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AISLAMIENTO, CARACTERIZACIÓN, SUPERVIVENCIA Y CONTROL QUÍMICO DE *BACILLUS CEREUS*

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AISLAMIENTO, CARACTERIZACIÓN, SUPERVIVENCIA Y CONTROL QUÍMICO DE *BACILLUS CEREUS*

Tesis doctoral realizada por D. Luis Alberto Hernández Herrero, Ingeniero Agrónomo, en el Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández de Elche, para la obtención del grado de Doctor.

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HACE CONSTAR:

Que el presente trabajo ha sido realizado bajo mi dirección, y recoge la labor realizada por el Ingeniero Agrónomo D. Luis Alberto Hernández Herrero para optar al grado de Doctor.

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Certifica:

Que da su conformidad a la lectura de la Tesis Doctoral presentada por D. Luis Alberto Hernández Herrero, titulada "Aislamiento, caracterización, supervivencia y control químico de *Bacillus cereus*" que se ha desarrollado dentro del programa de doctorado de "*Tecnología Agroalimentaria*" de este departamento, bajo la dirección del Dr. Manuel Valero Roche, la cual considera conforme en cuanto a forma y contenido para que sea presentada para su correspondiente exposición pública.

Y para que así conste a los efectos oportunos firmo el presente certificado en Orihuela a_____ de _____ de _____.

Dr. José Ramón Díaz Sánchez.

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A mi familia

PRÓLOGO

La presente Tesis Doctoral se ha elaborado siguiendo la normativa de la Universidad Miguel Hernández de Elche para la "Presentación de Tesis Doctorales como un conjunto de publicaciones", y se ha dividido en los siguientes apartados:

- Resumen.
- Introducción.
- Objetivos.
- Publicaciones, este apartado consta de tres artículos publicados:

  Valero, M., Hernández-Herrero, L. A., Fernández, P. S., Salmerón, M. C. 2002. Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. Food Microbiology. 19: 491-499.

  Valero, M., Hernández-Herrero, L. A., Giner, M. J. 2007. Survival, isolation and characterization of a psychrotrophic *Bacillus cereus* strain from a mayonnaise-based ready-to-eat vegetable salad. Food Microbiology. 24: 671-677.

  Hernández-Herrero, L. A., Giner, M. J., Valero, M. 2008. Effective cheminal control of psychrotrophic *Bacillus cereus* EPSO-35AS and INRA TZ415 spore outgrowth in carrot broth. Food Microbiology. 25: 714-721.

- Resumen de Resultados, Discusión y Conclusiones de las tres publicaciones.

- Bibliografía, en la que se reseñan todas las referencias que aparecen citadas en el texto, aunque también lo estén en las publicaciones.

Este documento no incluye el apartado “Materiales y Métodos”, puesto que éstos se encuentran descritos en las diferentes publicaciones.

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RESUMEN

RESUMEN

La contaminación por *Bacillus cereus* ha sido evaluada en un total de 97 muestras de pimientos frescos, pepinos, tomates, zanahorias, calabacines, ajos y cebollas, comúnmente usados como ingredientes para elaborar alimentos refrigerados mínimamente procesados y productos tales como gazpacho, salmorejo, ajoblanco, zanaranja y ensalada americana. Los recuentos medios no excedieron de 10^4 CFU/g en ningún caso. Todos los vegetales y productos procesados, excepto el ajo y la zanaranja, presentaron contaminación por este patógeno.

Un total de 37 presuntas cepas de *B. cereus* fueron aisladas, caracterizadas e identificadas por el sistema fenotípico API 50CH/20E y el programa informático APILAB Plus, combinado con test adicionales de movilidad, actividad oxidasa y crecimiento anaerobio. Confirmándose como *B. cereus* 33 cepas (89,2%), 2 (5,4%) como *B. licheniformis*, y las 2 (5,4%) cepas restantes fueron identificadas como *B. firmus*.

De las 33 cepas de *B. cereus* encontradas, 27 (81,8%) eran capaces de hidrolizar el almidón y 24 (72,7%) eran productoras de la enterotoxina diarreica en cultivos de TSB según el test de aglutinación pasiva invertida en látex para la enterotoxina de *B. cereus* (BCET-RPLA).

La resistencia térmica a 90 ºC de las esporas de las 33 cepas de *B. cereus* osciló entre 1,4 y 21,2 min. Las cepas incapaces de hidrolizar el almidón mostraron mayor resistencia térmica, con valores D_{90} superiores a 10,8 min. Todas las cepas productoras de enterotoxinas fueron capaces de hidrolizar el almidón.

Ninguna de las cepas fue capaz de crecer a 5 ºC. Sólo la cepa EPSO-35AS (el 3%) creció a 8 ºC, mientras que 29 cepas (el 87,9%) crecieron a 10 ºC. A las temperaturas de 12, 30 y 37 ºC crecieron todas las cepas, mientras que a 42 ºC sólo crecieron 19 cepas (el 57,6%).

