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Contaminação microbiológica e avaliação da segurança de alface na produção primária e varejo

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2015

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CONTAMINAÇÃO MICROBIOLÓGICA E AVALIAÇÃO DA SEGURANÇA DE ALFACE NA PRODUÇÃO PRIMÁRIA E VAREJO

Tese apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos (Área de concentração: Ciência e Tecnologia de Alimentos) como requisito para obtenção ao grau de Doutor em Ciência e Tecnologia de Alimentos.

Orientador: Eduardo César Tondo
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Aprovada em: ___/___/___

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RESUMO

Nos últimos anos, o consumo de vegetais frescos tem aumentado significativamente, em todo o mundo. Ao mesmo tempo, a ocorrência de Doenças Transmitidas por Alimentos (DTA) associadas a esses alimentos tem crescido de forma considerável, podendo levar as vítimas a consequências severas, inclusive à morte. Dados internacionais demonstram diversos microrganismos envolvidos nestes surtos, porém a *Salmonella* e a *Escherichia coli* O157:H7 têm sido frequentemente envolvidas. Este trabalho Tese teve o objetivo de avaliar a contaminação microbiológica e a segurança de alfaces convencionais produzida na região Sul do Brasil, desde a produção primária até o varejo. Para tanto, primeiramente, 128 amostras de adubo orgânico, solo, água de irrigação e lavagem, mãos de manipuladores, equipamentos, mudas e pés de alface foram coletadas em 3 propriedades rurais, durante o período de cultivo das alfaces. As amostras foram analisadas para quantificação de indicadores (*E. coli*) e para a presença de patógenos (*Salmonella* e *Escherichia coli* O157:H7). Paralelamante, um questionário de auto-avaliação, com 69 questões, foi aplicado com o objetivo de obter informações sobre os sistemas de gestão implementados nas propriedades rurais. Em seguida, foi realizada a comparação dos resultados microbiológicos das amostras coletadas, informações climáticas e de informações de sistemas de gestão das 03 propriedades produtoras de alfaces convencionais avaliadas neste trabalho e 03 propriedades produtoras de alfaces orgânicas avaliadas em outro trabalho. Os dados microbiológicos, as informações de gestão e informações a respeito de fatores climáticos foram analisados, no intuito de identificar as principais fontes de contaminação das alfaces. Posteriormente, 100 amostras de alface convencionais foram coletadas em hipermercados de Porto Alegre e analisadas para contagens de coliformes, *E. coli* e para a presença de *E. coli* O157:H7 e *Salmonella*. Em seguida, alfaces foram artificialmente contaminadas com *Salmonella* Enteritidis SE86 e *E. coli* ATCC8739 e armazenadas em diferentes temperaturas e intervalos de tempo (5, 10, 25 e 37 °C por 0, 2, 6, 24 e 48 h). Os dados obtidos foram modelados matematicamente, a fim de avaliar situações de risco dentro dos hipermercados. Os resultados obtidos na produção primária demonstraram baixas contagens de *E. coli* e ausência de patógenos em todas as amostras coletadas, porém um alto risco microbiológico foi identificado, para todas propriedades, através do questionário de auto-avaliação. A diferença entre ambos resultados pôde ser explicada pela adoção de algumas medidas como utilização de adubo adequadamente compostado, utilização de água de irrigação e lavagem apropriadas, ausência de animais nas propriedades, entre outros, porém não havia nenhum sistema de segurança de alimentos formalmente implementado. Os resultados também demonstraram que as propriedades orgânicas apresentaram um maior risco microbiológico e presença de patógenos quando comparadas com as propriedades convencionais, fato explicado devido à ocorrência de um evento de inundação, pela utilização de adubo orgânico produzido na própria propriedade, sem adequada compostagem. As alfaces coletadas nos hipermercados demonstraram 4% de prevalência de *E. coli* e presença de *Salmonella* em uma amostra. *Salmonella* e *E. coli* não se multiplicaram na alface a 5 e 10 °C durante 48 horas, sugerindo serem estas temperaturas adequadas para o armazenamento. Por outro lado, os mesmos microrganismos se multiplicaram a 25 e 37 °C, atingindo níveis elevados que podem oferecer risco à

saúde do consumidor, mesmo após uma higienização adequada. Como conclusão, a implementação de Boas Práticas Agrícolas, enfocando a compostagem adequada e qualidade microbiológica da água de irrigação e lavagem, é necessária para evitar a contaminação de alfaces na produção primária. Além disso, os resultados demonstraram a necessidade da manutenção dos vegetais folhosos frescos em cadeia refrigerada abaixo de 10 °C, desde a etapa de colheita até o consumo, a fim de evitar riscos de surtos alimentares relacionados a estes produtos.

ABSTRACT

The consumption of fresh produce is arising significantly, in the last years, worldwide. At the same time, foodborne outbreaks associated to these products are also increasing considerably, leading the victims to severe consequences, including death. International data demonstrated several microorganisms involved with these outbreaks, and *Salmonella* and *Escherichia coli* O157:H7 are frequently reported. This Thesis aimed to evaluate the microbial contamination and safety of conventional lettuces produced in Southern Brazil, from primary production to retail. In the first study, 128 samples of manure, soil, irrigation and washing water, worker's hands, equipment, seedlings and lettuces were taken from three conventional farms, during the growth period. Lettuces samples were analysed for indicators (*E. coli*) and for the presence of pathogens (*Salmonella* and *Escherichia coli* O157:H7). At the same time, a self-assessment questionaire, composed by 69 questions, was applied, willing tho get information about the management sistems implemented in the farms. In the second article, a comparison among the microbial results, climatic information and management systems of three conventional and three organic farms was carried out. Microbial data, information about management sistems and information about climatic conditions were analysed, with the aim to identify the major contamination sources of lettuces. In the third manuscript, 100 samples of conventional lettuces were taken from hypermarkets of Porto Alegre city, Southern Brazil, and analysed for coliforms and *E. coli* counts and for the presence of *E. coli* O157:H7 and *Salmonella*. After that, lettuces were artificially contaminated with *Salmonella* Enteritidis SE86 e *E. coli* ATCC8739 and stored at different temperatures and times (5, 10, 25, and 37°C for 0, 2, 6, 24, and 48 h). Data were modelled in order to evaluate risk situations inside hypermarkets. Results from primary production demonstrated low levels of *E. coli* and absence of pathogens in all analysed samples, but a high microbial risk was identified, for all farms, by the self-assessment questionaire. Differences between both results could be explained by the adoption of some adequate measures as the use of manure properly composted, use of water of good quality, absence of animals in the farms, but, at the same time, no formal management system was applied. Results also showed that organic farms demonstrated higher microbial risks based on questionnaire responses and presence of pathogens when compared with conventional ones, and these was explained mainly because a flooding event and the use of manure without adequate composting time was observed in the organic farms. Lettuces collected in the hypermarkets demonstrated 4% of *E. coli* prevalence and the presence of *Salmonella* in one sample. *Salmonella* and *E. coli* did not multiply on lettuce kept at 5 and 10 °C for 48 hours, suggesting that these could be adequate temperatures of storage. On the other hand, the same microorganisms multiply at 25 and 37 °C, reaching high levels that could represent risk to consumer's health, even after disinfection procedures. Concluding, the implementation of Good Agricultural Practices, focusing the adequate composting of manure and adequate microbial quality of irrigation and washing water is necessary in order to avoid contamination of lettuces in primary production. Besides that, results demonstrated the necessity of mantainence of fresh produce in cool chain below 10 °C, since harvest until consumption in order to avoid risks related to these products.

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1 INTRODUÇÃO

O aumento no consumo de vegetais frescos tem sido observado no mundo todo e isto se deve ao fato de os mesmos estarem associados a uma dieta saudável, à prevenção de doenças severas e ao seu conteúdo nutricional de vitaminas, fibras e sais minerais (WARRINER et al., 2009; JACXSENS et al., 2010; EFSA, 2014; CALLEJÓN et al., 2015). De acordo com dados da União Europeia (UE) e da Food and Agriculture Organizazion, o consumo mundial de vegetais frescos aumentou, anualmente, em torno de 4,5% no período de 1990 a 2004 (EU, 2007; FAO/WHO, 2008). Já dados da Food and Agriculture Organizazion (FAO/WHO, 2008), enquanto que nos Estados Unidos da América (EUA) o consumo aumentou 25% quando comparado o período entre 1997 a 1999 com o período de 1977 a 1997 (FDA, 2001). O mesmo pôde ser observado no Canadá, onde ocorreu um aumento de 26% no consumo de vegetais, no período de 1963 a 2010 (STATISTICS CANADA, 2011).

A ocorrência de Doenças Transmitidas por Alimentos (DTA) associadas a vegetais frescos também tem aumentado de forma considerável, nos últimos anos, em todo o mundo, podendo levar as vítimas a consequências severas, inclusive à morte (ARUSCAVAGE et al., 2006; FAO/WHO, 2008; WARRINER et al., 2009; FAOSTAT; EFSA, 2013;). Nos Estados Unidos, segundo o *Centers For Diseases and Control* (CDC), os vegetais frescos foram a segunda maior causa de DTA, ficando atrás apenas dos produtos de frango, no período de 2003 a 2008 (PAINTER et al., 2013). A *Salmonella* tem sido identificada como a bactéria mais frequentemente envolvida nestes surtos, correspondendo a 18% dos casos nos Estados Unidos e 20% na União Europeia, seguida da *Escherichia coli*, a qual foi responsável por 12,3% e 3,8 % dos surtos nestes mesmos locais, respectivamente, no período de 2004 a 2012 (CALLEJÓN et al., 2015). Diversos autores corroboram estes dados, evidenciando que a *Salmonella* e a *E. coli* têm sido os principais microrganismos causadores de surtos relacionados a produtos frescos (FDA, 1998; BUCK; WALCOTT; BEUCHAT, 2003; WARRINER et al., 2009; OILAMAT; HOLLEY, 2012).

Dentre os vegetais consumidos frescos, a alface (*Lactuca sativa*) tem ganho destaque, uma vez que tem sido apontado como o vegetal folhoso mais consumido no Brasil (CEAGESP 2012) e no mundo (FAOSTAT, 2013). Dados nacionais

demonstram um consumo elevado de alface, sendo que a comercialização no maior mercado brasileiro, CEAGESP (Companhia de Entrepostos e Armazéns Gerais de São Paulo), alcança mais de 21.000 toneladas anuais. Nesse mercado, a alface crespa representou cerca de 65% do consumo, seguido da alface americana (20%), da alface lisa (10%) e das outras variedades (AGRIANUAL, 2008).

A contaminação dos vegetais folhosos pode acontecer em diversas fases de sua produção e devido a vários fatores, tais como a inadequada compostagem do adubo orgânico, solo contaminado, água de irrigação contaminada por fezes de animais ou humanas, a presença de animais no campo, a ocorrência de fatores climáticos (chuvas intensas e inundações), contaminação pelos manipuladores durante a colheita. Além destes fatores, os vegetais também podem ser contaminados após a colheita pela água de enxágue contaminada, manipuladores com más condições de higiene, caixas e meios de transporte, poeira e pragas (GUO et al., 2002; BEUCHAT, 2002; LANG; SMITH, 2007; WHIPPS et al., 2008; ZHANG et al., 2009; ERICKSON et al., 2010; EFSA, 2014).

Em vista disso, o objetivo geral do presente estudo foi avaliar a contaminação microbiológica e a segurança da alface fresca produzida na região Sul do Brasil, desde a produção primária até o varejo.

Os objetivos específicos foram:

a) avaliar a situação atual das práticas agrícolas e sistemas de gerenciamento de propriedades produtoras de alface convencional no sul do Brasil a fim de identificar os maiores gargalos durante o período de cultivo relacionados a contaminações microbiológicas.

b) identificar as diferenças potenciais nos parâmetros microbiológicos entre propriedades de cultivo orgânico e entre os sistemas de cultivo.

c) avaliar a contaminação e a modelagem de crescimento de *Salmonella* e *Escherichia coli* em alfaces comercializadas em hipermercados no sul do Brasil.

2. REVISÃO BIBLIOGRÁFICA

2.1 DADOS DE MERCADO

Na última década a produção mundial de vegetais frescos cresceu por volta de 38% (FAOSTAT, 2013).

No Brasil, o vegetal fresco mais produzido é a alface, *Lactuca sativa*, representando por volta de 40% do total destes produtos, de acordo com Sala e Costa (2012), os principais tipos de alface cultivados em ordem de importância econômica são a crespa, americana, lisa e romana.

O sistema brasileiro de produção de alface está baseado nos "cinturões verdes", ou seja, áreas localizadas próximo aos centros urbanos onde estes vegetais são consumidos, ao contrário dos sistemas de produção existentes na Europa e Estados Unidos, os quais contam com um moderno sistema logístico necessariamente associado à cadeia de frio (SALA; COSTA, 2012).

2.2 SURTOS ALIMENTARES ASSOCIADOS A VEGETAIS FRESCOS

DTA associadas ao consumo de vegetais frescos têm sido cada vez mais notificadas no mundo. Por exemplo, esses alimentos foram considerados um dos cinco principais grupos associados à DTA na União Europeia, principalmente por serem consumidos crus (EFSA, 2014). Em 2011, um grave surto ocorrido na Alemanha e França, devido ao consumo de brotos de feno grego contaminados por uma cepa de *E. coli* enterohemorrágica, vitimou mais de 4.000 pessoas e causou 56 mortes (EFSA, 2014), demonstrando a importância da associação do consumo destes produtos com questões relacionadas à segurança dos alimentos.

Nos últimos anos, o aumento da notificação de surtos veiculados por vegetais frescos tem sido observado também nos Estados Unidos, correspondendo a 22,8% do total de surtos alimentares ocorridos naquele país, no período de 1998 a 2007 (CSPI, 2009). De acordo com dados americanos, foi estimado que 24% dos surtos ocorridos nos Estados Unidos, equivalendo a 20 milhões de doentes, foram causados por vegetais, processados ou enlatados, correspondendo a um custo

anual de mais de 38 bilhões de dólares (SCHARFF, 2010). De acordo com o *Food and Drug Administration* (FDA, 2009a), anualmente, os vegetais folhosos são responsáveis por cerca de 1/3 do total de surtos ocorridos. Dentre os vegetais frescos, os vegetais folhosos, como por exemplo, alfaces, repolhos, endívias, couve chinesa, espinafre, agrião, entre outros, têm sido frequentemente associados aos surtos, sendo que Painter et al (2013) demonstraram que eles foram identificados como a segunda maior causa de DTA, nos EUA, no período de 2003 a 2008. Apesar desses dados, vale salientar que os profissionais da saúde, como médicos e nutricionistas, consideram que os riscos de se contrair uma DTA pela ingestão de vegetais frescos são muito menores do que o benefício que a ingestão destes alimentos trará para a saúde, sabendo que são importantes na prevenção de diversas doenças, tais como câncer, doenças cardíacas, diabetes e obesidade (NYACHUBA, 2010).

Na Tabela 1 é possível observar os surtos causados por vegetais, em diversos países, no período entre 2005 a 2011.

Tabela 1. Surtos causados por vegetais frescos, de 2005 a 2011, em diversos países

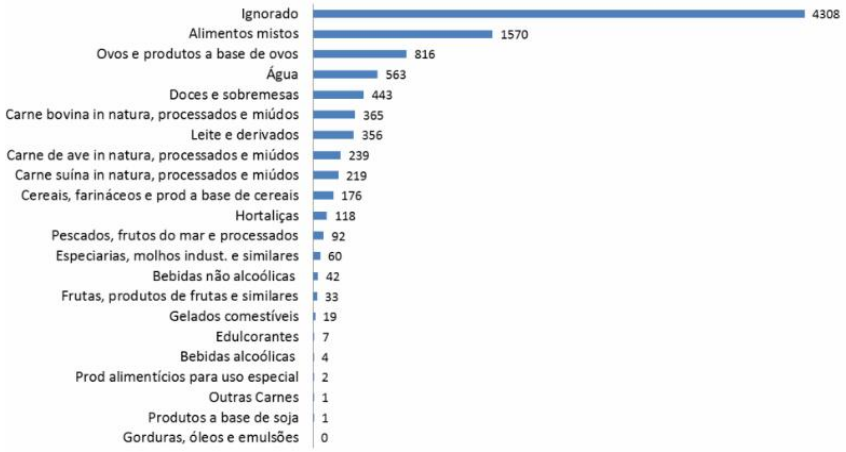
Local	**Ano**	**Patógeno**	**Produto Fresco**	**Casos (mortes)**	**Referência**
Canadá	2005	*Salmonella*	Broto de feijão	592	Rohekar et al., 2008
EUA	2005	*Salmonella*	Tomate	459	CDC, 2007
EUA	2006	*E. coli* O157:H7	Espinafre	199 (3)	CDC, 2006b
Austrália	2006	*Salmonella*	Broto de alfafa	125	Compton et al., 2008
EUA, Canadá	2006	*Salmonella*	Salada de frutas	41	Landry et al., 2007
EUA	2006	*Salmonella*	Tomate	183	CDC, 2006a
EUA	2006	*E. coli* O157:H7	Alface	81	FDA, 2007
Austrália	2006	*Salmonella*	Melão	115	Munnoch et al., 2008
EUA	2006	*E. coli* O157:H7	Espinafre	22	Grant et al., 2006
Europa	2007	*Salmonella*	Espinafre baby	354	Denny et al., 2007
América do Norte, Europa	2007	*Salmonella*	Manjericão	51	Pezzoli et al., 2007
Austrália, Europa	2007	*Shigella sonnei*	Cenoura baby	230	Lewis et al., 2007
Europa	2007	*Salmonella*	Broto de alfafa	45	Emberland et al., 2007
EUA, Canadá	2008	*Salmonella*	Pimentões	1442 (2)	CDC, 2008b; Mody et al.,
EUA, Canadá	2008	*E. coli* O157:H7	Alface	134	2011
Reino Unido	2008	*Salmonella*	Manjericão	32	Warriner and Namvar, 2010
EUA	2008	*Salmonella*	Melão	51	Elviss et al., 2009
EUA, Canadá	2008	*Salmonella*	Manteiga de amendoim	714 (9)	CDC, 2008a
EUA	2009	*Salmonella*	Broto de alfafa	235	CDC, 2009b
EUA	2010	*E. coli* O145	Alface	26	CDC, 2009a
EUA	2010	*Salmonella*	Broto de alfafa	44	CDC, 2010a
EUA	2010	*L. monocytogenes*	Aipo	10 (5)	CDC, 2010b
EUA	2011	*Salmonella*	Brotos de alfafa e mistos	140	FDA, 2010
EUA	2011	*Salmonella*	Melão	20	CDC, 2011b
EUA	2011	*Salmonella*	Papaya	106	CDC, 2011c
Europa	2011	*E. coli* O104:H4	Brotos de vegetais	3911(47)	CDC, 2011d
EUA	2011	*L. monocytogenes*	Melão	146 (31)	ECDC, 2011; EFSA, 2011
EUA	2011	*E. coli* O157:H7	Morango	15 (1)	CDC, 2011e
EUA	2011	*E. coli* O157:H7	Alface	60	FDA, 2011; CDC, 2011a

Fonte: Oilamat e Holey (2012)

É sabido que o número de surtos reportados não corresponde ao número real de surtos ocorridos em uma população, devido a problemas de subnotificação, falta de investigação epidemiológica ou outras questões ligadas aos sistemas de vigilância em saúde (O'BRIEN et al., 2002; EFSA, 2008; ARENDT et al., 2013).

No Brasil, surtos alimentares associados a vegetais ocupam a décima posição no *ranking* oficial do Ministério da Saúde (MS), tendo sido responsáveis por 118 do total de 9.718 surtos reportados no período de 2000 a 2014, como pode ser observado na Figura 1. Os alimentos mistos, ovos e produtos à base de ovos foram os principais alimentos relacionados aos surtos, porém, é importante ressaltar que não foi possível identificar os alimentos causadores de surtos em cerca de 50% dos casos investigados (Brasil, 2014).

Figura 1 - Numero de surtos alimentares associados ao grupo de alimentos no Brasil de 2000 a 2014



Fonte: Brasil (2014)

2.3 PATÓGENOS ASSOCIADOS A SURTOS COM VEGETAIS FRESCOS

Diversos são os patógenos que podem estar associados a DTA ocasionadas por vegetais frescos, especialmente pelo fato de estes produtos serem consumidos, na sua maioria sem nenhum tipo de tratamento térmico. Entre os patógenos, o que aparece em primeiro lugar é o *Norovirus*, responsável por 59% dos surtos com vegetais frescos ocorridos nos EUA e por 53% dos surtos ocorridos na UE no

período de 2004 a 2012, seguido pela *Salmonella*, com 18% e 20% dos surtos e da *E. coli*, com 12,2% e 3,8%, respectivamente (CALLEJÓN et al., 2015).

A tabela 2 apresenta um resumo dos surtos associados a vegetais frescos ocorridos nos EUA entre 2004 e 2012.

Tabela 2 - Resumo dos surtos associadoa a vegetais frescos e frutas nos Estados Unidos, 2004-2012

	Alimento envolvido									
	Vegetais					Frutas				
Patógeno	Saladas	Folhosos	Tomates	Outros	Brotos	Frutas vermelhas	Melão	Sucos	Outros	Total
Norovirus	97	62	5	9	0	5	9	3	33	223
Salmonella spp.	8	8	17	3	14	2	14	0	5	71
Escherichia coli	10	22	0	0	4	2	0	6	2	46
Campylobacter spp.	4	2	1	0	0	0	1	0	1	9
Shigella spp.	1	2	0	0	0	0	0	0	0	3
Clostridium spp.	0	0	0	0	0	0	0	0	0	0
Staphylococcus spp.	2	0	0	0	0	0	0	0	0	2
Yersinia spp.	0	0	0	0	0	0	0	0	0	0
Bacillus spp.	1	0	0	0	0	0	0	0	1	2
Giardia spp.	0	1	0	1	0	0	0	0	0	2
Cyclospora spp.	1	1	0	1	0	3	0	0	2	8
Cryptosporidium spp.	0	0	0	0	0	0	0	3	0	3
Outras viroses alimentares (vírus da hepatite A)	0	1	1	1	0	0	0	0	2	5
Outros microrganismos (*Listeria monocytogenes*)	0	0	0	0	2	0	1	0	0	3

Fonte: Center for Disease Control and Prevention's Outbreaknet Foodborne Outbreak Online Database. http://wwwn.cdc.gov/foodborneoutbreaks/, 2012.

A tabela 3 apresenta um resumo dos surtos associados a vegetais frescos e frutas na Europa entre 2004 a 2012.

Tabela 3 - Resumo dos surtos associados a vegetais frescos e frutas na Europa de acordo com a Diretiva 2003/99/EC, 2004-2012

	Alimento envolvido									
	Vegetais					Frutas				
Patógeno	Saladas	Folhosos	Tomates	Outros	Brotos	Frutas vermelhas	Melão	Sucos	Outros	Total
Norovirus	15	26	1	9	0	55	0	0	2	108
Salmonella spp.	8	12	1	3	11	0	1	1	3	40
Escherichia coli	3	0	0	1	3	0	0	0	0	7
Campylobacter spp.	2	1	0	0	0	0	0	0	0	3
Shigella spp.	1	0	0	3	0	0	0	0	1	5
Clostridium spp.	0	1	0	6	0	0	0	0	0	7
Staphylococcus spp.	0	0	0	3	1	0	0	0	0	4
Yersinia spp.	0	0	0	3	0	0	0	0	0	3
Bacillus spp.	2	0	0	3	0	1	0	0	0	5
Giardia spp.	0	0	0	0	0	0	0	0	0	0
Cyclospora spp.	0	0	0	0	0	0	0	0	0	0
Cryptosporidium spp.	1	0	0	0	0	0	0	0	0	0
Outras viroses alimentares	2	0	0	0	2	0	0	0	1	5
Outros microrganismos	0	0	0	9	0	0	0	0	0	9

Fonte: EFSA Summary Reports. www.efsa.europa.eu/en/zoonosesscdocs/zoonosescomsumrep.htm

A *Salmonella* tem sido reconhecida como um dos principais microrganismos causadores de DTA, em diversos países (SILVAPALASINGAM et al., 2004; CARRASCO; MORALES-RUEDA; GARCIA-GIMENO, 2011), assim como no Brasil (TONDO; RITTER; CASARIN, 2015). Cerca de 50% dos surtos causados por bactérias, tanto nos Estados Unidos, quanto na União Europeia, são causados por *Salmonella* (CALLEJÓN et al., 2015). Nos Estados Unidos, de acordo com dados do CDC (CDC, 2013), a maioria dos surtos de *Salmonella* causados pelo consumo de vegetais frescos foi associada ao consumo de tomates, sendo a *S.* Tyiphimurium e a *S.* Newport as mais envolvidas, enquanto que na União Europeia a maioria dos surtos foi provocada pelo consumo de saladas em geral e alface, sendo a *S.* Enteritidis e a *S.* Newport as mais envolvidas, respectivamente (EFSA, 2013).

