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# Modelling the microbial dynamics and antimicrobial resistance development of *Listeria* in viscoelastic food model systems of various structural complexities

Katherine M. Costello[a], Jorge Gutierrez-Merino[b], Madeleine Bussemaker[a], Marco Ramaioli[a], Maria Baka[c], Jan F. Van Impe[c], Eirini G. Velliou[a,*]

[a] Bioprocess and Biochemical Engineering Group (BioProChem), Department of Chemical and Process Engineering, University of Surrey, Guildford GU2 7XH, UK.
[b] School of Biosciences and Medicine, University of Surrey, Guildford, GU2 7XH, UK.
[c] Chemical and Biochemical Process Technology and Control Laboratory (BioTeC+), KU Leuven, Sustainable Chemical Process Technology, Campuses Ghent & Aalst, Gebroeders De Smetstraat 1, 9000 Ghent, Belgium.

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ABSTRACT

Minimal processing for microbial decontamination, such as the use of natural antimicrobials, is gaining interest in the food industry as these methods are generally milder than conventional processing, therefore better maintaining the nutritional content and sensory characteristics of food products.

The aim of this study was to quantify the impact of (i) structural composition and complexity, (ii) growth location and morphology, and (iii) the natural antimicrobial nisin, on the microbial dynamics of *Listeria innocua*.

More specifically, viscoelastic food model systems of various compositions and internal structure were developed and characterised, i.e. monophasic Xanthan gum-based and biphasic Xanthan gum/Whey protein-based viscoelastic systems. The microbial dynamics of *L. innocua* at 10 °C, 30 °C and 37 °C were monitored and compared for planktonic growth in liquid, or in/on (immersed or surface colony growth) the developed viscoelastic systems, with or without a sublethal concentration of nisin. Microscopy imaging was used to determine the bacterial colony size and spatial organisation in/on the viscoelastic systems.

Selective growth of *L. innocua* on the protein phase of the developed biphasic system was observed for the first time. Additionally, significant differences were observed in the colony size and distribution in the monophasic Xanthan gum-based systems depending on (i) the type of growth (surface/immersed) and (ii) the Xanthan gum concentration. Furthermore, the system viscosity in monophasic Xanthan gum-based systems had a protective role against the effects of nisin for immersed growth, and a further inhibitory effect for surface growth at a suboptimal temperature (10 °C).

These findings give a systematic quantitative insight on the impact of nisin as an environmental challenge on the growth and spatial organisation of *L. innocua*, in viscoelastic food model systems of various structural compositions/complexities. This study highlights the importance of accounting for system structural composition/complexity when designing minimal food processing methods with natural antimicrobials.

## 1. Introduction

In recent years, there has been an increasing consumer-driven demand for food products high in nutritional content and sensory characteristics (flavour/texture) which (i) have as few chemical preservatives as possible and (ii) have undergone minimal processing (Baka et al., 2015; Valdramidis and Koutsoumanis, 2016). Consequently, there is an increased interest in the food industry in minimal food processing to replace classical decontamination techniques. Natural antimicrobial compounds including bacteriocins produced by microorganisms such as lactic acid bacteria (LAB) are an emerging method of interest for microbial decontamination, and have been shown to act against food-related pathogenic bacteria (for example Ale et al., 2015; Bhatti et al., 2004; Gutierrez and Bourke, 2009; Mariam et al., 2014; Zapico et al., 1998). Additionally these antimicrobial compounds have the potential for use in combination with non-thermal processing technologies to inactivate food-borne bacteria (Liao et al., 2018; Muñoz et al., 2012; Ross et al., 2003).

* Corresponding author.
*E-mail address:* e.velliou@surrey.ac.uk (E.G. Velliou).

