Communication

Listeria monocytogenes Serogroup 1/2 Strains Have a Competitive Growth Advantage over Serotype 4b during Refrigerated Storage of an Artificially Contaminated Ready-To-Eat Pork Meat Product

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Abstract: *Listeria monocytogenes* is the bacterial causative agent of listeriosis, a life-threatening disease for humans, mainly transmitted through contaminated food. Human clinical isolates of the pathogen are frequently identified as serotype 4b strains; interestingly, however, serotype 4b (lineage I) is normally underrepresented among the food isolates in which serotype 1/2a (lineage II) is usually prevalent. The present study aimed to assess in situ dominance dynamics for the most commonly detected serotypes of *L. monocytogenes* implicated in foodborne listeriosis cases. A four-strain mixture comprised of *L. monocytogenes* serogroup 1/2 (i.e., serotypes 1/2a, 1/2b, and 1/2c) and serotype 4b food isolates was inoculated on a sliced ready-to-eat pork meat product, and dominance rates for the pathogenic strains were estimated based on serotype recoveries by utilizing multiplex polymerase chain reaction (mPCR), during storage of the product at 4 °C and 10 °C. The cumulative mPCR results showed that serotype 4b decreased at both storage temperatures, with the most abrupt decrease being noticed during storage at 10 °C. Irrespective of the storage temperature applied, *L. monocytogenes* strains of serogroup 1/2 predominated at the end of the meat product's storage period. Conclusively, the preliminary findings of this research suggested a competitive growth advantage of *L. monocytogenes* serogroup 1/2 strains over serotype 4b during the refrigerated shelf-life of foods, thus advancing our knowledge on the pathogen's behavior and contributing toward elucidating the manifested underrepresentation of serotype 4b in favor of serogroup 1/2 strains among the food isolates of the pathogen, particularly those recovered during detection and/or enumeration of *L. monocytogenes* in meat and products thereof.

Keywords: *Listeria monocytogenes*; multiplex PCR; pork meat; serogroup 1/2; serotype 4b; strain competition

1. Introduction

The bacterial infection in humans caused by *Listeria monocytogenes*, named listeriosis, is a life-threatening disease mainly of foodborne etiology with a high case fatality rate (ca. 20%) compared to other diseases caused by pathogenic bacteria found in foods [1] (pp. 20–149). There are currently 13 known serotypes of *L. monocytogenes* with the vast majority (>95%) of the reported human listeriosis cases attributed to strains within this pathogenic species, which are isolated from foods and patients and belong to serotypes 1/2a, 1/2b, 1/2c, and 4b [2–4]. Nevertheless, human clinical isolates of the pathogen, which in more than half of the cases are identified as serotype 4b (lineage I) [2,5], are

usually underrepresented among the food isolates in which serotype 1/2a (lineage II) is normally dominant [6–8]. The observed discrepancy in serotype prevalence between food and human clinical isolates of the pathogen remains an epidemiological paradox that has yet to be fully clarified.

Meat is mainly contaminated with *L. monocytogenes* serogroup 1/2 strains, while the most prevalent serotypes in meat are 1/2a and 1/2c (lineage II) [9–12]. In addition to these serotypes, 1/2b (lineage I) is a commonly detected serotype in pork meat and minced pork [13–16]. In an effort to encompass the variability encountered among the different serotypes of the pathogen, multiple *L. monocytogenes* isolates originating from the same and/or different matrices are used as a mixture or cocktail of strains for the inoculation of a food commodity and the study of the pathogen's behavior [17]. Although the growth potential of *L. monocytogenes* strain mixtures inoculated on various foods, such as fresh produce and animal-originated products, has been extensively studied [18–21], the differential microbial response and behavior of *L. monocytogenes* serotypes during shelf-life and storage at different temperatures has not been widely investigated in foods, with the notable exception of the investigation of the pathogen's strain competition in Katiki soft cheese [22] and ham [23]. In these instances, individual serotypes of the pathogen have been studied for their growth on the aforementioned products and the possible correlation of growth with serotype designation.

Considering the above and especially the documented underrepresentation of *L. monocytogenes* serotype 4b in foods [7], together with the organism's given psychrotrophic nature, the aim of this study was to assess in situ (i.e., on food) the dominance dynamics for the most commonly detected serotypes of the pathogen implicated in the majority of foodborne listeriosis cases, namely *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, and 4b, when intentionally introduced to a ready-to-eat (RTE) pork meat product and then kept under refrigeration at 4 °C or at temperature abuse storage conditions (i.e., storage at 10 °C).

