UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

AVALIAÇÃO QUANTITATIVA DO RISCO DE *Salmonella* spp. E DE *Escherichia coli* O157:H7 EM ALFACE NO RIO GRANDE DO SUL

SUSANA DE OLIVEIRA ELIAS

Orientador: Prof. Dr. Eduardo Cesar Tondo

Porto Alegre
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Susana de Oliveira Elias
Mestre em Microbiologia
Agrícola e do Ambiente

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AVALIAÇÃO QUANTITATIVA DO RISCO DE *Salmonella* spp. E DE *Escherichia coli* O157:H7 EM ALFACE NO RIO GRANDE DO SUL[1]

Autor: Susana de Oliveira Elias; Orientador: Prof. Dr. Eduardo Cesar Tondo

RESUMO: O consumo de vegetais e de frutas tem aumentado mundialmente, bem como os surtos alimentares envolvendo esses alimentos, especialmente a alface que é o vegetal folhoso mais consumido em nível mundial. Dessa forma, o objetivo desse estudo foi realizar uma avaliação quantitativa do risco de infecções causadas por *Salmonella* spp. e por *Escherichia coli* O157:H7 a partir do consumo de alface produzida e consumida no Rio Grande do Sul, visto que esses patógenos são os mais relacionados a surtos alimentares envolvendo vegetais folhosos em nível mundial. Para melhor compreender o comportamento desses patógenos na alface, eles foram inoculados nesse vegetal separadamente e armazenados sob condições isotérmicas de 5 a 40°C para *Salmonella* e de 5 a 42ºC para *E. coli* O157:H7, bem como sob condições não isotérmicas, simulando temperaturas encontradas da colheita até a venda da alface no Rio Grande do Sul. Dados experimentais demonstraram que ambas as bactérias podem se multiplicar em todas as temperaturas examinadas. Também foi proposto um parâmetro de tempo de multiplicação insignificante (ς), o qual fornece o tempo em que a alface pode ser exposta a uma temperatura específica e não apresentar uma multiplicação expressiva. O ς foi desenvolvido com base na equação do modelo primário de Baranyi e no conceito do potencial de crescimento. ς é o valor da fase lag adicionado do tempo necessário para população microbiana aumentar 0,5 log UFC/g. O ς da alface exposta a 37 °C foi de 1,3 h, enquanto que a 5 °C foi de 3,3 dias. Além dos modelos adequados, dados de prevalência e concentração são primordiais na avaliação de risco. Assim, foi realizada uma revisão sistemática da literatura para buscar esses dados. A prevalência mundial encontrada foi de 0,041 para ambos os patógenos na alface. Já a prevalência dos países desenvolvidos foi de 0,028 para *Salmonella* e de 0,125 para *E. coli* (EHEC), enquanto que nos países em desenvolvimento foi de 0,064 para *Salmonella* e 0,024 para *E. coli* (EHEC). A concentração de *Salmonella* em alface, em países em desenvolvimento, variou de 4,57 a 218,78 NMP/g, e para *E. coli* (EHEC) a concentração foi de < 3,0 NMP/g até > 1100 NMP/g. O modelo de avaliação quantitativa de risco microbiológico foi composto por nove módulos, desde o armazenamento da alface nas fazendas produtoras até o consumo. O risco médio (baseado no cenário mais comumente encontrado no Rio Grande do Sul) de infecção por *Salmonella* por mês foi de 0,017, enquanto que por *E. coli* O157:H7 foi de 0,006. Assim, de modo geral, o risco de infecção por *Salmonella* é maior do que por *E. coli* O157:H7 quando a alface é produzida e consumida nesse estado. Todos os cenários alternativos à correta higienização da alface (lavar as folhas de alface com água potável seguido de imersão em 200 ppm de cloro livre, por 15 minutos e enxaguar com água potável) aumentaram o risco. A principal redução do risco foi identificada no cenário que considerou o uso de refrigeração em todos os módulos do modelo. Análises de sensibilidade indicaram que, além da manutenção da cadeia fria e do procedimento correto de higienização, é importante reduzir a prevalência e a concentração dos patógenos na alface, a fim de diminuir o risco de infecção por essas bactérias. Por fim, a avaliação de risco desenvolvida nessa tese pode auxiliar no desenvolvimento de estratégias de intervenção para mitigar esse risco.

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QUANTITATIVE MICROBIAL RISK ASSESSMENT OF *Salmonella* spp. AND *Escherichia coli* O157:H7 ON LETTUCE IN RIO GRANDE DO SUL[1]

Author: Susana de Oliveira Elias
Advisor: Prof. Dr. Eduardo Cesar Tondo

ABSTRACT: The consumption of vegetables and fruits has increased worldwide, as well as foodborne outbreaks involving these foods, especially lettuce that is the most consumed leafy vegetable in the world. Thus, the objective of this study was to carry out a quantitative microbial risk assessment of *Salmonella* spp. and *Escherichia coli* O157: H7 on lettuce produced and consumed in Rio Grande do Sul, since these pathogens are the most related to foodborne outbreaks involving leafy vegetables worldwide. To study the behavior of these pathogens on lettuce, they were inoculated on this vegetable separately and stored under isothermal conditions of 5 to 40 °C for *Salmonella* and 5 to 42 °C for *E. coli* O157:H7, as well as under non-isothermal conditions, simulating temperatures from the harvest until the sale of lettuce in Rio Grande do Sul. Experimental data demonstrated that both bacteria can growth at all temperatures examined. A negligible growth time parameter (ς) has also been proposed, which provides the time that lettuce can be exposed to a specific temperature and does not present an expressive growth. The ς was developed based on the equation of the Baranyi primary model and the concept of growth potential. ς is the lag phase added value of the time required for microbial population to increase 0.5 log CFU/g. The ς of lettuce exposed at 37 ºC was 1.3 h, whereas at 5 ºC it was 3.3 days. In addition, prevalence and concentration data are paramount in the risk assessment studies. Thus, a systematic review of the literature was carried out to collect these data. The global prevalence found was 0.041 for both pathogens in lettuce. The prevalence of developed countries was 0.028 for *Salmonella* and 0.125 for *E. coli* (EHEC), while in developing countries it was 0.064 for *Salmonella* and 0.024 for *E. coli* (EHEC). The concentration of *Salmonella* in lettuce in developing countries ranged from 4.57 to 218.78 MPN/g, and for *E. coli* (EHEC) the concentration was < 3.0 MPN/g to > 1100 MPN/g. The quantitative microbial risk assessment model was composed by nine modules, from lettuce storage on farms to consumption. The average risk (based on the scenario most commonly found in Rio Grande do Sul) of *Salmonella* infection per month was 0.017, whereas for *E. coli* O157:H7 it was 0.006. Thus, in general, the risk of infection by *Salmonella* is higher than by *E. coli* O157:H7 when lettuce is produced and consumed in this State. All scenarios that were alternative to the correct hygiene of lettuce (washing lettuce leaves with drinking water followed by immersion in 200 ppm of free chlorine for 15 minutes and rinsing with potable water) increased the risk. The main risk reduction was identified in the scenario that considered the use of refrigeration in all modules of the model. Sensitivity analyzes indicated that, in addition to maintaining the cold chain and the correct hygienization procedure, it is important to reduce the prevalence and concentration of pathogens in lettuce, in order to reduce the risk of infection by these bacteria. Finally, the risk assessment developed in this thesis can help in the development of intervention strategies to mitigate this risk.

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LISTA DE ABREVIATURAS E SIGLAS

AR	Análise de risco
AQRM	Avaliação quantitativa de risco microbiológico
IC	Intervalo de confiança
EHEC	*E. coli* enterohemorrágica
ppm	Parte por milhão
SHU	Síndrome hemolítica-urêmica
STEC	*E. coli* que produz a toxina Shiga
VTEC	*E. coli* produtora de verotoxina

1. INTRODUÇÃO

O consumo de vegetais está aumentando em nível mundial. O número de surtos alimentares devido à ingestão desses alimentos também está cada vez maior. Uma das causas do aumento de ocorrências é o fato de grande parte dos vegetais serem consumidos crus, não havendo uma etapa de processamento que elimine totalmente agentes patogênicos, como, por exemplo, um tratamento térmico como o cozimento; o que vem despertando a preocupação de produtores, consumidores e de órgãos de fiscalização.

A alface é o vegetal folhoso mais consumido no Brasil e no mundo. Suas propriedades nutricionais, baixo custo e fácil acesso contribuem para esse fato. Porém, esse alimento pode ser contaminado por agentes patogênicos durante toda sua cadeia produtiva até seu consumo. Dessa forma, podem ocorrer diversas doenças transmitidas pelo consumo desse alimento, sendo que os principais agentes dos surtos alimentares ocorridos mundialmente são *Salmonella* e *Escherichia coli* O157:H7.

Salmonella é um dos principais patógenos transmitidos por alimentos em todo o mundo. Os surtos cujo agente etiológico é essa bactéria envolvem tanto alimentos de origem animal quanto vegetal, resultando na maioria dos casos em gastrenterite autolimitante. Já *E. coli* O157:H7 também tem sido relacionada a surtos de origem animal e vegetal, podendo desencadear doenças bastante graves, inclusive levando o paciente a óbito.

Assim, na tentativa de compreender os reais riscos do consumo de alface, possivelmente contaminada por *Salmonella* e por *E. coli* O157:H7, no Rio Grande do Sul, há a necessidade da utilização de ferramentas avançadas de investigação científica. Dentre essas ferramentas, uma das mais utilizadas atualmente é a Avaliação de Risco, pois permite que o impacto desde a produção da matéria-prima, do processamento até o consumo sejam avaliados e os resultados obtidos sejam utilizados para resolver um problema de segurança de alimentos.

A fim de realizar uma avaliação quantitativa de risco microbiológico adequada, diversas informações devem ser analisadas, de preferência com base científica. Em vista disso, é importante conhecer o cenário brasileiro e mundial, identificando os principais agentes patogênicos envolvidos em Doenças

Transmitidas por Alimentos (DTA), veiculadas por alface. Modelos matemáticos aplicados à microbiologia preditiva têm sido utilizados para descrever e simular o comportamento microbiano em diversos alimentos, e podem ser utilizados para uma melhor compreensão da multiplicação de *Salmonella* spp. e *E. coli* O157:H7 em alfaces.

Além disso, é importante determinar informações essenciais à realização da avaliação de risco, as quais geralmente são a prevalência e a concentração dos patógenos de interesse como é o caso de *Salmonella* spp. e de *E. coli* O157:H7 em alface, permitindo determinar o risco de infecção por esses micro-organismos, a partir do consumo de alfaces produzidas em regiões de interesse, como o estado do Rio Grande do Sul. A aplicabilidade desse estudo está na possibilidade da adoção de estratégias cientificamente baseadas para reduzir a contaminação e o risco de DTA provocadas por esses patógenos, devido ao consumo de alfaces, o que poderá levar a menores custos para a saúde pública e também auxiliará a manutenção do crescimento desse mercado, devido à diminuição do desperdício e ao aumento da vida de prateleira.

2. OBJETIVOS

2.1. Objetivo Geral

Realizar uma avaliação quantitativa do risco de infecções causadas por *Salmonella* spp. e por *Escherichia coli* O157:H7, a partir do consumo de alface produzida e consumida no Rio Grande do Sul.

2.2. Objetivos Específicos

2.2.1. Identificar os surtos notificados associados ao consumo de frutas e de vegetais no Brasil;

2.2.2. Avaliar a multiplicação de *Salmonella* spp. e de *E. coli* O157:H7 em alface contaminada artificialmente por esses patógenos em diferentes temperaturas (5°C, 10°C, 25°C, 37°C, 40ºC e 42ºC);

2.2.3. Determinar os parâmetros cinéticos de multiplicação (tempo de fase lag e taxa de multiplicação) de *Salmonella* spp. e de *E. coli* O157:H7 em alface contaminada artificialmente por esses patógenos, por meio de modelos preditivos primários; e descrever a taxa de multiplicação dos patógenos em função da temperatura de armazenamento (5 a 40°C ou 42ºC), utilizando-se modelos secundários;

2.2.4. Avaliar a adequação dos modelos gerados por meio dos parâmetros R^2 e RMSE;

2.2.5. Propor um parâmetro para fornecer o tempo que a alface pode ser exposta a uma certa temperatura e ainda ser considerada segura;

2.2.6. Determinar a prevalência e a concentração de *Salmonella* spp. e de *E. coli* O157:H7 em alface, por meio de revisão bibliográfica e meta-análise.

2.2.7. Determinar o risco de infecção por *Salmonella* spp. e por *E. coli* O157:H7 a partir do consumo de alface produzida no Rio Grande do Sul.

3. REVISÃO DA LITERATURA

3.1. Microbiologia da alface

A alface (*Lactuca sativa*), originária da Ásia a cerca de 4.500 antes de Cristo, foi trazida para o Brasil pelos portugueses no século XVI, e é o vegetal folhoso mais consumido nesse país e no mundo (Echer et al., 2016). Existem diversos tipos de alface, sendo a mais cultivada e consumida no Brasil o tipo “crespa”, o que é um fato único na alfacicultura mundial (Sala & Costa 2012). Esse vegetal é recomendado para fazer parte de uma dieta saudável por ser fonte de vitaminas, sais minerais e fibras, além de ser de baixo custo e de fácil acesso, devendo ser consumido cru para o aproveitamento dessas propriedades e para manutenção de suas características organolépticas (FAO, 2008).

Membros dos filos Proteobactérias, Firmicutes, Actinobacteria e Bacteroidetes dominam a microbiota da alface, embora as proporções de taxa individual possam variar de acordo com fenótipo, localização geográfica, época do ano e intervenção humana (Williams et al., 2013). Já os gêneros mais abundantes são: *Pantoea*, *Erwinia*, *Pseudomonas*, *Sphingomonas, Bacillus*, *Massilia*, *Arthrobacter*, *Rhizobium*, *Variovorax* e bactérias ácido-láticas. Dentre as principais funções dessa microbiota, está o fornecimento de proteção contra patógenos de plantas, principalmente por competição por nutrientes e por espaço (Rastogi et al., 2012; Schreiter et al., 2014; Williams et al., 2013).

Esses vegetais folhosos também podem ser vetores de patógenos humanos, levando a DTA. Embora os agentes patogênicos humanos, em geral, não façam parte da microbiota dessas plantas, *Escherichia coli* O157:H7 e *Salmonella*, por exemplo, sobrevivem durante longos períodos e podem até se multiplicar nesses vegetais (Elias et al., 2018; Williams & Marco 2014).

A origem dessa contaminação pode ser solo, água, animais selvagens e domésticos e até mesmo os humanos, sendo que ela pode ocorrer em toda a cadeia de produção, de transporte e de consumo, dependendo das boas práticas agrícolas, boas práticas de higiene, controle de temperatura e hábitos de consumo (Sant'Ana et al., 2014). Dessa forma, como os produtos frescos são normalmente consumidos

crus ou com um processamento mínimo, é importante manter a carga microbiana deles a mais baixa possível, a fim de prevenir DTA, visto que há a possibilidade das etapas anteriores ao consumo não serem capazes de garantir a redução de micro-organismos patogênicos até um nível seguro (Prado-Silva, et al., 2015; Wadamori et al., 2017).

Assim, durante as últimas décadas, o consumo e, consequentemente, o número de surtos envolvendo vegetais crus aumentou consideravelmente (Khalil & Frank, 2010). Como exemplo disso, no período de 1996 a 2005, o consumo de vegetais frescos aumentou 9% em comparação com a década anterior, contudo os surtos alimentares envolvendo vegetais folhosos aumentaram 38,6% (Herman et al., 2008). Além disso, os principais agentes bacterianos envolvidos nesses surtos foram *Salmonella* spp. e *Escherichia coli* O157:H7 (Wadamori et al., 2017; Yeni et al., 2016).

3.1.1 *Salmonella*

O gênero *Salmonella* está dividido em duas espécies principais: *Salmonella enterica* e *Salmonella bongori*. A sorologia dessas cepas é baseada nas diferenças de antígenos na superfície da célula bacteriana: O são os antígenos da membrana externa; H são os antígenos dos flagelos; e Vi são os antígenos capsulares. A combinação desses antígenos (fórmula antigênica) pode, portanto, ser única para cada cepa de *Salmonella*. O nome para cada tipo diferente é geralmente dado devido à doença causada ao hospedeiro ou ao local em que foi isolado pela primeira vez (Mahmoud, 2012).

Os humanos e os animais são reservatórios primários de *Salmonella*, mas esses micro-organismos são abundantes na natureza, assim *Salmonella* pode contaminar produtos frescos, tanto durante a produção através da água, solo, insetos ou outros animais, que estão contaminados com matéria fecal, quanto durante a preparação, por meio de contaminação cruzada (equipamentos, superfícies e manipuladores de alimentos) (Tondo et al., 2015). Além disso, dependendo da cepa, *Salmonella* pode ser tolerante e resistir a temperaturas baixas ou altas e ambientes ácidos extremos, consequentemente, pode não ser eliminada devido a condições inadequadas de armazenamento ou processamento (Yeni et al., 2016).

A salmonelose humana é a doença de origem alimentar bacteriana com maior ocorrência em nível mundial (Wadamori et al., 2017). Essa enfermidade geralmente causa uma gastrenterite autolimitante, porém infecções graves, tais como bacteremia, meningite, peritonite e miocardite têm sido reportadas (Belloso et al., 2011; Papamichalis et al., 2011; Sirinavin et al., 1999; Vidal et al., 2012;). A dose infectante típica varia de 10^6 a 10^8 UFC/g, porém a quantidade de células de *Salmonella* spp. que é necessária para causar uma infecção humana depende de vários fatores, como, por exemplo, o grau de resistência do hospedeiro e o estado fisiológico das células bacterianas, sendo que a dose pode ser inferior a 10 UFC/g de *Salmonella* spp. (Humphrey, 2004). No Brasil e no Rio grande do Sul, *Salmonella* spp. tem sido um dos principais patógenos envolvidos com surtos alimentares, desde a década de noventa (Brazil, 2017). Além disso, estudos têm reportado a presença de *Salmonella* spp. em alface, tanto em nível mundial quanto local (Waitt et al., 2013; Rodrigues et al., 2014).

3.1.2 *E. coli* O157:H7

A espécie *E. coli* - frequentemente utilizada como indicador de contaminação fecal, pois em sua maioria não causa doenças e faz parte da microbiota intestinal de animais e de humanos - apresenta seis grupos patogênicos (Olaniran et al., 2011). Entre esses grupos (enteropatogênicas, enterotoxigênicas, enteroinvasivas, enterohemorrágicas, enteroagregativas e difuso-aderentes), as doenças mais graves, como a diarreia sanguinolenta, a púrpura trombocitopênica trombótica, a colite hemorrágica e a síndrome hemolítica-urêmica (SHU) são causadas pelo grupo *E. coli* enterohemorrágica (EHEC), que também inclui *E. coli* que produzem toxinas Shiga (STEC - “Shiga-toxin-producing *E. coli*”) ou *E. coli* produtora de verotoxinas (VTEC – “Verotoxin-producing *E. coli*”) (Farrokh et al., 2013). Como os reservatórios primários dessas bactérias são os ruminantes, especialmente bovinos, esses agentes patogênicos podem contaminar produtos frescos durante a fase de cultivo, através de água contaminada com fezes de animais infectados ou durante a fase de preparação por meio de contaminação cruzada (equipamentos, superfícies e manipuladores) (Yeni et al., 2016).

Dentre as EHEC, *E. coli* O157:H7 tem sido bastante associada a surtos alimentares em humanos, causando desde diarreia simples a complicações graves

como a SHU, sendo a dose infectante desse patógeno de 5 a 50 UFC/g, o que torna seu controle difícil e essencial para a não ocorrência de surtos (Linden et al., 2013). Além disso, na América do Norte, Europa e outros países industrializados a maioria dos surtos relacionados às bactérias produtoras de toxina Shiga foi causado por cepas de *E. coli* O157:H7, sendo que dentre essas ocorrências várias estavam ligadas ao consumo de alface (Erickson et al., 2017). No Brasil, não há dados oficiais sobre surtos causados por essas bactérias, porém estudos vêm demonstrando sua presença em alguns alimentos (Bentancor et al., 2012; Loiko et al., 2016). Também, diversos casos de SHU têm sido relatados na última década, sem que o agente causador dessa síndrome tenha sido identificado (Guirro et al., 2013; Santos et al., 2017). Já no Rio Grande do Sul, *E. coli* O157:H7 tem sido isolada em água utilizada para a irrigação e lavagem de alfaces (Rodrigues et al., 2014; Decol et al., 2017).

3.2. Segurança dos alimentos

Alimentos seguros não causam danos à saúde do consumidor. Esses alimentos apresentam um risco aceitável aos consumidores, considerando a qualidade da matéria-prima, formas de produção e de distribuição, conservação e hábitos de consumo (Tondo & Bartz 2017). Existem diversas ferramentas que visam proporcionar a segurança dos alimentos durante sua produção e preparação as principais são: boas práticas agrícolas (BPA), boas práticas de fabricação (BPF), boas práticas (BP), sistema de análise de perigos e pontos críticos de controle (APPCC) e análise de risco (AR).

3.2.1 Análise de Risco (AR)

Em 1995, os países signatários da Organização Mundial do Comércio assinaram um acordo de Medidas Sanitárias e Fitossanitárias, sendo uma das principais consequências a adoção da AR no comércio internacional, para promover a segurança dos alimentos. Essa ferramenta é um método sistemático e altamente estruturado que se baseia em avaliações científicas, opiniões de especialistas de governos, das indústrias, das universidades e da comunidade em geral para possibilitar a tomada de decisões de modo consciente e adequado (FAO, 2007). Seus principais objetivos são reduzir os níveis de DTA e promover a segurança de

alimentos. De forma resumida, a AR avalia o contexto da produção de alimentos, identifica possíveis riscos presentes nos alimentos e fornece bases científicas para o estabelecimento ou não de medidas de controle (Tondo & Bartz, 2017).

Os componentes da AR são a Gestão do Risco, a Avaliação de Risco e a Comunicação do Risco. A Gestão de Riscos é o processo que considera os interesses das diversas partes envolvidas em um determinado problema de segurança de alimentos, devendo, de forma ideal, considerar toda a cadeia de produção do alimento em questão e consultar todas as partes interessadas relevantes para garantir que o problema seja abordado de forma integral e a tomada de decisões acertadas (FAO, 2007).

Já a Avaliação de Riscos é o embasamento científico da AR. A Avaliação de Riscos microbiológicos, em relação aos perigos biológicos, é definida como um processo sistemático e estruturado capaz de auxiliar na tomada de decisões pelos gestores do risco, com enfoque na caracterização e no entendimento da magnitude dos riscos, para desenvolver cenários de intervenção e controle desses riscos. Essa avaliação gera estimativas dos riscos identificados, podendo ser qualitativa ou quantitativa, sendo que a quantificação apresenta a vantagem de ser submetida à modelagem matemática por meio de *softwares* que possibilitem a aplicação de técnicas de microbiologia preditiva, identificando diferentes estratégias de intervenção (ILSI, 2012).

Por fim, a Comunicação de Riscos é a troca de informações interativa e de opiniões de todas as partes envolvidas na AR, ao longo de todo o processo (ILSI, 2012). Na AR, tanto os riscos quanto as medidas de controle devem ser claramente informados de forma eficiente e concisa, evitando exageros, pânico e má interpretação (Tondo & Bartz, 2017).

3.2.2 Avaliação quantitativa de risco

A Avaliação Quantitativa de Risco Microbiológico (AQRM) permite a estimativa quantitativa dos riscos microbiológicos à saúde pública, devido a uma combinação de alimento-patógeno (Oscar, 2011). Os resultados da AQRM podem ser utilizados no desenvolvimento de estratégias científicas para gerenciar riscos e salvaguardar a saúde pública (Sant'Ana et al., 2014).

Segundo o Codex Alimentarius (CODEX, 1999), a AQRM é um processo

de base científica, que envolve quatro etapas: identificação do perigo; avaliação da exposição; caracterização do perigo; e caracterização do risco. Assim, primeiramente se determina a combinação patógeno-alimento a ser considerada, baseando-se em informações epidemiológicas e biológicas pertinentes ao patógeno e ao alimento avaliados. Após, avalia-se a exposição da população considerada a esse patógeno, devido à ingestão do alimento. Essa avaliação baseia-se em modelos preditivos matemáticos que descrevem o comportamento microbiano ao longo da cadeia alimentar para estimar a quantidade do patógeno no momento do consumo. Já a caracterização do perigo fornece uma descrição quantitativa da severidade e da duração dos efeitos adversos decorrentes da ingestão do alimento contaminado, em geral essa etapa é baseada na relação dose-resposta. Por fim, a caracterização do risco 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, fornecendo as informações necessárias para a tomada de decisões dos gestores de risco (Sant'Ana & Franco 2009).

3.3. Capítulo de Microbiologia Preditiva em Alimentos aceito para ser publicado no livro Microbiologia e Sistemas de Gestão da Segurança de Alimentos.

Microbiologia Preditiva em Alimentos

Susana de Oliveira Elias e Eduardo Cesar Tondo

Introdução

A microbiologia preditiva é uma área da microbiologia de alimentos, a qual utiliza modelos matemáticos alimentados por dados de experimentos microbiológicos simples, para prever o comportamento dos micro-organismos nos alimentos. Ainda que os primeiros modelos preditivos tenham sido empregados no início do século 20, o grande desenvolvimento da microbiologia preditiva ocorreu nas últimas três décadas, como resultado dos avanços na área da informática. Existem diferentes tipos de modelos preditivos, os quais permitem predizer a multiplicação, a inativação e a sobrevivência dos micro-organismos em alimentos expostos a diferentes condições ambientais de tempo e de temperatura. Tais modelos também podem considerar fatores como o estado fisiológico das células microbianas, a interação com outros micro-organismos, além de fatores intrínsecos do próprio alimento, como pH, atividade de água e presença de conservadores.

Hoje em dia, os modelos preditivos tornaram-se uma ferramenta necessária para apoiar muitas decisões relativas à segurança e à qualidade dos alimentos, uma vez que eles podem fornecer respostas rápidas para perguntas específicas. Além disso, esses modelos estão sendo utilizados em sistemas de gestão da segurança de alimentos, como as BPF e APPCC, e em medidas de controle da segurança dos alimentos baseadas em risco. Eles também têm sido bastante utilizados para o desenvolvimento da avaliação quantitativa de risco, uma vez que podem modelar diferentes processos microbianos ao longo da cadeia alimentar e, assim, ajudar nos processos de tomada de decisão no âmbito da gestão de risco. A microbiologia preditiva ainda está em desenvolvimento, mas está se transformando em um importante instrumento para a melhoria da segurança e da qualidade dos alimentos.

Aspectos gerais da microbiologia preditiva

A microbiologia preditiva tem como objetivo predizer o comportamento microbiano em alimentos, a partir de modelos matemáticos adequados. Um modelo matemático é a descrição de um sistema real, utilizando equações matemáticas, que são simplificações do sistema com base em suas propriedades mais significativas (McMeekin et al., 2002).

Os modelos podem ser classificados de acordo com o tipo de estrutura e de variáveis. Os modelos primários estudam a variação da concentração microbiana em relação ao tempo (curvas de multiplicação e de inativação microbiana). Já os modelos secundários relacionam os parâmetros cinéticos derivados dos modelos primários (taxa de multiplicação, tempo de fase lag e população final máxima) a fatores ambientais (temperatura, pH, atividade de água). Finalmente, os modelos terciários são *software*, que utilizam os modelos primários e secundários, a fim de fornecer estimativas do comportamento microbiano sob condições específicas definidas pelos usuários (Whiting & Buchanan, 1993).

Em todos os casos, os modelos devem ser validados para que a confiabilidade de suas previsões seja comparada com os dados reais em alimentos. Com esse fim, diferentes estratégias têm sido utilizadas, tais como o cálculo de índices que avaliam o ajuste dos dados preditos pelos modelos matemáticos aos observados nos experimentos microbiológicos (coeficiente de determinação ou R^2, Raiz do Erro Quadrático Médio ou RMSE), e os que determinam a capacidade de predição dos modelos (fatores de viés e de acurácia) (Ross, 1996).

Aplicações

Algumas das potenciais aplicações da microbiologia preditiva estão resumidas a seguir (Forsythe, 2013; Membré & Lambert, 2008):

1) No APPCC

• auxílio na identificação dos perigos;

• identificação de Pontos Críticos de Controle (PCC);

• identificação de medidas corretivas;

• avaliação da interação das variáveis.

2) Avaliação de Risco e Gestão do Risco

• estimativa da dinâmica das populações microbianas ao longo da cadeia alimentar;

• avaliação da exposição a um patógeno específico;

• estudo de estratégias de gestão com base científica para garantir a segurança dos alimentos;

3) Estudos de vida útil ou de prateleira;

• previsão da multiplicação ou inativação de micro-organismos deteriorantes ou patogênicos nos alimentos.

4) Inovação e Desenvolvimento de um novo produto

• avaliação do impacto da deterioração microbiana de um produto;

• avaliação prévia do efeito do processamento na qualidade e na segurança do alimento;

• avaliação do efeito de outros fatores, ao longo da cadeia de alimentos;

5) Medidas de Higiene e Controle da Temperatura

• avaliação das consequências da aplicação da cadeia de frio na deterioração microbiana;

• otimização dos processos de inativação térmica e não-térmica;

6) Educação

• educação de equipes científicas e não-científicas;

• implementação e treinamento de sistemas de decisão baseados em computador.

7) Desenho Experimental

• estimativa do número de amostras a ser preparado;

• definição de intervalos dentro de cada fator a ser analisado.

Desenvolvimento de um novo modelo preditivo

Antes do desenvolvimento de um novo modelo preditivo ou de realizar modificações em um modelo já existente, recomenda-se que as seguintes perguntas (Q1 a Q8) sejam feitas e as respostas criticamente respondidas, conforme Quadro 1 (Pérez-Rodríguez & Valero, 2013).

Quadro 1: Passos a serem seguidos e perguntas a serem consideradas na construção e aplicação de um modelo preditivo.

Passo 1: Revisão da literatura e análise preliminar
Q1: Os dados e/ou modelos disponíveis na literatura são suficientes? Resposta Sim: rejeitar/parar a construção do novo modelo Resposta Não: ir para Q2
Q2: O modelo preditivo irá melhorar significativamente o conhecimento atual no campo de interesse? Sim: ir para próximo passo Não: depende da finalidade do modelo, em geral, quando já existem dados, só se deve construir um novo modelo se houver uma necessidade bastante específica
Passo 2: Planejamento e desenho experimental (mais detalhes a seguir)
Q3: Os recursos laboratoriais disponíveis são suficientes para realizar todas as análises em condições controladas? Sim: ir para Q4 Não: redefina o delineamento experimental, ou seja, a forma em que os tratamentos (níveis de um fator ou combinações de níveis de fatores) foram atribuídos às unidades experimentais
Q4: Os autores têm “a priori” o conhecimento sobre as principais condições ambientais que afetam a multiplicação/sobrevivência do perigo estudado e do alimento? Sim: ir para Q5 Não: vá para o passo 1
Q5: De acordo com essas condições, é possível desenvolver um desenho fatorial completo (que inclui todas as combinações entre os níveis dos fatores do experimento)? Sim: próximo passo Não: crie um delineamento fatorial incompleto (que inclui algumas das combinações entre os níveis dos fatores do experimento)
Passo 3: Desenvolvimento de modelo
Q6: O modelo matemático é abrangente e representa o comportamento observado do perigo estudado? Sim: próximo passo Não: revisar o processamento de dados e escolher uma outra equação
Passo 4: Validação do modelo
Q7: O modelo pode ser validado com medidas adicionais ou dados externos? Sim: próximo passo Não: vá para o passo 1
Passo 5: Aplicação do modelo
Q8: O modelo pode ser efetivamente aplicado nas indústrias de alimentos, serviços de alimentação ou pelas autoridades responsáveis? Sim: aplicação Não: vá para o passo 3

O desenho experimental dos modelos preditivos irá depender principalmente da aplicação do modelo em um cenário real. Os principais fatores a serem considerados nesse planejamento são abordados nas questões abaixo (Devlieghere, 2000):

• Qual é o principal objetivo do modelo preditivo?

O principal objetivo de um modelo preditivo pode ser verificar se determinado micro-organismo se multiplica em um certo alimento, numa faixa de temperatura. Como exemplo, pode-se citar o trabalho de Elias et al. (2016) que avaliou a multiplicação de *Salmonella* em maionese caseira exposta à uma faixa de temperatura de 7 a 37ºC. Essa faixa de temperatura abrangia as prováveis temperaturas de refrigeração e exposição da maionese caseira, em diferentes situações reais no Brasil.

• Quais são os principais fatores a serem controlados, de modo que este objetivo seja alcançado? Em quais níveis? E em quantas combinações?

Tradicionalmente, o desenho experimental completo que considera todas as combinações das diferentes variáveis deve ser o escolhido. Porém, deve-se levar em consideração que ele é frequentemente muito trabalhoso e caro. Assim, uma alternativa que pode ser adotada é um planejamento experimental eficaz para reduzir o número de análises, mantendo a qualidade dos dados.

Referindo-se ao exemplo anterior de Elias et al. (2016), o principal fator considerado capaz de influenciar a multiplicação de *Salmonella* em maionese caseira foi a temperatura. Dessa forma, os níveis (temperaturas) analisados foram 7, 10, 15, 20, 25, 30 e 37ºC, sendo que não foi necessário haver combinações de fatores, visto que nesse estudo apenas um fator foi testado.

• Quais as características do inóculo que será utilizado?

Em geral, os experimentos de microbiologia preditiva utilizam *pools* microbianos, ou seja, conjuntos de várias cepas (geralmente cinco) de uma mesma espécie microbiana, a fim de aumentar a representatividade do comportamento do micro-organismo estudado (Sousa et al., 2014). Aconselha-se utilizar cepas de diferentes origens, aumentando a chance de contemplar variações dentro da mesma espécie microbiana, ou seja, para compor o *pool*, pode-se utilizar cepas de referência (como as ATTCC), cepas de surtos e isoladas de alimentos e/ou pacientes infectados. Porém, existem casos em que pode ser utilizada apenas uma

cepa microbiana, como o do estudo previamente mencionado de Elias et al. (2016), no qual foi utilizada apenas a cepa *S.* Enteritidis SE86, por ser a principal causadora de surtos no estado do Rio Grande do Sul de 1999 a 2013. Dessa forma, o objetivo do estudo era avaliar o comportamento dessa cepa específica no principal alimento causador de surtos alimentares do RS (maionese caseira).