El estudio de la cinética de crecimiento de la cepa EPSO-35AS a 8 ºC mostró un crecimiento más rápido y una población final más elevada en TCB (caldo zanahoria tindalizado) que en NB (caldo nutritivo), siendo la fase de latencia 66 horas más corta.

Los parámetros cinéticos de crecimiento de la cepa psicrótrofa EPSO-35AS, en NB y en TCB fueron evaluados a diferentes temperaturas (8, 12 y 16 ºC) en combinación con la acidificación con ácido cítrico o zumo de limón a valores de pH comprendidos entre 4,7 y 5,5. Bajando el pH desde 7,4 en NB o 6,2 en TCB hasta 5,2 se inhibe el crecimiento bacteriano en ambos medios testados después de 60 días a 12 ºC o temperaturas inferiores.

Finalmente, se estudió la actividad antibacteriana de 4 compuestos naturales (aceite esencial de cinnamon, cinamaldehido, carvacrol y eugenol) en las cepas de *B. cereus* EPSO-35AS e INRA TZ415 en ambos medios y el mismo rango de temperaturas. La adición de aceite esencial de cinnamon o de cinamaldehido a concentraciones de 5 y 2 µL/100mL respectivamente, causaron la completa inhibición del crecimiento de ambas cepas psicrótrofas a 12 ºC. Así pues, la combinación de uno de estos compuestos y la temperatura de refrigeración, se puede usar para la conservación de los alimentos mínimamente procesados, en los cuales el principal ingrediente sea la zanahoria. Por el contrario, el carvacrol y eugenol a concentraciones de 5 y 35 µL/100mL respectivamente, sólo fueron capaces de prevenir el crecimiento de *B. cereus* EPSO-35AS durante el almacenamiento a 8 ºC.

1.- INTRODUCCIÓN

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El consumo de frutas y hortalizas en la dieta diaria tiene un efecto muy beneficioso para la salud, no sólo son una excelente fuente de vitaminas, minerales y fibra, sino que además poseen fotoquímicos que contribuyen a la salud. Estos componentes que se encuentran en las plantas, aunque no se consideran nutrientes esenciales, proporcionan una importante protección contra las toxinas, el cáncer y otros trastornos comunes del organismo. Otro aspecto importante de las frutas y hortalizas es que generalmente son bajas en calorías, y por lo tanto se adecuan a la tendencia actual del consumidor por realizar dietas más equilibradas.

Los hábitos de alimentación humana han cambiado mucho en las dos últimas décadas. El actual ritmo de vida, con escaso tiempo para preparar comidas equilibradas, ha provocado la demanda de productos cada vez con mayor grado de elaboración, dando lugar a distintas clasificaciones o gamas de acuerdo con el grado de procesamiento:

I Gama: vegetales en estado fresco.

II Gama: vegetales en conserva.

III Gama: vegetales congelados.

IV Gama: vegetales frescos mínimamente procesados (MPF), conservados bajo cadena de frío, listos para ser consumidos.

V Gama: alimentos cocidos, mantenidos bajo cadena de frío (REPFEDs).

El propósito de los alimentos mínimanente procesados refrigerados (IV Gama) es proporcionar al consumidor un producto hortícola muy parecido al fresco, con una vida útil prolongada y al mismo tiempo garantizar la seguridad de los mismos, manteniendo una sólida calidad nutritiva y sensorial.

También tienen como ventajas la reducción del espacio durante el transporte y almacenamiento, el menor tiempo de preparación de las comidas, y la calidad uniforme y constante de los productos durante todo el año.

Por otro lado, su conservación es crítica debido a los daños físicos producidos en los tejidos vegetales durante el proceso. Estos daños aceleran el metabolismo provocando el deterioro de las características sensoriales deseables, pérdida de nutrientes, así como el desarrollo de microorganismos que llevan a un rápido decaimiento de la calidad y acortamiento de la vida útil del producto. Este último efecto se ve aumentado en los alimentos poco ácidos, en los que la vida útil puede ser inferior a una semana, limitando en gran medida su comercialización en los mercados (Valero *et al.*, 2000). Además, necesitan una gran rotación y logística muy especializada y son más caros que el producto a granel, por lo que requieren consumidores con un poder adquisitivo medio.

Los alimentos mínimamente procesados y refrigerados están experimentando un fuerte crecimiento en todos los países europeos. El desarrollo de estos productos puede permitir la diversificación y el fomento de la industria de productos vegetales procesados, los cuales son muy importantes para la economía del sureste de España, aportando nuevos productos y desarrollando

nuevas tecnologías que prolonguen la vida útil del producto, minimizando la pérdida de calidad sensorial y nutritiva y garantizando la seguridad alimentaria.

No obstante, la presencia de microorganismos esporulados patógenos capaces de desarrollarse a temperaturas de refrigeración puede limitar el éxito de estos productos.

Una amplia gama de estos alimentos son sometidos a un tratamiento térmico moderado (temperaturas inferiores a 100 ºC, normalmente entre 65 y 95ºC), una conservación en refrigeración (a menos de 8 ºC) y bajos niveles de aditivos. Los alimentos así procesados (V Gama) no se pueden considerar estériles y pueden presentar una vida útil en torno a los 30 días.

