UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Programa de Pós-Graduação em Ciência dos Alimentos
Área de Bromatologia

Avaliação quantitativa do risco de *Salmonella* e *Listeria monocytogenes* em vegetais minimamente processados

Anderson de Souza Sant'Ana

Tese para obtenção do grau de
DOUTOR

Orientador:
Prof^a. Dr^a. Bernadette D.G.M. Franco

São Paulo
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Tese para obtenção do grau de Doutor

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São Paulo, _____ de _____.

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AVALIAÇÃO QUANTITATIVA DO RISCO DE *SALMONELLA* E *LISTERIA MONOCYTOGENES* EM VEGETAIS MINIMAMENTE PROCESSADOS

A ocorrência de surtos de doenças associadas aos vegetais minimamente processados (VMP) tem chamado a atenção para a sua segurança microbiológica. A avaliação quantitativa de riscos permite que o impacto das matérias-primas e processamento seja avaliado e os resultados obtidos sejam usados para gestão e comunicação do risco. Desta forma, o presente estudo objetivou quantificar o risco de infecções por *Salmonella* spp. e *Listeria monocytogenes* a partir do consumo de VMP no Brasil. Um total de quinhentas e doze amostras de VMP foram analisadas e foi possível enumerar e detectar *Salmonella* em 0,4% e 0,4% das amostras, respectivamente. *L. monocytogenes* foi enumerada e detectada em 0,97% e 3,1% das amostras analisadas, respectivamente. Os isolados de *Salmonella* spp. (n=4) e *L. monocytogenes* (n=69) foram confirmados por PCR e caracterizados por sorotipagem tradicional. Os isolados de *L. monocytogenes* foram caracterizados quanto ao ribotipo, resistência ao cloro, taxa de multiplicação (μ), capacidade de formação de biofilmes e presença de genes de virulência. O sorovar predominante entre *Salmonella* spp. foi *S. Typhimurium*. Em relação à *L. monocytogenes*, observou-se prevalência do sorotipo 4b e do ribogruppo DUP-1038 e presença de genes de virulência em 100% (*inlA*) e 97% (*inlC* e *inlJ*) dos isolados. A maioria dos isolados de *L. monocytogenes* foi resistente à exposição à 125 ppm de cloro livre, e todos foram capazes de aderir ao aço inox, atingindo concentrações acima de 4 log UFC/cm². Testes-desafio foram conduzidos para determinar o potencial de multiplicação (δ) de cepas de *Salmonella* e *L. monocytogenes* em nove diferentes tipos de VMPs armazenados a 7°C e 15°C por 6 dias. O armazenamento a 15°C por 6 dias resultou nos maiores aumentos nas populações de *L. monocytogenes* em couve picada (δ = 3,34) e rúcula (δ = 3,22), enquanto para *Salmonella*, as maiores populações foram observadas em rúcula (δ = 4,05) e escarola (δ = 2,80). Testes-desafios posteriores indicaram que a multiplicação dos dois patógenos em VMP foi mais pronunciada quando os mesmos foram embalados sob atmosfera modificada em comparação a embalagem em filmes perfurados. Modelos preditivos primários e secundários descrevendo a taxa de multiplicação e tempo de lag de *Salmonella* spp. e *L. monocytogenes* em VMP em função da temperatura de armazenamento (7, 10, 15, 20, 25 e 30°C) foram gerados. Verificou-se que os modelos gerados apresentaram a precisão necessária e foram adequados para modelagem da multiplicação dos dois patógenos em VMP. Os modelos de avaliação quantitativa de risco (AQR) foram construídos para determinar a probabilidade de infecção por *Salmonella* spp. e *L. monocytogenes* devido ao consumo de VMPs. Os modelos construídos com base nos dados levantados da literatura indicaram risco de infecção por *Salmonella* spp. e *L. monocytogenes* de 8.66×10^{-3} e 1.87×10^{-8} , respectivamente, sendo necessário que medidas de mitigação do risco sejam adotadas.

Palavras-chave: Vegetais minimamente processados, *Salmonella*, *Listeria monocytogenes*, microbiologia preditiva, avaliação de risco.

QUANTITATIVE RISK ASSESSMENT OF *SALMONELLA* AND *LISTERIA MONOCYTOGENES* IN MINIMALLY PROCESSED VEGETABLES

The occurrence of foodborne disease outbreaks linked to minimally processed vegetables (MPV) is concerning industries, consumers and governments worldwide. Quantitative risk assessments can estimate the impact of raw materials and processing practices and these estimates are used for risk management and risk communication. This study aimed at quantifying the risks of infection by *Salmonella* spp. and *Listeria monocytogenes* due to consumption of MPV in Brazil. A total of five hundred and twelve samples of MPV were analyzed and *Salmonella* was detected and enumerated in 0.4% and 0.4% of the samples, respectively. *L. monocytogenes* was enumerated and detected in 0.97% and 3.1% of the samples analyzed, respectively. Isolates of *Salmonella* spp. (n=4) and *L. monocytogenes* (n=69) were confirmed through PCR and characterized by traditional serotyping. The isolates of *L. monocytogenes* were characterized for their ribotype, resistance to chlorine, growth rate, (μ) and ability to form biofilms and presence of virulence factors. Among *Salmonella* spp., *S. Typhimurium* was the most prevalent serovar. Among *L. monocytogenes*, prevalence of serotype 4b and ribotype DUP-1038 was observed. Virulence gene *inlA* was present in 100% of the isolates, and genes *inlC* and *inlJ* in 97%. The majority of *L. monocytogenes* isolates were resistant to up to 125 ppm of free chlorine and all isolates were able to attach to stainless steel coupons, reaching populations of up to $4 \log_{10}$ CFU/cm². Challenge tests were carried out to determine the growth potential (δ) of *Salmonella* and *L. monocytogenes* in nine types of MPV stored at 7°C and 15°C for 6 days. The storage of MPV at 15°C for 6 days resulted in the greatest increases in *L. monocytogenes* populations in shredded collard green (δ = 3.34) and arugula (δ = 3.22), whereas for *Salmonella*, the highest populations were found in arugula (δ = 4.05) and escarole (δ = 2.80). Further challenge tests indicated that multiplication of both pathogens in MPV was more pronounced when these products were packaged under modified atmosphere in comparison to packaging in perforated films. Primary and secondary predictive models describing the growth rate and lag time of *Salmonella* and *L. monocytogenes* in MPV as a function of storage temperature (7-30°C) were generated. The generated models were accurate and suitable for modeling the growth of pathogens in MPVs. Quantitative risk assessment (QRA) models were built to determine the probability of infection by *Salmonella* and *L. monocytogenes* due to consumption of MPVs. The models built using data available in the literature indicated that the risks of infection by these pathogens were 8.66×10^{-3} and 1.87×10^{-8} , respectively, evidencing the need for adoption of risk mitigation measures.

Key-words: Minimally processed vegetables, *Salmonella*, *monocytogenes*, predictive microbiology, risk assessment.

1. INTRODUÇÃO

Vegetais minimamente processados (VMP) são definidos como aqueles preparados utilizando-se uma única etapa ou um número limitado de operações unitárias, como descascamento, corte, picagem, fatiamento, moagem e etc.. Para conservação dos VMP necessita-se refrigeração ou algum outro método que garanta sua estabilidade durante a vida de prateleira. Acompanhando uma tendência mundial, o consumo de VMP vem crescendo nos últimos anos também no Brasil. No Brasil, estima-se um crescimento no mercado em torno de 15% ao ano, com um movimento anual próximo a R\$ 450 milhões, só no mercado nacional.

O aumento do consumo de VMP se deve ao fato destes produtos apresentarem diversas vantagens em relação aos demais produtos de origem vegetal: a praticidade (prontos para o consumo), manutenção das características sensoriais e nutricionais do vegetal fresco, eliminação de desperdício, aquisição apenas da parte comestível do vegetal, possibilidade de compra de menores quantidades, conhecimento da procedência do produto e a opção de escolher marcas.

Apesar de frutas e vegetais frescos representarem importantes componentes de uma dieta saudável, a ocorrência de microrganismos patogênicos, como *Salmonella*, *Listeria monocytogenes* e *E. coli* 0157:H7 e surtos associados a estes produtos tem chamado a atenção para a sua segurança microbiológica. Os VMP foram implicados em 461 surtos de doenças transmitidas por alimentos (DTA's) nos EUA, entre 1990 e 2003, com mais de 20 mil casos reportados o que os figura entre os principais responsáveis por DTA's neste país. Estes produtos têm despertado a preocupação de produtores, consumidores e órgãos de vigilância, pois durante a sua fabricação não há nenhuma etapa que garanta a redução de patógenos potencialmente presentes a um nível satisfatório, apesar da existência da etapa de desinfecção. No Brasil, devido à fragilidade do sistema de vigilância epidemiológica, não existem relatos de surtos associados aos VMP, porém a incidência de patógenos como *Salmonella* e *L. monocytogenes* nestes produtos pode ser considerada preocupante do ponto de vista da possível ocorrência de surtos.

Nos diversos países do mundo é crescente a preocupação com as DTA's pelo fato causarem grandes prejuízos econômicos à saúde pública e algumas vezes a

morte. As indústrias de alimentos estão em contínua mudança com respeito aos perigos e riscos microbiológicos. Os microbiologistas são usualmente os responsáveis pela avaliação do impacto de tais mudanças no risco, indicando como mudanças no processamento e preparo de um determinado alimento podem afetar suas probabilidades de causar uma toxinfecção alimentar. A avaliação de riscos permite que o impacto das matérias-primas e processamento seja avaliado e os resultados obtidos sejam usados para gestão e comunicação do risco.

Devido à incidência de *Salmonella* spp. e *L. monocytogenes* a partir do consumo de VMP comercializados em São Paulo, Brasil, justifica-se analisar e determinar os riscos da ocorrência de infecções por estes patógenos a partir do consumo de VMP comercializados em São Paulo, Brasil. A aplicabilidade do estudo está na possibilidade da adoção de estratégias cientificamente baseadas para controlar e reduzir a contaminação e a incidência de patógenos nestes alimentos, o que poderá levar a menores custos para a saúde pública e também auxiliará a manutenção do crescimento deste mercado.

2. REVISÃO DE LITERATURA

2.1 Microbiologia de Vegetais Minimamente Processados (VMP):

Durante o plantio, colheita e transporte os vegetais estão sujeitos a contaminação por diversos microrganismos provenientes do solo, ar, água, vetores, manipulação humana e o esterco usado como adubo. O interesse pelos microrganismos nos VMP se deve a vários fatores: 1) Durante seu processamento, não há uma etapa de eliminação dos microrganismos (cozimento, por exemplo); 2) A extensão da vida-de-prateleira conseguida atualmente (10 a 14 dias) representa tempo suficiente para a multiplicação dos patógenos; 3) O uso de embalagem em atmosfera modificada, suprime a multiplicação de microrganismos deterioradores responsáveis pela sinalização do final da vida-de-prateleira e 4) Falhas no controle de temperatura durante a estocagem e elevadas populações de bactérias ou fungos que podem levar à redução da qualidade e da vida de prateleira (IFPA, 1999).

De acordo com De Roever (1998) os patógenos de importância para os VMP são: *Shigella* spp., *E. coli* 0157:H7, *E. coli* enterotoxigênica, *Vibrio cholerae*, *L. monocytogenes*, *Bacillus cereus*, vírus da Hepatite A, vírus Norwalk, *Cyclospora cayetanensis* e *Cryptosporidium parvum*. Ainda de acordo com este levantamento, *Salmonella* spp. é o microrganismo mais associado a surtos de DTA's nos EUA onde os VMP são os alimentos envolvidos. De acordo com dados do Centro de Vigilância Epidemiológica do estado de São Paulo (CVE, 2007), somente na cidade de São Paulo no ano de 2005, foram registrados mais de 84 surtos de DTA's, com mais de 860 casos e *Salmonella* spp. esteve entre os principais microrganismos envolvidos (mais de 150 casos). Apesar dos dados não permitirem que conclusões sejam tiradas (grande número de surtos não tiveram os agentes causadores e alimentos implicados determinados), pode-se considerar que os vegetais e a *Salmonella* sejam muitas vezes implicados nestes surtos.

Como a maioria dos vegetais possui pH superior a 4,5 e, portanto, favorável à multiplicação microbiana, as temperaturas de estocagem e comercialização são os

fatores determinantes da segurança destes produtos (De Roever, 1998). Nestes alimentos então, patógenos psicrotróficos ubíquos como *L. monocytogenes* (Gandhi e Chikindas, 2007) têm grande importância. De acordo com Farber et al. (1998), *L. monocytogenes* pode ter sua população aumentada em até 1,94 unidades logarítmicas após estocagem a 10°C por 12 dias, confirmando sua capacidade de se multiplicar sob refrigeração e nos VMP. Além disso, *L. monocytogenes*, é capaz de se multiplicar sob as baixas tensões de oxigênio que estão normalmente presentes nos VMP (Francis et al., 1999). A grande preocupação com a presença de *L. monocytogenes* nos alimentos se deve ao fato do microrganismo causar diversas doenças no homem, como septicemia, meningite e meningoencefalites, com taxa de mortalidade entre 20 e 25%. Indivíduos de risco são adultos imunocomprometidos e mulheres grávidas, nas quais o microrganismo pode levar ao aborto.

A qualidade microbiológica dos VMP tem sido objeto de diversas pesquisas ao redor do mundo e diversos patógenos tem sido isolados destes produtos, como *B. cereus* (Valero et al., 2002), *E.coli* e *Listeria* spp. (Lin et al., 1996), *Staphylococcus aureus* (Fang et al., 1999; Nguz et al., 2005) e *Salmonella* spp. (Salleh et al., 2003; Nguz et al., 2005), *L. monocytogenes* (Nguz et al., 2005; Francis e O'Beirne, 2006). No Brasil, a incidência de *Salmonella* e *Listeria monocytogenes* em VMP já foi comprovada. Porto e Eiroa (2001), analisando 250 amostras de vegetais em diferentes períodos do ano, encontraram contaminação por *L. monocytogenes* em 3,2% das amostras, com a população média do patógeno em alface sendo de 1,2 NMP/g e o maior período de isolamento ocorrendo no mês de janeiro (62,5%). Fröder et al. (2007), ao avaliar a qualidade microbiológica de 181 amostras de saladas minimamente processadas, comercializadas em São Paulo, encontraram 4 positivas para *Listeria* spp., sendo um isolado confirmado como *L. monocytogenes*. Já com relação a *Salmonella* spp. houve isolamento a partir de 4 amostras (3%). Maistro (2005), avaliando a qualidade microbiológica de 172 amostras de VMP comercializados em Campinas, SP, encontraram contaminação por *Salmonella* e *L. monocytogenes* em 1 e 3 amostras, respectivamente. A incidência de patógenos nos VMP, aliada à sua capacidade de sobrevivência ou multiplicação nesses produtos (Weissinger et al., 2000; Kärenlampi e Hanninen, 2004; Kim et al., 2006; Valero et al., 2003; Farber et al., 1998), tem levado ao estudo de diversos métodos para reduzir, eliminar ou controlar a ocorrência nos VMP. Dentre eles, destacam-se a irradiação (Martins et al., 2004; Goularte et al., 2004), a aplicação de ozônio (Selma

et al., 2007), compostos clorados (Gómez-López et al., 2007), que normalmente são aplicados como barreiras adicionais, em conjunto com a atmosfera modificada e refrigeração. Apesar dos poucos estudos relacionados à qualidade microbiológica dos VMP, pode-se comprovar a incidência de *Salmonella* e *L. monocytogenes* nos produtos produzidos e comercializados no Brasil, os quais podem representar sérios riscos à saúde pública. A quantificação destes patógenos nos VMP é de extrema importância para estudos de avaliação quantitativa de riscos, onde a variabilidade e incerteza devem sempre ser levadas em conta. Todavia, além da determinação da prevalência e enumeração dos patógenos nestes alimentos, a sua caracterização feno e genotípica é extremamente importante e pode fornecer informações úteis para a construção de modelos de avaliação quantitativa de risco.

2.2 Segurança dos Alimentos:

É considerado um alimento seguro aquele no qual, ao longo da cadeia produtiva, foram aplicadas medidas sanitárias e de higiene efetivas e eficazes e que, por isso, não representa riscos em níveis acima dos tolerados pelo consumidor, sempre e quando o mesmo for usado nas condições e para os fins a que se destina (PAS, 2004). Governos, indústrias e cientistas de alimentos têm procurado garantir a segurança dos alimentos através do desenvolvimento e aplicação de diversas ferramentas de gestão da inocuidade. Apesar das legislações de diversos países no mundo preconizarem a utilização de ferramentas como as boas práticas agrícolas, boas práticas de fabricação e o sistema APPCC, deve-se levar em conta que a sua implementação é voltada para uma linha de processamento de um produto específico para uma indústria específica, enquanto a avaliação de riscos é uma ferramenta com aplicação mais ampla e que foi desenvolvida para auxiliar os órgãos governamentais a tomarem decisões cientificamente embasadas. O desenvolvimento das técnicas de análise quantitativa de risco foi estimulado pelo desejo de melhor estimar-se o impacto potencial da qualidade e segurança dos alimentos junto à saúde pública e as consequências econômicas associadas (Whiting e Buchanan, 1997).

2.2.1 Análise de Riscos: A análise de risco (Risk analysis) é um processo constituído de três componentes: avaliação do risco, gestão do risco e comunicação do risco, que são relacionados entre si, mas constituem em distintas fases da análise de riscos (Codex, 2007).

2.2.2 Avaliação Quantitativa de Riscos: O Codex Alimentarius, através do documento “*Principles and guidelines for the conduct of microbiological risk assessment*” (CAC/GL30) (Codex, 2007), descreve os princípios gerais da avaliação quantitativa de risco e os tipos de informação necessárias em cada etapa de desenvolvimento do estudo. A partir dos onze princípios gerais descritos no documento, foram geradas diretrizes para aplicação da avaliação quantitativa de risco, traduzidas em cinco passos que devem ser seguidos de maneira a se desenvolver um estudo de avaliação de risco. Estes cinco passos são descritos abaixo:

1. Declaração do propósito da avaliação de risco: a primeira das etapas da avaliação de riscos deve ser a determinação do seu objetivo;
2. Identificação do perigo: é a segunda etapa da avaliação de riscos e tem por propósito identificar qual o perigo será o alvo do estudo. Para isso, são necessárias diversas informações sobre o microrganismo tais como: resultados de estudos clínicos, epidemiológicos, vigilância sanitária, com animais laboratoriais, características fisiológicas dos microrganismos e da sua interação com o alimento e o ambiente;
3. Avaliação da exposição: tem por objetivo estimar a quantidade dos agentes biológicos efetivamente ingeridos pelos consumidores, com vários níveis de incerteza e a probabilidade de sua ocorrência nos alimentos no momento do consumo. Os dois principais parâmetros a serem determinados nesta etapa são a frequência de contaminação dos alimentos pelo patógeno e seu nível nos alimentos com o passar do tempo (multiplicação, sobrevivência ou prevalência) e os padrões de consumo. Nesta etapa, modelos preditivos como os de Gompertz e

Barany podem encontrar grande aplicação de maneira a ajudar no entendimento da dinâmica dos microrganismos no alimento. Na modelagem primária são obtidos parâmetros cinéticos do crescimento microbiano, como tempo de lag e taxa de multiplicação de um microrganismo. Desta forma, com a microbiologia preditiva, o efeito de diferentes cenários durante diferentes condições de produção, manuseio e medidas de controle adotadas podem ser inseridas em modelos de risco de maneira a se prever o comportamento microbiano;

4. Caracterização do perigo: etapa que fornece uma descrição qualitativa ou quantitativa da severidade e duração dos efeitos adversos que podem ser resultados da ingestão de um microrganismo ou toxina presentes num alimento. Uma desejável característica desta etapa é o estabelecimento de uma relação dose-resposta, necessária para caracterização do perigo;
5. Caracterização do risco: é a etapa final da avaliação quantitativa do risco e envolve a integração dos resultados da identificação do perigo, caracterização do perigo e avaliação da exposição para obter uma estimativa do risco. Ele fornece uma estimativa qualitativa ou quantitativa da probabilidade e severidade dos efeitos adversos para uma determinada população, incluindo a descrição das incertezas associadas com estas estimativas. As estimativas por sua vez podem ser comparadas com os dados de prevalência da doença para sua avaliação. Além de fornecer as informações necessárias para tomada de decisões dos gestores de riscos, uma boa caracterização do risco também identifica dados chave atualmente indisponíveis que melhorariam a exatidão de futuras avaliações.

Diversos estudos de avaliação quantitativa de riscos têm sido desenvolvidos. Na *Tabela 1* é apresentada uma compilação dos estudos de avaliação de risco desenvolvidos até o momento. Pode-se observar que a maioria tem como alimento alvo da avaliação de risco, produtos de origem animal. Combinações envolvendo vegetais minimamente processados são recentes (2010 e 2011). Desta forma, a

determinação dos riscos associados ao consumo de VMP poderá auxiliar na definição de melhores estratégias para controlar e reduzir a contaminação e a incidência destes patógenos nestes alimentos, o que resultará em menores custos para a saúde pública e também auxiliará a manutenção do crescimento deste mercado.

Tabela 1. Combinações patógenos-alimentos usadas em estudos de avaliação de risco.

Combinação Patógeno-Alimento	Referência
<i>S. Enteritidis</i> e ovo líquido pasteurizado	Whiting & Buchanan (1997)
<i>E. coli</i> 0157:H7 e hambúrguer bovino	Cassin et al. (1998)
<i>L. monocytogenes</i> e peixe defumado	Lindqvist & Westöö (2000)
<i>C. jejuni</i> e carne fresca	Anderson et al. (2001)
<i>S. Typhimurium</i> DT 104 lingüiça de porco	Alban et al. (2002)
<i>Campylobacter</i> e frango	Rosenquist et al. (2003)
<i>Salmonella</i> e frango	Oscar (2004)
<i>Salmonella</i> e derivados de carne suína	Giovannini et al. (2004)
<i>Salmonella</i> e carne de frango	Parsons et al. (2005)
<i>Salmonella</i> e produtos contendo ovos	Mokhtari et al. (2006)
<i>L. monocytogenes</i> e contaminação cruzada	Pérez-Rodriguez et al. (2006)
<i>Campylobacter</i> e preparações à base de carne de frango	Uyttendaele et al. (2006)
<i>L. monocytogenes</i> e fatiados	Yang et al. (2006)
<i>Salmonella</i> e abate bovino	Tuominen et al. (2007)
<i>L. monocytogenes</i> e salmão defumado	Pouillot et al. (2007)
<i>Campylobacter</i> e carne de frango	Nauta et al. (2007)
<i>E. coli</i> 0157:H7 e leite não pasteurizado	Grace et al. (2008)
<i>V. parahaemolyticus</i> e mariscos	Yamamoto et al. (2008)
<i>Campylobacter</i> e contaminação cruzada durante a manipulação de frango	Lindqvist e Linblad (2008)
<i>E. coli</i> 0157:H7 e hambúrguer	Delignette-Muller et al. (2008)
<i>L. monocytogenes</i> e produtos cárneos pronto para o consumo	Ross et al. (2009)
<i>E. coli</i> produtora de toxina de Shiga e hambúrguer bovino	Signorini e Tarabla (2009)
<i>V. parahaemolyticus</i> e cavalo-marinho cru	Iwahori et al. (2010)
<i>L. monocytogenes</i> e produtos cárneos pronto para o consumo	Mataragas et al. (2010)
<i>E. coli</i> O157:H7, <i>S. enterica</i> and <i>L. monocytogenes</i> e vegetais folhosos	Tromp et al. (2010)
<i>Escherichia coli</i> O157:H7 e vegetais folhosos	Danyluk e Schaffner (2011)

3. OBJETIVOS

3.1 Objetivo Geral

Quantificar o risco de infecções por *Salmonella* spp. e *L. monocytogenes* a partir do consumo de VMP no Brasil, utilizando a metodologia descrita pelo *Codex Alimentarius*.

3.2 Objetivos Específicos

- ✓ Determinar a prevalência e quantificar o nível de contaminação de VMP obtidos no comércio de São Paulo, em relação a *Salmonella* spp. e *L.monocytogenes*;
- ✓ Caracterizar genotípica e fenotipicamente os isolados de *Salmonella* spp. e *L. monocytogenes*;
- ✓ Determinar o efeito de diferentes cenários de estocagem sobre a população final de *Salmonella* e *L. monocytogenes* inoculadas artificialmente em nove diferente tipos de VMPs;
- ✓ Determinar o efeito de diferentes cenários de estocagem e tipos de embalagem sobre a população final de *Salmonella* e *L. monocytogenes* contaminadas artificialmente com dois níveis de inóculos;
- ✓ Determinar os parâmetros cinéticos de multiplicação (taxa de multiplicação e tempo de lag) de três diferentes cepas de *Salmonella* e *L. monocytogenes* artificialmente inoculadas em VMPs durante estocagem entre 7-30°C, utilizando-se o modelo pred itivo de Barany, e, posteriormente, avaliar a influência da temperatura sobre os parâmetros cinéticos estudados, utilizando-se um modelo secundário (raíz quadrada);
- ✓ Determinação da probabilidade de infecção por *Salmonella* spp. e *L.monocytogenes* a partir do consumo de VMPs em São Paulo, Brasil.

4. ORGANIZAÇÃO DA TESE DE DOUTORADO

A apresentação desta tese de Doutorado foi dividida em sete capítulos, preparados na forma de artigos científicos. O **capítulo 1** corresponde a uma revisão de literatura sobre a avaliação quantitativa de risco e sua importância no contexto atual da segurança dos alimentos. No **capítulo 2** descrevem-se os resultados do estudo de prevalência e enumeração de *Salmonella* spp. em vegetais minimamente processados (VMP) comercializados na cidade de São Paulo, SP. O **capítulo 3** relata a prevalência, enumeração e caracterização feno e genotípica de *Listeria monocytogenes* isoladas das amostras de VMPs analisadas. O **capítulo 4** apresenta os resultados de testes-desafio, nos quais se avaliou o comportamento dos dois patógenos inoculados em nove tipos de VMPs durante o armazenamento em diferentes condições de tempo e temperatura. No **capítulo 5**, descrevem-se os resultados dos testes-desafio para os dois vegetais com maior consumo no Brasil (couve e alface), usando-se inóculos iniciais baixos (10^1 - 10^2 UFC/g) de ambos os patógenos e simulando as condições de armazenamento mais utilizadas por processadores e consumidores de VMP. No **capítulo 6**, são apresentados os resultados da determinação dos parâmetros cinéticos de multiplicação (taxa de multiplicação e tempo de lag) de *Salmonella* e *L. monocytogenes* em alface, correspondente a um dos vegetais mais consumidos no Brasil e no qual os dois patógenos se multiplicaram melhor, utilizando-se uma ampla faixa de temperatura (7 a 30°C). No **capítulo 7**, os dados gerados neste trabalho e aqueles levantados na literatura foram empregados para a construção de modelos de avaliação quantitativa de riscos, e determinação da probabilidade de infecção por *Salmonella* e *L. monocytogenes* devido ao consumo de VMPs.

CAPÍTULO 1

Revisão

“Avaliação quantitativa de risco microbiológico em alimentos: conceitos, sistemática e aplicações”

Artigo publicado na revista “Brazilian Journal of Food Technology”

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**AVALIAÇÃO QUANTITATIVA DE RISCO MICROBIOLÓGICO EM ALIMENTOS: CONCEITOS,
SISTEMÁTICA E APLICAÇÕES**
**MICROBIAL QUANTITATIVE RISK ASSESSMENT OF FOODS: CONCEPTS, SYSTEMATICS AND
APPLICATIONS**

Título abreviado: Avaliação quantitativa de risco.

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RESUMO

A segurança dos alimentos é um tema de crescente importância no mundo e preocupa governantes, indústrias e consumidores. Com o aumento do comércio mundial de alimentos, o estabelecimento de regras claras de comércio internacional e que protejam a saúde da população tornou-se prioritário. Em 1995, os países signatários da Organização Mundial do Comércio assinaram um acordo de Medidas Sanitárias e Fitossanitárias, cuja principal consequência foi a adoção da ferramenta de análise de risco no contexto da segurança dos alimentos. Um dos componentes dessa ferramenta é a avaliação de risco microbiológico, que permite verificar se uma determinada combinação microrganismo patogênico/alimento representa ou não um risco acima daquele considerado aceitável em um país. Trata-se de um processo que envolve quatro etapas: (i) identificação do perigo, (ii) caracterização do perigo, (iii) avaliação da exposição e (iv) caracterização do risco. Considerando a importância desta ferramenta na gestão da segurança dos alimentos, o objetivo desta revisão é apresentar os conceitos básicos da avaliação quantitativa de risco microbiológico em alimentos, sua importância no contexto atual de inocuidade dos alimentos e as possíveis aplicações, benefícios e limitações.

PALAVRAS-CHAVE: Avaliação quantitativa de risco, análise de risco microbiológico, inocuidade de alimentos.

SUMMARY

Food safety is an important issue everywhere and is a major concern of food industries, food safety authorities and governments. Due to the increasing international trade, clear trade rules and measures to protect consumers health became mandatory. In 1995, the World Trade Organization country members signed the Sanitary and Phyto-Sanitary Agreement, which specifies that measures should be based on assessment of the risks to human health. One of the consequences of this agreement was the adoption of risk analysis concepts. Risk assessment is one component of a risk analysis and allows determining if a risk associated to a pathogen-food combination is higher than that considered acceptable or adequate to protect consumer's health. Risk assessment is a science-based process, consisting of the four steps: (i) hazard identification; (ii) hazard characterization; (iii) exposure assessment and (iv) risk characterization. Due to its importance as a tool for food safety management, this review aimed at presenting the basic concepts of a microbiological risk assessment, its relevance in the accepted food safety context and possible applications, benefits and limitations.

KEY WORDS: Quantitative risk assessment, microbiological risk analysis, food safety.

1. INTRODUÇÃO

Com a intensificação do comércio internacional de alimentos, é fundamental que os sistemas de controle dos perigos e de gestão de sua segurança sejam estabelecidos com base em princípios claros e aceitáveis por todos os envolvidos. Devido à necessidade de harmonizar os procedimentos de controle relativos à segurança dos alimentos, os países signatários da Organização Mundial do Comércio assinaram em 1995 um acordo, denominado Acordo de Medidas Sanitárias e Fitosanitárias - SPS (*Sanitary and Phytosanitary Agreement*), cujo objetivo é proteger a saúde humana, animal e vegetal em seu território. Em função desse acordo, cada país membro da OMC tem o direito de utilizar as medidas que julgar necessárias para garantir o nível de proteção à saúde pública, sanidade animal e vegetal existentes no país. Estas medidas, todavia, não devem ter como objetivo o protecionismo no comércio ou se transformar em barreiras comerciais injustificáveis no comércio bilateral (WTO, 1995, STRINGER, 2005, WOOLRIDGE, 2008).

Uma das consequências mais marcantes do Acordo de Medidas Sanitárias e Fitosanitárias foi a adoção dos princípios básicos da Análise de Risco pela área de segurança de alimentos. Segundo a definição clássica do Codex Alimentarius, 1999, uma análise de risco é composta por três componentes: avaliação do risco, gestão do risco e comunicação do risco (*Figura 1*). Através de uma Análise de Risco, identifica-se um problema, avalia-se sua gravidade, considera-se as alternativas de gestão possíveis, seleciona-se a(s) alternativa(s) mais adequada(s), implementa-se essa(s) alternativa(s) de gestão e avalia-se seu impacto na solução do problema (DENNIS et al., 2008).

Figura 1. Estrutura de uma Análise de Risco (CODEX ALIMENTARIUS, 1999)

Enquanto a avaliação de risco microbiológico (*risk assessment*) associado a um perigo microbiológico com efeito adverso à saúde humana é subsidiada por informações científicas, a gestão do risco (*risk management*) é a etapa em que se implementa medidas de controle que efetivamente poderão reduzir o risco ao nível de proteção desejado. Para isso, é necessário avaliar todas as medidas de controle possíveis e selecionar as adequadas e passíveis de implementação. A comunicação do risco (*risk communication*) é a etapa em que se faz a troca de informações e opiniões a respeito dos riscos e dos fatores relacionados, no qual interagem os assessores de riscos, os gestores de riscos e as outras partes interessadas (FAO, 1997). Embora cada componente da análise de risco tenha objetivos distintos, as atividades são integradas, com a participação de todos os envolvidos no processo, sendo um processo sistemático e transparente (CAST, 2006, WHITING e BUCHANAN, 2008).

A análise de risco em alimentos e, particularmente, a avaliação do risco, é uma ferramenta de importância capital, principalmente para países em que o agronegócio representa uma significativa parcela da balança comercial, como o Brasil. Além de avaliar os riscos associados ao consumo dos alimentos produzidos no país e também dos importados, a análise do risco protege o país contra a imposição de barreiras comerciais “mascaradas” como medidas sanitárias e/ou fitossanitárias. Considerando a importância da avaliação de risco na gestão da segurança dos alimentos e o pouco conhecimento no país sobre o assunto, preparou-se a presente revisão, com o objetivo de apresentar os conceitos básicos da avaliação de risco e sua sistemática de aplicação, benefícios e limitações.

2. AVALIAÇÃO QUANTITATIVA DE RISCO MICROBIOLÓGICO

Segundo o *Codex Alimentarius* (CODEX, 1999), a avaliação de risco microbiológico é um processo de base científica, constituído das seguintes etapas: (i) identificação do perigo microbiológico, (ii) caracterização deste perigo, (iii) avaliação da exposição e (iv) caracterização do risco.

Uma avaliação de risco microbiológico inicia-se com determinação da combinação patógeno-alimento a ser considerada. Neste contexto, é preciso identificar o perigo microbiológico a ser considerado em um dado tipo de alimento de acordo com seu

envolvimento como agente de doenças nos consumidores, baseando-se em informações epidemiológicas e biológicas pertinentes ao patógeno e ao alimento avaliados (BUCHANAN, 1997, WOOLRIDGE, 2008).

Determinada a combinação patógeno-alimento que comporá a avaliação de risco, a etapa seguinte é a avaliação da exposição da população a esse patógeno em decorrência da ingestão do alimento considerado. A avaliação da exposição baseia-se em modelos preditivos matemáticos para descrever as respostas de multiplicação, sobrevivência e inativação do microrganismo, bem como a possibilidade de recontaminação em diferentes condições ambientais, com o objetivo de estimar a quantidade do patógeno no momento do consumo. Nas simulações, leva-se em conta a probabilidade de ocorrência do patógeno no alimento, o impacto do processamento e manuseio sobre esse patógeno, a frequência da exposição da população a esse patógeno, bem como a duração dessa exposição (CODEX, 1999, WOOLRIDGE, 2008).

A avaliação de dose-resposta estima a relação entre a quantidade do patógeno ingerido e a frequência e magnitude dos efeitos adversos na população. Considerando dados sobre taxas de infecção, morbidade e mortalidade, as avaliações de dose-resposta estimam a severidade dos efeitos adversos, permitindo avaliar seus impactos à saúde (LAMMERDING et al., 2001).

A caracterização do risco é a etapa final da avaliação do risco e envolve a integração dos resultados das avaliações de dose-resposta e de exposição, fornecendo uma estimativa da probabilidade de ocorrência do problema, bem como de sua magnitude (WOOLRIDGE, 2008).

Uma avaliação de risco pode ser qualitativa ou quantitativa (COLLEMAN e MARKS, 1999; ILSI, 2007). Enquanto a avaliação qualitativa lida com dados descritivos das informações, com resultados expressos em termos de probabilidade (baixa, média ou alta, por exemplo), nas avaliações quantitativas os resultados são expressos em termos numéricos, baseados em valores sobre prevalência e enumeração dos patógenos e equações matemáticas que descrevem o comportamento microbiano. Apesar das avaliações quantitativas serem as preferidas, algumas vezes não é possível desenvolvê-las devido à falta de dados que permitam a realização de estudos quantitativos e à falta de habilidade para lidar com as ferramentas matemáticas e

computacionais requeridas para o desenvolvimento de modelos de riscos. Além disso, estudos qualitativos são mais rápidos, simples e fáceis do que a avaliação quantitativa, além de serem mais facilmente entendidos pelos gestores de riscos. Segundo o *Codex Alimentarius* e WTO, ambos os tipos são válidos, desde que os requerimentos de cada um estejam atendidos (WOOLDRIDGE, 2008).

2.1. A AVALIAÇÃO DE RISCO MICROBIOLÓGICO NO CONTEXTO ATUAL DA INOCUIDADE DOS ALIMENTOS

Dentro do contexto atual do comércio mundial de alimentos, diversos termos e conceitos novos, relativos a sistemas e/ou critérios relacionados com a garantia da inocuidade dos alimentos, foram criados. O termo “ALOP”, do inglês “*Appropriate Level of Protection*”, refere-se ao nível de proteção da saúde humana, animal ou vegetal considerado apropriado em um país, que deve estabelecer as medidas sanitárias ou fitosanitárias necessárias para que esse nível de proteção em seu território seja assegurado. Um ALOP pode ser expresso em termos amplos, relativos aos objetivos da saúde pública, ou expressar quantitativamente a probabilidade de ocorrência de um efeito adverso à saúde causado por um perigo ou a incidência máxima de uma doença veiculada por alimentos. No estabelecimento de um ALOP, devem ser levados em conta fatores relacionados ao sistema produtivo, razões econômicas e a fundamentação científica do nível de proteção pretendido (SWARTE e DONKER, 2005).

Quando um país determina as metas de saúde pública em relação à incidência de doenças, esse procedimento não dá aos processadores de alimentos, produtores, manipuladores, comerciantes e consumidores informações sobre o que é necessário ser feito para que essas metas sejam atingidas. Para que tenham sentido, as metas necessitam ser transformadas em parâmetros que possam ser mensurados e avaliados pelas agências governamentais e utilizados pelos produtores no processamento de alimentos. Nesse contexto, surgiu o conceito de FSO, do inglês *Food Safety Objectives* (TUOMINEN et al., 2007, STRINGER, 2008)). O termo correspondente em português é *Objetivos de Inocuidade Alimentar*.

Um FSO é definido como “a frequência máxima e/ou concentração de um perigo microbiológico em um alimento no momento do seu consumo que esteja em

consonância com o nível de proteção à saúde pretendido” (ICMSF, 2002). Esta frequência máxima e/ou concentração de um perigo microbiológico determinada pelo FSO aplica-se para alimentos que estejam prontos para ser consumidos, mas não é aplicável para alimentos que ainda passarão por outras etapas (transporte, armazenamento, cozimento, etc) antes de consumidos. Os efeitos da estocagem e manuseio nos pontos de comercialização e consumo, bem como do tratamento térmico no preparo do alimento para o consumo sobre os microrganismos presentes nesses alimentos são imprevisíveis. O FSO determina uma meta que a cadeia produtiva deve alcançar, sem especificar a maneira como essa meta deve ser alcançada. Assim, o FSO dá flexibilidade para que, na cadeia produtiva, sejam utilizadas técnicas de processamento mais adequadas para cada situação, desde que o nível máximo do perigo especificado não seja ultrapassado. Por exemplo, a pasteurização garante a segurança do leite, mas no futuro essa segurança poderá ser obtida através de outras tecnologias, além da pasteurização. Isso é muito importante no comércio internacional, porque países diferentes podem usar tecnologias diferentes para alcançar um mesmo objetivo.

Enquanto os FSO são adotados para alimentos no momento do consumo, nos diferentes pontos da cadeia produtiva de alimentos adotam-se os POs, do inglês *Performance Objectives*. O termo em português é *Objetivos de Desempenho*. Um PO é definido como “a frequência máxima e/ou concentração de um perigo em um alimento em uma etapa específica da cadeia produtiva antes do consumo, que esteja de acordo com o ALOP, ou com o FSO”. Um PO é estabelecido com base no FSO, podendo ser mais ou menos rígido do que o FSO, dependendo se o perigo em questão permanece ou não no mesmo nível entre a etapa do PO e o consumo (VAN SCHOTHORST, 2005). O PO pode ser mais rígido do que o FSO quando pode haver contaminação ou multiplicação microbiana durante a distribuição, preparação, armazenamento e uso de um alimento em particular. Por outro lado, o PO pode ser mais tolerante que o FSO quando, por exemplo, o alimento necessitar de cozimento antes do consumo. Os POs podem ser estabelecidos pelo governo ou pela indústria.

Para avaliar se FSOs e POs estão adequados, utilizam-se *Critérios Microbiológicos* (MC, do inglês *Microbiological Criteria*), que devem indicar “a

aceitabilidade de um alimento ou lote de alimentos, baseada na presença/ausência de microrganismos ou na quantidade de microrganismos e/ou metabólitos, por unidade de massa, volume, área ou lote”. Os MC dependem da aplicação de um plano de amostragem adequado e do uso de métodos analíticos validados, que podem ser estabelecidos por órgãos governamentais e/ou pelas indústrias. Assim, um MC define a aceitabilidade de alimentos, ou de seus ingredientes, em um determinado ponto de toda a cadeia produtiva (CODEX, 1997).

Para avaliar o efeito de uma medida de controle na frequência e/ou concentração de um perigo num alimento, utilizam-se os *Critérios de Desempenho* (PC - *performance criteria*). Nesse caso, a medida de controle em avaliação é aquela que objetiva o atendimento do PO ou do FSO. Por outro lado, um *Critério de Processo* (Pc – *process criteria*) é um parâmetro de controle (temperatura, tempo, pH ou atividade de água, por exemplo) que pode ser aplicado para se atingir um PC (VAN SCHOTHORST, 2002). Todos estes conceitos podem ser aplicados em conjunto nas etapas de processamento dos alimentos de maneira a garantir que FSO e ALOP sejam atingidos.

A Figura 2 mostra um esquema indicando os pontos onde se aplicam ALOP, FSO, PO, MC, PC e Pc, envolvendo o país e a cadeia produtiva de alimentos. A Figura 3 mostra que os parâmetros ALOP, FSO, PO e MC são definidos pelo país, em nível nacional, enquanto as BPH (Boas Práticas de Higiene) e os sistemas HACCP (Hazard Analysis and Critical Control Points) são ferramentas de gerenciamento da segurança dos alimentos utilizadas nas plantas processadoras de alimentos (nível de processamento) de forma a se atingir o ALOP e os FSO e PO estabelecidos.

Figura 2. Esquema de cadeia produtiva mostrando a posição de ALOP, FSO, PO, MC, PC e Pc. (adaptado de ICMSF, 2006, WHITING e BUCHANAN, 2008)

Figura 3: Estruturação das ferramentas de gerenciamento da segurança dos alimentos (adaptado de ICMSF, 2006)

2.2. TIPOS DE AVALIAÇÃO DE RISCO MICROBIOLÓGICO

A escolha da estrutura e do tipo específico de avaliação de risco a ser realizada leva em conta o problema de saúde pública a ser estudado, as questões da gestão de riscos a serem respondidas e a disponibilidade de dados. Segundo CAST (2006) há quatro tipos de avaliação de risco (*Tabela 1*).

Tabela 1. Tipos de Avaliação de Risco Microbiológico, segundo CAST, 2006.

Tipos	Descrição	Aplicação	Exemplo
Ranqueamento	Compara os riscos relativos de diferentes perigos ou alimentos	Estabelecimento de prioridades de gestão dos riscos e de alocação de recursos e identificação dos estudos críticos necessários	<i>Listeria monocytogenes</i> em alimentos prontos para consumo
	Examina os fatores que influenciam o risco associado a uma determinada combinação alimento/patógeno em toda a cadeia produtiva	Identificação de fatores-chave que interferem na exposição, incluindo a frequência e nível de contaminação e o impacto de estratégias de intervenção sobre o risco avaliado	<i>Vibrio parahaemolyticus</i> em ostras cruas
Risco-risco	Avalia os riscos decorrentes da substituição de um determinado perigo por outro	Avaliação dos impactos de uma medida de controle destinada à redução de um determinado risco mas que pode causar aumento de outro risco ou do risco geral	Doença de veiculação hídrica versus efeitos nocivos dos derivados do cloro
Geográfico	Examina fatores que afetam a entrada, distribuição e impacto à saúde pública de um perigo novo em um determinado local em um determinado momento	Estimativa dos riscos potenciais decorrentes da falha do sistema de segurança dos alimentos	Desenvolvimento de uma nova variedade da Doença de Creutzfeldt-Jacob por passagem do príon da Encefalopatia Espongiforme Bovina do gado para humanos

A avaliação de risco do tipo “ranqueamento” coloca diferentes riscos em uma ordem crescente de gravidade, como por exemplo, salmonelose associada a diferentes alimentos, como ovos, carne bovina, carne suína ou aves. Esse tipo de avaliação de risco permite identificar a combinação patógeno/alimento que representa maior risco à saúde pública entre todas as combinações analisadas.

Na avaliação de risco do tipo “cadeia produtiva”, avalia-se quais fatores durante a produção e consumo de um alimento têm maior impacto no risco associado a esse alimento. Neste tipo de estudo, o impacto de diferentes estratégias de redução de risco pode também ser avaliado. Por exemplo, na combinação *Listeria monocytogenes*/queijo, estratégias de controle do risco podem incluir desde medidas para reduzir a contaminação do leite com o patógeno até a indicação da necessidade de mudanças nos hábitos de consumo. A decisão de quais medidas devem ser adotadas é tarefa dos gestores de risco, que necessitam levar em conta não apenas resultados da avaliação de risco mas também fatores econômicos e sociais.

A avaliação do tipo “risco-risco” pode ser exemplificada considerando-se produtos cárneos curados e cozidos, cuja conservação e inocuidade são garantidas pela combinação de cozimento a 74-75⁰C com a adição de nitrito. A presença do nitrito permite reduzir a intensidade do tratamento térmico necessário para garantir a segurança dos produtos, evitando alterações sensoriais e nutritivas indesejadas, e assim manter a segurança do produto pelo impedimento da germinação e multiplicação do *Clostridium botulinum*. Entretanto, o nitrito apresenta efeitos tóxicos (CULIK e KELLNER, 1995), gerando um novo risco para esses produtos. Nesse caso, os gestores de risco devem decidir, juntamente com as partes interessadas, qual risco será aceitável e quais são as alternativas de gestão possíveis.

Por último, na avaliação de risco do tipo “geográfico” analisa-se o risco da introdução um patógeno novo em uma região onde a ocorrência de doença relacionada a esse patógeno ainda não tenha sido relatada, como por exemplo, o mal da vaca louca (Síndrome Espongiforme Bovina). Nesse caso, a avaliação de risco depende de muitas informações, como sistemas de criação e alimentação dos animais, medidas de controle e prevenção adotadas, etc.

2.3. CONSTRUÇÃO DE UM MODELO DE AVALIAÇÃO QUANTITATIVA DE RISCO MICROBIOLÓGICO

De um modo geral, as seguintes informações básicas são necessárias para montagem de um modelo de avaliação quantitativa de risco: i) dados sobre a prevalência e concentração do patógeno nas matérias-primas; ii) dados sobre o efeito das diferentes etapas de processamento sobre a prevalência e concentração do patógeno no alimento; iii) dados de modelos preditivos previamente validados,

em condições reais ou simuladas, sobre o comportamento do patógeno nas diversas condições de processamento e estocagem; iv) dados sobre as condições do processamento (tempo, temperatura, atividade de água, pH, etc.) que podem influenciar a sobrevivência do patógeno; v) quantidade e frequência de ingestão do alimento em estudo, hábitos de consumo, características da população consumidora (faixa etária, condições de saúde, etc);

Em alguns casos, a avaliação do efeito das etapas do processamento sobre o patógeno, desde a produção até o consumo do alimento, requer que modelos de dose-resposta para se determinar o risco da doença em si sejam acoplados ao modelo de risco. Em outras vezes, a abordagem pode considerar a avaliação do comportamento do patógeno nos diferentes pontos da cadeia produtiva, como por exemplo, produção primária, processamento, transporte, e consumo. Evidentemente, quanto maior o número de etapas da cadeia produtiva dos alimentos e quanto maior o número de parâmetros que afetam a dinâmica do patógeno considerado, maior a complexidade do modelo, o que não pode ser um fator impeditivo para o desenvolvimento dos modelos de avaliação de risco. Todas as informações utilizadas e conclusões obtidas, bem como as limitações da avaliação, devem ser claramente apresentadas (transparência), de maneira que a avaliação possa ser atualizada sempre que novas informações estejam disponíveis.

Uma vez definidos os dados a serem utilizados em uma avaliação de risco, é preciso determinar: i) quais etapas e/ou módulos de toda a linha de processamento (*from farm to fork*) serão modelados; ii) dados de entrada, representados por distribuições estatísticas, acoplados ou não a modelos preditivos, suas variáveis e respectivas unidades; iii) dados de saída, suas variáveis e respectivas unidades. Na Figura 4, correspondente a um modelo de risco para *Escherichia coli* 0157:H7 em aparas de carne bovina, pode-se observar os módulos (linhas tracejadas com fundo cinza), as etapas de processamento e dados de entrada (linhas contínuas grossas com fundo claro), variáveis (linhas contínuas finas com fundo claro) e dados de saída (linhas tracejadas com fundo claro). O exemplo foi retirado e adaptado do trabalho de CUMMINS et al., 2008.

Figura 4. Esquema do modelo de risco para *E. coli* 0157:H7 em aparas de carne bovina (Cummins et al., 2008).

2.4. CARACTERIZAÇÃO DE VARIABILIDADE E INCERTEZA

Os termos variabilidade e incerteza referem-se a situações distintas. A variabilidade representa a heterogeneidade intrínseca de um fenômeno bem caracterizado, enquanto a incerteza está relacionada com conhecimento insuficiente de um fenômeno. Compreendendo melhor o fenômeno, à custa de mais pesquisa sobre ele, é possível diminuir a incerteza, mas não a variabilidade (CAST, 2006, LAMMERDING et al., 2001).

Tanto a variabilidade quanto a incerteza têm um papel importante no grau de confiança de uma estimativa de risco feita através do modelo desenvolvido. A variabilidade fornece informações valiosas sobre os extremos das distribuições, importantes na estimativa do risco microbiológico. Alguns exemplos de extremos são as populações de menor resistência à infecção (indivíduos imunocomprometidos), ou um número baixo de células ou esporos de patógenos termoresistentes, ou um número pequeno de embalagens contaminadas. Isoladamente cada um desses fatores contribui pouco para o risco, mas juntos podem aumentar a possibilidade de ocorrência de um surto, ou seja, aumentam o risco (MILLER et al., 1997).

A variabilidade é inerente a um fenômeno microbiológico, e deve ser sempre considerada nos modelos de avaliação quantitativa de risco, mesmo que não seja possível reduzi-la. Já a incerteza pode ser reduzida por medições e coletas de dados adicionais, o que deve ser feito sempre que possível. A relação entre grau de incerteza e a quantidade de dados disponíveis pode ser melhor compreendida no exemplo da Figura 5, relativa à prevalência estimada de *Salmonella* spp em alface, quando se encontra uma amostra positiva entre 142 amostras analisadas ou quando se encontra duas amostras positivas em 284 amostras analisadas. Apesar da proporção de amostras positivas ser a mesma nos dois casos (0,7%), no segundo a certeza e a confiança dos resultados obtidos são maiores, devido ao maior número de amostras analisadas (CAST, 2006).

Figura 5. Incerteza representada pela prevalência de *Salmonella* spp em 142 (A) e 284 (B) amostras analisadas de um alimento (adaptado de CAST, 2006).

Assim, com a sistemática da avaliação quantitativa de risco, fica evidente a necessidade de uma mudança no enfoque dos estudos de avaliação de incidência de patógenos em alimentos. Estes estudos não devem determinar somente a prevalência dos patógenos, mas devem também quantificá-los, analisando um número elevado e representativo de amostras. Estudos de avaliação de risco devem sempre considerar a variabilidade e todo esforço deve ser feito para que incertezas sejam reduzidas.

2.5. METODOLOGIA ANALÍTICA

Existem numerosas ferramentas para a modelagem de riscos para diferentes combinações patógeno/alimento. A simulação de Monte Carlo é a mais utilizada na avaliação de risco, na qual a estimativa de um ponto é substituída por uma distribuição probabilística. Nesse processo, realiza-se uma amostragem ao acaso de cada distribuição de probabilidade do modelo, centenas ou milhares de vezes, produzindo um novo cenário a cada iteração. Assim, gera-se um novo “ponto estimado” a cada iteração para cada um dos parâmetros do modelo, registrando-se o resultado. O processo é repetido tantas vezes quanto necessário para que o campo de cada distribuição de probabilidade individual seja suficientemente varrido. Em outras palavras, a modelagem de Monte Carlo seleciona um valor amostral a cada iteração que é baseada numa probabilidade de distribuição. Dessa forma,

baseando-se nos parâmetros da distribuição, os valores mais prováveis são selecionados com mais frequência que os valores menos prováveis (valores mínimo ou máximo, por exemplo, numa distribuição do tipo triangular), refletindo mais fielmente os eventos que aconteceriam na realidade (LAMMERDING et al., 2001). Ao realizar estudos de avaliação de risco, é necessário conhecer o número de iterações necessárias para cobrir todo o campo das distribuições utilizadas, caso contrário os resultados do modelo podem ser incorretos, fazendo com que as estimativas feitas não sejam confiáveis. Na Figura 6, apresenta-se um exemplo de simulação de Monte Carlo usando-se a distribuição triangular e 5000 iterações, sendo possível observar que quanto maior o número de iterações realizadas, maior é a cobertura do campo da distribuição estatística e mais confiáveis são os dados resultantes.

Além de permitir estimar riscos, a modelagem de Monte Carlo permite determinar quais variáveis do modelo são fortemente correlacionadas com os dados de saída, ou seja, com o risco avaliado (LAMMERDING et al., 2001).

Figura 6. Recriação de uma distribuição triangular usando-se a simulação de Monte Carlo (adaptado de LAMMERDING et al., 2001).

Existem diversas ferramentas computacionais para realização de estimativas de riscos empregando-se simulações de Monte Carlo, como Analytica (Lumina, USA), SAS (SAS, USA) e Mathematica (Wolfram, USA) (CAST, 2006), entre outras. No entanto, “@Risk” (Palisade Co., USA) e “Crystal Ball” (Decisioneering Inc., USA) são as mais adotadas atualmente em estudos de avaliação quantitativa de risco, por sua facilidade de operação (CASSIN et al., 1998; LINDQVIST, e WESTÖÖ, 2000; BEMRAH et al., 2002; OSCAR, 2004; GIOVANNINI et al., 2004; MAIJALA et al., 2005; DANYLUK et al., 2006; UYTTENDAELE et al., 2006).

2.6. ANÁLISE SENSITIVA

A análise sensitiva é uma ferramenta bastante utilizada para avaliar os resultados de um estudo de avaliação quantitativa de risco, que são complexos devido à variabilidade e incerteza inerentes a eles (CAST, 2006).

O gráfico de tornado é uma maneira comum de apresentar os resultados da análise sensitiva, que permite observar claramente a influência de diversos parâmetros do modelo sobre o parâmetro de saída a ser estimado. Na Figura 7, apresenta-se um exemplo de gráfico de tornado, elaborado para um estudo de avaliação quantitativa de risco de *Vibrio parahaemolyticus* (Vp) em ostras (USFDA 2005). As barras indicam os fatores que contribuem para o risco, e os valores apresentados após as barras indicam a porcentagem de contribuição de cada fator para o risco total. O gráfico evidencia claramente os fatores que afetam positivamente e negativamente a probabilidade de infecção por *Vibrio parahaemolyticus* (Vp) a partir do consumo de ostras. Fatores com valores positivos e mais próximos da parte superior do gráfico são os que mais impactam no aumento do risco, enquanto os fatores mais próximos da parte inferior indicam contribuições menos significativas. No exemplo citado, o tempo de refrigeração é o único fator que contribui na redução do risco da infecção por *V.parahaemolyticus*, enquanto todos os demais, que são valores positivos, contribuem para o aumento do risco, sendo a concentração do microrganismo no ambiente o que tem maior impacto.

Figura 7. Exemplo de gráfico tornado de um estudo de avaliação quantitativa de risco para *V. parahaemolyticus* e ostras (adaptado de Cast, 2006 e USFDA, 2005).

2.7. IMPORTÂNCIA, POSSÍVEIS APLICAÇÕES E LIMITAÇÕES DA AVALIAÇÃO DE RISCO:

A avaliação de risco é uma ferramenta que comprova que nenhum alimento é livre de riscos. Por vincular a presença de patógenos microbianos nos alimentos com indicadores de saúde pública (CAST, 2006), a avaliação de risco evidencia que cada etapa do processo produtivo dos alimentos, do campo à mesa, tem seu papel na garantia da segurança do produto final. Por analisar os riscos desde a obtenção da matéria-prima até o consumo, essa ferramenta permite identificar os pontos críticos dos quais são necessárias informações constantes, dando os subsídios necessários para a organização das estratégias de gestão do risco, ou seja, das medidas necessárias para controlar e reduzir os riscos associados a um alimento específico.

A avaliação de risco microbiológico permite avaliar a equivalência de diferentes processos tecnológicos para inativação de um perigo em alimento, verificado se asseguram o mesmo nível de proteção à saúde pública. Esta aplicação tem grande importância devido ao desenvolvimento constante de novos métodos para conservação dos alimentos, cuja eficácia precisa ser avaliada no contexto das diferentes opções tecnológicas disponíveis.

As observações das populações humana e/ou animal em relação às doenças são um ponto importante tanto para avaliações de risco como para estudos epidemiológicos. Enquanto a avaliação de risco é focada na dinâmica dos patógenos

ao longo da cadeia produtiva, usando modelos preditivos para estimar resultados em termos de saúde pública, os estudos epidemiológicos fornecem dados precisos do ponto de vista de saúde pública devido a disponibilidade de informações verdadeiras e não apenas estimadas. Assim, estudos epidemiológicos e avaliações de risco são atividades complementares (Figura 8) (HAVELAAR et al., 2007). Uma avaliação de risco pode ser validada pelos dados epidemiológicos, através da verificação dos modelos de risco desenvolvidos e dos resultados obtidos pela vigilância epidemiológica.

Figura 8. Interação entre avaliação quantitativa de risco e epidemiologia no estudo do impacto de patógenos nos alimentos (Havelaar et al, 2007).

Apesar dos avanços nos últimos anos nos campos da microbiologia preditiva, computação, microbiologia analítica e epidemiologia, a técnica de modelagem quantitativa de risco apresenta várias limitações: i) decisões baseadas em informações incompletas ou de má qualidade comprometem a utilidade do estudo devido à incerteza dos resultados; ii) os resultados de uma avaliação de risco nunca são definitivos e devem ser atualizados sempre que novas informações sejam disponibilizadas; iii) cientistas ligados à segurança de alimentos têm pouca familiaridade com a técnica de modelagem de riscos; iv) a avaliação de risco não pode prever riscos associados com patógenos para os quais não há dados epidemiológicos, clínicos e microbiológicos e v) modelos de risco são, na maioria das vezes, construídos a partir de dados de modelagem preditiva (sobrevivência,

inativação e/ou multiplicação) obtidos quando os patógenos não estão na presença da microbiota competidora naturalmente presente na maioria dos alimentos, o que pode comprometer a precisão dos resultados obtidos (CAST, 2006; LAMMERDING et al., 2001).

3. CONCLUSÕES E PERSPECTIVAS

A avaliação de risco é um processo governamental, desenvolvido para responder as questões formuladas pelos responsáveis pela gestão dos riscos identificados. No contexto atual do comércio internacional de alimentos, a avaliação de risco é uma ferramenta objetiva para ajustar as regulamentações de higiene e segurança dos alimentos e para determinar a efetividade das práticas de gestão dos riscos adotadas. O desenvolvimento e a melhor aplicação da análise de risco resultarão na adoção de medidas de controle dos perigos baseadas em critérios praticáveis, o que deve impactar positivamente a redução de problemas relacionados à inocuidade dos alimentos devido aos microrganismos patogênicos.

Segundo CAST, 2006, algumas recomendações em relação à avaliação de risco na área de segurança de alimentos são: i) focar em pesquisas que objetivem reduzir ao máximo as incertezas e a falta de dados nas avaliações de risco existentes; ii) estimular o desenvolvimento das ferramentas que dão suporte para o desenvolvimento dos modelos de risco, como investigação epidemiológica e metodologias analíticas quantitativas (com limites de detecção claramente determinados); iv) melhorar a disponibilização de dados sobre hábitos e padrões de consumo de um país/região; v) dar mais transparência às atividades e responsabilidades dos assessores de risco, gestores de riscos e partes interessadas, bem como melhorar a comunicação do risco; vi) estimular os microbiologistas de alimentos nas universidades, indústrias e governo a desenvolver seus estudos e interpretar seus resultados no contexto da avaliação de risco; vi) incentivar as universidades para formação de especialistas em análise de risco, com treinamento nas técnicas de avaliação, gestão e comunicação de riscos.

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REFERÊNCIAS

BEMRAH, N.; BERGIS, H.; COLMIN, C.; BEAUFORT, A.; MILLEMANN, Y.; DUFOUR, B.; BENET, J.J.; CERF, O.; SANAA, M. Quantitative risk assessment of human salmonellosis from the consumption of a turkey product in collective catering establishments. **International Journal of Food Microbiology**, Amsterdam, v.80, n.1, p.17-30, 2002.

BUCHANAN, R.L. National Advisory Committee on Microbiological Criteria for Foods "Principles of Risk Assessment for Illnesses caused by Foodborne Biological Agents". **Journal of Food Protection**, Des Moines, v.60, n.1, p.1417-1419, 1997.

CASSIN, M.H.; LAMMERDING, A.M.; TODD, E.C.D.; ROSS, W.; McCOLL, R.S. Quantitative risk assessment for *Escherichia coli* 0157:H7 in ground beef hamburgers. **International Journal of Food Microbiology**, Amsterdam, v.41, n.1, p.21-44, 1998.

CAST – Council for Agricultural Science and Technology. **Using risk analysis to inform microbial food safety decisions**. Issue Paper 31. CAST, Ames, Iowa, p.1-20, 2006.

CODEX - *Codex Alimentarius Commission*. **Principles and guidelines for the conduct of Microbiological Risk Assessment**, CAC/GL – 30, Roma, 1999. Disponível em: www.codexalimentarius.net Acesso em: 20 mar. 2009.

CODEX - *Codex Alimentarius Commission*. **Principles and guidelines for the conduct of Microbiological Risk Assessment**, CAC/GL – 30, Rome, 1999. Disponível em: www.codexalimentarius.net Acesso em: 20 mar. 2009.

CODEX - Codex Alimentarius Commission. **Principles for the establishment and application of microbiological criteria for foods**, CAC/GL – 21, Rome, 1997. Disponível em: www.codexalimentarius.net Acesso em: 17 mar. 2009.

