# Is *Listeria innocua* 2030c, a tetracycline-resistant strain, a suitable marker for replacing *L. monocytogenes* in challenge studies with cold-smoked fish?

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**Abstract**

The suitability of *Listeria innocua* 2030c, a tetracycline-resistant strain, to be used as an indicator for replacing *Listeria monocytogenes* in challenge studies with cold-smoked fish was ascertained. *L. innocua* 2030c was compared to serovars 4b and 1/2c of *L. monocytogenes*, the major types isolated from Portuguese cold-smoked fish products. Growth curves at 30°C, growth/survival patterns at 30°C under exposure to different times and concentrations of ozone and sensitivity to *Carnobacterium divergens* V41 and *C. piscicola* V1 and their bacteriocins V41 and V1, were determined. No important differences between *L. innocua* 2030c and *L. monocytogenes* 4b and 1/2c were found, therefore *L. innocua* 2030c can be considered a suitable indicator for replacing those *L. monocytogenes* strains in challenge studies. 

## 1. Introduction

*Listeria monocytogenes* is a pathogenic bacterium for immuno-compromised people and foetuses of pregnant women. Because of this pathogenicity it generally would not be possible to conduct actual challenge studies under any conditions other than a laboratory-simulated process. As challenge studies with inoculated fish for cold-smoking purposes were intended to be performed in a pilot plant rather than in a research laboratory it was necessary to replace *L. monocytogenes* by a non-pathogenic strain, and to ensure that it behaved equally to the pathogen under the same processing conditions.

*Listeria innocua* strains resistant to the antibiotics chloramphenicol and erythromycin have been used with success as indicators for evaluation of the lethality of thermal processes with respect to *L. monocytogenes* (Foegeding & Stanley, 1991).

Treatment of fish with ozone and application of bacteriocins are potential means of reducing *L. monocytogenes* levels in cold-smoked fish products. A suitable non-pathogenic marker should behave similarly to the pathogen *L. monocytogenes* when subjected to the same treatments while simultaneously being easily identified as non-natural cold-smoked fish flora. As all the strains of *L. monocytogenes* and *L. innocua* isolated from Portuguese cold-smoked fish were, until now, tetracycline-sensitive, *Listeria innocua* 2030c, a tetracycline-resistant strain, was chosen for this purpose.

For ascertaining the similarity of their behaviours, *L. innocua* 2030c was compared to serovars 4b and 1/2c of *L. monocytogenes*, the major types isolated from Portuguese cold-smoked fish products. Growth curves at 30°C, growth/survival patterns at 30°C, and on exposure to different times and concentrations of ozone, and sensitivity to *Carnobacterium divergens* V41 and *C. piscicola* V1 and their bacteriocins V41 and V1 were determined.

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## 2. Materials and methods

### 2.1. Ozone generation and measurements

Ozone was produced by a domestic ozone generator model PR1 (TRIOZON, Spain) using atmospheric air as the source of oxygen. The ozonated air produced at a constant flow rate by the apparatus was passed via a silicone tube to a pump, and then to a plastic chamber of 26 $dm^3$ volume. The ozone in the air flow produced by the apparatus (0.32 mg $l^{-1}$) was measured experimentally by the iodometric method as described Silva, Gibbs, and Kirby (1998). Also, the ozone concentration inside the chamber was calculated by a mass balance based on the total volume of the chamber, the air flow and the rate of production of ozone by the generator as described by Silva et al. (1998) (Fig. 1).

## 3. Growth curves

Pure cultures were grown in Tryptone soy yeast extract broth (TSB-YE) (Tryptone soy broth (Lab M) + 0.6% w/v yeast extract (Lab M)) incubated at 30°C. Curves were constructed using the drop counting technique (Miles & Misra, 1938) on Tryptone soy agar (TSA-YE: TSB-YE + 1.2% w/v of agar) (Lab M) plates and incubated at 30°C.

### 3.1. Treatments

#### 3.1.1. Ozone treatment

*L. innocua* 2030c was obtained from the culture collection of the Public Health Laboratory Service (PHLS) in London. From overnight cultures of *L. innocua* 2030c ($10^{8-9}$ CFU $ml^{-1}$) in TSB-YE (Lab M) serial decimal dilutions up to $10^{-9}$ were made in maximum recovery diluent (MRD) (Lab M). Cell suspensions were delivered onto pre-dried plates of TSA-YE (Lab M), using the drop counting technique (Miles & Misra, 1938). Each plate was divided into four equal spaces and two separate 20 μl aliquots were dropped in each space for each dilution. The inoculated plates were placed into an ozonation chamber and exposed to ozone for, respectively, 20, 40, 60, 80 and 90 min. After treatment the plates were incubated at 30°C for 24 h. This temperature was chosen because it is the temperature to be used in further experiments with cold-smoked fish previously inoculated with *L. innocua* 2030c. Results are the means of triplicate experiments.

