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Assessment of microbial risk factors and impact of meteorological conditions during production of baby spinach in the Southeast of Spain

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ABSTRACT

There is a timely need to evaluate the effect agricultural factors and meteorological conditions on fresh produce contamination. This study evaluated those risk factors and described, for the first time, the distribution of indicator microorganisms (*Escherichia coli*, *Enterococcus*, coliforms, and *Enterobacteriaceae*) and the prevalence of foodborne pathogens (Enterohaemorrhagic *E. coli*, *Listeria monocytogenes* and *Salmonella* spp.) in baby spinach grown in the Southeast of Spain. A longitudinal study was conducted on three farms (2011–2013). Results obtained for *E. coli* highlighted soil and irrigation water as important factors affecting the microbial safety of baby spinach. Significant differences in the proportion of *E. coli* positive samples were found between treated (46.1%) and untreated (100%) irrigation water. However, the microbial quality of irrigation water didn't affect *E. coli* prevalence in produce. All *E. coli* positive spinach samples were detected at the highest observed temperature range suggesting that ambient temperature affects the probability and extent of spinach contamination. *Salmonella* spp. was detected by RT-PCR in manure, soil, irrigation water and baby spinach but only two of them (manure and irrigation water) were confirmed by isolation in culture media. *Salmonella* RT-PCR positive samples showed higher levels of *E. coli* than *Salmonella* negative samples. This preliminary finding supports recent identification of *E. coli* as a suitable parameter for the hygiene criterion at the primary production of leafy greens.

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1. Introduction

Contamination of leafy greens with foodborne pathogens may occur at any step in the farm to consumer chain (growing, harvest, processing, wholesale storage, transportation, retailing and handling at home) from environmental, animal or human sources (FAO/WHO, 2008; FDA, 2009; EFSA, 2013). Recent publications highlighted several pre-harvest sources as the most probable origins of potential contamination including: contaminated water, soil amendments and fecal contamination from wildlife (Pachepsky et al., 2011; Doyle and Erickson, 2012; Ceuppens et al., 2014; Holvoet et al., 2014). Little data on the microbial quality and safety of baby leaves during pre-harvest is available generating the need for more studies on specific agricultural practices and microbiological risks.

The most common etiologic agents associated with produce outbreaks are *Escherichia coli* O157:H7 and *Salmonella* (Mootian et al., 2009). Recently, European Food Safety Authority (EFSA) highlighted *Salmonella* spp. and leafy greens eaten raw as salads as one of the five top raking food/pathogen combinations most often linked to human cases originating from Food of Non-Animal Origin (FoNAO) in the EU (EFSA, 2013). These microorganisms can persist in the environment for long periods of time, and they may spread to and contaminate distant locations. Recent studies have shown that bacterial survival in the field is significantly influenced by environmental and weather conditions. In the USA, precipitation has been identified as a predictor of spinach contamination with generic *E. coli*, indicating that the probability of contamination increases with an increase in the amount of rain over the past month (Park et al., 2014). Seasonal differences in the microbial

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concentrations on fresh produce have been also reported showing higher counts of indicators in the fall (September, October and November) compared to spring and winter (Ailes et al., 2008). In Belgium, Holvoet et al. (2014) found a direct correlation between indicator bacteria and pathogens in irrigation water with temperature and precipitation. These findings highlight the utility of weather databases to obtain hourly and daily weather information to predict contamination and demonstrate that environment and weather factors should be considered together to develop Good Agricultural Practices (GAPs) guidelines and measures to reduce produce contamination (Park et al., 2014). There is a clear increase in cases of salmonellosis when ambient temperatures increase (D'Souza et al., 2004; Kovats et al., 2005; Fleury et al., 2006; Semenza et al., 2012). For many years, there is great interest in determining the most common causes of the increase in produce-associated outbreaks in the summertime (FDA, 2001). However, the mechanisms underlying the observed seasonality in foodborne disease are not fully understood, but are likely to involve a complex interplay of multiple factors (Liu et al., 2013). *Salmonella* spp. is susceptible to climatic variables as it is vulnerable to sunlight and drying out, but their survival can be promoted at higher temperatures (McMichael et al., 2006). In fact, van Pelt et al. (2004) reported that above a 6 °C threshold, the risk of *Salmonella* infection increased in several European countries.

Due to the prohibitive cost of pathogen detection, many researchers use microbial indicators to characterize microbial contamination in the environment of field cultivation and fresh produce (Park et al., 2013). Although generic *E. coli* can form stable populations in temperate soil and water environments, its presence is indicative of conditions favorable for survival and persistence of pathogenic *E. coli* and *Salmonella* spp. (Park et al., 2013). Its presence on produce indicates fecal contamination and thus possible presence of pathogens carried in the intestinal tract of animals (Adams and Moss, 2000). This microorganism has been recognized as a good indicator for the presence of fecal contamination and a good index indicator for the presence of *Salmonella enterica* serovar Typhimurium (Natvig et al., 2002), *Salmonella* spp. (Park et al., 2013) and *E. coli* O157:H7 (Ogden et al., 2001).

