UNIVERSIDADE DE SÃO PAULO

Faculdade de Ciências Farmacêuticas

Programa de Pós-Graduação em Ciência dos Alimentos

Área de Bromatologia

Vegetais minimamente processados prontos para o consumo: influência da etapa de desinfecção na inativação de *Salmonella* Typhimurium, na ocorrência da contaminação cruzada e na avaliação quantitativa de risco microbiológico em relação a este patógeno

Daniele Fernanda Maffei

Tese para obtenção do Título de

DOUTOR

Orientador:

Profª. Drª. Bernadette Dora Gombossy de Melo Franco

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RESUMO

MAFFEI, D. F. **Vegetais minimamente processados prontos para o consumo: influência da etapa de desinfecção na inativação de *Salmonella* Typhimurium, na ocorrência da contaminação cruzada e na avaliação quantitativa de risco microbiológico em relação a este patógeno.** 2016. 127p. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2016.

Dados mundiais apontam haver uma associação entre o aumento do comércio de vegetais minimamente processados prontos para o consumo (VPC) e o aumento da ocorrência de surtos de enfermidades transmitidas por alimentos. Durante o processamento industrial de VPC, a desinfecção é a principal etapa de inativação de micro-organismos patogênicos presentes, mas nessa etapa também pode ocorrer contaminação cruzada, com transferência de contaminantes de produtos contaminados para não-contaminados. Neste trabalho, foram coletadas informações sobre as práticas empregadas na etapa de desinfecção em dez importantes indústrias produtoras de VPC no Estado de São Paulo, avaliando-se, em seguida, a influência dessas práticas na qualidade microbiológica dos produtos e na inativação de *Salmonella* Typhimurium, bem como na ocorrência de contaminação cruzada por este patógeno. Um modelo de avaliação quantitativa de risco microbiológico foi elaborado para estimar o impacto da contaminação cruzada durante a etapa de desinfecção no risco de infecção por *Salmonella* devido ao consumo de VPC. Observou-se que, em todas as indústrias visitadas, a desinfecção dos vegetais era feita com produtos à base de cloro em concentrações de 50 a 240 mg/L, que resultava em redução de até 1,2 log na carga microbiana dos vegetais que entravam na linha de processamento. Ao avaliar a influência das características da água de processamento (pH, temperatura, concentração de matéria orgânica e concentração de dicloroisocianurato de sódio) e do tempo de contato entre a água clorada e os vegetais na redução de *Salmonella*, observou-se que a concentração do produto à base de cloro foi o parâmetro que apresentou maior influência ($p<0.05$). Concentrações de dicloroisocianurato de sódio acima de 10 mg/L foram necessárias para controle da contaminação cruzada durante a etapa de lavagem. O modelo de avaliação de risco construído indicou quantitativamente haver uma relação entre a concentração de dicloroisocianurato de sódio na água de desinfecção e o risco de ocorrência de surtos causados por *Salmonella* em VPC. Cenários simulando uso de dicloroisocianurato de sódio em concentrações abaixo de 5 mg/L indicaram que mais de 96% dos casos preditos de infecção por *Salmonella* poderiam ser atribuídos à ocorrência de contaminação cruzada, enquanto que em cenários com concentrações acima de 50 mg/L, casos de infecção devidos à contaminação cruzada não foram preditos. Estes resultados mostram que o controle da qualidade da água e o monitoramento da concentração de sanitizante na etapa de desinfecção são essenciais para evitar a ocorrência de contaminação cruzada e garantir a produção de VPC seguros para o consumo.

Palavras-chave: vegetais prontos para o consumo, desinfecção, contaminação cruzada, *Salmonella*, avaliação quantitativa de risco microbiológico.

ABSTRACT

MAFFEI, D. F. **Minimally processed ready-to-eat vegetables: influence of washing-disinfection step on *Salmonella* Typhimurium inactivation, on occurrence of cross-contamination and on quantitative microbiological risk assessment regarding this pathogen.** 2016. 127p. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2016.

Surveillance data in several countries show an association between consumption of minimally processed ready-to-eat (RTE) vegetables and increased occurrence of foodborne diseases outbreaks. During RTE vegetables processing, washing-disinfection is the main step aiming to ensure inactivation of pathogenic microorganisms, but also is the step in which cross-contamination may occur, with transfer of contaminants from contaminated to non-contaminated products. In this study, we collected information on the practices employed during the washing-disinfection step in ten RTE vegetables processing plants located in the State of Sao Paulo, Brazil, and evaluated the influence of these washing practices on the microbial quality of the products and inactivation of *Salmonella* Typhimurium, as well as on the occurrence of cross-contamination by this pathogen. A quantitative microbial risk assessment model was built in order to estimate the impact of cross-contamination during the washing step on the risk of infection by *Salmonella* due to the consumption of RTE vegetables. In all visited processing plants, the disinfection step was done using chlorine-based products, in concentrations ranging from 50 to 240 mg/L, achieving a reduction of up to 1.2 log in the microbial load of vegetables entering the processing line. When the influence of washing water parameters (pH, temperature, organic load and sodium dichloroisocyanurate concentration) and time of contact between chlorinated water and vegetables on reduction of *Salmonella* were evaluated, sodium dichloroisocyanurate concentration influenced the most ($p<0.05$). Concentrations above 10 mg/L were necessary for avoiding cross-contamination during washing step. The risk assessment model indicated quantitatively a relationship between sodium dichloroisocyanurate concentration and the risk of illness caused by *Salmonella* in RTE vegetables. When simulation was done with less than 5 mg/L of sodium dichloroisocyanurate, most (>96%) of the illnesses arose from cross-contamination. However, when the concentration was 50 mg/L or higher, no illnesses arising from cross-contamination were predicted. These results show that controlling the quality of the water and monitoring the concentration of the sanitizer in the disinfection step are essential to avoid occurrence of cross contamination and ensure production of RTE vegetables that are safe for consumption.

Keywords: ready-to-eat vegetables, disinfection, cross-contamination, *Salmonella*, quantitative microbial risk assessment.

SUMÁRIO

1. INTRODUÇÃO

1.1 Vegetais prontos para o consumo

A busca por alimentos mais saudáveis constitui uma realidade em todo o mundo, o que vem causando mudanças importantes nos hábitos alimentares da população. O consumo de vegetais é altamente incentivado por autoridades de saúde em vários países, devido aos benefícios que proporcionam à saúde. O estilo de vida atual, caracterizado pela elevada carga de atividades diárias e pouco tempo para o preparo dos alimentos, tem estimulado a procura dos consumidores por produtos de origem vegetal, convenientes para o consumo e sem prejuízo para suas qualidades nutricional e sensorial (NASCIMENTO et al., 2014; VELDERRAIN-RODRÍGUEZ et al., 2015). Estes são chamados “vegetais minimamente processados” (VMP) ou “vegetais prontos para o consumo” (VPC).

VPC são vegetais que foram submetidos à seleção, corte (descascamento, fatiamento e trituração), desinfecção, centrifugação, embalagem e armazenamento sob refrigeração. Além de torná-los prontos para o consumo, esses procedimentos prolongam a vida útil, sem alterar as propriedades nutritivas e sensoriais (CODEX ALIMENTARIUS COMMISSION, 2003; NASCIMENTO et al., 2014). Pelo fato da maioria desses vegetais serem consumidos na forma crua, não necessitam de qualquer tratamento adicional antes do consumo. O mercado de VPC tem crescido anualmente no Brasil e em outros países ao longo das últimas décadas e vem ganhando espaço nos lares, redes de *fast food* e restaurantes (ABADIAS et al., 2008; OLIVEIRA et al., 2011; ROJAS-GRAU et al., 2011; SATO; MARTINS; BUENO, 2007).

Pesquisa realizada na cidade de São Paulo, SP, com 42 consumidores revelou que 64,3% dos entrevistados afirmaram adquirir VPC, enquanto outros 35,7% relataram não adquirir esse tipo de produto. Os principais motivos relatados para aquisição foram praticidade (88,9%) e higiene (29,6%), enquanto os principais motivos para não aquisição foram preço elevado (52%), descrédito na higiene do produto (22%) e preferência pelo produto a granel (19%) (SATO; MARTINS; BUENO, 2007). Outra pesquisa realizada com 246 consumidores em Belo Horizonte, MG, revelou que 77% dos entrevistados relataram não adquirir VPC, contra 23% que relataram adquirir esse tipo de produto. Assim como no estudo realizado em São

Paulo, os principais motivos pelos quais os consumidores de Belo Horizonte adquiriam estes produtos foram praticidade (46%), pouco tempo para preparo das refeições (21%) e higiene (11%), enquanto os principais motivos para não aquisição foram preço elevado (31,9%), preferência por preparar e/ou escolher os vegetais (23%) e desconfiança dos produtos ofertados (17,8%) (PEREZ et al., 2008).

Concomitante ao aumento no consumo mundial destes produtos, dados de vigilância sanitária em diversos países apontam uma crescente associação de consumo de vegetais *in natura* e minimamente processados com surtos de enfermidades transmitidas por alimentos, preocupando produtores, consumidores e agências governamentais responsáveis pelo controle de alimentos (BERGER et al., 2010; CALLEJÓN et al., 2015; HARRIS et al., 2003; LYNCH; TAUXE; HEDBERG, 2009). Conforme indicado na Tabela 1, micro-organismos patogênicos como *Salmonella* spp. e *Listeria monocytogenes* têm sido isolados de diferentes tipos de VPC comercializados no Brasil e em outros países ao longo da última década.

Tabela 1. Incidência de patógenos em amostras de vegetais prontos para o consumo.

País	Patógeno	Número de amostras		Referência
		Total	Positivas	
		n	n (%)	
Brasil	*L. monocytogenes*	181	1 (0,6)	Froder et al. (2007)
	Salmonella	133	4 (3,0)	
Brasil	*L. monocytogenes*	162	2 (1,2)	Oliveira et al. (2011)
	Salmonella	162	2 (1,2)	
Brasil	*L. monocytogenes*	172	2 (1,2)	Maistro et al. (2012)
	Salmonella	172	1 (0,6)	
Brasil	*L. monocytogenes*	512	16 (3,1)	Sant'Ana et al. (2012)
Brasil	*Salmonella*	512	2 (0,4)	Sant'Ana et al. (2011)
Coreia	*Salmonella*	129	1 (0,8)	Seo; Jang; Moon (2010)
Croácia	*L. monocytogenes*	100	1 (1,0)	Kovacevic et al. (2013)
Espanha	*L. monocytogenes*	236	2 (0,8)	Abadias et al. (2008)
	Salmonella	236	4 (1,7)	
Espanha	*L. monocytogenes*	70	3 (4,3)	Moreno et al. (2012)
Irã	*Salmonella*	20	1 (5,0)	Jeddi et al. (2014)
México	*Salmonella*	220	9 (4,1)	De Léon et al. (2013)

1.2 Fontes de contaminação

A contaminação dos vegetais pode ocorrer ao longo da cadeia produtiva, durante operações pré-colheita e pós-colheita. As fontes de contaminação pré-colheita incluem solo de cultivo, água de irrigação, fertilizantes usados para adubação, animais selvagens e domésticos no campo e manipulação, enquanto as fontes de contaminação pós-colheita incluem manipulação, uso de equipamentos e utensílios contaminados, além das operações de processamento em domicílio ou indústrias (BERGER et al., 2010; HARRIS et al., 2003).

O solo de cultivo é habitat de diversos tipos de micro-organismos, incluindo patógenos, que podem contaminar os vegetais através das sementes, raízes ou superfície. Micro-organismos oportunistas do solo têm acesso a tecidos internos por meio de lesões na casca ou abertura natural dos vegetais. Os fertilizantes empregados para adubação também podem ser fonte de contaminação, principalmente quando se emprega adubo orgânico composto por esterco animal. Tratamento térmico inadequado no processo de compostagem reduz a inativação de micro-organismos, propicia sua proliferação e aumenta o risco de contaminação (HARRIS et al., 2003; SANT'ANA et al., 2014).

A água de irrigação também pode ser uma fonte de contaminação. A água pode ser oriunda de fontes subterrâneas (aquíferos e poços) ou superficiais (rios e lagos ou poços), sendo susceptível à contaminação por despejo de dejetos. Alguns estudos têm reportado a presença de patógenos em águas de irrigação e sua transferência para os vegetais (CHIGOR; UMOH; SMITH, 2010; IJABADENIYI et al., 2011; OKAFO; UMOH; GALADIMA, 2003), mostrando o risco de contaminação. Além disso, o cultivo de vegetais em áreas abertas possibilita o acesso de animais (pássaros, insetos, roedores e animais selvagens e domésticos), que podem defecar nos campos de produção e ser via de contaminação.

Diversos micro-organismos, incluindo patógenos, são capazes de sobreviver no ambiente por períodos longos. Estudos conduzidos por Islam et al. (2004)[a,b] mostraram que *Salmonella* Typhimurium e *Escherichia coli* O157:H7 sobreviveram por longo tempo em solo e vegetais cultivados após contaminação experimental via adubo (esterco) ou água de irrigação. *S.* Typhimurium persistiu por 161 e 231 dias em solos nos quais alface e salsa, respectivamente, foram cultivadas, enquanto *E.coli* O157:H7 persistiu por 154 e 217 dias, respectivamente, nestes solos.

Durante a fase de pós-colheita, falhas de higiene durante a manipulação e processamento podem comprometer a segurança dos produtos. As condições do ambiente de armazenamento, tais como temperatura, circulação de ar e umidade relativa, devem ser controladas. O armazenamento dos produtos sob refrigeração (≤ 4 °C) é uma importante estratégia para reduzir a taxa metabólica dos vegetais e a multiplicação de micro-organismos, inclusive patógenos como *L. monocytogenes*, *E.coli* O157:H7 e *Salmonella* spp. (MATTHEWS, 2013). O reaproveitamento de caixas e utensílios não-higienizados utilizados durante a colheita e transporte de diferentes lotes de vegetais pode ocasionar contaminação cruzada.

1.3 Processamento mínimo e contaminação cruzada

O processamento mínimo de vegetais pode envolver diferentes etapas, tais como recepção e seleção da matéria-prima, pré-lavagem, processamento (corte, descascamento), desinfecção, enxague, centrifugação, embalagem, armazenamento e transporte/distribuição (Figura 1) (CENCI, 2011; CODEX ALIMENTARIUS COMMISSION, 2003), que podem comprometer a vida útil dos vegetais, pois expõem os tecidos internos e aceleram o metabolismo celular. O uso de embalagens adequadas, associado à refrigeração, é essencial para conservação deste tipo de produto (NANTES; LEONELLI, 2000).

Dentre as etapas de processamento citadas, a pré-lavagem e a desinfecção são as mais importantes, pois além de remover sujidades visíveis e reduzir a carga microbiana inicial dos vegetais, eliminam micro-organismos patogênicos que possam estar presentes. No entanto, durante estas etapas também pode ocorrer a transferência de micro-organismos de produtos contaminados para não-contaminados, resultando em contaminação cruzada, principalmente na ausência de sanitizantes na água de lavagem (GIL et al., 2009; GÓMEZ-LÓPEZ et al., 2015).

Vários estudos avaliaram a contaminação cruzada entre diferentes lotes de vegetais durante a etapa de lavagem (com ou sem o uso de sanitizantes), causadas por *E.coli* O157:H7 e *Salmonella* spp. (ALLENDE et al., 2008; HOLVOET et al. 2014; JENSEN et al. 2015; LÓPEZ-GÁLVEZ et al. 2009; LÓPEZ-GÁLVEZ et al. 2010; LUO et al., 2011, PEREZ-RODRIGUEZ et al. 2014, TOMÁS-CALLEJAS et al. 2012; ZHANG; PHELAN; DOYLE, 2009). Empregando ferramentas de avaliação quantitativa de risco microbiológico, Danyluk e Schaffner (2011) puderam estimar

que a contaminação cruzada durante a etapa de lavagem pode ter sido responsável por 95% a 100% dos casos de surtos por *E. coli* O157:H7 ocorridos nos Estados Unidos em 2006, associados ao consumo de espinafre.

Diversos produtos são utilizados para desinfecção de vegetais, sendo os compostos à base de cloro (ex. hipoclorito de sódio) os mais frequentes. Estes compostos apresentam um bom custo-benefício, pois possuem forte propriedade oxidante e baixo custo. No entanto, sua eficácia é afetada por diversos fatores, principalmente temperatura, pH, quantidade e tipo de matéria orgânica presente na água de lavagem, além de apresentarem o risco de formação de subprodutos halogenados cancerígenos devido à reação do cloro com outros compostos orgânicos (BENEFITS..., 2008; GIL et al., 2009; JOSHI et al., 2013). Diante disso, outros compostos químicos para desinfecção de vegetais, tais como ácido peracético, dióxido de cloro e ozônio, vêm ganhando interesse e sendo alvo de estudos (CHIATTONE; TORRES; ZAMBIAZI, 2008; LÓPEZ-GÁLVEZ et al., 2010; SREBERNICH, 2007).

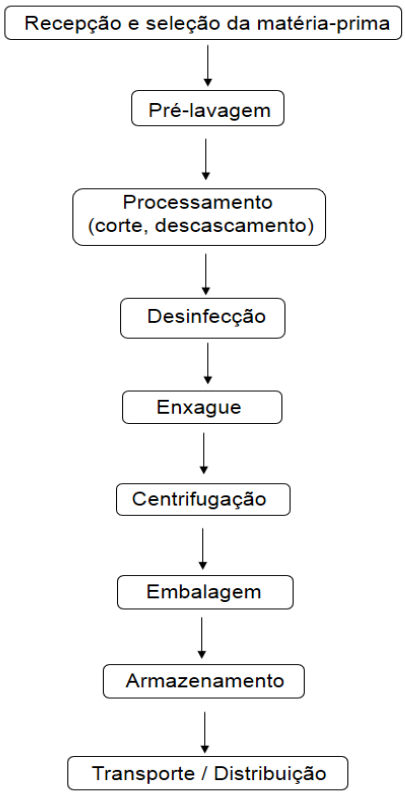

Figura 1. Fluxograma das etapas de processamento dos vegetais prontos para o consumo.

1.4 *Salmonella* spp.

Salmonelas são bactérias pertencentes à familia *Enterobacteriaceae.* São Gram-negativas, em forma de bacilo, anaeróbias facultativas, catalase-positivas, oxidase-negativas e não formadoras de esporos (ADAMS; MOSS, 2008; FORSYTHE, 2010). O gênero *Salmonella* compreende as espécies *S. enterica* e *S. bongori*, sendo que a espécie *S. enterica* contêm seis subespécies (S. *enterica* subsp. *enterica,* S. *enterica* subsp. *salamae*, S. *enterica* subsp. *arizonae*, S. *enterica* subsp. *diarizonae*, S. *enterica* subsp. *houtenae* e S. *enterica* subsp. *indica)* e mais de 2.600 sorovares conhecidos (ISSENHUTH-JEANJEAN et al., 2014).

As doenças causadas por *Salmonella* são especialmente graves em crianças, idosos e imunodeprimidos e costumam ser subdivididas em três grupos: i) febre tifóide, causada por *S. Typhi* e que só acomete o homem (não possui reservatório em animais). Os sintomas são graves e incluem septicemia (infecção generalizada), febre alta, diarréia e vômitos; ii) febre entérica, causada por *S. paratyphi* (A, B e C), cujos sintomas clínicos são mais brandos que os da febre tifóide, podendo evoluir para septicemia e desenvolver gastroenterite, febre e vômitos; iii) infecções entéricas (ou salmoneloses) causadas pelas demais salmonelas, que provocam infecção gastrointestinal com dores abdominais, diarréia, febre baixa e vômito, sendo raros os casos clínicos fatais (FRANCO; LANDGRAF, 2008; LI et al., 2013).

O gênero *Salmonella* é amplamente distribuído na natureza, tendo como principal reservatório o trato intestinal do homem e animais. A contaminação dos vegetais por *Salmonella* pode ocorrer inicialmente no campo, durante operações de cultivo e colheita. O uso de fertilizantes constituídos por esterco animal (fonte de micro-organismos patogênicos), bem como solo de cultivo e água de irrigação contaminados representam potenciais fontes de contaminação (BEUCHAT, 2002). Além disso, este patógeno pode se disseminar no ambiente de processamento dos vegetais, devido a falhas de higiene durante a manipulação, transporte, acondicionamento e preparo dos vegetais.

A presença de *Salmonella* em alimentos é inaceitável e representa potenciais riscos à saúde dos consumidores. Trata-se de um dos principais agentes etiológicos envolvidos em surtos de enfermidades transmitidas por alimentos reportados em diversos países (LI et al., 2013). No Brasil, a legislação em vigor (RDC nº. 12 de 02/01/2001) estabelece como critério microbiológico para hortaliças frescas ou

processadas ausência de *Salmonella* em 25 g de produto (AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA, 2001). No entanto, este patógeno tem sido isolado de amostras de vegetais “in natura” e minimamente processados analisados em diversas regiões do país (CEUPPENS et al., 2014; FRODER et al., 2007; MAISTRO et al., 2012; OLIVEIRA et al., 2011; ROCHA; SOARES; BESERRA, 2014; SANT’ANA et al., 2011; TAKAYANAGUI et al., 2006; TRESSELER et al., 2009).

1.5 Avaliação quantitativa de risco microbiológico

A análise de risco é uma importante ferramenta para o estabelecimento de padrões e recomendações relacionados à segurança dos alimentos, tendo três componentes: avaliação do risco, gestão do risco e comunicação do risco. Enquanto a avaliação do risco é subsidiada por informações científicas, a gestão do risco é a etapa em que se implementam medidas de controle visando reduzir o risco ao nível de proteção do consumidor desejado. Já a comunicação do risco é a etapa em que se faz a troca de informações a respeito dos riscos e dos fatores relacionados, no qual interagem os assessores de risco, os gestores de risco e todas as outras partes interessadas (APPLICATION..., 1995; SANT’ANA; FRANCO, 2009).

A avaliação de risco microbiológico é constituída das seguintes etapas: (i) identificação do perigo microbiológico; (ii) avaliação da exposição; (iii) caracterização do perigo e (iv) caracterização do risco (CODEX ALIMENTARIUS COMMISSION, 1999; APPLICATION..., 1995).

Uma avaliação de risco inicia-se com a determinação da combinação patógeno-alimento. Com base em informações científicas (dados epidemiológicos, microbiológicos etc.), identifica-se o perigo microbiológico a ser considerado em determinado tipo de alimento e seu envolvimento como causador de enfermidades (CODEX ALIMENTARIUS COMMISSION, 1999; RUZANTE et al, 2013). A etapa seguinte é a avaliação da exposição da população a esse patógeno, ou seja, a probabilidade de ingestão e a quantidade ingerida, de acordo com a multiplicação, sobrevivência e inativação do patógeno, bem como a possibilidade de recontaminação em diferentes condições ambientais. Nessa etapa, podem ser empregados modelos preditivos matemáticos e vários programas computacionais com diferentes bases de dados (CODEX ALIMENTARIUS COMMISSION, 1999; SANT’ANA; FRANCO, 2009). Na sequência, a etapa de caracterização do perigo,

também chamada avaliação dose-resposta, visa descrever a gravidade e duração dos efeitos adversos que podem resultar da ingestão do alimento contaminado com o microrganismo ou sua(s) toxina(s) (CODEX ALIMENTARIUS COMMISSION, 1999). Por fim, a caracterização do risco representa a integração dos resultados das etapas de identificação do perigo, caracterização do perigo (dose-resposta) e avaliação da exposição, fornecendo uma estimativa da probabilidade de ocorrência do problema, bem como de sua magnitude (CODEX ALIMENTARIUS COMMISSION, 1999; RUZANTE et al, 2013).

Uma avaliação de risco pode ser qualitativa ou quantitativa (CODEX ALIMENTARIUS COMMISSION, 1999; RUZANTE et al, 2013). Enquanto a avaliação qualitativa lida com dados descritivos, com resultados expressos em termos de probabilidade (ex. baixa, média ou alta), na avaliação quantitativa os resultados são expressos em termos numéricos, baseados em valores sobre prevalência e enumeração dos patógenos e equações matemáticas que descrevem o comportamento microbiano (RUZANTE et al, 2013; SANT'ANA; FRANCO, 2009). A Tabela 2 apresenta trabalhos que desenvolveram modelos de avaliação quantitativa de risco microbiológico em produtos vegetais.

Tabela 2. Modelos de avaliação quantitativa de risco microbiológico em produtos vegetais.

Micro-organismo	Produto	Referência
E.coli O157:H7	Alface	Ottoson et al. (2011)
E.coli O157:H7	Vegetais folhosos	Danyluk; Schaffner (2011)
E.coli O157:H7, *L. monocytogenes, Salmonella*	Vegetais folhosos	Franz et al. (2010)
E.coli O157:H7, *L. monocytogenes, Salmonella*	Vegetais folhosos	Tromp; Rijgersberg; Franz. (2010)
L. monocytogenes	Alface	Ding et al. (2013)
L. monocytogenes	Vegetais	United States (2003)
L. monocytogenes	Vegetais folhosos	Tian; Liu (2009)
L. monocytogenes e *Salmonella*	VPC (folhosos)	Sant'Ana et al. (2014)

Diante do aumento no consumo de vegetais minimamente processados prontos para o consumo e do risco de enfermidades causadas por micro-organismos patogênicos associados ao consumo destes produtos, faz-se necessário avaliar a influência das práticas de processamento na inativação de patógenos, na ocorrência de contaminação cruzada, bem como no risco de enfermidades devido ao consumo

destes produtos. Estes itens constituíram objetivos desta tese de doutorado, sendo que *Salmonella* foi selecionada como micro-organismo-desafio por estar frequentemente presente em surtos de enfermidades transmitidas por alimentos associados ao consumo de vegetais frescos (LYNCH; TAUXE; HEDBERG, 2009; MRITUNJAY; KUMAR, 2015) e alface (*Lactuca sativa* L) foi escolhida como vegetal em estudo por ser a hortaliça mais consumida no Brasil (AGRIANUAL, 2013).

2. OBJETIVOS

2.1 Objetivo geral

Avaliar a influência da etapa de desinfecção de vegetais na inativação de *Salmonella* Typhimurium, na ocorrência da contaminação cruzada e, com base nos resultados obtidos, elaborar um modelo de avaliação quantitativa de risco microbiológico para estimar o impacto da contaminação cruzada no risco de infecção por este patógeno devido ao consumo de vegetais prontos para o consumo.

2.2 Objetivos específicos

1. Realizar um levantamento das práticas empregadas por indústrias brasileiras durante a produção de vegetais prontos para o consumo, com foco especial nos procedimentos de desinfecção dos vegetais;

2. Utilizar os dados obtidos nas indústrias para avaliar a influência dos parâmetros da água de lavagem na inativação de *Salmonella* Typhimurium e na ocorrência de contaminação cruzada durante a etapa de desinfecção dos vegetais;

3. Construir um modelo de avaliação quantitativa de risco microbiológico para estimar o impacto da contaminação cruzada durante a etapa de lavagem de vegetais, no risco de infecção por *Salmonella* devido ao consumo de vegetais prontos para o consumo no Brasil.

3. ORGANIZAÇÃO DA TESE

A apresentação desta tese de doutorado foi dividida em quatro capítulos, a saber:

Capítulo 1: Esse capítulo corresponde ao artigo publicado em *LWT – Food Science and Technology*. Maffei, D.F., Alvarenga, V.O., Sant'Ana, A.S., Franco, B.D.G.M. (2016). Assessing the effect of washing practices employed in Brazilian processing plants on the quality of ready-to-eat vegetables. LWT – Food Science and Technology 69, 474-481. DOI: 10.1016/j.lwt.2016.02.001. O artigo descreve as práticas de processamento observadas nas indústrias brasileiras durante a produção de vegetais prontos para o consumo, bem como o efeito dos procedimentos de lavagem na qualidade microbiológica dos vegetais produzidos.

Capítulo 2: Descreve os experimentos e resultados da avaliação da influência dos parâmetros de processamento na inativação de *Salmonella* Typhimurium durante a etapa de desinfecção de alfaces (*Lactuca sativa* L). Esses experimentos foram necessários para viabilizar a construção do modelo de avaliação quantitativa de risco microbiológico mencionado no Objetivo 3 desta tese.