Além da *Salmonella*, outro microrganismo que tem sido bastante associado a surtos com vegetais frescos é a *E. coli*, em especial a *E. coli* O157:H7 (OILAMAT; HOLLEY, 2012; BERGER et al., 2010; HEATON; JONES, 2008; SIVAPALASINGAM et al., 2004), e a alface e suco de maçã não pasteurizado os produtos que mais veicularam este patógeno (FRIESMA et al., 2008; BERGER et al., 2010; BUCHHOLZ et al., 2011; ALTHAUS et al., 2012; KASE et al., 2012; ORUE et al., 2013). De acordo com Callejón et. al. (2015), alface contaminado com *E. coli* foi o vegetal folhoso que causou 8 do total de 22 surtos veiculados por vegetais frescos, nos Estados Unidos, no período entre 2004 e 2012.

A Tabela 4 demonstra a relação do número de mortes causadas por *Salmonella* e *E. coli* O157:H7, nos EUA, no período compreendido entre 2005 a 2011, em surtos causados por vegetais frescos.

Tabela 4 - Número de mortes causadas por *Salmonella* spp. e *E. coli* O157, nos EUA, entre 2005 e 2011

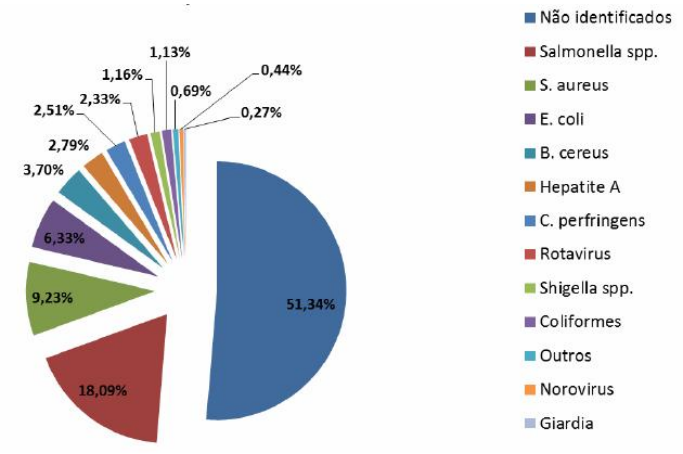
Local	Ano	Patógeno	Produto	Casos (mortes)
EUA, Canadá	2005	*Salmonella*	Tomates	459
EUA	2006	*E. coli* O157:H7	Espinafre	199 (3)
EUA, Canadá	2006	*Salmonella*	Salada de frutas	41
EUA	2006	*Salmonella*	Tomates	183
EUA	2006	*E. coli* O157:H7	Alface	81
EUA	2006	*E. coli* O157:H7	Espinafre	22
América do Norte, Europa	2007	*Salmonella*	Manjericão	51
EUA, Canadá	2008	*Salmonella*	Pimentões	1442 (2)
EUA, Canadá	2008	*E. coli* O157:H7	Alface	134
EUA	2008	*Salmonella*	Melão	51
EUA, Canadá	2008	*Salmonella*	Manteiga de amendoim	714 (9)
EUA	2009	*Salmonella*	Brotos de alfalfa	235
EUA	2010	*E. coli* O145	Alface	26
EUA	2010	*Salmonella*	Brotos de alfalfa	44
EUA	2010	*L. monocytogenes*	Minimamente processados (aipo)	10 (5)
EUA	2011	*Salmonella*	Brotos de alfalfa e mistos	140
EUA	2011	*Salmonella*	Melão	20
EUA	2011	*Salmonella*	Mamão papaya	106
EUA	2011	*L. monocytogenes*	Melão	146 (31)
EUA	2011	*E. coli* O157:H7	Morangos	15 (1)
EUA	2011	*E. coli* O157:H7	Alface	60

Fonte: Olaimat e Holley, 2012.

No Brasil, em 51,34% dos casos o agente causador dos surtos alimentares não foi identificado, normalmente devido à falta de amostras necessárias para a confirmação do patógeno. Como pode ser visualizado na Figura 2, a *Salmonella* também aparece como o principal microrganismo causador de surtos alimentares, sendo responsável por 18,9% do total de surtos notificados, seguida pelo

Staphylococcus aureus, com 9,23% dos surtos, e pela *E. coli*, com 6,33% dos surtos (GOMES; FRANCO; MARTINIS, 2013; BRASIL, 2014).

Figura 2 - Agentes etiológicos associados a surtos alimentares no Brasil de 2000 a 2014



Fonte: Brasil (2014).

2.4 FONTES DE CONTAMINAÇÃO EM PRODUTOS FRESCOS

Diversos são os fatores que podem influenciar na contaminação microbiológica intencional ou não intencional dos produtos frescos, tais como as variações nas práticas de cultivo (campo aberto, estufas, hidroponia), as condições de plantio, o contato direto das partes comestíveis da planta com o solo durante seu crescimento, a forma de colheita e processamento pós-colheita, além de fatores intrínsecos e extrínsecos (FAO, 2003; SUSLOW et al., 2003; PARK et al., 2012, 2013). A contaminação dos produtos frescos pode ocorrer, tanto durante as etapas de cultivo, as quais podem incluir o solo, o adubo orgânico, fezes de animais, água de irrigação, pragas, presença de animais domésticos e contaminação pelos manipuladores, quanto após a colheita, através dos equipamentos, caixas e veículos de transporte, pragas, poeira, água de enxágue (BEUCHAT, 2002; HEATON; JONES, 2008; WARRINER et al., 2009). Nesse sentido, fatores ambientais também

são importantes e devem ser considerados, tais como o clima, a localização da plantação e o tipo de produto (CAC, 2003).

Dentre todos os fatores citados acima, diversos autores têm relatado que a contaminação do solo, o uso de fertilizantes orgânicos, a fonte e qualidade da água de irrigação, bem como as mudanças climáticas são os fatores que mais têm impactado na prevalência e concentração de patógenos em produtos frescos (NATVIG et al., 2002; ISLAM et al., 2004a,b; FRANZ et al., 2005; SEMENOV et al., 2007; HUTCHISON; AVERY; MONAGHAN, 2008; GE; LEE; LEE, 2012; LIU; HOFSTRA; FRANZ, 2013). Ainda, de acordo com EFSA (2014), os principais fatores que podem possibilitar a contaminação de vegetais frescos por *Salmonella* são os fatores ambientais, tais como a proximidade de animais domésticos ou silvestres junto às áreas de plantio e as condições climáticas (chuvas, ocorrência de inundações), a utilização de adubo orgânico não corretamente compostado, uso de água contaminada para irrigação ou para diluição de pesticidas e contaminação cruzada por manipuladores ou equipamentos, durante a colheita ou pós-colheita.

2.4.1 Locais de plantio

Os locais de plantio podem interferir também de forma bastante significativa na contaminação dos produtos frescos, e a avaliação dos diversos fatores independentes de cada local de plantio deveria ser realizada de forma prévia, a fim de detectar possíveis fontes de contaminação (STRAWN et al., 2013a). De acordo com o *Codex Alimentarius* (CAC, 1969; CAC, 2003), as práticas de higiene estabelecidas para o cultivo de frutas e vegetais frescos para consumo humano estabelecem que a produção primária não deve ocorrer em áreas onde há contaminação conhecida, a fim de prevenir a transferência de patógenos aos produtos frescos. No entanto, cultivar produtos frescos somente em áreas onde não há contaminação não é um procedimetno fácil de ser realizado, especialmente devido ao fato de que os produtores, na maioria das vezes, não controlam ou não sabem como controlar as atividades que são executadas em áreas próximas à

plantação, nem conhecem o nível de contaminação presente nos locais de plantio (SUSLOW et al., 2003; JAMES, 2006; GIL et al., 2013).

Alguns autores descrevem a possibilidade de contaminação das plantações devido à presença de animais próximo às áreas plantadas, podendo a contaminação ocorrer de forma direta ou indireta, através de carreamento por água de chuva, aerosóis, poeira ou vetores como pássaros, roedores ou mesmo moscas (FAO, 2003; BRANDL, 2006; GELTING et al., 2011). Como exemplo disso, dois surtos causados por *E. coli* O157:H7 presente em vegetais folhosos foram associados à presença de gado próximo às áreas de plantio (JAY et al., 2007; SODERSTROM et al., 2008). Já no Brasil, Rodrigues et al. (2014) encontraram contaminação na água de irrigação por *E. coli* O157:H7, após a ocorrência de uma inundação, demonstrando assim que a localização topográfica do local de plantio também deve ser considerada de forma a prevenir possibilidades de inundações.

2.4.2 Presença de animais no local de cultivo

A presença de animais, sejam domésticos, tais como cachorros, gatos e animais de criação (gado, ovelha, galinha, cavalos) ou silvestres (sapos, cobras, pássaros, roedores, entre outros), pode representar um importante fator de contaminação dos produtos frescos, uma vez que suas fezes podem contaminar o solo e chegar até os produtos durante o seu cultivo (LOWELL; LANGHOLZ; STUART, 2010; OILAMAT; HOLLEY, 2012; EFSA, 2014). Vários autores demonstraram a presença de *Salmonella* nas fezes de diversas espécies de animais silvestres que, por terem acesso às áras de cultivo, podem levar a contaminação aos produtos frescos (LAPUZ et al., 2008; BENSKIN et al., 2009; LAWSON et al., 2010; RAMOS et al., 2010; VIEIRA-PINTO et al., 2011; CARLSON et al., 2011; ZOTTOLA et al., 2013), no entanto, a confirmação microbiológica de que a contaminação dos produtos frescos por *Salmonella* é devido à presença destes animais é bastante rara (EFSA, 2014), tendo sido comprovada apenas por alguns autores (SAGOO et al., 2003; JAY et al., 2007).

2.4.3 Solo e adubo orgânico

Patógenos tais como *Clostridium botulinum*, *Clostridium perfringens*, *Bacillus cereus* e *Listeria monocytogens*, podem ser naturalmente encontrados no solo (AVERY; KILLHAM; JONES, 2005; NICHOLSON; GROVES; CHAMBERS, 2005; OILAMAT; HOLEY, 2012; STRAWN et al., 2013b), no entanto, a capacidade de sobrevivência dos patógenos no ambiente irá influenciar na probabilidade de contaminação da plantação e na viabilidade destes patógenos no momento da colheita e durante o consumo do produto (OILAMAT; HOLEY, 2012). Conforme demonstrado na Tabela 5, o tempo de sobrevivência de microrganismos pode variar consideravelmente, dependendo do patógeno e do ambiente no qual ele se encontra.

Dependendo do tipo de solo e com a intenção de melhorar a produtividade e facilitar o crescimento das plantas, especialmente em sistemas de produções sucessivas, a adubação é uma prática comumente utilizada e pode ser realizada através da aplicação de adubos químicos ou orgânicos (EFSA, 2014), sendo estes últimos provenientes de fontes animais (fezes, esterco) ou vegetais. A utilização de adubo orgânico, como esterco e fezes de diversos tipos de animais, sem respeito ao adequado tempo de compostagem, pode ser um importante fator de introdução de outros importantes patógenos às plantações, tais como *Salmonella* e *E. coli* (BEUCHAT, 1996; NATVIG et al., 2002; AVERY; KILLHAM; JONES, 2005; OILAMAT; HOLEY, 2012; STRAWN et al., 2013b). Outro fator de contaminação que deve ser considerado é o local de armazenamento do adubo orgânico, o que muitas vezes é realizado próximo às áreas de plantio, permitindo a contaminação da plantação através do escoamento via água de chuva, através de vetores (pragas), poeira e aerosóis (SUSLOW et al., 2003; BRANDL, 2006; JAMES, 2006).

Tabela 5. Tempo de sobrevivência de patógenos entéricos no ambiente

Patógeno	Ambiente	Sobrevivência (dias)	Referências
E. coli O157:H7	Solo + esterco animal	30	Nicholson et al. (2005)
E. coli O157:H7	Solo + esterco animal	99	Nicholson et al. (2005)
E. coli O157:H7	esterco animal	60	Avery et al. (2005)
E. coli O157:H7	Chorume	60	Avery et al. (2005)
E. coli O157:H7	Resíduos de matadouros	60	Avery et al. (2005)
E. coli O157:H7	Lodo de esgoto	60	Avery et al. (2005)
E. coli O157:H7	Esterco ovino não aerado	>365	Kudva et al. (1998)
E. coli O157:H7	Esterco ovino aerado	120	Kudva et al. (1998)
E. coli O157:H7	Chorume não aerado	600	Kudva et al. (1998)
E. coli O157:H7	Chorume aerado	30	Kudva et al. (1998)
E. coli	Chorume + água suja	90	Nicholson et al. (2005)
Salmonella	Solo	968	Nicholson et al. (2005)
Salmonella	Solo + chorume bovino	300	Nicholson et al. (2005)
Salmonella	Solo + esterco animal	30	Nicholson et al. (2005)
Salmonella	Chorume + água suja	90	Nicholson et al. (2005)
Campylobacter	Solo + esterco animal	30	Nicholson et al. (2005)
Campylobacter	Chorume + água suja	90	Nicholson et al. (2005)
Listeria	Solo + esterco animal	30	Nicholson et al. (2005)
Listeria	Chorume + água suja	180	Nicholson et al. (2005)
Listeria monocytogenes	Lodo de esgoto	56	Everis (2004)
Vírus da Hepatite A	Água	>365	Seymour and Appleton (2001)
Vírus da Hepatite A	Solo	96	Seymour and Appleton (2001)

Fonte: Heaton e Jones, 2008.

Diversos autores já demonstraram a presença de *Salmonella* e *E. coli* em fezes e esterco de animais, com contagens variando de 10^2 a 10^7 UFC/g (PELL, 1997; HIMATHONGKHAM et al., 1999). Também Hutchison et al. (2008) demonstraram a presença de *Salmonella* em dejetos animais em um estudo

realizado no Reino Unido, apresentando contagens médias de 10^3 UFC/g a 10^7 UFC/g. Já na União Europeia e nos Estados Unidos, amostras de água em unidades de tratamento de chorume suíno, analisadas por McLaughlin e Brooks (2009), apresentaram contagens de 0,4 a 4 NMP/100 mL. Através destes estudos é possivel observar que a utilização de adubos inadequadamente compostados podem interferir de forma significativa para a contaminação dos produtos frescos com *Salmonella.*

A tabela 6 demonstra a prevalência de patógenos em diferentes tipos de adubo em várias partes do mundo.

Tabela 6. Prevalência de patógenos em diferentes tipos de adubo ou fezes de animais

Patógeno	Adubo orgânico ou fezes	País	Prevalência (%)	Referência
E. coli O157	Gado	Grã Bretanha	120/2553 (4,7)	Milnes et al., 2008
		Grã Bretanha	107/810 (13,2)	Hutchison et al., 2004
		Noruega	3/1541 (0,2)	Johnsen et al., 2001
		Nigéria	42/407 (10,3)	Ojo et al., 2010
	Ovelha	Grã Bretanha	21/2825 (0,7)	Milnes et al., 2008
		Grã Bretanha	5/24 (20,8)	Hutchison et al., 2004
		Nigéria	9/168 (5,4)	Ojo et al., 2010
	Suíno	Grã Bretanha	6/2114 (0.3)	Milnes et al., 2008
		Grã Bretanha	15/126 (11.9)	Hutchison et al., 2004
		Noruega	2/1976 (0.1)	Johnsen et al., 2001
		Nigéria	20/409 (4.9)	Ojo et al., 2010
		Canadá	12/359 (3.3)	Farzan et al., 2010
Salmonella	Gado	Grã Bretanha	36/2553 (1.4)	Milnes et al., 2008
		Grã Bretanha	62/810 (7.7)	Hutchison et al., 2004
		EUA	273/4977 (5.5)	Fedorka-Cray et al., 1998 Madden et al., 2007
		Irlanda	6/200 (3.0)	Milnes et al., 2008
	Ovelha	Grã Bretanha	30/2825 (1.1)	Hutchison et al., 2004
		Grã Bretanha	2/24 (8.3)	Pao et al., 2005
		EUA	17/287 (5.9)	Milnes et al., 2008
	Suíno	Grã Bretanha	124/529 (23.4)	Hutchison et al., 2004
		Grã Bretanha	10/126 (7.9)	Callaway et al., 2010
		EUA	44/600 (7.3)	Farzan et al., 2010
		Canadá	113/359 (31.5)	Orji et al., 2005
	Frango	Grã Bretanha	12/67 (17.9)	Murugkar et al., 2005
		Nigéria	15/120 (12.5)	Milnes et al., 2008
		Índia	34/231 (14.7)	Milnes et al., 2008

Campylobacter	Gado	Grã	364/667 (54.6)	
		Bretanha		Hutchison et al., 2004
		Grã	104/810 (12.8)	
		Bretanha		Chatre et al., 2010
		França	(16.5)	Madden et al., 2007
		Irlanda	52/220 (24.8)	Klein et al., 2010
		Austrália	30/32 (94)	Milnes et al., 2008
	Ovelha	Grã	312/713 (43.8)	
		Bretanha		Hutchison et al., 2004
		Grã	5/24 (20.8)	
		Bretanha		Salihu et al., 2009
		Nigéria	93/518 (18.0)	Workman et al., 2005
		Barbados	3/71 (4.2)	Milnes et al., 2008
	Suíno	Grã	366/528 (69.3)	
		Bretanha		Hutchison et al., 2004
		Grã	17/126 (13.5)	
		Bretanha		Workman et al., 2005
		Barbados	67/74 (90.5)	Farzan et al., 2010
	Frango	Canadá	131/359 (36.5)	Hutchison et al., 2004
		Grã	13/67 (19.4)	
		Bretanha		Workman et al., 200
		Barbados	13/67 (19.4)	Franchin et al., 2005
		Brasil	18/24 (75.0)	Hutchison et al., 2004
Listeria	Gado	Grã	241/810 (29.8)	
		Bretanha		Umeh et al., 2010
		Nigéria	20/150 (13.3)	Esteban et al., 2009
		Espanha	2277/6180 (36.8)	
			6/130 (4.6)	Kalender, 2003
		Turquia	10/220 (4.8)	Madden et al., 2007
		Irlanda	7/24 (29.2)	Hutchison et al., 2004
	Ovelha	Grã		
		Bretanha	6/150 (4.0)	Umeh et al., 2010
		Nigéria	511/3600 (14.2)	Esteban et al., 2009
		Espanha	5/170 (2.9)	
			25/126 (19.8)	Kalender, 2003
		Turquia		Hutchison et al., 2004
	Suíno	Grã	4/122 (3.3)	
		Bretanha	0/510 (0.0)	Farzan et al., 2010
		Canadá	13/67 (19.4)	Esteban et al., 2009
		Espanha		Hutchison et al., 2004
	Frango	Grã	5/150 (3.3)	
		Bretanha	24/206 (11.7)	Umeh et al., 2010
		Nigéria		Kalender, 2003
		Turquia		

Fonte: Oilamat e Holey, 2012.

A possibilidade de sobrevivência da *Salmonella* no solo de 7 até 25 semanas foi demonstrada por vários autores (GUO et al., 2002; LANG; SMITH, 2007; WHIPPS et al., 2008; ZHANG et al., 2009; ERICKSON et al., 2010;), enquanto que outros relataram a sobrevivência de *E. coli* O157:H7 de 45 até 100 dias no solo (NICHOLSON; GROVES; CHAMBERS, 2005), demonstrando a importância do correto manejo e compostagem dos adubos orgânicos, a fim de diminuir a possibilidade de introdução de bactérias patogênicas aos produtos frescos, no momento do plantio ou durante o ciclo de crescimento. Outros autores relataram que a adequada compostagem do adubo orgânico pode reduzir significativamente a quantidade inicial de *Salmonella* presente no adubo (LUNG et al., 2001; CEUSTERMANS et al., 2007), enquanto que outros demonstraram que no adubo

adequadamente compostado a *Salmonella* não se multiplica, mesmo no caso de uma recontaminação (KIM; JIANG, 2010).

Embora diversos autores tenham demonstrado que a *Salmonella* pode ser encontrada em vegetais folhosos cultivados em solo adubado com resíduos orgânicos provenientes de fezes (NATVIG et al., 2002; ARTHURSON; SESSITSCH; JADERLUND, 2011; ONGENG et al., 2011), de acordo com o EFSA (2014), a possibilidade de *Salmonella* ser encontrada em vegetais folhosos cultivados em solo que recebeu adubo orgânico contaminado diminui progressivamente, devido ao tempo entre a aplicação do adubo até o momento da colheita. Além disso, não é possível associar a ocorrência de um surto causado por vegetais frescos à contaminação do adubo, especialmente devido ao fato de que este é aplicado na lavoura várias semanas antes da colheita, sendo assim, é provável que o mesmo não esteja mais disponível no momento da colheita, o que possibilitaria a investigação (EFSA, 2014).

2.4.4 Água de irrigação

A qualidade da água de irrigação é de fundamental importância na prevenção da contaminação dos produtos durante o seu ciclo de produção (BRACKETT, 1999; ARUSCAVAGE et al., 2006; WARRINER et al., 2009) , uma vez que fezes, solo, adubo orgânico e outras fontes de patógenos podem ser carreados para a água de diversas maneiras, como chuva, inundações, presença de animais próximo às plantações (FDA, 1998; TYRREL et al., 2006). No entanto, a necessidade ou quantidade de irrigação pode variar devido a fatores como clima e tipo de solo (ENZA ZADEN, 2013; EFSA, 2014).

Diversas fontes de água podem ser utilizadas para irrigação e, conforme Leifert et al. (2008), o risco diminui, de acordo com a fonte de água, sendo maior em águas residuais não tratadas e diminuindo, sucessivamente, para água de superfície (lagos e rios), água subterrânea de poços rasos, água de chuva corretamente armazenada, água subterrânea de poços profundos e água potável. Na União Europeia, as principais fontes de água utilizadas para irrigação são, em ordem

decrescente, água de superfície (rios e lagos), água de poço ou de chuva armazenadas em reservatórios intermediários, água de poço e água potável, sendo esta última especialmente utilizada nos casos de hidroponia (EFSA, 2014). Águas de superfície que receberam água de esgoto tratado representaram 71% do total de águas utilizadas para irrigação no Reino Unido (TYRELL; KNOX; WEATHERHEAD, 2006) e, de acordo com Anonymous (2003), nos países em desenvolvimento, cerca de 10% das plantações é irrigada com águas residuais não tratadas, aumentando o potencial de contaminação, devido ao aumento na incidência de patógenos (STEELE; ODEMERU, 2004).

Vários estudos têm demonstrado a utilização de água de má qualidade microbiológica na irrigação de plantações ao redor do mundo. Na África do Sul, Gemmell e Schmidt (2011) encontraram 5,5 NPM/mL de *E. coli* em água de rio utilizada para irrigação de verduras. Já na Austrália foi demonstrado que 3 e 28% das amostras de água de açude e de estuário estavam contaminadas com *Salmonella* e *E. coli*, respectivamente (AHMED et al., 2009). Chigor et al. (2010) encontraram em água de rio utilizadas para irrigação de verduras, na Nigéria, 2,1% de amostras contaminadas com *E. coli* O157:H7. Nos Estados Unidos, *Salmonella* foi isolada em 79,2% de amostras de água de superfície (HALEY; COLE; LIPP, 2009) e, no Canadá, *E. coli* e *Salmonella* foram isoladas em 1,7 e 10,3% das amostras de água de rio utilizada para irrigação, respectivamente.

