A note on challenge trials to determine the growth of Listeria monocytogenes on mushrooms (Agaricus bisporus)

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Abstract

In the EU, food is considered safe with regard to Listeria monocytogenes if the number of micro-organisms does not exceed 100 colony forming units (cfu)/g throughout its shelf-life. Therefore, it is important to determine if a food supports growth of L. monocytogenes. Guidelines for conducting challenge tests for growth assessment of L. monocytogenes on foods were published by the European Union Reference Laboratory (EURL) in 2014. The aim of this study was to use these guidelines to determine if refrigerated, fresh, whole, closed-cap, prepackaged mushrooms (Agaricus bisporus) support the growth of L. monocytogenes. Three batches of mushrooms were artificially inoculated at approximately 100 cfu/g with a three-strain mix of L. monocytogenes and incubated for 2 days at 8°C followed by 4 days at 12°C. L. monocytogenes numbers were determined (in triplicate for each batch) on days 0, 2 and 6. Water activity, pH and total bacterial counts were also determined. There was no increase in the number of L. monocytogenes above the threshold of 0.5 log cfu/g in any of the replicates. In 8 of 9 replicates, the numbers decreased indicating that A. bisporus do not support the growth of L. monocytogenes. As the EU regulations allow < 100 cfu/g if the food cannot support growth of L. monocytogenes, the significance of this study is that mushrooms with < 100 cfu/g may be within the regulations and therefore, quantitative rather than qualitative determination may be required.

Keywords

Listeria monocytogenes • mushrooms • Agaricus bisporus • growth • challenge study • food safety

Introduction

Listeria monocytogenes is a food-borne pathogen that is widespread in the environment (Fox et al. 2009) and therefore, can contaminate food. It causes listeriosis, which is a relatively rare disease with a hospitalisation rate of 95% and a mortality rate of 20–30%, the third of all food-borne pathogens (Scallan et al. 2011). Susceptible groups include the young, elderly, pregnant women and those with pre-existing health issues (Gemer-Smidt et al. 2005; Farber and Peterkin 1991). L. monocytogenes is a very robust organism that can grow at refrigeration temperatures and survive low acid and high salt conditions and other stress factors (Gahan and Hill 2014). It is of particular concern in ready-to-eat foods as the heating step of cooking or some other inactivation process, which would normally kill L. monocytogenes, is absent. Listeriosis outbreaks have been commonly linked to dairy products, meat and fish products and vegetable produce in the past (Wang et al. 2013; Cartwright et al. 2013). According to the last EU summary report on zoonoses, zoonotic agents and food-borne outbreaks, 1642 confirmed human cases of listeriosis were reported in 2012 (0.41 cases per 100,000 population), while listeriosis represented the most severe food-borne human disease in terms of hospitalisation and fatal cases (12.1%) (EFSA 2014). According to regulation EC 2073-2005 (EC 2005), L. monocytogenes must be absent from 5 x 25 g samples of food unless the manufacturer can demonstrate that the numbers will not exceed a limit of 100 colony forming units (cfu)/g throughout its shelf-life with the exception of foods for medical purposes or infant formula where absence is required at all times. With a limit of 100 cfu/g, determining whether or not a food can support growth of L. monocytogenes becomes important. If growth is supported, it is important to show that the limit will not be exceeded throughout shelf-life. In the absence of data from the manufacturer on the ability to support growth, the ability to grow is assumed and absence in 5 x 25 g samples is required.

With recognised flaws such as artificial contamination, challenge studies are a method of determining whether or not a food supports growth of L. monocytogenes. In 2008, the European Union Reference Laboratory (EURL) published a technical guidance document for conducting challenge trials with L. monocytogenes on food (EC 2008). Following
application of this guidance document, it was recognised that
improvements could be made to it and therefore, an updated
technical guidance document was published in 2014 (EC 2014).
The aim of this study is to determine if refrigerated, fresh, whole,
closed-cap, prepackaged mushrooms (Agaricus bisporus)
have the ability to support growth of L. monocytogenes by
applying the recently published methodology for conducting
challenge trials.

Materials and methods

Sample collection
Refrigerated, fresh, whole, closed-cap, prepackaged
mushrooms (Agaricus bisporus; 3 batches of mushrooms
c.50 mm diameter - second flush mushrooms grown
on Phase III substrate) were obtained from a mushroom
supplier in Ireland. All mushroom samples were transported
to the laboratory by overnight refrigerated courier and tested
immediately on arrival at the laboratory.

Assessment of L. monocytogenes natural contamination
Before inoculation, a sample from each batch was removed
and tested by enrichment and enumeration for natural
contamination with L. monocytogenes using the ISO 11290-
1 and ISO 11290-2 methods (ISO 1997; 1998), except that
only Agar Listeria acc. to Ottavani & Agosti (ALOA) agar
(Biomérieux, UK) was used.

Bacterial strains, culture conditions and sample inoculation
A cocktail of three L. monocytogenes strains was used.
The cocktail comprised a reference strain from the EURL L.
monocytogenes strain collection, a strain originally isolated
from sliced mushrooms (strain 958) and a persistent strain
isolated from a cheese processing plant (strain 6179). The
three strains were grown independently at 37°C in brain–
heart infusion (BHI) broth for 18–20 h and from this culture
were inoculated into BHI and grown to stationary phase at
10°C for 4 days. Each strain was diluted independently in
maximum recovery diluent (MRD) and the dilutions added
together to give 30 ml of inoculation solution of approximately
10^3 cfu/ml. Inoculum (30 µl) was spread lightly on the cap
of each mushroom with a loop, not damaging the mushroom,
to give approximately 100 cfu/g. The mushrooms were dried in
laminar air flow for 10 min in a Petri dish. Incubation was at
8°C for 2 days followed by 12°C for 4 days. The mushrooms
were packed in trays of about 10 mushrooms and wrapped
with film as normally used for mushrooms for retail. Triplicate
analysis of each batch involving analysis of an individual
mushroom chosen at random from the pack at each sampling
time on days 0, 2 and 6 was undertaken.