Este moderado tratamiento térmico es suficiente para garantizar la inactivación de las bacterias en forma vegetativa, pero no de las esporas bacterianas (Graham *et al.*, 1996).

Cuando estos alimentos se envasan en anaerobiosis, el principal riesgo consiste en la presencia de *Clostridium botulinum*, capaz de desarrollarse y producir la toxina botulínica. Pero si son envasados en una atmósfera aerobia o en una atmósfera modificada que contenga oxígeno, el principal riesgo es la presencia de cepas psicrótrofas de *Bacillus cereus*, que son incluso más resistentes al calor que algunas cepas de *C. botulinum* (Harmon y Kautter, 1991).

1.1.- Características principales de *Bacillus cereus*.

Debido a la similitud entre las diferentes especies pertenecientes al género *Bacillus* se han realizado varias clasificaciones.

La clasificación más tradicional se realizó de acuerdo a sus características morfológicas, diámetro del bacilo, forma de las esporas, dimensión del esporangio debido a la espora y apariencia del protoplasma. *B. cereus* y las especies más relacionadas con él, se ubican en el Grupo I de esta clasificación (Gordon *et al*., 1973).

Actualmente *B. cereus* y estas especies estrechamente relacionadas se han clasificado de acuerdo a sus características fenotípicas y genómicas dentro del Grupo *B. cereus*, estando integrado este por *B. cereus, B. weihenstephanensis, B. mycoides, B. thuringiensis* y *B. anthracis* (Tourasse *et al*., 2006). A su vez, este grupo se ha subdividido de acuerdo con mayores grados de similitud entre especies apareciendo el subgrupo *B. cereus sensu lato*, dentro del cual se ubica *B. cereus, B. thuringiensis* y *B. anthracis* (Rasko *et al*., 2005).

Estas especies muestran un alto grado de similitud en sus propiedades fenotípicas y su ADN dificultando su identificación bioquímica (Martínez *et al*., 2011).

B. cereus se caracteriza por ser un bacilo voluminoso de 1-1,2 μm de largo y un diámetro que puede sobrepasar las 0,9 μm, formador de esporas elipsoidales

centrales o pericentrales, que raramente deforman el esporangio y termorresistentes. Es Gram positivo, catalasa positivo, oxidasa negativo, anaerobio facultativo, aunque requiere oxígeno para la producción de la toxina emética, y normalmente móvil, por flagelos peritricos, aunque existen variantes inmóviles (Pascual, 1992; Mcelroy *et al*., 2000; Gordon *et al*., 1973).

1.2.- Características de crecimiento.

Su temperatura óptima de crecimiento se encuentra entre 30 y 37 ºC. Su temperatura mínima de crecimiento se encuentra entorno a 5 ºC y su máxima a 55 ºC (Bourgeois *et al*., 1991).

El abanico para la producción de toxinas esta entre 15 y 40 ºC, pues las cepas productoras de toxina emética no germinan a temperaturas inferiores a 15ºC (Finlay *et al*., 2002).

Las cepas productoras de toxina emética son mesófilas, mientras que las cepas productoras de toxina diarreica son más heterogéneas abarcando cepas mesófilas y psicrótrofas. La mayoría de las cepas diarreicas tienen como temperatura mínima de crecimiento 7 ºC (Pielaat, 2005).

Sobrevive entre valores de pH comprendidos entre 4,5 y 9,3 (Bourgeois *et al*., 1991) y su pH óptimo está entre 6-7 (Finlay *et al*., 2002; Lake *et al*., 2004). La a_W está en torno a 0,92-0,95 para células vegetativas (Lake *et al*., 2004).

1.3.- Reservorios y contaminación.

B. cereus está muy extendido en la naturaleza, encontrándose en aire, agua y suelo, siendo este último el hábitat mayoritario de formación de la espora bacteriana (Vilain *et al*., 2006) y la fuente directa de la contaminación en alimentos (Guinebretiere *et al*., 2003; Guinebretiere y Nguyen-The, 2003). Las concentraciones de *B. cereus* en suelo pueden alcanzar valores de 10^5-10^6 esporas/gramo de suelo (te Giffel *et al*., 1995). De este ambiente natural pasa a los alimentos, especialmente a aquellos de origen vegetal. A través de la contaminación cruzada puede entonces difundirse a otros alimentos. Crece muy bien en la mayoría de los productos alimenticios: maíz, hortalizas, carne picada, embutidos de hígado, fiambre de carne, leche, carne cocida, y especialmente en arroz, y sobre todo si éste lleva proteínas animales, como podría ser el caso de un caldo de carne.

La espora de *B. cereus* es la más hidrófoba del género *Bacillus*, adhiriéndose a diferentes tipos de superficies (Husmark y Rönner, 1992; Husmark, 1993; Klavenes *et al.*, 2002), incluso acero inoxidable (Jullien *et al.*, 2002). Además, las esporas de *B. cereus* disponen de pelos y/o apéndices (Husmark y Rönner, 1992; Andersson *et al.*, 1995) que favorecen la adherencia (Husmark y Rönner, 1992).