Capítulo 3: Esse capítulo corresponde ao artigo publicado em *Letters in Applied Microbiology*. Maffei, D.F., Sant'Ana, A.S., Monteiro, G., Schaffner, D.W., Franco, B.D.G.M. (2016). Assessing the effect of sodium dichloroisocyanurate concentration on transfer of *Salmonella enterica* serotype Typhimurium in wash water for production of minimally processed iceberg lettuce (*Lactuca sativa* L). Letters in Applied Microbiology 62, 444-451. DOI: 10.1111/lam.12577. O artigo descreve os experimentos realizados para avaliar a ocorrência de contaminação cruzada por *Salmonella* Typhimurium durante a etapa de desinfecção dos vegetais, simulando diferentes cenários utilizando água clorada com dicloroisocianurato de sódio e não-clorada.

***Capítulo 4*:** Esse capítulo corresponde ao artigo submetido à publicação em *International Journal of Food Microbiology*: Maffei, D.F., Sant'Ana, A.S., Franco, B.D.G.M., Schaffner, D.W. (2016). Quantitative assessment of the impact of cross-

contamination during the washing processing step of ready-to-eat vegetables on the risk of illness caused by *Salmonella*. O artigo descreve o modelo de avaliação quantitativa de risco microbiológico construído para estimar o impacto da contaminação cruzada durante a etapa de desinfecção de vegetais, no risco de infecção por *Salmonella* devido ao consumo de vegetais prontos para o consumo no Brasil.

CAPÍTULO 1

Assessing the effect of washing practices employed in Brazilian processing plants on the quality of ready-to-eat vegetables

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Abstract

This study gathered information on the practices employed in Brazilian processing plants during production of ready-to-eat (RTE) vegetables and evaluated the effect of washing practices on the quality of RTE vegetables produced in these plants. Physicochemical analysis of water included temperature, pH, organic load and chlorine concentration and microbiological analysis included mesophilic bacteria, yeasts and molds, *Enterobacteriaceae*, total coliforms, *E.coli* and *Salmonella*. The ten selected processing plants were clustered in three groups: 1) plants A, B, D, H and I, where washing procedures included immersion in agitated tanks during pre-washing and washing-disinfection and use of disinfectant in the pre-washing step; 2) plant E, where vegetables were washed under running water in the pre-washing step, sodium hypochlorite was used as a disinfectant agent and processing of vegetables was only manual; and 3) plants C, F, G and J, where pre-washing and washing-disinfection were performed by immersion in water, followed by a rinsing step. Chlorine was the most used chemical agent for disinfection of vegetables. A 0.2-1.2 log reduction was achieved by the practices adopted in the plants, highlighting the importance of immediate refrigeration and control measures to avoid post-processing recontamination.

Keywords: ready-to-eat vegetables, processing plants, sanitization, washing.

1. Introduction

Over the last decades, the demand for fresh and convenient foods increased. People have less time to cook at home which reflects the increased popularity of ready-to-eat (RTE) foods, such as minimally processed vegetables (Abadias, Usall, Anguera, Solsona & Viñas, 2008; Chen, Zhu, Zhang, Niu & Du, 2010). Moreover, these products have gained ground in restaurants, hotels, fast food chains, catering services and other institutions (Rojas-Grau, Garner, & Martín-Belloso, 2011).

RTE vegetables may be subjected to minimal processing, such as peeling, cutting, slicing, shredding, washing, drying and packaging (Alzamora, Tapia, & López-Malo, 2000; Codex Alimentarius Commission, 2003), resulting in a diversity of products and packaging formats (Jung, Jang, & Matthews, 2014). As the majority of RTE vegetables require no further treatment before consumption, absence of a foodborne pathogens killing step can result in a potential public health problem. A number of foodborne illnesses associated to fresh produce have been reported (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004) and recent examples are *S.* Newport and *S.* Saintpaul in cucumbers, *S.* Enteritidis in bean, alfafa and spicy sprouts and *E.coli* O157:H7 in ready-to-eat salads, spinach and spring mix (Centers for Disease Control and Prevention, 2015). In Brazil, a number of outbreaks reported between 2000 and 2014 were associated with the consumption of vegetables (Brasil, 2014).

Vegetables can become contaminated with pathogenic microorganisms during harvesting and post-harvesting procedures, caused by soil, irrigation water, inadequately composted manure, air, wild and domestic animals, human handling, harvesting equipment, transport containers, vehicles, improper storage and packaging (Berger et al., 2010; Harris et al., 2003). Among these operations, washing seems to have a major relevance for the safety of these products. Although washing aims to remove debris and reduce microbial load, its effectiveness is limited, and reduction of certain microorganisms to a specific level of safety cannot be assured (Gil, Selma, López-Gálvez & Allende, 2009). Chemicals added to washing water are helpful, but the antimicrobial effectiveness depends on several factors (Prado-Silva et al., 2015). For instance, the effectiveness of chlorine at 50-200 mg/L, the most widely used sanitizer for disinfection of fresh produce, is typically less than 2 log CFU (Goodburn & Wallace, 2013).

Recent studies have shown that contamination of washing water used during RTE vegetable processing may lead to spread of contaminants within batches (Jensen, Friedrich, Harris, Danyluk & Schaffner, 2015; Holvoet et al., 2014; Perez-Rodriguez et al., 2014; Tomás-Callejas et al., 2012; Zhang, Ma, Phelan, & Doyle, 2009). On this matter, cross-contamination during washing may have been the potential cause of many reported foodborne outbreaks. Thereby, the knowledge about the washing practices and water characteristics employed in processing plants may help to understand and to prevent the occurrence of cross-contamination during washing of vegetables.

The objectives of this study were to gather information on the practices employed in ten selected Brazilian processing plants located in the State of Sao Paulo during production of RTE vegetables and to evaluate the effect of washing practices on the quality of RTE vegetables produced in these plants.

2. Materials and methods

2.1 *Assessment of washing practices in selected Brazilian processing plants during the production of RTE vegetables*

Ten major RTE vegetables processing plants, identified as A to J, located in the State of Sao Paulo, Brazil, were selected for the study. Three of them are located in Sao Paulo city and the other seven in inland towns. A questionnaire with 45 items focusing on the water usage during processing addressing washing method, water temperature, addition of disinfectant, water volume, amount of vegetables immersed in the water and reuse/discharge of water used in the pre-washing, washing-disinfection and rinsing steps was applied to these plants. Additional information on raw materials reception and storage, centrifugation of washed vegetables, packaging and transportation conditions was also collected. Prior to application in the ten processing plants, the questionnaire (Supplementary material) was validated by application in two plants. A flowchart with the processing steps in the ten visited RTE vegetable processing plants is shown in *Figure 1*.

2.2 *Collection of water and vegetable samples*

For the purpose of this study, pre-washing was considered the step in which vegetables were immersed in tanks with water (usually without disinfectant agents) aiming to remove soil and debris, prior to the washing-disinfection step. Washing-disinfection was considered the step in which vegetables were immersed in a tank with water containing disinfectant agents. Rinsing was the step in which pre-washed and disinfected vegetables were washed with clean water to eliminate residues of disinfectants.

A total of 34 water samples (250 mL) were collected in the ten visited processing plants during washing procedures, considering all industries presented a continuous processing. These samples included water from the tap water supply (n=10), water from pre-washing tanks (n=9), water from washing-disinfection tanks (n=10) and water from rinsing tanks (n=5). Water samples were collected in two bottles, one destined for chemical testing and the other for microbiological analysis. Thirty-six and forty-eight samples of vegetables were collected before and after processing, respectively, and comprised leafy greens [chicory (*Cichoriumintybus*), collard greens (*Brassica oleracea*), escarole (*Cichoriumendivia*), lettuce (*Lactuca sativa*), parsley (*Petroselinum crispum*), spinach (*Spinaciaoleracea*) and watercress (*Nasturtium officinale*)] and other vegetables [beet (*Beta vulgaris esculenta*), carrot (*Daucuscarota* subsp.*sativus*), onion (*Allium cepa*), potato (*Solanumtuberosum*), white carrot (*Arracaciaxanthorrhiza*) and zucchini (*Cucurbitapepo*)].

2.3 *Physicochemical analysis of water samples*

Water samples collected from the tanks were tested for temperature (˚C), pH, chlorine concentration (mg/L) and organic load concentration (mg/L). Temperature and pH were measured using a portable meter fitted with appropriate probes (HQ40d, Hach, USA). Organic load was determined by a gravimetric method (Teixeira, Tundisi, & Kutner, 1965; Tundisi, 1969) with modifications according to Wetzel and Likens (1991). Free chlorine was measured using a portable photometer (HI96771, Hanna Instruments, USA).

2.4 *Microbiological analysis of water samples*

In order to inactivate residual chlorine, 10% sterile sodium thiosulphate (Sigma-Aldrich ChemieGmbHSteinheim, Germany) was added to water samples before microbiological analysis. The samples were serially diluted in 0.1% peptone (Oxoid, England) and plated for enumeration of mesophilic bacteria (Morton, 2001) and *Enterobacteriaceae* (Kornacki & Johnson, 2001). MPN of total coliforms and *E.coli* were determined using Fluorocult®LMX Broth, double concentration (Hunt & Rice, 2005; Merck, 2005). Samples were also tested for *Salmonella* spp according to ISO 19250 (2010).

2.5 *Microbiological analysis of vegetables samples*

Twenty-five grams of each vegetable sample were transferred to a sterile plastic bag and mixed with 225 mL of 0.1% peptone water in a Stomacher 400 Lab-blender (Seward Medical, London, England) for 1 min. Serial dilutions were prepared in 0.1% peptone water and submitted to enumeration of mesophilic bacteria (Morton, 2001), yeasts and molds (Beuchat & Cousin, 2001) and *Enterobacteriaceae* (Kornacki & Johnson, 2001). Total coliforms and *E.coli* were also enumerated using the MPN technique and Fluorocult®LMX Broth (Kornacki & Johnson, 2001; Merck, 2005). Additionally, another 25 g sample of each sample was mixed with 225 mL of Buffered Peptone Water (BPW) (Difco, Sparks, Md., USA) for 1 min, following incubation at 37 ˚C for 24 h, for detection of *Salmonella* spp, according to ISO 6579:2002 (2007).

2.6 *Statistical analysis*

Microbial counts of water (CFU/mL and MPN/mL) and vegetable samples (CFU/g and MPN/g) were log transformed. The Student's t-test and the Mann–Whitney test were used, depending on variable distribution, to determine significant differences ($p<0.05$) among microbial counts in vegetable samples before and after processing. The software SigmaPlot version 12.5 (2013 Systat Software Inc.) was used for the statistical treatment. Cluster analyses were made using the software

XLSTAT (Addinsoft version 2015.1.01) to identify similarities among the practices adopted in the ten visited processing plants.

3. Results

Results from the questionnaire applied in the ten visited processing plants showed that in all plants, the raw materials were kept under refrigeration, varying from a minimum of 2 ˚C in plant D to a maximum of 8 ˚C, in plants B, G and J. In all plants, damaged vegetables were removed from the entering lots before processing. Despite present in all plants, the pre-washing step was performed differently: in plant E pre-washing was done under running water, in plants A, B, D and H pre-washing was done by immersion in agitated water and in plants C, F, G, I and J pre-washing was done by immersion in static water. At the pre-washing step, the water was chlorinated in plants A, B, D and J, and concentrations varied from 3 to 235 mg/L. In plants H and I, alkaline detergents were added in the water to remove soil and dirt.

Except for plant E, where peeling, cutting and shredding were done manually, the other plants used machines for this purpose, resulting in different types, sizes and cuts (e.g. grated, diced, sliced and stick vegetables or entire heads of leafy greens). Up to 86 varieties of ready-to-eat vegetables were produced in these processing plants.

All plants adopted a washing-disinfection step during processing. In seven plants (C, D, E, F, G, I and J) vegetables were immersed in a tank with water in static conditions, while in three plants (A, B and H), vegetables were immersed in agitated water tanks. The most common disinfectant agents were: sodium dichloroisocyanurate (plants A, B, C, D, F, G and J, concentration ranging from 75.5 to 155 mg/L), sodium hypochlorite (plant E, concentration 50 mg/L) and chlorine dioxide (plants H and I, concentration 240 mg/L). The contact time ranged from 2 to 20 min. In plant B, leafy greens were disinfected with ozonated water (1.0 g/L). The amount of water used for washing 1 kg of vegetables in the pre-washing and washing-disinfection tanks varied from 5 to 28 L (*Figure 2)*.

The rinsing step was performed in five processing plants: in plant A, rinsing was done with running water and in plants C, G, H and J vegetables were immersed in tanks containing non-chlorinated water. The other five plants did not include a rinsing step in the processing. In eight processing plants (A, B, C, F, G, H, I and J)

vegetables were submitted to a centrifugation step (average 1200 rpm for 1.5 min) for water removal. In plant A, washing water was changed uninterruptedly through a continuous circulation system. The other plants reported that the water was changed 3 to 7 times per day and the disinfectant concentration was adjusted after every water replacement. Reuse/recirculation of water was used in three plants: during pre-washing and washing-disinfection steps (plant B), during rinsing step (plant C) and during pre-washing step (plant J). In these plants, reused water was discharged when accumulation of debris became evident (~3-4 times/day).

In plant G, after rinsing and before centrifugation, some types of vegetables were immersed in water tanks containing ice cubes (prepared with potable but non-chlorinated water) and sodium metabisulphite as antioxidant. The processed vegetables were packaged for retail and for wholesale, in volumes varying from 30 to 300 g and from 1 to 5 kg, respectively. Most common packaging materials were polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC) and expanded polystyrene (EPS). Some plants used modified atmosphere in packages of leafy greens (e.g. lettuce) and vacuum in packages of other vegetables (e.g. minimally processed carrots and potatoes). All packages were stored under refrigeration (2-8 $^{\circ}$C) up to 48 h and distributed for retail under refrigeration (2-10 $^{\circ}$C) in plastic or cardboard boxes. However, only plants A, F, G, H and I monitored the temperature during distribution. The reported shelf life of RTE vegetables produced in the visited processing plants ranged from 5 to 7 days for leafy greens and 5 to 15 days for other vegetables (e.g. green beans, sweet potato, zucchini etc.).

Cluster analysis aiming at identifying similarities or dissimilarities among the practices observed in the ten visited processing plants resulted in three major groups (*Figure* 3): group 1 (plants A, B, D, H and I), group 2 (plant E) and group 3 (plants C, F, G and J). The similarities in plants of group 1 were immersion in agitated tanks during pre-washing step in A, B, D and H and during washing-disinfection step in A, B and H, use of a disinfectant in the pre-washing step in A, B, D, H and I, and refrigeration of water in A and D. Plant E (group 2) differed from the other processing plants as vegetables were washed under running water in the pre-washing step, sodium hypochlorite was used as a disinfectant agent and processing of vegetables was only manual. The similarities in plants of group 3 (C, F, G and J) were static immersion during pre-washing and washing-disinfection steps, followed by a rinsing step in C, G and J.

Results of temperature, pH, organic load and chlorine concentration in water are presented in boxplots (*Figure* 4). Only plants A and D used refrigerated water in all processing steps, and temperature varied from 6 to 14 ˚C. In plants B, C, E, F, G, H, I and J, the water used in all steps was at room temperature, with an average of 21±0.8 ˚C. The pH of water ranged from 6.4 to 8.3 for tap water, 5.8 to 8.2 for water in the pre-washing tanks, 5.6 to 8.3 for water in the washing-disinfection tanks and 6.5 to 7.7 for water in the rinsing tanks. Regarding organic load in the water, the highest concentration (50 mg/L) was detected in pre-washing tanks, which may be due to dirt and cell exudates from cut surfaces. On the other hand, in tanks of washing-disinfection the concentration of organic load ranged from 2.3 to 13.5 mg/L.

Different chlorine-based disinfectants were used in the washing-disinfection step: sodium dichloroisocyanurate or sodium hypochlorite in plants A, B, C, D, E, F, G and J (concentrations ranging from 50 to 155 mg/L), and chlorine dioxide in plants H and I (240 mg/L). Processing plant B also had a tank of ozonated water (1.0 g/L) for washing-disinfection of leafy greens exclusively. Measurement of free chlorine concentration in water samples containing chlorine dioxide or ozone was not possible with the portable photometer used in the study. In these cases, the technicians in the processing plants provided the data presented in Figure 4.

Microbiological testing of water (Tables 1 and 2) indicated that *Salmonella* was not detected in any sample (absence in 100 mL) and the lowest and highest counts of mesophilic bacteria and *Enterobacteriaceae* were found in tap water and water from the pre-washing tanks, respectively. Although microbial counts of mesophilic bacteria and *Enterobacteriaceae* seems to be higher in water samples collected from the pre-washing step, they were not statistically significant when the means of the ten plants were compared. Total coliforms were found in water from the pre-washing tanks in plants B, D, F, G, H and I, in water from the washing-disinfection tanks in plants H and I and in water from the rinsing tanks in plants G and H. *E.coli* was detected in water from the pre-washing tanks in plants F, G and H and in water from rinsing tanks in plant H, with counts of 6.9 MPN/100 mL, which is of concern, as this is the last washing step in RTE vegetables production. Considering that the proportion between the volume of water and the amount of vegetable immersed in this water affects the efficacy of disinfection process, this parameter was also measured. In the studied processing plants, the proportion ranged from 5 to 28 L of water for 1 kg of vegetables, and varied according to the type of vegetable and the

equipment used for washing, as different equipment require different water volume for efficient functioning.

Microbiological testing of vegetables before and after processing (Table 3) indicated that *Salmonella* was not detected in any tested sample (absence in 25 g), which is in agreement with Brazilian and other regulations (Brasil, 2001; Anonymous, 2014). *E.coli* was detected in 20 (55%) and 10 (21%) unprocessed and processed vegetables, respectively. These included three collard green samples from plants G, H and J, two chicory samples from plants E and F, two parsley samples from plants B and F, one scarole sample from processing plant J, one spinach from plant J and one grated carrot sample from plant J. Two samples (chicory and parsley) from processing plant F presented counts above 2 log CFU/g, which is the upper limit determined by the Brazilian Surveillance Agency for thermotolerant coliforms in vegetables (Brasil, 2001).

Reduction of the microbial load from unprocessed to processed vegetables seemed to be more effective in plant A (from 1.4 to 3.2 log-reduction) than in the other processing plants (Table 3). In plant A, the washing water and the processing environment were refrigerated and the washing steps were conducted with chlorinated water, highlighting the importance of these parameters during processing to assure the quality and safety of RTE vegetables.

Overall, the processing of vegetables caused a 0.2 to 1.2 log reduction in the initial microbial counts when compared unprocessed with processed vegetables (Table 4). These reductions were statistically significant ($p<0.05$) for mesophilic bacteria, yeasts and molds, *Enterobacteriaceae* and total coliforms. Although significant, these reductions can be overcome very quickly if the products are held at room temperature. Thus, immediate storage under refrigeration and practices to avoid recontamination are very important control measures. Microbial counts on RTE leafy greens seems to be higher than in other vegetables, but not statistically significant, which may be due structural differences on their surface (Table 4).

4. Discussion

The processing procedures (reception, pre-washing, peeling, cutting, shredding, washing-disinfection, rinsing, centrifugation, packaging, storage, transport and distribution) in the studied RTE vegetables processing plants were similar

(*Figure* 1). Unique procedures were observed in plant A, such as the refrigeration of whole processing environment and use of refrigerated and chlorinated water in all processing steps.

Each processing step during production of RTE vegetables plays an important role for the quality of the final product, but the washing steps are key steps, as they remove dirt and cells exudates from harvested produce, and reduce the microbial population from their surface (D'Acunzo, Cimmuto, Marinelli, Aurigemma & Giusti, 2012). However, if contaminated, wash water can become a source of cross-contamination, causing transfer of pathogenic and non pathogenic microorganisms from contaminated to non-contaminated batches (Holvoet et al, 2014; Jensen, Friedrich, Harris, Danyluk & Schaffner, 2015; Perez-Rodriguez et al., 2014; Tomás-Callejas et al., 2012; Zhang, Ma, Phelan, & Doyle, 2009). On this matter, Danyluk and Schaffner (2011) developed a quantitative microbial risk assessment using data published by Zhang et al. (2009) and hypothesized that 95% to 100% of the cases caused by *E. coli* 0157:H7 in the spinach outbreak occurred in the USA in 2006 could be explained by occurrence of cross-contamination.

In all visited plants, processing of vegetables included pre-washing and washing-disinfection steps, and different chlorine-based compounds were used for disinfection. Chlorine is the most widely used chemical agent for disinfection of fresh produce and presents a good cost-benefit ratio. The Brazilian Health Surveillance Agency recommends the use of chlorine-based products for disinfection of fresh produce, with concentrations ranging from 100 to 250 mg/L of free chlorine (Brazil, 2013).

Chlorine-based compounds have toxicity concerns due to reaction with organic materials that may cause the formation of halogenated by-products (Food and Agriculture Organization/World Health Organization, 2008; Joshi, Mahendran, Alagusundaram, Norton & Tiwari, 2013), causing the expansion of the use of sodium dichloroisocyanurate in Brazil due to less reactivity with organic materials (Salomão, Muller, Massaguer, & Aragão, 2011). In addition, sodium dichloroisocyanurate has a better stability in aqueous solution than sodium hypochlorite. Chlorine dioxide and ozone are powerful antimicrobial agents, but they are more expensive and require specific equipment and expertise to control the concentration in water (Chiattone, Torres, & Zambiazi, 2008; Joshi, Mahendran, Alagusundaram, Norton & Tiwari, 2013). Chlorine dioxide has been increasingly used as an alternative to sodium

hypochlorite since it does not react with organic compounds to generate undesirable carcinogenic chemicals (Chen & Zhu, 2011; López-Gálvez et al., 2010).

In the present study, we observed that RTE leafy greens produced by processing plant B (washed in ozonated water) showed lower microbial counts when compared to the plants where disinfection was carried out only with chlorine-based products. Mesophilic bacteria, yeasts and molds, *Enterobacteriaceae* and total coliforms populations in leafy greens washed in ozonated water were 2.6, 1.0, 3.2 and 1.4 log CFU/g lower than those found in leafy greens washed in chlorinated water (data not shown). *E.coli* and *Salmonella* were not detected in those RTE leafy greens samples washed in ozonated water. Alexopoulos et al. (2013) also observed a higher effectiveness of ozonation than chlorination of water for reduction of microbial counts in fresh-cut lettuce and green bell pepper. These authors noticed that dipping these vegetables in chlorinated water (20 mg/L) resulted in one log reduction of the total microbial counts in the first 15 min. However, when these vegetables were dipped in continuously ozonated water (0.5 mg/L), about 2 log decreases was achieved in the first 15 min and 3.5 log after 30 min of exposure.

The influence of using cold water and refrigeration in the processing environment also seems to have an influence in reducing microbial loads, since RTE vegetables produced in refrigerated processing plant A had lower contamination than in the other plants. The use of cold water during processing of vegetables lowers metabolism and respiration rate, which helps to prevent the internalization and infiltration of bacteria, increasing shelf life (Sapers, 2003). However, increased costs and health issues for employers due to work with refrigerated water were reported in most visited processing plants.

Although those parameters discussed above (washing of vegetables in ozonated or refrigerated water/environment) seem to have an influence on the reduction of microbial counts, there is no statistical test able to draw appropriate comparisons, due to the limit number of water and RTE vegetables samples collected at one unspecified point in time. That number is limited because the idea of this study was to collected samples at the same day of the visit in the ten selected processing plants, aiming to evaluate the microbiological quality of the RTE vegetables produced by those plants.

The differences in the proportion between the volume of water and the amount of vegetable immersed in this water in the studied processing plants had little or no

effect on the microbiological results, as microbial counts of water samples collected from tanks that used 28 L of water did not differ from those collected from tanks that used 5 L of water (data not shown). All ten visited processing plants were in accordance with Cenci, Gomes, Alvarenga and Junior (2006), who recommend the use of at least 5 to 10 L of water for washing 1 kg of product.

There was no linear correlation between microbial counts in the water samples and those obtained in RTE vegetables, i.e, the processing plants presenting higher microbial counts in water samples were not the ones that presented higher microbial counts in the produced RTE vegetables. In conclusion, data collected in this study contributes to a better understanding of the RTE vegetables processing chain, helping government and industry in the establishment of proper control measures to ensure safety of this increasingly important type of healthy, fresh and convenient food.

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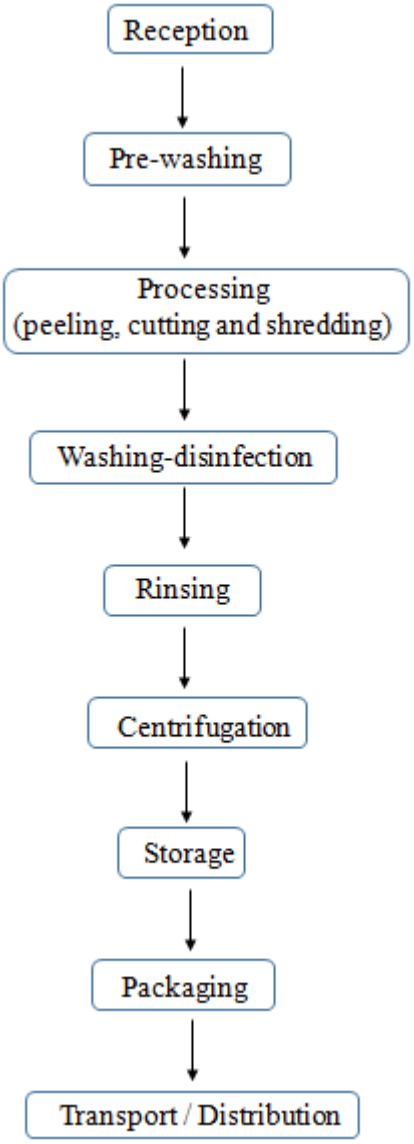

Figure 1. Flowchart showing the processing steps in the visited processing plants.

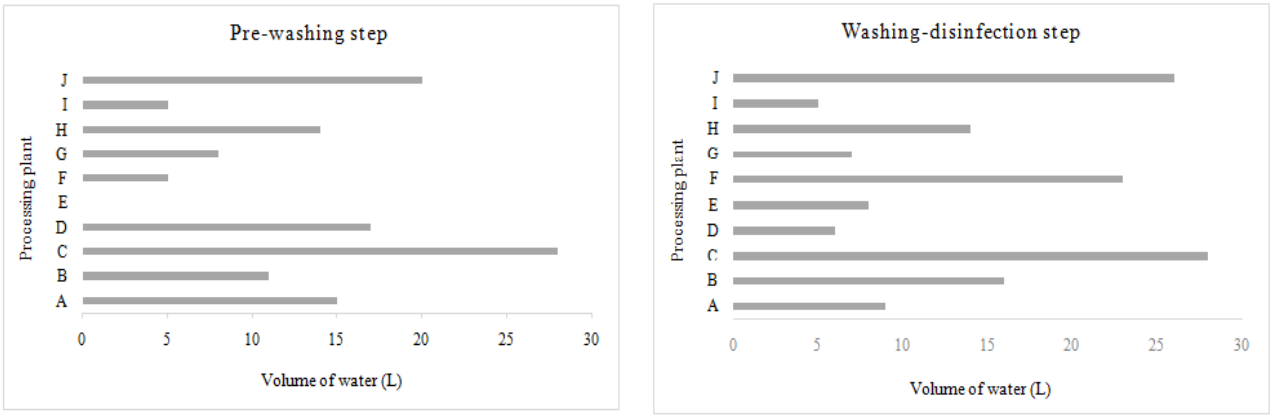

Figure 2. Volume of water used for washing 1 kg of vegetables in the visited processing plants, designated A, B, C, D, E, F, G, H, I and J.

