International Journal of Food Microbiology 171 (2014) 21–31

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

# Relationships among hygiene indicators and enteric pathogens in irrigation water, soil and lettuce and the impact of climatic conditions on contamination in the lettuce primary production

Kevin Holvoet [a,b], Imca Sampers [b,*], Marleen Seynnaeve [c], Mieke Uyttendaele [a]

[a] Laboratory of Food Microbiology and Food Preservation, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000 B-Ghent, Belgium
[b] Laboratory of Food Microbiology and -Biotechnology, Department of Industrial Biological Sciences, Faculty of Bioscience Engineering, Ghent University, Campus Kortrijk, Graaf Karel de Goedelaan 5, B-8500 Kortrijk, Belgium
[c] INAGRO, Provincial Research and Advisory Center for Agriculture and Horticulture, Ieperseweg 87, B-8800 Rumbeke-Beitem, Belgium

ARTICLE INFO

*Article history:*
Received 27 June 2013
Received in revised form 30 September 2013
Accepted 8 November 2013
Available online 18 November 2013

*Keywords:*
Lettuce
Indicator bacteria
Climate parameters
Pathogens
Seasonality
Primary production

ABSTRACT

Eight Belgian lettuce farms located in the West Flanders were sampled to establish the relationships between levels of indicator bacteria, detection of enteric zoonotic pathogens and the temperature and precipitation during primary production. Pathogenic bacteria (PCR EHEC positives, *Salmonella* spp. or *Campylobacter* spp.) and indicator bacteria (total psychrotrophic aerobic plate count (TPAC), total coliforms, *Escherichia coli*, enterococci) were determined over a period of one and a half year from seedling leaves, peat-soil of the seedling, lettuce crops, field soil and irrigation water. Neither *Salmonella* isolates nor PCR EHEC signals were detected from lettuce although one out of 92 field soil samples contained *Salmonella* spp. and five soil samples provided PCR positives for EHEC virulence factors (*vt1* or *vt2* and *eae* gene). A low prevalence of *Campylobacter* (8/88) was noted in lettuce. It was shown that irrigation water is a major risk factor with regard to the bacterial contamination of the fresh produce as the water samples showed on a regular basis *E. coli* presence (59.2% of samples ≥ 1 CFU/100 ml) and occasionally detection of pathogens (25%, n = 30/120), in particular *Campylobacter* spp. The highest correlations between indicator bacteria, pathogens, temperature and the amount of precipitation were observed for the water samples in contrast to the soil or lettuce samples where no correlations were observed. The high correlations between *E. coli*, total coliforms and enterococci in the water implicated redundancy between analyses. Presence of elevated levels of *E. coli* increased the probability for the presence of pathogens (*Campylobacter* spp., EHEC and *Salmonella* spp.), but had a low to moderate predictive value on the actual presence of pathogens. The presence of pathogens and indicator bacteria in the water samples showed a seasonal effect as they tend to be more present during the months with higher temperature.



## 1. Introduction

Severe foodborne disease outbreaks can be caused by pathogenic microorganisms associated with fresh produce (Delaquis et al., 2007). Foods of non-animal origin were associated with 10% of the outbreaks, 26% of the cases, 35% of the hospitalizations and 46% of the deaths in reported foodborne outbreaks in EU in the period 2007–2011 (EFSA, 2013a). Several publications showed that enteric diseases linked to consumption of fresh produce have dramatically increased in the last several decades (Mukherjee et al., 2007; Sivapalasingam et al., 2004). For this reason, fresh produce has been well recognized as a potential vehicle for transmission of pathogenic microorganisms known to cause human disease. Outbreaks associated with fresh produce result in considerable economic losses to farmers, distributors and the food industry (Golberg et al., 2011).

Primary production is probably the main concern in terms of introduction of hazards as pre-harvest contamination of vegetables can occur directly or indirectly via (wild) animals, insects, water, soil, dirty equipment and human handling. The contamination of produce can occur in the field by contaminated soil (such as the use of insufficiently composted manure), by the use of contaminated water for irrigation or pesticide application or by deposition of feces by wild animals (Ingham et al., 2005; Johannessen et al., 2005). Fecal bacteria (including enteric pathogens) are in particular in wet conditions and clouded weather (limited UV irradiation) able to survive for extended periods in soils (Islam et al., 2004), manure (Nicholson et al., 2005) and water (Chalmers et al., 2000; Steele and Odumeru, 2004) and thereby provide potential inoculum for contamination of the fresh produce.

* Corresponding author at: Laboratory of Food Microbiology and -Biotechnology, Department of Industrial Biological Sciences, Faculty of Bioscience Engineering, Ghent University, Campus Kortrijk, Graaf Karel de Goedelaan 5, B-8500 Kortrijk, Belgium. Tel.: +32 56 24 12 11; fax: +32 56 24 12 24.
*E-mail address:* imca.sampers@ugent.be (I. Sampers).

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http://dx.doi.org/10.1016/j.ijfoodmicro.2013.11.009

Enteric diseases have a seasonal pattern, with the highest incidence of illness during the summer months (Amin, 2002; Barwick et al., 2000). Warmer ambient temperature may contribute to the increased incidence of enteric diseases (Fleury et al., 2006). Changes in temperature and precipitation can influence environmentally mediated pathogen transmission pathways, playing an important role in driving seasonality in these diseases (Lal et al., 2012; Liu et al., 2013).

Intensive precipitation may increase surface and subsurface runoff, which might be an intermediate contamination pathway of pathogens from manure at livestock farms and from grazing pastures (Donnison and Ross, 2009; Parker et al., 2010). Rainfall is able to release fecal coliform and a variety of pathogenic microorganisms, which may be released into the environment in large numbers (Guber et al., 2006; Parker et al., 2010). Flooding or runoff can affect the microbial contamination of leafy vegetables through the spread of fecal waste onto the growing area, or through contaminated water. Fecal contamination of agricultural soils has been shown to increase after environmental flooding (Casteel et al., 2006).

To determine the microbial quality and hygiene, in particular in the framework of verification of good agricultural practices in lettuce crop production it is common practice to monitor the presence and levels of indicator bacteria such as total coliforms, enterococci and *Escherichia coli* in irrigation water or *E. coli* in the lettuce crops from the field. These microbial parameters are often used to indicate insufficient sanitary quality or potential fecal pollution. In addition, having *E. coli* of fecal (human or animal) origin has also been established as an “index” or “marker” organism (Mossel, 1978, 1982) to provide evidence of an increased likelihood of potential contamination of food or water by ecologically closely related pathogens such as Gram negative enteric pathogens encompassing human pathogenic EHEC, *Salmonella* spp. and *Campylobacter* spp. The detection of pathogens is expensive, time consuming, and complex due to pathogen variability (Savichtcheva and Okabe, 2006). Consequently, pathogens are most of the time not directly monitored in plant production areas or well or borehole waters. However, the extent of correlation among themselves and predictive value of these hygiene indicators for pathogen's presence has not been thoroughly established or quantified.

In the present field study, pathogenic bacteria (PCR enterohaemorrhagic (EHEC) positives (or *Salmonella* spp. or *Campylobacter* spp. isolates)) and indicator bacteria (total psychrotrophic aerobic plate count (TPAC), total coliforms, *E. coli*, enterococci) were determined in samples from the lettuce crops and primary production environment. Samples were taken from seedling leaves, peat-soil of the seedling, lettuce crop, field soil and irrigation water and analyzed for this broad scope of microbiological parameters. In parallel also climatic parameters (i.e., temperature, precipitation) close to the crop production fields were collected. The objectives of the study were to (i) determine risk factors for lettuce contamination with pathogenic bacteria and (ii) to establish correlations between the type and the levels of indicator bacteria, the detection of enteric zoonotic pathogenic bacteria and the effect of temperature, precipitation and seasonality on the bacterial contamination during lettuce primary production and this in order to check the extent of the parameters in correlation to the presence of pathogens.

