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1 # Occurrence of Hepatitis A and E and Norovirus GI and GII in ready-to-eat
2 # vegetables in Italy

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18 **Abstract**

19 Fresh vegetables and their ready-to-eat (RTE) salads have become increasingly recognized as
20 potential vehicles for foodborne diseases. The EU Reg. 1441/2007 establishes microbiological
21 criteria for bacterial pathogens for products placed on the market during their shelf-life (i.e.
22 *Salmonella* spp., *Listeria monocytogenes*) for pre-cut fruits and vegetables (RTE) whilst it does not
23 address the problem of contamination by enteric viruses.

24 In this study we investigated the contamination by hepatitis A virus (HAV), hepatitis E virus (HEV)
25 and norovirus (NoV) in 911 ready-to-eat vegetable samples taken from products at retail in Apulia
26 and in Lombardia.

27 The vegetable samples were tested using validated real-time PCR (RT-qPCR) assays, ISO
28 standardized virological methods and ISO culturing methods for bacteriological analysis.

29 The total prevalence of HAV and HEV was 1.9% (18/911) and 0.6% (6/911), respectively. None of
30 the samples analyzed in this study was positive for NoV, *Salmonella* spp. or *Listeria*
31 *monocytogenes*. The detection of HAV and HEV in RTE salads highlights a risk to consumers and
32 the need to improve production hygiene.

33 Appropriate implementation of hygiene procedures is required at all the steps of the RTE vegetable
34 production chain and this should include monitoring of emerging viral pathogens.

35 **Keywords:** green vegetables, viruses, molecular methods

36 **1. Introduction**

37 Leafy green vegetables and their ready-to-eat (RTE) salads are important components of the current
38 human diets but are accompanied by new food safety threats since they are eaten raw and usually
39 without any further washing/decontamination procedures (Little and Gillespie, 2008). Apart from
40 psychrotrophic pathogens and spoilage microorganisms, RTE vegetables can be contaminated with
41 a number of human pathogens, including parasites, bacteria and viruses.

42 In Italy, the prevalence of bacterial pathogens in leafy green vegetables has been estimated between
43 3.7 for fresh and 1.8% for RTE products (Losio et al., 2015). Numerous foodborne virus outbreaks
44 have been linked to the consumption of fresh produce, mostly attributable to enteric viruses such as
45 norovirus (NoV), hepatitis A virus (HAV), hepatitis E virus (HEV), rotavirus (RV) and astrovirus
46 (AstV) (European Food Safety Authority & European Centre for Disease Prevention and Control,
47 2013; Chiapponi et al., 2014; Collier et al., 2014; European Food Safety Authority, 2014; Kokkinos
48 et al., 2012; Terio et al., 2015; Wheeler et al., 2005).

49 Enteric viruses may contaminate vegetables, during cultivation before harvest or post-harvest.
50 During pre-harvest cultivation, there are various routes of contamination, which usually include
51 application of organic wastes as fertilizer, contamination of water used for irrigation with faecal
52 material, contact with inadequately-treated sewage or sewage-polluted water. In addition, direct
53 contamination by livestock, wild animals and birds should be considered (Heaton and Jones, 2008).

54 Water is the main critical vehicle of contamination in the farm-to-fork continuum. Spraying,
55 washing or immersion of fruits and vegetables in water are common practices during post-harvest
56 processing (Gandhi et al., 2010). Moreover, minimal processing may induce cross-contamination of
57 clean produce during cutting, washing and packaging (López-Velasco et al., 2010). In addition,
58 much emphasis has also been placed on the role of workers during collection, processing, storage,
59 distribution or final preparation (European Food Safety Authority BIOHAZ Panel, 2014; Koopmans
60 and Duizer, 2004; Rzezutka and Cook, 2004).

