UNIVERSIDAD DE LEÓN

FACULTAD DE VETERINARIA

DEPARTAMENTO DE HIGIENE Y TECNOLOGÍA DE LOS ALIMENTOS

"Estudio de los factores que determinan la respuesta de adaptación ácida y de protección cruzada frente al calor de *Salmonella* Typhimurium y *Salmonella* Senftenberg: mecanismos implicados"

"Factors influencing the acid tolerance and cross-protection responses of *Salmonella* Typhimurium and *Salmonella* Senftenberg: mechanisms involved"

Memoria presentada por

Avelino Álvarez Ordóñez

para optar al grado de Doctor por la Universidad de León

Universidad de León

INFORME DEL DIRECTOR DE LA TESIS
(Art. 11.3 del R.D. 56/2005)

Las Dras. D. Ana Bernardo Álvarez y Mercedes López Fernández como Directoras[1] de la Tesis Doctoral titulada "Estudio de los factores que determinan la respuesta de adaptación ácida y de protección cruzada frente al calor de *Salmonella* Typhimurium y *Salmonella* Senftenberg: mecanismos implicados" realizada por D. Avelino Álvarez Ordóñez en el Departamento de Higiene y Tecnología de los Alimentos, informan favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa.

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El Departamento de Higiene y Tecnología de los Alimentos en su reunión celebrada el día 18 de marzo de 2009 ha acordado dar su conformidad a la admisión a trámite de lectura de la Tesis Doctoral titulada "Estudio de los factores que determinan la respuesta de adaptación ácida y de protección cruzada frente al calor de *Salmonella* Typhimurium y *Salmonella* Senftenberg: mecanismos implicados", dirigida por la Dra. Dña. Ana Bernardo Álvarez y la Dra. Dña. Mercedes López Fernández, elaborada por D. Avelino Álvarez Ordóñez y cuyo título en inglés es el siguiente "Factors influencing the acid tolerance and cross-protection responses of *Salmonella* Typhimurium and *Salmonella* Senftenberg: mechanisms involved".

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Vº Bº

El Director del Departamento, El Secretario,

Fdo.: Carlos Alonso Calleja Fdo.: M. R. García Armesto

AGRADECIMIENTOS

Deseo expresar mi sincero agradecimiento a todas las personas que me han ayudado, acompañado y querido a lo largo de estos maravillosos años, lo que les ha convertido en acreedores a mi eterna gratitud.

A la Dra.Bernardo y a la Dra. López, por depositar en mí su confianza y por el esfuerzo y horas de trabajo que han invertido en enseñarme a amar "la ciencia".

A Ana, por guiarme en mis primeros pasos en un laboratorio y por poner a mi disposición toda su experiencia y conocimientos. Por lo que me ha aportado profesionalmente, pero sobre todo por lo mucho que me ha aportado personalmente, por haber sobrevivido a las miles de horas de despacho y laboratorio compartidas y haber sabido convertirse en mi mejor amiga.

Al Dr. Arenas, por su valiosa colaboración en los experimentos de determinación de la composición en ácidos grasos de las membranas bacterianas.

A Domingo, por realizar el diseño de la portada y por todos sus consejos artístico-técnicos, siempre útiles.

A Leticia, por soportar mi desorden en el laboratorio y mostrarme siempre la mejor disposición para ayudar en lo que hiciera falta.

Al resto de profesores y compañeros del Área de Tecnología de los Alimentos de la Universidad de León, porque tan importante como "trabajar bien" es "trabajar a gusto" y no podría haber encontrado un ambiente de trabajo mejor en ningún otro sitio. En especial, gracias a Noelia, por lo mucho que me ha ayudado y acompañado en los últimos meses, a Domingo y Ricardo, porque a pesar de su mal gusto futbolístico han conseguido que todos los días encontrase una razón para reírme a carcajadas, a Bernardo, mi inseparable compañero de "menú del día", por sus consejos y enseñanzas, a Tania, por escucharme en mis habituales visitas al primer piso, a Bea, con quien he compartido momentos memorables en congresos y cursos de doctorado, y a Almudena, Marta y Silvia, por su eficaz colaboración en alguno de los experimentos.

A los integrantes del Departamento de Producción Animal y Ciencia de los Alimentos de la Universidad de Zaragoza, por su hospitalidad, y en especial, al Dr.Condón y la Dra Mañas, por ayudarme a completar mi formación científica, a

Guillermo, por su colaboración en el laboratorio y a Diego, por su apoyo logístico en mis viajes a Holanda.

To all the members of the Laboratory of Food Microbiology of the University of Wageningen (The Netherlands), for make me feel as if I was at home, and especially to Prof. Dr. Tjakko Abee for his hospitality and brilliant supervision, and to Dr. Menno Van der Voort, for his help and advices.

A mi familia, especialmente a mis padres Avelino y Mª del Pilar y a mi hermano Lorenzo, por estar siempre a mi lado y confiar ciegamente en mí.

Este trabajo ha sido posible gracias a una beca del Programa de Formación de Profesorado Universitario del Ministerio de Educación y Ciencia.

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1.1.1.- Características generales y taxonomía

El género *Salmonella*, perteneciente a la familia *Enterobacteriaceae*, está constituido por bacilos Gram negativos, anaerobios facultativos, oxidasa-negativos y catalasa-positivos, generalmente móviles debido a la presencia de flagelos peritricos y capaces de catabolizar diversos carbohidratos y de descarboxilar la lisina (Holt y col., 1994; D´Aoust, 2000).

A través de los años se han propuesto varios esquemas de clasificación que han creado una cierta controversia y confusión en su compleja nomenclatura y taxonomía. Según la última edición del Manual de Bergey (Garrity y col., 2004) todas las salmonelas se agrupan en dos especies, *S. bongori*, grupo que contiene menos de 10 serovariedades, todas ellas muy poco frecuentes, y *S. enterica*, con más de 2.500 serovariedades, y dividido en seis subespecies (*enterica*, *arizonae*, *diarizonae*, *houtenae*, *indica* y *salamae*). Cada subespecie a su vez está subdividida en serovariedades, que se establecen según la presencia de diferentes antígenos somáticos (antígeno O), capsulares (V_i) y flagelares (H), detectados por reacciones de aglutinación frente a antisueros homólogos (D´Aoust, 1989a). La mayoría de las serovariedades aisladas del hombre y de los animales de sangre caliente pertenecen a *Salmonella enterica* subespecie *enterica*. Entre ellas cabe destacar las serovariedades *S.* Typhimurium y *S.* Enteritidis, por ser las más comúnmente asociadas con brotes de toxiinfección alimentaria (D´Aoust, 1989a; World Health Organization, 1995). Dado que las serovariedades no tienen nivel taxonómico de especie, sus nombres escapan al dominio del "Código Internacional de Nomenclatura Bacteriana" y, por ello, deben escribirse de la siguiente manera: *Salmonella enterica* subespecie *enterica* serovar … o de forma reducida *Salmonella* Typhimurium, *Salmonella* Enteritidis, o *S.* Typhimurium, *S.* Enteritidis (Brenner y col., 2000). Las serovariedades pertenecientes a las subespecies restantes y a *S.*

bongori, de baja incidencia en patología humana y animal, se designan con el nombre de la subespecie seguido por la fórmula antigénica, por ejemplo: *Salmonella enterica* subespecie *houtenae* 50:b:- (Brenner y col., 2000).

1.1.2.- Factores que condicionan el crecimiento y supervivencia en los alimentos

La capacidad de las salmonelas de crecer o sobrevivir bajo condiciones ambientales extremas representa un motivo de gran preocupación para la Salud Pública, ya que posibilita su presencia en diversos alimentos de origen animal. Por su importancia como agente etiológico de toxiinfecciones alimentarias las condiciones que permiten o limitan su desarrollo en los alimentos han sido ampliamente estudiadas, habiéndose desarrollado en los últimos años algunos modelos matemáticos que permiten predecir o estimar sus posibilidades de crecimiento o supervivencia bajo diversas condiciones ambientales (Blackburn y col., 1997; Mañas y col., 2003; Koutsoumanis y col., 2004).

Los microorganismos pertenecientes al género *Salmonella* se caracterizan por ser mesófilos (temperatura óptima de crecimiento 35-37ºC), aunque se ha descrito que son capaces de crecer en un amplio rango de temperaturas, desde 2 hasta 47ºC (D´Aoust, 1989a, 1991, 2000), e incluso se ha observado crecimiento a 54ºC para una cepa mutante de *S.* Typhimurium (Droffner y Yamamoto, 1992). Es importante tener en cuenta que las temperaturas límites de crecimiento dependen de las condiciones ambientales prevalentes durante el ensayo. Por ejemplo, se ha descrito previamente que la temperatura mínima de crecimiento puede ser diferente en alimentos que en medios de laboratorio (D´Aoust, 1989a, 1991), y que se ve incrementada a medida que el pH del medio de crecimiento se desvía de la neutralidad (Matches and Liston, 1972), lo que puede revestir gran importancia, ya que la refrigeración de los alimentos se considera uno de los métodos más adecuados para prevenir la salmonelosis.

En cuanto a su pH óptimo de crecimiento, se encuentra en torno a 6,5-7,5, aunque se ha observado crecimiento bacteriano en el rango de pH 3,6-9,5 (Chung y Goepfert, 1970; Holley y Proulx, 1986; Ruzickova, 1996), estando el pH mínimo de crecimiento también determinado por diversos factores ambientales, como la composición del medio de crecimiento (Booth y Kroll, 1989), la temperatura (Koutsoumanis y col., 2004) y el tipo de ácido utilizado (Perales y García, 1990; Jung y Beuchat, 2000). Cabe destacar que numerosos estudios han demostrado que las salmonelas pueden sobrevivir durante largos periodos de tiempo en alimentos ácidos. Por ejemplo, se han detectado células viables de diversas especies del género *Salmonella* tras 11-84 días en zumos de manzana, naranja, uva y piña (Parish y col., 1997, 2004; Oyarzábal y col., 2003), 19-68 días en yogur (El-Gazzar y Marth, 1992), 1 día en salsas para ensaladas (Beuchat y col., 2006) y 4 semanas en mayonesa (Leuschner y Boughtflower, 2001).

Otro de los principales factores que determinan el crecimiento microbiano en los alimentos es la actividad del agua (a_w). En general, las bacterias pertenecientes al género *Salmonella* son capaces de crecer a valores de $a_w \geq 0,93$ (D´Aoust, 2001), aunque se ha descrito que pueden sobrevivir en condiciones de a_w tan bajas como 0,84 (Payne y col., 2007), y que su crecimiento se ve favorecido por la adición de bajas concentraciones de sal (0,5-1%) (Matches y Liston, 1972), siendo capaces de crecer en concentraciones de cloruro sódico en torno al 2% (D´Aoust, 1989a; McKay y Peters, 1995) y de sobrevivir largos periodos de tiempo a concentraciones cercanas al 15% (Leyer y Johnson, 1993; Greenacre y Brocklehurst, 2006).

1.1.3.- Aspectos clínicos y epidemiológicos de la salmonelosis

Las salmonelas están ampliamente distribuidas en la naturaleza, encontrándose como microorganismos comensales y patógenos en el tracto gastrointestinal de humanos, mamíferos domésticos y salvajes, reptiles, aves e insectos, siendo agentes etiológicos de las conocidas salmonelosis, que pueden ocasionar “fiebres entéricas tifoideas y paratifoideas” (causadas por *S.* Typhi y *S.*

Paratyphi A, B y C) o "gastroenteritis no tifoideas", en las que están implicadas diferentes serovariedades. La epidemiología de la salmonelosis ha experimentado a lo largo de la historia importantes cambios (Tauxe, 1991). Mientras que en siglos pasados las fiebres entéricas tifoideas fueron las principales infecciones causadas por *Salmonella* spp. en humanos, la instauración de tratamientos de potabilización de agua de bebida, de sistemas de desagüe y de otras medidas higiénicas han conseguido prácticamente erradicarlas, adquiriendo una mayor importancia en los últimos años las gastroenteritis no tifoideas que cursan con síntomas que aparecen 8-72 horas después de la exposición a la fuente de contagio e incluyen náuseas, dolor abdominal, diarrea acuosa o ligeramente mucosa, fiebre de corta duración y vómitos (Yoshikawa, 1980; Hornick, 1988). Además, las salmonelosis no tifoideas pueden complicarse debido a la migración de los microorganismos a otras localizaciones somáticas a través de los vasos sanguíneos y linfáticos, causando un síndrome septicémico que puede ocasionar anomalías circulatorias, infección intracraneal y disfunción de diversos órganos vitales (Cohen y col., 1987).

Las salmonelas son los microorganismos patógenos más habitualmente identificados como agentes etiológicos de toxiinfecciones alimentarias en países desarrollados, y unos de los más frecuentes, junto con *Escherichia coli* y *Shigella* spp. en países en vías de desarrollo, en los que constituyen la segunda causa de morbilidad, después de los procesos respiratorios (Koohmaraie y col., 2005). Resulta además inquietante el incremento observado en los últimos años en el número de casos de salmonelosis no tifoidea registrados en Europa, especialmente en Dinamarca, Italia y Lituania (D´Aoust, 2000). En España, en las últimas décadas del siglo XX, más del 90% de todas las toxiinfecciones de origen bacteriano fueron causadas por *Salmonella* spp. Se produjeron una media de 468 brotes al año, que representa uno de los valores más elevados registrados en todo el mundo (Todd, 1996). Entre 1998 y 2001 se notificaron a la Red Nacional de Vigilancia Epidemiológica 14.815 casos de toxiinfección alimentaria, con 2.106 hospitalizaciones y 4 defunciones, estando el 85,5% de los casos asociados a *Salmonella* spp. (Carbó Malonda y col., 2005), prevalencia que se mantuvo a lo largo del año 2003 (Cevallos y col., 2005). Aunque se cree que las salmonelosis

humanas no tifoideas pueden ser causadas por unas dos mil serovariedades, en la mayoría de las ocasiones se han visto implicadas tan sólo cinco, que han sido identificadas por el Centro de Control de Enfermedades de Estados Unidos (CDC, 2002) como *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, *S.* Heidelberg y *S.* Javiana. Tradicionalmente, *S.* Typhimurium, una serovariedad asociada principalmente al ganado vacuno, ha sido la más frecuentemente aislada de brotes de salmonelosis humanas transmitidas por el consumo de alimentos (D´Aoust, 1989a; Tauxe, 1991). Sin embargo, a finales de la década de los 80 comenzó a detectarse un importante aumento de la serovariedad *S.* Enteritidis, asociada esencialmente al huevo y ovoproductos (D´Aoust, 1989a; Tauxe, 1991), aunque posteriormente *S.* Typhimurium volvió a recobrar parte de su protagonismo tradicional (Zeidler, 1997; Mazón y col., 1998). En cualquier caso, estas dos serovariedades superan la mitad de todos los episodios registrados (Dorronsoro y col., 1998; Mazón y col., 1998; Lawson y col., 2003; Beal y col., 2006), aunque, en la actualidad, nuevas serovariedades como *S.* Senftenberg están ganando protagonismo en brotes de infección alimentaria y nosocomial (L´Ecuyer y col., 1996; Kumar y Kumar, 2003).

Las salmonelas penetran en el organismo a través de la vía oral, de tal manera que deben sobrepasar la barrera defensiva que representa la acidez gástrica antes de adherirse e invadir las células del epitelio intestinal, en las que penetran y se multiplican, causando alteraciones histopatológicas (Millemann, 1998). La dosis infectiva es muy variable, y aunque probablemente se encuentre en torno a 10^5 microorganismos (Blaser y Newman, 1982), se han descrito cifras mucho más bajas, de tan sólo 100 células para *S.* Eastbourne (D´Aoust y col., 1975), 50 para *S.* Napoli (Greenwood y Hooper, 1983), 100-500 para *S.* Heidlberg (Fontaine y col., 1980) ó 1-6 para *S.* Typhimurium (D´Aoust, 1985), siendo destacable el hecho de que parece guardar una estrecha relación con su resistencia frente a la acidez (Waterman y Small, 1998).

La principal fuente de transmisión de la salmonelosis es el consumo de alimentos contaminados. Tradicionalmente, los alimentos más habitualmente implicados han sido las carnes (principalmente de ave, pero también de ganado porcino y bovino) y los huevos y ovoproductos (D´Aoust, 2000). A pesar de las

frecuentes advertencias realizadas por las agencias de protección del consumidor para poner de manifiesto el peligro que representa el consumo de carnes y pescados crudos o poco cocinados y la adición de huevos frescos a alimentos que no van a ser sometidos a un procesado térmico (bastante común en la industria panadera y repostera), tanto en los establecimientos de restauración colectiva como en los hogares, continúan extendidas estas malas prácticas (Klontz y col., 1995; World Health Organization, 1995; Scott, 1996), lo que pone de manifiesto que la educación del consumidor acerca de los riesgos asociados al consumo de alimentos continúa representando un gran reto (World Health Organization, 1995; Roels y col., 1997).

La leche cruda y los productos lácteos a base de leche cruda también constituyen un importante vehículo de *Salmonella* spp. (D´Aoust, 1989b), y aunque se han registrado brotes por consumo de leche pasteurizada, fueron atribuidos a errores humanos durante el procesado de la leche o a contaminaciones post-tratamiento, ya que un correcto tratamiento térmico de pasteurización debería ser capaz de eliminar las salmonelas de la leche (Sharp, 1987; D´Aoust, 1989b). Aún existen controversias sobre los beneficios que para las propiedades sensoriales y nutritivas de los quesos supone el emplear leche cruda en su elaboración y los peligros que su uso puede acarrear para la seguridad alimentaria. No obstante, el amplio número de brotes de salmonelosis asociados al consumo de quesos elaborados con leche cruda y la capacidad que muestran las salmonelas para sobrevivir durante los 60 días de maduración a los que por ley deben ser sometidos, hacen aconsejable el empleo de leche pasteurizada en la industria quesera (D´Aoust, 1989b).

También se han registrado casos clínicos de salmonelosis no tifoidea asociados al consumo de frutas y vegetales frescos contaminados (Parish, 1997; Boase y col., 1999; D´Aoust, 2000; Powell y Leudtke, 2000), pudiendo encontrarse en el origen de dichos brotes la utilización de abonos animales inadecuados para la fertilización de campos, de aguas contaminadas y efluentes en los sistemas de riego, o de agua no potable durante el lavado post-cosecha (D´Aoust, 2000). Por último, cabe destacar que en numerosas ocasiones se han descrito brotes de salmonelosis asociados al consumo de alimentos ácidos, como productos cárnicos fermentados

(Lake y col., 2002) o zumos de frutas (Parish, 1997; Boase y col., 1999; Powell y Leudtke, 2000).

1.2.- Utilización de ácidos orgánicos en la industria alimentaria

La mayor parte de las carnes, pescados, productos vegetales y lácteos tienen valores de pH situados en el intervalo 5,0-6,5 (Tabla 1), lo que les convierte en alimentos susceptibles a la acción de la mayoría de microorganismos alterantes y/o patógenos, por lo que la acidificación, de forma aislada o en combinación con otras tecnologías, constituye un método de conservación de alimentos de uso generalizado en la práctica industrial. La adición de ácidos como conservantes químicos es una técnica ampliamente utilizada en la industria alimentaria, y aunque algunos ácidos fuertes, como el clorhídrico (E-507), se utilizan en la formulación de varios alimentos, principalmente en quesos no madurados (Codex Stan 221, 2001), los ácidos más comúnmente utilizados son los ácidos orgánicos, compuestos genéricamente conocidos como ácidos débiles o ácidos carboxílicos (Cherrington y col., 1991), entre los que se incluyen los ácidos acético, ascórbico, cítrico, láctico y málico. De todos ellos, el *Codex Alimentarius* sólo reconoce como aditivo conservador al ácido acético, clasificando al resto como reguladores de la acidez (acético, cítrico, láctico y málico), antioxidantes (ascórbico y cítrico), emulsionantes (cítrico y málico) y agentes de retención del color (ascórbico). Sin embargo, aunque estos ácidos sean añadidos a los alimentos con otro objeto que la inhibición microbiana, poseen efectos antimicrobianos notables.

El ácido acético se utiliza como conservante en forma de vinagre desde hace más de 1.000 años (Nickol, 1979) y está presente de forma natural en la mayoría de las frutas y productos fermentados. Comercialmente se elabora mediante fermentación bacteriana del azúcar, melazas o alcohol o por síntesis química a partir de acetaldehído. Se caracteriza por presentar una mayor eficacia antimicrobiana frente a bacterias que frente a mohos y levaduras (Ingram y col., 1956), siendo especialmente efectivo ante *Listeria monocytogenes*, *Staphylococcus aureus*,

Salmonella spp., *Bacillus* spp. y *Clostridium* spp. Su utilización presenta como principales ventajas su bajo coste y toxicidad, mientras que entre sus mayores inconvenientes destacan el intenso sabor que transmite a los alimentos (Hartwing y McDaniel, 1995) y la posibilidad de causar delaminación de los materiales de envasado (Olafsson y col., 1993).

El ácido ascórbico se encuentra presente de forma natural en la mayoría de frutas y vegetales, siendo conocido comúnmente como vitamina C. Su nombre proviene del vocablo *scorbuticus* (escorbuto), enfermedad causada por su carencia en primates y algunas pocas especies más que carecen de la capacidad para sintetizarlo, por lo que deben obtenerlo por medio de su alimentación. Comercialmente se obtiene por fermentación bacteriana de la glucosa, seguida de una oxidación química, e industrialmente se emplea como antioxidante, principalmente en carne y productos cárnicos, en los que retrasa o previene la oxidación de la mioglobina.

El ácido cítrico es un constituyente natural de multitud de frutas, hallándose en grandes concentraciones en cítricos, kiwis y fresas. Comercialmente se obtiene mediante procesos fermentativos con levaduras y juega un importante papel en las industrias alimentaria y farmaceútica, utilizándose como regulador de la acidez, como compuesto aromático, para incrementar la capacidad gelificante en las mermeladas y para prevenir el pardeamiento enzimático en frutas y productos derivados. Además, por su actividad antimicrobiana frente a mohos y bacterias, también podría utilizarse como aditivo conservante, encontrándose muy extendido su uso por su agradable sabor, en contraste con el resto de ácidos orgánicos.

El ácido láctico se produce de forma natural durante la fermentación de algunos alimentos por medio de las bacterias ácido-lácticas. Aunque puede obtenerse por síntesis química, aproximadamente el 90% del ácido láctico se obtiene por procesos de fermentación. Tiene un amplio espectro de actividad antimicrobiana, siendo efectivo frente a *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* spp., *Bacillus* spp. y *Clostridium* spp. En la industria alimentaria se utiliza con diversos fines, tales como acidificar o regular el pH de los

Tabla 1. Valores de pH de algunos alimentos*

Producto	**pH**
C**arne**	
Carne de vaca	5,1-6,2
Jamón	5,9-6,1
Ternera	6,0
Pollo	6,2-6,4
Pescado y marisco	
Pescado	6,6-6,8
Pescado blanco	5,5
Salmón	6,1-6,3
Cangrejo	7,0
Atún	5,2-6,1
Vegetales	
Vegetales verdes	4,5-7,1
Espárragos	5,0-6,1
Judías	5,4-6,2
Repollos	5,2-6,3
Zanahorias	4,9-6,3
Patatas	5,6-6,2
Pimientos	5,0-7,0
Tomates	3,4-4,9
Frutas	
Manzanas	2,9-4,1
Melones	6,2-6,5
Naranjas, zumos	3,6-4,3
Setas	6,0-6,5
Productos lácteos	
Leche	6,3-6,5
Nata	6,5
Mantequilla	6,1-6,4
Yogur	4,6-5,0
Queso	5,2-7,0

* Adaptada de Lund y Eklund (2000)

alimentos, reducir su actividad del agua y potenciar el efecto de algunos antioxidantes como el ácido ascórbico. A todo ello hay que añadir su empleo como agente antimicrobiano en productos frescos o semiprocesados, así como en la descontaminación de canales en los mataderos (Smulders ang Greer, 1998; FDA, 2000; Fernández, 2007).

El ácido málico está presente de forma natural en la mayoría de frutas y vegetales, siendo especialmente abundante en las manzanas. Se usa habitualmente en la industria farmaceútica, en la fabricación de laxantes y de medicamentos indicados para el tratamiento de afecciones respiratorias, y en la industria alimentaria, como acidulante en gaseosas, refrescos y productos a base de fruta. En la elaboración del vino, durante la fermentación maloláctica, se transforma en ácido láctico, proporcionando una acidez más suave.

Merece una mención especial, por la frecuente implicación de las carnes de ave, ganado bovino y porcino en brotes de salmonelosis no tifoidea (apartado 1.1.3), el empleo de soluciones a base de ácidos orgánicos para la descontaminación de canales en los mataderos. Aunque su utilización ha sido aprobada en Estados Unidos, Canadá y Australia, donde es común el empleo de soluciones de ácido acético y láctico, que dan lugar a importantes reducciones en el número de bacterias contaminantes en la superficies de la canal (Dorsa y col., 1997; Smulders y Greer, 1998; Sofos y Smith, 1998), las regulaciones de la Unión Europea tan sólo permiten hasta el momento el tratamiento de canales con agua potable libre de aditivos (Smulders y Greer, 1998; del Río y col., 2007). Entre los principales inconvenientes que el Comité Científico Consultivo Veterinario de la Unión Europea encuentra en la utilización de estos compuestos en la descontaminación de canales de matadero cabe destacar el efecto negativo que su aprobación podría representar para la mentalidad del ganadero, que se vería tentado a rebajar su nivel de exigencia en cuanto a la aplicación de prácticas correctas de higiene en la granja, así como en el resto de eslabones de la cadena de producción (Smulders y Greer, 1998). Por ello, dicho comité de expertos no recomendó la introducción de técnicas de descontaminación durante el procesado de alimentos cárnicos, aunque dejó la puerta abierta para la reconsideración de su decisión a la espera de nuevos indicios que

hagan aconsejable su implantación. Además, también se debería tener en cuenta que la utilización de soluciones de ácidos orgánicos en el tratamiento de canales puede originar respuestas bacterianas adaptativas que causarían modificaciones en la resistencia microbiana a otras condiciones letales de estrés (Samelis y col., 2002; Stopforth y col., 2003), que generalmente se traducen en un incremento en su resistencia, no sólo frente a tratamientos térmicos y otros métodos de conservación de alimentos, sino también frente a las condiciones ácidas extremas del tracto gastrointestinal. Similares respuestas adaptativas pueden ser también inducidas en la industria alimentaria asociadas a la disminución progresiva del pH que se produce en productos fermentados, así como en la industria cárnica, durante la conversión del músculo en carne. Esta problemática ha sido revisada por nuestro grupo de investigación y ha dado lugar a una publicación en la revista "Alimentación, Equipos y Tecnología", que se refleja en el apartado 1.3., donde también se incluye un resumen sobre los principales mecanismos implicados en la actividad antimicrobiana de los ácidos orgánicos y los factores que condicionan su efectividad.

1.3.- Conservación de los alimentos mediante acidificación: actividad antimicrobiana de los ácidos orgánicos y factores que condicionan su efectividad

Ana Fernández, Avelino Álvarez-Ordóñez, Ana Bernardo, Guillermo J. Cebrián y Mercedes López

Alimentación, Equipos y Tecnología, 2007, 226, 46-49

Resumen

La acidificación, bien por fermentación o por la adición de ácidos débiles, se ha utilizado desde hace miles de años como método de conservación de los alimentos. La acidez puede ser un factor básico en la conservación, como en el caso de los alimentos fermentados, o tener un papel auxiliar, al combinar sus efectos con otros métodos, tales como conservantes químicos, calor, refrigeración o la disminución de la actividad del agua. Hasta hace relativamente poco tiempo la mayoría de los alimentos ácidos tenían un interés limitado en relación a la seguridad alimentaria al considerarse que el bajo pH ejercía un efecto inhibitorio sobre el crecimiento bacteriano. Sin embargo, en la última década se han documentado numerosos casos de enfermedades de origen alimentario de etiología diversa transmitidos por el consumo de alimentos ácidos, lo que fuerza a reconsiderar la creencia comúnmente aceptada de que los alimentos de bajo pH son microbiológicamente seguros desde un punto de vista sanitario. En este artículo se revisan los principales mecanismos implicados en la actividad antimicrobiana de los ácidos orgánicos y los factores que condicionan su efectividad.

Introducción

El pH es uno de los principales factores que influyen en la supervivencia y crecimiento de los microorganismos durante la distribución, procesado y almacenamiento de los alimentos. En el contexto de la conservación de los alimentos muchos microorganismos pueden cesar de crecer debido a los cambios del

pH citoplasmático que pueden ser ocasionados simplemente acidificando el medio exterior.

La acidez puede ser un factor básico en la conservación, como en el caso de los alimentos fermentados, o tener un papel auxiliar, al combinar sus efectos con otros métodos. En la fermentación, los productos que se originan en el transcurso del proceso ejercen su efecto conservador al reducir el pH y/o mediante la actividad antimicrobiana de los ácidos débiles que se forman. La adición de ácidos es también una técnica ampliamente utilizada por la Industria Alimentaria, y aunque algunos ácidos fuertes, como el clorhídrico (E-507), se usan en la formulación de varios alimentos (CODEX STAN 221-2001), los más comúnmente utilizados son los ácidos orgánicos.

Hasta hace relativamente poco tiempo la mayoría de los alimentos ácidos tenían un interés limitado en relación a la seguridad alimentaria, al considerarse que el bajo pH ejercía un efecto inhibitorio sobre el crecimiento bacteriano. Sin embargo, en la última década se han documentado numerosos casos de enfermedades de origen alimentario de etiología diversa transmitidos por el consumo de alimentos ácidos, lo que plantea la necesidad de adquirir un entendimiento más profundo sobre la capacidad de respuesta de los microorganismos a los ambientes ácidos.

En este artículo se revisa el estado actual de los conocimientos en relación a los principales mecanismos implicados en la actividad antimicrobiana de los ácidos orgánicos y a los factores que condicionan su efectividad.

Actividad antimicrobiana de los ácidos orgánicos

Los ácidos orgánicos ejercen su actividad antimicrobiana a través de un efecto del pH *per se,* debido a la concentración de iones hidrógeno libres, otro que depende del grado de disociación del ácido y un tercero relacionado con la naturaleza del mismo. La importancia relativa de cada uno de ellos ha sido muy discutida, aunque en general se acepta que el factor más relevante es el grado de disociación del ácido (Presser y col., 1997; Brul y Coote, 1999). El pH afecta al

grado de disociación de los ácidos, de acuerdo con la ecuación de Henderson-Hasselbach:

$$pH = pK_a + \log ([A^-] / [AH])$$

Donde: AH es el ácido sin disociar y A^- es la base conjugada.

Tradicionalmente, ha sido asumido que la forma no disociada de los ácidos orgánicos puede atravesar fácilmente la membrana de la célula bacteriana y una vez dentro, como el pH intracelular es más alto que el pKa del ácido, disociarse en aniones y protones (Salmond y col., 1984; Cherrington y col., 1991; Davidson, 2001) (Fig. 1). El aumento de la concentración interna de aniones provocará una alteración de la osmolaridad, que desencadena un mecanismo de compensación de la carga eléctrica que obliga a la célula bacteriana a aumentar los niveles de Na^+, K^+ y/o glutamato, lo que lleva a un incremento de la fuerza iónica intracelular y de la turgencia. Este proceso provoca un gran aumento de la presión mecánica sobre la pared del microorganismo, que, de no ser compensado, puede hacer que la célula se rompa (Foster, 1999). Además, los altos niveles de los aniones citoplasmátios van a ejercer un efecto sobre los procesos metabólicos por interferir en las reacciones enzimáticas (Roe y col., 2002).

Por otro lado, la acumulación de protones afectará directamente al pH intracelular microbiano (Carmelo y col., 1997; Arneborg y col., 2000), por lo que, con el fin de mantener el pH citoplasmático cercano a la neutralidad y así conservar la funcionalidad de las macromoléculas, la célula eliminará el exceso de hidrogeniones, hecho que, por un lado, provoca la disipación de la fuerza motriz de protones (ICMSF, 1980; Gould y col., 1983) y por otro, un gasto de adenosín trifosfato (ATP), que puede causar el agotamiento de la energía celular (Davidson, 2001). Si la célula no logra mantener el pH estable no podrá sintetizar componentes celulares y se producirá una reducción en la actividad de los sistemas de transporte, limitándose la entrada de iones esenciales y nutrientes, por lo que será incapaz de dividirse y crecer (Booth y Kroll 1989; Brown y Booth, 1991) o, al menos, se reducirá la tasa de crecimiento o aumentará la fase de latencia (Cheroutre-Vialette y col., 1998).

En otros casos, se ha propuesto que los ácidos orgánicos afectan a la estructura y la actividad del ADN y de las proteínas de la membrana citoplasmática, interfiriendo en el transporte de electrones, reduciendo la producción de ATP y/o disipando el gradiente eléctrico y de protones a través de las membranas celulares (Axe y Bailey, 1995; Stratford y Anslow, 1996; Davidson, 2001)**.** También se les ha atribuido la capacidad de interferir con el transporte de sustancias nutritivas y de influir en la síntesis de macromoléculas (Denyer y Stewart, 1998; Alakomi y col., 2000; Davidson, 2001).

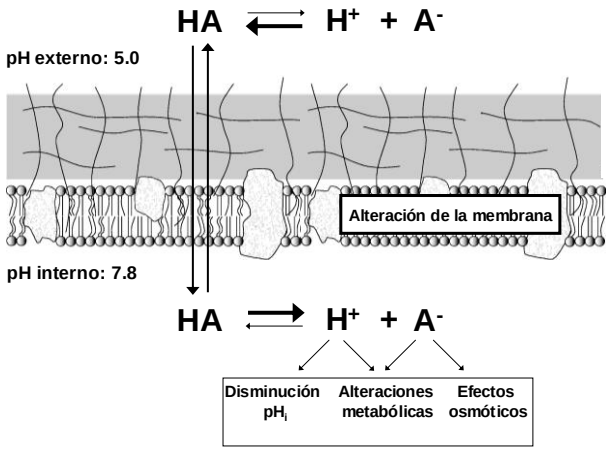

Fig. 1. Mecanismo general de acción de los ácidos orgánicos débiles.

En cuanto al efecto específico relacionado con la naturaleza del ácido, se ha comprobado que tienen mayor actividad a medida que se alarga la longitud de la cadena molecular, aunque los ácidos alifáticos de más de 10-11 átomos de carbono poseen muy poca aplicación potencial debido a su baja solubilidad en agua (ICMSF, 1983). Por otra parte, en algunos casos, como el del ácido cítrico, pueden presentar un efecto quelante, viéndose su actividad afectada por la concentración de iones metálicos presentes en el medio (Graham y Lund, 1986). En otros, como el del ácido sórbico y el ácido benzoico, se ha demostrado que son capaces de inhibir varias enzimas (Russell, 1991; Chipley, 1993; Sofos y Busta, 1993). Por otra parte, el

efecto relativo de los acidulantes depende también de la capacidad de los microorganismos para metabolizarlos (Chipley, 1993; Sofos y Busta, 1993).

Factores que condicionan la actividad antimicrobiana de los ácidos orgánicos

Son múltiples los factores que pueden interferir sobre la actividad antimicrobiana de los ácidos orgánicos, siendo la resistencia de los microorganismos muy variable, aunque en líneas generales, parece que las levaduras y los mohos toleran mejor la acidez que las bacterias y dentro de éstas, generalmente, las Gram negativas son más resistentes que las Gram positivas (Russell, 1991), probablemente debido a la existencia de la membrana externa (Nikaido y Varra, 1985). Existen también diferencias relacionadas con la especie y la forma celular, siendo las células vegetativas más sensibles que las esporas (Heinzel, 1998).

Los componentes y características del alimento pueden tener efectos aditivos o sinérgicos o, por el contrario, proteger a los microorganismos. En los alimentos sólidos, los ácidos orgánicos suelen ser menos efectivos que en los líquidos, porque la fijación de los microorganismos a las superficies puede incrementar su resistencia al pH ácido (Krisch y Szajani, 1997). Un alto contenido en grasa aumenta la concentración de los ácidos en la fase lipídica, limitando su disponibilidad para inhibir a los microorganismos que se encuentran en la fase acuosa (Sofos y Busta, 1993). La capacidad tampón del alimento es otro factor importante al limitar el descenso del pH (Lindsay, 2000). La actividad del agua del alimento también puede afectar al pKa de los ácidos orgánicos y por ello alterar su efecto antimicrobiano (Pethybridge y col., 1983; Rouse y col., 1988).

La aplicación de tratamientos térmicos, de pulsos eléctricos de alta intensidad o de altas presiones hidrostáticas puede originar cambios en la composición de la microbiota inicial, que a su vez influirán en la efectividad de los diferentes antimicrobianos empleados. Asimismo, el uso de atmósferas modificadas o el tiempo y la temperatura de almacenamiento pueden tener un gran efecto en relación con la efectividad antimicrobiana de los diversos ácidos orgánicos (Davidson, 2002).

La eficacia de los ácidos orgánicos está también relacionada con los niveles de contaminación inicial (Dickson, 1992; Greer y Dilts, 1992), al existir la posibilidad de que los hidrogeniones o las moléculas de ácido no disociadas interactúen con los microorganismos, pudiendo presentarse en la población una distribución de resistencias a la acidez, por lo que cuanto mayor sea el número de microorganismos presentes, mayor será la probabilidad de que alguno tenga una capacidad de tolerancia superior (Lund y Eklund, 2000).

La fase de crecimiento en la que se encuentren los microorganismos también puede influir en la eficacia de los ácidos orgánicos, siendo, en general, las células en fase estacionaria más resistentes (Kolter y col., 1993).

Otro aspecto que es necesario destacar y que puede contribuir a explicar las implicaciones de los alimentos ácidos en brotes de enfermedades es el hecho de que los microorganismos expuestos a diversos estreses ambientales pueden desarrollar respuestas de protección cruzada, que se manifiestan en la adquisición de una mayor resistencia frente a otras condiciones adversas (Leyer y Johnson, 1993; Bearson y col., 1997; Duffy y col., 2000; Mazzotta, 2001).

La exposición de los microorganismos a un estrés previo y los posibles daños subletales causados pueden aumentar o disminuir su resistencia a la acidez (Davidson y Harrison, 2002). Este tema ha sido objeto de múltiples investigaciones en la última década, habiéndose comprobado que el empleo de ácidos orgánicos puede inducir respuestas de resistencia frente a una acidificación posterior (Leyer y Johnson, 1993; Garren y col., 1998; Buchanan y Edelson, 1999; Wilde y col., 2000; Mazzotta, 2001), denominándose a este fenómeno tolerancia ácida. Esta respuesta adaptativa origina un aumento de la capacidad de supervivencia de los microorganismos tras su exposición a un pH letal, lo que puede ocasionar una mayor resistencia en las condiciones ácidas del estómago, causando de este modo la infección tras la ingestión del alimento (Bodmer y col., 2000; Russell, 2001).

En cualquier caso, estas respuestas de adaptación a la acidez, deberían preocupar, tanto a los manipuladores de alimentos como a los clínicos, ya que pueden determinar la supervivencia de los microorganismos en diversos ambientes

ácidos a los que pueden ser expuestos, no sólo durante el procesado de los alimentos, sino también en el cuerpo humano durante su paso a través del tracto gastrointestinal.

Referencias

Las referencias bibliográficas utilizadas en esta publicación se incluyen en el conjunto de referencias de la introducción (apartado 1.7).

1.4.- Respuesta de tolerancia ácida

Son numerosas las investigaciones en las que se ha descrito la aparición de una respuesta adaptativa frente a la acidez en diversos microorganismos, tanto Gram positivos (*Listeria* spp. (Hill y col., 1995; Greenacre y col., 2003), *Enterococcus faecalis* (Flahaut y col., 1996), *Enterococcus faecium* (Fernández, 2007), *Streptococcus mutans* (Hamilton y Buckley, 1991), *Lactobacillus acidophilus* (Lorca y col., 1998) y *Lactococcus lactis* (Hartke y col., 1996)), como Gram negativos (*Escherichia coli* (Garren y col., 1997; Cheng y col., 2003), *Aeromonas hydrophila* (Karem y col., 1994), *Vibrio parahaemolyticus* (Wong y col., 1998) y *Helicobacter pylori* (Mooney y col., 1990)). La respuesta de tolerancia ácida ha sido ampliamente estudiada en *S.* Typhimurium (Foster y Hall, 1990; Lee y col., 1994; Bearson y col., 1996, 1998; Greenacre y col., 2003), probablemente debido al buen conocimiento de su genoma y a la capacidad que posee de sobrevivir en un amplio rango de condiciones de estrés ácido que se encuentran tanto en los alimentos como en el hospedador durante la infección (Leyer y Johnson, 1992; Foster, 1995), habiéndose descrito también para otras serovariedades, como *S.* Enteritidis (Bacon y col., 2003a), *S.* Agona, *S.* Gaminara, *S.* Michigan, *S.* Montevideo, *S.* Poona (Yuk y Schneider, 2006) y *S.* Senftenberg (Kumar y Kumar, 2003).

El desarrollo de estas respuestas no sólo puede prolongar la supervivencia microbiana en varios alimentos ácidos, entre los que se incluyen quesos (Leyer y

Johnson, 1992), embutidos (Leyer y col., 1995), kétchup (Tsai y Ingham, 1997), yogur y otros productos lácteos fermentados (El-Gazzar y Marth, 1992; Gahan y col., 1996; Cheng y Chou, 2001) y zumos de frutas (Gahan y col., 1996; Parish, 1997; Cheng y Chou, 2001), sino que también puede incrementar la resistencia bacteriana a las condiciones ácidas del estómago, aumentando el riesgo de enfermedad. El papel que desempeña el pH del jugo gástrico como barrera frente a toxiinfecciones alimentarias es muy importante, habiéndose comprobado que la dosis infectiva de varios microorganismos desciende considerablemente si el inóculo se administra con bicarbonato sódico o en aquellos individuos que padecen aclorhidria (Koo y col., 2002; Smith, 2003; Tamplin, 2005). Además, aunque varios factores, como la capacidad bacteriana para invadir el epitelio intestinal o para sobrevivir al sistema inmunitario del hospedador, están involucrados en el desarrollo final de la enfermedad, numerosos estudios han encontrado una fuerte correlación entre la capacidad de los microorganismos para desarrollar respuestas de tolerancia ácida y su virulencia (García-Portillo y col., 1993; Wilmes-Riesenberg y col., 1996; Gahan y Hill, 1999). Por ello, este tema es de gran importancia a la hora de adoptar medidas que garanticen la seguridad alimentaria y, posiblemente, sea la base de los fallos existentes cuando se llevan a la práctica estrategias para la inactivación microbiana diseñadas a partir de estudios realizados con microorganismos obtenidos en situaciones no estresantes, por lo que parece necesario identificar aquellas condiciones en las que los microorganismos muestren su máxima resistencia a la acidez.

1.4.1.- Factores que condicionan la respuesta de tolerancia ácida en *Salmonella*

Es un hecho generalmente aceptado que la resistencia microbiana frente a los agentes físicos y químicos está influida por factores genéticos, existiendo numerosos estudios en los que se demuestra que la resistencia a la acidez de *Salmonella* varía entre serovariedades (Arvizu-Medrano y Escartín, 2005) e, incluso, entre cepas de la

misma serovariedad (Bacon y col., 2003a; de Jonge y col., 2003a; Samelis y col., 2003; Berk y col., 2005; Yuk y Schneider, 2006). En algunos de estos estudios se ha llegado a sugerir la existencia de una relación entre la resistencia intrínseca a la acidez y la capacidad patogénica de la cepa bacteriana, llegando a la conclusión de que los microorganismos que son capaces de desarrollar una mayor respuesta de tolerancia ácida son los que más posibilidades tienen de sobrevivir a las defensas del organismo y causar la infección. Así, Berk y col. (2005), trabajando con 37 cepas de *S.* Typhimurium aisladas de diferentes hábitats, encontraron que aquellas obtenidas a partir de aislamientos humanos eran las que poseían una mayor resistencia intrínseca a la acidez, sugiriendo la existencia de una correlación entre tolerancia ácida y patogenicidad en esta especie microbiana. También de Jonge y col. (2003a), estudiando 22 cepas de *S.* Typhimurium, observaron que varias de ellas pertenecientes a *S.* Typhimurium DT104, una de las salmonelas con mayor capacidad patogénica, poseían una mayor resistencia ácida en comparación con otras cepas menos virulentas. Sin embargo, también existen evidencias que indican que la respuesta está condicionada por ciertos factores ambientales que pueden actuar durante la adaptación a un medio ácido (fase de crecimiento, temperatura de cultivo, pH, composición del medio de cultivo, tiempo de adaptación…) o durante su exposición a valores extremos de pH (temperatura de tratamiento, pH, composición del medio de tratamiento) y, de hecho, en muchas ocasiones, su influencia puede llegar a superar la ejercida por los factores genéticos.

De acuerdo con los datos existentes en la bibliografía, la fase de crecimiento microbiano modifica la respuesta de tolerancia ácida desarrollada, resultando las células en fase de crecimiento exponencial más sensibles que las que se encuentran en fase estacionaria (Rees y col., 1995). Cabe destacar que, en el caso particular de *S.* Typhimurium, se ha señalado la existencia de dos sistemas de respuesta de tolerancia ácida bien diferenciados, que se manifiestan con características y mecanismos celulares completamente distintos en las células en fase exponencial y estacionaria (Apartado 1.6.1.).

En cuanto al efecto ejercido por la temperatura de cultivo, a pesar de la importancia que este parámetro ejerce sobre la resistencia microbiana frente a

diversos agentes físicos y químicos, que sepamos, tan sólo en una ocasión (Samelis y col., 2003) se ha estudiado su influencia sobre la resistencia ácida de *S.* Typhimurium, encontrando una disminución en la resistencia ácida al reducir la temperatura de crecimiento de 30 a 10ºC. Este descenso en la magnitud de la respuesta de tolerancia ácida cuando se utilizan bajas temperaturas de crecimiento ha sido descrito también para otros microorganismos, como *Escherichia coli* (Cheng y Kaspar, 1998; Semancheck y Golden, 1998; Samelis y col., 2003), y puede llegar a tener importantes repercusiones de tipo práctico, dado que la refrigeración podría constituir una técnica recomendable en el procesado de los alimentos ácidos.

En relación al efecto ejercido por otras condiciones que prevalecen durante la adaptación microbiana a la acidez, como el pH y la composición del medio de cultivo, cabe destacar que en la mayoría de los estudios de tolerancia ácida de *Salmonella* se han utilizado células adaptadas a la acidez mediante su crecimiento en medios de laboratorio a los que se les ha adicionado una cierta cantidad de glucosa, de tal manera que durante el crecimiento se produce un descenso gradual del pH extracelular hasta valores aproximados de 5,0-6,0, dependiendo fundamentalmente de la capacidad acidificante intrínseca del microorganismo (Wilde y col., 2000; Bacon y col., 2003a). En otras ocasiones, se han utilizado medios con bajo contenido en glucosa, en los que las células crecen a pH neutro hasta alcanzar la fase exponencial o estacionaria, siendo posteriormente expuestas a condiciones moderadamente ácidas (pH 4,4-5,8) durante cortos periodos de tiempo, para determinar finalmente su capacidad de supervivencia frente a un pH extremo (entre 3,0 y 3,8) (Foster y Hall, 1990; Leyer y Johnson, 1992; Bearson y col., 1996, 1998; Greenacre y col., 2003). Además, en estos últimos estudios se utilizaron diferentes tiempos de adaptación a la acidez, que oscilaron entre 30 minutos y 6 horas, y en aquellos casos en los que se estudió la influencia del tiempo de adaptación (Bearson y col., 1996; Greenacre y col., 2003), se comprobó que la máxima respuesta de tolerancia ácida adquirida dependía no sólo de la especie bacteriana, sino también del pH del medio de adaptación y del tipo de ácido utilizado. Por tanto, la gran variabilidad de condiciones experimentales utilizadas, unida a la existencia de escasos estudios en los que se realice una evaluación comparativa del efecto que

dichos factores ejercen sobre la magnitud de la respuesta de tolerancia ácida adquirida, no permiten establecer conclusiones sólidas al respecto. En este sentido, y a pesar de la importancia que tendría el conocimiento del comportamiento de *Salmonella*, tras su crecimiento en medios acidificados mediante el uso de ácidos orgánicos, los estudios realizados para aclarar este aspecto son muy escasos (Greenacre y col., 2003; Arvizu Medrano y Escartín, 2005; Yuk y Schneider, 2006). Greenacre y col. (2003), tras la exposición de células de *S.* Typhimurium durante 1-6 horas a valores de pH de 5,0; 5,5 y 5,8 en TSB acidificado con ácido acético y láctico, observaron que el ácido acético era más efectivo en la inducción de una respuesta de tolerancia ácida. Yuk y Schneider (2006), usando varios zumos de frutas para la obtención de células de diversas serovariedades (*S.* Agona, *S.* Gaminara, *S.* Michigan, *S.* Montevideo y *S.* Poona) adaptadas a la acidez, describieron que el tipo de ácido predominante en cada tipo de zumo influía en la magnitud de la respuesta bacteriana desarrollada, que se manifestaba por un incremento en la supervivencia a un tratamiento en jugo gástrico simulado (pH 1,5), aunque de forma variable en función de la temperatura de adaptación y de la serovariedad estudiada, obteniendo, en general, una respuesta de tolerancia ácida más intensa tras la adaptación en zumos de manzana y tomate, con un elevado contenido en ácido málico, que cuando ésta tuvo lugar en zumo de naranja, con un alto contenido en ácido cítrico. Sin embargo, Arvizu-Medrano y Escartín (2005), trabajando con *S.* Typhi y *S.* Typhimurium, observaron que, aunque la adaptación a la acidez mediante la exposición de las células durante 1 hora a un pH de 5,5 en TSB en presencia de ácido cítrico, láctico o clorhídrico ocasionaba en todos los casos un incremento en la resistencia a un tratamiento en TSB pH 3,0, la magnitud de la respuesta de tolerancia ácida desarrollada no estaba condicionada por el tipo de ácido utilizado.

En relación a la influencia que ejercen las condiciones prevalentes durante el tratamiento en condiciones ácidas extremas, la información disponible es aún más escasa, aunque parece que la temperatura condiciona el comportamiento microbiano, habiéndose demostrado en ocasiones que la resistencia a la acidez se ve incrementada a medida que disminuye la temperatura de tratamiento (Leistner, 2000;

Cheng y Chou, 2001). Leistner (2000) encontró que las salmonelas sobreviven mejor a temperaturas de refrigeración en algunos alimentos ácidos, como productos cárnicos fermentados o mayonesa, resultados que son muy similares a los obtenidos por Cheng y Chou (2001) en zumos de mango y espárrago para *Escherichia coli* O157:H7.