COLEMAN, M.; MARKS, H. Qualitative and quantitative risk assessment. **International Journal of Food Microbiology**, Amsterdam, v.10, n.4-5, p.289-297, 1999.

CULIK, J. and KELLNER, V. Toxic compounds arising by interaction of food constituents with food additives. Part A. Nitroso compounds. **Natural Toxic Compounds of Foods**. Boca Raton: CRC Press, 1995.

CUMMINS, E., NALLY, P., BUTLER, F., DUFFY, G., S.O'BRIEN. Development and validation of a probabilistic second-order exposure assessment model for

Escherichia coli 0157:H7 contamination of beef trimmings from Irish meat plants. **Meat Science**, Barking, v.79, n. 1, p.139-154, 2008.

DANYLUK, M.D.; HARRIS, L.J.; SCHAFFNER, D.W. Monte Carlo simulations assessing the risk of salmonellosis from consumption of almonds. **Journal of Food Protection**, Des Moines, v.69, n.7, p.1594-1599, 2006.

DENNIS, S.B., KAUSE, J., LOSIKOFF, M., ENGELJOHN, D.L., BUCHANAN, R.L. Using risk analysis for microbial food safety regulatory decision making. In: SCHAFFNER, D.W. (Ed.) **Microbial Risk Analysis of Foods**. Washington, DC, 2008. Chap. 5, p.137-176.

FAO/WHO - FOOD AND AGRICULTURE ORGANIZATION – WORLD HEALTH ORGANIZATION. **Application of risk analysis to food standards issues**. Report of a Joint FAO/WHO Consultation. World Health Organization. Geneva, 13-17 March. 39 p. 1997.

GIOVANNINI, A.; PRENCIPE, V.; CONTE, A.; MARINO, L.; PETRINI, A.; POMILIO, F.; RIZZI, V.; MIGLIORATI, G. Quantitative risk assessment of *Salmonella* spp. infection for the consumer of pork products in an Italian region. **Food Control**, Guildford, v.15, n.2, p.139-144, 2004.

HAVELAAR, A.H.; BRÄUNIG, J.; CHRISTIANSEN, K.; CORNU, M.; HALD, T.; MANGEN, M.-J.J.; MOLBAK, K.; PIELAAT, A.; SNARY, E.; VAN PELT, W.; VELTHUIS, A.; WAHLSTRÖM, H. Towards an integrated approach in supporting microbiological food safety decisions. **Zoonoses and Public Health**, Oxford, v. 54, n.3-4, p.103-117, 2008.

ICMSF - INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS. **Microorganisms in Foods 7: Microbiological Testing in Food Safety Management**. New York: Kluwer Academic/Plenum Publishers, 2002.

ICMSF - INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS. Guia Simplificado para Compreensão e Uso de Objetivos de Inocuidade Alimentar e Objetivos de Desempenho. 2006. Disponível em : <http://www.icmsf.iit.edu/pdf/FSO%20Objectives/GuiaSimplificadoPO.pdf>. Acesso em 20 mar. 2009.

LAMMERDING, A.M., FAZIL, A.M., PAOLI, G.M. Microbial Food Safety Risk Assessment. In: DOWNES, F.P., ITO, K. (EdS.) **Compendium of methods for the**

microbiological examination of foods. Washington DC: American Public Health Association, 2001. Chap. 29, p.267-282.

LINDQVIST, R.; WESTÖÖ, A. Quantitative risk assessment for *Listeria monocytogenes* in smoked or gravad salmon and rainbow trout in Sweden. **International Journal of Food Microbiology**, Amsterdam, v. 58, n.3, p.181-196, 2000.

MAIJALA, R.; RANTA, J.; SEUNA, E.; PELKONEN, S.; JOHANSSON, T. A quantitative risk assessment of the public health impact of the Finnish *Salmonella* control program for broilers. **International Journal of Food Microbiology**, Amsterdam, v.102, n.1, p.21-35, 2005.

MILLER, A.J.; WHITING, R.C.; SMITH, J.L. Use of risk assessment to reduce listeriosis incidence. **Food Technology**, Chicago, v.51, n.4, p.100-103, 1997.

OSCAR, T.P. A quantitative risk assessment model for *Salmonella* and whole chickens. **International Journal of Food Microbiology**, Amsterdam, v.93, n.2, p.231-247, 2004.

STRINGER, M. Food Safety Objectives – role in microbiological food safety management. **Food Control**, Guildford, v.16, n.9, p.775-777794, 2005

SWARTE, C., DONKER, R.A. Towards an FSO/ALOP based food safety policy. **Food Control**, Guildford, v.16, n.9, p.825-830, 2005.

TUOMINEN, P., RANTA, J., MAIJALA, R. Studying the effect of POs and MCs on the *Salmonella* ALOP with a quantitative risk assessment model for beef production. **International Journal of Food Microbiology**, Amsterdam, v.118, n.1, p.35-51, 2007.

UYTTENDAELE, M.; BAERT, K.; GHAFIR, Y.; DAUBE, G.; ZUTTER, L.; HERMAN, L.; DIERICK, K.; PIERARD, D.; DUBOIS, J.J.; HORION, B.; DEBEVERE, J. Quantitative risk assessment of *Campylobacter* spp in poultry based meat preparations as one of the factors to support the development of risk-based microbiological criteria in Belgium. **International Journal of Food Microbiology**, Amsterdam, v.111, n.2 p.149-163, 2006.

VAN SCHOTHORST, M. A proposed framework for the use of FSOs. **Food Control**, Guildford, v.16, n.9, p.811-816, 2005.

VAN SCHOTHORST, M., Implementing the results of an MRA: pathogen risk assessment. In: BROWN, M. and STRINGER, M. (Eds). **Microbiological risk**

assessment in food processing. Cambridge: Woodhead Publishing, 2002. Chap. 9, p.175-192.

U.S. Food and Drug Administration (USFDA). 2005. Quantitative Risk Assessment on the Public Health Impact of Pathogenic *Vibrio parahaemolyticus* in Raw Oysters. <<http://www.cfsan.fda.gov/~dms/vpra-toc.html>> Acesso em 15 Abr. 2009.

WHITING, R.C. and BUCHANAN, R.L. Using risk assessment principles in an emerging paradigm for controlling the microbial safety of foods. In: SCHAFFNER, D.W. (Ed.) **Microbial Risk Analysis of Foods**. Washington, DC, 2008. Chap. 2, p.29-50.

WOOLDRIDGE, M. Qualitative risk assessment. In: SCHAFFNER, D.W. (Ed.) **Microbial Risk Analysis of Foods**. Washington, DC, 2008. Chap. 1, p.1-28.

WTO - World Trade Organization (WTO). The WTO agreement on the application of sanitary and phytosanitary measures (SPS Agreement), 1995. Disponível em: www.wto.org/english/tratop_e/sps_e/spsagr_e.htm. Acesso em 15 mar. 2009.

CAPÍTULO 2

“Prevalence and counts of *Salmonella* spp. in minimally processed vegetables in Sao Paulo, Brazil”

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**Prevalence and counts of *Salmonella* spp. in minimally processed vegetables
in Sao Paulo, Brazil**

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Running title: *Salmonella* spp. in Minimally Processed Vegetables.

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Abstract

Minimally processed vegetables (MPV) may be important vehicles of *Salmonella* spp. and cause disease. This study aimed at detecting and enumerating *Salmonella* spp. in MPV marketed in the city of Sao Paulo, Brazil. A total of 512 samples of MPV packages collected in retail stores were tested for *Salmonella* spp. and total coliforms and *E. coli* as indication of the hygienic status. *Salmonella* spp. was detected in four samples, two using the detection method and two using the counting method, where the results were 8.8×10^2 CFU/g and 2.4×10^2 CFU/g. The serovars were *S. Typhimurium* (three samples) and *S. enterica* subsp. *enterica* O:47:z4,z23:- (one sample). Fourteen samples (2.7%) presented counts of *E. coli* above the maximum limit established by the Brazilian regulation for MPV (10^2 CFU/g). Therefore, tightened surveillance and effective intervention strategies are necessary in order to address consumers and governments concerns on safety of MPV.

Key-words: minimally processed vegetables, *Salmonella* spp., prevalence, enumeration, food safety.

1) Introduction

Minimally processed vegetables (MPV) are those submitted to simple operations (slicing or shredding and disinfection), aiming to preserve their freshness, nutritional quality and sensorial properties (Cruz et al., 2006). It is expected that in addition to health aspects, minimal processing addresses consumers' expectations for safe foods that are easy to use (Jacxsens et al., 2010).

The augmented demand and consumption of MPV and their increased association with foodborne diseases outbreaks intensify the concerns with the safety of these products (Olsen et al., 2000; Little et al., 2003; Little and Gillespie, 2008). Careful management of process hygiene is crucial to avoid the presence of microbial hazards since minimal processing is not an end-point preservation treatment. In this sense, the use of indicator microorganisms such as *Escherichia coli* has been used to indicate the hygiene during processing (Little and Gillespie, 2008).

Salmonella is the leading cause of foodborne diseases worldwide (Greig and Ravel, 2009). Although the majority of outbreaks in which *Salmonella* spp. is the etiological agent are linked with ingestion of contaminated foods of animal origin (Greig and Ravel, 2009; Oliveira et al., 2010), the association with vegetables has been increasing in the last few years (WHO, 2008; Little and Gillespie, 2008; Lynch et al., 2009). The pathogen is a major challenge for the microbiological safety of MPV (Olsen et al., 2000; Krtinić et al., 2010), and has been found in several types of vegetables in Brazil and other countries (Fröder et al., 2007; Abadias et al., 2008; Meldrum et al., 2009; Giusti et al., 2010). Despite the fact that the prevalence of *Salmonella* spp. in fresh vegetables may be as high as 35% (FDA, 2001) and that outbreaks due to this pathogen-food combination are quite frequent (Harris et al., 2003), to our knowledge no study reported counts of *Salmonella* spp. in MPV. Thus, this study aimed at detecting and enumerating *Salmonella* spp. in MPV available in retail stores in Sao Paulo, Brazil.

2) Material and Methods

2.1) Collection of MPV samples:

A total of 512 samples of MPV packages were purchased from retail stores of the five largest Brazilian supermarket chains in the city of São Paulo, Brazil. The samples belonged to the six most popular brands available in the market and only packages within expiration date stated in the label were collected. Each sample

belonged to a different lot of production and contained vegetables grown in Brazil. MPV containing lettuce, carrot, collard green and cabbage were prioritized since these are the most consumed vegetables in Brazil (IBGE, 2007). All vegetables had been submitted to a sanitization step during processing and thus considered ready-to-eat. The list of MPV types and number of samples are shown in *Table 1*.

2.2) Microbiological Analysis: After purchasing, MPV samples were kept under refrigeration (4-7°C) and analyzed within 24 h. Before testing, MPV packages were disinfected with 70% alcohol solution, opened under aseptic conditions and the vegetables analyzed for prevalence and enumeration of *Salmonella* spp. and counts of hygiene indicators (total coliforms and *Escherichia coli*).

Prevalence of *Salmonella* spp. was determined according to ISO 6579 (Anon, 2002). Briefly, 25 g of each sample were homogenized with 225 mL buffered peptone water (BPW) and incubated for 37±1°C for 18±2 h, followed by selective enrichment in Rappaport Vassiliadis soya peptone broth (RVS) (41,5±1°C for 24±3 h) and Muller-Kauffmann tetrathionate/novobiocin (MKTTn) broth (37±1°C for 24±3 h). Both broths were then plated on XLD- and Bismuth sulfide-agars and incubated at 37±1°C for 24±3 h. All culture media were from Merck (Darmstadt, Germany), except otherwise stated. For enumeration of *Salmonella* spp, the sample homogenate used for testing the presence of *Salmonella* spp (10⁻¹) was plated in three different plates of XLD agar (portions of 0.3, 0.3 and 0.4mL). Further decimal serial dilutions were prepared in 0.1% peptone water and aliquots of 0.1 mL were spread onto XLD agar plates, and incubated at 37±1°C for 24±3 h. Presumptive colonies in XLD and/or BS plates (five per plate in the case of enumeration) were then transferred to Nutrient Agar plates (Oxoid, Basingstoke, UK), incubated at 37±1°C for 24±3 h and then submitted to tests for fermentation of glucose, hydrolysis of urea, decarboxylation of lysine, production of α -galactosidase, production of acetoin (Voges-Proskauer test) and indol, as recommended by ISO 6579 (Anon, 2002). Colonies were also submitted to agglutination reaction using *Salmonella* polyvalent antisera (Probac, São Paulo, Brazil). Final confirmation was carried out using API20E (Biomérieux, Marcy l'Etoile, France) and PCR reaction, using primers ST 11 (AGC CAA CCA TTG CTA AAT TGG CGC A) and ST 15 (GGT AGA AAT TCC CAG CGG GTA CTG) selected from a *Salmonella*-specific fragment, JEO402-1 (Aabo et al., 1993) to amplify the 429 bp fragment specific for this microorganism. PCR tests were performed as described by Myint et al. (2006), and *Salmonella* Enteritidis ATCC 13076 and ultrapure water were

used as positive and negative controls, respectively. Results were expressed as absence or presence of *Salmonella* spp. per 25 g (prevalence) or as CFU/g (enumeration). Only colonies confirmed as *Salmonella* spp. by biochemical and genetic tests simultaneously were considered to be positive for this pathogen. Confidence intervals (95% CI) for the prevalence of *Salmonella* spp. were estimated based on cumulative binomial distribution. Serotyping of *Salmonella* spp. isolates based on O- and H-group antigens was carried out in the National Center for *Salmonella* Serotyping in *Fundação Oswaldo Cruz*, Rio de Janeiro, Brazil.

The enumeration of total coliforms and *Escherichia coli* was carried out using Petrifilm® EC (3M, Saint Paul, USA), following the manufacturers' instructions.

3) Results and Discussion

Out of 512 packages of MPV tested in this study, four were *Salmonella* spp. positive. Two (0.4%, with 95% confidence interval 0.05-1.4%) were positive in the prevalence study, and another two (0.4%) presented countable levels of this pathogen. In the prevalence study, *Salmonella* was detected in one pack of lettuce and arugula, and the serovars were *S. Typhimurium* and *S. enterica* subsp. *enterica* O:47:z4,z23:-, respectively. In the enumeration study, organic lettuce and mix of leafy vegetables (escarole and chicory) presented counts of the microorganism (8.8×10^2 CFU/g and 2.4×10^2 CFU/g, respectively) and both isolates belonged to serovar *S. Typhimurium*.

According to both Brazilian and international regulations (Brasil, 2001, Gilbert et al., 2000), the presence of *Salmonella* in ready-to-eat vegetables is unacceptable (absence/25 g) and may represent a risk for MPV consumers. Low prevalence of *Salmonella* in MPV as found in the present study has been reported by other authors in Brazil (Fröder et al., 2007), and in other countries as well (Abadias et al., 2008; Meldrum et al., 2009; Giusti et al., 2010). The prevalence of serovar *S. Typhimurium* is in agreement with data that show this serovar is one of the most common in humans or foods in Brazil (Geimba et al., 2004, Fernandes et al., 2006, Mürmann et al., 2008, Duarte et al., 2009).

In the period of this study, to the best of our knowledge neither cases of infection nor outbreaks linking consumption of MPV and *Salmonella* spp. have been reported in the State of São Paulo, Brazil. It is known that a dose in the range of 10^2 - 10^3 cells of *Salmonella* spp may cause disease in humans (Blaser and Newman

1982; Musher and Musher 2004), with higher attack rates and severity resulting from higher doses in most of cases (Mints et al., 1994, Hennessy *et al.* 1996). Although the counts of *Salmonella* found in some samples of MPV collected in this study could be enough to result in infection by this microorganism, several factors must be considered that affect the probability of infection by foodborne pathogens. One must consider that characteristics of the host, pathogen, food matrix and environment also play an important role in the probability of infection (Coleman and Marks, 1998). In addition, the heterogenic distribution of microorganisms in foods and their fate during commercialization and consumption steps will also influence the probability of occurrence of infection.

The populations of total coliforms in the tested MPV samples were above 10^4 CFU/g in 56% of the samples (*Table 2*). *E. coli* was also detected, but only 14 (2.7%) samples surpassed the limit of 10^2 CFU/g established by the Brazilian Surveillance Agency (ANVISA) for ready-to-eat vegetables (Brasil, 2001). These results indicate that the samples tested in this study presented better hygienic conditions than those reported in other studies in Brazil (Fröder et al., 2007) or in other countries (Abadias et al., 2008; Meldrum et al., 2009; Giusti et al., 2010). Interestingly, the counts of *E. coli* in the four MPV samples harboring *Salmonella* spp. were below 10^2 CFU/g, indicating that the use of this microorganism as surrogate for *Salmonella* in MPV is of little use.

The findings reported in this study show that tightened surveillance and effective intervention strategies are necessary in order to address consumers and governments concerns on safety of MPV.

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4) References

- Aabo, S., Rasmussen, O. F., Rossen, L., Sorensen, P. D., & Olsen, J. E. (1993). *Salmonella* identification by the polymerase chain reaction. *Molecular and Cellular Probes*, 7, 171-178.
- Abadias, M., Usall, J., Anguera, M., Solsona, C., & Viñas, I. (2008). Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology*, 123, 121-129.
- Anonymous (2002). ISO 6579:2002(E) - Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.. International Organization for Standardization. Switzerland.
- Blaser, M. J. & Newman, L. S. (1982). A review of human salmonellosis: I. infective dose. *Reviews of Infectious Diseases*, 4, 1096-1106.
- Brasil (2001). Ministério da Saúde, Agência Nacional de Vigilância Sanitária. Regulamento Técnico sobre Padrões Microbiológicos para Alimentos. RDC n.º 12, January 26, 2001. http://www.anvisa.gov.br/legis/resol/12_01rdc.htm. Accessed Nov 20, 2010.
- Coleman, M.E. & Marks, H.M. (1998). Qualitative and quantitative risk assessment. *Food Control*, 10, 289-297.
- Cruz, A. G., Cenci, S. A., & Maia, M. C. A. (2006). Quality assurance requirements in produce processing. *Trends in Food Science and Technology*, 17, 406-411.
- Duarte, D. A. M., Ribeiro, A. R., Vasconcelos, A. M. M., Santos, S. B., Silva, J. V. D., de Andrade, P. L. A. & Falcão, L. S. P. C. A. (2009). Occurrence of *Salmonella* spp. in broiler chicken carcasses and their susceptibility to antimicrobial agents. *Brazilian Journal of Microbiology*, 40, 569-573.
- FDA - Food and Drug Administration (2001). Center for Food Safety and Applied Nutrition, Office of Plant and Dairy Foods and Beverages. 2001 Jan 30. FDA survey of imported fresh produce. FY 1999 Field Assignment. <http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/FruitsVegetablesJuices/GuidanceComplianceRegulatoryInformation/ucm118891.htm> >. Accessed Feb 06, 2011.
- Fernandes, S. A., Tavechio, A. T., Ghilardi, Â C. R., Dias, Â M. G., De Almeida, I. A. Z. C., & De Melo, L. C. V. (2006). *Salmonella* serovars isolated from humans in São Paulo state, Brazil, 1996-2003. *Revista do Instituto De Medicina Tropical de Sao Paulo*, 48, 179-184.

Fröder, H., Martins, C. G., De Souza, K. L. O., Landgraf, M., Franco, B. D. G. M., Destro, M. T. (2007). Minimally processed vegetable salads: Microbial quality evaluation. *Journal of Food Protection*, 70, 1277-1280.

Geimba, M. P., Tondo, E. C., De Oliveira, F. A., Canal, C. W., & Brandelli, A. (2004). Serological characterization and prevalence of *spvR* genes in *Salmonella* isolated from foods involved in outbreaks in Brazil. *Journal of Food Protection*, 67, 1229-1233.

Gilbert, R. J., de Louvois, J., Donovan, T., Little, C., Nye, K., Ribeiro, C. D., Richards, J., Roberts, D., & Bolton, F.J. (2000). Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. PHLS advisory committee for food and dairy products. *Communicable Disease and Public Health / PHLS* 3, 163-167.

Giusti, M., Aurigemma, C., Marinelli, L., Tufi, D., De Medici, D., Di Pasquale, S., De Vito, C., & Boccia, A. (2010). The evaluation of the microbial safety of fresh ready-to-eat vegetables produced by different technologies in Italy. *Journal of Applied Microbiology*, 109, 996-1006.

Greig, J. D. & Ravel, A. (2009). Analysis of foodborne outbreak data reported internationally for source attribution. *International Journal of Food Microbiology*, 130, 77-87.

Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H. & Busta, F.F. (2003). Outbreaks associated with fresh produce: Incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety* 2, 78-141.

Hennessy, T. W., Hedberg, C. W., Slutsker, L., White, K. E., Besser-Wiek, J. M., Moen, M. E., Feldman, J., Coleman, W.W., Edmonson, L.M., MacDonald, K.L., & Osterholm, M.T. (1996). A national outbreak of *Salmonella* Enteritidis infections from ice cream. *New England Journal of Medicine* 334, 1281-1286.

IBGE – Instituto Brasileiro de Geografia e Estatística (2007). Sistema IBGE de Recuperação Automática – SIDRA. Banco de Dados Agregados. Disponível em: <<http://www.sidra.ibge.gov.br/bda/horti/default.asp?t=2&z=t&0=17&u1=1&u1=1&u3=1&u2=31>>. Accessed Feb 06, 2011.

Jacxsens, L., Luning, P. A., van der Vorst, J. G. A. J., Devlieghere, F., Leemans, R., & Uyttendaele, M. (2010). Simulation modelling and risk assessment as tools to

identify the impact of climate change on microbiological food safety - the case study of fresh produce supply chain. *Food Research International*, 43, 1925-1935.

Krtinić, G., Durić, P., & Ilić, S. (2010). Salmonellae in food stuffs of plant origin and their implications on human health. *European Journal of Clinical Microbiology and Infectious Diseases*, 1-5.

Little, C. L. & Gillespie, I. A. (2008). Prepared salads and public health. *Journal of Applied Microbiology*, 105, 1729-1743.

Little, C. L., Omotoye, R., & Mitchell, R. T. (2003). The microbiological quality of ready-to-eat foods with added spices. *International Journal of Environmental Health Research*, 13, 31-42.

Lynch, M. F., Tauxe, R. V., & Hedberg, C. W. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. *Epidemiology and Infection*, 137, 307-315.

Meldrum, R. J., Little, C. L., Sagoo, S., Mithani, V., McLauchlin, J., & de Pinna, E. (2009). Assessment of the microbiological safety of salad vegetables and sauces from kebab take-away restaurants in the United Kingdom. *Food Microbiology*, 26, 573-577.

Mintz, E. D., Cartter, M. L., Hadler, J. L., Wassell, J. T., Zingeser, J. A., & Tauxe, R. V. (1994). Dose-response effects in an outbreak of *Salmonella* Enteritidis. *Epidemiology and Infection*, 112, 13-23.

Mürmann, L., Dos Santos, M. C., Longaray, S. M., Both, J. M. C., & Cardoso, M. (2008). Quantification and molecular characterization of *Salmonella* isolated from food samples involved in salmonellosis outbreaks in Rio Grande do Sul, Brazil. *Brazilian Journal of Microbiology*, 39, 529-534.

Musher, D. M. & Musher, B. L. (2004). Contagious acute gastrointestinal infections. *New England Journal of Medicine*, 351, 2417-2427.

Myint, M. S., Johnson, Y. J., Tablante, N. L., & Heckert, R. A. (2006). The effect of pre-enrichment protocol on the sensitivity and specificity of PCR for detection of naturally contaminated *Salmonella* in raw poultry compared to conventional culture. *Food Microbiology*, 23, 599-604.

Oliveira, F. A., Pasqualotto, A. P., da Silva, W. P., & Tondo, E. C. (2010). Characterization of *Salmonella* Enteritidis isolated from human samples. *Food Research International*, DOI: 10.1016/j.foodres.2010.09.040

Olsen, S. J., MacKinnon, L. C., Goulding, J. S., Bean, N. H., & Slutsker, L. (2000). Surveillance for foodborne-disease outbreaks - United States, 1993-1997. *Morbidity and Mortality Weekly Report*, 49, 1-62.

Verhoeff-Bakkenes, L., Jansen, H. A. P. M., in 't Veld, P. H., Beumer, R. R., WHO – World Health Organization (2008). Drug-resistant *Salmonella*. <http://www.who.int/mediacentre/factsheets/fs139/en/>. Accessed 2010 Nov 20.

Table 1: Composition and number of minimally processed vegetables (MPV) samples analyzed in this study.

MPV	Number of Samples (%)
Salads ^a	214 (41.8)
Lettuce (<i>Lactuca sativa</i> L.)	152 (29.7)
Collard Greens (<i>Brassica oleracea</i> L.)	24 (4.7)
Arugula (<i>Eruca sativa</i> L.)	19 (3.7)
Mix for Yakisoba ^b	18 (3.5)
Watercress (<i>Nasturtium officinale</i>)	18 (3.5)
Chicory (<i>Cichorium intybus</i> L.)	16 (3.1)
Escarole (<i>Cichorium endivia</i>)	13 (2.6)
Cabbage (<i>Brassica oleracea</i> L. var. <i>Capitata</i>)	11 (2.1)
Spinach (<i>Spinacea oleracea</i> L.)	11 (2.1)
Swiss Chard (<i>Beta vulgaris</i> L. var. <i>cicla</i>)	9 (1.8)
Mix for Sukiyaki ^c	6 (1.2)
Colewort (<i>Geum urbanum</i> L.)	1 (0.2)

^aComposed by two or more of the following vegetables: lettuce (different varieties), watercress, chard, spinach, cabbage, carrot, arugula, chicory, escarole and tomatoes.

^bComposed by cauliflower, carrot, broccoli, cabbage and chard.

^cComposed by chard, cabbage, carrot, onion, green pepper, cauliflower and leek.

Table 2: Populations of Total Coliforms and *E. coli* in Minimally Processed Vegetables samples collected in São Paulo, Brazil.

Population (CFU/g)	$<10^2$	10^2-10^3	10^3-10^4	10^4-10^5	10^5-10^6	$>10^6$	Total
	Number of samples (%)						
Total Coliforms	99 (19.3)	102 (20)	80 (15.6)	103 (20.1)	119 (23,2)	9 (1.8)	512 (100)
<i>E. coli</i>	498 (97.2)	8 (1.6)	3 (0.6)	3 (0.6)	0 (0)	0 (0)	512 (100)

CAPÍTULO 3

“Prevalence, populations and pheno- and genotypic characteristics of *Listeria monocytogenes* isolated from ready-to-eat vegetables marketed in São Paulo, Brazil”

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Prevalence, populations and pheno- and genotypic characteristics of *Listeria monocytogenes* isolated from ready-to-eat vegetables marketed in São Paulo, Brazil

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Abstract

Listeria monocytogenes is a foodborne pathogen of great concern due to the high fatality rates of listeriosis. The consumption of RTE vegetables has increased in Brazil over the last two decades, but little is known about the risks associated to the consumption of these products. This study evaluated the prevalence and counts of *Listeria monocytogenes* in 512 packages of ready-to-eat vegetables marketed in Sao Paulo. The isolates were characterized for their serotypes, ribotypes, positivity for virulence genes *inlA*, *inlC* and *inlJ*, resistance to chlorine, growth rate variability and capability to form biofilm on stainless steel (AISI 304, #4) coupons. *L. monocytogenes* was detected in 3.1% of the samples. Only five samples presented countable levels, with counts between 1.0×10^1 and 2.6×10^2 CFU/g. Isolates belonged to serotypes 1/2b or 4b and most were positive for genes *inlC* and *inlJ*. Ribotypable isolates were grouped into four groups: 1038 (69.4%), 19175 (11.3%), 19191 (17.7%) and 18604 (one isolate). Most isolates survived to exposure to 125 ppm of a chlorine-based sanitizer for 3 min. All isolates were capable to attach to the coupons, reaching counts above $4 \log_{10}$ CFU/cm² and the growth rate (μ) at 25°C of the majority of the isolates varied between 0.1 and 0.2 log OD/h, but for few strains the μ was as high as 0.26 log OD/h. Results of this survey indicate that RTE vegetables may be vehicles of *L. monocytogenes* strains presenting little variability in serotype, ribotype and virulence factors but a major variability in resistance to chlorine sanitizers, capability of forming biofilm and growth rate. Data obtained is of foremost importance to serve as baseline for the development of scientific-based policies to control the incidence of *L. monocytogenes* in RTE vegetables in Brazil.

Key-words: *Listeria monocytogenes*, ready-to-eat vegetables, serotype, ribotype, biofilm, sanitizer.

1) Introduction

Listeria monocytogenes is a foodborne pathogen of great concern due to the high fatality rates of listeriosis (Vázquez-Boland et al., 2001b), and capability to survive stress conditions commonly encountered in foods such as high salt concentration, low temperature and low pH (Warriner and Namvar, 2009). Although surveillance data indicate that foodborne listeriosis outbreaks are mainly linked to poultry, beef, dairy products and ready-to-eat (RTE) foods, vegetables also have been vehicles of transmission (Behravesh et al., 2011, CDC, 2011, Todd and Notermans, 2011). Besides, the occurrence of sporadic cases of listeriosis linked to consumption of foods neither usually nor previously associated with this disease such as melons, hummus and vegetables is an additional concern for fresh produce industries and consumers (Wagner and Allerberger, 2003, Varma et al., 2007, Martins et al., 2010).

The consumption of RTE vegetables has increased in Brazil over the last two decades (Sato et al., 2007), but little is known about the risks associated to the consumption of these products. Although studies have reported that the occurrence of *L. monocytogenes* in RTE vegetables in several parts of the world may be as high as 25%, less studies either in Brazil or abroad have focused on the quantification of this microorganism in these products (Francis and O'Beirne, 2006, Crépet et al., 2007, Fröder et al., 2007, Lianou and Sofos, 2007, Little et al., 2007, Abadias et al., 2008, Cordano and Jacquet, 2009, Giusti et al., 2010, Oliveira et al., 2010). As *L. monocytogenes* is a challenge for the safety of RTE foods (Luber et al., 2011) and that RTE vegetables are consumed without a killing step before consumption, fresh produce play an important role in listeriosis epidemiology. In the context of risk assessment, the availability of data on the prevalence, counts and growth of foodborne pathogens is of foremost importance for proper building and refining of mathematical models for estimating risks (Crépet et al., 2007).

As virulence of *L. monocytogenes* is highly variable among strains (Velge and Roche, 2010), the differentiation between virulent and non-virulent strains is of great importance in evaluating the potential implications of the presence of this microorganism to food safety and public health (Jensen et al., 2008, Roche et al., 2009, Roberts et al., 2009). Conventional serotyping, useful in epidemiological studies due to the relationship between serotypes and foodborne listeriosis (Rocourt

and Bille, 1997, Thévenot et al., 2006, Ferreira et al., 2011), presents poor discriminatory power in comparison to DNA-based methods when information on the spreading and relatedness of strains are needed (Wiedmann, 2002). Automated ribotyping has been used in clonality surveys of *L. monocytogenes* isolated from foods and processing environment (Manfreda et al., 2005, Meloni et al., 2009, Williams et al., 2011), and for unveiling the relationship between a ribotype and likely threats to food safety (Wiedmann et al., 1997, Kathariou, 2002). The outputs of subtyping may be strengthened if virulence factors such as internalins, among others, with recognized role in pathogenesis of *L. monocytogenes* are sought (Vázquez-Boland et al., 2001a,b).

Studies have shown that the responses of *L. monocytogenes* to stressors in foods and processing environment are heterogeneous (Arguedas-Villa et al., 2010, Augustin et al., 2011, Lianou and Koutsoumanis, 2011). Sanitization of equipment and vegetables and chilling temperature are two relevant stressors during minimal processing of RTE vegetables, and responses of *L. monocytogenes* to these conditions may result either in inactivation, survival, and persistence in the processing environment or in growth in the product during cold storage. Then, knowledge on the behavior of *L. monocytogenes* is of great relevance for estimating the risks associated to RTE foods (Pal et al., 2008, Dupont and Augustin, 2009, Jacxsens et al., 2010).

This survey aimed at gathering data for risk assessments of *L. monocytogenes* in RTE vegetables in Brazil. The study was conducted with samples collected in the market in Sao Paulo, Brazil, containing at least one of the most consumed vegetables in the country (lettuce, carrot, collard green and cabbage) (IBGE, 2007). The samples were tested for prevalence and populations of *L. monocytogenes* and isolates were studied for their serotypes, ribotypes, presence of virulence genes, variability in growth rate, ability to form biofilm on stainless steel coupons and susceptibility to a chlorine-based product recommended for vegetables sanitization.

2) Material and Methods

2.1) Collection of samples:

A total of 512 packages of bagged RTE vegetables were purchased in shops of the five largest supermarket chains in the city of São Paulo, Brazil, between April and August 2008. Representative sampling was ensured by taking samples pertaining to the most consumed brands, coming from the main production sites in the neighborhood of the city and containing at least one of the most consumed vegetables in Brazil (lettuce, carrot, collard green and cabbage) (IBGE, 2007). The samples were minimally processed, *i.e.* they have been washed, shredded or sliced, disinfected with chlorinated or ozonized water and packed in plastic bags before commercialized. To avoid possible overgrowth of indigenous *L. monocytogenes*, only samples presenting less than two days of storage (2-8°C) were included in the study.

The RTE vegetables samples were of thirteen types: *a.* salad mix, containing at least two of the following components: lettuce (different varieties), watercress, chard, spinach, cabbage, carrot, arugula, chicory, escarole and tomatoes (n=214), *b.* lettuce (*Lactuca sativa* L.) (n=152), *c.* collard green (*Brassica oleracea* L.) (n=24), *d.* arugula (*Eruca sativa* L.) (n=19), *e.* mix for yakisoba, containing cauliflower, carrot, broccoli, cabbage and chard (n=18), *f.* watercress (*Nasturtium officinale*) (n=18), *g.* chicory (*Cichorium intybus* L.) (n=16), *h.* escarole (*Cichorium endivia*) (n=13), *i.* cabbage (*Brassica oleracea* L. var. *Capitata*) (n=11), *j.* spinach (*Tetragonia tetragonioides*) (n=11), *k.* Swiss chard (*Beta vulgaris* L. var. *cicla*) (n=9), *l.* mix for sukiyaki, containing chard, cabbage, carrot, onion, green pepper, cauliflower and leek (n=6) and *m.* colewort (*Geum urbanum* L.) (n=1). After purchase, samples were transported to the laboratory in isothermal boxes, maintained under refrigeration (<4°C) and analyzed within 24h.

2.2) Prevalence and enumeration of *L. monocytogenes*:

Before opening, packages of RTE vegetables were submitted to external disinfection with 70% alcohol solution. Samples were concomitantly submitted to ISO 11290-1 (Anon, 1996) and ISO 11290-2 (Anon, 1998) methods for prevalence and enumeration of *L. monocytogenes*, respectively. In the prevalence study, 25g of the sample were stomached with 225mL of Half-Fraser Broth (HF) without selective supplement and after 1h at 20±2°C, selective supplement (SR0166) was added and

the mixture was incubated at 30°C to complete 24h. An aliquot of 0.1 mL was transferred to tubes containing Fraser Broth (FB), and incubated at 37±1°C/48±3h. A loopful of the culture in FB was streaked onto Palcam (PC) and Oxford (OXA) agar plates, incubated at 37±1°C/24±3h and examined for the presence of presumptive *Listeria* colonies (dimpled brown/black colonies with black halo and brown colonies with aesculin hydrolysis halo, respectively). For enumeration, stomached samples in HF were serially diluted in 0.1% sterile peptone water, plated onto PC agar plates, incubated at 37±1°C/48±3h and examined for presence of presumptive *Listeria* colonies. Agar *Listeria* according to Ottaviani & Agosti (ALOA), as recommended in the amendment of ISO 11290-1 method, was not used in this study as PC agar presents similar performance (Wilis et al., 2006) and much lower cost. Five presumptive colonies recovered from each method (prevalence and enumeration) were randomly selected and streaked onto Tryptone soy agar supplemented with 0.6% of yeast extract (TSA-YE) and incubated at 37±1°C/24±3h. Identification of *L. monocytogenes* was done by biochemical tests and by a PCR for amplification of the listeriolysin (*hlyA*) gene, using primers A (CAT TAG TGG AAA GAT GGA ATG) and B (GTA TCC TCC AGA GTG ATC GA) (Blais et al., 2008). Results were expressed as presence or absence of *L. monocytogenes* per 25g (prevalence) or CFU/g (enumeration). Results of enumeration were based on the ratio between the total number of colonies in the PC agar plates and those confirmed as *L. monocytogenes*. All culture media and selective supplements were from Oxoid (Basingstoke, UK). Confidence intervals (95% CI) for the prevalence of *L. monocytogenes* in each group of vegetables, brands and overall prevalence were estimated based on cumulative binomial distribution.

2.3) Serotyping:

Serotyping of *L. monocytogenes* isolates was performed at the Instituto Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, using polyclonal somatic and flagellar antisera as described by Seeliger and Höhne (1979).

2.4) Multiplex PCR for virulence genes:

The presence of internalin virulence genes in *L. monocytogenes* isolates was investigated using a multiplex PCR and primers *inlA* (F: ACG AGT TAA CGG GAC AAA TGC; R: CCC GAC AGT GGT GCT AGA TT), *inlC* (F: AAT TCC CAC AGG ACA

CAA CC; R: CGG GAA TGC AAT TTT TCA CTA) and *inlJ* (F: TGT AAC CCC GCT ATC ACA GTT; R: AGC GGC TTG GCA GTC TAA TA) (Liu et al., 2007). *L. monocytogenes* ATCC 7644 and ultrapure water were used as positive and negative controls, respectively.

2.5) Ribotyping:

Ribotyping was performed using the RiboPrinter® microbial characterization system (DuPont Qualicon™ Inc., Wilmington, USA), and *EcoRI* restriction enzyme. The generated ribotype patterns were compared to those available in the equipment databank. An isolate was considered ribotyped when its ribotype pattern matched one of the patterns of the identification library of RiboPrinter® (DUP-IDs) with a similarity ≥ 0.84 . Isolates were grouped into ribogroups after combination of profiles within a similarity range, as determined using RiboPrinter's proprietary algorithm (≥ 0.93) (Bruce, 1996). Patterns were analyzed with Bionumerics version 6.0 software (Applied Maths, Sint-Martens-Latem, Belgium) and a dendrogram was generated based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and band based-Dice coefficient analysis (1% tolerance and optimization). Evolutionary lineage was assigned to strains based on their ribotype patterns according to Wiedmann et al. (1997). Simpson's index of discrimination (SID) was determined according to Hunter and Gaston (1988).

2.6) Susceptibility to a chlorine-based sanitizer:

The resistance of *L. monocytogenes* isolates to Sumaveg™ (JohnsonDiversey, Sao Paulo, Brazil), a sodium dichloroisocyanurate-based sanitizer frequently used for vegetable disinfection in Brazil, was tested using 96-well microtiter plates (model Y92096, MIDSCI, Saint Louis, MO, USA). Each isolate was cultivated in Tryptic Soy Broth supplemented with 6% Yeast extract (TSB-YE broth) at 37°C/24h and aliquots of 1 mL were centrifuged at 4°C for 10 min at 2810×g (Centrifuge 5417 C, Eppendorf AG, Hamburg, Germany). Pellets were washed twice with sterile distilled water and the optical density (OD_{630nm}) of the final suspension adjusted to 0.5 with distilled water, using an Ultrospec 200 UV/visible spectrophotometer (Pharmacia Biotech, Buckinghamshire, UK). The CFU/mL in the final suspension was determined by plating on TSA-YE 6% agar plates, and incubation at 37°C/24h. The sanitizer solutions were freshly prepared to achieve

concentrations of 0, 50, 100, 125, 150, 175 and 200 ppm of free chlorine and used within 30 min at room temperature (21°C) and protected from light. The concentrations of the sanitizer corresponded to those commonly used by vegetable processors in Brazil and were in accordance to the maximum chlorine concentration tolerated by the Brazilian legislation for vegetable sanitization, i.e., 250 ppm (Anon, 1999). Chlorine levels were measured using pHydrion test papers (Micro Chlorine, Johnson Wax Professional, Sturtevant, USA). For resistance testing, aliquots of 500 µL of the washed cultures were added to the wells of the microtiter plates containing the sanitizer solutions and homogenized. After 3 min of exposure, 100 µL of the homogenates were pour-plated onto Müller-Hinton Agar (MHA), in duplicates, and incubated at 37°C/24h. The experiments were repeated twice. No growth on MHA was interpreted as sensitivity to the tested concentration of the sanitizer. Growth of one single colony on MHA was interpreted as resistance to the tested concentration of the sanitizer. The detection limit of the method was 10² CFU/mL.

2.7) Biofilm formation in stainless steel coupons:

Biofilm formation was evaluated using AISI 304 #4 stainless steel coupons (0.366 µm roughness, 10 mm × 10 mm × 1 mm) under static conditions as previously described (Parizzi and Andrade, 2004, Careli et al., 2009). Before use, coupons were washed with neutral detergent, rinsed with distilled water, immersed in 70% ethanol for 1 h at room temperature, rinsed with distilled water again and sterilized at 121°C/15 min. For testing, 100 µL of the cultures of *L. monocytogenes*, obtained in TSB-YE broth at 37°C/24 h, were transferred to wells of 12-well microtiter plates (model Y92012, MIDSCI, Saint Louis, MO, USA) containing 3 mL of Luria-Bertani broth (LB) and, after gentle homogenization, the coupons were added to the wells. The microtiter plates were closed, sealed with plastic film and incubated at 37°C/24 h. The coupons were removed and transferred to 10 mL phosphate buffer salt (PBS) solution for 1 min for removal of planktonic cells. Sessile cells were enumerated by vortexing the coupons for 2 min in tubes containing 5 mL of PBS, followed by serial dilution, inoculation onto OXA agar plates and incubation at 37°C/48 h. The experiments were repeated twice and the results were expressed as CFU/cm².

2.8) Determination of growth rate of *L. monocytogenes* isolates:

Working with 96-well microtiter plates, one hundred μL of the cultures of *L. monocytogenes*, obtained in TSB-YE broth at $37^{\circ}\text{C}/24\text{ h}$, were added to duplicate wells containing 900 μL of TSB-YE broth. Microtiter plates were incubated at 25°C in a microplate incubator equipped with photometer (Multiskan FC Type 357, Thermo Fischer Scientific Instruments Co. Ltd., Shanghai, China). The optical density (OD) was measured at 620 nm every hour up to 20h. The experiment was replicated twice, with each experiment being performed with two wells for each isolate. The growth rate (μ) of each isolate was determined by linear regression of log of OD versus time of incubation (Krist et al., 1998). The best statistical distribution fitting the growth rate of *L. monocytogenes* strains was determined using the software @Risk version 5.7 (Palisade Corp., Ithaca, USA).

2.9) Statistical analysis:

One-factor analysis of variance (ANOVA) followed by Tukey's test were used to check significant differences ($p \leq 0.05$) regarding the ability of isolates to form biofilms. Statistical analyses were performed using Assistat version 7.5 free software (Campina Grande, Brazil) (Silva et al., 2002).

3) Results

The overall prevalence of *L. monocytogenes* in the 512 RTE vegetable samples was 3.1% (16/512) (Table 1). The positivity for salad mixes and lettuces, corresponding to the most consumed types of RTE vegetables in Brazil, was 0.9% and 2.0%, respectively. If only these two types of RTE vegetables had been included in the study, the prevalence of *L. monocytogenes* would be lower, i.e. 1.4% (5/366). Combining the type of vegetable and the number of samples of each type, mix for sukiyaki, escarole and mix of cabbages presented the highest positivity, i.e., 33.3%, 23.1% and 18.2%, respectively. Among 14 brands, five contained positive samples for *L. monocytogenes* (Table 2). Samples from Brand J presented the highest prevalence (60%), followed by those from brands A and B (4.8% and 6.2%, respectively).

Counting of *L. monocytogenes* was possible in only five samples (Table 3). Populations were low and varied between 1.0×10^1 and 2.6×10^2 CFU/g. The five samples belonged to five different brands and all contained more than one type of vegetable. Three of these samples (mix for yakisoba, mix for sukiyaki and mix of cabbages) were also positive for *L. monocytogenes* when submitted to the detection method.

Table 4 depicts the serotypes and ribotypes profiles of *L. monocytogenes* isolates according to the source and brands of the RTE vegetables. The great majority of isolates belonged to serotype 4b. Those belonging to serotype 1/2b were obtained from one single sample of mix for yakisoba. Most 4b isolates were recovered from RTE vegetables from brands A, B and J.

Seven isolates out of 69 recovered from RTE vegetables could not be ribotyped as their patterns did not match any pattern in the identification library of RiboPrinter® (DUP-IDs) with similarity ≥ 0.84 . However, *EcoRI* ribotyping confirmed that all tested isolates were *L. monocytogenes*. As shown in Table 4, most ribotyped isolates were grouped into DUP-ID 1038 (69.4%). Other detected DUP-IDs were 19191 (17.7%) and 19175 (11.3%). Only one isolate, obtained from a sample of mix of lettuces, belonged DUP-ID 18604.

The genetic relationships among *L. monocytogenes* isolates, determined by ribotyping are shown in Figure 1. Based on a similarity criterion of 83%, 27 different genetic profiles were obtained and isolates were grouped into three major clusters (A, B, and C). The largest cluster (B) comprehended 49 isolates, obtained from a variety of vegetables from brands A, B, E, F and J. Cluster A grouped six isolates recovered from samples of collard green, salad mix and cabbage from the same brand B (Table 4). All isolates in clusters A and B were serotype 4b, and the great majority belonged to DUP-ID 1038 (Table 4). In counterpart, cluster C grouped seven isolates, all belonging to serotype 1/2b and DUP-ID 19175 and originated from the same sample of mix for yakisoba, from brand G (Table 4).

The multiplex-PCR targeting internalin genes (*inlA*, *inlC* and *inlJ*) of *L. monocytogenes* recovered from the RTE vegetable samples confirmed the virulence potential of the isolates. Regarding the chlorine resistance, the findings of this study indicate a variable resistance even among clones of *L. monocytogenes*. All tested isolates (n=62) were resistant to exposure to the chlorine-based sanitizer for three minutes when the concentration was 50 ppm or 100 ppm. Twenty (32.2%), 25

(40.3%) and 9 (14.5%) isolates were inactivated by 125, 150 or 175 ppm of the sanitizer, respectively, but seven isolates (11.3%) were inactivated only when the concentration of the sanitizer was as high as 200 ppm. However, one isolate (#581) was resistant even to 200ppm of chlorine. Isolates belonging to ribotype cluster A presented similar resistance to the sanitizer, being inactivated with 125-150 ppm of chlorine. On the other hand, isolates in cluster B were variable: some were inactivated with 125 ppm of chlorine while others were resistant even to 200 ppm of chlorine. The isolates in cluster C tended to be more resistant than the other isolates, as they required at least 175 ppm of free chlorine for inactivation.

All *L. monocytogenes* isolates were capable to attach to stainless steel coupons, most of them achieving populations above $4 \log_{10}$ CFU/cm² (Figure 2). Populations above $6 \log_{10}$ CFU/cm² adhered to the coupons were found for 69.4% of the isolates. Isolates belonging to serotype 1/2b (ID numbers 2 to 8) presented a significantly higher average population of adhered cells when compared to isolates belonging to serotype 4b ($p < 0.05$).

The growth rates (μ) of the *L. monocytogenes* isolates in TSB-YE broth at 25°C are shown in Figure 3. Most isolates presented μ between 0.1 and 0.2 log OD/h, and only for few isolates μ was above 0.26 log OD/h. Besides the expected inter-strain variability, intra-strain variability was also observed. For instance, undistinguishable isolates, as determined by *EcoRI* ribotyping, such as isolates 2, 3, 4, 6, 7 and 8, presented different μ values, varying from approximately 0.10 to 0.21 log OD/h. The probability density for the growth rate for the *L. monocytogenes* isolates can be seen in Figure 4. A log-logistic distribution was the best ranked for fitting the data according to Anderson-Darling statistic test ($p > 0.05$). The generated distribution was skewed to the right and presented a mean of 0.179 and standard deviation of 0.0373, with 5% and 95% percentiles of 0.118 and 0.265, respectively.

4) Discussion

The detected prevalence of *L. monocytogenes* in the samples of RTE vegetables analyzed in this study (Table 1) is in the range reported in similar products in other locations (Berrada et al., 2006, Francis and O'Beirne, 2006, Lianou and Sofos, 2007, Little et al., 2007, Ponniah et al., 2010). The highest prevalence of *L. monocytogenes*

detected in mix for sukiyaki, escarole and cabbages should be interpreted with care since the number of samples was low, so that a larger result uncertainty is expected (Table 1). However, it should be taken into account that the sampling represented both qualitatively and quantitatively the different types of RTE vegetables available for purchase in supermarkets in Sao Paulo, highlighting their relevance as vehicles of *L. monocytogenes*. Another important output of the results is the relationship between prevalence of *L. monocytogenes* and amount of RTE vegetables consumed by the population. Despite the low occurrence of *L. monocytogenes* in lettuce and lettuce-based salads (Table 1), these are the most consumed RTE vegetables in Brazil and are always consumed raw (Sato et al., 2007). In counterpart, mix for sukiyaki and cabbage are sold as RTE vegetables but are frequently consumed cooked, probably reducing the consumer exposure to *L. monocytogenes*. Consequently, more consumers may be exposed to *L. monocytogenes* due to lettuce and lettuce-based salads than to mix for yakisoba and cabbages.

The five samples of RTE vegetables presenting countable levels of *L. monocytogenes* contained three or more components (Table 3), suggesting that this type of RTE vegetables are more prone to be contaminated with *L. monocytogenes*. An explanation for this finding could be that mixed vegetables are subjected to more manipulations during processing, thus more opportunity for introduction or dispersion of *L. monocytogenes*. Differentiated resistance to the sanitizing process of vegetables with different surface roughness may be an additional reason. Despite the inexistence of microbiological limits for *L. monocytogenes* in RTE vegetables in Brazil, the recovery of this microorganism at countable levels (10^1 - 10^2 CFU/g) is of concern because these vegetables can be used for preparation of other meals, resulting in cross-contamination of the end product. Furthermore, RTE vegetables may be added of other ingredients facilitating the growth of *L. monocytogenes* (Vermeulen et al., 2007, Uyttendaele et al., 2009). Thus, the infective dose for humans, which varies between 10^6 and 10^9 CFU depending on the strain and host (Takeuchi et al., 2006, Williams et al., 2007), can be reached in the food after a couple of hours at abuse temperature ($>8^\circ\text{C}$) (Skalin a and Nikolajeva, 2010).

The failure of isolation of *L. monocytogenes* in two out of five samples in which the enumeration was possible may be due to the intense growth of the background microbiota in the enrichment broth (even with selective agents added) or inhibition by selective agents in the enrichment broth. These conditions might have hampered the

growth of injured or stressed *L. monocytogenes* cells in the detection method, while in the enumeration method the inoculation was performed directly from the diluent broth (Half-Fraser, without selective agents) and plated onto PC or OXA plates. The higher sensitivity of sub-lethal cells to selective compounds found in culture media and its effects on the recovery of foodborne pathogens has been reported in the literature supporting this hypothesis (Jasson et al., 2007).

Although the reasons for the high prevalence of serotype 4b in human illness when compared to the other twelve serotypes of *L. monocytogenes* are still unclear, the prevalence of this serotype in RTE foods is of concern from a food safety perspective (Kathariou, 2002, Dumas et al., 2009). The findings of this study and recent data published in the literature (Martins and Germano, 2011, Barancelli et al., 2011) seem to indicate a trend of high prevalence of serotypes mostly associated with listeriosis in foods in state of São Paulo, Brazil (*Table 4*).

Previous studies in Brazil have characterized *L. monocytogenes* isolated from foods, environment or human cases and none have mentioned the occurrence of this ribotype (Chiarini et al., 2009, Bueno et al., 2010). The high prevalence of ribotype DUP-ID 1038 among *L. monocytogenes* isolated from the samples of RTE vegetables (*Table 4*) is another concern as this ribotype has been associated with several cases and outbreaks of listeriosis worldwide (Schlech et al., 1983, Linnan et al., 1988).

All isolates were positive for *inlA* gene, responsible for a species-specific surface protein with an important role in entrance of *L. monocytogenes* into host cells (Vázquez-Boland et al., 2001a, Orsi et al., 2007) and 97% were positive for *inlC* and *inlJ* genes, known to contribute in the late stage of infection (Engelbrecht et al., 1996) and passage through the intestinal barrier and several stages of infection (Sabet et al., 2005), respectively. Although results for presence of virulence genes showed most isolates were positive for *inlC* and *inlJ* genes, several studies indicated that strains displaying these genes were able to cause mortality in A/J mice via the intraperitoneal route (Liu et al., 2003, Sabet et al., 2005, Liu et al., 2007), but not necessarily in humans when ingested through foods (Vázquez-Boland et al., 2001b, Kathariou, 2002, Liu et al., 2007). Despite presenting several virulence genes playing a role in the pathogenicity of *L. monocytogenes* (Kathariou, 2002), the detection of *inlC* and *inlJ* genes is a quick approach to discriminate potential virulent strains of *L. monocytogenes* from avirulent ones (Liu et al., 2007).

The resistance of the *L. monocytogenes* isolates to exposure to the tested chlorine-based sanitizer for three minutes at concentration above 100 ppm is of great concern, as processors may use inadequate chlorine concentration, increasing costs without achieving the intended disinfection of their products. In addition, the resistant isolates are more likely to have a competitive advantage towards less resistant ones, prevailing both in the processing environment and in the final product (Beuchat et al., 2004). The persistence of *L. monocytogenes* in foods and food processing environment has been widely documented (Lomonaco et al., 2009, Chen et al., 2010, Ortiz et al., 2010) owing to their resistance to sanitizers, ability to form biofilms or competitive advantages when compared to other microorganisms (Aarnisalo et al., 2007, Harvey et al., 2007, Vermeulen et al., 2007). The results of these tests cannot direct infer on the behavior of the isolates under practical conditions (washing step), because factors such as the presence of organic material in the washing water were not considered. However, the findings obtained can be used for selection of the most resistant isolates that can be further used in validation studies of washing and disinfection procedures applied by RTE vegetables industries.

Although several factors are known to affect the formation of biofilms, the results obtained in this study (*Figure 2*) indicate that failures in sanitization may result in the attachment of *L. monocytogenes* to the stainless steel surfaces of equipment used for vegetables processing, persisting in these surfaces and compromising the safety of the end products (Van Houdt and Michiels, 2010). Persistence of *L. monocytogenes* in processing plants (Carpentier and Cerf, 2011) and the association of persistent strains with listeriosis outbreaks has been well documented (Anon, 2000). The results of ribotyping corroborate the persistence of *L. monocytogenes* in the processing environment of the tested RTE vegetables as clones were present in different types of vegetables and brands (*Table 4, Figure 1*). These data, in addition to findings on resistance to chlorine, reinforce the hypothesis that most resistant *L. monocytogenes* isolates can potentially attach to the surface of equipment, form biofilms and persist in the processing environment. Therefore, sanitization programs should be carefully validated and periodically evaluated in order to guarantee the inactivation of *L. monocytogenes*. Although the resistance of isolates to other sanitizers used in food processing has not been tested, the alternation of sanitizer with different active compounds could be an appropriate approach to ensure the inactivation of this microorganism. It should be highlighted that this procedure can

only be applied for sanitization of equipment and processing utilities as in Brazil only chlorine and ozone are allowed for washing/disinfection of RTE vegetables.

The growth rate is one of the most important kinetics parameters describing microbial behavior in foods (Pouillot and Lubran, 2011). Growth curves, based on the determination of OD of cultures grown in microtiter plates, allowed the estimation of growth rate for the whole set of *L. monocytogenes* isolates obtained from the RTE vegetables samples (Figure 3). This method has been successfully used in several studies on the determination of variability of growth kinetics of microorganisms (Krist et al., 1998, Lianou and Koutsoumanis, 2011). In addition, the determination of growth rate of pathogens in culture media has been extensively used for generate data used in tertiary models such as Pathogen Modeling Program (PMP) (<http://pmp.arserrc.gov/PMPOnline>). The lowest average growth rate value (0.1026 log OD/h) obtained for the *L. monocytogenes* isolates was in agreement with the estimation given by PMP (data not shown). Some isolates were characterized as fast growers (IDs 28, 35 and 59) and high inter-strain variability was observed for some isolates (IDs 18, 37 and 51) (Figure 3). The implication of these results is that while fast growers can prevail, those presenting great variability may lead to high or low counts depending on the environmental factors and their interactions. Taking into account that most risks result from the extremities of the statistical distributions, few RTE vegetable packages may contain high counts of the pathogen. Therefore, this information is of major importance when analyzing the potential implications of these findings for safety of these products.

In conclusion, results of this survey indicate that RTE vegetables may be vehicles of *L. monocytogenes* strains presenting little variability in serotype, ribotype and virulence factors but a major variability in resistance to chlorine sanitizers, capability of forming biofilm and growth rate. Variability among isolates regarding their responses to the environmental conditions is of great relevance for modeling the risks related to consumption of RTE vegetables. Data obtained is of foremost importance to serve as baseline for the development of scientific-based policies to control the incidence of *L. monocytogenes* in RTE vegetables and the risks it poses to the safety of these products in Brazil.

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5) References

- Aarnisalo, K., Lundén, J., Korkeala, H., Wirtanen, G., 2007. Susceptibility of *Listeria monocytogenes* strains to disinfectants and chlorinated alkaline cleaners at cold temperatures. *LWT - Food Science and Technology* 40, 1041-1048.
- Abadias, M., Usall, J., Anguera, M., Solsona, C., Viñas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology* 123, 121-129.
- Anonymous. 1996. ISO 11290-1:1996 - Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 1: Detection method. Switzerland.
- Anonymous. 1998. ISO 11290-2:1998 - Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 2: Enumeration method. Switzerland.
- Anonymous, 2000. Multistate outbreak of listeriosis--United States, 2000. *MMWR - Morbidity and Mortality Weekly Report* 49, 1129-1130.
- Anonymous. 2001. Ministério da Saúde. Agência Nacional de Vigilância Sanitária – Anvisa. Resolução RDC-12/01, de 2 de Janeiro de 2001. *Diário Oficial da República Federativa do Brasil*, Brasília, DF, 10 jan. 2001. Seção 1, p. 45.
- Arguedas-Villa, C., Stephan, R., Tasara, T., 2010. Evaluation of cold growth and related gene transcription responses associated with *Listeria monocytogenes* strains of different origins. *Food Microbiology* 27, 653-660.

- Augustin, J.-C., Bergis, H., Midelet-Bourdin, G., Cornu, M., Couvert, O., Denis, C., Huchet, V., Lemonnier, S., Pinon, A., Vialette, M., Zuliani, V., Stahl, V., 2011. Design of challenge testing experiments to assess the variability of *Listeria monocytogenes* growth in foods. *Food Microbiology* 28, 746-754.
- Barancelli, G. V., Camargo, T. M., Reis, C. M. F., Porto, E., Hofer, E., & Oliveira, C. A. F., 2011. Incidence of *Listeria monocytogenes* in cheese manufacturing plants from the northeast region of São Paulo, Brazil. *Journal of Food Protection* 74, 816-819.
- Behravesh, C.B., Jones, T.F., Vugia, D.J., Long, C., Marcus, R., Smith, K., Thomas, S., Zansky, S., Fullerton, K.E., Henao, O.L., Scallan, E., FoodNet Working Group, 2011. Deaths associated with bacterial pathogens transmitted commonly through food: Foodborne diseases active surveillance network (FoodNet), 1996-2005. *Journal of Infectious Diseases*, 204, 263-267.
- Berrada, H., Soriano, J. M., Picó, Y., Mañes, J., 2006. Quantification of *Listeria monocytogenes* in salads by real time quantitative PCR. *International Journal of Food Microbiology* 107, 202-206.
- Beuchat, L. R., Adler, B. B., Lang, M. M., 2004. Efficacy of chlorine and a peroxyacetic acid sanitizer in killing *Listeria monocytogenes* on iceberg and romaine lettuce using simulated commercial processing conditions. *Journal of Food Protection* 67, 1238-1242.
- Blais, B. W., Phillippe, L. M., Pagotto, F., & Corneau, N. (2008). Identification of presumptive positive *Listeria monocytogenes* from foods and environmental samples by the Polymerase Chain Reaction. *The compendium of analytical methods* (Vol. 3, MFLP-78). www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp78-02-eng.php.
- Brito, J. R. F., Santos, E. M. P., Arcuri, E. F., Lange, C. C., Brito, M. A. V. P., Souza, G. N., Cerqueira, M.M., Beltran, J.M., Call, J.E., Liu, Y., Porto-Fett, A.C., Luchansky, J.B., 2008. Retail survey of Brazilian milk and Minas Frescal cheese and a contaminated dairy plant to establish prevalence, relatedness, and sources of *Listeria monocytogenes* isolates. *Applied and Environmental Microbiology* 74, 4954-4961.
- Bruce, J., 1996. Automated system rapidly identifies and characterizes microorganisms in foods. *Food Technology* 50, 77-78.
- Bueno, V. F., Banerjee, P., Banada, P. P., José De Mesquita, A., Lemes-Marques, E. G., Bhunia, A. K., 2010. Characterization of *Listeria monocytogenes* isolates of food

and human origins from Brazil using molecular typing procedures and in vitro cell culture assays. *International Journal of Environmental Health Research* 20, 43-59.

Careli, R. T., Andrade, N. J., Soares, N. F., Júnior, J. I. R., Rosado, M. S., Bernardes, P. C., 2009. The adherence of *Pseudomonas fluorescens* to marble, granite, synthetic polymers, and stainless steel. *Ciencia e Tecnologia de Alimentos* 29, 171-176.

Carpentier, B., Cerf, O., 2011. Review - persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiology* 145, 1-8.

Centers for Disease Control and Prevention (CDC). Investigation Update: Multistate Outbreak of Listeriosis Linked to Whole Cantaloupes from Jensen Farms, Colorado. Available at: <http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/110211/index.html> Accessed Nov. 07, 2011.

Chen, B.-Y., Pyla, R., Kim, T.-J., Silva, J. L., Jung, Y.-S., 2010. Incidence and persistence of *Listeria monocytogenes* in the catfish processing environment and fresh fillets. *Journal of Food Protection* 73, 1641-1650.

Chiarini, E., Tyler, K., Farber, J. M., Pagotto, F., Destro, M. T., 2009. *Listeria monocytogenes* in two different poultry facilities: Manual and automatic evisceration. *Poultry Science* 88, 791-797.