Estas propiedades de adherencia permiten a la espora resistir los procesos de desinfección normales de la industria alimentaria, contaminando los alimentos durante su tratamiento, apareciendo incluso en los materiales de embalaje

(Pielaat *et al,* 2005), además de adherirse a las células epiteliales intestinales donde germina y produce las enterotoxinas (Granum, 1994a; Granum, 1994b).

La capacidad de *B. cereus* para sobrevivir en diversos ambientes, condiciones de estrés y su capacidad de adherirse a las superficies dificulta enormemente su eliminación en la industria alimentaria (Anderson *et al.*, 1995). Además, parece haber una asociación por "nicho térmico" en la industria de alimentos, es decir las cepas psicrótrofas viven en ambientes fríos, mientras que las cepas más termorresistentes viven en industrias donde se realizan procesos térmicos (Carlin *et al.*, 2010).

1.4.- La espora bacteriana.

La espora (del griego, semilla) o endospora bacteriana (llamada de esta manera debido a que la espora se forma dentro de la célula) es única por su capacidad para resistir temperaturas muy elevadas. Hay esporas bacterianas capaces de sobrevivir a temperaturas superiores a 121ºC e incluso cepas extraordinariamente resistentes que pueden sobrevivir a 135 ºC durante 4 horas. La endoespora es una forma de resistencia que presentan algunos géneros de bacterias *(Bacillus y Clostridium* son los más relevantes en alimentos) frente a condiciones ambientes desfavorables (desecación, falta de nutrientes, acumulación de sustancias tóxicas, etc.) (Brock y Madigan, 1991).

Las esporas se forman por invaginación de una doble capa de membrana celular, que se pliega sobre sí misma encerrando en su interior un genoma y una

pequeña porción de citoplasma. Entre ambas capas se sintetiza la fina pared de la espora y una corteza más gruesa (Davis *et al*., 1996).

La esporulación es el proceso de formación de la espora a consecuencia de la expresión de unos genes específicos. Para que se produzca la esporulación se han de cumplir unos requerimientos nutricionales que varían según las especies e incluso entre cepas. En general, es necesario un medio con un aporte limitado de hidratos de carbono, nitrógeno o fósforo. Esto indica que es un proceso que se ve favorecido en un medio con carencias nutricionales (Keynan y Sandler, 1983).

Las esporas son capaces de permanecer en dormancia por muchos años, pero pueden convertirse de nuevo en una célula vegetativa, proceso denominado germinación, en cuestión de minutos (Prescott *et al*., 1999).

Algunas esporas germinan espontáneamente en un medio favorable, pero otras permanecen durmientes hasta que algún agente traumático como el calor, pH bajo o algún compuesto con grupos sulfhídricos (-SH) o el envejecimiento las activa. Una vez dañada la cubierta, el agua y algún agente germinador (dipicolinato-Ca, Mn^{2+}, etc.) activan la hidrólisis de la corteza y el crecimiento de la célula vegetativa (Valero, 2000).

1.5.- Termorresistencia bacteriana y métodos para su determinación.

La resistencia térmica de la espora se atribuye en gran parte al dipicolinato-Ca, que puede constituir hasta el 15% de su peso. La mayoría de enzimas de la espora no son termoestables, por lo que la resistencia térmica de las esporas debe ser debida a la deshidratación del entorno intracelular (Valero, 2000).

Los equipos que se usan para determinar la termorresistencia de esporas son muy diversos, y pueden agruparse en métodos de calentamiento directo e indirecto (Brown, 1993).

En los métodos de calentamiento indirecto, la muestra no entra en contacto con el medio de calentamiento, recibiendo el calor a través de una barrera física que actúa como superficie intercambiadora (paredes de tubos de vidrio o metal, latas, intercambiadores de calor). La temperatura se eleva de forma exponencial, lo que implica que durante el tiempo que tarda la muestra en alcanzar la temperatura prevista, está expuesta a temperaturas que van ejerciendo una acción letal. Éste es quizás el principal factor limitante en este tipo de sistemas, porque las esporas pueden morir al alcanzar la temperatura del proceso.

En los métodos de calentamiento directo, la muestra está en íntimo contacto con el medio de calentamiento, siendo la forma más común el empleo de vapor de agua sobrecalentado. Tiene la ventaja de que el calor latente del vapor es capaz de producir un calentamiento extremadamente rápido. Otra forma de

calentamiento directo es el empleo de resistencias eléctricas introducidas dentro de la muestra.

A continuación describimos los métodos de calentamiento indirecto, ya que son los más utilizados en laboratorio:

1.5.1.- Método de los tubos TDT (tiempo de destrucción térmica).

Consiste en tubos de ensayo de vidrio de pequeño diámetro (7 a 10 mm) que se inoculan con volúmenes de 1 y 4 µL de tampón, medio de cultivo o alimento.

Es un método simple, disponible en cualquier laboratorio, y barato. En medios transparentes el desarrollo bacteriano o cualquier cambio en el alimento puede observarse sin necesidad de abrir los tubos, y en caso de ser necesario abrirlos se hace con facilidad. Su incubación no requiere un gran espacio, pero poseen el inconveniente de que llenar, cerrar y, en su caso, subcultivar los tubos es una tarea tediosa.

