AGRICULTURAL UNIVERSITY OF ATHENS
SCHOOL OF FOOD AND NUTRITIONAL SCIENCE
LABORATORY OF FOOD QUALITY CONTROL AND HYGIENE

PhD Thesis

Investigating the phenotypic response and underpinning mechanisms of inter-strain interactions of *Listeria monocytogenes* in foods and simulated food environments

Maria A. Gkerekou

Supervisor:
Dr. Panagiotis N. Skandamis, Professor, Agricultural University of Athens

Co-supervisors:
Dr. Eleftherios Drosinos, Professor, Agricultural University of Athens
Dr. Nikolaos Chorianopoulos, Associate Professor, Agricultural University of Athens

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Μελέτη του φαινότυπου και των εμπλεκόμενων μηχανισμών που ευθύνονται για τις δια-στελεχιακές αλληλεπιδράσεις του παθογόνου *Listeria monocytogenes* στα τρόφιμα και σε συστήματα προσομοίωσης τροφίμων

Maria A. Gkerekou

Thesis committee:
Dr. Panagiotis N. Skandamis, Professor, Agricultural University of Athens
Dr. Eleftherios Drosinos**,** Professor, Agricultural University of Athens
Dr. Nikolaos Chorianopoulos, Associate Professor, Agricultural University of Athens
Dr. Konstantinos Koutsoumanis, Professor, Aristotle University of Thessaloniki
Dr. Kalliopi Rantsiou, Professor, University of Torino
Dr. George Th. Tsangaris, Staff Research Scientist, Academy of Athens
Dr. Konstantinos Papadimitriou, Assistant Professor, Agricultural University of Athens

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Department of Food Science and Human Nutrition
Laboratory of Food Quality Control and Hygiene

Abstract

Listeria monocytogenes, a ubiquitous environmental microorganism, exhibits a remarkable ability to survive and grow in diverse environments, constituting a significant concern for ready-to-eat (RTE) products. Previous studies have identified the simultaneous presence of multiple *L. monocytogenes* strains, possibly introduced during processing. The coexistence in/on the same food products and subsequent invasion during ingestion adds complexity to the understanding of the pathogen's behaviour. Inter-strain interactions leading to growth inhibition have been demonstrated in substrates containing more than one *L. monocytogenes* strains.

The objectives of the thesis encompassed a comprehensive evaluation of different *L. monocytogenes* strains (C5, 6179, ScottA and PL25) behaviour during various storage conditions. Specifically, evaluated the growth and inter-strain interactions in/on different matrices, such as laboratory media, dairy-based model systems, and actual Ricotta and Camembert products. The study also examined the impact of co-culture on survival during exposure to simulated gastric fluid, growth dynamics in/on different media, and the role of cell proximity on the time to first division. Intracellular proteins and metabolic fingerprints were analysed to uncover mechanisms behind observed strain inhibition during co-culture.

Chapter 2, revealed that strain-dependent variations occur under different oxygen and substrate structure conditions. Liquid media under aerobic conditions favoured higher final populations. Notably, inter-strain interactions were more pronounced in liquid substrates, as well, while "weaker" strains consistently reached lower final populations, irrespective of oxygen conditions and strain combinations. **Chapter 3** highlighted nutritional composition differences affecting *L. monocytogenes* growth in/on Ricotta- and Camembert-based media. Inter-strain interactions were more pronounced in dairy-based broths, mitigated by agar addition and solidification, while anoxic conditions prolonged lag phases. **Chapter 4** explored matrix adaptation effects, indicating that adaptation did not significantly alter growth or inter-strain interactions. Cheese matrix influenced inter-strain interactions, contributing to an understanding of serotype dominance in safety-concerned foods. Camembert-induced habituation leads to acid sensitization, affecting exposure to simulated gastric fluid. **Chapter 5** demonstrated yeast extract's impact on pathogen's growth and revealed that nutrient renewal did not overcome suppression effects during co-culture. Cell-free spent media analysis showed complex differences, with no observed inter-strain interactions during growth. **Chapter 6** demonstrated that the density and relative proximity of observed cells, whether single or dual strains, influenced the time to the first division. Interestingly, the co-culture of different strains had no significant impact on time to first division. Finally, **Chapter 7** identified numerous intracellular proteins produced uniquely during co-culture, including the luxS enzyme associated with quorum sensing. "Moonlighting" proteins, multifunctional proteins, were also identified.

Overall, the thesis findings underscore how substrate characteristics, oxygen availability, and nutritional factors influence inter-strain interactions. The coexistence of multiple strains in the same food product is crucial for understanding listeriosis outbreaks, contributing valuable insights into the mechanisms of growth inhibition, which appear to involve a combination of contact-dependent inhibition and quorum sensing mechanisms.

Scientific area: Food Safety

Keywords: *L. monocytogenes*, inter-strain interactions, structure, oxygen availability, Ricotta, Camembert, simulated gastric fluid, FTIR-ATR, cell-free spent medium, intracellular proteins

Μελέτη του φαινότυπου και των εμπλεκόμενων μηχανισμών που ευθύνονται για τις δια-στελεχιακές αλληλεπιδράσεις του παθογόνου Listeria monocytogenes στα τρόφιμα και σε συστήματα προσομοίωσης τροφίμων

Τμήμα Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου
Εργαστήριο Ποιοτικού Ελέγχου και Υγιεινής Τροφίμων και Ποτών

Περίληψη

Ο παθογόνος *Listeria monocytogenes* είναι ένας πανταχού παρών περιβαλλοντικός μικροοργανισμός, που επιδεικνύει μια αξιοσημείωτη ικανότητα να επιβιώνει και να αναπτύσσεται σε διαφορετικά περιβάλλοντα, αποτελώντας σημαντική ανησυχία για τα έτοιμα προς κατανάλωση προϊόντα (ΕΚΤ). Προηγούμενες μελέτες έχουν εντοπίσει την ταυτόχρονη παρουσία πολλαπλών στελεχών *L. monocytogenes* στο ίδιο τελικό προϊόν, που πιθανώς το επιμόλυναν κατά την αρασκευή ή/και επεξεργασία του. Η συνύπαρξη στα ίδια τρόφιμα και η επακόλουθη λοίμωξη που μπορεί να προκληθεί κατά την κατάποση προσθέτει πολυπλοκότητα στην κατανόηση της συμπεριφοράς του παθογόνου. Αλληλεπιδράσεις μεταξύ στελεχών που οδηγούν σε αναστολή ανάπτυξης έχουν αποδειχθεί σε υποστρώματα που περιέχουν περισσότερα από ένα στελέχη *L. monocytogenes*.

Οι στόχοι της διατριβής περιελάμβάνουν μια ολοκληρωμένη αξιολόγηση της συμπεριφοράς διαφορετικών στελεχών του μικροοργανισμού *L. monocytogenes* (C5, 6179, ScottA και PL25) κατά τη διάρκεια διαφόρων συνθηκών συντήρησης. Συγκεκριμένα, η μελέτη αξιολόγησε την ανάπτυξη και τις αλληλεπιδράσεις μεταξύ των στελεχών σε διαφορετικές υποστρώματα, με βάση εργαστηριακά υλικά, συστήματα προσομοίωσης τροφίμων που βασίζονται σε γαλακτοκομικά προϊόντα και πραγματικά προϊόντα Ricotta και Camembert. Η μελέτη εξέτασε επίσης τον αντίκτυπο της συνκαλλιέργειας στην επιβίωση κατά την έκθεση σε συνθετικό γαστρικό υγρό, τη δυναμική ανάπτυξης σε διαφορετικά υποστρώματα υπό την αξιολόγηση του ρόλου της εγγύτητας των κυττάρων στο χρόνο πρώτου διπλασιασμού. Οι ενδοκυτταρικές πρωτεΐνες και τα μεταβολικά δακτυλικά αποτυπώματα που προέκυψαν από την ανάλυση με FTIR-ATR των cell-free υπερκειμένων, μετά από καλλιέργιεργεια μεμονομένων ή σε ζευγάρια στελεχών, αναλύθηκαν για να αποκαλυφθούν μηχανισμοί πίσω από την παρατηρούμενη αναστολή του στελέχους κατά τη διάρκεια της συγκαλλιέργειας.

Το **Κεφάλαιο 2** αποκάλυψε ότι η συμπεριφορά των στελεχών διαφοροποιήθηκε κάτω από διαφορετικές συνθήκες διαθεσιμότητας οξυγόνου και δομής υποστρώματος. Τα υγρά υποστρώματα, υπό αερόβιες συνθήκες, ευνόησαν την ανάπτυξη σε υψηλότερους τελικούς πληθυσμούς. Παράλληλα στα υγρά υποστρώματα ήταν πιο έντονες και οι αλληλεπιδράεις μεταξύ των στελεχών, ενώ τα «ασθενέστερα» στελέχη έφτασαν σταθερά σε χαμηλότερους τελικούς πληθυσμούς, ανεξάρτητα από τη διαθεσιμότητα οξυγόνου και τους συνδυασμούς στελεχών. Το **Κεφάλαιο 3** τόνισε την επηροή που μπορεί να έχιε η διατροφική σύνθεση του τροφίμου στην ανάπτυξη του *L. monocytogenes* σε υποστρώματα που βασίζονται σε Ricotta και Camembert. Οι αλληλεπιδράσεις μεταξύ των στελεχών ήταν πιο έντονες στα υγρά υποστρώματα με βάση τα γαλακτοκομικά προϊόντα. Η ένταση των αλληλεπιδράσεων μετριάστηκε από την προσθήκη άγαρ και τη στερεοποίηση, ενώ οι ανοξικές συνθήκες παρέτεινε την φάση προσαρμογής. Το **Κεφάλαιο 4** διερεύνησε την επίδραση της ανάπτυξης του παθογόνου στα προϊόντα Ricotta και Camembert, υποδεικνύοντας ότι η προσαρμογή δεν άλλαξε σημαντικά την ανάπτυξη ή τις αλληλεπιδράσεις μεταξύ των στελεχών. Η "προσαρμογή" μετά από ανάπτυξη στο Camembert οδήγησε σε μείωση της ανθεκτικότητας στο χαμηλό pH του συνθετικού γαστρικού υγρού. **Το Κεφάλαιο 5** κατέδειξε την επίδραση του εργαστηριακού συστατικού yeast extract, στην ανάπτυξη του παθογόνου και αποκάλυψε ότι η ανανέωση των θρεπτικών συστατικών δεν ήταν ικανή να ανατρέψει το φαινόμενο της καταστολής της ανάπτυξης κατά τη συγκαλλιέργεια. Η ανάλυση των προφίλ των cell-free υπερκειμένων έδειξε διαφοποποιήσεις μεταξύ των μεμονομέμων και σύνθετων καλλιεργειών και κατά την ανάπτυξη στα cell-free υπερκείμενα δεν παρατηρήθηκαν αλληλεπιδράσεις μεταξύ των στελεχών κατά την ανάπτυξη. Το **Κεφάλαιο 6** έδειξε ότι η πυκνότητα και η σχετική εγγύτητα των παρατηρούμενων κυττάρων, είτε κατά την παρατήρηση μεμονωμένων στελεχών είτε σε ζεύγη, επηρέασαν το χρόνο πρώτου διπλασιασμού. Τέλος, το **Κεφάλαιο 7** αναγνώρισε πολυάριθμες ενδοκυτταρικές πρωτεΐνες που παράγονται μοναδικά κατά τη συνκαλλιέργεια,

συμπεριλαμβανομένου του ενζύμου luxS που σχετίζεται με την ανίχνευση απαρτίας. Εντοπίστηκαν επίσης πρωτεΐνες "Moonlighting", που αναφέρονται στη βιβλιογραφία ως πολυλειτουργικές πρωτεΐνες.

Συνολικά, τα ευρήματα της διατριβής υπογραμμίζουν πώς τα χαρακτηριστικά του υποστρώματος, η διαθεσιμότητα οξυγόνου και οι διατροφικοί παράγοντες επηρεάζουν τις αλληλεπιδράσεις μεταξύ των στελεχών. Η συνύπαρξη πολλαπλών στελεχών στο ίδιο προϊόν διατροφής είναι ζωτικής σημασίας για την κατανόηση των κρουσμάτων λιστερίωσης, συμβάλλοντας σε πολύτιμες γνώσεις για τους μηχανισμούς αναστολής της ανάπτυξης, οι οποίοι φαίνεται να περιλαμβάνουν έναν συνδυασμό αναστολής εξαρτώμενης από την επαφή και μηχανισμών ανίχνευσης απαρτίας.

Επιστημονικός τομέας: Ασφάλεια Τροφίμων

Λέξεις κλειδιά: *L. monocytogenes*, αλληλεπιδράσεις μεταξύ στελεχών, δομή, διαθεσιμότητα οξυγόνου, Ricotta, Camembert, συνθετικό γαστρικό υγρό, FTIR-ATR, μέσο καλλιέργειας απαλαγμένο από κύτταρα, ενδοκυτταρικές πρωτεΐνες

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CHAPTER 1

General Introductions and Aims of the Thesis

Listeria monocytogenes and Listeriosis

Listeria monocytogenes is a Gram-positive, motile, non-sporulating, rod-shaped facultative anaerobe bacterium (Hain et al., 2012), which was initially described during an outbreak that caused mononuclear leukocytosis in rabbits and guinea pigs, in 1926. In 1970s recognized as the causative agent of a human systemic disease, called listeriosis and finally identified as a food-borne pathogen, in 1980s (Murray et al., 1926, Nwaiwu, 2020, Radoshevich and Cossart, 2018). The genus *Listeria* belongs to *Firmicutes* phylum, *Bacilli* class, *Bacillales* order, *Listeriaceae* family, which include 20 identified species (Table1; Nwaiwu, 2020), however only *L. monocytogenes* and *L. ivanovii* are pathogenic to humans and livestock (especially, small ruminants), respectively (Nwaiwu, 2020, Radoshevich and Cossart, 2018, Pizarro-Cerdá and Cossart, 2019). *Listeria* strains were historically characterized by serotyping. *L. monocytogenes* isolates can and have been assigned to their serotypes using a range of genotypic and phenotypic approaches, including ribotyping, pulse-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST). Four major evolutionary phylogenetic lineages (lineages I, II, III and IV) have been described for the species *L. monocytogenes*, comprising 13 different serotypes: 1/2b, 3b, 4b, 4d, 4e and 7 (lineage I) 1/2a, 3a, 3c and 1/2c (lineage II); 4a, 4c and 4b (line- ages III and IV) (Fig. 1). Traditionally, serotype 4b (lineage I) has been associated most frequently with human listeriosis cases. As mentioned above, using a multi-locus sequence typing scheme based on the sequence analysis of seven housekeeping genes, 63 different clonal complexes (CCs) have been identified within each evolutionary lineage: CC1, CC2, CC4 and CC6 (serotype 4b, lineage I) are significantly associated to human clinical isolates, while CC121 and CC9 (serotypes 1/2a and 1/2c, respectively, lineage II) are associated with food isolates (Orsi et al., 2011; Pizarro-Cerdá & Cossart, 2019; Radoshevich & Cossart, 2018). According to Orsi et al. (2011) lineage I is characterized by the lowest diversity among the lineages and most commonly isolated from various sources and overrepresented among human isolates. Lineage II is the most diverse lineage and its strains are common in foods, seem to be widespread in the natural and farm environments, and are also commonly isolated from animal listeriosis cases and sporadic human clinical cases, Lineage III and IV strains on the other hand are rare and predominantly isolated from animal sources.

Table1. The species of the genus Listeria (Nwaiwu, 2020)

Species	Year	Reference	Species	Year	Reference
L. thailandensis	2019	*Leclercq et al., 2019*	*L. weihenstephanensis*	2013	*Lang Halter et l., 2014*
L. costaricensis	2018	*Núñez- Montero et al., 2018*	*L. fleischmannii*	2013	*Bertsch et al., 2013*
L. goaensis	2018	*Doijad et al., 2018*	*L. rocourtiae*	2010	*Leclercq et al., 2013*
L. newyorkensis	2015	*Weller et al., 2015*	*L. marthii*	2010	*Graves et al., 2010*
L. booriae	2015	*Weller et al., 2015*	*L. grayi*	1992	*Rocourt et al., 1992*
L. riparia	2014	*den Bakker et al., 2014*	*L. ivanovii*	1984	*Seeliger et al., 1984*
L. grandensis	2014	*den Bakker et al., 2014*	*L. welshimeri*	1983	*Rocourt et al., 1983*
L. floridensis	2014	*den Bakker et al., 2014*	*L. seeligeri*	1983	*Rocourt et al., 1983*
L. cornellensis	2014	*den Bakker et al., 2014*	*L. innocua*	1983	*Seeliger et al., 1981*
L. aquatica	2014	*den Bakker et al., 2014*	*L. monocytogenes*	1940	*Pirie et al., 1987*

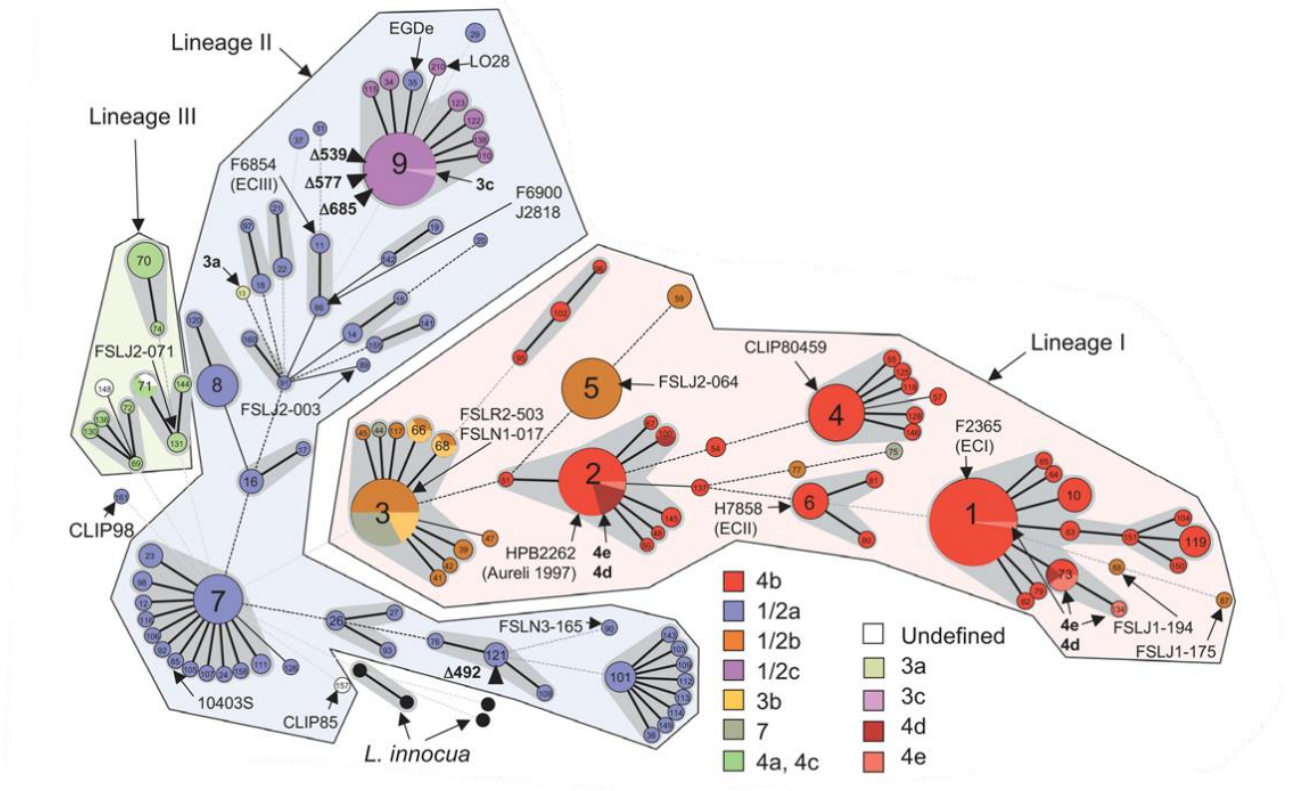


Figure 1. Minimum spanning tree analysis of 360 *L. monocytogenes* and four *L. innocua* strains based on MLST data. Each circle corresponds to a sequence type (ST). Grey zones surround STs that belong to the same clonal complex (CC; 24 CCs are visible in total). ST numbers are given inside the circles and are enlarged for the central genotypes that define the major CCs (e.g., ST9 defines the central genotype of CC9). The three major lineages are highlighted by polygons. Four *L. innocua* sequence types are also represented (black circles). The lines between STs indicate inferred phylogenetic relationships and are represented as bold, plain, discontinuous and light discontinuous depending on the number of allelic mismatches between profiles (1, 2, 3 and 4 or more, respectively); note that discontinuous links are only indicative, as alternative links with equal weight may exist. There were no common alleles between the three major lineages, *L. innocua*, ST161 (CLIP98) and ST157 (CLIP85); they are arbitrarily linked through ST7 by default. Circles and sectors were colored based on serotyping data according to the provided legend; in addition, rare serotypes (3a, 3c, 4d, 4e) are indicated directly on the Figure. Adapted from Ragon et al. (2008).

As mentioned above *L. monocytogenes* is Gram-positive bacilli that is non-spore- forming, facultatively anaerobic, catalase-positive, rod-shaped and circa 0.5 × 2-3 µm, isolated or arranged in small chains. The pathogen is unable to reduce nitrate to nitrite, hydrolyses esculin and has positive reaction in the Voges-Proskauer test, indicating ability to produce acetoin from the fermentation of glucose through the butanediol pathway. *L. monocytogenes* is a ubiquitous environmental pathogen and its remarkable adaptability to different physical and chemical stresses underpins its ability to survive and grow in wide range of different environments renders it a major concern for the food industry. *L. monocytogenes* is a psychrotolerant bacterium with an optimum growth temperature at 30 to 37°C, at pH 7.0. Nevertheless, it is able to grow at temperatures as low as 0.4°C up to 45°C, and over a wide pH range 4.2 - 9.5 (Junttila et al., 1988; Walker et al., 1990). It can grow at 10% NaCl, but can survive higher salt concentrations, while it is also

extremely tolerant to bile salt (Begley et al., 2002; Dowd et al., 2011). At 20 - 25°C, *L. monocytogenes* behaves as a flagellated environmental saprophyte (motile *via* 4 to 6 peritrichous flagella); at 37°C flagellar expression is repressed and *L. monocytogenes* activates a genetic program that allows bacterial life as a facultative intracellular pathogen (Kallipolitis & Ingmer, 2001; Pizarro-Cerdá & Cossart, 2019; Renier et al., 2011).

L. monocytogenes is a food-borne bacterial pathogen and the etiological agent of the invasive systemic disease called listeriosis. The incidence of listeriosis is low in the general population, despite the wide distribution of the microorganism in the environment and the relatively high frequency of isolation in foods, manifested as mild gastroenteritis. However, among the susceptible individuals the clinical manifestations are more severe including septicemia, meningitis, or other infections of the central nervous system in young, elderly, or immunocompromised individuals, while in pregnant women, infection may lead to spontaneous abortion, stillbirth, or fetal death (Buchanan et al., 2017; Ferreira et al., 2014; Orsi et al., 2011). Center of Disease Control and Prevention (CDC) has estimated that 1600 people get listeriosis each year, originated from a variety of ready-to-eat (RTE) products, and about 260 die (Table 2). According to the latest scientific report of EFSA, in 2021 reported 2,183 confirmed invasive human cases of *L. monocytogenes* infection to ECDC. These cases resulted in 923 hospitalizations and 196 deaths in the EU (Fig. 2). Also, in 2021 recorded 23 foodborne outbreaks (8 out of 23 were strong-evidence outbreaks) resulting in 104 cases of illness, 48 hospitalizations and 12 deaths. The EU notification rate was 0.49 per 100,000 population and the overall EU case fatality rate was 13.7% (EFSA, 2022).

Table 2. Listeriosis outbreaks (https://www.cdc.gov/listeria/outbreaks/)

Year	Product	Case counts	States	Hospitalizations	Deaths
2011	Cantaloupes	147	28	143	33
2012	Ricotta salata cheese	22	14	20	4
2013	Cheese	6	5	6	1
2014	Commercially produced, prepackaged caramel apples	35	12	34	7
2014	Bean sprouts	5	2	5	2
2014	Cheese	5	4	4	1
2014	Dairy products	8	2	7	1
2015	Soft cheese	30	10	28	3
2015	Ice cream	10	4	10	3
2016	Frozen vegetables	9	4	9	3
2016	Raw milk	2	2	2	1
2016	Packaged salads	19	9	19	1
2017	Vulto creamery soft raw milk cheese	8	4	8	2
2018	Pork products	4	4	4	0
2018	Deli ham	4	2	4	1
2019	Hard-boiled eggs	8	5	5	1

2019	*Listeria monocytogenes* infections	24	13	22	2
2019	Deli-sliced meats and cheeses	10	5	10	1
2020	Deli meats	12	4	12	1
2020	Enoki muchroonms	36	17	31	4
2021	Dole packaged salads	18	13	16	3
2021	Fresh express packaged salads	10	8	10	1
2021	Fully cooked chicken	3	2	3	1
2021	Queso fresco	13	4	12	1
2022	Enoki mushrooms	5	4	5	0
2022	Deli meat and cheese	16	6	13	1
2022	Brie and Camembert	6	6	5	0
2022	Ice cream	6	6	5	0
Up to August 2023	Ice cream	2	2	2	0
Up to August 2023	Leafy greens	19	16	18	0

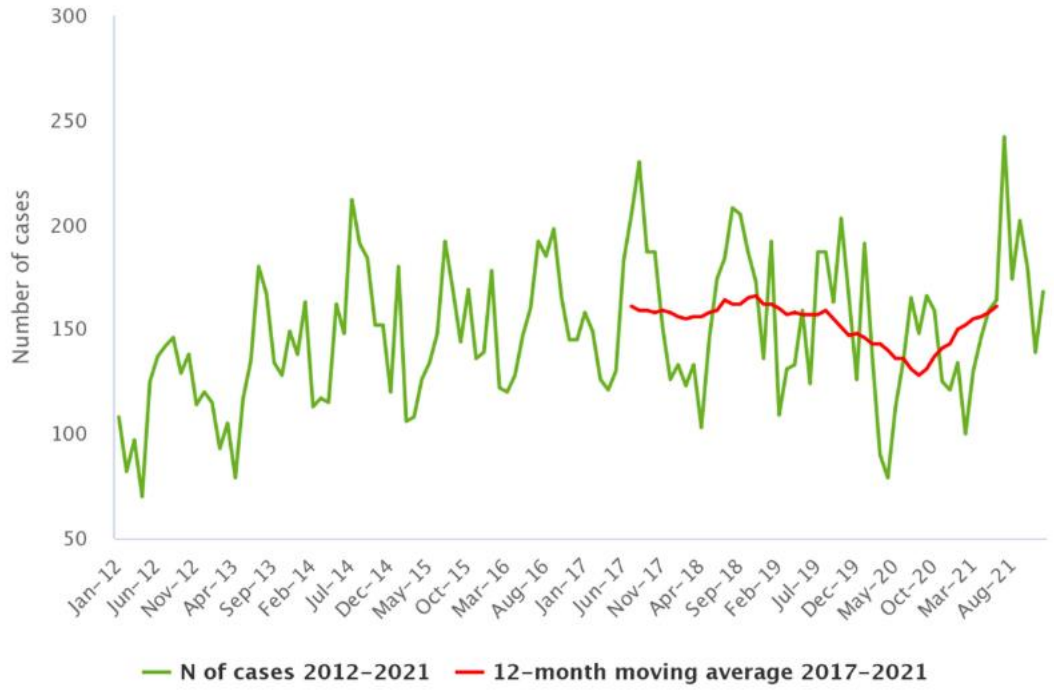


Figure 2. Trends in reported confirmed human cases of listeriosis in the EU by month, 2017–2021. Adapted from (EFSA, 2022).

Briefly, the primary route of infection is across the intestinal epithelium after consumption of contaminated food products by the host (Fig. 3). The adhesion and the subsequent invasion of the pathogen to the intestinal cells of the host require a number of bacterial surface proteins, including InlA and InlB.

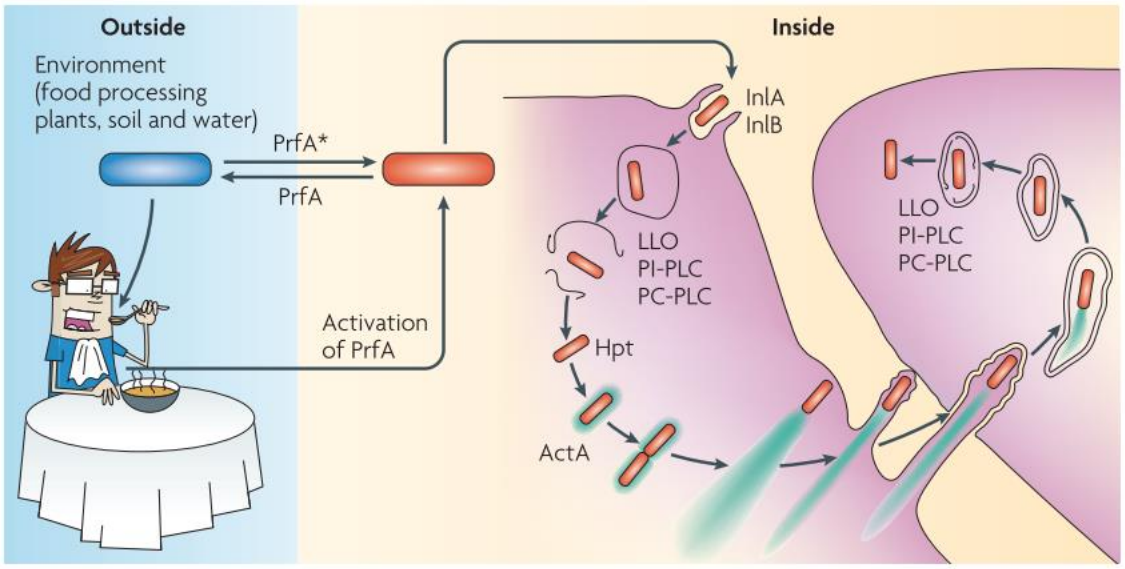


Figure 3. From saprophyte to intracellular pathogen. *Listeria monocytogenes* survives in a diverse array of environments, in habitats that include soil and water as well as food processing facilities. Central to the switch between life outside and life inside mammalian hosts is the transcriptional activator PrfA, which regulates the expression of many gene products that are required for bacterial virulence. Outside a host cell, PrfA exists in a low-activity state, with correspondingly low levels of virulence gene expres- sion. once inside the host, PrfA becomes activated (PrfA*) and induces the expression of gene products that are needed for host cell invasion (internalins InlA and InlB), phagosome lysis (listeriolysin o (LLo), phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphatidylcholine (PC)-PLC), intracellular growth (hexose-6-phosphate transporter (Hpt)), and cell-to-cell spread (actin assembly-inducing protein (ActA); actin polymerization is shown in turquoise). The intracellular life cycle is modified, with permission, from REF. 81© (1989) Rockefeller University Press. Adapted from (Freitag et al., 2009).

InlA binds E-cadherin, a host cell adhesion molecule, whereas InlB binds to the hepatocyte growth factor (HGF) receptor, Met. Binding to these receptors enables *L. monocytogenes* to gain entry into host cells through the exploitation of the host endocytic machinery. Once the bacterium is internalized inside the vacuole, it secrets the pore-forming cytolysin listeriolysin O (LLO) and two phospholipases, PlcA and PlcB, for vacuolar membrane rupture and translocate to the host cell cytoplasm, which are crucial steps in *L. monocytogenes* pathogenesis. During intracellular phase the bacteria proliferate by activating several bacterial metabolic pathways and using nutrients that are acquired from the host, inducing changes in the morphology of host cell organelles, thereby altering their function to promote infection. Another hallmark of the intracellular lifestyle of the pathogen is the capacity to use the polymerization of actin as a motility force, which it directs through the bacterial surface protein actin assembly-inducing protein (ActA), allowing *L. monocytogenes* to spread to neighboring cells (Fig. 3). Following entry into the bloodstream, unless their proliferation is controlled by the host innate immune response, most of the bacteria end up initially in the liver and spleen and secondarily may potentially cause fatal systemic or central nervous system infections (Fig. 4) (Freitag et al., 2009; Pizarro-Cerdá and Cossart, 2019b; Radoshevich and Cossart, 2018).

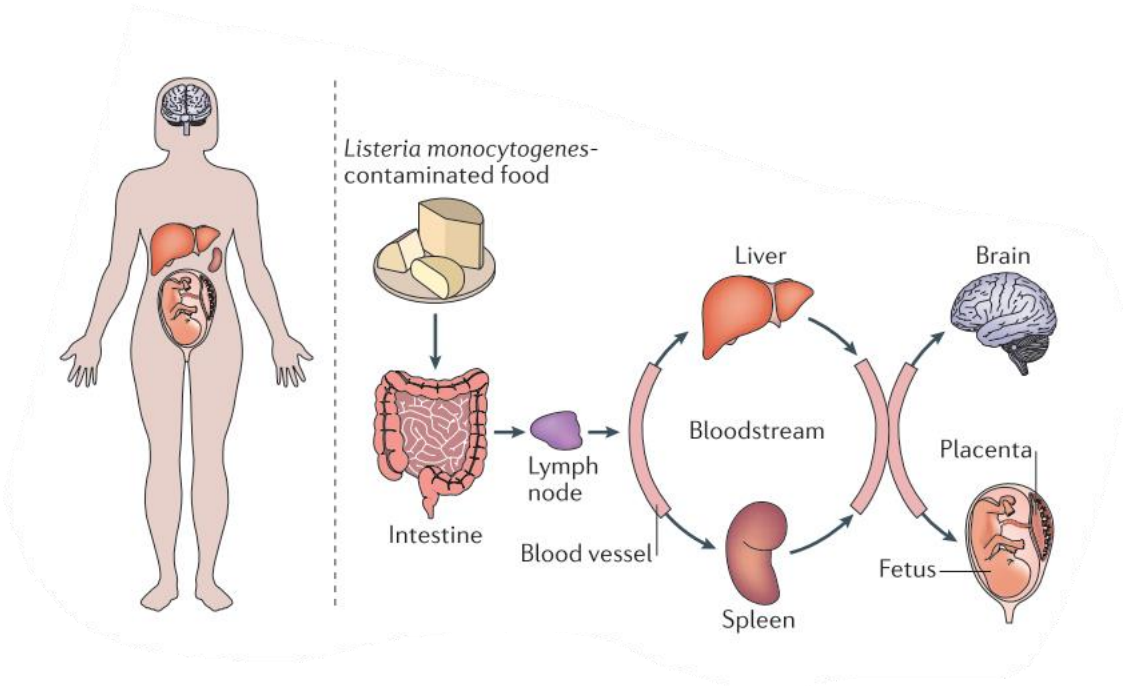


Figure 4. Schematic of Listeria monocytogenes infection of a human host. After ingestion of contaminated food, L. monocytogenes can traverse the intestinal barrier and spread into the bloodstream through the lymph nodes to disseminate to target tissues, such as the liver and spleen. In immunocompromised individuals, *L. monocytogenes* can cross the blood–brain barrier or fetoplacental barrier and cause potentially fatal meningitis, sepsis, premature birth or abortion. Adapted from (Radoshevich and Cossart, 2018).

Microbial competition

Microbes dominate the tree of life in species number and diversity. They inhabit the largest range of environments on earth, forming complex ecological webs. Interactions within these ecological webs can be either specific or nonspecific and can have positive, negative or no impact for the species involved (Faust and Raes, 2012; Ghoul and Mitri, 2016). Microbial interactions play a crucial role in the colonization and establishment of microorganisms in various environments, let alone the fate of pathogenic species contaminating foods. These interactions involve a range of ecological aspects, including physiochemical changes, metabolite exchange, signaling, and genetic exchange (Braga et al., 2016). Microbial interactions, specifically competition, play a significant role in microbial life, given that microbes are typically surrounded by different strains and species. Microbial competition may be expressed in different forms and mechanisms. Cells compete for the two main resources of microbial survival, nutrients and space, (i) indirectly through exploitative competition, which occurs through resource consumption (passive competition) and (ii) directly through interference competition, where individual cells damage one another (active, chemical warfare) (Cornforth and Foster, 2013; Ghoul and Mitri, 2016; Powell et al., 2004). Given such challenging living conditions, microbes have evolved many phenotypes with which they can outcompete and displace their neighbors: secretions to harvest resources, loss of costly genes whose

products can be obtained from others, poisoning neighboring cells, or colonizing spaces while preventing others from doing so (Table 3).

Table 3. Competitive Phenotypes in Microbes (Ghoul and Mitri, 2016).

Competitive Phenotype	Example of Molecule Type	Competitive Effect	References
Digestive enzyme secretion	Proteases	Enhanced access to nutrients	Diggle et al., 2007 Rendueles & Ghigo, 2012
Siderophore secretion	Pyoverdin	Enhanced access to nutrients	Griffin et al., 2004 Scholz & Greenberg, 2015
Altering metabolic regulation	-	Enhanced access to nutrients	Ackermann, 2015 Kotte et al., 2014 MacLean & Gudelj, 2006 Pfeiffer et al., 2001 Vulić & Kolter, 2001
Reduced expression of costly genes	Reduced or no secretion of molecules that act as public goods, e.g., digestive enzymes and siderophores	Exploitation of cost-bearing cells	Cordero et al., 2012 Diard et al., 2013 Diggle et al., 2007 Gore et al., 2009 Griffin et al., 2004
Production of structural and motility molecules	Surfactants, rhamnolipids, EPS, proteins, DNA, adhesion and anti-adhesion molecules	Enhanced access to space	An et al., 2006 Kim et al., 2014 Nadell & Bassler, 2011 Romero et al., 2011 Schluter et al., 2015 Whitchurch et al., 2017
Antibiotic production (non-contact-dependent)	Bacteriocins, toxins, peptides	Eliminate competitor	(Chao and Levin, 1981; Kerr et al., 2002; Riley and Gordon, 1999)
Type VI secretion systems (T6SS) (contact-dependent)	Stabbing structures that release lethal effector molecules and enzymes	Eliminate competitor	(Basler et al., 2013; Borgeaud et al., 2015; MacIntyre et al., 2010; Russell et al., 2014)
Production of nonbiocidal molecules	Surfactin, anti-adhesion molecules, nucleases, proteases	Disrupt other's competitive phenotype	(Jiang et al., 2011; Mowat et al., 2010; Rendueles and Ghigo, 2012; Valle et al., 2006)
Inhibit quorum sensing	Quorum sensing inhibitors or quenchers	Disrupt other's competitive phenotype	Christiaen et al., 2011 Musthafa et al., 2011

Exploitative competition involves the consumption of a limiting resource by one strain restricting its supply to the competitor. This occurs either through increased nutrient uptake or through the extracellular secretion of molecules that harvest nutrients (MacLean and Gudelj, 2006; Pfeiffer et al., 2001; Vulić and Kolter, 2001). Strains also compete to position themselves in prime locations within a niche while preventing others from accessing it (Kim et al., 2014). On the other hand, classical example of interference competition is the production of antimicrobials, which range in their killing spectrum from strain-specific bacteriocins to more broad-spectrum peptides and antibiotics (Chao and Levin, 1981; Riley and Gordon, 1999). Other

mechanisms involved in microbial competition, mediate antagonism between bacteria through contact-dependent inhibition (CDI), include type VI secretion systems (T6SS), whereby cells inject syringe-like protrusions containing toxins and other molecules into neighboring cells that then lyse, while quorum sensing (QS) allows groups of bacteria to synchronously alter behavior in response to changes in the population density and species composition of the vicinal community (Basler et al., 2013; Borgeaud et al., 2015; MacIntyre et al., 2010; Mukherjee and Bassler, 2019; Russell et al., 2014).

Interactions between *Listeria monocytogenes* strains

Listreria monoocytogenes is ubiquitous in a wide range of environmental niches and its ability to survive and grow in a wide range of harsh environmental conditions renders it a major food safety concern. Soil, manure, water and decaying plant material may act as pivotal reservoirs for the transmission of the bacterium (Kallipolitis et al., 2020; Linke et al., 2014; Muhterem-Uyar et al., 2018). It can be originally introduced to processing environments *via* raw materials in combination with unclean equipment and substantial deficiencies or temporal breakdowns in hygiene barrier efficiency (inadequate or wrong cleaning of floors and drains with high pressure water from hoses causing airborne spread of the pathogen, standing water) (Almeida et al., 2013; Kousta et al., 2010; Latorre et al., 2011, 2010; Melo et al., 2015a). Thereinafter, due to deficiencies in applied manufacturing protocols, such as inappropriate personnel movement and food workflows (Di Ciccio et al., 2020, 2012; Muhterem-Uyar et al., 2015; Viswanath et al., 2013) and/or unhygienic design of equipment like slicers, brining and packaging machines, where *L. monocytogenes* is able to adhere forming biofilms (Alvarez-Ordóñez et al., 2019; Latorre et al., 2010; Poimenidou et al., 2009; Shi and Zhu, 2009; Valderrama and Cutter, 2013) during the different stages of manufacturing and storage, the pathogen manage to colonize, spread and persist throughout the facility, resulting in cross-contaminations of the final products (Fox et al., 2011; Ho et al., 2007; Martínez-Suárez et al., 2016; Melero et al., 2019; Melo et al., 2015a; Muhterem-Uyar et al., 2015; Thévenot et al., 2006; Zoellner et al., 2018) (Fig. 5).

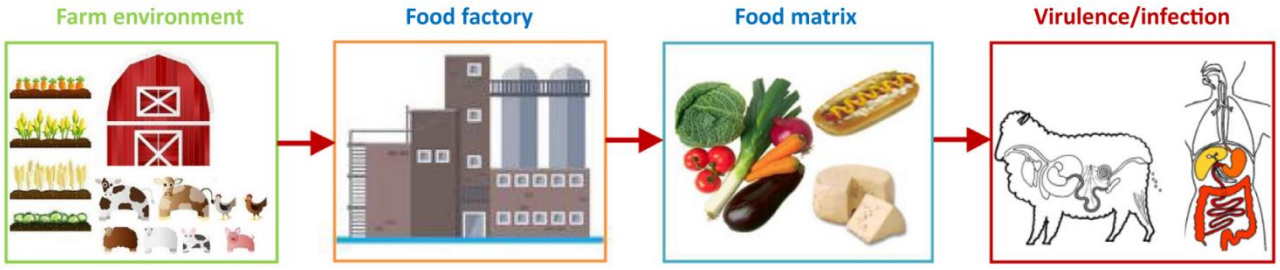


Figure 5. Simplified scheme of the transmission routes of Listeria monocytogenes from outdoor environments to the food industry environment, foodstuff and humans. Adapted from Kallipolitis et al. (2020).

While, Thévenot et al. (2006) support that the strains that are present in the final food product may be different than the strains that introduced to food processing facilities *via* the raw materials, recent

studies have shown that his may be due to the fact that more than one strains of *L. monocytogenes* may often co-exist either in the same final food product or in the same facility/surface. With regards to isolation of the pathogen by processing facilities, Destro et al. (1996), showed the presence and the dissemination of various *L. monocytogenes* strains at different sites in the same shrimp processing environment, which potentially resulted in a final product contaminated by multiple strains *via* cross-contamination. Moreover, a large number of studies have revealed the presence of more than one strains of the pathogen in the same final food product. Specifically, the research team of Danielsson-Tham et al. (1993) identified 2 to 4 different clones of *L. monocytogenes* from the same soft cheese sample, while Loncarevic et al. (1996) detected five clones of the pathogen in the same food sample, a gravad rainbow trout. Moreover, it appears that the various strains of *L. monocytogenes* are well-mixed in the environment, with multiple strains often co-existing in the same smoked salmon (Gendel and Ulaszek, 2000), raw meat and poultry (Ryser et al., 1996), latin-style fresh cheese (Kabuki et al., 2004) and Alheiras, a traditional Portuguese smoked meat sausage (Felício et al., 2007). Finally, underling all the above mentioned findings, studies have provided the proof-of-concept that multiple strains of *L. monocytogenes* may be involved and traced in case of listeriosis outbreaks. Specifically, researchers during outbreaks associated with ready-to-eat meat products, cantaloupe and a traditional curd cheese called “Quargel”, isolated two and four distinct PFGE patterns and two distinct MLST sequence types, respectively. To underline the above findings, in two of his studies Tham et al. (2002, 2007) reported a case of listeriosis involving two different *L. monocytogenes* strains, each isolated from different sites (blood or meninges) of the infected patient and a case involving isolates of different PFGE type from a single blood sample.

Until recently, the literature regarding the interaction between *L. monocytogenes* strains was limited and was focused mainly on their competition during biofilm formation and the selective enrichment. Specifically, in mixed-culture biofilms comprised by 1/2a and 4b strains, the presence of one serotype did not inhibited by the strains of the other serotype, however, the serotype 1/2a strains tested were generally more efficient at forming biofilms and predominated in the mixed-culture biofilms. Interestingly, the cocktail of serotype 4b strains survived and grew significantly better in mixed-culture biofilms containing a specific strain of serotype 1/2a than without it (Pan et al., 2009). Moreover, according to Gorski et al. (2006), during direct competition with each other in two-strain mixed cultures comprised by 1/2a and 4b strains of the pathogen, it was clear that some strains were more competitive or more fit than others, during the enrichment protocol, but the difference was not correlated with serotype and cannot be attributed to differences in growth rate alone. Due to their results, considering that all the strains produced similar numbers of cells in single-culture during the 48-h enrichment process, the researchers attributed the observed differences in fitness, in mixed cultures, to competition between *L. monocytogenes* strains, as well as any complexities added by the presence of foods and the source/niche of isolation. In the study of Bruhn

et al. (2005), different strains belonging to serotypes 1/2a and 4b and one *L. innocua* strain, had similar growth rates dung growth in brain heart infusion (BHI) broth but differed in their growth rate in the selective medium University of Vermont medium I (UVM I), when co-inoculated in the latter growth medium, some strains completely outgrew other strains. Inoculation at same cell densities resulted in *L. innocua* outcompeted *L. monocytogenes* 4b strains and 1/2a strains outcompeted 4b strains, indicating a bias influenced by the enrichment procedures. The results of the latter study demonstrated that the selective procedures used for isolation of *L. monocytogenes* may not allow a true representation of the types present in foods.

However, the last years Zilelidou et al. (2016a, 2016b, 2015) showed that difference in growth dynamics may also have different co-cultured *L. monocytogenes* strains during their coexistence in different food products/substrates, describing the phenomenon with the phrase “inter-strain competition”. The researchers observed growth inhibition, reduction of growth rate or even total suppression of growth of some strains (“weak competitors”; i.e. 6179 strain) due to the presence of a second strain (“strong competitors”; i.e. C5 and PL25 strains) during co-culture in TSB-YE, on TSA-YE, on ham-slices, on selective agar (ALOA) and in enrichment broth (after direct inoculation of the medium or *via* minced meat and ham slices). Outgrowth of strains by their competitors on ALOA resulted in limited to no recovery for “weak” strains and associated with the enrichment conditions (i.e. food type added to the enrichment broth) and the strain-combination. Most importantly, the observations about growth competition on food or nonselective agar surface did not necessarily coincide with the results of competition during enrichment. Also, Zilelidou et al. (2015) highlighted the importance of cell contact on growth inhibition and during *in vitro* virulence assays using human intestinal epithelial Caco2 cells showed a correlation between the invasion efficiency and growth inhibition: the strong growth competitor strains showed high invasiveness, while the invasion efficiency of the highly invasive strains was further increased in certain combinations by the presence of a low invasive strain. Finally, Zilelidou et al. (2016a) comment that co-culture of different *L. monocytogenes* strains did not have a profound role in the resistance of cells to gastric acid stress, but overall, the apparent resistance is more due to the initial population of each strain, at the time of exposure to the simulated gastric fluid (SGF), which was affected from the interactions of the strains during storage.

Many *Listeria* strains are reported to produce bactericidal substances (lytic particles), designated as listeriolysin S (LLS) and monocins, whose activity defined as similar to bacteriocins by Curtis and Mitchell (1992). LLS is a member of the family of thiazole/oxazole-modified microcins (TOMM’s), which has been described previously as an hemolytic and cytotoxic factor contributing to *L. monocytogenes* virulence, but the mechanism by which, LLS kills other bacteria is unknown (Cotter et al., 2008; Lee, 2020; Quereda et al., 2017). In 2008, Cotter et al. (2008) named the *L. monocytogenes* LLS gene cluster as pathogenicity island III (LIPI-3). The LLS operon consists of eight genes including the gene *llsA* which encodes for the actual LLS toxic

peptide, the genes *llsG* and *llsH* which encode for a putative transporter, the genes *llsB*, *llsY* and *llsD* which encode for putative post-translational modification enzymes involved in the production of thiazole/oxazole/ methyloxazole rings, the gene *llsP* which encodes for a putative protease, and the gene *llsX* which encodes for a protein of unknown function specific to the genus *Listeria* (Lee, 2020; Quereda et al., 2017). The LIPI-3 was discovered in a subset of lineage I strains (C5 and ScottA strains used in the present thesis belong to serotype 4b), suggesting that it could be associated to the higher virulence potential of these bacteria. Also, LIPI-3 was over-represented among the clones with higher infectious potential in the multilocus sequence typing-based survey of the isolates collected from France between 2005 and 201. On this note, LLS is an additional virulence factor produced by the isolates preferentially associated with epidemic listeriosis outbreaks and hypervirulence (Lee, 2020; Maury et al., 2016). Monocins are various high-molecular-weight bactericidal protein structures like bacteriocins resemble phage tail structures and result from the presence of incomplete, cryptic prophages, which produced intracellularly upon induction of the SOS response. The tail associated lytic proteins (used during infection for cell wall penetration) are toxic to certain *Listeria* species and act as biocins (Curtis and Mitchell, 1992; Klumpp and Loessner, 2013; Lee et al., 2016; Zink et al., 1995, 1994)

Fuqua and Winans (1994) used the term quorum sensing (QS) to describe the cell-to-cell communication as a mechanism which mediates cell-density-dependent gene expression. QS helps bacteria to understand changes in their environment and consequently to apply specific strategies that allow adaptation to environmental stress in space and time (Skandamis and Nychas, 2012). Bacteria behave as single cellular organisms at low cell densities, however, they may shift their behavior to 'multicellular' type by sensing that their population density has reached a threshold level. Bacterial cells produce extracellular signal molecules and sense the concentration of these molecules on the cell surface and subsequently, If the concentration of a signal molecule exceeds a threshold value, genes responsive to the molecule, which controls a variety of physiological activities, are induced, including biofilm formation, virulence factor production, cell adhesion, competence development and stress adaptation (Smith et al., 2004; Song et al., 2018). In *L. monocytogenes*, two QS systems, LuxS/autoinducer 2 (AI-2) and Agr/autoinducing peptide (AIP), have been identified. The LuxS/AI-2 appears in many species of Gram-negative and Gram-positive bacteria. Thus, AI-2 is considered a QS signaling molecule for interspecies communication. In *L. monocytogenes*, LuxS LuxS is the key enzyme in the AI-2 biosynthesis pathway and is involved in the repression of biofilm formation (Belval et al., 2006; Sela et al., 2006). The Agr/AIP consists of the four-gene operon agrBDCA, whose expression is driven by the P2 promoter upstream of agrB. Many studies have suggested that the Agr system contributes to biofilm formation and virulence in *L. monocytogenes* (Banerji et al., 2022; Yu et al., 2022).

Finally, studies have shown that contact-dependent inhibition (CDI) may occur mainly by Gram-negative bacteria, such as *E. coli,* which managed to compete another strain of the microorganism during mixed cultivation in shaking liquid culture (Aoki et al., 2005). In the same study, they confirmed that it is necessary the cells of the "strong" competitor, and not only their metabolome, have to be present, in order to occur the phenomenon of the growth inhibition. The researchers support the possibility that the secreted molecule, that is responsible for the inhibitions, is unstable and is only effective when delivered to target cells in close proximity. However, recent studies have found that CDI is not restricted to Gram-negative bacterial but also can be found in Gram-positive species such as *Listeria* (Heys et al., 2010). Specifically, CDI activity relies on the collaborative action of CdiB and CdiA, a two partner secretion system, where the CdiB is an outer membrane protein which is necessary for the presentation, to the cell surface, of CdiA exoprotein, which is responsible for the inhibitory effect on the cells of the competitive microorganism (C-terminus of CdiA). Rearrangement hotspots (Rhs) protein family is observed that it may be functionally analogous to CdiA (because of related C-terminal sequences) (Poole et al., 2011). Koskiniemi et al. (2013) proved that WapA (wall-associate protein A) proteins of *Bacillus* and *Listeria* species may be operate in the same way that Rhs operates, due to similar architecture and the C-terminal toxin domains. Furthermore, Schmitz-Esser et al. (2015) showed that Rhs proteins, are present to *L. monocytogenes* strain 6179 (ST121) renders it, according to the researchers, better competitor against other bacteria in the food production environment and increase its chances of becoming persistent.

Substrate's structure and microbial behavior

The safety and the quality of foods depend on a large degree on the extent to which they support microbial growth (Robins and Wilson, 1994). The survival, growth and interactions between the microbial communities of different (Ghoul and Mitri, 2016; Jia et al., 2020; Tirloni et al., 2019; Wimpenny et al., 1995) and/or the same species (Bruhn et al., 2005a; Gorski et al., 2006; Pan et al., 2006; Zilelidou et al., 2016b, 2015) and the predominance of a certain microorganism or strain are determined, apart from the intrinsic properties (pH, aw, etc.) of the substrate and the environmental (extrinsic) conditions surrounding the food product, by the structure of the substrate (Couvert et al., 2017; Henderson et al., 2019; E. Tirloni et al., 2019; Wimpenny et al., 1995). Food products are highly structured multiphase heterogeneous ecosystems, in which the chemical and physical conditions relevant to microbial growth can vary with position in the food microstructure (Robins and Wilson, 1994). As composite matrices of multiple constituents and phases are characterized by great complexity, so according to Wilson et al. (2002) food products may be classified in 6 categories with respect to their structural characteristics (liquids, aqueous gels, oil-in-water emulsions, water-in-oil emulsions, gelled emulsions and surface) (Table 4; Fig. 6). The compaction and the distribution

of water and oil (along with size of oil-droplets) in each of the above systems determine the available space for microbial growth, the nutrient and oxygen diffusion and thus, the motility of cells and mode of growth (planktonic *vs* immobilized) (Wilson et al., 2002; Boons et al., 2013). Microbial growth takes place in the aqueous phase of food products. In liquids and emulsions (with less than 83% of fat) bacterial cells adopt the planktonic way of growth and the motility enable them to move towards nutrient and/or oxygen-rich sites of the substrate and move away from areas where nutrients are depleted and metabolites have accumulated. Interestingly, it is the physical structure rather than chemical composition that makes butter-like water-in-oil emulsions less supportive of microbial growth than their oil-in-water equivalents such as dairy cream. The compartmentalization of the droplets of the aqueous phase in the butter greatly reduces both the access of contaminating organisms to the aqueous phase, and the availability of nutrients and space for their growth.

Table 4. Examples of food micro-architecture and representative foods (Wilson et al., 2002).

Micro-architecture	Food examples
Liquid	soups, juices: these are predominantly uniform liquids, although with some suspended material
Gel	pâté, jellies, cheeses such us cottage cheeses which are made from skimmed milk and hence are protein gels
Oil-in-water emulsion	dairy cream, milk, salad cream, mayonnaise
Water-in-oil emulsion	butter, margarine, low-fat spread
Gelled emulsion	Whole-milk cheese, sausage

Thus, to increase our understanding of the way in which organisms grow in real foods, we need to understand how interact (Robins and Wilson, 1994). By adding solidified agents to the liquid systems, e.g., due to addition of thickeners, or gelling (structure-inducing) agents, such as gelatin, pectin, starch, gums, etc., the bacterial growth changes from planktonic to colonial, resulting in immobilization of the cells within the gelled regions and constrain to grow as submerged colonies in three dimensions, having limited access to nutrients and oxygen, and increased exposure to metabolites accumulation (Skandamis and Jeanson, 2015; Wilson et al., 2002). Finally, if bacteria grow on the surface of food, such as meat, cheese or vegetables, growth is also colonial initially in two dimensions (mono-layer), whereas the centre of colony gradually develops in the third dimension most likely upward, depending on aeration and nutrient availability. Replenishment of nutrients takes place only from the bottom or the perimeter of the colony and soon cells in the centre of colony experience starvation and self-toxication (Skandamis and Jeanson, 2015). The growth of foodborne pathogenic bacteria has been measured in liquid media under a wide range of chemical conditions, and the consolidated results are available for predictive purposes'. The growth of bacteria in

liquid culture provides a baseline for their behavior (Robins and Wilson, 1994). To underline the importance of structure as factor that may influence the behavior of the microorganisms more and more studies try to incorporate microstructure into predictive models, because the majority of them is based on data from liquid media (Aspridou et al., 2014; Baka et al., 2017a; Velliou et al., 2013; Verheyen et al., 2018).

The growth rate of microorganisms in response to the different aforementioned forms of growth due to food structure, according to Brocklehurst et al. (1997), follows the order: planktonic cells > submerged colonies > surface colonies, order which is in line with the results of many studies (Aspridou et al., 2014; Boons et al., 2013; Noriega et al., 2010; Theys et al., 2008; Wilson et al., 2002).

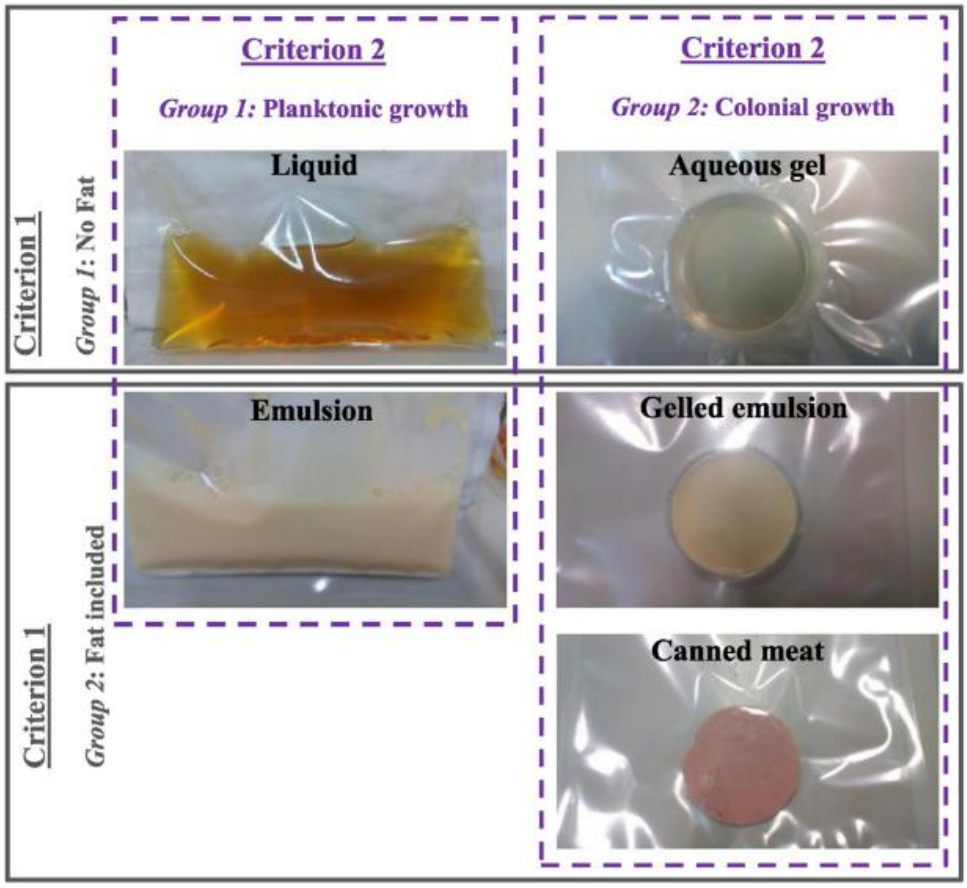


Figure 6. Demonstration of the model systems and schematic representation of the different criteria influencing microbial growth (typical example of the different structures as described by Wilson et al. (2002)). Adapted from Baka et al. (2016).

However, during culture of *Salmonella* Typhimurium and *Escherichia coli* as planktonic cells, immersed colonies and surface colonies under static conditions at temperatures in the range 8 to 22°C, the growth morphology has a negligible effect on the growth rate (Smet et al., 2015a). Additionally, the growth of *Listeria monocytogenes* in/on different structured fish-based (simulating fish patê) and meat-based (simulating Frankfurter sausages) substrates at 4, 8 and 12°C aerobically and vacuum packed, respectively, it was observed that the pathogen in/on the fish-based substrates grew faster in colonies on solid surfaces and

slower in liquid systems planktonically, while in/on the meat-based substrates grew faster on canned meat and real Frankfurters followed by liquids, aqueous gels, emulsions and gelled emulsions (Baka et al., 2017, 2016). Mitchell and Wimpenny (1997) studied the growth of both submerged spoilage and pathogenic bacteria in laboratory medium containing different concentrations of agar and presented that the agar concentration influenced both the morphology and dimensions of colonies, especially for motile bacteria. Motile species *Pseudomonas aeruginosa*, *S.* Typhimurium, *L. monocytogenes* and *E. coli* produced large diffused spherical colonies whose size was inversely related to the agar concentration. Above a critical agar concentration, around 0.65% (w/v), the colonies became from diffused and spherical to compact and lenticular. Contrarily, the non-motile bacteria showed no such relation between the size of their colony and the agar concentration. The agar is acting as a pore size net. At lower agar concentrations where the pore size is larger, motile cells may be well separated and able to move away from the colony, while above the critical agar concentration the trapped cells form lenticular colonies (the agar split along a particular fault line due to the hydrostatic pressure exerted by the growth) and continue to divide at rates governed by the speed with which nutrients diffused through the agar matrix to the colony. The structural properties of the substrate not only influence the vegetative forms of the bacteria but also appear to affect both the shape and the properties of the spores. In particular, the production of spores during culture of *Bacillus sp.* species, in liquid substrates based on the water, resulted in heterogeneous spore population including small and damaged spores, with lower resistance to heat and several chemicals and germinated more readily with several agents. However, between spores made in liquid and on plates, there were no major differences in the levels of dipicolinic acid (DPA), core water, small, acid-soluble spore proteins (SASP) and individual coat proteins or the cross-linking of a coat protein, characteristics which play role in the resistance to wet heat (Rose et al., 2007; Stecchini et al., 2009). Finally, food structure may impact the efficacy of some decontamination treatments like the cold atmospheric plasma (CAP). For both *S.* Typhimurium and *L. monocytogenes*, cells grown planktonically are easily inactivated, as compared to surface colonies. More stressing growth conditions, due to cell immobilization, result in more resistant cells during CAP treatment. The main difference between the inactivation support systems is the absence or presence of a shoulder phase. For experiments in the liquid carrier, which exhibit a long shoulder, the plasma components need to diffuse and penetrate through the medium. This explains the higher efficacies of CAP treatment on cells deposited on a solid(like) surface or on a filter. This research demonstrates that the food structure influences the cell inactivation behavior and efficacy of CAP, and indicates that food intrinsic factors need to be accounted when designing plasma treatment (Smet et al., 2017).

