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Impact of weather conditions, leaf age and irrigation water disinfection on the major epiphytic bacterial genera of baby spinach grown in an open field

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ABSTRACT

The effects of factors such as weather conditions, leaf age and irrigation water disinfection on the main bacterial genera (total bacterial, *Enterobacteriaceae* and *Pseudomonas*) of baby spinach were studied. Culture-dependent and independent quantification techniques were compared. Cultivation was carried out over two consecutive trials in commercial open field divided in two plots: 1) baby spinach irrigated with untreated surface water and 2) baby spinach irrigated with chlorine dioxide (ClO_2) treated water. In all the cases, higher concentrations of bacteria were detected using molecular quantification in comparison with culture dependent techniques. Based on the obtained results, wind speed, solar radiation and relative humidity seem to have an impact on the levels of total bacterial, *Enterobacteriaceae* and *Pseudomonas* during cultivation of baby spinach. However, further studies would be needed to confirm this tendency. Water disinfection treatments (ClO_2), when applied to irrigation water, impacted differently the bacterial genera evaluated in the present study. Thus, although no significant effects were observed in total bacterial enumerations of baby spinach irrigated with ClO_2 treated water; significant reductions were detected in *Enterobacteriaceae* (19%) and *Pseudomonas* spp. (14%) levels. These results were also confirmed using specific culture-dependent methods. On the other hand, leaf age did not influence the levels of the main bacterial genera of baby spinach. Considering that, a large proportion of foodborne and pathogenic bacteria associated to fresh produce belong to the *Enterobacteriaceae* family and *Pseudomonas* genera, reductions in these bacterial groups could be beneficial. However, these groups are very diverse, making difficult to link the measurement of *Enterobacteriaceae* and *Pseudomonas* levels with the presence/abundance of potential pathogenic and spoilage microorganisms.

1. Introduction

Recent findings have evidenced that the bacterial community of the phyllosphere appears to be affected by external factors including agricultural practices, weather conditions (daily cycles of temperature, solar radiation and relative humidity), and internal factors (plant species and leaf age) among others (Rastogi et al., 2012; Jensen et al., 2013; Williams et al., 2013; Wei et al., 2016). Overall, bacterial population of fruits and vegetables is large and diverse (Vorholt, 2012; Bulgarelli et al., 2013). In leafy greens, *Enterobacteriaceae* and *Pseudomonas* genera have been defined as the microbial "core" of the phyllosphere (Leff and Fierer., 2013; Rastogi et al., 2012; Lopez-Velasco et al., 2013). Considering that several spoilage and pathogenic bacteria belongs to the *Enterobacteriaceae* and *Pseudomonas* genera (Lee et al., 2013), it is of great relevance to evaluate if internal and external factors affect their population.

Intervention strategies, such as agricultural water disinfection treatments, have been recommended to growers to reduce the risk of microbiological contamination during primary production (EFSA, 2013; Allende and Monaghan, 2015). There are several commercially available treatments to efficiently treat irrigation water such as calcium hypochlorite, chloride dioxide (ClO_2) and UV-light (Gil et al., 2015). Among them, ClO_2 has been reported as an efficient water disinfection treatment for agricultural water (Suslow, 2010). The efficacy of ClO_2 for the inactivation of pathogenic bacteria in irrigation water has been widely confirmed (Yao et al., 2010; Scarlett et al., 2017; López-Gálvez et al., 2017, 2018). However, little is known about the impact of water disinfection treatments on the epiphytic bacteria of fresh produce.

Traditionally, the most relevant bacterial groups of fresh produce were enumerated by plate counts on selective medium (Oliveira et al., 2010; Medina-Martinez et al., 2015). However, conventional culture dependent techniques may not represent the most accurate methodology for the enumeration of the total population present on the phyllosphere of fruits and vegetables. Supporting this statement,

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several authors reported that culturable method only detect between 1 and 10% of the epiphytic bacterial species (Pace, 1997; Rastogi et al., 2010). A good tool for the detection and identification of higher percentage of bacteria is the use of molecular techniques. Among them, quantitative PCR (qPCR) based on the 16S rRNA gene, allows the quantification of relative and absolute levels of bacterial communities in environmental samples (Fierer et al., 2005; Truchado et al., 2017).

The purpose of this study was to determine the impact of different factors including weather conditions, leaf age and chlorine dioxide treated irrigation water on the quantitative levels of total bacterial, *Enterobacteriaceae* and *Pseudomonas* of baby spinach during cultivation. Cultivation-dependent and molecular techniques were compared for the quantification of the main bacterial genera in two trials that corresponded to two consecutive growing period.

