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# Effect of the substrate's microstructure on the growth of *Listeria monocytogenes*

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ABSTRACT

The effect of the microstructure of the medium on the growth of the food-borne pathogen *Listeria monocytogenes* was studied. The pathogen's growth kinetics was evaluated using liquid substrates and gels formed from different concentrations of sodium alginate (3.0% w/w) and gelatin (0–30.0% w/w). These results were further verified using a model dairy product with solid concentrations varying from 10.0 to 40.0% w/w. The pathogen's growth was faster in the liquid media than in the gels regardless of the gelling agent employed. The substrate's microstructure, apart from altering the growth pattern from planktonic to colonial, resulted in microbial growth suppression; however, each system affected the microorganism's growth in a different way. The suppressing effect of the substrate's microstructure on microbial growth was also dependent on temperature, while the presence of glucose in the solid medium accelerated microbial growth, thus reducing substantially the difference in growth kinetics between the gels and the liquid media. Any increase in the hydrocolloid concentration, which was also reflected in the rheological properties of the structured samples, resulted in a reduction of growth rate and in an increase of the lag phase of the pathogen. Overall, the gelation of the medium was found to exert a stress on the microorganism since the sol–gel transition, when the pathogen was already at the exponential growth phase, resulted in an additional lag phase or a decrease in the growth rate. The relationship between maximum specific growth rate and loss tangent of the gels ($tan\delta = G''/G'$) was explored, pointing to the possible use of a single structural parameter to describe food matrix effects on microbial growth kinetics.



## 1. Introduction

Over the last 30 years, *Listeria monocytogenes* has become increasingly important as a food-associated pathogen. Although, human listeriosis is not very usual, with the European Union incidence rate being 0.41 cases per 100,000 citizens, the EU case fatality rate was 17.8% among the majority of the confirmed cases. *L. monocytogenes* infections are also responsible for the highest hospitalization rates (91.6%) among all zoonoses under EU surveillance, while the majority of the cases were domestically acquired (EFSA/ECDC, 2014).

Food products constitute different types of ecosystems, depending on the environment and the microorganisms that are harbored (Mossel & Ingram, 1955), and as composite matrices of multiple constituents and phases are characterized by great structural complexity. In order to study microbial growth in such systems and to elucidate how specific microorganisms impact food safety and quality, the way that food microstructure (structure on micro-level) affects the growth of different microorganisms has to be explored (Robins & Wilson, 1994).

Microbial growth takes place in the aqueous phase of food products. The majority of food products are solid or semi-solid structured systems and are differentiated from liquid homogeneous media where microbial cell mobility is not constrained, displaying a planktonic growth pattern (Wilson et al., 2002). In many cases, however, structure in the aqueous phase of a food matrix is induced by gelation of different components in the medium. Food microstructure thus becomes a key element in order to control the microbial growth in foods, and thereby affects food safety in many ways, while it determines the method of antimicrobial agents' incorporation in formulated products (Corbo et al., 2009) as well as the redistribution of the added preservatives between the different phases in emulsions (Brocklehurst & Wilson, 2000).

The main effect of food microstructure on microbial growth is the fact that cells may become immobilized and their growth pattern turns from planktonic to colonial. Cell immobilization and growth in a structured medium may also cause alteration of cell morphology and physiological responses, affecting the thermal inactivation tolerance of the microorganisms (Mogollón, Marks, Booren, Orta-Ramirez & Ryser, 2009; Prachaiyo & McLandsborough, 2003), susceptibility to antibiotics (Jouenne, Tresse, & Junter, 1994; Junter, Coquet, Vilain, & Jouenne, 2002), protection from physicochemical stresses and cell membrane modification through the gene expression of some specific proteins

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(Junter, Coquet, Vilain, & Jouenne, 2002). Cell immobilization and colonial growth also modify the local conditions in the growth environment; e.g., cell metabolism changes the substrate composition around the colony (Wimpenny et al., 1995) and acidic metabolites are accumulated, causing a drop of pH inside and around the colony (Malakar et al., 2002; Walker, Brocklehurst, & Wimpenny, 1997), and creating pH gradients in the growth medium (Walker, Brocklehurst, & Wimpenny, 1997; Wimpenny et al., 1995). In the case of *L. monocytogenes*, this pH drop is able to stimulate an auto-induced acid tolerance response (Kroll & Patchett, 1992), resulting in reduced susceptibility to added growth inhibitory agents (Wilson et al., 2002), but also providing cross-protection against other environmental stresses as well as increased virulence, possibly attributed to enhanced ability to overcome the harsh *in vivo* conditions (O'Driscoll, Gahan, & Hill, 1996).

Food microstructure and immobilization of the microbial cells influence growth kinetics as they appear to exert additional stresses onto microbial growth (Brocklehurst, Mitchell, & Smith, 1997; Wilson et al., 2002). The microstructure of the growth medium may also influence the effect of the environment including temperature, water activity and pH on *L. innocua* growth (Antwi et al., 2006). Several studies have indeed shown that immobilized cells exhibit different metabolic activities (Skandamis, Tsigarida, & Nychas, 2000) and a slower growth rate compared to planktonic cells (Robins & Wilson, 1994). Wilson et al. (2002) have reported that cells of *Bacillus cereus* immobilized in gelatin gel exhibited a reduced growth rate compared to cells grown in broth. The same observations have been reported for *Aspergillus carbonarious* immobilized in gel where except for the reduced growth rate (estimated as biomass increase), the production of ochratoxin A was also reduced compared to that in liquid medium (Huang, Chapman, Wilson, & Hocking, 2009; Kapetanakou, Ampavi, Yanniotis, Drosinos, & Skandamis, 2011). Another feature that is related to immobilization and growth in a structured medium is a reduction in the growth yield (Brocklehurst, Parker, Gunning, Coleman, & Robins, 1995; Robins & Wilson, 1994). Moreover, Koutsoumanis, Kendall, and Sofos (2004) and Meldrum, Brocklehurst, Wilson, and Wilson (2003) have reported that cell immobilization may lead to narrower growth boundaries, concerning other environmental factors influencing bacterial growth.