A irrigação nas plantações pode ocorrer através de diferentes formas como por aspersão (*sprinklers*), gotejamento e inundação intencional ou por água de chuva, sendo que a irrigação por asperção e a inundação por água de chuva são as que oferecem maior risco de contaminar os produtos, enquanto que a irrigação por gotejamento parece ser a que oferece menor risco (FDA, 1998; SOLOMON; YARON; MATHEWS, 2002; FAO/WHO, 2008). Kisluk e Yaron (2012) demonstraram, em um experimento conduzido em uma estufa de cultivo de salsa com sistema de irrigação por *sprinklers* que, quando altos níveis de *Salmonella* estavam presentes na água de irrigação, quantidades similares do microrganismo foi encontrada na salsa. De forma similar, Solomon et al. (2002) demonstraram que 90% das alfaces foram contaminadas quando irrigadas por sistema de aspersão com

água contaminada com 7 Log UFC/mL de *E. coli* O157:H7, enquanto que, quando a irrigação foi realizada na superfiície do solo, a incidência caiu para 19%.

2.4.5 Trabalhadores

As atividades de colheita, pré-processamento e processamento podem representar um importante fator de contaminação para os vegetais frescos, uma vez que eles são, muitas vezes, colhidos manualmente (JAMES, 2006; GIL et al., 2013; EFSA, 2014), e também manipulados, nos serviços de alimentação, por colaboradores que podem estar contaminados (HALL et al., 2014). O fato de que diversas atividades realizadas com os produtos frescos necessitam do contato manual, tais como a colheita, lavagem pós-colheita, acondicionamento em caixas de transporte, processamento, entre outras, pode não só interferir na contaminação destes produtos por patógenos alimentares como também aumentar a população bacteriana (BRACKETT, 1999; DOYLE; ERICKSON, 2008; EFSA, 2011; OILAMAT; HOLLEY, 2012).

Microrganismos patogênicos, tais como os coliformes de origem fecal, podem ser isolados de vegetais frescos em diversos estágios de seu processamento e, uma vez que são considerados microrganismos indicadores, podem indicar contaminação e estar associados à ausência de higiene pessoal (WARRINER et al., 2009; BERGER et al., 2010). Os principais fatores de contaminação destes produtos pelos manipuladores são as falhas na higiene das mãos e na higiene pessoal (TODD et al., 2007; NODA; FUKUDA; NISHIO, 2008; EFSA, 2011a). Além disso, a contaminação cruzada através de luvas nitrílicas também pode influenciar de forma significativa a contaminação destes produtos (VERHAELEN et al., 2013).

2.4.6 Equipamentos

Durante todas as etapas de produção dos produtos frescos, desde o campo, até a chegada ao consumidor final, existe a possibilidade de contaminação. No entanto, de acordo com Yang et al. (2012), o manuseio pelos trabalhadores do campo, bem como o contato com equipamentos (facas de corte, caixas), fazem com

que as etapas finais de produção no campo possibilitem um maior risco de contaminação por patógenos alimentares. No momento da colheita as alfaces são cortadas manualmente por uma faca e, durante esta etapa já é possível que seja realizada uma pré-seleção, com a eliminação das folhas mais externas, por causa de danos ou mesmo sujeira. Este processo pode disseminar contaminação, conforme descrito por McEvoy et al. (2009) e Taormina et al. (2009) que demonstraram que uma única faca artificialmente contaminada com *E. coli* O157:H7 pode contaminar sucessivemente até 19 pés de alface.

Equipamentos de colheita automatizada, caixas e esteiras transportadoras podem também representar importantes fontes de contaminação (PRAZAK et al., 2002; JOHNSTON et al., 2006), demonstrando a importância da correta higienização destes equipamentos e superfícies, a fim de evitar a contaminação cruzada. No entanto, conforme FAO (2003), mais estudos são necessários, a fim de demonstrar a relação entre os diferentes tipos de equipamentos e a contaminação cruzada.

2.4.7 Água de enxágue

Normalmente, antes dos produtos serem colocados nas caixas de transporte, os mesmos são enxaguados, a fim de remover a sujeira mais pesada e, portanto, esta água pode representar uma fonte potencial de contaminação cruzada por microrganismos patogênicos (ALLENDE et al., 2008; LUO et al., 2011; BUCHHOLZ et al.; 2012; HOLVOET et al., 2012, 2014). O processo de lavagem realizado em tanques, também muito utilizado no Brasil, pode ser fonte de contaminação cruzada dos produtos frescos e inclusive representar um aumento no número de patógenos por falta de renovação.

Embora Holvoet et al. (2014) tenham demonstrado que somente uma pequena porção de microrganismos (em torno de 1,5%), incluindo *E. coli* e *E. coli* O157:H7, possa ser transferida através da água de lavagem para alfaces minimamente processadas, este fato permite comprovar a vulnerabilidade dos vegetais folhosos à contaminação cruzada, durante o enxágue final. Da mesma maneira, a manutenção da qualidade da água é outro fator importante a ser considerado durante a etapa de enxágue após a colheita, a fim de minimizar a

possibilidade de contaminação cruzada e, sendo assim, o tratamento desta água com produtos químicos antimicrobianos pode ajudar a manter a qualidade desta água e evitar a contaminação cruzada dos produtos (LOPEZ-GALVEZ et al., 2009; FDA, 2009).

2.5 DISTRIBUIÇÃO DA ALFACE

A distribuição da alface geralmente ocorre em feiras e supermercados, sendo que estes últimos podem ser desde pequenos, médios ou até grandes lojas e pode envolver várias etapas, incluindo o transporte, armazenamento, manuseio e embalagem (EFSA, 2014). Na União Europeia e nos Estados Unidos, normalmente todas as etapas de distribuição ocorrem em sistema refrigerado, desde a colheita até a comercialização final nos pontos de venda, enquanto que no Brasil, na maioria das vezes, as etapas de distribuição dos produtos frescos acontecem em temperatura ambiente (SALLA; COSTA, 2012).

O principal fator de contaminação citado por vários autores é a contaminação cruzada durante a distribuição dos vegetais folhosos (PATEL et al., 2010; RODRIGUEZ-LAZARO et al., 2012; ESCUDERO et al., 2012; VERHOEF et al., 2013; VERHAELEN et al., 2013; WANG et al., 2013). De acordo com o EFSA (2014), no que diz respeito à *Salmonella*, os principais fatores de risco de contaminação cruzada são o contato direto ou indireto entre os vegetais folhosos e alimentos crus de origem animal contaminados. Dessa maneira, é possível supor que, quanto maior for o local de distribuição e o volume de produtos a ser distribuído, o impacto social, caso estes venham a se contaminar, também pode ser bastante significativo.

2.8 MULTIPLICAÇÃO MICROBIANA EM ALFACE

Para avaliar a multiplicação microbiana em diversos alimentos a microbiologia preditiva tem sido aplicada, utilizando-se de modelos matemáticos para predizer a cinética de multiplicação dos microrganismos (FERRER et al., 2009). Estes modelos são divididos em primários, secundários e terciários.

O modelo primário utiliza como parâmetros o número inicial de células de microrganismos, a taxa de multiplicação, o tempo de fase *lag* e a densidade máxima da população, representando a dinâmica do desenvolvimento bacteriano ao longo do tempo, em condições ambientais e de cultivo pré-determinadas (MCKELLAR; LU, 2004), sendo o modelo de Baranyi o mais utilizado (BARANYI; ROBERTS, 1994). No modelo secundário, as respostas dos parâmetros do modelo primário em relação às mudanças no meio ambiente são simuladas (GUMUDAVELLI et al., 2007), sendo o modelo da raiz quadrada o mais utilizado. Enquanto que o modelo terciário utiliza um *software* de fácil aplicação, gerado a partir de um ou mais modelos primários e secundários (BARANYI; TAMPLIN, 2004). Neste último modelo, os programas *Combase* e PMP (*Pathogen Modelling Program*) os dois mais utilizados.

3 RESULTADOS

Os resultados deste estudo estão apresentados sob a forma de artigos científicos. Cada seção do capítulo de resultados da presente Tese corresponde a uma dessas publicações científicas.

"INSIGHTS IN AGRICULTURAL PRACTICES AND MANAGEMENT SYSTEMS LINKED TO MICROBIOLOGICAL CONTAMINATION OF LETTUCE IN CONVENTIONAL PRODUCTION SYSTEMS IN SOUTHERN BRAZIL"

Artigo publicado no periódico *International Journal of Food Contamination*, v. 2, p. 1-13, 2015. DOI 10.1186/s40550-015-0011-5

"MICROBIOLOGICAL QUALITY AND SAFETY ASSESSMENT OF LETTUCE PRODUCTION IN BRAZIL"

Artigo publicado no periódico *International Journal of Food Microbiology*, v.181, p.67-76, 2014.

CONTAMINATION AND GROWTH MODELLING OF SALMONELLA AND E. COLI ON CONVENTIONAL LETTUCES SOLD IN HYPERMARKETS OF SOUTHERN BRAZIL

Artigo a ser submetido no periódico *Food Control.*

3.1 ARTIGO 1

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Insights in agricultural practices and management systems linked to microbiological contamination of lettuce in conventional production systems in Southern Brazil

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Abstract

Background: Three conventional lettuce farms were evaluated in Southern Brazil using a standardized self-assessment questionnaire with 69 indicators and a microbiological sampling plan in order to assess the status of current agricultural practices and management systems. The use of both tools aimed to identify the foremost contamination sources and control measures during the crop production. A total of 128 samples were taken (manure, soil, water, workers' hands and equipment, lettuce seedlings and lettuce heads) in four visits during the growth cycle of lettuces. Samples were analysed for hygiene indicators (*E. coli*) and presence of pathogens (*Salmonella* spp. and *E. coli* O157).

Results: Microbiological results indicated that *E. coli* counts were very low in all analysed samples and no pathogens were detected. These results could be explained partially because all farms had toilets near to the fields, they did not raise animals near the crops, fields were located in areas where flooding was not possible, they used organic fertilizers adequately composted, and irrigation water demonstrated good microbiological quality. The microbial results for manure and soil indicated that the composting time was of utmost importance to maintain minimal contamination levels for the duration of the cultivation period, as long as the quality of irrigation water was very important to prevent further contamination of the crop. On the other hand, the self-assessment questionnaire identified a moderate to high risk level concerning microbiological contamination in all evaluated farms, because they had no formal good agricultural practices implemented, technical support, water control, inspections, food safety registers or sampling plan for microbiological or chemical analyses.

Conclusion: These different results are important in order to provide information about the actual status of contamination (microbial sampling plan) and possible food safety problems in the future based on the results given by the questionnaire. Furthermore, the results of this study also highlighted the necessity to provide more safety during the fresh produce cultivation, being formal good agricultural practices implementation an important start to the fresh produce farms in Brazil, as well as to adopt a higher level of control activities in order to achieve lower risk levels.

Keywords: Conventional lettuce; Good agricultural practices; Microbiological contamination

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Background

Fresh produce is frequently associated with healthy diets because their nutritional properties and global production and consumption has increased significantly in the last years around the world (FAOSTAT, 2013; Warriner et al., 2009; Aruscavage et al., 2006). Intensive production systems and the lack of reliable good agricultural practices in the field are some of the reasons for the worldwide increasing numbers of foodborne illnesses associated to fresh produce (EFSA, 2014; Oilamat and Holley, 2012; Warriner et al., 2009; Beuchat, 2006; Sivapalasingam et al. 2004; Beuchat, 1996). Fresh produce can become contaminated with pathogens at any step of the supply chain, mostly due to natural, human or environmental factors (Olaimat and Holley, 2012; Oliveira et al., 2012; Itohan et al., 2011; Taban and Halkman, 2011). As a consequence, several foodborne outbreaks associated with leafy greens have been reported as primarily caused by *Salmonella* spp. and pathogenic *Escherichia coli* (Callejón et al., 2015; Buchholz et al., 2011; Warriner et al., 2009; Delaquis et al., 2007; Stine et al., 2005; Buck et al., 2003).

In Brazil, as in many other countries, lettuce (*Lactuca sativa* L.) is one of the most consumed leafy vegetables, attributable to year round availability, low cost and nutritional factors (Abreu et al., 2010; Mocelin and Figueiredo, 2009; WHO et al. 2008; Mattos et al., 2007). The Brazilian lettuce cultivation system is predominantly done in open fields, which are located for the most part at urban surroundings. Generally the distribution system occurs without refrigeration at any step of the postharvest chain, in contrast to practices in the European Union and United States, where cold chain and advanced logistics systems are applied (Brasil, 2013; Salla and Costa, 2012).

Food Safety Management Systems, for example, Good Agricultural Practices (GAP), at farm level are able to prevent and reduce bacterial contamination of fresh produce (Morgharbel and Masson, 2005; CDC, 2003; FDA, 1998). A number of factors has been identified as sources of microbial contamination, for example: organic fertilizers, soil, workers and equipment and, most noteworthy, water. Water has been identified as one of the most important sources of contamination of fresh produce. Irrigation waters and the fresh produce rinsing waters are recurrently used devoid of any disinfecting treatment (Rodrigues et al., 2014; Olaimat and Holley, 2012; Salem et al., 2011; Allende et al., 2008; Beuchat, 2006; Anderson et al., 1997).

Based on these evidences, the objective of the present study was to evaluate the status of current agricultural practices and management systems of conventional lettuce farms in the State of Rio Grande do Sul (RS), Southern Brazil, in order to identify major bottlenecks during the crop growing time related to conceivable microbiological contaminations. Insights were disclosed by combining microbiological analyses with the diagnosis of the risk level at farm circumstances, the status of implemented control measures and assurance activities and the system outputs at three typical Brazilian farms.

Methods

Characterization of the farms

Three family managed, smallholdings (approximately 2 to 3 hectares of land) in which lettuce was grown in a conventional production system were involved in the present study. Further on these production units were denominated farm 1, 2 and 3. These farms were chosen because they had typical characteristics of small farms were conventional lettuces and other leafy greens are cultivated in Brazil and also due to their similar conditions in terms of lettuce production. Before sampling collection, the owners were contacted and agreed to cooperate in the research. One of the farms was located in the rural area of Porto Alegre, the capital city of Rio Grande do Sul, the southernmost State of Brazil. The other two farms were located in the rural area of Viamão, a city neighboring Porto Alegre. Their cultivation system was in a open field.

The lettuce seedlings used to start off the plantations were delivered to the farms by different commercial suppliers. There were no formal good agricultural practices implemented or any other voluntary standard certified at the farms in the course of the sampling period. The fertilization procedures of the production fields were similar in all three farms. Organic fertilizers, over 90 days composted chicken manure, were purchased from local suppliers. None of the farms produced any kind of organic fertilizer.

The lettuce fields were irrigated by overhead sprinkler systems and the water was pumped from ponds located adjacent and at a lower level of the cultivation areas.

In all three farms the workers' households were located near the fields (less than 100 meters apart) and were equipped with toilets. Besides the intensive rainfall during the sampling period, flooding did not occur or affect the production fields. The farmers, during the sampling period, did not have cattle, poultry or other livestock animals in breeding process at their premises.

Microbiological sampling plan

Sampling locations and collection

A microbiological sampling plan was used with the intent of identifying contamination sources in the current agricultural practices. The sampling locations were selected based on literature review related to potential risk factors which may contribute to the microbiological contamination of lettuce. These locations were

identified as critical sampling locations (CSL's), *i.e.*, sites in the production processes at which contamination, growth and/or survival of microorganisms may take place. In the present paper 12 CSL's were selected based on sources and potential risk factors of microbial contamination, starting from lettuce seedlings, soil and manure, irrigation and rinse waters, handlers, food contact equipment up to the final products (Rodrigues et al., 2014; Oilamat and Holley, 2012; Ilic et al., 2012).

The sampling period ranged from August to October 2012 and the microbial sampling plan was set up to obtain information about hygiene (*E. coli*) and safety levels (*Salmonella* spp., *E. coli* O157:H7). Samples of water, soil, manure, lettuce seedlings, lettuce heads, workers' hands and transport boxes were collected as previously described by Rodrigues et al. (2014).

All the samples were transported by car to the Laboratory of Microbiology and Food Control of the Institute of Food Science and Technology – ICTA/UFRGS inside thermal boxes. Analyses started in less than one hour after sampling.

Microbiological analyses

The analyses of microbiological parameters of each CSL are presented in Table 1. All the microbiological analyses were carried out according to Rodrigues et al. (2014).

Diagnostic instrument used to measure the food safety management systems

A questionnaire with 69 indicators was applied to gain insights into the level of the good agricultural practices and management system currently implemented on the farms, as previously described by Rodrigues et al. (2014). The questionnaires were answered by the farms' owners.

Weather conditions

Temperature and cumulative precipitation of the week prior to sampling and including the sampling day (8 days) were obtained from the National Institute for Meteorology of Brazil (Instituto Nacional de Meteorologia (INMET), http://www.inmet.gov.br/portal/). Table 2 shows the averages of temperature and precipitation during the sampling period.

Statistical analyses

Statistical analyses were performed with SPSS Statistics version 21 at $p < 0.050$. Bivariate correlations between the indicators were determined by calculating the Spearman's Rho coefficient using the raw enumeration data. Kruskal-Wallis or Mann-Whitney U tests were used to evaluate the influence of different factors. Pair wise tests were performed to identify the significant differences between individual categories when significant differences were found. In case of 'n' pair wise comparisons, Dunn-Sidak correction was applied, resulting in adjusted individual p' values: $p' = 1-(1-p)^{1/n}$, in which $p = 0.050$ to obtain a family-wise error rate of 5%.

Results

Microbiological contamination

The presence of *E. coli* in the collected samples from manure, manured soil before setting the lettuce plantlets into the field and soil along the growth cycle of the lettuce crops presented mostly counts below the detection limits (Table 3). The highest count of *E. coli* (2.00 $\log_{10}$ CFU/g) was observed in two samples: one sample of manure and another of soil (Table 3). There was no significant difference in *E. coli* counts between manured soil and soil samples for the duration of the sampling period (Kendall's tau-c, $p = 0.803$). There were no significantly differences in *E. coli* counts in manure among farms (Kruskal-Wallis Test, $p = 0.368$). *Salmonella* spp. and *E. coli* O157:H7 were not found in any sample. The *E. coli* concentration along the growth cycle in manure, manured soil and soil in the three farms is demonstrated in Figure 1.

Lettuce seedlings were collected only at the time of planting the seedlings in the field. *E. coli* counts ranged from <1.00 $\log_{10}$ CFU/g to 2.30 $\log_{10}$ CFU/g (average of 1.43 ± 0.75 $\log_{10}$ CFU/g). The highest count was observed on seedlings at farm 1. During the growth cycle of the lettuces, the *E. coli* distribution was similar (Kruskal-Wallis Test, $p = 0.560$) (Figure 2). However, the highest *E. coli* counts were observed two and one week before harvest. At harvest, all *E. coli* counts were below the detection limit (Figure 2). *E. coli* counts were similar on the lettuce head samples collected at all farms (Kruskal- Wallis Test, $p = 0.162$), ranging from $<1.00 \pm 0.00$ $\log_{10}$ CFU/g to 1.12 ± 0.14 $\log_{10}$ CFU/g. The rinsed lettuce heads presented *E. coli* counts below the detection limits and no pathogens were found on any sample of seedlings and lettuces.

Water samples collected from ponds, sprinklers and rinsing tanks presented low counts of *E. coli* and 88.5% of the samples counts were below the detection limit (Table 3). Counts of positive samples ranged from 1 to 1.4 $\log_{10}$ MPN/100 ml. No statistical differences were determined for *E. coli* among the three water sources during the growth cycle of the crop (Kruskal- Wallis Test, $p = 0.739$). No pathogens were detected in any analyzed sample. During the lettuce growth cycle, the distribution of *E. coli* showed no significant differences among farms and time of sampling (Kruskal- Wallis Test, $p = 0.212$). No pathogens were found in any water sample.

The samples of the transport boxes and workers' hands of the three farms were collected only at harvest. All samples showed *E. coli* counts below the detection limit (Table 3).

Table 1 Description of Critical Sampling Location (CSLs), samples, periodicity, microbiological parameters, methodologies, results interpretation and references

CSL	Description	Samples	Time	Microbiological parameters	Methodology	Interpretation of the results*	References
1	Manure	3 samples	T0	*E. coli*	ISO 21528-2:2004	10^3 cfu/g	MAPA/ IN n°46. (2011)
				E. coli O157:H7	ISO 16654:2001	A/25g	ND
				Salmonella spp.	ISO 6579:2002	A/25g	MAPA/ IN n°46. (2011)
2	Manured soil	3 samples → 3 x 3 pooled	T0	*E. coli*	ISO 21528-2:2004	10^3 cfu/g	MAPA/ IN n°46. (2011)
				E. coli O157:H7	ISO 16654:2001	A/25g	ND
				Salmonella spp.	ISO 6579:2002	A/25g	MAPA/ IN n°46. (2011)
3	Soil	3 samples → 3 x 3 pooled	T1	*E. coli*	ISO 21528-2:2004	10^3 cfu/g	MAPA/ IN n°46. (2011)
			T2	*E. coli* O157:H7	ISO 16654:2001	A/25g	ND
			T3	*Salmonella* spp.	ISO 6579:2002	A/25g	MAPA/ IN n°46. (2011)
4	Seedlings in soil	1 sample → 1 x 3 pooled	T0	*E. coli*	ISO 21528-2:2004	10^2 cfu/g	RDC n°12 (2001)
				E. coli O157:H7	ISO 16654:2001	A/25g	ND
				Salmonella spp.	ISO 6579:2002	A/25g	RDC n°12 (2001)
5	Seedling	1 sample	T0	*E. coli*	ISO 21528-2:2004	10^2 cfu/g	RDC n°12 (2001)
6	Lettuce	3 samples → 3 x 3 pooled	T1	*E. coli*	ISO 21528-2:2004	10^2 cfu/g	RDC n°12 (2001)
			T2	*E. coli* O157:H7	ISO 16654:2001	A/25g	ND
			T3	*Salmonella* spp.	ISO 6579:2002	A/25g	RDC n°12 (2001)
7	Lettuce after washing	3 samples → 3 x 3 pooled	T3	*E. coli*	ISO 21528-2:2004	10^2 cfu/g	RDC n°12 (2001)
				E. coli O157:H7	ISO 16654:2001	A/25g	ND
				Salmonella spp.	ISO 6579:2002	A/25g	RDC n°12 (2001)
8	Rinse water	100 ml	T3	*E. coli*	20 TH APHA (1998)	2 x10^2 MPN/100ml	CONAMA. n°357 de 2005
				E. coli O157:H7	ISO 16654:2001	A/25ml	ND
				Salmonella spp.	ISO 6579:2002	A/25ml	ND
9	Irrigation water source	100 ml	T0 T1	*E. coli*	20 TH APHA (1998)	2 x10^2 MPN/100ml	CONAMA. n°357 de 2005
			T2	*E. coli* O157:H7	ISO 16654:2001	A/25ml	ND
			T3	*Salmonella* spp.	ISO 6579:2002	A/25ml	ND
10	Irrigation water from tap	100 ml	T0 T1	*E. coli*	20 TH APHA (1998)	2 x10^2 MPN/100ml	CONAMA. n°357 de 2005
			T2	*E. coli* O157:H7	ISO 16654:2001	A/25ml	ND
			T3	*Salmonella* spp.	ISO 6579:2002	A/25ml	ND
11	Swab of farmers' hands	3 x 25 cm^2	T3	*E. coli*	ISO 21528-2:2004 and AOAC (1998)	≤ 0.7 log cfu/25 cm^2 (below detection)	Jacxsens. et al. (2010)
12	Swab of transport boxes of lettuce	3 x 50 cm^2	T3	*E. coli*	ISO 21528-2:2004	≤ 0.7 log cfu/25 cm^2 (below detection)	Jacxsens. et al. (2010)

A: absent; ND: not defined by official regulation.
T0: At planting. T1: Two weeks before harvest. T2: One week before harvest. T3: At harvest.