Al transferir el contenido de los tubos al medio de recuperación, siempre existe la posibilidad de perder supervivientes que queden adheridos a las paredes del tubo. Solo pueden usarse productos líquidos u homogeneizados y los períodos de inercia (tiempo que transcurre hasta que la muestra del tubo alcanza la temperatura de tratamiento o se enfría por completo en el baño de hielo) son importantes y difíciles de calcular (Stumbo, 1953; Odlaug y Pflug, 1977).

Odlaug y Pflug (1977) propusieron una variante de esta técnica, consistente en emplear tubos de aluminio cerrados con roscas de nylon, y con capacidad para 1 mL de muestra. Sin embargo, y a pesar del alto coeficiente de transmisión del calor del aluminio, persiste el problema de un alto periodo de inercia, lo cual limita su empleo a altas temperaturas.

1.5.2.- Método de los tubos TDT abiertos.

Consiste en utilizar tubos de 13 mm de diámetro y 100 mm de altura tapados con algodón, que se calientan en un baño de agua o aceite para temperaturas de hasta 100 ºC o con vapor bajo presión, si se trabaja a temperaturas superiores a 100 ºC. Son esos mismos tubos los que sirven para recuperar los microorganismos al añadirles el medio de cultivo, evitándose la posibilidad de perderlos.

La desventaja es que los periodos de tiempo requeridos para alcanzar las temperaturas de calentamiento y enfriamiento son altos, lo que limita su uso a altas temperaturas (Rodrigo y Martínez, 1988).

1.5.3.- Método de los tubos capilares.

Fue diseñado por Stem y Proctor (1954). Se emplean tubos capilares calibrados que se llenan con el medio inoculado, usando un auxiliar de micropipeteado. Se cierran a la llama y se calientan en baños de aceite

termostatado durante periodos predeterminados, enfriándose a continuación en un baño de agua y hielo.

Esta técnica posee como ventaja que es fácil de usar y el equipo necesario es muy simple, se requiere un bajo número de esporas y se pueden emplear tiempos de exposición cortos, del orden de segundos (Perkin *et al*., 1980).

El uso de capilares de paredes finas (0,15 mm) disminuye considerablemente los tiempos requeridos para el calentamiento y el enfriamiento o fases de inercia (Perkin *et al*., 1980). Sin embargo, el cierre, la apertura y el vaciado de los tubos es una tarea laboriosa, especialmente cuando se trabaja con productos viscosos.

Este método fue el elegido para nuestros ensayos de termorresistencia, utilizando tubos capilares de 10 µL y un baño de aceite de vaselina.

1.6.- Aislamiento, cuantificación y caracterización.

Para cuantificar la contaminación por *B. cereus* en los vegetales frescos, se utilizó la técnica de recuento en placa descrita por AFNOR (1996).

Para la detección y cuantificación de *B. cereus* en productos refrigerados mínimamente procesados y productos vegetales listos para comer, se utilizó el procedimiento de estimación estadística del “número más probable” (NMP).

Los aislados de *B. cereus* de cada muestra fueron confirmados con los tests bioquímicos API 50CH y API 20E, por comparación con perfiles tipo de la base de datos APILAB Plus V3.2.2 Versión B 01.93. También fueron realizadas la actividad oxidasa, la movilidad en un medio semi-sólido y el crecimiento anaerobio. Actualmente existen métodos más fiables para la confirmación de microorganismos como pueden ser las técnicas utilizadas en genética molecular e ingeniería genética, polimorfismos en la longitud de los fragmentos de restricción-reacción en cadena de la polimerasa RFLP-PCR y huella genética (genetic fingerprinting), pero estos métodos no son útiles para controles rutinarios. Además, el método utilizado es totalmente válido, ya que aunque a veces no permite la diferenciación entre especies estrechamente relacionadas, nos proporciona mucha información sobre las propiedades del microorganismo, que pueden ser útiles a la hora de combatirlo.

1.7.- Patogenicidad.

Este microorganismo causa dos tipos de intoxicación alimentaria, la diarreica y la emética. Ambas son generalmente leves, aunque también se producen intoxicaciones serias, e incluso en algunos casos letales (Dierick *et al*., 2005).

El tipo diarreico, fue reconocido después de un brote en un hospital de Oslo (Noruega), causado por una salsa de vainilla contaminada en 1948, y el tipo emético fue descrito hace 20 años, después de varios brotes en Londres (Granum, 1997; Montville, 1997).

El tipo de intoxicación diarreica está causado por una enterotoxina producida durante el crecimiento vegetativo de *B. cereus* en el intestino delgado (Granum, 1994b; Clavel *et al*., 2004). El síndrome emético esta causado por la toxina emética, llamada cereulide, producida en los alimentos antes de ser ingeridos (Kramer y Gilbert, 1989). Ambos tipos de enfermedades son normalmente causados por alimentos tratados térmicamente, donde las esporas supervivientes son el origen de la enfermedad (Granum, 1997). *Bacillus cereus* no es un microorganismo competitivo, pero crece bien después de un tratamiento térmico seguido de un enfriamiento. El tratamiento térmico causará la germinación de la espora, y en ausencia de flora competitiva se desarrolla sin ningún problema.

