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# Impact of the prevalence of different pathogens on the performance of sampling plans in lettuce products

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ABSTRACT

Prevalence and concentration of *Listeria monocytogenes*, *Salmonella* spp. and enteric pathogenic viruses (namely Hepatitis A—HAV, and noroviruses genogroup I—NoVGI and genogroup II—NoVGII) were determined in raw and RTE lettuce from a Spanish processing premise. Fifteen samplings were made from September 2010 to February 2012 ($n = 600$ samples). Sampling strategies for pathogen detection were suggested by the characterization of the uncertainty in prevalence associated with the performance of two-class attributes sampling plans ($c = 0$). A probabilistic model was run (1000 iterations) using a Bayesian approach with a conjugate beta distribution considering the impact of taking different number of samples on the proportion of positive samples and lots (within- and between-lot prevalence). No enumeration results were obtained for the pathogens tested. Presence of *L. monocytogenes* and NoVGII in RTE lettuce (10%) and NoVGI and NoVGII in unprocessed lettuce (10%) was obtained in the tested lots during cold season. Results evidenced that, as the number of samples increased, the probability of rejecting a contaminated lot became higher, yielding right-skewed distributions with values close to 1. According to our results, 25 samples would result in 80% of rejected lots, while 95% confidence level would be reached with $n > 100$. However, although those levels would imply a unrealistic high number of samples making the application of the sampling plan unfeasible, these results might be useful for food operators and risk managers to know the underlying distributions of microbial contamination together with potential control measures to be applied to assure a safer production of minimally processed vegetables.



## 1. Introduction

Consumption of fresh produce is becoming more popular in developed countries due to their convenience, ease of preparation and healthy and nutritional benefits (Alegre et al., 2010). Among the main fresh products, ready-to-eat (RTE) iceberg lettuce is one of the most consumed increasing in salad bar patronage and meals eaten outside the home (Buck et al., 2003; USDA, 2002). Severe foodborne disease outbreaks can be caused by pathogenic microorganisms associated with fresh produce (Delaquis et al., 2007). In the EU in 2009 and 2010, respectively 4.4% and 10% of the foodborne verified outbreaks were linked with the consumption of vegetables, fruits, berries, juices (and products thereof) (EFSA/ECDC, 2014). Contamination sources may come from the field by direct contact with animal waste, irrigated water, and inadequately treated manure (Johannessen et al., 2005). Further, fresh cut products are prone to contamination because the current industrial sanitizing treatments do not guarantee the total elimination of the pathogen when present (Beuchat, 1996; Parish et al., 2003; Abadias et al., 2008, Pérez-Rodríguez et al., 2011; Posada-Izquierdo et al., 2014).

Enteric pathogens such as *Salmonella* spp. appear to be particularly prone to lettuce contamination. Franz et al. (2007) reported that 18% of all lettuce-associated outbreaks were caused by *Salmonella* and 10% of all *Salmonella* outbreaks with fresh produce were related to lettuce. The presence of *Listeria monocytogenes* in leafy greens has been studied extensively through risk assessment (Franz et al., 2010; Oliveira et al., 2010) since this commodity may support the growth of *L. monocytogenes* (Carrasco et al., 2008; Francis and O'Beirne, 2005; Szabo et al., 2000). Other causal agents such as foodborne viruses, mainly noroviruses, are linked to the consumption of leafy greens. Until recently, no data was available about the prevalence of NoV on fresh produce. Nevertheless, NoV outbreaks have been reported linked to leafy greens (Ethelberg et al., 2010; Gallimore et al., 2005).

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**Table 1**
Results of the presence/absence of the studied pathogens in 25 g of unprocessed and RTE lettuce taken from different lots in the period 2010/2012 (15 sampling points).

| Microorganisms | Cold season (40 lots) | | Warm season (20 lots) | | Total |
|---|---|---|---|---|---|
| | Unprocessed lettuce | RTE lettuce | Unprocessed lettuce | RTE lettuce | |
| *Salmonella* spp. | 0/20 (0%) | 0/20 (0%) | 0/10 (0%) | 0/10 (0%) | 0/60 (0%) |
| *L. monocytogenes* | 0/20 (0%) | 2/20 (10%) | 0/10 (0%) | 0/10 (0%) | 2/60 (3.3%) |
| HAV[a] | 0/20 (0%) | 0/20 (0%) | 0/10 (0%) | 0/10 (0%) | 0/60 (0%) |
| NoVGI[b] | 2/20 (10%) | 0/20 (0%) | 0/10 (0%) | 0/10 (0%) | 2/60 (3.3%) |
| NoVGII[c] | 2/20 (10%) | 2/20 (10%) | 0/10 (0%) | 0/10 (0%) | 4/60 (6.6%) |

[a] Hepatitis A virus.
[b] Norovirus Group I.
[c] Norovirus Group II.