Weather parameters

Regarding weather parameters (temperature and precipitation), results were significantly different (Kruskal-Wallis Test, $p < 0.001$) among the farms and the sampling days throughout the sampling period (Table 2). At farm 1, on the first day of sampling (T0), the highest count of *E. coli* found on soil seedling samples was 2.30 $\log_{10}$ CFU/g. On that day the amount of rain fall was, statistically, the lowest in comparison to the other sampling days (Mann-Whitney *U* Test, $p < 0.001$) (Table 2; Figure 1). On the other farms, no statistical differences were observed both for *E. coli* counts and rain fall volumes during the sampling period.

Temperature at transplanting day was similar to temperatures observed at two and one week before harvest (Mann-Whitney *U* Test, $p = 0.446$, $p = 0.64$, respectively) and significantly different from the harvest day (Mann-Whitney *U* Test, $p = 0.002$). Between the sampling periods of one and two weeks before harvest, temperatures were as well significantly different (Mann-Whitney *U* Test,

Table 2 Mean and standard deviation of temperature and precipitation during sampling period in three farms producing conventional lettuces in Southern Brazil

Farm	Visit	Temperature* (°C)	Precipitation* (mm)
1	T0	18.01 ± 2.58^{a}	0.58 ± 1.31^{a}
	T1	19.02 ± 2.46^{b}	4.84 ± 12.80^{b}
	T2	19.24 ± 1.96^{c}	9.65 ± 13.97^{c}
	T3	17.71 ± 2.33^{d}	23.38 ± 35.06^{d}
2	T0	19.02 ± 2.46^{a}	4.84 ± 12.80^{a}
	T1	16.92 ± 3.15^{b}	1.49 ± 3.71^{b}
	T2	21.70 ± 1.99^{c}	5.66 ± 6.93^{c}
	T3	19.40 ± 1.90^{d}	3.70 ± 5.24^{d}
3	T0	19.02 ± 2.46^{a}	4.84 ± 12.80^{a}
	T1	16.92 ± 3.15^{b}	1.49 ± 3.71^{b}
	T2	21.70 ± 1.99^{c}	5.66 ± 6.93^{c}
	T3	19.40 ± 1.90^{d}	3.70 ± 5.24^{d}

^{a,b,c,d} : Different letters indicate statistically significant differences between the different sampling period.

$p = 0.004$), however no significant difference was observed in *E. coli* counts on samples.

The rain fall amounts were similar between the transplanting day, one week before harvest and at harvest (Mann-Whitney *U* Test, $p = 0.064$ and $p = 0.426$, respectively). However, two weeks before harvest the amount of rain fall was statistically higher when compared to one week before and at harvest (Mann-Whitney *U* Test, $p < 0.01$ for both), but the *E. coli* counts remained similar.

Diagnosis of the current good agricultural practices and management system

The context of the farmers appraised revealed that the conventional lettuce farms had a high risk context towards microbiological safety and crop hygiene. The calculated averages for product and process characteristics reached an index of 3.0 for all the three farms, because they have similar products and production practices (Table 4).

Indicators of organization & chain processing scored 2.46 (farm 1), 2.69 (farm 2) and 2.54 (farm 3), indicating moderate to high level of risk (Table 4). The riskiness of the organization of the farms was very similar, except for the indicators 'technical staff of the farm' and 'variability in workforce'. Farm 1 had a stable workforce and additionally technological insights were as well present. At farm 2 also a good technological staff was present, but the activities had to rely on part time working personnel. For farm 3 the situation was rather the opposite. Working personnel at the premises was already an effective and a stable workforce for a long period of time. Nonetheless, the technological knowledge was not present. The indicators at level 2 (moderate risk) were 'extent of power in supplier relationships' and 'logistic facilities' for all three farms. However, all the other indicators were classified as at high risk level (level 3) for the three farms (sufficiency of operator competences, extent of management commitment, degree of employee involvement, level of formalization, sufficiency supporting information systems, food safety information exchange, and inspections of food safety authorities).

The indicated levels of the control activities in the good agricultural practices of the farms are specified in Table 4. The mean score of the design or set-up of control activities was 1.53. An indication that these activities were absent (level 1) or conducted on a basic level, using historical and common knowledge (level 2), and no sector information or information from suppliers was applied (level 3), nor tailored to the farms own situation (level 4).

The profiles were very similar for all the three farms, though farm 3 differs from farms 1 and 2 on 'partial physical intervention' (rinsing step), because rinsing of the lettuce crops was not conducted at farm 3. Farms were operating mainly at basic level (level 1) with regards to items related to 'equipment hygienic design maintenance program', 'sanitation program', 'packaging equipment', 'water control', 'sampling for microorganisms', 'analyzing methods for pathogens' and 'corrective actions'. An indication that all these control activities were not in place on the three farms (Table 4).

The indicators 'storage facilities', 'personal hygiene', 'raw materials control', 'fertilizer program', 'irrigation method' were classified at level 2. That level suggests that these activities were performed based on the knowledge of the farmers and not based on inputs from guidelines, sector organizations or government (Table 4).

For the farms at which rinsing of the lettuce heads was implemented after harvest (farms 1 and 2), the rinsing was also done based on their individual knowledge. Supplier control of the seedlings and manure composting were well achieved (level 3, best situation) because all farms bought seedlings from the same supplier and fertilizers had been already composted over 90 days before arrival to the farms.

Moreover, the actual operation of control activities was lower (averages of 1.43 for all three farms – Table 4) compared to the design or set-up of the control measures. This situation is indicating that the control measures were not implemented and applied in practice. Only the indicator 'compliance to producers' received a level 2, because the growers comply to their own working method.

Also assurance activities such as 'translation of stakeholder requirements', 'use of feedback information', 'validation activities' and 'verification activities', 'documentation system' and 'record keeping' were not present or had

Table 3 Sampling location, sample type, number of samples and results for microbiological analysis

			Hygiene indicators				Pathogen indicator	
			E. coli (mean and stdv)	Number of samples per *E. coli* counts			*Salmonella*	*E. coli* O157:H7
CSL	**Sample**	**n**		<1.0 log	≥1.0 and < 2.0 log	≥2.0 and < 3.0 log	A/P*	A/P*
1	Manure	9	1.11 ± 0.33 cfu/g	8	0	1	A	A
2	Manured soil	9	<1.00 ± 0.00 cfu/g	9	0	0	A	A
3	Soil	27	1.05 ± 0.20 cfu/g	23	3	1	A	A
4	Seedlings in soil	3	1.43 ± 0.75 cfu/g	2	0	1	A	A
5	Seedlings	3	1.00 ± 0.00 cfu/g	2	1	0	A	A
6	Lettuce	27	1.06 ± 0.22 cfu/g	23	3	1	A	A
7	Lettuce after washing	6	1.00 ± 0.00 cfu/g	5	1	0	A	A
8	Rinse water	2	1.00 ± 0.00 MPN/100 ml	2	0	0	A	A
9	Irrigation water source	12	1.03 ± 0.012 MPN/100 ml	10	2	0	A	A
10	Irrigation water from tap	12	1.04 ± 0.12 MPN/100 ml	10	1	1	A	A
11	Swab of farmers' hands	9	1.00 ± 0.00 cfu/25 cm^2	9	0	0	-	-
12	Swab of transport boxes of lettuce	9	1.00 ± 0.01 cfu/25 cm^2	9	0	0	-	-
Total		128		112	11	5	-	-

* A: absent in 25 g or 25 ml; P: presence in 25 g or 25 ml; stdv: standard deviation.

not been yet developed. An indication that the farms could not demonstrate that they were working correctly (mean level of 1 for all).

The system output of the current good practices for the conventional lettuce farms was also low (mean 1 for all the three farms). The reason for this was that no information was available about the system output: no inspection or audit was performed, no samples (for microbiological or chemical analyses) were taken, no visual quality was evaluated, and no non-conformities were recorded or evaluated. Consequently no actual evaluation of the system output could be completed (Table 4).

Discussion

In the present study low levels of microbiological contamination were found in samples collected from small farms producing conventional lettuces in Southern Brazil, even though a high risk context towards microbiological safety and crop hygiene was verified in all of them based on the self-assessment questionnaire. These different results may indicate that some good agricultural practices were in place, however no formal control was applied.

For example, low levels of contamination and the absence of *Salmonella* spp. and *E. coli* O157:H7 observed in manure were attributed to the fact that all farms purchased manure from commercial suppliers, which was already composted for over 90 days. Several authors described that adequate composting time will effectively reduce contamination (Oliveira et al., 2012; Fischer-Arndt et al., 2010; James, 2006; Millner, 2003; MAFF, 2000) and particular pathogens like *E. coli* and *Salmonella* spp. can

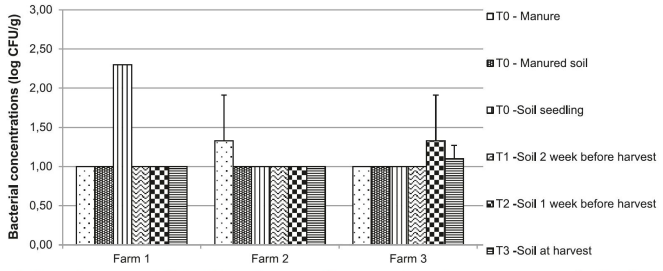

Figure 1 Overview of the performance of *Escherichia coli* enumeration among farms in manure, manured soil, soil seedling, soil two and one week before harvest and at harvest.

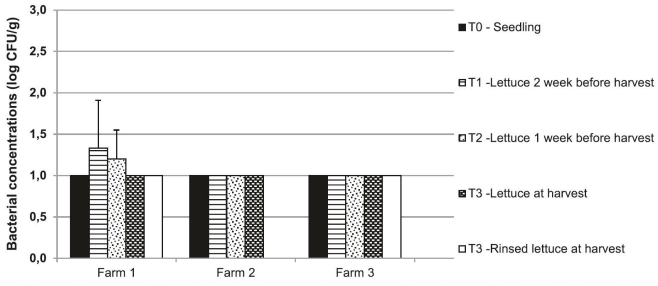

Figure 2 Overview of the performance of *Escherichia coli* enumeration among farms in seedling, lettuce two and one week before harvest, lettuce at harvest and rinsed lettuce at harvest.

survive at maximum to 90 days in soil as well as in manure (Heaton and Jones, 2007; Nicholson et al., 2005).

Moreover, the composted manures used at farms were added to the soil at least two weeks prior setting the seedlings into the field and after that no more manure was applied to supply nutrients to the lettuce plants. Also in the evaluation of the current good agricultural practices related to manure management, indicator 'organic fertilizer program', a moderate level 2 was given for the three farms, indicating that they used and manipulated manure based on generic knowledge from their suppliers (Table 4).

At planting and at harvest, all *E. coli* counts were below the detection limit (<1.00 $\log_{10}$ CFU/g), demonstrating good quality of lettuce seedlings and final product (lettuce) in attendance to the parameters of the Brazilian legislation (Brasil, 2001) that sets 10^2 CFU/g as the maximum acceptable limit for *E. coli* counts. The fact that no *E. coli* was detected on lettuces can be attributed to the low pressure of *E. coli* in the manure, manured soil around the crop and low contamination of the irrigation water. Corroborating these results, EFSA (2014) reported that several reasons can be attributed to the variation in *E. coli* numbers on leafy greens and the relationship between primary production practices and numbers of *E. coli* in final product is very variable. Even though, it is difficult to define which is the main cause of this variation, the microbial quality of manure and irrigation water are frequently cited (EFSA, 2014).

In the present study, the water supply was considered a high risk (Table 4), especially because the water came from ponds (Richardson et al., 2009) and there was no further treatment, however water sampled from ponds, sprinklers and rinsing tanks presented low levels of contamination by *E. coli*. All analyzed samples were in accordance with the Brazilian regulation for irrigation of vegetables (CONAMA, 2005), which establishes a limit of 2×10^2 CFU/100 ml for thermotolerant coliforms. Similarly, no *Salmonella* spp. or *E. coli* O157:H7 were isolated from any of the analysed water samples. In a different study conducted in organic farms of the same region of Brazil (Rodrigues et al., 2014), the presence of *Salmonella* spp. and *E. coli* O157:H7 was detected in two samples (irrigation and rinsing tank water), after a flooding event. It is important to mention that in the farms investigated in the present study, the water supply (ponds) and the crop fields were located in elevated areas were flooding could not occur. Other authors observed the influence of flooding in the variation of pathogens levels (Liu et al., 2013, Castro-Ibañez et al., 2013; Cevallos-Cevallos et al., 2012; Tirado et al., 2010; Franz et al., 2005; Girardin et al., 2005; Rose et al., 2001).

Water has been identified as the source of microbial contamination of several foodborne outbreaks involving leafy vegetables around the world (Itohan et al., 2011; Delaquis et al., 2007; Beuchat, 1996). Pathogenic bacteria such as *E. coli* O157:H7 are often associated with outbreaks of waterborne diseases, resulting from inadequate treatments of the water used for irrigation and rinsing of fruits and vegetables (Levantesi et al., 2012; Moyne et al., 2011). Furthermore, in the present study, farms 1 and 2 used the same irrigation water source to rinse the lettuces after harvest. At farm 3 no rinsing of the lettuce heads did take place. The results indicated that no significant differences were observed for *E. coli* counts before, after or without the rinsing procedure, even though the water supply was considered a high risk of contamination (Table 4) and there was no water control.

No pathogens were identified in any crop sample and no increases in the microbial counts were as well observed after the rinsing process, demonstrating just the opposite in our study of what was ascertained in a study conducted by Antunes (2009).

Regarding organization and chain characteristics (Table 4), the technological staff present in farm 1 had received technical support provided by local government (city), while in

Table 4 Scores and calculated mean attributed to the indicators of food safety management system

Indicators	Farm1	Farm 2	Farm 3	Description of situation
I. Context factors (overall)[a]				
Product and process characteristics				
Risk of raw materials microbial	3	3	3	Seedlings and manure purchased from commercial suppliers without any Good Agricultural Practice implemented. Irrigation water without any treatment. Seedlings in direct contact with soil.
Risk of final product microbial	3	3	3	The lettuces crops growing in direct contact with soil and without covering.
Production system	3	3	3	Open cultivation field and contact with soil.
Climate conditions	3	3	3	The farms were located in subtropical areas, with uncontrolled climate conditions.
Water supply	3	3	3	All producers used water from ponds, without treatment.
Mean product and process	*3,00*	*3,00*	*3,00*	
Organization and chain				
Presence of technological staff	2	3	3	Farm 1 had technical support provided by government department (of the city). Farm 2 and 3 had no technical support.
Variability in workforce composition	1	3	1	Farm 2 had a high turnover of employees and temporary operators were commonly used. Farm 1 and 3 had low turnover, with occasonaly temporary operators.
Sufficiency of operator competences	3	3	3	Operators with no training in food safety control, only practice experience in the field.
Extent of management commitment	3	3	3	All three farms had no written food safety policy and no official quality team.
Degree of employee involvement	3	3	3	There was no safety control sistems implemented in the farms.
Level of formalization	3	3	3	No meetings sistem implemented for instructions communication exist in all producers.
Sufficiency supporting information systems	3	3	3	None of the producers had standard information system for food safety control decisions.
Severity of stakeholders Requirements of	3	3	3	Steakholders did not ask for any QA requirements.
Extent of power in supplier relationships	2	2	2	All farms required from their manure suppliers to compost the manure as a prerequisit for purchase.
Food safety information exchange	3	3	3	No sistematic exchange of information on food safety issues were done with the suppliers of the three producers.
Logistic facilities	2	2	2	Transport of the final products to the distributer done by trucks in protected conditions (covered) but room temperature.
Inspections of food safety authorities	3	3	3	Never a inspection were done in the three farms.
Supply source of initial materials	1	1	1	Only local suppliers of major initial materials
Mean organisation and chain	*2,46*	*2,69*	*2,54*	
II. Control activities design[b]				
Hygienic design of equipment and facilities	1	1	1	None specific hygienic design required for equipement and facilities among the producers.
Maintenance and calibration program	1	1	1	No manteinance and calibration program apllied in any of the producers.
Storage facilities	2	2	2	Storage was made in ambient conditions in all farms.
Sanitation program(s)	1	1	1	The producers had no specific sanitation program implemented.

Table 4 Scores and calculated mean attributed to the indicators of food safety management system *(Continued)*

Personal hygiene requirements	2	2	2	No specific hygiene instructions were followed by the operators but washing facilities and toillets were available next to the field in all farms.
Incoming material control	2	2	2	Incoming material control was done by visual inspections based on historical experience in all farms.
Packaging equipment	2	2	2	Use of non specific plastic boxes to pack the lettuce.
Supplier control	2	2	2	The farms had no specific pre requisites for supplier selection.
Organic fertilizer program	2	2	2	Pre composted manure purchased from local suppliers in all producers.
Water control	1	1	1	There was no water control in all farms.
Irrigation method	2	2	2	All producers used sprinkler as the irrigation method.
Partial physical intervention	2	2	1	General partial physical intervention applied by washing the lettuce and external leaves removed
Analytical methods to assess pathogens	1	1	1	The presence of pathogens were never analyzed by any of the producers.
Sampling plan for microbial assessment	1	1	1	The producers had no sampling plan implemented.
Corrective actions	1	1	1	The farms had no corrective actions described.
Mean control activities design	*1,53*	*1,53*	*1,53*	
III. Control activities operation[b]				
Actual availability of procedures	1	1	1	The procedures were not documented in all the three farms.
The actual of compliance to procedures	2	2	2	The operators executed tasks according to their own experience and ad-hoc basis.
Actual hygienic performance of equipment and facilities	1	1	1	The hygienic design is not considered to be important for food safety.
Actual storage/cooling capacity	1	1	1	The farms had no cooling storage facility available.
Actual process capability of partial physical intervention	2	2	2	The partial physical intervention were done without standard parameters and no control charts.
Actual process capability of packaging	2	2	2	Packaging were done without regular parameters and based on the lettuce size.
Actual performance of analytical equipment	1	1	1	No analytical analyses were done in all farms.
Mean control Activities operation	*1,43*	*1,43*	*1,43*	
IV. Assurance activities[b]				
Translation of stakeholder requirements into own HSMS requirements	1	1	1	Stakeholder requirements were not present in all three farms.
The systematic use of feedback information to modify HSMS	1	1	1	The farms had no HSMS implemented.
Validation of preventive measures	1	1	1	The producers had no preventive measures implemented and validated.
Validation of intervention processes	1	1	1	Intervention processes have never been validated and were done based on their own knowledge.
Verification of people related performance	1	1	1	The producers had no documented procedures described, so no verification was done.
Verification of equipment and methods related performance	1	1	1	No procedures of verification for equipment and methods were preformed in all producers.
Documentation system	1	1	1	Documentation were not available in all the farms.
Record keeping system	1	1	1	no record keeping system were present in all three farms.
Mean assurance activities	*1,00*	*1,00*	*1,00*	
Food safety management system Output[c]				
Food safety Management System evaluation	1	1	1	No inspection or audit of the Food Safety Management System were done in all produceres.

Table 4 Scores and calculated mean attributed to the indicators of food safety management system *(Continued)*

Seriousness of remarks of remarks	1	1	1	Audits on HSMS were never performed.
Hygiene related and microbiological food safety	1	1	1	No records of hygine related and microbiological food safety complains were available in the farms.
Chemical safety complaints of customers	1	1	1	Chemical complains records were not avalilable in the producers.
Typify the visual quality complaints	1	1	1	No records about quality complaints were available in the farms.
Product sampling microbiological performance	1	1	1	The microbiological performance is not known once no microbiological analyses were done on regular basis.
Judgment criteria microbiological	1	1	1	Microbiological analyses were not performed in the farms.
Non conformities	1	1	1	The performance of the HSMS was not possible once no coformities registration were available
Mean food safety output	*1,00*	*1,00*	*1,00*	

[a]I Context factors: product and process characteristics and organization and chain characteristics were evaluated based on three risk levels: level 1 (low risk); level [2](medium risk); and level 3 (high risk).
[b]II Control activities design: evaluates the designs of control activities; III evaluates the actual operation or implementation of control activities; IV evaluates the assurance activities in good agricultural practices based on four levels: level 1 (non-existing or not implemented); level 2 (activities done at basic level based on own knowledges and historical information); level 3 (activities implemented based on sector information or guidelines); level 4 (activities adapted and tailored to the specific situation on the farm).
[c]IV Food safety system output indicators: evaluation based on external or governmental audits, records, microbial and chemical analysis: level 1 (not done or no information available); level 2 (limited information available); level 3 (more systematic information is available); level 4 (systematic informations available and good results are obtained).

farms 2 and 3 no technical support was given. At the same time, farm 1 and 3 had a stable workforce, while farm 2 demonstrated a high turnover. Some authors described that the stability of the workforce can help the companies to prevent food safety questions and problems (Kirezieva et al., 2013a; Luning et al., 2011). At the same time the other organization characteristics demonstrated that all farms were operating in a very low level of organization, what is common in family based companies (Lunning et al, 2011; Powell et al., 2011), with the operators without any king of food safety training, no safety control systems implemented or written, no standard information about safety control systems, stakeholders without any quality assurance required, transport of the final product without temperature control and no inspection done by official authorities. It is well known that a trained workforce can help the companies to implement the good agricultural practices, once the employees know their responsibilities with the food safety issues (Kirezieva et al., 2013b) and that governmental inspections are also important to assure the compliance of the companies with the good practices (Jafee and Masakure, 2005; Kierzieva et al., Kirezieva et al. 2013a). It has also been demonstrated that the practice of keeping registration and documents in the primary production level is not usual in other countries (Jevsnik et al., 2008; Nieto-Montenegro et al., 2008), however, this could be a good procedure to be implemented in Brazilian farms in order to reach higher food safety levels.

It might be assumed that the studied conventional lettuce farms were in a moderate to high level of risk in microbiological contamination due to product and process characteristics (Kirezieva et al., 2013b), once the seedling where purchased from commercial suppliers without formal good agricultural practices implemented, lettuce crops were in direct contact with soil, farmers located in subtropical areas without climate conditions control, there was no treatment of irrigation water, and the cultivation was in open fields (Table 4). That context level found in the three farms suggests that a medium to advanced level of good agricultural practices and management system should be present in order to have a good system output as described by authors such as Osés et al. (2012) and Kirezieva et al. (2013b). However, the good practices and management of all investigated farms were informal and very basic, which may implicate in a high risk of food safety problems (Uyttendaele et al., 2014). Moreover, in the conventional lettuce farms investigated, there was no system output because of the lack of registered information and controls. This results could be explained because in Brazil there is no governmental requirement for that and producers are not stimulated to make quality records. A similar situation was observed in organic farms in the same region of Brazil (Rodrigues et al., 2014). Different circumstances was reported by Kirezieva et al. (2015) for companies located in the European Union where lower to moderate risk of production and supply chain context was found because, among other factors, controlled water sources were used and the cultivation was done in a protected area. The microbial load and pressure in the conventional farms analysed in the present study were lower compared to the samples collected in organic farms studied by Rodrigues et al. (2014), who reported higher *E. coli* counts and also the presence of *Salmonella* and *E. coli* O157:H7.

The major differences between conventional and organic farms studied in Southern Brazil were the manure and composting of manure, which was conducted by the organic farms themselves with uncontrolled manner while a good manure management and control was evaluated for the conventional farms. Also, no animals were present on conventional farms what may contributed in the reduction of *E. coli* pressure on the water sources. Furthermore, a good water quality was verified in conventional farms, what was not the case in the organic ones (Rodrigues et al., 2014).

Conclusions

The use of the risk based sampling plan in combination with the diagnostic questionnaire allowed to analyse the microbiological aspects and the status of management systems of conventional lettuce farms in Southern Brazil.