Cordano, A. M., Jacquet, C., 2009. *Listeria monocytogenes* isolated from vegetable salads sold at supermarkets in Santiago, Chile: Prevalence and strain characterization. *International Journal of Food Microbiology* 132, 176-179.

Crépet, A., Albert, I., Dervin, C., Carlin, F., 2007. Estimation of microbial contamination of food from prevalence and concentration data: Application to *Listeria monocytogenes* in fresh vegetables. *Applied and Environmental Microbiology* 73, 250-258.

Cruz, A. G., Cenci, S. A., Maia, M. C. A., 2006. Quality assurance requirements in produce processing. *Trends in Food Science and Technology* 17, 406-411.

Dumas, E., Meunier, B., Berdagué, J. -L., Chambon, C., Desvaux, M., Hébraud, M., 2009. The origin of *Listeria monocytogenes* 4b isolates is signified by subproteomic profiling. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1794, 1530-1536.

Dupont, C., Augustin, J.-C., 2009. Influence of stress on single-cell lag time and growth probability for *Listeria monocytogenes* in half Fraser broth. *Applied and Environmental Microbiology* 75, 3069-3076.

- Engelbrecht, F., Chun, S.-., Ochs, C., Hess, J., Lottspeich, F., Goebel, W., Sokolovic, Z., 1996. A new *PrfA*-regulated gene of *Listeria monocytogenes* encoding a small, secreted protein which belongs to the family of internalins. *Molecular Microbiology* 21, 823-837.
- Ferreira, V., Barbosa, J., Stasiewicz, M., Vongkamjan, K., Switt, A. M., Hogg, T., Gibbs, P., Teixeira, P., Wiedmann, M., 2011. Diverse geno- and phenotypes of persistent *Listeria monocytogenes* isolates from fermented meat sausage production facilities in Portugal. *Applied and Environmental Microbiology* 77, 2701-2715.
- Francis, G. A., O'Beirne, D., 2006. Isolation and pulsed-field gel electrophoresis typing of *Listeria monocytogenes* from modified atmosphere packaged fresh-cut vegetables collected in Ireland. *Journal of Food Protection* 69, 2524-2528.
- Fröder, R., Martins, C. G., Sousa, K. L. O., Landgraf, M., Franco, B. D. G. M., Destro, M. T., 2007. Minimally processed vegetable salads: microbial quality evaluation. *Journal of Food Protection* 70, 1277-1280.
- Gil, M. I., Selma, M. V., López-Gálvez, F., Allende, A., 2009. Fresh-cut product sanitation and wash water disinfection: Problems and solutions. *International Journal of Food Microbiology* 134, 37-45.
- Giusti, M., Aurigemma, C., Marinelli, L., Tufi, D., De Medici, D., Di Pasquale, S., De Vito, C., Boccia, A., 2010. The evaluation of the microbial safety of fresh ready-to-eat vegetables produced by different technologies in Italy. *Journal of Applied Microbiology*, 109, 996-1006.
- Harvey, J., Keenan, K. P., Gilmour, A., 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. *Food Microbiology* 24, 380-392.
- Hofer, E., Falavina Dos Reis, C. M., Hofer, C. B., 2006. Serovars of *Listeria monocytogenes* and related species isolated from human clinical specimens. *Revista da Sociedade Brasileira de Medicina Tropical* 39, 32-37.
- Hofer, E., Ribeiro, R., Feitosa, D. P., 2000. Species and serovars of the genus *Listeria* isolated from different sources in Brazil from 1971 to 1997. *Memorias do Instituto Oswaldo Cruz* 95, 615-620.
- Hunter, P. R., Gaston, M. A., 1988. Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. *Journal of Clinical Microbiology* 26, 2465-2466.
- IBGE (Instituto Brasileiro de Geografia e Estatística), 2011. Sistema IBGE de Recuperação Automática – SIDRA. Banco de Dados Agregados. Available in:

<http://www.sidra.ibge.gov.br/bda/horti/default.asp?t=2&z=t&0=17&u1=1&u1=1&u3=1&u2=31>. Accessed in 14 Oct. 2011.

Jacxsens, L., Luning, P. A., van der Vorst, J. G. A. J., Devlieghere, F., Leemans, R., Uyttendaele, M., 2010. Simulation modelling and risk assessment as tools to identify the impact of climate change on microbiological food safety - the case study of fresh produce supply chain. *Food Research International* 43, 1925-1935.

Jasson, V., Uyttendaele, M., Rajkovic, A., Debevere, J., 2007. Establishment of procedures provoking sub-lethal injury of *Listeria monocytogenes*, *Campylobacter jejuni* and *Escherichia coli* O157 to serve method performance testing. *International Journal of Food Microbiology* 118, 241–249.

Jensen, A., Thomsen, L. E., Jørgensen, R. L., Larsen, M. H., Roldgaard, B. B., Christensen, B. B., Vogel, B.F., Gram, L., Ingmer, H., 2008. Processing plant persistent strains of *Listeria monocytogenes* appear to have a lower virulence potential than clinical strains in selected virulence models. *International Journal of Food Microbiology* 123, 254-261.

Kathariou, S., 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection* 65, 1811-1829.

Krist, K.A., Ross, T., McMeekin, T.A., 1998. Final optical density and growth rate; effects of temperature and NaCl differ from acidity. *International Journal of Food Microbiology* 43, 195–203.

Lianou, A., Koutsoumanis, K. P., 2011. Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior. *Food Microbiology* 28, 828-837.

Lianou, A., Sofos, J. N., 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *Journal of Food Protection* 70, 2172-2198.

Linnan, M. J., Mascola, L., Xiao Dong Lou, Goulet, V., May, S., Salminen, C., Hird, D., Yonekura, M., Hayes, P., Weaver, R., 1988. Epidemic listeriosis associated with Mexican-style cheese. *New England Journal of Medicine* 319, 823-828.

Little, C. L., Taylor, F. C., Sagoo, S. K., Gillespie, I. A., Grant, K., McLauchlin, J., 2007. Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK. *Food Microbiology* 24, 711-717.

Liu, D., Ainsworth, A. J., Austin, F. W., Lawrence, M. L., 2003. Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative

transcriptional regulator and internalin genes. *Journal of Medical Microbiology* 52, 1065-1070.

Liu, D., Lawrence, M. L., Austin, F. W., Ainsworth, A. J., 2007. A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*. *Journal of Microbiological Methods* 71, 133-140.

Lomonaco, S., Decastelli, L., Nucera, D., Gallina, S., Manila Bianchi, D., Civera, T., 2009. *Listeria monocytogenes* in gorgonzola: Subtypes, diversity and persistence over time. *International Journal of Food Microbiology* 128, 516-520.

Luber, P., Crerar, S., Dufour, C., Farber, J., Datta, A., Todd, E. C. D., 2011. Controlling *Listeria monocytogenes* in ready-to-eat foods: Working towards global scientific consensus and harmonization - recommendations for improved prevention and control. *Food Control* 22, 1535-1549.

Manfreda, G., De Cesare, A., Stella, S., Cozzi, M., Cantoni, C., 2005. Occurrence and ribotypes of *Listeria monocytogenes* in gorgonzola cheeses. *International Journal of Food Microbiology* 102, 287-293.

Martins, E. A., Leal Germano, P. M., 2011. *Listeria monocytogenes* in ready-to-eat, sliced, cooked ham and salami products, marketed in the city of São Paulo, Brazil: Occurrence, quantification, and serotyping. *Food Control* 22, 297-302.

Martins, I. S., Faria, F. C. C., Miguel, M. A. L., Dias, M. P. S. C., Cardoso, F. L. L., Magalhães, A. C. G., Mascarenhas, L.A., Nouér, S.A., Barbosa, A.V., Vallim, D.C., Hofer, E., Rabello, R.F., Riley, L.W., Moreira, B.M., 2010. A cluster of *Listeria monocytogenes* infections in hospitalized adults. *American Journal of Infection Control* 38, e31-e36.

Meloni, D., Galluzzo, P., Mureddu, A., Piras, F., Griffiths, M., Mazzette, R., 2009. *Listeria monocytogenes* in RTE foods marketed in Italy: Prevalence and automated *EcoRI* ribotyping of the isolates. *International Journal of Food Microbiology* 129, 166-173.

Oliveira, M.A., Abeid Ribeiro, E.G., Morato Bergamini, A.M., Pereira De Martinis, E.C., 2010. Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. *Food Microbiology* 27, 19-23.

Orsi, R. H., Ripoll, D. R., Yeung, M., Nightingale, K. K., Wiedmann, M., 2007. Recombination and positive selection contribute to evolution of *Listeria monocytogenes* inIA. *Microbiology* 153, 2666-2678.

- Ortiz, S., López, V., Villatoro, D., López, P., Dávila, J. C., Martínez-Suárez, J. V., 2010. A 3-year surveillance of the genetic diversity and persistence of *Listeria monocytogenes* in an Iberian pig slaughterhouse and processing plant. *Foodborne Pathogens and Disease* 7, 1177-1184.
- Pal, A., Labuza, T. P., Diez-Gonzalez, F., 2008. Comparison of primary predictive models to study the growth of *Listeria monocytogenes* at low temperatures in liquid cultures and selection of fastest growing ribotypes in meat and turkey product slurries. *Food Microbiology* 25, 460-470.
- Parizzi, S. Q. F., Andrade, N. J., Silva, C. A. S., Soares, N. D. F. F., Silva, E. A. M., 2004. Bacterial adherence to different inert surfaces evaluated by epifluorescence microscopy and plate count method. *Brazilian Archives of Biology and Technology* 47, 77-83.
- Ponniah, J., Robin, T., Paie, M. S., Radu, S., Ghazali, F. M., Kqueen, C. Y., Nishibuchi, M., Nakaguchi, Y., Malakar, P. K., 2010. *Listeria monocytogenes* in raw salad vegetables sold at retail level in Malaysia. *Food Control* 21, 774-778.
- Pouillot, R., Lubran, M. B., 2011. Predictive microbiology models vs. modeling microbial growth within *Listeria monocytogenes* risk assessment: What parameters matter and why. *Food Microbiology* 28, 720-726.
- Roberts, A. J., Williams, S. K., Wiedmann, M., Nightingale, K. K., 2009. Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, *inlA* transcript levels, and swarming motility in vitro. *Applied and Environmental Microbiology* 75, 5647-5658.
- Roche, S. M., Kerouanton, A., Minet, J., Le Monnier, A., Brisabois, A., Velge, P., 2009. Prevalence of low-virulence *Listeria monocytogenes* strains from different foods and environments. *International Journal of Food Microbiology* 130, 151-155.
- Rocourt, J., Bille, J., 1997. Foodborne listeriosis. *World Health Statistics Quarterly* 50, 67-73.
- Sabet, C., Lecuit, M., Cabanes, D., Cossart, P., Bierne, H., 2005. LPXTG protein *InlJ*, a newly identified internalin involved in *Listeria monocytogenes* virulence. *Infection and Immunity* 73, 6912-6922.
- Sato, G.S., Martins, V.A., Bueno, C.R.F., 2007. Análise exploratória do perfil do consumidor de produtos minimamente processados na cidade de São Paulo. *Informações Econômicas* 37, 62-71.

- Schlech III, W. F., Lavigne, P. M., Bortolussi, R. A., 1983. Epidemic listeriosis - evidence for transmission by food. *New England Journal of Medicine* 308, 203-206.
- Seeliger, H. P. R., Höhne, K., 1979. Serotyping of *Listeria monocytogenes* and related species, p.31-49. *In* Bergan, T., and J. R. Norris (ed.), *Methods in Microbiology*, vol. 13. Elsevier, New York, NY.
- Silva, F.A.S., Azevedo, C.A.V., 2002. Versão do programa computacional Assistat para o sistema operacional Windows. *Revista Brasileira de Produtos Agroindustriais* 4, 71-78.
- Skalina, L., Nikolajeva, V., 2010. Growth potential of *Listeria monocytogenes* strains in mixed ready-to-eat salads. *International Journal of Food Microbiology* 144, 317-321.
- Takeuchi, K., Mytle, N., Lambert, S., Coleman, M., Doyle, M. P., Smith, M. A., 2006. Comparison of *Listeria monocytogenes* virulence in a mouse model. *Journal of Food Protection* 69, 842-846.
- Thévenot, D., Delignette-Muller, M.-L., Christieans, S., Leroy, S., Kodjo, A., Vernozzy-Rozand, C., 2006. Serological and molecular ecology of *Listeria monocytogenes* isolates collected from 13 French pork meat salting-curing plants and their products. *International Journal of Food Microbiology* 112, 153-161.
- Todd, E. C. D., Notermans, S., 2011. Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes*. *Food Control* 22, 484-1490.
- Uyttendaele, M., Busschaert, P., Valero, A., Geeraerd, A. H., Vermeulen, A., Jacxsens, L., Goh, K., K., De Loy, A., Van Impe, J.F., Devlieghere, F., 2009. Prevalence and challenge tests of *Listeria monocytogenes* in Belgian produced and retailed mayonnaise-based deli-salads, cooked meat products and smoked fish between 2005 and 2007. *International Journal of Food Microbiology* 133, 94-104.
- Van Houdt, R., Michiels, C. W., 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology* 109, 1117-1131.
- Varma, J. K., Samuel, M. C., Marcus, R., Hoekstra, R. M., Medus, C., Segler, S., Anderson, B.J., Jones, T.F., Shiferaw, B., Haubert, N., Megginson, M., McCarthy, P.V., Graves, L., Gilder, T.V., Angulo, F.J., 2007. *Listeria monocytogenes* infection from foods prepared in a commercial establishment: A case-control study of potential sources of sporadic illness in the United States. *Clinical Infectious Diseases* 44, 521-528.

- Vázquez-Boland, J. A., Domínguez-Bernal, G., González-Zorn, B., Kreft, J., Goebel, W., 2001a. Pathogenicity islands and virulence evolution in *Listeria*. *Microbes and Infection* 3, 571-584.
- Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., Kreft, J., 2001b. *Listeria* pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews* 14, 584-640.
- Velge, P., Roche, S.M., 2010. Variability of *Listeria monocytogenes* virulence: A result of the evolution between saprophytism and virulence? *Future Microbiology* 5, 1799-1821.
- Vermeulen, A., Smigic, N., Rajkovic, A., Gysemans, K., Bernaerts, K., Geeraerd, A., Van Impe, J., Debevere, J., Devlieghere, F., 2007. Performance of a growth-no growth model for *Listeria monocytogenes* developed for mayonnaise-based salads: Influence of strain variability, food matrix, inoculation level, and presence of sorbic and benzoic acid. *Journal of Food Protection* 70, 2118-2126.
- Wagner, M., Allerberger, F., 2003. Characterization of *Listeria monocytogenes* recovered from 41 cases of sporadic listeriosis in Austria by serotyping and pulsed-field gel electrophoresis. *FEMS Immunology and Medical Microbiology* 35, 227-234.
- Warriner, K., Namvar, A., 2009. Why is the hysteria with *Listeria*? *Trends in Food Science and Technology* 20, 245-254.
- Wiedmann, M., 2002. Molecular subtyping methods for *Listeria monocytogenes*. *Journal of AOAC International* 85, 524-531.
- Wiedmann, M., Bruce, J. L., Keating, C., Johnson, A. E., McDonough, P. L., Batt, C. A., 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infection and Immunity* 65, 2707-2716.
- Wilis, C., Baalham, T., Greenwood, M., Presland, F., 2006. Evaluation of a new chromogenic agar for the detection of *Listeria* in food. *Journal of Applied Microbiology* 101, 711-717.
- Williams, D., Irvin, E. A., Chmielewski, R. A., Frank, J. F., Smith, M. A., 2007. Dose-response of *Listeria monocytogenes* after oral exposure in pregnant guinea pigs. *Journal of Food Protection* 70, 1122-1128.
- Williams, S. K., Roof, S., Boyle, E. A., Burson, D., Thippareddi, H., Geornaras, I., Sofos, J. N., Wiedmann, M., Nightingale, K., 2011. Molecular ecology of *Listeria*

monocytogenes and other *Listeria* species in small and very small ready-to-eat meat processing plants. Journal of Food Protection 74, 63-77.

Table 1: Prevalence of *Listeria monocytogenes* in ready-to-eat vegetables marketed in Sao Paulo, Brazil.

Type of vegetable	Analyzed samples n (%)	Positive samples n (%)	95% CI ^a
Salad mix ^b	214 (41.8)	2 (0.93)	1.1-3.3
Lettuce	152 (29.7)	3 (2.0)	0.4-5.7
Collard Greens	24 (4.7)	1 (4.2)	0.1-21.1
Arugula	19 (3.7)	0 (0)	NA ^c
Mix for Yakisoba	18 (3.5)	1 (5.6)	0.1-27.3
Watercress	18 (3.5)	1 (5.6)	0.1-27.3
Chicory	16 (3.1)	0 (0)	NA ^c
Escarole	13 (2.6)	3 (23.1)	5.1-53.8
Cabbage	11 (2.1)	2 (18.2)	0.2-51.8
Spinach	11 (2.1)	1 (9.1)	0.2-41.3
Swiss Chard	9 (1.8)	0 (0)	NA ^c
Mix for Sukiyaki	6 (1.2)	2 (33.3)	4.3-77.2
Colewort	1 (0.2)	0 (0)	NA ^c
Total	512 (100)	16 (3.1)	1.8-5.0

^a95% confidence interval; ^bMixes of different varieties of lettuce, watercress, chard, spinach, carrot, arugula, chicory, escarole and tomato. ^cNA = not applicable

Table 2: Prevalence of *Listeria monocytogenes* in ready-to-eat vegetables marketed in Sao Paulo, Brazil, according to the brand.

Brand	Analyzed samples	Positive samples	95% CI ^a
	n	n (%)	
A	124	6 (4.8)	1.8-10.2
B	81	5 (6.2)	2.0-13.8
C	80	0 (0)	NA ^b
D	72	0 (0)	NA
E	49	1 (2.1)	0.05-10.8
F	36	0 (0)	NA
G	31	1 (3.2)	0.08-16.8
H	20	0 (0)	NA
I	9	0 (0)	NA
J	5	3 (60)	14.7-94.7
L	2	0 (0)	NA
M	1	0 (0)	NA
N	1	0 (0)	NA
O	1	0 (0)	NA

^a95% confidence interval, ^bNA = not applicable.

Table 3: Enumeration of *Listeria monocytogenes* in ready-to-eat vegetables marketed in Sao Paulo, Brazil.

Type of vegetable	Composition	Brand	Population (CFU/g)
Salad mix	Crispy and butter head lettuces, arugula and radicchio	A	1.6×10^1
Cabbage	Mix of green and red cabbages and carrots	B	1.0×10^1
Salad mix	Iceberg and crisp lettuces, carrot and red cabbage	F	1.0×10^1
Mix for Yakisoba	Cauliflower, carrot, broccoli, cabbage and chard	G	2.6×10^2
Mix for Sukiyaki	Chard, cabbage, carrot, onion, green pepper, cauliflower and leek	J	1.0×10^1

Table 4: Serotypes and ribotypes clusters of *Listeria monocytogenes* isolated from RTE vegetables marketed in Sao Paulo, Brazil, according to the source and brand.

Source	Isolate (ID) ^a	Brand	Serotype	Ribotype	Cluster
Collard green	359 (17), 362 (18), 364 (19)				
Salad mix	391 (20), 405 (22)	B	4b	1038	A
Cabbages	400 (21)				
Salad mix	101 (1)	F	4b	1038	
Watercress	480 (35)	B	4b	19191	
Lettuce	532 (46), 561 (49), 562 (50), 563 (51)	A	4b	1038	
Cabbages	336 (10)	B	4b	19191	
Mix for Yakisoba	504 (42)	E	4b	1038	
	335 (9), 436 (31)	J			
Mix for Sukiyaki	564 (52), 567 (53)	A	4b	1038	
	437 (32), 497 (40), 500 (41)	J		19191	
	524 (43), 525 (44), 526 (45), 550 (47), 552 (48), 590 (61), 594 (62)	A	4b	1038	
Escarole	461 (33), 462 (34)	B		19191	B
	579 (54), 581 (56), 583 (58), 586 (59), 588 (60)	A	4b	1038	
Spinach	580 (55), 582 (57)			19191	
	413 (23)			18604	
Lettuce	419 (27)	J	4b	19191	
	415 (24), 417 (25), 418 (26)			1038	
Salad mix	424 (29), 425 (30), 492 (37), 493 (38), 495 (39)	J	4b	1038	
	423 (28), 491 (36)			19191	
	347 (11)			19191	
Collard green	348 (12), 349 (13), 353 (14)	A	4b	1038	
	356 (15), 358 (16)	B		1038	
Mix for Yakisoba	221 (2), 223 (3), 224 (4), 283 (5), 293 (6), 294 (7), 295 (8)	G	1/2b	19175	C

^a Numbers outside parenthesis indicate the reference number of the isolates, while those within parenthesis correspond to ID numbers of these isolates in Figures 2 and 3.

Figure 1: Dendrogram of the 62 *Listeria monocytogenes* isolates obtained from ready-to-eat vegetables marketed in Sao Paulo, Brazil. Dendrogram was produced using Band based-Dice coefficient analysis and UPGMA algorithm.

Figure 2: Adhesion and biofilm formation on stainless steel coupons by 62 *L. monocytogenes* isolates obtained from ready-to-eat vegetables marketed in Sao Paulo, Brazil, expressed as log CFU/cm².

Figure 3: Growth rate (μ) of 62 *L. monocytogenes* isolates obtained from ready-to-eat vegetables marketed in Sao Paulo, Brazil, in TSB-YE broth at 25°C.

Figure 4: Distribution of growth rate (μ) of 62 *L. monocytogenes* isolates, obtained from ready-to-eat vegetables marketed in Sao Paulo, Brazil, in TSB-YE broth at 25°C.

Figure 1:

Figure 2:

Figure 3:

Figure 4:

CAPÍTULO 4

“Growth potential of *Salmonella* spp. and *Listeria monocytogenes* in nine types of ready-to-eat vegetables stored at variable temperature conditions during shelf-life”

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Growth potential of *Salmonella* spp. and *Listeria monocytogenes* in nine types of ready-to-eat vegetables stored at variable temperature conditions during shelf-life

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Running title: Growth of *Salmonella* spp. and *Listeria monocytogenes* in RTE vegetables.

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Abstract:

Growth potential (δ) is defined as the difference between the population of a microorganism at the end of shelf-life of specific food and its initial population. The determination of δ of *Salmonella* and *L. monocytogenes* in RTE vegetables can be very useful to determine likely threats to food safety. However, little is known on the behavior of these microorganisms in several RTE vegetables. Therefore, the aim of this study was to determine the δ of both pathogens in nine different types of RTE vegetables (escarole, collard green, spinach, watercress, arugula, grated carrot, green salad, and mix for yakisoba) stored at refrigeration (7°C) and abuse temperature (15°C). The population of aerobic microorganisms and lactic acid bacteria, including those showing antimicrobial activity has been also determined. Results indicated that *L. monocytogenes* was able to grow ($\delta \geq 0.5 \log_{10}$) in more storage conditions and vegetables than *Salmonella*. Both microorganisms were inhibited in carrots, although a more pronounced effect has been observed against *L. monocytogenes*. The highest δ values were obtained when the RTE vegetables were stored 15°C/6 days in collard greens ($\delta = 3.34$) and arugula ($\delta = 3.22$) (*L. monocytogenes*) and arugula ($\delta = 4.05$) and escarole ($\delta = 2.80$) (*Salmonella*). In most vegetables and storage conditions studied, the counts of total aerobic microorganisms raised significantly despite the temperature of storage ($p < 0.05$). Counts of lactic acid bacteria were higher in vegetables partially or fully stored at abuse temperature with recovery of isolates showing antimicrobial activity. In conclusion, the results of this study show that *Salmonella* and *L. monocytogenes* may grow and reach high populations in RTE vegetables depending on storage conditions and the definition of effective intervention strategies are needed to control their growth in these products.

Key-words: *Salmonella*, *Listeria monocytogenes*, ready-to-eat vegetables, challenge testing, growth potential.

1) Introduction

Surveillance data have shown that vegetables have been implicated in foodborne disease outbreaks caused by a variety of pathogenic microorganisms (Campbell et al., 2001, Long et al., 2002, Sivapalasingam et al., 2004, Anon, 2008a, Anon, 2010). As a result, numerous studies have been performed to determine the occurrence of microorganisms such as *Salmonella* (Elviss et al., 2009, Quiroz-Santiago et al., 2009, Giusti et al., 2010, Gorski et al., 2011, Sant'Ana et al., 2011a), pathogenic *Escherichia coli* (Arthur et al., 2007, Mora et al., 2007, Bohaychuk et al., 2009, Rúgeles et al., 2010) and *Listeria monocytogenes* (Little et al., 2007, Cordano and Jacquet, 2009, Oliveira et al., 2010, Ponniah et al., 2010, Sant'Ana et al., 2011b) in different types of vegetables.

The contamination of vegetables by *Salmonella* spp. and *L. monocytogenes* might occur either in the field or during handling or processing (Ailes et al., 2008, Caponigro et al., 2010) as salmonella is present in the intestinal tract of humans and animals (Adley et al., 2011, Arguello et al., 2011, Rostagno and Callaway, 2011), and *L. monocytogenes* is a markedly ubiquitous microorganism being isolated from diverse sources (Gandhi and Chikindas, 2007, Fox et al., 2011). Despite present in the vegetables, the capability of these microorganisms to survive, grow and cause disease will depend upon plant-microbe and microbe-microbe interactions (Brandl, 2006) and their responses to stressors during minimal processing and storage (Capozzi et al., 2009).

Regardless the low prevalence and counts of *L. monocytogenes* and *Salmonella* in RTE vegetables reported in several surveys (Little et al., 2007, Cordano and Jacquet, 2009, Elviss et al., 2009, Oliveira et al., 2010, Gorski et al., 2011, Sant'Ana et al., 2011a,b), populations as high as 10^6 CFU/g may be reached depending on storage conditions (Koseki et al., 2005a,b, Crépet et al., 2009). Although the infectious dose for *Salmonella* is highly variable (Blaser and Newman 1982, Mints et al., 1994, Musher and Musher 2004), the infection by *L. monocytogenes* seems to be mostly associated with ingestion of high doses of this pathogen (Takeuchi et al., 2006, Williams et al., 2007). Given this, the knowledge of growth potential (δ) in RTE vegetables can be very useful to determine the likely threats these microorganisms may pose to food safety and to develop effective

measures aiming to control the occurrence and growth of pathogens in these products.

Hence, the purpose of this study was to determine the growth potential (δ) of *Salmonella* and *L. monocytogenes* in nine types of RTE vegetables stored at different temperature conditions. In addition, the populations of aerobic microorganisms and lactic acid bacteria, including those showing antimicrobial activity were also investigated as they may influence the survival and growth of the pathogens.

2) Material and Methods

2.1) Microorganisms and preparation of cell suspensions:

Five strains of *L. monocytogenes* and five strains of *Salmonella* spp were used in the study. The *L. monocytogenes* strains were isolated from RTE vegetables marketed in Sao Paulo, Brazil (Sant'Ana et al., 2011b), and belonged serotypes 4b (strains 349, 564, 586 and 480) and 1/2b (strain 221) and ribotypes DUP 1038 (strains 349, 564 and 586), DUP 19191 (strain 480) and DUP 19175 (strain 221). Three *Salmonella* spp strains were also isolated from RTE vegetables marketed in Sao Paulo, Brazil (Sant'Ana et al., 2011a) and belonged to serovars *S. Typhimurium* (strain 227), *S. Typhi* (strain 386) and *S. enterica* subsp. *enterica* O:47:z4,z23:- (strain 994). Two *Salmonella* spp strains (strains 2494 and 5711), from human and animal origin, kindly provided by Dr. Ernesto Hofer from Oswaldo Cruz Institute (Rio de Janeiro, Brazil), belonged to serovars *S. Infantis* IOC 2494 and *S. Concord* IOC 5711.

The strains of *Salmonella* spp. and *L. monocytogenes* were separately grown in 10 mL of tryptic soy broth (TSB) and TSB supplemented with 0.6% of yeast extract (TSB-YE), respectively, at 37°C for 24h. One mL aliquots were transferred to fresh broths for incubation for extra 24h, twice. The cultures were centrifuged at 8°C for 10 min at 2810 × *g* (Mikro 22R, Hettich Zentrifugen, Germany), supernatants were discharged and pellets were washed three times with phosphate buffered saline pH 6.0. Pools of each pathogen were prepared mixing equal volumes of each washed suspension, to achieve an optical density at 630nm equal to 0.5, corresponding to 10⁸ CFU/mL, as checked by plate counting on TSA and TSA-YE.

2.2) Vegetables:

Packages of RTE vegetables with no more than one day of storage after processing were acquired from supermarkets in the city of Sao Paulo, Brazil. Nine types vegetables were selected either based on their consumption in Brazil (IBGE, 2011), prevalence of *Salmonella* spp. (Sant'Ana et al., 2011a) and *L. monocytogenes* (Sant'Ana et al., 2011b). All the RTE vegetables used (escarole, collard green, spinach, watercress, arugula, grated carrot, green salad (crisp, romaine and butter lettuces), and mix for yakisoba (broccoli, cabbage, cauliflower, leek, carrots and chard) were packaged under modified atmosphere. The packages were transported to the laboratory in isothermal boxes and kept under refrigeration until the experiments were performed.

2.3) Inoculation of vegetables, packaging and storage conditions:

Portions of 25g of each RTE vegetable were placed in plastic bags (62 μm thickness, O_2 permeability of $1.375 \text{ m}^3 \text{ m}^{-2} \text{ day}^{-1}$ at 23°C and water steam permeability of $3.5 \text{ g water m}^{-2} \text{ day}^{-1}$ at 38°C and 90% relative humidity) and spot inoculated with 0.5 mL of the pools of *Salmonella* and *L. monocytogenes* properly diluted to achieve a final concentration of 10^3 CFU/g in the products, according to the guidelines for challenge tests described in Anon (2003). The bags containing the inoculated samples were sealed in a vacuum sealing machine AP 500 (Tecmaq, Sao Paulo, Brazil) under modified atmosphere containing 5% O_2 , 15% CO_2 and 80% N_2 (White Martins, Osasco, Brazil), which corresponds the gaseous composition commonly used by RTE vegetables processors in Sao Paulo.

Packages of RTE vegetables were exposed to three different storage conditions: I (100% of shelf-life at $7^\circ\text{C} \pm 1^\circ\text{C}$), II (30% at $7^\circ\text{C} \pm 1^\circ\text{C}$ and 70% at $15^\circ\text{C} \pm 1^\circ\text{C}$) and III (100% at $15^\circ\text{C} \pm 1^\circ\text{C}$). These storage scenarios were chosen with the purpose to understand the behavior of *Salmonella* and *L. monocytogenes* under optimal storage conditions ($\leq 7^\circ\text{C}$) and under partial or full abuse temperature during shelf-life (15°C). The shelf-life established by processors of RTE vegetables sold in Sao Paulo (6 days) was set as the maximum storage time. A total of 288 bags of RTE vegetables were prepared. Control samples comprehended vegetables spot inoculated with 0.5 mL of sterile distilled water. Experiments were carried out twice. The pH values of the vegetables were determined using a pH meter (Láctea, LCP-210, Brazil) according to Scott et al. (2001).

2.4) Enumeration and detection of *Salmonella* spp. and *L. monocytogenes*:

Salmonella spp. and *L. monocytogenes* were enumerated in three packages of RTE vegetables at time “0” (after inoculation) and at the end of shelf-life (day “6”). In addition, at time “0”, two packages were submitted to tests for presence-absence of both pathogens. Enumerations of *Salmonella* spp. and *L. monocytogenes* were performed by homogenizing 25 g of samples with 225mL of 0.1% peptone water, following decimal dilutions and inoculation in duplicate plates of Mannitol Lysine Crystal Violet Brilliant Green (MLCB) agar (*Salmonella* spp.) and Oxford (OXA) agar added of selective supplement (SR0206) (*L. monocytogenes*). MLCB and OXA plates were incubated at $37\pm1^{\circ}\text{C}$ for 24h and 48 h, respectively. Mauve colonies with black centers in MLCB agar were counted as *Salmonella*, while brown colored colonies with aesculin hydrolysis in OXA were enumerated as *L. monocytogenes*. Up to 3 colonies per sample were selected for further confirmation of *Salmonella* spp and *L. monocytogenes* by polyvalent serotyping and biochemical tests, respectively (Anon, 1996, 2002). The final results were expressed as $\log_{10}$ CFU/g. Samples of vegetables used for enumeration were kept frozen at -20°C until the results were obtained. When no colonies were recovered by the quantification method (below the detection limit of 10^1 CFU/g), the samples were submitted to tests for presence-absence of *Salmonella* spp and *L. monocytogenes* using ISO 6579 (Anon, 2002) and ISO 11290-1 (Anon, 1996) methods, respectively.

2.5) Determination of growth potential (δ) of *Salmonella* spp. and *L. monocytogenes*:

The growth potential (δ) of *Salmonella* spp. and *Listeria monocytogenes* in each type of RTE vegetable was determined by the difference between the counts of each microorganism at the end (day “6”) ($\log_{10}$ CFU/g) and at the beginning (time “0”) ($\log_{10}$ CFU/g) of shelf-life (Beaufort, 2011). The vegetable was considered as supporting growth of the pathogen when δ was higher than $0.5 \log_{10}$, which corresponds to the uncertainty of the microbiological methods (AFSSA, 2004, Anon, 2008a,b,c). The pathogens were considered not able to grow in the RTE vegetable when δ values were negative or lower than $0.5 \log_{10}$ (AFSSA, 2004, Anon, 2008a,b,c).

2.6) Enumeration of aerobic microorganisms and lactic acid bacteria:

Aerobic mesophilic microorganisms and lactic acid bacteria were quantified in two different packages of each type of vegetable and condition studied at the beginning (time “0”) and at the end of shelf-life (time “6”). Twenty five grams of each vegetable were homogenized with 225 mL of 0.1% peptone water, following decimal dilutions and pour plating in Plate Count (PCA) and Man, Rogosa and Sharpe (MRS) agars, for counts of aerobes and lactic acid bacteria, respectively. MRS agar plates were overlaid with 15% agar-agar to create an environment with low oxygen tension. PCA and MRS plates were incubated at $37\pm1^{\circ}\text{C}$ for $48\pm3\text{h}$, and growing colonies were counted. Results were expressed as $\log_{10}$ CFU/g.

2.7) Determination of antimicrobial activity of lactic acid bacteria:

The isolates of lactic acid bacteria were screened for antimicrobial activity using the method of Todorov and Dicks (2005) with modifications. After incubation, MRS agar plates were overlaid with a thin layer of Brain Heart Infusion (BHI) containing 1% agar (w/v) inoculated with 10^5 - 10^6 CFU/mL of *L. monocytogenes* Scott A. MRS plates were incubated at $37\pm1^{\circ}\text{C}$ for 24h and further examined for the presence of halos of inhibition. Any colonies showing ability to inhibit *L. monocytogenes* Scott A were selected, inoculated in MRS broth and purified by streaking onto MRS agar. Bacteriocin screening was performed by method described by Ivanova et al. (1998). After incubation at $30\pm1^{\circ}\text{C}$ for 24h in MRS broth, cell free supernatants were prepared, and pH was adjusted to 6.0 with 1M NaOH to eliminate the antimicrobial activity due to acid production by the lactic acid bacteria. Antimicrobial activity due to production of proteinaceous bacteriocins was checked testing the sensitivity of the supernatant to 1mg/mL of Proteinase K (Sigma-Aldrich, Saint Louis, USA), α -Chymotrypsin from bovine pancreas type II (Sigma) and Protease type XIV from *Streptomyces griseus* (Sigma) for 2h. Antimicrobial activity was monitored according to the method described by Van Reenen et al. (1998). All the culture media used in this study were from Oxoid (Basingstoke, UK) unless otherwise stated, while all the enzymes were from Sigma-Aldrich (Saint Louis, USA).

2.7) Statistical Analysis:

The populations of total aerobic microorganisms and lactic acid bacteria at the beginning and at the end of shelf-life of RTE vegetables were assessed for significant

statistical ($p \leq 0.05$) differences using Shapiro-Wilk test followed by t test. The populations of total aerobic microorganisms and lactic acid bacteria obtained in different storage scenarios were checked for significant statistical ($p \leq 0.05$) differences using one-factor analysis of variance (ANOVA) followed by Tukey's test. Statistical analyses were carried out in Assistat version 7.5 free software (Campina Grande, Brazil) (Silva et al., 2002).

3) Results and Discussion

Inappropriate storage temperature has been reported as one of the three most important faults contributing for the occurrence of outbreaks due to consumption of salads (Little and Gillespie, 2008). The occurrence of foodborne diseases outbreaks linked to vegetables have led to increased concerns over the safety of RTE vegetables (Harris et al., 2003, Ragaert et al., 2004, Sivapalasingam et al., 2004, Perez et al., 2008, Lynch et al., 2009, Jacxsens et al., 2010). Two main outputs of the growth potential tests (δ) are the determination of the "growth or no growth" (positive or negative values of δ) of a microorganism under packaging and storage conditions studied and their classification into categories of potential risks when assessed along with data on the prevalence of pathogens (Uyttendaele et al., 2009, Skalina and Nikolajeva, 2010, Beaufort, 2011). Thus, the determination of the growth potential (δ) of *Salmonella* spp. and *L. monocytogenes* in different types of RTE vegetables stored at recommended (7°C) and abuse temperature (15°C) conditions may contribute for the assessment of consumer's exposure to these microorganisms. Control samples ensured that no vegetables used in the experiments were previously contaminated with *Salmonella* spp. and *L. monocytogenes*. In addition, none of the control samples taken at the end of shelf-life indicated the presence of *Salmonella* spp. and *L. monocytogenes*.

As shown in Table 1, the pH values of the nine RTE vegetables studied (6.2-7.2) were in the range for optimum growth of *Salmonella* spp. and *L. monocytogenes* (Nguyen-the and Carlin, 1994). However, no relationship between pH values and growth potential (δ) has been found. In fact, it is known that the growth of foodborne pathogens at the surface of vegetables is dependent upon several factors and their complex interactions, such as epiphytic fitness, the physicochemical environment of

plant surfaces, biofilm formation, and bacteria-bacteria and vegetable-bacteria relations (Brandl, 2006).

The fate of foodborne pathogens such as *Salmonella* spp. and/or *L. monocytogenes* has been studied in several vegetables such as endives (Carlin et al., 1995), lettuces (Kakiomenou et al., 1998, Koseki et al., 2005a,b), carrots (Finn and Upton, 1997, Kakiomenou et al., 1998), cabbage (Finn and Upton, 1997), peppers and tomatoes (Ma et al., 2010), herbs (Hsu et al., 2006), spinach (Pirovani et al., 2000), and radish (Islam et al., 2004) but not in RTE collard greens, escarole, watercress, arugula and mix for yakisoba. Therefore, the current study reports for the first time the growth potential of *Salmonella* and *L. monocytogenes* in these RTE vegetables. Overall, data obtained indicate that growth potential (δ) of both pathogens was not related to the type of vegetable (*Table 1*). Amongst the nine vegetables studied, seven were leafy (escarole, collard greens, cabbage, spinach, watercress, arugula and green salad), one was root (carrot) and one (yakisoba) was a mix of inflorescent (broccoli and cauliflower), leafy (cabbage), root (carrot) and stalk (chard) vegetables.

From *Table 1* it can be depicted that *L. monocytogenes* grew better ($\delta \geq 0.5 \log_{10}$) than *Salmonella* spp in the studied vegetables, which can be explained by the psychrophilic behavior of *L. monocytogenes* (Gandhi and Chikindas, 2007). When the vegetables were stored at 7°C during the whole shelf-life (condition I), an increase in the counts of *L. monocytogenes* was observed in escarole, collard green, watercress and yakisoba. However, this increase was not greater than the uncertainty of the enumeration method ($\sim 0.5 \log_{10}$). At storage condition I, a complete inhibition of *L. monocytogenes* was observed only in carrots. In these samples, the populations of *L. monocytogenes* were lower than the limit of enumeration of the method used (10^1 CFU/g). However, after 6 days of shelf-life the survival of this microorganism was confirmed by the positive results obtained in the detection method (data not shown). The anti-listerial activity of raw carrots has been linked to antimicrobial compounds found in the carrot tissue, which have been found to show lethal effects in low temperatures (4 and 10°C) (No riega et al., 2010). As the levels of *L. monocytogenes* in vegetables are barely above 10^3 CFU/g (Crépet et al., 2007), if carrots are stored at 7°C inhibition of this pathogen during shelf-life is expected. Still at 7°C, *L. monocytogenes* was able to grow ($\delta \geq 0.5 \log_{10}$) in green salad, spinach and arugula. Despite this, the increase in the population of this microorganism was

not higher than 1 and 2 log₁₀, highlighting the suppressant effect of temperature on the growth of this bacterium. The results shown in *Table 1* indicate that shredding (lettuce) or not (arugula and spinach) seems to not correlate with growth potential of this pathogen in the vegetables. Some authors have reported that the attachment and further survival and growth of pathogen are more prominent when vegetables are cut or shredded during minimal processing (Gleeson and O'Beirne, 2005, Ells and Hansen, 2006, Brandl, 2008). In addition to providing sites for the attachment of microorganisms (Patel and Sharma, 2010, López-Gálvez et al., 2010, Goldberg et al., 2011), cutting and shredding causes release of exudates from vegetables nourishing the attached bacteria that may grow and reach high populations (Teplitski et al., 2011, Koseki et al., 2005a,b).

At 7°C, *Salmonella* was able to grow ($\delta \geq 0.5 \log_{10}$) in escarole and arugula during shelf-life (condition I). The increase in the population of this microorganism in escarole and arugula were ≥ 1 and $\geq 2 \log_{10}$, respectively (*Table 1*). Green salad, spinach and yakisoba stored at 7°C did not support the growth of *Salmonella* during shelf-life. At 7°C, *Salmonella* was inhibited in collard greens, cabbage, watercress and carrots. Even though the presence of antimicrobial compounds in carrots and cabbage has been reported (Gogo et al., 2010, Noriega et al., 2010), it is known that inhibitory compounds found in carrots are particularly effective against *L. monocytogenes* (Noriega et al., 2010), while cabbage antimicrobial activity is more pronounced against *Salmonella* and Gram-negative bacteria (Gogo et al., 2010). Besides, the fact that cabbage did not support the growth of *Salmonella* at all storage conditions may be related to the low attachment strength of this bacterium to surface of cabbages. According to Patel and Sharma (2010), *Salmonella* presented the overall lower attachment strength on cabbage (0.12) when compared to Iceberg (0.23) and Romaine lettuce (0.34) after 24h at 10°C.

When the growth potential (δ) of both pathogens was investigated at partial or full abuse temperature during shelf-life (*Table 1*), results indicated that *L. monocytogenes* was able to grow in all RTE vegetables studied, excepting carrots when partially or fully exposed to abuse temperature (15°C) during shelf-life, i.e., conditions II and III. In these scenarios, the increase in the populations of *L. monocytogenes* at the end of shelf-life was $\geq 2 \log_{10}$ in collard green, arugula and green salad (conditions II and III) and watercress (condition III). On the other hand, an increase of *L. monocytogenes* population $\leq 2 \log_{10}$ was observed in escarole,

cabbage, spinach and yakisoba (conditions II and III) and watercress (condition II). The highest growth potential (δ) values for *L. monocytogenes* were observed at storage condition III in collard greens ($\delta = 3.34$) and arugula ($\delta = 3.22$), while the lowest values were observed in cabbage. At abuse storage conditions (II and III), negative values of growth potential (δ) of *L. monocytogenes* were only observed in carrots. Although cabbage extract has been shown highly inhibitory for Gram-negative microorganisms, it presents some suppressant effects against the growth of Gram-positive microorganisms such as *L. monocytogenes* (Gogo et al., 2010). On the other hand, the growth of this microorganism in all the other types of RTE vegetables with rises in its population of up to 3 log₁₀ CFU/g concerns these vegetables are consumed raw. Although the highest increase in the population of *L. monocytogenes* has been observed in collard greens at condition III, the exposure of consumers to these microorganisms will depend, among other factors, of the mode of preparation.

Another major issue regarding the presence of *L. monocytogenes* in RTE vegetables is their further use to prepare salad-based meals that are not stored at adequate refrigeration temperatures nor consumed quickly, providing adequate nutritional and environmental conditions for the growth of this pathogen. This is of particular concern since the growth of *L. monocytogenes* in salad-based meals has been reported in the literature (Uyttendaele et al., 2009, Skalina and Nikolajeva, 2010). Whereas vegetables have been placed in a category of low risk based on probability of representing a listeriosis hazard (Warriner and Namvar, 2009), the implications of the growth potential of *L. monocytogenes* in these products to food safety should not be underestimated.

The exposure of the RTE vegetables to abuse temperature (15°C) remarkably influenced the growth potential (δ) of *Salmonella* (Table 1). The microorganism was able to grow in escarole, collard green, spinach, watercress, arugula, green salad and yakisoba partially exposed at abuse temperature (15°C) (condition II). In these vegetables and storage condition, the increase in *Salmonella* population was not higher than 2 log₁₀, except in arugula in which the growth of the microorganism was 2.81 log₁₀ (Table 1). At storage condition III (15°C/6 days), *Salmonella* was able to grow in all vegetables ($\delta \geq 0.5$ log₁₀), except on cabbage where δ was ≤ 0.5 log₁₀. The highest δ values for *Salmonella* spp. were obtained at storage condition III in arugula ($\delta = 4.05$), followed by escarole ($\delta = 2.80$), collard green ($\delta = 2.62$) and green salad

($\delta = 2.61$). Even though the detection of a single cell of *Salmonella* in foods is unacceptable from a regulatory point of view (zero tolerance) (Anon, 2001), the determination of its growth potential in vegetables is of major importance as *Salmonella* is the leading etiological agent of outbreaks of fresh produce illnesses (Tauxe et al., 1997, Harris et al., 2003, Little and Gillespie, 2008).

In order to investigate the role of background microbiota of RTE vegetables on the behavior of *Salmonella* and *L. monocytogenes*, the populations of total aerobic microorganisms and lactic acid bacteria at the beginning and end of shelf-life were investigated (Table 2). Populations of total aerobic microorganisms varied from 5.5 to 8.1 log₁₀ CFU/g and 7.1 to 10 log₁₀ CFU/g at the beginning and at the end of shelf-life, respectively. In most vegetables and storage conditions studied, the counts of total aerobic microorganisms raised significantly despite the temperature of storage ($p < 0.05$). On the other hand the effect of storage temperature on the populations of aerobic microorganisms on a same type of vegetable varied depending on the vegetable. Overall, the more time stored at abuse temperature (15°C) the higher the counts of total aerobic microorganisms at the end of shelf-life (day "6"). The populations of total counts of aerobic mesophilic microorganisms are in agreement with data reported in the literature, which indicate that populations of this group of microorganisms may vary from 10² up to 10⁸ CFU/g depending on the kind of vegetable, among other factors (Nguyen-the and Carlin, 1994, Abadias et al., 2008, Caponigro et al., 2010).

The populations of lactic acid bacteria increased significantly in most of storage scenarios and vegetables studied, excepting in collard greens, watercress (both at storage conditions I and II) and yakisoba (storage condition I) (Table 2). Lactic acid bacteria have been frequently isolated from RTE vegetables packaged under modified atmosphere (Rudi et al., 2002, Fröder et al., 2007, Abadias et al., 2008, Caponigro et al., 2010). Furthermore, the populations of lactic acid bacteria were higher when the vegetables were partially or fully stored at abuse temperature (15°C) ($p < 0.05$). Several authors have shown that vegetables may contain different species of lactic acid bacteria with antimicrobial activity (Liao and Fett, 2001, Allende et al., 2007, Trias et al., 2008, Ponce et al., 2008). In the current study, lactic acid bacteria showing antimicrobial activity were only recovered when the vegetables were partially (II) or fully (III) kept at abuse temperature during shelf-life, with populations between 4.7 and 8.6 log₁₀ CFU/g. However, the antimicrobial activity

presented by the tested lactic acid bacteria could not be associated to production of bacteriocins (data not shown). Possible mechanisms of inhibition may be explained by competition for nutrients or production of toxic metabolites such as organic acids (Matamoros et al., 2009, Aguilar and Klotz, 2010).

In conclusion, the results obtained in this study highlight that *Salmonella* and *L. monocytogenes* are able to grow and reach high populations in several types of RTE vegetables. It has been found that arugula, collard greens and green salad constituted the best substrates for the growth of these pathogens, which has been greatly potentialized whenever exposition at abuse temperature (15°C) has been tested. Although field operations are likely to represent the main source of contamination of fresh vegetables (Farrar and Guzewich, 2009, Matthews, 2009), conditions and practices during processing, commercialization and consumption will define the fate of pathogens and likely risks to consumers. Currently, little information on the behavior of *Salmonella* and *L. monocytogenes* in these RTE vegetables has been available. Thus, the knowledge of the behavior of these microorganisms in RTE vegetables at variable storage conditions either through the use of growth potential tests or modeling approach may be helpful to define effective intervention strategies to control bacterial growth in these products.

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4) References

Abadias, M., Usall, J., Anguera, M., Solsona, C., Viñas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology* 123, 121-129.

- Adley, C., Dillon, C., Morris, C. P., Delappe, N., Cormican, M., 2011. Prevalence of *Salmonella* in pig ear pet treats. *Food Research International* 44, 193-197.
- AFSSA – Agence Française de Sécurité Sanitaire des Aliments, 2004. *Compte Rendu des Essais ISO sur L'Incertitude de Mesure 2003/2004*. p.1-19.
- Aguilar, C., Klotz, B., 2010. Effect of the temperature on the antagonistic activity of lactic acid bacteria against *Escherichia coli* and *Listeria monocytogenes*. *Journal of Food Safety* 30, 996-1015.
- Ailes, E. C., Leon, J. S., Jaykus, L.-A., Johnston, L. M., Clayton, H. A., Blanding, S., Kleinbaum, D. G., Backer, L. C., Moe, C. L., 2008. Microbial concentrations on fresh produce are affected by postharvest processing, importation, and season. *Journal of Food Protection* 71, 2389-2397.
- Allende, A., Martínez, B., Selma, V., Gil, M. I., Suárez, J. E., Rodríguez, A., 2007. Growth and bacteriocin production by lactic acid bacteria in vegetable broth and their effectiveness at reducing *Listeria monocytogenes in vitro* and in fresh-cut lettuce. *Food Microbiology* 24, 759-766.
- Anonymous, 1996. ISO 11290-1:1996 - Microbiology of food and animal feeding stuffs -- Horizontal method for the detection and enumeration of *Listeria monocytogenes* -- Part 1: Detection method. Switzerland.
- Anonymous, 2001. Ministério da Saúde, Agência Nacional de Vigilância Sanitária. Regulamento Técnico sobre Padrões Microbiológicos para Alimentos. RDC n.º 12, January 26, 2001. http://www.anvisa.gov.br/legis/resol/12_01rdc.htm. Accessed Aug. 22, 2011.
- Anonymous, 2002. ISO 6579:2002(E) - Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.. International Organization for Standardization. Switzerland.
- Anonymous, 2003. Microbiological challenge testing. *Comprehensive Reviews in Food Science and Food Safety* 2, 46-50.
- Anonymous, 2008a. Outbreak of *Salmonella* serotype Saintpaul infections associated with multiple raw produce items - United States, 2008. *Morbidity and Mortality Weekly Report* 57, 929–934.
- Anonymous, 2008b. EU Community Reference Lab for *L. monocytogenes* EU Community Reference Lab for *L. monocytogenes* (2008). Working document November 2008. Technical Guidance Document on shelf-life studies for *Listeria monocytogenes* in ready-to-eat foods.

- Anonymous, 2008c. Guidance document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. SANCO/1628/2008 ver. 9.3 (26112008). Brussels.
- Anonymous, 2010. Surveillance for foodborne disease outbreaks - United States, 2007. Morbidity and Mortality Weekly Report 59, 973-979.
- Arguello, H., Carvajal, A., Collazos, J. A., García-Feliz, C., Rubio, P., 2011. Prevalence and serovars of *Salmonella enterica* on pig carcasses, slaughtered pigs and the environment of four Spanish slaughterhouses. Food Research International doi:10.1016/j.foodres.2011.04.017
- Arthur, L., Jones, S., Fabri, M., Odumeru, J., 2007. Microbial survey of selected Ontario-grown fresh fruits and vegetables. Journal of Food Protection 70, 2864-2867.
- Beaufort, A., 2011. The determination of ready-to-eat foods into *Listeria monocytogenes* growth and no growth categories by challenge tests. Food Control 22, 1498-1502.
- Blaser, M. J., Newman, L. S., 1982. A review of human salmonellosis: I. infective dose. Reviews of Infectious Diseases 4, 1096-1106.
- Bohaychuk, V. M., Bradbury, R. W., Dimock, R., Fehr, M., Gensler, G. E., King, R. K., Rieve, R., Barrios P. R., 2009. A microbiological survey of selected Alberta-grown fresh produce from farmers' markets in Alberta, Canada. Journal of Food Protection 72, 415-420.
- Brandl, M. T., 2006. Fitness of human enteric pathogens on plants and implications for food safety. Annual Review of Phytopathology 44, 367-392.
- Brandl, M. T., 2008. Plant Lesions Promote the Rapid Multiplication of *Escherichia coli* O157:H7 on Postharvest Lettuce. Applied and Environmental Microbiology 74, 5285-5289.
- Campbell, J. V., Mohle-Boetani, J., Reporter, R., Abbott, S., Farrar, J., Brandl, M., Mandrell, R., Werner, S.B., 2001. An outbreak of *Salmonella* serotype Thompson associated with fresh cilantro. Journal of Infectious Diseases 183, 984-987.
- Caponigro, V., Ventura, M., Chiancone, I., Amato, L., Parente, E., Piro, F., 2010. Variation of microbial load and visual quality of ready-to-eat salads by vegetable type, season, processor and retailer. Food Microbiology 27, 1071-1077.

- Capozzi, V., Fiocco, D., Amodio, M.L., Gallone, A., Spano, G., 2009. Bacterial stressors in minimally processed food. *International Journal of Molecular Sciences* 10, 3076-3105.
- Carlin, F., Nguyen-the, C., Silva, A. A., 1995. Factors affecting the growth of *Listeria monocytogenes* on minimally processed fresh endive. *Journal of Applied Bacteriology* 78, 636-646.
- Cordano, A. M., Jacquet, C., 2009. *Listeria monocytogenes* isolated from vegetable salads sold at supermarkets in Santiago, Chile: Prevalence and strain characterization. *International Journal of Food Microbiology* 132, 176-179.
- Crépet, A., Stahl, V., Carlin, F., 2009. Development of a hierarchical Bayesian model to estimate the growth parameters of *Listeria monocytogenes* in minimally processed fresh leafy salads. *International Journal of Food Microbiology* 131, 112-119.
- Ells, T. C., Hansen, L. T., 2006. Strain and growth temperature influence *Listeria* spp. attachment to intact and cut cabbage. *International Journal of Food Microbiology* 111, 34-42.
- Elviss, N. C., Little, C. L., Hucklesby, L., Sagoo, S., Surman-Lee, S., de Pinna, E., Threlfall, E. J., Food, Water and Environmental Surveillance Network, 2009. Microbiological study of fresh herbs from retail premises uncovers an international outbreak of salmonellosis. *International Journal of Food Microbiology* 134, 83-88.
- Farrar, J., Guzewich, J., 2009. Identification of the source of contamination. In: Sapers, G. M., Solomon, E. B., Matthews, K. R. (Eds.), *The Produce Contamination Problem – Causes and Solutions*. Elsevier, Oxford, pp. 49-78.
- Finn, M. J., Upton, M. E., 1997. Survival of Pathogens on Modified-Atmosphere-Packaged Shredded Carrot and Cabbage. *Journal of Food Protection* 60, 1347-1350.
- Fox, E., Hunt, K., O'Brien, M., Jordan, K., 2011. *Listeria monocytogenes* in Irish farmhouse cheese processing environments. *International Journal of Food Microbiology* 145, S39-S45.
- Fröder, H., Martins, C. G., De Souza, K. L. O., Landgraf, M., Franco, B. D. G. M., Destro, M. T., 2007. Minimally processed vegetable salads: Microbial quality evaluation. *Journal of Food Protection* 70, 1277-1280.
- Gálvez, A., Abriouel, H., López, R. L., Omar, N. B., 2007. Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology* 120, 51-70.

- Gandhi, M., Chikindas, M.L., 2007. *Listeria*: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology* 113, 1-15.
- Giusti, M., Aurigemma, C., Marinelli, L., Tufi, D., De Medici, D., Di Pasquale, S., De Vito, C., Boccia, A., 2010. The evaluation of the microbial safety of fresh ready-to-eat vegetables produced by different technologies in Italy. *Journal of Applied Microbiology* 109, 996-1006.
- Gleeson, E., O'Beirne, D., 2005. Effects of process severity on survival and growth of *Escherichia coli* and *Listeria innocua* on minimally processed vegetables. *Food Control* 16, 677-685.
- Gogo, L. A., Shitandi, A. A., Lokuruka, M. N. I., Sang, W., 2010. Antimicrobial effect of juice extract from fermented cabbage against select food-borne bacterial pathogens. *Journal of Applied Sciences Research* 6, 1807-1813.
- Goldberg, D., Kroupitski, Y., Belausov, E., Pinto, R., Sela, S., 2011, *Salmonella* Typhimurium internalization is variable in leafy vegetables and fresh herbs. *International Journal of Food Microbiology* 145, 250-257.
- Gorski, L., Parker, C. T., Liang, A., Cooley, M. B., Jay-Russell, M. T., Gordus, A. G., Atwill, E. R., Madrell, R. E., 2011. Prevalence, distribution, and diversity of *Salmonella enterica* in a major produce region of California. *Applied and Environmental Microbiology* 77, 2734-2748.
- Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H., Busta, F.F., 2003. Outbreaks associated with fresh produce: Incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety* 2, 78-141.
- Hsu, W. Y., Simonne, A., Jitareerat, P., 2006. Fates of seeded *Escherichia coli* O157:H7 and *Salmonella* on selected fresh culinary herbs during refrigerated storage. *Journal of Food Protection* 69, 1997-2001.
- IBGE - Instituto Brasileiro de Geografia e Estatística, 2011. Sistema IBGE de Recuperação Automática – SIDRA. Banco de Dados Agregados. Available in: <http://www.sidra.ibge.gov.br/bda/horti/default.asp?t=2&z=t&0=17&u1=1&u1=1&u3=1&u2=31>. Accessed on August 25, 2011.
- Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P., Jiang, X., 2004. Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. *Applied and Environmental Microbiology* 70, 2497-2502.

Ivanova, I., Miteva, V., Stefanova, T., Pantev, A., Budakov, I., Danova, S., Moncheva, P., Nikolova, I., Dousset, X., Boyaval, P., 1998. Characterization of a bacteriocin produced by *Streptococcus thermophiles* 81. *International Journal of Food Microbiology* 42, 147-158.

Jacxsens, L., Luning, P. A., van der Vorst, J. G. A. J., Devlieghere, F., Leemans, R., Uyttendaele, M., 2010. Simulation modeling and risk assessment as tools to identify the impact of climate change on microbiological food safety - the case study of fresh produce supply chain. *Food Research International* 43, 1925-1935.

Kakiomenou, K., Tassou, C., Nychas, G.-J., 1998. Survival of *Salmonella enteritidis* and *Listeria monocytogenes* on salad vegetables. *World Journal of Microbiology and Technology* 14, 383-387.

Koseki, S., Isobe, S., 2005a. Growth of *Listeria monocytogenes* on iceberg lettuce and solid media. *International Journal of Food Microbiology* 101, 217-225.

Koseki, S., Isobe, S., 2005b. Prediction of pathogen growth on iceberg lettuce under real temperature history during distribution from farm to table. *International Journal of Food Microbiology* 104, 239-248.

Liao, C. H., Fett, W. F., 2001. Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. *Journal of Food Protection* 64, 1110-1115.

Little, C. L., Gillespie, I. A., 2008. Prepared salads and public health. *Journal of Applied Microbiology* 105, 1729-1743.

Little, C. L., Taylor, F. C., Sagoo, S. K., Gillespie, I. A., Grant, K., McLauchlin, J., 2007. Prevalence and level of *Listeria monocytogenes* and other listeria species in retail pre-packaged mixed vegetable salads in the UK. *Food Microbiology* 24, 711-717.

Long, S. M., Adak, G. K., O'Brien, S. J., Gillespie, I. A., 2002. General outbreaks of infectious intestinal disease linked with salad vegetables and fruit, England and wales, 1992-2000. *Communicable Disease and Public Health / PHLS* 5, 101-105.

López-Gálvez, F., Gil, M. I., Truchado, P., Selma, M. V., Allende, A., 2010. Cross-contamination of fresh-cut lettuce after a short-term exposure during pre-washing cannot be controlled after subsequent washing with chlorine dioxide or sodium hypochlorite. *Food Microbiology* 27, 199-204.

- Lynch, M. F., Tauxe, R. V., Hedberg, C. W., 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. *Epidemiology and Infection* 137, 307-315.
- Ma, L., Zhang, G., Gerner-Smidt, P., Tauxe, R. V., Doyle, M. P., 2010. Survival and growth of *Salmonella* in salsa and related ingredients. *Journal of Food Protection* 73, 434-444.
- Matamoros, S., Pilet, M. F., Gigout, F., Prévost, H., Leroi, F., 2009. Selection and evaluation of seafood-borne psychrotrophic lactic acid bacteria as inhibitors of pathogenic and spoilage bacteria. *Food Microbiology* 26, 638-644.
- Matthews, K. R., 2009. Leafy vegetables. In: Sapers, G. M., Solomon, E. B., Matthews, K. R. (Eds.), *The Produce Contamination Problem – Causes and Solutions*. Elsevier, Oxford, pp. 165-188.
- Mintz, E. D., Cartter, M. L., Hadler, J. L., Wassell, J. T., Zingesser, J. A., Tauxe, R. V., 1994. Dose-response effects in an outbreak of *Salmonella* Enteritidis. *Epidemiology and Infection* 112, 13-23.
- Mora, A., León, S. L., Blanco, M., Blanco, J. E., López, C., Dahbi, G., Echeita, A., González, E. A., Blanco J., 2007. Phage types, virulence genes and PFGE profiles of Shiga toxin-producing *Escherichia coli* O157:H7 isolated from raw beef, soft cheese and vegetables in Lima (Peru). *International Journal of Food Microbiology* 114, 204-210.
- Musher, D. M., Musher, B. L., 2004. Contagious acute gastrointestinal infections. *New England Journal of Medicine* 351, 2417-2427.
- Nguyen-the, C., Carlin, F., 1994. The microbiology of minimally processed fresh fruits and vegetables. *Critical Reviews in Food Science and Nutrition* 34, 371-401.
- Noriega, E., Newman, J., Saggars, E., Robertson, J., Laca, A., Díaz, M., Brocklehurst, T. F., 2010. Antilisterial activity of carrots: Effect of temperature and properties of different carrot fractions. *Food Research International* 43, 2425-2431.
- Oliveira, F. A., Pasqualotto, A. P., Silva, W. P., Tondo, E. C., 2011. Characterization of *Salmonella* Enteritidis isolated from human samples. *Food Research International* doi:10.1016/j.foodres.2010.09.040
- Oliveira, M.A., Abeid Ribeiro, E. G., Morato Bergamini, A. M., & Pereira De Martinis, E. C., 2010. Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. *Food Microbiology* 27, 19-23.