2. Materials and methods

2.1. Experimental design

Baby spinach (*Spinacea oleracea* L.) was cultivated in a commercial field located in Pozo de la Higuera (Almería, Spain). For treatments, the field was divided was divided in two plots (0.5 and 0.8 ha each). Two trials were consecutively evaluated: Trial 1 between November–December and Trial 2 between February–March. Experimental design was as previously described by López-Gálvez et al. (2018). Briefly, baby spinach (*Spinacea oleracea* L.) was grown according to standard commercial practices used by the grower (Primaflor S.A.T., Pulpí, Spain). Irrigation and fertilization practices were carried out following commercial conditions recommended by the grower, using an overhead-sprinkler system for irrigation. The two plots used for the study showed very similar characteristics regarding soil texture and topography. One field was irrigated with untreated surface water (Control) and the other with ClO_2-treated water (Treated) in one trial and reversed in the second trial. Chlorine dioxide water treatment was applied and monitored as described by López-Gálvez et al. (2018). The concentration of ClO_2 in irrigation water was maintained at a residual dose of about 0.25 mg/L.

Additionally, weather parameters including solar radiation, relative humidity (RH) and temperature during the growing period were obtained from the nearby Pozo de la Higuera – weather station (37° 35′ 25,7" N, 1° 43′ 32″W), using the local climatological database (SIAM, 2015).

2.2. Sampling material

Baby spinach was collected at different leaf ages during the whole cultivation period of 47 days for Trial 1 and 44 days for Trial 2. After the true leaves appeared, baby spinach were collected 3 weeks (3Ws), 2 weeks (2Ws) and 1 week (1W) before harvest and also when baby spinach reached commercial maturity stage (0 W). These time intervals studied corresponded to similar days after planting in Trial 1 and Trial 2, respectively: 28 days (3Ws), 30–35 days (2Ws), 40–42 days (1W) and 47-44 days (0W).

At each time, plants from different positions, distributed homogeneously in each field, were collected. Samples of 100 g each were hand harvested using scissors from the base of petioles, placed them in sterile plastic bags and stored in polystyrene boxes under refrigerated conditions. The scissor and stainless steel shovel were wiped with ethanol (70%) between treatments. Samples kept in the polystyrene boxes under refrigerated conditions were transported (approximately 135 km) to the CEBAS-CSIC laboratory (Murcia, Spain) and stored at 4 °C. Once in the laboratory, samples were processed within 4 h after the arrival.

2.3. Culture-based analyses

Sample preparation was performed as previously described by Truchado et al. (2017). Briefly, 40 g of baby spinach were sonicated with 200 ml of 2% sterile buffered peptone water (BPW; Scharlau Chemie, Barcelona, Spain) supplemented with 0.1% of Tween-80 (Sigma Aldrich, St Louis, MO, USA) for 7 min. Serial dilutions were performed as needed and plated on three culture media: plate count agar (PCA; Scharlau Chemie, Barcelona, Spain) incubated at 30 °C for 48 h to estimate total bacterial enumerations, *Pseudomonas* agar (Oxoid, Basingstoke, Hampshire, England) incubated at 30 °C for 24 h for the estimation of *Pseudomonas* spp. and *Enterobacteriaceae* agar (Oxoid) incubated at 37 °C for 48 h for quantification of *Enterobacteriaceae*. The remaining homogenates of each baby spinach sample were further used for DNA extraction.

2.4. DNA extraction

Homogenized baby spinach was centrifuged at 3000 g for 10 min and the resulting pellet was stored at −20 °C for genomic DNA extraction. Genomic DNA of baby spinach samples was extracted using the FastDNA® SPIN Kit for soil and the FastPrep® 24-Instrument (MPBiomedicals, Germany), according to the manufacturer's indications. The quality and concentration of DNA extracts were determined by spectrophotometric measurement at 260/280 nm and 260/230 nm using a NanoDrop®ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

2.5. Quantitative-PCR procedure

Quantitative real-time PCR (qPCR) using the 16SrRNA gene as biomarker was performed to assess the abundance of total bacteria, *Pseudomonas* and *Enterobacteriaceae* using an ABI 7500 Sequence Detection System (ABI, Applied Biosystems, Madrid, Spain). Primers, reaction volumes, concentrations and the applied cycling parameters used are listed in Table 1. The amplification and detection were carried out in 96-well plates using KAPA SYBR FAST Universal qPCR Master mix kit (KapaBiosystems, Massachusetts, USA). In all cases, a non-template control (NTC) was included using DNAse free water instead of the DNA template. Melting curve analysis of the PCR products was conducted following each assay to confirm the fluorescence signal originated from a specific PCR product. The qPCR data were normalized based on the DNA concentrations. Standard curves were made using known concentrations of genomic DNA isolated from overnight cultures

Table 1
Primers used for the qPCR analyses.