#### 3.1.2. Bacteriocins

*L. innocua* 2030c, *L. monocytogenes* 4b and 1/2c strains were sent to École Nationale d'Ingénieurs des Techniques des Industries Agricole et Alimentaires (ENITIAA), Nantes, France. They were tested for their sensitivity to *C. divergens* V41 and *C. piscicola* V1 and their bacteriocins V41 and V1, following the protocol of Pilet et al. (1995) (Duffes, Corre, Leroi, Dousset, & Boyaval, 2000).

## 4. Results and discussion

All the strains tested were equally sensitive to *C. divergens* V41 and *C. piscicola* V1 and their bacteriocins V41 and V1 (data not shown, reported by ENITIAA) therefore, *L. innocua* 2030c can be considered a suitable marker for replacing *L. monocytogenes* in experiments with application of these bacteriocin producers and/or their bacteriocins.

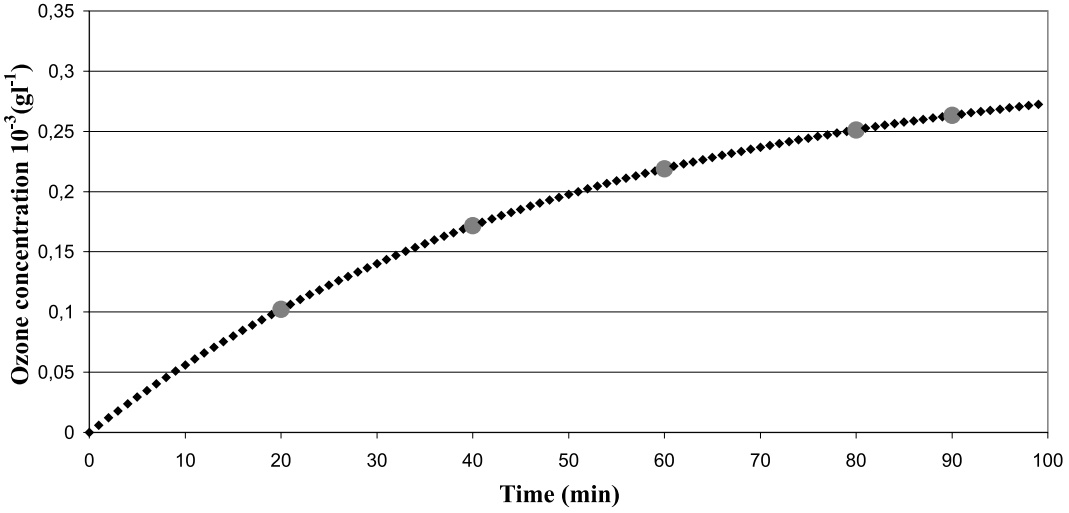


Fig. 1. Theoretical curve of ozone concentration in the air inside the desiccator.

Growth curves at 30°C are shown in Fig. 2. A slightly longer lag phase was observed in *L. innocua* and *L. monocytogenes* 1/2c growth compared to *L. monocytogenes* 4b strain. All the strains reached stationary growth phase with about 9.4–9.5 log CFU $ml^{-1}$ and the length of this phase was similar (8–15 h). *L. monocytogenes* 1/2c numbers then declined faster than *L. innocua* 2030c numbers and the latter declined faster than *L. monocytogenes* 4b strain. Therefore, *L. innocua* 2030c can provide an additional margin of safety as an indicator organism with respect to *L. monocytogenes* 1/2c but not with respect to *L. monocytogenes* 4b strain. Nevertheless, as differences in the number were ca. 0.5 log CFU $ml^{-1}$ they were considered not important for future applications on cold-smoking fish processing and storage experiments.

Exposure to gaseous ozone was shown to be effective in reducing bacterial levels. However, survival rates of the three strains were not linearly related to ozonation time (Fig. 3), the higher death rate (about 3 log) occurring during the first 20 min of ozone exposure and stabilising during the following 70 min. Several other studies using ozone have also shown that death rate kinetics for a variety of bacteria and viruses exhibit a biphasic process over an extended time period (Broadwater, Hoehn, & King, 1973; Ishikaki, Shinriki, & Matsuyama, 1986; Silva et al., 1998). Silva et al. (1998) after testing three Gram-negative and two Gram-positive bacteria found that the higher death rate occurred after 15 min of exposure to ozone when the level of ozone inside the chamber was about $0.22 \times 10^{-3}$ g $l^{-1}$.

