UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA

AGRÍCOLA E DO MEIO AMBIENTE

ENVOLVIMENTO DOS GENES *rpoS* E *dps* NA RESISTÊNCIA AO HIPOCLORITO DE SÓDIO E DO GENE *ompR* NA RESISTÊNCIA TÉRMICA DE *Salmonella* Enteritidis SE86

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Abril de 2012

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"Se eu vi mais longe, foi por estar de pé sobre ombros de gigantes".

Isaac Newton

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A pessoa mais otimista do mundo, meu maridão, meu amor, meu amigo Rafael: pela paciência, pelas conversas, pela ajuda com a tese, pelo companheirismo, pela tranquilidade, pela força diária e por me fazer acreditar. Te amo!

A todos que de alguma forma contribuíram para realização deste trabalho e para minha evolução pessoal.

Envolvimento dos genes *rpoS e dps* na resistência ao hipoclorito de sódio e do gene *ompR* na resistêncía térmica de *Salmonella* Enteritidis SE86[1]

Autor: Ana Carolina Ritter
Orientador: Eduardo César Tondo

RESUMO

Salmonella é um dos principais agentes de Doenças Transmitidas por Alimentos (DTA) em todo o mundo. No Rio Grande do Sul, uma cepa de *Salmonella* Enteritidis vem sendo identificada como o principal agente causador de DTA na última década, a qual foi nominada SE86. Quando comparada com outros sorovares de *Salmonella*, a SE86 demonstrou maior capacidade de adaptação ácida e térmica, além de maior resistência contra diferentes sanificantes. O presente estudo teve como objetivo investigar o envolvimento dos genes *rpoS e dps* na resistência ao hipoclorito de sódio e do gene *ompR* na adaptação ácida e resistência térmica da cepa *S.* Enteritidis SE86. Os genes avaliados foram atenuados pela técnica de *Knockout* e a expressão dos mesmos foi investigada pela técnica *Epitope Tagging* - 3 x FLAG. Os resultados demonstraram que a cepa mutante SE86, sem a presença do gene *dps* (Δ*dps*), demonstrou uma maior sensibilidade em relação à cepa SE86 *Wild Type* (WT), após a exposição ao hipoclorito de sódio a 200 ppm. As proteínas Rpos e Dps foram ativamente expressas nas mesmas condições de exposição ao hipoclorito de sódio, demonstrando assim a relação destes genes com o estresse oxidativo provocado por hipoclorito de sódio na SE86. O envolvimento do gene *ompR* na resistência térmica foi investigado após a adaptação ácida de SE86 WT e do mutante Δ*ompR.* As células ácido adaptadas do mutante Δ*ompR* foram completamente inativadas após 240 minutos de exposição a temperatura de 52º C, enquanto que as células WT resistiram até 300 minutos de exposição. A expressão da proteína OmpR foi observada por *Western blotting* e confirmou a relação da adaptação ácida com a maior resistência térmica da SE86.

Involvement of *rpoS* and *dps* genes in the resistance to sodium hypochlorite and *ompR* gene in the thermal resistance of *Salmonella* Enteritidis SE86[1]

Author: Ana Carolina Ritter
Advisor: Eduardo César Tondo

ABSTRACT

Salmonella is one of the main agents of foodborne diseases worldwide. In Rio Grande do Sul, a clone of *Salmonella* Enteritidis has been identified as the main cause of foodborne diseases in the last decade, which was named SE86. The the present study aimed to investigate the involvement of *rpoS* and *dps* genes in resistance to sodium hypochlorite and *ompR* gene in acid adaptation and thermal resistance of the strain *S*. Enteritidis SE86. The genes evaluated were attenuated by *knockout technique* and their expression was determined by tagged (3XFLAG) strains. The results demonstrated that strain *S*. Enteritidis SE86 without the presence of *dps* gene (Δ*dps*) showed a higher sensitivity to the wild type strain SE86 (WT) when exposed to sodium hypochlorite at 200 ppm. Furthermore, RpoS and Dps proteins were actively expressed in these experimental conditions, thus demonstrating the relationship of these genes with oxidative stress in *S*. Enteritidis SE86 caused by sodium hypochlorite. The involvement of *ompR* gene in thermal resistance was observed after the acid adaptation of *S*. Enteritidis SE86 WT and Δ*ompR* since cells adapted acid without the presence of *ompR* have been completely inactivated when exposed to 240 min at temperature of 52 °C, while WT cells resisted until 300 min of exposure. The expression of the OmpR protein by western blotting confirmed the necessity of the acid adaptation for that *S*. Enteritidis SE86 resists to high temperatures.

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1 INTRODUÇÃO

Salmonella é responsável por sérios problemas de saúde pública e significativas perdas econômicas, em todo o mundo. No Rio Grande do Sul (RS), *Salmonella* (*S.*) tem sido identificada como o principal microrganismo responsável pelas Doenças Transmitidas por Alimentos (DTA) por quase duas décadas.

Embora diversos sorovares de *Salmonella* tenham sido encontrados em diferentes alimentos no RS, o sorovar Enteritidis foi identificado como o causador de mais de 95% das salmoneloses alimentares investigadas pela Secretaria de Saúde do Estado, nos últimos anos. Sabendo que existem 2.610 sorovares de *Salmonella* e que todos eles são considerados potencialmente patogênicos, o estudo das características que permitam que apenas o sorovar *S.* Enteritidis provoque a maioria das salmoneloses investigadas no RS torna-se de grande interesse, sendo o foco da presente pesquisa.

Em trabalhos anteriores realizados no Laboratório de Microbiologia e Controle de Alimentos do ICTA/UFRGS, foi possível identificar a presença do gene *spv*R (*Salmonella* Plasmid Virulence) em 82,7% das *S.* Enteritidis envolvidas em salmoneloses alimentares ocorridas no RS, de 1999 a 2000. Os mesmos isolados também foram caracterizados segundo sua resistência a

antimicrobianos, demonstrando altas porcentagens de sensibilidade à maioria das drogas testadas. Em seguida, Oliveira et al. (2006) demonstraram que os isolados de *S.* Enteritidis envolvidos em surtos ocorridos no RS, em 2001 e 2002, apresentaram praticamente os mesmos perfis de resistência que os isolados do período anterior. Confirmando a baixa variabilidade desses isolados, foi realizada a caracterização genotípica, por PCR-ribotipificação e seqüenciamento de DNA, das cepas isoladas em 1999 e 2000, e, por PCR-ribotipificação e RAPD, dos isolados de 2001 e 2002. Os resultados demonstraram que um perfil molecular foi predominante em mais de 95% das *S.* Enteritidis isoladas nas salmoneloses ocorridas no RS (Oliveira et al., 2007), e essa cepa foi denominada SE86. Malheiros et al. (2007) avaliaram a cinética de crescimento de alguns sorovares de *Salmonella* em salada de batata com maionese caseira, o principal alimento envolvido em surtos alimentares no RS. Os pesquisadores observaram que *S.* Enteritidis SE86 apresentou capacidade de multiplicação mais rápida que os outros sorovares de *Salmonella,* nas primeiras seis horas de cultivo. Além disso, Malheiros et al. (2008) avaliaram a resistência ácida e térmica da *S.* Enteritidis SE86 e observaram que esse microrganismo apresentou uma maior capacidade de adaptação ácida e maior resistência térmica quando comparada com outros dois sorovares não envolvidos em surtos alimentares no RS, porém isolados da mesma região.

Tondo et al. (2010) avaliaram a resistência da *S.* Enteritidis SE86, S. Typhimurium e S. Bredeney a desinfetantes comumente utilizados nas indústrias de alimentos e concluíram que o ácido peracético, hipoclorito de sódio e quaternário de amônio foram capazes de inativar todos os

microrganismos quando as concentrações recomendadas pelos fabricantes foram utilizadas. No entanto, *S.* Enteritidis SE86 apresentou maior resistência que os demais sorovares testados na concentração de 200ppm de hipoclorito de sódio. Esse resultado assumiu grande importância, uma vez que esse produto nessa condição é provavelmente o principal desinfetante e a principal concentração utilizada nos serviços de alimentação e indústrias de alimentos no RS e no Brasil.

Dando sequência à investigação desse importante patógeno alimentar, o presente estudo teve como objetivo investigar o envolvimento da expressão dos genes *rpoS e dps* na resistência ao hipoclorito de sódio e do gene *ompR* na adaptação ácida e resistência térmica da cepa *S.* Enteritidis SE86.

2 REVISÃO BIBLIOGRÁFICA

2.1 *Salmonella* e Salmonelose

O gênero *Salmonella* pertence à família *Enterobacteriaceae* e compreende bastonetes Gram negativos, não esporulados, anaeróbios facultativos, não fermentadores de lactose (Koneman et al. 1997; Spector e Kenyon, 2011). A maioria, com exceção das *Salmonella* Pullorum e *Salmonella* Gallinarum, é móvel através de flagelos peritríquios (Jay, 2005).

As *Salmonella* sp*.* multiplicam-se, preferencialmente, em uma temperatura de incubação entre 35 e 37° C, mas existem relatos de crescimento em temperaturas entre 5 e 47° C (Franco e Landgraf, 1996; Malheiros et al., 2008).

Todas os sorovares de *Salmonella* são considerados potencialmente patogênicas, sendo o grau de virulência dependente da própria cepa, do hospedeiro e do ambiente (Chatti et al., 2008).

As doenças causadas por *Salmonella* sp. são conhecidas como salmoneloses e podem ser divididas em dois grupos: a febre tifóide, causada por *Salmonella* Typhi e *Salmonella* Paratyphi (A, B e C) e as enterocolites, causadas pelos demais sorovares de *Salmonella* (Forsythe, 2002).

A febre tifóide só acomete o homem e, normalmente, é transmitida por alimentos ou água contaminada com material fecal humano. O reservatório

de *S.* Typhi é o homem e, há casos onde algumas pessoas podem se tornar portadores assintomáticos. A febre tifóide é a salmonelose mais grave, uma vez que pode levar à infecção sistêmica, causando a morte em muitos indivíduos acometidos (Shinohara et al., 2008).

A doença mais comumente causada por *Salmonella* sp., a enterocolite, é resultante, também, da ingestão de alimentos ou água contaminados. É uma das mais freqüentes infecções de origem alimentar no mundo (Geimba et al., 2004; Carrasco et al., 2011). Apesar de a enterocolite ser a mais branda das manifestações causadas por *Salmonella* sp., a alta freqüência dos casos é responsável por milhares de mortes todo ano (Malorny et al, 2007). Somente nos Estados Unidos da América, estima-se que 800 a 4000 mortes ocorram devido à salmonelose (Reis et al., 2003). Os sintomas mais comumente associados às enterocolites são a diarreia sanguinolenta, vômito, febre, dores abdominais e a desidratação. Tais enfermidades têm sido responsáveis por grandes problemas de saúde pública e prejuízos econômicos decorrentes do tratamento de casos clínicos e perdas de dias produtivos da população (Costalunga e Tondo, 2002; Tondo e Bartz, 2011).

2.2 Salmoneloses no Mundo e no Brasil

O aumento da incidência de surtos de salmoneloses, em diversos países, tem chamado à atenção dos órgãos de saúde pública, sendo que as investigações epidemiológicas identificam o consumo de ovos ou de alimentos contendo ovos, como responsável pela maioria desses surtos. Sendo assim, os

alimentos de origem animal são considerados os maiores veículos de *Salmonella (*Paula et al., 2005).

Salmonella foi reconhecida como um dos principais microrganismos causadores de DTA em diversas partes do mundo, com estimativa de incidência anual de 1,3 bilhões de casos e 3 milhões de mortes (Thong et al., 1995; Carrasco et al., 2011). A Salmonelose foi responsável por 131.468 casos humanos confirmados na União Europeia (UE) em 2008, representando a doença zoonótica mais freqüentemente relatada em humanos, seguidas da campilobacteriose (Carrasco et al., 2011).

Na Inglaterra, a carne de frango tem sido referida como um importante veículo de *S.* Enteritidis, enquanto que outros países do Reino Unido citam ovos ou alimentos contendo ovos como o principal veículo do microrganismo (Kantama & Jayanetra, 1996).

No Brasil, uma legislação do Ministério da Saúde (Resolução nº 12 de 02 de janeiro de 2001 – MS) exige ausência de *Salmonella* sp. em 25 g de amostra de alimentos comercializados, sendo que a pesquisa desse microrganismo é preconizada para praticamente todos os alimentos (BRASIL, 2001).

O Estado do Paraná dispõe de dados importantes, embora a subnotificação também seja uma realidade nesse Estado. Entre 1978 e 1997, 67,1% dos surtos de DTA ocorridos no Paraná foram de origem bacteriana, sendo que *Salmonella* sp. foi isolada em 20,3% dos surtos. A partir de 1995, a salmonelose passou a ser a principal DTA notificada neste Estado (Camargo et al., 1998). O laboratório LAPA/LACEN analisou, no período de janeiro de 1996

a abril de 1997, 161 alimentos envolvidos em surtos ocorridos no Paraná. Os agentes etiológicos responsáveis por esses surtos foram identificados em 52 (32,3%) amostras, sendo a *Salmonella* sp. indicada como responsável por 44,2% dos surtos. Os alimentos mais envolvidos nesses surtos foram carnes e derivados (28,9%) e maionese caseira (23,1%) (Camargo et al., 1998a). Em 1998, 57,1% dos surtos analisados laboratorialmente identificaram *Salmonella* sp. (Camargo et al. , 1999). Os alimentos mais freqüentemente envolvidos em surtos alimentares no Paraná, em 1998, foram as preparações mistas, entre as quais figurava a salada de maionese caseira.

No Rio Grande do Sul, Costalunga e Tondo (2002) relataram que *Salmonella* sp. foi responsável por 35,7% dos 323 surtos alimentares investigados no período de 1997 a 1999, sendo a maionese caseira o alimento mais envolvido nesses surtos, tanto na forma de saladas (32,4%) como na forma de ingrediente de outros alimentos de preparação caseira (2,2%). No período entre 2000 e 2001, os surtos de salmonelose investigados no Estado, demonstraram que a população envolvida foi de 14.253 pessoas expostas, das quais 3.400 ficaram doentes, 1938 foram hospitalizadas e 2 pessoas morreram. Os locais mais frequentes da ocorrência de surtos foram residências (61,5%) e estabelecimentos comerciais (13,7%). O principal alimento envolvido continuou sendo a maionese caseira (48,1%), seguida de carnes e produtos cárneos (22,6%) (Silveira e Tondo, 2006). No período de 2002 a 2004 o número de pessoas envolvidas em surtos no RS aumentou para 23.725, sendo que destas 1.878 foram hospitalizadas e uma pessoa morreu (Wagner et al., 2012).

2.3 O sorovar *S*. Enteritidis

Os sorovares *S*. Typhimurium e o *S*. Enteritidis foram identificados como os mais comumente implicados em infecções alimentares humanas em diversas partes do mundo (Babu et al., 2006).

Até meados da década de 80, *S*. Enteritidis era isolada de humanos e animais com pouca freqüência na maioria dos países (Castilla, 2003). Porém, no final dessa década ocorreu uma mudança expressiva neste cenário, fazendo com que a prevalência desse sorovar em infecções humanas ultrapassasse *S*. Typhimurium que, até então, ocupava o primeiro lugar. Ainda, Castilla (2003) relatou que esta mudança foi observada nos países da Europa e, posteriormente, em países das Américas. No Brasil, o primeiro caso conhecido ocorreu em 1981, o qual foi relatado por Mota et al. (1981) na cidade de Curitiba, PR. A partir de 1993, a salmonelose por *S*. Enteritidis passou a ser frequente em outras regiões do país, incluindo o Noroeste do estado de São Paulo, Sorocaba, Campinas e Santo André (Peresi et al., 1998; Fernandes, 2003).

De acordo com dados da Secretaria da Saúde do Estado do Rio Grande do Sul, em 1990 *S*. Typhimurium foi isolada de 86% dos alimentos envolvidos em salmoneloses e não houve relatos de *S*. Enteritidis. No entanto, no ano de 1993, este sorovar foi encontrado em 64% dos alimentos relacionados aos surtos, enquanto a *S*. Typhimurium foi isolada em apenas 4% desses alimentos (Tavechio et al., 1996). Os mesmos autores, durante o período de 1999 a 2000, também encontraram como principal sorovar proveniente de salmoneloses ocorridas no RS, *S*. Enteritidis.

Nadvorny et al. (2004) relataram que no ano de 2000, *Salmonella*

foi também a principal causadora de surtos alimentares no RS, sendo 72,2% dos surtos igualmente associados ao consumo de alimentos preparados com ovos, especialmente a maionese caseira. Manipulação inadequada dos alimentos e utilização de produtos sem inspeção sanitária constituíram os principais fatores associados com a ocorrência de salmonelose nos surtos confirmados no RS, em 2000.

Geimba et al., (2004) observaram que o gene *spv*R (*Salmonella* Plasmid Virulance) esteve presente em 82,7% das cepas de *S.* Enteritidis envolvidas em salmoneloses alimentares ocorridas no RS, em 1999 a 2000.

Oliveira et al. (2007) e Oliveira et al., (2009) analisaram por PCR-ribotipificação, seqüenciamento de DNA e RAPD cepas de *S.* Enteritidis isoladas no período de 1999 a 2002 e verificaram que estes isolados apresentaram uma baixa variabilidade.

