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Evaluation of *Listeria* challenge testing protocols: A practical study using cooked sliced ham

Linda Everis*, Gail Betts

Campden BRI, Chipping Campden, Glos GL556LD, United Kingdom

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ABSTRACT

A comparison was made of two approaches to microbiological challenge testing. Sliced cooked ham was challenge tested with *Listeria monocytogenes* using the protocol detailed in the Technical Guidance Document on shelf life studies for *L. monocytogenes* in ready-to-eat foods produced by Agence Française de Securité Sanitare des Aliments (AFSSA now known as ANSES) which required testing of three batches of product, an inoculum level of no more than 100 cfu/g, cultures pre-adapted to chill conditions and a storage regime of 7 d at 8 °C followed by 14 d at 12 °C. This was compared to a more standard industry type of approach using a single batch of product, an inoculum level of between 100 and 1000 cfu/g, cultures grown overnight at optimal temperature and a storage regime of 21 d at 8 °C. The results of the trial showed that the standard Industry approach gave similar results to that of the more complex AFSSA approach with regards to growth potential, lag time and time for a 2 log increase.

1. Introduction

Products are formulated, manufactured and processed in order to minimise the potential for growth of pathogens and target spoilage organisms. However, it is possible that a final product could contain pathogens or spoilage organisms, which could grow in the formulation, or may be present due to process deviation or hygiene problems. Under these circumstances it may be necessary to demonstrate whether the undesirable organisms could grow to unacceptable levels during the intended shelf life (Betts, 2010).

Microbiological challenge tests are designed to assess the potential for growth of specific food borne pathogens or spoilage organisms in a food product from point of manufacture through to consumption. This involves the inoculation of a product with relevant microorganism(s) and storing under a range of controlled environmental conditions in order to assess the risk of food poisoning or to establish product stability (Betts, 2010).

The use of challenge testing to assess product safety and stability has increased over the past few years, particularly with respect to food pathogens such as *Listeria monocytogenes*, where evidence is required to demonstrate the potential for growth of this organism throughout product shelf life. According to EU Regulations EC 2073/2005 (as amended), chilled ready-to-eat (RTE) products other than those intended for infants or for special medical purposes should have no more than 100 cfu/g *L. monocytogenes* per gram present at the end of shelf life. Food business operators (FBO) producing RTE products able to support growth must therefore be able to demonstrate that the product will not exceed the limit of 100 cfu/g throughout its shelf life. When this is not demonstrated, a criterion of absence of *L. monocytogenes* applies at the end of the manufacturing process.

There are a limited number of ways in which FBO can demonstrate compliance with the Regulations. These include the use of predictive modelling tools to assess the potential for likelihood of growth, historical microbiological data and challenge testing.

Specific guidance is freely available to help FBO's demonstrate compliance with the EU Regulation (Anon, 2005; CFA, 2010). Detailed guidance has also been given for laboratories who conduct challenge tests and durability studies (AFSSA, 2008), which is also described in by Beaufort, 2011. The AFSSA document describes how cultures should be prepared, including adaptation to chill temperatures, the levels at which to inoculate and the temperature at which to store the inoculated products. This protocol is very detailed and requires the inoculation and sampling of three batches of product and, in addition to enumeration of *Listeria*, requires the levels of naturally present microflora to be assessed as well as sodium chloride, pH and water activity throughout the trial. There are also details given on maximum permitted inoculation levels and how to interpret the results, including standard deviations that must be met within replicates and batches.

* Corresponding author. Tel.: +44 01386 842063; fax: +44 01386 842100.
E-mail address: l.everis@campden.co.uk (L. Everis).

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Whilst the target inoculation level recommended by AFSSA is more representative of the likely level of *L. monocytogenes* present in real foods, it does present issues with respect to reliability of data. There are some reports which show large variability in lag times and growth rate that may be observed in small cell populations. François et al. (2006) evaluated the variability in growth of *L. monocytogenes* when inoculated at levels of 10, 100, 1000 or 10,000 cells. They found the variability of growth of *L. monocytogenes* was high for low inoculum levels and recommended that levels of 1000 cfu per sample or higher were used to accurately measure the growth response of *L. monocytogenes* in challenge test studies. Interestingly, AFSSA originally proposed an inoculum level of 1000 cfu/ml (AFSSA, 2001). However, they revised this downwards in order to meet more precisely the requirements of EU Regulations EC 2073/2005 (as amended).

An alternative approach is needed in order to meet the requirements of EU Regulations EC 2073/2005 (as amended), and to use a high enough inoculum to avoid the variability issues of low inoculum levels. Due to the growth kinetics of bacterial cells, it is proposed that increasing from 1 cell to 100 cells takes the same number of generations as increasing from 100 to 10,000 cells or from 1000 to 100,000 cells, i.e. a 2-log increase in numbers. Therefore, if a challenge test procedure could evaluate the time taken to exceed a 2-log increase in numbers, rather than the more precise increase from 1 to 100 cells, then it would be easier on a practical basis, could overcome the issues of low inoculum levels, but would yield the same result. Such an approach is used routinely throughout the food industry (Betts, 2010). It is simpler in terms of the inoculum requirements, and generally requires the testing of replicate samples from a single batch of product rather than three.