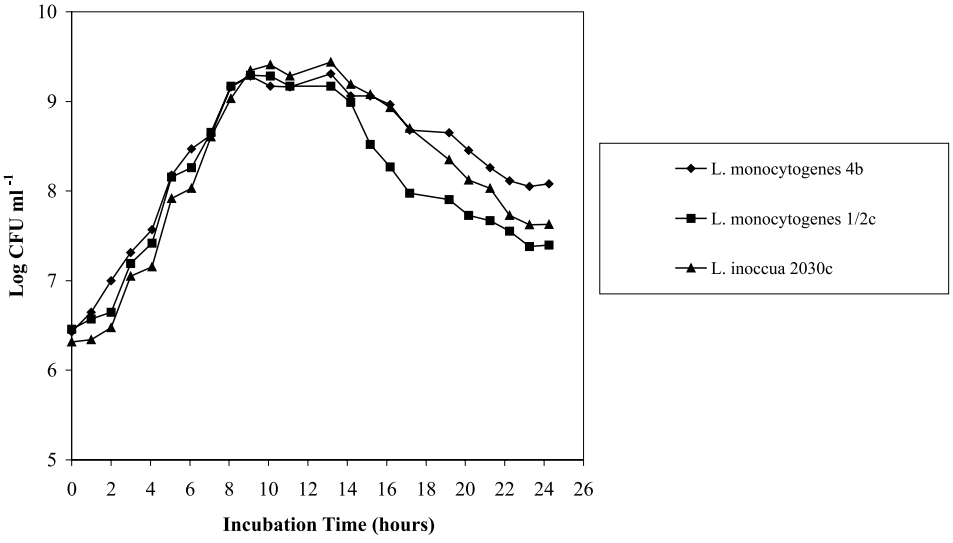


Fig. 2. *Listeria* spp. growth curves (at 30°C).

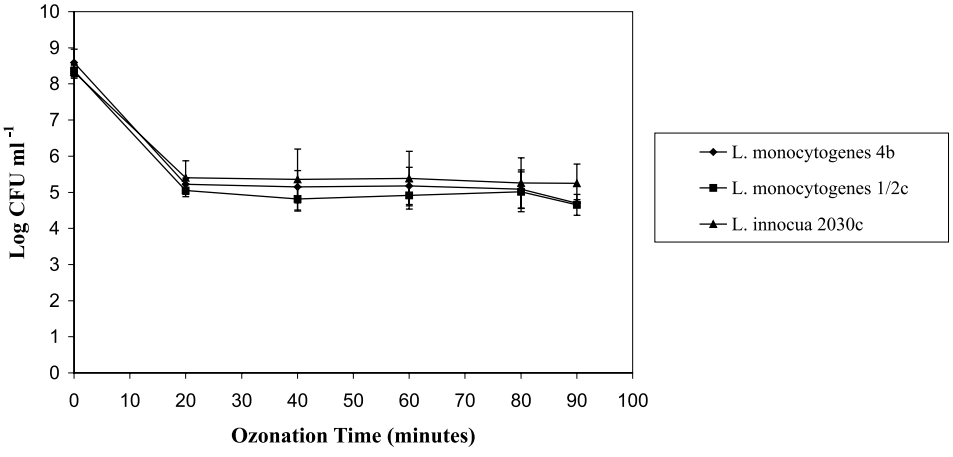


Fig. 3. *Listeria* spp. survivor curves during ozone exposure.

The theoretical curve obtained for the ozone concentration in the air inside the chamber (Fig. 1) shows the ozone concentration after 20 and 90 min reaching, respectively, $0.1 \times 10^{-3}$ and $0.26 \times 10^{-3}$ g $l^{-1}$. A small decrease in the number of *L. monocytogenes* strains was noticeable after 90 min of ozone exposure whereas the number of *L. innocua* 2030c remained stable, therefore these differences will provide an additional margin of safety when using *L. innocua* 2030c as an indicator organism as it was more resistant to ozone than the *L. monocytogenes* strains.

Thus, for gaseous ozone treatments, the 20 min, when the level of ozone reached $0.1 \times 10^{-3}$ g $l^{-1}$, can be considered the best time of exposure to reduce the number of all strains by about 3.5 log CFU $ml^{-1}$.

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