O aumento repentino no isolamento de *S.* Enteritidis pode ser atribuído ao sucesso das campanhas de erradicação de *S.* Gallinarum e *S.* Pullorum, causadoras do tifo aviário e da diarréia bacilar em frangos. Essas campanhas possibilitaram a abertura de um novo nicho ecológico que vem sendo ocupado, desde então, por este sorovar (Baumler et al., 2000).

Em vista disso, a investigação de características fisiológicas, moleculares e de resistência dos principais sorovares envolvidos em surtos alimentares assume grande importância, objetivando o maior entendimento desses microrganismos e, se possível, a prevenção de novos surtos.

2.4 Resposta de *Salmonella* ao Estresse

Salmonella sp., assim como outros microrganismos enteropatogênicos, possuem uma surpreendente capacidade de sobreviver a condições rigorosas encontradas no ambiente natural e no organismo hospedeiro. A capacidade de adaptação e sobrevivência ao estresse está diretamente relacionada à habilidade de alguns microrganismos em causar doenças (Audia et al., 2001).

Humphrey (2004) reporta que *Salmonella* sp*.* apresentam um sistema regulatório complexo, o qual media sua resposta para o ambiente externo, podendo ser ativado sob condições de estresse, tais como o pH estomacal, o ambiente intestinal, mudança de temperatura, baixo nível de oxigênio e água. Segundo este autor, essa regulação está associada aos fatores sigma, os quais regulam reconhecimento dos sítios de ligação da RNA polimerase. Alguns tipos de fatores sigma direcionam a ligação da RNA polimerase para genes que codificam proteínas de estresse, as quais aumentam as possibilidades da bactéria sobreviver a mudanças ambientais. A adaptação pode ser induzida tanto na fase exponencial quanto na fase estacionária, promovendo, em ambas as situações, maior resistência à acidez letal e outros tipos de estresse. Na indução durante a fase exponencial, quando as células são expostas a pHs moderados (4,5 a 5,8), a sobrevivência a pH 3,0 pode aumentar 1000 vezes em relação às células não adaptadas. Em geral, essa proteção ocorre devido a uma série de proteínas de choque ácido (ASPs), as quais são induzidas durante a adaptação (60 ASPs, segundo análise de proteomas) (Audia et al., 2001).

Salmonella, antes e durante a infecção do hospedeiro, encontra inúmeras variações ambientais, as quais podem ser interpretadas como situações de estresse para os microrganismos. Muitas dessas variações são potencialmente letais para Salmonella, porém a sobrevivência do microrganismo depende de seus mecanismos de adaptação ou resistência (Velkinburgh e Gunn, 1999). Genes importantes para resistência, adaptação aos fatores de estresse e de virulência podem ser ativados e expressados no primeiro contato com o hospedeiro, desde que os mesmos estejam presentes no genoma bacteriano. A maioria dos genes de virulência de Salmonella são agrupados em regiões distribuídas ao longo do cromossomo, chamadas de ilhas de patogenicidade (SPI). Até recentemente, cinco SPIs foram identificados no cromossoma de Salmonella (Asten e Dijk, 2005).

2.4.1 Resposta de Tolerância ácida (ATR)

A ATR (Acid Tolerance Response- ATR) é um fenômeno complexo fortemente afetado pela interação de diferentes fatores ambientais que prevalecem durante tanto adaptação ácida. Salmonella apresenta na fase estacionária, dois tipos de sistemas de resposta de tolerância ácida. Um deles é uma resposta induzida pela acidez, independente do fator sigma, e o outro dependente desse fator, o qual não requer indução ácida (Lee et al., 1995; Bang et al., 2000). A ATR induzida pelo ácido está associada a um aumento da proteína regulatória OmpR que é responsável pela expressão de proteínas de choque ácido, as quais protegerão a maquinaria celular contra a acidez letal (Foster, 2000). Ao contrário da ATR induzida pelo ácido, o fator sigma

alternativo (σ^s), codificado pelo gene *rpo*S é induzido automaticamente para virulência/resistência ao estresse geral. Quando as células entram em fase estacionária, aumenta a concentração de σ^s, estimulando a expressão de uma série de genes, denominados de regulon σ^s, cujo objetivo é proteger a célula de vários tipos de estresse (Audia et al., 2001) tais como deficiência de nutrientes e condições ácidas (Bearson et al., 1998).

2.4.1.1 Regulação gênica por *rpoS*

O σ^s, ou *rpoS*, é uma subunidade sigma da RNA polimerase que é induzido e pode substituir parcialmente o fator sigma vegetativo σ^{70} (*rpoD*) sob condições de estresse. Como consequência, a transcrição de numerosos σ^{s-} dependentes são ativados (Figura I). Além de RpoS ser responsável pela ATR não induzida por acidez na fase estacionária em *Salmonella* (item 2.4.1), esta proteína tem demonstrado envolvimento na indução ácida de ATR na fase exponencial de crescimento, controlando assim a expressão de pelo menos 10 ASPs (proteínas choque ácido), que protegem a célula do estresse ácido (Alvarez-Ordónez, et al., 2011).
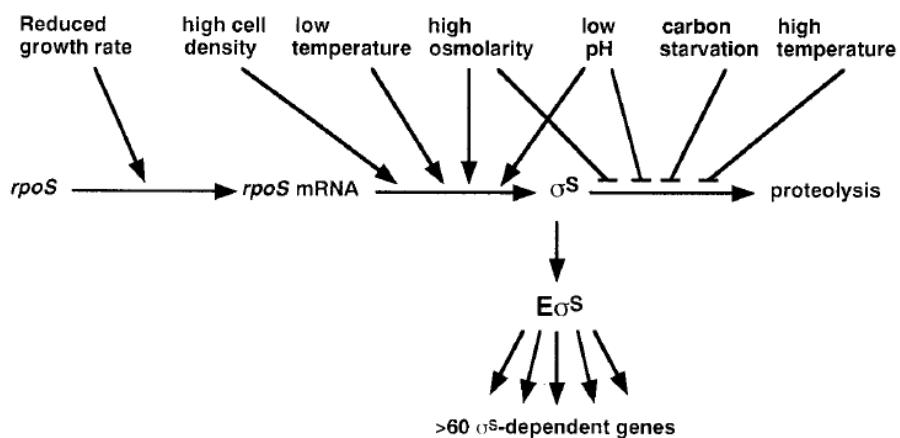
FIGURA I- Vários níveis de regulação do σ^s são diferentemente afetados por várias condições de estresse. Um aumento do nível de σ^s na célula pode ser obtido através estimulação da síntese do σ^s nos níveis de transcrição, pela tradução do mRNA ou ainda por inibição de proteólises (adaptado de Hengge-Aronis, 1996).

2.4.1.2 Regulação gênica por *ompR*

A ATR induzida pelo ácido está associada a um aumento da proteína regulatória OmpR que é responsável pela expressão de proteínas de choque ácido, as quais protegerão a maquinaria celular contra a acidez letal. Cerca de 10 ASPs não são produzidos em um mutante defectivo *ompR*. A proteína OmpR é uma importante reguladora de vários aspectos da fisiologia da célula, além de controlar a expressão de SsrA/SsrB, dois componentes reguladores, cujos genes residem na ilha de patogenicidade-2 de *Salmonella*. OmpR induz a si mesmo por choque ácido ao nível transcricional (Foster, 2000).

Segundo Bang et al. (2002), o pH ácido induz OmpR vindas de um promotor diferente daquele utilizado para expressão basal. A transcrição vinda

deste promotor é induzida por histonas como proteínas HNS (proteínas reguladoras da transcrição). O sensor quinase-EnvZ colabora para produzir níveis ótimos de OmpR-P, necessários para a auto-indução. Ainda, segundo o mesmo autor, a proteína OmpR regula a expressão de genes codificadores de porinas- OmpF e OmpC, responsáveis pela difusão de pequenos metabolitos como açúcares, aminoácidos e íons (Figura II).
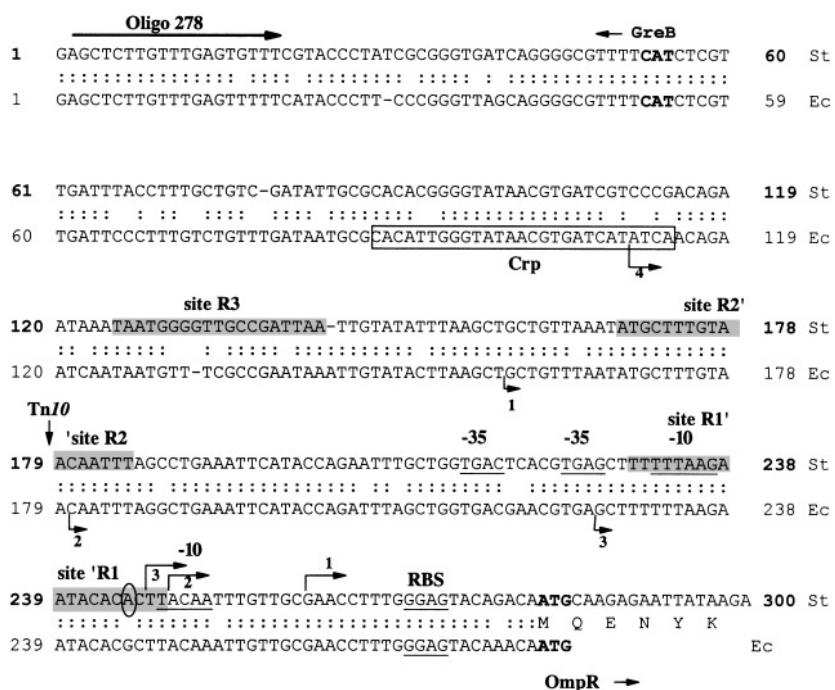
FIGURA II. A figura acima representa a seqüência de nucleotídeos da região regulatória de *ompR em S.* Thyphimurium. O topo da seqüência representa a região *upstream* de *ompR* de *Salmonella*. Em negrito as marcas ATG revelam o começo da região de codificação de OmpR. O início da transcrição são mostrados com setas para cima e para baixo. O promotor *ompR* possui três sítios de ligação potenciais (R1, R2 e R3). O "A" circulado representa o inicio do sitio de transcrição (adaptado de Bang et al., 2002).

2.4.2 Capacidade Adaptativa

Muitos sorovares de *Salmonella* tornam-se mais resistentes ao ácido após a adaptação a alguns tipos de estresses gerados para esses microrganismos, como acidez do alimento. Segundo Leyer e Johnson (1992), a

adaptação ácida aumentou a resistência de *Salmonella* sp. a vários tipos de ácidos orgânicos, além de aumentar a sua sobrevivência em alguns alimentos como leite fermentado e queijo. Esses autores demonstraram que *S.* Typhimurium ácido-adaptada em HCl pH 5,8 tornou-se mais resistente ao ácido lático, ácido propiônico e ácido acético pH 3,85. De maneira semelhante, Tosun e Gönül (2003) demonstraram que *S.* Typhimurium ácido-adaptada em HCl pH 5,8 tornou-se mais resistente ao pH 3,45 (HCl) do que culturas não adaptadas.

A adaptação ácida, que é normalmente transitória, adquire interesse maior, especialmente quando a segurança dos alimentos é levada em consideração, porque pode propiciar a sobrevivência de *Salmonella* (Leyer; Johnson, 1992) em alimentos acidificados. Adicionalmente, espécies de patógenos ácido-adaptados podem exibir proteção cruzada para outras condições de estresses, como o estresse térmico e o estresse à irradiação (Lou e Yosef, 1997; Ryu e Beuchat, 1999).

A sobrevivência de patógenos ácido-adaptados em baixo pH pode aumentar também sua resistência a altas temperaturas (Samelis et al., 2003; Malheiros et al., 2008). A cocção permanece como uma das medidas de controle mais importantes para a eliminação de patógenos dos alimentos. Para que ela seja efetiva, o tempo e a temperatura de cocção devem ser controlados e estes também dependem da resistência térmica dos microrganismos, além da composição e características físico-químicas dos alimentos (Buchanan e Edelson, 1999). Segundo a Resolução 216/2004 da ANVISA, a cocção deve atingir 70º C em todas as partes dos alimentos, porém é sabido que

freqüentemente isso não ocorre, podendo expor os microrganismos a temperaturas insuficientes para sua inativação (Malheiros et al., 2007).

A tolerância térmica e ácida em espécies patogênicas merece atenção especial também devido à possibilidade do microrganismo passar pelo baixo pH estomacal depois de adaptado ao estresse no alimento, aumentando suas chances de estabelecer a infecção (Audia et al., 2001). A resposta de tolerância ácida é altamente variável entre espécies (Lin et al., 1995) ou cepas da mesma espécie (Humphrey et al., 1993; Yuk e Schneider, 2006), em relação ao tipo de acidulante usado na adaptação ácida (Buchanan e Edelson, 1999a) e quando se utiliza a fermentação da glicose, a qual reduz o pH gradualmente durante o cultivo (Ryu e Beuchat, 1999).

Wilde et al. (2000) relataram que a tolerância ácida e térmica de isolados de *Salmonella enterica* sorovar Enteritidis PT4 foi nitidamente mais elevada quando as células foram cultivadas, durante a fase estacionária, em triptona de soja comercial (TSB) ou infusão cérebro-coração (BHI), contendo glicose, do que quando as células foram cultivadas em qualquer caldo nutriente de triptona durante a incubação por 24 h. A redução do pH ocorreu até aproximadamente 5,8 - 4,7 durante a incubação por 24 h e isso resultou em adaptação, que, consequentemente, aumentou a tolerância ácida e térmica. Muitos trabalhos têm sido realizados utilizando a fermentação da glicose para indução ácida (Buchanan e Edelson ,1999b; Wilde et al., 2000; Samelis et al., 2003; Tetteh e Beuchat, 2003). Segundo Samelis et al. (2003), o cultivo de *Salmonella* até atingir a fase estacionária em meio de cultura contendo 1% de glicose induzirá a ATR dependente do pH, aumentando a sobrevivência do

microrganismo em ácidos orgânicos. Desde modo, esse método é apropriado para produção de culturas ácido-adaptadas.

Além de *Salmonella*, outros microrganismos patogênicos, tais como *Escherichia coli* O157:H7 (Leyer et al., 1995; Gahan et al., 1996), *Listeria monocytogenes* e *Shigella* (Gahan et al., 1996) demonstraram capacidade de adaptação ácida, ressaltando a importância de estudos relacionados a adaptação ao estresse para segurança dos alimentos (Malheiros et al., 2007).

2.5 Resistência térmica

O tratamento térmico é um dos principais métodos utilizados nas indústrias de alimentos para eliminar microrganismos patogênicos. Por esta razão, uma extensa investigação nesta área focada nos efeitos de fatores ambientais sobre a resistência térmica bacteriana tem sido realizada.

Os estudos sobre os mecanismos envolvidos na tolerância térmica bacteriana estão concentrados em desvendar o papel de conhecidas proteínas de choque térmico (HSPs), que são sintetizadas em resposta a vários tipos de estresse ambientais e, provavelmente, funcionam como chaperonas em processos fisiológicos normais das células. Segundo Tang et al., (2007), algumas HSPs como GroEL e DnaK estão envolvidas na resistência térmica, bem como na respostas imune do hospedeiro, pois são as mais abundantes proteínas expressadas por *Salmonella* dentro dos macrófagos. Farr e Kogoma (1991), ao analisarem proteínas GroES, GroEL e DnaK,

concluíram que, além do choque térmico, o estresse oxidativo também induz estas proteínas. Estes autores observaram ainda que o crescimento em temperaturas acima de 40°C requer concomitante super expressão do gene *dnaK* e que GroE e DnaK são proteínas-chave na proteção das *Salmonella* contra estresse térmico.

Cabe ressaltar que os microrganismos apresentam diferença no seu comportamento frente à resistência ao calor após a exposição a certos estresses ambientais, tais como o ácido e frio (Álvarez-Ordonez et al, 2008). No caso particular de *Salmonella* sp., existem vários estudos sobre a influência do estresse ácido sobre a sua resistência ao calor, porém não existe um consenso sobre quais mecanismos que levam estes microrganismos a apresentarem uma maior tolerância térmica após a exposição moderada ao ácido (Leyer e Johnson, 1993; Wilde et al, 2000.; Tosun e Gonul, 2003).

2.6 Resistências a sanificantes

Sanificantes são substâncias ou preparações químicas capazes de destruir microrganismos patogênicos, em curto espaço de tempo, quando aplicados em superfície (McDonnel e Russel, 1999). Sua escolha deve ser precedida de uma análise detalhada, levando-se em conta aspectos como uso autorizado do produto pela legislação, grau de toxicidade, poder corrosivo, efeito residual sobre os alimentos, efeito sobre o ambiente e o custo (Machado et al., 2011).