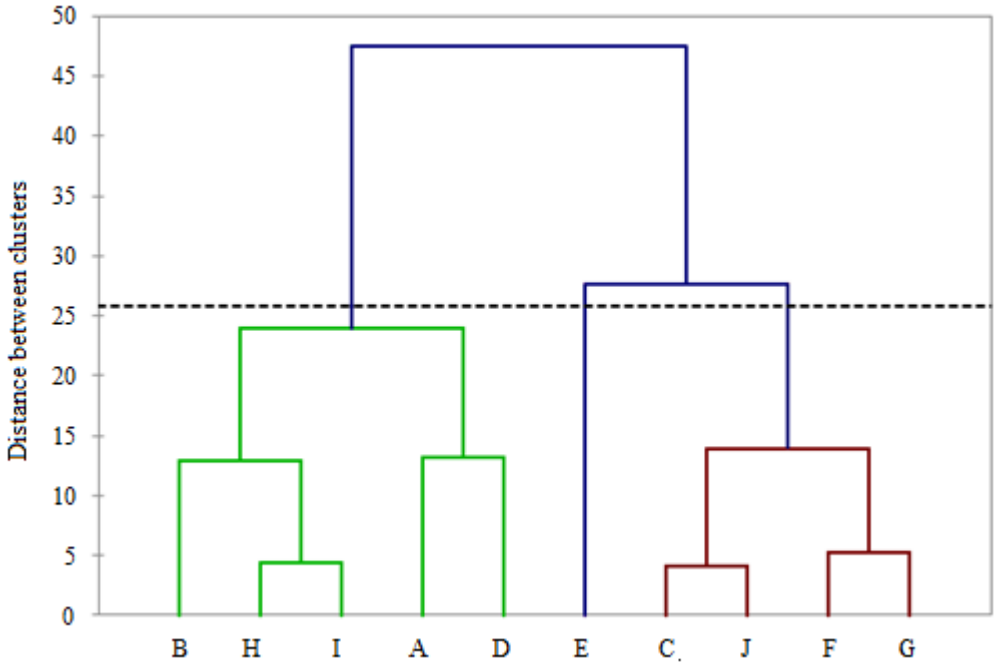

Figure 3. Cluster analysis for the practices employed in the visited processing plants, designated A, B, C, D, E, F, G, H, I and J.

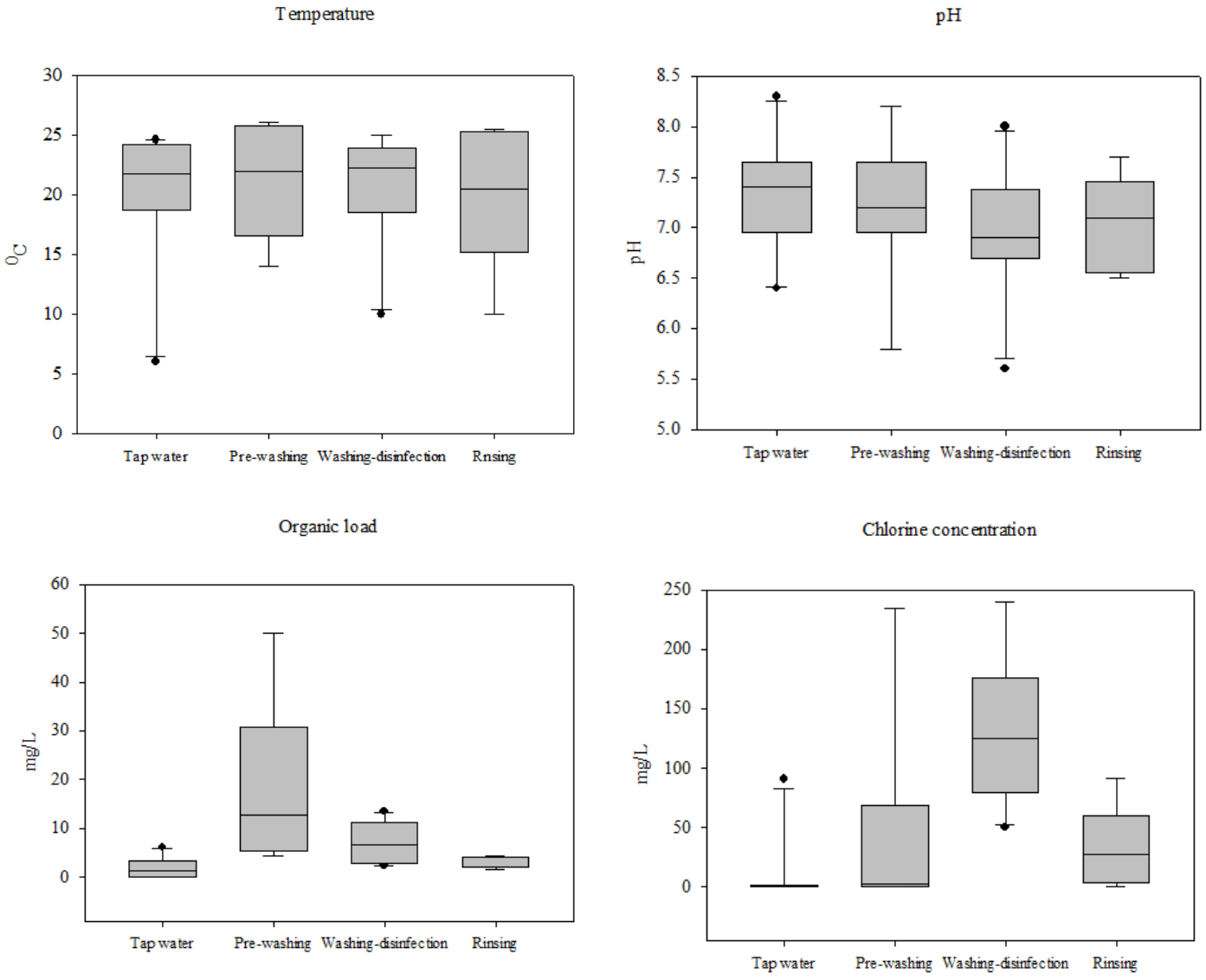

Figure 4. Boxplots showing the physicochemical parameters of water samples collected in the visited processing plants.

Table 1. Counts of mesophilic bacteria and *Enterobacteriaceae* in water samples collected in ten vegetables processing plants.

Processing plant	Mesophilic bacteria				*Enterobacteriaceae*			
	Tap water	Pre-washing	Washing-disinfection	Rinsing	Tap water	Pre-washing	Washing-disinfection	Rinsing
A	2.0±0.1	1.7±0.3	1.9±0.2	1.6±0.1	< 1	< 1	< 1	< 1
B	1.7±0.1	3.4±0.6	1.0±0.1	n.a.	1.8±0.2	1.5±0.5	< 1	n.a.
C	1.5±0.1	1.6±0.8	1.2±0.3	1.1±0.1	< 1	< 1	< 1	< 1
D	1.4±0.1	3.7±0.9	1.1±0.4	n.a.	< 1	2.1±0.3	< 1	n.a.
E	1.0±0.0	1.0±0.0	3.4±0.2	n.a.	< 1	< 1	< 1	n.a.
F	1.4±0.2	4.0±0.5	1.0±0.1	n.a.	< 1	1.8±0.3	< 1	n.a.
G	< 1	3.9±0.6	< 1	1.1±0.2	< 1	3.7±0.4	< 1	1.0±0.0
H	1.4±0.3	3.5±0.3	3.3±0.4	3.5±0.3	< 1	3.1±0.2	2.8±0.3	2.9±0.3
I	1.1±0.1	1.4±0.2	1.0±0.0	n.a.	< 1	1.3±0.2	1.2±0.2	n.a.
J	1.1±0.1	1.3±0.3	1.3±0.3	1.4±0.5	< 1	< 1	< 1	< 1

Results expressed as mean±SD (log CFU/mL)
“n.a.” not applicable (step not performed in the plant)

Table 2. Counts of total coliforms and *E.coli* in water samples collected in ten vegetables processing plants.

Processing plant	Total coliforms				*E.coli*			
	Tap water	Pre-washing	Washing-disinfection	Rinsing	Tap water	Pre-washing	Washing-disinfection	Rinsing
A	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1
B	< 1.1	16	< 1.1	n.a.	< 1.1	< 1.1	< 1.1	n.a.
C	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1
D	< 1.1	>23	< 1.1	n.a.	< 1.1	< 1.1	< 1.1	n.a.
E	< 1.1	< 1.1	< 1.1	n.a.	< 1.1	< 1.1	< 1.1	n.a.
F	< 1.1	>23	< 1.1	n.a.	< 1.1	>23	< 1.1	n.a.
G	< 1.1	>23	< 1.1	12	< 1.1	>23	< 1.1	< 1.1
H	< 1.1	>23	>23	>23	< 1.1	>23	< 1.1	6.9
I	< 1.1	5.1	3.6	n.a.	< 1.1	< 1.1	< 1.1	n.a.
J	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1

Results expressed as MPN/100 mL
“n.a.” not applicable (step not performed in the plant)

Table 3. Counts of mesophilic bacteria, *Enterobacteriaceae,* yeasts and molds, total coliforms and *E.coli* in vegetables before and after processing.

Processing plants	Mesophilic bacteria (log CFU/g)		*Enterobacteriaceae* (log CFU/g)		Yeasts and Molds (log CFU/g)		Total coliforms (log MPN/g)		*E.coli* (log MPN/g)	
	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed
A	6.2±0.2	3.0±0.5	5.6±0.3	2.9±0.8	5.2±0.1	3.6±0.2	> 3.0	1.6±0.6	1.4±0.0	< 1
B	6.2±0.1	4.4±1.8	5.1±0.1	2.8±1.6	4.6±0.2	4.4±1.0	> 3.0	2.3±0.8	> 3.0	0.5±0.0
C	6.4±0.1	3.8±0.5	5.3±0.2	3.4±0.5	5.4±0.7	4.9±1.0	> 3.0	1.9±0.6	< 1	< 1
D	3.1±0.0	3.9±0.7	2.6±0.0	3.2±1.2	3.5±0.0	3.9±0.8	> 3.0	2.0±0.3	< 1	< 1
E	6.3±0.1	6.2±0.1	5.0±0.2	4.9±0.3	5.8±0.2	5.6±0.4	> 3.0	> 3.0	2.3±0.8	0.9±0.0
F	6.3±0.1	5.5±0.5	5.1±0.1	4.8±0.6	5.8±0.1	5.0±0.5	> 3.0	> 3.0	0.9±0.6	2.5±0.2
G	5.7±0.6	4.7±0.5	4.6±1.0	3.0±1.1	5.4±0.6	5.0±1.1	> 3.0	3.0±0.2	1.7±1.1	0.5±0.0
H	6.2±0.1	5.3±0.6	5.5±0.4	4.9±0.4	5.7±0.7	4.4±0.4	> 3.0	2.5±0.9	1.1±0.5	1.0±0.0
I	5.8±0.6	5.3±0.3	4.7±0.9	4.3±0.9	5.6±0.2	5.2±0.6	2.4±0.9	1.4±0.4	1.1±0.2	< 1
J	6.4±0.5	5.9±0.7	6.0±0.4	5.0±0.4	5.3±0.8	5.2±0.6	> 3.0	> 3.0	1.8±0.4	1.2±0.2

Results expressed as mean±SD

Table 4. Effect of processing on counts of mesophilic bacteria, yeasts and molds, *Enterobacteriaceae,* total coliforms and *E.coli* counts in leafy greens and other vegetables.

Samples	Mesophilic bacteria (log CFU/g)		Yeasts and Molds (log CFU/g)		*Enterobacteriaceae* (log CFU/g)		Total coliforms (log MPN/g)		*E.coli* (log MPN/g)	
	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed
RTE vegetables	6.0±0.6*	4.9±1.2*	5.3±0.7*	4.8±0.8*	5.0±0.7*	3.9±1.2*	2.9±0.4*	2.4±0.7*	1.6±0.8	1.3±0.7
Leafy greens	6.2±0.2*	5.0±1.2*	5.5±0.6	5.0±0.8	5.2±0.5*	4.1±1.2*	3.0±1.4*	2.6±0.7*	1.7±0.8	1.3±0.7
Other vegetables**	5.8±0.9*	4.8±1.2*	5.1±0.8	4.6±0.8	4.8±1.0*	3.8±1.2*	2.8±0.6*	2.2±0.7*	1.2±0.2	1.0 ±0.0

Results expressed as mean±SD
Statistical analysis performed by the Student's t-test and the Mann–Whitney test
*Significant differences ($p<0.05$) observed between unprocessed and processed vegetables for the microbial groups evaluated
**Other vegetables included bulbs, roots and tubers

Supplementary Material

Questionnaire applied in the Brazilian RTE vegetables processing plants.

Reception

Stored under refrigeration?

At what temperature?

Manual produce selection/classification?

Pre-washing

Is it done?

Method: ( ) running water ( ) immersed ( ) shaken ( )
sprinkled

Any disinfectant used?

What concentration?

Water volume?

Water temperature?

Amount of immersed vegetables?

Processing

Manual or mechanical?

Number (variety) of vegetables processed?

Ambient temperature in processing plant?

Washing-disinfection

Method: immersed ( ) shaken ( ) sprinkled ( )

How many times?

Any disinfectant used?

What concentration?

Water volume?

Water temperature?

Amount of immersed vegetables?

Contact time of vegetables with disinfectant solution?

Reuse/recirculation of water

Is it done?

How many times?

Applied some treatment for reuse?

Any disinfectant used?

What composition and concentration?

Discharge of water

Is it done?

Partial water change (how many times)?

Total water change (how many times)?

Applied some treatment after discharge?

Any disinfectant used?

What composition and concentration?

Rinsing

Is it done?

Method: ( ) running water ( ) immersed ( ) shaken ( ) sprinkled

Water temperature during this stage?

Centrifugation

Is it done?

For how long and rpm?

Packaging

Temperature?

Packaging method?

Type of packaging?

Amount per package?

Transport/distribution

Temperature?

Temperature recorded during transport?

Type of material used for transport?

How long after packaging (expedition)?

CAPÍTULO 2

Influência dos parâmetros de processamento na inativação de *Salmonella* Typhimurium durante a etapa de lavagem e desinfecção de alfaces (*Lactuca sativa* L).

Resumo

A etapa de desinfecção é considerada uma das mais importantes durante o processamento dos vegetais prontos para o consumo, mas também pode ser via de contaminação cruzada, principalmente se a qualidade da água de desinfecção não for adequadamente controlada. Este capítulo apresenta os experimentos realizados para avaliar a influência dos parâmetros da água de processamento na inativação de *Salmonella* Typhimurium durante a etapa de desinfecção de alfaces (*Lactuca sativa* L.). Os parâmetros testados incluíram proporção entre volume de água e quantidade de vegetais imersos, temperatura e pH da água, concentração de cloro, concentração de matéria orgânica e tempo de contato dos vegetais com a solução de desinfecção. Dentre estes parâmetros, apenas a concentração de cloro (dicloroisocianurato de sódio) apresentou influência significativa ($p \leq 0,05$) na redução das contagens de *S*. Typhimurium, sendo que os demais parâmetros testados não apresentaram influência no intervalo testado.

Palavras-chave: alface, desinfecção, *Salmonella* Typhimurium, vegetais prontos para o consumo.

1. Introdução

Durante o processamento dos vegetais prontos para o consumo, a desinfecção é considerada uma das etapas mais importantes, pois visa reduzir a carga microbiana e eliminar micro-organismos patogênicos que possam estar presentes nos vegetais. No entanto, nesta etapa também pode ocorrer a contaminação cruzada, ou seja, a transferência de micro-organismos de produtos contaminados para produtos não-contaminados (GIL et al., 2009; GÓMEZ-LÓPEZ et al., 2015).

Diversos fatores podem exercer influência na eficácia do processo de desinfecção de vegetais e na contaminação cruzada durante esta etapa. Assim, foi preciso avaliar quais parâmetros de processamento deveriam ser selecionados para realização dos testes de contaminação cruzada necessários para construção do modelo de avaliação quantitativa de risco microbiológico proposto como objetivo desta pesquisa. Para isso, os parâmetros de processamento observados nas indústrias brasileiras visitadas durante a produção de vegetais minimamente processados prontos para o consumo (VPC), descritos no Capítulo 1, foram reproduzidos em laboratório, a fim de avaliar a influência de cada um deles na inativação de *S.* Typhimurium durante a etapa de desinfecção de alfaces (*Lactuca sativa* L). Os parâmetros avaliados foram: proporção entre volume de água e quantidade de vegetais imersos, temperatura e pH da água, concentração de cloro, concentração de matéria orgânica e tempo de contato dos vegetais com a solução de desinfecção.

Como os produtos vegetais podem apresentar carga microbiana natural elevada, foi essencial empregar nos procedimentos laboratoriais uma técnica de marcação que permitisse distinguir as cepas de *S.* Typhimurium, utilizadas para a contaminação experimental dos vegetais, das outras salmonelas ou outros micro-organismos eventualmente presentes. A técnica selecionada para este estudo foi a transformação das cepas de *S.* Typhimurium por eletroporação, empregando-se um vetor (pGFPuv) que carrega o plasmídio que expressa a Proteína Verde-Fluorescente (GFP).

2. Materiais e métodos

2.1 Transformação das cepas de Salmonella *Typhimurium*

O trabalho foi realizado com as cepas de *S.* Typhimurium #277 e #386, isoladas de vegetais minimamente processados comercializados no Brasil (SANT'ANA et al., 2011) e pertencentes à coleção de culturas do Laboratório de Microbiologia de Alimentos da FCF/USP e uma cepa referencia (*S.* Typhimurium ATCC 14028). As cepas foram transformadas para expressar a Proteína Verde-Fluorescente (Green Fluorescent Protein - GFP) segundo Sambrook et al. (1989), empregando-se o vetor pGFPuv, da Clontech Laboratories, Inc, USA (Figura 1). Os experimentos foram realizados no laboratório de Biologia Molecular e Biotecnologia de Leveduras, Departamento de Tecnologia e Bioquímico-Farmacêutica, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, que possui autorização da Comissão Interna de Biossegurança (CIBio) da instituição para realização de tais procedimentos.

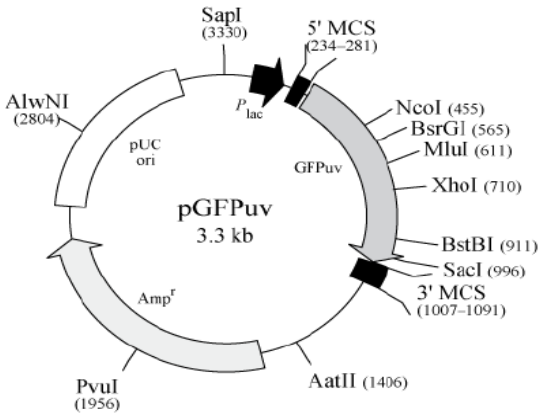

Figura 1. Mapa do Vetor pGFPuv / Fonte: Clontech

Para a transformação, 3 a 5 colônias de cada cepa de *S.* Typhimurium foram inoculadas em 10 mL de caldo TSB (Tryptic Soy Broth, Difco) e incubadas a 37 °C por 18-24 h, sob agitação a 170 rpm. Esta pré-cultura foi diluída em caldo TSB até obtenção de uma suspensão com DO_{600nm} = 0.2 e incubada a 37 °C, sob agitação, até que a cultura atingisse a DO_{600nm} = 0,5. Na sequência, a cultura foi resfriada em gelo por 20 minutos e centrifugada a 4 °C, 5.000 rpm (RCF: 3001xg), por 15 minutos. O sobrenadante foi descartado e o sedimento ressuspenso em água estéril

gelada (7 °C) e centrifugado novamente. Após duas lavagens, o sedimento celular final foi ressuspenso em 1 mL de glicerol 10% gelado (7 ^{0}°C) e essa suspensão foi distribuída em alíquotas de 40 µl e armazenadas a -80 °C até sua utilização nos experimentos de eletroporação.

As alíquotas de 40 µl foram descongeladas, misturadas (aspiração/expiração) com 1 µl da suspensão do vetor pGFPuv (Clontech Laboratories, Inc, USA) e transferidas para cubetas de eletroporação resfriadas. A transformação foi feita no equipamento para eletroporação (Eletroporador MicroPulser Bio-Rad) programado para pulsos a 2,5 kV/cm. Finda a operação, a cubeta foi removida do aparelho, e após a adição de 1 mL de caldo TSB, o conteúdo foi transferido para um tubo de ensaio e incubado a 37 °C, com agitação a 100 rpm por 1 hora. Em seguida, aproximadamente 100 µl da suspensão de bactérias foram transferidas para placas com ágar MLCB (Mannitol Lysine Crystal Violet Brilliant Green Agar, Oxoid*)* contendo carbenicilina (50 µg/mL) e incubadas a 37 °C por 18-24h. Colônias resistentes ao antibiótico e que apresentavam cor verde fluorescente sob luz ultra-violeta (UV, 366 nm) (Figura 2) foram consideradas transformadas e foram selecionadas para os testes posteriores. As sem fluorescência verde foram também semeadas em placas com ágar MLCB contendo o antibiótico, como controle negativo.

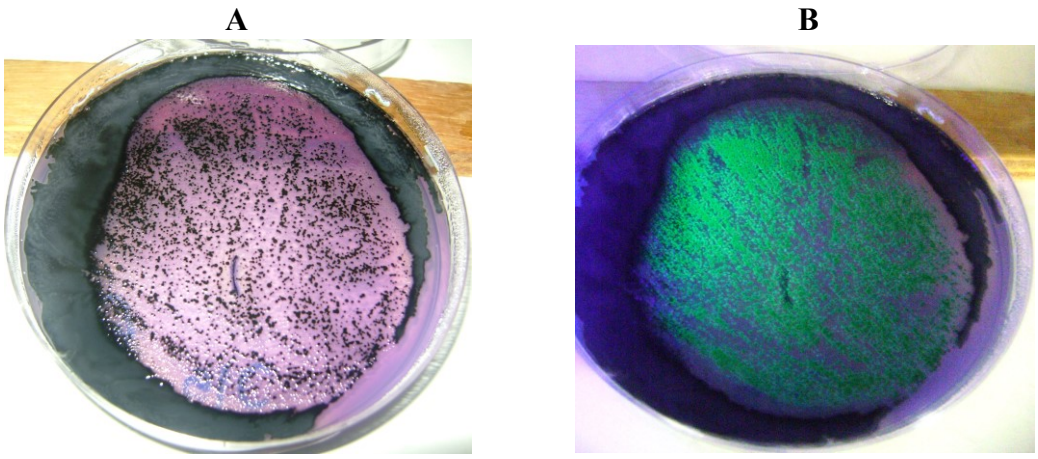

Figura 2. Placas apresentando colônias de *S.* Typhimurium eletroporadas com o plasmídio pGFPuv, expostas à luz comum (**A**) e à luz UV (**B**).

A estabilidade do plasmídio nas células transformadas foi avaliada através da inoculação de 2-3 colônias em 10 mL de caldo TSB, incubadas a 37 °C por 18-24

h, semeadas em MLCB (com e sem antibiótico) e incubadas a 37 °C por 18-24 h para observação do crescimento de colônias.

*2.2 A*valiação da influência dos parâmetros de processamento sobre a eficiência da inativação de *Salmonella* na etapa de lavagem e desinfecção de alfaces.

2.2.1 Contaminação experimental das alfaces

A contaminação experimental das alfaces foi realizada com as cepas de *S.* Typhimurium contendo o plasmídio pGFPuv, cultivadas em 100 mL de caldo TSB acrescentado de carbenicilina (50 µg/mL) a 37 °C por 24 h, submetidas a três lavagens consecutivas com água peptonada (centrifugação a 4 °C por 10 min a 2810 *x*g) e ajustadas de forma a se obter uma suspensão com 10^8 UFC/mL. Esta suspensão foi adicionada à água destilada estéril usada para a contaminação experimental dos vegetais. As alfaces a serem contaminadas foram previamente lavadas em água corrente e cortadas com faca estéril em pedaços de aproximadamente 3 x 3 cm. Os pedaços foram imersos em 4 litros de água destilada contaminada com um *pool* das três cepas em estudo e mantidos por 30 minutos para adesão do patógeno à superfície.

2.2.2 Proporção entre volume de água e quantidade de alfaces imersas

O primeiro parâmetro avaliado foi a proporção entre o volume de água empregada para a desinfecção e a quantidade de alfaces imersas nesta água, reproduzindo todas as proporções observadas nos tanques de desinfecção nas 10 indústrias de processamento visitadas (1 Kg vegetal para 5 a 28 litros de água), descritas no Capítulo 1. Os vegetais experimentalmente contaminados (10^6 UFC/g) foram transferidos para recipientes contendo solução de dicloroisocianurato de sódio (Sumaveg, Johnson Diversey) a 250 mg/L, correspondente à concentração máxima permitida para higienização de vegetais (SÃO PAULO, 2013). Após 15 min de contato, os vegetais foram centrifugados em centrifuga manual previamente higienizada. Foram coletadas de 5 a 10 amostras de 25 g de cada alface, que foram homogeneizadas com 225 mL de água peptonada 0,1% (diluição inicial) e submetidas a diluições decimais seriadas, empregando o mesmo diluente. A

enumeração de *Salmonella* foi feita através da semeadura das diluições em Ágar MLCB contendo carbenicilina (1 µl/mL), com incubação a 37 °C por 24 h. Os resultados foram expressos em UFC/g.

2.2.3 Parâmetros da água de desinfecção

Para avaliar a influência dos parâmetros da água de desinfecção na inativação de *S.* Typhimurium foram realizados alguns experimentos de triagem para determinar quais as variáveis críticas, que podem exercer influência na eficiência do processo de desinfecção. Para isso, foi realizado um delineamento fatorial 2^4, compreendendo 16 tratamentos (Tabela 1), combinando as variáveis pH (5,6 e 8,0), matéria orgânica (2,3 e 13,5 mg/L), concentração de dicloroisocianurato de sódio (50 e 250 mg/L) e tempo de contato dos vegetais com a solução de desinfecção (2 e 20 minutos). Os experimentos foram realizados em duas condições de temperatura da água de desinfecção: ambiente (25 °C) e refrigeração (10 °C), com 16 ensaios para cada temperatura. Os valores testados correspondem ao maior (+1) e menor (-1) valor das variáveis obtidas nos tanques de desinfecção das indústrias visitadas.

As alfaces experimentalmente contaminadas (10^6 UFC/g) foram transferidas para recipientes contendo a solução de desinfecção nas condições definidas no delineamento experimental. Para cada condição testada, cinco amostras de 25 g foram coletadas, centrifugadas na centrifuga manual para remoção do liquido, e homogeneizadas com 225 mL de água peptonada 0,1% (diluição inicial) e submetidas a diluições decimais seriadas. A enumeração de *Salmonella* foi feita através da semeadura das diluições em Ágar MLCB contendo carbenicilina (1 µl/mL), com incubação a 37 °C por 24 h e os resultados foram expressos em UFC/g. Para ajuste do pH das soluções de desinfecção foram utilizados ácido clorídrico (HCl 1N) e hidróxido de sódio (NaOH 1N). A matéria orgânica adicionada à água (2,3 e 13,5 mg/L) consistiu de uma pasta de vegetais triturados previamente preparada. Os recipientes contendo a solução sanitizante nas condições testadas foram mantidos em temperatura controlada a 25 e 10 °C.

2.3 Análise estatística

A análise estatística dos dados foi realizada com o auxílio dos softwares Sigma Stat, versão 3.11 (Systat Software, USA) e Statistica 12.0 (Stat Software, USA) com intervalo de confiança de 95% (p<0,05). Os valores foram submetidos a testes estatísticos apropriados, de acordo com a distribuição das variáveis.

3. Resultados e discussão

Os resultados indicaram que a proporção entre volume de água e quantidade de alface imersa nesta água não influenciou significativamente (p>0.05) na redução das contagens de *Salmonella* nos pedaços de alface. As dez diferentes proporções testadas (1 Kg vegetal para 5 a 28 L de água), empregando-se a mesma concentração de dicloroisocianurato de sódio (250 mg/L) e o mesmo tempo de contato (15 min), apresentaram redução de aproximadamente 2 log (dados não apresentados). Dessa forma, a proporção a ser utilizada nos experimentos seguintes foi estabelecida como 1:10, ou seja, 1 kg alface para 10 L de água ou 100g alface para 1 L de água. Esta proporção atende a recomendação feita por Cenci et al. (2006), de 5 a 10 L água/Kg de produto.