## 2. Materials and methods

### 2.1. Selection of lettuce production farms

Eight Belgian lettuce producing farms comprising four greenhouse farms and four open field farms, all located in West-Flanders' region, were selected and agreed upon to take part in this study. All the farms were certified for the Belgian quality assurance standards Flandria and IKKB Standard (Integral Chain of Quality Management) (IKKB, 2010). In order to tackle problems during export of vegetables, IKKB Standard was benchmarked to GLOBALGAP (GlobalGap, 2012). Six farms were using open well water for irrigation and two farms used bore hole water. All farms used overhead sprinklers for irrigation of crops. The dimensions of the farms ranged from 1 ha up to 120 ha.

### 2.2. Sampling plan

The greenhouse farms having year-round production were each sampled during three lettuce crop production cycles distributed over the whole year. Because of temperate climate, the open field farms only had lettuce production going on from May to the end of September. In that restricted time period for each of the open field farms also three lettuce crop production cycles were monitored. This resulted in an accumulation of obtained results in the summer period. The complete study took place in two phases, the start-up with one crop cycle at open field farm in April–May 2011 whereas the remainder of the open field and greenhouse farms was taken up from September 2011 with the last sampling occurring (at a greenhouse farm) in December 2012. A production cycle is the time required to follow a lettuce crop from seedling at the start until its harvest and takes approximately 5–14 weeks depending upon the season. During a production cycle, 4 visits (and thus sampling times) were included: at the start during the planting of the lettuce seedling, next two weeks before harvest, one week before harvest and at harvest of the lettuce crop. Samples were collected from seedling or lettuce crop, soil and irrigation water and if applicable (at harvest) from food handler's hands or crates. All the samples were stored and transported in the dark at <4 °C to the lab for further handling (cutting/pooling) and subsequent microbial analysis. Samples were analyzed within 4 to 24 h.

### 2.3. Sampling of seedlings, soil, lettuce crop and irrigation water

The first sampling time for a lettuce crop production cycle took place at the start during the planting of the lettuce seedling. Before planting, nine samples were taken from the potting peat-soil of the seedling ($9 \times \pm$ 300 g), the seedlings ($9 \times$ one seedling) itself and soil of the planting field ($9 \times \pm$ 300 g). During the next three sampling visits in the lettuce crop production cycle (two and one week before harvest and at harvest) sampling of lettuce crops ($9 \times 1$ crop), planting field soil and irrigation water (5 l) occurred. Upon each visit, nine crops of lettuce were cut by a sterile knife and put directly into a sterile bag using disinfected gloves. At harvest in addition, nine samples were also taken from the ready to market rinsed lettuce crops.

For enumeration purposes, the nine peat-soil samples, planting field soil samples and the nine lettuce crops s were randomly pooled by three in the lab. The three crops were pooled as follows: each crop was cut in two, three halves were discarded, the remaining three halves were cut in pieces of 3 cm and the pieces were mixed thoroughly. The seedling samples were all nine pooled together due to the low mass of the seedlings. Next 10 g of each pooled sample was weighed in a stomacher bag and homogenized (for the lettuce and seedling by using a stomacher) for 1 min in 90 ml peptone physiological salt water (PPS) as a starting point for serial tenfold dilution in PPS and plating for enumeration of indicator organisms. This resulted in three enumerations of indicator organisms for the peat-soil, planting field soil and lettuce per sampling visit and one result for the seedlings. For detection of pathogens in the peat-soil of the seedlings, in the field soil and for the lettuce crops, all nine samples were joined to one sample resulting in a single detection result per visit per sample type. 25 g was taken from the pooled samples and put in a stomacher bag with the respective enrichment media.

Water samples were collected during the lettuce crop production cycle. Samples were taken from the water source and if possible at the water tap (outlet of the irrigation sprinkling system to the crops). Rinsing water was collected as well when sampling during harvest. Five liter samples were collected into sterile bottles according to ISO 19458:2006 (ISO, 2006b). The pH and temperature of the water were measured directly after sampling at the farm.

### 2.4. Climatic parameters

The climate parameters, accumulative precipitation and mean outside temperature of the week before sampling were collected from the Belgian Royal Meteorological Institute (RMI) automatic weather stations located closest to the lettuce production farm. The observations from the weather stations of Beitem (8.1 km from open field farm 7, 1.1 km for greenhouse 6), Gits (5.5 km from greenhouse 4, 2.7 km from greenhouse 1, 10 km from greenhouse 2), Izegem (7.7 km from farm 5), Poperinge (5.7 km from open field farm 8) and Wingene (3.2 km from greenhouse 3) were used.

### 2.5. Selection and methods of analysis for microbial parameters

Depending on its relevance and its common use in the assessment of the microbial quality of lettuce, soil or water, various microbial parameters were selected (Table 1). Coliforms, *E. coli* and enterococci were taken up as hygiene indicator organisms, coliforms and enterococci were only used for water analysis. The psychrotrophic (22 °C) aerobic total plate count (TPAC) was determined to assess its functionality as an overall utility indicator and correlation to other indicator organisms. For lettuce crops (and seedlings) as well as for the irrigation water samples, the pathogens *Salmonella* spp., EHEC *E. coli*, (i.e., *E. coli* strains possessing the vtx-coding genes *vt1* or *vt2* and the intimin-coding gene *eae*) and *Campylobacter* spp. were analyzed. For the soil, only *Salmonella* spp. and EHEC were included as pathogens in the analysis (Table 1).

#### 2.5.1. Solid samples: lettuce, soil and seedlings (leaves and peat-soil)

For the enumeration of TPAC, the reference method ISO 4833:2003 was applied (ISO, 2003), with the exception that the plates were incubated at 22 °C for five days instead of 30 °C for three days. RAPID'*E. coli* 2/Agar (BioRad, France), a selective chromogenic medium incubated for 24 h at 44 °C was used for the enumeration of *E. coli* (AFNOR, 2004). All indicator samples were plated in duplicate.

*Campylobacter* spp. detection was based upon ISO 10272-1:2006 (ISO, 2006a). 25 g of lettuce was homogenized for 1 min in 225 ml of Bolton broth (Oxoid, UK) and incubated under microaerophilic conditions for 48 h at 41.5 °C. The mCCDA plates (Oxoid, UK) incubated for 24–48 h at 41.5 °C were used for the isolation of *Campylobacter*. Presumptive colonies were confirmed as *Campylobacter* spp. by catalase, oxidase test and microscopy.

For the detection of *Salmonella* spp. and EHEC, 25 g of lettuce or soil was homogenized for 1 min in 225 ml of buffered peptone water (BioRad, France) and incubated for 18 ± 2 h at 37 °C.

During the start-up (one crop cycle at open field farm in the period April–May 2011), the Vidas Easy SLM Assay (BioMérieux, France) was used for the detection of *Salmonella* spp. (AFNOR, 2005). In case of a positive sample, ISO 6579:2002 (ISO, 2002) was used for further isolation of presumptive *Salmonella* colonies and confirmation. VIDAS UP Assay (BioMérieux, France) was used for the detection of *E. coli O157* (AFNOR, 2009). The enrichment broth of the positive samples was transferred to the Belgian national expert lab at the Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University (Merelbeke, Belgium) for isolation of presumptive EHEC colonies, confirmation of *vt1* or *vt2* and *eae* genes and if possible determining the serogroup of the EHEC colonies (O26, O103, O111, O145, O157) using the alternative culture method of Posse et al. (2008).

From September 2011, after the non-selective pre-enrichment the validated method of GeneDisc® Rapid Microbiology System (Pall Cooperation, USA) was used to screen for positive samples of *Salmonella* spp. and EHEC. In agreement with the Pall company (Washington, USA), dedicated GeneDisc® plates were made for the purpose of this study to screen in parallel for specific gene sequences of human pathogenic verotoxin producing *E. coli* virulence factors (*vt1*, *vt2*, *eae*, *aggR*) and *Salmonella* specific gene *iroB* while also including inhibition control and negative control. Except for the *aggR* gene the EHEC and *Salmonella* spp. target genes used were similar as those included in the commercially available GeneDisc® plates (Beutin et al., 2009). If the GeneDisc® mulitiplex PCR provided a positive PCR signal for *Salmonella* spp. ISO 6579:2002 (ISO, 2002) was used for further isolation of presumptive *Salmonella* spp. colonies and confirmation. Only culture confirmed samples were recorded and taken up as *Salmonella* spp. positive samples for further statistical analysis in this study.

