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Short communication

Microbiological survey of raw and ready-to-eat leafy green vegetables marketed in Italy

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ABSTRACT

The presence of foodborne pathogens (*Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7, thermotolerant *Campylobacter*, *Yersinia enterocolitica* and norovirus) in fresh leafy (FL) and ready-to-eat (RTE) vegetable products, sampled at random on the Italian market, was investigated to evaluate the level of risk to consumers. Nine regional laboratories, representing 18 of the 20 regions of Italy and in which 97.7% of the country's population resides, were involved in this study. All laboratories used the same sampling procedures and analytical methods. The vegetable samples were screened using validated real-time PCR (RT-PCR) methods and standardized reference ISO culturing methods.

The results show that 3.7% of 1372 fresh leafy vegetable products and 1.8% of 1160 "fresh-cut" or "ready-to-eat" (RTE) vegetable retailed in supermarkets or farm markets, were contaminated with one or more foodborne pathogens harmful to human health.

1. Introduction

The last decade has witnessed an increase in the demand for fresh leafy (FL) green vegetables and other ready-to-eat (RTE) products due to a change in eating habits arising from healthier lifestyle choices. It is widely recognized that leafy green vegetables are an important component of any healthy diet, providing vitamins, minerals, and phytonutrients.

In Italy, there are approximately 450 companies, comprising 80 processing plants, involved in the production of RTE vegetables; in 2011 domestic vegetable production stood at 3.7 million tons (2% of the vegetable market) (Ortofrutta italiana/Dossier, 2011). Since then, a continuous rise in demand places Italy today behind Great Britain as the two leaders in the fresh vegetable market of Europe (www.ismeaservizi.it).

A downside to the potential health benefits is that raw, leafy, green vegetable products are considered important vehicles in the transmission of foodborne pathogens, including *Salmonella* spp., thermotolerant *Campylobacter*, *Listeria monocytogenes* and certain enteric viruses (Park et al., 2012). These may contaminate vegetables during any one of the many stages of production, with the pathogens cycling through the environment and into the food chain via manure, insects, water and soil (Hou et al., 2013).

Both in Europe and the USA, recent outbreaks of foodborne illnesses have revealed that a close correlation exists between pathogen contamination and leafy green vegetable consumption (Mercanoglu Taban and Halkman, 2011). Although contaminated samples of RTE foods are found occasionally in Europe, as documented by the Rapid Alert Systems for Food and Feed (European Commission, 2013), a number of previous laboratory surveys indicate that pathogenic organisms are uncommon in fresh products (Althaus et al., 2012; Koseki et al., 2011). With reference to Italy, only very limited data exist on the microbial safety of ready-to-eat vegetable products (De Giusti et al., 2010). Fresh, ready-to-eat vegetables have a very short shelf-life (a maximum

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of 7 days), and since classical laboratory culture procedures give results after only 5–7 days, it follows that rapid and sensitive molecular methods are those most useful for detecting pathogens and assessing product safety in a timely manner. However, the plethora of molecular methods, in general, provides poorly comparable results, which means that the data obtained are not always useful for risk analyses. For this reason, all alternative techniques including molecular methods, have to be validated following the reference standard: ISO 16140: 2003 "Microbiology of food and animal feed stuffs — Protocol for the validation of alternative methods" (Lombard and Leclercq, 2011).

The purpose of this study was to evaluate the presence of foodborne pathogens such as *Salmonella* spp., *L. monocytogenes*, *Escherichia coli* O157:H7, thermotolerant *Campylobacter*, *Yersinia enterocolitica* and norovirus GI and GII serogroups, in fresh leafy and ready-to-eat vegetables available on the open market in Italy.

2. Material and methods

Nine governmental regional veterinary institutes named Istituti Zooprofilattici Sperimentali (IZSs), namely Lombardia and Emilia Romagna (IZSLER), Piemonte and Liguria (IZSPLV), Veneto and Trentino Alto Adige (IZSVe), Sardegna (IZSSa), Lazio and Toscana (IZSLT), Campania and Calabria (IZSME), Puglia and Basilicata (IZSPB), Umbria and Marche (IZSUM), Sicilia (IZSPa) and the National Institute of Health, Microbiological Foodborne Hazard Unit (ISS Rome, Italy), were involved in the survey conducted in 18 of the 20 regions comprising Italy, and where 97.7% of the country's population resides.

Samples were obtained at production/processing plants, wholesale markets and supermarkets; on each occasion 5 to 15 samples were taken. The inherent variability of the Italian farming landscape and the mild and largely frost-free Mediterranean climate means that a wide variety of fresh vegetable products, remains uniformly available throughout the year. The samples obtained fall into two main categories: (a) fresh leafy vegetables (FL) sold traditionally in bulk packaging in supermarkets or farm markets, from which the customer can choose a portion. These types of vegetables are selected, washed, dried and cut by the buyers and can be eaten raw in salads or cooked, depending on the species and local eating traditions; (b) "fresh-cut" or "ready-to-eat" vegetables (RTE) are sold in packets, in this case the vegetables have been washed, selected and cut by producers for their customers. The RTE vegetables are sold in small amounts (80–500 g). A total of 2532 samples (FL vegetables n = 1372; RTE vegetables n = 1160) were collected and analysed between 2011 and 2012.

