorganisms [3,4] without negatively affecting the organoleptic properties of products and their overall quality [5,6]. Carbon dioxide (CO_2) is a non-flammable, non-toxic, physiologically safe, chemically inert and readily available gas [7,8]. It reaches the supercritical phase at very mild conditions of pressure and temperature (7.3 MPa, 31 °C), acquiring physical properties such as density, viscosity, and diffusivity with intermediate characteristics between liquids and gases.

Standard cultivation-based methods have earlier been applied to validate the inactivation efficacy of SC-CO_2 treatment on microorganisms spiked on food products as well as the efficiency of other innovative pasteurization techniques [9,10]. Even if these methods for years have been considered the golden standard for microbial count, intrinsic limitations have been acknowledged: first, non-viable bacteria cannot be detected; in addition, some microbial species cannot reliably grow on selective media [11]. Due to these limitations, these methods may induce strong underestimations of actual microbial concentration in the analyzed samples. Under environmental stress conditions (e.g. nutrient limitation, pressure, temperature) many bacterial species may become uncultivable even though remaining viable and potentially becoming more resistant to stress [12,13], leading to potential undesired regrowth phenomena in the processed foodstuffs.

Recently, several approaches have been proposed to discriminate and quantify microbial cells based on their physiological status. Among them, flow cytometry (FCM) combined with

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http://dx.doi.org/10.1016/j.procbio.2014.12.014
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fluorescent markers has been applied for cultivation-independent analysis of bacterial viability in disinfection experiments [14], food biotechnology [15] and environmental microbiology [16–19]. Propidium monoazide quantitative PCR (PMA-qPCR) is another viability test method based on cell membrane permeability [20], and has been applied to quantify intact and permeabilized cells in environmental matrices [21] and in disinfection experiments [22].

The high mortality rate of listeriosis (around 20%) [23] and the persistence of *Listeria monocytogenes* in food-associated environments [24] makes its elimination or reduction a compulsory step before marketing potentially contaminated products. Some countries such as the USA [25], Australia, and New Zealand [26] have adopted a zero tolerance policy for *L. monocytogenes*, where the criterion "not detected in 25 g" is applied before the product leaves the production plant [27]. Introducing culture-independent techniques for microbiological risks assessment will help to overcome the limitations of conventional cultivation methods. Here, we assessed the relative performance of cultural and culture-independent methods for detection and quantification of viable *L. monocytogenes* cells spiked on dry cured ham after SC-CO_2 treatment.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. monocytogenes ATCC19115 (DSMZ, Braunschweig, Germany) was grown on brain heart infusion (BHI) medium (Becton Dickson) at 37 °C for 16 h. One colony was picked up and inoculated into 10 mL of the corresponding broth medium. Bacterial culture was incubated at 37 °C with constant shaking (200 rpm) to stationary phase (16 h). Cells were collected by centrifugation at 6000 rpm for 10 min and re-suspended in 5 mL of phosphate buffered saline solution (PBS, Sigma–Aldrich Co., Milan, Italy).

2.2. Dry cured ham surface contamination

Slices of dry cured ham surface were purchased from a local market, cut in 2 g with a rectangular shape (surface area of about 2 cm^2) and spiked with 50 μL of *L. monocytogenes* at a concentration of 10^9 CFU/mL i.e. 7.5 log(CFU/g) ham. The samples were left 1 h in a sterile chamber at room temperature to let the microbial suspension absorb on dry cured ham and then were loaded in a SC-CO_2 multi-batch apparatus.

2.3. SC-CO_2 treatment

SC-CO_2 treatment was performed in a multi-batch apparatus as described by Mantoan and Spilimbergo [28]. Briefly, the system consisted of 10 identical 15 mL-capacity reactors operating in parallel. All reactors, immersed in the same temperature-controlled water bath to maintain the desired temperature constant during the entire experimental runs, were connected to an on–off valve for independent slow depressurization over approximately 1 min at different treatment times. The solid samples were loaded into the reactors and pressurized with CO_2. To achieve a substantial reduction of *L. monocytogenes* spiked on dry cured ham surface, previously established optimal process conditions [4] were applied: pressure of 8 and 12 MPa, temperature of 45 and 50 °C, treatment time from 5 to 45 min. The operating parameters were continuously recorded by a real time acquisition data system (NATIONAL INSTRUMENTS, field point FP-1000 RS 232/RS 485) and monitored by LabVIEW™ 5.0 software (National Instruments, Milan, Italy). Each process condition was tested in triplicate and results were reported as mean values ± standard deviations.