61 Currently, the European legislation on the safety of pre-cut (ready-to-eat) fruit and vegetables
62 requires the assessment of *Salmonella* spp. and *Listeria monocytogenes* contamination at the end of
63 the production chain (at retail). In addition, the *Escherichia coli* count is required during the
64 manufacturing processes (Commission Regulation (EC) No. 1441/2007). The regulations do not
65 take into consideration the risks deriving from food contamination by enteric or emerging viral
66 pathogens, although there is a growing attention and interest for this important risk, as evidenced by
67 recent NoV and HAV outbreaks in Europe (European Centre for Disease Prevention and Control
68 and the European Food Safety Authority, 2014; Müller et al., 2016). Limited data exist in the
69 literature on the prevalence of foodborne viruses in RTE vegetables. In Italy, a study on RTE at
70 retail has been reported recently, revealing a very low rate (< 0.1%) of NoV contamination (Losio
71 et al., 2015). The purpose of this study was to investigate the presence of enteric viruses (HAV,
72 HEV and NoV) in RTE vegetables available for sale in Italy.
73

74 **2. Materials and Methods**

75 *2.1 Sampling*

76 A total of 911 samples of bagged ready-to-eat vegetables belonging to different brands and
77 purchased from supermarkets in Apulia and Lombardy regions, Italy, were collected during 2014-
78 2015. They included 619 mixed salads, 53 carrot (*Daucus carota* subsp. *sativus*), 40 valerian
79 (*Valeriana officinalis*), 104 rocket (*Eruca vesicaria*), 10 spinach (*Spinacia oleracea*), 18 Iceberg
80 (*Lactuca sativa*) and 67 Romaine lettuce (*Lactuca sativa* L var. longifolia). All samples were
81 labelled as "Pre-washed and ready-to-eat". All samples were obtained in their original packaging
82 and analysed before the expiration date (up to 8 days). Samples were transported to the laboratory
83 in refrigerated boxes (< 8°C) and analysed on the day of purchase.
84

85 *2.2 Isolation of Salmonella* spp. *and Listeria monocytogenes*

86 Vegetable samples were subjected to isolation of the pathogenic foodborne microorganisms
87 *Salmonella* spp. and *Listeria monocytogenes*, using EN/ISO 6579 and EN/ISO 11290-1,
88 respectively.

89

90 *2.3 Virus concentration and nucleic acid extraction*

91 Viral RNA was extracted following the ISO/TS 15216-2:2013 method for NoV and HAV detection
92 in foodstuffs. In brief, 25g of each sample were cut into small pieces and homogenized with TGBE
93 buffer pH 9.5 (100 mM Tris–HCl, 50 mM glycine, 1% beef extract) and 10 µl of process control
94 virus material (Mengovirus). The eluate was concentrated with 5X PEG/NaCl solution (50% (w/v)
95 PEG 8000, 1.5 M NaCl) and the viral nucleic acids were extracted and purified using commercial
96 kits (NucliSENS miniMAG kit, bioMérieux) according to the manufacturer's instructions.

97

98 *2.4 Reverse transcriptase-polymerase chain reaction*

99 Reverse transcription of viral RNA was performed using High-Capacity cDNA Reverse
100 transcription Kit (Applied Biosystems, Italy), containing 10X RT Buffer II, 5 mM $MgCl_2$, 1 mM
101 dNTPs, 2.5 µM random hexamers, 20 U RNAse inhibitor and 50 U Reverse transcriptase according
102 to the manufacturer's instructions. The reaction conditions were 42°C for 30 min and 99°C for 5
103 min. The obtained cDNA was used for specific real-time PCR (RT-qPCR) for each target virus:
104 HAV, HEV, NoV GI and NoV GII.

105 The RT-qPCR reactions were performed in duplicate. All tests included negative controls for virus
106 and for an internal amplification control (IAC).