A ocorrência de cepas resistentes a desinfetantes pode representar desafios econômicos para a indústria de alimentos e trazer

implicações para a saúde pública, ainda mais quando se trata de microrganismos com resistência cruzada a desinfetantes e a antibióticos. A resistência varia de microrganismo para microrganismo e tipicamente resulta de alterações celulares que implicam em acúmulo de biocidas, incluindo alterações do envelope celular que limita a absorção ou a expressão de mecanismos de efluxo. É de extrema importância salientar a adaptação aos desinfetantes que seleciona microrganismos intrinsecamente resistentes ao composto desinfetante aplicado. A adaptação pode ocorrer em superfícies com enxague deficiente, deixando baixas concentrações de desinfetantes nas superfícies (Tondo et al., 2010; Machado et al., 2011).

O cloro e suas várias formas, provavelmente, são os compostos mais comumente utilizados para a desinfecção em indústrias de alimentos e serviços de alimentação (Tondo e Bartz, 2011). Tais compostos podem incluir cloro líquido, hipocloritos, compostos clorados orgânicos e inorgânicos e tem apresentado amplo espectro germicida, devido à sua ação sobre a membrana celular, inibição de enzimas envolvidas no metabolismo da glicose, danos no DNA e oxidação de proteínas celulares (Schmidt, 2003). A quantidade de cloro livre que estará presente na solução dependerá do pH. Em pH igual a 8,0, cerca de 22% do cloro estão na forma ativa, enquanto que, em pH igual a 6,0, cerca de 96% do cloro estará na forma ativa (Souza e Daniel, 2005).

A ampla utilização do hipoclorito de sódio pode ser atribuída, dentre várias razões, devido ao seu baixo custo e amplo espectro de ação. Segundo a Portaria 78/2009, que está em vigor no Estado do Rio Grande do Sul, para desinfecção de alimentos hortifrutigranjeiros, deve-se utilizar solução

clorada entre 100 e 250 ppm, por 15 minutos. Concentrações de aproximadamente 200 ppm, por 15 minutos, têm sido recomendadas e utilizadas na desinfecção de panos de limpeza, equipamentos e utensílios, desde que enxaguadas com água potável, após aplicação do desinfetante (Brasil, 2009; Oliveira et al., 2012). O *Food and Drug Administration* recomenda o uso do hipoclorito de sódio como agente desinfetante para superfícies que entram em contato com alimentos em concentrações acima de 200 ppm. Concentrações entre 100 e 200 ppm têm sido recomendadas para desinfecção de utensílios e equipamentos (Tondo e Bartz, 2011; Oliveira et al., 2012).

Tondo et al. (2010) avaliaram a resistência de três sorovares de *Salmonella* diferentes sanificantes e demonstraram que *S*. Enteritidis (SE86) apresentou maior resistência ao hipoclorito de sódio a 400 ppm, além de ser a única cepa bacteriana capaz de sobreviver por até 15 minutos de exposição à 200 ppm desse desinfetante.

2.7 Estresse Oxidativo

As células bacterianas são expostas a multiplos agentes oxidantes quando passam através do hospedeiro. Espécies reativas de oxigênio (ROS), tais como superóxido ($O_2{}^{\cdot-}$) e peróxido de hidrogênio (H_2O_2) são originários do metabolismo aeróbico endógeno ou como parte de um ataque das células do sistema imune (fagocitose). Estes compostos reagem fortemente com moléculas bacterianas, causando danos aos ácidos nucléicos, proteínas, lipídios e carboidratos (Cabiscol et al, 2000;. Farr & Kogoma, 1991;

Imlay, 2008), que sem mecanismos de proteção, seriam rapidamente destruidas pela acumulação de danos oxidativos.

Para combater o estresse oxidativo, sorovares de *Salmonella* desenvolveram duas grandes vias de respostas a este estresse. Uma delas, o regulador *oxyR,* responde especificamente à presença de H_2O_2, enquanto que o regulador SoxRS, é responsável pelas mudanças no estado redox- celular (Spector e Canyon, 2011). Apesar de esses dois reguladores controlarem a maioria das proteínas relacionadas ao estresse oxidativo, eles não são os únicos envolvidos. Alguns genes como *katG*, *gorA* e *dps*, que são regulados tanto pelo fator sigma (σS) como oxyR, também demonstram um papel importante na resistência ao estresse oxidativo (Spector e Canyon, 2011).

Dps é uma proteína citoplasmática, parte do sistema de regulação do fator sigma, que protege DNA celular contra os danos que podem ser causados pelo stress oxidativo devido à exposição à peróxido de hidrogênio, HOCl e acidez (Pacello et al., 2008, Martinez e Kolter, 1997). O gene *dps* é responsável por codificar uma proteína de ligação ao DNA não específica, cuja expressão é induzida em fase estacionária (Arnold et al., 2001). Young et al (2007) revelou que, nos casos em que *Salmonella* foi exposta a múltiplos estresses, incluindo o stress oxidativo, Dps foi identificada como uma das principais proteínas expressas.

3 RESULTADOS

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

3.1 "*Salmonella* and Salmonellosis in Southern Brazil: a review of the last decade"

Capítulo aceito para publicação no livro "*Salmonella*: Classification, Genetics and disease Outbreaks", editado pela *Nova Science Publishers, Inc*, Nova York, USA. ISBN: 978-161-942-928-4.

3.2 "Investigation of *rpoS* and *dps* genes in sodium hypochlorite resistance of *Salmonella* Enteritidis SE86 isolated from foodborne illness outbreaks in Southern Brazil"

Artigo publicado no periódico *Journal of Food Protection*, v.75, p.437 - 442, 2012.

3.3 Expression of *ompR* gene in the acid adaptation and thermal resistance of *S.* Enteritidis SE86

Artigo a ser submetido para publicação no periódico Food Microbiology.

3.1 Capítulo de livro

3.1.1 "*Salmonella* and Salmonellosis in Southern Brazil: a review of the last decade"

Salmonella: Classification, Genetics and Disease Outbreaks

Retail Price: ~~$140.00~~

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You Pay: **$126.00**

Editors: Adelaide S. Monte and Paulo Eduardo De Santos

Book Description:
Salmonella are ubiquitous enteric bacteria, responsible for thousands of deaths worldwide. In this book, the authors present current research in the study of the classification, genetics and disease outbreak cases relating to salmonella. Topics include the pre- and post-harvest intervention strategies for controlling salmonella contamination in broiler production; salmonella enterica survival to biocides and antibiotics; salmonella newport contamination in produce; genome comparisons of salmonella; salmonella in sub-Antarctica and Antarctica; and hazard of salmonella in the intact shell egg. (Imprint: Nova Biomedical)

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Chapter

SALMONELLA AND SALMONELLOSIS IN SOUTHERN BRAZIL: A REVIEW OF THE LAST DECADE

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ABSTRACT

Salmonella was identified as the first cause of food-borne diseases in the last decade in Brazil. Currently, Brazil is an important producer of pork and is the major poultry and beef exporter in the world. Brazilian animal products have been recognized worldwide, and to attend international standards the Brazilian food industries have been putting considerable effort in order to prevent food contamination, especially by *Salmonella*. A representative part of the Brazilian eggs, beef, poultry, and pork meats is produced in the State of Rio Grande do Sul (RS), in southern Brazil, where *Salmonella* was pointed out as the first cause of food-borne diseases for almost two decades. In this region, homemade mayonnaise was identified as the food preparation mostly involved with salmonellosis, and both meat and meat products were also identified as frequent vehicles for salmonellosis. A specific strain of *Salmonella* Enteritidis (named SE86) was identified as the responsible agent in more than 95 % of investigated food-borne diseases throughout the last decade. SE86 strains isolated from several outbreaks demonstrated the same DNA banding patterns when typed by PCR-methods and PFGE, suggesting that it is spread out through RS. SE86 demonstrated high percentages of sensitivity to many antibiotics; however, an increasing resistance to nalidixic acid and ampicillin has been identified. After exposure to sub-letal pHs, SE86 demonstrated a greater acid adaptation and also to be more acid-and-thermal-resistant than *S.* Typhimurium and *S.* Bredeney also isolated in RS State. Acid-adapted SE86 presents higher virulence than non-acid-adapted SE86 and acid-adapted *S.* Typhimurium in germ-free mice. This strain has shown to be able to form biofilms on stainless steel and polypropylene, and was more resistant to 200 and 400 ppm sodium hypochlorite than others *Salmonella* serovars, but was not more resistant than other *S.* Enteritidis isolated in countries such as Albania, Morocco, Pakistan and Zimbabwe. SE86 mutants (*dps, rpoS*) were constructed in order to investigate the involvement of these genes with oxidative stress caused by sodium hypochlorite exposure. The results demonstrated that both genes are expressed during such exposure; however, *dps* was expressed in much higher amounts, increasing survival rates of SE86. Poultry meat produced in RS State demonstrated *Salmonella* prevalence of 4.4 % to 15 %, whilst expressively higher percentages of *Salmonella* were found in pork meat samples. Strains isolated from poultry and pork belonged to diverse serovars and, in general, demonstrated different patterns and higher antimicrobial resistance than SE86 isolated from salmonellosis.

INTRODUCTION

Brazil and General Data Concerning Food-Borne Outbreaks

Brazil is the biggest country in South America and the fifth biggest country in the world, with a population of approximately 190 million people distributed in 27 States. The southern region of Brazil is composed by three States called Paraná, Santa Catarina, and Rio Grande do Sul. This region is expressively industrialized and presents high standards of life quality. The southernmost State of Brazil is Rio Grande do Sul, a 10.7-million-people State, composed of 496 cities and presenting an area of 268,781.896 Km^2, bordering Argentina and Uruguay. According to data of the Ministry of Health, during the period from 2000 to 2011, *Salmonella* sp. was identified as the major cause of reported food-borne diseases in Brazil [1]. The most common food vehicles were identified as being mixed food preparations, as well as eggs and egg-based products, respectively. Beef and poultry meat were the fifth and seventh foods most frequent involved in salmonellosis. Private residences were the places where the largest number of outbreaks occurred, followed by restaurants, pastry shops and schools [1]. Among the 3,487 etiologic agents identified in food-borne diseases, *Salmonella* sp. was identified in 42.27 % do the outbreaks, followed by *Staphylococcus aureus* (20.34 %) and *Escherichia coli* (10.46 %) [1].

Salmonella and Salmonellosis in Southern Brazil

Based on the official data of the Division of Sanitary Surveillance of the RS State (DVS/RS), since 1993, *Salmonella* spp. became the major cause of reported food-borne diseases in RS State [2]. Official data also demonstrated that during the period of 1887 to 1993 *S. aureus* was the agent most identified in food-borne diseases in RS; however, *Salmonella* overcame *S. aureus*, probably as a consequence of mandatory good manufacturing practices, as well

as training courses, implemented as an obligation for all people working in food services in the city of Porto Alegre, the capital of RS State. According to the same regulation body, the training course was responsible for the decrease of contamination originated from food handlers, justifying the reductions of outbreaks caused by *S. aureus.*

Epidemiological data on salmonellosis occurred in RS State in the periods from 1997 to 1999, 2000 to 2001, and 2002 to 2004, are summarized in Table 1. Data were published elsewhere [3, 4, 5] after analysing official data of DVS/RS. The following factors were analysed: total number of confirmed outbreaks, number of people involved, number of people hospitalized, incidence of outbreaks according to the period of the year, age and sex of the involved people, food vehicle, food storage conditions, local where the disease occurred, and possible causes of the *Salmonella* outbreaks. Salmonellosis data indicated that more than 8,200, 14,200, and 23,700 people were involved, and approximately 1,500, 1,900 and 1,800 had to be hospitalized during the three periods analysed, respectively. The highest number of salmonellosis occurred in springtime, and this fact could be explained by inadequate food refrigeration during this season presenting mild temperatures. The higher incidence of outbreaks in springtime also occurred with other etiological agents of food-borne diseases, confirming the negligence regarding control of temperature. Such carelessness is frequently observed during winter and is prolonged into spring when temperature in RS State is frequently lower than 10 ºC [3].

The most affected age groups were those comprised in between 16- and 50-years old, and the most common food vehicle was potato salad made with homemade mayonnaise prepared with raw eggs. The second food vehicle was the group of meat and meat products. The principal causes for salmonellosis was the usage of raw materials not submitted to regulatory inspection (22.92 %, from 1997 to 1999), mainly eggs and foods maintained at room temperature for more than 2 hours (1997 to 2004).

The majority of the outbreaks took place in private homes, and in second place came food services. In the years of 2000 and 2001 two persons died, and

in the period from 2002 to 2004 one person died. The incidence of male and female was practically the same, during all periods observed.

Table 1. Characteristics of salmonellosis occurred in Rio Grande do Sul State, southern Brazil, during 1999 to 2004

Period	Involved people	Hospitalized people	Age group affected (years old)	The most common food vehicle	Local of occurrence	Reference
1997-1999	8,217	1,557	16 - 50	Home-made mayonnaise (42.45%)	Private home (43.70%) and commercial food establishments (25.21%)	Costalunga and Tondo (2002)
2000-2001	14,253	1,938	20-49	Home-made mayonnaise (48.1%)	private homes (61.5%), and commercial food establishments (13.7%)	Silveira and Tondo (2006)
2002-2004	23,725	1,878	20-49	Home-made mayonnaise (53.51%)	private homes (55.81%) and commercial food establishments (12.1%)	Wagner et al. (submitted)

Salmonellosis Caused by *S.* Enteritidis

Geimba et al. [6], when analyzing *Salmonella* strains (n=75), isolated from foods involved with food-borne outbreaks occurred in RS from 1999 to 2000 and found 98% of *S. enterica* serovar Enteritidis. Only two isolates were serotyped as *S.* Derby and *S.* Typhimurium. Among the strains of S. Enteritidis, 83 % were positive for the presence of the *Salmonella* Plasmid Virulence (*spv*R) regulatory gene, and a positive correlation ($P <0.05$) between *S.* Enteritidis and the presence of *spv*R gene was demonstrated. Oliveira et al. [7] investigated 85 *Salmonella* isolated from foods responsible for salmonellosis in RS in the period of 2001-2002, and observed that 93 % were also *S.* Enteritidis. Only six isolates were serotyped as *S.* Javiana (1), *S.* Infantis (1), *S.* Agona (1), *S.* Typhimurium (1), and *S. enterica* subspecies enterica 1, 4, 5 [7]. Comparing to other

Salmonella serotypes involved in cases of salmonellosis in RS in 1990, *S.* Typhimurium was isolated from 86 % of the foods involved in salmonellosis, while *S.* Enteritidis was not found. However, in the year of 1993, *S.* Enteritidis was isolated from 64 % of the suspected foods, surpassing *S.* Typhimurium, which was isolated from only 4% of the analysed food [*3*]. Similar findings were verified by Usera et al. (*8*) describing the surpassing of *S.* Typhimurium by *S.* Enteritidis in 1990, becoming the most common *Salmonella* serotype reported in the United States.

Asymptomatic infections in poultry flocks were first noticed in the late 1970s, and in the 1980s they spread rapidly throughout the United Kingdom, the United States, South America and other regions of the world [*9*]. During this period the proportion of salmonellosis cases attributed to *S.* Enteritidis increased substantially, becoming the predominant cause of gastroenteritis in many countries. It is suggested that this rapid increase of the prevalence of *S.* Enteritidis may have been due to the successful campaign to eradicate *S.* Gallinarum and *S.* Pullorum, which are the causative agents of fowl typhoid and pullorum disease in chickens [*10*]. These campaigns may have opened an ecological niche that has since been occupied by *S.* Enteritidis [6].

Clonal Relationship of *S.* Enteritidis in the RS State

Using PCR-ribotyping, Oliveira et al. [7] demonstrated that isolates of *S.* Enteritidis responsible for several cases of salmonellosis in RS during 2001-2002, which were grouped in only two banding profiles (R1 and R2), being that profile R1 comprised 92.4 % of the strains. The same isolates were typed by RAPD and demonstrated four banding patterns (A to D), and the result was that profile A comprised 81 % of the strains. Later Oliveira et al. [11] investigated the clonal relationship of 152 isolates of *S.* Enteritidis involved in food-borne outbreaks isolated in the same State from 1999 to 2002, and concluded that a specific strain of *S.* Enteritidis was involved in the majority of the investigated

cases. The DNA typing methods used were PCR ribotyping, Pulsed Field Gel Eletrophoresis (PFGE) and DNA sequencing.

The amount of *Salmonella* sp. present in different foods implicated in salmonellosis occurred in 2005 in RS was studied. After that, the isolates of *Salmonella* were characterized by means of phenotypic and genotypic methods. Food samples (n=19) involved with cases of salmonellosis were analysed by the Central Laboratory of the Department of Health of RS State (FEPPS/LACEN/RS), demonstrating to be positive for *Salmonella.* Food samples were submitted to estimation of *Salmonella* using the Most Probable Number (MPN) technique. Higher counts ($>10^7$ MPN.g^{-1}) of *Salmonella* were detected mostly in foods containing mayonnaise. All strains were identified as *S*. Enteritidis, and presented a unique macrorestriction profile, demonstrating the predominance of one clonal group in foods involved in the salmonellosis outbreaks. A low frequency of antimicrobial resistant *S*. Enteritidis strains was observed and nalidixic acid was the only resistance marker detected [12].