Otras características que pueden condicionar la supervivencia en condiciones extremas son el pH y la composición química del medio de tratamiento. En general, en los diferentes estudios desarrollados sobre la respuesta bacteriana de tolerancia ácida se han utilizado una gran diversidad de medios de tratamiento (jugo gástrico simulado, alimentos ácidos, medios de laboratorio acidificados,…) y de valores de pH (entre 1,5 y 3,8), pero no hemos encontrado ninguno que evalúe el efecto del pH de tratamiento sobre la magnitud de la respuesta desarrollada. Por el contrario, se ha demostrado que la resistencia bacteriana a condiciones ácidas extremas se ve incrementada cuando se utilizan matrices alimentarias, especialmente, medios con un alto contenido graso o proteico, habiéndose descrito que los brotes de salmonelosis en los que se encuentra una baja dosis infectiva están a menudo asociados con el consumo de alimentos con un elevado contenido en grasa, como chocolate y queso, o un elevado contenido proteico, como carne picada o clara de huevo (Waterman y Small, 1998). Además, la presencia en el medio de tratamiento de algunos aminoácidos también puede modificar la resistencia ácida, posibilitando el desarrollo de una respuesta de tolerancia ácida. Numerosos estudios han demostrado que la inclusión en el medio de tratamiento de arginina, lisina y ácido glutámico incrementa la supervivencia de *Escherichia coli* en condiciones ácidas extremas (Castaine-Cornet y col., 1999; Castaine-Cornet y Foster, 2001; Iyer y col., 2003; Foster, 2004). Sin embargo, en el caso de *Salmonella* este efecto protector sólo ha sido señalado bajo ciertas condiciones de cultivo (Park y col., 1996; de Jonge y col., 2003a; Kieboom y Abee, 2006). Park y col. (1996) demostraron que las células de *S.* Typhimurium adaptadas a la acidez mediante su incubación a pH 4,4 durante 1 hora veían incrementada su resistencia a un tratamiento a pH 3,0 cuando éste era realizado en presencia de lisina. En el estudio llevado a cabo por de Jonge y col. (2003a) se describió que las células de *S.* Typhimurium crecidas en un medio

nutritivo en presencia de glucosa a pH 5,0 hasta alcanzar la fase estacionaria mostraban una mayor tolerancia ácida cuando el medio de tratamiento (un medio mineral mínimo) era suplementado con arginina o lisina. Por último, Kieboom y Abee (2006) observaron que la supervivencia a un tratamiento a pH 2,5 en un medio mineral de las células de *S.* Typhimurium crecidas en Luria Broth (LB) suplementado con glucosa a pH 5,0 se veía incrementada cuando el tratamiento se realizaba en presencia de arginina.

1.5.- Respuestas de protección cruzada

El consumidor actual demanda alimentos de elevada calidad nutritiva y sensorial y con características similares a las del producto fresco. La industria alimentaria se encuentra, por tanto, en una encrucijada, ya que por un lado los cambios en las tendencias y hábitos de consumo de la población le obligan a desarrollar nuevas tecnologías de procesado y conservación de los alimentos y a lanzar al mercado nuevos productos, con sabores más naturales, libres de aditivos o que sufren tratamientos térmicos más suaves, y por otro, debe garantizar la seguridad alimentaria de esos productos sometidos a tratamientos de conservación más lábiles. Por ello, en los últimos años se está realizando un importante esfuerzo investigador con la finalidad de adecuar los métodos de conservación de los alimentos a las tendencias del mercado. Uno de los enfoques que más atención está recibiendo es la "tecnología de los procesos combinados" (Leistner, 2000), que consiste en la aplicación simultánea o sucesiva de varios métodos de conservación, que, actuando de forma sinérgica o aditiva, garanticen la consecución del grado de inactivación deseado. La exposición bacteriana a diferentes condiciones ambientales de estrés desencadena determinados mecanismos de mantenimiento de la homeostasis interna que provocan un importante incremento en las demandas energéticas, pudiendo llegar a la muerte celular por un proceso de agotamiento metabólico (Gould, 1995; Leistner, 2000).

Los métodos de conservación utilizados más frecuentemente en la tecnología de los procesos combinados son: temperaturas extremas (tratamientos térmicos y de refrigeración), reducción de la actividad del agua, acidificación, modificaciones del potencial redox, empleo de aditivos, como nitratos, sorbatos y sulfitos, y utilización de cultivos iniciadores, sobre todo de bacterias ácido-lácticas, habiéndose descrito más de 60 posibles "barreras" que podrían emplearse en la industria alimentaria para mejorar la estabilidad y/o calidad del producto final (Leistner, 1999). En algunos casos no sólo se utilizan por su efecto antimicrobiano, sino también por su contribución a la mejora de la calidad organoléptica de los alimentos, aunque, con carácter general, la intensidad de las distintas barreras aplicadas debe mantenerse en un rango óptimo que tenga en cuenta tanto la seguridad como la calidad sensorial del alimento (Leistner, 1994). Sin embargo, la frecuente implicación de estos productos en brotes de toxiinfección alimentaria pone en entredicho su seguridad (Gibbs, 1999; Sharp y Reilly, 2000). Se trata, en la mayoría de los casos, de toxiinfecciones en las que se han visto implicados microorganismos psicrotrofos o mesófilos, capaces de multiplicarse o sobrevivir a las temperaturas de refrigeración comúnmente utilizadas durante el almacenamiento y distribución de los alimentos mínimamente procesados (Abee y Wouters, 1999). Entre las diversas causas que pueden contribuir a este problema cabe destacar la capacidad que poseen los microorganismos de desarrollar respuestas adaptativas cuando son expuestos a condiciones subletales de estrés, mediante la activación de mecanismos generales de supervivencia que ocasionan un incremento en su posterior resistencia, no sólo frente al agente inductor de la respuesta, sino también frente a otros agentes estresantes, entre los que se incluirían los diferentes procedimientos de conservación de los alimentos, como los tratamientos térmicos moderados, la acidificación o la adición de sal, así como el uso de ciertos agentes de desinfección y limpieza (Lou y Yousef, 1997; Abee y Wouters, 1999; Hill y col., 2002; van Schaick y Abee, 2005). Por todo ello, la industria alimentaria debe buscar la manera de evitar el desarrollo de respuestas adaptativas bacterianas, que no sólo pueden posibilitar la supervivencia de microorganismos patógenos y alterantes, sino que, además, pueden provocar la inducción de factores de virulencia en los microorganismos patógenos, facilitando la selección de una flora potencialmente más peligrosa para la salud pública (Abee y

Wouters, 1999; Jorgensen y col., 2000; Hill y col., 2002). Además, dada la tendencia actual a reducir la intensidad de los diferentes tratamientos de conservación, la posibilidad de que se desarrollen estos fenómenos adaptativos es cada vez más elevada, con las consiguientes consecuencias para la seguridad y estabilidad de los alimentos. La solución al problema se alcanzará cuando se consiga adquirir un conocimiento completo de las bases moleculares que sustentan los fenómenos de inactivación y resistencia microbiana frente a los distintos agentes de conservación de los alimentos, lo que permitiría establecer las pautas de actuación adecuadas. En este sentido, ha alcanzado notoriedad la tecnología de procesos combinados de tipo "multitarget", expuesta por Leistner (1995), en la que los diferentes tratamientos de conservación aplicados deberían ejercer un efecto sinérgico, al actuar cada uno de ellos sobre una diana celular diferente (membrana citoplasmática, ADN, sistemas enzimáticos, etc.), por lo que, tras ser combinados, perturbarían la homeostasis celular a varios niveles, haciendo más difícil la reparación del daño ocasionado. Sin embargo, la consecución de este objetivo es difícil, tanto más si se tiene en cuenta que las bases moleculares de los mecanismos de inactivación son aún desconocidas, constituyendo en su conjunto un fenómeno muy complejo que además se encuentra sometido a la influencia de diferentes factores, en ocasiones interrelacionados entre sí y que pueden llegar a modificar la respuesta microbiana.

1.5.1.- Adaptación ácida de *Salmonella* y protección cruzada frente a tratamientos térmicos

El calor constituye uno de los principales métodos de conservación de los alimentos, bien sea en la elaboración de alimentos pasterizados, comercialmente estériles o mediante el cocinado previo al consumo, y actualmente sigue siendo, sin duda, una de las principales estrategias empleadas por la industria alimentaria para aumentar la vida útil de los alimentos y obtener productos microbiológicamente seguros. La optimización de los tratamientos térmicos requiere disponer de datos precisos del grado, características y biovariabilidad de la resistencia al calor de los

microorganismos, teniendo en cuenta también las posibles respuestas de adaptación que pueden desarrollar cuando han sido sometidos a condiciones adversas. La aparición de estas respuestas, que pueden manifestarse en un aumento de la resistencia al calor, tiene también una importancia trascendental sobre los diversos modelos desarrollados en microbiología predictiva a la hora de asegurar la calidad microbiológica de los alimentos, especialmente en el caso de los microorganismos patógenos, que podrán prevalecer en los productos alimentarios, si no se ha considerado esta circunstancia. Sin embargo, la evaluación de la resistencia microbiana al calor se realiza, generalmente, utilizando microorganismos obtenidos en óptimas condiciones de crecimiento, ya que existe la creencia generalizada de que en esas condiciones presentan su máximo nivel de resistencia al calor, y desde luego, parece necesario conocer su historial previo, sobre todo en aquellos casos en que han sido sometidos a determinadas condiciones adversas, ya que las células estresadas no se comportan de igual forma que aquellas crecidas en medios ricos y en condiciones óptimas.

Hasta hace relativamente poco tiempo, los alimentos ácidos tenían un interés limitado en relación a su seguridad alimentaria, al considerarse que su bajo pH ejercía un efecto inhibitorio sobre el crecimiento bacteriano. Sin embargo, en las últimas décadas se han producido numerosos casos de toxiinfección alimentaria de etiología diversa, incluida la salmonelosis, en los que el vehículo de transmisión fueron alimentos ácidos, entre ellos sidra y zumo de manzana (Tauxe y col., 1997; Mead y col., 1999; CDC, 2004), zumo de naranja (CDC, 1997, 2004; Krause y col., 2001), algunos productos cárnicos (CDC, 1995, 2001; Bremer y col., 2004) y salsa de tomate (CDC, 2004). Estos incidentes llevaron a la U.S. Food and Drug Administration (FDA) y al U.S. Departament of Agriculture Food Safety and Inspection Service (DAFSIS) a proponer una regulación de Análisis de Peligros y Puntos de Control Críticos (APPCC) que incluía criterios de actuación para garantizar su seguridad (Red, 1995; FDA, 1998), haciendo obligatorio implementar un sistema que logre la reducción de 5 unidades logarítmicas del microorganismo más resistente de importancia sanitaria. La inclusión de un tratamiento térmico en el proceso de elaboración de algunos productos podría lograr con éxito la deseada

reducción en el número de patógenos, incrementando su seguridad, pero la posibilidad de que alguno de estos microorganismos desarrollen respuestas de protección cruzada frente al calor constituye un serio problema que debería ser tenido en cuenta en el diseño de tratamientos térmicos apropiados. Estas respuestas han sido descritas en numerosas ocasiones en algunos patógenos alimentarios, como *Listeria monocytogenes* (Farber y Pagotto, 1992; Lou y Yousef, 1996; Juneja y col., 1998) y *Escherichia coli* (Cheville y col., 1996; Ryu y Beuchat, 1998, 1999; Buchanan y Edelson, 1999; Rowe y Kirk, 1999; Duffy y col., 2000), siendo también muy abundantes las investigaciones realizadas en los últimos años sobre el desarrollo de estas respuestas de protección cruzada frente al calor en *Salmonella* (Leyer y Johnson, 1993; Wilde y col., 2000; Mazzotta, 2001; Bacon y col., 2003b; Tosun y Gönül, 2003).

1.5.1.1.- Factores que condicionan la respuesta de protección cruzada frente al calor

Es bien sabido que la termorresistencia de los microorganismos es una propiedad compleja que no sólo está determinada genéticamente, sino también por una multitud de factores ambientales cuya influencia puede ser incluso mayor que la debida al genotipo, entre los que cabe destacar todos aquellos que actúan durante el crecimiento y desarrollo de las células o la aplicación de determinados tratamientos previos, como choques térmicos, osmóticos o ácidos o el almacenamiento a refrigeración.

Sin embargo, y a pesar de la importancia que para el diseño de tratamientos térmicos adecuados tendría el conocimiento del desarrollo de estas respuestas de protección cruzada frente al calor en determinadas condiciones de crecimiento celular (temperatura de crecimiento, pH y composición del medio de cultivo,…), los estudios llevados a cabo para clarificar estos aspectos son muy escasos y aún más en el caso concreto de las salmonelas. Además, la gran variedad de condiciones

experimentales empleadas por los diferentes grupos de investigación que han abordado esta problemática hace difícil la extracción de conclusiones firmes acerca de la influencia ejercida por estos factores sobre la respuesta bacteriana de protección cruzada frente al calor.

En concreto, en los trabajos desarrollados para estudiar el comportamiento frente al calor de los microorganismos adaptados a la acidez, en muchas ocasiones se han utilizado para la obtención de células control (no adaptadas a la acidez) algunos medios comerciales (BHI, TSB) que contienen aproximadamente un 0,2% de glucosa, y, para inducir la adaptación, estos mismos medios con una determinada proporción de glucosa añadida (Thippareddi y col., 1995; Ryu y Beuchat, 1998, 1999; Riordan y col., 2000). Por ello, en ambos casos, el crecimiento microbiano originaría un descenso progresivo del pH del medio que condicionaría la resistencia térmica observada, hecho que podría explicar que en algunos casos no se obtuvieran estas respuestas de protección cruzada (Thippareddi y col., 1995; Riordan y col., 2000).

En otras investigaciones, tras el crecimiento microbiano en condiciones neutras, la adaptación a la acidez se llevó a cabo exponiendo a las células a valores bajos de pH durante periodos predeterminados de tiempo (Farber y Pagotto, 1992; Leyer y Johnson, 1993; Lou y Yousef, 1996; Williams e Ingham, 1998; Rowe y Kirk, 1999; Jung y Beuchat, 2000; Evrendilek y Zhang, 2003), estando la respuesta adaptativa condicionada por el tiempo de exposición y el valor de pH utilizados para la adaptación. Farber y Pagotto (1992) observaron para *L. monocytogenes* que a un pH de 4,0, el tiempo óptimo de adaptación, al que se desarrollaba la máxima protección cruzada, era de una hora, y que con tiempos superiores se producía una disminución de la resistencia térmica. Por el contrario, Evrendilek y Zhang (2003), trabajando con *Escherichia coli* O157:H7 adaptada a un pH de 3,6, encontraron que la termorresistencia aumentaba a medida que lo hacía el tiempo de exposición hasta 6 horas. Estos mismos autores describieron también que el valor de pH mostraba una gran influencia sobre la magnitud de la respuesta de protección cruzada obtenida, que resultó máxima a pH 3,6, el menor de los valores de pH estudiados (3,6; 5,2; 7,0). Resultados similares fueron obtenidos por Lou y Yousef (1996), que trabajando

con células de *L. monocytogenes* expuestas a un intervalo de pH entre 4 y 7 durante una hora, observaron que el valor de pH que originaba una mayor protección frente a un tratamiento térmico posterior era de 4,5.

En otras ocasiones, la adaptación a la acidez se ha conseguido mediante el crecimiento en medios tamponados con un pH moderadamente ácido (Duffy y col., 2000) o previamente ajustados con ácido clorhídrico (Mazzotta, 2001; Tosun y Gönül, 2003). Sin embargo, la obtención de las células mediante la acidificación del medio de crecimiento con diversos ácidos orgánicos, que reflejaría de forma más real las condiciones en las que habitualmente se desarrollan en las condiciones de procesado de los alimentos, ha sido una técnica escasamente empleada, a pesar de que los ácidos orgánicos se consideran más efectivos como agentes antimicrobianos que el ácido clorhídrico. Que nosotros sepamos, tan sólo Juneja y col. (1998) y Fernández (2007) han descrito para *L. monocytogenes* y *E. faecium*, respectivamente, un incremento en la termorresistencia para las células adaptadas a la acidez tras su crecimiento en presencia de varios ácidos orgánicos.

Aunque es un hecho bien conocido que la temperatura de crecimiento afecta a la termorresistencia bacteriana que, en general, alcanza los niveles más bajos al obtener las células a bajas temperaturas, que nosotros sepamos, no se han realizado estudios acerca de la influencia que la temperatura de crecimiento ejerce sobre las respuestas de protección cruzada frente al calor inducidas en condiciones ácidas moderadas, ni se ha investigado si estas respuestas se producen en células obtenidas a temperaturas de refrigeración, a pesar del amplio uso de las mismas en las etapas previas al procesado térmico de los alimentos. Asimismo, ha recibido escasa atención el estudio del efecto ejercido por las características físico-químicas del medio de calentamiento, a pesar de que es bien conocido que el pH, la a_w, o el contenido en grasa, proteína o determinados agentes antimicrobianos del medio de tratamiento condicionan la termorresistencia bacteriana y por ello, podrían modificar la magnitud de la respuesta. En este sentido, la gran mayoría de los estudios llevados a cabo se han realizado utilizando medios de laboratorio, siendo muy escasa la información disponible en relación al desarrollo de estas respuestas adaptativas en los propios alimentos. Que sepamos, tan sólo Mazzotta (2001), trabajando con

Listeria monocytogenes, *Escherichia coli* O157:H7 y un combinado de 5 cepas de *Salmonella* spp. constituido por *S.* Typhimurium, *S.* Enteritidis, *S.* Gaminara, *S.* Rubislaw y *S.* Hartford utilizando zumos de naranja, manzana y uva ha demostrado que la composición del medio de calentamiento ejercía un gran efecto sobre la magnitud de la respuesta desarrollada, que resultó superior en los zumos de naranja y manzana.

1.5.2.- Adaptación ácida de *Salmonella* y protección cruzada frente a otros agentes estresantes

La adaptación bacteriana a condiciones ácidas subletales puede incrementar la resistencia bacteriana frente a otros tratamientos frecuentemente utilizados en la industria alimentaria. Aunque estas respuestas han recibido menos atención, algunos estudios han demostrado que la exposición de *Salmonella* a ambientes ácidos ocasiona un aumento en su posterior resistencia frente a la sal, estrés oxidativo, luz ultravioleta, cristal violeta y polimixina B (Foster y Hall, 1990; Leyer y Johnson, 1993; Lee y col., 1995; Kwon y col., 2000; Greenacre y Brocklehurst, 2006; Gabriel y Nakano, 2009).

En general, aunque las salmonelas poseen una elevada resistencia intrínseca frente al cloruro sódico (ver apartado 1.1.2.), pueden ver incrementada su supervivencia en medios de elevada salinidad tras su exposición a condiciones subletales de estrés, incluido el estrés ácido (Leyer y Johnson, 1993; Greenacre y Brocklehurst, 2006). Este hecho podría representar un serio problema para la seguridad alimentaria en algunos alimentos, como los quesos y otros productos fermentados, en los que un elevado número de condiciones estresantes, entre las que se encuentran la acidificación, la adición de sal y el descenso de la actividad del agua, se combinan con el objetivo de conseguir un nivel óptimo de inactivación microbiana. Leyer y Johnson (1993) observaron que las células de *S.* Typhimurium adaptadas a un pH de 5,8 durante un corto período de tiempo veían incrementada su supervivencia en un tampón que contenía cloruro sódico a una concentración de 2,5

M. Sin embargo, el efecto protector ejercido por la sal parece estar condicionado por el tipo de ácido utilizado para la adaptación de las células. Así, Greenacre y Brocklehurst (2006), trabajando con *S.* Typhimurium, demostraron que la adaptación celular durante 2 horas a un pH de 5,5 en presencia de ácido acético ocasionaba una respuesta de protección cruzada frente a la sal, presente en el medio de tratamiento (TSBG) a una concentración de 2,5 M, mientras que esta respuesta no se produjo cuando para la adaptación a la acidez se utilizó ácido láctico. Tampoco esta respuesta de protección cruzada fue observada por Kwon y col. (2000) al utilizar una mezcla de ácidos grasos de cadena corta.

En alimentos fermentados, debido a la síntesis de agentes oxidantes por las bacterias constituyentes de los cultivos iniciadores, y en los fagolisosomas, formando parte de la respuesta del hospedador frente a la infección, se encuentran condiciones de estrés oxidativo que también imponen una barrera frente al desarrollo microbiano. Sin embargo, también se ha comprobado que la acidez puede desencadenar una respuesta de protección cruzada frente a este tipo de estrés, aunque en los escasos trabajos realizados con *Salmonella* se han obtenido resultados contradictorios. Algunos autores al usar ácido clorhídrico (Lee y col., 1995) o mezclas de ácidos grasos de cadena corta (Kwon y col., 2000) han observado una respuesta de protección cruzada frente a un tratamiento letal con peróxido de hidrógeno. Por el contrario, en otros estudios no se ha observado ningún efecto tras la exposición de *S.* Typhimurium a ambientes ácidos moderados (Foster y Hall, 1990) o, incluso, se ha encontrado un incremento en su sensibilidad frente al estrés oxidativo tras la exposición bacteriana a la acción del ácido láctico (Greenacre y col., 2006).

Se han demostrado también respuestas de protección cruzada en *Salmonella* frente a otros agentes estresantes, como la luz ultravioleta, el cristal violeta o la polimixina B. Así, Gabriel y Nakano (2009) describieron que la resuspensión de *S.* Enteritidis en zumo de manzana incrementaba la posterior resistencia bacteriana frente a la luz ultravioleta, y Leyer y Johnson (1993), trabajando con *S.* Typhimurium, demostraron que la adaptación a la acidez incrementaba la supervivencia microbiana frente a varios agentes tensoactivos, como el cristal

violeta y la polimixina B. Sin embargo, en algunos casos la acidificación puede causar el efecto contrario, sensibilizando a las células frente a ciertos agentes estresantes de interés alimentario, hecho que podría ser aprovechado en el diseño de adecuados procesos combinados para el control microbiano. Por ejemplo, este parece ser el caso de los tratamientos de alcalinización, habiéndose demostrado en varios estudios que la adaptación a la acidez sensibiliza a las bacterias frente a condiciones alcalinas letales y viceversa (Rowbury y col., 1993, 1996; Rowbury y Hussain, 1996; Sampathkumar y col., 2004).

1.6.- Mecanismos implicados en las respuestas de tolerancia ácida y de protección cruzada

1.6.1.- Bases moleculares de la respuesta de tolerancia ácida

Aunque las respuestas microbianas frente al estrés ácido han sido extensamente investigadas en bacterias Gram negativas, especialmente en *Escherichia coli* y *Salmonella* spp. (Abee y Wouters, 1999; Foster, 2000; Audia y col., 2001; Rowbury, 2001; Stingl y col., 2002), los mecanismos involucrados en el desarrollo de estas respuestas aún no son bien conocidos. Se sabe que los microorganismos desarrollan diferentes estrategias que pueden contribuir a su supervivencia en condiciones ácidas. Estos sistemas incluyen: (a) la homeostasis del pH intracelular, (b) la síntesis de proteínas del choque ácido (Acid Shock Proteins - ASPs) y (c) la existencia de modificaciones en la composición en ácidos grasos de la membrana. En la figura 2 se muestra un esquema de los principales mecanismos de respuesta al estrés ácido en enterobacterias.

El pH intracelular bacteriano tiene una gran importancia en el metabolismo celular, al determinar la actividad de los sistemas enzimáticos y modular la cinética de transporte de nutrientes y metabolitos. Por ello, las bacterias para sobrevivir frente a descensos acusados del pH extracelular (pH_{ex}) han desarrollado ciertas

estrategias que les permiten regular su pH intracelular (pH_{in}) a través de **sistemas homeostáticos** complejos, aunque los mecanismos seguidos por microorganismos Gram positivos y Gram negativos son completamente distintos. Mientras que en los primeros se produce un descenso progresivo del pH intracelular a medida que disminuye el pH extracelular, manteniendo así un gradiente de pH (pH_{ex}-pH_{in}) constante, las bacterias Gram negativas intentan mantener un pH_{in} relativamente constante, en torno a 7,6-7,8, y, como consecuencia, sufren cambios en el gradiente de pH a medida que se modifica el pH_{ex} (Slonczwesky y col., 1981; Foster, 2000; Siegumfeldt y col., 2000). Para mantener el pH_{in} constante expulsan protones del interior de la célula a través de varios sistemas transportadores K^+/H^+ (Kroll y Booth, 1983; Booth, 1999), aunque también utilizan otros sistemas especializados, como la glutamato decarboxilasa, la lisina decarboxilasa y la arginina decarboxilasa, que desempeñan un importante papel en el mantenimiento de la homeostasis del pH intracelular. El sistema glutamato decarboxilasa, que funciona decarboxilando el ácido glutámico en ácido gamma-aminobutírico (GABA), ha sido identificado como uno de los sistemas más efectivos en *Escherichia coli*, pero parece estar ausente en *Salmonella* (Bearson y col., 1997; Foster, 2000). La lisina decarboxilasa (CadA) actúa en cooperación con un sistema transportador lisina/cadaverina (CadB) y contribuye al mantenimiento del pH_{in} y al desarrollo de una respuesta de tolerancia ácida en *Salmonella* Typhimurium (Park y col., 1996). CadA actúa decarboxilando la lisina intracelular, produciendo cadaverina y consumiendo un protón, y posteriormente, la cadaverina (carga +2) es intercambiada por lisina extracelular (carga +1) a través del transportador CadB. Como consecuencia, se produce un incremento del pH_{in}, por el consumo de un protón en el proceso, y se genera un potencial de membrana, debido a la pérdida neta de cargas positivas, lo que conducirá a la síntesis de ATP, por mediación de la F_0F_1-ATPasa. De forma análoga, la arginina decarboxilasa (AdiA) convierte la arginina intracelular en agmatina, consumiendo un protón, con el consiguiente incremento del pH_{in}. La agmatina es expulsada de la célula mediante un transportador arginina/agmatina (AdiC), generando también un potencial de membrana (Abee y Wouters, 1999). El sistema arginina decarboxilasa está bien documentado en *Escherichia coli* (Castaine-Cornet y col., 1999; Foster, 2000; Iyer y col., 2003), pero en el caso de *S.* Typhimurium, tan

sólo Kieboom y Abee (2006) han conseguido demostrar su participación en el mantenimiento de la homeostasis celular en ambientes ácidos extremos, y únicamente cuando el crecimiento microbiano tuvo lugar en anaerobiosis. Por tanto, el hecho de que *S.* Typhimurium posea varios sistemas homeostáticos basados en la decarboxilación de aminoácidos sugiere que este microorganismo podría ser capaz de sobrevivir a muy bajos valores de pH en presencia de diversos aminoácidos en el medio extracelular.

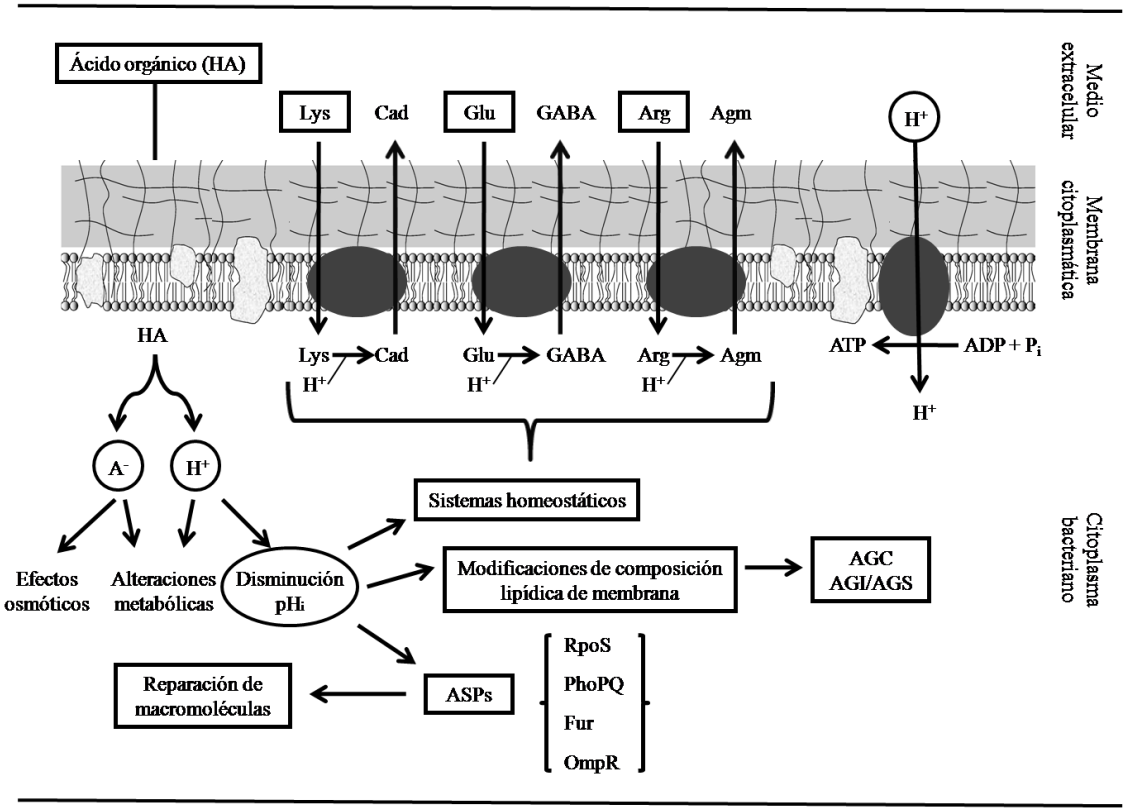

Fig. 2. Bases moleculares de la respuesta de tolerancia ácida en enterobacterias.

Las bacterias también responden a las condiciones de estrés ácido mediante la **síntesis de proteínas de choque ácido (ASPs)** que protegen a la célula frente a condiciones más extremas de acidez. *S.* Typhimurium posee dos sistemas independientes de tolerancia ácida bien diferenciados, uno se activa en fase exponencial y otro en fase estacionaria, responsables de la reparación de los daños macromoleculares ocasionados por el estrés ácido. En la Figura 3 se muestra un

esquema de los sistemas de tolerancia ácida implicados en cada fase. En la respuesta de tolerancia ácida en fase exponencial se han identificado cuatro proteínas reguladoras, el factor alternativo σ^S, codificado por el gen *rpoS*, el regulador férrico Fur y los dos componentes reguladores del sistema PhoPQ, cada una de las cuales, tras ser activada en ambientes ácidos, controla la síntesis de un grupo diferente de ASPs (Foster y Hall, 1992; Foster, 1993; Hall y Foster, 1996; Bearson y col., 1998; Fang y col., 1999), habiéndose demostrado que una mutación en cualquiera de estos reguladores previene la tolerancia ácida desarrollada en fase logarítmica, aunque no ejerce efecto alguno sobre la adquirida en fase estacionaria (Foster, 2000).

En cuanto a la respuesta de tolerancia ácida en fase estacionaria parecen estar implicados dos sistemas, uno dependiente del factor alternativo σ^S, no inducible por la acidez, y otro independiente del mismo e inducible en condiciones ácidas, en la que el regulador transcripcional OmpR se encuentra involucrado (Lee y col., 1994; Foster, 2000). La entrada de las células en la fase estacionaria activa al factor alternativo σ^S (RpoS), que actúa como un regulador central de la respuesta al estrés en bacterias Gram negativas (Foster, 2000; Dodd y Aldsworth, 2002), habiéndose demostrado que su deleción ocasiona un descenso en la supervivencia bacteriana frente a diversas condiciones de estrés y una disminución de la virulencia en *Salmonella* (Jorgensen y col., 2000; Dodd y Aldsworth, 2002; Rychlik y Barrow, 2005). En la degradación del factor alternativo σ^s, que es muy rápida en células en fase exponencial, se requiere la intervención de la ClpXP, una proteasa activada por la MviA, chaperona que actúa como un sensor de las perturbaciones de la fisiología celular, interactuando con el factor alternativo σ^s, probablemente en el residuo 173, favoreciendo su reconocimiento por la proteasa ClpXP. Tras un choque ácido, la fosforilación del residuo 58 (aspartato) de la MviA provoca una reducción de la capacidad degradativa, produciéndose un acúmulo del factor alternativo σ^s (Foster, 2000).

El descenso del pH intracelular afecta negativamente a diversas macromoléculas, como el ADN o proteínas citoplasmáticas, en cuya reparación participan las ASPs, sintetizadas tras la activación de ciertos reguladores transcripcionales. Estas proteínas de estrés son principalmente chaperonas y

proteasas. Las chaperonas, entre las que destacan DnaK y GroEL, forman parte de la maquinaria celular de plegamiento, transporte, reparación y degradación de proteínas, participando en la reparación de las proteínas desnaturalizadas y en la evacuación de las dañadas (Zou y col., 1998; Lorca y col., 2002; Frees y col., 2003; Takaya y col., 2004). En cuanto a la reparación del ADN celular, ésta es llevada a cabo por diversas proteasas (RecA, Smn y Uvr), que se encargan de la eliminación de errores o del restablecimiento de la horquilla de replicación (Sancar, 1996; Cox y col., 2000; Jeong y col., 2008). Asimismo, el regulador transcripcional Ada, con actividad ADN metil-transferasa, también ha sido identificado recientemente como uno de los componentes de la respuesta bacteriana frente al estrés ácido en *S.* Typhimurium, colaborando en la reparación de daños en el ADN (Bearson y col., 1998; Foster, 2000).

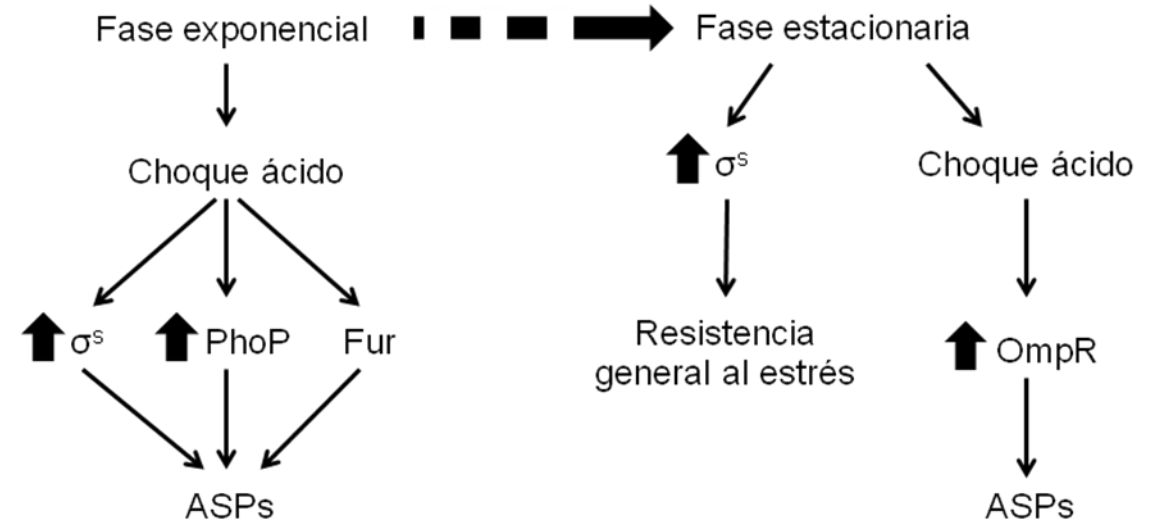

Fig. 3. Sistemas de tolerancia ácida en fase exponencial y fase estacionaria de *S.* Typhimurium (adaptada de Foster, 2000).

La membrana celular es una de las principales defensas con las que cuentan los microorganismos en su lucha contra los cambios ambientales, siendo numerosas

las investigaciones en las que se han descrito **modificaciones en la composición en ácidos grasos y en la fluidez de la membrana bacteriana** tras su exposición a diversas condiciones de estrés, incluyendo la acidificación (Russell y col., 1995; Brown y col., 1997; de Jonge y col., 2003a; Wang y col., 2005). Debido a sus diferentes puntos de fusión, generalmente superiores para los ácidos grasos saturados (AGS) que para los insaturados (AGI), un aumento en la relación AGI/AGS se asocia con un incremento en la fluidez de membrana y, en muchos microorganismos, se ha comprobado una disminución en dicha relación a medida que lo hace el pH del medio de cultivo, lo que se traduce en un incremento en la rigidez de la membrana (Brown y col., 1997). Como consecuencia, pueden producirse cambios en el flujo de protones, de tal forma que en las células adaptadas a la acidez la membrana será menos permeable frente a los mismos. Además, también se ha comprobado que durante la adaptación a la acidez se induce la síntesis de ácidos grasos cíclicos derivados del ciclopropano (AGC), lo que constituye un factor decisivo en la protección celular frente a diversos agentes estresantes (Brown y col., 1997; Chang y Cronan, 1999). La síntesis de AGC es considerada una modificación post-sintética de la bicapa lipídica de la membrana celular, que tiene lugar principalmente cuando se produce la entrada en la fase estacionaria de crecimiento por medio de la acción de la AGC-sintasa, que cataliza la adición de un grupo metileno (proveniente de la S-adenosil-L-metionina) a un doble enlace de la fracción insaturada de los fosfolípidos de membrana (Brown y col., 1997; Grogan y Cronan, 1997). La conversión de los ácidos grasos insaturados (AGI) de la membrana bacteriana en sus correspondientes derivados cíclicos (AGC) se cree que puede proteger frente a condiciones ácidas extremas, tratamientos térmicos y otros agentes estresantes (Brown y col., 1997; Kim y col., 2005). Sin embargo, la contribución de los AGC a las propiedades físicas de la membrana bacteriana aún no ha sido discernida en su totalidad, sobre todo en lo que se refiere a los cambios en la fluidez de membrana en respuesta a estímulos ambientales, aunque algunos estudios han sugerido que el incremento en el contenido en AGC puede ocasionar un aumento en la estabilidad de las propiedades estructurales y dinámicas de la membrana (Duforc y col., 1984), y un descenso de la fluidez de la misma (Yatvin y col., 1986; Magnuson y col., 1993; Annous y col., 1999).

La importancia de los cambios en la composición en ácidos grasos de la membrana celular y de la síntesis de AGC en la respuesta bacteriana de tolerancia ácida ha sido ampliamente estudiada en *Escherichia coli* (Brown y col., 1997; Chang y Cronan, 1999; de Jonge y col., 2003b). Sin embargo, hemos encontrado muy pocas referencias para *S.* Typhimurium, aunque parece probable que la síntesis de ácidos grasos cíclicos juegue un papel importante en el desarrollo de respuestas de tolerancia ácida en este microorganismo (de Jonge y col., 2003a; Kim y col., 2005). En concreto, de Jonge y col. (2003a) han asociado el incremento en el contenido de AGC en células obtenidas en fase estacionaria con el desarrollo de una respuesta adaptativa frente al estrés ácido en este microorganismo, y Kim y col. (2005) demostraron que mutantes de *S.* Typhimurium en el gen *cfa,* que carecen de la actividad AGC-sintasa, mostraban una mayor sensibilidad frente a tratamientos ácidos de inactivación, indicando la importancia de este tipo de ácidos grasos en la respuesta frente al estrés ácido en *Salmonella* spp.

1.6.2.- Mecanismos involucrados en las respuestas de protección cruzada

El importante desarrollo en los últimos años de técnicas de biología molecular que permiten conocer el perfil completo de expresión génica bacteriana bajo diferentes condiciones ambientales ha posibilitado profundizar en el conocimiento de los mecanismos de inactivación por medio de diferentes agentes estresantes, y de las estrategias microbianas de respuesta al estrés.

En las últimas décadas se han realizado importantes avances en el conocimiento de las bases moleculares de las respuestas bacterianas frente a diversos agentes estresantes, pero aún no se ha conseguido esclarecer los mecanismos responsables de las respuestas de protección cruzada, y en concreto, de aquellas inducidas tras la exposición a condiciones ácidas subletales.

En lo que respecta a la protección cruzada frente al calor, gran número de estudios han descrito que tras un tratamiento térmico se produce un aumento rápido

de la síntesis de una serie de proteínas, conocidas como proteínas de choque térmico (denominadas con su acrónimo en inglés HSPs), que juegan un importante papel en la adaptación y supervivencia de las células frente a los cambios ambientales y metabólicos (Bukau, 1993; Georgopoulos y col., 1994; Yura y col., 2000). El calor produce alteraciones en la estructura terciaria de las proteínas citoplasmáticas, y las HSPs, en su mayoría chaperonas y proteasas (GroEL, DnaK, DnaJ, GrpE, recA,…), colaboran en el plegamiento, ensamblaje y transporte proteico, así como en la degradación y eliminación de proteínas desnaturalizadas. Sin embargo, el estrés térmico no es el único agente inductor de la síntesis de HSPs, sino que otras condiciones adversas, como bajas temperaturas, irradiación, cambios en la presión osmótica, presencia de etanol y metales pesados, estrés ácido y estrés oxidativo, incrementan la expresión de varias HSPs (Bukau, 1993; Yura, 1993; Georgopoulos y col., 1994; Gross, 1996), hecho que podría constituir la base de las respuestas bacterianas de protección cruzada frente al calor. Además, la síntesis de la mayoría de las HSPs se encuentra regulada por el factor alternativo σ^S y otros reguladores transcripcionales que han sido previamente identificados como componentes de la respuesta bacteriana de tolerancia ácida (Abee y Wouters, 1999; Foster, 2000; Dong y col., 2008). De todos modos, es muy probable que otros mecanismos activados durante la adaptación a la acidez, como las modificaciones en la composición en ácidos grasos de la membrana celular, se encuentren también involucrados en la subsecuente respuesta de protección cruzada frente al calor. Numerosos estudios han demostrado que las condiciones de cultivo y en especial la temperatura de crecimiento (Suutari y Laakso, 1994; Russell y col., 1995; Kadner, 1996; Annous y col., 1997, 1999; Casadei y col., 2002; Wang y col., 2005), el pH (Russell y col., 1995; Brown y col., 1997; de Jonge y col., 2003a; Sampathkumar y col., 2004; Wang y col., 2005), la concentración de etanol (Teixeira y col., 2002) y la osmolaridad del medio (Guillot y col., 2000), así como la entrada en la fase estacionaria de crecimiento (Russell y col., 1995; Kadner, 1996; Casadei y col., 2002), modifican la composición en ácidos grasos de las membranas bacterianas. Sin embargo, se han llevado a cabo escasas investigaciones encaminadas a clarificar la importancia de estas modificaciones de membrana en las respuestas bacterianas de protección cruzada frente a tratamientos térmicos (que nosotros sepamos, ninguna

para *S.* Typhimurium y *S.* Senftenberg). Tan sólo Annous y col. (1999) y Sampathkumar y col. (2004) han descrito un incremento en la termorresistencia bacteriana en aquellas condiciones de crecimiento que daban lugar a una disminución de la fluidez de membrana. Annous y col. (1999), trabajando con *Pediococcus* spp., demostraron que el crecimiento bacteriano a bajas temperaturas daba lugar a un incremento en el contenido de la membrana celular en ácidos grasos insaturados, y, por ello, a un aumento en la fluidez de la membrana, que fue responsabilizado de la menor termorresistencia observada en esas condiciones. Sampathkumar y col. (2004) observaron que las células de *S.* Enteritidis pre-expuestas a condiciones alcalinas mostraban una mayor resistencia térmica, que fue asociada a un descenso en la fluidez de la membrana bacteriana ocasionado por un incremento en la proporción de ácidos grasos saturados de la misma.

La respuesta microbiana frente al estrés osmótico es asimismo muy compleja. Parece que los microorganismos acumulan iones y ciertos compuestos osmoprotectores, que penetran al interior de las células por mediación de ciertas porinas de membrana (OmpC y OmpF), cuyo nivel de expresión varía en respuesta a diferentes agentes estresantes, entre los que destaca la acidificación (Foster y Hall, 1990; Leyer y Johnson, 1993; Bremer y Krämer, 2000), habiéndose observado que las células de *S.* Typhimurium expuestas a ambientes ácidos moderados muestran un marcado incremento en la expresión de la porina OmpC, hecho que podría explicar la aparición de fenómenos de protección cruzada. Los agentes oxidativos actúan formando radicales libres que causan daños en el ADN y en las proteínas citoplasmáticas (Storz y Zheng, 2000), y para evitarlos se produce la síntesis de varias enzimas protectoras: catalasas, alkil hidroperoxidasas, glutatión reductasas y la proteína Dps, la mayoría de las cuales forman parte del regulon *oxyR* (Dukan y Touati, 1996; Storz y Zheng, 2000), o bien tiene lugar un descenso en la permeabilidad de la membrana celular frente a los agentes oxidantes que penetrarán con menor facilidad en el citoplasma, ejerciendo un menor efecto inhibitorio.

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2.

JUSTIFICACIÓN Y OBJETIVOS

2.- JUSTIFICACIÓN Y OBJETIVOS

La salmonelosis supone un grave problema para la Salud Pública y, en contra de lo que cabría esperar por la continua mejora de los procedimientos de elaboración y conservación de los alimentos así como de los sistemas preventivos de control, su incidencia aumenta cada año de forma alarmante. En este sentido, cabe señalar que nos encontramos en un momento crucial, dado que se están produciendo cambios en las tendencias y hábitos de consumo que han llevado al desarrollo de nuevas tecnologías de procesado y conservación de los alimentos. Los consumidores, cada día más exigentes, demandan productos alimenticios libres de aditivos, más frescos y con sabores más naturales, por lo que los procesos térmicos utilizados tradicionalmente se han ido sustituyendo por otros de menor intensidad o incluso, en aquellos casos en los que es posible, se han eliminado. Ello ha dado lugar a la presencia en el mercado de toda una gama de alimentos mínimamente procesados, que ha propiciado un incremento en la aparición de episodios de enfermedades transmitidas por alimentos, haciéndose necesario el diseño de estrategias encaminadas a reducir los riesgos que plantea su consumo.

Uno de los métodos más comúnmente empleado por la industria alimentaria para controlar el crecimiento de microorganismos patógenos es la acidificación mediante la adición de ácidos orgánicos. Sin embargo, los microorganismos expuestos a condiciones ácidas pueden desarrollar respuestas adaptativas que se manifiestan en un aumento de su resistencia frente a la acidez y otras condiciones adversas, lo que puede ocasionar su supervivencia tras la aplicación de las subsecuentes barreras comúnmente utilizadas en la práctica industrial.

Por todo ello, resulta importante comprobar si el crecimiento microbiano en condiciones ácidas moderadas puede inducir respuestas de tolerancia ácida y de protección cruzada frente a diversos agentes estresantes de interés en la industria alimentaria, así como conocer los mecanismos moleculares involucrados en el desarrollo de dichas respuestas adaptativas, con la finalidad de obtener información que nos permita una selección y combinación inteligente de los procesos

combinados en base a sus dianas celulares de actuación, con el fin de conseguir un mayor efecto antimicrobiano.

Esta Tesis Doctoral se ha centrado en determinar la respuesta adaptativa de dos serovariedades del género *Salmonella* de gran interés tecnológico y sanitario, *S.* Typhimurium y *S.* Senftenberg, frente al estrés ácido originado tras el crecimiento a diferentes valores de pH y en presencia de diferentes ácidos orgánicos (acético, ascórbico, cítrico, láctico y málico) y ácido clorhídrico. *S.* Typhimurium resulta especialmente peligrosa por su elevada resistencia frente a diversos agentes antimicrobianos, estando implicada como agente etiológico en múltiples casos de toxiinfección alimentaria por consumo de leche cruda y pasteurizada, tarta de queso, huevos crudos y cocidos, helados, carne de ave y jamón, aunque las fuentes de contagio más habituales son las carnes contaminadas, y, en particular, embutidos y hamburguesas. *S.* Senftenberg está cobrando en estos últimos años un gran interés como agente causante de toxiinfecciones alimentarias, siendo frecuente su aislamiento a partir de alimentos de origen animal, y de ambientes marinos y pescados. A la creciente importancia sanitaria de *S.* Senftenberg habría que añadir su interés tecnológico dado que, por su elevada termorresistencia en relación con la que presentan el resto de serovariedades del género *Salmonella*, es un buen modelo biológico para el estudio del comportamiento de las especies de este género frente a los tratamientos térmicos. Se ha estudiado la influencia que diversos parámetros ambientales ejercen sobre el desarrollo de repuestas de adaptación a la acidez, determinando la cinética de inactivación de las células adaptadas a la acidez tras ser sometidas a tratamientos térmicos y a condiciones ácidas extremas en medios de laboratorio y algunos alimentos como extracto de carne y zumos de frutas, con el fin de comprobar la aparición de respuestas de tolerancia ácida y de protección cruzada frente al calor, que no sólo pueden prolongar la supervivencia microbiana en varios alimentos ácidos, sino que también pueden incrementar la resistencia bacteriana a las condiciones ácidas del tracto gastrointestinal, disminuyendo la dosis infectiva e incrementando el riesgo de contraer la enfermedad. Asimismo, se ha determinado la influencia que la adaptación a la acidez ejerce sobre el comportamiento de

Salmonella frente a otros tratamientos habitualmente empleados por la industria alimentaria, utilización de sal, alcalinización y estrés oxidativo.

La correcta aplicación de la tecnología de los procesos combinados requiere un conocimiento profundo de los mecanismos implicados en las respuestas bacterianas frente a diversos agentes estresantes, y, aunque en los últimos años se ha avanzado mucho en este sentido, aún no se conocen en su totalidad las bases moleculares de la respuesta bacteriana al estrés ácido. En esta Tesis Doctoral se ha estudiado la implicación de las modificaciones de la composición lipídica de la membrana celular y de algunos sistemas homeostáticos, como la arginina decarboxilasa, la lisina decarboxilasa y la glutamato decarboxilasa en el desarrollo de respuestas de tolerancia ácida y de protección cruzada frente al calor en *S.* Typhimurium y *S.* Senftenberg.

En concreto, los objetivos específicos planteados en el desarrollo de esta tesis fueron los siguientes:

1.- Determinar el efecto inhibitorio de diferentes ácidos (acético, ascórbico, cítrico, láctico, málico y clorhídrico) y la influencia ejercida por el pH, la temperatura y la composición del medio de cultivo sobre el crecimiento de *S.* Typhimurium y *S.* Senftenberg.