107

108 *2.5 Hepatitis A virus qPCR*

109 This assay was performed using the primers and conditions described in the ISO/TS 15216-2:2013
110 method with the inclusion of an internal amplification control (IAC). The reaction contained 1X
111 iTaq^{TM} Universal Probes Supermix (Bio-Rad), 0.5 µM primer HAV68, 0.9 µM primer HAV240;

112 0.25 µM probe HAV150 (-) (FAM labelled) (Costafreda et al., 2006), 50 nM IAC probe (VIC
113 labelled) and 300 copies of IAC (Martinez-Martinez et al., 2011). 20 µl of cDNA was added to
114 make a final reaction volume of 50 µl. The thermocycling conditions were 5 min at 95°C, followed
115 by 45 cycles of 15 s at 95°C and 1 min at 60°C and 1 min at 65°C.
116
117 *2.6 Hepatitis E virus qPCR*
118 This assay used the primers and conditions described by Jothikumar et al. (2006) with the inclusion
119 of an internal amplification control (IAC). The reaction contained 1X iTaq^{TM} Universal Probes
120 Supermix (Bio-Rad), 0.25 µM for each primer (JVHEVF and JVHEVR), 0.1 µM probe JVHEV-P
121 (labelled with FAM), 50 nM IAC probe (labelled with VIC) and 300 copies of IAC (Martinez-
122 Martinez et al., 2011). 20 µl of cDNA was added to make a final reaction volume of 50 µl. The
123 thermocycling conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at
124 60°C.
125
126 *2.7 Norovirus GI and GII qPCR*
127 This assay was performed using the primers and conditions described in the ISO/TS 15216-2:2013
128 method with the inclusion of an internal amplification control (IAC). The reaction was carried out
129 using 1X iTaq^{TM} Universal Probes Supermix (Bio-Rad), 250nM probe (labelled with FAM), 500nM
130 forward primer and 900nM reverse primer, 50nM IAC probe (labelled with VIC) and 300 copies of
131 IAC (Martinez-Martinez et al., 2011).
132 Primers targeted the ORF2 region; for NoV GI: forward primer QNIF4, reverse primer NV1LCR
133 and probe NV1LCpr were employed; for NoV GII, forward primer QNIF2d, reverse primer
134 COG2R and probe QNIFS were used (da Silva et al., 2007; Kageyama et al., 2003; Loisy et al.,
135 2005; Svraka et al., 2007).
136 20 µl of cDNA was added to make a final reaction volume of 50 µl. The thermocycling conditions
137 were 5 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C and 1 min at 65°C.

138

139 *2.8 Extraction efficiency*

140 For each sample, two aliquots of 5µl nucleic acid (NA) extract were added to adjacent wells of a
141 96-well optical reaction plate and made up to 25µl with Mengovirus-specific TaqMan reaction mix.
142 A dilution series prepared from the Mengovirus process control material was also tested. The
143 percentage extraction efficiency for each sample was determined by comparing the Ct values for the
144 sample NA extract with those for the Mengovirus dilution series. Any sample with an extraction
145 efficiency of <1% was subjected to retesting, in a first instance by re-extracting the viral RNA from
146 stored homogenate, then by testing the stored RTE salads. Results for any sample providing three
147 extraction efficiency results of <1% were considered invalid.

148

149 **3. RESULTS**

150

151 *3.1. Isolation of Salmonella spp. and Listeria monocytogenes*

152 The pathogenic foodborne microorganisms *Salmonella* spp. and *L. monocytogenes,* were not
153 detected in any RTE salads.

154

155 *3.2 Detection of foodborne viruses by RT-qPCR*

156 The mean viral extraction efficiency of the process was > 1% for each sample.

157 The results obtained from virological analysis of 911 RTE vegetable samples are summarized in
158 Table 1.

159 HAV and HEV were detected in 2.6% of the tested samples (24/911): HAV was the most prevalent
160 pathogenic enteric virus, detected in 1.9% of samples (18/911), and followed by HEV in 0.6% of
161 samples (6/911).

162 In detail, 14 of the HAV positive samples were mixed salad samples (2.3% of mixed salad
163 samples), 1 valerian (2.5% of valerian samples) and 3 rocket samples (1.9% of rocket samples).