Além disso, ao realizar experimentos microbiológicos é importante que se certifique do estado fisiológico do micro-organismo escolhido. A fim de garantir que seu metabolismo esteja funcionando adequadamente (metabolismo ativado), o que em geral é alcançado através de duas multiplicações seguidas, em um meio de cultura rico, como, por exemplo, BHI (infusão de coração e cérebro) ou TSB (caldo de soja tripticaseína) (Sant'Ana et al., 2012). Aconselha-se que experimentos que objetivem avaliar a multiplicação de micro-organismos tenham inóculos iniciais de aproximadamente de 10^2 a 10^3 UFC/g, enquanto que experimentos que objetivem avaliar a inativação ou sobrevivência tenham inóculos iniciais de 10^7 UFC/g (ICMSF, 2015).

• Qual é o substrato (alimento ou meio de cultura) a ser utilizado?

A escolha do substrato que será utilizado no desenvolvimento do modelo é muito importante. Em geral, quando se compara as predições de modelos realizados em meios de cultura com aquelas realizadas em alimentos, observa-se uma multiplicação mais rápida nos meios de cultura. Vários fatores podem contribuir para essa diferença, por exemplo: a matriz alimentar, muitas vezes é semi-sólida ou sólida, enquanto os meios de cultura podem ser líquidos e agitados, aumentando a disponibilidade de oxigênio aos micro-organismos. A presença da microbiota acompanhante do próprio alimento pode inibir a multiplicação do micro-organismo-alvo, ou mesmo os fatores ambientais e intrínsecos dos alimentos podem ter o mesmo efeito inibidor, o que não ocorrerá nos meios de cultura. Dessa forma, o uso de meios que mimetizam as estruturas do alimento real de interesse implica em vantagens do ponto de vista experimental, como melhor controle, facilidade de operação e repetitividade das análises (Noriega et al., 2008; Wilson et al., 2002), porém podem se afastar da realidade do alimento.

• Quantidade de pontos a serem coletados e métodos de análise?

Em relação a quantidade de dados que devem ser coletados ao longo de toda a curva cinética (curva de multiplicação ou de sobrevivência), em geral, são necessários 15 pontos de coleta, sendo 20 uma quantidade ótima e menos que 10, uma quantidade que pode ser responsável por um ajuste incompleto que gere incertezas. A distribuição desses pontos também é muito importante, eles devem representar o comportamento completo da curva (Poschet & Van Impe 1999) e serem coletados, principalmente nos pontos de inflexão dela. Ainda que a microbiologia preditiva possa ser aplicada também em cálculos da vida de prateleira e estudos de desafios, nesses experimentos um número menor de pontos pode ser coletado (5 a 7 pontos), porém isso pode aumentar a incerteza dos resultados, uma vez que o número de pontos de coleta está abaixo de 10 (ICMSF, 2015). Essa possibilidade de menor número de coletas baseia-se no fato que em experimentos de vida de prateleira não se espera a multiplicação excessiva de micro-organismos, os quais devem permanecer em fase lag, praticamente em todo o experimento.

Atualmente, há diversos métodos que permitem a coleta de dados utilizados na microbiologia preditiva, porém o mais utilizado é a contagem microbiana em placas. Como outros métodos que também podem ser utilizados pode-se citar a turbidimetria, citometria de fluxo, métodos eletroquímicos (impedância e condutância), análise de imagem e microscopia (Forsythe, 2013).

Tipos de modelos

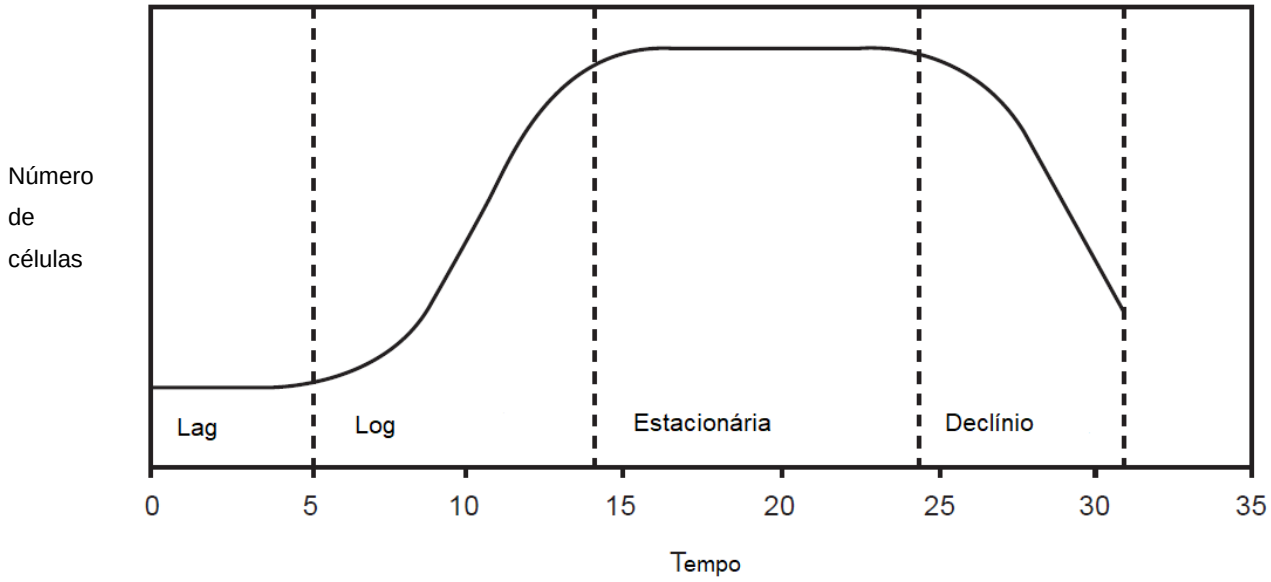
Como já mencionado os modelos preditivos são divididos em primários, secundários e terciários, essa classificação depende principalmente do tipo de predição gerada pelo modelo. A seguir mais detalhes sobre cada um dos modelos serão discutidos:

1) Modelos primários: destinam-se a descrever a cinética de um processo com poucos parâmetros. Eles são utilizados para investigar o aumento (multiplicação) ou diminuição (inativação) na densidade da população microbiana em relação ao tempo.

a) Modelos de multiplicação: a curva de multiplicação dos micro-organismos é caracterizada pelas seguintes fases: lag (adaptação), log (exponencial), estacionária e declínio, conforme Figura 1. A fase lag é o período onde os micro-organismos estão se adaptando ao novo meio e não apresentam

multiplicação microbiana; na fase log ocorre a multiplicação exponencial dos micro-organismos, aumentando expressivamente o número de células em um período reduzido; na fase estacionária há um equilíbrio entre o número de células que se multiplicam e as que morrem; enquanto durante o declínio o número de células que morrem é muito maior do que as que se multiplicam, ocorrendo a diminuição da população, devido ao esgotamento de nutrientes e de espaço (Zwietering et al., 1990).

Figura 1: Curva de multiplicação microbiana típica, compreendendo as quatro fases: lag, log, estacionária e de declínio.



O modelo proposto por Baranyi e Roberts (1994) é um dos modelos primários de multiplicação mais amplamente utilizados, ele é calculado por meio da seguinte fórmula:

$$N_t = N_{max} - \ln [1 + (\exp (N_{max} - N_0) - 1\exp (-\mu_{max} A(t)]$$

Onde:

N_t = tamanho da população (logaritmo)

N_{max} = população máxima (logaritmo)

N_0 = tamanho da população inicial (logaritmo)

μ_{max} = taxa máxima de multiplicação específica

$A(t)$ = integral de ajuste da função

Esse modelo adaptou melhor os dados experimentais do que outros

modelos existentes, como o Gompertz, principalmente na previsão da fase lag e da fase log. Além disso, sua grande utilização, se deve em parte, por estar disponível em *softwares* como, por exemplo, o Combase, que é gratuito e de fácil utilização.

b) Modelos de inativação: descrevem os padrões de inativação dos micro-organismos quando expostos a um processo ou agente letal, os quais podem ser físicos ou químicos. Os principais modelos são o linear e o não-linear.

O modelo linear (Figura 2) foi proposto por Bigelow & Esty (1920), assumindo uma curva cinética de primeira ordem, representada pela seguinte fórmula:

$$\log S_t = -\frac{t}{D}$$

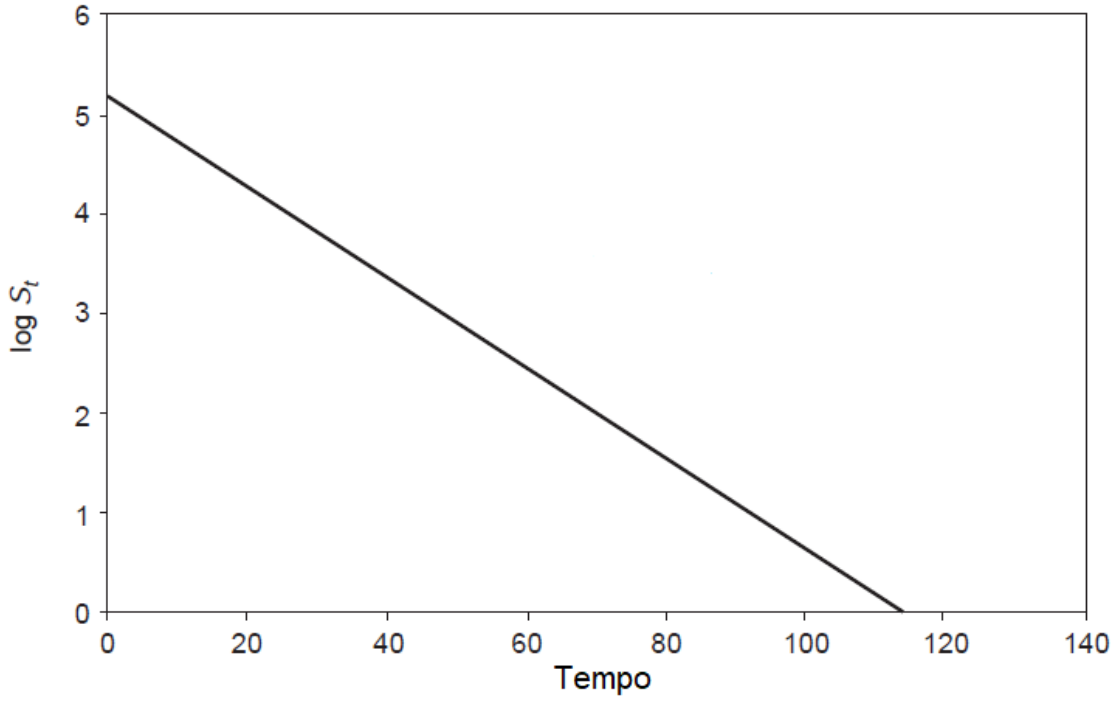
Onde:

$S_t = N_t / N_0$ (N_t = tamanho da população; N_0 = tamanho da população inicial)

t = tempo

D = valor D (tempo necessário para reduzir a população microbiana em 90% ou um ciclo logaritmo)

Figura 2: Representação do modelo de inativação log-linear.



Como exemplos de modelos não-lineares pode-se citar: bifásico, multi-exponencial, Weibull e Geeraerd. Alguns desses, além da cinética linear, apresentam um patamar inicial (“shoulder”), no qual pouca ou nenhuma inativação ocorre, e também uma cauda final (“tail”), que representa as células resistentes ao tratamento (residuais ou sobreviventes). Como um exemplo de modelo de inativação mais complexo, destaca-se o modelo desenvolvido por Geeraerd et al. (2000), que inclui o patamar inicial e a cauda, baseando-se no estado fisiológico das células e na densidade populacional residual (região da cauda), sendo calculado pela fórmula a seguir:

$$N = \left[(N_0 - N_{res}) \exp(-k_{\max}\ t) \frac{\exp(-k_{\max}\ t_L)}{1 + \exp((-k_{\max} t_L) - 1) \exp(-k_{\max} t)} + N_{res} \right]$$

Onde:

N = número de micro-organismos sobreviventes no tempo t

N_0 = carga microbiana inicial

k_{max} = taxa máxima de decaimento específico

t_L = tempo anterior à inativação

N_{res} = densidade populacional residual.

2) Modelos secundários: descrevem o efeito das condições ambientais (físico-químicas e fatores biológicos) sobre os valores dos parâmetros de um modelo primário, principalmente a taxa de multiplicação e o tempo de fase lag. Existem três tipos principais de modelos: polinomiais (superfície de resposta), raiz quadrada e Arrhenius. O modelo da raiz quadrada (Ratkowsky et al., 1982), que é baseado na relação linear entre a raiz quadrada da taxa de multiplicação e a temperatura, é um dos mais utilizados por incluir o conceito de zero biológico, o qual é a temperatura quando a taxa de multiplicação é zero; a fórmula encontra-se a seguir:

$$\sqrt{\mu_{\max}} = b \cdot (T - T_{\min})$$

Onde:

μ_{max} = taxa máxima de multiplicação específica

b = constante

T = temperatura

T_{min} = temperatura mínima teórica em que a multiplicação é detectada.

3) Modelos terciários: geralmente são programas de computador, que apresentam uma interface amigável, permitindo entradas escolhidas pelo usuário e que calculam saídas gráficas simplificadas. Existem diversos modelos terciários disponíveis com distintas funções (Quadro 2) dentre os quais se destacam o PMP e o ComBase (Pérez-Rodríguez, 2014).

O mais antigo dos programas é o PMP (*Pathogen Modeling Program*) que foi desenvolvido pelo Departamento de Agricultura dos EUA (USDA), em 1994, sendo atualizado continuamente ao longo dos anos. Ele pode ser conseguido no endereço https://pmp.errc.ars.usda.gov/default.aspx ou utilizado na versão *on-line* (https://pmp.errc.ars.usda.gov/PMPOnline.aspx), ambos gratuitos. O PMP destina-se principalmente à predição de bactérias patogênicas, incluindo modelos de resfriamento, multiplicação, inativação e transferência (contaminação cruzada). Os usuários podem introduzir valores de temperatura, pH, concentração de sais de sódio, nível inicial das bactérias e se o ambiente é aeróbio ou anaeróbio; enquanto que o programa calcula os parâmetros cinéticos (fase lag, taxa de multiplicação, tempo de geração, densidade populacional máxima, valor D), juntamente com uma representação gráfica do comportamento microbiano.

O ComBase foi desenvolvido, em 2003, pelo *Institute of Food Research* (Norwich, Reino Unido). Ele pode ser acessado gratuitamente, registrando-se no site www.combase.cc. Esse aplicativo possui as seguintes funções:

Browser: ferramenta de busca de modelos preditivos dinâmicos e estáticos já existentes;

ComBase Predictor: permite a previsão da multiplicação e da inativação de diferentes bactérias deteriorantes e patogênicas. Os modelos estáticos e dinâmicos baseiam-se em dados cinéticos gerados em meios de cultura, sendo possível introduzir valores de nível inicial das bactérias, temperatura, pH e atividade de água ou concentração de cloreto de sódio; enquanto que o programa calcula os parâmetros cinéticos (taxa máxima, valor D), juntamente com uma representação gráfica do comportamento microbiano.

Food Models: permite a previsão do comportamento de *Clostridium perfringens* em carnes e de *Salmonella* em ovos.

DMFit: nessa ferramenta o usuário pode colocar seus dados experimentais de tempo e de concentração dos micro-organismos e ajustar esses dados aos modelos disponíveis no *software*.

Resources: indicam outras ferramentas disponíveis de microbiologia preditiva.

Help: auxilia o usuário na compreensão das ferramentas disponíveis no ComBase.

Quadro 2: Lista de modelos terciários (ferramentas) disponíveis e suas respectivas características.

Ferramenta	Disponível em	Características
ComBase *Predictor*	http://www.combase.cc/index.php/en/	Modelos de multiplicação e inativação sob condições estáticas e dinâmicas
E. coli Inactivation in Fermented Meats Model	http://www.utas.edu.au/tia/centres/food-systems/fact-sheets-and-tools/fact-sheets-and-tools/predictive-models/e.-coli-inactivation-in-fermented-meats-model	Eficácia do processo de fermentação da carne na inativação de *E. coli*
Fish Shelf Life Prediction Program (FSLP) e FISHMAP	http://www.azti.es/network/shelf-life-prediction-software/	Predição de vida de prateleira em peixes
Food Product Modeller	http://www.mirinz.org.nz/prod/foodprodmod.asp	Avaliação de processos de refrigeração, congelamento, descongelamento e aquecimento para diversos produtos
Food Spoilage and Safety Predictor (FSSP)	http://fssp.food.dtu.dk/	Modelos preditivos de vida de prateleira para frutos do mar
Foodrisk	http://foodrisk.org/	Banco de dados e modelos preditivos
Campden BRI	https://www.campdenbri.co.uk/services/predictive-microbiology.php	Modelos preditivos
GInaFit	https://cit.kuleuven.be/biotec/software/GinaFit	Ajuste dos modelos de inativação microbiana em dados experimentais do usuário

GroPIN *Modeling* DataBase	http://www.aua.gr/psomas/gropin/	Banco de dados de modelagem preditiva para modelos cinéticos e probabilísticos
ICRA	http://icra.foodrisk.org/	Catálogo de modelos dinâmicos para o risco microbiano
Listeria Control Model TM	https://clcm.corbion.com/	Modelos preditivos para *L. monocytogenes* em função dos conservadores PURAC
Microbial Risk Viewer (MRV)	http://mrviewer.info/	Modelos cinéticos de multiplicação/não multiplicação microbiana
Microhibro	http://www.microhibro.com/	Modelos preditivos e sistema de avaliação de risco quantitativo
Pathogen Modeling Program (PMP)	https://pmp.errc.ars.usda.gov/PMPonline.aspx	Modelos de multiplicação e inativação para bactérias
Refrigeration Index Calculator	http://ricalculator.mla.com.au/	Multiplicação de *E. coli* no resfriamento da carne
Risk Management Tool in Poultry	http://tools.fstools.org/poultryRMTool/	Calcula o risco de *Campylobacter* e *Salmonella* no processamento de frango
Risk Ranger	http://www.foodsafetycentre.com.au/riskranger.php	Estima o risco semi-quantitativo
Shelf Stability Predictor	https://meathaccp.wisc.edu/ST_calc.html	Modelos de *L. monocytogenes* e *Staphylococcus aureus* em carne
Simprevius	https://symprevius.eu/en	Modelos preditivos e avaliação de risco
SOPHY	http://sophy-project.eu/	Predição da segurança, qualidade e vida de prateleira de produtos prontos para consumo
THERM	https://meathaccp.wisc.edu/therm/	Modelos de *Salmonella, E. coli* O157:H7 e *S. aureus* em carnes, em geral
UGPM (*Unified Growth Prediction Model*)	http://www.aua.gr/psomas/	Modelos preditivos em condições de temperatura dinâmica

Validação dos modelos

A validação pode ser definida como o processo que avalia a capacidade de um modelo prever o comportamento microbiano do sistema real. Assim, modelos que foram desenvolvidos utilizando meios de cultura sempre precisam ser validados na matriz alimentícia para sua aplicação (Ross et al., 2000). Existem diferentes índices estatísticos que podem ser aplicados na validação. Os índices de ajuste de qualidade baseados na proximidade entre observações e previsões também são utilizados para fins de validação. Os principais índices são o coeficiente de determinação (R^2) e a raiz do erro quadrático médio (RMSE). O primeiro informa sobre a proporção da variabilidade total explicada pelo modelo, de modo que quanto mais próximo de um (1) for o R^2, melhor o modelo representa as observações. Já a RMSE é uma medida padronizada dos resíduos do modelo que pode ser utilizada para avaliar o quão bem o modelo descreve as observações. Um valor próximo de zero da RMSE significa melhor adequação do modelo para descrever os dados experimentais (Nunes, et al., 2015).

Os fatores de viés (B_f) e de acurácia (A_f) avaliam se os modelos preditivos podem descrever corretamente observações independentes obtidas nas matrizes dos alimentos. O B_f é uma proporção da média das predições pelas observações. O valor de um (1) significa que as observações são igualmente distribuídas acima e abaixo das predições, valores <1 significam que os valores preditos são menores que os observados (modelo subestima os valores reais), enquanto que valores >1 indicam que os valores preditos são maiores do que os observados (modelo superestima os valores reais). Valores aceitáveis de B_f estão entre 0,75-1,25. O A_f é a média dos valores absolutos da relação entre predições e observações, informando quão próximas são as predições das observações. O valor de 1 indica concordância perfeita entre predição e observação, enquanto que valores >1 significam predições maiores que as observações (Ross 1996).

Tendências e Perspectivas

Além dos modelos cinéticos previamente discutidos, novas abordagens estão sendo desenvolvidas como, por exemplo, os modelos de transferência (contaminação cruzada), de multiplicação/não multiplicação (condições limite), de interação entre espécies e de célula-única (inicia o modelo com apenas uma célula

do micro-organismo). Já os modelos de escala “ômica” são construídos a partir de dados experimentais obtidos em pesquisa genômica, proteômica e metabolômica. Embora esses modelos sejam promissores em relação à capacidade de predição, eles ainda são poucos e limitados a micro-organismos e situações específicas. Pesquisas adicionais são necessárias, para complementar as informações disponíveis, produzindo modelos mais adequados para serem aplicados a situações reais relativas à segurança e qualidade dos alimentos (Brul et al., 2008). Também as redes neurais, ferramentas da inteligência artificial, estão sendo aplicadas a diversos tipos de modelos preditivos. Essas redes são inspiradas no funcionamento dos neurônios, no cérebro humano, sendo os modelos constituídos por três tipos de neurônios computacionais: entrada, oculto e saída (Oscar, 2009).

Além disso, a avaliação quantitativa do risco microbiano (QMRA) é uma área relativamente recente, que requer modelos de microbiologia preditiva (para estimar o efeito quantitativo das etapas determinadas ao longo da cadeia alimentar). Apesar dos avanços acima mencionados, ainda há várias questões metodológicas a serem respondidas sobre como os modelos podem ser aplicados para obter uma estimativa de risco mais precisa. Portanto, o desenvolvimento de novos e inovadores modelos preditivos é necessário para melhorar os estudos de QMRA e preencher as lacunas de dados existentes (Oscar, 2011). A meta-análise é uma das estratégias propostas focada em uma análise sistemática de uma grande coleção de dados com a intenção de gerar informações padronizadas e resumidas para produzir uma estimativa global de uma intervenção ou um tratamento específico (van Besten & Zwietering 2012).

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4. ARTIGOS

A apresentação dos resultados dessa Tese de Doutorado foi dividida em quatro artigos. O artigo 1 é uma análise dos surtos alimentares notificados ocorridos no Brasil, envolvendo frutas e vegetais no período de 2008 a 2014. No artigo 2, são apresentados os resultados da determinação dos parâmetros cinéticos de multiplicação de *Escherichia coli* O157:H7 e de *Salmonella* em alface, utilizando uma ampla faixa de temperatura (5 a 42ºC). O artigo 3 corresponde a uma meta-análise a partir de uma revisão de literatura sobre a prevalência e a concentração de *E. coli* O157:H7 e de *Salmonella* em alface local e mundialmente. Por fim, no artigo 4, os dados gerados nesse estudo e aqueles buscados na literatura foram empregados na construção de modelos de avaliação quantitativa de riscos, para a determinação da probabilidade de infecção por *E. coli* O157:H7 e por *Salmonella,* devido ao consumo de alface no Rio Grande do Sul.

4.1. Artigo 1

Artigo a ser submetido:

Reported foodborne outbreaks in Brazil associated to fruits and vegetables: 2008 through 2014

Susana de Oliveira Elias[a], Luana Tombini Decol[a], Eduardo Cesar Tondo[a]

[a] Departamento de Ciências dos Alimentos – Universidade Federal do Rio Grande do Sul, Instituto de Ciência e Tecnologia de Alimentos – Av. Bento Gonçalves, 9500 Prédio 43212 Agronomia CEP: 91505-970 – Porto Alegre – RS – Brasil, Telefone: +55 (51) 3308-6677

e-mail: susanaelias@gmail.com

e-mail: decolnutri@gmail.com

e-mail: tondo@ufrgs.br

Corresponding Author: Susana de Oliveira Elias

E-mail: susanaelias@gmail.com

Postal Address: Avenida Bento Gonçalves, 9500 – Campus do Vale – Prédio 43212 – Sala 205. CEP 91501-970 - Porto Alegre/RS/Brasil

ABSTRACT

Foodborne disease outbreaks linked to fruits and vegetables have been increasing in occurrence worldwide. Therefore, the aim of this study was to identify the reported foodborne outbreaks associated with fruits and vegetables consumption in Brazil from 2008 to 2014. The 30 produce-related outbreaks included 2926 cases of illnesses. Also, the total number of hospitalizations was 347, while no deaths were reported for that same period. Only bacterial pathogens were identified as etiological agents. Among these, *Salmonella* was the most frequent one (30% of outbreaks) followed by *Staphylococcus aureus* (23.3%), *Escherichia coli* (10%), *Bacillus cereus* (6.6%), and thermotolerant coliforms (3.3%), while non-determined etiological agents performed 26.6% of outbreaks. The most commonly food vehicles implicated in outbreaks were generically named as fruits and vegetables (46.6% of outbreaks). The term salad was used generic and specifically like salads (2 outbreaks), raw/cooked salads (4 outbreaks), vegetable salad, Tropical salad, Caesar salad, raw salad of cabbage and tomato. Only one outbreak was related exclusively with fruit (fruit pulp), other outbreaks were related with cooked carrot, lettuce, cucumber, watermelon/cabbage, and chard/beet. Contamination sources and issues related to the future control of produce–related foodborne disease outbreaks also are discussed.

KEYWORDS: produce, pathogens, *Salmonella*, foodborne illness, disease surveillance, notification

1. INTRODUCTION

The consumption of fruits and vegetables continues to rise worldwide due to healthy lifestyle recommendations (Callejón et al., 2015). In Brazil, for example, one of the best-known education tools designed to help people follow a healthy diet is the Food Guide for the Brazilian Population, which recommends eating 3 to 6 servings of fruits and vegetables per day (totaling 400 grams/day) (Brazil, 2008). However, these products can be contaminated at any point in the food chain. According to the World Health Organization (WHO), a hazard can exist in production systems due to several factors: postharvest practices, water, local environment, fertilizer, workers health and hygiene, and consumption patterns and practices (WHO, 2008). Besides that, most produce might promote the growth of many microorganisms. For example, Elias et al. (2018) demonstrated that *Salmonella* and *Escherichia coli* O157:H7 grows on lettuce exposed to isothermal and non-isothermal conditions between 5 to 42ºC.

In general, produce is consumed raw or with minimal processing, and because of that it is important to keep the microbial load of fresh produce as low as possible to prevent foodborne illnesses (Wadamori et al., 2016). Meanwhile, several pathogens have been isolated from fruits and vegetables worldwide. Ceuppens et al. (2014) and Rodrigues et al. (2014) isolated *Salmonella* from lettuce in Brazil. Also, Byrne et al. (2016) isolated *Listeria monocytogenes* in 2.22% of raw vegetables and 5.56% of ready-to-eat vegetables in Brazil. In this way, the rate of foodborne illness caused by the consumption of these products is increasing, representing a significant public health and financial issue all around the world (Yeni et al., 2016).

In Brazil, food safety is a responsibility shared at all levels of government. The agencies responsible for food control are the Ministry of Agriculture, Livestock and Food Supply and the Ministry of Health, through the National Health Surveillance Agency (ANVISA) (Gomes et al., 2013). In addition, public laboratories are responsible for conducting analyses of food available for consumption in routine sanitary surveillance programs, and of food suspected of involvement in foodborne disease outbreaks. All this information collected by these mentioned organizations may be used by health authorities and food industry professionals to target prevention efforts against pathogens and food that could cause outbreaks (Nunes et al., 2013). Therefore, the aim of this study was to identify the reported foodborne

outbreaks associated with consumption of fruits and vegetables in Brazil from 2008 to 2014.

2. MATERIALS AND METHODS

The available annual summary data on reported foodborne outbreaks in Brazil from 2008 to 2014 published by the National Sanitary Surveillance Agency (ANVISA) were examined. The total number of foodborne outbreaks reported annually included all outbreaks due to food or water.

Information obtained for each investigated outbreak included the notification date, the Brazilian state of occurrence, area (urban or countryside), place where food was eaten, location of food preparation, number of cases, and age and gender of the affected individuals, number of hospitalizations, number of deaths, main symptoms, mean of illness incubation period, outbreak probable cause, food involved, the etiological agent identified in the food sample and/or the biological sample, and the criterion used to conclude the outbreak. Some reports did not have completed all these information (unknown). In these case, the outbreak knowledge depended on the data available during this period. All analyses were conducted using Microsoft Excel™ 2016 (Microsoft, Redmond, WA).

3. RESULTS AND DISCUSSION

The total number of notified food and water-borne outbreaks in Brazil between 2008-2014 was 5138. In this period, the total number of foodborne outbreaks linked to fruit and vegetables was 30 representing approximately 0.6% of the total notified outbreaks and an yearly average of 4.3 outbreaks (Table 1). This average was similar to Canada and New Zealand, which reported yearly averages of 3 (2001-2009) and 5.5 (2002-2012), respectively, of foodborne outbreaks linked to produce contamination. In Japan and in USA were reported between 2002 and 2012 higher yearly averages: 7.7 and 56.9, respectively (Kozak, et al., 2013; Wadamori et al., 2017).

Pires et al. (2012) showed that 4.4% of foodborne outbreaks in Latin America between 2000-2010 were related to contaminated vegetables consumption. According to Wadamori et al. (2017) in the USA from 1998 to 2007, vegetables contributed to 33% (228 outbreaks) of outbreaks of foodborne illnesses, and about

50% (345 outbreaks) to portions of produce including salads. In New Zealand, 716 food poisoning outbreaks occurred in 2012, 13.3% of which were from leafy vegetables, 10% from root vegetables; 6.7% from fruits/nuts, and 3.3% from stalk vegetables (3.3%). Also, Greig & Ravel (2009) studied the food vehicle for foodborne outbreaks reported internationally between 1988 and 2007 based on available public reports and found that approximately 12% (498/4093) of these outbreaks were associated with produce.

These values were higher than those of Brazil (0.6%). An explanation is that in the majority of foodborne outbreaks in Brazil, it is not possible to identify the food related to the outbreak (66.4% the food source was ignored or inconclusive). When the food is identified, in the first place, comes the mixed food (8.6%) (Brazil, 2017[a]). The Table 1 shows in detail the data collected from the foodborne outbreaks between 2008-2014 related with produce in Brazil.

The notification date of Brazilian produce outbreaks from 2008 to 2014 was shown in the Figure 1-A that presents the frequency of outbreaks by year, while Figure 1-B by month. In both figures is not possible to observe a clear trend in the data. In 2008 and 2009, 17 outbreaks occurred (more than half of total), while in 2010 and 2012 only 1 outbreak in each year was registered. For the United States, the absolute number of outbreaks due to fresh produce ranged from 23 to 60 per year, also not showing a clear trend along 2004-2012. In fact, there were substantial increases in 2006 (57 outbreaks), 2008 (51 outbreaks), and 2011 (60 outbreaks) (CDC's database, 2016).

Meanwhile, for the European Union, the number of outbreaks oscillated between 10 and 42 during this period, highlighting increases in 2006 (29 outbreaks), 2009 (34 outbreaks), and 2010 (44 outbreaks). Hence, they also lack a clear tendency (EFSA Summary Reports, 2015). In relation to the months of outbreaks occurrence in Brazil, both in September (5 outbreaks) and in March (4) there is a predominance of outbreaks. In these months the season changes from winter to spring and summer to autumn, respectively. There was no reported outbreak in December (spring to summer) in this period of data analysis.

Figure 2 presents the Brazilian regions of foodborne outbreaks. In the Northeast, 12 foodborne outbreaks occurred from 2008 to 2014. This region, formed by 9 states, has the largest coastline in the country, the second largest population,

and faces periods of drought. The Southeast region presented 9 outbreaks. It is composed by 4 states, has the largest population in the country, besides being the most industrialized and economically developed. The North region was responsible for 6 outbreaks, it is formed by 7 states, being the largest region in territorial dimension, and the Amazon rainforest occupies this territory. The South region and the Central-West one presented 2 and 1 outbreaks, respectively, both are formed by 3 states, however in the Central-West there is the Federal District where is located the national capital of Brazil (IBGE, 2017). Besides this, 24 outbreaks occurred in urban areas, 1 in peri-urban (North), and 3 in countryside (South and Northeast) (Table 1).

The place where contaminated food was consumed is shown in Figure 3. Job refectory and restaurants/bakeries were the places with most occurrences (7 each). In second place, it was several (more than 1 local) with 5 outbreaks and in third place come the hospital/health units and residences (3 each). Other sites presented only 1 foodborne outbreak occurrence (church refectory, hawker, nursery/school, rest home, social events). In relation to location of food preparation, in general, the food was consumed in the place where it was prepared. In the case of several places of consume the locations that prepared the food were: industry (2), artisanal production, restaurant, snack bar/bakery/confectionery. Additionally, institutions and foodservices are considered main locations for foodborne outbreaks also in USA; and due to, current lifestyle, the number of people consuming food outside their homes are increasing, which could lead to increased exposure to pathogens associated with foodborne illnesses (Nsoesie et al., 2014).

In Brazil, all places that prepare and serve food, except at home, must follow the regulation RDC nº 216/2004 (Brazil, 2004). RDC nº 216/2004 establishes good practices procedures for food services in order to guarantee the hygienic and sanitary conditions of the prepared food. In relation to the industry, the RDC nº275/2002 that establishes standard operating procedures that contribute to guarantee hygienic-sanitary conditions necessary for the processing/industrialization of food, complementing the good manufacturing practices (Brazil, 2002). These regulations are capable to guarantee the food safety; however, they are not always followed, and the outbreak may happen.

The total number of cases was 2926, on average it had approximately 100

cases in each outbreak due to produce contamination between 2008 and 2014. In relation to the age group, no reports on diseased infants, with less than 1 year old, thought, in all other categories of age cases were reported. The age group from 20 to 49 years old had the most patients (Figure 4). This is the age range (20-49) of the more economically active population, which usually takes meals outside the home, which correlates with the fact that most outbreaks also occurred outside the home (Figure 3). Besides this most patients were male, but it is important to highlight that the identification of gender and age was made in only 24% (707) of the cases.

The most frequent symptoms reported in the studied outbreaks were diarrhea (29), vomit (26), abdominal pain (24) and nausea (23), while the less frequent were headache (14), fever (12), and neurological symptoms (2) (Figure 5). All these symptoms are related with foodborne disease, mainly the more cited. The average of illness incubation time was 10 h (1 h to 26 h). This period range can be associated with infections or intoxication caused by foodborne pathogens (Forsythe, 2013). Also, the total number of hospitalizations was 347, while the total number of obituaries was zero in this period (2008-2014).