In case of a positive PCR EHEC signal (defined as *E. coli* strains possessing the vtx-coding genes *vt1* or *vt2* and the intimin-coding gene *eae*) a protocol involving CHROMagar™ (CHROMagar™, USA) (Hirvonen et al., 2012; Tzschoppe et al., 2012) was taken up in the attempt to isolate the EHEC presumptive colonies, confirm and allocate serotype to the EHEC isolates as recommended by ISO TS 13136 (ISO, 2012). For the isolation protocol the 18 h incubated buffered peptone water enrichment broth was plated by means of a 4 × 4 streaking on CHROMagar™ and five presumptive EHEC isolates (mauve colonies) for each enrichment broth were picked, purified on Tryptic Soy Agar

**Table 1**
Description of the sampling plan (the sample types, sampling time and microbiological parameters) for each lettuce crop production unit under investigation (whether at open field farm or greenhouse).

| Description | Samples | Time | Microbiological parameters |
|---|---|---|---|
| Seedling peat-soil | 9 samples ➔ 3 × 3 pooled | Before planting | TPAC[a], *E. coli*<br>EHEC[b], *Salmonella* spp. |
| Seedling's leaves | 9 samples ➔ 1 × 9 pooled | Before planting | TPAC[a], *E. coli* |
| Soil | 9 samples ➔ 3 × 3 pooled | Before planting<br>Harvest − 2 weeks<br>Harvest − 1 week<br>Harvest | TPAC[a], *E. coli*<br>EHEC[b], *Salmonella* spp. |
| Lettuce crop | 9 samples ➔ 3 × 3 pooled | Harvest − 2 weeks<br>Harvest − 1 week<br>Harvest | TPAC[a], *E. coli*<br>EHEC[b], *Salmonella* spp., *Campylobacter* spp. |
| Market-ready lettuce | 9 samples ➔ 3 × 3 pooled | Harvest | TPAC[a], *E. coli*<br>EHEC[b], *Salmonella* spp., *Campylobacter* spp. |
| Open well water or groundwater and sprinkler water (if possible) | 5 | Harvest − 2 weeks<br>Harvest − 1 week<br>Harvest | TPAC[a], *E. coli*, coliforms, enterococci<br>EHEC[b], *Salmonella* spp., *Campylobacter* spp. |
| Rinsing water (at harvest wash) | 5 | At harvest | TPAC[a], *E. coli*, coliforms, enterococci<br>EHEC[b], *Salmonella* spp., *Campylobacter* spp. |

[a] TPAC: Total psychrotrophic aerobic plate count.
[b] EHEC: PCR EHEC positive signal: positive signals for vtx-coding genes *vt1* or *vt2* and the intimin-coding gene *eae*.

and identified by BBL™ Crystal™ Identification Systems (BD, USA) for *E. coli*. The suspected colonies were transferred to the Belgian national expert lab at the Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University (Merelbeke, Belgium) to confirm the presence of *vt1 or vt2* and *eae* genes and for further isolation and confirmation of the serogroup of the EHEC (O26, O103, O111, O145, O157) colonies using the method of (Posse et al., 2008).

Although there is no single or combination of marker(s) that fully define a pathogenic EHEC, strains positive for verocytotoxin 2 gene (*vtx*2) and *eae* (intimin production) genes are associated with higher risk of more severe illness than other virulence gene combinations (EFSA, 2013b). In the present study the option was taken to consider all positive GeneDisc® PCR EHEC samples (positive signals for the vtx-coding genes *vt1* or *vt2* and the intimin-coding gene *eae*) as pathogen positive samples for further statistical analysis (also if no EHEC colonies were isolated).

### 2.5.2. Water samples

The colony count of the cultivable microorganisms in water was performed according to ISO 6222:1999 (i.e., plating of 1 ml samples on PCA and incubation for 72 h at 22 °C) (ISO, 1999). The enumeration of *E. coli* and coliform bacteria was performed according to ISO 9308-1 (i.e., membrane filtration of 100 ml) with the exception that the tergitol 7 medium was replaced by Rapid'*E. coli* 2 chromogenic media (BioRad, France) (ISO, 2000a) to provide more convenient and reliable enumeration in particular in the highly bacterial contaminated surface waters. Enterococci were enumerated (i.e., membrane filtration of 100 ml) according to ISO 7899-2 (ISO, 2000b) using a 44 h incubation of filters at 36 °C on Slanetz and Bartley medium (Oxoid, UK), followed by the transfer of filters for another 2 h incubation at 44 °C on bile-aesculine-azide agar (Sigma, US).

For pathogen detection in the water samples, two times 1 l of irrigation water (from the well or taken at the sprinkler's tap) was filtered (i.e., membrane filtration, 0.45 μm), the filters of the first liter filtration were incubated in 100 ml buffered peptone water for 18 ± 2 h at 37 °C as a prior enrichment for GeneDisc® PCR detection of EHEC and *Salmonella* spp. Attempted culture confirmation of samples was analogous as mentioned above for the soil and lettuce samples. From the second liter filtration, the filters were incubated in 100 ml Bolton broth at 41.5 ± 1 °C for 48 ± 4 h under microaerophilic conditions as a prior enrichment step to detect *Campylobacter* based upon ISO 10272-1 (2006a) by isolation on mCCDA as mentioned above (ISO, 2005).

### 2.6. Data processing and statistical methods

Many of the *E. coli* enumerations for lettuce, soil or water were expected and were indeed shown to be negative, i.e., values below the detection limit (0.7 log CFU/g or 0 log CFU/100 ml respectively). So for some parts of the statistical analysis the *E. coli* data were transferred into classes (Table 2), while for other parts (e.g., determining the difference between the raw data of the *E. coli* and the presence of pathogens) the results with values below the detection limit were allocated at a value of 0.7 log CFU/g or 0 log/100 ml.

For the median, minimum and maximum calculations, only samples with numbers above the detection limit were included in the analysis. The outside temperature and precipitation used in statistical analysis are the mean temperature and accumulative precipitation calculated from the daily data of temperature and precipitation collected from the nearest RMI weather station during the seven days prior to and including the sampling day at the farm.

IBM SPSS statistics 20 and Microsoft Excel were used for statistical analysis. Spearman rank order correlation coefficients ($r_s$) were calculated among densities of indicator bacteria in soil and lettuce samples, weekly mean outside temperature and accumulated precipitation. For the irrigation water samples, additionally pH and temperature were measured of the irrigation water upon sampling and included to calculate the correlations. Binary logistic regression was employed to determine probability of pathogen occurrence from indicator bacteria and climatic parameters, odds ratio and Nagelkerke r square were displayed. Pathogen's occurrence included the occurrence of any pathogen including the presence of either alone or together in the sample of *Salmonella* or *Campylobacter* isolates or positive GeneDisc® PCR EHEC samples (positive signals for the vtx-coding genes *vt1* or *vt2* and the intimin-coding gene *eae*). Significant difference between *E. coli* classes and the presence or absence of pathogens were determined using Kendall's Tau-c test. The Kolmogorov–Smirnov test and Levene's test were used to assess normality and equality of variance ($P \geq 0.05$) respectively. If normality or equality of variance could not be assumed, Mann Whitney U test was used to determine the difference between the raw data of the indicators, climate parameters and the presence of pathogens ($P < 0.05$). For the comparison of *E. coli* prevalence per production system and per sample type Chi square or Fishers exact test was used in case one group that contained less than 5 samples ($P < 0.05$).

