Inoculation density is affecting growth conditions of *Listeria monocytogenes* on fresh cut lettuce

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Abstract

*Listeria monocytogenes* is a particular risk for the ready-to-eat food sector because of its ability to grow in various environmental conditions. In the literature, growth and survival of *L. monocytogenes* on food is tested using inoculation densities ranging from less than $10^2$ to over $10^5$ CFU g$^{-1}$. Inoculation densities on food have been rarely tested as a factor for growth. In this study, inoculation densities from $10^2$ to $10^5$ of *L. monocytogenes* were tested on iceberg lettuce (*Lactuca sativa*) in modified atmospheres and air in model packages at 4 and 8 °C to identify any potential inoculation density effects. On days 0, 2, 5 and 7, *L. monocytogenes* was extracted from the lettuce surface and enumerated via selective media. The resulting growth curves identified a significant inoculation density effect at 4 and 8 °C with significantly higher amounts of growth (1–2 logs) when lettuce was inoculated at $10^2$ CFU g$^{-1}$ as opposed to $10^4$ and $10^5$ CFU g$^{-1}$. In contrast, the use of different atmospheres had limited influence on growth of *L. monocytogenes*. In conclusion, greater emphasis on inoculation density of *L. monocytogenes* should be taken in inoculation experiments when confirmation of growth or the efficacies of growth inhibiting treatments are tested on ready-to-eat food such as lettuce.

Graphical abstract

**Keywords** Modified atmosphere packaging · Multiplex PCR · Leafy vegetables · Food safety · Refrigeration

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Extended author information available on the last page of the article
Introduction

The global fresh fruit and vegetable industry has increased production by nearly 40% between 2000 and 2014 (FAO 2017). With this rise in consumption also comes a rise in food related illnesses especially in foods that are minimally processed (Omac et al. 2015). The majority of these foods comprise of considerable amounts of natural microbes, some of which have the potential to be pathogenic such as Listeria monocytogenes (Francis et al. 1999). The minimally processed food industry, particularly the ones that handle fresh cut products, rely on various techniques to minimize contamination that are implemented as part of the hazard analysis critical control point (HACCP) procedures. Examples of this include the use of low storage temperatures and modified atmospheres (Francis and O’Beirne 1997). However, contamination by L. monocytogenes is still a common occurrence (Leong et al. 2017). In the US, the CDC has estimated 1600 annual cases of listeriosis and recorded 98 Listeria related deaths from the years 2000 to 2015 (CDC 2016).

Listeria monocytogenes has the ability to grow at refrigeration temperatures and in high salt foods, all while being found in various environments including soil, water, vegetation, livestock, food processing and storage facilities (Harris et al. 2003). This ubiquity allows easy entry into the food processing chain. In combination with its ability to grow under adverse conditions, L. monocytogenes is able to persist in food processing environments for long periods, despite sanitation efforts (Leong et al. 2014).

Numerous studies into the effect of inoculum size on the growth of L. monocytogenes have been carried out under in vitro conditions such as in microtiter plate based assays, where variables such as water activity, pH, environmental stresses and temperature can be easily controlled (Gysemans et al. 2007; Koutsoumanis and Sofos 2005; Robinson et al. 2001; Tienungoon et al. 2000; Vermeulen et al. 2009). These studies have allowed for the development of mathematical models which can accurately predict bacterial growth. However, many of these models have inadequacies when cell densities are below a certain limit (Vermeulen et al. 2009), particularly when under environmental stresses which can lead to reduced cell viability (Aguirre and Koutsoumanis 2016). Under these conditions, applications of very low cell numbers may result in the absence of cells capable to grow and multiply. These models have also shown that an increase in the inoculum size can result in a change of the normal growth limits to more extreme inhibitory conditions (Aguirre and Koutsoumanis 2016; Dupont and Augustin 2009; Koutsoumanis and Sofos 2005; Robinson et al. 2001). While the information gained from these models is of great value, there is a trend to move away from the broth-based models, as Koseki and Iseobe (2005) described that L. monocytogenes is able to deviate markedly from the liquid media data when grown on lettuce.

The aim of this study was to evaluate the growth behaviour of L. monocytogenes at four different inoculation densities and three atmospheres on lettuce in model packages at temperatures that resemble storage at retail and consumer level. The main hypothesis of this study was that inoculation at low densities that reflect EU limits may result in growth performances by L. monocytogenes that would be fundamentally different to higher inoculation density experiments commonly used to test growth inhibition and pathogen inactivation. Three atmosphere conditions were compared to clarify if different O2 and CO2 concentrations could result in distinct growth performances. Findings from this study may influence how growth of L. monocytogenes is being assessed on ready-to-eat products in the future.

