Universidade Federal do Rio Grande do Sul
Instituto de Ciências e Tecnologia de Alimentos
Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos (PPGCTA)

TESE DE DOUTORADO

Luana Tombini Decol

QUALIDADE MICROBIOLÓGICA DA ÁGUA DE IRRIGAÇÃO E SEU IMPACTO SOBRE A SEGURANÇA NA PRODUÇÃO DE ALFACES

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RESUMO

Estudos recentes têm demonstrado o risco de contaminação microbiológica em frutas e vegetais irrigados com água não tratada, a qual é a mais utilizada na agricultura, tanto em nível mundial, quanto no Brasil. O objetivo da primeira parte deste trabalho foi avaliar a qualidade microbiológica das fontes de água superficiais de irrigação mais utilizadas no sul do Brasil e seu impacto sobre a segurança de alfaces. Para tanto, foram realizadas coletas mensais de água de irrigação e alfaces irrigadas de julho de 2014 a agosto de 2015, em quatro propriedades da região metropolitana de Porto Alegre. Na água de irrigação, foi verificada prevalência de 100% dos indicadores Coliformes Totais e *Enterococcus* spp., e de 84,8% do indicador *E. coli* genérica. Já para as amostras de alface, verificou-se também a prevalência de 100% para Coliformes Fecais e 38,3% para *E. coli* Genérica. Não houve diferença significativa entre os níveis dos indicadores para as diferentes fontes de água avaliadas. As amostras com contagens de *E. coli* acima de 100 UFC/100mL foram submetidas à análise de presença de patógenos entéricos. *E. coli* O157:H7 foi identificada em 13 das 64 amostras analisadas, sendo nove amostras de açude e quatro amostras de riachos. *Salmonella* spp. foi identificada em seis das 64 amostras, das quais quatro foram amostras de açude e duas de riachos. *Salmonella* spp. foi identificada em 4 das 27 amostras avaliadas de alface. As altas contagens de *Enterococcus* spp. e *E. coli* genérica apresentaram uma correlação positiva com a presença de *Salmonella* spp. Foi verificada a influência de fatores climáticos e práticas agrícolas sobre os níveis de contaminação na alface. Na busca por fontes alternativas de água para utilização na agricultura, a água residual urbana vem sendo considerada uma boa opção. Entretanto, este tipo de água geralmente possui uma alta carga de contaminação microbiana. Em um segundo momento, foi avaliado a eficácia da aplicação de dióxido de cloro (ClO_2) no tratamento de águas residuais urbanas com tratamento secundário. Verificou-se que o desinfetante foi capaz de melhorar a qualidade microbiológica da água, reduzindo significativamente o indicador *E. coli* quantificado pelo método tradicional em placas. Entretanto, esta diferença não foi observada quando a *E. coli* foi quantificada pelo método PMA-qPCR, indicando que o tratamento de água residual com ClO_2 pode induzir as bactérias a entrar no estado viável mas não cultivável (VNC). A proporção de amostras positivas para a presença de patógenos foi baixa quando comparada à água sem tratamento (7 em 8) e com tratamento com ClO_2 (1 em 8). Apesar dos bons resultados na redução da contaminação, foi observado um acúmulo significativo de cloratos nas amostras de alface, o que pode apresentar risco químico ao consumidor. Uma vez que foi identificada a presença de *E. coli* O157:H7 em fontes de água de irrigação no sul do Brasil, objetivou-se, em um terceiro momento do presente trabalho, avaliar a capacidade de adaptação e multiplicação de quatro isolados e um *pool* de *E. coli* O157:H7 quando inoculados em água de açude filtrada, a 26°C, por até 72 h. Os isolados identificados como Ec1(açude) e Ec2 (riacho) apresentaram diferenças significativas nos parâmetros de taxa máxima de crescimento e fase Lag. Os isolados também foram testados quanto ao comportamento frente ao desinfetante hipoclorito de sódio aplicado em água de irrigação filtrada, nas concentrações de 5 mg/L e 7 mg/L de cloro residual. Quando avaliada a redução pelo método tradicional em placas foi verificado uma redução de 100% dos isolados, após 30 min de contato com a água de irrigação tratada com o

desinfetante a 7 mg/L, com uma concentração de 5 mg/L foram identificadas redução média de 3.15 (± 0.02) Log cfu / mL após 30 min. Já pelo método de quantificação por PMA-qPCR, não foi observada redução significativa entre as concentrações testadas, indicando um possível efeito bacteriostático do hipoclorito de sódio quando utilizado nas condições testadas. A partir dos resultados obtidos na presente tese, observa-se um alto risco de contaminação de vegetais irrigados por águas superficiais, sendo necessária a adoção de medidas de controle, em nível de produção primária.

Palavras-chaves: Irrigação; Hortaliças folhosas; Micro-organismos indicadores; Patógenos bacterianos; Produção primária; Desinfetante; PMA-qPCR.

ABSTRACT

Recent studies indicated microbial contamination risk in fruits and vegetables irrigated with untreated water, which is the most common source of irrigation water worldwide and in Brazil. The objective of the first part of this work was to evaluate microbial quality of superficial irrigation water sources used in Southern Brazil and its impact on lettuce safety. Samples of irrigation water and lettuces were taken once a month, from July 2014 to August 2015, from four rural farms at the metropolitan region of Porto Alegre city. For irrigation water, 100% prevalence of the indicators total coliforms and *Enterococcus* spp., and 84.8% for the indicator generic *E. coli* was determined. For lettuce samples, it was determined 100% prevalence of total coliforms and 38.3% of generic *E. coli.* There was no significant difference among the indicator levels for the different water sources evaluated. For samples with more than 100 cfu/100mL of generic *E. coli*, the presence of enteric pathogens was checked. *E. coli* O157:H7 was detected in 13 from 64 analyzed samples, 9 in pounds and 4 in streams. *Salmonella* spp. was found in 6 from 64 samples, 4 in pond and 2 in streams. On lettuce samples, *Salmonella* spp. was in 4 from 27 samples evaluated. High *Enterococcus* spp. and *E. coli* counts were positively related to *Salmonella* presence. It was verified the influence of climatic factors and agricultural practices on lettuce contamination levels. Urban residual water is considered a good alternative for using in agriculture. However, this kind of water usually carries a high level of microbial contamination. So, the efficacy of chlorine dioxide (ClO_2) in municipal wastewater with a secondary treatment was evaluated. Results show that the disinfectant improved water microbial quality, with significant reduction of generic *E. coli* when measured by the traditional plate count method. However, the same was not observed when generic *E. coli* was measured by PMA-qPCR method, which indicates that ClO_2 treatment in residual water can induce bacteria to stay viable but not cultivable state (VBNC). The rate of positive samples for the presence of pathogens was low when waters before treatment (7 in 8) and after ClO_2 treatment (1 in 8) were compared. Despite good results in water decontamination, significant chlorate accumulation was observed on lettuce samples, what may represent a chemical risk to the consumers. Once identified the presence of *E. coli* O157:H7 in irrigation water in Southern Brazil, the adaptation and multiplication capacity of 4 strains and a pool of *E. coli* O157:H7 was evaluated when inoculated in sterile irrigation water at 26°C up to 72 h. The isolates Ec1 (from pond) and Ec4 (from stream) significantly differed for the maximum growth rate and lag phase. These isolates were also tested for their behavior against sodium hypochlorite applied in sterile irrigation water at concentrations of 5 mg/L and 7 mg/L of residual chlorine. Reduction of 100% from the isolates after 30 min in contact with the disinfectant at 7mg/L was observed. The concentration of 5 mg / L, caused a mean reduction of 3.15 (± 0.02) Log cfu / mL after 30 min by the plate count method. However, there was no significant reduction among the tested concentrations by using the PMA-qPCR method, indicating a possible bacteriostatic effect of sodium hypochlorite under the tested conditions. The

results obtained in this work shows high contamination risk of vegetables when irrigated by superficial waters and highlight the need of control measures in primary production.

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1. INTRODUÇÃO

O consumo de vegetais folhosos, como as alfaces, vem aumentando, assim como tem aumentado o envolvimento desses produtos com Doenças Transmitidas por Alimentos (DTA) em diversas partes do mundo (FDA, 2012; CDC, 2016 EFSA, 2017). Por exemplo, de 2007 a 2016, 1,4 % dos alimentos identificados em surtos de DTA, no Brasil, foram frutas, produtos de frutas e similares, e hortaliças (ANVISA, 2016). Na Europa, frutas e vegetais foram associadas a 10 % dos surtos notificados (EFSA, 2015) sendo os vegetais folhosos os produtos frescos mais frequentemente associados a surtos com *Salmonella* spp e *Escherichia coli* O157:H7 (*E. coli* O157:H7). Nos Estados Unidos, 16 % dos surtos ocorridos em 2014, foram associados a vegetais e frutas contaminados, e destes 3,36 % envolveram vegetais verdes folhosos (CDC, 2016).

A contaminação microbiológica dos vegetais pode ocorrer em várias etapas da cadeia produtiva. Dentre as principais fontes de contaminação estão o solo, fertilizantes, água de lavagem e água de irrigação (JOHANNESSEN et al., 2002; INGHAM et al., 2005; PARK et al., 2014; CASTRO-IBÁÑEZ et al., 2015a). A água de irrigação tem sido destacada em diversas partes do mundo como um importante veículo de patógenos entéricos para a produção primária de vegetais folhosos (PARK et al., 2014; RODRIGUES et al., 2014; PACHEPSKY et al., 2011; GIL et al., 2015).

Estudos recentes têm demonstrado o risco de contaminação microbiana em frutas e vegetais nas etapas de pré-colheita devido à água de irrigação sem tratamento, a qual ainda é a fonte de água mais utilizada para este fim (GORSKI, et al., 2011; RODRIGUES et al., 2014; EFSA, 2014; CASTRO-IBÁÑEZ et al., 2015b). Estudos demonstraram ainda, que a água de irrigação

sem tratamento, aumentando significativamente a ocorrência de patógenos nestes alimentos (CAI et al., 1988; MELLOUL et al., 2001; MANAS et al., 2009).

Dentre as principais fontes de água utilizadas para a irrigação, pode-se destacar os poços artesianos, águas de chuva armazenadas e protegidas, rios e açudes, sendo esta ordem a de menor para maior risco de contaminação (FERGUSON et al., 2012; AHMED et al., 2012; CASTRO-IBANEZ et al., 2015b). Outro tipo de água que vem aumentando a sua utilização na agricultura, principalmente em regiões semiáridas, é a água residual urbana e/ou industrial tratada (E.P.H.C. 2006; BECERRA-CASTRO et al., 2015). Tal tipo de água, quando não tratada adequadamente e utilizada para irrigação na agricultura, pode apresentar alto risco à saúde aos trabalhadores do campo e consumidores (PEDRERO et al., 2010; UNESCO-WWAP, 2017). Águas residuais geralmente contêm bactérias entéricas patogênicas, vírus e parasitas entéricos (WHO, 2006). Portanto, é necessário um tratamento de desinfecção eficaz para assegurar a qualidade microbiológica da mesma (WHO, 2006).

Para reduzir o risco na utilização destas fontes de águas, pode-se utilizar inúmeros métodos de desinfecção, sendo a desinfecção por agentes químicos derivados do cloro as mais utilizadas globalmente (HUANG et al., 2008; WARRINER et al., 2009; VAN HAUTE et al., 2015). O uso frequente de compostos liberadores de cloro pode ser atribuído ao seu menor custo, grande eficiência na ação desinfetante e também disponibilidade residual para continuar agindo no sistema de distribuição, após a aplicação na água (SUSLOW et al., 2001; TOMAS-CALLEJAS et al., 2012).

Apesar do alto poder bactericida destacado em diversos estudos, os desinfetantes químicos liberadores de cloro podem induzir as bactérias a um

estado conhecido como viável mas não cultivável (VNC) (OLIVER et al., 2005; KIBBEE AND ÖRMECI, 2017). O estado VNC permite que bactérias permaneçam viáveis na água, mas não sejam detectadas por métodos convencionais de análise, uma vez que não se multiplicam em meios de cultura e não são, consequentemente, detectadas, podendo ocorrer uma subestimativa da real contaminação (GENSBERGER et al., 2014; TRUCHADO et al., 2016). Uma vez no alimento, essas bactérias podem se recuperar e ativar seu metabolismo novamente, podendo se multiplicar e causar surtos alimentares.

Atualmente, o uso de técnicas moleculares para quantificar micro-organismos indicadores de contaminação fecal, como a *E. coli* genérica em amostras ambientais, tem sido bem aceito e utilizado, devido a sua maior sensibilidade, rapidez e especificidade quando comparadas as técnicas tradicionais (AHMED et al., 2012; FERGUSON et al., 2012; TRUCHADO et al., 2016). Um exemplo disso é a técnica de PCR em tempo real aliada a um pré-tratamento da amostra com Propidium Monoazida (PMA). Este pré-tratamento permite que somente os micro-organismos viáveis, mesmo em estado VNC, sejam quantificados (TRUCHADO et al., 2016; RUDI et al., 2005; VARMA et al., 2009).

Neste contexto, em um primeiro momento, o presente estudo avaliou a qualidade microbiológica da água de irrigação e seu impacto sobre a segurança na produção de alfaces. Baseados no grande potencial existente para a utilização de águas residuais urbanas e/ou industriais na agricultura, foi avaliada a eficiência do desinfetante dióxido de cloro (ClO_2) aplicado em águas residuais urbanas, para a redução de contaminação microbiológica. Visando a utilização do mesmo para irrigação por aspersão de alfaces do tipo *baby*, no sul

da Espanha. A partir da constatação da presença do patógeno *E. coli* O157:H7 nas fontes de água de irrigação. Objetivou-se investigar a capacidade de multiplicação e sobrevivência de isolados de *E. coli* O157:H7, provenientes de fontes distintas de água de irrigação, a soluções de hipoclorito de sódio aplicado a água de açude, visando uma possível forma de controle desse patógeno.

2. OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo do presente estudo foi avaliar a qualidade microbiológica da água de irrigação, a fim de determinar o seu impacto sobre a segurança na produção de alfaces.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar a qualidade microbiológica da água de irrigação de diferentes fontes superficiais e seu impacto sobre a segurança na produção de alfaces irrigadas por estas fontes;

- Investigar a correlação entre a quantidade de micro-organismos indicadores (Coliformes Totais, *Enterococcus* spp. e *E. coli* genérica) e a presença ou ausência de *E. coli* O157:H7 e *Salmonella* spp. em água de irrigação e alfaces;

- Avaliar a influência de fatores climáticos e ambientais na contaminação de água de irrigação e alfaces;

- Avaliar a adequação do tratamento com dióxido de cloro (ClO_2) em água residual urbana utilizada para irrigação por aspersão, através da redução de *E. coli* genérica, quantificada por método convencional e molecular, e dos patógenos entéricos *Salmonella* spp. e *E. coli,* produtoras de shiga-toxina;

- Avaliar a capacidade de multiplicação de isolados de *E. coli* O157:H7 em água de irrigação;

- Avaliar o efeito bactericida ou bacteriostático do hipoclorito de sódio, pelos métodos convencional e molecular de quantificação, utilizando baixas concentrações, aplicado em água de irrigação frente a isolados de *E. coli* O157:H7.

3. REVISÃO BIBLIOGRÁFICA

3.1 CULTURA DA ALFACE

A alface *(Lactuca sativa* L.) é uma planta anual, originária de clima temperado, pertencente à família *Asteraceae*, certamente uma das hortaliças mais populares e consumidas no Brasil e no mundo (EMBRAPA, 2009).

Atualmente, no mercado brasileiro, há cinco principais tipos de alface, a Americana, Crespa, Lisa, Mimosa e Romana, estes tipos se diferenciam pelas folhas e na formação da cabeça (HENZ et al., 2009; SALA et al., 2012).

Segundo a Confederação da Agricultura e Pecuária do Brasil (APB, 2017) e Associação Brasileira do Comércio de Sementes e Mudas (2018), a cadeia produtiva de hortaliças movimenta, no país, cerca de R$ 55 bilhões ao ano, com uma área de 820.000 hectares destinados à produção. Cerca de 18 hortaliças diferentes são produzidas por ano no Brasil, dentre eles estão o tomate, cebola, melancia e alface, as quais são responsáveis por 50% desse total. No Brasil, em 2016, a alface apresentou uma produtividade de 18,6 t/ha e ocupou uma área de produção total de 91.172 ha (APB, 2017).

No Brasil, o cultivo da alface, na sua maioria, tem sido em campo aberto (SALA et al., 2012), entretanto, há pelo menos quatro sistemas produtivos: o cultivo convencional e o orgânico, ambos em campo aberto; o cultivo no sistema hidropônico e no solo com proteção, tendo suas diferenças, tanto no manejo da cultura como na pós-colheita (HENZ et al., 2009).

O cultivo no sistema tradicional é o mais importante em termos de área e de produção, concentrando-se geralmente perto dos grandes centros urbanos (FILGUEIRA, 2005). Ao contrário dos sistemas de produção americano e europeu, que contam com sistema de distribuição com cadeia de

frio, o modelo brasileiro baseia-se na produção de alface em “cinturões verdes”, localizados próximos aos centros consumidores desta folhosa, a fim de facilitar o escoamento do produto. Uma vez que o sistema de frio é praticamente inexistente na maioria dos pequenos produtores brasileiros (BARTZ et al., 2015; SALA & COSTA, 2012) os produtores, colhem as hortaliças nas primeiras horas da manhã ou ao fim do dia, quando a temperatura ambiente é menor. As hortaliças colhidas são acomodadas à sombra de árvores, galpões e/ou caminhões sem refrigeração. A distribuição ocorre, dependendo da região, às primeiras horas da manhã seguinte (MORETTI & MATTOS 2005; EMBRAPA 2007; SALA & COSTA 2012; SUINAGA et al., 2013).

A cadeia de frio no pós-colheita de hortaliças contribui para diminuir as perdas na qualidade, sendo que isto ocorre devido a três fatores: 1) redução da atividade biológica do vegetal; 2) redução da perda de água do vegetal e 3) diminuição da atividade de micro-organismos deteriorantes (EMBRAPA, 2007). Além disso, a manutenção de hortaliças em baixas temperaturas contribui diretamente na segurança, uma vez que impede a multiplicação de micro-organismos patogênicos que podem ser introduzidos na plantação e se manter no produto final, devido a falhas nas Boas Práticas Agrícolas (BPA) (BARTZ et al., 2015; FAO/WHO, 2008).

3.2 PRINCIPAIS FONTES DE CONTAMINAÇÃO NA PRODUÇÃO DE VEGETAIS FRESCOS

Estudos demonstram a importância do controle na produção primária dos vegetais frescos, tendo em vista que a água de irrigação, água de lavagem, solo e adubos podem ser importantes fontes de contaminação

microbiológica (HOLVOET et al., 2014; RODRIGUES et al., 2014). Em vista disso, é importante conhecer a procedência e a qualidade da água de irrigação e água de lavagem, se as sementes são certificadas, o tipo de adubo utilizado, bem como o processamento, manipulação, armazenamento e transporte desses produtos (SOON et al., 2012).

3.2.1 ÁGUA DE IRRIGAÇÃO

De acordo com a UNESCO-WWAP (2017), a agricultura é a atividade que mais utiliza água, correspondendo a 70% do consumo mundial. As águas utilizadas para irrigação podem ser advindas de fontes, como água potável de tratamento urbano, águas superficiais, águas subterrâneas ou águas residuais urbanas e industriais tratadas (E.P.H.C. 2006; FERGUSON et al., 2012; AHMED et al., 2012; CASTRO-IBANEZ et al., 2015b).

Por ser tratada, a água municipal é a de melhor qualidade, mas está disponível apenas em algumas regiões desenvolvidas e possui custo mais elevado. Em seguida, com relação a qualidade microbiológica, estão as águas subterrâneas, água da chuva e águas de superfície (UYTTENDAELE et al., 2015). Por sua qualidade aceitável e baixo custo, estas fontes estão sendo cada vez mais utilizadas para irrigação (SUSLOW et al., 2003; JAMES 2006; MAROUELLI; SILVA 2015). No entanto, a qualidade e a sustentabilidade dos reservatórios estão ameaçados em algumas regiões. Isso resulta da degradação e poluição de rios, destruição de zonas úmidas e contaminação química e microbiológica da água (REID et al., 2003).

Por fim, as águas residuais urbanas e industriais tratadas vem aumentando a sua utilização na agricultura, principalmente em regiões áridas e

semiáridas que apresentam escassez de água (E.P.H.C. 2006; BECERRA-CASTRO et al., 2015; UNESCO-WWAP, 2017).

A utilização deste tipo de água vem sendo fortemente incentivada, uma vez que as mudanças climáticas, o aumento da população e produção agrícola estão impactando nas fontes de água doce no mundo (UNESCO-WWAP, 2017). Se adequadamente tratada e aplicada de forma segura, as fontes de água doce são uma valiosa fonte hídrica e de nutrientes para a agricultura (E.P.H.C. 2006; BECERRA-CASTRO et al., 2015; UNESCO-WWAP, 2017).

A água quando contaminada por efluentes não tratados, principalmente esgoto doméstico, é um dos principais meios de transmissão e disseminação de doenças ao homem, e essas doenças podem ser causadas por protozoários, helmintos, vírus, fungos e bactérias (MAROUELLI et al., 2011). No caso de uso da água para irrigação, uma água de baixa qualidade pode acarretar na contaminação dos alimentos irrigados, comprometendo a qualidade do produto e, principalmente, a saúde humana, uma vez que hortaliças e frutas, especialmente aquelas consumidas cruas, podem servir de veículo de transmissão de uma série de doenças aos consumidores (ALLENDE & MONAGHAN, 2015; UYTTENDAELE et al., 2015).

A presença de micro-organismos patogênicos, tais como a *E. coli* 0157:H7 e *Salmonella* spp., em hortaliças irrigadas com água contaminada tem sido crescente, e, por essa razão, a avaliação da água de irrigação adquire grande importância (AHMED et al., 2012; FERGUSON et al., 2012; OLIVEIRA et al., 2012; CEUPPENS et al., 2014; ALLENDE e MONAGHAN, 2015; UYTTENDAELE et al., 2015).

No Brasil, a resolução n.º 20/86, do Conselho Nacional do Meio Ambiente (CONAMA, 1986), Art. 26, estabelece que “as águas utilizadas para a irrigação de hortaliças ou plantas frutíferas que se desenvolvam rentes ao solo e que são consumidas cruas, sem remoção de casca ou película, não devem estar poluídas por excrementos humanos, ressaltando-se a necessidade de inspeções sanitárias periódicas”. Cabe ressaltar, que conforme estudos já descritos, a água de irrigação é uma importante fonte de contaminação no cultivo de hortaliças.

Na Europa, está sendo elaborado um documento centrado na orientação de implementação de requisitos de higiene na produção primária de frutas e hortaliças. Neste guia, a água de irrigação é classificada com base no risco microbiológico e nos limites recomendados para *E. coli*, que é considerado o melhor indicador de contaminação de origem fecal, por diversos estudos e centros de referência (PACHEPSKY et al., 2011; FERGUSON et al., 2012; GAYEON et al., 2013; PAHL et al. 2013; RODRIGUES et al., 2014; ALLENDE & MONAGHAN, 2015; CASTRO-IBANEZ et al., 2015a; UYTTENDAELE et al., 2015; AHDB, 2016; DECOL et al., 2017). Baseados nestes guias e recomendações, a Comunidade Europeia, vem projetando estratégias de prevenção e controle de risco na produção de vegetais consumidos crus (ALLENDE & MONAGHAN, 2015; PARKER et al., 2012).

3.2.2 SISTEMAS DE IRRIGAÇÃO

Diferentes sistemas de irrigação podem ser utilizados na produção de hortaliças. Todos os sistemas apresentam características distintas, com custos variáveis, vantagens e desvantagens.

A agricultura utiliza diferentes métodos de irrigação, sendo os sistemas de irrigação superficial e por aspersão os mais utilizados no Brasil (MOROUELLI et al., 2011). A escolha do tipo de sistema dependerá do tipo de solo, clima, cultura, disponibilidade de energia e condições socioeconômicas a que ele se destina (EMBRAPA, 2008; MOROUELLI et al., 2011).

3.2.3 IRRIGAÇÃO SUPERFICIAL

Também conhecido como método de irrigação por gotejamento, este método compreende os sistemas por sulco, faixa, corrugação e inundação, nos quais a condução e a distribuição da água são realizadas diretamente sobre o solo, onde se encontram as raízes das plantas (EMBRAPA, 2004; PACHEPSKY et al., 2011). Este método requer um maior investimento inicial e menor uso de energia e tem sido utilizado em hortaliças que requerem irrigação frequentes como ervilha, pimentão e tomate (EMBRAPA, 2004).

Na irrigação por gotejamento, o risco de transferência de patógenos para plantas é minimizado, devido a menor exposição da água irrigada com o produto e redução de respingos em solos contaminados. Este método certamente proporciona maior grau de proteção da saúde dos trabalhadores rurais e consumidores, especialmente quando os métodos são automatizados (HAMILTON et al., 2006; SONG et al., 2006; CEVALLOS-CEVALLOS et al., 2012).

3.2.4 IRRIGAÇÃO POR ASPERSÃO

A irrigação por aspersão caracteriza-se pela pulverização do jato de água no ar, visando o umedecimento da área superficial da planta. Existe uma

série de modelos de aspersores, os quais podem ser convencionais, portáteis, semi-portáteis e fixos (EMBRAPA, 2004: PACHEPSKY et al., 2011). Este sistema requer menor uso de mão-de-obra e possibilita a melhor distribuição de água sobre a superfície do solo. O sistema de aspersão convencional é o mais utilizado na produção de hortaliças brasileiras, especialmente em pequenas áreas de produção (EMBRAPA, 2004).

Na irrigação por aspersão há uma maior contaminação de culturas, pois a parte comestível do vegetal é exposta diretamente à água (PACHEPSKY et al., 2011). As gotas em aerossol da água, quando contaminadas por patógenos, podem contaminar a hortaliças, o solo e serem transportados pelo vento, criando um risco à saúde para os trabalhadores e residentes nas proximidades da área irrigada (MARITES et al., 2010; BARKER-REID et al., 2009).

Assim a irrigação por aspersão é melhor aplicada nas fases iniciais do crescimento da planta, maximizando a oportunidade da morte do patógeno por fatores ambientais, até a data de colheita (UYTTENDAELE et al., 2015). Os fatores chaves que podem acarretar a morte bacteriana, quando os mesmos estão presentes na superfície de folhas, são: exposição a raios ultravioleta (UV), baixa umidade, altas temperaturas e intensidade dos ventos sobre às folhas (WOOD et al., 2010; PACHEPSKY et al., 2011; OLIVEIRA 2012; GU et al., 2013; UYTTENDAELE et al., 2015).

Estudos têm demonstrado que, ao utilizar uma fonte de água não controlada, a realização da irrigação por aspersão, próxima ao período de colheita, representa um maior risco a segurança dos vegetais (PARK et al., 2013; PARK et al., 2014; UYTTENDAELE et al., 2015; DECOL et al., 2017). De

acordo com PARK et al. (2013), quando o tempo desde a última irrigação até a colheita foi maior que cinco dias, a redução da contaminação foi de uma a quatro vezes maior do que quando foi realizada a irrigação em um intervalo de tempo menor. Com isso sugere-se que, a irrigação seja suspensa próxima a colheita para que possa assegurar a redução da contaminação e a segurança do consumidor (UYTTENDAELE et al., 2015).

3.3 DESINFECÇÃO DA ÁGUA

Sendo a água uma matéria-prima importante na cadeia da produção primária de vegetais, participando de operações como irrigação, aplicação de fertilizantes e pesticidas, higienização, entre outras atividades, a garantia da sua qualidade é de extrema importância (STEELE e ODUMERU, 2004; HOLVOET et al., 2012; VAN HAUTE et al., 2015). Quando a água utilizada é de baixa qualidade ou com tratamento ineficiente, pode se tornar um importante veículo de patógenos entéricos, que podem contaminar a produção das hortaliças (ALLENDE e MONAGHAN, 2015; UYTTENDAELE et al., 2015).

Atualmente, diferentes desinfetantes químicos estão disponíveis para a desinfecção de água, seja para consumo humano, como para agricultura. Dentre os compostos químicos mais utilizados estão o hipoclorito de sódio, dióxido de cloro, ozônio, peróxido de hidrogênio e ácido peracético. Além dos desinfetantes químicos, métodos físicos, como radiação ultravioleta (UV) e filtração, podem ser utilizados na desinfecção da água (ZIMMER & SLAWSON, 2002; SANZ et al., 2007; VAN HAUTE et al., 2015).

O tratamento de diferentes fontes de água varia conforme a sua finalidade de uso. Na maioria dos casos, o sistema de tratamento se inicia com

remoção da matéria orgânica e/ou inorgânica e partículas solúveis. Os processos são compostos por etapas físicas, como a sedimentação, floculação, filtração, entre outros, e por processos biológicos, como a oxidação biológica de ferro e manganês, por exemplo. Após estes processos, geralmente é realizada alguma etapa para inativação de micro-organismos por aplicação de desinfetantes químicos e/ou radiação UV (METCALF et al., 2007; CRITTENDEN et al., 2005). Dentre os diversos desinfetantes químicos existentes e empregados no mundo, os mais utilizados são os desinfetantes a base de cloro livre (FAO/WHO, 2008).

3.3.1 COMPOSTOS CLORADOS

A cloração da água é a técnica de desinfecção mais aplicada, e em muitos países é amplamente utilizada, tanto na água para consumo humano como na indústria de alimentos. Isso se deve ao seu baixo custo, boa eficiência contra células vegetativas de bactérias e pode ser implementado em operações de qualquer tamanho (SUSLOW, 2001; PARISH et al., 2003; EPA, 2004; TOMAS-CALLEJAS et al., 2012; VAN HAUTE et al., 2015).

O cloro é encontrado na forma de gás de cloro (Cl_2), como hipoclorito de sódio (NaOCl) ou hipoclorito de cálcio ($Ca(OCl)_2$). Estes compostos ao serem dissolvidos em água formam o ácido hipocloroso que é a sua forma mais ativa e íons hipoclorito (OCl^-) (FAO/WHO, 2008). A forma mais ativa para desinfecção, o ácido hipocloroso, ocorre predominante nos valores de pH 5 a 7. Em pH abaixo ou acima desta faixa há predominância das formas Cl_2 e OCl^-, respectivamente, sendo essas formas de menor poder oxidante e desinfetante (MEYER et al., 1994).

Além do pH da água, a concentração de matéria orgânica também influencia na eficiência do processo de desinfecção pelo cloro. O cloro reage com a matéria orgânica ficando imobilizado, diminuindo sua concentração em solução, e consequentemente sua ação desinfetante (EUROPEAN UNION, 1998; EPA, 2004; GÓMEZ-LÓPEZ, 2014). Assim, quanto maior a concentração de matéria orgânica em solução, maior será a necessidade de doses mais elevadas, a fim de manter um nível de cloro livre, disponível para inativação microbiana (GÓMEZ-LÓPEZ, 2014).

Devido à alta reatividade com a matéria orgânica que o cloro apresenta e a formação de subprodutos da desinfecção como os trihalometanos (THM), reconhecidos como possivelmente carcinogênicos para humanos (AYYILDIZ, et al., 2009; NIKOLAOU & LEKKAS, 2001; RODRIGUEZ & SERODES, 2001). Na busca por uma nova alternativa para a substituição ao cloro na desinfecção de águas, o dióxido de cloro (ClO_2) vem sendo utilizado. O ClO_2 apresenta uma menor reatividade com a matéria orgânica e apresenta uma maior capacidade de oxidação, aumentando assim seu poder bactericida (FAO/WHO, 2008; VAN HAUTE et al., 2015; HASSEMBERG et al., 2017; VAN HAUTE et al., 2017).

O dióxido de cloro (ClO_2) é um gás amarelo esverdeado à temperatura ambiente que é altamente solúvel em água, podendo ser 10 vezes mais solúvel em água que o cloro (EFSA, 2005). Este composto pode ser produzido pelas seguintes reações químicas: na mistura de uma solução de cloro com uma solução de clorito de sódio, pela acidificação de cloratos com ácido clorídrico ou sulfúrico, pela redução de cloratos em meio ácido, reagindo ácidos com cloritos e pela eletrólise, usando cloreto de sódio, clorito de sódio e água (Dychdala, 2001). Como o dióxido de cloro é instável na forma de gás, é quase

sempre formulado dissolvido em água a uma concentração de 0,5-10 g / L (FAO/WHO, 2008).

A ação bactericida do ClO_2 é reconhecida desde o início dos anos 1990. O mecanismo de ação pelo qual, o ClO_2, realiza a inativação de micro-organismos ocorre devido a uma alteração na parede celular alterando e/ou interrompendo o transporte de nutrientes através da mesma e pela capacidade de penetrar na célula bacteriana e interromper a síntese proteica da mesma, causando a morte celular (EFSA, 2005; FAO/WHO, 2008).

Como desinfetante, o ClO_2 apresenta uma vantagem quando comparado ao cloro na inativação de micro-organismos patogênicos, incluindo vírus e protozoários (VAN HAUTE et al., 2015; HASSEMBERG et al., 2017). Outra vantagem é a maior estabilidade frente a uma maior variabilidade do pH da água a ser tratada (FAO/WHO, 2008).

Além da sua maior estabilidade, o ClO_2 apresenta a formação de subprodutos da desinfecção menos halogenados, como os compostos cloritos (ClO_2^-) e cloratos (ClO_3^-) (LÓPEZ-GÁLVEZ et al., 2010; GIL et al., 2016). Atualmente, não existem evidências suficientes de que o ClO_2, assim como os cloritos e cloratos sejam carcinogênicos aos seres humanos. De acordo com OMS e a Agência de Proteção Ambiental dos Estados Unidos (EPA), estão classificados no grupo de “substâncias não classificáveis em termos de carcinogenicidade humana” (IARC, 2001; USEPA, 2009).

3.4 DTA RELACIONADOS A CONSUMO DE VEGETAIS FRESCOS

Durante o período de 2007 a 2014 foram registrados 30 surtos no Brasil envolvendo vegetais, frutas e similares. Destes, 6.67% foram associadas as

saladas cruas, 53.3% a saladas cruas mistas, 13.3% saladas cozidas, 3.3% a frutas e 23.3% o tipo de vegetal ou fruta envolvido no surto não foi registrado. Os principais micro-organismos identificados foram *E. coli* em 33.7%, *Shigella* spp. em 6.1% e *Salmonella* spp. 4.77% (GT-SINAN, 2015). A investigação e a associação dos surtos registrados a alimentos como vegetais e frutas no Brasil ainda enfrentam dificuldades técnicas e financeiras. Portanto, o cruzamento de dados entre o alimento que causou o surto e a sua origem (distribuição, produtor e outros) ainda é de difícil realização.

Em países como os Estados Unidos, por exemplo, no período de 2002 a 2011 ocorreram 230 surtos alimentares envolvendo vegetais, sendo que 10.806 pessoas adoeceram. (CSPI,2014). Um exemplo destes casos, é o recente surto que está sendo investigado em 15 estados americanos e Canadá, envolvendo o consumo de vegetais verdes folhosos contaminados com *E. coli* O157:H7. Até 12 de dezembro de 2017, 13 pessoas adoeceram e 15% delas relatam ter consumido alface do tipo romana, entretanto até o momento nenhum fornecedor ou distribuidor foi identificado como fonte de contaminação (CDC, 2018a). Outro exemplo é o ocorrido no ano de 2014, envolvendo vegetais, no estado de Columbia nos EUA, onde aproximadamente 225 pessoas ficaram doentes depois de consumirem pepino infectado por *Salmonella* Newport (CDC, 2015b).

As *E. coli* produtoras de Shiga toxina são micro-organismos que tem sido frequentemente relacionados a surtos de DTA nos Estados Unidos. Outro exemplo, ocorreu em 2014 envolvendo *Escherichia coli* O157:H7 e afetou 33 pessoas em cinco estados americanos, as quais ingeriram espinafre orgânico e salada mix de folhas verdes contaminados. Das pessoas acometidas 46%

foram hospitalizadas e duas pessoas desenvolveram Síndrome Hemolítica Urêmica (HUS) que é uma das complicações causadas pela Shiga toxina produzida por esta bactéria (CDC, 2015c).

Da mesma forma, na Europa, diversos micro-organismos patogênicos, tais como *Salmonella* spp., *E. coli* O157:H7, *Listeria monocytogenes* têm sido veiculados por vegetais, causando importantes surtos alimentares (RUBINO et al., 2011).

No ano de 2013, na União Europeia, ocorreram 5.196 casos de surtos alimentares envolvendo alimentos ou água contaminados por micro-organismos, sendo a *Salmonella* spp. responsável por 22,5% (EFSA, 2015). Um surto em particular mobilizou a União Européia em 2011, na Alemanha, brotos de feijão contaminados com uma variante de *E. coli* enteroagregativa e produtora de Shiga toxina 2 (O104:H4), acometeu 2,987 mil pessoas, destas, 1.855 desenvolveram HUS e 53 evoluíram a óbito (RKI, 2011).

Países desenvolvidos com os Estados Unidos, o Canadá e a União Européia, diferentemente do Brasil, conseguem realizar o mapeamento do surto mais rapidamente. Na maioria dos casos identificam o alimento e o micro-organismo causador do surto, bem como a origem deste alimento através do processo de rastreabilidade. Nestes países também é obrigatório a notificação para as autoridades quando há uma DTA envolvendo um paciente.

Os mecanismos de garantia de segurança e qualidade como as Boas Práticas Agrícolas (BPA), Análise de Perigos e Pontos Críticos de Controle (APPCC) e Rastreabilidade são fundamentais na cadeia de vegetais frescos e visam estabelecer medidas preventivas na produção primária (campo) e na

manipulação da hortaliça após a colheita. Garantindo assim um alimento seguro do campo até a mesa (MALDONADE, 2014; EMBRAPA, 2004).

3.5 MÉTODOS MOLECULARES DE DETECÇÃO E QUANTIFICAÇÃO

Métodos de cultivos convencionais utilizados para monitorar e quantificar a presença de micro-organismos são extremamente demorados, necessitam de mão-de-obra e não são capazes de identificar um micro-organismo quando ele se encontra no estado viável mas não cultivável, podendo levar a uma subestimação dos indicadores, ou a um resultado falso negativo da presença de patógenos (FERGUSON et al., 2012; LOFF et al., 2014).