Patel, J., Sharma, M., 2010. Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. *International Journal of Food Microbiology* 139, 41-47.

Perez, R., Ramos, A. M., Binoti, M. L., De Sousa, P. H. M., Machado, G. D. M., Cruz, I. B., 2008. Profile of minimally processed vegetable consumers in Belo Horizonte, Brazil. *Horticultura Brasileira* 26, 441-446.

Pirovani, M. E., Güemes, D. R., Di Pentima, J. H., Tessi, M. A., 2000. Survival of *Salmonella* Hadar after washing disinfection of minimally processed spinach. *Letters in Applied Microbiology* 31, 143-148.

Ponce, A. G., Moreira, M. R., del Valle, C. E., Roura, S. I., 2008. Preliminary characterization of bacteriocin-like substances from lactic acid bacteria isolated from organic leafy vegetables. *LWT – Food Science and Technology* 41, 432-441.

Ponniah, J., Robin, T., Paie, M. S., Radu, S., Ghazali, F. M., Kqueen, C. Y., Mitsuaki Nishibuchi, M., Nakaguchi, Y., Malakar, P.K., 2010. *Listeria monocytogenes* in raw salad vegetables sold at retail level in Malaysia. *Food Control* 21, 774-778.

Quiroz-Santiago, C., Rodas-Suárez, O. R., Vázquez Q., C. R., Fernández, F. J., Quiñones-Ramírez, E. I., Vázquez-Salinas, C., 2009. Prevalence of *Salmonella* in vegetables from Mexico. *Journal of Food Protection* 72, 1279-1282.

Ragaert, P., Verbeke, W., Devlieghere, F., Debevere, J., 2004. Consumer perception and choice of minimally processed vegetables and packaged fruits. *Food Quality and Preference* 15, 259-270.

Rostagno, M. H., Callaway, T. R., 2011. Pre-harvest risk factors for *Salmonella enterica* in pork production. *Food Research International* doi:10.1016/j.foodres.2011.04.041

Rudi, K., Flateland, S. L., Hanssen, J. F., Bengtsson, G., Nissen, H., 2002. Development and evaluation of a 16S ribosomal DNA array-based approach for describing complex microbial communities in ready-to-eat vegetable salads packed in a modified atmosphere. *Applied and Environmental Microbiology* 68, 1146-1156.

Rúgeles, L. C., Bai, J., Martínez, A. J., Vanegas, M. C., Gómez-Duarte, O. G., 2010. Molecular characterization of diarrheagenic *Escherichia coli* strains from stools samples and food products in Colombia. *International Journal of Food Microbiology* 138, 282-286.

Sant'Ana, A. S., Igarashi, M. C., Langraf, M., Destro, M. T., Franco, B. D. G., 2011b. Low occurrence and populations of *Listeria monocytogenes* in ready-to-eat

vegetables marketed in São Paulo, Brazil, with predominance of serotype 4b and ribotype DUP-1038 and-variable growth kinetics, susceptibility to chlorine and ability to form biofilm. Applied and Environmental Microbiology. Submitted.

Sant'Ana, A. S., Landgraf, M., Destro, M. T., Franco, B. D. G. M., 2011a. Prevalence and counts of *Salmonella* spp. in minimally processed vegetables in São Paulo, Brazil. Food Microbiology 28, 1235-1237.

Scott, V.N., Clavero, R.S., Troller, J.A., 2001. Measurement of water activity (a_w), acidity, and Brix. In Downes, F.P., & Ito, K. (Eds.), *Compendium of methods for the microbiological examination of foods* (pp. 649-659). Washington, DC: APHA.

Silva, F.A.S., Azevedo, C.A.V., 2002. Versão do programa computacional Assistat para o sistema operacional Windows. Revista Brasileira de Produtos Agroindustriais 4, 71-78.

Silva, N., Silveira, N. F. A., Yokoya, F., Okazaki, M. M., 2003. Occurrence of *Escherichia coli* 0157:H7 in vegetables and its resistance to the disinfectants used in fresh produce. Ciência e Tecnologia de Alimentos 23, 167-173.

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

Skalina, L., Nikolajeva, V., 2010. Growth potential of *Listeria monocytogenes* strains in mixed ready-to-eat salads. International Journal of Food Microbiology 144, 317-321.

Takeuchi, K., Mytle, N., Lambert, S., Coleman, M., Doyle, M. P., Smith, M. A., 2006. Comparison of *Listeria monocytogenes* virulence in a mouse model. Journal of Food Protection 69, 842-846.

Tauxe, R. V., 1997. Emerging foodborne diseases: An evolving public health challenge. Emerging Infectious Diseases 3, 425-434.

Teplitski, M., Noel, J. T., Alagely, A., Danyluk, M., 2011. Functional genomics studies shed light on the nutrition and gene expression of non-typhoidal *Salmonella* and enterovirulent *E. coli* in produce. Food Research International doi:10.1016/j.foodres.2011.06.020

Todorov, S. D., Dicks, L. M. T., 2005. *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against gram-negative bacteria. Enzyme and Microbial Technology 36, 318-326.

- Trias R, Bañeras L, Badosa E, Montesinos E., 2008. Bioprotection of Golden Delicious apples and Iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. *International Journal of Food Microbiology* 123, 50-60.
- Uyttendaele, M., Busschaert, P., Valero, A., Geeraerd, A. H., Vermeulen, A., Jacxsens, L., Goh, K. K., De Loy, A., Van Impe, J. F., Devlieghere, F., 2009. Prevalence and challenge tests of *Listeria monocytogenes* in Belgian produced and retailed mayonnaise-based deli-salads, cooked meat products and smoked fish between 2005 and 2007. *International Journal of Food Microbiology* 133, 94-104.
- Van Reenen, C. A., Chikindas, M. L., Van Zyl, W. H., Dicks, L. M. T., 2002. Characterization and heterologous expression of a class IIa bacteriocin, plantaricin 423 from *Lactobacillus plantarum* 423, in *Saccharomyces cerevisiae*. *International Journal of Food Microbiology* 81, 29–40.
- Warriner, K., Namvar, A., 2009. What is the hysteria with *Listeria*? *Trends in Food Science and Technology* 20, 245-254.
- Williams, D., Irvin, E. A., Chmielewski, R. A., Frank, J. F., Smith, M. A., 2007. Dose-response of *Listeria monocytogenes* after oral exposure in pregnant guinea pigs. *Journal of Food Protection* 70, 1122-1128.

Table 1: Growth potential (δ) of *Salmonella* spp. and *Listeria monocytogenes* in nine types of RTE vegetables under different storage conditions.

Vegetables	pH	Storage conditions ^a	δ ($\log_{10}$) ^b	
			<i>L. monocytogenes</i>	<i>Salmonella</i> spp.
Escarole	6.2	I	0.49	1.03
		II	1.82	1.91
		III	1.92	2.80
Collard green	6.8	I	0.30	-0.71
		II	2.61	0.57
		III	3.34	2.62
Cabbage	7.2	I	-0.21	-0.92
		II	0.64	-0.53
		III	0.85	0.31
Spinach	6.2	I	0.88	0.13
		II	1.19	1.49
		III	1.83	1.68
Watercress	6.3	I	0.21	-0.03
		II	1.13	1.75
		III	2.69	2.39
Arugula	6.6	I	1.85	2.11
		II	3.18	2.81
		III	3.22	4.05
Carrot	6.7	I	-3.61	-0.44
		II	-2.32	-0.51
		III	-1.37	0.93

Cont.

Green salad	6.4	I	0.51	0.46
		II	2.39	1.19
		III	2.62	2.61
Yakisoba ^c	6.7	I	0.38	0.05
		II	0.97	0.92
		III	1.59	1.33

^aStorage conditions: I [100% of shelf-life (6 days) at 7°C±1°C], II [30% of shelf-life (6 days) at 7°C±1°C and 70% at 15°C±1°C] and III [100% of shelf-life (6 days) at 15°C±1°C]; ^bGrowth was assigned when δ value was higher than 0.5 log₁₀; ^cComposed by cauliflower, carrot, broccoli, cabbage and chard.

Table 2: Populations of aerobic microorganisms and lactic acid bacteria in nine different types of RTE vegetables after storage at variable temperature conditions^{a,b}.

Vegetables	Storage conditions	Total plate count (log ₁₀ CFU/g)		Lactic acid bacteria (log ₁₀ CFU/g)	
		Day “0”	Day “6”	Day “0”	Day “6”
Escarole	I		7.48±0.05 ^{a,B}		6.29±0.03 ^{a,C}
	II	5.46±0.03 ^b	8.77±0.29 ^{a,A}	5.09±0.25 ^b	7.47±0.01 ^{a,A}
	III		8.28±0.08 ^{a,AB}		6.90±0.16 ^{a,B}
Collard green	I		7.62±0.03 ^{a,B}		6.33±0.01 ^{b,C}
	II	5.91±0.03 ^b	9.91±0.01 ^{a,A}	5.02±0.41 ^b	6.72±0.06 ^{b,B}
	III		9.93±0.02 ^{a,A}		8.94±0.02 ^{a,A}
Cabbage	I		8.65±0.07 ^{a,A}		7.12±0.03 ^{a,B}
	II	7.67±0.09 ^b	8.72±0.02 ^{a,A}	6.00±0.16 ^b	7.97±0.03 ^{a,A}
	III		7.82±0.05 ^{b,B}		7.15±0.03 ^{a,B}
Spinach	I		7.56±0.10 ^{b,C}		6.92±0.03 ^{a,C}
	II	7.08±0.08 ^b	8.55±0.04 ^{a,A}	6.54±0.08 ^b	7.10±0.03 ^{a,B}
	III		8.17±0.03 ^{a,B}		7.51±0.03 ^{a,A}
Watercress	I		7.11±0.41 ^{b,B}		3.00±2.00 ^{b,A}
	II	7.15±0.06 ^b	9.00±0.08 ^{a,A}	5.92±0.51 ^b	6.29±0.03 ^{b,A}
	III		10.0±0.04 ^{a,A}		8.66±0.08 ^{a,A}
Arugula	I		8.93±0.07 ^{a,A}		7.47±0.02 ^{a,B}
	II	8.11±0.16 ^b	9.03±0.03 ^{a,A}	6.17±0.01 ^b	8.53±0.01 ^{a,A}
	III		9.01±0.03 ^{a,A}		8.61±0.12 ^{a,A}

Cont.

Carrot	I		7.91±0.04 ^{a,B}		7.18±0.03 ^{a,C}
	II	6.15±0.02 ^b	8.86±0.10 ^{a,A}	5.88±0.01 ^b	7.81±0.01 ^{a,B}
	III		8.55±0.09 ^{a,A}		8.63±0.09 ^{a,A}
Green salad	I		8.74±0.13 ^{a,B}		7.47±0.07 ^{a,A}
	II	5.79±0.04 ^b	9.26±0.07 ^{a,A}	5.13±0.05 ^b	7.06±0.02 ^{a,B}
	III		9.11±0.05 ^{a,AB}		7.12±0.04 ^{a,B}
Yakisoba	I		7.33±0.10 ^{b,A}		3.71±1.59 ^{b,A}
	II	6.80±0.09 ^b	7.22±0.26 ^{b,A}	5.04±0.14 ^b	6.22±0.08 ^{a,A}
	III		7.61±0.04 ^{a,A}		5.77±0.01 ^{a,A}

^aDifferent lowercase letters in the same rows indicate significant difference ($p < 0.05$) between the samples at day “0” and “6” according to *t* test. ^bDifferent superscript letters in a column indicate significant difference ($p < 0.05$) among the same type of vegetable stored at different conditions in the end of shelf-life (day “6”) according to Tukey’s test.

CAPÍTULO 5

“Influence of storage scenarios and type of packaging on growth potential of *Salmonella* spp. and *Listeria monocytogenes* in ready-to-eat lettuce and collard green”

Artigo formatado para publicação em “Food Control”

Influence of storage scenarios and type of packaging on growth potential of *Salmonella* spp. and *Listeria monocytogenes* in ready-to-eat lettuce and collard green

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Running title: Growth of *Salmonella* and *L.monocytogenes* in lettuce and collard green.

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Abstract:

This study aimed at determining the effects of different storage scenarios on the growth potential (δ) of *Salmonella* spp. and *Listeria monocytogenes* in ready-to-eat (RTE) mixes of iceberg and crisp lettuces (*Lactuca sativa*) and collard greens (*Brassica oleracea*). After purchase, the vegetables were submitted to minimal processing, experimentally contaminated to achieve 10^1 and 10^2 CFU/g, packed in modified atmosphere and perforated films and submitted to the following storage scenarios: I= 100% of the shelf-life (6 days) at 7°C; II= 70% of shelf-life at 7°C and 30% at 15°C; III= 30% at 7°C and 70% at 15°C; IV=100% at 15°C. *Salmonella* grew better in the mix of lettuces than in collard greens, while the opposite occurred with *L. monocytogenes*. Keeping the RTE vegetables at 15°C, during whole shelf-life (scenario IV) or part of it (scenarios II and III) markedly influenced the growth of both pathogens. Inhibition of growth of *Salmonella* spp and *L. monocytogenes* was more pronounced in samples packed with perforated films than in those submitted to modified atmosphere. The findings indicate that even contamination as low as 10^1 CFU/g can lead to high populations if there is abuse in the storage temperature (15°C). These results highlight the importance of good hygienic practices and attention to the cold chain for the control of *Salmonella* and *L. monocytogenes* in RTE vegetables.

Keywords: *Salmonella*, *Listeria monocytogenes*, lettuce, collard green, ready-to-eat, growth potential.

1) Introduction

The consumption of fruits and vegetables is a key factor for a healthy diet (Dembitsky et al., 2011, Hervert-Hernández et al., 2011). However, factors such as fast spoilage, difficult access to fresh products and lack of variety and convenience are major barriers for a larger consumption of these products. Minimal processing technology was developed to overcome these challenges, contributing to the increased demand for these products (Francis et al., 1999, Cruz et al., 2006).

The market of ready-to-eat (RTE) vegetables has increased significantly in the last two decades, but the fresh produce industry face big technological challenges because of the association of these products with foodborne disease outbreaks (Harris et al., 2003, Sivapalasingam et al., 2004, Little and Gillespie, 2008, Lynch et al., 2009). In consequence, several measures to prevent contamination of vegetables in the field, cross-contamination and recontamination during processing and growth of microorganisms during commercialization and consumption have been proposed (Doyle and Erickson, 2011, Jacobsen and Bech, 2011, López-Gálvez et al., 2009, López-Gálvez et al., 2010, Luo et al., 2011, Trias et al., 2008, Mahmoud et al., 2010, Oliveira et al., 2010).

Contributing strategies to safeguard food safety at commercialization and consumption levels include increase of consumer awareness of the risks associated to these products (Tsakiridou et al., 2011, Ragaert et al., 2004) and use of appropriate packaging systems to control the growth of microbial pathogens, such as modified atmosphere (MA) and new packaging materials (Francis et al., 1999, Mangaraj et al., 2009, Oliveira et al., 2010, Finn and Upton, 1997). MA consists in the alteration of the inner atmosphere in which a food product is packaged using a combination of gases such as oxygen, dioxide carbon and nitrogen (Rojas-Graü et al., 2009, Mangaraj et al., 2009). This technique has been successfully employed to delay the biochemical, microbial and chemical processes that may shorten the shelf-life of RTE vegetables (Rojas-Graü et al., 2009, Mangaraj et al., 2009). Alternatively, perforated films have been used to pack fresh vegetables at lower cost than MA. Perforations increase the gas permeability of the film, and the exchange rates of oxygen and carbon dioxide with the external environment are improved, avoiding the

negative effects of anaerobic conditions inside the packages (Rai et al., 2009, Rodrigues-Aguilera and Oliveira, 2009, Rai et al., 2011).

Although several studies have been performed to understand the behavior of foodborne pathogens in RTE vegetables (Oliveira et al., 2010, Koseki and Isobe, 2005a,b), to the best of our knowledge no studies have described the fate of pathogens in vegetables packaged in perforated films. Due to the increasing use of this kind of films for packaging of RTE vegetables in Brazil, this study was carried out to determine the growth potential of *Salmonella* and *L. monocytogenes* experimentally added to lettuce and collard green samples packed in perforated films and stored under different storage scenarios.

2) Material and Methods

2.1) Preparation of inocula:

The study was carried out with two strains of *Salmonella* Typhimurium (#277 and 386) isolated from RTE vegetables in Brazil (Sant'Ana et al., 2011a) and one collection strain (*S. Enteritidis* ATCC 13076) and three strains of *Listeria monocytogenes* (#221, 349 and 480) also isolated from RTE vegetables in Brazil (Sant'Ana et al., 2011b). *L. monocytogenes* belonged to serotypes 1/2b, 4b and 4b and ribotypes DUP 1038, 19175 and 19191, respectively. Suspensions of *Salmonella* spp and *L. monocytogenes* were prepared growing the strains separately in Tryptic soy broth (TSB) and TSB supplemented with 0.6% of yeast extract (TSB-YE) at 37°C for 24h, respectively. The broths were centrifuged at 6°C for 10 min at 2810 × *g* (Mikro 22R, Hettich Zentrifugen, Germany), and the pellets were suspended in phosphate buffered saline pH 6.0. The procedure was repeated three times. Pools of cells were prepared by mixing equal volumes of each cell suspension, adjusted to contain 10⁸ CFU/mL using a spectrophotometer (OD₆₃₀=0.5). Cell concentrations were checked by plating in Tryptic Soy Agar (TSA) (*Salmonella*) and TSA-YE (*L. monocytogenes*).

2.2) Minimal processing and inoculation of vegetables:

Fresh lettuces (iceberg - *Lactuca sativa* var. *capitata* and crisp - *Lactuca sativa* var. *crispa*) and collard greens (*Brassica oleracea*) were acquired in supermarkets in the city of Sao Paulo, Brazil. These vegetables were chosen either based on high consumption in Brazil (IBGE, 2007), on the incidence of *Salmonella* and *L. monocytogenes* (Sant'Ana et al., 2011a,b) and on previous studies on the growth potential of these pathogens in fresh produce (Sant'Ana et al., 2012). The core of lettuces and stalks of collard greens were removed, and only internal and undamaged leaves were used. The two types of lettuces were mixed in the same proportion (1:1) to compose the mix of most consumed type of lettuces in Brazil. Leaves were washed in chlorinated tap water and shredded into strips with a disinfected knife. The vegetables were sanitized with Sumaveg solution (JohnsonDiversey, Sao Paulo, Brazil) at 100ppm for 15 min, with the concentration of chlorine being checked with pH paper test strips (pHydrion, Micro Chlorine, Johnson Wax Professional, Sturtevant, USA). Then, vegetables were rinsed in sterile distilled water and let dry in a laminar flow cabinet for 30 min. Inoculation of vegetables with *Salmonella* and *L. monocytogenes* was carried out in a biosafety cabinet. Four liters of sterile distilled water were added to sterile plastic bags, hold in buckets, and inoculated with each *Salmonella* and *L. monocytogenes* suspension. Two separate sets were prepared: one to achieve a final concentration in the vegetables of 10^1 CFU/g and the other to achieve 10^2 CFU/g. The vegetables were soaked into the contaminated water for 15 min, and then spinned in a sanitized salad spinner (Model DD1056Y, Casa da Moda, Brazil). Control samples were obtained by the same experimental approach, soaking the vegetables in sterile water only.

2.3) Packaging of RTE vegetables and storage scenarios:

Twenty five grams of each experimentally contaminated RTE vegetable were packaged in plastic films under modified atmosphere and in perforated films. The selected plastic film is the one commonly used to pack RTE vegetables in Brazilian industries, being composed by bi-orientated polypropylene (BOPP) (outer layer) and a blend of low density polyethylene (LDPE) with linear low-density polyethylene (LLDPE) or a coextruded

structure of LDPE and LLDPE (inner layer). The features of the plastic film were: thickness: 62 μm , O_2 permeability: $1.375 \text{ m}^3 \text{ m}^{-2} \text{ day}^{-1}$ at 23°C and water steam permeability: $3.5 \text{ g water m}^{-2} \text{ day}^{-1}$ at 38°C and 90% relative humidity. Modified atmosphere packaging was obtained in a vacuum sealing machine AP 500 (Tecmaq, Sao Paulo, Brazil), and flush of a gaseous mixture containing 5% O_2 , 15% CO_2 and 80% N_2 (White Martins, Osasco, Brazil), the same used by RTE vegetable processors in Sao Paulo. Four holes ($\varnothing = 0.3\text{mm}$ each) were done in each side of the plastic films, at the top and bottom, before vegetable packaging.

The packaged RTE vegetables were stored simulating the recommended temperature ($\leq 7^\circ\text{C}$) and abuse temperature (15°C), for six days (shelf-life), under four storage scenarios: scenario I (100% of shelf-life at $7^\circ\text{C} \pm 1^\circ\text{C}$), scenario II (70% of shelf-life at $7^\circ\text{C} \pm 1^\circ\text{C}$ and 30% of shelf-life at $15^\circ\text{C} \pm 1^\circ\text{C}$), scenario III (30% of shelf-life at $7^\circ\text{C} \pm 1^\circ\text{C}$ and 70% of shelf-life at $15^\circ\text{C} \pm 1^\circ\text{C}$) and scenario IV (100% of shelf-life at $15^\circ\text{C} \pm 1^\circ\text{C}$). The samples were prepared and triplicates and the experiments were repeated two times for the two types of vegetables (fresh lettuces and collard greens), totalizing 384 packages.

2.4) Microbiological analysis and determination of growth potential (δ):

Salmonella spp. and *L. monocytogenes* were enumerated in the inoculated vegetable samples at the beginning (T_0) and at the end (T_f) of shelf-life, in the four studied storage scenarios. For enumeration of *Salmonella* spp., samples were stomached with 0.1% peptone water, submitted to decimal serial dilutions and surface plated on mannitol lysine crystal violet brilliant green (MLCB) agar, and incubated at 37°C for 24h. *L. monocytogenes* was counted in Oxford agar (OXA) supplemented with selective supplement SR0206, incubated at 37°C for 48h. Up to three characteristic colonies of each pathogen per condition studied were picked and submitted to confirmation by polyvalent serotyping (*Salmonella* spp.) or biochemical tests (*L. monocytogenes*) (Anonymous, 1998). All culture media were from Oxoid (Basingstoke, UK) unless otherwise stated.

The growth potential (δ) of the pathogens was estimated calculating the difference between the counts in T_f and T_0 , expressed in $\log_{10}$ (Anon, 2008). When δ was equal or higher than the uncertainty of the microbiological method ($\geq 0.5 \log_{10}$),

results were interpreted as capability of growth in the tested condition. A δ below 0.5 $\log_{10}$ was interpreted as absence of growth.

3) Results and Discussion

Tables 1 and 2 depict the growth potentials (δ) of *Salmonella* and *L. monocytogenes* in the experimentally contaminated RTE lettuces and collard green, respectively. The effect of the temperature on growth is clearly observed, regardless the packaging system, type of vegetable or inoculum level. The lowest growth potential (δ) values were observed at storage condition I (7°C), while at storage condition IV (15°C) δ values were up to four times higher.

Previous studies have shown that growth of pathogens such as *Salmonella* and *L. monocytogenes* in fresh produce is limited when the temperature is below 10°C (Koseki et al., 2005 a,b, Carrasco et al., 2008, Oliveira et al., 2010). Koseki et al. (2005b) reported that despite not growing in RTE lettuce at 5°C, *Salmonella* could grow when the temperature was 10°C or above. Growth rates and lag times at 10°C and 15°C were 0.02 and 0.13 and 35.1 and 6.45 hours, respectively. For *L. monocytogenes*, Koseki et al. (2005a) observed growth in RTE lettuce at 5°C, reporting that growth rates at 5°C and 15°C were 0.021 and 0.090, respectively and the lag times were 60.1 hours and 10.2 hours, respectively.

Data from this study indicate that keeping RTE lettuces and collard green at 15°C, during whole shelf-life (scenario IV) or part of it (scenarios II and III) markedly influenced the δ of both pathogens. These results highlight the importance of the cold chain in controlling the growth of *Salmonella* and *L. monocytogenes* in RTE vegetables. According to Little and Gillespie (2008), inadequate storage temperature is one of the three main faults contributing for the occurrence of salads outbreaks. Therefore, exposure of these products to temperature abuse anytime of shelf-life may lead to the growth of pathogens such as *Salmonella* and *L. monocytogenes* to populations likely to represent a food safety problem.

The growth potential (δ) of *Salmonella* and *L. monocytogenes* varied according to the type of vegetable. *Salmonella* grew better in the mix of lettuces than in collard greens, while the opposite occurred with *L. monocytogenes*. These results corroborate recent data published by Patel and Sharma (2010), who reported

stronger attachment strength of *Salmonella* on Iceberg and Romaine lettuces than on cabbage. Attachment to the phyllosphere of vegetables is a key point driving further survival and growth of foodborne pathogens in fresh produce (Brandl, 2006, Teplitski et al., 2012).

The δ values when the vegetables were packed in perforated films were lower than those obtained when the vegetables were packaged under modified atmosphere, regardless the type of vegetable. Data from literature has indicated that the survival and even growth of *Salmonella* and *L. monocytogenes* are favored under modified atmosphere (Francis and O'Beirne, 1997, Francis and O'Beirne, 1998, Scifò et al., 2009, Horev et al., 2012). This behavior seems to be related to the direct bacteriostatic effect of carbon dioxide on the background microbiota, leading to significant qualitative and quantitative changes in the microbial ecology, resulting in the prevalence of undesired pathogenic bacteria (Devlieghere et al., 2001, Francis and O'Beirne, 1997, Francis and O'Beirne, 1998, Devlieghere and Debevere, 2000). In perforated packages, due to the high oxygen tension, rapid growing aerobic microorganisms will prevail and growth of pathogens will be slowed down (Oliveira et al., 1998, Jacksens et al., 2001, Mangaraj et al., 2009).

The results are of concern because lettuce and collard green are amongst the most consumed RTE vegetables in Brazil (IBGE, 2007). Several studies in Brazil and abroad have reported the occurrence of *Salmonella* and *L. monocytogenes* in several types of RTE vegetables, including lettuce and collard green (Fröder et al., 2007, Little et al., 2007, Abadias et al., 2008, Cordano and Jacquet, 2009, Sant'Ana et al., 2011). A previous study has also indicated that arugula; collard greens and mix of lettuces were the vegetables that best supported the growth of *Salmonella* and *L. monocytogenes* (Sant'Ana et al., 2012).

Despite the fact that perforated packages, which are increasingly being used, inhibit in some extent the growth of *Salmonella* and *L. monocytogenes* in RTE vegetables, proper producers and consumers practices need to be stressed.

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4) References

- Anonymous. 1998. ISO 11290-2:1998 - Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 2: Enumeration method. Switzerland.
- Anonymous, 2008. Guidance document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. SANCO/1628/2008 ver. 9.3 (26112008). Brussels.
- Brandl, M. T., 2006. Fitness of human enteric pathogens on plants and implications for food safety. Annual Review of Phytopathology 44, 367-392.
- Carrasco, E., Pérez-Rodríguez, F., Valero, A., García-Gimeno, R. M., Zurera, G., 2008. Growth of *Listeria monocytogenes* on shredded, ready-to-eat iceberg lettuce. Food Control 19, 487-494.
- Dembitsky, V. M., Poovarodom, S., Leontowicz, H., Leontowicz, M., Vearasilp, S., Trakhtenberg, S., Gorinstein, S., 2011. The multiple nutrition properties of some exotic fruits: Biological activity and active metabolites. Food Research International 44, 1671-1701.
- Devlieghere, F., Debevere, J., 2000. Influence of dissolved carbon dioxide on the growth of spoilage bacteria. LWT - Food Science and Technology 33, 531-537.
- Devlieghere, F., Debevere, J., Van Impe, J., 1998. Concentration of carbon dioxide in the water-phase as a parameter to model the effect of a modified atmosphere on microorganisms. International Journal of Food Microbiology 43, 105-113.
- Doyle, M. P., Erickson, M. C., 2011. Opportunities for mitigating pathogen contamination during on-farm food production. International Journal of Food Microbiology, doi: 10.1016/j.ijfoodmicro.2011.02.037
- Finn, M. J., Upton, M. E., 1997. Survival of pathogens on modified-atmosphere-packaged shredded carrot and cabbage. Journal of Food Protection 60, 1347-1350.
- Francis, G. A., O'Beirne, D., 1998. Effects of storage atmosphere on *Listeria monocytogenes* and competing microflora using a surface model system. International Journal of Food Science and Technology 33, 465-476.
- Francis, G. A., O'Beirne, D. O., 1997. Effects of gas atmosphere, antimicrobial dip and temperature on the fate of *Listeria innocua* and *Listeria monocytogenes* on

minimally processed lettuce. *International Journal of Food Science and Technology* 32, 141-151.

Francis, G. A., Thomas, C., O'Beirne, D., 1999. The microbiological safety of minimally processed vegetables. *International Journal of Food Science and Technology* 34, 1-22.

Hervet-Hernández, D., García, O. P., Rosado, J. L., Goñi, I., 2011. The contribution of fruits and vegetables to dietary intake of polyphenols and antioxidant capacity in a Mexican rural diet: Importance of fruit and vegetable variety. *Food Research International* 44, 1182-1189.

Horev, B., Sela, S., Vinokur, Y., Gorbatshevich, E., Pinto, R., Rodov, V., 2011. The effects of active and passive modified atmosphere packaging on the survival of *Salmonella enterica* serotype Typhimurium on washed romaine lettuce leaves. *Food Research International*, doi: 10.1016/j.foodres.2011.05.037

IBGE - Instituto Brasileiro de Geografia e Estatística, 2011. Sistema IBGE de Recuperação Automática – SIDRA. Banco de Dados Agregados. Available in: <http://www.sidra.ibge.gov.br/bda/horti/default.asp?t=2&z=t&0=17&u1=1&u1=1&u3=1&u2=31>. Accessed on September 07, 2011.

Jacobsen, C. S., Bech, T. B., 2011. Soil survival of salmonella and transfer to freshwater and fresh produce. *Food Research International*, doi:10.1016/j.foodres.2011.07.026

Koseki, S., Isobe, S., 2005a. Growth of *Listeria monocytogenes* on iceberg lettuce and solid media. *International Journal of Food Microbiology* 101, 217-225.

Koseki, S., Isobe, S., 2005b. Prediction of pathogen growth on iceberg lettuce under real temperature history during distribution from farm to table. *International Journal of Food Microbiology* 104, 239-248.

Little, C. L., Gillespie, I. A., 2008. Prepared salads and public health. *Journal of Applied Microbiology* 105, 1729-1743.

Little, C. L., Taylor, F. C., Sagoo, S. K., Gillespie, I. A., Grant, K., McLauchlin, J., 2007. Prevalence and level of *Listeria monocytogenes* and other listeria species in retail pre-packaged mixed vegetable salads in the UK. *Food Microbiology* 24, 711-717.

López-Gálvez, F., Allende, A., Selma, M. V., Gil, M. I., 2009. Prevention of *Escherichia coli* cross-contamination by different commercial sanitizers during

washing of fresh-cut lettuce. *International Journal of Food Microbiology* 133, 167-171.

López-Gálvez, F., Gil, M. I., Truchado, P., Selma, M. V., Allende, A., 2010. Cross-contamination of fresh-cut lettuce after a short-term exposure during pre-washing cannot be controlled after subsequent washing with chlorine dioxide or sodium hypochlorite. *Food Microbiology* 27, 199-204.

Luo, Y., Nou, X., Yang, Y., Alegre, I., Turner, E., Feng, H., Abadias, M., Conway, W., 2011. Determination of free chlorine concentrations needed to prevent *Escherichia coli* O157:H7 cross-contamination during fresh-cut produce wash. *Journal of Food Protection* 74, 352-358.

Mahmoud, B. S. M., Bachman, G., Linton, R. H., 2010. Inactivation of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* and *Shigella flexneri* on spinach leaves by X-ray. *Food Microbiology* 27, 24-28.

Mangaraj, S., Goswami, T. K., Mahajan, P. V., 2009. Applications of Plastic Films for Modified Atmosphere Packaging of Fruits and Vegetables: A Review. *Food Engineering Reviews* 1, 133-158.

Mintz, E. D., Cartter, M. L., Hadler, J. L., Wassell, J. T., Zingesser, J. A., Tauxe, R. V., 1994. Dose-response effects in an outbreak of *Salmonella* Enteritidis. *Epidemiology and Infection* 112, 13-23.

Oliveira, F. A. R., Fonseca, S. C., Oliveira, J. C., Brecht, J. K., Chau, K. V., 1998. Development of perforation-mediated modified atmosphere packaging to preserve fresh fruit and vegetable quality after harvest. *Food Science and Technology International* 4, 339-352.

Oliveira, M., Usall, J., Solsona, C., Alegre, I., Viñas, I., Abadias, M., 2010. Effects of packaging type and storage temperature on the growth of foodborne pathogens on shredded 'Romaine' lettuce. *Food Microbiology* 27, 375-380.

Patel, J., Sharma, M., 2010. Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. *International Journal of Food Microbiology* 139, 41-47.

Ragaert, P., Verbeke, W., Devlieghere, F., Debevere, J., 2004. Consumer perception and choice of minimally processed vegetables and packaged fruits. *Food Quality and Preference* 15, 259-270.

- Rai, D. R., Jha, S. N., Wanjari, O. D., Patil, R. T., 2009. Chromatic Changes in Broccoli (*Brassica oleracea italica*) under Modified Atmospheres in Perforated Film Packages. Food Science and Technology International. 15, 387-395.
- Rai, D. R., Kaur, P., Patil, R. T., 2011. Quality changes in fresh-cut *Capsicum* (*Capsicum annuum*) shreds under modified atmospheres during simulated retail and home storage. Journal of Food Processing and Preservation 35, 402–409.
- Rojas-Graü, M. A., Oms-Oliu, G., Soliva-Fortuny, R., Martín-Belloso, O., 2009. The use of packaging techniques to maintain freshness in fresh-cut fruits and vegetables: A review. International Journal of Food Science and Technology 44, 875-889.
- Sant'Ana, A. S., Barbosa, M. S., Landgraf, M., Destro, M. T., Franco, B. D. G. M., 2012. Growth potential of *Salmonella* spp. and *Listeria monocytogenes* in nine types of ready-to-eat vegetables stored at variable temperature conditions during shelf-life. International Journal of Food Microbiology. Submitted.
- Sant'Ana, A. S., Landgraf, M., Destro, M. T., Franco, B. D. G. M., 2011a. Prevalence and counts of *Salmonella* spp. in minimally processed vegetables in São Paulo, Brazil. Food Microbiology 28, 1235-1237.
- Sant'Ana, A. S., Igarashi, M. C., Langraf, M., Destro, M. T., Franco, B. D. G., 2011b. Prevalence, populations and pheno- and genotypic characteristics of *Listeria monocytogenes* isolated from ready-to-eat vegetables marketed in São Paulo, Brazil. International Journal of Food Microbiology. Submitted.
- Scifò, G. O., Randazzo, C. L., Restuccia, C., Fava, G., Caggia, C., 2009. *Listeria innocua* growth in fresh cut mixed leafy salads packaged in modified atmosphere. Food Control 20, 611-617.
- Teplitski, M., Noel, J. T., Alagely, A., Danyluk, M., 2011. Functional genomics studies shed light on the nutrition and gene expression of non-typhoidal *Salmonella* and enterovirulent *E. coli* in produce. Food Research International doi:10.1016/j.foodres.2011.06.020
- Trias, R., Bañeras, L., Badosa, E., Montesinos, E., 2008. Bioprotection of golden delicious apples and iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. International Journal of Food Microbiology 123, 50-60.
- Tsakiridou, E., Mattas, K., Tsakiridou, H., Tsiamparli, E., 2011. Purchasing fresh produce on the basis of food safety, origin, and traceability labels. Journal of Food Products Marketing 17, 211-226.

Table 1: Growth potential (δ) of *Salmonella* spp. and *L. monocytogenes* inoculated in RTE mix of lettuces, packed under modified atmosphere and perforated films, and submitted to four storage scenarios.

Vegetable	Initial inoculum level (CFU/g)	Type of packaging	Storage scenarios ^a	δ^b <i>Salmonella</i> spp.	δ^b <i>L. monocytogenes</i>
Mix of lettuces	10 ¹	Modified atmosphere	I	0.90	-0.34
			II	1.18	1.92
			III	2.68	2.38
			IV	3.04	3.24
		Perforated film	I	-0.27	-0.32
			II	1.81	0.90
			III	1.79	1.06
			IV	2.98	2.03
	10 ²	Modified atmosphere	I	1.38	1.27
			II	2.79	1.97
			III	3.57	3.31
			IV	4.10	4.50
		Perforated film	I	-0.41	0.79
			II	1.74	2.36
			III	2.04	3.08
			IV	2.82	3.06

^aStorage scenarios: I (100% of shelf-life at 7°C±1°C), II (70% at 7°C±1°C and 30% at 15°C±1°C), III (30% at 7°C±1°C and 70% at 15°C±1°C) and IV (100% of shelf-life at 15°C±1°C); ^bAbility to grow was assigned when $\delta \geq 0.5 \log_{10}$.

Table 2: Growth potential (δ) of *Salmonella* spp. and *L. monocytogenes* inoculated in RTE collard greens, packed under modified atmosphere and perforated films, and submitted to four storage scenarios.

Vegetable	Initial inoculum level (CFU/g)	Type of packaging	Storage scenarios ^a	δ^b <i>Salmonella</i> spp.	δ^b <i>L. monocytogenes</i>
Collard greens	10 ¹	Modified atmosphere	I	1.14	1.04
			II	1.38	1.92
			III	1.54	3.81
			IV	2.80	4.32
		Perforated film	I	0.16	-0.14
			II	0.44	1.84
			III	0.73	3.08
			IV	1.89	3.85
	10 ²	Modified atmosphere	I	1.47	1.80
			II	1.57	3.78
			III	2.37	4.08
			IV	4.13	5.57
		Perforated film	I	-0.29	1.43
			II	1.75	2.89
			III	1.57	3.66
			IV	2.57	4.88

^aStorage conditions: I (100% of shelf-life at 7°C±1°C), II (70% at 7°C±1°C and 30% at 15°C±1°C), III (30% at 7°C±1°C and 70% at 15°C±1°C) and IV (100% of shelf-life at 15°C±1°C); ^bAbility to grow was assigned when $\delta \geq 0.5 \log_{10}$.

CAPÍTULO 6

“Modeling the growth rate and lag time of different strains of *Salmonella enterica* and *Listeria monocytogenes* in ready-to-eat lettuce”

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Modeling the growth rate and lag time of different strains of *Salmonella enterica* and *Listeria monocytogenes* in ready-to-eat lettuce

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Abstract

The growth parameters (growth rate, μ and lag time, λ) of three different strains each of *Salmonella enterica* and *Listeria monocytogenes* in minimally processed lettuce (MPL) and their changes as a function of temperature were modeled. MPL were packed under modified atmosphere (5% O₂, 15% CO₂ and 80% N₂), stored at 7-30°C and samples collected at different time intervals were enumerated for *S. enterica* and *L. monocytogenes*. Growth curves and equations describing the relationship between μ and λ as a function of temperature were constructed using the DMFit Excel add-in and through linear regression, respectively. The predicted growth parameters for the pathogens observed in this study were compared to ComBase, Pathogen modeling program (PMP) and data from the literature. High R^2 values (0.97 and 0.93) were observed for average growth curves of different strains of pathogens grown on MPL. Secondary models of μ and λ for both pathogens followed a linear trend with high R^2 values (>0.90). Root mean square error (RMSE) showed that the models obtained are accurate and suitable for modeling the growth of *S. enterica* and *L. monocytogenes* in MP lettuce. The current study provides growth models for these foodborne pathogens that can be used in microbial risk assessment.

Key-words: Ready-to-eat vegetables, lettuce, *Salmonella*, *Listeria monocytogenes*, variability, predictive modeling.

1. Introduction

Ready-to-eat minimally processed vegetables (MPV) have gained more importance in the last 20 years due to consumers' demand for fresh, convenient, preservative-free foods that may promote health (Zink, 1997; Jacxsens et al., 2010). In Brazil, the MPV market increases yearly, with lettuce representing the most consumed leafy vegetable in the country (Sato et al., 2007).

Concurrent with the increase in their production and consumption, ready-to-eat vegetables have been associated with progressively more disease outbreaks (Harris et al., 2003; Sivapalasingam et al., 2004; Little and Gillespie, 2008; Lynch et al., 2009). Between 1999 and 2008, 6,062 foodborne disease outbreaks were reported in Brazil, of which 114 involved vegetables (Anonymous, 2010). Moreover, several surveys have been carried out to evaluate the microbiological quality of MPV in Brazil (Fröder et al., 2007; Silva et al., 2007; Oliveira et al., 2011) and abroad (Abadias et al., 2008; Ailes et al., 2008; Meldrum et al., 2009; Caponigro et al., 2010; Giusti et al., 2010) and have reported on the prevalence and/or countable levels of pathogens such as *Salmonella* and *Listeria monocytogenes* in these products (Fröder et al., 2007; Little et al., 2007; Abadias et al., 2008; Cordano and Jacquet, 2009; Giusti et al., 2010; Oliveira et al., 2010; Quiroz-Santiago et al., 2009; Sant'Ana et al., 2011).

Salmonella is the major bacterial challenge for the safety of a wide variety of foods around the world (Greig and Ravel, 2009), and it is the main etiological agent of outbreaks in which fresh produce items are implicated (Tauxe, 1997; Harris et al., 2003; Little and Gillespie, 2008). In Brazil, salmonellae are responsible for approximately 47% of the notified foodborne disease outbreaks (Anonymous, 2010). The primary source of *Salmonella* appears to be animal feces, while contaminated soil and water used for irrigation may also contribute to its spread to vegetables (Krtinić et al., 2010).

L. monocytogenes is a ubiquitous foodborne pathogen with a known ability to grow under refrigeration temperatures (Hanning et al., 2008) and to survive and adapt to adverse environments (Gandhi and Chikindas, 2007). Although it causes fewer foodborne diseases outbreaks than *Salmonella* (Greig and Ravel, 2009), *L. monocytogenes* represents a major food safety concern due to the high mortality rate of those sickened with listeriosis (Bennion et al., 2008).

In spite of the importance of estimating microbial growth kinetics in the context of quantitative risk assessment (QRA), simple addition of modeling data into QRA models might not be enough for obtaining more accurate models. Several studies have shown substantial differences in the growth kinetics among different strains of foodborne pathogens (Guillier and Augustin, 2006; Lindqvist, 2006; Koutsoumanis, 2008; Pal et al., 2008; Valík et al., 2008; Augustin et al., 2011; Lianou and Koutsoumanis, 2011). It is recognized that intra-species variability of microbial growth kinetics may influence the accuracy of outputs from risk assessment models (Delignette-Muller and Rosso, 2000). Therefore, information on the variability of growth kinetics among different strains of foodborne pathogens and how these parameters are affected by variations in the environment should be developed to help obtain reliable outputs from risk assessment (Lianou and Koutsoumanis, 2011). That is why, in this study, the growth of three different strains of *S. enterica* and *L. monocytogenes* in minimally processed lettuce as a function of temperature (7 to 30°C) were modeled separately using both primary and secondary models.

2. Material and Methods

2.1. Strains and preparation of cell suspensions

Three different strains of *S. enterica* and *L. monocytogenes* were used in this study. *S. enterica* strains were from serotype Typhimurium (# 277 and 386) and Enteritidis ATCC 13076 while the *L. monocytogenes* strains were of serotype 4b (#: 413, 494 and 581). Except for the *Salmonella* ATCC strain, the other two strains used in this study were isolated from MPV marketed in Sao Paulo, Brazil.

Each strain of *S. enterica* and *L. monocytogenes* was grown separately in 10 mL of Tryptic Soy Broth (Oxoid, Basingstoke, UK) (TSB) and TSB supplemented with 0.6% yeast extract (Oxoid, Basingstoke, UK) (TSB-YE), respectively. Broth tubes were incubated at 37°C/24h, following two successive inoculations. The third inoculation was carried in 90mL of the culture medium, following centrifugation at 2810 *g* at 4°C for 10 min (Mikro 22R, Hettich Zentrifugen, Germany) for removing debris and residual culture media. After centrifugation, the supernatant was discarded and pellets were washed with sterile 0.1% peptone water. Centrifugation and washing procedures were repeated three times and cells were re-suspended in

sterile 0.1% peptone water. Final cell concentrations were adjusted at optical density 0.5 (OD₆₃₀) (10^8 CFU/mL) using an Ultrospec 200 UV/visible spectrophotometer (Pharmacia Biotech, Buckinghamshire, UK). Each suspension was separately prepared just before the experiments.

2.2. Minimal processing of lettuce and its inoculation with pathogens

The two most consumed varieties of lettuce in Brazil (iceberg - *Lactuca sativa* var. *capitata* and crisp - *Lactuca sativa* var. *crispa*) were purchased from produce outlets located in the city of Sao Paulo, Brazil, and transported to the lab within one hour. The external, damaged leaves and core were discarded before washing. The leaves were individually washed in tap water and were cut into strips of approximately 2 cm² width with a disinfected knife. The two varieties of lettuce were mixed in the same proportion and shredded leaves were immersed into a disinfected 20L plastic basin containing Sumaveg solution (JohnsonDiversey, Sao Paulo, Brazil) at 100 ppm for 15 min. Chlorine levels were checked using pH paper test strips (pHydrion, Micro Chlorine, Johnson Wax Professional, Sturtevant, USA). Shredded lettuce leaves were then transferred into a previously disinfected plastic basin containing sterile distilled water, rinsed and allowed to dry in a laminar flow cabinet for at least 30 min.

Sterile distilled water (4 L) in pails lined with sterile plastic bags was inoculated with each suspension to reach a final concentration of 10^1 - 10^2 CFU/g of each pathogen strain. Shredded lettuce leaves were inoculated by dipping into solutions containing different strains of *S. enterica* and *L. monocytogenes* for 15 min. Removal of excess water was done with the aid of a previously sanitized salad spinner (Model DD1056Y, Casa da Moda, Brazil) in a biosafety cabinet. Control samples were prepared by repeating the same procedure above in non-inoculated sterile distilled water.

2.3. Packaging, storage of minimally processed lettuce and enumeration of pathogens

Twenty-five grams of contaminated, minimally processed lettuce was packaged in bags made of multilayer films (external layer: bi-oriented polypropylene; internal layer: blend or co-extruded structure of low-density polyethylene with linear low-

density polyethylene) under modified atmosphere (5% O₂, 15% CO₂ and 80% N₂ – White Martins, Osasco, Brazil) using vacuum-sealing machine AP 500 (Tecmaq, Sao Paulo, Brazil). This plastic material has been used by several fresh vegetable processing facilities in Brazil and has 62 µm thickness, O₂ permeability of 1.375 m³ m⁻² day⁻¹ at 23°C and water steam permeability of 3.5 g water m⁻² day⁻¹ at 38°C and 90% relative humidity. Bags of lettuce were stored at 7, 10, 15, 20, 25 and 30°C, and samples were collected at different time intervals and were analyzed for *S. enterica* or *L. monocytogenes*. The time intervals between sampling varied depending on the temperature of storage and ended once the microorganisms reached stationary phase.

S. enterica was enumerated by stomaching the vegetables (25 g) and the diluents, following pour-plate of 1 mL of serial dilutions prepared in 0.1% sterile peptone water in mannitol lysine crystal violet brilliant green (MLCB) agar (Oxoid, Basingstoke, UK) in duplicates. MLCB plates were further incubated at 37°C for 24h. *L. monocytogenes* was enumerated in the same approach, following pour-plate of 1 mL of serial dilutions prepared in 0.1% sterile peptone water in Oxford agar (Oxoid, Basingstoke, UK) with incubation at 37°C for 48h. Approximately 3-5 colonies per point along the growth curve were selected for further confirmation by polyvalent serotyping (*Salmonella enterica*) or biochemical tests as described in ISO 11290-2 (*L. monocytogenes*) (Anonymous, 1998). The limit of enumeration of the method used was 10¹ CFU/g. Experiments were replicated twice for each strain and temperature studied. Results were expressed as CFU/g and data were used for primary predictive modeling.

2.4. Modeling growth parameters of three different strains of *S. enterica* and *L. monocytogenes* in minimally processed lettuce

Growth curves for each temperature and strain were built separately by fitting data to the Baranyi model (Baranyi and Roberts, 1994) (equations 1-3) using DMFit version 2.1 Excel® add-in (www.ifr.ac.uk/safety/DMfit).

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

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

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

Where: $\ln(N(t))$ = log of cell concentration at time t [h] (CFU/g); $\ln(N_0)$ = log of initial cell concentration (CFU/g); $\mu_{\max}$ = exponential growth rate (log CFU/g/h); $\ln(N_{\max})$ = log of maximum cell concentration; q_0 [-] = parameter expressing the physiological state of cells when $t=t_0$; λ = lag time (h).

The Ratkowsky model (Ratkowsky et al., 1982) was used to describe μ and λ as a function of storage temperature (Equation 4):

$$\sqrt{r} = b(T - T_0) \quad \text{Equation 4}$$

In this model, $\sqrt{r}$ is the square root of maximum growth rate, b is the slope of the regression line, T is temperature and T_0 is a conceptual minimum temperature for microbial growth, where T was given in °C. For λ , transformation of data to natural logarithm (Ln) was needed to describe its relationship with storage temperature.

2.5. Model evaluation

Computer programs (ComBase Predictor [<http://modelling.combase.cc/>] and the Pathogen Modeling Program 7.0 – PMP [<http://pmp.arserrc.gov/PMPOnline.aspxi>]) were used to estimate μ and λ for further comparison with the data obtained in this study. The PMP and ComBase models were run with the following selected parameters: pH = 6.8, initial level of log 3 CFU/mL of the pathogen, and a_w = 0.995 (equal to 0.9 g/dL of NaCl). The pH and a_w values were chosen based on previous measurements carried out in our laboratory as described by Scott et al. (2001). PMP models used were “*Salmonella* aerobic growth” and “*L. monocytogenes* anaerobic growth” while ComBase models used were “salmonellae with CO₂ (%)” and “*L. monocytogenes* with CO₂ (%)”. The aerobic growth model for *Salmonella* was chosen because PMP does not include anaerobic or modified atmosphere growth models for

this pathogen. In the case of the ComBase models, CO₂ was set at 15%, *i.e.*, the initial % of CO₂ used in modified atmosphere. The minimal temperature used for predictions for the PMP *Salmonella* models were 10°C as these models did not allow selection of a lower temperature. Additionally, our data were compared with the μ and λ reported in selected literature (Koseki and Isobe, 2005a,b), here referred to as “Koseki-derived model.” Procedures previously adopted to transform data obtained in this study for secondary modeling were also used to deal with the data obtained from literature and from ComBase and PMP programs.

2.6. Statistical Analysis

The μ and λ of the different strains of *Salmonella* and *L. monocytogenes* were checked for significant statistical differences ($p \leq 0.05$), employing one-factor analysis of variance (ANOVA) followed by Duncan’s test. Growth parameters for both pathogens were also compared to each other. Statistical analyses were carried out in Assistat version 7.5 free software (Campina Grande, Brazil) (Silva and Azevedo, 2002). In addition, fitting of the models was evaluated by the coefficient of determination (R^2) and their performance was checked by the root mean square error (RMSE).

3. Results

S. enterica and *L. monocytogenes* survived, but did not grow on minimally processed lettuces stored at 5°C. Growth curves started with an initial population of 10¹-10² CFU/g and final populations of *S. enterica* and *L. monocytogenes* varied between 10⁵-10⁶ CFU/g and 10⁴-10⁷ CFU/g, respectively. The average R^2 values for growth curves of the three different strains of *S. enterica* and *L. monocytogenes* inoculated on minimally processed lettuce leaves and fitted to Baranyi model were 0.97 and 0.93, respectively (figures not shown). Control samples did not show growth of *S. enterica* or *L. monocytogenes* during storage at different temperatures and periods.

Table 1 shows the average growth kinetic parameters for each of the three different strains of *S. enterica* and *L. monocytogenes* on packed minimally processed lettuce. At 7 and 10°C *S. enterica* presented lower λ in comparison to *L.*

monocytogenes ($p<0.05$). With the rise in temperature, λ did not differ among the pathogens ($p>0.05$) except at 20°C. *S. enterica* grew faster than *L. monocytogenes* over the temperature range ($p<0.05$), except at 20°C. When growth parameters among strains of the same pathogen were evaluated, significant differences were observed for *S. enterica* only at 10°C (growth rate) and 20°C (lag time) ($p<0.05$). The highest variations in μ and λ in salmonellae were observed when the growth took place at 30°C. The μ varied between 0.32 log CFU/g/h and 0.44 log CFU/g/h in *S. Typhimurium* strains (#277 and 386). In addition, λ values varied from 1.1 hour for *S. Enteritidis* ATCC 13076 to 2.4 hour for *S. Typhimurium* (#386) (Table 1). It was found that *S. Enteritidis* ATCC 13076 presented slightly higher λ in comparison to strains 277 and 386 at 7 and 10°C, while the strain 386 (*S. Typhimurium*) presented higher λ in the range of 15-30°C. *S. Typhimurium* 277 presented an overall higher μ that was more evident at 25-30°C.

Significant differences were observed at 20°C (μ) and 30°C (λ) ($p<0.05$) for *L. monocytogenes* (Table 1). *L. monocytogenes* strain 581 presented slightly higher lag time at 7-10°C, while strain 413 presented higher μ between 15-30°C.

The relationship between the storage temperature of minimally processed lettuce and the average growth parameters (μ and λ) for each strain of *S. enterica* and *L. monocytogenes* is shown in Figure 1. The relationship between the growth parameters and temperature for both pathogens followed a linear trend with high R^2 values (>0.90). Certain data points were out of the 95% confidence intervals, although the mean values are within the 95% CI (Figure 1).

Figure 2 presents a comparison of linear regression among the average growth parameters of *S. enterica* and *L. monocytogenes* obtained in this study, PMP, ComBase and Koseki. The model for λ of *S. enterica* (Figure 2-A) derived from Koseki was very close to the model obtained in this study, mainly at the points of 15, 20 and 25°C. In this case, the ComBase and PMP-derived models presented higher slopes. On the other hand, secondary models for *L. monocytogenes* derived from ComBase, PMP and Koseki followed a similar pattern, with most of the data falling within the 95% CI of average data obtained in this study (Figure 2-B). Models for μ of *S. enterica* (Figure 2-C) derived from ComBase and PMP showed the greatest slope, followed by the model derived from Koseki. The secondary model for μ of *S. enterica* obtained in this study did not follow the same trend as the three other models.

Conversely, the secondary model for *L. monocytogenes* derived from Koseki followed the same trend of the model derived from data obtained in this study, while models derived from PMP and ComBase presented the greatest slope (*Figure 2-D*).

Equations describing the relationship of growth parameters and temperature for *S. enterica* and *L. monocytogenes* grown in packed minimally processed lettuce from 7 to 30°C are shown in *Equations 5-6 and 7-8*, respectively:

$$\sqrt{\mu} = 0.0178(T - 6.65) \quad \text{Equation 5}$$

$$\ln(\lambda) = -0.118(T + 34.84) \quad \text{Equation 6}$$

$$\sqrt{\mu} = 0.0144(T - 1.96) \quad \text{Equation 7}$$

$$\ln(\lambda) = -0.1445(T + 35.3) \quad \text{Equation 8}$$

RMSE values obtained for μ and λ models of *S. enterica* were 0.033 and 0.214, respectively. RMSE values for *L. monocytogenes* models were 0.015 (μ) and 0.271 (λ), respectively. It can be noticed that RMSE values for μ models were lower than for λ , no matter the pathogen.

4. Discussion

Despite the increasing association of vegetables with foodborne disease outbreaks in recent years (Lynch et al., 2009), few studies have focused on predicting the growth of pathogens in fresh vegetables (Koseki and Isobe, 2005a,b; Crépet et al., 2009; Ding et al., 2010) when compared to meat and dairy products (McMeekin, 2007; Poirazi et al., 2007; Vissers et al., 2007; Oscar, 2009; Cornu et al., 2010; Limbo et al., 2010; McMeekin et al., 2010; Schvartzman et al., 2010; Lindqvist and Lindblad, 2011). Therefore, there is a need for more data on the growth of foodborne pathogens in these products.

Studies on the growth modeling of pathogens are built with a higher starting initial population (*i.e.*, $> 10^3$ - 10^4 CFU/g) (Koseki and Isobe, 2005a,b; Ding et al., 2010) of pathogens than that of naturally-contaminated foods, such as fresh vegetables (*i.e.*, $< 10^1$ - 10^2 CFU/g) (Sagoo et al., 2003; Little et al., 2007; Cordano and Jacquet, 2009; Oliveira et al., 2010; Sant'Ana et al., 2011). However, predictive models should match the complexity of foods of concern, including low pathogens

contamination levels, in order to provide more realistic outputs (Mejlholm et al., 2010). Therefore, herein the growth kinetic parameters (μ and λ) of three different strains of *S. enterica* and *L. monocytogenes* on packed MPV were predicted with low initial starting populations (10^1 - 10^2 CFU/g). The average growth curves of three different strains of these pathogens presented higher R^2 values (>0.93) than those found in the separate curves for each strain (0.83 and 0.90). R^2 is defined as the proportion of the variability in the data set that is explained by the statistical model (Myers et al. 2009). Therefore, the improvement in curve fitting for the averaged data indicate that when mean values of data points were calculated, marginal values that caused lower R^2 values in separate curves were pulled into the center (Figures 1 and 2). Thus, high R^2 were obtained in average growth curves because the regression line approximated the real data points.

As shown in Table 1, *S. enterica* grew faster than *L. monocytogenes* in the temperature range tested (7-30°C) at which MPV can be exposed from farm to fork ($p<0.05$). Overall, *S. enterica* presented μ 1.5 to 3.5 times higher than *L. monocytogenes*. Koseki et al. (2005 a,b) reported μ of approximately 0.02 and 0.04 log CFU/g/h at 10°C, respectively, for *S. enterica* and *L. monocytogenes* growing on iceberg lettuce. In our study, the average μ of both pathogens at 10°C was 0.045 and 0.024 log CFU/g/h, respectively. Marked differences in λ were found at low temperatures (7 and 10°C), when *L. monocytogenes* presented larger values in comparison to *S. enterica* ($p<0.05$). It is known that even during minimal processing, microorganisms are exposed to stressors such as cold, sanitizers, modified atmosphere and the presence of background microbiota (Capozzi et al., 2009). Even though both pathogens present several mechanisms to overcome hurdles faced during food processing (Gandhi and Chikindas, 2007; Spector and Kenyon, 2011), *L. monocytogenes* is noticeably more prone to inhibition by indigenous microorganisms present in MPV, such as lactic acid bacteria, than is *Salmonella* (Liao and Fett, 2001; Hanning et al., 2008; Dominguez and Schaffner, 2008). Therefore, considering the similar responses of both pathogens to other stressors found during minimal processing (i.e., sanitizer and modified atmosphere), it may be hypothesized that the inhibitory effect of indigenous microbiota on *L. monocytogenes* supplants the ability of this pathogen to adapt to chilling temperatures (Gandhi and Chikindas, 2007; Capozzi et al., 2009). Altogether, these factors may have substantiated the faster

growth of *S. enterica* compared to *L. monocytogenes*, in minimally processed lettuce under the conditions tested in the present study

Temperature is a major environmental factor affecting microbial growth kinetics in foods (McMeekin et al., 2008). Regression lines of secondary models depicted in *Figure 2* were drawn based on the mean growth kinetics parameters of *S. enterica* and *L. monocytogenes* strains while the data points added were obtained from models of each different strain of the pathogens. It can be noticed that most data points were generally within the 95% confidence interval of the averaged values. Modeling of μ was successfully performed using a linear relationship between the square root of the parameter and temperature as described by Ratkowsky et al. (1982). However, the natural logarithm transformation of lag time (λ) resulted in better fit than the square root transformation for this parameter and temperature.

Data on secondary modeling obtained herein were compared to those available in the literature (Koseki et al., 2005a,b) and to tertiary models such as ComBase (Baranyi and Tamplin, 2004) and PMP. These tertiary models are the most recognized software of modeling used to predict growth parameters of several foodborne microorganisms under a variety of conditions. Literature data used for comparison were comprised of models previously described by Koseki and Isobe (2005a,b), since these are the only papers dealing with the growth kinetics of *S. enterica* and *L. monocytogenes* in MPV in the wide range of temperatures these products may be subjected from farm to fork. As can be seen in *Figure 2*, secondary models derived from ComBase and PMP basically follow the same slope pattern regarding μ and λ . Overall, our λ models for *S. enterica* and *L. monocytogenes* were closer to ComBase, PMP and Koseki-derived models than our μ models (*Figure 2*). Differences among our models, ComBase and the PMP-derived models can be due to the use of data obtained from culture media for building tertiary models. When growing in culture media, microorganisms are not exposed to factors such as the structure of foods and the presence of background microflora, among other issues. The differences between foods and culture media might result in the stress of bacterial cells, leading to faster μ (and greater slopes) when growth takes place in culture media. This hypothesis can be supported by the fact that μ is a characteristic of a microorganism growing in a particular environment, while λ is dependent upon the history and physiological state of the bacterial cells (Baranyi et al., 1995).

RMSE is a measure of the precision of a predictive model, and accounts for the differences between predicted and observed values. The lower the RMSE value for a model, the more precise the data were described (Ross, 1996). Therefore, the RMSE values obtained in this study are very close to those found in models available in the literature, which indicates that the models built here are generally suitable for modeling the growth of *S. enterica* and *L. monocytogenes* in MP lettuce. In addition, the current study adds to the available literature data on the growth of foodborne pathogens in MPV and can be used to build risk assessment models.