Em relação aos parâmetros físico-químicos da água de desinfecção, a Tabela 1 apresenta as contagens de *Salmonella* e reduções decimais obtidas nas diferentes condições testadas na triagem experimental. Os resultados revelaram que a condição de número 03 (pH: 5,6, matéria orgânica: 13,5 mg/L, dicloroisocianurato de sódio: 50 mg/L por 2 min a 25 °C) ocasionou a menor redução de contagem: 0,5 log enquanto que a condição de número 14 (pH: 8,0, matéria orgânica: 2,3 mg/L, dicloroisocianurato de sódio: 250 mg/L por 20 min a 10 °C) ocasionou o maior nível de redução: 2,0 log. Observa-se que enquanto a condição n.03 consistiu em pH baixo (ácido), alta concentração de matéria orgânica e baixa concentração de dicloroisocianurato de sódio por pouco tempo de contato, a condição 14 foi exatamente o oposto, ou seja, pH alto (alcalino), baixa concentração de matéria orgânica, alta concentração de dicloroisocianurato de sódio e maior tempo de contato.

O efeito das variáveis estudadas na redução das contagens de *Salmonella* está apresentado nas Tabelas 2 a 5. Com base nos resultados da análise de

variância, e considerando significativos os parâmetros com p≤0,05, observou-se que somente a concentração de dicloroisocianurato de sódio apresentou influência significativa na redução da contagem (p= 0.04).

Para fins de construção do modelo de avaliação quantitativa de risco microbiológico proposto como objetivo desta tese, selecionou-se as condições de processamento que apresentaram maior e menor nível de redução decimal (planejamento experimental ns. 03 e 14) para realização dos testes de contaminação cruzada, apresentados no Capítulo 3.

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Tabela 1. População de *Salmonella* nas amostras de alface submetidas à desinfecção nas condições estabelecidas no delineamento experimental.

Tratamento	Parâmetros da água de desinfecção				Temperatura ambiente (25 °C)		Temperatura de refrigeração (10 °C)	
	pH	Matéria orgânica (mg/L)	Concentração de cloro (mg/L)	Tempo de contato (min)	Resposta* (log UFC/g)	Redução (log)	Resposta* (log UFC/g)	Redução (log)
1	-1 (5,6)	-1 (2,3)	-1 (50)	-1(2)	5,26±0,3	0,7	5,17±0,1	0,8
2	1 (8,0)	-1 (2,3)	-1 (50)	-1(2)	4,73±0,1	1,2	4,69±0,1	1,3
3	-1 (5,6)	1 (13,5)	-1 (50)	-1(2)	5,53±0,1	**0,5**	4,93±0,1	0,7
4	1 (8,0)	1 (13,5)	-1 (50)	-1(2)	5,10±0,1	0,9	4,98±0,2	1,0
5	-1 (5,6)	-1 (2,3)	1 (250)	-1(2)	4,81±0,1	1,2	4,32±0,2	1,7
6	1 (8,0)	-1 (2,3)	1 (250)	-1(2)	4,62±0,1	1,4	4,29±0,1	1,7
7	-1 (5,6)	1 (13,5)	1 (250)	-1(2)	4,82±0,1	1,2	4,59±0,2	1,4
8	1 (8,0)	1 (13,5)	1 (250)	-1(2)	4,65±0,1	1,3	4,33±0,2	1,7
9	-1 (5,6)	-1 (2,3)	-1 (50)	1(20)	5,23±0,2	0,7	5,13±0,1	1,0
10	1 (8,0)	-1 (2,3)	-1 (50)	1(20)	4,44±0,3	1,2	4,60±0,3	1,4
11	-1 (5,6)	1 (13,5)	-1 (50)	1(20)	5,39±0,1	0,6	5,24±0,1	0,9
12	1 (8,0)	1 (13,5)	-1 (50)	1(20)	5,06±0,1	0,9	4,90±0,1	1,1
13	-1 (5,6)	-1 (2,3)	1 (250)	1(20)	4,78±0,1	1,2	4,32±0,2	1,7
14	1 (8,0)	-1 (2,3)	1 (250)	1(20)	4,51±0,1	1,5	3,99±0,3	**2,0**
15	-1 (5,6)	1 (13,5)	1 (250)	1(20)	4,77±0,1	1,3	4,35±0,3	1,6
16	1 (8,0)	1 (13,5)	1 (250)	1(20)	4,63±0,1	1,5	4,00±0,1	1,8

*Resposta expressa como média da contagem de 5 amostras (25g) de vegetais por condição testada.

Tabela 2. ANOVA fatorial dos parâmetros testados para alface crespa minimamente processada em água a 25 °C.

Parâmetros	Soma dos quadrados	Graus de liberdade	Quadrado médio	F-calc	p-valor
pH	0,5075	1	0,5075	157,17	0,0506
Matéria orgânica (mg/L)	0,1556	1	0,1556	48,19	0,0910
Concentração cloro (mg/L)	**0,6155**	**1**	**0,6155**	**190,61**	**0,0460**
Tempo de contato (min)	0,0325	1	0,0325	10,08	0,1942
1*2	0,0323	1	0,0323	10,02	0,1947
1*3	0,1073	1	0,1073	33,24	0,1093
1*4	0,0031	1	0,0031	0,97	0,5041
2*3	0,1004	1	0,1004	31,12	0,1129
2*4	0,0026	1	0,0026	0,81	0,5332
3*4	0,0044	1	0,0044	1,39	0,4474
1*2*3	0,0111	1	0,0111	3,44	0,3146
1*2*4	0,0138	1	0,0138	4,27	0,2866
1*3*4	0,0006	1	0,0006	0,19	0,7360
2*3*4	0,0002	1	0,0002	0,09	0,8137

Tabela 3. Efeito das variáveis testadas para alface crespa minimamente processada em água a 25 °C.

Parâmetros	Efeito	Erro padrão	t-valor	p-valor	Lim. de conf. -95%	Lim. de conf. +95%
pH	-0,35	0,02	-12,5	0,0506	-0,71	0,00
Matéria orgânica (mg/L)	0,19	0,02	6,94	0,0910	-0,16	0,55
Concentração cloro (mg/L)	**-0,39**	**0,02**	**-13,8**	**0,0460**	**-0,75**	**-0,03**
Tempo de contato (min)	-0,09	0,02	-3,17	0,1942	-0,45	0,27
1*2	0,08	0,02	3,16	0,1947	-0,27	0,45
1*3	0,16	0,02	5,76	0,1093	-0,19	0,52
1*4	-0,02	0,02	-0,98	0,5041	-0,38	0,33
2*3	-0,15	0,02	-5,57	0,1129	-0,51	0,20
2*4	0,02	0,02	0,90	0,5332	-0,33	0,38
3*4	0,03	0,02	1,18	0,4474	-0,32	0,39
1*2*3	-0,05	0,02	-1,85	0,3146	-0,41	0,30
1*2*4	0,05	0,02	2,06	0,2866	-0,30	0,41
1*3*4	0,01	0,02	0,44	0,7360	-0,34	0,37
2*3*4	-0,00	0,02	-0,30	0,8137	-0,36	0,35

Tabela 4. ANOVA fatorial dos parâmetros testados para alface crespa minimamente processada em água a 10 °C.

Parâmetros	Soma dos quadrados	Graus de liberdade	Quadrado médio	F-calc	p-valor
pH	0,3504	1	0,3504	13,49	0,1692
Matéria orgânica (mg/L)	0,0526	1	0,0526	2,02	0,3899
Concentração cloro (mg/L)	1,7838	1	1,7838	68,67	0,0764
Tempo de contato (min)	0,0258	1	0,0258	0,99	0,5007
1*2	0,0197	1	0,0197	0,75	0,5436
1*3	0,0115	1	0,0115	0,44	0,6255
1*4	0,0334	1	0,0334	1,28	0,4600
2*3	0,0023	1	0,0023	0,08	0,8154
2*4	0,0000	1	0,0000	0,00	0,9901
3*4	0,0701	1	0,0701	2,70	0,3479
1*2*3	0,0713	1	0,0713	2,74	0,3456
1*2*4	0,0026	1	0,0026	0,10	0,8034
1*3*4	0,0000	1	0,0000	0,00	0,9777
2*3*4	0,0187	1	0,0187	0,72	0,5513

Tabela 5. Efeito das variáveis testadas para alface crespa minimamente processada em água a 10 °C.

Parâmetros	Efeito	Erro padrão	t-valor	p-valor	Lim. de conf. -95%	Lim. de conf. +95%
pH	-0,29	0,08	-3,67	0,1692	-1,31	0,72
Matéria orgânica (mg/L)	0,11	0,08	1,42	0,3899	-0,90	1,13
Concentração cloro (mg/L)	-0,66	0,08	-8,28	0,0764	-1,69	0,35
Tempo de contato (min)	-0,08	0,08	-0,99	0,5007	-1.10	0,94
1*2	0,07	0,08	0,87	0,5436	-0,95	1,09
1*3	0,05	0,08	0,66	0,6255	-0,97	1,07
1*4	-0,09	0,08	-1,13	0,4600	-1,11	0,93
2*3	-0,02	0,08	-0,29	0,8154	-1,04	0,99
2*4	0,00	0,08	0,01	0,9901	-1,02	1,02
3*4	-0,13	0,08	-1,64	0,3479	-1,15	0,89
1*2*3	-0,13	0,08	-1,65	0,3456	-1,15	0,89
1*2*4	-0,02	0,08	-0,31	0,8034	-1,04	0,99
1*3*4	-0,00	0,08	-0,03	0,9777	-1,02	1,02
2*3*4	-0,06	0,08	-0,85	0,5513	-1,09	0,95

CAPÍTULO 3

Assessing the effect of sodium dichloroisocyanurate concentration on transfer of *Salmonella enterica* serotype Typhimurium in wash water for production of minimally processed iceberg lettuce (*Lactuca sativa* L)

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Significance and impact of the study

In this study, the impact of sodium dichloroisocyanurate in the wash water on transfer of *Salmonella* Typhimurium from inoculated lettuce to wash water and then to other non-inoculated lettuces washed sequentially in the same water was evaluated. The use of chlorinated water, at concentration above 10 mg l^{-1}, effectively prevented *Salm*. Typhimurium transfer under several different washing scenarios. Conversely, when non-chlorinated water was used, *Salm*. Typhimurium transfer occurred in up to at least ten non-inoculated batches of lettuce washed sequentially in the same water.

Abstract

This study evaluated the impact of sodium dichloroisocyanurate (5, 10, 20, 30, 40, 50 and 250 mg l^{-1}) in wash water on transfer of *Salmonella* Typhimurium from contaminated lettuce to wash water and then to other non-contaminated lettuces washed sequentially in the same water. Experiments were designed mimicking conditions commonly seen in minimally processed vegetables (MPV) processing plants in Brazil. Scenarios were: 1) Washing one inoculated lettuce portion in non-chlorinated water, followed by washing ten non-inoculated portions sequentially. 2) Washing one inoculated lettuce portion in chlorinated water followed by washing five non-inoculated portions sequentially. 3) Washing five inoculated lettuce portions in chlorinated water sequentially, followed by washing five non-inoculated portions sequentially. 4) Washing five non-inoculated lettuce portions in chlorinated water sequentially, followed by washing five inoculated portions sequentially and then by washing five non-inoculated portions sequentially in the same water. *Salm*. Typhimurium transfer from inoculated lettuce to wash water and further dissemination to non-inoculated lettuces occurred when non-chlorinated water was used (scenario 1). When chlorinated water was used (scenarios 2, 3 and 4) no measurable *Salm*. Typhimurium transfer occurred if the sanitizer was ≥10 mg l^{-1}. Use of sanitizers in correct concentrations is important to minimize the risk of microbial transfer during MPV washing.

Keywords: *Salmonella*, disinfection, food safety, minimally processed vegetables, bacterial transfer, cross contamination.

Introduction

Fruits and vegetables are an important part of a healthy diet. Consumers have been encouraged to consume healthy and convenient foods, which has been reflected in an increased demand for minimally processed vegetables (MPV). MPV may be defined as fresh vegetables that have been subjected to minimal processing, such as peeling, cutting, slicing, shredding, washing, drying, packaging and refrigerated storage (CAC 2003). MPV provide convenience to consumers while maintaining shelf life and preserving the nutritive and sensorial properties of the food.

Concurrent with the increase in production and consumption, MPV have been associated with foodborne disease outbreaks, becoming a major concern for **c**onsumers, governments and the food industry. A number of foodborne illnesses associated to fresh produce have been reported in several countries (Berger *et al*. 2010; Callejón *et al*. 2015; CDC 2015).

Washing is an important step during minimal processing of vegetables as it can reduce microbial populations and remove dirt and debris. Wash water can however become a source of cross contamination, and pathogenic microorganisms can be spread throughout a batch of MPV. This study aimed to evaluate the transfer of *Salmonella* during the washing step for production of minimally processed iceberg lettuce (*Lactuca sativa* L**)**, simulating different processing scenarios using chlorinated and non-chlorinated water. The selected scenarios indicate probable cross contamination pathways and reflect washing practices commonly seen in MPV processing plants in Sao Paulo, Brazil (unpublished data), where different batches of vegetables are washed sequentially in the same water. *Salmonella* was selected as it has caused several outbreaks associated with the consumption of fresh and minimally processed vegetables (Lynch *et al*. 2009; da Silva Felício *et al*. 2015; Vestrheim *et al*. 2015), and lettuce was used as MPV because this is the most frequently consumed leafy vegetable in Brazil (Anon 2013).

Results and Discussion

Figure 1 shows that washing one lettuce portion containing 6.6±0.1 log CFU g^{-1} of *Salmonella enterica* serotype Typhimurium in non-chlorinated wash water (scenario 1) resulted in contamination of the water such that subsequent washing of non-inoculated lettuces in this water resulted in counts of 5.4±0.2 log CFU g^{-1} in the first washed lettuce portion. Subsequently washed portions also contained high *Salm*. Typhimurium levels (3.7±0.1 to 4.3±0.1 log CFU g^{-1}). After the wash water became contaminated with *Salm*. Typhimurium, the counts in the water remained ca. 4.8±0.1 log CFU ml^{-1} until the last (10^{th}) portion of lettuce was washed (Figure 1). These results highlight the potential survival of this bacterium in the water and further transfer to incoming non-inoculated vegetables.

Similar findings were reported in other studies. Holvoet *et al*. (2014) observed that washing lettuce portions contaminated with *Escherichia coli* (4.0 log CFU g^{-1}) in two subsequent non-chlorinated water baths caused a rapid transfer and high level of this bacterium in the water (4.0 and 3.5 log CFU per 100 ml in the first and second bath, respectively), and the artificially contaminated water (3.0, 4.0 and 5.0 log CFU per 100 ml) caused a cross contamination in the subsequently washed lettuce portions in levels of ca. 1.0 up to 1.9 log CFU g^{-1}. These authors also noticed a limited reduction of *E.coli* after washing a contaminated lettuce portion ca. 4.0 log CFU g^{-1} in both baths of potable water (0.33±0.1 and 0.16±0.1 log reduction). Jensen *et al*. (2015) conducted experiments washing lettuce leaf pieces (one contaminated and ten non-contaminated) simultaneously in non-chlorinated wash water and observed that most *E.coli* O157:H7 artificially inoculated in the contaminated samples were transferred to the wash water (90-99%), and this contaminated water cross contaminated all the initially non-contaminated lettuce pieces.

Recent studies have focused on evaluation and modelling of the transfer of pathogenic bacteria (*Salmonella* and *E.coli*), norovirus and phages from contaminated to non-contaminated portions of vegetables during washing (Danyluk and Schaffner 2011; Holvoet *et al*. 2014; Perez-Rodriguez *et al*. 2014; Jensen *et al*. 2015). Although these studies shed light on the degree of pathogen transfer and mechanisms involved in the process, only few studies (López-Gálvez *et al*. 2009; Zhang *et al*. 2009; López-Gálvez *et al*. 2010; Tomás-Callejas *et al*. 2012) have

considered the impact of disinfecting agents added to the wash water on cross contamination, especially regarding *Salmonella*.

López-Gálvez *et al*. (2009) evaluated the risk of cross contamination by non-pathogenic *E.coli* during washing of minimally processed lettuce using sodium hypochlorite (40 mg l^{-1} of free chlorine), Tsunami (500 mg l^{-1}), Citrox (5000 mg l^{-1}) and Purac (20,000 mg l^{-1}). They observed that chlorine and Tsunami were effective in reducing the inoculum levels in the processing water to the detection limit, but Citrox and Purac were not effective even at the highest manufacturer's recommended doses. In another study, López-Gálvez *et al*. (2010) observed that a sanitation step using active chlorine dioxide (3 mg l^{-1}) or sodium hypochlorite (100 mg l^{-1} of free chlorine) applied to minimally processed lettuce cross contaminated by *E.coli* during washing, did not inactivate *E. coli* cells on the vegetable tissue, but inactivated most *E. coli* cells that passed from inoculated product to wash water.

Zhang *et al*. (2009) studied the efficacy of free chlorine and total peracid by using sodium hypochlorite (30 and 50 mg l^{-1}), peroxyacetic acid and mixed peracid (10, 20, and 30 mg l^{-1}) on reduction of *E.coli* O157:H7 in water containing or not 10% organic load. These researchers observed that all sanitizers were effective in reducing bacterial counts in processing water, and in reducing the transfer of the bacteria from an inoculated leaf to non-inoculated leaves in the processing water. Tomás-Callejas *et al*. (2012) studied the efficacy of sodium hypochlorite (free chlorine at 25 mg l^{-1}) and active chlorine dioxide (3 mg l^{-1}) to prevent *E.coli* O157:H7 and *Salmonella* cross contamination on minimally processed Baby Red Chard during washing-disinfection, rinsing and de-watering steps. No colonies of *E.coli* O157:H7 and *Salmonella* were recovered from the non-inoculated leaves regardless of the sanitizer used, but were detected by PCR-based methods. Furthermore, transference of viable *Salmonella* from inoculated leaves to the processing water was detected, with large populations recovered from the centrifugation effluent water.

All of the studies cited above evaluated the effect of different sanitizer (mainly sodium hypochlorite) on cross contamination during washing, and most mixing contaminated and non-contaminated vegetables simultaneously. The present study mimicked washing procedures observed in some Brazilian MPV processing plants, where subsequent portions of vegetables may be washed in the same water used to wash previous portions. If these previous portions are contaminated, cross contamination may occur among different batches of MPV. Additional washing

scenarios were also evaluated (scenarios 2, 3 and 4), using a chlorine-based sanitizer commonly used in Brazil (sodium dichloroisocyanurate), and not yet widely evaluated in the published literature (Figure 2).

In scenario 2, the initial counts of *Salm*. Typhimurium were reduced by 0.5 to 2.0 log depending on the concentration of the sanitizer in the wash water (Table 1). Counts before and after washing differed significantly ($p<0.05$; by paired t-test) for all chlorine concentration tested. As expected, the highest concentration (250 mg l^{-1} of free chlorine) was the most effective, confirmed by ANOVA with post-hoc Tukey's test. *Salm*. Typhimurium in the non-inoculated lettuce portions introduced in the water containing sodium dichloroisocyanurate was below the quantification and detection limit (<2 log CFU g^{-1} and absence in 25 g), regardless the concentration of the sanitizer. However, when the free chlorine concentration was only 5 mg l^{-1}, *Salm*. Typhimurium could not be counted (<2 log CFU g^{-1}) but was detected by enrichment (presence in 25 g) in the first non-inoculated lettuce portion entering the process.

In scenario 3, where five inoculated lettuce portions were washed in water containing 50 mg l^{-1} (washing condition 1) and 250 mg l^{-1} of free chlorine (washing condition 2), followed by washing of five non-inoculated portions, *Salm*. Typhimurium transfer was not observed (<2 log CFU g^{-1} and absence in 25 g after enrichment). These data indicate that even if additional contaminated lettuce portions are washed in the water in the beginning of the washing process, the chlorinated water (50 and 250 mg l^{-1}) effectively prevents *Salm*. Typhimurium transfer from the inoculated to the non-inoculated lettuce portions. The same results were obtained for scenario 4 (<2 log CFU g^{-1} of *Salm*. Typhimurium and absence in 25 g). In these scenarios (2, 3 and 4), *Salm*. Typhimurium was not recovered from water samples (<2 log CFU ml^{-1}).

Chlorine-based products for washing of vegetables have been used much more as a strategy to reduce microbial load in the produce than to avoid cross contamination (Gil *et al*. 2009). Chlorine is a very potent and low-cost sanitizer with robust oxidizing properties, recommended by health authorities around the world for disinfection of fruits and vegetables, usually at concentrations of 50-200 mg l^{-1} (Gil *et al*., 2009; Brazil 2013). However, the use of chlorinated water for production of RTE vegetables has limitations, such as inactivation of active agents by high level of organic load and risk of carcinogenic trihalomethanes formation, and dependence on neutral pH for optimal activity (FAO/WHO 2008).

Data presented in our study will be extremely useful for produce processors and regulators to aid in setting standards for sanitizer in wash water to avoid cross contamination. Our data will be especially useful for those considering use of sodium dichloroisocyanurate, as very few peer reviewed published articles have studied this compound for it's efficacy in fresh produce washing *(*Nicholl and Prendergast 1998; Nascimento *et al.* 2003*)*. Our study showed that the use of sodium dichloroisocyanurate in the wash-water, at concentrations at least 10 mg l^{-1}, effectively prevented *Salm*. Typhimurium transfer from inoculated lettuce to the wash-water and then to incoming non-inoculated lettuce leaves under several different washing scenarios. Conversely, when vegetables were washed in non-chlorinated water, cross contamination not only occurred, but also continued to occur over at least ten non-inoculated batches washed sequentially in the same water. These results highlight the potential survival of *Salm*. Typhimurium in the water and further transfer to incoming non-contaminated vegetables. Better control of MPV outbreaks can be expected if sanitizers in proper concentrations are applied during washing of MPV.

Materials and methods

Strains and electro-*transformation of Salmonella to* express Green Fluorescent Protein

The study was conducted with a cocktail of three carbenicillin sensitive *Salm*. Typhimurium strains: two strains (#277 and #386) isolated from MPV sold in Sao Paulo, Brazil (Sant'Ana *et al*. 2011) and one reference strain (ATCC 14028). The three strains were electro-transformed to express Green Fluorescent Protein (GFP) and resistance to ampicillin (Sambrook *et al*. 1989), to distinguish them from other bacteria possibly present in the vegetables. Before electroporation, strains were tested for sensitivity to carbenicillin by checking absence of growth inoculation in 10 mL of TSB containing carbenicillin (50 µg ml^{-1}), at 37 °C for 24 h.

Each strain was grown overnight at 37 °C in 10 mL of Tryptic Soy Broth (TSB) (Oxoid, Basingstoke, UK) and OD_{600nm} (Ultrospec 2000, Pharmacia Biotech, Cambridge, UK) was measured from time to time until proper dilution resulted in OD_{600nm} = 0.2. This culture was incubated at 37 °C under agitation at 170 rpm

(Innova 4000, New Brunswick Scientific, Edison, NJ) until OD_{600nm} = 0.5. After cooling to 4 °C, the culture was centrifuged at 3000 ×g for 15 min at 4 °C (6-16 K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The resulting pellet was washed twice in sterile cold water (10 °C), resuspended in 1 mL of 10% glycerol (Synth, Diadema, Brazil) and divided in 40 µL aliquots in sterile microcentrifuge tubes. The content of the tubes was mixed with 1 µL of the pGFPuv Vector plasmid (Clontech, Mountain View, CA) in a 0.2 cm gap width cuvette and an electric pulse of 2.5 kV cm^{-1} was applied (MicroPulser, Bio-Rad Laboratories, Hercules, CA) for electro-transformation. Sterile TSB was added to the cuvette and electro-transformed cells were incubated at 37 ºC for 1 h, and plated onto Mannitol Lysine Crystal Violet Brilliant Green Agar (MLCB) (Oxoid) supplemented with carbenicillin disodium salt (50 µg ml^{-1}) (Sigma Aldrich, Rehovot, Israel). *Salm*. Typhimurium colonies presenting fluorescence under UV light 366 nm were inoculated into 50 mL of TSB supplemented with carbenicillin (50 µg ml^{-1}), following incubation at 37 °C for 24 h. The cultures were frozen in microcentrifuge tubes (30% glycerol, 70% TSB) and stored -70 °C in an ultra-low-temperature freezer (Model 910, Thermo Fisher Scientific, Rockford, IL) until use. The plasmid stability of transformed *Salm*. Typhimurium strains was tested by growing them in 10 mL of TSB at 37 °C for 24 h, followed by plating on MLCB Agar with and without carbenicillin (50 µg ml^{-1}). Carbenicillin was required for expression of GFP, indicating that the plasmid of transformed *Salm*. Typhimurium strains was stable. Furthermore, the presence of the plasmid did not seem to affect the survival capacity and resistance of the transformed *Salm*. Typhimurium strains, since these were able to survive in non-chlorinated water even after washing up to ten batches of vegetables, and were not resistant in chlorinated water (i.e. inactivation was possible at low chlorine concentrations).

Experimental contamination of lettuce

The frozen cultures of electro-transformed *Salm*. Typhimurium strains were thawed, inoculated into 100 mL of TSB supplemented with carbenicillin (50 µg ml^{-1}) and incubated at 37 °C for 24 h. Cells were washed twice (2810 × g for 5 min at 4 ºC) and the pellet was suspended in 0.1% peptone (Oxoid, England). The *Salm*. Typhimurium cell suspensions were combined in equal volumes to achieve ca. 10^8 log CFU ml^{-1}. Exact counts were determined by enumeration in MLCB supplemented with carbenicillin (50 µg ml^{-1}).

Whole unprocessed heads of iceberg lettuce were purchased in local supermarkets in the city of Sao Paulo, Brazil, and kept under refrigeration no longer than 24 h until the experiments were performed. Injured or damaged leaves were removed and the remaining leaves were individually washed in running tap water provided by the Sao Paulo municipality and were cut (approximately 3 cm width) with a sterile knife. The washed lettuce pieces were experimentally contaminated with *Salm*. Typhimurium by immersion in 4 l of distilled water containing the cocktail of the three electro-transformed strains (10^6 CFU ml^{-1}) for 30 min. Tests for presence of *Salmonella* spp. in the incoming lettuce samples prior to inoculation were all negative (data not shown).

Scenarios simulating *Salmonella* transfer during washing of lettuce

The following washing scenarios were investigated (Figure 2): 1) Washing one inoculated lettuce portion in non-chlorinated water, followed by washing ten non-inoculated lettuce portions sequentially. 2) Washing one inoculated lettuce portion in chlorinated water followed by washing five non-inoculated lettuce portions sequentially. 3) Washing five inoculated lettuce portions in chlorinated water sequentially, followed by washing five non-inoculated lettuce portions sequentially. 4) Washing five non-inoculated lettuce portions in chlorinated water sequentially, followed by washing five inoculated lettuce portions sequentially and then by washing five non-inoculated lettuce portions sequentially in the same water. These scenarios indicate probable cross contamination pathways and reflect washing practices commonly seen in MPV processing plants in Sao Paulo, Brazil (unpublished data), where different batches of vegetables are washed sequentially in the same water.