164 Two mixed salad samples resulted HEV positive (0.3 % of mixed salad samples), 2 rocket samples
165 (1.9%) and 2 spinach samples (20%). None of the samples analysed in this study tested positive for
166 NoV.
167

168 **4. DISCUSSION**

169 Over the years, foodborne NoV and HAV outbreaks have mainly been linked to shellfish
170 consumption in the EU although more recently other matrices such as leafy vegetables and frozen
171 red fruits (Chiapponi et al., 2014; Severi et al., 2015; Terio et al., 2015) have been associated with
172 these infections. Foodborne transmission of HEV has been increasingly reported in sporadic cases
173 and small outbreaks associated with the consumption of raw or uncooked boar or deer meat, of liver
174 and liver sausages (Colson et al., 2010; Di Bartolo et al., 2015; Guillois et al., 2015), although HEV
175 has also been detected in fresh produce (Kokkinos et al., 2012).
176 In this study, *Salmonella* spp. and *L. monocytogenes* were not detected in RTE salads. Our findings
177 are in general agreement with another investigation performed in Italy in which a low prevalence
178 (0.3-0.5%) of contamination by *Salmonella* spp. and *L. monocytogenes* was reported (Losio et al.,
179 2015).
180 On the opposite, in our study the virological investigations detected the presence of HAV and HEV
181 in 1.9% and 0.6% of the analysed samples, respectively. To our knowledge, this is the first report
182 documenting the presence of HAV and HEV in RTE salads in Italy. One limitation of this study
183 was the inability to determine the viability of the viruses detected in the samples; indeed, although
184 the laboratory-adapted HAV strains can grow on foetal rhesus monkey kidney cells (Kingsley and
185 Richards, 2001), wild-type HAV strains are unable to replicate *in vitro.* Likewise, there is no
186 specific cell culture line for laboratory cultivation of HEV and cultivation of human NoV is
187 fastidious (Ettayebi et al., 2016; Jones et al., 2015). On the other hand, the fact that RT-PCR
188 methods can detect inactivated viruses may not be a critical factor for RTE vegetables risk

189 assessment since a small number of viable virus particles are sufficient to cause illness (Fiore, 2004;
190 US Food and Drug Administration, 2012).
191 Whether the virus detected in RTE salads is viable or not, the presence of viral acid nucleic
192 revealed by the molecular assays represents a clear index of contamination and potential risk for
193 consumers considering that RTE salads do not undergo any treatment able to guarantee the virus
194 inactivation. Moreover, this contamination represents an unacceptable risk that food handlers have
195 to evaluate in the risk assessment plan (EC regulation N° 178/2002).
196 This study indicates that attention should be paid to enteric viruses and emerging viral pathogens in
197 order to guarantee safety of RTE vegetables. Indeed, screening for enteric viruses, which are not
198 correlated to the presence of *E. coli* and *Salmonella* spp. (Ethelberg et al., 2010; Fong and Lipp,
199 2005; Krog et al., 2014; Westrell et al., 2010), is crucial when assessing the health risks related with
200 RTE salads, as already pointed out by the EU Regulation 1235/2012 for frozen strawberry.
201 For this reason, with a few exceptions in HACCP plans food businesses usually establish bacteria as
202 a food safety hazard, whilst viruses are not considered as hazards. In addition, the measures taken to
203 reduce the growth of, or eliminate bacteria, do not necessarily lead to a decrease in virus
204 prevalence. On some occasions, these measures may even preserve viral particles, as is the case of
205 refrigeration (Croci et al., 2002; Fiore, 2004).
206 Most of the available literature regarding the use of sanitizers during RTE production, such as
207 chlorine, has concluded that washing with water or with disinfectant solutions reduces the natural
208 microbial populations on the surface of the product by only 2 to 3 log units (Allende et al., 2007;
209 Beuchat et al., 2004; Gómez-López et al., 2007; Gonzalez et al., 2004; Inatsu et al., 2005; Selma et
210 al., 2008; Ukuku et al., 2005) and it has been reported that current industrial sanitizing treatments
211 do not guarantee the total elimination of pathogens when present (Abadias et al., 2008; Beuchat,
212 1996; Parish et al., 2003; Pérez-Rodríguez et al., 2011; Posada-Izquierdo et al., 2014).
213 Studies on the efficacy of disinfectants for inactivating viruses show that viruses are relatively
214 resistant to chlorine decontamination (Seymour and Appleton, 2001), that viruses are generally