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Nisin is one such natural antimicrobial; it is generally recognised as safe by the Food and Drug Administration (FDA), and it is one of the only bacteriocins approved by the European Union for use in foods, thus it is currently used in the food industry as a preservative (Abee et al., 1994; Collins et al., 2010; Gharsallaoui et al., 2016). However, the efficiency of nisin for microbial decontamination is still unclear, with the majority of studies still conducted in liquid broths despite most food products being solids (Boziaris et al., 1998; Collins et al., 2010; Kim et al., 2001; Li et al., 2010). There are studies that have been performed in real food products (for example Benkerroum and Sandine, 1988; Bhatti et al., 2004; Bukvički et al., 2014; Colak et al., 2008; Eshamah et al., 2014; Govaris et al., 2010; Pawar et al., 2000; Zapico et al., 1998), which can be informative only for the specific food system under study. *A fundamental understanding of the correlation of structural effects with the antimicrobial activity of nisin is currently lacking.*

Cells in a solid system grow as colonies and experience a completely different environment as compared to (liquid) planktonic growth. More specifically, in a solid system, microorganisms evolve as colonies and due to diffusional limitations of oxygen and nutrients, as well as the accumulation of (acidic) metabolic products around the colony, microorganisms may experience a self-induced (acid) stress that could affect their overall kinetics and response to various environmental factors (Aspridou et al., 2014; Noriega et al., 2013; Velliou et al., 2011a, 2011b). Additionally, microorganisms grown as colonies could display/develop a different level of antimicrobial resistance due to environmental stress adaptation and cross protection (Baka et al., 2013; Velliou et al., 2010, 2012, 2013; Yousef and Juneja, 2003). For example, significant differences in microbial kinetics have been reported in food model systems as compared to liquid broths for both growth (Antwi et al., 2008; Brocklehurst et al., 1997; Noriega et al., 2010a; Skandamis and Jeanson, 2015; Theys et al., 2009; Wilson et al., 2002) and inactivation studies (Baka et al., 2017b; Velliou et al., 2013; Wang et al., 2017). This phenomenon of environmental stress adaptation and cross protection is a significant concern, particularly for minimal food processing, as these treatments are naturally milder than traditional food processing and could lead to an increased microbial resistance and survival, resulting in unsafe food products (FDA, 2000; NovelQ, 2011). Therefore, it is important to conduct systematic kinetic experiments in food model systems of controlled composition, complexity and rheology, in order to obtain a fundamental understanding of the impact of system structure on the development of antimicrobial resistance in microbial species.

The most commonly used food model systems are monophasic and homogeneous and are composed by the addition of gelling agents such as gelatin, agar, Xanthan gum, and κ-carrageenan, in a nutrient broth such as Tryptic Soy Broth (TSB) or Brain Heart Infusion (BHI) (for example, Aspridou et al., 2014; Das et al., 2015; Mertens et al., 2009; Skandamis et al., 2007; Theys et al., 2009; Velliou et al., 2013; Wang et al., 2017). There are very few studies in more complex food models where, to better mimic the complex structure of a food product, a secondary phase is added, creating a heterogeneous system. More specifically, by combining a protein gelling agent (i.e. gelatin, sodium caseinate) and a polysaccharide gelling agent (i.e. dextran, sodium alginate) in suitable concentration, ratio, and ionic strength, a biphasic heterogeneous system is formed (Boons et al., 2013a, 2014, Léonard et al., 2013, 2015).

The aim of this work was to obtain a systematic comparative study of the kinetics, colony size and spatial organisation of *Listeria innocua*, as influenced by the presence of nisin on (surface growth) or in (immersed growth) viscoelastic food models of various structural complexities, i.e., (i) Xanthan gum-based monophasic systems of various viscosities and (ii) a Xanthan gum/Whey protein isolate biphasic system.