Técnicas moleculares vem sendo desenvolvidas para tornar a quantificação de micro-organismos indicadores, como a *E. coli*, em amostras ambientais mais sensíveis, rápidas e específicas do que os métodos tradicionais de contagem em placa (AHMED et al., 2012; FERGUSON et al., 2012; PITKÄNEN et al., 2013; TRUCHADO et al., 2016).

Atualmente, a técnica de PCR em tempo real é considerada a mais adequada para detecção e confirmação de patógenos devido à sua precisão e diagnóstico precoce (PICARDEAU et al., 2014). Entretanto, quando utilizada para quantificar micro-organismos ainda não é capaz de diferenciar células viáveis de células mortas, ou resíduo de DNA de células mortas que podem persistir em amostras ambientais, ocorrendo assim uma superestimação da real concentração de indicadores (RUDI MOEN, et al., 2005; VARMA et al., 2009).

Para evitar esta superestimação da quantificação ou falsos positivos, pesquisas estão utilizando como estratégia um pré tratamento da amostra com

propídium monoazide (PMA). Este tratamento ocorre antes da extração de DNA, fazendo com que o PMA consiga se ligar ao DNA de células mortas que apresentam comprometimento da membrana celular ou possível resíduo de DNA presente. Após a ligação do PMA ao DNA, não ocorre a amplificação do mesmo durante a análise de PCR em tempo real, possibilitando assim a quantificação somente das células viáveis da amostra (NOCKER et al., 2007; NOCKER e CAMPER, 2009; VARMA et al., 2009; FITTIPALDI et al., 2012; TRUCHADO et al., 2016).

Diversos estudos já vêm comprovando o sucesso da integração do PMA com o método de reação em cadeia da polimerase (PCR) em tempo real para quantificação de *E. coli* em amostras ambientais, principalmente amostras de água (VARMA et al., 2009; VAN FRANKENHUYZEN et al., 2013; GENSBERGER et al., 2014; TRUCHADO et al., 2016). Possibilitando assim quantificar mais rapidamente e eficientemente indicadores de contaminação fecal como por exemplo a *E. coli* genérica, (TRUCHADO et al., 2016).

Para a investigação de patógenos entéricos que podem estar presentes em amostras ambientais, tanto na água como em produtos frescos, diversas técnicas alternativas as técnicas convencionais vêm sendo estudadas e desenvolvidas. A exemplo destas técnicas podemos citar a técnica de reação em cadeia da polimerase (PCR) convencional ou em tempo real, e amplificação isotérmica mediada por loop (LAMP) que combinam amplificação de DNA isotérmico com detecção de bioluminescência, como a análise desenvolvida pela 3M, Molecular Detection System (MDS®) (CROWLEY et al., 2012; FERGUSON et al., 2012; LOFF et al., 2014).

O advento dos métodos de identificação de micro-organismos patogênicos por métodos moleculares permitiu o rápido e preciso diagnóstico clínico, análise de segurança dos alimentos e desenvolvimento de estudos epidemiológicos moleculares de doenças de origem alimentar (YANG et al., 2013).

4. RESULTADOS

Os resultados da presente Tese são apresentados na forma de quatro artigos científicos, os quais são:

ARTIGO 1. *Microbial Quality of Irrigation Water used in Leafy Green Production in Southern Brazil and its Relationship with Produce Safety*

Publicado na revista científica internacional *Food Microbiology*, v.65, pg.105 - 113, 2017.

ARTIGO 2. *Correlation between indicator microorganisms and prevalence of Salmonella spp. in different irrigation water sources and irrigated lettuces in Southern of Brazil*

Será submetido a revista científica internacional *Food Microbiology.*

ARTIGO 3. *Suitability of reclaimed wastewater disinfected with chlorine dioxide for sprinkler irrigation of baby lettuce*

Foi submetido a revista científica internacional *Food Control.*

ARTIGO 4. *E. coli* O157:H7 *multiplication and resistance to chlorine applied in irrigation water*

Será submetido a revista científica internacional *Food Microbiology.*

4.1 Artigo 1

Microbial quality of irrigation water used in leafy green production in Southern Brazil and its relationship with produce safety

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ABSTRACT

Irrigation water has been recognized as an important microbial risk factor for fruits and vegetables in many production areas, but there is still a lack of information about how the microbiological quality of different irrigation water sources and climatic conditions influence the safety of vegetables produced in Brazil. This study evaluated the distribution of generic E. coli and the prevalence of *E. coli* O157:H7 in two different water sources (ponds and streams bordering farmlands and urban areas) used for irrigation and on commercially produced lettuces in Southern Brazil. We also evaluated the effect of agricultural factors and meteorological conditions in the potential contamination of water and produce samples. A longitudinal study was conducted on four farms during a year (July 2014 to August 2015). The results showed generic *E. coli* prevalence of 84.8% and 38.3% in irrigation water samples and on lettuces, respectively, indicating irrigation water as an important source of contamination of lettuces. No significant differences were detected in the counts of *E. coli* between the two different surface water sources. The climatic conditions, particularly rainfall and environmental temperature, have influenced the high concentration of *E. coli*. The highest loads of *E. coli* in irrigation water and on lettuces were found during the warmest time of the year. *E. coli* O157:H7 was detected by qualitative polymerase chain reaction (qPCR) in 13 water samples but only 4 were confirmed by isolation in culture media.

Key-Words: Irrigation water; Fresh produce; Indicator microorganisms; Foodborne pathogens; Primary production.

1. Introduction

Leafy greens have been associated with foodborne outbreaks worldwide (EFSA AND ECDC, 2015; CDC, 2014). In Brazil, following the general global tendency, almost 3% of the foodborne outbreaks have been associated with fruits and vegetables in the last years (ANVISA, 2014). In an attempt to understand the most probable sources of contamination of leafy greens, recent studies have evaluated potential risk factors during primary production (Castro-Ibañez et al., 2015a; Ceuppens et al., 2014, 2015; Benjamin et al., 2013). In most of the cases, obtained results showed a strong evidence of contamination of produce from irrigation water. Additionally, recent reviews identified contamination events where water was recognized as a risk factor in the production and harvesting of fresh produce (Allende and Monaghan, 2015; Uyttendaele et al., 2015).

Irrigation water used in growing fields originates from a variety of water sources and much knowledge is needed to relate risk factors associated with the transfer coefficients for pathogens by source, concentration and use (Leaman et al., 2014). Among the main water sources used for irrigation, it can be identified, from lower to higher contamination risk, the wells, rainwater harvesting, rivers, and reservoirs (Castro-Ibañez et al., 2015b; Ahmed et al., 2012; Ferguson et al., 2012).

Mostly due to the low prevalence of pathogens in environmental and fresh produce samples, numerous published research papers rely on the enumeration of microbial indicators as a good strategy to characterize microbial contamination (Allende and Monaghan, 2015). However, there are controversial opinions regarding the relationship between the microbial quality of irrigation water and the food safety of fresh produce (Leaman et al., 2014). While some studies reported a correlation between numbers of *E. coli* in irrigation water and irrigated leafy vegetables, other researchers

did not find a significant relation between fecal indicators on fresh produce with those found in irrigation water (Castro-Ibañez et al., 2015a; Gayeon et al., 2013; Pahl et al., 2013; Pachepsky et al., 2011). On the other hand, recently published research papers found a higher prevalence of foodborne pathogens in water and fresh produce samples with higher loads of *E. coli* (Castro-Ibañez et al., 2015a; Ceuppens et al., 2014).

A study focused on the microbial quality and safety assessment of lettuce production in the Southern of Brazil identified contamination of vegetables with high counts of enteric microorganisms probably originating from irrigation water (Ceuppens et al., 2014). Although the recognized importance of irrigation water on the safety of vegetables, there is still a lack of information about how the microbiological quality of different irrigation water sources and climatic conditions influence the safety of vegetables produced in Brazil. This leads to the necessity of additional research to better understand microbial risks associated to irrigation water.

The present study aims to describe the distribution of generic *E. coli* and the prevalence of *E. coli* O157:H7 in irrigation water in commercial fields from the Southern Brazil. The impact of meteorological conditions on the microbial quality of irrigation and fresh produce samples was also evaluated. Moreover, the relationship between the distribution of generic *E. coli* and the presence of foodborne pathogen *E. coli* O157:H7 in samples was established.

2. Materials and methods

2.1. Growing fields

Four commercial leafy green growers agreed to participate in this study. All farms were located in the “green belt” from metropolitan region of Porto Alegre city in the State of Rio Grande do Sul at the Southern of Brazil and were small family farms.

The specific location was kept confidential to protect the identity of the farmers. The dimension of the farms was characteristic of the local commercial growing field and ranged between 0.1 and 0.5 ha. Two different irrigation water sources commonly used in the local commercial growing fields were involved in this study including natural ponds (Ponds) and streams bordering farmlands and urban areas (Streams). In all the cases, sprinkle irrigation was used. Irrigation was usually carried out once in the morning during autumn, winter and spring, while during summer irrigation was performed twice, early in the morning and late in the evening.

2.2. Sampling scheme

A systematic sampling plan was developed to identify potential risk factors for microbial contamination linked to irrigation water in the production of whole lettuce. The sampling sites were selected based on a previously published research paper focused on the potential risk factors that contribute to microbiological contamination on lettuce (Ceuppens et al., 2014). For each water source and growing field, the sampling plan included sample collection during a year period where samples were taken monthly from July 2014 to August 2015. Water and lettuce samples were taken in duplicate and triplicate, respectively. A total of 219 water samples were collected during the study: where 138 were water samples and 81 were lettuce samples. All samples were taken to laboratory in thermal boxes and kept for 2 – 12 hours at <4 °C until microbiological analyses. Physicochemical parameters of the irrigation water (pH and temperature) were recorded at each sampling point.

2.3. Sampling methodology

The protocol previously described by Holvoet et al. (2014) was followed. For lettuce, 9 samples of approximately 100 g each were randomly collected from different locations in the field following a zig-zag pattern started from a randomly selected side

of the field. Once in the laboratory, samples (100 g each) were randomly pooled into 3 samples (25 g each). In the case of water, samples were collected from different water sources: natural ponds (Ponds) and streams bordering farmlands and urban areas (Streams). Two liter samples were collected into sterile bottles according to ISO 19459:2006 (ISO, 2006). Microbial analyses were conducted within 2–14 h from the time of sample collection.

2.4. Microbiological analyses

The microbial quality of lettuce samples were evaluated by diluting 25 g of each sample or sample pool in 225 mL of 1% buffered peptone water. Water samples (100 mL) were filtered using a cellulose nitrate membrane filters (0.45 µM diameter, Microsart®, Sartorius, Brazil). *E. coli* was monitored as previously described (Holvoet et al., 2014). Briefly, *E. coli* were enumerated in lettuce and samples using Chromocult Agar (Merck, Brazil) after incubation for 24 h at 37 °C. The detection limits were 10 cfu/g in case of lettuce and 100 cfu/100 mL in case of water samples.

Presence or absence of *E. coli* O157:H7 were determined in water samples with more than 100 cfu/100 mL (Ceuppens et al., 2015). Screening of positive samples was analyzed using PCR technique. In this case, bacterial DNA was extracted following the protocol described by the NMKL Method nº 174 (NMKL, 2002). Briefly, 900 µl of the enrichment broth were transferred to a tube containing 600 µl of Percoll (Sigma-Aldrich®) 40%. Then, the tube was centrifuged for 1 min at 13.200 rpm. The fluid on the top of the tube was removed and 0.1 mL was leaved at the bottom. The remaining volume was transferred to a tube contain 1.2 mL sterile distilled water and vortexed by 1 min. The tube was centrifuged for 5 min at 10.000 rpm. The fluid on the top of the tube was removed, leaving 0.1 mL at the bottom, 1 mL of sterile distilled water was added and the tube vortexed by 1 min. This last step was repeated again. Then, the fluid

on the top was discarded, leaving 0.2 mL in the tube, which was incubated for 20 min at 95 °C in a heating block. After incubation, the tubes were placed on ice for 5 min and centrifuged for 1 min at 10.000 rpm. The tubes were stored at −20 °C until PCR analysis. The Real-Time PCR was performed according the primers and cycling conditions described in the ISO 13136:2012 (ISO, 2012). The software StepOne Plus® (LifeTechnologies®, Carlsbad, United States).

2.5. Meteorological parameters

Weather data for ambient temperature, precipitation and solar radiation were obtained during the sampling period from the Brazilian National Institute of Meteorology (INMET). Temperature and solar radiation data from 24 h before the sampling point were considered while in the case of precipitation, data from 7 days prior sampling were taken and used to correlate microbiological results and climatic conditions following procedures previously described (Castro-Ibáñez et al., 2015; Ceuppens et al., 2014).

2.6. Domestic animals

The presence of domestic animals was farm dependent, whit three farms performing mix crop-livestock farming while all of them had pets around the farm. To determine the agricultural practices related to domestic animals and pets a questionnaire was given to the growers (**Table 1**).

2.7. Statistical analysis

Non-zero microbial loads were log-transformed and stored along with zero counts in an Excel spreadsheet (Microsoft Corporation, Redmon, WA, USA). Results were compiled and graphs were made using Sigma Plot 12.0 Systat Software, Inc. (Addilink Software Scientific, S.L. Barcelona). For calculation and graphical presentation of the median and interquartile range (IQR) of microbial counts only

positive samples (i.e., with numbers above the detection limit) were included. All analyses were performed with IBM SPSS Statistics 18 at a significance level of 5% ($p = 0.05$). Mann-Whitney U and Kuskal-Wallis tests were used to respectively determine the difference between the positives counts of the indicators with respect to presence/absence of pathogens and to define differences in counts between different irrigation water source and management practices. The Pearson correlation coefficient was calculated ($p < 0.01$) between *E. coli* and irrigation water and lettuce samples as well as the means of the outside temperature, the accumulated precipitation, and UV radiation.

3. Results and discussion

3.1. Microbial quality of irrigation water sources and their impact on the microbial quality of lettuce

Irrigation water samples were taken from two surface water sources, including ponds and streams. Surface water and groundwater represents the most commonly used water sources for irrigation (Gleick, 2000). Surface water has been classified as the riskiest irrigation water sources by several international agencies, because, among other reasons, it may include discharges of treated or untreated wastewater (Allende and Monaghan, 2015). In this study, irrigation water samples showed an *E. coli* prevalence of 84.8%. Among them, irrigation water from ponds showed a prevalence of 82.8%, while all the water samples (100%) obtained from the farm using streams were positive for *E. coli*. *E. coli* levels of positive samples ranged from 2.1 to 5.4 log cfu/100 mL (**Fig. 1**). Obtained results are very similar to those previously reported by Ceuppens et al. (2014). However, *E. coli* prevalence and loads reported in different production areas from Europe were much lower than those found in this study. In Spain and Belgium, *E.*

coli prevalence previously reported for irrigation water were 52% and 59%, respectively (Castro-Ibañez et al., 2015a; Holvoet et al., 2014). Counts of *E. coli* in surface irrigation water reported from European production areas ranged between 1.0 and 1.5 log CFU/100 mL, which were also lower than values obtained in the present study (Castro-Ibañez et al., 2015a; Holvoet et al., 2014). When the microbial quality of different irrigation water sources was compared it was observed that water obtained from ponds showed higher prevalence and *E. coli* loads than water from streams, although no significant differences have been found (**Fig. 1**). Differences in the microbial quality of irrigation water sources have been also reported by Castro-Ibañez et al. (2015a) who showed that water obtained from ponds had higher *E. coli* loads than the water obtained from irrigation heads. They also reported significant differences in the microbial quality between untreated and treated irrigation water.

Guidelines and regulation focused on microbial quality specifications of irrigation water have been already published in some countries or states and by international agencies. Most of these guidelines select *E. coli* as the best indicator of fecal contamination and acceptable limits vary between 10 and 126 *E. coli* cfu/100 mL (Uyttendaele et al., 2015). At the European level, a draft guidance document focused on the implementation of hygiene requirements for fresh fruits and vegetables at primary production is being elaborated. In this guide, irrigation water is classified based on the microbial risk and recommended limits of *E. coli* are included. In the case of leafy greens irrigated using sprinkle irrigation, the *E. coli* limit is established in 100 cfu/ 100 mL (AHDB, 2016). In Brazil, current legislation established fecal coliform limit is 200 cfu/100 mL of irrigation water for vegetables which are consumed raw (BRASIL, 1986). Based on the obtained results, the irrigation water source used in Southern Brazil were above the Brazilian limit and, consequently, can be considered not appropriate to

irrigate crops likely to be eaten uncooked, such as lettuce, which irrigation water comes into direct contact with the edible portion of the crop.

E. coli prevalence in lettuce was 38.3%, which is much higher than previously reported values for leafy greens ranging between 5% and 6.6% in Belgium, Southwestern and Western United States and Southeast of Spain (Holvoet et al., 2014; Park et al., 2014; Castro-Ibañez et al., 2015a). In the present study, the *E. coli* counts on positive samples of lettuce ranged from 1.3 to 4.5 log cfu/g (**Fig. 1A**). These values were again much higher than previously reported *E. coli* numbers on leafy greens produced outside Brazil (Holvoet et al., 2014; Park et al., 2014; Castro-Ibañez et al., 2015a). Several authors reported a good correlation between concentration of *E. coli* in irrigation water and microbial contamination of leafy greens (Park et al., 2013; Ceuppens et al., 2014; Castro-Ibañez et al., 2015a). Results obtained in the present study showed a good correlation between the *E. coli* levels of positive samples of irrigation water and lettuce (**Fig. 1B**).

E. coli O157:H7 was detected by qPCR analysis in 13 out of 62 samples of irrigation water (20.9%), from these, 9 samples were obtained from ponds and 4 samples were obtained from streams (**Table 2**). Prevalence of *E. coli* O157:H7 in irrigation water samples in this study was higher than that reported by Ceuppens et al. (2014), which showed a prevalence of 2.1% when 48 irrigation water samples, taken from similar water sources in southern Brazil, were analyzed. Outside Brazil, Holvoet et al. (2014), in Belgium, reported a prevalence of STEC (through PCR *E. coli* entero-haemorrhagic) of 9% (6/68) in irrigation water samples taken from rainfall. These results corroborate the hypothesis that irrigation water represents a main risk factor for introduction of pathogens during primary production of leafy greens (Holvoet et al., 2014; Park et al., 2014; Castro-Ibañez et al., 2015a). However, only 4 samples of

irrigation water were confirmed by isolation in culture media. This fact has been already discussed in other studies, where environmental and fresh produce samples are analyzed, reporting the challenge in recovering viable cells. These studies reported that the presence of indigenous competing microbiota on selective agars can inhibit the growth of pathogenic microorganisms (Castro-Ibañez et al., 2015a; Delbeke et al., 2015).

Although the high counts of generic *E. coli* found in the irrigation water samples in the present study, *E. coli* levels were not significantly correlated with the presence of *E. coli* O157:H7 (Mann-Whitney U Test, p = 0.979) (**Fig. 2**). However, the correlation between generic *E. coli* counts and the presence of pathogens (as *E. coli* O157:H7 and *Salmonella*) in environmental and fresh produce samples has been already reported (Holvoet et al., 2014; Park et al., 2014; Castro-Ibañez et al., 2015a).

3.2. Impact of weather conditions and agricultural practices on the microbial quality of irrigation water sources

Consequences of climate change have been identified as having potential for increasing bacterial contamination of food and water (Tirado et al., 2010) and an important impact on levels of indicator and pathogenic bacteria on leafy greens at harvest (Park et al., 2015; Castro-Ibañez et al., 2015b). Agricultural practices such as irrigation systems have been shown to influence microbial contamination of leafy greens (Castro-Ibañez et al., 2015a). In the current study, the impact of climatic factors and agricultural practices on the microbial quality of irrigation water and fresh produce were evaluated.

3.2.1. Rainfall

Some investigations have reported that rainfalls increased the counts of indicator and pathogenic microorganisms during primary production of leafy greens (Holvoet et al., 2014; Castro-Ibáñez et al., 2015b; Park et al., 2015). Besides the increase of the indicator counts, flooding events have been already associated with the presence of pathogenic microorganisms as *E. coli* O157: H7 in irrigation water (Ceuppens et al., 2014; Castro-Ibáñez et al., 2015b). However, in the present study, no significant differences were observed between the *E. coli* levels in irrigation water when different amount of rainfall were recorded (**Fig. 3**). This fact could be due to the low amount of rainfall observed during the sampling interval. On the other hand, a positive correlation was found between the counts of *E. coli* on lettuces and the precipitation levels ($p = 0.406$). The highest *E. coli* counts were found when accumulative precipitation levels of 6–12 mm of water were registered; although high volumes of rain or flooding were not registered during the evaluated period (**Fig. 3**). According to these results, Holvoet et al. (2014) found significant, but very weak correlation between the *E. coli* enumeration data on lettuce samples and accumulative precipitation ($p = 0.190$).

3.2.2. Temperature and seasonality

Obtained results showed significantly higher *E. coli* levels in irrigation water and leafy greens when higher ambient temperatures were recorded (**Fig. 4**). The observed correlation among the *E. coli* counts in the irrigation water and ambient temperature as $p = 0.454$. These findings are in agreement with previous studies which demonstrated that the highest levels of *E. coli* and highest prevalence of pathogens in water and on produce were observed during periods of the year when the environmental temperatures and irrigation water temperatures were high (Park et al., 2012; Strawn et al., 2013; Castro-Ibañez et al., 2015a; Park et al., 2015).

Regarding the relationship between the temperature and the *E. coli* levels in the fresh produce, a weak but significant correlation was found in this study ($p = 0.353$); although it was higher than the correlation previously described ($p = 0.120$) by Holvoet et al. (2014). However, in agreement with our results, Castro-Ibañez et al. (2015a) and Park et al. (2015) noticed that higher counts of *E. coli* were found when ambient temperature were high.

Based on the previous results it could be expected that seasonality also influenced the levels of *E. coli*, being summertime the time of the year (i.e. from December 2014 to April 2015) when irrigation water showed the highest microbial counts of *E. coli* (**Fig. 5**). According to our results, Holvoet et al. (2014) also described that the highest loads of *E. coli* and the highest prevalence of pathogens in irrigation water were observed in the time of the year when the outside temperature were the highest (May to September). In the Southern of Brazil, during summertime, producers use to irrigate twice a day their crops on fields, which might increase the risk of microbial contamination if contaminated irrigation water is used. This fact has been already highlighted by other authors who found relationship between the increased frequency of irrigation and the increase in indicator microorganisms counts (Ceuppens et al., 2014; Da Silva et al., 2007).

3.2.3. Presence of domestic animals

The presence of farm and wild animals near the water source can significantly contribute to the increment of contamination with *E. coli* on produce (Ceuppens et al., 2014). According to Liu et al. (2013) and Park et al. (2013), the presence of wild or farm animals near to vegetable production or water sources increases the risk of microbial contaminating the environment. In this study, domestic animals (i.e. cattle, kitchen and pigs) and pets (i.e. dogs, cats) were commonly present in all the commercial

fields sampled in this study. Results obtained from the survey indicated that in most of the cases (75%), animals were allowed to graze near the water sources or the lettuce fields which may contribute to the high prevalence of *E. coli* observed in the present study. In 100% of the farms pets were present, while in 75% of the farms mixed crop-livestock farming was carried out (**Table 3**). The presence of animal faeces was also observed around the lettuce crop in 75% of cases. In all the farms there was an absence of physical protection around the water sources (**Table 3**).

In general, the correlation reported by several studies between high levels of precipitation and increased contamination by faecal microorganisms in untreated surface water has been mainly attributed to the presence of livestock near to water sources used for irrigation (CDC, 2014; Castro-Ibáñez et al., 2015b; Ceuppens et al., 2014). The use of manure composted or the presence of animal faeces in the plantation, or in its surroundings, may assist in increasing the contamination in the event of rainfall. This can occur when these contaminants are on the soil surface and, during rains they come into contact with the vegetables through droplets splashing from the soil, which is near the ground (Liu et al., 2013).

3.2.4. Irrigation regime

In this study, significant differences were found between the *E. coli* levels of lettuce when harvest was performed after 6, 12 or 24 h from the last irrigation. When harvest was performed 24 h or more after the last irrigation event, the *E. coli* levels were significantly lower than the rest of time intervals between irrigation and harvest (**Fig. 6**). These results agreed with previous reports, which identified an association between contamination of generic *E. coli* and short irrigation lapse time between irrigation and harvest (Park et al., 2014). According to this, Park et al. (2013) reported that the odds of

spinach contamination were reduced to approximately 1 in 4 when the time since the last irrigation was longer than 5 days.

4. Conclusions

Results obtained in the present study confirmed irrigation water as an important risk factor for microbial contamination during primary production of lettuce. The two irrigation water sources analyzed in this study showed high counts of *E. coli*, above the recommended microbiological limits for irrigation water, which probably impacted the microbial quality of the produce. Additionally *E. coli* O157: H7 was found in more than 20% of the water samples, which is probably the highest prevalence reported in a systematic sampling study. Regarding environmental factors (climate and location), rainfall and temperature have influenced the counts of *E. coli* found in the product. However, caution is needed when trying to extrapolate the obtained results to different geographical locations mostly due to the limited number of tested samples.

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References

Agência Nacional de Vigilância Sanitária – ANVISA, 2012. Vigilância Epidemiológica das Doenças Transmitidas por Alimentos – VE-DTA. São Paulo. Available at: http://www.anrbrasil.org.br/new/pdfs/2014/3_PAINEL_1_ ApresentacaoRejaneAlvesVigilanciaEpidemiologica-VE-DTA-Agosto_2014_ PDF.pdf (Acessed January 2016).

AHDB, 2016. Agriculture and Horticulture Development Board. 'Keep it Clean' January 2016 workshop. Programme drafted in consultation with Research & Development technical committees of the British Leafy Salads Association; Baby Leaf Growers' Association; Plant Propagators Ltd.; the NFU Watercress Growers Association and BritishHerbs.Availableat: http://horticulture.ahdb.org.uk/sites/default/files/Keep%20It%20Clean%20Microbials%20Workshops%20January%202016%20handout.pdf. (Acessed July 2016).

Ahmed, W., Richardson, K., Sidhu, J. P. S., Toze, S., 2012. *Escherichia coli* and *Enterococcus* spp. in Rainwater Tank Samples: Comparison of Culture-Based Methods and 23S rRNA Gene Quantitative PCR Assays. Environ. Sci Technol, 46, 11370−11376.

Allende, A. and Monaghan, J., 2015. Irrigation Water Quality for Leafy Crops: A Perspective of Risks and Potential Solutions. Int. J. Environ. Res. Public Health Res. 12, 7457-7477.

Benjamin, L., Atwill, E.R., Jay-Russell, M., Cooley,M., Carychao, D., Gorski, L.,Mandrell, R.E., 2013. Occurrence of generic *E. coli*, *E. coli* O157 and *Salmonella* spp. in water and sediment from leafy green produce farms and streams on the Central California coast. Int. J. Food Microbiol. 165, 65-76.

BRASIL. Ministry of Environment. Resolution no. 20 of 18 June 1986. Determining the classification of waters, fresh, brackish and salt marshes of the National Territory Official Gazette, Executive, Brasília, DF, publish in 30 of July 1986. Available at: http://www.mma.gov.br/port/conama/res/res86/res2086.html(Acessed January 2016)

Castro-Ibáñez, I., Gil, M.I., Tudela, J.A., Ivanek, R., Allende, A., 2015a. Assessment of microbial risk factors and impact of meteorological conditions during production of baby spinach in the Southeast of Spain. Food Microbiol. 49, 173-181.

Castro-Ibáñez, I., Gil, M.I., Tudela, J.A., Allende, A., 2015b. Microbial safety considerations of flooding in primary production of leafy greens: A case study. Food Res. Int. 68, 62–69.

Centers for Disease Control and Prevention, 2014. Surveillance for Foodborne Disease Outbreaks, United States (2012). Annual Report US Department of Health and Human Services, Atlanta, Georgia. Available at: http://www.cdc.gov/foodsafety/pdfs/foodborne-disease-outbreaks-annual-report-2012-508c.pdf (Acessed January 2016).

Ceuppens, S., Hessel, C.T., Rodrigues, R.deQ., Bartz, S., Tondo, E.C., Uyttendaele, M, 2014. Microbiological quality and safety assessment of lettuce production in Brazil. Int. J. Food Microbiol. 181, 67–76.

Ceuppens, S., Johannessen, G.S., Allende, A., Tondo, E.C., El-Tahan, F., Sampers, I., Jacxsens, L., Uyttendaele, M., 2015. Risk factors for *Salmonella*, shiga toxin-producing *Escherichia coli* and *Campylobacter* occurrence in primary production of leafy greens and strawberries. Int. J. Environ. Res. Public Health 12, 9809–9831.

Da Silva, S.R.P., Verdin, S.E.F., Pereira, D.C., Schatkoski, A.M., Rott, M.B., Corcao, G., 2007. Microbiological quality of minimally processed vegetables sold in Porto Alegre, Brazil. Braz. J. Microbiol. 38, 594–598.

De Boer, E., and Heuvelink, A. E., 2000. Methods for the detection and isolation of Shiga toxin-producing *Escherichia coli*. J. Appl. Microbiol. 88, 133S–143S.

Delbeke, S., Ceuppens, S., Holvoet, K., Samuels, E., Sampers, I., Uyttendaele, M., 2015. Multiplex real-time PCR and culture methods for detection of Shiga toxinproducing *Escherichia coli* and *Salmonella* Thompson in strawberries, a lettuce mix and basil. Int. J. Food Microbiol., 193, 1-7.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA Journal 2015;13(12):4329, 191. Available at: 10.2903/j.efsa.2015.4329.

EFSA Panel on Biological Hazards (BIOHAZ), 2014. Scientific Opinion on the Risk Posed by Pathogens in Food of Non-animal Origin. Part 2 (*Salmonella* and Norovirus in leafy greens eaten raw as salads). EFSA Journal, 11(3600), Available at: www.efsa.europa.eu/efsajournal (Acessed February 2016).

Ferguson, A. S., Layton, A. C., Mailloux, B. J., Culligan, P. J., Williams, D. E., Smartt, A. E., Sayler, G. S., Feighery, J., Mckay, L. D., Knappett, P. S.K., Alexandrova E., Arbit, T., Emch, M., Escamilla, V., Ahmed, K. M., Alam, MD. J., Streatfield, P. K., Yunus, M., Geen, A. V., 2012. Comparison of fecal indicators with pathogenic bacteria and rotavirus in groundwater. Sci.Total Environ**,** 431, 314–322.

Gayeon, W., Schlegel, P.J., Schrock, J.M., LeJeune, J.T., 2013. Absence of direct association between coliforms and *Escherichia coli* in irrigation water and on produce. J. Food Prot. 6, 928-1108.

Gleick, P.H., 2000. The world's water 2000–2001: the Biennial Report on Freshwater Resources. Island Press, Washington, DC.

Holvoet, K., Sampers, I., Seynnaeve, M., & Uyttendaele, M., 2014. 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. Int. J. Food Microbiol, 171, 21–31.

ISO, 2006. Water Quality. Sampling for Microbiological Analysis. ISO 19458:2006.

ISO, 2012. Microbiology of Food and Animal Feed — Real-time Polymerase Chain Reaction (PCR)-based Method for the Detection of Food-borne Pathogens. Horizontal Method for the Detection of Shiga Toxin-producing *Escherichia coli* (STEC) and the Determination of O157, O111, O26, O103 and O145 Serogroups. ISO 13136:2012.

Leaman, S., Gorny, J., Wetherington, D., Bekris, H., 2014. CPS Symposium: Agricultural Water: Five Year Research Review. Center for Produce Safety, Davis, California. Available at: http://www.pma.com/~/media/pma-files/food-safety/cps/cps-research-symposium-water-report72014.pdf?la=en (Acessed January 2016).

Liu, C., Hofstra, N., Franz, E., 2013. Impacts of climate change on the microbial safety of pre-harvest leafy green vegetables as indicated by *Escherichia coli* O157 and *Salmonella* spp.. Int. J. Food Microbiol. 163, 119–128.

NMKL 174, 2002, 2nd Ed. *Shigella* spp. PCR-metod för påvisning i livsmedel. *Shigella* spp. PCR method for detection in foods.

Pachepsky, Y., Shelton, D.R., McLain, J.E.T., Patel, J., Mandrell, R.E., 2011. Irrigation waters as a source of pathogenic microorganisms in produce: a review. In: In: Donald, L.S. (Ed.), Advances in Agronomy. vol. 113. Academic Press, pp. 73–138

Pahl, D.M., Telias, A., Newell, M., Ottesen, A.R., Walsh, C., 2013. Comparing source of agricultural contact water and the presence of fecal indicator organisms on the surface of 'Juliet' grape tomatoes. J. Food Prot. 6, 928-1108.

Park, S., Navratil, S., Gregory, A., Bauer, A., Srinath, I., Jun, M., Szonyi, B., Nightingale, K., Anciso, J., Ivanek, R., 2013. Generic *Escherichia coli* contamination of spinach at the preharvest level: the role of farm management and environmental factors. Appl. Environ. Microbiol. 79, 4347–4358.

Park, S., Navratil, S., Gregory, A., Bauer, A., Srinath, I., Szonyi, B., Nightingale, K., Anciso, J., Jun, M., Han, D., Lawhon, S., Ivanek, R., 2014. Farm management, environment, and weather factors jointly affect the probability of spinach contamination by generic Escherichia coli at the preharvest stage. Appl. Environ. Microbiol. 80, 2504-2515.

Park, S., Navratil, S., Gregory, A., Bauer, A., Srinath, I., Szonyi, B., Nightingale, K., Anciso, J., Jun, M., Han, D., Lawhon, S., Ivanek, R., 2015. Count of generic *Escherichia coli* on spinach at the preharvest level determined by the multifactorial effect of ambient temperature, precipitation, farm management and environmental factors. Appl. Environ. Microbiol. 81, 793-814.

Strawn, L.K., Fortes, E.D., Bihn, E.A., Nightingale, K.K., Grohn, Y.T., Worobo, R.W., Wiedmann, M., Bergholz, P.W., 2013. Landscape and meteorological factors affecting prevalence of three food-borne pathogens in fruit and vegetable farms. Appl. Environ. Microbiol. 79, 588-600.

Tirado, M.C., Clarke, R., Jaykus, L.A., McQuatters-Gollop, A., Franke, J.M., 2010. Climate change and food safety: a review. Food Res. Int. 43, 1745–1765.

Uyttendaele, M., Jaykus, L.A., Amoah, P., Chiodini, A., Cunliffe, D., Jacxsens, L., Holvoet, K., Korsten, L., Lau, M., McClure, P., Medema, G., Sampers, I., Jasti, P. R., 2015. Microbial Hazards in Irrigation Water: Standards, Norms, and Testing to Manage Use of Water in Fresh Produce Primary Production. Comp. Rev. Food Sci. Food Saf. 14, 336–356.

Table 1. Questionnaire submitted to the growers to evaluate the influence of domestic animals in the microbial quality of irrigation water and leafy greens.

Parameter	**Description of situation**	**Answer**
Presence of animals	• Farm animals (cow, horse, chicken, pig and others)	Yes/No
	• Pets (dog, cat and others)	
	• Wild animals (bird, mouse, rabbit, opossum and others)	Yes/No
		Yes/No
Presence of animal faeces	• Presence of faeces around the water source.	Yes/No
	• Presence of faeces around the plantation.	Yes/No
Existence of physical protection	• Prevents contact of the animal and the animal faeces with source water.	Yes/No
	• Prevents contact of the animal and the animal faeces with the planting.	Yes/No
Other intervention strategy		

Table 2. Prevalence of foodborne pathogens in samples from irrigation water

	***E. coli* O157:H7**	
	qPCR	**Confirmed***
Ponds	9/50	3/9
Streams	4/12	1/4
Total	**13/62**	**4/13**

*Samples were confirmed by isolation in selective culture media and conventional PCR.

Table 3. Responses provided by the growers answering to the questionnaire about the presence of domestic animals.

	Farms			
	Farm 1	**Farm 2**	**Farm 3**	**Farm 4**
Presence animals				
• Farm animal	Yes	Yes	Yes	-
• Pets animal	Yes	Yes	Yes	Yes
• Wild animal	Yes	Yes	Yes	Yes
Presence of animal faeces				
• Source water	Yes	No	Yes	No
• Plantation	Yes	Yes	Yes	No
Existence of physical protection				
• Source water	No	No	No	No
• Plantation	Yes	No	No	No

Figures

Figure 1A. Boxplots representing (blue) *E. coli* counts (log cfu/100mL) in positive water samples and (green) *E. coli* counts (log cfu/g) in positive lettuce samples as a function of the two different source irrigation water. In this study, positive samples are defined as samples contaminated above detection limit (0 log cfu/100 and mL 2 log cfu/g, for water and lettuce, respectively). In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Significant differences were determined by Mann-Whitney test ($p<0.005$) and are represented with different letters.

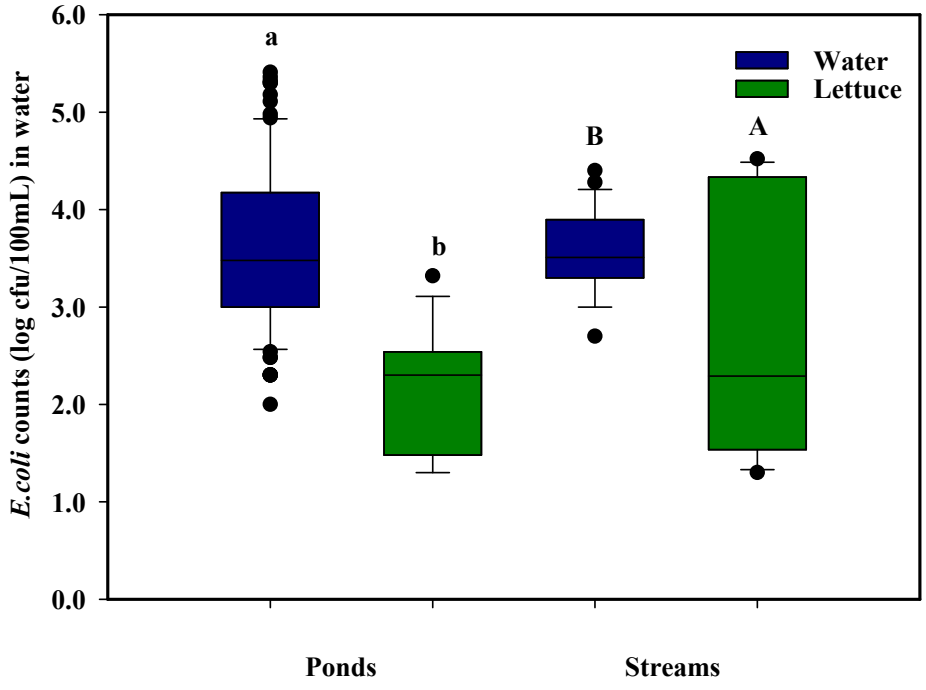


Figure 1B. Scatter plots showing the relationship between *E. coli* counts in water (log cfu/100 mL) and lettuce samples (log cfu/g). Confidence intervals at 95% (broken lines) and central regression lines are represented.