Although all farms had no formal good agricultural practices implemented and there was no technical support in any of them, the microbial parameters showed very low levels of contamination, including the final products (lettuce heads). These results are plausible for the reason that Brazilian regulatory bodies do not enforce the implementation of good agricultural practices, nonetheless farmers are frequently aware that farm organization and hygienic procedures are necessary in order to maintain food safety and good productive levels. As an example, all analyzed farms had toilets near to the fields, providing adequate personnel hygienic practices. Further, the farms did not raise animals such as cows, pigs and hens, ultimate sources of cross contamination of the fields, remarkably, as a consequence of rain falls. In addition, the fields were located in areas where flooding was not possible. Another important aspect to take into account, concerning the organic fertilizer that was appropriately composted, not impacting on the contamination of the crops. Similarly, the good quality of the irrigation waters used, evidenced by the microbial analyses, did not influence the contamination of the final product.

Good practices should be applied during all food chain, farm to fork. It has been observed that in the last years, outbreaks caused by fresh produce are increasing around the world, suggesting that, in that particular step of the chain, primary production, more efforts are needed in order to get more safety.

Even though the fact that all the microbial results were very low and no pathogen was determined in any of the analysed samples, attention should be given to the results of the self-assessment questionnaire that indicated moderate to high risk levels at all farms. These different results are important in order to provide information about the actual status of contamination (microbial sampling plan) and possible food safety problems in the future based on the results given by the questionnaire. Furthermore, the results of this study also highlighted the necessity to provide more safety during the fresh produce cultivation, based on the bottlenecks identified by the self-assessment questionnaire, being formal good agricultural practices implementation an important start to the fresh produce farms in Brazil, as well as to adopt a higher level of control activities in order to achieve lower risk levels.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SB carried out initial contact with producers, performed sampling collection, carried out the microbial analyses, interviewed the producers with the self-assessment questionnaire, elaborated critical analyses based on results, drafted the manuscript. Carried out revisions on manuscript. CTH - Helped with the microbial and statistical analyses, participated in scientific discussions. RQ - Helped with the sample collection. AP - Prepared sampling material and contributed with for microbial analyses, participated in scientific discussions. FP - Prepared sampling material and contributed with for microbial analyses, participated in scientific discussions. LJ - Planned the sampling collection and general organization of experiments. Helped with the interpretation of the self-assessment questionnaire results and discussion. Participated in scientific discussions. MU - Planned the sampling collection and general organization of experiments. participated in scientific discussions. RJB - Revised the manuscript and added inputs. ECT organised research team in all activities of the manuscript. Planned the sampling collection and general organization of experiments. participated in scientific discussions. Contributed with laboratory infra-estructure. Participated in elaboration of the manuscript. All authors read and approved the final manuscript.

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3.1 ARTIGO 2

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Microbiological quality and safety assessment of lettuce production in Brazil

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ABSTRACT

The microbiological quality and safety of lettuce during primary production in Brazil were determined by enumeration of hygiene indicators *Escherichia coli*, coliforms and enterococci and detection of enteric pathogens *Salmonella* and *E. coli* O157:H7 in organic fertilizers, soil, irrigation water, lettuce crops, harvest boxes and worker's hands taken from six different lettuce farms throughout the crop growth cycle. Generic *E. coli* was a suitable indicator for the presence of *Salmonella* and *E. coli* O157:H7, while coliforms and enterococci were not. Few pathogens were detected: 5 salmonellae and 2 *E. coli* O157:H7 from 260 samples, of which only one was lettuce and the others were manure, soil and water. Most (5/7) pathogens were isolated from the same farm and all were from organic production. Statistical analysis revealed the following environmental and agro-technical risk factors for increased microbial load and pathogen prevalence in lettuce production: high temperature, flooding of lettuce fields, application of contaminated organic fertilizer, irrigation with water of inferior quality and large distances between the field and toilets. Control of the composting process of organic fertilizers and the irrigation water quality appear most crucial to improve and/or maintain the microbiological quality and safety during the primary production of lettuce.

1. Introduction

Awareness about healthy diets and specifically the importance of daily consumption of fruits and vegetables has been increasing worldwide over the last decades. Since regular consumption of vegetables may reduce the risk of cancer, cardiovascular diseases and obesity, their consumption is promoted by governmental health agencies (de Lima et al., 2008; Legnani et al., 2010; Neutzling et al., 2010; Perozzo et al., 2008). Lettuce (*Lactuca sativa* L.) is the leafy vegetable most consumed in Brazil, making up approximately 40% of the total volume traded in fresh produce supply companies (Sala and Costa, 2012). Unfortunately, fresh produce is not free of health risks, because contamination with pesticides and pathogens is regularly reported. Leafy greens were reported as the vehicle in 40 foodborne outbreaks corresponding with 1455 disease cases in the US between 1996 and 2008 and prepared salads caused 82 outbreaks (4% of the total) in the UK between 1992 and 2006 (Anderson et al., 2011; Little and Gillespie, 2008). As much as 20% (15/75) of the leafy greens sampled on the retail level in Spain contained unacceptable pesticide residues (above the maxima residue limits), with lettuce being the more contaminated than spinach and chard (Gonzalez-Rodriguez et al., 2008). Specifically for lettuce, the most severe microbiological threats are contamination with enterohemorrhagic *Escherichia coli* and *Salmonella enterica* (Anderson et al., 2011). Furthermore, *Salmonella* was the most important pathogen in food-borne outbreaks in Brazil between 2000 and 2012 (Gomes et al., 2013; Tondo and Ritter, 2012). Although the microbiological quality of ready-to-eat lettuce in the state of São Paulo, Brazil, was among the better of the sampled retail salads, there was room for improvement. The limit of 100 CFU/g *E. coli* established by the Brazilian Surveillance Agency was surpassed in 2.7 to 73.0% of lettuce samples and 0.4% to 3.0% contained *Salmonella* (BRASIL, 1986; Froder et al., 2007; Sant'Ana et al., 2011). However, few data on the microbial quality and safety of lettuce crops during primary production are available. The microbiological quality of ready-to-eat lettuce put on the market depends primarily on the quality of the lettuce crop and is impacted by good agricultural practices. Organic farming has been implemented to provide agricultural products which meet the consumer's increasing demand. In Brazil, organic farming is mostly practiced in horticulture, namely 4.5% in comparison with 1.8% of organic production of general agriculture (IBGE, 2006). Furthermore, organic production is predominant in the south region of Brazil, the state of Rio Grande do Sul (the study area) having the third largest production of organic vegetables in the country. Since no chemical fertilizers, pesticides or fungicides are applied by organic farmers, organic fresh produce scores much higher than the conventional in the consumers' perception (Hoefkens et al., 2009). People generally believe that organic vegetables have a higher nutrient and vitamin content and contain no or less chemical and biological contaminants. However, as a consequence of the use of manure or

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manure-derived organic fertilizers, it has been proposed that the microbiological quality of organic produce is lower than that of conventional production (Maffei et al., 2013; Oliveira et al., 2010).

In this study, the microbiological quality and safety of lettuce in Brazil were assessed to reveal its current status. The microbiological characteristics of the agricultural production environment were determined and the agricultural practices were recorded by interviews and questionnaires to provide insight into possible contamination routes and sources, allowing recommendation of specific interventions to improve the quality and safety of lettuce. Six different lettuce farms were sampled, of which three worked according to conventional production principles and three adhered to organic agricultural practices, so that potential differences in microbiological parameters between farms and between production systems could be investigated.

2. Materials and methods

2.1. Sampling and microbiological analyses

The microbiological quality and safety in the primary production of lettuce were assessed in the rural area of Rio Grande do Sul, the southernmost State in Brazil (Fig. 1). Six farms were sampled between December 2011 and October 2012. Each farm was sampled four times throughout the lettuce crop growth cycle, namely at planting (four weeks before harvest), two weeks before harvest, one week before harvest and at harvest. Three farms produced lettuce according to the conventional production system and three according to the organic production (certified by the Organism of Social Control (OSC) and by the Participative Organization of Organic Compliance (OPAC) of the Southern region of Brazil). Relevant environmental, technical and agricultural information was collected for the statistical analysis and interpretation of the microbiological results. Background information on the agricultural practices and environmental factors was obtained by visual inspection of the farm during the visits and a questionnaire interview with the farm supervisor (the original questionnaire (in Portuguese) is available as a Supplementary Figure). Climatic conditions, i.e. the average week temperature and the cumulative precipitation of the week prior to sampling and including the sampling day (8 d), were obtained from the National Institute for Meteorology of Brazil (Instituto Nacional de Meteorologia (INMET), http://www.inmet.gov.br/portal/).

Distributed equally over the six lettuce farms, 260 samples were taken (Table 1): 18 of manure (200 g from the on-farm composting location), 72 of soil (3 samples of 200 g pooled), 6 of soil on the seedling (200 g), 6 of seedling (500 g), 54 of lettuce (3 crops pooled), 15 of rinsed lettuce (3 crops pooled), 48 of irrigation water (5 L), 5 of rinse water at harvest (5 L), 18 of transport boxes for harvested lettuce (1 swab of 50 cm^2 in 5 mL of 0.1% peptone water) and 18 of workers' hands (1 swab of the entire hand surface in 5 mL of 0.1% peptone water). Large sample volumes and pooling of samples were done to obtain a more representative sample and after mixing a smaller subsample (25 g, 25 mL or 1 mL) were analysed for the following microbiological parameters: detection (presence/absence per 25 g or 25 mL) of the pathogens *E. coli* O157:H7 (ISO 16654:2001) and *Salmonella* spp. (ISO 6579:2002) and enumeration (per gram or 100 mL) of the hygiene indicators *E. coli* and total coliforms, which were enumerated in solid samples (i.e. manure, soil, seedling, lettuce, rinsed lettuce, transport boxes and hands) by plating 1 mL on Petrifilm™ plates of the appropriate tenfold dilutions in 0.1% peptone water, while in water samples the Most Probable Number (MPN) method using the multiple tube technique was applied (APHA, 1998). In water samples, *Enterococcus* spp. was enumerated per 100 mL as an additional faecal hygiene indicator (APHA, 1998).

2.2. Statistical analyses

All analyses were performed with SPSS Statistics version 21 at a significance level of 5% ($p = 0.050$). The 95% confidence intervals for pathogen prevalence were calculated according to the Wilson score method without continuity correction (Wilson, 1927). Comparison of the pathogen prevalence in different data groups was done using the Likelihood Ratio calculated in the Chi-Squared Test of Independence with the Monte Carlo option (10,000 repetitions, 99% confidence level). Raw data for *E. coli*, coliforms and enterococci were not normally distributed, so non-parametric tests were used for statistical analysis (Kolmogorov–Smirnov test, $p \leq 0.001$ for all). Ordinal classes were defined for the indicators *E. coli*, coliforms and enterococci (Table 2) to cope with the large number

Fig. 1. Map of Brazil showing the location of six lettuce farms which participated in the current sampling study by the dotted circle.

Table 1
Sampling scheme performed on six farms throughout the cultivation cycle of lettuce to assess the microbial quality and safety.

Sample types	Sampling time				Samples per farm	Total samples
	Planting	Two weeks before harvest	One week before harvest	Harvest		
Fertilizer	Composted manure (3)	–	–	–	3	18
Soil	Soil (3) Soil from the lettuce seedling (1)	Soil (3)	Soil (3)	Soil (3)	13	78
Lettuce	Lettuce seedling (1)	Lettuce (3)	Lettuce (3)	Lettuce (3) Lettuce after harvest and washing (3)[a]	13	75[a]
	–	–	–			
Water	Irrigation water reservoir (1) Irrigation water at application point (1)	Irrigation water reservoir (1) Irrigation water at application point (1)	Irrigation water reservoir (1) Irrigation water at application point (1)	Irrigation water reservoir (1) Irrigation water at application point (1)	9	53[a]
	–	–	–	Rinse water (1)[a]		
Contact surfaces	–	–	–	Swab of hands (3) Swab of transport boxes (3)	6	36
	–	–	–			
Total					44	260

[a] On one farm harvested lettuce was not washed, so only 15 instead 18 samples of rinsed lettuce and 5 instead of 6 rinse water samples were obtained.

of results below the detection limits and to investigate their relation with pathogens, which were calculated using Kendall's Tau-c. Bivariate correlations between the indicators were determined by calculating the Spearman's Rho coefficient using the raw enumeration data. The influence of various factors was assessed using Kruskal–Wallis or Mann–Whitney *U* tests. If significant differences were found, pair wise tests were performed to identify the significant differences between individual categories. In case of *n* pair wise comparisons, Dunn–Sidak correction was applied, resulting in adjusted individual p' values: $p' = 1 - (1 - p)^{1/n}$, in which p was set to 0.050 to obtain a family-wise error rate of 5%. The performance of enumeration of indicator microorganisms and subsequent classification as a screening tool to identify samples with pathogens was evaluated by Receiver Operating Characteristic (ROC) curve analysis in SPSS. Logistic regression was applied to identify the factors of influence considered simultaneously (multivariate statistics), their relative importance and possible interactions on the presence of pathogens. Backward likelihood ratio model selection was performed on all significant factors in univariate analysis for significant main effects and all possible interactions between the obtained effects were checked one-by-one by addition to the final model and added if significant.

3. Results

3.1. Data evaluation

Since many indicator enumerations were negative, i.e. below the detection limit, the raw data were transformed by defining ordinal classes (Table 2). A new class was only started for minimum 5 samples; otherwise the samples were added to the next lowest class. A small number of pathogens was detected (Table 3), resulting in the overall prevalence of 1.9% (95% confidence interval (CI): 0.8%–4.4%) for *Salmonella* and 0.8% for *E. coli* O157:H7 (95% CI: 0.2%–2.8%). More specifically, *Salmonella* prevalence was 5.6% in manure (95% CI: 1.0%–25.8%), 2.6% in soil (95% CI: 0.7%–8.9%), 1.9% in water (95% CI: 0.3%–9.9%) and 1.3% in lettuce (95% CI: 0.2%–7.2%). *E. coli* O157:H7 was only isolated from water samples, namely 3.8% (95% CI: 1.0%–12.8%).

3.2. Correlation between different hygiene indicators

There was a significant but weak linear correlation between the faecal indicators *E. coli* and coliforms in all sample types (Spearman's Rho bivariate correlation coefficient 0.480; $p < 0.001$; $N = 260$; Fig. 2). This correlation was strongest in manure samples (correlation coefficient 0.873; $p < 0.001$; $N = 18$), then in soil samples (correlation coefficient 0.380; $p = 0.001$; $N = 78$), next in water samples (correlation coefficient 0.310; $p = 0.024$; $N = 49$), the weakest in lettuce samples (correlation coefficient 0.266; $p = 0.021$; $N = 75$) and finally no significant relation was found in samples of hands ($p = 0.926$; $N = 18$) and boxes (no *p*-value because *E. coli* was always undetected; $N = 18$). Enterococci were not significantly correlated with *E. coli* ($p = 0.431$; $N = 49$) and coliforms ($p = 0.369$; $N = 49$). This means that enterococci are an independent indicator of *E. coli* and coliforms, but that the latter are dependent indicators and thus enumeration either suffices.

3.3. Evaluation of indicators to predict pathogen presence

The presence of pathogens, i.e. *Salmonella* or *E. coli* O157:H7, was significantly correlated with the *E. coli* class (Kendall's Tau-c, $p < 0.001$), meaning that samples belonging to higher *E. coli* classes were associated with increased pathogen prevalence (Fig. 3A). In contrast, pathogen presence was not correlated with the coliforms (Kendall's Tau-c, $p = 0.304$; Fig. 3B) and enterococci (Fig. 3C).

Receiver Operating Characteristic (ROC) curve analysis showed that both the concentration and classification of *E. coli* had significant predictive power to predict the presence of pathogens *Salmonella* and *E. coli* O157:H7 ($p = 0.001$ and $p < 0.001$, respectively), while coliforms and enterococci classes and counts did not ($p = 0.315$, $p = 0.617$, $p = 0.773$ and $p = 0.661$, respectively). The area under the curve (AUC) for *E. coli* classes and counts was respectively 0.898 and 0.873 with standard errors of resp. 0.032 and 0.035, which are moderate to high, suggesting that *E. coli* is an indicator with substantial predictive power in this situation.

3.4. Influence of environmental factors on microbiological parameters during lettuce farming

The presence of *E. coli* O157:H7 and *Salmonella* was associated with significantly higher average temperatures during the week of sampling (Mann–Whitney *U* Test, $p = 0.018$, Fig. 4A), but the cumulative week precipitation did not differ significantly (Mann–Whitney *U* Test, $p = 0.330$, Fig. 4B). Fields of organic producers 2 and 3 were flooded two days before harvest with irrigation water from the close-by water pond due to heavy rainfall (28.6 mm on the day of sampling). The probability of detecting pathogens increased fourfold within one week of a flooding event for all sample types together, albeit this difference was just above the limit of statistical significance (Chi-Squared Test of Independence, Likelihood Ratio, $p = 0.058$; Fig. 4C). Flooding had no influence on the concentrations of *E. coli*, coliforms and enterococci (Mann–Whitney *U* Test, $p = 0.332$, $p = 0.143$ and $p = 0.541$, respectively). Interestingly, *E. coli* O157:H7 was only isolated from water samples taken during the flooding event ($N = 2$; Fig. 4C) and never during normal precipitation conditions. Specifically for water samples ($N = 53$), flooding significantly increased pathogen prevalence from 0.0 to 50.0% (Chi-Squared Test of Independence, Likelihood

Table 2
Definition of classes for the enumeration results of indicators *E. coli*, coliforms and enterococci.

E. coli class	Solid samples	Water samples
1 (=undetected)	<1.0 log CFU/g	<0.04 log MPN/100 mL
2	≥1.0 and <2.0 log CFU/g	≥0.04 and ≤1.4 log MPN/100 mL
3	≥2.0 and <3.0 log CFU/g	>1.4 log MPN/100 mL
4	≥3.0 and <4.0 log CFU/g	–
5	≥4.0 log CFU/g	–
Coliforms class	Solid samples	Water samples
1 (=undetected)	<1.0 log CFU/g	<0.04 log MPN/100 mL
2	≥1.0 and <2.0 log CFU/g	≥0.04 and ≤1.4 log MPN/100 mL
3	≥2.0 and <3.0 log CFU/g	>1.4 log MPN/100 mL
4	≥3.0 and <4.0 log CFU/g	–
5	≥4.0 and <5.0 log CFU/g	–
6	≥5.0 log CFU/g	–
Enterococci class	Solid samples	Water samples
1 (=undetected)	ND	<2.0 log CFU/100 mL
2	ND	≥2.0 and <3.0 log CFU/100 mL
3	ND	≥3.0 log CFU/100 mL

ND = not determined.

Ratio, $p = 0.001$) and the average concentration of *E. coli* tenfold from 0.48 log MPN/100 mL (standard deviation 0.54 log MPN/100 mL, $N = 47$) to 1.46 log MPN/100 mL (standard deviation 0.43 log MPN/100 mL, $N = 6$) (Mann-Whitney U Test, $p < 0.001$), while flooding had no significant impact on the counts of coliforms and enterococci (Mann–Whitney *U* Test, $p = 0.207$ and $p = 0.541$).

3.5. Influence of agro-technical factors on microbiological parameters during lettuce farming

The distance from the lettuce fields to toilet facilities differed considerably among the farms: 50 m (producer 5), 100 m (producer 6), 150 m (producer 4), 200 m (producer 1) and 500 m (producer 2 and 3). Increasing distance was significantly linked with increased concentrations of *E. coli* and coliforms (Kruskal–Wallis Test, $p < 0.001$ for both) and higher pathogen prevalence (Mann-Whitney U Test, $p = 0.005$, Fig. 5A), but the distance to toilets was unrelated to enterococci counts (Kruskal–Wallis Test, $p = 0.052$).

All farmers used pond water for irrigation with sprinklers, except producer 2 which used ground water pumped up from a dug well for drip irrigation. Water from ponds and the dug well did not differ in the average number of *E. coli* and coliforms (Mann–Whitney *U* Test, $p = 0.246$ and $p = 0.536$, respectively). However, pathogens were more frequently found in irrigation water from the dug well (2/9) than in pond water (1/44), but this observation could not be confirmed statistically due to the low number of pathogen-positive samples ($N = 3$; Fig. 5B).

All farmers used composted organic material of various origins as fertilizer. Producer 2 applied organic fertilizer made from horse manure, which contained the highest average numbers of *E. coli* (5.64 log CFU/g, standard deviation ± 0.35 log CFU/g) and coliforms (6.78 ± 0.10 log CFU/g). Moreover, composted horse manure was the only fertilizer from which *Salmonella* was isolated (1/3; Fig. 5C). Producer 3 used on-farm composted vegetable materials, which contained medium numbers of faecal indicators, namely 3.43 ± 0.41 log CFU/g *E. coli* and 4.74 ± 0.06 log CFU/g coliforms. Organic fertilizers made from poultry manure were applied by producers 1, 4, 5 and 6. These contained the lowest bacterial numbers on average, but showed large variations in the microbial load depending on the producer, namely 4.32 ± 0.11 log CFU/g *E. coli* and 4.63 ± 0.11 log CFU/g coliforms for producer 1, no (<1.00 log CFU/g) *E. coli* and 2.96 ± 0.88 log CFU/g coliforms for producer 4, 1.67 ± 1.15 log CFU/g *E. coli* and 3.93 ± 0.11 log CFU/g coliforms for producer 5 and finally no *E. coli* and no coliforms (<1.00 log CFU/g) for producer 6. Due to the low number of samples

Table 3
Overview of samples which contained pathogens (presence per 25 g or 25 mL).

Producer	Sample	Pathogen	*E. coli*		Coliforms		Enterococci		Average week temperature (°C)	Average week precipitation (mm)	Flooding (within sampling week)	Irrigation water	Manure type	Distance to toilets (m)
			Concentration (log CFU/g or log MPN/100 mL)	Class	Concentration (log CFU/g or log MPN/100 mL)	Class	Concentration (log CFU/100 mL)	Class						
1	Manured soil	*Salmonella*	2.0	3	3.2	4	ND	ND	22.6	4.8	No	Pond	Poultry	200
2	Manure	*Salmonella*	5.6	5	6.7	6	ND	ND	24.3	1.6	No	Dug well	Horse	500
2	Manured soil	*Salmonella*	4.4	5	5.7	6	ND	ND	24.3	1.6	No	Dug well	Horse	500
2	Lettuce	*Salmonella*	3.1	4	3.1	4	ND	ND	26.2	13.3	No	Dug well	Horse	500
2	Irrigation water (source)	*Salmonella*	1.0	2	1.3	2	<2.0	1	27.0	3.6	Yes	Dug well	Horse	500
2	Irrigation water (tap)	*E. coli* O157:H7	>1.4	3	>1.4	3	<2.0	1	27.0	3.6	Yes	Dug well	Horse	500
3	Rinse water	*E. coli* O157:H7	>1.4	3	>1.4	3	<2.0	1	27.0	3.6	Yes	Pond	Vegetable	500

ND = not determined.