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5. References

- Abadias, M., Usall, J., Anguera, M., Solsona, C., & Viñas, I., (2008). Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology*, 123, 121-129.
- Ailes, E.C., Leon, J.S., Jaykus, L.-A., Johnston, L.M., Clayton, H.A., Blanding, S., Kleinbaum, D.G., Backer, L.C., & Moe, C.L., (2008). Microbial concentrations on fresh produce are affected by postharvest processing, importation, and season. *Journal of Food Protection*, 71, 2389-2397.
- Anonymous, (1998). ISO 11290-2:1998 - Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 2: Enumeration method. Switzerland.
- Anonymous, (2010). Análise Epidemiológica dos Surtos de Doenças Transmitidas por Alimentos no Brasil. Coordenação de Vigilância das Doenças de Transmissão Hídrica e Alimentar. Secretaria de Vigilância em Saúde. Ministério da Saúde. Available at: http://portal.saude.gov.br/portal/arquivos/pdf/surtos_dta_15.pdf Accessed on: April 07, 2011.
- Augustin, J.-C., Bergis, H., Midelet-Bourdin, G., Cornu, M., Couvert, O., Denis, C., Huchet, V., Lemonnier, S., Pinon, A., Vialette, M., Zuliani, V., & Stahl, V., (2011).

Design of challenge testing experiments to assess the variability of *Listeria monocytogenes* growth in foods. *Food Microbiology*, 28, 746-754.

Baranyi, J.; Roberts, T.A.. A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology*, V. 23, p. 277-294, 1994.

Baranyi, J., & Tamplin, M. L., (2004). ComBase: A common database on microbial responses to food environments. *Journal of Food Protection*, 67, 1967-1971.

Baranyi, J., Robinson, T. P., Kaloti, A., & Mackey, B. M., (1995). Predicting growth of *Brochothrix thermosphacta* at changing temperature. *International Journal of Food Microbiology*, 27, 61-75.

Bennion, J. R., Sorvillo, F., Wise, M. E., Krishna, S., & Mascola, L., (2008). Decreasing listeriosis mortality in the United States, 1990-2005. *Clinical Infectious Diseases*, 47, 867-874.

Caponigro, V., Ventura, M., Chiancone, I., Amato, L., Parente, E., & Piro, F., (2010). Variation of microbial load and visual quality of ready-to-eat salads by vegetable type, season, processor and retailer. *Food Microbiology*, 27, 1071-1077.

Capozzi, V., Fiocco, D., Amodio, M.L., Gallone, A., & Spano, G., (2009). Bacterial stressors in minimally processed food. *International Journal of Molecular Sciences*, 10, 3076-3105.

Cordano, A. M., & Jacquet, C., (2009). *Listeria monocytogenes* isolated from vegetable salads sold at supermarkets in Santiago, Chile: Prevalence and strain characterization. *International Journal of Food Microbiology*, 132, 176-179.

Cornu, M., Billoir, E., Bergis, H., Beaufort, A., & Zuliani, V., (2010). Modeling microbial competition in food: Application to the behavior of *Listeria monocytogenes* and lactic acid flora in pork meat products. *Food Microbiology*, 28, 639-647.

Crépet, A., Stahl, V., & Carlin, F., (2009). Development of a hierarchical Bayesian model to estimate the growth parameters of *Listeria monocytogenes* in minimally processed fresh leafy salads. *International Journal of Food Microbiology*, 131, 112-119.

Delignette-Muller, M. L., & Rosso, L., (2000). Biological variability and exposure assessment. *International Journal of Food Microbiology*, 58, 203-212.

Ding, T., Jin, Y.-G., & Oh, D.-H., (2010). Predictive model for growth of *Listeria monocytogenes* in untreated and treated lettuce with alkaline electrolyzed water. *World Journal of Microbiology and Biotechnology*, 26, 863-869.

- Dominguez, S.A., & Schaffner, D.W., (2008). Modeling the growth of *Salmonella* in raw poultry stored under aerobic conditions. *Journal of Food Protection*, 71, 2429-2435.
- Fröder, H., Martins, C. G., De Souza, K. L. O., Landgraf, M., Franco, B. D. G. M., & Destro, M. T., (2007). Minimally processed vegetable salads: Microbial quality evaluation. *Journal of Food Protection*, 70, 1277-1280.
- Gandhi, M., & Chikindas, M.L., (2007). *Listeria*: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology*, 113, 1-15.
- Giusti, M., Aurigemma, C., Marinelli, L., Tufi, D., De Medici, D., Di Pasquale, S., De Vito, C., & Boccia, A., (2010). The evaluation of the microbial safety of fresh ready-to-eat vegetables produced by different technologies in Italy. *Journal of Applied Microbiology*, 109, 996-1006.
- Greig, J. D. & Ravel, A., (2009). Analysis of foodborne outbreak data reported internationally for source attribution. *International Journal of Food Microbiology*, 130, 77-87.
- Guillier, L., & Augustin, J.-C., (2006). Modeling the individual cell lag time distributions of *Listeria monocytogenes* as a function of the physiological state and the growth conditions. *International Journal of Food Microbiology*, 111, 241-251.
- Hanning, I. B., Johnson, M. G., & Ricke, S. C., (2008). Pre-cut prepackaged lettuce: A risk for listeriosis? *Foodborne Pathogens and Disease*, 5, 731-746.
- Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H. & Busta, F.F., (2003). Outbreaks associated with fresh produce: Incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety*, 2, 78-141.
- Jacxsens, L., Luning, P. A., van der Vorst, J. G. A. J., Devlieghere, F., Leemans, R., & Uyttendaele, M., (2010). Simulation modeling and risk assessment as tools to identify the impact of climate change on microbiological food safety - the case study of fresh produce supply chain. *Food Research International*, 43, 1925-1935.
- Koseki, S., & Isobe, S., (2005a). Growth of *Listeria monocytogenes* on iceberg lettuce and solid media. *International Journal of Food Microbiology*, 101, 217-225.
- Koseki, S., & Isobe, S., (2005b). Prediction of pathogen growth on iceberg lettuce under real temperature history during distribution from farm to table. *International Journal of Food Microbiology*, 104, 239-248.

- Koutsoumanis, K., (2008). A study on the variability in the growth limits of individual cells and its effect on the behavior of microbial populations. *International Journal of Food Microbiology*, 128, 116-121.
- Krtinić, G., Crossed D Signurić, P., & Ilić, S., (2010). Salmonellae in food stuffs of plant origin and their implications on human health. *European Journal of Clinical Microbiology and Infectious Diseases*, 29, 1321-1325.
- Lianou, A., & Koutsoumanis, K.P., (2011). Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior. *Food Microbiology*, 28, 828-837.
- Liao, C.-H., & Fett, W.F., (2001). Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. *Journal of Food Protection*, 64, 1110-1115.
- Limbo, S., Torri, L., Sinelli, N., Franzetti, L., & Casiraghi, E., (2010). Evaluation and predictive modeling of shelf life of minced beef stored in high-oxygen modified atmosphere packaging at different temperatures. *Meat Science*, 84, 129-136.
- Lindqvist, R., (2006). Estimation of *Staphylococcus aureus* growth parameters from turbidity data: characterization of strain variation and comparison of methods. *Applied and Environmental Microbiology*, 72, 4862-4870.
- Lindqvist, R., & Lindblad, M., (2011). Time to growth and inactivation of three STEC outbreak strains under conditions relevant for fermented sausages. *International Journal of Food Microbiology*, 145, 49-56.
- Little, C. L. & Gillespie, I. A., (2008). Prepared salads and public health. *Journal of Applied Microbiology*, 105, 1729-1743.
- Little, C. L., Taylor, F. C., Sagoo, S. K., Gillespie, I. A., Grant, K., & McLauchlin, J., (2007). Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK. *Food Microbiology*, 24, 711-717.
- Lynch, M. F., Tauxe, R. V., & Hedberg, C. W., (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. *Epidemiology and Infection*, 137, 307-315.
- McMeekin, T. A., (2007). Predictive microbiology: Quantitative science delivering quantifiable benefits to the meat industry and other food industries. *Meat Science*, 77, 17-27.

- McMeekin, T., Bowman, J., McQuestin, O., Mellefont, L., Ross, T., & Tamplin, M., (2008). The future of predictive microbiology: Strategic research, innovative applications and great expectations. *International Journal of Food Microbiology*, 128, 2-9.
- McMeekin, T., Tamplin, M., Ross, T., & Koseki, S., (2010). The way forward with predictive microbiology in the dairy industry. *Australian Journal of Dairy Technology*, 65, 91-97.
- Mejlholm, O., Gunvig, A., Borggaard, C., Blom-Hanssen, J., Mellefont, L., Ross, T., Leroi, F., Else, T., Visser, D., & Dalgaard, P., (2010). Predicting growth rates and growth boundary of *Listeria monocytogenes* - an international validation study with focus on processed and ready-to-eat meat and seafood. *International Journal of Food Microbiology*, 141, 137-150.
- Meldrum, R. J., Little, C. L., Sagoo, S., Mithani, V., McLauchlin, J., & de Pinna, E., (2009). Assessment of the microbiological safety of salad vegetables and sauces from kebab take-away restaurants in the United Kingdom. *Food Microbiology*, 26, 573-577.
- Myers, R. H., Montgomery, D. C., & Anderson-Cook, C. M., (2009). Response Surface Methodology: Process and product optimization using designed experiments, third ed. John Wiley and Sons, New York.
- Oliveira, M. A., Souza, V.M., Bergamini, A. M. M., & Martinis, E.C.P., (2011). Microbiological quality of ready-to-eat minimally processed vegetables consumed in Brazil. *Food Control*, 22, 1400-1403.
- Oliveira, M.A., Abeid Ribeiro, E.G., Morato Bergamini, A.M., & Martinis, E.C.P., (2010). Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. *Food Microbiology*, 27, 19-23.
- Oscar, T.P., (2009). General regression neural network and Monte Carlo simulation model for survival and growth of *Salmonella* on raw chicken skin as a function of serotype, temperature, and time for use in risk assessment. *Journal of Food Protection*, 72, 2078-2087.
- Pal, A., Labuza, T. P., & Diez-Gonzalez, F., (2008). Comparison of primary predictive models to study the growth of *Listeria monocytogenes* at low temperatures in liquid cultures and selection of fastest growing ribotypes in meat and turkey product slurries. *Food Microbiology*, 25, 460-470.

- Poirazi, P., Leroy, F., Georgalaki, M. D., Aktypis, A., De Vuyst, L., & Tsakalidou, E., (2007). Use of artificial neural networks and a gamma-concept-based approach to model growth of and bacteriocin production by *Streptococcus macedonicus* ACA-DC 198 under simulated conditions of Kasseri cheese production. *Applied and Environmental Microbiology*, 73, 768-776.
- Quiroz-Santiago, C., Rodas-Suárez, O.R., Vázquez Q., C.R., Fernández, F. J., Quiñones-Ramírez, E. I., & Vázquez-Salinas, C., (2009). Prevalence of *Salmonella* in vegetables from Mexico. *Journal of Food Protection*, 72, 1279-1282.
- Ratkowsky, D. A., Olley, J., McMeekin, T. A., & Ball, A., (1982). Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology*, 149, 1-5.
- Ross, T., 1996. Indices for performance evaluation of predictive models in food microbiology. *Journal of Applied Bacteriology*, 81, 501-508.
- Sagoo, S. K., Little, C. L., Ward, L., Gillespie, I. A., & Mitchell, R. T., (2003). Microbiological study of ready-to-eat salad vegetables from retail establishments uncovers a national outbreak of salmonellosis. *Journal of Food Protection*, 66, 403-409.
- Sant'Ana, A.S., Landgraf, M., Destro, M.T., & Franco, B.D.G.M., (2011). Prevalence and counts of *Salmonella* spp. in minimally processed vegetables in Sao Paulo, Brazil. *Food Microbiology*, 28, 1235-1237.
- Sato, G.S., Martins, V.A., & Bueno, C.R.F., (2007). Análise exploratória do perfil do consumidor de produtos minimamente processados na cidade de São Paulo. *Informações Econômicas*, 37, 62-71.
- Schvartzman, M. S., Belessi, X., Butler, F., Skandamis, P., & Jordan, K., (2010). Comparison of growth limits of *Listeria monocytogenes* in milk, broth and cheese. *Journal of Applied Microbiology*, 109, 1790-1799.
- Scott, V.N., Clavero, R.S., & Troller, J.A. (2001). Measurement of water activity (a_w), acidity, and Brix. In Downes, F.P., & Ito, K. (Eds.), *Compendium of methods for the microbiological examination of foods* (pp. 649-659). Washington, DC: APHA.
- Silva, F.A.S., & Azevedo, C.A.V., (2002). Versão do programa computacional Assistat para o sistema operacional Windows. *Revista Brasileira de Produtos Agroindustriais*, 4, 71-78.
- Silva, S.R.P., Verdin, S.E.F., Pereira, D.C., Schatkoski, A.M., Rott, M.B., & Corção, G., (2007). Microbiological quality of minimally processed vegetables sold in Porto Alegre, Brazil. *Brazilian Journal of Microbiology*, 38, 594-598.

- Sivapalasingam, S., Friedman, C. R., Cohen, L., & Tauxe, R. V., (2004). Fresh produce: A growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection*, 67, 2342-2353.
- Spector, M.P., & Kenyon, W.J., (2011). Resistance and survival strategies of *Salmonella enterica* to environmental stresses. *Food Research International*, doi:10.1016/j.foodres.2011.06.056.
- Tauxe, R. V., (1997). Emerging foodborne diseases: An evolving public health challenge. *Emerging Infectious Diseases*, 3, 425-434.
- Valík, L., Medved'ová, A., Bajúsová, B., & Liptáková, D., (2008). Variability of growth parameters of *Staphylococcus aureus* in milk. *Journal of Food and Nutrition Research*, 47, 18-22.
- Vissers, M.M.M., Te Giffel, M.C., Driehuis, F., De Jong, P., & Lankveld, J.M.G., (2007). Predictive modeling of *Bacillus cereus* spores in farm tank milk during grazing and housing periods. *Journal of Dairy Science*, 90, 281-292.
- Zink, D.L., (1997). The Impact of Consumer Demands and Trends on Food Processing. *Emerging Infectious Diseases*, 3, 467-469.

Table 1: Growth kinetic parameters (μ , log CFU/g/h, and λ , h) of three different strains of *Salmonella enterica* and *L. monocytogenes* on packed minimally processed lettuce stored at 7-30°C ^{a,b}.

Pathogen	Growth parameters	Strains	7	10	15	20	25	30
<i>Salmonella</i> spp.	μ	277	0.053±0.010 ^a	0.0497±0.001 ^a	0.149±0.04 ^a	0.148±0.04 ^a	0.328±0.002 ^a	0.44±0.09 ^a
		386	0.061±0.008 ^a	0.042±0.002 ^b	0.124±0.01 ^a	0.138±0.16 ^a	0.300±0.007 ^a	0.32±0.03 ^a
		ATCC	0.0635±0.006 ^a	0.045±0.001 ^{a,b}	0.094±0.05 ^a	0.149±0.01 ^a	0.315±0.001 ^a	0.36±0.03 ^a
		Average	0.06±0.0045 ^A	0.05±0.0031 ^A	0.12±0.022 ^A	0.16±0.015 ^A	0.31±0.011 ^A	0.37±0.05 ^A
<i>L. monocytogenes</i>	μ	413	0.0165±0.003 ^a	0.0225±0.006 ^a	0.0656±0.04 ^a	0.172±0.02 ^a	0.152±0.003 ^a	0.24±0.01 ^a
		494	0.0191±0.008 ^a	0.0272±0.009 ^a	0.0605±0.05 ^a	0.069±0.01 ^b	0.122±0.001 ^a	0.20±0.02 ^a
		581	0.0141±0.001 ^a	0.0233±0.010 ^a	0.0495±0.01 ^a	0.094±0.01 ^b	0.110±0.002 ^a	0.19±0.01 ^a
		Average	0.02±0.002 ^B	0.02±0.002 ^B	0.06±0.007 ^B	0.11±0.04 ^A	0.13±0.017 ^B	0.22±0.02 ^B
<i>Salmonella</i> spp.	λ	277	25.2±1.2 ^a	12.8±2.3 ^a	4.7±2.1 ^a	4.4±0.1 ^{a,b}	1.7±0.4 ^a	1.5±0.2 ^a
		386	26.5±1.6 ^a	11.8±3.1 ^a	7.4±2.0 ^a	5.4±0.6 ^a	2.2±0.7 ^a	2.4±1.1 ^a
		ATCC	28.4±2.1 ^a	14.3±0.9 ^a	5.4±0.9 ^a	3.8±0.5 ^b	1.8±0.1 ^a	1.1±0.4 ^a
		Average	26.7±1.3 ^B	12.9±1.0 ^B	5.9±1.1 ^A	4.5±0.7 ^A	1.9±0.2 ^B	1.6±0.6 ^A
<i>L. monocytogenes</i>	λ	413	45.5±11.4 ^a	39.1±20.1 ^a	8.4±7.7 ^a	6.9±2.6 ^a	4.3±0.5 ^a	1.4±0.4 ^b
		494	56.6±13.7 ^a	44.6±1.8 ^a	14.4±4.4 ^a	5.4±0.9 ^a	3.6±0.3 ^a	2.5±0.3 ^{a,b}
		581	76.2±10.0 ^a	48.7±8.2 ^a	8.4±1.5 ^a	5.7±1.4 ^a	3.2±0.1 ^a	3.4±0.7 ^a
		Average	59.4±12.7 ^A	44.1±3.9 ^A	10.4±2.8 ^A	5.9±0.6 ^A	3.7±0.5 ^A	2.4±0.8 ^A

^aDifferent superscript letters in the same column indicate significant differences ($p<0.05$) for growth rate or lag time of different strains of the same pathogen according to Duncan's test. ^bDifferent superscript capital letters in the same row indicate significant differences ($p<0.05$) for average values of growth rate or lag time between *Salmonella enterica* and *L. monocytogenes* according to Duncan's test.

Figure Captions:

Figure 1: Relationship between average growth parameters (μ and λ) of *Salmonella enterica* (A and C) and *L. monocytogenes* (B and D) strains and temperature of storage of minimally processed lettuce.

Figure 2: Comparison of the linear regression models for (μ and λ) derived from ComBase, PMP, Koseki (symbols) and average data obtained in this study (solid lines).

Figure 1:

Figure 2:

CAPÍTULO 7

“Risk of infection with *Salmonella* and *Listeria monocytogenes* due to consumption of ready-to-eat leafy vegetables in São Paulo, Brazil”

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Risk of infection with *Salmonella* and *Listeria monocytogenes* due to consumption of ready-to-eat leafy vegetables in São Paulo, Brazil

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Abstract

The current study was carried out to estimate the risks of infection due to consumption of RTE vegetables contaminated with *Salmonella* and *L. monocytogenes* in Brazil. The risk assessment model was composed of five different modules comprising the retail-consumption steps. Scenarios were simulated using prevalence and concentration levels reported in RTE vegetables in Brazil and reduction in prevalence and concentration levels. In addition, scenarios in which temperature during transportation and storage are majorly maintained below 7°C were also evaluated. Models built in Excel spreadsheets were run (100,000 iterations) using @Risk software. The two outputs were risk of infection per month and number of cases of infection. The MQRA models predicted that the mean risk of infection per month of *Salmonella* is 8.66E-03, while for *L. monocytogenes* the risk was 1.87E-08. The reduction of prevalence of *Salmonella* from 1.7% to 0.7% resulted in a decrease of risk of infection per month of up 6-7 times. In the case of *L. monocytogenes*, the reduction of prevalence from 2.2% to 0.22% resulted in decrease of risk of infection by this microorganism from 1.87E-08 to 2.90E-09. The risks and number of cases predicted in scenarios in which temperature was kept below 7°C (#5-8) were reduced for both pathogens studied when compared to scenarios #1-4. The scenario #8 (reduction of prevalence and concentration of pathogens and temperature <7°C) led to the lowest number of cases of infection due by *Salmonella* and *L. monocytogenes* (185 and 3.81E-05 cases, respectively). The results suggest that effective mitigation strategies need to be adopted. More data is needed to improve the accuracy of risk assessment models developed.

Key-words: Risk assessment, *Salmonella*, *Listeria monocytogenes*, ready-to-eat vegetables, risk characterization, produce.

1) Introduction

The increased association of fresh produce with foodborne disease outbreaks in the last 15 years is concerning consumers, governments and industries worldwide. Although this increase may also be due to improvements in microbiological methods and surveillance programs (Harris et al., 2003), it highlights that efforts must be done to ensure the microbiological safety of fresh produce. Among fresh produce, ready-to-eat (RTE) vegetables play an important role in the epidemiology of produce-borne illness as several outbreaks have been linked to these products (Harris et al., 2003, Sivapalasingam et al., 2004, Little and Gillespie, 2008, Lynch et al., 2009).

Epidemiological investigations have shown that *Salmonella* and pathogenic *E. coli* are the main foodborne bacterial pathogens involved in produce outbreaks (Sivapalasingam et al., 2004, Takkinen et al., 2005, Friesema et al., 2007, Little and Gillespie, 2008, Lynch et al., 2009). In Brazil, *Salmonella* is the most important foodborne pathogen, accounting for 47% of foodborne disease outbreaks notified, while pathogenic *E. coli* (enteropathogenic and enterotoxigenic) is responsible for less than 0.1% of outbreaks (Anonymous, 2010). On the other hand, *Listeria monocytogenes*, an important challenge for the safety of RTE foods, was not directly associated with foodborne illness in Brazil so far (Martins et al., 2010).

The prevalence of *Salmonella* in RTE vegetables marketed in Brazil has been reported in the literature (Fröder et al., 2007, Oliveira et al., 2011, Sant'Ana et al., 2011). Although the occurrence of Shiga toxin-producing *E. coli* has been reported in food-producing animals (Oliveira et al., 2008), the prevalence of this microorganism in vegetables (Silva et al., 2003) and in meat products in Brazil is insignificant (Silva et al., 2001, Bergamini et al., 2007). *L. monocytogenes* has been isolated from food commodities and patients in the country (Lemes-Marques et al., 2007, Miyasaki et al., 2009, Oliveira et al., 2010, Barancelli et al., 2011).

Among a total of 6,062 foodborne disease outbreaks reported between 1999 and 2008 in Brazil, 144 were linked to consumption of vegetables (Anonymous, 2010). Although the association of foodborne diseases and consumption of RTE vegetables is not clear in Brazil, this relation has been reported in other countries (Takkinen et al., 2005, Friesema et al., 2007, Little and Gillespie, 2008, Lynch et al., 2009). This association might not have been shown in Brazil yet because the

attribution of disease outbreaks with foods is not an easy task (Pires et al., 2009, Greig and Ravel, 2009).

Microbiological quantitative risk assessment (MQRA) consists of four steps: 1) hazard identification; 2) exposure assessment; 3) hazard characterization and 4) risk characterization (Codex, 1999). MQRA is a holistic approach on the processing of foods, allowing the quantitative estimation of the risks posed to public health by a food-pathogen combination (Oscar, 2011). The development of MQRA models has increased in the last 15 years, however, few models on produce-pathogens combinations are available (Franz et al., 2010, Tromp et al., 2010, Danyluk and Schaffner, 2011). The development of MQRA models focusing on this combination is of foremost importance because RTE vegetables are mostly eaten raw, without a killing step before consumption and pathogens prevail in these foods. Then, the outputs of MQRA can be used in the development of scientific-based strategies to manage risks and safeguard public health. Given the above and considering that the consumption of RTE vegetables is growing in Brazil (Sato et al., 2007), the current study was carried out to estimate the risks of infection due to consumption of RTE vegetables contaminated with *Salmonella* and *L. monocytogenes* in São Paulo, Brazil.

2) Material and Methods

The risk assessment model comprised five different modules from finished product leaving the produce processing facility until consumption (*Table 1*).

2.1) Produce leaving processing facilities to retail:

Data on prevalence of *Salmonella* and *L. monocytogenes* on RTE vegetables were gathered from published literature. Only information on leafy green vegetables was considered (*Table 2*), and the prevalence of both pathogens was represented in the model by Beta distribution (*Table 1*). Few data on populations of both pathogens in RTE vegetables in Brazil were available in the literature. Only one study enumerated *Salmonella* in RTE leafy vegetables (Sant'Ana et al., 2011). In that study, *Salmonella* was recovered from two out of 477 packages of RTE leafy vegetables, with populations of 8.8×10^2 and 2.4×10^2 CFU/g, respectively.

Populations of 1.0×10^1 and 1.6×10^1 CFU/g of *L. monocytogenes* were reported in two samples from a total of 477 packages of RTE vegetables analyzed (Sant'Ana et al., 2012a). Few other studies presented enumeration data of *L. monocytogenes* in RTE vegetables in Brazil (Porto and Eiroa, 2001, Oliveira et al. 2010), however, the populations found (0.4, 1.2 and 3.2 MPN/g) were lower than the levels reported by Sant'Ana et al. (2012a). Given the above, the concentration of both pathogens was modeled using Pert distribution with the average counts found by Sant'Ana et al. (2011, 2012a) inserted as maximum values. The minimal value (0.001 CFU/g) was a user input, while the most likely value (0.04 CFU/g) was estimated considering that 1 CFU was present in 25g of positive samples according to the prevalence study (Table 2).

Temperature and time during transportation of RTE vegetables from industry to retail were modeled based on data obtained from Pereira (2008) and Pereira et al. (2010). These authors recorded temperature of chilled and frozen food products during deliveries for 9 different retail shops, which last about 9h in total (Pereira, 2008; Pereira et al., 2010). Taking into account that RTE vegetables consumed in large cities of Brazil are originated from production sites located in surrounding areas of metropolitan regions ($< 100\text{Km}$), maximum transportation time was assumed to be 9h. By using this time, it was ensured that the foods delivered in the last retail shop were represented in the model. The minimum time was 2h and corresponded to average time for the first delivery in a working day. The most likely value (5h) was the average value for the remaining (seven) deliveries done after the first and last deliveries, respectively (Pereira, 2008). A pert distribution was used to model transportation time from processing facilities to retail (Table 1). Temperature was based on the data collected with thermocouples attached outside the primary package of foods used as models (Pereira, 2008). Maximum (10.3°C), minimum (3°C) and mean (most probable) (7.6°C) values reported by this author were used to describe transportation temperature from industry to retail using a pert distribution (Table 1).

The growth of *Salmonella* and *L. monocytogenes* during transportation and everywhere in the risk assessment model was described by the relationship between growth rate and temperature represented by the linear regression model proposed by Ratkowsky et al. (1982):

$$\sqrt{r} = b(T - T_0) \quad \text{Equation 1}$$

Where: $\sqrt{r}$ is the square root of maximum growth rate (μ), b is the slope of the regression line, T is temperature and T_0 is a conceptual minimum temperature for microbial growth, where T was given in $^{\circ}\text{C}$. The logarithmic growth of *Salmonella* (Equation 2) and *L. monocytogenes* (Equation 3) during transportation was modeled using previous published data (Sant'Ana et al., 2012b):

$$\sqrt{\mu} = 0.0178T - 0.11837 \quad \text{Equation 2}$$

$$\sqrt{\mu} = 0.0144T - 0.028224 \quad \text{Equation 3}$$

As the minimum temperature of growth used in the development of predictive models for *Salmonella* and *L. monocytogenes* in leafy greens was 7°C (Sant'Ana et al., 2012b), in the model it was assumed that growth would only occur above this temperature (Table 1). Once the logarithmic growth was determined, the growth of both pathogens during transportation was calculated by multiplying logarithmic growth by time of transportation. The level after transportation was the sum of initial concentration of each pathogen and their growth during this step.

2.2) Arrival and storage at retail:

In a survey of microbiological quality of RTE vegetables carried out by Maistro (2006) temperature of displays in the supermarkets were recorded. Although BestFit (Palisade Co, Ithaca, NY) was used to fit statistical distributions to temperature, no good adjustment to represent these data was obtained (data not shown). Thus, frequencies of temperature recorded at retail level were calculated and a discrete distribution was included in the risk assessment model to represent this event (Figure 1, Table 1). The storage time was modeled using uniform distribution, simulating two scenarios of purchase: *i*) it was assumed that 70% of RTE vegetables are purchased within 5 days and *ii*) the remaining packages (30%) are purchased in the last 3 days of shelf-life. The maximum shelf-life of RTE vegetables of 8 days was based on observation of labels of these products. In the first scenario (*i*), minimum value was 0h while maximum was 120h (5 days), whereas the second scenario (*ii*) was

modeled considering 0 and 72h as minimum and maximum values plus 120h. These formulas were based on calculations previously reported by Nauta et al. (2003). The logarithmic growth of *Salmonella* and *L. monocytogenes* and their level after retail storage were calculated as described in section 2.1.

2.3) Transportation from retail to home:

No data on time and temperature during consumer transportation in Brazil has been found. Therefore, a pert distribution was used to model data in this module and the values were mainly based on assumptions (*Table 1*). Minimum temperature, most likely and maximum temperature in this module was 7°C, 12°C and 20°C, respectively. Time of transportation was modeled as described by Nauta et al. (2003) (mean: 42.8 min; standard deviation: 18.7 min), using a Gamma distribution with 5.24 and 8.17 as parameters (*Table 1*). The logarithmic growth and growth during transportation from retail to home were calculated as described in section 2.1.

2.4) Home storage module:

Temperature during storage in home refrigerators was modeled using a pert distribution with minimum, most likely and maximum values of 3.1°C, 6°C and 10.8°C, respectively, extracted from (Silva et al., 2008). Storage time was modeled taking into account that consumer behavior on storage of foods in home refrigerator is influenced by shelf-life shown in the label in the moment of purchase as proposed by Nauta et al. (2003). A uniform distribution with 0 and 192h as minimum and maximum values was used to model storage time at home. As the time of purchase and maximum shelf-life are considered in the calculations proposed by Nauta et al. (2003), times of purchase (I and II) was calculated using time of transportation, storage time at retail and time of transportation from retail to home. Time of purchase used in the calculations of the model was determined using a discrete distribution. The logarithmic growth and level after home storage were calculated as described in section 2.1.

2.5) Consumption of RTE vegetables node, determination of dose-response relationship, probability of illness and number of cases:

The amount of RTE vegetable consumed by Brazilian population is unknown. Therefore, it was assumed 25 g, 50 g and 75 g, as minimum, most likely and maximum values, respectively. The level of pathogens was calculated by summing their levels at the end of each module of the QMRA model (*Table 1*). The dose of pathogens per serving is calculated by multiplying amounts of vegetables consumed and the level of pathogen (*Table 1*). The exposure (number of servings of RTE vegetables intake per month) was obtained from Perez et al. (2008). The dose-response relationship for infection by *Salmonella* was estimated using a beta-Poisson model as proposed by (WHO/FAO, 2002). The parameters α and β were modeled using pert distribution. Minimum, most likely and maximum values were obtained from WHO/FAO (2002). The dose-response relationship for infection by *L. monocytogenes* was determined using an exponential model (Buchanan et al., 1997). The r parameter was represented in the model by a pert distribution with values described by Mataragas et al. (2010). The outputs of the MQRA model were the risk of infection per month and number of cases in the exposed population (*Table 1*). The determination of number of cases of infection due to *Salmonella* and *L. monocytogenes* was calculated considering the population of Sao Paulo city, Brazil (IBGE, 2011) and assumption that approximately 23% of population eats RTE vegetables (Perez et al., 2008).

2.6) Evaluation of different scenarios:

The QMRA model was used to simulate risk of infection and number of cases due to consumption of RTE vegetables starting with lower prevalence and initial populations of *Salmonella* and *L. monocytogenes* than the actual scenario (*Table 3*). Scenarios evaluating the impact of stricter control of temperature during transportation from industry to retail, during retail and home storage on risks and number of cases of infection were also studied (*Table 3*).

2.7) Simulation settings and analysis of models outputs:

The QMRA model was built in an Excel spreadsheet (Microsoft, Redmond, WA) and simulated using @Risk software (Palisade, Ithaca, NY). A total of 100,000 iterations for each scenarios created were run using Monte Carlo sampling and with the random generator seed fixed at 1 to ensure that results can be repeated, allowing comparisons of different scenarios.

3) Results and Discussion:

The current study was carried out to estimate the risks of infection by *Salmonella* and *L. monocytogenes* due to contamination of RTE vegetables consumed in Brazil. The five modules composing the MQRA model are shown in *Table 1*. Few studies were found reporting the prevalence and levels of foodborne pathogens in the field, none in Brazil (Mukherjee et al., 2004, Johnston et al., 2005, Schwaiger et al., 2011). Moreover, information on the behavior of foodborne pathogens in leafy vegetables during field operations is scarce (Islam et al., 2004a, Islam et al., 2004b, Fonseca et al., 2011, Tomás-Callejas et al., 2011). Thus, the fate of *Salmonella* and *L. monocytogenes* in the field and processing operations was not assessed in the current model. In fact, it was assumed that RTE leafy vegetables leaving the processing facilities were contaminated with *Salmonella* and *L. monocytogenes* either in the field or during processing operations (cross-contamination, for example). Although the current model does not include these operations in the calculations, research should be done to generate these data because it is known that field and processing contamination seem to play an important role in the occurrence of foodborne disease outbreaks (Tauxe et al., 1997). The lack of data in these operations was reported recently by Danyluk and Schaffner (2011). The availability of these data would be very useful for the improvement of predictions and accurateness of MQRA models developed in this study.

As can be seen in *Table 1*, in the first module of MQRA model (transportation from produce facility to retail) data on prevalence and concentration of both pathogens were gathered from surveys carried out in Brazil (Porto and Eiroa, 2001, Simões et al., 2001, Maistro, 2006, Fröder et al., 2007, Tresseler et al., 2009, Oliveira et al., 2010, Oliveira et al., 2011, Sant'Ana et al., 2012a). Although these

samples were collected in retail shops, in the MQRA model data on prevalence and levels of pathogens (*Table 2*) were assumed to be found in RTE vegetables just before transportation from processing facilities to retail (*Table 1*). In the current study, prevalence data were represented by beta distribution with α ($s + 1$; where s represents the number of positive samples) and β ($n - s + 1$; where n represents the number of samples analyzed) parameters being 20 and 1098 for *Salmonella*, and 27 and 1181 for *L. monocytogenes*, respectively (*Table 1*). Surveys carried out in Brazil have reported that the prevalence of *Salmonella* in RTE vegetables is majorly below 3% (*Table 2*). The prevalence of *L. monocytogenes* has been reported in levels of up to 5%, with most surveys showing prevalence of about 2% (*Table 2*). In addition, studies reporting levels of *Salmonella* and *L. monocytogenes* in RTE vegetables were sought. Data on concentration of *Salmonella* in RTE vegetables was found in only one study in Brazil (Sant'Ana et al., 2011), whereas *L. monocytogenes* have been reported in countable levels more frequently (Porto and Eiroa, 2001, Oliveira et al., 2010, Sant'Ana et al., 2012a). The maximum levels of *Salmonella* (2.7 log CFU/g) and *L. monocytogenes* (1.1 log CFU/g) found in these studies were used in the MQRA model to represent the possibility that few packages might harbor high levels of these microorganisms (*Table 1*). Although it is known that the risk of foodborne disease outbreaks is dependent upon concentrations of pathogens at the time of consumption (dose ingested), the results obtained in the models built indicated that this correlation is not always true for initial prevalence and concentration levels. Although it has been reported that few packages presenting high levels of pathogens (extremities of statistical distributions) might lead to most risks (Miller et al., 1997), the results of the current models (scenario #1) indicate that even low prevalence and concentration levels might lead to high risk of infection (*Figure 2*). The same pattern of weak correlation was observed for *L. monocytogenes* (figures not shown). These results show that depending on temperature profile to which the RTE vegetables are exposed from processing until consumption might be greatly responsible for the occurrence of foodborne disease outbreaks. A similar pattern has been found by Gonzales-Barron et al. (2012) when characterizing the risk of infection by *S. Typhimurium* due to consumption of Irish pork sausages. Although the QMRA models were created with prevalence and concentration data available currently (*Table 2*), it should be highlighted that more surveys aiming at determining the populations of *Salmonella* and *L. monocytogenes* in fresh produce should be done

using more sensible methods such as MPN or PCR-based techniques (Oliveira et al., 2010). These data would be very useful to improve MQRA models and to reduce uncertainty involved in their predictions (Lammerding et al., 2001).

Still in module 1 (*Table 1*), even knowing that temperature data used were not recorded during transportation of vegetables, but cooked ham (Pereira, 2008, Pereira et al., 2010), it was considered that these data represent well the temperature observed during transportation of chilled foods. This was the only study in which temperature was fully recorded during transportation and delivery. The temperature recorded at surface of primary packages were selected and used in the models because it was observed that temperature of products did not change in the same magnitude of the containers (although large variations occurred between deliveries, mostly when the doors of the truck were opened to unload the products) (Pereira, 2008, Pereira et al., 2010).

The changes (growth) in numbers of pathogens in the modules of RTE vegetables commercialization and consumption chain were modeled using the predictive models generated in experiments to consider the variability in growth rate of three different strains of *Salmonella* and *L. monocytogenes* isolated from RTE vegetables (*Equations 2 and 3*) (Sant'Ana et al., 2012a). The fact that the predictive models used do not consider growth of pathogens at temperature below 7°C does not indicate they will not grow or survive in RTE vegetables. It is the best approach to be used because it assumes that during the shelf-life of RTE vegetables (~8 days) stored below 7°C, the increase in populations of *Salmonella* and particularly *L. monocytogenes* is insignificant or will not happen. Thus, everywhere in the model when a temperature below 7°C was selected during iterations, a growth rate (μ) of zero was assigned and no increase in the initial concentration (module 1) was considered (*Table 1*).

The temperature in retail storage module was represented by data recorded by Maistro (2006). As can be seen in *Figure 1*, a great percentage of data is above 7°C, which indicates the storage of RTE vegetables in retail was not proper. Although these data is limited in order to represent temperature in a wide number of retail shops of Brazil, this study was the single one to provide data on temperature of displays of RTE vegetables in retail shops. The events involving purchase and consumption of RTE vegetables were based on the approaches developed by Nauta et al. (2003). This approach can be quite adequate to represent the behavior of

consumers regarding the purchase and consumption of foods before or until the completion of shelf-life. In this study, the relation between purchase and consumption of RTE vegetables was assessed considering that 70% of vegetables are consumed in less than 5 days, while the 30% remaining are consumed the last 3 days of shelf-life (total shelf-life of 8 days). However, no output describing the risks associated with any of these percentages was assessed.

The main outputs of the MQRA models (risks of infection per month and numbers of cases of infection) developed are showed in *Table 3*. The first scenario (#1) represents the current knowledge regarding RTE vegetable and the pathogens studied, while scenarios #2-4 consider a reduction of prevalence and concentrations (only on maximum values in the case of this last input) of both pathogens (*Table 3*). In the practice, the scenarios (#2-4) would represent the application of intervention measures either in the field or during processing to reduce the prevalence and populations of pathogens reported in *Table 2*. The lower prevalence and concentrations of pathogens tested in these scenarios (#2-4) consider a reduction of these parameters of 10 times. For example, a reduction of prevalence of *Salmonella* from 1.7% to 0.17%, while the decrease in these parameters for *L. monocytogenes* were from 2.2% to 0.22% (*Table 3*). It was not discussed which measures could be adopted or their efficiency to achieve these levels, because it was assumed that measures to reach these targets need to applied either in the field or processing, operations that were not included in this model.

Table 3 shows that *Salmonella* represents a major health hazard due to consumption of RTE vegetables than *L. monocytogenes*. For example, in scenario #1 (that represents the current knowledge of the pathogens-RTE vegetable combination); the mean risk of infection per month of *Salmonella* is 8.66E-03, while for *L. monocytogenes* a risk of 1.87E-08 was predicted (*Table 3*). According to the models developed, scenario #1 would result in the highest number of cases of infection (22,546 and 4.87E-02, for *Salmonella* and *L. monocytogenes*, respectively). Although these numbers can be considered high, it should be highlighted that the current MQRA model did not evaluate the probability of illness (*i.e.*, any derangement in the whole body function or any of its parts), but only the probability of infection (*i.e.*, the invasion of pathogens in the body of hosts), which involves a series of complex events dependent upon interaction of infective agent, host and food determinants,

among others (Put et al., 1988). Therefore, the results should be evaluated carefully as not all cases of infection predicted might result in disease.

Outbreaks involving *Salmonella* and fresh produce have been reported worldwide (Sivapalasingam et al., 2004, Takkinen et al., 2005, Friesema et al., 2007, Little and Gillespie, 2008, Lynch et al., 2009). Although the results of the simulations indicated that the risk of infection caused by *L. monocytogenes* is low (scenario #1: 1.87E-08), the potential of this food-pathogen combination should not be underestimated because *L. monocytogenes* is widely spread in the environment (Gandhi and Chikindas, 2007) and it is resistant to chlorine (main chemical used during produce washing) (Aarnisalo et al., 2007). The microorganism is also able to form biofilms which may allow its persistence in food processing (Harvey et al., 2007) and to grow under chilling temperatures (Gandhi and Chikindas, 2007). RTE vegetables have been considered a category of low risk for listeriosis (Warriner and Namvar, 2009), however, the recent outbreak involving *L. monocytogenes* and cantaloupe in the USA might change this evaluation in some extent.

The analysis of scenarios #2-4 shows that the reduction of prevalence of *Salmonella* from 1.7% to 0.7% would lead to reduction in risk of infection per month of up 6-7 times (Table 3). The simulations show that the reduction of prevalence and concentration is very similar in decreasing the mean risk of infection. In the case of *L. monocytogenes*, the reduction of prevalence from 2.2% to 0.22% resulted in decrease of risk of infection by this microorganism from 1.87E-08 to 2.90E-09. The reduction of population of *L. monocytogenes* from 1.11 log CFU/g to -0.39 log CFU/g would mean a decrease of risk of infection per month from 1.87E-08 to 1.62E-09 (Table 3).

Among the first scenarios studied (#1-4), the lowest number of cases of infection were predicted in scenario #4 (Table 3). This finding indicates that interventions to reduce both prevalence and populations of pathogens would be the most effective to reduce potential threats to food safety due to consumption of RTE vegetables. Therefore, intervention strategies should be applied in order to avoid the contamination of vegetables and further its spreading in a batch of vegetables during processing. Danyluk and Schaffner (2011) reported that according to predictions of their risk assessment model for leafy greens and *E. coli* 0157:H7 up to 95.4% of cases found could be due to cross-contamination during washing step. Thus, cross-contamination during disinfection seems to play a critical role in RTE vegetable

safety because depending on operating conditions, this step might serve as a point of contamination rather than a reduction step (Gil et al., 2009).

A further action to reduce the risk of infection by *Salmonella* and *L. monocytogenes*, i.e., ensuring that temperature is majorly kept $<7^{\circ}\text{C}$ during transportation and storage were evaluated (scenarios #5-8). These scenarios were evaluated because it is known that maintenance of temperature during commercialization and transportation of food products is much unknown. Besides food handlers and consumers might not be aware on how slight changes in temperature might impact microbial growth and risks. The risks and number of cases predicted by these scenarios (#5-8) were reduced for both pathogens studied when compared to scenarios #1-4 (Table 3). Although this reduction was small when scenario #5 is compared to #1, it was large when scenarios #1 and 8 were compared. The outputs of the models indicated that in scenario #8, a total of 185 and $3.81\text{E-}05$ cases of infection due to *Salmonella* and *L. monocytogenes* would be observed (Table 3). This scenario (#8) comprehends both the reduction of prevalence and concentration of pathogens and the maintenance of temperature during the production, commercialization and consumption steps below 7°C . The fact that no scenario would result in low risks (<0) of infection due to *Salmonella* indicate that measures must be taken in the field and processing to avoid their occurrence. This is of particular importance because RTE leafy vegetables are consumed raw. Efforts should be concentrated in avoiding contamination and reducing the prevalence, concentration and growth of pathogens in the field, processing and retail steps. Consumer, transportation companies and retail shops workers should be trained on the importance of keeping RTE vegetables adequately refrigerated.

Epidemiological data have indicated that most outbreaks of salmonellosis and listeriosis are associated with foods of animal origin (Greig and Ravel, 2009). Despite this a recent trend on the occurrence of outbreaks linked to consumption of fresh produce has been observed (Lynch et al., 2009). Currently, few risk assessment models focusing on pathogens and fresh produce have been developed (Franz et al., 2010, Tromp et al., 2010, Danyluk and Schaffner, 2011). However, as these products normally have close contact with soil during field operations (Harris et al., 2003), and knowing that their microbiological safety relies mainly on washing (actually the only step able to inactivate pathogens) (Gil et al., 2009), the association of these products with foodborne disease outbreaks should not be underestimated. However, due to

the crescent reports of outbreaks involving produce-pathogens combinations (Lynch et al., 2009), more studies that take into account diverse variables and factors affecting the fate of pathogens and risks of infection should be developed. This need is confirmed by the recent large and severe outbreak involving *L. monocytogenes* and consumption of cantaloupes in the USA (CDC, 2011). More data is needed to improve the accuracy of risk assessment models developed herein. Despite this, results obtained with the data available concerns mainly regarding *Salmonella*, which is the main foodborne pathogen linked to foodborne disease outbreaks in Brazil. The results suggest that not only more data is needed, but also mitigation strategies need to be taken into account.

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4) References:

- Aarnisalo, K., Lundén, J., Korkeala, H., Wirtanen, G., 2007. Susceptibility of *Listeria monocytogenes* strains to disinfectants and chlorinated alkaline cleaners at cold temperatures. *LWT - Food Science and Technology* 40, 1041-1048.
- Anonymous, (2010). Análise Epidemiológica dos Surtos de Doenças Transmitidas por Alimentos no Brasil. Coordenação de Vigilância das Doenças de Transmissão Hídrica e Alimentar. Secretaria de Vigilância em Saúde. Ministério da Saúde. Available at: http://portal.saude.gov.br/portal/arquivos/pdf/surtos_dta_15.pdf Accessed on: Nov 29, 2011.
- Barancelli, G. V., Camargo, T. M., Reis, C. M. F., Porto, E., Hofer, E., Oliveira, C. A. F. (2011). Incidence of *Listeria monocytogenes* in cheese manufacturing plants from the northeast region of São Paulo, Brazil. *Journal of Food Protection*, 74, 816-819.
- Bergamini, A. M. M., Simões, M., Irino, K., Amaral, T. A. T. G., & Guth, B. E. C. (2007). Prevalence and characteristics of Shiga toxin-producing *Escherichia coli*

(STEC) strains in ground beef in São Paulo, Brazil. *Brazilian Journal of Microbiology*, 38, 553-556.

Buchanan, R. L., Damert, W. G., Whiting, R. C., Van Schothorst, M. (1997). Use of epidemiologic and food survey data to estimate a purposefully conservative dose-response relationship for *Listeria monocytogenes* levels and incidence of listeriosis. *Journal of Food Protection*, 60, 918-922.

Centers for Disease Control and Prevention (CDC). (2011). Multistate Outbreak of Listeriosis Linked to Whole Cantaloupes from Jensen Farms, Colorado. Available in: <http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html> Accessed on Nov 28, 2011.

Codex - (Codex Alimentarius Commission) (1999). Principles and guidelines for the conduct of Microbiological Risk Assessment. CAC/GL-30. 1-6.

Danyluk, M. D., Schaffner, D. W. (2011). Quantitative assessment of the microbial risk of leafy greens from farm to consumption: Preliminary framework, data, and risk estimates. *Journal of Food Protection*, 74, 700-708.

Fonseca, J. M., Fallon, S. D., Sanchez, C. A., Nolte, K. D. (2011). *Escherichia coli* survival in lettuce fields following its introduction through different irrigation systems. *Journal of Applied Microbiology*, 110, 893-902.

Franz, E., Tromp, S. O., Rijgersberg, H., Van Der Fels-Klerx, H. J. (2010). Quantitative microbial risk assessment for *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in leafy green vegetables consumed at salad bars. *Journal of Food Protection*, 73, 274-285

Friesema, I., Schimmer, B., Stenvers, O., Heuvelink, A., de Boer, E., van der Zwaluw, K., de Jager, C., Notermans, D., van Ouwerkerk, I., de Jonge, R., van Pelt, W. (2007). STEC O157 outbreak in the Netherlands, September-October 2007. *Euro Surveillance*, 12, E0711011.

Fröder, H., Martins, C. G., De Souza, K. L. O., Landgraf, M., Franco, B. D. G. M., Destro, M. T. (2007). Minimally processed vegetable salads: Microbial quality evaluation. *Journal of Food Protection*, 70, 1277-1280.

Gandhi, M., & Chikindas, M.L., (2007). *Listeria*: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology*, 113, 1-15.

Gil, M. I., Selma, M. V., López-Gálvez, F., Allende, A., 2009. Fresh-cut product sanitation and wash water disinfection: Problems and solutions. *International Journal of Food Microbiology* 134, 37-45.

- Gonzales-Barron, U. A., Redmond, G., Butler, F. (2010). A risk characterization model of *Salmonella* Typhimurium in Irish fresh pork sausages. Food Research International, doi:10.1016/j.foodres.2010.04.036
- Greig, J. D. & Ravel, A. (2009). Analysis of foodborne outbreak data reported internationally for source attribution. International Journal of Food Microbiology, 130, 77-87.
- Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H., Busta, F.F. (2003). Outbreaks associated with fresh produce: Incidence, growth, and survival of pathogens in fresh and fresh-cut produce. Comprehensive Reviews in Food Science and Food Safety, 2, 78-141.
- Harvey, J., Keenan, K. P., Gilmour, A., 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. Food Microbiology 24, 380-392.
- Instituto Brasileiro de Geografia e Estatística – IBGE. (2011). Estimativa Populacional 2011. – São Paulo. Available at: <http://www.ibge.gov.br/home/estatistica/populacao/estimativa2011/estimativa.shtm> Accessed on Nov 15, 2011.
- Islam, M., Doyle, M. P., Phatak, S. C., Millner, P., Jiang, X. (2004a). Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. Journal of Food Protection, 67, 1365-1370
- Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P., Jiang, X. (2004b). Persistence of *Salmonella enterica* serovar Typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water. Foodborne Pathogens and Disease, 1, 27-35.
- Johnston, L. M., Jaykus, L. -, Moll, D., Martinez, M. C., Anciso, J., Mora, B., et al. (2005). A field study of the microbiological quality of fresh produce. Journal of Food Protection, 68, 1840-1847.
- Lammerding, A. M., Fazil, A. M., Paoli, G. M. (2001). Microbial Food Safety Risk Assessment. In: Downes, F. P., Ito, K. (Eds.). Compendium of methods for the microbiological examination of foods. Washington DC: American Public Health Association, p. 267-282.
- Lemes-Marques, E. G., Cruz, C. D., Destro, M. T. (2007). Pheno- and genotypic characterization of *Listeria monocytogenes* clinical isolates from the southwestern

region of the state of São Paulo, Brazil. Brazilian Journal of Microbiology, 38, 287-292.

Little, C. L., Gillespie, I. A. (2008). Prepared salads and public health. Journal of Applied Microbiology, 105, 1729-1743.

Lynch, M. F., Tauxe, R. V., & Hedberg, C. W. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. Epidemiology and Infection, 137, 307-315.

Maistro, L.C. (2006). Avaliação Microbiológica Visando a Utilização e Comparação de Métodos Rápidos e Convencionais em Vegetais Folhosos Minimamente Processados. Thesis – PhD in Food Science. University of Campinas. Campinas, Brazil, 270 p.

Martins, I. S., Da Conceição Faria, F. C., Miguel, M. A. L., De Sá Colao Dias, M.P., Cardoso, F. L. L., De Gouveia Magalhães, A. C., et al. (2010). A cluster of *Listeria monocytogenes* infections in hospitalized adults. American Journal of Infection Control, 38, e31-e36.

Mataragas, M., Zwietering, M. H., Skandamis, P. N., Drosinos, E. H. (2010). Quantitative microbiological risk assessment as a tool to obtain useful information for risk managers - specific application to *Listeria monocytogenes* and ready-to-eat meat products. International Journal of Food Microbiology, 141, S170-S179.

Miller, A.J., Whiting, R.C., Smith, J.L. (1997). Use of risk assessment to reduce listeriosis incidence. Food Technology, 51, 100-103.

Miyasaki, K. N., Chiarini, E., San'Ana, A. S., Destro, M. T., Landgraf, M., Franco, B. D. G. M. (2009). High prevalence, low counts and uncommon serotypes of *Listeria monocytogenes* in lingüiça, a Brazilian fresh pork sausage. Meat Science, 83, 523-527.

Mukherjee, A., Speh, D., Dyck, E., Diez-Gonzalez, F. (2004). Preharvest evaluation of coliforms, *Escherichia coli*, *Salmonella*, and *Escherichia coli* O157:H7 in organic and conventional produce grown by Minnesota farmers. Journal of Food Protection, 67, 894-900.

Nauta, M. J., Litman, S., Barker, G. C., Carlin, F. (2003). A retail and consumer phase model for exposure assessment of *Bacillus cereus*. International Journal of Food Microbiology, 83, 205-218.

- Oliveira, M. A., Souza, V.M., Bergamini, A. M. M., & Martinis, E.C.P., (2011). Microbiological quality of ready-to-eat minimally processed vegetables consumed in Brazil. *Food Control*, 22, 1400-1403.
- Oliveira, M. G., Brito, J. R. F., Gomes, T. A. T., Guth, B. E. C., Vieira, M. A. M., Naves, Z. V. F., Vieira, M.A.M., Naves, Z.V.F., Vaz, T.M.I., Irino, K. (2008). Diversity of virulence profiles of Shiga toxin-producing *Escherichia coli* serotypes in food-producing animals in Brazil. *International Journal of Food Microbiology*, 127, 139-146.
- Oliveira, M.A., Abeid Ribeiro, E.G., Morato Bergamini, A.M., & Martinis, E.C.P., (2010). Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. *Food Microbiology*, 27, 19-23.
- Oscar, T. P. (2011). Plenary lecture: Innovative modeling approaches applicable to risk assessments. *Food Microbiology*, 28, 777-781.
- Pereira, V. F., Doria, E. C. B., Carvalho Jr., B. C., Neves Filho, L. C., Silveira Jr., V. (2010). Evaluation of temperatures in a refrigerated container for chilled and frozen food transport. *Ciencia e Tecnologia de Alimentos*, 30, 158-165.
- Pereira, V.F. (2008). Evaluation of temperatures in the chilled and frozen food transport. Dissertation – Master degree in Food Engineering. University of Campinas. Campinas, Brazil, 173 p.
- Perez, R., Ramos, A. M., Binoti, M. L., De Sousa, P. H. M., Machado, G. D. M., & Cruz, I. B. (2008). Profile of minimally processed vegetable consumers in Belo Horizonte, Brazil. *Horticultura Brasileira*, 26, 441-446.
- Pires, S. M., Evers, E. G., Van Pelt, W., Ayers, T., Scallan, E., Angulo, F. J., et al. (2009). Attributing the human disease burden of foodborne infections to specific sources. *Foodborne Pathogens and Disease*, 6, 417-424.
- Porto, E., Eiroa, M.N.U. (2001). Occurrence of *Listeria monocytogenes* in vegetables. *Dairy, Food and Environmental Sanitation*, 21, 12-16.
- Putt, S.N.H.; Shaw, A.P.M.; Woods, A.J.; Tyler, L.; James, A.D. (1988). Veterinary epidemiology and economics in Africa - A manual for use in the design and appraisal of livestock health policy. Available in: <http://www.fao.org/Wairdocs/ILRI/x5436E/x5436e00.htm#Contents> Accessed: Nov 30, 2011.

- Ratkowsky, D. A., Olley, J., McMeekin, T. A., & Ball, A., (1982). Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology*, 149, 1-5.
- Sant'Ana, A.S., Igarashi, M.C., Landgraf, M., Destro, M.T., Franco, B.D.G.M. (2012a). Prevalence, populations and pheno- and genotypic characteristics of *Listeria monocytogenes* isolated from ready-to-eat vegetables marketed in São Paulo, Brazil. *International Journal of Food Microbiology*, submitted.
- Sant'Ana, A.S., Franco, B.D.G.M., Schaffner, D.W. (2012b). Modeling the growth rate and lag time of different strains of *Salmonella enterica* and *Listeria monocytogenes* in ready-to-eat lettuce. *Food Microbiology*, doi:10.1016/j.fm.2011.11.003
- Sant'Ana, A.S., Landgraf, M., Destro, M.T., & Franco, B.D.G.M., (2011). Prevalence and counts of *Salmonella* spp. in minimally processed vegetables in Sao Paulo, Brazil. *Food Microbiology*, 28, 1235-1237.
- Sato, G.S., Martins, V.A., & Bueno, C.R.F., (2007). Análise exploratória do perfil do consumidor de produtos minimamente processados na cidade de São Paulo. *Informações Econômicas*, 37, 62-71.
- Schwaiger, K., Helmke, K., Hölzel, C. S., Bauer, J. (2011). Comparative analysis of the bacterial flora of vegetables collected directly from farms and from supermarkets in Germany. *International Journal of Environmental Health Research*, 21, 161-172.
- Silva, D.L.D., Celidonio, F. A., Oliveira, K. M. P. (2008). Verification of the temperature of domestic refrigerators to minimize the deterioration and possible foodborne illnesses. *Higiene Alimentar*, 22, 42-45.
- Silva, N., Silveira, N.F.A., Contreras, C., Beraquet, N.J., Yokoya, F., Nascimento, C.A., Oliveira, V.M., Tse, C.L. (2001). Occurrence of *Escherichia coli* 0157:H7 in meat products and sensibility of the detection methods. *Ciência e Tecnologia de Alimentos*, 21, 223-227.
- Silva, N., Silveira, N.F.A., Yokoya, F., Okazaki, M.M. (2003). Occurrence of *Escherichia coli* 0157:H7 in vegetables and its resistance to the disinfectants used in fresh produce. *Ciência e Tecnologia de Alimentos*, 23, 167-173.
- Simões, M., Pisani, B., Marques, E. G. L., Prandi, M. A. G., Martini, M. H., Chiarini, P. F. T., et al. (2001). Hygienic-sanitary conditions of vegetables and irrigation water from kitchen gardens in the municipality of Campinas, SP. *Brazilian Journal of Microbiology*, 32, 331-333.

- Sivapalasingam, S., C. R. Friedman, L. Cohen, Tauxe, R.V. (2004). Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection*, 67, 2342–2353.
- Takkinen, J., Nakari, U., Johansson, T., Niskanen, T., Siitonen, A., Kuusi, M. (2005). A nationwide outbreak of multiresistant *Salmonella* Typhimurium var Copenhagen DT104B infection in Finland due to contaminated lettuce from Spain, May 2005. *Euro Surveillance*, 10, E050630.
- Tauxe, R. V., (1997). Emerging foodborne diseases: An evolving public health challenge. *Emerging Infectious Diseases*, 3, 425-434.
- Tomás-Callejas, A., López-Velasco, G., Camacho, A. B., Artés, F., Artés-Hernández, F., Suslow, T. V. (2011). Survival and distribution of *Escherichia coli* on diverse fresh-cut baby leafy greens under pre-harvest through postharvest conditions. *International Journal of Food Microbiology*, 151, 216-222.
- Tresseler, J. F. M., Figueiredo, E. A. T., Figueiredo, R. W., Machado, T. F., Delfino, C. M., Sousa, P. H. M. (2009). Microbiological quality evaluation minimally processed vegetables. *Ciencia e Agrotecnologia*, 33, 1722-1727.
- Tromp, S. O., Rijgersberg, H., Franz, E. (2010). Quantitative microbial risk assessment for *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in leafy green vegetables consumed at salad bars, based on modeling supply chain logistics. *Journal of Food Protection*, 73, 1830-1840.
- Warriner, K., Namvar, A., 2009. Why is the hysteria with *Listeria*? *Trends in Food Science and Technology* 20, 245-254.
- WHO/FAO - World Health Organization and Food and Agriculture Organization of the United Nations. (2002). Risk Assessments of *Salmonella* in Eggs and Broiler Chickens. Microbiological Risk Assessment Series. Rome. 329 p.

Table 1: The risk assessment models of infection by *Salmonella* and *L. monocytogenes* due to consumption of RTE vegetables in Brazil.

Notation	Event	Values	Unities	Source
1- Transportation from produce processing facility to retail module				
P_i	Prevalence	<i>Salmonella</i> =RiskBeta(20,1098) <i>L. monocytogenes</i> =RiskBeta(27,1181)	%	see Table 2
C_i	Concentration	<i>Salmonella</i> =RiskPert(-3,-1.4,1.1) <i>L. monocytogenes</i> =RiskPert(-3,-1.4,1.1)	log CFU/g	Sant'Ana et al. (2011, 2012a)
T_1	Temperature during transportation	=RiskPert(3,7.6,10.3)	°C	Pereira et al. 2010, Pereira, 2008.
t_1	Time of transportation	=RiskPert(2,5,9)	h	Pereira et al. 2010, Pereira, 2008.
Lg_1	Logarithmic growth	<i>Salmonella</i> =If($T_1 > 7$, $(0.0178 \times T_1 - 0.11837)^2, 0$) <i>L. monocytogenes</i> =If($T_1 > 7$, $(0.0178 \times T_1 - 0.11837)^2, 0$)	log CFU/g/h	Sant'Ana et al. (2012b)
G_{tra1}	Growth during transportation 1	= $t_1 \times Lg_1$	log CFU/g	Calculated
L_{at1}	Level after transportation 1	= $C_i + G_{tra}$	log CFU/g	Calculated
2- Retail storage module				
T_2	Storage temperature	=RiskDiscrete({5\7\8\9\10\11\12\13\15},{0.017\0.051\0.33\0.10\0.17\0.12\0.16\0.017\0.017})	°C	Maistro (2005)
t_{270}	Storage time at retail I – 70%	=RiskUniform(0,120)	h	Assumption
t_{230}	Storage time at retail II – 30%	=RiskUniform(0,72)+120	h	Assumption
Lg_2	Logarithmic growth	<i>Salmonella</i> =If($T_2 > 7$, $(0.0178 \times T_2 - 0.11837)^2, 0$) <i>L. monocytogenes</i> =If($T_2 > 7$, $(0.0178 \times T_2 - 0.11837)^2, 0$)	log CFU/g/h	Sant'Ana et al. (2012b)
G_{St70}	Growth during retail storage – 70%	= $t_{270} \times Lg_2$	log CFU/g	Calculated
G_{St30}	Growth during retail storage – 30%	= $t_{230} \times Lg_2$	log CFU/g	Calculated
L_{ar}	Level after retail storage	=RiskDiscrete(G_{St70} : G_{St30} ,{0.7\0.3})	log CFU/g	Calculated

Table 1: continued.