**Table 2**
*E. coli* classes defined for grouping of *E. coli* results for soil, lettuce and irrigation water.

| *E. coli* class | Soil and lettuce (log CFU/g) | Water (log CFU/100 ml) |
|---|---|---|
| Class 1 | <0.7 log (undetected) | <0 (undetected) |
| Class 2 | ≥0.7 and <2 | ≥0 and <1 |
| Class 3 | ≥2 and <3 | ≥1 and <2 |
| Class 4 | ≥3 | ≥2 |

## 3. Results

### 3.1. Prevalence of pathogens and hygiene-indicator bacteria in lettuce primary production

Pathogenic bacteria (PCR EHEC positives, *Salmonella* spp. or *Campylobacter* spp. isolates) and indicator bacteria (TPAC, total coliforms, *E. coli*, enterococci) were determined from various sampling locations in eight lettuce primary production farms in West Flanders, Belgium. From April 2011 to December 2012, 740 samples were collected encompassing 23 samples of seedlings, 57 samples of seedling's peat-soil, 264 lettuce crop samples, 276 the crop's field soil samples and 120 irrigation water samples (Table 3).

**Table 3**
Prevalence of hygiene indicators and pathogens in lettuce primary production.

| | n | | | Median | Minimum | Maximum |
|---|---|---|---|---|---|---|
| Lettuce | 264 | TPAC[a] | 100%[b] | 6.2 | 5.0 | 8.5 |
| | 264 | *E. coli* | 5%[b] | 1.0 | 0.7 | 2.0 |
| | 88 | Pathogens | 9%[c] | | | |
| Seedling | 23 | TPAC[a] | 100% [b] | 6.0 | 4.6 | 6.9 |
| | 23 | *E. coli* | 4%[b] | 0.7 | 0.7 | 0.7 |
| Soil seedling | 57 | TPAC[a] | 100%[b] | 8.0 | 6.1 | 9.3 |
| | 57 | *E. coli* | 4%[b] | 2.1 | 0.7 | 3.9 |
| | 23 | Pathogens | 0%[c] | | | |
| Soil | 276 | TPAC[a] | 100%[b] | 7.1 | 6.0 | 8.9 |
| | 276 | *E. coli* | 37%[b] | 1.2 | 0.7 | 3.2 |
| | 92 | Pathogens | 7%[c] | | | |
| Water | 120 | TPAC[a] | 100%[b] | 5.5 | 2.3 | 7.8 |
| | 120 | *E. coli* | 59%[b] | 1.5 | 0.0 | 3.6 |
| | 120 | Coliforms | 30%[b] | 1.7 | 0.0 | 4.1 |
| | 120 | Enterococcus | 37%[b] | 1.6 | 0.0 | 3.6 |
| | 120 | Pathogens | 28%[c] | | | |

Indicators, in log CFU/g or log CFU/100 ml, pathogens, presence per 25 g or per one liter. For the median, minimum and maximum calculations, only samples with numbers higher than the detection limit were included in the analysis.

[a] TPAC: Total psychrotrophic aerobic plate count.

[b] Proportion of samples with *E. coli* or TPAC > detection limit (LOD), LOD for *E. coli* ≥ 0.7 log CFU/g or ≥0 log CFU/100 ml, coliforms ≥ 0 log CFU/100 ml, enterococci ≥ 0 log CFU/100 ml).

[c] Proportion of pathogens' positive samples (*Salmonella*, PCR EHEC positives or *Campylobacter*).

Neither *Salmonella* isolates nor PCR EHEC signals (positive signals for the vtx-coding genes *vt1* or *vt2* and the intimin-coding gene *eae*) were detected from lettuce. One soil sample (1/92) contained *Salmonella* and five soil samples provided PCR positives for EHEC virulence factors (Table 4). However, in only three of these five soil samples isolation of EHEC cultures succeeded. In the three samples, four isolates were confirmed as respectively *E. coli* O157 (*vt2*, *eae* positive), *E. coli* O103 (*vt1*, *eae*) and one sample which contained two serotypes, namely *E. coli* O157 (*vt1*, *vt2*, *eae*) and *E. coli* O26 (*vt1*, *eae*) (Table 4). A low prevalence of *Campylobacter* (9%, n = 8/88) was noted in lettuce.

Pathogens were occasionally detected not only in water, in particular *Campylobacter* spp. (22.5%, n = 23/120) but also in some occasions PCR EHEC signals (5.0%, n = 6/120) and in another water sample *Salmonella* spp. was isolated. Of the six PCR EHEC signals in water, only in two water samples EHEC could be isolated, *E. coli* O26 (*vt1*, *eae*) and *E. coli* O111 (*vt1*, *eae*) (Table 4).

Samples containing numerable *E. coli* (≥5 CFU/g or ≥1 CFU/100 ml) were significantly more present in the environmental samples (soil and water) compared to the product samples (lettuce crops and seedlings) ($P < 0.05$). Of the environmental samples, the peat-soil of the seedling had the highest prevalence of numerable *E. coli* (96.5%) (≥5 CFU/g or ≥1 CFU/100 ml) while *E. coli* was numerable in only 37% of the field soil samples and 57.8% of the irrigation water samples. The lowest prevalence of *E. coli* number exceeding the detection level of 5 CFU/g was found for the product samples (5% in lettuce crops and 4% in the seedlings) (Table 3).

### 3.2. Correlation between the various hygiene-indicator bacteria and enteric bacterial pathogens

Spearman rank correlation was conducted using all non-categorical microbial data gathered during the survey.

In the case all sample types (soil, lettuce and water) were grouped together, a significant ($P < 0.05$) but moderate to low correlation between *E. coli* and TPAC (0.355) was found. Stronger correlations were observed for the subset of the irrigation water samples. In that case, all four indicator organisms (*E. coli*, coliforms, enterococci and TPAC) were significantly correlated with each other ($P < 0.05$ for all) with the strongest correlations observed between *E. coli* and coliforms (0.918), between *E. coli* and enterococci (0.846) and between coliforms and enterococci (0.748), followed by moderate relationships between TPAC and *E. coli* (0.437), coliforms (0.447) and enterococci (0.470) (Table 5).

For the irrigation water samples alone, the presence of pathogens was significantly correlated with the *E. coli* class (Kendall's Tau-c, $P < 0.001$) (Fig. 1c). This is not the case for the subset of solid (soil or produce) samples, although still more pathogens were present in class 2 compared to class 1 (Fig. 1a, b). When the raw data of enumeration of *E. coli* and TPAC were analyzed for soil and produce samples, no significant difference was found for the two parameters between the absence or presence of pathogens (Mann–Whitney U test, $P < 0.05$) (Fig. 1d, e). In contrast, *E. coli*, coliforms, enterococci and TPAC were significantly higher when pathogens were present in the water (Mann–Whitney U test, $P < 0.05$) (Fig. 1f). These findings were complemented by the binary logistic regression results (odds ratios) between pathogen and indicator bacteria suggesting generally weak but significant associations between the different indicators *E. coli*, coliforms and enterococci and pathogens (Table 6).

### 3.3. Correlation between microbial parameters and climatic conditions (outside temperature, precipitation), temperature and pH of the irrigation water

There were no significant correlations between *E. coli* numeration (raw data used) in soil samples and the accumulative precipitation and the outside temperature of the last week before sampling. However, for the soil samples (36.6% of samples) containing numerable *E. coli* levels it was noted that the outside temperature was significantly higher than the observed outside temperature in the subset of soil samples showing absence of numerable *E. coli* (Mann–Whitney U test, $P < 0.05$). In contrast, pathogens in soil were detected in a period when lower outside temperature and lower precipitation occurred (Fig. 2a).