Materials and methods

Preparation of fresh-cut vegetable (iceberg lettuce)

Iceberg lettuce (Class I, Spain) was obtained locally from a fruit and vegetable supplier at the day of testing and prepared as published recently (Scollard et al. 2016). Outer layers and stem were manually removed, damaged leaves and the core of the heads were excluded. Inner leaves were sliced with a disinfected sharp stainless steel knife and cut into 20 mm pieces. The cut lettuce (10 g) was transferred into 35 μm thick orientated polypropylene (OPP) packaging bags (18 × 10 cm) which had a permeability to O2 of 5.7 nmol m−2 s−1 kPa−1 and to CO2 of 19 nmol m−2 s−1 kPa−1 (Amcor Flexibles, Gloucester, UK).

Preparation of Listeria cultures and inoculation of lettuce

For all incubation tests a combination of three L. monocytogenes strains from the Listeria strain collection at Teagasc Food Research Centre (Moorepark, Ireland) were used in order to follow the European Guidelines for challenge tests on ready-to-eat foods (EURL Lm 2014) as described previously (Scollard et al. 2016). Strain 6179 was repeatedly isolated from a food processing plant, strain 959 was isolated from a vegetable produce facility and strain 1382 is a EURL Lm reference strain. Individual strains were grown separately in tryptone soya broth (50 mL TSB Oxoid CM0129, Thermo Scientific, Waltham, MA) at either 4 or 8 °C (cultures to be incubated as under test conditions; EURL Lm). Equal numbers of each strain were then mixed together and diluted in phosphate buffered saline (PBS, Oxoid BR014,
Inoculation of lettuce at $10^2$, $10^3$, $10^4$ and $10^5$ CFU g$^{-1}$ was conducted uniformly with 0.1 mL of L. monocytogenes suspension per lettuce package, resembling inoculation densities used in the literature ranging from $10^5$ (Scollard et al. 2016) to $10^5$ CFU g$^{-1}$ in fresh-cut produce (Scollard et al. 2014).

Modified atmosphere packaging (MAP), package sealing and storage conditions, gas analysis

Packs were flushed after inoculation with the following gas atmospheres: (a) air, (b) 8 kPa CO$_2$, 4 kPa O$_2$, 88 kPa N$_2$ or (c) 15 kPa CO$_2$, 1 kPa O$_2$, 84 kPa N$_2$ (MAP) and heat sealed using a vacuum packer (Multivac Mobil 3000, Wolfertschwenden, Germany) as described previously (Scollard et al. 2016). The packs were then incubated at either 4 or 8°C for up to 7 days.

On each sampling day (day 0, 2, 5, 7) oxygen content within the packages were analysed with a gas analyser (PBI-Dansensor, PBI Development, Denmark, Model TIA-III LV) using an injection needle to penetrate the packs.

Enumeration of Listeria and total cell count

Bacterial cell counts were carried out on day 0 (day of inoculation) and days 2, 5 and 7 from three replicate packs. The lettuce samples (10 g) from each package were homogenised for 120 s at 260 rpm in 20 mL of PBS (twofold dilution) using a Seward laboratory stomacher (Model 400, Worthing, UK). Subsequently, depending on inoculation density bacterial suspensions were either concentrated ten times via centrifugation at 4000×g to obtain a detection limit of 0.5 CFU g$^{-1}$ food (Scollard et al. 2016), used neat or were diluted in PBS and plated on Listeria selective agar (LSA, Oxford CM0856, Oxoid, with supplement SR0206; Thermo Scientific) during the testing period. CFU of L. monocytogenes (black colony with halo) were determined after incubation at 37°C for 48 h.

Listeria serogrouping via PCR

Colonies on LSA were confirmed as L. monocytogenes and serogrouped by multiplex PCR as described in Doumith et al. (2004) to establish the relative abundance of the individual strains from the mixed inoculum at day 7 (for details, please see Supplementary Information SM1 and Supplementary Table S1 in Supplementary Information). DNA was extracted from presumptive L. monocytogenes isolates, with 50 colonies randomly taken from each of the day 7 inoculation densities in order to obtain the spread of the three serogroups.