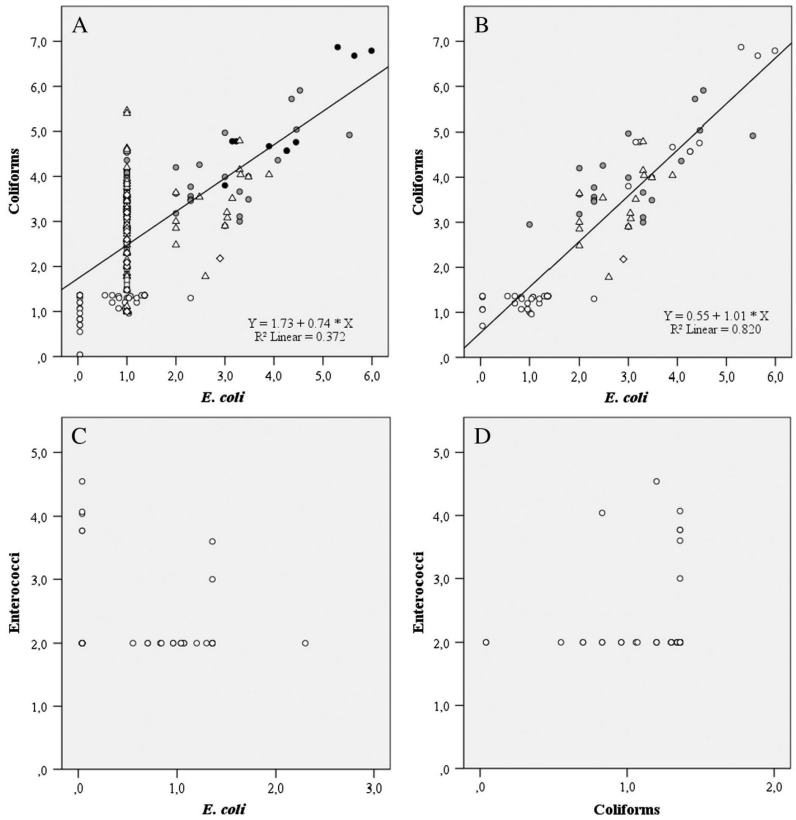

Fig. 2. Scatter plots presenting the linear correlation between the faecal indicators *E. coli* and coliforms in all samples (A) and only in samples with countable *E. coli* (B), the correlation between *E. coli* and enterococci (C) and coliforms and enterococci (D) in all sample types, namely manure (black dots), soil (grey dots), water (white dots), lettuce (white triangles), boxes (white squares) and hands (white diamonds).

($N = 3$) per producer, no statistical tests could be performed to compare the microbiological parameters of different organic fertilizer types, but the existence of large variation in microbiological quality, and thus potentially in safety, among the different types of organic fertilizers and even among different batches of the same type is strikingly apparent. In addition to this organic fertilizer, the conventional farms (producers 4, 5 and 6) applied additional commercial mineral fertilizers. The time of application was also different: composted manure was applied at the time of planting and 20 days later by organic producers 1, 2 and 3, while conventional farmers 4, 5 and 6 administered the organic fertilizer one week before planting and during growth when deemed necessary.

Some lettuce farmers also held farm animals: producer 1 kept pigs, cows and chickens and producer 4 had horses. No influence of keeping farm animals on *E. coli,* coliforms and enterococci was found (Mann–Whitney *U* Test, $p = 0.706$, $p = 0.716$ and $p = 0.517$, respectively), which was not entirely surprising because the animals were always kept in stables away from the lettuce fields. Pathogens were isolated from farms with (1/88) and without (6/172) farm animals, but this difference was not statistically significant (Chi-Squared Test of Independence, Likelihood Ratio, $p = 0.429$).

Rinsing of harvested lettuce crops did not affect the microbial load of lettuce, since the average of *E. coli* and coliforms remained similar (Mann–Whitney *U* Test, $p = 0.605$ and $p = 0.986$, respectively). It must be noted that rinsing appeared to spread the bacteria more homogenously over the lettuces, resulting in a more narrow distribution by reducing the maximum and increasing the minimum count. Moreover, *E. coli* O157:H7 was isolated once from rinse water, indicating a potential safety problem.

3.6. Multivariate analysis of environmental and agro-technical factors on the presence of pathogens

Multivariate statistics (i.e. logistic regression) was applied to identify the factors of influence considered simultaneously, their relative importance and possible interactions, in contrast to the univariate analyses above. Logistic regression modelling retained the concentration of generic *E. coli*, flooding and the irrigation water source as significant predictors for the presence of pathogens (in order of decreasing significance), while the following other factors were not significant in the model: manure type, toilet distance, farm type (organic or conventional

production), sample type, average week temperature, cumulative week precipitation, coliforms, enterococci and the presence of farm animals. The magnitude of the effects of the predictor variables was estimated by the logistic regression model, showing that an increase of the generic *E. coli* level with 1 log CFU per g or 100 mL increased the odds of detecting pathogens 2.0-fold, irrigation water from the dug well of producer 2 was associated with a 4.8-fold increase and flooding events caused a 6.9-fold increase.

3.7. Evolution of the microbiological parameters during the growth cycle of lettuce

The microbial load (*E. coli* and coliforms) of the agricultural environment varied significantly during the lettuce crop growth cycle of lettuce (Kruskal–Wallis Test, both $p < 0.001$). The number of coliforms in the agricultural environment and in lettuce itself significantly decreased during the growth cycle of lettuce (Kruskal–Wallis Test, $p < 0.001$ and $p = 0.003$, respectively), while the number of *E. coli* only decreased in the environment and not on lettuce (Kruskal–Wallis Test, $p < 0.001$ and $p = 0.256$, respectively). This suggests that the organic fertilizer applied at planting was the main source of *E. coli* and coliforms in the soil, i.e. poultry manure (producers 1, 4, 5, 6), horse manure (producer 2) and vegetable remains (producer 3) (Fig. 6).

4. Discussion

4.1. Microbial indicators for the presence of pathogens

In the present study, only *E. coli* was correlated with enteric pathogens *Salmonella* and *E. coli* O157:H7, while coliforms and enterococci were not. This result is in agreement with several other studies which showed generic *E. coli* to be a suitable indicator for *Salmonella* and *E. coli* O157:H7 contamination, performing better than enterococci (Lau and Ingham, 2001; Natvig et al., 2002; Ogden et al., 2001), especially when the pathogen prevalence is very low (Park et al., 2013). *E. coli* is the only valid indicator for faecal contamination of fresh produce, because several genera of coliforms are common contaminants of non-faecal sources including plant materials, e.g. *Klebsiella*, *Enterobacter* and *Citrobacter* species (Doyle and Erickson, 2006; Tortorello, 2003). However, occasionally no relation has been found between *E. coli* and pathogens, e.g. *E. coli* was not linked to *Salmonella* in water in California, US (Benjamin et al., 2013), so the validity of *E. coli* as a predictor for pathogens should be checked for each specific situation prior to its use.

4.2. Organic versus conventional farming systems

Several studies have pointed out that organic farming is associated with higher microbiological risks than its conventional counterpart (Arbos et al., 2010; Loncarevic et al., 2005; Maffei et al., 2013; Oliveira et al., 2010), while others have found these differences in microbiological quality of fresh produce from organic and conventional farming systems not statistically significant (Boraychuk et al., 2009; Mukherjee et al., 2006; Neto et al., 2012). In our study, all pathogens (5 salmonellae and 2 *E. coli* O157:H7) were isolated from organic and generic *E. coli* were detected more frequently and at higher average concentrations in lettuce samples from organic farms (23.1% and 3.22 log CFU/g) than conventional farms (16.7% and 2.27 log CFU/g) (Mann–Whitney *U* Test, $p = 0.002$). This corresponds well with a Spanish study, in which 22.2% of organic and 12.5% of conventional lettuce contained *E. coli* (Oliveira et al., 2010) and with a Brazilian study which found *E. coli* in 50.0% of organic and 37.5% of conventional lettuce samples (Maffei et al., 2013). Although this study appears to deliver supporting evidence to the claim that organic farming holds increased microbiological risks, the observed differences originate at least partially from different climatic conditions, since organic farms were sampled at days with significantly higher temperature (25.5 °C versus 19.1 °C) and lower cumulative week precipitation (6.1 mm and 6.9 mm) (Mann–Whitney *U* Test, $p < 0.001$ and $p = 0.003$, respectively). Higher temperatures give rise to higher bacterial numbers and thus also pathogens in the environment and more rainfall decreases the need for irrigation with water of low(er) quality and may cause dilution or wash-out of bacteria. Conventional farmers indeed reported lower frequency of applying irrigation water to their crops in comparison to the organic farmers due to more rainfall during the cultivation period. Weather and environmental conditions were previously related with higher contamination in vegetables (da Silva et al., 2007; Liu et al., 2013; Natvig et al., 2002). Contamination of vegetables with *E. coli* and *Salmonella* spp. present in soil was only observed in the warmer >20 °C season in Wisconsin, US (Liu et al., 2013; Natvig et al., 2002). Interestingly, another study has shown that the microbial load, including *E. coli* and coliforms, of fresh produce sampled at retail was higher during months with higher temperatures in Porto Alegre, the same Brazilian area where the lettuce farms in our study are located (da Silva et al., 2007). These authors suggested that increased use of contaminated

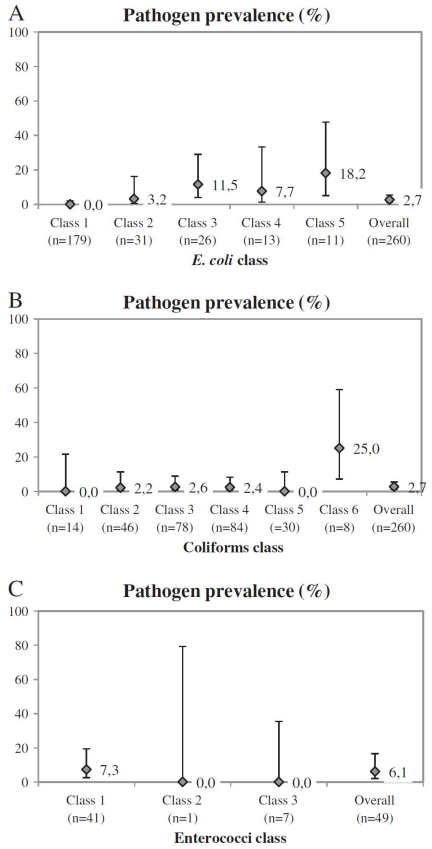

Fig. 3. Pathogen (*Salmonella* and *E. coli* O157:H7) prevalence in samples belonging to different *E. coli* classes (A), coliform classes (B) and enterococci classes (C); error bars indicate the 95% confidence intervals.

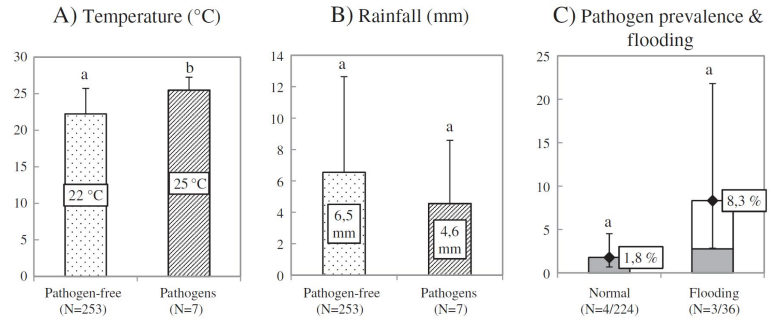

Fig. 4. Associations between climatic conditions and the presence of pathogens: (A) Average week temperature with error bars indicating the standard deviations for samples without (dotted bars) and with (hatched bars) pathogens, (B) cumulative week precipitation with error bars indicating the standard deviations for samples without (dotted bars) and with (hatched bars) pathogens and (C) flooding of the lettuce fields within the week of sampling. The overall prevalence of pathogens in the occurrences and the absence of flooding is indicated by black diamonds and the 95% confidence intervals by error bars, with specific indication of the proportions of *Salmonella* (in grey bars) and *E. coli* O157:H7 (in white bars). Different letters indicate statistically significant differences.

water, amongst other reasons, may be responsible for the observed effects.

Fig. 6 shows the considerable variation observed among the individual producers, which points to further agro-technical factors causing differences in the microbiological parameters in lettuce farming. The following factors and farming practices were associated with higher microbial load and pathogen prevalence in organic farms in comparison with the conventional ones: application of more contaminated organic fertilizers, irrigation with water of inferior quality, flooding events and larger distances to toilets. In contrast, rinsing of harvested lettuce and keeping animals on the farm had no effect on the microbiological quality of lettuce in the present study. In agreement, research has shown that the use of portable toilets decreased the levels of generic *E. coli* levels in spinach (Park et al., 2013) and that washing of harvested vegetables in water without sanitizers did not alter *E. coli* and coliform counts (Natvig et al., 2002; Neto et al., 2012). In contrast to the reports in literature that domestic and wild animal intrusion significantly increased contamination with *E. coli* (Park et al., 2013), no significant effect of animals was found in this study. The reason for this is probably that farm animals, if present, were always kept indoors or well separated from the lettuce fields, while on all farms without exception, dogs and/or cats had free access to the fields. Wildlife activity was not recorded, but none of the farms took any precautions to prevent it.

4.3. Quality of organic fertilizers

Application of composted manure as a soil fertilizer is a common practice in both conventional and organic vegetable crop production worldwide. Although manure is a well-known source of human pathogens, organic fertilizers derived thereof are valuable nutrient resources after an appropriate treatment such as composting (Johannessen et al., 2004). Contamination of agricultural soil with generic *E. coli* and enteric pathogens such as *E. coli* O157:H7 and *Salmonella* occurs through application of untreated manure and through wildlife defecation of the fields (Ingham et al., 2004). After introduction into the soil, these harmful microorganisms can survive for a considerable time, several weeks to months, depending on environmental conditions and the initial concentration (Erickson et al., 2010; Ibenyassine et al., 2006; Islam et al.,

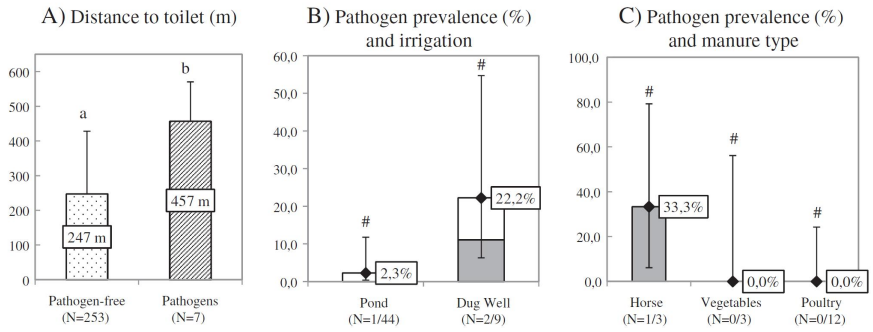

Fig. 5. Associations between agro-technical factors and the presence of pathogens: (A) Distance between toilet facilities and lettuce fields for samples without (dotted bars) and with (hatched bars) pathogens, (B) Type of irrigation water type and (C) manure type of the organic fertilizer. The overall prevalence of pathogens is indicated by black diamonds and the 95% confidence intervals by error bars, with specific indication of the proportions of *Salmonella* (in grey bars) and *E. coli* O157:H7 (in white bars). Different letters indicate statistically significant differences; # indicates too few (pathogen) data to perform statistical tests.

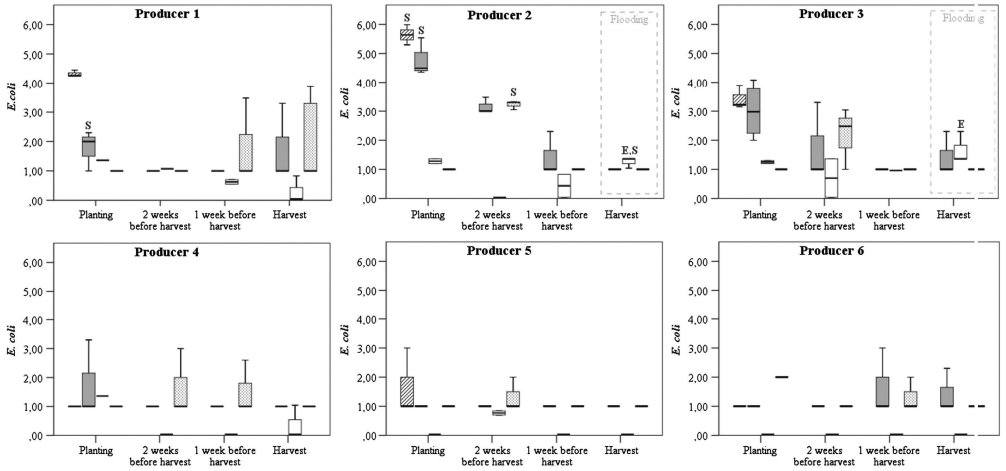

Fig. 6. Boxplots showing the temporal variation of *E. coli* counts in manure (hatched boxes), soil (grey boxes), water (white boxes) and lettuce (dotted boxes) samples during the growth cycle of lettuce from planting of the seedlings to harvest of the crops in the six farms producing lettuce according to the organic (producer 1, 2, 3) or the conventional (producer 4, 5, 6) system. Occurrence of flooding of the lettuce fields is marked with a grey dashed box and isolation of pathogens *Salmonella* and *E. coli* O157:H7 is marked with the letters S and E respectively at the corresponding sample type, sampling time and producer (for more information on these positive samples see Table 3).

2004a,b). Subsequently, transfer of these pathogens from the contaminated soil to the produce may occur (Mootian et al., 2009). Therefore, the application time of the organic fertilizers is a critical factor for preventing contamination of fresh produce with faecal bacteria in the case of untreated or insufficiently composted manure (Ingham et al., 2004). This study confirms that control of the composting process is crucial, noticeable by the considerable differences in the *E. coli* numbers found in composted poultry manure purchased from different sources, ranging from none (<1.0 log CFU/g) to 4.5 log CFU/g. Horse manure which was composted on the farm was a major source of pathogens. *Salmonella* was isolated from the composted horse manure, in the soil after application of this fertilizer and two weeks later on the lettuce grown in the contaminated soil.

4.4. Irrigation water quality and flooding

According to the current Brazilian legislation, thermotolerant coliforms should not be present in 100 mL of irrigation water for vegetables which are to be consumed raw (BRASIL, 1986). In our study, total coliforms were present in 100% (53/53) of the irrigation water samples and *E. coli* in 60% (32/53), so the majority (between 60% and 100%) of irrigation water samples contained thermotolerant coliforms and thus did not comply with the Brazilian legislation. Water quality was significantly worse at organic farms where 100% (27/27) of the irrigation water samples contained *E. coli* in comparison with 19% (5/26) in conventional farms (Mann–Whitney *U* Test, $p < 0.001$). Other Brazilian studies have also identified contamination of vegetables with high levels of coliforms originating from irrigation water (Abreu et al., 2010; Guimaraes et al., 2003). Besides the organic fertilizers, irrigation water was the other major source of pathogens detected in this study, which corresponds with the results of other researchers (Gelting et al., 2011; Ibenyassine et al., 2006; Ingham et al., 2004; Islam et al., 2004b; Natvig et al., 2002; Ogden et al., 2001; Park et al., 2013). Strikingly, in the current study flooding had always occurred within the week when irrigation water samples were found to contain pathogens, and more specifically *E. coli* O157:H7 was only detected after flooding. This suggests that contamination of irrigation water in ponds occurred mainly by surface run-off water. Flooding events are associated with damage to crops and contamination of crops with pathogens, hazardous chemicals and pesticides due to surface run-off and remobilization of contaminated river sediments and upstream contaminated terrestrial areas such as grazing areas (Tirado et al., 2010; Gelting et al., 2011). The observed contamination of ground water from the dug well of producer 2 may be the consequence of infiltration of rainwater contamination with faecal microorganisms from the composting site which was very close (20 m). In addition, no form of physical or geographic protection was present to avoid spread or leakage of composting manure, so the most probable contamination route was through rain water from the horse manure to the underlying ground water. This hypothesized contamination route is comparable to that of a spinach farm on which the irrigation water well was contaminated with *E. coli* O157:H7 originating from wild swine and cattle faeces in river water due to groundwater–surface water interaction (Gelting et al., 2011).

5. Conclusion

Salmonella and *E. coli* O157:H7 were mainly found in the primary production environment of lettuce, i.e. three times in water and three times in manure and manured soil. Only once was *Salmonella* detected on lettuce, so this results in a prevalence of 1.3% (95% CI: 0.2%–7.2%) for *Salmonella* and 0.0% (95% CI: 0.0%–4.9%) for *E. coli* O157:H7 on lettuce crops. All pathogens were isolated from organic farms, suggesting lower microbiological quality and safety, but these differences originated at least partially from (i) differences in climatic conditions during sampling, i.e. higher temperatures, lower precipitation and flooding events and (ii) differences in agro-technical practices, i.e. application of more contaminated organic fertilizers, especially on-farm composted horse manure, fertilizing at the time of planting and during lettuce growth, using irrigation water of lower quality and large distance between the toilet facilities and the fields.

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3.3 ARTIGO 3

Contamination and Growth Modelling of *Salmonella* and *E. coli* on Conventional Lettuces Sold in Hypermarkets of Southern Brazil

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Abstract

Samples of conventional lettuces (*Lactuca sativa*) (n = 100) were taken from hypermarkets in Southern Brazil and analysed for coliforms, *E. coli* and the presence of *Salmonella* and *E. coli* O157:H7. Growth modelling was also performed using two microorganisms, *E. coli* ATCC 8739 and *Salmonella* Enteritidis SE865, inoculated on lettuces and kept at 5 °C, 10 °C, 25 °C, and 37 °C for 0, 2, 6, 24, and 48 hours. Microbiological results demonstrated that only 4% of the lettuces samples presented *E. coli* counts above the detection limit and on1% *Salmonella* was present. *E. coli* O157:H7 was not detected in any sample. Modelling experiments demonstrated that the growth rates of *E. coli* at 25 ^{o}C and 37 ^{o}C were bigger than growth rates of *S*. Enteritidis SE86. *Salmonella* and *E.coli* increased 0.6 and 0.75 log CFU/g after 6 h of incubation at 25 ^{o}C, and 1.84 and 2.85 log CFU/g, respectively, after the same time at 37 ^{o}C. When incubatet for 48 h at 25 ^{o}C, *Salmonella* and *E. coli* increased their initial population in approximately 1.53 and 1.68 log CFU/g, respectively, and at 37 ^{o}C, the icrease was 1.53 and 1.68 log CFU/g, respectively, during the same time. The micoorganism reached contamination levels that could represent risk for the consummers, even after adequate sanitation procedures.These results permited us to conclude that besides the temperature control and storage of the lettuces during all chain process, attention should be made in order to apply preventive mesures during the primary production as the implementation of the Good Agricultural Practices.

Key words: Lettuce, Hypermarkets, Modelling, *Salmonella, E. coli*.

1. Introduction

Leafy greens are widely consumed worldwide especially because they may contribute with healthier habits and prevent diseases, as cancer, diabetes, obesity and heart diseases (Lopéz-Galvéz et al., 2010; Ignarro, Balestrieri, & Napoli, 2007; Liu, 2003). As an example of the importance of vegetables for human diet, the World Health Organization (WHO) recommends the consumption of 400 g per day of fruit and vegetables. At the same time, the association of foodborne outbreaks to fresh produce is increasing around the world, according to the numbers reported by European Union (EU) and the United States (Callejón et al., 2015; EFSA, 2014; FAOSTAT, 2013; Oilamat & Holley, 2012; FAO/WHO, 2008).

Several pathogens can be transmitted by fresh produce (EFSA, 2014; Berger et al., 2010;), being *Salmonella* and pathogenic *Escherichia coli* frequently reported (Callejón et al., 2015; Buchholz et al., 2011; Warriner et al., 2009; DELAQUIS; BACH; DINU, 2007). The consumption of fresh produce in EU, as reported by EFSA (2013), showed that the risk of association of fresh produce with *Salmonella* is a major issue regarding human cases of infection linked with food of non-animal origin. Another example is the 2011 outbreak occurred in Germany and France resulting in 54 deaths after the consumption of raw sprouts contaminated with *Escherichia coli* O104:H4 (EFSA 2014; Soon et al., 2013), highlighting the importance of improve the food safety in fresh produce.

The primary production can play an important role in contamination of fresh produce, once they can be contaminated by different sources, as soil, manure, irrigation water, workers, equipment, animals, among others (EFSA, 2014; Rodrigues et al., 2014; Oilamat and Holley, 2012; Oliveira et al., 2012; Warriner et al., 2009).

Contaminated vegetables can reach final consumers and cause foodborne illnesses especially because some of these products are eaten raw, as lettuces (EFSA, 2014; Oilamat and Holley, 2012; Lynch et al., 2009).