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Four *L. monocytogenes* strains (LQC15138; serotype 1/2a, LQC15164; ser. 1/2b, LQC15169; ser. 1/2c, and LQC15189; ser. 4b, Supplementary Table S1) were isolated from naturally contaminated fresh minced pork meat during a survey conducted by Andritsos et al. [24] and were retrieved from the microbial culture collection of the Laboratory of Food Quality Control and Hygiene (LQC; Agricultural University of Athens, Athens, Greece). Strains were deposited and stored at −20 °C in brain heart infusion (BHI) broth (Biolife, Milan, Italy) supplemented with 20% glycerol (Merck, Darmstadt, Germany). Resuscitation took place by means of individually subculturing the strains twice in BHI broth (1% inoculum) and then incubating at 37 °C for 16–18 h, which corresponded to the mid-exponential growth phase of the cells for each strain (Supplementary Figure S1). Afterwards, the microbial cells were harvested (10,844 × g, 15 min, 4 °C; Heraeus Megafuge 1.0R Benchtop Centrifuge, Heraeus Instruments, Hanau, Germany) and washed twice with $\frac{1}{4}$ Ringer solution (Merck), as previously described by Mataragas et al. [25].

2.2. Inoculation and Storage of the Meat Product

A bacterial suspension was prepared by pooling an equal volume (10 mL) of each strain of the different *L. monocytogenes* serotypes in a final volume of 1 L maximum recovery diluent (MRD; Biolife). The mixture was surface inoculated (ca. 10^5 CFU/mL) on both sides (1 mL of the bacterial suspension on a 10 × 10 cm surface) of an RTE sliced, cooked, and cured pork shoulder (each slice weighing 25 g) with an initial pH of 6.36 to 6.48, supplied by a local meat industry supplier. The inoculum was spread on the product's surface by manually massaging the exterior of the protective packaging film. Following a 10 min cell attachment period at 4 °C, inoculated and noninoculated (control) slices of meat product were individually packaged with a low oxygen permeable film (90 µM

polyamide-polyethylene), which is typically used as a protective barrier for products under vacuum, using a tabletop vacuum packaging machine (Henkovac 1900, Hertogenbosch, The Netherlands). Half the quantity of the individually vacuum-packaged, inoculated and noninoculated, slices of meat product was stored at 4 °C and the other half at 10 °C.

2.3. Sampling, Microbiological Analysis, and pH of the Meat Product

The meat product was sampled at appropriate time intervals depending on the storage temperature. Three randomly selected individually packaged slices, inoculated or not, from each temperature storage condition were microbiologically analyzed for the detection and enumeration of *Listeria* spp. other than *L. monocytogenes* (in noninoculated slices; i.e., controls) and for the enumeration of *L. monocytogenes* (in inoculated slices) after 0, 3, 8, 13, 15, 18, 21, 24, 27, and 29 and 0, 1, 2, 3, 4, 5, 7, 8, 9, 13, and 15 days of storage at 4 °C and 10 °C, respectively. Samples were also analyzed for total viable counts (TVCs) and lactic acid bacteria (LAB) counts during storage at 4 °C (at Days 0, 8, 13, 18, 24, 29) and 10 °C (at Days 0, 3, 7, 15). At the beginning of the storage experiment (Day 0) only samples taken from 4 °C were analyzed.

The microbiological analysis of the product samples was performed according to the detailed methodology described elsewhere [24,26]. Briefly, the presence and enumeration of *Listeria* spp., as well as *L. monocytogenes* counts, were determined on PALCAM agar (Biolife) incubated aerobically at 37 °C for 48 h, while TVC and LAB were enumerated on plate count agar (PCA; Merck) and de Man, Rogosa, and Sharp (MRS) agar (Biolife), respectively, both plating media being incubated aerobically at 30 °C for 72 h. Hence, three serial decimal dilutions were plated in triplicate for enumeration purposes, whereas the detection of *Listeria* spp. was performed in duplicate plates of PALCAM agar.

The pH of the meat product was measured in triplicate by using a digital pH meter (WTW, pH 526, Weilheim, Germany), according to the ISO 2917 reference method [27], after the microbiological analysis was concluded. In this way, the accurate recording of pH changes for the meat product throughout its storage was achieved.