2.- Comprobar si las células crecidas en un ambiente moderadamente ácido, hasta un pH de 4,5, desarrollan respuestas adaptativas de tolerancia ácida y de protección cruzada frente al calor y evaluar la efectividad de los ácidos orgánicos en la inducción de las mismas.

3.- Examinar si estas respuestas se expresan en todo el intervalo de temperaturas de crecimiento y el efecto ejercido por la aplicación secuencial de la refrigeración.

4.- Comprobar la capacidad de supervivencia de las células adaptadas a la acidez frente a un pH extremo y al calor en matrices alimentarias (extracto de carne y zumos de naranja y manzana).

5.- Determinar la importancia de los sistemas arginina, lisina y glutamato decarboxilasa como mecanismos homeostáticos implicados en la respuesta de tolerancia ácida.

6.- Evaluar la influencia del pH y de la temperatura de crecimiento sobre las modificaciones en la composición en ácidos grasos de la membrana celular y valorar sus repercusiones sobre la subsecuente resistencia a un pH extremo y al calor.

7.- Determinar si las células adaptadas a la acidez son capaces de desarrollar respuestas de protección cruzada frente a otros agentes estresantes (cloruro sódico, alcalinización y estrés oxidativo).

3.

Comparison of acids on the induction of an Acid Tolerance Response in Salmonella Typhimurium, consequences for food safety

Avelino Álvarez-Ordóñez, Ana Fernández, Ana Bernardo and Mercedes López

Meat Science, 2009, 81, 65-70

ABSTRACT

Salmonella Typhimurium inactivation at pH 3.0 in Brain Heart Infusion (BHI) and Meat Extract (ME) was studied using stationary-phase cells grown in non-acidified BHI (pH 7.4) and ME (pH 6.6), and acidified BHI and ME at pH values of 6.4, 5.4 and 4.5 with acetic, ascorbic, citric, lactic, malic and hydrochloric acids. Cells grown in buffered BHI (pH 7.0) were used as non-acid adapted control cells. Acid adapted *S.* Typhimurium cells obtained in both media (BHI and ME) were more resistant to extremely acidic conditions when ME was used as challenge medium, although the ability of *S.* Typhimurium to survive extreme pH conditions also depended on growth medium and type of acidulant used. Acid adapted cells grown in BHI developed a higher Acid Tolerance Response (ATR) than those grown in ME. When cells were grown in acidified BHI, no bacterial inactivation was observed after three hours of acid challenge in ME. Furthermore, when cells were grown in acidified ME at pH values of 6.4 and 5.4, D-values obtained using ME as challenge medium were, respectively, 6–9 and 10–15 fold higher than those found when BHI was used as challenge medium. In all cases, the order of acids in inducing the ATR was citric > acetic > lactic > malic ≥ hydrochloric > ascorbic. These findings represent a concern for food safety as the increase in the acid resistance of acid adapted cells could allow for *S.* Typhimurium survival in the strong acidic environment of the gastrointestinal tract.

INTRODUCTION

Salmonella spp. are the cause of foodborne illness involving several products of animal origin. The retail ground meat and meat products are contaminated with the bacteria mainly due to their ubiquity in the natural environment and routine slaughter practices which increases the level of bacterial contamination by up to 20% (Forsythe and Hayes, 1998). In order to control the growth of microorganisms in carcasses and fresh meat, several decontamination techniques have been proposed, which include spraying with diluted solutions of organic acids, mainly acetic and lactic acids. These treatments, which are routinely used in US slaughtering plants, have not been allowed until now by the European Union meat hygiene regulations (del Río et al., 2007). The immediate effects of these decontamination techniques are 1- to 3-log reductions in meat surface contaminants (Dorsa et al., 1997; Smulders and Greer, 1998; Sofos and Smith, 1998), but their long-term residual effects are not completely understood. The use of organic acids must be considered with some degree of caution, since it causes a decrease in the pH value of the meat to final values ranging from 3.3 to 5.8 as a function of the type of organic acid used, the treatment time, the acid concentration and the combination with other decontamination techniques (Kanellos and Burriel, 2005; Mehyar et al., 2005; del Río et al., 2007) which may induce an acid adaptive response which frequently results in enhanced resistance to more extreme stresses, including lethal acidity, phenomenon which has been termed Acid Tolerance Response (ATR) (Foster and Hall, 1991; Hill et al., 1995). An important consequence of this bacterial response is the fact that acid adapted cells could be more resistant to the strong acidic environment found in the gastrointestinal tract, increasing the risk of foodborne human salmonellosis (Koo et al., 2002). Furthermore, the acidification naturally occurring in the meat during muscle to meat conversion or the decline of pH with the passing of time in fermented meat products could produce moderate acidic conditions which may also give way to an ATR, a concern for the food industry.

ATR has been reported in various foodborne pathogens, such as *Listeria monocytogenes* (Kroll and Patchett, 1992; Hill et al., 1995; Gahan and Hill, 1999), *Escherichia coli* O157:H7 (Garren et al., 1997; Buchanan and Edelson, 1999; Leenanon and Drake, 2001) and *Salmonella* spp. (Foster and Hall, 1990; Leyer and Johnson, 1992; Lee et al., 1994; Bearson et al., 1996, 1998; Bacon et al., 2003; Greenacre et al., 2003; Yuk and Schneider, 2006). These studies have been carried out growing the cells in media slightly acidified, generally supplemented with glucose or using cells exposed to a mildly acidic environment for several times. In the particular case of *Salmonella* spp. studies carried out to clarify the effect of organic acids on the ATR are scarce (Greenacre et al., 2003; Yuk and Schneider, 2006). It has been shown that the type of organic acid used influenced the ATR observed. Greenacre et al. (2003), using TSB acidified with acetic or lactic acid, found that acetic acid was more efficient than lactic acid for the induction of an ATR in *S.* Typhimurium. Yuk and Schneider (2006) evaluating the ATR of five *Salmonella* serovars adapted in TSB, containing 1.25% glucose, and several fruit systems (apple, orange and tomato juices) showed that cells adapted in apple and tomato juices, with a predominant content of malic acid, showed increased acid resistance compared to those adapted in orange juice, with a predominant content of citric acid. However, it is important to note that in these studies the acid adaptation was achieved by exposure of the bacterial cultures to the acidic environment for short time periods, and to date there is no information on the effect of acid adaptation by means of bacterial growth in media acidified with organic acids or in acidified food environments on the subsequent ATR of *Salmonella* spp., although it has been pointed out that this response could be influenced by the methodology used to obtain acid adapted cells (Cheng et al., 2003; Greenacre et al., 2003; Yuk and Schneider, 2006).

In this study, the kinetic inactivation of *S.* Typhimurium (CECT 443) at pH 3.0 in meat based media, BHI (Brain Heart Infusion) and ME (Meat Extract), was evaluated after its growth in different media (BHI and ME) acidified up to pH 4.5 with different organic acids (acetic, ascorbic, citric, lactic, malic) and hydrochloric

acid in order to clarify the possible consequences of acidification of meat during decontamination treatments on the acid resistance of this pathogenic bacterium.

MATERIAL AND METHODS

Bacterial strain, media and culture conditions

Salmonella enterica serovar Typhimurium strain (CECT 443) used in this study was obtained from Colección Española de Cultivos Tipo (CECT) (Spanish Type Culture Collection). Brain Heart Infusion (BHI, Oxoid) and Meat Extract (ME) were used as growth and challenge media. BHI agar (BHIA) was used as recovery medium. To prepare the ME, pork was purchased, cut and trimmed. Afterwards, 375 g of meat was placed in glass bottles and 750 mL of distilled water added. The mixture was treated at 121 ºC for 15 min, filtered through a sterile cloth and sterilized by autoclaving (121 ºC, 15 min). After preparation, extract was stored frozen (-18 ± 2 ºC) prior to use.

The lyophilized cultures were revived in BHI and incubated for 24 h at 37 ºC. Pure cultures were maintained on BHIA plates at 4 ºC. Subcultures were prepared by transferring an isolated colony from a plate into a test tube containing 10 mL of sterile BHI followed by incubation at 37 ºC for 24 h. These fresh subcultures were used to produce acid adapted and non-acid adapted cells.

Preparation of acid adapted cells

Flasks containing 50 mL of sterile BHI (pH 7.4) and ME (pH 6.6) non-acidified and acidified at pH values of 6.4, 5.4 and 4.5 with several acids (acetic (Prolab), ascorbic (Merck), citric (Sigma), lactic (Merck), malic (Scharlau) and hydrochloric acids (Panreac)) were inoculated with the subculture to a final concentration of 10^3 cells/mL. These pH values were chosen since they are representative of the slightly acidic environment that organisms may find in many foods. To obtain non-acid adapted control samples, buffered BHI adjusted to pH 7.0

by addition of Sorensen buffer 0.2 M (bisodium (Merck)-monopotassium (Panreac) phosphate) was used.

These cultures were then incubated at 37 ºC for 24 h, except those grown in BHI adjusted to pH 4.5, which were incubated for 36 h. This resulted in cultures that were in late stationary-phase of growth. Initial and final pH values of each culture condition are shown in Table 2.

Growth curves and calculation of growth parameters

Samples (1 mL) of the cultures obtained at different assayed conditions were decimally diluted in sterile 0.1% (w/v) peptone solution (Oxoid) and appropriate dilutions were plated in duplicate on BHIA. Plates were incubated at 37 ºC for 48 h, and the number of colonies enumerated. Viable counts were converted to $\log_{10}$ values. Growth curves generated by fitting the data to the Gompertz equation (Buchanan et al., 1997) were used to calculate lag phase duration, exponential growth rate, generation time and maximum population density, as well as the time needed to reach the stationary-phase.

Assessment of acid tolerance

Aliquots of 5 mL of cells adapted to each combination pH/acid and non-adapted cells were harvested by centrifugation at 8000 g for 5 min at 4 ºC (Eppendorf centrifuge 5804R). The supernatant liquid was discarded and cells were resuspended in 50 mL of BHI or ME adjusted to a pH value of 3.0 with HCl, incubated at room temperature and survival was monitored periodically for up to 3 h. Ten-fold serial dilutions were produced in sterile 0.1% (w/v) peptone solution and suitable dilutions were plated in duplicate on BHIA. Viable cell densities at each point in time were enumerated following incubation of the plates at 37 ºC for 48 h (longer incubation times did not have any influence on the count). Survivors were counted with a modified Image Analyser Automatic Counter (Protos Analytical Measuring Systems, Cambridge, UK) as described by Ibarz et al. (1991). Three different freshly grown cultures of *S.* Typhimurium were used for each experimental run.

Survivor curves and statistical analysis

D-values (min) were determined by plotting the log number of survivors against time for each culture. The line that best fits survivor plots was determined by linear regression (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA), and the negative reciprocal of the slope was used for the D-value.

D-values were compared using Student's t-test (Steel and Torrie, 1986) (Statistica for Windows version 4.5, Statsoft, Inc, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Salmonella Typhimurium inactivation at pH 3.0 in BHI and ME was studied using stationary-phase cells. Viable counts of *S.* Typhimurium were monitored during its growth in buffered BHI (pH 7.0), non-acidified BHI (pH 7.4) and ME (pH 6.6) and acidified BHI and ME up to pH 4.5 with different organic acids (acetic, ascorbic, citric, lactic and malic) and hydrochloric acid. The estimations of the growth parameters of interest are shown in Table 1. An example of growth curves obtained in non-acidified ME and acidified ME at pH values of 6.4, 5.4 and 4.5 with all the acids tested is shown in Fig. 1. It is noteworthy that the lowest pH value (4.5) did not support the growth of this pathogenic microorganism in ME, whereas in BHI only acetic and lactic acids were effective in inhibiting growth. At higher pH values a similar growth pattern was observed in both media, although this microorganism grew faster and attained a higher maximum population in BHI than in ME, especially at pH 5.4. The fact that *S.* Typhimurium growth was inhibited at pH 4.5 in meat extract is not enough to state that decontamination treatments using organic acids are effective in preventing food poisoning. The pH values of treated carcasses described in the literature are quite heterogeneous, ranging from 3.3 to 5.8 (Kanellos and Burriel, 2005; Mehyar et al., 2005; del Río et al., 2007). This could be due to the influence of different factors, such as the type of acid, treatment time, acid concentration and combination with other decontamination techniques. Furthermore,

Table 1. Growth parameters, as mean value ± standard deviation, obtained by fitting the data to the Gompertz equation for cells of *S.* Typhimurium (CECT 443) in the different conditions which supported the bacterial growth.

Growth conditions			Growth parameters			
Growth media	**Acid**	**Initial pH**	**MPD**	**GT**	**LPD**	**TSP**
BUFFERED BHI	-	7.0	9.1±0.3	0.32±0.02	0.80±0.07	10.0±0.5
BHI	-	7.4	9.2±0.4	0.31±0.04	0.78±0.09	10.0±0.5
	ACETIC	6.4	8.7±0.3	0.58±0.04	0.83±0.06	12.0±1.0
	ASCORBIC	6.4	8.6±0.5	0.35±0.05	1.21±0.10	10.0±0.5
	CITRIC	6.4	8.8±0.4	0.36±0.03	1.37±0.11	10.0±0.5
	LACTIC	6.4	8.5±0.3	0.32±0.03	1.19±0.12	10.0±0.5
	MALIC	6.4	8.8±0.5	0.34±0.02	1.03±0.08	10.0±0.5
	HCl	6.4	8.6±0.2	0.32±0.05	0.94±0.06	10.0±0.5
	ASCORBIC	5.4	8.3±0.2	0.49±0.04	1.13±0.12	12.0±1.0
	CITRIC	5.4	8.6±0.4	0.54±0.05	0.97±0.07	12.0±0.5
	LACTIC	5.4	8.3±0.4	0.48±0.03	0.99±0.08	12.0±1.0
	MALIC	5.4	8.5±0.2	0.46±0.04	1.82±0.15	12.0±1.0
	HCl	5.4	8.4±0.3	0.42±0.04	0.91±0.06	10.0±0.5
	ASCORBIC	4.5	7.6±0.5	1.14±0.09	4.12±0.25	24.0±1.5
	CITRIC	4.5	8.1±0.4	0.75±0.10	6.13±0.43	22.0±2.0
	MALIC	4.5	8.1±0.6	0.64±0.08	3.99±0.28	18.0±1.5
	HCl	4.5	8.4±0.4	0.52±0.05	3.25±0.31	14.0±1.5
MEAT EXTRACT	-	6.6	8.8±0.5	0.41±0.05	1.53±0.11	10.0±0.5
	ACETIC	6.4	8.3±0.4	0.43±0.04	1.95±0.14	11.0±1.0
	ASCORBIC	6.4	8.6±0.3	0.53±0.04	1.59±0.13	11.0±1.0
	CITRIC	6.4	8.7±0.5	0.43±0.03	1.48±0.14	10.0±0.5
	LACTIC	6.4	8.7±0.5	0.41±0.04	1.48±0.16	10.0±0.5
	MALIC	6.4	8.7±0.3	0.39±0.05	2.15±0.18	10.0±0.5
	HCl	6.4	8.7±0.4	0.42±0.04	1.39±0.10	10.0±0.5
	ASCORBIC	5.4	7.2±0.3	0.61±0.07	2.54±0.22	15.0±1.0
	CITRIC	5.4	7.6±0.4	0.69±0.06	2.04±0.17	14.0±1.5
	LACTIC	5.4	7.5±0.2	0.61±0.05	2.26±0.19	15.0±1.5
	MALIC	5.4	7.9±0.5	0.62±0.07	2.05±0.21	14.0±1.0
	HCl	5.4	7.8±0.4	0.53±0.05	1.91±0.16	13.0±1.0

MPD: máximum population density (log cfu/mL)
GT: generation time (h)
LPD: lag phase duration (h)
TSP: time needed to reach the stationary-phase (h)

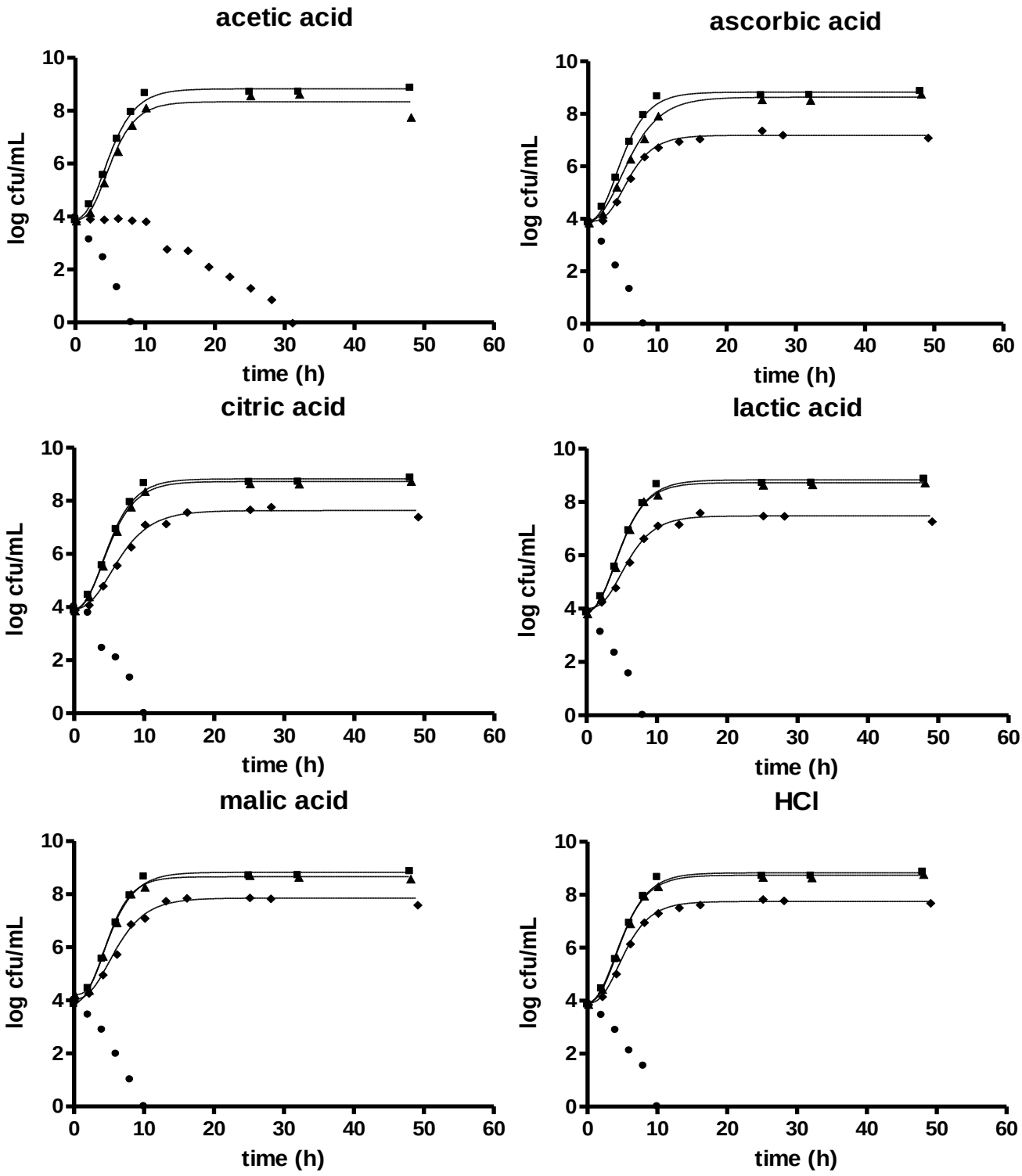

Fig. 1. Growth curves of *Salmonella* Typhimurium (CECT 443) in non-acidified ME (■) and ME acidified at pH values of 6.4 (▲), 5.4 (♦) and 4.5 (●) with different acids (one of three trials). The symbols represent the experimental data obtained, while the lines were the result of the adjustment of the data through the Gompertz equation.

several studies have previously reported that the pH of treated carcasses is usually increased during storage, due to the buffering capacity of the meat tissue, which could restore the pH at the meat surface (Mehyar et al., 2005; del Río et al., 2007). Under these acidic conditions, a contamination with *S.* Typhimurium cells could occur, and they could survive and develop an ATR resulting in a higher acid resistance.

D-values obtained under all conditions tested are reported as the mean of three independent experiments ± the standard deviation (Table 2). A representative example of survival curves obtained when BHI (pH 3.0) was used as challenge medium is shown in Fig. 2. The data fitted properly into a first order kinetic. The goodness of fit was determined by both visual inspection and R^2 value (between 0.94 and 0.99; data not shown).

It can be seen that both, the composition of growth and challenge medium, as well as the type of acidulant used had a marked effect on the resistance of *S.* Typhimurium to a lethal pH.

In all cases, the use of ME as challenge medium protected *S.* Typhimurium against acid inactivation. No bacterial inactivation was observed for cells grown in acidified BHI after three hours of acid challenge in ME. Cells grown in buffered BHI and non-acidified BHI displayed an increase in acid tolerance, showing D-values about five-fold higher than those obtained when cells were challenged in BHI. This effect was more pronounced for cells grown in ME (non acidified and acidified), with D-values around 6-15 fold higher. These results clearly demonstrate that the use of a food source for the acid challenge could provide a protection against low pH. Although it is unknown how food can protect bacteria from extreme acidic conditions, it has been reported that the presence of amino acids in the acid challenge media could play a significant role (Lin et al., 1995; Waterman and Small, 1996). Furthermore, Waterman and Small (1998) found that several acid sensitive enteric pathogens survived better in extreme pH conditions when the microorganisms were inoculated onto ground beef or boiled egg white, postulating that the protein content of foods may protect bacteria against the killing effects of

Table 2. D-values (min) for acid challenged cells of *S.* Typhimurium (CECT 443) grown in buffered BHI, non-acidified BHI and Meat Extract and acidified BHI and Meat Extract at different pH values with several acids.

Acid adaptation conditions				D-values (min)	
				Acid challenge media pH 3.0	
Growth media	Acid	Initial pH	Stationary-phase pH	BHI	Meat extract
BUFFERED BHI	-	7.0	7.0	9.57 ± 1.70^{a}	42.11 ± 6.46^{a}
BHI	-	7.4	6.25	25.39 ± 3.74^{b}	121.85 ± 9.30^{b}
	ACETIC	6.4	5.37	56.37 ± 6.51^{cd}	NI
	ASCORBIC	6.4	5.37	25.72 ± 3.27^{b}	NI
	CITRIC	6.4	5.34	63.97 ± 6.82^{ce}	NI
	LACTIC	6.4	5.33	41.97 ± 3.60^{f}	NI
	MALIC	6.4	5.59	37.76 ± 5.60^{fg}	NI
	HCl	6.4	5.20	32.53 ± 4.57^{bg}	NI
	ASCORBIC	5.4	4.93	26.30 ± 2.63^{b}	NI
	CITRIC	5.4	5.01	76.76 ± 9.09^{e}	NI
	LACTIC	5.4	4.91	56.58 ± 4.47^{c}	NI
	MALIC	5.4	5.10	43.61 ± 4.79^{df}	NI
	HCl	5.4	4.80	43.00 ± 2.46^{f}	NI
	ASCORBIC	4.5	4.61	26.23 ± 2.76^{b}	NI
	CITRIC	4.5	4.76	78.47 ± 11.03^{e}	NI
	MALIC	4.5	4.76	42.64 ± 2.82^{f}	NI
	HCl	4.5	4.61	43.47 ± 6.72^{dfg}	NI
MEAT EXTRACT	-	6.6	6.15	10.58 ± 0.98^{a}	73.66 ± 4.09^{cd}
	ACETIC	6.4	5.94	11.72 ± 2.09^{a}	89.54 ± 9.16^{ce}
	ASCORBIC	6.4	5.85	9.05 ± 1.06^{a}	72.79 ± 5.87^{cdf}
	CITRIC	6.4	5.91	9.61 ± 0.90^{a}	89.43 ± 7.92^{e}
	LACTIC	6.4	5.98	9.05 ± 0.92^{a}	63.30 ± 7.92^{df}
	MALIC	6.4	6.00	9.99 ± 0.68^{a}	62.61 ± 5.10^{f}
	HCl	6.4	5.90	8.90 ± 0.85^{a}	67.43 ± 4.65^{df}
	ASCORBIC	5.4	5.06	10.16 ± 2.20^{a}	104.69 ± 10.91^{be}
	CITRIC	5.4	5.06	13.23 ± 2.70^{a}	190.74 ± 14.50^{g}
	LACTIC	5.4	5.11	9.72 ± 0.60^{a}	106.58 ± 17.63^{be}
	MALIC	5.4	5.16	10.46 ± 0.89^{a}	128.95 ± 16.25^{b}
	HCl	5.4	5.13	10.24 ± 2.12^{a}	111.45 ± 7.25^{b}

$^{a-g}$: D-values (mean of three experiments ± SD) with different superscript in the same column are significantly different ($P<0.05$).
NI: no bacterial inactivation after three hours of acid challenge.

gastric fluid. Our results confirm these findings. After determination of the nitrogen fractions of both media, it was found that although the Total Nitrogen and the Non-Protein Nitrogen contents (% p/p) were higher in BHI than in ME (2.01 *vs* 0.91 and 1.95 *vs* 0.72, respectively), the Protein Nitrogen content (% p/p) was three times higher in ME (0.18) than in BHI (0.06).

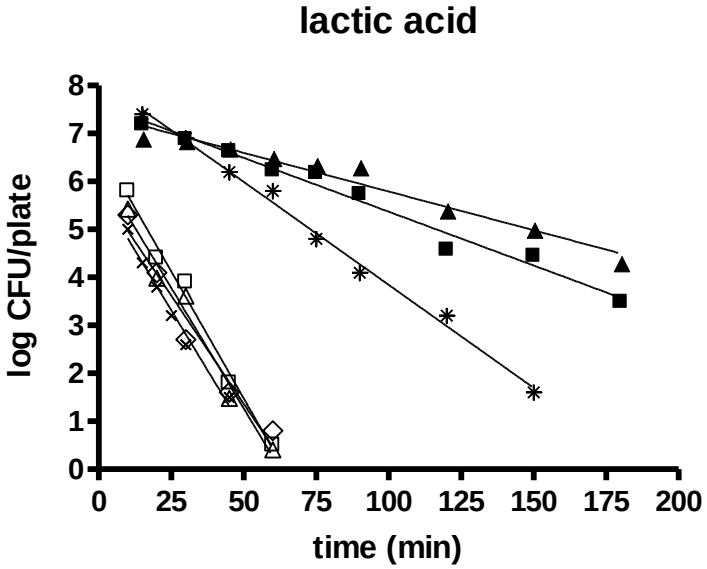

Fig. 2. Survival curves in BHI pH 3.0 of *Salmonella* Typhimurium (CECT 443) grown in buffered BHI (×); non-acidified BHI (*) and Meat Extract (◊); acidified BHI (closed symbols) and Meat Extract (opened symbols) at pH values of 6.4 (■, □) and 5.4 (▲, △) with lactic acid (one of three trials).

The composition of the growth medium also influenced the acid inactivation of *S.* Typhimurium. When BHI was used as challenge medium D-values obtained for cells grown in buffered BHI (pH 7.0), non-acidified ME (pH 6.6) and acidified ME (pH 5.1-6.0) were 9.57, 10.58 and 8.90-13.23 min, respectively, whereas cells grown in non-acidified BHI (pH 7.4) exhibited a higher acid resistance (D-value 25.39 min), probably due to the fact that BHI is a medium which contains 0.2% glucose, and therefore, the growth of *S.* Typhimurium causes a decrease in the extracellular pH to an approximate pH value of 6.2. This work confirms that the inclusion of a fermentable carbohydrate in a growth medium is an effective means of

inducing bacterial acid tolerance, as previously reported (Buchanan and Edelson, 1996; Wilde et al., 2000). All bacterial cultures grown in acidified BHI, with D-values ranging from 25.72 to 78.47 min, showed a greater tolerance to a lethal pH of 3.0 than non-acid adapted control cells (D-value 9.57 min), although the ability of *S.* Typhimurium to survive at an extreme pH (3.0) was dependent on the acid used to acidify the growth medium, especially when cells were obtained in acidified BHI and challenged in BHI. The order of ATR of acid adapted Salmonella cells was citric > acetic > lactic > malic ≥ HCl > ascorbic. Citric and acetic acids were also the organic acids which caused the highest ATR when cells were grown and challenged in ME. This could have an important impact for food safety because citric, acetic and lactic acids are the most commonly used in carcass decontamination treatments (Smulders and Greer, 1998) and as preservatives in the food industry. The differences in the bacterial ATR as a function of the acid used may be linked to the maintenance of the internal pH (pH_i) of the cell. Certain organic acids may enter the cell more easily than others and therefore alter the pH_i of the cell more quickly (Greenacre et al., 2003). Evidence for the involvement of pH_i maintenance in acid tolerance has been found (Foster and Hall, 1991; Cotter et al., 1999).

Overall results confirm that ATR is a complex phenomenon strongly affected by the interaction of different environmental factors which prevail during both acid adaptation and acid inactivation. The interaction of all these factors as well as unknown factors contributing to the acid resistance of microorganisms could explain the apparent contradictory results described in the literature with regard to the effects of organic acids on bacterial ATR. Thus, whereas Baik et al. (1996) did not find an ATR in *S.* Typhimurium cells after adaptation in presence of acetate, benzoate or propionate, others (Greenacre et al., 2003; Yuk and Schneider, 2006) observed a specific effect of the type of acidulant on the ATR developed.

Microorganisms encounter several environmental conditions in foods which may provide a means by which they become acid adapted. In addition to the decline of pH over time associated with food fermentation, bacteria could be exposed to a sublethal pH during the muscle to meat conversion process. Furthermore, rinsing with organic acid solutions to reduce bacterial populations on carcass surfaces

provides the opportunity for bacterial exposure to these microenvironments and as a result bacterial acid tolerance may be developed (Samelis et al., 2001). On the other hand, the use of acid solutions as antimicrobial sprays could offer an environment in which the mixing of organic acids in meat runoff with water could result in washing mixes with pH values of 4.5-5.5 (Samelis et al., 2002; Stopforth et al., 2003) in which microorganisms could become acid adapted and acquire an enhanced potential in resisting further acidification. The successful induction of an ATR in bacteria exposed to the declining pH values may result in an increase in the ability for bacteria to endure otherwise fatal low pH exposures associated with stomach or intestinal and intracellular environments (Wilmes-Riesenberg et al., 1996).

The mechanisms involved in the development of bacterial ATR are still not completely understood, although it is generally acknowledged that various genes and proteins such as alternative RpoS and acid shock proteins (ASPs) are involved (Lee et al., 1995; Foster, 2000). Thus, RpoS has already been shown to be involved in the acid resistance of *Shigella flexneri*, *Escherichia coli* (Gorden and Small, 1993) and *S.* Typhimurium (Fang et al., 1992). The ASPs and regulatory proteins involved in stationary-phase ATR are different from those involved in log-phase ATR. Stationary-phase *Salmonella* cells possess two types of Acid Tolerance Response (ATR) systems. The first is an acid inducible response (σ^s-independent) and the second is a σ^s-dependent response which does not require acid induction (Lee et al., 1995). In addition to RpoS and ASPs, modifications in membrane fluidity and fatty acid composition have also been shown to be involved in the adaptation to acid stress (Brown et al., 1997), although it has been shown recently for *S.* Typhimurium that the modifications in the membrane fatty acid composition were not dependent on either the pH or the acid used to acidify the growth medium (Álvarez-Ordóñez et al., 2008). Furthermore, there are some enzymatic systems, such as arginine decarboxylase and glutamate decarboxylase, which may contribute to the increased acid resistance of acid adapted cells (Lin et al., 1996; Kieboom and Abee, 2006), although their relative contribution seems to be dependent on the type of acid (Lin et al., 1996).

In conclusion, *S.* Typhimurium was shown to exhibit a significant ATR following its culture in acidic environments, which protects the cells from severe acid stress (pH 3.0). Susceptibility to pH 3.0 is dependent on growth and challenge medium composition and type of acidulant used. When ME was used as challenge medium, a large increase in *S.* Typhimurium survival was observed, regardless of the growth conditions. These findings suggest that some types of meat products could provide environments that enhance survival of *S.* Typhimurium acid adapted cells, playing a significant role in human salmonellosis. However, it would be advisable to carry out further studies to clarify how these meat products can protect *S.* Typhimurium from extreme acidic environments.

ACKNOWLEDGEMENTS

This work was supported by the Junta de Castilla y León (project number LE045A06). Álvarez-Ordóñez A. and Fernández A. gratefully acknowledge the finantial support from the Spanish Ministry of Education and the University of León, respectively.

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4.

Acid Tolerance in Salmonella Typhimurium *induced by culturing in the presence of organic acids at different growth temperatures*

Avelino Álvarez-Ordóñez, Ana Fernández, Ana Bernardo and Mercedes López

Submitted for publication

ABSTRACT

The influence of growth temperature (10-25-37-45ºC) and acidification of the culture medium up to pH 4.25 with acetic, citric, lactic and hydrochloric acids on the growth and subsequent acid resistance at pH 3.0 of *Salmonella* Typhimurium CECT 443 was studied. The minimum pH value which allowed for *S.* Typhimurium growth within the temperature range of 25-37ºC was 4.5 when the pH was reduced using citric and hydrochloric acids, and 5.4 and 6.4 when lactic acid and acetic acid were used, respectively. At high (45ºC) or low (10ºC) temperatures, the growth pH boundary was increased about one pH unit. The order of acids in causing an inhibition or a delay in its growth was: acetic > lactic > citric > hydrochloric. The growth temperature strongly modified the acid resistance of the resulting cells. In all cases, D-values were lower for cells grown at 10ºC and significantly increased with increasing growth temperature up to 37ºC, at which D-values obtained were up to 10 times higher. A further increase in growth temperature rendered cells with similar D-values to those found at 25ºC. The growth of cells in acidified media, regardless of the pH value, caused an increase in their acid resistance at the four incubation temperatures, although the magnitude of the ATR observed depended on the growth temperature. Acid adapted cultures at 10ºC showed D-values ranging from 5.75 to 6.91 min, which turned out to be about 2 times higher than those corresponding to non-acid adapted cultures, while higher temperatures induced an increase in D-values of at least 3.5 times. Another finding was that while no significant differences between the effect of the acids were observed at 10 and 45ºC, when cells were grown at 25 and 37ºC, citric acid generally turned out to be the acid which induced a stronger ATR. Results obtained in this study show that growth temperature is an important factor affecting *S.* Typhimurium acid resistance and could contribute to find new strategies based on intelligent combinations of hurdles, which could prevent the development or survival of *Salmonella* spp. in foods. The fact that the lower temperatures markedly decrease the acid resistance and also increase the growth pH boundary of *S.* Typhimurium suggests the convenience to reduce the temperature during food processing when it relies on acidity as the primary means of controlling the growth and survival of this pathogenic microorganism.

INTRODUCTION

Salmonella Typhimurium represents a concern with regard to food safety due to its ability to growth in a wide range of adverse environmental conditions. Moreover, *S.* Typhimurium which contaminates foods may be derived from environments in which its previous growth occurs at moderate acidic conditions and, consequently, it may develop adaptive responses which enhance its resistance to other stress conditions occurring during food processing (Foster and Hall, 1990; Leyer and Johnson, 1993; Tosun and Gönül, 2003). One of the most important and intensively studied stress responses in *S.* Typhimurium is the Acid Tolerance Response (ATR) which increases its subsequent ability to survive in high acid foods, as well as in the extreme acid conditions of the gastrointestinal tract, increasing the risk of illness (Leyer and Johnson, 1992; Waterman and Small, 1998; Greenacre et al., 2003; Yuk and Schneider, 2006). However, in order to study this phenomenon a wide variation in the techniques used to produce acid adapted cells has been used. In most cases the acid adaptation was achieved by a sudden exposure to sublethal acid conditions or by growing cells in media supplemented with glucose. In contrast, few studies have been performed by growing cells in media acidified with organic acids or in acidified foods, in spite of the fact that it has been demonstrated that *S.* Typhimurium ATR is influenced by the conditions which prevail during its growth, with the composition and the pH of the growth medium and the type of acidulant being among them (Bearson et al., 1996; 1998; Greenacre et al., 2003; Yuk and Schneider, 2006). It is important to note that all of these studies were performed using acid adapted cells grown to stationary phase at optimal growth temperature and, to the best of our knowledge, the effects of growth temperature on the subsequent *S.* Typhimurium ATR has been only studied by Samelis et al. (2003), who found that, for cells grown in TSBYE supplemented with 1% glucose at 10 and 30ºC, the ATR was minimum for cells incubated at 10ºC. Thus, it is still unknown whether, and to what extent, ATR is inducible at non-optimal growth temperatures in the presence of organic acids, in spite of the fact that the ATR expressed in response to these conditions is of great relevance for food processing. Therefore, this

study was conducted to evaluate *S.* Typhimurium ATR after its growth within a wide range of temperatures (10-45ºC) up to pH 4.5 in the presence of several organic acids (acetic, citric and lactic) and hydrochloric acid.

MATERIAL AND METHODS

Bacterial strain and culture conditions

The *Salmonella enterica* serovar Typhimurium strain CECT 443 used throughout these experiments was obtained from Colección Española de Cultivos Tipo (CECT) (Spanish Type Culture Collection). The lyophilized cultures were grown in tubes containing 10 mL of Brain Heart Infusion (BHI) (Oxoid) at 37ºC for 24 hours followed by streaking on BHI agar (BHIA) plates and incubating under the same conditions. Stationary phase inocula were prepared inoculating 10 mL of fresh BHI with an isolated colony and incubating it at 37ºC for 24 h. The suspension was then used to inoculate, approximately to a final concentration of 10^3 cells/mL, flasks containing 50 mL of sterile BHI (pH 7.4) non-acidified and acidified at pH values of 6.4, 5.4, 4.5 and 4.25 with acetic (Prolab), citric (Sigma), lactic (Merck) and hydrochloric (Panreac) acids. In order to obtain non-acid adapted cells, buffered BHI adjusted to pH 7.0 by addition of Sorensen buffer 0.2 M (bisodium–monopotassium phosphate (Merck)) was used. These cultures were then incubated at different growth temperatures (10, 25, 37 and 45ºC) for the time needed to reach a late stationary phase of growth.

Growth curves and calculation of growth parameters

At appropriate intervals, samples (1mL) were removed from each culture condition assayed. Number of viable cells in the suspensions was estimated by duplicate plating from 10-fold serial dilutions, prepared in 0.1% (w/v) peptone water (Oxoid), on BHIA and counting the colonies after 24 h of incubation at 37ºC. The number of viable cells, expressed as $\log_{10}$ CFU/mL, was plotted against time and afterwards, growth curves generated by fitting the data to the Gompertz equation

(Buchanan et al., 1997) were used to calculate lag phase duration, exponential growth rate, generation time and maximum population density, as well as the time needed to reach the stationary-phase.

Assessment of acid tolerance

Aliquots of 5 mL of cell cultures obtained as described above were harvested by centrifugation (8000 g, 5 min, 4ºC) (Eppendorf centrifuge 5804R) and the pellets were resuspended into flasks containing 50 mL of BHI with pH adjusted to 3.0 with HCl and incubated at room temperature. Survival was monitored before incubation (0 h) and at appropriate intervals for up to 3 h. The number of viable cells was determined by direct plating on BHIA using peptone water for decimal dilution. The plates were incubated at 37ºC for 48 h (longer incubation times did not have any influence on the count). After incubation, colony-forming units were counted with a modified Image Analyser Automatic Counter (Protos Analytical Measuring Systems, Cambridge, UK) as described by Ibarz et al. (1991). All experiments were conducted in triplicate on three different fresh cultures.

Survival curves and statistical analysis

D-values (min) were determined by plotting the log number of survivors against time. The line that best fits survivor plots was determined by linear regression (GraphPad Prism version 4.00 for Windows, San Diego, USA), and the negative reciprocal of the slope was used for the D-value. Student's t-test (Steel and Torrie, 1986) (Statistica for Windows version 4.5, Statsoft, Tulsa, USA) was used to determine significant differences ($p < 0.05$) between D-values.

RESULTS

***Influence of temperature, pH and type of acidulant on the growth kinetics of S.* Typhimurium**

The growth of *S.* Typhimurium in buffered BHI (pH 7.0), non-acidified BHI (pH 7.4) and BHI acidified with acetic, citric, lactic and hydrochloric acids was

monitored at different temperatures (10-45ºC) and pH values (4.25-7.4). A total of 60 combined treatments were examined. The data obtained for viable counts were fitted to the Gompertz equation so as to obtain the main kinetic growth parameters, generation time (GT), lag phase duration (LPD), time needed to reach the stationary-phase of growth (TSP) and maximum population density (MPD) (Table 1). The determination coefficient (R^2) values of the Gompertz equation, higher than 0.95, show the good fitness of the curves to the set of data. An example of growth curves obtained in non-acidified BHI and acidified BHI at pH values of 6.4 and 4.5 with citric acid under the different temperatures tested is shown in Fig.1.

The three variables studied (incubation temperature, growth medium pH and type of acidulant), individually and in combination, influenced the growth of *S.* Typhimurium. The acidification of the growth medium increased GT, LPD and TSP and decreased MPD, with the order of acids in causing an inhibition or a delay in *S.* Typhimurium growth being: acetic > lactic > citric > hydrochloric.

Our results also show that *S.* Typhimurium was capable of growing at all range of temperatures tested, with the optimal growth temperature being 37ºC. Higher and lower temperatures caused a delay in bacterial growth, regardless of the acid condition tested, which was linked to a decreased MPD and an increased GT, LPD and TSP, especially at 10ºC, in which very high LPD (47.7-59.5 h), GT (12.8-14.5 h) and TSP (300-320 h) were obtained.

The minimum pH value at which growth was observed varied according to the incubation temperature and acidulant used. In the temperature range 25-37ºC *S.* Typhimurium was capable of growing up to pH 4.5 when the pH of the medium was reduced using citric and hydrochloric acids. When acetic acid and lactic acid were used, the minimum pH tested which allowed for growth was 6.4 and 5.4, respectively. The inhibitory effects of the acidulants were enhanced under reduced (10ºC) or elevated (45ºC) temperatures. Thus, acidification to pH $\leq$ 6.4 with acetic acid and to pH $\leq$ 5.4 with citric, lactic and hydrochloric acids was sufficient to inhibit *S.* Typhymurium growth.

Table 1. Growth parameters, as mean value ± standard deviation, obtained by fitting the data to the Gompertz equation

Growth condition	Growth parameter	Temperature (ºC)			
		10	25	37	45
B-BHI	MPD	8.9	9.1	9.2	8.5
NA-BHI		9.1	9.2	9.3	8.6
$A\text{-}BHI_{acetic\ pH\ 6.4}$		-	8.6	8.8	-
$A\text{-}BHI_{citric\ pH\ 6.4}$		8.3	8.6	8.7	8.3
$A\text{-}BHI_{lactic\ pH\ 6.4}$		8.5	8.5	8.9	8.4
$A\text{-}BHI_{HCl\ pH\ 6.4}$		8.4	8.6	8.8	8.4
$A\text{-}BHI_{acetic\ pH\ 5.4}$		-	-	-	-
$A\text{-}BHI_{citric\ pH\ 5.4}$		-	8.5	8.5	-
$A\text{-}BHI_{lactic\ pH\ 5.4}$		-	8.4	8.3	-
$A\text{-}BHI_{HCl\ pH\ 5.4}$		-	8.5	8.4	-
$A\text{-}BHI_{citric\ pH\ 4.5}$		-	8.2	8.5	-
$A\text{-}BHI_{lactic\ pH\ 4.5}$		-	-	-	-
$A\text{-}BHI_{HCl4.5}$		-	8.4	8.4	-
B-BHI	GT	13.2	0.7	0.3	0.4
NA-BHI		12.8	0.7	0.2	0.4
$A\text{-}BHI_{acetic\ pH\ 6.4}$		-	1.0	0.7	-
$A\text{-}BHI_{citric\ pH\ 6.4}$		14.1	0.8	0.5	0.5
$A\text{-}BHI_{lactic\ pH\ 6.4}$		14.5	0.8	0.4	0.5
$A\text{-}BHI_{HCl\ pH\ 6.4}$		13.5	0.8	0.4	0.5
$A\text{-}BHI_{acetic\ pH\ 5.4}$		-	-	-	-
$A\text{-}BHI_{citric\ pH\ 5.4}$		-	0.9	0.5	-
$A\text{-}BHI_{lactic\ pH\ 5.4}$		-	1.2	0.7	-
$A\text{-}BHI_{HCl\ pH\ 5.4}$		-	0.8	0.4	-
$A\text{-}BHI_{citric\ pH\ 4.5}$		-	1.4	0.8	-
$A\text{-}BHI_{lactic\ pH\ 4.5}$		-	-	-	-
$A\text{-}BHI_{HCl4.5}$		-	1.2	0.6	-
B-BHI	LPD	50.4	1.5	0.7	1.5
NA-BHI		47.7	1.3	0.6	1.4
$A\text{-}BHI_{acetic\ pH\ 6.4}$		-	3.9	1.8	-
$A\text{-}BHI_{citric\ pH\ 6.4}$		59.5	2.3	1.5	1.8
$A\text{-}BHI_{lactic\ pH\ 6.4}$		50.9	1.8	1.6	1.7
$A\text{-}BHI_{HCl\ pH\ 6.4}$		52.8	1.5	1.0	1.6
$A\text{-}BHI_{acetic\ pH\ 5.4}$		-	-	-	-
$A\text{-}BHI_{citric\ pH\ 5.4}$		-	2.8	1.1	-
$A\text{-}BHI_{lactic\ pH\ 5.4}$		-	9.2	1.9	-
$A\text{-}BHI_{HCl\ pH\ 5.4}$		-	2.1	1.1	-
$A\text{-}BHI_{citric\ pH\ 4.5}$		-	8.9	6.5	-
$A\text{-}BHI_{lactic\ pH\ 4.5}$		-	-	-	-
$A\text{-}BHI_{HCl4.5}$		-	4.2	3.6	-
B-BHI	TSP	310.0	16.0	10.0	12.0
NA-BHI		300.0	16.0	10.0	12.0
$A\text{-}BHI_{acetic\ pH\ 6.4}$		-	20.0	13.0	-
$A\text{-}BHI_{citric\ pH\ 6.4}$		320.0	18.0	10.0	14.0
$A\text{-}BHI_{lactic\ pH\ 6.4}$		320.0	18.0	11.0	14.0
$A\text{-}BHI_{HCl\ pH\ 6.4}$		320.0	18.0	10.0	14.0
$A\text{-}BHI_{acetic\ pH\ 5.4}$		-	-	-	-
$A\text{-}BHI_{citric\ pH\ 5.4}$		-	22.0	12.0	-
$A\text{-}BHI_{lactic\ pH\ 5.4}$		-	24.0	14.0	-
$A\text{-}BHI_{HCl\ pH\ 5.4}$		-	20.0	11.0	-
$A\text{-}BHI_{citric\ pH\ 4.5}$		-	24.0	20.0	-
$A\text{-}BHI_{lactic\ pH\ 4.5}$		-	-	-	-
$A\text{-}BHI_{HCl4.5}$		-	22.0	14.0	-

B-BHI: Buffered BHI pH 7.0
NA-BHI: non-acidified BHI (pH 7.4)
A-BHI: acidified BHI with different acids at different pH values
MPD: maximum population density (log cfu/mL)
GT: generation time (h)
LPD: lag phase duration (h)
TSP: time needed to reach the stationary-phase (h)
- : no bacterial growth observed

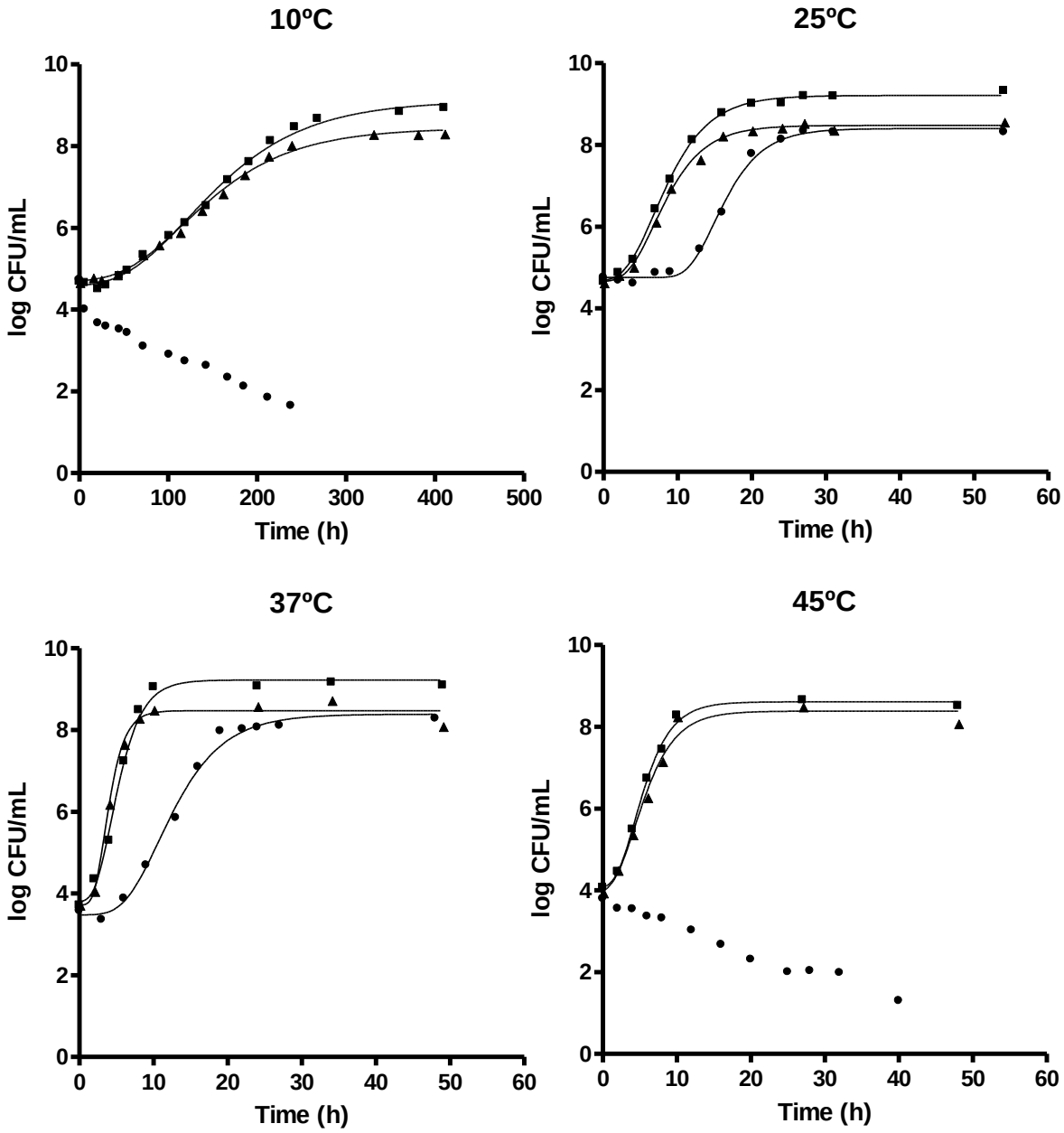

Fig. 1. Growth curves of *Salmonella* Typhimurium (CECT 443) in non-acidified BHI (■) and BHI acidified with citric acid at pH values of 6.4 (▲) and 4.5 (●) obtained under different growth temperatures (one of three trials). The symbols represent the experimental data obtained, while the lines were the result of the adjustment of the data through the Gompertz equation.