1.7.1.- Toxina diarreica.

La toxina diarreica o enterotoxina se produce durante la fase de crecimiento exponencial, pero la cantidad máxima de toxina se encuentra al principio de la fase estacionaria. La lisis de la célula no es necesaria para la producción de la toxina. Es termolábil (se inactiva a 60 ºC), y sensible a la pronasa y a la tripsina. Actúa rompiendo la integridad de la membrana plasmática de las células epiteliales, pues estimula el sistema AMP cíclico-adenilato ciclasa, lo que provoca la acumulación de líquido en el intestino delgado (Bourgenois *et al*., 1991; Stenfors Arnesen *et al*., 2008).

B. cereus produce un gran número de citotoxinas y enzimas que pueden contribuir a la enfermedad diarreica, entre las cuales destacan tres: Hbl (Hemolisina), Nhe (Enterotoxina no hemolítica) y CytK (Citotoxina K), estando sus masas moleculares entre los 39 y 105 KDa (Lund y Granum., 1996; Lund *et al*., 2000). Las cepas productoras de la toxina diarreica son capaces de hidrolizar el almidón.

El síndrome diarreico es similar al producido por *Clostridium perfringens,* y se caracteriza por diarrea líquida, dolores abdominales y ocasionalmente náuseas (Tabla 1). El tiempo de incubación es superior a 6 horas, normalmente de 8-16 horas, siendo la media de 12 horas, aunque en raros casos se han dado tiempos de incubación superiores. La duración de la enfermedad normalmente es de 12-24 horas, aunque se han descrito casos de varios días (Kramer y Gilbert, 1989). El número de microorganismos necesario para producir la intoxicación diarreica es de 10^5-10^8 células (Granum, 2007).

Esta intoxicación está asociada a alimentos proteicos, salsas, aderezos y vegetales (Kramer y Gilbert, 1989), productos cárnicos, potajes, pudín y productos lácteos (Granum, 2007).

1.7.2.- Toxina emética.

Cereulide, la toxina emética, es un péptido cíclico de masa molecular 1,2 KDa, resiste las condiciones ácidas (no es destruido por el ácido gástrico), la proteolisis (tripsina y pepsina) y un amplio rango de temperaturas, manteniéndose

estable a 126 ºC durante 90 minutos (termoestable) y a 4 ºC durante dos meses (Agata *et al*., 1994; Agata *et al*., 2002; Ehling-Shulz *et al*., 2004).

Los síntomas, náuseas y vómitos, son parecidos a los producidos por *Staphylococcus aureus.* El tiempo de incubación es de 1 a 6 horas después de la ingestión, pero también se han observado tiempos inferiores (0,5 h) y superiores a estos (Tabla 1). La duración de la enfermedad suele esta comprendida entre 6-24 horas (Ehling-Shulz *et al*., 2004). La intoxicación producida por esta toxina es más grave y más aguda que la producida por la toxina diarreica, inhibiendo la oxidación de ácidos grasos de las células del duodeno, y por lo tanto, paralizando la actividad mitocondrial de estas células (Mikkola *et al*., 1999).

Esta intoxicación está asociada a alimentos farináceos, tales como el arroz cocido (Gilbert y Kramer, 1984; Kramer y Gilbert, 1989) y la pasta (Granum, 2007).

Tabla 1. Características de los dos tipos de enfermedades alimentarias causadas por *Bacillus cereus*. Adaptada por Granum (2007). [a] Clavel *et al*., (2004). [b] Agata *et al*., (1994); Agata *et al*., (1995); Shinagawa *et al*., (1995). [c] Lund *et al*., (2000). [d]Mahler *et al*., (1997); Dierick *et al*., (2005).

Características	Enfermedad diarreica	Enfermedad emética
Tipo de toxina:	Proteína; enterotoxinas: Hbl, Nhe, CytK	Péptido cíclico; toxina emética (cereulide)
Lugar donde se produce la toxina:	En el intestino delgado del huésped	En los alimentos
Dosis para la infección:	10^5-10^8 UFC (total), en caso de tratarse de esporas se requiere un menor número [a]	Normalmente se encuentra en los alimentos implicados 10^5-10^8 células/g, pero la existencia de células vivas no es requisito para la intoxicación Cereulide: 8-10 µg/kg de peso corporal (ensayos en animales) [b]
Tiempo de incubación:	8-16 h (ocasionalmente más de 24 h)	0,5-6 h
Duración de los síntomas:	12-24 h (ocasionalmente varios días)	6-24 h
Síntomas:	Dolor abdominal, diarrea líquida y ocasionalmente náuseas y rara vez la muerte [c]	Náuseas y vómitos. Rara vez puede ser letal (posiblemente debido a daños en el hígado) [d]
Alimentos frecuentemente implicados:	Alimentos proteicos: productos cárnicos, potajes, vegetales, pudín, salsas, aderezos, leche y productos lácteos	Alimentos ricos en almidón: arroz frito o cocido, pasta, productos de pastelería

Se han encontrado diferencias en la distribución de las dos enfermedades entre países, debido posiblemente a las costumbres alimenticias de cada país. Mientras que en Japón y Reino Unido predomina la enfermedad emética (Shinagawa *et al*., 1995), en Norteamerica y norte de Europa predomina la enfermedad diarreica (Kotiranta *et al*., 2000).