2.4. Dynamics of *L. monocytogenes* Strains on Meat Product

The dynamics of *L. monocytogenes* strains intentionally introduced to the pork meat product under study were assessed by monitoring strain dominance through serotype prevalence at different time intervals, expressed as serotype isolation rates, during storage of the product at 4 °C and 10 °C. For this reason, *L. monocytogenes* was isolated from the lowest countable dilution (15–150 colonies) on PALCAM agar at specific time intervals. At least 10% of the colonies grown on each of the three countable plates of PALCAM agar were isolated from all three individually tested packaged slices (i.e., biological replicates) of meat product (i.e., in total, 110 and 120 colonies recovered during the meat product's storage at 4 °C and 10 °C, respectively). The selection of colonies was based on the use of Harrison's disk [28]. When there were less than 15 colonies on PALCAM agar, all colonies were picked. Isolated colonies were streaked for a purity check on tryptic soy agar supplemented with 0.6% yeast extract (TSAYE; Biolife). Following 16–18 h of incubation at 37 °C, streaked colonies of *L. monocytogenes* were serotyped. Serotyping and subsequent differentiation of the isolates were performed by multiplex polymerase chain reaction (mPCR) based on the protocol proposed by Doumith et al. [3], the latter being slightly modified [29]. After the assignment of serotypes, dominance rates regarding strain prevalence at different time intervals were estimated, during storage of the product at 4 °C and 10 °C, using Equation (1) below:

$$\text{Dominance rate} = (\text{number of recovered } L. \text{ monocytogenes strains per serotype} / \text{total number of } L. \text{ monocytogenes isolates}) \times 100, \quad (1)$$

The total number of *L. monocytogenes* isolates at each time interval (in days of storage) originated from the three sample (biological) replicates performed (i.e., slices of meat

product), plated thrice from each of the three serial decimal dilutions prepared from each replicate, in one independent trial of the experimental procedure (Appendix A; Figure A1).

2.5. Statistical Analysis

Data regarding the serotype prevalence and dominance rates for each strain of *L. monocytogenes*, during storage at different temperatures of the inoculated product, were plotted and analyzed in Excel[®] 2019 (Microsoft, Redmont, WA, USA). Bacterial counts for the populations of microorganisms during storage of the product were initially expressed as the mean $\pm$ standard deviation in log CFU/g and checked for normality with the calculation of the Anderson–Darling (A–D) test statistic [30]. The microbial counts were then subjected to a pairwise Student's t-test for statistically significant differences between replicates and storage at different temperatures, with significance set at $p < 0.05$.

3. Results and Discussion

There was no natural contamination of the meat product with *Listeria* spp. (i.e., <10 CFU/g and not detected in 25 g of product), whereas all single *L. monocytogenes* strains combined together and inoculated on the product had similar growth kinetics and reached almost identical final populations (ca. 7.8–8.1 log CFU/mL) in BHI broth (Supplementary Figure S1). Similarities in growth capacity and the bacterial growth of the individually grown strains prior to their mixing and inoculation, as monitored by recording the population of each monoculture on BHI agar, were verified previously also in a relevant research study by Locatelli et al. [31] and suggested that strain competition among *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, and 4b was merely the result of bacterial interactions on the meat product, among strains of the same species with almost the same growth potential. Average initial populations for TVCs, LAB, and *L. monocytogenes* on cooked, cured pork shoulder were 5.7 ± 0.7 , 5.2 ± 0.1 and 5.6 ± 0.6 log CFU/g, respectively. All microbial counts were normally distributed around the mean according to the calculated A-D test statistic. Bacterial counts for these microbiological parameters were not statistically different between replicates ($p > 0.05$), but the respective microbial populations during the meat product's storage at 4 °C and 10 °C differed statistically ($p < 0.05$). LAB counts during storage of the artificially contaminated meat product at the two different storage conditions (Figure 1) were far from reaching their maximum values, and so, there was no possibility for the Jameson effect to occur in any case [32,33]. Therefore, the observed serotype prevalence of *L. monocytogenes* during storage of the meat product was not the result of the interaction and competition between LAB and the pathogen as a common limiting source [34].

Recoveries (i.e., isolation rates) and dominance rates for the main *L. monocytogenes* serotypes during storage of the inoculated RTE product are presented in Table 1 and Figure 2, respectively. The data in Figure 2 clearly depict that serotype 4b decreased at both storage temperatures, with the most abrupt decrease highlighted during storage at 10 °C. Despite the storage temperature applied, serotypes of serogroup 1/2 (ser. 1/2a, 1/2b, and 1/2c) dominated the *L. monocytogenes* consortium at the end of the meat product's storage period (Table 1; Day 29 at 4 °C and Day 15 at 10 °C).