A 25 g portion of each sample was cultured in specific broth media and then used to test for the presence of *Salmonella* spp., *L. monocytogenes*, *E. coli* O157:H7 and thermotolerant *Campylobacter* using the appropriate commercially available RT-PCR kits denominated as "PCR Adiafood AES Chemunex®, Detection Systems" (Marcy, l'Etoile-France). All the kits were originally validated following the requirements as set out in the reference standard ISO 16140: 2003, and certified by AFNOR (Association Française de Normalisation, La Plaine Saint Denis, France). DNAs from these enrichment broths were tested using the appropriate RT-PCR methods. While awaiting the RT-PCR results (3–4 h), the enrichment broths were maintained at 4 °C.

RT-PCR positive results for *Salmonella* spp., *L. monocytogenes*, and thermotolerant *Campylobacter*, were confirmed using the corresponding reference analytical microbiological method, respectively the ISO 6579:2002/Corr.1:2004, the ISO 11290–1:2004 and the ISO 10272–1:2006.

Conversely, the presence of pathogenic *Y. enterocolitica* was assayed simultaneously using both the microbiological ISO 10273:2003 reference method and an "in house" RT-PCR method targeting the virulence *ail* gene (Mäde et al., 2008). The same procedure was followed for *E. coli* O157:H7, for which magnetic immune-separation and ISO 16654:2001 were applied in parallel with the *E. coli* O157:H7PCR Adiafood AES Chemunex® alternative method.

The isolated *E. coli* strains were screened for the presence of the verotoxins *vtx1* and *vtx2* and for the cell adhesion factor, namely, the *eae*-intimin gene. These pathogenicity genes (*vtx1*, *vtx2* and *eae*) were detected using the RT-PCR methods obtained via the European Union Reference Laboratory VTEC website (http://www.iss.it/vtec).

The isolated strains of *Salmonella* spp. were further characterised for the identification of the serotype following the White–Kauffmann–Le Minor scheme.

The presence of norovirus genotypes GI and GII, mostly involved with human cases of disease, was investigated using a non-validated reverse transcription RT-PCR (rRT-PCR) (Lees, 2010; Pavoni et al., 2013).

All the RT-PCRs based on commercial kits, were performed following the manufacturer's instructions. In order to be able to compare the results, all laboratories used the same instruments (Stratagene Mx3005P, Agilent, USA).

To evaluate the variability of the results obtained by the different participating laboratories, six collaborative studies (CSs), one for each pathogen, were organized according to the standard ISO 16140:2003 method. All nine laboratories (IZSs) involved in the monitoring plan, including the Microbiological Foodborne Hazard Unit (ISS Rome, Italy), participated in the CSs. All the laboratories were accredited according to the UNI CEI EN ISO/IEC 17025:2005 "General requirements for the competence of testing and calibration laboratories" standard. The CSs were organized by the various IZSs, which distributed the samples to all other laboratories. More specifically, the CS for *L. monocytogenes* was implemented by the IZSLER; for thermotolerant *Campylobacter* by the IZSPLV; and for *Salmonella* spp. and *E. coli* O157:H7 by the IZSVe. The Microbiological Foodborne Hazard Unit (ISS Rome, Italy) provided protocols, DNA reference material and reagents for the "in house" *Y. enterocolitica* and norovirus GI and GII rRT-PCRs, including an Internal Amplification Control (IAC) system for *Y. enterocolitica* RT-PCR. The results, from each CS, were analysed by the organizing IZS and made available in a final report to all participants. The detailed procedures for each CS were previously agreed upon amongst all IZSs. All IZSs received, for each CS, 24 blind samples with a unique numerical sequence assigned to each laboratory. Standardized forms for recording the results anonymously were provided.

3. Results

The results obtained from the analysis of these 2532 samples (1372 FL and 1160 "fresh-cut" or "ready-to-eat" samples) are summarised in Table 1.

Eighteen of the samples were RT-PCR positive for *Salmonella* spp.; of these, twelve were detected in FL vegetables and six in RTE vegetables. Four positives were also confirmed using the ISO method. Amongst these it was possible to serotype two strains of *S.* Veneziana, one in each of two samples, one from central Italy (round chicory — *Cichorium intybus L.*), the other from northern Italy (rocket — *Eruca sativa*); one strain of *S.* Kasenyi (escarole endive — *C. intybus* L. var. *Milano*) was detected in a third sample, this from the south of Italy. The serotype of the fourth strain was not identified.

Twenty-one samples (0.8%), i.e. seventeen raw plants (FL) and four RTE salads, respectively, were positive for *L. monocytogenes* of these nine were also positive using the microbiological standard method.

Eighteen samples (0.7%), twelve FL vegetables and six RTE salads, were positive for thermotolerant *Campylobacter* RT-PCR; four of them were confirmed using the ISO method: one sample of sugarloaf (*C. intybus* L. var. *Milano*) and one sample of escarole endive (*Cichorium endivia* L. var. *Latifolium* Hegi) were positive for the presence of *C. jejuni*; one sample of chicory (*C. intybus* L.) and one sample of mache (*Valerianella locusta* (L.) Laterrade) were positive for the presence of *C. lari*. All four FL vegetables formed the ingredients of a single RTE mixed product that was negative for *Campylobacter* RT-PCR (data not shown). *C. lari* were found in the remaining fourteen RT-PCR positive samples.