In most of the outbreaks the food vehicle was generically indicated as fruits and vegetables (14). The term salad was used generically and specifically like salads (2), raw/cooked salads (4), vegetable salad, Tropical salad, Caesar salad, raw salad of cabbage and tomato. Only one outbreak was related exclusively with fruit (fruit pulp), other outbreaks were related with cooked carrot, lettuce, cucumber, watermelon/cabbage, and chard/beet (Table 1). This lack of a classification standard has been a major problem to associate the pathogens that cause gastrointestinal illnesses in the population and the specific food that serves as a vehicle for these infections. Besides, this lack of association between etiology and food vehicle, suggests that cross-contamination, environmental contamination and food handler contamination may be common along the food chain (Greig & Ravel 2009).

The Figure 6 shows the etiological agents of foodborne outbreaks linked to produce from 2008 to 2014. *Salmonella* was the pathogen that caused most of the outbreaks. Callejón et al. (2015) reported that *Salmonella* was the leading cause of multistate produce outbreaks in the United States and it was the pathogen involved in the majority of sprouts-associated outbreaks. Also, Yeni et al, (2016) mentioned that the rise in the notifications concerning pathogenic microorganisms in

fresh produce worldwide is mainly due to *Salmonella* spp in fruits and vegetables, herbs and spices. Lastly, Kozak et al (2013) reviewed the foodborne disease outbreaks linked to produce consumption in Canada from 2001 through 2009 and found that *Salmonella* was the most frequent agent (50% of outbreaks).

Many studies were conducted to find an explanation for the increase in *Salmonella* outbreaks. Currently, the internalization theory is gaining strength since several studies have indicated that *Salmonella* spp. is capable of replication to relatively high levels on or within the plant (Rossbach et al., 2017; Wiedemann et al., 2015; Deering et al., 2012). This internalization occurs in several leafy vegetables and fresh herbs; the level of internalization largely varies among plants and within the same species (Liu et al., 2017).

The other pathogens identified in the present study were *Staphylococcus aureus* (7 outbreaks), *Escherichia coli* (3 outbreaks), *Bacillus cereus* (2 outbreaks), and thermotolerant coliforms (1 outbreaks), while not determined etiological agent was applied by 8 outbreaks (Figure 6). In Brazil, until 2016 most foodborne outbreaks were caused by bacteria, mainly *Salmonella* spp., followed by *Escherichia coli*, *Staphylococcus aureus, Bacillus cereus,* and coliforms (Brazil, 2016). However, for approximately 70% of the registered outbreaks, the etiological agent of the disease could not be determined, mostly due to the lack of suspected food samples to investigate or because Brazilian official laboratories usually investigate only classical foodborne micro-organisms, but not emergent etiological agents (Brazil, 2016; Ritter & Tondo, 2014). Also, the absence of the etiologic agent can be the result of the use of antibiotics by the affected population, in the case of clinical analysis (Nunes et al., 2013).

The probable causes outbreaks were mostly inadequate storage and handling. Only one outbreak was related exclusively with improper raw material, and was caused by salad contaminated by *Escherichia coli*. Finally, the criterion used to conclude the outbreak were laboratory clinical analysis, laboratory food analysis or clinical epidemiological, which includes information on symptoms, dietary habits and existence of family members or other consumers with the same symptoms. The majority of outbreaks were concluded by food analysis (11) and clinical analysis/clinical epidemiological (10). However, one food analysis and five clinical analysis/clinical epidemiological were not able to identify the foodborne outbreak

etiological agent. The other outbreaks were completed by clinical analysis (6), and food analysis/clinical epidemiological (1). In two outbreaks these analyses were not possible to perform.

Foodborne diseases control and prevention has been improved considerably in Brazil in the last few decades. Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Control Points (HACCP) have been implemented in several food industries and food services and the Sanitary and Epidemiological Surveillance Services are now better structured and prepared than before (Ritter & Tondo 2014). However, as in many other countries, there is still a subnotification of foodborne diseases and lack of more complete official epidemiological data (Gomes et al., 2013). The number of food disease outbreaks reported in Brazil is most likely underestimated, for a variety of reasons reported outbreaks represent only a small portion of all actual outbreaks. According to do Carmo et al. (2005), over 3 million hospitalizations due to foodborne diseases occurred in the country from 1999 to 2004, and 25,281 fatalities from 1999 to 2002. Most of the outbreaks involved in these cases were probably never investigated or reported.

According to Kozak et al. (2013) an estimated of 11 million episodes of foodborne disease occur annually in Canada; for every case of enteric illness reported, 313 to 347 cases go unreported. Also, in the United States, although there are several surveillance systems for foodborne illnesses at the local, state and territorial levels, these systems capture only a fraction of the foodborne illness burden mainly due to few affected individuals seeking medical care and lack of reporting to appropriate authorities (McCabe-Sellers & Beattie, 2004; Nsoesie et al., 2014).

Besides the subnotification, the consumption of fruits and vegetables by Brazilian families, regardless of the income range, is low, ranging from 3% to 4%, between 1974 and 2003 (Brazil, 2008), which may contribute to the low number of outbreaks due to ingestion of this type of food. Thus, to attain the recommended minimum consumption, the average current consumption of the Brazilian population of fruits and vegetables should increase by at least 3 times. However, Brazilians have a relatively healthy diet, the Family Budgets Survey (Pesquisa de Orçamentos Familiares - POF), conducted by the IBGE between May 2008 and May 2009, shows that fresh or minimally processed foods and culinary preparations made with these

foods still correspond, in terms of the total calories consumed, to almost two thirds of the food intakes of Brazilians (Brazil, 2011).

As the world trend is to increase the consumption of fruits and vegetables, it is important to adopt control measures, such as good agricultural practices and good manufacturing practices. In Rio Grande do Sul, the southernmost state of Brazil, there is a regulation Portaria Nº 90/2017 that disposes the Technical Regulation of Good Manufacturing Practices and Standard Operating Procedures for the industrialization of minimally processed fruits and vegetables and provides the Checklist of Good Manufacturing Practices in Producer/Industrializer Establishments of minimally processed fruits and vegetables (Brazil, 2017[b]). Besides this, although there is no possible way to completely eliminate microbial foodborne pathogens from fresh produce, there are methods available to reduce pathogens from fresh produce: physical (brushing, rinsing), chemical (hypochlorite, acidified sodium chlorite, chlorine dioxide, trisodium phosphate, quaternary ammonium compounds, acids, hydrogen peroxide, and ozone), and biological (using microbial antagonists as a biocontrol agent), it is essential to use these methods to guarantee the food safety when consuming fresh produce (Yeni et al., 2016).

This study identified important data gaps in the region and highlighted the importance of the implementation of effective food safety surveillance programs that allow the identification of food safety problems and investigation of sources of disease. Efforts should be made to improve the outbreak notification and investigation system and the laboratory capabilities so that biological and food samples may be collected in a timely and correct manner to identify the etiological agent. Also, quantitative data on microbiological hazards in foods are needed if risk assessments programs are to be implemented.

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Table 1: Summary of outbreaks associated with vegetables and fruits in Brazil, 2008–2014.

Notification date	Brazilian state of occurrence	Place where food was consumed	Location of food preparation	Nº of cases	Age	Sex	Number of hospitali-zations	Area	Main symptoms	Median of illness incubation period (h)	Outbreak probable cause	Food involved	Etiological agent identified	Criterion used to conclude the outbreak
3/9/2008	MG	Residence	Residence	3	20/ 50+	2M,1F	0	Urban area	N, D, A, F	ND	Inadequate conservation	Fruits and vegetable	*Salmonella*	Clinical analysis
3/27/2008	RS	Restaurant/ Bakery	Restaurant	11	20-49	11M, 0F	0	Countryside	N, V, D, A	11	ND	Fruits and vegetable	*Salmonella*	Food analysis
5/16/2008	PR	Hospital / Health Unit	Restaurant	93	20/ 50+	16M, 6F	0	Urban area	N, V, D, A	5	ND	Fruits and vegetable	*Bacillus cereus*	Clinical analysis; epidemiological
6/10/2008	MT	Social Events	Residence	200	5-50+	11M, 19F	0	Urban area	N, V, D, H, A, F	6	Inadequate conservation and handling	Tropical salad	ND	Clinical analysis; epidemiological
9/18/2008	PA	Residence	Residence	16	5-50+	3M, 5F	0	Urban area	V, D, A, F	6	Inadequate conservation and handling	Fruits and vegetable	ND	Inconclusive
10/3/2008	PE	Restaurant/ Bakery	Restaurant	14	10/ 50+	4M, 2F	0	Countryside	N, V, D, H, A	ND	Inadequate conservation and handling	Fruits and vegetable	*Escherichia coli*	Food analysis
11/19/2008	MA	Several	Artisanal production	43	1/ 50+	17M, 24F	10	Urban area	N, V, D, H, A, NS, F	ND	Inadequate conservation	Fruits and vegetable	ND	Clinical analysis; epidemiological
11/24/2008	MA	Several	Industry	43	1/ 50+	17M, 24F	10	Urban area	N, V, D, H, A, NS, F	17	Inadequate handling	Fruits and vegetable	*Salmonella*	Clinical analysis
3/20/2009	TO	Hospital / Health Unit	Hospital / Health Unit	123	20-49	1M, 10F	0	Urban area	N, V, D, H, A	ND	Inadequate handling	Raw / cooked salads	*Staplylococcus aureos*	Clinical analysis; Food analysis
4/11/2009	RJ	Restaurant/ Bakery	Restaurant	3	1/ 50+	2M, 1F	0	Urban area	D	ND	ND	Fruits and vegetable	*Staplylococcus aureus*	Clinical analysis; epidemiological
4/14/2009	RJ	Restaurant/ Bakery	Restaurant	2	20-49	1M, 1F	0	Urban area	V, D	ND	Inadequate handling	Caesar salad	*Staphylococus aureus*	Clinical analysis; epidemiological
5/14/2009	PE	Hospital / Health Unit	Hospital / Health Unit	41	ND	0M, 0F	0	Urban area	N, V, D	ND	Inadequate conservation and handling	Fruits and vegetable	ND	Clinical analysis; epidemiological
7/10/2009	AL	Restaurant/	Restaurant	21	20-	9M,	3	Urban area	D, A	9	Inadequate	Fruits and	*Bacillus cereus*	Clinical

		Bakery			49	1F					conservation	vegetable		analysis; epidemiological
7/10/2009	MG	Job refectory	Refectory	550	10-49	22M, 0F	0	Urban area	N, V, D, H, A, F	15	Inadequate handling	Fruits and vegetable	*Staphylococcus aureus*	Food analysis
8/6/2009	TO	Residence	Residence	4	1-49	3M, 1F	0	Urban area	N, V, A	1	ND	Fruits and vegetable	ND	Clinical analysis; epidemiological
8/10/2009	TO	Several	Restaurant	180	1/ 50+	8M, 42F	1	Urban area	N, V, D, H, A, F	20	Inadequate conservation and handling	Fruits and vegetable	*Salmonella*	Clinical analysis
9/14/2009	TO	Hawker	Hawker	2	20/ 50+	1M, 1F	0	Urban area	N, V, D, A	1	Inadequate conservation and handling	Fruits and vegetable	ND	Clinical analysis; epidemiological
9/22/2010	BA	Church refectory	Church kitchen	49	20-49	7M, 36F	37	Urban area	N, V, D, H, A, F	8	ND	Raw salad of Cabbage and tomato	Thermotolerant Coliform	Clinical analysis
2/10/2011	PE	Restaurant/ Bakery	Restaurant	400	1-49	3M, 0F	2	Urban area	N, V, D	ND	Inadequate conservation and handling	Vegetable salad	*Escherichia coli*	Food analysis
6/8/2011	PA	Job refectory	Restaurant	186	20-49	151M, 0F	186	Peri-urban	N, V, D, A	ND	ND	Salads	*Staphylococcus aureus*	Clinical analysis; epidemiological
6/26/2012	RJ	Nursery / School	Nursery / School	3	1-4	2M, 1F	3	Urban area	V, D	6	ND	Cooked carrot	*Staphylococcus aureus*	Clinical analysis
2/15/2013	MG	Several	Snack Bar / Bakery / Confectionery	90	5/ 50+	17M, 21F	90	Urban area	N, V, D, H, A, F	26	ND	Lettuce	*Salmonella Enteritidis*	Food analysis
2/28/2013	MG	Rest home	Rest home kitchen	10	20/ 50+	6M, 4F	5	Urban area	N, V, D, F	ND	ND	Fruit pulp	*Salmonella Enteritidis*	Clinical analysis
3/22/2013	PE	Job refectory	Refectory	472	10/ 50+	41M, 0F		ND	N, V, D, A	ND	Inadequate conservation and handling	cooked salads	*Staphylococcus aureus*	Food analysis
9/27/2013	PE	Job refectory	Residence	24	10/ 49	3M, 0F	0	Urban area	N, V, D, A	12	Improper raw material	Salad	*Escherichia coli*	Food analysis
1/16/2014	MG	Restaurant/ Bakery	Restaurant	95	20/ 50+	8M, 2F	0	ND	N, V, D, H, A	12	Inadequate handling	Watermelon and cabbage	ND	Inconclusive
1/21/2014	PE	Job refectory	Restaurant	65	20-49	7M, 0F	0	Countryside	N, D, H, A	ND	Inadequate conservation	Cucumber	*Salmonella*	Food analysis
5/15/2014	RJ	Job	Restaurant	55	20/	55M,	0	Urban area	N, V, D,	9	Inadequate	Chard and	ND	Food analysis

		refectory			50+	0F			H, A, F		handling	beet		
9/18/2014	PE	Several	Industry	90	10/ 50+	39M, 0F	0	Urban area	N, V, D, H, A, F	11	Inadequate handling	Raw salad	*Salmonella*	Food analysis
10/17/2014	PE	Job refectory	Industry	38	20/ 50+	37M, 1F	0	Urban area	N, V, D, H, A	ND	Improper raw material, inadequate handling	Raw salad	*Salmonella*	Food analysis

Nº = number
M = male, F = female
ND = not determined
Main symptoms: N = nausea, V = vomit, D = diarrhea, H = headache, A = abdominal pain, NS = neurological symptoms, F = fever

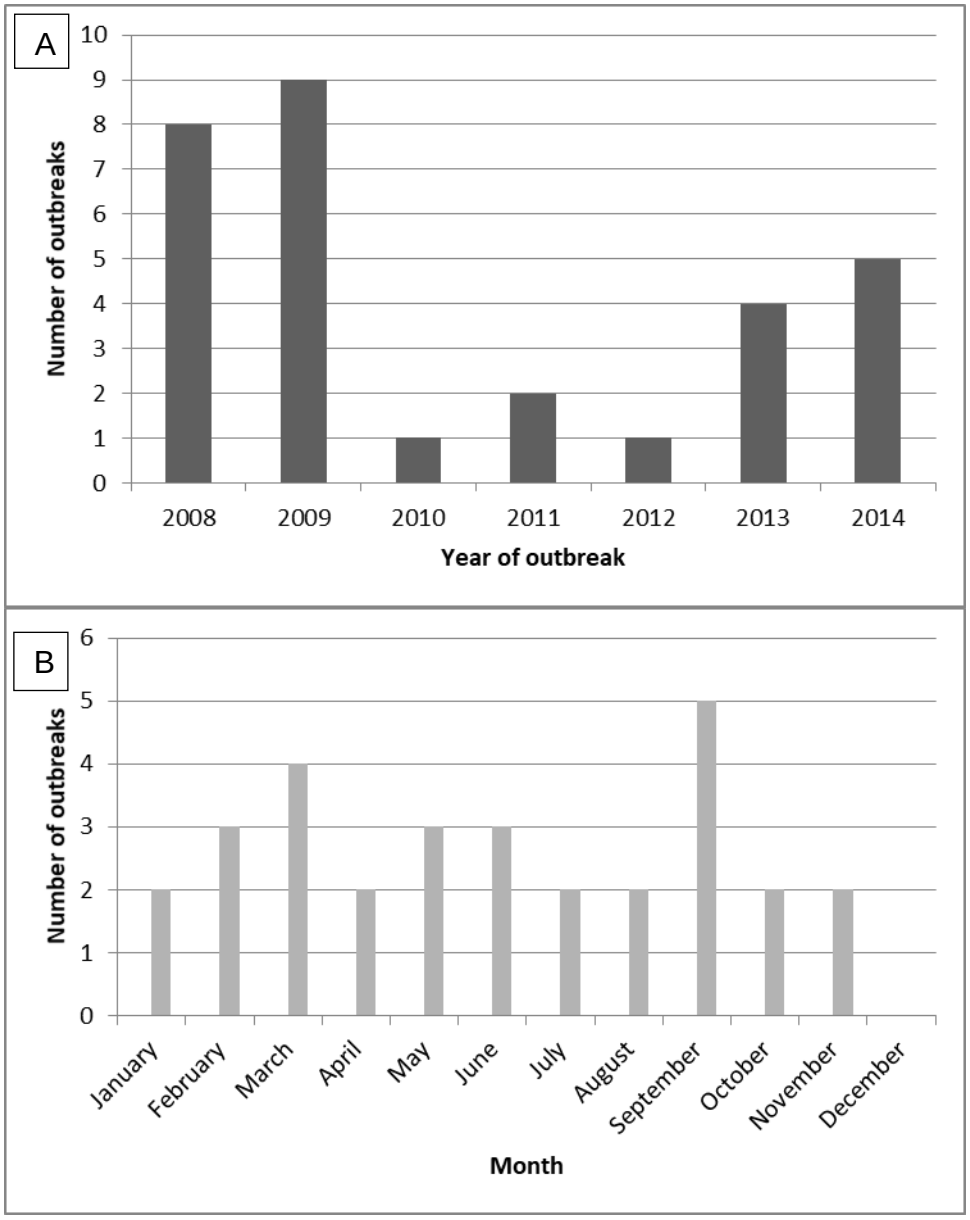

Figure 1: Frequency of foodborne outbreaks linked to produce contamination between 2008 and 2014; A = year and B = month.

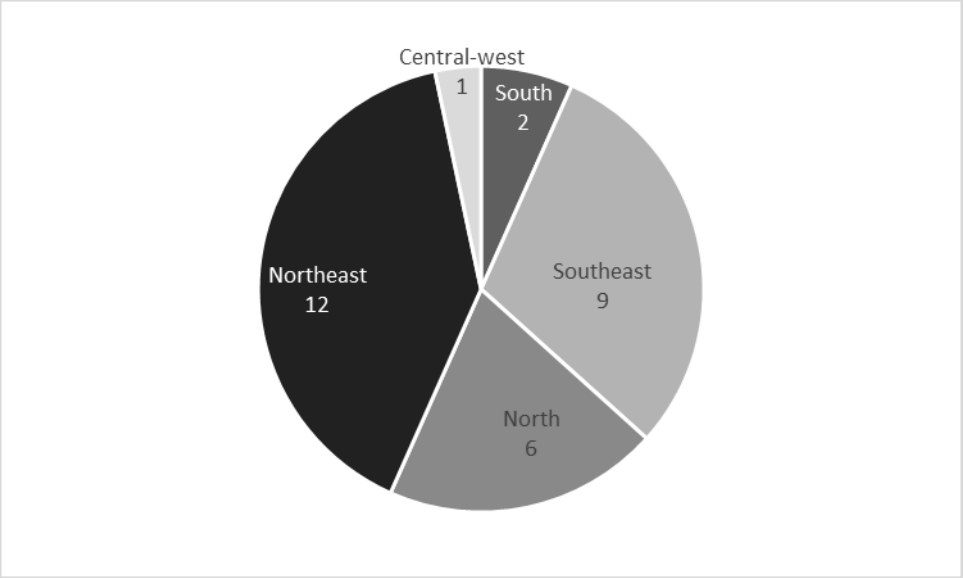

Figure 2: Brazilian region of foodborne outbreak occurrence between 2008 and 2014.

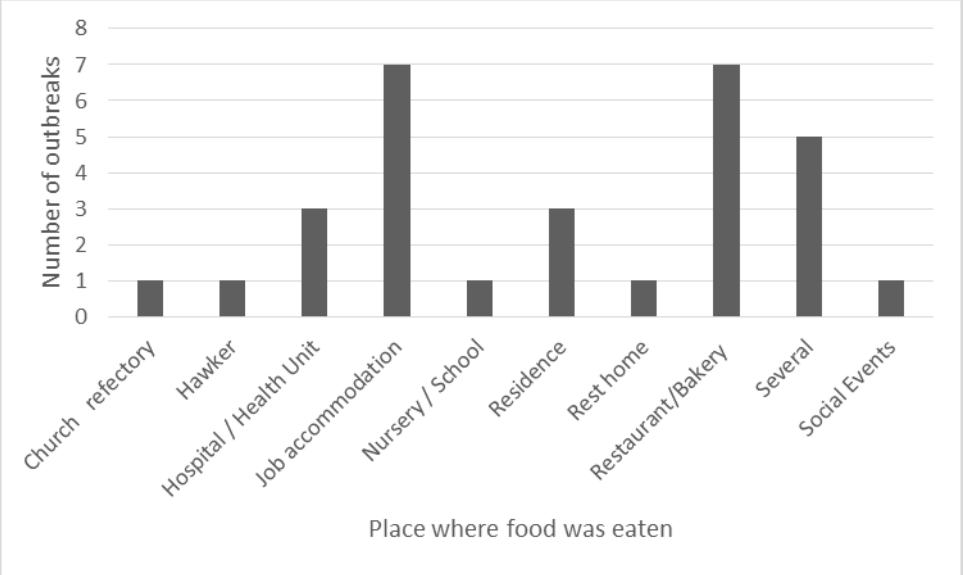

Figure 3: Frequency of place where food was eaten, and the number of foodborne outbreaks linked to produce contamination between 2008 and 2014.

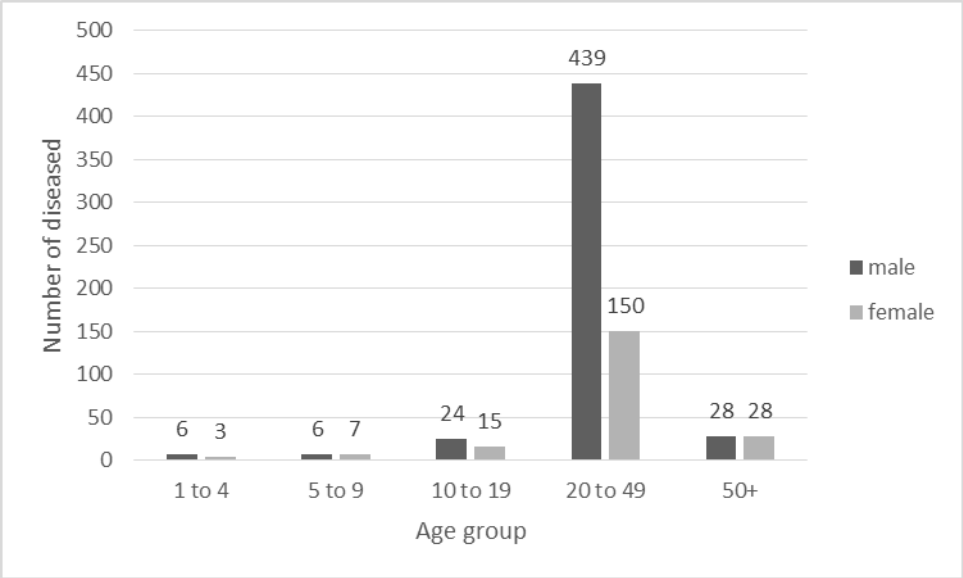

Figure 4: Frequency of age group and gender of diseased people by foodborne outbreaks linked to produce contamination between 2008 and 2014.

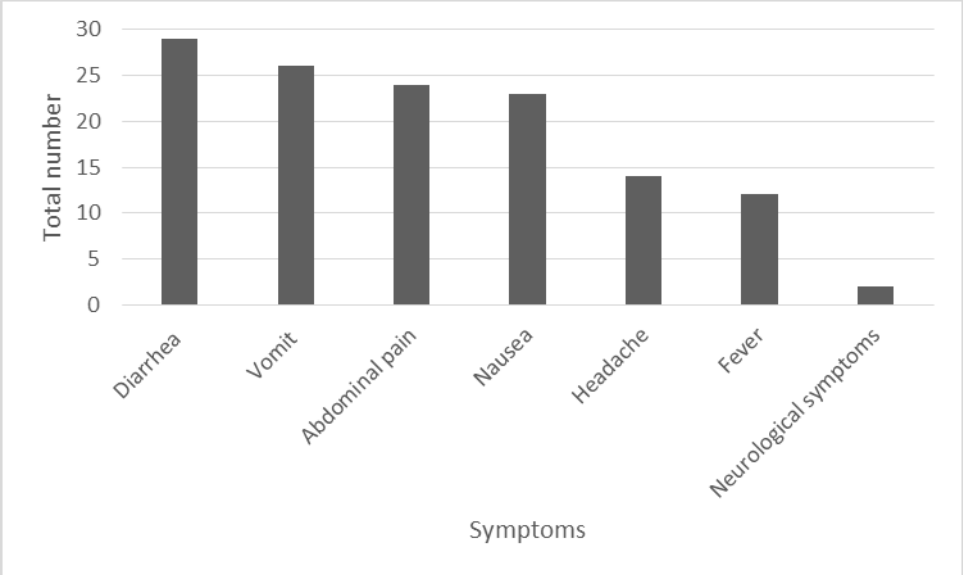

Figure 5: Frequency of symptoms of foodborne outbreaks linked to produce contamination between 2008 and 2014.

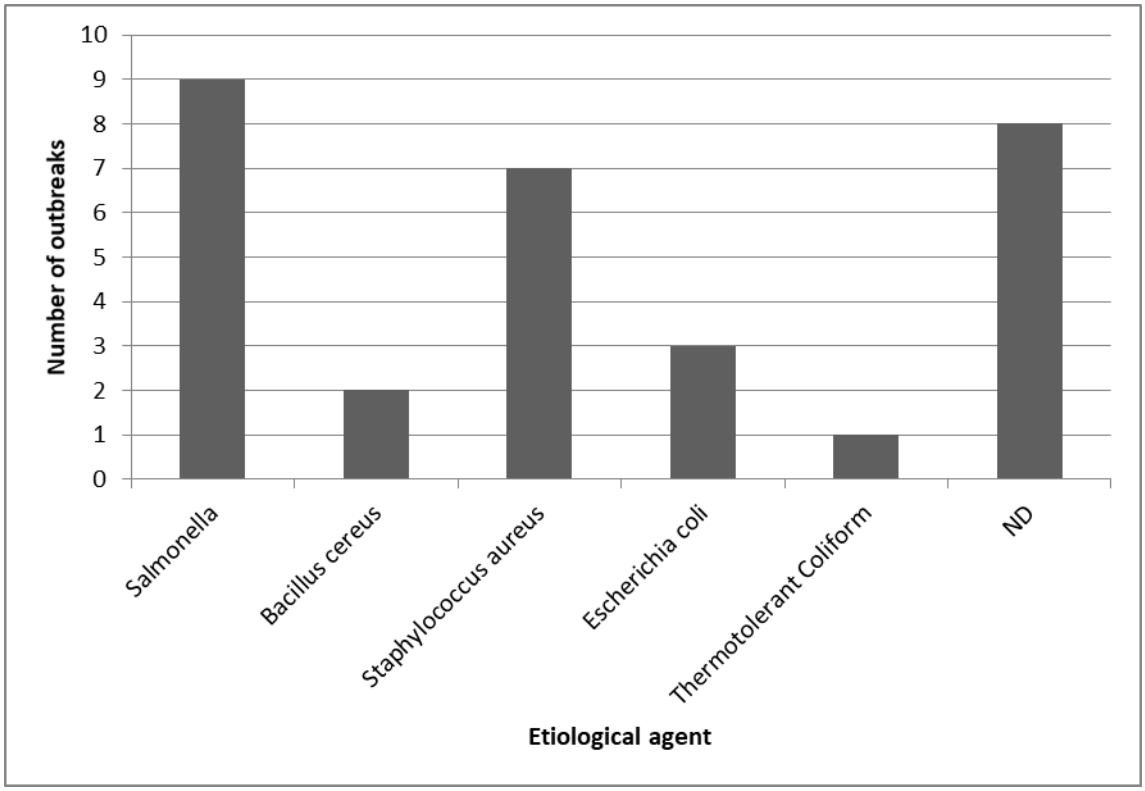

Figure 6: Foodborne pathogens linked to outbreaks caused by contaminated produce in the Brazil between 2008 and 2014. ND = not determined.

4.2. Artigo 2

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Assessment of *Salmonella* spp. and *Escherichia coli* O157:H7 growth on lettuce exposed to isothermal and non-isothermal conditions

Susana de Oliveira Elias[a], Tiago Baptista Noronha[b], Eduardo Cesar Tondo[c]

[a] Departamento de Ciências dos Alimentos – Universidade Federal do Rio Grande do Sul, Instituto de Ciência e Tecnologia de Alimentos – Av. Bento Gonçalves, 9500 Prédio 43212 Agronomia CEP: 91505-970 – Porto Alegre – RS – Brasil, Telefone: +55 (51) 3308-6677 e-mail: susanaelias@gmail.com

[b] Departamento de Ensino, Pesquisa e Extensão – Instituto Federal de Educação, Ciência e Tecnologia Sul-rio-grandense – Rua General Balbão, 81 – CEP 96745-000 – Charqueadas – RS – Brasil, Telefone: +55 (51) 3658-3775 e-mail: tiagonoronha@charqueadas.ifsul.edu.br

[c] Departamento de Ciências dos Alimentos, UFRGS/ICTA, Av. Bento Gonçalves, 9500 Prédio 43212 Agronomia CEP: 91505-970 – Porto Alegre/RS/Brasil, Telefone: +55 (51) 3308-6677 e-mail: tondo@ufrgs.br

Corresponding Author: Susana de Oliveira Elias

E-mail: susanaelias@gmail.com

Postal Address: Avenida Bento Gonçalves, 9500 – Campus do Vale – Prédio 43212 – Sala 205. CEP 91501-970 - Porto Alegre/RS/Brasil

ABSTRACT – This study aimed to assess the growth of *Salmonella* and *Escherichia coli* O157:H7 on lettuce exposed to isothermal and non-isothermal conditions. Pathogens were inoculated on lettuce separately and stored under isothermal condition at 5ºC, 10ºC, 25ºC, 37ºC for both bacteria, at 40°C for *Salmonella* and 42ºC for *E. coli* O157:H7. Growth curves were built by fitting the data to the Baranyi's DMFit, generating R^2 values greater than 0.92 for primary models. Secondary models were fitted with Ratkowsky equations, generating R^2 values higher than 0.91 and RMSE lower than 0.1. Experimental data showed that both bacteria could grow at all temperatures. Also, the growth of both pathogens under non-isothermal conditions was studied simulating temperatures found from harvest to supermarkets in Brazil. Models were analysed by R^2, RMSE, bias factor (Bf) and accuracy factor (Af). *Salmonella* and *E. coli* O157:H7 were able to grow in this temperature profile and the models could predict the behavior of these microorganisms on lettuce under isothermal and non-isothermal conditions. Based on the results, a negligible growth time (ς) was proposed to provide the time which lettuce could be exposed to a specific temperature and do not present an expressive growth of bacteria. The ς was developed based on Baranyi's primary model equation and on growth potential concept. ς is the value of lag phase added of the time necessary to population grow 0.5 log CFU/g. The ς of lettuce exposed to 37ºC was 1.3 h, while at 5°C was 3.3 days.

KEYWORDS: leafy greens; predictive modeling; negligible growth time (ς); temperature; vegetable; microbial pathogen.

1. INTRODUCTION

The World Health Organization reported that the low intake of fruit and vegetables is one of the major risk factors to account for much of the morbidity and mortality for noncommunicable diseases worldwide (WHO, 2003). Different strategies have been adopted to increase vegetable consumption in many countries, including Brazil. For example, Brazilian government launched the Resolution 408/2008 promoting health nutrition, based on agricultural products preferentially produced by family agriculture, which included an affordable supply of fruits and vegetables for population (Brazil, 2008).

Besides this, the global production of fruit and vegetables grew by 94% from 1980 to 2004 (Olaimat and Holley, 2012). Lettuce (*Lactuca sativa*) is the most produced and consumed leafy green globally (Paulsen and Andersen 2016). In Brazil, this leafy vegetable is the most consumed, being responsible for approximately 40 % of the total volume traded in fresh produce supply companies (Ceuppens et al., 2014). This high consumption is attributed to the continuous availability, cost, and nutritional factors (Rodrigues et al. 2014). However, independently the way lettuce is prepared and served, it is always consumed raw, being possibly contaminated by various pathogens. Further, washing of lettuces generally is able to inactivate only 1 to 2 log CFU/g of microorganism, what may be not sufficient to reduce pathogen population until safe levels. Moreover, in some cases, cross-contamination can occur during washing step (Jensen et al., 2013; Jensen et al., 2015; Prado-Silva et al.,2015).

Currently, foodborne pathogens are a major concern in the global food market. Simultaneously, the foodborne outbreaks associated with consumption of fresh produce have increased worldwide (Olaimat and Holley, 2012). The human pathogens involved in these outbreaks include bacteria, viruses and parasites (Callejón et al., 2015; Jung et al., 2014). Microbial contamination of lettuce can occur throughout the production chain, transportation and consumption, depending on the good agricultural practices, good hygiene practices, control of temperature, and consumers habits (Sant'Ana et al., 2014). Consequently, a high percentage of the population can be exposed to foodborne pathogens due to the intake of these products, if appropriate preventive measures were not applied.

As previously stated, the processing applied on fresh produce do not complete eliminate the microbial contamination. Then to maintain the safety of leafy produce it is essential to control the time and temperature to avoid the growth of pathogens. The storage time and temperatures of lettuce before eating are highly variable, depending on availability of equipment, cultural habits and environmental conditions. In Brazil, normally, lettuces are not kept under refrigeration after harvest until consumption (Ascal and Tondo, 2015) and environmental temperatures can easily exceed 40 ^{o}C. At the same time, the recommended temperature for storage of processed fresh produce in Brazilian food services is below 5 °C (Brazil, 2004). However, this condition is hardly reached and maintained during distribution in supermarkets or food services, increasing the risk of microbial growth (Ascal and Tondo, 2015; Koseki and Isobe, 2005; McKellar et al. 2013). Then, in the present study, in order to assess the safety of lettuces, we firstly identified the main pathogens involved in foodborne diseases vehiculated by leafy vegetables worldwide. Secondly, growth models were built to simulate and predict the behavior of the most important pathogens on lettuce exposed to isothermal and non-isothermal conditions based on time and temperature scenarios of lettuce chain found in Brazil. Finally, a parameter was proposed in order to provide the time which lettuce could be exposed to a certain temperature and it would still be considered safe.