Phylogenetic target	Name	Sequence (5′-3′)	qPCR cycling parameters	Reference
Total bacteria	534 F	CCAGCAGCCGCGGTAAT	2 min at 50 °C, 3 min at 95 °C, 40 cycles of 30s at 95 °C, 30 s at 53 °C, 1min at 72 °C	Rastogi et al., 2010
	783 R	ACCMGGGTATCTAATCCKG		
Enterobacteriaceae	En-lsu-3F 5′	TGCCGTAACTTCGGGAGAAGGCA	2 min at 50 °C, 3 min at 95 °C, 40 cycles of 30s at 94 °C, 20 s at 60 °C, 50s at 72 °C	Matsuda., 2009*
	En-lsu-3R	TGCCGTAACTTCGGGAGAAGGCA		
Pseudomonas	Pse435F	ACTTTAAGTTGGGAGGAAGGG	2 min at 50 °C, 3 min at 95 °C, 40 cycles of 30s at 95 °C, 30 s at 60 °C, 50s at 72 °C	Roosa et al., 2015*
	Pse 686R	ACACAGGAAATTCCACCACCC		

*With some modifications in qPCR cycling parameters.

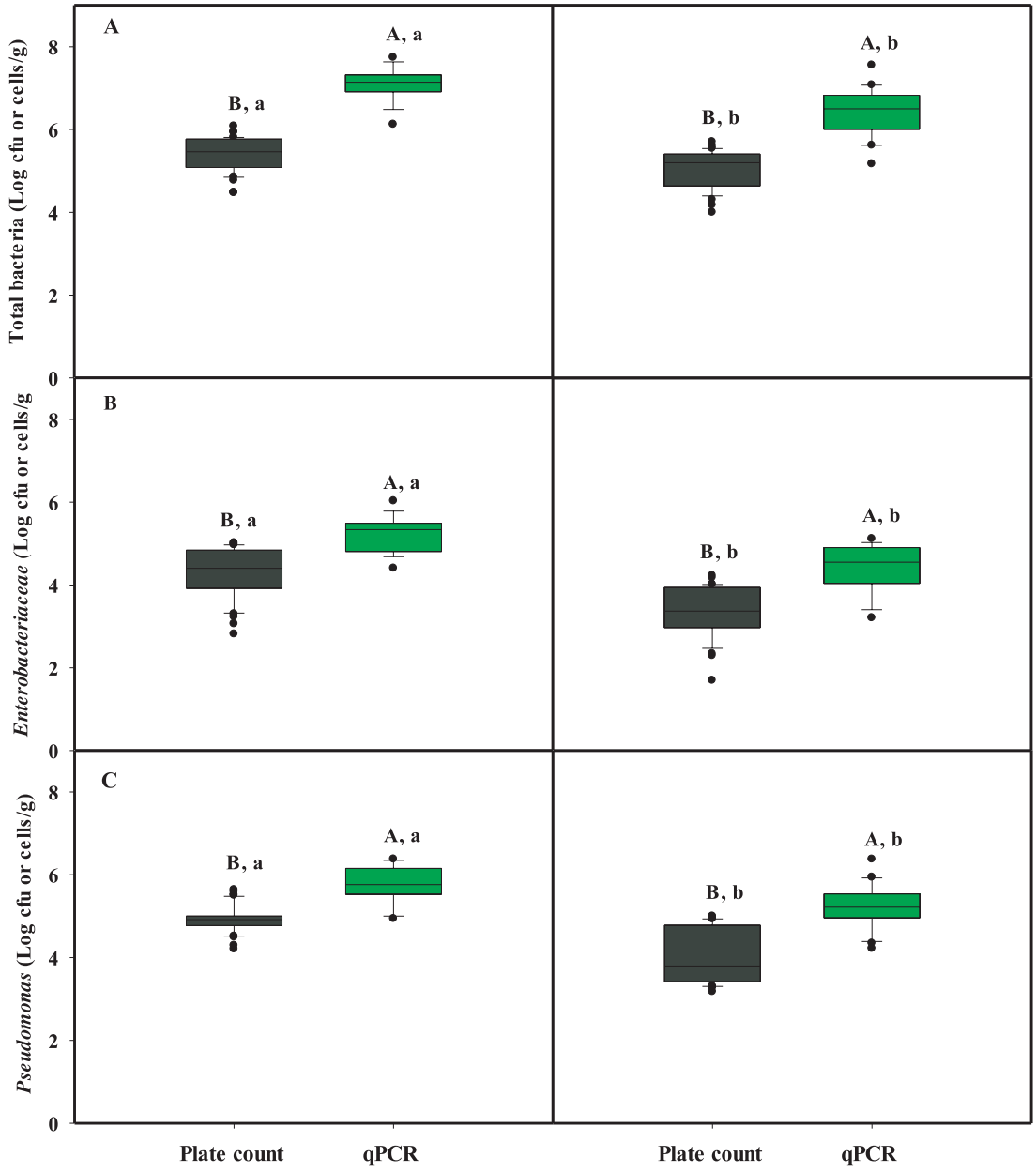

Fig. 1. Box-plot representing the mean of total bacteria (A), *Enterobacteriaceae* (B) and *Pseudomonas* (C) levels measured by culture-dependent methods (Log CFU/g) and culture-independent molecular methods (qPCR, Log cells/g) of baby spinach during the whole growing period. Box-plot with different letters indicates significant difference at $P < 0.05$. Box-plot with different upper case letters indicate significant difference between culture-dependent methods (plate count) and culture-independent methods (qPCR) (qPCR) for the same trial. Lower case letters indicate significant difference between trials for the same enumeration method. Trial 1: November–December (A) and Trial 2: February–March (B).

