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Microbial safety considerations of flooding in primary production of leafy greens: A case study

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ABSTRACT

This study evaluated the effects of a flood event, floodplain and climatic parameters on microbial contamination of leafy greens grown in the floodplains. Additionally, correlations between pathogenic bacteria and levels of indicator microorganisms have been also determined. To diagnose the microbial contamination after the flood event, sampling was carried out in weeks 1, 3, 5 and 7 after the flooding in four flooded lettuce fields. To assess the impact of flooding on the microbial contamination of leafy greens, indicator microorganisms (coliforms, *Escherichia coli* and *Enterococcus*) and pathogenic microorganisms (*Salmonella* spp., VTEC (*E. coli* O157:H7 and other verocytotoxin producing *E. coli*, O26, O103, O111, O145) and *Listeria monocytogenes*) were evaluated. Irrigation water, soil and lettuce samples showed levels of coliforms and *E. coli* higher than 5 and 3 log cfu/g or 100 mL, respectively when sampled 1 week after flooding. However, bacterial counts drastically declined three weeks after the flooding. Climatic conditions after flooding, particularly the solar radiation (6–8 MJ/m^2), affected the survival of bacteria in the field. *L. monocytogenes* was not detected in lettuce samples, except for 2 samples collected 3 weeks after the flooding. The presence of *Salmonella* was detected in irrigation water, soil and lettuce by multiplex PCR one week after the flooding, but only 2 samples of soil and 1 sample of water were confirmed by colony isolation. Verotoxigenic *E. coli* was detected in soil and lettuce samples by multiplex PCR. Therefore, the implication of flood water as the source of VTEC contamination of soil and lettuce was not clear. *E. coli* counts in irrigation water were positively correlated with those in lettuce. A significant correlation ($P < 0.005$) was found between the presence of pathogens and *E. coli* counts, highlighting a higher probability of detection of pathogens when high levels of *E. coli* are found. The results obtained in the present study confirm previous knowledge which defined flooding as a main risk factor for the microbial contamination of leafy greens.

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1. Introduction

Climate change affects the frequency, intensity and duration of extreme water-related weather events such as excessive rainfall and flooding (Pachauri, 2012; Semenza & Menne, 2009). Flooding accounts for about 40% of all natural disasters that occurred worldwide 20 years ago (French & Roy, 1989). Recently, Cann, Thomas, Salmon, Wyn-Jones, and Kay (2013) have reported that out of all outbreaks associated with extreme water-related weather events, heavy rainfall and flooding were by far the most common climate change effects. The increasing intensity of heavy rainfall is projected to make extreme river floods even more frequent in some areas, especially in central, northern and north-eastern Europe (IPCC, 2012).

Consequences of climate change have been identified as having potential for increasing bacterial contamination of food and water (Tirado, Clarke, Jaykus, McQuatters-Gollop, & Frank, 2010). A study conducted during flooding in the US in 2001 identified an increased incidence of gastrointestinal illness due to flooding (Salvato, Nemerow, & Agardy, 2003). Flooding may have multiple food safety consequences, particularly if the agricultural land is adjacent to livestock farms and industrial and residential areas (Miraglia et al., 2009). These events may lead to the contamination of soil, irrigation water and produce with pathogens from the contaminated floodplain sources (Confalonieri et al., 2007). Flooding may have an impact on the persistence and patterns of occurrence of bacteria and it affects the ecology of microbes (Tirado et al., 2010). In a study carried out by Orozco et al. (2008), the presence of *Escherichia coli* and *Salmonella Newport* was demonstrated in tomato samples during and after a flooding event. Consequently, fresh produce grown in contaminated land after flooding has been recognized as a potential vehicle for transmission of pathogenic microorganisms (CAC, 2003; EFSA, 2013). The consequence of outbreaks associated with fresh produce result in considerable economic losses to farmers, distributors and the food industry (Golberg, Kroupitski, Belausov, Pinto, & Sela, 2011). Recently, a guide has been established to assess growers on the intervention strategies to mitigate these risks (CDC, 2011). This guide specified that after a flood event, health authorities should follow risk assessment measures to determine the safe use of previously flooded outdoor areas. In general, product contamination is reduced with longer intervals between flooding and the harvest of the plant (FDA, 2011).