Oxygen availability and microbial behavior

Both, different structures and different ways of packaging may lead to different levels of oxygen availability, an environmental factor that studies have shown that influence microbial physiology and *via* the widely used modified atmosphere packaging (MAP) or vacuum packaging, oxygen restriction is a method of controlling microbial growth (Noriega et al., 2010a). However, the facultative nature of *Listeria monocytogenes* enables it not only to overcome the barrier of anaerobiosis but grow at similar cell density to that grown under aerobic conditions (Buchanan and Klawitter, 1991; Couvert et al., 2019; Lungu et al., 2009; Pine et al., 1989). Moreover, Buchanan and Klawitter (1991) found that under aerobic conditions at low pH in tryptose phosphate broth, *L. monocytogenes* survived for extended periods at low temperatures (5 and 10°C), grew at intermediate temperatures (19 and 28°C) but was inactivated at 37°C. However, under anaerobic conditions *L. monocytogenes* recovered and survived for extended periods at 37°C. O_2 restriction also enhanced growth at 19°C. Therefore, they concluded that O_2 and temperature likely interact to influence the survival of *L. monocytogenes* in low pH environments. Apart from growth, culture under anaerobic conditions may influence its ability to resist and survive against a number of other environmental stresses. Oxygen restriction of *L. monocytogenes,* prior to infection of Caco-2 cells, appears to be an environmental signal to trigger the initial colonization in the intestine and mediate an advantage during *in vivo* growth increasing the invasiveness of the pathogen (Bo Andersen et al., 2007) and induce its acid tolerance renders it more resistant to organic and inorganic acids (Lungu et al., 2009; Sewell et al., 2015). Specifically, anaerobic growth induced an acid tolerance response, causing cells to be more resistant to organic and inorganic acids and to pH 2.5 simulated gastric juice (SGJ) compared to aerobically grown cells. Then latter observation was most pronounced in exponential phase cells. However, exposure of stationary phase cells to pH 3.5 SGJ enhanced bile tolerance, suggesting a link between acid and bile tolerance (Sewell et al., 2015). Also, studies have shown that many strains of the pathogen during culture under anaerobic conditions developed resistance to bile increasing the infectious potential of *L. monocytogenes* (Wright et al., 2016; White et al., 2015). Four strains of the pathogen evaluated for changes in viability and proteome expression following exposure to bile in aerobic or anaerobic conditions. Viability for F2365 (serotype 4b), EGD-e (serotype 1/2a), and 10403S (serotype 1/2a) increased following exposure to 10% porcine bile under anaerobic conditions. However, HCC23 (serotype 4a) exhibited no difference in bile resistance between aerobic and anaerobic conditions, indicating that oxygen availability does not influence resistance in this strain. Mimicking conditions within the duodenum White et al. (2015) studied the ability of *L. monocytogenes* to resist bile under anaerobic conditions, at acidic pH. Exposure to bile salts at acidic pH increased toxicity of bile, resulting in a significant reduction in survival for strains representing six different serotypes. Anaerobic culture of the pathogen increased bile resistance, but a significant increase was only observed in virulent strains when exposed to bile at pH 5.5. Exposure to pH 3.0 prior to bile decreased

viability amongst avirulent strains in bile in acidic conditions. The researchers resulted that the ability of *L. monocytogenes* to sense and respond to oxygen availability may influence the expression of stress response mechanisms, and this response may correspond to disease outcome. Recovery under anaerobic conditions was more preferable for the heat treated cells of the pathogen (George et al., 1998), while prior alkaline adaptation, which may induced by the use of sanitizers in the food processing environment, may enhance the proliferation in low oxygen packaging conditions that often are used in foods as growth limiting hurdle (Nilsson et al., 2013). Regarding the role of oxygen on microbial interactions, Thomas and Wimpenny (1996) showed that decrease of oxygen availability may influence the growth of *P. putida* in a mixed culture with different strains of *Salmonella.* Specifically, during co-cultivation of *Salmonella* and *P. putida,* as immerged colonies at 20^{o}C, the latter microorganism affected by the present of the pathogen and managed to grow to lower final population compared with the cell density that reached when singly cultivated under the same conditions. Finally, an important aspect worth mentioned is the nutritional requirements and metabolism under anoxic conditions. Pine et al. (1989) in a thorough study resulted that no strain of *Listeria* grew on sucrose, maltose or lactose. The metabolic profile of *L. monocytogenes* is affected by the availability of oxygen. *L. monocytogenes* has been shown to produce similar metabolites under anaerobic conditions formed by CO2 or N2 (Jydegaard-Axelsen et al., 2004). In brain heart infusion broth (BHI), Under aerobic growth the end products consisted of lactate (28%), acetate (23%), acetoin (26%) and carbon dioxide (23%), while under anaerobic conditions lactate (79%) is the major end product, but small amounts of acetate (2%), formate (5.4%), ethanol (7.8%) and carbon dioxide (2.3%) (Müller-Herbst et al., 2014). During growth in a chemically defined medium by Premaratne et al. (1991), acetic and lactic acid were the major products in aerobic cultures, and acetoin was only produced aerobically. Lactic acid was the major product in anaerobic cultures, and formic acid was only produced anaerobically. There was no significant difference in ethanol production for the different atmospheres.

Microbial heterogeneity on time to first division

Population level analysis reflects the dominant biological mechanism operating within individual cells in a population (Altschuler and Wu, 2010). Genetically identical bacterial cells, even when experience the same environmental conditions, exhibit unpredictable variation in their phenotypes (Choudhary et al., 2023; Evans and Zhang, 2020; Koutsoumanis and Lianou, 2013; Papagianeli et al., 2022). Heterogeneity of bacterial populations may result from both phenotypic and genotypic variations, during interaction of internal and environmental factors, as well as from random fluctuations of the biochemical and physiological characteristics. Cell heterogeneity improves the survival of bacterial populations under heterogeneous or variable environmental conditions, as well as under the effect of stress factors. Thus, under diverse

environmental conditions bacterial physiological heterogeneity reveals the need of describing bacterial population dynamics at single-cell level (Magdanova and Golyasnaya, 2013). The growth and evolution of these populations in turn depend on a complex interplay between single-cell properties, the symbiosis or competition between the cells, so the ability to encapsulate the cells within a closed environment allows studies of bacterial interactions such as quorum sensing or competition between different strains (Barizien et al., 2019).

Quantitative microbial risk assessment (QMRA) have to take into account the heterogeneity in kinetic parameters, because pathogenic bacteria, when are present in food, they are often found in very low numbers and the distribution of individual lag times within cell populations cannot derived by observations at the cell population level (Elfwing et al., 2004; Kutalik et al., 2005a; Niven et al., 2008). As “lag time” is the incipient stage of a bacterial growth cycle, in which cells are adjusting to the new environmental conditions, before initiating exponential growth. Lag is a dynamic, organized adaptive and evolvable process that protects bacteria from threats, promotes reproductive fitness and it is broadly relevant to the study of bacterial evolution, host-pathogen interactions, antibiotic tolerance and food safety (Bertranda, 2019). The duration of the lag phase depends on the previous history of the cells as well as the current environment. The term “time to first division” includes both the lag time and the time for the division process, with other words, the time needed for the individual cell to start dividing into two daughter cells. Pin and Baranyi (2008) showed that the more time *Escherichia coli* cells spent in the stationary phase prior to inoculation, the longer was the time to first division and the more widely distributed. However, the age of cells did not affect the distribution of the second generation time, indicating that the main effect of the age of the cells is on the lag period prior to the first division. Apart from the previous history of the population, the inoculum size also seems to influence lag phase. Two classes of inoculum size effect on population lag may be envisaged (a) cooperative or inhibitory effects of high cell concentrations or (b) statistical effects at low cell concentrations arising from the variability in individual lag times. There is little specific information about the possible effects of cell–cell interactions on lag time although cell signaling has been shown to affect the emergence of cells from dormancy and the lag time of populations in biofilms (Robinson et al., 2001). Smelt et al. (2002) during the evaluation of lag phase’s duration of sublethal heat-treated and untreated *Lactobacillus plantarum* cells showed that the effect of inoculum size on the shelf life (i.e., time to spoilage) is not only due to the number of generations needed to attain the spoilage level but also the effect of inoculum size on the apparent lag phase. According to Gay et al. (1996) differences in the initial cell concentration combined with certain storage and/or culture conditions, concerning the storage of inoculum and the temperature of pre-inoculation incubation, seems to increase the duration of the lag phase at low initial bacterial populations. The authors speculated that in a smaller population there is less likelihood of individuals with relatively shorter lag time. Additionally, the duration of the lag phase and by extension the

duration of time to first division of stressed/injured bacteria cells, expected to be influenced during culture, especially, under growth-limiting conditions (Robinson et al., 2001). The lag time of *Listeria monocytogenes* growing under suboptimal conditions (low nutrient concentrations, pH 6, and 6.5°C) was extended when the inoculum was severely stressed by starvation and the inoculum size was very small (Augustin et al., 2000). When *L. monocytogenes* was inoculated in substrates with unfavorable characteristics and/or under suboptimal conditions (different salt concentration and pH), during the extended lag phase, a high initial inoculum seems to be more likely to initiate growth faster (Pascual et al., 2001).

Along with optical density (D'Arrigo et al., 2006; Métris et al., 2003; Robinson et al., 2001; Wu et al., 2000) and flow cytometry (Bannenberg et al., 2021; Smelt et al., 2002), an effective method to determine the time to first division is the combination of microscopy and imaging (Elfwing et al., 2004; Koutsoumanis and Lianou, 2013; Kutalik et al., 2005b; Niven et al., 2008, 2006; Papagianeli et al., 2022). This method provides direct observation of single cell growth and by extension time to first division can be obtained by determining when the first cell doubling occurs (Wu et al., 2000). However, not all the developed protocols including microscopy can work with all the different cell morphologies or cell observation during slide culture method (when inoculate a piece of agar on a slide) is has its limitations because the late-dividing cells in a stressed population may be overgrown by the more dynamic ones (Niven et al., 2006).

Aims of the present study

Listeria monocytogenes is a ubiquitous environmental microorganism that "*knows how to survive*". As a saprophytic bacterium thriving in diverse environments, managing to survive and grow in a wide range of harsh environmental conditions renders it a major concern for ready-to-eat (RTE) products. Previous studies have described the simultaneous presence and dissemination of multiple *L. monocytogenes* strains, which may have been introduced *via* raw materials at various time-points in the processing environment. The pathogen may persist and spread, possibly ending to multiple strains co-existing in/on the same food products and subsequently during ingestion, where the pathogen switch to an invasive intracellular bacterium. Zilelidou et al. (2016a, 2016b, 2015) has already prove that during simultaneous present of more than one strains in the same substrate may occur inter-strains interaction resulting in growth inhibition for one strain of the dual composite. Taking the latter into consideration, the present work aimed to investigate the behavior of different *L. monocytogenes* strains during co-culture in/on differently structured substrates based on different nutritional and physicochemical characteristics under different levels of oxygen availability. Additionally, in the present study performed a number of different experiments in an attempt to describe to potential mechanisms behand the observed growth inhibition of some strains during co-culture.

The main objectives of this thesis were the following:

1) To evaluate the effect of: i) oxygen availability (under aerobic, hypoxic, or anoxic conditions) and ii) substrate's structure (liquid, semi-solid, or solid media) based on different concentrations of agar, on growth and inter-strain interactions of different *L. monocytogenes* strains, that may manifest, during co-culture of two strains of the pathogen. **(Chapter 2)**
2) To evaluate the effect of oxygen availability and the matrix structure on growth and the subsequently occurred inter-strain interactions of the pathogen *L. monocytogenes* in/on different dairy-based model systems produced by Ricotta and Camembert. **(Chapter 3)**
3) To evaluate the inter-strain interactions and matrix-adaptation of different *L. monocytogenes* strains during their growth on Ricotta and Camembert products and their subsequent survival after exposure to simulated gastric fluid (SGF). **(Chapter 4)**
4) To investigated if the occurred interactions during co-culture of different *L. monocytogenes* strains in the same substrate, apart from the contact-dependent inhibition, are due to the restriction of the nutrients or the production of metabolic factors. **(Chapter 5)**
5) To evaluated the impact of cell proximity of different co-cultured *L. monocytogenes* strains on time to first division and was developed a program to identify and monitor the individual cells during an image sequence. **(Chapter 6)**

6) To record the intracellular proteins in order to investigate if the observed inhibition of some L. monocytogenes strains during co-culture is due to a protein or enzyme and potentially describe the underling mechanism. **(Chapter 7)**

Figure 7 outlines the questions addressed to each chapter of this thesis and how the different chapters connected with each other.

Substrates with different structures and different nutritional characteristics stored under different levels of oxygen availability

CHAPTER 2

In vitro evaluation of the effect of oxygen availability (aerobic, hypoxic or anoxic conditions) and substrate's structure (liquid, semi-solid, or solid media), using TSB-YE solidified with the appropriate concentrations of agar, on growth and inter-strain interactions between different *L. monocytogenes* strains.

L. monocytogenes strains, C5 (belonging to serotype 4b) and 6179 (belonging to serotype 1/2a) were selected due to the remarkable interaction which was observed during their co-culture. The strains were cultured in TSB-YE under aerobic conditions (constant shaking) at 7°C (**Chapters 5 and 7**) or on TSA-YE surface at 37°C (**Chapter 6**).

CHAPTER 3 and 4

Evaluation of the effect of oxygen availability and substrate's structure on growth and inter-strain interactions between different strains of the pathogen *L. monocytogenes* in/on different **dairy-based model systems** produced by Ricotta and Camembert, in **Ricotta** and on **Camembert** products.

CHAPTER 4

Evaluation of the survival by simulated gastric digestion (exposure to SGF at pH 2.0, 2h, 37°C) of singly-cultured and co-cultured cells of different *L. monocytogenes* strains after storage in Ricotta and on Camembert.

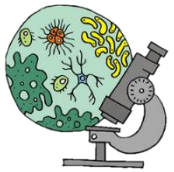
CHAPTER 5

Investigation of whether the occurred interactions during co-culture of *L. monocytogenes* strains, are also due to nutrients deficiency or the production of metabolic factors, apart from the contact-mediated inhibition.

CHAPTER 6

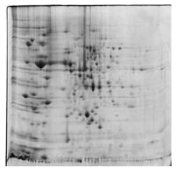
Assessing the impact of cell proximity of co-cultured *L. monocytogenes* strains on time to first division (with time-lapse microscopy).

Correlation of the occurred inter-strain interactions at single-cell level with the behavior at population level.



CHAPTER 7

Investigation of possible protein-mediated underpinning mechanism of inter-strain interaction: recording the intracellular proteins to examine whether the observed growth inhibition of selected *L. monocytogenes* strains during co-culture is due to a protein or enzyme.



Studies performed in an attempt to describe the underlying mechanism

Figure 7. Outline of research topics addressed in this thesis

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CHAPTER 2

Evaluation of oxygen availability on growth and inter-strain interactions of L. monocytogenes in/on liquid, semi-solid and solid laboratory media

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Evaluation of oxygen availability on growth and inter-strain interactions of *L. monocytogenes* in/on liquid, semi-solid and solid laboratory media

Maria A. Gkerekou, Konstantina G. Athanaseli, Anastasia E. Kapetanakou, Eleftherios H. Drosinos and Panagiotis N. Skandamis*

Laboratory of Food Quality Control and Hygiene, Department of Food Technology & Human Science, Agricultural University of Athens, Greece.

Abstract

The coexistence and interactions among *Listeria monocytogenes* strains in combination with the structural characteristics of foods, may influence their growth capacity and thus, the final levels at the time of consumption. In the present study, we aimed to evaluate the effect of oxygen availability in combination with substrate micro-structure on growth and inter-strain interactions of *L. monocytogenes*. *L. monocytogenes* strains, selected for resistance to different antibiotics (to enable distinct enumeration), belonging to serotypes 4b (C5,ScottA), 1/2a (6179) and 1/2b (PL25) and were inoculated in liquid (Tryptic Soy Broth supplemented with Yeast Extract - TSB-YE) and solid (TSB-YE supplemented with 0.6% and 1.2% agar) media (2-3 log CFU/mL, g or cm^2), single or as two-strain cultures (1:1 strain-ratio). Aerobic conditions (A) were achieved with constant shaking or surface inoculation for liquid and solid media respectively, while static incubation or pour plated media corresponded to hypoxic environment (H). Anoxic conditions (An) were attained by adding 0.1% w/V sodium thioglycolate and paraffin overlay (for solid media). Growth was assessed during storage at 7^{o}C (n=3x2). Inter-strain interactions, as manifested by the difference in the final population between singly and co-cultured strains. The extent of suppression increased with reduction in agar concentration, while the impact of oxygen availability was dependent on strain combination. During co-cultivation, in liquid and solid media, 6179 was suppressed by C5 by 4.0 (in TSB-YE under H) to 1.8 log units (in solid medium under An), compared to the single culture, which attained population of *ca.* 9.4 log CFU/mL or g. The growth of 6179 was also inhibited by ScottA by 2.7 and 1.9 log units, in liquid culture under H and An, respectively. Interestingly, in liquid medium under A and An, ScottA was suppressed by C5, by 3.3 and 2.3 log units, while in solid media, growth inhibition was less pronounced. Investigating growth interactions in different environments could assist in explaining the dominance of *L. monocytogenes* certain serotypes.

Keywords: L. monocytogenes, inter-strain interactions, structure, oxygen availability

Introduction

Listeria monocytogenes is a foodborne pathogen that "*knows how to survive*" (Gandhi and Chikindas, 2007). The ubiquity of this pathogen and its ability to survive and grow in a wide range of harsh environmental conditions renders it a major concern for ready-to-eat (RTE) products. According to the latest report of European Food Safety Authority, the confirmed invasive human cases of listeriosis for 2018 were 2549 (EFSA, 2019).

The pathogen can be introduced to the processing environments *via* raw materials, thereinafter may persist and spread in the processing environment, resulting in cross-contamination of the final products (Martínez-Suárez et al., 2016; Thévenot et al., 2006; Zoellner et al., 2018). The simultaneous presence and dissemination of multiple *L. monocytogenes* strains in the processing plants has been confirmed by studies that have isolated more than one strains from different sites of the same facility, e.g., from the same shrimp processing plant (Destro et al., 1996). As expected, this may result in more than one strain being isolated from different dairy (i.e. soft cheese, Latin style fresh cheese), fishery (i.e. gravad rainbow trout, smoked salmon) or meat (i.e. raw meat, poultry Alheiras, a traditional Portuguese smoked meat sausage) final products (Danielsson-Tham et al., 1993; Felício et al., 2007; Gendel and Ulaszek, 2000; Kabuki et al., 2004; Loncarevic et al., 1996; Ryser et al., 1996). The above findings are further underpinned by studies with epidemiological evidence that multiple strains of *L. monocytogenes* may be traced in listeriosis outbreaks caused by the consumption of ready-to-eat meat products, cantaloupe and a traditional curd cheese called "Quargel", where two and four distinct PFGE patterns and two distinct MLST sequence types, were isolated, respectively (Gilmour et al., 2010; Laksanalamai et al., 2014; Rychli et al., 2014a). Furthermore, studies investigating the behavior of multiple strains in the same matrix (Zilelidou et al., 2015; 2016a), suggest that some strains may survive better than others against food processing-related stresses (i.e., persistent strains adapted to food-processing facilities *via* natural selection) or may be "opportunistic" competitors, due to adaptation to a particular habitat (Destro et al., 1996; Lunden et al., 2003; Thévenot et al., 2006).

During growth, the interactions between microbial communities of different (Ghoul and Mitri, 2016; Jia et al., 2020; Tirloni et al., 2019; Wimpenny et al., 1995) and/or same species (Bruhn et al., 2005; Gorski et al., 2006; Pan et al., 2006; Zilelidou et al., 2015, 2016a) and the predominance of a certain microorganism or strain are, among others (intrinsic food properties and environmental conditions), determined by the micro-structure and the adaptation of each microorganism/strain to that certain environment (Couvert et al., 2017; Henderson et al., 2019; Lundén et al., 2003; Tirloni et al., 2019; Wimpenny et al., 1995). According to Wilson et al. (2002), foods may be classified into 6 categories with respect to their structural characteristics and/or the sites where microbial growth occurs, namely liquids, aqueous gels, oil-in-water emulsions, water-in-oil emulsions, gelled emulsions and surface. The compaction and the distribution of water and oil-droplets (including their size) in each of the above systems determine the available space for microbial growth, the

nutrient and oxygen diffusion and thus, the cell motility and mode of growth (planktonic *vs* immobilized) (Boons et al., 2013; Wilson et al., 2002). In liquids and emulsions, with less than 83% of fat, the bacterial growth is planktonic, rendering bacterial cells able to move away from areas, where nutrients are depleted and metabolites have accumulated. By adding solidified agents to liquid systems, the bacterial growth changes from planktonic to colonial. The immobilized cells grow as immerged or surface colonies, having limited access to nutrients and oxygen and increased exposure to accumulated growth-limiting metabolites (Panagiotis N Skandamis and Jeanson, 2015). The growth rate of microorganisms in response to food structure follows the order: broth > immerged colonies > surface colonies. Surface colonies are known to have the slowest growth rate because bacterial cells are more directly exposed to environmental changes and during spread and building of the colony, cells soon experience starvation, as the replenishment of nutrients takes place from the bottom of the colony (Aspridou et al., 2014; Brocklehurst et al., 1997; Noriega et al., 2010b; Theys et al., 2008; Wilson et al., 2002).

Variations in food structure and packaging atmospheres may lead to different levels of oxygen availability, an environmental factor that is also known to impact microbial physiology and *via* the widely used MAP or vacuum packaging, it is considered a method of controlling microbial growth (Noriega et al., 2010a). However, *L. monocytogenes* as a facultative anaerobic microorganism, is capable to overcome the barrier of anaerobiosis and grow at a similar cell density to that grown under aerobic conditions (Buchanan and Klawitter, 1991; Couvert et al., 2019; Lungu et al., 2009; Pine et al., 1989), whereas culture or storage under anaerobic conditions may influence its ability to resist and survive a number of stresses, such as acid and bile stress and increase the invasiveness to Caco-2 cells (Andersen et al., 2007; Lungu et al., 2009; Sewell et al., 2015; White et al., 2015; Wright and Pendarvis, 2016). The same applies to stresses that are related to the processing environment, including heat or alkaline stress, or even stresses associated with the use of sanitizers (George et al., 1998; Nilsson et al., 2013).

Many studies have elucidated the behavior of multiple *L. monocytogenes* strains, yet separately, in response to various environmental conditions (Antwi et al., 2007; Baka et al., 2017; Møller et al., 2013; Noriega et al., 2008a, 2009). However, interactions between strains, that may co-exist in a food product, appeared to be influenced by a specific set of environmental conditions that may occur in different habitats (Buchanan and Bagi, 1999, Zilelidou et al., 2016a; 2016b). It is therefore, important to study the interactions between different strains co-existing in foods, because the relative levels of each strain during storage may reflect the population of each strain at the end of the enrichment and subsequently their probability of isolation on ALOA plates, according to ISO11280 (Zilelidou et al., 2016b). Masking of certain strains throughout the detection process may hinder the tracing of the actual causative agent (strain) of an outbreak, during epidemiological investigations, which may have slower growth during storage or enrichment procedure, but might be more virulent.

Based on the above, the objectives of the present study were to evaluate the effect of: i) oxygen availability (under aerobic, hypoxic, or anoxic conditions) and ii) microstructure (liquid, semi-solid, or solid media) produced by different concentrations of agar, on growth and inter-strain interactions of different *L. monocytogenes* strains, that may manifest, during co-culture of two strains of the pathogen.

Materials and methods

L. monocytogenes strains

Four *L. monocytogenes* strains, from the microorganism collection of the Laboratory of Quality Control and Hygiene in Agricultural University of Athens, were used in the present study (Table 1). Strains were selected, based on their antibiotic resistance to streptomycin (Streptomycin Sulfate Biochemica, AppliChem) or rifampicin (Rifambicin, AppliChem) for enabling selective enumeration of each strain in co-culture, according to the method described by de Blackburn & Davies (1994) (Table 1). Briefly, the wild strains were incubated in Tryptic Soy Broth (LAB004, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract (MC001, Lab M Limited, United Kingdom) (TSB-YE; pH: 7.3 ± 0.2) for 24 h at 37°C. Subsequently, an equal amount of TSB-YE was added with the corresponding antibiotic (different concentrations tested) and the cultures re-incubated for another 24 h (37°C). After 48 h of incubation, aliquots (0.1 mL) were spread on Tryptic Soy Agar (LAB011, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract (TSA-YE; pH: 7.3 + 0.2), supplemented with the corresponding antibiotic. Streptomycin was used at 1000 μg/mL and rifampicin at 50 μg/mL. Single colonies of each strain were selected and were maintained at -20°C in TSB-YE, 20% glycerol and the appropriate concentration of rifampicin or streptomycin depending on the strain. The concentration of antibiotics used, for the preparation of the (selective) enumeration media, were the lowest in which the second strain was unable to grow, i.e., the one that was not resistant to this antibiotic. The selection of strains aimed to include strains of different serotype and origin (outbreak and animals) and strains characterized as persistent in dairy processing environments (Fox et al., 2011) (Table 1). Both ScottA (streptomycin resistant strain and rifampicin resistant strain) and C5 belong to serotype 4b, while 6179 and PL25 belong to serotypes 1/2a and 1/2b, respectively.

Inoculum preparation

All strains were maintained on TSA-YE containing rifampicin (TSA-YE+R) or streptomycin (TSA-YE+S) at 4°C and sub-cultured once a month. A single colony from a TSA-YE+S or TSA-YE+R stock culture of the target strain was transferred to 10 mL TSB-YE+S or TSB-YE+R and incubated for 24 h at 30°C and subsequently, 100 μL of each culture was transferred to fresh TSB-YE+S or TSB-YE+R for 18 h incubation at 30°C to obtain stationary-phase cells with a density of *ca.* 10^9 CFU/mL. Following activation, strains were harvested by centrifugation (3600 rpm for 10 min at 4°C) (Megafuge 1.0R, Heraeus, Buckinghamshire, England), washed

twice and re–suspended in 10 mL of ¼ strength Ringers' solution (LAB M, Lancashire, UK). The level of the inoculum was determined by plating 0.1 mL of the appropriate decimal dilution of each strain on TSA-YE+S or TSA-E+R and incubation at 37°C for 48 h.

Media preparation, inoculation and growth conditions

For the preparation of the liquid systems (L) was used TSB-YE. According to Noriega et al. (2008), depending on the oxygen availability for cell growth, three types of experimental conditions were assayed: aerobic, hypoxic and anoxic. *Aerobic conditions* (A) were tested in 50 mL falcon tubes containing 30 mL of sterile medium. The tubes were inoculated and stored on an orbital shaker (Shaker KS 130 basic, IKA-Werke GmbH & Co. KG, Germany), at 240 rpm. *Hypoxic conditions* (H) were tested in 120 mL of sterile medium (full glass bottle), inoculated with the appropriate dose of inoculum, closed with N20 Butyl rubber stoppers and crimp caps (Macherey-Nagel GmbH & Co. KG, Germany) and stored without shaking. *Anoxic conditions* (An) were generated by depleting initial dissolved oxygen from 120 mL medium (full glass bottle) by the addition of 0.1% w/V sodium thioglycolate and the bottles were inoculated and stored under the same conditions as those described for the hypoxic conditions. Usually, the study of the behavior of *L. monocytogenes* under anoxic conditions is achieved by removing the dissolved oxygen by flushing sterile nitrogen. However, the use of nitrogen gas could not be applied to solid substrates, so we chose sodium thioglycolate, based on the modified protocol of Vasconcelos and Deneer (1994) (*see supplementary material*).

For the preparation of the semi-solid (SS) and solid systems (S), was used TSB-YE and different concentrations of agar (MC002, Lab M Limited, United Kingdom). *Aerobic conditions* were assayed in Petri dishes filled with 20 g of TSB-YE supplemented with 1.2% agar. Inoculation was performed on the surface of the medium, while petri dishes were sealed with parafilm before storage to prevent dehydration. Moreover, for the attainment of *hypoxic conditions,* duran flasks with 250 mL of TSB-YE supplemented with 0.6% or 1.2% agar were autoclaved at 121^{o}C for 15 min. Inoculation was performed when the media were at a temperature of about 38 to 40°C, with single and dual-strain cultures. Following inoculation, 20 mL of each inoculated medium quickly distributed into petri dishes. The petri dishes left to cool further and closed with parafilm. Finally, the preparation of the samples for the evaluation of growth and inter-strain interactions on solid substrates under *anoxic conditions* was similar to that of samples stored under hypoxic conditions. The only difference was the addition of 0.1% w/V of sodium thioglycolate before the inoculation of the media. Following inoculation, 12 g of each inoculated medium, quickly distributed into falcon tubes. The falcon tubes left to cool further and the anoxic conditions attained, during storage, with a paraffin overlay.

The above media were inoculated either with one or two strains (strain ratio of 1:1) listed in Table 1, at approximately 2-3 log CFU/mL or cm^2 or g. The dual-strain combinations were: C5+6179, C5+ScottA, C5+PL25, 6179+ScottA and ScottA+PL25. Each strain in the paired cultures was resistant to a different

antibiotic, enabling the selective enumeration of either strain in the co-culture, as described in §2.1 (Fig. 1). The inoculated samples were stored at 7°C, in high precision (± 0.5°C) incubation chambers (MIR 153, Sanyo Electric Co., Osaka, Japan). Three independent storage experiments were performed and duplicate samples were used for each trial (*n*=6).

Microbiological analysis

Sampling liquid medium

On various days during storage at 7°C, to determine the growth curves of the different strains and the same strains in the co-cultures, 3-5 mL of each liquid culture was removed under aseptic conditions. Following decimal dilutions in ¼ strength Ringer's solution, aliquots of 0.1 mL and/or 1 mL of diluted sample were spread on selective and non-selective culture media. The population of *L. monocytogenes* strains was enumerated on TSA-YE and TSA-YE+S or/and TSA-YE+R, at 37°C for 48 h. Average numbers of colonies *per* plate were used to calculate the viable-cell concentrations, expressed as log CFU/mL.

Sampling semi-solid and solid media

On various days during storage at 7°C, 12-15 g of each semi-solid and solid sample was removed from their containers (the petri dishes and the falcon tubes) and placed in plastic bags, in which 3-fold sterile ¼ strength Ringer's solution was added under aseptic conditions. The samples were homogenized in a stomacher (Interscience, France) for 60 s. Following decimal dilutions in ¼ strength Ringer's solution, aliquots of 0.1 mL and/or 1 mL of diluted sample were spread on selective and non-selective culture media. The population of *L. monocytogenes* strains was enumerated on TSA-YE and TSA-YE+S or/and TSA-YE+R after 48 h of incubation at 37°C. Data were expressed as log CFU/g in case of inoculation inside the solidified substrate or log CFU/cm^2, in case of surface inoculation.

pH and a_w measurements

The pH values of the samples were recorded at every sampling by using a digital pH meter (pH 526, Metrohm Ltd, Switzerland) *via* immersion of pH electrode in the homogenate. Water activity (a_w) was monitored by a digital aw meter (Hydrolab rotronic, Switzerland) at the beginning, the middle, and the end of storage.

Statistical analysis and primary modelling

Statistical analysis was performed with *STATGRAPHICS® Centurion XVII* computer package (Statpoint Technologies Inc., USA). During analysis of variance (ANOVA), Tukeys' HSD multiple range tests was used to evaluate the differences in the growth kinetics between the single and co-cultures among the different combinations of structure and oxygen availability, while for all pairwise comparisons the Student's t-test was

used. Differences were considered to be significant at P-values<0.05. The obtained bacterial growth data, per singly or co-cultured strains, were fitted to the Baranyi-Roberts model with DMFit Excel Add-In software. Maximum specific growth rate (μ_{max}; $days^{-1}$) and lag time (λ; days) were determined.

Results

Growth of single L. monocytogenes strains

The lag time of the strain PL25 in the solid medium was significantly longer under hypoxic than under aerobic conditions lasting 2.53 days. At liquid and semi-solid media the growth rate was higher under hypoxic than under anoxic conditions, while under anoxic conditions the growth rate increased by increasing the structure density with the addition of 1.2% agar (resulting in 0.93 day^{-1} at S/An) (Table 2). The final population of the strain under the different storage conditions studied, ranged from 8.3 (S/A) to 9.3 log CFU/mL or g or cm^2 (S/H) (Table 2). In the liquid and semi-solid cultures of strain PL25, the decrease of oxygen levels resulted in lower final populations (Table 2). Also, during culture in solid medium under anoxic conditions, strain PL25 showed lower levels of final population (8.4 log CFU/g), compared to the growth density under hypoxic conditions (9.3 log CFU/g), although they illustrated similarly high growth rate (Table 2).

Strain C5 showed similar growth to all different combinations of matrix structure and oxygen availability. The duration of lag phase in liquid and solid media increased with the restriction of oxygen, from 0.29 to 1.87 days and from 0 to 2.64 days, respectively (Table 3). In liquid substrate the growth rate increased as a result of the increase in oxygen availability, from anoxic to hypoxic and aerobic conditions (P<0.05) (Table 3). However, the different agar concentrations, representing matrices of varying density, and hence oxygen diffusion, impacted microbial growth rate, but only under hypoxic and anoxic conditions. In particular under hypoxic and anoxic conditions increasing the agar concentration, concomitantly increased the growth rate of *L. monocytogenes* (P<0.05) (Table 3). Regardless structure, the final cell density in anoxic environment was significantly lower than that under aerobic or low oxygen conditions (Table 3). In parallel, regardless of oxygen availability, in or on solid media the final population was higher than the population in the liquid media (Table 3).

In liquid and solid matrices, the lag time of single ScottA cultures, was extended to 1.99 and 2.67 days, respectively, with the reduction or removal of oxygen, while under hypoxic conditions, the lag phase was statistically shorter in liquid than in solid media (Table 4). The growth rate of the singly cultured strain ScottA increased with addition of the maximum percentage of agar concentration (1.2%), both under hypoxic and anoxic conditions (Table 4). Furthermore, in all three studied matrix structures, strain ScottA reached lower cell density under anoxic conditions, at the end of storage (8.7 (L/An), 9.2 (SS/An) and 8.1 (S/An) log CFU/mL or g, respectively) (Table 4).

Likewise, the lag time of strain 6179 increased with solidification of substrate (addition of 1.2% agar) under hypoxic conditions ($P<0.05$) (Table 5). The growth rate increased with increasing agar concentration, under anoxic conditions and on the surface or inside solid substrate the growth's rate increasing trend occurred with decreasing oxygen availability. The condition S/An induced the highest growth rate (0.83 day^{-1}) (Table 5). Similarly to the other studied strains, strain 6179 reached final cell density between 8.7 (SS/H) and 9.4 (S/H) log CFU/mL or g or cm^2 (Table 5). Both under hypoxic and anoxic conditions the final cell density was significantly higher in solid media compared to the cell density in liquid substrates.

Overall, in solid media, under aerobic conditions, the lag time of strains PL25 (1/2b), C5 (4b) and ScottA (4b) was shorter than the lag time under hypoxic or/and anoxic conditions (Tables 2 to 4). Culture of 3 out of 4 single strains (C5, 6179, ScottA), under hypoxic conditions, resulted in significantly longer lag phase in solid than in liquid media (Tables 3 to 5), while all single strains, under anoxic conditions, had significantly faster growth rate in solid compared to liquid media (Tables 2 to 5). All the single strains in liquid media, under aerobic conditions, reached higher final populations compared to their final population under anoxic conditions (Tables 2 to 5; Figures 2Ic to 5Ic). Interestingly, in semi-solid substrate strain 6179 (1/2a) reached higher final population under anoxic conditions, contrary to the other three studied strains which reached higher population under hypoxic conditions. Finally, under hypoxic conditions, in liquid media, the studied strains reached lower final population compared to the population that observed in solid media, while during culture under aerobic or anoxic conditions, the relative difference of the final cell density between the different matrix-structures tested was strain-dependent.

Growth of co-cultured L. monocytogenes

Strain-to-strain interactions occurred in the form of one strain (dominant) significantly suppressing the final population density of the second "weaker" strain (Figs 2 to 5).

Co-cultivation of strain PL25 with strains C5 and ScottA (Fig. 2) in TSB-YE, regardless of oxygen availability, did not affect the behavior of PL25, which grew similarly as in the single culture. Furthermore, in co-culture with C5 in semi-solid medium, under both hypoxic and anoxic conditions, strain PL25 had lower cell density at the end of storage (Table 2, Fig. 2). The observed differences in co-culture on solid matrices, under aerobic conditions, were deescalated as a result of oxygen depletion (Table 2, Fig. 2).

Proceeding to the evaluation of the behavior of strain C5 co-cultivated with strains 6179, ScottA and PL25, the presence of a second strain did not affect the growth of C5 under aerobic and hypoxic conditions, regardless of the matrix structure (L, SS, S). In contrast, during growth under anoxic conditions in liquid substrate, the lag time increased when the strains were co-cultured, especially in the mixed culture with the strain ScottA ($P<0.05$), while C5 (8.8 log CFU/mL), in the presence of PL25, reached lower final population (7.5 log CFU/mL) (Table 3, Fig. 3). In the two-strain culture of C5 with PL25, in solid substrates under anoxic conditions, the presence of the second strain seemed to affect the growth rate of C5, in comparison to single

culture (Table 3). However, in both types of substrates (SS and S), the final population of C5 in the co-culture with the strains 6179, ScottA and PL25 was close to the final population of the single culture. Consequently, it can be said that the growth suppressing effect of strain PL25 on strain C5 in liquid culture under anoxic conditions, is moderated in structured media (Fig. 3Ic-3IIIc).

Regarding the co-culture of strain ScottA with the other three strains, the presence of strain 6179 in/on the same substrates, regardless of oxygen availability, had no impact on growth kinetics of strain ScottA (Table 4; Fig. 4), except for the lower growth rate of co-cultured (0.60 day^{-1}) compared to the singly cultured (0.75 day^{-1}) ($P<0.05$), in semi-solid medium under hypoxic conditions (Table 4; Fig. 4). Nonetheless, despite the lower growth rate of ScottA in the co-culture with 6179 (SS/H), it attained population of 9.2 log CFU/g at the end of storage, i.e., cell density close to that of the single culture of the same strain (9.6 log CFU/g) (Fig. 4) ($P<0.05$). On the contrary, co-culture of ScottA with PL25, caused a pronounced reduction ($P<0.05$) (*max.* slightly >1.0 log unit in the case of L/A) on the final population of ScottA, suggesting that PL25 tended to cease growth of ScottA when the former reached the stationary phase (Fig. 4). Under hypoxic conditions, the presence of PL25 caused a consistent suppression of the maximum population density of ScottA, by 0.7-0.9 log units both in liquid and structured media, as compared to the levels observed for ScottA under identical conditions in single culture (Table 4; Fig. 4Ib–4IIIb). Finally, in the co-culture of ScottA with C5, an even more pronounced effect of the second strain on ScottA was observed, in all cases, but especially in liquid media regardless of oxygen availability (Fig. 4Ia-4IIb), where ScottA reached markedly (>2.0 log units) lower final populations. Notably, growth suppression, induced by co-culture, decreased with increasing of agar concentration, especially under anoxic conditions (Table 4; Fig. 4).

Similarly to the observations for the impact of co-culture on growth of ScottA, the final population levels of strain 6179 in mixed cultures with strains C5 and ScottA, were also markedly suppressed in all the different combinations of structure and oxygen availability, but mostly in liquid cultures (Fig. 5). Notably, there was no evident influence on lag time and growth rate, suggesting again that the competitors caused strain 6179 to enter stationary phase earlier and at lower levels than when cultured alone (Fig. 5). Of the two strains, combined in the mixed cultures with 6179, strain C5 caused more pronounce growth suppression to 6179, than ScottA. Its effect was more evident under aerobic and hypoxic conditions (Fig. 5a-5b). Moreover, strain ScottA also markedly reduced the final cell density of 6179 by at least 0.6 log units (S/An) (Table 5). Consistently with the aforementioned results about the impact of strain competition on ScottA, the magnitude of the suppression of 6179 final population, compared to single culture, by C5, i.e., the strain with the maximum impact on 6179 in the co-culture, or ScottA, was maximized in liquid cultures (suppression by 4.0 and 2.7 log units, respectively (L/H)), but decreased with the addition of agar i.e., upon transition from liquid to semi-solid and solid state (Table 5; Fig. 5).

Comparing the effect of the three media structures of the study (L, SS, S) on inter-strain interactions,

the inhibition was greater in liquid substrates and the "weaker" strains reached lower final populations compared to those observed in the semi-solid and solid media, regardless of strain combination and oxygen availability (Figs 3, 4 and 5).

pH and a_w measurements

The initial pH was 7.01 ± 0.08 and until the end of storage decreased to 5.79 ± 0.27, regardless of structure or oxygen availability and no clear strain or strain combination pattern was observed. Finally the a_w remained 0.99 throughout storage (data not shown).

Discussion

The study in principle, sought to evaluate the influence of structure and nutrient availability, under different levels of oxygen availability (Fig. 1), on *L. monocytogenes* strain-to-strain interactions, as compared to the behavior of single strains. Using TSB-YE as a common base of all substrates, the same type and quantity of nutrients across all conditions was ensured. Under each set of experimental conditions tested, single strains showed similar growth behavior (duration of lag time and growth rate) and reached similar cell density at the end of storage, and altering structure and oxygen availability impacted similarly the different strains in single culture. As a result none of them showed *a priori* any noteworthy fitness advantage, which would highlight it as potentially strong competitor in co-culture with another strain (Figs. 2 to 5). The characteristics of the studied semi-solid and solid substrates (studied agar concentrations, initial pH and a_w of the TSB-YE) allowed both single and co-cultured strains (Figs 2 to 5) to counteract the influence of matrix structure, which in turn determines the mode of microbial growth (planktonic *vs* colonial). The agar concentrations used (0.6 and 1.2%), likely enabled unconstrained formation of spherical colonies, because of the large pores in the matrix induced by agar concentrations lower or close to 1% (Kobanova et al., 2012; Mitchell and Wimpenny, 1997). Such a setting also facilitates the access of external colony cells to replenishing nutrients, as opposed to the more constrained growth environments, created by higher agar concentrations (or substrates structured with other coagulants like *κ*-carrageenan or gelatin), that create smaller pores (Aspridou et al., 2014; Varghese et al., 2014). Furthermore, it is conceivable that the presence of 0.25% glucose in TSB-YE, enhances growth of *L. monocytogenes* and possibly moderates the differences between growth of planktonic cells and cells in colonies (Aspridou et al., 2014). As a result, our observations do not follow the clearly separated ranking as: broth > immerged colonies > surface colonies, which many studies suggest (Brocklehurst et al., 1997; Noriega et al., 2010b; Theys et al., 2008; Wilson et al., 2002), but are in line with studies, which reported no major differences in the growth kinetics between different modes of growth (Smet et al., 2015a; 2015b).

The potentially different food structures and the spatial distribution of the contaminating hazards

per food, as well as the packaging conditions (air or vacuum), result in cells being exposed to microenvironments with various gradients of oxygen availability. However, the facultative anaerobic nature of the pathogen strengthens the microorganism to overcome the limited oxygen availability and grow. Specifically, the microorganism managed to grow in all cases, regardless of oxygen availability, with similar lag times and growth rates reaching almost similar levels of final population in/on TSB-YE (Tables 2 to 5; Figs. 2 to 5) and both in vacuum packed liquid and frankfurters-based aqueous gel and liquid and synthetic meat (solid medium) (Baka et al., 2016; Noriega et al., 2009). All the individual or some combined characteristics of the substrate, may manipulate the growth rate of the pathogen so that growth cannot be categorized by only the criterion of structure. Different environments can affect the strains of the pathogen, in different ways.

The impact of the above-mentioned parameters on inter-strain interactions was manifested by the difference in the final population between singly and co-cultured strains of *L. monocytogenes*, during storage (Figs. 2 to 5). Strains PL25 and C5 were identified as strong competitors in mixed cultures with the other strains of the study, an observation that is consistent with the results of Zilelidou et al. (2015, 2016a, 2016b). In addition, the growth of C5 (serotype 4b) impacted the growth of 6179 (serotype 1/2a) and ScottA (serotype 4b) (Tables 4 and 5; Figs 4 and 5) and in co-culture of 6179 with ScottA, strain 6179 was also inhibited by ScottA (Table 5; Fig. 5). In an attempt to explain how structure affects the behavior of a "weak" strain in liquid co-culture (ScottA (+C5), Fig. 4; 6179 (+C5) and 6179 (+ScottA), Fig. 5), we speculate that due to cell motility, cells of competing strains have higher chances to interact, possibly expressing contact mediated inhibition, while in parallel, they are exposed to the metabolic-end products of the faster growing strain. Thus, the inter-strain interactions between co-cultured strains, according to our results, is more likely to be evident, or occur earlier during storage, in liquid media as compared to semi-solid and solid substrates. In or on solidified structures, using a rather low initial inoculation level for both single and mixed cultures (2-3 log CFU/mL, g or cm^2), resulted in distant dispersion of cells throughout the inoculated matrices that apparently gave rise to well-separated colonies during storage. For instance, in less than 10000 CFU/ml initial population densities it is reasonably assumed that each strain practically grows individually, undisturbed by the presence of any adjacent competitor (Malakar et al., 2002). Nonetheless, the interaction (if any) may gradually occur during colony formation, depending on the size and final proximity of colonies. In conclusion, from our observations regarding the structure, since liquid foods are more dynamic systems and microorganisms have more freedom, the interactions seem to be more intense. We could therefore conclude that perhaps in liquid foods it is less likely to isolate the weakly interacting strain than in the structured, where the population of the "weak" strain may have chances to attain similar levels to those of the "strong" strain.

Concerning the impact of oxygen availability on strain competition, Zilelidou et al. (2015, 2016a),

evaluated inter-strain interactions, between the strains that we used in the present study, in static TSB-YE culture (according to Smet et al., (2015a), we consider Zilelidou's static aerobic culture in TSB-YE, an in-between aeration condition) i.e., resembling initially aerobic conditions and gradual generation of hypoxic conditions, on TSA-YE surface and on commercially vacuum-packed ham slices. They attributed their observations to differences in the nutritional composition between TSA-YE/TSB-YE and ham slices, the accessibility of nutrients by microorganisms (as determined by the viscosity of the liquid vs solid growth substrate), as well as the availability of oxygen due to different headspace atmosphere (i.e., aerobic conditions in TSB-YE, or on TSA-YE *versus* oxygen-deprived conditions in vacuum ham packages). Our results indicate that in co-culture in TSB-YE of 6179 with C5 or ScottA, the extent of inhibition of strain 6179 by C5 and ScottA was significantly different between aerobic and hypoxic conditions (Table 5). Inter-strain interactions in co-cultivation on TSA-YE under aerobic conditions, were evident here (Figs 2 to 5) as opposed to the findings of Zilelidou et al. (2015, 2016a). This inconsistency may be due to different storage conditions in terms of oxygen availability (i.e., culture under shaking aerobic conditions *versus* static aerobic conditions in the other study) and temperature (7^{o}C *vs* 10^{o}C). Differences in the extent of strain-to-strain interactions among the studied oxygen availabilities may be smaller than the impact of structure.

Several studies have highlighted that different combinations of plating and enrichment media can reveal remarkable diversity in microbiota composition and *L. monocytogenes* subtypes co-existing in a sample (Dunbar et al., 1997; Loncarevic et al., 1996; Lund et al., 1991; Pritchard and Donnelly, 1999). More importantly, isolation bias may occur at the level of species (*L. monocytogenes vs L. innocua*) and strain (1/2a vs 4b) (Burn et al., 2005; Gorski et al., 2006), due to variations in strain fitness originally in food and then during enrichment and isolation on plates. Any systematic bias firstly due to the storage conditions (substrate and environmental conditions), that may favor the growth of some strains and offer them a quantitative advantage over others at the begging of enrichment and subsequently during isolation of a foodborne pathogen may result in mismatches between clinical and food isolates. Some hyper-virulent, but slowly growing strains may be outcompeted by faster, albeit less virulent strains (Maury et al. 2016). The apparent competitive advantage of strains belonging to serotype 4b (C5 and ScottA) under hypoxic conditions, could be due to lower oxygen availability. There are studies (Buncic et al., 2001; Davis et al., 2019; Swaminathan et al., 2007) which have shown that the strains belonging to this serotype are those that are more frequently isolated from clinical samples. The restriction of oxygen when the pathogen enters the intestinal mucosa is one of the stimuli of virulence, in order to colonize it (Müller-Herbst et al., 2014). However, strain-to-strain variability in the growth potential and tolerance to food-related stresses may also occur within the same serotype (e.g., C5 *vs* ScottA) (Buncic et al., 2001). During co-culture of *Carnobacterium pisciocola* with *L. monocytogenes*, the latter was suppressed because of the higher relative growth rates of *C. pisciocola* (Buchanan and Bagi, 1999). The greater "fitness" of an organism can be advantageous when two

microorganisms or strains may contaminate simultaneously the same substrate; however both Zilelidou et al. (2015) and the present study, show that inter-strain interactions leading to growth suppression of one strain, can even occur between strains with the same growth potential, suggesting contact-mediated inhibition as the potentially predominant key factor. Zilelidou et al. (2015), observed reduced growth of ScottA in the presence of PL25 strain, when the two strains were cultured separately. Notably, the inhibition of ScottA was greater when the co-cultivation with PL25 took place without a membrane preventing cell contact between strains. According to Cornforth and Foster (2013), the two major bacterial responses to ecological competition are sensing nutrient limitation, or direct cell damage. Studies have shown that contact-dependent inhibition (CDI) may occur mainly in Gram-negative bacteria, such as *E. coli*, and this has been demonstrated in shaking liquid culture (Aoki et al., 2005). The same study suggested that growth inhibition among a 'weak' and a 'strong' strain requires that cell come in direct contact, and not only *via* their metabolome, e.g., when inoculating the weak strain in the spent medium of the strong one, or separating competing strains by an impermeable membrane. Aoki et al. (2005) support the possibility that the secreted molecule, responsible for the inhibition phenotype, is unstable and is only effective when delivered to target cells in close proximity. However, recent studies have found that CDI is not restricted to Gram-negative bacterial but may also occur in Gram-positive bacteria, including *Listeria* (Haeys et al., 2010). Schmitz-Esser et al. (2015) showed that Rhs proteins, present in *L. monocytogenes* strain 6179 (ST121) renders it better competitor against other bacteria in the food processing environment and increases the likelihood of becoming persistent in the processing plant. Strain 6179 has been characterized as persister in the food processing environment (Fox et al., 2011) but here, 6179 was rather a poor competitor. In addition, to CDI, there are many *L. monocytogenes* strains that are known to produce monocins. Monocins are high molecular weight bactericidal protein structures like bacteriocins, which are produced intracellularly by *Listeria sp*. and have an antibiotic effect against *L. monocytogenes*, upon induction of the SOS response. Monocins released to the environment by cell lysis aim to kill competitive bacteria (Curtis and Mitchell, 1992; Lee et al., 2016, Ortel, 1989).

Conclusions

Our results suggest that competition, between certain strains of *L. monocytogenes,* may occur and that both matrix structure (liquid, solid and semi-solid) and oxygen availability (resulting from different substrate structures, packaging conditions or contamination site) could play a key role in the recovery and prevalence of certain *L. monocytogenes* strains in different products. The findings emphasize the need to characterize and describe the way that the intrinsic characteristics of growth matrix and packaging atmosphere may influence the inter-strain competition since they may be critical factors contributing to the dominance of specific *L. monocytogenes* strains, when a food is contaminated with multiple strains. Future

studies will reveal the way that nutrients, in combination with structure and oxygen availability, may manipulate the growth and inter-strain interactions and how co-culture and adaptation on different substrates may influence the survival during gastric transit. Also it would be very interesting to study whether the strains, which proved to be strong competitors in our study, express and produce the proteins, which are involved in the CDI.

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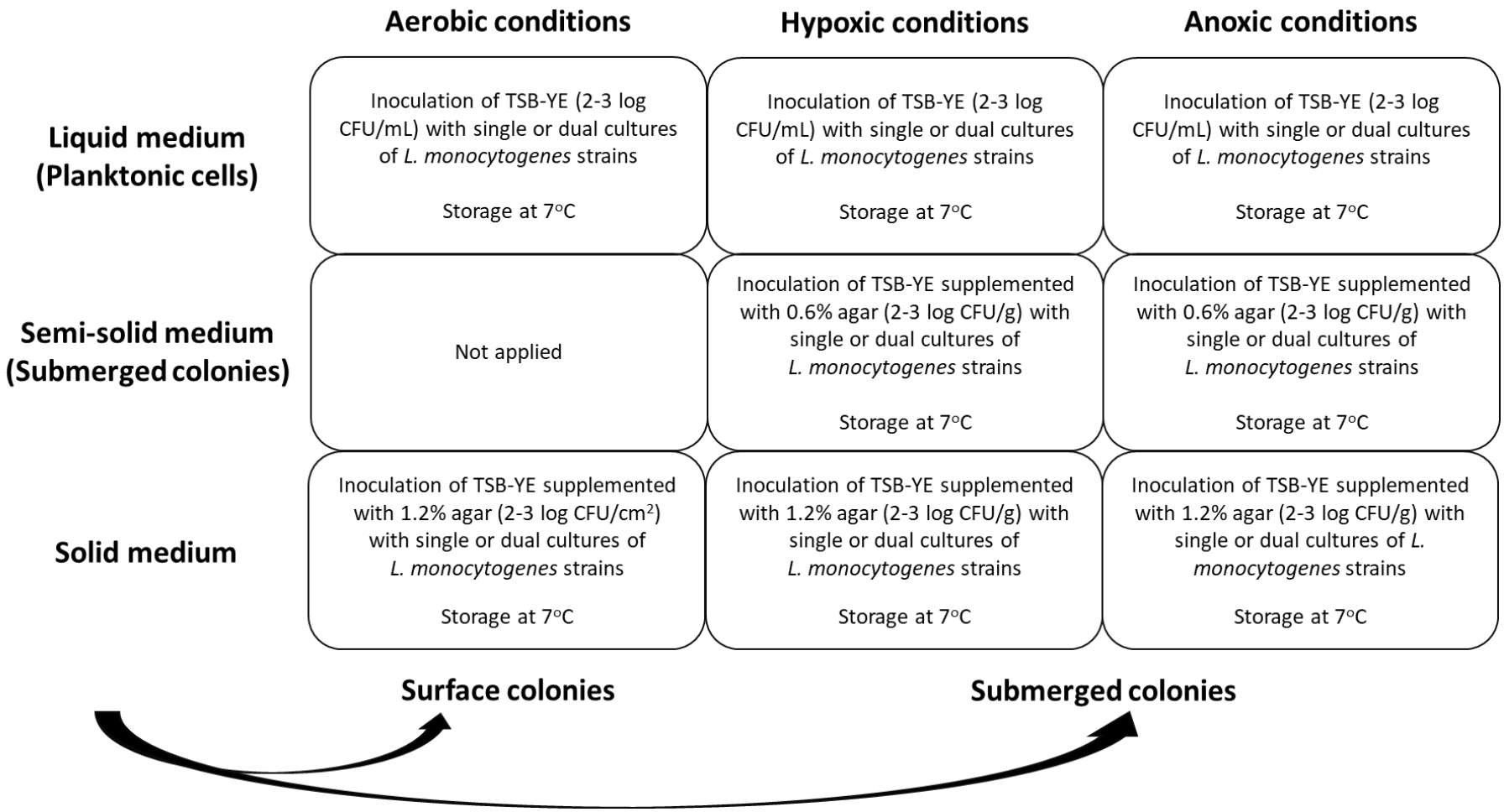

Figure 1. Schematic representation of the experimental design.

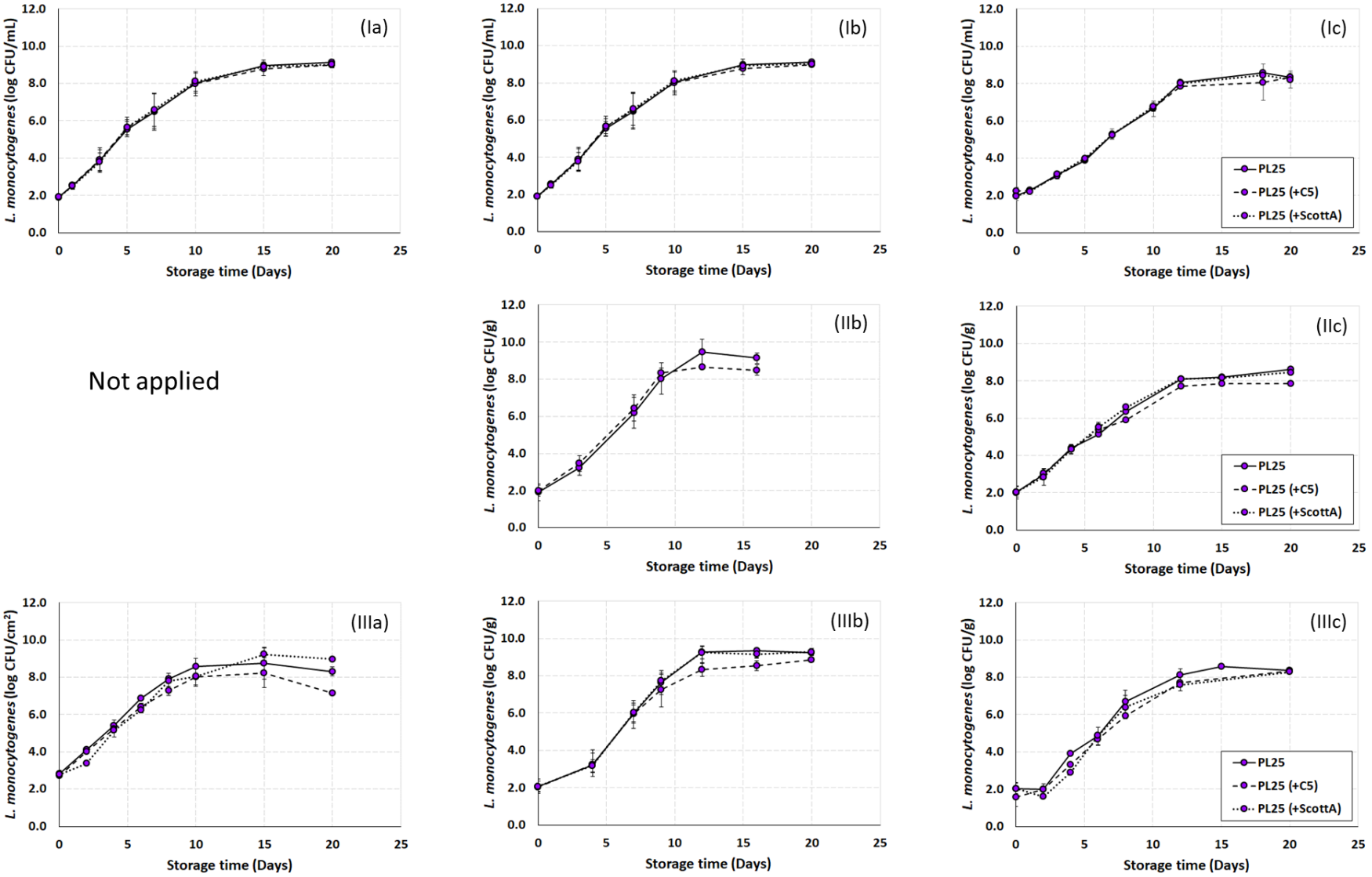

Figure 2. Growth curves of *L. monocytogenes* strain PL25 singly and co-cultured with strains C5 and ScottA, during storage in I) liquid (0% agar), II) semi-solid (0.6% agar), III) solid (1.2% agar) culture media under a) aerobic, b) hypoxic, and c) anoxic conditions, at 7°C.

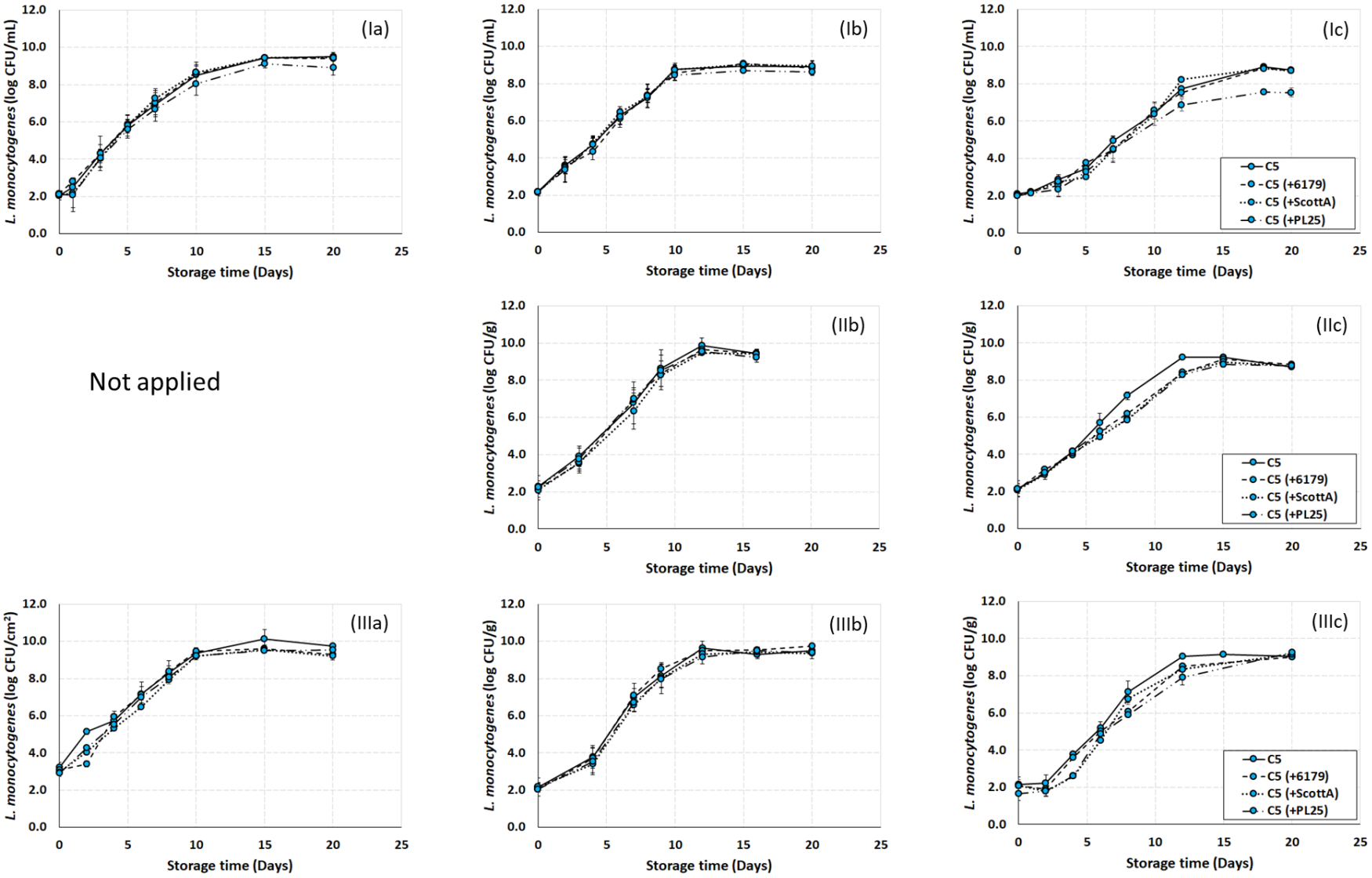

Figure 3. Growth curves of *L. monocytogenes* strain C5 singly and co-cultured with strains 6179, ScottA, and PL25 during storage in I) liquid (0% agar), II) semi-solid (0.6% agar), III) solid (1.2% agar) culture media under a) aerobic, b) hypoxic, and c) anoxic conditions, at 7°C.