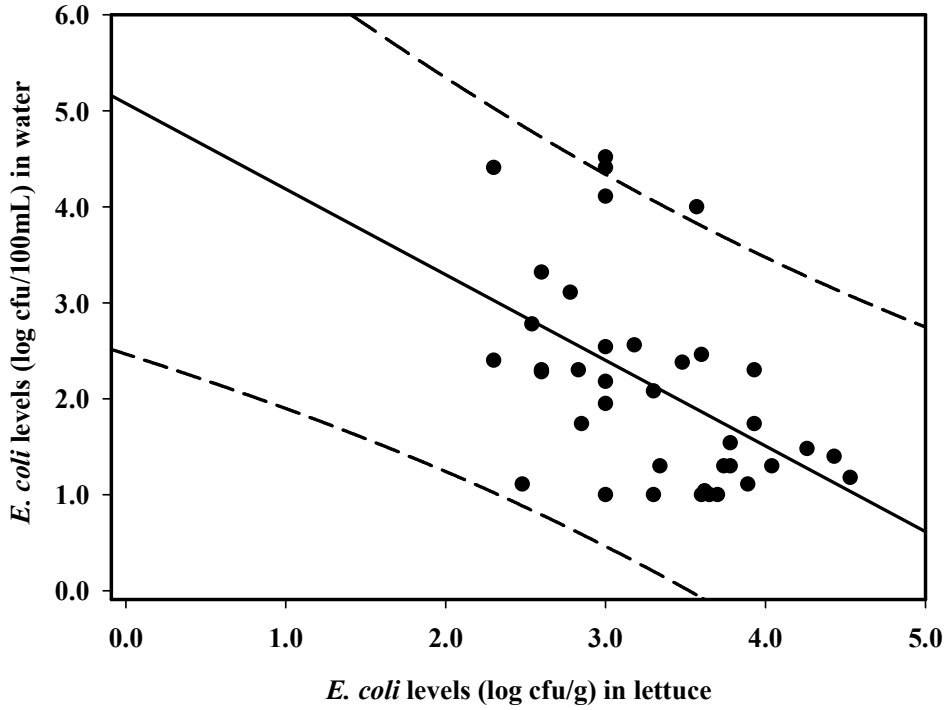


Figure 2. Boxplots representing *E. coli* counts (log cfu/100mL) and detection of *E. coli* O157:H7 in positive water samples by Real Time PCR (qPCR). In this study, positive samples are defined as samples contaminated above detection limit (0 log cfu/100). In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median.

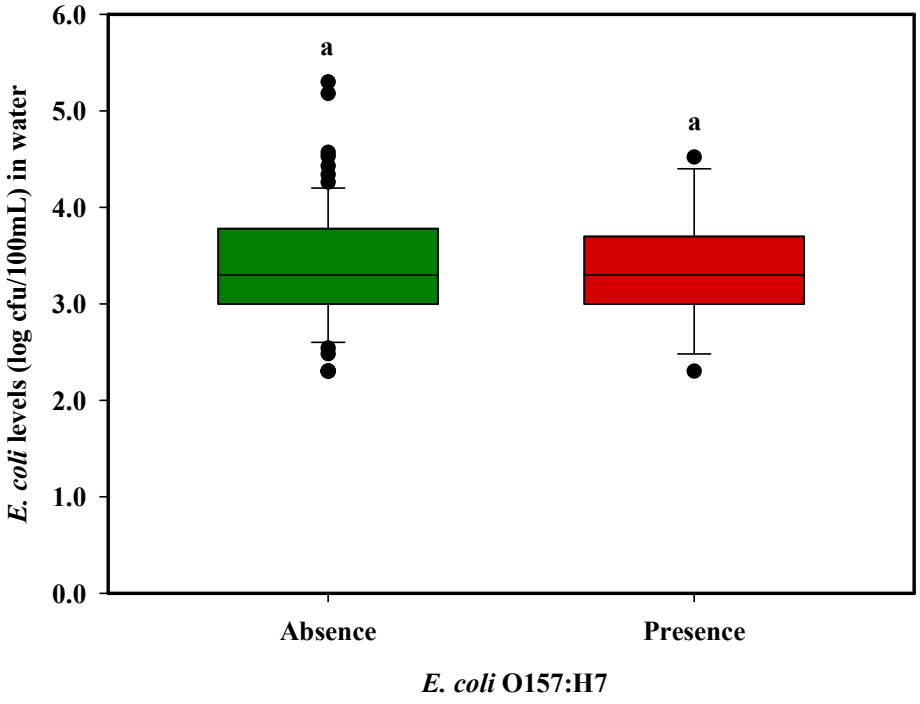


Figure 3. Boxplots representing (A) *E. coli* counts (log cfu/100mL) in positive water samples and (B) *E. coli* counts (log cfu/g) in positive lettuce samples as a function of the mean precipitation during the week before sample collection (mm). In this study, positive samples are defined as samples contaminated above detection limit (0 log cfu/100mL and 2 log cfu/g, for water and lettuce respectively). In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Significant differences were determined by Mann-Whitney test ($p<0.005$) and are represented with different letters.

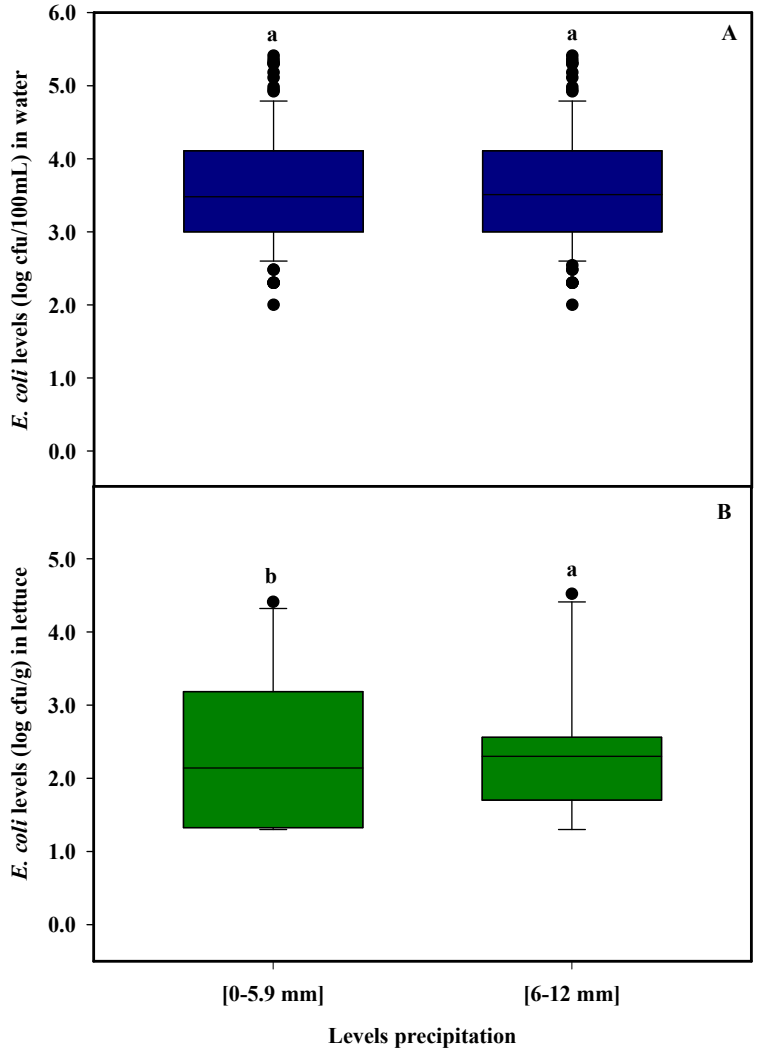


Figure 4. Boxplots representing (A) *E. coli* counts (log cfu/100mL) in positive water samples and (B) *E. coli* counts (log cfu/g) in positive lettuce samples as a function of the mean ambient temperature before 24h sample collection (^{0}C). In this study, positive samples are defined as samples contaminated above detection limit (0 log cfu/100 and mL 2 log cfu/g for water and lettuce, respectively). In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Significant differences were determined by Mann-Whitney test ($p<0.005$) and are represented with different letters.

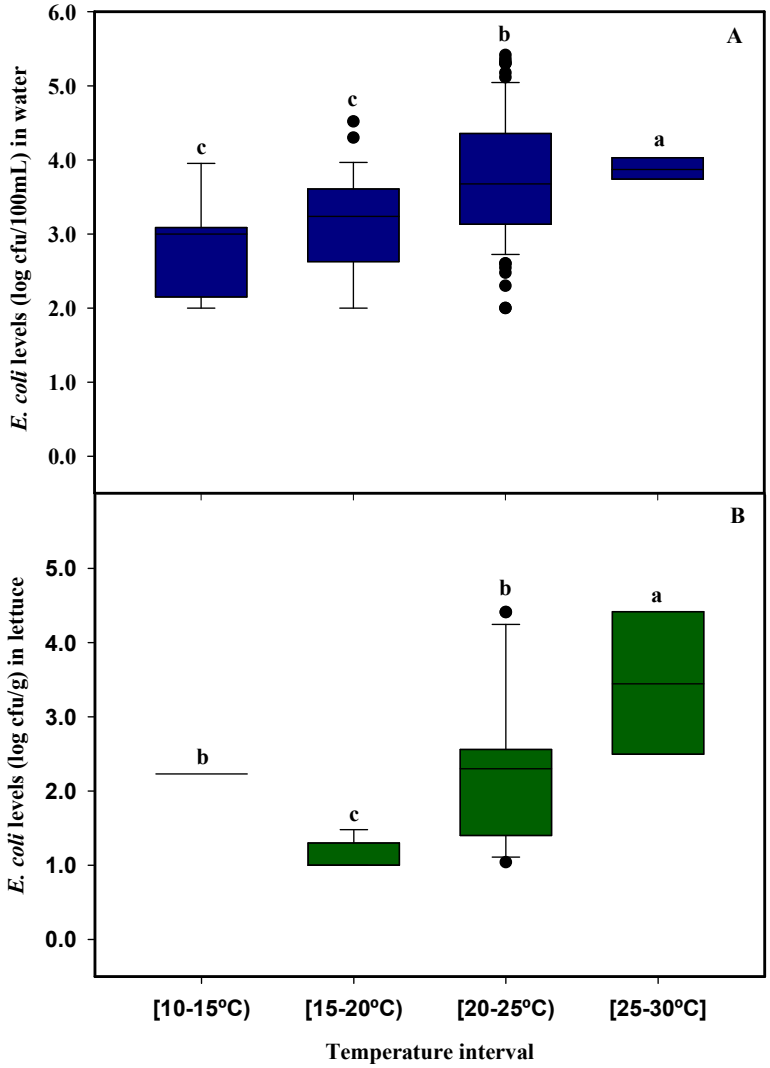


Figure 5. Boxplots representing *E. coli* counts (log cfu/100mL) in positive water samples as a function a months of the year collection. In this study, positive samples are define as samples contaminated above detection limit (0 log cfu/100 mL). In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median.

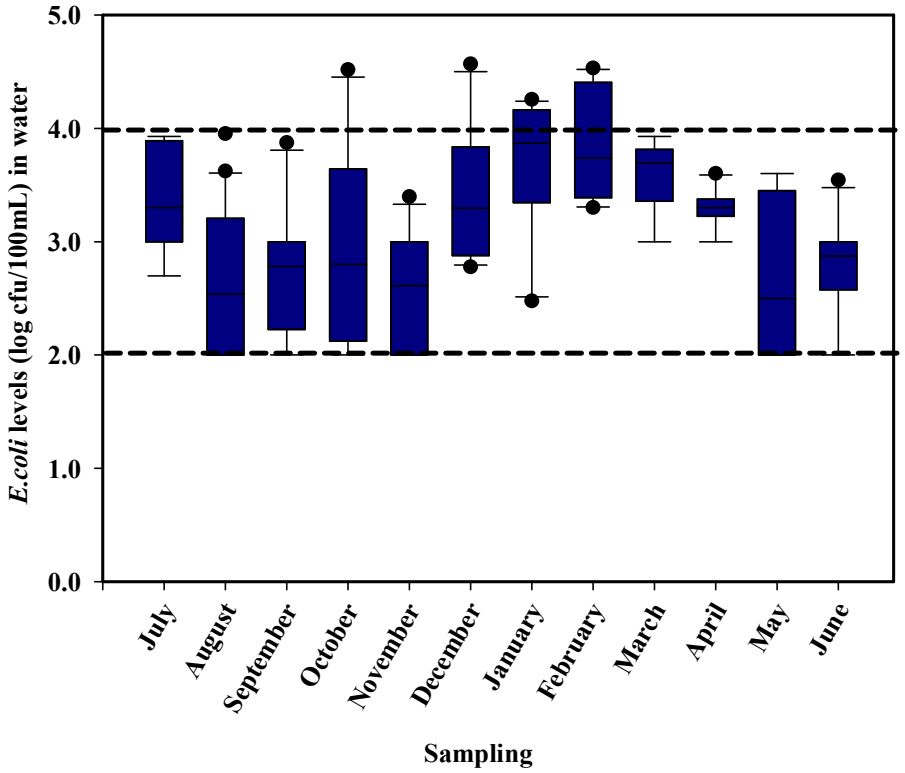
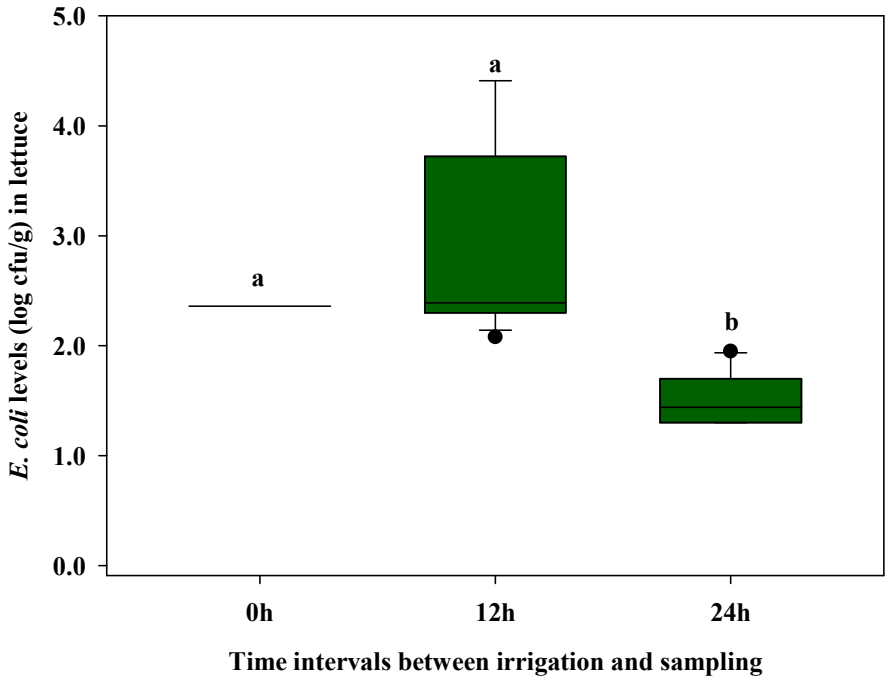


Figure 6. Boxplots representing *E. coli* counts (log cfu/g) in positive lettuce samples as a function of time intervals between last irrigation and sample collection (h). In this study, positive samples are defined as samples contaminated above detection limit (2 log cfu/g). In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Significant differences were determined by Mann-Whitney test ($p<0.005$) and are represented with different letters.



4.1 Artigo 2

Correlation between indicator microorganisms and prevalence of *Salmonella* spp. in different irrigation water sources and irrigated lettuces in Southern of Brazil

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ABSTRACT

Several studies in different countries have identified the irrigation water as an important source of contamination of leafy greens eaten raw. However, there are still few studies concerning the microbiological contamination of different irrigation water sources of lettuces in Brazil. The present study evaluated indicator microorganisms and their relation to *Salmonella* spp. in different sources of irrigation water and on irrigated lettuces. Samples of irrigation water and irrigated lettuce heads were monthly collected, for one year, in ponds and streams of 4 lettuce producer farms in Southern Brazil. As expected, results demonstrated a prevalence of 100% of total coliforms in water samples and on lettuces, and 100% of *Enterococcus* spp. in water samples. No significant statistic difference was found when comparing counts of indicators from both water sources. *Salmonella* spp. was identified in 6.4 % of the water samples (4 samples from natural ponds and 2 samples from streams) and on 14.8 % of the lettuce samples. A positive correlation was identified between the presence of *Salmonella* and *Enterococcus* spp. High prevalence of this indicator correlated to the presence of *Salmonella* spp. in irrigation water and on irrigated lettuces, highlighting irrigation water as an important source of microbiological contamination of leafy greens in primary production.

***Key-Words*:** *Leafy greens; Source water; pathogenic microorganisms; Indicator microorganisms; Primary production*.

1. Introduction

The microbiological contamination of fresh vegetables may occur at several stages of the production, processing and distribution chain. Among the main sources of contamination of leafy greens are soil, seeds and seedlings, fertilizers, irrigation water and process wash water (Decol et al., 2017; Castro-Ibáñez et al., 2015a; Gil et al., 2015; Ceuppens et al., 2014; Park et al., 2014; Rodrigues et al., 2014; Pachepsky et al., 2011; Ingham et al., 2005; Johannessen et al., 2002). Irrigation water have been identified as a critical factor and selectin of suitable water sources is key to avoid contamination with foodborne pathogens (Allende and Monaghan, 2015). Even though several sources of water are used for irrigation, the main sources are the artesian wells, stored and protected rainwaters, rivers and ponds, in which such order indicates the sources concerning lower to higher risk (Castro-Ibanez et al., 2015b; Ahmed et al., 2012; Ferguson et al., 2012). Irrigation water can be spread on leafy vegetables through different irrigation systems. The most commonly used systems are drip irrigation and sprinkle irrigation. The latter is considered of higher risk of contamination because water get in contact with the edible parts of the plant (Pachepsky et al., 2011).

Leafy greens harbor several types of microorganisms including bacteria, molds and yeasts. Most of the microorganisms present in the plants are not pathogenic and are part of the background microbiota of the plant (Marine et al., 2015). However, human pathogenic bacteria such as *Salmonella* have been associated with foodborne outbreaks involving fresh produce and can be present on vegetables (EFSA AND ECDC, 2015; CDC, 2014). The ability of foodborne pathogens to colonize and persist on plant tissues represents a significant food safety risk (Uyttendaele et al., 2015; Sivapalasingam et al., 2004).

Mostly due to the low prevalence of pathogens in the environment and on fresh produce samples, enumeration of indicator microorganisms have been proposed as a good strategy to characterize microbial contamination (Allende and Monaghan, 2015). However, there are controversial opinions concerning the correlation between indicator microorganisms and specific foodborne pathogens and the adequacy of using indicators to highlight food safety aspects of fresh products (Leaman et al., 2014).

The purpose of the present study was to assess the microbial counts of specific indicator microorganisms (total coliforms and *Enterococcus*) to characterize different water sources use to irrigate leafy green in the Southern of Brazil. It was also intuited to assess the correlation between these specific microbial indicators and the presence of *Salmonella* spp. in water sources.

2. Material and Methods

2.1. Characterization of farms and sampling collection

During the period from July 2014 to August of 2015, 12 sampling collections were carried out in 4 small farms of cultivation of lettuces, located in the metropolitan area of Porto Alegre, Southern Brazil. All the producers used natural surface water sources for irrigation, i.e. natural ponds (Ponds) and streams bordering farmlands and urban areas (Streams). Details about the characteristics of the producers were previously described (Decol et al., 2017). Sampling was carried out following the methods described by Holvoet et al. (2014) and resulted in 138 samples from two different sources of irrigation water (116 ponds and 22 streams), and 81 samples of lettuce (55 irrigated by pond water and 26 irrigated by streams water), totalizing 219 samples, collected in one year. Approximately 18 samples were collected and analysed every month. After the collection, the samples were transported in thermal boxes at

refrigeration temperatures (< 7ºC) to the Food Microbiology and Food Control Laboratory (ICTA/UFRGS) and were analysed within 24 hours. Samples of 2 L of water were collected from each source (ponds and streams) in sterile glass jars. The samples were analysed following ISO 19459:2006 recommendations (ISO, 2006). For lettuce, 9 samples of approximately 100 g each were randomly collected from different locations of the fields, following a zig-zag pattern of collection starting from a randomly selected side of each field. In the laboratory, 3 samples of 100 g tissue were randomly pooled and 25 g of the pooled sample were microbiologically analysed following methods of AFNOR (2004).

2.2. Enumeration of total coliforms and Enterococcus spp.

The samples were analysed according to a protocol previously described by Holvoet et al., (2014) for the qualification of total coliforms and *Enterococcus*. Each water sample of 100 mL was vacuum pumped and filtered using a cellulose nitrate membrane (0.45µM of pore diameter, Microsart®, Sartorius, Brazil). Agar Chromocult (Merck, Brazil) incubated for 24 h at 37 °C was used for the enumeration of total coliforms, while Agar Enterococcosel (Oxoid, Basingstoke, Hampshire, UK) incubated for 24 h at 37 ºC was used for the enumeration of *Enterococcus*.

In order to quantify total coliforms, 25g of lettuce were added to 225 mL of 1% peptone water (Oxoid, Basingstoke, Hampshire, UK) and serial decimal dilutions were conducted. After that, 1 mL of each dilution was plated on Agar Chromocult (Merck, Brazil) which was incubated for 24 h at 37 °C. The limit of detection was 10 cfu/g for lettuce and 100 cfu/100mL for water samples.

2.3. Isolation of Salmonella

In a previous study, *E. coli* counts of the lettuce and water samples included in this study were assayed (Decol et al., 2017). Lettuce and water samples showing *E. coli* counts higher than 100 cfu/g or 100 mL in the previous study (Decol et al., 2017) were further evaluated for the presence of *Salmonella* spp..

For the analysis, 1 L of water was filtered using cellulose nitrate membranes (0.45µM pore diameter, Microsart®, Sartorius, Brazil). Afterwards, the membranes were placed in 225 mL of 1 % peptone water (Oxoid, Basingstoke, Hampshire, UK) and incubated for 18-24 h at 37 °C. For the detection of *Salmonella* on lettuce samples, 25 g of each lettuce were added to 225 mL of 1 % peptone water (Oxoid, Basingstoke, Hampshire, UK), and incubated for 18-24 h at 37 °C. Next, the samples were analyzed by the Molecular Detection System (MDS, 3M™), according to the manufacturer's instructions and previous studies of Loff et al. (2014). Samples presenting positive results with MDS were confirmed through ISO 6579:2007 and conventional PCR analysis by Zhang et al., (2008) with modifications. For the PCR, the genomic DNA of the environment samples was extracted using the Master Pure™ Complete DNA and RNA purification kit (Epi-center, Madison, USA), following the manufacturer's instructions.

2.4. Statistical analyses

Non-zero microbial loads were log-transformed and stored along with zero counts in an Excel spreadsheet (Microsoft Corporation, Redmon,WA, USA). Results were compiled and graphs were made using Sigma Plot 12.0 Systat Software, Inc. (Addilink Software Scientific, S.L. Barcelona). For calculation and graphical presentation of the median of microbial counts, only positive samples (i.e., with numbers above the

detection limit) were included. All analyses were performed with IBM SPSS Statistics 21 at a significance level of 5% (p = 0.05). Mann-Whitney U and Kuskal-Wallis tests were used, respectively, in order to determine the difference between positive counts of indicators and the presence/absence of *Salmonella*. The Pearson correlation coefficient was calculated ($p<0.01$) between total coliforms in irrigation water and lettuce samples.

3. Results and Discussion

3.1. Enumeration of total coliforms and Enterococcus spp. in different water sources used as irrigation water

A 100 % prevalence of total coliforms and *Enterococcus* spp. was found in the 138 samples of irrigation water. The counts ranged from 2.48 to 6.61 log cfu/100mL and 2.00 to 5.82 log cfu/100mL, respectively (**Fig. 1** and **2**). Total coliforms results are similar to those reported by Rodrigues et al. (2014) and Castro-Ibanez et al. (2015a), in Southern Brazil and in Spain, respectively, who found 100 % of irrigation water samples were positive for total coliforms. Marine et al., (2015), in the mid-Atlantic region of The United States, evaluated the prevalence of total coliforms in irrigation waters source (ponds and rivers) from 32 farms (organic and conventional farms) and detected a 70.8% prevalence. Already, Holvoet et al. (2014), in Belgium, analyzed 120 samples of irrigation water from open wells and bore holes and reported a prevalence of 30% for total coliforms, with median counts of 1.7 log cfu/100mL.

The differences between the prevalence for total coliform already reported in the literature is probably due to the type of irrigation water source analysed. For example, Rodrigues et al. (2014), Castro-Ibanez et al. (2015a) and Marine et al. (2015) analysed different types of superficial water, which were exposed to several sources of contamination (e.g. animals, soil, wind, etc.). However, Holvoet et al. (2014) analysed

water from wells and bore holes, which is considered less risky water sources regarding microbiological contamination (Castro-Ibanez et al., 2015b; Ahmed et al., 2012; Ferguson et al., 2012).

The prevalence of *Enterococcus* spp. found in the present work was higher than the one reported by Castro-Ibanez et al. (2015a) in Spain. The authors indicated a prevalence of 60 % and average counts around 1.1 log cfu/100 mL. In Australia, Ahmed et al. (2012), analised 50 different rainwater tanks for *Enterococcus* spp. and reported a prevalence of 98%. In the mid-Atlantic region of United States, Micallef et al. (2013) investigated the diversity, distribution and antibiotic resistance of *Enterococcus* spp. in large and small-scale farms of tomatoes, and reported a prevalence of 29% for *Enterococcus* spp. in different types of water sources used (pond and ground water). The same authors also reported that the highest concentrations of *Enterococcus* spp. occurs on the small-scale farms. The differences in agricultural practices between large and small-scale production systems may account for these disparities. Some of these differences included fertilization methods, use of protection systems and the accessibility of wild and domestic animals to the field.

The present study analysed small properties with showed several of the above mentioned characteristics, including uncontrolled sources of fertilization and access of wild and domestic animals to the field. These might have influenced the high prevalence of indicator microorganisms determined in the tested samples.

Enterococci are enteric, commensal bacteria that colonize the digestive tracts of a wide range of vertebrate hosts and are, therefore widespread in the environment and in agricultural settings (Maheux et al., 2011; Fisher and Phillips, 2009; Wyn-Jones et al., 2001; Franz et al., 1999). The United States Environmental Protection Agency (USEPA, 2002) includes the enumeration of enterococci as an indicator of fecal pollution, which

can be linked to the presence of enteric pathogens in recreational waters. These microorganisms are also used as indicators of microbiological quality of fresh produce (Ailes et al., 2008; Johnston et al., 2006). However, the usefulness of enterococci as indicators of the risk of waterborne diseases for humans is limited by their broad environmental distribution. Thus, environmental water quality assessment may benefit from focusing on a group of *Enterococcus* spp. that is associated with sources of fecal pollution rather than relying on the entire *Enterococcus* genus (Bonds et al., 2006; Frahm, E. and Obst, U., 2003).

Enterococcus species that can be associated with fecal pollution are *E. faecalis* and *E. faecium* and they have been consistently identified as predominant enterococcal species in warm-blooded animal feces and sewage, but not from environmental sources (Castillo-Rojas et al., 2013). In the present study, enumeration of the *Enterococcus* group was performed without differentiation of the species. Therefore confirmation of the fecal origin of the positive samples was not performed. The results obtained did not show significant differences between the different water sources used for irrigation for *Enterococcus* spp. counts (**Fig. 2).**

The concentration of *Enterococcus* in human and animal feces is smaller than the concentration of other indicator microorganisms, such as *E. coli* (Byappanahalli et al., 2012) and the survival of each group is different and depends on several factors (Aragonés et al., 2016; Byappanahalli et al., 2012). Some authors reported that *Enterococcus* spp. present a more demanding metabolism than *E. coli* and, in most cases, they cannot multiply in aquatic environments, however these microorganisms are more resistant to environmental factors than *E. coli* (Maheux et al., 2011; WHO, 1996). Based on this, some studies associate the presence of *E. coli* in water with recent fecal

contamination, while *Enterococcus* are associated with older contamination of the water source (Da Silva et al., 2008; Leclerc et. al., 2002; Edberg et al., 2000).

The high prevalence of indicator microorganisms in both sources of irrigation water evaluated in the present study confirms the hypothesis that surface water sources with no protection or treatment are the ones which present higher risk of microbiological contamination (Uyttendaele et al., 2015; Ahmed et al., 2012; Ferguson et al., 2012). Despite this risk of contamination has already been identified in several studies (Castro-Ibanez et al., 2015b; Holvoet et al., 2014; Park et al., 2014; Ceuppens et al., 2014; Ahmed et al., 2012; Ferguson et al., 2012; Arthur et al.,2004), surface water still is the most common source of water for irrigation (EFSA, 2014; Rodrigues et al., 2014; Gorski, et al., 2011).

3.2. Enumeration of total coliforms on lettuces and their correlation with its presence in irrigation waters

The prevalence of total coliforms on the 81 lettuce samples was100 %, presenting counts from 1.3 to 8.1 log cfu/g (**Fig. 3)**. In the Southern of the United States, Johnston et al. (2005) evaluated the quality of fresh produce in different stages of production and identified counts ranging from 1.0 to 3.5 log cfu/g. Marine et al. (2015) in addition to the water source evaluated for total coliforms, evaluated these indicator microorganisms on leafy greens and found a prevalence of 60.6%. In the same region, the mid-Atlantic region in The United States, Pagadala et al. (2015) evaluated 259 tomato samples collected in organic and conventional farms and detected a prevalence of 90.3% for total coliforms.

Despite the high prevalence found in the present study, the high levels of total coliform counts cannot be directly correlated with an increase if food safety risk.

Differences in total coliforms counts can be attributable to differences in soil management practices. One example is the case of farms that use organic manure as fertilizers, which helps to preserve natural nutrients in the soil and thus preserves the soil microorganism ecosystem (Pagadala et al., 2015; Mendes et al, 2013).

A good correlation between the total coliform counts found in irrigation water and levels of total coliforms found on lettuces was demonstrated (p=0.429) (**Fig. 3**). These findings confirm that irrigation water is an important vehicle of microorganisms in the production of leafy greens.

3.3. Presence of Salmonella spp. and correlation with indicator microorganisms

In the present study, samples showing levels of *E. coli* higher than 2 log cfu/g or 100 mL where further investigated for the presence of pathogens. The correlation of generic *E. coli* counts higher than 100 cfu/g or 100 mL with the presence of foodborne pathogens in environmental samples was demonstrated by several studies (Castro-Ibáñez et al, 2015a; Ceuppens et al., 2015; Holvoet et al., 2014; Johannessen et al., 2014; Park et al., 2014). However, in a previous study published by Decol et al., 2017, samples showing levels of *E. coli* counts higher than 2 log cfu/g or 100 mL did not correlate with the presence of *E. coli* O157:H7 in irrigation water samples.

In this study, irrigation water and lettuce samples (62 samples of water and 27 of lettuces) were analyzed for the presence of *Salmonella* spp.. Prevalence was 6.4 % for irrigation water and 14.8 % for lettuce samples (**Table 1**). This *Salmonella* spp. prevalence was higher than that prevously reported by Rodrigues et al. (2014), who found a prevalence of 1.9 % in irrigation water samples and 1.3 % on lettuce samples collected also at small farms in Southern Brazil. Lower prevalence was also reported by Castro-Ibáñez et al. (2015b) and Ceuppens et al. (2015), which showed a *Salmonella*

prevalence of 2 % and 3.1 % on lettuces in Spain and Belgium, respectively. On the other hand, Abdel-Moneim et al. (2014) reported that 42 % and 39 % of irrigation water samples and lettuce samples were contaminated with *Salmonella* spp. in Egypt. High prevalence of pathogens such as *Salmonella*, in surface water sources represent a great risk of contamination, and this risk increases in the case of vegetables like lettuces that are consumed raw after being irrigated. This is even more evident if sprinkler irrigation is used as contaminated water comes into contact with the edible parts of the plant. In the present study, the use of sprinkler irrigation versus drip irrigation, may have influenced the high prevalence of *Salmonella* spp. of the sampled lettuces.

The presence of *Salmonella* in irrigation water found in our study confirms that irrigation water is an important risk factor for the introduction of pathogens in the primary production of leafy vegetables consumed raw (Castro-Ibáñez et al, 2015b; Ceuppens et al., 2014; Pachepsky et al., 2011; Park et al., 2012; Harwood et al., 2005; Schets et al., 2005). However, of the 10 positive samples for *Salmonella* spp. identified by molecular methods (MDS^{TM} and conventional PCR), only 1 sample of irrigation water was confirmed by isolation on culture media (**Table 1**). This fact has been previously discussed in other studies, where environment and fresh produce samples are analyzed, highlighting the challenge in recovering viable cells. These studies reported that the presence of indigenous competing microbiota on selective agars can inhibit the growth of pathogenic microorganisms (Castro-Ibáñez et al., 2015a; Delbeke et al., 2015) or stressed cells cannot be able to grow because they are in a "viable but not cultivable" state.

Our results demonstrated a positive correlation between the counts of *Enterococcus* spp. (4 to 5 log cfu/ 100mL) and *Salmonella* (Mann-Whitney U Test, $p<0.05$) (**Fig. 4**). This correlation was not identified in other studies (Castro-Ibáñez et

al, 2015a; Holvoet et al., 2014; Rodrigues et al., 2014). Even though other studies have identified the *Enterococcus* spp. as a good fecal indicator for water samples (Schets et al., 2010; Sazakli et al., 2007), in the present work the identification of the *Enterococcus* species was not performed, and because of that we cannot suggest a potential source of contamination.

This study found a significant positive correlation (Mann-Whitney U Test, $p<0.05$) between the presence of *Salmonella* and the counts of generic *E. coli* counts. Other studies already reported this positive correlation between the presence of enteric pathogens and counts of generic *E. coli* (Castro-Ibáñez et al, 2015a; Ceuppens et al., 2015; Holvoet et al., 2014; Johannessen et al., 2014).

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 (Wilkes et al., 2009; Harwood et al., 2005; Schets et al., 2005). Variations in pathogen input as the prevalence in population water and resistance to environmental conditions (Payment and Locas, 2011). As a result, there is clearly no indicator that may be suitable for all pathogens for all environmental scenarios (Yates, 2007; Harwood et al., 2005). However, the probability of detection of any pathogen is higher at higher levels of indicators (Holvoet et al., 2014; Savichtcheva and Okabe, 2006).

4. Conclusion

The results obtained in the present study confirmed irrigation water as an important source of microbial contamination in the primary production of lettuces. The two water sources showed high levels of total coliforms and *Enterococcus* spp.. High prevalence of *Salmonella* spp. was also found in irrigation waters, suggesting an important impact on safety of lettuces. The positive correlation between high counts of

Enterococcus with the presence of *Salmonella* was demonstrated, suggesting that *Enterococcus,* besides *E. coli,* can be a good indicator for the presence of *Salmonella* in irrigation water.

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References

Abdel-Moneim, A., Ceuppens, S., El-Tahan, F., Uyttendaele, M., 2014. Microbiological safety of strawberry and lettuce during primary production and retail in Egypt. J. Food Process. Technol. 5,308.

Ahmed, W., Richardson, K., Sidhu, J. P. S., Toze, S., 2012. *Escherichia coli* and *Enterococcus* spp. in rainwater tank samples: comparison of culture-based methods and 23S rRNA gene quantitative PCR assays. Environ. Sci. Technol. 46, 11370−11376.

Ailes, E.C., Leon, J.S., Jaykus, L.A., Johnston, L.M., Clayton, H.A., Blanding, S., Kleinbaum, D.G., Backer, L.C., Moe, C.L., 2008. Microbial concentrations on fresh produce are affected by postharvest processing, importation, and season. J. Food Protec. 71, 2389–2397.

Allende, A., Monaghan, J.M., 2015. Irrigation water quality for leafy crops: A perspective of risks and potential solutions. Int. J. Environ. Health. Res. 12, 7457−7477.

Aragonés, L., López, I., Palazón, A., López-Úbeda, R., García, C., 2016. Evaluation of the quality of coastal bathing waters in Spain through fecal bacteria *Escherichia coli* and *Enterococcus*. Sc. Total Env., 566–567, 288–297.

Arthur, L., Jones, S., Fabri, M., Odumeruz, J. 2007. Microbial survey of selected Ontario- rown fresh fruits and vegetables. J. Food Protec. 70, 2864–2867.

Benjamin, L., Atwill, E.R., Jay-Russell, M., Cooley, M., Carychao, D., Gorski, L., Mandrell, R.E., 2013. Occurrence of generic *Escherichia coli*, *E. coli* O157 and *Salmonella* spp. in water and sediment from leafy green produce farms and streams on the Central California coast. Int. J. Food Microbiol. 165, 65–76.

Bonds, B., Christensen, A.B., Flaherty, D.K., 2006. *Enterococci* species in Gulf Coast marine water samples as measured by the Environmental Protection Agency Method 1600. Tex. J. Sci. 58, 141–146.

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., Harwood, V.J., 2012. *Enterococci* in the environment. Microbiol. Mol. Biol. Rev. 76 (4), 685–706.

Castillo-Rojas, G., Mazari-Hiríart, M., de León, S.P., Amieva-Fernández, R.I., Agis-Juárez, R.A., Huebner, J., López-Vidal, Y., 2013. Comparison of *Enterococcus faecium* and *Enterococcus faecalis* Strains Isolated from Water and Clinical Samples: Antimicrobial Susceptibility and Genetic Relationships. *PLoS ONE*, *8*(4), 59491.

