Food Microbiology 36 (2013) 355–364

Contents lists available at SciVerse ScienceDirect

Food Microbiology

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

Effect of cell immobilization on heat-induced sublethal injury of *Escherichia coli*, *Salmonella* Typhimurium and *Listeria innocua*

Estefanía Noriega [a,b], Eirini Velliou [a,b], Eva Van Derlinden [a,b], Laurence Mertens [a,b], Jan F.M. Van Impe [a,b,*]

[a] *CPMF[2] – Flemish Cluster Predictive Microbiology in Foods, Belgium[1]*
[b] *BioTeC – Chemical and Biochemical Process Technology and Control, Department of Chemical Engineering, KU Leuven, Leuven, Belgium*

ARTICLE INFO

Article history:
Received 18 February 2013
Received in revised form
15 May 2013
Accepted 14 June 2013
Available online 4 July 2013

Keywords:
Sublethal injury
Mild heating
Cell colony
Food model systems
Xanthan gum
Escherichia coli
Salmonella Typhimurium
Listeria innocua

ABSTRACT

The occurrence of sublethally injured cells in foods poses major public health concerns and is an essential aspect when assessing the microbial response to food preservation strategies, yet there is limited research dealing with its specific implications for mild heating. All available studies so far have been performed in broths colonized by planktonic cells, although their susceptibility to lethal agents has often been reported to be markedly different to the stress tolerance of cell colonies developed in solid foods. In this work, the effect of planktonic and colony growth, as well as the influence of colony density on sublethal injury induced by mild heating of *Escherichia coli*, *Salmonella* Typhimurium and *Listeria innocua* were assessed in food model systems. Detection of injured survivors relied on their inability to form visible colonies on salt-based selective media, which do not affect the growth of healthy cells. Sublethal injury (SI) increased rapidly with shorter exposure times and afterwards, decreased progressively, suggesting a mechanism of cumulative damage triggering lethal instead of SI. Cell arrangement affected the degree of SI, higher values being generally found for gelified systems, although the effect of colony density depended on the target microorganism. This information is essential for optimizing the design of food safety assurance systems.

1. Introduction

Food-borne microorganisms are systematically exposed to super- and sub-optimal stresses, arising from either the food environment itself or hurdle strategies implemented throughout the food chain, which may cause physiological alterations of varying degrees, termed sublethal injury (Mossel and Van Netten, 1984; Brashears et al., 2001; Jasson et al., 2007; Wesche et al., 2009).

Sublethal injury (SI) typically affects cell wall or membrane permeability (structural injury), but may also cause extensive damage to various functional cell components (metabolic injury) (Brashears et al., 2001). Due to their impaired metabolism, injured microorganisms may express different physiological and nutritional requirements, as compared to normal cells, and may lose characteristic growth capabilities (Czechowicz et al., 1996). In this sense, sublethal cell damage is macroscopically detectable by the inability to form visible colonies in media containing selective agents (e.g., sodium chloride), to which uninjured cells normally show resistance (Hurst, 1977; Gilbert, 1984; Semanchek and Golden, 1998; Besse et al., 2000; Mackey, 2000; Besse, 2002; Osmanagaoglu, 2005; Kurbanoglu and Algur, 2006; Jasson et al., 2007). This sensitivity to selective media is indicative of membrane damage, to such an extent that the membrane-bound electron transport chain might be altered, resulting in major metabolic alterations (Camper and McFeters, 1979; Ray, 1979; Domek et al., 1987). Accordingly, the difference in plate counts between non-selective media, which support cell recovery and represent both uninjured and injured cells, and the corresponding selective media, to which injured cells become sensitive, is a traditional means to quantify SI as a proportion or percentage of the entire population (Busta, 1976; Restaino et al., 1980; Busch and Donnelly, 1992; Czechowicz et al., 1996; Semanchek and Golden, 1998; Brashears et al., 2001).