2.4. Sample homogenization

Untreated and SC-CO_2-treated dry cured ham samples, spiked with *L. monocytogenes* to mimic superficial contamination, were collected and resuspended in 4 mL of PBS in a sterile plastic bag (Reinforced Round Bag-400, International P.B.I., Milan, Italy) and homogenized in a Stomacher 400 instrument (International P.B.I., Milan, Italy) at 230 rpm for 2 min [29]. The resulting homogenate was used for plate counts, FCM and PMA-qPCR experiments.

2.5. Plate counts

L. monocytogenes detection was performed on untreated and treated homogenized samples serially diluted 1:10 in PBS and spread-plated on O.A. *Listeria* agar (Ottaviani and Agosti; Liofilchem, Teramo, Italy). The plates were incubated at 37 °C for 48 h[30]. Three independent experiments were performed for each treatment condition. Bacterial inactivation was expressed as $\log(N/N_0)$, with N and N_0 defined as CFU/g (colony forming unit per gram) present in the treated and untreated sample, respectively. The detection limit of plate counts was 30 CFU/g.

2.6. Flow cytometry

Untreated and treated homogenized samples were diluted 1:10 in PBS. 1 mL of untreated and treated samples were stained with 10 μL SYBR-I (1:30,000 final concentration in DMSO; Merck, Darmstadt, Germany) and 10 μL PI (1 mg/mL; Invitrogen, Carlsbad, CA, USA). Peak excitation and emission wavelengths were λ_{ex} = 495 nm and λ_{em} = 525 nm for SYBR-I and λ_{ex} = 536 nm and λ_{em} = 617 nm for PI. Samples were incubated at room temperature in the dark for 15 min. FCM analyses were performed with an Apogee-A40 flow cytometer (Apogee Flow Systems, Hertfordshire, UK) equipped with an Argon laser emitting at 488 nm. The instrument directly quantifies the suspension concentration without using reference beads. For each cell crossing the focus point of the laser, light scattering signal (FALS) and two fluorescence signals (green, FL1 and red, FL3) were collected. FALS was collected on a 256-channel linear scale while fluorescence signals were collected with logarithmic amplifier gain. FALS was used to distinguish *Listeria* cells and natural flora from food debris. To exclude electronic noise, thresholds were set on green or red fluorescence histograms. The detection limit of FCM was approximately 2000 cells/g.

2.7. PMA-qPCR

The untreated and treated samples were stained with 50 μM PMA (Biotium Inc., Hayward, CA, USA) and incubated at room temperature in the dark for 5 min. Subsequently the samples were exposed to blue light (470 nm) for 15 min using a PhAST BLUE Instrument (GenIUL, Terrassa, Spain). After photo-induced cross-linking, the samples were centrifugated at 6000 × *g* for 10 min. Genomic DNA (gDNA) was extracted from unstained and stained samples using QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Milan, Italy), according to the manufacturer's instructions for gram positive bacteria. Identification and quantification of *L. monocytogenes* cells was performed using primers and TaqMan probe targeting *hlyA* gene as previously described [31]. The reaction mixture contained 1× iQ™ Multiplex Powermix (Bio-Rad Laboratories, Milan, Italy), 200 nM each primer, 200 nM probe and 2 μL template gDNA (or 2 μL distilled H_2O for the no-template control) in a total volume of 25 μL. Each TaqMan PCR assay was performed in triplicate using a CFX96 Real Time PCR Detection System (Bio-Rad Laboratories, Milan, Italy) with the following cycling program: 3 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C for 40 cycles. PCR results were analyzed using CFX Manager 1.1 software (Bio-Rad

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