Castro-Ibáñez, I., Gil, M.I., Tudela, J.A., Ivanek, R., Allende, A., 2015a. Assessment of microbial risk factors and impact of meteorological conditions during production of baby spinach in the Southeast of Spain. Food Microbiol. 49, 173−181.

Castro-Ibáñez, I., Gil, M.I., Tudela, J.A., Allende, A., 2015b. Microbial safety considerations of flooding in primary production of leafy greens: A case study. Food Res. Int. 68, 62−69.

Centers for Disease Control and Prevention, 2014. Surveillance for Foodborne Disease Outbreaks, United States (2012). Annual Report. US Department of Health and Human Services, Atlanta, Georgia. Available at: http://www.cdc.gov/ foodsafety/pdfs/foodborne-disease-outbreaks-annual-report-2012-508c.pdf (Acessed January 2016).

Ceuppens, S., Hessel, C.T., Rodrigues, R. de Q., Bartz, S., Tondo, E.C., Uyttendaele, M., 2014. Microbiological quality and safety assessment of lettuce production in Brazil. Int. J. Food Microbiol. 181, 67–76.

Ceuppens, S., Johannessen, G.S., Allende, A., Tondo, E.C., El-Tahan, F., Sampers, I., Jacxsens, L., Uyttendaele, M. 2015. Risk factors for *Salmonella*, Shiga Toxin-Producing *Escherichia coli* and *Campylobacter* occurrence in primary production of leafy greens and strawberries. Int. J. Environ. Res. Public Health, 12, 9809–9831.

Da Silva, M.E.Z., Santana, R.G., Guilhermetti, M., Filho, I.C., Endo, E.H., Ueda-Nakamura, T. 2008. Comparison of the bacteriological quality of tap water and bottled mineral water. Int. J. Hyg. and Environ. Health. 211, 504−509.

Decol, L.T., Casarin, L.S., Hessel, C.T., Batista, A.C.F., Allende, A., Tondo, E.C. 2017. Microbial quality of irrigation water used in leafy green production in Southern Brazil and its relationship with produce safety. Food Microbiol. 65, 105−113.

Delbeke, S., Ceuppens, S., Hessel , C.T., Castro-Ibanez, I., Jacxsens, L., De Zutter, L., Uyttendaele, M. 2015. Microbial safety and sanitary quality of strawberry primary production in Belgium: Risk factors for *Salmonella* and Shiga toxin producing *Escherichia coli* (STEC) contamination. Appl. Environ. Microb., 81, 2562–2570.

Edberg, S.C., Rice, E.W., Karlin, R.J., Allen, M.J. 2000. *Escherichia coli*: the best biological drinking water indicator for public health protection. J. of Appl. Microbiol. 88, 106−116.

EFSA Panel on Biological Hazards (BIOHAZ), 2014. Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* and Norovirus in leafy greens eaten raw as salads). EFSA Journal, 11, 3600. Available at: www.efsa.europa.eu/efsajournal (Acessed February 2016).

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA Journal, 13, 4329. Available at: 10.2903/j.efsa.2015.4329 (Acessed February 2016).

Fisher, K., Phillips, C., 2009. The ecology, epidemiology and virulence of *Enterococcus*. J. Microbiol. 155, 1749–1757.

Ferguson, A.S., Layton, A.C., Mailloux, B.J., Culligan, P.J., Williams, D.E., Smartt, A.E., Sayler, G.S., Feighery, J., Mckay, L.D., Knappett, P.S.K., Alexandrova E., Arbit, T., Emch, M., Escamilla, V., Ahmed, K.M., Alam, MD.J., Streatfield, P.K., Yunus, M., Geen, A.V., 2012. Comparison of fecal indicators with pathogenic bacteria and rotavirus in groundwater. Sci. Total Environ. 431, 314–322.

Frahm, E., Obst, U., 2003. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. J. Microbiol. Methods, 52, 123–131.

Franz, C.M.A.P., Stiles, M.E., Schleifer, K.H., Holzapfel, W.H., 2003. *Enterococci* in foods-A conundrum for food safety. Int. J. Food Microbiol. 88, 105–122.

Gil, M.I.; Selma, M.V.; Suslow, T.; Jacxsens, L.; Uyttendaele, M.; Allende, A. 2015. Pre-and Postharvest Preventive Measures and Intervention Strategies to Control

Microbial Food Safety Hazards of Fresh Leafy Vegetables. Crit. Rev. in Food Sci. and Nut. 55, 453–468.

Gorski, L., Parker, C.T., Liang, A., Cooley, M.B., Jay-Russell, M.T., Gordus, A.G., Atwill, E.R., Mandrell, R.E. 2011. Prevalence, Distribution, and Diversity of *Salmonella enterica* in a Major Produce Region of California. Appl. Environ. Microbiol. 77(8), 2734.

Harwood, V.J., Levine, A.D., Scott, T.M., Chivukula, V., Lukasik, J., Farrah, S.R., 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl.Environ. Microbiol. 71, 3163–3170.

Holvoet, K., Sampers, I., Seynnaeve, M., Uyttendaele, M., 2014. 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. Int. J. Food Microbiol. 171, 21–31.

Ingham, S.C., Fanslau, M.A., Engel, R.A., Breuer, J.R., Breuer, J.E., Wright, T.H., Reith-Rozelle, J.K., Zhu, J. 2005. Evaluation of fertilization-to-planting and fertilization-to-harvest intervals for safe use of noncomposted bovine manure in Wisconsin vegetable production. J. Food Prot. 68, 1134–1142.

ISO, 2006. Water quality. sampling for microbiological analysis. ISO 19458:2006.

Johannessen, G.S., Loncarevic, S., Kruse, H. 2002. Bacteriological analysis of fresh produce in Norway. Int. J. Food Microbiol. 77, 199–204.

Johannessen, G., Uyttendaele, M., Ceuppens, S. 2014. Microbial safety and hygiene of fresh produce. Veg-i-Trade Closing Event, Taking safety of fresh produce to the next level. Brussels, 11-12 June.

Johnston, L. M., L. A. Jaykus, D. Moll, J. Anciso, B. Mora, and C. L. Moe. 2006. A field study of the microbiological quality of fresh produce of domestic and Mexican origin. Int. J. Food Microbiol. 112:83–95.

Johnston, L.M., Jaykus, L.A., Moll, D., Martinez, M.C., Anciso, J. Mora, B., Moe C.L. 2005. A field study of the microbiological quality of fresh produce. J. Food Prot. 68, 1840–1847.

Leaman, S., Gorny, J., Wetherington, D., Bekris, H. 2014. CPS symposium: agricultural water: five year research review. Center for Produce Safety, Davis, California. Available at: http://www.pma.com/~/media/pma-files/food-safety/cps/cps-research-symposium-water-report72014.pdf?la=en (Acessed January 2016).

Leclerc, H., Moreau, A. 2002. Microbiological safety of natural mineral water. FEMS Microb. Rev. 26, 207–222.

Loff, M., Mare, L., Kwaadsteniet, M., Khan, W., 2014. 3M™Molecular Detection system versus MALDI-TOF mass spectrometry and molecular techniques for the identification of *Escherichia coli* 0157:H7, *Salmonella* spp. & *Listeria* spp. J. Microbiol. Methods. 101, 33–43.

Marine, S.C., Pagadala, S., Wangd, F., Pahl, D.M., Melendez, M.V., Klinef, W.L., Oni, R.A., Walsh, C.S., Everts, K.L., Buchanan, R.L., Micallef, S.A. 2015. Growing season, but not farming system, a food safety risk determinant for leafy greens in the mid-Atlantic region. Appl. Environ. Microbiol. 81, 2395–2407.

Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.L. T., Huppé, V., Bérubé, E., Boudreau, D.K., Picard, F. J., Huletsky, A., Bergeron, M.G. 2011. Method for rapid and sensitive detection of *Enterococcus* sp. and *Enterococcus faecalis*/*faecium* cells in potable water samples. Water Research 45, 2342− 2354.

Mendes, R., Garbeva, P., Raaijmakers, J.M., 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol. Rev. 37, 634–663.

Micallef, S.A., Goldstein, R.E. R., George, A., Ewing, L., Tall, B.D., Boyer, M.S., Joseph, S.W., Sapkota, A.R. 2013. Diversity, distribution and antibiotic resistance of *Enterococcus* spp. recovered from tomatoes, leaves, water and soil on U.S. Mid-Atlantic farms. Food Microbiol. 36, 465–474.

Pachepsky, Y., Shelton, D.R., McLain, J.E.T., Patel, J., Mandrell, R.E., 2011. Irrigation waters as a source of pathogenic microorganisms in produce: a review. In: Donald, L.S. (Ed.), Advances in Agronomy, vol. 113. Academic Press, pp. 73–138.

Pagadala, S., Marine, S.C., Micallef, S.A., Wang, F., Pahl, D.M., Melendez, M.V., Kline, W.L., Oni, R.A., Walsh, C.S., Everts, K.L., Buchanan, R.L., 2015. Assessment of region, farming system, irrigation source and sampling time as food safety risk factors for tomatoes. Int. J. Food Microbiol. 196, 98–108.

Park, S., Szonyi, B., Gautam, R., Kendra, N., Anciso, J., Ivanek, R., 2012. Risk factors for microbial contamination in fruits and vegetables at the preharvest level: a systematic review. J. Food Prot. 75, 2055–2081.

Park, S., Navratil, S., Gregory, A., Bauer, A., Srinath, I., Szonyi, B., Nightingale, K., Anciso, J., Jun, M., Han, D., Lawhon, S., Ivanek, R., 2014. Farm management, environment, and weather factors jointly affect the probability of spinach contamination by generic *Escherichia coli* at the preharvest stage. Appl. Environ. Microbiol. 80, 2504–2515.

Payment, P., Locas, A., 2011. Pathogens in water: value and limits of correlation with microbial indicators. Ground Water, 49, 4–11.

Rodrigues, R.Q.L.M.R., Paula,C.M., Hessel, C.T., Jacksens, L., Uyttensaele, M., Bender,R.J., Tondo, E.C., 2014. Microbiological contamination linked to implementation of good agricultural practices in the production of organic lettuce in Southern Brazil. Food Control, 42, 152–164.

Savichtcheva, O., Okabe, S., 2006. Alternative indicators of fecal pollution: relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. Water Research, 40, 2463–2476.

Sazakli, E., Alexopoulos, A., Leotsinidis, M., 2007. Rainwater harvesting, quality assessment and utilization in Kefalonia Island, Greece. Water Res. 41, 2039–2047.

Schets, F.M., Italiaander, R., Van den Berg, H.H., De Roda Husman, A.M., 2010. Rainwater harvesting: quality assessment and utilization in The Netherlands. J. Water Health 8, 224–235.

Sivapalasingam, S., Friedman, C.R., Cohen, L., Tauxe, R.V. 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. J. Food Prot. 67, 2342–2353.

USEPA (Environmental Protection Agency Office of Water). 2002. Method 1106.1: Enterococci in Water by Membrane Filtration Using Membrane-Enterococcus-Esculin Iron Agar (mE-EIA). EPA 821-R-02e021. U.S., Washington, DC.

Uyttendaele, M., Jaykus, L.A., Amoah, P., Chiodini, A., Cunliffe, D., Jacxsens, L., Holvoet, K., Korsten, L., Lau, M., McClure, P., Medema, G., Sampers, I., Jasti, P.R., 2015. Microbial hazards in irrigation water: standards, norms, and testing to manage use of water in fresh produce primary production. Comp. Rev. Food Sci. Food Saf. 14. 336–356.

WHO (World Health Organization). Guidelines for drinking water quality. 1996. Health criteria and other supporting information. 2nd ed. Vol. 2. Geneva.

Wilkes, G., Edge, T., Gannon, V., Jokinen, C., Lyautey, E., Medeiros, D., Neumann, N., Ruecker, N., Topp, E., Lapen, D.R, 2009. Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium oocysts*, *Giardia cysts*, and hydrological indices for surface waters within an agricultural landscape. Water Research, 43, 2209-2223.

Wyn-Jones, A., Sellwood, J., 2001. Enteric viruses in the aquatic environment. J. Appl. Microbiol. 91, 945–962.

Yates, M.V., 2007. Classical indicators in the 21st century--far and beyond the coliform. Water Envir. Res., 79, 279–286.

Zhang, D., Zhang, H., Yang, L., Guo, J., Li, X., Feng, Y. 2009. Simultaneous detection of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica* and *Escherichia coli* O157:H7 in food samples using multiplex PCR method. J. Food Saf. 29, 348–363.

Table 1. Prevalence of *Salmonella* spp. in samples from irrigation water and fresh lettuce.

Sample type	***Salmonella* spp.**		
Irrigation Water	**MDS™**	**PCR**	**Confirmed[a]**
Pond	4/50	4/4	1/4
Streams	2/12	2/2	0/4
Lettuce	**MDS™**	**PCR**	**Confirmed[a]**
Pond	2/12	2/2	0/2
Streams	2/15	2/2	0/2

[a]Samples were confirmed by isolation in selective culture media and conventional PCR.

Figures

Figure 1. Boxplots representing (light grey) counts of Total Coliforms (log cfu/100mL) in positive samples of ponds source water and (dark gray) counts of Total Coliforms (log cfu/100mL) in positive samples of streams source water. In this study, we considered as positive, samples with counts above the detection limit (0 log cfu/100). In boxplot, the lower and upper parts of the boxes represent the quartiles (25th and 75th percentile), with the inner line in the box representing the median. Statistical differences determined by the statistical test Mann-Whitney ($p<0.005$) are represented by different letters.

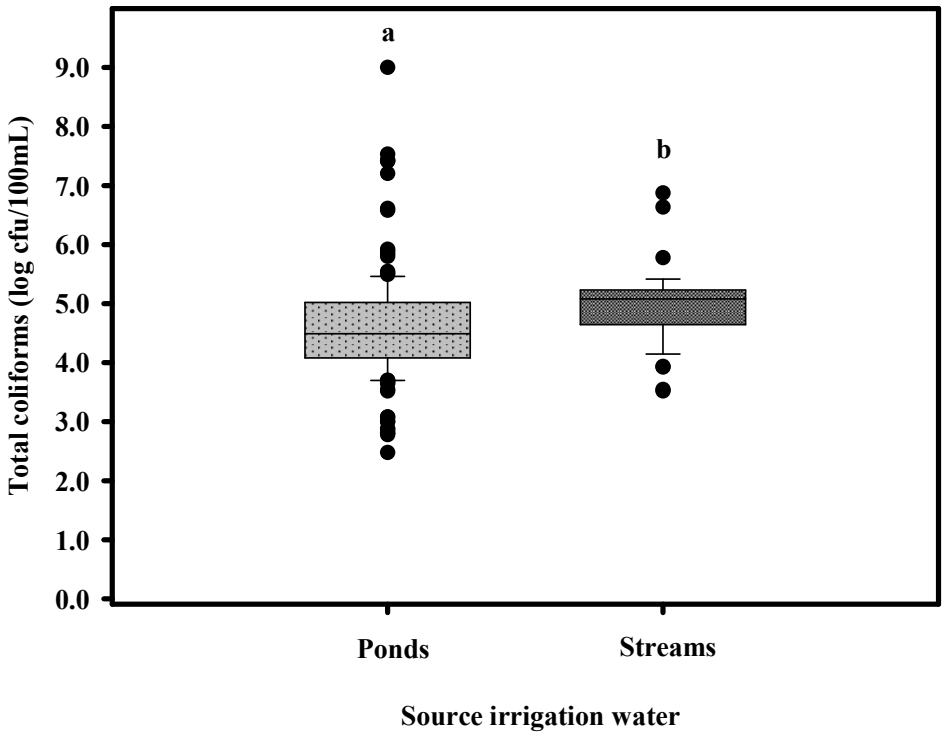


Figure 2. Boxplots representing (light grey) counts of *Enterococcus* (log cfu/100mL) in positive samples of ponds source water and (dark gray) counts of *Enterococcus* (log cfu/100mL) in positive samples of streams source water. In this study, we considered as positive, samples with counts above the detection limit (0 log cfu/100). In boxplot, the lower and upper parts of the boxes represent the quartiles (25th and 75th percentile), with the inner line in the box representing the median. Statistical differences determined by the statistical test Mann-Whitney ($p<0.005$) are represented by different letters.

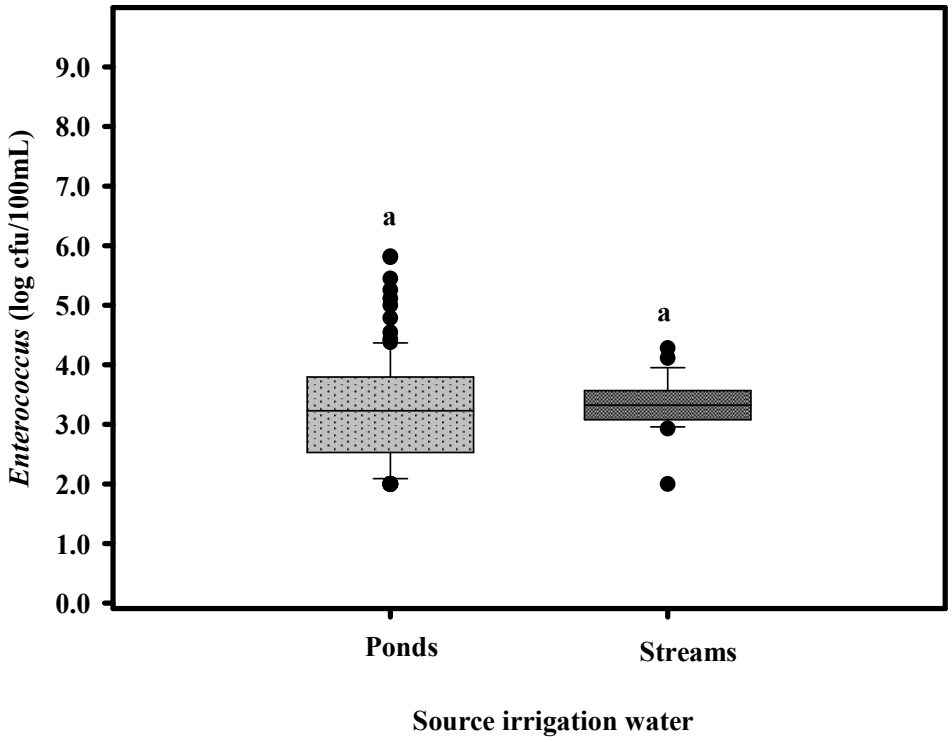


Figure 3. Scatter plots representing the correlation between the counts of Total Coliforms in irrigation water (log cfu/100mL) and lettuce (log cfu/g). Confidence interval 95% and central regression line are represented.

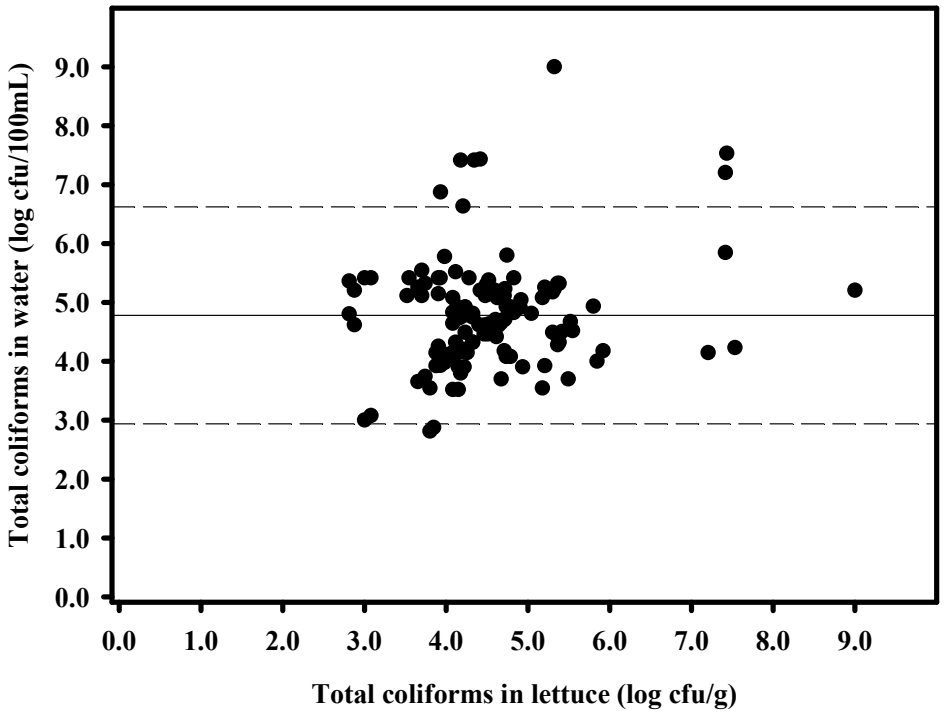
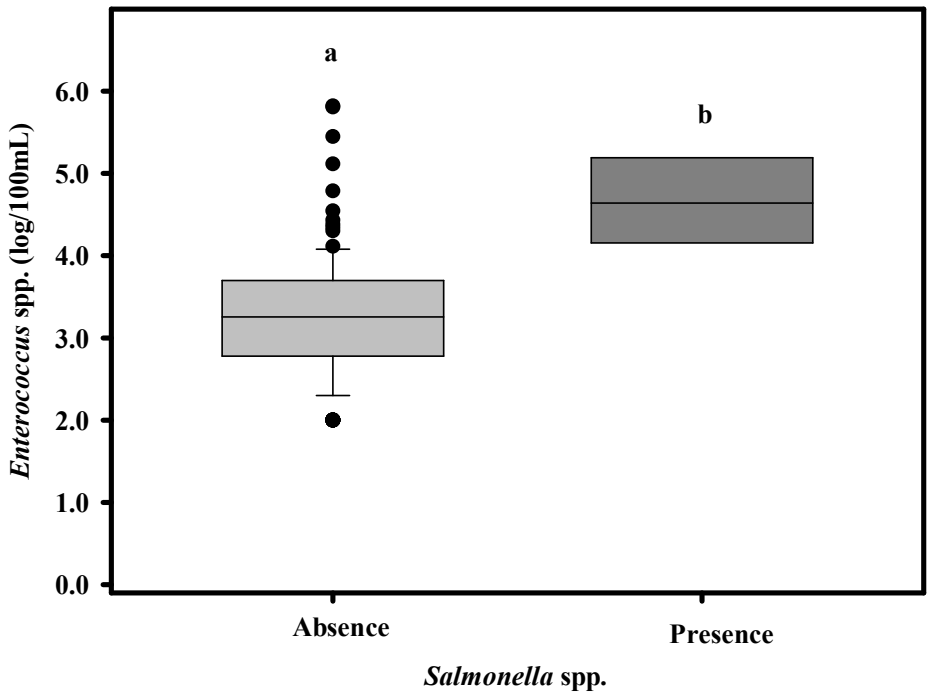


Figure 4. Boxplots representing *Enterococcus* spp. counts (log cfu/100mL) and detection of *Salmonella* spp. in positive irrigation water and lettuce samples by MDS^{TM}. In this study, positive samples are defined as samples contaminated above detection limit (0 log cfu/100). In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median.



4.1 Artigo 3

Running Title: ClO_2 disinfection of wastewater to be used as agricultural water.

Suitability of chlorine dioxide as a tertiary treatment for municipal wastewater and use of reclaimed water for overhead irrigation of baby lettuce

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ABSTRACT

Reclaimed wastewater used for agricultural irrigation should meet specific microbiological standards in order to avoid contamination of the irrigated produce. Different disinfection treatments (e.g. chlorine dioxide (ClO_2)) can be used as tertiary treatments to improve the microbiological quality of secondary treated wastewater. The objective of this study was to evaluate the suitability of ClO_2 for the treatment of secondary treated municipal wastewater and its use for overhead irrigation of greenhouse grown baby lettuce. The impact of reclaimed water in commercially grown baby lettuce was evaluated considering *E. coli* concentration, the presence of pathogenic bacteria, and the occurrence of specific disinfection by-products (i.e. chlorates) in water and baby lettuces. *E. coli* was quantified in all samples using both conventional plating methods and a quantitative real time PCR (qPCR) method with propidium monoazide (PMA) pre-treatment to differentiate between viable and death bacteria. Concentration of cultivable *E. coli* was significantly lower ($p < 0.05$) in tertiary-treated reclaimed water using ClO_2 (ClO_2W) when compared with secondary-treated municipal wastewater (SW). However, no significant differences were observed between treatments when *E. coli* loads were quantified using the PMA-PCR method. These results could indicate that ClO_2 treatment of wastewater water did not kill the bacteria present in the water but it induced bacteria into a VBNC state in which the bacteria are dormant and no longer form colonies. The proportion of samples positive for the presence of pathogenic bacteria was lower in ClO_2W (1 out of 8) compared with SW (7 out of 8). In baby lettuce samples, analyzed by plating during the last sampling days (4 and 0 days before harvest), significantly lower *E. coli* counts ($p < 0.05$) were detected in plants irrigated with ClO_2W compared with those irrigated using SW. A link between higher *E. coli* counts and the presence of pathogens was observed when lettuce samples were analyses

by the PMA-qPCR technique (Mann-Whitney U Test, $p<0.05$). When the accumulation of chlorates was evaluated, baby lettuce irrigated with ClO_2W showed significantly higher concentration of chlorates that lettuce irrigated with SW. Based on the obtained results, when conventional plating quantification methods are use, ClO_2 seems to be a suitable disinfection treatment to reduce microbial loads in secondary-treated wastewater under the conditions of the present study. However, when molecular methods are used to enumerate viable bacteria, its efficacy seems to be overestimated. Additionally, accumulation of chlorates in the tissue might represent a chemical risk for consumers.

Keywords: *Fresh produce; fruits and vegetables; agricultural water; water disinfection; foodborne pathogens; disinfection by-products; chemical risk.*

1. Introduction

Water reclamation and reuse for irrigated agriculture are priority innovation areas. In the European Commission (EC), water reuse is indicated as an important topic for the circular economy, a regenerative system in which resource input and waste, emission, and energy leakage are minimized. Currently, reclaimed water is mostly used in agricultural irrigation, especially in semi-arid and arid regions to overcome water scarcity (Becerra-Castro et al., 2015). Limited awareness of potential benefits among stakeholders and the general public, and lack of a supportive and coherent framework for water reuse are major reason for reducing this practice in the EU (EC, 2017a). Wastewater usually contains pathogenic microorganisms, many of which are able to survive in the environment and be transmitted to humans (EPA, 2004; Steele & Odumeru, 2004; Uyttendaele et al., 2015; López-Gálvez et al. 2016). Although reclamation treatments can improve the microbiological quality of water, the effluents of wastewater treatment plants can be vehicle of microbiological and chemical hazards that can affect the safety of irrigated vegetables (Pérez-Sautu et al., 2012). In fact, microbiological safety of irrigation water is one of the most important factors to be considered for the safe production of leafy greens (Allende & Monaghan, 2015; Uyttendaele et al., 2015; Decol et al., 2017).

Chemical disinfection treatments are often used as tertiary treatments for the reclamation of municipal wastewater but also to ensure the safety of surface water used for agricultural irrigation. Among the chemical disinfectants, chlorine is one of the most commonly used biocides for irrigation water disinfection. However, chlorine is highly reactive with organic compounds and causes the generation of toxic and carcinogenic disinfection by-products (e.g. chlorates and trihalomethanes) (Ayyildiz, et al., 2009; Nikolaou & Lekkas, 2001; Rodriguez & Serodes, 2001). Chlorine dioxide (ClO_2) has

been defined as a potential alternative to chlorine for disinfection of agricultural water. It presents lower reactivity with organic compounds in wastewater (Veschetti et al., 2003; Van Haute et al., 2015; Van Haute et al., 2017), and higher oxidation capacity, increasing the bactericidal capability (Hassemberg et al., 2017). Furthermore, ClO_2 leads to the formation of less halogenated DBPs than chlorine (López-Gálvez et al., 2010). However, the use of ClO_2 can lead to the presence of other DBPs such as chlorites (ClO_2^-) and chlorates (ClO_3^-) in the treated water (Gil et al., 2016). Bactericidal effectiveness of ClO_2 depends on several factors, including disinfectant dose, contact time, water temperature, pH, and organic load (Junli et al., 1997; Ayyildiz, et al., 2009).

Currently, drip and sprinkler irrigation are the most used irrigation systems in commercial growing systems (Oron, 2002; Pachepsky et al., 2011). Current Spanish and US legislation classify reclaimed water based on specific microbiological standards and each category is allowed to be used for specific crops and irrigation systems (Real Decreto 1620/2007, 2007). For instance, only reclaimed water with less than 2 log CFU *E. coli* / 100 mL can be applied in direct contact with the edible part of the crop using overhead irrigation.

The aim of present study is to evaluate the suitability of ClO_2 for the reduction of the microbiological contamination present in secondary-treated wastewater used for overhead irrigation of commercially grown baby lettuce. We assessed the effect of ClO_2 on *E. coli* concentration as fecal contamination indicator, and on the presence of the bacterial pathogens Shiga-toxigenic *Escherichia coli* (STEC) and *Salmonella* spp. These pathogenic microorganisms were selected because they have been reported as the most important foodborne pathogens on leafy greens (Decol et al., 2017; Castro-Ibañez et al., 2015; EFSA and ECDC, 2015; CDC, 2014; Ahmed et al., 2012; Ferguson et al.,

2012). Additionally, the potential occurrence of DBPs (i.e. chlorates) in water and in the irrigated plants due to the ClO_2 treatment was evaluated.

2. Materials and methods

2.1. Experimental setup

Ten-days-old lettuce plants (baby red oak leaf lettuce) were obtained from a local nursery (Semilleros Jimenado S.A., Torre Pacheco, Spain), and were grown in a greenhouse located next to the wastewater treatment plant (WWTP) of Murcia, (Spain) (37°47′48″ N, 0°57′33″ W). Data acquisition including climatological data and the irrigation head's layout were described in previous works (López-Gálvez et al., 2014). In the experiments, two types of water were used for overhead irrigation of the plants. The first water type was secondary effluent from the WWTP (SW). Secondary treatment of municipal wastewater was performed as described in López-Gálvez et al. (2016). The second irrigation water type consisted in secondary effluent from the WWTP disinfected using chlorine dioxide (ClO_2W). The plants were grown on trays with peat as substrate. A total of eight lettuce trays with 294 plants each were used for each irrigation water type.

Two preliminary tests were carried out in order to serve as the basis for adjusting the doses of ClO_2 and to adapt the experimental conditions. During November and December 2016, a final experiment that lasted 21 days was performed using optimum conditions stablished in the preliminary tests. Throughout the final experiment, minimum and maximum temperature inside the greenhouse were 14.4 °C and 28.3 °C, respectively, with an average of 17.2 °C. The relative humidity (RH) in the greenhouse ranged from 52.6 % to 91.6 % with an average of 76.8 %. The approximate total amount of irrigation water applied throughout the experiment was 1.6 m^3 per treatment.

During the experiments, concentration of culturable and viable *E. coli*, as well as the presence of pathogenic bacteria were assessed in water and lettuce samples. Additionally, residual ClO_2 concentration and other physicochemical parameters of the irrigation water (pH, temperature, oxidation reduction potential (ORP), absorbance at 254 nm (UV254), and chlorates (ClO_3^-)) were analyzed.

2.2. Preparation, measurement, and application of chlorine dioxide

The company Servicios Técnicos de Canarias (STC S.L.U., Las Palmas de Gran Canaria, Spain) provided reagents and instructions for the preparation of the stable chlorine dioxide solution AGRI DIS® (ClO_2). A concentrated ClO_2 solution (7000 ppm) was prepared weekly and was kept in an opaque plastic jerry can at ambient temperature. Chronoamperometric measurement of ClO_2 concentration was performed using the equipment ChlordioXense® (Palintest, Gateshead, United Kingdom). Using tap water, the concentrated ClO_2 solution was diluted to reach the desired ClO_2 concentration to be applied to the secondary treated wastewater. This diluted solution was daily prepared in an opaque plastic jerry can just before starting the irrigation of the plants. To treat the irrigation water with ClO_2, the diluted ClO_2 solution was pumped directly to the ClO_2W pipes using a peristaltic pump. The residual concentration of ClO_2 was measured in each irrigation event at the end of the SW and ClO_2W irrigation heads. Pipe length and contact time from the ClO_2 application point to the sprinklers were ≈50 m and ≈6 min, respectively. The ClO_2 doses applied were selected based on the results of the preliminary trials.

2.3. Sample collection

Water sampling was performed every 2 to 7 days, during the lettuce growing cycle. Irrigation water was sampled during the irrigation of lettuce from the irrigation heads located at the end of the irrigation lines (**Fig. 1**). Each sampling day, three to five 2-L water samples were collected using sterile plastic jars. For microbiological analysis, in order to quench residual ClO_2, 5 mL of sodium thiosulfate (17.5 g/L) were added to the ClO_2W samples. A total of n=74 water samples (38 SW, and 36 ClO_2W samples) were collected in eight different sampling days along the growing cycle of the crop. For pathogenic bacteria analysis, one 10-L sample per treatment was collected each sampling day (n=16). Additionally, for the measurement of chlorates, three samples (45 mL) per treatment (n=74) were taken each sampling day.

Lettuce sampling was performed 5 times during the last two weeks of the lettuce growing cycle. A total of n=30 lettuce samples were collected (15 samples per treatment) in five sampling days. The lettuce samples were always taken before irrigation. In each sampling day, three samples (60 g each) per treatment were aseptically taken and were transported to the lab in refrigerated conditions.

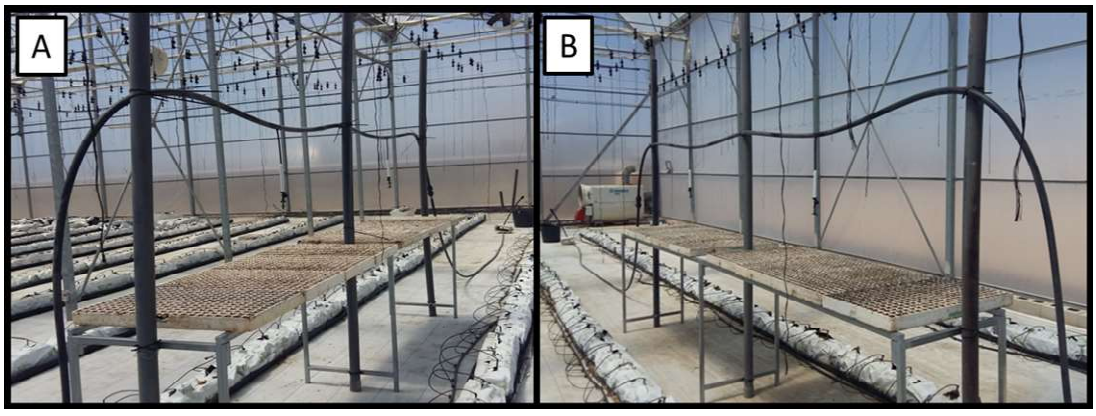

Figure 1. Irrigation system for sprinkle in tables for lettuce cultivation, (A) system receiving ClO_2 treatment (ClO_2W) and (B) system control (SW).

2.4. E. coli analysis

Culturable *E. coli* quantification was carried out in water and lettuce samples. For water samples, depending on the expected *E. coli* concentration, pour plating (1 mL) and/or membrane filtration (10 and 100 mL) was used. Samples were filtered through 0.45 µm membrane filters (Sartorius, Madrid, Spain) using a filter holder manifold (Millipore, Madrid, Spain). Chromocult coliform agar (Merck, Darmstadt, Germany) was used for membrane incubation and pour plating. Plates were incubated for 24 h at 37 °C before interpretation of the results. Dark blue-violet colonies were considered positives for *E. coli*. Lettuce samples were homogenized in 100 mL of sterile 0.1% buffered peptone water (BPW, Scharlab, Barcelona, Spain) for the quantification of culturable *E. coli*. The homogenate was serially diluted and 1 mL aliquots were pour plated using Chromocult coliform agar. Incubation of the plates and interpretation of results was performed as explained before for water samples.

Molecular quantification of *E. coli* in irrigation water and baby lettuce was performed following the combined use of propidium monoazide (Biotium Inc, Hayward, CA, USA) and quantitative polymerase chain reaction (q-PCR) (PMA-qPCR) as previously described in Truchado et al. (2016a) with some modifications. Three water samples (200 mL) per treatment (SW and ClO_2W) and sampling day were centrifuged at 3000 *g* for 20 min. In the case of lettuce samples, three samples (25 g each) were homogenized in 100 mL of sterile 0.1% BPW using a Stomacher at low speed for 1 min. The homogenate of each sample was centrifuged at 3000 *g* for 10 min. Then, each obtained pellet was activated with PMA (20 µM) and kept at -20 ºC until the DNA extraction was performed. Master Pure TM Complete DNA and RNA purification kit (Epicenter, Madison, USA) following the manufacturer's instructions was used. For molecular quantification, primers and probes for detecting genes of *E. coli* 23S rRNA as

well as PMA-qPCR procedure were identical to those described in Truchado et al. (2016a).

2.5. *Detection of pathogenic microorganisms*

For the detection of Shiga-toxigenic *E. coli* (STEC), *E. coli* O157:H7 and *Salmonella* spp. in water samples, 10-L samples were filtered at the greenhouse through Modified Moore Swabs (MMS) as previously described (Sbodio et al., 2013). The MMS were transferred aseptically into sterile stomacher plastic bags and transported to the lab in refrigerated conditions. Once in the lab, 200 mL of 20 g/L buffered peptone water (Scharlab, Barcelona, Spain) was added to the bags that were incubated for 24 h at 37 °C for enrichment. After incubation, a volume of 7 mL was transferred into 15 mL centrifuge tubes and stored at −20 °C with 30% glycerol until the analyses were performed.

For the lettuce samples analyses, the homogenate described in paragraph 2.3.1 was supplemented with 125 mL of BPW (40 g/L) and the samples were homogenized by massaging by hand the stomacher bags. Afterwards, bags were incubated for 24 h at 37 °C for enrichment, and then a volume of 7 mL was transferred into 15 mL centrifuge tubes and stored at −20 °C with 30% glycerol until the analyses were performed.