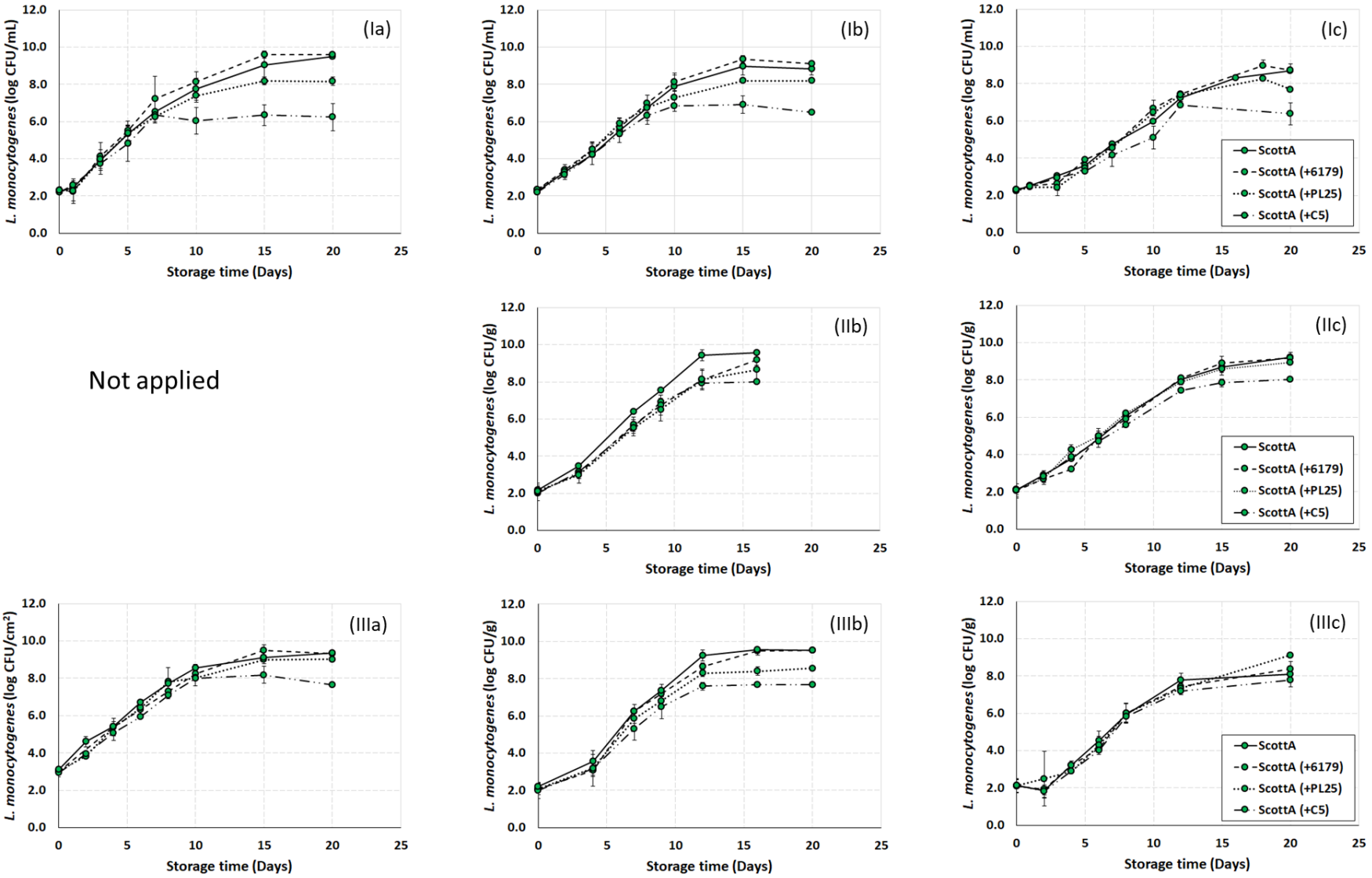

Figure 4. Growth curves of *L. monocytogenes* strain ScottA singly and co-cultured with strains C5, 6179 and PL25, during storage in I) liquid (0% agar), II) semi-solid (0.6% agar), III) solid (1.2% agar) culture media under a) aerobic, b) hypoxic, and c) anoxic conditions, at 7°C.

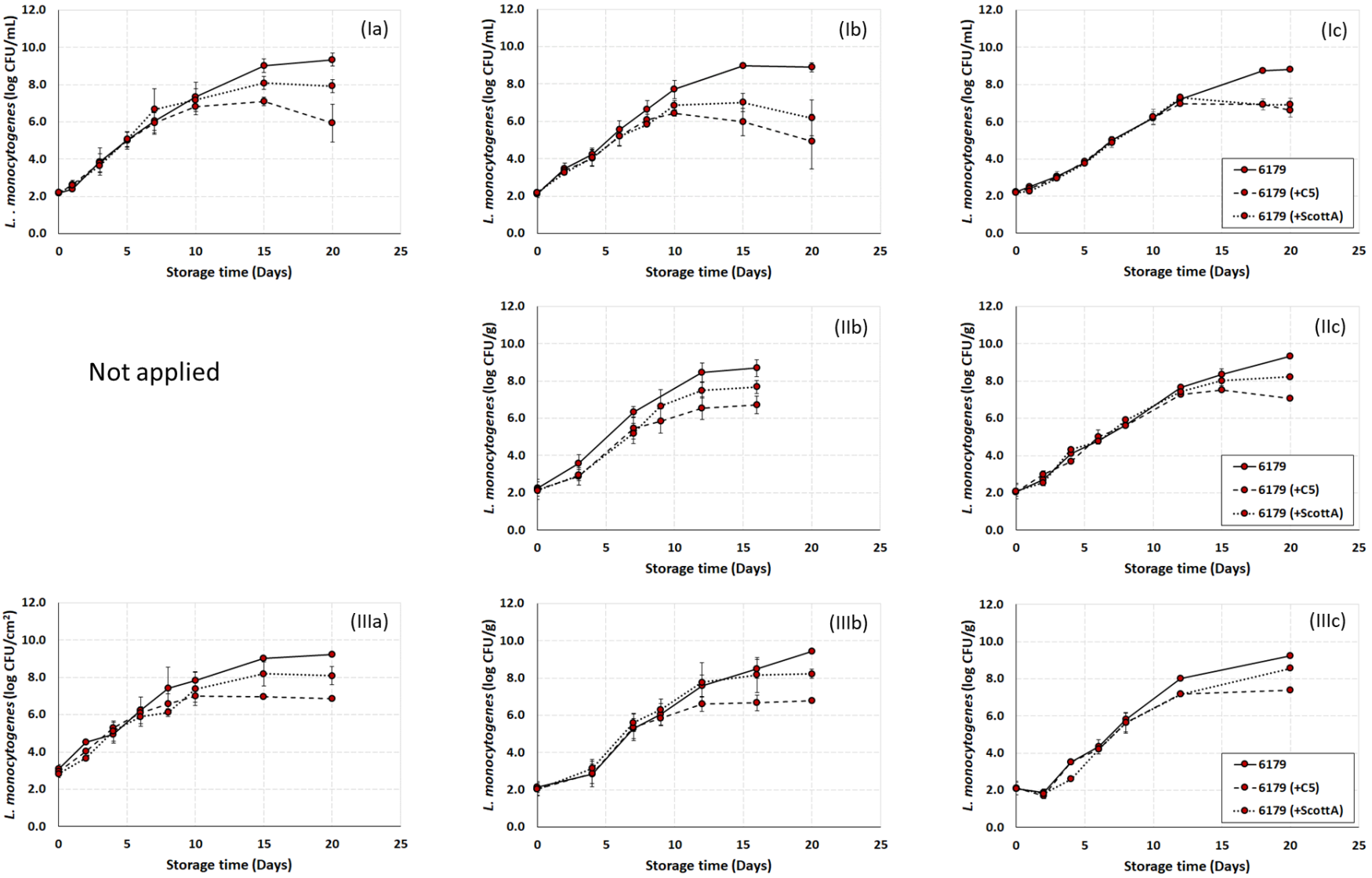

Figure 5. Growth curves of *L. monocytogenes* strain 6179 singly and co-cultured with strains C5 and ScottA, during storage in I) liquid (0% agar), II) semi-solid (0.6% agar), III) solid (1.2% agar) culture media under a) aerobic, b) hypoxic, and c) anoxic conditions, at 7°C.

Table 1. *Listeria monocytogenes* strains used in the study.

Strain	Serotype	MLST	Source	Year of isolation	Reference	Antibiotic resistance (µg/mL)*
C5	4b	ST2	Dairy farm environment isolation	2007	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Streptomycin (2000)
6179	1/2a	ST121	Cheese	1999	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Rifampicin (>800)
ScottA	4b	ST290	Human isolate	1983	Research Institute ATO-DLO, Wageningen, Netherlands	Streptomycin (4000) Rifampicin (>800)
PL25	1/2b (3b, 7)**	ST59	Animal origin	2009	Agricultural University of Athens, Department of food science and human nutrition, Laboratory of Food Quality Control and Hygiene, Athens, Greece	Rifampicin (>800)

*Approximate MIC was considered as the minimum tested concentration (µg/mL) of antibiotic at which no bacterial growth was observed after 24 hours at 30°C. Bacterial growth was confirmed through measurements of optical density (OD_{600}). The streptomycin concentrations were 0, 125, 250, 500, 1000, 2000, 4000 µg/mL. Rifampicin was evaluated at 0, 200, 400, 800 µg/mL.

**The serovar-specific group was characterized by multiplex PCR according to Doumith et al., (2004) and the serovars in parenthesis were omitted due to Multilocus Sequence Typing (MLST) classification.

Table 2. Estimated growth kinetics (lag time and growth rate) and observed final population of *L. monocytogenes* strain PL25 in single and co-culture with strains C5 and ScottA, in liquid, semi-solid and solid media under aerobic, hypoxic and anoxic conditions, at 7°C.

		LAG TIME (Days)			GROWTH RATE (Days^{-1})			FINAL POPULATION (log CFU/mL or cm^2 or g)		
		Aerobic (A)	**Hypoxic (H)**	**Anoxic (An)**	**Aerobic (A)**	**Hypoxic (H)**	**Anoxic (An)**	**Aerobic (A)**	**Hypoxic (H)**	**Anoxic (An)**
Liquid (L)	**Single PL25**	0.33 ± 0.40^{Aa}	0.62 ± 0.74^{Aa}	1.07 ± 0.01^{Aa}	0.68 ± 0.08^{ABa}	0.72 ± 0.05^{Ba}	0.54 ± 0.06^{Aa}	9.1 ± 0.2^{Bb}	8.6 ± 0.2^{Aa}	8.4 ± 0.2^{Aa}
	PL25 (+C5)	0.24 ± 0.29^{Aa}	0.31 ± 0.53^{Aa}	1.42 ± 0.15^{Bb}	0.68 ± 0.11^{ABa}	0.74 ± 0.04^{Ba}	0.52 ± 0.07^{Aa}	9.0 ± 0.1^{Bb}	8.4 ± 0.2^{Aa}	8.4 ± 0.2^{Ab}
	PL25 (+ScottA)	0.35 ± 0.39^{Aa}	0.58 ± 0.62^{Aa}	1.11 ± 1.24^{Aab}	0.70 ± 0.09^{Aa}	0.70 ± 0.06^{Aa}	0.56 ± 0.09^{Aa}	9.0 ± 0.2^{Bb}	8.6 ± 0.1^{Aa}	8.2 ± 0.45^{Aa}
Semi-solid (SS)	**Single PL25**	-	1.83 ± 1.38^{Aa}	0.00 ± 0.00^{Aa}	-	0.90 ± 0.10^{Ba}	0.54 ± 0.09^{Aa}	-	9.1 ± 0.3^{Bb}	8.6 ± 0.0^{Ab}
	PL25 (+C5)	-	0.92 ± 0.56^{Bab}	0.00 ± 0.00^{Aa}	-	0.90 ± 0.05^{Ba}	0.55 ± 0.05^{Aa}	-	$8.5 \pm 0.3^{Ba*}$	$7.9 \pm 0.0^{Aa*}$
	PL25 (+ScottA)	-	1.36 ± 0.64^{Aab}	0.33 ± 0.47^{Aa}	-	0.79 ± 0.09^{Aa}	$0.68 \pm 0.02^{Aab*}$	-	9.0 ± 0.3^{Bb}	$8.5 \pm 0.0^{Aa*}$
Solid (S)	**Single PL25**	0.19 ± 0.27^{Aa}	2.53 ± 0.28^{Ba}	1.94 ± 1.99^{ABa}	0.70 ± 0.02^{Aa}	0.98 ± 0.19^{Aa}	0.93 ± 0.35^{Ab}	8.3 ± 0.2^{Aa}	9.3 ± 0.2^{Bb}	8.4 ± 0.0^{Aa}
	PL25 (+C5)	0.00 ± 0.00^{Aa}	2.23 ± 0.73^{Bb}	0.44 ± 0.62^{Aa}	$0.62 \pm 0.04^{Aa*}$	0.87 ± 0.19^{Aa}	0.57 ± 0.16^{Aa}	$7.2 \pm 0.0^{Aa*}$	$8.9 \pm 0.1^{Cb*}$	$8.3 \pm 0.0^{Bb*}$
	PL25 (+ScottA)	$0.83 \pm 0.01^{Aa*}$	2.23 ± 0.33^{Bb}	2.56 ± 1.13^{Bb}	0.68 ± 0.01^{Aa}	0.99 ± 0.06^{Ab}	0.96 ± 0.32^{Ab}	$9.0 \pm 0.1^{Ba*}$	9.3 ± 0.2^{Cc}	$8.3 \pm 0.0^{Aa*}$

Values with different uppercase letters, that correspond to the same singly or co-cultured strain, in the same row and within the same structured medium, are significantly different ($P<0.05$).

Values with different lower letters, that correspond to the same singly or co-cultured strain in the same column and within the same condition of oxygen availability, are significantly different ($P<0.05$).

Star indicates statistical difference between the growth kinetics of the co-cultured strains in comparison to the singly cultured under the same conditions of structure and oxygen availability (*: $P<0.05$).

Dashes indicate the treatment combinations that were non applicable.

Table 3. Estimated growth kinetics (lag time and growth rate) and observed final population of *L. monocytogenes* strain C5 in single and co-culture with strains 6179, ScottA and PL25, in liquid, semi-solid and solid media under aerobic, hypoxic and anoxic conditions, at 7°C.

		LAG TIME (Days)			GROWTH RATE (Days^{-1})			FINAL POPULATION (log CFU/mL or cm^2 or g)		
		Aerobic (A)	**Hypoxic (H)**	**Anoxic (An)**	**Aerobic (A)**	**Hypoxic (H)**	**Anoxic (An)**	**Aerobic (A)**	**Hypoxic (H)**	**Anoxic (An)**
Liquid (L)	**Single C5**	0.29 ± 0.33^{Aa}	0.36 ± 0.63^{Aa}	1.87 ± 0.99^{Ba}	0.71 ± 0.07^{Ba}	0.73 ± 0.02^{Ba}	0.54 ± 0.01^{Aa}	9.5 ± 0.2^{Bb}	8.9 ± 0.2^{Aa}	8.8 ± 0.0^{Aa}
	C5 (+6179)	0.48 ± 0.42^{Aa}	0.71 ± 0.64^{Aa}	2.71 ± 0.01^{Bc}	0.74 ± 0.08^{Aa}	0.74 ± 0.09^{Aa}	0.59 ± 0.03^{Aa}	9.4 ± 0.1^{Ba}	8.8 ± 0.1^{Aa}	8.7 ± 0.1^{Aa}
	C5 (+ScottA)	0.60 ± 0.32^{Aa}	0.18 ± 0.32^{Aa}	$3.63 \pm 0.79^{Bb*}$	0.77 ± 0.06^{Aa}	0.71 ± 0.09^{Aa}	$0.66 \pm 0.05^{Aa*}$	9.4 ± 0.1^{Cb}	9.0 ± 0.3^{Ba}	8.7 ± 0.1^{Aa}
	C5 (+PL25)	0.41 ± 0.38^{Aa}	0.51 ± 0.44^{Aa}	3.07 ± 0.61^{Bb}	0.68 ± 0.09^{ABa}	0.73 ± 0.09^{Ba}	0.50 ± 0.02^{Aa}	$8.9 \pm 0.4^{Ba*}$	$8.6 \pm 0.2^{Ba*}$	$7.5 \pm 0.3^{Aa*}$
Semi-solid (SS)	**Single C5**	-	1.31 ± 1.14^{Aab}	0.28 ± 0.40^{Aa}	-	0.86 ± 0.13^{Aab}	0.66 ± 0.18^{Aab}	-	9.5 ± 0.2^{Bb}	8.7 ± 0.0^{Aa}
	C5 (+6179)	-	1.22 ± 1.06^{Aa}	0.00 ± 0.00^{Aa}	-	0.86 ± 0.11^{Aab}	0.59 ± 0.04^{Aa}	-	9.5 ± 0.2^{Bb}	8.8 ± 0.0^{Ab}
	C5 (+ScottA)	-	1.24 ± 1.14^{Aab}	0.00 ± 0.00^{Aa}	-	0.88 ± 0.14^{Aa}	0.58 ± 0.01^{Aa}	-	9.4 ± 0.2^{Bb}	8.8 ± 0.0^{Aa}
	C5 (+PL25)	-	0.56 ± 0.76^{Aa}	0.00 ± 0.00^{Aa}	-	0.87 ± 0.15^{Aa}	0.54 ± 0.02^{Aa}	-	$9.2 \pm 0.3^{Bb*}$	8.8 ± 0.0^{Ab}
Solid (S)	**Single C5**	0.00 ± 0.00^{Aa}	2.40 ± 0.30^{ABb}	2.64 ± 2.48^{Ba}	0.63 ± 0.04^{Aa}	1.07 ± 0.12^{Ab}	1.17 ± 0.50^{Ab}	9.8 ± 0.1^{Ca}	9.5 ± 0.1^{Bb}	9.0 ± 0.0^{Ab}
	C5 (+6179)	1.29 ± 0.30^{Aa}	2.35 ± 0.09^{Ba}	1.18 ± 0.39^{Ab}	0.89 ± 0.18^{ABb}	1.12 ± 0.1^{Bb}	0.67 ± 0.11^{Aa}	$9.3 \pm 0.3^{Ba*}$	$9.7 \pm 0.1^{Cc*}$	9.0 ± 0.0^{Ac}
	C5 (+ScottA)	0.97 ± 0.23^{Aa}	2.33 ± 0.09^{Bb}	3.38 ± 0.55^{Cb}	0.71 ± 0.08^{Aa}	1.08 ± 0.24^{Aa}	1.14 ± 0.22^{Ab}	$9.2 \pm 0.2^{Aa*}$	9.4 ± 0.3^{Ab}	$9.2 \pm 0.0^{Ab*}$
	C5 (+PL25)	0.00 ± 0.00^{Aa}	2.00 ± 0.85^{Ba}	$0.86 \pm 1.21A^{Ba}$	0.67 ± 0.01^{ABa}	0.97 ± 0.22^{Ba}	$0.60 \pm 0.18^{Aa*}$	9.6 ± 0.0^{Ab}	9.4 ± 0.1^{Cb}	$9.2 \pm 0.0^{Bc*}$

Values with different uppercase letters, that correspond to the same singly or co-cultured strain in the same row and within the same structured medium, are significantly different ($P<0.05$).

Values with different lower letters, that correspond to the same singly or co-cultured strain in the same column and within the same condition of oxygen availability, are significantly different ($P<0.05$).

Star indicates statistical difference between the growth kinetics of the co-cultured strains in comparison to the singly cultured under the same conditions of structure and oxygen availability (*: $P<0.05$).

Dashes indicate the treatment combinations that were non applicable.

Table 4. Estimated growth kinetics (lag time and growth rate) and observed final population of *L. monocytogenes* strain ScottA in single and co-culture with strains C5, 6179 and PL25, in liquid, semi-solid and solid media under aerobic, hypoxic and anoxic conditions, at 7°C.

		LAG TIME (Days)			GROWTH RATE (Days^{-1})			FINAL POPULATION (log CFU/mL or cm^2 or g)		
		Aerobic (A)	**Hypoxic (H)**	**Anoxic (An)**	**Aerobic (A)**	**Hypoxic (H)**	**Anoxic (An)**	**Aerobic (A)**	**Hypoxic (H)**	**Anoxic (An)**
Liquid (L)	**Single ScottA**	0.50 ± 0.51^{Aa}	0.65 ± 0.81^{Aa}	1.99 ± 1.15^{Bab}	0.61 ± 0.15^{Aa}	0.63 ± 0.11^{Aa}	0.49 ± 0.06^{Aa}	9.5 ± 0.1^{Ab}	8.9 ± 0.4^{Aa}	8.7 ± 0.1^{Ba}
	ScottA (+C5)	1.64 ± 1.36^{Aa}	0.98 ± 0.72^{Aa}	1.85 ± 0.59^{Aa}	0.78 ± 0.03^{Bb}	0.66 ± 0.08^{Ba}	$0.35 \pm 0.11^{Aa*}$	$6.2 \pm 0.7^{Aa*}$	$6.5 \pm 0.1^{Aa*}$	$6.4 \pm 0.6^{Aa*}$
	ScottA (+6179)	0.60 ± 0.52^{Aa}	0.69 ± 0.72^{Aa}	3.39 ± 0.84^{Bb}	0.70 ± 0.13^{Aa}	0.64 ± 0.10^{Aa}	$0.62 \pm 0.08^{Aa*}$	9.6 ± 0.1^{Cb}	9.1 ± 0.1^{Ba}	8.7 ± 0.1^{Ab}
	ScottA (+PL25)	0.44 ± 0.44^{Aa}	0.11 ± 0.19^{Aa}	$4.03 \pm 1.28^{Bb*}$	0.60 ± 0.07^{Aa}	0.56 ± 0.03^{Aa}	$0.62 \pm 0.01^{Aa*}$	$8.2 \pm 0.2^{Ba*}$	$8.2 \pm 0.0^{Ba*}$	$7.7 \pm 0.1^{Aa*}$
Semi-solid (SS)	**Single ScottA**	-	1.57 ± 1.30^{Aab}	0.68 ± 0.92^{Aa}	-	0.75 ± 0.04^{Ba}	0.52 ± 0.07^{Aa}	-	9.6 ± 0.0^{Bb}	9.2 ± 0.3^{Ab}
	ScottA (+C5)	-	0.68 ± 0.96^{Aa}	1.23 ± 1.74^{Aa}	-	0.67 ± 0.16^{Aa}	0.55 ± 0.02^{Aab}	-	$8.0 \pm 0.1^{Ac*}$	$8.0 \pm 0.0^{Ab*}$
	ScottA (+6179)	-	0.68 ± 0.59^{Aa}	0.19 ± 0.27^{Aa}	-	$0.60 \pm 0.12^{Aa*}$	0.54 ± 0.06^{Aa}	-	$9.2 \pm 0.3^{Aa*}$	9.2 ± 0.0^{Ac}
	ScottA (+PL25)	-	1.11 ± 1.00^{Aab}	0.30 ± 0.42^{Aa}	-	0.64 ± 0.08^{Aab}	0.56 ± 0.07^{Aa}	-	$8.7 \pm 0.5^{Ab*}$	$8.9 \pm 0.0^{Bb*}$
Solid (S)	**Single ScottA**	0.00 ± 0.00^{Aa}	2.53 ± 0.52^{Bb}	2.67 ± 2.05^{Bb}	0.58 ± 0.04^{Aa}	0.91 ± 0.13^{Bb}	0.92 ± 0.39^{Bb}	9.4 ± 0.1^{Aa}	9.5 ± 0.0^{Bb}	8.1 ± 0.7^{Ab}
	ScottA (+C5)	0.18 ± 0.26^{Aa}	1.92 ± 1.09^{ABa}	2.81 ± 2.12^{Ba}	0.54 ± 0.05^{Ab}	0.77 ± 0.13^{Aa}	0.88 ± 0.37^{Ab}	$7.7 \pm 0.0^{Ab*}$	$7.7 \pm 0.1^{Ab*}$	$7.8 \pm 0.0^{Bb*}$
	ScottA (+6179)	0.00 ± 0.00^{Aa}	2.27 ± 1.23^{ABa}	2.79 ± 2.01^{Bb}	0.54 ± 0.03^{Aa}	0.91 ± 0.27^{Aa}	0.91 ± 0.43^{Aa}	9.3 ± 0.1^{Ba}	9.5 ± 0.1^{Cb}	8.4 ± 0.1^{Aa}
	ScottA (+PL25)	$0.53 \pm 0.76^{Aa*}$	2.03 ± 0.84^{Ab}	0.69 ± 0.98^{Aa}	0.65 ± 0.16^{Aa}	0.81 ± 0.15^{Ab}	0.56 ± 0.03^{Aa}	$9.0 \pm 0.1^{Bb*}$	$8.6 \pm 0.1^{Aab*}$	$9.1 \pm 0.1^{Bc*}$

Values with different uppercase letters that correspond to the same singly or co-cultured strain, in the same row and within the same structured medium, are significantly different ($P<0.05$).

Values with different lower letters that correspond to the same singly or co-cultured strain, in the same column and within the same condition of oxygen availability, are significantly different ($P<0.05$).

Star indicates statistical difference between the growth kinetics of the co-cultured strains in comparison to the singlcultured under the same conditions of structure and oxygen availability (*: $P<0.05$).

Dashes indicate the treatment combinations that were non applicable.

Table 5. Estimated growth kinetics (lag time and growth rate) and observed final population of *L. monocytogenes* strain 6179 in single and co-culture with strains C5 and ScottA, in liquid, semi-solid and solid media under aerobic, hypoxic and anoxic conditions, at 7°C.

		LAG TIME (Days)			GROWTH RATE (Days^{-1})			FINAL POPULATION (log CFU/mL or cm^2 or g)		
		Aerobic (A)	**Hypoxic (H)**	**Anoxic (An)**	**Aerobic (A)**	**Hypoxic (H)**	**Anoxic (An)**	**Aerobic (A)**	**Hypoxic (H)**	**Anoxic (An)**
Liquid (L)	**Single 6179**	0.40 ± 0.46^{ABa}	0.33 ± 0.57^{Aa}	1.45 ± 0.26^{Ba}	0.56 ± 0.09^{Aa}	0.59 ± 0.06^{Aa}	0.47 ± 0.01^{Aa}	9.3 ± 0.4^{Ba}	8.9 ± 0.3^{Aa}	8.8 ± 0.1^{Aa}
	6179 (+C5)	0.22 ± 0.37^{Aa}	0.86 ± 0.60^{ABa}	1.48 ± 0.16^{Bab}	0.55 ± 0.13^{Aa}	0.58 ± 0.11^{Aa}	0.50 ± 0.08^{Aa}	$5.9 \pm 1.0^{ABa*}$	$4.9 \pm 1.5^{Aa*}$	$6.6 \pm 0.4^{Ba*}$
	6179 (+ScottA)	0.74 ± 0.71^{Aa}	0.49 ± 0.85^{Aa}	1.71 ± 0.27^{Aab}	0.65 ± 0.27^{Aa}	0.56 ± 0.08^{Aa}	0.51 ± 0.10^{Aa}	$7.9 \pm 0.4^{Ba*}$	$6.2 \pm 1.0^{Aa*}$	$6.9 \pm 0.3^{Aa*}$
Semi-solid (SS)	**Single 6179**	-	0.32 ± 0.55^{Aa}	0.61 ± 0.86^{Aa}	-	0.55 ± 0.11^{Aa}	0.52 ± 0.01^{Aa}	-	8.7 ± 0.4^{Aa}	9.3 ± 0.0^{Bb}
	6179 (+C5)	-	1.26 ± 1.10^{Aa}	0.00 ± 0.00^{Aa}	-	0.62 ± 0.17^{Aa}	$0.48 \pm 0.01^{Aa*}$	-	$6.7 \pm 0.5^{Ab*}$	$7.1 \pm 0.0^{Bb*}$
	6179 (+ScottA)	-	0.94 ± 1.23^{Aa}	0.00 ± 0.00^{Aa}	-	0.65 ± 0.16^{Aa}	$0.59 \pm 0.00^{Aab*}$	-	$7.7 \pm 0.4^{Ab*}$	$8.2 \pm 0.0^{Bb*}$
Solid (S)	**Single 6179**	0.00 ± 0.00^{Aa}	1.59 ± 0.51^{Ab}	2.54 ± 2.38^{Aa}	0.52 ± 0.14^{Aa}	0.62 ± 0.04^{Aa}	0.83 ± 0.30^{Ab}	9.2 ± 0.1^{Aa}	9.4 ± 0.1^{Bb}	9.2 ± 0.1^{Ab}
	6179 (+C5)	0.19 ± 0.27^{Aa}	2.33 ± 0.28^{Ba}	2.39 ± 2.17^{Bb}	0.54 ± 0.09^{Aa}	0.74 ± 0.20^{Aa}	0.83 ± 0.37^{Aa}	$6.9 \pm 0.1^{Ab*}$	$6.8 \pm 0.1^{Ab*}$	$7.4 \pm 0.1^{Bb*}$
	6179 (+ScottA)	0.28 ± 0.39^{Aa}	1.69 ± 0.76^{ABa}	2.59 ± 1.68^{Bb}	0.52 ± 0.17^{Aa}	0.70 ± 0.18^{Aa}	0.80 ± 0.32^{Ab}	$8.1 \pm 0.5^{Ab*}$	$8.2 \pm 0.2^{ABb*}$	$8.6 \pm 0.0^{Bc*}$

Values with different uppercase letters that correspond to the same singly or co-cultured strain, in the same row and within the same structured medium, are significantly different ($P<0.05$).

Values with different lower letters that correspond to the same singly or co-cultured strain, in the same column and within the same condition of oxygen availability, are significantly different ($P<0.05$).

Star indicates statistical difference between the growth kinetics of the co-cultured strains in comparison to the singly cultured under the same conditions of structure and oxygen availability (*: $P<0.05$).

Dashes indicate the treatment combinations that were non applicable.

CHAPTER 3

Studying the effect of oxygen availability and matrix structure on population density and inter-strain interactions of Listeria monocytogenes in different dairy model systems

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Studying the effect of oxygen availability and matrix structure on population density and inter-strain interactions of *Listeria monocytogenes* in different dairy model systems

Maria A. Gkerekou, Lamprini A. Adam, Georgios K. Papakostas and Panagiotis N. Skandamis*

Laboratory of Food Quality Control and Hygiene, Department of Food Science and Human Nutrition, Agricultural University of Athens, Greece.

Abstract

Due to the ubiquitous character of *Listeria monocytogenes*, multiple strains of the pathogen may end up co-existing in/on the same final products and could potentially cause infection during consumption. Such multiple strain contamination may occur in different stages of the food supply chain. The present study evaluated the effect of oxygen availability and matrix structure on inter-strain interactions of *L. monocytogenes* that may occur at high population levels in/on different dairy model systems. *L. monocytogenes* strains C5 and ScottA (4b), 6179 (1/2a) and PL25 (1/2b) selected as resistant to different antibiotics (enabling selective enumeration of each strain in co-culture) and were inoculated (2.0 - 3.0 log CFU/mL, g or cm^2) in Ricotta and Camembert broth (1 dairy product : 2 ¼ Ringer solution) and in/on dairy-based structured media (dairy broth supplemented with 0.6 and 1.4% agar), in single and two-strain cultures (1:1 strain ratio). Bacterial growth was assessed during storage at 7ºC, under aerobic, hypoxic and anoxic conditions. Every experimental treatment was tested with three biological replicates and two technical repeats (n=3x2). The simultaneously presence of different strains of the pathogen in/on the same substrate did not affect neither the duration of the lag phase nor the growth rate of the co-cultured strains. The observed inter-strain interactions were related with the maximum population reached or decreased during storage and occurred after the "critical" population density of *c.a.* 6.0 log CFU/mL, g or cm^2. The phenomenon was more pronounced in/on Ricotta than in Camembert-based substrates, indicating that the composition and the available nutrients of the substrate may affect the interactions that expressed as difference in the final population level between singly and co-cultured strains. Under aerobic and hypoxic conditions, most of the observed interactions were more pronounced in dairy-based broths and were mitigated with the addition of agar. The elimination of oxygen resulted in a prolonged lag time, which lasted at least 5 days and no observed interactions by the end of storage, due to low microbial counts. Investigating inter-strain interactions during growth in/on different substrates, which may have undergone temperature abuse during their transport along the supply chain or during storage in household refrigerators, could assist in explaining the mismatch between clinical and food samples, during outbreak investigations.

Keywords: pathogenic microorganism; inter-strain interactions; Ricotta, Camembert; structure; oxygen availability

Introduction

Post-processing contamination of dairy products with *Listeria monocytogenes*, is ascribed to flawed hygiene practices, the high occurrence of the pathogen in the processing environment and its ability to adhere onto food processing surfaces and form biofilms (Alvarez-Ordóñez, Coughlan, Briandet, & Cotter, 2019; Melero et al., 2019; Poimenidou et al., 2009; Rückerl et al., 2014; Tirloni et al., 2020). Given that many dairy products are of high risk, due to their physicochemical characteristics (moisture, pH and a_w) and their categorization as Ready-To-Eat products, since 1979's outbreak in Maryland (USA) until 2017, they have repeatedly identified as the source of several human listeriosis outbreaks (www.cdc.gov/listeria/outbreaks/index.html) (Martinez-Rios and Dalgaard, 2018; Shamloo et al., 2019). Interestingly, during the reported listeriosis outbreak occurred in Austria, Germany and the Czech Republic in 2009 and 2010, the investigations were traced back to a traditional Austrian curd cheese called ''Quargel'', which was contaminated with two distinct *L. monocytogenes* strains of serotype 1/2a (Rychli et al., 2014). Moreover, previous studies have shown that different strains of *L. monocytogenes* may be introduced at various time-points in the processing environment (Chambel et al., 2007; Martín et a;., 2014; Ortiz et al., 2010), ending to multiple strains co-existing in the same food, as reported in Latin-style fresh cheese, traditional Portuguese smoked meat sausage, called Alheiras or smoked salmon (Felício, Hogg, Gibbs, Teixeira, & Wiedmann, 2007; Gendel & Ulaszek, 2000; Kabuki, Kuaye, Wiedmann, & Boor, 2004). More importantly, Tham et al. (2002) and Tham, Lopez, Valladares, Helmersson, Österlund and Danielsson-Tham (2007) showed that different strains of *L. monocytogenes* may co-exist during infection, as they have been isolated from different sites (blood and meninges) of the infected patient or from a single blood sample, during investigation of listeriosis cases.

Cheeses and other dairy products constitute multiphase systems characterized by great complexity and their chemical composition and structural properties may affect the growth characteristics of the microbial communities (Mertens et al., 2011; Noriega, Laca, & Díaz, 2008; Smet, Noriega, Van Mierlo, Valdramidis, & Van Impe, 2015b; Theys et al., 2008; Velliou et al., 2013; Wilson et al., 2002). Different compositions of protein (casein), water, fat and salt result in different semi-solid or solid structures, representing different cheeses or other dairy products, where bacterial cells are constrained to grow as colonies due to limited space by the physical structure (Hills et al., 2001; Møller et al., 2012; Pappa et al., 2007; Wilson et al., 2002). At colony level, microorganisms experience a significantly different biochemical and structural environment in comparison to liquid systems (planktonic growth). Cells experience diffusional limitations of oxygen and nutrients, while metabolic products accumulated around the colony, affecting microbial kinetics and microbial environmental response (Skandamis & Jeanson, 2015; Wilson et al., 2002). Additionally, spatial organization, colony size and species location may affect the inter- and intra-species interaction and communication and subsequently the environmental stress within the colony, reflecting to

growth kinetics (Aspridou, Moschakis, Biliaderis, & Koutsoumanis, 2014; Costello et al., 2020; Jeanson, Floury, Gagnaire, Lortal, & Thierry, 2015; Zilelidou, Manthou, & Skandamis, 2016a).

The existing studies report that interactions during co-culture of different species may occur at high population density of a dominant strain which forces a second "weaker" strain to cease growth, possibly (among other) due to competition for resources or the accumulation of toxic metabolites by the dominant species, a phenomenon known as "Jameson effect" and appear to be influenced by the substrate characteristics (Gkerekou, Athanaseli, Kapetanakou, Drosinos, & Skandamis, 2021; Zilelidou et al., 2016a). Considering all the above, it is important to identify the factors (intrinsic, extrinsic and structure-related/implicit) and the extent to which they can influence inter-strain interactions, after a critical population reached due to temperature abuse during their transport along the supply chain or during storage in household refrigerators. Moreover, from food safety aspect, the relative levels of each strain during storage may reflect the population of each strain at the end of enrichment and subsequently their probability of isolation on ALOA plates (i.e., the critical detection step), according to ISO 11280 (Zilelidou et al., 2016b). Thus, potential masking of certain strains throughout the detection process may hinder the tracing of the actual causative agent (strain) of an outbreak, during epidemiological investigations, which may have slower growth during storage or enrichment procedure, but may be more virulent. Given the above, the present study aimed to evaluate the effect of oxygen availability and the matrix structure on growth and the subsequently occurred inter-strain interactions of the pathogen *L. monocytogenes* in/on different dairy-based model systems produced by Ricotta and Camembert.

Materials and methods

L. monocytogenes strains

Four *L. monocytogenes* strains were selected, based on their antibiotic resistance to streptomycin (Streptomycin Sulfate Biochemica, AppliChem) or rifampicin (Rifampicin, AppliChem) (Table 1), for the attainment of selective enumeration of each strain during co-culture. The strains were obtained from the microorganism collection of the Laboratory of Food Quality Control and Hygiene of Agricultural University of Athens and their selection to the antibiotics was made according to the method described by de W. Blackburn and Davies (1994) (Table 1). The wild strains incubated twice in Tryptone Soy Broth (LAB004, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract (MC001, Lab M Limited, United Kingdom) (TSB-YE, pH: 7.3 ± 0.2) for 24h at 37°C, however during the second incubation were added different concentrations of the corresponding antibiotics. For the selection of the antibiotic resistant strains, the studied concentrations for streptomycin were 0, 125, 250, 500, 1000, 2000 and 4000 μg/mL, while rifampicin was evaluated at 0, 200, 400 and 800 μg/mL (de W. Blackburn & Davies, 1994). The strains were maintained at -20°C in TSB-YE with 20% glycerol and the appropriate concentration of rifampicin or

streptomycin depending on the strain. Streptomycin was used at 1000 μg/mL and rifampicin at 50 μg/mL. The concentration of antibiotics used, during their maintenance and for the preparation of the (selective) enumeration media, was the lowest in which the second strain (the one that was not resistant to the particular antibiotic) was unable to grow. Both ScottA (streptomycin resistant strain and rifampicin resistant strain) and C5 were selected as strains belong to serotype 4b, while 6179 and PL25 belong to serotypes 1/2a and 1/2b, respectively (Table 1). The selection of strains aimed to include strains of different serotype and origin (outbreak and animals) and strains characterized as persistent in dairy processing environment (Fox, Leonard, & Jordan, 2011) (Table 1).

Inoculum preparation

During the experiments, all strains were maintained on Tryptone Soy Agar (LAB011, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract, containing rifampicin (50 μg/mL; TSA-YE/R) or streptomycin (1000 μg/mL; TSA-YE/S) at 4°C and sub-cultured once a month. A single colony from a TSA-YE/S or TSA-YE/R stock culture of the target strain was transferred to 10 mL TSB-YE/S or TSB-YE/R and incubated for 24 h at 30°C and subsequently, 100 μL of each culture was transferred to fresh TSB-YE/S or TSB-YE/R for 18 h incubation at 30°C to obtain stationary-phase cells with a density of *ca.* 10^9 CFU/mL. Following activation stage, strains were harvested by centrifugation (3600 rpm or 2246 g for 10 min at 4°C) (Megafuge 1.0R, Heraeus, Buckinghamshire, England), washed twice and finally re-suspended in 10 mL of ¼ strength Ringer's solution (LAB M, Lancashire, UK). The level of the inoculum was determined by plating 0.1 mL from the appropriate decimal dilution of each strain on TSA-YE/S or TSA-YE/R and incubation at 37°C for 48 h.

Dairy-model systems preparation

Commercial packages of Ricotta (RBr; Granarolo, Bologna, Italy) and Camembert (CBr; Alpenhain, Munich, Pfaffing, Germany) were purchased from a local supermarket (Athens, Greece) close to their production date to ensure the lowest initial population of indigenous microbiota depending on their production process.

The dairy-based broths were prepared by homogenizing, with hand blender (at maximum speed; Multi Mix 700W, Izzy), 1 part of the selected dairy products (Ricotta and Camembert) with 2 parts of sterile ¼ strength Ringer's solution. Subsequently, the dairy-based broths were heated up to 70°C and remained at this temperature for 15 min (in water-bath), aiming to eliminate the possible endogenous microflora (*ca.* 2.0 – 3.0. log CFU/g), which may affect the growth of the pathogen and subsequently the evaluation of inter-strain interactions. As described by Noriega et al., (2008), the behavior of the pathogen in the dairy-based broths assayed under aerobic, hypoxic and anoxic conditions. Specifically: (i) aerobic conditions (A) were tested in 50 mL falcon tubes containing 30 mL of sterile dairy-based broth which inoculated and stored on an orbital shaker at 240 rpm (Shaker KS 130 basic, IKA-Werke GmbH & Co. KG, Germany), (ii) hypoxic conditions

(H) were tested in sterile glass bottles containing 120 mL of dairy-based broths (full bottle), which inoculated, closed with N20 Butyl rubber stoppers and crimp caps (Macherey-Nagel GmbH & Co. KG, Germany) and stored without shaking, and (iii) anoxic conditions (An) were generated by depleting initial dissolved oxygen from glass bottles containing 120 mL of dairy-based broths (full bottle) by the addition of 0.1% w/v sodium thioglycollate, while the bottles were stored without shaking. Usually, in liquid substrates the removal of oxygen is achieved by flushing sterile nitrogen (Noriega et al., 2008), however, the use of gas nitrogen could not be applied to solid substrates and alternatively sodium thioglycollate was used for the attainment of anoxic conditions. For each assay (aerobic, hypoxic and anoxic conditions) were prepared 20 containers (5 single-cultures and 5 co-cultures x 2 technical replicates).

The structured dairy-based media were prepared by adding certain agar concentration (MC002, Lab M Limited, United Kingdom) to dairy-based broths, aiming to simulate the level of hardness of the chosen dairy products. Texture profile analysis (TPA) was performed in order to select the appropriate agar concentrations. TPA was performed initially to Ricotta and Camembert, while in second stage, to structured media which were produced by adding different concentrations of agar (0.4 - 1.8%) (Fig. 1). A Shimadzu testing instrument (AGS-500 NG, Shimadzu Corporation, Japan) was used, equipped with a 5 kg load cell and a plunger with a diameter of 6 mm attached to the moving crosshead. The speed of the crosshead was set at 2.5 cm min^{-1} in both upward and downward directions. The samples (cylinder of 10 cm diameter and 6 cm high; n=9) were placed on a flat holding plate at 20^{o}C and the plunger inserted 20 mm below the sample surface and two consecutive bites were taken (Kaminarides and Stachtiaris, 2000). Hardness (maximum load; F_{max}; N) was chosen among the textual characteristic that has been used by other studies to evaluate and categorize different structures (Baka, Noriega, Van Langendonck, & Van Impe, 2016; Floury et al., 2009; Kaya, 2002; Noriega et al., 2008). Among the tested agar concentrations 0.6 and 1.4% showed the closest F_{max} values to the respective ones of Ricotta and Camembert (Fig. 1). Thus, the structured dairy-based media were prepared by: (i) Ricotta and Camembert-based broths, namely Ricotta or Camembert: sterile ¼ strength Ringer's solution in a ratio 1:1 after heating in water-bath at 70^{o}C for 15 min and (ii) a second, equal to above, part of ¼ strength Ringer's solution (proportions used for preparation of dairy-based broths) was used to dissolve the appropriate percentage of agar (0.6% or 1.4%; Figs. 1 and 2) for resembling the different dairy products and mixture was autoclaved (121^{o}C for 15 min). The two mixtures were mixed, rendering to similar ingredients concentration as the dairy-based broths. By choosing agar as the hardening agent was possible to achieve homogeneous inoculation, because the temperature between gelling and melting point enable the inoculation without the pathogen lose its viability (38 - 39^{o}C), using a solidifying agent which is well-known that it cannot be utilized by bacteria (Jeanson, Floury, Gagnaire, Lortal, & Thierry, 2015; Noriega et al., 2008). Similarly as in dairy-based broths different levels of oxygen conditions were tested: (i) aerobic conditions: Petri dishes filled with 20 mL of Ricotta or Camembert-based medium with 1.4% agar

concentration (coded as 1.4Ric and 1.4Cam, respectively), while inoculation was performed on the surface of the medium and the Petri dishes were sealed with parafilm before storage (calculation of plate's area and surface inoculation with 2.0 - 3.0 log CFU/cm^2), (ii) hypoxic conditions: Duran bottles with 250 mL of Ricotta and Camembert-based media (after the mixing of the two mixtures, as described above) allowed to cool at a temperature of 38 - 40°C, were inoculated with single or dual-cultures of the selected strains and 20 mL of each inoculated medium quickly distributed into Petri dishes (sealed with parafilm) and (iii) anoxic conditions: The preparation was similar to that of samples stored under hypoxic conditions, having as only difference the addition of 0.1% w/v of sodium thioglycollate before the inoculation of the media, while after inoculation, 12 g of each inoculated medium, quickly distributed into falcon tubes and overlaid with 2 mL paraffin. Sodium thioglycollate has been widely used for the culture of anaerobic bacteria, because of its ability to remove molecular oxygen from the medium, thus, creating an oxygen limiting environment (Makariti, Grivokostopoulos, & Skandamis, 2021). Both under hypoxic and anoxic conditions the preparation of the two different dairy-based substrates aiming to simulated the structure of Ricotta, by adding 0.6 % agar (0.6Ric and 0.6Cam) and the structure of Camembert, by adding 1.4% agar (Figs. 1 and 2). The experimental design aimed to evaluate whether the growth behavior and more importantly the inter-strain interactions were affected by the nutrients of the different dairy-based products under of the influence of the same structure and oxygen availability or the other way around, i.e. by the difference in the structure or oxygen availability under the influence of the same nutrients.

Inoculation and storage conditions

All samples were inoculated, with single or dual cultures (strain ratio 1:1) of different *L. monocytogenes* strains listed in Table 1, at approximately 2.0 - 3.0 log CFU/mL or cm^2 or g. The dual-strain combinations were: C5+6179, C5+ScottA, C5+PL25, 6179+ScottA and ScottA+PL25. Each strain of the studied combinations was resistant to a different antibiotic for the attainment of selective enumeration of each strain in the co-culture, as mentioned in §2.1 (Fig. 2). The inoculated samples were stored at 7°C, in high precision (± 0.5°C) incubation chambers for 25 days (MIR 153, Sanyo Electric Co., Osaka, Japan). The duration of storage was similar to the shelf-life of the commercial products, so that the results illustrate how inter-strain interactions may occur to a realistic scenario during which the products may suffer temperature abuse due to failures along the supply chain. Three independent storage experiments were performed and duplicate samples were used for each trial (n=6).

Microbiological analysis

Microbiological analysis was performed at specific time intervals throughout storage at 7^{o}C. Specifically, in order to determine the growth curves of the different strains and the same strains in the co-cultures, 3 - 5 mL of each dairy-based broth was removed under aseptic conditions. Regarding the dairy-based structured

samples, 12 - 15 g of each dairy-based structured medium were removed from their containers (the Petri dishes and the falcon tubes) and placed in plastic bags, in which 3-fold sterile ¼ strength Ringer's solution was added under aseptic conditions. The samples were homogenized in a stomacher at 240 rpm (Stomacher® 400 Circulator, Seward, UK) for 60 s. Following decimal dilutions in ¼ strength Ringer's solution, aliquots of 0.1 and/or 1 mL of diluted sample were spread on selective and non-selective culture media. Population of *L. monocytogenes* strains was enumerated, after 48 h at 37°C, on TSA-YE and TSA-YE/S or/and TSA-YE/R. Average numbers of colonies *per* plate were used to calculate the viable-cell concentrations, expressed as log CFU/mL for the dairy-based broths, while the viable-cell concentrations, expressed as log CFU/g in case of inoculation inside the solidified substrate or log CFU/cm^2, in the case of the surface inoculation, with enumeration limit of 100 CFU/mL, g or cm^2.

pH and a_w measurements

The pH values of samples were recorded at every sampling point by using a digital pH meter (pH 526, Metrohm Ltd, Switzerland) *via* immersion of pH electrode in the homogenate. Water activity (a_w) was monitored by a digital aw meter (Hydrolab rotronic, Switzerland) at the beginning, the middle, and the end of storage. Each of the different dairy-based samples (liquids or structured) were placed to cover the bottom of a plastic container similar to a small petri dish and on the top of this container was placed the probe of the device for the measurement of the a_w.

Statistical analysis

Statistical analysis was performed with *STATGRAPHICS® Centurion XVII* computer package (Statpoint Technologies Inc., USA). During analysis of variance (ANOVA), Tukeys' HSD multiple range tests was used to evaluate the differences in the growth kinetics between the single and co-cultures among the different combinations of structure and oxygen availability, while for all pairwise comparisons the Student's t-test was used. Differences were considered to be significant for p-values < 0.05. The obtained bacterial growth data, per single or co-cultured strains, were fitted to the Baranyi-Roberts model with DMFit Excel Add-In software. Maximum specific growth rate (μ_{max}; $days^{-1}$) and lag time (λ; days) were determined. In the experimental cases that pathogens' population showed a decrease after stationary phase, the experimental data in the decay phase were identified by visual inspection, removed from the data which used for the model fitting and the primary model was run without taking into account these certain measurements.

Results and Discussion

Effect of matrix nutritional composition on the growth and inter-strain interactions of L. monocytogenes strains in/on Ricotta and Camembert-based substrates

Considering the risk of temperature abuse due to failures along the supply chain and the fact that transportation, retail display or household refrigeration are outside the manufacturer's direct control and often deviates from specifications, the results of the present study indicate that the compositional characteristics of the different dairy-based substrates (see §Supplementary material) may influence the growth of single strains. Subsequently, if the pathogen manages to reach high enough population density during storage, strain-to-strain interactions may also occur in the case of simultaneously presence of more than one strains. In all the studied structures, under aerobic and hypoxic conditions, every singly-cultured strain of *L. monocytogenes* reached lower level of final population in/on Ricotta-based (Figs. 3A – 6A; Table 2), than in/on Camembert-based media (Figs. 3B – 6B; Table 3). Under anoxic conditions, all the singly-cultured strains reached higher final population, in Ricotta-based substrates (Figs. 3A - 6A). Identifying the differences of the two dairy products, which may explain the differences in the level of final population of the pathogen, salt, protein and carbohydrate content, are likely the key determinants affecting the growth of *L. monocytogenes*. Both Ricotta and Camembert consist of caseins and whey proteins which may serve as nitrogen sources (7.5 and 18 g, respectively). In Ricotta, lactose may serve as carbon source (3 g), while in Camembert, the lactose content is negligible (<0.5 g), due to its consumption by the starter culture at the initial stages of Camembert production process and ripening (see §Supplementary material) (Fox, Guinee, Cogan, & McSweeney, 2016; Perko, 2002). Galactose and glucose concentrations in Camembert are very low too (Fox et al., 2016). Margolles, Mayo and de los Reyes-Gavilán (2000) and Pine, Malcom, Brooks and Daneshvar (1989) have shown that there is variability in the lactose utilization between different strains of *L. monocytogenes*. However, they have also shown that lactose-positive and lactose-negative strains of the pathogen, during culture in milk, managed to grow, reaching similar final population levels, suggesting the possible use of an alternative carbon source. In milk or dairy products, the pathogen may use either the available glucose present in milk (50 mg/L) (Fox, Uniake-Lowe, McSweeney, & O'Mahony, 2009; Pine et al., 1989) or the glucose moiety of the lactose molecule (Crespo Tapia et al., 2020; Dalet, Arous, Cenatiempo, & Héchard, 2003; Pine et al., 1989). The presence of amino acids is required for the growth of the pathogen (Premaratne, Lin, & Johnson, 1991; Verheul et al., 1995; Verheul, Rombouts, & Abee, 1998) and may originate either from hydrolysis of caseins by proteases produced by the pathogen, during colonization of a substrate (Shumi, Hossain, & Anwar, 2014), or by the additional proteolytic action of the coagulant (addition of calf (traditionally) rennet), the plasmin (an important indigenous proteinases present in milk), the starter culture and the mold *Penicillium candidum*, during cheese production and ripening. The difference of salt content between the two dairy products, according to their nutritional declaration, due to the preparation process of the substrates, is almost eliminated (see §Supplementary material) and reduced to levels that do not affect the behavior of the pathogen. Additionally, neither pH nor a_w changed such significantly during storage as to affect the behavior of the pathogen or explain the observed differences in its behavior in/on

the substrates produced from the different dairy products. At the begging of storage the pH of Ricotta and Camembert was 6.8 ± 0.2 and 6.2 ± 0.2 and by the end of storage was decreased to 6.3 ± 0.2 and 6.1 ± 0.2, respectively, while a_w was constant at 0.99 throughout storage.

After the middle of storage, when both strains of the dual culture had exceeded 6.0 log CFU/mL or g or cm^2, the impact of the co-culture on the population density of some strains was increasingly pronounced, judging from the difference in the final population of a singly-cultured strain and the final population of the same strain during its co-culture with another strain. Similarly to the observations made by Zilelidou et al. (2015, 2016a) and Gkerekou et al. (2021), who studied the inter-strain interactions of the same strains during storage on gam slices and in/on TSB-YE and TSA-YE, strains PL25 (1/2b) and C5 (4b) were identified as 'strong' competitors (Figs. 5A - 6A and 5B - 6B), suggesting that irrespectively of structure, especially under aerobic and hypoxic conditions, certain strains, may manage to grow unaffected by the presence of a second strain. In contrast, growth of strains 6179 (1/2a) and ScottA (4b), is consistently suppressed by the presence of high cell number of a 'stronger' strain (Figs 3A - 4A and 3B - 4B). A major result of the study is that in mixed cultures, most of the observed inter-strain interactions were more pronounced in/on Ricotta (Figs. 3A - 4A) than in Camembert-based substrates (Figs. 3B - 4B), indicating that the available nutrients of each matrix may affect the level of the final population reached and subsequently, the difference between the final cell density of singly and co-cultured strains. Specifically, in Ricotta-based broth growth of strain 6179 was significantly suppressed by C5 and ScottA (Fig. 3A), while, in Camembert-based broth strain 6179 reached 2.1 and 1.7 log units lower population density during co-culture with C5, under aerobic and hypoxic conditions, respectively (Fig. 3B). Moreover, in Ricotta-based broth, during co-culture the presence of C5, 6179 and PL25 significantly decreased the maximum population density of ScottA by 3.3 (RicBr/A and H), 2.9 (RicBr/A) and 2.3 log units (RicBr/A), respectively (Fig. 4A), while in Camembert-based broth the difference between singly and co-cultured reached population of ScottA was up to 1.2 (CamBr/H), 0.8 (CamBr/H) and 1.9 (CamBr/A), respectively (Fig. 4B). Considering the physicochemical and nutritional characteristics of the two dairy products, described above, along with their manufacturing technology, Camembert-based media are substrates which, due to the considerable proteolytic activity, becomes rich in soluble nitrogen content (Maćej, Jovanović, & Denin, 2001). This, likely explains the enhanced growth capacity of singly-cultured *L. monocytogenes* strains and the diminished interactions during co-culture compared to Ricotta-based media, under aerobic and hypoxic conditions (Figs. 3A - 6A and 3B - 6B). Substrates that have undergone proteolysis are reported to stimulate growth of *L. monocytogenes,* as it happens when the pathogen coexists with the highly proteolytic *Pseudomonas* (Marshall & Schmidt, 1991). Additionally, due to the lower moisture content of Camembert, the resulting broth was thicker, compared to that produced by Ricotta after mixing with same volume of ¼ Ringer's solution (one part cheese and two parts ¼ Ringer's solution; see §2.3). Finally, the homogenization that took place during the preparation of the substrates, perhaps, reduced the diameter of

the fat globules resulting in their greater and more uniform dispersion throughout the mass of the substrate, presumably creating higher number of smaller spaces within the aqueous phase of the substrate (Pricope-Ciolacu et al., 2013). According to Zilelidou et al. (2015), contact of cells may be the key factor for the manifestation of the inter-strain interactions. During co-culture in TSB-YE of strains PL25 and ScottA, the inhibition of the latter strain was increased due to contact of the two strains, while the prevention of contact of the strains by membrane resulted in limited inhibition. So, the intensity of the observed inter-strain interactions, during growth in/on the different dairy-based substrates, depend on the combination of high population density and the conditions affecting the motility of the pathogen, which are in turn, associated with the characteristics of the growth substrate. In the likelihood of temperature abuse of products contaminated with more than one strains, the time of storage will determine the relative population levels of each strain to be ingested. Furthermore, factors that alter the strain-specific growth attributes, such as the substrate characteristics or the presence of a second strain, may also compromise the tracking of the total strains initially present in the food, due to the relative behavior of co-existing strains either at the enrichment stage (Bruhn et al. 2005, Gorski et al, 2006, Zilelidou et al. 2016a) or within the host, especially concerning serotypes 1/2a (prevalent in foods) and 4b (prevalent in food-borne outbreaks). There are hyper virulent strains, which may be outcompeted by faster, albeit less virulent strains (Maury et al. 2016). The latter strains lack the growth fitness advantage and thus, it is less likely to be isolated by enrichment, a fact that could mask the outcome of outbreak investigations, leading to mismatches between clinical and food isolates.

Effect of matrix structure on growth and inter-strain interactions of L. monocytogenes strains in/on Ricotta- and Camembert-based substrates

In addition to the evaluation of inter-strain interactions in/on substrates based on different dairy products, we were also able to evaluate the effect of the structure by keeping the same substrate's components (same dairy product) and level of oxygen availability (comparison between structures in the same column; Figs. 3 - 6). In both Ricotta and Camembert-based media, under anoxic conditions, all the singly-cultured strains reached significantly lower final population in the substrate with the maximum agar concentration (1.4%) (Figs. 3A - 6A and 3B - 6B; Tables 3 and 4). The presence of even a low concentration of oxygen, seems to suffice for the pathogen to counteract the impact of the different studied structures, resulting in similar growth rate between the different modes of growth, i.e., planktonic, immobilized or submerged in the matrix (partially or fully constrained). Contrarily to the well-established ranking of growth rate between the three modes of growth, which follow the order: broth > immerged colonies > surface colonies (Aspridou et al., 2014; Noriega, Laca, & Díaz, 2010; Theys et al., 2008; Wilson et al., 2002), the similar growth rate of strains grown in/on different dairy-based substrates pointed out that substrate

characteristics, like structure density, the particular thickening agent etc., impact growth capacity regardless the mode of growth. At agar concentrations up to the critical value of 0.65% (w/v), the bacterial colonies rather grow spherically due to the unconstrained motility of bacteria through the lower agar strength of the matrix, while at higher agar concentrations, their shape becomes lenticular due to the more pronounced restriction of motility (Mitchell and Wimpenny, 1997). However, according to Kabanova, Stulova and Vilu (2012), even in/on substrates with a concentration of agar close to 1% (w/v), the pores that are formed are quite large, resulting in adequate nutrient diffusion to the colonies. As such, the limited cellular mobility in the substrates with the studied agar concentrations (0.6 and 1.4% w/v), may leave the growth rate unaffected by the agar level and the dairy product from which they were prepared (Mitchell and Wimpenny, 1997). Similarly to our results, no major differences have been reported between the growth kinetics of different modes of growth of *Salmonella* Typhimurium and *Escherichia coli* (planktonic, immerged and surface colonies), under static conditions, while both *S.* Typhimurium and *L. monocytogenes* managed to grow similarly in liquid and solidified media (with the addition of 5% gelatin) at 20°C under static conditions, as well (Smet et al., 2015; Smet, Van Derlinden, Mertens, Noriega, & Van Impe, 2015). However, it is worth noting that Baka, Vercruyssen, Cornette and Van Impe (2017) observed higher growth rate of *L. monocytogenes* cultured in aqueous gel than in fish-based juice.

Under aerobic and hypoxic conditions the observed interactions, were more pronounced in dairy-based broths and were mitigated with the addition of agar and the solidification of the dairy-based substrates (Figs 3A - 6A and 3B - 6B). In the absence of oxygen, no interactions were observed until the end of storage, except for the dual cultures of strains ScottA and C5 with strain PL25, where the presence of the latter resulted in restriction of strains ScottA and C5 growth, thus remaining at the level of initial population. However, the addition of agar, where the structure from broth become semi-solid or solid, resulted in the elimination of the observed interactions (Figs. 3A - 6A). Interestingly, it appears that a denser substrate structure, such as that of Camembert, where the strains most likely tend to grow as colonies, influences the behavior of some strains in the co-culture Cell motility, which is more pronounced in liquid substrates, is also a key parameter that may affect the interactions between communities at high cell density, along with the metabolic products of the one population that may also affect the behavior of the other(s), the depletion of nutrients and the presence of competing bacterial cells (Malakar et al., 2003). According to Cornforth and Foster (2013), the two major bacterial responses to ecological competition are sensing nutrient limitation, and direct cell damage. Studies have shown that contact-dependent inhibition (CDI) may occur mainly in Gram-negative bacteria, such as *E. coli*, and this has been demonstrated in shaking liquid culture (Aoki et al., 2005). The same study suggested that growth inhibition among a 'weak' and a 'strong' strain requires that cell come in direct contact, and not only *via* their metabolome, e.g., when inoculating the weak strain in the spent medium of the strong one, or separating competing strains by an impermeable membrane. Aoki et al.

(2005) support the possibility that the secreted molecule, responsible for the inhibition phenotype, is unstable and is only effective when delivered to target cells in close proximity. However, recent studies have found that CDI is not restricted to Gram-negative bacterial but may also occur in Gram-positive bacteria, including *Listeria* (Hayes, Aoki, & Low, 2010). Increasing the complexity of the structure developing semi-solid and solid substrates, increases the complexity of the interactions which are highly influenced by the propinquity (spatial distribution) (Jeanson et al., 2015). Apart from the density of the substrate, heterogeneity of matrix components is also important because food aggregates and fat particles increase the length of the diffusion path for most solutes rendering colony-to-colony interactions less evident (Jeanson et al., 2015). The fact that in liquid foods bacterial cells have the ability to move more than in highly structured foods results in more evident interactions between two strains and thus, less chances to isolate the 'weaker' strain (the one suppressed by the other). Conversely, in a solid food, where the weak strain appears to reach higher final populations, both strains may have comparable chances of detection.

Growth and inter-strain interactions of L. monocytogenes strains in/on Ricotta- and Camembert-based substrates under different availability of oxygen

The presence of even a limited oxygen creates a more favorable environment for the growth of pathogen and this may lead to expression of interactions between the different populations at the latest stages of growth. Contrarily, under anoxic conditions, singly and co-cultured *L. monocytogenes* strains presented a prolonged lag phase compared with the other studied levels of oxygen availability. The non-occurrence of interactions during co-culture of strain 6179 with strains C5 and ScottA (Figs. 3A and 3B), ScottA with C5, 6179 (Figs. 4A and 4B) and PL25 (Fig. 4B), under anoxic conditions, did not necessarily prove, that the interactions were not expressed at all, under the present conditions, but may occur later during storage when the strains reach high enough population. Gkerekou et al. (2021), observed that singly-cultured *L. monocytogenes* counteracted the elimination of oxygen and reached similar final populations as grown under presence of oxygen and inter-strain interactions occurred both in TSB-YE and TSB-YE supplemented with agar, due to the presence of glucose in the substrate, which can be consumed, by the pathogen, regardless of oxygen availability. Zilelidou et al. (2016a) attributed the observed differences in inter-strain interactions to difference in nutritional composition of TSB-YE, TSA-YE and vacuum-packed ham slices, combined with the differences of oxygen availabilities coming from the different way of storage. In support of our results, in studies by Noriega et al. (2008) and Noriega, Laca, & Díaz (2009) where they evaluated the growth of *Listeria innocua,* they observed that under anoxic conditions both in model cheese and synthetic meat (both broth and structured, as well) the cell density was lower that the cell density under aerobic conditions. The behavior of *L. monocytogenes* under anoxic conditions it is important to be studied because the restriction of oxygen is one of the stimuli of virulence during the entrance of the pathogen in

the intestine, in order to colonize it (Müller-Herbst et al., 2014). Within natural, mixed-strain *L. monocytogenes* populations, different strains may show different behavior with respect to extreme environmental conditions and different physiological responses of the pathogen in low oxygen environments may be observed, even in the same serotype (Buncic et al., 2001), because there are isolates that may do not fit in the general patterns.

