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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 8ºC followed by 4 days at 12ºC. *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 (Gerner-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

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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 of 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 (ISO 1997; 1998).

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 $\log_{10}$ of *L. monocytogenes* numbers was calculated at each sampling time. Growth potential was calculated as the difference between the $\log_{10}$ 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).

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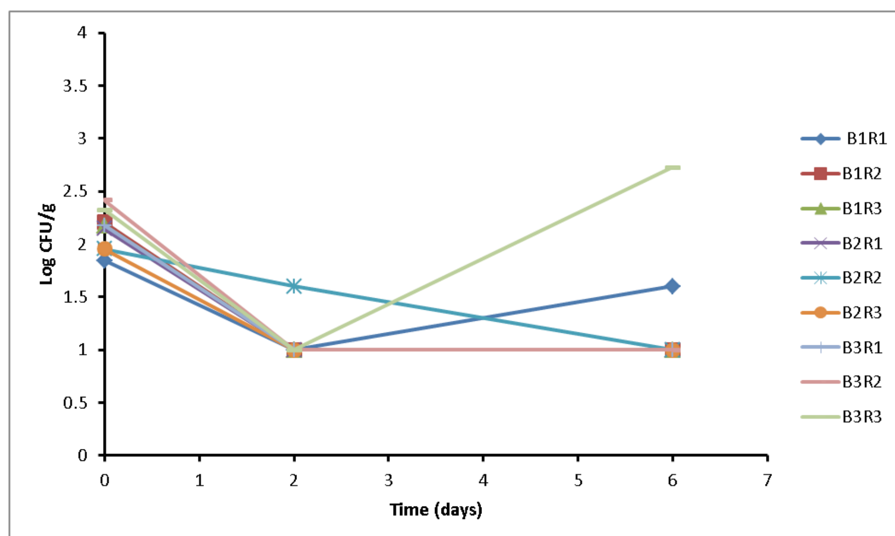

Figure 1. The behaviour of Listeria monocytogenes as determined on mushrooms. B1R1; batch 1 replicate 1, and so on. The dashed line indicates the limit of enumeration. CFU: colony forming units.

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

Time (days)	Log_{10} TBC, cfu/g*	pH	Water activity
Uninoculated	Not determined	6.87 ± 0.05	0.996 ± 0.001
0	3.82 ± 0.53	6.86 ± 0.02	0.994 ± 0.002
2	4.77 ± 0.20	6.92 ± 0.05	0.993 ± 0.005
6	5.94 ± 0.89	6.78 ± 0.03	0.996 ± 0.003

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

Discussion

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).

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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.

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