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Using experimental data, several studies have attempted to develop recommended intervals between field contamination and harvest but vary significantly in their designation of a safe time period (Doyle & Erickson, 2008). Most of the information related to potential sources of preharvest contamination has been acquired from experimental studies in the laboratory or field trials in which they have demonstrated, after artificial inoculation, the persistence of foodborne pathogens for different periods of time (Tomás-Callejas et al., 2011). However, to evaluate the microbial contamination risk after flooding is a challenge because of difficulties in developing an adequate experimental design. This is mostly because of the sporadic nature of these events, which make it difficult to repeat the sampling in a specific setting. Thus, attempting to establish a safe interval between the flood and the harvest to avoid microbial risks is challenging. The objective of the study was to evaluate the effects of a flood event, floodplain and climatic parameters on microbial contamination of leafy greens grown in the flood plains. The relationships between indicator and pathogenic microorganisms were also established.

2. Materials and methods

2.1. Sampling area

At the end of September 2012, an extreme event of heavy rainfall occurred in the south-east of Spain, which caused flooding in most of the adjacent lands and growing fields. This area is characterized by a dry climate with an average rainfall of 40.7 mm in September during the last 12 years. However, in this water-related event, the rainfall was 84 mm in 24 h. This region has been subjected to several flooding events (Table 1).

2.2. Climatic parameters

Climatic data were obtained from the nearby Barranda climatic station (38° 35′ 6″ N, −1° 40′47,994″ W) and Purias climatic station (37° 33′ 53,95″ N, −1° 41′ 49,2″ W) located within 10 km of the fields, using the local climatological database (SIAM, 2014). Relevant climate parameters, such as temperature, rainfall and solar radiation were collected daily during the sampling period.

2.3. Sampling plan

Four growing fields of iceberg lettuce (*Lactuca sativa* L.) affected by flooding were selected and sampled for this study (Supplementary information): *Field 1* (38° 35′ 6″ N, −1° 40′ 47,994″ W), located in Lorca (Murcia, Spain) with a surface area of 3.5 ha without a watercourse in the surroundings; *Field 2* (37° 33′ 53,95″ N, −1° 41′ 49,2″ W), located in Lorca (Murcia, Spain) with a surface area of 6.2 ha and a watercourse at 250 m; *Field 3* (38° 2′ 38,62″ N, 1° 58′ 35,52″ W), located in Caravaca (Murcia, Spain), with a surface area of 7.6 ha and a watercourse at 316 m; and *Field 4* (38° 2′ 31,45″ N, 1° 58′ 34,52″ W), located in Caravaca (Murcia, Spain) with a surface area of 4.8 ha and a watercourse at 210 m. Sampling was carried out approximately in weeks 1, 3, 5 and 7 after the flooding. Soil in this growing area was sand-clay organically manured.

Table 1
Flooding events recorded in the south-east area of Spain in the last 4 years. Data represent the monthly average rainfall (mm) between 2009 and 2012 and the rainfall (mm) per month and per 24 h of specific years.

Dates	Monthly average rainfall (2009–2012)	Rainfall/month (specific year)	Rainfall/24 h (specific year)
March	26.4 ± 31.3	107.4 (2009)	67.2 (2009)
May	37.0 ± 33.6	115.6 (2009)	48.7 (2009)
August	14.5 ± 28.1	92.2 (2010)	59.7 (2010)
September	40.7 ± 41.8	137.5 (2009)	96.8 (2009)
		88.5 (2012)	84.0 (2012)