Conclusions

During listeriosis outbreaks by the consumption of contaminated food products, with two or more strains of the pathogen, it is important the products that may favor the manifestation of interactions to be described. The results of the present study revealed that after the pathogen reaches a critical population density, the nutritional characteristics of the matrix could affect the occurred inter-strain interactions and in combination with the structure of the substrate and the presence or not of oxygen, may be reduced and/or even eliminated. In case of temperature abuse takes place along the supply chain or during storage in households' refrigerators, the importance lies in the fact that in substrates (in this case dairy), with more moisture and looser structure, in the presence of oxygen, the interactions appear to be more pronounced. As a result, during co-culture, the strain affected by the presence of the second one to reach lower final population, a fact that may lead to its non-isolation during listeriosis outbreak investigations. The latter is less likely to occur, according to our study, on substrates with a denser structure, lower humidity that the pathogen can found at a spot inside the food product or in a package where oxygen is limited. The evaluated, by the present study, parameters are not the only ones that differ among the food products and may affect the behavior of the pathogen. Thus, it is necessary the growth and inter-strain interactions to be studied under more food-related parameters (i.e. pH, a_w, fat content etc.) as an important aspect, contributing to mismatches between clinical isolates and infection sources and aiming listeriosis outbreaks investigations to be even more sufficient.

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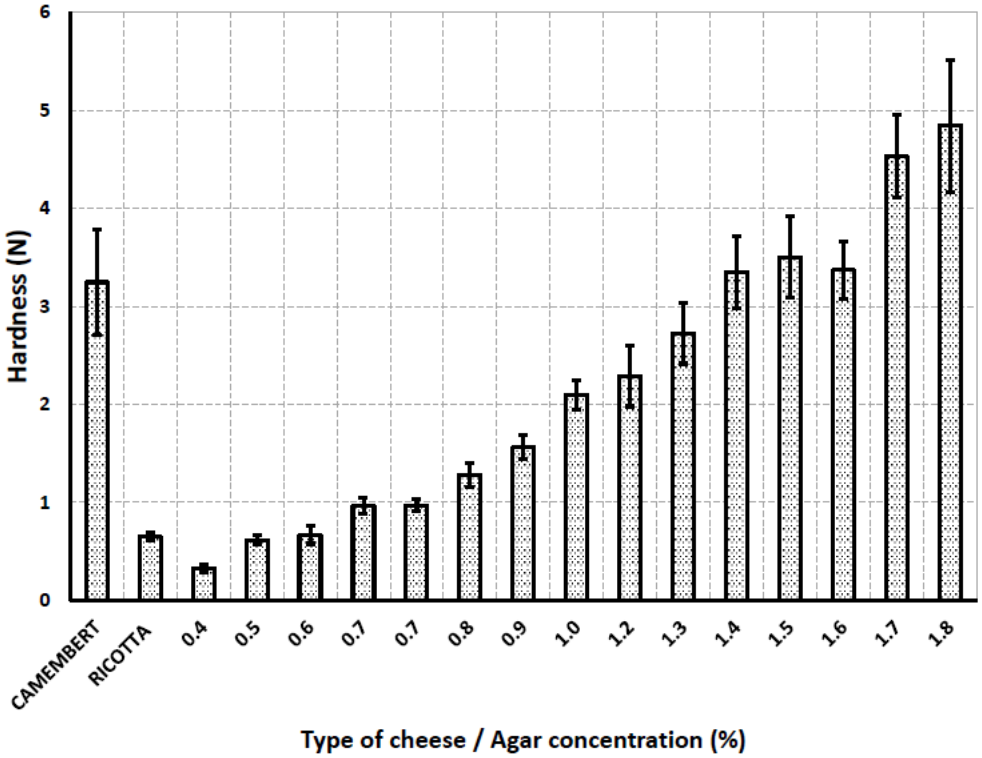

Figure 1. Values of hardness obtained by the Texture Profile Analysis (TPA) on the surfaces of Ricotta, Camembert and the structured media produced by different concentrations of agar.

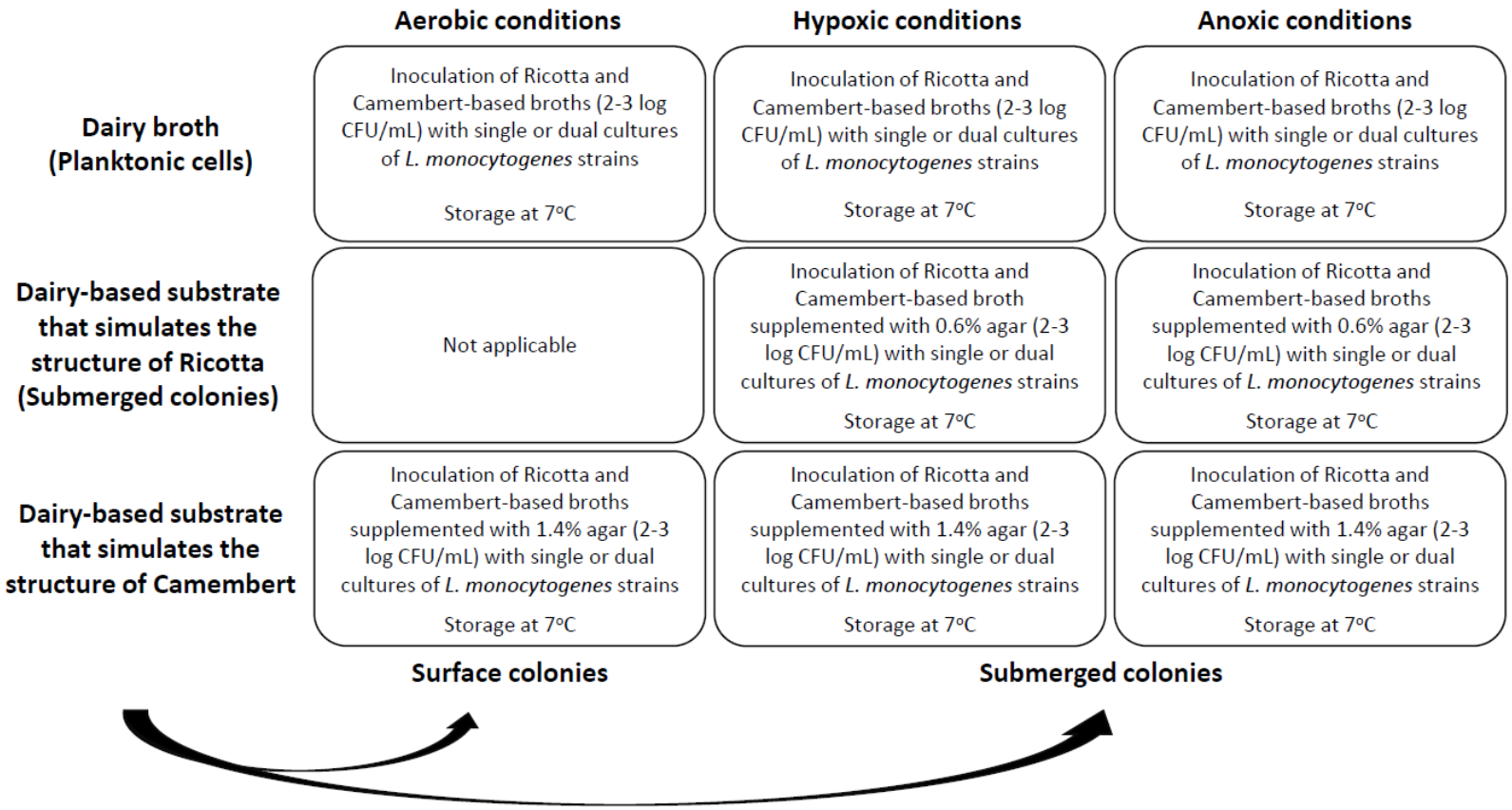

Figure 2. Schematic representation of the experimental design.

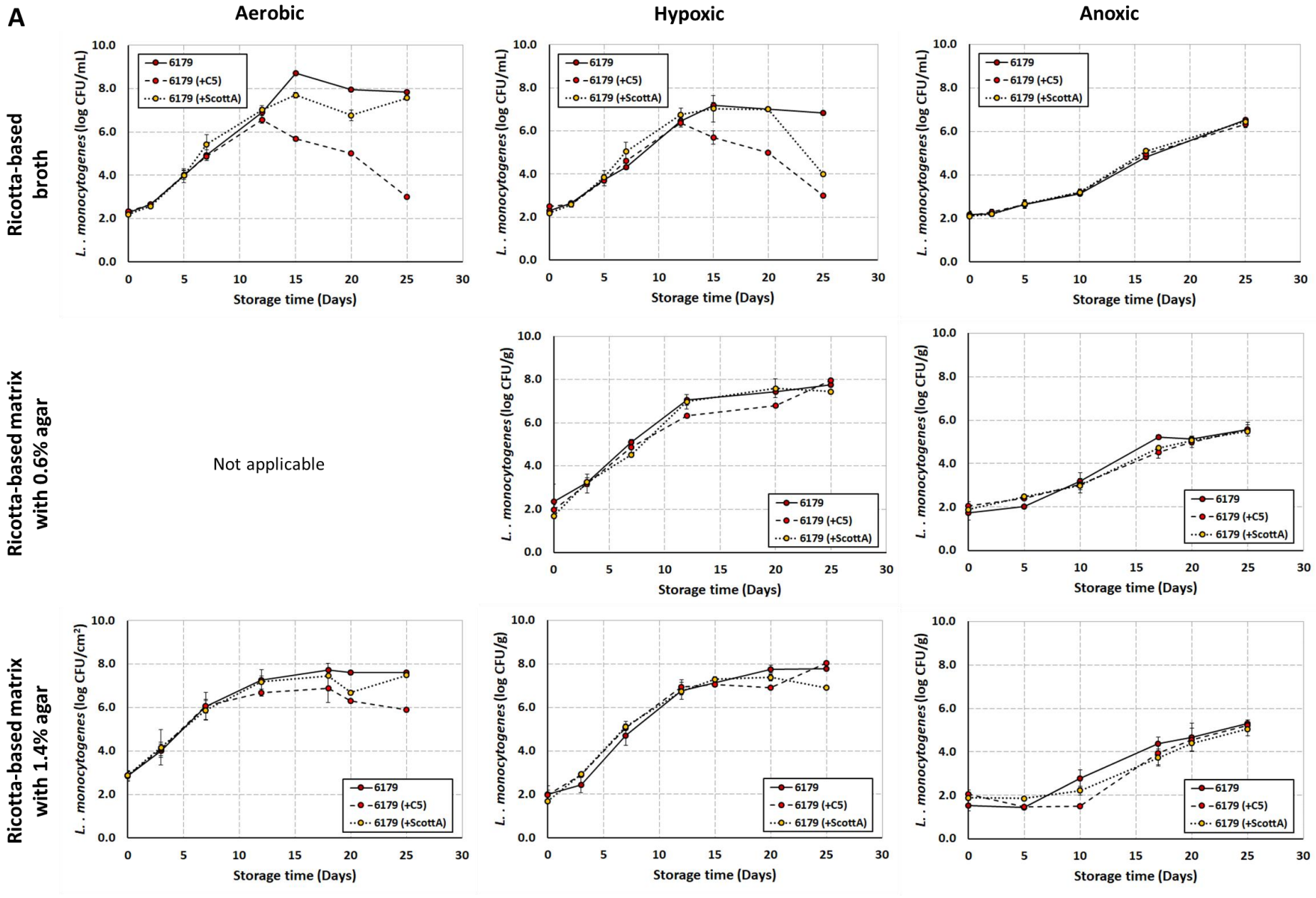

Figure 3A. Growth curves of *L. monocytogenes* strain 6179, singly and co-cultured with strains C5 and ScottA in Ricotta broth (per mL) and Ricotta-based substrates with the addition of 0.6 (per g) and 1.4% agar (per cm^2 or g), under aerobic, hypoxic and anoxic conditions and storage at 7°C.

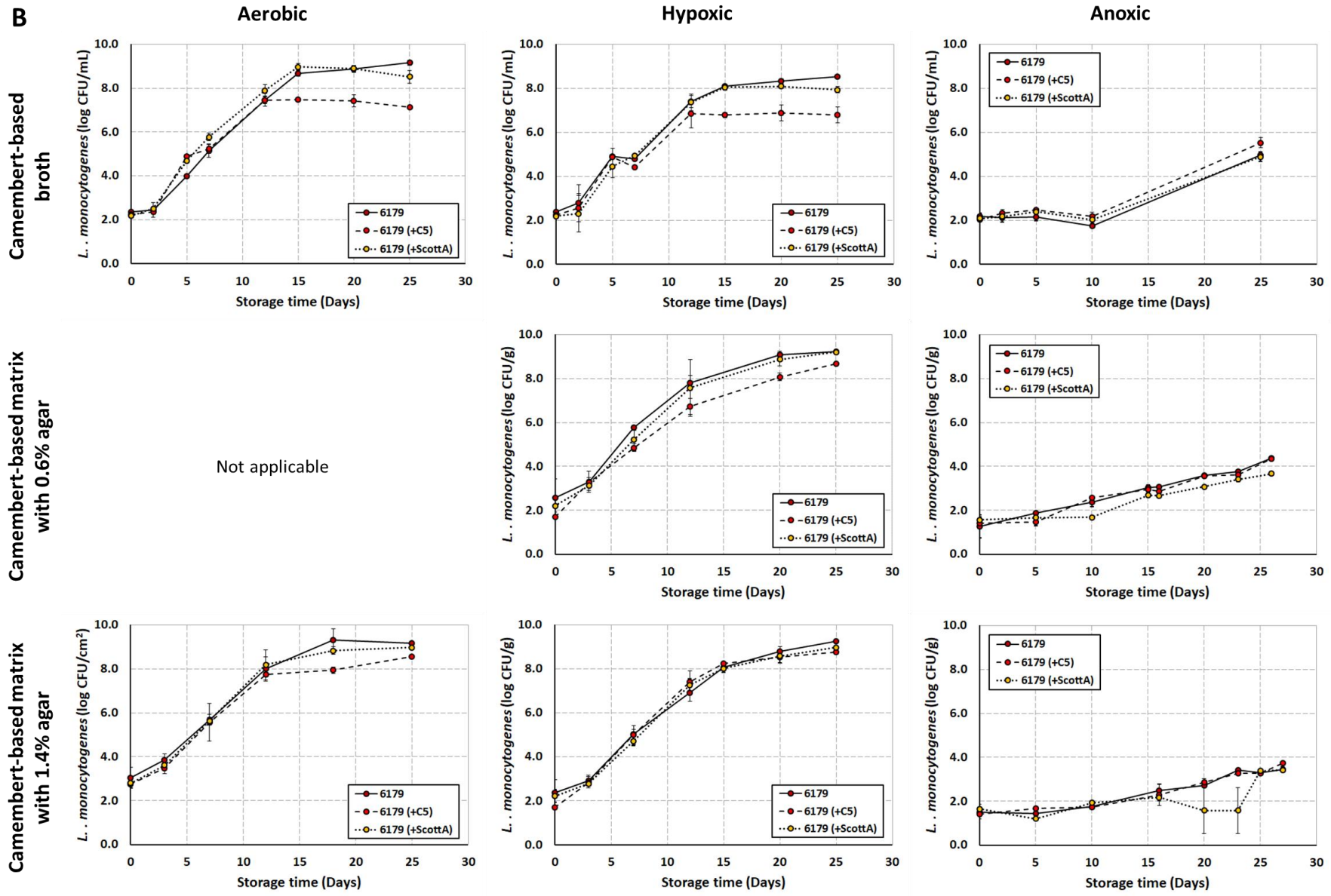

Figure 3B. Growth curves of *L. monocytogenes* strain 6179, singly and co-cultured with strains C5 and ScottA in Camembert broth (per mL) and Camembert-based substrates with the addition of 0.6 (per g) and 1.4% agar (per cm^2 or g), under aerobic, hypoxic and anoxic conditions and storage at 7°C.

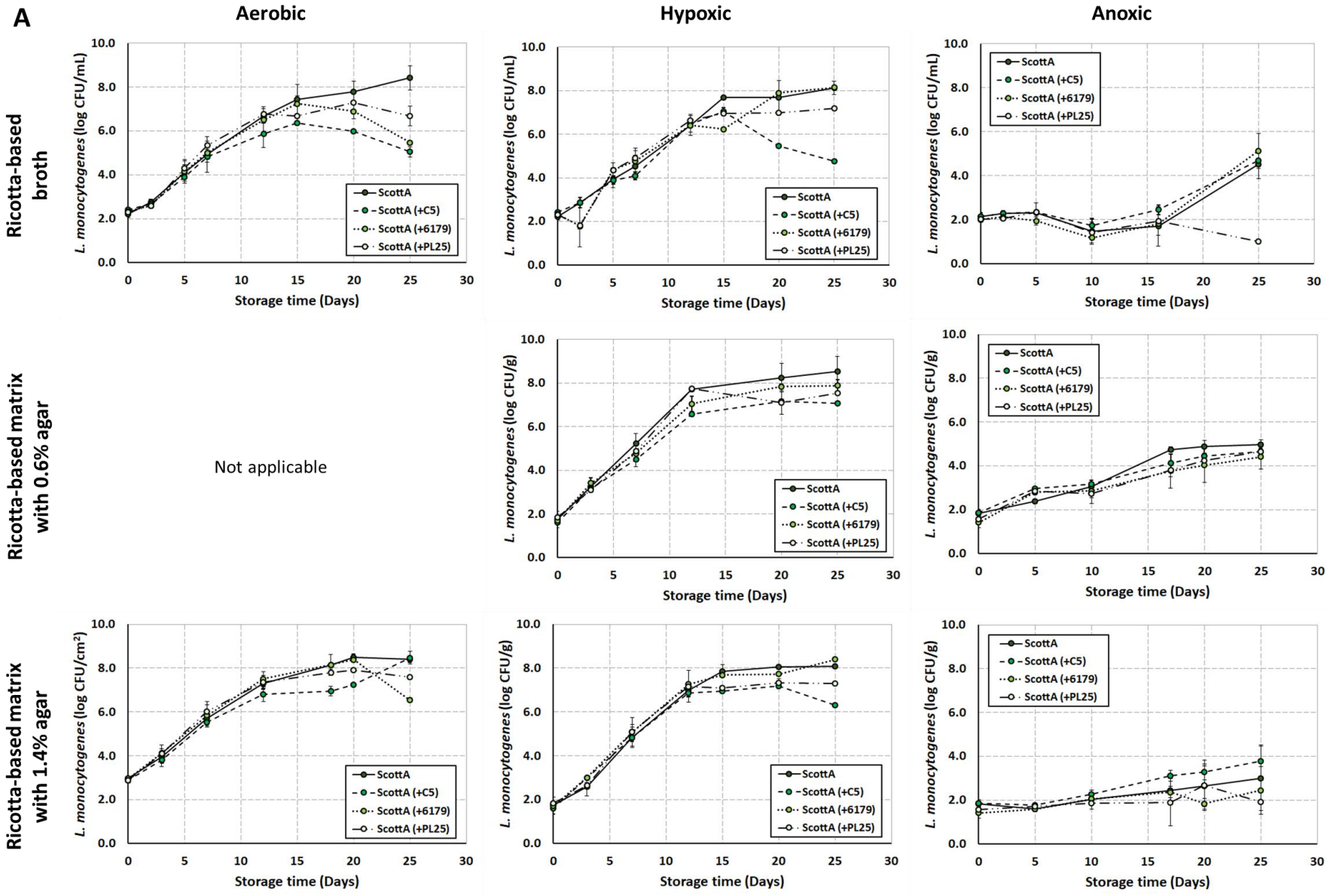

Figure 4A. Growth curves of *L. monocytogenes* strain ScottA, singly and co-cultured with strains C5, 6179 and PL25 in Ricotta broth (per mL) and Ricotta-based substrates with the addition of 0.6 (per g) and 1.4% agar (per cm^2 or g), under aerobic, hypoxic and anoxic conditions and storage at 7°C.

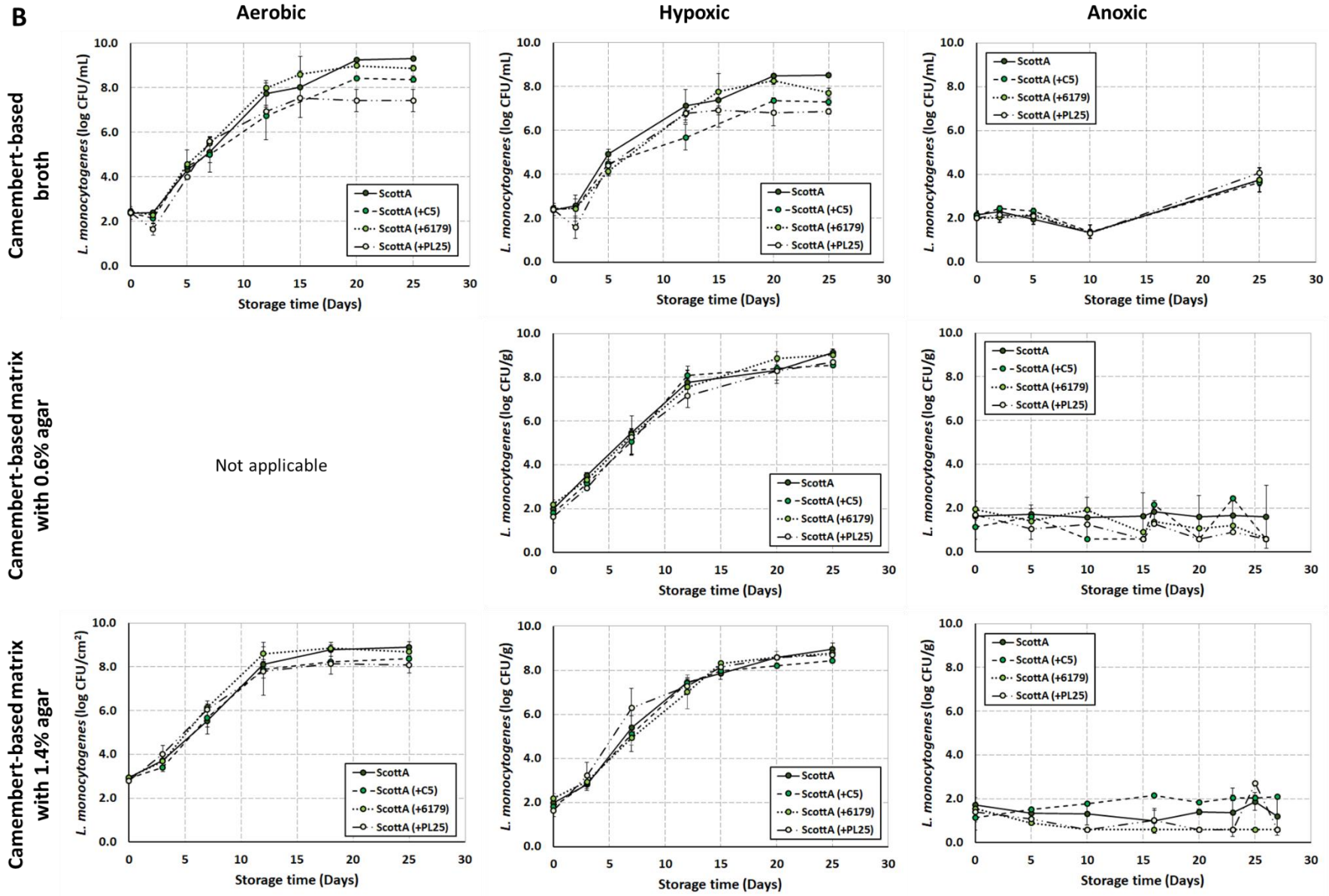

Figure 4B. Growth curves of *L. monocytogenes* strain ScottA, singly and co-cultured with strains C5, 6179 and PL25 in Camembert broth (per mL) and Camembert-based substrates with the addition of 0.6 (per g) and 1.4% agar (per cm^2 or g), under aerobic, hypoxic and anoxic conditions and storage at 7°C.

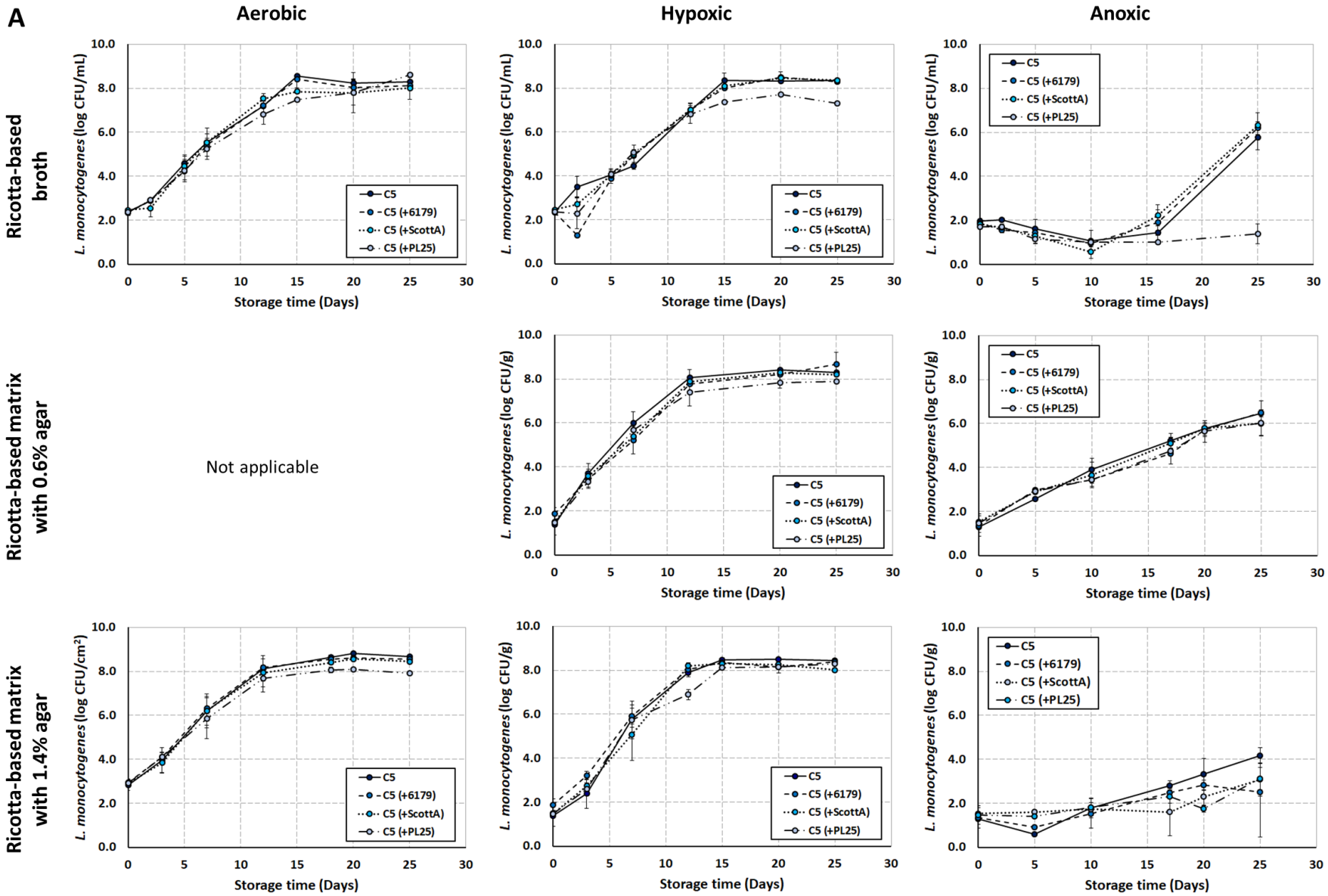

Figure 5A. Growth curves of *L. monocytogenes* strain C5, singly and co-cultured with strains 6179, ScottA and PL25 in Ricotta broth (per mL) and Ricotta-based substrates with the addition of 0.6 (per g) and 1.4% agar (per cm^2 or g), under aerobic, hypoxic and anoxic conditions and storage at 7°C.

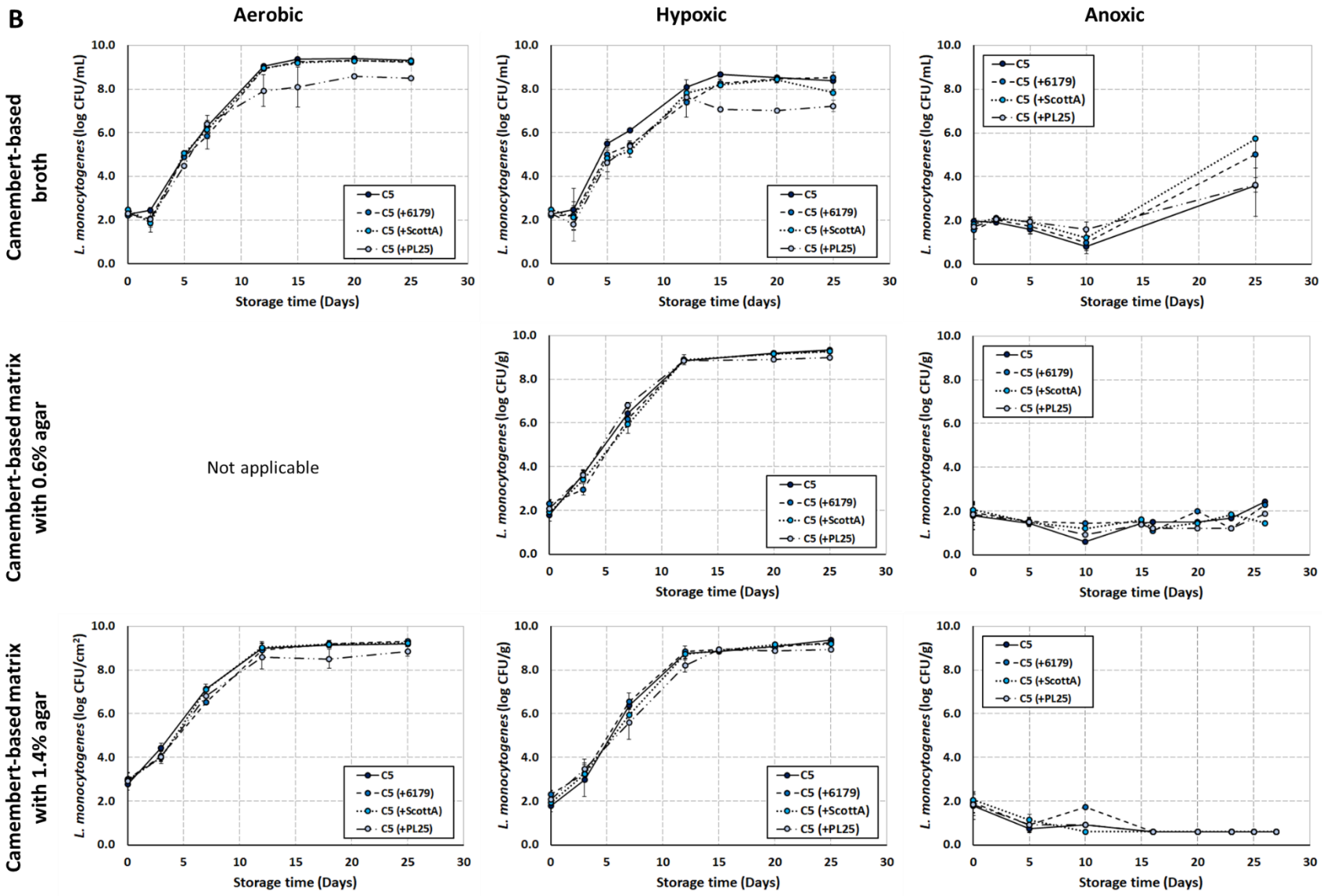

Figure 5B. Growth curves of *L. monocytogenes* strain C5, singly and co-cultured with strains 6179, ScottA and PL25 in Camembert broth (per mL) and Camembert-based substrates with the addition of 0.6 (per g) and 1.4% agar (per cm^2 or g), under aerobic, hypoxic and anoxic conditions and storage at 7°C.

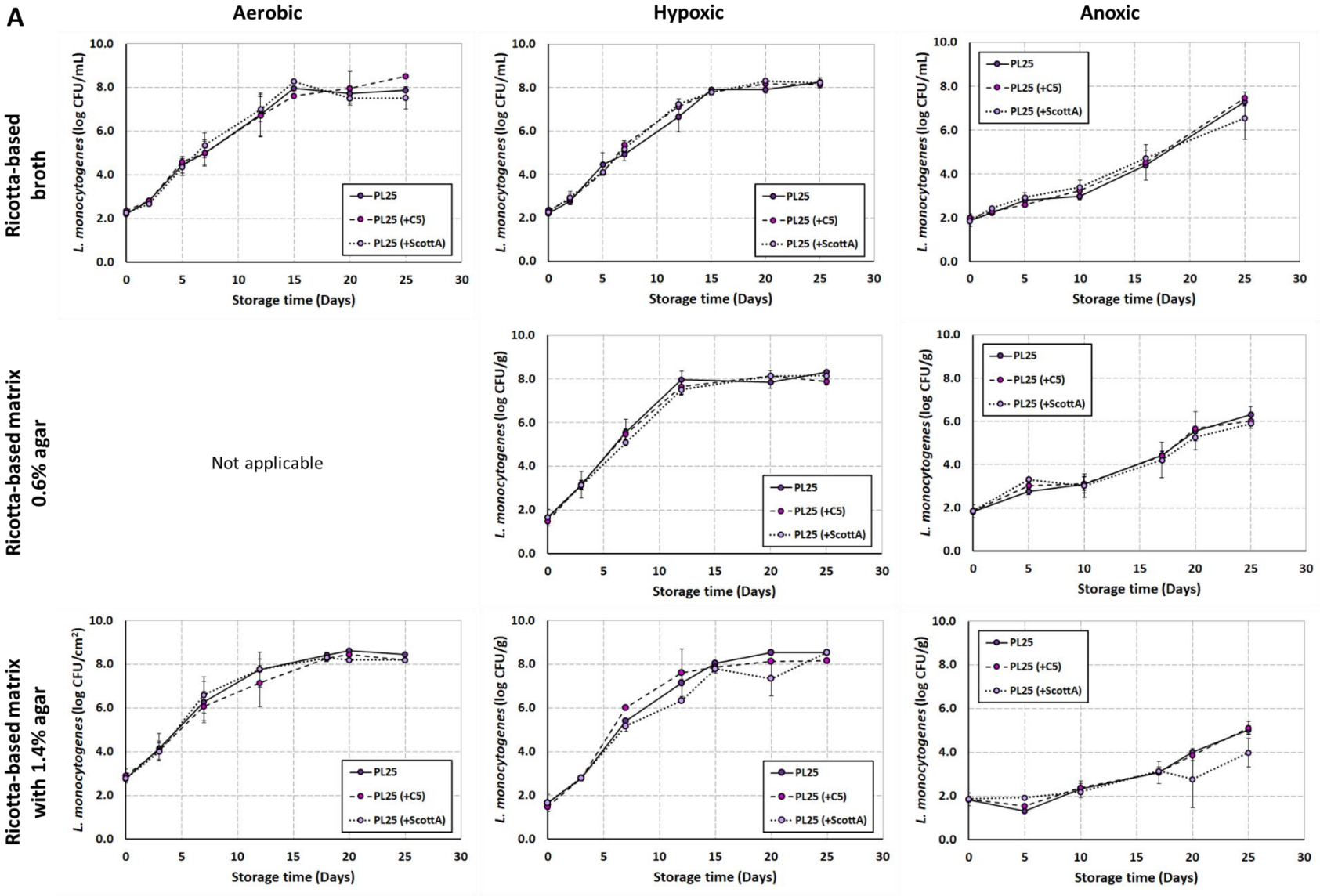

Figure 6A. Growth curves of *L. monocytogenes* strain PL25, singly and co-cultured with strains C5 and ScottA in Ricotta broth (per mL) and Ricotta-based substrates with the addition of 0.6 (per g) and 1.4% agar (per cm2 or g), under aerobic, hypoxic and anoxic conditions and storage at 7°C.

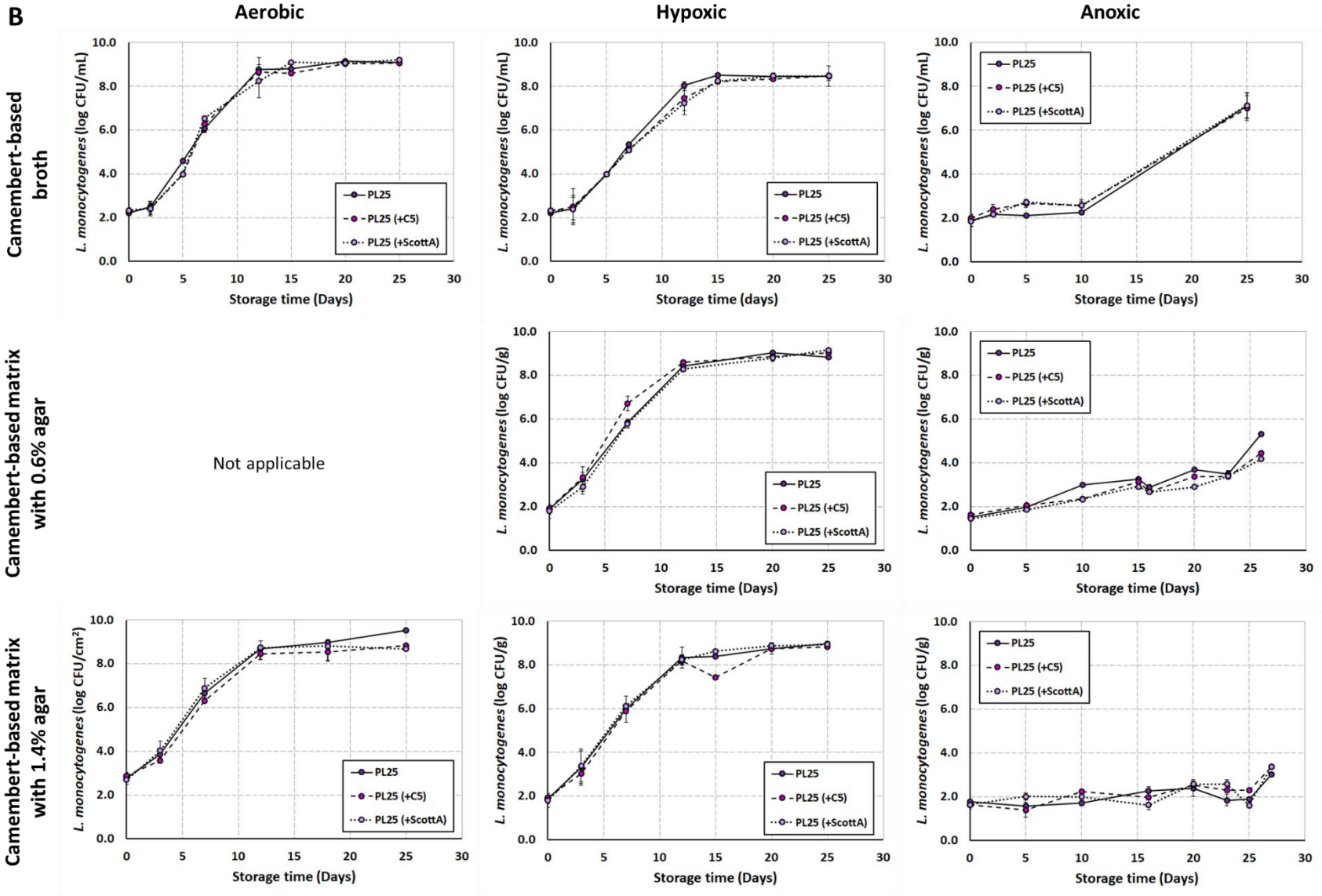

Figure 6B. Growth curves of *L. monocytogenes* strain PL25, singly and co-cultured with strains C5 and ScottA in Camembert broth (per mL) and Camembert-based substrates with the addition of 0.6 (per g) and 1.4% agar (per cm2 or g), under aerobic, hypoxic and anoxic conditions and storage at 7°C.

Table 1. *Listeria monocytogenes* strains used in the study.

Strain	Serotype	MLST	Source	Year of isolation	Reference	Antibiotic resistance (μg/mL)*
C5	4b	ST2	Dairy farm environment isolation	2007	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Streptomycin (2000)
6179	1/2a	ST121	Cheese	1999	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Rifampicin (>800)
ScottA	4b	ST290	Human isolate	1983	Research Institute ATO-DLO, Wageningen, Netherlands	Streptomycin (4000) Rifampicin (>800)
PL25	1/2b (3b, 7)**	ST59	Animal origin	2009	Agricultural University of Athens, Department of Food Science and Human Nutrition, Laboratory of Food Quality Control and Hygiene, Athens, Greece	Rifampicin (800)

*Approximate MIC was considered as the minimum tested concentration (μg/mL) of antibiotic at which no bacterial growth was observed after 24 hours at 30°C. Bacterial growth was confirmed through measurements of optical density (OD_{600}). The streptomycin concentrations were 0, 125, 250, 500, 1000, 2000, 4000 μg/mL. Rifampicin was evaluated at 0, 200, 400, 800 μg/mL.

**The serovar-specific group was characterized by multiplex PCR according to Doumith et al., (2004) and the serovars in parenthesis were omitted due to Multilocus Sequence Typing (MLST) classification.

Table 2. Lag phase and growth rate estimates and observed final population of *L. monocytogenes* strains 6179, ScottA, C5 and PL25, singly and co-cultured in Ricotta broth, Ricotta-based substrate that simulates Ricotta's structure (with addition of 0.6% agar) and Ricotta-based substrate that simulates Camembert's structure (with addition of 1.4% agar), under aerobic, hypoxic and anoxic conditions, at 7°C.

Substrate	Type of culture*	Lag time (Days)		Growth rate ($Days^{-1}$)		Final population (log CFU/mL, cm^2 or g)		
		Aerobic	Hypoxic	Aerobic	Hypoxic	Aerobic	Hypoxic	Anoxic
Ricotta-based broth	**Single 6179**	1.3 ± 0.6^{Aa}	2.1 ± 1.1^{Aa}	0.46 ± 0.07^{Aa}	0.44 ± 0.04^{Aa}	7.8 ± 0.2^{Ca}	6.8 ± 0.0^{Ba}	6.5 ± 0.1^{Ac}
	6179 (+C5)	1.2 ± 1.7^{Aa}	2.7 ± 0.8^{Aa}	0.45 ± 0.06^{Aa}	0.52 ± 0.14^{Aa}	$3.0 \pm 0.0^{Aa**}$	$3.0 \pm 0.0^{Aa**}$	$6.3 \pm 0.1^{Bc*}$
	6179 (+ScottA)	2.5 ± 0.4^{Aa}	2.1 ± 1.8^{Aa}	0.72 ± 0.21^{Aa}	0.66 ± 0.36^{Aa}	7.6 ± 0.0^{Ca}	$4.0 \pm 0.0^{Aa**}$	6.4 ± 0.0^{Bc}
Ricotta-based substrate (0.6% agar)	**Single 6179**	-	0.5 ± 0.6^{a}	-	0.51 ± 0.01^{a}	-	7.7 ± 0.0^{Bb}	5.6 ± 0.2^{Ab}
	6179 (+C5)	-	0.0 ± 0.0^{a}	-	$0.40 \pm 0.00^{a**}$	-	$8.0 \pm 0.1^{Bb*}$	5.6 ± 0.3^{Ab}
	6179 (+ScottA)	-	0.0 ± 0.0^{a}	-	$0.44 \pm 0.01^{a*}$	-	$7.4 \pm 0.0^{Bc*}$	5.5 ± 0.1^{Ab}
Ricotta-based substrate (1.4% agar)	**Single 6179**	0.5 ± 0.5^{Aa}	1.6 ± 0.1^{Ba}	0.48 ± 0.08^{Aa}	0.51 ± 0.05^{Aa}	7.6 ± 0.0^{Ba}	7.8 ± 0.1^{Cb}	5.3 ± 0.2^{Aa}
	6179 (+C5)	0.0 ± 0.0^{Aa}	0.6 ± 0.8^{Aa}	0.48 ± 0.09^{Aa}	0.49 ± 0.03^{Aa}	$5.9 \pm 0.0^{Bb**}$	$8.0 \pm 0.0^{Cb*}$	5.2 ± 0.3^{Aa}
	6179 (+ScottA)	0.8 ± 1.4^{Aa}	0.3 ± 0.4^{Aa}	0.51 ± 0.16^{Aa}	0.50 ± 0.00^{Aa}	7.5 ± 0.1^{Ca}	$6.9 \pm 0.0^{Bb**}$	5.1 ± 0.3^{Aa}
Ricotta-based broth	**Single ScottA**	0.4 ± 0.7^{Aa}	0.7 ± 1.4^{Aa}	0.40 ± 0.08^{Aa}	0.38 ± 0.08^{Aa}	8.4 ± 0.6^{Ba}	8.1 ± 0.2^{Ba}	4.5 ± 0.5^{Ab}
	ScottA (+C5)	1.6 ± 1.4^{Aa}	1.4 ± 0.1^{Ab}	0.49 ± 0.31^{Aa}	0.36 ± 0.01^{Aa}	$5.1 \pm 0.2^{Ba**}$	$4.8 \pm 0.0^{Aa**}$	4.7 ± 0.1^{Ab}
	ScottA (+6179)	0.8 ± 1.1^{Aa}	0.9 ± 1.3^{Aa}	0.44 ± 0.06^{Aa}	0.37 ± 0.14^{Aa}	$5.5 \pm 0.0^{Aa**}$	8.2 ± 0.0^{Bb}	5.1 ± 0.8^{Ab}
	ScottA (+PL25)	1.2 ± 0.1^{Aa}	0.9 ± 1.3^{Aa}	0.53 ± 0.06^{Aa}	0.46 ± 0.04^{Aa}	$6.7 \pm 0.4^{Ba**}$	$7.2 \pm 0.0^{Ca**}$	$1.0 \pm 0.0^{Aa**}$
Ricotta-based substrate (0.6% agar)	**Single ScottA**	-	0.3 ± 0.4^{a}	-	0.54 ± 0.06^{b}	-	8.5 ± 0.5^{Bb}	5.0 ± 0.3^{Ab}
	ScottA (+C5)	-	0.0 ± 0.0^{a}	-	0.42 ± 0.01^{b}	-	$7.1 \pm 0.0^{Bc**}$	4.7 ± 0.1^{Ab}
	ScottA (+6179)	-	0.0 ± 0.0^{a}	-	0.44 ± 0.01^{a}	-	$7.9 \pm 0.3^{Ba*}$	4.4 ± 0.6^{Ab}
	ScottA (+PL25)	-	1.1 ± 0.3^{a}	-	0.56 ± 0.03^{a}	-	$7.6 \pm 0.0^{Bb**}$	4.6 ± 0.2^{Ac}
Ricotta-based substrate (1.4% agar)	**Single ScottA**	0.2 ± 0.3^{Aa}	1.2 ± 1.2^{Aa}	0.38 ± 0.04^{Aa}	0.52 ± 0.10^{Bb}	8.4 ± 0.1^{Ba}	8.1 ± 0.1^{Ba}	3.0 ± 1.2^{Aa}
	ScottA (+C5)	0.5 ± 0.8^{Aa}	0.0 ± 0.0^{Aa}	0.28 ± 0.14^{Aa}	0.48 ± 0.01^{Ac}	8.5 ± 0.3^{Cb}	$6.3 \pm 0.0^{Bb**}$	3.8 ± 0.7^{Aa}
	ScottA (+6179)	0.0 ± 0.0^{Aa}	0.4 ± 0.6^{Aa}	0.42 ± 0.06^{Aa}	0.53 ± 0.04^{Aa}	$6.5 \pm 0.1^{Bb**}$	$8.4 \pm 0.0^{Cc*}$	2.5 ± 1.1^{Aa}
	ScottA (+PL25)	0.5 ± 0.9^{Aa}	1.6 ± 0.8^{Aa}	0.49 ± 0.14^{Aa}	0.61 ± 0.23^{Aa}	$7.6 \pm 0.0^{Cb**}$	$7.3 \pm 0.0^{Ba**}$	$1.9 \pm 0.5^{Ab*}$

Table 2. *Continued*

Ricotta-based broth	**Single C5**	0.3 ± 0.4^{Aa}	1.1 ± 1.5^{Aa}	0.47 ± 0.12^{Aa}	0.42 ± 0.12^{Aa}	8.3 ± 0.2^{Ba}	8.3 ± 0.0^{Ba}	5.8 ± 0.6^{Ab}
	C5 (+6179)	0.9 ± 1.2^{Aa}	1.7 ± 0.5^{Aa}	0.47± 0.05^{Aa}	0.50 ± 0.00^{Aa}	8.1 ± 0.0^{Ba}	8.3 ± 0.0^{Ba}	6.2 ± 0.3^{Ab}
	C5 (+ScottA)	1.1 ± 0.9^{Aa}	1.5 ± 0.5^{Aa}	0.51 ± 0.01^{Aa}	0.44 ± 0.05^{Aa}	8.0 ± 0.5^{Ba}	8.4 ± 0.0^{Bc}	6.3 ± 0.6^{Ab}
	C5 (+PL25)	0.0 ± 0.0^{Aa}	1.1 ± 1.5^{Aa}	0.39 ± 0.06^{Aa}	0.46 ± 0.13^{Aa}	8.6 ± 0.1^{Cb*}	7.3 ± 0.0^{Ba**}	1.4 ± 0.4^{Aa**}
Ricotta-based substrate (0.6% agar)	**Single C5**	-	0.4 ± 0.6^{a}	-	0.74 ± 0.14^{a}	-	8.3 ± 0.0^{Ba}	6.5 ± 0.02^{Ac}
	C5 (+6179)	-	0.3 ± 0.4^{a}	-	0.51 ± 0.05^{a}	-	8.7 ± 0.6^{Bb}	6.5 ± 0.5^{Ab}
	C5 (+ScottA)	-	0.0 ± 0.0^{a}	-	0.56 ± 0.01^{a}	-	8.2 ± 0.0^{Bb*}	6.0 ± 0.5^{Ab*}
	C5 (+PL25)	-	0.0 ± 0.0^{a}	-	0.59 ± 0.07^{a}	-	7.9 ± 0.1^{Bb**}	6.0 ± 0.6^{Ac}
Ricotta-based substrate (1.4% agar)	**Single C5**	0.9 ± 0.7^{Aa}	1.6 ± 0.9^{Aa}	0.56 ± 0.14^{Aa}	0.81 ± 0.19^{Aa}	8.7 ± 0.0^{Cb}	8.4 ± 0.0^{Ba}	4.2 ± 0.4^{Aa}
	C5 (+6179)	1.0 ± 0.6^{Aa}	1.1 ± 0.3^{Aa}	0.57 ± 0.19^{Aa}	0.68 ± 0.17^{Aa}	8.6 ± 0.1^{Bb}	8.4 ± 0.0^{Ba}	2.5 ± 2.0^{Aa*}
	C5 (+ScottA)	1.2 ± 1.2^{Aa}	0.6 ± 0.8^{Aa}	0.59 ± 0.22^{Aa}	0.71 ± 0.16^{Aa}	8.4 ± 0.0^{Cb*}	8.0 ± 0.0^{Ba*}	3.1 ± 0.7^{Aa**}
	C5 (+PL25)	0.8 ± 0.6^{Aa}	0.0 ± 0.0^{Aa}	0.51 ± 0.15^{Aa}	0.59 ± 0.06^{Aa}	7.9 ± 0.0^{Ba**}	8.3 ± 0.1^{Cc}	3.1 ± 0.5^{Ab**}
Ricotta-based broth	**Single PL25**	0.0 ± 0.0^{Aa}	0.0 ± 0.0^{Aa}	0.41 ± 0.11^{Aa}	0.41 ± 0.09^{Aa}	7.9 ± 0.7^{ABa}	8.3 ± 0.0^{Ba}	7.3 ± 0.2^{Ac}
	PL25 (+C5)	0.0 ± 0.0^{Aa}	0.6 ± 0.8^{Aa}	0.39± 0.08^{Aa}	0.44 ± 0.07^{Aa}	8.5 ± 0.1^{Cb}	8.1 ± 0.0^{Bb}	7.5 ± 0.3^{Ac}
	PL25 (+ScottA)	0.0 ± 0.0^{Aa}	0.6 ± 0.8^{Aa}	0.43 ± 0.08^{Aa}	0.45 ± 0.02^{Aa}	7.5 ± 0.5^{ABa}	8.2 ± 0.2^{Ba}	6.5 ± 1.0^{Ac}
Ricotta-based substrate (0.6% agar)	**Single PL25**	-	0.6 ± 0.9^{a}	-	0.62 ± 0.03^{Ac}	-	8.3 ± 0.1^{Ba}	6.3 ± 0.4^{Ab}
	PL25 (+C5)	-	0.0 ± 0.0^{a}	-	0.59 ± 0.02^{Ab}	-	7.9 ± 0.2^{Ba*}	6.0 ± 0.3^{Ab}
	PL25 (+ScottA)	-	0.5 ± 0.8^{a}	-	0.53 ± 0.05^{Ab}	-	8.2 ± 0.1^{Ba}	5.9 ± 0.2^{Ab*}
Ricotta-based substrate (1.4% agar)	**Single PL25**	0.0 ± 0.0^{Aa}	0.0 ± 0.0^{Aa}	0.48 ± 0.14^{Aa}	0.54 ± 0.02^{Ab}	8.4 ± 0.0^{Bb}	8.6 ± 0.0^{Cb}	5.0 ± 0.2^{Aa}
	PL25 (+C5)	0.0 ± 0.0^{Aa}	0.0 ± 0.0^{Aa}	0.41 ± 0.12^{Aa}	0.66 ± 0.12^{Bb}	8.2 ± 0.0^{Ba*}	8.2 ± 0.0^{Bb**}	5.1 ± 0.3^{Aa}
	PL25 (+ScottA)	0.9 ± 1.2^{Aa}	2.1 ± 0.4^{Ab*}	0.64 ± 0.29^{Aa}	0.69 ± 0.05^{Ac}	8.2 ± 0.0^{Bb*}	8.6 ± 0.0^{Cb}	4.0 ± 0.6^{Aa*}

* Single culture or co-culture.

For each one of the studied strains of *L. monocytogenes* (6179, ScottA, C5 and PL25), respectively:

Values with different uppercase letters that correspond to the same singly or co-cultured strain, in the same row (within the same substrate), are significantly different ($p<0.05$) (comparison of lag time, growth rate and final population between the different levels of oxygen availability, in/on the same substrate).

Values with different lower letters that correspond to the same singly or co-cultured strain, in the same column (within the same condition of oxygen availability), are significantly different ($p<0.05$) (comparison of lag time, growth rate and final population between the different levels of structure, under the same level of oxygen availability).

Star indicates statistical difference between the growth kinetics of the co-cultured strains in comparison to the singly cultured under the same conditions of structure and oxygen availability (*: $p<0.05$).

Dashes indicate the treatment combinations that were not applicable.

The units of final population refer to the way that the inoculum calculated and the inoculation performed on the surface of the solid dairy-based substrates (cm^2), inside the mass of the dairy-based substrates (g) or in the dairy-based broth substrates (mL).

Table 3. Lag phase and growth rate estimates and observed final population of *L. monocytogenes* strains 6179, ScottA, C5 and PL25, in singly and co-cultured in Camembert broth, Camembert-based substrate that simulates Ricotta's structure (with addition of 0.6% agar) and Camembert-based substrate that simulates Camembert's structure (with addition of 1.4% agar), under aerobic, hypoxic and anoxic conditions, at 7°C.

Substrate	Type of culture*	Lag time (Days)		Growth rate ($Days^{-1}$)		Final population (log CFU/mL, cm^2 or g)		
		Aerobic	Hypoxic	Aerobic	Hypoxic	Aerobic	Hypoxic	Anoxic
Camembert broth	**Single 6179**	2.0 ± 0.2^{Aa}	1.1 ± 1.5^{Aa}	0.52 ± 0.03^{Aa}	0.51 ± 0.02^{Aa}	9.2 ± 0.1^{Ca}	8.5 ± 0.0^{Ba}	5.0 ± 0.0^{Ac}
	6179 (+C5)	0.7 ± 1.0^{Aa}	1.0 ± 1.5^{Aa}	0.57 ± 0.07^{Aa}	0.49 ± 0.07^{Aa}	$7.1 \pm 0.0^{Cb**}$	$6.8 \pm 0.4^{Ba**}$	$5.5 \pm 0.0^{Ac**}$
	6179 (+ScottA)	0.8 ± 1.2^{Aa}	1.2 ± 1.7^{Aa}	0.61 ± 0.10^{Aa}	0.57 ± 0.15^{Aa}	$8.5 \pm 0.3^{Cb**}$	$7.9 \pm 0.1^{Ba**}$	4.9 ± 0.2^{Ac}
Camembert-based substrate (0.6% agar)	**Single 6179**	-	0.6 ± 0.8^{a}	-	0.56 ± 0.07^{Aa}	-	9.2 ± 0.0^{Bb}	4.4 ± 0.1^{Ab}
	6179 (+C5)	-	0.0 ± 0.0^{a}	-	0.44 ± 0.04^{Aa}	-	$8.7 \pm 0.1^{Bb**}$	4.3 ± 0.1^{Ab}
	6179 (+ScottA)	-	1.1 ± 1.1^{a}	-	0.53 ± 0.28^{Aa}	-	9.2 ± 0.1^{Bc}	$3.7 \pm 0.0^{Ab**}$
Camembert-based substrate (1.4% agar)	**Single 6179**	1.0 ± 0.8^{Aa}	0.4 ± 0.5^{Aa}	0.49 ± 0.08^{Aa}	0.45 ± 0.10^{Aa}	9.2 ± 0.1^{Ba}	9.2 ± 0.0^{Bb}	3.5 ± 0.1^{Aa}
	6179 (+C5)	2.2 ± 1.1^{Aa}	0.8 ± 0.3^{Aa}	0.56 ± 0.05^{Aa}	0.54 ± 0.08^{Aa}	$8.6 \pm 0.1^{Ba**}$	$8.8 \pm 0.1^{Bb**}$	3.7 ± 0.1^{Aa}
	6179 (+ScottA)	1.5 ± 0.2^{Aa}	2.1 ± 0.5^{Aa}	0.53 ± 0.09^{Aa}	0.53 ± 0.01^{Aa}	$9.0 \pm 0.1^{Ba*}$	$9.0 \pm 0.0^{Bb*}$	3.4 ± 0.1^{Aa}
Camembert broth	**Single ScottA**	2.0 ± 0.2^{Aa}	1.1 ± 1.3^{Aa}	0.55 ± 0.12^{Aa}	0.48 ± 0.13^{Aa}	9.3 ± 0.1^{Cb}	8.5 ± 0.2^{Ba}	3.7 ± 0.5^{Ab}
	ScottA (+C5)	1.7 ± 0.0^{Aa}	0.9 ± 1.2^{Aa}	0.45 ± 0.21^{Aa}	0.31 ± 0.07^{Aa}	$8.4 \pm 0.1^{Ca**}$	$7.3 \pm 0.2^{Ba**}$	3.6 ± 0.1^{Ac}
	ScottA (+6179)	1.9 ± 0.1^{Aa}	2.0 ± 1.5^{Aa}	0.59 ± 0.00^{Aa}	0.47 ± 0.12^{Aa}	$8.9 \pm 0.1^{Ca**}$	$7.7 \pm 0.2^{Ba**}$	3.7 ± 0.2^{Ab}
	ScottA (+PL25)	2.3 ± 0.0^{Bb}	0.0 ± 0.0^{Aa}	0.77 ± 0.02^{Ba}	0.48 ± 0.05^{Aa}	$7.4 \pm 0.5^{Ca**}$	$6.9 \pm 0.1^{Ba**}$	4.1 ± 0.2^{Ab}
Camembert-based substrate (0.6% agar)	**Single ScottA**	-	0.5 ± 0.7^{a}	-	0.54 ± 0.04^{a}	-	9.1 ± 0.1^{Bc}	1.6 ± 0.0^{Aa}
	ScottA (+C5)	-	0.3 ± 0.4^{a}	-	0.57 ± 0.00^{b}	-	$8.5 \pm 0.0^{Bb**}$	$0.6 \pm 0.0^{Aa*}$
	ScottA (+6179)	-	0.7 ± 1.0^{a}	-	0.52 ± 0.23^{a}	-	9.0 ± 0.0^{Bc}	$0.6 \pm 0.0^{Aa*}$
	ScottA (+PL25)	-	0.1 ± 0.2^{a}	-	0.50 ± 0.07^{a}	-	$8.7 \pm 0.0^{Bb*}$	$0.6 \pm 0.0^{Aa*}$
Camembert-based substrate (1.4% agar)	**Single ScottA**	1.1 ± 0.8^{Aa}	1.1 ± 0.7^{Aa}	0.49 ± 0.05^{Aa}	0.56 ± 0.06^{Aa}	8.9 ± 0.2^{Ba}	8.9 ± 0.2^{Bb}	1.2 ± 0.6^{Aa}
	ScottA (+C5)	2.2 ± 0.2^{Aa}	1.9 ± 0.7^{Aa}	0.58 ± 0.06^{Aa}	0.64 ± 0.12^{Ab}	$8.4 \pm 0.1^{Ba**}$	$8.4 \pm 0.1^{Bb*}$	$2.1 \pm 0.0^{Ab**}$
	ScottA (+6179)	1.7 ± 0.4^{Aa}	1.5 ± 0.5^{Aa}	0.61 ± 0.10^{Aa}	0.49 ± 0.12^{Aa}	$8.7 \pm 0.1^{Ba*}$	8.8 ± 0.0^{Bb}	$0.6 \pm 0.0^{Aa*}$
	ScottA (+PL25)	0.3 ± 0.4^{Aa}	0.7 ± 0.2^{Aa}	0.49 ± 0.12^{Aa}	0.76 ± 0.17^{Aa}	$8.1 \pm 0.3^{Ba**}$	8.7 ± 0.0^{Cb}	$0.6 \pm 0.0^{Aa*}$

Table 3. *Continued*

Camembert broth	**Single C5**	2.2 ± 0.6^{Aa}	1.2 ± 1.7^{Aa}	0.81 ± 0.03^{Ab}	0.80 ± 0.19^{Aa}	9.3 ± 0.1^{Bb}	8.4 ± 0.4^{Ba}	3.6 ± 1.4^{Ac}
	C5 (+6179)	2.6 ± 1.0^{Aa}	1.1 ± 1.5^{Aa}	0.85 ± 0.02^{Aa}	0.57 ± 0.16^{Aa}	9.2 ± 0.1^{Ba}	8.5 ± 0.1^{Ba}	5.0 ± 0.7^{Ac}
	C5 (+ScottA)	2.2 ± 0.2^{Aa}	$2.3 \pm 0.4^{Aa*}$	0.84 ± 0.12^{Aa}	0.73 ± 0.07^{Aa}	9.3 ± 0.1^{Ca}	$7.8 \pm 0.1^{Ba*}$	$5.7 \pm 0.1^{Ac*}$
	C5 (+PL25)	2.3 ± 0.3^{Aa}	1.0 ± 1.4^{Aa}	0.91 ± 0.11^{Aa}	0.69 ± 0.12^{Aa}	$8.5 \pm 0.0^{Ca*}$	$7.2 \pm 0.3^{Ba*}$	3.6 ± 0.3^{Ac}
Camembert-based substrate (0.6% agar)	**Single C5**	-	0.5 ± 0.7^{a}	-	0.72 ± 0.09^{a}	-	9.3 ± 0.0^{Bb}	2.4 ± 0.0^{Ab}
	C5 (+6179)	-	2.1 ± 0.0^{a}	-	0.79 ± 0.10^{a}	-	9.3 ± 0.0^{Bb}	2.3 ± 0.0^{Ab}
	C5 (+ScottA)	-	1.4 ± 0.6^{a}	-	0.71 ± 0.04^{a}	-	9.3 ± 0.0^{Bc}	$1.4 \pm 0.0^{Ab*}$
	C5 (+PL25)	-	1.2 ± 0.1^{a}	-	0.82 ± 0.06^{a}	-	$9.0 \pm 0.1^{Bb*}$	$1.9 \pm 0.0^{Ab*}$
Camembert-based substrate (1.4% agar)	**Single C5**	0.4 ± 0.5^{Aa}	1.2 ± 1.7^{Aa}	0.68 ± 0.02^{Aa}	0.86 ± 0.25^{Aa}	9.2 ± 0.0^{Ba}	9.4 ± 0.0^{Cb}	0.6 ± 0.0^{Aa}
	C5 (+6179)	1.4 ± 0.5^{Aa}	2.2 ± 0.5^{Aa}	0.63 ± 0.10^{Aa}	0.96 ± 0.26^{Aa}	9.3 ± 0.0^{Ca}	$9.2 \pm 0.0^{Bb*}$	0.6 ± 0.0^{Aa}
	C5 (+ScottA)	1.6 ± 0.3^{Aa}	1.8 ± 0.1^{Aa}	$0.77 \pm 0.00^{Aa*}$	0.80 ± 0.22^{Aa}	9.2 ± 0.2^{Ba}	$9.2 \pm 0.1^{Bb*}$	0.6 ± 0.0^{Aa}
	C5 (+PL25)	0.9 ± 1.3^{Aa}	1.7 ± 0.3^{Aa}	0.66 ± 0.13^{Aa}	0.72 ± 0.22^{Aa}	$8.8 \pm 0.2^{Bb*}$	$8.9 \pm 0.0^{Bb*}$	0.6 ± 0.0^{Aa}
Camembert-based broth	**Single PL25**	0.7 ± 0.9^{Aa}	2.4 ± 0.5^{Bb}	0.67 ± 0.00^{Aa}	0.67 ± 0.04^{Ab}	9.1 ± 0.0^{Ca}	8.4 ± 0.2^{Ba}	7.1 ± 0.6^{Ac}
	PL25 (+C5)	1.1 ± 1.6^{Aa}	1.2 ± 1.7^{Aa}	0.72 ± 0.11^{Aa}	0.52 ± 0.19^{Aa}	9.1 ± 0.1^{Bb}	8.5 ± 0.5^{Ba}	7.0 ± 0.6^{Ac}
	PL25 (+ScottA)	1.0 ± 1.4^{Aa}	1.6 ± 0.9^{Aa}	0.69 ± 0.20^{Aa}	0.51 ± 0.15^{Aa}	9.2 ± 0.1^{Cb}	8.5 ± 0.1^{Ba}	7.1 ± 0.6^{Ac}
Camembert-based substrate (0.6% agar)	**Single PL25**	-	0.4 ± 0.6^{a}	-	0.53 ± 0.02^{a}	-	8.8 ± 0.0^{Bb}	5.3 ± 0.0^{Ab}
	PL25 (+C5)	-	1.2 ± 0.5^{a}	-	$0.84 \pm 0.17^{a*}$	-	$9.0 \pm 0.0^{Bb*}$	$4.5 \pm 0.1^{Ab**}$
	PL25 (+ScottA)	-	$1.2 \pm 0.0^{a*}$	-	$0.66 \pm 0.04^{a**}$	-	$9.2 \pm 0.1^{Bc*}$	$4.2 \pm 0.1^{Ab**}$
Camembert-based substrate (1.4% agar)	**Single PL25**	1.2 ± 0.3^{Aa}	0.5 ± 0.7^{Aa}	0.66 ± 0.06^{Aa}	0.62 ± 0.06^{Aa}	9.5 ± 0.0^{Cb}	9.0 ± 0.1^{Bc}	3.0 ± 0.1^{Aa}
	PL25 (+C5)	$1.9 \pm 0.3^{Aa*}$	1.3 ± 1.0^{Aa}	0.66 ± 0.05^{Aa}	0.73 ± 0.19^{Aa}	$8.8 \pm 0.0^{Ba**}$	$8.8 \pm 0.0^{Bb*}$	$3.4 \pm 0.1^{Aa*}$
	PL25 (+ScottA)	1.2 ± 0.8^{Aa}	0.5 ± 0.8^{Aa}	0.73 ± 0.03^{Aa}	0.65 ± 0.14^{Aa}	$8.7 \pm 0.0^{Ba**}$	$8.9 \pm 0.0^{Cb*}$	$3.4 \pm 0.1^{Aa*}$

* Single culture or co-culture.