SI is responsible for important limitations in food diagnostics related to cell enumeration and detection. For instance,

* Corresponding author. BioTeC – Chemical and Biochemical Process Technology and Control, Department of Chemical Engineering, KU Leuven, Willem de Croylaan 46, B-3001 Leuven, Belgium. Tel.: +32 16 32 14 66; fax: +32 16 32 29 91.
E-mail addresses: estefania.noriegafernandez@cit.kuleuven.be (E. Noriega), eirini.velliou@cit.kuleuven.be (E. Velliou), eva.vanderlinden@cit.kuleuven.be (E. Van Derlinden), laurence.mertens@cit.kuleuven.be (L. Mertens), jan.vanimpe@cit.kuleuven.be, Beatrice.Degeest@cit.kuleuven.be (J.F.M. Van Impe).
[1] www.cpmf2.be.

0740-0020/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fm.2013.06.015

conventional media used in food diagnostics contain a wide variety of selective compounds, which are potentially harmful to injured cells (Restaino et al., 2001; Adams, 2005; Jasson et al., 2007), and this results in an underestimation of survivors levels. Additionally, the food environment itself can act as a toxic medium due to its formulation, pH and presence of salts or antimicrobial compounds (Kilimann et al., 2006). SI may be also responsible for false negative results, since injured cells respond to stress by entering a physiological state that requires specific reparative processes (Agranovski et al., 2003). Accordingly, damaged survivors may show a longer lag phase, compared to healthy cells, due to time needed to repair (Van Houteghem et al., 2008), not reaching often the detection threshold of analytical methods. On the other hand, sufficient recovery time under optimal environmental conditions, for instance during food storage, may allow for the complete restoration of cell activity, depending on the degree of injury (Brashears et al., 2001; Bozoglu et al., 2004; Vermeiren et al., 2006). In fact, systematic exposure to multiple hurdles along the food chain, either intrinsic or extrinsic to foods, that microorganisms need to overcome to ensure their survival, may trigger stress adaptation (Skandamis et al., 2008). Many bacterial pathogens have exhibited increased resistance to commonly applied microbial control strategies, for example cooking, and retained or enhanced their virulence, as a consequence of sublethal injury. Therefore, the presence of sublethally injured cells in foods poses major public health concerns and is a crucial aspect in the assessment of the microbial response to food preservation strategies (Wesche et al., 2009).

Thermal processing has been one of the most widespread and efficient preservation techniques to extend product shelf-life. Nevertheless, detrimental changes in organoleptic and nutritional properties caused by severe heating (Arnoldi, 2002; Devlieghere et al., 2004) and increased consumer demand for minimally processed foods, have encouraged the development of gentle microbial reduction strategies, such as mild heat treatment. However, a relatively short exposure to moderately elevated temperatures may induce sublethal injury (Wuytack et al., 2003; Miller et al., 2006; Gabriel and Nakano, 2010), with very limited research dealing with its specific implications for the microbiological safety of mild-heated foods. All available studies so far have been performed in model or real liquid foods colonized by planktonic or freely suspended cells, which are routinely used for assessing the efficacy of food preservation strategies (Hodges, 2011). However, planktonic cells are often more susceptible to lethal agents compared to cell colonies developed in solid matrices (Perrot et al., 1998; Hodges, 2011). Intra- and inter-colony interactions may also arise in solid matrices as a consequence of the initial cell density, affecting overall microbial physiology (Malakar et al., 2002, 2003). Low inoculum levels normally lead to colony overgrowth, resulting in less active or even dead cells at the colony centre (high-density colonies), whereas high inoculum levels result in much smaller and closer colonies (low-density colonies) (Malakar et al., 2003). Accordingly, planktonic cells, low- and high-density colonies may reasonably show different physiological states, which presumably affect their ability to survive in challenging environmental conditions.