More specifically, in this study, the growth dynamics of *L. innocua* were monitored in/on these viscoelastic systems at the lower and upper limit of optimal temperature range, i.e., 30 °C and 37 °C, and at suboptimal temperature, i.e., 10 °C (Ryser and Marth, 2007). The systems were rheologically characterized with respect to the viscoelastic response and temperature stability, and the heterogeneous structure of the biphasic gels was visualised using confocal laser scanning microscopy (CLSM). The distribution and size of the microbial colonies were investigated using CLSM or light microscopy, for all viscoelastic food model systems. *Listeria* is investigated in the present study because it is associated with ready-to-eat foods and with high mortality rates (EFSA, 2017). For example, 16.2% of listeriosis cases in 2016 resulted in fatality, while by comparison *E. coli* infections had a 0.3% fatality rate, and *Salmonella* infections a 0.1% fatality rate in the same year (EFSA, 2017). Furthermore, some *Listeria* species (*L. monocytogenes* and *L. innocua*) are shown to have a tolerance to nisin (Bergholz et al., 2013; Collins et al., 2010; Zhou et al., 2014), with a small proportion demonstrating resistance to some clinically relevant antibiotics (Escolar et al., 2017; Gómez et al., 2014; Granier et al., 2011; Nielsen et al., 2017). While *L. monocytogenes* is known to be more susceptible to antimicrobials than other *Listeria* species, it is also known that resistance genes may be transferred between bacterial species (Charpentier and Courvalin, 1999; Chen et al., 2010; Gómez et al., 2014; Nielsen et al., 2017). It is therefore important to investigate the stress response and adaptation of *Listeria* species to novel processing technologies to minimise the risk of increasing resistance.

Xanthan gum and Whey protein isolate were used in this study as they are both widely used in the food industry and they are thermally stable at a wide range of temperatures. More specifically, Xanthan gum is used in products such as sauces, dressings, bakery products (Kang and Pettitt, 1993), and thickeners to manage swallowing disorders (Mowlavi et al., 2016). Whey proteins are used for their nutritional and functional properties as nutrient supplements in infant formula, dietetic and health foods, and as emulsifiers (Bryant and McClements, 2000; de Wit, 1998). Therefore, Xanthan gum and Whey protein isolate model systems are a good surrogate to mimic the structure of a real food product, and they have not been widely used in microbial growth studies. Furthermore, based on the ratio of the protein/polysaccharide, a range of mono- and biphasic model systems can be created to represent the internal structure of a wide range of food products with which *Listeria* species are associated, for example soft cheeses, cheeses in various stages of ripening, pâtés, and meats (Lopez et al., 2006; Noriega et al., 2010a; Norton and Frith, 2001).

## 2. Materials and methods

### 2.1. Inoculum preparation

Stock cultures of *L. innocua* (ATCC 33090), a surrogate for the foodborne pathogen *L. monocytogenes*, were stored at −80 °C in Tryptic Soy Broth (TSB, Oxoid Ltd., UK), supplemented with 15% v/v glycerol. A loopful of thawed culture was inoculated in 15 ml TSB supplemented with 0.6% w/v yeast extract (Oxoid Ltd., UK) (TSBYE) for 9.5 h at 37 °C. 20 μl was subsequently transferred to fresh 15 ml TSBYE and cultured for 15 h at 37 °C until early stationary phase was reached (approximately $10^9$ CFU/ml).

### 2.2. Preparation of viscoelastic food model systems

For monophasic viscoelastic systems, Xanthan gum (XG) (Xantural® 75; CP Kelco, UK) was added to TSBYE at concentrations of 3%, 5% or 7% w/v and mechanically stirred for at least 5 min until fully homogenised (Omni Mixer Homogenizer, Omni International Inc., USA). These concentrations of XG were selected to be of higher viscosity than previously investigated (Ale et al., 2015; Boons et al., 2013b; Mertens et al., 2009; Velliou et al., 2013), and to represent a variety of viscoelastic food products. Additionally, it was experimentally impossible to prepare reproducible surface growth systems for XG concentrations below 3% due to their comparatively low viscosity. The homogenised