Effect of growth conditions on S. Typhimurium *acid resistance in BHI pH 3.0*

In order to study the effects of acidification and growth temperature on the subsequent acid resistance of *S.* Typhimurium, stationary phase cells grown at 10,

25, 37 and 45ºC in non-acidifed BHI (pH 7.4), buffered BHI (pH 7.0) and BHI acidifed with acetic (pH 6.4), citric (pH 6.4 and 4.5), lactic (pH 6.4 and 5.4) and hydrochloric (pH 6.4 and 4.5) acids, were challenged in BHI pH 3.0. The survival curves obtained followed a first-order inactivation kinetic. As a consequence, D-values (time in min for a 10-fold reduction in survival counts) were useful parameters for resistance comparison purposes. Table 2 shows the decimal reduction times obtained, expressed as the mean value of three independent experiments and standard deviations. An example of survival curves obtained for non-acid adapted cells and acid adapted cells grown at pH 6.4 in the presence of citric acid is shown in Fig. 2, which are representative of those obtained throughout this investigation.

Table 2. D-values (min) for acid treated (pH 3.0) cells of *Salmonella* Typhimurium (CECT 443) grown at 10, 25, 37 and 45 ºC in buffered BHI (non-acid adapted cells), non-acidified BHI and acidified BHI at different pH values with several acids.

GROWTH MEDIUM	**GROWTH TEMPERATURE**			
	10ºC	25ºC	37ºC	45ºC
B-BHI	$2.61\pm0.40^{a}{}_{1}$	$4.50\pm0.45^{a}{}_{2}$	$10.14\pm1.34^{a}{}_{3}$	$4.90\pm0.50^{a}{}_{2}$
NA-BHI	$3.68\pm0.47^{b}{}_{1}$	$11.75\pm0.90^{b}{}_{2}$	$21.18\pm3.12^{b}{}_{3}$	$14.68\pm0.90^{b}{}_{4}$
A-BHI$_{\text{acetic pH 6.4}}$	NG	$15.69\pm1.25^{c}{}_{1}$	$60.12\pm9.34^{cd}{}_{2}$	NG
A-BHI$_{\text{citric pH 6.4}}$	$6.09\pm0.96^{c}{}_{1}$	$21.97\pm0.73^{d}{}_{2}$	$61.83\pm7.31^{cf}{}_{3}$	$24.86\pm5.13^{c}{}_{2}$
A-BHI$_{\text{citric pH 4.5}}$	NG	$24.62\pm1.40^{e}{}_{1}$	$78.42\pm16.13^{c}{}_{2}$	NG
A-BHI$_{\text{lactic pH 6.4}}$	$5.75\pm0.83^{c}{}_{1}$	$16.42\pm1.41^{c}{}_{2}$	$45.91\pm4.38^{de}{}_{3}$	$22.56\pm4.42^{c}{}_{2}$
A-BHI$_{\text{lactic pH 5.4}}$	NG	$18.07\pm1.32^{c}{}_{1}$	$65.28\pm12.08^{cd}{}_{2}$	NG
A-BHI$_{\text{HCl pH 6.4}}$	$6.91\pm0.70^{c}{}_{1}$	$15.50\pm2.50^{bc}{}_{2}$	$38.76\pm5.68^{e}{}_{3}$	$23.45\pm7.27^{bc}{}_{2}$
A-BHI$_{\text{HCl pH 4.5}}$	NG	$22.16\pm0.79^{de}{}_{1}$	$46.32\pm8.15^{def}{}_{2}$	NG

B-BHI: Buffered BHI pH 7.0
NA-BHI: non-acidified BHI (pH 7.4)
A-BHI: acidified BHI with different acids: acetic, citric, lactic and HCl at different pH values
$^{a\text{-}f}$: D-values (mean of three experiments ± SD) with different superscript in the same column are significantly different (P<0.05)
$_{1\text{-}4}$: D-values (mean of three experiments ± SD) with different subscript in the same row are significantly different (P<0.05)
NG: no bacterial growth

The acid resistance of the resulting cells depended on the growth temperature. In all cases, D-values were lower for cells grown at 10ºC and significantly increased with increasing growth temperature up to 37ºC, at which D-values obtained were of up to 10 times higher. A further increase in growth

temperature (45ºC), resulted in cells with an acid resistance similar to their counterparts grown at 25ºC. Of particular interest from our data is the fact that under all culture conditions which allowed for *S.* Typhimurium growth, the relationship between D-values and growth temperature in the range 10-37ºC followed an exponential kinetic, which depended on the growth medium used (Fig. 3).

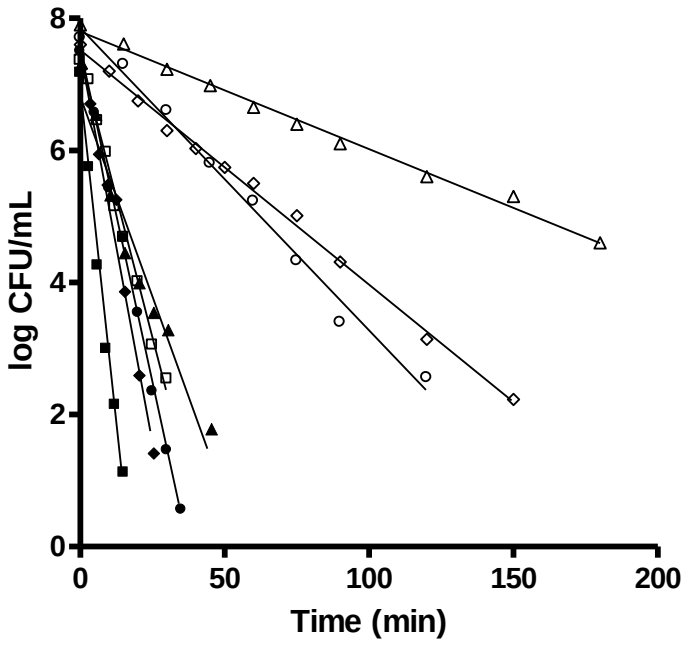

Fig. 2. Survival curves in BHI pH 3.0 for S. Typhimurium (CECT 443) cells grown in buffered BHI (closed symbols) and acidified BHI with citric acid at pH 6.4 (opened symbols) under several growth temperatures: 10 (■,□), 25 (●,○), 37 (▲,Δ) and 45 ºC (♦, ◊).

Acid adapted cells and those grown in non-acidified BHI were more resistant to a lethal pH than their non-acid adapted counterparts at the four incubation temperatures assayed, although the degree of acid tolerance acquired by non-acidified cells was lower than that found for acid adapted cells, especially at the lowest temperature tested. Whereas the growth of *S.* Typhimurium in non-acidified BHI within the temperature range 25-45ºC increased its acid tolerance to about 2.5

times, this increase only represented about 1.5 times at 10ºC. Acid adapted cultures at 10ºC showed D-values of about 2 times higher than those corresponding to non-acid adapted cultures, while higher temperatures induced an increase in the D-values of at least 3.5 times.

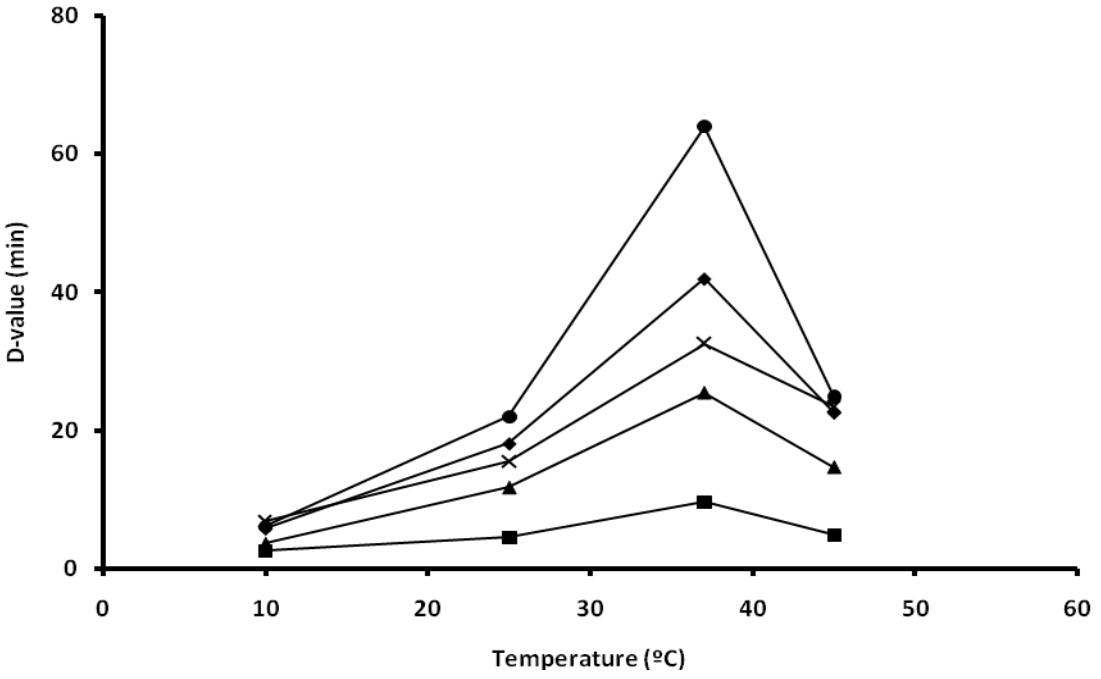

Fig. 3. Relationship between D-values and growth temperature for *S.* Typhimurium cells grown in buffered BHI (■), non-acidified BHI (▲) and acidified BHI at pH 6.4 with several acids: A-BHI$_{\text{citric pH 6.4}}$ (●),A-BHI$_{\text{lactic pH 6.4}}$ (♦) and A-BHI$_{\text{HCl pH 6.4}}$ (×).

Our results also show that the acidification of the medium at pH values lower than 6.4 did not cause any further increase in the acid resistance found. From Table 2 it can also be seen that the growth temperature conditioned the efficacy of the acidulants in inactivating *S.* Typhimurium at pH 3.0. Whereas no significant differences between the effects of the acids were observed at 10 and 45ºC, when cells were grown at 25 and 37ºC, citric acid generally turned out to be the acid which induced a stronger ATR.

DISCUSSION

The results obtained in this study showed that the ability of *S.* Typhimurium to growth under acidic conditions was affected by the type of acid used to acidify the growth medium. The order of acids in causing an inhibition or a delay in *S.* Typhimurium growth was: acetic > lactic > citric > hydrochloric. These results agree with those previously reported by Jung and Beuchat (2000), who testing the ability of *S.* Typhimurium to grow in TSB acidified with acetic, lactic, citric and malic acids at pH values of 5.4, 4.4, 4.0 and 3.7 found that, regardless of the pH value, the order of acids in inhibiting bacterial growth was: acetic > lactic > citric > malic. The inhibitory effect of organic acids has widely been reported to be caused by their undissociated form. In contrast to inorganic acids, weak acids penetrate bacterial cell membranes as undissociated molecules (through permeases or porins) and their intracellular dissociation reduces cytoplasmatic pH, affecting the metabolic activity of the cell (Cherrington et al., 1991; Davidson, 2001). Acetic acid due to its low molecular weight and its great liposolubility can easily penetrate bacterial membranes, whereas lactic and citric acids diffuse slowly.

The minimum pH value which allowed for *S.* Typhimurium growth within the temperature range of 25-37ºC was 4.5 when the pH was reduced using citric and hydrochloric acids, and 5.4 and 6.4 when lactic acid and acetic acid were used, respectively. At higher (45ºC) or lower (10ºC) temperatures, the growth pH boundary was increased about one pH unit. Results previously reported by Koutsoumanis et al. (2004) also showed that the minimum pH allowing for *S.* Typhimurium growth increased as temperature decreased in the range of 35-10ºC.

Acid adapted cells and, to a lesser extent, non-acidified cells of *S.* Typhimurium showed an improved survival in BHI pH 3.0 compared with non-acid adapted cells. Our results show that the magnitude of the ATR developed was greatly influenced by the conditions in which the cells were habituated to acidic environments. It was found that *S.* Typhimurium was capable of acquiring an ATR after its growth in BHI (pH 7.4) containing 0.2% glucose by reducing the culture pH

to a final value of approximately 6.2 (data not shown). However, the degree of acid tolerance acquired by cells grown in BHI acidified with the different acids tested was clearly higher, although the extent of this increase was not significantly affected by reducing the growth medium pH from 6.4 to 4.5.

Acid adaptation response has been extensively reported for *S.* Typhimurium (Foster and Hall, 1990; Lee et al., 1994; Bearson et al., 1996, 1998; Greenacre et al., 2003; Álvarez-Ordóñez et al., 2009), and represents a great concern for food industry, since during food processing, microorganisms encounter several mildly acidic conditions which may induce an ATR and may also be responsible for an increased ability to survive other lethal stresses, including otherwise fatal low pH exposures associated with stomach and intracellular environments (Wilmes-Riesenberg et al., 1996; Wilde et al., 2000; Tosun and Gonul, 2003). Moreover, several studies have previously shown an increased virulence and invasion for acid adapted cultures of *Salmonella* spp. (Humphrey et al., 1996; Wilmes-Riesenberg et al., 1996; Gahan and Hill, 1999).

Growth temperature is an important factor modifying bacterial cell composition and physiology, and, therefore, affecting the bacterial resistance to environmental stresses, including acid resistance. However, up to date little effort has been made to study the influence of growth temperature on bacterial acid resistance. Results of this study show that the susceptibility of *S.* Typhimurium to acid stress is dependent on growth temperature. The most acid resistant cells were those grown at their optimal temperature, 37ºC, decreasing the acid resistance at temperatures both above and below this temperature, especially at 10ºC. The variations observed in this study in the extent of acid tolerance of *S.* Typhimurium as a function of growth temperature agree with the report by Samelis et al. (2003), who found that *S.* Typhimurium cells grown at 10ºC showed a reduced acid resistance in comparison to cells obtained at 30ºC. These results also agree with other previous studies performed on other pathogenic bacteria, such as *E. coli* O157:H7 (Cheng and Kaspar, 1998; Semancheck and Golden, 1998) and *Listeria monocytogenes* (Patchett et al., 1996; Samelis et al., 2003).

A further finding from this study was that the growth temperature conditioned the efficacy of organic acids in inhibiting *S.* Typhimurium in BHI pH 3.0, with citric acid being the most effective acid in conferring an ATR, although this effect was only observed at 25 and 37ºC. These findings evidence the need to further expand the knowledge on the ATR expressed in response to different environmental conditions in order to facilitate the design and increase the accuracy of risk assessment studies.

Recent progress has been made with regard to the characterization of systems which protect cells against acid stress. However, the mechanisms involved are not completely understood, although evidence for the involvement of internal pH maintenance has been previously found. Thus, the differences in the bacterial ATR as a function of the acid used could be associated with the different ability shown by acids to alter the internal pH of the cells (Foster and Hall, 1991; Greenacre et al., 2003). Furthermore, the induction of modifications in membrane fatty acid composition and, consequently, in membrane fluidity has also been implicated in bacterial ATR (Brown et al., 1997; Chang and Cronan, 1999; Kim et al., 2005; Álvarez-Ordóñez et al., 2008). Given the essential role of envelopes in cell inactivation and survival, it is feasible that changes in membrane fatty acid composition and in the physical behavior of the membranes may exert an effect on bacterial resistance to several stresses, including acid stress. Our research group has previously shown for *S.* Typhimurium (Alvarez-Ordóñez et al., 2008) that the acidification of the growth medium caused a decrease in the unsaturated to saturated fatty acids ratio (UFA/SFA) and an increase in CFA. Moreover, a membrane adaptation corresponding to an increase in the UFA/SFA ratio and, consequently, in membrane fluidity and a decrease in cyclopropne fatty acids (CFA) levels at low growth temperature (10ºC) was observed. As the growth temperature increased to 37ºC, for all culture conditions, a progressive decrease in the UFA/SFA ratio was observed, decreasing thereafter. With regard to the proportion of CFA obtained an opposite trend was found. These changes in membrane fatty acid composition are linked to *S.* Typhimurium resistance to extreme acidic environments and could explain the results presented here. In Fig. 4 the relationship between the D-values

and the CFA content and the UFA/SFA ratio observed for *S.* Typhimurium acid adapted cells under the different growth temperatures is shown.

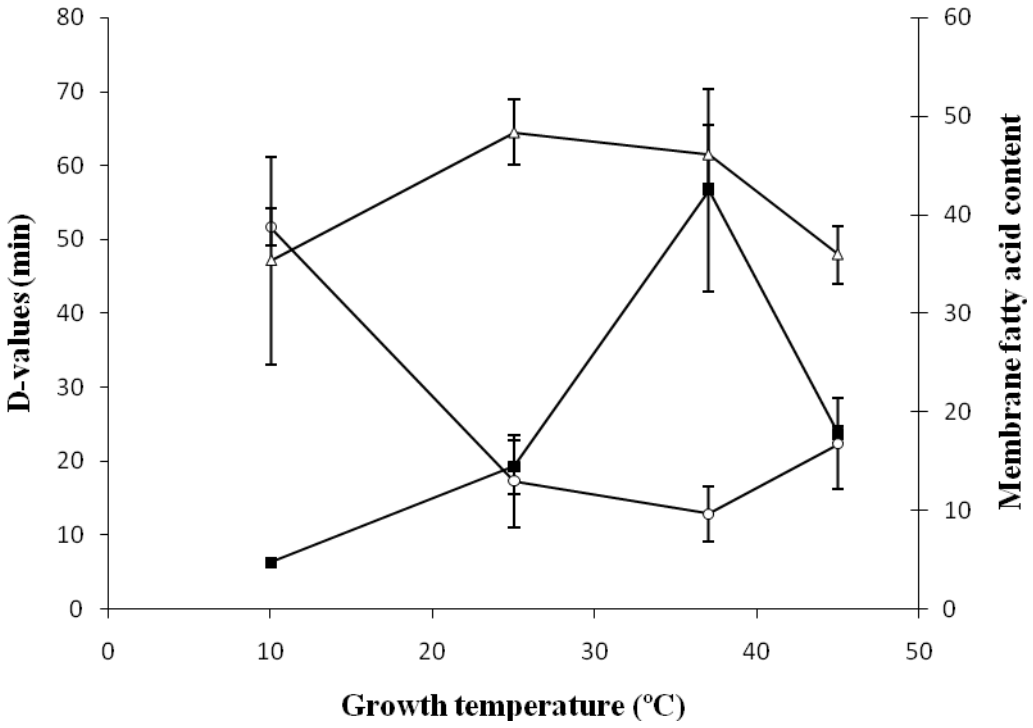

Fig. 4. Relationship between D-values (■) and membrane fatty acid composition (Δ: CFA[1] content, expressed as % of total fatty acids; ○: (UFA/SFA)*100[1], expressed both UFA and SFA as % of total fatty acids) for *S.* Typhimurium acid adapted cells grown under different temperatures. The data presented are the mean values obtained for acid adapted cells grown under the different acidic conditions.

[1]: Data from Álvarez-Ordóñez et al. (2008)

It can be seen that under the growth conditions which led to a higher acid resistance, the bacterial membranes showed the lowest UFA/SFA ratios, and the highest CFA contents. Thus, the higher resistance of acid adapted cells could be attributed to a lower permeability of the membrane. In the same way, the presence of CFA could increase the membrane rigidity and stability, and therefore could be also involved in the acquisition of enhanced acid resistance. Other previously published studies also stated that increased levels of cyclopropane fatty acids may enhance the

survival of microbial cells exposed to low pH (Brown et al., 1997; Chang and Cronan, 1999; Kim et al., 2005). However, changes observed in the membrane fatty acid composition are not enough to explain the great acid resistance found for cells grown at 37ºC, and therefore, other molecular mechanisms must be involved in *Salmonella* response to acid stress. Thus, it is worth noting that acid adaptation conditions may also influence the expression level of various systems designed to alleviate acid stress through the consumption of protons by amino acid decarboxylases (Foster, 2000; Kieboom and Abee, 2006). Studies carried out in our laboratory have confirmed the implication of arginine and lysine decarboxylases in *S.* Typhimurium ATR (unpublished data) after its adaptation at 37ºC.

To sum up, the results of this study demonstrate that the ability of *S.* Typhimurium to survive in extreme acidic environments can sustantially vary as a function of prior growth conditions. The fact that lower temperatures markedly decrease the acid resistance and increase the growth pH boundary of *S.* Typhimurium suggests the convenience to reduce the temperature during food processing in order to control the growth and survival of this pathogenic microorganism.

ACKNOWLEDGEMENTS

Álvarez-Ordóñez A. and Fernández A. gratefully acknowledge the finantial support from the Spanish Ministry of Education and the University of León, respectively.

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5.

Arginine and lysine decarboxylases and the Acid Tolerance Response of Salmonella Typhimurium

Avelino Álvarez-Ordóñez, Ana Fernández, Ana Bernardo and Mercedes López

Submitted for publication

ABSTRACT

Salmonella Typhimurium inactivation at pH 2.5 in Mineral Medium (MM) and MM supplemented with 0.01% (p/v) arginine, lysine or glutamic acid was studied using stationary-phase cells grown in buffered BHI pH 7.0 (non-acid adapted cells) and acidifed BHI up to pH 4.5 with acetic, citric, lactic and hydrochloric acids (acid adapted cells). In all cases, acid adapted cells, with D-values ranging from 23.34 to 86.90 min, showed a significantly higher acid resistance than non-acid adapted cells, with D-values between 8.90 and 10.29 min. Whereas the conditions used for acid adaptation did not exert a significant effect on the acid resistance of the *S.* Typhimurium resulting cells, the inclusion of lysine and arginine in the challenge medium protected them against acid inactivation, reaching D-values of about 2 and 3 times higher, respectively, than those found in MM or MM supplementd with glutamic acid. None of these three amino acids significantly modified the acid resistance of non-acid adapted cells. The relative expression level of *adi* (encoding the arginine decarboxylase), *adiY* (encoding the transcriptional activator of *adi*), *cadA* (encoding the lysine decarboxylase) and *cadB* (encoding the lysine/cadaverine transport protein) was examined by quantitative PCR. Acid adapted cells showed higher relative expression levels for both systems, arginine decarboxylase and lysine decarboxilase, which demonstrates that the induction of specialized pH-homeostatic systems plays an important role in *S.* Typhimurium protection against acid stress. However, the increased acid resistance showed by acid adapted cells challenged in MM arginine or lysine free suggests the existence of other microbial survival strategies.

INTRODUCTION

The significance of *Salmonella* Typhimurium as a foodborne pathogen has been long recognized (Brennan et al., 1999; D´Aoust, 2000; Hall, 2002; CDC, 2003, 2004; Boccia et al., 2004; Mazurek et al., 2004). The capacity of this microorganism to cause illness is related to its ability to survive harsh conditions encountered in the natural environment and within the host organism during pathogenesis. One critical host defence which must be defeated by enteropathogenic microorganisms in order to cause disease is the acid stress barrier present in the stomach, with a pH value of 2.0, approximately (Audia et al., 2001). Previous studies have shown that *Salmonella* spp. growth in a moderately acid environment induces an adaptive response which results in an enhanced resistance to more extreme acid conditions (Bacon et al., 2003; Yuk and Schneider, 2006; Álvarez-Ordóñez et al., 2009). An important consequence of this so-called Acid Tolerance Response (ATR) is the fact that acid adapted cells could be more resistant to the strong acidic environment found in the stomach, increasing the risk of human salmonellosis (Wilmes-Riesenberg et al., 1996). It has been previously shown that the composition of the challenge medium greatly influences *S.* Typhimurium acid resistance (Waterman and Small, 1998; Álvarez-Ordóñez et al., 2009). These authors have stated that the protein content of foods could allow bacteria to survive extremely acidic environments. This protective effect could be related to the existence of several homeostatic systems which use extracellular amino acids to enhance the bacterial intracellular pH. However, data available on literature show that whereas for some microorganisms, such as *Escherichia coli*, the inclusion of arginine, lysine and glutamic acid in the challenge medium is responsible for an increased acid resistance (Castaine-Cornet et al., 1999; Castaine-Cornet and Foster, 2001; Iyer et al., 2003; Foster, 2004), the reports found for *S.* Typhimurium are scarce and indicate that these arginine and lysine-dependent survival systems are only activated under specific culture conditions (Park et al., 1996; de Jonge et al., 2003; Kieboom and Abee, 2006).

The aim of this study was to determine the acid inactivation kinetics of *S.* Typhimurium CECT 443 acid adapted cells grown in the presence of several organic acids (acetic, citric and lactic) and hydrochloric acid and challenged at extreme low pH values in a mineral medium, with and without arginine, lysine and glutamic acid. The relative expression level of several genes, such as *adi* (encoding the arginine decarboxylase), *adiY* (encoding the transcriptional activator of *adi*), *cadA* (encoding the lysine decarboxylase) and *cadB* (encoding the lysine/cadaverine transport protein), potentially linked to *S.* Typhimurium acid adaptive response, was also determined in order to contribute to elucidate the molecular mechanisms involved in *S.* Typhimurium response to extreme acidic conditions.

MATERIAL AND METHODS

Bacterial strain and culture conditions

The *Salmonella enterica* serovar Typhimurium strain CECT 443 was obtained from Colección Española de Cultivos Tipo (CECT) (Spanish Type Culture Collection). Revitalized cultures were stored on Brain Heart Infusion Agar (BHIA; Oxoid) plates at 4 ºC and then were activated by transferring an isolated colony from BHIA to Brain Heart Infusion (BHI; Oxoid) and incubated at 37 ºC for 24 h to give a stock suspension of 10^9 cfu/mL. Flasks containing 50 mL of sterile BHI (pH 7.4) non-acidified and acidified with acetic (Prolab) (pH 6.4), citric (Sigma) (pH 6.4 and 4.5), lactic (Merck) (pH 6.4 and 5.4) and hydrochloric (Panreac) (pH 6.4 and 4.5) acids were inoculated with the subculture to a final concentration of 10^3 cells/mL. In order to obtain non-acid adapted cells, buffered BHI adjusted to pH 7.0 by addition of Sorensen buffer 0.2 M (bisodium (Merck) – monopotassium (Panreac) phosphate) was used. The phosphate-buffer system maintained the pH value within ± 0.1 pH units during the incubation period. These cultures were then incubated at 37ºC for 36 h. Previous growth studies indicated that all cultures were in a late stationary-phase of growth at this time period (Álvarez-Ordóñez et al., 2009). These cultures

were used to determine the acid resistance and the expression level of genes potentially involved in the acid resistance response of *S.* Typhimurium.

Assessment of acid resistance

The defined Mineral Medium (MM) was that of de Jonge et al. (2003), which contains per litre of distilled water: 1 g $(NH_4)_2SO_4$ (Panreac), 10 g K_2HPO_4 (Panreac), 4.5 g KH_2PO_4 (Panreac), 0.2 g $MgCl_2$ (Panreac), 40 mg EDTA (Panreac), 15 mg thiamine (Sigma), 2 mg $FeSO_4$ (Panreac), 4 g glucose (Fluka), adjusted to pH 2.5 using 1M HCl (Panreac).

Aliquots of 5 mL of cell cultures obtained as previously described were harvested by centrifugation at 8000 g for 5 min at 4ºC, and the cellular pellet was resuspended in 50 mL of MM pH 2.5. In order to assess the protective effect of arginine (Sigma), lysine (Sigma) and glutamic acid (Sigma) these amino acids were added to MM at a final concentration of 0.01% (p/v). Populations were monitored by removing samples over a 3-hour period at room temperature. Ten-fold serial dilutions were produced in sterile 0.1% (w/v) peptone solution (Oxoid) and suitable dilutions were plated in duplicate on BHIA. Plates were then incubated at 37 ºC for 48 h and survivors were counted using an automatic cfu counter (Protos Analytical Measuring Systems, Cambridge, UK) following the specifications described by Ibarz et al. (1991).

Three different freshly grown cultures of *S.* Typhimurium were used for each experimental run.

Survival curves and statistical analysis

D-values (min) were determined by plotting the log number of survivors against time for each culture. The line that best fits survivor plots was determined by linear regression (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA), and the negative reciprocal of the slope was used for the D-value.

D-values were compared using Student´s *t*-test (Steel and Torrie, 1986) (Statistica for Windows version 4.5, Statsoft, Inc, Tulsa, OK, USA).

Assessment of expression level of genes potentially involved in S. Typhimurium *acid resistance*

From cultures in stationary growth phase aliquots of 1 mL were removed, immediately frozen in liquid nitrogen and then stored at -25ºC until their use for RNA isolation. The time between removal from incubator and freezing of cells was approximately 60 seconds. After a centrifugation at 10000 g - 4ºC for 1 min, RNA from pellet cells was extracted using TRI reagent (Ambion). After precipitation of nucleic acids, the residual DNA was removed with TURBO DNA-free system (Ambion), as recommended by the supplier, and after phenol-chloroform extraction and precipitation, the RNA was quantified by measuring the OD_{260}. Extracted RNA samples were stored until cDNA synthesis in 70% ethanol (Panreac), 83 mM sodium acetate (Panreac) buffer (pH 5.2) at -25ºC. Afterwards, cDNA was synthesized from RNA by using Superscript III reverse transcriptase (Invitrogen), 5 pmol of gene-specific reverse primers (Table 1), each deoxynucleoside triphosphate (Ambion) at a concentration of 0.5 mM and 0.5 μg of total RNA. Reverse transcription was performed at 55ºC for 60 min and then the reverse transcriptase was inactivated by incubation at 70ºC for 15 min. Primers were designed by using Primer3 (www. genome.wi.mit.edu). Quantitative PCR was performed with the synthesized cDNA by using an ABI Prism 7000 with SYBR Green technology (PE Applied Biosystems). Each 50 μL reaction mixture contained 1X SYBR Green master mixture (Applied Biosystems), the gene-specific reverse and forward primers at a concentration of 400 nM (Table 1), and the reverse-transcribed RNA. Amplification was initiated at 95ºC for 10 min, and this was followed by 40 cycles of 95ºC for 15 s and 55ºC for 60 s. Cycle threshold (*Ct*) values were obtained by manually setting the baseline and threshold values at which fluorescence was appreciably above the background fluorescence for each reaction in the exponential phase of amplification for all reactions. All experiments were performed in triplicate. Relative transcript levels were calculated by using the relative expression software tool (REST) based on the comparative ΔΔ*Ct* method described by Pfaffl (2001) and Pfaffl et al. (2002). The expression of the *rrsA* gene was used as a reference to determine the induction levels.

Table 1. Primers used for the assessment of gene expression by qPCR.

GENE	SEQUENCE (5´ - 3´)
adi	F: AACAGTCCGCAGGTGGGTTA
	R: AATTTCCGTGCCTTCGGTTT
adiY	F: CAACGCCGATCCCTTATTGA
	R: AATTTCATCCGGTGCTTCCA
cadA	F: ACGCCTGAAAAGCGAATCTG
	R: CATGCCATGCGCTATCTGAA
cadB	F: TCCAGACCGGCGTTCTTTAT
	R: ATGCCGGGAAGAACGTAGAA
rrsA	F: CAACGAGCGCAACCCTTATC
	R: TCCCCACCTTCCTCCAGTTT

RESULTS

Salmonella Typhimurium inactivation at pH 2.5 in Mineral Medium (MM), with or without arginine, lysine or glutamic acid (0.01% p/v), was studied using stationary-phase cells grown in non-acidified BHI (pH 7.4) and BHI acidified with acetic (pH 6.4), citric (pH 6.4 and 4.5), lactic (pH 6.4 and 5.4) and hydrochloric (pH 6.4 and 4.5) acids. Cells grown in buffered BHI (pH 7.0) were used as non-acid adapted cells. Survival curves obtained fitted properly into a first order kinetic. The goodness of fit was determined both by visual inspection and R^2 value, which ranged from 0.94 to 0.99 (data not shown). Fig. 1 shows a representative example of survival curves for non-acid adapted cells and acid adapted cells grown at pH 6.4 in the presence of lactic acid. D-values obtained in all conditions tested, reported as the mean of three independent experiments ± the standard deviations, are shown in Table 2.

In all cases, acid adapted cells, with D-values ranging from 23.34 to 86.90 min, showed a significantly higher acid resistance than non-acid adapted cells, with D-values of between 8.90 and 10.29 min. It can be seen that neither the pH value of

the growth medium nor the type of acid used during the acid habituation had a significant effect ($p<0.05$) on the acid resistance of acid adapted *S.* Typhimurium. The inclusion of arginine, lysine or glutamic acid in the challenge medium did not modify the acid resistance of non-acid adapted cells. However, the magnitude of the Acid Tolerance Response (ATR) developed was dependent on the composition of the challenge medium. Thus, whereas acid adapted cells challenged in MM and MM supplemented with glutamic acid showed D-values of about 3 times higher than non-acid adapted cells, D-values increased up to about 6 and 8 times when the challenge medium was supplemented with lysine and arginine, respectively.

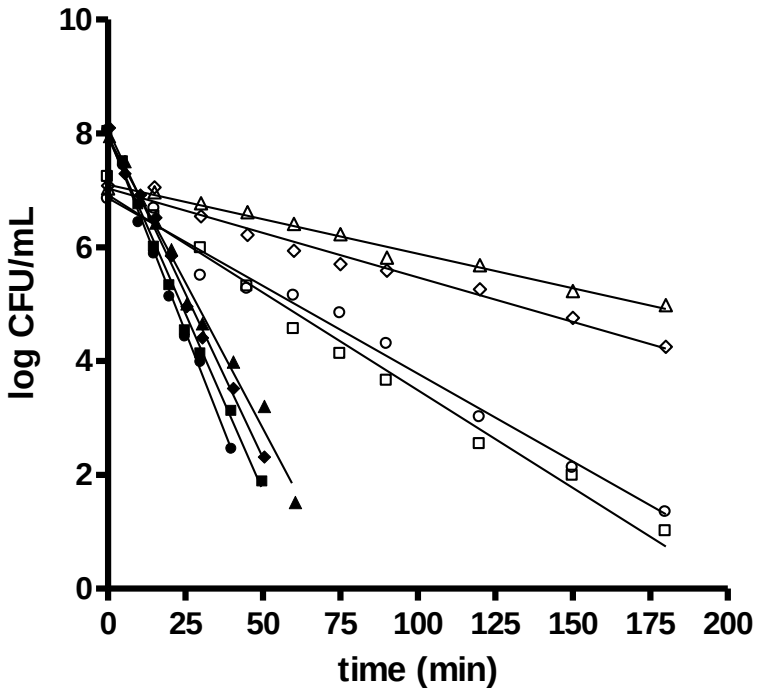

Fig. 1. Survival curves of *Salmonella* Typhimurium (CECT 443) grown in buffered BHI (closed symbols) and in acidified BHI at pH 6.4 with lactic acid (opened symbols) and challenged in MM pH 2.5 (■, □), MM pH 2.5 supplemented with arginine (▲, △), MM pH 2.5 supplemented with lysine (♦, ◊) and MM pH 2.5 supplemented with glutamic acid (●, ○) (one of three trials).

RNA from stationary-phase *S.* Typhimurium was extracted and, subsequently, the expression of *adi*, *adiY*, *cadA* and *cadB* was examined by

quantitative PCR. Table 3 shows the mean relative expression values for each gene under the different growth conditions. Of particular interest in our data is the fact that the arginine decarboxylase system, including the *adi* gene (encoding the arginine decarboxylase) and the *adiY* gene (encoding the transcriptional activator of *adi*), was found to be significantly induced in *S.* Typhimurium grown under acidic conditions. Thus, acid adapted cells showed relative expression levels for *adi* of up to 110 times higher than non-acid adapted cells. The lysine decarboxylase system also was found to be up-regulated under acid growth conditions, but to a lower extent. Both *cadA* (encoding the lysine decarboxylase) and *cadB* (encoding the lysine/cadaverine transport protein) showed higher relative expression levels in acid adapted *S.* Typhimurium cells.

Table 2. D-values (min) for acid challenged cells of *S.* Typhimurium (CECT 443) grown in buffered BHI, non-acidified BHI and acidified BHI at different pH values with several acids.

GROWTH MEDIA	**ACID CHALLENGE MEDIA (pH 2.5)**			
	MM	MM+ARG	MM+LYS	MM+GLU
B-BHI	$8.98\pm0.93^{a}{}_{1}$	$10.29\pm1.52^{a}{}_{1}$	$9.67\pm0.97^{a}{}_{1}$	$8.90\pm1.70^{a}{}_{1}$
NA-BHI	$27.32\pm5.02^{b}{}_{1}$	$73.03\pm5.95^{b}{}_{2}$	$55.70\pm5.30^{b}{}_{3}$	$28.18\pm6.09^{bc}{}_{1}$
A-BHI$_{\text{acetic pH 6.4}}$	$24.69\pm4.33^{b}{}_{1}$	$76.74\pm6.66^{b}{}_{2}$	$57.85\pm5.33^{b}{}_{3}$	$23.95\pm2.69^{b}{}_{1}$
A-BHI$_{\text{citric pH 6.4}}$	$25.46\pm5.24^{b}{}_{1}$	$80.87\pm9.60^{b}{}_{2}$	$57.75\pm7.63^{b}{}_{3}$	$23.34\pm2.90^{b}{}_{1}$
A-BHI$_{\text{citric pH 4.5}}$	$28.43\pm3.29^{b}{}_{1}$	$80.44\pm11.31^{b}{}_{2}$	$56.91\pm5.56^{b}{}_{3}$	$28.96\pm2.67^{bc}{}_{1}$
A-BHI$_{\text{lactic pH 6.4}}$	$31.03\pm2.89^{b}{}_{1}$	$86.90\pm8.48^{b}{}_{2}$	$68.24\pm7.18^{b}{}_{3}$	$33.61\pm4.20^{c}{}_{1}$
A-BHI$_{\text{lactic pH 5.4}}$	$27.30\pm3.48^{b}{}_{1}$	$76.53\pm7.54^{b}{}_{2}$	$62.26\pm7.78^{b}{}_{2}$	$32.22\pm3.83^{c}{}_{1}$
A-BHI$_{\text{HCl pH 6.4}}$	$27.48\pm2.97^{b}{}_{1}$	$84.34\pm7.10^{b}{}_{2}$	$60.87\pm6.74^{b}{}_{3}$	$30.18\pm2.43^{c}{}_{1}$
A-BHI$_{\text{HCl pH 4.5}}$	$28.01\pm3.22^{b}{}_{1}$	$76.16\pm6.80^{b}{}_{2}$	$55.68\pm7.19^{b}{}_{3}$	$30.07\pm3.96^{bc}{}_{1}$

B-BHI: Buffered BHI pH 7.0
NA-BHI: non-acidified BHI (pH 7.4)
A-BHI: acidified BHI with several acids at different pH values
MM: mineral medium (de Jonge et al., 2003)
MM+ARG: mineral medium supplemented with 0.01% (p/v) arginine
MM+LYS: mineral medium supplemented with 0.01% (p/v) lysine
MM+GLU: mineral medium supplemented with 0.01% (p/v) glutamic acid
$^{a\text{-}c}$: D-values (mean of three experiments ± SD) with different superscript in the same column are significantly different ($P<0.05$).
$_{1\text{-}3}$: D-values (mean of three experiments ± SD) with different subscript in the same row are significantly different ($P<0.05$).

Table 3. Change in relative gene expression in *Salmonella* Typhimurium CECT 443 as measured by qPCR[a]

Growth conditions	Fold change in gene expression[b]			
	adi	*adiY*	*cadA*	*cadB*
B-BHI	1.0	1.0	1.0	1.0
NA-BHI	95.3	9.1	18.7	3.8
A-BHI$_{\text{acetic pH 6.4}}$	85.6	3.9	12.5	2.2
A-BHI$_{\text{citric pH 6.4}}$	93.2	1.4	14.7	2.3
A-BHI$_{\text{citric pH 4.5}}$	110.2	6.7	16.6	6.6
A-BHI$_{\text{lactic pH 6.4}}$	108.0	1.8	35.3	1.6
A-BHI$_{\text{lactic pH 5.4}}$	99.1	4.6	24.0	7.3
A-BHI$_{\text{HCl pH 6.4}}$	58.6	2.7	12.9	3.9
A-BHI$_{\text{HCl pH 4.5}}$	33.3	1.9	21.0	6.2

[a] The *rrsA* gene, encoding the 16S ribosomal RNA, was used as a housekeeping gene for normalization
[b] Relative gene expression of non-acid adapted control cells was set at 1.0. The standard deviation of the mean change in the experiment was < 20%

DISCUSSION

Salmonella spp. continues to persist as a concern in the food industry and Public Health due to their increasing implication in outbreaks linked to the consumption of food products. Acid adaptation could contribute to the epidemiology of salmonellosis, as this adaptive response may increase their ability to survive in extreme acid conditions, such as that found in the gastrointestinal tract. Acid adaptation responses have been reported for many Gram-negative and Gram-positive bacteria, including *Salmonella* spp. (Bacon et al., 2003; Greenacre et al., 2003; Yuk and Schneider, 2006; Álvarez-Ordóñez et al., 2009), *Escherichia coli* (Leenanon and Drake, 2001; Yuk and Marshall, 2004) and *Listeria monocytogenes* (Gahan and Hill, 1999; Greenacre et al., 2003). These studies reported an Acid Tolerance Response (ATR) for microbial cells after their exposure to a mild acid environment. Our

results showed that acid adapted *S.* Typhimurium obtained under the different acidic growth conditions had greater acid resistance during its subsequent exposure to MM pH 2.5 than non-acid adapted cells. The ATR developed was not dependent on the pH value of the growth medium or on the type of acid used to obtain acid adapted cells. Although on some occasions a specific effect of the type of acidulant has been reported (Greenacre et al., 2003; Yuk and Schneider, 2006), our different results could be attributed to the different experimental conditions used for acid adaptation and acid challenge. Thus, in a recent study carried out in our laboratory for *S.* Typhimurium (Álvarez-Ordóñez et al., 2009) a different effectiveness of organic acids when BHI was used for both adaptation and acid challenge was observed, but these differences were not found when a meat extract was used for acid adaptation.

Our results also show that acid adapted *S.* Typhimurium survived better at pH 2.5 in the presence of arginine and lysine, but glutamic acid did not exert any protective effect. Álvarez-Ordóñez et al. (2009) previously showed that the use of a meat extract as challenge medium protected *S.* Typhimurium against acid inactivation and stated that this protective effect could be due to the higher protein content found in this medium. Similar results were previously obtained by Waterman and Small (1998) using microorganisms inoculated onto ground beef or boiled egg white. Although it is unknown how food can protect bacteria from extreme acid conditions, it has been reported that the presence of amino acids in the acid challenge media could play a significant role (Lin et al., 1995; Waterman and Small, 1996). It should be noted that the acid resistance found for non-acid adapted cells challenged in the presence of amino acids was not significantly different than that corresponding to cells challenged in MM. Therefore, we can conclude that arginine and lysine-dependent systems are only active in acid environments, and this could explain why several authors using *S.* Typhimurium non-stressed cells have not found any amino acid-dependent acid resistance response for this microorganism (Lin et al., 1995; Foster, 1999). Our findings agree with results previously reported by Park et al. (1996), de Jonge et al. (2003) and Kieboom and Abee (2006). Park et al. (1996) showed that *S.* Typhimurium cells pre-exposed to acidic conditions will effectively undergo an acid-shock-induced acid tolerance in the presence of lysine,

and proposed a pH-homeostatic mechanism as responsible of the enhanced acid resistance based on the activity of lysine decarboxylase, with the operon *cadBA* being acid inducible. In the study by de Jonge et al. (2003) it was shown that stationary phase cultures of *S.* Typhimurium, grown in a nutrient-rich medium in the presence of glucose at pH 5.0, survived better at pH 2.5 in the presence of arginine or lysine. Kieboom and Abee (2006) found that *S.* Typhimurium grown in Luria Broth supplemented with glucose at pH 5.0 and challenged at pH 2.5 showed an enhanced acid tolerance in the presence of arginine. These latter authors also observed a similar protective response when cells were grown or challenged under anoxic conditions. Our results, in addition to data previously reported, clearly confirm that arginine and lysine-dependent pH-homeostatic systems are present in *S.* Typhimurium, but they are only active under acidic conditions, or after exposure to other stresses, such as exposure to anaerobic environments (Kieboom and Abee, 2006).

In this study we checked the relative expression level of *adi* (encoding the arginine decarboxylase) *adiY* (encoding the transcriptional activator of *adi*) *cadA* (encoding the lysine decarboxylase) and *cadB* (encoding a lysine/cadaverine antiporter). Relative expression levels found for both the arginine decarboxylase and lysine decarboxylase related genes, were significantly higher for acid adapted cells - an observation which agrees with their increased acid resistance in the presence of arginine and lysine. As far as we known, only Park et al. (1996) have previously shown lysine decarboxylase to be involved in *S.* Typhimurium ATR. These authors described a model for how the *cadBA* system works. During periods of acid stress, lysine enters the cell and is converted into cadaverine by lysine decarboxylase (CadA) with the consumption of a proton. Cadaverine is then expelled from the cell by CadB in exchange for lysine in the medium and thereby alkalinizing the cytoplasm. With regard to arginine decarboxylase system, it has been shown to be composed of an arginine decarboxylase which converts arginine into agmatine in the cytoplasm with the consumption of a proton. Afterwards, agmatine is expelled from the cell by an arginine/agmatine antiporter, thus causing a rise in the intracellular pH due to proton consumption (Abee and Wouters, 1999). An enhanced relative

expression level for *adi* and *adiY* under acid growth conditions was previously found by Kieboom and Abee (2006), who also described how *S.* Typhimurium disrupted in the arginine decarboxylase gene showed a reduced acid resistance in comparison to the wild type strain.

However, acid adapted cells challenged in the absence of arginine or lysine also developed an ATR, although at lower levels, and this suggests that other protective mechanisms different from pH-homeostatic systems, must be involved in *S.* Typhimurium ATR. It has been previously found that acid adaptation and acid shock result in the induction of several acid shock proteins (ASPs) regulated by the alternative sigma factor RpoS, which is thought to contribute to acid survival. Moreover, other regulatory proteins, such as Fur, and the two-component systems PhoPQ and OmpR/EnvZ regulate a subset of ASPs, contributing to acid tolerance (Audia et al., 2001). Furthermore, macromolecular repair mechanisms, such as O6-methylguanine DNA methyltransferase activity of the Ada regulator, can also be involved in acid resistance responses (Foster, 2000). In addition to these strategies, modifications in membrane fatty acid composition linked to a decreased membrane fluidity could play an important role (Brown et al., 1997; Chang and Cronan, 1999; Kim et al., 2005). We have recently demonstrated for *S.* Typhimurium that the acidification of the growth medium causes a decrease in the unsaturated to saturated fatty acid ratio and in the C18:1 relative concentration and an increase in the cyclopropane fatty acids content, which could modify its subsequent acid resistance (Álvarez-Ordóñez et al., 2008).

To sum up, the results of this study show an increased acid resistance for *S.* Typhimurium acid adapted cells challenged in MM pH 2.5 in the presence of arginine and lysine linked to an enhanced expression of genes encoding for arginine and lysine decarboxylase systems, indicating that the induction of these specialized pH-homeostatic systems plays an important role in *S.* Typhimurium protection against acid stress. However, the ATR observed for acid adapted cells in the absence of amino acids in the challenge medium suggests the existence of other survival strategies. Therefore, further studies would be needed in order to achieve a complete understanding of the molecular aspects of *S.* Typhimurium ATR.

ACKNOWLEDGEMENTS

This work was supported by the Junta de Castilla y León (project number LE002A07). Álvarez-Ordóñez A. gratefully acknowledges the finantial support from the Spanish Ministry of Education.

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6.

Modifications in membrane fatty acid composition of Salmonella Typhimurium *in response to growth conditions and their effect on heat resistance*

Avelino Álvarez-Ordóñez, Ana Fernández, Mercedes López, Ricardo Arenas and Ana Bernardo

International Journal of Food Microbiology, 2008, 123, 212-219

ABSTRACT

The effects of growth temperature (in the range 10-45 °C) and acidification up to pH 4.5 of the culture medium (Brain Heart Infusion, BHI) with different organic acids (acetic, citric and lactic) and hydrochloric acid on membrane fatty acid composition and heat resistance of *Salmonella* Typhimurium CECT 443 were studied. The heat resistance was maximal in cells grown at 45 °C (cells grown in non-acidified BHI showed a D_{58}-value of 0.90 min) and decreased with decreasing growth temperature up to 10 °C (D_{58}-value of 0.09 min). The growth of cells in acidified media caused an increase in their heat resistance. In general, acid adapted cells showed D-values of between 1.5 and 2 times higher than the corresponding for non-acid adapted control cells. This cross-protection response, which has important implications in food processing, was not dependent on the pH value and the acid used to acidify the growth medium. A membrane adaptation corresponding to an increase in the unsaturated to saturated fatty acids ratio (UFA/SFA) and membrane fluidity was observed at low growth temperature. Moreover, the acidification of the growth medium caused a decrease in UFA/SFA ratio and in the C18:1 relative concentration, and an increase in cyclopropane fatty acids (CFA) content mainly due to the increase in cyc19 relative concentration. Thus, acid adapted cells showed CFA levels 1.5 times higher than non-acid adapted control cells. A significant proportion of unsaturated fatty acids were converted to their cyclopropane derivatives during acid adaptation. These changes in membrane fatty acid composition result in cells with decreased membrane fluidity. A clear relation between membrane fatty acid composition and heat resistance was observed. In general, D-values were maximum for cells with low UFA/SFA ratio, and, consequently, with low membrane fluidity. Moreover, CFA formation played a major role in protecting acid adapted cells from heat inactivation. However, changes observed in membrane fatty acid composition are not enough to explain the great thermotolerance of cells grown at 45 °C. Thus, other mechanisms, such as the synthesis of Heat Shock Proteins, could be responsible for this increase in the bacterial heat resistance.