of *E. coli* CECT 5947 and *Pseudomonas* putida CECT 4064. *Enterobacteriaceae* standard was prepared from the mixture of several species belonging to this family, i.e *E. coli* CECT 434, 471, 515, 516, 533 and *Yersinia enterocolitica* CECT 4054. All strains were obtained from the Spanish Type Culture Collection (CECT) (Valencia, Spain). Cultures were inoculated in Brain Heart Infusion (BHI) broth (Scharlau Chemie, Barcelona, Spain) and incubated without agitation at 37 °C for 24 h. DNA was isolated using the PrepSEQ™ Rapid Spin sample preparation kit (Applied Biosystems, Madrid, Spain) following the manufacturer's instructions. Standard curves were prepared by plotting threshold cycles (Ct) vs. total number of bacteria (CFU/mL). Cell counts before DNA extraction were determined by the standard plate count method (Elizaquivel et al., 2011).

2.6. Statistical analysis

Bacterial numbers determined by plate count and qPCR assays were expressed as Log cells/g of baby spinach. IBM SPSS Statistics 19 was used for statistical analysis. Shapiro-Wilk test and Levene's test were performed to assess the normality of the data ($P > 0.05$) respectively. If normality or equality of variance could not be assumed, Mann Whitney *U* test was used to determine the differences between the raw data ($P < 0.05$). Except when otherwise stated, *P* values below 0.05 were considered statistically significant. Spearman rank analyses were used to determine correlations between total bacterial, *Enterobacteriaceae* and *Pseudomonas* and the weather parameters (i.e. temperature, RH and solar radiation).

Table 2
Weather parameters recorded during the growing period of baby spinach in Trial 1 (November–December) and Trial 2 (February–March). Data are the mean ± standard deviation obtained from the climatological database of Murcia, Spain (SIAM).

	Trial 1	Trial 2
Diurnal temperature range (ºC)	13.12 ± 3.45	12.73 ± 3.75
Wind speed (m/s)	1.09 ± 0.5	2.59 ± 1.1
Relative humidity (%)	97.4 ± 16.0	80.2 ± 17.5
Solar radiation (W/m^2)	74.8 ± 8.7	48.5 ± 11.5
Precipitation (mm)	ND[a]	ND

[a] ND, no detected.

Table 3
Spearman rank correlations between microbiological levels of baby spinach and weather parameters.

		Total bacteria	*Enterobacteriaceae*	*Pseudomonas*
Wind Speed	Coeficiente	−0.658	−0.789	−0.881
	Sig	0.058	0.210	0.004
Relative humidity	Coeficiente	0.762[a]	−0.238	0.762[a]
	Sig	0.028	0.570	0.028
UV Radiation	Coeficiente	−0.952[b]	0.476	−0.714[a]
	Sig	0.000	0.233	0.047

[a] Significant correlations at $P < 0.05$.
[b] Significant correlations at $P < 0.01$.

3. Results

3.1. Major bacterial species of baby spinach detected by culture-dependent and culture-independent techniques in two consecutive trials

Average loads of culturable aerobic bacteria in baby spinach were 5.4 ± 0.4 and 5.0 ± 0.4 log cfu/g in the first and second trials, respectively. On the other hand, when levels of total aerobic bacteria were determined using qPCR quantification, the average number of total bacteria in the phyllosphere of baby spinach was 7.1 ± 0.5 and 6.4 ± 0.6 log cells/g for the first and second trials, respectively. Using both quantification techniques, levels of bacteria in the first trial were higher than in the second trial. The same trend was observed when levels of *Enterobacteriaceae* and *Pseudomonas* were quantified, but the number of bacteria detected using a culture-independent technique were up to 10-fold higher than those detected by plate counts. Our results agree with those previous publications that reported differences between cultivation-dependent and independent quantification techniques for the characterization of the phyllosphere of leafy greens (Rastogi et al., 2012; Jackson et al., 2013; Williams et al., 2013; Williams and Marco, 2014). The discrepancy between both quantification techniques can be attributed to the presence of non-viable and viable but non-culturable (VBNC) bacteria present in the phyllosphere (Wilson and Lindow, 2000). It is well known that the surface of the leaves is a hostile habitat for microorganisms due to exposure to different abiotic and biotic stresses. Stress conditions can inflict sub-lethal injuries to bacterial species that have acquired multiple complex mechanisms of stress resistance as a temporary state called VBNC (Oliver, 2005; Li et al., 2014). This state is characterized by a low metabolic activity in which cells can persist for extended periods without division. Bacteria in the VBNC state cannot be detected by culture-based techniques (Ducret et al., 2014; Dinu and Bach, 2011). In this study, we cannot determine that proportion of bacteria is killed or just stressed and non-cultivable, due to qPCR assay cannot distinguish between viable and dead cells. This limitation could have been avoided if the samples had been pretreated with DNA intercalating dyes, such as propidium monoazide (PMA), prior to the qPCR (Truchado et al., 2016).