During the period from 1999 to 2006, the Official Laboratory of RS (FEPPS/LACEN/RS) isolated 931 *S*. Enteritidis isolates in 190 outbreaks officially investigated. Among them, S. Enteritidis isolates from 80 different outbreaks were randomly chosen to be analysed - the researchers picked ten isolates from each year of the period comprised from 1999 to 2006. The 80 *S*. Enteritidis samples were isolated from blood (n=12) and feces (n=68) of salmonellosis victims in RS, and were analysed by means of PCR ribotyping and XbaI macrorestriction PFGE. Results identified only three closely related PCR ribotyping patterns (R1, R2, and R3) and just one PFGE profile - this last one, however, grouped 97 % of the strains [13]. These findings confirmed the occurrence of the same DNA banding profile among *S*. Enteritidis isolated from foods, blood and feces of the victims of salmonellosis from 1999 to 2006 in RS, suggesting that a specific strain of *S*. Enteritidis was responsible for the majority of the cases of salmonellosis in this Brazilian State in the last years; this strain has been named *S*. Enteritidis SE86.

Acid and Thermal Resistance of S. Enteritidis SE86

After identifying the involvement of a specific strain of *S*. Enteritidis (SE86) in more than 95 % of the salmonellosis outbreaks investigated in RS between 1999 and 2006, the characteristics of this food pathogen were investigated. Malheiros et al. [14] have compared the growth kinetics, as well as the acid and the thermal resistance of the SE86 strain, with those of the *S*. Typhimurium and *S*. Bredeney strains; these last ones were not involved in foodborne outbreaks, but have also been isolated in RS. Each serovar was inoculated separately in nutrient broth (CN) and in potato salad prepared with homemade mayonnaise (SMC), and then incubated at 30 ºC and 9.5 ºC. In CN at 30 ºC, similar growing characteristics were found for all serovars. However, when growing in homemade mayonnaise, SE86 demonstrated higher counts at the first 6 hours. These results are interesting because SMC has been identified as the major food vehicle responsible for salmonellosis in RS [3, 4]. In CN and in SMC at 9.5 ºC, there was no detectable growth of any *Salmonella* serovars during the first 24 hours, suggesting that such temperature was adequate to control the multiplication of these microorganisms.

The acid and thermal resistances of *Salmonella* serovars were evaluated. The three serovars were cultivated separately in Nutrient Broth (NB), as well as Nutrient Broth supplemented with 1 % glucose (NBG) - the later medium was used in order to induce acid-adapted cells. Thus, the three serovars were exposed to different pH (3.5, 4.0 and 4.5) and temperatures (52, 56 and 60 ºC). Results indicated that *S*. Bredeney demonstrated higher resistance to pH 3.5 and 4.0; nevertheless, SE86 presented a better capacity for acid adaptation than *S*. Typhimurium and *S*. Bredeney. At pH 4.5, all serovars demonstrated a similar behavior, remaining at the same levels of viable cells for 300 minutes. At 52 ºC, *S*. Bredeney presented higher survivor rates, even though acid adaptation was able to protect only SE86. At 56 ºC and 60 ºC, non-adapted and acid-adapted SE86 strains were more thermally resistant than other serovars tested. SDS-PAGE analysis demonstrated differences in the protein profiles of non-adapted and acid-adapted cells of all serovars [15].

Using simulated gastric fluid (FGS) and germ-free mices, Perez et al. [16] evaluated the survival in the ability of intestinal invasion of SE86 and *S.* Typhimurium (ST99), whether submitted or not to acid adaptation. The microorganisms were grown in culture media supplemented with glucose in order to promote acid adaptation due to pH reduction. After the growth, approximately 8 log of SE86 and ST99 (either acid-adapted or non-adapted) were exposed to FGS with pH 1.5, and then inoculated in germ-free adult male Wistar rats. Germ-free mice were also inoculated with approximately 2 log, and the animals were observed at aseptic conditions for twelve days. Animal feces, as well as portions of the gastrointestinal tract, were examined by means of microbiological analysis, and the rats were also investigated for intestinal morphological abnormalities. The mice were analyzed by means of histopathology and then submitted to the mortality curve. The results indicated that acid-adapted SE86 had a significant higher survival rate ($p < 0.05$) than non-adapted SE86, non-adapted ST99 and also than acid-adapted ST99 in the FGS. The *in vivo* experiments demonstrated that acid-adapted SE86 and acid-adapted ST99 were capable of causing intestinal morphological abnormalities, and were then recovered from feces, jejunum and ileum-cecal junctions of rats. Acid-adapted SE86 showed higher counts in the ileum-cecal junction than the other strains, suggesting that acid adaptation influenced the virulence of this microorganism. All strains were able to rapidly multiply in germ-free animals, but mortality caused by acid-adapted SE86 was more intense. Histopathological analyses revealed greater severity of the infection caused by SE86, a fact which was confirmed by the death of animals starting at the fourth day of infection - unlike the ST99 which did not cause the death of animals until twelve days after inoculation.

The SE86 Biocide Resistance

The resistance to biocides of SE86, *S.* Typhimurium and *S.* Bredeney was evaluated using the suspension test as recommended by the Brazilian Ministry

of Agriculture. Peracetic acid, quaternary ammonium and sodium hypochlorite were chosen to be tested because they are sanitizers commonly used in the Brazilian food industries. Based on the results, it was possible to observe that, when the concentrations indicated by the manufacturers were used, biocides were able to inactivate all of the microorganisms tested. However, SE86 was resistant to 400 ppm sodium hypochlorite and survived for up to 15 minutes of exposure to 200 ppm of this biocide, a fact which was not demonstrated by the other strains [17]. The same microorganisms were used in order to test biofilm formation capability on surfaces of material commonly used in food industries and food services. Coupons of stainless steel and polyethylene were immersed in bacterial suspensions of SE86, *S.* Typhimurium, and *S.* Bredeney during 15, 30 and 60 minutes, and then were submitted to different concentrations of peracetic acid, sodium hypochlorite, and quaternary ammonium. Hydrophobicity of the surfaces was evaluated by means of measurements of contact angle using the sessile drop method, and bacterial adhesion was accompanied by means of bacterial counts and scanning electron microscopy (SEM). When evaluating the results, it was possible to observe that the three serovars indicated similar counts of adhered cell to both materials (5.0 to 6.5 log cfu cm^{-2}). The time of exposure did not influence the counts of adhered cells on both surfaces; however, SEM revealed larger clusters of SE86 on both materials, which were not found in the other serovars. SE86 demonstrated a lower sessile drop angle when on polyethylene, indicating hydrophilic properties of this material. The biocides were not able to inactivate all the microorganisms adhered on both surface materials. At least 1 log cfu cm^{-2} of all serovars tested remained viable after the exposure to different sanitizer concentrations. In general, higher counts of survivors were observed on polyethylene disinfected with different concentrations of biocides. *S.* Bredeney and *S.* Typhimurium were more resistant than SE86 to peracetic acid, whilst SE86 demonstrated smaller reduction rates after sodium hypochlorite exposure. When adhered to polyethylene, the serovars *S.* Typhimurium and SE86 were more resistant to quaternary ammonium than *S.* Bredeney in all concentrations tested, and the numbers of SE86 remained almost unaltered. On stainless steel disinfected by

quaternary ammonium, *S.* Bredeney presented higher numbers of survivors [18].

Since SE86 demonstrated to be resistant to high concentrations of sodium hypochlorite [17,18] , the resistance of this Brazilian strain was compared to other strains of *S.* Enteritidis isolated worldwide, i. e. from Albania, Zimbabwe, Morocco and Pakistan. All the strains were chosen because they were responsible for salmonellosis and were submitted to 200 ppm sodium hypochlorite in suspension tests for 5, 10, 15, and 20 minutes, according recommended by the Brazilian Ministry of Agriculture. The results showed that none of the *S.* Enteritidis were totally inactivated after 20 minutes of exposure and the reduction rates were similar. Only the strain isolated in Albania demonstrated to be significantly more sensitive [19].

The involvement of the *rpo*S and *dps* genes in the resistance to 200 ppm sodium hypochlorite was also investigated in SE86. Mutants of SE86 were constructed by means of the Knockout method (Datsenko and Wanner, 2000) and tagged by using the 3X FLAG method (Uzzau, et al. 2001). The survival of the Wild Type (WT) strain, as well as of the attenuated strains, was determined by bacterial counts. Tagged proteins (Dps and RpoS) were detected by means of SDS-PAGE and also immunoblotting with anti-FLAG antibodies. The SE86 strain which lacked *dps* demonstrated greater sensitivity compared to the WT SE86 exposed to sodium hypochlorite, with a 2-log reduction after one minute, whilst WT demonstrated a reduction of only one log. The RpoS and Dps proteins were actively expressed under the conditions tested, strongly suggesting that these SE86 genes are related to oxidative stress resistance, since both were expressed during the exposure of the microorganism to 200 ppm sodium hypochlorite [19].

Antimicrobial Resistance of *Salmonella*

Salmonella is not only a public health concern due to the number of salmonellosis cases that it causes, but also because many strains are resistant

to a number of antimicrobial agents. The concern is that if such strains cause food poisoning in humans they can be difficult to treat, making the probability of human death more likely. The resistance of *Salmonella* sp. to antibiotics is increasing rapidly throughout the world, and the indiscriminate and incorrect use of antibiotics has facilitated the emergence of resistance in many serovars. For example, we can mention the recent food-borne outbreaks in the USA and Europe caused by both multi-antibiotic-resistant *S.* Heidelberg and *S.* Kentucky, respectively. Other examples are related to several cases involving the clone of the *S.* Typhimurium DT104, which is characterized by chromosomal resistance to ampicillin, tetracycline, streptomycin, chloramphenicol, and sulphonamides. Infections with *S.* Typhimurium DT104 are associated with more severe illness, higher rates of admission to hospitals and increased mortality. Although the antimicrobial resistance in *S.* Enteritidis has been considered low when compared to the dramatic increase of resistance demonstrated by some *S.* Typhimurium isolates (Yang et al. 2002), attention should be given to the frequent isolation of multi-resistant *S.* Enteritidis [20]. The surveillance of the rising antimicrobial resistance of *S.* Enteritidis is especially important, since this serotype has become the prevalent agent of human salmonellosis in many countries in the last years, including Brazil. Over the last decade in the RS State, the resistance of *S.* Enteritidis isolated from food-borne illnesses, pork and poultry has been the subject of several studies and are presented below.

Antimicrobial Resistance of *S.* Enteritidis Isolated in Food-Borne Outbreaks in the RS State

S. Enteritidis strains isolated from foods involved in salmonellosis in 1999 - 2000 (n=73) demonstrated to be predominantly resistant to streptomycin (37 %), gentamicin (14 %), and nalidixic acid (14 %), while intermediate resistance was observed most often for tetracycline (53 %), neomycin (30 %), and gentamicin (15 %). Multiple resistance was demonstrated in 17 of the strains (23 %), and one isolate exhibited resistance to four drugs (neomycin,

kanamycin, streptomycin, and nalidixic acid). Most strains demonstrated to be fully susceptible to the ten drugs tested [21]. *S.* Enteritidis strains (n=79) isolated in 2001 to 2002 showed the highest percentages of resistance for gentamicin (13 %), streptomycin (11 %), and nalidixic acid (21 %), while the most expressive intermediate resistances were demonstrated for kanamycin (29 %), neomycin (18. 7%) and streptomycin (14 %). Full susceptibility for all ten drugs tested was found in 62 % of the strains. The highest rates of sensitivity were demonstrated for ampicillin (95 %), tetracycline (91 %), and chloramphenicol (99 %). No resistance was observed regarding sulfamethoxazole/trimetoprim and sulfazotrim. Comparing the antibiotic resistance of the bacteria isolated in 2001 to those isolated in 2002, an increase in the resistance percentage of the isolates was observed concerning tetracycline (0 % in 2001 to 5 % in 2002), kanamycin (2 % in 2001 to 3 % in 2002), nalidixic acid (19 % in 2001 to 24 % in 20002), and chloramphenicol (0 % in 2001 to 3 % in 2002). Results of the intermediate resistance for neomycin, kanamycin, gentamycin, and streptomycin also increased from 2001 to 2002. Overall, resistance was verified in 30 strains (38 %) [22]. Among the 80 *S.* Enteritidis isolated from salmonellosis in RS from 1999 to 2006, only three isolates (4 %) were fully susceptible to all the antibiotics tested. The major resistances were observed for ampicillin (81 %), streptomycin (19 %), and nalidixic acid (25%) [13]. Paula et al. [23] have studied 130 *S.* Enteritidis responsible for food-borne salmonellosis occurred in RS from 2003 to 2006, and have reported that the higher percentages of resistance were demonstrated for ampicillin (100 %) and nalidixic acid (48 %). Intermediate resistance was found for neomycin (49 %) and tetracycline (25 %). Multiple drug resistance was observed in 63 % of isolates. Analysing the results published by Geimba et al. [21], Oliveira et al. [22], Oliveira et al. [11], and Paula et al. [23], it is possible to observe increasing resistance rates for nalidixic acid and ampicillin regarding *S.* Enteritidis associated with salmonellosis in RS (Figure 1).
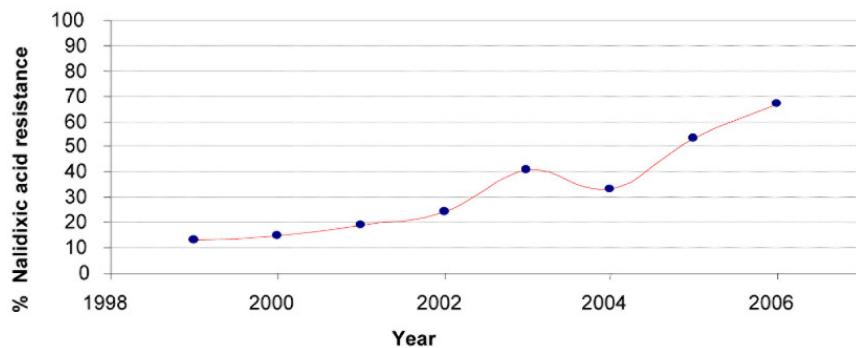
Figure 1. Resistance to nalidixic acid in the *S.* Enteritidis responsible for food-borne salmonellosis outbreaks occurred in the Rio Grande do Sul State, southern Brazil, from 1999 to 2006 (Paula et al., 2006).

The raising resistance for nalidixic acid and the resistance for ciprofloxacin are issues of concern, because several studies have reported increasing numbers of *Salmonella* sp. strains resistant to quinolones in Germany [24], England and Wales [25] and Spain [26]. Emphasizing this concern, the CLSI added a guideline recommending clinical laboratories to routinely test for nalidixic acid resistance in extraintestinal *Salmonella* isolates in order to alert physicians about the emerging resistance (Clinical and Laboratory Standards Institute [27]. Quinolones are widely used in food animal production and are known to select for quinolone-resistant *Salmonella* sp. in animals. At the same time, quinolones are one of the few available therapies for serious *Salmonella* infections, particularly in adults.

The prevalence of resistant samples to tetracycline and streptomycin could be explained by the dissemination of resistance genes, *strA* and *strB* tet (A), as observed by Pezzella, Ricci, and Digiannatale [28] in a study about *Salmonella* strains isolated from animals in Italy. Hyeon et al. [29] also demonstrated resistance to streptomycin (22.2 %) and tetracycline (16.7 %) among *S.* Enteritidis isolated from retail meats products in South Korea in 2009. Tetracycline and streptomycin are common antimicrobials administered in veterinary medicine and their frequent use may have contributed to the resistance rates found in the RS State and South Korea. On the other hand, chloramphenicol resistance observed among *S.* Enteritidis isolated in southern Brazil could not be explained by the use of this drug in animal feed, because

chloramphenicol was banned from animal feed production in Brazil more than a decade ago.

Salmonella in Pork Production and Pork Products in the RS State

The southern region of Brazil is the most important area for pork production in the country. Its pork output accounted for two thirds of the Brazilian total production, which consisted of 2.8 million tons in 2006. Approximately 70 % of the pork produced in Brazil is consumed in the form of processed meat products, such as sausages, bacon and ham. Pork contamination by Salmonella can occur at multiple stages along the production chain. Moreover, the level of Salmonella infection of pigs on farms can be increased during transport, lairage and slaughtering-plant operations [30].

The slaughter of Salmonella carrier pigs is considered the first critical point for the contamination of pork products. The risk represented by positive animals tends to increase when this pathogen is found in carcass portions consumed by the population [31].

Castagna et al. [31] assessed the implication of the prevalence of Salmonella-positive pigs at slaughter on the contamination of ground meat used for fresh sausage production in the RS State. Mandibular lymph node/tonsil pools (LT) and intestinal contents (CI) of 16 animals were collected in each one of the three samplings conducted at an abattoir. On the day after, 99 samples of the ground meat, produced with carcass parts of pigs slaughtered in the previous day, were collected immediately before the casing. The mean prevalence of Salmonella carrier pigs at slaughter was 83.33 %, while 93.94 % of the ground meat samples were positive. The most prevalent serovars were Panama, Bredeney, and Typhimurium. Results demonstrated that the slaughter of Salmonella-positive pigs implicated on the contamination of fresh pork sausage.