Statistical analysis

A non-parametric test (Kruskal–Wallis; as data did not meet the criteria for normality) was selected to identify significant amounts of growth (P ≤ 0.05) within growth curves over the 7 day period from the experiments at different atmospheres and temperatures, using SPSS (IBM, Armonk, NY). The remaining data including the cumulated areas of growth curves were tested via ANOVA (Tukey posthoc test) as they conformed to normality (Shapiro–Wilk) and homoscedasticity (Levene).

Differences between growth curves were determined via the analysis of the areas below growth curves between samplings (days x increase in log$_{10}$ CFU/2; for days 0–2; 2–5, 5–7). In order to obtain independent replicates, growth curves from different atmospheres were combined to compare different inoculation densities at 4 and 8°C, respectively.

Results

Growth at 8°C

In air at 8°C, growth was significant (P ≤ 0.05) for inoculation densities $10^2$–$10^4$ CFU g$^{-1}$ and ranged from 2.7 logs to 1.5 and 1.3 logs ($10^3$, $10^2$ and $10^4$ CFU g$^{-1}$ inoculation, respectively). Growth at the highest inoculation density was below 0.2 logs and not significant (Fig. 1a; see Supplementary Table S2 in Supplementary Information). Under modified atmosphere with 1 kPa oxygen amounts of growth were highest at the lowest inoculation densities, similar to the results found in air. Likewise, all but the highest inoculation density amounts of growth were significant. However, growth under 1 kPa O$_2$ MAP at $10^2$ CFU g$^{-1}$ inoculation was slightly lower than in air (2.4 logs). This trend continued under $10^3$ and $10^4$ CFU g$^{-1}$ inoculation (1.1 and 0.9 logs, respectively), while growth at the highest inoculation density was slightly higher than in air (0.4 logs) but again not significant (P > 0.05; Fig. 1b, see Supplementary Table S2 in Supplementary Information). Under modified atmosphere with 4 kPa oxygen, inoculation densities from $10^2$ to $10^4$ CFU g$^{-1}$ demonstrated significant amounts of growth. Their highest levels were again with the lowest inoculation density ($10^2$ CFU g$^{-1}$) at 1.9 compared to 1.6 and 1.1 logs for the $10^3$ and $10^4$ CFU g$^{-1}$ inoculation densities, respectively (Fig. 1c, see Supplementary Table S2 in Supplementary Information). Again, the highest inoculation density resulted in the lowest amount of growth that did not reach significance (0.5 log).

Comparison of inoculation densities at 8°C with all atmospheres combined revealed that all four inoculation densities had significantly different amounts of growth.
with $10^5$ being the lowest and $10^2$ CFU g$^{-1}$ being the highest (Table 1). In contrast, CFUs at day 7 from the three different atmospheres were not significantly different, when the same inoculation densities were compared, thus atmospheres had no significant effect on *L. monocytogenes* growth (data not shown).

**Growth at 4 °C**

In an air atmosphere at 4 °C, growth was significant ($10^2$–$10^4$ CFU g$^{-1}$, 1.8, 1.1 and 0.9 logs) except for the highest inoculation level where the growth rate of 0.4 log didn’t reach significance ($10^5$ CFU g$^{-1}$; Fig. 2a, see Supplementary Table S2 in Supplementary Information). Under 1 kPa O$_2$ all amounts of growth were significant ($10^2$–$10^5$ CFU g$^{-1}$) at 1.0, 1.1, 0.7 and 0.6 logs respectively (Fig. 2b; see Supplementary Table S2 in Supplementary Information). Under 4 kPa O$_2$ MAP growth at 1.7, 1.1 and 0.9 logs were identified to be significant for the $10^2$–$10^4$ CFU g$^{-1}$ inoculations, while growth of $10^5$ CFU g$^{-1}$ at 0.7 log didn’t reach significance (Fig. 2c, see Supplementary Table S2 in Supplementary Information).

Furthermore, when comparison of inoculation densities at 4 °C with all atmospheres were combined it revealed that the amounts of growth were significantly higher at $10^2$ CFU g$^{-1}$ inoculation over the $10^4$ and $10^5$ inoculation (Table 1). Likewise, a significant difference was also found when the $10^3$ inoculation curve was compared with the $10^5$ inoculation curve (Table 1). In contrast, CFUs at day 7 across the three different atmospheres were not significantly different (for each inoculation density, respectively, data not shown).