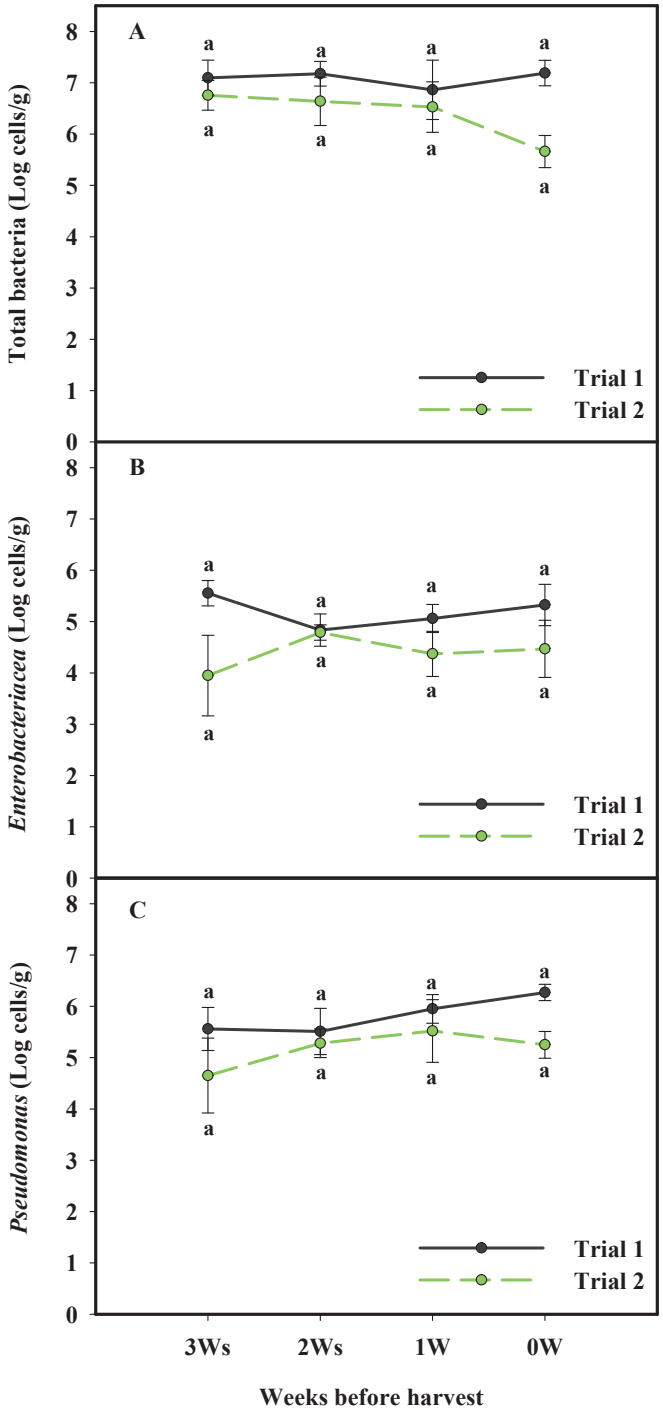

Fig. 2. Influence of maturity stage on total bacteria (A), *Enterobacteriaceae* (B) and *Pseudomonas* (C) levels (Log cells/g) of baby spinach in Trial 1 (November–December) and Trial 2 (February–March). Values are the mean of 5 replicates and standard deviation. Bars with different letters indicate significant difference during growing cycle at $P < 0.01$.

3.2. Influence of weather conditions and leaf age on the major bacterial species of baby spinach in two consecutive trials

To determine the changes in the major bacterial species of baby spinach cultivated in open field using overhead irrigation two consecutive trials were compared (Fig. 1). It was observed that levels of total bacterial (Fig. 1A), *Enterobacteriaceae* (Fig. 1B) and *Pseudomonas* (Fig. 1C) were always higher in Trial 1 than in Trial 2.