One measure for verification of the safety of the products along the food processing is the establishment of microbial testing at different points throughout the production chain to detect foodborne pathogens and verify lot acceptance. When establishing a microbiological criterion (MC) in relation to a Performance Objective (PO) and/or a Food Safety Objective (FSO) knowledge of the contamination distribution should be gained. For vegetables, as solid matrices, microbial contamination is normally present at very low levels and heterogeneously distributed so that the probability of pathogen detection dramatically decreases. Random sampling, when available, could be a simple method to provide detection of positive samples (i.e. homogeneous lot contamination), but it was previously shown that systematic sampling is in many cases more effective in detecting clusters of microorganisms (Jongenburger et al., 2011a). In order to take a representative sample, the sampling strategy is important, especially when the microorganisms are distributed heterogeneously or localizedly (Jongenburger et al., 2011b).

There is a lack of information about the adequate sampling method to follow in leafy greens as well as the evaluation of between-lot and within-lot variability to detect bacterial pathogens and enteric viruses. The present work aimed at determining the effect that the concentration and prevalence of several pathogens (*L. monocytogenes*, *Salmonella* spp. and enteric pathogenic viruses) in lettuce have in the performance of two-class attribute sampling plans as a model for leafy green vegetables.

## 2. Materials and methods

### 2.1. Sampling strategy

This study was conducted in a Spanish RTE lettuce processing premise during September 2010 to February 2012. The samples collected in each sampling were raw and RTE lettuces belong to the same lot (raw lettuce used for obtaining RTE salads and RTE lettuce packages produced in the same day under the same conditions). A total of 300 lettuce samples (150 raw and 150 RTE—processed—lettuces) were taken during fifteen independent samplings separated by at least two weeks each. On each sampling, 5 raw lettuces and 5 RTE lettuces were collected.

### 2.2. Microbiological analysis

The outer wrapper of each pack was disinfected with 70% ethanol before analyzing the sample. Using an appropriate sterilized material each product was aseptically cut into several pieces. Afterwards, a sample unit of 25 g was obtained from different parts of the sample portion randomly selected. Samples were tested for *Salmonella* spp., *L. monocytogenes* and the main enteric pathogenic viruses (NoVGI, NoVGII and HAV).

The presence and enumeration of *L. monocytogenes* were conducted according to ISO 11290-1:1996/Amd 1:2004 (ISO, 1996, 2004a) and ISO11290-2/Amd 1:2004 (ISO, 1998, 2004b), respectively. The presence of *Salmonella* spp. was carried out according to ISO 6579 (ISO, 2002). The enumeration of mesophilic bacteria was conducted according to ISO 4833 (ISO, 2013).

The concentration and extraction of viruses from the lettuce were performed as previously described (Dubois et al., 2006, Kokkinos et al., 2012) including a sample process control virus-murine norovirus (MNoV-1) (Diez-Valcarce et al., 2011a). The nucleic acid extraction was performed using the NucliSENS® miniMAG® kit (bioMérieux) according to the manufacturer's instructions (Kokkinos et al., 2012) and the detection of each given virus was performed by a specific RT real-time PCR (RTqPCR) (Kokkinos et al., 2012). An internal amplification control (IAC) (Diez-Valcarce et al., 2011b) was included in every assay. For the human noroviruses specific RTqPCR assays, chimeric RNA positive controls were used (Martínez-Martínez et al., 2011). For a proper interpretation of the results four different signals were assayed: The target virus, the SPCV control, the target IAC and the SPCV IAC, and when at least one of the two replicate targets (for HAV, NoVGI and NoVGII) was detected, these lettuce samples were considered to be positive (D'Agostino et al., 2011).

### 2.3. Data treatment and probabilistic model

Outcomes from the microbiological analyses of unprocessed and RTE lettuces were expressed as CFU/g or log CFU/g, absence or presence in 25 g according to the type of analysis carried out. Although uncertantity in the perfomance on the microbiological methods can be observed, the recovery capacity of the microbiological methods was assumed to be 100%, so theoretical limits were applied based on the dilution factor used for analysis. The limit of quantification (LOQ) for the enumeration method for *L. monocytogenes* corresponded to 10 CFU/g. For investigation methods, the limit of detection (LOD) for bacteria was established at 1 CFU/25 g since results were obtained as the presence or absence in 25 g of the analyzed sample. For virus, LOD was established at 30 genome equivalents in 25 g of the analyzed sample.

To derive suitable two-class sampling plans, a probabilistic model was built described as follows. Due to the lack of quantitative data, the underlying frequency distributions for the pathogens within and between lots were unknown. Therefore, the proportion ($p$) of positive samples (named within-lot) and lots (named between-lot) were used, considering the LOD and LOQ for each pathogen. The uncertainty on $p$ was estimated based on a Bayesian approach utilizing a conjugate beta distribution to derive the corresponding posterior distribution in each case, based on Eq. (1).

$$p = \mathrm{Beta}(\alpha + s, \beta + n - s) \quad (1)$$

Where $\alpha$ and $\beta$ are the distribution parameters, $n$ corresponds to the total number of analyzed lots or samples, and $s$ is the total number of positive lots or samples.

Assessment of sampling plans was carried out considering a two-class attribute sampling plan with $c = 0$ as microorganisms to be detected are pathogenic, and they should be absent. Different values of $n$ were assessed, determining the probability of detecting (or rejecting) the contaminated lots. The calculations were performed considering a