2. MATERIALS AND METHODS

2.1. Pathogens selection

The identification of pathogens involved in foodborne diseases with vegetables was carried out using scientific literature. Literature searches were performed using PubMed database to identify potentially relevant publications, prioritizing peer-reviewed journals in English. The keywords included in the literature search were: lettuce, leafy vegetables, leafy greens, salad and foodborne outbreaks. The published studies dates were between January 1, 2000 and January 1, 2016.

The criteria considered for the literature search of the pathogens on lettuce consisted of occurrence of reported foodborne outbreaks associated with fresh leafy vegetables consumption. About one hundred scientific articles were found in the database. Only 36 studies were chosen to select the pathogens, since they

obeyed the prerequisites established previously. The identified microorganisms involved in these outbreaks were *Bacillus* spp., *Campylobacter* spp., *Clostridium perfringens*, *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, Hepatitis A virus, Norovirus, Norwalk, *Cryptosporidium parvum*, *Cyclospora* spp., *Enterocytozoon* spp. and *Giardia* spp. Viruses and parasites were not considered since they cannot grow on food.

For this study, *E*. *coli* O157:H7 and *Salmonella* spp. were selected to perform in-deep studies. Once these pathogens were the most involved bacteria in outbreaks associated with leafy vegetables (Berger et al., 2010; Buss et al., 2016; Callejón et al., 2015; Doyle and Erickson, 2008; Duedu et al., 2014; Eraky et al., 2014; Jung et al., 2014; Loutreul et al., 2014; Mohamed et al., 2016; Olaimat, and Holley, 2012; Pérez-Rodríguez et al., 2014; Sant'Ana et al., 2014; Yoon et al., 2009).

2.2. Lettuce

Crisp lettuce (*L. sativa* var. *crispa*), the most consumed one in Brazil (Sala and da Costa, 2012) was purchased at a local supermarket of Porto Alegre city, Brazil. The outer leaves, the core, and all visible dirtiness were removed from lettuce and discarded before experiments. The intact leaves were cut into 4 cm X 4 cm pieces, using a sterile surgical knife and a disinfected metallic template. Each 10 g of leaves were placed into sterile plastic bags separately.

2.3. Pathogens inoculation on lettuce

Scientific literature analysis identified *Salmonella* spp. and *E. coli* O157:H7 as the pathogens more related to foodborne diseases involving leafy vegetables. Based on these, both microorganisms were used to study the microbial behavior on lettuces exposed to different temperatures. Before the inoculation, lettuce was tested for the presence of *Salmonella* spp. and of *E. coli* O157:H7 following ISO 6579:2002 and ISO 16654:2001, respectively, in order to ensure these microorganisms were absent.

The *Salmonella* spp. pool was composed by six strains isolated from food in Brazil: *Salmonella* Enteritidis SE86, *Salmonella* Typhimurium L12031, *Salmonella* Typhimurium 1T2, *Salmonella* Anatum, *Salmonella* Newport and *Salmonella* Saint Paul. The *E. coli* O157:H7 pool included four strains isolated from different sources, two were isolated from bovines in the States of Rio Grande do Sul and São Paulo (Brazil), and the other two were isolated from manure and from lettuce washing

water, respectively, in Porto Alegre city (Brazil).

Each *Salmonella* and *E. coli* O157:H7 strain was grown separately in 5 mL of Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, UK), at 37 °C for 24 h; this step was repeated once. Each microorganism was cultivated again in BHI broth, was centrifuged at 4 °C, for 10 min, at 2810 *g*, the supernatants were discharged and the pellets were washed with 0.1 % peptone water (Oxoid, Basingstoke, UK). This procedure was repeated 3 times and then, after that, cells were re-suspended with 0.1 % peptone water. All the *Salmonella* and *E. coli* O157:H7 strains were mixed in two different pools.

The final cell concentration of 10^8 CFU/mL was adjusted through optical density 0.5 (OD_{630nm}), using Ultrospec™ 3100 *pro* (Amersham Biosciences, UK) and confirmed by plating on BHI agar (Oxoid, Basingstoke, UK). Decimal serial dilutions using 0.1 % peptone water were prepared, and a *Salmonella* pool was inoculated on lettuce (1 mL in each bag) in order to obtain a final cell concentration of nearly 10^2 CFU/g. The *E. coli* O157:H7 pool was inoculated on lettuce (1 mL in each bag) in order to reach a final concentration of nearly 10^4 CFU/g, a higher inoculum was used following Koseki et al. (2005) and because this bacterium was described as fastidious (Paula et al., 2014). All samples were gently massaged by hand to ensure that bacteria were evenly distributed in the food matrix.

2.4. Storage under isothermal conditions and enumeration of pathogens on lettuce

Inoculated portions (10 g) of lettuce were stored at 5 °C, 10 °C, 25 °C and 37 °C for both bacteria, and at 40 °C for *Salmonella* and 42 ºC only for *E. coli* O157:H7. The period of incubation was finished when the bacteria reached the stationary phase. The temperatures were chosen because they simulate the following scenarios: recommended temperature of Brazilian regulation for storage and distribution of processed vegetable produce (<5 °C), abuse refrigeration temperature (10 °C), common environmental temperature (25 °C) in Brazil, the ideal growth temperature (37 °C) for the bacteria (Smet et al., 2015) and summer temperature (≥40 ºC) in Brazil.

Sampling was carried out at varying time intervals, depending on the storage temperature. At each time point, 10 g of sample were homogenized with 90 mL of 0.1 % peptone water, using a stomacher (Seward, London, UK) for 2 min.

Afterwards, the sample was decimal diluted in 0.1 % peptone water. Then, aliquots were plated onto Xylose Lysine Deoxycholate agar (XLD, Oxoid, Basingstoke, UK) for *Salmonella;* for *E. coli* O157:H7 was used sorbitol MacConkey agar (SMAC, Oxoid, Basingstoke, UK) supplemented with Cefixime-Tellurite (CT) selective supplement (Oxoid, Basingstoke, UK), all plates were incubated at 37 °C for 24 h. In the case of pathogen enumeration, at least three typical colonies per sampling point were selected for confirmation using *S. enterica* polyvalent serum against O antigen (Probac, São Paulo, BR) and *E. coli* O157 serum (Probac, São Paulo, BR). The lower enumeration limit of the method used was 10 CFU/g. All the bacterial counts were carried out in triplicate. The experiments were repeated at least three times and the results were expressed as log CFU/g.

2.5. Modeling of pathogens growth on lettuce under isothermal conditions

The predictive primary model described by Baranyi and Roberts (1994) was used in order to calculate the growth kinetic parameters of pathogens on lettuce. The growth curves for each temperature and pathogen were built separately by fitting the experimental data to the DMFit tool in ComBase software (browser.combase.cc/DMFit.aspx). The following parameters were obtained: maximum growth rate (μ_{max}), lag time (λ), and maximum population density (N_{max}) using the Baranyi and Roberts (1994) model (Equation 1-3)

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

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

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

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

The predictive secondary model was built using the square root equation described by Ratkowsky et al. (1982) to describe μ as a function of storage

temperature (Equation 4). The Equation 5 (Ratkowsky et al., 1983) was used to model growth rate of *Salmonella* above the optimum growth temperature.

$$\sqrt{\mu} = a(T - T_0) \quad (4)$$

$$\sqrt{\mu} = a(T - T_0)\{1 - \exp[c(T - T_{max})]\} \quad (5)$$

where: $\sqrt{\mu}$ is the square root of maximum growth rate, a is the regression coefficient of the square root of growth rate constant versus temperatures below the optimal temperature, T (°C) is temperature and T_0 (°C) is a conceptual minimum temperature for microbial growth. T_{max} is the maximum temperature for microbial growth, whereas c is a parameter to enable the model to fit the data for temperatures above the optimal temperature.

2.6. Storage under non-isothermal conditions

2.6.1 Use of secondary model to predict pathogens growth on the worst scenario of lettuce distribution

The prediction capability of secondary model was evaluated using the worst scenario created on the information provided by Ascal and Tondo (2015). The scenario was construct considering the steps of lettuce distribution from harvest to retail and binomial time-temperature abuses. The non-isothermal conditions evaluated were: I) 30 °C for 3 h; II) 25 °C for 9 h; III) 35 ºC for 2 h; IV) 15 ºC for 8 h and V) 20 °C for 8 h. The first condition (I) simulated the summer temperature during lettuce harvest. The condition II was used in order to simulate storage in the farm at room temperature. The third period (III) was set to simulate transportation from farm to distribution centers, during a summer day. The condition IV was used to simulate storage inside distribution centers, while condition V simulated lettuce exposure on retail.

Lettuce was inoculated with *Salmonella* spp. and *E. coli* O157:H7 pools as described in subsection 2.3. Then the contaminated lettuce was stored under these non-isothermal conditions and counts were done as described in subsection 2.4. Sampling was carried out during the whole storage period in programed intervals. At the same time points in which samples were collected for pathogen enumeration, temperature of lettuce was also recorded.

2.7. Modeling the growth of pathogens on lettuce under non-isothermal conditions

Salmonella and *E. coli* O157:H7 growth under non-isothermal conditions were modeled through the application of a set of differential equations as described by Baranyi et al. (1995) (Equation 6 and 7):

$$\frac{dq}{dt} = \mu_{max} q, \qquad q(0) = q_0 \tag{6}$$

$$\frac{dx}{dt} = \mu_{max} \frac{q}{1+q} \left(1 - \frac{x}{x_{max}}\right) x, \qquad x(0) = x_0 \tag{7}$$

where: μ_{max} (1/h) is the maximum growth rate, x_{max} is the maximum population density, x_0 is the initial population concentration, $x(t)$ is the natural logarithm of cell concentration, q_0 and q (dimensionless) are the amounts related to the critical compounds needed for growth and depict the physiological state of the cells in the instant of inoculation and later period, respectively.

The model for growth rate shown represented by Equation 4 and 5 was substituted in the Equation 6 and 7, with all parameters of the differential equations being temperature dependent. The differential equation was solved through the Runge-Kutta (2,3) pair of Bogacki and Shampine method available in MATLAB® version R2016b (Mathworks, Natick, USA). This allowed to be estimated *Salmonella* and *E. coli* O157:H7 population concentration under non-isothermal storage of lettuce.

2.8. Statistical analysis applied to isothermal and non-isothermal models

Measures of coefficient of determination (R^2) and Root Mean Square Error (RMSE) were used to evaluate the performance of the isothermal models built in this study. The R^2 is generally considered as an overall measure of the prediction calculated by developed model, it could assume a value between 0 to 1, and a value equals 1 for this measure indicate the best performance of the model (Wang et al., 2013). However, this is only one criterion to assess the goodness of fit of the model. If R^2 is low (<0.7), the mathematical model is not good; on the other hand, if R^2 is high (>0.9), it means that other criteria should be analyzed (Granato et al., 2014). So, it is prudent to check RMSE values when evaluating R^2 (Nunes et al., 2015). The RMSE is used to offer a standard measurement of goodness-of-fit of a model to the data used to produce it. A RMSE equals 0 indicates the best possible fit between

predicted and observed values (Wang et al., 2013).

Bias and Accuracy factors jointly with RMSE were calculated to evaluate the predictive capacity of the proposed non-isothermal models. Equations 8 and 9 represents Bias and Accuracy factors, respectively, where $\mu_{max\ predicted}$ and $\mu_{max\ observed}$ are the values of growth rate estimated by the predictive model and obtained in the experiments, correspondently, and *n* is the number of data points used for the calculations of both factors (Wang et al., 2013). The closer to 1 the better the model's performance, considering both factors (Ross, 1996).

$$Bias\ factor = 10^{(\sum \log(\mu_{\max\ predicted}/\mu_{\max\ observed})/n)} \quad (8)$$

$$Accuracy\ factor = 10^{(\sum |\log(\mu_{\max\ predicted}/\mu_{\max\ observed})|/n)} \quad (9)$$

where: $\mu_{\max\ predicted}$ (1/h) is the maximum growth rate predicted, $\mu_{\max\ observed}$ (1/h) is the maximum growth rate observed, n is the number of observations.

In addition, ComBase Predictor (http://modelling.combase.cc) and Pathogen Modeling Program (PMP) version 8.0 (download from https://pmp.errc.ars.usda.gov/PMPDownload/PMP80Setup.exe) were used to estimate the growth parameters of *E. coli* and *Salmonella* with the similar intrinsic parameters of lettuce for further comparison with the data obtained in this study. In ComBase, pH=6.4, aw=0.996, initial population of *Salmonella* and *E. coli* equal to 2.4 and 3.88 log CFU/mL, respectively were used. In PMP, pH=6.4, Sodium Chloride=0.5 % [g/dL], initial population of *Salmonella* and *E. coli* equal to 3.0 and 3.9 log CFU/mL, respectively were used.

2.9. Negligible growth time (ς) parameter

Sant'Ana et al. (2012, 2013), Álvarez-Ordóñez et al. (2015) and Jesus et al. (2016) reported that the growth potential (δ) is the difference between the microbial counts at the end of the shelf life and at the beginning, being that foods with δ higher than 0.5 log CFU/g are considered capable of supporting growth under storage. Based on this, microbial growth up to 0.5 log CFU/g is considered acceptable for the shelf life of products, in other words, it is used as a limit to consider a food incapable of supporting microbial growth under the storage conditions studied. In the present study, negligible growth time (ς) was defined as the

time of lag phase (λ) added of the time necessary to obtain an increase of 0.5 log CFU/g, and this was named Δy (Equation 10-13). In order to calculate ς, 0.5 log CFU/g were transformed in ln CFU/g and applied in Equation1, where the variable Time was isolated, then ς is the time in which a determine food can be exposed to a specific temperature and be considered safe.

$$\varsigma = \frac{1}{\mu_{max}} \ln\left[\left(\frac{k\Delta x - \Delta x}{k - \Delta x}\right)\left(\frac{1+q_0}{q_0}\right) - \frac{1}{q_0}\right] \quad (10)$$

$$\Delta x = e^{\Delta y} \quad (11)$$

$$k = e^{y_{max} - y_0} \quad (12)$$

$$q_0 = \frac{1}{e^{\lambda \mu_{max}} - 1} \quad (13)$$

where: ς negligible growth time [h]; μ_{max} = maximum growth rate (ln CFU/g/h); Δx [-] = linear growth ratio; Δy = the growth limit (ln CFU/g); y_{max} = the maximum population (ln CFU/g); y_0 = the initial population (ln CFU/g); q_0 [-] = parameter expressing the initial physiological state of cells; λ=lag time (h).

3. RESULTS AND DISCUSSION

3.1. Growth modelling of *E. coli* O157:H7 and *Salmonella* spp. on lettuce under isothermal and non-isothermal conditions

Salmonella spp. growth curves on lettuce started with an initial concentration of nearly 2 log CFU/g, and reached a final concentration of approximately 6 log CFU/g after 10, 110 and 310 h at 25, 10 and 5 °C, respectively (Figure 1A and 1B). For the other temperatures, approximately 8 log CFU/g, were reached after 10 h at 37 °C and 11 h at 40 ºC (Figure 1B). For *E. coli* O157:H7, all growth curves started with an initial concentration of nearly 4 log CFU/g and reached a final concentration of approximately 6 log CFU/g after 7, 150 and 250 h at 25, 10, and 5 °C, respectively (Figure 1C and 1D). For the other temperatures, approximately 7 and 8 log CFU/g were obtained after 6 h at 37 °C and 7 h at 42 ºC, respectively (Figure 1D).

In Figure 1 (A-D) it is possible to observe the good fit between the experimental data and the Baranyi model; the curves obtained from DMFit at all temperatures showed a high correlation coefficient (R^2 > 0.92). Besides this, the

primary growth parameters (growth rate, lag time and maximum population density) of the developed models on lettuce were compared with those predicted by Combase and PMP (Tables 1 and 2). In general, the parameters values of model of *Salmonella* on lettuce were similar to Combase and of *E. coli* O157:H7 to PMP (except the maximum population density). In relation to growth rate the models of *Salmonella* on lettuce showed greater values at 10 and 25°C and lesser ones at 37 and 40 °C. In contrast, all models of *E. coli* O157:H7 on lettuce present lower values of growth rate when compared to PMP (except to 25 °C). Models of *Salmonella* on lettuce obtained shorter lag time than predicted by the programs. Also, models of *E. coli* O157:H7 on lettuce showed this same behavior when compared to PMP except at 37 °C. For the maximum population density, all predict values were higher than the calculated ones for both pathogens on lettuce (except *Salmonella* modelled at 37 °C).

It can be observed that the temperature had a considerable influence on the microbial behavior, because the primary parameters were different for each temperature. This may be explained by the lettuce microbiota that can compete for nutrients and space, resulting in less growth of pathogen mainly at low temperatures (Oliveira et al., 2015). However, aiming to verify if natural microflora of lettuces would influence pathogen counts, PCA (plate count agar) counts were carried out at the same time to the counts performed on XLD and SMAC-CT. The results demonstrated that the counts were similar, indicating that the majority of growing microorganisms were those artificially inoculated (results not shown).

Furthermore, the antimicrobial properties (plant defense substances) of lettuce could cause reduction in bacterial counts; Posada-Izquierdo et al. (2016) assessing the growth of *E. coli* O157:H7 and *Salmonella* in leafy extracts, showed that spinach and chard matrix supported more growth of these pathogens than lettuce. Besides this, Koseki and Isobe (2005) studied the growth of *E. coli* O157:H7 and *Salmonella* on lettuce also observed maximum population density about 6 log CFU/g at 10 and 25ºC. Furthermore, in the review of Delaquis et al. (2007) and McKellar and Delaquis (2011) several studies of the growth of *E. coli* O157:H7 on lettuce at temperatures from 0 to 22ºC showed that the behavior of this bacteria at low temperatures is quite variable.

The data obtained in primary model (the values of growth rate) were used to elaborate a secondary model (Table 3), which allowed the prediction of the

parameter growth rate described on the basis of the temperature variation. Besides, the Table 3 showed R^2 and RMSE calculated for each model, the values of these parameters demonstrated the models adequacy. The developed model of *Salmonella* spp. on lettuce was able to assess its growth under various temperatures, ranging from 5 to 40 °C. While for *E. coli* O157:H7 the developed model on lettuce could assess the growth of this pathogen under various temperatures, ranging from 5 to 42 °C.

Therefore, the models of pathogens on lettuce were used to predicte their behavior in the worst scenario of lettuce distribution from the farm to the retail (section 2.6). *Salmonella* stored under non-isothermal conditions (Figure 2 A) attained about 8 log CFU/g after 30 h of storage. The first condition simulated the summer temperature during lettuce harvest and had an increase of 0.4 log CFU/g. The second condition was used in order to simulate storage in the farm at room temperature and showed a growth of 2.4 log CFU/g. The third period was set to simulate transportation from farm to distribution centers, during a summer day and presented an increment of 1.2 log CFU/g. The fourth condition was used to simulate storage inside distribution centers and had an addition of 0.75 log CFU/g. Finally, the fifth condition simulated lettuce exposure on retail and showed an increase of 0.8 log CFU/g. While *E. coli* O157:H7 stored under the same conditions (Figure 2 B) reached about 7 log CFU/g. The condition I (30 °C for 3 h) had an increase of 0.32 log CFU/g; the situation II (25 °C for 9 h) showed a growth of 1.2 log CFU/g; the condition III (35 ºC for 2 h) presented an increment of 0.5 log CFU/g; the situation IV (15 ºC for 8h) had an addition of 0.23 log CFU/g and the final condition (20 °C for 8 h) showed an increase of 0.75 log CFU/g. In brief, *Salmonella* growth approximately 5.5 log CFU/g, while *E. coli* O157:H7 increased 3 log CFU/g. This may be happened because *Salmonella* initial inoculum was lower than *E. coli* O157:H7, permitting that *Salmonella* could grow more, since both pathogens attained its maximum population density.

The non-isothermal model of *Salmonella* on lettuce presented a RMSE of 0.26, Bias of 0.99 and Accuracy equals 1.05. For *E. coli* O157:H7 the RMSE was 0.23, Bias and Accuracy were 1.02 and 1.03, respectively. According to Ross (1999) a good model present Bias factor between 0.9-1.05, and Accuracy factor until 1.5. Then the developed models are slightly fail-safe, in other words, they may over-

estimate the growth rate, but they are adequate to be used.

Koseki and Isobe (2005) studied the growth of *E. coli* O157:H7 and *Salmonella* on lettuce under dynamic temperature conditions during distribution from farm to table in Japan, as well as McKellar et al. (2012;2013) studied this conditions in Canada. These resarches showed less growth of pathogens comparing with the present study done in Brazil, because the temperaturas in Brazil are higher than in those contries.

3.2. Negligible growth time parameter (ς)

The negligible growth time parameter (ς) was developed to obtain the maximum period that a lettuce could be exposed to a certain temperature and not show an expressive bacterial growth, in other words, ς is the time of lag phase (λ) added of the time necessary to obtain an increase of 0.5 log CFU/g in the microbial population. The ς values are showed in Table 4, it is possible to note that *Salmonella* presented lower values because, in general, its lag time were lower and its growth rates were greater than *E. coli* O157:H7. Then the negligible growth time to keep lettuce without refrigeration, considering the main pathogens, was 1.31 h (that is, 1 h and 18 min). In this way, it is recommended to maintain lettuce under refrigeration throughout its food chain, considering that the possible treatments before consumption are not able to complete eliminate the microbial hazards that could grow (Prado-Silva et al., 2015).

Besides this, once ς is the time of lag phase (λ) added of the time necessary to obtain an increase of 0.5 log CFU/g in the microbial population it can be used to obtain the maximum period which a determined food could be exposed to a specific temperature and would still be considered safe, being applicable for any type of food. Also, since the ς equation provides the time that a microorganism takes to attain a determined cell concentration, it can be adapted for other situations when is permitted or desirable that the microorganism reach high counts, as in fermented foods and foods with probiotic. In order to use the equation in these situations it is necessary to change the Δy, using the final cell concentration desired.

In conclusion, the developed models were suitable to assess the growth of both *Salmonella* spp. and *E. coli* O157:H7 on lettuce stored at 5 to 40 °C and 5 to 42 °C, respectively under isothermal and non-isothermal conditions. Finally, the ς was developed in this study to calculate the safe shelf life for a food product when

exposed to a determined temperature. In the case of lettuce and considering *Salmonella* and *E. coli* O157:H7, ς value was 3.3 days at 5 °C, while at 37 ºC it was 1.31 h.

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Table 1: Estimated maximum growth rate, lag time and maximum population density (MPD) of *Salmonella* inoculated on the lettuce (R^2 0.92 - 0.99) and calculated from Combase and Pathogen Modelling Program (PMP) stored at different temperatures.

Temperature	Growth rate (log CFU/h)			Lag time (h)			MPD (log CFU/g)		
(°C)	Lettuce [a,b]	Combase	PMP	Lettuce [a,b]	Combase	PMP	Lettuce [a,b]	Combase	PMP
5	0.02±0.006	ND[c]	ND	63.24±8.7	ND	ND	5.82±0.2	ND	ND
10	0.05±0.014	0.035	0.01	24.60±4.4	25.6	62.5	5.96±0.1	8.5	9.22
25	0.63±0.230	0.488	0.27	1.85±0.9	2.0	4.2	5.64±0.1	8.5	9.20
37	0.82±0.051	0.919	ND	0.85±0.4	1.2	ND	8.20±0.1	8.5	ND
40	0.79±0.031	0.876	ND	1.12±0.3	1.2	ND	8.58±0.1	8.5	ND

[a] Mean value of triplicate trials.
[b] Temperatures 5-37°C from Veys et al., 2016
[c] Not determined because the program did not allow this analysis.

Table 2: Estimated maximum growth rate, lag time and maximum population density (MPD) of *E. coli* O157:H7 inoculated on the lettuce (R^2 0.93 - 0.99) and calculated from Combase and Pathogen Modelling Program (PMP) stored at different temperatures.

Temperature	Growth rate (log CFU/h)			Lag time (h)			MPD (log CFU/g)		
(°C)	Lettuce [a,b]	Combase	PMP	Lettuce [a,b]	Combase	PMP	Lettuce [a,b]	Combase	PMP
5	0.01±0.007	ND[c]	0.020	125.65±22.7	ND	213.24	5.91±0.1	ND	9.40
10	0.02±0.004	0.034	0.056	52.10±7.5	24.8	53.88	5.80±0.5	8.7	9.40
25	0.71±0.312	0.495	0.492	2.72±0.9	2.0	3.22	6.14±0.3	8.7	9.40
37	0.79±0.098	0.836	1.013	1.80±0.5	1.2	1.38	6.80±0.2	8.7	9.40
42	0.89±0.099	0.680	1.050	1.10±0.3	1.2	1.41	8.31±0.4	8.7	9.40

[a] Mean value of triplicate trials.
[b] Temperatures 10-37°C from Veys et al., 2016
[c] Not determined because the program did not allow this analysis.

Table 3: Secondary model represented by Ratkowsky equations, showing the relationship between growth rate and temperature of *E. coli* O157:H7 and *Salmonella* inoculated on lettuce under isothermal conditions.

Bacteria	Secondary model	R^2	RMSE
E. coli **O157:H7**	$\sqrt{\mu} = 0.025(T - 0.408)$	0.91	0.10
Salmonella	$\sqrt{\mu} = 0.0339(T - 1.92)\{1 - \exp[0.089(T - 53.09)]\}$	0.98	0.002

R^2 is coefficient of determination;
RMSE is root mean square error;
μ is maximum growth rate (log CFU/g/h);
T is temperature (°C).

Table 4: Negligible growth time (ς) parameter (h) calculated to *E. coli* O157:H7 and *Salmonella*.

Temperatureº C	*E. coli* O 157:H7	*Salmonella*
5	161.60	80.53
10	70.16	31.55
25	3.21	2.40
37	2.24	1.31
40	ND [a]	1.58
42	1.50	ND [a]

[a] Not determined.

Figure 1: Growth curves of *Salmonella* spp. (A and B) and *E. coli* O157:H7 (C and D) on lettuce, isothermal primary models, fitting data to DMFit from Combase software. Each symbol represents a mean of triplicate results.

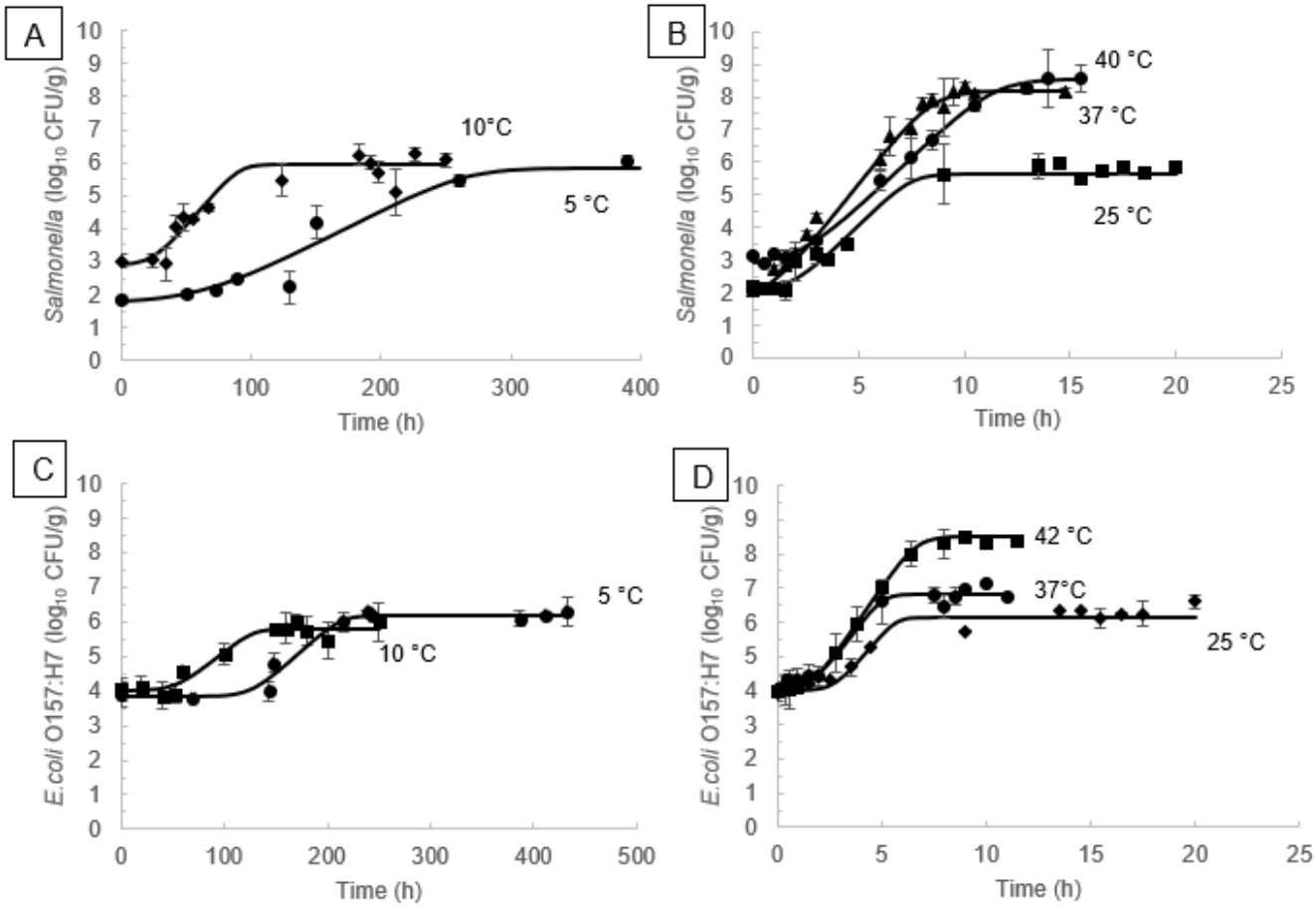
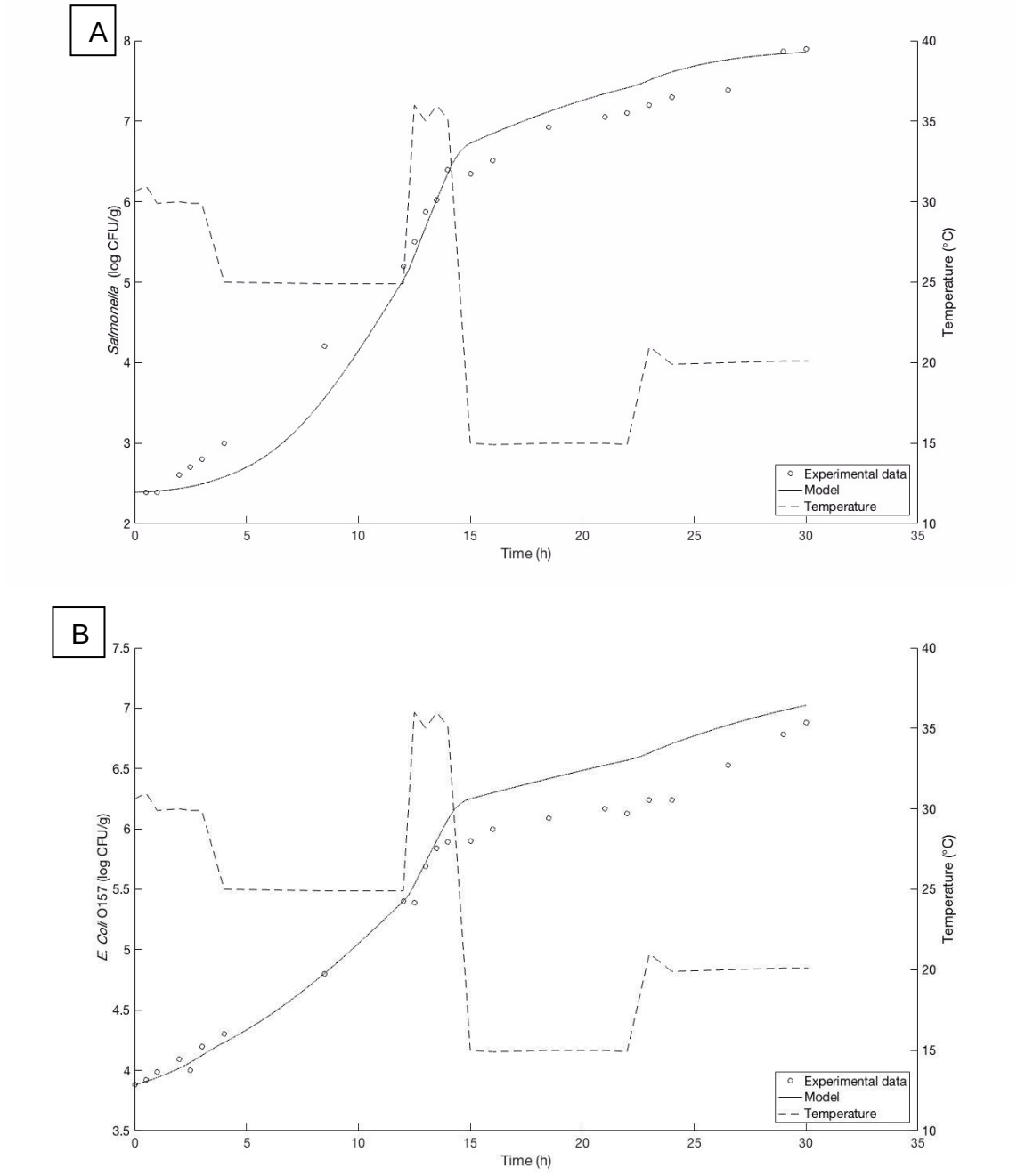


Figure 2: The observed growth of *Salmonella* spp. (A) and *E. coli* O157:H7 (B) on lettuce under non-isothermal simulated storage conditions. The growth of pathogens was predicted by the Baranyi model with a value of y_{max} = 8.5 log CFU/g for *Salmonella* and y_{max} = 8.3 log CFU/g for *E. coli* O157:H7 (values observed in the experiments).



4.3. Artigo 3

A ser submetido.

