International Journal of Food Microbiology 152 (2012) 175–180

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Effect of microbial loading on the efficiency of cold atmospheric gas plasma inactivation of *Salmonella enterica* serovar Typhimurium

A. Fernández, N. Shearer, D.R. Wilson, A. Thompson *

Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, UK

ARTICLE INFO

Article history:
Received 21 October 2010
Received in revised form 24 January 2011
Accepted 28 February 2011
Available online 8 March 2011

Keywords:
Cold atmospheric gas plasma
Salmonella Typhimurium
Microbial inactivation
Initial cell concentration

ABSTRACT

In recent years the application of cold atmospheric gas plasma (CAP) aimed at the removal of microbial contamination from fresh and minimally processed food has received increased attention. For CAP to be successfully adopted by the food production industry, factors which affect its potential for microbial inactivation must be evaluated. In this study, we examined the effects of initial microbial concentration, present on filter discs, on the inactivation of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) with nitrogen CAP. It was found that the rate of inactivation of *S.* Typhimurium is inversely proportional to initial bacterial concentration, with the D-value observed at the highest cell concentration assayed (10^8 CFU/filter) being 14 fold higher than seen at the lowest starting concentration (10^5 CFU/filter). Addition of increasing concentrations of *Pseudomonas fluorescens* cells to a *Salmonella* population of 10^5 CFU/filter resulted in an exponential decrease in the rate of killing of the *Salmonella* cells. However, whilst the addition of heat-killed *S.* Typhimurium cells to 10^5 CFU/filter live *S.* Typhimurium cells resulted in a significant decrease in the killing rate, this effect was dose independent. This suggests that although biomass plays a role in the protection against CAP inactivation seen at high cell densities, dead cells and their components released during the heating period are not as effective as viable cells. Fluorescence microscopy showed that, unlike the single dispersed cells observed at low cell densities, at higher cell densities bacteria were present in a multilayered structure. This phenomenon could explain the reduced inactivation by the plasma, since the top layer may present a physical barrier that protects underlying cells. In conclusion, this work clearly shows a link between bacterial cell density and the efficacy of CAP inactivation, making an important contribution to the understanding of this alternative food processing technology, which should be taken into account in both further studies and in the practical application of this technique to the food industry.

1. Introduction

The demand for minimally processed, prepacked, ready-to-eat fruit, vegetables and salads has grown rapidly in recent years. This is due to increasing consumer requirements for high quality convenience foods containing fewer preservatives and with higher nutritive value and fresh sensory attributes (Rico et al., 2007; Zeuthen, 2002).

Concomitant with this trend there has been an increase in the number of recorded outbreaks of foodborne illness associated with fresh products (FDA, 2008). This is due to the fact that these products are consumed raw, without further preparation or cooking which would usually remove microbial contamination. Microbiological safety is therefore a key issue for the entire product range.

The European Food Safety Authority (EFSA) (2009) report on "Foodborne Outbreaks in the European Union in 2007" stated that *Salmonella* is by far the most commonly reported causative agent of foodborne outbreaks. Overall in the EU, *Salmonella* Typhimurium and *Salmonella* Enteritidis are the serovars most frequently associated with human illness. *Salmonellae* have been found to be responsible for food poisoning outbreaks associated with a range of food-produce including sprouted seeds, cantaloupe melons, tomatoes, unpasteurised citrus juices, rocket and lettuce (Jones and Heaton, 2006).

Traditional means to control food spoilage and microbiological safety hazards, such as sterilization, curing or freezing, are not compatible with consumer demands for fresh-like convenience food. Therefore, research efforts are directed towards developing alternative, robust antimicrobial processes (a "kill step") suitable for application to minimally processed foods (Zeuthen, 2002).

An emerging antimicrobial technology for decontaminating infected surfaces is the use of non-thermal ionized gases (cold gas plasmas). Briefly, plasma is composed of gas molecules which have been dissociated by an energy input. It is constituted of particles in permanent interaction. The particles include photons, electrons, positive and negative ions, atoms, free radicals and excited or non-excited molecules that, in combination, have the ability to inactivate microorganisms (Keudell et al., 2010; Yang et al., 2009). Cold atmospheric gas plasma (CAP) treatment, based on the use of ionized gases generated at room temperature and atmospheric pressure, are of particular interest in

* Corresponding author. Tel.: +44 160325 5077; fax: +44 1603507723.
E-mail address: arthur.thompson@bbsrc.ac.uk (A. Thompson).

0168-1605/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2011.02.038

microbial decontamination of food surfaces since they do not require extreme conditions, offering the possibility of treating the surfaces of vegetable tissues (Kayes et al., 2007; Niemira and Sites, 2008; Vleugels et al., 2005).

Implementation of an emerging technology such as CAP, in food preservation, depends on reliable estimations of its efficacy against pathogenic and food spoilage microorganisms. To reach this objective it is necessary to understand the factors which affect microbial resistance. Among these factors, the effect of initial cell concentration is known to be one of the most relevant in food surface treatment strategies (Deng et al., 2005; Moreau et al., 2000). In general, during inactivation of microorganisms on surfaces, the rate of inactivation is inversely proportional to the initial cell concentration (Shintani, 2000). A literature search revealed that there is no information available on the effect of microbial surface loading for killing of *S.* Typhimurium by gas plasmas, in spite of the fact that this bacterium is of great concern for food safety.