The designed scenarios aimed to evaluate the consequence of washing one or more contaminated lettuce portions in the same water where non-contaminated portions are washed (at the beginning or during the process), as well to compare the cross contamination pathways using or not chlorinated water. The lettuce to wash water ratio was 1:10 (i.e. 100 g portions of shredded lettuce to 1 l of water), a proportion commonly used by Brazilian processing plants. Each lettuce portion represented a lot. Preliminary tests with larger amounts of lettuce (500 g) and wash water (5 l) resulted in the same *Salm*. Typhimurium transfer to the wash water and to the lettuce (data not shown). The chlorine sanitizer was sodium dichloroisocyanurate (Sumaveg, Johnson Diversey, Sao Paulo, SP, Brazil) which is widely used in Brazil instead of sodium hypochlorite, due to good stability in aqueous solution and greater efficacy in the presence of higher organic loads (Salomão *et al*. 2011). The free chlorine concentration in the wash water was measured and adjusted manually using a portable photometer (HI96771, Hanna Instruments, Ann Arbor, MI). Samples of water were collected in cuvettes (10 ml) and added of appropriate reagents (HI 95771-01) for the colorimetric determination of chlorine, following the manufacturer's instructions.

Two washing conditions were tested for each scenario: (1) wash water: pH 5.6, organic load 13.5 mg l^{-1} and free chlorine concentration 50 mg l^{-1}; contact: 2 min at 25 °C, and (2) wash water: pH 8.0, organic load 2.3 mg l^{-1} and free chlorine concentration 250 mg l^{-1}; contact: 20 min at 10 °C. These conditions represent minimum and maximum range of physical and chemical parameters observed during the washing step of vegetables in Brazilian processing plants (Maffei *et al*. 2016), and overlap with the Brazilian recommendations on the use of chlorine-based products for disinfection of fresh produce at maximum concentration of 250 mg l^{-1} of chlorine (Brazil 2013). The simulation of these washing parameters allowed evaluation of the occurrence of cross contamination under water conditions observed in washing procedures used in Brazilian processing plants. A preliminary study also assessed the influence of wash water parameters (pH, organic load, temperature, free chlorine concentration and time of contact) on inactivation of *Salm*. Typhimurium during washing step and indicated that chlorine concentration was the most important parameter for reduction of *Salm*. Typhimurium, while the other parameters were less relevant within the analyzed interval (data not shown). There is a number of publications on the influence of the physicochemical parameters of water on the

activity of disinfectants (Park *et al*. 2004; Pirovani *et al*. 2004; Stopforth *et al*. 2008; López-Gálvez *et al*. 2010). A meta-analysis conducted by Prado-Silva *et al*. (2015) including data from 40 studies on the effect of sanitizing treatments of fresh produce concluded that in addition to chlorine concentration, parameters such as washing time and temperature can significantly affect the mean log reduction of sanitizing treatments.

Scenario 2 was selected to perform an extra washing condition with lower chlorine concentrations (5, 10, 20, 30 and 40 mg l^{-1}) and physicochemical parameters selected to provide a better efficacy of the sanitizer: wash water at 25 °C, with a pH 7.0, no organic load and a contact time of 10 min.

Enumeration of *Salmonella*

The washed shredded lettuce was transferred to a sanitized salad spinner (SecaSaladaPlick, Sao Paulo, Brazil) and spun for 1 min inside a biosafety laminar hood (VLFS-12, Veco, Campinas, SP, Brazil) to remove excess water. At least two 25 g samples of each washed portion were homogenized with 225 mL of 0.1% peptone for 1 minute in a Stomacher 400 Lab-blender (Seward Medical, London, England). Serial decimal dilutions were prepared in 0.1% peptone and plated onto MLCB Agar supplemented with carbenicillin (50 µg ml^{-1}). Plates were incubated at 37 °C for 24 h and typical *Salm*. Typhimurium colonies expressing GFP under UV light 366 nm were counted. Additionally, another 25 g sample of each portion of washed lettuce was homogenized with 225 mL of Buffered Peptone Water (BPW) (Difco, Sparks, Md., USA) and tested for *Salmonella* according to ISO 6579 (2002), adding carbenicillin (50 µg ml^{-1}) to all culture media. Results were expressed as presence or absence of *Salmonella* in 25 g of lettuce.

Enumeration of *Salm*. Typhimurium in wash water (50 ml) was done by plating onto MLCB Agar supplemented with carbenicillin (50 µg ml^{-1}) and incubated at 37 °C for 24 h. The sanitizer in wash water was inactivated by addition of 1 mol l^{-1} sterile sodium thiosulfate (Sigma-Aldrich ChemieGmbHSteinheim, Germany) after chlorine treatment, before plating.

Data analysis

All experiments were repeated at least twice, and plating was done in duplicate. Average counts of *Salm*. Typhimurium in lettuce and wash water were expressed as CFU g^{-1} or CFU ml^{-1}, respectively, and log transformed. A paired t-test was used to determine whether counts of *Salm*. Typhimurium before and after washing differed significantly ($p<0.05$). ANOVA followed by Tukey's test were performed to determine significant differences in *Salm*. Typhimurium counts in lettuces washed in wash water with different chlorine concentrations. SigmaPlot version 12.5 ((Systat Software Inc., San Jose, CA) was used for the statistical analysis.

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Conflict of interest

No conflict of interest declared.

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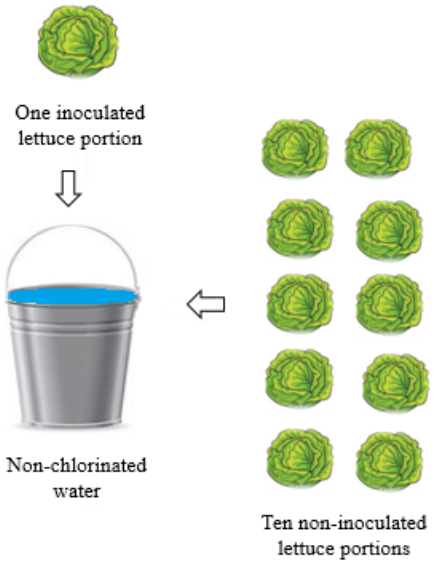
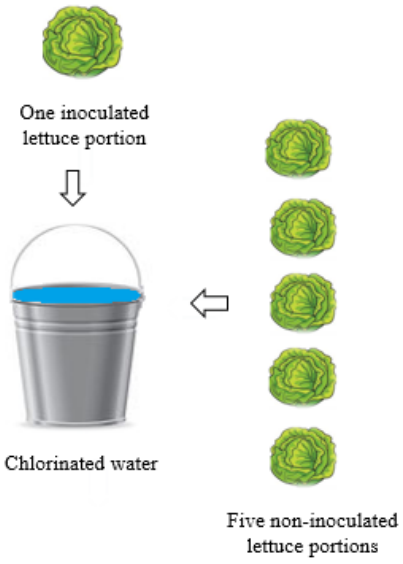
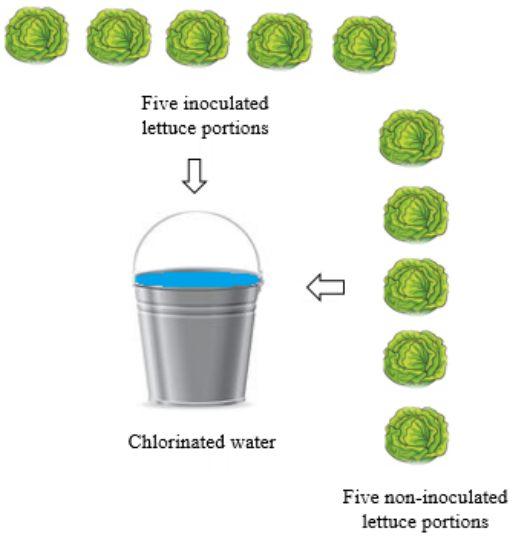
Scenario i) Washing one inoculated lettuce portion in non-chlorinated water, followed by washing ten non-inoculated lettuce portions sequentially.



Scenario ii) Washing one inoculated lettuce portion in chlorinated water followed by washing five non-inoculated lettuce portions sequentially.



Scenario iii) Washing five inoculated lettuce portions in chlorinated water sequentially, followed by washing five non-inoculated lettuce portions sequentially.



Scenario iv) Washing five non-inoculated lettuce portions in chlorinated water sequentially, followed by washing five inoculated lettuce portions sequentially and then by washing five non-inoculated lettuce portions sequentially.

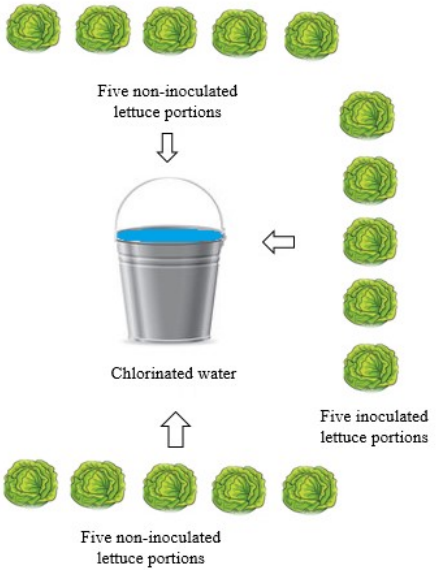


Figure 1. Scenarios simulating *Salmonella* Typhimurium transfer during washing of lettuce. See text for more details.

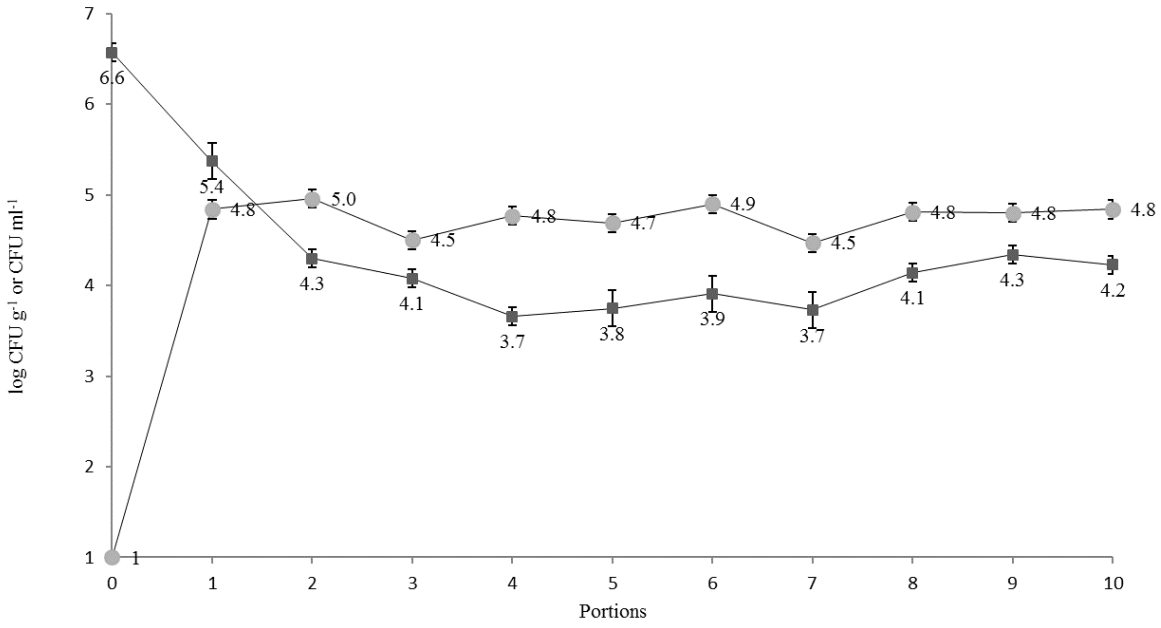


Figure 2. Transfer of *Salmonella* Typhimurium to ten consecutive portions of lettuce washed in non-chlorinated water contaminated with the pathogen by washing one lettuce portion containing ca. 10^6 CFU g^{-1}. Counts in log CFU g^{-1} for lettuce (—■—) and log CFU ml^{-1} for water (—●—).

Table 1. *Salmonella* counts in lettuces after washing in water with different chlorine concentrations (scenario 2).

Free chlorine concentration ($mg\ l^{-1}$)	Counts of *Salmonella* ($\log CFU\ g^{-1}$)						
	Inoculated lettuce before washing	Inoculated lettuce after washing	1st non-inoculated lettuce portion	2nd non-inoculated lettuce portion	3rd non-inoculated lettuce portion	4th non-inoculated lettuce portion	5th non-inoculated lettuce portion
250*	6.2±0.1	4.2±0.3	<2	< 2	< 2	< 2	< 2
50**	6.1±0.1	5.3±0.3	< 2	< 2	< 2	< 2	< 2
40***	5.7±0.1	4.9±0.1	< 2	< 2	< 2	< 2	< 2
30***	5.8±0.2	5.0±0.1	< 2	< 2	< 2	< 2	< 2
20***	6.1±0.1	5.4±0.1	< 2	< 2	< 2	< 2	< 2
10***	5.7±0.1	5.0±0.1	< 2	< 2	< 2	< 2	< 2
5***	5.9±0.1	5.4±0.1	< 2	< 2	< 2	< 2	< 2

Results expressed as mean±SD.
S. Typhimurium was detected (presence in 25 g, after enrichment step) in the 1st non-inoculated lettuce portion only when the chlorine concentration in water was 5 mg l^{-1}
* Wash water: pH 8.0, organic load 2.3 mg l^{-1}; contact: 20 min at 10 °C
** Wash water: H 5.6, organic load 13.5 mg l^{-1}; contact: 2 min at 25 °C
*** Wash water: pH 7.0, no organic load; contact: 10 min at 25 °C

CAPÍTULO 4

Quantitative assessment of the impact of cross-contamination during the washing processing step of ready-to-eat vegetables on the risk of illness caused by *Salmonella*

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Abstract

The purpose of this study was to develop a quantitative microbial risk assessment (QMRA) model to estimate the risk of illness caused by *Salmonella* in ready-to-eat vegetables, based on common practices in Brazilian processing plants. The risk assessment model was composed of five modules: in field, washing, retail storage, home storage and dose-response. Fifty thousand iterations of a @Risk model built in Excel were run for each of sixty scenarios. These scenarios considered different initial pathogen concentrations, fractions of contaminated produce and chlorine concentrations. For chlorine, seven fixed concentrations (0, 5, 10, 25, 50, 150 and 250 ppm) and three triangular distributions were considered [RiskTriang(0,5,10 ppm), RiskTriang(0,80,250 ppm) and RiskTriang(10,120,250 ppm)]. The outputs were risk of infection, number of illnesses and percent of illness arising from cross-contamination. The QMRA model indicated quantitatively that higher chlorine concentrations resulted in lower risk of illness. When simulation was done with less than 5 ppm of chlorine, most (>96%) of the illnesses arose from cross-contamination, but when chlorine concentration was 50 ppm or higher, no illnesses arising from cross-contamination were predicted. Proper control of sanitizer concentration is mandatory to reduce initial contamination and avoid cross-contamination during washing of vegetables, reducing the risk of illness.

Keywords: risk assessment, *Salmonella*, ready-to-eat vegetables, cross-contamination, produce washing.

1. Introduction

An increased number of foodborne disease outbreaks have been associated with fresh and fresh-cut produce during the past decade concomitant with an increased consumption of these products (Doyle and Erickson, 2008; Jung et al., 2014). Ready-to-eat (RTE) fresh-cut produce is often consumed raw and typically requires no further preparation before consumption, increasing risk of infection if contaminating organisms are present (Berger et al., 2010).

Salmonella is the most common foodborne pathogen in Brazil, accounting for ~38% of reported foodborne disease outbreaks occurring between 2000 and 2014 (Anonymous, 2014). Despite the lack of information associating outbreaks to consumption of vegetables, this bacterium is a frequent contaminant of RTE vegetables marketed in Brazil (Froder et al., 2007; Maistro et al., 2012; Oliveira et al., 2011; Sant'Ana et al., 2011).

Washing is an important step to reduce microbial populations and remove dirt and debris during production of RTE vegetables. Wash water can be a source of cross-contamination if the water is not properly disinfected (Gómez-López et al., 2015). Previous studies have demonstrated that pathogenic microorganisms such as *Escherichia coli* O157:H7 and *Salmonella* can be transferred from contaminated to non-contaminated vegetables during washing in the same water (Allende et al., 2008; Holvoet et al., 2014; Jensen et al., 2015; López-Gálvez et al., 2009; Luo et al., 2011; Perez-Rodriguez et al., 2014; Tomás-Callejas et al., 2012, Zhang et al., 2009). Danyluk and Schaffner (2011) developed a quantitative assessment of the microbial risk of leafy greens showing that 95% to 100% of the cases caused by *E. coli* 0157:H7 in the spinach outbreak occurred in the USA in 2006 could be explained by occurrence of cross-contamination.

Quantitative microbial risk assessment (QMRA) is a scientifically based process used to estimate the risk of foodborne disease and to support management decisions for the reduction of food safety risks (Boone et al., 2010). It typically consists of four steps: i) hazard identification; ii) exposure assessment; ii) hazard characterization (dose-response relationship) and iv) risk characterization (Codex Alimentarius Commission, 1999). The purpose of this study was to develop a quantitative microbial risk assessment model to estimate the impacts of cross-contamination

during vegetable washing on the microbial risk of illness by *Salmonella* in ready-to-eat vegetables based on typical Brazilian industry practices.

2. Materials and methods

The risk assessment model contains five modules or stages: (i) in field, (ii) washing step, (iii) retail storage, (iv) home storage and (v) consumption, dose-response and risk of infection by *Salmonella*. Table 1 provides an overview of the simulation variables and distributions used in the risk assessment model, discussed in detail in the Results and Discussion section. The first column indicates the spreadsheet cell reference of the variable on that line of the table. The second column describes the variable or section (in bold) describing each line of the risk assessment. The third column represents the value of the cells as a number, a formula, or a @Risk function. The fourth column shows the unit of the variable, and the last column indicates the source of the information used to establish the variable, based on user input, literature citation or calculated from other cells in the spreadsheet.

The risk assessment model was built in an Excel spreadsheet (Microsoft, Redmond, WA) and simulated using @Risk software version 6 (Palisade Corporation, Ithaca, NY). Fifty thousand iterations were run for each scenario using Latin Hypercube sampling and random number seed fixed at 1 to ensure that results could be directly compared. Pathogen starting concentration (0 and 1 log CFU/g), fraction of contaminated produce (0.01, 0.1 and 1.0%) and chlorine concentration were chosen to simulate sixty unique scenarios. Chlorine concentrations were defined using fixed concentrations (0, 5, 10, 25, 50, 150 and 250 ppm) and three different triangular distributions [RiskTriang(0,5,10 ppm), RiskTriang(0,80,250 ppm) and RiskTriang(10,120,250 ppm)].

3. Results and Discussion

The first section of Table1 (in field) describes the model for *Salmonella* in vegetables in the field. The model assumes that all contamination arises in the field. Variables were derived from user input (starting contamination level, number of days that the produce remains in the field after contamination and fraction of contaminated

produce) or determined by calculation (contamination level at harvest and fraction of non-contaminated produce). Starting prevalence and concentration of *Salmonella* in vegetables were assumed to be similar to those reported by Danyluk and Schaffner (2011) for *Escherichia coli* O157:H7 and represent worst-case assumptions. Data from Islam et al. (2004) regarding the persistence of *Salmonella* on lettuce grown in fields contaminated by poultry manure, two types of dairy manure or irrigation water were used to create a normal distribution to estimate the log reduction per day for *Salmonella* in the field.

For the second section in Table 1 (washing step), data extracted from Maffei et al. (2016a) on the quality of RTE vegetables in Brazilian processing plants were used to create a triangular distribution, with minimum, most likely and maximum chlorine concentrations during washing. Data from another study by Maffei et al. (2016b) on the effect of washing leafy greens in chlorinated water on transfer of *Salmonella* were re-analyzed to assess the relationship between chlorine concentration and log reduction of *Salmonella*, as well to evaluate the transfer rate of the pathogen from contaminated to non-contaminated leaves washed in the same water.

To evaluate the relationship between chlorine concentration and log reduction of *Salmonella,* data from Maffei et al. (2016b) were divided into two regions: <10 or ≥10 ppm of chlorine (Figure 1). The average log reduction values were predicted by linear regression as a function of chlorine concentration. The standard deviations from each log reduction were combined to create a standard deviation for all chlorine concentrations. The means obtained from linear regression and the overall standard deviation were used to create a normal distribution of log reduction as a function of chlorine concentration (Table 1).

Linear regression was used to predict upper and lower limits for cross-contamination for *Salmonella* transfer between contaminated and non-contaminated leaves, based on data published by Maffei et al. (2016b). Cross-contamination at 0 ppm of chlorine corresponded to the counts of *Salmonella* on lettuce samples. Cross-contamination at 5 ppm of chlorine corresponded to the limit of detection for counts (upper limit) and the enrichment detection limit (lower limit). Cross-contamination at 10 ppm of chlorine corresponded to the enrichment detection limit. A uniform distribution was used to estimate the cross contamination, where the upper and lower limits were based on the regression equations for upper and lower limits (Figure 2).

The level on contaminated and cross-contaminated portions after washing and chosen level were determined by calculation.

The third section in Table 1 (retail storage) represents the expected change in *Salmonella* level during retail storage. Maistro et al. (2012) observed that the storage temperature of minimally processed vegetables sold in Brazilian supermarkets ranged from 8.1 to 11.3 °C, with standard deviations ranging from 1 to 2.7 °C. These results were used to calculate the mean of a normal distribution for retail temperature and standard deviation.

Data from Maffei et al. (2016a) for the labeled shelf life of leafy green vegetables produced by Brazilian processing plants were used to represent retail storage time, via a triangular distribution. The growth rate of *Salmonella* as a function of temperature was determined using data from ComBase Predictor [http://modelling.combase.cc/] with the following assumptions: pH 6.8, a_w 0.995, CO_2 15% and temperature 7, 10, 13 and 16 °C. The pH, a_w and CO_2 parameters were those specified by Sant'Ana et al. (2012). Change of level of contamination during retail storage and level after retail storage were determined by calculation.

The fourth section in Table 1 (home storage) represents the expected change in *Salmonella* level during storage at the consumer's home. Data from Marklinder et al. (2004) and Silva et al. (2008) were considered for use in setting home storage temperature. Marklinder et al. (2004) measured the temperature at which RTE salads were stored in Swedish households and modeled those data using a Gamma distribution (7.15,1.03). Silva et al. (2008) collected data on the temperature of domestic refrigerators in Brazilian households and reported only minimum and maximum temperature values (3.0 and 10.8°C). Data from both studies were compared and data from the Swedish study were elected as they encompassed essentially the same range as the Brazilian study, with more details than only minimum and maximum temperatures. Data provided by Marklinder et al. (2004) were used to express the home storage time (days) as a triangular distribution, where "0" means that RTE vegetables packages are consumed immediately after purchase. The growth of *Salmonella* during home storage was obtained using the same growth model used for retail storage.

The fifth section in Table 1 comprises data on consumption, dose-response and risk of infection by *Salmonella*. As the serving size of RTE vegetables consumed in Brazil is unknown, the "mixed salad" data from Agudo (2004) were used, and

modeled using a RiskNormalAlt distribution. A Normal distribution can be specified based on values at two percentiles, in this case, 20% at 45 g and 80% at 90 g to represent the serving size for Brazilian RTE vegetable consumption.

The level of *Salmonella* per serving was calculated multiplying the amount of vegetable consumed by the concentration of the pathogen in the vegetable. The dose-response relationship was estimated using a Beta-Poisson model as proposed by the World Health Organization/Food and Agriculture Organization of the United Nations (2002). The probability of illness by a single dose is combined with exposure (i.e. the number of servings used per iteration) in a binomial distribution to predict the number of illnesses arising from those servings.

The main outputs of the QMRA model were risk of infection, number of illnesses and percent of illness arising from cross-contamination. The results showed that the higher the chlorine concentration, the lower the risk of illness, regardless the starting concentration of *Salmonella* or the initial fraction of contaminated produce. In scenarios for different chlorine concentration (Tables 2 and 3), when washings with 0 and 5 ppm of chlorine were simulated, less than five servings were needed to cause 1 illness and most (96.5% to 99.9%) of the illnesses would arise from cross-contamination. When washing with 10 ppm of chlorine was simulated, from 20 to 40 servings were needed to cause 1 illness and from 77.6% to 99.8% of the illnesses were predicted to arise from cross-contamination. On the other hand, when washing with 25 ppm of chlorine was simulated, the number of servings needed to cause 1 illness was considerably higher (125 to 1,666 servings). No illnesses arising from cross-contamination were observed when the simulated chlorine concentration was 50 ppm or higher.

Considering that the active chlorine concentration in water is influenced by many factors, such as batch size and organic load, three additional scenarios using a triangular distribution for chlorine concentration were considered, with minimum, most likely and maximum chlorine concentrations (Tables 4 and 5). When chlorine concentration RiskTriang(0,5,10) was simulated, it was predicted that most (97.1% to 99.9%) of the illnesses would arise from cross-contamination (Table 5). The predicted number of serving needed to cause 1 illness increased substantially (Table 4) when chlorine concentration was raised to RiskTriang(0,80,250). No illnesses arising from cross-contamination were observed when a chlorine concentration of RiskTriang(10,120,250) was simulated (Table 5). The results also showed that the

lower the prevalence of *Salmonella* in the incoming servings, the higher the percentage of illness arising from cross-contamination (Tables 3 and 5). This finding can be understood by realizing that a higher concentration of pathogens in a single dose may be more likely to cause an individual illness, but if those cells are distributed over multiple servings, even if the probability of illness per serving is less, the total number of potential illnesses will be higher.

Similar results were obtained by Danyluk and Schaffner (2011) for *E. coli* 0157:H7. Based on data published by Zhang et al. (2009), Danyluk and Schaffner (2011) developed a QMRA and hypothesized that 95% to 100% of the cases caused by *E. coli* 0157:H7 in the spinach outbreak in the USA in 2006 could be linked to cross-contaminated pieces. It was predicted that the lower the prevalence of *E. coli* 0157:H7 in the incoming vegetables, the higher the percentage of cross contaminated pieces associated with predicted cases of infection caused by this bacterium.

Outbreaks involving *Salmonella* and fresh produce have been reported worldwide (Berger et al., 2010, Callejón et al., 2015; Kozac et al., 2013)**,** becoming a major concern for consumers, governments and the food industry. The use of sanitizers during washing of vegetables is a well-known important tool for reducing microbial contamination and avoiding cross-contamination between clean and contaminated products (Gil et al., 2009; Prado-Silva et al., 2015). Application of chlorine at 50-200 ppm is the most widely used technique for disinfection of fresh produce, but this procedure has limitations, such as inactivation of active agents by high level of organic load and dependence on neutral pH for optimal activity (FAO/WHO, 2008; Goodburn and Wallace, 2013). A continuous control of chlorine concentration and water quality in fresh-cut processing plants is very important to ensure the safety of these products.

Information regarding consumption of RTE vegetables by the Brazilian population was found in only two studies. Perez et al. (2008), reported that 23% of the interviewed individuals in Belo Horizonte, MG, consumed this type of vegetables, but a much higher consumption (64.3%) was reported in Sao Paulo, SP (Sato et al., 2007).

The number of cases of infection by the consumption of RTE vegetables contaminated with *Salmonella* was estimated using the most recent data on the population of Sao Paulo city Brazil (IBGE, 2014). The simulated number of cases of

infection per month, assuming that 64.3% of the population eats RTE vegetables at least once per month (Sato et al., 2007), is shown in Table 6. Considering a scenario simulating the triangular distribution with 10, 120 and 250 ppm of chlorine, which corresponds to the most representative for Brazilian processing plants, (Maffei et al., 2016a), and the initial prevalence and concentration of *Salmonella* in RTE vegetables, the population of Sao Paulo city would experience from 459 to 55,837 illnesses per month, which seems quite high.

Validation of these findings using Brazilian public health data is not possible as reports regarding foodborne diseases in Brazil are scarce and inconsistent. Only a few Brazilian Federative States have a structured foodborne diseases surveillance system and regular reports to the health authorities (Gomes et al., 2013). Consequently, it is not possible to determine the degree to which the obtained results overestimate or underestimate the risk.