Notation	Event	Values	Unities	Source
3- Transportation from retail to home module				
T_3	Temperature	=RiskPert(7,12,20)	°C	Assumption
t_3	Time	=RiskGamma(5.24,8.17)/60	h	Nauta et al. (2003)
Lg_3	Logarithmic growth	$Salmonella = \text{If}(T_3 > 7, (0.0178 \times T_3 - 0.11837)^2, 0)$ $L. monocytogenes = \text{If}(T_3 > 7, (0.0178 \times T_3 - 0.11837)^2, 0)$	log CFU/g/h	Sant'Ana et al. (2012b)
G_{tra2}	Growth during transportation	$= t_3 \times Lg_3$	log CFU/g	Calculated
L_{at2}	Level after transportation 2	$= G_{tra2}$	log CFU/g	Calculated
4- Home storage module				
T_4	Storage temperature	=RiskPert(3.04,6,10.8)	°C	Silva et al., 2008
t_4	Storage time	=RiskUniform(0,192)	h	Assumption
t_{p1}	Time of purchase I	$= t_1 + t_2 70 + t_3$	h	Calculated
t_{p2}	Time of purchase II	$= t_1 + t_2 30 + t_3$	h	Calculated
$\%^{pur70}$	% of packages purchased < 5 days	=0.7	%	Assumption
$\%^{pur30}$	% of packages purchased > 5 days	=0.3	%	Assumption
PD^{usd}	Time of purchase used	=RiskDiscrete(t_{p1} : t_{p2} , $\%^{pur70}$: $\%^{pur30}$)	h	Calculated
PSL	Product shelf-life	192	h	Assumption
Lg_4	Logarithmic growth	$Salmonella = \text{If}(T_3 > 7, (0.0178 \times T_3 - 0.11837)^2, 0)$ $L. monocytogenes = \text{If}(T_3 > 7, (0.0178 \times T_3 - 0.11837)^2, 0)$	log CFU/g/h	Sant'Ana et al. (2012b)
G_{hs}	Growth during home storage	$= \text{If}(t_4 + PD^{usd} > \text{PSL}, 0, Lg_4)$	log CFU/g	Calculated
L_{ahSt}	Level after home storage	$= G_{hs}$	log CFU/g	Calculated
T_{lcons}	Total level before consumption	$= L_{at1} + L_{ar} + L_{at2} + L_{ahSt}$	log CFU/g	Calculated

Table 1: continued.

Notation	Event	Values	Unities	Source
<i>5- Consumption, dose-response and risk of infection module - Salmonella</i>				
S	Serving size	$=\text{RiskPert}(25,50,75)$	g	Assumption
CFU	Level of pathogen (non-log)	$=10^{T_{lcons}}$	CFU/g	Calculated
D	Dose per serving	$= S \times CFU$	CFU	Calculated
α	Parameter alpha	$=\text{RiskPert}(0.0763,0.1324,0.2274)$	-	WHO/FAO (2002)
β	Parameter beta	$=\text{RiskPert}(38.4,51.4,57.9)$	-	WHO/FAO (2002)
P_{id}	Probability of infection single dose	$=1-(1+ D/\alpha)^{-\beta}$	-	Calculated
E	Exposure (number of servings/month)	$=\text{RiskDiscrete}(\{1\backslash 2\backslash 12\backslash 30\},\{0.43\backslash 0.23\backslash 0.27\backslash 0.07\})$	Servings	Perez et al. (2008)
R_m	Risk of infection per month	$=\text{RiskOutput}()+1-(1- P_i \times P_{id})^E$	-	Calculated
P_{sp}	Population Sao Paulo city	$=1.13\text{E}+07$	Inhabitants	IBGE (2011)
$\%_{eat}$	% of population eating RTE vegetables	$=23$	%	Perez et al., 2008
P_{eat}	Population of Sao Paulo eating RTE vegetables	$=2.60\text{E}+06$	Inhabitants	Calculated
N_c	Number of cases in population exposed	$=R_m \times P_{eat}$	Cases	Calculated
<i>6- Consumption, dose-response and risk of infection module – L. monocytogenes</i>				
S	Serving size	$=\text{RiskPert}(25,50,75)$	g	Assumption
CFU	Level of pathogen (non-log)	$=10^{T_{lcons}}$	CFU/g	Calculated
D	Dose per serving	$= S \times CFU$	CFU	Calculated
r	Parameter r	$=\text{RiskPert}(1.11 \times 10^{-15}, 4.47 \times 10^{-11}, 1.36 \times 10^{-9})$	-	Mataragas et al. (2010)
P_{id}	Probability of infection single dose	$=1-e^{(-r \times D)}$	-	Calculated
E	Exposure (number of servings/month)	$=\text{RiskDiscrete}(\{1\backslash 2\backslash 12\backslash 30\},\{0.43\backslash 0.23\backslash 0.27\backslash 0.07\})$	Servings	Perez et al. (2008)
R_m	Risk of infection per month	$=1-(1- P_i \times P_{id})^E$	-	Calculated
P_{sp}	Population Sao Paulo city	$=1.13\text{E}+07$	Inhabitants	IBGE (2011)

Table 1: continued.

$\%_{eat}$	% of population eating RTE vegetables	=23	%	Perez et al., 2008
P_{eat}	Population of Sao Paulo eating RTE vegetables	=2.60E+06	Inhabitants	Calculated
N_c	Number of cases in population exposed	$=R_m \times P_{eat}$	-	Calculated

Table 2: Data published in literature and selected for this study on the prevalence of *Salmonella* and *L. monocytogenes* in RTE leafy vegetables in Brazil.

Microorganisms	Samples		Reference
	Total	Positive	
<i>Salmonella</i>	166	4	Simões et al. (2001)
	172	1	Maistro (2005)
	111	4	Fröder et al. (2007)
	56	6	Tresseler et al. (2009)
	134	2	Oliveira et al., (2011)
	477	2	Sant'Ana et al. (2011)
<i>Total</i>	1,116	19	-
<i>L. monocytogenes</i>	150	8	Porto and Eiroa (2001)
	172	2	Maistro (2005)
	111	1	Fröder et al. (2007)
	162	2	Oliveira et al. (2010)
	134	2	Oliveira et al. (2011)
	477	11	Sant'Ana et al. (2011)
<i>Total</i>	1,206	26	-

Table 3: Outputs of the QMRA model depicting the probability of infection and number of cases due to consumption of RTE vegetables contaminated with *Salmonella* and *L. monocytogenes* in Sao Paulo, Brazil^a.

Scenarios ^b	Description	Microorganism	Output 1		Output 2	
			(Risk of infection/month)	(Number of cases of infection)		
			Mean	95%	Mean	95%
1	$P=1.7\%$ $C=2.74 \log \text{ CFU/g}$	<i>Salmonella</i>	8.66E-03	4.83E-02	22,546	125,637
	$P=2.2\%$ $C=1.11 \log \text{ CFU/g}$	<i>L. monocytogenes</i>	1.87E-08	5.94E-09	4.87E-02	1.54E-02
	$P=0.17\%$ $C=2.74 \log \text{ CFU/g}$	<i>Salmonella</i>	1.33E-03	6.83E-03	3,465	17,775
2	$P=0.22\%$ $C=1.11 \log \text{ CFU/g}$	<i>L. monocytogenes</i>	2.90E-09	8.35E-10	7.56E-03	2.17E-03
	$P=1.7\%$ $C=-1.04 \log \text{ CFU/g}$	<i>Salmonella</i>	1.84E-03	6.57E-03	4,795	17,098
3	$P=2.2\%$ $C=-0.39 \log \text{ CFU/g}$	<i>L. monocytogenes</i>	3.24E-09	1.62E-09	8.44E-03	4.23E-03
	$P=0.17\%$ $C=-1.04 \log \text{ CFU/g}$	<i>Salmonella</i>	2.77E-04	9.97E-04	721	2,594
4	$P=0.22\%$ $C=-0.39 \log \text{ CFU/g}$	<i>L. monocytogenes</i>	4.91E-10	2.36E-10	1.27E-03	6.14E-04

Table 3: continued.

5	$P=1.7\%$ $C=2.74 \log \text{ CFU/g}$	Salmonella	5.50E-03	2.94E-02	14,323	76,609
	$P=2.2\%$ $C=1.11 \log \text{ CFU/g}$	<i>L. monocytogenes</i>	4.57E-10	1.71E-09	1.189E-03	4.47E-03
	$P=0.17\%$ $C=2.74 \log \text{ CFU/g}$	Salmonella	8.41E-04	4.09E-03	2,188	10,637
6	$P=0.22\%$ $C=1.11 \log \text{ CFU/g}$	<i>L. monocytogenes</i>	6.75E-11	2.49E-10	1.75E-04	6.48E-04
	$P=1.7\%$ $C=-1.04 \log \text{ CFU/g}$	Salmonella	4.74E-04	2.07E-03	1,234	5,400
	$P=2.2\%$ $C=-0.39 \log \text{ CFU/g}$	<i>L. monocytogenes</i>	9.91E-11	4.63E-10	2.58E-04	1.20E-03
8	$P=0.17\%$ $C=-1.04 \log \text{ CFU/g}$	Salmonella	7.09E-05	3.24E-04	185	843
	$P=0.22\%$ $C=-0.39 \log \text{ CFU/g}$	<i>L. monocytogenes</i>	1.46E-11	6.70E-11	3.81E-05	1.74E-04

^aEach scenario was run in @Risk using 100,000 iterations with generator seed fixed at 1; ^bScenario 1 was run with data representing the real world, while scenarios 2-8 represent changes either in prevalence, concentration or temperature during transportation and storage of RTE vegetables.

Figure 1: Discrete distribution representing the temperature of retail storage of RTE vegetables as recorded by Maistro (2005).

Figure 2: Statistical distribution of the growth rate (μ) of *Salmonella* (A) and *L. monocytogenes* (B) in RTE leafy vegetables.

12. CONCLUSÕES GERAIS

As análises microbiológicas de quinhentas e doze amostras de VMPs comercializados na cidade de São Paulo indicaram que *Salmonella* e *L. monocytogenes* ocorrem em diversos tipos de vegetais, oriundos das diferentes regiões abastecedoras do mercado consumidor, inclusive com algumas amostras apresentando populações dos patógenos acima de 10^2 UFC/g. Todas as cepas de *Salmonella* isoladas pertenciam a espécie *S. enterica*, que foi isolada de diversos alimentos no Brasil anteriormente. A enumeração de *L. monocytogenes* com populações maiores que 10^1 UFC/g em cinco amostras e prevalência em 3.1% dos VMPs analisados preocupa, principalmente se tais vegetais forem usados para preparação de saladas não cozidas e mantidas sob refrigeração por períodos prolongados. Entre os isolados de *L. monocytogenes*, foi observada a incomum ocorrência de sorotipos 4b, os quais são normalmente isolados de pacientes. Além disso, foi observada uma grande predominância do ribotipo DUP-ID 1038, que é o mais envolvido em surtos de listeriose transmitidos por alimentos no mundo. Mesmo isolados considerados clones através da ribotipagem apresentaram respostas variadas frente ao cloro normalmente utilizado para sanificação dos vegetais e equipamentos, capacidade de formação de biofilmes e taxa de multiplicação. Estes resultados indicam a importância da caracterização das cepas isoladas frente a fatores estressantes normalmente encontrados no processamento dos alimentos em questão.

Os testes-desafio demonstraram que tanto *Salmonella* quanto *L. monocytogenes* podem se multiplicar rapidamente sob temperatura de abuso (15°C) e demonstram a importância de se manter controle restrito de temperatura ($<7^{\circ}\text{C}$) durante toda a cadeia produtiva, comercialização e consumo. Os resultados dos testes-desafio permitem concluir que tais microrganismos não se multiplicam a populações elevadas porque principalmente a vida de prateleira de VMPs no Brasil não ultrapassa 8 dias em sua maioria, ao contrário de países como EUA, onde a vida de prateleira dos VMPs pode chegar a 15 dias. Desta forma, a redução da vida de prateleira funciona como uma estratégia de gestão do risco, impedindo que patógenos potencialmente contaminando os VMPs se multipliquem e possam causar infecções e doenças. Ainda de acordo com os testes-desafio, o uso de filmes

perforados para embalar os VMPs, como ocorre em diversos casos no Brasil, parece auxiliar no controle do desenvolvimento de patógenos como *Salmonella* e *L. monocytogenes* que podem contaminar tais alimentos.

Os modelos preditivos desenvolvidos numa ampla faixa de temperatura (7-30°C) permitem a sua utilização para modelagem da multiplicação de *Salmonella* e *L. monocytogenes* em modelos de avaliação quantitativa de risco, representando condições sob as quais os VMPs podem ser expostos desde sua produção até consumo.

Os modelos de avaliação de risco construídos indicaram que *Salmonella* representa um desafio para a segurança dos VMPs. Apesar de mais dados serem necessários para melhorar a precisão dos modelos, os resultados obtidos já servem para que mais atenção e, posteriormente, estratégias de gestão de riscos sejam desenvolvidas e validadas para reduzir os riscos de infecção por *Salmonella* devido ao consumo de VMPs. Isto é particularmente preocupante por VMPs serem consumidos crus e a dose infectante de *Salmonella*, ser baixa em alguns casos. A análise dos cenários estudados fornece uma estimativa de quanto os riscos podem ser mitigados com a redução da prevalência e concentração inicial dos patógenos estudados. Além disso, fica claro como pequenas variações de temperatura podem contribuir para o aumento da probabilidade de infecções, e desta forma, verifica-se o quão importante é treinamento e conscientização dos envolvidos em toda cadeia produtiva sobre o controle da temperatura como estratégia para garantir alimentos seguros. Os modelos de avaliação de risco desenvolvidos deverão ser atualizados com a geração de mais dados e informações das partes interessadas, o que levará a melhoria de suas predições, que poderão ser usadas como base para definição de estratégias de intervenção e gestão cientificamente embasadas.

13. REFERÊNCIAS

- ALBAN, L., OLSEN, A.-M., NIELSEN, B., SORENSEN, R., JESSEN, B. Qualitative and quantitative risk assessment for human salmonellosis due to multi-resistant *Salmonella* Typhimurium DT104 from consumption of Danish dry-cured pork sausages. **Preventive Veterinary Medicine**, v.52, p.251-265, 2002.
- ANDERSON, S. A., YEATON, R. W., CRAWFORD, L. M. Risk assessment of the impact on human health of resistant *Campylobacter jejuni* from fluoroquinolone use in beef cattle. **Food Control**, v.12, p.13-25, 2001.
- CASSIN, M. H., LAMMERDING, A. M., TODD, E. C. D., ROSS, W., MCCOLL, R. S. Quantitative risk assessment for *Escherichia coli* O157:H7 in ground beef hamburgers. **International Journal of Food Microbiology**, v.41, p.21-44, 1998.
- CENTRO DE VIGILÂNCIA EPIDEMIOLÓGICA DO ESTADO DE SÃO PAULO (CVE). **Surtos de doenças transmitidas por alimentos notificados ao CVE**. Disponível em: http://www.cve.saude.sp.gov.br/htm/hidrica/hidri_estat.html Acessado em 28 de dezembro de 2011, 2007.
- CODEX ALIMENTARIUS. **Principles and guidelines for the conduct of microbiological risk assessment**. CAC/GL-30/1999. Rome: Codex Alimentarius. Disponível em: www.codexalimentarius.net. Acesso em 20 de Novembro de 2011. 1999.
- DANYLUK, M. D., SCHAFFNER, D. W. Quantitative assessment of the microbial risk of leafy greens from farm to consumption: Preliminary framework, data, and risk estimates. **Journal of Food Protection**, v.74, p.700-708, 2011.
- DE ROEVER, C. Microbiological safety evaluations and recommendations on fresh produce. **Food Control**, v.9, p.321-347, 1998.
- DELIGNETTE-MULLER, M. L., CORNU, M. Quantitative risk assessment for *Escherichia coli* O157:H7 in frozen ground beef patties consumed by young children in French households. **International Journal of Food Microbiology**, v.128, p.158-164, 2008.
- FANG, T.J., CHEN, C.Y., KUO, W.Y. Microbiological quality and incidence of *Staphylococcus aureus* and *Bacillus cereus* in vegetarian food products. **Food Microbiology**, v.16, p.385-391, 1999.

FARBER, J.M., WANG, S.L., CACI, Y., ZHANG, S. Changes in populations of *Listeria monocytogenes* inoculated on packaged fresh-cut vegetables. **Journal of Food Protection**, v.61, p.192-195, 1998.

Francis, A.G., Thomas, C.O., O'Beirne, D. The microbiological safety of minimally processed vegetables. **International Journal of Food Science and Technology**, v.34, p.1-22, 1999.

FRANCIS, G.A., O'BEIRNE, D. Isolation and pulsed-field gel electrophoresis typing of *Listeria monocytogenes* from modified atmosphere packaged fresh-cut vegetables collected in Ireland. **Journal of Food Protection**, v.69, p.2524-2528, 2006

FRÖDER, H, MARTINS, C.G., SOUZA, K.L.O., LANDGRAF, M., FRANCO, B.D.G.M., DESTRO, M.T. Minimally processed vegetables: microbiological quality evaluation. **Journal of Food Protection**, v.70, p.1277-1280, 2007.

GANDHI, M., CHIKINDAS, M.L. *Listeria*: A foodborne pathogen that knows how to survive. **International Journal of Food Microbiology**, v.113, p.1-15, 2006.

GIOVANNINI, A., PRENCIPE, V., CONTE, A., MARINO, L., PETRINI, A., POMILIO, F., RIZZI, V., MIGLIORATI, G. Quantitative risk assessment of *Salmonella* spp. infection for the consumer of pork products in an Italian region. **Food Control**, v.15, p.139-144, 2004.

GÓMEZ-LOPEZ, V.M. DEVLIEGHERE, F., DEBEVERE, J. Shelf-life extension of minimally processed carrots by gaseous chlorine dioxide. **International Journal of Food Microbiology**, v.16, p.221-227, 2007.

GOULARTE, L., MARTINS, C.G., MORALES-AIZPURÚA, I. C., DESTRO, M. T., FRANCO, B.D.G.M., VIZEU, D.M., HUTZLER, B.W., LANDGRAF, M. Combination of minimal processing and irradiation to improve the microbiological safety of lettuce (*Lactuca sativa*, L.). **Radiation Physics and Chemistry**, v.71, p.157-161, 2004.

GRACE, D., OMORE, A., RANDOLPH, T., KANG'ETHE, E., NASINYAMA, G. W., MOHAMMED, H. O. Risk assessment for *Escherichia coli* O157:H7 in marketed unpasteurized milk in selected east African countries. **Journal of Food Protection**, v.71, p.257-263, 2007.

IFPA - International Fresh-Cut Produce Association. Food Safety Guidelines for the Fresh-Cut Produce Industry, 1999.

Iwahori, J., Yamamoto, A., Suzuki, H., Yamamoto, T., Tsutsui, T., Motoyama, K., SAWADA, M., MATSUSHITA, T., HASEGAWA, A., OSAKA, K., TOYOFUKU, H., KASUGA, F. Quantitative risk assessment of *Vibrio parahaemolyticus* in finfish: A

model of raw horse mackerel consumption in Japan. **Risk Analysis**, v.30, p.1817-1832, 2010.

KARENLAMPI, R., HANNINEN, M.L. Survival of *Campylobacter jejuni* on various fresh produce. **International Journal of Food Microbiology**, v.97, p.187-195, 2004.

KIM, H., RYU, J.H., BEUCHAT, L.R. Survival of *Enterobacter sakasaki* on fresh produce as affected by temperature, and effectiveness of sanitizers for its elimination. **International Journal of Food Microbiology**, v.111, p.134-143, 2006.

LIN, C.M., FERNANDO, S.Y., WEI, C. Occurrence of *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* and *E. coli* 0157:H7 in vegetable salads. **Food Control**, v.7, p.135-140, 1996.

LINDQVIST, R., LINDBLAD, M. Quantitative risk assessment of thermophilic *Campylobacter* spp. and cross-contamination during handling of raw broiler chickens evaluating strategies at the producer level to reduce human campylobacteriosis in Sweden. **International Journal of Food Microbiology**, v.121, p.41-52, 2008.

LINDQVIST, R., WESTOO, A. Quantitative risk assessment for *Listeria monocytogenes* in smoked or gravad salmon and rainbow trout in Sweden. **International Journal of Food Microbiology**, v.58, p.181-196, 2000.

MAISTRO, L.C. **Avaliação microbiológica visando a utilização e comparação de métodos rápidos e convencionais em vegetais folhosos minimamente processados**. Campinas. 2005. Tese (Doutorado em Ciência de Alimentos) – Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas.

MARTINS, C.G., BEHRENS, J.H.; DESTRO, M.T., FRANCO, B.D.G.M., VIZEU, D.M., HUTZLER, B., LANDGRAF, M. Gamma radiation in the reduction of *Salmonella* spp inoculated on minimally processed watercress (*Nasturtium officinalis*). **Radiation Physics and Chemistry**, v.71, p.87-91, 2004..

MATARAGAS, M., ZWIETERING, M. H., SKANDAMIS, P. N., DROSINOS, E. H. Quantitative microbiological risk assessment as a tool to obtain useful information for risk managers - specific application to *Listeria monocytogenes* and ready-to-eat meat products. **International Journal of Food Microbiology**, v.141, p.S170-S179, 2010.

MOKHTARI, A., MOORE, C.M., YANG, H., JAYKUS, L.A., MORALES, R., CATES, S.C., COWEN, P. Consumer-phase *Salmonella enterica* serovar Enteritidis risk assessment for egg-containing food products. **Risk Analysis**, v.26, p.753-768, 2006.

NAUTA, M. J., JACOBS-REITSMA, W. F., HAVELAAR, A. H. A risk assessment model for *Campylobacter* in broiler meat. **Risk Analysis**, v.27, p.845-861, 2007.

NGUZ, K., SHINDANO, J., SAMAPUNDO, S., HUYGHEBAERT, A. Microbiological evaluation of fresh-cut organic vegetables produced in Zambia. **Food Control**, v.16, p.623-628, 2005.

OSCAR, T.P. A quantitative risk assessment model for *Salmonella* and whole chickens. **International Journal of Food Microbiology**, v.93, p.231-247, 2004

PARSONS, D. J., ORTON, T. G., D'SOUZA, J., MOORE, A., JONES, R., DODD, C. E. R. A comparison of three modelling approaches for quantitative risk assessment using the case study of *Salmonella* spp. in poultry meat. **International Journal of Food Microbiology**, v.98, p.35-51, 2005.

PÉREZ-RODRÍGUEZ, F., TODD, E. C. D., VALERO, A., CARRASCO, E., GARCÍA, R. M., ZURERA, G. Linking quantitative exposure assessment and risk management using the food safety objective concept: An example with *Listeria monocytogenes* in different cross-contamination scenarios. **Journal of Food Protection**, v.69, p.2384-2394, 2006.

PORTO, E., EIROA, M.N.U. Occurrence of *Listeria monocytogenes* in vegetables. **Dairy, Food and Environmental Sanitation**, v.21, p.282-286, 2001.

POUILLOT, R., MICONNET, N., AFCHAIN, A.-L., DELIGNETTE-MULLER, M. L., BEAUFORT, A., ROSSO, L., DENIS, J.-B., CORNU, M. Quantitative Risk Assessment of *Listeria monocytogenes* in French Cold-Smoked Salmon: I. Quantitative Exposure Assessment. **Risk Analysis**, v.27, p.683–700, 2007.

Programa Alimentos Seguros – PAS. **Análise de riscos na gestão da segurança de alimentos**. Ações Especiais – PAS. Vassouras. SENAI-DR/RJ. 44 p, 2004.

ROSENQUIST, H., NIELSEN, N. L., SOMMER, H. M., NØRRUNG, B., & CHRISTENSEN, B. B. Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. **International Journal of Food Microbiology**, v.83, p.87-103, 2003.

ROSS, T., RASMUSSEN, S., FAZIL, A., PAOLI, G., SUMNER, J. Quantitative risk assessment of *Listeria monocytogenes* in ready-to-eat meats in Australia. **International Journal of Food Microbiology**, v.131, p.128-137, 2009.

SALLEH, N.A., RUSUL, G., HASSAN, Z., REEZAL, A., ISA, S.H., NISHIBUCHI, M., RADU, S. Incidence of *Salmonella* spp in raw vegetables in Selangor, Malaysia. **Food Control**, v.14, p.475-479, 2003.

- SELMA, M.V., BELTRÁN, D., ALLENDE, A., CHACÓN-VERA, E., GIL, M.I. Elimination by ozone of *Shigella sonnei* in shredded lettuce and water. **Food Microbiology**, v.24, p.492-499, 2007
- SIGNORINI, M., TARABLA, H. Quantitative risk assessment for verocytotoxigenic *Escherichia coli* in ground beef hamburgers in Argentina. **International Journal of Food Microbiology**, v.132, p.153-161, 2009.
- TROMP, S. O., RIJGERSBERG, H., FRANZ, E. Quantitative microbial risk assessment for *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in leafy green vegetables consumed at salad bars, based on modeling supply chain logistics. **Journal of Food Protection**, v.73, p.1830-1840, 2010.
- TUOMINEN, P., RANTA, J., MAIJALA, R. Studying the effects of POs and MCs on the *Salmonella* ALOP with a quantitative risk assessment model for beef production. **International Journal of Food Microbiology**, v.118, p.35-51, 2007.
- UYTTENDAELE, M., BAERT, K., GHAFIR, Y., DAUBE, G., DE ZUTTER, L., HERMAN, L., DIERICK, K., PIERARD, D., DUBOIS, J.J., HORION, B., DEBEVERE, J. Quantitative risk assessment of *Campylobacter* spp. in poultry based meat preparations as one of the factors to support the development of risk-based microbiological criteria in Belgium. **International Journal of Food Microbiology**, v.111, p.149-163, 2006.
- VALERO, M., FERNÁNDEZ, P.S., SALMERÓN, M.C. Influence of pH and temperature on growth of *Bacillus cereus* in vegetable substrates. **International Journal of Microbiology**, v.82, p.71-79, 2003.
- WEISSINGER, W.R., CHANTARAPANONT, W., BEUCHAT, L.R. Survival and growth of *Salmonella* Baildon in shredded lettuce and diced tomatoes, and effectiveness of chlorinated water as a sanitizer. **International Journal of Food Microbiology**, v.62, p.123-131, 2000.
- WHITING, R. C., BUCHANAN, R. L. Development of a quantitative risk assessment model for *Salmonella* Enteritidis in pasteurized liquid eggs. **International Journal of Food Microbiology**, v.36, p.111-125, 1997.
- YAMAMOTO, A., IWAHORI, J., VUDDHAKUL, V., CHARERNJIRATRAGUL, W., VOSE, D., OSAKA, K., SHIGEMATSU, M., TOYOFUKU, H., YAMAMOTO, S., NISHIBUCHI, M., KASUGA, F. Quantitative modeling for risk assessment of *Vibrio*

parahaemolyticus in bloody clams in southern Thailand. **International Journal of Food Microbiology**, v.124, p.70-78, 2008.

YANG, H., MOKHTARI, A., JAYKUS, L.-., MORALES, R. A., CATES, S. C., COWEN, P. Consumer phase risk assessment for *Listeria monocytogenes* in deli meats. **Risk Analysis**, v.26, p.89-103, 2006.

ANEXOS

Revisão

Avaliação quantitativa de risco microbiológico em alimentos: conceitos, sistemática e aplicações

Review

Microbial quantitative risk assessment of foods: concepts, systematics and applications

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Resumo

A segurança dos alimentos é um tema de crescente importância no mundo e preocupa governantes, indústrias e consumidores. Com o aumento do comércio mundial de alimentos, o estabelecimento de regras claras de comércio internacional e que protejam a saúde da população tornou-se prioritário. Em 1995, os países signatários da Organização Mundial do Comércio assinaram um acordo de Medidas Sanitárias e Fitossanitárias, cuja principal consequência foi a adoção da ferramenta de análise de risco no contexto da segurança dos alimentos. Um dos componentes dessa ferramenta é a avaliação de risco microbiológico, que permite verificar se uma determinada combinação microrganismo patogênico/alimento representa ou não um risco acima daquele considerado aceitável em um país. Trata-se de um processo que envolve quatro etapas: (i) identificação do perigo; (ii) caracterização do perigo; (iii) avaliação da exposição; e (iv) caracterização do risco. Considerando a importância desta ferramenta na gestão da segurança dos alimentos, o objetivo desta revisão é apresentar os conceitos básicos da avaliação quantitativa de risco microbiológico em alimentos, sua importância no contexto atual de inocuidade dos alimentos e as possíveis aplicações, benefícios e limitações.

Palavras-chave: Avaliação quantitativa de risco;
Análise de risco microbiológico; Inocuidade de alimentos.

Summary

Food safety is an important issue the world over and is a major concern of food industries, food safety authorities and governments. Due to increasing international trade, clear trade rules and measures to protect consumer health have become mandatory. In 1995, the member countries of the World Trade Organization signed the Sanitary and Phyto-Sanitary Agreement, which specifies that the measures should be based on assessment of the risks to human health. One of the consequences of this agreement was the adoption of the risk analysis concepts. Risk assessment is one component of a risk analysis and allows one to evaluate if a risk associated with a determined pathogen-food combination is higher than that considered acceptable or adequate to protect consumer health. Risk assessment is a science-based process, consisting of the following four steps: (i) hazard identification; (ii) hazard characterization; (iii) exposure assessment and (iv) risk characterization. Due to its importance as a tool in food safety management, this review aimed at presenting the basic concepts of a microbiological risk assessment, its relevance in the accepted food safety context and possible applications, benefits and limitations.

Key words: Quantitative risk assessment; Microbiological risk analysis;
Food safety.

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Avaliação quantitativa de risco microbiológico em alimentos: conceitos, sistemática e aplicações

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1 Introdução

Com a intensificação do comércio internacional de alimentos, é fundamental que os sistemas de controle dos perigos e de gestão de sua segurança sejam estabelecidos com base em princípios claros e aceitáveis por todos os envolvidos. Devido à necessidade de harmonizar os procedimentos de controle relativos à segurança dos alimentos, os países signatários da Organização Mundial do Comércio assinaram em 1995 um acordo denominado Acordo de Medidas Sanitárias e Fitossanitárias - SPS (*Sanitary and Phytosanitary Agreement*), cujo objetivo é proteger a saúde humana, animal e vegetal em seu território. Em função desse acordo, cada país membro da OMC tem o direito de utilizar as medidas que julgar necessárias para garantir o nível de proteção à saúde pública, sanidade animal e vegetal existentes no país. Estas medidas, todavia, não devem ter como objetivo o protecionismo no comércio ou se transformar em barreiras comerciais injustificáveis no comércio bilateral (WTO, 1995; STRINGER, 2005; WOOLRIDGE, 2008).

Uma das consequências mais marcantes do Acordo de Medidas Sanitárias e Fitossanitárias foi a adoção dos princípios básicos da Análise de Risco pela área de segurança de alimentos. Segundo a definição clássica do Codex Alimentarius, 1999, uma análise de risco é composta por três componentes: avaliação do risco, gestão do risco e comunicação do risco (Figura 1). Através de uma Análise de Risco, identifica-se um problema, avalia-se sua gravidade, consideram-se as alternativas de gestão possíveis, seleciona(m)-se a(s) alternativa(s) mais adequada(s), implementa(m)-se essa(s) alternativa(s) de gestão e avalia-se seu impacto na solução do problema (DENNIS et al., 2008).

Enquanto a avaliação de risco microbiológico (*risk assessment*) associado a um perigo microbiológico com efeito adverso à saúde humana é subsidiada por informações científicas, a gestão do risco (*risk management*) é a etapa em que se implementam medidas de controle que efetivamente poderão reduzir o risco ao nível de proteção

desejado. Para isso, é necessário avaliar todas as medidas de controle possíveis e selecionar as adequadas e passíveis de implementação. A comunicação do risco (*risk communication*) é a etapa em que se faz a troca de informações e opiniões a respeito dos riscos e dos fatores relacionados, no qual interagem os assessores de riscos, os gestores de riscos e as outras partes interessadas (FAO; WHO, 1997). Embora cada componente da análise de risco tenha objetivos distintos, as atividades são integradas, com a participação de todos os envolvidos no processo, sendo um processo sistemático e transparente (CAST, 2006; WHITING e BUCHANAN, 2008).

A análise de risco em alimentos, particularmente a avaliação do risco, é uma ferramenta de importância capital, principalmente para países em que o agronegócio representa uma significativa parcela da balança comercial, como o Brasil. Além de avaliar os riscos associados ao consumo dos alimentos produzidos no País e também dos importados, a análise do risco protege o País contra a imposição de barreiras comerciais "mascaradas" como medidas sanitárias e/ou fitossanitárias. Considerando a importância da avaliação de risco na gestão da segurança dos alimentos e o pouco conhecimento no País sobre o assunto, preparou-se a presente revisão, com o objetivo de apresentar os conceitos básicos da avaliação de risco e sua sistemática de aplicação, benefícios e limitações.

2 Avaliação quantitativa de risco microbiológico

Segundo o *Codex Alimentarius* (CODEX, 1999), a avaliação de risco microbiológico é um processo de base científica, constituído das seguintes etapas: (i) identificação do perigo microbiológico; (ii) caracterização deste perigo; (iii) avaliação da exposição e (iv) caracterização do risco.

Uma avaliação de risco microbiológico inicia-se com a determinação da combinação patógeno-alimento a ser considerada. Neste contexto, é preciso identificar o perigo microbiológico a ser considerado em um dado tipo de alimento de acordo com seu envolvimento como agente de doenças nos consumidores, baseando-se em informações epidemiológicas e biológicas pertinentes ao patógeno e ao alimento avaliados (BUCHANAN, 1997; WOOLRIDGE, 2008).

Determinada a combinação patógeno-alimento que comporá a avaliação de risco, a etapa seguinte é a avaliação da exposição da população a esse patógeno em decorrência da ingestão do alimento considerado. A avaliação da exposição baseia-se em modelos preditivos matemáticos para descrever as respostas de multiplicação, sobrevivência e inativação do microrganismo, bem como a possibilidade de recontaminação em diferentes condições ambientais, com o objetivo de estimar a quantidade do patógeno no momento do consumo.

Figura 1. Estrutura de uma Análise de Risco (CODEX ALIMENTARIUS, 1999).

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Nas simulações, leva-se em conta a probabilidade de ocorrência do patógeno no alimento, o impacto do processamento e manuseio sobre esse patógeno, a frequência da exposição da população a esse patógeno, bem como a duração dessa exposição (CODEX, 1999; WOOLRIDGE, 2008).

A avaliação de dose-resposta estima a relação entre a quantidade do patógeno ingerido e a frequência e magnitude dos efeitos adversos na população. Considerando dados sobre taxas de infecção, morbidade e mortalidade, as avaliações de dose-resposta estimam a severidade dos efeitos adversos, permitindo avaliar seus impactos à saúde (LAMMERDING et al., 2001).

A caracterização do risco é a etapa final da avaliação do risco e envolve a integração dos resultados das avaliações de dose-resposta e de exposição, fornecendo uma estimativa da probabilidade de ocorrência do problema, bem como de sua magnitude (WOOLRIDGE, 2008).

Uma avaliação de risco pode ser qualitativa ou quantitativa (COLEMAN e MARKS, 1999; ILSI, 2007). Enquanto a avaliação qualitativa lida com dados descritivos das informações, com resultados expressos em termos de probabilidade (baixa, média ou alta, por exemplo), nas avaliações quantitativas os resultados são expressos em termos numéricos, baseados em valores sobre prevalência e enumeração dos patógenos e equações matemáticas que descrevem o comportamento microbiano. Apesar das avaliações quantitativas serem as preferidas, algumas vezes não é possível desenvolvê-las devido à falta de dados que permitam a realização de estudos quantitativos e à falta de habilidade para lidar com as ferramentas matemáticas e computacionais, requeridas para o desenvolvimento de modelos de riscos. Além disso, estudos qualitativos são mais rápidos, simples e fáceis do que a avaliação quantitativa, além de serem mais facilmente entendidos pelos gestores de riscos. Segundo o *Codex Alimentarius* e WTO, ambos os tipos são válidos, desde que os requerimentos de cada um estejam atendidos (WOOLDRIDGE, 2008).

2.1 A avaliação de risco microbiológico no contexto atual da inocuidade dos alimentos

Dentro do contexto atual do comércio mundial de alimentos, diversos termos e conceitos novos, relativos a sistemas e/ou critérios relacionados com a garantia da inocuidade dos alimentos, foram criados. O termo "ALOP", do inglês "*Appropriate Level of Protection*", refere-se ao nível de proteção da saúde humana, animal ou vegetal considerado apropriado em um país, que deve estabelecer as medidas sanitárias ou fitossanitárias necessárias para que esse nível de proteção em seu território seja assegurado. Um ALOP pode ser expresso em termos amplos, relativos aos objetivos da saúde

pública, ou expressar quantitativamente a probabilidade de ocorrência de um efeito adverso à saúde causado por um perigo ou a incidência máxima de uma doença veiculada por alimentos. No estabelecimento de um ALOP, devem ser levados em conta fatores relacionados ao sistema produtivo, razões econômicas e a fundamentação científica do nível de proteção pretendido (SWARTE e DONKER, 2005).

Quando um país determina as metas de saúde pública em relação à incidência de doenças, esse procedimento não dá aos processadores de alimentos, produtores, manipuladores, comerciantes e consumidores informações sobre o que é necessário ser feito para que essas metas sejam atingidas. Para que tenham sentido, as metas necessitam ser transformadas em parâmetros que possam ser mensurados e avaliados pelas agências governamentais e utilizados pelos produtores no processamento de alimentos. Nesse contexto, surgiu o conceito de FSO, do inglês *Food Safety Objectives* (TUOMINEN et al., 2007; STRINGER, 2008). O termo correspondente em português é Objetivos de Inocuidade Alimentar.

Um FSO é definido como "a frequência máxima e/ou concentração de um perigo microbiológico em um alimento no momento do seu consumo que esteja em consonância com o nível de proteção à saúde pretendido" (ICMSF, 2002). Esta frequência máxima e/ou concentração de um perigo microbiológico determinada pelo FSO aplica-se para alimentos que estejam prontos para ser consumidos, mas não é aplicável para alimentos que ainda passarão por outras etapas (transporte, armazenamento, cozimento, etc.) antes de serem consumidos. Os efeitos da estocagem e manuseio nos pontos de comercialização e consumo, bem como do tratamento térmico no preparo do alimento para o consumo sobre os microrganismos presentes nesses alimentos são imprevisíveis. O FSO determina uma meta que a cadeia produtiva deve alcançar, sem especificar a maneira como essa meta deve ser alcançada. Assim, o FSO dá flexibilidade para que, na cadeia produtiva, sejam utilizadas técnicas de processamento mais adequadas para cada situação, desde que o nível máximo do perigo especificado não seja ultrapassado. Por exemplo, a pasteurização garante a segurança do leite, mas no futuro essa segurança poderá ser obtida através de outras tecnologias, além da pasteurização. Isso é muito importante no comércio internacional, porque países diferentes podem usar tecnologias diferentes para alcançar um mesmo objetivo.

Enquanto os FSO são adotados para alimentos no momento do consumo, nos diferentes pontos da cadeia produtiva de alimentos adotam-se os POs, do inglês *Performance Objectives*. O termo em português é *Objetivos de Desempenho*. Um PO é definido como "a frequência máxima e/ou concentração de um perigo em um alimento em uma etapa específica da cadeia produtiva

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antes do consumo, que esteja de acordo com o ALOP, ou com o FSO". Um PO é estabelecido com base no FSO, podendo ser mais ou menos rígido do que o FSO, dependendo se o perigo em questão permanece ou não no mesmo nível entre a etapa do PO e o consumo (Van SCHOTHORST, 2005). O PO pode ser mais rígido do que o FSO quando pode haver contaminação ou multiplicação microbiana durante a distribuição, preparação, armazenamento e uso de um alimento em particular. Por outro lado, o PO pode ser mais tolerante que o FSO quando, por exemplo, o alimento necessitar de cozimento antes do consumo. Os POs podem ser estabelecidos pelo governo ou pela indústria.

Para avaliar se FSOs e POs estão adequados, utilizam-se *Critérios Microbiológicos* (MC, do inglês *Microbiological Criteria*) que devem indicar "a aceitabilidade de um alimento ou lote de alimentos, baseada na presença/ausência de microrganismos ou na quantidade de microrganismos e/ou metabólitos, por unidade de massa, volume, área ou lote". Os MC dependem da aplicação de um plano de amostragem adequado e do uso de métodos analíticos validados, que podem ser estabelecidos por órgãos governamentais e/ou pelas indústrias. Assim, um MC define a aceitabilidade de alimentos, ou de seus ingredientes, em um determinado ponto de toda a cadeia produtiva (CODEX, 1997).

Para avaliar o efeito de uma medida de controle na frequência e/ou concentração de um perigo num alimento, utilizam-se os *Critérios de Desempenho* (PC - *performance criteria*). Nesse caso, a medida de controle em avaliação é aquela que objetiva o atendimento do PO ou do FSO. Por outro lado, um *Critério de Processo* (Pc - *process criteria*) é um parâmetro de controle (temperatura, tempo, pH ou atividade de água, por exemplo) que pode ser aplicado para se atingir um PC (Van SCHOTHORST, 2002). Todos estes conceitos podem ser aplicados em conjunto nas etapas de processamento dos alimentos de maneira a garantir que FSO e ALOP sejam atingidos.

A Figura 2 mostra um esquema indicando os pontos em que se aplicam ALOP, FSO, PO, MC, PC e Pc, envolvendo o país e a cadeia produtiva de alimentos. A Figura 3 mostra que os parâmetros ALOP, FSO, PO e MC são definidos pelo país, em nível nacional, enquanto as BPH (Boas Práticas de Higiene) e os sistemas HACCP (*Hazard Analysis and Critical Control Points*) são ferramentas de gerenciamento da segurança dos alimentos utilizadas nas plantas processadoras de alimentos (nível de processamento) de forma a se atingir o ALOP e os FSO e PO estabelecidos.

2.2 Tipos de avaliação de risco microbiológico

A escolha da estrutura e do tipo específico de avaliação de risco a ser realizada leva em conta o problema de saúde pública a ser estudado, as questões

Figura 2. Esquema de cadeia produtiva mostrando a posição de ALOP, FSO, PO, MC, PC e Pc. (adaptado de ICMSF, 2006; WHITING e BUCHANAN, 2008).

Figura 3. Estruturação das ferramentas de gerenciamento da segurança dos alimentos (adaptado de ICMSF, 2006).

da gestão de riscos a serem respondidas e a disponibilidade de dados. Segundo CAST (2006) há quatro tipos de avaliação de risco (Tabela 1).

A avaliação de risco do tipo "ranqueamento" coloca diferentes riscos em uma ordem crescente de gravidade, como por exemplo, salmonelose associada a diferentes alimentos, como ovos, carne bovina, carne suína ou aves. Esse tipo de avaliação de risco permite identificar a combinação patógeno/alimento que representa maior risco à saúde pública entre todas as combinações analisadas.

Na avaliação de risco do tipo "cadeia produtiva", avalia-se quais fatores durante a produção e consumo de um alimento têm maior impacto no risco associado a esse alimento. Neste tipo de estudo, o impacto de diferentes estratégias de redução de risco pode também ser avaliado. Por exemplo, na combinação *Listeria monocytogenes*/queijo, estratégias de controle do risco podem incluir desde medidas para reduzir a contaminação do leite com o patógeno até a indicação da necessidade de mudanças nos hábitos de consumo. A decisão de quais medidas devem ser adotadas é tarefa

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Tabela 1. Tipos de avaliação de risco microbiológico, segundo Cast, 2006.

Tipos	Descrição	Aplicação	Exemplo
Ranqueamento	Compara os riscos relativos de diferentes perigos ou alimentos	Estabelecimento de prioridades de gestão dos riscos e de alocação de recursos e identificação dos estudos críticos necessários	<i>Listeria monocytogenes</i> em alimentos prontos para consumo
Cadeia produtiva	Examina os fatores que influenciam o risco associado a uma determinada combinação alimento/patógeno em toda a cadeia produtiva	Identificação de fatores-chave que interferem na exposição, incluindo a frequência e nível de contaminação e o impacto de estratégias de intervenção sobre o risco avaliado	<i>Vibrio parahaemolyticus</i> em ostras cruas
Risco-risco	Avalia os riscos decorrentes da substituição de um determinado perigo por outro	Avaliação dos impactos de uma medida de controle destinada à redução de um determinado risco mas que pode causar aumento de outro risco ou do risco geral	Doença de veiculação hídrica versus efeitos nocivos dos derivados do cloro
Geográfico	Examina fatores que afetam a entrada, distribuição e impacto à saúde pública de um perigo novo em um determinado local em um determinado momento	Estimativa dos riscos potenciais decorrentes da falha do sistema de segurança dos alimentos	Desenvolvimento de uma nova variedade da Doença de Creutzfeldt-Jacob por passagem do príon da Encefalopatia Espongiforme Bovina do gado para humanos

dos gestores de risco, que necessitam levar em conta não apenas resultados da avaliação de risco mas também fatores econômicos e sociais.

A avaliação do tipo “risco-risco” pode ser exemplificada considerando-se produtos cárneos curados e cozidos, cuja conservação e inocuidade são garantidas pela combinação de cozimento a 74-75 °C com a adição de nitrito. A presença do nitrito permite reduzir a intensidade do tratamento térmico necessário para garantir a segurança dos produtos, evitando alterações sensoriais e nutritivas indesejadas, e assim mantê-la pelo impedimento da germinação e multiplicação do *Clostridium botulinum*. Entretanto, o nitrito apresenta efeitos tóxicos (CULIK e KELLNER, 1995), gerando um novo risco para esses produtos. Nesse caso, os gestores de risco devem decidir, juntamente com as partes interessadas, qual risco será aceitável e quais são as alternativas de gestão possíveis.

Por último, na avaliação de risco do tipo “geográfico”, analisa-se o risco da introdução de um patógeno novo em uma região onde a ocorrência de doença relacionada a esse patógeno ainda não tenha sido relatada como, por exemplo, o mal da vaca louca (Síndrome Espongiforme Bovina). Neste caso, a avaliação de risco depende de muitas informações, como sistemas de criação e alimentação dos animais, medidas de controle e prevenção adotadas, etc.

2.3 Construção de um modelo de avaliação quantitativa de risco microbiológico

De um modo geral, as seguintes informações básicas são necessárias para a montagem de um modelo de avaliação quantitativa de risco: i) dados sobre a prevalência e concentração do patógeno nas matérias-primas; ii) dados sobre o efeito das diferentes etapas de processamento sobre a prevalência e concentração do patógeno no alimento; iii) dados de modelos preditivos previamente validados, em condições reais ou simuladas, sobre o comportamento do patógeno nas diversas condições de processamento e estocagem; iv) dados sobre as condições do processamento (tempo, temperatura, atividade de água, pH, etc.) que podem influenciar a sobrevivência do patógeno e v) quantidade e frequência de ingestão do alimento em estudo, hábitos de consumo, características da população consumidora (faixa etária, condições de saúde, etc.).

Em alguns casos, a avaliação do efeito das etapas do processamento sobre o patógeno, desde a produção até o consumo do alimento, requer que modelos de dose-resposta para se determinar o risco da doença em si sejam acoplados ao modelo de risco. Em outras vezes, a abordagem pode considerar a avaliação do comportamento do patógeno nos diferentes pontos da cadeia produtiva como, por exemplo, produção primária, processamento, transporte e consumo. Evidentemente, quanto maior o número de etapas da cadeia produtiva

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dos alimentos e quanto maior o número de parâmetros que afetam a dinâmica do patógeno considerado, maior a complexidade do modelo, o que não pode ser um fator impeditivo para o desenvolvimento dos modelos de avaliação de risco. Todas as informações utilizadas e conclusões obtidas, bem como as limitações da avaliação, devem ser claramente apresentadas (transparência), de maneira que a avaliação possa ser atualizada sempre que novas informações estejam disponíveis.

Uma vez definidos os dados a serem utilizados em uma avaliação de risco, é preciso determinar: i) quais etapas e/ou módulos de toda a linha de processamento (*from farm to fork*) serão modelados; ii) dados de entrada, representados por distribuições estatísticas, acoplados, ou não, a modelos preditivos, suas variáveis e respectivas unidades e iii) dados de saída, suas variáveis e respectivas unidades. Na Figura 4, correspondente a um modelo de risco para *Escherichia coli* 0157:H7 em aparas de carne

Figura 4. Esquema do modelo de risco para *E. coli* 0157:H7 em aparas de carne bovina (adaptado de CUMMINS et al., 2008).

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bovina, pode-se observar os módulos (linhas tracejadas com fundo cinza), as etapas de processamento e os dados de entrada (linhas contínuas grossas com fundo claro), as variáveis (linhas contínuas finas com fundo claro) e os dados de saída (linhas tracejadas com fundo claro). O exemplo foi retirado e adaptado do trabalho de Cummins et al., 2008.

2.4 Caracterização de variabilidade e incerteza

Os termos variabilidade e incerteza referem-se a situações distintas. A variabilidade representa a heterogeneidade intrínseca de um fenômeno bem caracterizado, enquanto a incerteza está relacionada com o conhecimento insuficiente de um fenômeno. Compreendendo melhor o fenômeno, à custa de mais pesquisa sobre ele, é possível diminuir a incerteza, mas não a variabilidade (CAST, 2006; LAMMERDING et al., 2001).

Tanto a variabilidade quanto a incerteza têm um papel importante no grau de confiança de uma estimativa de risco feita através do modelo desenvolvido. A variabilidade fornece informações valiosas sobre os extremos das distribuições, importantes na estimativa do risco microbiológico. Alguns exemplos de extremos são as populações de menor resistência à infecção (indivíduos imunocomprometidos), ou um número baixo de células ou esporos de patógenos termorresistentes, ou um número pequeno de embalagens contaminadas. Isoladamente cada um desses fatores contribui pouco para o risco, mas juntos podem aumentar a possibilidade de ocorrência de um surto, ou seja, aumentam o risco (MILLER et al., 1997).

A variabilidade é inerente a um fenômeno microbiológico e deve ser sempre considerada nos modelos de avaliação quantitativa de risco, mesmo que não seja possível reduzi-la. Já a incerteza pode ser reduzida por medições e coletas de dados adicionais, o que deve ser feito sempre que possível. A relação entre o grau de incerteza e a quantidade de dados disponíveis pode ser melhor compreendida no exemplo da Figura 5, relativa à prevalência estimada de *Salmonella* spp. em alface, quando se encontra uma amostra positiva entre 142 amostras analisadas, ou quando se encontram duas amostras positivas em 284 amostras analisadas. Apesar da proporção de amostras positivas ser a mesma nos dois casos (0,7%), no segundo a certeza e a confiança dos resultados obtidos são maiores, devido ao maior número de amostras analisadas (CAST, 2006).

Assim, com a sistemática da avaliação quantitativa de risco, fica evidente a necessidade de uma mudança no enfoque dos estudos de avaliação de incidência de patógenos em alimentos. Estes estudos não devem determinar somente a prevalência dos patógenos, mas devem também quantificá-los, analisando um número elevado e representativo de amostras. Estudos de avaliação de risco devem sempre considerar a variabilidade, e todo esforço deve ser feito para que incertezas sejam reduzidas.

Figura 5. Incerteza representada pela prevalência de *Salmonella* spp. em 142 (a) e 284 (b) amostras analisadas de um alimento (adaptado de CAST, 2006).

2.5 Metodologia analítica

Existem numerosas ferramentas para a modelagem de riscos para diferentes combinações patógeno/alimento. A simulação de Monte Carlo é a mais utilizada na avaliação de risco. Nela, a estimativa de um ponto é substituída por uma distribuição probabilística. Nesse processo, realiza-se uma amostragem ao acaso de cada distribuição de probabilidade do modelo centenas ou milhares de vezes, produzindo um novo cenário a cada iteração. Assim, gera-se um novo “ponto estimado” a cada iteração para cada um dos parâmetros do modelo, registrando-se o resultado. O processo é repetido tantas vezes quanto necessárias para que o campo de cada distribuição de probabilidade individual seja suficientemente varrido. Em outras palavras, a modelagem de Monte Carlo seleciona um valor amostral a cada iteração, que é baseada numa probabilidade de distribuição. Dessa forma, baseando-se nos parâmetros da distribuição, os valores mais prováveis são selecionados com mais frequência que os valores menos prováveis (valores mínimo ou máximo, por exemplo, numa distribuição do tipo triangular), refletindo mais fielmente os eventos que aconteceriam na realidade (LAMMERDING et al., 2001). Ao realizar estudos de avaliação de risco, é necessário conhecer o número de iterações necessárias para cobrir todo o campo das distribuições utilizadas, caso contrário os resultados do modelo podem ser incorretos, fazendo com que as estimativas feitas não sejam confiáveis. Na Figura 6, apresenta-se um exemplo de simulação de Monte Carlo usando-se a distribuição triangular e 5000 iterações, sendo possível observar que quanto maior o número de iterações realizadas maior é a cobertura do campo da distribuição estatística e mais confiáveis são os dados resultantes.

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Figura 6. Recriação de uma distribuição triangular usando-se a simulação de Monte Carlo (adaptado de LAMMERDING et al., 2001).

Além de permitir estimar riscos, a modelagem de Monte Carlo permite determinar quais variáveis do modelo são fortemente correlacionadas com os dados de saída, ou seja, com o risco avaliado (LAMMERDING et al., 2001).

Existem diversas ferramentas computacionais para a realização de estimativas de riscos empregando-se simulações de Monte Carlo, como Analytica (Lumina, USA), SAS (SAS, USA) e Mathematica (Wolfram, USA) (CAST, 2006), entre outras. No entanto, “@Risk” (Palisade Co., USA) e “Crystal Ball” (Decisioneering Inc., USA) são as mais adotadas atualmente em estudos de avaliação quantitativa de risco, por sua facilidade de operação (CASSIN et al., 1998; LINDQVIST e WESTÖÖ, 2000; BEMRAH et al., 2002; OSCAR, 2004; GIOVANNINI et al., 2004; MAIJALA et al., 2005; DANYLUK et al., 2006; UYTENDAELE et al., 2006).

2.6 Análise Sensitiva

A análise sensitiva é uma ferramenta bastante utilizada para avaliar os resultados de um estudo de avaliação quantitativa de risco, que são complexos devido à variabilidade e incerteza inerentes a eles (CAST, 2006).

O gráfico de tornado é uma maneira comum de apresentar os resultados da análise sensitiva, que permite observar claramente a influência de diversos parâmetros do modelo sobre o parâmetro de saída a ser estimado. Na Figura 7, apresenta-se um exemplo de gráfico de tornado, elaborado para um estudo de avaliação quantitativa de risco de *Vibrio parahaemolyticus* (Vp) em ostras (USFDA, 2005). As barras indicam os fatores que contribuem para o risco, e os valores apresentados após as barras indicam a porcentagem de contribuição de cada fator para o risco total. O gráfico evidencia claramente os fatores que afetam positiva e negativamente a probabilidade de infecção

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por *Vibrio parahaemolyticus* (Vp) a partir do consumo de ostras. Fatores com valores positivos e mais próximos da parte superior do gráfico são os que mais impactam no aumento do risco, enquanto os fatores mais próximos da parte inferior indicam contribuições menos significativas. No exemplo citado, o tempo de refrigeração é o único fator que contribui na redução do risco da infecção por *V. parahaemolyticus*, enquanto todos os demais, que são valores positivos, contribuem para o aumento do risco, sendo a concentração do microrganismo no ambiente o que tem maior impacto.

2.7 Importância, possíveis aplicações e limitações da avaliação de risco

A avaliação de risco é uma ferramenta que comprova que nenhum alimento é livre de riscos. Por vincular a presença de patógenos microbianos nos alimentos com indicadores de saúde pública (CAST,

2006), a avaliação de risco evidencia que cada etapa do processo produtivo dos alimentos, do campo à mesa, tem seu papel na garantia da segurança do produto final. Por analisar os riscos desde a obtenção da matéria-prima até o consumo, essa ferramenta permite identificar os pontos críticos dos quais são necessárias informações constantes, dando os subsídios necessários para a organização das estratégias de gestão do risco, ou seja, das medidas necessárias para controlar e reduzir os riscos associados a um alimento específico.

A avaliação de risco microbiológico permite avaliar a equivalência de diferentes processos tecnológicos para a inativação de um perigo em alimento, verificando se asseguram o mesmo nível de proteção à saúde pública. Esta aplicação tem grande importância devido ao desenvolvimento constante de novos métodos para conservação dos alimentos, cuja eficácia precisa ser avaliada no contexto das diferentes opções tecnológicas disponíveis.

As observações das populações humana e/ou animal em relação às doenças são um ponto importante tanto para avaliações de risco como para estudos epidemiológicos. Enquanto a avaliação de risco é focada na dinâmica dos patógenos ao longo da cadeia produtiva, usando modelos preditivos para estimar resultados em termos de saúde pública, os estudos epidemiológicos fornecem dados precisos do ponto de vista de saúde pública, devido à disponibilidade de informações verdadeiras e não apenas estimadas. Assim, estudos epidemiológicos e avaliações de risco são atividades complementares (Figura 8) (HAVELAAR et al., 2007). Uma avaliação de risco pode ser validada pelos dados epidemiológicos, através da verificação dos modelos

Figura 7. Exemplo de gráfico tornado de um estudo de avaliação quantitativa de risco para *V. parahaemolyticus* em ostras (adaptado de CAST, 2006 e USFDA, 2005).

Figura 8. Interação entre avaliação quantitativa de risco e epidemiologia no estudo do impacto de patógenos nos alimentos (HAVELAAR et al., 2007).

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de risco desenvolvidos e dos resultados obtidos pela vigilância epidemiológica.

Apesar dos avanços nos últimos anos nos campos da microbiologia preditiva, computação, microbiologia analítica e epidemiologia, a técnica de modelagem quantitativa de risco apresenta várias limitações: i) decisões baseadas em informações incompletas ou de má qualidade comprometem a utilidade do estudo devido à incerteza dos resultados; ii) os resultados de uma avaliação de risco nunca são definitivos e devem ser atualizados sempre que novas informações sejam disponibilizadas; iii) cientistas ligados à segurança de alimentos têm pouca familiaridade com a técnica de modelagem de riscos; iv) a avaliação de risco não pode prever riscos associados com patógenos para os quais não há dados epidemiológicos, clínicos e microbiológicos e v) modelos de risco são, na maioria das vezes, construídos a partir de dados de modelagem preditiva (sobrevivência, inativação e/ou multiplicação) obtidos quando os patógenos não estão na presença da microbiota competidora naturalmente presente na maioria dos alimentos, o que pode comprometer a precisão dos resultados obtidos (CAST, 2006; LAMMERDING et al., 2001).

3 Conclusões

A avaliação de risco é um processo governamental, desenvolvido para responder às questões formuladas pelos responsáveis pela gestão dos riscos identificados. No contexto atual do comércio internacional de alimentos, a avaliação de risco é uma ferramenta objetiva para ajustar as regulamentações de higiene e segurança dos alimentos e para determinar a efetividade das práticas de gestão dos riscos adotadas. O desenvolvimento e a melhor aplicação da análise de risco resultarão na adoção de medidas de controle dos perigos baseadas em critérios praticáveis, o que deve impactar positivamente a redução de problemas relacionados à inocuidade dos alimentos devido aos microrganismos patogênicos.

Segundo Cast, 2006, algumas recomendações em relação à avaliação de risco na área de segurança de alimentos são: i) focar em pesquisas que objetivem reduzir ao máximo as incertezas e a falta de dados nas avaliações de risco existentes; ii) estimular o desenvolvimento das ferramentas que dão suporte para o desenvolvimento dos modelos de risco, como investigação epidemiológica e metodologias analíticas quantitativas (com limites de detecção claramente determinados); iv) melhorar a disponibilização de dados sobre hábitos e padrões de consumo de um país/região; v) dar mais transparência às atividades e responsabilidades dos assessores de risco, gestores de riscos e partes interessadas, bem como melhorar a comunicação do risco; vi) estimular os microbiologistas de alimentos nas universidades, indústrias e governo a desenvolver seus estudos e interpretar seus resultados no

contexto da avaliação de risco e vi) incentivar as universidades para formação de especialistas em análise de risco, com treinamento nas técnicas de avaliação, gestão e comunicação de riscos.

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Referências

- BEMRAH, N.; BERGIS, H.; COLMIN, C.; BEAUFORT, A.; MILLEMANN, Y.; DUFOUR, B.; BENET, J. J.; CERF, O.; SANAA, M. Quantitative risk assessment of human salmonellosis from the consumption of a turkey product in collective catering establishments. **International Journal of Food Microbiology**, Amsterdam, v. 80, n. 1, p. 17-30, 2002.
- BUCHANAN, R. L. National Advisory Committee on Microbiological Criteria for Foods "Principles of Risk Assessment for Illnesses caused by Foodborne Biological Agents". **Journal of Food Protection**, Des Moines, v. 60, n. 1, p. 1417-1419, 1997.
- CASSIN, M. H.; LAMMERDING, A. M.; TODD, E. C. D.; ROSS, W.; MCCOLL, R. S. Quantitative risk assessment for *Escherichia coli* 0157:H7 in ground beef hamburgers. **International Journal of Food Microbiology**, Amsterdam, v. 41, n. 1, p. 21-44, 1998.
- COUNCIL FOR AGRICULTURAL SCIENCE AND TECHNOLOGY - CAST. **Using risk analysis to inform microbial food safety decisions**. Issue Paper 31. CAST, Ames, Iowa, p.1-20, 2006.
- CODEX ALIMENTARIUS COMMISSION - CODEX. **Principles and guidelines for the conduct of Microbiological Risk Assessment**. CAC/GL – 30. Rome, 1999. Disponível em: <www.codexalimentarius.net>. Acesso em: 20 mar. 2009.
- CODEX ALIMENTARIUS COMMISSION - CODEX. **Principles for the establishment and application of microbiological criteria for foods**. CAC/GL – 21. Rome, 1997. Disponível em: www.codexalimentarius.net. Acesso em: 17 mar. 2009.
- COLEMAN, M.; MARKS, H. Qualitative and quantitative risk assessment. **International Journal of Food Microbiology**, Amsterdam, v. 10, n. 4-5, p. 289-297, 1999.
- CULIK, J.; KELLNER, V. Toxic compounds arising by interaction of food constituents with food additives. Part A. Nitroso compounds. **Natural Toxic Compounds of Foods**. Boca Raton: CRC Press, 1995.
- CUMMINS, E.; NALLY, P.; BUTLER, F.; DUFFY, G.; O'BRIEN, S. Development and validation of a probabilistic second-order exposure assessment model for *Escherichia coli* 0157:H7 contamination of beef trimmings from Irish meat plants. **Meat Science**, Barking, v. 79, n. 1, p. 139-154, 2008.
- DANYLUK, M. D.; HARRIS, L. J.; SCHAFFNER, D. W. Monte Carlo simulations assessing the risk of salmonellosis from consumption

Revisão

Avaliação quantitativa de risco microbiológico em alimentos: conceitos, sistemática e aplicações

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of almonds. **Journal of Food Protection**, Des Moines, v. 69, n. 7, p. 1594-1599, 2006.