Salmonella spp. and *Escherichia coli* O157:H7 prevalence and levels on lettuce: a systematic review and meta-analysis

Susana de Oliveira Elias[a], Tiago Baptista Noronha[b], Eduardo Cesar Tondo[a]

[a] Departamento de Ciências dos Alimentos – Universidade Federal do Rio Grande do Sul, Instituto de Ciência e Tecnologia de Alimentos – Av. Bento Gonçalves, 9500 Prédio 43212 Agronomia CEP: 91505-970 – Porto Alegre – RS – Brasil, Telefone: +55 (51) 3308-6677 e-mail: susanaelias@gmail.com

e-mail: tondo@ufrgs.br

[b] Departamento de Ensino, Pesquisa e Extensão – Instituto Federal de Educação, Ciência e Tecnologia Sul-rio-grandense – Rua General Balbão, 81 – CEP 96745-000 – Charqueadas – RS – Brasil, Telefone: +55 (51) 3658-3775 e-mail: tiagonoronha@charqueadas.ifsul.edu.br

Corresponding Author: Susana de Oliveira Elias

E-mail: susanaelias@gmail.com

Postal Address: Avenida Bento Gonçalves, 9500 – Campus do Vale – Prédio 43212 – Sala 205. CEP 91501-970 - Porto Alegre/RS/Brasil

ABSTRACT

Lettuce is the most consumed leafy vegetable in the world and frequently is implicated with foodborne disease (FBD) outbreaks, being *Salmonella* and *Escherichia coli* O157:H7 the most common bacteria causing these illnesses. Estimates of prevalence and levels of these pathogens on lettuce are scarce in developed or developing countries, which hinders risk assessment attempts. This article is a systematic review and meta-analysis of reported prevalence and levels of *Salmonella* spp. and *E. coli* O157:H7 on lettuce using the most reliable available data worldwide. The relevant literature was reviewed, and trained reviewers examined the results for inclusion of articles in the meta-analysis. Relevant data (prevalence and/or level of *Salmonella* spp. and *E. coli* O157:H7 on lettuce, sample characteristic, country of origin, and *Salmonella* identified serovars) were extracted, and a meta-analysis was performed in Open Meta-Analyst, Task Order # 2 software. Only one article reported the detection of *E. coli* O157:H7 on lettuce. Then, were also considered studies that found other enterohemorrhagic (EHEC) *E. coli* on lettuce. The average prevalence of these group on lettuce was 0.041 (95% CI: 0.005–0.078). Also, only one article demonstrated concentration of *E. coli* (EHEC) on lettuces equal to < 3.0 MPN/g - > 1100 MPN/g. The mean prevalence of *Salmonella* on lettuce was 0.041 (95% CI: 0.030–0.052). Subgroup analyses were conducted to examine how prevalence of *Salmonella* on lettuce varied by study characteristics. In relation to studied countries the prevalence range of 0.001 in Japan to 0.5 in Burkina Faso. Also, the mean prevalence in developing countries was 0.064 (95% CI: 0.041–0.087), while in developed countries was 0.028 (95% CI: 0.014–0.042). Three articles reported concentration of *Salmonella* on lettuce. The values varied from 0.054±0.058 viable counts/g until 218.78 MPN/g. Despite a relatively low prevalence, consumption of lettuce is inherently risky because it usually is eaten raw, without thermal treatment to inactivate pathogens. This potential risk further supports performance of quantitative risk assessments to quantify the probability of FBD caused by *Salmonella* spp. and *E. coli* O157:H7 transmitted to lettuce.

KEYWORDS: foodborne pathogen, leafy green, concentration, bacteria, vegetable, *Lactuca sativa*

1.INTRODUCTION

Leafy green vegetables are an important component of a healthy diet, providing important vitamins, minerals, and fibers. There is an international increase in their production and consumption, aiming to promote better nutrition (Taban and Halkman, 2011). Along with the increased consumption, the number of foodborne outbreaks caused by pathogens associated with leafy green vegetables has increased worldwide in recent decades. However, the proportion of these outbreaks has augmented beyond that could be explained by an increased consumption (Callejón et al., 2015).

Despite strategies to prevent the contamination of fresh produce, produce-related outbreaks have occurred in many countries, and leafy green vegetables are thought to pose the greatest risk (WHO/FAO 2008). Additionally, lettuce, which usually is eaten raw, is the most consumed leafy vegetable in the world and frequently is implicated with outbreaks, being *Salmonella* and *Escherichia coli* O157:H7 the most common bacteria causing the outbreaks (Ilic et al., 2012; Wadamori et al,2017).

Humans and animals are the primary reservoirs of *Salmonella* species, but these microorganisms are abundant in nature, as well *Salmonella* can contaminate fresh produce, both during the production through water, soil, insects or other animals, which are contaminated with faecal matter, and during the preparation, through cross contamination (equipment, surfaces, food handlers). Moreover, depending on the serotype, *Salmonella* can be tolerant to low or high temperatures and extreme acidic environments, consequently may not be eliminated due to inappropriate storage or handling conditions (Yeni et al., 2016).

Currently, there are six pathogenic groups of *E. coli*, that could be also tolerant to low pH as well *Salmonella*. Among these groups, the most severe diseases like bloody diarrhea, thrombotic thrombocytopenic purpura, hemorrhagic colitis, and hemolytic-uremic syndrome are caused by enterohemorrhagic *E. coli* (EHEC) group, which also includes the Shiga-toxin-producing *E. coli* (STEC) serotypes or verocytotoxin-producing *E. coli* (VTEC). As primary reservoirs are ruminants, especially cattle, these pathogens can contaminate fresh produce during both the production phase through contaminated water with infected animal faeces,

or during the preparation phase through cross contamination (equipment, surfaces, handlers) (Yeni et al., 2016). Furthermore, *E. coli* serovar O157:H7 is a prevalent foodborne pathogen that has caused numerous outbreaks of human gastroenteritis linked to the consumption of lettuce in the United States, Europe, and other industrialized countries (Erickson et al., 2017).

Although some studies have been identified *Salmonella* and/or *E. coli* O157:H7 on lettuce, data of prevalence and concentration of contamination are scarce in developed or developing countries. These data are indispensable to carry out quantitative microbial risk assessments, which are necessary to quantify the potential risk involving lettuce consumption and improve the evidence base for food safety regulations and public health policies. Therefore, the objective of this systematic review was to estimate the prevalence and levels of *Salmonella* spp. and *E. coli* O157:H7 on lettuce using the most reliable available data worldwide.

2.MATERIALS AND METHODS

Systematic review and meta-analysis were used to estimate *Salmonella* spp. and *Escherichia coli* O157:H7 prevalence and concentration on lettuce, identifying data gaps.

2.1 Systematic review search strategy and selection criteria

A search was carried out using the terms ‘‘lettuce’’ OR ‘‘*Lactuca sativa*’’ AND “*Salmonella*” OR “*Escherichia coli* O157:H7” in the PubMed and Web of Science platforms. No date restrictions were applied. Endnote version X6 (Thomson Reuters) was used to collect publications (Table 1). All articles found were checked for duplicates, using Endnote and Mendeley (https://www.mendeley.com/). The search focus on the prevalence and the concentration of *Salmonella* spp. and *E. coli* O157:H7 on lettuce. Articles were collected and included when they were published in English, Spanish or Portuguese and relevant search terms appeared in the title, abstract, or key words. Publications were excluded if they were review articles and book chapters; had incomplete information on the prevalence and concentration of pathogens on lettuce; or used sanitized lettuce or artificial contamination (Figure 1).

Full-text articles were accessed whenever possible, and when these were not available, abstracts and article titles were evaluated for relevance. When abstracts were the only text available but deemed relevant, additional effort was done

in order to access full articles; otherwise articles were excluded.

2.2 Data extraction

Data for *Salmonella* spp. and *E. coli* O157:H7 prevalence and concentration on lettuce were extracted from the studies identified through the systematic review of the literature and included in the study database independently by a single trained reviewer and validated by a second reviewer. Data extracted included type of lettuce, sample size, sample pooled, sample source, laboratory detection technique, country of origin, *Salmonella* spp. or *E. coli* O157:H7 prevalence on lettuce, level of contamination in CFU/g or MPN/g, and identified *Salmonella* serovars. Data on farms that were sampled repeatedly, and samples that were analyzed with different methods were included in the meta-analysis as unique values.

2.3 Data analysis

The meta-analysis and Forest plotting of pathogens prevalence as well as estimation of the subgroups effects were done using the Open Meta-Analyst, Task Order # 2 software (available at https://www.brown.edu/academics/public-health/research/evidence-based-medicine/research-initiatives/software-0). The data were analyzed in binary random model effects by the DerSimonian-Laird method at 95% confidence interval. Individual models were used for analysis of each pathogen.

The heterogeneity across the studies estimated in the random-effects model was quantified using inverse variance index (I^2). The I^2 values at 25%, 50% and 75% were considered as low, moderate and high heterogeneity, respectively (Higgins et al.,2003). Subgroup analyses were performed, differentiating by the country of origin, region of origin, country economic status, sample source, laboratory detection technique, sample size, and sample pooled.

Pathogen concentration values were extracted when possible. However, limited data prevented the development of a meta-analysis of estimates the levels of pathogens on lettuce.

3. RESULTS AND DISCUSSION

In this study, a rigorous and a transparent approach was used to identify publications reporting the prevalence and/or the concentration of *Salmonella* spp. and *E. coli* O157:H7 on lettuce. All 1296 articles were screened to search this

information of pathogens on lettuce (Figure 1). Most of these studies investigated but did not detect the pathogens on lettuce. When these bacteria were detected, sometimes artificially contamination had been used, or the detection occurred on sanitized lettuce, or yet it was not clarified in which type of vegetable the detection was made. Then, these articles were excluded. After the screening, one study was also excluded due to be impossible to access the full article (Rude et al., 1984). We considered exclusively publications that detected *Salmonella* spp. and *E. coli* O157:H7 on lettuce *in natura*.

Only one article presented the detection of *E. coli* O157:H7 on lettuce (Khandaghi et al., 2010). Because of this, also were considered studies that found STEC or VTEC on lettuce. Thus, five articles published between 2010 and 2017, written in English, and carried out in five different countries (Spain, Germany, Iran, Malaysia, and Pakistan) were considered. The sample source were farms (2) and retail (3), and the analyses were done using traditional and molecular methods (Table 1 supplementary material). Only one of these five manuscripts showed results about concentration of STEC on lettuce, and the results ranged from less than 3.0 MPN/g to more than 1100 MPN/g (Kuan et al., 2017). These results reflect the scarcity of data on the detection and levels of *E. coli* O157:H7 on lettuce, indicating the importance of further studies focusing on these themes and making possible in deep researches as microbiological risk assessments.

Figure 2 shows that the average prevalence of *E. coli* (including O157:H7, VTEC, STEC) on lettuce as calculated from the reviewed publications (n = 5) was 0.041 (95% CI: 0.005–0.078), which means approximately 4 positives samples in 100 tested. The I^2 was 23.6% (p=0.264), meaning low heterogeneity. In other words, all studies analysed had similar prevalence of pathogen on lettuce. Then, even though few articles were found they obtained similar results which may suggest their application in future studies.

The average prevalence in developed countries was 0.125 (95% CI: 0.038–0.212) (n = 2), while in developing countries the average prevalence was 0.024 (95% CI: -0.005–0.053) (n = 3, Figure 3). In both cases the heterogeneity among the publications concerning prevalence of *E. coli* on lettuce depending on the economic country status was 0%. The developed countries showed a higher prevalence than those countries in development. This may be explained because

microbiological detection techniques used in developed countries generally are more sensitive than those applied in developing countries, providing higher positive results.

In relation to the prevalence of *Salmonella* on lettuce, 31 articles were found. They were published between 1976 and 2017; 26 studies were written in English, four in Portuguese, and one in Spanish. The articles represented four different continents (Africa, America, Asia, and Europe), and 17 countries. The sample source were farms, retail, industry, and the methods of analyses were based on microbial culture and molecular detection (Table 2 supplementary material).

Figure 4 shows that the calculated average prevalence of *Salmonella* on lettuce based on the results of 31 reviewed publications was 0.041 (95% CI: 0.030–0.052), which means approximately 4 positives samples in 100 tested, the same value found to *E. coli* (EHEC). The I^2 was 92.85%, ($p<0.001$) it means high heterogeneity among the publications was found concerning prevalence of *Salmonella* on lettuce.

Factors affecting variation (e.g., season, livestock presence, irrigation water type, flooding, fertilizer type, and hygiene practices) that were not identified by our data extraction may have caused this heterogeneity or there may have been few studies inputs to reflect the true variation in prevalence. Besides, no studies were obtained from most populous nations such as China, India, Indonesia, Nigeria, Bangladesh, and Russia, which may be due to a large data gap or to the selection criterion of limiting the search to English, Portuguese and Spanish-language publications. Furthermore, the high heterogeneity is common for systematic reviews, because of the limited number of published reports (Christidis et al., 2016; Paudyal et al., 2017; Pintar et al., 2015).

Figure 5 presents the average prevalence of *Salmonella* on lettuce in relation with country of origin. In Brazil it was 0.013 (95% CI: -0.000–0.027) (n = 6). In Canada it was 0.002 (95% CI: -0.002–0.006) (n = 1). In Turkey it was 0.052 (95% CI: -0.022–0.125) (n = 3). In Mexico it was 0.084 (95% CI: 0.004–0.164) (n = 2). In Spain it was 0.052 (95% CI: -0.021–0.125) (n = 3). In Malaysia it was 0.364 (95% CI: 0.079–0.648) (n = 1). In Italy it was 0.355 (95% CI: -0.286–0.996) (n = 2). In Japan it was 0.001 (95% CI: -0.000–0.003) (n = 1). In Chile it was 0.033 (95% CI: -0.031–0.098) (n = 1). In United States it was 0.006 (95% CI: -0.003–0.015) (n = 2). In Egypt it was 0.033 (95% CI: -0.012–0.079) (n = 1). In Costa Rica it was 0.133 (95% CI:

0.012–0.255) (n = 1). In Senegal it was 0.066 (95% CI: 0.046–0.087) (n = 2). In Thailand it was 0.200 (95% CI: 0.076–0.324) (n = 1). In Netherlands it was 0.020 (95% CI: -0.037–0.077) (n = 2). In Burkina Faso it was 0.500 (95% CI: 0.281–0.719) (n = 1). In Philippines it was 0.250 (95% CI: 0.140–0.360) (n = 1). The heterogeneity among the publications concerning prevalence of *Salmonella* on lettuce depending on the country of origin ranged from 0% (Brazil, United States) until 99,48% (Italy). In countries where there was only 1 study, the heterogeneity could not be calculated. The prevalence range of 0.001 in Japan to 0.5 in Burkina Faso. The country with higher number of studies was Brazil. This might have occurred, due to the language selection (English, Portuguese and Spanish).

Figure 6 presents the average prevalence of *Salmonella* on lettuce in relation with region of origin. In Central and South America, it was 0.036 (95% CI: 0.014–0.058) (n = 10). In United States and Canada, it was 0.002 (95% CI: -0.001–0.006) (n = 3). In Asia it was 0.063 (95% CI: 0.025–0.101) (n = 7). In Europe it was 0.135 (95% CI: 0.070–0.200) (n = 7). In Africa it was 0.079 (95% CI: 0.027–0.130) (n = 4). The heterogeneity among the publications concerning prevalence of *Salmonella* on lettuce depending on the region of origin varied from 0% (United States and Canada) until 97,76% (Europe). The higher prevalence found in Europe (1012 samples tested), while the lower was in United States and Canada (788 samples tested), both regions are considered developed, which is related to higher per capita income, life expectancy, and literacy. Central and South America, Asia, and Africa - that are considered in developing regions - had similar prevalence.

Figure 7 demonstrated that the average prevalence of *Salmonella* in developing countries was 0.064 (95% CI: 0.041–0.087) (n = 20), while developed countries had average prevalence of 0.028 (95% CI: 0.014–0.042) (n = 11). In both cases, the heterogeneity was high (79,6% in developing and 96.36% in developed countries). The developing countries showed higher prevalence than those developed. This may have occurred due to good agricultural practices, that are more frequently apply in developed countries.

Figure 8 presents the average prevalence of *Salmonella* in relation with sample size. Studies with less than 99 samples had prevalence of 0.080 (95% CI: 0.050–0.111) (n = 20). While articles with more than 99 had average prevalence of 0.031 (95% CI: 0.018–0.043) (n = 11). The heterogeneity among the publications

concerning prevalence of *Salmonella* on lettuce depending on the sample size was high in large samples (96,87%) and moderate in smaller samples (71.44%).

Figure 9 presents the average prevalence of *Salmonella* on lettuce in relation with pooled samples. Articles with no pooled samples had prevalence of 0.043 (95% CI: 0.031–0.054) (n = 24), while studies with pooled samples had average prevalence of 0.036 (95% CI: -0.000–0.072) (n = 7). The heterogeneity among the publications concerning prevalence of *Salmonella* on lettuce depending on the pooled samples was high in no pooled sample studies (94,3%) and moderate in pooled sample studies (48.87%).

Figure 10 presents the average prevalence of *Salmonella* on lettuce in relation with sample source. When samples were collected at farms, the average prevalence of *Salmonella* was 0.021 (95% CI: 0.007–0.035) (n = 11). In studies which the samples were collected at more than one place the prevalence was 0.019 (95% CI: 0.007–0.030) (n = 5). At retail it was 0.169 (95% CI: 0.103–0.236) (n = 13), while at the industry it was 0.010 (95% CI: -0.049–0.236) (n = 2). The heterogeneity among the publications concerning prevalence of *Salmonella* on lettuce depending on the sample source varied from 9.84% (industry) until 96,17% (retail). Retail showed the higher prevalence, while other sources presented similar values.

Figure 11 presents the average prevalence of *Salmonella* in relation with laboratory detection techniques. Studies that used only culture method had prevalence of 0.057 (95% CI: 0.040–0.074) (n = 19). While articles that used culture and molecular methods had average prevalence of 0.029 (95% CI: 0.013–0.044) (n = 12). In both cases the heterogeneity among the publications concerning prevalence of *Salmonella* on lettuce depending on the laboratory detection technique was high (94.91% in culture and 92.85% in culture and molecular methods). In general, molecular methods may overestimate the presence of live microorganisms by providing a false-positive result, whereas culture methods may underestimate prevalence because of the presence of viable but nonculturable cells. In this meta-analysis, more studies included the use of a culture detection method versus a molecular detection method, and the results showed the opposite of expected, that is, the prevalence of culture methods was higher.

In general, the prevalence data had high and moderate heterogeneity among studies. It is difficult to identify the specific factors that might have contributed

to this heterogeneity of the data. The estimation of the effects of origin, economic status, size, pool, source and analyses methods on average prevalence also revealed that the findings from most studies were highly heterogeneous. The prevalence data could be factual with extensive varieties depending on many issues of hygienic conditions. Besides this, high heterogeneity is common in metanalysis studies (Higgins et al.,2003).

Table 2 shows three articles reporting concentration of *Salmonella* on lettuce. The values varied from 0.054±0.058 viable counts/g until 218.78 MPN/g. In these studies, the levels were based on a total of 99 positive samples for *Salmonella*. Each study adopted different methods and units of measuring, making difficult to calculate mean values.

The risk of consuming *Salmonella* and *E. coli* O157:H7 through lettuce can be estimated with a quantitative microbial risk assessment by using the prevalence and levels determined in the present meta-analysis, adding information about lettuce consumption and washing. The worldwide prevalence found in this study was 0.041 for both pathogens on lettuce. When the countries were divided by social economic status, the prevalence of developed countries was 0.028 for *Salmonella* and 0.125 for *E. coli* (EHEC), while developing countries was 0.064 for *Salmonella* and 0.024 for *E. coli* (EHEC).

Then the results of this study can be used to identify and quantify risk associated with the consumption of lettuce when performing risk assessments, and to identify data gaps for future research. Efforts to systematically collect the available evidence are critical to inform public health risk management and decision-making, and prioritization of interventions.

Based on our knowledge, the present meta-analysis provides the best available estimates for the prevalence and levels of *Salmonella* and *E. coli* O157:H7 on lettuce. However, a significant gap exists in the enumeration of these pathogens on lettuce internationally, mainly on the *E. coli* O157:H7 data.

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Table 1: Electronic search strategies for research databases and search results.

Database	*Salmonella*	*E. coli* O157:H7	Search strategy
PubMed MEDLINE	334	298	Abstract
Web of Science (Science Citation Index)	996	933	title/keywords/abstract

Table 2: Concentration of *Salmonella* on lettuce.

Reference	Concentration of *Salmonella* on lettuce
Ercolani, 1976	0.054[a]±0.058 Viable counts/g (fresh weight)
Vital et al., 2014	4.57–218.78 MPN/g
Wijnands et al., 2014	0.281 (0.041-1.31) CFU/g

[a]mean

Figure 1: Flow diagram of the literature search and selection of eligible studies.

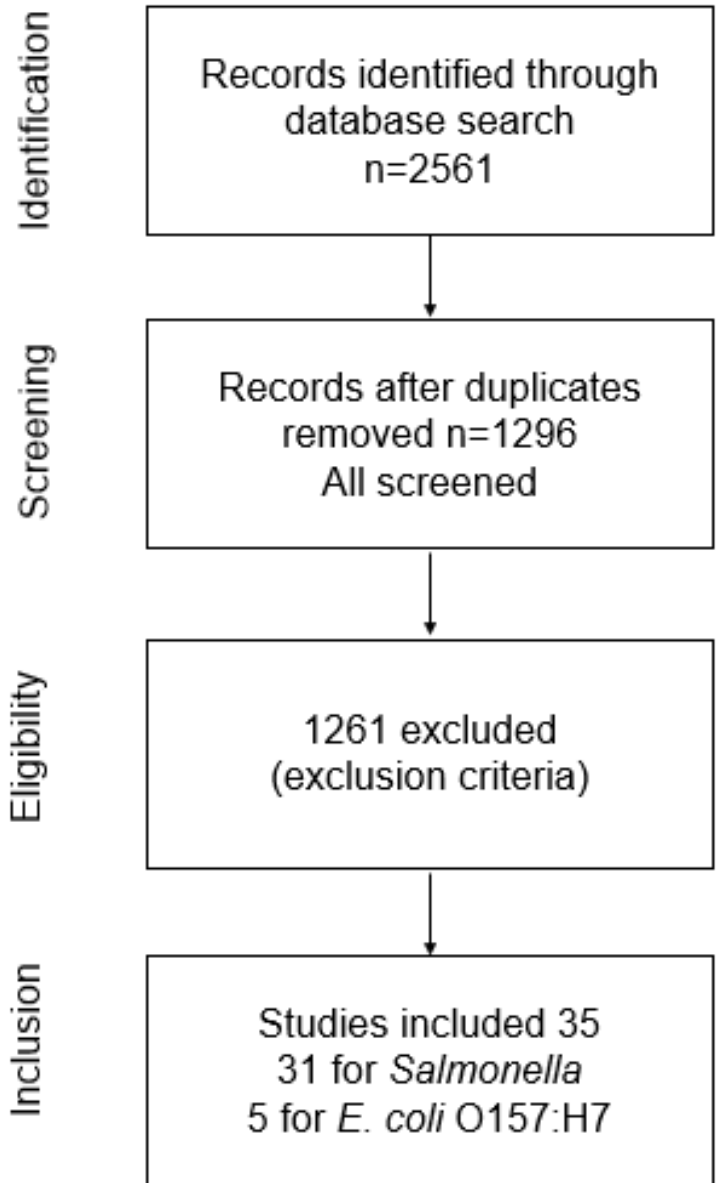


Figure 2: Prevalence of *E. coli* (including O157:H7, VTEC, STEC) on lettuce (Random Effects Model, I^2=23.6%, p=0.264). X-axis is the proportion of the organism reported in individual studies as listed along the Y-axis, with the range of proportion in 95% confidence interval (CI). Studies given higher weights are indicated by larger marker. Ev = number of positive sample; Trt = total sample number.

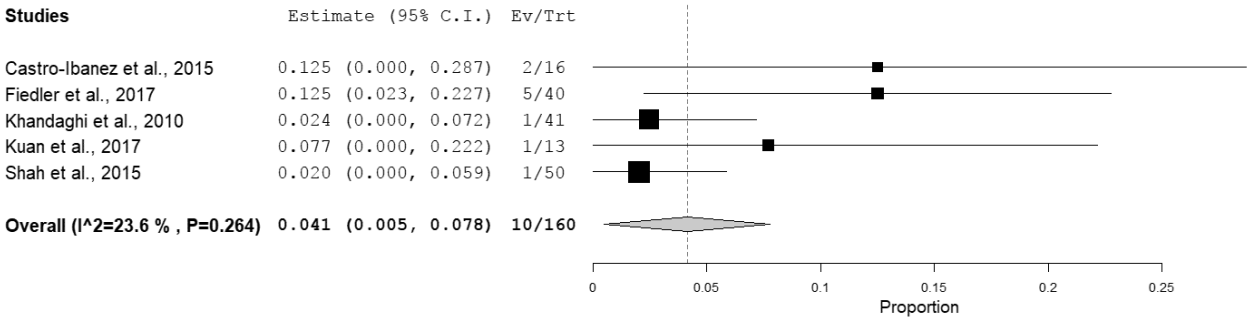


Figure 3: Prevalence of *E. coli* (including O157:H7, VTEC, STEC) on lettuce in relation with country economic status. X-axis is the proportion of the organism reported in individual studies as listed along the Y-axis, with the range of proportion in 95% confidence interval (CI). Studies given higher weights are indicated by larger marker. Ev = number of positive sample; Trt = total sample number.

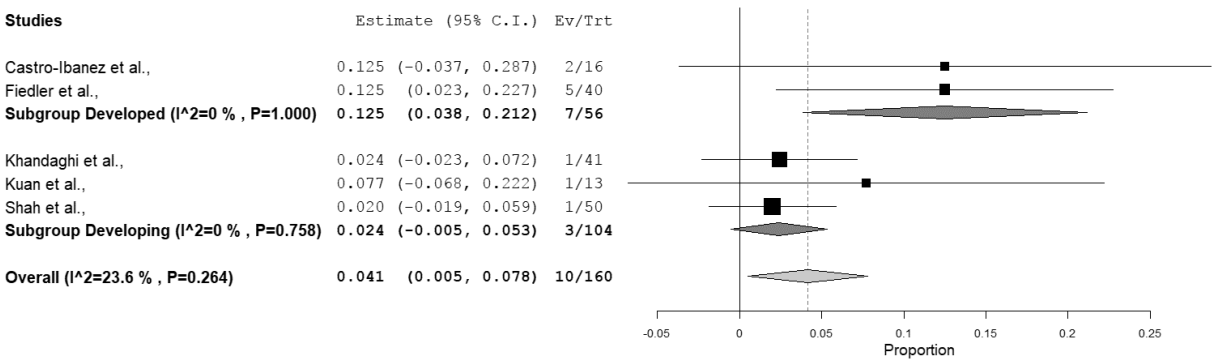


Figure 4: Prevalence of *Salmonella* on lettuce (Random Effects Model, I^2=92.85%, p<0.001). X-axis is the proportion of the organism reported in individual studies as listed along the Y-axis, with the range of proportion in 95% confidence interval (CI). Studies given higher weights are indicated by larger marker. Ev = number of positive sample; Trt = total sample number.

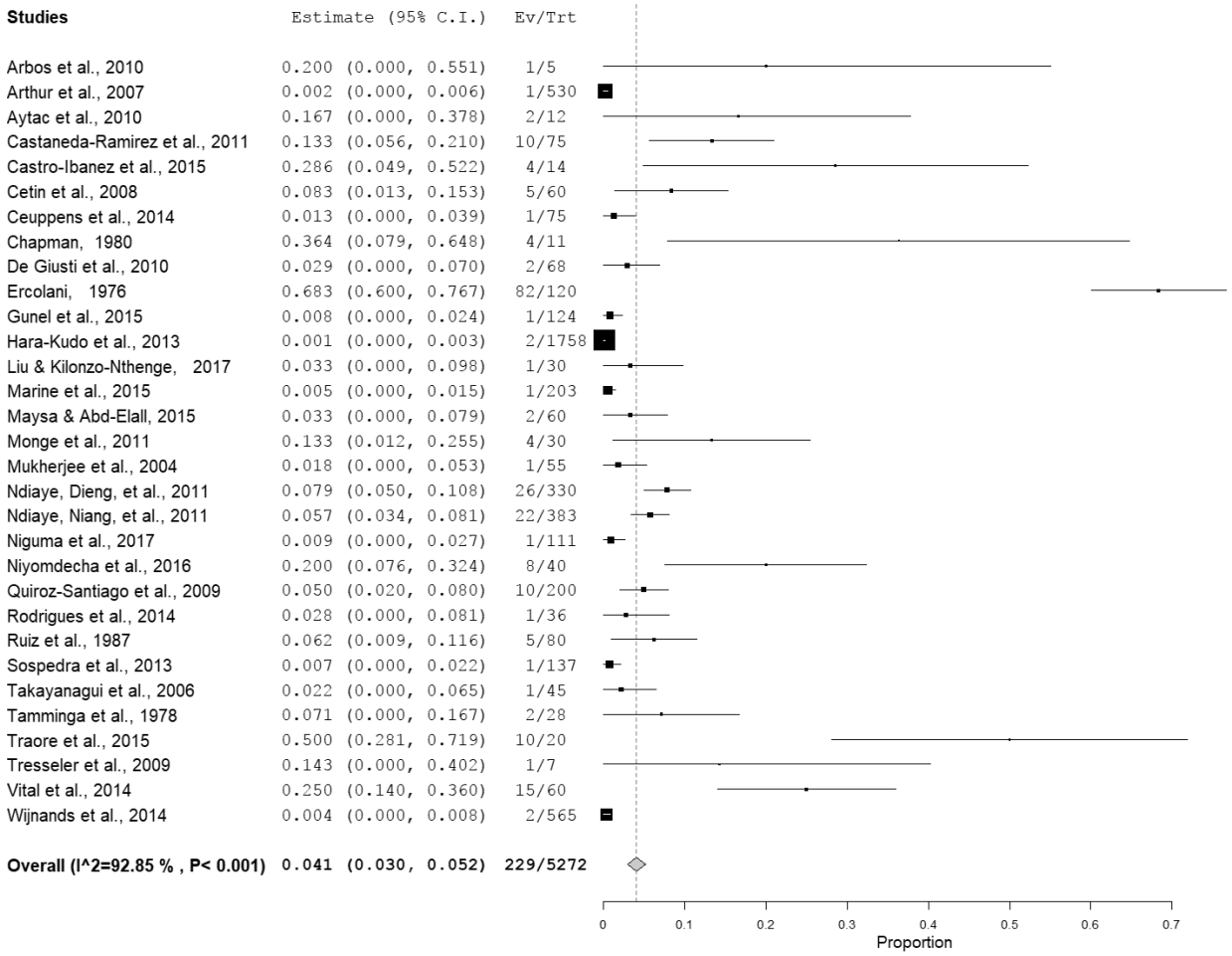


Figure 5: Prevalence of *Salmonella* on lettuce in relation with country of origin. X-axis is the proportion of the organism reported in individual studies as listed along the Y-axis, with the range of proportion in 95% confidence interval (CI). Studies given higher weights are indicated by larger marker. Ev = number of positive sample; Trt = total sample number.

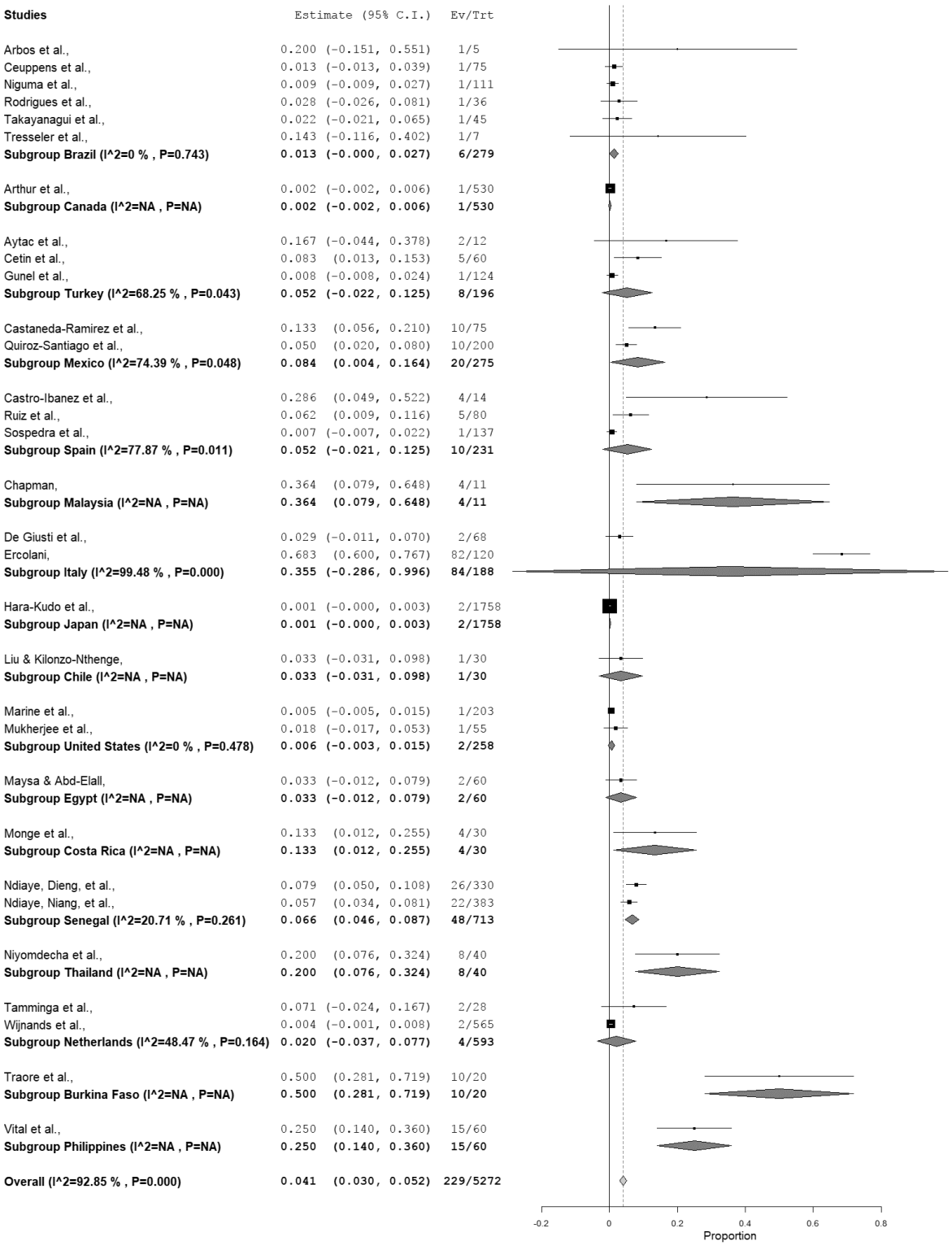


Figure 6: Prevalence of *Salmonella* on lettuce in relation with region of origin. X-axis is the proportion of the organism reported in individual studies as listed along the Y-axis, with the range of proportion in 95% confidence interval (CI). Studies given higher weights are indicated by larger marker. Ev = number of positive sample; Trt = total sample number.