Although validation with Brazilian public health data is not feasible at this moment, some comparisons with similar risk assessments are possible. Sant'Ana et al. (2014) developed a QMRA model to estimate the risks of infection due to consumption of RTE vegetables contaminated with *Salmonella* and *Listeria monocytogenes* in Sao Paulo, Brazil, focusing on the retail and consumption steps. Their model did not include in field and processing steps and prevalence and concentration levels of pathogens were based on published data. These authors predicted that the mean risk of *Salmonella* infection per month in the exposed population is 5.7E-03 (0.0057) per serving, with 14,958 cases of infection per month. It should be highlighted that Sant'Ana et al. (2014) did not focus the entire RTE chain and did not evaluate the probability of illness, but the probability of infection, and not all cases of infection predicted might result in disease. The predictions of illness in the present study for chlorine concentrations according to RiskTriang (0,80,250) and RiskTriang (10,120,250) are consistent with the predicted number of illness by *Salmonella* in the exposed population obtained by Sant'Ana et al. (2014).

Despite the little consistency of the available public health data in Brazil related to foodborne diseases associated to RTE vegetables, the developed QMRA is useful. The results indicate that proper control of chlorine concentration during washing of vegetables is essential to reduce initial contamination and avoid cross-contamination, and that this practice has an important influence on the reduction of the risk of illness due to consumption of these products. To help reduce foodborne disease risk caused

by RTE vegetables, it is mandatory that processing plants avoid scenarios in which the chlorine concentration in the washing water is below 10 ppm.

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Table 1. Overview of simulation variables and parameters.

Cell	Variable	Value	Unit	Reference
B2	**In field**			
B3	Starting level	_	Log CFU/g	User input
B4	Days in the field after contamination	=RiskUniform(1,60)	Days	User input
B5	Log reduction in field	=RiskNormal(-0.0175,0.00862)	Log CFU/g/day	Islam et al., 2004
B6	Level at harvest	=B3-(B4*B5)	Log CFU/g	Calculated
B7	Fraction contaminated on incoming services	_	Percent	User input
B8	Fraction non-contaminated	=1-B7	Percent	User input
B9	**Washing step**			
B10	Chlorine concentration	=RiskTriang(0,80,250)	ppm	Maffei et al., 2016a
B11	Log reduction on contaminated portions after washing <10 ppm	=B10*0.04+0.3	Log CFU/g	Maffei et al., 2016b
B12	Log reduction on contaminated portions after washing ≥10 ppm	=B10*0.0056+0.5952	Log CFU/g	Maffei et al., 2016b
B13	Log reduction on contaminated portions after washing, chosen	=IF(B10<10,B11,B12)	Log CFU/g	Maffei et al., 2016b
B14	Log reduction SD	0.175	Log CFU/g	Maffei et al., 2016b
B15	Log reduction on contaminated portions after washing, with sd	=RiskNormal(B13,B14)	Log CFU/g	Maffei et al., 2016b
B16	Log % Transfer to non-contaminated portions (cross-contamination), upper	=-0.6798*B10-0.6	Log CFU/g	Maffei et al., 2016b
B17	Log % Transfer to non-contaminated portions (cross-contamination), lower	=-1.3596*B10-0.6	Log CFU/g	Maffei et al., 2016b

Table 1. Continued.

Cell	Variable	Value	Unit	Reference
B18	Log % Transfer to non-contaminated portions (cross-contamination), actual	=RiskUniform(B17,B16)	Log CFU/g	Maffei et al., 2016b
B19	Level on contaminated portions after washing	=B6-B13	Log CFU/g	Calculated
B20	Level on cross-contaminated portions after washing	=B6+B18	Log CFU/g	Calculated
B21	Choose contaminated or non-contaminated	=RiskBinomial(1,B7)	Log CFU/g	Calculated
B22	Chosen level	=IF(B21=0,B20,B19)	Log CFU/g	Calculated
B23	**Retail storage**			
B24	min retail temperature	8.1	°C	Maistro et al., 2012
B25	max retail temperature	11.3	°C	Maistro et al., 2012
B26	mean retail temperature	=RiskUniform(B24,B25)	°C	Calculated
B27	sd min retail temperature	1	°C	Calculated
B28	sd most likely retail temperature	1	°C	Calculated
B29	sd max retail temperature	2.7	°C	Calculated
B30	sd retail temperature	=RiskTriang(B27,B28,B29)	°C	Calculated
B31	retail temperature act	=RiskNormal(B26,B30)	°C	Calculated
B32	Time	=RiskTriang(3.5,7.7)	Days	User input
B33	Growth model *b* parameter	0.0243	Log CFU/hr/°C	ComBase Predictor
B34	Growth model T_0parameter	2.66	°C	ComBase Predictor
B35	Square root growth rate	=B33*(B31-B34)	sq rt(log CFU/hr)	Calculated
B36	Growth rate	=B35*B35	Log CFU/hr	Calculated
B37	Below min temp corrected growth rate	=IF(B35>0;B35*B35;0)	Log CFU/hr	Calculated

Table 1. Continued.

Cell	Variable	Value	Unit	Reference
B38	Hours to days corrected growth rate	=B37*24	Log CFU/day	Calculated
B39	Change during retail storage	=B38*B32	Log CFU/change	Calculated
B40	Level after retail storage	=B22+B39	Log CFU/g	Calculated
B41	**Home storage**			
B42	Temperature	=RiskGamma(7.15,1.03)	°C	Marklinder et al., 2004
B43	Time	=RiskTriang(0,1,4)	Days	Marklinder et al., 2004
B44	Growth model *b* parameter	0.0243	Log CFU/hr/°C	ComBase Predictor
B45	Growth model T_0 parameter	2.66	°C	ComBase Predictor
B46	Square root growth rate	=B44*(B42-B45)	sq rt(log CFU/hr)	Calculated
B47	Growth rate	=B46*B46	Log CFU/hr	Calculated
B48	Below min temp corrected growth rate	=IF(B46>0;B46*B46;0)	Log CFU/hr	Calculated
B49	Hours to days corrected growth rate	=B48*24	Log CFU/day	Calculated
B50	Change during home storage	=B49*B43	Log CFU/change	Calculated
B51	Level after home storage	=B40+B50	Log CFU/g	Calculated
B52	**Consumption, dose-response and risk of infection**			
B53	Serving size	=RiskNormalAlt(20%,45,80%,90)	g	Agudo, 2004
B54	Level of pathogen (non-log)	=10^B51	CFU/g	Calculated
B55	Level per serving, uncorrected	=B54*B53	CFU	Calculated
B56	Level per serving, with zeros	=IF(B55<1,0,TRUNC(B55))	CFU	Calculated
B57	Dose-response alpha	0.1324	No units	WHO/FAO, 2002

Table 1. Continued.

Cell	Variable	Value	Unit	Reference
B58	Dose-response beta	51.45	No units	WHO/FAO, 2002
B59	Probability of infection single dose	=1-(1+B56/B58)^-B57	Percent	Calculated
B60	Exposure (number of servings per iteration)	1	Servings	User input
B61	Risk of infection per number of servings per iteration (illness)	=RiskBinomial(B60,B59)	Illnesses	Calculated
B62	Was there illness?	= IF(B61>0,1,0)	No units	Calculated
B63	Was there cross-contamination?	=IF(B21=0,1,0)	No units	Calculated
B64	Number of illness due to cross-contamination?	=IF(B63+B62=2,B61,0)	Illnesses	Calculated
B65	Population of Sao Paulo city	11.896893	Inhabitants	IBGE, 2014
B66	% of population consuming RTE vegetables	64.3	%	Sato et al., 2007
B67	Population of Sao Paulo consuming RTE vegetables	=B65*B66	Inhabitants	Calculated
B68	Number of cases in population exposed	=B61*B67	Cases	Calculated

_, user inputs that are point values and have been omitted from this table.

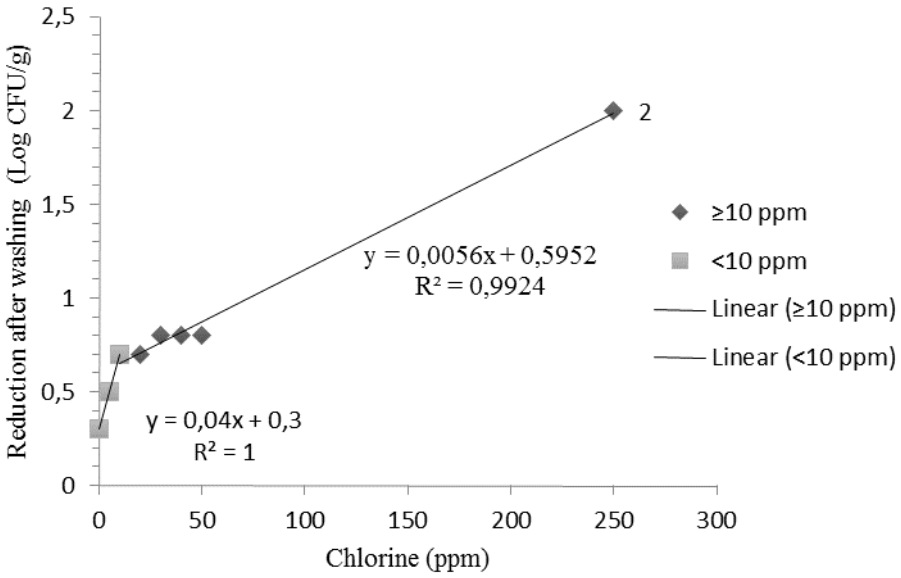

Figure 1. Log CFU/g reduction of *Salmonella* in RTE vegetables after washing contaminated portions with water containing ≥ 10 ppm and < 10 ppm of chlorine.

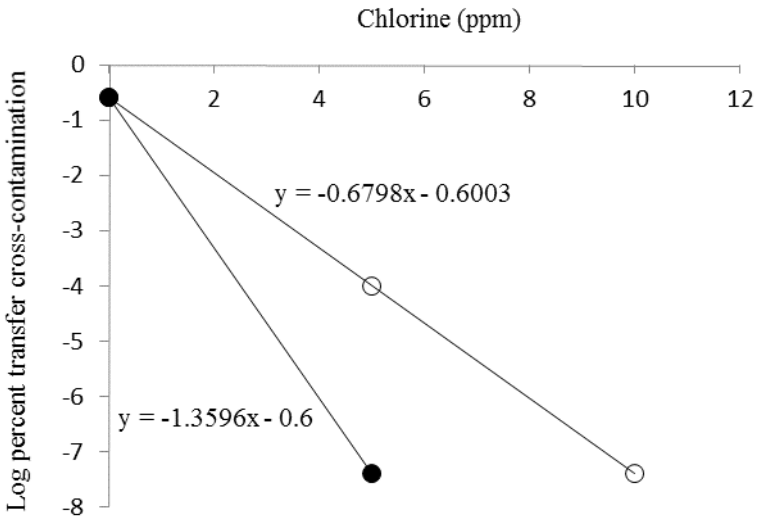

Figure 2. Log percent transfer of *Salmonella* in RTE vegetables via cross-contamination during washing (values refer to plate counts and detection limits for counting and enrichment).

Table 2. Number of servings needed to cause 1 illness.

Percent of contaminated produce	1.0%		0.1%		0.01%	
Pathogen starting level (log CFU/g in product in the field)	0	1	0	1	0	1
Chlorine concentration						
0	1.4	1.2	1.4	1.2	1.4	1.2
5	4.7	3.3	4.8	3.4	4.8	3.4
10	31.4	20.5	39.2	23.8	40	24.2
25	140.0	125.3	1,666	1,351	8,333	8,333
50	143.6	127.5	1,785	1,428	12,500	12,500
150	151.9	135.8	1,851	1,562	12,500	12,500
250	165.0	144.5	1,923	1,785	16,666	12,500

Table 3. Number of illness per 50,000 servings, where each iteration is a serving. Percent of illnesses arising from cross-contamination is shown in parenthesis.

Percent of contaminated produce	1.0%		0.1%		0.01%	
Pathogen starting level (log CFU/g in product in the field)	0	1	0	1	0	1
Chlorine concentration						
0	36,286 (98.9)	39,815 (98.9)	36,277 (99.9)	39,810 (99.9)	36,272 (99.9)	39,807 (99.9)
5	10,610 (96.5)	15,118 (97.3)	10,367 (99.6)	14,879 (99.7)	10,349 (99.9)	14,859 (99.9)
10	1,589 (77.6)	2,440 (83.6)	1,276 (98.5)	2,095 (98.3)	1,252 (99.7)	2,064 (99.8)
25	357 (0.6)	399 (0.5)	30 (6.6)	37 (5.4)	6 (33.3)	6 (33.3)
50	348 (0)	392 (0)	28 (0)	35 (0)	4 (0)	4 (0)
150	329 (0)	368 (0)	27 (0)	32 (0)	4 (0)	4 (0)
250	303 (0)	346 (0)	26 (0)	28 (0)	3 (0)	4 (0)

Table 4. Number of servings needed to cause 1 illness (using RiskTriang for chlorine concentration)

Percent of contaminated produce	1.0%		0.1%		0.01%	
Pathogen starting level (log CFU/g in product in the field)	0	1	0	1	0	1
Chlorine concentration						
RiskTriang(0,5,10)	4	3	4	3	4	3
RiskTriang(0,80,250)	133	118	684	537	1,162	819
RiskTriang(10,120,250)	152	137	1,562	1,428	16,666	16,666

Table 5. Number of illness per 50,000 servings, where each iteration is a serving, using RiskTriang for chlorine concentration. Percent of illness arising from cross-contamination shown in parenthesis.

Percent of contaminated produce	1.0%		0.1%		0.01%	
Pathogen starting level (log CFU/g in product in the field)	0	1	0	1	0	1
Chlorine concentration						
RiskTriang(0,5,10)	12,524 (97.1)	16,538 (97.6)	12,302 (99.7)	16,320 (99.7)	12,281 (99.9)	16,298 (99.9)
RiskTriang(0,80,250)	374 (10.4)	423 (13.4)	73 (54.7)	93 (62.3)	43 (93)	61 (95)
RiskTriang(10,120,250)	328 (0)	364 (0)	32 (0)	35 (0)	3 (0)	3 (0)

Table 6. Estimated number of cases of infection by the consumption of ready-to-eat vegetables contaminated with *Salmonella* in the population of Sao Paulo city per month.

Percent of contaminated produce	1.0%		0.1%		0.01%	
Pathogen starting level (log CFU/g in product in the field)	0	1	0	1	0	1
Fixed chlorine concentration						
0	5,464,073	6,374,752	5,464,073	6,374,752	5,464,073	6,374,752
5	1,627,596	2,318,092	1,593,688	2,249,912	1,593,688	2,249,912
10	243,621	373,156	195,145	321,416	191,416	316,103
25	54,641	61,051	4,592	5,662	918	918
50	53,271	59,998	4,286	5,357	612	612
150	50,360	56,331	4,133	4,897	612	612
250	46,362	52,939	3,978	4,286	459	612
Triangular distribution for chlorine concentration						
0,5,10	1,961,462	2,549,901	1,912,426	2,549,901	1,865,781	2,549,901
0,80,250	57,517	64,828	11,184	14,245	6,583	9,340
10,120,250	50,327	55,837	4,897	5,357	459	459

4. CONCLUSÕES GERAIS

Os experimentos realizados e o modelo de avaliação de risco microbiológico construído com base nos resultados obtidos demonstraram quantitativamente que quanto maior a concentração de cloro na água de lavagem dos vegetais, menor o risco de casos de infecção por *Salmonella* devido ao consumo de vegetais minimamente processados prontos para o consumo. Quando cenários com concentração de dicloroisocianurato de sódio abaixo de 5 mg/L foram simulados, estimou-se que mais de 96% dos casos de infecção poderiam ser provenientes de contaminação cruzada. Por outro lado, quando cenários com concentração de dicloroisocianurato de sódio acima de 50 mg/L foram simulados, casos de infecção provenientes de contaminação cruzada não foram preditos. Estes resultados confirmam que o controle da qualidade da água e o monitoramento da concentração de sanitizante são essenciais para evitar a ocorrência de contaminação cruzada e garantir a produção de vegetais minimamente processados prontos para o consumo que sejam seguros.

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ANEXOS

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Assessing the effect of washing practices employed in Brazilian processing plants on the quality of ready-to-eat vegetables

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ABSTRACT

This study gathered information on the practices employed in Brazilian processing plants during production of ready-to-eat (RTE) vegetables and evaluated the effect of washing practices on the quality of RTE vegetables produced in these plants. Physicochemical analysis of water included temperature, pH, organic load and chlorine concentration and microbiological analysis included mesophilic bacteria, yeasts and molds, *Enterobacteriaceae*, total coliforms, *Escherichia coli* and *Salmonella*. The ten selected processing plants were clustered in three groups: 1) plants A, B, D, H and I, where washing procedures included immersion in agitated tanks during pre-washing and washing-disinfection and use of disinfectant in the pre-washing step; 2) plant E, where vegetables were washed under running water in the pre-washing step, sodium hypochlorite was used as a disinfectant agent and processing of vegetables was only manual; and 3) plants C, F, G and J, where pre-washing and washing-disinfection were performed by immersion in water, followed by a rinsing step. Chlorine was the most used chemical agent for disinfection of vegetables. A 0.2–1.2 log reduction was achieved by the practices adopted in the plants, highlighting the importance of immediate refrigeration and control measures to avoid post-processing recontamination.

1. Introduction

Over the last decades, the demand for fresh and convenient foods increased. People have less time to cook at home which reflects the increased popularity of ready-to-eat (RTE) foods, such as minimally processed vegetables (Abadias, Usall, Anguera, Solsona, & Viñas, 2008; Chen, Zhu, Zhang, Niu, & Du, 2010). Moreover, these products have gained ground in restaurants, hotels, fast food chains, catering services and other institutions (Rojas-Grau, Garner, & Martín-Belloso, 2011).

RTE vegetables may be subjected to minimal processing, such as peeling, cutting, slicing, shredding, washing, drying and packaging (Alzamora, Tapia, & López-Malo, 2000; Codex Alimentarius Commission, 2003), resulting in a diversity of products and packaging formats (Jung, Jang, & Matthews, 2014). As the majority of RTE vegetables require no further treatment before consumption, absence of a food borne pathogens killing step can result in a potential public health problem. A number of food borne illnesses associated to fresh produce have been reported (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004) and recent examples are *S.* Newport and *S. Saintpaul* in cucumbers, *S. Enteritidis* in bean, alfafa and spicy sprouts and *Escherichia coli* O157:H7 in ready-to-eat salads, spinach and spring mix (Centers for Disease Control and Prevention, 2015). In Brazil, a number of outbreaks reported between 2000 and 2014 were associated with the consumption of vegetables (Brasil, 2014).

Vegetables can become contaminated with pathogenic microorganisms during harvest and post-harvest, caused by soil, irrigation water, inadequately composted manure, air, wild and domestic animals, human handling, harvesting equipment, transport containers, vehicles, improper storage and packaging (Berger et al., 2010; Harris et al., 2003). Among these operations, washing seems to have a major relevance for the safety of these products.

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Although washing aims to remove debris and reduce microbial load, its effectiveness is limited, and reduction of certain microorganisms to a specific level of safety cannot be assured (Gil, Selma, López-Gálvez, & Allende, 2009). Chemicals added to washing water are helpful, but the antimicrobial effectiveness depends on several factors (Prado-Silva, Cadavez, Gonzales-Barron, Rezende, & Sant'Ana, 2015). For instance, the effectiveness of chlorine at 50–200 mg/L, the most widely used sanitizer for disinfection of fresh produce, is typically less than 2 log CFU (Goodburn & Wallace, 2013).

Recent studies have shown that contamination of washing water used during RTE vegetables processing may lead to spread of contaminants within batches (Jensen, Friedrich, Harris, Danyluk, & Schaffner, 2015; Holvoet et al., 2014; Perez-Rodriguez et al., 2014; Tomás-Callejas et al., 2012; Zhang, Ma, Phelan, & Doyle, 2009). On this matter, cross-contamination during washing may have been the potential cause of many reported food borne outbreaks. Thereby, the knowledge about the washing practices and water characteristics employed in processing plants may help to understand and to prevent the occurrence of cross-contamination during washing of vegetables.

The objectives of this study were to gather information on the practices employed in ten selected Brazilian processing plants located in the State of Sao Paulo during production of RTE vegetables and to evaluate the effect of washing practices on the quality of RTE vegetables produced in these plants.

2. Materials and methods

2.1. Assessment of washing practices in selected Brazilian processing plants during the production of RTE vegetables

Ten major RTE vegetables processing plants, identified as A to J, located in the State of Sao Paulo, Brazil, were selected for the study. Three of them are located in Sao Paulo city and the other seven in inland towns. A questionnaire with 45 items focusing the water usage during processing addressing washing method, water temperature, addition of disinfectant, water volume, amount of vegetables immersed in the water and reuse/discharge of water used in the pre-washing, washing-disinfection and rinsing steps was applied to these plants. Additional information on raw materials reception and storage, centrifugation of washed vegetables, packaging and transportation conditions was also collected. Prior to application in the ten processing plants, the questionnaire (Supplementary material) was validated by application in two plants. A flowchart with the processing steps in the ten visited RTE vegetables processing plants is shown in Fig. 1.

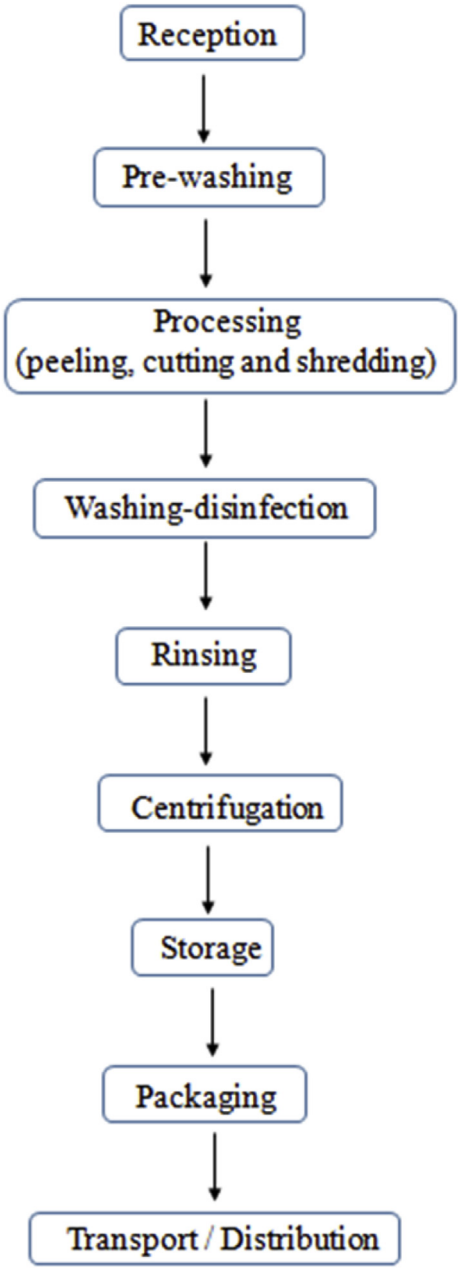

Fig. 1. Flowchart showing the processing steps in the visited processing plants.

2.2. Collection of water and vegetables samples

For the purpose of this study, pre-washing was considered the step in which vegetables were immersed in tanks with water (usually without disinfectant agents) aiming to remove soil and debris, prior to the washing-disinfection step. Washing-disinfection was considered the step in which vegetables were immersed in a tank with water containing disinfectant agents. Rinsing was the step in which pre-washed and disinfected vegetables were washed with clean water to eliminate residues of disinfectants.

A total of 34 water samples (250 mL) were collected in the ten visited processing plants during washing procedures, considering all industries presented a continuous processing. These samples included water from the tap water supply (n = 10), water from pre-washing tanks (n = 9), water from washing-disinfection tanks (n = 10) and water from rinsing tanks (n = 5). Water samples were collected in two bottles, one destined for chemical testing and the other for microbiological analysis. Thirty-six and forty-eight samples of vegetables were collected before and after processing, respectively, and comprised leafy greens [chicory (*Cichorium intybus*), collard greens (*Brassica oleracea*), escarole (*Cichorium endivia*), lettuce (*Lactuca sativa*), parsley (*Petroselinum crispum*), spinach (*Spinacia oleracea*) and watercress (*Nasturtium officinale*)] and other vegetables [beet (*Beta vulgaris esculenta*), carrot (*Daucus carota* subsp. *sativus*), onion (*Allium cepa*), potato (*Solanum tuberosum*), white carrot (*Arracacia xanthorrhiza*) and zucchini (*Cucurbita pepo*)].

2.3. Physicochemical analysis of water samples

Water samples collected from the tanks were tested for temperature (°C), pH, chlorine concentration (mg/L) and organic load

concentration (mg/L). Temperature and pH were measured using a portable meter fitted with appropriate probes (HQ40d, Hach, USA). Organic load was determined by a gravimetric method (Teixeira, Tundisi, & Kutner, 1965; Tundisi, 1969) with modifications according to Wetzel and Likens (1991). Free chlorine was measured using a portable photometer (HI96771, Hanna Instruments, USA).

2.4. Microbiological analysis of water samples

In order to inactivate residual chlorine, 10% sterile sodium thiosulphate (Sigma–Aldrich ChemieGmbHSteinheim, Germany) was added to water samples before microbiological analysis. The samples were serially diluted in 0.1% peptone (Oxoid, England) and plated for enumeration of mesophilic bacteria (Morton, 2001) and *Enterobacteriaceae* (Kornacki & Johnson, 2001). MPN of total coliforms and *E. coli* were determined using Fluorocult®LMX Broth, double concentration (Hunt & Rice, 2005; Merck, 2005). Samples were tested also for *Salmonella* spp according to ISO 19250 (2010).

2.5. Microbiological analysis of vegetables samples

Twenty-five grams of each vegetable sample were transferred to a sterile plastic bag and mixed with 225 mL of 0.1% peptone water in a Stomacher 400 Lab-blender (Seward Medical, London, England) for 1 min. Serial dilutions were prepared in 0.1% peptone water and submitted to enumeration of mesophilic bacteria (Morton, 2001), yeasts and molds (Beuchat & Cousin, 2001) and *Enterobacteriaceae* (Kornacki & Johnson, 2001). Total coliforms and *E. coli* were also enumerated using the MPN technique and Fluorocult®LMX Broth (Kornacki & Johnson, 2001; Merck, 2005). Additionally, another 25 g sample of each sample was mixed with 225 mL of Buffered Peptone Water (BPW) (Difco, Sparks, Md., USA) for 1 min, following incubation at 37 °C for 24 h, for detection of *Salmonella* spp, according to ISO 6579:2002 (2007).

2.6. Statistical analysis

Microbial counts of water (CFU/mL and MPN/100 mL) and vegetables samples (CFU/g and MPN/g) were log transformed. The Student's t-test and the Mann–Whitney test were used, depending on variable distribution, to determine significant differences ($p < 0.05$) among microbial counts in vegetables samples before and after processing. The software SigmaPlot version 12.5 (2013 Systat Software Inc.) was used for the statistical treatment. Cluster analyses were made using the software XLSTAT (Addinsoft version 2015.1.01) to identify similarities among the practices adopted in the ten visited processing plants.

3. Results

Results from the questionnaire applied in the ten visited processing plants showed that in all plants, the raw materials were kept under refrigeration, varying from a minimum of 2 °C in plant D to a maximum of 8 °C, in plants B, G and J. In all plants, damaged vegetables were removed from the entering lots before processing. Despite present in all plants, the pre-washing step was performed differently: in plant E pre-washing was done under running water, in plants A, B, D and H pre-washing was done by immersion in agitated water and in plants C, F, G, I and J pre-washing was done by immersion in static water. At the pre-washing step, the water was chlorinated in plants A, B, D and J, and concentrations varied from 3 to 235 mg/L. In plants H and I, alkaline detergents were added in the water to remove soil and dirt.

Except for plant E, where peeling, cutting and shredding were done manually, the other plants used machines for this purpose, resulting in different types, sizes and cuts (e.g. grated, diced, sliced and stick vegetables or entire heads of leafy greens). Up to 86 varieties of ready-to-eat vegetables were produced in these processing plants.