215 more resistant than bacteria (Allwood et al., 2004), that virus sensitivity to disinfectants may vary
216 widely between different virus species (Fraisse et al., 2011) and that sanitizing agents or
217 disinfection techniques must be evaluated, usually on a case-by-case basis (Gil et al., 2009).
218 In our study, NoV was not detected in any of the 911 RTE samples analysed. The results of our
219 investigation are comparable with data reported in a previous Italian study in which NoV was
220 detected in 1/1372 (< 0.1%) fresh leafy samples but not in 1160 RTE vegetable samples (Losio et
221 al., 2015). Although in these relatively small-scale studies the rate of NoV contamination was low,
222 the impact of NoV contamination in fresh and RTE vegetables has been clearly outlined by the
223 2013 report of the European Food Safety Authority (European Food Safety Authority & European
224 Centre for Disease Prevention and Control, 2013) that noted an increase in the number of NoV
225 outbreaks caused by consumption of vegetables in Europe in 2011. The settings that were most
226 often reported were restaurants, households, schools and kindergartens and canteen or workplace
227 catering and residential institutions, and many NoV outbreaks were traced to food that was handled
228 by one infected worker during food preparation (European Food Safety Authority & European
229 Centre for Disease Prevention and Control, 2011).
230 Viral contamination may occur in several times and points of the production chain. As viruses are
231 not able to replicate extracellulary, their presence can only be accounted for by pre-harvesting
232 contaminations of vegetables or by post-harvesting re-contaminations by food handlers (Berger et
233 al., 2010; Heaton and Jones, 2008; Koopmans et al., 2002; Wu et al., 2005). Several factors, chiefly,
234 the epidemiological patterns of viral infections (i.e. seasonal fluctuations in their prevalence,
235 incubation times, duration of virus shedding after the clinical signs) should also be considered and
236 could explain, at least in part, our findings.
237 The samples that tested positive for HAV and HEV were mixed salads, valerian, rocket and
238 spinach. Mixed salads, valerian and rocket are characterized by a production process that requires
239 strong manipulation during collection, whilst spinaches pose difficulties for washing of their leaves.

240 Water quality used for agricultural irrigation and the hands of the workers in the field affects the
241 microbiological characteristics of products and therefore may pose a risk to consumer health.
242 Appropriate implementation of food-safety management systems including Good Agricultural
243 Practices (GAP), Good Hygiene Practices (GHP) and Good Manufacturing Practices (GMP) should
244 be the primary objective of operators producing RTE salads. Compliance with hygiene
245 requirements, in particular hand hygiene, is an absolute necessity for food handlers at all stages of
246 the vegetable production and supply chain to reduce the risks of foodborne virus contamination.
247 It is possible that the results of our and of other studies were biased by the sampling procedure. The
248 EU regulations do not provide indications for the virological risks and the sampling plan adopted in
249 our study was based on similar literature (Losio et al., 2015; Serracca et al., 2012). Indeed, the need
250 for adequate sampling plans for virological investigations in food matrices has arisen in recent years
251 along with the understanding of the risks posed by viruses in food products (Perez-Rodriguez et al.,
252 2014). Evaluation of the microbiological contamination status can be useful to define risk-based
253 planning for official controls, while implementation of the management practices and knowledge of
254 the prevalence of foodborne viruses in RTE vegetables represent a contribution for assessing
255 consumer exposure.
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521

1 **Table 1**

2 *Summary of virological results (no. of positive samples) obtained, for Ready-to-Eat vegetables,*

3 *using the reference ISO method, in Italy.*

Type of samples		**Total no. 911**	**Positive for HAV**	**Positive for HEV**	**Positive for NoV**
Mixed salads		619	**14**	**2**	0
Carrot	*Daucus carota* subsp. *sativus*	53	0	0	0
Valerian	*Valeriana officinalis*	40	**1**	0	0
Rocket	*Eruca vesicaria*	104	**3**	**2**	0
Spinach	*Spinacia oleracea*	10	0	**2**	0
Iceberg lettuce	*Lactuca sativa*	18	0	0	0
Romaine Lettuce	*Lactuca sativa* L var. longifolia	67	0	0	0

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