Mariot et al. [32] studied the incidence of Salmonella sp. in four surface points (ham, belly, loin and neck) of 239 pork carcasses processed in a big exporter pig-slaughtering plant (3200 animals per day) in RS. Salmonella spp.

was isolated in 30 % of the carcasses after bleeding. Other steps also demonstrated *Salmonella* contamination on the pork carcasses. As examples, *Salmonella* sp. isolation was verified after washing (17.95 %), after dehairing (16.65 %), and also after the first polishing (20 %). *Salmonella* sp. was not detected after the scalding and singeing steps, suggesting that these steps are important to control *Salmonella* in pork production.

Bessa et al. [33] evaluated the prevalence of *Salmonella*-positive pigs at slaughterhouses under federal inspection in RS. Samples of feces and lymph nodes of 300 animals were collected in three different slaughterhouses and submitted to bacteriological analysis. The prevalence of *Salmonella* carrier animals was 55.66 %, being 17.6 % of the animals *Salmonella*-positive in lymph nodes, 18.3 % in feces, and 19.6 % in both materials. Twenty-six different serovars were identified among 226 *Salmonella* isolates. The most prevalent serovars were Typhimurium (24.3 %), Agona (19.9 %), Derby (13.2 %) e Bredeney (12 %).

Schwartz et al. [34] analyzed the prevalence of *Salmonella* in slaughtered swine and the results of serology and *Salmonella* isolation were compared in order to determine the most important stage of the infection (on-farm or transport, and pre-slaughter) in relation to the number of carrier animals found at slaughter. Forty herds from three different swine raisers in the southern region of Brazil were sampled at slaughter. Blood and mesenteric lymph nodes were collected from 20 swine in each herd. *Salmonella* was isolated from 62.5 % to 85.0 % of the animals, while seroprevalence varied from 73.8 % to 83.2 % in the three swine raisers. Serovars Agona, Typhimurium and Panama were the most prevalent among the *Salmonella* isolates. Isolation and serological results demonstrated that *Salmonella* infection occurred during the on-farm stage, since a high seroprevalence was detected at slaughter.

Muller et al. [35] compared the prevalence of *Salmonella* positive pigs at the beginning of the finishing phase and at slaughter in order to identify the possible sources of contamination in the farms. In three finishing farms, environmental swabs from the barns and from the feed silos were collected during the sanitary emptiness. Feces samples from all pigs were *Salmonella*-negative at the

beginning of the finishing phase; in two farms seropositive animals were found. In two farms, residual environmental contamination was detected, and in the third farm one of the feed batches was *Salmonella*-positive. At slaughter, over 90 % of the animals were positive and, in all cohorts, a variable number (12 % - 92 %) of carriers was detected. From this on, it was concluded that the finishing phase was critical for the amplification of *Salmonella* infection, and the residual environmental contamination in the farms, as well as *Salmonella*-positive feed batches, were the probable sources of infection.

A total of 336 samples of fresh pork sausage randomly obtained from supermarkets and butcher shops in Porto Alegre city, the capital of the RS State, were examined for the presence of *Salmonella* sp. *Salmonella enterica* was detected in 24.4 % of the samples, with a most probable number count ranging from 0.03 MPN g^{-1} to 460 MPN g^{-1}. Strains belonging to the most isolated *S. enterica* serovars (Brandenburg, Panama, Derby, and Typhimurium) were further analysed for antimicrobial resistance, and the results demonstrated that the resistance for tetracycline was the most prevalent among the *Salmonella* isolates. *S*. Panama and *S*. Typhimurium presented the greatest number of resistance phenotypes [30].

The survival of *Salmonella* sp. in pig slurry submitted to treatment in successive stabilization ponds on a pig-breeding farm was investigated. The isolated *Salmonella* strains were tested for their resistance against 14 antibiotics, using the agar diffusion method. Of a total of 20 samples taken from different points in the stabilization ponds system, 13 were positive for *Salmonella* sp. in the beginning, and only one at the end of the system. Most of the isolated *Salmonella* (161/163) belonged to sorovar Typhimurium. These strains were resistant to sulfonamide (100 %), tetracycline (9.4 %), sulfamethoxazole/trimethoprin (84.5 %), ampicillin (76.4 %), cloramphenicol (29.2 %), streptomycin (90.1 %), nalidixic acid (77.6 %), tobramycin (13.7 %), neomycin (5 %), amikacin (3.7 %), cefaclor (25.5 %), gentamicin (6.2 %) and amoxacilin/clavulanic acid (5 %). Most *S*. Typhimurium strains (94.5 %) were resistant to four or more antibiotics. The multi-resistance level and the pattern

variability of these strains were similar in the beginning and at the end of the stabilization ponds system [36].

Salmonella Isolated from Pork and Resistance to Biocides

The effectiveness of six biocides (quaternary ammonium, glutaraldehyde, iodophor, sodium hypochlorite, phenol and peracetic acid) in strains of _Salmonella_ sp. isolated from pigs was evaluated. The disinfectants were evaluated against 8 porcine _S_. Typhimurium strains presenting different antibiotic resistance profiles, with a contact time of five minutes, with and without organic matter. All disinfectants were effective in the absence of organic matter. However, when organic matter was included on the assay, only sodium hypochlorite, phenol and peracetic acid were effective. Furthermore, sodium hypochorite, phenol and peracetic acid were also the most effective against porcine _S_. Typhimurium strains after five minutes of contact. These results indicate that the effectiveness of the tested disinfectants was more related to the presence of organic matter and exposure time than to the resistance profile presented by the tested strains [37].

The sensitivity of 96 _S_. Typhimurium strains isolated from slaughter pigs in the RS State was evaluated. The isolates were tested against quaternary ammonium and iodophor, which represent two commercial disinfectants commonly used in animal production. The tested disinfectants were used in the concentration recommended by the fabricant and in a sub-concentration in order to simulate a possible field situation. Dilution suspension tests were conducted observing the inactivation of each _S_. Typhimurium isolate after 5, 15, 30 and 60 minutes of contact with each compound. All tested isolates were inactivated by the quaternary ammonium compound in both concentrations. Four isolates revealed resistance to iodophor in the recommended concentration, and 59 isolates were resistant when a sub-concentration was used [38].

Salmonella in Brazilian Poultry

Brazil is the third biggest poultry producer in the world, staying behind only China and the USA. However, concerning the exportations, Brazil is the biggest exporter in the world since 2006, when this country exported 2,900 thousand tons of poultry meat. Approximately 40 % of the poultry meat in the world comes from Brazil [39]. Even though poultry meat produced in Brazil presents high quality standards, the growth of the Brazilian poultry industry in the 1990s, as well as the increasing number of birds reared in high density environments, have created favorable conditions for the presence of *Salmonella* in some flocks [40].

The control of *Salmonellae* in Brazil is regulated by the Poultry National Health Plan developed by the Ministry of Agriculture, Livestock and Food Supply. In 2003, a vaccination for broiler breeders using killed vaccine against *S*. Enteritidis was approved. It has been suggested that the presence of one serovar in a flock will reduce the accumulation of others. For example, it is believed that either the reduction of *S*. Gallinarum resulted in the increase of *S*. Enteritidis in flocks or the increased presence of *S*. Enteritidis displaced other serovars. In broiler production, when cecal tonsil colonization is established by *Salmonella*, such bacteria are consistently excreted in the feces [40].

Santos et al. [41] evaluated samples of broiler carcasses produced in poultry companies located at the highlands region of RS, which were all submitted to a microbiological monitoring program carried out during the years 1995 and 1996. During this period, 1,344 carcasses were analysed, from which 15.10 % were positive for *Salmonella* sp. (111 of the later identified as *S*. Enteritidis). The 111 isolates of *S*. Enteritidis were phage-typed and the results indicated that 93.3 % of them belonged to the PT4. The same isolates were evaluated for RAPD, and it was verified that 91.8 % were classified as pattern A [40]. However, lesser percentages of *Salmonella*-positive samples were verified by the Brazilian Sanitary Agency (ANVISA), who analyzed 180 poultry carcasses produced in RS, demonstrating that 95.56 % of them were negative for *Salmonella* sp [42].

Salmonella strains were isolated from ill and shedding birds in several regions of Brazil between 1962 and 1991. Serotyping of 2123 isolates showed 90 serovars pertaining to 14 serogroups. There was a predominance of groups O:9 (40.0%), O:4 (33.3%), O:7 (10.6%) and O:3,10 (6.7%). Major serovar diversity was found in the serogroup O:7, which accounted for 22 different types, followed by the serogroups O:4, O:3,10 and O:9, with 19, 15 and 10 serotypes respectively .

An average of 10.8 serovars was isolated per year. S. Gallinarum, S. Pullorum, S.Typhimurium, S. Heidelberg, S. Enteritidis and S. Infantis were the most frequent serovars found over 30 years, representing 65 % to 67 % of the total of isolates [43].

Antimicrobial Resistance of *Salmonella* sp. Isolated from Poultry

The antimicrobial resistance of 96 *S.* Enteritidis isolated from salmonellosis outbreaks (n=43) and poultry-related products not involved with food-borne outbreaks (n=53), in the period from 1995 up to 2003, was investigated. Although 43.75 % of the samples were sensitive to all drugs tested, and resistance to sulfonamide (34.37 %), trimethoprim-sulfamethoxazole (25.00 %), nalidixic acid (14.58 %), streptomycin (2.08 %), gentamicin and tetracycline (1.04 %) was identified. *S.* Enteritidis resistance to nalidixic acid (14.58 %) was identified only in strains isolated from poultry, and this result could be explained because nalidixic acid is commonly used in poultry therapy in Brazil [44]. Ribeiro et al. [45] evaluated the occurrence of *Salmonella* sp. in raw broiler parts and analysed the antimicrobial resistance profile of the microorganisms isolated. The study was carried out using 61 broiler chicken parts (wings, whole legs, boneless breasts and backs) collected in the period from September to December of 1996 in a processing plant located in southern Brazil. Regarding the antimicrobial resistance, the results indicated that 88 % of the *Salmonella* isolates were resistant to one or more antimicrobial agents, presenting eight different patterns of resistance. Multiple resistance was not observed in five of

the 21 *S.* Enteritidis isolates, while 3 were susceptible to all antimicrobials tested, and two were resistant only to tetracycline. Multiple resistance was also found in the *S.* Typhimurium, and in all three *S.* Hadar isolates. Three *S.* Enteritidis isolates were susceptible to all antimicrobials tested, and two were resistant only to tetracycline. The results emphasized the need for the responsible use of antimicrobials in animal production.

During the years of 1999, 2000 and 2001, the antimicrobial resistance of *S.* Enteritidis isolated from clinical and environmental poultry samples was evaluated in southern Brazil. Among the 79 isolated samples, 81 % were resistant to at least one of the antimicrobial agents tested, showing 22 different resistance patterns. Tetracycline showed the highest percentage of resistance (64.5 %) among the antimicrobial agents used. Resistance to drugs at different levels was found as the following: ampicillin (1.2 %), kanamycin (1.2 %), ciprofloxacin (2.5 %), enrofloxacin (8.8 %), gentamicin (21.5 %), streptomycin (20.2 %), nitrofurantoin (26.6 %), and nalidixic acid (30.4 %). None of the *S.* Enteritidis strains were resistant to chloramphenicol, norfloxacin, and polimycin B. Among the 64 *S.* Enteritidis strains that showed resistance, 67.2 % were resistant to two or more antimicrobial drugs. Twenty-one isolates (32.8 %) were resistant to only one of the antimicrobial agents, 14 to tetracycline, three to nalidixic acid, three to nitrofurantoin, and one to gentamycin. These levels of antimicrobial resistance suggest a high occurrence of tetracycline-resistant *S.* Enteritidis strains, as well as resistance to two or more antimicrobial drugs [46].

CONCLUSION

Salmonella sp. was recognized as the first cause of food-borne illnesses in Brazil in the last decade, being that, in the southern region, this microorganism has been identified as the first cause for food-borne outbreaks for almost two decades. A specific strain of *S.* Enteritidis (SE86) was identified as the microorganism responsible for more than 95 % of salmonellosis in RS. When compared to other *Salmonella* serovars, SE86 demonstrated higher acid-and-

thermal adaptation capability and also has demonstrated to be able to grow faster in home-made mayonnaise, the food vehicle most involved with salmonellosis in RS. When exposed to sub-letal pHs, SE86 increased its virulance in germ-free mices, which was not verified for *S.* Typhimurium and *S.* Bredeney. The antibiotic surveillance of the *S.* Enteritidis isolated in RS demonstrated an increasing resistance for nalidixic acid and ampicilim, even though high levels of suceptibility were reported for many antibiotics. SE86 was tested against different sanitizers and demonstrated to be more resistant than other *Salmonella* serovars, mainly to sodium hypochlorite, the most used biocide in RS. The involvement of *rpo*S and *dps* genes with this resistance was studied and it was demonstrated that both genes are expressed during sodium hypochlorite exposure. However, the mutant *dps* demonstrated lesser survival rates than the wildtype SE86. All those characteristics of SE86 could be related to the frequent involvement of this strain with salmonellosis outbreaks in RS. Also corroborating this possibility is the fact of the presence of some other *Salmonella* serovars in pork and poultry meat produced in RS, which were not responsible for salmonellosis outbreaks.

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3.2 ARTIGO 1

3.2.1 "Investigation of *rpoS* and *dps* genes in sodium hypochlorite resistance of *Salmonella* Enteritidis SE86 isolated from foodborne illness outbreaks in Southern Brazil"

Investigation of *rpoS* and *dps* Genes in Sodium Hypochlorite Resistance of *Salmonella* Enteritidis SE86 Isolated from Foodborne Illness Outbreaks in Southern Brazil

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ABSTRACT

In Rio Grande do Sul, southern Brazil, *Salmonella* Enteritidis is one of the principal microorganisms responsible for foodborne disease. The present study was conducted to compare the sodium hypochlorite resistance of *Salmonella* Enteritidis SE86 with that of other strains of *Salmonella* Enteritidis isolated from different regions of the world and to investigate the involvement of the *rpoS* and *dps* genes in resistance to this disinfectant. We tested five *Salmonella* Enteritidis wild-type (WT) strains isolated from different countries, two mutant strains of *Salmonella* Enteritidis SE86, and two tagged (3XFLAG) strains of *Salmonella* Enteritidis SE86 for their resistance to sodium hypochlorite (200 ppm). The survival of the WT and attenuated strains was determined based on bacterial counts, and tagged proteins (Dps and RpoS) were detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with anti-FLAG antibodies. None of the WT strains of *Salmonella* Enteritidis were totally inactivated after 20 min. The SE86 strain lacking *dps* was more sensitive to sodium hypochlorite than was the WT SE86 strain, with a 2-log reduction in counts after 1 min. The RpoS and Dps proteins were actively expressed under the conditions tested, indicating that in *Salmonella* Enteritidis SE86 these genes, which are expressed when in contact with sodium hypochlorite, are related to oxidative stress.

In Brazil, 6,349 official notifications of foodborne outbreaks were recorded from 1999 to 2009, resulting in 123,917 ill people and 70 deaths, and 42.5% the outbreaks were caused by *Salmonella (24)*. Brazil is populated by approximately 190 million people distributed in 27 states. As in other parts of the world, only a small number of foodborne outbreaks are officially reported to regulatory agencies. The great majority (72%) of Brazilian foodborne illnesses cases were reported from only three states of southern Brazil: Rio Grande do Sul (RS; 30%), São Paulo (22%), and Paraná (12%) *(24)*.

Costalunga and Tondo *(9)* reported that salmonellosis accounted for 36% of foodborne disease outbreaks investigated in RS between 1997 and 1999, and Silveira and Tondo *(34)* stated that salmonellosis was at the top of the foodborne disease list of RS in 2000 and 2001. Geimba et al. *(15)* reported that more than 97% of the cases of salmonellosis that occurred in RS from 1999 to 2002 were caused by *Salmonella* Enteritidis. Further studies have revealed that the majority of foodborne outbreaks were caused by a specific strain: *Salmonella* Enteritidis SE86 *(27)*. This microorganism was able to grow faster *(21)* and

to adapt better than other *Salmonella* serovars (*Salmonella* Typhimurium and *Salmonella* Bredeney) after exposure to sublethal pH and was more resistant to acid and high temperature *(20)*.

Humphrey *(17)* reported that *Salmonella* possesses a complex regulatory system, which mediates its responses to environmental stresses and can be activated under unfavorable conditions such as extremes in pH and temperature and low levels of oxygen. The sigma factors regulate the specificity of RNA polymerase and gene expression related to environmental stresses *(17)*. SigmaS factor, encoded by the gene *rpoS*, is maximally induced in the early stationary phase and controls the expression of more than 40 genes and operons. The activity of *rpoS* also can be induced in the exponential growth phase by lack of nutrients, heat, osmotic shock, and oxidative stress *(11)*. As part of the regulatory system of *rpoS*, the *dps* gene encodes for a nonspecific DNA binding protein whose expression is induced in the stationary growth phase *(3)*. The *dps* gene is responsible for protecting bacteria against oxidative damage both in vivo and in vitro *(22, 29)*. Dps can be added to the set of evolutionarily conserved antioxidant proteins used by *Salmonella* for resistance to different types of stresses.