For each one of the studied strains of *L. monocytogenes* (6179, ScottA, C5 and PL25), respectively:

Values with different uppercase letters that correspond to the same singly or co-cultured strain, in the same row (within the same substrate), are significantly different ($p<0.05$) (comparison of lag time, growth rate and final population between the different levels of oxygen availability, in/on the same substrate).

Values with different lower letters that correspond to the same singly or co-cultured strain, in the same column (within the same condition of oxygen availability), are significantly different ($p<0.05$) (comparison of lag time, growth rate and final population between the different levels of structure, under the same level of oxygen availability).

Star indicates statistical difference between the growth kinetics of the co-cultured strains in comparison to the singly cultured under the same conditions of structure and oxygen availability (*: $p<0.05$).

Dashes indicate the treatment combinations that were not applicable.

The units of final population refer to the way that the inoculum calculated and the inoculation performed on the surface of the solid dairy-based substrates (cm^2), inside the mass of the dairy-based substrates (g) or in the dairy-based broth substrates (mL).

Supplementary material

Description of the dairy products used in the present study

Ricotta and Camembert are both RTE dairy products which have caused listeriosis outbreaks in the past, however they are very different matrices produced *via* different technological procedures (Martinez-Rios and Dalgaard, 2018; Shamloo et al., 2019). Ricotta, is not classified as cheese, because is obtained from whey (from bovine milk) and cream coagulated by combination of temperature and acidity, resulting in a fresh soft dairy product of low salt content (0.3 g), high pH value (5.75 – 6.55), high moisture (80%) and water activity approximately 0.99, with self-life up to 1 month (Fox, Guinee, Cogan, & McSweeney, 2017; Hough, Puglieso, Sanchez, & Da Silva, 1999; Kapetanakou, Gkerekou, Vitzilaiou, & Skandamis, 2017; Tirloni, Stella, Bernardi, Dalgaard, & Rosshaug, 2019). On the other hand, Camembert is a surface mould-ripened soft cheese (bloomy rind cheese; Batty et al., 2019), which has also high pH (6.01 to 6.67), 55% moisture and water activity approximately 0.97, with a self-life up to 1 month, depending on the packaging (Fox, Guinee, Cogan, & McSweeney, 2016; Kapetanakou et al., 2017) (Table 1). The physicochemical characteristics of the dairy products, along with their shelf-life that was higher than 5 days renders the selected products non-compliant with Regulation (EC) 2073/2005 and able to support the growth of *L. monocytogenes.*

Table 1. *Nutritional declaration of studied dairy products, according to the commercial label.*

Nutritional values per 100 g

	Ricotta	**Camembert**
Energy	623 kJ / 150 kcal	1231 kJ / 297 kcal
Fat	12 g	25 g
- of which saturates	8.4 g	17 g
Carbohydrate	3 g	<0.5 g
- of which sugars	3 g	<0.5 g
Protein	7.5 g	18 g
Salt	0.3 g	2 g

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CHAPTER 4

The impact of inter-strain interactions on growth and acid resistance of L. monocytogenes strains on different dairy products

Unpublished manuscript

The impact of inter-strain interactions on growth and acid resistance of *L. monocytogenes* strains on different dairy products

Maria A. Gkerekou, Maria K. Georgoulia, Konstantina G. Athanaseli and Panagiotis N. Skandamis*

Laboratory of Food Quality Control and Hygiene, Department of Food Science and Human Nutrition, Agricultural University of Athens, Greece.

Abstract

Contamination of cheese with multiple *Listeria (L.) monocytogenes* strains has been previously demonstrated. However, scarce information exists on the survival and/or growth of the pathogen if contamination with multiple strains occur, let alone how the relative population of each strain during co-culture may influence its survival after exposure in simulated gastric fluid (SGF), which is the first digestion stage. The objectives of the present study were the evaluation of the inter-strain interactions and matrix-adaptation of *L. monocytogenes* strains on their growth on Ricotta and Camembert and their subsequent survival in SGF. Antibiotic-resistant (for selective enumeration), matrix-adapted (MA) and non-adapted (NA) *L. monocytogenes* strains (C5, ScottA (serotype 4b); 6179 (1/2a); PL25 (1/2b)), were inoculated in single or two-strain cultures (1:1 strain-ratio) at approximately 2.0 - 3.0 log CFU/g on Ricotta and Camembert (10g) cheeses. Adaptation of cells was performed in cheese broth (1:1 cheese in Maximum Recovery Diluent) (7^{o}C / 48h). Growth and survival of *L. monocytogenes* were assessed during aerobic storage of cheese samples at 7^{o}C. Survival of middle exponential and early stationary bacteria cells was evaluated after 10, 20, 40, 60 and 120 minutes in SGF (pH 2.0; 37°C) (n=3x2). Matrix-adaptation did not affect neither the growth of strains nor the occurred interstrain interactions compared with the results from the NA single-cultures and co-cultures. On Camembert, only the growth of ScottA influenced by the presence of C5. On Ricotta, significant ($P<0.05$) growth inhibition of certain strains in mixed cultures was observed as manifested by the final population levels of the pathogen. NA ScottA (5.2 ± 0.1 log CFU/g) and NA and MA 6179 (5.5 ± 0.8 log CFU/g and 5.1 ± 0.8 log CFU/g, respectively) were suppressed by the presence of C5, compared to the corresponding single cultures, which reached 8.0 ± 0.5, 6.9 ± 0.5 and 8.1 ± 0.1 log CFU/g, respectively. Habituation of the pathogen on Camembert resulted in acid sensitization against subsequent exposure against SGF due to the site of the contamination. Regarding Ricotta, ScottA, displayed increased survival compared to C5 and PL25, even though it was outcompeted during storage. The results reveal how cheese matrix may affect the outcome of inter-strain interactions and therefore could assist in explaining the dominance of certain serotypes in foods of safety concern for *L. monocytogenes*.

Keywords: Listeria monocytogenes; Inter-strain interactions; Ricotta; Camembert; Simulated gastric fluid

Introduction

World production of cheese is ~19 x 10^6 tonnes per annum, with Europe being first both in production (8634 x 10^3 tonnes) and consumption (17.1 kg per caput) (Fox et al., 2017). Cheeses and other dairy products are categorized as Ready-To-Eat (RTE) products that can be consumed without prior cooking, resulting in foodborne illnesses and outbreaks associated with these products to be considered of great importance and they need to be addressed in the most cost-effective manner, because of their impact on public health and consumer's confidence in the food industry (Le et al., 2014; Swaminathan and Gerner-Smidt, 2007). In U.S. from 1986 to 2008 and in Canada from 2004 to mid-2009 there were 137 and 15 recalls of various types of cheeses, of which 108 (79%) and 11 (73%) were *Listeria*-related. The three most common types of cheeses involved in these recalls were fresh soft cheeses, hard cheeses (which represent the largest market share), and soft-ripened cheeses. In Europe, since 1987 and the incident associated with the consumption of soft cheese in Switzerland (smear cheese; Vacherin Mont d'Or) (Kousta et al., 2010; Martinez-Rios and Dalgaard, 2018; Schoder et al., 2013), until nowadays, approximately 14 listeriosis outbreaks have been associated with different types of cheeses (Büla et al., 1995; Ianache and Ceausu, 2018; Kousta et al., 2010; Melo et al., 2015b; Swaminathan and Gerner-Smidt, 2007), compelling cheese producers consider *Listeria monocytogenes* as the main biological hazard (Melo et al., 2015b). Specifically, for soft-ripened cheeses, the risk for listeriosis per serving is estimated to be 50- to 160-fold greater for cheeses made from unpasteurized milk than pasteurized milk (Jackson et al., 2018). Listeriosis is a serious invasive infection, which can cause severe illness and potentially death, especially to the susceptible individuals including the elderly population, the immunocompromised, the pregnant women and the newborns/infants. Thus, for the production of most of the dairy products is used pasteurized milk, however, post-processing contamination of dairy products with *L. monocytogenes*, is ascribed to flawed hygiene practices, the high occurrence of the pathogen in the processing environment and its ability to adhere onto food processing surfaces and form biofilms (Alvarez-Ordóñez, Coughlan, Briandet, & Cotter, 2019; Melero et al., 2019; Poimenidou et al., 2009; Rückerl et al., 2014; Tirloni et al., 2020). *L. monocytogenes* is a widely distributed environmental bacterium possesses characteristics that favor it as foodborne pathogen. It grows at low temperature, down to freezing point, which means that it may grow in refrigerated foods, enable it a major biological hazard for the minimally processed RTE products (Swaminathan and Gerner-Smidt, 2007). In parallel, the enhanced tolerance to different stresses such as high salinity, low pH, starvation, low water activity, presence of antibiotics and/or disinfectants etc. equips the pathogen with the ability to survive, persist and grow both in the processing food environment as well as inside the host. The development of stress adaptive responses is of great importance with regard to food safety because equips the pathogen with the necessary "armory" to overcome the hostile host-defense systems in the human gastrointestinal

tract (GIT), including the acidic conditions encountered during gastric passage, that are the last hurdle which can prevent the infection of the host.

Previous studies have shown that different strains of *L. monocytogenes* may be introduced at various time-points in the same processing environment *via* raw materials (Chambel et al., 2007; Martín et al., 2014; Ortiz et al., 2010; Thévenot et al., 2006), ending to multiple strains co-existing in the same food product. Danielsson-Tham et al. (1993) identified 2 to 4 different clones of *L. monocytogenes* from the same soft cheese sample, while multiple strains, also, isolated from the same sample of latin-style fresh cheese (Kabuki et al., 2004). Studies have, already, provided the proof-of-concept that multiple strains of *L. monocytogenes* may be involved and traced in case of listeriosis outbreaks, as happened during the reported listeriosis outbreak occurred in Austria, Germany and the Czech Republic in 2009 and 2010 and the US multistate listeriosis outbreak in 2015 involving ice-cream manufactured by Blue Bell creameries. The investigations were traced back to a traditional Austrian curd cheese called ''Quargel'', which was contaminated with two distinct *L. monocytogenes* strains of serotype 1/2a and ice cream bars and ice cream cookie sandwiches, in which identified 3 serotypes (1/2a, 1/2b, and 3b) that together had 15 PFGE pattern combinations among patient, food, and environmental *L. monocytogenes* isolates, respectively (Conrad et al., 2023; Rychli et al., 2014b; Weissfeld et al., 2017). Taken together, this information suggests that since a single food can carry more than one strain of *L. monocytogenes*, the ingestion of multiple strains might also occur. The variability and the competitive fitness of different strains contaminating the same food product are crucial during ingestion and the evolution of the potential infection. Towards storage, the different population level of each strain during co-culture (Gkerekou et al., 2022, 2021; Zilelidou et al., 2016b, 2015) defines the population of each strain upon entry in the gastric fluid phase. As a result, even with similar inactivation rates, the populations of two competing strains in SGF could be different at each time point due to differences in their initial cell density (Zilelidou et al., 2016a). Thus, during the present study evaluated the effect of physicochemical characteristics and matrix adaptation on/in different dairy products (Ricotta and Camembert) on inter-strain interactions of different *L. monocytogenes* strains and the subsequent survival in simulated gastric fluid.

Materials and methods

L. monocytogenes strains

In the present study were used four *L. monocytogenes* strains, which were selected based on their antibiotic resistance to streptomycin (Streptomycin Sulfate Biochemica, AppliChem) or rifampicin (Rifambicin, AppliChem) (Laboratory of Quality Control and Hygiene in Agricultural University of Athens) (Table 1). The selection to the different antibiotics was performed according to the method described by de Blackburn & Davies (1994) and was enable the selective enumeration of each strain in co-culture.

Streptomycin was used at 1000 μg/mL and rifampicin at 50 μg/mL, while the concentration of antibiotics used, for the preparation of the (selective) enumeration media, were the lowest in which the second strain was unable to grow, i.e., the one that was not resistant to this antibiotic. Additionally, the selection of strains aimed to include strains of different serotype and origin (outbreak and animals) and strains characterized as persistent in dairy processing environments (Fox et al., 2011) (Table 1). Both ScottA (streptomycin resistant strain and rifampicin resistant strain) and C5 belong to serotype 4b, while 6179 and PL25 belong to serotypes 1/2a and 1/2b, respectively.

Inoculum preparation

All strains were maintained at 4ºC on Tryptic Soy Agar (LAB011, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract (TSA-YE; pH: 7.3 ± 0.2) and the appropriate concentration of rifampicin (TSA-YE+R) or streptomycin (TSA-YE+S) and sub-cultured once a month. A single colony from a TSA-YE/S or TSA-YE/R stock culture of the target strain was transferred to 10 mL Tryptic Soy Broth (LAB004, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract (MC001, Lab M Limited, United Kingdom) (TSB-YE; pH: 7.3 ± 0.2) and the appropriate concentration of rifampicin (TSB-YE+R) or streptomycin (TSB-YE+S) and incubated for 24 h at 30°C. Subsequently, 100 μL of each culture were transferred to fresh TSB-YE/S or TSB-YE/R for 18 h incubation at 30ºC to obtain stationary-phase cells with a density of *ca.* 10^9 CFU/mL. Following activation, strains were harvested by centrifugation (2463 x g for 10 min at 4°C) (Megafuge 1.0R, Heraeus, Buckinghamshire, England), washed twice and re-suspended in 10 mL of ¼ strength Ringers' solution (non-adapted cells) (LAB M, Lancashire, UK) or in 10 mL dairy-based broth prepared by homogenizing one part of the selected dairy products (Ricotta and Camembert) and one part of sterile Maximum Recovery Diluent (MRD; LAB103, Lab M Limited, United Kingdom; pH: 7.0 ± 0.2) (adapted cells). Before the inoculation of the samples with the adapted strains, adaptation of cells performed in the dairy-based broth at 7ºC for 48 h. Strain adaptation performed in order to evaluate the occurred inter-strain interactions by reducing the adaptation period of bacterial cells, belonging to the different strains of the pathogen, to the new environment (lag time). The level of the inoculum was determined by plating 0.1 mL of the appropriate decimal dilution of each strain on TSA-YE/S or TSA-E/R and incubation at 37°C for 48 h.

Storage experiments

Commercial packages of dairy products Ricotta (Granarolo, Bologna, Italy) and Camembert (Alpenhain, Munich, Pfaffing, Germany) (Table 2), were purchased from local supermarket (Athens, Greece) close to their production date to ensure the lowest level of endogenous microbiota, depending on their production process. Ricotta and Camembert are both RTE dairy products which have caused listeriosis outbreaks in the past, however they are very different matrices produced *via* different technological procedures (Martinez-Rios and Dalgaard, 2018; Shamloo et al., 2019). The physicochemical characteristics of

the dairy products (pH > 5.0 and a_w > 0.94; Table 2), along with their shelf-life that is higher than 5 days renders the selected products non-compliant with the regulated criteria of Regulation (EC) 2073/2005 and suitable to support the growth of *L. monocytogenes.* Portions of 10 g of Ricotta were placed into sterile plastic containers (60 mL of volume), were inoculated, throughout the mass, with aliquots of diluted inoculum so as the final concentration of the pathogen to be *ca.* 2.0-3.0 log CFU/g and the containers were loosely closed in order to maintain aerobic conditions. Camembert cheeses were sliced into smaller pieces (6 x 2 cm), which weighted 10 g. Following portioning, all slices were surface inoculated (*ca.* 2.0 - 3.0 log CFU/cm^2) by placing the inoculum on both sides. Cell attachment and evaporation of excess liquid took place at 4°C for 15 min. Samples were placed in plastic bags with gas permeability *ca.* 25, 90, and 6 cm^3/m^2 per day/105 Pa for CO_2, O_2 and N_2, at 20°C and 50% relative humidity (Flexo-Pack S.A., Athens, Greece) and packaged under aerobic conditions. Finally, all dairy samples were stored at 7°C in high precision (± 0.5°C) incubation chambers (MIR-153, Sanyo Electric Co., Osaka, Japan), thus mimicking temperature abuse. The selection of packaging type per dairy product was in accordance with its commercial packaging on the shelf by the time of purchase. Three independent storage experiments were performed and duplicate samples were used in each trial (n=6).

Microbiological analysis

On various days during storage at 7°C, to determine the growth curves of the studied strains and the same strains in the co-cultures, samples of the dairy products were removed from their container under aseptic conditions. Following decimal dilutions in ¼ strength Ringer's solution, aliquots of 0.1 mL and/or 1 mL of diluted sample were spread on selective and non-selective culture media. Population of *L. monocytogenes* strains was enumerated on TSA-YE and TSA-YE/S or/and TSA-YE/R, at 37°C for 48 h. Total viable counts (TVC) were estimated on TSA-YE after incubation at 30°C for 72 h. Average numbers of colonies *per* plate were used to calculate the viable-cell concentrations, expressed as log CFU/g or cm^2.

pH and a_w measurements

The pH values of dairy samples were recorded at every sampling point by using a digital pH meter (pH 526, Metrohm Ltd, Switzerland), while water activity (a_w) was monitored by a digital a_w meter (Hydrolab rotronic, Switzerland) at the beginning, the middle, and the end of storage.

Simulated gastric fluid experiments

The impact of the occurred or not inter-strain interactions on the subsequent acid resistance of the pathogen was evaluated during growth of singly and co-cultured strains C5, ScottA and PL25, in Ricotta at 7°C, while the impact of storage in/on different dairy products at 7°C, was evaluated during growth of singly and co-cultured strains ScottA and PL25. For the first assessment, the selection of the strain combinations

was intended to evaluate whether the occurrence of inter-strain interactions (ScottA+C5) or not (ScottA+PL25), during storage under the same conditions of temperature, oxygen availability (incorporation into the mass of the product) and substrate components (Ricotta), may have any impact on the subsequent survival to SGF. The second assessment was aimed to evaluate the impact of growth of singly and co-cultured strains in/on different substrates, having different oxygen and nutrient availabilities (Ricotta and Camembert), on the survival during exposure to SGF.

As such, commercial packages of Ricotta and Camembert cheeses were purchased, inoculated, and stored similarly to the storage experiments. SGF was prepared according to (Molly et al., 1994; Naim et al., 2004) with the following formulation: 0.4 g/L glucose (Riedel de Haën, Switzerland), 3.0 g/L yeast extract (Lab M Limited, United Kingdom), 1.0 g/L Bacto Peptone (Lab M Limited, United Kingdom), 4.0 g/L porcine mucin (Sigma-Aldrich Co., USA), 0.5 g/L cysteine (Sigma-Aldrich Co., USA), 0.08 g/L NaCl (Merck KGaA, Germany), 0.4 g/L $NaHCO_3$ (PanReac AppliChem, Spain), 0.04 g/L K_2HPO_4 (Merck KGaA, Germany), 0.04 g/L KH_2PO_4 (Merck KGaA, Germany), 0.008 g/L $CaCl_2 \cdot 2H_2O$ (Merck KGaA, Germany), 0.008 g/L $MgSO_4 \cdot 7H_2O$ (Mallinckrodt Pharmaceuticals, Ireland), 1.0 g/L xylan (Sigma-Aldrich Co., USA), 3.0 g/L soluble starch (Merck KGaA, Germany), 2.0 g/L pectin (Sigma-Aldrich Co., USA), and 1 mL/L Tween 80 (Scharlab S.L., Spain) *per* liter of distilled water. The solution was autoclaved and cooled to approximately 37°C, followed by addition of 3.0 g/L pepsin (≥ 400 units/mg protein) (Sigma-Aldrich Co., USA) from porcine stomach mucosa and pH adjustment to pH value 2.0 (12N HCl).

On standard time points, the whole content of each package was removed, placed into a plastic stomacher with filter bag (Interscience, France) in which, 10-fold (according to weight) volumes of pre-warmed SGF at 37°C were added, and homogenized for 30 sec. The time points during storage at 7°C, of simulated gastric digestion exposure, were selected according to growth curves of each singly and co-cultured pathogen's strain (in the middle of exponential phase and at early stationary phase). *L. monocytogenes* population was assessed initially (0 min) and after 5, 10, 20, 40, 60, and 120 min of exposure to SGF by plating samples onto selective (TSA-YE/S or TSA-YE/R) and non-selective (TSA-YE) culture media. In cases where population levels were expected to be close or below the enumeration limit of 10 CFU/g or cm^2, aliquots (25 mL) of each homogenized dairy product were added in 225 mL of Half Frasre Broth in order to estimate the presence or absence of the pathogen, following a modified enrichment procedure of ISO 11290-1: 1996 amd 1:2004. The pH of the homogenate (dairy product + SGF) was also monitored in parallel to the population of *L. monocytogenes* during SGF challenge. Two independent experiments were performed and triplicate samples were used for each trial (*n=6*).

Statistical analysis and primary modelling

Statistical analysis was performed with *STATGRAPHICS® Centurion XVII* computer package (Statpoint Technologies Inc., USA). During analysis of variance (ANOVA), Tukeys' HSD multiple range tests was used to evaluate the differences in the growth kinetics between the single and co-cultures during storage in/on the different dairy products, while for all pairwise comparisons was used the Student's t-test. Differences were considered to be significant at p-values <0.05. The obtained bacterial growth data, per single or co-cultured strain, were fitted to the Baranyi-Roberts model with DMFit Excel Add-In software. Maximum specific growth rate (μ_{max}; $days^{-1}$) and lag time (λ; days) were determined.

Results and Discussion

Growth of adapted and non-adapted singly and co-cultured L. monocytogenes strains in/on Ricotta and Camembert

The major observation, due to matrix adaptation prior to inoculation, was the decreased impact of strains C5 and PL25 on growth of ScottA during co-culture in Ricotta (Fig. 2; Supplementary material). Adaptation of strains did not differentiate the growth rate of both singly and co-cultured strains during growth in/on both dairy products, while in some cases reduced the duration of the lag phase (Fig. 1; Table 3; Supplementary materials).

Comparing the behavior of the pathogen during storage in/on the different dairy products, the singly-cultured non-adapted *L. monocytogenes* strains 6179, ScottA and C5 had lower growth rate and the final population of strain PL25 was significantly higher during growth on Camembert (Fig. 1; Table 3). Interestingly, singly-cultured non-adapted strain 6179 reached significantly lower final population during growth on Camembert (Fig. 1; Table 3). According to Wilson et al. (2002) cheeses (Camembert) and dairy products (Ricotta) produced from whole-fat milk categorized as gelled emulsions. Ricotta is a "semi-solid" substrate in which the pathogen may grow both planktonic and as immerged colonies, under aerobic or/and hypoxic conditions depending on the site of the bacterial cells inside the mass of the product. Camembert considered "solid" substrate on the surface of which the bacterial cells may grow as colonies, under aerobic conditions due to packaging (Table 2). The individual behavior of the different *L. monocytogenes* strains may be due to impact of the different nutritional characteristics of the substrates, which are mutually dependent with the difference in the physicochemical characteristics (Table 2), the density/structure of the substrates, which affect the mode of pathogen's growth (planktonic *vs* colonial) (Gkerekou et al., 2022), and the availability of oxygen, depending on the packaging and/or the site of the contamination (inside the mass *vs* on the surface; see § and Table 2). Moreover, beyond nutrient composition, matrix plays an important role on nutrient release and their bioavailability (Fardet et al., 2019). The impact and the differences of these two particular substrates has been extensively discussed by Gkerekou et al. (2022). Contrary to Gkerekou et al. (2022), in the present study the substrates consist entirely of the dairy products, however, the differences

pointed out by the latter study, applied to the present study, as well. Both substrates considered to be rich in different nutrients (Table 2), yet sufficient to support the growth of the pathogen. Both dairy products, consists of caseins and whey proteins, fulfilling the pathogens' need for amino acids which produced by hydrolysis of caseins, by proteases produced by the pathogen (Shumi and Anwar, 2014) or by the additional proteolytic action of the coagulant, the plasmin, the starter culture and the mold *Penicillium candidum,* in case of Camembert, during cheese production and ripening. In Ricotta, under aerobic and hypoxic conditions lactose, the available glucose present in milk (50 mg/L) and/or the glucose moiety of the lactose molecule may serve as carbon source for the growth of the pathogen (Crespo Tapia et al., 2020; Dalet et al., 2003; Fox, 2009; Pine et al., 1989). The growth rate of most of the strains whose behavior was studied in the present study seems to follow the pattern confirmed by many researchers who argue that the pathogen has a higher growth rate when growing planktonic, in case of Ricotta, than when growing in colony form, during storage on Camembert (Z. Aspridou et al., 2014; Theys et al., 2008; Wilson et al., 2002). Specifically, in liquid substrates the environment is uniform and the bacterial cells are able to move towards the sites rich in nutrients and move away from sites with accumulated metabolites, while during growth as surface colony the pathogen have access to the nutrients only from the substrate under the colony and the concentration of metabolites around the colony result in environmental alterations, like the local pH drop (Panagiotis N. Skandamis and Jeanson, 2015). The latter phenomenon, along with the lower a_w (Table 2), may account for the lower growth rate of the strains on Camembert, as mentioned above. Additionally, the heterogeneity of the used dairy products, originate from the presence of fat, with Camembert having double the percentage of Ricotta, causes additional stress resulting in slower growth rates (T. . Brocklehurst et al., 1997; Wilson et al., 2002). It is important not to overlook the fact that some behavioral variations of the strains may be maneuver by the biochemical history and adaptive changes undergoing in/on the isolation niche (Cooper et al., 1968).

The impact on growth of one strain by the presence of another, which is also referred as inter-strain interactions (Gkerekou et al., 2022, 2021; Zilelidou et al., 2016b, 2015), was evaluated by comparing the estimated growth kinetics (lag time and growth rate) and the observed final population level between singly- and co-cultured strains (Table 3). The co-culture of non-adapted *L. monocytogenes* strains in Ricotta did not affect the duration of the lag phase, while the growth rate of 6179 and ScottA decreased by the presence of C5 and the growth rate of C5 decreased by the presence of ScottA and PL25 (Fig. 1; Table 3). In Ricotta, strain 6179 reached significantly lower final population during co-culture with strain C5, by 1.4 log units and strain ScottA affected, mainly, by the presence of C5 and PL25 reaching decreased final cell density by 2.7 and 1 log units, respectively (Fig. 1; Table 2). On Camembert, the co-culture of non-adapted strains of the pathogen did not have any impact neither on the duration of the lag phase nor on the growth rate. The level of the final population by the end of storage was significantly lower during co-culture of ScottA with strains

C5 and 6179, by 2.3 and 0.6 log units and during co-culture of strain PL25 with strain C5, by 0.6 log units, respectively (Fig. 1; Table 2).

After a number of studies (Gkerekou et al., 2022, 2021; Zilelidou et al., 2016b, 2015) it seems that after a critical population density close to 6.0 log CFU/mL, some strains of the pathogen, regardless of growth conditions, seem to arise as strong competitors (*L. monocytogenes* strains C5 and PL25; Table 1). According to Zilelidou et al. (2016, 2015) and Gkerekou et al. (2021, 2022) *L. monocytogenes* strains C5 and PL25 are not affected by the presence of the other strains during co-culture in TSB-YE, in/on TSA-YE, in dairy-based broths, in/on dairy-based structured substrates and on ham slices. However, the intensity of growth suppression of weak strains by the presence of strong competitors, appears to be influenced by the bacterial cell mode of growth and/or the substrate characteristics. Interestingly, in the present study, the impact of strain C5 on growth of strain ScottA was similar regardless of the substrate. The two dairy products, used in the present study, differ in many characteristics, so the different extent of interactions between the same pairs of strains or the interactions between different pairs of strains manifested during co-culture cannot directly attributed to specific substrate characteristics or storage conditions.

Survival of single and co-cultured L. monocytogenes strains during exposure to SGF

Regardless of the growth phase in which the different single and co-cultured strains were exposed to the SGF (pH 2.0, 37°C), the reduction of pathogens' population on Camembert cheese pieces was faster than the reduction in Ricotta samples, the first minutes of the challenge (Fig. 2). Specifically, during the first 5 min of exposure to SGF of mid-exponential cells, on Camembert cheese the population reduction was more than 3.4 log units, while the reduction in Ricotta samples was less than 1.3 log units (Fig. 2A, 2B, 2E, 2F and 2I). The latter observation applies, also, to the reduction of early-stationary phase cells. On Camembert, the reduction the first 10 min of the challenged was ranged from 2.7 to 3.5 log units, compared to the observed reduction in Ricotta samples, which reached up to 2.8 log units, the first 20 min of the challenge (Fig. 2C, 2D, 2G, 2H and 2J). With respect to compositional characteristics of foods, high-fat products frequently implicated in outbreaks of listeriosis (Linnan et al., 1988; Lyytikäinen et al., 2000) and due to the presence of fat has been long believed that pathogenic cells become entrapped into hydrophobic lipid moieties evading the lethal effect of gastric phase (Waterman and Small, 1998). However, according to the results of the present study, the survival of the pathogen appears to be closely related to the site of contamination. Camembert pieces were surface inoculated resulting in the bacterial cells coming in direct contact with the low pH of SGF, in contrast with the bacterial cells found in the mass of Ricotta's samples. Moreover, according to Figure 2 early-stationary cells, regardless of substrate (Camembert or Ricotta), managed to maintain higher population density than middle-exponential cells, by the end of challenge (120 min). The latter observation could be associated probably with their high levels prior to SGF challenge, however, the enhanced survival could be connected, also, with the growth phase of cells since according to Samelis et al.

(2003) they are much more resistant at stationary phase compared to exponential. Bacterial cells possess a generalized stress resistance (GSR) that is expressed upon entry into stationary phase and seems to develop a pH-independent resistance to low pH (Davis et al., 1994; Samelis et al., 2003; Sewell et al., 2015). O'Driscoll et al. (1996) observed that *L. monocytogenes* cells grown overnight were naturally tolerant to pH 3.5 without prior acid induction. Interestingly, upon inoculation of fresh culture medium acid tolerance was rapidly lost, until mid-exponential phase where the pathogen presented its maximum sensitivity, however, as the culture proceeded into the stationary-phase the tolerance returned. Additionally, Phan-thanh and Alige (2000) proved the natural acid tolerance displayed by *L. monocytogenes* at the onset of the stationary phase.

In most of the different combinations of strains, substatre and growth-phase prior to SGF exposure, the *L. monocytogenes* strains grew in co-culture, although the survival in SGF of early stationary cells increased compared to the observed survival of mid-exponential cells, their inactivation kinetics did not significantly differ from the kinetics seen with their respective single-cultures (Fig. 2). Singly- and co-cultured, with PL25 in Ricotta, early-stationary cells of ScottA seems to display similar acid resistance, however co-cultured ScottA reached lower population by the end of the exposure to SGF due to the lower initial population prior to challenge. The viability and the competitive fitness of different *L. monocytogenes* strains contaminating the same substrate are also crucial for food ingestion and the evolution of a possible infection. During the evaluation of the effect of co-culture on the survival of *L. monocytogenes* strains after exposure in SGF, it was illustrated that the effect of co-culture, partly, lies in the fact that due to co-culture at different growth phases the population of each strain is different. So, the occurred inter-strain interactions determine the population of each strain upon entry in the gastric fluid. As a result, despite the similar inactivation rates, the populations of two competing strains in SGF could be different at each time point due to differences in their initial cell density (Fig. 2H). Zilelidou et al. (2016a) also observed increased inactivation of strain 6179, after co-culture with strains C5, due to the lower initial population prior to the exposure to SGF. The inoculum size can affect bacterial inactivation kinetics with lower population prior to challenge resulting in facter inactivation (Barmpalia-Davis et al., 2008; Johnston et al., 2000), as in the case of co-cultured ScottA with PL25 (Fig. 2H). Interestingly, co-culture could also have and direct effect on survival after exposuse to SGF. Co-cultured with C5, ScottA seems to be influenced by the presence of the second strains and presented enhanced resistance to low pH, reaching similar population with the singly-cultured even though the co-cultured ScottA was exposed to the SGF at a lower initial population (Fig. 2H). In adiition, co-cultured, with PL25 in Ricotta, mid-exponetial cells of ScottA and co-cultured, with ScottA both in Ricotta and Camembert, early-stationary cells of PL25 even though exposused to SGF at the same initial population that the respective singly-cultured strains, they presented a trend to be more sensitive to the low pH during the exposure to the SGF. In accordance with the results of the present study, ScottA displayed increased acid resistance compared to C5 and PL25 after 6 or 8 days of co-incubation with the latter strains

in TSB-Y. Despite having lower initial populations than C5 and PL25, ScottA showed an overall higher survival rate in SGF as indicated by the smoother slope ofits inactivation curve (Zilelidou et al., 2016a).

Conclusions

The results of the present study, combined with the previous observations (Gkerekou et al., 2022, 2021) demonstrate that the occurrence of multiple *L. monocytogenes* strains in a single food sample can complicate downstream investigations and effective source attribution not only due to genetic and phenotypic diversity between strains or the proved inter-strain competition but also due to substrate characteristics which seems to influence the occurred interactions. The succession of steps included in this study did not entirely simulate the passage ofcontaminated food through the GIT *in vivo,* however the low pH during the gastric phase is the first and most important hurdle/stresses that confront the foodborne pathogens. L. monocytogenes faces various stresses before it reaches enterocytes, and such stresses affect the behavior ofthe pathogen.

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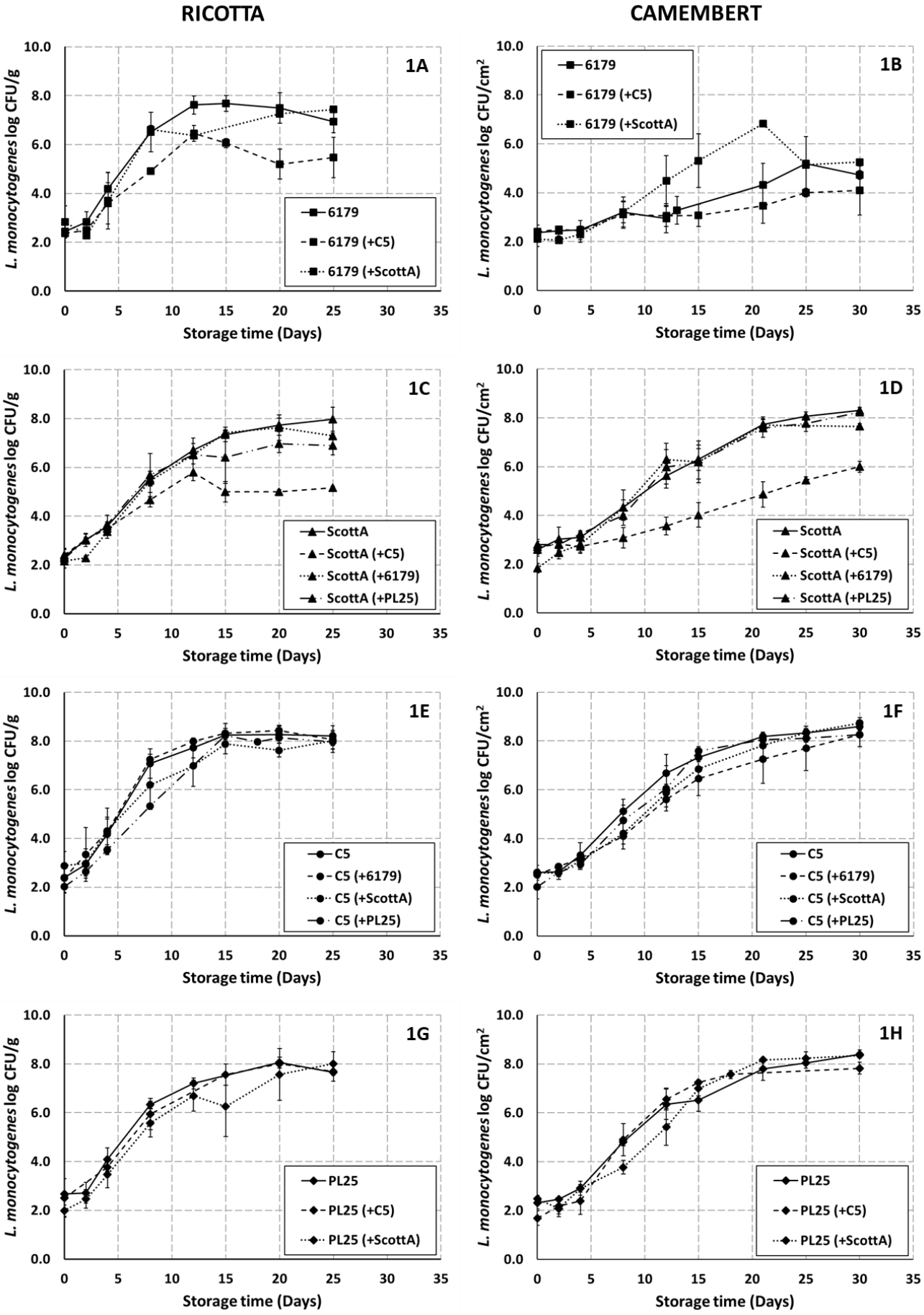

Figure 1. Growth curves of singly- and co-cultured *L. monocytogenes* strains 6179 (1A and 1B), ScottA (1C and 1D), C5 (1E and 1F) and PL25 (1G and 1H) in/on Ricotta and on Camembert, at 7°C.

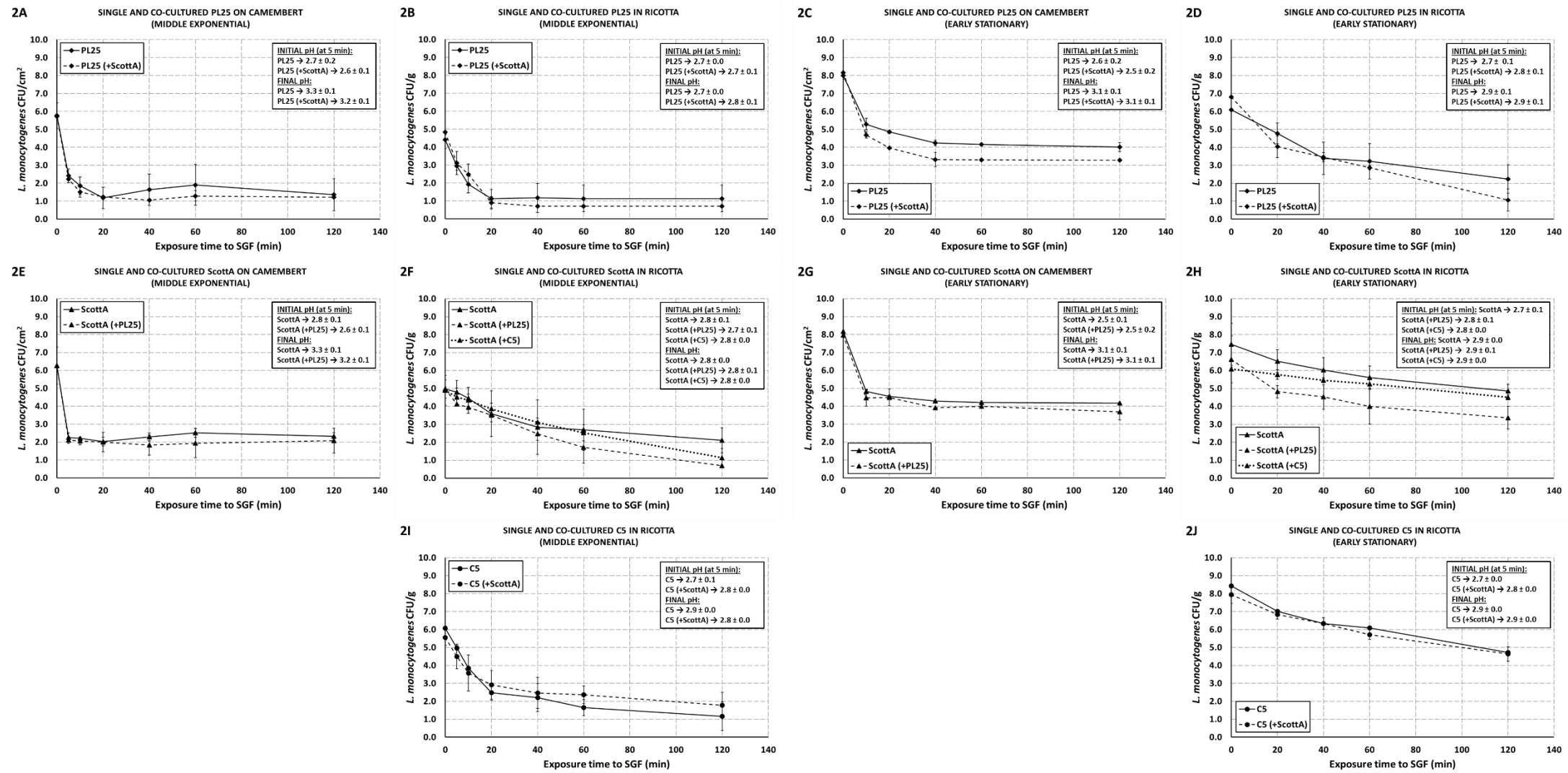

Figure 2. Survival of *L. monocytogenes* strains PL25, ScottA and C5 in SGF (pH 2.0, 37°C), after co-culture both until mid-exponetial (2A, 2B, 2E, 2F and 2I) and early stationary (2C, 2D, 2G, 2H and 2J) phases at 7°C, in/on Ricotta (2B, 2D, 2F, 2H 2I and 2J) and on Camembert (2A, 2C, 2E and 2G).

Table 1. *Listeria monocytogenes* strains used in the study.

Strain	Serotype	MLST	Source	Year of isolation	Reference	Antibiotic resistance (μg/mL)*
C5	4b	ST2	Dairy farm environment isolation	2007	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Streptomycin (2000)
6179	1/2a	ST121	Cheese	1999	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Rifampicin (>800)
ScottA	4b	ST290	Human isolate	1983	Research Institute ATO-DLO, Wageningen, Netherlands	Streptomycin (4000) Rifampicin (>800)
PL25	1/2b (3b, 7)**	ST59	Animal origin	2009	Agricultural University of Athens, Department of Food Science and Human Nutrition, Laboratory of Food Quality Control and Hygiene, Athens, Greece	Rifampicin (800)

*Approximate MIC was considered as the minimum tested concentration (μg/ml) of antibiotic at which no bacterial growth was observed after 24 hours at 30°C. Bacterial growth was confirmed through measurements of optical density (OD_{600}). The streptomycin concentrations were 0, 125, 250, 500, 1000, 2000, 4000 μg/ml. Rifampicin was evaluated at 0, 200, 400, 800 μg/ml.

**The serovar-specific group was characterized by multiplex PCR according to Doumith et al., (2004) and the serovars in parenthesis were omitted due to Multilocus Sequence Typing (MLST) classification.

Table 2. Description, physiochemical characteristics and nutritional declaration of studied dairy products, according to the literature, commercial label and experimental measurements.

Product	Description*	Nutritional value per 100 g**	pH***	a_w***	Moisture**	Self-life**	Packaging
Ricotta	• Not classified as cheese • Obtained from whey (from bovine milk) • Cream coagulated by combination of temperature and acidity • Fresh soft dairy product of low salt content	Energy: 623 kJ / 150 kcal Fat : 12 g - of which saturates: 8.4 g Carbohydrates: 3 g -of which sugars: 3 g Protein: 7.5 g Salt: 0.3 g	5.75 - 6.55	0.99	80%	up to 1 month	Aerobic conditions
Camembert	• Surface mould-ripened soft cheese (bloomy rind cheese; Batty et al., 2019)	Energy: 1231 kJ / 297 kcal Fat : 25 g - of which saturates: 17 g Carbohydrates: <0.5 g -of which sugars: <0.5 g Protein: 18 g Salt: 2 g	6.01 - 6.67	0.97	55%	up to 1 month - depending on the packaging	Aerobic conditions

*According to literature

**According to commercial label

***According to experimental measurements during the present study

Table 3: Estimated growth kinetics (lag time and growth rate) and observed final population of non-adapted *L. monocytogenes* strains 6179, ScottA, C5 and PL25 in single and co-culture in/on different dairy products, at 7°C.

	LAG TIME (Days)		GROWTH RATE (Days^{-1})		FINAL POPULATION (log CFU/cm^2 or g)	
	Ricotta	**Camembert**	**Ricotta**	**Camembert**	**Ricotta**	**Camembert**
Single 6179	2.1 ± 1.1	-	0.66 ± 0.16	-	6.9 ± 0.5^{b}	4.7 ± 0.2^{a}
6179 (+C5)	1.5 ± 1.6	-	0.44 ± 0.13*	-	5.5 ± 0.8^{a**}	4.1 ± 1.0^{a}
6179 (+ScottA)	2.7 ± 1.8	-	0.53 ± 0.00	-	7.4 ± 0.1^{b}	5.3 ± 0.1^{a*}
Single ScottA	0.6 ± 0.7^{a}	2.7 ± 3.8^{a}	0.43 ± 0.09^{b}	0.24 ± 0.09^{a}	8.0 ± 0.5^{a}	8.3 ± 0.1^{a}
ScottA (+C5)	0.0 ± 0.0^{a}	4.8 ± 5.3^{a}	0.28 ± 0.05^{a*}	0.14 ± 0.03^{a}	5.2 ± 0.1^{a**}	6.0 ± 0.3^{b**}
ScottA (+6179)	1.1 ± 1.5^{a}	0.0 ± 0.0^{a}	0.42 ± 0.01^{a}	0.32 ± 0.10^{a}	7.3 ± 0.1^{a*}	7.7 ± 0.1^{b**}
ScottA (+PL25)	0.8 ± 1.1^{a}	1.9 ± 0.8^{a}	0.47 ± 0.20^{a}	0.28 ± 0.05^{a}	6.9 ± 0.4^{a**}	8.2 ± 0.1^{b}
Single C5	1.4 ± 1.1^{a}	2.0 ± 0.1^{a}	0.68 ± 0.07^{b}	0.40 ± 0.06^{a}	8.2 ± 0.4^{a}	8.6 ± 0.2^{a}
C5 (+6179)	2.1 ± 1.8^{a}	1.1 ± 1.6^{a}	0.81 ± 0.06^{b}	0.28 ± 0.05^{a}	8.1 ± 0.4^{a}	8.3 ± 0.5^{a}
C5 (+ScottA)	0.0 ± 0.0^{a}	2.1 ± 0.3^{b}	0.40 ± 0.10^{a**}	0.33 ± 0.05^{a}	8.0 ± 0.3^{a}	8.7 ± 0.2^{b}
C5 (+PL25)	0.2 ± 0.3^{a}	2.2 ± 1.3^{a}	0.42 ± 0.05^{a**}	0.39 ± 0.16^{a}	8.0 ± 0.4^{a}	8.3 ± 0.5^{a}
Single PL25	1.0 ± 0.8^{a}	2.2 ± 0.5^{a}	0.53 ± 0.10^{a}	0.39 ± 0.10^{a}	7.6 ± 0.4^{a}	8.4 ± 0.2^{b}
PL25 (+C5)	1.6 ± 1.6^{a}	1.3 ± 0.6^{a}	0.54 ± 0.10^{a}	0.39 ± 0.07^{a}	7.7 ± 0.1^{a}	7.8 ± 0.2^{a**}
PL25 (+ScottA)	0.0 ± 0.0^{a}	4.1 ± 1.5^{b}	0.35 ± 0.20^{a}	0.42 ± 0.07^{a}	8.0 ± 0.5^{a}	8.3 ± 0.0^{a}

Values with different lowercase letters, that correspond to the same singly or co-cultured strain in/on the two different dairy products (in the same row) are significantly different (P<0.05).

Star indicates statistical difference between the growth kinetics of the co-cultured strains in comparison to the singly cultured under the same conditions of structure and oxygen availability (*: P<0.05).

Dashes indicate the treatment combinations that were non applicable.

Supplementary material

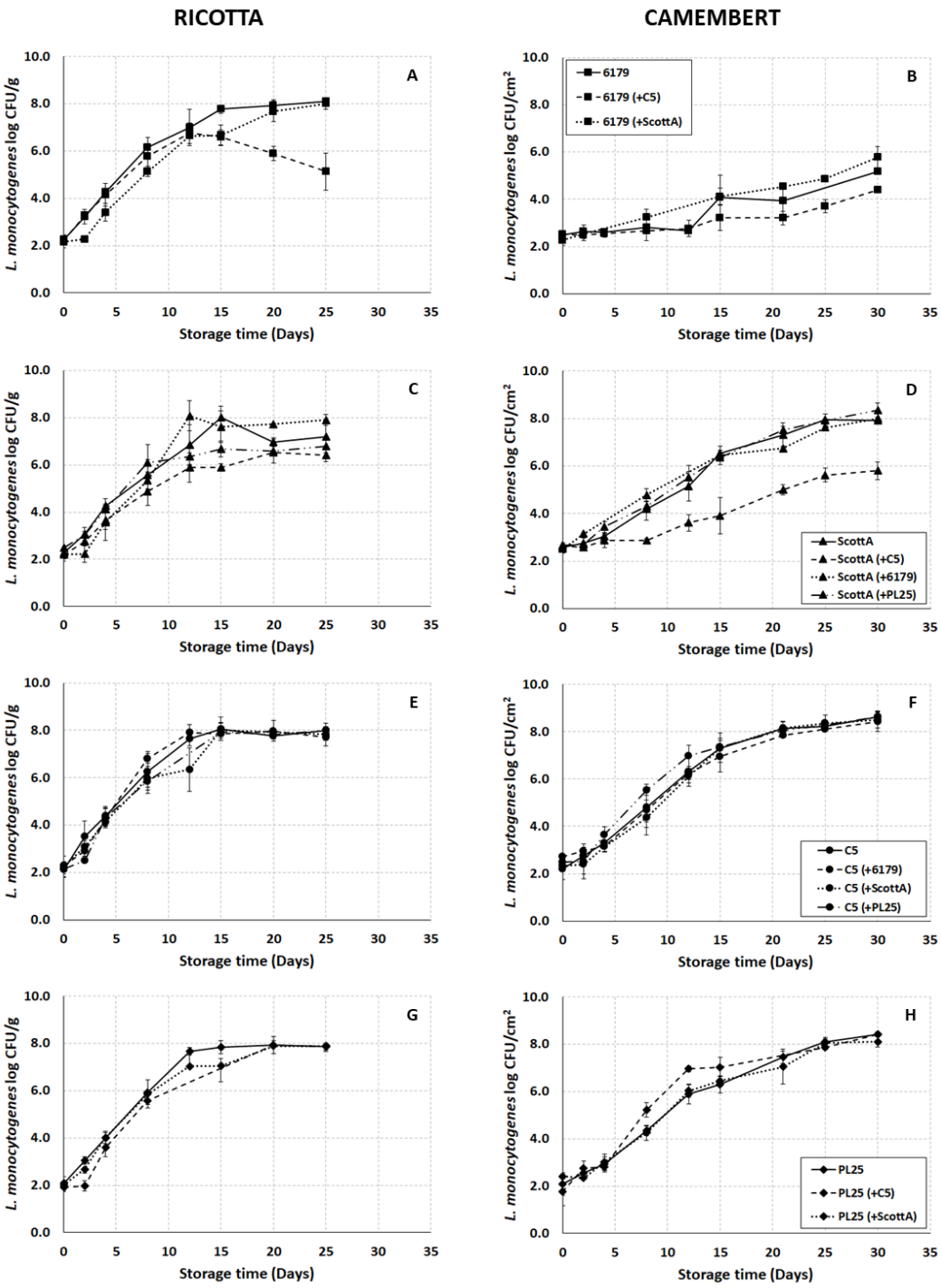

Figure: Growth curves of singly and co-cultured adapted *L. monocytogenes* strains 6179 (A-B), ScottA (C-D), C5 (E-F) and PL25 (G-H) in/on Ricotta and Camembert products during storage at 7°C.

Table: Estimated growth kinetics (lag time and growth rate) and observed final population of adapted *L. monocytogenes* strains 6179, ScottA, C5 and PL25 in single and co-culture in/on different dairy products, at 7°C.

	LAG TIME (Days)		GROWTH RATE (Days^{-1})		FINAL POPULATION (log CFU/cm^2 or g)	
	Ricotta	**Camembert**	**Ricotta**	**Camembert**	**Ricotta**	**Camembert**
Single 6179	0.2 ± 0.3	-	0.48 ± 0.14	-	8.1 ± 0.1^{b}	5.2 ± 0.1^{a}
6179 (+C5)	0.0 ± 0.0	-	0.47 ± 0.06	-	5.1 ± 0.8^{a**}	4.4 ± 0.1^{a**}
6179 (+ScottA)	1.6 ± 0.7	-	0.50 ± 0.01	-	8.0 ± 0.2^{b}	5.8 ± 0.4^{a}
Single ScottA	0.0 ± 0.0^{a}	3.2 ± 3.8^{a}	0.43 ± 0.12^{b}	0.22 ± 0.07^{a}	7.2 ± 0.5^{a}	7.9 ± 0.1^{b}
ScottA (+C5)	0.0 ± 0.0^{a}	4.0 ± 2.1^{a}	0.36 ± 0.09^{a}	0.13 ± 0.02^{a}	6.4 ± 0.3^{a}	5.8 ± 0.4^{a**}
ScottA (+6179)	1.7 ± 1.8^{a*}	1.1 ± 1.5^{a}	0.54 ± 0.06^{a}	0.38 ± 0.12^{a}	7.9 ± 0.2^{a}	8.0 ± 0.0^{a}
ScottA (+PL25)	0.9 ± 1.2^{a}	1.4 ± 0.0^{a}	0.62 ± 0.30^{a}	0.27 ± 0.01^{a}	6.8 ± 0.3^{a}	8.3 ± 0.3^{b**}
Single C5	0.2 ± 0.4^{a}	1.2 ± 1.4^{a}	0.57 ± 0.13^{b}	0.37 ± 0.03^{a}	8.0 ± 0.1^{a}	8.6 ± 0.2^{b}
C5 (+6179)	0.4 ± 0.7^{a}	3.5 ± 3.1^{a}	0.53 ± 0.17^{a}	0.40 ± 0.10^{a}	7.7 ± 0.1^{a*}	8.4 ± 0.4^{b}
C5 (+ScottA)	0.0 ± 0.0^{a}	3.8 ± 3.7^{a}	0.43 ± 0.11^{a}	0.47 ± 0.20^{a}	7.8 ± 0.5^{a}	8.5 ± 0.3^{a}
C5 (+PL25)	0.3 ± 0.5^{a}	0.9 ± 0.9^{a}	0.47 ± 0.12^{a}	0.41 ± 0.06^{a}	8.0 ± 0.2^{a}	8.6 ± 0.2^{b}
Single PL25	0.6 ± 0.4^{a}	0.0 ± 0.0^{a}	0.55 ± 0.80^{b}	0.32 ± 0.03^{a}	7.9 ± 0.2^{a}	8.4 ± 0.1^{b}
PL25 (+C5)	0.8 ± 1.1^{a}	0.0 ± 0.0^{a}	0.52 ± 0.07^{a}	0.31 ± 0.07^{a}	7.9 ± 0.2^{a}	8.4 ± 0.1^{b}
PL25 (+ScottA)	0.0 ± 0.0^{a}	1.6 ± 0.4^{b**}	0.45 ± 0.06^{a}	0.29 ± 0.04^{a}	7.9 ± 0.2^{a}	8.1 ± 0.2^{a*}

CHAPTER 5

Studying the metabolic factors that may impact the growth of co-cultured Listeria monocytogenes strains at low temperature

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Studying the metabolic factors that may impact the growth of co-cultured *Listeria monocytogenes* strains at low temperature

Maria A. Gkerekou[a], Eleftheria H. Kaparakou[b], Petros A. Tarantilis[b] and Panagiotis N. Skandamis[a]*

Laboratory of Food Quality Control and Hygiene, Department of Food Science and Human Nutrition, Agricultural University of Athens, Greece[a]

Laboratory of General Chemistry, Department of Food Science and Human Nutrition, Agricultural University of Athens, Greece[b]

Abstract

The simultaneous presence of more than one strains of *Listeria monocytogenes* in the same food product may affect the growth capacity of each strain. The present study evaluated the metabolites composition that may potentially influence the growth of individual *L. monocytogenes* strains in a dual strain composite. Based on previous studies, *L. monocytogenes* strains, C5 (4b) and 6179 (1/2a) were selected due to the remarkable interaction, which was observed during their co-culture. The selected strains were inoculated (2.0 - 3.0 log CFU/mL) in Tryptic Soy Broth with 0.6% Yeast Extract (TSB-YE) in single and two-strain cultures (1:1 strain ratio). Bacterial growth was assessed during storage at 7°C, under aerobic conditions (AC). Their resistance to different antibiotics enabled the selective enumeration of each strain in the co-culture. After reaching stationary phase, single and dual cultures were centrifuged and filtered. The cell-free spent medium (CFSM) was either characterized by Fourier transform infrared (FTIR-ATR) spectrometry or re-inoculated, after the addition of concentrated TSB-YE (for nutrient replenishment), with single and two-strain cultures for the evaluation of growth under the influence of metabolites produced from the same singly and co-cultured strains in the different combinations of strains and CFSM origin (7°C/AC) (n=2x3). By the end of storage, singly-cultured C5 and 6179 had reached 9.1 log CFU/mL, while in dual culture, 6179 was affected by the presence of C5 attaining only 6.4 ± 0.8 log CFU/mL. FTIR-ATR spectra of CFSM produced by singly-cultured 6179 and the co-culture were almost identical. Characteristic peaks in FTIR-ATR spectrum of CFSM of singly-cultured C5 at 1741, 1645 and 1223 cm^{-1} represent functional groups which were not present in the CFSM of the co-culture. These molecules may be located intracellularly or mounted on bacterial cell surface and removed from the supernatant during cell filtration of the co-culture. Both singly- and co-cultured 6179 managed to grow similarly regardless of CFSM origin. Contrarily, both singly- and co-cultured C5 managed to outgrow 6179 in CFSM which contained high concentration of C5 metabolites, while in CFSM produced by singly-cultured 6179, C5 did not grow, suggesting that the produced metabolites of strain 6179 appears to be harmful to strain C5. However, during co-culture, C5 may produce molecules that counteract the inhibitory effect of 6179. The findings shed more light on the mechanism

behind the inter-strain interactions of *L. monocytogenes* indicating that both contact of cells and extracellular metabolites may influence the behavior of the different co-existing strains.

Keywords: Listeria monocytogenes; Inter-strain interactions; FTIR-ATR; Cell-free spent medium

Introduction

Listeria monocytogenes is a foodborne pathogen that "*knows how to survive*" (Gandhi and Chikindas, 2007). The ubiquity of this pathogen and its ability to survive and grow in a wide range of harsh environmental conditions renders it a major concern for ready-to-eat (RTE) products. Previous studies have described the simultaneous presence and dissemination of multiple *L. monocytogenes* strains, which may have been introduced *via* raw materials at various time-points in the processing environment (Chambel et al., 2007; Martín et al., 2014; Martínez-Suárez et al., 2016; Ortiz et al., 2010; Thévenot et al., 2006; Zoellner et al., 2018). The pathogen may persist and spread, possibly ending to multiple strains co-existing in/on the same food products (Felício et al., 2007; Gendel and Ulaszek, 2000; Kabuki et al., 2004; Rychli et al., 2014a). Considering possible temperature abuse along the supply chain and the fact that transportation, retail display or household refrigeration are outside the manufacturer's direct control, pathogen may markedly grow even at illness causing levels. Recent studies have shown that if the pathogen grows, strain-to-strain interactions may also occur between cells in close proximity, in the case of simultaneously presence of more than one strains. Specifically, according to Zilelidou et al. (2015), Zilelidou, Manthou et al. (2016) and Gkerekou et al. (2021, 2022) the interactions appear to take the form of competition, since these authors observed that some of the co-cultured strains grew similarly as in single-culture in or on the surface of laboratory substrates, food-based substrates and/or food products and characterized them as "strong" competitors. Contrarily, some strains during co-culture were affected by the presence of the second strain and after the critical population density of 6.0 log CFU/mL, the "weak" strains reached lower final population levels compared to the population that reached during single-culture. Exploring the mechanism of how different strains interact from a food safety perspective, is important, because the relative levels of each strain during storage may also determine the population of each strain at the end of an enrichment step and subsequently their probability of isolation on ALOA plates (i.e., the critical detection step), according to ISO 11280 (Zilelidou, Karmiri et al., 2016). Thus, potential masking of certain strains throughout the detection process may hinder the tracing of the actual causative agent (strain) of an outbreak, during epidemiological investigations, which may have slower growth during storage or enrichment procedure, yet more virulent.