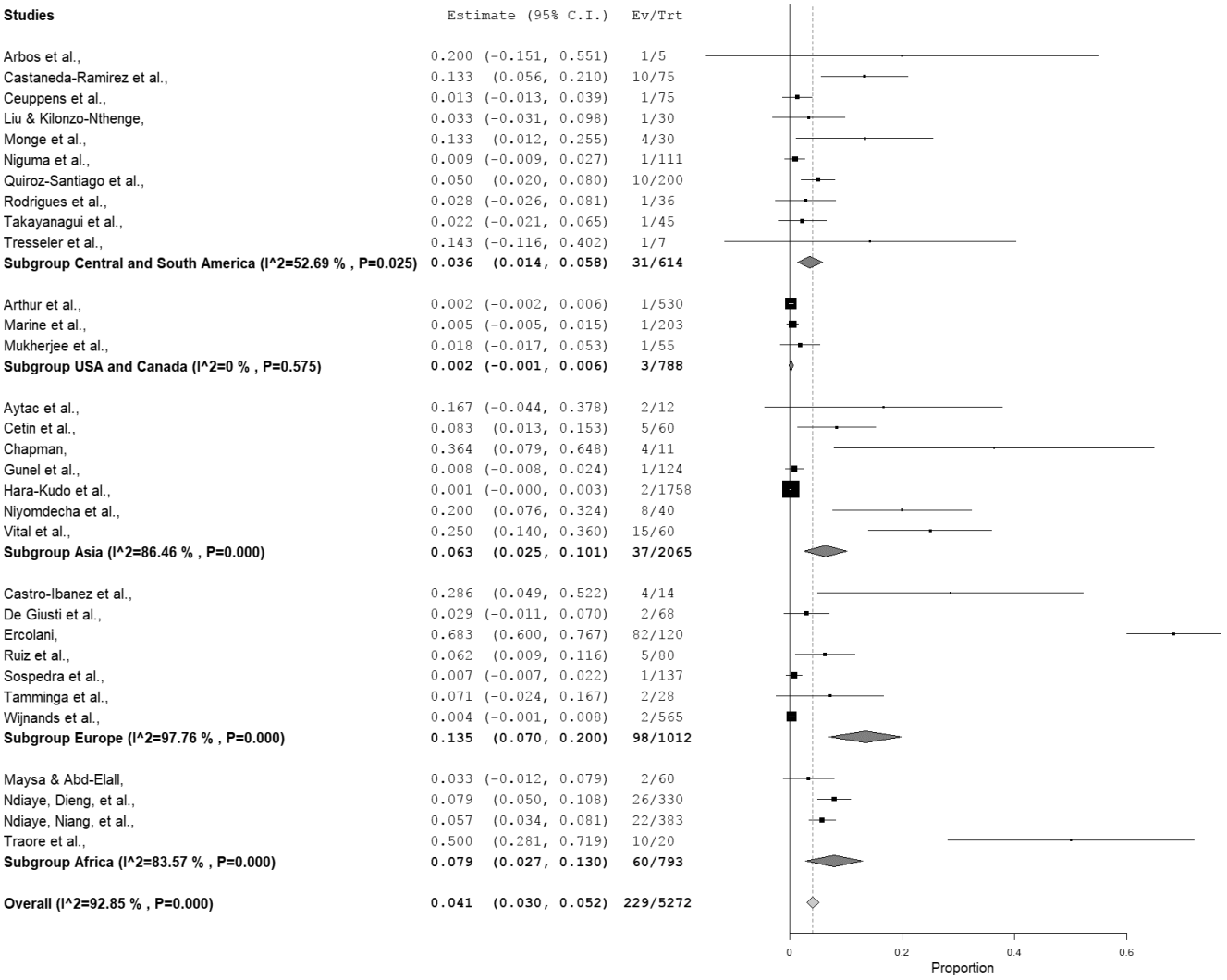


Figure 7: Prevalence of *Salmonella* on lettuce in relation with country economic status. X-axis is the proportion of the organism reported in individual studies as listed along the Y-axis, with the range of proportion in 95% confidence interval (CI). Studies given higher weights are indicated by larger marker. Ev = number of positive sample; Trt = total sample number.

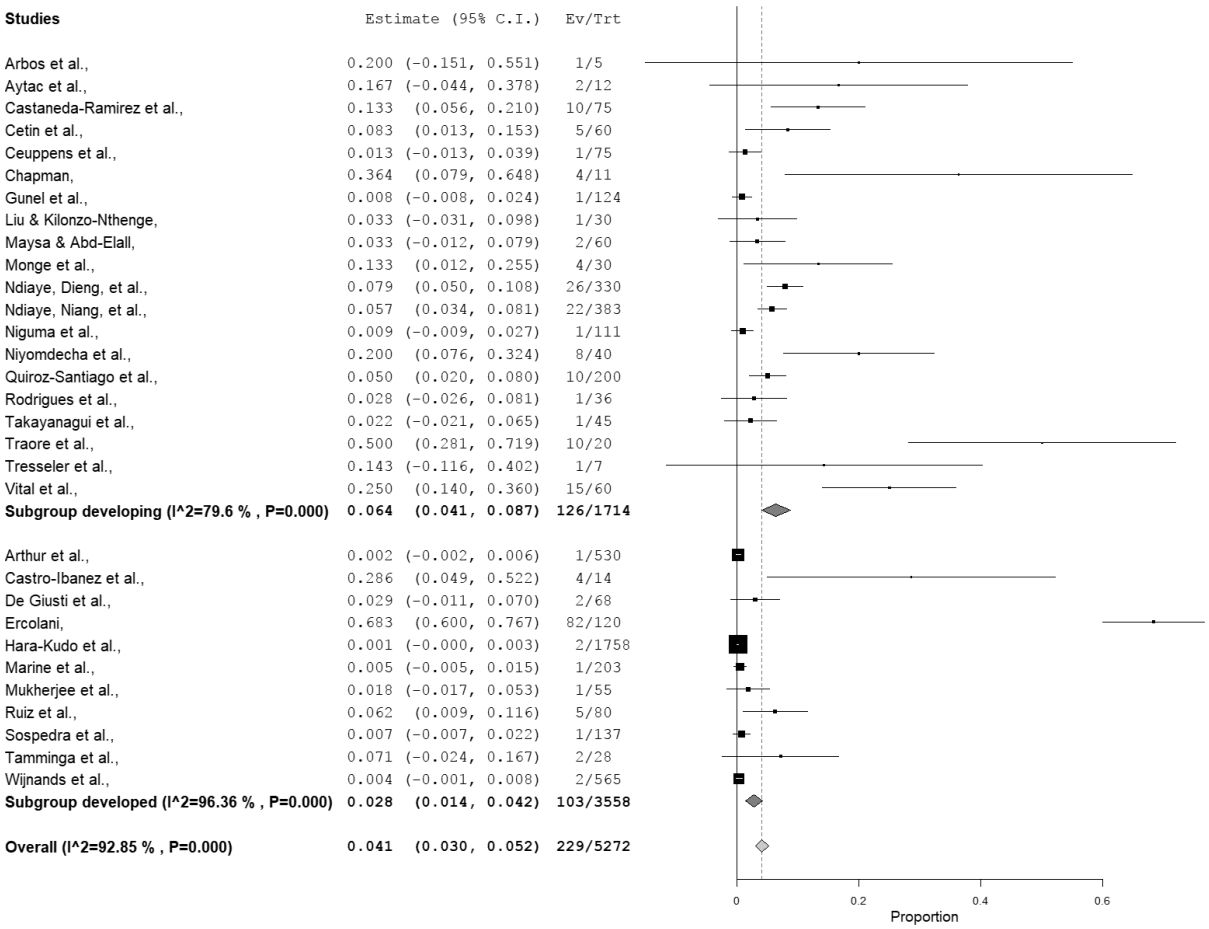


Figure 8: Prevalence of *Salmonella* on lettuce in relation with sample size. X-axis is the proportion of the organism reported in individual studies as listed along the Y-axis, with the range of proportion in 95% confidence interval (CI). Studies given higher weights are indicated by larger marker. Ev = number of positive sample; Trt = total sample number.

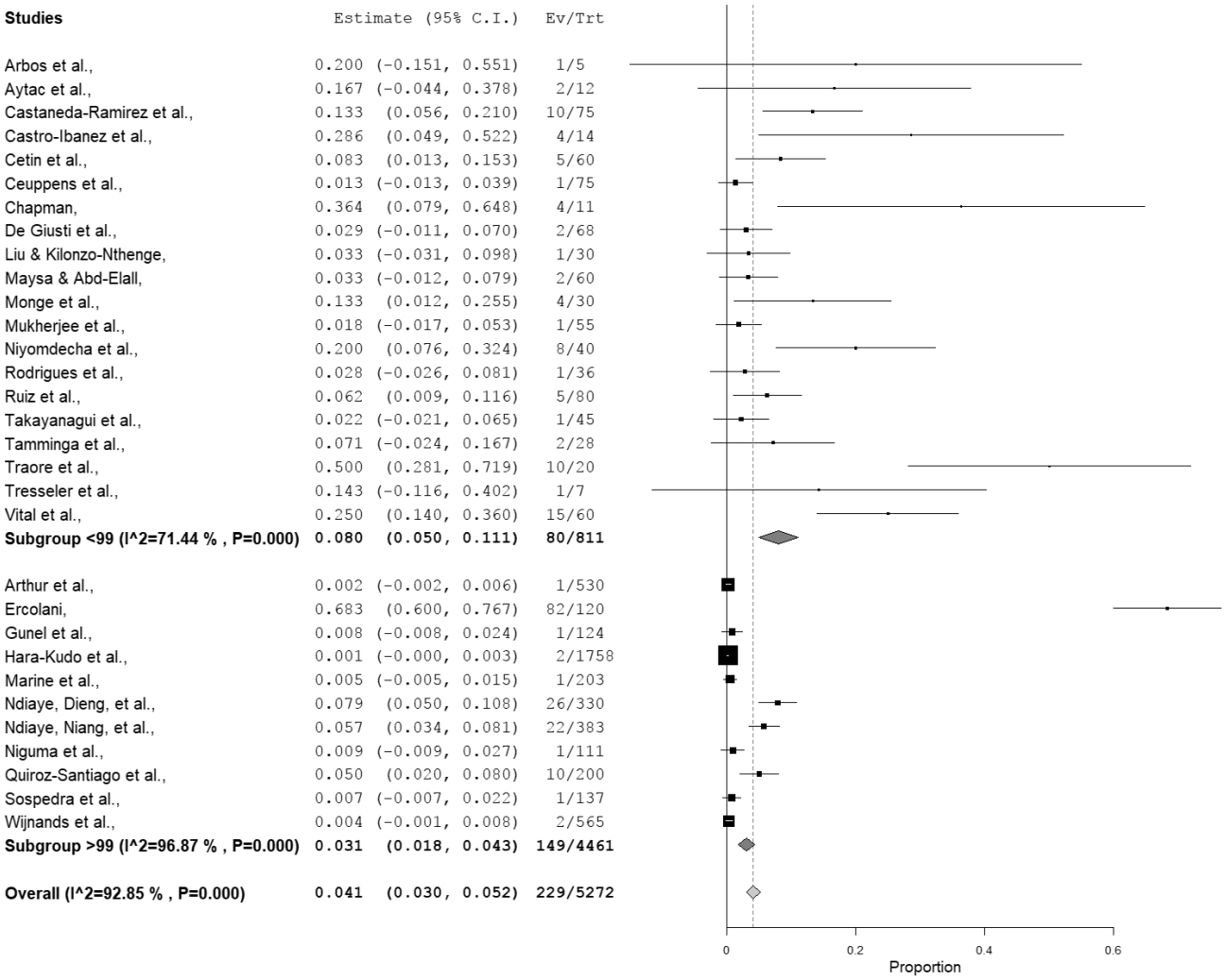


Figure 9: Prevalence of *Salmonella* on lettuce in relation with sample pooled. X-axis is the proportion of the organism reported in individual studies as listed along the Y-axis, with the range of proportion in 95% confidence interval (CI). Studies given higher weights are indicated by larger marker. Ev = number of positive sample; Trt = total sample number.

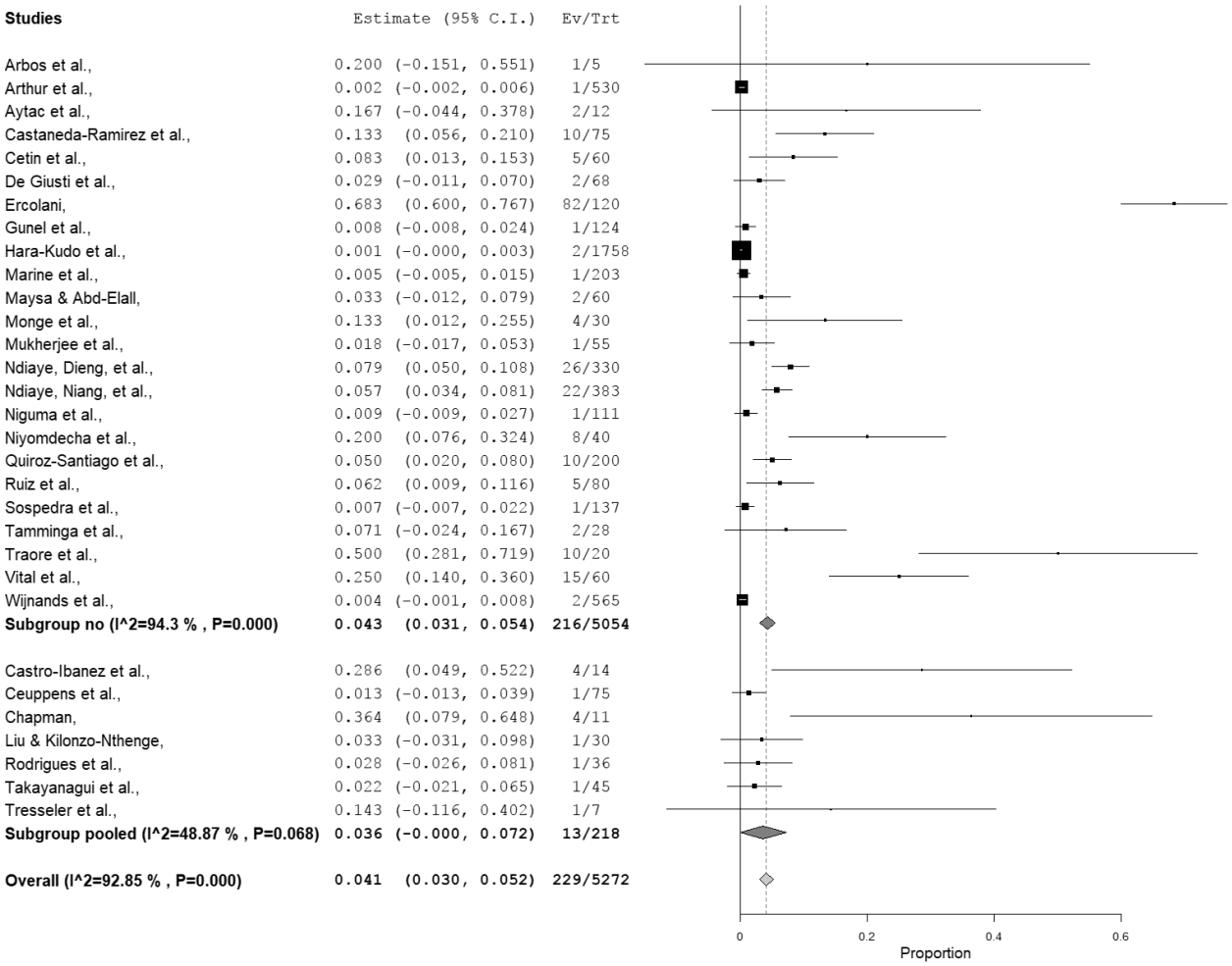


Figure 10: Prevalence of *Salmonella* on lettuce in relation with sample source. X-axis is the proportion of the organism reported in individual studies as listed along the Y-axis, with the range of proportion in 95% confidence interval (CI). Studies given higher weights are indicated by larger marker. Ev = number of positive sample; Trt = total sample number. Several = more than one source.

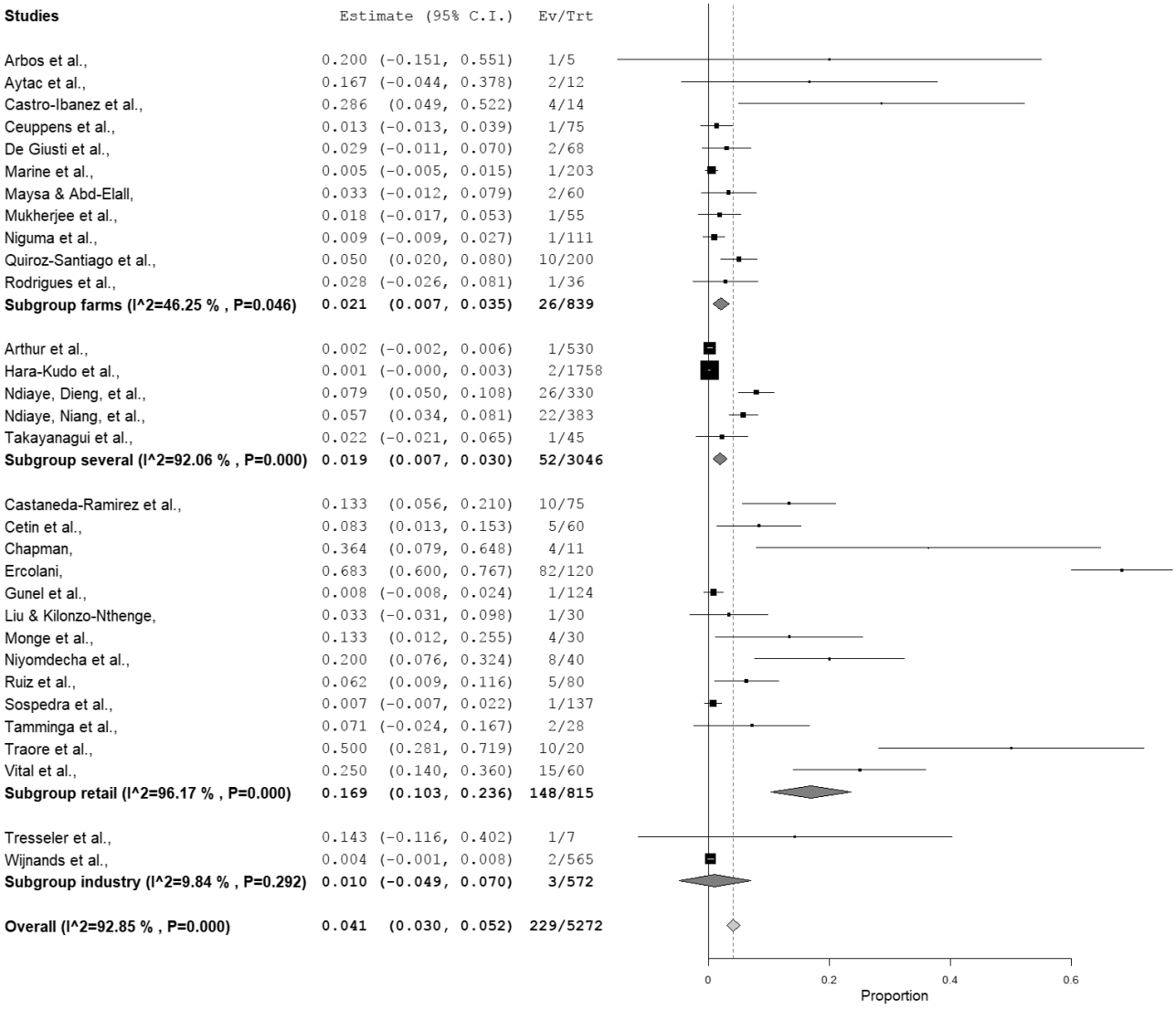
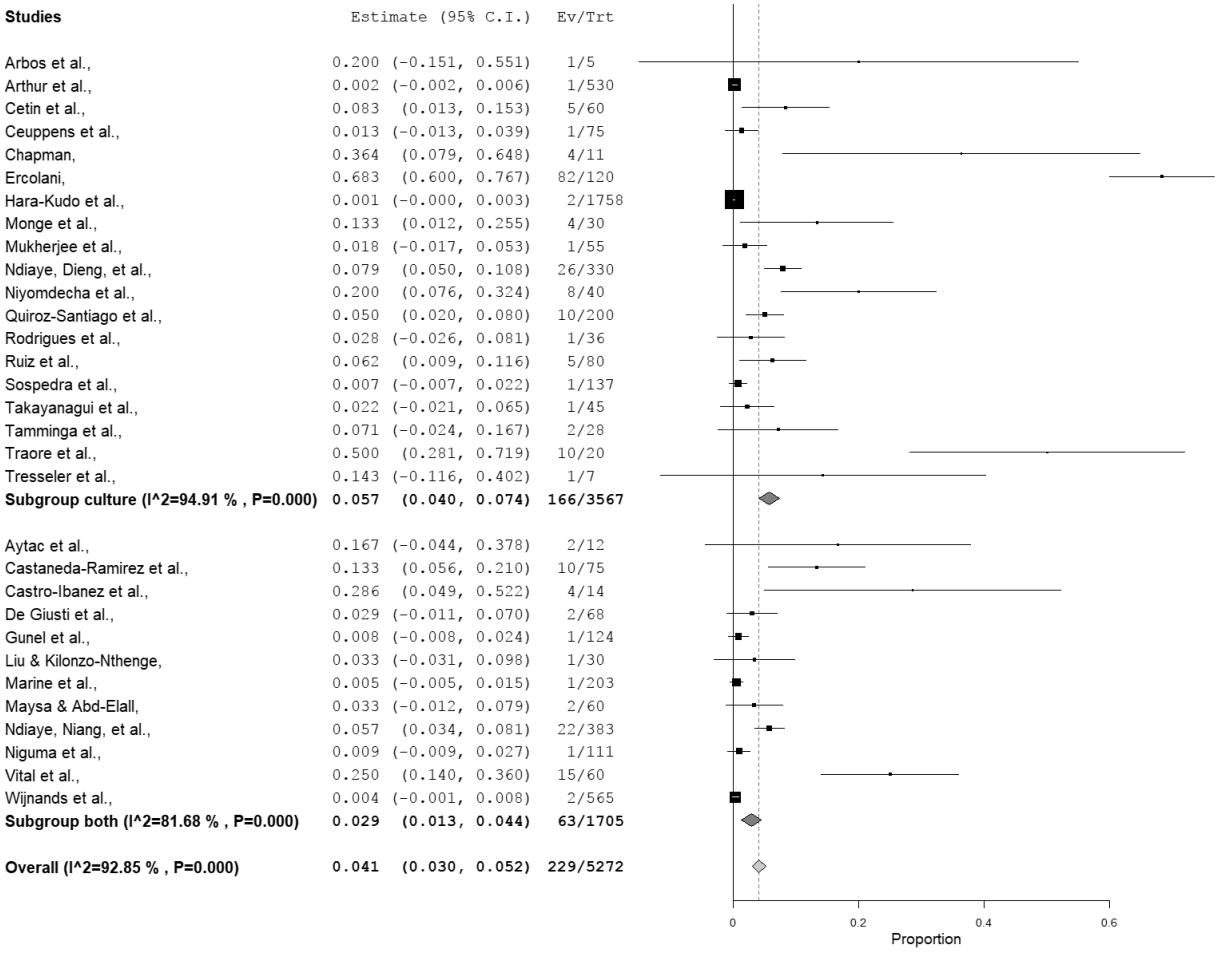


Figure 11: Prevalence of *Salmonella* on lettuce in relation with laboratory detection technique. X-axis is the proportion of the organism reported in individual studies as listed along the Y-axis, with the range of proportion in 95% confidence interval (CI). Studies given higher weights are indicated by larger marker. Ev = number of positive sample; Trt = total sample number. Both = culture and molecular.



Supplementary material

Table 1: Articles used for EHEC *E. coli* prevalence and concentration determination.

Reference	Prevalence	Weights	*E. coli*	Sample source	Sample pooled	Method of analyses	Country of origin	Region	Status	Concentration
Castro-Ibanez et al., 2015	2/16	4.781%	VTEC	farms	yes	Both	Spain	Europe	Developed	-
Fiedler et al., 2017	5/40	11.021%	STEC	retail	no	Both	Germany	Europe	Developed	-
Khandaghi et al., 2010	1/41	35.027%	O157:H7	farms	no	Both	Iran	Asia	Developing	-
Kuan et al., 2017	1/13	5.900%	STEC	retail	no	Both	Malaysia	Asia	Developing	< 3.0–> 1100(MPN/g)
Shah et al., 2015	1/50	43.270%	STEC	retail	no	Both	Pakistan	Asia	Developing	-

Table 2: Articles used for *Salmonella* prevalence and concentration determination.

Reference	Prevalence	Weights	*Salmonella*	Sample source	Sample pooled	Method of analyses	Country of origin	Region	Status	Concentration
Arbos et al., 2010	1/5	0.096%	-	farms	no	culture	Brazil	Central and South America	developing	-
Arthur et al., 2007	1/530	7.355%	*Salmonella* Schwarzengrund	several	no	culture	Canada	USA and Canada	developed	-
Aytac et al., 2010	2/12	0.258%	-	farms	no	Both	Turkey	Asia	developing	-
Castaneda-Ramirez et al., 2011	10/75	1.582%	-	retail	no	Both	Mexico	Central and South America	developing	-
Castro-Ibanez et al., 2015	4/14	0.207%	-	farms	pooled	Both	Spain	Europe	developed	-
Cetin et al., 2008	5/60	1.833%	-	retail	no	culture	Turkey	Asia	developing	-
Ceuppens et al., 2014	1/75	5.224%	-	farms	pooled	culture	Brazil	Central and South America	developing	-
Chapman, 1980	4/11	0.144%	-	retail	pooled	culture	Malays-ia	Asia	developing	-
De Giusti et al., 2010	2/68	3.700%	*Salmonella* Umbilo	farms	no	Both	Italy	Europe	developed	-
Ercolani, 1976	82/120	1.395%	63.4% for *S.* Typhimurium, 41.6% for *S.*	retail	no	culture	Italy	Europe	developed	5.4±5.8 Viable counts/100 g

			Schottmuelleri, 37.8% for *S*. Typhi, 34.1% for *S*. Anatum, 8.5% for *S*. Dublin, and 8.5% for *S*. Thompson.							(fresh weight).
Gunel et al., 2015	1/124	6.426%	*S*. Mikawasima	retail	no	Both	Turkey	Asia	developing	-
Hara-Kudo et al., 2013	2/1758	7.406%	-	several	no	culture	Japan	Asia	developed	-
Liu & Kilonzo-Nthenge, 2017	1/30	2.077%	-	retail	pooled	Both	Chile	Central and South America	developing	-
Marine et al., 2015	1/203	7.012%	-	farms	no	Both	United States	USA and Canada	developed	-
Maysa & Abd-Elall, 2015	2/60	3.246%	*S*. Typhimurium	farms	no	Both	Egypt	Africa	developing	-
Monge et al., 2011	4/30	0.726%	-	retail	no	culture	Costa Rica	Central and South America	developing	-
Mukherjee et al., 2004	1/55	4.175%	-	farms	no	culture	United States	USA and Canada	developed	-
Ndiaye, Dieng, et al., 2011	26/330	4.860%	- *S*. Kunduchi; *S*. Caledon; *S*. Kingston; *S*. Schwarzengrund ; *S*. Banana; *S*. Montevideo; *S*. Mbandaka; *S*. Manchester; *S*. Molade; *S*. Ekotedo; *S*. Sinstorf; *S*. Westhampton; *S*. New-haw; *S*.	several	no	culture	Senegal	Africa	developing	-

			Gaminara; *S.* Tilène							
Ndiaye, Niang, et al., 2011	22/383	5.543%	-	several	no	Both	Senegal	Africa	developing	-
Niguma et al., 2017	1/111	6.220%	-	farms	no	Both	Brazil	Central and South America	developing	-
Niyomdecha et al., 2016	8/40	0.702%	2 *S.* Panama; *S.* Schwarzengrund; *S.* Rissen; *S.* Stanley; *S.* Hvittingfoss; *S.* Weltevreden; *S.* Bangkok	retail	no	culture	Thaila-nd	Asia	developing	-
Quiroz-Santiago et al., 2009	10/200	4.729%	-	farms	no	culture	Mexico	Central and South America	developing	-
Rodrigues et al., 2014	1/36	2.654%	-	farms	pooled	culture	Brazil	Central and South America	developing	-
Ruiz et al., 1987	5/80	2.695%	*S.* Cleveland; *Salmonella* group B; *Salmonella* spp.; 2 *S.* Kapemba	retail	no	culture	Spain	Europe	developed	-
Sospedra et al., 2013	1/137	6.584%	-	retail	no	culture	Spain	Europe	developed	-
Takayanagui et al., 2006	1/45	3.441%	-	several	pooled	culture	Brazil	Central and South America	developing	-
Tamminga et al., 1978	2/28	1.112%	-	retail	no	culture	Netherl-ands	Europe	developed	-

Traore et al., 2015	10/20	0.240%	4 *S*. Korlebu; 1 *S*. Gerland; 4 *S*. Colindale; 1 *S*. Bredeney	retail	no	culture	Burkina Faso	Africa	developing	-
Tresseler et al., 2009	1/7	0.173%	-	industry	pooled	culture	Brazil	Central and South America	developing	-
Vital et al., 2014	15/60	0.875%	-	retail	no	Both	Philippi-nes	Asia	developing	0.66–2.34 log10MPN/g
Wijnands et al., 2014	2/565	7.308%	*Salmonella* Typhimurium DTI04	industry	no	Both	The Netherl-ands	Europe	developed	0.281 CFU/g 0.041-1.31

4.4. Artigo 4

Artigo a ser submetido.

Risk of infection with *Salmonella* and *Escherichia coli* O157:H7 due to consumption of lettuce in southern Brazil

Susana de Oliveira Elias[a], Tiago Baptista Noronha[b], Liesbeth Jacksens[c], Eduardo Cesar Tondo[a]

[a] Departamento de Ciências dos Alimentos – Universidade Federal do Rio Grande do Sul, Instituto de Ciência e Tecnologia de Alimentos – Av. Bento Gonçalves, 9500 Prédio 43212 Agronomia CEP: 91505-970 – Porto Alegre – RS – Brasil, Telefone: +55 (51) 3308-6677

[b] Departamento de Ensino, Pesquisa e Extensão – Instituto Federal de Educação, Ciência e Tecnologia Sul-rio-grandense – Rua General Balbão, 81 – CEP 96745-000 – Charqueadas – RS – Brasil, Telefone: +55 (51) 3658-3775 e-mail: tiagonoronha@charqueadas.ifsul.edu.br

[c] Ghent University Campus Ufo, Rectorate Sint-Pietersnieuwstraat 25 B-9000 Ghent Belgium e-mail: liesbeth.Jacxsens@ugent.be

Corresponding Author: Susana de Oliveira Elias

E-mail: susanaelias@gmail.com

Postal Address: Avenida Bento Gonçalves, 9500 – Campus do Vale – Prédio 43212 – Sala 205. CEP 91501-970 - Porto Alegre/RS/Brasil

ABSTRACT

Lettuce is the main produced and consumed leafy vegetable in Brazil. There is an increased number of foodborne outbreaks linked with the consumption of this fresh produce and *Salmonella* and *Escherichia coli* O157:H7 pathogens. Then, this study was carried out to estimate the risks of infection due to consumption of lettuce contaminated with these pathogens in Southern Brazil. The quantitative microbial risk assessment (QMRA) model comprised nine modules from storage of lettuce in producer farms until consumption. Scenarios were simulated using prevalence, concentration, and exposure levels lower than those found in Brazil. Different procedures of washing and disinfection as well as cold chain (≤5ºC) in all distribution steps were also tested. Models built in Excel spreadsheet were simulated using @Risk® software (100,000 iterations for each scenario created was run using Monte Carlo sampling). Sensitivity analysis of the real-world model and the cold chain scenario were performed. In general, the QMRA simulations show that overall risks of foodborne disease due to consumption of lettuce are higher for *Salmonella* than for *E. coli* O157:H7. The mean risk of *Salmonella* infection per month was 0.017, while for *E. coli* O157:H7 was 0.006. All alternative scenarios to clean lettuce, increase the risk. Then, the suggested procedure is washing leaves with potable water followed by immersion in 200 ppm of sodium hypochlorite for 15 minutes and rinsing with potable water again. The major risk reduction was due to cold chain scenario, which presented mean risk of *Salmonella* infection per month of 3.16E-07, while for *E. coli* O157:H7 was 2.27E-08. Sensitivity analyses indicated that in addition to the maintenance of the cold chain and the washing and disinfection procedures, it is important to reduce the prevalence and concentration of pathogens on lettuce in fields, in order to decrease the risk of infection by these bacteria. The developed QMRA model estimated the risk associated with consumption of *Salmonella* and *E. coli* O157:H7–contaminated lettuce and can guide the evaluation and development of intervention strategies to mitigate the risk.

KEYWORDS: QMRA; foodborne pathogen; leafy green; bacteria; vegetable; *Lactuca sativa*

1.INTRODUCTION

Lettuce is the main produced and consumed leafy vegetable in Brazil and the Grand Rapids (crispa) type represents the main varietal segment growing in the country. That Brazilian preference for the crispa type is a unique event in world market of lettuce (Sala & Costa 2012). Although healthy benefits are attributed to the consumption of lettuce, foodborne diseases can happen if this vegetable is contaminated by pathogens, and that fact has been highlighted by the increased number of foodborne outbreaks linked with the consumption of this fresh produce worldwide (FAO/WHO 2008, Gil et al., 2015).

Salmonella spp. and *Escherichia coli* O157:H7 are the most common pathogenic bacteria that contaminate lettuce, causing many outbreaks in the world (Callejón et al., 2015). In Brazil, a total of 7170 foodborne disease outbreaks were reported between 2007 and 2017, and *E. coli* was the most identified foodborne pathogen, accounting for 525 of foodborne disease outbreaks, while *Salmonella*, which occupied the second place, and was responsible for 515 outbreaks (Anonymous, 2017). Rio Grande do Sul (RS), the southernmost State of Brazil, is one of the Brazilian states that most investigates and reports foodborne diseases outbreaks. Shigatoxin-producing *E. coli* (*E. coli* O157:H7) and *Salmonella* has been isolated from lettuce cultivated in this state, and from water used to irrigate lettuce fields, indicating risk of illnesses due to consumption of this vegetable (Ceuppens et al., 2014; Decol et al.,2017; Missiaen, 2015; Rodrigues et al., 2014).