A significant but very low correlation of 0.12 and 0.19 was observed between the raw *E. coli* enumeration data of the lettuce samples and respectively the outside temperature and the accumulative precipitation

**Table 4**
Prevalence of pathogens in lettuce primary production.

| Amount of samples (n)[a] | Sample type | *E. coli* class | *E. coli* count (log CFU/g or log CFU/100 ml) | Pathogen | PCR screening[b] | Confirmed by culture | Serotype |
|---|---|---|---|---|---|---|---|
| 1 | Soil | 2 | 1.4[c,f] | EHEC | *vt2*, *eae* | YES | O157 |
| 1 | Soil | 2 | 1.0 ± 0[c,e] | EHEC | *vt2*, *eae* | NO | |
| 1 | Soil | 2 | 1.0[c,f] | EHEC | *vt1*, *vt2*, *eae* | YES | O103, O157 |
| 1 | Soil | 2 | 1.0[c,f] | EHEC | *vt1*, *eae* | YES | O26 |
| 1 | Soil | 2 | 1.5 ± 0.7[c] | EHEC | *vt1*, *eae* | NO | |
| 1 | Water | 4 | 3.6 | EHEC | *vt1*, *eae* | NO | |
| 1 | Water | 4 | 2.6 | EHEC | *vt1*, *eae* | YES | O111 |
| 1 | Water | 4 | 2.0 | EHEC | *vt1*, *eae* | YES | O26 |
| 1 | Water | 3 | 1.6 | EHEC | *vt1*, *vt2*, *eae* | NO | |
| 1 | Water | 3 | 1.0 | EHEC | *vt1*, *eae* | NO | |
| 1 | Water | 1 | <0 | EHEC | *vt1*, *vt2*, *eae* | NO | |
| 1 | Soil | 1 | <0.7 | *Salmonella* spp. | | | |
| 1 | Water | 3 | 1.0 | *Salmonella* spp. | | | |
| 7 | Water | 4 | 2.6 ± 0.4[d] | *Campylobacter* spp. | | | |
| 15 | Water | 3 | 1.4 ± 0.3[d] | *Campylobacter* spp. | | | |
| 2 | Water | 2 | 0.6 ± 0.4[d] | *Campylobacter* spp. | | | |
| 3 | Water | 1 | <0[d] | *Campylobacter* spp. | | | |
| 2 | Lettuce | 2 | 0.8 ± 0.1[d] | *Campylobacter* spp. | | | |
| 6 | Lettuce | 1 | <0.7[d] | *Campylobacter* spp. | | | |

[a] Number of samples in the respectively *E. coli* class showing presence of PCR EHEC positives, *Salmonella* spp., *Campylobacter* spp.
[b] PCR screening with GeneDisc or method of Posse et al. (2008).
[c] Mean *E. coli* count ± standard deviation of the individual three soil samples (pooled together for PCR EHEC detection). Only the samples with *E. coli* levels higher than the detection limit were used in the calculations.
[d] Mean *E. coli* count ± standard deviation of samples in the respective class for *Campylobacter* spp.
[e] 1/3 samples below detection limit (<0.7 log CFU/g).
[f] 2/3 samples below detection limit (< 0.7 log CFU/g).

**Table 5**
Spearman's Rho correlation coefficients between water samples for the combinations which showed significant correlations (P < 0.05).

| Water samples | | *E. coli* | Coliforms | Enterococcus | TPAC[a] | $T_{outside}$ | $T_{water}$ | $pH_{water}$ | Precipitation |
|---|---|---|---|---|---|---|---|---|---|
| *E. coli* | Coefficient | | 0.92 | 0.85 | 0.44 | 0.39 | 0.51 | | 0.25 |
| | N[b] | | 120 | 120 | 120 | 120 | 116 | | 120 |
| Coliforms | Coefficient | 0.92 | | 0.78 | 0.45 | 0.30 | 0.44 | 0.28 | 0.29 |
| | N[b] | 120 | | 120 | 120 | 120 | 116 | 105 | 120 |
| Enterococcus | Coefficient | 0.85 | 0.78 | | 0.47 | 0.29 | 0.42 | 0.27 | 0.28 |
| | N[b] | 120 | 120 | | 120 | 120 | 116 | 105 | 120 |
| TPAC | Coefficient | 0.44 | 0.45 | 0.47 | | 0.25 | 0.36 | | |
| | N[b] | 120 | 120 | 120 | | 120 | 116 | | |
| $T_{outside}$ | Coefficient | 0.38 | 0.30 | 0.29 | 0.25 | | 0.68 | | |
| | N[b] | 118 | 120 | 120 | 120 | | 116 | | |
| $T_{water}$ | Coefficient | 0.51 | 0.44 | 0.42 | 0.36 | 0.68 | | | |
| | N[b] | 116 | 116 | 116 | 116 | 116 | | | |
| $pH_{water}$ | Coefficient | | 0.28 | 0.27 | | | | | |
| | N[b] | | 105 | 105 | | | | | |
| Precipitation | Coefficient | 0.25 | 0.29 | 0.28 | | | | | |
| | N[b] | 120 | 120 | 120 | | | | | |

The outside temperature and precipitation included are the mean temperature and accumulative precipitation calculated from the daily data of temperature and precipitation collected from the nearest RMI weather station during the seven days prior to and including the sampling day at the farm.
[a] TPAC: Total psychrotrophic aerobic plate count.
[b] N: amount of samples.

(Spearman rank, P < 0.05). No significant difference in temperature and precipitation was observed between the subset of lettuce samples (5.7% of samples) with presence of numerable *E. coli* (>5 CFU/g) and the samples showing absence of numerable *E. coli* (Mann–Whitney U test, P > 0.05). A higher (not significant) outside temperature (14.4 °C ↔ 17.3 °C) and precipitation (11.6 mm ↔ 15.3 mm) were noted when pathogens were detected in the lettuce samples compared to the ones without pathogens (Mann–Whitney U test, P > 0.05) (Fig. 2b).

For the water samples, the indicators (*E. coli*, coliforms and enterococci) showed moderate significant correlations with the mean outside temperature of the last week before harvest (between 0.3 and 0.4), but lower correlations with the accumulative precipitation (between 0.25 and 0.28) (Table 5). There was also a significantly higher outside

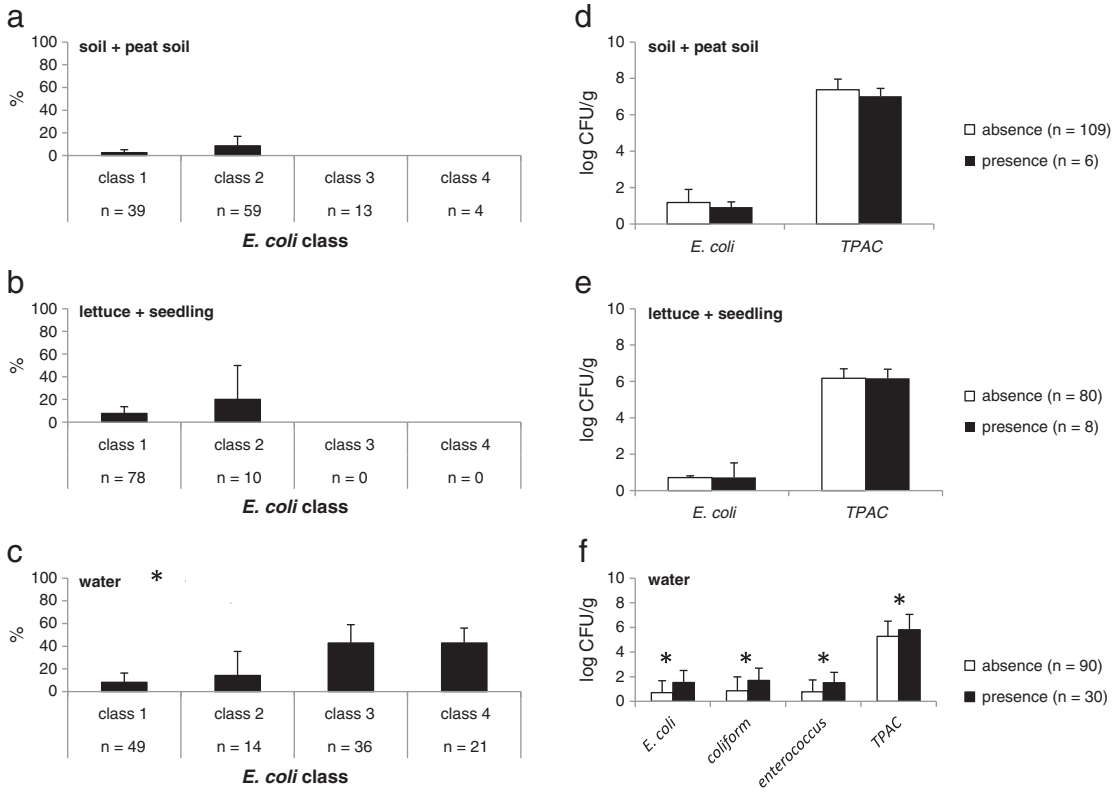



**Fig. 1.** Pathogen detection based on the class of *E. coli* bacteria (Table 2) (a–c). Detection is quantified in terms of percentage of the total number of samples in the corresponding class for the different samples (Kendall's Tau-c test performed to indicate significant difference). a: Soil (field soil + peat-soil) (P > 0.05), b, produce (lettuce + seedling) (P > 0.05) and c: irrigation water samples (P < 0.05). Bars represent the 95% confidence interval. The mean value of the *E. coli*, TPAC, coliforms or enteroccus enumeration (if analyzed) in the subset of samples with either absence or presence of pathogens is shown in d for soil (soil + peat soil) (P > 0.05), e for produce (lettuce + seedling) (P > 0.05) and f for the irrigation water samples (P < 0.05). Mann–Whitney U test performed to indicate significant difference (P < 0.05). Bars represent the standard deviation. * significant difference.