Lettuce (*lactuca sativa* L.) is one of the most consumed leafy greens around the world, as well as in Brazil, because of its low cost and availability during all year (Oliveira et al., 2010; Mocelin & Figueiredo, 2009; WHO, 2008; Mattos at al., 2007). Lettuce cultivation systems can be done by conventional, organic and hydroponic methods (EFSA, 2014), although in Brazil, conventional method in open fields is the most used (EMBRAPA, 2013; Chaves et al., 2000). Generally, in this country lettuces are produced in small familiar farms, but highly distributed by hypermarkets, being bought by thousands of people, mostly without refrigeration.

The aim of this study was to assess the microbiological contamination of conventional lettuces (*Lactuca sativa* L.) sold in hypermarkets of Porto Alegre, Southern Brazil, and modelling the growth of *Salmonella* and *Escherichia coli* in these products.

2. Material and method

2.1 Lettuce sampling

From November 2013 to November 2014, 100 conventional curly lettuces (*Lactuca sativa*) were sampled in five different hypermarkets of Porto Alegre city, Southern Brazil. Most of hypermarkets were located inside big shopping malls, receiving more than 20.000 people per day. The lettuces were purchased at room temperature, individually pre-packaged in polyethylene bags, and were immediately transported to the Laboratory for analysis. Questions about the suppliers, hypermarket information

and management features regarding lettuces were asked to the hypermarket managers, in order to obtain data about practices that could influence the contamination or bacterial growth on lettuces. This procedure was done by face-to-face interviews at the time of sampling. The temperature of lettuces was measured at sampling time using a thermometer (Dellt) put in the middle of one lettuce head. Each sample unit was established as one lettuce head. In the Laboratory, the lettuces were cut in pieces with a sterile knife, homogenized into a sterile plastic bag and weighted for the followed microbial analyses.

2.2 Microbial analyses

For Coliforms and *E. coli* analyses, 10 g of each lettuce were placed into a sterile plastic bag containing 90 ml of 0.1% peptone water added with 0.9% NaCl. The sample was homogenized using a stomacher (Seward) for 30 s. After that, decimal dilutions were prepared, and triplicate samples of 1 mL were placed on Petrifilm (3M) dishes and incubated for 24 hours, at 37 °C.

The presence of *Salmonell*a spp. was determined according to ISO 6579:2002 standard (ISO, 2002). The isolates positively identified as *Salmonella* spp. were sent to the reference Laboratory of *Enterobacteriaceae* at the Bacteriology Department of the Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ) in order to perform serological confirmation.

The presence of *E. coli* O157:H7 was investigated according to the methodology described by ISO 16654:2001 standard (ISO, 2001). Suspected colonies were submitted to agglutination reaction using *E.coli* O157 antiserum (DIFCO).

Presumptive colonies were submitted to multiplex-PCR (Paton and Paton, 1998) in order to confirm *E. coli* O157 identity.

2.3 Growth Modelling

For growth modelling studies, *E. coli* ATCC 8739 and *Salmonella* Enteritidis SE86 were used. *S.* Enteritidis SE86 was isolated from cabbage responsible for a foodborne outbreak occurred in 1999 in Southern Brazil. This microorganism was identified as responsible for several Salmonellosis outbreaks since 1999, in the State of Rio Grande do Sul (RS), Southern Brazil (Tondo and Ritter, 2012) and is one of the most important foodborne pathogen of Southern Brazil, during the last 15 years (Tondo et al., 2015).

Before artificial inoculation of lettuces, both strains were kept at - 20 °C in 10% glycerol. In the day before the inoculum *Salmonella* was subcultivated in Brain Heart Infusion (BHI) (HiMidea) and *E. coli* was cultivated in Tryptone Soya Broth (TSB) (OXOID), for 24 h, at 37 °C. Decimal dilutions were done using 0.1% peptone water in order to reach a final concentration of 10^4 - 10^5 CFU/mL of each microorganism.

Curly lettuces were purchased from the same hypermarkets investigated in the prevalence study. Outer leaves and the core were removed. The leaves were cut with a sterile knife and homogenized in a sterile bowl. In sterile plastic bags, 10 g of leaves were weighted and inoculated with 100 µL broth with a final concentration of 10^4 - 10^5 CFU/mL of each strain, separately. The bags were incubated at the following temperatures: 5 °C, 10 °C, 25 °C and 37 °C for 0, 2, 6, 24, and 48 hours. Each experiment was repeated two times (trials) with three replicates per trial at each time interval.

At each sampling interval, 90 mL of 0.1% peptone water was added to the plastic bag containing inoculated lettuces and the vegetables were homogenized using a stomacher (Seward) for 30 s. The sample was serially diluted in 0.1% peptone water and plated out (20uL), in triplicate, by the droplet-method on appropriate selective agar plates (Silva et al., 2007). For *Salmonella*, the selective medium used was XLD (HiMedia), while for *E. coli* was used ChromoCult Agar (CC) (Merck). Counts of both microorganisms also were done using Nutrient Agar (NA) (HiMedia). The plates were incubated at 37 °C, for 24 hours.

The predictive primary model described by Baranyi and Roberts, (1994) was used in this study in order to calculate the growth kinetic parameters of *S.* Enteritidis SE86 and *E. coli* on lettuce. The growth curves for each temperature were built by fitting data to the Combase's DMFit (http://browser.combase.cc/DMFit.aspx). The following parameters were obtained: maximum growth rate, lag time and maximum population density.

In order to evaluate if the microbial growth on lettuces reached risk levels, the following formula described by Zweitering et al. (2010) was used:

$H_0 - \sum R + \sum I \leq FSO$

Where H_0: is the initial level of contamination; $\sum R$: is the reductions feasible in the process; $\sum I$: is the total increase of the microbial population; and FSO is the Food Safety Objective, i.e. the maximum concentration of hazard at the consumption point.

We assumed the following numbers: initial population of *Salmonella* of 1 log CFU/g, reduction rates of 2.0 log CFU/g (ICMSF, 2015; Sapers, 2003; Brackett, 1999; Beuchat, 1998), and FSO = absence of pathogens *Salmonella* or *E. coli* O157:H7 in 25 g (ICMSF, 2015).

2.4 Statistical analysis

Statistical analyses were performed with SPSS Statistics version 21 at $p< 0.050$. Bivariate correlations between the indicators were determined by calculating the Spearman's Rho coefficient using the raw enumeration data. Kruskal-Wallis or Mann-Whitney U tests were used to evaluate the influence of different factors. Pair wise tests were performed to identify the significant differences between individual categories when significant differences were found. In case of ´n´ pair wise comparisons, Dunn-Sidak correction was applied, resulting in adjusted individual p' values: $p' = 1-(1-p)^{1/n}$, in which $p = 0.050$ to obtain a family-wise error rate of 5 %.

3. Results

Hypermarket Lettuce Management information

According to the questions answered by the hypermarket managers, lettuce trade organization was very similar among all hypermarkets investigated. For example, all of them had three to five lettuce suppliers, located around Porto Alegre city. Depending on the necessity, hypermarkets ordered lettuces during the evening before the selling day, and the lettuces were delivered next morning (around six to seven o'clock), by supplier's trucks, at room temperature. It was common for all the hypermarkets to sell lettuces from more than one supplier at the same day on the same shelves. The lettuce heads arrived individually packaged in plastic bags, inside farmers' plastic crates and went directly to the shelves to be sold or to the fridge (5 to 10 °C) to be stored until the moment of selling. Traceability was possible by the name of each supplier printed on individual plastic bags. At the end of each day, the

lettuces not sold were stored again inside fridges overnight under controlled temperature (5 to 10 °C). In all hypermarkets, the lettuces were available to the consumers at room temperature. During the day, the lettuce heads could stay on shelves for until 15 hours to be sold and after that could go to the refrigeration for the next selling day. The lettuce's temperature inside hypermarkets, at the sampling moments, ranged from 20 to 23.5 °C.

Bacterial Contamination

Table 1 demonstrates the microbial mean numbers and standard deviation of lettuces analysed in each hypermarket.

Figure 1 demonstrates the coliform counts obtained in each hypermarket. The raw data for coliform counts were normally distributed among the hypermarkets ($p = 0.069$; $p < 0.05$). All lettuce samples had coliforms, ranging from 1.00 log CFU/g to 6.13 log CFU/g, being the average 3.60 ± 0.10 log CFU/g. The average numbers did not differ statistically from each other

Only four lettuce samples had *E. coli* above the detection limit (4/100, 4%) , CI 95% = 1.97 – 9.84), being three of them of 1.30 log CFU/g and one of 3.53 log CFU/g. No linear statistically relationship between *E. coli* and coliform counts were observed ($y=0.01+0.02*x$, $R^2= 0.002$) and the raw data for *E. coli* were not normally distributed ($p = 0.001$; $p < 0.05$). *Salmonella enterica* subspecie *enterica* (O:9,12) was found on one sample and *E. coli* O157:H7 was not detected.

The correlation between indicators (coliforms and *E. coli*) and the presence of *Salmonella* was not statistically significant (Kendall's tau-c, $p = 0.867$; Kendall's tau-c, $p = 1.00$, respectively). Corroborating these results, the ROC curves for coliforms and

E. coli and the presence of *Salmonella* have areas under the curves 0.576 and 0.480, respectively, being none predictive values. The sample where *Salmonella* was found had no *E. coli* (data not shown). Higher *E. coli* counts were not correlated to high temperatures (Kruskal- Wallis Test, p = 0.905).

Growth modelling

In order to study the growth of the microorganisms found on lettuces, *S.* Enteritidis SE86 and *E. coli* ATCC8739 were used. The growth of these microorganisms were modelled under different temperatures, which were chosen based on the information obtained by questions answered by hypermarkets managers and measurements. These temperatures were 5, 10, 25 and 37 °C, considering that fridges temperatures ranged from 5 to 10 °C, lettuce heads demonstrated 20 to 23.5 °C on shelves at sampling, and a hot day in the Southern Brazil can easily reach 37 °C, what corresponds to the optimum growth temperature for *Salmonella* and *E. coli* (Worst Scenario).

At 5 °C and 10 °C *S*. Enteritidis SE86 and *E. coli* ATCC8739 did not show a significant growth until 48 hours, fitting with a linear model (Baranyi and Roberts model, 1994). Figure 2 shows the representative data on the growth of *S*. Enteritidis SE86 and *E. coli* ATCC8739 on lettuce at 25 °C and 37 °C, with fitted growth curves to the Baranyi and Roberts model (1994). The curves obtained at these temperatures, except for *S*. Enteritidis SE86 at 37 °C, showed a high correlation coefficient (R2> 0.95). Estimated maximum growth rate, lag time and maximum population density (MPD) of *S*. Enteritidis SE86 and *E. coli* ATCC8739 inoculated on the lettuces are shown on Table 2. Growth rates of *E. coli* ATCC8739 at 25 °C and

37 °C were bigger than the growth rates of *S*. Enteritidis SE86, however, the MPD were similar for both microorganisms, when compared the same temperatures (6.32 ± 0.01 and 6.10 ± 0.13 CFU/g at 25 °C; 7.25 ± 0.36 and 7.23 ± 0.05 at 37 °C, respectively). Within 6 h of incubation at 25^{o}C, *S*. Enteritidis SE86 and *E. coli* ATCC8739 increased their counts in 0.6 and 0.75 log CFU/g, respectively, while at 37 ^{o}C, the counts increased 1.84 and 2.85 log CFU/g, respectively. After 48 hours of incubation at 25 ^{o}C, *S*. Enteritidis SE86 and *E. coli* increased their initial population in approximately 1.53 and 1.68 log CFU/g, respectively, while at 37 ^{o}C the increases were around 2.12 and 2.85 log CFU/g, respectively (Figure 2). Table 3 demonstrated that the FSO was not reached after the growth of *S.* Enteritdis SE86 on lettuces exposed to 25 ^{o}C, for 48 hours, and to 37 ^{o}C, for 6 and 48 hours, even considering a reduction rate of 2.0 log after washing and disinfection.

4. Discussion

According to EFSA (2014), *E. coli* can be used as a hygiene criterion at primary production of leafy greens in order to validate Good Agricultural Practices (GAP). In the present study, 100 lettuces were sampled on hypermarkets of Southern Brazil, and four samples (4%) demonstrated *E. coli* counts above the detection limit, ranging from 1 Log UFC/g to 3.53 Log UFC/g. *E. coli* contamination on lettuces can be vary variable, depending on where the samples were taken. For example, Maffei et al. (2013) in a study conducted in São Paulo, Brazil, found 40% of conventional lettuce samples contaminated with *E. coli*, and counts ranged from 1 to 2 log CFU/g. In a study conducted in Maracaibo, Venezuela, among 150 leafy vegetable samples composed by 50 lettuce, 50 parsley and 50 cilantro samples, 10% presented *E. coli*,

being that on lettuces only one sample presented counts above the detection limit (1 x 10^3 UFC/g) (Rincón et al., 2010). On the other hand, in another study conducted in Egypt (Uyttendaele et al., 2014), the contamination of fresh produce with *E. coli* was very high, being that 73% of 120 lettuces and strawberries samples were contaminated.

In the present study, *Salmonella* was found only on one lettuce sample, representing 1 % of prevalence. In another study conducted in Brazil that investigated 130 lettuce samples from retail market, no *Salmonella* was found (Maffei et al., 2013). Different results were found by Uyttendaele et al. (2014) in Egypt, were the prevalence of *Salmonella* on 30 lettuces sold in retail markets investigated was 43 %. It is important to notice that in the latter study the lowest percentage of *Salmonella* was found on lettuces purchased in hypermarkets (25%), being the highest concentrations (50%) obtained on lettuces from open markets, demonstrating a trend that produce sold in hypermarkets could have a lower level of contamination. Some previous studies also demonstrated that street markets may have poor microbial quality and can, so far, constitute important sources of food pathogens (Tandon et al., 2011; Berdegue et al., 2005). The poor hygienic procedures found can be a result of the lack of education and training (Emongor and Kirsten, 2006). Other issues as absence of proper sanitary facilities and lack of clean water to wash utensils and hands can lead to the contamination of produce by pathogens (Colen et al., 2012; Koseki et al., 2011). Hypermarket managers interviewed in the present study reported that all personnel working with vegetables were trained on GMP, and were working in facilities with good hygiene standards, had potable water to wash their hands and all food handlers used adequate uniforms. These factors can explain the low levels of contamination

observed on investigated lettuces, when compared with other studies (Uyttendaele et al., 2014). Other factor that may contributed to this is that all lettuces analysed were pre-packaged into plastic bags since farms, lowing the risk of cross-contamination among lettuce heads or by food handlers inside hypermarkets.

Salmonella and *E. coli* growth on lettuces

According to the modeling results of the present study, lettuces artificially contaminated with *S*. Enteritidis SE86 and *E. coli* stored under 5 or 10 ºC did not supported bacterial growth until 48 hours. Similar results were demonstrated by other researchers (Sant'Ana 2013; Koseki and Isobe, 2005). Tian et al. (2012), in a modeling study conducted with different vegetables (romaine lettuce, iceberg lettuce, perilla leaves, and sprouts) stored at 4 ºC demonstrated that no significant growth of *S*. Typhimurium, and *E. coli* O157:H7 occurred after 15 days. Furthermore, other researchers demonstrated reduction trends on microbial population exposed to 7 ^{o}C on shredded lettuces, at normal atmosphere (Bülent Ergönül, 2011; Oliveira et al., 2010). However, another study Elexson et al. (2011) showed the growth of *Salmonella* at 4 ºC on lettuce and cucumber slices and Koseki and Isobe (2005) inoculated *Salmonella* on lettuces and stored at 10 ºC, observing a lag phase of 35.06 h and growth rate of 0.02.

Sant'Ana et al. (2013) studied the effects of different storage scenarios on the potential growth of *Salmonella* strains and *Listeria monocytogenes* on ready-to-eat mixes of different lettuces varieties, finding that a low contamination of 10^{1} CFU/g can reach high populations at 15 ºC. Eckner (2015) recovered inoculated *Salmonella* from basil leaves for up to 18 days, at 20 ºC to 22 ºC. Bülent Ergönül (2011) found

that during storage at 20 ºC the final count of *S*. Typhimurium on shredded lettuce was the same as the initial count, whereas the average *E. coli* counts increased approximately 1.5 log CFU/g, after seven days of storage. Other authors (Tian et al., 2012) observed that the populations of *S*. Typhimurium and *E. coli* O157:H7 on different vegetables (romaine lettuce, iceberg lettuce, perilla leaves, and sprouts) stored at 15 ºC increased significantly, approximately 2.0 log CFU/g after 1 day.

Considering the temperature of 25ºC, an usual environmental temperature in tropical countries as Brazil, *Salmonella* SE86 and *E. coli* on lettuce showed a lag time of 1.15 ± 0.55 h and 3.28 ± 4.86 h, respectively. Despite the difference on the lag time, the growth rate and final population density were quite similar for both microorganisms. It can be observed that in the first hours *E. coli* grew faster than *Salmonella*. For example, *Salmonella* took 8.64h to increase 1 log CFU/g, while for *E.coli* it was 6.72 h, at 25 ^{o}C. Similar results were obtained by Koseki and Isobe (2005) who demonstrated that lettuce inoculated with *Salmonella* and *E. coli* O157:H7 and stored at 25 ºC showed lag time of 2.78 h and growth rate of 0.41 and lag time of 4.44 h and growth rate of 0.44, respectively. Oliveira et al. (2010) inoculated *Salmonella* and *E. coli* O157:H7 on shredded lettuces packaged with modified atmosphere conditions, and demonstrated that the loads increased 2.44 and 4.19 log units, after 3 days, at 25 ºC.

At 37 ºC, *Salmonella* SE86 and *E. coli* demonstrated expressive growing rates, increasing 1.84 and 2.85 log CFU/g in 6 hours. This can be explained because 37 ºC is usually indicated as the optimum temperature for these microorganisms, however attention should be paid, because this temperature may be common in Brazil and other tropical countries, especially during summertime. Also, it is important to

highlight that these bacterial count increases can be reached inside hypermarkets if the lettuces are kept on shelves for 6 hours, at hot days (37 ºC).

Considering the increase of the *S*. Enteritidis SE86 population on lettuce at 25 ^{o}C and 37 ^{o}C, the adequacy to Food Safety Objetive (FSO, absence in 25g) was calculated using the formula $H_0 - \sum R + \sum I \leq FSO$. *E. coli* O157:H7 was not included because this microorganism was not found on lettuces collected in hypermarkets. For the calculation, we assumed an initial contamination of 1log CFU/g of *S*. Enteritidis SE86 and a reduction rate of 2.0 log, because it is well established that conventional washing and sanitizing methods are not capable of reducing microbial counts by more than 1 to 2 log CFU/g (ICMSF, 2015; Sapers, 2003; Brackett, 1999; Beuchat, 1998).

As demonstrated in Table 3 adequacy to FSO was not reached, considering the increase of *S.* Enteritidis SE86 population after growth during 6h and 48h at 37 ^{o}C and 48h at 25 ^{o}C, suggesting risk to consumers. This can be assumed once the requirement for *Salmonella* is absence in 25 g according the Brazilian legislation (Brazil, 2001).

In conclusion, this study demonstrated that lettuces purchased in hypermarkets in Southern Brazil can be contaminated with *Salmonella* and *E. coli*. Moreover, our results showed that these microorganisms were able to growth on lettuces submitted to similar temperature conditions found in hypermarkets. It was possible to conclude that lettuces stored <10 ^{o}C did not support *Salmonella* and *E. coli* growth for 48 hours, indicating to be an appropriate temperature for store lettuces at safety conditions. However, lettuces exposed to 25 ^{o}C and 37 ^{o}C for periods of 6 hours or more could support bacterial growth above safe limits, even considering the use of

appropriate disinfection methods. Based on the results, it was concluded that Good Agricultural Practices should be implemented at primary production in order to prevent lettuce contamination and that leafy greens should be properly stored at refrigeration at all steps of the chain, including the post harvest at farm, and disinfected before consumption in order to be safe.

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Table 1: Results of microbial quality of lettuce

Hypermarket	n	Temperature (ºC)	Microbial Results			
			Coliforms (n) mean and stdv log CFU/g	*E.coli* (n) mean and stdv log CFU/g	*Salmonella* (n) presence in 25 g	*E.coli* O157:H7 (n) presence in 25 g
A	20	23,5	(20/20) 3.29 ± 0.83	(1/20) 1.30	(0/20)	(0/20)
B	20	21,5	(20/20) 3.06 ± 0.64	(1/20) 1.30	(0/20)	(0/20)
C	20	20	(20/20) 3.41 ± 0.51	(1/20) 3.53	(0/20)	(0/20)
D	20	21	(20/20) 4.03 ± 0.77	(0/20)	(1/20)	(0/20)
E	20	23	(20/20) 4.18 ± 1.47	(1/20) 1.00	(0/20)	(0/20)

Table 2. Estimated maximum growth rate, lag time and maximum population density (MPD) of *Salmonella* and *E.coli* inoculated on the lettuce.

Temperature (ºC)	*Salmonella*[a]			*E.coli*[a]		
	Growth rate (log CFU/h)	Lag time	Maximum population density (MPD) (log CFU/g)	Growth rate (log CFU/h)	Lag time	Maximum population density (MPD) (log CFU/g)
5	ND[b]	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND	ND
25	0.12 ± 0.01	1.15 ± 0.55	6.32 ± 0.01	0.29 ± 0.46	3.28 ± 4.86	6.10 ± 0.13
37	0.35 ± 0.24	0.49 ± 3.17	7.25 ± 0.36	0.70 ± 0.06	0.00[c]	7.23 ± 0.05

[a] Mean value of triplicate trials

[b] Not determined

[c] Not showed

Table 3. Adequacy to Food Safety Objetive (FSO) of *Salmonella* Enteritidis SE86 after growth during 6 and 48 hours on lettuce exposed to 25°C and 37°C assuming an initial contamination of 1 log CFU/g and a reduction rate of 2.0 log CFU/g.

Time of growth (h)	Temperature (°C)	Growth increase	$H_0 - \sum R + \sum I \leq FSO$	Adequacy to FSO
6	25	0.6	1 log – 2.0 + 0.6 = - 0.4 log	Yes
6	37	1.84	1 log – 2.0 + 1.84 = 0.84 log	No
48	25	1.53	1 log – 2.0 + 1.53 = 0.53 log	No
48	37	2.12	1 log – 2.0 + 2.12 = 1.12 log	No

Figure 1: Boxplot for Coliform counts (log CFU/g) per hypermarket sampled.