The aim of this study was to assess the streamlined industry type approach with the more detailed AFSSA approach to ascertain if a simplified and more economical approach to *Listeria* challenge testing would give similar results to the more complex and therefore more expensive approach. A cooked sliced ham product was chosen as this product has been implicated with issues of contamination with *L. monocytogenes*. Contamination rates over 30% in deli meats in Europe have been reported (Todd & Notermans, 2011). This type of deli meat has been linked to outbreaks of listeriosis, such as the deli meat outbreak in Canada in 2008, which led to 58 cases with 20 deaths (Todd & Notermans, 2011). Uyttendale et al., 2009 studied the prevalence of *Listeria* in ready-to-eat cooked meats and also the potential for growth via challenge tests. They found that 61/92 cooked meat challenge tests demonstrated growth potential of this organism in the product during the target shelf life. They concluded that these products allow the growth of *Listeria* but have a low prevalence (1.1%) and as such recommended that they be considered intermediate risk foods with respect to growth of *L. monocytogenes*.

2. Materials and methods

Two different challenge test procedures were used in this study.

Industry Approach: Cultures were grown at 30 °C for 24 h, inoculated into a single batch of product at approximately 10^3 cfu/g and stored at 8 °C.

AFFSA Approach: Cultures were grown at 37 °C for 24 h then 5 °C for 7 d, inoculated into triplicate batches of product at approximately 50 cfu/g and stored at 8 °C for 7 d then 12 °C for 14 d.

2.1. Products

The AFSSA approach requires 3 batches of product to be tested. Therefore, 3 batches of cooked sliced ham (batches A, B and C) were sourced directly from a manufacturer. The ham was produced and sliced 2 days prior to the trial start date. The ham was packaged in a 70% N_2/30% CO_2 mix.

2.2. Organisms

Three strains of *L. monocytogenes* were used in this trial. These were *L. monocytogenes* CRA 1170 isolated from chicken, CRA 16458 isolated from a meat factory and CRA 6600 Type strain NCTC 11994.

Each of the 3 cultures were inoculated into Tryptone Soya Broth (TSB, Oxoid CM0129) and incubated at either 30 °C for 24 h, or 37 °C for 24 h followed by a subculture into fresh TSB and incubation at 5 °C for 7 days.

2.3. Sample inoculation

A cocktail of each of the 3 strains grown under both conditions was prepared containing an equal concentration of each strain. A microscopic count was performed on each of the 3 strains and dilutions prepared in Maximum Recovery Diluent (MRD) (LabM LAB103) to give the correct level in the inoculum. The Industry Approach samples were inoculated at a target level of 10^3 cfu/g and the AFSSA samples with target 50–100 cfu/g. Samples of product were inoculated with 0.1 ml using a syringe through a double sided foam pad on the outside of the packaging. The product weight was 90 g.

2.4. Storage

Products were stored at either 8 °C throughout the 21 day trial (Industry) or at 8 °C for 7 d followed by 12 °C for 14 d (AFSSA).

2.5. Sample preparation

Triplicate samples of product from each of the test protocols were enumerated for level of *L. monocytogenes* on days 0, 7, 10, 14, 18 and 21.

2.6. Microbiological analysis

For enumeration, the whole sample was weighed out aseptically and a 1:1 dilution was prepared using Maximum Recovery Diluent (MRD) (LabM LAB103). A sample, 2 ml, of this dilution was then added to 8 ml MRD, to give a 1:10 dilution. A decimal dilution series was prepared using MRD. The spread plate technique was used for the enumeration of *L. monocytogenes*.

For enumeration of *L. monocytogenes*, pre-poured plates of chromagenic *Listeria* agar base (Oxoid CM1080 & SR0227) were used. The plates were allowed to dry, inverted and incubated at 37 °C for 48 ± 4 h, and all typical colonies counted.

In order to be able to assess the time for a 2 log increase the data was input to DMFit web edition (http://ifrsvwwwdev.ifrn.bbsrc.ac.uk/CombasePMP/GP/DMFit.aspx).

Growth curves were then fitted to the data using DM-fit. This enabled the lag time and growth rate to be calculated and smooth lined graphs to be produced. The model chosen for use was the complete Baranyi and Roberts model (Baranyi & Roberts, 1994). This model has 4 main parameters: initial level, lag time, maximum growth rate and final level, and 2 curvature parameters: mCurv = 10 and nCurv = 1 which describe the curvature of the sigmoid curve at the beginning and at the end of the growth phase. These fitted data were then entered into Minitab and scatterplots were then produced so the time for a 2 log increase could be calculated.