Aliquots of 1 mL from each frozen sample were added to 9 mL of selective enrichment broth specific for the target pathogens. Modified Buffered Peptone Water (20 g/L) supplemented with pyruvate (Scharlau, Barcelona, Spain; mBPWp) incubated for 24 h at 42 °C was used for STEC and *E. coli* O157:H7. Tetrathionate Broth (TT; Scharlau) incubated for 24 h at 37 °C was used for *Salmonella*. Prevalence of pathogenic microorganisms in water (n= 16) and lettuce samples (n=30) were performed using the Salmonella-STEC GeneDisc Pack in a Genedisc Cycler multiplex

PCR (Pall® Corporation, WA, USA) following manufacturer instructions. Confirmation of presumptive positive samples was performed by isolation in selective culture media. For STEC, CHROMagar STEC (CHROMagar, Paris, France) incubated for 24 h at 37 °C was used. For *E. coli* O157:H7, two culture media were used: CT-SMAC (Scharlab, Barcelona, Spain) and CHROMagar O157 (CHROMagar, Paris, France), followed by further confirmation using a latex test (Oxoid, Basingstoke, UK). For *Salmonella*, the IBISA method (AES Chemunex, Bruz, France) was used, followed by further confirmation using a latex test (Oxoid, Basingstoke, UK).

2.6. Physicochemical analysis of irrigation water

Temperature, pH and ORP were measured using a multimeter (pH and redox 26, Crison, Barcelona, Spain). For measuring the UV254, water was filtered through 0.45 µm syringe nylon filters (Fisherbrand-Fisher Scientific, Waltham, USA), and a UV-VIS spectrophotometer (Jasco V-630, Tokyo, Japan) and quartz cuvettes with a path length of 1 cm (Hellma, Müllheim, Germany) were used.

2.7. Presence of chlorates in irrigation water and in lettuce

Chlorates (ClO_3^-) content in water and lettuce was analysed by LC-MS as described in Gil et al. (2016), using an analytical standard of chlorate (RTC, ICS-004-100, Fluka, Sigma-Aldrich, Spain) for quantification. Areas of the peaks detected by MS were used for the quantification of chlorates. Results were expressed in mg/L and in mg/kg for water and lettuce samples, respectively.

2.8. Statistical analysis

Counts derived from microbiological analyses were log transformed and entered in an Excel spreadsheet (Microsoft Excel, 2016). Results were compiled and graphs were made using Sigma Plot 11.0 Systat Software, Inc. (Addilink Software Scientific, S.L. Barcelona). SPSS statistics 21 (IBM, Armonk, NY, USA) was used for statistical analysis at a significance level of 5% ($p = 0.05$). The Kolmogorov–Smirnov test and Levene's test were used to assess normality and equality of variance, respectively. When data was not following a normal distribution, nonparametric tests were applied. Mann–Whitney U and Kruskal–Wallis tests were used to determine the difference between the raw data of the indicators and the presence of pathogens. The Pearson's correlation coefficient was calculated ($p<0.01$) to assess links between physicochemical characteristics of wastewater (SW and ClO_2W).

3. Results and discussion

3.1. ClO_2 treatment

In our study, the calculated initial ClO_2 concentration applied to the secondary-treated wastewater ranged between 3.3 and 9.2 mg/l (**Fig. 2**). These initial ClO_2 doses were necessary to reduce the levels of *E. coli* of reclaimed water below the 2 log CFU / 100 mL, which is the recommended *E. coli* threshold for irrigation water of leafy vegetables included in the guidance document for addressing microbiological risks in fresh fruits and vegetables at primary production recently published by the EC (EC, 2017b). **Fig. 2** shows the residual ClO_2 levels in reclaimed water after a contact time in the irrigation network of approximately 6 minutes. In all the cases, the ClO_2 residual of wastewater as measured in the irrigation head was less than 1 mg/l (<0.02 to 0.33 mg/L) to avoid any potential damage in the vegetable tissue (WEAH, 2016).

UV254 was measured as an indicator of the organic matter content of the irrigation water. UV254 of SW ranged between 0.05 and 1.35 cm^{-1} (**Table 1)**. Based on previous studies the increasing order of reactivity of some disinfectants commonly used in the treatment of wastewater with organic matter is: peracetic acid<ClO_2<chlorine<ozone (Hassemberg et al., 2017; Van Haute et al., 2017; Veschetti et al., 2003). Even though there are several references reporting that ClO_2 is less reactive with organic matter than chlorine (Van Haute et al., 2015; FAO/WHO, 2008; Rodriguez & Serodes, 2001), in the present study, the concentration of organic matter in the water influenced the residual ClO_2 concentration in ClO_2W. For example, in the days when a lower concentration of organic matter was observed (day 4: 0.05 cm^{-1}; day 6: 0.08 cm^{-1}), higher ClO_2 residuals were detected (0.26 and 0.33 mg/L) (**Fig. 2**). A significant negative correlation of - 0,508 ($p < 0.01$) was found between residual ClO_2 concentration and UV254. Similar results were obtained in other studies (Hassemberg et al., 2017; Haute et al., 2017; Praeger et al., 2016; Tomàs-Callejas et al., 2012).

3.2. Effect of the disinfection treatment in the microbiological characteristics of irrigation water

Different studies have reported the strong bactericidal capability of ClO_2 in the treatment of drinking water, food processing water, and wastewater (Banach et al., 2017; Kibbee and Örmeci, 2017; Al-Otoum at al., 2016; Praeger et al., 2016; Volk et al., 2002). In our study, when SW and ClO_2W samples were analyzed by conventional plate count methods, statistically significant differences ($p <0.05$) in *E. coli* counts were observed (**Fig. 3A)**. *E. coli* counts of SW and ClO_2W samples ranged between 2.00 and 4.76 log CFU/100 mL (IQR=3.32 – 3.82) and between 0.70 and 3.49 log CFU/100 mL (IQR=1.59 – 2.18), respectively. Considering the mean counts of SW and ClO_2W, it

was possible to calculate a mean reduction of 2.21 log CFU/100 mL. However, when *E. coli* levels were quantified using the PMA-qPCR method, no statistically significant differences in *E. coli* enumerations were observed comparing SW and ClO_2W (p>0.05) (**Fig. 3B**).

For the SW and ClO_2W samples the *E. coli* levels as determined by PMA-qPCR were 3.17 to 6.27 log cells/100 mL (IQR 4.19 – 5.10) and 2.71 to 5.46 log cells/100 mL (IQR 3.66 – 4.54), respectively (**Fig. 3B**). In the study performed by López-Gálvez et al. (2018a) in an open commercial field were baby spinach were cultivated, similar trends were observed. On the other hand, we observed a difference of 1.07 and 2.22 log CFU/100 mL, respectively, for the samples SW and ClO_2W, when comparing the results of the different quantification methods used. In agreement with ours results, some studies have reported that the levels of *E. coli* cells quantified by PMA-qPCR assay in different water samples such as drinking water, wastewater, irrigation water and seawater were higher than those obtained by cultivation based techniques (Gensberger et al., 2014; Van Frankenhuyzen et al., 2013; Truchado et al., 2016b; Li et al., 2014). PMA is a DNA-dye, which allows the differentiation between viable and dead cells, avoiding an overestimation of results by qPCR (Gensberger et al., 2014; Truchado et al., 2016b). Therefore, the observed differences between PMA-qPCR and plate counts can be due to the presence of viable but not cultivable (VBNC) bacteria. The obtained results could indicate a bacteriostatic action of ClO_2, which can induce the entrance of *E. coli* cells into a VBNC state. Oliver et al., (2015) reported that VBNC stage can be induced when microorganisms are exposed to chemical disinfectants. The presence of VBNC bacteria in reclaimed wastewater due to water reclamation processes has been previously demonstrated (Kibbee and Örmeci, 2017; Lin et at., 2016; Zhang et al., 2015). Recently, Kibbee and Örmeci (2017) have evaluated the levels of *E. coli*

present in secondary wastewater effluent after chlorine disinfection, showing that high numbers of VBNC *E. coli* survive chlorination. Therefore, the use of conventional plate counting methods might lead to an overestimation of the efficacy of the water disinfection treatments (Zhang et al., 2015).

Several studies have shown that different factors may influence the bactericidal effect of ClO_2 (Ayyildiz, et al., 2009; Junli et al., 1997). Some of the most important are temperature, pH, and presence of organic matter. The temperature of water can strongly influence the microbial inactivation capacity of ClO_2. Barbeau et al. (2005) observed that 0.25 mg/L of free ClO_2 were sufficient to inactivate 99% of *E. coli* in water after 16 seconds, at 30 °C. However, when the temperature of water was 5 °C, the same reduction was reached only after 110 seconds. Huang et al. (1997) reported that 10 °C increase of the water temperature doubles the inactivation power of ClO_2 against microorganisms. Although these studies reported the influence of temperature on the microbiological inactivation capacity of ClO_2, this influence was not observed in the present study. The reductions of *E. coli* observed in reclaimed wastewater demonstrated no correlation with the water temperature measured of – 0,381 with ($p<0,01$) and this may be due to the narrow range of temperature variation in the irrigation water (15.2 to 19.3 °C).

Globally, water demand is predicted to increase significantly over the coming decades. According to UNESCO-WWAP (2017), more than 70% of the water that is consumed all over the world is used for agricultural irrigation. Therefore, there is a big potential for the application of reclaimed for agricultural irrigation (WHO, 2006). To evaluate the microbiological suitability of reclaimed municipal wastewater for agricultural irrigation, the recommendations and microbiological limits described in guidelines and regulations should be used. For example, World Health Organization

(WHO, 2006) recommends that wastewaters used for irrigation of agricultural crops likely to be eaten raw should have a level of fecal coliforms $\leq 10^3$ CFU/100 mL. In the United States, the Food and Drug Administration (FDA) established a limit of 23 CFU/100 mL for *E. coli* concentration in agricultural irrigation water, when there is direct contact of irrigation water with produce (Sugano et al., 2016). In Italy, the limit for *E. coli* concentration in treated wastewater used for agricultural irrigation is 10 CFU/100 mL (Decreto Ministeriale, 2003). Spanish legislation for reuse of reclaimed water establishes a maximum concentration of 10^2-10^3 CFU *E. coli* per 100 mL in irrigation water (number of sample units (n) = 10, threshold value for the number of *E. coli* (m) = 100 cfu/100 mL, maximum value for the number of *E. coli* (M) = 1000 cfu/100 mL, number of sample units where the *E. coli* count may be between m and M (c) = 3) when there is direct contact of the water with produce that is going to be consumed raw (Real Decreto 1620/2007, 2007).

In the present study, *E. coli* concentration was above the 2 log/100 mL threshold in all the SW samples analyzed. On the other hand, due to the treatment with ClO_2, 69.4% of the ClO_2W analyzed samples (25 out of 36) were in accordance with the Spanish legislation for *E. coli* (Real Decreto 1620/2007, 2007) based on conventional plate counting techniques. Therefore, even after ClO_2 treatment, 30.6% of the ClO_2W samples were not acceptable for being used in overhead irrigation of raw consumed leafy green vegetables according to the Spanish legislation.

3.3. Effect of the disinfection treatment in the microbiological characteristics of baby lettuce

The baby lettuce samples irrigated with SW and ClO_2W beared culturable *E. coli* counts ranging between 0.70 and 2.90 log CFU/g (IQR 0.69 – 1.30) and between 0.70

and 1.40 log CFU/g (IQR 0.69 – 0.69), respectively, throughout the sampling period (**Figure 3A**). A significant statistical difference ($p < 0.05$) in culturable *E. coli* counts was observed between baby lettuce samples irrigated with SW and ClO_2W. This difference could be explained by the exposure of SW-irrigated plants to higher microbial contamination during the entire growing period. On the opposite, irrigation with ClO_2W, bearing lower *E. coli* concentrations, would have resulted in lower contamination of the plants. Overall, results demonstrated that higher numbers of cultivable *E. coli* in irrigation water resulted in higher numbers of cultivable *E. coli* on lettuce. Similar results were demonstrated by Makkaew et al. (2016) investigating the influence of different levels of *E. coli* contamination present in wastewater stabilization ponds in South Australia. In the mentioned study, wastewater was used to irrigate three different varieties of lettuce (Iceberg, Cos and Oak leaf). Their results verified a significant correlation between *E. coli* contamination present in wastewater and contamination of irrigated lettuce. These authors also reported that the morphology of the lettuce affects the degree of contamination, because dense foliage of variety Oak leaf showed a greater retention of *E. coli* contamination when compared with the other types of lettuces (Makkaew et al., 2016; Amahmid et al., 1999). In our study, we used the variety baby red Oak leaf, whose morphology may have contributed to the retention of *E. coli*.

E. coli enumerations using PMA-qPCR rendered counts in SW and ClO_2W irrigated lettuce of 2.17 to 3.83 log cells/g (IQR 2.83 – 3.25) and 2.18 to 3.31 log cells /g (IQR 2.55 – 2.98), respectively (**Fig. 4B).** PMA-qPCR analysis resulted in log counts around 2 logarithmic units higher than those from the same samples analyzed by the plating method. Truchado et al. (2016a) also observed *E. coli* counts 2 log units higher on lettuce samples analyzed by PMA-qPCR method compared to the conventional

cultivation techniques. As previously described, the difference observed between plate count and PMA-qPCR methods could be due to the presence of VBNC *E. coli* in the treated irrigation water used in this study, but also to the stress provoked by the environmental conditions. The phyllosphere is a hostile habitat for microorganisms due to nutrient limitation, shift in temperature, and solar radiation exposure, which can induce the VBNC state in bacteria (Wilson and Lindow, 2002). This phenomenon should be considered because bacteria can persist for long periods of time in this state and they could retain their virulent potential (Dinu and Bach, 2011).

3.4. Correlation between E. coli levels and presence/absence of pathogens

The prevalence of pathogens was detected by a multiplex PCR in 9 out of 16 samples of all irrigation water samples (56.20 %). Among the positive samples, 8 samples were confirmed using selective media and latex test. Seven out of eight corresponded to SW (1 *Salmonella* and 6 STEC) while only one sample of ClO_2W was positive for STEC. For the lettuce samples, the presence of pathogens was detected in 4 out of 33 samples (13.33 %), by a multiplex PCR. Only 1 lettuce sample irrigated with SW was confirmed for the presence of STEC using selective media and latex test. *E. coli* O157:H7 was not confirmed in any water or lettuce samples (**Table 2**).

Fig. 5 shows the correlation between the *E. coli* levels enumerated using both plate count and PMA-qPCR methods and the presence/absence of pathogenic bacteria in irrigation water samples. In most of the cases, positive samples for pathogenic bacteria showed significantly higher *E. coli* levels than samples negative for the presence of pathogenic bacteria when both quantification methods were used (plate count and PMA-qPCR) (Mann-Whitney U Test, $p<0.05$) (**Fig. 5B)**. The only exception was for SW when *E. coli* was enumerated using conventional plating methods, which could be due

to the high levels of *E. coli* found in samples both positive and negative for pathogenic bacteria. Corroborating these findings Truchado et al. (2016b) and Ferguson et al. (2012) reported that molecular techniques are suitable techniques to predict the potential presence of pathogenic bacteria in water samples (i.e. groundwater, and secondary reclaimed water, respectively).

Other studies also found a significant correlation between *E. coli levels* and the presence of STEC and *Salmonella* (Ceuppens et al., 2014; Park et al., 2014). López-Gálvez et al. (2014) found a significant correlation between the counts of *E. coli* and the presence of *Salmonella* spp. in two types of water (reclaimed and surface water) used in drip irrigation of hydroponic tomato. Castro-Ibáñez et al. (2015), also found a significant correlation between the counts of *E. coli* and the presence of *Salmonella* spp. in irrigation water from ponds, soil and baby spinach. Holvoet et al. (2014) reported that high levels of *E. coli* increased the probability of detecting pathogens like STEC, *Salmonella* and *Campylobacter* in environmental samples of lettuce farms. This finding supports the hypothesis that considers *E. coli* as a good microbial indicator of fecal contamination and the value of this microorganism as a predictive tool for pathogen presence.

3.5. Occurrence of chlorates in water and in baby lettuce

The use of disinfectants can lead to the presence of disinfection by-products (DBPs) that can pose threat to human health. ClO_2 has recently been used as an alternative to chlorine, as it does not lead to the formation of chlorinated DBPs (Pereira et al., 2008). Despite its numerous advantages, ClO_2 has by-products like chlorite and chlorate ions (ClO_2^-, ClO_3^-), that may pose a significant human health risk (Gordon et al., 1990).

When irrigation water samples were analyzed for the presence of ClO_3^-, SW showed very low levels (0.00-0.01 mg/L), while the concentration in ClO_2W ranged between 1.44 and 5.94 mg/L (**Fig. 6)**. In other studies, lower chlorates concentrations were detected in irrigation water treated with disinfectants (López-Gálvez et al., 2018a; López-Gálvez et al., 2018b; Nitsopoulos et al., 2014). However, in those studies, water with a much better microbiological and physicochemical quality was used and, therefore, lower disinfectant concentration was needed for the treatment. In our study, due to the characteristics of the treated water, a high initial concentration of ClO_2 was needed to obtain the desired microbiological reduction (**Fig. 2**). The calculated Pearson's correlation coefficient, showed a significant correlation of 0.65 ($p < 0,01$) between the presence of ClO_3^- and initial ClO_2 concentration, which would explain the high initial consumption of ClO_2 in the treatment.

According to Korn et al. (2002), lower concentration of ClO_2 and lower levels of organic matter can reduce the presence of chlorates in treated water. In the present study, it was verified that the high amount of organic matter present in the water may have influenced the increase of chlorate formation, although there was no significant correlation between the factors.

In the baby lettuce samples irrigated with ClO_2W, the obtained concentration of ClO_3^- ranged between 1.13 and 8.49 mg/kg (**Fig. 6**). These levels are much higher than the maximum residue limit for chlorate allowed in the European Union in food (0.01 mg/kg; EC, 2005), although these levels are currently being revised by the EC (EC, 2014). In a previous study from our group (López-Gálvez et al., 2018a), lower chlorate concentrations were detected in the crop (baby spinach) cultivated in open field and irrigated by overhead irrigation with ClO_2-treated surface water. However, much lower disinfectant concentrations were used compared with the present study, leading to lower

chlorate concentration in irrigation water and in the crop. Other studies performed using irrigation water treated with chlorine-based disinfectants reported lower chlorate concentration in the crop compared with our study, probably due to the lower concentrations of disinfectants applied (Nitsopoulos et al., 2014; López-Gálvez et al., 2018b).

4. Conclusions

The ClO_2 treatment was able to improve the microbiological quality of the reclaimed water used for irrigation, reducing culturable *E. coli* concentration and the prevalence of pathogenic bacteria. However, when viable bacteria was enumerated by molecular techniques combined with the use of dies, no significant differences were observed between untreated and treated water, indicating a potential bacteriostatic action of ClO_2, which can induce the entrance of *E. coli* cells into a VBNC state. Based on these results it could be concluded that the use of plate count methods to estimate the efficacy of disinfection methods could lead to an overestimation of the microbial reductions. Plants irrigated with treated water beared lower concentrations of culturable *E. coli* than the plants irrigated with the untreated secondary effluent of the WWTP. Detection of pathogens in lettuce was almost null, and, as a consequence, the potential effect of the treatment on the occurrence of pathogens in the lettuce plants could not be detected. In spite of the microbiological results, the accumulation of chlorates in the vegetables tissue as a consequence of the ClO_2 treatment exceeded the maximum recommended limits of chlorates for drinking water (0.7 mg/L), making the treatment unsuitable to be applied under the conditions evaluated in the present study.

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References

Ahmed, W., Richardson, K., Sidhu, J. P. S., Toze, S., 2012. *Escherichia coli* and *Enterococcus* spp. in rainwater tank samples: comparison of culture-based methods and 23S rRNA gene quantitative PCR assays. Environ. Sci. Technol. 46, 11370−11376.

Allende, A., Monaghan, J.M., 2015. Irrigation water quality for leafy crops: A perspective of risks and potential solutions. Int. J. Environ. Health. Res. 12, 7457−7477.

Amahmid, O., Asmama, S. & Bouhoum, K., 1999. The effect of wastewater reuse in irrigation on the contamination level of food crops by Giardia cysts and Ascaris eggs. International Journal of Food Microbiology, 49 (1), 19–26.

Armon, R., Gold, D., Brodsky, M., Oron, G. 2002. Surface and subsurface irrigation with effluents of different qualities and presence of *Cryptosporidium oocysts* in soil and on crops. Water Science and Technology 46 (3), 115–122.

Ayyildiz, O., Ileri, B., Sanik, S., 2009. Impacts of water organic load on chlorine dioxide disinfection efficacy. J. of Hazardous Materials, 168,1092–1097.

Banach, J.L., Van Bokhorst-van de Veen, H., Van Overbeek, L.S., Van der Zouwen, P.S., Van der Fels-Klerx, H.J., Groot, M.N.N., 2017. The efficacy of chemical sanitizers on the reduction of *Salmonella Typhimurium* and *Escherichia coli* affected by bacterial cell history and water quality. Food Control, 81,137–146.

Barbeau, B., Desjardins, R., Mysore, C., Prevost, M., 2005. Impacts of water quality on chlorine and chlorine dioxide efficacy in natural waters. Water Res. 39, 2024–2033.

Becerra-Castro, C., Lopes, A.R., Vaz-Moreira, I., Silva, E.F., Manaia, C. M., Nunes, O.C., 2015. Wastewater reuse in irrigation: A microbiological perspective on implications in soil fertility and human and environmental health. Environ. International, 75, 117–135.

BOE. 2007. Spanish Royal Decision, 1620/2007: Royal Decree where is regulated the legal regime for reuse of purified waste waters. Boletín Oficial del Estado No. 294.

Bonetta, S., Pignata, C., Lorenzi, E., Ceglia, M., Meucci, L., Bonetta, Sa., Gilli., G., Carraro, E., 2016. Detection of pathogenic *Campylobacter*, *E. coli* O157:H7 and *Salmonella* spp. in wastewater by PCR assay. Environ Sci Pollut Res., 23, 15302–15309.

Ceuppens, S., Hessel, C.T., Rodrigues, R. de Q., Bartz, S., Tondo, E.C., Uyttendaele, M., 2014. Microbiological quality and safety assessment of lettuce production in Brazil. Int. J. Food Microbiol. 181, 67–76.

Cirelli, GL, Consoli S, Di Grande V., 2008. Long-term storage of reclaimed water: the case studies in Sicily (Italy). Desalination, 218, 62–73.

Colwell, R.R. and Gray, D.J., 2000. Nonculturable Microorganisms in the Environment. American Society for Microbiology, Washington, DC.

Decol, L.T., Casarin, L.S., Hessel, C. T., Batista, A.C. F., Allende, A., Tondo, E.C., 2017. Microbial quality of irrigation water used in leafy green production in Southern Brazil and its relationship with produce safety. Food Microbiology, 65, 105–113.

Decreto Ministeriale 12 giugno 2003, n. 185.Regolamento recante norme tecniche per il riutilizzo delle acque reflue in attuazione dell'articolo 26, comma 2, del D.Lgs. 11 maggio 1999, n. 15. (G.U. 23 luglio 2003, n. 169).

Dinu L-D Bach S., 2011. Induction of viable but nonculturable *Escherichia coli* O157:H7 in the phyllosphere of lettuce: a food safety risk factor. Appl Environ Microbiol, 77: 8295–8302.

EC, European Commission, 2005. Regulation (EC) No 396/2005 (2005) of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. Official Journal of the European Union, L 70/1, 16/03/2005.

EC, European Commission, 2014. Standing Committee of the Food Chain and Animal Health on 12-13 June 2014, Statement as regards the presence of chlorate in food and feed. Available at https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_eu-1119-2014_scfcah-statement.pdf. Accessed January 2018-

EC, European Commission, 2017a. Water re-use factsheet. EC - Water Reuse - Background and policy context UN – Water and Jobs. Available at http://ec.europa.eu/environment/water/pdf/water_reuse_factsheet_en.pdf. Accessed January 2018.

EC, European Commission, 2017b. Commission notice on guidance document on addressing microbiological risks in fresh fruits and vegetables at primary production through good hygiene (2017/C 163/01). Official Journal of the European Union, C 163/1, 23/05/2017.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA Journal, 13, 4329. Available at: 10.2903/j.efsa.2015.4329 (Acessed February 2016).

Ferguson, A.S., Layton, A.C., Mailloux, B.J., Culligan, P.J., Williams, D.E., Smartt, A.E., Sayler, G.S., Feighery, J., McKay, L., Knappett, P.S.K., Alexandrova, E., Arbit, T., Emch, M., Escamilla, V., Ahmed, K.M., Alam, M.J., Streatfield, P.K., Yunus, M., van Geen, A., 2012. Comparison of fecal indicators with pathogenic rotavirus in groundwater. Sci. Total Environ. 431, 314–322.

Fonseca, J.M., Fallon, S.D., Sanchez, C.A., Nolte, K.D., 2011. Escherichia coli survival in lettuce fields following its introduction through different irrigation systems. Journal of Applied Microbiology, ISSN 1364–5072.

Gil, M.I., Marín, A., Andujar, S. and Allende A., 2016. Should chlorate residues be of concern in fresh-cut salads? Food Control, 60: 416–421.

Hassenberg, K., Geyer, M., Mauerer, M., Praeger, U., Herppich, W.B., 2017. Influence of temperature and organic matter load on chlorine dioxide efficacy on *Escherichia coli* inactivation. LWT - Food Science and Technology, 79,349–354.

Holvoet, K., Sampers, I., Seynnaeve, M., Uyttendaele, M., 2014. 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. Int. J. Food Microbiol. 171, 21–31.

Junli, H., Li, W., Nanqi, R., Fang, M., Juli, 1997. Disinfection effect of chlorine dioxide on bacteria in water. Wat. Res. 31, n°3, 607–613.

Kibbee, R.J. and Örmeci, B., 2017. Development of a sensitive and false-positive free PMA-qPCR viability assay to quantify VBNC *Escherichia coli* and evaluate disinfection performance in wastewater effluent. J. Microbiol. Methods, 132, pp. 139–147.

Korn, C., Andrews, R.C., Escobar, M.D., 2002. Development of chlorine dioxide-related by-product models for drinking water treatment. Water Res 36: 330–342.

Li, D., Tong, T., Zeng, S., Lin,Y., Wu, S., He, M., 2014. Quantification of viable bacteria in wastewater treatment plants by using propidium monoazide combined with quantitative PCR (PMA-qPCR). J. Environ. Sci., 26 (2), pp. 299–306.

Lin, Y.W., Li, D., Gu, A.Z., Zeng, S.Y. He, M., 2016. Bacterial regrowth in water reclamation and distribution systems revealed by viable bacterial detection assays. Chemosphere 144, 2165–2174.

López-Gálvez, F., Allende, A., Martinez-Sanchez, A., Tudela, J.A., Selma, M.V., Gil, M.I., 2010. Suitability of aqueous chlorine dioxide versus sodium hypochlorite as an effective sanitizer for preserving quality of fresh-cut lettuce while avoiding by product formation. Postharvest Biol. Tec., 55, 53–60.

López-Gálvez, F., Allende, A., Pedrero-Salcedo, F., Alarcon, J.J., Gil, M.I., 2014. Safety assessment of greenhouse hydroponic tomatoes irrigated with reclaimed and surface water. International Journal of Food Microbiology, 191, 97–102.

López-Gálvez, F., Andújar, S., Marín, A., Tudela, J.A., Allende, A., Gil, M.I., 2018b. Disinfection by-products in baby lettuce irrigated with electrolyzed water. Journal of the Science of Food and Agriculture, DOI: 10.1002/jsfa.8796.

López-Gálvez, F., Gil, M. I., Pedrero-Salcedo, F., Alarcón, J. J., Allende, A., 2016. Monitoring generic *Escherichia coli* in reclaimed and surface water used in hydroponically cultivated greenhouse peppers and the influence of fertilizer solutions. Food Control, 67, 90–95.

López-Gálvez, F., Gil, M.I., Meireles, A., Truchado, P., Allende, A., 2018b. Demonstration tests of irrigation water disinfection with chlorine dioxide in open field cultivation of baby spinach. J. of the Sc. of Food and Agriculture, DOI: 10.1002/jsfa.8794.

Makkaew, P., Miller, M., Cromar, N. J., Fallowfield, H. J., 2016. The influence of the microbial quality of wastewater, lettuce cultivars and enumeration technique when estimating the microbial contamination of wastewater irrigated lettuce. Journal of Water and Health, in press.

Mendes Silva, D., Domingues, L., 2015. On the track for an efficient detection of *Escherichia coli* in water: a review on PCR-based methods. Ecotoxicol. Environ. Saf. 113, 400–411.

Nikolaou, A.D., Lekkas T.D., 2001. The role of natural organic matter during formation of chlorination by-products: a review. Acta Hydrochim. Hydrobiol. 29, 63–77.

Nitsopoulos, A., Glaumer, T., & Friedle, A., 2014. Chlorate- a contaminant in foodstuff and drinking water. Agilent technologies Labor Friedle GMBH. Available at http://www.chem.agilent.com/Library/posters/Public/EPRW_Poster_Chlorat_2014_V6.pdf.

Oliver, J. D., 2000. Public health significance of viable but nonculturable bacteria. In: Non-Culturable Microorganisms in the Environment (eds. R. R. Colwell & D. J. Grimes). American Society for Microbiology Press, Washington, D.C.

Oliver, J. D.; Dagher, M.; Linden, K., 2005. Induction of *Escherichia coli* and *Salmonella typhimurium* into the viable but nonculturable state following chlorination of wastewater. J. Water Health, 3, 249−257.

Oron, G., 2002. Effluent reuse in agricultural production in modern and traditional irrigation technologies in the eastern Mediterranian. Chap. 9 International Development Research Centre, (Available at: http://www.idrc.ca/EN/Resources/Publications/Pages/DRCBookDetails.aspx?PublicationID=276. Accessed 20 April 2017).

Pachepsky, Y., Shelton, D.R., McLain, J.E.T., Patel, J., Mandrell, R.E., 2011. Irrigation waters as a source of pathogenic microorganisms in produce: a review. In: Donald, L.S. (Ed.), Advances in Agronomy, vol. 113. Academic Press, pp. 73–138.

Pachepsky, Y., Shelton, D.R., McLain, J.E.T., Patel, J., Mandrell, R.E., 2011. Irrigation waters as a source of pathogenic microorganisms in produce: a review. Adv. Agron. 113, 73–138.

Park, S., Navratil, S., Gregory, A., Bauer, A., Srinath, I., Szonyi, B., Nightingale, K., Anciso, J., Jun, M., Han, D., Lawhon, S., Ivanek, R., 2014. Farm management, environment, and weather factors jointly affect the probability of spinach contamination by generic *Escherichia coli* at the preharvest stage. Appl. Environ. Microbiol. 80, 2504–2515.

Praeger, U., Herppich, W.B., Hassenberg, K., 2016. Aqueous chlorine dioxide treatment of horticultural produce: Effects on microbial safety and produce quality e a

review. Critical Reviews in Food Science and Nutrition. (Available at: http://dx.doi.org/10.1080/10408398.2016.1169157. Acessed 20 July 2017)

Real Decreto 1620/2007, 2007. de 7 de diciembre, por el que se establece el regimen jurídico de la reutilización de las aguas depuradas, (BOE num. 294, 8 de diciembre de 2007).

Rodriguez, M.J., Serodes J.B., 2001. Spatial and temporal evolution of trihalomethanes in three water distribution systems, Water Res. 35,1572–1586.

, C.A. Sanchez, C.A.,

Sbodio, A., Maeda, S., López-Velasco, G., Suslow, T. V., 2013. Modified Moore swab optimization and validation in capturing *E. coli* O157:H7 and *Salmonella enterica* in large volume field samples of irrigation water. Food Res. International 51, 654–662.

Schaefer, K., Exall, K., Marsalek, J., 2004. Water reuse and recycling in Canada: a status and needs assessment. Canadian Water Resources Journal, 29 (3), 195–208.

Shen, C., Luo, Y., Nou, X., Wang, Q., & Millner, P., 2013. Dynamic effects of free chlorine concentration, organic load, and exposure time on the inactivation of *Salmonella*, *Escherichia coli* O157:H7 and Non-O157 Shiga toxin-producing *E. coli*. Journal Food Protection, 76, 386–393.

Steele, M. and Odumeru, J. 2004. Irrigation water as source of foodborne pathogens on fruit and vegetables. J Food Prot 67, 2839–2849.

Sugano, J., Uyeda, J., Nakamura-Tengan, L., Hollyer, J., Motomura, S., Kahana, J., Murakami, M., Mencher, F., Miyamoto, B., Gushiken, E., Akahoshi, K., Wong, K., Reppun, F., Fiedler, K., Sibonga, S., 2016. Dissecting the Food Safety Modernization Act (FSMA) and Produce Rule & Good Agricultural Practices

(GAP).University of Hawaii at Manoa. College of Tropical Agriculture and Human Resources.

Swietlik, J., & Sikorska, E., 2004. Application of fluorescence spectroscopy in the studies of natural organic matter fractions reactivity with chlorine dioxide and ozone. Water Research, 38, 3791–3799.

Tomás-Callejas, A., López-Velasco, G., Valadez, A. M., Sbodio, A., Artés-Hernández, F., Danyluk, M. D., et al. 2012. Evaluation of current operating standards for chlorine dioxide in disinfection of dump tank and flume for fresh tomatoes. Journal of Food Protection, 75(2), 304–313.

Truchado, P., López-Gálvez, F., Gil, M.I., Pedrero-Salcedo, F., Alarcon, J.J., Allende, A., 2016b. Suitability of different *Escherichia coli* enumeration techniques to assess the microbial quality of different irrigation water sources. Food Microb 58, 29–35.

U.S. Environmental Protection Agency (EPA). 2004. Guidelines for water reuse. Office of Wastewater Management Office of Water, Washington, D. Office of Research and Development Cincinnati. AvaliableAvailable from: https://nepis.epa.gov/Exe/tiff2png.cgi. Accessed 5 July 2017.

UNESCO-WWAP (United Nations World Water Assessment Programme), 2017. The United Nations World Water Development Report 2017. Wastewater: The Untapped Resource. Paris.

Uyttendaele, M., Jaykus, L-A., Amoah, P., Chiodini, A., Cunliffe, D., Jacxsens, L., Holvoet, K., Korsten, L., Lau, M., McClure, P., Medema, G., Sampers, I., Jasti, P. R., 2015. Microbial hazards in irrigation water: standards, norms, and testing to manage use of water in fresh produce primary production. Comp. Rev. Food Sci. Food Saf. 14. 336–356.

Van Frankenhuyzen, J.K., Trevors, J.T., Flemming, C.A., Lee, H., & Habash, M.B., 2013. Optimization, validation, and application of a real-time PCR protocol for quantification of viable bacterial cells in municipal sewage sludge and biosolids using reporter genes and *Escherichia coli*. Journal of Industrial Microbiology and Biotechnology, 40, 1251–126.

Van Haute, S., López-Gálvez, F., Gómez-López, V. M., Eriksson, M., Devlieghere, F., Allende, A., 2015. Methodology for modeling the disinfection efficiency of fresh-cut leafy vegetables wash water applied on peracetic acid combined with lactic acid. International Journal of Food Microbiology, 208, 102–113.

Van Haute, S., Tryland, I., Escudero, C., Vanneste, M., Sampers, I., 2017. Chlorine dioxide as water disinfectant during fresh-cut iceberg lettuce washing: Disinfectant demand, disinfection efficiency, and chlorite formation. LWT - Food Science and Technology, 75, 301–304.

Veschetti, E., Cutilli, D., Bonadonna, L., Briancesco, R., Martini, C., Cecchini, G., 2003. Pilot-plant comparative study of peracetic acid and sodium hypochlorite wastewater disinfection. Water Research, 37(1), 78–94.

Volk, J.R., C., Hofmann, Chauret, G.A. Gagnon, G. Ranger, Andrews, R.C., 2002. Implementation of chlorine dioxide disinfection: Effects of the treatment change on drinking water quality in a full-scale distribution system. C. J. Environ. Eng. Sci., 1: 323–330.

Water quality for agriculture by R.S. Ayers Soil and Water Specialist (Emeritus) University of California Davis, California, USA and D.W. Westcot Senior Land and Water Resources Specialist California Regional Water Quality Control Board Sacramento, California, USA FAO irrigation and drainage paper 29 Rev. Reprinted 1989, 1994.

WEAH, Water Education Alliance for Horticulture, Treatment Technologies: Chlorine dioxide, 2016. Available at: http://watereducationalliance.org/keyinfo.asp. Accessed January 2018.

Wilson, M., Lindow, S.E., 2000. Viable but nonculturable cells in plant-associated bacterial populations. Non-culturable Microorganisms microorganisms in the Environment environment (Colwell RR Grimes DJ, eds), pp. 229–241. ASM Press, Washington, D.C.

World Health Organization. 2006. WHO guidelines for the safe use of wastewater, excreta and grey water. Wastewater use in agriculture. Available from: http://whqlibdoc.who.int/publications/2006/9241546832_eng.pdf. Accessed 5 July 2017.

Yang, Y., Luo, Y., Millner, P., Shelton, D., Nou, X., 2012. Enhanced chlorine efficacy against bacterial pathogens in wash solution with high organic loads. J. of Food Proc. and Preservation, 36, 560–566.

Zhang, S., Ye, C., Lin, H., Lv, L. & Yu, X., 2015. UV disinfection induces a VBNC state in *Escherichia coli* and *Pseudomonas aeruginosa*. Environmental Science and Technology, 49, 1721-1728.

Zhou, B., Luo, Y., Turner, E., Wang, Q., Schneider, K., 2014. Evaluation of current industry practices for maintaining tomato dump tank water quality during packing house operations. J. of Food Processing and Preservation, 38, 2201–2208.

Table 1. Temperature, pH and oxidation reduction potential (ORP) of untreated (SW) and ClO_2 treated (ClO_2W) secondary effluent from a wastewater treatment plant used to irrigate baby lettuce grown in a commercial greenhouse.