**Table 6**
Binary logistic regressions between pathogens and indicators *E. coli*, coliforms, enterococcus and TPAC and between the parameters $T_{outside}$ and $T_{water}$ (only significant results ($P < 0.05$) are shown).

| Irrigation water samples | All pathogens | | *Campylobacter* | |
|---|---|---|---|---|
| | Nagelkerke's $r^2$ | Odds ratio | Nagelkerke's $r^2$ | Odds ratio |
| *E. coli* | 0.164 | 2.169 | 0.120 | 1.931 |
| Coliforms | 0.140 | 1.885 | 0.103 | 1.719 |
| Enterococcus | 0.150 | 2.213 | 0.125 | 2.070 |
| TPAC | 0.045[a] | 1.456[a] | 0.066 | 1.615 |
| $T_{outside}$ | 0.086 | 1.141 | 0.100 | 1.159 |
| $T_{water}$ | 0.123 | 1.158 | 0.135 | 1.172 |

[a] Not significant ($P > 0.05$).

temperature (13.4 °C ↔ 15.7 °C) when pathogens were present (Mann–Whitney U test, $P < 0.05$) (Fig. 2c). This is confirmed by binary logistics, as the outside temperature showed a weak but significant odds ratio of approximately 1.2 for the water samples (Table 6).

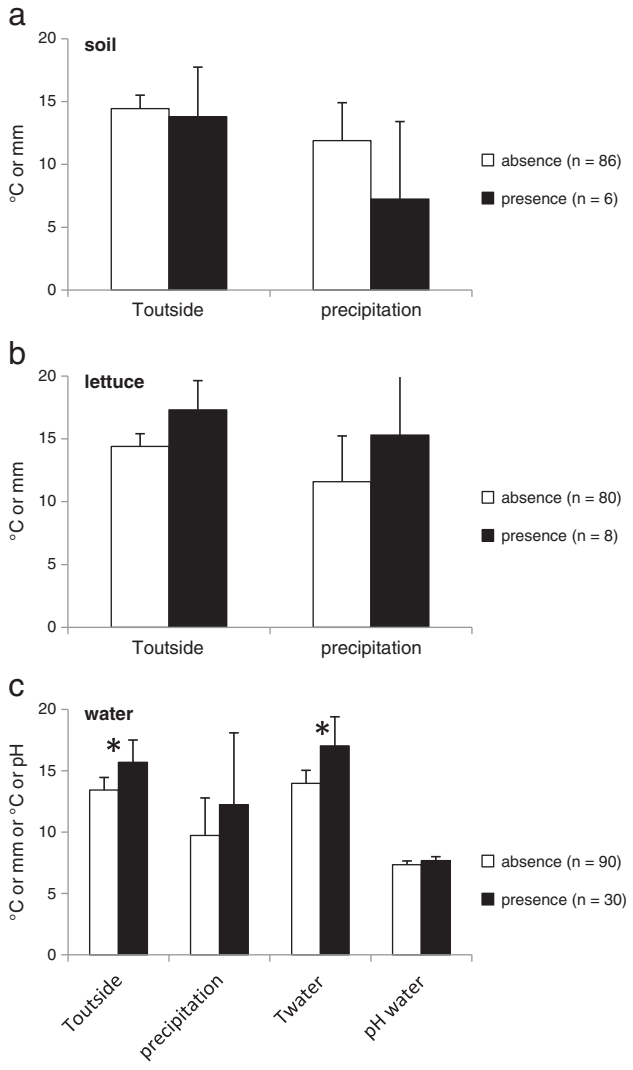



**Fig. 2.** The potential impact of the climatic parameters ($T_{outside}$, precipitation) and the $T_{water}$, and $pH_{water}$ on the presence or absence of pathogens in the various sample types of lettuce primary production. a: soil (field soil), b: lettuce and c irrigation water samples. Mann–Whitney U test performed to indicate significant difference ($P < 0.05$). Bars are 95% confidence interval. * significant difference.

Concerning the water parameters, the water samples showed moderate correlations between the water temperature and the microbial indicators *E. coli* (0.512), coliforms (0.437), enterococci (0.421) and TPAC (0.358) (Table 5). There was also a significantly higher water temperature (13.9 °C ↔ 17.0 °C) when pathogens were present (Mann–Whitney U test, $P < 0.05$) (Fig. 2c). This was confirmed by the odds ratio of 1.2 (Table 6).

The pH showed only significant correlations to coliforms and enterococci (±0.28) and was approximately the same (pH 7.4) if pathogens were present compared to the absence of pathogens.

During the year-around survey also a seasonal effect was established. *E. coli* was present in the soil in 10/12 months and of these ten months also during four months (July, September, October and December) *E. coli* was present in numerable levels in the lettuce crop (Fig. 3a). However, these months did not cluster in a particular season of the year. It is noteworthy that the irrigation water samples showed the highest concentrations of *E. coli*, coliforms and enterococci from May to September whereas in the months of March and April, the first two months of spring season lower levels of *E. coli* were found in the irrigation water (Fig. 3b).

Pathogens were only detected in samples taken in the time period from May to November (Fig. 3d). This corresponds with the a higher outside temperature as the temperature during sampling was the highest (from 13.7 °C in May to a maximum of 19 °C in August and 11.7 °C in November) in those months (Fig. 3e). The presence of pathogens in these months was mostly attributed to the detection of *Campylobacter* in irrigation water samples (Fig. 3e). The prevalence of *Campylobacter* in water ranged from 1/9 samples in November up to 2/3 samples in month June (Fig. 3d).