Analysis method for L. monocytogenes
A total of 5 g of mushroom cap from where the inoculum
was spread was cut and analysed. The size of the piece
cut was consistent as the mushrooms were of a consistent
size. Listeria analysis by ISO 11290-1 for detection and ISO
11290-2 for enumeration (plating on ALOA only) were used

Additional analyses
Water activity was analysed using an Aqua Lab water
activity meter (Series 3 TB, Decagon Devices Inc., Pullman,
WA, USA.), total bacterial count (TBC) was measured by
spreading appropriate dilutions on Plate Count Agar (plates
were incubated for 3 days at 30°C) and the pH was measured
at each time point by inserting a pH probe (Hanna pH 211,
Woonsocket, RI, USA.) into the mushroom.

Calculation of growth potential
The log, of L. monocytogenes numbers was calculated at
each sampling time. Growth potential was calculated as the
difference between the log, of the numbers on day 6 and 0. If
the numbers (in any of the replicates) were 0.5 log higher on
day 6 than on day 0, growth was possible.

Results

L. monocytogenes numbers prior to inoculation
No L. monocytogenes were detected in any of the batches
prior to the challenge study.

Inoculum level
The variation in the inoculum used in each batch was < 0.5
log (data not shown) and the level of inoculation was 126 ±
49, 106 ± 29 and 206 ± 55 cfu/g for each batch, respectively.

L. monocytogenes growth
For 8 of the 9 replicates, there was a decrease in the numbers
of L. monocytogenes over the incubation time. For the 9th
replicate, there was an increase, but the increase was 0.4 log
cfu/g on day 6, indicating no growth in any of the replicates
(Figure 1).

Total bacterial count
The TBC increased over time (Table 1).

pH and water activity
The addition of the inoculum had little impact on the pH or the
water activity (Table 1).
It was concluded that in this challenge trial, which was conducted in accordance with the EU guidelines of 2014 (EC 2014), mushrooms did not support the growth of *L. monocytogenes*. In fact, the numbers of *L. monocytogenes* decreased in most cases. The inoculation had little effect on the pH or water activity values and the TBC values were not sufficiently high enough to inhibit the growth of *L. monocytogenes*.

In previous experiments, Leong *et al.* (2013) showed growth of *L. monocytogenes* on mushrooms. González-Fandos *et al.* (2001) also evaluated the potential of *L. monocytogenes* to grow in whole mushrooms stored at 4 and 10°C and they reported growth of between 1 and 2 log units, respectively, during the first 48 h of incubation. In addition, Hoelzer, Pouillot and Dennis (2012) suggested that fresh mushrooms would be among the commodities that support the growth of *L. monocytogenes*. On the other hand, Chikthimmah, LaBorde and Beelman (2007) showed that mushrooms do not support the growth of *L. monocytogenes*. However, in the experiments listed above, the EURL guidance document was not followed. The different inoculation and preparation methods and varying storage temperatures and conditions used may have influenced the results. The recently published EURL *Lm* Technical Guidance Document for conducting shelf-life studies on *L. monocytogenes* in ready-to-eat foods (EC 2014) is a valuable document that will give food business operators the opportunity to have challenge studies undertaken in a timely and cost-effective manner and will guarantee a more homogeneous approach, making the comparison of results among laboratories easier.

Although a recall of sliced white mushrooms occurred in Canada in 2012 (Canadian Food Inspection Agency 2014), no illnesses were reportedly associated with the recall and the grower/producer decided to recall the product voluntarily due to the possibility of contamination. Similarly, a recall of sliced crimini mushrooms occurred in Canada in 2014 (Canadian Food Inspection Agency 2014) with no associated illnesses reported. No *L. monocytogenes* outbreaks have historically been associated with mushrooms, although contamination occurs sporadically (FSAI 2006).

**Table 1.** TBC, pH and water activity on the three batches of mushrooms (average ± standard deviation).

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Log$_{10}$ TBC, cfu/g*</th>
<th>pH</th>
<th>Water activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated</td>
<td>Not determined</td>
<td>6.87 ± 0.05</td>
<td>0.996 ± 0.001</td>
</tr>
<tr>
<td>0</td>
<td>3.82 ± 0.53</td>
<td>6.86 ± 0.02</td>
<td>0.994 ± 0.002</td>
</tr>
<tr>
<td>2</td>
<td>4.77 ± 0.20</td>
<td>6.92 ± 0.05</td>
<td>0.993 ± 0.005</td>
</tr>
<tr>
<td>6</td>
<td>5.94 ± 0.89</td>
<td>6.78 ± 0.03</td>
<td>0.996 ± 0.003</td>
</tr>
</tbody>
</table>

* TBC, cfu/g: total bacterial count, colony forming units/g

**Discussion**

![Figure 1. The behaviour of *Listeria monocytogenes* as determined on mushrooms. B1R1; batch 1 replicate 1, and so on. The dashed line indicates the limit of enumeration. CFU: colony forming units.](image-url)
Viswanath et al. (2013) showed that mushrooms can be contaminated with *L. monocytogenes* with an occurrence of 1.2%, although there was no quantification of the level of contamination. As the regulations allow < 100 cfu/g if the food cannot support growth of *L. monocytogenes*, the results of the current study demonstrate that quantification of *L. monocytogenes* on mushrooms is necessary as numbers below 100 cfu/g will not increase during the shelf-life and therefore, mushrooms with < 100 cfu/g would be within European regulation.

**Acknowledgements**

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**References**