**Oxygen in atmospheres**

In air, the O$_2$ content across the various inoculation densities declined from just below 21 kPa (day 0; 4 and 8 °C) to between 11.3 and 13.7 kPa (4 and 8 °C) by day 7 (see Supplementary Figs. S1a and S2a in Supplementary Information). In 1 kPa O$_2$, the oxygen content across the various inoculation densities increased from approximately 1.0 kPa (day 0; 4 and 8 °C) to between 9.8 and 9.0 kPa by day 7 (4 and 8 °C; see Supplementary Figs. S1b and S2b in Supplementary Information). In 4 kPa O$_2$, the oxygen content across the various inoculation densities increased from around 4.0 kPa (day 0; both temperatures) to between 9.3 and 11.7 kPa by day 7 (both temperatures; see Supplementary Figs. S1c and S2c in Supplementary Information). At no stage were significantly different oxygen concentrations found (same day, same temperature) when different inoculation densities were compared.

**Serogrouping**

To compare the growth capabilities of the individual *L. monocytogenes* strains within the three strain mix a multiplex PCR was employed to generate serogroup specific banding patterns. On day 7 at 8 °C incubation and 4 kPa O$_2$ MAP, 41% of the randomly selected CFUs ($10^2$ inoculation) harbourd a PCR pattern that resembled strain 6179, 35% of the CFUs were identified as strain 959, while 24%
Environmental conditions and the amount of the inoculant have been shown to significantly affect the growth of \emph{L. monocytogenes} in vitro in the past (Koutsoumanis and Sofos 2005; Robinson et al. 2001). Thus, the initial inoculation density of \emph{L. monocytogenes} when conducting experiments into its growth or response under different physicochemical conditions is of great importance. While information on environmental conditions and the amount of the inoculum density on growth abilities at refrigeration temperatures on fresh cut produce such as lettuce have been added to spinach (Omac et al. 2015), the growth of \emph{Listeria} cells did not change even when they were outnumbered 100 times by the indigenous bacterial population.

The current study demonstrated significantly higher amounts of growth at starting concentrations of 10^5 CFU g^{-1} when compared to higher inoculation rates, despite the abundance of indigenous bacteria on lettuce at around 10^5 CFU g^{-1} food (data not shown). This effect was independent of the model atmosphere, but was accelerated at higher storage temperature (8 °C over 4 °C). The results from this and previous studies suggest that the presence of indigenous bacteria on the lettuce are unlikely to inhibit growth of \emph{L. monocytogenes} when added at higher inoculation levels at the beginning of the experiment. Nevertheless, growth of indigenous bacteria to levels of 10^6 CFU g^{-1} during the course of the experiment could play a growth limiting role for \emph{L. monocytogenes} towards the end of storage. Another possible explanation could be that when cell densities of \emph{L. monocytogenes} reach 10^6 CFU g^{-1}, intra-species competition may play a greater role as predicted by Quinto et al. (2016). Further in detailed investigations would be needed to support or refuse these theories. Foods of higher nutrient content may be more useful to test these theories as they would have the potential to allow higher amounts of growth. As for now, it is unclear if the observed inoculation density effect would be the same on nutrient rich ready-to-eat food products.

Within the literature, growth of \emph{L. monocytogenes} appears to be reduced at lower temperatures (< 5 °C) when compared to that of higher temperatures (≥ 8 °C) (Francis and O’Beirne 1998), the growth of the \emph{Listeria} cells did not change even when they were outnumbered 100 times by the indigenous bacterial population.
In this study, the same effect can be seen at inoculation densities $10^2$–$10^4$ but not at $10^5$ CFU g$^{-1}$. Nevertheless, at both tested temperatures all of the three lowest inoculation densities ($10^2$–$10^4$ CFU g$^{-1}$) maintained significant amounts of growth ($P \leq 0.05$) across the three atmospheres. When growth curves of different atmospheres were combined, growth was significantly higher in the $10^5$ CFU g$^{-1}$ inoculated bags over the $10^4$ and $10^3$ CFU g$^{-1}$ inoculated bags at both temperatures, while at $8^\circ C$ each increase in inoculation density resulted in a significantly lower amount of growth. Consequently, inoculation densities play a pivotal role for the analysis of growth of *L. monocytogenes* on leafy vegetables like lettuce at temperatures commonly found in domestic storage or during transport.