The contamination of lettuce can occur at any point, from the farm to the consumer. As fresh produce is normally consumed raw or with minimum processing, it is important to keep the microbial load of fresh produce as low as possible to prevent foodborne illnesses (Wadamori et al., 2017). Then, quantitative microbial risk assessment (QMRA) can be a very useful tool to help in this prevention. QMRA allows the quantitative estimation of the risks posed to public health by a food-pathogen combination (Oscar, 2011). The outputs of QMRA can be used in the development of scientific-based strategies in order to manage risks and safeguard public health (Sant'Ana et al., 2014). Given the above and considering the increasing consumption of lettuce in Brazil (Sala & Costa 2012), this study was carried out to estimate the risks of infection due to consumption of lettuce contaminated with

Salmonella and *E. coli* O157:H7 in Southern Brazil.

2.MATERIALS AND METHODS

2.1. Models development

The risk assessment model comprised nine different modules from storage of lettuce in the producer farms until consumption. Table 1 summarizes the cells on Excel spreadsheet used for subsequent risk calculations. The first column (symbol) represents the spreadsheet cell designation of the variable on that line of the table. This label is needed to understand how the variable links to the other variables in the risk assessment. The next column (event) is a text description of the variable. The third column (values) is either a number, a simple formula, or an @RISK formula representing the value of the cells. The fourth column (unities) represents the units of the variables. The last column (source) represents the source of the information used to determine the value of the variables. The source can be an assumption or a literature citation or calculated from other cells in the spreadsheet.

2.1.1. Producer storage

Data on prevalence and levels of *Salmonella* and *E. coli* O157:H7 on lettuce were gathered from a systematic review metanalysis (Elias et al., submitted). Few data on prevalence and populations of both pathogens on lettuce in Brazil were available in the literature. Because of this, the values chosen to be used represent the developing countries in the systematic review metanalysis. The prevalence of both pathogens was represented in the model by Beta distribution, while the concentration was modeled using Pert distribution (Table 1).

Temperature and time during producer storage of lettuce were modeled based on data obtained from Ascal, 2015. This author studied the distribution chain of lettuce from farms until retail in an important supermarket network in Southern Brazil. The data from storage time depends on when lettuce was harvested. The most common process is harvesting at evenings and transporting in the next morning. Then, generally, the lettuce remains at environment temperature for approximately 10h (most probable), waiting to be transported. Sometimes lettuce is harvested and transported in the same day, then the minimum transport period was considered 1h, while the maximum period was assumed 12h. These values were reported by Ascal (2015) and were used to describe storage time in the farms using a

Pert distribution (Table 1). The temperatures were based on the data collected by INMET (National Institute of Meteorology) (INMET, 2017), and represent the annual averages of temperature in RS that were: 33.56ºC (maximum), 3.55ºC (minimum) and 18.12ºC (most probable). These values were used to describe storage temperature in the farms using a Pert distribution (Table 1).

The growth of *Salmonella* and *E. coli* O157:H7 was described by the relationship between growth rate and temperature represented by the models in the study of Elias et al. (2018). The growth of both pathogens during storage was calculated by multiplying predicted growth by time of storage at a given temperature. The concentration after transport was the sum of initial concentration of each pathogen and subsequent growth during this step.

2.1.2. Transport from producer to the distribution center

Time and temperature during transportation of lettuce from producers to distribution center were modeled based on data obtained from Ascal (2015). Considering different producers in different places that produce lettuce to the referred supermarket network the mean distance from farm to the main distribution center was 133.11 km, while the maximum route was 289 km, and the minimum was 16 km. Then, the transportation time was considered 1.8h (most probable), 0.5h (minimum), and 4h (maximum). A Pert distribution was used to model transportation time from farms to distribution center (Table 1). The temperature during the transportation was based on the data collected by INMET, already described in Section 2.1.1, because some vehicles do not have refrigeration. The growth of *Salmonella* and *E. coli* O157:H7 and their respective levels after transportation were calculated as described in Section 2.1.1

2.1.3. Arrival and storage at distribution center

Time and temperature during storage of lettuce at distribution center were modeled based on data obtained from Ascal (2015). The storage time reported in this study was 10h (most probable), ranging from 1h (minimum) to 12h (maximum). A Pert distribution was used to model storage time at distribution center (Table 1). The temperature during the storage was 15ºC (most probable temperature inside cold chambers), 10ºC (minimum), and 20ºC (maximum). A Pert distribution was used to model storage temperature at distribution center (Table 1). The growth of *Salmonella* and *E. coli* O157:H7 and their respective levels after storage were calculated as

described in Section 2.1.1

2.1.4. Transportation from distribution center to markets

Time and temperature during transportation of lettuce from distribution center to markets were modeled based on data obtained from Ascal (2015). Considering all markets of this network in the RS the mean distance from distribution center to them was 39 km, while the maximum route was 291 km, and the minimum was 1.6 km. Then, the most probable transportation time was considered 0.65h, 0.05h (minimum), and 4h (maximum). A Pert distribution was used to model transportation time from distribution center to markets (Table 1). The temperature during the transportation was based on the data collected by INMET, the same as cited in Section 2.1.1, because the vehicles do not have refrigeration. The growth of *Salmonella* and *E. coli* O157:H7 and their respective levels after transportation were calculated as described in Section 2.1.1

2.1.5. Market storage

Time and temperature during storage of lettuce at market were modeled based on data obtained from Ascal (2015) and Missiaen (2015). The latter studied lettuces marketed in Southern Brazil. The storage time at markets reported in these studies were one day, all markets are supplied of lettuces every day. A uniform distribution was used to model storage time at market (Table 1). The most probable temperature during the storage was considered 22.5ºC, while the minimum was 20ºC, and the maximum 25ºC. A Pert distribution was used to model storage temperature at markets (Table 1). The growth of *Salmonella* and *E. coli* O157:H7 and their respective levels after storage were calculated as described in Section 2.1.1.

2.1.6. Transportation from retail to home

No consumer time and temperature transportation data in Brazil are currently available. A Pert distribution was used in this module and the values were based on assumptions by the authors (Table 1). Minimum, most likely and maximum temperatures in this module were assumed to be 10ºC, 20ºC and 30ºC, 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 bacterial growth during transportation from retail to consumers home was calculated as described in Section 2.1.1.

2.1.7. Home storage

Temperature during storage in home refrigerators was modeled using a Pert distribution with minimum, most likely and maximum values of 3.1ºC, 6ºC and 10.8ºC, respectively, as extracted from Silva et al. (2008). Storage time was modeled by assuming that consumers behavior on storage of foods in their home refrigerator is influenced by the organoleptic properties. Borghi et al. (2009) studied the storage of lettuce *in natura*, and found that the maximum shelf-life considering organoleptic properties of lettuce in these cited conditions was approximately 120h. A uniform distribution with 0 and 120h as minimum and maximum values, respectively, was used to model storage time at home. The logarithmic growth and level after home storage were calculated as described in Section 2.1.1.

2.1.8. Washing and disinfection

It was assumed that lettuce is washed and disinfected before the consumption at home, using the same procedures preconized to the Brazilian food services described by the RS regulation Portaria 78/2009 (Rio Grande do Sul, 2009). The process considered the washing of lettuce using potable water followed by immersion in 200 ppm of sodium hypochlorite for 15 minutes and rinsing with potable water. Silveira et al. (2017) studied the *Salmonella* reduction, using this procedure, reported levels of 5.83±0.82 log CFU/g, while De Paula (2014) demonstrated reductions levels of 6.27±0.50 log CFU/g for *E. coli* O157:H7, using the same process; in both studies bacteria were artificially inoculated on lettuce. These levels of reductions were used in the present study. A normal distribution was used to model the washing and disinfection of lettuce. The concentration after sanitation was calculated by subtracting the concentration after home storage of each pathogen and subsequent reduction after washing and disinfection steps.

2.1.9. Consumption of lettuce, determination of dose-response relationship, probability of illness and number of cases

The typical serving size of lettuce as consumed by the Brazilian population was studied by Carlos et al. (2008); the values were 20g, 30g and 50g, as minimum, most likely and maximum serving sizes, respectively. The distribution used was triangular (Krzyzanowski et al., 2016). The level of pathogens was calculated by summing or subtracting their levels at the end of each module of the QMRA model (Table 1). The dose of pathogens per serving was calculated by multiplying amounts of vegetables consumed and the level of pathogen (Table 1). The exposure (number

of servings of lettuce intake per month) was obtained from Mattos et al. (2000), Krzyzanowski et al. (2016), and Souza et al. (2013), who reported that the consumption of lettuce in Brazil occurs every day. The dose-response relationship for infection by *Salmonella* was estimated using a beta-Poisson model as proposed by WHO/FAO (2002). Also, the values of parameters α and β were obtained from WHO/FAO (2002). The dose-response model for *E. coli* O157:H7 was based on the model developed by Cassin et al. (1998), using *E. coli* O157:H7 in ground beef. Cassin et al. (1998) proposed a beta-binomial model that predicts the probability of illness from a particular dose. We simplified the Cassin et al. (1998) beta-binomial model, converting it back into a simple beta-Poisson model (WHO/FAO, 2002) that specifies a mean population risk (Danyluk et al., 2011). The outputs of the QMRA model were the risk of infection per month (probability of infection per month due to consumption of lettuce) and number of cases (number of people that consumed lettuce and get infected per month) in the exposed population (Table 1). The determination of number of cases of infection due to *Salmonella* and *E. coli* O157:H7 was calculated considering the population of RS, Brazil (IBGE, 2017) and assuming that approximately 16% of population eats lettuce (Souza et al., 2013).

2.2. Evaluation of different scenarios

The QMRA model was used to simulate risk of infection and number of cases due to consumption of lettuce contaminated with *Salmonella* and *E. coli* O157:H7 in several scenarios showed in Table 2 and 3, respectively. The scenario 1 was composed by all inputs of Table 1 and represent the real-world conditions. The scenarios 2-4 represented the decrease in prevalence and/or levels of *Salmonella* and *E. coli* O157:H7. Scenario 2 used half of the prevalence of these pathogens (3,2% and 1,2%, respectively). Scenario 3 used half of the concentrations values for *Salmonella*: RiskPert (0, 0.33, 1.17), while for *E. coli* O157:H7 was used: RiskPert (0, 0.235, 1.52). Scenario 4 combined the reductions of both scenarios 2 and 3. Also, scenario 5 considered less consumption frequency of lettuce by the population. The exposure adopted by scenario 5 was half of the exposure used in the real-world scenario (15 days per month).

Furthermore, different washing and disinfection procedures were tested: scenario 6 considered washing of lettuce using only potable water [reducing 0.97±0.18 for *Salmonella*, and 1,49±0.18 for *E. coli* O157:H7 according to Silveira et

al. (2017) and De Paula et al. (2015)]; scenario 7 considered washing with potable water followed by immersion in 200 ppm of sodium hypochlorite for 1 minute and rinsing with potable water [reducing 5.11±0.82 for *Salmonella*, and 3.27±0.27 for *E. coli* O157:H7 according to Silveira et al. (2017) and De Paula et al. (2015)]; and scenario 8 considered washing of lettuce with potable water followed by immersion in 200 ppm of sodium hypochlorite for 5 minutes and rinsing with potable water [reducing 4.41±0.48 for *Salmonella*, and 2.76±0.26 for *E. coli* O157:H7 according to Silveira et al. (2017) and De Paula et al. (2015)]. The scenario 9 evaluated the impact of control of temperature (maximum temperature 5ºC) from storage at farms until home storage on risks and on number of cases of infection, modeled using a Pert distribution with 1ºC, 3ºC and 5ºC as minimum, most likely and maximum values. The last scenario (10) considered lettuces submitted to entire cold chain control with the “only washing” procedure.

2.3. Simulation settings and analysis of models outputs

The QMRA model was built in an Excel spreadsheet (Microsoft, Redmond, WA) and simulated using @Risk software version 7.5 (Palisade Corporation). A total of 100,000 iterations for each scenario created was run using Monte Carlo sampling and with the random generator seed fixed at 1 to ensure that results could be repeated, allowing comparisons of different scenarios. Spearman’s correlation coefficients were used for sensitivity analysis of the real-world model and the scenario 9 (cold chain) to determine the effect of input variables on the probability of illness per serving and on the number of illness cases in RS per month.

3. RESULTS AND DISCUSSION

The present study was conducted to estimate the risks of infection by *Salmonella* and *E. coli* O157:H7 due to contamination of lettuce consumed in Southern Brazil, based on the distribution chain of lettuces in RS. The nine modules composing the QMRA model are presented in Table 1. Although there are some studies that identified these pathogens in the field in RS, none reported the behavior of these bacteria in leafy vegetables during field operations (Ceuppens et al., 2014; Decol et al.,2017; Rodrigues et al., 2014). Thus, the fate of *Salmonella* and *E. coli* O157:H7 in the field operations was not assessed in the current model. The risk

factors influencing the pathogens occurrence and growth in primary production of leafy greens are: temperature, rainfall, flooding, the presence domestic and wild animals, irrigation water sources, topography, fertilizers, and hygiene practices of farmers (Castro-Ibanez et al., 2015; Ceuppens et al., 2015; Holvoet et al., 2014). However, the scarcity of data about this issue in Brazil does not permit its consideration in the present model. These data would be useful for improving the accuracy of QMRA models developed in this study, as well as could help in the development of risk management strategies. Moreover, to the best of our knowledge, this is the first study examining microbial growth and reductions during distribution chain of lettuce in Brazil.

In the first module of QMRA model (producer storage) we presented the prevalence and concentration of pathogens. These data were extracted from Elias et al. (submitted) that reported the prevalence and levels of pathogens on lettuce. Few studies were found describing this data in the fields in Brazil, because of that all developing countries data were considered in the present QMRA. Besides this, some samples of Elias et al. (submitted) study were collected in retail shops, but the current QMRA model assumes that these data represent prevalence and levels of pathogens as found on lettuce just after harvest. In relation to temperatures, the only Brazilian study in which temperature was measured on the lettuce head was Missiaen (2015). In the other cases it was used environmental or equipment (cold chamber, refrigerators) temperatures. Even with these data limitations, the present study is important, since few QMRA have been performed in developing countries (Abia et al., 2016; Murmann et al., 2011). Furthermore, risk assessment is a valuable alternative when surveillance data are nonexistent or sparse, and the development of a QMRA offers a scientific basis approach for risk management, providing ranks of the most effective risk management options (Enger et al.,2012; Pouillot et al.,2012).

The increase in pathogen concentration in the modules of the current study were modeled using the predictive models generated in experiments in order to consider the variability of growth rates of six strains of *Salmonella* and four strains of *E. coli* O157:H7 isolated from food in Brazil (Elias et al., 2018). Using this approach, no growth of *Salmonella* and *E. coli* O157:H7 on lettuce was assumed if product temperature was below 1.92ºC or 0.408ºC, respectively. Thus, in the QMRA model, when a temperature below T0 was selected during iterations, zero growth was

assigned and no increase in the initial concentration (module 1) was assumed (Table 1).

The main outputs of the QMRA models (risks of infection per month per serving and numbers of cases of infection in the population exposed) developed are shown in Tables 2 (*Salmonella* results) and 3 (*E. coli* O157:H7 results). In general, the QMRA simulations show that overall risks of foodborne disease due to consumption of lettuce are higher for *Salmonella* than for *E. coli* O157:H7. This can be explained, because the prevalence of *Salmonella* on lettuce is higher than *E. coli* O157:H7. Also, the growth rates of *Salmonella* (in most tested temperatures) are slightly higher than *E. coli* O157:H7 ones. The first scenario (real-world) represents the current knowledge regarding lettuce and the pathogens studied, while scenarios 2-4 consider a reduction of prevalence and/or concentrations of each pathogen. These scenarios (2-4) would represent the application of intervention measures in the field before harvest to reduce the prevalence and levels of pathogens. Table 2 shows in scenario 2 the reduction of prevalence of *Salmonella* from 6.4% to 3.2%, representing 56.5% of reduction in the risk of infection per month per serving in relation to scenario 1. A similar reduction was seen (58%) when the prevalence and the concentration were reduced by half (scenario 4). However, less than 5% of reduction was observed when only the concentration was reduced by half (scenario 3). In table 3 is possible to observe that reductions in the scenarios 2-4 were similar. In the scenario 2, *E. coli* O157:H7 prevalence was reduced from 2.4% to 1.2% and the number of cases of infection per month in the population exposed reduced 47,2%. When prevalence and concentration were reduced (scenario 4) by half, the number of cases reduced 48,1%, and in scenario 3 the reduction was nearly 10%. This indicates that interventions to reduce prevalence of pathogens would be more effective than measures to reduce the concentration of pathogens on lettuces, regarding the risk of illnesses caused by both microorganisms.

Scenario 5 simulated a reduction of approximately 50 % in the population exposure, reducing lettuce consumption to 3-4 times per week instead of eating lettuce every day. A decrease of 50.6% in the risk of infection by *Salmonella* per month per serving was observed in relation to scenario 1 (Table 2). Similarly, the number of cases of infection by *E. coli* O157:H7 per month in the population exposed was reduced by 51% when the consumption was reduced by half (Table 3). It means

that people who consume lettuce fewer times a week have a lower risk of contamination by these pathogens. It is important to highlight that the number of infection cases do not represent the number of illnesses, but the number of people contaminated by the microorganisms. The development of disease will depend on the immunity of the person contaminated with the pathogen, the infections dose and the severity of the strain (Pouillot et al., 2016).

The scenarios 6-8 showed different procedures to clean lettuce before eating. The scenario 1 represents the procedure recommended to food services described by the regulation of RS (washing leaves with potable water followed by immersion in 200 ppm of sodium hypochlorite for 15 minutes and rinsing with potable water). The scenario 6 considered only the procedure of washing the lettuce using potable water; while the scenario 7 encompassed the washing followed by immersion in 200 ppm of sodium hypochlorite for only 1 minute and rinsing with potable water; and scenario 8 considered washing followed by immersion in 200 ppm of sodium hypochlorite for 5 minutes and rinsing. In tables 2 and 3, is possible to observe the scenarios 6-8 increased the risk of infection by *Salmonella* and *E. coli* O157:H7 per month per serving in relation to scenario 1. Thus, it is strongly recommended to adopt the procedure recommended by Portaria 78/2009 (Rio Grande do Sul, 2009), is being the most effective method to reduce counts of both pathogens.

The scenario 9 considered that lettuce was kept from 1ºC to 5ºC during all steps of the QMRA model, simulating a well-controlled cold chain. In Tables 2 and 3, it is possible to observe that this condition was very effective to decrease the risk of infection, since the number of cases of infection by *Salmonella* and *E. coli* O157:H7 per month in the population exposed was reduced to less than 1 case per month. In other words, the number of *Salmonella* cases would be approximately 7 per year, while for *E. coli* O157:H7 would be nearly 1 case every 2 years. In majority of cities of Brazil, lettuce is not kept refrigerated, from the harvest to the final consumer, which makes this vegetable distribution one of the major bottlenecks to the expansion of this sector (Sala & Costa 2012). Even considering that ready-to-eat (RTE) vegetables have to be kept under cold chain in Brazil, it is noticed that a great percentage of displays in the supermarkets the temperature is above 7ºC, which indicates the storage of Brazilian RTE vegetables in retail stores is seldom what experts would recommend (Sant'Ana et al., 2014). Then, it is strongly recommended to maintain the

lettuce until 5ºC to keep it safe for consumption, also increasing the quality and shelf-life of this vegetable. Besides that, the adoption of a cold chain could contribute to the increase of acceptance and sales of lettuce.

The last scenario (10) joined the cold chain with the “only washing” procedure to clean lettuce representing the safer and the riskier procedures, respectively. In tables 2 and 3 it is possible to observe that this scenario showed the lowest risk of infection by *Salmonella* and *E. coli* O157:H7 per month per serving when compared to the other scenarios with exception of scenario 9 (cold chain). Based on our results, the best alternative to reduce the risk of infection by *Salmonella* and *E. coli* O157:H7 is to keep lettuce refrigerated ≤5ºC and carry out washing and disinfection procedures established by Portaria 78/2009 (Rio Grande do Sul, 2009).

The sensitivity of the baseline model (scenario 1) outcomes to input values and model parameters, determined by Spearman’s rank order correlation, revealed that the mean number of illness cases per year was most sensitive to time of market storage (t5), for both pathogens (Figures 1A and 2A). In second place was temperature during producer storage (T1), followed by the *Salmonella* prevalence on lettuce (Figure 1A), while to *E. coli* O157:H7 occurred the opposite, prevalence in second place, followed by temperature during producer storage (T1) (Figure 2A). The same sensitivity analysis was applied to scenario 9 (cold chain), and revealed that the mean number of illness cases per year was most sensitive to reduction by washing and disinfection (Rw) and concentration of pathogens on lettuce for both bacteria (Figures 1B and 2B). Then, in addition to the maintenance of the cold chain and the washing and disinfection procedures, it is important to reduce the prevalence and concentration of pathogens on lettuce in the fields, in order to decrease the risk of infection by these bacteria.

More data are always needed to improve the accuracy of risk assessment models (Sant’Ana et al., 2014), including those developed here. Despite this need, the results obtained by our study demonstrated that *Salmonella* and *E. coli* O157:H7 represent measurable risks in lettuce in Southern Brazil. Our results suggest that *Salmonella* and *E. coli* O157:H7 risk of infection by lettuces can be best mitigated adopting cold chain to all steps of lettuce distribution and by performing correct washing and disinfection procedure before consumption.

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Table 1: The risk assessment models of infection by *Salmonella* and *E. coli* O157:H7 due to consumption of lettuce in Southern Brazil.

Symbol	Event	Values	Unities	Source
1- Producer storage				
Pi	Prevalence	*Salmonella* RiskBeta (127, 1589) *E. coli* O157:H7 RiskBeta (4, 102)	%	Elias et al., submitted
Ci	Concentration	*Salmonella* RiskPert (0, 0.66, 2.34) *E. coli* O157:H7 RiskPert (0, 0.47, 3.04)	Log MPN/g	Elias et al., submitted
T1	Temperature during storage1	RiskPert (3.55,18.12, 33.56)	°C	INMET, 2017
t1	Time of storage1	RiskPert (1, 10, 12)	H	Ascal, 2015
b	Parameter b growth model	*Salmonella* 0.0339 *E. coli* O157:H7 0.025	$\sqrt{\text{Log CFU/day/°C}}$	Elias et al., 2018
T0	Parameter T0 growth model	*Salmonella* 1.92 *E. coli* O157:H7 0.408	°C	Elias et al., 2018
Lg1	Logarithmic growth	*Salmonella* (0.0339x(If(T1 - T0) < 0,0, (T1 - T0)))2 *E. coli* O157:H7 (0.025x(If(T1 - T0) < 0,0, (T1 - T0)))2	log CFU/g/h	Elias et al., 2018
G1	Growth during storage1	t1xLg1	log cfu/g	Calculated
L1	Level after storage1	Ci+G1	log cfu/g	Calculated
2- Transportation from producer to distribution center				
T2	Temperature during transportation1	RiskPert (3.55,18.12, 33.56)	°C	INMET, 2017
t2	Time of transportation1	RiskPert(0.5, 1.8, 4)	H	Ascal, 2015
b	Parameter b growth model	*Salmonella* 0.0339 *E. coli* O157:H7 0.025	$\sqrt{\text{Log CFU/day/°C}}$	Elias et al., 2018
T0	Parameter T0 growth model	*Salmonella* 1.92 *E. coli* O157:H7 0.408	°C	Elias et al., 2018
Lg2	Logarithmic growth	*Salmonella* (0.0339x(If(T2 - T0) < 0,0, (T2 - T0)))2 *E. coli* O157:H7 (0.025x(If(T2 - T0) < 0,0, (T2 - T0)))2	log CFU/g/h	Elias et al., 2018
G2	Growth during transportation1	t2xLg2	log cfu/g	Calculated
L2	Level after transportation1	L1+G2	log cfu/g	Calculated
3- Arrival and storage at distribution center				
T3	Temperature during dc storage	RiskPert (10, 15, 20)	°C	Ascal, 2015
t3	Time of dc storage	RiskPert (1, 10, 12)	H	Ascal, 2015
b	Parameter b growth model	*Salmonella* 0.0339 *E. coli* O157:H7 0.025	$\sqrt{\text{Log CFU/day/°C}}$	Elias et al., 2018
T0	Parameter T0 growth model	*Salmonella* 1.92 *E. coli* O157:H7 0.408	°C	Elias et al., 2018
Lg3	Logarithmic growth	*Salmonella* (0.0339x(If(T3 - T0) < 0,0, (T1 - T0)))2 *E. coli* O157:H7 (0.025x(If(T3 - T0) < 0,0, (T1 - T0)))2	log CFU/g/h	Elias et al., 2018

G3	Growth during dc storage	t3xLg3	log cfu/g	Calculated
L3	Level after dc storage	L2+G3	log cfu/g	Calculated
4- Transportation from distribution center to market				
T4	Temperature during transportation2	RiskPert (3.55,18.12, 33.56)	°C	INMET, 2017
t4	Time of transportation2	RiskPert(0.05, 0.65, 4)	H	Ascal, 2015
b	Parameter b growth model	*Salmonella* 0.0339 *E. coli* O157:H7 0.025	$\sqrt{\text{Log CFU/day/°C}}$	Elias et al., 2018
T0	Parameter T0 growth model	*Salmonella* 1.92 *E. coli* O157:H7 0.408	°C	Elias et al., 2018
Lg4	Logarithmic growth	*Salmonella* (0.0339x(If(T4 - T0) < 0,0, (T1 - T0)))2 *E. coli* O157:H7 (0.025x(If(T4 - T0) < 0,0, (T1 - T0)))2	log CFU/g/h	Elias et al., 2018
G4	Growth during transportation2	t4xLg4	log cfu/g	Calculated
L4	Level after transportation2	L3+G4	log cfu/g	Calculated
5- Market storage				
T5	Temperature during market storage	RiskPert(20, 22.5 , 25)	°C	Ascal, 2015; Missiaen, 2015
t5	Time of market storage	RiskUniform(0,17)	H	Ascal, 2015; Missiaen, 2015
b	Parameter b growth model	*Salmonella* 0.0339 *E. coli* O157:H7 0.025	$\sqrt{\text{Log CFU/day/°C}}$	Elias et al., 2018
T0	Parameter T0 growth model	*Salmonella* 1.92 *E. coli* O157:H7 0.408	°C	Elias et al., 2018
Lg5	Logarithmic growth	*Salmonella* (0.0339x(If(T5 - T0) < 0,0, (T1 - T0)))2 *E. coli* O157:H7 (0.025x(If(T5 - T0) < 0,0, (T1 - T0)))2	log CFU/g/h	Elias et al., 2018
G5	Growth during market storage	t5xLg5	log cfu/g	Calculated
L5	Level after market storage	L4+G5	log cfu/g	Calculated
6- Transportation from retail to home				
T6	Temperature during transportation3	RiskPert(10,20,30)	°C	Assumption
t6	Time of transportation3	RiskGamma(5.24,8.17)/60	H	Nauta et al. (2003)
b	Parameter b growth model	*Salmonella* 0.0339 *E. coli* O157:H7 0.025	$\sqrt{\text{Log CFU/day/°C}}$	Elias et al., 2018
T0	Parameter T0 growth model	*Salmonella* 1.92 *E. coli* O157:H7 0.408	°C	Elias et al., 2018
Lg6	Logarithmic growth	*Salmonella* (0.0339x(If(T6 - T0) < 0,0, (T1 - T0)))2 *E. coli* O157:H7 (0.025x(If(T6 - T0) < 0,0, (T1 - T0)))2	log CFU/g/h	Elias et al., 2018
G6	Growth during transportation3	t6xLg6	log cfu/g	Calculated

L6	Level after transportation3	L5+G6	log cfu/g	Calculated
7- Home storage				
T7	Temperature during home storage	RiskPert(3.04,6,10.8)	°C	Silva et al., (2008)
t7	Time of home storage	RiskUniform(0,120)	H	Borghi et al, 2009
b	Parameter b growth model	*Salmonella* 0.0339 *E. coli* O157:H7 0.025	$\sqrt{\text{Log CFU/day/°C}}$	Elias et al., 2018
T0	Parameter T0 growth model	*Salmonella* 1.92 *E. coli* O157:H7 0.408	°C	Elias et al., 2018
Lg7	Logarithmic growth	*Salmonella* (0.0339x(If(T7 - T0) < 0,0, (T1 - T0)))2 *E. coli* O157:H7 (0.025x(If(T7 - T0) < 0,0, (T1 - T0)))2	log CFU/g/h	Elias et al., 2018
G7	Growth during home storage	t7xLg7	log cfu/g	Calculated
L7	Level after home storage	L6+G7	log cfu/g	Calculated
8- Washing and disinfection				
Rw	Log reduction by washing and disinfection	*Salmonella* RiskNormal(5.83,0.82) *E. coli* O157:H7 RiskNormal(6.27,0.50)	log cfu/g	Silveira et al., 2017; de Paula 2014
L8	Log concentration after washing	L7-Rw	log cfu/g	Calculated
9- Consumption of lettuce, determination of dose-response relationship, probability of illness and number of cases				
S	Serving size	RiskTriangle (20; 30; 50)	G	Carlos et al. 2008;
CFU	Level of pathogen (non-log)	10^L8	CFU/g	Calculated
D	Dose per serving	SxCFU	CFU	Calculated
α	Parameter alpha	*Salmonella* 0.1324 *E. coli* O157:H7 0.267	No units	WHO/FAO 2002 Cassin et al., 1998.
β	Parameter beta	*Salmonella* 51.45 *E. coli* O157:H7 229.2928	No units	WHO/FAO 2002 Cassin et al., 1998.
Pisd	Probability of infection single dose	1-(1+D/β)ˆ-α	%	Calculated
E	Exposure (number of servings/month)	RiskDiscrete({28\30\31},{0.083\0.417\0.5}	Servings	Mattos et al., 2000; Krzyzanowski et al., 2016; Souza et al, 2013
Rim	Risk of infection per month	RiskOutput() + 1 - (1 - Pi x Pisd)^E		Calculated
	Population RS	11322895	Inhabitants	IBGE, 2017
%eat	% of population eating lettuce	16	%	Souza et al, 2013
Peat	Population RS eating lettuce	1811663.2	Inhabitants	Calculated
Nc	Number of cases in population exposed	RimxPeat		Calculated

Table 2: Outputs of the QMRA model depicting the risk of infection per month per serving and number of cases of infection per month in the population exposed due to consumption of lettuce contaminated with *Salmonella* in Southern Brazil[a].

Scenarios		Risk of infection per month per serving		Number of cases of infection per month in the population exposed	
		Mean	Upper 95%	Mean	Upper 95%
1	Real	0.017183	0.023348	31129.81	42298.71
2	-50% prevalence	0.007467	0.009571	13527.69	17339.43
3	-50% concentration	0.016629	0.023228	30126.15	42081.31
4	-50% prevalence and concentration	0.007233	0.009527	13103.76	17259.72
5	-50% exposure	0.008493	0.011531	15386.46	20890.29
6	Only washing	0.02105	0.024645	38135.51	44648.44
7	1 min disinfection	0.018075	0.023609	32745.81	42771.56
8	5 min disinfection	0.018881	0.023844	34206.01	43197.3
9	Cold chain	3.16E-07	1.03E-06	0.572486	1.866013
10	Cold chain and only washing	0.00185	0.004781	3351.577	8661.562

[a]Each scenario was run in @Risk using 100,000 iterations with generator seed fixed at 1.

[b]Scenario 1 was run with data representing the real world. Scenario 2 represents change in prevalence of pathogen; scenario 3 represents change in concentration of pathogen; scenario 4 represents change in prevalence and concentration of pathogen. Scenario 5 represents change in exposure of population. Scenario 6 represents only lettuce washing; scenarios 7-8 represent change in time of disinfection. Scenario 9 represent strict temperature conditions during all steps of lettuce chain studied. Scenario 10 represent strict temperature conditions and the only lettuce washing.

Table 3: Outputs of the QMRA model depicting the risk of infection per month per serving and number of cases of infection per month in the population exposed due to consumption of lettuce contaminated with *E. coli* O157:H7 in Southern, Brazil.