Chlorine-releasing compounds have been widely used in the food industry and food services worldwide because of

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their broad spectrum efficacy against microorganisms and their affordability. In Brazil, chlorine is the most commonly used biocide in food industries and food services for disinfection of vegetables, fruits, equipment, and utensils, and 200 ppm is the concentration most widely used, as stipulated by Brazilian regulations (1). Tondo et al. (36) found that sodium hypochlorite inactivated biofilms of various *Salmonella* serovars on polyethylene and stainless steel surfaces. These authors determined that *Salmonella* Enteritidis SE86 was more resistant than *Salmonella* Bredeney and *Salmonella* Typhimurium to 200, 400, and 800 ppm of sodium hypochlorite, which suggests that this characteristic of *Salmonella* Enteritidis SE86 could be related to its frequent identification as the cause of salmonellosis. Sodium hypochlorite and hydrogen peroxide inactivate microorganisms by cellular oxidation (38). The present study was conducted to compare the sodium hypochlorite resistance of *Salmonella* Enteritidis SE86 with that of other strains of *Salmonella* Enteritidis isolated from other countries and to investigate the genetic basis of this resistance, which explain the frequent involvement of *Salmonella* Enteritidis SE86 in outbreaks.

MATERIALS AND METHODS

Bacterial strains. The five *Salmonella* Enteritidis strains investigated had been identified as responsible for foodborne outbreaks. Strain SSM2047 was isolated in Albania, strain SSM1813 was isolated in Pakistan, strain SSM4242 was isolated in Morocco, and strain SSM1644 was isolated in Zimbabwe. *Salmonella* Enteritidis SE86 was isolated from a cabbage responsible for an outbreak of salmonellosis in RS, Brazil, in 1999. This strain was characterized with phenotypic and genotypic methods by Geimba et al. (15) and Oliveira et al. (27, 28) and has the same genotypic profile as *Salmonella* Enteritidis strains involved in more than 95% of the investigated salmonellosis cases in RS from 1999 to 2006 (28). Strains SSM2047, SSM1813, SSM4242, and SSM1644 are stored in the Laboratorio di Microbiologia from Università di Sassari (Sassari, Italy). Strain SE86 was provided by the Laboratório de Microbiologia e Controle de Alimentos of Instituto de Ciência e Tecnologia de Alimentos (Porto Alegre, RS, Brazil). Until used, all strains were stored at −70°C in Luria-Bertani medium with 40% glycerol. *Listeria monocytogenes* ATCC 7641 was used as a negative control for the sodium hypochlorite resistance experiments.

Mutants of *Salmonella* Enteritidis SE86 (Δ*dps* and Δ*rpoS*) were constructed in the Laboratorio di Microbiologia (Università degli Studi di Sassari, Sassari, Italy) using the method described by Datsenko and Wanner (10). The construct was verified by PCR analysis. The SSM5327 (*dps*::Kan) and SSM5333 (*rpoS*::Kan) mutations were transferred into a clean *Salmonella* Enteritidis SE86 background by P22 transduction.

Expression of the *dps* and *rpoS* genes. *Salmonella* Enteritidis SE86 was tagged with the eight amino acid FLAG epitope tag peptide. Strains SSM5348 (*rpoS*::3XFLAG *cat*::FLAG) and SSM5350 (*dps*::3XFLAG *cat*::FLAG) of serovar *Salmonella* Enteritidis were obtained using the method described by Uzzau et al. (37). The 3XFLAG epitope is a sequence of three tandem FLAG epitopes (22 amino acids). For each tagged mutant, a pair of primers was designed to amplify a 3XFLAG and kanR coding sequence by using plasmid pSUB11 (37). The 3′ ends of these oligonucleotides were complementary to the first 20 nucleotides of the pSUB11 3XFLAG coding region (GACTACAAAGACCAT-

GACGG, forward primers) and to the 20 nucleotides of the pSUB11 priming site 2 (CATATGAATATCCTCCTTAG, reverse primers). The 5′ ends of the oligonucleotides were designed to be homologous to the last 40 nucleotides of each tagged gene, not including the stop codon (forward primers), and to the 40 nucleotides immediately downstream of the gene stop codon (reverse primers). The Cat protein was used as an internal marker because it is very stable. A constitutively expressed epitope-tagged gene such as *cat* was used as a positive control or internal reference (37).

Evaluation of the resistance to sodium hypochlorite. Four *Salmonella* Enteritidis strains (SSM2047, SSM1813, SSM4242, and SSM1644) were incubated separately in brain heart infusion (BHI; Sigma, St. Louis, MO) at 37°C for about 18 h. After incubation, the cultures were diluted in 0.1% peptone water (pH 7.2) to approximately 10^6 CFU/ml, and 0.1 ml of each suspension was inoculated into 9 ml of 200 ppm of sodium hypochlorite solution supplemented with 0.9 ml of 1% bovine serum albumin (23). After 1, 3, 5, 10, 15, and 20 min at room temperature, 1 ml of each bacterial suspension was serially diluted in tubes containing 9 ml of 0.1% peptone water supplemented with 0.1% sodium thiosulfate. After vigorous vortexing, survival was quantified by plating 20 μl of each appropriate dilution on BHI agar and incubating for 24 h at 37°C. Quantification of survivors was performed according to the technique described by Silva et al. (33). The lower detection limit was 1.69 log CFU/ml, and each experiment was conducted twice, with duplicate counts. *L. monocytogenes* ATCC 7641 was used as a negative control in this experiment. The concentration of free chlorine used in experiments was determined with a chlorine test kit (CHEMetrics, Inc., Calverton, VA).

Western blotting of tagged genes. After exposure to sodium hypochlorite, 1-ml aliquots of the cultures were centrifuged (18,000 × *g*, 10 min, 4°C). Protein extracts were boiled for 5 to 10 min, and an aliquot of each sample was resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis for detection of 3XFLAG-tagged proteins by Western blotting. The nitrocellulose membranes were blocked with 5% (wt/vol) nonfat dried milk in phosphate-buffered saline (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl) containing 0.05% Tween 20, washed, and incubated with mouse anti-FLAG M2-peroxidase (HRP) mAbs (Sigma) diluted 1:1,000. The detection was performed using H_2O_2 and $CoCl_2$. Blots were scanned, and the density of the signals was analyzed with the National Institutes of Health public domain software Image J (http://rsb.info.nih.gov/nih-image/). All relative density values corresponded to arbitrary values from Image J software analysis of corresponding bands from Western blots and were normalized by reference to immunodetection of the control protein catalase. Western blots were evaluated in biological and technical duplicates.

Statistical analysis. All experiments performed to evaluate the survival of microorganisms were repeated at least twice, and all counts were conducted in duplicate. The Tukey test was used to compare the differences between the mean values. The differences were considered significant when P values were less than 0.05.

RESULTS AND DISCUSSION

None of the strains of *Salmonella* Enteritidis were totally inactivated after 20 min of exposure to 200 ppm of sodium hypochlorite (Fig. 1). In general, initial cell counts
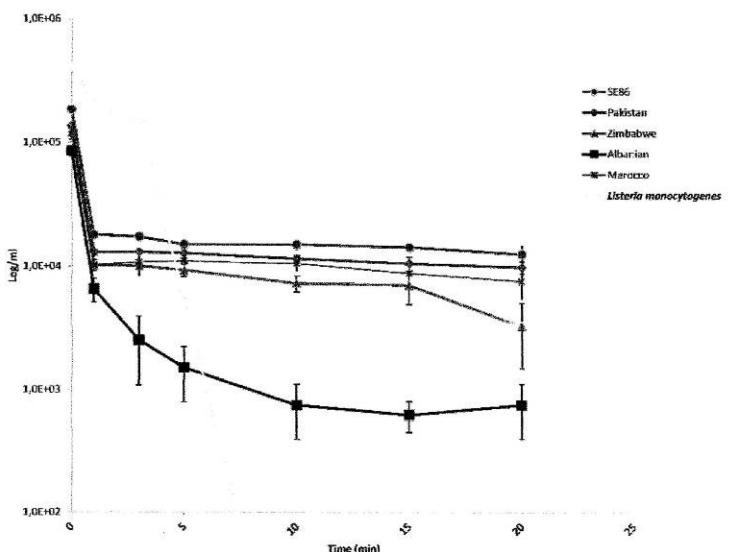
FIGURE 1. *Survival after exposure to 200 ppm of sodium hypochlorite of* Salmonella *Enteritidis strains that have caused food-associated outbreaks in various countries.*

of approximately 10^5 CFU were reduced by 1 log CFU after 1 min of exposure to the sanitizer. However, counts of viable cells remained almost unaltered during the exposure. The strain from Albania (SSM2047) was most sensitive; the viable cell counts were reduced by almost 2.5 log CFU after 20 min. Greater inactivation was reported by Riazi and Matthews *(31)*, who found >3-log reductions of *Salmonella* Enteritidis after exposure to 256 ppm of sodium hypochlorite for 20 min.

Various characteristics, e.g., growth rate, thermal and acid resistance *(26)*, and resistance to disinfection methods, may contribute to the emergence of a microorganism as a food pathogen. Malheiros et al. *(21)* found that *Salmonella* Enteritidis SE86 multiplied faster than *Salmonella* Typhimurium and *Salmonella* Bredeney in the first 6 h of incubation in homemade mayonnaise. This food was the main vehicle responsible for salmonellosis in RS from 1997 to 2001 *(9, 34)*. Malheiros et al. *(20)* also found that *Salmonella* Enteritidis SE86 had a greater capacity for acid adaptation and thermal resistance than did other *Salmonella* serovars after exposure to sublethal pH. Tondo et al. *(36)* found that *Salmonella* Enteritidis SE86 was more resistant to sodium hypochlorite than were other *Salmonella* serovars; however, in our study resistance of *Salmonella* Enteritidis SE86 to sodium hypochlorite was similar to that of other *Salmonella* Enteritidis serovars from other countries. Several researchers have reported very low genetic diversity among *Salmonella* Enteritidis strains *(8, 19)*. Pang et al. *(30)* reported that a clone of *Salmonella* Enteritidis has spread worldwide, causing salmonellosis in various countries. This fact could explain the similar resistance to sodium hypochlorite verified in the present study.

Susceptibility of *Salmonella* Enteritidis SE86 mutants to sodium hypochlorite. The effect of sodium hypochlorite on the survival of wild-type (WT) *Salmonella*

Enteritidis SE86 and *Salmonella* Enteritidis SE86 mutants is shown in Figure 2. The *Salmonella* Enteritidis SE86 mutant lacking *rpoS* had behavior similar to that of WT SE86, with counts of 10^5 CFU after 20 min of exposure to the sanitizer. These results are in contrast to those of Dukan and Touati *(13)*, who investigated the behavior of an *rpoS*-deficient *Escherichia coli* mutant. This strain was much more sensitive to hypochlorous acid. However, unlike Dukan and Touati, we tested our strains against sodium hypochlorite rather than hypochlorous acid. In aqueous environments, a balance is maintained between these two forms (collectively referred to as free chlorine), deionized hypochlorous acid (HOCl) and hypochlorite ions (ClO$_2$). The ratio of these forms depends on pH and temperature. HOCl is the more reactive of the two forms *(13)*.

As expected, *Salmonella* Enteritidis SE86 lacking *dps* was significantly more sensitive to sodium hypochlorite ($P < 0.05$) than was the WT SE86, with a 2-log reduction after 1 min of exposure and a 3-log reduction after 20 min of exposure. In several studies with *E. coli* strains lacking *dps*, the authors concluded that when this gene is not present, the cell suffers more oxidative damage, suggesting that this gene is involved in protecting DNA against this type of damage *(2, 3, 5, 7, 13, 25)*.

The Dps protein in *Salmonella* Typhimurium has up to 95% homology with the Dps protein in other enteric gram-negative bacteria, including *E. coli*. The nucleotide sequence of the promoter region of *Salmonella dps* shares 95% identity with the promoter region of *E. coli dps*, suggesting that the *Salmonella dps* promoter may behave similarly to the *E. coli dps* promoter *(39)*. Altuvia et al. *(3)* found that bacterial strains lacking *dps* have impaired oxidative stress responses and are unable to survive when there is insufficient food for long periods.

As a control, we exposed the mutant strains to peptone water without sodium hypochlorite (results not shown),

FIGURE 2. *Survival of* Salmonella *Enteritidis SE86 mutants exposed to 200 ppm of sodium hypochlorite.*
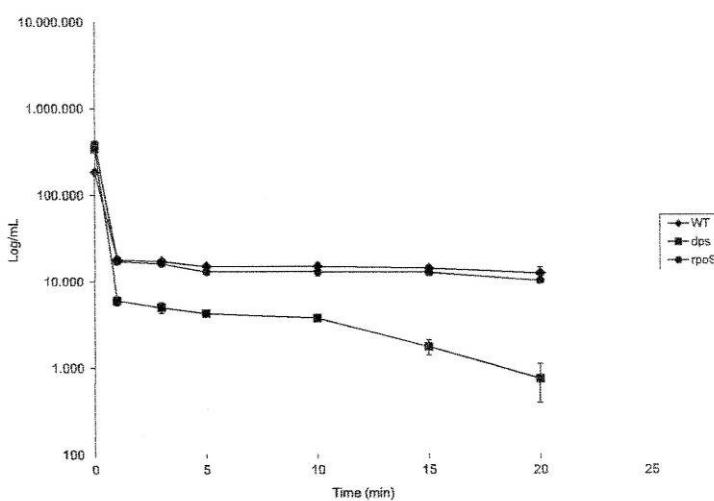
using the same times and conditions, to determine the influence of these genes on the sensitivity to sodium hypochlorite. The sensitivity of the mutant strains was similar to that of the WT strain, suggesting that the *rpoS* and *dps* genes are involved in sodium hypochlorite resistance.

Immunodetection of epitope-tagged proteins. Chlorinated compounds have a wide spectrum of activity, affecting the cell membrane, inhibiting enzymes involved in the metabolism of glucose, causing damage to DNA, and oxidizing cellular proteins *(32)*. The phenotypic characteristics of a pathogen are determined by their genetic makeup, and determinants of virulence may be present in the chromosome, usually encoded in pathogenicity islands, plasmids, and bacteriophages *(14)*. Audia et al. *(6)* and Dong et al. *(12)* found that the expression of the *rpoS* gene is related to the exposure of food pathogens to sublethal stress factors, such as temperature, acid, and oxidation.

To investigate the involvement of tagged mutants in resistance to disinfectants, bacterial strains were exposed to sodium hypochlorite for various periods. The expression of the RpoS protein in response to exposure to 200 ppm of sodium hypochlorite was confirmed by Western blot (Fig. 3). The expression of RpoS remained virtually constant for the first 10 min of exposure, briefly increased after 15 min, and then decreased after 20 min. These results indicate that the *rpoS* gene is induced during exposure to sodium hypochlorite and probably coordinated the expression of other genes involved in stress responses.

In several studies, high oxidative stress resistance was entirely mediated by SigmaS *(7, 13, 35)*. Induction of numerous stress resistance genes in the stationary phase depends on the Sigma factor, which is encoded by *rpoS*. The *rpoS* gene regulates the expression of DNA repair enzymes, such as the exonuclease xthA, the methyl transferase ada, and the nonspecific DNA binding protein Dps *(18)*. Dps was actively expressed under the conditions tested in our experiments (Fig. 4). Dps also had greater expression than rpoS in bacteria exposed to the same conditions, especially

in the first minutes of exposure. Dps is a cytoplasmic protein that protects cellular DNA against damage caused by oxidative stress due to exposure to hydrogen peroxide, HOCl, and acid *(13, 29, 39)*. Young et al. *(39)* found that when *Salmonella* was exposed to multiple stresses, including oxidative stress, a DNA binding protein in the stationary phase (Dps) was one of the main overexpressed proteins. The positive control used in the
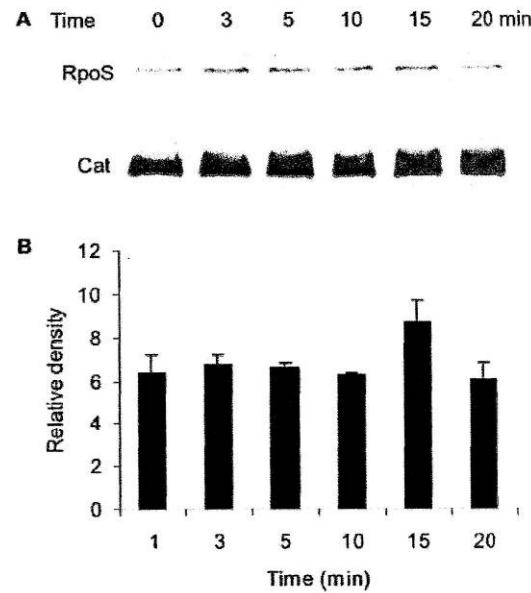
FIGURE 3. *Immunodetection of 3XFLAG-tagged proteins in* Salmonella *Enteritidis SE86 (*rpoS::3XFLAG cat::3XFLAG*). (A) Western blot of 3XFLAG-tagged proteins, RpoS and catalase (Cat), as the control. (B) Densitometric analysis of bands from immunodetection of RpoS protein (all values of relative density correspond to arbitrary values from Image J software analysis normalized in reference to immunodetection of control protein Cat).*

A Time   0   3   5   10   15   20 min

Cat

Dps

B
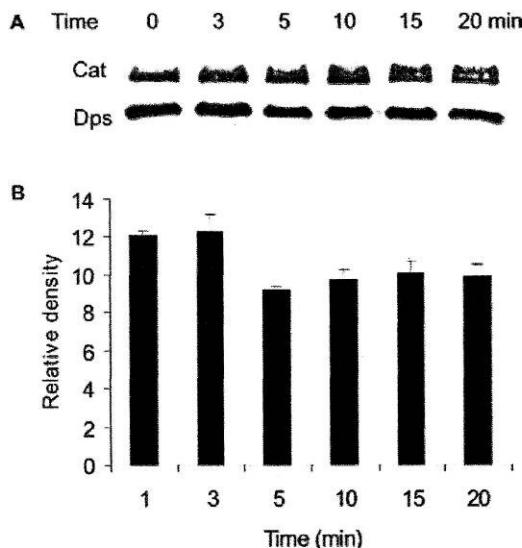
FIGURE 4. *Immunodetection of 3XFLAG-tagged proteins in Salmonella Enteritidis SE86 (dps::3XFLAG cat::3XFLAG). (A) Western blot of 3XFLAG-tagged proteins Dps and catalase (Cat), as the control. (B) Densitometric analysis of bands from immunodetection of Dps protein (all values of relative density correspond to arbitrary values from Image J software analysis normalized in reference to immunodetection of control protein Cat).*

present study, the *cat* gene, was detected at all times and under all conditions analyzed, as described by others *(16, 37)*. As a negative control, mutant strains were exposed to peptone water without sodium hypochlorite (Fig. 5) to evaluate the difference in protein expression.