Therefore, in this work we examined the effect of initial bacterial concentration on the efficacy of inactivation of *S.* Typhimurium by a cold atmospheric plasma jet and examined the hypothesis that a biomass factor could compromise the CAP induced inactivation of *S.* Typhimurium.

2. Material and methods

2.1. Bacterial strain, media and culture conditions

All experiments were performed using the fully virulent *S.* Typhimurium strain 4/74 (Wray and Soijka, 1978). In order to examine the role of high concentrations of viable cells on the plasma resistance of *S.* Typhimurium, *Pseudomonas fluorescens* A3 (Garrood et al., 2004) was also used. For fluorescence microscopy *S.* Typhimurium strain JH3016 (SL1344 rpsM::gfp+) (Hautefort et al., 2003) was used.

Bacteria stored as frozen stocks were revitalized in luria bertani broth (LB) (Difco) and incubated for 24 h at 25 °C. Subcultures were then prepared by inoculating 0.1 mL into a test tube containing 10 mL of sterile LB broth followed by static incubation at 20 °C for 24 h. Dilutions of harvested cells were prepared in peptone salt dilution fluid (PSDF) and 30 μL aliquots were deposited onto 0.2 μm retention, 25 mm diameter Whatman polycarbonate membrane filters (Fisher Scientific, Loughborough, UK) placed on LB agar plates (LBA, Difco). The filters were then allowed to dry for 15 min in a laminar flow cabinet before plasma treatment.

2.2. Thermal inactivation of S. Typhimurium cells

In order to examine the effect of biomass on the plasma resistance of *S.* Typhimurium, heat-killed *S.* Typhimurium cells were used. To provide heat-killed cells, a bacterial suspension was diluted to 10^8 CFU/mL in preheated (60 °C) PSDF and then heated at 60 °C for 5 min. Complete heat inactivation of samples was confirmed by duplicate plating on plate count agar (PCA) (Difco).

2.3. Plasma inactivation procedure

Plasma treatments were carried out in a commercially available nitrogen plasma jet (CP121 Plasma Demonstrator, OMVE BV, Netherlands) described elsewhere (Mastwijk et al., 2009). The experimental set-up used in this work is shown in Fig. 1.

The CAP system used is based on a copper wire electrode configured as a large bandwidth, high impedance voltage probe. Its electrical potential is perturbed by the afterglow of a jet of excited state nitrogen produced by a high voltage discharge typically sampled at a rate of 1 kHz. The electrode is located 25 mm above the gas outlet. A grid near the electrode, held at the same potential, is used to remove space charge. The system is operated at atmospheric pressure. Under these experimental conditions the temperature of the samples, which was room temperature initially, never exceeded 35 °C. Experiments were performed at a nitrogen throughput of 12 standard litres per minute, power setting 3 (approx. 1 W output power).

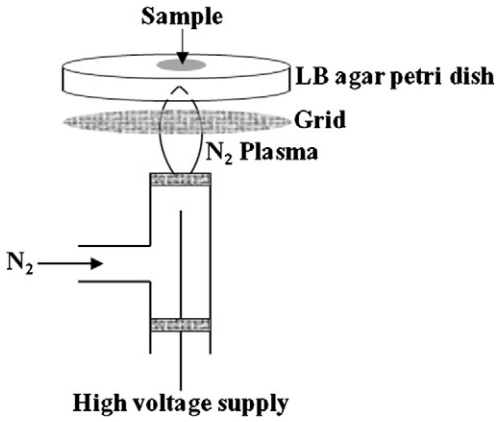

Fig. 1. Overview of the experimental set-up used for the CAP treatment.

Inoculated membrane filters were exposed to plasma conditions for a period up to 15 min, which was the time required to obtain 2 log cycles of inactivation at the highest cell concentration assayed. As a control, bacteria were exposed to nitrogen (discharge turned "off") according to the same time series. It was found that in all cases cell viability remained constant throughout the nitrogen treatment period (data not shown).

Following plasma exposure, each membrane filter was carefully removed from the agar with sterile forceps and placed into stomacher bags containing 10 mL of PSDF, which was used to recover plasma-treated cells from the membrane filters through agitation using a stomacher (Lab System, England). Diluted aliquots were spread on PCA to allow enumeration of surviving bacteria. CFUs of mixed suspensions containing *Salmonella* and *Pseudomonas* cells were determined on xylose lysine deoxycholate agar (XLD agar, Oxoid) to select for *Salmonella*. Preliminary results showed that XLD yielded the same rate of *Salmonella* recovery than the non-selective media TSA (data not shown). The PCA and XLD plates were incubated at 25 °C for 48 h and 37 °C for 24 h, respectively, before enumeration. All experiments were conducted in triplicate using three biologically independent cultures.

2.4. Data analysis

D-values were calculated from the negative inverse slope of the straight portion of survival curves, obtained from a plot of the log number of survivors vs. their corresponding treatment times, using the following equation:

$$\log[N / N_0] = -t / D$$

where:

N = bacterial population at any time, t
N_0 = initial bacterial population
D = decimal reduction time, or time in minutes for the bacterial survival CFU to be reduced by 1 log cycle

The best fit was determined by linear regression (GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego, California, USA). Two-tailed Student's t test was used to determine significant differences between D-values at $p<0.05$ (Steel and Torrie, 1986).

The relationship between D-values and the initial cell concentration ($\log(D) = 0.414 \times \log(N_0) - 2.797$; where N_0 is the initial concentration) was determined from the regression line obtained by plotting log