## 4. Discussion

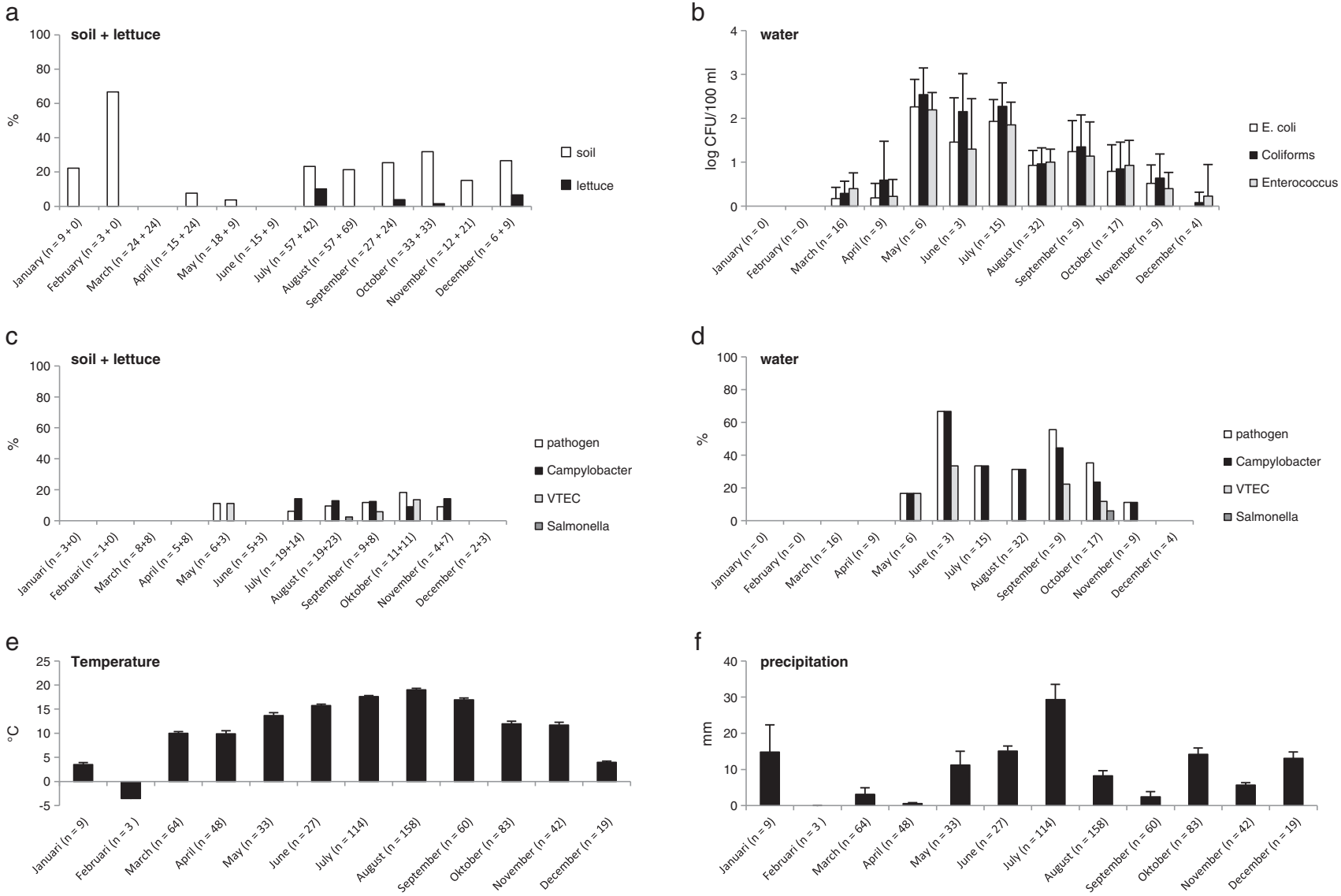
### 4.1. The prevalence of pathogens in lettuce primary production

The prevalence of *Campylobacter* on the lettuce (9%) was quite similar to Park and Sanders (1992), who detected *Campylobacter* in lettuce (3.1%), radish (2.7%), green onions (2.5%) and parsley (2.4%) from samples collected at farms in Canada. Bohaychuk et al. (2009) found no *Campylobacter* in more than 600 vegetable samples from farmer markets in Canada including 128 lettuce samples. In the Netherlands only 13 of 5640 (0.2%) vegetable and fruit samples sampled at retail (fresh-cut or crops) were positive for *Campylobacter* (Verhoeff-Bakkenes et al., 2011).

*Salmonella* and EHEC were absent on the lettuce samples ($n = 0/88$; CI 0–4, 2%) which is in agreement with other studies (e.g., Johannessen et al., 2002; Loncarevic et al, 2005; Oliveira et al., 2010; Bohaychuk et al., 2009). From the 2011 European Union Summary Report on Trends and Sources of Zoonoses EU reported by EFSA (2013c), it was noted that no positive *Salmonella* samples were detected in 1606 samples of fresh-cut fruit or vegetable samples and no VTEC in a total of 2887 samples of fresh-cut or none-cut vegetable samples. However, studies executed in Spain found *Salmonella* spp. (1.7% ($n = 236$)) in minimally processed fresh-cut vegetables (Abadias et al., 2008).

EFSA (2013a) ranked *Salmonella* spp. and leafy vegetables eaten raw as the highest concern in a list of various pathogen-commodity combinations based on reported foodborne outbreaks in EU and the ability for growth, consumption etc. Also in a FAO report on leafy greens, *Salmonella* was mentioned as the most important pathogen (FAO, 2008). However, the present sampling scheme is too limited to make conclusions on the absence of any potential pathogen in the lettuce in particular for pathogens characterized by low prevalence (<0.1 to 1%) such as *Salmonella* and EHEC in fresh produce.

The proportion of samples with numerable *E. coli* on the seedling and lettuce crops (5%) was slightly lower compared to findings of other studies conducted in developed countries. Bohaychuk et al. (2009) found numerable *E. coli* in 18% of the 128 lettuce samples




The outside temperature and precipitation included is the mean temperature and accumulative precipitation calculated from the daily data of temperature and precipitation collected from the nearest RMI weather station during the seven days prior to and including the sampling day at the farm.

**Fig. 3.** Seasonality of presence of indicator organisms and pathogen detection in the various sample types in lettuce primary production and characteristics of climatic conditions in function of the month throughout the sampling season. a: *E. coli* presence (% > 5 CFU/g) in soil (n = 276) and lettuce samples (n = 264), b: *E. coli*, coliform and enterococcus enumeration in irrigation water samples (n = 120); c: Pathogens' presence in soil (n = 92) and lettuce samples (n = 88), % presence per 25 g of sample; d: Pathogens' presence in water samples (n = 120), % presence in 1 liter sample; e: Outside temperature (n = 660); and f: Precipitation (n = 660). Bars are the 95% confidence intervals and n = the amount of samples.

sampled from the farmer markets in Canada. Loncarevic et al. (2005) enumerated *E. coli* in 9% of the lettuce samples (16/179) obtained from an organic farm in Norway and *E. coli* was detected in 22.5% of the organic lettuce samples and in 9% of the conventional lettuce samples sampled from farmers in Spain (Oliveira et al., 2010).

Overall the prevalence of *E. coli* in the irrigation water source was high (75% ≥ 1 CFU/100 ml) and on some occasions elevated levels of *E. coli* were detected (65% ≥ 10 CFU/100 ml and 26% ≥ 100 CFU/100 ml). A lower prevalence of *E. coli* was observed for the irrigation tap and rinse water (38% ≥ 1 CFU/100 ml). Furthermore, 35% of the water sources showed to be positive for at least one pathogen (*Salmonella*, *Campylobacter* isolates or PCR EHEC signals).

In total 6 out of 8 farms used open wells to stock irrigation water and they claimed that the open well water contained only collected rainfall water and no surface water. Upon collection, the rainfall water is assumed to be low in microbiological contamination but the bacterial load may increase during runoff along roof or soil surfaces and during the storage in the open well (Helmreich and Horn, 2009; Schets et al., 2010). Microbial contamination may originate from fecal contamination by birds and mammals that have access to catchment areas or water storage (Sazakli et al., 2007).

The prevalence of *Campylobacter* (30.9%) or *E. coli* (75%) in the irrigation water source in the present study was high. Comparable results were found in collected rainwater in Denmark. Albrechtsen (2002) found a prevalence of 79% and 12% respectively for *E. coli* and *Campylobacter*, also in Australia 25% of the samples were found positive for *Campylobacter* and 60% for *E. coli* (Ahmed et al., 2008, 2010). Similar *Campylobacter* levels were observed in collected rainwater in New Zealand (37%) (Savill et al., 2001) and in the Netherlands (27%) (Schets et al., 2010). The *Campylobacter* levels were also comparable to surface water studies (Wilkes et al., 2009; Brennhovd et al., 1992; Arvanitidou et al., 1995; Horman et al., 2004). In contrast, Economou et al. (2012) found no *Campylobacter* in Greek surface water, although the water source was located in an area characterized by high agricultural and livestock activity such as cattle and poultry farms.

The presence of *Salmonella* (1 out of 68 samples; 1.4%) in the irrigation water source was very low compared to the presence of *Campylobacter* and compared to other publications, in which a prevalence of *Salmonella* of more than 16% in collected rainwater in Australia and approximately 10% in surface water in Greece and Canada was noted (Economou et al., 2012; Wilkes et al., 2009).