In order to determine if the weather conditions were responsible for

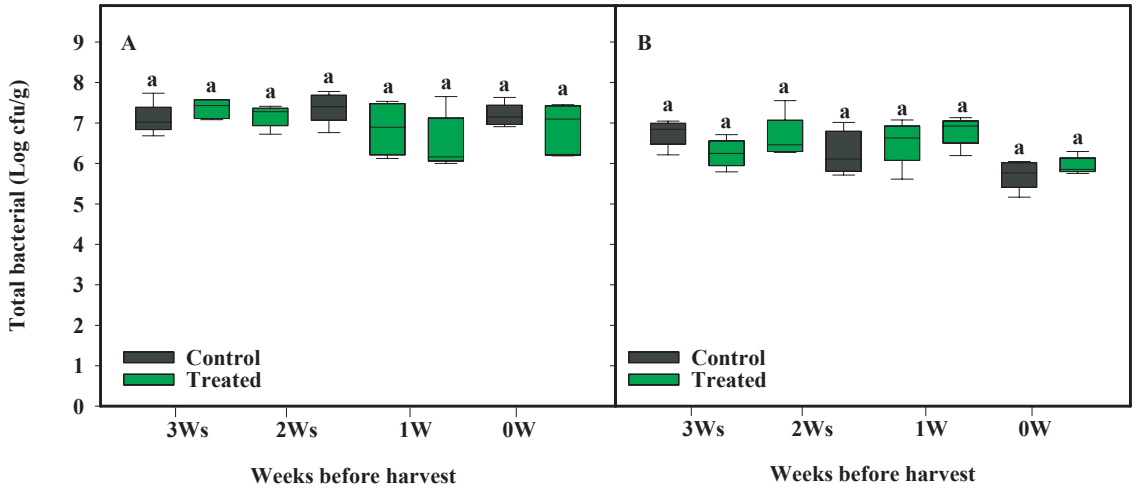

Fig. 3. Box-plot representing total bacterial counts (Log cells/g) of baby spinach irrigated with untreated (Control) and ClO_2 treated water (Treated) during the growing period in Trial 1 (**A**, November–December) and Trial 2 (**B**, February–March). Box-plot with different letters indicates significant difference at $P < 0.05$. Bottom and top of the boxes represent the 25th and 75th percentiles. The boxplot whiskers represent the minimum and maximum values.

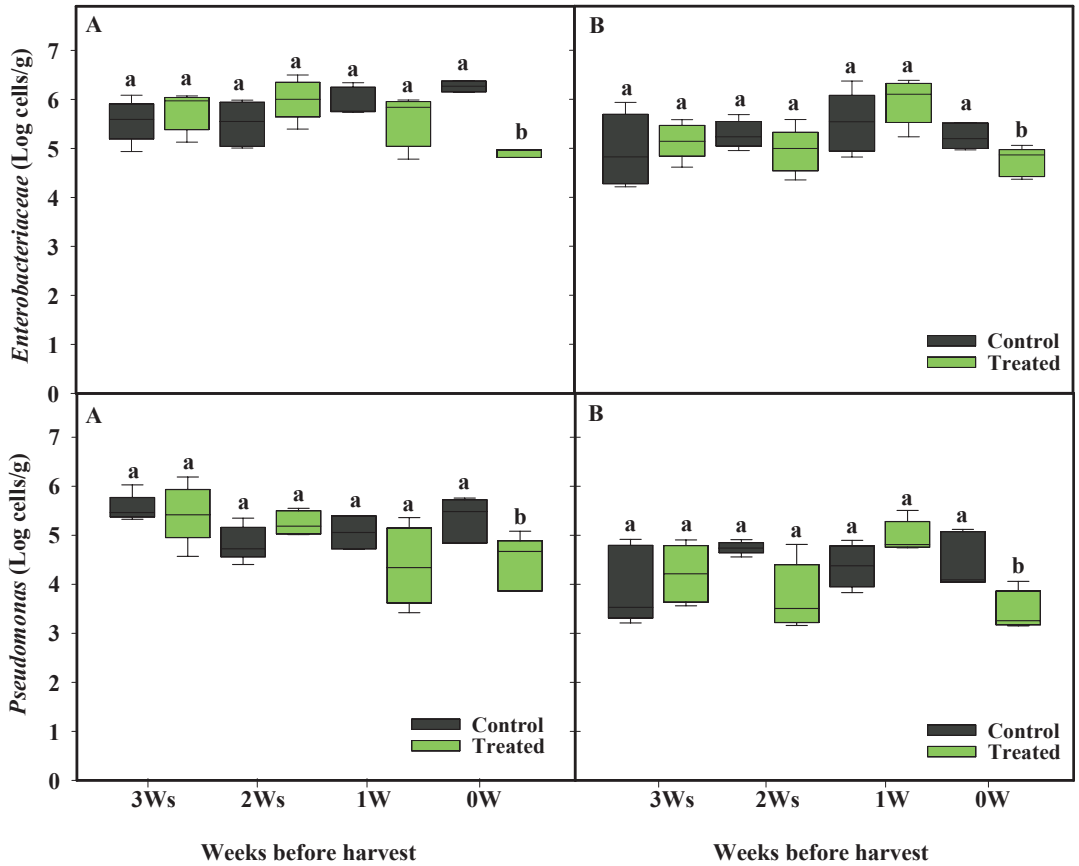

Fig. 4. Box-plot representing *Enterobacteriaceae* and *Pseudomonas* levels (Log cells/g) of baby spinach irrigated with untreated (Control) and ClO_2 treated water (Treated) during the growing period in Trial 1 (**A**; November–December) and Trial 2 (**B**; February–March). Box-plot with different letters indicate significant difference at $P < 0.05$. Bottom and top of the boxes represent the 25th and 75th percentiles. The boxplot whiskers represent the minimum and maximum values.