To effectively reduce the prevalence of foodborne pathogens in baby leaves at the pre-harvest level, both the contamination routes and meteorological factors affecting pathogens' survivability should be considered (Park et al., 2014). The aim of the present study was to describe, for the first time, the distribution of indicator microorganisms (generic *E. coli*, total coliforms, *Enterobacteriaceae* and *Enterococcus*) and the prevalence of foodborne pathogens (*Listeria monocytogenes*, Enterohaemorrhagic *E. coli* and *Salmonella* spp.) with respect to the potential risk factors in the production of baby spinach grown in the Southeast of Spain. The Southeast area of Spain is considered the garden of Europe, as it is a leading European horticultural area. The impact of weather factors on the contamination of baby spinach with generic *E. coli* and foodborne pathogens at the pre-harvest level was also evaluated. Moreover, the relationship between the distribution of indicator microorganisms and the presence of foodborne pathogens in a sample was established.

2. Materials and methods

2.1. Production farms

Three of the biggest Spanish leafy green growers agreed to participate in this study. All farms were located in the southeast of Spain between Murcia and Almeria. The specific location was kept confidential to protect the identity of the farmers. The dimension of the farms ranged between 2 and 4 ha. Irrigation water on these farms was from ponds; overhead sprinkler irrigation was used for irrigation. Irrigation was usually carried out every day in the morning.

2.2. Sampling plan

The study took place between November 2011 and April 2013. A systematic longitudinal sampling plan was developed to identify potential risk factors for microbial contamination in the production of baby spinach. The sampling sites were selected based on the literature review of potential risk factors that contribute to microbiological contamination, particularly in leafy greens (Pachepsky et al., 2011; Olaimat and Holley, 2012; Park et al., 2012). For each selected farmer, the sampling plan included sample collection during 3 production cycles distributed throughout a growing season, which excludes summer (from May to August) because there is no summer production in this area. The duration of the production cycle, considered from the day of seeding until the day of harvest, varied depending on the part of the season and it was on average 8 and 5 weeks in winter and spring, respectively. During each complete production cycle, 4 visits were carried out and 4 codes were used for sampling-time identification: T1 = at planting, T2 = 2 weeks before harvest, T3 = one week before harvest and T4 = at harvest. The sampling was carried out over the three individual production cycles during the growing season for the three farms with a total of 540 samples collected during the study: 27 samples of manure, 27 samples of seeds, 120 samples of soil, 150 samples of water, 108 samples of baby spinach, 81 samples of surfaces and 27 samples of worker' hands.

2.3. Sampling methodology

The protocol previously described by Holvoet et al. (2014) was followed. For solid samples (soil, seeds, manure and baby spinach), 9 samples of approximately 100 g each were randomly collected. In the case of soil and fresh produce, samples were taken from different locations in the field following a zig-zag pattern started from a randomly selected side of the field. Soil samples were taken at the surface (0–5 cm depth) within a 20 cm diameter around each sampled plant using a spade previously disinfected with 70% ethyl alcohol. Manure and seed samples were taken at random sites at the company storehouse. Once in the laboratory, the solid samples (100 g each) were randomly pooled into 3 samples (25 g each). In the case of water, samples were collected from ponds and at the irrigation head (outlet of the irrigation system to the produce). Four liter samples were collected into sterile bottles according to ISO 19459:2006 (ISO, 2006). For sampling of surfaces (conveyor belt, blade and boxes) and worker's hands, sterile swabs were used for swabbing of 50 cm^2 of surface area and both hands, respectively. The swab was immersed in 5 mL buffered peptone water and transported to the lab. Microbial analyses were conducted within 2–14 h from the time of sample collection.

2.4. Microbial analysis

2.4.1. Indicator microorganisms

Counts of indicator microorganisms were monitored as previously described (Holvoet et al., 2014). *E. coli*, total coliforms and *Enterococcus* were enumerated in 100 mL water samples using cellulose nitrate membrane filters (0.45 μM diameter, Microsart®, Sartorius, Spain). *Enterobacteriaceae* were only determined in surface and worker's hands samples. Chromocult Agar (AES Chemunex, France, Europe) was used for the enumeration of *E. coli* and total coliforms after incubation for 24 h at 37 °C in solid, water and surface samples. *Enterococcus* were incubated on Slanetz and

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