Days before harvest	SW			ClO_2W		
	pH	Temperature (°C)	ORP (mV)	pH	Temperature (°C)	ORP (mV)
20	6.43	19.3	472	6.36	19.1	477
18	6.34	16.7	482	6.38	16.7	493
17	6.31	19.2	463	6.57	20.0	460
13	7.60	-	-	7.75	-	-
11	8.13	16.5	431	7.95	16.9	432
6	8.01	15.2	514	7.89	15.3	791
4	7.58	16.9	450	7.51	17.0	698
0	-	-	-	-	-	-

Table 2. Presence and absence of pathogen microorganisms in baby lettuce and untreated (SW) and ClO_2 treated (ClO_2W) secondary effluent from a wastewater treatment plant used to irrigate baby lettuce grown in a commercial greenhouse.

Samples type	***Salmonella***		**STEC**		***E. coli* O157:H7**	
Water	Genedisc®	Confirmed	Genedisc®	Confirmed	Genedisc®	Confirmed
SW	5/8	1/5	7/8	6/7	6/8	0/6
ClO_2W	1/8	0/1	1/8	1/1	1/8	0/1
Lettuce	Genedisc®	Confirmed	Genedisc®	Confirmed	Genedisc®	Confirmed
SW	2/15	0/2	2/15	1/2	1/15	0/1
ClO_2W	2/15	0/2	1/15	0/1	1/15	0/1

Figures

Figure 2. Initial and residual chlorine dioxide (ClO_2) concentration in ClO_2 treated (ClO_2W) secondary effluent from a wastewater treatment plant used to irrigate baby lettuce grown in a commercial greenhouse

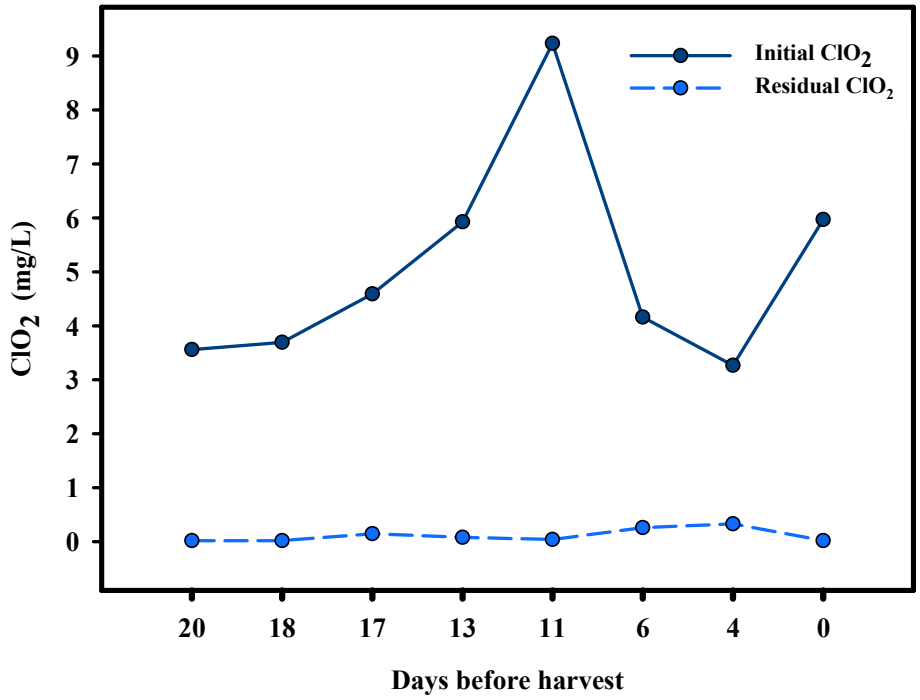


Figure 3. Boxplot representing *E. coli* counts (log CFU/100 mL) in untreated (SW) and ClO_2 treated (ClO_2W) secondary effluent from a wastewater treatment plant used to irrigate baby lettuce. (A) Counts obtained by conventional plate count method. (B) Counts obtained by PMA–qPCR molecular quantification method. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Different letters indicate significant differences ($p<0.05$).

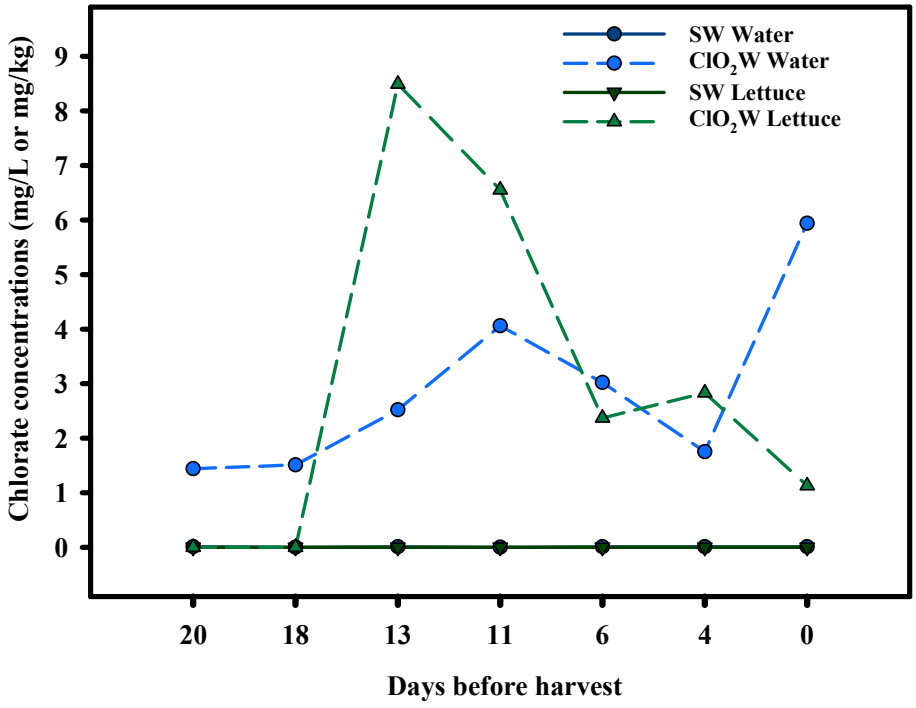


Figure 4. Boxplot representing *E. coli* counts (log CFU/g) in baby lettuces irrigated with untreated (SW) and ClO_2 treated (ClO_2W) secondary effluent from a wastewater treatment plant used to irrigate baby lettuce. (A) Counts obtained by conventional plate count method. (B) Counts obtained by PMA–qPCR molecular quantification method. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Different letters indicate significant differences ($p<0.05$).

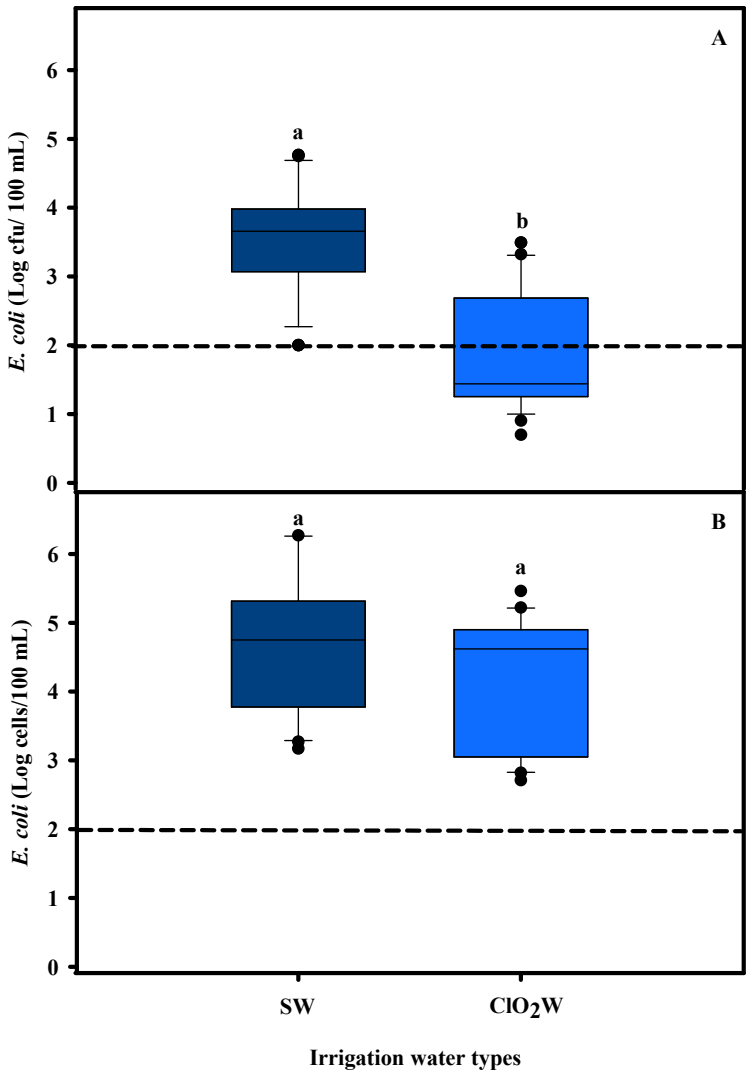


Figure 5. Boxplot representing *E. coli* counts (log CFU/100mL) in the subset of water samples with either absence or presence of pathogens in untreated (SW) and ClO_2 treated (ClO_2W) secondary effluent from a wastewater treatment plant used to irrigate baby lettuce. (A) *E. coli* counts obtained by conventional plate counting method. (B) *E. coli* counts obtained by PMA–qPCR quantification method. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box represents the median. Different letters indicate significant differences ($p<0.05$).

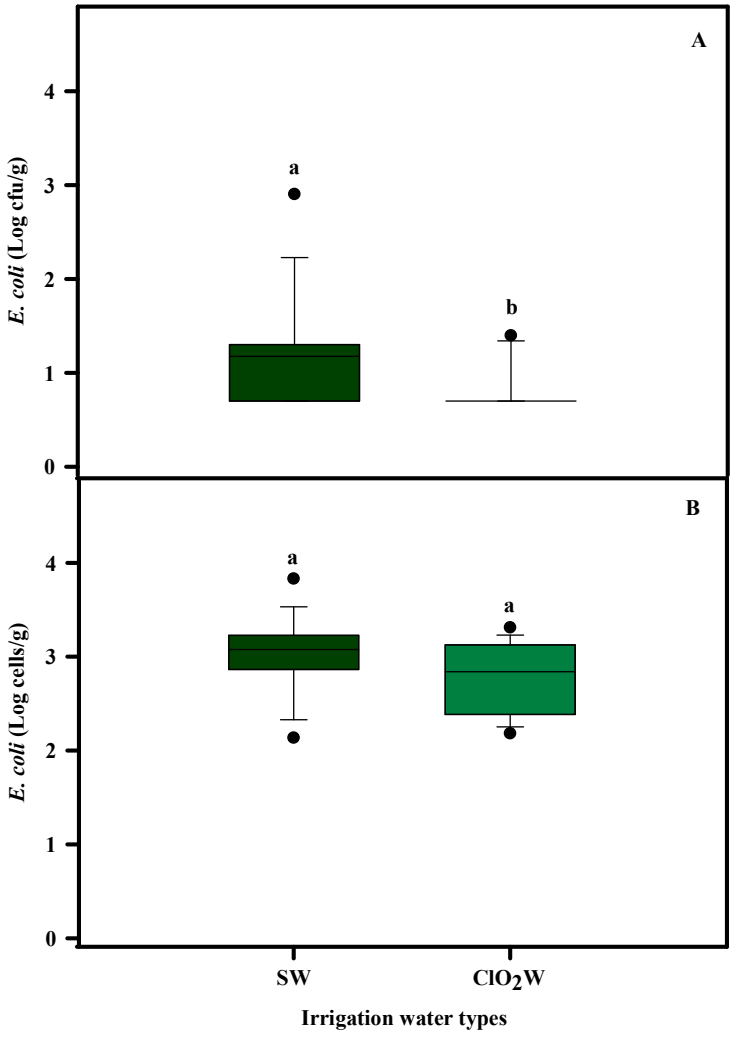
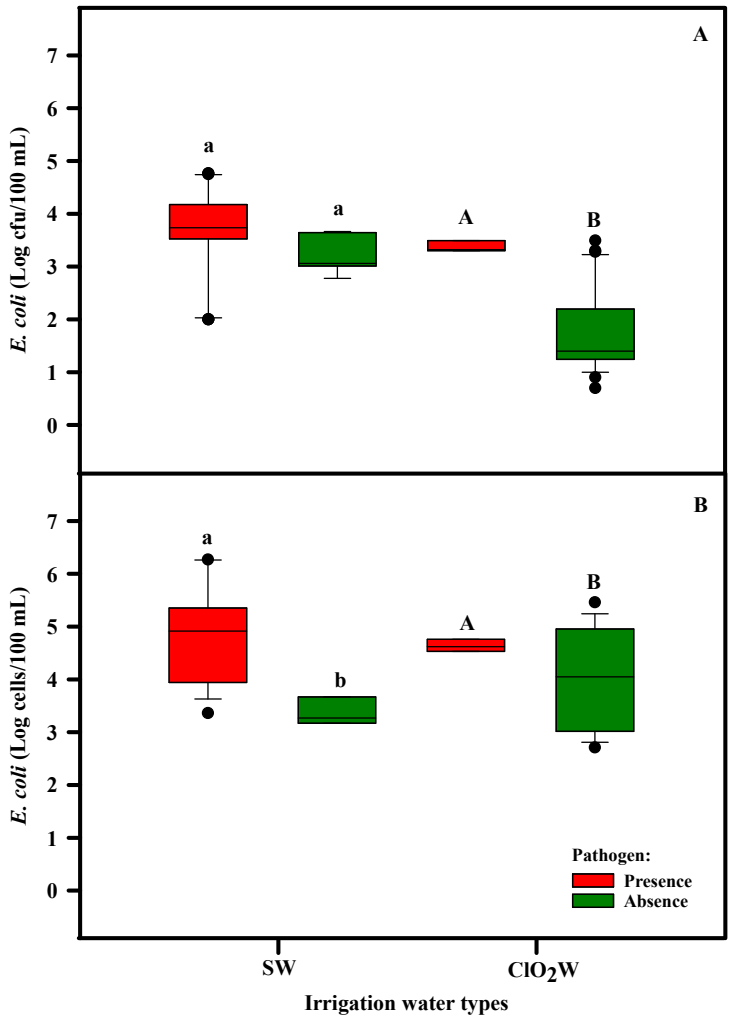


Figure 6. Chlorate concentration in baby lettuce (mg/kg) and untreated (SW) and ClO_2 treated (ClO_2W) secondary effluent (mg/L) from a wastewater treatment plant used to irrigate baby lettuce grown in a commercial greenhouse.



4.1 Artigo 4

Survival and growth of *E. coli* O157:H7 in irrigation water and the efficacy of sodium hypochlorite as a disinfection treatment

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Abstract

Irrigation water is considered an important vehicle of contamination of fresh produce by pathogenic microorganisms, such as *E. coli* O157:H7. Chemical disinfection treatments have been proposed as an intervention strategy to reduce contamination of irrigation water. Chlorine is one of the most commonly used disinfection treatments for irrigation water. The objective of the present study was to evaluate the suitability of *E. coli* O157:H7 strains to survive and grow in irrigation water. Additionally, the inactivation capacity of sodium hypochlorite applied at two different concentrations of free chlorine (5 mg/L and 7 mg/L) and at four different contact times (1, 5, 15 and 30 mins) was evaluated. That inactivation capacity of sodium hypochlorite against four different *E. coli* O157:H7 strains was determined taking into account the cultivable bacteria, but also the viable but non cultivable (VBNC) cells. Enumeration of cultivable *E. coli* O157:H7 showed that its suitability to survive in irrigation water for up to 72 h. The tested *E. coli* O157:H7 strains showed similar sensibility to sodium hypochlorite (5 mg/L) except for one of the strains when conventional plate count methods were used. The use of 7 ppm of free chlorine for 30 min completely inactivated all the bacteria present in the irrigation water. However, when the VBNC *E. coli* O157:H7 were enumerated, bacterial inactivation was much lower. This could be due to the induction of the VBNC state of *E. coli* O157:H7, indicating the reduced antimicrobial capacity of the disinfection treatment. The obtained results corroborate previous studies stating that the use of conventional enumeration methods to estimate the efficacy of disinfection methods could lead to an overestimation of the microbial reductions.

Key-Words: *Fresh produce; Leafy greens, pathogenic microorganisms, free chlorine, cultivable but non viable cells*.

1. Introduction

Escherichia coli O157:H7 (*E. coli* O157:H7) belongs to the group of enterohemorrhagic *E. coli* (EHEC), which produces verotoxins (shiga-like toxins) causing outbreaks of hemorrhagic colitis and hemolytic ureamic syndrome (Law, 2000). Besides this, it is an important bacterial pathogen associated with foodborne illness caused by the consumption of fresh produce (EFSA, 2015; ECDC, 2015; CDC, 2016; Ahmed et al., 2012; Ferguson et al., 2012; Sivapalasingam et al., 2004).

Fresh produce may become contaminated at every stage of the production, processing and distribution chain. At the pre-harvest level, irrigation water has been defined as a major risk factor in the contamination of leafy crops eaten raw as salads (Decol et al., 2017; Castro-Ibañez et al., 2015; Uyttendaele et al., 2015; Ceuppens et al., 2014; CPS, 2014; Holvoet et al., 2014). Among the main water sources used for irrigation, surface water is considered one of the riskiest water sources when compared to other sources such as borehole water and rainwater (Castro-Ibanez et al., 2015; Ahmed et al., 2012; Ferguson et al., 2012). Despite the higher contamination risk, the surface water, is still the most commonly irrigation water used in the production of fresh produce in the Southern of Brazil, (Decol et al., 2017; Ceuppens et al., 2014).

Previous studies highlighted the high prevalence of foodborne pathogens that can be present in surface water used for irrigation (Decol et al., 2017; Castro-Ibañez et al., 2015; Ceuppens et al., 2014). Most of the Good Agricultural Practices (GAP) include several intervention strategies to reduce contamination of irrigation water. One of the most recommended strategies is the application of water treatments (Pachepsky et al., 2011). Chlorine is one of the most commonly used sanitizer for drinking water, irrigation water and process wash water of fresh and fresh-cut produce mostly because of its bactericidal effectiveness, low cost, convenience, and relatively long-lived

residual (Gómez-López et al., 2014; Raudales et al., 2014). However, the susceptibility of bacterial pathogens to chlorine is variable. The ability of pathogenic microorganisms, such as *E. coli* O157:H7, to remain physiologically active and develop resistance to chlorine has significant public health implications (Ryu and Beuchat, 2005; Stephanie et al., 2004; Lisle eta al., 1998). However, studies demonstrated that concentrations of 0.5 to 10 mg / L of free chlorine in fresh produce wash water is sufficient for the reduction of microbial contamination to below the detection level for traditional technical (Luo, et al., 2018; Chen, et al., 2017; Gómez-López, et al., 2014; Luo, et al., 2011).

Considering the increasing involvement of fresh produce in foodborne illnesses caused by *E. coli* O157:H7 (CDC, 2016; EFSA AND ECDC, 2015), and the role of irrigation water as a vector of this bacterial contamination, the present focus on: (i) the evaluation of the capacity of adaptation and multiplication of strains of *E. coli* O157:H7 in irrigation water at isothermal conditions; (ii) the efficacy of chlorine treatments to inactivate *E. coli* O157:H7 in irrigation water; and (iii) the ability of *E. coli* O157:H7 to entry in a dormant state, also known as viable but non cultivable (VBNC) state.

2. Materials and methods

2.1. Pathogen selection

For this study, four *E. coli* O157:H7 strains, isolated from irrigation water were selected to perform in deep studies (Decol et al., 2017; Rodrigues et al., 2014).

2.2. Irrigation water

Irrigation water was obtained from a water reservoir at a commercial farm. Water samples (5 L each) were collected in a filtered plastic bottle. After the collection, water samples were transported in a thermal box under refrigeration temperatures (< 7ºC) to the Food Microbiology and Food Control Laboratory (ICTA/UFRGS). Once in

the lab, the water was filtered to remove the natural microbiota using a filtered syringe filter with a 0.22 µm pore size Mixed Cellulose Esters membrane (Merck, Darmstadt, Germany) and stored in a sterile bottle at -18 °C until use. For the experiments, 10 mL aliquots were placed in sterile falcon tubes. The **Table 1** shows the physicochemical characteristics of filtered irrigation water used in these experiments.

2.3. Bacterial strains and inoculum preparation

Four *E. coli* O157:H7 strains, three of them isolated (EC1, EC2, EC3) from water reservoirs and one (EC4) of them isolated from a stream bordering farmlands and urban areas were used in this study. Strains were grown separately in 5 mL of Brain Heart Infusion (BHI) broth (BHI, Oxoid, Basingstoke, UK), at 37 °C for 24 h; this step was repeated once. After incubation, the broth was centrifuged at 5000 *g* for 10 min at 4 °C. The supernatants were discharged and the pellets were washed with 0.1% peptone water (Oxoid, Basingstoke, UK). This procedure was repeated three times and then, after that, cells were re-suspended with 0.1% peptone water. The final cell concentration of 10^8 cfu/mL was adjusted through optical density 0.5 (OD630nm), using Ultrospec™ 3100 pro (Amersham Biosciences, UK) and confirmed by plating Mac-Conkey Sorbitol (SMAC) (OXOID, Basingstoke, UK). Plates were incubated at 37 °C for 24 h. Decimal serial dilutions using 0.1% peptone water were prepared. Growth of the four different *E. coli* O157:H7 strains was characterized in filtered irrigation water. Irrigation water was inoculated to a final cell concentration of 10^4 CFU/mL. The same was repeated for a cocktail of the 4 *E. coli* O157:H7 strains (Cocktail). To test the efficacy of sodium hypochlorite to inactivate of the *E. coli* O157:H7 strains in irrigation water, the water was inoculated following the same procedure up to a concentration of 10^6 CFU/mL of each of the *E. coli* O157:H7 strains and the four-strain cocktail.

2.4. Modeling of E. coli O157:H7 growth in irrigation water under isothermal conditions

Aliquots of 10 mL of filtrated irrigation water were inocculated with the different *E. coli* O157:H7 strains and the four-strain cocktail as previously mentioned. The falcon tubes were incubated at 26°C, mimicking the water temperature during the growing season in this region of Southern of Brazil. (Decol et al., 2017). Tubes were incubated until the strain reached the stationary phase. Sampling was carried out at varying time intervals. At each time point, 1mL of the sample was decimal diluted in 0.1% peptone water. Then, aliquots were plated in sorbitol MacConkey agar (SMAC, Oxoid, Basingstoke, UK) in triplicate and the plates incubated at 37 °C for 24 h. The experiments were repeated at least three times and the results were expressed as log CFU/mL.

The predictive primary model described by Baranyi and Roberts (1994) was used in order to calculate the growth kinetic parameters of strains on irrigation water. The growth curves for each temperature and pathogen were built separately by fitting the experimental data to the DMFit tool in ComBase software (browser.combase.cc/ DMFit.aspx). The following parameters were obtained: maximum growth rate (mmax), lag time (l), and maximum population density (Nmax) using the Baranyi and Roberts (1994) model (Equations (1) -(3)).

$$\ln(N(t)) = \ln(N_0) + \mu_{\max} A(t) - \ln\left[1 + \frac{e^{\mu_{\max} A(t)} - 1}{e^{(N_{\max} - N_0)}}\right] \quad (1)$$

$$A(t) = t + \frac{1}{\mu_{\max}} \ln\left(\frac{e^{(-\mu_{\max} t)} + q_0}{1 + q_0} \right) \quad (2)$$

$$\lambda = \frac{\ln\left(1 + \frac{1}{q_0}\right)}{\mu_{\max}} \quad (3)$$

where: ln(N(t)) ¼ ln of cell concentration at time t [h] (CFU/g); ln(N0) ¼ ln of initial cell concentration (CFU/g); mmax ¼ maximum growth rate (log CFU/g/h); ln(Nmax) ¼ ln of maximum cell concentration; q_0 [-] ¼ parameter expressing the initial physiological state of cells; l ¼ lag time (h).

2.5. Hypochlorite resistance test

To test the efficacy of sodium hypochlorite for inactivation strains of *E. coli* O157:H7 in irrigation water a commercial solution of sodium hypochlorite (2,0 – 2,5 % p/p) was selected. Two different solutions, containing different concentrations of free chlorine, (5 mg/l and 7 mg/l) were prepared using 10 mL filtered irrigation water in falcon tubes. The solutions were prepared at the time of use. Free chlorine was determined based on the N,N-diethyl-p-phenylendiamine (DPD) method (APHA, 1998) using a commercial kit Chlorine Cell Test (Merck, Darmstadt, Germany) and a spectrophotometer (λ=515) (Ultrospec 3100 Pro, Amersham, Biosciences, UK).To test the efficacy of sodium hypochlorite to inactivate of the E. coli O157:H7 strains and the four-strain cocktail was evaluated in four different contact times (1, 5, 15 and 30 mins).

The COD was determined by the standard photometric method (APHA, 1998) using the spectrophotometer (λ=600) and the commercial kit COD Cell Test (Merck,

Darmstadt, Germany). The method is based on the photometric determination of the concentration of $Cr_3þ$ ions resulting from the oxidation of organic matter by a hot mixture of $K_2Cr_2O_7$ acidified by H_2SO_4 that contains Ag_2SO_4 as catalyst, containing $HgSO_4$ as chloride masker.

Free chlorine and COD measurements were performed at different time intervals including 0, 1, 5, 15 and 30 minutes after the bacteria was inoculated in the filtered irrigation water and the water treated with sodium hypochlorite (**Fig. 1**). However, no significant variations were observed in these parameters at the evaluated times.

2.6. Microbiological analysis

Microbial analyses of the cultivable and VBNC bacteria after treatment with the selected free chlorine concentrations (5 and 7 mg/L) were performed using two different methodologies at the same time intervals (1, 5, 15, 30 min). The reaction of the free chlorine after each time interval was stoped by means of adding sodium thiosulfate (0.5%). Then, samples of 1mL each were decimal diluted in 0.1% peptone water and plated in sorbitol MacConkey agar (SMAC, Oxoid, Basingstoke, UK). Plates were incubated at 37 °C for 24 h. All the bacterial counts were carried out in triplicate. The experiments were repeated at least two times and the results were expressed as log CFU/mL.

For the molecular quantification of the VBNC *E. coli* O157:H7 cells, qPCR combined with propidium monoazide (Biotium Inc, Hayward, CA, USA) (q-PCR) (PMA-qPCR) was used as previously described in Truchado et al. (2016) with some modifications. Briefly, samples (9 mL each) were centrifuged at 13.000 rpm for 10 min at 4°C. Then, each obtained pellet was activated with PMA (20 µM) and kept at -20 ºC until the DNA extraction was performed. Master Pure^{TM} Complete DNA and RNA

purification kit (Epicenter, Madison, USA) following the manufacturer's instructions was used. For molecular quantification, primers and probes for detecting genes of *E. coli* 23S rRNA as well as PMA-qPCR procedure were identical to those described in Truchado et al. (2016).

2.7. Statistical analysis

Counts derived from microbiological analyses were log transformed and entered in an Excel spreadsheet (Microsoft Excel, 2016). Results were compiled and graphs were made using Sigma Plot 11.0 Systat Software, Inc. (Addilink Software Scientific, S.L. Barcelona). For the analysis of results the modelling of *E. coli* O157:H7 growth in irrigation water under isothermal conditions, measures of coefficient of determination (R^2) and Root Mean Square Error (RMSE) were used to evaluate the performance of the isothermal models built in this study. The R^2 is generally considered as an overall measure of the prediction calculated by the developed model. The R^2 could assumes a value between 0 and 1, and a value equal 1 for this measure indicates the best performance of the model (Wang et al., 2013). However, this is only one criterion to assess the goodness of fit of the model. If R^2 is low (<0.7), the mathematical model is not good; on the other hand, if R^2 is high (>0.9), it means that other criteria should be analyzed (Granato et al., 2014). So, it is prudent to check RMSE values when evaluating R^2 (Nunes et al., 2015). The RMSE is used to offer a standard measurement of goodness-of-fit of a model to the data used to produce it. A RMSE equals to 0 indicates the best possible fit between predicted and observed values (Wang et al., 2013).

For the analysis of results the resistance of commercial sodium hypochlorite using the software IBM SPSS version 21 (Chicago, USA). The Shapiro-Wilk test is used to analyse the normality. Mean statistical differences between treatments, strains

and quantification techniques were analyzed by Mann–Whitney U and Kruskal–Wallis. All tests were used at significance level of 5% (p = 0.05).

3. Results and discussion

3.1. E. coli O157:H7 Survival and Growth in Irrigation Water

Growth curves of each of the strains as well as the Cocktail of *E. coli* O157:H7 started with an initial concentration of about 4.0 log CFU/mL and reached a final concentration of approximately 7.0 log CFU/mL after 72 h at 26°C (**Fig. 2**). The predictive primary model Baranyi and Roberts (1994) was used to determine the primary growth parameters. Although the Barany model was developed for food application, a good correlation was observed with environmental samples such as irrigation water.

It is possible to observe the good fit between the experimental data and the Barany model; the curves obtained from DMFit at 26°C, showed a high correlation coefficient ($R^2 > 0.96$) and the RMSE medium of 0.223. Besides this, the primary growth parameters (growth rate, lag time and maximum population density) obtained using the DMFit software were compared with those predicted by the Combase software (**Table 2**). The values obtained with the Growth Model from Combase software were 0.587 (log cfu/h) for the growth rate, 2.00 (h) for the lag time and 8.7 (log cfu/mL) of maximal population density.

It was also noted, when comparing the different strains in relation of primary growth parameters, that the isolates EC1 and EC4, obtained from different water sources, presented the greatest differences between the parameters growth rate and lag time, being 0.27 ± 0.038 and 0.34 ± 0.028 (log cfu/h), and, 5.79 ± 1.05 and 4.13 ± 0.64 (h), respectively. Another result was that, when inoculating the strains in the form of a

four strains-cocktail, the lag time was reduced to 2.03 ± 2.21 (h) (**Table 2**). This might indicate that the microorganisms in the cocktail form have a faster adaptation to the irrigation water than that of the single strains.

This result demonstrates the ability of different *E. coli* O157:H7 strains to survive and grow in irrigation water with the specific characteristics of the water used in this study. This behaviour was already been observed by other authors. Van der Linden et al. (2014) observed multiplication of *Salmonella* and *E. coli* O157: H7 in different types of irrigation water (ponds and groundwater) for up to 14 days. Miyagi et al. (2011), also reported that *E. coli* STEC O157 was able to multiply in sterilized sea water and survived for at least 15 days.

The survival and multiplication of *E. coli* O157 in surface waters depends on several biological, physical and chemical factors. These include, amongst others, the presence of resident aquatic microbiota, the availability of nutrients, ultraviolet (UV) light and temperature (Vital et al., 2008). Van der Linden et al. (2014), compared different temperatures, 4 °C and 20 ° C, and verified that at 20 °C occurred a higher multiplication of the pathogens in different types of waters. Vital et al. (2008) also observed a strong influence of temperature on the ability *of E. coli* O157: H7 to multiply in sterilized natural freshwater at low carbon concentrations when the initial inoculation concentration is not higher (3 Log cfu/mL). In the present study, the water temperature of 26 °C was used, considering the most common ambient temperature during the growing season of leafy greens in this area of Southern of Brazil, as previously reported (Decol et al., 2017).

The ability of *Escherichia coli* O157:H7 to survive and grow in surface water may increase the potential for dissipation of the organism to facilitate cycles of livestock re-infection and lead to human infection (Avery et al., 2008). In addition, the rapid

multiplication capacity observed in this study, medium Lag time 5.18 h for strains and 2.03 h for cocktail for software DMFit, puts at risk all plant products that will be irrigated. Especially when using the spraying system, because the water comes in direct contact with the edible part, and when it is carried out near the harvest (Uyttendaele et al., 2015; Marouelli and Silva, 2011; Pachepsky et al., 2011; Marouelli et al., 2008). Oliveira et al. (2012) observed that *E. coli* O157:H7 survived on the surface of lettuce leaves after overhead irrigation with contaminated water for five weeks. This study verified that the transfer of *E. coli* O157:H7 to lettuce leaves occurred from contaminated soil or irrigation water. Barker-Reid et al. (2009), observed persistence of *E. coli* in lettuce irrigated with contaminated water.

These results suggest that when using irrigation water without treatment or guarantee of microbiological quality, the interval between the last irrigation and the harvest is respected. According to studies of Park et al. (2014) and Decol et al. (2017), who identified an association between contamination of generic *E. coli* and short irrigation lapse time between irrigation and harvest. Park et al., (2013) reported that the odds of spinach contamination were reduced to approximately 1 in 4 when the time since the last irrigation was longer than 5 days.

3.2. Inactivation of E. coli O157: H7 *by sodium hypochlorite in irrigation water*

The inactivation of four different *E. coli* O157:H7 strains and a Cocktail by a sodium hypochlorite solution was evaluated in the same type of irrigation water. **Figures 3** and **4** show the results obtained when 2 different concentrations of free chlorine (5 and 7 mg/L) were applied at three time intervals (0, 1, 5, 15 and 30 minutes). Enumeration of *E. coli* O157:H7 using plate count methods showed that the addition of 5 mg/L of free chlorine reduced the initial population of the bacteria by about 2.34 (±

0.14) Log cfu / mL for the single strains Ec1, Ec3, Ec4 as well as for the Cocktail after 1 min of contact time. A reduction of approximately 3.15 (± 0.02) Log cfu / mL was observed after 30 min contact time (**Table 3**). Differently, the strain Ec2 presented higher reduction after 1 min, of 3.79 (± 0.30) Log cfu / mL, and after 5 min. it was completely reduced.

When the same strains were tested using the solution with free chlorine concentrations at 7 mg /L in filtered irrigation water, more significant initial reductions were observed. The strains Ec1 and Ec2, which presented the initial medium reduction of 6.01 (± 0.32) Log cfu / mL after 1min contact time, and at the end of 30 min were completely reduced. The strain Ec4 showed the highest susceptibility to the sanitizer at this concentration, after 1 min of contact time it was completely reduced. Differently, the strain Ec3 and Cocktail presented lower initial reductions, but at the end of the 30 min contact time were completely reduced (**Table 3**).

Several studies have shown that low concentrations of free chlorine are effective in reducing the microbial contamination in fresh produce wash water (Luo, et al., 2018; Chen, et al., 2017; Zhou et al., 2015; Gómez-López, et al., 2014; Luo, et al., 2011). Luo, et al. (2018), evaluated the minimal effective free chlorine concentration to prevent pathogen presence during commercial washing water of Romaine lettuce, Iceberg lettuce, and diced cabbage and confirm that maintaining at least 10 mg/L free chlorine in wash water strongly reduced the likelihood of bacterial survival and thus potential cross contamination of washed produce.

Another study was conducted by Gomez-Lopez et al. (2014), to evaluate pathogen survival during the dynamic changes in free chlorine concentration affected by adding *Escherichia coli* O157:H7 inoculated to spinach juice and continuously replenishing chlorine. When a free available chlorine concentration of 5 mg/L was

maintained, no pathogens were detected in the wash water during the entire 1-hour testing period.

However, when quantified using the PMA-qPCR assay for the same samples for the chlorine concentrations at 5 mg / L and 7 mg / L no statistically significant differences in reduction of the different strains were observed (p= 0.460). For the treatment using the chlorine concentration at 5 mg / L the medium reduction after 30 min the contact is the 1.48 (± 0.25) Log cfu / mL, and in the treatment using chlorine concentration at 7 mg / L the medium reduction after 30 min the contact is the 1.80 (± 0.31) Log cfu / mL (**Table 3**). These results are the accordance with other studies, when levels of *E. coli* detected with the PMA-PCR method were higher than those detected by plate culture (López-Gálvez et al., 2018; Truchado et al., 2016).

The differences between the quantification techniques, conventional plate count and PMA-qPCR assay, has been reported by several studies (Truchado et al., 2018; Truchado et al., 2016; Li et al., 2014; Van Frankenhuyzen et al., 2013). This result may be due to the fact that the PMA is a DNA-dye, which allows the differentiation between viable and dead cells (Truchado et al., 2016; Gensberger et al., 2014). Therefore, the observed differences between qPCR and plate counts can attributed to the presence of VBNC bacteria. The obtained results corroborate previous studies that describe a bacteriostatic action of the disinfection treatments, which can induce the entrance of *E. coli* O157:H7 cells into a VBNC state (Truchado et al., 2018). VBNC state is produced when the microorganism is confronted to adverse environment conditions, such as scarcity of nutrients, non-optimal temperatures, solar irradiation, high osmotic pressure, heavy metals and etc (Fakruddin et al., 2013; Zhao et al., 2013; Oliver, 2010; Colwell and Grimes, 2000). This state can also be induced when microorganisms are exposed to chemical disinfectants with chlorine and chlorine dioxide (Fakruddin et al., 2013; Zhao

et al., 2013; Oliver et. al., 2005). Recently, Kibbee and Örmeci (2017) have evaluated the levels of *E. coli* present in secondary wastewater effluent after chlorine disinfection, showing that high numbers of VBNC *E. coli* survive chlorination.

The *E. coli* generic and pathogenic species have been shown to survive sub-lethal environmental stress conditions by entering into the VBNC (Pienaar et al., 2016; Zhao et al., 2013; Liu et al., 2010; Asakura et al., 2007; Arana et al., 2004). This characteristic allows to many species of pathogenic *E. coli* to survive treatment and persist in processed food, in treatment for drinking water, as well as in the environment (Aurass et al., 2011; Sardessai et al., 2005). Another important factor, is that the VBNC state is reversible (Pienaar et al., 2016; Li et al., 2014).

After a stress period, the pathogenic bacteria can return to its viable state and express their virulence genes (Ding et al., 2017; Pienaar et al., 2016). Lothigius et al. (2010) observed in study that state VBNC allowed after the enterotoxigenic *E. coli* is exposed for a three month in both fresh and salt water, after this, the cell wall remained intact and there was expression of both metabolic and virulence genes. Other interesting fact is was observed by Yaron and Matthews (2002), in a study of toxin genes (stx1 and stx2) that could still be expressed in the VBNC state of *E. coli* O157:H7 by reverse transcription PCR. However, interestingly, expression of the virulence gene in VBNC cells does not necessarily indicate that the cells will produce toxins (Zhao et al., 2017).