these differences, climatological parameters including diurnal temperature range (DTR, ºC), wind speed (m/s), solar radiation (W/m^2), RH (%) and precipitation (mm) of each trial were acquired. Significant differences between the two trials were observed for wind speed, solar radiation, and RH, while similar values were detected for DTR in both trials (Table 2). No significance correlation was establish for the precipitation rate, mostly because of the low rainfall rate during the two trials. When Spearman correlation between weather conditions and total bacterial, *Enterobacteriaceae* and *Pseudomonas* levels of baby spinach were calculated, significant differences were observed (Table 3). The level of *Enterobacteriaceae* and *Pseudomonas* were negatively correlated with wind speed, suggesting that wind speed may lower the level of these families on the baby spinach. Although few studies have evaluated the association between wind speed and epiphytic bacteria. The influence of the wind speed on the bacterial community in the crops has been already reported (Hirano and Upper, 1983, 2000). A

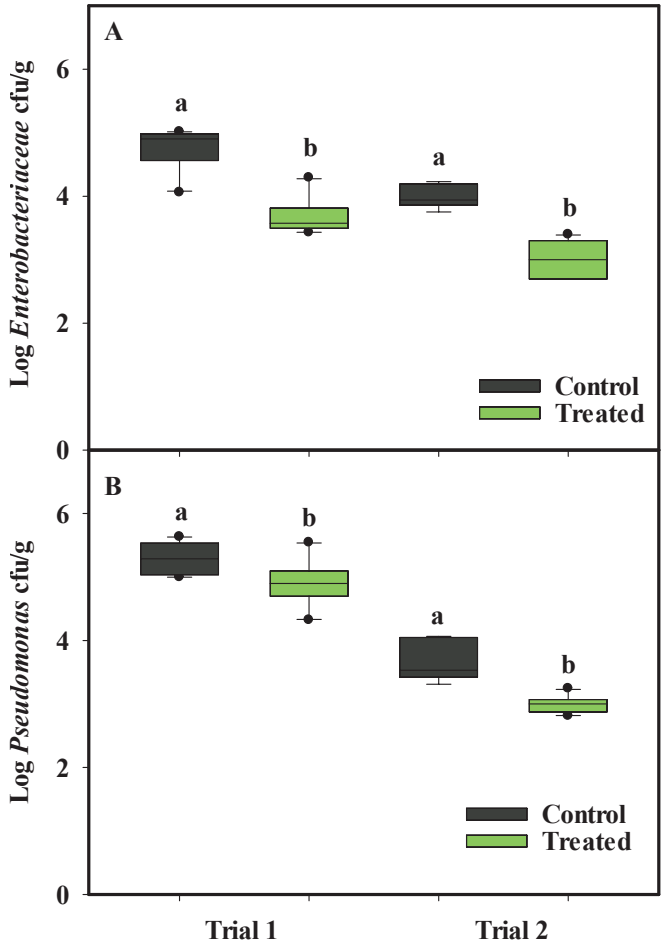

Fig. 5. Box-plot representing *Enterobacteriaceae* (A) and *Pseudomonas* (B) loads (Log cfu/g) of baby spinach irrigated with untreated (Control) and ClO_2 treated water (Treated) during the growing period in Trial 1 (November–December) and Trial 2 (February–March). Box-plot with different letters indicate significant difference at $P < 0.05$. Bottom and top of the boxes represent the 25th and 75th percentiles. The boxplot whiskers represent the minimum and maximum values.

negative significant correlation was also found between solar radiation and the levels of total bacteria and *Pseudomonas* indicating that high solar radiation reduced the levels of total bacterial and *Pseudomonas* of baby spinach. This is in accordance with previous reported studies which demonstrated that UV radiation reduces the levels of bacteria on the tissue (Heaton and Jones, 2008; Wood et al., 2010). Castro-Ibañez et al. (2015a) also noticed that the lowest level of *E. coli* and coliforms of baby spinach were detected at the highest solar radiation. On the other hand, several studies, carried out in open field as well as under protected cultivation, reported that levels of microorganisms in plants grown in shaded areas were higher than those grown under full solar exposure (Dreux et al., 2007; Wood et al., 2010; Wei et al., 2016). Recently, Truchado et al. (2017) observed that on the phyllosphere of pigmented baby lettuce solar radiation affected the relative abundance of *Gammaproteobacteria* class, which includes the *Pseudomonas* genera.