All plants adopted a washing-disinfection step in the processing. In seven plants (C, D, E, F, G, I and J) vegetables were immersed in a tank with water in static conditions, while in three plants (A, B and H), vegetables were immersed in agitated water tanks. The most common disinfectant agents were: sodium dichloroisocyanurate (plants A, B, C, D, F, G and J, concentration ranging from 75.5 to 155 mg/L), sodium hypochlorite (plant E, concentration 50 mg/L) and chlorine dioxide (plants H and I, concentration 240 mg/L). The contact time ranged from 2 to 20 min. In plant B, leafy greens were disinfected with ozonated water (1.0 g/L). The amount of water used for washing 1 kg of vegetables in the pre-washing and washing-disinfection tanks varied from 5 to 28 L (Fig. 2).

The rinsing step was performed in five processing plants: in plant A, rinsing was done with running water and in plants C, G, H and J vegetables were immersed in tanks containing non-chlorinated water. The other five plants did not include a rinsing step in the processing. In eight processing plants (A, B, C, F, G, H, I and J) vegetables were submitted to a centrifugation step (average 1200 rpm for 1.5 min) for water removal. In plant A, washing water was changed uninterruptedly through a continuous circulation system. The other plants reported that the water was changed 3–7 times per day and the disinfectant concentration was adjusted after every water replacement. Reuse/recirculation of water was used in three plants: during pre-washing and washing-disinfection steps (plant B), during rinsing step (plant C) and during pre-washing step (plant J). In these plants, reused water was discharged when accumulation of debris became evident (~3–4 times/day).

In plant G, after rinsing and before centrifugation, some types of vegetables were immersed in water tanks containing ice cubes (prepared with potable but non-chlorinated water) and sodium metabisulphite as antioxidant. The processed vegetables were packaged for retail and for wholesale, in volumes varying from 30 to 300 g and from 1 to 5 kg, respectively. Most common packaging materials were polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC) and expanded polystyrene (EPS). Some plants used modified atmosphere in packages of leafy greens (e.g. lettuce) and vacuum in packages of other vegetables (e.g. minimally processed carrots and potatoes). All packages were stored under refrigeration (2–8 °C) up to 48 h and distributed for retail under refrigeration (2–10 °C) in plastic or cardboard boxes. However, only plants A, F, G, H and I monitored the temperature during distribution. The reported shelf life of RTE vegetables produced in the visited processing plants ranged from 5 to 7 days for leafy greens and 5–15 days for other vegetables (e.g. green beans, sweet potato, zucchini etc.).

Cluster analysis aiming at identifying similarities or dissimilarities among the practices observed in the ten visited processing plants resulted in three major groups (Fig. 3): group 1 (plants A, B, D, H and I), group 2 (plant E) and group 3 (plants C, F, G and J). The similarities in plants of group 1 were immersion in agitated tanks during pre-washing step in A, B, D and H and during washing-disinfection step in A, B and H, use of a disinfectant in the pre-washing step in A, B, D, H and I, and refrigeration of water in A and D. Plant E (group 2) differed from the other processing plants as vegetables were washed under running water in the pre-washing step, sodium hypochlorite was used as a disinfectant agent and processing of vegetables was only manual. The similarities in plants of group 3 (C, F, G and J) were static immersion during pre-washing and washing-disinfection steps, followed by a rinsing step in C, G and J.

Results of temperature, pH, organic load and chlorine

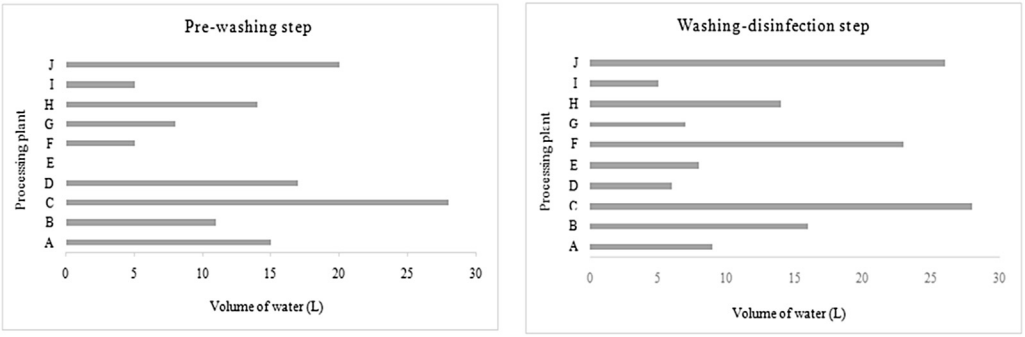

Fig. 2. Volume of water used for washing 1 kg of vegetables in the visited processing plants, designated A, B, C, D, E, F, G, H, I and J.

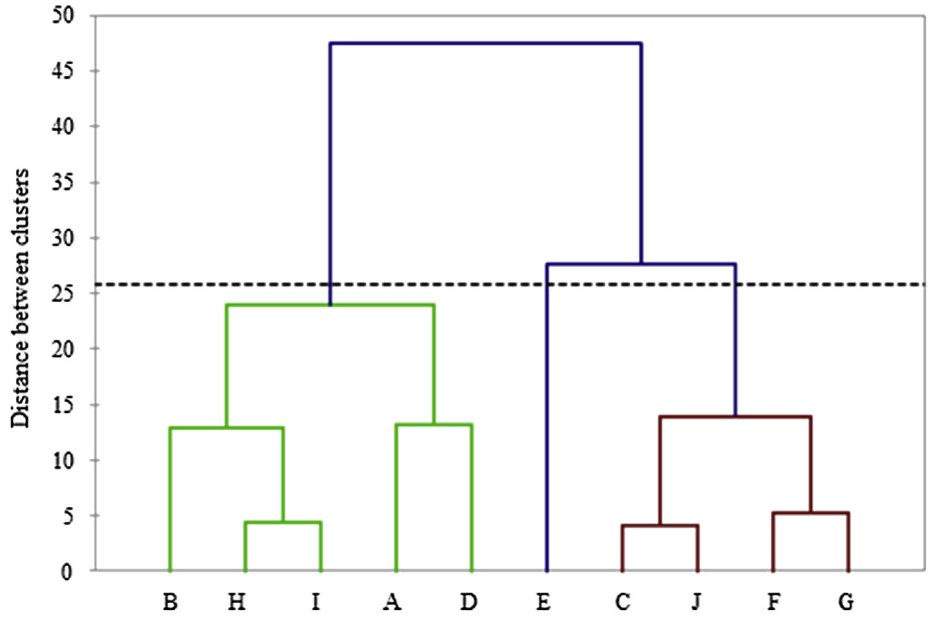

Fig. 3. Cluster analysis for the practices employed in the visited processing plants, designated A, B, C, D, E, F, G, H, I and J.

concentration in water are presented in boxplots (Fig. 4). Only plants A and D used refrigerated water in all processing steps, and temperature varied from 6 to 14 °C. In plants B, C, E, F, G, H, I and J, the water used in all steps was at room temperature, with an average of 21 ± 0.8 °C. The pH of water ranged from 6.4 to 8.3 for tap water, 5.8 to 8.2 for water in the pre-washing tanks, 5.6 to 8.3 for water in the washing-disinfection tanks and 6.5 to 7.7 for water in the rinsing tanks. Regarding organic load in the water, the highest concentration (50 mg/L) was detected in pre-washing tanks, which may be due to dirt and cell exudates from cut surfaces. On the other hand, in tanks of washing-disinfection the concentration of organic load ranged from 2.3 to 13.5 mg/L.

Different chlorine-based disinfectants were used in the washing-disinfection step: sodium dichloroisocyanurate or sodium hypochlorite in plants A, B, C, D, E, F, G and J (concentrations ranging from 50 to 155 mg/L), and chlorine dioxide in plants H and I (240 mg/L). Processing plant B also had a tank of ozonated water (1.0 g/L) for washing-disinfection of leafy greens exclusively. Measurement of free chlorine concentration in water samples containing chlorine dioxide or ozone was not possible with the portable photometer used in the study. In these cases, the technicians in the processing plants provided the data presented in Fig. 4.

Microbiological testing of water (Tables 1 and 2) indicated that *Salmonella* was not detected in any sample (absence in 100 mL) and the lowest and highest counts of mesophilic bacteria and *Enterobacteriaceae* were found in tap water and water from the pre-washing tanks, respectively. Although microbial counts of mesophilic bacteria and *Enterobacteriaceae* seems to be higher in water samples collected from the pre-washing step, they were not statistically significant when a mean of the ten plants were compared. Total coliforms were found in water from the pre-washing tanks in plants B, D, F, G, H and I, in water from the washing-disinfection tanks in plants H and I and in water from the rinsing tanks in plants G and H. *E. coli* was detected in water from the pre-washing tanks in plants F, G and H and in water from rinsing tanks in plant H, with counts of 6.9 MPN/100 mL, which is of concern, as this is the last washing step in RTE vegetables production. Considering that the proportion between the volume of water and the amount of vegetable immersed in this water affects the efficacy of disinfection process, this parameter was also measured. In the studied processing plants, the proportion ranged from 5 to 28 L of water for 1 kg of vegetables, and varied according to the type of vegetable and the equipment used for washing, as different equipment require different water volume for efficient functioning.

Microbiological testing of vegetables before and after processing (Table 3) indicated that *Salmonella* was not detected in any tested

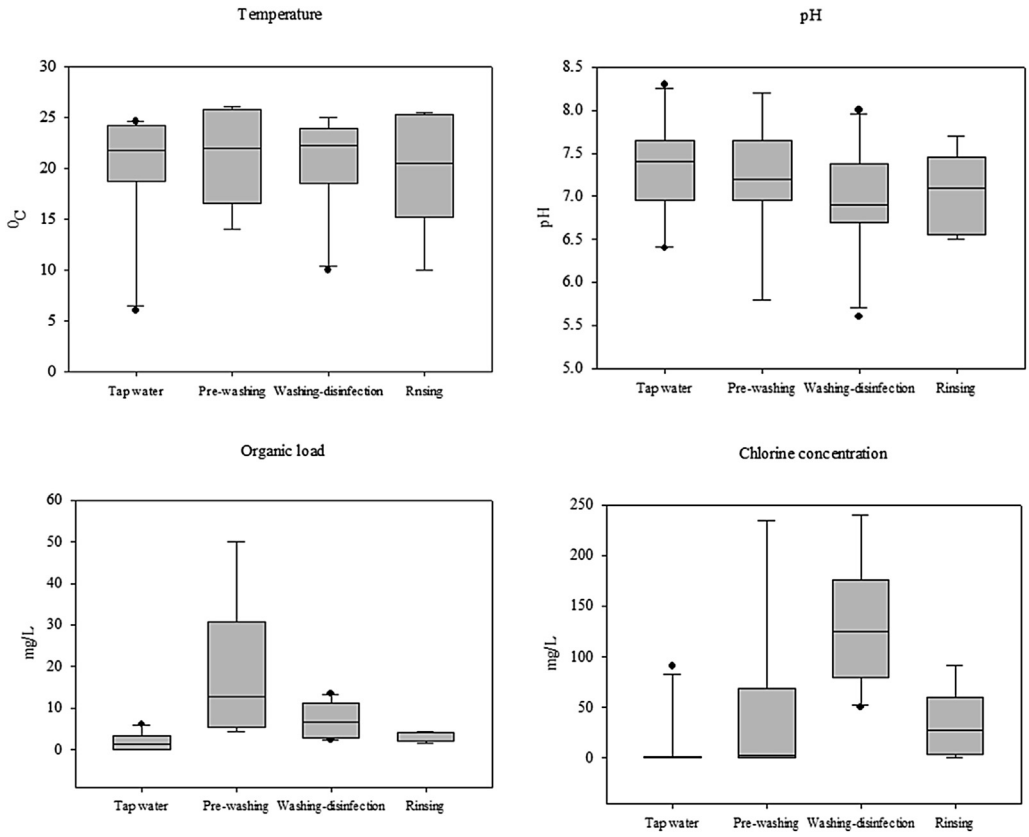

Fig. 4. Boxplots showing the physicochemical parameters of water samples collected in the visited processing plants.

Table 1
Counts of mesophilic bacteria and *Enterobacteriaceae* in water samples collected in ten vegetables processing plants.

Processing plant	Mesophilic bacteria				*Enterobacteriaceae*			
	Tap water	Pre-washing	Washing-disinfection	Rinsing	Tap water	Pre-washing	Washing-disinfection	Rinsing
A	2.0 ± 0.1	1.7 ± 0.3	1.9 ± 0.2	1.6 ± 0.1	<1	<1	<1	<1
B	1.7 ± 0.1	3.4 ± 0.6	1.0 ± 0.1	n.a.	1.8 ± 0.2	1.5 ± 0.5	<1	n.a.
C	1.5 ± 0.1	1.6 ± 0.8	1.2 ± 0.3	1.1 ± 0.1	<1	<1	<1	<1
D	1.4 ± 0.1	3.7 ± 0.9	1.1 ± 0.4	n.a.	<1	2.1 ± 0.3	<1	n.a.
E	1.0 ± 0.0	1.0 ± 0.0	3.4 ± 0.2	n.a.	<1	<1	<1	n.a.
F	1.4 ± 0.2	4.0 ± 0.5	1.0 ± 0.1	n.a.	<1	1.8 ± 0.3	<1	n.a.
G	<1	3.9 ± 0.6	<1	1.1 ± 0.2	<1	3.7 ± 0.4	<1	1.0 ± 0.0
H	1.4 ± 0.3	3.5 ± 0.3	3.3 ± 0.4	3.5 ± 0.3	<1	3.1 ± 0.2	2.8 ± 0.3	2.9 ± 0.3
I	1.1 ± 0.1	1.4 ± 0.2	1.0 ± 0.0	n.a.	<1	1.3 ± 0.2	1.2 ± 0.2	n.a.
J	1.1 ± 0.1	1.3 ± 0.3	1.3 ± 0.3	1.4 ± 0.5	<1	<1	<1	<1

Results expressed as mean ± SD (log CFU/mL).
"n.a." not applicable (step not performed in the plant).

sample (absence in 25 g), which is in agreement with Brazilian and other regulations (Brasil, 2001; Anonymous, 2014). *E. coli* was detected in 20 (55%) and 10 (21%) unprocessed and processed vegetables, respectively. These included three collard green samples from plants G, H and J, two chicory samples from plants E and F, two parsley samples from plants B and F, one scarole sample from processing plant J, one spinach from plant J and one grated carrot sample from plant J. Two samples (chicory and parsley) from processing plant F presented counts above 2 log CFU/g, which is the upper limit determined by the Brazilian Surveillance Agency for thermotolerant coliforms in vegetables (Brasil, 2001).

Reduction of the microbial load from unprocessed to processed vegetables seemed to be more effective in plant A (from 1.4 to 3.2 log-reduction) than in the other processing plants (Table 3). In plant A, the washing water and the processing environment were refrigerated and the washing steps were conducted with chlorinated water, highlighting the importance of these parameters during processing to assure the quality and safety of RTE vegetables.

Overall, the processing of vegetables caused a 0.2 to 1.2 log reduction in the initial microbial counts when compared unprocessed with processed vegetables (Table 4). These reductions were statistically significant ($p < 0.05$) for mesophilic bacteria, yeasts and molds, *Enterobacteriaceae* and total coliforms. Although significant, these reductions can be overcome very quickly if the products are held at room temperature. Thus, immediate storage under refrigeration and practices to avoid recontamination are very important control measures. Microbial counts on RTE leafy greens seems to be higher than in other vegetables, but not statistically significant, which may be due structural differences on their surface (Table 4).

Table 2
Counts of total coliforms and *E.coli* in water samples collected in ten vegetables processing plants.

Processing plant	Total coliforms				*E.coli*			
	Tap water	Pre-washing	Washing-disinfection	Rinsing	Tap water	Pre-washing	Washing-disinfection	Rinsing
A	<1.1	<1.1	<1.1	<1.1	<1.1	<1.1	<1.1	<1.1
B	<1.1	16	<1.1	n.a.	<1.1	<1.1	<1.1	n.a.
C	<1.1	<1.1	<1.1	<1.1	<1.1	<1.1	<1.1	<1.1
D	<1.1	>23	<1.1	n.a.	<1.1	<1.1	<1.1	n.a.
E	<1.1	<1.1	<1.1	n.a.	<1.1	<1.1	<1.1	n.a.
F	<1.1	>23	<1.1	n.a.	<1.1	>23	<1.1	n.a.
G	<1.1	>23	<1.1	12	<1.1	>23	<1.1	<1.1
H	<1.1	>23	>23	>23	<1.1	>23	<1.1	6.9
I	<1.1	5.1	3.6	n.a.	<1.1	<1.1	<1.1	n.a.
J	<1.1	<1.1	<1.1	<1.1	<1.1	<1.1	<1.1	<1.1

Results expressed as MPN/100 mL.
"n.a." not applicable (step not performed in the plant).

Table 3
Counts of mesophilic bacteria, *Enterobacteriaceae*, yeasts and molds, total coliforms and E.coli in vegetables before and after processing.

Processing plants	Mesophilic bacteria (log CFU/g)		*Enterobacteriaceae* (log CFU/g)		Yeasts and Molds (log CFU/g)		Total coliforms (log MPN/g)		*E. coli* (log MPN/g)	
	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed
A	6.2 ± 0.2	3.0 ± 0.5	5.6 ± 0.3	2.9 ± 0.8	5.2 ± 0.1	3.6 ± 0.2	>3.0	1.6 ± 0.6	1.4 ± 0.0	<1
B	6.2 ± 0.1	4.4 ± 1.8	5.1 ± 0.1	2.8 ± 1.6	4.6 ± 0.2	4.4 ± 1.0	>3.0	2.3 ± 0.8	>3.0	0.5 ± 0.0
C	6.4 ± 0.1	3.8 ± 0.5	5.3 ± 0.2	3.4 ± 0.5	5.4 ± 0.7	4.9 ± 1.0	>3.0	1.9 ± 0.6	<1	<1
D	3.1 ± 0.0	3.9 ± 0.7	2.6 ± 0.0	3.2 ± 1.2	3.5 ± 0.0	3.9 ± 0.8	>3.0	2.0 ± 0.3	<1	<1
E	6.3 ± 0.1	6.2 ± 0.1	5.0 ± 0.2	4.9 ± 0.3	5.8 ± 0.2	5.6 ± 0.4	>3.0	>3.0	2.3 ± 0.8	0.9 ± 0.0
F	6.3 ± 0.1	5.5 ± 0.5	5.1 ± 0.1	4.8 ± 0.6	5.8 ± 0.1	5.0 ± 0.5	>3.0	>3.0	0.9 ± 0.6	2.5 ± 0.2
G	5.7 ± 0.6	4.7 ± 0.5	4.6 ± 1.0	3.0 ± 1.1	5.4 ± 0.6	5.0 ± 1.1	>3.0	3.0 ± 0.2	1.7 ± 1.1	0.5 ± 0.0
H	6.2 ± 0.1	5.3 ± 0.6	5.5 ± 0.4	4.9 ± 0.4	5.7 ± 0.7	4.4 ± 0.4	>3.0	2.5 ± 0.9	1.1 ± 0.5	1.0 ± 0.0
I	5.8 ± 0.6	5.3 ± 0.3	4.7 ± 0.9	4.3 ± 0.9	5.6 ± 0.2	5.2 ± 0.6	2.4 ± 0.9	1.4 ± 0.4	1.1 ± 0.2	<1
J	6.4 ± 0.5	5.9 ± 0.7	6.0 ± 0.4	5.0 ± 0.4	5.3 ± 0.8	5.2 ± 0.6	>3.0	>3.0	1.8 ± 0.4	1.2 ± 0.2

Results expressed as mean ± SD.

Table 4
Effect of processing on counts of mesophilic bacteria, yeasts and molds, *Enterobacteriaceae*, total coliforms and *E.coli* counts in leafy greens and other vegetables.

Samples	Mesophilic bacteria (log CFU/g)		Yeasts and Molds (log CFU/g)		*Enterobacteriaceae* (log CFU/g)		Total coliforms (log MPN/g)		*E. coli* (log MPN/g)	
	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed
RTE vegetables	6.0 ± 0.6*	4.9 ± 1.2*	5.3 ± 0.7*	4.8 ± 0.8*	5.0 ± 0.7*	3.9 ± 1.2*	2.9 ± 0.4*	2.4 ± 0.7*	1.6 ± 0.8	1.3 ± 0.7
Leafy greens	6.2 ± 0.2*	5.0 ± 1.2*	5.5 ± 0.6	5.0 ± 0.8	5.2 ± 0.5*	4.1 ± 1.2*	3.0 ± 1.4*	2.6 ± 0.7*	1.7 ± 0.8	1.3 ± 0.7
Other vegetables**	5.8 ± 0.9*	4.8 ± 1.2*	5.1 ± 0.8	4.6 ± 0.8	4.8 ± 1.0*	3.8 ± 1.2*	2.8 ± 0.6*	2.2 ± 0.7*	1.2 ± 0.2	1.0 ± 0.0

Results expressed as mean ± SD.
Statistical analysis performed by the Student's t-test and the Mann–Whitney test.
*Significant differences ($p < 0.05$) observed between unprocessed and processed vegetables for the microbial groups evaluated.
**Other vegetables included bulbs, roots and tubers.

4. Discussion

The processing procedures (reception, pre-washing, peeling, cutting, shredding, washing-disinfection, rinsing, centrifugation, packaging, storage, transport and distribution) in the studied RTE vegetables processing plants were similar (Fig. 1). Unique procedures were observed in plant A, such as the refrigeration of whole processing environment and use of refrigerated and chlorinated water in all processing steps.

Each processing step during production of RTE vegetables plays an important role for the quality of the final product, but the washing steps are key steps, as they remove dirt and cells exudates from harvested produce, and reduce the microbial population from their surface (D'Acunzo, Cimmuto, Marinelli, Aurigemma, & Giusti, 2012). However, if contaminated, wash water can become a source of cross-contamination, causing transfer of pathogenic and non pathogenic microorganisms from contaminated to non-contaminated batches (Holvoet et al., 2014; Jensen et al., 2015; Perez-Rodriguez et al., 2014; Tomás-Callejas et al., 2012; Zhang et al., 2009). On this matter, Danyluk and Schaffner (2011) developed a quantitative microbial risk assessment using data published by Zhang et al. (2009) and hypothesized that 95%–100% of the cases caused by *E. coli* 0157:H7 in the spinach outbreak occurred in the USA in 2006 could be explained by occurrence of cross-contamination.

In all visited plants, processing of vegetables included pre-washing and washing-disinfection steps, and different chlorine-based compounds were used for disinfection. Chlorine is the most widely used chemical agent for disinfection of fresh produce and presents a good cost-benefit ratio. The Brazilian Health Surveillance Agency recommends the use of chlorine-based products for disinfection of fresh produce, with concentrations ranging from 100 to 250 of free chlorine (Brasil, 2013).

Chlorine-based compounds have toxicity concerns due to

reaction with organic materials that may cause the formation of halogenated by-products (Food and Agriculture Organization/World Health Organization, 2008; Joshi, Mahendran, Alagusundaram, Norton, & Tiwari, 2013), causing the expansion of the use of sodium dichloroisocyanurate in Brazil due to less reactivity with organic materials (Salomão, Muller, Massaguer, & Aragão, 2011). In addition, sodium dichloroisocyanurate has a better stability in aqueous solution than sodium hypochlorite. Chlorine dioxide and ozone are powerful antimicrobial agents, but they are more expensive and require specific equipment and expertise to control the concentration in water (Chiattone, Torres, & Zambiazi, 2008; Joshi et al., 2013). Chlorine dioxide has been increasingly used as an alternative to sodium hypochlorite since it does not react with organic compounds to generate undesirable carcinogenic chemicals (Chen & Zhu, 2011; López-Gálvez et al., 2010).

In the present study, we observed that RTE leafy greens produced by processing plant B (washed in ozonated water) showed lower microbial counts when compared to the plants where disinfection was carried out only with chlorine-based products. Mesophilic bacteria, yeasts and molds, *Enterobacteriaceae* and total coliforms populations in leafy greens washed in ozonated water were 2.6, 1.0, 3.2 and 1.4 log CFU/g lower than those found in leafy greens washed in chlorinated water. *E. coli* and *Salmonella* were not detected in those RTE leafy greens samples washed in ozonated water. Alexopoulos et al. (2013) also observed a higher effectiveness of ozonation than chlorination of water for reduction of microbial counts in fresh-cut lettuce and green bell pepper. These authors noticed that dipping these vegetables in chlorinated water (20 mg/L) resulted in one log reduction of the total microbial counts in the first 15 min. However, when these vegetables were dipped in continuously ozonated water (0.5 mg/L), about 2 log decreases was achieved in the first 15 min and 3.5 log after 30 min of exposure.

The influence of using cold water and refrigeration in the processing environment also seems to have an influence in reducing microbial loads, since RTE vegetables produced in refrigerated processing plant A were lower than in the other plants. The use of cold water during processing of vegetables lowers metabolism and respiration rate, which helps to prevent the internalization and infiltration of bacteria, increasing shelf life (Sapers, 2003). However, increased costs and health issues for employers due to work with refrigerated water were reported in most visited processing plants.

Although those parameters discussed above (washing of vegetables in ozonated or refrigerated water/environment) seems to have an influence on the reduction of microbial counts, there is no statistical test able to draw appropriate comparisons, due to the limit number of water and RTE vegetables samples collected at one unspecified point in time. That number is limited because the idea of this study was to collected samples at the same day of the visit in the ten selected processing plants, aiming to evaluate the microbiological quality of the RTE vegetables produced by those plants.

The differences in the proportion between the volume of water and the amount of vegetable immersed in this water in the studied processing plants had little or no effect on the microbiological results, as microbial counts of water samples collected from tanks that used 28 L of water did not differ from those collected from tanks that used 5 L of water (data not shown). All ten visited processing plants were in accordance with Cenci, Gomes, Alvarenga, and Junior (2006), who recommend the use of at least 5–10 L of water for washing 1 kg of product.

There was no linear correlation between microbial counts in the water samples and those obtained in RTE vegetables, i.e, the processing plants presenting higher microbial counts in water samples were not the ones that presented higher microbial counts in the produced RTE vegetables. In conclusion, data collected in this study contributes to a better understanding of the RTE vegetables processing chain, helping government and industry in the establishment of proper control measures to ensure safety of this increasingly important type of healthy, fresh and convenient food.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2016.02.001.

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Letters in Applied Microbiology

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ORIGINAL ARTICLE

Assessing the effect of sodium dichloroisocyanurate concentration on transfer of *Salmonella enterica* serotype Typhimurium in wash water for production of minimally processed iceberg lettuce (*Lactuca sativa* L.)

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Significance and Impact of the Study: In this study, the impact of sodium dichloroisocyanurate in the wash water on transfer of *Salmonella* Typhimurium from inoculated lettuce to wash water and then to other noninoculated lettuces washed sequentially in the same water was evaluated. The use of chlorinated water, at concentration above 10 mg l^{-1}, effectively prevented *Salm*. Typhimurium transfer under several different washing scenarios. Conversely, when nonchlorinated water was used, *Salm*. Typhimurium transfer occurred in up to at least 10 noninoculated batches of lettuce washed sequentially in the same water.

Keywords
bacterial transfer, cross-contamination, disinfection, food safety, minimally processed vegetables, *Salmonella*.

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Abstract

This study evaluated the impact of sodium dichloroisocyanurate (5, 10, 20, 30, 40, 50 and 250 mg l^{-1}) in wash water on transfer of *Salmonella* Typhimurium from contaminated lettuce to wash water and then to other noncontaminated lettuces washed sequentially in the same water. Experiments were designed mimicking the conditions commonly seen in minimally processed vegetable (MPV) processing plants in Brazil. The scenarios were as follows: (1) Washing one inoculated lettuce portion in nonchlorinated water, followed by washing 10 noninoculated portions sequentially. (2) Washing one inoculated lettuce portion in chlorinated water followed by washing five noninoculated portions sequentially. (3) Washing five inoculated lettuce portions in chlorinated water sequentially, followed by washing five noninoculated portions sequentially. (4) Washing five noninoculated lettuce portions in chlorinated water sequentially, followed by washing five inoculated portions sequentially and then by washing five noninoculated portions sequentially in the same water. *Salm.* Typhimurium transfer from inoculated lettuce to wash water and further dissemination to noninoculated lettuces occurred when nonchlorinated water was used (scenario 1). When chlorinated water was used (scenarios 2, 3 and 4), no measurable *Salm.* Typhimurium transfer occurred if the sanitizer was ≥10 mg l^{-1}. Use of sanitizers in correct concentrations is important to minimize the risk of microbial transfer during MPV washing.