When analysing the behaviour of the different strains and the four strain-cocktail, no significant difference (p= 0.340) was observed, despite the difference of origin (rural properties and water sources), the behaviour and the resistance of the strains were similar to the different exposure conditions (free chlorine and time contact). Several studies have shown that different factors may influence the bactericidal effect of free chlorine and consider the most important are temperature, pH, and presence of organic

matter. (Luo, et al., 2018; Chen, et al., 2017; Zhou et al., 2015; Gómez-López, et al., 2014; Luo, et al., 2011; Ayyildiz, et al., 2009). In the present study, the concentration of organic matter no present significant variations, and could interfere in the effectiveness of free chlorine during the times tested.

4. Conclusions

This study corroborated previous studies which demonstrated the capacity of *E. coli* O157:H7 strains to survive and grow in surface irrigation water. This fact is very important because the risk of using irrigation water without treatment or guarantee of microbiological contamination, since these pathogens (*E. coli* O157:H7) have the capacity to adapt to the environmental conditions. The strains of *E. coli* O157:H7 demonstrate the ability to entry in a viable but non cultivable state. This fact is observed from the viable bacteria enumerated by molecular techniques combined with the use of dies (PMA-qPCR), no significant differences were observed between free chlorine concentration and time tested. The significative difference between quantification techniques observed in this study prove that the use of plate count methods to estimate the efficacy of disinfection methods could lead to an overestimation of microbial reductions. This result corroborated previous studies, indicating a potential bacteriostatic action of sodium hypochlorite present when applied in surface irrigation water.

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References

Ahmed, W., Richardson, K., Sidhu, J. P. S., Toze, S., 2012. *Escherichia coli* and *Enterococcus* spp. in Rainwater Tank Samples: Comparison of Culture-Based Methods and 23S rRNA Gene Quantitative PCR Assays. Environmental Science & Technology, 46, 11370−11376.

Arana, I., Seco, C., Epelde, K., Muela, A., 2004. Fernández-Astorga, A., Barcina, I. Relationships between *Escherichia coli* cells and the surrounding medium during survival processes. Antonie van Leeuwenhoek, 86(2):189–199.

Asakura, H., Panutdaporn, N., Kawamoto, K., Igimi, S., Yamamoto, S. and Makino, S. Proteomic Characterization of Enterohemorrhagic *Escherichia coli* O157:H7 in the Oxidation-Induced Viable but Non-Culturable State. Microb.and Immunology, 51: 875–881, 2007.

Aurass, P., Prager, R., and Flieger,A. EHEC/EAEC O104: H4 strain linked with the 2011 German outbreak of haemolytic uremic syndrome enters into the viable but non-culturable state in response to various stresses and resuscitates upon stress relief. Environ. Microbiol. 13, 3139–3148, 2011.

Avery, L.M., Williams, A.P., Killham, K., Jones, D.L. Survival of *Escherichia coli* O157:H7 in waters from lakes, rivers, puddles and animal-drinking troughs. Science of The Total Environment. V. 389, (25), 378-385, 2008.

Ayyildiz, O., Ileri, B., Sanik, S., 2009. Impacts of water organic load on chlorine dioxide disinfection efficacy. J. of Hazardous Materials, 168,1092–1097.

Barker-Reid, F., Harapas, D., Engleitner, S., Kreidl, S., Holmes, R., Faggian, R., 2009. Persistence of *Escherichia coli* on injured iceberg lettuce in the field, overhead irrigated with contaminated water. J Food Prot., 72(3):458-64.

Baranyi J. and Roberts T.A. A dynamic approach to predicting bacterial growth in food. Int. J. Food Microbiol. 23, 277-294, 1994.

Castro-Ibáñez, I., Gil, M.I., Tudela, J.A., Allende, A., 2015. Microbial safety considerations of flooding in primary production of leafy greens: A case study. Food Research International. 68, 62−69.

CDC (Centers for Disease Control and Prevention). 2016. Reports of Selected *E. coli* Outbreak Investigations. https://www.cdc.gov/ecoli/outbreaks.html

Ceuppens, S., Hessel, C.T., Rodrigues, R.de Q., Bartz, S., Tondo, E.C., Uyttendaele, M., 2014. Microbiological quality and safety assessment of lettuce production in Brazil. Int. J. Food Microbiol. 181, 67−76.

Chen, X., Hung, Y.C., 2017. Effects of organic load, sanitizer pH and initial chlorine concentration of chlorine-based sanitizers on chlorine demand of fresh produce wash waters. Food Control, 77, 96-101.

Colwell, R.R., Grimes, D.J., (2000). Nonculturable microorganisms in the environment. Herndon, VA: ASM press.

Decol, L.T., Casarin, L.S., Hessel, C.T., Batista, A.C.F., Allende, A., Tondo, E.C., 2017. Microbial quality of irrigation water used in leafy green production in Southern Brazil and its relationship with produce safety. Food Microbiol. 65, 105−113.

Ding, T., Suo1, Y., Xiang, Q., Zhao, X., Chen, S., Ye, X., Liu, D. Significance of Viable but Nonculturable *Escherichia coli*: Induction, Detection, and Control. J. Microbiol. Biotechnol. 27(3), 417–428, 2017.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2015. The European Union summary report on trends and

sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA Journal, 13, 4329. Available at: 10.2903/j.efsa.2015.4329 (Acessed February 2016).

Fakruddin, M., Mannan, K., Andrews, S. Viable but nonculturable bacteria: food safety and public health perspective. ISRN Microbiology, Article ID 703813, 2013.

Ferguson, A. S., Layton, A. C., Mailloux, B. J., Culligan, P. J., Williams, D. E., Smartt, A. E., Sayler, G. S., Feighery, J., Mckay, L. D., Knappett, P. S.K., Alexandrova E., Arbit, T., Emch, M., Escamilla, V., Ahmed, K. M., Alam, MD. J., Streatfield, P. K., Yunus, M., Geen, A. V., 2012. Comparison of fecal indicators with pathogenic bacteria and rotavirus in groundwater. Science of the Total Environment**,** 431, 314–322.

Gómez-López, V.M., Lannoo, A.S., Gil, M.I., Allende, A., 2014. Minimum free chlorine residual level required for the inactivation of *Escherichia coli* O157:H7 and trihalomethane generation during dynamic washing of fresh-cut spinach. Food Control, 42, 132−138.

Granato, D., Calado, V.M.A., Jarvis, B., 2014. Observations on the use of statistical methods in food science and technology. Food Res. Int. 55, 137−149.

Holvoet, K., Sampers, I., Seynnaeve, M., & Uyttendaele, M., 2014. 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. International Journal of Food Microbiology, 171, 21–31.

Junli, H., Li, W., Nanqi, R., Fang, M., Juli, 1997. Disinfection effect of chlorine dioxide on bacteria in water. Wat. Res. 31, n°3, 607–613.

Kibbee, R.J. and Örmeci, B., 2017. Development of a sensitive and false-positive free PMA-qPCR viability assay to quantify VBNC *Escherichia coli* and evaluate

disinfection performance in wastewater effluent. J. Microbiol. Methods, 132, pp. 139–147.

Law, D. Virulence factors of *Escherichia coli* O157:H7 and other Shiga-toxin producing *E. coli,* 2000. J. Appl. Microb. 88, 729–745.

Li, L., Mendis, N., Trigui, H., Oliver, J.D., Faucher, S.P., 2014. The importance of the viable but non-culturable state in human bacterial pathogens. Front Microbiol, 5:258.

Lisle, J.T., Broadway, S.C., Prescott, A.M., Pyle, B.H., Fricker, C., McFeters, G. A. ,1998. Effects of starvation on physiological activity and chlorine disinfection resistance in *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 64, 4658–4662.

Liu, Y., Wang, C., Tyrrell, G., Li, X.F., 2010. Production of Shiga-like toxins in viable but nonculturable *Escherichia coli* 0157:H7. Water Res., 44(3):711–718.

López-Gálvez, F., Gil, M.I., Meireles, A., Truchado, P., Allende, A. 2018. Demonstration tests of irrigation water disinfection with chlorine dioxide in open field cultivation of baby spinach. J.Sc. Food Agric., Accepted.

Lothigius, Å., Sjöling, Å., Svennerholm, A.M., Bölin, I., 2010. Survival and gene expression of enterotoxigenic *Escherichia coli* during long-term incubation in sea water and freshwater. J Appl Microbiol. 108(4):1441–1449.

Luo, Y., Nou, X., Yang, Y., Alegre, I., Turner, E., Feng, H., Abadias, M., Conway, W., 2011. Determination of Free Chlorine Concentrations Needed to Prevent *Escherichia coli* O157:H7 Cross-Contamination during Fresh-Cut Produce Wash. J. Food Protec., Vol. 74:3, 352–358.

Luo, Y., Zhou, B., Van Haute, S., Nou, X., Zhang, B., Teng, Z., Turner, E.R., Wang, Q., Millner, P.D., 2018. Association between bacterial survival and free chlorine

concentration during commercial fresh-cut produce wash operation. Food Microbiology, 70, 120-128.

Miyagi, K. et al., 2001. Survival of Shiga Toxin-Producing *Escherichia coli* O157 in Marine Water and Frequent Detection of the Shiga Toxin Gene in Marine Water Samples from an Estuary Port. Epidem. and Infec. v. 126, p. 129−133.

Marouelli, W.A., Silva, W.L.C., 2011. Seleção de sistemas de irrigação para hortaliças. Circular Técnica 98. EMBRAPA Hortaliças, Dez. (2ª edição). Acesso em: 05 de fevereiro 2015.

Nunes, C.A., Alvarenga, V.O., Sant'Ana, A.S., Santos, J.S., Granato, D., 2015. The use of statistical software in food science and technology: advantages, limitations and misuses. Food Res. Int. 75, 270−280.

Oliver, J. D., 2005. The viable but nonculturable state in bacteria. J. Microbiol. 43, 93–100.

Oliver, J.D., 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol. Rev. 34: 415-425.

Oliveira, M., Viñas, I., Usall, J., Anguera, M., Abadias, M., 2012. Presence and survival of *Escherichia coli* O157:H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water. International Journal of Food Microbiology. 156, 133–140.

Pachepsky, Y., Shelton, D.R., McLain, J.E.T., Patel, J., Mandrell, R.E., 2011. Chapter two: irrigation waters as a source of pathogenic microorganisms in produce: a review. In: Donald, L.S. (Ed.), Advances in Agronomy, vol. 113. Academic Press, pp. 73-138.

Pienaar, J.A., Singh, A., Barnard, T.G., 2016. The viable but nonculturable state in pathogenic *Escherichia coli*: A general review. Afr J Lab Med. 2016;5(1), a368.

Raudales, R.E., Parke, J.L., Guy, C.L., Fisher, P.R., 2014. Control of waterborne microbes in irrigation: A review. Agricultural Water Management, 143, 9–28.

Rodrigues, R.Q.L.M.R., Paula, C.M., Hessel, C.T., Jacksens, L., Uyttensaele, M., Bender, R.J., Tondo, E.C., 2014. Microbiological contamination linked to implementation of good agricultural practices in the production of organic lettuce in Southern Brazil. Food Control, 42, 152-164.

Ryu, J.H., Beuchat, L.R., 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: effect of exopolysaccharide and curli production on its resistance to chlorine. Appl. Environ. Microbiol. 71, 247–254.

Sardessai Y., 2005. Viable but non-culturable bacteria: their impact on public health. Curr. Sci. 89(10):1650.

Sivapalasingam, S., Friedman, C.R., Cohen, L., Tauxe, R.V., 2004. Fresh produce: A growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. J. Food Prot., 67, 2342–2353.

Stephanie, L.R., Cash, J.N., Siddiq, M., Ryser, E.T., 2004. A Comparison of Different Chemical Sanitizers for Inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* in Solution and on Apples, Lettuce, Strawberries, and Cantaloupe. Journal of Food Protection, Vol. 67, No. 4, pp. 721-731.

Truchado, P., López-Gálvez, F., Gil, M.I., Pedrero-Salcedo, F., Alarcon, J.J., Allende, A., 2016. Suitability of different *Escherichia coli* enumeration techniques to assess the microbial quality of different irrigation water sources. Food Microb 58, 29–35.

Truchado, P., Hernandez, N., Gil, M.I., Ivanek, R., Allende, A., 2018. Correlation between *E. coli* levels and the presence of foodborne pathogens in surface irrigation water: Establishment of a sampling program. Water Research, 128, 226-233.

Uyttendaele, M., Jaykus, L.A., Amoah, P., Chiodini, A., Cunliffe, D., Jacxsens, L., Holvoet, K., Korsten, L., Lau, M., McClure, P., Medema, G., Sampers, I., Jasti, P. R., 2015. Microbial Hazards in Irrigation Water: Standards, Norms, and Testing to Manage Use of Water in Fresh Produce Primary Production. Comprehensive Reviews in Food Science and Food Safety.Vol.14.

Vital, M.; Hammes, F.; Egli, T., 2008. *Escherichia coli* O157 can grow in natural freshwater at low carbon concentrations. Environ. Microbiol. 10, 2387–2396.

Van Der Linden et al., 2014. Enteric Pathogen Survival Varies Substantially in Irrigation Water from Belgian Lettuce Producers. Int. J. Res. Pub. Health. v.10, p. 10105−10124.

Van Frankenhuyzen, J.K., Trevors, J.T., Flemming, C.A., Lee, H., & Habash, M.B., 2013. Optimization, validation, and application of a real-time PCR protocol for quantification of viable bacterial cells in municipal sewage sludge and biosolids using reporter genes and *Escherichia coli*. Journal of Industrial Microbiology and Biotechnology, 40, 1251–126.

Wang, H.Y., Wen, C.F., Chiu, Y.H., Lee, I.N., Kao, H.Y., 2013. *Leuconostoc mesenteroides* growth in food products: prediction and sensitivity analysis by adaptive network based fuzzy inference systems. PLoS One 8, 1−16.

Yaron, S., and Matthews, K., 2002. A reverse transcriptase-polymerase chain reaction assay for detection of viable *Escherichia coli* O157: H7: investigation of specific target genes. J. Appl. Microbiol. 92, 633–640.

Zhao F, Bi X, Hao Y, Liao, X., 2013. Induction of viable but nonculturable *Escherichia coli* 0157:H7 by high pressure CO_2 and its characteristics. PLoS One, 8(4):62388.

Zhao, X., Zhong, J., Wei, C., Lin, C.W., Ding, T., 2017. Current Perspectives on Viable but Non-culturable State in Foodborne Pathogens. Front. Microbiol. 8:580.

Table 1. Physicochemical parameters of irrigation water at the time of collection

Parameter	**Value**
COD (mgO_2/l)	22.0
Total organic carbon (mg/l)	17.1
Turbidity (NTU)	18.10
pH	6.0
Conductivity (µS/cm)	45.7
Temperature (°C)	25.0
Total coliforms (log CFU/mL)	4.62
Elements and salts	
-Na (mg/l)	4.67
-Ca (mg/l)	4.06
-K (mg/l)	1.48
-Fe (mg/l)	4.29
-S (mg/l)	0.77
-Mg (mg/l)	1.35

Table 2. Estimated the primary grow parameters of *E. coli* O157:H7 inoculated in the filtered irrigation water and the correlation with Combase at 26°C

Strains	Growth rate (log cfu/h)[a]	Lag time (h)[a]	MPD (log CFU/g)[a]	R^2 [b]	RMSE[c]
EC1	0.27 ± 0.038	5.79 ± 1.05	7.29 ± 0.09	0.991	0.156
EC2	0.32 ± 0.084	5.40 ± 1.67	6.78 ± 0.13	0.966	0.262
EC3	0.32 ± 0.084	5.40 ± 1.67	6.78 ± 0.13	0.966	0.262
EC4	0.34 ± 0.028	4.13 ± 0.64	7.05 ± 0.06	0.994	0.118
Cocktail	0.28 ± 0.060	2.03 ± 2.21	7.28 ± 0.16	0.961	0.316

[a] Mean value of triplicate trials.
[b] R-square
[c] RMSE =root mean square error

Table 3. Log reductions of *E. coli* O157:H7 on filtered irrigation water treated with sodium hypochlorite

Treatment[a]	Time (min)[b]	Log reduction[c]									
		Plate count (Log cfu/mL)[d]					PMA-qPCR(Log cells/mL)[e]				
		Ec1	Ec2	Ec3	Ec4	Cocktail	Ec1	Ec2	Ec3	Ec4	Cocktail
5 mg/L	1	2.34±0.4A[f]	3.79±0.3A	2.42±0.1A	2.46±0.02A	2.15±0.2A	1.08±0.2A	0.89±0.2A	1.65±0.4A	1.47±0.2A	0.73±0.3A
	5	2.77±0.1A	6.43±0.7B	1.88±0.2A	1.81±0.2AB	1.79±0.2B	1.05±0.1A	1.03±0A	1.13±0.4B	1.26±0.1A	0.84±0A
	15	3.28±0.2B	6.43±0B	2.71±0.7AB	2.94±0.03C	1.66±0.3B	1.04±0.1A	1.07±0.2A	1.52±0.1A	1.67±0.2A	0.28±0.3B
	30	3.28±0.2B	6.43±0B	3.52±0.1B	3.31±0.1C	2.47±0.3C	1.79±0.1A	1.22±0.1A	1.44±0.2A	1.67±0.2A	1.27±0.1A
7 mg/L	1	6.21±0.9A	5.81±0.9A	3.57±0.2A	6.80±0A	1.95±0.7A	0.58±0.8A	0.67±0.5A	1.07±0.3A	1.12±0.7A	1.08±0.5A
	5	6.83±0B	5.92±0.7A	3.43±0.2A	6.80±0A	2.57±0B	0.61±0.9A	0.78±0.6A	1.52±0.3B	1.46±1.1B	0.79±0A
	15	6.83±0B	6.43±0B	6.00±1.2BC	6.80±0A	3.79±0.1C	0.44±0.4A	0.70±0.2A	2.05±1.5BC	1.21±0.8AB	1.16±0.7A
	30	6.83±0B	6.43±0B	6.85±0C	6.80±0A	6.11±0D	1.68±0.1B	1.64±0.1B	2.11±0.2C	2.14±0C	1.44±0.1B

[a]Free chlorine concentrations: 5 mg/l and 7 mg/l in irrigation water; [b]Time intervals of contact with chorine treatment and strains; [c]Log reduction= means of bacterial population before treatment – means of bacterial population after treatment; [d]Quantification using plating count in sorbitol MacConkey agar; [e] Quantification using the molecular technical PMA-qPCR. [f]Means with different uppercase letters in the same column are significantly different ($p < 0.05$). Results of three replicates ± standard desviation.

Figures

Figure 1. Chlorine concentrations (mg/L) and COD variation (mgO_2L^{-1}) in filtered irrigation water during test of commercial sodium hypochlorite resistance of strains *E. coli* O157:H7.

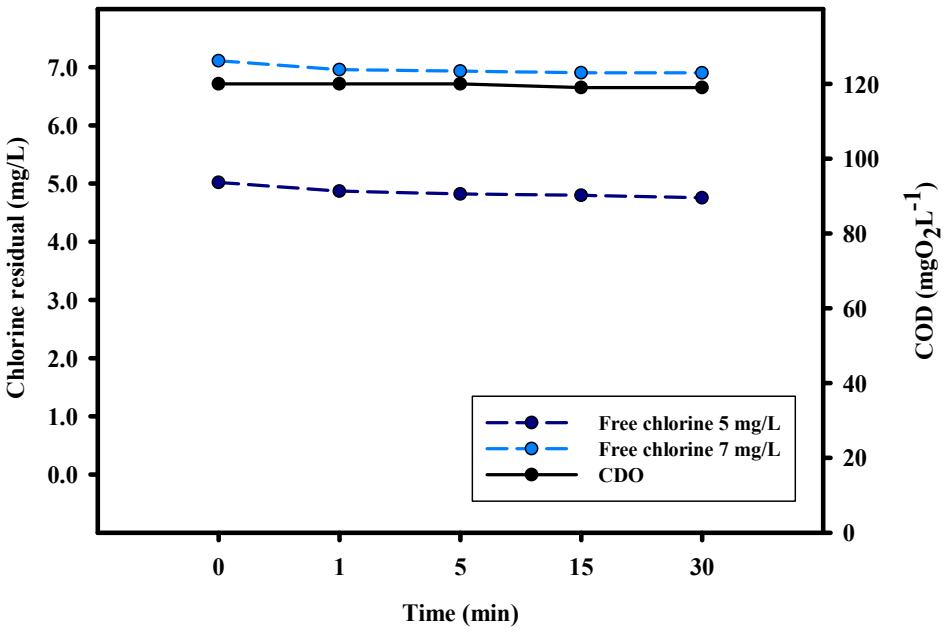


Figure 2. Growth curves of *E. coli* O157:H7 in filtered irrigation water for 72 h at 26°C (Log cfu/mL). The orange line representing the fitting data to DMFit from Combase software. A) representing growth curves of strain Ec1, B) representing growth curves of strain Ec2, C) representing growth curves of strain Ec3, D) representing strain growth curves of Ec4 and E) representing growth curves of Cocktail.

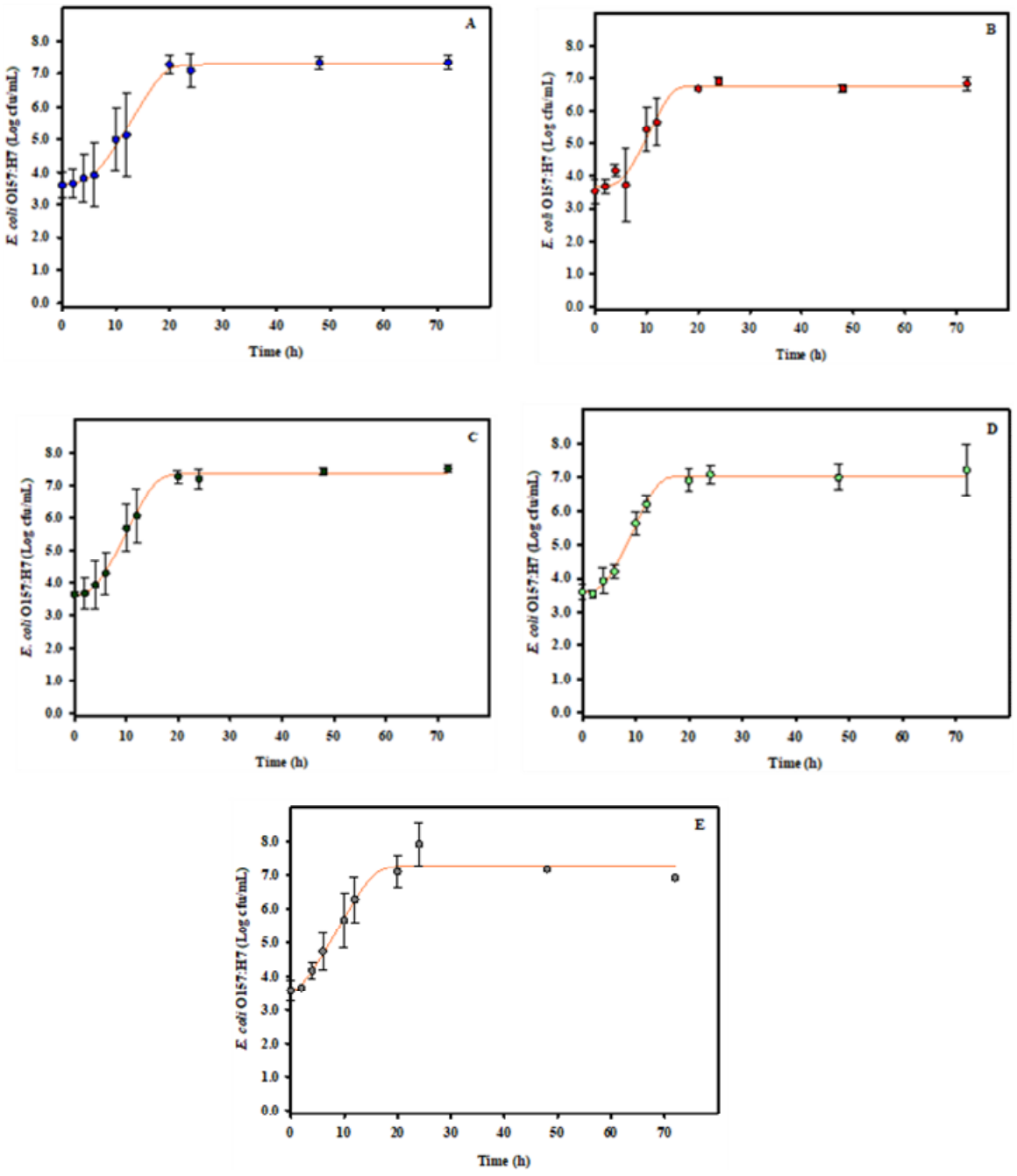


Figure 3. Changes in *E. coli* O157:H7 counts, obtained by conventional plate count method (Log cfu/mL), exposed to filtered irrigation water with sodium hypochlorite at 26°C. A) free chlorine 5 mg/L and B) free chlorine 7 mg/L.

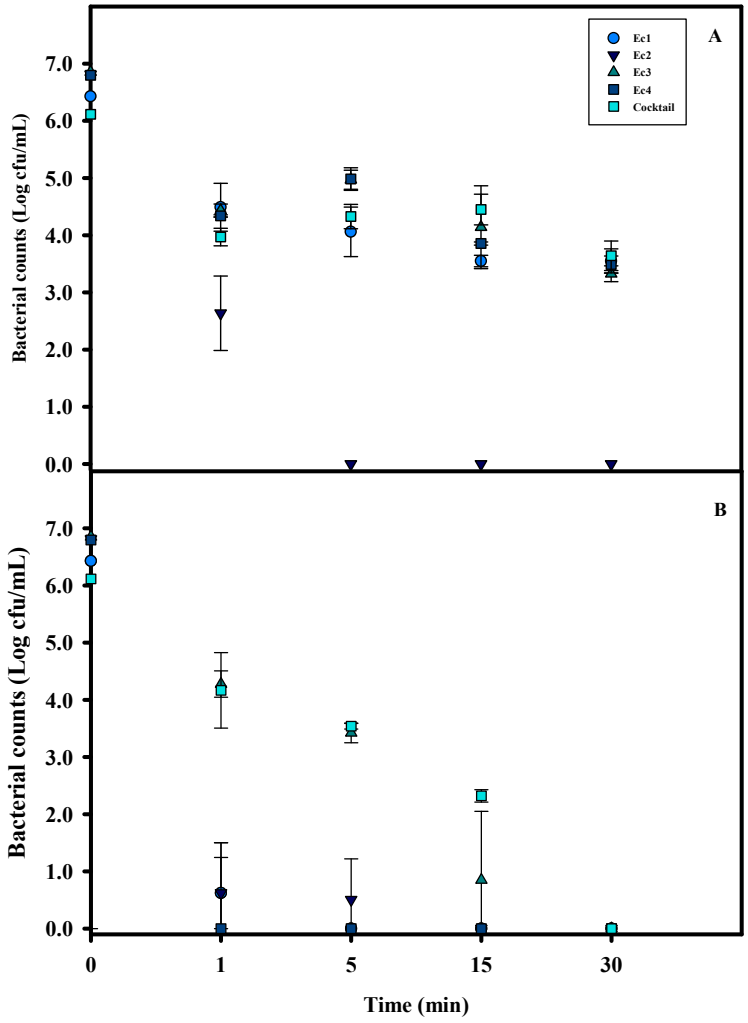
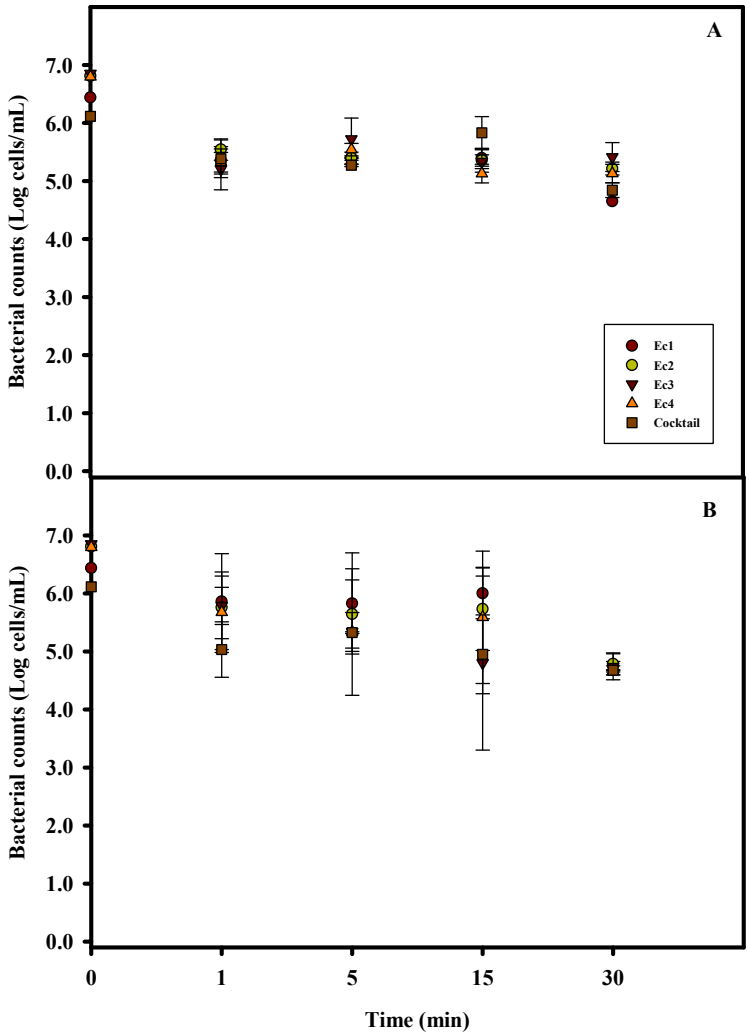


Figure 4. Changes in *E. coli* O157:H7 counts, obtained by PMA–qPCR molecular quantification method (Log cells/mL), exposed to filtered irrigation water with sodium hypochlorite at 26°C. A) free chlorine 5 mg/L and B) free chlorine 7 mg/L.



5. DISCUSSÃO GERAL

Águas superficiais como as de rios, riachos e açudes são as fontes mais comumente utilizadas para irrigação (ALLENDE & MONAGHAN, 2015) e, de acordo com alguns estudos e agências internacionais, são as que apresentam o maior risco de contaminação quando utilizadas para irrigação de vegetais consumidos frescos (ALLENDE & MONAGHAN, 2015; UYTTENDAELE et al., 2015; GLEICK, 2000). Na presente Tese, os artigos 1 e 2, demonstraram que as fontes de água analisadas apresentaram uma alta prevalência de micro-organismos indicadores, como Coliformes Totais, *E. coli* genérica e *Enterococcus* spp. Por exemplo, *E. coli* foi encontrada em 84,8% das amostras de açudes e em 100% nas amostras de riacho, enquanto *Enterococcus* spp. e Coliformes Totais estiveram prevalentes em 100% das amostras de água de ambas origens. Entretanto, ao comparar as contagens dos indicadores, principalmente *E. coli* genérica, entre as diferentes fontes, não foi verificada diferença estatística significativa. A alta prevalência de micro-organismos indicadores, em ambas as fontes de água de irrigação avaliadas no presente estudo, confirma a hipótese de que as fontes de água superficiais sem proteção ou tratamento são as que apresentam maior risco de contaminação microbiológica (UYTTENDAELE et al., 2015; AHMED et al., 2012; FERGUSON et al., 2012).

Atualmente, já são encontrados guias e regulamentações publicados por países e agencias internacionais, que recomendam padrões para a qualidade microbiana de água de irrigação. A maioria destes guias indicam a *E. coli* genérica como o melhor indicador de contaminação fecal e determinam limites aceitáveis que variam entre 10 e 126 UFC/100mL (UYTTENDAELE et al.,

2015). No caso de vegetais folhosos, como a alface, irrigados por aspersão, o limite recomendado para *E. coli* genérica é 100 UFC/100mL (AHDB, 2016). Já no Brasil, a legislação estabelece um limite de coliformes fecais de 2 log UFC/100mL para água de irrigação de vegetais consumidos crus (BRASIL, 1986).

As amostras positivas para *E. coli* genérica apresentaram contagem que variaram de 2.1 a 5.4 Log/ 100mL. Com base nestes resultados as fontes de água analisadas se encontram acima do limite brasileiro e internacional, consequentemente, podem ser consideradas não apropriadas para irrigar vegetais consumidos crus, como as alfaces, principalmente utilizando o método de irrigação por aspersão. Além disso, foi encontrada uma correlação positiva entre os níveis de *E. coli* genérica das amostras de água de irrigação e alfaces, indicando que a água de irrigação possui impacto importante na qualidade e segurança dos vegetais irrigados pelas mesmas.

Outro dado importante demostrado no presente trabalho, foi a alta prevalência dos patógenos entéricos *E. coli* O157:H7 e *Salmonella* spp. em amostras de água e alface, os quais apresentaram contagens acima de 2 log UFC/100mL de *E. coli* genérica. Além disso, foi verificada uma correlação significativa entre as contagens dos indicadores *Enterococcus* spp. e *E. coli* genérica com a presença de *Salmonella* spp. Esse fato está em concordância com estudos já publicados que correlacionam as altas contagens de indicadores de origem fecal, principalmente a *E. coli* genérica, como um bom preditivo para a presença de patógenos entéricos em amostras de origem ambiental (CASTRO-IBÁÑEZ et al, 2015a; CEUPPENS et al., 2014; HOLVOET et al., 2014; PARK et al., 2014).

Estudos tem relatado como uma boa estratégia a quantificação de micro-organismos indicadores para caracterizar a contaminação microbiana na produção de vegetais. Uma vez que, a maioria dos patógenos bacterianos se apresenta em baixa concentração e fastidiosos em amostras ambientais (ALLENDE & MONAGHAN, 2015).

Além da contaminação microbiana, foi possível verificar também, que fatores climáticos (estações do ano, temperatura ambiental e os níveis de precipitação) e práticas agrícolas (presença de animais e regime de irrigação) influenciaram nos níveis contaminação das alfaces.

Visando uma água de qualidade e segura para utilização na irrigação por aspersão de alfaces do tipo *baby* cultivadas em estufa. Foi testada a eficácia do desinfetante ClO_2 quando aplicado em águas residuais urbanas, que é uma fonte de água comumente utilizada para irrigação nas regiões áridas e semiáridas (BECERRA-CASTRO et al., 2015). No artigo 3, níveis aceitáveis de *E. coli* genérica (<2 log CFU/100mL, segundo EC, 2017) foram obtidos, quando águas residuais foram tratadas com quantidades de 3.3 e 9.2 mg/L de ClO_2. Para avaliar a capacidade de redução microbiana do ClO_2 quando aplicado neste tipo de água, foram utilizados dois métodos de quantificação de *E. coli* genérica, um convencional, em placas com meio de cultura, e outro molecular, utilizando PMA-qPCR. No método convencional em placas, foi verificada diferença estatística significativa ($p < 0.05$) entre as amostras controle (SW) e tratamento (ClO_2) em amostras de água e alfaces. Pelo método molecular PMA-qPCR, não foi encontrada diferença estatística significativa ($p > 0.05$) entre SW e ClO_2 nas amostras de água e alfaces. Esta diferença entre os métodos de quantificação de *E. coli* genérica, já foi observada em outros estudos (VAN

FRANKENHUYZEN et al., 2013; GENSBERGER et al., 2014; LI et al., 2014; TRUCHADO et al., 2016b) e pode ser explicada pela capacidade do método molecular PMA-qPCR poder quantificar células viáveis mas não cultiváveis (VNC), o que não é possível pelo método convencional com placas. Através da quantificação das células bacterianas no estado VNC demonstrando que o ClO_2 apresentou um efeito bacteriostático nas condições avaliadas.

No presente estudo (artigo 3), a concentração de *E. coli* foi acima do limite de 2 log UFC/100 mL em todas as amostras antes do tratamento com ClO_2 (SW). Por outro lado, após o tratamento com ClO_2, 69.4% das amostras analisadas estavam em conformidade com a legislação espanhola para *E. coli* (REAL DECRETO 1620/2007, 2007), com base na quantificação pelo método tradicional. Portanto, mesmo após o tratamento com ClO_2, 30.6% das amostras de ClO_2W não estavam aceitáveis para serem utilizadas na irrigação por aspersão de vegetais folhosos consumidos crus de acordo com a legislação espanhola. Demonstrando que o método de desinfecção com ClO_2 não foi eficiente na redução de *E. coli* genérica presente nas amostras de água residual urbana com tratamento secundário.

Com relação a presença de patógenos, foi encontrada uma prevalência de 56,20 % (9/16) em amostras de água. Sendo 7 correspondentes as amostras de controle (1 *Salmonella* e 6 STEC) e somente 1 amostra positiva STEC nas amostras que receberam tratamento com ClO_2. Para as amostras de alface apenas 1 amostra de SW foi confirmada para STEC. Além da redução significativa de patógenos após a aplicação do ClO_2, foi verificada uma correlação significativa (Mann-Whitney U Test, $p<0.05$) entre a concentração de *E. coli* genérica, em ambos os métodos de quantificação utilizados, e a

presença de patógenos. Este achado vai ao encontro ao relatado por estudos como de Truchado et al. (2016) e Ferguson et al. (2012), confirmando que as técnicas moleculares são eficazes para predizer a presença de bactérias patogênicas em amostras de água.

A utilização do ClO_2 pode levar a presença de subprodutos de desinfecção, como os cloretos e cloratos (NIKOLAOU & LEKKAS, 2001; RODRIGUEZ & SERODES, 2001; AYYILDIZ, et al., 2009). Apesar de uma menor reação a matéria orgânica, quando comparado ao cloro, no presente estudo foi observada uma concentração de cloratos entre 1.44 e 5.94 mg/L, na água de irrigação após o tratamento com ClO_2. Este resultado é superior a demais estudos que avaliaram a utilização deste desinfetante no tratamento de água (NITSOPOULOS et al., 2014; LÓPEZ-GÁLVEZ et al., 2018a; LÓPEZ-GÁLVEZ et al., 2018b). Entretanto, nos demais estudos a qualidade microbiológica e físico-química da água era superior a utilizada no presente trabalho, principalmente em relação a concentração de matéria orgânica. Em amostras de alface, irrigadas com ClO_2W foi observada uma concentração de cloratos que variou de 1.13 a 8.49 mg / kg. Estes níveis são muito superiores ao limite máximo de residual de cloratos permitido na União Europeia nos alimentos (0.01 mg / kg; CE, 2005).