Since growth at storage temperature of $4^\circ C$ at $10^5$ CFU g$^{-1}$ was not significant when tested in air and 4 kPa O$_2$, the results could be wrongly interpreted that lettuce is not supporting growth of *L. monocytogenes* at optimal storage temperature. Thankfully, recent guidelines on growth studies with ready-to-eat foods recommend inoculation densities of $10^2$–$10^5$ CFU g$^{-1}$ food (EURL Lm 2014; Oliveira et al. 2010; NACMCF 2010; Spanu et al. 2014). At the same time, inactivation studies use inoculation densities of up to $10^7$ CFU g$^{-1}$ food (NACMCF 2010). A great variety of inoculation densities are employed to study either maximum increases or decreases of several logs. As a consequence, many studies choose inoculation densities of around $10^2$–$10^3$ CFU food (EURL Lm 2014; Oliveira et al. 2010; NACMCF 2010; Spanu et al. 2014). At the same time, at $8^\circ C$, each increase in inoculation density resulted in a significantly lower amount of growth. Consequently, inoculation densities play a pivotal role for the analysis of growth of *L. monocytogenes* on leafy vegetables like lettuce at temperatures commonly found in domestic storage or during transport.

The reduction of oxygen in fresh-cut lettuce packages has shown to reduce product browning (Smyth et al. 1998) and therefore has the potential to extend the shelf life of lettuce. However, this form of MAP may not necessarily reduce growth of *L. monocytogenes* on fruits and vegetables (Beuchat and Brackett 1991; Bourke and O’Beirne 2004; Francis and O’Beirne 1997; Oliveira et al. 2010). Nevertheless, Kakamennou et al. (1998) demonstrated *L. monocytogenes* growth reductions on lettuce and carrots under a modified atmosphere of approximately 5 kPa CO$_2$ and varying O$_2$ of 2 or 5 kPa. Therefore, the comparison of different atmospheres in this study was needed to compare growth under different inoculation densities. The present study found that the applied modified atmospheres had no significant effect on *L. monocytogenes* growth on lettuce. However, at the highest inoculation density one temperature and one atmosphere setting resulted in significant growth (1 kPa O$_2$ at $4^\circ C$) while on all other occasions growth at $10^5$ CFU g$^{-1}$ was not significant. Over the tested 7 day period, oxygen concentrations in the model packs changed towards a concentration of $9$–$13$ kPa, thus differences in the atmospheres were only prominent at the first few days of the experiments. Findings from this study suggest that modified atmosphere may have an indifferent to limited impact on *L. monocytogenes* growth and that
in most cases other factors as tested in this study tend to have a greater impact. Findings from the current study are therefore in accord with the majority of studies conducted under similar conditions.

The preliminary screening of *L. monocytogenes* colonies after 7 days of incubation suggested that all three strains in the inoculation mix showed similar growth on lettuce at a starting density of $10^2$ CFU g$^{-1}$. However, to make more conclusive assumptions, further investigations would be needed to confirm that strains from the applied strain mix show no preferential growth on lettuce at various environmental conditions. Nevertheless, the preliminary results from this study appear to be supported by previous findings from Francis and O’Beirne (2005), where specific *L. monocytogenes* serogroups were not considerably differently effected by the conditions of their growth on lettuce at 8 °C. Possible explanations for the lack of any selection effects may be that iceberg lettuce and the incubation conditions used were not stressful enough for the development of clear differences between the serogroups tested. Francis and O’Beirne (2005) showed that harsher conditions can lead to significant differences in growth between the *L. monocytogenes* serogroups.

This study has shown that *L. monocytogenes* can grow from inoculation densities of $10^2$ CFU g$^{-1}$ to beyond acceptable regulatory levels on lettuce at low and abuse temperatures that can be found within the food processing, transport and storage environment. Since the data within this study demonstrates a significantly larger amount of growth at lower inoculation densities under both the abuse and widely recommended storage temperatures, these findings may be useful for the adaptation of predictive models of contamination at low cell densities from a persistent source within a cold storage chain. The US Department of Agriculture Pathogen Modeling Program is considering various inoculation densities on different food sources of animal origin but growth of *L. monocytogenes* of up to $10^5$ CFU g$^{-1}$ food appear to be identical (https://pmp.rrc.ars.usda.gov/PMPOnline.aspx). Reduced growth at inoculation densities from as little as $10^3$ over $10^2$ CFU g$^{-1}$ food suggests that an inherent bias could exist in growth experiments especially at higher storage temperature (8 °C). Considerations on inoculation densities are currently focussed on the ease of testing and not on inoculation bias. Consequently, future studies on growth and inhibition of *L. monocytogenes* on ready-to-eat food need to be carried out at $10^2$ CFU g$^{-1}$ food as well to avoid potential underestimation of its growth potential. This is particularly important for leafy vegetables like lettuce, where strong *Listeria* growth inhibitors cause product browning and thus milder anti-listerial treatments are applied.

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