Previous studies have addressed the problem of outcompeting *Listeria* spp. against *L. monocytogenes* [31,35–38]. However, interactions among *L. monocytogenes* strains have not yet been fully investigated, and studies have mainly focused on competition during selective enrichment [39,40]. In our inoculation study, it became obvious that strains of serogroup 1/2 dominated the meat product at the end of its storage period and outcompeted serotype 4b, irrespective of the initial concentration of serotypes and the storage temperature applied on the product as well, even though serotypes 1/2b and 1/2c were more prevalent than serotype 1/2a and clearly prevailed against serotype 4b during the product's storage at 10 °C (Figure 2b). This is a clear indication of a possible growth advantage that serotypes 1/2a, 1/2b, and 1/2c might have over serotype 4b under refrigeration conditions of storage, implying an association of the growth of *L. monocytogenes*

with serotype designation. Our findings go hand-in-hand with the observations made by Bruhn et al. [41] that a competitive growth advantage exists in lineage II strains over lineage I strains of *L. monocytogenes*. Moreover, strain evolution or persistence has proven to be temperature dependent during storage at different temperatures of Katiki soft cheese inoculated with a mixture of strains, since different strains of *L. monocytogenes* predominated at different temperatures [22]. On the other hand, there are studies that failed to show a straight correlation of the growth of *L. monocytogenes* with serotype designation for the pathogen [23,42]. Furthermore, Pan et al. [43] already substantiated that *L. monocytogenes* serotype 1/2a strains were more efficient at forming biofilms and predominated in mixed-culture biofilms against serotype 4b in biofilm communities, although this was not examined in the present study.

Figure 1. Bacterial counts of TVC (■), LAB (▲), *L. monocytogenes* (◆), and changes in pH (●) during storage of an RTE cooked, cured meat product inoculated with a cocktail of *L. monocytogenes* serotypes. (a) Storage of the meat product at 4 °C; (b) storage of the meat product at 10 °C. Symbols and error bars represent the average value and standard deviation, respectively, of three replicates.

Table 1. Recoveries for *L. monocytogenes* strains of different serotypes during the refrigerated at 4 °C or temperature abused at 10 °C storage of an artificially contaminated RTE pork meat product.

Storage Temp. (°C)	Storage Time (Days)	Number of Isolated Strains	Recoveries (%) of <i>L. monocytogenes</i> Strains ¹			
			ser. 1/2a	ser. 1/2b	ser. 1/2c	ser. 4b
4	0 ²	16	3 (18.8)	4 (25.0)	2 (12.5)	7 (43.8)
	13	42	6 (14.3)	11 (26.2)	12 (28.6)	13 (31.0)
	21	20	4 (20.0)	5 (25.0)	7 (35.0)	4 (20.0)
	29	32	8 (25.0)	7 (21.9)	14 (43.8)	3 (9.4)
	Total number (%) of strains per serotype	110	21 (19.0)	27 (24.6)	35 (31.8)	27 (24.6)
10	0 ²	16	3 (18.8)	4 (25.0)	2 (12.5)	7 (43.8)
	2	24	7 (29.2)	6 (25.0)	4 (16.7)	7 (29.2)
	7	27	8 (29.6)	6 (22.2)	10 (37.0)	3 (11.1)
	9	41	7 (17.1)	11 (26.8)	18 (43.9)	5 (12.2)
	15	28	3 (10.7)	15 (53.6)	7 (25.0)	3 (10.7)
	Total number (%) of strains per serotype	120 ³	25 (20.8)	38 (31.7)	39 (32.5)	18 (15.0)

¹ Recoveries of the pathogen per serotype from one independent trial with three sample replicates of the meat product. ² At Day 0, only samples of the product stored at 4 °C were analyzed. ³ The number of total isolated strains at 10 °C does not include the isolated strains (16) from Day 0.

Figure 2. Dominance rates for *L. monocytogenes* serotypes 1/2a (■), 1/2b (▲), 1/2c (◆), and 4b (●) during storage of an RTE cooked, cured meat product inoculated with a cocktail of *L. monocytogenes* strains. (a) Storage of the meat product at 4 °C; (b) storage of meat product at 10 °C. Symbols represent the dominance rates for the respective *L. monocytogenes* serotype on each sampling day, calculated through Equation (1) (see the text). Serotyped *L. monocytogenes* strains on each sampling day originate as the result of pathogen isolation from the three replicates performed and the subsequent differentiation by mPCR; the error bars represent the standard deviation of these three replicates.