INTRODUCTION

Salmonella spp. are a leading cause of bacterial foodborne disease all over the world, causing a diversity of illnesses that include typhoid fever, gastroenteritis and septicemia (D'Aoust, 2000). Although more than 1500 *Salmonella* serovars exist, the most frequently isolated serotype is *Salmonella* Typhimurium, which accounts for about 35% of reported human isolates (Wilmes-Riesenberg et al., 1996). Heat treatment has long been recognized as a primary method for preserving foods. Several factors, such as growth medium (Annous and Kozempel, 1998; Casadei et al., 1998), growth temperature (Rowan and Anderson, 1998; Martínez et al., 2003), growth phase (Rees et al., 1995; Martínez et al., 2003), water activity (Fernández et al., 2007) and pH (Annous and Kozempel, 1998), may influence the heat resistance of microorganisms. Furthermore, bacterial thermal resistance could be influenced by the microbial response to other physiological stresses. *S.* Typhimurium encounters many diverse and extreme environments, and, as a result it has developed responses to combat these adverse conditions (Foster and Spector, 1995). One of the most studied stress response in *Salmonella* is the Acid Tolerance Response (ATR), induced to protect it against severe acid stress (Greenacre et al., 2003; Yuk and Schneider, 2006). Furthermore, this ATR also appears to provide protection to other stresses, such as heat (Ingham and Uljas, 1998; Ryu and Beuchat, 1998; Buchanan and Edelson, 1999; Duffy et al., 2000; Mazzotta, 2001), osmotic and oxidative stress (Lee et al., 1995; O'Driscoll et al., 1996; Lou and Yousef, 1997; Wilde et al., 2000), and ionizing radiations (Buchanan et al., 1999). This cross-protection is of a great interest in food preservation where multiple stresses are often used, showing a concern for microbiological safety of processed foods.

Although several researches have been carried out to try to elucidate the molecular and physiological changes associated with *Salmonella* stress and cross-protection responses, the mechanisms involved are not completely understood, although it is generally acknowledged that several genes, such as *rpoS*, and proteins, like some Acid Shock Proteins –ASP– and Heat Shock Proteins –HSP– are implicated (Foster and Spector, 1995; Foster, 2000; Dodd and Aldsworth, 2002).

Probably other macromolecules, such as membrane fatty acids, are also involved in these bacterial responses. However, few studies (to our knowledge, none for *S.* Typhimurium) have been carried out to clarify its potential role. It has been shown that variations in growth temperature (Suutari and Laakso, 1994; Russell et al., 1995; Kadner, 1996; Annous et al., 1997, 1999; Casadei et al., 2002; Wang et al., 2005), pH (Russell et al., 1995; Brown et al., 1997; de Jonge et al., 2003; Sampathkumar et al., 2004; Wang et al., 2005), ethanol concentration (Teixeira et al., 2002) and external osmolality (Guillot et al., 2000), as well as transition to the stationary phase (Russell et al., 1995; Kadner, 1996; Casadei et al., 2002) lead to changes in membrane fatty acid composition. Furthermore, it has been shown that membrane fatty acid composition and membrane fluidity affect the bacterial thermotolerance (Annous et al., 1999; Sampathkumar et al., 2004). Annous et al. (1999) showed for *Pediococcus* spp. that cells obtained at low temperatures had an increase in the concentration of unsaturated fatty acids of the cytoplasmic membrane, resulting in a higher membrane fluidity, which was linked with a reduced thermal resistance. Sampathkumar et al. (2004) showed an increased thermotolerance in *Salmonella* Enteritidis when it was pretreated at alkaline pH values, which are linked to an increased saturated and cyclic to unsaturated fatty acid ratio. The aim of this work was to study the effects of acidic and growth temperature conditions on the thermotolerance and membrane fatty acid profile of *S.* Typhimurium CECT 443 in an attempt to clarify the role of membrane composition and fluidity in *Salmonella* stress and cross-protection responses.

MATERIAL AND METHODS

Bacterial strain and culture conditions

The *Salmonella enterica* serovar Typhimurium strain CECT 443 used in this study was obtained from Colección Española de Cultivos Tipo (CECT) (Spanish Type Culture Collection). The lyophilized cultures were revived in Brain Heart Infusion (BHI) (Oxoid) and incubated for 24 h at 37 °C. Pure cultures were

maintained on BHI agar (BHI-Agar) plates at 4 °C. Precultures were prepared by transferring an isolated colony from a plate into a test tube containing 10 mL of sterile BHI followed by incubation at 37 °C for 24 h. Flasks containing 50 mL of sterile BHI (pH 7.4) non-acidified and acidified at pH values of 6.4, 5.4 and 4.5 with several acids: acetic (Prolab), citric (Sigma), lactic (Merck) and hydrochloric (Panreac) were inoculated with the subculture to a final concentration of 10^3 cells/mL. These pH values were chosen since they are representative of the slightly acidic environment that organisms could find in many foods. In order to obtain non-acid adapted control cells, buffered BHI adjusted to pH 7.0 by addition of Sorensen buffer of 0.2 M (bisodium (Merck)–monopotassium (Panreac) phosphate) was used. These cultures were then incubated at different growth temperatures (10, 25, 37 and 45 °C) for the time needed to reach the late stationary phase of growth and were used to determine the thermotolerance and membrane fatty acid composition. When acidified BHI was used as growth medium the experiments were carried out, for each acid, only at the highest and the lowest pH values which allowed *S.* Typhimurium growth.

Heat treatments

Heat treatments were carried out in a thermoresistometer TR-SC as described by Condón et al. (1993). Once the temperature (58 °C) of the heat treatment medium (350 mL of BHI) was stabilized (T±0.05 °C) an inoculum of each culture was injected. During heating, samples of 0.2 mL were removed at predetermined time intervals and were plated in BHI-Agar. Plates were then incubated at 37 °C for 48 h and survivors were counted with a modified Image Analyser Automatic Counter (Protos Analytical Measuring Systems, Cambridge, UK) as described elsewhere (Ibarz et al., 1991).

Heat resistance parameters

D-values (min) were determined by plotting the log number of survivors against time. The line that best fitted survivor plots was determined by linear regression (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA), and the negative reciprocal of the slope was used for D-

value determinations. D-values were compared using Student's t-test (Steel and Torrie, 1986) (Statistica for Windows version 4.5, Statsoft, Inc., Tulsa, OK, USA).

Fatty acid analysis

The fatty acid analyses were carried out from 1 L of each bacterial culture obtained as previously described. Cultures were centrifuged at 4 °C for 10 min at 8000 g (Eppendorf centrifuge 5804R) and pellets were resuspended in 50 mL of sterile Mili Q water. This washing phase was repeated once.

In order to extract the cell lipids, the method of Bligh and Dyer (1959) was used. To obtain a monophasic system, 3.75 mL of a methanol:chloroform (2:1, v/v) (Panreac) mixture was added for each mL of bacterial suspension in Mili Q water. Samples were kept at room temperature for 1 h with shaking. In order to convert the system into a diphasic state, 2.5 mL of a chloroform:Mili Q water (1:1 v/v) mixture was added for each mL of bacterial suspension and samples were again kept at room temperature for 1 h with shaking. Afterwards, samples were centrifuged at 1000 g for 5 min and the chloroform-rich phase (bottom) containing the cellular lipids was removed and the solvent was evaporated. The lipid residue was immediately dissolved in hexane (Merck) and stored at -18 °C under a nitrogen atmosphere, adding BHT (Sigma) as antioxidant until chromatographic analyses were carried out. Methylation of the fatty acids to yield their fatty acid methyl esters (FAME) was achieved in an alkaline medium using $NaOCH_3$ (Merck) as described elsewhere (Mossoba, 2001). The membrane fatty acid composition of the bacteria was determined on a gas chromatographer (HP 6890, Hewlett Packard, Avondale, PA) equipped with a mass selective detector (HP 5973) using a capillary column (60 m, 0.25 mm, 0.20 μm). The fatty acid methyl esters were first identified by comparing their retention times with those of known standards (Supelco). The identities of the fatty acid methyl esters were confirmed by comparing their mass spectra with the data bank (Hewlett Packard). Results were expressed as relative percentages of each fatty acid, which were calculated as the ratio of the surface area of the considered peak to the total area of all peaks.

RESULTS

Influence of growth conditions on the thermotolerance of S. Typhimurium

In order to study the effects of acidification and growth temperature on the heat resistance of *S.* Typhimurium, cells grown at 10, 25, 37 and 45 °C in non-acidified BHI (pH 7.4), buffered BHI (pH 7.0) and BHI acidified with acetic, citric, lactic and hydrochloric acids (pH 6.4; 5.4 and 4.5) were heated at 58 °C in BHI. Survival curves obtained fitted properly into a first order kinetic. The goodness of fit was determined both by visual inspection and R^2 value, which ranged from 0.91 to 0.99 (data not shown). An example of survival curves obtained at 58 °C for cells grown at 37 °C in buffered BHI, non-acidified BHI and acidified BHI up to pH 4.5 with citric acid is shown in Fig. 1. D-values calculated are shown in Table 1 as mean values and standard deviation.

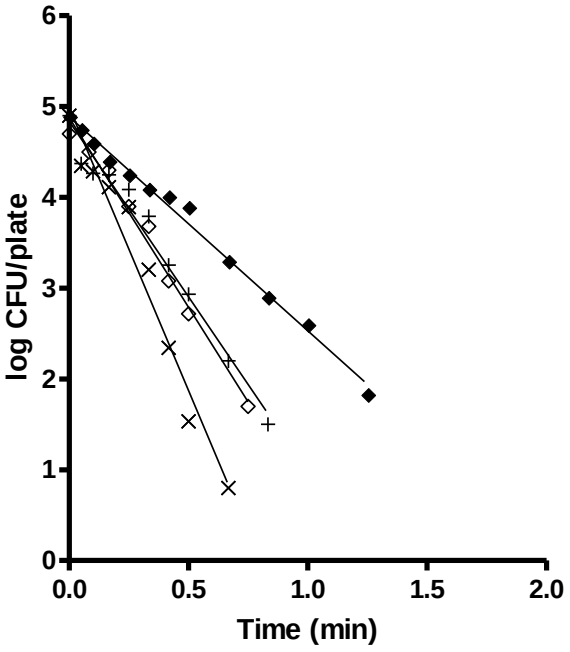

Fig. 1. Survival curves at 58 ºC for *S.* Typhimurium (CECT 443) cells grown at 37ºC in buffered BHI (non-acid adapted control cells) (x), non-acidified BHI (+), and acidified BHI with citric acid at pH values of 6.4 (♦) and 4.5 (◊).

In all cases, *S.* Typhimurium cells showed minimum D-values when cells were grown at 10 °C and its heat resistance significantly increased (P<0.05) with increasing growth temperature up to 45 °C, showing D-values around ten times higher. A shift in growth temperature from 10 to 37 °C increased D-values around three times, and D-values obtained at 45 °C were three times higher than those obtained at 37 °C. The relationship between D-values and growth temperature is shown in Fig. 2. Under all culture conditions which allowed the growth of *S.* Typhimurium at all the temperatures tested this relationship followed an exponential kinetic which depended on the growth medium used (Table 2).

Table 1. D-values (min) for heat treated (58ºC) cells of *S.* Typhimurium (CECT 443) grown at 10, 25, 37 and 45 ºC in buffered BHI (non-acid adapted control cells), non-acidified BHI and acidified BHI at different pH values with several acids.

GROWTH MEDIUM	**GROWTH TEMPERATURE**			
	10ºC	25ºC	37ºC	45ºC
B-BHI	$0.04\pm0.005^{a}{}_{1}$	$0.06\pm0.010^{a}{}_{2}$	$0.18\pm0.015^{a}{}_{3}$	$0.46\pm0.080^{a}{}_{4}$
NA-BHI	$0.09\pm0.015^{bc}{}_{1}$	$0.07\pm0.010^{ab}{}_{1}$	$0.28\pm0.060^{bd}{}_{2}$	$0.90\pm0.050^{b}{}_{3}$
A-BHI$_{\text{acetic pH 6.4}}$	NG	$0.09\pm0.010^{b}{}_{1}$	$0.37\pm0.030^{bc}{}_{2}$	NG
A-BHI$_{\text{citric pH 6.4}}$	$0.10\pm0.010^{c}{}_{1}$	$0.11\pm0.005^{c}{}_{1}$	$0.45\pm0.055^{c}{}_{2}$	$1.02\pm0.180^{bc}{}_{3}$
A-BHI$_{\text{citric pH 4.5}}$	NG	$0.12\pm0.005^{c}{}_{1}$	$0.24\pm0.030^{d}{}_{2}$	NG
A-BHI$_{\text{lactic pH 6.4}}$	$0.07\pm0.010^{b}{}_{1}$	$0.12\pm0.005^{c}{}_{2}$	$0.30\pm0.050^{bd}{}_{3}$	$0.70\pm0.105^{c}{}_{4}$
A-BHI$_{\text{lactic pH 5.4}}$	NG	$0.08\pm0.010^{ab}{}_{1}$	$0.36\pm0.060^{bc}{}_{2}$	NG
A-BHI$_{\text{HCl pH 6.4}}$	$0.07\pm0.005^{b}{}_{1}$	$0.11\pm0.010^{c}{}_{2}$	$0.37\pm0.040^{bc}{}_{3}$	$0.78\pm0.140^{bc}{}_{4}$
A-BHI$_{\text{HCl pH 4.5}}$	NG	$0.11\pm0.005^{c}{}_{1}$	$0.37\pm0.070^{bc}{}_{2}$	NG

B-BHI: Buffered BHI pH 7.0
NA-BHI: non-acidified BHI (pH 7.4)
A-BHI: acidified BHI with different acids: acetic, citric, lactic and HCl at different pH values
$^{a-d}$: D-values (mean of three experiments ± SD) with different superscript in the same column are significantly different (P<0.05).
$_{1-4}$: D-values (mean of three experiments ± SD) with different subscript in the same row are significantly different (P<0.05).
NG: no bacterial growth

Thermal resistance for cells grown at 37 °C in non-acidified BHI (pH 7.4) and BHI acidified with several acids (acid adapted cells), with D_{58}-values ranging from 0.24 to 0.45 min, was significantly higher than that corresponding to non-acid

adapted control cells (D_{58} = 0.18 min). This behaviour turned out to be similar at the different temperatures tested (10, 25 and 45 °C). In general, acid adapted cells (grown in non-acidified or acidified BHI) showed D-values 1.5–2 times higher than those corresponding to non-acid adapted control cells (grown in buffered BHI). Our results also show that neither the type of acidulant used nor the pH value of the growth medium significantly modified the cross-protection response found.

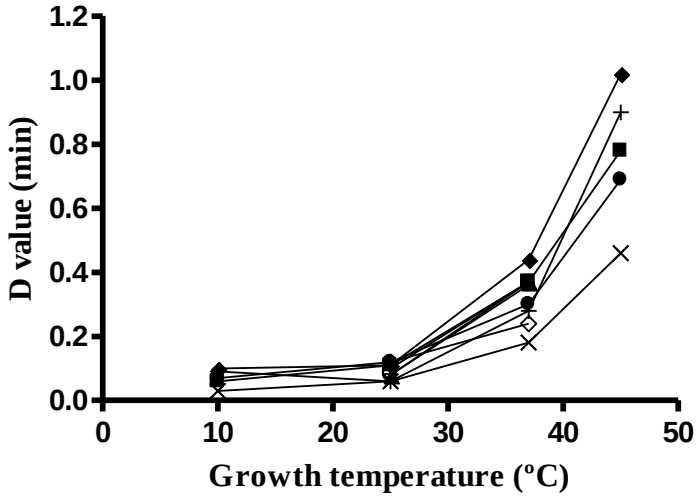

Fig. 2. Relationship between D-values and growth temperature for *S.* Typhimurium cells grown in buffered BHI (non-acid adapted control cells) (x), non-acidified BHI (+), and acidified BHI at different pH values with several acids: A-$BHI_{acetic\ pH\ 6.4}$ (▲), A-$BHI_{citric\ pH\ 6.4}$ (♦), A-$BHI_{citric\ pH\ 4.5}$ (◊), A-$BHI_{lactic\ pH\ 6.4}$ (●), A-$BHI_{lactic\ pH\ 5.4}$ (○), A-$BHI_{HCl\ pH\ 6.4}$ (■) and A-$BHI_{HCl\ pH\ 4.5}$ (□).

Membrane fatty acid composition of S. Typhimurium: *influence of growth temperature*

The membrane fatty acid composition of *S.* Typhimurium CECT 443 cultured under the different growth conditions was determined using a chromatographic method (Table 3). Fourteen fatty acids were found when cells were grown in buffered BHI (non-acid adapted control cells) at 37°C. The seven main peaks were identified as tetradecanoic (myristic) acid (C14:0), hexadecanoic

(palmitic) acid (C16:0), hexadecenoic (palmitoleic) acid (C16:1), methylenhexadecanoic acid (cyc17), octadecanoic (stearic) acid (C18:0), octadecenoic (oleic or vaccenic) acids (C18:1) and methylenoctadecenoic (dihydrosterculic or lactobacillic) acids (cyc19). Their relative percentages were between 3 and 31%, corresponding to more than 93% of all fatty acids observed. Seven minor fatty acids were also detected at lower relative concentrations: C8:0, C10:0, C12:0, C13:0, C17:0, C19:0 and C20:0. For cells grown under the other conditions studied a qualitatively similar fatty acid composition was found with the same main peaks but with different relative percentages. The minor fatty acids observed in non-acid adapted control cells were not present in all the growth conditions studied and some new minor fatty acids were found in these cultures (C11:0, C15:0, C18:2 and C18:3).

Table 2. Equations that describe the relationship between the heat resistance (D-value) of *S.* Typhimurium and the growth temperature.

GROWTH CONDITIONS	EQUATION	R^2
B-BHI	$D = 0{,}0033 * e^{(0{,}11*Tg)}$	0.99
NA-BHI	$D = 0{,}0016 * e^{(0{,}14*Tg)}$	0.98
$A\text{-}BHI_{citric\ pH\ 6.4}$	$D = 0{,}0098 * e^{(0{,}10*Tg)}$	0.99
$A\text{-}BHI_{lactic\ pH\ 6.4}$	$D = 0{,}0107 * e^{(0{,}09*Tg)}$	0.99
$A\text{-}BHI_{HCl\ pH\ 6.4}$	$D = 0{,}0120 * e^{(0{,}09*Tg)}$	0.99

B-BHI: Buffered BHI pH 7.0
NA-BHI: non-acidified BHI (pH 7.4)
A-BHI: acidified BHI with different acids: citric, lactic and HCl at pH 6.4
Tg: growth temperature

Total saturated fatty acids (SFA), total unsaturated fatty acids (UFA) and total cyclic fatty acids (CFA) were used to determine the differences among membrane fatty acids of *S.* Typhimurium cells grown under the different conditions. The UFA/SFA ratio was used as an indirect indicator of the membrane fluidity. It has been previously reported that membranes with high UFA/SFA ratio show a high

Table 3. Membrane fatty acid composition of *S.* Typhimurium CECT 443 cells grown under different conditions

Growth conditions		C14:0	C16:0	C16:1	cyc17	C18:0	C18:1	cyc19	Minor FA	SFA	UFA	CFA	UFA / SFA
Temperature	**Media**												
10 ºC	B-BHI	7.75	26.31	10.46	5.52	11.41	24.65	5.03	9.84	54.23	35.11	10.55	0.65
	NA-BHI	6.43	30.33	5.21	17.10	9.64	22.60	5.99	2.69	48.10	28.81	23.10	0.60
	A-BHI$_{\text{citric pH 6.4}}$	5.27	26.84	2.50	32.93	3.53	13.35	10.69	4.89	36.53	19.85	43.62	0.54
	A-BHI$_{\text{lactic pH 6.4}}$	7.69	27.34	3.22	25.28	4.18	17.81	13.63	0.85	40.06	21.02	38.91	0.52
	A-BHI$_{\text{HCl pH 6.4}}$	10.04	23.54	4.25	14.01	9.07	20.80	9.46	10.42	50.99	25.05	23.47	0.49
25 ºC	B-BHI	6.72	27.53	3.80	18.72	8.05	24.26	6.97	3.96	46.25	28.06	25.69	0.61
	NA-BHI	4.46	31.28	4.32	25.83	0.39	13.87	19.20	0.66	36.78	18.19	45.03	0.49
	A-BHI$_{\text{acetic pH 6.4}}$	5.88	38.11	1.64	33.73	0.82	4.72	13.92	1.18	45.99	6.36	47.65	0.14
	A-BHI$_{\text{citric pH 6.4}}$	5.56	33.02	1.60	33.13	0.66	5.41	19.26	1.35	40.60	7.01	52.39	0.17
	A-BHI$_{\text{citric pH 4.5}}$	5.30	36.17	1.41	26.20	0.81	8.55	20.42	1.13	43.41	9.96	46.63	0.23
	A-BHI$_{\text{lactic pH 6.4}}$	6.38	33.68	1.49	29.90	0.74	5.80	20.05	1.96	42.76	7.29	49.95	0.17
	A-BHI$_{\text{lactic pH 5.4}}$	5.77	36.52	1.24	27.74	0.88	7.06	19.70	1.10	44.27	8.30	47.43	0.19
	A-BHI$_{\text{HCl pH 6.4}}$	5.95	34.92	2.34	23.18	2.22	8.95	19.54	2.90	45.99	11.29	42.72	0.25
	A-BHI$_{\text{HCl4.5}}$	4.09	38.63	0.57	34.33	1.81	2.33	17.56	0.68	45.21	2.90	51.88	0.06
37 ºC	B-BHI	5.90	31.77	7.18	23.26	2.82	15.47	6.82	6.78	47.27	22.66	30.07	0.48
	NA-BHI	5.69	29.50	4.78	26.56	1.76	10.91	19.52	1.29	38.24	15.68	46.07	0.41
	A-BHI$_{\text{acetic pH 6.4}}$	5.75	39.71	1.88	33.10	1.04	3.01	15.15	0.35	46.86	4.89	48.25	0.10
	A-BHI$_{\text{citric pH 6.4}}$	6.42	33.42	1.39	26.56	2.19	4.30	22.49	3.23	45.26	5.70	49.05	0.13
	A-BHI$_{\text{citric pH 4.5}}$	7.21	39.73	1.23	28.14	2.71	2.38	15.36	3.24	52.88	3.62	43.50	0.07
	A-BHI$_{\text{lactic pH 6.4}}$	5.18	38.66	1.04	27.23	0.87	6.44	19.79	1.79	46.50	7.47	46.02	0.16
	A-BHI$_{\text{lactic pH 5.4}}$	7.18	33.75	1.12	26.43	3.44	4.59	18.17	5.31	49.69	5.71	44.60	0.11
	A-BHI$_{\text{HCl pH 6.4}}$	5.07	35.43	1.54	28.75	0.78	5.84	20.96	1.63	42.91	7.38	49.71	0.17
	A-BHI$_{\text{HCl pH 4.5}}$	7.74	35.55	1.21	25.26	2.83	6.72	16.48	4.21	50.33	7.93	41.74	0.16
45 ºC	B-BHI	3.58	40.88	3.46	27.86	2.71	7.39	12.71	1.41	48.58	10.85	40.57	0.22
	NA-BHI	3.61	39.14	1.49	22.23	2.90	10.19	17.18	3.27	48.92	11.68	39.40	0.24
	A-BHI$_{\text{citric pH 6.4}}$	5.25	38.12	0.81	21.17	5.14	13.07	13.41	3.02	50.64	14.78	34.58	0.29
	A-BHI$_{\text{lactic pH 6.4}}$	2.52	38.92	2.05	19.88	7.47	6.55	19.44	3.17	52.08	8.61	39.32	0.17
	A-BHI$_{\text{HCl pH 6.4}}$	5.37	35.81	0.46	20.28	8.15	10.09	13.68	6.15	54.74	11.30	33.96	0.21

B-BHI: Buffered BHI pH 7.0 NA-BHI: non-acidified BHI (pH 7.4) A-BHI: acidified BHI with different acids: acetic, citric, lactic and HCl at different pH values
C14:0: tetradecanoic (myristic) acid C16:0: hexadecanoic (palmitic) acid C16:1: hexadecenoic (palmitoleic) acid
cyc17: methylenhexadecanoic acid C18:0: octadecanoic (stearic) acid C18:1: octadecenoic (oleic or vaccenic) acids
cyc19: methylenoctadecenoic (dihydrosterculic or lactobacillic) acids Minor FA: Minor fatty acids
SFA: Total saturated fatty acids UFA: Total unsaturated fatty acids CFA: Total cyclic fatty acids

fluidity (Casadei et al., 2002). As expected, growth of *S.* Typhimurium at different temperatures resulted in differences in membrane fatty acid composition. The proportion of CFA obtained for all the culture conditions was not affected by the growth temperature in the range 25-45 ºC. However, minimum CFA levels were observed at 10 ºC. The UFA/SFA ratio observed for non-acid adapted control cells was higher (0.65 and 0.61) in cells grown at the lowest temperatures (10 and 25 ºC, respectively), and decreased with increasing growth temperature, reaching a minimum at 45 °C (0.22). This fact was due to changes in the proportion of unsaturated fatty acids, whereas the proportion of saturated fatty acids remained almost constant. At low temperatures (10 and 25 °C) the proportion of unsaturated fatty acids, mainly C18:1, increased, modifying the physical properties of the bacterial membrane. A similar behaviour was observed for *S.* Typhimurium cells grown in non-acidified BHI, in which the UFA/SFA ratio was inversely proportional to growth temperature. However, when cells were grown in acidified BHI, the UFA/SFA ratio found at 25, 37 and 45 ºC was quite similar and very low (between 0.06 and 0.29). *S.* Typhimurium cells grown in acidified BHI at 10 ºC showed ratios significantly higher (between 0.49 and 0.54).

***Effect of the acid adaptation on membrane fatty acid composition of S.* Typhimurium**

The membrane fatty acid composition of acid adapted and non-acid adapted control cells at the different growth temperatures assayed is also shown in Table 3. The UFA/SFA ratio observed for non-acid adapted control cells (0.48) and cells grown in non-acidified BHI (0.41) was significantly higher than those found for *S.* Typhimurium cells grown in acidified BHI (between 0.07 and 0.17) when the growth temperature used was 37 ºC.

Moreover, the acidification of the growth medium also induced changes in the proportion of CFA observed. Acid adapted cells (grown in acidified or non-acidified BHI at 37 °C) showed CFA levels 1.5 times higher than non-acid adapted control cells. The effect of acid adaptation on C18:1 and cyc19 relative concentrations is shown in Fig. 3. These fatty acids were greatly affected by the

acidification of the growth medium which rendered a significant decrease in C18:1 content and a concomitant increase in cyc19 level probably due to the fact that a significant proportion of the UFA (C18:1) was converted to CFA (cyc19) during acid adaptation by the addition of a methyl group from S-adenosyl-L-methionine across the *cis* double bond.

It is important to note that the modifications in the membrane fatty acid composition observed for *S.* Typhimurium acid adapted cells were not dependent on the pH value or on the acid used to acidify the growth medium.

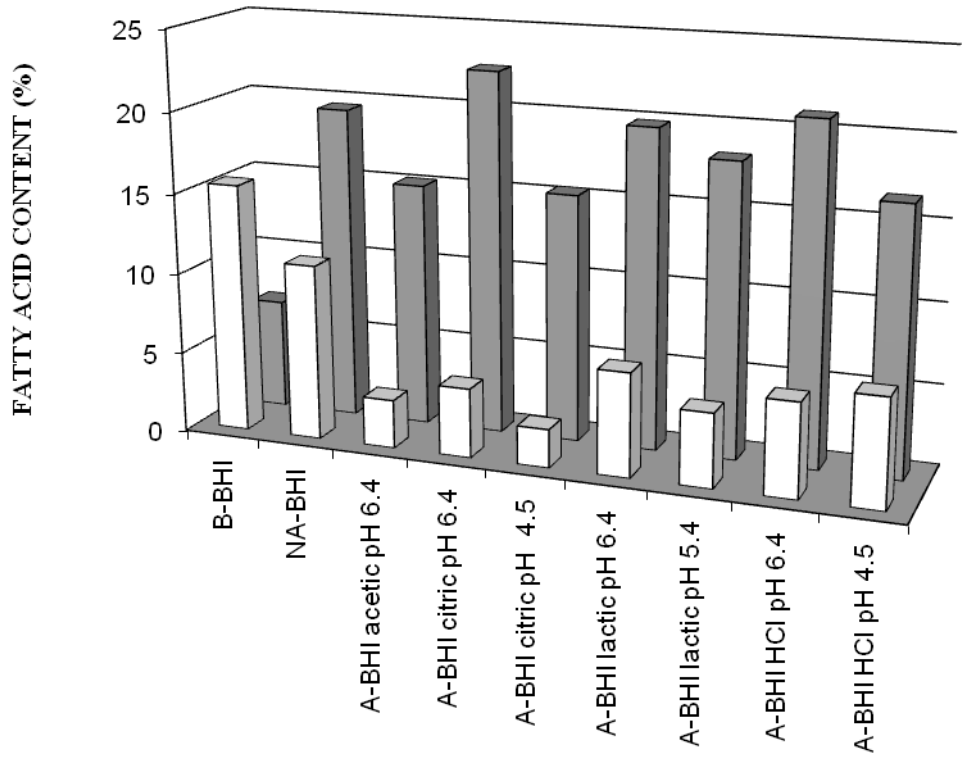

Fig. 3. Influence of acid adaptation on the membrane content in C18:1 (white) and cyc19 (grey) for *S.* Typhimurium cells grown at 37ºC.

S. Typhimurium cells grown at 10 and 25 °C showed the same behaviour as those obtained at 37 °C. However, the acidification of the growth medium did not greatly affect the membrane fatty acid profile of *S.* Typhimurium cells grown at 45

°C. At this growth temperature the membrane content in SFA, UFA and CFA was almost the same for acid and non-acid adapted control cells (Table 3).

DISCUSSION

Influence of growth conditions on the thermotolerance of S. Typhimurium

It is generally assumed that microorganisms grown at higher temperatures have greater resistance to heat (Ng et al., 1969; Dega et al., 1972; Pagán et al., 1999). However, there is not agreement on the growth temperature at which the microbial heat resistance is maximal. Whereas some authors (Ng et al., 1969; Dega et al., 1972; Pagán et al., 1999) have reported a gradual increase in heat resistance in response to a rise in the growth temperature, Juneja et al. (1998), Mañas et al. (2003) and Martínez et al. (2003) found that cells grown at temperatures above 37 °C showed a similar or even lower heat resistance than those grown at 37 °C for *L. monocytogenes*, *S.* Typhimurium and *E. faecium*, respectively. In our case, *S.* Typhimurium heat resistance was maximal in cells grown at 45 °C and decreased with decreasing growth temperature up to 10 °C.

On the other hand, the growth of *S.* Typhimurium in acidified BHI or in non-acidified BHI caused an acid adaptation which increased its heat resistance. BHI is a medium which contains 0.2% glucose. Thus, the growth of *S.* Typhimurium causes a decrease in the extracellular pH to a final pH value of around 6.2, which probably causes the development of an acid adaptation response in *S.* Typhimurium cells. Increased thermal tolerance resulting from bacterial responses to exposure to an acidic environment has been reported for *L. monocytogenes* (Farber and Paggoto, 1992; Lou and Yousef, 1996; Juneja et al., 1998), *E. coli* (Ryu and Beuchat, 1998, 1999; Buchanan and Edelson, 1999; Rowe and Kirk, 1999; Duffy et al., 2000) and *Salmonella* spp. (Leyer and Johnson, 1993; Wilde et al., 2000; Mazzotta, 2001; Bacon et al., 2003;Tosun and Gonul, 2003). This cross-protection response has

important implications in food processing, as *Salmonella* could survive after heat treatments under certain conditions.

Membrane fatty acid composition of S. Typhimurium: *influence of growth temperature*

The main fatty acids observed for *S.* Typhimurium cells (Table 3) were similar to those previously described for *S.* Enteritidis (Sampathkumar et al., 2004) and other gram negative bacteria as *E. coli* (Casadei et al., 2002).

A membrane adaptation of *S.* Typhimurium, corresponding to an increase in UFA/SFA ratio and membrane fluidity and a decrease in CFA levels was observed at low growth temperature. These results agreed with those previously reported for *L. acidophilus* (Wang et al., 2005), *E. coli* (Casadei et al., 2002) and *Pediococcus* spp. (Annous et al., 1999). On the other hand, the opposite effect was described by other authors (FernándezMurga et al., 2000, Guillot et al., 2000). For instance, Guillot et al. (2000) reported an increase in the UFA/SFA ratio of *L. lactis* cells when the growth temperature increased from 30 to 40 °C. The primary response in *S.* Typhimurium to a decrease in growth temperature is a marked increase in the level of unsaturation of the membrane lipids. These changes are characteristic of an organism which uses the so-called anaerobic pathway of fatty acid biosynthesis, in which most of the increase in unsaturated fatty acids is in C18:1 (Russell et al., 1995). Thus, the changes in the composition of the cell membrane in response to growth temperature serve the purpose of maintaining a degree of fluidity, which is compatible with life.

Effect of the acid adaptation on membrane fatty acid composition of S. Typhimurium

A membrane adaptation was observed in *S.* Typhimurium cells adapted to low pH, as shown by a decrease in UFA/SFA ratio and in the C18:1 relative concentration, and by an increase in CFA content, by means of a rise in cyc19 relative concentration. These changes in membrane fatty acid composition result in *S.* Typhimurium cells with decreased membrane fluidity. A decrease in UFA and an increase in SFA in acid adapted cells were previously reported by Brown et al.

(1997). On the contrary, other authors have found an increase in UFA/SFA ratio at low pH values for other microorganisms, such as *L. acidophilus* (Wang et al., 2005), *L. reuteri* (Palmfeldt and Hahn Hagerdal, 2000), *O. oeni* (Bastianini et al., 2000) and *S. thermophilus* (Béal et al., 2001). An important role of CFA in microbial acid adaptation responses was previously described by Brown et al. (1997), who found a great increase in CFA content in acid habituated cells for five E. coli strains. However, the acidification of the growth medium did not greatly affect the membrane fatty acid profile of cells grown at 45 °C. A possible explanation for this last observation is the fact that this growth temperature is so extreme that causes great membrane fatty acid changes, which disguise the real effect of the acid adaptation on the membrane fatty acid composition.

Relationship between the membrane fatty acid composition and the thermotolerance of S.* Typhimurium *grown at different pH values and temperatures

S. Typhimurium cells under the different growth conditions assayed showed important modifications in membrane fatty acid composition that were linked to their heat resistance. Acid adapted cells developed a cross-protection response, showing higher heat resistance than non-acid adapted control cells. This great thermotolerance was associated with a low UFA/SFA ratio, a high CFA content, a low C18:1 and a high cyc19 relative concentration. Low UFA/SFA ratio has previously been linked to less membrane fluidity (Casadei et al., 2002; Wang et al., 2005). It is generally admitted that cells regulate their lipid composition in order to achieve a degree of fluidity compatible with life (Teixeira et al., 2002). Our results show that D-values obtained were higher for cells with low membrane fluidity. Similar results have been previously found for *Pediococcus* spp. (Annous et al., 1999) and *S.* Enteritidis (Sampathkumar et al., 2004).

The increase of CFA levels in acid adapted cells also seems to be important for the development of cross-protection responses against heat treatments. Previous researchers concluded that the synthesis of CFA in cell membrane phospholipids during acid adaptation is an important factor in the protection from several stress

conditions (Brown et al., 1997; Chang and Cronan, 1999). The formation of CFA is considered to be a postsynthetic modification of the phospholipid bilayer which predominantly occurs as cultures enter into the stationary phase. The CFA are formed by CFA synthase through the addition of a methylene group from S-adenosyl-L-methionine to the *cis* double bond of the UFA moiety of the phospholipid (Brown et al., 1997). In our case, a significant proportion of the UFA (C18:1) were converted to CFA (cyc19) during acid adaptation. This conversion of the unsaturated fatty acids to their cyclopropane derivatives is believed to serve as a protective measure against low pH, thermal inactivation and other stress conditions (Yatvin et al., 1986; Brown et al., 1997). However, CFA contribution to membrane properties is not yet understood, especially concerning the modifications of membrane fluidity in response to environmental stress. Duforc et al. (1984) have indicated that the presence of a cyclopropane ring within membrane fatty acids increases the stability of the structural and dynamic properties of biological membranes. Other studies have suggested that an increase in CFA content could cause a decrease in membrane fluidity (Yatvin et al., 1986, Magnuson et al., 1993; Annous et al., 1999), which could explain the increase in thermal resistance of *S.* Typhimurium cells with high CFA levels.

On the other hand, changes observed in the membrane fatty acid composition are not enough to explain the influence of growth temperature on the thermotolerance of *S.* Typhimurium and the great heat resistance observed for cells grown at 45 °C. Although minimum CFA levels were observed at 10 °C, the growth temperature did not strongly affect the proportion of CFA found in the temperature range 25 - 45 °C. These results therefore suggest that the conversion of UFA in CFA does not play a role in temperature adaptation responses. Non-acidified and non-acid adapted control cells showed UFA/SFA ratio inversely proportional to growth temperature. Thus, at high temperatures the decrease in UFA/SFA ratio observed and, consequently, in the membrane fluidity could explain the increase in D-values found for *S.* Typhimurium in response to the increase in growth temperature. Nevertheless, when cells were grown in acidified BHI, little differences in UFA/SFA ratio, and, consequently, in membrane fluidity were observed at 25, 37

and 45 °C. Therefore, other mechanisms must be responsible for this thermal resistance increase, such as the synthesis of Heat Shock Proteins (Gottesman et al., 1997; Yura et al., 2000; Periago et al., 2002). Thus, these results need to be complemented by an analysis of the proteomic responses of cells to different culture conditions in relation to their thermotolerance especially at high growth temperatures.

ACKNOWLEDGEMENTS

This work was supported by the Junta de Castilla y León (project number LE002A07). Álvarez-Ordóñez A. and Fernández A. gratefully acknowledge the financial support from the Spanish Ministry of Education and the University of León, respectively.

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7.

Relationship between membrane fatty acid composition and heat resistance of acid and cold stressed Salmonella Senftenberg CECT 4384

Avelino Álvarez-Ordóñez, Ana Fernández, Mercedes López and Ana Bernardo

Food Microbiology, 2009, 26, 347-353

ABSTRACT

This study evaluates the adaptative response to heat (63 ºC) and the modifications in membrane fatty acid composition of *Salmonella* Senftenberg after its growth in an acidified medium and after its exposure to combinations of acid and cold stresses. Cells were grown in Brain Heart Infusion (BHI) buffered at pH 7.0 and acidified up to pH 4.5 (fresh cultures) and kept at refrigeration temperature (4 ºC) for 7 days (refrigerated cultures). The results indicate that previous adaptation to a low pH increased the bacterial heat resistance, but combinations of sublethal stresses reduced *S.* Senftenberg heat tolerance, specially when the growth medium pH was decreased. Acid-adapted cells showed D_{63}-values ranging from 3.10 to 6.27 min, while non-acid-adapted cells showed D_{63}-values of 1.07 min. As pH decreased, over the pH range studied (7.4-4.5), D_{63}-values of the resulting cells increased. However, refrigerated acid-adapted cells showed lower D_{63}-values, which ranged from 0.95 to 0.49 min. A linear relationship between the thermotolerance of *S.* Senftenberg cells and the previous growth medium pH was found in both fresh and refrigerated cultures, which allowed us to predict changes in heat resistance of *S.* Senftenberg that occur at any pH value within the range used in the present study in which most foodstuffs are included. Both acidification of the growth medium and refrigeration storage of cells induced modifications in membrane fatty acid composition, which were clearly linked to their heat resistance. Acid-adapted cells, regardless of the pH value of the growth medium, showed the lowest UFA/SFA ratio and a CFA content 1.5-2-fold higher than that observed for non-acid-adapted cells. On the other hand, the UFA/SFA ratio found for *S.* Senftenberg cells exposed to a cold stress was 1.2-1.8-fold higher than that observed for non-refrigerated cultures. This increase in the UFA/SFA ratio was specially high for acid-adapted cells. The highest thermotolerance was observed for cells with low UFA/SFA ratio, and, consequently, having a low membrane fluidity. However, changes observed in CFA content did not explain the great heat sensitivity of refrigerated acid-adapted cells.

INTRODUCTION

Salmonella is one of the most prevalent pathogens linked to outbreaks in foodborne disease (D'Aoust, 2000). Although *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most frequent causative agents of human salmonellosis, other serovars, such as *Salmonella* Senftenberg, have been recently implicated in foodborne gastroenteritis (L'Ecuyer et al., 1996; Kumar and Kumar, 2003). This fact is of great importance since *S.* Senftenberg is a persistent contaminant in slaughterhouses (Sogaard and Nielsen, 1979; Liebana et al., 2001) and it has also been revealed as one of the predominant serovars isolated from marine environments and seafood (Martínez-Urtaza et al., 2004).

Heat treatment is one of the principal methods used in food industry to eliminate pathogen microorganisms from food products. For this reason, extensive investigation in this area focused on the effects of environmental factors on the bacterial heat resistance has been performed. As was shown, microorganisms show modifications in their heat resistance after exposure to certain environmental and preservation stresses, such as acid and cold stresses (Annous and Kozempel, 1998; Rowan and Anderson, 1998; Martínez et al., 2003; Álvarez-Ordóñez et al., 2008). In the particular case of *Salmonella* spp. there are several studies on the influence of acid stress on their heat resistance and there seems to exist an accordance with the fact that these microorganisms exhibit a ''heat induced tolerance'' after exposure to moderate acidic environments (Leyer and Johnson, 1993; Wilde et al., 2000; Mazzotta, 2001; Bacon et al., 2003; Tosun and Gonul, 2003). The effect of cold stress has been less studied, although from data in literature for *S.* Typhimurium and *S.* Enteritidis it can be deduced that their exposure to low temperatures is accompanied by a decrease in the subsequent heat resistance (Humphrey, 1990; Mañas et al., 2003; Álvarez-Ordóñez et al., 2008). However, as far as we know, little attention has been paid to the combined effects of both stresses on the bacterial thermal resistance in spite of its great practical interest in food preservation where multiple stresses are often used.

The mechanisms involved in the bacterial thermal tolerance are not fully understood and the majority of studies are focused on the role of stress proteins (heat shock proteins) and the regulation of gene expression in response to environmental changes (Foster and Spector, 1995; Foster, 2000; Dodd and Aldsworth, 2002). However, a link between the membrane fatty acid composition and the bacterial heat resistance has also been found (Annous et al., 1999; Sampathkumar et al., 2004; Álvarez-Ordóñez et al., 2008). In general, these authors have shown that cells with a decreased concentration of unsaturated fatty acids or with an increased content of saturated fatty acids have a decreased membrane fluidity, which is linked to a higher heat resistance. Furthermore, in a previous study performed in our laboratory we found that the formation of cyclic fatty acids plays an important role in protecting acid-adapted *S.* Typhimurium cells from heat inactivation (Álvarez-Ordóñez et al., 2008).

To the best of our knowledge, there is no information available either on the behaviour against heat of acid and cold stressed cells or on the changes in membrane fatty acid composition under these stressful conditions for *S.* Senftenberg, even though this microorganism not only represents a potential concern for food safety but also is frequently used as a biological indicator to assess the lethality of heat treatments with regard to *Salmonella* spp. because of its greater heat resistance. Thus, the aim of this study is to evaluate the subsequent thermal resistance of *S.* Senftenberg following its exposure to combinations of acid and cold stresses. A further aim is to check the effect of acid adaptation and cold storage on *S.* Senftenberg membrane fatty acid profile in an attempt to clarify the role of membrane composition and fluidity in its heat resistance.

MATERIAL AND METHODS

Bacterial strain and culture conditions

The *Salmonella enterica* serovar Senftenberg strain CECT 4384 was obtained from Colección Española de Cultivos Tipo (CECT) (Spanish Type Culture

Collection). Revitalized cultures were stored on Brain Heart Infusion Agar (BHIA; Oxoid) plates at 4 ºC and then were activated by transferring an isolated colony from BHIA to Brain Heart Infusion (BHI; Oxoid) and incubated at 37 ºC for 24 h to give a stock suspension of 10^9 cfu/mL. Non-acidified and acid adapted cells were obtained by inoculating a portion of the activated culture into flasks containing 50 mL of BHI (pH 7.4) non-acidified and acidified at pH values of 6.4, 5.4 and 4.5 with hydrochloric acid (Panreac), respectively. In order to obtain non-acid adapted cells, BHI was buffered at pH 7.0 using a Sorensen buffer 0.2 M (bisodium (Merck)–monopotassium (Panreac) phosphate). All cultures were then incubated at 37 ºC until a late stationary phase of growth was reached (36 h) and were used to determine the thermotolerance and membrane fatty acid composition.

Cold adaptation consisted of maintaining cultures at 4 ºC for 7 days. Preliminary studies confirmed that in all cases cell viability remained constant throughout the cold storage period.

Growth curves and calculation of growth parameters

Samples (1 mL) of the cultures obtained at different assayed conditions were decimally diluted in sterile 0.1% (w/v) peptone solution (Oxoid) and appropriate dilutions were plated in duplicate on BHIA. Plates were incubated at 37 ºC for 48 h, and the number of colonies enumerated. Viable counts were converted to $\log_{10}$ values. Growth curves generated by fitting the data to the Gompertz equation (Buchanan et al., 1997) were used to calculate lag phase duration, exponential growth rate, generation time and maximum population density, as well as the time needed to reach the stationary phase.

Determinations of thermoresistance

Heat resistance experiments (63 ºC) were performed on 0.2 mL aliquots of cell cultures heated in 350 mL of BHI in a thermoresistometer TR-SC (Condón et al., 1993). After different heating times, 0.2 mL samples were collected and immediately pour-plated in BHIA. Plates were then incubated at 37 ºC for 48 h and survivors were counted by means of an automatic cfu counter (Protos Analytical

Measuring Systems, Cambridge, UK) following the specifications described by Ibarz et al. (1991).

D-values (time in min for survival count to drop 1 log cycle) for three different cultures were calculated as the negative reciprocals of the slopes of the regression lines obtained by plotting the log number of survivors against time (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA). The statistical significance ($p \leq 0.05$) of the differences between the D-values was tested using the Student's t test as described by Steel and Torrie (1986) (Statistica for Windows version 4.5, Statsoft, Inc., Tulsa, OK, USA).

Fatty acid analysis

The membrane fatty acid composition was determined using a chromatographic method as described elsewhere (Álvarez-Ordóñez et al., 2008). Lipids were extracted with chloroform and methanol from 1 L of each bacterial culture obtained as described above, following the Bligh and Dyer (1959) method. Afterwards, the chloroform rich phase obtained containing the cellular lipids was removed and the solvent was evaporated. Lipids were methylated in an alkaline medium using $NaOCH_3$ (Merck). The resulting fatty acid methyl esters (FAME) were analyzed on a gas chromatographer (HP 6890, Hewlett Packard, Avondale, PA) equipped with a mass selective detector (HP 5973) using a capillary column (60 m, 0.25 mm, 0.20 mM). The FAME were identified by comparing their retention times to those corresponding to known standards (Supelco) and by comparing their mass spectra to the data bank (Hewlett Packard). The results obtained from a single experiment are expressed as relative percentages of each fatty acid.

RESULTS

***Influence of acid adaptation and cold storage on the heat resistance of S.* Senftenberg**

In order to study the effects of acid and cold adaptation on the subsequent heat resistance of *S.* Senftenberg, cells were grown at 37 ºC in non-acidified BHI pH

7.4 (non-acidified cells), buffered BHI pH 7.0 (non-acid-adapted cells) and BHI acidified with hydrochloric acid at pH values of 6.4, 5.4 and 4.5 (acid-adapted cells). Fig. 1 shows an example of growth curves obtained in all conditions assayed. It is worth noting that *S.* Senftenberg grew well at all pH values tested. The effects of pH on kinetic parameters were relatively small at pH values ≥ 5.4. The acidification of the growth medium at pH 4.5 doubled the generation time (0.57 h) and the time needed to reach the stationary phase (16 h), tripled the lag phase duration (6.61 h) and decreased the maximum population density about a half log cycle.

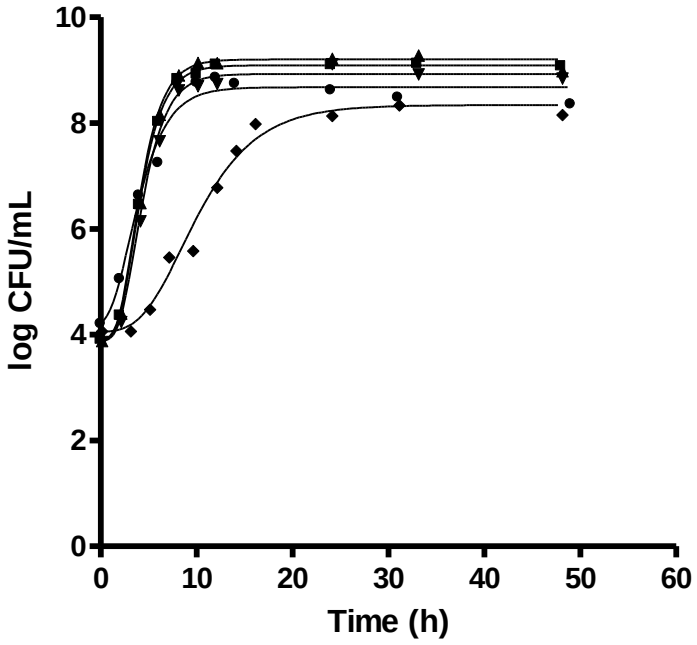

Fig. 1. Growth curves of *Salmonella* Senftenberg (CECT 4384) in buffered BHI (■), non-acidified BHI (▲) and BHI acidified with hydrochloric acid at pH values of 6.4 (▼), 5.4 (●) and 4.5 (♦) (one of three trials). The symbols represent the experimental data obtained, while the lines were the result of the adjustment of the data through the Gompertz equation.