Microbial competition may be expressed in different forms and mechanisms. Cells compete for the two main resources of microbial survival, i.e., nutrients and space, (i) indirectly through exploitative competition, which occurs through resource consumption (passive competition) and (ii) directly through

interference competition, where individual cells damage one another (active, chemical warfare) (Cornforth and Foster, 2013; Ghoul and Mitri, 2016; Powell et al., 2004). Interestingly, according to Zilelidou et al. (2015) contact of cells may be the key factor for the manifestation of the inter-strain interactions, as the inhibition of the "weak" strain increased due to contact of the two strains, while the prevention of contact of the strains by membrane resulted in limited-to-no inhibition. The above mentioned phenomenon indirectly confirmed by Gkerekou et al. (2021, 2022), who described the enhanced inhibition between co-cultured *L. monocytogenes* strains in liquid (broth) substrates where bacterial cells were able to move, compared with the co-culture inside or on solid substrate, where bacterial cells were constrained to grow as colonies due to limited space available by the physical structure. Contact-dependent inhibition (CDI) may occur mainly in Gram-negative bacteria, such as *Escherichia coli*, and it has been demonstrated in shaking liquid culture (Aoki et al., 2005). The same study suggested that growth inhibition among a "weak" and a "strong" strain requires that cells come in direct contact, and not only *via* their metabolome, e.g., when inoculating the weak strain in the spent medium of the strong one, or separating competing strains by an impermeable membrane. Aoki et al. (2005) supported the hypothesis that the secreted molecule, responsible for the inhibition phenotype, is unstable and is only effective when delivered to target cells in close proximity. Moreover, recent studies have found that CDI is not restricted to Gram-negative bacteria but may also occur in Gram-positive bacteria, including *Listeria* (Hayes et al., 2010). However, the observed interactions and the inhibition of some strains, regardless cell-contact (Zilelidou et al., 2015) or constriction due to substrate structure (Gkerekou et al., 2021, 2022; Zilelidou, Manthou et al., 2016) indicate the presence of an additional way of interaction between the bacterial cells.

Naumann et al. (1991) and Naumann (2006) introduced Fourier Transform Infrared spectroscopy (FTIR) to microbiology, producing spectral fingerprints of intact bacteria and its constituents like DNA/RNA, membrane and cell-wall components including proteins, fatty acids, carbohydrates, nucleic acids, and lipopolysaccharides. FTIR has been successfully applied for detection, discrimination identification and classification of bacteria at serogroup and genus level without any prior preselection by other taxonomic criteria. The advantage of FTIR spectroscopy is that it can reflect the entirety of cell constituents (in the form of chemical groups or types of molecular bonds) and can reveal structures not readily detectable by other methods (LCMS, GC-MS, NMR, MALDI-TOF/MS; Oyedeji et al., 2021), like the secondary protein structure, giving the phenotypic and genetic fingerprint of a sample under study (Al-Mariri et al., 2019; Beekes et al., 2007; Davis and Mauer, 2010; Helm et al., 1991; Oust et al., 2006; Rebuffo et al., 2006; Romanolo et al., 2015). As such, FTIR has proven useful also in metabolomics for the characterization of the organic substances resulting from the metabolism of the studied organisms growing in a culture medium (Junot and Fenaille, 2019; Oyedeji et al., 2021).

Considering all the above in the present study investigated if the occurred interactions during co-culture of different *L. monocytogenes* strains in the same substrate, apart from the contact-dependent inhibition, are due to the restriction of the nutrients or the production of metabolic factors.

Materials and methods

L. monocytogenes strains

L. monocytogenes strains C5 and 6179 were selected based on previous studies, as ones that had shown evident strain-to-strain interaction (Gkerekou et al., 2021, 2022; Zilelidou et al., 2015; Zilelidou, Manthou et al., 2016). Furthermore, their innate resistance to streptomycin (Streptomycin Sulfate Biochemica, AppliChem) and rifampicin (Rifampicin, AppliChem) (Table 1), seemed not to affect their growth capacity and was useful for the selective enumeration of each strain during co-culture. The strains were obtained from the microorganism collection of the Laboratory of Food Quality Control and Hygiene of Agricultural University of Athens and their selection to the antibiotics was made according to the method described by de W. Blackburn and Davies (1994) (Table 1). The strains were maintained at -20°C in Tryptone Soy Broth (LAB004, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract (MC001, Lab M Limited, United Kingdom) (TSB-YE, pH: 7.1 ± 0.2), 20% glycerol and the appropriate concentration of rifampicin or streptomycin, depending on the strain. Streptomycin was used at 1000 μg/mL and rifampicin at 50 μg/mL. The concentration of antibiotics used, during their maintenance and for the preparation of the (selective) enumeration media, was the lowest in which the second strain (the one that was not resistant to the particular antibiotic) was unable to grow (Table 1).

Inoculum preparation

During the experiments, both strains were maintained on Tryptone Soy Agar (LAB011, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract, containing rifampicin (50 μg/mL; TSA-YE/R) or streptomycin (1000 μg/mL; TSA-YE/S) at 4°C and sub-cultured once a month. A single colony from a TSA-YE/S or TSA-YE/R stock culture of the target strain was transferred to 10 mL TSB-YE/S or TSB-YE/R and incubated for 24 h at 30°C and subsequently, 100 μL of each culture was transferred to fresh TSB-YE/S or TSB-YE/R for 18 h incubation at 30°C to obtain stationary-phase cells with a density of *ca.* 10^9 CFU/mL. Following activation stage, strains were harvested by centrifugation (2463 x g for 10 min at 4°C) (Megafuge 1.0R, Heraeus, Buckinghamshire, England), washed twice and finally re-suspended in 10 mL of ¼ strength Ringer's solution (LAB M, Lancashire, UK). The level of the inoculum was determined by plating 0.1 mL from the appropriate decimal dilution of each strain on TSA-YE/S or TSA-YE/R and incubation at 37°C for 48 h.

Contact-dependent inhibition

During the first part of the study, the contact-dependent inhibition experiment of Zilelidou et al. (2015) was reproduced, with modifications regarding the used strains (C5 and 6179; Table 1), the temperature (7°C) and the oxygen availability (aerobic conditions). In detail, bacterial cultures were prepared by inoculation of TSB-YE with singly-cultured C5 and 6179, at approximately 2.0 - 3.0 log CFU/mL. Polyethylene tetraphthalate (PET) track-etched membrane inserts of 0.4 μm pore size (Thermo Fischer Scientific, Denmark) were placed in 6-well culture plates and 2 mL of 6179 culture were added to the upper chamber of the well and 2 mL of C5 culture were added to the lower chamber (ensuring no contact between strains). Growth of strains in single cultures was also recorded in separate wells in addition to growth of strains in direct contact (1:1 strain ratio). The effect of culture the cells in the upper chamber in comparison to the lower chamber was also tested. The different cultures were incubated at 7°C for 20 days. Sampling was performed at day 0, 3, 5, 7, 10 and 20. Each experiment was performed in two independent duplicate trials (n=4).

Effect of substrate's nutrients composition on growth and inter-strain interactions of L. monocytogenes

Additionally, during the first part of the present study, apart from TSB-YE (described in §2.1), which was used at previous studies (Gkerekou et al., 2021; Zilelidou et al., 2015; Zilelidou, Manthou et al., 2016), TSB without Dextrose (LAB205, Lab M Limited, United Kingdom) and TSB-YE without Dextrose (TSB w/o Dextrose supplemented with 0.6% Yeast Extract; pH: 7.3 ± 0.2) were also inoculated with singly and co-cultured C5 and 6179, reproducing the conditions previously studied by Gkerekou et al. (2021) of culture in liquid substrate, under aerobic conditions. Specifically, 50 mL falcon tubes containing 40 mL of sterile TSB without Dextrose, TSB-YE without Dextrose or TSB-YE, were inoculated either with single or both strains (strain ratio of 1:1) listed in Table 1, at approximately 2.0 - 3.0 log CFU/mL and stored at 7°C for 20 days, in high precision (± 0.5°C) incubation chambers (MIR 153, Sanyo Electric Co., Osaka, Japan), under aerobic conditions produced by constant shaking on an orbital shaker, at 240 rpm (Shaker KS 130 basic, IKA-Werke GmbH & Co. KG, Germany) (Noriega et al., 2008a). Each experiment was performed in two triplicate trials (n=6).

Cell-free spent medium preparation, inoculation and growth conditions

In parallel with the investigation of the influence of substrate's nutrient composition on growth and inter-strain interactions between different strains of the pathogen (see §2.4), during the first part of the present study, the samples and the substrates, which were evaluated/used during the second part of the present study, were prepared by single and dual inoculation of *L. monocytogenes* strains C5 and 6179 in TSB-YE, under aerobic conditions (Fig. 1). Specifically, 50 mL falcon tubes containing 40 mL of sterile TSB-YE were inoculated either with single or both strains (strain ratio of 1:1) listed in Table 1, at approximately 2.0 - 3.0 log CFU/mL and stored at 7°C, in high precision (± 0.5°C) incubation chambers (MIR 153, Sanyo Electric Co.,

Osaka, Japan) on an orbital shaker, at 240 rpm (Shaker KS 130 basic, IKA-Werke GmbH & Co. KG, Germany) (Noriega et al., 2008). At every biological replicate of the following experimental setups were prepared 72 falcon tubes of which 24 tubes were inoculated with strain C5, 24 tubes were inoculated with strain 6179 and the last 24 tubes inoculated with both strains. At the 17th day of storage both singly- and co-cultured strains had reached stationary phase, while during co-culture, strain 6179 affected by the presence of C5, as previous described by Gkerekou et al. (2021) (Fig. 1). At that point, cultures of the 17th day of storage were used as samples (9 samples at §2.5.1 and 9 samples at §2.5.2) or substrates (54 samples at §2.5.3) during the second part of the present study.

Effect of nutrient depletion on inter-strain interactions of L. monocytogenes

After 17 days of storage, as described previously in §2.5, 9 samples (3 of every inoculation scheme) centrifuged (4752 x g at 4ºC for 20 min), their supernatant was discarded, the biomass was re-suspended in fresh TSB-YE and the samples further stored for an additional 20 days at 7ºC, under aerobic conditions (on orbital shaker, at 240 rpm, as described in §2.4).

Investigation of metabolic factors of single and dual-cultures of L. monocytogenes by FTIR-ATR spectroscopy

In parallel, cultures of 9 different samples (3 samples of every inoculation scheme, as described in §2.5) were centrifuged (4752 x g at 4ºC for 20 min), their supernatant was filtered with sterile syringe filters (PES, 0.22 μm, Millex®) and 10 mL of each produced cell-free spent medium (CFSM) and fresh TSB-YE (which was used as control/background during measurements) were stored at -80ºC in urine boxes (120 mL of volume). After storage for at least 48 h at -80ºC, the different CFSM were lyophilized for 48 h (Virtis 25 EL Freemobile laboratory lyophilizer) and stored at 4°C until the performance of the spectroscopic analysis. FTIR analysis was performed using an IROS 05 FTIR spectrometer (Ostec Enterprise Ltd, Moscow, Russia) equipped with ATR (Attenuated Total Reflectance) crystal. The FTIR-ATR spectra of the different samples were obtained by placing the lyophilized samples on the ATR crystal and pressing them so as to have the best possible contact with the crystal. The spectrometer was programmed to collect spectra over the wavenumber range 4000 to 600 cm^{-1} and the scans per measurement were 32 with a resolution of 4 cm^{-1}. Reference spectra were acquired by collecting a spectrum from the cleaned blank crystal and the lyophilized fresh TSB-YE, prior to the measurement of each sample replicate. At the end of each sampling, the crystal surface was first cleaned with analytical grade acetone and finally dried with tissue. The range of FTIR-ATR spectra that was used for further analysis was between 3500 and 1000 cm^{-1}. Three technical replicates (three FTIR-ATR spectra) of each sample were collected from two biological replicates and each sample from both the technical and the biological replicates measured three times, by using different sub-sample (n=54).

Spectrum processing was performed using the software OMNIC ver.9.1 (ThermoFisher Scientific Inc., Waltham, MA, USA). Pre-treatments were performed with "automatic smoothing" (5-point moving second-degree polynomial) and "baseline correction" (second-degree polynomial, twenty iterations) functions. Finally, using the "Statistical Spectra" function, the average of the three spectra for each sample was calculated, and each average spectrum was normalized (absorbance axis from a value 0 to 1).

Investigation of metabolic factors production that may affect the growth and inter-strains interactions of L. monocytogenes during co-culture by culture in cell-free spent medium

On the 17th day of storage, the last 54 samples (18 samples of every inoculation scheme, as described in paragraph §2.5) were centrifuged, filtrated and the produced CFSM re-inoculated with singly and co-cultured *L. monocytogenes* strains C5 and 6179 (studied all the different combinations of strain and CFSM). In 27 out of the 54 samples of CFSM (9 samples of CFSM produced by singly-cultured C5 (C5-CFSM), 9 samples of CFSM produced by singly-cultured 6179 (6179-CFSM) and 9 samples of CFSM produced by co-cultured C5 and 6179 (C5+6179-CFSM)) were added 5 mL of concentrated TSB-YE (referred from now on as enriched CFSM) for nutrient replenishment. The amount of concentrated TSB-YE added was selected aiming to the resulted growth substrate having comparable concentration of nutrients to the fresh TSB-YE in the final volume of the culture medium (35 mL). Subsequently, 3 samples of enriched C5-CFSM inoculated with singly-cultured C5, 3 samples of enriched C5-CFSM inoculated with singly-cultured 6179 and 3 samples of enriched C5-CFSM inoculated with co-cultured C5 and 6179 and stored at 7ºC, under aerobic conditions (on orbital shaker, at 240 rpm). Similarly with the latter experimental scheme were inoculated the enriched 6179-CFSM and the enriched C5+6179-CFSM (Fig. 1, Supplementary material). In parallel, the same number of samples was inoculated as described above, but without the addition of the concentrated TBS-YE in the different CFS culture media (C5-CFSM, 6179-CFSM, C5+6179-CFSM), and the samples finally stored at 7°C, under aerobic conditions (on orbital shaker at 240 rpm) (Fig. 1, Supplementary material). Two independent storage experiments were performed and triplicate samples were used for each trial (*n*=6).

Microbiological analysis

For both parts of the present study, on various days during storage at 7°C, to determine the growth curves of the different strains and the same strains in the co-culture, up to 3 mL of each liquid culture was removed under aseptic conditions. Following decimal dilutions in ¼ strength Ringer's solution, aliquots of 0.1 mL and/or 1 mL of diluted sample were spread on selective and non-selective culture media. The population of *L. monocytogenes* strains was enumerated on TSA-YE and TSA-YE/S or/and TSA-YE/R, at 37ºC for 48 h. Average numbers of colonies *per* plate were used to calculate the viable-cell concentrations, expressed as log CFU/mL.

pH measurements

The pH values of the samples were recorded at every sampling by using a digital pH meter (pH 526, Metrohm Ltd, Switzerland) *via* immersion of pH electrode in the homogenate.

Statistical analysis

For all the pairwise comparisons was used the Student's t-test and were performed with *STATGRAPHICS® Centurion XVII* computer package (Statpoint Technologies Inc., USA). Differences were considered to be significant for p-values < 0.05.

Results and Discussion

Effect of substrate's nutrients composition on growth and inter-strain interactions of L. monocytogenes

The substrates that were used in the present study, differ in the presence of glucose (with and without dextrose) and the presence of yeast extract, which is a mixture of amino acids, peptides, water soluble vitamins and carbohydrates. The presence of yeast extract resulted in higher final population for both singly-cultured strains compared with the final population reached during culture in TSB without dextrose. The presence of amino acids is required for the growth of the pathogen (Premaratne et al., 1991; Verheul et al., 1995; 1998) so the addition of yeast extract may enhance the growth capacity. Substrates that have undergone proteolysis and are rich in peptides and amino acids, are reported to stimulate growth of *L. monocytogenes*, as it happens when the pathogen coexists with the highly proteolytic Pseudomonas (Marshall and Schmidt, 1991). According to Figures 2A and 2B, strain 6179 seems to grow slower in the absence of glucose, while during co-culture, the growth inhibition of strain 6179 by the presence of C5 was more pronounced. Specifically, in TSB without dextrose and in TSB-YE without dextrose the difference in the final population between the singly and co-cultured 6179 was 3.6 and 6.3 log units, respectively (Figs. 2A and 2B). During co-culture in TSB-YE, the growth inhibition of strain 6179 by strain C5 was 2.7 log units. The presence of up to 2.5 g/L of glucose appears to enhance the yield of microbial biomass, proportionally (Schneebeli and Egli, 2013). Moreover, according to Crespo Tapia et al. (2018) the addition of 1% of glucose in the medium enhanced the growth of *L. monocytogenes* at 30^{o}C, compared to non-supplemented Nutrient Broth, under both aerobic and anaerobic conditions. However, it is reported that the addition of glucose in TSB-YE stimulated the growth of several 4b strains (C5; Table 1), leading to a growth rate higher than that of 1/2a strains (6179; Table 1) (Fig. 1) (Pan et al., 2010). Considering all the above, we can assume, that in the present study, the growth of 6179 in co-culture may be enhanced by the presence of glucose, which results in the difference between single-culture and co-culture being reduced in relation to the substrates that did not contain glucose (Figs. 1, 2A and 2B).

Effect of nutrient depletion on inter-strain interactions of L. monocytogenes

Both singly- and co-cultured C5 had similar growth rate and reached 9.1 ± 0.4 and 9.0 ± 0.3 log CFU/mL, respectively (Fig. 1). However, the growth of 6179 in co-culture, yet having the same growth rate as the single culture, was suppressed by the presence of the other strain (C5). When strain C5 exceeded the critical population density of 6.0 log CFU/mL, 6179 stopped growing, reaching lower population density in the co-culture compared with the population level that reached during the single culture (Fig. 1). The renewal of nutrients by the addition of fresh TSB-YE did not seem to be enough, to enable strain 6179 to overcome the suppressing effect of the presence of the strain C5 and the difference in the final population during the co-culture remained constant throughout the extra storage time. The latter indicates that nutrient deficiency does not appear to explain inter-strain interactions (Fig. 3). Among the different mechanisms that have been proposed to explain the interactions between different populations that may coexist in a food product, "Jameson Effect" has been used to describe the non-specific competition for nutrients (Jameson, 1962; Ross et al., 2000). According to numerous studies, different species or strains within a microbial community race to consume the available nutrients of the substrate to maximize their population density. When those resources are depleted by a single "dominant" species or strain, the race is "over" and the growth of the "weaker" species or strain practically ceases (Baka et al., 2014; Buchanan and Bagi, 1997; Costa et al., 2020; Guillier et al., 2008; Mellefont et al., 2008). The naturally occurred indigenous flora of the food products is often considered the "dominant" community, while the possible contamination by a pathogen is considered the "weaker" population (commonly at much lower levels), and microbial interactions seem to contribute to the limitation of pathogen's growth. Studies have tried both to quantify the effect of the interaction and to incorporate it in the already existing predictive models (Mellefont et al., 2008; Østergaard et al., 2014). According to Mellefont et al. (2008), during co-culture in TSB-YE with equal initial population levels and similar growth capacity of *L. monocytogenes*, *E. coli*, *Pseudomonas fluorescence* and *Lactobacillus plantarum, Listeria* reached lower maximum population density by approximately 0.9 log CFU/mL, in the mixed than in the single culture. The authors concluded that nutrient depletion (associated with "Jameson Effect") may be the main explanation for most of their observations and, secondarily, the individual kinetic parameters of the microorganisms, which paired in the different co-cultures combined with their level during the inoculation. However, "Jameson effect" seems rather unsuitable to describe the observations of the present study (Figs. 1 and 3).

Investigation of the production of metabolic/secreted factors that may affect the growth of L. monocytogenes during co-culture

The inter-strain interactions were evaluated by comparison of the growth behavior and final population level of each singly-cultured strain with the same strain in the co-culture. Thereby, based on the growth curves of Figure 1, it was shown that *L. monocytogenes* strain 6179 was affected by the presence of

strain C5 and reached lower final population compared with the population level of the same strain in single culture. According to Figure 4, the inter-strain interactions are attributed to the cell contact; however apart from contact, there seems to be an additional mechanism, which may inhibit the growth of certain strains during co-culture (Fig. 4). Specifically, singly- and co-cultured C5, with or without the presence of inserts, reached similar final population levels (9.6 ± 0.0, 9.7 ± 0.1 and 9.6 ± 0.1 log CFU/mL, respectively). Nevertheless, co-cultured 6179 reached 0.8 and 5.2 log units lower population, with and without the presence of inserts, respectively, compared with the population of the same singly-cultured strain by the end of storage (Fig. 4). Beyond the CDI and the competition for nutrient resources, another mechanism of competition between different strains coexisting in/on the same food product is the production of metabolic by-products by one strain, which may inhibit the growth of the others. The chemical composition of the CFSM of the individual single cultures and that of co-culture was characterized by FTIR-ATR spectroscopy and compared to assess if the interactions may be due to the production of specific secreted compounds. The obtained FTIR-ATR spectra reflected the biochemical composition of the CFSM and considered the molecular fingerprints of the metabolome of the singly-cultured C5 and 6179 and their co-culture. The possible assignments of the vibration modes are given in detail in Table 2. Generally, the metabolome of *L. monocytogenes* is composed of amino acids, sugars, organic acids, alcohols, nucleotides and some other primary and secondary metabolites (Zhao et al., 2020). According to the obtained results, the individual spectral profiles of the metabolome became more complex and the differences or the similarities between the CFSM produced by the singly- and the co-cultured strains became more evident as storage progressed (Fig. 5). Evaluating the spectra of the CFSM on the 17th day of storage, where the interaction between the two strains of the co-culture was very pronounced, the profile of the CFSM produced from the singly-cultured C5 was more complex, while the spectra from the CFSM produced from the singly-cultured 6179 and the co-culture shared a lot of similarities (Table 2; Fig. 5). A metabolic compound, represented by the bands 1457 and 1454 cm^{-1} and assigned to C-H deformation of >CHO_2 in lipids proteins, was obtained only from the CFSM of the single-cultured C5 and 6179, respectively, and was absent from the CFSM of the co-culture, probably indicating that the present of a second population may affect the production of this particular metabolite. Additionally, in the CFSM of the singly-cultured C5 there were recorded derivatives which were not detected in the CFSM of the co-culture, at 1741, 1645 and 1223 cm^{-1}. The latter bands were ascribed to C=O stretches of ester functional groups from lipid triglycerides and fatty acids, to C=O stretching vibrations between the amide bonds of amino acids of the proteins (Amide I band of α-helical structures of proteins) and to P=O asymmetric stretching of phosphodiesters in phospholipids, respectively. Interestingly, the compound at 1645 cm^{-1} was a metabolic by-product, produced by singly-cultured C5 later during storage and was absent from the profile of the CFSM of the co-culture, indicating that this compound may have been infused into or mounted on the cells of strain 6179 and thus, was excluded from the spectrum of co-culture

due to the removal of the cells by filtration. The secreted compounds represented by the measurements at 1740-1739 and 1218-1216 cm^{-1} were evident in the spectra of the CFSM produced by both the singly and the co-cultured strains on the 5th day of storage, where the different cultures were at the same growth phase and no interactions had occurred during co-culture; however, later on, they were detected only in the CFSM of the singly-cultured C5 (1741 and 1223 cm^{-1}) (Fig. 5). Finally, some metabolites represented by bands at 1518 and 1075 cm^{-1} and assigned to the amide II band of proteins and to P=O symmetric stretching in DNA, RNA and phospholipids, C-O-C, C-O dominated by ring vibrations in various polysaccharides, respectively, were produced earlier during storage by the co-cultured strains. A hypothesis could be that their premature production is related to the presence of the second population and subsequently their increased concentration earlier during storage may be related to the fact that the co-cultured *L. monocytogenes* strain 6179 was "forced" to enter the stationary phase at a lower population, compared to its single culture. The 17th day of storage, when all different cultures have reached the stationary phase, the above mentioned specific compounds were recorded in all spectra (Fig. 5).

The major findings of the present study are located in three out of the four main areas of the spectrum. Derivatives represented by the bands at 1741, 1645 (singly-cultured C5 at 17th day of storage) and 1518 cm^{-1} (co-culture at 5th day of storage) belong to the second region of the spectrum (1800 -1500 cm^{-1}) absorbed by the amide bonds of proteins and peptides of cells. Bands at 1223, 1457 (singly-cultured C5 at 17th day of storage) and 1454 cm^{-1} (singly-cultured 6179 at 17th day of storage) belong to the third region of the spectrum (1500 - 1200 cm^{-1}) comprised by absorptions of both proteins and fatty acids of the cells (Fig. 5; Table 2). The bacterial extracellular proteome or exoproteome, i.e., an important subset of the total proteome, is characterized by its dynamic nature, undergoing variations and adjustments and varying in composition within each species (Cabrita et al., 2014; Dumas et al., 2008; Rychli et al., 2016). Secreted proteins are one of the main tools used by bacteria to interact with their environment, could be released extracellularly, e.g, as a soluble (free) protein and undergo more pronounced alterations than the cytoplasmic proteins (Cabrita et al., 2014; Dumas et al., 2008, 2009). Most studies in the literature investigate and compare the secretome of different strains of the pathogen, especially the production of extracellular proteins, under conditions encountered by the pathogen during host infection (at 37°C), biofilm formation or the food processing environment (at room temperature, 20 - 22°C) (Dumas et al., 2009, 2008; Lee and Wang, 2020; Renier et al., 2013; Trost et al., 2005). *L. monocytogenes'* metabolism is strongly influenced by the environmental conditions, so its metabolic profile is associated with biological activity at a given time and under certain environmental conditions. However, temperature is one of the most important environmental factors, as temperatures occurring in the food chain affect both growth and metabolism (Renier et al., 2013). During storage at 11°C, proteins both with and without secretion signal were included in the exoproteome, functioning as specific virulent factors in cell envelope and cellular processes (with

secretion signal), or involved in glycolysis and detoxification and adaptation to atypical conditions (without secretion signal) (Cabrita et al., 2013; Ramnath et al., 2003). Among the detected proteins, OppA (possibly involved in oligopeptide transport) helps the bacterium to counteract the low diffusion rate of solutes, during growth at low temperatures, This may be done by mediating oligopeptide transport intracellularly, thus enabling the efficient uptake of peptides (Cabrita et al., 2013). Interestingly, after growth at 11°C, proteins OppA and flagelin (FlaA) were detected only in the serotype 1/2a *L. monocytogenes* strain and not in the exoproteome of the serotype 4b strain (different from those used in the present study), possible explaining the persistence of particular strains in the food industry environment (Cabrita et al., 2014).

Band at 1223 cm^{-1} is characteristic of asymmetric stress vibrations of phosphodiester bonds (>P=0), which occur between nucleotides in DNA and RNA molecules, as well as in the phospholipids of cell membranes (Candoğan et al., 2021). Among the strategies deployed by *L. monocytogenes* to adapt environmental changes, modifications of fatty acyl chains of membrane lipids are essential for keeping enough fluidity compatible with integrity and functionality of the membrane (Chihib et al., 2003). During growth at 8°C, different *L. monocytogenes* strains expressed pathways involved in peptidoglycan synthesis, while repressed metabolic pathways related to the synthesis of the polar and pyruvate families of amino acids, which are precursors for the synthesis of iso-branched-chain fatty acids, which decrease membrane fluidity (Cordero et al., 2016; Mansilla et al., 2004). The psychotropic feature of *L. monocytogenes* has been attributed to its relevant proportion of branched-chained fatty acids, which presumably contributes to maintaining an appropriate membrane fluidity at temperatures near to 0°C (Juneja and Davidson, 1993; Püttmann et al., 1993). Membrane's fatty acid composition is dominated to an unusual extent (90% or more) by anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{15:0}$ and the major change in fatty acid composition during growth at low temperatures is the increase in the content of anteiso-$C_{15:0}$ (Julotok et al., 2010). However, according to Gianotti et al. (2008), the straight long chain fatty acids, palmitic (C16:0) and stearic (C18:0), were the principal fatty acids released in the culture medium after 2 h incubation at 37°C by two different *L. monocytogenes* strains.

The last secreted compound represented by absorption at 1073 - 1078 cm^{-1} belongs to the forth region of the spectrum (from 1200 to 900 cm^{-1}), which includes derivatives mainly of bacterial cell wall polysaccharides.

During culture in the different enriched CFSM, no considerable inter-strain interactions were observed, as both co-cultured strains grew similarly and at the same population density as in single culture. Both singly and co-cultured C5 and 6179 had lower growth rate and reached lower final population by the end of storage, compared with the growth capacity of the same strains in fresh TSB-YE (Fig. 6). Since a sufficient amount of nutrients has been added to the CFSM (see §2.5.3), the difference in growth capacity may be due to the presence of metabolites or the lower initial pH. However, during culture in all the

different CFSM, the initial pH was only ≈1 pH unit lower than the optimum for growth pH (Fig. 6). It is obvious that the overall presence of metabolites in the culture medium seems to influence the growth of the pathogen, because both strains had different growth kinetics than during growth in fresh TSB-YE (Figs. 1 and 6). Even though C5 outgrew 6179 during co-culture in various studied substrates (Fig. 1; Gkerekou et al., 2021, 2022; Zilelidou et al., 2015; Zilelidou, Manthou et al., 2016), in this study, when cultured in the enriched CFSM produced from singly-cultured 6179, it did not manage to grow (Fig. 6). The produced metabolites of strain 6179 appears to be harmful to strain C5, particularly, at the concentration used in the present experimental design *via* the CFSM. The concentration of the metabolites considered to be critical because in the CFSM produced by co-culture, the metabolites of 6179 are present, but due to the lowest population density, they may not constitute a substantial percentage of the total metabolome. The derivatives represented by the bands 1374 (C5-CFSM) and 1341 cm^{-1} (C5+6179-CFSM) that belong to the mixed region of the spectrum absorbed by the amide bonds of proteins and peptides of cells, were observed in the CFSM profiles of the singly-cultured C5 and the co-culture, in which strain C5 managed to grew. The latter indicates that may be a characteristic secreted compound enable strain C5 to cope with the presence of strain 6179 (Figs. 5 and 6, Table 2). Early studies by Kalmokoff et al. (1999) proved that some *L. monocytogenes* strains had inhibitory activity, which increased inversely to temperature, against different *Listeria* sp. including *L. monocytogenes*. Many *Listeria* strains are reported to produce bactericidal substances (lytic particles), designated as listeriolysin S (LLS) and monocins, whose activity defined as similar to bacteriocins by Curtis and Mitchell (1992). LLS is a member of the family of thiazole/oxazole-modified microcins (TOMM's), which has been described previously as an hemolytic and cytotoxic factor contributing to *L. monocytogenes* virulence, but the mechanism by which, LLS kills other bacteria is unknown (Cotter et al., 2008; S. Lee, 2020; Quereda et al., 2017). In 2008, Cotter et al. (2008) named the *L. monocytogenes* LLS gene cluster as pathogenicity island III (LIPI-3). The LIPI-3 was discovered in a subset of lineage I strains (C5 belong to serotype 4b; Table 1), suggesting that it could be associated to the higher virulence potential of these bacteria. Monocins resemble phage tail structures and result from the presence of incomplete, cryptic prophages. The tail associated lytic proteins (used during infection for cell wall penetration) are toxic to certain *Listeria* species and act as biocins (Klumpp and Loessner, 2013; Zink et al., 1994, 1995). The strain 6179 used in the present study is fully sequenced, so it is known that it harbors the *monocin,* but does not harbor *Listeriolysin S locus*; however, such information is not available for strain C5, due to the fact that is partially sequenced (Table 1). Robinson et al. (2001) concluded that the addition of spent medium to TSB containing 1.6 M NaCl shortened detection times, and decreased lag time variability, but no change was recorded in the probability of growth initiation. Cells may produce a chemical or physicochemical change *in situ*, or there may be production of certain substances during culture, as the requirement for specific signal molecules in recovery from stress has been reported in some bacteria. When one of the studied *L.*

monocytogenes strains (isolated from cheese) was incubated for 2 h in its own CFSM, the extent of fatty acid release increased up to 900 mg/L (Gianotti et al., 2008). Interestingly, singly and co-cultured 6179 grew in all different CFSM (Fig. 6), indicating that the factors which affect its behavior during co-culture, could be also on the surface of the "*competitive*" cells and not secreted in the environment. As discussed above, characteristic derivatives are present in the CFSM produced by C5 (1741, 1645 and 1223 cm^{-1}, Fig. 5 and Table 2) and absent by the CFSM produced after co-culture of the studied strains (Fig. 5 and Table 2). The "*surfaceome*", includes surface proteins involved in important biological processes, such as bacterial growth, responses to environmental stress, host invasion, and interference with the immune system, including autolysins, N-acetylglucosamine deacetylase PgdA, proteases, penicillin binding proteins and the lipoprotein PrsA-2, among others (Bierne and Cossart, 2007; Zhang et al., 2013). Given that the growth and co-culture of strains was done at low temperature (7°C) which, as mentioned above, affects the profile and composition of the metabolome, the competition may be also due to the effect of the one strain on the ability of the other to grow at chilled conditions. Kagkli et al. (2009) inoculated Katiki Domokou with different single-strains and cocktail inoculum (consisted of the different studied strains) and after the evaluation of the different inactivation kinetics and survival under different storage temperatures (5, 10, 15 and 20 °C), concluded that some strains may have better survival capacity, however the rest of the studied strains more or less affected by the presence of the others strains. Interestingly, not all strains survived at all temperatures until the end of storage, and differences were observed even within the low and high temperatures (Kagkli et al., 2009). Overall, competition may be expressed either by the production of certain metabolites by the strong strain and/or by the suppression of certain metabolic pathways of the weak strain, resulting in decreased cold tolerance.

Conclusions

The phenomenon of inter-strain interactions between different strains of *L. monocytogenes* has been proven (Gkerekou et al., 2022, 2021; Zilelidou et al., 2015; Zilelidou, Manthou et al., 2016) and its study is worth being advanced. With the present work, it was shown that the interactions are not due to the absence of nutrients, while highlighted the differences of the secreted compounds between single and co-culture. The present results constitute the first solid indications that apart from the role of CDI, the interactions, may be also due to metabolic derivatives, which are either located on the surface of the cells or secreted in the growth medium. Both the characterization of the secreted proteins and the proteins located on the bacterial wall surface during co-culture and possibly linked to CDI, appear to be the next steps in the investigation of the underpinning mechanism(s) of inter-strain interactions. The in-depth description of the phenomenon is important, because differential expression/production of metabolites may have an impact

on pathogenicity behavior and niche adaptation of cells and might be a key and worth further investigating strain-specific feature**.**

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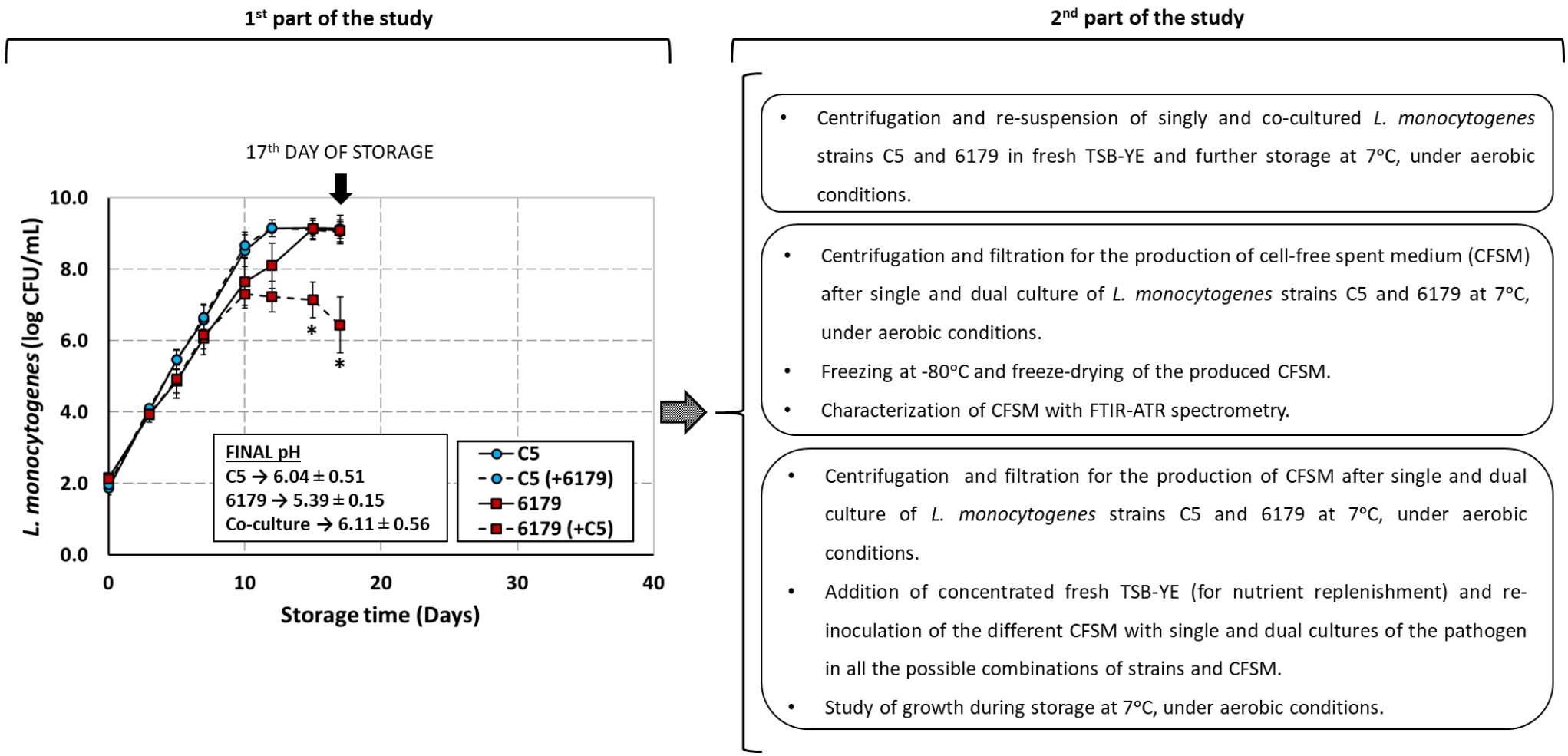


Figure 1. Growth curves of singly- and co-cultured *L. monocytogenes* strains C5 and 6179 in TSB-YE, under aerobic conditions and storage at 7°C and **schematic representation of the experimental design**.

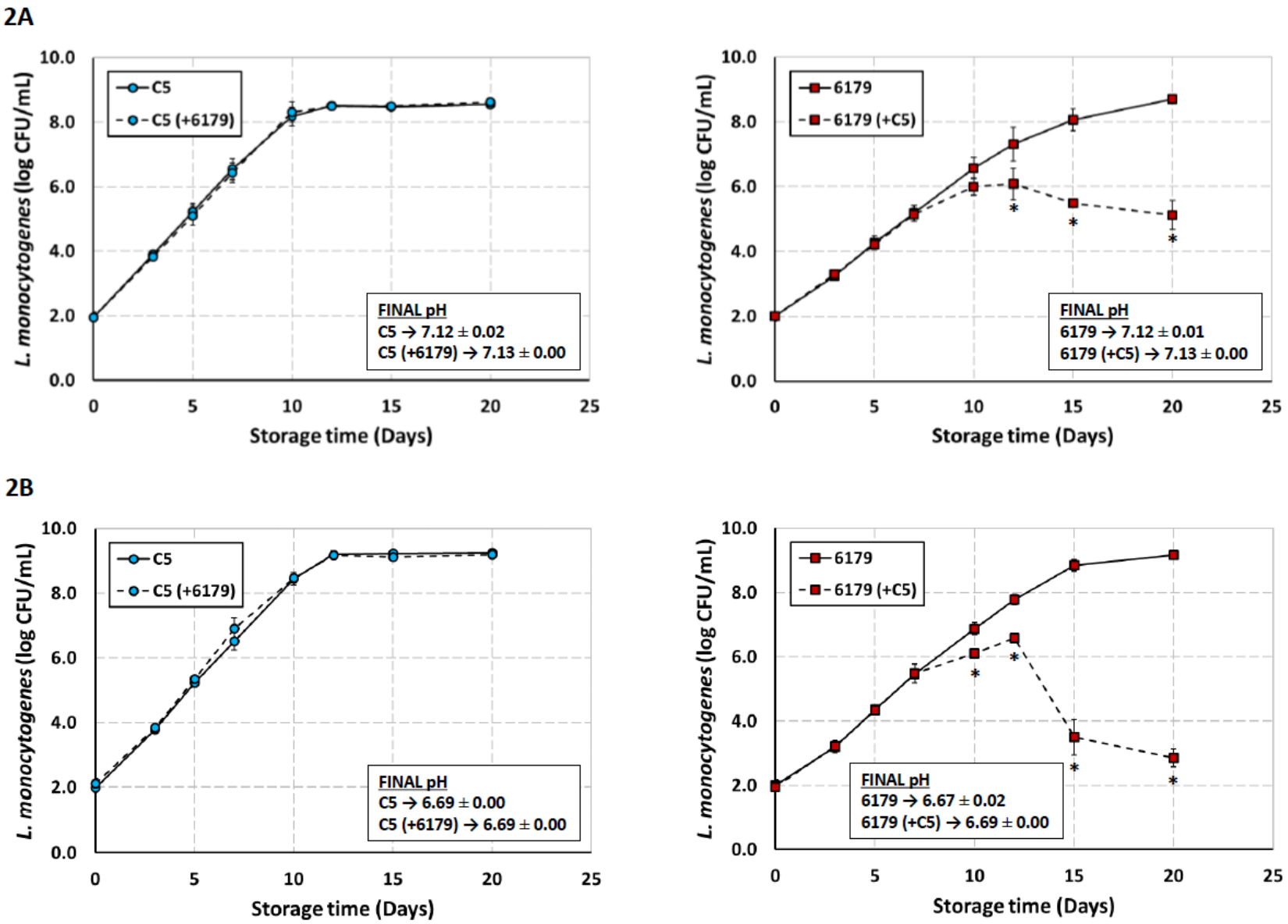


Figure 2. Growth curves of singly- and co-cultured *L. monocytogenes* strains C5 and 6179 in TSB without dextrose (A) and in TSB-YE without dextrose (B), under aerobic conditions and storage at 7°C.

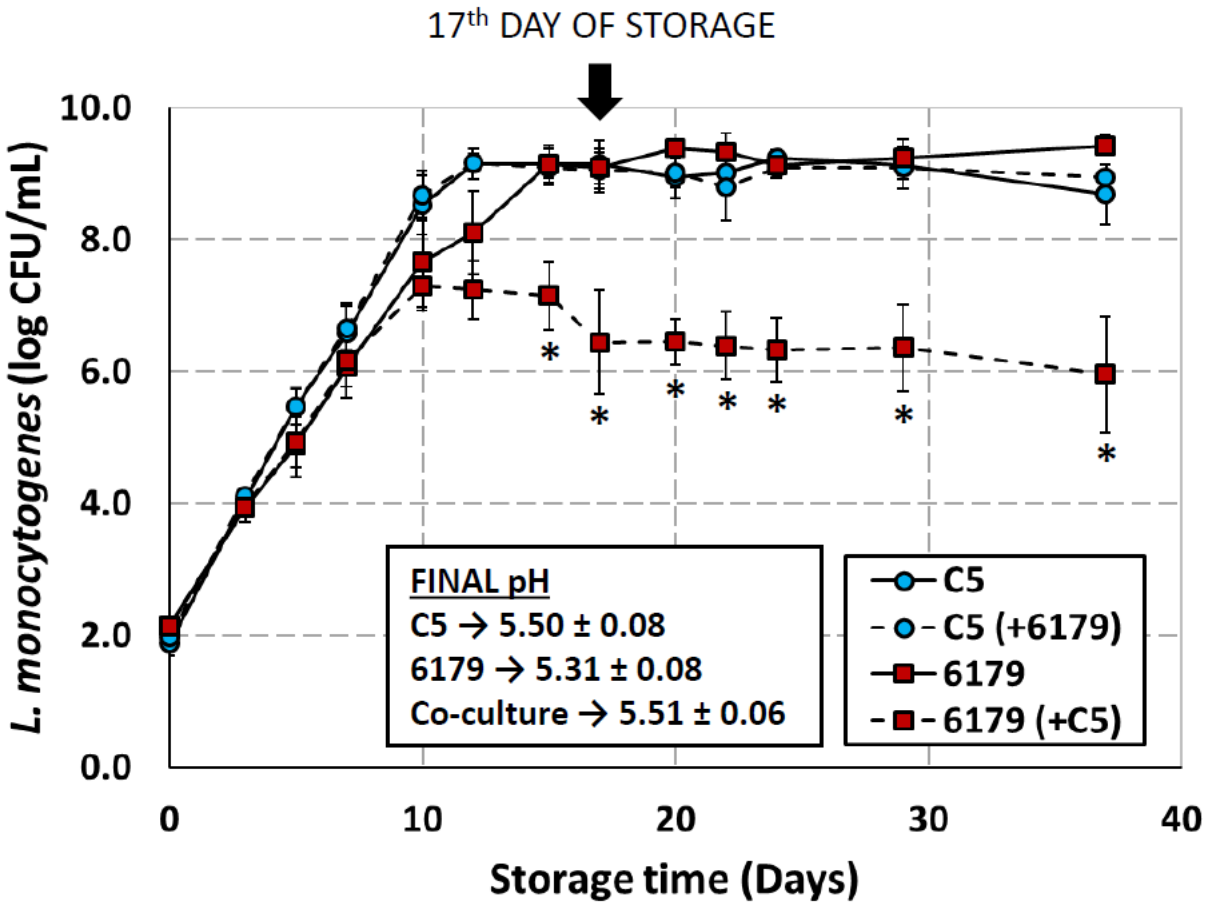


Figure 3. Growth curves of singly- and co-cultured *L. monocytogenes* strains C5 and 6179 in fresh TSB-YE after the 17th day of storage, under aerobic conditions and further storage at 7°C.

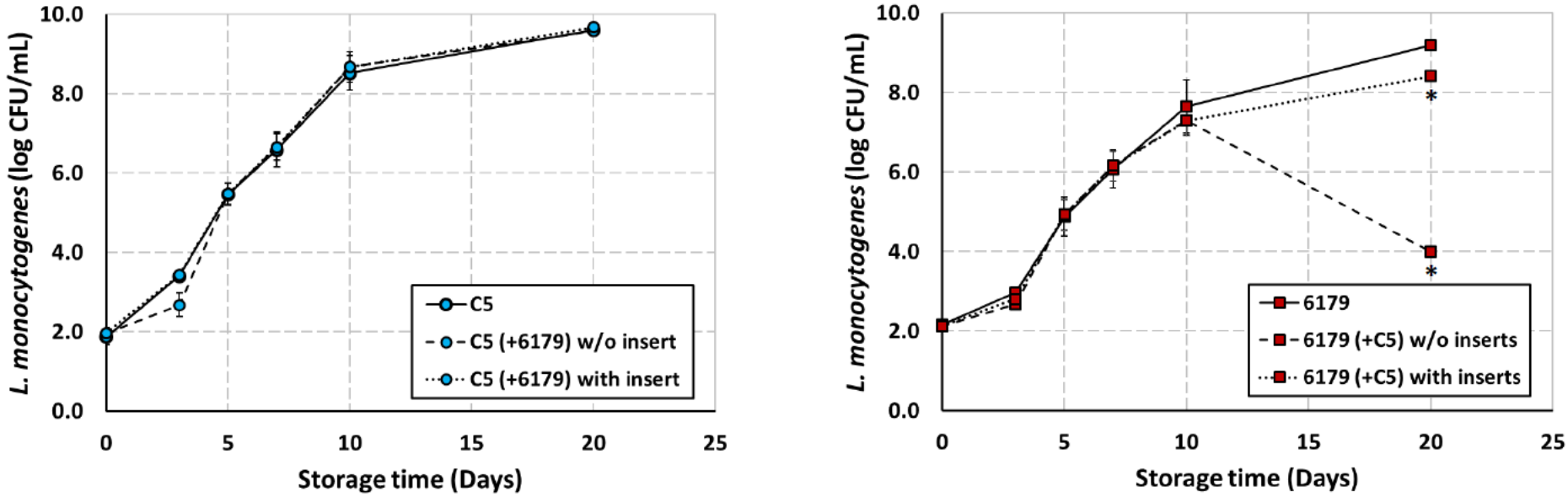
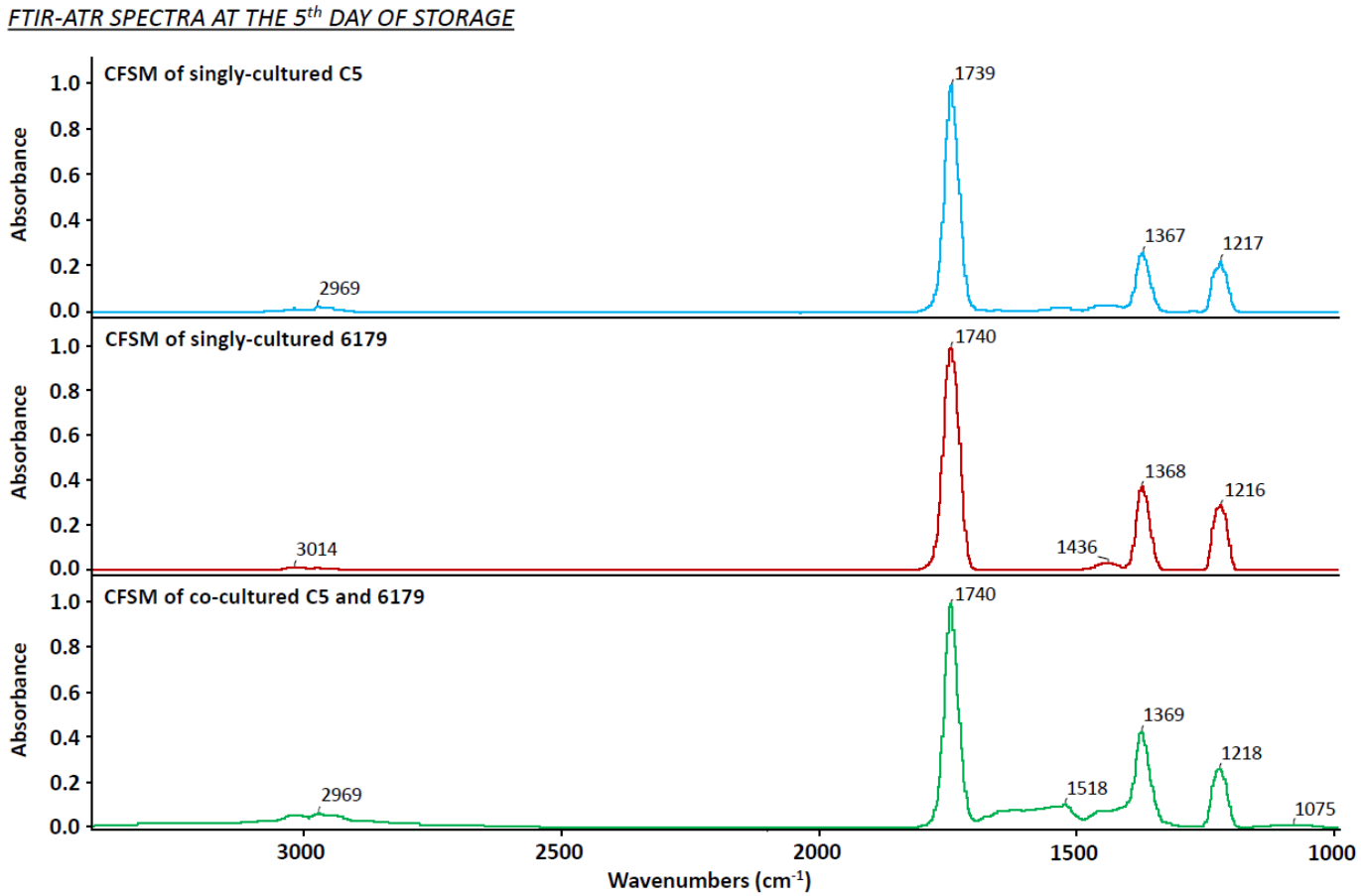


Figure 4. Growth curves of singly- and co-cultured *L. monocytogenes* strains C5 and 6179 in 6-well culture plates with and without inserts, under aerobic conditions and storage at 7°C.



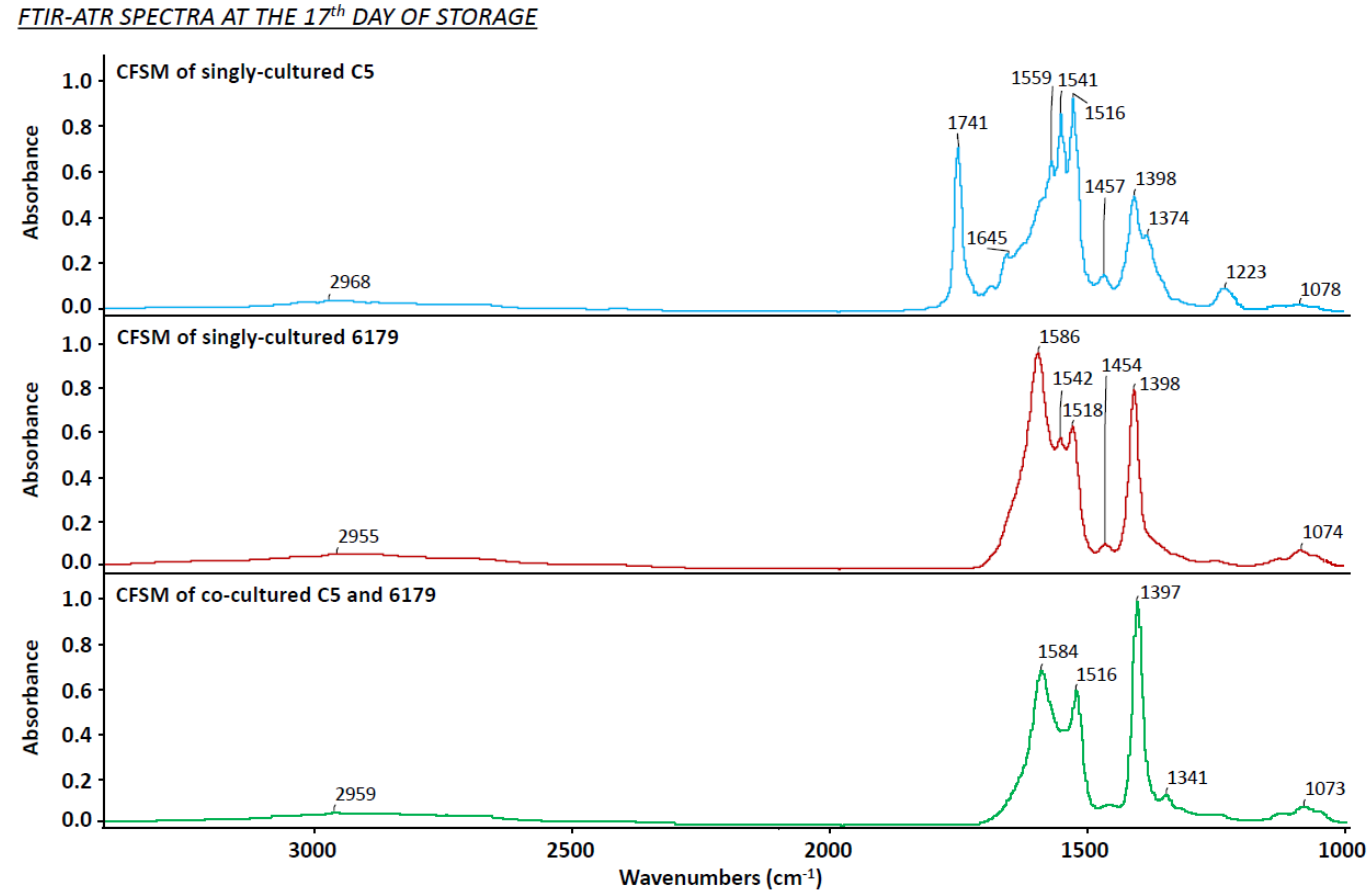


Figure 5. FTIR-ATR spectra of cell free spent media (CFSM) produced by filtration after single culture and co-culture of *L. monocytogenes* strains C5 and 6179 after 5 and 17 days of storage, under aerobic conditions at 7°C.

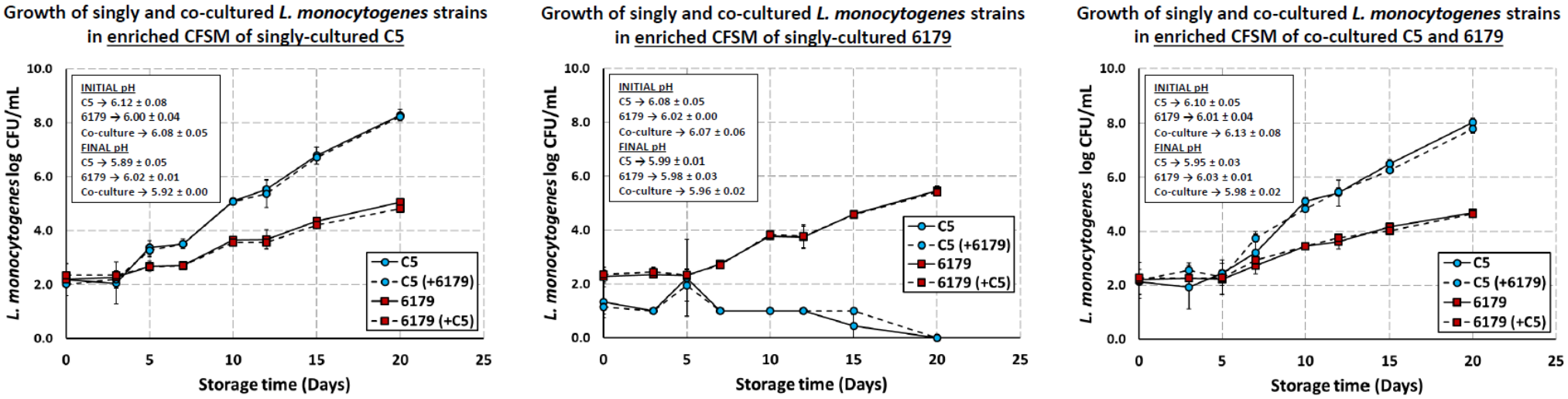


Figure 6. Growth curves of singly- and co-cultured *L. monocytogenes* strains C5 and 6179 in enriched cell free spent media (CFSM) produced by filtration after single culture and co-culture of *L. monocytogenes* strains C5 and 6179 after 17 days of storage, under aerobic conditions and storage at 7°C.

Table 1. *Listeria monocytogenes* strains used in the study.

Strain*	Serotype	MLST	Source	Year of isolation	Reference	Antibiotic resistance (μg/mL)**	Assembly level	Accession number
C5	4b	ST2	Dairy farm environment isolation	2007	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Streptomycin (2000)	Contig	NZ_MDQI00000000
6179	1/2a	ST121	Cheese	1999	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Rifampicin (>800)	Complete Genome	CP098509 HG813249

*Strain selection due to their pronounced inter-strain interaction according to Gkerekou et al. (2021, 2022).

**Approximate MIC was considered as the minimum tested concentration (μg/mL) of antibiotic at which no bacterial growth was observed after 24 hours at 30°C. Bacterial growth was confirmed through measurements of optical density (OD_{600}). The streptomycin concentrations were 0, 125, 250, 500, 1000, 2000, 4000 μg/mL. Rifampicin was evaluated at 0, 200, 400, 800 μg/mL.

Table 2. Assignment of functional groups associated with major vibration bands in mid IR spectra of CFSM of singly- and co-cultured *L. monocytogenes* strain C5 and 6179, after 5 and 17 days of storage at 7°C, under aerobic conditions.

CFSM spectra of singly- and co-cultured *L. monocytogenes* strains		
Sample	**Wavenumber (cm^{-1})**	**Molecular vibrations of functional groups and the biomolecule contributor***
CFSM spectrum of singly-cultured C5 the 5th day of storage	2969	C-H asymmetric stretching of -CH_3 in fatty acids
	1739	>C=O stretching of lipid esters
	1367	Mixed region: fatty acid bending vibrations, proteins, and phosphate-carrying compounds
	1217	P=O asymmetric stretching of phosphodiesters in phospholipids
CFSM spectrum of singly-cultured C5 the 17th day of storage	2968	C-H asymmetric stretching of -CH_3 in fatty acids
	1741	>C=O stretching of lipid esters
	1645	Amide I of α-helical structures of proteins
	1559, 1541 and 1516	Amide II band of proteins
	1457	C-H deformation of >CH_2 in lipids proteins
	1398	C=O symmetric stretching of COO- group in aminoacids, fatty acids
	1374	Mixed region: fatty acid bending vibrations, proteins, and phosphate-carrying compounds
	1223	P=O asymmetric stretching of phosphodiesters in phospholipids
	1078	P=O symmetric stretching in DNA, RNA and phospholipids, C-O-C, C-O dominated by ring vibrations in various polysaccharides
CFSM spectrum of singly-cultured 6179 the 5th day of storage	3014	Fatty acid region
	1740	>C=O stretching of lipid esters
	1436	C-H deformation of >CH_2 in lipids proteins
	1368	Mixed region: fatty acid bending vibrations, proteins, and phosphate-carrying compounds
	1216	P=O asymmetric stretching of phosphodiesters in phospholipids

CFSM spectrum of singly-cultured 6179 the 17th day of storage	2955	C-H asymmetric stretching of -CH_3 in fatty acids
	1586, 1542 and 1518	Amide II band of proteins
	1454	C-H deformation of >CH_2 in lipids proteins
	1398	C=O symmetric stretching of COO- group in aminoacids, fatty acids
	1074	P=O symmetric stretching in DNA, RNA and phospholipids, C-O-C, C-O dominated by ring vibrations in various polysaccharides
CFSM of co-cultured C5 and 6179 the 5th day of storage	2969	C-H asymmetric stretching of -CH_3 in fatty acids
	1740	>C=O stretching of lipid esters
	1518	Amide II band of proteins
	1369	Mixed region: fatty acid bending vibrations, proteins, and phosphate-carrying compounds
	1218	P=O asymmetric stretching of phosphodiesters in phospholipids)
	1075	P=O symmetric stretching in DNA, RNA and phospholipids, C-O-C, C-O dominated by ring vibrations in various polysaccharides
CFSM of co-cultured C5 and 6179 the 17th day of storage	2959	C-H asymmetric stretching of -CH_3 in fatty acids
	1584 and 1516	Amide II band of proteins
	1397	C=O symmetric stretching of COO- group in aminoacids, fatty acids
	1341	Mixed region: fatty acid bending vibrations, proteins, and phosphate-carrying compounds
	1073	P=O symmetric stretching in DNA, RNA and phospholipids, C-O-C, C-O dominated by ring vibrations in various polysaccharides

Literature: Burgula et al. (2007), Davis & Mauer (2010), Mauer & Reuhs (2008), and Naumann (2006).

CHAPTER 6

Evaluating the impact of co-culture of different L. monocytogenes strains on time of first division at single-cell level

Unpublished manuscript

Evaluating the impact of co-culture of different *L. monocytogenes* strains on time of first division at single-cell level

Maria A. Gkerekou[a], Vasiliki Papadopoulou[a], Marianna Arvaniti[a], Antonios N. Psomas[b] and Panagiotis N. Skandamis[a]*

Laboratory of Food Quality Control and Hygiene, Department of Food Science and Human Nutrition, Agricultural University of Athens, Greece[b]

Abstract

Quantitative risk assessment have to count bacterial population dynamics at single-cell level due to physiological heterogeneity under diverse environmental conditions. The study evaluated the impact of co-culture of different *Listeria monocytogenes* strains on time to first division at single-cell level by optical microscopy and the growth and co-culture at population-level. *L. monocytogenes* strains, C5(4b) and 6179(1/2a) were selected due to the remarkable interaction during co-culture and were inoculated on 4.84cm^2 piece of Tryptic Soy Agar with 0.6% Yeast Extract, in single and two-strain cultures (1:1 strain ratio), at two population levels (dense (DP) and sparse proximity (SP)). Bacterial growth was recorded with phase-contrast optical microscopy during incubation at 37°C and images were acquired every 5min for 2h. By image analysis and a program, written in-house, using the x- and y-coordinates identified the individual cells in each field and tracked throughout the image sequence. Regardless of cell proximity, a greater percentage of singly-cultured 6179 cells managed to divide for the first time, while the same strain showed a lower percentage of cells that did not divide or divided for the second time, compared with the singly-cultured C5, within the 2h of incubation. For both strains, the time at which 50% of the cells divided for the first time remained the same regardless of the relative proximity of the cells. The 50% of singly-cultured C5 in SP managed to divide for the first time within the first 30 - 40 min of incubation, while the 50% of cells in DP divided for the first time within the first 40 - 50 min. Singly-cultured 6179 observed to be slower than C5, thus the 50% of singly-cultured 6179 cells divided for the first time within the first 50 - 60 min and 60 - 70 min of incubation during culture in SP and DP, respectively. The co-culture of the different strains had no effect on the time to first division. The latter conclusion arose from the fact that the time of first division of the co-culture recorded in the middle of time to first division of the single cultures, both for cells in DP (50-60 min) and SP (40-50 min). The findings indicate that cell proximity may influence the behavior of the different co-existing strains even at a single-cell level.