Prevalence of EHEC (6 out of 68 samples; 9%) (six EHEC PCR positive samples resulting in two confirmed isolates: *E. coli* O26 and *E. coli* O111) was difficult to compare to other literature reports as in most cases only *E. coli* O157 was analyzed and another methodology was used (PCR detection compared to isolation). Nevertheless, *E. coli* O157 was detected (4 out of 144 samples) in private water supplies (supposed to be drinking water) in the Netherlands (Schets et al., 2005). River water containing *vt2* genes used for irrigation was a possible source for an outbreak of *E. coli* O157 in Sweden involving iceberg lettuce (Soderstrom et al., 2008) and a spinach outbreak in USA (Jay et al., 2007). The irrigation water being most likely contaminated by upstream cattle grazing.

Water showed to be a risk factor for introduction of pathogens in the primary production of lettuce in this survey, as pathogens were found in 25% of all water samples (open well (21/51), groundwater (3/17), sprinkler tap water (2/41) and rinsing water at harvest (4/11)). In Belgium (and all farms included in the study) overhead irrigation with a sprinkler system is used, generally known as the irrigation method with the highest risk for contamination as the water is applied on top of the edible part of the lettuce crops (Hamilton et al., 2006; Song et al., 2006). Pathogenic bacteria can be reduced between irrigation and harvest due to UV radiation, drying, or competition with commensal microbiota (Brandl and Amundson, 2008; Ottoson et al., 2011). Increasing the interval from the time of contamination to the point of harvest significantly decreased the likelihood that the pathogen would be present in the harvested product (Fonseca et al., 2011; Moyne et al., 2011).

However, the summer of 2012 was characterized by quite some rainfall (Fig. 3f) and this on a regular basis, which resulted in a low need for irrigation of lettuce crops during open field production and thus only restricted potential for transfer of bacterial contamination from the water source to the crop through direct contact via sprinkler irrigation. However, in greenhouse production there is a continuous need for irrigation and thus contact of water with crop leaves upon irrigation. It could be noted that in greenhouses the irrigation water is regularly vaporized minimizing or excluding splashing in contrast to rainfall or open field sprinkler irrigation showing more intensive impact of water droplets on the lettuce (and surrounding soil). Nevertheless, not only sprinkler irrigation but also natural rainfall events – depending upon the density – enhance the risk of splashing manure and soil particles to the lettuce (Cevallos-Cevallos et al., 2012; Liu et al., 2013). Still, the occurrence of elevated *E. coli* levels numerated on the lettuce was low (5%) (1 out of 264 samples ≥100 CFU/g, 14 out of 264 samples ≥5 CFU/g). This might also be influenced by the sampling method in the present study. Sampling was performed using pooled samples of cut halves of lettuce crops whereas the sampling of e.g., outer leaves only may be more vulnerable to finding elevated levels of *E. coli*. To the knowledge of the authors, hitherto, no harmonized protocol or ISO or CEN guidelines for sampling of lettuce crops is present.

### 4.2. Correlation between microbial indicators and pathogens

The correlation between the different indicators *E. coli*, enterococci and coliforms for the water (ranging from 0.79 to 0.92) was slightly higher compared to the correlation of approximately 0.75 observed by Wilkes et al. (2009). Economou et al. (2012) found lower correlations between *E. coli* and total coliforms (0.54). The correlation between TPAC and the other indicators was low compared to the correlation between *E. coli*, enterococci and total coliforms. These results confirmed other publications who demonstrated that TPAC is not a good indicator of overall sanitary quality and fecal contamination during production for both environmental samples and lettuce samples (Economou et al., 2012; Holvoet et al., 2012). The high correlation (between 0.79 and 0.92) between *E. coli*, total coliforms and enterococci in the irrigation water indicated that it was not necessary to enumerate all hygiene indicators as they were strongly correlated to each other. *E. coli* is preferable as indicator of unsanitary conditions in comparison to coliforms due to its fecal origin.

Most publications found weak or no correlations in water between different pathogens and hygiene indicator bacteria. Wilkes et al. (2009) found slightly lower odds ratios for the pathogens (between 1.4 and 1.7) compared to the present study (between 1.9 and 2.2) indicating a higher probability for pathogens if higher levels of *E. coli*, enterococci and coliforms were present. Significant correlations have not been found in New Zealand river water between *Campylobacter* spp. and *E. coli* (Savill et al., 2001) and between *E. coli* and *Salmonella* in Greece (Economou et al., 2012). Vereen et al. (2007) on the other hand found a correlation of 0.35 in surface water in USA between fecal coliform and *Campylobacter*.

Microbial indicators of fecal contamination do not necessarily reflect the input of enteric pathogens, however, some predictive value has been reported especially in water between the fecal indicators and pathogens (Harwood et al., 2005; Schets et al., 2005; Wilkes et al., 2009). Variations in pathogen input (i.e., prevalence in population), dilution, retention, and die-off result in conditions where relationships/correlations between any pathogen and any indicator are random, site-specific, or time-specific (Payment and Locas, 2011). As a result, there is clearly no indicator that may be suitable for all pathogens for all environmental scenarios (Harwood et al., 2005; Yates, 2007). However, the probability of detection of any pathogen is high at high levels of indicators (Savichtcheva and Okabe, 2006).

### 4.3. Correlation between microbial parameters and climatic parameters

The highest levels of *E. coli* and the highest prevalence of pathogens in the water were observed in that time of the year when the outside temperature and water temperature were the highest (between May and October). This was in agreement with other publications, who noted a positive association between temperature and bacterial indicator levels in the water (Isobe et al., 2004; Shehane et al., 2005). Many studies have demonstrated these positive associations between temperature and foodborne illness (Checkley et al., 2000; Singh et al., 2001). In the UK, a correlation was found between gastrointestinal disease and the temperature in the month preceding the illness (Bentham and Langford, 2001). Another study in Australia found a positive association between temperature in the previous month and the number of notifications of cases of *Salmonella* in the current month (D'Souza et al., 2004). In several European countries including The Netherlands, England, Switzerland and Spain, a linear correlation was found between temperature and the number of reported cases of Salmonellosis (Kovats et al., 2004). The monthly presence of *Campylobacter* in the irrigation water showed the same seasonal trend as to the presence of *Campylobacter* in Belgian chicken meat preparations (Habib et al., 2008).

There was a significant correlation between precipitation and hygiene indicator contamination for the water samples. This is confirmed by many other studies, which have shown a correlation between increased precipitation accompanied by increased runoff or discharge of (untreated) wastewater and the concentration of fecal indicator organisms or pathogens in water. After changes in rainfall patterns, an increase in fecal indicator densities in the water was observed by several authors (Dorner et al., 2007; Shehane et al., 2005). In the United States, the highest *E. coli* concentrations were found in the period that corresponded with a period of greater rainfall intensity (Schilling et al., 2009). Due to the heavy rainfall, the irrigation water becomes contaminated and irrigation with water of bad quality may give rise to a contaminated product (Castillo et al., 2004; Ensink et al., 2007).

Although these parameters cannot predict the presence of pathogens, temperature, precipitation and *E. coli* concentration provide some information concerning the most critical periods for possible pathogen contamination of the produce and water. These periods must induce a higher state of awareness to prevent contamination of the produce by e.g., contaminated irrigation water.

## Acknowledgments

We would like to thank Anne-Sophie Vandevoorde for the assistance in the water sampling and Prof. Dr. Lieven De Zutter from the Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University for his assistance and support during this study. Our special appreciation goes to the personnel from Howest and the Accredited Lab (Laboratory of Food Preservation and Food Microbiology, Faculty of Bioscience Engineering, Ghent University), who assisted in the purchase of reagents and microbial analysis.

This research was conducted for the Veg-i-Trade project "Impact of Climate Change and Globalization on Safety of Fresh Produce—Governing a Supply Chain of Uncompromised Food Sovereignty" (www.veg-i-trade.org). Veg-i-Trade is funded under the Seventh Framework Program of the European Commission (Grant number: 244994).

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