Following the growth in these media at 37 ºC for 36 h, resistance determinations at 63 ºC in BHI were then performed for fresh cultures and after their storage at 4 ºC for 7 days (refrigerated cultures). In all cases, the populations of viable cells remained static throughout the storage period, regardless of the prior growth conditions.

Fig. 2 shows an example of survival curves obtained under all conditions assayed. Survival curves obtained properly fitted into a first order inactivation kinetic. The goodness of fit was determined both by visual inspection and R^2 value, which ranged from 0.95 to 0.99 (data not shown). D-values calculated are also included in Fig. 2 as the mean of three independent experiments ± standard deviation.

Acid-adapted cells were more heat resistant (D_{63}-values ranging from 3.10 to 6.27 min) than their non-acid-adapted (D_{63}-value of 1.07 min) and non-acidified (D_{63}-value of 1.05 min) counterparts. The results also showed a specific effect of the growth medium pH on the heat tolerance of the resulting *S.* Senftenberg cells. The D_{63}-values obtained linearly increased as the pH decreased (Fig. 3), reaching D_{63}-values of approximately 6-fold higher when the pH value of the growth medium was acidified from 7.4 to 4.5.

When bacterial cultures were exposed to a subsequent cold storage, acid-adapted cells turned out to be more heat sensitive, reaching D_{63}-values of about 1.2-2.2-fold lower than those corresponding to non-acid adapted and non-acidified cells. The inactivation rate at 63 ºC of refrigerated *S.* Senftenberg cells followed a linear relationship with the previous growth medium pH (Fig. 3).

In both cases, these results give satisfactory fits as indicated by the high R^2-values found, 0.973 and 0.970 for fresh and refrigerated cultures, respectively. Of particular interest in our data is the fact that it is possible to predict changes in heat resistance of fresh and refrigerated cultures of *S.* Senftenberg which occur at any adaptation pH value within the range used in the present study, in which most foodstuffs are included.

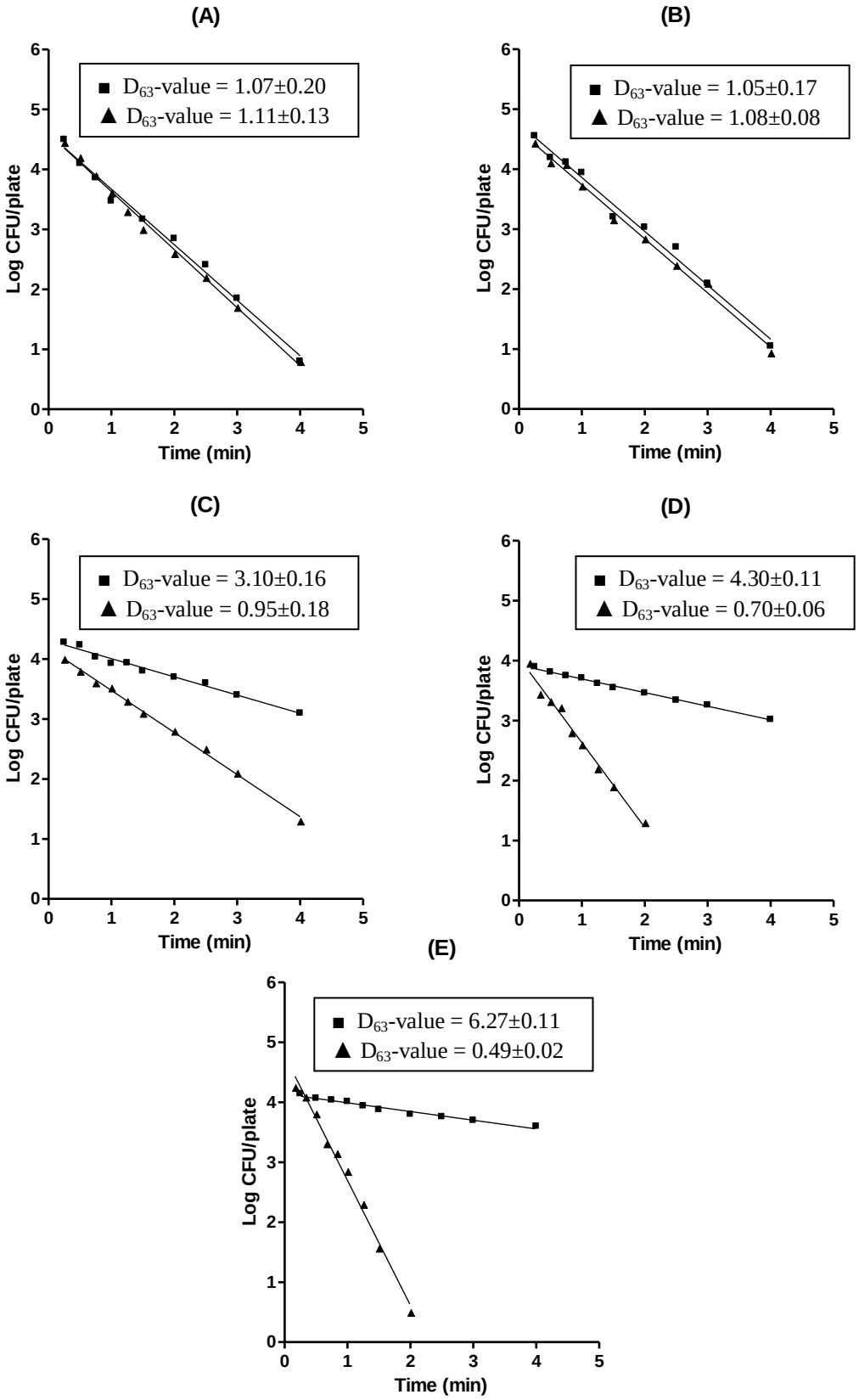

Fig. 2. Survival curves obtained at 63 ºC for *Salmonella* Senftenberg (CECT 4384) cells grown in buffered BHI (A), non-acidified BHI (B), and BHI acidified with hydrochloric acid at pH values of 6.4 (C), 5.4 (D) and 4.5 (E) for fresh (■) and refrigerated cultures (▲) (one of three trials).

Influence of acid adaptation and cold storage on membrane fatty acid composition of S. Senftenberg

The membrane fatty acid composition of *S.* Senftenberg CECT 4384 cells obtained under the different environmental conditions assayed is shown in Table 1. In all cases, thirteen fatty acids were found. The six main peaks were identified as tetradecanoic (myristic) acid (C14:0), hexadecanoic (palmitic) acid (C16:0), hexadecenoic (palmitoleic) acid (C16:1), octadecanoic (stearic) acid (C18:0), octadecenoic (oleic or vaccenic) acids (C18:1) and methylenoctadecenoic (dihydrosterculic or lactobacillic) acids (cyc19). Their relative percentages were between 3 and 36%, corresponding to more than 93% of all fatty acids observed. Seven minor fatty acids were also detected at lower relative concentrations: C8:0, C10:0, C12:0, C15:0, C17:0, cyc17 and C18:2.

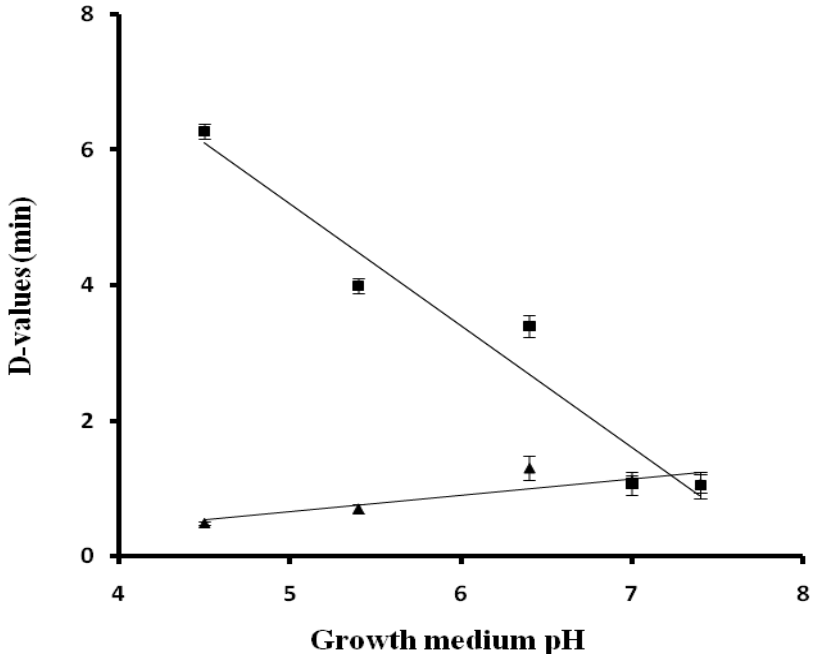

Fig. 3. Relationship between D_{63}-values and pH value of the growth medium for fresh (■) and refrigerated (▲) *Salmonella* Senftenberg (CECT 4384) cultures.

Table 1. Membrane fatty acid composition of *Salmonella* Senftenberg CECT 4384 cells under the different conditions tested.

Environmental conditions	C14:0	C16:0	C16:1	C18:0	C18:1	cyc19	Minor FA	SFA	UFA	CFA	UFA/SFA
Fresh cultures											
B-BHI	9.08	32.76	5.51	9.45	26.50	11.00	5.13	55.96	32.58	11.46	0.58
NA-BHI	6.52	32.50	3.81	11.26	29.51	11.61	4.79	54.85	33.31	11.84	0.61
A-BHI$_{HCl\ pH\ 6.4}$	5.21	32.63	3.43	10.21	23.68	19.66	5.18	53.02	27.12	19.86	0.51
A-BHI$_{HCl\ pH\ 5.4}$	5.28	29.88	3.74	12.38	24.58	18.22	5.92	51.93	29.36	18.71	0.57
A-BHI$_{HCl\ pH\ 4.5}$	6.59	30.74	4.27	10.92	19.56	21.15	6.77	52.68	24.67	22.65	0.47
Refrigerated cultures											
B-BHI	7.43	30.54	4.76	10.58	28.61	12.84	5.24	52.46	34.33	13.21	0.65
NA-BHI	4.93	31.45	4.11	11.52	30.80	14.85	2.34	50.13	34.91	14.96	0.70
A-BHI$_{HCl\ pH\ 6.4}$	3.39	25.77	3.37	11.27	36.44	15.65	4.11	43.13	41.13	15.74	0.95
A-BHI$_{HCl\ pH\ 5.4}$	3.70	26.36	3.44	11.77	33.26	16.79	4.68	44.67	38.28	17.05	0.86
A-BHI$_{HCl\ pH\ 4.5}$	3.23	26.12	4.60	10.67	31.22	19.43	4.73	43.51	36.70	19.79	0.84

B-BHI: Buffered BHI pH 7.0
NA-BHI: non-acidified BHI (pH 7.4)
A-BHI: acidified BHI with HCl at different pH values
C14:0: tetradecanoic (myristic) acid
C16:0: hexadecanoic (palmitic) acid
C16:1: hexadecenoic (palmitoleic) acid
C18:0: octadecanoic (stearic) acid
C18:1: octadecenoic (oleic or vaccenic) acids
cyc19: methylenoctadecenoic (dihydrosterculic or lactobacillic) acids
Minor FA: Minor fatty acids
SFA: Total saturated fatty acids
UFA: Total unsaturated fatty acids
CFA: Total cyclic fatty acids

So as to determine the differences among membrane fatty acid composition of *S.* Senftenberg cells, total saturated fatty acids (SFA), total unsaturated fatty acids (UFA) and total cyclic fatty acids (CFA) were estimated. At the same time the UFA/SFA ratio was used as an indirect indicator of the membrane fluidity, since both are directly related (Casadei et al., 2002).

For non-refrigerated cultures the UFA/SFA ratio was similar under all conditions tested, ranging from 0.47 to 0.61, although minimum ratios were obtained for acid-adapted *S.* Senftenberg cells (grown in acidified BHI), with ratios ranging from 0.47 to 0.57. Our results also show that the acidification of the growth medium induces changes in the proportion of CFA. Acid-adapted cells showed CFA levels 1.5-2-fold higher than non-acid-adapted and non-acidified cells. C18:1 and cyc19 were greatly affected by the acidification of the growth medium which rendered a significant decrease in C18:1 content and a concomitant increase in cyc19 level probably due to the fact that a significant proportion of the UFA (C18:1) was converted to CFA (cyc19) during acid adaptation period.

The storage under refrigeration conditions caused large modifications in the membrane fatty acid profile of *S.* Senftenberg cells, specially in acid adapted cells. The UFA/SFA ratio, ranging from 0.65 to 0.95, was clearly higher than that corresponding to fresh cultures, although the magnitude of the increase was not the same for all growth conditions. Thus, whereas acid adapted cells showed ratios 1.5-1.9-fold higher, a milder increase was found in non-acid adapted and non-acidified cells. The high UFA/SFA ratio found for acid adapted cells, ranging from 0.84 to 0.95, resulted in an increase in membrane fluidity due to both a decrease in SFA and an increase in UFA. It is important to note that C18:1 relative concentration was greatly affected by the cold storage in acid adapted cells (Fig. 4), showing an increase of about 1.4-1.6-fold than that corresponding to fresh cultures. On the other hand, the cold stress caused a slight decrease in CFA content in acid adapted cells, although the CFA content found was only 1.1-1.5-fold higher than that corresponding to non-acid adapted and non-acidified cells, which, at the same time, showed higher CFA levels than those found for fresh cultures.

DISCUSSION

Acidification is an important preserving method which influences the growth and survival of microorganisms. The minimal pH at which salmonellae can initiate growth is not well defined, since it varies depending on several factors, including the composition of the growth medium and the type of acidulant used (Álvarez-Ordóñez et al., 2009). Our findings indicate that the acidification of the growth medium (BHI) up to pH 4.5, although did not markedly affect the growth kinetic parameters of *S.* Senftenberg, increased the thermotolerance of the resulting cells. This so-called cross-protection response could have serious implications for food safety, since some type of fermented, acidified or acid foods could provide environments which could promote the development of acid adapted cells that are capable of surviving a subsequent heat treatment. Increased thermal tolerance resulting from bacterial responses to exposure to an acidic environment has been previously reported for other pathogenic microorganisms, such as *Listeria monocytogenes* (Farber and Pagotto, 1992; Lou and Yousef, 1996; Juneja et al., 1998), *Escherichia coli* (Ryu and Beuchat, 1998, 1999; Buchanan and Edelson, 1999; Rowe and Kirk, 1999; Duffy et al., 2000; Evrendilek and Zhang, 2003), *S.* Typhimurium (Leyer and Johnson, 1993; Bacon et al., 2003; Tosun and Gonul, 2003) and *S.* Enteritidis (Wilde et al., 2000; Mazzotta, 2001). However, this study would appear to be the first in examining this *S.* Senftenberg response.

Our results also show that the cross-protection response observed was dependent on the pH value of the growth medium. The maximum adaptive response of *S.* Senftenberg to heat was found after its growth in a medium of pH 4.5. In spite of the differences in experimental procedures, these results agree with those reported by Lou and Yousef (1996) and Evrendilek and Zhang (2003) who found that the magnitude of this cross-protective effect on the heat tolerance was affected by the pH of the media in which bacteria were habituated to acidic environments. Lou and Yousef (1996) found that a decrease in the pH of the medium from 5.5 to 4.5 increased *L. monocytogenes* heat resistance by 10-fold. Evrendilek and Zhang

(2003) also found that after exposure of *E. coli* O157:H7 cells to pH values of 3.6, 5.2 and 7.0, the lowest pH used gave rise to the most heat resistant cells.

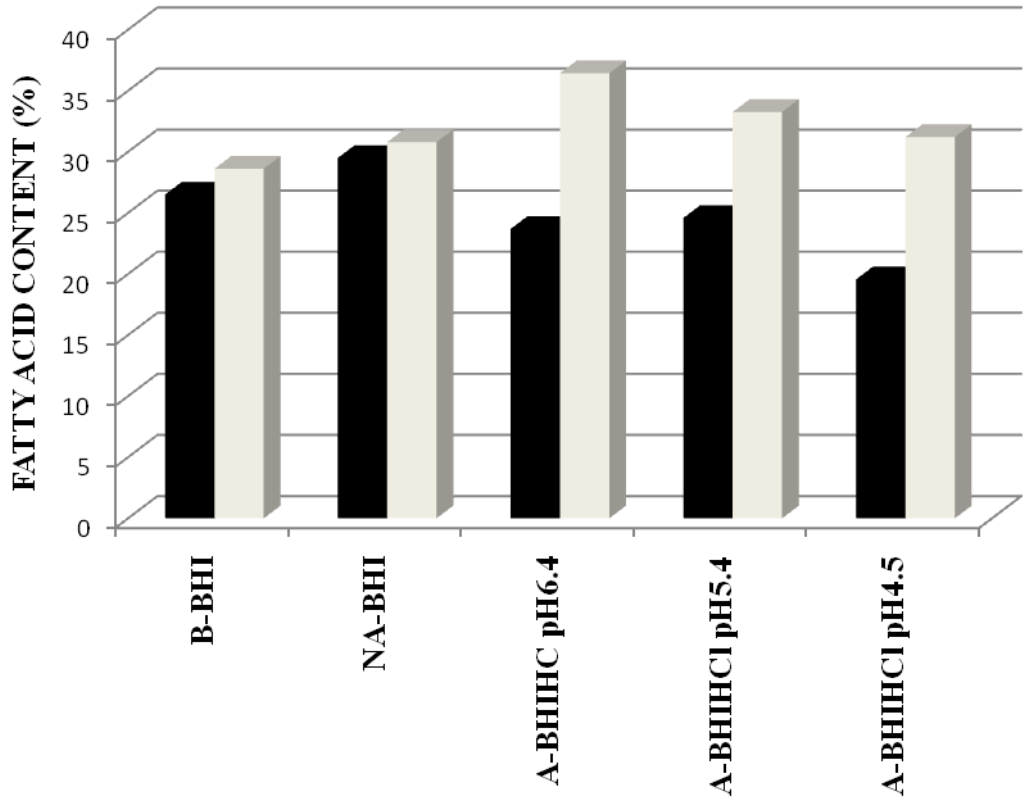

Fig. 4. Membrane content in C18:1 for fresh (black) and refrigerated (grey) *Salmonella* Senftenberg (CECT 4384) cultures.

Previous studies available in literature on the influence of refrigeration storage on the microbial heat resistance are contradictory. Whereas some authors have reported that cold storage gives rise to an increase in thermal tolerance for *Aeromonas hydrophila* (Condón et al., 1992), *Staphylococcus aureus* (Kornacki and Marth, 1989) and *E. coli* O157:H7 (Jackson et al., 1996; Evrendilek and Zhang, 2003), others have found a decrease in heat resistance for *E. coli* O157:H7 (Katsui et al., 1981, 1982; Uyttendaele et al., 2001), *S.* Enteritidis (Humphrey, 1990) and *L. monocytogenes* (Gay and Cerf, 1997; Bayles et al., 2000). It has also been reported

in other cases that cold storage does not modify the thermal tolerance for *Salmonella* spp. (Read et al., 1968) and *S. aureus* (Kennedy et al., 2005). Our results could explain, at least in part, this apparent discrepancy. We have found that heat resistance of acid adapted cells following cold stress at 4 ºC for 7 days was lower than that corresponding to fresh cultures, whereas non-acid adapted and non-acidified cells showed a similar thermal tolerance in both fresh and refrigerated cultures. Moreover, our results also show that heat sensitivity of acid adapted cells increased as growth medium pH decreased.

In spite of the fact that the refrigeration is frequently combined with the acidification in the food industry, little attention has been paid to the thermal inactivation of acid adapted microorganisms exposed to low temperatures. As far as we know, only Ingham and Uljas (1998) have studied the influence of the storage for 24 h at 4 ºC of *E. coli* O157:H7 in apple juice acidified at pH 3.4 with malic acid and they found a significant decrease in its heat resistance under these conditions. These results agree with those reported here for *S.* Senftenberg. The fact that refrigeration under acidic conditions is capable of increasing the lethality of a subsequent heat treatment could be of great practical interest, as thermal treatment could be optimized in order to avoid its negative effect on the sensorial quality of some foods.

Various studies have indicated that growth conditions, such as the composition of the growth medium (Annous et al., 1999), the growth phase of the cells (Russell et al.,1995; Kadner,1996; Casadei et al., 2002), the incubation temperature (Kadner, 1996; Annous et al., 1999; Casadei et al., 2002; Wang et al., 2005), and the pH value (Russell et al., 1995; Brown et al., 1997; Sampathkumar et al., 2004; Wang et al., 2005) markedly affect the bacterial membrane fatty acid composition. However, little is known on the influence of these modifications in membrane composition on the bacterial resistance to subsequent stresses.

The main fatty acids observed for *S.* Senftenberg cells in this study were similar to those previously described for *S.* Typhimurium (Álvarez-Ordóñez et al., 2008), *S.* Enteritidis (Sampathkumar et al., 2004) and other Gram negative bacteria as *E. coli* (Casadei et al., 2002).

Acid adaptation of *S.* Senftenberg caused a decrease in UFA/SFA ratio and in the C18:1 relative concentration, and a concomitant increase in CFA content, specially in cyc19 relative concentration. These results agree with those previously reported by Brown et al. (1997) and Álvarez-Ordóñez et al. (2008), who found a decrease in UFA, an increase in SFA and a large increase in CFA content for acid habituated cells of *E. coli* and *S.* Typhimurium, respectively. On the other hand, several studies have found an increased UFA/SFA ratio at low pH values for other microorganisms, such as *Lactobacillus acidophilus* (Wang et al., 2005), *Lactobacillus reuteri* (Palmfeldt and Hahn Hagerdal, 2000), *Oenococcus oeni* (Bastianini et al., 2000) and *Streptococcus thermophilus* (Béal et al., 2001).

The storage under refrigeration conditions caused an increase in the UFA/SFA ratio and consequently in the membrane fluidity. Previous studies were performed in order to clarify the effect of growth temperature on the membrane fatty acid composition. In the majority of them the bacterial growth at low temperatures was associated with an increased content in UFA and in membrane fluidity for *S.* Typhimurium (Álvarez-Ordóñez et al., 2008), *L. acidophilus* (Wang et al., 2005), *E. coli* (Casadei et al., 2002) and *Pediococcus* spp. (Annous et al., 1999). On the other hand, the opposite effect was described on some occasions for *L. acidophilus* (Fernández Murga et al., 2000) and *Lactococcus lactis* (Guillot et al., 2000). However, as far as we know there are no studies on the influence of the storage at refrigeration temperatures on the bacterial membrane fatty acid composition for stationary-phase cells. In our case, the changes in membrane fatty acid composition observed after the cold storage of *S.* Senftenberg were qualitatively similar to those previously observed for *S.* Typhimurium cells after their growth at 10 ºC (Álvarez-Ordóñez et al., 2008).

The modifications in *S.* Senftenberg membrane fatty acid composition were clearly linked to its heat resistance. In general, the D-values obtained were higher for cells with low membrane fluidity, as has been previously found for *S.* Typhimurium (Álvarez-Ordóñez et al., 2008), *S.* Enteritidis (Sampathkumar et al., 2004) and *Pediococcus* spp. (Annous et al., 1999).

Acid adapted cells showed a higher heat resistance than non-acid adapted cells for fresh cultures, which are associated with a low UFA/SFA ratio and a high CFA content. The conversion of a significant proportion of the UFA to CFA during acid adaptation could serve as a protective measure against heat inactivation. It is important to note that several studies have suggested that an increase in CFA content could cause a decrease in membrane fluidity (Yatvin et al., 1986; Magnuson et al., 1993; Annous et al., 1999), which could contribute to explain the increase in thermal resistance of *S.* Senftenberg cells with high CFA levels. Thus, the synthesis of CFA in cell membrane during acid adaptation could be an important factor in the protection against several stress conditions, such as low pH and thermal inactivation (Brown et al., 1997; Chang and Cronan, 1999). However, the CFA levels observed for refrigerated cultures were significantly higher under the conditions in which bacterial thermotolerance was minimal (refrigerated acid adapted cells), showing similar values to those corresponding to acid adapted fresh cultures. In this case, it seems probable that the extremely high UFA/SFA ratio shown by these cells (between 0.84 and 0.95) causes a maximum degree of membrane fluidity, responsible for their lower heat resistance, in spite of the high CFA levels expressed. Therefore, the UFA/SFA ratio, and consequently the bacterial membrane fluidity, seems to be the main factor reflecting heat resistance of *S.* Senftenberg cells, specially after exposure to cold stress, although the formation of cyclic fatty acids could also play a decisive role in protecting bacteria from heat inactivation. Further studies on the effect of simultaneous application of several stresses would be necessary to clarify the role played by membrane adaptations on the cross-protection response to heat.

On the other hand, the great sensitivity to heat showed by refrigerated cultures could have important practical consequences. According to the European Food Safety Authority (EFSA) (2006), risk mitigation during food processing requires the implementation of the principles elaborated in the Hazard Analysis and Critical Control Point (HACCP) system, the maintenance of the cold chain, and the application of the hurdle concept, which states that several inhibitory factors (hurdles), that individually are unable to inhibit microbial growth, will nevertheless

be effective when combined. Therefore, methods based on the hurdle technology to reduce or inhibit salmonellae are of primary importance in the food industry. Evidence provided in this study shows that the combination of acidification and refrigeration could represent an effective measure for the control of *Salmonella* spp. in food products. The physiological explanation of this synergistic effect is also provided by the membrane fatty acid composition observed for *S.* Senftenberg cells after refrigeration storage. The low temperatures caused an increase in UFA/SFA ratio, which was clearly greater for acid adapted cells, responsible for the lower heat resistance shown under these environmental conditions.

ACKNOWLEDGEMENTS

This work was supported by the Junta de Castilla y León (project number LE002A07). Álvarez-Ordóñez A. gratefully acknowledges the financial support from the Spanish Ministry of Education. The authors want to thank Ricardo Arenas for his assistance during the chromatographic experiments.

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8.

A comparative study of thermal and acid inactivation kinetics in fruit juices of acid adapted Salmonella Typhimurium *and Salmonella* Senftenberg

Avelino Álvarez-Ordóñez, Ana Fernández, Ana Bernardo and Mercedes López

Submitted for publication

ABSTRACT

Acid and heat inactivation in orange and apple juices of *Salmonella* Typhimurium and *Salmonella* Senftenberg grown in buffered BHI (pH 7.0) and acidified BHI up to pH 4.5 with acetic, citric, lactic and hydrochloric acids was evaluated. Acid adaptation induced an adaptive response which increased their subsequent resistance to extreme pH conditions (pH 2.5) and to heat, although the magnitude of these responses varied among serovars and fruit juices. The acid resistance found in orange juice for acid adapted cells (D-values of 28.27-34.54 min for *S.* Senftenberg and 30.02-39.23 min for *S.* Typhimurium) resulted to be about 2-3-times higher than that corresponding to non-acid adapted cells. In apple juice, acid adapted *S.* Senftenberg cells survived better than those of *S.* Typhimurium, obtaining mean D-values of 114.8±12.2 and 41.9±2.4 min, respectively. The thermotolerance of non-acid adapted *S.* Typhimurium in orange juice (D_{58}-value: 0.028 min) and apple juice (D_{58}-value: 0.10 min) was approximately double for acid-adapted cells. This cross-protection to heat was more strongly expressed in *S.* Senftenberg. D_{58}-values obtained for non-acid adapted cells in orange juice (0.11 min) and apple juice (0.19 min) increased approximately 10 and 5-times, respectively after their growth in acidified media. However, it is worth noting that several acid adapted *S.* Typhimurium resulted to be more thermotolerant than non-acid adapted *S.* Senftenberg. The conditions prevailing during bacterial growth and heat treatment did not significantly influence the z-values observed (6.03±0.29 for *S.* Typhimurium and 7.04±0.30ºC for *S.* Senftenberg). The enhanced acid resistance found for both serovars could enable them to survive for prolonged time periods in the gastrointestinal tract, increasing the risk of illness. Furthermore, it should be taken into account that microbial growth in acidified media also induces a cross-protection response against heat which should be also considered for the design of pasteurization processes for acid foods.

INTRODUCTION

Fruit juices, such as apple and orange juices, with pH values ranging from 3.3 to 4.1 (Mattick and Moyer, 1983), have been long considered as safe foods, since the growth of pathogenic bacteria is thought to be limited due to their low pH, caused by naturally occurring organic acids (Parish, 1997). However, they have been implicated as the food source in several foodborne outbreaks of salmonellosis in which *Salmonella* Typhimurium has been identified as the causative agent (Parish, 1997, 1998; Boase et al., 1999; D´Aoust et al., 2001; Mazzotta, 2001).

Although recent studies within our research group have demonstrated that media acidified with several organic acids (ascorbic, acetic, citric, lactic and malic) at pH values lower than 4.5 did not support the growth of *S.* Typhimurium (Álvarez-Ordóñez et al., 2009a), it has been previously reported that growth can occur at pH values as low as 4.0 (Lin et al., 1995; Ruzickova, 1996; D´Aoust, 2000). Furthermore, it has been described that *Salmonella* can survive for long time periods in some acid foods. Thus, viable cells have been detected after up to 12 weeks in apple, orange, pineapple, and white grape juice concentrates (Parish et al., 1997; Oyarzábal et al., 2003), 19-68 days in yogurt (El-Gazzar and Marth, 1992) and 4 weeks in mayonnaise (Leuschner and Boughtflower, 2001). Taking into account the potential of fruit juices to serve as vehicles of *Salmonella* spp. in infection outbreaks, the Food and Drug Administration (FDA) has proposed a hazard analysis and critical control point (HACCP) regulation in which juice producers are required to implement a system capable of inactivating pathogenic bacteria to safety levels (FDA, 1998). However, in order to reach this objective it is necessary to understand the factors affecting microbial resistance, also bearing in mind that microorganisms exposed to several environmental stresses can develop protective mechanisms which could increase their resistance to subsequent processing steps, difficulting their elimination from foods. Among these stresses, the previous exposure to acidic conditions is known to be one of the most relevant. Numerous studies have reported that acid adapted *S.* Typhimurium shows an increased acid resistance (Bearson et al., 1996, 1998; Greenacre et al., 2003; Álvarez-Ordóñez et al., 2009a) and thermal

tolerance (Mazzotta, 2001; Tosun and Gönül, 2003; Álvarez-Ordóñez et al., 2008). However, little attention has been paid to the examination of these adaptive responses for *Salmonella* Senftenberg in spite of the fact that this microorganism not only represents a potential concern for food safety (L´Ecuyer et al., 1996; Gupta et al., 1999; Bairy et al., 2000) but it is also frequently used as a biological indicator to assess the lethality of heat treatments, because of its greater heat resistance related to other *Salmonella* serovars (Horan et al., 2004). As far as we know, only Kumar and Kumar (2003) and Álvarez-Ordóñez et al. (2009b) have reported a protective effect of acid adaptation on the subsequent acid and thermal inactivation of *S.* Senftenberg, respectively. Moreover, and despite the practical significance for food industry and Public Health of the increased resistance of acid adapted foodborne pathogens, studies carried out to determine the influence of environmental factors on the protection provided against heat treatments or extreme acid exposures are scarce. The influence of different environmental conditions on these adaptive responses for *S.* Typhimurium has been previously studied in our laboratory and we have found that the acid tolerance acquired was dependent on the growth medium pH and the type of acid used to acidify the growth medium (Álvarez-Ordóñez et al., 2009a). It has been also demonstrated that neither the type of acidulant nor the pH of the growth medium significantly modified the magnitude of the cross-protection response against heat shown by *S.* Typhimurium acid adapted cells (Álvarez-Ordóñez et al., 2008). However, in spite of the great interest that these bacterial responses have for fruit processors, we could not find any reference to indicate that the thermal and acid resistance of acid adapted *S.* Typhimurium and *S.* Senftenberg in fruit juices has been investigated, although it is well known that the composition of the treatment medium is a factor which markedly influences bacterial resistance (Palop et al., 2000; Leguérinel et al., 2007; Álvarez-Ordóñez et al., 2009a). Thus, the aim of this study was to determine the acid and thermal inactivation kinetics of *S.* Typhimurium and *S.* Senftenberg grown in the presence of different organic acids (acetic, lactic and citric) and hydrochloric acid using orange and apple juices as treatment media in order to find out how these conditions could influence the ultimate level of resistance of these pathogenic microorganisms.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Salmonella enterica serovar Typhimurium strain CECT 443 and *Salmonella enterica* serovar Senftenberg strain CECT 4384 were obtained from Colección Española de Cultivos Tipo (CECT) (Spanish Type Culture Collection). The lyophilized cultures were revived in Brain Heart Infusion (BHI, Oxoid) and incubated for 24 hours at 37ºC. Pure cultures were maintained on BHI agar (BHIA) plates at 4ºC. Precultures were prepared by transferring an isolated colony from a plate into a test tube containing 10 mL of sterile BHI followed by incubation at 37ºC for 24 h. Flasks containing 50 mL of sterile BHI (pH 7.4) non-acidified and acidified at pH values of 6.4, 5.4 and 4.5 with several acids: acetic (Prolab), citric (Sigma), lactic (Merck) and hydrochloric (Panreac) were inoculated with the subculture to a final concentration of 10^3 cells/mL. In order to obtain non-acid adapted cells, buffered BHI adjusted to pH 7.0 by addition of Sorensen buffer 0.2 M (bisodium (Merck) – monopotassium (Panreac) phosphate) was used. These cultures were then incubated at 37ºC for 36 h, time in which cells were in a late stationary-phase of growth.

Production of growth curves and calculation of growth parameters

In order to follow the bacterial growth, aliquots (1 mL) of the cultures obtained as described above were removed at several time intervals, decimally diluted in sterile peptone solution 0.1% (w/v) (Oxoid), and plated on BHIA. After the incubation of plates at 37ºC for 48 h, viable counts were determined, converted to $\log_{10}$ values and plotted *vs* time. The experimental data were fitted to the Gompertz equation (Buchanan et al., 1997) so as to determine the main growth parameters, such as lag phase duration, exponential growth rate, generation time, maximum population density and time needed to reach the stationary-phase (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA).

Assessment of acid tolerance

Aliquots of 5 mL of cultures were harvested by centrifugation at 8000 g for 5 min at 4ºC (*Eppendorf centrifuge 5804R).* The cell pellets were resuspended in 50 mL of commercial orange or apple juices adjusted to a pH value of 2.5 with HCl 1N, incubated at room temperature and survival was periodically monitored for up to 4 h. Samples were decimally diluted in sterile 0.1% peptone solution and appropriate dilutions were plated in duplicate on BHIA. Plates were incubated at 37ºC for 48 h and the number of colonies was counted with a modified Image Analyser Automatic Counter (Protos Analytical Measuring Systems, Cambridge, UK) as described elsewhere (Ibarz et al., 1991). Longer incubation times had no influence on the counting. All experiments were performed in triplicate using three different fresh cultures.

Heat treatments

Heat treatments were carried out in a thermoresistometer TR-SC as described by Condón et al. (1993). Once the temperature (50, 54 and 58ºC for *S.* Typhimurium and 55, 58 and 63ºC for *S.* Senftenberg) of the heat treatment medium (350 mL of commercial orange and apple juices) was stabilized (T±0.05ºC), an inoculum of each culture was injected. Throughout heating, samples of 0.2 mL were removed at predetermined time intervals and were plated on BHIA. Plates were then incubated at 37ºC for 48 h and survivors were enumerated. The heat resistance experiments at 58ºC were conducted in triplicate using three different fresh cultures and the results are expressed as means ± standard deviations.

D and z-values and statistical analysis

D-value (time required for a 10-fold reduction in viable cells) was determined by plotting the log number of survivors for each culture against time. The line that best fits survivor plots was determined by linear regression (GraphPad Prism version 4.00 for Windows), and the negative reciprocal of the slope was used to calculate the D-value. Statistical significance between D-values was determined using the

Student`s “t” test (Steel and Torrie, 1986a) (Statistica for Windows version 4.5, Statsoft, Inc, Tulsa, OK, USA).

Z-value (change in temperature required for a 10-fold reduction of D-value) was determined as the negative reciprocal of the slope of the regression line of the thermal death time curve (log D-values *vs* temperature). In order to statistically compare z-values, the homogeneity test of the slopes of these graphs was used (Steel and Torrie, 1986b) (Statistica for Windows version 4.5).

RESULTS

Viable counts of *S.* Typhimurium and *S.* Senftenberg were monitored throughout their growth at 37ºC in buffered BHI (pH 7.0), non acidified BHI (pH 7.4) and BHI acidified up to pH 4.5 with acetic, citric, lactic and hydrochloric acids. It is important to note that the minimum pH value which allowed for the growth of both serovars depended on the type of acidulant used. Acetic acid had a more potent inhibitory effect than lactic acid which, in turn, was more inhibitory than citric and hydrochloric acids. Whereas the minimum pH tested which allowed for growth was 6.4 and 5.4 in the presence of acetic acid and lactic acid, respectively, both pathogenic microorganisms were capable of growing up to pH 4.5 when the pH of the medium was reduced using citric and hydrochloric acids. An example of growth curves obtained in non-acidified BHI, buffered BHI and BHI acidified up to pH 4.5 with citric acid is shown in Fig. 1. These graphs are representative of those obtained when the acidification was performed with all acids tested. Under all the conditions assayed, the behaviour of both serovars was not significantly different, although *S.* Senftenberg grew faster than *S.* Typhimurium, especially at pH values $\leq$ 5.4. Generally, the stationary phase was attained after about 8-12 h at pH 6.4, 10-14 h at pH 5.4 and 16-22 h at pH 4.5. Generation times also increased with the acidification of the growth medium. The average generation times at pH values of 7.4, 6.4, 5.4 and 4.5 were 0.31±0.02, 0.35±0.09, 0.50±0.11 and 0.68±0.16 h, respectively. For

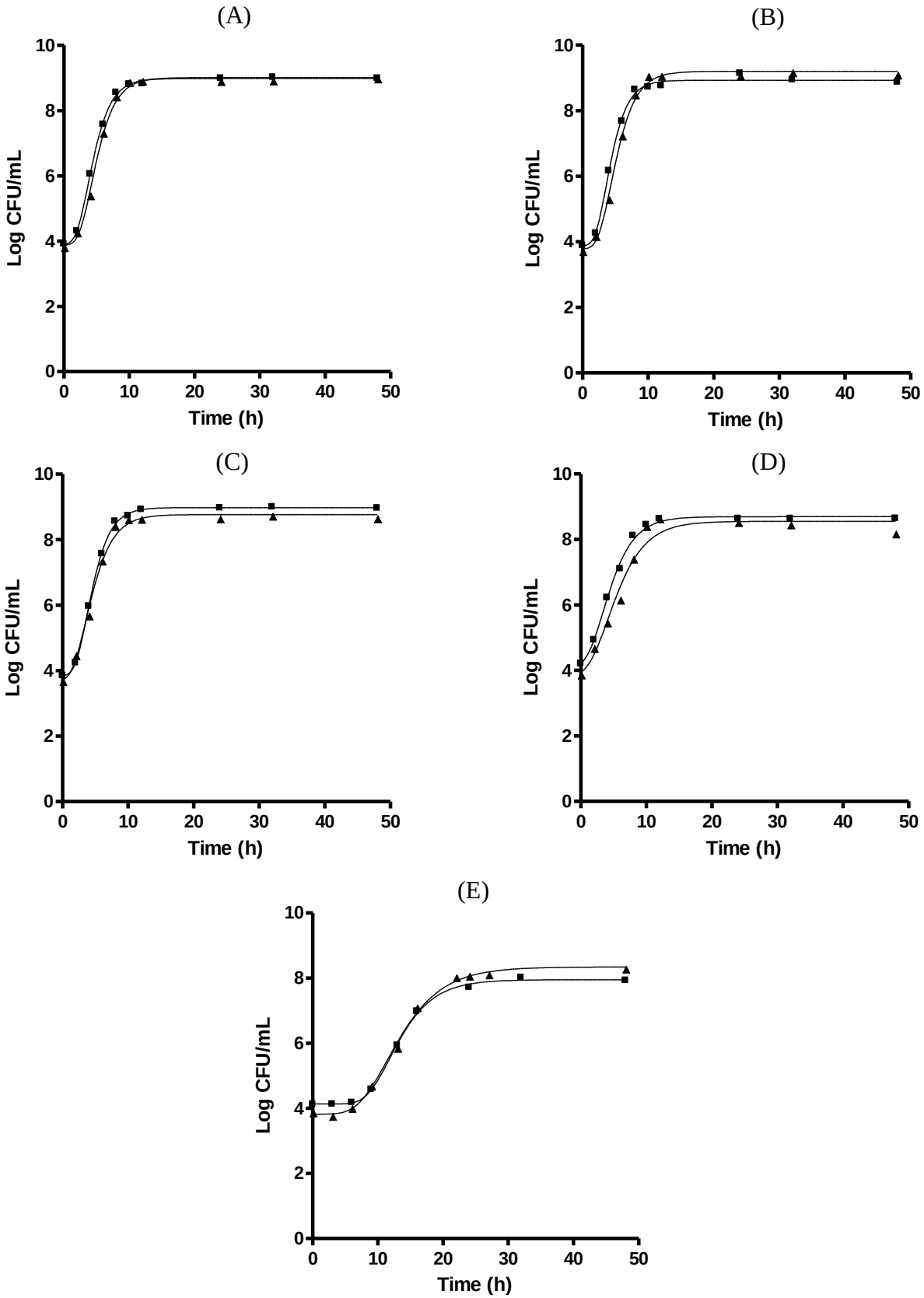

Fig. 1. Growth curves of *S.* Senftenberg (■) and *S.* Typhimurium (▲) in buffered-BHI (A), non-acidifed BHI (B) and acidified BHI at pH 6.4 (C), 5.4 (D) and 4.5 (E) with citric acid. The symbols represent the experimental data obtained, while the lines are the result of the adjustment of the data through the Gompertz equation.

cells grown at pH 4.5 the maximum population density decreased approximately about 0.5-1 log cycle.

Influence of acid adaptation conditions on the bacterial Acid Tolerance Response (ATR)

S. Typhimurium and *S.* Senftenberg inactivation at pH 2.5 in orange and apple juices was studied using stationary phase cells grown at 37ºC for 36 h in buffered BHI, non-acidified BHI and BHI acidified with the different acids up to the minimum pH tested which allowed for bacterial growth (acetic pH 6.4; citric pH 6.4 and 4.5; lactic pH 6.4 and 5.4; hydrochloric pH 6.4 and 4.5).

Survival curves obtained fitted properly into a first order inactivation kinetic. The goodness of fit was determined by both visual inspection and R^2 value, which ranged from 0.95 to 0.99 (data not shown). An example of survival curves obtained in orange and apple juices for *S.* Typhimurium and *S.* Senftenberg grown in buffered BHI, non-acidified BHI and BHI acidified with lactic acid is shown in Fig. 2. The D-values obtained under all conditions tested, expressed as mean values ± standard deviations, are shown in Table 1. Non-acid adapted cells showed D-values significantly lower ($p<0.05$) than those found for acid adapted cells (grown in non-acidified BHI or in acidified BHI) for both microorganisms. Of particular interest in our data is the fact that, whereas neither the type of acidulant nor the pH value of the acidified BHI showed a significant influence on the magnitude of the ATR developed, the composition of the challenge medium did exert. The acid resistance found for both serovars was higher when apple juice was used as challenge medium, although these differences were more marked for *S.* Senftenberg, which showed D-values for acid adapted cells ranging from 54.89 (for cells grown in non-acidified BHI) to 132.45 min (for lactic pH 5.4 acid adapted cells). These values were significantly higher than those corresponding to *S.* Typhimurium acid adapted cells, with D-values ranging from 35.33 to 44.03 min. However, when orange juice was used as challenge medium no significant differences ($p>0.05$) were found. These results show the importance of the challenge medium composition in the protection against acid inactivation, especially for *S.* Senftenberg, which showed D-values of

about 2.5 and 3.5-4 times higher in apple juice than those obtained in orange juice for cells grown in non-acidified BHI and acidified BHI, respectively.

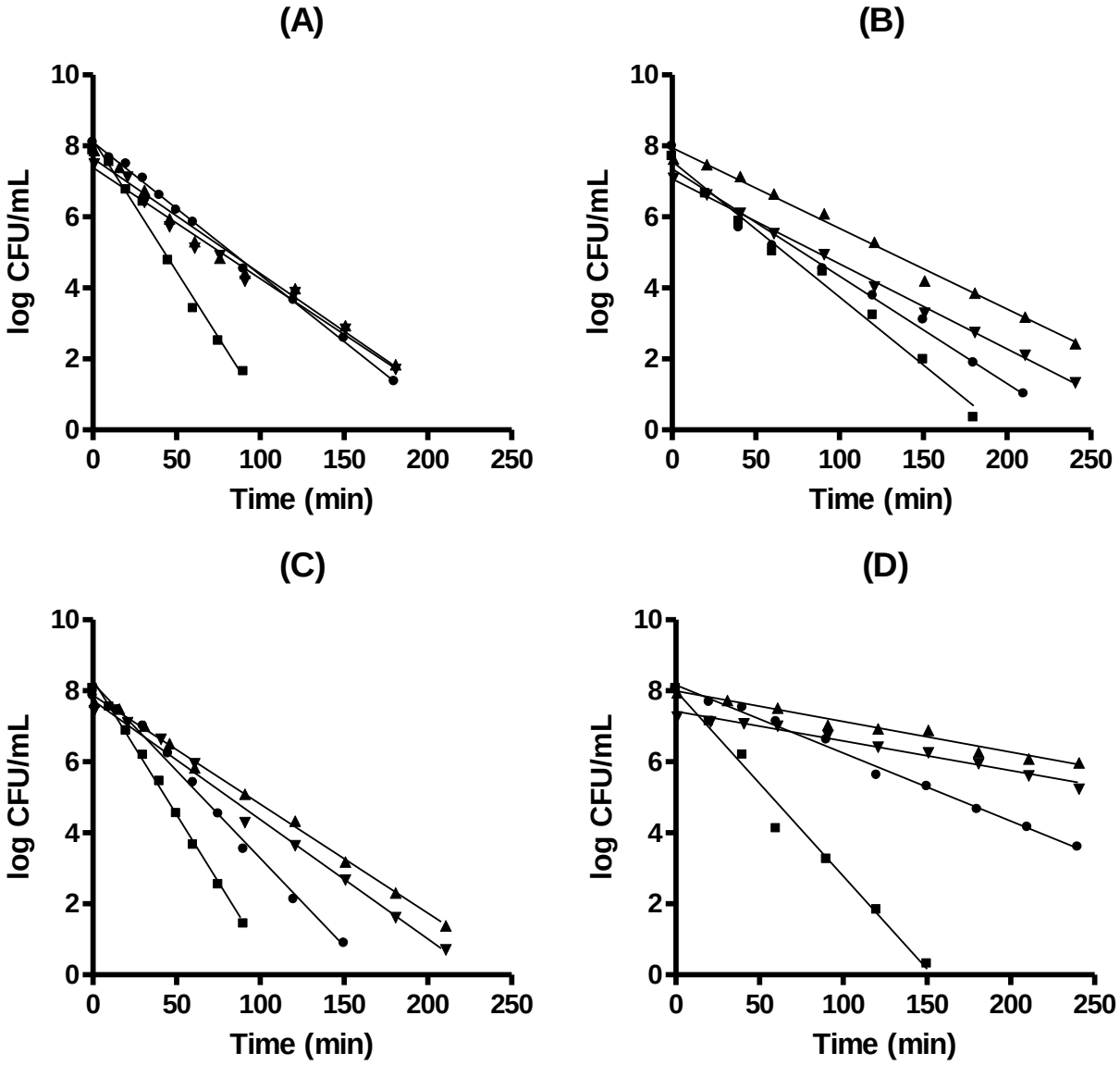

Fig. 2. Survival curves at pH 2.5 for cells grown in buffered BHI (■), non-acidified BHI (●), and acidified BHI with lactic acid at pH values of 6.4 (▲) and 5.4 (▼). *S.* Typhimurium cells challenged in orange juice (A) and apple juice (B) adjusted to pH 2.5. *S.* Senftenberg cells challenged in orange juice (C) and apple juice (D) adjusted to pH 2.5.

Influence of acid adaptation on the bacterial heat resistance

D and z-values obtained in the range of temperatures tested for *S.* Typhimurium (50, 54 and 58ºC) and *S.* Senftenberg (55, 58 and 63ºC) grown at 37ºC in non-acidified BHI (pH 7.4), buffered BHI (pH 7.0) and BHI acidified with acetic (pH 6.4), citric (pH 6.4 and 4.5), lactic (6.4 and 5.4) and hydrochloric (pH 6.4 and 4.5) acids using apple and orange juices as heating media are shown in Table 2. D_{58}-values are expressed as mean values ± standard deviations for three different cultures. The survivor curves for both *Salmonella* serovars were linear, regardless of heating temperature or the type of fruit juice used, and were consistent with a first-order inactivation kinetic, with R^2 values higher than 0.94 (data not shown). An example of survival curves obtained at 58ºC in orange and apple juices for *S.* Typhimurium and *S.* Senftenberg grown in buffered BHI, non-acidified BHI and BHI acidified up to pH 5.4 with lactic acid is shown in Fig. 3. These graphs are representative of those obtained under all conditions assayed.

Table 1. D-values (min), as mean value ± standard deviation, for acid treated cells in orange juice or apple juice adjusted at pH 2.5 of *S.* Typhimurium and *S.* Senftenberg grown in buffered BHI, non-acidified BHI and acidified BHI at different pH values with several acids.