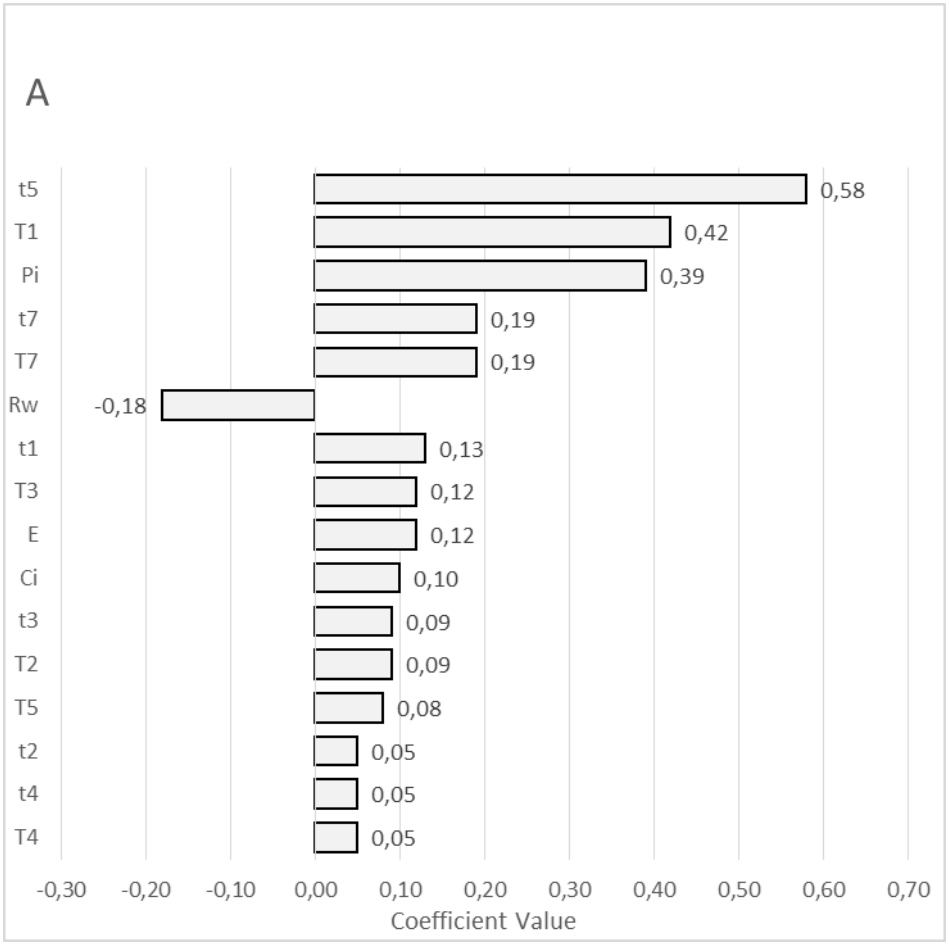
Scenarios		Risk of infection per month per serving		Number of cases of infection per month in the population exposed	
		Mean	Upper 95%	Mean	Upper 95%
1	Real	0.006093	0.016284	11038.46	29501.12
2	-50% prevalence	0.003218	0.003713	5829.932	6726.705
3	-50% concentration	0.005446	0.015584	9866.318	28232.96
4	-50% prevalence and concentration	0.003162	0.003713	5728.479	6726.705
5	-50% exposure	0.002988	0.007985	5413.25	14466.13
6	Only washing	0.010835	0.020848	19629.37	37769.55
7	1 min disinfection	0.009852	0.019787	17848.51	35847.38
8	5 min disinfection	0.010228	0.020158	18529.69	36519.51
9	Cold chain	2.27E-08	7.81E-08	0.041125	0.141491
10	Cold chain and washing	0.000461	0.001892	835.1767	3427.667

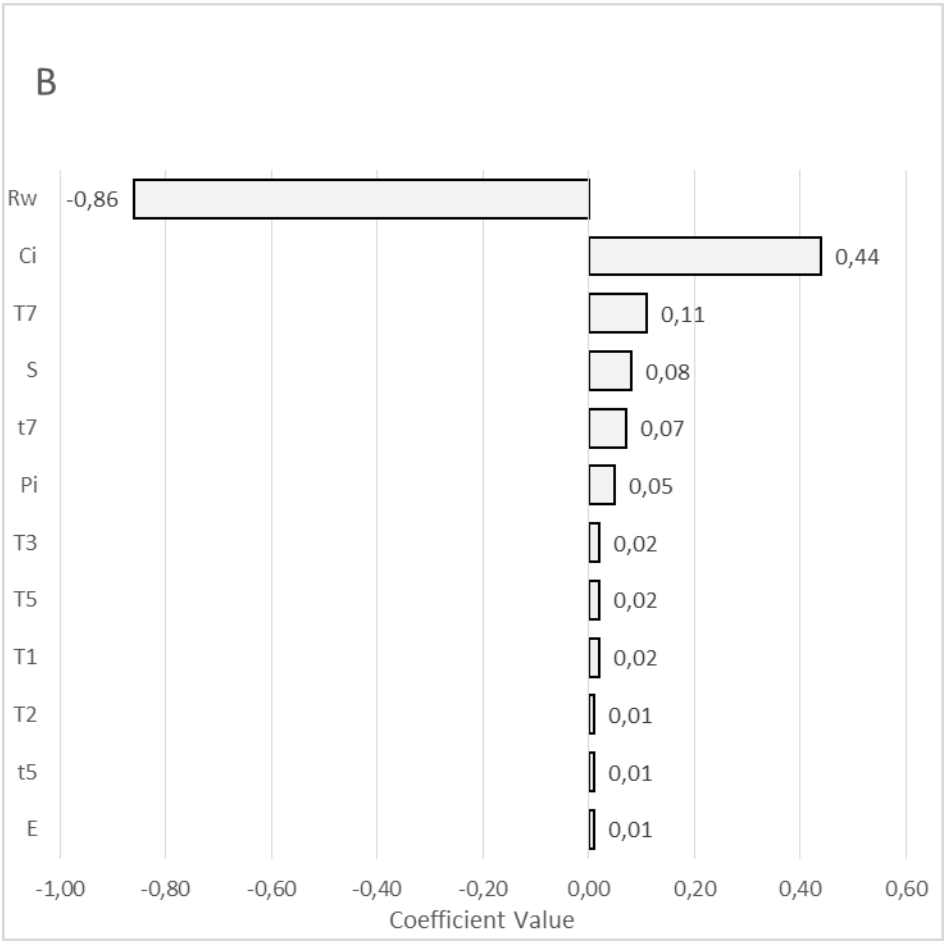
[a]Each scenario was run in @Risk using 100,000 iterations with generator seed fixed at 1.

[b]Scenario 1 was run with data representing the real world. Scenario 2 represents change in prevalence of pathogen; scenario 3 represents change in concentration of pathogen; scenario 4 represents change in prevalence and concentration of pathogen. Scenario 5 represents change in exposure of population. Scenario 6 represents only lettuce washing; scenarios 7-8 represent change in time of disinfection. Scenario 9 represent strict temperature conditions during all steps of lettuce chain studied. Scenario 10 represent strict temperature conditions and the only lettuce washing.

Figure 1. Tornado graph showing the most important parameters and variables affecting the estimated number of illness cases per month due to consumption of *Salmonella*–contaminated lettuce. Spearman's correlation coefficients were obtained from @Risk sensitivity analyses and were shown next to each bar.

A: real-world scenario; B: cold-chain scenario.




B
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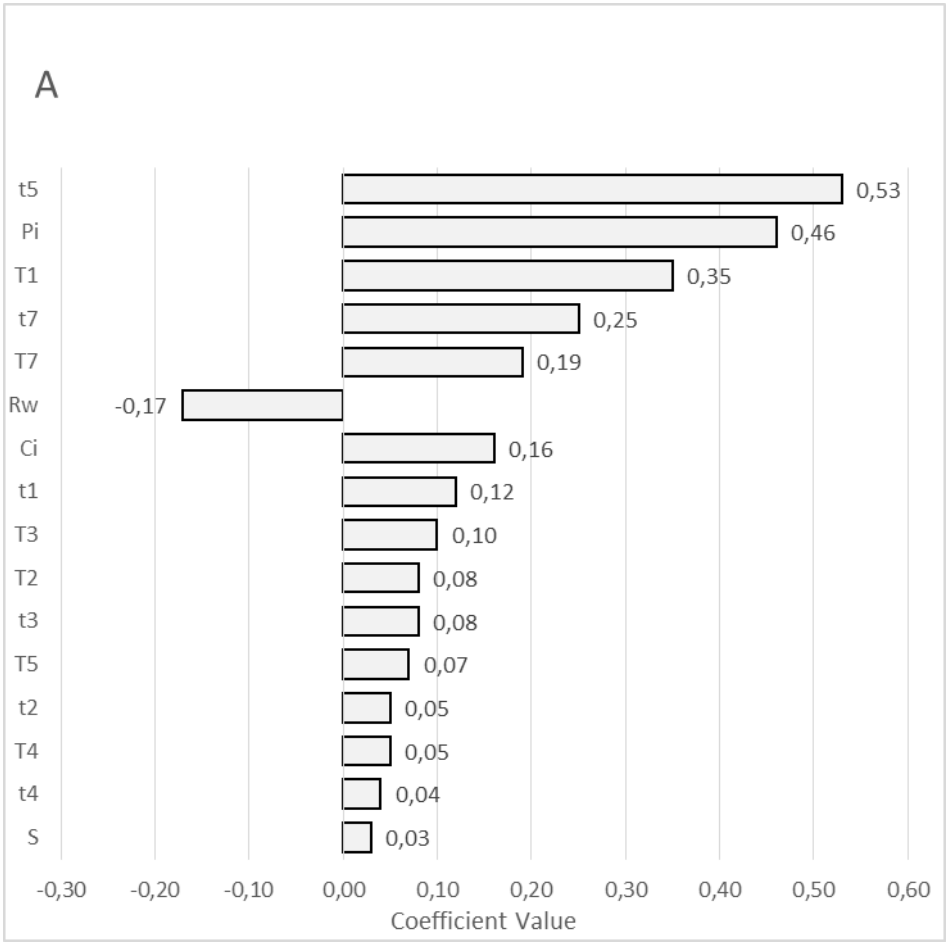
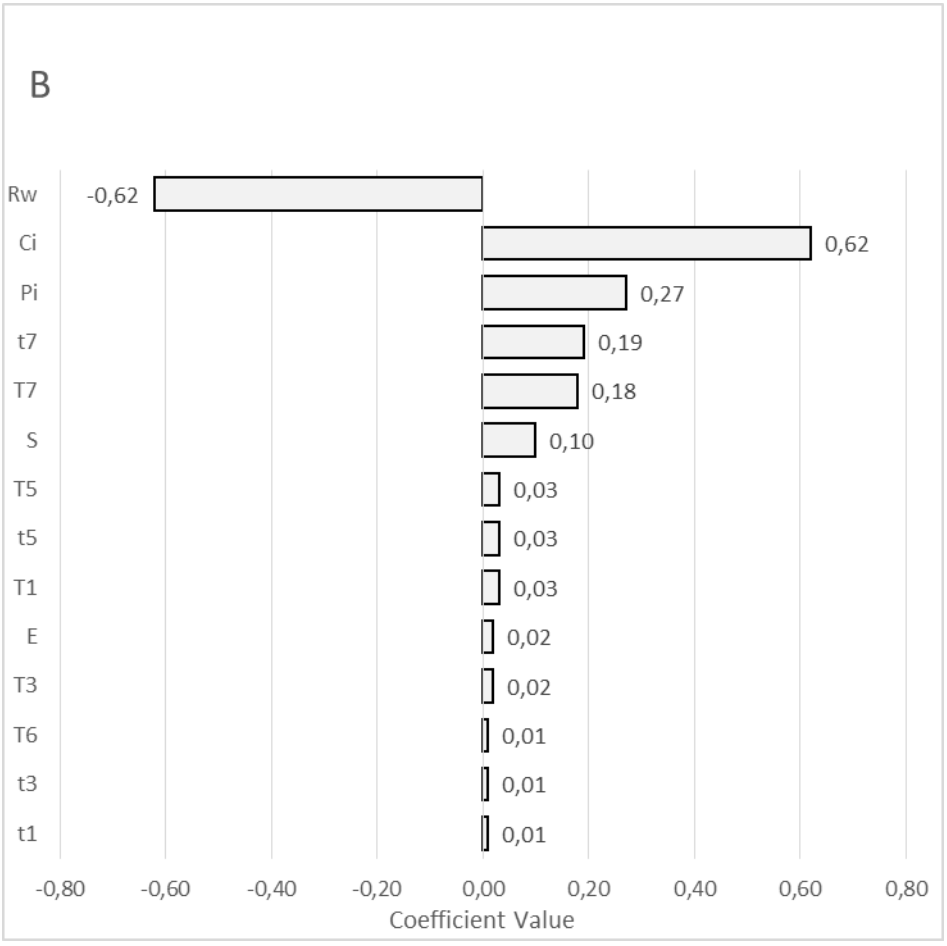
0,60
Coefficient Value

Figure 2. Tornado graph showing the most important parameters and variables affecting the estimated number of illness cases per month due to consumption of *E. coli* O157:H7–contaminated lettuce. Spearman's correlation coefficients were obtained from @Risk sensitivity analyses and were shown next to each bar.

A: real-world scenario; B: cold-chain scenario.




B
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-0,80
-0,60
-0,40
-0,20
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0,60
0,80
Coefficient Value

4.5. Resultados não apresentados na forma de artigo.

Semi-quantitative risk assessment for main pathogens on lettuce: risk estimation by the Risk Ranger®

The identification of pathogens involved in foodborne diseases with leafy vegetables was carried out using scientific literature and expert opinion. Literature searches were performed using PubMed database to identify potentially relevant publications, prioritizing peer-reviewed journals in English. The keywords included in the literature search were: lettuce, leafy vegetables, leafy greens, salad and foodborne outbreaks. The published studies dates were between January 1, 2000 and January 1, 2016.

The Risk Ranger® software was used to develop a first estimate of relative risk of identified pathogens involved in vegetable outbreaks. This tool was developed to be generic but robust, and to include all elements that affect food safety risks; it was downloaded from: http://www.foodsafetycentre.com.au/riskranger.php (Ross & Sumner, 2002; Sumner et al., 2005).

The criteria considered for the evaluation of the pathogens on lettuce consisted of occurrence of reported foodborne outbreaks associated with fresh leafy vegetables consumption. About one hundred scientific articles were found in the database. Only 10 studies were chosen to select the pathogens, since they obeyed the prerequisites established previously. The microbials selected were *E. coli* O157:H7, *Salmonella* spp, *Listeria monocytogenes*, *Campylobacter* spp, *Shigella* spp, *Clostridium perfringens*, Norovirus, Hepatitis A virus, *Cryptosporidium parvum*, *Cyclospora* spp and *Giardia* spp.

The semi-quantitative risk assessment for the main pathogens identified was carried out using Risk Ranger®. This is a tool which can be used to give weight to the different risks in the evaluated food and aid to pre-screen the risks that certain pathogens can represent to this food (Ross & Sumner, 2002). In this study, it was used as a preliminary assessment for the safety of lettuce, identifying the main foodborne pathogens that represent risk for the lettuce production chain.

The program is composed by 11 questions divided in 3 blocks. The first block has 2 questions about the hazard severity of the pathogen evaluated and the susceptibility of the population that will eat the food. The second block enquires

about frequency of consumption, proportion and size of the population that will consume this food. In this assessment, it was assumed that lettuce was consumed weekly and by most (75%) population in Brazil (Coelho & Rodrigues, 2007). Also, the size of Brazilian population assumed was 206,081,432 people (IBGE, 2016). The third block of Risk Ranger focuses on the probability of contamination of raw product per serving and in the processing until consumption. It was assumed that the effect of processing on lettuce slightly reduces the hazards, such as, the effect of preparation before eating; also, there is a minor potential for recontamination after processing; and the post-processing control system is controlled (Jensen et al., 2013; Jensen et al., 2015; Prado-Silva et al.,2015; Sant'Ana et al., 2014). The results of the evaluated pathogens and the selected input values are shown in Table 1 and 2.

For the pathogens, *Campylobacter* spp, *C. perfringens*, *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* spp, *Shigella* spp, (Table 1) and *C. parvum*, *Cyclospora* spp, *Giardia* spp, Hepatitis A virus and Norovirus (Table 2), the severity, susceptible population and infective dose were chosen in agreement of their characteristics (Forsythe, 2013). While the probability of contamination of lettuce was determined by scientific literature (Berger et al, 2010; Buss et al., 2016; Doyle & Erickson, 2008; Duedu et al., 2014; Eraky et al., 2014; Loutreul et al., 2014; Mohamed et al., 2016; Olaimat & Holley, 2012; Pérez-Rodríguez et al., 2014; Sant'Ana et al., 2014; Yoon et al., 2009).

The outputs of the program were the probability of illness per day per consumer, the total predicted illnesses/annum in population of interest and the risk ranking. Using Norovirus results as an example (Table 2), the first output was $1.07x10^{-4}$, meaning that one person in 10,000 people would become ill per day, due to consumption of lettuce contaminated with Norovirus. The second output estimated 570,000 illnesses per year, meaning that this quantity of viruses would happen considering a population of approximately 206,000,000 people. Tables 1 and 2 show the risk ranking for the identified pathogens. The highest risk rankings were observed for *Salmonella* spp. and *E. coli* O157:H7 which scored 67, followed by the parasites with 63, *Campylobacter* spp, *L. monocytogenes*, *Shigella* spp, and Hepatitis A virus with 61, Norovirus with 60, and finally *C. perfringens* with 44. The risk ranking values are interpreted as a semi-logarithmic scale designed by the authors of the program, where the maximum rank (100) represents that all the meals are contaminated with a

lethal dose and consumed daily. While the lowest rank (0) would mean that one meal in ten billion will be contaminated with a lethal dose and consumed within one hundred years. Also, an increment of six in the ranking corresponds approximately to a 10-fold increase in risk (Sumner et al., 2005).

For this study, *E. coli* O157:H7 and *Salmonella* spp. were selected to perform in-deep studies (articles 2, 3 and 4). These pathogens had the greatest risk ranking and are the most involved bacteria in outbreaks associated with leafy vegetables (Callejón et al., 2015; Jung et al., 2014; Wadamori et al., 2017).

Table 1: Risk Ranger®[a] applied to lettuce and bacterial pathogens.

Risk Ranger parameters	*Campylobacter*	*Clostridium perfringens*	*E. coli* O157:H7	*Listeria monocytogenes*	*Salmonella*	*Shigella*
Hazard severity	Moderate	Mild	Moderate	Severe	Moderate	Moderate
How susceptible is the population of interest?	General	General	General	Slight	General	General
Frequency of consumption	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly
Proportion of population consuming the product	Most	Most	Most	Most	Most	Most
Size of consuming population	206,081,432[b]	206,081,432	206,081,432	206,081,432	206,081,432	206,081,432
Probability of contamination of raw product per serving	1%	1%	1%	1%	1%	1%
Effect of processing	Reduces	Reduces	Reduces	Reduces	Reduces	Reduces
Is there potential for recontamination after processing?	Yes – minor 1%	Yes – minor 1%	Yes – minor 1%	Yes – minor 1%	Yes – minor 1%	Yes – minor 1%
How effective is the post processing control system?	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled
What increase in the post-processing contamination level would cause infection or intoxication to the average consumer?	Moderate	Significant	Slight	Significant	Slight	Moderate
Effect of preparation before eating	Slightly reduces	Slightly reduces	Slightly reduces	Slightly reduces	Slightly reduces	Slightly reduces
Probability of illness per day per consumer of interest	$2.14\ 10^{-5}$	$2.14\ 10^{-7}$	$2.14\ 10^{-4}$	$1.07\ 10^{-6}$	$2.14\ 10^{-4}$	$2.14\ 10^{-5}$
Total predicted illnesses/ annum in population of interest	$1.14\ 10^{3}$	$1.14\ 10^{3}$	$1.14\ 10^{4}$	$1.14\ 10^{3}$	$1.14\ 10^{4}$	$1.14\ 10^{3}$
Risk ranking	61	44	67	61	67	61

[a] http://www.foodsafetycentre.com.au/docs/RiskRanger.xls.

[b] Population in Brazil at 31/08/2016. http://www.ibge.gov.br/home/mapa_site/mapa_site.php#populacao (Last visited 16th/Feb/2017).

Table 2: Risk Ranger®[a] applied to lettuce associated with viral and parasite pathogens.

Risk Ranger parameters	*Cryptosporidium parvum*	*Cyclospora*	*Giardia*	*Hepatitis A virus*	*Norovirus*
Hazard severity	Moderate	Moderate	Moderate	Moderate	Mild
How susceptible is the population of interest?	Extreme	Extreme	Extreme	General	General
Frequency of consumption	Weekly	Weekly	Weekly	Weekly	Weekly
Proportion of population consuming the product	Most	Most	Most	Most	Most
Size of consuming population	206,081,432[b]	206,081,432	206,081,432	206,081,432	206,081,432[b]
Probability of contamination of raw product per serving	0.1%	0.1%	0.1%	0.1%	10%
Effect of processing	Reduces	Reduces	Reduces	Reduces	Reduces
Is there potential for recontamination after processing?	Yes – minor 1%	Yes – minor 1%	Yes – minor 1%	Yes – minor 1%	Yes – minor 1%
How effective is the post processing control system?	Controlled	Controlled	Controlled	Controlled	Controlled
What increase in the post-processing contamination level would cause infection or intoxication to the average consumer?	Slight	Slight	Slight	Moderate	Moderate
Probability of illness per day per consumer of interest	$4.27\ 10^{-2}$	$4.27\ 10^{-2}$	$4.27\ 10^{-2}$	$2.14\ 10^{-5}$	$1.07\ 10^{-4}$
Total predicted illnesses/ annum in population of interest	$2.28\ 10^{5}$	$2.28\ 10^{5}$	$2.28\ 10^{5}$	$1.14\ 10^{5}$	$5.70\ 10^{5}$
Risk ranking	63	63	63	61	60

[a] http://www.foodsafetycentre.com.au/docs/RiskRanger.xls.

[b] Population in Brazil at 31/08/2016. http://www.ibge.gov.br/home/mapa_site/mapa_site.php#populacao (Last visited 16th/Feb/2017).

5. DISCUSSÃO GERAL

O consumo de vegetais e de frutas é cada vez maior em nível mundial, sendo que o número de surtos alimentares associados a esses produtos também tem aumentado mundialmente. No período de 2008 a 2014, foram notificados 30 surtos alimentares envolvendo esses alimentos no Brasil. Esse valor foi similar a alguns países como Canadá e Nova Zelândia, mas abaixo dos valores encontrados em outros, como, por exemplo, Estados Unidos e Japão. A subnotificação dos surtos alimentares é um fenômeno mundial e, mesmo em países cujo sistema de vigilância sanitária é bem estruturado, o número de surtos notificados é sempre menor do que o número total de ocorrências de DTA (Kozak, et al., 2013; Nsoesie et al., 2014; Wadamori et al., 2017). Em relação aos surtos ocorridos no Brasil, o agente patogênico mais identificado foi *Salmonella,* enquanto que apenas um dos 30 surtos foi relacionado unicamente a ingestão de alface contaminada. A padronização na classificação do tipo de alimento envolvido no surto é um grande problema no Brasil. Na maioria dos surtos notificados, a identificação foi realizada de modo bem genérico, utilizando a nomenclatura “frutas e vegetais”. Também foram utilizadas expressões como “salada crua ou cozida”, ou simplesmente salada. Isso pode ter contribuído para o baixo número de surtos identificados, relacionados unicamente a alface. Além disso, *E. coli* O157:H7 não foi identificada em nenhum desses surtos, o que pode ter ocorrido devido ao fato da análise desse micro-organismo não ser realizada na rotina dos laboratórios que recebem as amostras provenientes de surtos alimentares. Dessa forma, esforços devem ser feitos para melhorar o sistema de notificação e de investigação de surtos, bem como as capacidades laboratoriais para que as amostras biológicas e de alimentos possam ser coletadas de forma rápida e correta para identificar o agente etiológico causador dos surtos.

Salmonella e *E. coli* O157:H7, os principais patógenos relacionados aos surtos devido à ingestão de alface em nível mundial, foram inoculados separadamente sobre alfaces e se multiplicaram em todas as temperaturas examinadas, tanto em condições isotérmicas, quanto em temperaturas variáveis. Então, os modelos desenvolvidos nesse estudo, para ambos os patógenos, foram capazes de predizer a multiplicação de *Salmonella* spp. e de *E. coli* O157:H7 na alface, nas faixas de temperatura de 5 a 40ºC e de 5 a 42ºC, respectivamente.

Esses modelos foram utilizados para prever o comportamento de ambos os patógenos, simulando a distribuição das alfaces (da fazenda ao varejo), sem cadeia de frio e com temperaturas de verão no Rio Grande do Sul. Assim, após 30h nesse cenário, *Salmonella* atingiu 8 log UFC/g, enquanto que *E. coli* O157:H7 alcançou 7 log UFC/g, totalizando aproximadamente 5,5 log UFC/g e 3 log UFC/g de aumento na população, respectivamente. Esse menor incremento na população de *E. coli* O157:H7 pode ter ocorrido porque o inóculo inicial deste experimento foi maior que o de *Salmonella*, permitindo que *E. coli* O157:H7 pudesse se multiplicar menos, uma vez que ambos os agentes patogênicos atingiram a sua densidade populacional máxima. Esse inóculo maior para *E. coli* O157:H7 foi adotado, visto que essa bactéria não é uma boa competidora e como os experimentos foram realizados inoculando os patógenos na alface sem estar higienizada, os agentes patogênicos deveriam competir com a microbiota do vegetal (Paula et al., 2014).

Além disso, nesse estudo foi desenvolvido um parâmetro chamado de tempo de multiplicação insignificante (ς) que fornece o tempo máximo em que a alface pode ser exposta a uma temperatura específica e não apresentar uma multiplicação expressiva dos patógenos estudados. O ς foi desenvolvido com base na equação do modelo primário de Baranyi (Baranyi & Roberts 1994) e no conceito de potencial de crescimento (Sant'Ana et al., 2012), ou seja, ς é o valor da fase lag adicionado do tempo necessário para população microbiana aumentar 0,5 log UFC/g. O ς da alface exposta a 37 °C foi de 1,3 h, enquanto que a 5 °C foi de 3,3 dias. Assim, é recomendado manter a alface sob refrigeração ao longo de sua cadeia de produção, distribuição até seu consumo, visto que os possíveis tratamentos de higienização aplicáveis antes de ser consumida não serão capazes de eliminar completamente os perigos microbianos que podem se multiplicar rapidamente, dependendo da temperatura de armazenamento. Também, o ς pode ser aplicado em estudos futuros para qualquer tipo de matriz alimentar, apenas sendo necessário que se utilize o modelo de Baranyi para calcular os parâmetros de multiplicação.

Além dos modelos preditivos adequados os dados de prevalência e de concentração dos patógenos são essenciais para realizar uma avaliação de risco quantitativa. Assim, como esses dados são escassos, foi realizada uma revisão sistemática e meta-análise da prevalência e da concentração de *Salmonella* e de *E.*

coli O157:H7 em alface. Os valores de prevalência e de concentração dos patógenos utilizados no modelo de avaliação de risco corresponderam aos estudos realizados nos países em desenvolvimento e essa escolha foi realizada devido à semelhança sócio-econômica dessas nações com o Brasil.

A prevalência média de *E. coli* EHEC na alface foi de 0,041 (95% IC: 0,005–0,078) em nível mundial, já nos países em desenvolvimento foi de 0,024 (95% IC: -0,005-0,053), e a concentração foi igual a < 3,0 NMP/g até > 1100 NMP/g. A prevalência média de *Salmonella* em alface foi de 0,041 (95% IC: 0,030–0,052) mundialmente, enquanto que em países em desenvolvimento foi de 0,064 (95% IC: 0,041–0,087). Em relação à concentração de *Salmonella* em alface, os valores variaram de 0,054 ± 0,058 contagens de células viáveis/g até 218,78 NMP/g, mundialmente, e nos países em desenvolvimento ela variou de 4,57 a 218,78 NMP/g. Além disso, apenas um estudo relatou a detecção de *E. coli* O157:H7 em alface, por isso se utilizou *E. coli* EHEC, grupo no qual *E. coli* O157:H7 faz parte. Também somente um trabalho quantificou *E. coli* EHEC na alface. Ao se considerar *Salmonella,* mais estudos foram encontrados tanto de prevalência, quanto de concentração na alface, mesmo assim esses dados não foram abundantes, indicando a necessidade de mais trabalhos focados nesse tema para possibilitar pesquisas mais aprofundadas como outras avaliações de risco microbiológico.

O modelo de avaliação quantitativa de risco microbiológico proposto nesse estudo foi composto por nove módulos: armazenamento no produtor; transporte do produtor para o centro de distribuição; chegada e armazenamento no centro de distribuição; transporte do centro de distribuição para o mercado; armazenamento no mercado; transporte do varejo para casa do consumidor; armazenamento doméstico; lavagem e desinfecção; consumo de alface, determinação da relação dose-resposta, probabilidade de infecção e número de casos. O estudo não pôde avaliar as operações do campo (desde a aquisição das sementes/mudas até a colheita), pois faltavam muitos dados em relação à produção primária da alface, o que aumentaria as incertezas do modelo, inviabilizando sua construção. Mesmo com limitações de dados, o presente estudo é importante, visto que poucas AQRM são realizadas em países desenvolvidos ou em desenvolvimento e que esses estudos são uma alternativa valiosa quando os dados de vigilância são escassos, pois o desenvolvimento de uma AQRM oferece uma abordagem científica

para a gestão de riscos, fornecendo, muitas vezes, as opções mais eficazes para a resolução de problemas de segurança de alimentos (Abia et al., 2016; Enger et al., 2012; Murmann et al., 2011; Pouillot et al., 2012).

O risco de infecção por *Salmonella* por mês foi de 0,017, ou seja, a cada 1000 pessoas que consumirem alface 17 irão encontrar esse alimento contaminado com esse patógeno; enquanto que por *E. coli* O157:H7 foi de 0,006 (6 em cada 1000), considerando o cenário baseado nos dados mais comumente encontrados no Rio Grande do Sul. Assim, de modo geral o risco de infecção por *Salmonella* é maior do que por *E. coli* O157:H7 quando a alface é consumida. Isso pode ter ocorrido devido à prevalência de *Salmonella* ser maior do que a de *E. coli* O157:H7 na alface, também as taxas de multiplicação daquele patógeno foram maiores do que as deste micro-organismo na maioria das temperaturas examinadas. Além desse cenário que foi considerado “mundo-real” por representar o conhecimento atual sobre a alface e sobre os patógenos estudados, foram criados outros nove cenários: redução da prevalência dos patógenos; redução da concentração dos patógenos; redução da prevalência e da concentração dos patógenos; redução da exposição da população que consume alface; realização apenas do procedimento de lavagem da alface; realização da lavagem da alface mais 1 min de desinfecção e enxague em água potável; realização da lavagem da alface mais 5 min de desinfecção e enxague em água potável; cadeia de frio (até 5ºC) durante todos os módulos do modelo; combinação da cadeia de frio com a realização apenas do procedimento de lavagem da alface.

Todos os cenários alternativos à correta higienização da alface preconizada na Portaria 78/2009 (Rio Grande do Sul, 2009), aumentam o risco de infecção pelos patógenos. Então, o procedimento que deve ser seguido é lavar as folhas com água potável, após imergi-las em 200 ppm de Cloro livre por 15 min e enxaguar com água potável. Além disso, a principal redução do risco foi por meio do cenário da cadeia de frio durante todos os módulos do modelo. Dessa forma, recomenda-se fortemente que a cadeia de frio seja adotada na distribuição e armazenamento das alfaces desde o produtor até a casa do consumidor, o que aumentaria não só a segurança ao consumir esse alimento, mas também a qualidade e a vida de prateleira da alface.

As análises de sensibilidade realizadas indicaram que, além da

manutenção da cadeia de frio e do procedimento correto de higienização, é importante reduzir a prevalência e a concentração dos patógenos na alface, a fim de diminuir o risco de infecção por essas bactérias. Assim com a adoção de todas essas medidas o risco de infecção por esses patógenos irá diminuir consideravelmente.

Por fim, a avaliação de risco desenvolvida no presente trabalho pode auxiliar no desenvolvimento de estratégias de intervenção para mitigar esse risco. Esses resultados podem ser utilizados pelos profissionais da área alimentar e por órgãos públicos, como, por exemplo, a Vigilância Sanitária na definição de estratégias para conscientização dos consumidores e dos manipuladores de alimentos e para fornecer bases científicas para preparação e para implementação de normas e de legislações. Essas ações podem impactar grandemente a saúde pública, já que a implementação de programas e de ações cientificamente embasados, podem contribuir para a redução dos surtos. Assim, menos recursos financeiros públicos e privados seriam gastos, além de se reduzir impactos sociais, como a mortalidade, associados às DTA.

6. CONCLUSÃO E CONSIDERAÇÕES FINAIS

Foram identificados 30 surtos notificados associados ao consumo de frutas e de vegetais no Brasil no período entre 2008 a 2014, sendo que *Salmonella* foi o agente etiológico mais identificado e a alface foi relacionada a um desses surtos. Também *Salmonella* spp. e *Escherichia coli* O157:H7 foram identificados como os principais patógenos envolvidos em DTA, veiculadas por alface mundialmente.

Assim modelos preditivos de multiplicação de *Salmonella* spp. e de *E. coli* O157:H7 em alface foram desenvolvidos numa ampla faixa de temperatura (5 a 42ºC), permitindo sua utilização para modelagem do comportamento desses patógenos em modelos de avaliação de risco, representando condições sob as quais a alface pode ser exposta desde sua produção até o consumo. Dados experimentais mostraram que ambas as bactérias se multiplicam em todas as temperaturas examinadas.

Também foi proposto um parâmetro (ς) para fornecer o tempo que a alface pode ser exposta a uma certa temperatura e ainda ser considerada segura para o consumo, considerando os patógenos estudados. O ς da alface exposta a 37 °C foi de 1,3 h, enquanto que a 5 °C foi de 3,3 dias.

A fim de realizar a avaliação quantitativa do risco de infecções causadas por *Salmonella* spp. e por *E. coli* O157:H7 a partir do consumo de alface no Rio Grande do Sul, foi necessário determinar a prevalência e a concentração desses patógenos na alface, por meio de uma revisão bibliográfica. A prevalência nos países em desenvolvimento foi de 0,064 para *Salmonella* e de 0,024 para *E. coli* (EHEC). Em relação à concentração de *Salmonella* em alface, nos países em desenvolvimento variou de 4,57 a 218,78 NMP/g, e para *E. coli* (EHEC) a concentração foi de < 3,0 NMP/g até > 1100 NMP/g.

Os modelos de avaliação de risco construídos indicaram que ambos os patógenos, especialmente *Salmonella*, representam um desafio para a segurança da alface consumida no Rio Grande do Sul. Apesar de mais dados serem necessários para melhorar a precisão dos modelos, os resultados obtidos já são suficientes para que mais atenção e, posteriormente, estratégias de gestão de risco sejam desenvolvidas e implementadas para reduzir os riscos de infecção por esses

patógenos devido ao consumo de alface. Isso é particularmente preocupante devido ao fato desse alimento ser consumido cru e da dose infectante de ambos os patógenos poder ser baixa. Também a análise dos cenários demostrou a importância do procedimento de higienização ser realizado conforme previsto na legislação (lavar as folhas com água potável seguido de imersão em 200 ppm de Cloro livre por 15 minutos e enxaguar com água potável). Além disso, a principal redução do risco foi com o cenário da cadeia de frio durante todos os módulos do modelo, deixando claro que pequenas variações de temperatura podem contribuir para o aumento da probabilidade de infecções. Dessa forma, ratifica-se a importância do treinamento e da conscientização dos envolvidos em toda a cadeia produtiva e de distribuição sobre o controle da temperatura como estratégia para garantir alimentos seguros. As análises de sensibilidade indicaram que, além da manutenção da cadeia de frio e do procedimento correto de higienização, é importante reduzir a prevalência e a concentração dos patógenos na alface, a fim de diminuir o risco de infecção por essas bactérias, o que poderia auxiliar nessa redução é a implementação das boas práticas agrícolas, que juntamente com a adoção da cadeia de frio iriam reduzir consideravelmente tanto a prevalência, quanto a concentração dos patógenos na alface.

Por fim, a avaliação de risco desenvolvida nessa tese pôde auxiliar no desenvolvimento de estratégias de intervenção e gestão para mitigar esse risco. Entretanto, os modelos desenvolvidos deverão ser atualizados com a geração de novos dados, o que levará a melhoria de suas predições.

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