Keywords: Listeria monocytogenes; time to first division; phase-contrast optical microscopy; inter-strain interactions

Introduction

Listeria monocytogenes is a ubiquitous environmental microorganism and according to previous studies multiple strains may simultaneously introduced in the food processing environment *via* raw materials and inadequate hygiene practices (Chambel et al., 2007; Martín et al., 2014; Martínez-Suárez et al., 2016; Ortiz et al., 2010; Thévenot et al., 2006; Zoellner et al., 2018). The ability to survive and grow in a wide range of harsh environmental conditions and to adhere food processing surfaces unable the pathogen to persist and spread, resulting in multiple strains co-existing in/on the same food products (Felício et al., 2007; Gendel and Ulaszek, 2000; Kabuki et al., 2004; Rychli et al., 2014a). Recent studies have shown that strain-to-strain interactions may occur between cells in close proximity and appear to take the form of competition (Gkerekou et al., 2022, 2021; Zilelidou et al., 2016b, 2015). Exploring the mechanism of how different strains interact from a food safety perspective, is important, because the relative levels of each strain during storage may also determine the population of each strain at the end of an enrichment step and subsequently their probability of isolation on ALOA plates (i.e., the critical detection step), according to ISO 11280 (Zilelidou, Karmiri et al., 2016).

Population level analysis reflects the dominant biological mechanism operating within individual cells in a population (Altschuler and Wu, 2010). Genetically identical bacterial cells, even when experience the same environmental conditions, exhibit unpredictable variation in their phenotypes (Choudhary et al., 2023). Heterogeneity of bacterial populations may result from both phenotypic and genotypic variations, during interaction of internal and environmental factors, as well as from random fluctuations of the biochemical and physiological characteristics. Cell heterogeneity improves the survival of bacterial populations under heterogeneous or variable environmental conditions, as well as under the effect of stress factors. Thus, under diverse environmental conditions bacterial physiological heterogeneity reveals the need of describing bacterial population dynamics at single-cell level (Magdanova and Golyasnaya, 2013). The growth and evolution of these populations in turn depend on a complex interplay between single-cell properties, the symbiosis or competition between the cells, so the ability to encapsulate the cells within a closed environment allows studies of bacterial interactions such as quorum sensing or competition between different strains (Zhang et al., 2022). Quantitative microbial risk assessment have to take into account the heterogeneity in kinetic parameters, because pathogenic bacteria, when are present in food, they are often found in very low numbers and the distribution of individual lag times within cell populations cannot derived by observations at the cell population level (Elfwing et al., 2004; Kutalik et al., 2005a; Niven et al., 2008). As “lag time” is the incipient stage of a bacterial growth cycle, in which cells are adjusting to the new environmental conditions, before initiating exponential growth. Lag is a dynamic, organized adaptive and evolvable process that protects bacteria from threats, promotes reproductive fitness and it is broadly relevant to the study of bacterial evolution, host-pathogen interactions, antibiotic tolerance and food safety

(Bertranda, 2019). The term “time to first division” includes both the lag time and the time for the division process, with other words, the time needed for the individual cell to start dividing into two daughter cells. An effective method to determine the time to first division is the combination of microscopy and imaging. This method provides direct observation of single cell growth and by extension time to first division can be obtained by determining when the first cell doubling occurs (Wu et al., 2000). So, during the present study evaluated the impact of cell proximity on time to first division of different co-cultured *L. monocytogenes* strains which, as mentioned above, seems to be important in the manifestation of inter-strain interactions, and was developed a program to identify and monitor the individual cells during an image sequence.

Materials and methods

L. monocytogenes strains and inoculum preparation

L. monocytogenes strains C5 and 6179 were obtained from the microorganism collection of the Laboratory of Food Quality Control and Hygiene of Agricultural University of Athens and were selected based on their pronounced strain-to-strain interaction during co-culture in/on substrates with variable structures (Gkerekou et al., 2022, 2021; Zilelidou et al., 2016b, 2015) (Table 1). Their resistance to different antibiotics enables the selective enumeration during co-culture. The concentration of antibiotics used during their storage and for the preparation of the (selective) enumeration media, was the lowest in which the second strain (the one that was not resistant to the particular antibiotic) was unable to grow. The strains were maintained at -20^{o}C in Tryptone Soy Broth (LAB004, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract (MC001, Lab M Limited, United Kingdom) (TSB-YE, pH: 7.1 ± 0.2), 20% glycerol and the appropriate concentration of streptomycin (1000 μg/mL) and rifampicin (50 μg/mL), while during the experiments, both strains were maintained on Tryptone Soy Agar (LAB011, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract and streptomycin (1000 μg/mL; TSA-YE/S) or rifampicin (50 μg/mL; TSA-YE/R), at 4^{o}C and sub-cultured once a month. A single colony from a TSA-YE/S or TSA-YE/R stock culture of the target strain was transferred to 10 mL TSB-YE/S or TSB-YE/R and incubated for 24 h at 30^{o}C and subsequently, 100 μL of each culture was transferred in fresh TSB-YE/S or TSB-YE/R for 18 h incubation at 30^{o}C to obtain stationary-phase cells with a density of *ca.* 10^{9} CFU/mL. Following activation stage, strains were harvested by centrifugation (2246 g for 10 min at 4^{o}C) (Megafuge 1.0R, Heraeus, Buckinghamshire, England), washed twice and finally re-suspended in 10 mL of ¼ strength Ringer’s solution (LAB M, Lancashire, UK). The level of the inoculum was determined by plating 100 μL from the appropriate decimal dilution of each strain on TSA-YE/S or TSA-YE/R and incubation at 37^{o}C for 48 h.

Evaluating inter-strains interactions between cells with different relative proximity at single-cell level

Preparation of samples and proximity determination

The preparation of samples performed according to Arvaniti et al. (2023). Under aseptic condtions, a piece of TSA-YE (22 x 22 mm) (Fig. 1A), transferred onto a microscope slide and inoculated with 10 µL of singly-cultured C5 or 6179 were transferred onto a microscope slide containing TSA-YE, while for the evaluation of the impact of co-culture on time to first division the TSA-YE piece was inoculated with 5 µL of each stain. The inoculum was spread oner the agar surface by a sterile microbiological loop and the sample remained for 15 min in laminar flow cabinet fro cell attachment end evaporation of the excess liquid. Then, a coverslip placed over the agar and sealed with silicone around the perimeter (Fig. 1B).

To assess the effect of the different relative cell proximity on time to first division, two density levels were studied, which were achieved by different initial inoculum. The aim was to record the behavior of a large population, in the attempt to simulate the population density after the "critical" population threshold of 6.0 log CFU/mL (Gkerekou et al., 2022). Specifically, as samples with cells in *sparse proximity (SP)* were defined those which inoculated with 10 µL (single cultures) or 5 µL (of each strain during co-culture) from an initial inoculum of 10^8 CFU/mL and monitored areas of the samples with 0.03 to 0.14 cells per cm^2 of exported image. With the specific experimental design was achieved the recording of approximately 100 cells per image. Similarly, as samples with cells in *dense proximity (DS)* were defined those which inoculated from an initial inoculum of 10^9 CFU/mL and selected for monitoring areas of the samples with 0.34 to 0.51 cells per cm^2 of exported image. The latter, experimental design was managed to have a distinctly denser image with a clearly greater number of cells, which led to a reduction of the relative distance between the cells, but at the same time the number of cells and the density did not prevent the monitoring of at least the first two divisions.

Incubation, monitoring conditions and image analysis

As described by Arvaniti et al. (2023), following sample preparation, both singly- and co-cultured cells incubated for 2h at ~37°C (36.9 to 37.2°C), on heating microscope stage (Fig. 1C) connected with pump for temperature control and by using K-type thermocouple placed on the surface of the microscope slide to ensure the accurate temperature adjiustment (Fig. 1D). Image sequences were acquired every 5 min, using an inverted optical microscope (Leica Dmi8) equipped with an oil immersion 63x phase contrast objective with a numeric aperture value of 1.25, a DFC 7000T camera (Leica) and LAS X software (Leica). The quality of the images was improved by developing an autofocus procedure with an extended depth of focus (EDF) system. The above procedure allows for multiple (in the present study 59) serial images in different z-axis planes to be captured and then combines the best focal areas of the serial images into a single in-focus image (z-stack). Individual final images were compiled to give a sequence of frames for the field of view, which was further transformed into a video showing the behavior of the same cell over time throughout the

experiment. Two (in the case of singly-cultured strains) to four (in the case of co-cultured strains) independent time-lapses were performed for the determination of the time to first and second division for both cells in SP and DP and from each studied time-lapse were selected three to five (depending on the studied cell proximity) subfields to track in total 200 to 400 individual cells (100 cells per time-lapse). Every digital image taken during a time-lapse was analyzed by ImageJ software and the individual cells identified in each image by an object number, but the same cell was rarely given the same identifier in successive images. To overcome this "obstacle", was developed a program, written in-house with Visual Basic, which tracked individual cells throughout the sequence of images by using the x- and y-coordinates and generated coherent time courses for each cell. The output of the program is a diagram with points placed exactly in the position of the individual cells in the each image sequence (Fig. 2).

Evaluating inter-strain interactions at population level during incubation at 37˚C

During this assessment was reproduced the single culture and the co-culture of the studied strains, with and without contact, in TSB-YE (Gkerekou et al., 2023, 2021) and on TSA-YE (Gkerekou et al., 2021), under aerobic conditions, with modification regarding the temperature (37°C). Initially, were prepared falcon tubes containing 30 mL of fresh TSB-YE (evaluation of cells' in contact growth) and petri plates containing 20 mL of TSA-YE. In parallel, bacterial cultures were prepared by inoculation of TSB-YE with singly-cultured C5 and 6179, at approximately 2.0 log CFU/mL. Polyethylene tetraphthalate (PET) track-etched membrane inserts of 0.4 μm pore size (Thermo Fischer Scientific, Denmark) were placed in 6-well culture plates and 2 mL of 6179 culture were added to the upper chamber of the well and 2 mL of C5 culture were added to the lower chamber (ensuring no contact between strains). The effect of culture the cells in the upper chamber in comparison to the lower chamber was also tested. All samples, eventually, were inoculated with single or dual cultures (strain ratio 1:1) of strains C5 and 6179 (Table 1), at approximately 2.0 log CFU/mL or cm^2. The inoculated samples were stored at 37°C, in high precision (± 0.5°C) incubation chambers for 24 h (MIR 153, Sanyo Electric Co., Osaka, Japan), on an orbital shaker at 240 rpm for the liquid samples (Shaker KS 130 basic, IKA-Werke GmbH & Co. KG, Germany).

Microbiological analysis was performed after 0, 2, 5, 8, 12 and 24 h of storage. At every sample point from the liquid samples were removed 1 mL or 100 μL, while regarding the solid samples was removed the hole content of the Petri dishes and placed in stomacher bags, in which 3-fold sterile ¼ strength Ringer's solution was added under aseptic conditions. The latter samples were homogenized in a stomacher at 240 rpm (Stomacher® 400 Circulator, Seward, UK) for 60 s. Following decimal dilutions in ¼ strength Ringer's solution, aliquots of 0.1 and/or 1 mL of diluted sample were spread on selective and non-selective culture media. Population of *L. monocytogenes* strains was enumerated, after 48 h at 37°C, on TSA-YE and TSA-YE/S or/and TSA-YE/R. Average numbers of colonies *per* plate were used to calculate the viable-cell concentrations, expressed as log CFU/mL for the liquid cultures and as log CFU/cm^2 for the surface

inoculated samples, with enumeration limit of 100 CFU/mL or cm^2. Two independent storage experiments were performed and triplicate samples were used for each trial (*n*=6).

Statistical analysis

The obtained bacterial growth data, per single or co-cultured strains, were fitted to the Baranyi-Roberts model with DMFit Excel Add-In software. Maximum specific growth rate (μ_{max}; $hours^{-1}$) and lag time (λ; hours) were determined.

Results and Discussion

Regardless of whether the observed cells consisted of one or two strains of the pathogen, it appeared that density and relative proximity affected the time to first division, with cells that were cultured in DP to divide later than the cells that were in SP from each other (Fig. 3). Most of the studies in literature have evaluated the effect of initial population density on the duration of the lag phase and the subsequent effect on growth behavior for relatively low initial inoculum size compared with the present study, concluding that the duration of the lag phase depends inversely on the size of the inoculum, namely as the number of cells increases, lag duration decreases (Augustin et al., 2000; Bertranda, 2019; Kutalik et al., 2005a). According to Gay et al. (1996) differences in the initial cell concentration combined with certain storage and/or culture conditions, concerning the storage of inoculum and the temperature of pre-inoculation incubation, seems to increase the duration of the lag phase at low initial bacterial populations. The authors speculated that in a smaller population there is less likelihood of individuals with relatively shorter lag time. When *L. monocytogenes* was inoculated in substrates with unfavorable characteristics and/or under suboptimal conditions, during the extended lag phase, a high initial inoculum seems to be more likely to initiate growth faster (Pascual et al., 2001). The latter study, also, highlighted the importance of the cells' phase during the inoculation, reporting that exponential phase cells are more susceptible to stressful growth conditions than robust stationary phase cells, so the lag time of the pathogen results to be extended when the inoculum size is small and the need for even higher initial population for the pathogen to exit the lag phase and start divining. However, at high initial inoculum populations the lag phase may also be influenced by other factors related to population density, like the cell-to-cell communication *via* chemical signaling, also known as quorum sensing. Bacterial cell have the ability to produce and sense diffusible signal molecules named autoinducers the concentration of which may determine the growth behavior (Koutsoumanis et al., 2004; Robinson et al., 2001). Regarding the singly-cultured strains, the 50% of C5 cells in SP managed to divide for the first time within the first 30 to 40 min of incubation, while the 50% of cells in DP divided for the first time within the first 40 to 50 min. Singly-cultured 6179 observed to be slower than C5, thus the 50% of singly-cultured 6179 cells divided for the first time within the first 50 to 60 min and 60 to

70 min of incubation during culture in SP and DP, respectively (Fig. 3). Time to first division includes the lag time which determined by the two hypothetical quantities, the amount of work that a cell has to perform to adapt to new conditions and the rate at which it can perform that work (Robinson et al., 2001), so the observed difference on time to first division, between the different strains, may be due to the different work that each strain needs in order to adapt to the new substrate/environment, since the two strains seems to have similar growth rates (Table 2).

The co-culture of the different strains had no effect on the time to first division. The latter conclusion arose from the fact that the time of first division of the co-culture recorded in the middle of time to first division of the single cultures, both for cells in DP (50-60 min) and SP (40-50 min) (Fig. 3). The experimental procedure had ensured the uniform distribution of the different strains on the sample (the agar piece; Fig. 1) and afterwards, from each selected frame used multiple crops from different parts of the image to ensure the representation of similar number of cells from each strain. Additionally, the behavior of the strains during co-culture on TSA-YE, at population level, confirmed that the different strains did not interact and grew without the present of one strain to significantly impact the growth of the other and *vice versa* (Fig. 4). At population level, is recorded the growth kinetics of the subpopulation which has shorter lag phase and starts to divide having the faster growth rate, so there is not absolute connection between the observation of the lag phase's duration at single-cell level with the lag phase, at population level (Koutsoumanis and Lianou, 2013). In the present study, the behavior of the strains during co-culture, at population level, just confirms the observations at single-cell level. Interestingly, the results of the present study revealed that inter-strain interactions may influenced by storage/incubation temperature, since, during growth in TSB-YE was observed 1.3 log units difference between singly and co-cultured 6179 which was decreased during growth on TSA-YE (Fig. 4). During co-culture of the same strains, under aerobic conditions at 7°C, the observed interactions were more pronounced in TSB-YE (3.4 log units) and dairy-based broths and were mitigated with the addition of agar and the solidification of the substrates (2.3 log units difference of singly- and co-cultured 6179 on TSA-YE) (Gkerekou et al., 2022, 2021). For an inoculation level of 100 CFU/mL and above, interactions between colonies can be expected (Jeanson et al., 2015; Wimpenny et al., 1995) and as mentioned above may be due to cell–cell communication, which is accomplished through the production, release, detection and group-level response of an universal extracellular signaling molecule called autoinducer 2 (AI-2) responsible for interspecies communication termed quorum sensing (QS) (Song et al., 2018; Zhang et al., 2017). The signal molecules are secreted at a basal level during bacterial growth and as the density of a bacterial population increases, the AI-2 concentration in the external environment also accumulates and once a critical threshold concentration is reached, bacteria respond by changes in gene expression regulating a variation of bacterial processes and/or characteristics (Skandamis and Nychas, 2012; Song et al., 2018; Zhang et al., 2017). However, incubation temperature of microorganisms appears to

influence AI-2 production, differently. According to Ahmed et al. (2008) the optimal growth temperature for *Streptococcus intermedius* (39°C) was possibly not the best for AI-2 production (highest levels at 37°C) and at 35°C, AI-2 displayed the lowest values. In *Edwardsiella tarda* and *Escherichia coli* AI-2 production was reported to decrease with increasing temperature, while for *Lactobacillus* the activity of AI-2 in relation with temperature was species specific (DeLisa et al., 2001; Yeo et al., 2015; Zhang et al., 2008). The latter theory is also supported by the detection of the S-ribosylhomocysteine lyase during the investigation of the produced proteins during the co-culture of the same strains (C5 and 6179) at 7°C, under aerobic conditions (unpublished data).

Overall, the 76.2% and 70.5% of singly-cultured C5 cells, in SP and DP, managed to divide at least once, within the first 2h of incubation, while the corresponding numbers for singly-cultured 6179 were 95.3% and 91.2%, respectively (Table 3). Additionally, the 23.8% and 29.5% of singly-cultured C5 cells, in SP and DP, did not divided within first 2h incubation, while the corresponding numbers for singly-cultured 6179 were 4.7% and 8.8%, respectively (Table 3). The results may indicate that the population of C5 consists of 2 sub-populations, one that is faster than 6179 and one that may be so slow that it's time to first division did not recorded within the first 2 h of incubation. On the contrary, the greater percentage of the singly-cultured cells of 6179 presented less variability on time to first division. By decreasing the relative distance between cells (cells in DP), the percentages of both cells that managed to divide at least once and cells that performed second division, within the first 2h of incubation, either remained the same (percentage of cells that divided for the first time during co-culture) or in most cases decreased (Table 3). For Wu et al. (2000) the number of cells that did not divided within the period of experiment (3.5 h) was less than 5% of the total cell population, and they hypothesized that these cells can be considered dead, metabolically inactive or injured.

Conclusions

With the present work, it was shown at singly-cell level that the relative proximity of cells may influence the time to first division *via* the use of a method which enable the observation and the recording of a large number of cells simultaneously. Moreover, the results obtain by the co-culture of the studied strains at population-level, indicate that the phenomenon of inter-strain interactions and the underpinning mechanism(s) may affected by the temperature. The phenomenon of inter-strain interactions between different strains of *L. monocytogenes* has been proven (Gkerekou et al., 2022, 2021; Zilelidou et al., 2015; Zilelidou, Manthou et al., 2016) and its study is worth being advanced. The in-depth description of the phenomenon is important, because differential expression/production of metabolites may have an impact on pathogenicity behavior and niche adaptation of cells and might be a key and worth further investigating strain-specific feature**.**

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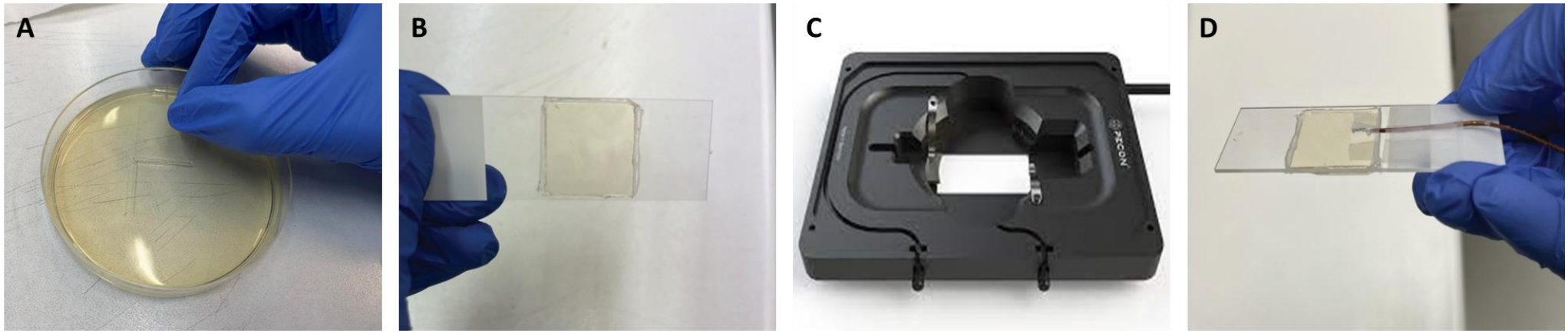

Figure 1. Schematic representation of sample preparation prior to moniting.

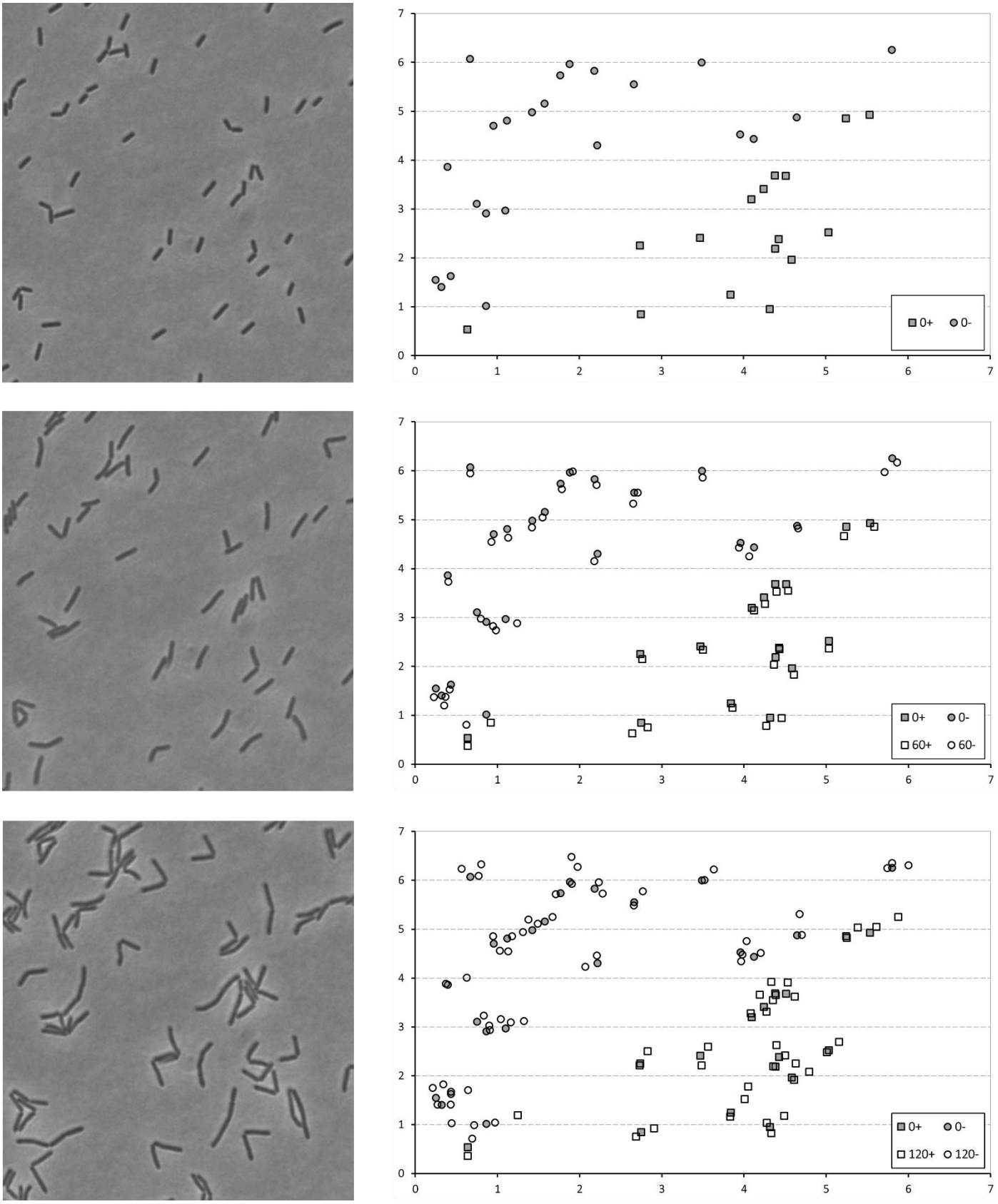

Figure 2. Representative output of the program, written in-house, regarding the place of individual cells in the different images of a time-lapse, towards incubation time.

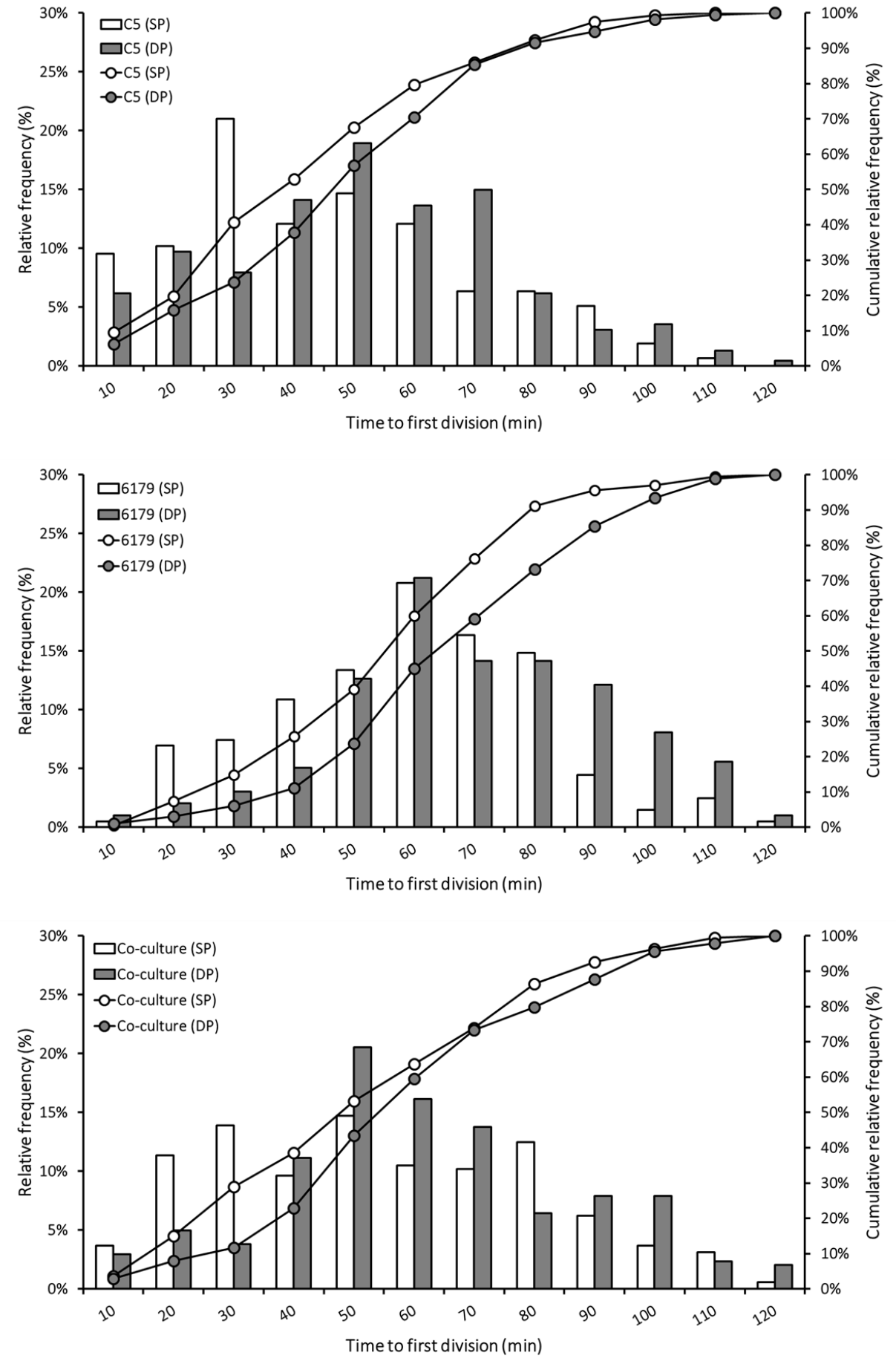

Figure 3. Relative Frequency (%) and Cumulative Relative Frequency (%) of the different times of first division of the singly-cultured and co-cultured *L. monocytogenes* strains C5 and 6179, distributed in sparse and dense proximity.

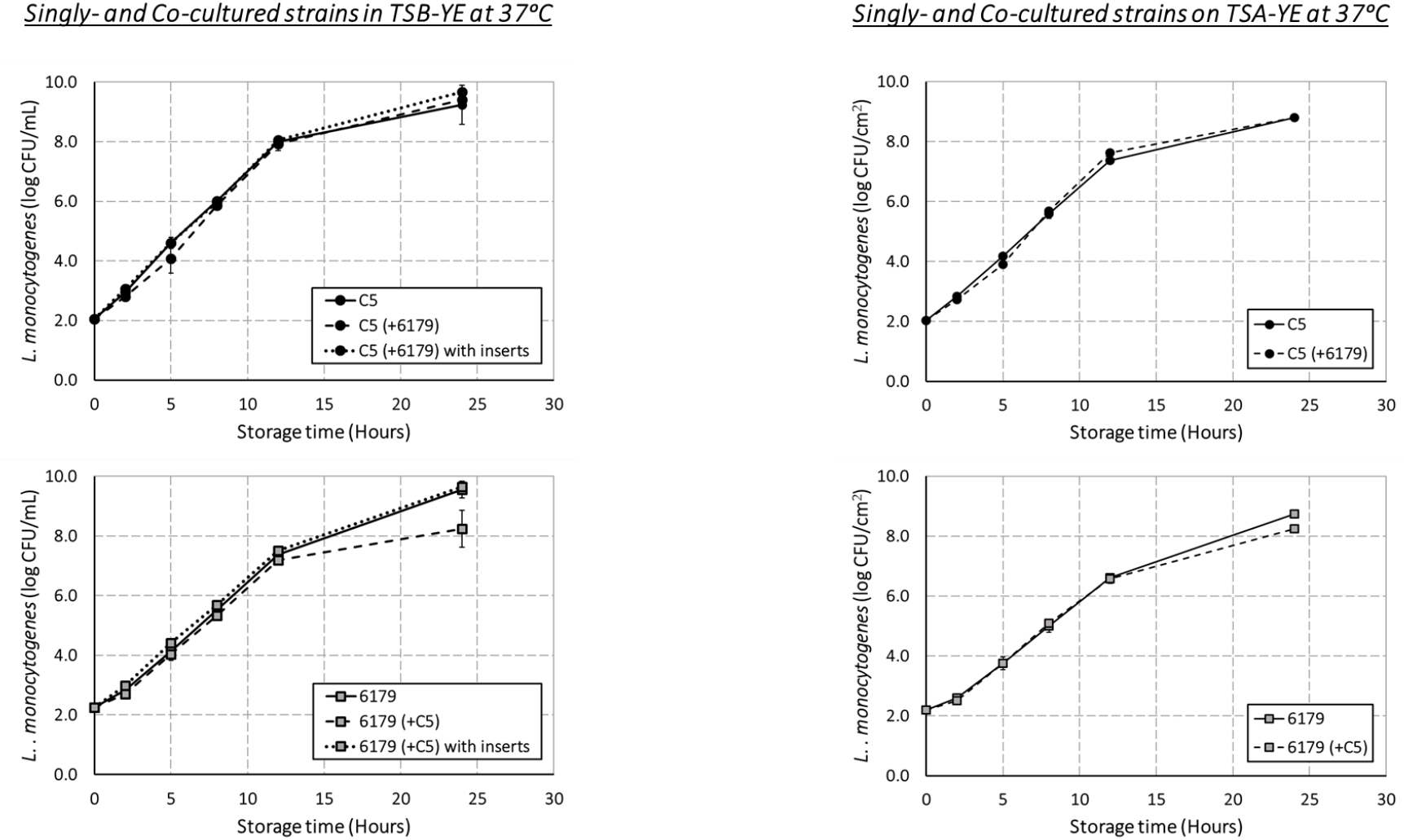

Figure 2. Growth curves of singly- and co-cultured *L. monocytogenes* strains C5 and 6179 in TSB-YE at 37°C, under aerobic conditions.

Table 1. *Listeria monocytogenes* strains used in the study.

Strain*	Serotype	MLST	Source	Year of isolation	Reference	Antibiotic resistance (μg/mL)**	Assembly level	Accession number
C5	4b	ST2	Dairy farm environment isolation	2007	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Streptomycin (2000)	Contig	NZ_MDQI00000000
6179	1/2a	ST121	Cheese	1999	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Rifampicin (>800)	Complete Genome	CP098509 HG813249

*Strain selection due to their pronounced inter-strain interaction according to Gkerekou et al. (2021, 2022).

**Approximate MIC was considered as the minimum tested concentration (μg/mL) of antibiotic at which no bacterial growth was observed after 24 hours at 30°C. Bacterial growth was confirmed through measurements of optical density (OD_{600}). The streptomycin concentrations were 0, 125, 250, 500, 1000, 2000, 4000 μg/mL. Rifampicin was evaluated at 0, 200, 400, 800 μg/mL.

Table 2. Estimated growth kinetics (lag time and growth rate) and observed final population of singly- and co-cultured *L. monocytogenes* strain C5 and 6179, in TSB-YE under aerobic conditions, at 7^{o}C.

Substrate	Type of culture*	Lag time (Hours)	Growth rate ($Hours^{-1}$)	Final population (log CFU/mL or cm^2)
TSB-YE	**Single C5**	0.3 ± 0.3	0.52 ± 0.03	9.2 ± 0.7
	Single 6179	0.8 ± 0.4	0.46 ± 0.02	9.5 ± 0.3
	C5 (+6179)	0.9 ± 0.4	0.53 ± 0.02	9.4 ± 0.2
	6179 (+C5)	1.1 ± 0.3	0.46 ± 0.01	8.2 ± 0.6
	C5 (+6179) with inserts	0.1 ± 0.1	0.51 ± 0.01	9.7 ± 0.0
	6179 (+C5) with inserts	0.4 ± 0.1	0.45 ± 0.00	9.7 ± 0.1
TSA-YE	**Single C5**	0.3 ± 0.1	0.46 ± 0.00	8.8 ± 0.0
	Single 6179	1.1 ± 0.0	0.41 ± 0.01	8.7 ± 0.1
	C5 (+6179)	0.9 ± 0.1	0.51 ± 0.01	8.8 ± 0.1
	6179 (+C5)	1.2 ± 0.3	0.41 ± 0.00	8.2 ± 0.1

* Single culture or co-culture.

Table 3. Singly- and co-cultured *L. monocytogenes* cells, in sparse and dense proximity, that managed to perform first and second division or did not divide, within the first 2 hours of incubation.

Percentages (%)	C5		6179		Co-culture	
	Sparse	**Dense**	**Sparse**	**Dense**	**Sparse**	**Dense**
Cells that divided for the **1st time** within the first **2 h** of incubation at **37°C**	76.2	70.5	95.3	91.2	78.4	78.9
Cells that did not divided within the first **2 h** of incubation at **37°C**	23.8	29.5	4.7	8.8	21.6	21.1
Cells that divided for the **2nd time** within the first **2 h** of incubation at **37°C**, over those that divided	77.7	66.5	55.4	31.8	61.5	54.5
Cells that did not divided for the **2nd time** within the first **2 h** of incubation at **37°C**, over those that divided	22.3	33.0	45.0	68.2	38.5	45.5

CHAPTER 7

Identifying the intracellular proteins which may be involved in the inter-strain interaction phenomenon between different L. monocytogenes strains during co-culture

Unpublished manuscript

Identifying the intracellular proteins which may be involved in the inter-strain interaction phenomenon between different *L. monocytogenes* strains during co-culture

Maria A. Gkerekou[a], Stavros Proikakis[ab], George T. Tsangaris[b] and Panagiotis N. Skandamis[a]*

Laboratory of Food Quality Control and Hygiene, Department of Food Science and Human Nutrition, Agricultural University of Athens, Greece[a]

Proteomics Research Unit, Biomedical Research Foundation, Academy of Athens, Athens, Greece[b]

Abstract

Due to the ubiquitous character of *Listeria monocytogenes* multiple strains of the pathogen may end up co-existing in/on the same final products affecting their growth capacity. The present study recorded the intracellular proteins of singly and co-cultured strains, aiming to identify proteins that may potentially influence the growth of individual *L. monocytogenes* strains in a dual strain composite. Based on previous studies, *L. monocytogenes* strains, C5 (4b) and 6179 (1/2a) were selected due to the remarkable interaction, which was observed during their co-culture. The selected strains were inoculated (2.0 log CFU/mL) in Tryptic Soy Broth with 0.6% Yeast Extract (TSB-YE) in single and two-strain cultures (1:1 strain ratio). Bacterial growth was assessed during storage at 7°C, under aerobic conditions (AC). Their resistance to different antibiotics enabled the selective enumeration of each strain in the co-culture. The 17th day of storage, single and dual cultures were centrifuged and performed protein extraction from the biomass of the individual samples and two dimensional gel electrophoresis (2-DE). Subsequently, the gel spots of the individual proteins were characterized by Matrix-Assisted Laser Desorption Tandem TOF Mass Spectrometer (MALDI-TOF-MS) analysis. According to our results, identified 36, 33 and 67 intracellular proteins from singly-cultured C5, 6179 and the co-culture, respectively. Interestingly, 41 out of the 67 proteins were uniquely recorded during co-culture. The identified proteins belong to 21 function categories with 48.4% of them belonging in 3 groups, translation, carbohydrate transport and metabolism and nucleotide transport and metabolism. Among the identified proteins, recorded the luxS enzyme, which has being connected to the population density regulation *via* quorum sensing and multiple "moonlighting" proteins a subset of multifunctional proteins that are primarily intracellular, but perform a second biochemical function in other cellular locations, mostly on the cell surface. The findings shed more light on the mechanism behind the inter-strain interactions of *L. monocytogenes* indicating that growth inhibition *via* quorum sensing mechanism or multifunctional proteins acting as antimicrobials may influence the behavior of the co-existing strains.

Keywords: Listeria monocytogenes, inter-strain interactions, intracellular proteins, quorum sensing, moonlight proteins

Introduction

Foodborne diseases continue to be public health concern and a factor causing industry losses. Special concerns are addressed towards *L. monocytogenes,* a Gram-positive, non-sporulating, facultative anaerobic bacterium, which is considered as one of the most severe foodborne pathogens. *L. monocytogenes* is the aetiological agent of the invasive systemic illness listeriosis caused by the consumption of contaminated leafy greens, ice cream, deli meat and cheeses like Brie and Camembert and enoki mushrooms according to CDC for the year 2022. In the European Union (EU), listeriosis is the fifth most commonly reported zoonosis in humans, with a notification rate of 0.49 cases per 100,000 population in 2021, 14% higher than the rate of 0.43 in 2020. According to the latest available report from EFSA and ECDC (EFSA, 2022), a total of 2,183 reported cases of human invasive listeriosis (923 hospitalized and 196 deaths) were observed in the EU, with meat products from bovines or pigs, fruits and vegetables, and sheep's milk cheeses accounting for the highest values (from 2 to 5%). Although the number of infection per year are moderately low, the mortality among infected individuals is very high, reaching 20 to 30 %, mainly in immunocompromised individuals, elderly, pregnant women and newborns (de Noordhout et al., 2014; Radoshevich and Cossart, 2018).

L. monocytogenes is ubiquitous in the environment, thus previous studies have described the simultaneous presence and dissemination of multiple *L. monocytogenes* strains, which may have been introduced *via* raw materials at various time-points in the processing environment (Chambel et al., 2007; Martín et al., 2014; Martínez-Suárez et al., 2016; Ortiz et al., 2010; Thévenot et al., 2006; Zoellner et al., 2018). The pathogen may persist and spread, possibly ending to multiple strains co-existing in/on the same food products as reported in Latin-style fresh cheese, traditional Portuguese smoked meat sausage, called Alheiras or smoked salmon (Felício et al., 2007; Gendel and Ulaszek, 2000; Kabuki et al., 2004; Rychli et al., 2014a). More importantly, Tham et al. (2002, 2007) showed that different strains of *L. monocytogenes* may co-exist during infection, as they have been isolated from different sites (blood and meninges) of the infected patient or from a single blood sample, during investigation of listeriosis cases. Studies have already proved that if more than one strains of the pathogen may end up contaminate the same product, inter-strain interactions may occur and the growth of the "weak" strain will be inhibited by the presence of the "strong" strain, reaching lower population density, which will continue to grow unaffected by the presence of the second strain (Gkerekou et al., 2021, 2022; Zilelidou, Manthou, et al., 2016; Zilelidou et al., 2015). Exploring the mechanism of how different strains interact from a food safety perspective, is important, because the relative levels of each strain during storage may also determine the population of each strain at the end of an enrichment step and subsequently their probability of isolation on ALOA plates (i.e., the critical detection step), according to ISO 11280 (Zilelidou, Karmiri, et al., 2016). Thus, potential masking of certain strains throughout the detection process may hinder the tracing of the actual causative agent (strain) of an

outbreak, during epidemiological investigations, which may have slower growth during storage or enrichment procedure, yet more virulent. Microbial competition may be expressed in different forms and mechanisms, including directly through interference competition, where individual cells damage one another (active, chemical warfare) (Cornforth and Foster, 2013; Ghoul and Mitri, 2016; Powell et al., 2004). Microbes have evolved many phenotypes with which they can outcompete and displace their neighbors. Secretions to harvest resources, loss of costly genes whose products can be obtained from others or stabbing and poisoning neighboring cells (Ghoul and Mitri, 2016). Considering the above, the aim of the present study was to record the intracellular proteins in order to investigate if the observed inhibition of some *L. monocytogenes* strains during co-culture is due to a protein or enzyme and potentially describe the underling mechanism.

Materials and methods

L. monocytogenes strains

L. monocytogenes strains C5 and 6179 were selected based on previous studies, as ones that had shown evident strain-to-strain interaction (Gkerekou et al., 2021, 2022; Zilelidou et al., 2015; Zilelidou, Manthou et al., 2016). Furthermore, their innate resistance to streptomycin (Streptomycin Sulfate Biochemica, AppliChem) and rifampicin (Rifampicin, AppliChem) (Table 1), seemed not to affect their growth capacity and was useful for the selective enumeration of each strain during co-culture. The strains were obtained from the microorganism collection of the Laboratory of Food Quality Control and Hygiene of Agricultural University of Athens and their selection to the antibiotics was made according to the method described by de W. Blackburn and Davies (1994) (Table 1). The strains were maintained at -20°C in Tryptone Soy Broth (LAB004, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract (MC001, Lab M Limited, United Kingdom) (TSB-YE, pH: 7.1 ± 0.2), 20% glycerol and the appropriate concentration of rifampicin or streptomycin, depending on the strain. Streptomycin was used at 1000 μg/mL and rifampicin at 50 μg/mL. The concentration of antibiotics used, during their maintenance and for the preparation of the (selective) enumeration media, was the lowest in which the second strain (the one that was not resistant to the particular antibiotic) was unable to grow (Table 1).

Inoculum preparation

During the experiments, both strains were maintained on Tryptone Soy Agar (LAB011, Lab M Limited, United Kingdom) supplemented with 0.6% Yeast Extract, containing rifampicin (50 μg/mL; TSA-YE/R) or streptomycin (1000 μg/mL; TSA-YE/S) at 4°C and sub-cultured once a month. A single colony from a TSA-YE/S or TSA-YE/R stock culture of the target strain was transferred to 10 mL TSB-YE/S or TSB-YE/R and incubated for 24 h at 30°C and subsequently, 100 μL of each culture was transferred to fresh TSB-YE/S or

TSB-YE/R for 18 h incubation at 30°C to obtain stationary-phase cells with a density of *ca.* 10^9 CFU/mL. Following activation stage, strains were harvested by centrifugation (2463 x g for 10 min at 4°C) (Megafuge 1.0R, Heraeus, Buckinghamshire, England), washed twice and finally re-suspended in 10 mL of ¼ strength Ringer's solution (LAB M, Lancashire, UK). The level of the inoculum was determined by plating 100 µL from the appropriate decimal dilution of each strain on TSA-YE/S or TSA-YE/R and incubation at 37°C for 48 h.

Storage experiment

Falcon tubes (50 mL of volume) containing 40 mL of sterile TSB-YE were inoculated either with single or both strains (strain ratio of 1:1) listed in Table 1, at approximately 2.0 log CFU/mL and stored at 7°C for 17 days, in high precision (± 0.5°C) incubation chambers (MIR 153, Sanyo Electric Co., Osaka, Japan), under aerobic conditions produced by constant shaking on an orbital shaker, at 240 rpm (Shaker KS 130 basic, IKA-Werke GmbH & Co. KG, Germany) (Gkerekou et al., 2021; Noriega et al., 2008a). Each experiment was performed in two triplicate trials (n=6).

Intracellular protein extraction

At the 17th day of storage both singly- and co-cultured strains had reached stationary phase, while during co-culture, strain 6179 affected by the presence of C5, as previous described by Gkerekou et al. (2021) (Fig. 1). Both singly-cultured and co-cultured cells were harvested by centrifugation (2463 x g for 15 min at 4°C) (Megafuge 1.0R, Heraeus, Buckinghamshire, England), washed twice with 10 mL of ¼ strength Ringer's solution (LAB M, Lancashire, UK), re-suspended in 1 mL sample buffer No.1 (50 mM Tris-HCl pH 7.3) and finally transferred in an eppendorf tube. Subsequently, the cells were lysed with sonication (3 min, 60 W; Q125 Sonicator Qsonica) for 1 min with a 1 min break and the different samples remained in ice water throughout the process which lasted 5 min. Then, the samples were centrifuged at 13000 x g for 20 min at 4°C. At the end of the centrifugation, 700 µL of supernatant was transferred to a new eppendorf tube, 200 µL of TCA (Trichloroacetic acid) were added and after vortexing, the samples were incubated on ice for 1 h. After incubation, the samples were centrifuged again, the supernatant was discarded and 800 µL of frozen acetone were added to the samples followed by vortex. The samples stored at -20°C overnight. The next day they were centrifuged at 13000 x g for 15 min at 4°C. After the discard of the supernatant, the samples were left at room temperature allowing the evaporation of the acetone.

As previously described by Vaiopoulou et al. (2015), 200 µL sample buffer No.2 consisting of 7 M urea, 50 mM Tris-HCl (pH 8.8), 2 M thiurea, 2% CHAPS, 0.4% dithioerythritol (DTE) were added to the samples and the samples were treated with sonication 3 times for 1 min duration (60W, continuous) in order to break the mixture of proteins and acetone. For the next steps of the analysis, in each of the samples were calculated 500 µg of protein, by Bradford assay. Then, 9 µL of protease inhibitors mixture (Roche Diagnostics, Basel, Swiss) and sample buffer No.2 were added into the samples until final volume of 197.5 µL

and stored at -20°C overnight. The next day, 2.5 µL IPG buffer pH 3 - 10 (Amersham Biosciences) were added into the samples that were placed into sample cups at both acidic and basic ends of 18 cm stationary gradient strips pH 3 - 10 NL (Bio-Rad Lab, Hercules, CA). The strips had been previously rehydrated overnight in rehydration trays with 500 µL of a buffer (rehydration buffer) consisting of 8 M urea, 2% CHAPS and 0.4% DTE at each strip. First dimensional electrophoresis focusing started at 250 V and the voltage was gradually increased to 5000 V at 3 V/min where it was kept constant for 28 h (approximately a total of 100,000 Vh). After the first dimension electrophoresis, each of the strips was treated with a solution consisting of 12.5 mL Tris-HCl 1.5 M pH 8.8, 20mL of acrylamide solution 30% SDS, 17 mL of distilled water and 500 µL of a SDS solution 20%. After that, 500 µL of an APS solution 10% and 50 µL of TEMED were added to the solution in order to start the polymerization and create the 12% SDS-polyacrylamide gels (180 × 200 × 1.5 mm^3) for the second dimension electrophoresis. The vertical electrophoresis was carried out with a run of 40 mA/gel, using PROTEIN-II multicell apparatuses (Bio-Rad). After second dimension electrophoresis (2D), the gels were fixed in 50% methanol containing 5% phosphoric acid overnight. The fixative solution was washed off by agitation in distilled water for 45 min. For the visualization of the protein spots, Coomassie Blue G-250 staining solution (Novex, San Diego, CA) was used on gels overnight. The gels were scanned in a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA) with the use of the scanning application/tool of the PD-Quest v8.0 software (Bio-Rad, Hercules, CA) and they stored on computer for further analysis (Cravatt et al., 2007).

Gel image analysis

Gel images were analyzed as already described by Zografos et al. (2019). All gels' protein spots analyzed, were detected, aligned, matched and using the PD-Quest v8.0 image processing software, according to the instructions of the manufacturer. Manual inspection of the spots was used for the verification of matching's accuracy.

Protein identification by mass MALDI-TOF-MS

For Matrix-Assisted Laser Desorption Tandem TOF Mass Spectrometer (MALDI-TOF-MS) analysis, proteins spots of interest were manually annotated using Melanie 4.02 software and excised from 2-DE gels using Proteiner SPII (Bruker Daltonics, Bremen, Germany) (Fig. 2). Gel pieces were then inserted into 96-well microtiter plates that were filled with 180 µL of 30% acetonitrile (ACN) in 50 mM ammonium bicarbonate. In-gel digestion was performed at room temperature for 16 h using 0.01 µg/µL trypsin (Roche Diagnostics, Basel, Switzerland). Next 10 µL of 50% ACN containing 0.1% trifluoroacetic acid (TFA) were added to each dried gel piece and the digested peptides were extracted from the gel. Tryptic peptide mixtures, 1µL of volume, were applied on an anchor chip MALDI plate mixed with 1 µL of matrix solution, consisting of 0.08% α-cyano-4-hydroxycinnamic acid (CHCA, Sigma) and internal standard peptides des-Arg-bradykinin (904.4681

Da, Sigma) and adrenocorticotropic hormone fragment 18-39 (2465.1989 Da, Sigma) in 50% distilled water, 50% ACN and 0.1% TFA. Peptide mixtures were analyzed in a MALDI-TOF MS as already described by Kolialexi et al. (2010). Laser shots (n = 400) of intensity between 40% and 60% were collected and summarized and the peak list was created by the Flexanalysis v2.2 software (Bruker). Smoothing was performed using the Savitzky-Golay algorithm (width 0.2 m/z, cycle number 1). S/N was calculated with the SNAP algorithm and a threshold ratio of 2.5 was allowed. Peptide matching and protein searches were performed automatically with using the MASCOT Server 2 (Matrix Science). Peptide masses were compared with the theoretical peptide masses of all available proteins from *L. monocyotgenes* in the UniProt database. Stringent criteria were used for protein identification with a maximum allowed mass error of 25 ppm and a minimum of 4 matching peptides. Probability score with $P < 0.05$ was the criterion for affirmative protein identification. Monoisotopic masses were used and one missed trypsin cleavage site was calculated for proteolytic products. The search parameters included a potential alteration of the residue mass due to the existence of carbamidomethylation and oxygenation. Redundant proteins which were found in databases with different names and accession numbers have been eliminated. If more than one protein was identified under one spot, the single protein member with the highest protein score was singled out from the multiprotein family.

Results and discussion

Interestingly, among the proteins produced during co-culture of the different *L. monocytogenes* strains (C5 and 6179) recorded the enzyme S-ribosylhomocysteine lyase or luxS (Table 2). To date there are no studies, elucidating the underlying mechanisms for competition between *L. monocytogenes* strains, so to our knowledge, this is the first study which indicate population density regulation *via* the mechanism of quorum sensing (QS) and particularly the production of autoinducer AI-2 (a furanosyl borate diester) by LuxS QS system, during inter-strain inhibitory interactions. So far, in the literature, only inhibitory intra- and inter-species interactionç have been attributed to QS. AI-2 is produced from S-adenosylmethionine (SAM) in three enzymatic steps. Briefly, SAM donates a methyl group and is converted to S-adenosylhomocysteine (SAH). The toxic SAH is then quickly removed by a nucleosidase to produce S-ribosylhomocysteine (SRH). Finally, SRH is transformed to homocysteine and 4, 5-dihydroxy-2, 3- pentanedione (DPD), which is the precursor of AI-2 that forms as a result of the spontaneous rearrangement of DPD, by S-ribosylhomocysteine lyase (LuxS) (Song et al., 2018). Among others, Dourou et al. (2011) showed the presence of AI-2 signaling compounds in the cell-free culture supernatants of *Yersinia enterocolitica*-like GTE 112, *Serratia proteamaculans* 00612, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844 and the effect on growth of two *Salmonella* Enteritidis and two *S.* Typhimurium strains. During single-culture and co-culture of *Lactobacillus acidophilus* and *L. monocytogenes* EGD-e, the growth of the latter significantly affected by the co-culture. Both after incubation

with viable *L. monocytogenes* cells and by addition of cell-free culture supernatant of *L. monocytogenes* the transcriptional levels of *luxS* of *L. acidophilus* were increased, whereas incubation with heat killed cells of *L. monocytogenes* had no effect on the transcriptional level. This could indicate that the up-regulation of *luxS* is due to a response to a secreted compound produced by *L. monocytogenes* cells (Moslehi-Jenabian et al., 2011). Rios-Covian et al. (2018) found a slight but significantly lower final counts of *L. monocytogenes* and its *luxS* gene was over-expressed during co-culture with *Bifidobacterium.* During co-culture of different *L. monocytogenes* and *L. innocua* isolates in different enrichment media, without significant differences regarding the growth characteristics, the overgrowth of *L. innocua* demonstrated the possibility of an inhibitory interaction between these two species (Cornu et al., 2002). Moreover, in TSB-YE one co-culture of *L. innocua* and *L. monocytogenes* resulted in growth inhibition of the latter, while in pasteurized milk was always observed inhibition of the pathogen when *L. innocua* was present in higher concentrations (Carvalheira et al., 2010). While species-specific QS apparently allows recognition of self in a mixed population, it seems likely that bacteria also need a mechanism or mechanisms to detect the presence of other species. Additionally, it is conceivable that it is useful for bacteria to have the ability to calculate the ratio of self to other in mixed populations, and in turn, to specifically modulate behavior based on fluctuations in this ratio. Quorum sensing is a mechanism of cell-to-cell communication and is mediated by extracellular chemical signals generated by the bacteria when specific cell densities are reached, usually *ca.*10^6 cfu/ml. When the concentration of the signal (and cell population) is sufficiently high, the target gene or genes are either activated or repressed. Quorum sensing increases the ability of the bacteria to have access to nutrients or to more favorable environmental niches and enhances bacterial defenses against eukaryotic hosts, competing bacteria, and environmental stresses or modulate a number of cellular functions (genes), including sporulation, biofilm formation, bacteriocin production or virulence response (Carvalheira et al., 2010; Federle and Bassler, 2003; Smith et al., 2004). Telesensing processes are now known to be influenced by environmental cues, including temperature, ligand concentration, pH, and water and oxygen availability (Bollinger et al., 2001; Roux et al., 2009; Shrout et al., 2006; Surette and Bassler, 1998).

All the intracellular proteins identified during the present study from both singly- and co-cultured cells of the pathogen after 17 days of storage at 7°C, under aerobic conditions, are listed in Table 2 and summarized by Venn diagram (Fig. 3). STRING analysis highlighted a network characterized by 32, 34 and 66 nodes and 142, 112 and 480 edges for C5, 6179 and the co-culture, respectively (Fig. 4). Specifically, were identified 91 proteins in total, belonging to 21 categories of function classification (Fig. 3; Table 2). A great percentage of the recorded proteins (41 out of 91; Fig. 3) appear to have been uniquely produced during co-culture, as a result of the coexistence of the different strains, while only 8 and 12 were recorded solely by singly-cultured C5 and 6179, respectively (Fig. 3). The category with the highest percentage of identified

proteins is “Translation” (26.4%), followed by “Carbohydrate transport and metabolism” and “Nucleotide transport and metabolism”, 11% each (Fig. 5; Table 2). Half of the “Translational” proteins were produced only during co-culture (12 out of 24) and most of them are ribosomal proteins (8 out of 13) (Table 2). According to Hurtado-Rios et al. (2022) some ribosomal proteins involved in protein translation have also shown other functionalities, including inhibiting infectious bacteria, viruses, parasites, fungi, and tumor cells. Therefore, they may be considered antimicrobial peptides (AMPs). The antimicrobial activity of ribosomal proteins may be associated with an increase in intracellular reactive oxidative species (ROS) in target cells, which, in turn, could affect membrane integrity and cause their inactivation and death (Chen et al., 2021; Qu et al., 2020). The ribosomal proteins that may present antimicrobial activity belong to the broader group of proteins called “moonlighting” proteins. Moonlighting proteins comprise a subset of multifunctional proteins that are primarily intracellular, but perform a second biochemical function in other cellular locations, mostly on the cell surface. Many of the known moonlighting proteins are cytosolic enzymes, chaperones, or other proteins and abundance of moonlighting functions exhibited by glycolytic enzymes and ribosomal proteins (Amblee and Jeffery, 2015; Henderson and Martin, 2011; Jeffery, 2019, 1999). Apart from the ribosomal proteins, in the present study, have been recorded additional proteins which have been characterized as “moonlighting” by different researchers and are marked in Table 2 with an asterisk (Table 2). Among the proteins that have been characterized as “moonlighting”, glucose-6-phosphate isomerase, triosephosphate isomerase and purine nucleoside phosphorylase DeoD-type are produced both by the singly-cultured C5 strain and during co-culture and could be responsible for the growth inhibition of 6179 strain during the co-culture (Fig. 1). Glucose-6-phosphate isomerase (GPI) was identified as novel adhesive moonlighting proteins of *L. crispatus* ST1. These proteins are released from the *L. crispatus* surface after cell trauma, under conditions of alkaline stress, or in the presence of the antimicrobial peptide LL-37 produced by human cells (Kainulainen et al., 2012). Apart from moonlighting proteins, uniquely during co-culture were also identified various recordings belonging to different functional categories (Table 2). PrsA2 is lipid modified and membrane-associated (cell wall integrity, swimming motility) but a significant amount of is also secreted. The PrsA2 secretion chaperone has been shown to contribute to multiple aspects of *L. monocytogenes* physiology and virulence (the expression of *prsA2* is directly regulated by the transcriptional activator PrfA), enabling the pathogen to survive under multiple stress conditions that likely interfere with protein folding at the membrane-cell wall interface (Alonzo et al., 2009; Cahoon et al., 2022; Cahoon and Freitag, 2015). Pyrophosphatase (Ppax) plays a role in DNA repair, modulating the intracellular pyrophosphate pool and has been found only in response to cold stress (D’Onofrio et al., 2023). RecA protein have a key role in DNA replication, recombination and repair and has been implicated in the regulation of bacterial virulence. Specifically, RecA regulated the expression of virulence genes by modulating transcriptional regulator PrfA

activity. Additionally, RecA has been implicated in the regulation of stress, including responses to heat shock and DNA damage (D'Onofrio et al., 2023).

Conclusions

The phenomenon of inter-strain interactions between different strains of *L. monocytogenes* has been proven (Gkerekou et al., 2022, 2021; Zilelidou et al., 2015; Zilelidou, Manthou et al., 2016) and its study is worth being advanced. The present results constitute the first solid indication of QS mediated growth inhibition. Along with the identification of the intracellular proteins which performed during the present study, future characterization of the secreted proteins and the proteins located on the bacterial wall surface during co-culture, appear to be the next steps in the investigation of the underpinning mechanism(s) of inter-strain interactions and how are they related to each other (QS, Contact-dependent inhibition). The in-depth description of the phenomenon is important, because differential expression/production of metabolites may have an impact on pathogenicity behavior and niche adaptation of cells and might be a key and worth further investigating strain-specific feature**.**

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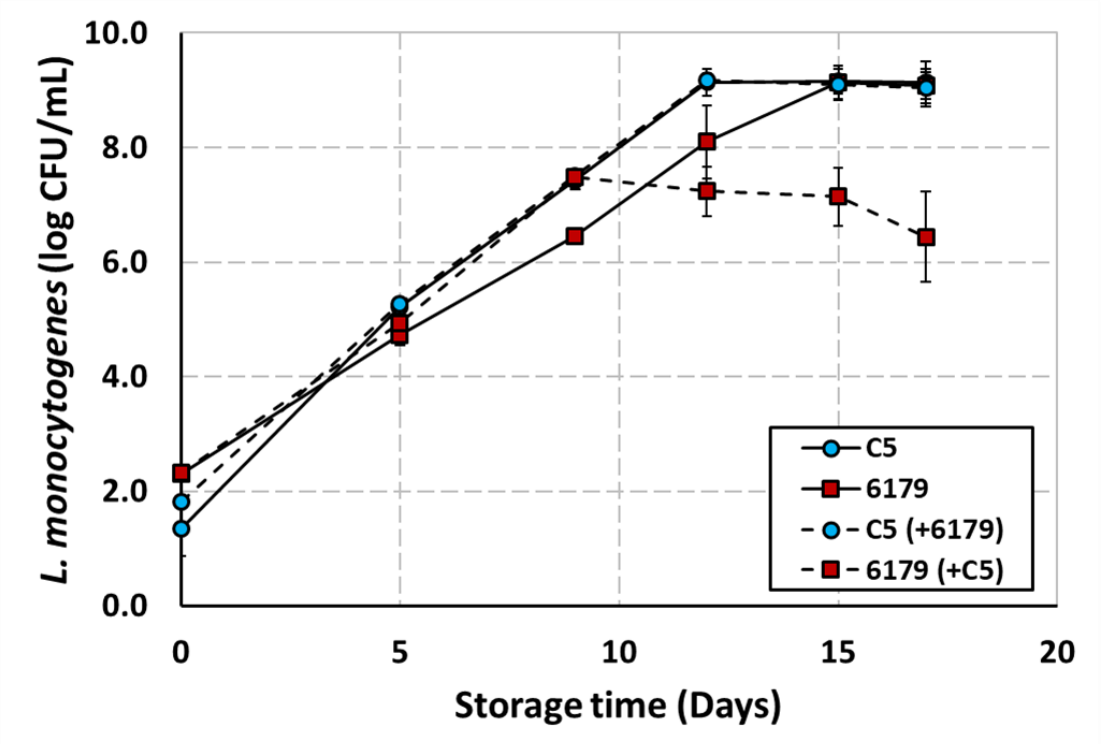
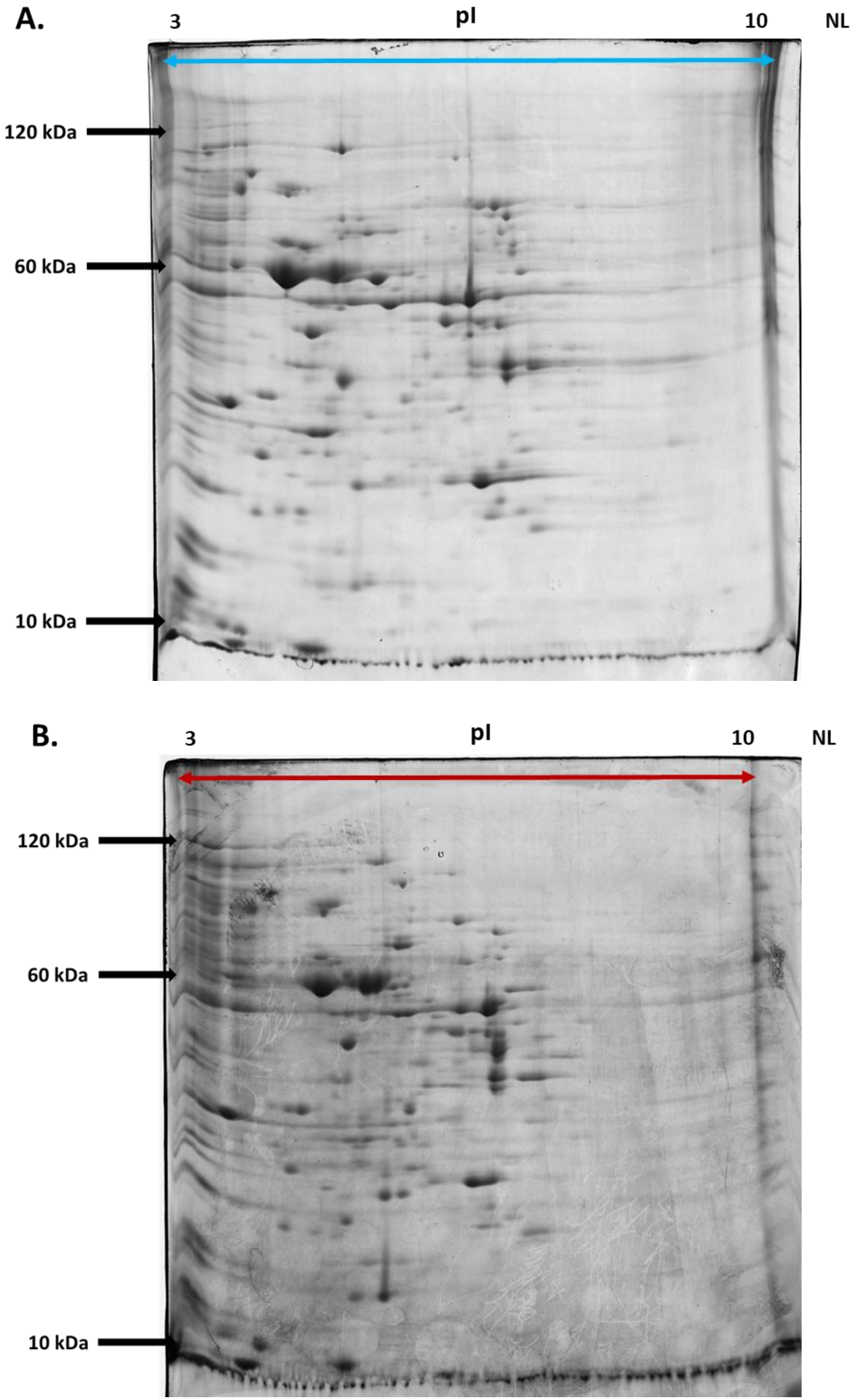


Figure 1. Growth curves of singly- and co-cultured *L. monocytogenes* strains C5 and 6179 in TSB-YE at 37°C, under aerobic conditions.