Growth condition	S. Typhimurium		S. Senftenberg	
	Orange juice	Apple juice	Orange juice	Apple juice
B-BHI	$14.43\pm1.76^{a}_{1}$	$21.49\pm4.03^{a}_{2}$	$12.15\pm1.91^{a}_{1}$	$20.60\pm1.95^{a}_{2}$
NA-BHI	$27.82\pm2.12^{b}_{1}$	$35.33\pm3.31^{b}_{2}$	$19.07\pm2.20^{b}_{3}$	$54.89\pm4.37^{b}_{4}$
$A\text{-}BHI_{acetic\ pH\ 6.4}$	$36.42\pm4.20^{c}_{1}$	$37.41\pm3.94^{b}_{1}$	$29.91\pm2.64^{c}_{1}$	$103.36\pm12.12^{c}_{2}$
$A\text{-}BHI_{citric\ pH\ 6.4}$	$32.98\pm4.43^{bc}_{1}$	$43.95\pm5.03^{b}_{2}$	$29.29\pm2.94^{c}_{1}$	$102.53\pm17.07^{c}_{3}$
$A\text{-}BHI_{citric\ pH\ 4.5}$	$38.49\pm5.48^{c}_{12}$	$42.47\pm3.99^{b}_{1}$	$28.27\pm4.32^{c}_{2}$	$102.06\pm13.34^{c}_{3}$
$A\text{-}BHI_{lactic\ pH\ 6.4}$	$32.02\pm4.73^{bc}_{1}$	$40.13\pm5.05^{b}_{1}$	$34.54\pm2.81^{c}_{1}$	$125.35\pm14.15^{c}_{2}$
$A\text{-}BHI_{lactic\ pH\ 5.4}$	$30.02\pm4.34^{bc}_{1}$	$44.03\pm4.50^{b}_{2}$	$31.35\pm3.06^{c}_{1}$	$132.45\pm15.65^{c}_{3}$
$A\text{-}BHI_{HCl\ pH\ 6.4}$	$39.23\pm4.52^{c}_{12}$	$43.66\pm5.27^{b}_{1}$	$29.55\pm4.37^{c}_{2}$	$118.05\pm12.25^{c}_{3}$
$A\text{-}BHI_{HCl\ pH\ 4.5}$	$35.14\pm3.64^{c}_{1}$	$41.53\pm3.43^{b}_{1}$	$33.37\pm5.31^{c}_{1}$	$119.57\pm20.83^{c}_{1}$

B-BHI: Buffered BHI pH 7.0.
NA-BHI: Non-acidified BHI.
A-BHI: Acidified BHI with different acids: acetic, citric, lactic and HCl at different pH values.
$^{a\text{-}c}$: D-values (mean of three experiments ± SD) with different superscript in the same column are significantly different ($P<0.05$).
$_{1\text{-}4}$: D-values (mean of three experiments ± SD) with different subscript in the same row are significantly different ($P<0.05$).

Cells grown in acidified BHI showed a higher thermal tolerance than their non-acid adapted counterparts. This adaptive response was more strongly expressed in *S.* Senftenberg heated in orange juice, with D_{58}-values (ranging from 1.01 to 1.38 min) about 10 times higher than those found for non-acid adapted cells (D_{58}-value of 0.11 min). When apple juice was used as heating medium, acid adaptation increased *S.* Senftenberg heat resistance about 5 times. The composition of heating medium did not influence the magnitude of the cross-protection response observed for *S.* Typhimurium, finding an acid adaptation-linked increase in D_{58}-values of about 1.5-2 times. Of particular interest in our data is the fact that although the composition of the heating medium significantly influenced the heat resistance of both serovars, a different pattern was observed between them. Whereas *S.* Typhimurium showed a greater survival in apple juice, regardless of the growth conditions, only non-acid adapted *S.* Senftenberg cells survived better in this fruit juice.

The growth of both *Salmonella* serovars in non-acidified BHI (pH 7.4) also caused an acid adaptation which increased about 2-3 and 1.2 times the heat resistance observed for *S.* Senftenberg and *S.* Typhimurium, respectively. Our results also show that the type of acidulant did not significantly modify the cross-protection response found. In general, the decrease of the growth medium pH from 6.4 to 4.5 did not show any significant effect on the thermal inactivation, although a trend to obtain higher D-values as the growth medium pH decreased was found for *S.* Senftenberg when citric and hydrochloric acids were used.

It can be concluded from our data that *S.* Senftenberg generally exhibits a greater heat resistance than *S.* Typhimurium, although the magnitude of the differences observed was dependent on the heating medium composition and the conditions which prevailed during growth. D_{58}-values found for non-acid adapted *S.* Senftenberg cells treated in apple juice (0.19 min) and orange juice (0.11 min) were two and four times higher, respectively, than those corresponding to *S.* Typhimurium cells. When cells were grown in non-acidified BHI, D_{58}-values of 0.41 *vs* 0.13 min in apple juice and 0.34 *vs* 0.039 min in orange juice were found. The differences encountered turned out to be even higher (about 6 and 30-fold in apple juice and orange juice, respectively) for cells grown in BHI acidified with the

Table 2. D and z-values for *Salmonella* Typhimurium and *Salmonella* Senftenberg cells grown in buffered BHI, non-acidified BHI and acidified BHI at different pH values with several acids, and heated in fruit juices.

Heating medium	Growth conditions	*Salmonella* Typhimurium				*Salmonella* Senftenberg			
		D-value (min)			z-value (ºC)	D-value (min)			z-value (ºC)
		D_{50}	D_{54}	D_{58}		D_{55}	D_{58}	D_{63}	
Orange Juice	B-BHI	0.66	0.11	$0.028\pm0.004^{a}{}_{1}$	$5.83^{a}{}_{1}$	0.37	$0.11\pm0.01^{a}{}_{1}$	0.025	$6.92^{a}{}_{1}$
	NA-BHI	0.66	0.14	$0.039\pm0.005^{bc}{}_{1}$	$6.51^{a}{}_{1}$	1.05	$0.34\pm0.04^{b}{}_{1}$	0.072	$6.93^{a}{}_{1}$
	A-BHI$_{\text{acetic pH 6.4}}$	0.98	0.18	$0.043\pm0.004^{bcd}{}_{1}$	$5.89^{a}{}_{1}$	2.69	$1.30\pm0.11^{c}{}_{1}$	0.18	$6.70^{a}{}_{1}$
	A-BHI$_{\text{citric pH 6.4}}$	0.87	0.21	$0.037\pm0.003^{b}{}_{1}$	$5.83^{a}{}_{1}$	2.68	$1.03\pm0.10^{d}{}_{1}$	0.17	$6.65^{a}{}_{1}$
	A-BHI$_{\text{citric pH 4.5}}$	0.69	0.14	$0.037\pm0.003^{b}{}_{1}$	$6.30^{a}{}_{1}$	4.11	$1.25\pm0.14^{cd}{}_{1}$	0.24	$6.53^{a}{}_{1}$
	A-BHI$_{\text{lactic pH 6.4}}$	0.94	0.17	$0.047\pm0.005^{cd}{}_{1}$	$6.15^{a}{}_{1}$	3.28	$1.28\pm0.11^{c}{}_{1}$	0.24	$7.03^{a}{}_{1}$
	A-BHI$_{\text{lactic pH 5.4}}$	1.04	0.20	$0.050\pm0.006^{cd}{}_{1}$	$6.07^{a}{}_{1}$	3.38	$1.01\pm0.09^{d}{}_{1}$	0.23	$6.94^{a}{}_{1}$
	A-BHI$_{\text{HCl pH 6.4}}$	0.97	0.20	$0.054\pm0.006^{d}{}_{1}$	$6.38^{a}{}_{1}$	3.22	$1.14\pm0.13^{cd}{}_{1}$	0.22	$6.88^{a}{}_{1}$
	A-BHI$_{\text{HCl pH 4.5}}$	0.74	0.16	$0.043\pm0.004^{bcd}{}_{1}$	$6.47^{a}{}_{1}$	3.70	$1.38\pm0.15^{c}{}_{1}$	0.25	$6.83^{a}{}_{1}$
Apple Juice	B-BHI	2.58	0.39	$0.10\pm0.01^{a}{}_{2}$	$5.67^{a}{}_{1}$	0.43	$0.19\pm0.02^{a}{}_{2}$	0.034	$7.20^{a}{}_{1}$
	NA-BHI	3.29	0.49	$0.13\pm0.02^{ab}{}_{2}$	$5.70^{a}{}_{1}$	1.05	$0.41\pm0.05^{b}{}_{1}$	0.086	$7.36^{a}{}_{1}$
	A-BHI$_{\text{acetic pH 6.4}}$	4.08	0.60	$0.16\pm0.01^{bc}{}_{2}$	$5.69^{a}{}_{1}$	2.93	$0.92\pm0.10^{cd}{}_{2}$	0.26	$7.74^{a}{}_{1}$
	A-BHI$_{\text{citric pH 6.4}}$	4.17	0.68	$0.20\pm0.03^{cd}{}_{2}$	$6.07^{a}{}_{1}$	2.74	$0.79\pm0.09^{c}{}_{2}$	0.19	$7.01^{a}{}_{1}$
	A-BHI$_{\text{citric pH 4.5}}$	2.99	0.51	$0.16\pm0.02^{bc}{}_{2}$	$6.29^{a}{}_{1}$	3.12	$1.03\pm0.08^{d}{}_{1}$	0.25	$7.38^{a}{}_{1}$
	A-BHI$_{\text{lactic pH 6.4}}$	4.25	0.74	$0.23\pm0.02^{d}{}_{2}$	$6.32^{a}{}_{1}$	3.27	$1.05\pm0.11^{d}{}_{1}$	0.25	$7.24^{a}{}_{1}$
	A-BHI$_{\text{lactic pH 5.4}}$	4.34	0.79	$0.18\pm0.02^{c}{}_{2}$	$5.79^{a}{}_{1}$	2.91	$0.81\pm0.09^{c}{}_{1}$	0.21	$7.14^{a}{}_{1}$
	A-BHI$_{\text{HCl pH 6.4}}$	4.51	0.71	$0.19\pm0.02^{cd}{}_{2}$	$5.82^{a}{}_{1}$	3.01	$0.97\pm0.08^{cd}{}_{1}$	0.21	$6.98^{a}{}_{1}$
	A-BHI$_{\text{HCl pH 4.5}}$	4.13	0.73	$0.16\pm0.02^{bc}{}_{2}$	$5.67^{a}{}_{2}$	3.42	$1.14\pm0.12^{d}{}_{1}$	0.27	$7.32^{a}{}_{1}$

B-BHI: Buffered BHI pH 7.0.

NA-BHI: Non-acidified BHI.

A-BHI: Acidified BHI with different acids: acetic, citric, lactic and HCl at different pH values.

$^{a-d}$: D-values (mean of three experiments ± SD) with different superscript in the same column (different growth conditions and the same heating medium) are significantly different ($P<0.05$).

$_{1-2}$: D-values (mean of three experiments ± SD) with different subscript in the same column (the same growth conditions and different heating medium) are significantly different ($P<0.05$).

different acids tested. However, it is worth noting that several acid adapted *S.* Typhimurium turned out to be more thermotolerant than non-acid adapted *S.* Senftenberg. For instance, *S.* Typhimurium cells grown in BHI acidifed with lactic acid at pH 6.4 and heated in apple juice exhibited a D_{58}-value of 0.23 min, while non-acid adapted *S.* Senftenberg showed D_{58}-values of 0.11 and 0.19 in orange juice and apple juice, respectively.

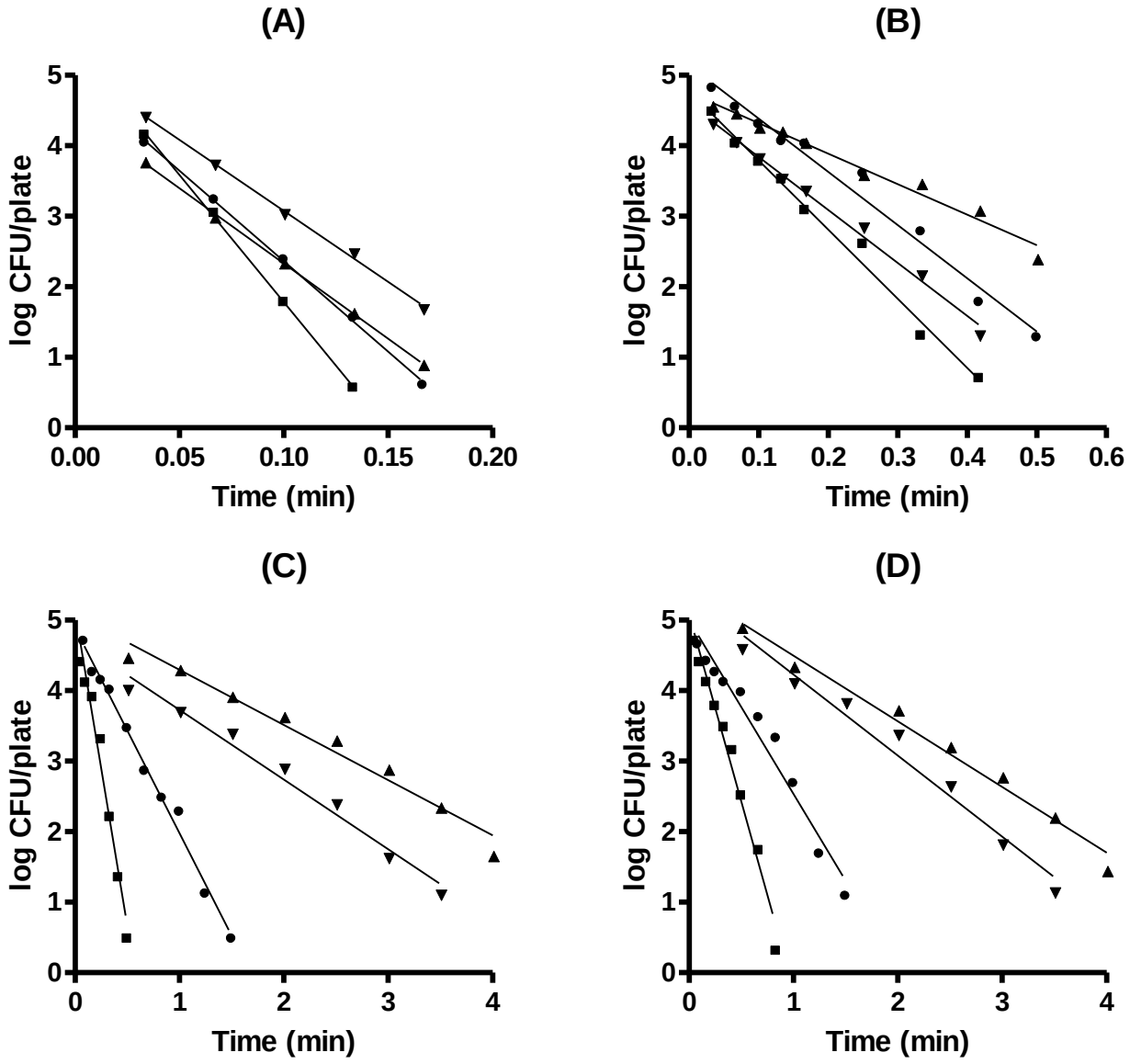

Fig. 3. Survival curves at 58ºC for cells grown in buffered BHI (■), non-acidified BHI (●), and acidified BHI with lactic acid at pH values of 6.4 (▲) and 5.4 (▼). *S.* Typhimurium cells heated in orange juice (A) and apple juice (B). *S.* Senftenberg cells heated in orange juice (C) and apple juice (D).

Z-values calculated under all conditions tested are shown in Table 2. The thermal death time curves of *S.* Typhimurium and *S.* Senftenberg grown in buffered BHI, non-acidified BHI and acidified BHI up to pH 5.4 with lactic acid are shown in Fig. 4. It can be seen that the conditions used to obtain acid adapted cells did not significantly ($p>0.05$) affect z-values calculated for each bacterium. Also of interest was the fact that the heating medium did not modify this parameter either. However, the average z-value determined for *S.* Typhimurium (6.03±0.29ºC) was significantly lower than that corresponding to *S.* Senftenberg (7.04±0.30ºC).

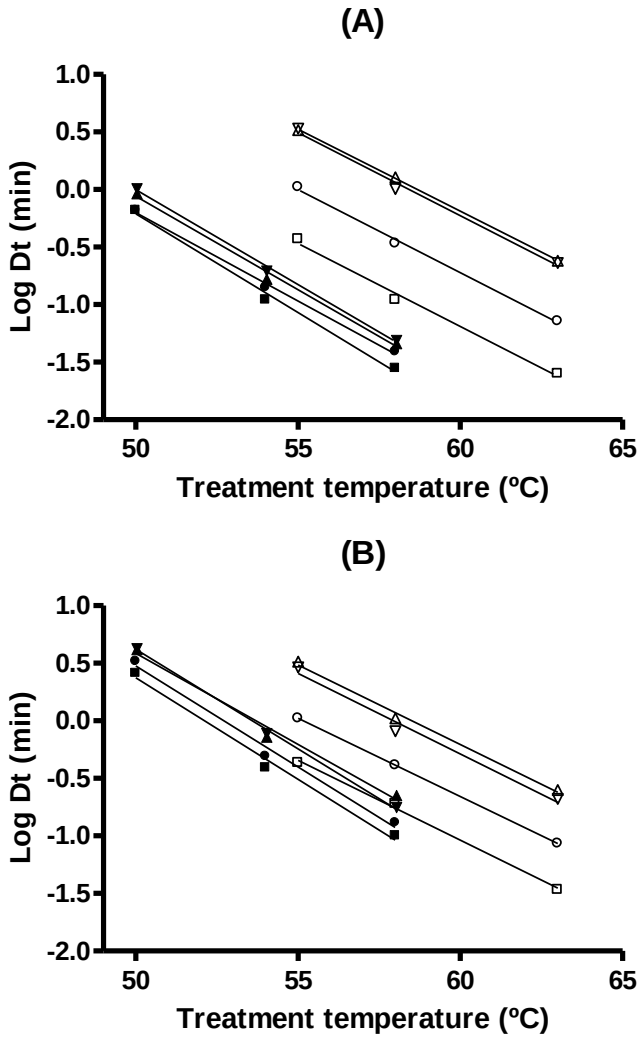

Fig. 4. Thermal death time curves of *S.* Typhimurium (closed symbols) and *S.* Senftenberg (opened symbols) cells grown in buffered BHI (■,□), non-acidified BHI (●,○), and acidified BHI with lactic acid at pH values of 6.4 (▲,Δ) and 5.4 (▼,▽). Cells treated in orange juice (A) and apple juice (B).

DISCUSSION

Recent food-borne disease outbreaks attributed to the consumption of unpasteurized orange and apple juices and other acid foods contaminated with *Salmonella* spp. highlight the need for knowledge on the behavior of these pathogenic bacteria in acidic food systems. Although there also exist a lot of factors affecting the bacterial acid resistance, the previous exposure to acidic conditions is probably one of the most important. Acid adaptation could lead to the development of an Acid Tolerance Response (ATR), which has been previously reported for various foodborne pathogens, including *Salmonella* spp. (Bearson et al., 1996, 1998; Bacon et al., 2003a; Greenacre et al., 2003; Yuk and Schneider, 2006). *S.* Typhimurium is also known to express this adaptive response (Bearson et al., 1996, 1998; Greenacre et al., 2003; Álvarez-Ordóñez et al., 2009a), but information available on the examination of the ability of *S.* Senftenberg to survive in extreme acidic conditions is very scarce (Kumar and Kumar, 2003).

In this study we have shown that acid adapted *S.* Typhimurium and *S.* Senftenberg showed a greater acid resistance when they were exposed to a lethal pH (2.5) in orange or apple juice than those grown in buffered BHI (non-acid adapted cells). Cells grown in non-acidified BHI (pH 7.4) also showed an enhanced survival under all conditions tested, probably due to the fact that BHI is a medium which contains 0.2% glucose and, therefore, the microbial growth causes a decrease in the extracellular pH to an approximate pH value of 6.2 (Álvarez-Ordóñez et al., 2008). Our results also show that the ATR acquired by cells adapted to acidic conditions during their growth in acidified BHI was not influenced by either the type of acidulant or the pH value of the growth medium. However, the composition of the challenge medium showed a significant effect on *S.* Typhimurium and *S.* Senftenberg ability to survive the acid challenge at pH 2.5. Higher D-values were obtained in all cases in apple juice than in orange juice, especially for *S.* Senftenberg, probably due to the fact that citric acid, the main organic acid present

in orange juice, shows a greater antimicrobial activity than malic acid, major organic acid of apple juice (Deng et al., 1999). However, the inducible ATR is an extremely complex phenomenon that, in addition to be conditioned by the presence of several compounds which may protect bacteria (Park et al., 1996; de Jonge et al., 2003; Kieboom and Abee, 2006) or in contrast may sensitize them (Chen et al., 2004) throughout the acid challenge, it is also influenced by genetic and other environmental factors (Berk et al., 2005). Therefore, comparisons of acid survival results in various research groups and microorganisms are difficult because of the use of different experimental conditions. Earlier studies have indicated that growth conditions significantly influence the degree of protection throughout the acid challenge. It has been previously reported for *S.* Typhimurium (Greenacre et al., 2003; Yuk and Schneider, 2006) that the ATR developed was dependent on the type of acid used to induce acid adaptation. In a recent study performed in our laboratory (Álvarez-Ordóñez et al., 2009a) we have shown that the effect of growth medium pH, as well as the efficacy of organic acids, on the induction of this adaptive response in *S.* Typhimurium was conditioned by the composition of growth and challenge media. For instance, citric, acetic and lactic acids were shown to be more effective than malic, hydrochloric and ascorbic acids when cells were grown in BHI and challenged in BHI pH 3.0, while no differences among organic acids were found when meat extract was used as growth medium. Furthermore, a specific effect of growth medium pH was only observed when cells were grown and challenged in meat extract. It is important to note that the previously mentioned studies, showing an ATR dependent on the type of acid used, were performed using different treatment media, such as simulated gastric fluid-pH 1.5 (Yuk and Scheneider, 2006) and TSB pH 3.0 (Greenacre et al., 2003).

Overall results confirm that the ATR is a complex phenomenom strongly affected by food matrixes and microbial characteristics, in addition to the interaction of different environmental factors. Although much effort has been made in the study of the mechanisms of induction of bacterial acid tolerance responses, these are not fully understood. It has been suggested that the induction of various genes and proteins, such as alternative *rpoS* and acid shock proteins (Bearson et al., 1997;

Foster, 2000), and some enzymatic systems, such as arginine decarboxylase and glutamate decarboxylase (Lin et al., 1995; Kieboom and Abee, 2006), as well as the existence of changes in membrane fatty acid composition (Brown et al., 1997; Yuk and Marshall, 2004; Álvarez-Ordóñez et al., 2008) are involved.

The enhanced acid resistance of *Salmonella* spp. acid adapted cells in fruit juices could represent an important concern for food safety as their possible survival in the stomach may enhance their likelihood of colonizing the intestine and thus, increasing the risk of infection. The relatively high acid resistance showed by acid adapted cells of both serovars, with mean D-values of 32.9±3.5 min in orange juice, and 41.9±2.4 and 114.8±12.2 min in apple juice for *S.* Typhimurium and *S.* Senftenberg, respectively, together with the short gastric emptying times of liquid foods could help to explain the occurrence of salmonellosis outbreaks associated with fruit juices consumption.

The practical significance of the increased acid resistance shown by these food-borne pathogens highlights the need to sufficiently pasteurize fruit juices to ensure their inactivation. However, it should also be taken into account that the growth of *S.* Typhimurium and *S.* Senftenberg in acidified media induces a cross-protection response against heat. This response has been reported in several occasions for various *Salmonella* serovars (Wilde et al., 2000; Mazzotta, 2001; Bacon et al., 2003b), including *S.* Typhimurium (Tosun and Gönül, 2003; Álvarez-Ordóñez et al., 2008) and *S.* Senftenberg (Álvarez-Ordóñez et al., 2009b). However, few studies have been conducted to examine the influence of some environmental factors, such as the composition of the growth and heating medium, on the thermal inactivation of acid adapted cells. Results obtained in this study show that the cross-protection response observed was not markedly influenced by the conditions prevailing during acid adaptation, although a trend to obtain higher D-values as the growth medium pH decreased was found for *S.* Senftenberg grown in BHI acidified with citric and hydrochloric acids. These results agree with those previously reported for both microorganisms (Álvarez-Ordóñez et al., 2008, 2009b). Our results also show that the magnitude of the cross-protection response developed was dependent on the juice composition, showing *S.* Senftenberg cells treated in orange

juice as the largest adaptive response. Moreover, the heating medium composition also affected the rate of inactivation of non-acid adapted and non-acidified cells of both microorganisms, which survived better in apple juice than in orange juice.

Recent progress has been made regarding to the molecular mechanisms involved in the acquisition of acid-induced heat resistance. It is generally acknowledged that exposure to acidic environments induces the synthesis of sets of characteristic stress proteins, and this has been hypothesized to contribute to cross-protection effects (Foster, 1995; Buchanan and Edelson, 1999). Moreover, an increase in cyclopropane fatty acid levels of the bacterial membrane, and changes in the unsaturated to saturated fatty acids ratio have previously been linked to these adaptive responses (Brown et al., 1997; Álvarez-Ordóñez et al., 2008, 2009b).

It is worth noting that D values observed in this study for both microorganisms are significantly lower than those described in literature using different foods and laboraratory culture media as heating media (Doyle and Mazzotta, 2000). This lower heat resistance could be attributed to the low pH value of orange and apple juices (3.6 and 3.4, respectively), since it is well known that the acidification of the heating medium causes a decrease in bacterial heat resistance (Blackburn et al., 1997; Casadei et al., 2001; Mañas et al., 2003). Results obtained in this study confirm further evidence that the bacterial survival during heating is a function of the composition rather than the pH of the environment. However, data available on the effect of organic acids on the microbial thermal tolerance do not often agree. Several authors have reported that citric acid turns out to be more effective in reducing the bacterial heat resistance than malic acid (Powers, 1976; Lynch and Potter, 1988, Palop et al., 1996), whereas others have found the opposite effect (Smelt, 1980; Blocher and Busta, 1983; Mazzotta, 2001). Our results show that, whereas the rate of inactivation of *S.* Typhimurium grown in all conditions assayed and *S.* Senftenberg grown in buffered BHI and non-acidified BHI was higher in orange juice than in apple juice, the acid adapted populations of *S.* Senftenberg better survived in orange juice, although, generally, no significant statistical differences could be found.

For both *Salmonella* serovars the test of homogeneity of the slopes indicated no significant differences ($p>0.05$) between the z-values obtained in orange juice or apple juice. For *S.* Typhimurium a mean z-value of 6.16±0.27ºC and 5.89±0.27ºC in orange juice and apple juice was obtained, respectively. The z-values for *S.* Senftenberg were slightly higher in apple juice (7.26±0.23ºC) than in orange juice (6.82±0.16ºC). These z-values turned out to be included in the range reported for these microorganisms in literature (Orta-Ramírez et al., 1997; Veeramuthu et al., 1998; Murphy et al., 2000, 2002), but they were to some extent higher than those found in other studies on liquid whole egg, egg yolk and egg white and milk (Doyle and Mazzotta, 2000), ranging from 3.3 to 5.3ºC. It is important to note that our data were obtained in orange and apple juices, with pH values of 3.6 and 3.4, respectively, and the acidification of the heating medium is known to cause an increase in the z-values obtained (Sanz Pérez et al., 1982; Casadei et al., 2001). Our data agree with those previously reported by Mazzotta (2001) for a *Salmonella* five strain-composite (*S.* Typhimurium, *S.* Enteritidis, *S.* Gaminara, *S.* Rubislaw, *S.* Hartford), who found a mean z-value of 5.8±0.3ºC when cells were treated in apple, orange and white grape juices adjusted to pH 3.9.

Based in the data shown here, it appears that for each culture condition used, *S.* Senftenberg showed a higher thermotolerance than *S.* Typhimurium, although it is interesting to note that under several test conditions D-values found for *S.* Typhimurium acid adapted cells were higher than those found for *S.* Senftenberg non-acid adapted cells (Table 2). This further implies that heat treatments based on data obtained for non-stressed target microorganisms could lead to underestimating the time needed to achieve a particular level of inactivation. Therefore, the effect of environmental stresses should be considered when food preservation measures are developed, and in the particular case of the design of pasteurization processes in fruit juices is recommendable to consider the heat resistance of acid adapted cells, which will include an extra safety factor to the minimal requirements for pasteurization.

To sum up, the existence of an ATR in *S.* Typhimurium and *S.* Senftenberg in acidified fruit juices has been shown in this study, which could represent a great

concern for juice producers and Public Health. Thus, a proper design of heat treatments of pasteurization is needed in order to guarantee food safety of these products. However, it should be taken into account that acid adaptation may increase bacterial heat resistance. The magnitude of the cross-protection response also varies in both serovars and was determined by growth conditions and those prevailing during heating. These findings highlight the convenience of carrying out studies focused on the influence of environmental factors on the heat resistance of a target microorganism in order to find the conditions in which its thermal tolerance is maximal, with the aim of guaranteeing safety of processed foods.

ACKNOWLEDGMENTS

This work was supported by the Junta de Castilla y León (project number LE002A07). Álvarez-Ordóñez A. gratefully acknowledges the finantial support from the Spanish Ministry of Education.

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9.

Acid adaptation influences Salmonella Typhimurium *resistance to osmotic, oxidative and alkaline stresses*

Avelino Álvarez-Ordóñez, Ana Fernández, Ana Bernardo and Mercedes López

Submitted for publication

ABSTRACT

The influence of acid adaptation on the subsequent sensitivity of *S.* Typhimurium to osmotic, oxidative and alkaline stresses was studied using cells grown in buffered BHI (pH 7.0) and BHI acidified up to pH 4.5 with acetic, citric, lactic and hydrochloric acids. BHI containing 2.5 M NaCl, or 30 mM H_2O_2, or NaOH (to reach a pH value of 11.0), or 2.5% TSP (pH 11.0) was used as treatment medium. Acid adapted *S.* Typhimurium showed an increased vulnerability to the toxicity of salt and hydrogen peroxide. Non-acid adapted cells showed D-values of 493.45 and 31.85 min in BHI-2.5 M NaCl and BHI-30 mM H_2O_2, respectively, which turned out to be about 1.5-2 and 1.5-2.5 times higher than those observed for acid adapted cells. These findings may be useful for food preservation regimes, as the synergistic effect of acid and osmotic and oxidative stresses could contribute to control *S.* Typhimurium survival in some foods, such as cheese and other fermented products. On the other hand, a cross-protection response to alkaline stress was found, although this response was not apparent when the alkaline treatment was carried out in the presence of TSP, which showed a higher antimicrobial effect. Thus, when *S.* Typhimurium cells were treated in the presence of NaOH, D-values found for acid adapted cells, ranging from 7.94 to 9.86 min, were significantly higher than those corresponding to non-acid adapted cells (D-value of 5.09 min). This fact suggests that although the antimicrobial effect of TSP is mainly due to its alkaline pH, there is also another unknown specific effect. These findings may help to improve food safety by an intelligent selection and combination of hurdles. However, further studies are needed in order to elucidate the targets of different preservative factors within the microbial cells.

INTRODUCTION

It is well known that *S.* Typhimurium is capable of developing adaptive responses to acid stress which allow for its survival in lethal acidic conditions (Foster and Hall, 1990; Lee et al., 1994; Bearson et al., 1996, 1998; Greenacre et al., 2003; Álvarez-Ordóñez et al., 2009). This so-called Acid Tolerance Response (ATR) also provides protection against other food-related stresses and has become a major concern with regard to food safety. It is also well known that acid adapted *S.* Typhimurium exhibits cross-protection against heat treatments (Mazzota, 2001; Tosun and Gönül, 2003; Álvarez-Ordóñez et al., 2008). However, less attention has been paid to the effect of acid adaptation on other stressful conditions which prevail during food processing, such as osmotic, oxidative and alkaline stresses, in spite of the fact that this information could be very useful in order to control its survival in acidic foods. Data available in literature with regard to the behavior of *S.* Typhimurium acid adapted cells after exposure to salt indicate that the type of acidulant influences the bacterial response observed. Greenacre and Brocklehurst (2006) found a decrease in salt tolerance for acid adapted *S.* Typhimurium in the presence of lactic acid, and the opposite effect when acetic acid was used, and Foster and Hall (1990) and Leyer and Johnson (1993) found that hydrochloric acid protects it against salt stress. The influence of acid adaptation on the resistance to oxidative stress has been investigated on several occasions but the results obtained are contradictory. Thus, whereas Leyer and Johnson (1993) and Greenacre et al. (2006) have found that acid adaptation sensitized *S.* Typhimurium to oxidative damage, other authors (Lee et al., 1995; Kwon et al., 2000) have identified an ATR-linked tolerance to hydrogen peroxide. Finally, there are no data available on the behavior of *S.* Typhimurium acid adapted cells in alkaline environments, although it has been previously shown that acid adaptation increases the sensitivity of *S.* Enteritidis to alkaline stress (Sampathkumar et al., 2004). It is important to note that in all of these studies, cells were not adapted by growing them at low pH values in the presence of organic acids in spite of the fact that cells adapted under these conditions may accurately represent those occurring in their natural environment, since organic acids

are commonly used as preservatives in the food industry and in decontamination treatments. Therefore, the aim of this work was to determine the response of *S.* Typhimurium to osmotic (NaCl), oxidative (hydrogen peroxide) and alkaline (sodium hydroxide or trisodium phosphate) stresses after its growth in the presence of organic acids (acetic, citric and lactic) and hydrochloric acid up to pH 4.5.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Salmonella enterica serovar Typhimurium strain (CECT 443) used in this study was obtained from Colección Española de Cultivos Tipo (CECT) (Spanish Type Culture Collection). The lyophilized cultures were revived in BHI and incubated for 24 hours at 37ºC. Pure cultures were maintained on BHIA plates at 4ºC. Subcultures were prepared by transferring an isolated colony from a plate into a test tube containing 10 mL of sterile BHI followed by incubation at 37ºC for 24 h. These fresh subcultures were used to produce acid adapted and non-acid adapted cells.

Flasks containing 50 mL of sterile BHI (pH 7.4) non-acidified and acidified at pH values of 6.4, 5.4 and 4.5 with acetic (Prolab), citric (Sigma), lactic (Merck), and hydrochloric acids (Panreac) were inoculated with the subculture to a final concentration of 10^3cells/mL. So as to obtain non-acid adapted control samples, buffered BHI adjusted to pH 7.0 by addition of Sorensen buffer 0.2 M (bisodium (Merck)-monopotassium (Panreac) phosphate) was used. Afterwards, cultures obtained as described above were incubated at 37ºC during the time needed to reach the late stationary-phase of growth. When acidified BHI was used as growth medium the experiments were carried out, for each acid, only at the highest and the lowest pH values which allowed for *S.* Typhimurium growth.

Study of the response of acid adapted cells to osmotic, oxidative and alkaline stresses

Aliquots of 5 mL of stationary-phase cells were harvested by centrifugation at 8000 g for 5 min at 4ºC (*Eppendorf centrifuge 5804R).* The supernatant liquid was discarded and cells were resuspended in 50 mL of BHI supplemented with (i) 2.5 M NaCl (ii) 30 mM H_2O_2 (iii) NaOH (pH 11.0) and (iv) 2.5% trisodium phosphate (TSP). Then, after incubation at room temperature, survival was monitored periodically. Samples (0.1 mL) were collected after different treatment times, ten-fold serial dilutions were produced in sterile 0.1% (w/v) peptone solution (Oxoid) and were plated in duplicate on BHIA. Viable cell densities at each point in time were enumerated following incubation of the plates at 37ºC for 48 h (longer incubation times did not have any influence on the counting). Survivors were counted with a modified Image Analyser Automatic Counter (Protos Analytical Measuring Systems, Cambridge, UK) as described elsewhere (Ibarz et al., 1991). All experiments were performed in triplicate on three different fresh cultures.

Survivor curves and statistical analysis

D-values (min) were determined by plotting the log number of survivors against time for each culture. The line that best fits survivor plots was determined by linear regression (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA), and the negative reciprocal of the slope was used for the D-value.

D-values were compared using Student´s *t*-test (Steel and Torrie, 1986) (Statistica for Windows version 4.5, Statsoft, Inc, Tulsa, OK, USA).

RESULTS AND DISCUSSION

The subsequent resistance of *S.* Typhimurium to osmotic, oxidative and alkaline stresses was assessed using cells grown in non-acidified BHI (pH 7.4), buffered BHI (pH 7.0) and BHI acidified with acetic (pH 6.4), citric (pH 6.4 and 4.5), lactic (pH 6.4 and 5.4) and hydrochloric acids (pH 6.4 and 4.5) and challenged in BHI containing 2.5 M NaCl, or 30 mM H_2O_2, or NaOH (to reach a pH value of

11.0), or 2.5% TSP (pH 11.0). In all cases, survival curves obtained fitted properly into a first order kinetic (R^2 ranging from 0.92 to 0.99; data not shown). An example of inactivation curves obtained under all challenge conditions for cells grown in buffered BHI and BHI acidified at pH 6.4 and 5.4 with lactic acid is shown in Fig. 1. D-values calculated are shown in Table 1, expressed as mean values of three independent experiments ± standard deviations. Under all culture conditions tested *S.* Typhimurium turned out to be extremely resistant to salt, as shown by the fact that only 1.5-3 log reductions were achieved after 12 hours of treatment. D-values obtained for cells grown in buffered BHI (493.45 min) and non-acidified BHI (461.05 min) were significantly higher than those found for acid adapted cells, with D-values ranging from 245.20 to 330.25 min. Our results agree with those reported by Greenacre and Brocklehurst (2006), who showed that, for *S.* Typhimurium, acid adaptation in the presence of lactic acid rendered cells hypersensitive to NaCl. However, these authors also found that acetic acid provided cells cross-protected against NaCl stress. In other studies an increase has been reported in salt tolerance for *S.* Typhimurium acid adapted cells (Leyer and Johnson, 1993), and it has also been found that a mixture of short-chain fatty acids at concentrations in the small intestine was unable to provide cross-protection to salt (Kwon et al., 2000). These contradictory results could be due to the existence of intraspecific differences (Faleiro et al., 2003) or to the different experimental conditions used to produce acid adapted cells. It is important to note that, for acid adapted cells, neither the pH value of the culture medium nor the type of acidulant significantly modified the response found. The mechanism of bacterial response to osmotic stress is not fully understood but it is well known that it involves the accumulation of ions and osmoprotectant compounds in which uptake the OmpC and OmpF membrane porins are involved (Bremer and Krämer, 2000). The levels of these porins vary in response to different demands and stresses, including acid stress (Leyer and Johnson, 1993; Bremer and Krämer, 2000). Leyer and Johnson (1993) have previously reported that, for *S.* Typhimurium, under mildly acidic conditions the expression of OmpC was enhanced and OmpF was repressed. Either way, it seems reasonable to suggest that both acid and osmotic stresses probably act on the same cellular target -the bacterial membrane- since a great influence of acid adaptation on the membrane fatty acid

composition and physical fluidity has been previously reported (Brown et al., 1997; Álvarez-Ordóñez et al., 2008).

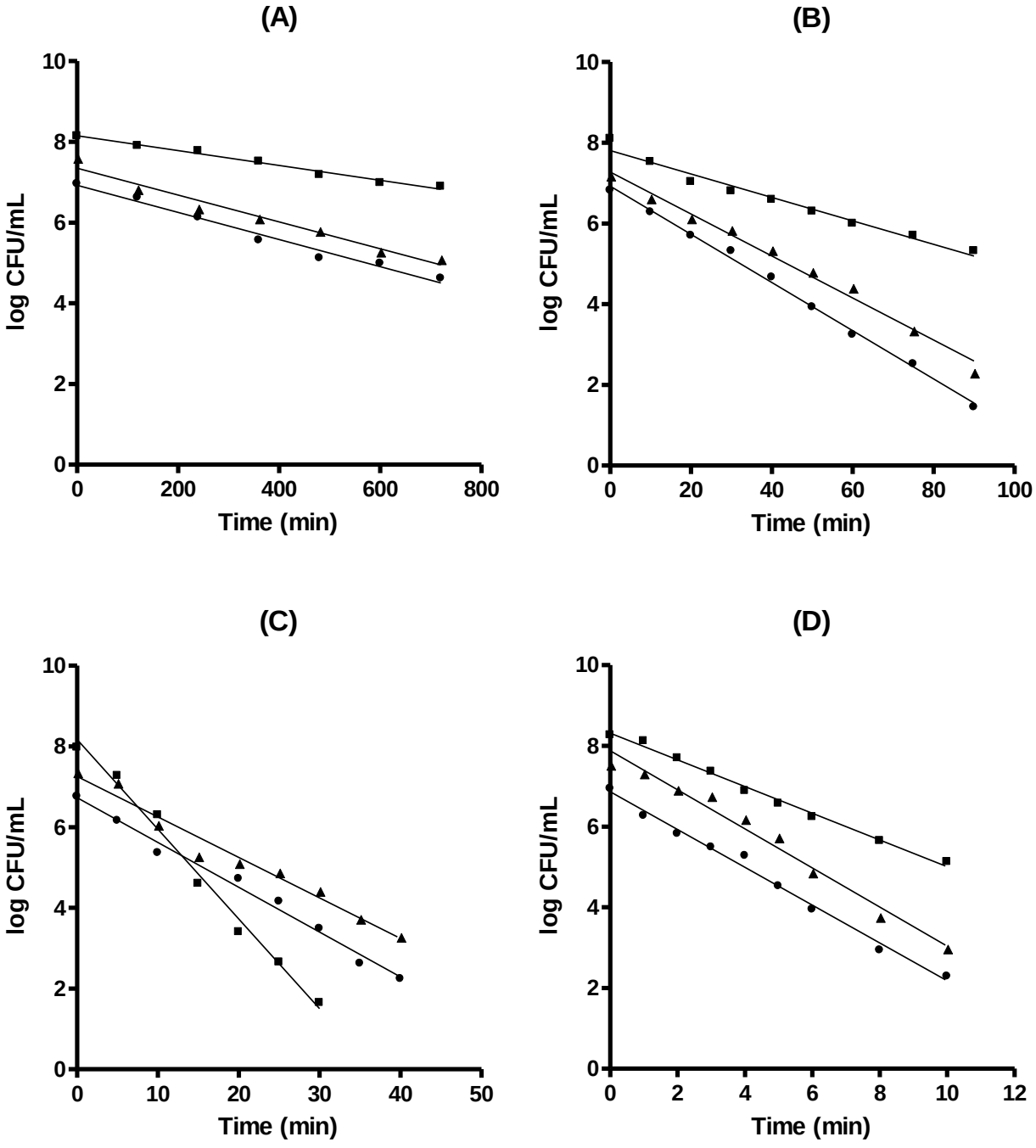

Fig. 1. Survival curves in BHI containing (A) NaCl 2.5 M (B) H_2O_2 30 mM (C) NaOH pH 11.0 and (D) TSP 2.5% for *S.* Typhimurium (CECT 443) cells grown in buffered BHI (■) and acidified BHI with lactic acid at pH 6.4 (▲) and 5.4 (●).

Table 1. D-values (min) for cells of *S.* Typhimurium (CECT 443) grown at 37ºC in buffered BHI (non-acid adapted control cells), non-acidified BHI and acidified BHI at different pH values with several acids and treated in BHI containing NaCl 2.5 M, H_2O_2 30 mM, NaOH pH 11.0, or TSP 2.5%.

GROWTH MEDIUM	NaCl 2.5 M	H_2O_2 30 mM	NaOH pH 11.0	TSP 2.5%
B-BHI	493.45±70.07^{a}	31.85±3.67^{a}	5.09±1.00^{a}	2.77±0.77^{ab}
NA-BHI	461.05±64.42^{a}	28.55±3.70^{ad}	8.07±0.54^{b}	3.00±0.55^{b}
A-BHI$_{\text{acetic pH 6.4}}$	245.20±36.49^{b}	12.10±1.25^{b}	9.18±1.42^{b}	1.87±0.40^{a}
A-BHI$_{\text{citric pH 6.4}}$	311.65±42.36^{b}	20.45±2.36^{c}	9.86±1.76^{b}	2.04±0.32^{ab}
A-BHI$_{\text{citric pH 4.5}}$	309.07±32.14^{b}	20.06±2.16^{c}	8.33±1.21^{b}	1.88±0.25^{a}
A-BHI$_{\text{lactic pH 6.4}}$	330.25±42.92^{b}	17.12±2.66^{c}	9.02±1.32^{b}	1.72±0.23^{a}
A-BHI$_{\text{lactic pH 5.4}}$	325.25±38.96^{b}	16.10±1.81^{c}	8.79±0.59^{b}	1.85±0.50^{ab}
A-BHI$_{\text{HCl pH 6.4}}$	281.25±47.02^{b}	20.90±3.08^{cd}	8.11±0.84^{b}	1.68±0.35^{a}
A-BHI$_{\text{HCl pH 4.5}}$	300.85±27.65^{b}	18.75±3.40^{c}	7.94±0.78^{b}	2.16±0.47^{ab}

B-BHI: Buffered BHI pH 7.0
NA-BHI: non-acidified BHI (pH 7.4)
A-BHI: acidified BHI with different acids: acetic, citric, lactic and HCl at different pH values
$^{a-d}$: D-values (mean of three experiments ± SD) with different superscript in the same column are significantly different ($P<0.05$).

Hydrogen peroxide is an antimicrobial agent frequently found by microorganisms in the food environment from different origin. It is produced not only by starter bacteria added during the manufacturing of several fermented products but also during the metabolic burst that occurs in the phagolysosome. Results obtained (Fig. 1 and Table 1) show that *S.* Typhimurium acid adaptation causes an increase in its sensitivity to hydrogen peroxide, which could be exploited in preservation regimes. Cells grown in buffered BHI (D-value of 31.85 min) and non-acidified BHI (D-value of 28.55 min) showed a significantly higher resistance to H_2O_2 than acid adapted cells, with D-values ranging from 12.10 to 20.90 min. The lowest D-values were obtained for *S.* Typhimurium cells produced in the presence of

acetic acid, probably due to its greater ability to enter the cell. No significant differences among the rest of acids and pH values tested were found. Our results agree with those previously reported by Greenacre et al. (2006), who described that acid adapted cells in the presence of lactic acid were vulnerable to oxidative damage and displayed a hypersensitive phenotype compared with unadapted cells. On the contrary, several studies have identified an ATR-linked resistance to hydrogen peroxide for acid adapted cells in the presence of HCl (Foster and Hall, 1990; Lee et al., 1995) or a mixture of short-chain fatty acids (Kwon et al., 2000). It is important to bear in mind that hydrogen peroxide mode of action includes the formation of oxidative radicals which damage the DNA and the cytoplasmic proteins (Storz and Zheng, 2000). Once inside the cell, its action is partly counteracted by several enzymes, such as catalase, alkyl hydroperoxidase, glutathione reductase, and the DNA-binding protein Dps, most of them under *oxyR* regulation (Dukan and Touati, 1996; Storz and Zheng, 2000). Results obtained by Greenacre et al. (2006), who found that five members of the *oxyR* regulon were down-regulated during *S.* Typhimurium acid adaptation in the presence of lactic acid, indicate that the lower resistance showed by acid adapted cells could be attributed to a lower amount of these protective enzymes.

Salmonella spp. are commonly exposed to alkaline pH environments in polluted water from extreme alkaline sewage, chemical industry and agricultural effluents (Rowbury et al., 1989), as well as in some foods, such as egg whites (Humphrey et al., 1991), and some food processing treatments. The trisodium phosphate (TSP) is an antimicrobial agent which exerts its action by means of its alkaline pH, and it has been recently approved by the United States Department of Agriculture (USDA) for its use as a food ingredient and for the reduction of *Salmonella* contamination during poultry processing (Federal Register, 1994; Sampathkumar et al., 2004; del Río et al., 2006). Our results on the resistance of acid adapted *S.* Typhimurium cells to alkaline stress are also shown in Fig. 1 and Table 1. It is important to note that the influence of acid adaptation on the subsequent resistance to pH 11.0 was different as a function of the composition of the challenge medium. Whereas D-values found for acid adapted cells challenged in

the presence of NaOH, ranging from 7.94 to 9.86 min, were significantly higher than those corresponding to non-acid adapted cells (D-value of 5.09 min), the opposite effect was found when TSP was used. *S.* Typhimurium was more sensitive to 2.5% TSP than to its equivalent treatment with NaOH, although the magnitude of the differences depended on the growth conditions. Thus, whereas D-values found for non-acid adapted cells were about two times lower when cells were challenged in the presence of TSP, acid adapted cells showed D-values about 4-5 times lower than those found when the alkaline treatment was performed with NaOH. Several studies have previously reported that acid adaptation sensitizes the cells to alkaline stress and vice versa for *E. coli* (Rowbury et al., 1993, 1996; Rowbury and Hussain, 1996), *E. faecalis* (Flahaut et al., 1997) and *S.* Enteritidis (Sampathkumar et al., 2004). As far as we know, this is the first study reporting a cross-protection response for *S.* Typhimurium acid adapted cells against alkaline stress in the presence of NaOH. The differences found between both alkaline agents suggest that the TSP has a specific antimicrobial effect. These findings do not agree with the results previously obtained for *S.* Enteritidis by Sampathkumar et al. (2003), who reported that the effect of TSP was similar to that of an equivalent alkaline pH treatment and the antimicrobial activity of TSP was lost when the pH of the treatment solution containing TSP was adjusted to 7.0, and concluded that *S.* Enteritidis could respond in a similar way when it is exposed to a sublethal concentration of TSP or its equivalent alkaline pH. However, these authors also reported that TSP exerts a specific effect on the bacterial outer membrane related to the formation of chelates with divalent cations in the bacterial outer membrane, leading to increased membrane permeability, membrane damage, release of intracellular contents and cell death, which could contribute to explain why acid adapted cells challenged in the presence of TSP showed D-values about 4-5 times lower than those found when the alkaline treatment was performed in the presence of NaOH.

To sum up, we have shown that acid adaptation sensitizes *S.* Typhimurium to osmotic and oxidative stresses and to alkaline treatments in the presence of TSP. On the other hand, a cross-protection response between acid and alkaline stress was found when the alkaline treatment was carried out in the presence of NaOH. These

findings uphold that food preservation measures should be based on the intelligent selection and combination of hurdles, and highlight the need for a better understanding of the mechanisms of microbial adaptation to stress in order to design effective food safety systems.

ACKNOWLEDGMENTS

Álvarez-Ordóñez A. gratefully acknowledges the finantial support from the Spanish Ministry of Education.

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10.

RESUMEN Y CONCLUSIONES

RESUMEN

El incremento de la demanda por parte del consumidor de alimentos de elevada calidad sensorial, más frescos y saludables, supone un reto de gran importancia para la industria alimentaria, que se ve obligada a implementar nuevas tecnologías de conservación de los alimentos y a combinar varios agentes de conservación a bajas intensidades, con consecuencias menos agresivas para las características organolépticas de los alimentos, en lo que se conoce como tecnología de procesos combinados. Sin embargo, las condiciones imperantes durante el procesado y distribución de alimentos mínimamente procesados pueden permitir la supervivencia de algunos microorganismos que han podido desarrollar respuestas adaptativas, aumentando su resistencia frente a diferentes agentes estresantes que, en condiciones normales, serían letales (Abee y Wouters, 1999; Gibbs, 1999; Sharp y Reilly, 2000), hecho que incrementa el riesgo de expansión de diferentes toxiinfecciones de origen alimentario, entre las que destaca la salmonelosis.