Introduction

Fruits and vegetables are an important part of a healthy diet. Consumers have been encouraged to consume healthy and convenient foods, which has been reflected in an increased demand for minimally processed vegetables (MPV). MPV may be defined as fresh vegetables that have been subjected to minimal processing, such as peeling,

cutting, slicing, shredding, washing, drying, packaging and refrigerated storage (CAC 2003). MPV provide convenience to consumers while maintaining the shelf life and preserving the nutritive and sensorial properties of the food.

Concurrent with the increase in production and consumption, MPV have been associated with foodborne disease outbreaks, becoming a major concern for consumers, governments and the food industry. A number of foodborne illnesses associated with fresh produce have been reported in several countries (Berger *et al.* 2010; Callejón *et al.* 2015; CDC 2015).

Washing is an important step during minimal processing of vegetables as it can reduce microbial populations and remove dirt and debris. Wash water can, however, become a source of cross-contamination, and pathogenic micro-organisms can be spread throughout a batch of MPV. This study aimed to evaluate the transfer of *Salmonella* during the washing step for production of minimally processed iceberg lettuce (*Lactuca sativa* L.), simulating different processing scenarios using chlorinated and nonchlorinated water. The selected scenarios indicate probable cross-contamination pathways and reflect washing practices commonly seen in MPV processing plants in Sao Paulo, Brazil (unpublished data), where different batches of vegetables are washed sequentially in the same water. *Salmonella* was selected as it has caused several outbreaks associated with the consumption of fresh and minimally processed vegetables (Lynch *et al.* 2009; da Silva Felício *et al.* 2015; Vestrheim *et al.* 2015), and lettuce was used as MPV because this is the most frequently consumed leafy vegetable in Brazil (Anon 2013).

Results and discussion

Figure 1 shows that washing one lettuce portion containing 6·6 ± 0·1 log CFU g^{-1} of *Salmonella enterica* serotype Typhimurium in nonchlorinated wash water (scenario 1) resulted in contamination of the water such that subsequent washing of noninoculated lettuces in this water resulted in counts of 5·4 ± 0·2 log CFU g^{-1} in the first washed lettuce portion. Subsequently washed portions also contained high *Salm*. Typhimurium levels (3·7 ± 0·1 to 4·3 ± 0·1 log CFU g^{-1}). After the wash water became contaminated with *Salm*. Typhimurium, the counts in the water remained approx. 4·8 ± 0·1 log CFU ml^{-1} until the last (10th) portion of lettuce was washed (Fig. 1). These results highlight the potential survival of this bacterium in the water and further transfer to incoming noninoculated vegetables.

Similar findings were reported in other studies. Holvoet *et al.* (2014) observed that washing lettuce portions contaminated with *Escherichia coli* (4·0 log CFU g^{-1}) in two subsequent nonchlorinated water baths caused a rapid transfer and high level of this bacterium in the water (4·0 and 3·5 log CFU per 100 ml in the first and second bath respectively) and the artificially contaminated water (3·0, 4·0 and 5·0 log CFU per 100 ml) caused a cross-contamination in the subsequently washed lettuce portions in levels of approx. 1·0 up to 1·9 log CFU g^{-1}. These authors also noticed a limited reduction of *E. coli* after washing a contaminated lettuce portion of approx. 4·0 log CFU g^{-1} in both baths of potable water (0·33 ± 0·1 and 0·16 ± 0·1 log reduction). Jensen *et al.* (2015) conducted experiments washing lettuce leaf pieces (one contaminated and 10 noncontaminated) simultaneously in nonchlorinated wash water and observed that most *E. coli* O157:H7 artificially inoculated in the contaminated samples was transferred to the wash water (90–99%), and this contaminated water cross-contaminated all the initially noncontaminated lettuce pieces.

Recent studies have focused on evaluation and modelling of the transfer of pathogenic bacteria (*Salmonella* and *E. coli*), norovirus and phages from contaminated to noncontaminated portions of vegetables during washing

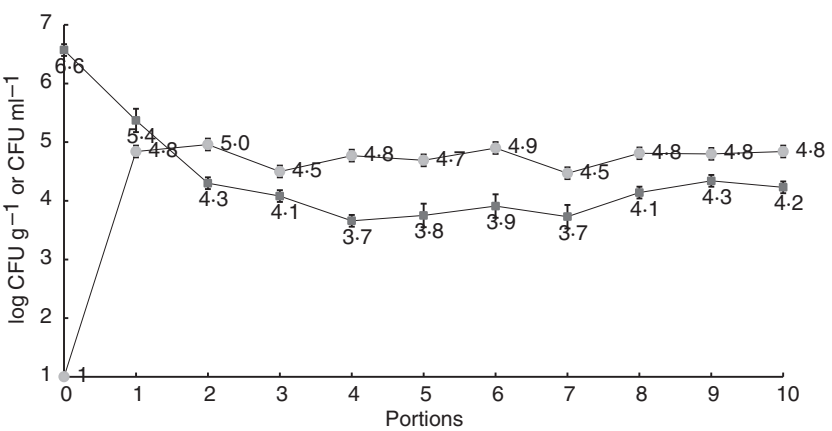

Figure 1 Transfer of *Salmonella* Typhimurium to 10 consecutive portions of lettuce washed in nonchlorinated water contaminated with the pathogen by washing one lettuce portion containing approx. 10^6 CFU g^{-1}. Counts in log CFU g^{-1} for lettuce (–■–) and log CFU ml^{-1} for water (–●–).

(Danyluk and Schaffner 2011; Holvoet *et al.* 2014; Perez-Rodriguez *et al.* 2014; Jensen *et al.* 2015). Although these studies shed light on the degree of pathogen transfer and mechanisms involved in the process, only few studies (López-Gálvez *et al.* 2009, 2010; Zhang *et al.* 2009; Tomás-Callejas *et al.* 2012) have considered the impact of disinfecting agents added to the wash water on cross-contamination, especially regarding *Salmonella*.

López-Gálvez *et al.* (2009) evaluated the risk of cross-contamination by nonpathogenic *E. coli* during washing of minimally processed lettuce using sodium hypochlorite (40 mg l^{-1} of free chlorine), Tsunami (500 mg l^{-1}), Citrox (5000 mg l^{-1}) and Purac (20 000 mg l^{-1}). They observed that chlorine and Tsunami were effective in reducing the inoculum levels in the processing water to the detection limit, but Citrox and Purac were not effective even at the highest manufacturer's recommended doses. In another study, López-Gálvez *et al.* (2010) observed that a sanitation step using active chlorine dioxide (3 mg l^{-1}) or sodium hypochlorite (100 mg l^{-1} of free chlorine) applied to minimally processed lettuce cross-contaminated by *E. coli* during washing, did not inactivate *E. coli* cells on the vegetable tissue, but inactivated most *E. coli* cells that passed from inoculated product to wash water.

Zhang *et al.* (2009) studied the efficacy-free chlorine and total peracid using sodium hypochlorite (30 and 50 mg l^{-1}), peroxyacetic acid and mixed peracid (10, 20, and 30 mg l^{-1}) on reduction of *E. coli* O157:H7 in water with or without 10% organic load. These researchers observed that all sanitizers were effective in reducing the bacterial counts in processing water, and in reducing the transfer of the bacteria from an inoculated leaf to noninoculated leaves in the processing water. Tomás-Callejas *et al.* (2012) studied the efficacy of sodium hypochlorite (free chlorine at 25 mg l^{-1}) and active chlorine dioxide (3 mg l^{-1}) to prevent *E. coli* O157:H7 and *Salmonella* cross-contamination on minimally processed Baby Red Chard during washing-disinfection, rinsing and de-watering steps. No colonies of *E. coli* O157:H7 and *Salmonella* were recovered from the noninoculated leaves regardless of the sanitizer used, but were detected by PCR-based methods. Furthermore, transference of viable *Salmonella* from inoculated leaves to the processing water was detected, with large populations recovered from the centrifugation effluent water.

All of the studies cited above evaluated the effect of different sanitizer (mainly sodium hypochlorite) on cross-contamination during washing, and most mixing contaminated and noncontaminated vegetables simultaneously. This study mimicked washing procedures observed in some Brazilian MPV processing plants, where subsequent portions of vegetables may be washed in the same water used to wash previous portions. If these previous portions are contaminated, cross-contamination may occur among different batches of MPV. Additional washing scenarios were also evaluated (scenarios 2, 3 and 4), using a chlorine-based sanitizer commonly used in Brazil (sodium dichloroisocyanurate), and not yet widely evaluated in the published literature (Fig. 2).

In scenario 2, the initial counts of *Salm*. Typhimurium were reduced by 0·5–2·0 log depending on the concentration of the sanitizer in the wash water (Table 1). Counts before and after washing differed significantly ($P < 0{\cdot}05$; by paired *t*-test) for all chlorine concentration tested. As expected, the highest concentration (250 mg l^{-1} of free chlorine) was the most effective, confirmed by ANOVA with *post hoc* Tukey's test. *Salmonella* Typhimurium in the noninoculated lettuce portions introduced in the water containing sodium dichloroisocyanurate was below the quantification and detection limit (<2 log CFU g^{-1} and absence in 25 g), regardless of the concentration of the sanitizer. However, when the free chlorine concentration was only 5 mg l^{-1}, *Salm* Typhimurium could not be counted (<2 log CFU g^{-1}) but was detected by enrichment (presence in 25 g) in the first noninoculated lettuce portion entering the process.

In scenario 3, where five inoculated lettuce portions were washed in water containing 50 mg l^{-1} (washing condition 1) and 250 mg l^{-1} of free chlorine (washing condition 2), followed by washing of five noninoculated portions, *Salm*. Typhimurium transfer was not observed (<2 log CFU g^{-1} and absence in 25 g after enrichment). These data indicate that even if additional contaminated lettuce portions are washed in the water in the beginning of the washing process, the chlorinated water (50 and 250 mg l^{-1}) effectively prevents *Salm*. Typhimurium transfer from the inoculated to the noninoculated lettuce portions. The same results were obtained for scenario 4 (<2 log CFU g^{-1} of *Salm*. Typhimurium and absence in 25 g). In these scenarios (2, 3 and 4), *Salm*. Typhimurium was not recovered from water samples (<2 log CFU ml^{-1}).

Chlorine-based products for washing of vegetables have been used much more as a strategy to reduce microbial load in the produce than to avoid cross-contamination (Gil *et al.* 2009). Chlorine is a very potent and low-cost sanitizer with robust oxidizing properties, recommended by health authorities around the world for disinfection of fruits and vegetables, usually at concentrations of 50–200 mg l^{-1} (Gil *et al.* 2009; Brazil 2013). However, the use of chlorinated water for production of RTE vegetables has limitations, such as inactivation of active agents by high level of organic load and risk of carcinogenic trihalomethane formation, and dependence on neutral pH for optimal activity (FAO/WHO 2008).

Data presented in our study will be extremely useful to produce processors and regulators to aid in setting standards for sanitizer in wash water to avoid cross-contamination. Our data will be especially useful for those

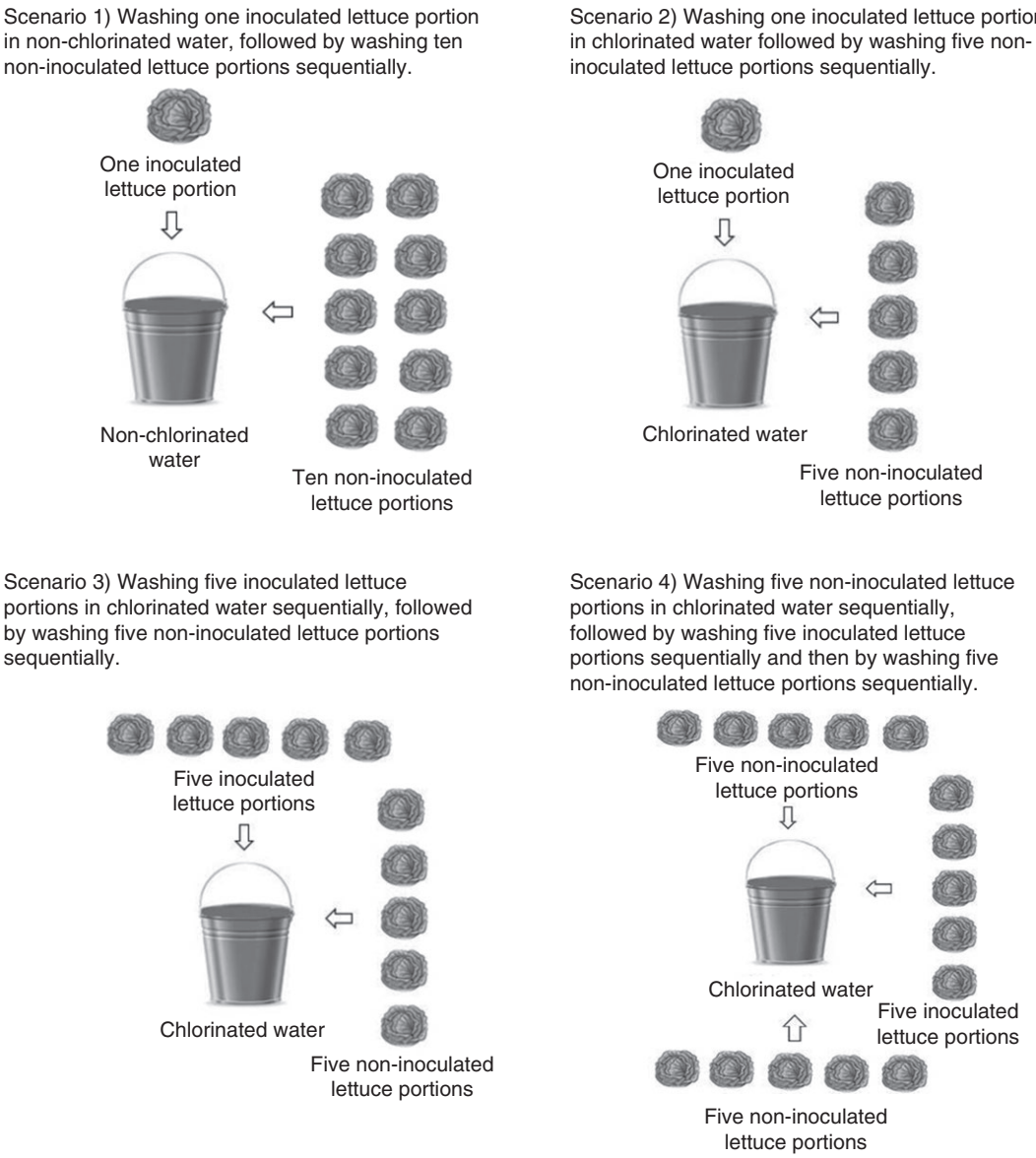

Figure 2 Scenarios simulating *Salmonella* Typhimurium transfer during washing of lettuce. See text for more details.

considering the use of sodium dichloroisocyanurate, as very few peer reviewed published articles have studied this compound for its efficacy in fresh produce washing (Nicholl and Prendergast 1998; Nascimento *et al.* 2003). Our study showed that the use of sodium dichloroisocyanurate in the wash water, at concentrations at least 10 mg l^{-1}, effectively prevented *Salm*. Typhimurium transfer from inoculated lettuce to the wash water and then to incoming noninoculated lettuce leaves under several different washing scenarios. Conversely, when vegetables were washed in nonchlorinated water, cross-contamination not only occurred but also continued to occur over at least 10 non-inoculated batches washed sequentially in the same water. These results highlight the potential survival of *Salm*. Typhimurium in the water and further transfer to incoming noncontaminated vegetables. Better control of MPV outbreaks can be expected if sanitizers in proper concentrations are applied during washing of MPV.

Materials and methods

Strains and electro-transformation of *Salmonella* to express green fluorescent protein

The study was conducted with a cocktail of three carbenicillin sensitive *Salm*. Typhimurium strains: two strains

Table 1 *Salmonella* counts in lettuces after washing in water with different chlorine concentrations (scenario 2)

	Counts of *Salmonella* (log CFU g^{-1})						
Free chlorine concentration (mg l^{-1})	Inoculated lettuce before washing	Inoculated lettuce after washing	1st noninoculated lettuce portion	2nd noninoculated lettuce portion	3rd noninoculated lettuce portion	4th noninoculated lettuce portion	5th noninoculated lettuce portion
250*	6·2 ± 0·1	4·2 ± 0·3	<2	<2	<2	<2	<2
50†	6·1 ± 0·1	5·3 ± 0·3	<2	<2	<2	<2	<2
40‡	5·7 ± 0·1	4·9 ± 0·1	<2	<2	<2	<2	<2
30‡	5·8 ± 0·2	5·0 ± 0·1	<2	<2	<2	<2	<2
20‡	6·1 ± 0·1	5·4 ± 0·1	<2	<2	<2	<2	<2
10‡	5·7 ± 0·1	5·0 ± 0·1	<2	<2	<2	<2	<2
5‡	5·9 ± 0·1	5·4 ± 0·1	<2	<2	<2	<2	<2

Results expressed as mean ± SD.

Salmonella Typhimurium was detected (presence in 25 g, after enrichment step) in the 1st noninoculated lettuce portion only when the chlorine concentration in water was 5 mg l^{-1}.

*Wash water: pH 8·0, organic load 2·3 mg l^{-1}; contact: 20 min at 10°C.

†Wash water: pH 5·6, organic load 13·5 mg l^{-1}; contact: 2 min at 25°C.

‡Wash water: pH 7·0, no organic load; contact: 10 min at 25°C.

(#277 and #386) isolated from MPV sold in Sao Paulo, Brazil (Sant'Ana *et al.* 2011) and one reference strain (ATCC 14028). The three strains were electro-transformed to express green fluorescent protein (GFP) and resistance to ampicillin (Sambrook *et al.* 1989), to distinguish them from other *Salmonella* possibly present in the vegetables. Before electroporation, strains were tested for sensitivity to carbenicillin by checking absence of growth inoculation in 10 ml of TSB containing carbenicillin (50 μg ml^{-1}), at 37°C for 24 h.

Each strain was grown overnight at 37°C in 10 ml of Tryptic Soy Broth (TSB) (Oxoid, Basingstoke, UK) and OD600nm (Ultrospec 2000; Pharmacia Biotech, Cambridge, UK) was measured from time to time until proper dilution resulted in OD600nm = 0·2. This culture was incubated at 37°C under agitation at 170 rev min^{-1} (Innova 4000; New Brunswick Scientific, Edison, NJ) until OD600nm = 0·5. After cooling to 4°C, the culture was centrifuged at 3000 ***g*** for 15 min at 4°C (6–16 K; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The resulting pellet was washed twice in sterile cold water (10°C), resuspended in 1 ml of 10% glycerol (Synth, Diadema, Brazil) and divided into 40 μl aliquots in sterile microcentrifuge tubes. The content of the tubes was mixed with 1 μl of the pGFPuv Vector plasmid (Clontech, Mountain View, CA) in a 0·2 cm gap width cuvette and an electric pulse of 2·5 kV cm^{-1} was applied (MicroPulser; Bio-Rad Laboratories, Hercules, CA) for electro-transformation. Sterile TSB was added to the cuvette and electro-transformed cells were incubated at 37°C for 1 h, and plated onto Mannitol Lysine Crystal Violet Brilliant Green Agar (MLCB) (Oxoid) supplemented with carbenicillin disodium salt (50 μg ml^{-1}) (Sigma Aldrich, Rehovot, Israel). *Salmonella* Typhimurium colonies presenting fluorescence under UV light (366 nm) were inoculated into 50 ml of TSB supplemented with carbenicillin (50 μg ml^{-1}), following incubation at 37°C for 24 h. The cultures were frozen in microcentrifuge tubes (30% glycerol, 70% TSB) and stored at −70°C in an ultra-low-temperature freezer (Model 910; Thermo Fisher Scientific Inc., Rockford, IL) until use. The plasmid stability of transformed *Salm*. Typhimurium strains was tested by growing them in 10 ml of TSB at 37°C for 24 h, followed by plating on MLCB Agar with and without carbenicillin (50 μg ml^{-1}). Carbenicillin was required for the expression of GFP, indicating that the plasmid of transformed *Salm*. Typhimurium strains was stable. Furthermore, the presence of the plasmid did not seem to affect the survival capacity and resistance of the transformed *Salm*. Typhimurium strains, as these were able to survive in nonchlorinated water even after washing up to 10 batches of vegetables, and were not resistant in chlorinated water (i.e. inactivation was possible at low chlorine concentrations).

Experimental contamination of lettuce

The frozen cultures of electro-transformed *Salm*. Typhimurium strains were thawed, inoculated into 100 ml of TSB supplemented with carbenicillin (50 μg ml^{-1}) and incubated at 37°C for 24 h. Cells were washed twice (2810 ***g*** for 5 min at 4°C) and the pellet was suspended in 0·1% peptone (Oxoid). The *Salm*. Typhimurium cell suspensions were combined in equal volumes to achieve approx. 10^8 log CFU ml^{-1}. Exact counts were determined

by enumeration in MLCB supplemented with carbenicillin (50 μg ml^{-1}).

Whole unprocessed heads of iceberg lettuce were purchased in local supermarkets in the city of Sao Paulo, Brazil, and kept under refrigeration for no longer than 24 h until the experiments were performed. Injured or damaged leaves were removed and the remaining leaves were individually washed in running tap water provided by the Sao Paulo municipality and were cut (approx. 3 cm width) with a sterile knife. The washed lettuce pieces were experimentally contaminated with *Salm*. Typhimurium by immersion in 4 l of distilled water containing the cocktail of the three electro-transformed strains (10^6 CFU ml^{-1}) for 30 min. Tests for presence of *Salmonella* spp. in the incoming lettuce samples prior to inoculation were all negative (data not shown).

Scenarios simulating *Salmonella* transfer during washing of lettuce

The following washing scenarios were investigated (Fig. 2): (1) Washing one inoculated lettuce portion in nonchlorinated water, followed by washing 10 noninoculated lettuce portions sequentially. (2) Washing one inoculated lettuce portion in chlorinated water followed by washing five noninoculated lettuce portions sequentially. (3) Washing five inoculated lettuce portions in chlorinated water sequentially, followed by washing five noninoculated lettuce portions sequentially. (4) Washing five noninoculated lettuce portions in chlorinated water sequentially, followed by washing five inoculated lettuce portions sequentially and then by washing five noninoculated lettuce portions sequentially in the same water. These scenarios indicate probable cross-contamination pathways and reflect washing practices commonly seen in MPV processing plants in Sao Paulo, Brazil (unpublished data), where different batches of vegetables are washed sequentially in the same water. The designed scenarios aimed to evaluate the consequence of washing one or more contaminated lettuce portions in the same water where noncontaminated portions are washed (at the beginning or during the process), as well to compare the cross-contamination pathways using or not using the chlorinated water. The lettuce to wash water ratio was 1 : 10 (i.e. 100 g portions of shredded lettuce to 1 l of water), a proportion commonly used by Brazilian processing plants. Each lettuce portion represented a lot. Preliminary tests with larger amounts of lettuce (500 g) and wash water (5 l) resulted in the same *Salm*. Typhimurium transfer to the wash water and to the lettuce (data not shown). The chlorine sanitizer was sodium dichloroisocyanurate (Sumaveg, Johnson Diversey, Sao Paulo, Brazil) which is widely used in Brazil instead of sodium hypochlorite, due to good stability in aqueous solution and greater efficacy in the presence of higher organic loads (Salomão *et al.* 2011). The free chlorine concentration in the wash water was measured and adjusted manually using a portable photometer (HI96771; Hanna Instruments, Ann Arbor, MI). Samples of water were collected in cuvettes (10 ml) and added with appropriate reagents (HI 95771-01) for the colorimetric determination of chlorine, following the manufacturer's instructions.

Two washing conditions were tested for each scenario: (1) wash water: pH 5·6, organic load 13·5 mg l^{-1} and free chlorine concentration 50 mg l^{-1}; contact: 2 min at 25°C, and (2) wash water: pH 8·0, organic load 2·3 mg l^{-1} and free chlorine concentration 250 mg l^{-1}; contact: 20 min at 10°C. These conditions represent minimum and maximum range of physical and chemical parameters observed during the washing step of vegetables in Brazilian processing plants (Maffei *et al.* 2016), and overlap with the Brazilian Health Surveillance Agency recommendations on the use of chlorine-based products for disinfection of fresh produce at maximum concentration of 250 mg l^{-1} of chlorine (Brazil 2013). The simulation of these washing parameters allowed evaluation of the occurrence of cross-contamination under water conditions observed in washing procedures used in Brazilian processing plants. A preliminary study also assessed the influence of wash water parameters (pH, organic load, temperature, free chlorine concentration and time of contact) on inactivation of *Salm*. Typhimurium during washing step and indicated that chlorine concentration was the most important parameter for reduction of *Salm*. Typhimurium, while the other parameters were less relevant within the analysed interval (data not shown). There are a number of publications on the influence of the physicochemical parameters of water on the activity of disinfectants (Park *et al.* 2004; Pirovani *et al.* 2004; Stopforth *et al.* 2008; López-Gálvez *et al.* 2010). A meta-analysis conducted by Prado-Silva *et al.* (2015) including data from 40 studies on the effect of sanitizing treatments of fresh produce concluded that in addition to chlorine concentration, parameters such as washing time and temperature can significantly affect the mean log reduction of sanitizing treatments.

Scenario 2 was selected to perform an extra washing condition with a lower chlorine concentration (5, 10, 20, 30 and 40 mg l^{-1}) and physicochemical parameters selected to provide a better efficacy of the sanitizer: wash water at 25°C, with a pH 7·0, no organic load and a contact time of 10 min.

Enumeration of *Salmonella*

The washed shredded lettuce was transferred to a sanitized salad spinner (SecaSaladaPlick, Sao Paulo, Brazil)

and spun for 1 min inside a biosafety laminar hood (VLFS-12; Veco, Campinas, Brazil) to remove excess water. At least two 25 g samples of each washed portion were homogenized with 225 ml of 0·1% peptone for 1 min in a Stomacher 400 Lab-blender (Seward Medical, London, UK). Serial decimal dilutions were prepared in 0·1% peptone and plated onto MLCB Agar supplemented with carbenicillin (50 μg ml^{-1}). Plates were incubated at 37°C for 24 h and typical *Salm*. Typhimurium colonies expressing GFP under UV light (366 nm) were counted. Additionally, another 25 g sample of each portion of washed lettuce was homogenized with 225 ml of Buffered Peptone Water (BPW) (Difco, Sparks, MD) and tested for *Salmonella* according to ISO 6579 (2002), adding carbenicillin (50 μg ml^{-1}) to all culture media. Results were expressed as presence or absence of *Salmonella* in 25 g of lettuce.

Enumeration of *Salm*. Typhimurium in wash water (50 ml) was done by plating onto MLCB Agar supplemented with carbenicillin (50 μg ml^{-1}) and incubated at 37°C for 24 h. The sanitizer in wash water was inactivated by addition of 1 mol l^{-1} sterile sodium thiosulphate (Sigma-Aldrich ChemieGmbH, Steinheim, Germany) after chlorine treatment, and before plating.

Data analysis

All experiments were repeated at least twice, and plating was done in duplicate. Average counts of *Salm*. Typhimurium in lettuce and wash water were expressed as CFU g^{-1} or CFU ml^{-1}, respectively, and log transformed. A paired *t*-test was used to determine whether counts of *Salm*. Typhimurium before and after washing differed significantly ($P < 0{\cdot}05$). ANOVA followed by Tukey's test were performed to determine significant differences in *Salm*. Typhimurium counts in lettuces washed in wash water with different chlorine concentrations. SIGMAPLOT ver. 12.5 (Systat Software Inc., San Jose, CA) was used for the statistical analysis.

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Conflict of Interest

No conflict of interest declared.

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