There is an assumption that selective and/or chromogenic plating media may play a role in the recovery of *L. monocytogenes*, and an effect of the composition and structure of the solid culture medium on the pathogen's strain recovery might exist. Hadjilouka et al. [29], based on the findings from Andritsos et al. [24] on minced pork meat, pointed out that serotype 1/2a was mostly recovered from agar Listeria acc. to Ottaviani and Agosti (ALOA), while RAPID[®]L.mono agar was able to recover all the serotype 4b strains isolated in the study. The spatial distribution, limited diffusion of inhibitors, and growth kinetics of the competing strains might be influenced by the microenvironment of the solid medium [36,37,40,43], whereas agar concentration and oxygen availability may affect the extent of suppression in interstrain competition between different serotypes of *L. monocytogenes* [44]. Besides, the relatively low prevalence and recovery of serotype 1/2c recorded in the aforementioned study [29] could be explained by the different ecological niche of this specific serotype, which is different from those of serotypes 1/2a, 1/2b, and 4b, as suggested by Ochiai et al. [16]. Finally, it should be noted that the role of possible LAB interaction with *L. monocytogenes*, through the production of specific bacteriocins [45], and the effect of pH changes on serotype prevalence during storage of the meat product remain relatively unclear in the present study, as their influence was not specifically addressed.

4. Conclusions

Multiple *L. monocytogenes* strains can be present in the same food matrix as a result of cross-contamination with different serotypes of the pathogen. In this context, taking also into account the probability of occurrence in foods as discussed in the Introduction, four *L. monocytogenes* strains were selected among a collection of isolates recovered from naturally contaminated minced pork meat samples. Strain selection took place, primarily, on the basis of the microorganism's serotypes recovered from the minced meat samples, and the selected strains represented the most commonly detected serotypes of the pathogen in foods. The resulting mixture of strains used to inoculate the meat product, further used in our study, simulated the worst real-case contamination scenario and achieved a wide degree of biodiversity during the postprocessing contamination of an RTE meat product with *L. monocytogenes*. At this point, we feel it should be acknowledged that the higher and lower initial concentrations of serotypes 4b and 1/2c, respectively, in the mixture of strains, might also result in noticeable changes to the growth potential for each pathogen's

serotype [17,46]. However, given the fact that the meat product is able to support the growth of *L. monocytogenes*, we could say that the observed disproportion of the serotype distribution at the beginning of storage not only hindered, but, characteristically, marked the existence of population dynamics during the strain competition of the pathogen, as revealed by the final distribution and prevalence (%) at the end of the meat product's storage period (Table 1).

In conclusion, although the present findings are considered to be more preliminary in nature and the results of this study are limited by the scope of investigation due to the lack of a vigorous statistical analysis using multiple biological replicates from more than one independent trial, they indicate, however, a strain-specific competitive advantage in the growth of *L. monocytogenes*. Foods contaminated with *L. monocytogenes* strains of multiple serotypes present differences in the dominance dynamics of the pathogen's serotypes, due to strain competition during storage at different temperatures, which can lead to the suppression or predominance of certain serotypes. Intraspecies interactions are thus inferred. In this way, the observed underrepresentation of serotype 4b in favor of serogroup 1/2 strains (ser. 1/2a, 1/2b, and 1/2c) among the *L. monocytogenes* strains isolated from food, particularly those recovered during detection and/or enumeration of the pathogen in meat and the products thereof, may be partially explained. Further studies are needed to elucidate the mechanisms that exist at the molecular level, through gene expression, which will simulate the transition of the pathogen from food to human host and will contribute towards our greater understanding regarding the observed underrepresentation of *L. monocytogenes* among the food isolates. These studies may shed light on how certain serotypes of the pathogen become capable of adapting and outcompeting or surviving better than others in the food matrix.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11136096/s1>, Figure S1: Growth of *L. monocytogenes* strains under study in brain heart infusion (BHI) broth, representing different serotypes of the pathogen, with bacterial counts performed on BHI agar, Table S1: *Listeria monocytogenes* strains used in the study.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Figure A1. Diagrammatic illustration of the experimental design. The study involved the microbiological analysis of sample replicates ($n = 3$) and the plating in triplicate of three serial decimal dilutions in one independent trial of the experiment.

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