In the case of RH, a positive significant correlation was observed with total bacterial and *Pseudomonas* levels, indicating that, as for many other bacterial groups, conditions of high RH favors the growth of *Pseudomonas* in leafy greens. In agreement with our results, Medina-Martinez et al. (2015) reported that *Pseudomonas* growth showed a negative relationship with dry conditions, reduced rainfalls, and minimum temperature fluctuations during the growing period of pigmented baby lettuce. Regarding the *Enterobacteriaceae* family, no significant correlations were found with solar radiation and HR (Table 3). Castro-Ibañez et al. (2015b) reported that low coliform levels were usually associated with low RH environments and vice versa. However, further studies are needed to clarify the effect of solar radiation and RH on the *Enterobacteriaceae* family present in the phyllosphere. Based on these results, it could be concluded that the differences found in the levels of bacterial population present in baby spinach between the two trials could be partially attributed to differences in weather conditions during the two growing periods.

On the other hand, levels of total bacteria, *Enterobacteriaceae* and *Pseudomonas* were enumerated using qPCR, no significant differences were observed between both growing cycles (Fig. 2). For total bacterial, the average level per gram of baby spinach slightly increased when increasing leaf age from 7.1 ± 0.3 to 7.2 ± 0.3 Log cells/g in Trial 1. However, in Trial 2, the level of total bacterial populations decreased when increasing leaf age from 6.7 ± 0.3 to 5.7 ± 0.3 Log cells/g plant (Fig. 2A). *Enterobacteriacea*e levels showed a similar tendency in the two trials with similar levels at the beginning and at the end of the growing period (Fig. 2B). In the case of *Pseudomonas* (Fig. 2C), average levels from the less mature leave to the more mature one were about 5.6 ± 0.4 to up to 6.3 ± 0.7 log cells/g in Trial 1. A similar tendency was observed in Trial 2, where counts increased from 4.6 ± 0.6 log cells/g to 5.2 ± 0.7 log cells/g as the plants grown. In accordance with our results, Tydings et al. (2011) observed that bacterial community present in the cotyledons of spinach plants was similar to those of young leaves. Medina-Martinez et al. (2015) did not find any correlations between growing stage and the levels of mesophilic aerobic bacteria, *Pseudomonas* and coliforms on pigmented baby lettuce. In addition to that, previous studies have reported that the impact of leaf age on the phyllosphere communities is not fully understood (William et al., 2013; Dees et al., 2015).

3.3. Impact of irrigation with ClO_2 treated water on the major bacterial species of baby spinach

The impact of ClO_2 treated water on the levels of total bacteria and the main genera of phyllosphere-associated bacterial community was evaluated. Fig. 3 shows the changes in the size of total bacterial populations detected in baby spinach irrigated with untreated and ClO_2 treated water during cultivation. As it can be observed, ClO_2 treated water did not affect the total bacteria population of baby spinach and only slight changes were observed between treated and untreated samples.

Regarding *Enterobacteriaceae* and *Pseudomonas*, no significant differences were observed during cultivation in Trial 1 (Fig. 4). However, significant differences in *Enterobacteriaceae* and *Pseudomonas* levels were observed between baby spinach irrigated with treated and untreated water at commercial maturity stage (Fig. 4). Results indicated that baby spinach irrigated with ClO_2 treated water showed significantly reduced levels of *Enterobacteriaceae* (1.2- and 1.3-folds in Trial 1 and Trial 2, respectively) and *Pseudomonas* (1.2- and 1.1-folds in Trial 1 and Trial 2, respectively) when compared with the untreated one. To validate the qPCR findings, we also analyzed the levels of *Pseudomonas* and *Enterobacteriaceae* using plate count at commercial maturity stage (Fig. 5). The obtained results also showed a reduction in the levels of these epiphytic bacterial genera. Regarding food quality, *Pseudomonas* cause a breakdown of the peptic polymers in plant cells and able to grow at refrigeration conditions after processing (Membré and Burlot, 1994; Conte et al., 2008). Concerning food safety, human pathogenic bacteria in leafy greens have been associated to members of the family *Enterobacteriaceae* (Brandl, 2006; Teplitski et al., 2011). Therefore, a reduction of these two genera (*Enterobacteriaceae* and *Pseudomonas*) could be interesting to reduce spoilage and prevent safety problems associated with these genera.

4. Conclusions

The present study shows the effect of some factors including weather conditions, plant age and chlorine dioxide disinfected water for irrigation on the levels of total bacteria, *Enterobacteriaceae* and *Pseudomonas* during cultivation of baby spinach. Solar radiation and RH affected negatively and positively, respectively the level of the total bacterial and *Pseudomonas* populations of baby spinach. Regarding agricultural practices, the use of ClO_2 treated irrigation water was able to reduce the presence of *Enterobacteriaceae* and *Pseudomonas*, in commercial baby spinach, independently of the weather conditions. This is a remarkable finding because a large proportion of foodborne and pathogenic bacteria associated to fresh produce belong to the *Enterobacteriaceae* genera and *Pseudomonas* family, respectively. Therefore, the reduction of these bacterial groups might have a beneficial impact in the microbial quality and safety of leafy greens.

Acknowledgments

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