A.
3
pI
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B.
3
pI
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NL
120 kDa
60 kDa
10 kDa

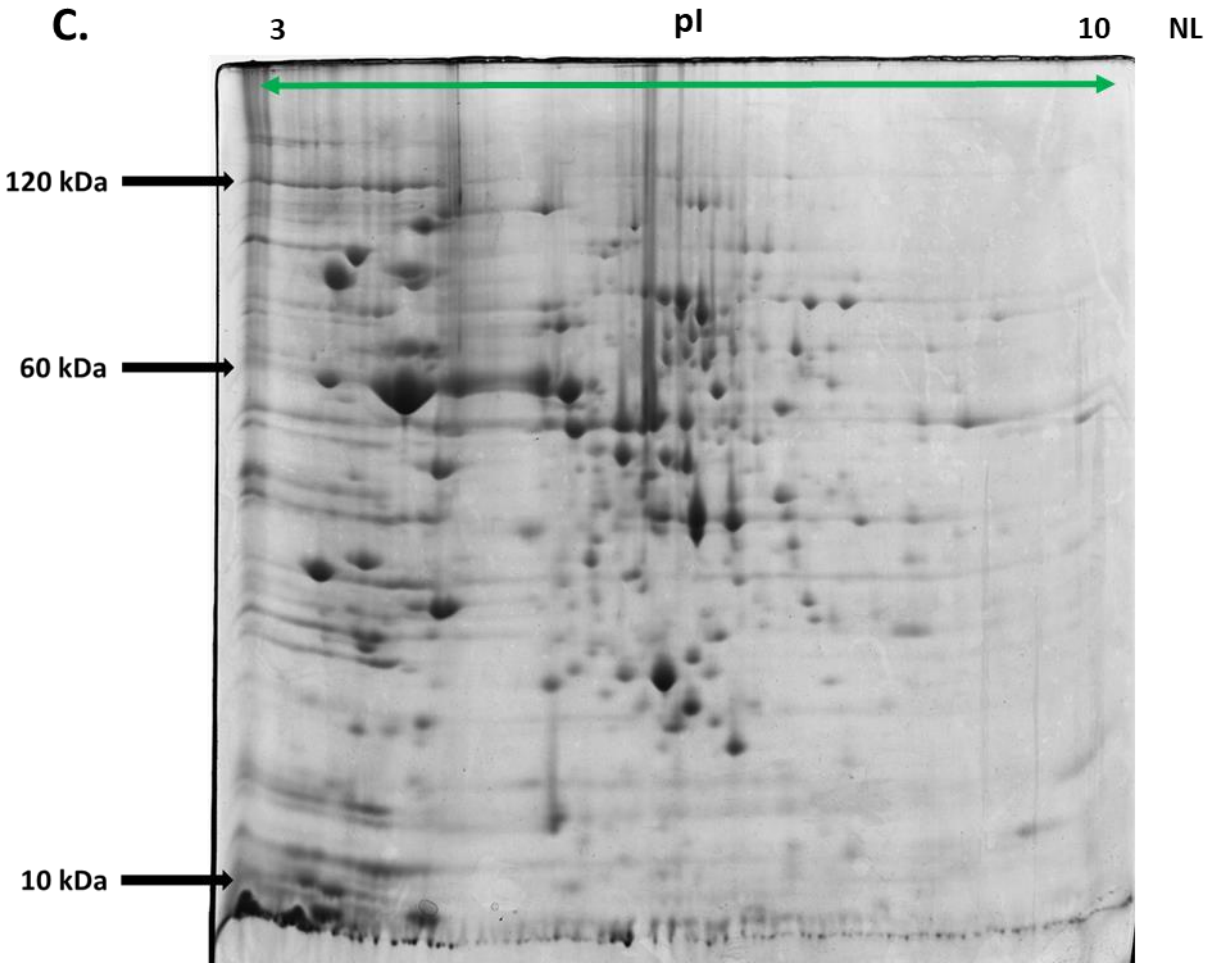


Figure 2. Proteins extracted by the singly-cultured (A and B) and co-cultured (C) strains of *L. monocytogenes* C5 (A) and 6179 (B) analyzed by 2-DE. The gels were stained with Coomassie blue and protein spots were excised and further analyzed by MALDI-TOF-MS, as described in the Materials and Methods section.

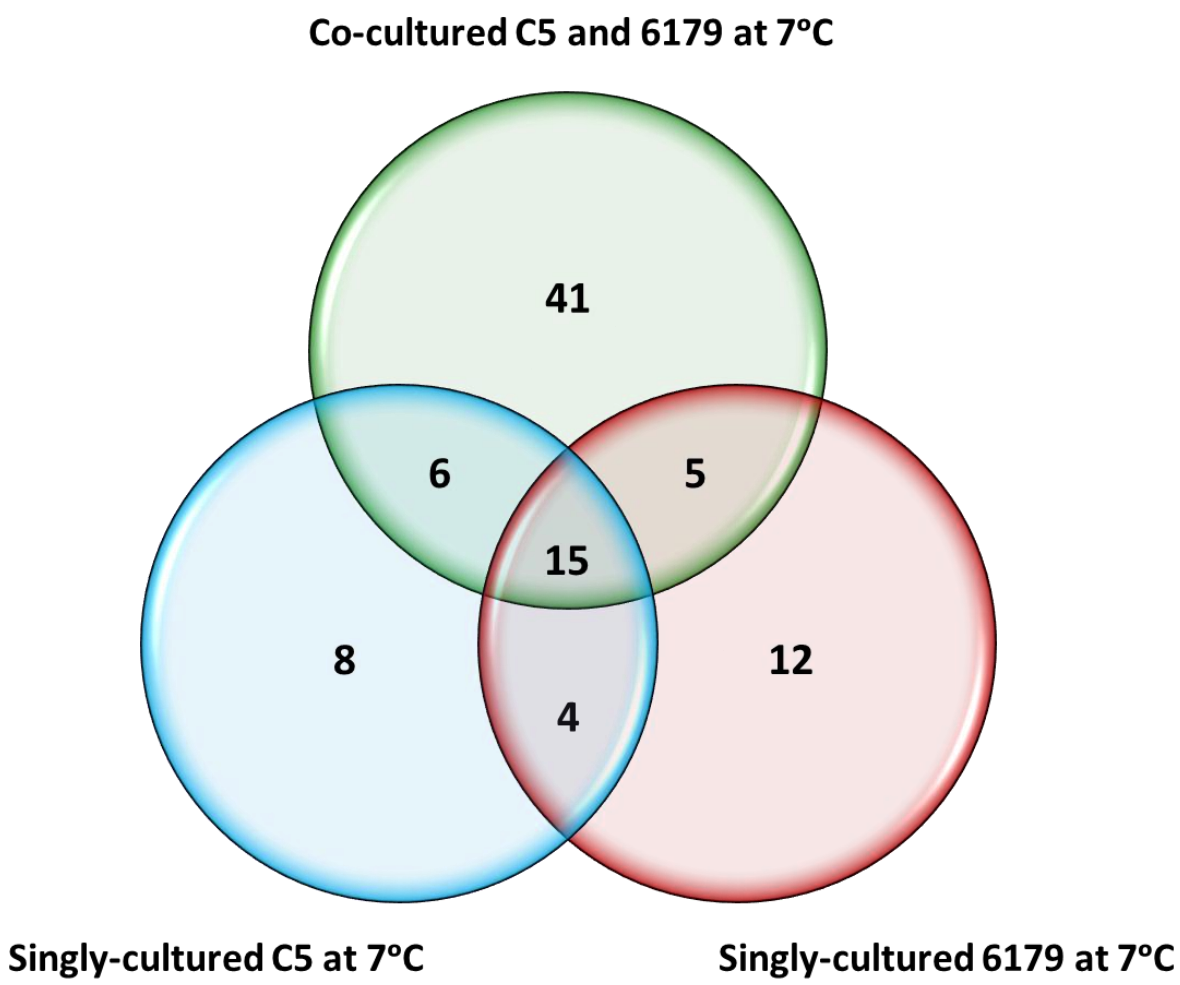
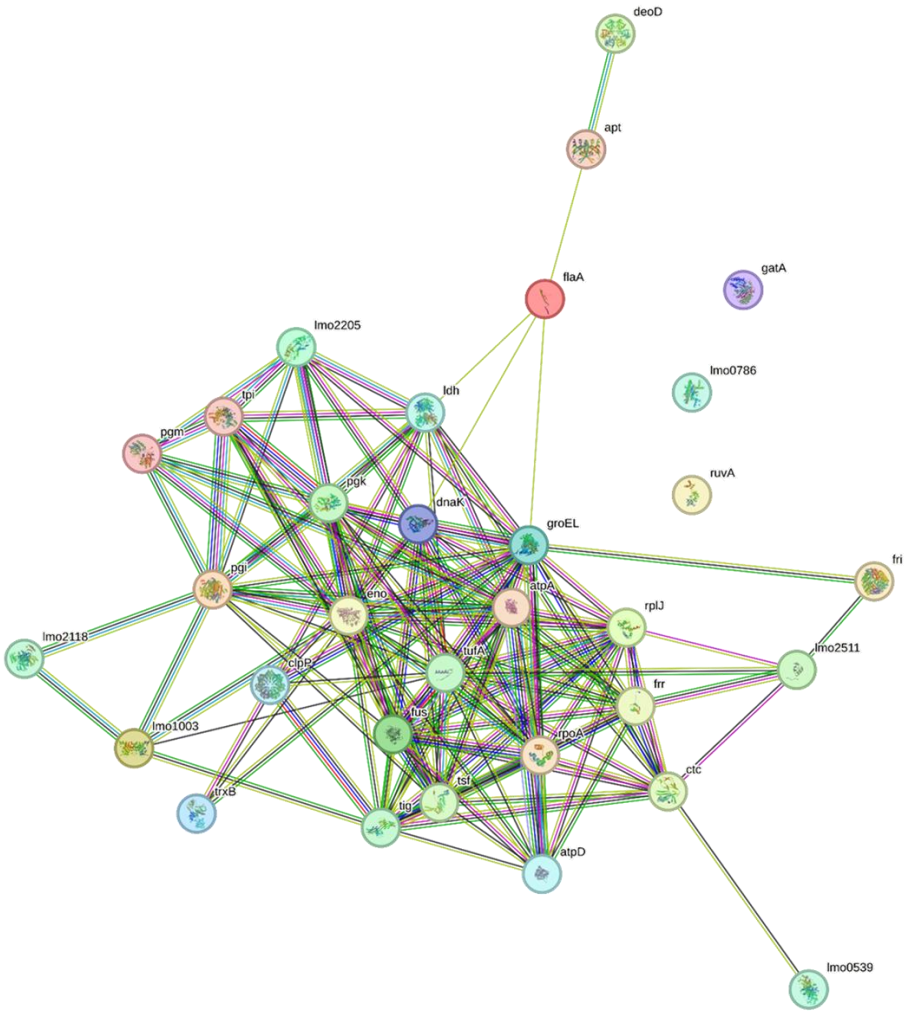


Figure 3. List of identified proteins of singly-cultured and co-cultured *L. monocytogenes* strains C5 and 6179 in TSB-YE at 7°C summarized by Venn diagram.

A.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flaA
gatA
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lmo0786
tpi
ldh
pgm
ruvA
pgk
dnaK
groEL
fri
pgi
eno
atpA
rplJ
lmo2118
tufA
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frr
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tsf
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tig
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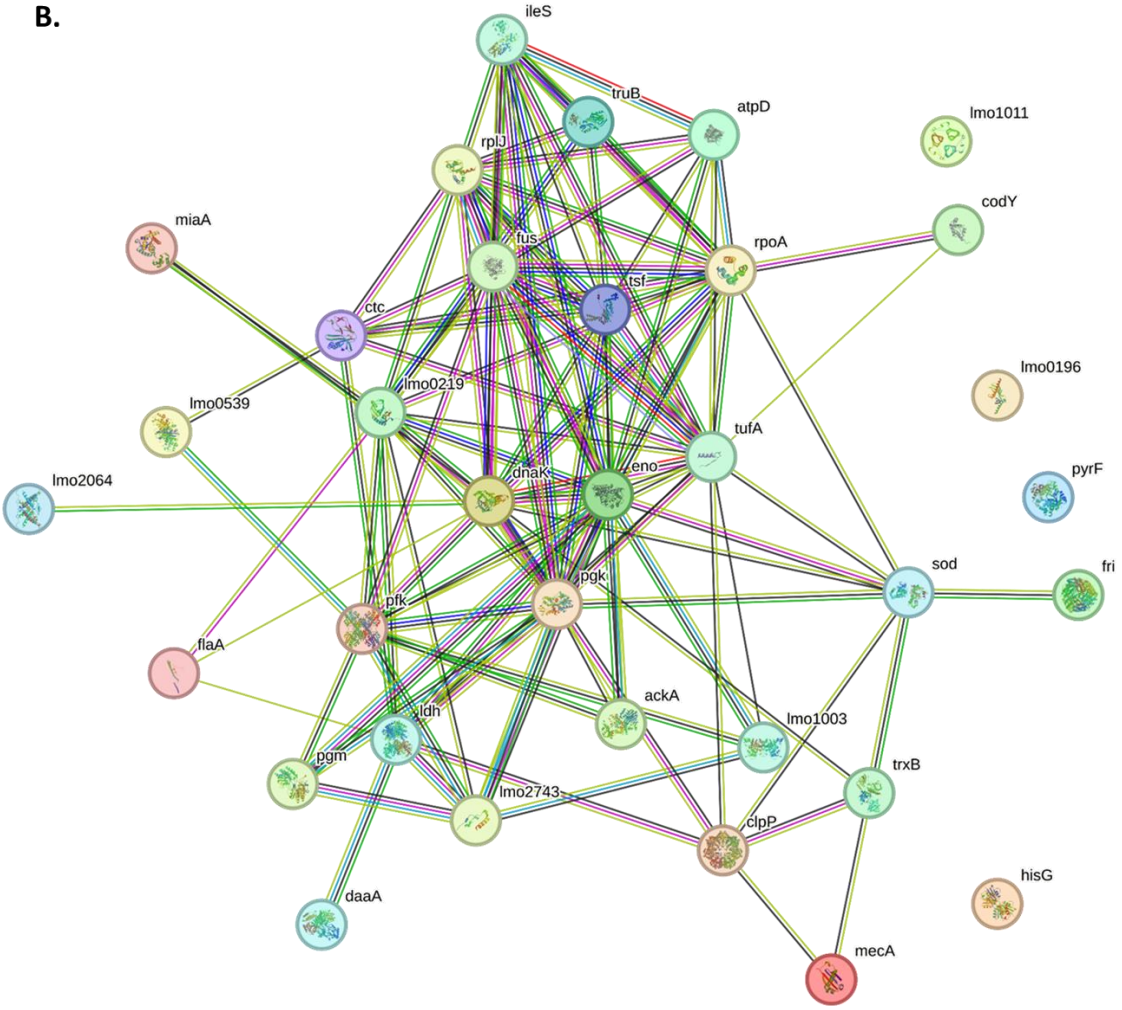
B.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atpD
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rplJ
codY
miaA
fus
rpoA
tsf
ctc
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tufA
pyrF
lmo2064
dnaK
eno
sod
fri
pgk
pfk
flaA
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ldh
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pgm
trxB
lmo2743
clpP
hisG
daaA
mecA

C.

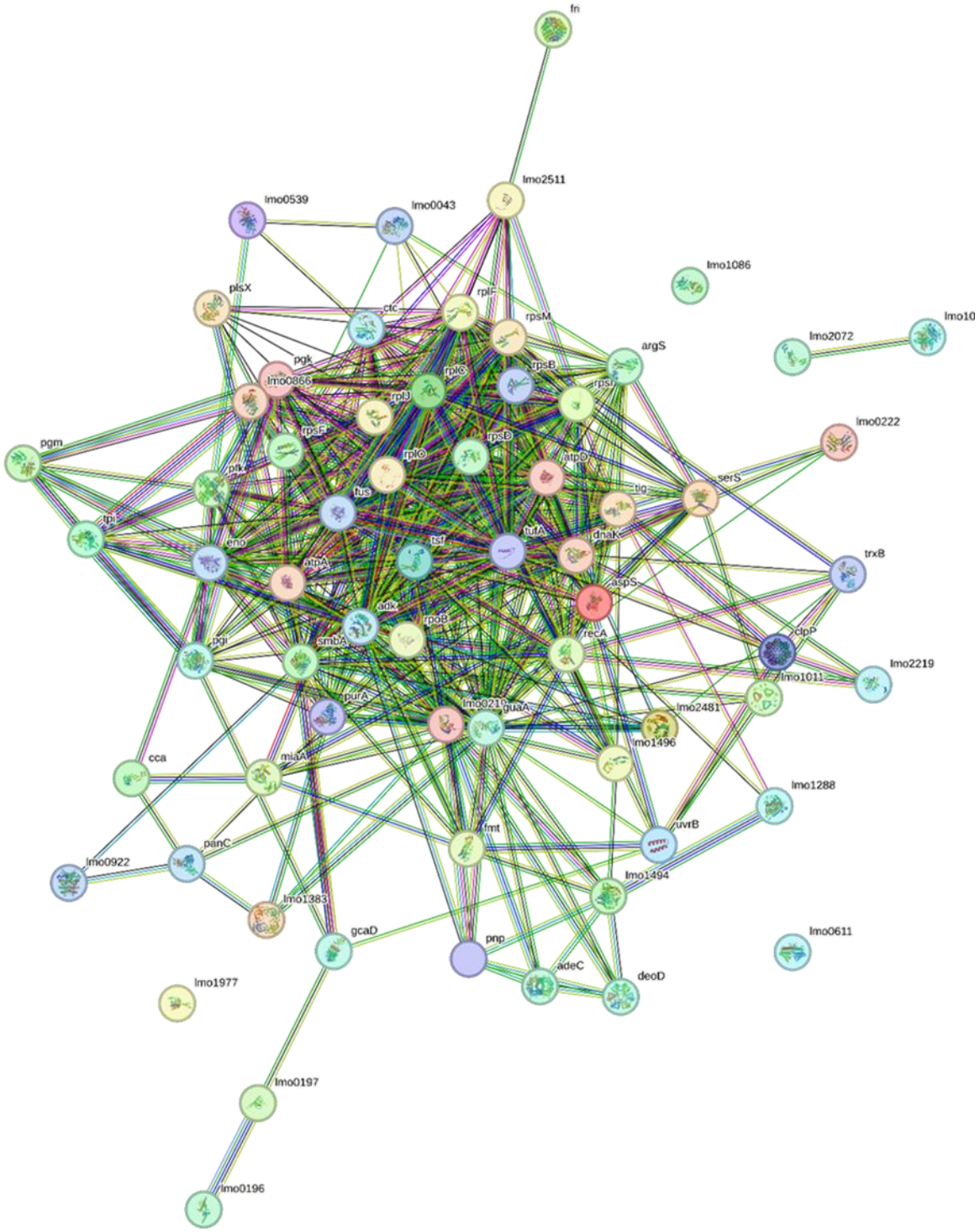

Figure 4. Network diagrams created using STRING v12.0 to show the protein–protein interactions of the identified proteins from singly-cultured (A and B) and co-cultured (C) *L. monocytogenes* strains C5 nad 6179 in TSB-YE at 7°C. Nodes represent proteins, while edges indicate their interactions. The edges are color-coded to represent different types of interactions, including known (pink and light blue), predicted (green, red, and blue), and other (yellow, black, and grey) interactions.

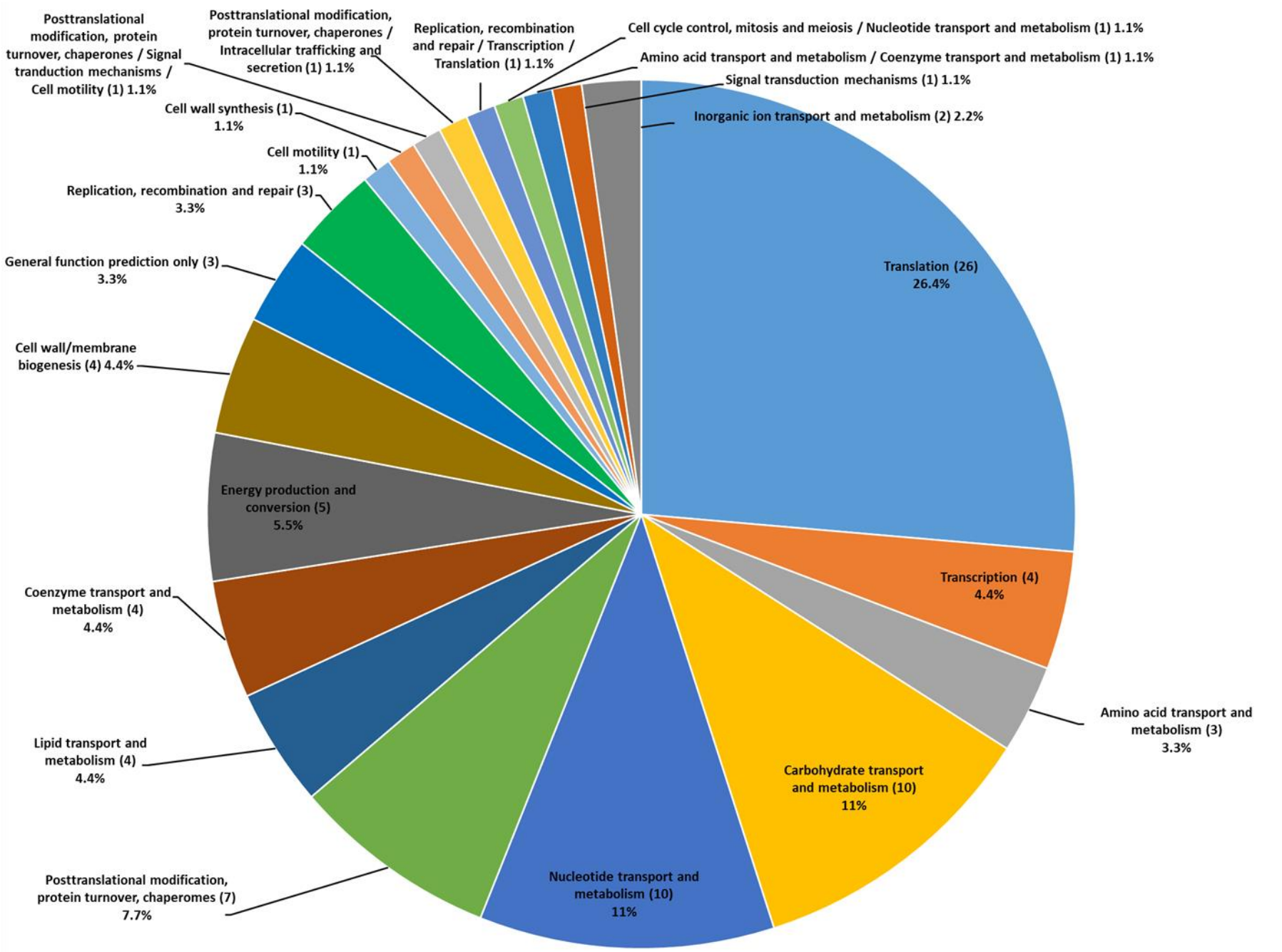

Figure 5. Pie chart illustrating the distribution of the 91 unique identified proteins by their predicted function classification.

Table 1. *Listeria monocytogenes* strains used in the study.

Strain*	Serotype	MLST	Source	Year of isolation	Reference	Antibiotic resistance (μg/mL)**	Assembly level	Accession number
C5	4b	ST2	Dairy farm environment isolation	2007	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Streptomycin (2000)	Contig	NZ_MDQI00000000
6179	1/2a	ST121	Cheese	1999	Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland	Rifampicin (>800)	Complete Genome	CP098509 HG813249

*Strain selection due to their pronounced inter-strain interaction according to Gkerekou et al. (2021, 2022).

**Approximate MIC was considered as the minimum tested concentration (μg/mL) of antibiotic at which no bacterial growth was observed after 24 hours at 30°C. Bacterial growth was confirmed through measurements of optical density (OD_{600}). The streptomycin concentrations were 0, 125, 250, 500, 1000, 2000, 4000 μg/mL. Rifampicin was evaluated at 0, 200, 400, 800 μg/mL.

Table 2. List of proteins produced by singly- and co-cultured *L. monocytogenes strains* C5 and 6179 during culture in TSB-YE under aerobic conditions at 7°C.

Function classification[a]	Locus[a]	Gene[a]	Protein name[b]	Produced by			UniProt[c]
				C5	6179	Co-culture	
Translation	*lmo2608*	*rpsM*	30S ribosomal protein S13			+	P66383
	lmo1658	*rpsB*	30S ribosomal protein S2			+	Q8Y6M6
	lmo1596	*rpsD*	30S ribosomal protein S4			+	Q8Y6T6
	lmo0044	*rpsF*	30S ribosomal protein S6			+	Q8YAR9
	lmo2596	*rpsI*	30S ribosomal protein S9*			+	Q8Y459
	lmo0250	*rplJ*	50S ribosomal protein L10	+	+	+	P66042
	lmo2613	*rplO*	50S ribosomal protein L15			+	Q8Y447
	lmo0211	*rplY/ctc*	50S ribosomal protein L25	+	+	+	Q8YAD3
	lmo2632	*rplC*	50S ribosomal protein L3			+	Q8Y440
	lmo2617	*rplF*	50S ribosomal protein L6			+	Q8Y444
	lmo2561	*argS*	Arginine--tRNA ligase			+	Q8Y493
	lmo1519	*aspS*	Aspartate--tRNA ligase			+	Q8Y709
	lmo1905	*cca*	CCA-adding enzyme			+	Q8Y5Z8
	lmo2654	*fusA*	Elongation factor G *	+	+	+	Q8Y421
	lmo1657	*tsf*	Elongation factor Ts *	+	+	+	Q8Y6M7
	lmo2653	*tuf*	Elongation factor Tu *	+	+	+	Q8Y422
	lmo1755	*gatA*	Glutamyl-tRNA(Gln) amidotransferase subunit A *	+			Q8Y6D2
	lmo2019	*ileS*	Isoleucine--tRNA ligase		+		Q8Y5N8

	lmo1823	*fmt*	Methionyl-tRNA formyltransferase			+	Q8Y676
	lmo2511	*hpf*	Ribosome hibernation promotion factor	+		+	Q927Y2
	lmo1314	*frr*	Ribosome-recycling factor *	+			Q8Y7G7
	lmo2747	*serS*	Serine--tRNA ligase			+	Q8Y3T4
	lmo1294	*miaA*	tRNA dimethylallyltransferase		+	+	Q8Y7I3
	lmo1328	*truB*	tRNA pseudouridine synthase B		+		Q8Y7F3
Transcription	*lmo2606*	*rpoA*	DNA-directed RNA polymerase subunit alpha	+	+		P66699
	lmo0258	*rpoB*	DNA-directed RNA polymerase subunit beta			+	Q9RLT9
	lmo1280	*codY*	GTP-sensing transcriptional pleiotropic repressor CodY		+		Q8Y7J7
	lmo1496	*greA*	Transcription elongation factor GreA			+	P64277
Amino acid transport and metabolism	*lmo1011*	*dapH*	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransfe		+	+	Q8Y8A1
	lmo0568	*hisG*	ATP phosphoribosyltransferase		+	+	Q8Y9G0
	lmo0043	*arcA*	Arginine deiminase			+	Q8YAS0
Carbohydrate transport and metabolism	*lmo2205*	*gpmA*	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	+			Q8Y571
	lmo2456	*gpmI*	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	+	+	+	Q8Y4I4
	lmo1571	*pfkA*	ATP-dependent 6-phosphofructokinase		+	+	Q8Y6W0
	lmo2455	*eno*	Enolase *	+	+	+	P64074
	lmo2367	*pgi*	Glucose-6-phosphate isomerase *	+		+	Q8Y4R7
	lmo1003	*ptsI*	Phosphoenolpyruvate-protein phosphotransferase	+	+		O31149
	lmo2458	*pgk*	Phosphoglycerate kinase *	+	+	+	Q8Y4I2
	lmo2743	*tal1*	Probable transaldolase 1		+		Q8Y3T8

	lmo0539	*lacD*	Tagatose 1,6-diphosphate aldolase	+	+	+	Q8Y9I9
	lmo2457	*tpiA1*	Triosephosphate isomerase 1 *	+		+	Q8Y4I3
Nucleotide transport and metabolism	*lmo1742*	*ade*	Adenine deaminase			+	Q8Y6E5
	lmo1524	*apt*	Adenine phosphoribosyltransferase	+			P0A2X5
	lmo2611	*adk*	Adenylate kinase			+	Q8Y449
	lmo0055	*purA*	Adenylosuccinate synthetase			+	Q8YAR1
	lmo1096	*guaA*	GMP synthase [glutamine-hydrolyzing]			+	Q8Y822
	lmo1953	*pnp*	Polyribonucleotide nucleotidyltransferase			+	Q8Y5V2
	lmo1856	*deoD*	Purine nucleoside phosphorylase DeoD-type*	+		+	Q8Y644
	lmo1313	*pyrH*	Uridylate kinase			+	P65927
	lmo1494	*mtnN*	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase			+	Q8Y729
	lmo1832	*pyrF*	Orotidine 5'-phosphate decarboxylase		+		P58641
Posttranslational modification, protein turnover, chaperones	*lmo0222*	*hslO*	33 kDa chaperonin			+	Q8YAC4
	lmo2068	*groEL*	60 kDa chaperonin*	+			Q9AGE6
	lmo1473	*dnaK*	Chaperone protein DnaK*	+	+	+	P0DJM2
	lmo2219	*prsA2*	Foldase protein PrsA 2			+	Q8Y557
	lmo1583	*tpx*	Thiol peroxidase		+		Q8Y6U8
	lmo2478	*trxB*	Thioredoxin reductase	+	+	+	O32823
	lmo1267	*tig*	Trigger factor	+		+	Q8Y7L0
Lipid transport and metabolism	*lmo0611*	*acpD*	FMN-dependent NADH-azoreductase 1			+	Q8Y9C1
	lmo0786	*azoR2*	FMN-dependent NADH-azoreductase 2	+			Q8Y8V6

	lmo1809	*plsX*	Phosphate acyltransferase			+	Q8Y688
	lmo1086	*tarI*	Ribitol-5-phosphate cytidylyltransferase			+	Q8Y832
Coenzyme transport and metabolism	*lmo2211*	*cpfC*	Coproporphyrin III ferrochelatase	+	+	+	Q8Y565
	lmo1046	*moaC*	Cyclic pyranopterin monophosphate synthase			+	Q8Y871
	lmo0922	*coaA*	Pantothenate kinase			+	Q8Y8I0
	lmo1901	*panC*	Pantothenate synthetase			+	Q8Y602
Energy production and conversion	*lmo1581*	*ackA1*	Acetate kinase 1		+		Q8Y6V0
	lmo2531	*atpA2*	ATP synthase subunit alpha 2	+		+	Q8Y4C0
	lmo2529	*atpD2*	ATP synthase subunit beta 2	+	+	+	Q8Y4C1
	lmo1383	*fni*	Isopentenyl-diphosphate delta-isomerase			+	Q8Y7A5
	lmo0210	*ldh1*	L-lactate dehydrogenase 1 *	+	+		P33380
Cell wall/membrane biogenesis	*lmo0198*	*glmU*	Bifunctional protein GlmU			+	Q8YAD4
	lmo2064	*mscL*	Large-conductance mechanosensitive channel		+		Q8Y5J6
	lmo0196	*spoVG1*	Putative septation protein SpoVG 1		+	+	Q8YAD5
	lmo0197	*spoVG2*	Putative septation protein SpoVG 2 *			+	Q92F70
General function prediction only	*lmo2481*	*ppaX*	Pyrophosphatase PpaX			+	Q8Y4G3
	lmo2072	*rex*	Redox-sensing transcriptional repressor Rex			+	P60384
	lmo1977	*rnz*	Ribonuclease Z			+	Q8Y5S8
Replication, recombination and repair	*lmo1533*	*ruvA*	Holliday junction ATP-dependent DNA helicase RuvA	+			Q8Y6Z7
	lmo1398	*recA*	Protein RecA			+	P0DJP0
	lmo2489	*uvrB*	UvrABC system protein B			+	Q8Y4F5

Cell motility	*lmo0690*	*flaA*	Flagellin	+	+		Q02551
Cell wall synthesis	*lmo2118*	*glmM*	Phosphoglucosamine mutase	+			Q8Y5E6
Posttranslational modification, protein turnover, chaperones / Signal transduction mechanisms / Cell motility	*lmo2190*	*mecA*	Adapter protein MecA		+		Q9RGW9
Posttranslational modification, protein turnover, chaperones / Intracellular trafficking and secretion	*lmo2468*	*clpP*	ATP-dependent Clp protease proteolytic subunit	+	+	+	Q9RQI6
Replication, recombination and repair / Transcription / Translation	*lmo0866*	*cshA*	ATP-dependent RNA helicase CshA			+	Q8Y8N0
Cell cycle control, mitosis and meiosis / Nucleotide transport and metabolism	*lmo0219*	*tilS/hprT*	Bifunctional protein TilS/HprT		+	+	Q8YAC7
Amino acid transport and metabolism / Coenzyme transport and metabolism	*lmo1619*	*dat*	D-alanine aminotransferase		+		P0DJL9
Signal transduction mechanisms	*lmo1288*	*luxS*	S-ribosylhomocysteine lyase			+	Q8Y7I9
Inorganic ion transport and metabolism	*lmo0943*	*dps*	DNA protection during starvation protein	+	+	+	Q8Y8G1
	lmo1439	*sodA*	Superoxide dismutase [Mn]*		+		P28764

* Proteins characterized as "*moonlighting*" by *Mujahid et al. (2007), Portillo et al. (2011) and Schaumburg et al. (2004)*

[a] Functional classification and unique alphanumeric identifier used to represent the *locus* and gene according to Galperin et al. (2021), Glaser et al. (2001), Lenz et al. (2003), listiwiki.uni-goettingen.de and UniProt database (Release 2023_03).

[c] Full protein name recommended by the UniProt database consortium (Release 2023_03).

[d] Unique alphanumeric identifier of each entry in UniProt database (Release 2023_03).

CHAPTER 8

General discussion – Concluding remarks

General discussion – Concluding remarks

Listeria monocytogenes is a ubiquitous environmental microorganism that "*knows how to survive*". As a saprophytic bacterium thriving in diverse environments, managing to survive and grow in a wide range of harsh environmental conditions renders it a major concern for ready-to-eat (RTE) products. Previous studies have described the simultaneous presence and dissemination of multiple *L. monocytogenes* strains, which may have been introduced *via* raw materials at various time-points in the processing environment. The pathogen may persist and spread, possibly ending to multiple strains co-existing in/on the same food products and subsequently during ingestion, where the pathogen switch to an invasive intracellular bacterium. Zilelidou et al. (2016a, 2016b, 2015) has already prove that during simultaneous present of more than one strains in the same substrate may occur inter-strains interaction resulting in growth inhibition for one strain of the dual composite. Thus, in the present thesis described how substrate's structure, the different levels of oxygen availability due to the different types of packaging and/or the site of the contamination and the nutritional characteristics may influence the occurred inter-strains interactions. According to the results of **Chapters 2**, **3** and **4,** in combination with the studies of Zilelidou et al. (2016b, 2015) it seems that after a critical population density close to 6.0 log CFU/mL *L. monocytogenes* strains C5 (belonging to serotype 4b) and PL25 (belonging to serotype 1/2b), regardless of growth conditions, seem to arise as strong competitors and their growth and the final population level did not affected by the presense of the second strains during the co-culture. On the other hand, during co-culture strains 6179 (belonging to serotype 1/2a) and ScottA (belonging to serotype 4b), under many of the studied conditions, showed that influenced by the present of the second strain, reaching lower final population compared with the population density that reached during single-culture, under the same experimental conditions. In **Chapter 2**, comparing the effect of the three media structures of the study (liquid, semi-solid and solid substrates based on TSB-YE), on inter-strain interactions, the inhibition was more pronounced in liquid substrates and strains 6179 and ScottA reached lower final populations compared to those observed in the semi-solid and in/on solid media, regadless of oxygen availability. In **Chapter 3,** during the evaluation of inter-strain interactions in different dairy-based substrates of different structure, under aerobic and hypoxic conditions the observed interactions, were more pronounced in dairy-based broths and were mitigated with the addition of agar and the solidification of the dairy-based substrates. In the absence of oxygen, no interactions were observed until the end of storage, except for the dual cultures of strains ScottA and C5 with strain PL25, where the presence of the latter resulted in restriction of strains' ScottA and C5 growth, thus remaining at the level of initial population. However, the addition of agar, where the structure from broth become semi-solid or solid, resulted in the elimination of the observed interactions. The results of **Chapter 4** highlight the importance

of evaluating the behavior of foodborne pathogens in real foods and under as "realistic" conditions as possible. As previously (**Chapter 2** and **3**), in Ricotta observed growth inhibition of strains 6179 and ScottA, however on Camembert both singly and co-cultured strain 6179 did not manage to reach high population density, by the end of storage. Cells of competing strains grown planktonically have higher chances to interact, possibly expressing contact mediated inhibition, while in parallel, are exposed to the metabolic-end products of the faster growing strain. Thus, the inter-strain interactions between co-cultured strains, according to our results, is more likely to be evident or occur in liquid/broth media as compared to semi-solid and solid substrates. Studies have shown that contact-dependent inhibition (CDI) may occur mainly in Gram-negative bacteria, such as *Escherichia coli*, and this has been demonstrated in shaking liquid culture (Aoki et al., 2005). The same study suggested that growth inhibition among a 'weak' and a 'strong' strain requires that cell come in direct contact, and not only via their metabolome, e.g., when inoculating the weak strain in the spent medium of the strong one, or separating competing strains by an impermeable membrane. Aoki et al. (2005) support the possibility that the secreted molecule, responsible for the inhibition phenotype, is unstable and is only effective when delivered to target cells in close proximity. However, recent studies have found that CDI is not restricted to Gram-negative bacterial but may also occur in Gram-positive bacteria, including Listeria (Haeys et al., 2010).

A major result of **Chapter 3** is that in mixed cultures, most of the observed inter-strain interactions were more pronounced in/on Ricotta than in/on Camembert-based substrates, indicating that the available nutrients and the physicochemical characteristics of each matrix may affect the level of the final population reached and subsequently, the difference between the final cell density of singly and co-cultured strains. Substrates that have undergone proteolysis (as Camembert) are reported to stimulate growth of *L. monocytogenes,* as it happens when the pathogen coexists with the highly proteolytic *Pseudomonas* (Marshall & Schmidt, 1991). Moreover, due to the lower moisture content of Camembert, the resulting broth was thicker, compared to that produced by Ricotta, while the homogenization that took place during the preparation of the substrates, perhaps, reduced the diameter of the fat globules resulting in their greater and more uniform dispersion throughout the mass of the substrate, presumably creating higher number of smaller spaces within the aqueous phase of the substrate, making it more "difficult" for the strains of the dual cultures to interact. The growth behavior of singly- and co-cultured strains observed during growth under axonic conditions in **Chapter 3** is due to the nutritional characteristics of the substate and their abiliy to consume the available nutrients under the specific storage conditions. Beyond nutrient composition, matrix plays an important role on nutrient release and their bioavailability (Fardet et al., 2019). The importance of nutrients was also studied in **Chapter 5**, in which was assessed the impact of the presence of glucose (TSB with and TSB without dextrose) and the presence of yeast extract (a mixture of amino acids, peptides, water soluble vitamins and carbohydrates; TSB-YE without dextrose)

on inter-strain interactions. The presence of yeast extract resulted in higher final population for both singly-cultured strains (C5 and 6179) compared with the final population reached during culture in TSB without dextrose. In the absence of glucose during co-culture, the growth inhibition of strain 6179 by the presence of C5 was more pronounced. Specifically, in TSB without dextrose and in TSB-YE without dextrose the difference in the final population between the singly and co-cultured 6179 was 3.6 and 6.3 log units, respectively. During co-culture in TSB-YE, the growth inhibition of strain 6179 by strain C5 was 2.7 log units. Among the different mechanisms that have been proposed to explain the interactions between different populations that may coexist in a food product, "Jameson Effect" has been used to describe the non-specific competition for nutrients (Jameson, 1962; Ross et al., 2000). According to numerous studies, different species or strains within a microbial community race to consume the available nutrients of the substrate to maximize their population density. When those resources are depleted by a single "dominant" species or strain, the race is "over" and the growth of the "weaker" species or strain practically ceases (Baka et al., 2014; Buchanan and Bagi, 1997; Costa et al., 2020; Guillier et al., 2008; Mellefont et al., 2008). However, "Jameson effect" seems rather unsuitable to describe the observations of the present thesis. The renewal of nutrients in the middle of storage, by the addition of fresh TSB-YE, did not seem to be enough to enable strain 6179 to overcome the suppressing effect of the presence of the strain C5 and the difference in the final population during the co-culture remained constant throughout the extra storage time. The latter indicates that nutrient deficiency does not appear to explain inter-strain interactions (**Chapter 5**).

In the present thesis, in **Chapter 4**, also evaluated the survival of strains co-cultured in/on different dairy products prior to the exposure to simulated gastric fluid (SGF) until mid-exponetial and until early-stationary phases. Regardless of the growth phase (both mid-exponential and early stationary cells were exposused to SGF) at which the different single and co-cultured strains were exposed to the SGF (pH 2.0, 37°C), the reduction of pathogens' population on Camembert cheese pieces was faster than the reduction in Ricotta samples, the first minutes of the challenge, due to the site of contamination. Camembert pieces were surface inoculated resulting in the bacterial cells coming in direct contact with the low pH of SGF, in contrast with the bacterial cells found in the mass of Ricotta's samples. On one hand, the effect of co-culture, partly, lies in the fact that due to co-culture at different growth phases the population of each strain is different. So, the occurred inter-strain interactions determine the population of each strain upon entry in the gastric fluid. As a result, despite the similar inactivation rates, the populations of two competing strains in SGF could be different at each time point due to differences in their initial cell density. On the other hand, co-culture could also have and direct effect on survival after exposuse to SGF. It was observed that strain ScottA influenced by the presence of the second strains and showed enhanced resistance to low pH, reaching similar population with the singly-cultured even though the co-

cultured ScottA was exposed to the SGF at a lower initial population. In addition, co-cultured strains, even though exposed to SGF at the same initial population that the respective singly-cultured strains ScottA and PL25, they presented a trend to be more sensitive to the low pH during the exposure to the SGF.

As mentioned above contact of cells seems to be a key parameter for the phenomenon of inter-strain interactions. Specifically, singly- and co-cultured C5, with or without the presence of inserts, reached similar final population levels (**Chapter 5**). Nevertheless, co-cultured 6179 reached 0.8 and 5.2 log units lower population, with and without the presence of inserts, respectively, compared with the population of the same singly-cultured strain by the end of storage. Growth inhibition even in the presence of the inserts suggests an additional mechanism beyond the CDI and the competition for nutrient resources, like the production of metabolic by-products by one strain, which may inhibit the growth of the others. The chemical composition of the cell-free spent medium (CFSM) of the individual single cultures and that of co-culture of strains C5 and 6179 was characterized by Fourier transform infrared (FTIR) attenuated total internal reflection (ATR) spectroscopy and compared, to assess whether the interactions may be due to the production of specific secreted compounds. The obtained FTIR-ATR spectra reflected the biochemical composition of the CFSM and considered the molecular fingerprints of the metabolome of the singly-cultured C5 and 6179 and their co-culture (**Chapter 5**). According to the obtained results, the individual spectral profiles of the metabolome became more complex and the differences or the similarities between the CFSM produced by the singly- and the co-cultured strains became more evident as storage progressed. Evaluating the spectra of the CFSM on the 17th day of storage, where the interaction between the two strains of the co-culture was very pronounced, the profile of the CFSM produced from the singly-cultured C5 was more complex, while the spectra from the CFSM produced from the singly-cultured 6179 and the co-culture shared a lot of similarities. Among the results obtained from the evaluation of the different spectra, a metabolic compound, represented by the bands 1457 and 1454 cm^{-1} was obtained only from the CFSM of the single-cultured C5 and 6179, respectively, and was absent from the CFSM of the co-culture, probably indicating that the present of a second population may affect the production of this particular metabolite. Additionally, in the CFSM of the singly-cultured C5 there were recorded derivatives which were not detected in the CFSM of the co-culture, at 1741, 1645 and 1223 cm^{-1}. The compound at 1645 cm^{-1} was a metabolic by-product, produced by singly-cultured C5 later during storage and was absent from the profile of the CFSM of the co-culture, indicating that this compound may have been infused into or mounted on the cells of strain 6179 and thus, was excluded from the spectrum of co-culture due to the removal of the cells by filtration. Finally, some metabolites represented by bands at 1518 and 1075 cm^{-1} were produced earlier during storage by the co-cultured strains. A hypothesis could be that their premature production is related to the presence of the second population and subsequently their increased concentration earlier during storage may

be related to the fact that the co-cultured *L. monocytogenes* strain 6179 was "forced" to enter the stationary phase at a lower population, compared to its single culture. The 17th day of storage, when all different cultures have reached the stationary phase, the above mentioned specific compounds were recorded in all spectra (**Chapter 5**). In parallel, in **Chapter 5**, was assessed the growth capacity of singly- and co-cultured strains in CFSM produced by the same singly- and co-cultured strains (inoculated all the combinations of singly-cultured and co-cultured strains in the different CFSM). It was obvious from the obtained results that the overall presence of metabolites in the culture medium seems to influence the growth of the pathogen, because both strains had different growth kinetics than during growth in fresh TSB-YE. Interenstlingly, even though C5 outgrew 6179 during co-culture in various studied substrates (**Chapters 2, 3, 4** and **5**), in this assessment, when cultured in the enriched CFSM produced from singly-cultured 6179, it did not manage to grow. The concentration of the metabolites considered to be critical because in the CFSM produced by co-culture, the metabolites of 6179 are present, but due to the lowest population density, they may not constitute a substantial percentage of the total metabolome. Singly and co-cultured 6179 grew in all different CFSM, indicating that the factors which affect its behavior during co-culture, could be also on the surface of the "*competitive*" cells and not secreted in the environment. Finally, during culture in the different CFSM, no considerable inter-strain interactions were observed, as both co-cultured strains grew similarly and at the same population density as in single culture (**Chapter 5**).

In **Chapter 6**, was evaluated the impact of relative cell proximity on time to first division *via* optical microscopy at single-cell level (singly- and co-cultured strains C5 and 6179 on agar at 37°C). Specifically, in the present chapter the experimental design aiming to capture the effect of co-culture on time to first division, using two relatively high but distinct levels of cell densities (cells in sparse and dense proximity, SP and DP, respectively) in an attempt to simulate the conditions above the critical population (approximately 6.0 log CFU/mL) where interactions are usually observed. Regardless of whether the observed cells consisted of one or two strains of the pathogen, it appeared that density and relative proximity affected the time to first division, with cells that were cultured in DP to divide later than the cells that were in SP from each other. The term "time to first division" includes both the lag time and the time for the division process, with other words, the time needed for the individual cell to start dividing into two daughter cells and ususally, as the number of cells increases, lag duration decreases (Augustin et al., 2000; Bertranda, 2019; Kutalik et al., 2005a). However, at high initial inoculum populations the lag phase may also be influenced by other factors related to population density, like the cell-to-cell communication *via* chemical signaling, also known as quorum sensing (QS). Bacterial cell have the ability to produce and sense diffusible signal molecules named autoinducers the concentration of which may determine the growth behavior (Koutsoumanis et al., 2004; Robinson et al., 2001). The co-culture of the

different strains had no effect on the time to first division. The latter conclusion arose from the fact that the time of first division of the co-culture recorded in the middle of time to first division of the single cultures, both for cells in DP (50-60 min) and SP (40-50 min). Also, in **Chapter 6**, evaluated the growth and inter-stain interactions at population-level during incubation at 37°C. Interestingly, the results of the present chapter revealed that inter-strain interactions may influenced by storage/incubation temperature, since, during growth in TSB-YE was observed 1.3 log units difference between singly and co-cultured 6179 which was significantly decreased during growth on TSA-YE, indirectly confirming the behavior of co-cultured strains at single-cell level (**Chapter 6**). During co-culture of the same strains, under aerobic conditions at 7°C, the observed interactions were more pronounced in TSB-YE (3.4 log units) and were mitigated with the addition of agar and the solidification of the substrate (2.3 log units difference of singly- and co-cultured 6179 on TSA-YE) (**Chapter 2**).

Finally, in the last chapter of the present thesis (**Chapter 7**) recorded the intracellular proteins of singly- and co-cultured strains C5 and 6179 after storage at 7°C until the manifestation of inter-strain interactions, in order to investigate whether the observed inhibition of some *L. monocytogenes* strains during co-culture is due to a protein or an enzyme and potentially describe the underling mechanism. Interestingly, among the proteins produced during co-culture of the different *L. monocytogenes* strains (C5 and 6179) recorded the enzyme S-ribosylhomocysteine lyase or luxS. To date there are no studies, elucidating the underlying mechanisms for competition between *L. monocytogenes* strains, so to our knowledge, this is the first study which indicate population density regulation *via* the mechanism of QS and particularly the production of autoinducer AI-2 (a furanosyl borate diester) by LuxS QS system, during inter-strain inhibitory interactions. So far, in the literature, only inhibitory intra- and inter-species interactionç have been attributed to QS. QS increases the ability of the bacteria to have access to nutrients or to more favorable environmental niches and enhances bacterial defenses against eukaryotic hosts, competing bacteria, and environmental stresses or modulate a number of cellular functions (genes), including sporulation, biofilm formation, bacteriocin production or virulence response (Carvalheira et al., 2010; Federle and Bassler, 2003; Smith et al., 2004). Telesensing processes are now known to be influenced by environmental cues, including temperature, ligand concentration, pH, and water and oxygen availability (Bollinger et al., 2001; Roux et al., 2009; Shrout et al., 2006; Surette and Bassler, 1998). Moreover, in the present chapter, have been recorded additional proteins which have been characterized as “moonlighting” by different researchers. Moonlighting proteins comprise a subset of multifunctional proteins that are primarily intracellular, but perform a second biochemical function in other cellular locations, mostly on the cell surface, acting as antimicrobial peptides. Among the proteins that have been characterized as “moonlighting”, glucose-6-phosphate isomerase, triosephosphate isomerase and purine nucleoside phosphorylase DeoD-type are produced both by the singly-cultured C5 strain and during co-culture and could be responsible for the growth

inhibition of 6179 strain during the co-culture. Interestingly, different studies have linked CDI to both the activity of QS (Garcia, 2018, Majerczyk et al. 2014, 2016) and of "*moonlighting*" proteins (Campanini et al., 2015, Johnson et al. 2016).

The findings of the present thesis highlight how substrate structure, oxygen availability, and nutritional characteristics may influence inter-strain interactions and shed more light on the mechanism behind the inter-strain interactions of *L. monocytogenes* indicating that growth inhibition may be a combination of contact-dependent inhibition and quorum sensing mechanism. The occurrence of multiple strains in the same food is an important aspect contributing to mismatches between clinical isolates and infection sources during listeriosis outbreak investigations.

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CHAPTER 9

List of publications

Presentations in Conferences & Competences

Acknowledgements

Publications

Publications leading to the present thesis

- **Gkerekou, M. A.**, Athanaseli, K. G., Kapetanakou A. E., Skandamis, P. N. (2021). Evaluation of oxygen availability on growth and inter-strain interactions of L. monocytogenes in/on liquid, semi-solid and solid laboratory media. *International Journal of Food Microbiology 341, 109052.*

- **Gkerekou, M. A.**, Adam, L. A., Papakostas G., K., Skandamis, P. N. (2022). Studying the effect of oxygen availability and matrix structure on population density and inter-strain interactions of Listeria monocytogenes in different dairy model systems. *Food Research International 156, 111118.*

- **Gkerekou, M. A.**, Kaparakou, E. H., Tarantilis, P. A., Skandamis, P. N. (2023). Studying the metabolic factors that may impact the growth of co-cultured Listeria monocytogenes strains at low temperature. *Food Research International, 171(May), 113056.*

Publications regarding additional research projects during the course of the PhD

- Kapetanakou, A. E., **Gkerekou, M. A.**, Vitzilaiou, E. S., Skandamis, P. N. (2017). Assessing the capacity of growth, survival, and acid adaptive response of Listeria monocytogenes during storage of various cheeses and subsequent simulated gastric digestion. *International Journal of Food Microbiology 246, 50-63.*

- Makariti, I., Kapetanakou A. E., **Gkerekou, M.**, Bertoli, M., Dremetsika, C., Kalaitzoglou, I., Skandamis, P. N. (2019). Using the gamma concept in modelling fungal growth: A case study on brioche-type products. *Food Microbiology 45, 231-244.*

- Emilia Papakonstantinou, Konstantinos Galanopoulos, Anastasia E. Kapetanakou , **Maria Gkerekou** and Panagiotis N. Skandamis (2022). Short-Term Effects of Traditional Greek Meals: Lentils with Lupins, Trahana with Tomato Sauce and Halva with Currants and Dried Figs on Postprandial Glycemic Responses—A Randomized Clinical Trial in Healthy Humans. *Int. J. Environ. Res. Public Health, 19(18), 11502.*

Presentations in Conferences & Competences

Presentations in Conferences & Competences leading to the present thesis

- 3-Year Scholarship from The State Scholarships Foundation (IKY) - This research is co-financed by Greece and the European Union (European Social Fund- ESF) through the Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the project "Strengthening Human Resources Research Potential via Doctorate Research" (MIS-5000432), implemented by the State Scholarships Foundation (IKY)
  (file:///C:/Users/DELL/Downloads/3.pinakas_epilegenton_fysikes_phd_FINAL.pdf)

- **Gkerekou M. A.**, Georgoulia M. K., Kapetanakou A. E., Drosinos E. H., Skandamis P.N. (2017). Growth differences and inter-strain interactions between matrix-adapted and non-adapted *L. monocytogenes* strains on different types of cheeses. *7ο Συνέδριο MIKROBIOKOSMOS, 7-9 Απριλίου.* ***(Oral presentation)***

- **Maria A. Gkerekou**, Maria K. Georgoulia, Anastasia E. Kapetanakou, Eleftherios H. Drosinos, Panagiotis N. Skandamis (2017). The role of inter-strain interactions on the growth of matrix-adapted and non-adapted *L. monocytogenes* strains on different types of cheeses. *Annual Meeting of the International Association of Food Protection (IAFP), Tampa, Florida, USA, 9-12 July.* ***(Poster presentation)***

- **Maria A. Gkerekou**, Maria K. Georgoulia, Eleftherios H. Drosinos, Panagiotis N. Skandamis (2018). The impact of inter-strain interactions and matrix adaptation on growth and acid resistance of *L. monocytogenes* strains on different types of cheeses. *IAFP's European Symposium, Stockholm, Sweden, Europe, 25-27 April (poster award competition).* ***(Poster presentation)***

- **Γκερέκου Μ.**, Γεωργούλια Μ., Αθανασέλη Κ., Δροσινός Ε., Σκανδάμης Π. Ν. (2018). Μελέτη των διαστελεχιακών αλληλεπιδράσεων στην ανάπτυξη του μικροοργανισμού *Listeria monocytogenes* σε μαλακά τυροκομικά προϊόντα και στη μετέπειτα επιβίωσή του κατά την έκθεση σε συνθετικό γαστρικό υγρό. *DAIRY EXPO, Αθήνα, 10-12 Νοεμβρίου.* ***(Προφορική παρουσίαση)***

- **Μαρία Α. Γκερέκου**, Κωνσταντίνα Γ. Αθανασέλη, Ελευθέριος Χ. Δροσινός, Παναγιώτης Ν. Σκανδάμης (2019). Μελέτη της επίδρασης της διαθεσιμότητας οξυγόνου σε υποστρώματα διαφορετικής δομής που προσομοιάζουν προϊόντα ζωικής προέλευσης, στις δια-στελεχιακές αλληλεπιδράσεις του παθογόνου μικροοργανισμού *Listeria monocytogenes*. *6ο Πανελλήνιο Συνέδριο «το κρέας και τα προϊόντα του», Θεσσαλονίκη, 1-3 Φεβρουαρίου.* ***(Προφορική παρουσίαση)***

- **Maria A. Gkerekou**, Konstantina G. Athanaseli, Eleftherios H. Drosinos, Panagiotis N. Skandamis (2019). Evaluation of oxygen availability and substrate structure on growth and inter-strain interactions of *L. monocytogenes*. *IAFP's European Symposium, Nantes, France, Europe, 24-26 April.* ***(Poster presentation)*** ***Travel scholarship:*** *Student Travel Scholarship Award, IAFP's European Symposium, 2019, Nantes, France*

- **Maria A. Gkerekou**, Lamprini A. Adam, Georgios K. Papakostas, Eleftherios H. Drosinos, Panagiotis N. Skandamis (2020). Evaluation of oxygen availability and different structured dairy model systems on growth and inter-strain interactions of *L. monocytogenes. Annual meeting of Association of Food Protection (IAFP), Cleveland, USA, 25-28 October.* ***(Poster presentation)***

- **Maria A. Gkerekou**, Maria K. Georgoulia, Konstantina G. Athanaseli, Lamprini A. Adam, Eleftherios H. Drosinos, Panagiotis N. Skandamis (2021). The impact of oxygen availability, structure and nutrients of different substrates on inter-strain interactions and acid resistance of *L. monocytogenes strains. 9th Conference of MIKROBIOKOSMOS, Athens, Greece, 16-18 December.* ***(Poster presentation)***

- **Maria A. Gkerekou**, Eleftheria H. Kaparakou, Petros A. Tarantilis and Panagiotis N. Skandamis (2022). Studying the production of metabolic factors that may influence the growth of *Listeria monocytogenes* during co-culture of different strains of the pathogen. FoodMicro2022, August 28 to 31, Athens, Greece. ***(Poster presentation)***

- **Maria A. Gkerekou**, Vasiliki Papadopoulou, Marianna Arvaniti, Antonios N. Psomas and Panagiotis N. Skandamis (2023). Evaluating the impact of co-culture of different *L. monocytogenes* strains on time of first division at single-cell level. *12th International Conference on. Predictive Modelling in Food, June 13-16, 2023 Sapporo, Japan.* ***(Poster presentation)***

Presentations in Conferences & Competences regarding additional research projects during the course of the PhD

- **1st prize** in the 6th National contest ECOTROPHELIA for the product name *"Veggie it"*, as **team member**. **Qualified** in the corresponding European competition in SIAL exposition in Paris, October 2016. Team members: A. E. Kapetanakou, N. Grivokostopoulos, I. P. Makariti**,** Danae L. Siderakou, **M. A. Gkerekou**, Dimitra Tagkouli, Katerina-Aithra Sterioti.
- Makariti, I., Kapetanakou, A. E., Bertoli M.-A., Dremetsika, C., **Gkerekou, M.**, Skandamis P. N. (2016). Molecular characterization and in vitro determination of fungal growth from sweet brioche-like

products. *25th International ICFMH Symposium Food Micro, Dublin, Ireland, 3-6 September.* ***(Poster presentation)***

- **Best poster award**: Makariti, I., Kapetanakou, A. E., Bertoli M.-A., Dremetsika, C., **Gkerekou, M.**, Skandamis P. N. (2016). Molecular Identification and In Vitro Evaluation of Fungal Growth from Sweet Brioche-Like Products. *IAFP's European Symposium, Athens, Greece, 11-13 May.* ***(Poster presentation)***
- Ifigeneia P. Makariti, Nikos C. Grivokostopoulos, Artemis Zavitsanou, Danae L. Siderakou, **Maria A. Gkerekou**, Spiros Paramithiotis, Panagiotis N. Skandamis (2017). Field assessment of the effect of natural nitrite substitute on the growth of spoilage organisms and Clostridium sporogenes in cooked meat products. *10th International Conference of predictive Modelling Food (ICPMF), Cordoba, Spain, 26-29 September.* ***(Poster presentation)***
- **Best presentation award** in the 8th National contest ECOTROPHELIA for the product name *"PYRAMEAL"*, as **supervisor**.
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- Participation in the 12th National contest ECOTROPHELIA with the product name *"The European Boba Company: REFRESH"*, as **co-supervisor** of the 1st team consisted of students belonging to 5 European Universities (France, Montpellier SupAgro and AgroParisTech; Ausria, BOKU; Slovenia, University of Ljubljana; Spain, UPV Universitat Politècnica de València; Greece, Agricultural University of Athens).

Investigating the phenotypic response and underpinning mechanisms of inter-strain interactions of Listeria monocytogenes in foods and simulated food environments

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