Apesar dos resultados microbiológicos, o acúmulo de cloratos no tecido vegetal das alfaces, como consequência do tratamento com ClO_2, excedeu os limites máximos recomendados de cloratos para a água potável (0.7 mg/L) e o permitido pela União Europeia para alimentos (0.01 mg/kg), tornando o tratamento inadequado a ser aplicado nas condições avaliadas.

Nos estudos realizados por Rodrigues et al. (2014) e Decol et al. (2017), foi detectada a presença de *E. coli* O157:H7 em fontes distintas de água de irrigação no sul do Brasil. Ao ser detectado o patógeno em água de irrigação se indicou um risco de contaminação do produto final. Neste contexto, o presente trabalho (artigo 4) buscou caracterizar a capacidade de adaptação e multiplicação de quatro isolados de *E. coli* O157:H7 (Ec1, Ec2, Ec3, Ec4) e um coquetel (*Cocktail*) desses mesmos micro-organismos provenientes de fontes distintas de água de irrigação (açude e riacho), quando inoculados em água de irrigação filtrada a temperatura de 26°C, por até 72 h. Para determinar os parâmetros de crescimento foi utilizado o modelo matemático preditivo de Barany & Roberts (1994), no qual foi obtido uma boa correlação entre o modelo matemático e o experimento realizado ($R^2 > 0.96$). Isto demonstra que, mesmo tendo sido desenvolvido para utilização em alimentos, o modelo apresentou bons resultados quando utilizado em condições de amostras ambientais.

Com relação aos parâmetros, taxa de crescimento e fase Lag, foi possível verificar uma diferença significativa entre os isolados Ec1 e Ec4 que são provenientes de fontes de águas distintas, açude e riacho, respectivamente. Também foi possível observar que os isolados ao serem inoculados na forma de *Cocktaill* apresentam um tempo de adaptação (fase Lag) menor que ao serem inoculados separadamente. Os resultados obtidos demonstram uma rápida habilidade de adaptação e multiplicação destes patógenos em água de irrigação quando não está presente uma microbiota competidora e, principalmente, em temperaturas mais elevadas como as verificadas no período de verão. Além disso, foi observada uma rápida

capacidade de multiplicação neste estudo, fase de Lag média 5.18 h para isolados e 2.03 h para *Cocktail*.

Após ser determinada a capacidade de adaptação e multiplicação da *E. coli* O157:H7 em água de irrigação, foi investigada a resistência dos mesmos isolados frente ao desinfetante hipoclorito de sódio, quando aplicado na água de irrigação filtrada. Foram utilizadas duas concentrações de cloro residual final, 5 mg/L e 7 mg/L a temperatura de 26°C. Ao ser realizada a quantificação pelo método tradicional em placas, não foi observada uma redução inicial significativa ao ser utilizado a concentração de 5 mg/L de cloro livre, e ao final de 30 min de contato os isolados e do *Cocktail* apresentaram uma redução média de 3,15 (± 0,02) Log UFC / mL. Quando utilizado a concentração de 7 mg/L foi verificada uma maior redução nos primeiros minutos de contato e ao final do 30 min foi obtida uma completa redução.

Diversos estudos realizados em água de lavagem na indústria de vegetais minimamente processados indicaram que baixas concentrações de cloro livre (5 mg/L a 10 mg/L) foram efetivos para a redução da contaminação microbiana abaixo do limite de detecção, pelo método convencional em placa (LUO, et al., 2011; GÓMEZ-LÓPEZ, et al., 2014; ZHOU et al., 2015; CHEN, et al., 2017; LUO, et al., 2018).

No presente estudo, quando realizada a quantificação pelo método molecular PMA-qPCR, não foi observada diferença estatística significativa entre as concentrações testadas. Esta diferença entre as técnicas utilizadas se deve a capacidade da técnica molecular de quantificar bactérias quando estão no estado VNC. Portanto, este resultado sugere um efeito bacteriostático do hipoclorito de sódio, quando utilizado nas condições testadas. Além disso, com

base nos resultados, pode-se verificar que o uso do método de contagem de placas para estimar a eficácia dos métodos de desinfecção pode levar a uma subestimação das contagens bacterianas.

6. CONCLUSÃO GERAL

- Os resultados obtidos no presente estudo confirmaram que a água de irrigação pode contribuir com a contaminação microbiológica, durante a produção primária de alfaces. Entretanto, não foi observada diferença estatística significativa na contaminação entre as diferentes fontes de água de irrigação avaliadas. Também foi observado que a alta contaminação detectada nas fontes de água de irrigação impactou na qualidade microbiológica da alface irrigada pelas mesmas;

- A presença dos patógenos entéricos *E. coli* O157:H7 e *Salmonella* spp. nas amostras analisadas demonstraram a disseminação destes micro-organismos nas fontes de água;

- Foi verificada uma correlação significativa entre as altas contagens dos micro-organismos indicadores *Enterococcus* spp. e *E. coli* genérica e a presença de *Salmonella* spp., porém isso não ocorreu com *E. coli* O157:H7;

- Com relação aos fatores climáticos (estações do ano, temperatura ambiental e os níveis de precipitação) e práticas agrícolas (presença de animais e regime de irrigação), foi verificado sua influência nas contagens de *E. coli* genérica das alfaces.

- O dióxido de cloro demonstrou ser capaz de melhorar a qualidade microbiológica da água de reuso utilizada para irrigação por aspersão de alfaces cultivadas em estufa na Espanha. Este resultado positivo se deve pela redução de contagens de *E. coli* genérica, após a aplicação do ClO_2 nas concentrações entre 3.3 e 9.2 mg/L, verificado pelo método de cultivo em

placa, e pela redução da prevalência de bactérias patogênicas. Apesar dos resultados microbiológicos, o acúmulo de cloratos no tecido vegetais, como consequência do tratamento com ClO_2 excedeu os limites máximos recomendados de cloretos para a água potável (0,7 mg/L) e alimentos (0,01 mg/kg) recomendados pela Comunidade Europeia, tornando o tratamento inadequado para ser aplicado nas condições avaliadas.

- As cepas de *E. coli* O157:H7 isoladas apresentaram capacidade de sobrevivência e multiplicação na água de irrigação mantida em temperatura de 26°C. Tal fato demonstra o alto risco de contaminação de vegetais irrigados por essas águas, sendo necessária a adoção de medidas de controle, em nível de produção primária.

- Ao avaliar o efeito do hipoclorito de sódio aplicado nas concentrações de 5 mg/L e 7 mg/L em água de irrigação, frente as cepas de *E. coli* O157:H7, pelo método de quantificação PMA-qPCR, foi verificado possível efeito bacteriostático do desinfetante quando utilizado nas condições testadas.

- Considerando o crescente envolvimento de vegetais consumidos crus em doenças transmitidas por alimentos causadas pelos patógenos entéricos *Salmonella* spp. e *E. coli* O157 e a evidência de que fontes de água de irrigação estão contaminadas por esses patógenos, é importante que sejam conduzidos novos estudos para avaliar diferentes tecnologias de tratamento, bem como a necessidade de implementar estratégias de controle, sobretudo as Boas Práticas Agrícolas, a fim de evitar surtos veiculados por alfaces contaminados através da água de irrigação.

7. REFERÊNCIAS BIBLIOGRÁFICAS

AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA – ANVISA. **Vigilância Epidemiológica das Doenças Transmitidas por Alimentos – VE-DTA**. São Paulo, 07 de agosto de 2014.

AHMED, W.; RICHARDSON K.; SIDHU J. P. S.; TOZE S. *Escherichia coli* and *Enterococcus* spp. in Rainwater Tank Samples: Comparison of Culture-Based Methods and 23S rRNA Gene Quantitative PCR Assays. **Environmental Science & Technology**, 46, 11370−11376, 2012.

AHDB, Agriculture and Horticulture Development Board, 2016. 'Keep it Clean' January 2016 Workshop. Programme Drafted in Consultation with Research and Development Technical Committees of the British Leafy Salads Association; Baby Leaf Growers' Association; **Plant Propagators Ltd. the NFU Watercress Growers Association and British Herbs.** Disponível em: http://horticulture.ahdb.org.uk/sites/ default/files/Keep%20It%20Clean%20Microbils%20Workshops%20January%20 2016%20handout. pdf . Acessado em: Julho de 2016.

ALLENDE, A.; MONAGHAN, J.M. Irrigation water quality for leafy crops: A perspective of risks and potential solutions. **Int. J. Environ. Health. Res.**, 12, 7457−7477, 2015.

ASSOCIAÇÃO BRASILEIRA DO COMÉRCIO DE SEMENTES E MUDAS. Manual Técnico Cultivo de Hortaliças, 88pg. 2011.

AYYILDIZ, O.; ILERI, B.; SANIK, S. Impacts of water organic load on chlorine dioxide disinfection efficacy. **J. of Hazardous Materials**, 168,1092–1097, 2009.

BARANYI J. AND ROBERTS T.A. A dynamic approach to predicting bacterial growth in food. **Int. J. Food Microbiol.** 23, 277-294, 1994.

BARKER-REID, F. et al. Persistence of *Escherichia coli* on Injured iceberg Lettuce in the Field, Overhead Irrigated with Contaminated Water. **Journal of Food Protection**. v.73, p. 458-464, 2009.

BARTZ, S.; HESSEL, C.T.; RODRIGUES, R.Q.; POSSAMAI, A.; PERINI, F.O.; JACXSENS, L.; UYTTENDAELE, M.; BENDER, R.J.; TONDO, E.C. Insights in agricultural practices and management systems linked to microbiological contamination of lettuce in conventional production systems in Southern Brazil. **International Journal of Food Contamination,** 2:7, 2015.

BECERRA-CASTRO, C.; LOPES, A. R.; VAZ-MOREIRA, I.; SILVA, E. F.; MANAIA, C. M.; NUNES, O. C. Wastewater reuse in irrigation: A microbiological perspective on implications in soil fertility and human and environmental health. **Environment International**, 75, 117−135, 2015.

BRASIL. CONSELHO NACIONAL DO MEIO AMBIENTE. Resolução CONAMA Nº 20, de 18 de junho de 1986. Publicado no **Diário Oficial da União** de 30 de jul de 1986. Disponível em

http://www.mma.gov.br/port/conama/res/res86/res2086.html. Acesso em: 27 de maio de 2013.

CAI, S. W.; ZHOU, S. Y.; WANG, J. Q.; LI, S. Y.; ZHU, X. L.; WANG, J. J.; XUE, J. R. A bacteriological and helminthological investigation of a sewage-irrigated area in a Beijing suburb. **Biomed. Environ. Sci**. 1, 332–338, 1988.

CASTRO-IBÁÑEZ, M.I.; GIL, J.A.; TUDELA, A.; ALLENDE. Microbial safety considerations of flooding in primary production of leafy greens: A case study. **Food Research International,** 2015a.

CASTRO-IBÁÑEZ, I.; GIL, M.I.; TUDELA, J.A.; IVANEK, R.; ALLENDE, A. Assessment of microbial risk factors and impact of meteorological conditions during production of baby spinach in the Southeast of Spain. **Food Microbiology,** 49, 173-181, 2015b.

CENTERS FOR DISEASE CONTROL AND PREVENTION. **Multistate Outbreak of Shiga Toxin-producing *Escherichia coli* O157:H7 Infections Linked to Organic Spinach and Spring Mix Blend (Final Update)** Disponível em: http://www.cdc.gov/ecoli/2012/O157H7-11-12/index.html. Acesso em: 20 de fev. de 2015.

CENTERS FOR DISEASE CONTROL AND PREVENTION. **Update: Multistate Outbreak of *E. coli* O157:H7 Infections.** Disponível em: https://www.cdc.gov/media/releases/2018/s0110-update-ecoli.html. Acesso em: 25 de fev. de 2018.

CENTERS FOR DISEASE CONTROL AND PREVENTION. Surveillance for Foodborne Disease Outbreaks, United States, 2014, Annual Report. Atlanta, Georgia: US Department of Health and Human Services, CDC, 2016.

CENTRO DE INFORMAÇÕES ESTRATÉGICAS EM VIGILÂNCIA EM SAÚDE (GT-SINAN). **Secretaria de Vigilância em Saúde.** Departamento de Vigilância Epidemiológica. Ministério da Saúde, 2015.

CEUPPENS, S.; HESSEL, C.T.; RODRIGUES, R. DE Q.; BARTZ, S.; TONDO, E.C.; UYTTENDAELE, M. Microbiological quality and safety assessment of lettuce production in Brazil. **Int. J. Food Microbiol**. 181, 67–76, 2014.

CEVALLOS-CEVALLOS, J.M. et al. Dispersal of *Salmonella Typhimurium* by rain splash onto tomato plants. **Journal of Food Protection**. v. 75, p :472–479, 2012.

CONFEDERAÇÃO DA AGRICULTURA E PECUÁRIA DO BRASIL (APB). **Mapeamento e qualificação da cadeia produtiva das hortaliças do Brasil**. Brasília: CNA, pag. 79, 2017.

CRITTENDEN J.C.; TRUSSELL R.R.; HAND D.W.; HOWE K.J.; TCHOBANOGLOUS G. Water treatment: principles and design. **Second Edition. Wiley.** New Jersey, US, 2005.

CROWLEY, E.; BIRD, P.; FISHER, K.; JUENGER, M.; HUFFMAN, T.; BOYLE, M.; BENZINGER, M.J.; AGIN, J.; GOINS, D.; ZOOK, C.; DAVID, J. P1-102: comparative evaluation of the 3M™ molecular detection assay *Escherichia coli*

O157:H7 for the detection of *Escherichia coli* O157:H7 in foods. 2012. Acesso em:http://solutions.
3mdeutschland.de/3MContentRetrievalAPI/BlobServlet?lmd=1354703207000&-locale=de_DE&assetType=MMM_Image&assetId=1319243049213&blobAttribute= ImageFile. Acesso em: 20 de abril 2015.

DECOL, L.T.; CASARIN, L.S.; HESSEL, C.T.; BATISTA, A.C.F.; ALLENDE, A.; TONDO, E.C. Microbial quality of irrigation water used in leafy green production in Southern Brazil and its relationship with produce safety. **Food Microbiol.** 65, 105−113, 2017.

DYCHDALA, G.R. Chlorine and chlorine compounds. In: Block SS, ed. **Disinfection, sterilization, and preservation**. 5th ed. Philadelphia, PA, Lean and Febiger, 2001.

EUROPEAN COMMISSION (EC). Commission notice on guidance document on addressing microbiological risks in fresh fruits and vegetables at primary production through good hygiene (2017/C 163/01). **Official Journal of the European Union**, C 163/1, 23/05/2017.

EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA. Manual de Boas Práticas Agrícolas e Sistema APPCC. Brasília, DF: Embrapa Informação Tecnologica, 2004. (Disponível em:<http:// www.infoteca.cnptia.embrapa.br/bitstream/doc/111882/1/MANUALBOASPRATICASAGRICappcc.pdf>. Acesso em: 20 de fev. de 2018.

EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA. **Embrapa Milho e Sorgo:** Sistemas de Produção, 1. 2º ed., 2008. Disponível em: http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Milho/CultivodoMilho_2ed/imetodos.htm Acesso em: 27 de maio 2016.

EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA. **Tipos de Alface Cultivados no Brasil.** Comunicado Técnico 75. 2009. Acesso em: 23 de fevereiro 2015.

EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA. **Cultivo da Videira.** Sistema de Produção, 2004. Acesso em: 23 de fevereiro 2015.

E.P.H.C. 2006. Australian guidelines for water recycling. Managing health and environmental risks. Phase 1. National water quality management strategy 21. Natural Resource Management **Ministerial Council**. Environment Protection and Heritage Council, Australian Health Ministers Conference Council, Canberra, Australia. Available from: http://www. environment.gov.au/resource/national-water-quality-management-strategyaustralian-guidelines-water-recycling-anaging-0. Accessed 2014 September 25.

EUROPEAN FOOD SAFETY AUTHORITY. Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the Commission related to: Treatment of poultry carcasses with chlorine dioxide, acidified sodium chlorite, trisodium phosphate and peroxyacids. **The EFSA Journal**, 297:1–27, 2005.

EUROPEAN FOOD SAFETY AUTHORITY. Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (Salmonella and Norovirus in leafy greens eaten raw as salads). EFSA Panel on Biological Hazards (BIOHAZ). **EFSA Journal,** 12(3):3600, 2014. Disponível em: http://www.efsa.europa.eu/en/ efsajournal/pub/3600.htm. Acessado em: 20 de fevereiro 2015.

EUROPEAN FOOD SAFETY AUTHORITY. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. **EFSA Journal**, 13(1):3991, 2015. Disponível em: http://www.efsa.europa.eu/ en/efsajournal/doc/3991.pdf. Acessado em: 20 de fevereiro 2015.

EUROPEAN FOOD SAFETY AUTHORITY AND EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. **EFSA Journal,** 15(12):5077, 228 pp., 2017. Available from: https://doi.org/10.2903/j.efsa.2017.5077.

EUROPEAN UNION. Drinking water standards - **Council Directive 98/83/EC on the quality of water intented for human consumption**, 1998.

FERGUSON, A. S.; LAYTON, A. C.; MAILLOUX, B. J.; CULLIGAN, P. J.; WILLIAMS, D. E.; SMARTT, A. E.; SAYLER, G. S.; FEIGHERY, J.; MCKAY, L. D.; KNAPPETT, P. S.K.; ALEXANDROVA E.; ARBIT, T.; EMCH, M.; ESCAMILLA, V.; AHMED, K. M.; ALAM, MD. J.; STREATFIELD, P. K.; YUNUS, M.; GEEN, A VAN. Comparison of fecal indicators with pathogenic bacteria and rotavirus in groundwater. **Science of the Total Environment,** 431, 314–322, 2012.

FILGUEIRA, F. A. R. **Novo manual de olericultura: agrotecnologia moderna na produção e comercialização de hortaliças.** 2. ed. Viçosa. MG: Ed. UFV, 2005. 412 p.

FITTIPALDI, M.; NOCKER, A.; CODONY, F. Progress in understanding preferential detection live cells using viability dyes in combination with DNA amplification. **Journal of Microbial Methods**, 91, 276–289, 2012.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS/ WORLD HEALTH ORGANIZATION. Microbiological hazards in fresh leafy vegetables and herbs: Meeting Report. **Microbiological Risk Assessment Series**, No. 14. Rome. 151pp., 2008.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. **Statistical Yearbook.** World Food and Agriculture. Rome, 2012. p. 290. Disponível em: http://www.fao.org/docrep/018/i3107e/i3107e00.htm Acesso em: 19 de maio 2013.

FRANCO, R.A.M.; VANZELA, L.S.; HERNANDEZ, F.B.T. Avaliação biológica da qualidade da água para irrigação do córrego Três Barras, Marinópolis, SP. In: CONGRESSO NACIONAL DE IRRIGAÇÃO E DRENAGEM, 16, 2006, Goiânia. Anais...Brasília: Associação Brasileira de Irrigação e Drenagem, 2006.

GAYEON, W.; SCHLEGEL, P.J.; SCHROCK, J.M.; LEJEUNE, J.T. Absence of direct association between coliforms and *Escherichia coli* in irrigation water and on produce. **J. Food Prot.** 6, 928-1108, 2013.

GENSBERGER, E., T.; POLT, M.; KONRAD-KÕSZLER, P., K.; SESSITSH, A.; KOSTIC, T. Evaluation of quantitative PCR combined with PMA treatment for molecular assessment of microbial water quality. **Water Research**. 67, 367-376, 2014.

GIL, M. I.; SELMA, M. V.; SUSLOW, T.; JACXSENS, L.; UYTTENDAELE, M.; ALLENDE, A. Pre-and Postharvest Preventive Measures and Intervention Strategies to Control Microbial Food Safety Hazards of Fresh Leafy Vegetables. **Critical Reviews in Food Science and Nutrition**, 55, 453–468, 2015.

GIL, M.I.; MARÍN, A.; ANDUJAR, S.; ALLENDE A. Should chlorate residues be of concern in fresh-cut salads? **Food Control**, 60: 416–421, 2016.

GLEICK, P.H. **The world's water 2000–2001: The biennial report on freshwater resources.** Washington, DC: Island Press., 2000.

GÓMEZ-LÓPEZ, V.M.; LANNOO, A.-S.; GIL, M.I.; ALLENDE, A. Minimum free chlorine residual level required for the inactivation of *Escherichia coli* O157:H7 and trihalomethane generation during dynamic washing of fresh-cut spinach. **Food Control** 42, 132–138, 2014.

GORSKI, L.; PARKER, C. T.; LIANG, A.; COOLEY, M. B., JAY-RUSSELL, M. T.; GORDUS, A. G.; ATWILL, E. R.; MANDRELL, R. E. Prevalence, Distribution, and Diversity of Salmonella enterica in a Major Produce Region of California. **Appl. Environ. Microbiol** 77(8): 2734, 2011.

GU, G.Y. et al. Factors affecting the occurrence of *Escherichia coli* O157 contamination in irrigation ponds on produce farms in the Suwannee River Watershed. **Canadian Journal of Microbiology**, v. 59, p. 175-182, 2013.

HAMILTON, A.J. et al. Quantitative microbial risk assessment models for consumption of raw vegetables irrigated with reclaimed water. **Applied and Environmental Microbiology**,v. 72, p. 3284–3290, 2006.

HASSENBERG, K.; GEYER, M.; MAUERER, M.; PRAEGER, U.; HERPPICH, W.B. Influence of temperature and organic matter load on chlorine dioxide efficacy on *Escherichia coli* inactivation. **LWT - Food Science and Technology**,79, 349–354, 2017.

HENZ, G.P; SUINAGA F. **Comunicado Técnico 75:** Tipos de Alface Cultivados no Brasil. EMBRAPA. Brasília, DF, nov. 2009. Disponível em: http://www.cnph.embrapa.br/paginas/serie_documentos/publicacoes2009/cot_75.pdf Acesso em: 12 de maio 2013.

HOLVOET, K. et al. Horticultural assessment scheme: insight in prevalence and distribution of microbial contamination to evaluate water management in fresh produce processing industry. **Journal of Food Protection**, v. 75, n. 4, p. 671-681. 2012.

HUANG, Y.R.; HUNG, Y.C.; HSU, S.Y.; HUANG, Y.W.; HWANG, D.F. Application of electrolyzed water in the food industry. **Food Control.** 19:329–345, 2008.

INGHAM, S.C.; FANSLAU, M.A.; ENGEL, R.A.; BREUER, J.R.; BREUER, J.E.; WRIGHT, T.H.; REITH-ROZELLE, J.K.; ZHU, J. Evaluation of fertilization-to-planting and fertilization-to-harvest intervals for safe use of noncomposted bovine manure in Wisconsin vegetable production. **J. Food Prot**. 68, 1134–1142, 2005.

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (IARC). Chlorinated drinking-water; chlorination by-products; some other halogenated compounds; cobalt and cobalt comppunds. Lyon, IARC, pp. 45-359, **IARC Monographs on the Evaluation of Carcinogenic Risks to Humans**, Vol.52.

JAMES, J. Overview of Microbial Hazards in Fresh Fruit and Vegetables Operations. **Microbial Hazard Identification in Fresh Fruit and Vegetables**. J. W. Sons. Hoboken, USA. 2006.

JOHANNESSEN, G.S.; LONCAREVIC, S.; KRUSE, H. Bacteriological analysis of fresh produce in Norway. **Int. J. Food Microbiol**. 77, 199–204, 2002.

KIBBEE, R.J. & ÖRMECI, B. Development of a sensitive and false-positive free PMA-qPCR viability assay to quantify VBNC *Escherichia coli* and evaluate disinfection performance in wastewater effluent. **J. Microbiol. Methods**, 132, pp. 139–147, 2017.

LI, D.; TONG, T.; ZENG, S.; LIN, Y.; WU, S.; HE, M. Quantification of viable bacteria in wastewater treatment plants by using propidium monoazide combined with quantitative PCR (PMA-qPCR). **J. Environ. Sci.**, 26 (2), pp. 299–306, 2014.

LOFF, M.; MARE, L.; KWAADSTENIET, M.; KHAN, W. 3M™Molecular Detection system versus MALDI-TOF mass spectrometry and molecular techniques for the identification of *Escherichia coli* 0157:H7, *Salmonella* spp. & *Listeria* spp. **J. Microbiol. Methods**. 101, 33–43, 2014.

LÓPEZ-GÁLVEZ, F.; ALLENDE, A.; MARTINEZ-SANCHEZ, A.; TUDELA, J.A.; SELMA, M.V.; GIL, M.I. Suitability of aqueous chlorine dioxide versus sodium hypochlorite as an effective sanitizer for preserving quality of fresh-cut lettuce while avoiding by product formation. **Postharvest Biol. Tec.**, 55, 53–60, 2010.

LÓPEZ-GÁLVEZ, F.; GIL, M.I.; MEIRELES, A.; TRUCHADO, P.; ALLENDE, A. Demonstration tests of irrigation water disinfection with chlorine dioxide in open field cultivation of baby spinach. **J. of the Sc. of Food and Agriculture**, DOI: 10.1002/jsfa.8794, 2018a.

LÓPEZ-GÁLVEZ, F.; ANDÚJAR, S.; MARÍN, A.; TUDELA, J.A.; ALLENDE, A.; GIL, M.I. Disinfection by-products in baby lettuce irrigated with electrolyzed water. **Journal of the Science of Food and Agriculture**, 2018b.

MALDONADE, I. R. **Manual de boas práticas na produção de Alface** – Brasília, DF: Embrapa Hortaliças, 44 p, 2014.

MANAS, P.; CASTRO, E.; DE LAS HERAS, J. Irrigation with treated wastewater: effects on soil, lettuce (Lactuca sativa L.) crop and dynamics of microorganisms. **J. Environ. Sci. Health Part A Toxic-Hazard. Subst. Environ. Eng**. 44:1261–1273, 2009.

MARITES, M. et al. Risk analysis integrating livelihood and economic impacts of wastewater irrigation on health. Wastewater irrigation and health: assessing and mitigating risk in low-income countries. **Earthscan-International Development Research Centre**. London: p. 127-148, 2010.

MAROUELLI, W. A.; SILVA, W. L. C. **Circular Técnica 98**. Seleção de sistemas de irrigação para hortaliças. EMBRAPA Hortaliças, Dez. 2011 (2ª edição). Acesso em: 05 de fevereiro 2015.

MELLOUL, A. A.; HASSANI, L.; RAFOUK, L. Salmonella contamination of vegetables irrigated with untreated wastewater. **World J. Microbiol. Biotechno**l. 17, 207–209, 2001.

METCALF & EDDY INC. AN AECOM COMPANY, ASANO, T.; BURTON, F.; LEVERENZ, H.; TSUCHIHASHI, R.; TCHOBANOGLOUS, G. Water reuse: issue, technologies, and applications. **McGraw Hill Professional**. New York, US, 2007

MORETTI, C.L.; MATTOS, L.M. Processamento mínimo de alface crespa. v.25, p. 1-7. **Comunicado Técnico EMBRAPA**. v.1, p. 1-6, 2005.

NIKOLAOU, A.D.; LEKKAS T.D. The role of natural organic matter during formation of chlorination by-products: a review. **Acta Hydrochim. Hydrobiol**. 29, 63–77, 2001.

NOCKER, A.; SOSSA, K.E.; CAMPER, A.K. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. **J. Microbiol. Meth**. 70, 252–260, 2007.

NOCKER, A. & CAMPER, A.K. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. **FEMS Microbiol. Lett.** 291, 137–142, 2009.

OLIVEIRA, M. et al. Presence and survival of Escherichia coli O157:H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water. **International Journal of Food Microbiology**, p.133–140. 2012.

OLIVER, J. D.; DAGHER, M.; LINDEN, K. Induction of *Escherichia coli* and *Salmonella typhimurium* into the viable but nonculturable state following chlorination of wastewater. **J. Water Health**, 3, 249−257, 2005.

PACHEPSKY, Y.; SHELTON, D.R.; MCLAIN, J.E.T.; PATEL, J.; MANDRELL, R.E. Irrigation Waters as a Source of Pathogenic Microorganisms in Produce: A Review. In: **Advances in Agronomy**, Volume 113 pp. 73–138, Academic Press (San Diego, CA), 2011.

PAHL, D.M.; TELIAS, A.; NEWELL, M.; OTTESEN, A.R.; WALSH, C. Comparing Source of Agricultural Contact Water and the Presence of Fecal Indicator Organisms on the Surface of 'Juliet' Grape Tomatoes. **J. Food Prot.**, 6, 928-1108, 2013.

PARISH, M. E.; BEUCHAT, L. R.; SUSLOW, T. V.; HARRIS, L. J.; GARRETT, E. H.; FARBER, J. N.; BUSTA, F. F. Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. **Compr. Rev. Food Sci. Food Safety**. 2:161–173, 2003.

PARK, S.; NAVRATIL, S.; GREGORY, A.; BAUER, A.; SRINATH, I.; JUN, M.; SZONYI, B.; NIGHTINGALE, K.; ANCISO, J.; IVANEK, R. Generic *Escherichia coli* contamination of spinach at the preharvest level: the role of farm management and environmental factors. **Appl. Environ. Microbiol**. 79, 4347–4358, 2013.

PARK, S.; NAVRATIL, S.; GREGORY, A.; BAUER, A.; SRINATH, I.; SZONYI, B.; NIGHTINGALE, K.; ANCISO, J.; JUN, M.; HAN, D.; LAWHON, S.; IVANEK, R. Farm management, environment, and weather factors jointly affect the probability of spinach contamination by generic *Escherichia coli* at the preharvest stage. **Appl. Environ. Microbiol.** 80, 2504-2515, 2014.

PARK, S.; NAVRATIL, S.; GREGORY, A.; BAUER, A.; SRINATH, I.; SZONYI, B.; NIGHTINGALE, K.; ANCISO, J.; JUN, M.; HAN, D.; LAWHON, S.; IVANEK, R. Count of generic *Escherichia coli* on spinach at the preharvest level determined by the multifactorial effect of ambient temperature, precipitation, farm management and environmental factors. **Appl. Environ. Microbiol**. 81, 793–814, 2015.

PARKER, J.S.; WILSON, R.S.; LEJEUNE, J.T.; RIVERS, L.; DOOHAN, D. An expert guide to understanding grower decisions related to fresh fruit and vegetable contamination prevention and control. **Food Control,** 26, 107–116, 2012.

PEDRERO, F.; KALAVROUZIOTIS, I.; ALARC´ON, J.J.; KOUKOULAKIS, P.; ASANO T. Use of treated municipal wastewater in irrigated agriculture. Review of some practices in Spain and Greece. **Agr Water Manag.** 97:1233–41, 2010.

PICARDEAU, M. et al. Rapid tests for diagnosis of leptospirosis: Current tools and emerging technologies. **Diagnostic Microbiology and Infectious Disease**. v. 78, p. 1–8, 2014.

PITKÄNEN, T.; RYU, H.; ELK, M.; HOKAJÄRVI, A. M.; RÄSÄNEN, P.; SIPONEN, S. Detection of fecal bacteria and source tracking identifiers in environmental waters using rRNA-based RT-qPCR and rDNA-based qPCR assays. **Environmental Science and Technology**, 47, 13611–13620, 2013.

REAL DECRETO 1620/2007, 2007. de 7 de diciembre, por el que se establece el regimen jurídico de la reutilización de las aguas depuradas, (BOE num. 294, 8 de diciembre de 2007).

REID, D.C. et al. The quality of drinking water from private water supplies in Aberdeenshire, UK. **Water Research**. v. 37, p. 245 – 254, 2003.

ROBERT KOCH INSTITUT: Report: Final evaluation and presentation of epidemiological findings of the EHEC O104:H4 outbreak, Germany. Edited by RKI **Department for Infectious Disease Epidemiology Division** 35. Berlin: RKI-Print Shop; 2011. p. 2011. This report provides a complete overview of the epidemiology of the STEC O104:H4 outbreak in Germany while also describing the efforts of the Robert Koch institute in tracing the outbreak source, 2011.

RODRIGUEZ, M.J.; SERODES J.B. Spatial and temporal evolution of trihalomethanes in three water distribution systems, **Water Res**. 35,1572–1586, 2001.

RODRIGUES, R. Q. L. M. R.; PAULA, C.M.; HESSEL, C.T.; JACKSENS, L.; UYTTENSAELE, M.; BENDER,R.J.; TONDO, E.C. Microbiological contamination linked to implementation of good agricultural practices in the production of organic lettuce in Southern Brazil. **Food Control,** 42, 152-164, 2014.

RUBINO, S.; CAPPUCCINELLI P.; KELVIN, D.J. *Escherichia coli* (STEC) serotype O104 outbreak causing haemolytic syndrome (HUS) in Germany and France. **J Infect Dev Ctries** 5(6):437-440. 2011.

RUDI, K.; MOEN, B.; DRØMTORP, S. M.; HOLCK, A. L. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. **Applied Environmental Microbiology**, 71, 1018–1024, 2005.

SALA F.C.; COSTA C.P. Retrospectiva e tendência da alfacicultura brasileira. **Horticultura Brasileira** v. 30, n.2, p.187-194, abr.- jun.2012.

SANZ, E. N.; DAVILA, I. S.; BALAO, J. A. A.; ALONSO, J. M. Q. Modelling of reactivation after UV disinfection: Effect of UV-C dose on subsequent photoreactivation and dark repair. **Water Res.** 41:3141–3151, 2007.

SOON, J.M. et al. Field application of farm-food safety risk assessment (FRAMp) tool for small and medium fresh produce farms. **Food Chemistry**, 136 p.1603-1609, 2012.

SONG, I. et al. Comparison of crop contamination by microorganisms during subsurface drip and furrow irrigation. **Journal of Environmental Engineering**, 125, 2006.

SUINAGA, F.A. et al. Desempenho produtivo de cultivares de alface crespa. **Comunicado Técnico EMBRAPA**. v.1, p. 1-15, 2013.

SUSLOW, T. V. Water disinfection: A practical approach to calculating dose values for preharvest and postharvest applications. **ANR Catalog Publ**. no. 7256. Univ. of California, Davis, CA., 2001.

SUSLOW, T.V.; ORIA, M.P.; BEUCHAT, L.R.; GARRETT, E.H.; PARISH, M.E.; HARRIS, L.J.; FARBER, J.N.; BUSTA, F.F. Production practices as risk factors in microbial food safety of fresh and fresh cut produce**. Compr Rev Food Sci Food Saf.** 2:38–77, 2003.

TOMAS-CALLEJAS, A.; LOPEZ-GALVEZ, F.; SBODIO, A.; ARTES, F.; ARTES-HERNANDEZ, F.; SUSLOW, T. V. Chlorine dioxide and chlorine effectiveness to prevent *Escherichia coli* O157:H7 and *Salmonella* cross-contamination on fresh-cut **Red Chard. Food Control**. 23:325–332, 2012.

TRUCHADO, P.; GIL, M.I.; KOSTIC, T.; ALLENDE, A. Optimization and validation of a PMA qPCR method for *Escherichia coli* quantification in primary production. **Food Control,** 62: 150-156, 2016.

UNESCO-WWA, United Nations World Water Assessment Programme. The United Nations World Water Development Report 2017. **Wastewater: The Untapped Resource**. Paris, 2017.

UNITED STATES ENVIROMENTAL PROTECTION AGENCY (EPA). **Edition of the Drinking Water Standards and Health Advisories. Washington** D. C.: USEPA, Office of Water, 2009.

UYTTENDAELE, M.; JAYKUS, L-A.; AMOAH, P.; CHIODINI, A.; CUNLIFFE, D.; JACXSENS, L.; HOLVOET, K.; KORSTEN, L.; LAU, M.; MCCLURE, P.; MEDEMA, G.; SAMPERS, I.; JASTI, P. R. Microbial hazards in irrigation water: standards, norms, and testing to manage use of water in fresh produce primary production. **Comp. Rev. Food Sci. Food Saf.** 14. 336–356, 2015.

VAN FRANKENHUYZEN, J.K.; TREVORS, J.T.; FLEMMING, C.A.; LEE, H.; HABASH, M.B. Optimization, validation, and application of a real-time PCR protocol for quantification of viable bacterial cells in municipal sewage sludge and biosolids using reporter genes and *Escherichia coli*. **J. Ind. Microbiol. Biotech.** 40 (11), 1251–1261, 2013.

VAN HAUTE, S.; SAMPERS, I.; JACXSENS, L.; UYTTENDAELE, M. Selection Criteria for Water Disinfection Techniques in Agricultural Practices. **Critical Reviews in Food Science and Nutrition**, 55:1529–1551, 2015.

VAN HAUTE, S.; TRYLAND, I.; ESCUDERO, C.; VANNESTE, M.; SAMPERS, I. Chlorine dioxide as water disinfectant during fresh-cut iceberg lettuce washing: Disinfectant demand, disinfection efficiency, and chlorite formation. **LWT - Food Science and Technology**, 75, 301–304, 2017.

VARMA, M.; FIELD, R.; STINSON, M.; RUKOVETS, B.; WYMER, L.; HAUGLAND, R. Quantitative real-time PCR analysis of total and propidium monoazide – resistant fecal indicator bacteria in wastewater. **Water Research**, 43, 4790–4801, 2009.

YANG, Y.; LUO, Y.; MILLNER, P.; SHELTON, D.; NOU, X. Enhanced chlorine efficacy against bacterial pathogens in wash solution with high organic loads. **J. of Food Proc. and Preservation**, 36, 560–566, 2012.

WARRINER, K.; HUBER, A.; NAMVAR, A.; FAN, W.; DUNFIELD, K. Chapter 4. Recent advances in the microbial safety of fresh fruits and vegetables. **Advances in Food and Nutrition Research.** 57, 155–208, 2009.

WHO. World Health Organization. 2006. WHO guidelines for the safe use of wastewater, excreta and grey water. **Wastewater use in agriculture**. Available from:http://whqlibdoc.who.int/publications/2006/9241546832_eng.pdf. Accessed 5 July 2017.

WOOD, J.D. et al. Population dynamics of *Escherichia coli* inoculated by irrigation into the phyllosphere of spinach grown under commercial production conditions. *International Journal of Food Microbiology*. v. 143, p 198–204, 2010.

ZIMMER, J. L. & SLAWSON, R. M. Potential repair of Escherichia coli DNA following exposure to UV radiation from both medium- and low-pressure UV sources used in drinking water treatment. **Appl. Environ. Microbiol**. 68: 3293–3299, 2002.