To our knowledge, the present study is the first in which sodium hypochlorite resistance has been compared among *Salmonella* Enteritidis strains involved in foodborne outbreaks in different parts of the world. This study is also the first to include investigation of the involvement of *Salmonella* Enteritidis *dps* and *rpoS* in the resistance to sodium hypochlorite. The *Salmonella* Enteritidis strains from different countries had similar responses to sodium hypochlorite. Focusing on *Salmonella* Enteritidis strain SE86 isolated in southern Brazil, we found that the *rpoS* and *dps* genes were important for survival after exposure to sodium hypochlorite, indicating the involvement of these genes in responses to oxidative stress. These results suggest that *rpoS* and *dps* may be important factors in the frequent involvement of *Salmonella* Enteritidis SE86 in salmonellosis outbreaks in southern Brazil.

The *Salmonella* Enteritidis SE86 mutant without *dps* survived less well than did the WT SE86 and the SE86 mutant without *rpoS*, suggesting that *dps* is more involved in the oxidative stress response than is *rpoS*. Other researchers have found that *rpoS* in *Salmonella* Enteritidis is involved in acid resistance *(4)*. More studies are necessary to understand the role of these genes in the behavior of *Salmonella* Enteritidis SE86.

Line 1   Line 2

RpoS

Cat

Dps
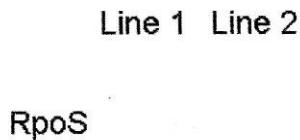
FIGURE 5. *Immunodetection of 3XFLAG-tagged proteins. Line 1, Salmonella Enteritidis SE86 (rpoS::3XFLAG cat::3XFLAG) exposed to peptone water for 10 min; line 2, Salmonella Enteritidis SE86 (dps::3XFLAG cat::3XFLAG) exposed to peptone water for 10 min.*

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3.3 ARTIGO 2

3.3.1 Expression of *ompR* gene in the acid adaptation and thermal resistance of *S*. Enteritidis SE86

Expression of *ompR* gene in the acid adaptation and thermal resistance of *Salmonella* Enteritidis SE86

Ana Carolina Ritter, Donatella Bacciu, Lucélia Santi, Salvatore Rubino, Sergio Uzzau, Eduardo Cesar Tondo

ABSTRACT

The objective of this study was to evaluate the involvement of *ompR* gene in the acid adaptation and thermal resistance of *S*. Enteritidis SE86. In this study, we tested one mutant strains of *S*. Enteritidis SE86 (Δ*ompR*) that was attenuated by knockout technique and the OmpR expression was determined by SE86 *ompR* tagged (3XFLAG). The mutant strains were cultivated separately in Nutrient Broth and Nutrient Broth supplemented with 1 % glucose (NBG) and were exposed to different temperatures (37, 52, and 60ºC). The latter medium was used to induce acid-adapted cells. The survival of the SE86 WT and attenuated strain were determined by bacterial counts, and the tagged protein (*ompR*::3XFLAG cat::FLAG) was detected by SDS-PAGE and immunoblotting with anti-FLAG antibodies. The results demonstrated that when exposed at 52ºC the acid-adapted SE86 WT cells were completely inactivated after 300 minutes, however, non-adapted cells (WT and Δ*ompR*) and acid-adapted Δ*ompR* demonstrated. At 60ºC, the acid-adapted SE86 Δ*ompR* also demonstrated higher sensitivity that SE86 WT, being totally inactivated at 15 min, while the WT cells were inactivated in 20 minutes. The behavior of non-adapted cells was similar, because they were completely inactivated in 15 min. The acid adapted cells showed increased expression of OmpR when exposed at 52 and 60ºC, confirmed the necessity of the acid adaptation for that *S*. Enteritidis SE86 resists to high temperatures.

Introduction

Salmonella (*S.*) is one of the most common causes of human food poisoning worldwide. The genus encompasses more than 2,600 serotypes (Guibourdenche et al., 2010); of which *S.* Enteritidis and *S.* Typhimurium are the two mostly involved serovars with salmonellosis (Carrasco et al., 2011).

Before and during host infection, *Salmonella* finds numerous environmental variations, which can be interpreted as stressful situations for those microorganisms. Many of these environmental variations are potentially lethal to *Salmonella*, but the survival of the organism depends on their mechanisms of adaptation or resistance (Spector and Kenyon, 2011). Adaptation to stress of *Salmonella* cells can be induced in the exponential and stationary phases. During the both phases an increased resistance to lethal acid and thermal stress can occur (Audia et al., 2001). In order to promote acid adaptation, *Salmonella* serotypes expresses two low-pH-inducible systems named "Acid Tolerance Response" - ATR. They are classified based on the growth phase at which each becomes induced. Most studies have focused on the log-phase ATR system induced when exponentially growing cells suddenly undergo a rapid transition to low pH (Bang, 2000). More than 50 acid shock proteins (ASP) are produced during this response (Alvarez-Ordónez,et al., 2011). The regulatory gene *rpoS,* encoding an alternative sigma factor, is required for log-phase acid tolerance and control the production of clusters of ASPs (Alvarez-Ordónez,et al., 2011; Spector and Kenyon, 2011). The second ATR system, related to the stationary-phase ATR, is induced by exposing stationary-phase cells to low pH (Bang et al., 2000, Bang et al., 2002). It is distinct from the general stress response system that is induced by entry into stationary phase regardless of the culture pH. The general stress response system requires stationary-phase induction of the alternative sigma factor, while the acid-induced stationary-phase ATR does not. Thus, OmpR is a stationary-phase ASP. However, RpoS, responsible for the stationary-phase induction of many proteins, was not required for this induction (Bang et al., 2000). The importance of OmpR for optimal functionality of the stationary-phase ATR in

Salmonella is well known, but further studies are necessary to clarify which OmpR-regulated genes are involved in this adaptive response (Alvarez-Ordonez et al., 2011).

Our previous studies have demonstrated the acid adaptation capacity of *S.* Enteritidis involved with salmonellosis occurred in southern Brazil (Malheiros et al. 2007, 2008; Perez et al., 2010,2012). Even though different *Salmonella* serovars has been isolated from foods in this Country, it was observed that a specific *S.* Enteritidis strain (named SE86) was the microorganism most involved with salmonellosis in the last decade in Rio Grande do Sul (RS) State, southernmost State of Brazil (Geimba *et al.* 2004; Oliveira *et al.* 2010). In "*in vitro*" studies, SE86 has demonstrated an increased acid and thermal resistance after the acid exposure (Malheiros *et al.* 2008; Perez et al., 2010). In an "*in vivo*" study, the intestinal invasion ability of SE86 and *S.* Typhimurium (ST99) submitted or not to acid adaptation was compared. Results demonstrated that acid-adapted SE86 showed higher cell counts in the ileum-cecal junction than the ST99, suggesting that acid adaptation influenced in the virulence of this microorganism (Perez et al., 2012). Based on these data, the aim of this study was to evaluate the involvement of *ompR* gene in the acid adaptation and thermal resistance of *S.* Enteritidis SE86.

Material and Methods

Bacterial strains. The *S.* Enteritidis SE86 strain was isolated from a cabbage responsible for an outbreak of salmonellosis which occurred in RS State, Brazil, in 1999. This bacterium was characterized by phenotypic and genotypic methods by Geimba et al. (2004) and Oliveira et al. (2007), and showed the same genotypic profile of *S.* Enteritidis strains involved in more than 95% of the investigated salmonellosis of RS State during 1999 to 2006 (Oliveira et al., 2010). The SE86 strain was provided by the Laboratory of Food Microbiology of Food Science and Technology Institute - ICTA/UFRGS, Brazil. Before experiments, all strains were stored at −70º C in LB medium with the addition of 40% glycerol. The mutant of *S.* Enteritidis SE86 (Δ*ompR*) was constructed in

the Laboratorio di Microbiologia of Università di Sassari, Italy, using Knockout method, described by Datsenko and Wanner (2000). The construct was verified by PCR analysis. The SSM 5337 (ompR::Kan) mutation was transferred into a clean *S*. Enteritidis SE86 background by P22 transduction.

Expression of the *ompR* gene. *S*. Enteritidis SE86 was tagged with the 8 aa FLAG epitope tag peptide. Strain SSM5358 (*ompR*::3XFLAG cat::FLAG) of serovar *S.* Enteritidis was obtained using the method described by Uzzau et al. (2001). The 3XFLAG epitope is a sequence of three tandem FLAG epitopes (22 aa). For tagged mutant, a pair of primers was designed to amplify a 3XFLAG- and kanR-coding sequence by using plasmid pSUB11 (Uzzau et al., 2001). The 3' ends of these oligonucleotides were complementary to the first 20 nt of the pSUB11 3XFLAG coding region (GACTACAAAGACCATGACGG, forward primers) and to the 20 nt of the pSUB11 priming site 2 (CATATGAATATCCTCCTTAG, reverse primers). The 5' ends of the oligonucleotides were designed to be homologous to the last 40 nt of each tagged gene, not including the stop codon (forward primers), and to the 40 nt immediately downstream of the gene stop codon (reverse primers). The Cat protein was used as an internal marker because it is very stable. A constitutively expressed epitope-tagged gene such as cat was used as a positive control or internal reference (Uzzau et al., 2001).

Acid adaptation: For acid adaptation, strains were cultivated in Nutrient Broth supplemented with 10 g/L glucose (NBG; Merck, Darmstadt, Germany). The cultures were maintained for 18 h at 37^0 C in static condition according to Tetteh and Beuchat (2003). After 18 hours of incubation, the culture pH was measured using a pH meter model PHS-3B (PHTEK). Each strain was also incubated in Nutrient broth (NB) (Synth, São Paulo, Brazil) without glucose, at the same conditions, in order to produce non-adapted *Salmonella* cells (Malheiros et al., 2008).

Determination of thermal resistance: Aliquots of 1.0 ml (approximately 8.0 log CFU ml^{-1}) of the acid-adapted and non adapted cultures were transferred to Erlenmeyer flasks containing 99 ml of pre-warmed NB kept in a water bath (Schott CT 52, Mainz, Germany). The flasks were incubated at temperatures of 37, 56 ºC for 300 minutes and 60° C for 20 minutes. Aliquots of 1 ml were taken and serially diluted in 9 ml of 1 g l-1 peptone water (Vetec, Rio de Janeiro, Brazil). After vigorous vortexing, 20 µl of appropriate dilution were plated, 120 by drop culture method, on BHI agar (Merck, Darmstadt, Germany) and incubated for 24 h at 37º C. The quantification of survivors was carried out according to the method described by Silva (2007). The lowest detection limit of acid survivors was 1.69 log CFU ml^{-1}, and each experiment was carried out at least two times, with duplicate counts (Malheiros et al., 2008).

Western blotting of tagged gene. After the exposure to specific temperature described above, 1 ml aliquots were taken and centrifuged (18000 g, 10 min, 4º C). Protein extracts were then boiled for 5–10 min, and an aliquot of each sample was resolved by 12% SDS-PAGE for detection of 3XFLAG-tagged proteins by western blotting. The nitrocellulose membranes were blocked with 5% (w/v) non-fat dried milk in PBS (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), containing 0.05% Tween 20, washed and incubated with mouse anti-FLAG M2-peroxidase (HRP) mAbs (Sigma) diluted 1:1000. The detection was carried out using H_2O_2 and $CoCl_2$.

Statistical analysis. All survival experiments were repeated at least twice, and all counting results were done in duplicates. The Tukey Test was carried out aiming to compare the differences between the mean values. The differences were considered significant with P values < 0.05.

Results

pH reduction during acid-adaptation

The glucose fermentation by *S.* Enteritidis SE86 growing in NBG resulted in pH decreasing, and this was used to achieve acid adaptation. *Salmonella* grown in NBG was considered acid-adapted, while *Salmonella* cultivated in NB was considered non-adapted. Before inoculum, the initial pH values of NBG were 6.60±0.02 and after *S.* Enteritidis SE86 growth, the final pH values presented mean values of 4.49±0.02. The pH of NB without glucose before and after inoculum was approximately 6.80.

Susceptibility to different temperatures of non-adapted and acid-adapted Δ*ompR S.* Enteritidis SE86

After four hours of exposure at 37° C, acid-adapted SE86, with or without *ompR* gene, demonstrated higher counts when compared with non-adapted SE86. The difference observed between counts was almost 2 logs cfu (figure 1) but statistically not show significant difference (P<0.05).

Figure 2 demonstrates the effect of acid adaptation on the survival of SE86 wild type (WT) and SE86 mutant *ompR* exposed to 52º C for 300 minutes. Acid-adapted SE86 WT cells were completely inactivated after 300 minutes, however, non-adapted cells (WT and Δ*ompR*) and acid-adapted Δ*ompR* demonstrated higher sensitivity (and significant difference (P<0.05)), because were completely inactivated in 240 minutes.

At 60º C, the acid-adapted SE86 Δ*ompR* also demonstrated higher sensitivity that SE86 WT (Figure 3), being totally inactivated at 15 min, while the WT cells were inactivated in 20 minutes. The behavior of non-adapted cells was similar, because they were completely inactivated in 15 min.

Immunodetection of epitope-tagged OmpR protein of non-adapted and acid-adapted SE86 at different temperatures

In order to verify the expression of OmpR protein as a consequence of acid adaptation and thermal resistance, the *ompR* gene was tagged and subjected

to the same experiments described above. The expression of the OmpR protein, in response to the exposure to 37º C, 52º C and 60º C was confirmed by western blot (Figure 4, 5 and 6).

After exposure of 60, 120 and 180 minutes at 37º C, acid-adapted and non-adapted cells expressed similar amounts of OmpR protein (Figure 3). However, higher expression of OmpR protein were observed in acid-adapted SE86 *ompR* tagging than non-adapted cells during the exposure at 52° C and 60º C (Figures 5 and 6).

Discussion

Several virulence factors are involved in the adaptive response to the environmental factors because pathogens are exposed to a variety of stresses. It has been demonstrated that alterations in temperature, osmolarity, nutrient availability, pH and oxygen tension contribute to the differential regulation of the virulence genes of *Salmonella* (Sirsat et al., 2011; Alvarez-Ordónez and Pietro, 2010). In this study we confirmed the involvement of the OmpR protein in the acid adaption of *Salmonella*, a fact already reported by several studies. As an example, Bang et al. (2000) have observed that acid adaptation induced the production of OmpR, which in its phosphorylated state, could trigger the expression of diverse genes necessary for the acid-induced stationary-phase ATR of *S.* Typhimurium. The *ompR* gene is known to associate the acid adaptation with the increased acid shock resistance of *Salmonella* (specially the sorovar Typhimurium) since several groups of acid shock proteins (ASPs) are induced during ATR in order to prevent or repair the macromolecular damage caused by acid stress (Audia et al., 2001). An extensive research effort has been made in the last decades to identify and characterize these stress proteins in *S.* Typhimurium and several regulatory genes controlling the expression of different subsets of ASPs have been described, including the alternative sigma factor RpoS, the iron regulator Fur, the two-component signal transduction system PhoP/PhoQ and the OmpR response regulator (Foster, 2000). Most of the identified ASPs are involved in cellular regulation, molecular chaperoning,

energy metabolism, transcription, translation, synthesis of fimbriae, regulation of the cellular envelopes, colonization and virulence (Bearson et al., 2006).