Uno de los métodos más comúnmente empleado por la industria alimentaria para controlar el crecimiento microbiano es la acidificación mediante la adición de ácidos orgánicos. Sin embargo, la implicación de los alimentos ácidos en algunos brotes de toxiinfección, incluida la salmonelosis (Parish, 1997; D´Aoust, 2000), obliga a reconsiderar la creencia de que los alimentos con bajo pH son microbiológicamente seguros.

Aunque en alguna ocasión se ha estudiado la adaptación ácida de *Salmonella* y sus implicaciones en la respuesta frente a otros agentes estresantes (Foster y Hall, 1990; Leyer y Johnson, 1993; Bearson y col., 1998; Wilde y col., 2000; Mazzotta, 2001; Bacon y col., 2003a, b; de Jonge y col., 2003; Greenacre y col., 2003; Tosun y Gönül, 2003; Greenacre y Brocklehurst, 2006; Yuk y Schneider, 2006), la realidad es que se conoce muy poco acerca de la variabilidad de estas respuestas en función de distintos factores, a pesar de la importancia que este conocimiento supone para controlar de una forma adecuada el desarrollo de este patógeno.

Esta Tesis Doctoral se ha centrado en determinar la respuesta adaptativa de dos serovariedades del género *Salmonella* de gran interés tecnológico y sanitario, *S.* Typhimurium y *S.* Senftenberg, frente al estrés ácido originado tras el crecimiento a diferentes valores de pH y en presencia de diferentes ácidos orgánicos (acético, ascórbico, cítrico, láctico y málico) y ácido clorhídrico, estudiando la influencia que el desarrollo de esta respuesta adaptativa ejerce sobre su comportamiento frente a diversos tratamientos habitualmente empleados por la industria alimentaria, calor, utilización de sal, alcalinización y estrés oxidativo. Asimismo, se ha determinado la cinética de inactivación de las células adaptadas a la acidez tras su exposición a un pH extremo, en medios de laboratorio y algunos alimentos, como extracto de carne y zumos de frutas, con el fin de comprobar la aparición de respuestas de tolerancia ácida, que no sólo pueden prolongar la supervivencia microbiana en varios alimentos ácidos, sino que también pueden incrementar la resistencia bacteriana frente a las condiciones ácidas del tracto gastrointestinal, disminuyendo la dosis infectiva e incrementando el riesgo de contraer la enfermedad.

La tecnología de los procesos combinados se considera actualmente como la posibilidad más prometedora para reducir el riesgo microbiológico en los alimentos pero, para su correcta aplicación, se hace necesario un conocimiento profundo de los mecanismos implicados en las respuestas bacterianas frente a los diversos factores de estrés y, aunque se ha avanzado mucho en estos últimos años para comprender estas respuestas, los mecanismos involucrados aún no están completamente elucidados. Nuestra contribución, en este sentido, ha consistido en el estudio de la implicación de las modificaciones en la composición lipídica de la membrana celular y de algunos sistemas homeostáticos, como la arginina decarboxilasa, la lisina decarboxilasa y la glutamato decarboxilasa, en la adquisición de estas respuestas.

La consecución de estos objetivos se refleja concretamente de la forma siguiente. En los capítulos 3, 4 y 5 se describe la respuesta de tolerancia ácida en *S.* Typhimurium, los factores ambientales que la condicionan y la importancia de los sistemas homeostáticos dependientes de la presencia de aminoácidos (lisina decarboxilasa y arginina decarboxilasa) en el medio de tratamiento. Los capítulos 6 y 7 incluyen los trabajos relacionados con el estudio de la influencia que la

adaptación a la acidez ejerce sobre la resistencia al calor de *S.* Typhimurium y *S.* Senftenberg, así como el efecto que sobre la misma ejercen las modificaciones en la composición en ácidos grasos de la membrana bacteriana. En el capítulo 8 se presentan los resultados obtenidos para *S.* Typhimurium y *S.* Senftenberg sobre las respuestas de tolerancia ácida y de protección cruzada frente al calor en algunos alimentos ácidos (zumo de naranja (pH 3,6) y zumo de manzana (pH 3,4)). Por último, en el capítulo 9 se describe el efecto de la adaptación ácida de *S.* Typhimurium sobre su posterior resistencia frente a la sal, el estrés oxidativo y la alcalinización.

Respuesta de tolerancia ácida en Salmonella Typhimurium y *Salmonella* Senftenberg

El hecho de que ambas serovariedades sean capaces de crecer en un intervalo amplio de pH conlleva la posibilidad de su desarrollo en numerosas situaciones que ocurren durante el procesado de los alimentos. La disminución de pH a lo largo del tiempo en alimentos fermentados o durante la conversión del músculo en carne, así como la utilización de ácidos orgánicos como aditivos o como agentes antimicrobianos para la descontaminación de canales en mataderos, son ejemplos de ambientes ácidos subletales que pueden dar lugar al desarrollo de respuestas bacterianas de adaptación a la acidez (Samelis y col., 2001) con el consecuente aumento de resistencia a valores de pH más extremos.

Para la determinación de la cinética de inactivación de *S.* Typhimurium y *S.* Senftenberg a un pH letal (2,5-3,0) se utilizaron células en fase estacionaria tras su crecimiento en Brain Heart Infusion (BHI) acidificado a valores de pH de 6,4, 5,4 y 4,5 empleando diversos ácidos orgánicos (acético, ascórbico, cítrico, málico y láctico) y ácido clorhídrico (células adaptadas a la acidez), BHI no acidificado (células no acidificadas) y BHI tamponado a pH 7,0 (células no adaptadas a la acidez). En los experimentos destinados a evaluar la influencia de la temperatura de crecimiento, se utilizó un amplio rango de temperaturas, 10, 25, 37 y 45ºC. Para estudiar el efecto de la composición del medio de tratamiento se utilizaron medios de laboratorio, BHI y un medio mineral mínimo (MM) descrito por de Jonge y col. (2003), así como un extracto de carne y zumos de frutas (naranja y manzana).

En líneas generales, ambas serovariedades mostraron una gran capacidad para crecer en condiciones ácidas, presentando un comportamiento prácticamente similar, si bien *S.* Senftenberg fue capaz de crecer de forma más rápida, especialmente a valores de pH ≤ 5,4. En todos los casos, la acidificación del medio de cultivo ocasionó un incremento en los tiempos de generación, en la duración de la fase de latencia y en el tiempo requerido para alcanzar la fase estacionaria de crecimiento y un descenso en la densidad de la población alcanzada. El pH límite de crecimiento estuvo condicionado por el tipo de ácido utilizado. De este modo, no se detectó crecimiento en presencia de ácido acético a pH ≤ 5,4 y de ácido láctico a pH ≤ 4,5 para ninguna de las dos serovariedades, siendo, en general, el orden de eficacia inhibitoria el siguiente: acético > láctico > cítrico ≥ málico ≥ ascórbico ≥ clorhídrico. Cabe destacar que la temperatura de cultivo influyó de forma notable en el crecimiento de *S.* Typhimurium en condiciones ácidas, de tal manera que a temperaturas alejadas de la óptima de crecimiento (37ºC) se potenció el efecto inhibitorio de los ácidos orgánicos, viéndose incrementado el pH límite de crecimiento a las temperaturas de 10 y 45ºC hasta valores de pH ≤ 5,4.

En todas las condiciones experimentales ensayadas, las curvas de supervivencia obtenidas se ajustaron a una cinética de inactivación de primer orden, por lo que el valor D (tiempo necesario en minutos para reducir a la décima parte la población microbiana) resultó un parámetro adecuado para comparar la resistencia que exhibieron las células en las diferentes condiciones.

Los resultados mostrados en los capítulos 3, 4 y 5 ponen de manifiesto que en todas las condiciones utilizadas para la obtención de las células adaptadas a la acidez se produjo un aumento de su resistencia a un pH letal (2,5 ó 3,0). Incluso las células no acidificadas vieron incrementada su resistencia a la acidez, en comparación con las células crecidas en BHI tamponado, ya que durante su crecimiento se produce un descenso progresivo del pH extracelular hasta un valor aproximado de 6,0. Los resultados obtenidos también muestran que la magnitud de la respuesta de tolerancia ácida desarrollada estuvo condicionada por diversos factores ambientales. En general, la adaptación de ambas serovariedades a la acidez mediante su crecimiento en BHI (pH 7,4) originó una respuesta de tolerancia ácida claramente inferior a la

desarrollada cuando el crecimiento microbiano tuvo lugar en presencia de diferentes ácidos orgánicos a valores inferiores de pH (6,4; 5,4 y 4,5). Para estas últimas células, el efecto de las condiciones utilizadas para la adaptación (pH y tipo de ácido) estuvo condicionado por la composición y pH del medio de tratamiento. Así, mientras que al realizar el tratamiento en BHI y extracto de carne a pH 3,0 la disminución del pH del medio de adaptación dio lugar a células más resistentes a la acidez, este efecto no se observó cuando el tratamiento se llevó a cabo a pH 2,5 en zumo de naranja y de manzana y en un medio mineral mínimo, detectándose solamente un efecto específico del tipo de ácido en BHI o en extracto de carne ajustados a pH 3,0, resultando, de forma general, los ácidos cítrico, acético y láctico los que ocasionaron una mayor respuesta adaptativa. Este hecho podría ser de gran importancia para la seguridad alimentaria, ya que estos compuestos son frecuentemente utilizados en tratamientos de descontaminación de canales en los mataderos y como aditivos en la industria alimentaria (Smulders y Greer, 1998).

La temperatura de crecimiento modificó la resistencia mostrada por *S.* Typhimurium frente a un tratamiento en BHI a pH 3,0, que resultó de menor cuantía para las células crecidas a 10ºC y aumentó de forma exponencial hasta 37ºC, temperatura a la que se obtuvieron valores D del orden de 5-10 veces superiores que los correspondientes a 10ºC. Las células crecidas a 45ºC mostraron menor resistencia, que resultó similar a la de las obtenidas a 25ºC. El hecho de que la obtención de *S.* Typhimurium a 10ºC ocasione un incremento en el pH límite de crecimiento y una disminución de la resistencia bacteriana a condiciones ácidas extremas, unido a la menor intensidad de la respuesta de tolerancia ácida desarrollada a esta temperatura pone de manifiesto que la refrigeración puede ser una medida eficaz durante el procesado y distribución de los alimentos de pH ácido moderado en el control de la salmonelosis.

La respuesta de tolerancia ácida se manifestó, tanto en medios de laboratorio, BHI (capítulos 3 y 4) y MM (capítulo 5), como en matrices alimentarias (extracto de carne -capítulo 3- y zumos de naranja y manzana -capítulo 8), observándose que la velocidad de inactivación a un determinado valor de pH estuvo determinada por la composición del medio de tratamiento. Cuando el choque ácido fue realizado a pH

2,5, ambas serovariedades sobrevivieron mejor en zumo de manzana que en zumo de naranja, especialmente *S.* Senftenberg, que presentó valores D del orden de 4 veces más altos. En el caso de las células expuestas a un pH de 3,0 la resistencia ácida encontrada fue claramente superior cuando el tratamiento fue realizado en extracto de carne que cuando se utilizó BHI, no llegando a observarse ningún efecto letal incluso tras tres horas de tratamiento en extracto de carne para las células crecidas en BHI acidificado. Estos resultados demuestran que determinados componentes de los alimentos pueden ejercer un efecto protector frente a la inactivación en condiciones ácidas extremas y sugieren que la fracción proteica de los alimentos puede ser responsable de la protección desarrollada. En este sentido, existen numerosos estudios, la mayoría llevados a cabo con *Escherichia coli*, en los que se describe que la presencia en el medio de tratamiento de ciertos aminoácidos, como arginina, lisina o ácido glutámico, incrementa la resistencia bacteriana a la acidez (Castaine-Cornet y col., 1999; Castaine-Cornet y Foster, 2001; Iyer y col., 2003; Foster, 2004). Sin embargo, son escasas las investigaciones que han llegado a esta misma conclusión en *Salmonella*, habiéndose demostrado únicamente la existencia de una resistencia ácida dependiente de la presencia de aminoácidos en el medio de tratamiento tras su crecimiento en condiciones específicas de cultivo (anaerobiosis y medios acidificados) (Park y col., 1996; de Jonge y col., 2003; Kieboom y Abee, 2006). Los resultados recogidos en el capítulo 5 demuestran que la inclusión de arginina y lisina en un medio mínimo (MM) origina un incremento significativo de la resistencia ácida en las células de *S.* Typhimurium adaptadas a la acidez, lo que pone de manifiesto la importancia de los sistemas homeostáticos "arginina decarboxilasa" y "lisina decarboxilasa" en el desarrollo de estas respuestas de tolerancia ácida, hecho que fue posteriormente confirmado por el incremento observado en el nivel relativo de expresión de los genes involucrados en la regulación de dichos sistemas homeostáticos (*adi*, *adiY*, *cadA* y *cadB*). No obstante, la existencia de una respuesta de tolerancia ácida, aunque menos intensa, en ausencia de estos aminoácidos en el medio de tratamiento pone de manifiesto que la respuesta bacteriana al estrés ácido es un fenómeno complejo en el que otros mecanismos moleculares diferentes de la activación de sistemas homeostáticos pueden estar implicados, tales como la síntesis de proteínas de choque ácido,

encargadas de la reparación de macromoléculas dañadas, o la inducción de cambios en la composición en ácidos grasos de la membrana bacteriana (Brown y col., 1997; Abee y Wouters, 1999; Foster, 2000).

Respuesta de protección cruzada frente a tratamientos térmicos

La adaptación a la acidez de *S.* Typhimurium (capítulos 6 y 8) y *S.* Senftenberg (capítulos 7 y 8) ocasionó un incremento en su resistencia frente a un tratamiento térmico posterior en todas las condiciones estudiadas, aunque en líneas generales, tanto la termorresistencia como la respuesta de protección cruzada desarrollada, no estuvieron condicionadas por el tipo de ácido utilizado para acidificar el medio de crecimiento.

El crecimiento de ambas serovariedades en BHI sin acidificar (pH 7,4) ocasionó una disminución progresiva del pH extracelular hasta un valor aproximado de 6,0, probablemente responsable de la respuesta de protección cruzada frente al calor observada en estas condiciones, resultando, no obstante de menor magnitud que la mostrada por las células crecidas en BHI acidificado con los distintos ácidos hasta valores de pH de 4,5. Esta respuesta de protección cruzada ha sido previamente descrita para diversas serovariedades de *Salmonella* (Leyer y Johnson, 1993; Wilde y col., 2000; Mazzotta, 2001; Bacon y col., 2003b; Tosun y Gönül, 2003), pero en la mayoría de los trabajos se ha evaluado la termorresistencia adquirida determinando el grado de supervivencia microbiana a un único tiempo de tratamiento. En este trabajo hemos demostrado que la inactivación térmica de *S.* Typhimurium y *S.* Senftenberg, tras su adaptación ácida en las diferentes condiciones estudiadas, sigue una cinética de primer orden.

Asimismo, hemos comprobado que la termorresistencia adquirida por las células estuvo condicionada por diferentes factores previos y simultáneos al tratamiento térmico, así como por factores genéticos. *S.* senftenberg no sólo resultó ser más termorresistente que *S.* Typhimurium, sino que también desarrolló una respuesta de protección cruzada frente al calor de mayor intensidad, si bien, en algunas ocasiones las células de *S.* Typhimurium adaptadas a la acidez mostraron una resistencia térmica superior a la observada para las de *S.* Senftenberg no

adaptadas. Por ejemplo, las células de *S.* Typhimurium tras su crecimiento en BHI-pH 6,4 en presencia de ácido láctico y su tratamiento en zumo de manzana mostraron un valor D_{58} de 0,23 min, mientras que para las células de *S.* Senftenberg no adaptada a la acidez se observaron valores D_{58} de 0,11 y 0,19 min en zumo de naranja y de manzana, respectivamente. Este hecho evidencia los inconvenientes que puede presentar para el cálculo de tratamientos efectivos de pasteurización la utilización de microorganismos indicadores o de datos basados en estudios de termorresistencia sobre células obtenidas en condiciones óptimas de crecimiento.

El aumento de la temperatura de crecimiento desde 10 a 45 ºC ocasionó un incremento exponencial de la termorresistencia mostrada por *S.* Typhimurium, aunque no condicionó la magnitud de la respuesta de protección cruzada desarrollada (capítulo 6). En el caso de *S.* Senftenberg, la refrigeración (4 ºC) previa al tratamiento térmico ocasionó un marcado descenso de la termorrestencia observada para las células adaptadas a la acidez, sin modificar sensiblemente la de los cultivos no adaptados (capítulo 7), lo que pone de manifiesto que la refrigeración previa al procesado térmico de los alimentos y la utilización de ambas tecnologías (acidificación y refrigeración) en la elaboración de alimentos mínimamente procesados puede constituir una medida eficaz para el control de la salmonelosis, hecho también confirmado por los resultados obtenidos en el capítulo 4 en relación a la respuesta de tolerancia ácida.

El pH del medio de cultivo no condicionó la magnitud de la respuesta de protección cruzada desarrollada, aunque la termorresistencia de *S.* Senftenberg tendió a aumentar a medida que disminuía el pH extracelular.

En cuanto a la influencia de los factores simultáneos al tratamiento térmico, hemos comprobado que la composición del medio de calentamiento modificó de forma considerable la termorresistencia mostrada por ambas serovariedades, de tal manera que, los valores D obtenidos en BHI fueron claramente superiores a los encontrados en zumo de naranja y de manzana, probablemente debido a su bajo pH. No obstante, también se detectaron diferencias en la velocidad de inactivación microbiana en ambos zumos, sobreviviendo mejor, en líneas generales, en zumo de

manzana, aunque la respuesta de protección cruzada desarrollada por *S.* Senftenberg fue máxima cuando el tratamiento térmico tuvo lugar en zumo de naranja. Cabe destacar que los valores z obtenidos no se vieron significativamente afectados por ninguna de las diferentes condiciones de crecimiento y de tratamiento estudiadas, obteniéndose un valor medio de 6,03±0,29 y 7,04±0,30 ºC para *S.* Typhimurium y *S.* Senftenberg, respectivamente.

Los resultados descritos en los capítulos 6 y 7 sobre las modificaciones en la composición en ácidos grasos de la membrana celular han puesto de manifiesto que el pH y la temperatura de crecimiento originan una serie de cambios en la composición lipídica que repercuten en la termorresistencia de *S.* Typhimurium y *S.* Senftenberg. La adaptación a la acidez ocasionó un descenso en la proporción de ácidos grasos insaturados (UFA) y un incremento en el contenido en ácidos grasos saturados (SFA) y en ácidos grasos cíclicos derivados del ciclopropano (CFA), mientras que las bajas temperaturas dieron lugar a un acusado aumento en el contenido en UFA y en la relación UFA/SFA, indicador indirecto de la fluidez de la membrana bacteriana (Casadei y col., 2002). En líneas generales, ambas serovariedades mostraron su máxima termorresistencia en aquellas condiciones ambientales en que la relación UFA/SFA y, por tanto, la fluidez de membrana, fueron mínimas, hecho que concuerda con los resultados previamente descritos para otros microorganismos, como *Pediococcus* spp. (Annous y col., 1999) y *S.* Enteritidis (Sampathkumar y col., 2004). Además, el incremento en la proporción en CFA observado en las células adaptadas a la acidez parece jugar también un papel importante en el desarrollo de respuestas de protección cruzada frente al calor. Sin embargo, los cambios observados no permitieron explicar la termorresistencia obtenida en todas las condiciones experimentales estudiadas, como, por ejemplo, la elevada resistencia al calor mostrada por las células de *S.* Typhimurium crecidas a 45ºC, lo que sugiere la existencia de otros mecanismos moleculares implicados en estas respuestas de protección cruzada (Gottesman y col., 1997; Yura y col., 2000).

Efecto de la adaptación a la acidez sobre la resistencia de Salmonella frente a otros agentes estresantes (sal, estrés oxidativo y alcalinización)

Los resultados que se describen en el capítulo 9 de esta Tesis Doctoral muestran que la adaptación de *S.* Typhimurium a la acidez ocasiona una sensibilización frente a la sal y al estrés oxidativo. Este efecto sinérgico puede ser de gran importancia práctica para el control de la salmonelosis en quesos y otros productos fermentados, en los que la acidificación y el estrés osmótico y oxidativo desempeñan un importante papel para prevenir el crecimiento microbiano.

Por el contrario, la supervivencia de las células adaptadas a la acidez frente a tratamientos en medios alcalinos, en presencia de hidróxido sódico o fosfato trisódico (TSP), se vio condicionada por el tipo de agente utilizado, manifestándose solamente una respuesta de protección cruzada cuando el tratamiento alcalino fue realizado en BHI pH 11,0 en presencia de hidróxido sódico, lo que sugiere la existencia de una actividad antimicrobiana intrínseca del TSP independiente de la ocasionada por la alcalinización del medio extracelular.

Las respuestas adaptativas frente al estrés ácido descritas en esta Tesis Doctoral para *Salmonella* Typhimurium y *Salmonella* Senftenberg tienen importantes consecuencias para la seguridad alimentaria y evidencian la necesidad de realizar en los próximos años un mayor esfuerzo investigador centrado en la adquisición de un conocimiento más completo de los mecanismos moleculares responsables de la inactivación microbiana por los diferentes agentes estresantes con el objetivo de poder realizar una selección inteligente de los métodos de conservación que podrían ser utilizados de forma sinérgica en la tecnología de procesos combinados.

Los resultados obtenidos durante el periodo de desarrollo de esta Tesis Doctoral se englobaron en dos proyectos de investigación financiados por la Junta de Castilla y León ("Estudio de la respuesta de adaptación ácida de *Salmonella* Typhimurium inducida por el pH y diversos ácidos orgánicos y sus implicaciones en el control de la salmonelosis" (2006) e "Influencia de la respuesta de adaptación ácida sobre la aparición de brotes de salmonelosis en alimentos sometidos a tratamientos térmicos moderados" (2007-2008)) y han dado lugar a las siguientes

publicaciones, algunas ya aceptadas en revistas relacionadas en el "Journal of Citation Reports" y otras enviadas para su publicación:

- Comparison of acids on the induction of an Acid Tolerance Response in *Salmonella* Typhimurium, consequences for food safety. *Meat Sience* (2009), 81, 65-70.

- Acid tolerance in *Salmonella* Typhimurium induced by culturing in the presence of organic acids at different growth temperatures. *Submitted for publication.*

- Arginine and lysine decarboxylases and the Acid Tolerance Response of *Salmonella* Typhimurium. *Submitted for publication.*

- Modifications in membrane fatty acid composition of *Salmonella* Typhimurium in response to growth conditions and their effect on heat resistance. *International Journal of Food Microbiology* (2008), 123, 212-219.

- Relationship between membrane fatty acid composition and heat resistance of acid and cold stressed *Salmonella* Senftenberg CECT 4384. *Food Microbiology* (2009), 26, 347-353.

- A comparative study of thermal and acid inactivation kinetics in fruit juices of acid adapted *Salmonella* Typhimurium and *Salmonella* Senftenberg. *Submitted for publication.*

- Acid adaptation influences *Salmonella* Typhimurium resistance to osmotic, oxidative and alkaline stresses. *Submitted for publication.*

- Conservación de los alimentos mediante acidificación: actividad antimicrobiana de los ácidos orgánicos y factores que condicionan su efectividad. *Alimentación, Equipos y Tecnología* (2007), 226, 46-49.

Además, parte de los resultados obtenidos se han presentado en 4 comunicaciones en el XV Congreso Nacional de Microbiología de los Alimentos

(2006), el IV Congreso Nacional de Ciencia y Tecnología de los Alimentos (2007) y FoodMicro (2008). También cabe destacar que a lo largo del periodo de disfrute de la beca del Programa Nacional de Formación de Profesorado Universitario (FPU) (2005-2009) he realizado 3 estancias de investigación, una de ellas en el Área de Tecnología de los Alimentos de la Universidad de Zaragoza, bajo la dirección de la Dra. Mañas, en la que estudié los mecanismos de inactivación microbiana por pulsos eléctricos de alto voltaje, y otras dos en el "Laboratory of Food Microbiology" de la Universidad de Wageningen (Países Bajos), bajo la supervisión del Prof. Dr. Tjakko Abee en las que participé en los trabajos desarrollados sobre la importancia del factor alternativo σ^B en la esporulación y propiedades de las esporas de *Bacillus cereus*, y sobre el papel de la respuesta SOS en la capacidad mutagénica y en la resistencia de *Listeria monocytogenes* frente a diferentes agentes estresantes.

CONCLUSIONES

1. El crecimiento de *Salmonella* Typhimurium y *Salmonella* Senftenberg en presencia de ácidos orgánicos hasta un valor de pH de 4,5 origina respuestas adaptativas que se manifiestan en un incremento de su resistencia a la acidez extrema y al calor, aunque su magnitud está condicionada por diferentes factores ambientales (temperatura, pH y composición del medio de cultivo y de tratamiento), exhibiendo en líneas generales *S.* Senftenberg una mayor resistencia ácida y térmica que *S.* Typhimurium.

2. El pH del medio de crecimiento y el tipo de ácido condicionan de forma marcada la resistencia a la acidez, resultando más efectivos para la inducción de la misma los bajos pHs y los ácidos acético, cítrico y láctico, hecho que podría cuestionar su uso como agentes descontaminantes de canales o como aditivos en la industria alimentaria

3. El aumento en la temperatura de crecimiento de 10 a 37 ºC incrementa de forma exponencial las respuestas adaptativas frente a la acidez extrema y

al calor, aunque temperaturas más altas, 45ºC, sólo originan un incremento en la termorresistencia. Este comportamiento, unido a la sensibilización frente al calor observada tras la aplicación secuencial de la acidificación y la refrigeración, pone de manifiesto que la utilización conjunta de ambos procesos en la elaboración de alimentos mínimamente procesados puede llegar a ser una medida eficaz para el control de la salmonelosis.

4. La expresión de ambas respuestas adaptativas fue más pronunciada en alimentos que en medios de laboratorio. La elevada resistencia ácida encontrada en zumos de naranja y manzana, con tiempos de reducción decimal de hasta 130 minutos, indica la posibilidad de supervivencia de las salmonelas en el ambiente ácido del estómago, lo que podría justificar la frecuente implicación de estos productos en brotes de salmonelosis, haciendo aconsejable la implantación de tratamientos de pasteurización en el procesado de los zumos. Sin embargo, y a pesar de que las células adaptadas a la acidez exhibieron un incremento en su termorresistencia de hasta 10 veces, los parámetros de inactivación obtenidos, tiempos de reducción decimal a 58ºC entre 0,03 y 1,3 minutos y valores z entre 5,8 y 7,7ºC, ponen de manifiesto que la utilización de tratamientos térmicos de pasteurización convencionales proporcionaría un margen de seguridad suficiente, aunque en todo caso habría que considerar que los procesos diseñados a partir de estudios de termorresistencia sobre células obtenidas en condiciones óptimas de crecimiento podrían resultar inadecuados.

5. El alto grado de supervivencia exhibido por las células adaptadas a la acidez en extracto de carne a pH 3,0 sugiere que la fracción proteica de los alimentos desempeña un importante efecto protector. De hecho, hemos comprobado que la inclusión de arginina y lisina en un medio mínimo protege frente a la inactivación de las salmonelas a un pH ácido letal, a través de la activación de los sistemas homeostáticos arginina decarboxilasa y lisina decarboxilasa. Sin embargo, también hemos constatado que otros mecanismos moleculares, como las modificaciones

en la composición en ácidos grasos de la membrana celular, están también involucrados en estas respuestas adaptativas, mostrando las células la máxima tolerancia ácida y térmica en aquellas condiciones ambientales que originan un aumento en el contenido en ácidos grasos cíclicos derivados del ciclopropano (CFA) y una disminución en la relación entre ácidos grasos insaturados y ácidos grasos saturados (UFA/SFA)

6. El crecimiento de *Salmonella* Typhimurium en condiciones ácidas moderadas origina una respuesta de protección cruzada frente a un tratamiento de alcalinización en presencia de hidróxido sódico y una sensibilización frente al cloruro sódico y al estrés oxidativo, que puede tener importantes repercusiones para el control de la salmonelosis en quesos y otros productos fermentados.

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11.

SUMMARY AND CONCLUDING REMARKS

SUMMARY

Consumers demand fresher and more natural foods. This prompts food manufacturers to implement new food preservation methods and to combine several mild preservation agents (hurdle technology) in order to minimize the quality losses. However, several studies have indicated that mild processing conditions may induce adaptive responses which frequently result in an enhanced resistance to other environmental stresses (Abee and Wouters, 1999; Gibbs, 1999; Sharp and Reilly, 2000), increasing the risk of foodborne illnesses, including salmonellosis.

Acidification with organic acids is a preservation measure commonly used in food industry in order to control the microbial growth. However, the recent involvement of acid foods in several salmonellosis outbreaks (Parish, 1997; D´Aoust, 2000) questions their microbiological safety. Although *Salmonella* acid adaptation response and its implications for the bacterial resistance to other stress conditions have been extensively studied (Foster and Hall, 1990; Leyer and Johnson, 1993; Bearson et al., 1998; Wilde et al., 2000; Mazzotta, 2001; Bacon et al., 2003a, b; de Jonge et al., 2003; Greenacre et al., 2003; Tosun and Gönül, 2003; Greenacre and Brocklehurst, 2006; Yuk and Schneider, 2006), little is known on the variability of these adaptive responses as a function of different environmental conditions, in spite of its importance for the control of this pathogenic bacterium.

This Thesis studied the acid stress response of *Salmonella* Typhimurium and *Salmonella* Senftenberg grown in media acidified with several organic acids (acetic, ascorbic, citric, lactic and malic) and hydrochloric acid. The influence of acid adaptation on *S.* Typhimurium and *S.* Senftenberg resistance to several food preservation treatments commonly used in food industry, such as heat, salt, alkaline and osmotic stresses, was also tested. Finally, the inactivation kinetic of acid adapted cells after their exposition to extreme acidic conditions in laboratory media and several foods, such as meat extract and fruit juices was determined, with the aim of confirming the development of an Acid Tolerance Response (ATR), which not only could enhance the microbial survival in acidic foods, but may also increase the

resistance to the acidic conditions of the gastrointestinal tract, causing a decrease in the infective dose and increasing the risk of gastrointestinal illness.

Hurdle technology is used in industrialized as well as in developing countries for an effective preservation of foods, but it is important to note that for the intelligent application of hurdle technology a complete knowledge of the molecular mechanisms involved in the bacterial stress response is needed, and, although several researches have been carried out to try to elucidate the molecular and physiological changes associated with *Salmonella* stress and cross-protection responses, the mechanisms involved are not completely understood. This Thesis contributes to elucidate them since the importance of some homeostatic systems, such as arginine decarboxylase, lysine decarboxylase and glutamate decarboxylase and the role of modifications in membrane fatty acid composition on the ATR and the cross-protections responses of *S.* Typhimurium and *S.* Senftenberg were also studied.

In chapters 3, 4 and 5, *S.* Typhimurium Acid Tolerance Response (ATR) is described, paying attention to the influence of several environmental factors and to the importance of amino acid dependent homeostatic systems (arginine decarboxylase, lysine decarboxylase and glutamate decarboxylase). In chapters 6 and 7 the effect of acid adaptation on *S.* Typhimurium and *S.* Senftenberg thermal resistance and the role of modifications in membrane fatty acid composition on the cross-protection developed are discussed. In chapter 8 the existence of acid tolerance and cross-protection responses to heat is demonstrated for *S.* Typhimurium and *S.* Senftenberg in orange and apple juices. Finally, in chapter 9, the influence of acid adaptation on *S.* Typhimurium resistance to salt, oxidative and alkaline stresses is assessed.

Salmonella Typhimurium and _Salmonella_ Senftenberg Acid Tolerance Response (ATR).

Both *S.* Typhimurium and *S.* Senftenberg are able to growth in a wide range of pH values. This fact may promote their survival or development under several acid conditions prevailing during food processing. In addition to the decline of pH

over time associated with food fermentation, bacteria could be exposed to a sublethal pH during the muscle to meat conversion process. Furthermore, rinsing with organic acid solutions to reduce bacterial populations on carcass surfaces provides the opportunity for bacterial exposure to these microenvironments and as a result bacterial acid tolerance may be developed (Samelis et al., 2001).

The inactivation kinetics of *S.* Typhimurium and *S.* Senftenberg in lethal acidic conditions (pH 2.5-3.0) were studied on stationary-phase cells after their growth in Brain Heart Infussion (BHI) acidified at pH values of 6.4, 5.4 and 4.5 using several organic acids (acetic, ascorbic, citric, malic and lactic) and hydrochloric acid (acid adapted cells), non-acidified BHI (non-acidified cells) and buffered BHI pH 7.0 (non-acid adapted cells). The influence of growth temperature on the ATR of *S.* Typhimurium was assessed in the range 10-45ºC. In order to study the effect of challenge medium composition, several laboratory media, BHI and a mineral medium (MM) described by de Jonge et al. (2003), as well as some food products, such as a meat extract and fruit juices (apple and orange juices) were used to perform the treatments.

In general, both *Salmonella* serovars showed a great ability to grow under acidic conditions, although it is important to note that *S.* Senftenberg grew faster, especially at pH ≤ 5.4. The acidification of the growth medium caused an increase in the generation time, in the lag phase duration and in the time needed to reach the stationary-phase, and a decrease in the maximum population density. The growth pH boundary was conditioned by the type of acidulant. Thus, no bacterial growth was observed in the presence of acetic acid and lactic acid at pH ≤ 5.4 and ≤ 4.5, respectively, with the order of acids in inhibiting the bacterial growth being: acetic > lactic > citric > malic ≥ ascorbic ≥ hydrochloric. It is worth noting that the growth temperature markedly influenced *S.* Typhimurium growth under acidic conditions. Thus, at 10 and 45ºC the inhibitory effect of organic acids was enhanced, and the growth pH boundary was increased, allowing for bacterial growth exclusively at pH values ≥ 6.4.

Survival curves obtained fitted properly into a first order inactivation kinetic, and thus, the D-value (time required for a 10-fold reduction in viable cells) turned

out to be a suitable parameter to compare the microbial acid resistance exhibited under the different conditions tested.

Results described in chapters 3, 4 and 5 show that, in all cases, acid adapted cells were significantly more resistant to an acid challenge (pH 2.5 or 3.0) than non-acid adapted cells. Non-acidified cells also showed an increased acid resistance, since their growth caused a mild decrease in extracellular pH to an approximate value of 6.0. Results obtained also show that the magnitude of the ATR developed was conditioned by several environmental factors, such as pH, type of acid, growth temperature and composition of the challenge medium. In general, the growth in non-acidified BHI (pH 7.4) caused an ATR clearly lower than that induced in the presence of organic acids at lower pH values (6.4; 5.4 and 4.5). For cells grown in acidified BHI the influence of the conditions prevailing during acid adaptation (pH and type of acidulant) was dependent on the pH value and the composition of the challenge medium. Thus, whereas a decrease in the growth medium pH caused an increase in the bacterial acid resistance when the acid challenge was performed in BHI and meat extract at pH 3.0, this effect was not apparent when the treatment was carried out at pH 2.5 in orange and apple juices and MM. In the same way, a specific effect of the type of acid was only found when BHI or meat extract at pH 3.0 were used as challenge medium. Under these conditions, acetic, citric and lactic acids caused the strongest adaptive response. This fact could show an important impact for food safety because acetic, citric and lactic acids are the most commonly used in carcass decontamination treatments and as preservatives in the food industries (Smulders and Greer, 1998).

Growth temperature conditioned *S.* Typhimurium acid resistance at pH 3.0 in BHI. In all cases, D-values were minimum for cells grown at 10ºC and significantly increased with increasing temperature up to 37ºC, at which D-values obtained were around 5-10 times higher. However, cells grown at 45ºC showed a decreased acid resistance, with D-values similar to those found for *S.* Typhimurium cells grown at 25ºC. This fact, in addition to the enhanced growth pH boundary and the decreased ATR found for cells grown at 10ºC show that refrigeration is a suitable preservation

measure to control salmonellosis throughout processing and distribution of acid foods.

With regard to the effect of the conditions prevailing during the acid challenge, *S.* Typhimurium and *S.* Senftenberg developed an ATR both in laboratory media, such as BHI (chapters 3 and 4) and MM (chapter 5), and acidic food products, such as meat extract (chapter 3) and orange and apple juices (chapter 8). However, the pH and the composition of the challenge medium not only exerted a great effect on the ATR, but also conditioned the influence shown by other environmental factors, such as growth medium pH and type of acidulant used to obtain acid adapted cells. When the acid challenge was carried out at pH 2.5, both *Salmonella* serovars showed maximum D-values after their treatment in apple juice, especially acid adapted *S.* Senftenberg cells, with D-values 4 times higher than those found in orange juice or MM. The acid resistance shown by cells exposed to pH 3.0 was clearly higher when meat extract was used as challenge medium. Thus, no bacterial inactivation was observed for cells grown in BHI after three hours of acid challenge in meat extract. Therefore, our results demonstrate that several food components could exert a protective effect against acid inactivation in *Salmonella* spp. The higher protein content found in the meat extract suggests that the protein fraction of foods is responsible of this bacterial protection. It is important to note that several studies, most of them performed on *Escherichia coli*, have previously described an increased acid resistance when the challenge medium is supplemented with some amino acids, such as arginine, lysine or glutamic acid (Castaine-Cornet et al., 1999; Castaine-Cornet and Foster, 2001; Iyer et al., 2003; Foster, 2004). However, the reports found for *S.* Typhimurium are scarce and indicate that these amino acid dependent survival systems are only activated under specific culture conditions (anaerobic and acidic growth) (Park et al., 1996; de Jonge et al., 2003; Kieboom and Abee, 2006). In our case, results described in chapter 5 show that the inclusion of arginine and lysine in the challenge medium (MM) causes a significant increase in the acid resistance of *S.* Typhimurium acid adapted cells, and this highlights the importance of "arginine decarboxylase" and "lysine decarboxylase" homeostatic systems for the development of an ATR in *S.* Typhimurium, fact

confirmed by the enhanced relative expression levels found in acid adapted cells for the genes involved in the regulation of these homeostatic systems. Nevertheless, the fact that acid adapted cells challenged in the absence of amino acids also developed an ATR shows that bacterial acid stress response is a complex phenomenon in which other molecular mechanisms may be involved, such as the synthesis of shock proteins, which repair macromolecular damages, and the induction of changes in the membrane fatty acid composition (Brown et al., 1997; Abee and Wouters, 1999; Foster, 2000).

Cross-protection responses against heat treatments

S. Typhimurium (chapters 6 and 8) and *S.* Senftenberg (chapters 7 and 8) acid adaptation caused an increase in their subsequent resistance to a heat treatment under all conditions assayed. Although the bacterial growth in non-acidified BHI (pH 7.4) caused a progressive decrease in the extracellular pH to an approximate pH value of 6.0, which resulted in the induction of a cross-protection response to heat, this adaptive response turned out to be weaker than that developed by cells grown in acidified BHI at pH 6.4; 5.4 and 4.5. However, in general, the heat resistance and the magnitude of the cross-protection response developed were not dependent on the type acid used to obtain acid adapted cells.

A cross-protective response to heat induced by acid adaptation has been previously described for *Salmonella* spp. (Leyer and Johnson, 1993; Wilde et al., 2000; Mazzotta, 2001; Bacon et al., 2003b; Tosun and Gönül, 2003). However, most of these studies have evaluated the bacterial heat resistance only at one point in time and until now little is known about the inactivation kinetics of *Salmonella* acid adapted cells. In this Thesis, we have shown that survival curves fitted properly into a first order inactivation kinetic.

Our results also show that the acid-induced thermotolerance acquired was conditioned by several factors which act before and throughout the heating, as well as by genetic factors. In general, *S.* Senftenberg was more heat resistant and showed a stronger cross-protection response to heat, although in some cases *S.* Typhimurium acid adapted cells showed a higher thermotolerance than *S.* Senftenberg non-acid

adapted cells. For instance, *S.* Typhimurium cells grown in BHI acidifed with lactic acid at pH 6.4 and heated in apple juice exhibited a D_{58}-value of 0.23 min, while non-acid adapted *S.* Senftenberg showed D_{58}-values of 0.11 and 0.19 min in orange juice and apple juice, respectively. This fact highlights the disadvantages of using biological indicators or microorganisms obtained under optimal growth conditions for the design of appropriate pasteurization treatments.

With regard to the influence of growth temperature (10-45ºC), *S.* Typhimurium cells showed minimum D-values when cells were grown at 10 °C and their heat resistance significantly increased with increasing growth temperature up to 45°C, showing D-values around ten times higher. Under all culture conditions which allowed for bacterial growth the relationship between D-values and growth temperature followed an exponential kinetic which depended on the growth medium used (chapter 6). Furthermore, a refrigeration (4ºC) prior to heating caused a marked decrease in the thermotolerance of *S.* Senftenberg acid adapted cells, but this was not found for non-acid adapted cells (chapter 7). This fact demonstrates that the combination of refrigeration and acidification in the production of minimally processed foods, and the use of low temperatures as a previous step to the pasteurization of acid foods could represent suitable measures for the control of salmonellosis. This asseveration is also supported by the results described in chapter 4 on the influence of growth temperature on *S.* Typhimurium ATR.

In general, the decrease of the growth medium pH from 6.4 to 4.5 did not show any significant effect on the thermal inactivation rate of the resulting cells, although a trend to obtain higher D-values as the growth medium pH decreased was found for *S.* Senftenberg when citric and hydrochloric acids were used.

Our results also show that the heating medium composition modified the heat resistance showed by both *Salmonella* serovars. Thus, D-values obtained were clearly higher for cells treated in BHI than for those heated in orange or apple juices, probably due to the low pH value of both fruit juices. Furthermore, significant differences among D-values were also found among fruit juices. Thus, the highest heat resistance was obtained, generally, when apple juice was used as heating medium. It is important to note that the heating temperature did not modify the

influence shown by acid adaptation on *S.* Typhimurium and *S.* Senftenberg heat resistance. Thus, no statistically significant differences were found between z-values obtained under all experimental conditions.

Results described in chapters 6 and 7 show that changes in growth pH and growth temperature cause modifications in membrane fatty acid composition which are linked to the heat resistance observed under the different experimental conditions. For both *Salmonella* serovars, acid adaptation caused a decrease in the membrane content in unsaturated fatty acids (UFA) and an increase in the content in saturated fatty acids (SFA) and cyclic fatty acids (CFA). Furthermore, under low temperatures an enhanced UFA content and, consequently, UFA/SFA ratio (an indirect indicator of the membrane fluidity) was found, especially for *S.* Senftenberg acid adapted cells. In general, D-values found for both *Salmonella* serovars were maximum for cells with low UFA/SFA ratio and, consequently, with low membrane fluidity. These findings agree with results previously obtained for other microorganisms, such as *Pediococcus* spp. (Annous et al., 1999) and *S.* Enteritidis (Sampathkumar et al., 2004). Moreover, the increase of CFA levels in acid adapted cells also seems to be important for the development of cross-protection responses against heat treatments. However, changes observed in the membrane fatty acid composition are not enough to explain the heat resistance obtained under all experimental conditions, such as, for instance, the great thermotolerance obtained for *S.* Typhimurium cells grown at 45ºC, and this fact suggests the existence of other molecular mechanisms involved in these adaptive responses, such as the synthesis of heat shock proteins (Gottesman et al., 1997; Yura et al., 2000).

Cross-protection response to other stressful agents of relevance in food industry (salt, oxidative and alkaline stresses)

Results described in chapter 9 show that acid adaptation sensitizes *S.* Typhimurium to salt and oxidative stresses. The synergic effect of acidification and salt and hydrogen peroxide treatments may show an impact on food preservation regimes for the control of salmonellosis in cheese and other fermented products, where a number of stresses, including acidification, salt addition, oxidative stress

and decreased water activity probably contribute to achieve the bacterial inactivation.

On the other hand, *S.* Typhimurium acid adaptation causes a cross-protection response to alkaline treatments, although this response is only developed when the alkaline challenge is performed at pH 11.0 in the presence of sodium hydroxide. When the challenge medium is alkalinized at pH 11.0 using trisodium phosphate (TSP) no significant differences were found between D-values obtained for acid adapted cells and non-acid adapted cells. This fact suggests that although the antimicrobial effect of TSP is mainly due to its alkaline pH, there is also an unknown specific effect exerted by TSP.

In conclusion, results obtained in this Thesis demonstrate the importance of *S.* Typhimurium and *S.* Senftenberg adaptive responses to acid stress, which are responsible of the induction of cross-protection responses against several stressful environments. These adaptive responses are, therefore, a great concern for food safety and evidence the need of carrying out further studies focused on the molecular mechanisms involved in bacterial inactivation by food related stresses, which should definitely help to make an intelligent selection of food preservation measures to be combined with a synergistic antimicrobial effect throughout the implementation of the hurdle technology.

Results obtained in this Thesis were included in two research projects funded by "Junta de Castilla y León" ("Estudio de la respuesta de adaptación ácida de *Salmonella* Typhimurium inducida por el pH y diversos ácidos orgánicos y sus implicaciones en el control de la salmonelosis – 2006" and "Influencia de la respuesta de adaptación ácida sobre la aparición de brotes de salmonelosis en alimentos sometidos a tratamientos térmicos moderados – 2007/2008") and resulted in the following publications, some of them already accepted in international peer reviewed journals:

- Comparison of acids on the induction of an Acid Tolerance Response in *Salmonella* Typhimurium, consequences for food safety. *Meat Sience* (2009), 81, 65-70.

- Acid tolerance in *Salmonella* Typhimurium induced by culturing in the presence of organic acids at different growth temperatures. *Submitted for publication.*

- Arginine and lysine decarboxylases and the Acid Tolerance Response of *Salmonella* Typhimurium. *Submitted for publication.*

- Modifications in membrane fatty acid composition of *Salmonella* Typhimurium in response to growth conditions and their effect on heat resistance. *International Journal of Food Microbiology* (2008), 123, 212-219.

- Relationship between membrane fatty acid composition and heat resistance of acid and cold stressed *Salmonella* Senftenberg CECT 4384. *Food Microbiology* (2009), 26, 347-353.

- A comparative study of thermal and acid inactivation kinetics in fruit juices of acid adapted *Salmonella* Typhimurium and *Salmonella* Senftenberg. *Submitted for publication.*

- Acid adaptation influences *Salmonella* Typhimurium resistance to osmotic, oxidative and alkaline stresses. *Submitted for publication.*

- Conservación de los alimentos mediante acidificación: actividad antimicrobiana de los ácidos orgánicos y factores que condicionan su efectividad. *Alimentación, Equipos y Tecnología* (2007), 226, 46-49.

Furthermore, part of the results obtained were presented in several National and International congresses (XV Congreso Nacional de Microbiología de los Alimentos -2006, IV Congreso Nacional de Ciencia y Tecnología de los Alimentos -2007, and FoodMicro -2008).

It is worth noting that during the PhD period I have performed three research stays. The aim of the first stay (2006), performed in the Food Technology Department of the University of Zaragoza (Spain), under the supervision of Dr.

Mañas, was to study the molecular mechanisms involved in *E. coli* and *S. aureus* inactivation by pulsed electric fields. In the other two research stays, which were carried out in the Laboratory of Food Microbiology of Wageningen University (The Netherlands) under the supervision of Prof. Dr. Tjakko Abee, the work performed was part of the programme "Food Functionality and Safety" of the Top Institute Food & Nutrition, and was focused on the study of the effects of *sigB*, *rsbY* and *rsbK* deletion on the spore properties of *Bacillus cereus* ATCC 14579 (2007) and the role of the SOS response on *Listeria monocytogenes* EGD-e and LO28 stress resistance and mutagenic capacity.

CONCLUSSIONS

1. The growth of *Salmonella* Typhimurium and *Salmonella* Senftenberg up to pH 4.5 in the presence of organic acids causes an adaptive response, which results in an increase in their subsequent resistance to extreme acidic conditions and to heat. The magnitude of these adaptive responses depends on several environmental factors, such as growth temperature, pH and composition of growth and challenge media, with *S.* Senftenberg generally being more acid and heat resistant than *S.* Typhimurium.

2. The growth medium pH and the type of acidulant influence the bacterial acid resistance. Acetic, citric and lactic acids are more effective in the induction of an Acid Tolerance Response, especially at the lowest pH values. This fact shows an important impact for food safety as these organic acids are commonly used in carcass decontamination treatments and as preservatives in the food industry.

3. The acid and heat resistance exponentially increase as growth temperature rises from 10 to 37ºC. However, higher growth temperatures (45ºC) only cause an enhanced thermotolerance. This behavior, in addition to the higher sensibility to heat shown by cells sequentially exposed to acidic environments and refrigeration temperatures, shows that the combination

of both preservative methods could be a suitable measure for the control of salmonellosis in minimally processed foods.

4. The expression of both adaptive responses was higher in foods than in laboratory media. The extremely high acid resistance found in orange and apple juices, with D-values as high as 130 min, may allow for bacterial survival in the acidic environment of the stomach. This could explain the frequent implication of fruit juices in salmonellosis outbreaks and makes it advisable to implement suitable pasteurization treatments in their processing. However, although acid adapted cells showed an increased thermotolerance, the inactivation parameters obtained (D_{58}-values between 0.03 and 1.3 min, and z-values between 5.8 and 7.7ºC) show that the conventional pasteurization treatments should be adequate to inactivate *Salmonella* to safe levels. Nevertheless, food processors should take into account that data obtained from cells grown under optimal conditions may be inappropriate in designing effective heat treatments.

5. The extremely high survival shown by acid adapted cells at pH 3.0 in meat extract suggests that the protein content of foods protects bacteria against the killing effects of gastric fluid. In fact, the inclusion of arginine and lysine in a minimal challenge medium protects *Salmonella* against lethal pH conditions. This protective effect was shown to be due to the activation of arginine decarboxylase and lysine decarboxylase homeostatic systems. However, other molecular mechanisms, such as the modifications in membrane fatty acid composition, are also involved in the development of these adaptive responses. Thus, the highest acid and heat resistant cells show an increase in the content in cyclic fatty acids (CFA) and a decrease in the unsaturated fatty acids to saturated fatty acids ratio (UFA/SFA)

6. *S.* Typhimurium acid adaptation causes a cross-protection response to an alkaline treatment in the presence of sodium hydroxide, and sensitizes it to osmotic and oxidative stresses. These synergic effects may be of

interest for the control of salmonellosis in cheese and other fermented foods.

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