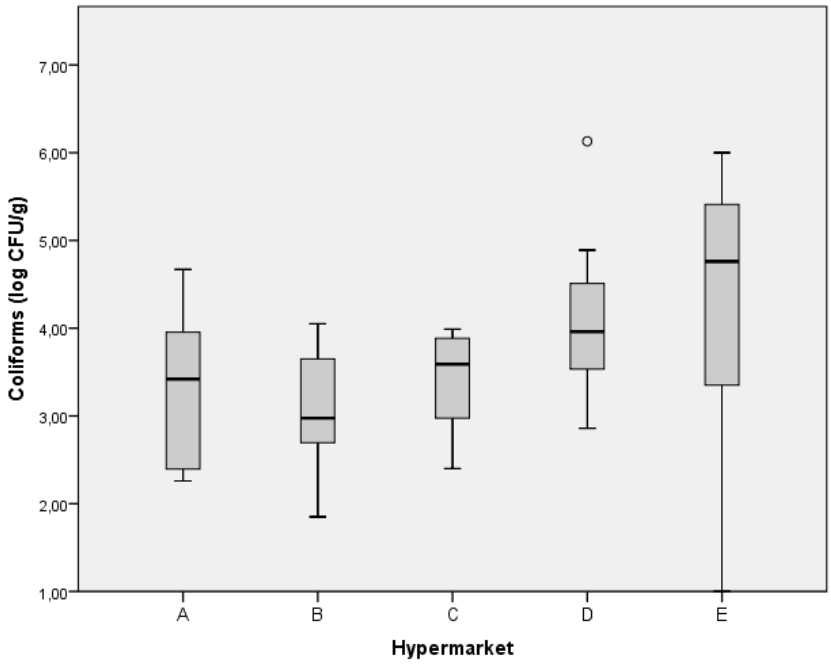
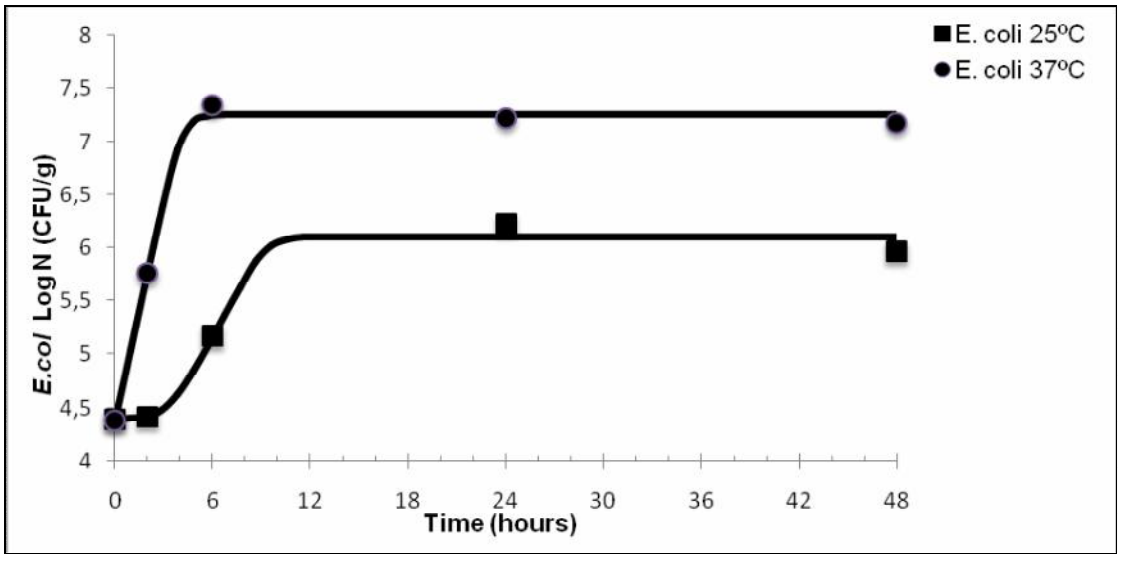
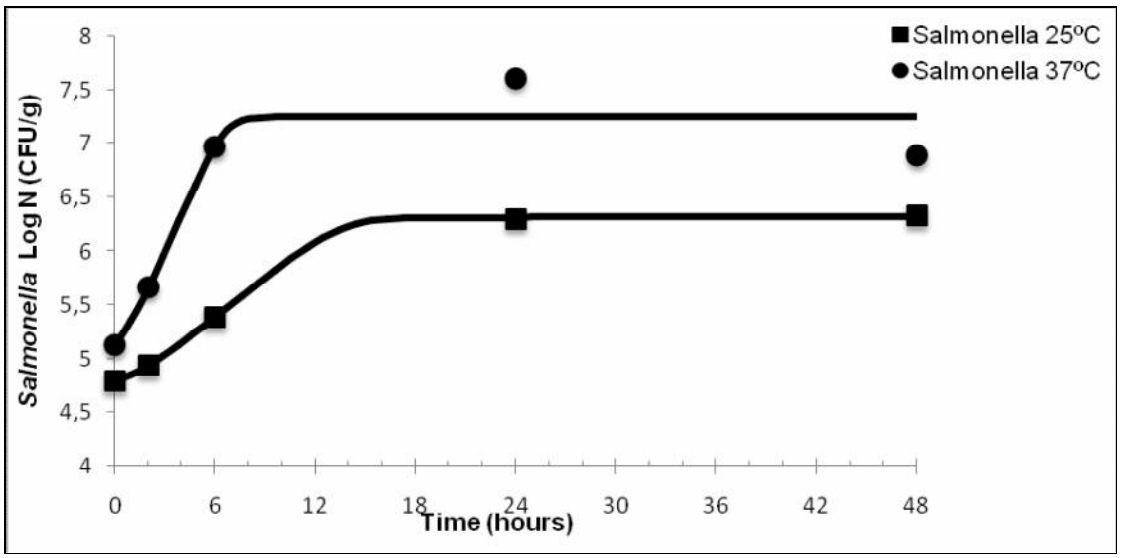


Figure 2: The observed growth of E.coli and Salmonella on lettuce at 25ºC and 37ºC.




Salmonella 25ºC
Salmonella 37ºC
Salmonella Log N (CFU/g)
8
7,5
7
6,5
6
5,5
5
4,5
4
0
6
12
18
24
30
36
42
48
Time (hours)

4 DISCUSSÃO GERAL

No primeiro artigo, que analisou as etapas da produção primária, foram encontrados baixos níveis de contaminação microbiológica e ausência de patógenos em todas as amostras analisadas, embora os resultados do questionário de auto-avaliação tenham indicado um alto risco relacionado à segurança microbiológica nos três produtores investigados.

Estes resultados podem ser explicados por alguns fatores, como por exemplo a aquisição do adubo orgânico compostado por no mínimo 90 dias por todos os produtores, o que pode explicar os baixos índices de contaminação, uma vez que diversos autores relatam que a compostagem adequada é fundamental para reduzir a contaminação e a presença de patógenos como *E. coli* e *Salmonella* (MAFF, 2000; MILLNER, 2003; JAMES, 2006; FISCHER-ARNDT et al., 2010; OLIVEIRA et al., 2012). Além disso, o adubo não era armazenado próximo aos locais de plantio e, durante o período de amostragem, inundações não foram observadas, fatos estes que poderiam, conforme relatado por Gil et al. (2013) ter interferido de forma considerável para a contaminação.

De acordo com o estudo do EFSA (2014) diversas podem ser as rasões para a variação no número de *E.coli* nos vegetais folhosos e a relação entre a produção primária e o produto final pode ser bastante variável, porém é difícil definir qual a principal causa desta variação, sendo a qualidade microbiológica do adubo orgânico e da água de irrigação são frequentemente citados. No primeiro artigo, as mudas e a alface (produto final) apresentaram contagens abaixo do limite de detecção para *E. coli*, demonstrando boa qualidade de ambos. Este fato também pode ser atribuído às baixas contagens de *E. coli* encontradas no adubo, no solo adubado e na água de irrigação e água de lavagem final, os quais apresentaram baixos níveis de contaminação.

Outro fato importante a ser citado é que de acordo com os resultados do questionário de auto-avaliação a água de irrigação foi considerada de alto risco, conforme também já relatado por outros autores, por ser água proveniente de superfície e não possuir tratamento (RICHARDSON et al., 2009; GIL et al., 2013). Além disso, ela tem sido identificada como fonte de contaminação microbiológica em

surtos envolvendo vegetais frescos (BEUCHAT, 1996; DELAQUIS; BACH; DINU, 2007; ITOHAN; PETERS; KOLO, 2011; MOYNE et al., 2011; LEVANTESI et al., 2012). Porém, todas as amostras de água investigadas no primeiro artigo apresentaram baixas contagens de contaminação por *E. coli* e nenhum patógeno, diferente do encontrado em outro estudo realizado na mesma região do país em produtores orgânicos (Rodrigues et al., 2014) que detectou a presença destes dois patógenos em amostras de água de irrigação e água de lavagem após um evento de inundação.

A ausência de Boas Práticas Agrícolas (BPA) de maneira formal em todas as propriedades investigadas também pode explicar os resultados do diagnóstico relacionados às características da organização que demonstraram que todos os produtores estavam operando num nível muito baixo de organização o que, de acordo com alguns autores (LUNNING et al, 2011; POWELL; JACOB; CHAPMAN, 2011), é comum em companhias com base familiar, que não recebem suporte técnico e treinamento e que não têm o hábito de produzir registros, o que também é comum em outros países (JAVSNIK et al., 2008; NIETO-MONTENEGRO; BROWN; LABORDE, 2008). Cabe salientar que, conforme já observado por Kirezieva et al. (2013b), a mão de obra capacitada e que conheça os riscos relacionados à segurança de alimentos pode ajudar os estabelecimentos a implementar as BPA, assim como a fiscalização realizada por órgãos governamentais, inexistente no Brasil neste setor, que pode contribuir para garantir que os produtores estão aplicando as BPA conforme o determinado (JAFEE; MASAKURE, 2005; KIERZIEVA et al., 2013a).

O questionário de auto-avaliação também demonstrou um moderado para alto risco relacionado à contaminação microbiológica nas propriedades investigadas no primeiro artigo devido às características do produto e do processo (KIREZIEVA et al., 2013b), o que pode ocasionar um alto risco de problemas relacionados à segurança de alimentos (UYTTENDAELE et al., 2014), sugerindo que um nível médio a avançado de gerenciamento de BPA deveria ser implementado a fim de diminuir o risco relacionado aos produtos, conforme já descrito por outros autores (OSÉS et al., 2011; KIREZIEVA et al., 2013b). Diferentes resultados foram encontrados em produtores da União Europeia (KIREZIEVA et al., 2015), onde

baixos a moderados riscos no contexto da produção e cadeia de suprimentos foram identificados, em especial devido ao controle das fontes de água utilizadas e a proteção dos locais de cultivo frente a contaminações externas.

No segundo artigo, que comparou a contaminação nas etapas de produção primária de propriedades de cultivo orgânico e convencional, a presença de *E. coli* foi relacionada à presença dos patógenos entéricos como *Salmonella* e *E. coli* O157:H7, enquanto que a presença de coliformes totais e enterococus não. Estes resultados corroboram o que já foi relatado anteriormente por outros estudos que demonstraram que a *E.coli* genérica é adequada como um microrganismo indicador de contaminação por patógenos (LAU; INGHAM, 2001; OGDEN et al., 2001; NATVIG et al., 2002), especialmente quando a prevalência de patógenos é muito baixa (PARK et al., 2013).

A *E.coli* só é válida como indicador de contaminação fecal em produtos frescos porque diversos gêneros de coliformes são contaminantes ambientais, tais como as espécies *Klebsiella*, *Enterobacter* e *Citrobacter* (TORTORELLO, 2003; DOYLE; ERICKSON, 2006). Entretanto, em alguns casos a *E.coli* não foi relacionada à presença de patógenos, como por exemplo, em um estudo realizado com água na Califórnia, EUA, a presença de *E. coli* não foi relacionada à presença de *Salmonella* (BENJAMIN et al., 2013), sendo assim, a validação da *E. coli* como um indicador de patógenos deve ser confirmada para cada situação específica antes do seu uso.

Diversos autores têm demonstrado associação a um maior risco microbiológico nas produções orgânicas do que nas produções convencionais (LONCAREVIC; JOHANNESSEN; RORVIK, 2005; ARBOS et al., 2010; OLIVEIRA et al., 2010; MAFFEI; SILVEIRA; CATANOZI, 2013), enquanto que outros não demonstraram diferenças significativas na qualidade microbiológica de produtos frescos de produtores orgânicos ou convencionais (MUKHERJEE et al., 2006; BORAYCHUK et al., 2009; NETO et al., 2012). No segndo artigo da presente Tese todos patógenos (5 *Salmonella* e 2 *E. coli* O157:H7) foram isolados nas propriedades orgânicas, nas quais também a *E. coli* genérica apareceu mais frequentemente e em maiores concentrações nas amostras de alface quando comparadas às propriedades de cultivo convencional. Resultados semelhantes foram encontrados em um estudo realizado na Espanha, no qual 22% das alfaces

orgânicas e 12,5% das alfaces convencionais estavam contaminadas com *E. coli* (OLIVEIRA et al., 2010) e também com outro estudo realizado no Brasil no qual a *E. coli* foi encontrada em 50% e 37,5% das amostras de alfaces orgânicas e convencionais, respectivamente (MAFFEI; SILVEIRA; CATANOZI, 2013).

Embora os resultados do segundo artigo pareçam evidenciar que a produção orgânica representa um maior risco microbiológico, as diferenças observadas se originaram, ao menos parcialmente, nas condições climáticas, uma vez que as amostras das propriedades orgânicas foram coletadas em dias com temperaturas significativamente mais altas do que nas propriedades convencionais e, conforme já relatado por outros autores, as condições climáticas e ambientais foram relacionadas com altas contaminações em vegetais (NATVIG et al., 2002; DA SILVA et al., 2007; LIU; HOFSTRA; FRANZ, 2013). Além disso, os produtores convencionais relataram uma menor frequência de irrigação em relação aos produtores orgânicos devido à maior quantidade de chuva durante o período de cultivo, fator que pode explicar também as baixas contagens microbianas devido à "lavagem" das plantas, assim como da diminuição da necessidade de irrigação.

Os seguintes fatores e práticas de cultivo foram associados a uma maior contagem microbiana e prevalência de patógenos nas propriedades orgânicas em comparação com as convencionais: aplicação de adubo orgânico mais contaminado, irrigação com água de qualidade inferior, ocorrência de inundação e maiores distâncias até os sanitários. Em contraste, a lavagem da alface após a colheita e a presença de animais nas propriedades não tiveram efeito na qualidade microbiológica dos alfaces conforme os resultados do segundo artigo. Corroborando estes resultados, foi demonstrado que o uso de banheiros móveis diminuiu os níveis de *E.coli* genérica em amostras de espinafre (PARK et al.,2013) e que a lavagem após a colheita em água sem desinfetantes não alterou as contagens de *E. coli* e coliformes (NATVIG et al., 2002; NETO et al., 2012).

Ao contrário do encontrado na literatura (PARK et al.,2013), de que a presença de animais domésticos e silvestres aumenta de forma significativa a contaminação por *E. coli*, nossos resultados demonstraram que a presença de animais não teve influência, provavelmente porque, se havia animais presentes, os

mesmos eram mantidos afastados das áreas de cultivo, uma vez que todos os produtores possuíam cães e gatos.

A aplicação de adubo orgânico compostado como fertilizante para o solo é uma prática comum tanto nas produções orgânicas e convencionais ao redor do mundo e, embora estes sejam fontes de patógenos, também são importantes fontes de nutrientes (JOHANNESSEN et al., 2004). A contaminação do solo por patógenos como *E coli* O157:H7 e *Salmonella* ocorre devido à aplicação de adubo orgânico não compostado e por fezes de animais (INGHAM et al., 2004) e estes microrganismos podem sobreviver por um tempo considerável no solo, chegando a várias semanas ou meses (ISLAM et al., 2004a,b; IBENYASSINE et al., 2006; ERICKSON et al., 2010), podendo vir a contaminar os produtos (MOOTIAN; WU; MATTHEWS, 2009). Os resultados do segundo artigo confirmam que a adequada compostagem é crucial, especialmente devido às diferenças nos números de *E. coli* e da presença de patógenos como *Salmonella* encontrados nas amostras de adubo provenientes das propriedades orgânicas.

Também os resultados do segundo artigo demonstraram que a qualidade da água foi significativamente pior nas propriedades orgânicas, onde 100% das amostras de água de irrigação continham *E. coli* em comparação com 19% das amostras provenientes das propriedades convencionais, tendo sido considerada a segunda principal fonte de contaminação. Outros estudos realizados no Brasil também identificaram a contaminação de vegetais com altos níveis de coliformes provenientes da água de irrigação (GUIMARAES et al., 2003; ABREU et al., 2010), assim como outros autores também identificaram a água como uma das principais fontes de contaminação (OGDEN et al., 2001; NATVIG et al., 2002; ISLAM et al., 2004b; INGHAM et al., 2004; IBENYASSINE et al., 2006; GELTING et al., 2011; PARK et al., 2013).

A ocorrência da inundação na semana em que uma amostra de água foi coletada em uma propriedade orgânica pode explicar o fato da presença da *E. coli* O157:H7 na água, a qual só foi detectada após a ocorrência da inundação, sugerindo que a contaminação da água de irrigação do açude ocorreu devido à esta inundação. Inundações já foram associadas a danos e a contaminação das plantações por patógenos por outros autores (TIRADO et al., 2010; GELTING et al.,

2011). Da mesma forma, a contaminação da água do poço em um dos produtores orgânicos pode ter sido consequência da infiltração da água da chuva contaminada com microrganismos originários do adubo que ficava armazenado bem próximo a este poço. Esta hipótese de contaminação é comparável com a encontrada numa fazenda de espinafre, na qual a água de irrigação também estava contaminada com *E. coli* O157:H7 originária de fezes provenientes de animais devido à mistura da água do solo com a água de irrigação (GELTING et al., 2011).

O terceiro artigo visou avaliar a contaminação presente em alfaces vendidas em hipermercados de Porto Alegre, além de investigar a possibilidade de multiplicação de patógenos em alfaces em diferentes temperaturas.

Conforme os resultados deste último artigo, apenas 4% das alfaces avaliadas apresentaram contaminação por *E. coli* acima do limite de detecção e em uma das amostras *Salmonella* foi detectada, representando um percentual de 1% de prevalência. Em outro estudo realizado no Brasil, Maffei et al. (2013) encontraram 40% das amostras de alface contaminadas com *E. coli* e nenhuma *Salmonella*, enquanto que na Venezuela, de 50 amostras de alface, apenas uma apresentou *E. coli* acima dos limites de detecção (RINCÓN et al., 2010). Já em outro estudo realizado no Egito por Uyttendaele et al. (2014), das 120 amostras de alface e morangos analisadas, 73% apresentaram contaminação por *E. coli* e a prevalência de *Salmonella* em alfaces comercializadas foi de 43%, bem diferente do encontrado em nosso estudo. Cabe ressaltar que no estudo de Uyttendaele et al. (2014), as alfaces comercializadas em hipermercados apresentaram um percentual muito inferior de *Salmonella* quando comparado às alfaces comercializadas em mercados de rua (25% e 50%, respectivamente), corroborando resultados de outros artigos que também demonstraram maior contaminação em produtos provenientes de mercados de rua (TANDON; WOOLVERTON; LANDES, 2011; BERDEGUE et al., 2005). As melhores condições de higiene relatadas pelos gerentes dos hipermercados pode ter contribuido para uma menor contaminação, além do fato de as alfaces chegarem sempre pré embaladas em sacos plásticos individuais.

Os resultados da modelagem do terceiro artigo demonstraram que os patógenos não suportaram crescimento nas temperaturas de 5 e 10 ºC, resultados

também observados por outros pesquisadores (SANT'ANA et al., 2013; KOSEKI; ISOBE; 2005), inclusive utilizando temperaturas inferiores (TIAN et al., 2012).

Quando submetidas à temperatura de 25 ºC, tanto *Salmonella* quanto *E. coli* apresentaram crescimento significativo, demonstrando uma fase lag de 1,15 ± 0.55 h and 3.28 ± 4.86 h, respectivamente. O incremento de 1 Log na população de *Salmonella* nesta temperatura, foi observado após 8,64 horas, enquanto que para a *E. coli* este aumento de população ocorreu em menor tempo, após 6,72 horas. Outros autores demonstraram resultados similares aos encontrados em nosso estudo (KOSEKI; ISOBE; 2005), onde *Salmonella* e *E. coli* O157:H7f inoculadas em alface apresentaram uma fase lag de 2,78 e 4,44 horas respectivamente, quando armazenadas a 25 ºC. Também Oliveira et al. (2010), em um estudo realizado com alface picada embalada em atmosfera modificada demonstraram um aumento de 2,44 e 4,19 Log após 3 dias incubadas a 25 ºC.

Na temperatura de 37 ºC, considerada a temperatura ótima de crescimento para a *Salmonella* e *E. coli*, estes patógenos também demonstraram taxas de crescimento significativas, ficando em 1,84 e 2,85 Log após 6 horas. Cabe ressaltar que a temperatura de 37 ºC também é comum nos meses mais quentes do ano no Brasil e, sendo assim, se as alfaces permanecerem expostas nos supermercados a esta temperatura, as mesmas poderão representar risco aos consumidores devido ao aumento da taxa microbiana.

A equação para avaliação do Objetivo de Segurança de Alimentos (FSO) foi aplicada para *Salmonella* em função dos resultados de modelagem encontrados no terceiro artigo nas temperaturas de 25 e 37 ^{o}C, visando avaliar os riscos aos quais a população poderia estar exposta caso houvesse uma contaminação inicial de 1 Log no alface adquirido nos hipermercados e se este fosse sofrer uma higienização na casa dos consumidores antes do consumo, o que, conforme a literatura (ICMSF, 2015; SAPERS, 2003; BRACKETT, 1999; BEUCHAT, 1998), reduziria a contaminação em até 2 Log, a fim de atingir o objetivo de segurança de ausência de *Salmonella* em 25 g de alimento, conforme descrito na RDC 12 (Brasil, 2001). De acordo com os resultados demonstrados na Tabela 3 do último artigo, o objetivo de

segurança não foi atingido, considerando o aumento da população de *Salmonella* após 6 a 37 ^{o}C e após 6 e 48 horas exposta a 25 ^{o}C.

5 CONCLUSÕES

De forma geral, a partir dos resultados obtidos na presente Tese, foi possível concluir que, embora o diagnóstico de auto-avaliação tenha demonstrado alto risco microbiológico na produção primária de alfaces convencionais, os resultados das análises microbiológicas demonstraram baixas contagens e ausência de patógenos. Estes resultados, embora importantes, não podem ser considerados conclusivos, uma vez que foi observada a ausência de procedimentos de prevenção de contaminação previstos na aplicação das Boas Práticas Agrícolas e que diversos fatores, tais como a baixa contaminação do adubo e da água, a quantidade de chuva e a não presença de animais nos locais de plantio, possam ter contribuído para a baixa contaminação.

Da mesma forma, foi possível concluir também que, a partir da compraração dos resultados de avaliações semelhantes realizadas em propriedades produtoras de alfaces convencionais e orgânicas, estas últimas apresentaram maior contaminação e presença de patógenos, podendo representar um maior risco aos consumidores, especialmente devido ao apelo comercial e nutricional que estes produtos vêm ganhando, nos últimos anos. Esta diferença teve relação parcial com as condições climáticas encontradas durante a coleta de amostras das propriedades orgânicas, as quais foram realizadas em época do ano com temperatura maior, menor intensidade de chuva e onde houve ocorrência de inundação. Além disso, técnicas agrícolas mais precárias também interferiram de forma significativa para os resultados das propriedades orgânicas, especialmente pela utilização de adubo orgânico mais contaminado, pela aplicação do adubo no momento do plantio e durante o crescimento dos pés de alface, pela pior qualidade microbiológica da água de irrigação utilizada e pela maior distância dos sanitários até os locais de plantio

Além disso, embora os resultados desta Tese tenham demontrado também baixa contaminação nas alfaces convencionais comercializadas nos hipermercados

investigados, um patógeno foi identificado. Este resultado, quando avaliado sob o ponto de vista do Objetivo da Segurança de Alimentos (FSO), indica que a alface pode representar risco de surtos alimentares, especialmente devido ao fato de a cadeia produtiva e comercial não prever etapas refrigeradas como obrigatórias, uma vez que as alfaces são transportadas e expostas à venda sob temperatura ambiente. Neste sentido, os resultados dos experimentos demonstraram que nas temperaturas de 5 e 10 ^{o}C não houve multiplicação significativa dos microrganismos testados, porém quando as alfaces foram mantidas a 25 e 37 ^{o}C, estes microrganismos puderam se multiplicar rapidamente, alcançando níveis de risco potencial. Sendo assim, se o alface adquirido no supermercado estiver contaminado com apenas 1 Log de microrganismos patogênicos, como a *Salmonella*, por exemplo, e for realizada a correta desinfecção do mesmo com soluções cloradas, ainda assim é possível que haja risco de contaminação do consumidor, pois a desinfecção pode não ser suficiente para eliminar os patógenos até um nível seguro. Dessa forma, a recomendação de implementação das BPA nas propriedades rurais pode ser importante para a prevenção da contaminação dos alfaces, enquanto que a manutenção dos vegetais folhosos frescos em cadeia refrigerada é importante para prevenir a multiplicação de microrganismos até quantidades que representem risco.

6 PERSPECTIVAS

- Estudar aprofundadamente os procedimentos realizados em supermercados relativos aos vegetais, desde o recebimento até a venda;
- Estudar a realidade de indústrias de vegetais minimamente processados;
- Estudar aprofundadamente a qualidade de águas de irrigação, suas fontes e métodos de aplicação (gotejamento ou aspersão).

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