1.8.- Mecanismos de control.

La técnica de “carrera de obstáculos” se basa en las interacciones entre los diferentes sistemas de conservación tradicionales, tales como los tratamientos térmicos, las bajas temperaturas, la acidificación, el potencial de oxidación-reducción, la actividad de agua y la adición de conservantes. El efecto antimicrobiano puede ser mejorado por la combinación de varios sistemas formando una estrategia global de conservación. De acuerdo con este concepto, la combinación de dos o más tratamientos de conservación a bajas intensidades individuales minimiza su impacto sobre las propiedades sensoriales y nutritivas del alimento (Leistner, 1999; Leistner, 2000).

1.8.1.- La esterilización térmica.

La destrucción de los microorganismos es el objetivo fundamental en los procesos de esterilización de alimentos, con el fin de conservar dicho alimento durante el mayor periodo posible de tiempo. Para ello, se utilizan técnicas tan variadas como: presión hidrostática, agentes químicos, radiaciones ultravioleta, radiaciones ionizantes, etc. Entre todos, la aplicación de calor húmedo es la más usada, por ser muy eficaz, económica y fácil de controlar (Valero, 2000).

1.8.2.- La refrigeración.

La temperatura es uno de los factores que más afectan al crecimiento y supervivencia microbiana. A medida que aumenta la temperatura, aumentan las

reacciones enzimáticas y el crecimiento es más rápido, hasta llegar a un valor en el cual las proteínas, ácidos nucleicos y otros componentes se dañan de manera irreversible, deteniéndose las funciones celulares. Por tanto, para cada microorganismo existe una **temperatura máxima**, a partir de la cual no existe crecimiento, una **temperatura óptima** en la cual el crecimiento es rápido y una **temperatura mínima**, por debajo de la cual tampoco existe crecimiento (Madigan *et al*., 1999).

Se comprobó la capacidad de crecimiento de 33 cepas de *B. cereus* en tubos con TSB a 2, 8, 10, 12, 30, 37 y 42 ºC. De estas cepas, 32 pertenecen a la colección de aislados de vegetales frescos y REPFEDs (Valero *et al*., 2002). La cepa restante fue aislada durante el segundo estudio en ensalada americana.

De las 33 cepas, sólo la aislada de ensalada americana fue capaz de crecer a baja temperatura (8 ºC), por lo cual en teoría es la cepa más peligrosa. Debido a esto, los últimos dos trabajos se centran en esta cepa psicrótrofa de *B. cereus* aislada en ensalada americana.

1.8.3.- Efecto del pH.

El pH interno de muchos microorganismos se acerca a la neutralidad. Una variación drástica del pH medioambiental afecta al crecimiento microbiano originando cambios estresantes en el pH citoplasmático. Aunque los microorganismos frecuentemente crecen en amplios rangos de pH, cada especie tiene definido un rango de pH para el crecimiento y un pH de crecimiento optimo.

Sin embargo, el pH mínimo para su crecimiento varía entre cepas y también depende de los acidulantes, pues los ácidos orgánicos atraviesan mejor la membrana celular que los inorgánicos. A pesar de su tolerancia a la acidez, la muerte bacteriana se produce por inhibición de la actividad de las enzimas y de las proteínas transportadoras de la membrana si el pH interno de la célula vegetativa disminuye por debajo de 5,0-5,5.

1.8.4.- Los antimicrobianos.

En los últimos años ha aumentado el interés de la industria alimentaria por sustituir los conservantes sintéticos por naturales. Los compuestos antimicrobianos naturales incluyen enzimas, compuestos producidos por cultivos iniciadores y compuestos de origen vegetal tales como especias, extractos, aceites esenciales y sus compuestos aislados (Davidson, 1997). Es conocido que algunas especias y sus aceites esenciales poseen diferentes grados de actividad antimicrobiana (Shelef, 1983; Zaika, 1988; Beuchat y Golden, 1989; Ting y Deibel, 1992; Juven *et al.*, 1994; Tassou *et al.*, 1995; Chang, 1995; Sivropoulou *et al.*, 1996; Chaibi *et al.*, 1997; Wan *et al.*, 1998; Lachowicz *et al.*, 1998). El inconveniente de los antimicrobianos naturales radica en que su elevado flavor limita su uso en determinados alimentos (Ultee *et al*., 2000; Frutos y Hernández-Herrero, 2004).