In the present study, we exposed acid-adapted and non-adapted mutants of SE86 (lacking *ompR*) to 52° C and 60° C and observed the expression of the OmpR, demonstrating the involment of this protein with the thermal resistance. Interestingly, at 52º C and also at 60 ℃, the acid-adapted mutants of SE86 demonstrated similar behavior of non-adapted SE86 cells, which were completely inativated in 240 and 15 min, respectively (Figure 1 and 2). The acid-adapted SE86 WT demonstrated the same behavior of SE86 studied by Malheiros et al. (2008), being inactivated after 300 minutes of exposure at 52ºC. Several studies have demonstrated that acid adaptation increases the thermal resistance of *Salmonella* (Wilde et al., 2000; Gönül and Tosun, 2002; Malheiros et al., 2008), however, most authors have attributed this behavior to the heat-shock proteins (HSPs), responsible for the thermal shock resistance of *Salmonella*. Classical HSPs are molecular chaperones (DnaK, DnaJ, GroEL and GroES) or adenosine 5′-triphosphate-dependent proteases (ClpC, ClpP, ClpX and FtsH), and these proteins are usually controlled by the classic heat-shock sigma factor (σH) and an extracytoplasmic response regulated by the extracytoplasmic function (ECF) sigma factor (σE) (Ades, 2008; Alba and Gross, 2004; Guisbert et al., 2008; Spector and Kenyon, 2011). Interestingly, several σH- and σE regulated genes encoding cytoplasmic and periplasmic chaperones and proteases appear to contribute to *Salmonella* virulence (Spector and Kenyon, 2011). The molecular mechanisms of σH and σE activation differ, but both mechanisms allow for a rapid response to the thermal stress (Spector and Kenyon, 2011).

The expression of the OmpR protein was observed in SE86 *ompR* tagged exposed to the same conditions of acid adaptation and thermal resistance that the mutant SE86 lacking *ompR.* Furthermore, we could observe an increasing expression of OmpR in acid-adapted cells when compared to non-adapted cells (Figure 4 and 5). At 37° C there was no difference between treatments, which leads us to believe that temperatures like 52º C and 60º C are responsible for the increasing of OmpR expression. Two genes regulated by OmpR, as *ompF*

and *ompC*, have already demonstrated to be related to high temperature exposure, as demonstrated by Begic and Worobec (2006). These authors evaluated the expression of *S. marcescens ompF* and *ompC* genes in the presence of different environmental factors, including osmotic pressure, temperature, pH and salicylate and concluded that high temperature followed by pH were responsible for the predominant expressions of these genes.

In conclusion, the results of the present study show that the *ompR* is involved with the thermal resistance, after acid-adaptation of SE86. However, further Two-Dimensional (2D) Gel Electrophoresis and proteomic studies are necessary to confirm the involvement of other genes regulated by the OmpR protein with this behavior.

Acknowledgements

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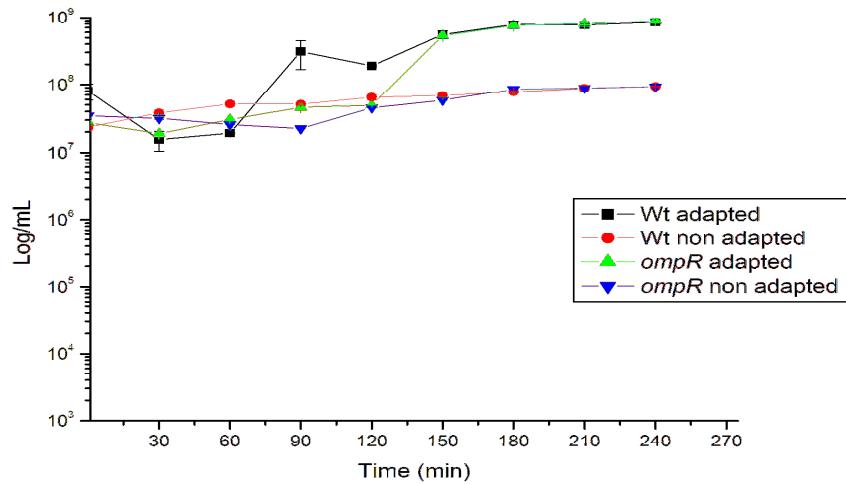
FIGURE 1. Survival of nonadapted *Salmonella* Enteritidis SE86 Wild Type (▲) and acid-adapted *Salmonella* Enteritidis SE86 Wild Type (♦), nonadapted *Salmonella* Enteritidis SE86 lacking *ompR* (●) and acid-adapted *Salmonella* Enteritidis SE86 lacking *ompR* (■) exposed to 52º C in Nutrient Broth.
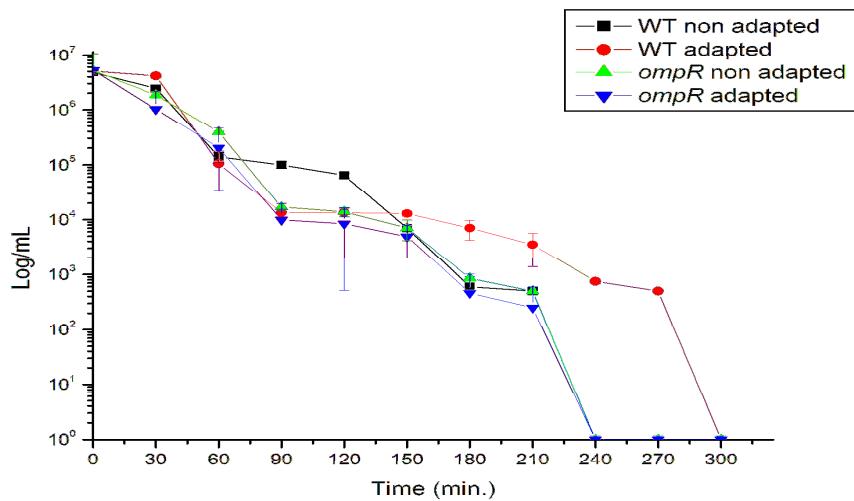
FIGURE 2. Survival of nonadapted *Salmonella* Enteritidis SE86 Wild Type (▲) and acid-adapted *Salmonella* Enteritidis SE86 Wild Type (♦), nonadapted *Salmonella* Enteritidis SE86 lacking *ompR* (●) and acid-adapted *Salmonella* Enteritidis SE86 lacking *ompR* (■) exposed to 52º C in Nutrient Broth.
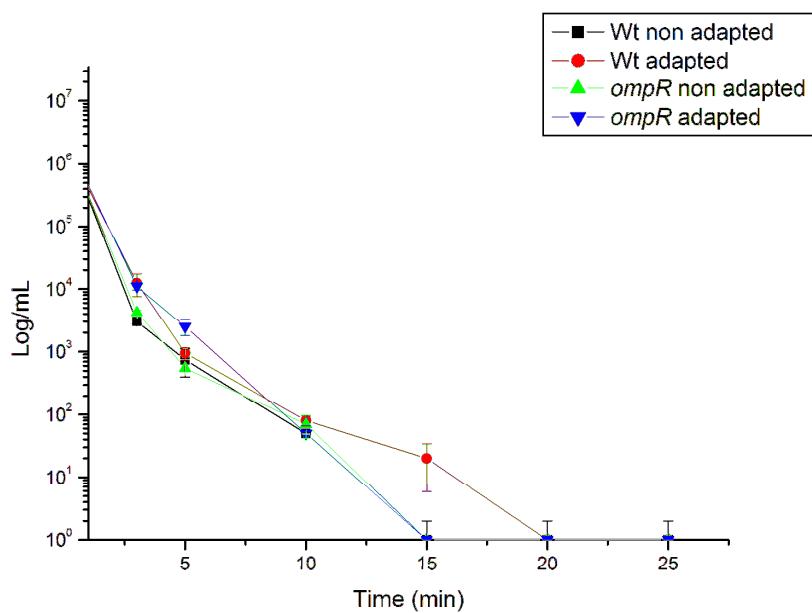
FIGURE 3. Survival of nonadapted *Salmonella* Enteritidis SE86 Wild Type (▲) and acid-adapted *Salmonella* Enteritidis SE86 Wild Type (♦), nonadapted *Salmonella* Enteritidis SE86 lacking *ompR* (●) and acid-adapted *Salmonella* Enteritidis SE86 lacking *ompR* (■) exposed to 60ºC in Nutrient Broth.
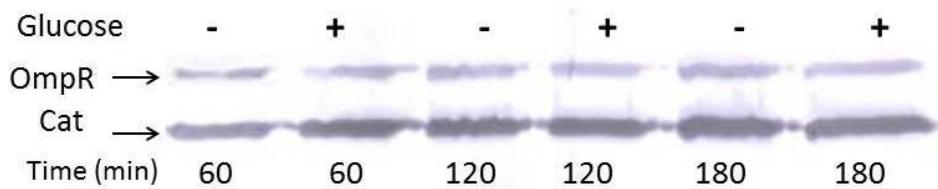

FIGURE 4: Western blot of *S.* Enteritidis SE86 3xFLAG-tagged OmpR protein and catalase Cat protein as control (OmpR::3xFLAG cat::3xFLAG) exposed to 37º C.
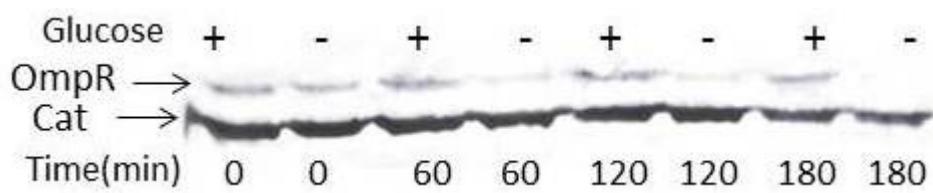
FIGURE 5: Western blot of *S*. Enteritidis SE86 3xFLAG-tagged OmpR protein and catalase Cat protein as control (OmpR::3xFLAG cat::3xFLAG) exposed to 52º C.
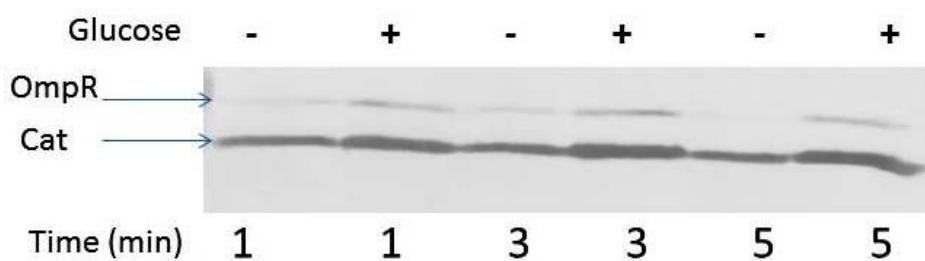
**FIGURE 6**: Western blot of *S*. Enteritidis SE86 3xFLAG-tagged OmpR protein and catalase Cat protein as control (OmpR::3xFLAG cat::3xFLAG) exposed to 60º C.

4 DISCUSSÃO GERAL

Salmonella sp. foi reconhecida como a primeira causa de DTA no Brasil na última década, sendo que, na região Sul, esse microrganismo tem sido identificado como a primeira causa de DTA há quase duas décadas. Uma cepa específica de S. enteritidis (SE86) foi identificada como o microrganismo responsável por mais de 95% dos casos de salmonelose no RS (Geimba et al, 2004; Oliveira et al., 2009). Quando comparado com outros sorovares de Salmonella, S. Enteritidis SE86 demonstrou maior capacidade de adaptação ácida e térmica, além de maior resistência contra diferentes sanificantes, principalmente o hipoclorito de sódio (Malheiros et al., 2008; Tondo et al., 2010 Machado et al., 2011). Para melhor entendimento dos mecanismos que levaram a SE86 a desenvolver tais resistências, o presente trabalho analisou três genes, conhecidos por sua ligação com diferentes estresses sofridos por Salmonella sp.

Apesar de o gene dps ser controlado pelo fator sigma (rpoS), neste estudo pode-se observar que a resistência de S. Enteritidis SE86 ao hipoclorito de sódio está principalmente ligada a presença do gene dps, visto que as células de SE86 dps mutante (Δdps) demonstraram taxas de sobrevivência

menores do que o SE86 não mutada (WT). Alguns estudos já associaram o gene *dps* ao estresse oxidativo (Nair e Finkel 2004; Halsey et al., 2004; Pacello et al., 2008) durante a fase estacionária de *Salmonella*, porém, sua relação com a resistência a sanificantes ainda não havia sido reportada.

No presente estudo, a diferença entre cepas de *S*. Enteritidis isoladas de diferentes países, frente à resistência ao hipoclorito de sódio, também foi observada. Os resultados demonstraram que nenhuma das cepas foi totalmente inativada após 20 minutos de exposição. A resistência bacteriana ao hipoclorito de sódio vem sendo relatada por diversos estudos (Dukan et al., 1996; Tondo et al., 2010; Machado et al., 2011). Oliveira et al.(2012), ao avaliarem diferentes protocolos de higienização de alfaces em restaurantes de Porto Alegre, observaram que o hipoclorito de sódio é um dos sanificantes mais utilizados para desinfecçao de folhosos. Além disso, os mesmos autores observaram que a concentração recomendada pela legislação brasileira (Portaria Nº78/2009) é eficaz para inativar mesófilos aeróbios e coliformes totais, porém o tempo de exposição deve ser de 30 minutos, o dobro recomendado pela legislação vigente. Além disso, três dos quatro isolados analisados demonstraram grande similaridade quanto à sensibilidade ao sanificante analisando, exceto uma cepa, proveniente da Albânia. Estes dados reforçam a necessidade dos estudos da expressão de genes de resistência a sanificantes, pois podem auxiliar na prevenção e controle de *S*. Enteritidis em escala mundial.

O gene *ompR* é conhecido por estar associado à resistência ácida em *Salmonella* sp. (Bang et al., 2000; Álvarez-Ordónez et al., 2011). O

aumento da expressão da proteína OmpR, após contato com o ácido, é responsável pela expressão de muitas outras proteínas de choque ácido, as quais protegem a célula do microrganismo na fase estacionária (Bang et al., 2000). Ao submeter o mutante de *S*. Enteritidis SE86 (ΔompR) à adaptação ácida e posterior choque térmico, foi possível observar a relação destes dois fatores na sobrevivência deste microrganismo, visto que as células de SE86 ΔompR ácido adaptadas demonstraram maior sensibilidade em relação às células de SE86 não mutadas, quando colocadas em contato com as temperaturas de 52º C e 60º C. A diferença na expressão da proteína OmpR nas células de SE86 ácido adaptada e não ácido adaptadas confirmaram a relação deste gene com a adaptação ácida e com a exposição da SE86 a temperaturas de 52 e 60º C. De acordo com Samelis et al. (2003), o uso de ácidos orgânicos nas indústrias de alimentos, tais como o ácido acético, tem sido muito difundido devido a sua capacidade bactericida. Esse tipo de ácido pode ser naturalmente formado ou adicionado como conservador em alimentos. No estado do RS, a salada de batata com maionese caseira é tradicionalmente preparada com diferentes doses de vinagre (ácido acético) o que pode resultar em diminuição do pH e consequente adaptação ácida das bactérias presentes nesse produto (Costalunga e Tondo, 2002; Malheiros et al., 2007a).

Os resultados do presente trabalho confirmam os diversos estudos que relataram no aumento da resistência térmica de diversos patógenos alimentares após adaptação ácida (Humphrey et al., 1993; Audia et al., 2001, Malheiros et al., 2008). A temperatura ainda é uma das medidas primárias

pelas quais microrganismos patogênicos são eliminados de alimentos. A Portaria Nº78/2009 da ANVISA recomenda que a cocção deve atingir 70º C em todas as partes dos alimentos, porém é sabido que, frequentemente, isso não ocorre, podendo expor os microrganismos a temperaturas sub-letais. Tal exposição, combinada com contato prévio dos microrganismos a pequenas quantidades de ácidos, podem induzir a ativação de genes responsáveis pela resistência térmica destes microrganismos.

Através dos dados obtidos nesta pesquisa, é possível concluir que o estudo da expressão de genes de resistência em *S.* Enteritidis deve ser mais explorado, visto que muitos genes ainda possuem ação desconhecida no comportamento deste patógeno frente a estresses ambientais. Através do estudo do comportamento dos genes *dps*, *rpoS* e *ompR*, foi possível avançar no entendimento das razões que contriubuem para que a cepa SE86 seja a maior causadora de surtos no Rio Grande do Sul, contribuindo assim para prevenção de novos casos de salmonelose.

5 CONCLUSÕES

- As proteínas Dps e RpoS foram expressas após contato com hipoclorito de sódio.

- A mutação do gene *dps* esteve associada à queda de resistência da cepa SE86 ao hipoclorito de sódio 200ppm.

- A mutação do gene *rpoS* não esteve associada à resitência da cepa SE86 ao hipoclorito de sódio 200ppm.

- A proteína OmpR foi mais expressa na cepa SE86 após a adaptação ácida.

- A cepa com mutação no gene *ompR* ácido adaptada foi mais sensível nas temperaturas de 52º C e 60º C do que a cepa SE86 WT.

- O gene *ompR* está envolvido com a resistência térmica de *S.* Enteritidis SE86, após a adaptação ácida.

6 PERSPECTIVAS

1. Avaliar o comportamento do gene *dps* frente a diferentes sanificantes utilizados em indústrias de alimentos através das cepas SE86 mutadas (∆dps e *dps*::3XFLAG cat::FLAG);

2. Realizar estudos proteômicos através de SDS-PAGE -2D com a cepa SE86 *ompR* mutante (∆*ompR*) a fim de verificar quais proteínas controladas por *ompR* estão relacionadas com a resistência térmica.

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