Commercial harvest of the lettuce was carried out 10 weeks after the flooding. At each sampling time, 9 samples of soil and 9 whole head iceberg lettuces were randomly collected from different locations in the field following a zig-zag pattern which started from one of the sides of the field. Soil samples were taken at the surface (0–5 cm depth) within a 20 cm diameter by using a spade. Nine samples of irrigation water were taken from the irrigation systems at each sampling time, except for Field 1, where because of the severity of the flood event, the systems were not available and samples were taken from the water reservoir. Samples of lettuce in Field 1 were also not available because of the severity of the flood event. All samples were stored and transported in the dark at 4 °C to the lab (max. 40 km) for further handling (cutting/pooling). The sampling methodology used in this study followed the protocol previously described by Holvoet, Sampers, Seynnaeve, and Uyttendaele, (2014). Summarizing, 9 soil samples (100 g each) and 9 lettuce samples from the edible leaves were randomly pooled by 3 in the lab. Microbial analyses were conducted within 2–14 h.

2.4. Microbial analysis

2.4.1. Indicator microorganisms

Soil and lettuce samples of 25 g each were homogenized in a 1:10 dilution of sterile 0.1% buffered peptone water (BPW; AES Chemunex, BioMérieux SA, France). Water samples (2 L each) were collected into sterile bottles according to ISO 19458 (ISO, 2006). Serial dilutions of samples were performed and plated on the appropriate culture media.

Coliforms, *E. coli* and *Enterococcus* were enumerated in 100 mL water samples using cellulose nitrate membrane filters (0.45 μM diameter, Microsart®, Sartorius, Madrid, Spain), while coliforms and *E. coli*, were quantified in soil and lettuce samples. Chromocult Agar (AES Chemunex), a selective chromogenic medium, was used for the enumeration of *E. coli* and total coliforms after incubation for 24 h at 37 °C. Enumeration of coliforms and *E. coli* in water samples were performed according to ISO 9308-1 (ISO, 2000a) with the exception that the Tergitol 7 medium was replaced by Chromocult Agar. *Enterococcus* was enumerated according to ISO 7899-2 (ISO, 2000b). Briefly, filters were incubated on Slanetz and Bartley medium (Oxoid, Hampshire, UK) for 44 h at 37 °C. Then, filters were transferred to bile-aesculine-azide agar (Sigma Chemical, St. Louis, MO) for 2 h at 44 °C.

2.4.2. Pathogenic microorganisms

Presence or absence of *Salmonella* spp., VTEC (*E. coli* O157:H7 and other verocytotoxin producing *E. coli*, O26, O103, O111, O145) and *Listeria monocytogenes*, were determined in all the samples as previously described (Desroche, Chablain, Guzzo, & Arbault, 2009; Holvoet et al., 2014). Samples of 25 g of soil and lettuce were homogenized for 1 min in 225 ml of BPW (AES Chemunex) and incubated for 18 ± 2 h at 37 °C for enrichment. In this case, the 9 samples of each type of sample (soil and lettuce) were further pooled in one sample. Water samples (1 L each) were filtered and the filters were incubated in 100 ml BPW at 37 °C for 18–20 h for enrichment. Then, 50 μL of all enriched samples were used to extract and purify the bacterial DNA using a commercial extraction kit (Extraction Pack Food for *Salmonella*, STEC, EHEC, O157:H7 and *Listeria* detection, Pall®, France). Part of the enriched samples was also kept at −80 °C for further analysis. Once the DNA was extracted, samples were analyzed using the validated method of GeneDisc® Rapid Microbiology System (GeneSystems, France). Commercially available GeneDisc® plates were used for the screening in parallel of specific gene sequences of human pathogenic verotoxin producing *E. coli* virulence factors (*stx1*, *stx2*, *eae*), *E. coli* O157:H7 ($rfbE_{O157}$ and $fliC_{H7}$) and *Salmonella* spp. specific genes (*iroB*), while also including inhibition control and negative control (Beutin, Jahn, & Fach, 2009). In the case of a positive PCR signal for pathogen presence by the GeneDisc® multiplex PCR, isolation and confirmation of colonies was attempted. Before isolation, 1 mL of frozen (30% glycerol) enriched samples was subjected to second non-selective enrichment in 10 mL of BPW (AES Chemunex) at 37 °C for

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