DENNIS, S. B.; KAUSE, J.; LOSIKOFF, M.; ENGELJOHN, D. L.; BUCHANAN, R. L. Using risk analysis for microbial food safety regulatory decision making. In: SCHAFFNER, D. W. (Ed.). **Microbial Risk Analysis of Foods**. Washington, DC, 2008. Chap. 5, p.137-176.

FOOD AND AGRICULTURE ORGANIZATION – FAO; WORLD HEALTH ORGANIZATION - WHO. **Application of risk analysis to food standards issues**. Report of a Joint FAO/WHO Consultation. World Health Organization. Geneva, 13-17 March 1997. 39 p.

GIOVANNINI, A.; PRENCIPE, V.; CONTE, A.; MARINO, L.; PETRINI, A.; POMILIO, F.; RIZZI, V.; MIGLIORATI, G. Quantitative risk assessment of *Salmonella* spp. infection for the consumer of pork products in an Italian region. **Food Control**, Guildford, v. 15, n. 2, p. 139-144, 2004.

HAVELAAR, A. H.; BRÄUNIG, J.; CHRISTIANSEN, K.; CORNU, M.; HALD, T.; MANGEN, M. J. J.; MOLBAK, K.; PIELAAT, A.; SNARY, E.; VAN PELT, W.; VELTHUIS, A.; WAHLSTRÖM, H. Towards an integrated approach in supporting microbiological food safety decisions. **Zoonoses and Public Health**, Oxford, v. 54, n. 3-4, p. 103-117, 2008.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS - ICMSE. **Microorganisms in Foods 7: Microbiological Testing in Food Safety Management**. New York: Kluwer Academic/Plenum Publishers, 2002.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS – ICMSE. **Guia Simplificado para Compreensão e Uso de Objetivos de Inocuidade Alimentar e Objetivos de Desempenho**. 2006. Disponível em: <<http://www.icmsf.iit.edu/pdf/FSO%20Objectives/GuiaSimplificadoPO.pdf>>. Acesso em: 20 mar. 2009.

LAMMERDING, A. M.; FAZIL, A. M.; PAOLI, G. M. Microbial Food Safety Risk Assessment. In: DOWNES, F. P.; ITO, K. (Eds.). **Compendium of methods for the microbiological examination of foods**. Washington DC: American Public Health Association, 2001. Chap. 29, p. 267-282.

LINDQVIST, R.; WESTÖÖ, A. Quantitative risk assessment for *Listeria monocytogenes* in smoked or gravad salmon and rainbow trout in Sweden. **International Journal of Food Microbiology**, Amsterdam, v. 58, n. 3, p. 181-196, 2000.

MAIJALA, R.; RANTA, J.; SEUNA, E.; PELKONEN, S.; JOHANSSON, T. A quantitative risk assessment of the public

health impact of the Finnish *Salmonella* control program for broilers. **International Journal of Food Microbiology**, Amsterdam, v. 102, n. 1, p. 21-35, 2005.

MILLER, A. J.; WHITING, R. C.; SMITH, J. L. Use of risk assessment to reduce listeriosis incidence. **Food Technology**, Chicago, v. 51, n. 4, p. 100-103, 1997.

OSCAR, T. P. A quantitative risk assessment model for *Salmonella* and whole chickens. **International Journal of Food Microbiology**, Amsterdam, v. 93, n. 2, p. 231-247, 2004.

STRINGER, M. Food Safety Objectives – role in microbiological food safety management. **Food Control**, Guildford, v. 16, n. 9, p. 775-777794, 2005

SWARTE, C.; DONKER, R. A. Towards an FSO/ALOP based food safety policy. **Food Control**, Guildford, v. 16, n. 9, p. 825-830, 2005.

TUOMINEN, P.; RANTA, J.; MAIJALA, R. Studying the effect of POs and MCs on the *Salmonella* ALOP with a quantitative risk assessment model for beef production. **International Journal of Food Microbiology**, Amsterdam, v. 118, n. 1, p. 35-51, 2007.

VAN SCHOTHORST, M. A proposed framework for the use of FSOs. **Food Control**, Guildford, v. 16, n. 9, p. 811-816, 2005.

VAN SCHOTHORST, M. Implementing the results of an MRA: pathogen risk assessment. In: Brown, M.;Stringer, M. (Eds.). **Microbiological risk assessment in food processing**. Cambridge: Woodhead Publishing, 2002. Chap. 9, p.175-192.

U.S. FOOD AND DRUG ADMINISTRATION (USFDA). 2005. **Quantitative Risk Assessment on the Public Health Impact of Pathogenic *Vibrio parahaemolyticus* in Raw Oysters**. 2005. <<http://www.cfsan.fda.gov/~dms/vpra-toc.html>>. Acesso em: 15 abr. 2009.

WHITING, R. C.; BUCHANAN, R. L. Using risk assessment principles in an emerging paradigm for controlling the microbial safety of foods. In: SCHAFFNER, D. W. (Ed.). **Microbial Risk Analysis of Foods**. Washington, DC, 2008. Chap. 2, p. 29-50.

WOOLDRIDGE, M. Qualitative risk assessment. In: SCHAFFNER, D. W. (Ed.) **Microbial Risk Analysis of Foods**. Washington, DC, 2008. Chap. 1, p.1-28.

WORLD TRADE ORGANIZATION (WTO). **The WTO agreement on the application of sanitary and phytosanitary measures (SPS Agreement), 1995**. Disponível em: <www.wto.org/english/tratop_e/sps_e/spsagr_e.htm>. Acesso em: 15 mar. 2009.

Short communication

Prevalence and counts of *Salmonella* spp. in minimally processed vegetables in São Paulo, Brazil

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ABSTRACT

Minimally processed vegetables (MPV) may be important vehicles of *Salmonella* spp. and cause disease. This study aimed at detecting and enumerating *Salmonella* spp. in MPV marketed in the city of São Paulo, Brazil. A total of 512 samples of MPV packages collected in retail stores were tested for *Salmonella* spp. and total coliforms and *Escherichia coli* as indication of the hygienic status. *Salmonella* spp. was detected in four samples, two using the detection method and two using the counting method, where the results were 8.8×10^2 CFU/g and 2.4×10^2 CFU/g. The serovars were *Salmonella* Typhimurium (three samples) and *Salmonella enterica* subsp. *enterica* O:47:z4,z23:- (one sample). Fourteen samples (2.7%) presented counts of *E. coli* above the maximum limit established by the Brazilian regulation for MPV (10^2 CFU/g). Therefore, tightened surveillance and effective intervention strategies are necessary in order to address consumers and governments concerns on safety of MPV.

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1. Introduction

Minimally processed vegetables (MPV) are those submitted to simple operations (slicing or shredding and disinfection), aiming to preserve their freshness, nutritional quality and sensorial properties (Cruz et al., 2006). It is expected that in addition to health aspects, minimal processing addresses consumers' expectations for safe foods that are easy to use (Jacxsens et al., 2010).

The augmented demand and consumption of MPV and their increased association with foodborne disease outbreaks intensify the concerns with the safety of these products (Olsen et al., 2000; Little et al., 2003; Little and Gillespie, 2008). Careful management of process hygiene is crucial to avoid the presence of microbial hazards since minimal processing is not an end-point preservation treatment. In this sense, the use of indicator microorganisms such as *Escherichia coli* has been used to indicate the hygiene during processing (Little and Gillespie, 2008).

Salmonella is the leading cause of foodborne diseases worldwide (Greig and Ravel, 2009). Although the majority of outbreaks in which *Salmonella* spp. is the etiological agent are linked with ingestion of contaminated foods of animal origin (Greig and Ravel, 2009; Oliveira et al., 2010), the association with vegetables has been

increasing in the last few years (WHO, 2008; Little and Gillespie, 2008; Lynch et al., 2009). The pathogen is a major challenge for the microbiological safety of MPV (Olsen et al., 2000; Krtinić et al., 2010), and has been found in several types of vegetables in Brazil and other countries (Fröder et al., 2007; Abadias et al., 2008; Meldrum et al., 2009; Giusti et al., 2010). Despite the fact that the prevalence of *Salmonella* spp. in fresh vegetables may be as high as 35% (FDA, 2001) and that outbreaks due to this pathogen–food combination are quite frequent (Harris et al., 2003), to our knowledge no study reported counts of *Salmonella* spp. in MPV. Thus, this study aimed at detecting and enumerating *Salmonella* spp. in MPV available in retail stores in São Paulo, Brazil.

2. Materials and methods

2.1. Collection of MPV samples

A total of 512 samples of MPV packages were purchased from retail stores of the five largest Brazilian supermarket chains in the city of São Paulo, Brazil. The samples belonged to the six most popular brands available in the market and only packages within expiration date stated in the label were collected. Each sample belonged to a different lot of production and contained vegetables grown in Brazil. MPV containing lettuce, carrot, collard green and cabbage were prioritized since these are the most consumed vegetables in Brazil (IBGE, 2007). All vegetables had been submitted to a sanitization step during processing and thus

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Table 1

Composition and number of minimally processed vegetables (MPV) samples analyzed in this study.

MPV	Number of samples (%)
Salads ^a	214 (41.8)
Lettuce (<i>Lactuca sativa</i> L.)	152 (29.7)
Collard Greens (<i>Brassica oleracea</i> L.)	24 (4.7)
Arugula (<i>Eruca sativa</i> L.)	19 (3.7)
Mix for Yakisoba ^b	18 (3.5)
Watercress (<i>Nasturtium officinale</i>)	18 (3.5)
Chicory (<i>Cichorium intybus</i> L.)	16 (3.1)
Escarole (<i>Cichorium endivia</i>)	13 (2.6)
Cabbage (<i>Brassica oleracea</i> L. var. <i>Capitata</i>)	11 (2.1)
Spinach (<i>Spinacea oleracea</i> L.)	11 (2.1)
Swiss Chard (<i>Beta vulgaris</i> L. var. <i>cicla</i>)	9 (1.8)
Mix for Sukiyaki ^c	6 (1.2)
Colewort (<i>Geum urbanum</i> L.)	1 (0.2)

^a Composed by two or more of the following vegetables: lettuce (different varieties), watercress, chard, spinach, cabbage, carrot, arugula, chicory, escarole and tomatoes.

^b Composed by cauliflower, carrot, broccoli, cabbage and chard.

^c Composed by chard, cabbage, carrot, onion, green pepper, cauliflower and leek.

considered ready-to-eat. The list of MPV types and the number of samples are shown in Table 1.

2.2. Microbiological analysis

After purchasing, MPV samples were kept under refrigeration (4–7 °C) and analyzed within 24 h. Before testing, MPV packages were disinfected with 70% alcohol solution, opened under aseptic conditions and the vegetables analyzed for prevalence and enumeration of *Salmonella* spp. and counts of hygiene indicators (total coliforms and *E. coli*).

Prevalence of *Salmonella* spp. was determined according to ISO 6579 (Anonymous, 2002). Briefly, 25 g of each sample were homogenized with 225 mL buffered peptone water (BPW) and incubated for 37 ± 1 °C for 18 ± 2 h, followed by selective enrichment in Rappaport Vassiliadis soya peptone broth (RVS) (41.5 ± 1 °C for 24 ± 3 h) and Muller–Kauffmann tetrathionate/novobiocin (MKTn) broth (37 ± 1 °C for 24 ± 3 h). Both broths were then plated on XLD- and Bismuth sulfide-agars and incubated at 37 ± 1 °C for 24 ± 3 h. All culture media were from Merck (Darmstadt, Germany), except otherwise stated. For enumeration of *Salmonella* spp., the sample homogenate used for testing the presence of *Salmonella* spp. (10⁻¹) was plated in three different plates of XLD agar (portions of 0.3, 0.3 and 0.4 mL). Further decimal serial dilutions were prepared in 0.1% peptone water and aliquots of 0.1 mL were spread onto XLD agar plates, and incubated at 37 ± 1 °C for 24 ± 3 h. Presumptive colonies in XLD and/or BS plates (five per plate in the case of enumeration) were then transferred to Nutrient Agar plates (Oxoid, Basingstoke, UK), incubated at 37 ± 1 °C for 24 ± 3 h and then submitted to tests for fermentation of glucose, hydrolysis of urea, decarboxylation of lysine, production of β-galactosidase, production of acetoin (Voges–Proskauer test) and indol, as recommended by ISO 6579 (Anonymous, 2002). Colonies were also submitted to agglutination reaction using *Salmonella* polyvalent antisera (Probac, São Paulo, Brazil). Final confirmation was carried out using API20E (Biomérieux, Marcy l'Etoile, France) and PCR reaction, using primers ST 11 (AGC CAA CCA TTG CTA AAT TGG CGC A) and ST 15 (GGT AGA AAT TCC CAG CGG GTA CTG) selected from a *Salmonella*-specific fragment, JEO402-1 (Aabo et al., 1993) to amplify the 429 bp fragment specific for this microorganism. PCR tests were performed as described by Myint et al. (2006), and *Salmonella* Enteritidis ATCC 13076 and ultrapure water were used as positive and negative controls, respectively. Results were expressed as absence or presence of *Salmonella* spp.

per 25 g (prevalence) or as CFU/g (enumeration). Only colonies confirmed as *Salmonella* spp. by biochemical and genetic tests simultaneously were considered to be positive for this pathogen. Confidence intervals (95% CI) for the prevalence of *Salmonella* spp. were estimated based on cumulative binomial distribution. Serotyping of *Salmonella* spp. isolates based on O- and H-group antigens was carried out in the National Center for *Salmonella* Serotyping in Fundação Oswaldo Cruz, Rio de Janeiro, Brazil.

The enumeration of total coliforms and *E. coli* was carried out using Petrifilm® EC (3M, Saint Paul, USA), following the manufacturers' instructions.

3. Results and discussion

Out of 512 packages of MPV tested in this study, four were *Salmonella* spp. positive. Two (0.4%, with 95% CI 0.05–1.4%) were positive in the prevalence study, and another two (0.4%) presented countable levels of this pathogen. In the prevalence study, *Salmonella* was detected in one pack of lettuce and arugula, and the serovars were *Salmonella* Typhimurium and *Salmonella enterica* subsp. *enterica* O:47:z4,z23:-, respectively. In the enumeration study, organic lettuce and mix of leafy vegetables (escarole and chicory) presented counts of the microorganism (8.8 × 10² CFU/g and 2.4 × 10² CFU/g, respectively) and both isolates belonged to serovar *S. Typhimurium*.

According to both Brazilian and international regulations (Brasil, 2001; Gilbert et al., 2000), the presence of *Salmonella* in ready-to-eat vegetables is unacceptable (absence/25 g) and may represent a risk for MPV consumers. Low prevalence of *Salmonella* in MPV as found in the present study has been reported by other authors in Brazil (Fröder et al., 2007), and in other countries as well (Abadias et al., 2008; Meldrum et al., 2009; Giusti et al., 2010). The prevalence of serovar *S. Typhimurium* is in agreement with data that show this serovar is one of the most common in humans or foods in Brazil (Geimba et al., 2004; Fernandes et al., 2006; Mürmann et al., 2008; Duarte et al., 2009).

In the period of this study, to the best of our knowledge neither cases of infection nor outbreaks linking consumption of MPV and *Salmonella* spp. have been reported in the State of São Paulo, Brazil. It is known that a dose in the range of 10²–10³ cells of *Salmonella* spp. may cause disease in humans (Blaser and Newman, 1982; Musher and Musher, 2004), with higher attack rates and severity resulting from higher doses in most of cases (Mintz et al., 1994; Hennessy et al., 1996). Although the counts of *Salmonella* found in some samples of MPV collected in this study could be enough to result in infection by this microorganism, several factors must be considered that affect the probability of infection by foodborne pathogens. One must consider that characteristics of the host, pathogen, food matrix and environment also play an important role in the probability of infection (Coleman and Marks, 1998). In addition, the heterogenic distribution of microorganisms in foods and their fate during commercialization and consumption steps will also influence the probability of occurrence of infection.

The populations of total coliforms in the tested MPV samples were above 10⁴ CFU/g in 56% of the samples (Table 2). *E. coli* was also detected, but only 14 (2.7%) samples surpassed the limit of 10² CFU/g established by the Brazilian Surveillance Agency (ANVISA) for ready-to-eat vegetables (Brasil, 2001). These results indicate that the samples tested in this study presented better hygienic conditions than those reported in other studies in Brazil (Fröder et al., 2007) or in other countries (Abadias et al., 2008; Meldrum et al., 2009; Giusti et al., 2010). Interestingly, the counts of *E. coli* in the four MPV samples harboring *Salmonella* spp. were below 10² CFU/g, indicating that the use of this microorganism as surrogate for *Salmonella* in MPV is of little use.

Table 2 Populations of total coliforms and *E. coli* in minimally processed vegetables samples collected in São Paulo, Brazil.

Population (CFU/g)	Number of samples (%)						Total
	<10 ²	10 ² –10 ³	10 ³ –10 ⁴	10 ⁴ –10 ⁵	10 ⁵ –10 ⁶	>10 ⁶	
Total coliforms	99 (19.3)	102 (20)	80 (15.6)	103 (20.1)	119 (23.2)	9 (1.8)	512 (100)
<i>E. coli</i>	498 (97.2)	8 (1.6)	3 (0.6)	3 (0.6)	0 (0)	0 (0)	512 (100)

The findings reported in this study show that tightened surveillance and effective intervention strategies are necessary in order to address consumers and governments concerns on safety of MPV.

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References

- Aabo, S., Rasmussen, O.F., Rossen, L., Sorensen, P.D., Olsen, J.E., 1993. *Salmonella* identification by the polymerase chain reaction. *Molecular and Cellular Probes* 7, 171–178.
- Abadias, M., Usall, J., Anguera, M., Solsona, C., Viñas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology* 123, 121–129.
- Anonymous, 2002. ISO 6579:2002(E) – Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection of *Salmonella* spp. International Organization for Standardization, Switzerland.
- Blaser, M.J., Newman, L.S., 1982. A review of human salmonellosis: I. infective dose. *Reviews of Infectious Diseases* 4, 1096–1106.
- Brasil, 2001. Ministério da Saúde, Agência Nacional de Vigilância Sanitária. Regulamento Técnico sobre Padrões Microbiológicos para Alimentos. RDC n.º 12, January 26, 2001. <http://www.anvisa.gov.br/legis/resol/12_01rdc.htm> (accessed 20.11.10).
- Coleman, M.E., Marks, H.M., 1998. Qualitative and quantitative risk assessment. *Food Control* 10, 289–297.
- Cruz, A.G., Cenci, S.A., Maia, M.C.A., 2006. Quality assurance requirements in produce processing. *Trends in Food Science and Technology* 17, 406–411.
- Duarte, D.A.M., Ribeiro, A.R., Vasconcelos, A.M.M., Santos, S.B., Silva, J.V.D., de Andrade, P.L.A., Falcão, L.S.P.C.A., 2009. Occurrence of *Salmonella* spp. in broiler chicken carcasses and their susceptibility to antimicrobial agents. *Brazilian Journal of Microbiology* 40, 569–573.
- FDA – Food and Drug Administration, 2001. FDA Survey of Imported Fresh Produce. FY 1999 Field Assignment. 2001 Jan 30. Center for Food Safety and Applied Nutrition, Office of Plant and Dairy Foods and Beverages. <<http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/FruitsVegetablesJuices/GuidanceComplianceRegulatoryInformation/ucm118891.htm>> (accessed 06.02.11).
- Fernandes, S.A., Tavechio, A.T., Ghilardi, Â.C.R., Dias, Â.M.G., De Almeida, I.A.Z.C., De Melo, L.C.V., 2006. *Salmonella* serovars isolated from humans in São Paulo state, Brazil, 1996–2003. *Journal of the São Paulo Institute of Tropical Medicine* 48, 179–184.
- Fröder, H., Martins, C.G., De Souza, K.L.O., Landgraf, M., Franco, B.D.G.M., Destro, M.T., 2007. Minimally processed vegetable salads: microbial quality evaluation. *Journal of Food Protection* 70, 1277–1280.
- Geimba, M.P., Tondo, E.C., De Oliveira, F.A., Canal, C.W., Brandelli, A., 2004. Serological characterization and prevalence of *spvR* genes in *Salmonella* isolated from foods involved in outbreaks in Brazil. *Journal of Food Protection* 67, 1229–1233.
- Gilbert, R.J., de Louvois, J., Donovan, T., Little, C., Nye, K., Ribeiro, C.D., Richards, J., Roberts, D., Bolton, F.J., 2000. Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. PHLS advisory committee for food and dairy products. *Communicable Disease and Public Health/PHLS* 3, 163–167.
- Giusti, M., Aurigemma, C., Marinelli, L., Tufi, D., De Medici, D., Di Pasquale, S., De Vito, C., Boccia, A., 2010. The evaluation of the microbial safety of fresh ready-to-eat vegetables produced by different technologies in Italy. *Journal of Applied Microbiology* 109, 996–1006.
- Greig, J.D., Ravel, A., 2009. Analysis of foodborne outbreak data reported internationally for source attribution. *International Journal of Food Microbiology* 130, 77–87.
- Harris, L.J., Farber, J.N., Beuchat, L.R., Parish, M.E., Suslow, T.V., Garrett, E.H., Busta, F.F., 2003. Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety* 2, 78–141.
- Hennessy, T.W., Hedberg, C.W., Slutsker, L., White, K.E., Besser-Wiek, J.M., Moen, M.E., Feldman, J., Coleman, W.W., Edmonson, L.M., MacDonald, K.L., Osterholm, M.T., 1996. A national outbreak of *Salmonella* Enteritidis infections from ice cream. *New England Journal of Medicine* 334, 1281–1286.
- IBGE – Instituto Brasileiro de Geografia e Estatística, 2007. Sistema IBGE de Recuperação Automática – SIDRA. Banco de Dados Agregados. Disponível em: <<http://www.sidra.ibge.gov.br/bda/horti/default.asp?t=2&z=t&0=17&u1=1&u3=1&u2=31>> (accessed 06.02.11).
- Jacksens, L., Luning, P.A., van der Vorst, J.G.A.J., Devlieghere, F., Leemans, R., Uyttendaele, M., 2010. Simulation modelling and risk assessment as tools to identify the impact of climate change on microbiological food safety – the case study of fresh produce supply chain. *Food Research International* 43, 1925–1935.
- Krtinić, G., Durić, P., Ilić, S., 2010. *Salmonellae* in food stuffs of plant origin and their implications on human health. *European Journal of Clinical Microbiology and Infectious Diseases*, 1–5.
- Little, C.L., Gillespie, I.A., 2008. Prepared salads and public health. *Journal of Applied Microbiology* 105, 1729–1743.
- Little, C.L., Omotayo, R., Mitchell, R.T., 2003. The microbiological quality of ready-to-eat foods with added spices. *International Journal of Environmental Health Research* 13, 31–42.
- Lynch, M.F., Tauxe, R.V., Hedberg, C.W., 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiology and Infection* 137, 307–315.
- Meldrum, R.J., Little, C.L., Sagoo, S., Mithani, V., McLauchlin, J., de Pinna, E., 2009. Assessment of the microbiological safety of salad vegetables and sauces from kebab take-away restaurants in the United Kingdom. *Food Microbiology* 26, 573–577.
- Mintz, E.D., Cartter, M.L., Hadler, J.L., Wassell, J.T., Zingales, J.A., Tauxe, R.V., 1994. Dose-response effects in an outbreak of *Salmonella* Enteritidis. *Epidemiology and Infection* 112, 13–23.
- Mürmann, L., Dos Santos, M.C., Longaray, S.M., Both, J.M.C., Cardoso, M., 2008. Quantification and molecular characterization of *Salmonella* isolated from food samples involved in salmonellosis outbreaks in Rio Grande do Sul, Brazil. *Brazilian Journal of Microbiology* 39, 529–534.
- Musher, D.M., Musher, B.L., 2004. Contagious acute gastrointestinal infections. *New England Journal of Medicine* 351, 2417–2427.
- Myint, M.S., Johnson, Y.J., Tablante, N.L., Heckert, R.A., 2006. The effect of pre-enrichment protocol on the sensitivity and specificity of PCR for detection of naturally contaminated *Salmonella* in raw poultry compared to conventional culture. *Food Microbiology* 23, 599–604.
- Oliveira, F.A., Pasqualotto, A.P., da Silva, W.P., Tondo, E.C., 2010. Characterization of *Salmonella* Enteritidis isolated from human samples. *Food Research International*. doi:10.1016/j.foodres.2010.09.040.
- Olsen, S.J., MacKinnon, L.C., Goulding, J.S., Bean, N.H., Slutsker, L., 2000. Surveillance for foodborne-disease outbreaks – United States, 1993–1997. *Morbidity and Mortality Weekly Report* 49, 1–62.
- WHO – World Health Organization, 2008. Drug-resistant *Salmonella*. <http://www.who.int/mediacentre/factsheets/fs139/en/> (accessed 20.11.10).

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Modeling the growth rate and lag time of different strains of *Salmonella enterica* and *Listeria monocytogenes* in ready-to-eat lettuce

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ABSTRACT

The growth parameters (growth rate, μ and lag time, λ) of three different strains each of *Salmonella enterica* and *Listeria monocytogenes* in minimally processed lettuce (MPL) and their changes as a function of temperature were modeled. MPL were packed under modified atmosphere (5% O₂, 15% CO₂ and 80% N₂), stored at 7–30 °C and samples collected at different time intervals were enumerated for *S. enterica* and *L. monocytogenes*. Growth curves and equations describing the relationship between μ and λ as a function of temperature were constructed using the DMFit Excel add-in and through linear regression, respectively. The predicted growth parameters for the pathogens observed in this study were compared to ComBase, Pathogen modeling program (PMP) and data from the literature. High R^2 values (0.97 and 0.93) were observed for average growth curves of different strains of pathogens grown on MPL. Secondary models of μ and λ for both pathogens followed a linear trend with high R^2 values (>0.90). Root mean square error (RMSE) showed that the models obtained are accurate and suitable for modeling the growth of *S. enterica* and *L. monocytogenes* in MP lettuce. The current study provides growth models for these foodborne pathogens that can be used in microbial risk assessment.

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1. Introduction

Ready-to-eat minimally processed vegetables (MPV) have gained more importance in the last 20 years due to consumers' demand for fresh, convenient, preservative-free foods that may promote health (Zink, 1997; Jacxsens et al., 2010). In Brazil, the MPV market increases yearly, with lettuce representing the most consumed leafy vegetable in the country (Sato et al., 2007).

Concurrent with the increase in their production and consumption, ready-to-eat vegetables have been associated with progressively more disease outbreaks (Harris et al., 2003; Sivapalasingam et al., 2004; Little and Gillespie, 2008; Lynch et al., 2009). Between 1999 and 2008, 6062 foodborne disease outbreaks were reported in Brazil, of which 114 involved vegetables (Anonymous, 2010). Moreover, several surveys have been carried out to evaluate the microbiological quality of MPV in Brazil (Fröder et al., 2007; Silva et al., 2007; Oliveira et al., 2011) and abroad

(Abadias et al., 2008; Ailes et al., 2008; Meldrum et al., 2009; Caponigro et al., 2010; Giusti et al., 2010) and have reported on the prevalence and/or countable levels of pathogens such as *Salmonella* and *Listeria monocytogenes* in these products (Fröder et al., 2007; Little et al., 2007; Abadias et al., 2008; Cordano and Jacquet, 2009; Giusti et al., 2010; Oliveira et al., 2010; Quiroz-Santiago et al., 2009; Sant'Ana et al., 2011).

Salmonella is the major bacterial challenge for the safety of a wide variety of foods around the world (Greig and Ravel, 2009), and it is the main etiological agent of outbreaks in which fresh produce items are implicated (Tauxe, 1997; Harris et al., 2003; Little and Gillespie, 2008). In Brazil, salmonellae are responsible for approximately 47% of the notified foodborne disease outbreaks (Anonymous, 2010). The primary source of *Salmonella* appears to be animal feces, while contaminated soil and water used for irrigation may also contribute to its spread to vegetables (Krtinić et al., 2010).

L. monocytogenes is a ubiquitous foodborne pathogen with a known ability to grow under refrigeration temperatures (Hanning et al., 2008) and to survive and adapt to adverse environments (Gandhi and Chikindas, 2007). Although it causes fewer foodborne diseases outbreaks than *Salmonella* (Greig and Ravel, 2009), *L. monocytogenes* represents a major food safety concern due to the high mortality rate of those sickened with listeriosis (Bennion et al., 2008).

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In spite of the importance of estimating microbial growth kinetics in the context of quantitative risk assessment (QRA), simple addition of modeling data into QRA models might not be enough for obtaining more accurate models. Several studies have shown substantial differences in the growth kinetics among different strains of foodborne pathogens (Guillier and Augustin, 2006; Lindqvist, 2006; Koutsoumanis, 2008; Pal et al., 2008; Valík et al., 2008; Augustin et al., 2011; Lianou and Koutsoumanis, 2011). It is recognized that intra-species variability of microbial growth kinetics may influence the accuracy of outputs from risk assessment models (Delignette-Muller and Rosso, 2000). Therefore, information on the variability of growth kinetics among different strains of foodborne pathogens and how these parameters are affected by variations in the environment should be developed to help obtain reliable outputs from risk assessment (Lianou and Koutsoumanis, 2011). That is why, in this study, the growth of three different strains of *Salmonella enterica* and *L. monocytogenes* in minimally processed lettuce as a function of temperature (7–30 °C) were modeled separately using both primary and secondary models.

2. Material and methods

2.1. Strains and preparation of cell suspensions

Three different strains of *S. enterica* and *L. monocytogenes* were used in this study. *S. enterica* strains were from serotype Typhimurium (# 277 and 386) and Enteritidis ATCC 13076 while the *L. monocytogenes* strains were of serotype 4b (#: 413, 494 and 581). Except for the *Salmonella* ATCC strain, the other two strains used in this study were isolated from MPV marketed in Sao Paulo, Brazil.

Each strain of *S. enterica* and *L. monocytogenes* was grown separately in 10 mL of Tryptic Soy Broth (Oxoid, Basingstoke, UK) (TSB) and TSB supplemented with 0.6% yeast extract (Oxoid, Basingstoke, UK) (TSB-YE), respectively. Broth tubes were incubated at 37 °C/24 h, following two successive inoculations. The third inoculation was carried in 90 mL of the culture medium, following centrifugation at 2810g at 4 °C for 10 min (Mikro 22R, Hettich Zentrifugen, Germany) for removing debris and residual culture media. After centrifugation, the supernatant was discarded and pellets were washed with sterile 0.1% peptone water. Centrifugation and washing procedures were repeated three times and cells were re-suspended in sterile 0.1% peptone water. Final cell concentrations were adjusted at optical density 0.5 (OD₆₃₀) (10⁸ CFU/mL) using an Ultrospec 200 UV/visible spectrophotometer (Pharmacia Biotech, Buckinghamshire, UK). Each suspension was separately prepared just before the experiments.

2.2. Minimal processing of lettuce and its inoculation with pathogens

The two most consumed varieties of lettuce in Brazil (iceberg – *Lactuca sativa* var. *capitata* and crisp – *L. sativa* var. *crispa*) were purchased from produce outlets located in the city of Sao Paulo, Brazil, and transported to the lab within 1 h. The external, damaged leaves and core were discarded before washing. The leaves were individually washed in tap water and were cut into strips of approximately 2 cm² width with a disinfected knife. The two varieties of lettuce were mixed in the same proportion and shredded leaves were immersed into a disinfected 20 L plastic basin containing Sumaveg solution (JohnsonDiversey, Sao Paulo, Brazil) at 100 ppm for 15 min. Chlorine levels were checked using pH paper test strips (Hydriion, Micro Chlorine, Johnson Wax Professional, Sturtevant, USA). Shredded lettuce leaves were then transferred into a previously disinfected plastic basin containing sterile

distilled water, rinsed and allowed to dry in a laminar flow cabinet for at least 30 min.

Sterile distilled water (4 L) in pails lined with sterile plastic bags was inoculated with each suspension to reach a final concentration of 10¹–10² CFU/g of each pathogen strain. Shredded lettuce leaves were inoculated by dipping into solutions containing different strains of *S. enterica* and *L. monocytogenes* for 15 min. Removal of excess water was done with the aid of a previously sanitized salad spinner (Model DD1056Y, Casa da Moda, Brazil) in a biosafety cabinet. Control samples were prepared by repeating the same procedure above in non-inoculated sterile distilled water.

2.3. Packaging, storage of minimally processed lettuce and enumeration of pathogens

Twenty-five grams of contaminated, minimally processed lettuce was packaged in bags made of multilayer films (external layer: bi-oriented polypropylene; internal layer: blend or co-extruded structure of low-density polyethylene with linear low-density polyethylene) under modified atmosphere (5% O₂, 15% CO₂ and 80% N₂ – White Martins, Osasco, Brazil) using vacuum-sealing machine AP 500 (Tecmaq, Sao Paulo, Brazil). This plastic material has been used by several fresh vegetable processing facilities in Brazil and has 62 µm thickness, O₂ permeability of 1.375 m³ m⁻² day⁻¹ at 23 °C and water steam permeability of 3.5 g water m⁻² day⁻¹ at 38 °C and 90% relative humidity. Bags of lettuce were stored at 7, 10, 15, 20, 25 and 30 °C, and samples were collected at different time intervals and were analyzed for *S. enterica* or *L. monocytogenes*. The time intervals between sampling varied depending on the temperature of storage and ended once the microorganisms reached stationary phase.

S. enterica was enumerated by stomaching the vegetables (25 g) and the diluents, following pour-plate of 1 mL of serial dilutions prepared in 0.1% sterile peptone water in mannitol lysine crystal violet brilliant green (MLCB) agar (Oxoid, Basingstoke, UK). MLCB plates were further incubated at 37 °C for 24h. *L. monocytogenes* was enumerated in the same approach, following pour-plate of 1 mL of serial dilutions prepared in 0.1% sterile peptone water in Oxford agar (Oxoid, Basingstoke, UK) with incubation at 37 °C for 48 h. Approximately 3–5 colonies per point along the growth curve were selected for further confirmation by polyvalent serotyping (*S. enterica*) or biochemical tests as described in ISO 11290-2 (*L. monocytogenes*) (Anonymous, 1998). The limit of enumeration of the method used was 10¹ CFU/g. Experiments were replicated twice for each strain and temperature studied. Results were expressed as CFU/g and data were used for primary predictive modeling.

2.4. Modeling growth parameters of three different strains of *S. enterica* and *L. monocytogenes* in minimally processed lettuce

Growth curves for each temperature and strain were built separately by fitting data to the Baranyi model (Baranyi and Roberts, 1994) (Equations (1)–(3)) using DMFit version 2.1 Excel® add-in (www.ifr.ac.uk/safety/DMfit).

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

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

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

Where: $\ln(N(t))$ = log of cell concentration at time t [h] (CFU/g); $\ln(N_0)$ = log of initial cell concentration (CFU/g); $\mu_{\max}$ = exponential growth rate (log CFU/g/h); $\ln(N_{\max})$ = log of maximum cell concentration; q_0 [-] = parameter expressing the physiological state of cells when $t = t_0$; λ = lag time (h).

The Ratkowsky model (Ratkowsky et al., 1982) was used to describe μ and λ as a function of storage temperature (Equation (4)):

$$\sqrt{r} = b(T - T_0) \quad (4)$$

In this model, $\sqrt{r}$ is the square root of maximum growth rate, b is the slope of the regression line, T is temperature and T_0 is a conceptual minimum temperature for microbial growth, where T was given in °C. For λ , transformation of data to natural logarithm (Ln) was needed to describe its relationship with storage temperature.

2.5. Model evaluation

Computer programs (ComBase Predictor [<http://modelling.combase.cc/>]) and the Pathogen Modeling Program 7.0 – PMP [<http://pmp.arserrc.gov/PMPOnline.aspx>]) were used to estimate μ and λ for further comparison with the data obtained in this study. The PMP and ComBase models were run with the following selected parameters: pH = 6.8, initial level of log 3 CFU/mL of the pathogen, and $a_w = 0.995$ (equal to 0.9 g/dL of NaCl). The pH and a_w values were chosen based on previous measurements carried out in our laboratory as described by Scott et al. (2001). PMP models used were “*Salmonella* aerobic growth” and “*L. monocytogenes* anaerobic growth” while ComBase models used were “*salmonellae* with CO₂ (%)” and “*L. monocytogenes* with CO₂ (%)”. The aerobic growth model for *Salmonella* was chosen because PMP does not include anaerobic or modified atmosphere growth models for this pathogen. In the case of the ComBase models, CO₂ was set at 15%, i.e., the initial % of CO₂ used in modified atmosphere. The minimal temperature used for predictions for the PMP *Salmonella* models

were 10 °C as these models did not allow selection of a lower temperature. Additionally, our data were compared with the μ and λ reported in selected literature (Koseki and Isobe, 2005a,b), here referred to as “Koseki-derived model.” Procedures previously adopted to transform data obtained in this study for secondary modeling were also used to deal with the data obtained from literature and from ComBase and PMP programs.

2.6. Statistical analysis

The μ and λ of the different strains of *Salmonella* and *L. monocytogenes* were checked for significant statistical differences ($p \leq 0.05$), employing one-factor analysis of variance (ANOVA) followed by Duncan's test. Growth parameters for both pathogens were also compared to each other. Statistical analyses were carried out in Assistat version 7.5 free software (Campina Grande, Brazil) (Silva and Azevedo, 2002). In addition, fitting of the models was evaluated by the coefficient of determination (R^2) and their performance was checked by the root mean square error (RMSE).

3. Results

S. enterica and *L. monocytogenes* survived, but did not grow on minimally processed lettuces stored at 5 °C. Growth curves started with an initial population of 10^1 – 10^2 CFU/g and final populations of *S. enterica* and *L. monocytogenes* varied between 10^5 – 10^6 CFU/g and 10^4 – 10^7 CFU/g, respectively. The average R^2 values for growth curves of the three different strains of *S. enterica* and *L. monocytogenes* inoculated on minimally processed lettuce leaves and fitted to Baranyi model were 0.97 and 0.93, respectively (figures not shown). Control samples did not show growth of *S. enterica* or *L. monocytogenes* during storage at different temperatures and periods.

Table 1 shows the average growth kinetic parameters for each of the three different strains of *S. enterica* and *L. monocytogenes* on packed minimally processed lettuce. At 7 and 10 °C *S. enterica* presented lower λ in comparison to *L. monocytogenes* ($p < 0.05$). With the rise in temperature, λ did not differ among the pathogens ($p > 0.05$) except at 20 °C. *S. enterica* grew faster than *L. monocytogenes* over the temperature range ($p < 0.05$), except at 20 °C. When growth parameters among strains of the same

Table 1

Growth kinetic parameters (μ , log CFU/g/h and λ , h) of three different strains of *S. enterica* and *L. monocytogenes* on packed minimally processed lettuce stored at 7–30 °C^{a,b}.

Pathogen	Growth parameters	Strains	Temperature (°C)					
			7	10	15	20	25	30
<i>Salmonella</i> spp.	μ	277	0.053 ± 0.010 ^a	0.0497 ± 0.001 ^a	0.149 ± 0.04 ^a	0.148 ± 0.04 ^a	0.328 ± 0.002 ^a	0.44 ± 0.09 ^a
		386	0.061 ± 0.008 ^a	0.042 ± 0.002 ^b	0.124 ± 0.01 ^a	0.138 ± 0.16 ^a	0.300 ± 0.007 ^a	0.32 ± 0.03 ^a
		ATCC	0.0635 ± 0.006 ^a	0.045 ± 0.001 ^{a,b}	0.094 ± 0.05 ^a	0.149 ± 0.01 ^a	0.315 ± 0.001 ^a	0.36 ± 0.03 ^a
		Average	0.06 ± 0.0045 ^A	0.05 ± 0.0031 ^A	0.12 ± 0.022 ^A	0.16 ± 0.015 ^A	0.31 ± 0.011 ^A	0.37 ± 0.05 ^A
<i>L. monocytogenes</i>	μ	413	0.0165 ± 0.003 ^a	0.0225 ± 0.006 ^a	0.0656 ± 0.04 ^a	0.172 ± 0.02 ^a	0.152 ± 0.003 ^a	0.24 ± 0.01 ^a
		494	0.0191 ± 0.008 ^a	0.0272 ± 0.009 ^a	0.0605 ± 0.05 ^a	0.069 ± 0.01 ^b	0.122 ± 0.001 ^a	0.20 ± 0.02 ^a
		581	0.0141 ± 0.001 ^a	0.0233 ± 0.010 ^a	0.0495 ± 0.01 ^a	0.094 ± 0.01 ^b	0.110 ± 0.002 ^a	0.19 ± 0.01 ^a
		Average	0.02 ± 0.002 ^B	0.02 ± 0.002 ^B	0.06 ± 0.007 ^B	0.11 ± 0.04 ^A	0.13 ± 0.017 ^B	0.22 ± 0.02 ^B
<i>Salmonella</i> spp.	λ	277	25.2 ± 1.2 ^a	12.8 ± 2.3 ^a	4.7 ± 2.1 ^a	4.4 ± 0.1 ^{a,b}	1.7 ± 0.4 ^a	1.5 ± 0.2 ^a
		386	26.5 ± 1.6 ^a	11.8 ± 3.1 ^a	7.4 ± 2.0 ^a	5.4 ± 0.6 ^a	2.2 ± 0.7 ^a	2.4 ± 1.1 ^a
		ATCC	28.4 ± 2.1 ^a	14.3 ± 0.9 ^a	5.4 ± 0.9 ^a	3.8 ± 0.5 ^b	1.8 ± 0.1 ^a	1.1 ± 0.4 ^a
		Average	26.7 ± 1.3 ^B	12.9 ± 1.0 ^B	5.9 ± 1.1 ^A	4.5 ± 0.7 ^A	1.9 ± 0.2 ^B	1.6 ± 0.6 ^A
<i>L. monocytogenes</i>	λ	413	45.5 ± 11.4 ^a	39.1 ± 20.1 ^a	8.4 ± 7.7 ^a	6.9 ± 2.6 ^a	4.3 ± 0.5 ^a	1.4 ± 0.4 ^b
		494	56.6 ± 13.7 ^a	44.6 ± 1.8 ^a	14.4 ± 4.4 ^a	5.4 ± 0.9 ^a	3.6 ± 0.3 ^a	2.5 ± 0.3 ^{a,b}
		581	76.2 ± 10.0 ^a	48.7 ± 8.2 ^a	8.4 ± 1.5 ^a	5.7 ± 1.4 ^a	3.2 ± 0.1 ^a	3.4 ± 0.7 ^a
		Average	59.4 ± 12.7 ^A	44.1 ± 3.9 ^A	10.4 ± 2.8 ^A	5.9 ± 0.6 ^A	3.7 ± 0.5 ^A	2.4 ± 0.8 ^A

^a Different superscript letters in the same column indicate significant differences ($p < 0.05$) for growth rate or lag time of different strains of the same pathogen according to Duncan's test.

^b Different superscript capital letters in the same row indicate significant differences ($p < 0.05$) for average values of growth rate or lag time between *Salmonella enterica* and *L. monocytogenes* according to Duncan's test.

pathogen were evaluated, significant differences were observed for *S. enterica* only at 10 °C (growth rate) and 20 °C (lag time) ($p < 0.05$). The highest variations in μ and λ in salmonellae were observed when the growth took place at 30 °C. The μ varied between 0.32 log CFU/g/h and 0.44 log CFU/g/h in *S. Typhimurium* strains (#277 and 386). In addition, λ values varied from 1.1 h for *S. Enteritidis* ATCC 13076–2.4 h for *S. Typhimurium* (#386) (Table 1). It was found that *S. Enteritidis* ATCC 13076 presented slightly higher λ in comparison to strains 277 and 386 at 7 and 10 °C, while the strain 386 (*S. Typhimurium*) presented higher λ in the range of 15–30 °C. *S. Typhimurium* 277 presented an overall higher μ that was more evident at 25–30 °C.

Significant differences were observed at 20 °C (μ) and 30 °C (λ) ($p < 0.05$) for *L. monocytogenes* (Table 1). *L. monocytogenes* strain 581 presented slightly higher lag time at 7–10 °C, while strain 413 presented higher μ between 15 and 30 °C.

The relationship between the storage temperature of minimally processed lettuce and the average growth parameters (μ and λ) for each strain of *S. enterica* and *L. monocytogenes* is shown in Fig. 1. The relationship between the growth parameters and temperature for both pathogens followed a linear trend with high R^2 values (>0.90). Certain data points were out of the 95% confidence intervals, although the mean values are within the 95% CI (Fig. 1).

Fig. 2 presents a comparison of linear regression among the average growth parameters of *S. enterica* and *L. monocytogenes*

obtained in this study, PMP, ComBase and Koseki. The model for λ of *S. enterica* (Fig. 2A) derived from Koseki was very close to the model obtained in this study, mainly at the points of 15, 20 and 25 °C. In this case, the ComBase and PMP-derived models presented higher slopes. On the other hand, secondary models for *L. monocytogenes* derived from ComBase, PMP and Koseki followed a similar pattern, with most of the data falling within the 95% CI of average data obtained in this study (Fig. 2-B). Models for μ of *S. enterica* (Fig. 2-C) derived from ComBase and PMP showed the greatest slope, followed by the model derived from Koseki. The secondary model for μ of *S. enterica* obtained in this study did not follow the same trend as the three other models. Conversely, the secondary model for *L. monocytogenes* derived from Koseki followed the same trend of the model derived from data obtained in this study, while models derived from PMP and ComBase presented the greatest slope (Fig. 2-D).

Equations describing the relationship of growth parameters and temperature for *S. enterica* and *L. monocytogenes* grown in packed minimally processed lettuce from 7 to 30 °C are shown in Equations (5)–(6) and (7)–(8), respectively:

$$\sqrt{\mu} = 0.0178(T - 6.65) \quad (5)$$

$$\ln(\lambda) = -0.118(T + 34.84) \quad (6)$$

$$\sqrt{\mu} = 0.0144(T - 1.96) \quad (7)$$

Fig. 1. Relationship between average growth parameters (μ and λ) of *Salmonella enterica* (A and C) and *Listeria monocytogenes* (B and D) strains and temperature of storage of minimally processed lettuce.

Fig. 2. Comparison of the linear regression models for (μ and λ) derived from ComBase, PMP, Koseki (symbols) and average data obtained in this study (solid lines).

$$\ln(\lambda) = -0.1445(T + 35.3) \quad (8)$$

RMSE values obtained for μ and λ models of *S. enterica* were 0.033 and 0.214, respectively. RMSE values for *L. monocytogenes* models were 0.015 (μ) and 0.271 (λ), respectively. It can be noticed that RMSE values for μ models were lower than for λ , no matter the pathogen.

4. Discussion

Despite the increasing association of vegetables with foodborne disease outbreaks in recent years (Lynch et al., 2009), few studies have focused on predicting the growth of pathogens in fresh vegetables (Koseki and Isobe, 2005a,b; Crépet et al., 2009; Ding et al., 2010) when compared to meat and dairy products (McMeekin, 2007; Poirazi et al., 2007; Vissers et al., 2007; Oscar, 2009; Cornu et al., 2010; Limbo et al., 2010; McMeekin et al., 2010; Schwartzman et al., 2010; Lindqvist and Lindblad, 2011). Therefore, there is a need for more data on the growth of foodborne pathogens in these products.

Studies on the growth modeling of pathogens are built with a higher starting initial population (i.e., $>10^3$ – 10^4 CFU/g) (Koseki and Isobe, 2005a,b; Ding et al., 2010) of pathogens than that of naturally-contaminated foods, such as fresh vegetables (i.e., $<10^1$ – 10^2 CFU/g) (Sagoo et al., 2003; Little et al., 2007; Cordano and Jacquet, 2009; Oliveira et al., 2010; Sant'Ana et al., 2011). However, predictive models should match the complexity of foods of concern, including low pathogens contamination levels, in order to provide more realistic outputs (Mejlholm et al., 2010). Therefore, herein the

growth kinetic parameters (μ and λ) of three different strains of *S. enterica* and *L. monocytogenes* on packed MPV were predicted with low initial starting populations (10^1 – 10^2 CFU/g). The average growth curves of three different strains of these pathogens presented higher R^2 values (>0.93) than those found in the separate curves for each strain (0.83 and 0.90). R^2 is defined as the proportion of the variability in the data set that is explained by the statistical model (Myers et al., 2009). Therefore, the improvement in curve fitting for the averaged data indicate that when mean values of data points were calculated, marginal values that caused lower R^2 values in separate curves were pulled into the center (Figs. 1 and 2). Thus, high R^2 were obtained in average growth curves because the regression line approximated the real data points.

As shown in Table 1, *S. enterica* grew faster than *L. monocytogenes* in the temperature range tested (7–30 °C) at which MPV can be exposed from farm to fork ($p < 0.05$). Overall, *S. enterica* presented μ 1.5–3.5 times higher than *L. monocytogenes*. Koseki and Isobe (2005a, b) reported μ of approximately 0.02 and 0.04 log CFU/g/h at 10 °C, respectively, for *S. enterica* and *L. monocytogenes* growing on iceberg lettuce. In our study, the average μ of both pathogens at 10 °C was 0.045 and 0.024 log CFU/g/h, respectively. Marked differences in λ were found at low temperatures (7 and 10 °C), when *L. monocytogenes* presented larger values in comparison to *S. enterica* ($p < 0.05$). It is known that even during minimal processing, microorganisms are exposed to stressors such as cold, sanitizers, modified atmosphere and the presence of background microbiota (Capozzi et al., 2009). Even though both pathogens present several mechanisms to overcome

hurdles faced during food processing (Gandhi and Chikindas, 2007; Spector and Kenyon, 2011), *L. monocytogenes* is noticeably more prone to inhibition by indigenous microorganisms present in MPV, such as lactic acid bacteria, than is *Salmonella* (Liao and Fett, 2001; Hanning et al., 2008; Dominguez and Schaffner, 2008). Therefore, considering the similar responses of both pathogens to other stressors found during minimal processing (i.e., sanitizer and modified atmosphere), it may hypothesized that the inhibitory effect of indigenous microbiota on *L. monocytogenes* supplants the ability of this pathogen to adapt to chilling temperatures (Gandhi and Chikindas, 2007; Capozzi et al., 2009). Altogether, these factors may have substantiated the faster growth of *S. enterica* compared to *L. monocytogenes*, in minimally processed lettuce under the conditions tested in the present study.

Temperature is a major environmental factor affecting microbial growth kinetics in foods (McMeekin et al., 2008). Regression lines of secondary models depicted in Fig. 2 were drawn based on the mean growth kinetics parameters of *S. enterica* and *L. monocytogenes* strains while the data points added were obtained from models of each different strain of the pathogens. It can be noticed that most data points were generally within the 95% confidence interval of the averaged values. Modeling of μ was successfully performed using a linear relationship between the square root of the parameter and temperature as described by Ratkowsky et al. (1982). However, the natural logarithm transformation of lag time (λ) resulted in better fit than the square root transformation for this parameter and temperature.

Data on secondary modeling obtained herein were compared to those available in the literature (Koseki and Isobe, 2005a, b) and to tertiary models such as ComBase (Baranyi and Tamplin, 2004) and PMP. These tertiary models are the most recognized software of modeling used to predict growth parameters of several foodborne microorganisms under a variety of conditions. Literature data used for comparison were comprised of models previously described by Koseki and Isobe (2005a, b), since these are the only papers dealing with the growth kinetics of *S. enterica* and *L. monocytogenes* in MPV in the wide range of temperatures these products may be subjected from farm to fork. As can be seen in Fig. 2, secondary models derived from ComBase and PMP basically follow the same slope pattern regarding μ and λ . Overall, our λ models for *S. enterica* and *L. monocytogenes* were closer to ComBase, PMP and Koseki-derived models than our μ models (Fig. 2). Differences among our models, ComBase and the PMP-derived models can be due to the use of data obtained from culture media for building tertiary models. When growing in culture media, microorganisms are not exposed to factors such as the structure of foods and the presence of background microflora, among other issues. The differences between foods and culture media might result in the stress of bacterial cells, leading to faster μ (and greater slopes) when growth takes place in culture media. This hypothesis can be supported by the fact that μ is a characteristic of a microorganism growing in a particular environment, while λ is dependent upon the history and physiological state of the bacterial cells (Baranyi et al., 1995).

RMSE is a measure of the precision of a predictive model, and accounts for the differences between predicted and observed values. The lower the RMSE value for a model, the more precise the data were described (Ross, 1996). Therefore, the RMSE values obtained in this study are very close to those found in models available in the literature, which indicates that the models built here are generally suitable for modeling the growth of *S. enterica* and *L. monocytogenes* in MP lettuce. In addition, the current study adds to the available literature data on the growth of foodborne pathogens in MPV and can be used to build risk assessment models.

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References

- Abadias, M., Usall, J., Anguera, M., Solsona, C., Viñas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology* 123, 121–129.
- Ailes, E.C., Leon, J.S., Jaykus, L.-A., Johnston, L.M., Clayton, H.A., Blanding, S., Kleinbaum, D.G., Backer, L.C., Moe, C.L., 2008. Microbial concentrations on fresh produce are affected by postharvest processing, importation, and season. *Journal of Food Protection* 71, 2389–2397.
- Anonymous, 1998. ISO 11290-2:1998-Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes* – Part 2: Enumeration method. Switzerland.
- Anonymous, 2010. Análise Epidemiológica dos Surtos de Doenças Transmitidas por Alimentos no Brasil. Coordenação de Vigilância das Doenças de Transmissão Hídrica e Alimentar. Available at: Secretaria de Vigilância em Saúde. Ministério da Saúde http://portal.saude.gov.br/portal/arquivos/pdf/surtos_dta_15.pdf (accessed 07.04.11).
- Augustin, J.-C., Bergis, H., Midelet-Bourdin, G., Cornu, M., Couvert, O., Denis, C., Huchet, V., Lemonnier, S., Pinon, A., Vialette, M., Zuliani, V., Stahl, V., 2011. Design of challenge testing experiments to assess the variability of *Listeria monocytogenes* growth in foods. *Food Microbiology* 28, 746–754.
- Baranyi, J., Roberts, T.A., 1994. A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology* 23, 277–294.
- Baranyi, J., Tamplin, M.L., 2004. ComBase: a common database on microbial responses to food environments. *Journal of Food Protection* 67, 1967–1971.
- Baranyi, J., Robinson, T.P., Kaloti, A., Mackey, B.M., 1995. Predicting growth of *Brochothrix thermosphacta* at changing temperature. *International Journal of Food Microbiology* 27, 61–75.
- Bennion, J.R., Sorvillo, F., Wise, M.E., Krishna, S., Mascola, L., 2008. Decreasing listeriosis mortality in the United States, 1990–2005. *Clinical Infectious Diseases* 47, 867–874.
- Caponigro, V., Ventura, M., Chiancone, I., Amato, L., Parente, E., Piro, F., 2010. Variation of microbial load and visual quality of ready-to-eat salads by vegetable type, season, processor and retailer. *Food Microbiology* 27, 1071–1077.
- Capozzi, V., Fiocco, D., Amodio, M.L., Gallone, A., Spano, G., 2009. Bacterial stressors in minimally processed food. *International Journal of Molecular Sciences* 10, 3076–3105.
- Cordano, A.M., Jacquet, C., 2009. *Listeria monocytogenes* isolated from vegetable salads sold at supermarkets in Santiago, Chile: prevalence and strain characterization. *International Journal of Food Microbiology* 132, 176–179.
- Cornu, M., Billoir, E., Bergis, H., Beaufort, A., Zuliani, V., 2010. Modeling microbial competition in food: Application to the behavior of *Listeria monocytogenes* and lactic acid flora in pork meat products. *Food Microbiology* 28, 639–647.
- Crépet, A., Stahl, V., Carlin, F., 2009. Development of a hierarchical Bayesian model to estimate the growth parameters of *Listeria monocytogenes* in minimally processed fresh leafy salads. *International Journal of Food Microbiology* 131, 112–119.
- Delignette-Muller, M.L., Rosso, L., 2000. Biological variability and exposure assessment. *International Journal of Food Microbiology* 58, 203–212.
- Ding, T., Jin, Y.-C., Oh, D.-H., 2010. Predictive model for growth of *Listeria monocytogenes* in untreated and treated lettuce with alkaline electrolyzed water. *World Journal of Microbiology and Biotechnology* 26, 863–869.
- Dominguez, S.A., Schaffner, D.W., 2008. Modeling the growth of *Salmonella* in raw poultry stored under aerobic conditions. *Journal of Food Protection* 71, 2429–2435.
- Fröder, H., Martins, C.G., De Souza, K.L.O., Landgraf, M., Franco, B.D.G.M., Destro, M.T., 2007. Minimally processed vegetable salads: microbial quality evaluation. *Journal of Food Protection* 70, 1277–1280.
- Gandhi, M., Chikindas, M.L., 2007. *Listeria*: a foodborne pathogen that knows how to survive. *International Journal of Food Microbiology* 113, 1–15.
- Giusti, M., Aurigemma, C., Marinelli, L., Tufi, D., De Medici, D., Di Pasquale, S., De Vito, C., Boccia, A., 2010. The evaluation of the microbial safety of fresh ready-to-eat vegetables produced by different technologies in Italy. *Journal of Applied Microbiology* 109, 996–1006.
- Greig, J.D., Ravel, A., 2009. Analysis of foodborne outbreak data reported internationally for source attribution. *International Journal of Food Microbiology* 130, 77–87.
- Guillier, L., Augustin, J.-C., 2006. Modeling the individual cell lag time distributions of *Listeria monocytogenes* as a function of the physiological state and the growth conditions. *International Journal of Food Microbiology* 111, 241–251.
- Hanning, I.B., Johnson, M.G., Riche, S.C., 2008. Pre-cut prepackaged lettuce: a risk for listeriosis? *Foodborne Pathogens and Disease* 5, 731–746.
- Harris, L.J., Farber, J.N., Beuchat, L.R., Parish, M.E., Suslow, T.V., Garrett, E.H., Busta, F.F., 2003. Outbreaks associated with fresh produce: Incidence, growth,

- and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety* 2, 78–141.
- Jacxsens, L., Luning, P.A., van der Vorst, J.G.A.J., Devlieghere, F., Leemans, R., Uyttendaele, M., 2010. Simulation modeling and risk assessment as tools to identify the impact of climate change on microbiological food safety - the case study of fresh produce supply chain. *Food Research International* 43, 1925–1935.
- Koseki, S., Isobe, S., 2005a. Growth of *Listeria monocytogenes* on iceberg lettuce and solid media. *International Journal of Food Microbiology* 101, 217–225.
- Koseki, S., Isobe, S., 2005b. Prediction of pathogen growth on iceberg lettuce under real temperature history during distribution from farm to table. *International Journal of Food Microbiology* 104, 239–248.
- Koutsoumanis, K., 2008. A study on the variability in the growth limits of individual cells and its effect on the behavior of microbial populations. *International Journal of Food Microbiology* 128, 116–121.
- Krtinić, G., Crossed D Signurić, P., Ilić, S., 2010. Salmonellae in food stuffs of plant origin and their implications on human health. *European Journal of Clinical Microbiology and Infectious Diseases* 29, 1321–1325.
- Lianou, S., Koutsoumanis, K.P., 2011. Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior. *Food Microbiology* 28, 828–837.
- Liao, C.-H., Fett, W.F., 2001. Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. *Journal of Food Protection* 64, 1110–1115.
- Limbo, S., Torri, L., Sinelli, N., Franzetti, L., Casiraghi, E., 2010. Evaluation and predictive modeling of shelf life of minced beef stored in high-oxygen modified atmosphere packaging at different temperatures. *Meat Science* 84, 129–136.
- Lindqvist, R., 2006. Estimation of *Staphylococcus aureus* growth parameters from turbidity data: characterization of strain variation and comparison of methods. *Applied and Environmental Microbiology* 72, 4862–4870.
- Lindqvist, R., Lindblad, M., 2011. Time to growth and inactivation of three STEC outbreak strains under conditions relevant for fermented sausages. *International Journal of Food Microbiology* 145, 49–56.
- Little, C.L., Gillespie, I.A., 2008. Prepared salads and public health. *Journal of Applied Microbiology* 105, 1729–1743.
- Little, C.L., Taylor, F.C., Sagoo, S.K., Gillespie, I.A., Grant, K., McLauchlin, J., 2007. Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK. *Food Microbiology* 24, 711–717.
- Lynch, M.F., Tauxe, R.V., Hedberg, C.W., 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiology and Infection* 137, 307–315.
- McMeekin, T.A., 2007. Predictive microbiology: quantitative science delivering quantifiable benefits to the meat industry and other food industries. *Meat Science* 77, 17–27.
- McMeekin, T., Bowman, J., McQuestin, O., Mellefont, L., Ross, T., Tamplin, M., 2008. The future of predictive microbiology: Strategic research, innovative applications and great expectations. *International Journal of Food Microbiology* 128, 2–9.
- McMeekin, T., Tamplin, M., Ross, T., Koseki, S., 2010. The way forward with predictive microbiology in the dairy industry. *Australian Journal of Dairy Technology* 65, 91–97.
- Mejlholm, O., Gunvig, A., Borggaard, C., Blom-Hanssen, J., Mellefont, L., Ross, T., Leroi, F., Else, T., Visser, D., Dalgaard, P., 2010. Predicting growth rates and growth boundary of *Listeria monocytogenes* – an international validation study with focus on processed and ready-to-eat meat and seafood. *International Journal of Food Microbiology* 141, 137–150.
- Meldrum, R.J., Little, C.L., Sagoo, S., Mithani, V., McLauchlin, J., de Pinna, E., 2009. Assessment of the microbiological safety of salad vegetables and sauces from kebab take-away restaurants in the United Kingdom. *Food Microbiology* 26, 573–577.
- Myers, R.H., Montgomery, D.C., Anderson-Cook, C.M., 2009. *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*, third ed. John Wiley and Sons, New York.
- Oliveira, M.A., Abeid Ribeiro, E.G., Morato Bergamini, A.M., Martinis, E.C.P., 2010. Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. *Food Microbiology* 27, 19–23.
- Oliveira, M.A., Souza, V.M., Bergamini, A.M.M., Martinis, E.C.P., 2011. Microbiological quality of ready-to-eat minimally processed vegetables consumed in Brazil. *Food Control* 22, 1400–1403.
- Oscar, T.P., 2009. General regression neural network and Monte Carlo simulation model for survival and growth of *Salmonella* on raw chicken skin as a function of serotype, temperature, and time for use in risk assessment. *Journal of Food Protection* 72, 2078–2087.
- Pal, A., Labuza, T.P., Diez-Gonzalez, F., 2008. Comparison of primary predictive models to study the growth of *Listeria monocytogenes* at low temperatures in liquid cultures and selection of fastest growing ribotypes in meat and turkey product slurries. *Food Microbiology* 25, 460–470.
- Poirazi, P., Leroy, F., Georgalaki, M.D., Aktypis, A., De Vuyst, L., Tsakalidou, E., 2007. Use of artificial neural networks and a gamma-concept-based approach to model growth of and bacteriocin production by *Streptococcus macedonicus* ACA-DC 198 under simulated conditions of Kasser cheese production. *Applied and Environmental Microbiology* 73, 768–776.
- Quiroz-Santiago, C., Rodas-Suárez, O.R., Vázquez Q., C.R., Fernández, F.J., Quiñones-Ramírez, E.I., Vázquez-Salinas, C., 2009. Prevalence of *Salmonella* in vegetables from Mexico. *Journal of Food Protection* 72, 1279–1282.
- Ratkowsky, D.A., Olley, J., McMeekin, T.A., Ball, A., 1982. Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology* 149, 1–5.
- Ross, T., 1996. Indices for performance evaluation of predictive models in food microbiology. *Journal of Applied Bacteriology* 81, 501–508.
- Sagoo, S.K., Little, C.L., Ward, L., Gillespie, I.A., Mitchell, R.T., 2003. Microbiological study of ready-to-eat salad vegetables from retail establishments uncovers a national outbreak of salmonellosis. *Journal of Food Protection* 66, 403–409.
- Sant'Ana, A.S., Landgraf, M., Destro, M.T., Franco, B.D.G.M., 2011. Prevalence and counts of *Salmonella* spp. in minimally processed vegetables in Sao Paulo, Brazil. *Food Microbiology* 28, 1235–1237.
- Sato, G.S., Martins, V.A., Bueno, C.R.F., 2007. Análise exploratória do perfil do consumidor de produtos minimamente processados na cidade de São Paulo. *Informações Econômicas* 37, 62–71.
- Schvartzman, M.S., Belessi, X., Butler, F., Skandamis, P., Jordan, K., 2010. Comparison of growth limits of *Listeria monocytogenes* in milk, broth and cheese. *Journal of Applied Microbiology* 109, 1790–1799.
- Scott, V.N., Clavero, R.S., Troller, J.A., 2001. Measurement of water activity (aw), acidity, and Brix. In: Downes, F.P., Ito, K. (Eds.), *Compendium of Methods for the Microbiological Examination of Foods*. APHA, Washington, DC, pp. 649–659.
- Silva, F.A.S., Azevedo, C.A.V., 2002. Versão do programa computacional Assistat para o sistema operacional Windows. *Revista Brasileira de Produtos Agroindustriais* 4, 71–78.
- Silva, S.R.P., Verdin, S.E.F., Pereira, D.C., Schatkoski, A.M., Rott, M.B., Corção, G., 2007. Microbiological quality of minimally processed vegetables sold in Porto Alegre, Brazil. *Brazilian Journal of Microbiology* 38, 594–598.
- Sivapalasingam, S., Friedman, C.R., Cohen, L., Tauxe, R.V., 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection* 67, 2342–2353.
- Spector, M.P., Kenyon, W.J., 2011. Resistance and survival strategies of *Salmonella enterica* to environmental stresses. *Food Research International*. doi:10.1016/j.foodres.2011.06.056.
- Tauxe, R.V., 1997. Emerging foodborne diseases: an evolving public health challenge. *Emerging Infectious Diseases* 3, 425–434.
- Valík, L., Medvedová, A., Bajúsová, B., Liptáková, D., 2008. Variability of growth parameters of *Staphylococcus aureus* in milk. *Journal of Food and Nutrition Research* 47, 18–22.
- Vissers, M.M.M., Te Giffel, M.C., Driehuis, F., De Jong, P., Lankveld, J.M.G., 2007. Predictive modeling of *Bacillus cereus* spores in farm tank milk during grazing and housing periods. *Journal of Dairy Science* 90, 281–292.
- Zink, D.L., 1997. The impact of consumer demands and trends on food processing. *Emerging Infectious Diseases* 3, 467–469.