The objective of the present study was to assess the effect of planktonic and colony growth, as well as the influence of colony density on sublethal injury induced by mild heating of *Escherichia coli* and *Salmonella* Typhimurium (Gram-negative) and *Listeria innocua* (Gram-positive) in food model systems. Early stationary-phase cells, previously grown in broth or a xanthan gum-based system, were treated at 54 °C during different exposure times. The gelified medium was inoculated with 10^3 or 10^8 CFU/mL – the relevant thresholds for intra- and inter-colony interactions (Malakar et al., 2002, 2003). In order to estimate the heat-induced sublethal injury, treated cells were recovered on non-selective and selective media, the latter based on the corresponding maximum non-inhibitory concentration of sodium chloride. Survival curves of total and uninjured populations were fit to the inactivation model of Whiting et al. (1996) and the evolution of sublethal injury towards exposure time was estimated, as well as the cumulative sublethal damage for the overall treatment.

2. Materials and methods

2.1. Microorganisms and preculture conditions

E. coli K12 MG1655 was acquired from the *E. coli* Genetic Stock Center at Yale University and stored at −80 °C in Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) supplemented with 25% (v/v) glycerol (Acros Organics, NJ, USA). Inoculum was prepared by transferring a loopful of the stock culture into 20 mL of BHI. After 9.5 h at 37 °C under static conditions (Binder KB-series incubator; Binder Inc., NY, USA), 20 μL of the stationary phase culture were inoculated into 20 mL of fresh BHI and incubated for 15 h under the same conditions.

Salmonella enterica serovar Typhimurium SL1344 was kindly provided by the Institute of Food Research (Norwich, UK) and stored at −80 °C in Luria Bertani (LB) broth with 25% (v/v) glycerol. A stock culture was prepared from this frozen culture by streaking onto a Tryptone Soy Agar plate (Oxoid Ltd., Basingstoke, UK), which was incubated for 24 h at 37 °C and subsequently stored at 4 °C. From this stock culture, which was refreshed monthly, one colony was suspended in 20 mL of Tryptone Soy Broth (TSB; Oxoid Ltd., Basingstoke, UK) and incubated for 9.5 h at 37 °C under static conditions. Subsequently, 20 μL of the cell suspension were transferred into 20 mL of fresh TSB and incubated for 15 h under the same conditions.

A stock culture of *L. innocua* ATCC 33090 strain was kindly donated by the National Institute of Nutrition and Seafood Research (Nofima, Norway) and stored at −80 °C in cryovials (Microbank, Pro-Lab Diagnostics, Canada). Cells were reactivated by transferring one frozen bead into 10 mL of TSB supplemented with 0.6% (w/v) yeast extract (Merck, Darmstadt, Germany) (TSBYE) and incubating at 37 °C overnight. Inoculum was prepared in TSBYE and grown to early-stationary phase as described above for *E. coli* and *S.* Typhimurium.

Cell cultivation under the previously defined conditions yielded early-stationary phase populations of *E. coli*, *S.* Typhimurium and *L. innocua* with a concentration of approximately 10^9 CFU/mL. These cell cultures were used both to inoculate the gelified model systems and to evaluate the heat-induced sublethal injury of planktonic cells.

2.2. Gelified food model systems: preparation and growth conditions

Regarding the formulation of the gelified food model systems, xanthan gum (Xantural® 75; CP Kelco, Surrey, UK) was added to the corresponding broth (BHI, TSB or TSBYE) at a concentration of 1.5% (w/v) and mechanically stirred for at least 30 min (OST 20 basic, IKA Werke GmbH & Co.). Then, 10 mL of the homogenous mixture were dispensed in 50 mL gamma-sterilized conical tubes (TPP, Switzerland) with special pipettes for high viscous media (MICROMAN®, Gilson Inc., USA) and centrifuged for 5 min at 5752 × *g* (Eppendorf 5810 R) to remove entrapped air bubbles. After autoclaving at 121 °C for 25 min, the gelified medium was centrifuged again at 5752 × *g* for 3 min (Mertens et al., 2009).

Initial cell density in the gelified systems was adjusted to 10^3 and 10^8 CFU/mL by diluting a sample of the corresponding stationary-phase broth culture (see Section 2.1), in order to get