So far, most available predictive models for microbial growth are based on experimental data from liquid laboratory media and thus do not consider the significant effect of differentiated diffusion rates of nutrients, oxygen and metabolic end products, and of the mechanical constraints that microbial cells may experience due to the microstructure of the substrate (Robins & Wilson, 1994). This constitutes a *completeness error*, as described by Ross, McMeekin, and Baranyi (1999), and limits the ability of any microbial growth model to accurately predict the safety and quality of structured real food products, leading to fail-safe predictions and thereby causing economical losses. As a result, an extensive amount of research has been carried out recently to study structured systems using (mostly) agar or gelatin as solidifying agents in order to mimic the microstructure of gelled food products like pâté, cheeses or emulsion gels, like sausages (Noriega, Laca, & Díaz, 2008, 2009; Wilson et al., 2002). However, gels made from different gelling agents are differentiated regarding their properties and microstructure. Furthermore, the gel microstructure properties are affected by the concentration of the biopolymer used and by processing and storage conditions. A different microstructure may influence the impact each gel network exerts on microbial cells. To tackle this, diverse microstructures should be studied in order to clarify the influence of microstructure and to identify a universal parameter to describe the effect of substrate's microstructure on the microbial growth kinetics. Sodium alginate and gelatin are typical biopolymers that form self-supporting gels and are used in many food and pharmaceutical products as structuring hydrocolloids.

Alginates are polysaccharides produced by brown seaweeds and algae. They are linear copolymers of D-mannuronic acid and L-guluronic acid. Each chain normally contains continuous blocks of the constituent sugars and regions where the two residues alternate (Smidsrød, 1970). Divalent cation ($Ca^{2+}$) binding and gel formation are the most important features of alginates. This property is attributed to a specific and strong interchain interaction between stretches of guluronic acid blocks (G-blocks) and the divalent ions leading to the formation of junction zones. Grant, Morris, Rees, Smith and Thom (1973) have proposed the egg-box model to describe this gelling mechanism. Each cross-linking $Ca^{2+}$ ion interacts with two adjacent G residues as well as with two adjacent residues in the opposing chain; this physical ionotropic interaction connecting alginate chains results in hydrogel formation (Donati & Paoletti, 2009). Gelatin is readily dissolved in water when heated to 40–50 °C and it remains in random coil conformation, unless the solution is cooled below 30 °C where a reverse coil to helix transition takes place. Then the gelatin molecules reassemble to a triple-helix conformation and form a thermoreversible viscoelastic gel network stabilized by extensive interchain H-bonding (Djabourov, Leblond, & Papon, 1988).

The aim of the present work was to explore the effect of growth medium microstructure on the growth of the food-borne pathogen *L. monocytogenes*. Specifically, the main objectives were: i) to study the growth kinetics of *L. monocytogenes* when inoculated in gels formed by different biopolymers, ii) to evaluate the influence of each gelling system on the growth of the pathogen, and iii) to investigate the factors affecting the influence of microstructure on microbial growth such as the storage temperature and the presence and concentration of glucose in the growth medium. The endmost aim of the study was to investigate structural parameters that could be included in predictive growth models, to take into account the influence of microstructure on microbial proliferation and thus to simulate the environment of real food product matrices.

## 2. Materials and methods

### 2.1. Microorganism and inoculum preparation

*L. monocytogenes* FSL R2-500 (serotype 4b), which was isolated from a Mexican type cheese during a listeriosis outbreak (in 2000) in North Carolina, United States, was used. The isolate was kindly provided by Dr. Martin Wiedmann (Cornell University, Ithaca, New York) and deposited in the strain collection of the Laboratory of Food Microbiology and Hygiene of Aristotle University of Thessaloniki. The stock culture was stored frozen (−70 °C) onto Microbank™ porous beads (Pro-Lab Diagnostics, Ontario, Canada), whereas the working culture was stored at 5 °C on Brain Heart Infusion Agar (BHIA, LAB M, Lancashire, United Kingdom) slants and was renewed bimonthly. The inoculum was activated by transferring a loopful from the BHIA slant into 9 mL of Brain Heart Infusion Broth (BHI, LAB M) and incubated at 30 °C for 24 h. Aliquots of the activated culture were transferred into 9 mL of fresh BHI, incubated at 30 °C for 24 h and then the cultures were used for the preparation of the test inoculum. The initial concentration of the inoculum was determined by viable plate counting on BHIA.

For the experiments with milk powder, after the activation of the culture, portions of the activated culture were transferred into 9 mL of fresh BHI and incubated at 30 °C. 24 h-cultures were centrifuged at 6000 rpm for 20 min at 4 °C. The harvested cells were then washed with 9 mL of quarter-strength Ringer's solution (Lab M) and centrifuged as described previously. The harvested cells of the washed cultures were resuspended in 9 mL of Ringer's solution.

### 2.2. Kinetic analysis of microbial growth

In all the experiments, the samples were analyzed in appropriate time intervals to obtain the effective kinetic analysis of microbial growth. Two or four independent experiments were conducted at each set of growth conditions.