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Prevalence, populations and pheno- and genotypic characteristics of *Listeria monocytogenes* isolated from ready-to-eat vegetables marketed in São Paulo, Brazil

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ABSTRACT

Listeria monocytogenes is a foodborne pathogen of great concern due to the high fatality rates of listeriosis. The consumption of RTE vegetables has increased in Brazil over the last two decades, but little is known about the risks associated to the consumption of these products. This study evaluated the prevalence and counts of *L. monocytogenes* in 512 packages of ready-to-eat vegetables marketed in São Paulo. The isolates were characterized for their serotypes, ribotypes, positivity for virulence genes *inlA*, *inlC* and *inlJ*, resistance to chlorine, growth rate variability and capability to form biofilm on stainless steel (AISI 304, #4) coupons. *L. monocytogenes* was detected in 3.1% of the samples. Only five samples presented countable levels, with counts between 1.0×10^1 and 2.6×10^2 CFU/g. Isolates belonged to serotypes 1/2b or 4b and most were positive for genes *inlC* and *inlJ*. Ribotypable isolates were grouped into four groups: 1038 (69.4%), 19175 (11.3%), 19191 (17.7%) and 18604 (one isolate). Most isolates survived to exposure to 125 ppm of a chlorine-based disinfectant for 3 min. All isolates were capable to attach to the coupons, reaching counts above $4 \log_{10}$ CFU/cm² and the growth rate (μ) at 25 °C of the majority of the isolates varied between 0.1 and 0.2 log OD/h, but for few strains the μ was as high as 0.26 log OD/h. Results of this survey indicate that RTE vegetables may be vehicles of *L. monocytogenes* strains with limited variation in serotype, ribotype and virulence factors but varying significantly in resistance to chlorine disinfectants, capability of forming biofilm and growth rate. Data obtained is of foremost importance to serve as baseline for the development of scientific-based policies to control the incidence of *L. monocytogenes* in RTE vegetables in Brazil.

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1. Introduction

Listeria monocytogenes is a foodborne pathogen of great concern due to the high fatality rates of listeriosis (Vázquez-Boland et al., 2001b), and capability to survive stress conditions commonly encountered in foods such as high salt concentration, low temperature and low pH (Warriner and Namvar, 2009). Although surveillance data indicate that foodborne listeriosis outbreaks are mainly linked to poultry, beef, dairy products and ready-to-eat (RTE) foods, vegetables have also been identified as vehicles of transmission (Behraves et al., 2011; CDC, 2011; Todd and Notermans, 2011). Besides, the occurrence of sporadic cases of listeriosis linked to the consumption of foods neither usually nor previously associated with this disease such as melons, hummus and vegetables is an additional concern for fresh produce industries and consumers (Wagner and Allerberger, 2003; Varma et al., 2007; Martins et al., 2010).

The consumption of RTE vegetables has increased in Brazil over the last two decades (Sato et al., 2007), but little is known about the risks associated with the consumption of these products. Although studies have reported that the occurrence of *L. monocytogenes* in RTE vegetables in several parts of the world may be as high as 25%, less studies either in Brazil or abroad have focused on the quantification of this microorganism in these products (Francis and O'Beirne, 2006; Crépet et al., 2007; Fröder et al., 2007; Lianou and Sofos, 2007; Little et al., 2007; Abadias et al., 2008; Cordano and Jacquet, 2009; Giusti et al., 2010; Oliveira et al., 2010). As *L. monocytogenes* is a challenge for the safety of RTE foods (Luber et al., 2011) and that RTE vegetables are consumed without a killing step before consumption, fresh produce plays an important role in listeriosis epidemiology. In the context of risk assessment, the availability of data on the prevalence, counts and growth of foodborne pathogens is of foremost importance for proper building and refining of mathematical models for estimating risks (Crépet et al., 2007).

As virulence in *L. monocytogenes* is highly variable among strains (Velge and Roche, 2010), the differentiation between virulent and non-virulent strains is of great importance in evaluating the potential

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implications of the presence of this microorganism to food safety and public health (Jensen et al., 2008; Roche et al., 2009; Roberts et al., 2009). Conventional serotyping although useful in epidemiological studies due to the relationship between serotypes and foodborne listeriosis (Rocourt and Bille, 1997; Thévenot et al., 2006; Ferreira et al., 2011), presents poor discriminatory power in comparison to DNA-based methods when information on the spreading and relatedness of strains are needed (Wiedmann, 2002). Automated ribotyping has been used in clonality surveys of *L. monocytogenes* isolated from foods and processing environment (Manfreda et al., 2005; Meloni et al., 2009; Williams et al., 2011), and for identifying the relationship between a ribotype and likely threats to food safety (Wiedmann et al., 1997; Kathariou, 2002). The outputs of subtyping may be strengthened if virulence factors such as internalins, among others, with recognized roles in pathogenesis of *L. monocytogenes* are sought (Vázquez-Boland et al., 2001a,b).

Studies have shown that the responses of *L. monocytogenes* to stressors in foods and the processing environment are heterogeneous (Arguedas-Villa et al., 2010; Augustin et al., 2011; Lianou and Koutsoumanis, 2011). Sanitization of equipment, disinfection of vegetables and chilling temperature are relevant stressors during minimal processing of RTE vegetables. The responses of *L. monocytogenes* to these conditions may result either in inactivation, survival, and persistence in the processing environment or growth in the product during cold storage. Then knowledge of the behavior of *L. monocytogenes* is of great relevance for estimating the risks associated with RTE foods (Pal et al., 2008; Dupont and Augustin, 2009; Jacxsens et al., 2010).

This survey aimed at gathering data for risk assessments of *L. monocytogenes* in RTE vegetables in Brazil. The study was conducted with samples collected in the market in São Paulo, Brazil, containing at least one of the most consumed vegetables in the country (lettuce, carrot, collard green and cabbage) (IBGE, 2011). The samples were tested for prevalence and populations of *L. monocytogenes* and isolates were studied for their serotypes, ribotypes, presence of virulence genes, variability in growth rate, ability to form biofilm on stainless steel coupons and susceptibility to a chlorine-based product recommended for vegetable disinfection.

2. Materials and methods

2.1. Collection of samples

A total of 512 packages of bagged RTE vegetables were purchased in shops of the five largest supermarket chains in the city of São Paulo, Brazil, between April and August 2008. Representative sampling was ensured by taking samples pertaining to the most consumed brands, coming from the main production sites in the neighborhood of the city and containing at least one of the most consumed vegetables in Brazil (lettuce, carrot, collard green and cabbage) (IBGE, 2011). The samples were minimally processed, i.e. they have been washed, shredded or sliced, disinfected with chlorinated or ozonized water and packed in plastic bags for retail commercialization. To avoid possible overgrowth of indigenous *L. monocytogenes*, only samples available in the retail displays for no more than two days after processing were included in the study.

The RTE vegetables samples were of thirteen types: a. salad mix, containing at least two of the following components: lettuce (different varieties), watercress, chard, spinach, cabbage, carrot, arugula, chicory, escarole and tomatoes ($n=214$), b. lettuce (*Lactuca sativa* L.) ($n=152$), c. collard green (*Brassica oleracea* L.) ($n=24$), d. arugula (*Eruca sativa* L.) ($n=19$), e. mix for yakisoba, containing cauliflower, carrot, broccoli, cabbage and chard ($n=18$), f. watercress (*Nasturtium officinale*) ($n=18$), g. chicory (*Cichorium intybus* L.) ($n=16$), h. escarole (*Cichorium endivia*) ($n=13$), i. cabbage (*Brassica oleracea* L. var. *Capitata*) ($n=11$), j. spinach (*Tetragonia tetragonioides*) ($n=11$), k. Swiss chard (*Beta vulgaris* L. var. *cicla*) ($n=9$), l. mix for sukiyaki, containing

chard, cabbage, carrot, onion, green pepper, cauliflower and leek ($n=6$) and m. colewort (*Geum urbanum* L.) ($n=1$). After purchase, samples were transported to the laboratory in isothermal boxes, maintained under refrigeration ($<4^{\circ}\text{C}$) and analyzed within 24 h.

2.2. Prevalence and enumeration of *L. monocytogenes*

Before opening, packages of RTE vegetables were submitted to external disinfection with 70% alcohol solution. Samples were concomitantly submitted to ISO 11290-1 (Anon, 1996) and ISO 11290-2 (Anon, 1998) methods for prevalence and enumeration of *L. monocytogenes*, respectively. In the prevalence study, 25 g of the sample was stomached with 225 mL of Half-Fraser Broth (HF) without selective supplement and after 1 h at $20 \pm 2^{\circ}\text{C}$, selective supplement (SR0166) was added and the mixture was incubated at 30°C to complete 24 h. An aliquot of 0.1 mL was transferred to tubes containing Fraser Broth (FB), and incubated at $37 \pm 1^{\circ}\text{C}/48 \pm 3$ h. A loopful of the culture in FB was streaked onto Palcam (PC) and Oxford (OXA) agar plates, incubated at $37 \pm 1^{\circ}\text{C}/24 \pm 3$ h and examined for the presence of presumptive *Listeria* colonies (dimpled brown/black colonies with black halo and brown colonies with esculin hydrolysis halo, respectively). For enumeration, stomached samples in HF were serially diluted in 0.1% sterile peptone water, and 1 mL of the first dilution (10^{-1}) and 0.1 mL of the following dilutions ($>10^{-2}$) were plated onto PC agar plates. PC plates were incubated at $37 \pm 1^{\circ}\text{C}/48 \pm 3$ h and examined for the presence of presumptive *Listeria* colonies. When 1 mL was inoculated onto PC plates, the aliquot was divided in 0.3, 0.3 and 0.4 mL and the counts obtained in the three plates were summed to reflect the counts of *Listeria* in the first dilution. Thus, the limit of quantification of this method was 10^1 CFU/g. Agar *Listeria* according to Ottaviani & Agosti (ALOA), as recommended in the amendment of ISO 11290-1 method, was not used in this study as PC agar presents similar performance (Wilis et al., 2006) and much lower cost. Five presumptive colonies recovered from each method (prevalence and enumeration) were randomly selected and streaked onto Tryptone soy agar supplemented with 0.6% of yeast extract (TSA-YE) and incubated at $37 \pm 1^{\circ}\text{C}/24 \pm 3$ h. Identification of *L. monocytogenes* was done by biochemical tests and a PCR for amplification of the listeriolysin (*hlyA*) gene, using primers A (CAT TAG TGG AAA GAT GGA ATG) and B (GTA TCC TCC AGA GTG ATC GA) (Blais et al., 2008). Results were expressed as presence or absence of *L. monocytogenes* per 25 g (prevalence) or CFU/g (enumeration). Results of enumeration were based on the ratio between the total number of colonies in the PC agar plates and those confirmed as *L. monocytogenes*. All culture media and selective supplements were from Oxoid (Basingstoke, UK). Confidence intervals (95% CI) for the prevalence of *L. monocytogenes* in each group of vegetables, brands and overall prevalence were estimated based on cumulative binomial distribution (Agresti and Coull, 1998; Brown et al., 2001).

2.3. Serotyping

Serotyping of *L. monocytogenes* isolates was performed at the Instituto Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, using polyclonal somatic and flagellar antisera as described by Seeliger and Höhne (1979).

2.4. Multiplex PCR for virulence genes

The presence of internalin virulence genes in *L. monocytogenes* isolates was investigated using a multiplex PCR and primers *inlA* (F: ACG AGT TAA CGG GAC AAA TGC; R: CCC GAC AGT GGT GCT AGA TT), *inlC* (F: AAT TCC CAC AGG ACA CAA CC; R: CGG GAA TGC AAT TTT TCA CTA) and *inlJ* (F: TGT AAC CCC GCT ATC ACA GTT; R: AGC GGC TTG GCA GTC TAA TA) (Liu et al., 2007). *L. monocytogenes* ATCC 7644 and ultrapure water were used as positive and negative controls, respectively.

2.5. Ribotyping

Ribotyping was performed using the RiboPrinter® microbial characterization system (DuPont Qualicon™ Inc., Wilmington, USA), and *EcoRI* restriction enzyme. The generated ribotype patterns were compared to those available in the equipment databank. An isolate was considered ribotyped when its ribotype pattern matched one of the patterns of the identification library of RiboPrinter® (DUP-IDs) with a similarity ≥ 0.84 . Isolates were grouped into ribogroups after combination of profiles within a similarity range, as determined using RiboPrinter's proprietary algorithm (≥ 0.93) (Bruce, 1996). Patterns were analyzed with Bionumerics version 6.0 software (Applied Maths, Sint-Martens-Latem, Belgium) and a dendrogram was generated based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and band based-Dice coefficient analysis (1% tolerance and optimization). Evolutionary lineage was assigned to strains based on their ribotype patterns according to Wiedmann et al. (1997). Simpson's index of discrimination (SID) was determined according to Hunter and Gaston (1988).

2.6. Susceptibility to a chlorine-based disinfectant

The resistance of *L. monocytogenes* isolates to Sumaveg™ (JohnsonDiversey, São Paulo, Brazil), a sodium dichloroisocyanurate-based sanitizer frequently used for vegetable disinfection in Brazil, was tested using 96-well microtiter plates (model Y92096, MIDSCI, Saint Louis, MO, USA). Each isolate was cultivated in Tryptic Soy Broth supplemented with 6% Yeast extract (TSB-YE broth) at 37 °C/24 h and aliquots of 1 mL were centrifuged at 4 °C for 10 min at 2810 × g (Centrifuge 5417 C, Eppendorf AG, Hamburg, Germany). Pellets were washed twice with sterile distilled water and the optical density (OD_{630 nm}) of the final suspension adjusted to 0.5 with distilled water, using an Ultrospec 200 UV/visible spectrophotometer (Pharmacia Biotech, Buckinghamshire, UK). The CFU/mL in the final suspension was determined by plating on TSA-YE 6% agar plates, and incubation at 37 °C/24 h. The disinfectant solutions were freshly prepared to achieve concentrations of 0, 50, 100, 125, 150, 175 and 200 ppm of free chlorine and used within 30 min at room temperature (21 °C) and protected from light. The concentrations of the disinfectant corresponded to those commonly used by vegetable processors in Brazil and were in accordance to the maximum chlorine concentration tolerated by the Brazilian legislation for vegetable disinfection, i.e., 250 ppm (Anon, 1999). Chlorine levels were checked using Hydrion micro chlorine test papers (Micro Chlorine, Johnson Wax Professional, Sturtevant, USA). For resistance testing, aliquots of 500 µL of the washed cultures were added to the wells of the microtiter plates containing the disinfectant solutions and homogenized. After 3 min of exposure, 100 µL of the homogenates was pour-plated onto Müller-Hinton Agar (MHA), in duplicate, and incubated at 37 °C/24 h. The experiments were repeated twice. No growth on MHA was interpreted as sensitivity to the tested concentration of the disinfectant. Growth of one single colony on MHA was interpreted as resistance to the tested concentration of the disinfectant. The detection limit of the method was 10² CFU/mL.

2.7. Biofilm formation in stainless steel coupons

Biofilm formation was evaluated using AISI 304 #4 stainless steel coupons (0.366 µm roughness, 10 mm × 10 mm × 1 mm) under static conditions as previously described (Parizzi et al., 2004; Careli et al., 2009). Before use, coupons were washed with neutral detergent, rinsed with distilled water, immersed in 70% ethanol for 1 h at room temperature, rinsed with distilled water again and sterilized at 121 °C/15 min. For testing, 100 µL of the cultures of *L. monocytogenes*, obtained in TSB-YE broth at 37 °C/24 h, were transferred to wells of 12-well microtiter plates (model Y92012, MIDSCI, Saint

Louis, MO, USA) containing 3 mL of Luria-Bertani broth (LB) and, after gentle homogenization, the coupons were added to the wells. The microtiter plates were closed, sealed with plastic film and incubated at 37 °C/24 h. The coupons were removed and transferred to 10 mL phosphate buffer salt (PBS) solution for 1 min for removal of planktonic cells. Sessile cells were enumerated by vortexing the coupons for 2 min in tubes containing 5 mL of PBS, followed by serial dilution, inoculation onto OXA agar plates and incubation at 37 °C/48 h. The experiments were repeated twice and the results were expressed as CFU/cm².

2.8. Determination of growth rate of *L. monocytogenes* isolates

Working with 96-well microtiter plates, 100 µL of the cultures of *L. monocytogenes*, obtained in TSB-YE broth at 37 °C/24 h, was added to duplicate wells containing 900 µL of TSB-YE broth. Microtiter plates were incubated at 25 °C in a microplate incubator equipped with photometer (Multiskan FC Type 357, Thermo Fischer Scientific Instruments Co. Ltd., Shanghai, China). The optical density (OD) was measured at 620 nm every hour up to 20 h. The experiment was replicated twice, with each experiment being performed with two wells for each isolate. The growth rate (μ) of each isolate was determined by linear regression of log of OD versus time of incubation (Krist et al., 1998). The best statistical distribution fitting the growth rate of *L. monocytogenes* strains was determined using the software @Risk version 5.7 (Palisade Corp., Ithaca, USA).

2.9. Statistical analysis

One-factor analysis of variance (ANOVA) followed by Tukey's test was used to check significant differences ($p \leq 0.05$) regarding the ability of isolates to form biofilms. Statistical analyses were performed using Assistat version 7.5 free software (Campina Grande, Brazil) (Silva and Azevedo, 2002).

3. Results

The overall prevalence of *L. monocytogenes* in the 512 RTE vegetable samples was 3.1% (16/512) (Table 1). The prevalence in salad mixes and lettuces, corresponding to the most consumed types of RTE vegetables in Brazil, was 0.9% and 2.0%, respectively. If only salad mixes and lettuces were collected in this study, the prevalence of *L. monocytogenes* would be lower, i.e. 1.4% (5/366). Combining the type of vegetable and the number of samples of each type, mix

Table 1
Prevalence of *Listeria monocytogenes* in ready-to-eat vegetables marketed in São Paulo, Brazil.

Type of vegetable	Analyzed samples n (%)	Positive samples n (%)	95% CI ^a
Salad mix ^b	214 (41.8)	2 (0.93)	1.1–3.3
Lettuce	152 (29.7)	3 (2.0)	0.4–5.7
Collard Greens	24 (4.7)	1 (4.2)	0.1–21.1
Arugula	19 (3.7)	0 (0)	NA ^c
Mix for yakisoba	18 (3.5)	1 (5.6)	0.1–27.3
Watercress	18 (3.5)	1 (5.6)	0.1–27.3
Chicory	16 (3.1)	0 (0)	NA ^c
Escarole	13 (2.6)	3 (23.1)	5.1–53.8
Cabbage	11 (2.1)	2 (18.2)	0.2–51.8
Spinach	11 (2.1)	1 (9.1)	0.2–41.3
Swiss chard	9 (1.8)	0 (0)	NA ^c
Mix for sukiyaki	6 (1.2)	2 (33.3)	4.3–77.2
Colewort	1 (0.2)	0 (0)	NA ^c
Total	512 (100)	16 (3.1)	1.8–5.0

^a 95% confidence interval.

^b Mixes of different varieties of lettuce, watercress, chard, spinach, carrot, arugula, chicory, escarole and tomato.

^c NA = not applicable.

for sukiyaki, escarole and mix of cabbages presented the highest prevalence, i.e., 33.3%, 23.1% and 18.2%, respectively. Among 14 brands, five contained positive samples for *L. monocytogenes* (Table 2). Samples from Brand J had the highest prevalence (60%), followed by those from brands A and B (4.8% and 6.2%, respectively).

Counting of *L. monocytogenes* was possible in only five samples (Table 3). Populations were low and varied between 1.0×10^1 and 2.6×10^2 CFU/g. The five samples belonged to five different brands and all contained more than one type of vegetable. Three of these samples (mix for yakisoba, mix for sukiyaki and mix of cabbages) were also positive for *L. monocytogenes* when submitted to the detection method (ISO 11290-1).

Table 4 depicts the serotypes and ribotypes profiles of *L. monocytogenes* isolates according to the source and brands of the RTE vegetables. The great majority of isolates belonged to serotype 4b. Those belonging to serotype 1/2b were obtained from one single sample of mix for yakisoba. Most 4b isolates were recovered from RTE vegetables from brands A, B and J.

Seven isolates out of 69 recovered from RTE vegetables could not be ribotyped as their patterns did not match any pattern in the identification library of RiboPrinter® (DUP-IDs) with similarity ≥ 0.84 . However, *EcoRI* ribotyping confirmed that all tested isolates were *L. monocytogenes*. As shown in Table 4, most ribotyped isolates were grouped into DUP-ID 1038 (69.4%). Other detected DUP-IDs were 19191 (17.7%) and 19175 (11.3%). Only one isolate, obtained from a sample of mix of lettuces, belonged DUP-ID 18604.

The genetic relationships among *L. monocytogenes* isolates, determined by ribotyping are shown in Fig. 1. Based on a similarity criterion of 83%, 27 different genetic profiles were obtained and isolates were grouped into three major clusters (A, B, and C). The largest cluster (B) contained 49 isolates, obtained from a variety of vegetables from brands A, B, E, F and J. Cluster A grouped six isolates recovered from samples of collard green, salad mix and cabbage from the same brand B (Table 4). All isolates in clusters A and B were serotype 4b, and the great majority belonged to DUP-ID 1038 (Table 4). In contrast, cluster C grouped seven isolates, all belonging to serotype 1/2b and DUP-ID 19175 and originated from the same sample of mix for yakisoba, from brand G (Table 4).

The multiplex-PCR targeting internalin genes (*inlA*, *inlC* and *inlJ*) of *L. monocytogenes* recovered from the RTE vegetable samples confirmed the virulence potential of the isolates. Regarding the chlorine resistance, the findings of this study indicate a variable resistance even among clones of *L. monocytogenes*. All tested isolates ($n=62$) were resistant to exposure to the chlorine-based disinfectant for 3 min when the concentration was 50 ppm or 100 ppm. Twenty (32.2%), 25 (40.3%) and 9 (14.5%) isolates were inactivated by 125,

Table 3

Enumeration of *Listeria monocytogenes* in ready-to-eat vegetables marketed in São Paulo, Brazil.

Type of vegetable	Composition	Brand	Population (CFU/g)
Salad mix	Crispy and butter head lettuces, arugula and radicchio	A	1.6×10^1
Cabbage	Mix of green and red cabbages and carrots	B	1.0×10^1
Salad mix	Iceberg and crisp lettuces, carrot and red cabbage	F	1.0×10^1
Mix for yakisoba	Cauliflower, carrot, broccoli, cabbage and chard	G	2.6×10^2
Mix for sukiyaki	Chard, cabbage, carrot, onion, green pepper, cauliflower and leek	J	1.0×10^1

150 or 175 ppm of the disinfectant, respectively, but seven isolates (11.3%) were inactivated only when the concentration of the disinfectant was as high as 200 ppm. However, one isolate (#581) was resistant even to 200 ppm of chlorine. Isolates belonging to ribotype cluster A presented similar resistance to the disinfectant, being inactivated with 125–150 ppm of chlorine. On the other hand, isolates in cluster B were variable: some were inactivated with 125 ppm of chlorine while others were resistant even to 200 ppm of chlorine. The isolates in cluster C tended to be more resistant than the other

Table 4

Serotypes and ribotypes clusters of *Listeria monocytogenes* isolated from RTE vegetables marketed in São Paulo, Brazil, according to the source and brand.

Source	Isolate (ID) ^a	Brand	Serotype	Ribotype	Cluster
Collard green	359 (17), 362 (18), 364 (19)	B	4b	1038	A
Salad mix	391 (20), 405 (22)				
Cabbages	400 (21)				
Salad mix	101 (1)	F	4b	1038	B
Watercress	480 (35)	B	4b	19191	
Lettuce	532 (46), 561 (49), 562 (50), 563 (51)	A	4b	1038	
Cabbages	336 (10)	B	4b	19191	
Mix for Yakisoba	504 (42)	E	4b	1038	
Mix for Sukiyaki	335 (9), 436 (31)	J	4b	1038	
	564 (52), 567 (53)	A			
	437 (32), 497 (40), 500 (41)	J		19191	
Escarole	524 (43), 525 (44), 526 (45), 550 (47), 552 (48), 590 (61), 594 (62)	A	4b	1038	
	461 (33), 462 (34)	B		19191	
Spinach	579 (54), 581 (56), 583 (58), 586 (59), 588 (60)	A	4b	1038	
	580 (55), 582 (57)			19191	
Lettuce	413 (23)	J	4b	18604	
	419 (27)			19191	
	415 (24), 417 (25), 418 (26)			1038	
Salad mix	424 (29), 425 (30), 492 (37), 493 (38), 495 (39)	J	4b	1038	
	423 (28), 491 (36)			19191	
Collard green	347 (11)	A	4b	19191	
	348 (12), 349 (13), 353 (14)			1038	
	356 (15), 358 (16)	B		1038	
Mix for yakisoba	221 (2), 223 (3), 224 (4), 283 (5), 293 (6), 294 (7), 295 (8)	G	1/2b	19175	C

Table 2

Prevalence of *Listeria monocytogenes* in ready-to-eat vegetables marketed in São Paulo, Brazil, according to the brand.

Brand	Analyzed samples n	Positive samples n (%)	95% CI ^a
A	124	6 (4.8)	1.8–10.2
B	81	5 (6.2)	2.0–13.8
C	80	0 (0)	NA ^b
D	72	0 (0)	NA
E	49	1 (2.1)	0.05–10.8
F	36	0 (0)	NA
G	31	1 (3.2)	0.08–16.8
H	20	0 (0)	NA
I	9	0 (0)	NA
J	5	3 (60)	14.7–94.7
L	2	0 (0)	NA
M	1	0 (0)	NA
N	1	0 (0)	NA
O	1	0 (0)	NA

^a 95% confidence interval.

^b NA = not applicable.

^a Numbers outside parenthesis indicate the reference number of the isolates, while those within parenthesis correspond to ID numbers of these isolates in Figs. 2 and 3.

Fig. 1. Dendrogram of the 62 *Listeria monocytogenes* isolates obtained from ready-to-eat vegetables marketed in São Paulo, Brazil. A, B and C correspond to the different clusters obtained. Dendrogram was produced using Band based-Dice coefficient analysis and UPGMA algorithm (1% tolerance and optimization).

Fig. 2. Adhesion and biofilm formation on stainless steel coupons by 62 *L. monocytogenes* isolates obtained from ready-to-eat vegetables marketed in São Paulo, Brazil, expressed as log CFU/cm². The correspondence between reference numbers of the isolates and ID numbers is shown in Table 4.

isolates examined and required at least 175 ppm of free chlorine for inactivation.

All *L. monocytogenes* isolates were capable of attachment to stainless steel coupons, most of them achieving populations above 4 log₁₀-CFU/cm² (Fig. 2). Populations above 6 log₁₀-CFU/cm² adhered to the coupons were found for 69.4% of the isolates. Isolates belonging to serotype 1/2b (ID numbers 2 to 8) presented a significantly higher average population of adhered cells when compared to isolates belonging to serotype 4b ($p < 0.05$).

The growth rates (μ) of the *L. monocytogenes* isolates in TSB-YE broth at 25 °C are shown in Fig. 3. Most isolates presented μ between 0.1 and 0.2 log OD/h and only for few isolates μ was above 0.26 log OD/h. Besides the expected inter-strain variability, intra-strain variability was also observed. For instance, undistinguishable isolates, as determined by *EcoRI* ribotyping, such as isolates 2, 3, 4, 6, 7 and 8, presented different μ values, varying from 0.10 to 0.21 log OD/h. The probability density for the growth rate for the *L. monocytogenes* isolates can be seen in Fig. 4. A log-logistic distribution was the best ranked for fitting the data according to Anderson–Darling statistic test ($p < 0.05$). The generated distribution was skewed to the right and presented a mean of 0.179 and standard deviation of 0.0373, with 5% and 95% percentiles of 0.118 and 0.265, respectively.

4. Discussion

The detected prevalence of *L. monocytogenes* in the samples of RTE vegetables analyzed in this study (Table 1) is in the range reported in similar products in other locations (Berrada et al., 2006; Francis and O'Beirne, 2006; Lianou and Sofos, 2007; Little et al., 2007; Ponniah et al., 2010). The highest prevalence of *L. monocytogenes* detected in mix for sukiyaki, escarole and cabbages should be interpreted with care since the number of samples was low, so that a larger result uncertainty is expected (Table 1). However, it should be taken into account that the sampling represented both qualitatively and quantitatively the

different types of RTE vegetables available for purchase in supermarkets in São Paulo, highlighting their relevance as vehicles of *L. monocytogenes*. Another important output of the results is the relationship between prevalence of *L. monocytogenes* and the amount of RTE vegetables consumed by the population. Despite the low occurrence of *L. monocytogenes* in lettuce and lettuce-based salads (Table 1), these are the most consumed RTE vegetables in Brazil and are always consumed raw (Sato et al., 2007). In contrast, mix for sukiyaki and cabbage are sold as RTE vegetables but are frequently consumed cooked, probably reducing consumer exposure to *L. monocytogenes*. Consequently, more consumers may be exposed to *L. monocytogenes* due to lettuce and lettuce-based salads than to mix for yakisoba and cabbages.

The five samples of RTE vegetables with countable levels of *L. monocytogenes* contained three or more components (Table 3), suggesting that this type of RTE vegetables are more prone to be contaminated with *L. monocytogenes*. An explanation for this finding could be that mixed vegetables are subjected to more manipulations during processing, thus there is greater opportunity for the introduction or dispersion of *L. monocytogenes*. Differentiated resistance to the disinfection process of vegetables with different surface roughness may be an additional reason. Despite the inexistence of microbiological limits for *L. monocytogenes* in RTE vegetables in Brazil, the recovery of this microorganism at countable levels (10^1 – 10^2 CFU/g) is of concern because these vegetables can be used for preparation of other meals, resulting in cross-contamination of the end product or if stored for some time may result in growth of the pathogen in the product. Furthermore, RTE vegetables may be added of other ingredients facilitating the growth of *L. monocytogenes* (Vermeulen et al., 2007; Uyttendaele et al., 2009). Thus, populations in which the probability of infection is increased such as 10^6 – 10^9 CFU depending on the strain and host (Takeuchi et al., 2006; Williams et al., 2007), could potentially be reached after a couple of hours of storage at abuse temperature (> 8 °C) (Skalina and Nikolajeva, 2010).

Fig. 3. Growth rate (μ) of 62 *L. monocytogenes* isolates obtained from ready-to-eat vegetables marketed in São Paulo, Brazil, in TSB-YE broth at 25 °C. The correspondence between reference numbers of the isolates and ID numbers is shown in Table 4.

Fig. 4. Distribution of growth rate (μ) of 62 *L. monocytogenes* isolates, obtained from ready-to-eat vegetables marketed in São Paulo, Brazil, in TSB-YE broth at 25 °C. Where RiskLoglogistic(γ ; β ; α), with γ : continuous location parameter; β : continuous scale parameter ($\beta > 0$) and α : continuous shape parameter.

The failure of isolation of *L. monocytogenes* in two out of five samples in which the enumeration was possible may be due to the intense growth of the background microbiota in the enrichment broth (even with selective agents added) or inhibition by selective agents in the enrichment broth. These conditions might have hampered the growth of injured or stressed *L. monocytogenes* cells in the detection method, while in the enumeration method the inoculation was performed directly from the diluent broth (Half-Fraser, without selective agents) and plated onto PC or OXA plates. The higher sensitivity of sub-lethally injured cells to selective compounds found in culture media and its effects on the recovery of foodborne pathogens has been reported in the literature supporting this hypothesis (Jasson et al., 2007).

Although the reasons for the high prevalence of serotype 4b in human illness when compared to the other twelve serotypes of *L. monocytogenes* are still unclear, the prevalence of this serotype in RTE foods is of concern from a food safety perspective (Kathariou, 2002; Dumas et al., 2009). The findings of this study show a high prevalence of serotypes commonly associated with listeriosis among *L. monocytogenes* isolated from RTE vegetables commercialized in the state of São Paulo, Brazil (Table 4). Similar results have been reported by Martins and Leal Germano (2011) and Barancelli et al. (2011), working with RTE deli meats and cheese processing plants of the state of São Paulo, respectively.

Previous studies in Brazil have characterized *L. monocytogenes* isolated from foods, environment or human cases and none have mentioned the occurrence of this ribotype (Chiarini et al., 2009; Bueno et al., 2010). The high prevalence of ribotype DUP-ID 1038 among *L. monocytogenes* isolated from the samples of RTE vegetables (Table 4) is another concern as this ribotype has been associated with several cases and outbreaks of listeriosis worldwide (Schlech et al., 1983; Linnan et al., 1988).

All isolates were positive for the *inlA* gene, responsible for a species-specific surface protein with an important role in entrance of *L. monocytogenes* into host cells (Vázquez-Boland et al., 2001a; Orsi et al., 2007) and 97% were positive for *inlC* and *inlJ* genes, known to contribute in the late stage of infection (Engelbrecht et al., 1996) and passage through the intestinal barrier and several stages of infection (Sabet et al., 2005), respectively. Although results for the presence of virulence genes showed most isolates were positive for *inlC* and *inlJ* genes, several studies indicated that strains displaying these genes were able to cause mortality in A/J mice via the intraperitoneal route (Liu et al., 2003; Sabet et al., 2005; Liu et al., 2007), but not necessarily in humans when ingested through foods (Vázquez-Boland et al., 2001b; Kathariou, 2002; Liu et al., 2007). Despite

presenting several virulence genes playing a role in the pathogenicity of *L. monocytogenes* (Kathariou, 2002), the detection of *inlC* and *inlJ* genes is a quick approach to discriminate potential virulent strains of *L. monocytogenes* from avirulent ones (Liu et al., 2007).

The resistance of the *L. monocytogenes* isolates to exposure to the tested chlorine-based disinfectant for 3 min at concentrations above 100 ppm is of concern, as processors may use inadequate chlorine concentrations increasing costs without achieving the intended disinfection of their products. The results of these tests cannot directly infer on the behavior of the isolates under practical processing conditions (disinfection step), because factors such as the presence of organic material in the disinfection water were not considered. However, the findings obtained can be used for selection of the most resistant isolates that can be further used in validation studies of disinfection procedures applied by RTE vegetable industries. This is based on the assumption that the resistant isolates are more likely to have a competitive advantage towards less resistant ones, prevailing both in the processing environment and potentially in the final product (Beuchat et al., 2004). Although the resistance of isolates to other disinfectants used in food processing has not been tested, the alternation of disinfectant with different active compounds could be an appropriate approach to ensure the inactivation of this microorganism. It should be highlighted that this procedure can only be applied for sanitization of equipment and processing utilities as in Brazil only chlorine and ozone are allowed for disinfection of RTE vegetables.

The persistence of *L. monocytogenes* in foods and the food processing environment has been widely documented (Lomonaco et al., 2009; Chen et al., 2010; Ortiz et al., 2010). The resistance of this microorganism to disinfectants (Aarnisalo et al., 2007), its ability to form biofilms (Harvey et al., 2007) or competitive advantages towards other microorganisms (Vermeulen et al., 2007) were considered to favor the persistence of *L. monocytogenes* in food processing environments. However, recently, Carpentier and Cerf (2011) concluded that the persistence of *L. monocytogenes* in food processing plants and premises seems to be associated to the presence of harborage sites that are difficult to eliminate and allow the microorganism to survive and grow.

Although several factors are known to affect the formation of biofilms, the results obtained in this study (Fig. 2) indicate that failures in disinfection may result in the attachment of *L. monocytogenes* to the stainless steel (or other) surfaces of equipment used for vegetable processing and further compromising the safety of the end products (Van Houdt and Michiels, 2010). The association of persistent strains of *L. monocytogenes* with listeriosis outbreaks has been well documented (Anon, 2000). Thus, the isolation of clones of *L. monocytogenes* from different types of vegetables, processed in different industries (Table 4, Fig. 1), may indicate their persistence either in the field or in the processing environment of the tested RTE vegetables due to presence of harborage sites. Thus, in addition to usual quality control and validation of disinfection programs to ensure the inactivation of *L. monocytogenes*, persistence of this microorganism in processing plants can be avoided by eliminating harborage sites.

The growth rate is one of the most important kinetics parameters describing microbial behavior in foods (Pouillot and Lubran, 2011). Growth curves, based on the determination of OD of cultures grown in microtiter plates, allowed the estimation of growth rate for the whole set of *L. monocytogenes* isolates obtained from the RTE vegetables samples (Fig. 3). This method has been successfully used in several studies on the determination of variability of growth kinetics of microorganisms (Krist et al., 1998; Lianou and Koutsoumanis, 2011). In addition, the determination of growth rate of pathogens in culture media has been extensively used for generate data used in tertiary models such as Pathogen Modeling Program (PMP) (<http://pmp.arserrc.gov/PMPOnline>). The lowest average growth rate value (0.1026 log OD/h) obtained for the *L. monocytogenes* isolates was in

agreement with the estimation given by PMP (data not shown). Some isolates were characterized as fast growers (IDs 28, 35 and 59) and high inter-strain variability was observed for some isolates (IDs 18, 37 and 51) (Fig. 3). The implication of these results is that while fast growers can prevail, those presenting great variability may lead to high or low counts depending on the environmental factors and their interactions. Taking into account that most risks result from the extremities of the statistical distributions, few RTE vegetable packages may contain high counts of the pathogen. Therefore, this information is of major importance when analyzing the potential implications of these findings for safety of these products.

In conclusion, results of this survey indicate that RTE vegetables may be vehicles of *L. monocytogenes* strains presenting little variability in serotype, ribotype and virulence factors but a major variability in resistance to chlorine disinfectants, capability of forming biofilm and growth rate. Variability among isolates regarding their responses to the environmental conditions is of great relevance for modeling the risks related to consumption of RTE vegetables. Data obtained is of foremost importance to serve as baseline for the development of scientific-based policies to control the incidence of *L. monocytogenes* in RTE vegetables and the risks it poses to the safety of these products in Brazil.

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References

- Aarnisalo, K., Lundén, J., Korkeala, H., Wirtanen, G., 2007. Susceptibility of *Listeria monocytogenes* strains to disinfectants and chlorinated alkaline cleaners at cold temperatures. *LWT-Food Science and Technology* 40, 1041–1048.
- Abadias, M., Usall, J., Anguera, M., Solsona, C., Viñas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology* 123, 121–129.
- Agresti, A., Coull, B.A., 1998. Approximate is better than “exact” for interval estimation of binomial proportions. *The American Statistician* 52, 119–126.
- Anonymous, 1996. ISO 11290-1:1996 – Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection and Enumeration of *Listeria monocytogenes* – Part 1: Detection Method. Switzerland.
- Anonymous, 1998. ISO 11290-2:1998 – Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection and Enumeration of *Listeria Monocytogenes* – Part 2: Enumeration Method. Switzerland.
- Anonymous, 1999. Centro de Vigilância Sanitária da Secretaria de Estado da Saúde. Portaria CVS 6-99, de 10 de março de 1999. Diário Oficial do Estado de São Paulo, 12 de março de 1999. Seção 1, pp. 24–27.
- Anonymous, 2000. Multistate outbreak of listeriosis—United States, 2000. *MMWR—Morbidity and Mortality Weekly Report* 49, 1129–1130.
- Arguedas-Villa, C., Stephan, R., Tasara, T., 2010. Evaluation of cold growth and related gene transcription responses associated with *Listeria monocytogenes* strains of different origins. *Food Microbiology* 27, 653–660.
- Augustin, J.-C., Bergis, H., Midelet-Bourdin, G., Cornu, M., Couvert, O., Denis, C., Huchet, V., Lemonnier, S., Pinon, A., Viallette, M., Zuliani, V., Stahl, V., 2011. Design of challenge testing experiments to assess the variability of *Listeria monocytogenes* growth in foods. *Food Microbiology* 28, 746–754.
- Barancelli, G.V., Camargo, T.M., Reis, C.M.F., Porto, E., Hofer, E., Oliveira, C.A.F., 2011. Incidence of *Listeria monocytogenes* in cheese manufacturing plants from the north-east region of São Paulo, Brazil. *Journal of Food Protection* 74, 816–819.
- Behraves, C.B., Jones, T.F., Vugia, D.J., Long, C., Marcus, R., Smith, K., Thomas, S., Zansky, S., Fullerton, K.E., Henao, O.L., Scallan, E., FoodNet Working Group, 2011. Deaths associated with bacterial pathogens transmitted commonly through food: foodborne diseases active surveillance network (FoodNet), 1996–2005. *Journal of Infectious Diseases* 204, 263–267.
- Berrada, H., Soriano, J.M., Picó, Y., Mañes, J., 2006. Quantification of *Listeria monocytogenes* in salads by real time quantitative PCR. *International Journal of Food Microbiology* 107, 202–206.
- Beuchat, L.R., Adler, B.B., Lang, M.M., 2004. Efficacy of chlorine and a peroxyacetic acid sanitizer in killing *Listeria monocytogenes* on iceberg and romaine lettuce using simulated commercial processing conditions. *Journal of Food Protection* 67, 1238–1242.
- Blais, B.W., Phillippe, L.M., Pagotto, F., Corneau, N., 2008. Identification of presumptive positive *Listeria monocytogenes* from foods and environmental samples by the Polymerase Chain Reaction. *The compendium of analytical methods*, Vol. 3 www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp78-02-eng.php MFLP-78.
- Brown, L.D., Cai, T.T., DasGupta, A., 2001. Interval estimation for a binomial proportion. *Statistical Science* 16, 101–133.
- Bruce, J., 1996. Automated system rapidly identifies and characterizes microorganisms in foods. *Food Technology* 50, 77–78.
- Bueno, V.F., Banerjee, P., Banada, P.P., José De Mesquita, A., Lemes-Marques, E.G., Bhunia, A.K., 2010. Characterization of *Listeria monocytogenes* isolates of food and human origins from Brazil using molecular typing procedures and in vitro cell culture assays. *International Journal of Environmental Health Research* 20, 43–59.
- Careli, R.T., Andrade, N.J., Soares, N.F., Júnior, J.L.R., Rosado, M.S., Bernardes, P.C., 2009. The adherence of *Pseudomonas fluorescens* to marble, granite, synthetic polymers, and stainless steel. *Ciencia e Tecnologia de Alimentos* 29, 171–176.
- Carpentier, B., Cerf, O., 2011. Review – persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiology* 145, 1–8.
- Centers for Disease Control and Prevention (CDC), 2011. Investigation update: multi-state outbreak of listeriosis linked to whole cantaloupes from Jensen Farms, Colorado. Available at: <http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/110211/index.html>. Accessed Nov. 07.
- Chen, B.-Y., Pyla, R., Kim, T.-J., Silva, J.L., Jung, Y.-S., 2010. Incidence and persistence of *Listeria monocytogenes* in the catfish processing environment and fresh fillets. *Journal of Food Protection* 73, 1641–1650.
- Chiarini, E., Tyler, K., Farber, J.M., Pagotto, F., Destro, M.T., 2009. *Listeria monocytogenes* in two different poultry facilities: manual and automatic evisceration. *Poultry Science* 88, 791–797.
- Cordano, A.M., Jacquet, C., 2009. *Listeria monocytogenes* isolated from vegetable salads sold at supermarkets in Santiago, Chile: prevalence and strain characterization. *International Journal of Food Microbiology* 132, 176–179.
- Crépet, A., Albert, I., Dervin, C., Carlin, F., 2007. Estimation of microbial contamination of food from prevalence and concentration data: application to *Listeria monocytogenes* in fresh vegetables. *Applied and Environmental Microbiology* 73, 250–258.
- Dumas, E., Meunier, B., Berdagué, J.-L., Chambon, C., Desvaux, M., Hébraud, M., 2009. The origin of *Listeria monocytogenes* 4b isolates is signified by subproteomic profiling. *Biochimica et Biophysica Acta-Proteins and Proteomics* 1794, 1530–1536.
- Dupont, C., Augustin, J.-C., 2009. Influence of stress on single-cell lag time and growth probability for *Listeria monocytogenes* in half Fraser broth. *Applied and Environmental Microbiology* 75, 3069–3076.
- Engelbrecht, F., Chun, S., Ochs, C., Hess, J., Lottspeich, F., Goebel, W., Sokolovic, Z., 1996. A new PrfA-regulated gene of *Listeria monocytogenes* encoding a small, secreted protein which belongs to the family of internalins. *Molecular Microbiology* 21, 823–837.
- Ferreira, V., Barbosa, J., Stasiewicz, M., Vongkamjan, K., Switt, A.M., Hogg, T., Gibbs, P., Teixeira, P., Wiedmann, M., 2011. Diverse geno- and phenotypes of persistent *Listeria monocytogenes* isolates from fermented meat sausage production facilities in Portugal. *Applied and Environmental Microbiology* 77, 2701–2715.
- Francis, G.A., O’Beirne, D., 2006. Isolation and pulsed-field gel electrophoresis typing of *Listeria monocytogenes* from modified atmosphere packaged fresh-cut vegetables collected in Ireland. *Journal of Food Protection* 69, 2524–2528.
- Fröder, R., Martins, C.G., Sousa, K.L.O., Landgraf, M., Franco, B.D.G.M., Destro, M.T., 2007. Minimally processed vegetable salads: microbial quality evaluation. *Journal of Food Protection* 70, 1277–1280.
- Giusti, M., Aurigemma, C., Marinelli, L., Tufi, D., De Medici, D., Di Pasquale, S., De Vito, C., Boccia, A., 2010. The evaluation of the microbial safety of fresh ready-to-eat vegetables produced by different technologies in Italy. *Journal of Applied Microbiology* 109, 996–1006.
- Harvey, J., Keenan, K.P., Gilmour, A., 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. *Food Microbiology* 24, 380–392.
- Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson’s index of diversity. *Journal of Clinical Microbiology* 26, 2465–2466.
- IBGE (Instituto Brasileiro de Geografia e Estatística), 2011. Sistema IBGE de Recuperação Automática—SIDRA. Banco de Dados Agregados. Available in: <http://www.sidra.ibge.gov.br/bda/horti/default.asp?c=2&z=t&t0=17&u1=1&u1=1&u3=1&u2=31>. Accessed in 14 Oct. 2011.
- Jacksens, L., Luning, P.A., van der Vorst, J.G.A.J., Devlieghere, F., Leemans, R., Uyttendaele, M., 2010. Simulation modelling and risk assessment as tools to identify the impact of climate change on microbiological food safety—the case study of fresh produce supply chain. *Food Research International* 43, 1925–1935.
- Jasson, V., Uyttendaele, M., Rajkovic, A., Debever, J., 2007. Establishment of procedures provoking sub-lethal injury of *Listeria monocytogenes*, *Campylobacter jejuni* and *Escherichia coli* O157 to serve method performance testing. *International Journal of Food Microbiology* 118, 241–249.
- Jensen, A., Thomsen, L.E., Jørgensen, R.L., Larsen, M.H., Roldgaard, B.B., Christensen, B.B., Vogel, B.F., Gram, L., Ingmer, H., 2008. Processing plant persistent strains of *Listeria monocytogenes* appear to have a lower virulence potential than clinical strains in

- selected virulence models. *International Journal of Food Microbiology* 123, 254–261.
- Kathariou, S., 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection* 65, 1811–1829.
- Krist, K.A., Ross, T., McMeekin, T.A., 1998. Final optical density and growth rate; effects of temperature and NaCl differ from acidity. *International Journal of Food Microbiology* 43, 195–203.
- Lianou, A., Koutsoumanis, K.P., 2011. Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior. *Food Microbiology* 28, 828–837.
- Lianou, A., Sofos, J.N., 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *Journal of Food Protection* 70, 2172–2198.
- Linnan, M.J., Mascola, L., Lou, X.L., Goulet, V., May, S., Salminen, C., Hird, D.W., Yonekura, M.L., Hayes, P., Weaver, R., Audurier, A., Plikaytis, B.D., Fannin, S.L., Kleks, A., Broome, C.V., 1988. Epidemic listeriosis associated with Mexican-style cheese. *New England Journal of Medicine* 319, 823–828.
- Little, C.L., Taylor, F.C., Sagoo, S.K., Gillespie, I.A., Grant, K., McLauchlin, J., 2007. Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK. *Food Microbiology* 24, 711–717.
- Liu, D., Ainsworth, A.J., Austin, F.W., Lawrence, M.L., 2003. Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. *Journal of Medical Microbiology* 52, 1065–1070.
- Liu, D., Lawrence, M.L., Austin, F.W., Ainsworth, A.J., 2007. A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*. *Journal of Microbiological Methods* 71, 133–140.
- Lomonaco, S., Decastelli, L., Nucera, D., Gallina, S., Manila Bianchi, D., Civera, T., 2009. *Listeria monocytogenes* in gorgonzola: subtypes, diversity and persistence over time. *International Journal of Food Microbiology* 128, 516–520.
- Luber, P., Crerar, S., Dufour, C., Farber, J., Datta, A., Todd, E.C.D., 2011. Controlling *Listeria monocytogenes* in ready-to-eat foods: working towards global scientific consensus and harmonization – recommendations for improved prevention and control. *Food Control* 22, 1535–1549.
- Manfreda, G., De Cesare, A., Stella, S., Cozzi, M., Cantoni, C., 2005. Occurrence and ribotypes of *Listeria monocytogenes* in gorgonzola cheeses. *International Journal of Food Microbiology* 102, 287–293.
- Martins, E.A., Leal Germano, P.M., 2011. *Listeria monocytogenes* in ready-to-eat, sliced, cooked ham and salami products, marketed in the city of São Paulo, Brazil: occurrence, quantification, and serotyping. *Food Control* 22, 297–302.
- Martins, I.S., Faria, F.C.C., Miguel, M.A.L., Dias, M.P.S.C., Cardoso, F.L.L., Magalhães, A.C.G., Mascarenhas, L.A., Nouér, S.A., Barbosa, A.V., Vallim, D.C., Hofer, E., Rabello, R.F., Riley, L.W., Moreira, B.M., 2010. A cluster of *Listeria monocytogenes* infections in hospitalized adults. *American Journal of Infection Control* 38, e31–e36.
- Meloni, D., Galluzzo, P., Mureddu, A., Piras, F., Griffiths, M., Mazzette, R., 2009. *Listeria monocytogenes* in RTE foods marketed in Italy: prevalence and automated *EcoRI* ribotyping of the isolates. *International Journal of Food Microbiology* 129, 166–173.
- Oliveira, M.A., Abeid Ribeiro, E.G., Morato Bergamini, A.M., Pereira De Martinis, E.C., 2010. Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. *Food Microbiology* 27, 19–23.
- Orsi, R.H., Ripoll, D.R., Yeung, M., Nightingale, K.K., Wiedmann, M., 2007. Recombination and positive selection contribute to evolution of *Listeria monocytogenes* *inlA*. *Microbiology* 153, 2666–2678.
- Ortiz, S., López, V., Villatoro, D., López, P., Dávila, J.C., Martínez-Suárez, J.V., 2010. A 3-year surveillance of the genetic diversity and persistence of *Listeria monocytogenes* in an Iberian pig slaughterhouse and processing plant. *Foodborne Pathogens and Disease* 7, 1177–1184.
- Pal, A., Labuza, T.P., Diez-Gonzalez, F., 2008. Comparison of primary predictive models to study the growth of *Listeria monocytogenes* at low temperatures in liquid cultures and selection of fastest growing ribotypes in meat and turkey product slurries. *Food Microbiology* 25, 460–470.
- Parizzi, S.Q.F., Andrade, N.J., Silva, C.A.S., Soares, N.D.F.F., Silva, E.A.M., 2004. Bacterial adherence to different inert surfaces evaluated by epifluorescence microscopy and plate count method. *Brazilian Archives of Biology and Technology* 47, 77–83.
- Ponniah, J., Robin, T., Paie, M.S., Radu, S., Ghazali, F.M., Kqueen, C.Y., Nishibuchi, M., Nakaguchi, Y., Malakar, P.K., 2010. *Listeria monocytogenes* in raw salad vegetables sold at retail level in Malaysia. *Food Control* 21, 774–778.
- Pouillot, R., Lubran, M.B., 2011. Predictive microbiology models vs. modeling microbial growth within *Listeria monocytogenes* risk assessment: what parameters matter and why. *Food Microbiology* 28, 720–726.
- Roberts, A.J., Williams, S.K., Wiedmann, M., Nightingale, K.K., 2009. Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, *inlA* transcript levels, and swarming motility in vitro. *Applied and Environmental Microbiology* 75, 5647–5658.
- Roche, S.M., Kerouanton, A., Minet, J., Le Monnier, A., Brisabois, A., Velge, P., 2009. Prevalence of low-virulence *Listeria monocytogenes* strains from different foods and environments. *International Journal of Food Microbiology* 130, 151–155.
- Rocourt, J., Bille, J., 1997. Foodborne listeriosis. *World Health Statistics Quarterly* 50, 67–73.
- Sabet, C., Lecuit, M., Cabanes, D., Cossart, P., Bienne, H., 2005. LPXTG protein *InlJ*, a newly identified internalin involved in *Listeria monocytogenes* virulence. *Infection and Immunity* 73, 6912–6922.
- Sato, G.S., Martins, V.A., Bueno, C.R.F., 2007. Análise exploratória do perfil do consumidor de produtos minimamente processados na cidade de São Paulo. *Informações Econômicas* 37, 62–71.
- Schlech III, W.F., Lavigne, P.M., Bortolussi, R.A., 1983. Epidemic listeriosis—evidence for transmission by food. *New England Journal of Medicine* 308, 203–206.
- Seeliger, H.P.R., Höhne, K., 1979. Serotyping of *Listeria monocytogenes* and related species. In: Bergan, T., Norris, J.R. (Eds.), *Methods in Microbiology*, vol. 13. Elsevier, New York, NY, pp. 31–49.
- Silva, F.A.S., Azevedo, C.A.V., 2002. Versão do programa computacional Assistat para o sistema operacional Windows. *Revista Brasileira de Produtos Agroindustriais* 4, 71–78.
- Skalina, L., Nikolajeva, V., 2010. Growth potential of *Listeria monocytogenes* strains in mixed ready-to-eat salads. *International Journal of Food Microbiology* 144, 317–321.
- Takeuchi, K., Mytelle, N., Lambert, S., Coleman, M., Doyle, M.P., Smith, M.A., 2006. Comparison of *Listeria monocytogenes* virulence in a mouse model. *Journal of Food Protection* 69, 842–846.
- Thévenot, D., Delignette-Muller, M.-L., Christieans, S., Leroy, S., Kodjo, A., Vernozzy-Rozand, C., 2006. Serological and molecular ecology of *Listeria monocytogenes* isolates collected from 13 French pork meat salting-curing plants and their products. *International Journal of Food Microbiology* 112, 153–161.
- Todd, E.C.D., Notermans, S., 2011. Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes*. *Food Control* 22, 484–490.
- Uyttendaele, M., Busschaert, P., Valero, A., Geeraerd, A.H., Vermeulen, A., Jaxsens, L., Goh, K.K., De Loy, A., Van Impe, J.F., Devlieghere, F., 2009. Prevalence and challenge tests of *Listeria monocytogenes* in Belgian produced and retailled mayonnaise-based deli-salads, cooked meat products and smoked fish between 2005 and 2007. *International Journal of Food Microbiology* 133, 94–104.
- Van Houdt, R., Michiels, C.W., 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology* 109, 1117–1131.
- Varma, J.K., Samuel, M.C., Marcus, R., Hoekstra, R.M., Medus, C., Segler, S., Anderson, B.J., Jones, T.F., Shiferaw, B., Haubert, N., Megginson, M., McCarthy, P.V., Graves, L., Gilder, T.V., Angulo, F.J., 2007. *Listeria monocytogenes* infection from foods prepared in a commercial establishment: a case-control study of potential sources of sporadic illness in the United States. *Clinical Infectious Diseases* 44, 521–528.
- Vázquez-Boland, J.A., Domínguez-Bernal, G., González-Zorn, B., Kreft, J., Goebel, W., 2001a. Pathogenicity islands and virulence evolution in *Listeria*. *Microbes and Infection* 3, 571–584.
- Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., Kreft, J., 2001b. *Listeria* pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews* 14, 584–640.
- Velge, P., Roche, S.M., 2010. Variability of *Listeria monocytogenes* virulence: a result of the evolution between saprophytism and virulence? *Future Microbiology* 5, 1799–1821.
- Vermeulen, A., Smigic, N., Rajkovic, A., Gysemans, K., Bernaerts, K., Geeraerd, A., Van Impe, J., Debevere, J., Devlieghere, F., 2007. Performance of a growth-no growth model for *Listeria monocytogenes* developed for mayonnaise-based salads: influence of strain variability, food matrix, inoculation level, and presence of sorbic and benzoic acid. *Journal of Food Protection* 70, 2118–2126.
- Wagner, M., Allerberger, F., 2003. Characterization of *Listeria monocytogenes* recovered from 41 cases of sporadic listeriosis in Austria by serotyping and pulsed-field gel electrophoresis. *FEMS Immunology and Medical Microbiology* 35, 227–234.
- Warriner, K., Namvar, A., 2009. Why is the hysteria with *Listeria*? *Trends in Food Science and Technology* 20, 245–254.
- Wiedmann, M., 2002. Molecular subtyping methods for *Listeria monocytogenes*. *Journal of AOAC International* 85, 524–531.
- Wiedmann, M., Bruce, J.L., Keating, C., Johnson, A.E., McDonough, P.L., Batt, C.A., 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infection and Immunity* 65, 2707–2716.
- Wilis, C., Baalham, T., Greenwood, M., Presland, F., 2006. Evaluation of a new chromogenic agar for the detection of *Listeria* in food. *Journal of Applied Microbiology* 101, 711–717.
- Williams, D., Irvin, E.A., Chmielewski, R.A., Frank, J.F., Smith, M.A., 2007. Dose-response of *Listeria monocytogenes* after oral exposure in pregnant guinea pigs. *Journal of Food Protection* 70, 1122–1128.
- Williams, S.K., Roof, S., Boyle, E.A., Burson, D., Thippareddi, H., Geornaras, I., Sofos, J.N., Wiedmann, M., Nightingale, K., 2011. Molecular ecology of *Listeria monocytogenes* and other *Listeria* species in small and very small ready-to-eat meat processing plants. *Journal of Food Protection* 74, 63–77.