Se estudiaron las actividades antimicrobianas de 4 compuestos naturales. Uno de ellos es un aceite esencial, aceite esencial de cinnamon; los otros tres,

son compuestos químicos aislados de aceites esenciales, eugenol, carvacrol y cinamaldehído, este último componente mayoritario del aceite esencial de cinnamon.

Se eligieron estos compuestos naturales para proporcionar información sobre su uso potencial en alimentos mínimamente procesados y convertirse así en una alternativa a los conservantes tradicionales de alimentos. Además, con estos compuestos naturales se evita la aparición de resistencias a antibióticos, problemas de alergias y toxicidad a los consumidores.

1.9.- Evaluación de la cinética de crecimiento de *Bacillus cereus*.

Para evaluar el crecimiento de *B. cereus* se ha utilizado un medio sintético, caldo nutritivo (NB), de pH 7,4 y un medio natural, caldo de zanahoria tindalizado (TCB), de pH 6,2, tal como se describe en Valero y Giner (2006). Este último, ha sido escogido por ser la zanahoria un vegetal poco ácido ampliamente utilizado como ingrediente en los alimentos mínimamente procesados (Valero y Salmerón, 2003). Por ello el TCB ha sido utilizado a modo de sistema modelo de este tipo de alimentos. Además, en el TCB no ha sido detectado ningún factor inhibidor que afecte al crecimiento de *B. cereus*, por lo que se asegura una buena proliferación microbiana.

Aunque en general los medios de cultivo naturales son más estresantes para los microorganismos que los medios sintéticos, nuestros resultados previos indicaron que el medio natural podía tener un efecto estimulante sobre el

crecimiento de cepas psicrótrofas de *B. cereus*, por lo que era interesante comparar estos dos medios de cultivo.

La acidificación de los productos mínimamente procesados es una forma de aumentar la vida útil de estos alimentos, por ello se acidifico con zumo de limón y ácido cítrico hasta valores de pH comprendidos entre 4,7 y 5,5. Acidificar con zumo de limón responde al interés de potenciar el uso de aditivos naturales, en este caso un acidulante, frente a los aditivos sintéticos.

Para el ajuste de las curvas de crecimiento y el cálculo de sus parámetros se ha utilizado el programa D-model, el cual utiliza la ecuación de Baranyi *et al*. (1993).

1.10.- Contextualización.

El desarrollo de la presente tesis se ha llevado a cabo en tres fases:

En un primer estudio se investigó con la finalidad de detectar, cuantificar, aislar, identificar bioquímicamente y confirmar la presencia de *B. cereus* en un amplio rango de muestras de vegetales frescos y productos mínimamente procesados. Como resultado de estos ensayos se obtuvo una colección de cepas de *B. cereus* aisladas a partir de vegetales y productos mínimamente procesados y refrigerados, a las cuales se le realizaron ensayos de termorresistencia.

En un segundo estudio se realizaron ensayos de crecimiento a diferentes temperaturas sobre la colección de cepas de *B. cereus, e*specialmente sobre la cepa psicrótrofa EPSO-35AS aislada a partir de ensalada americana, realizándose sobre ésta un nuevo estudio de temorresistencia. Resulta de gran interés conocer la resistencia térmica de una amplia representación de cepas de *B. cereus*, tanto mesófilas como psicrótrofas, aisladas a partir de distintos alimentos. Estos resultados contribuirán a disponer de valores de termorresistencia más precisos, que ayudarán a establecer tratamientos térmicos más efectivos y adecuados para garantizar la ausencia de riesgos microbiológicos en estos productos.

Por último, se estudió la capacidad de las esporas de la cepa EPSO-35AS activadas térmicamente para crecer en un rango de temperatura de 8 a 16 ºC en caldo nutritivo (NB) y caldo de zanahoria tindalizado (TCB), tanto a pH natural como acidificado. El estudio también fue dirigido a determinar la actividad antimicrobiana de 4 compuestos naturales (aceite esencial de cinnamon, cinamaldehído, carvacrol y eugenol) sobre la cinética de crecimiento de células germinadas de esporas. Finalmente, los resultados encontrados fueron comparados con los de la cepa psicrótrofa de *B. cereus* INRA TZ415, aislada de alimentos vegetales mínimamente procesados en la Estación Tecnológica de Productos Vegetales (Institut Nacional de la Recherche Agronomique, Avignon, Francia).

2.- OBJETIVOS

2.- OBJETIVOS.

Los objetivos de la presente tesis doctoral son los siguientes:

1.- Realizar el aislamiento, cuantificación e identificación bioquímica de *B. cereus* en un amplio rango de muestras de vegetales frescos y productos refrigerados mínimamente procesados.

2.- Determinar la resistencia térmica a 90 ºC de esporas bacterianas pertenecientes a cada una de las cepas aisladas.

3.- Poner de relieve la presencia de cepas psicrótrofas de *B. cereus* entre los diferentes aislados obtenidos.

4.- Comprobar la capacidad de crecimiento a bajas temperaturas de dichas cepas en medio sintético y substrato vegetal a base de zanahoria.

5.- Determinar los efectos inhibitorios de la acidificación, adición de antimicrobianos naturales y la temperatura de incubación sobre el crecimiento de *B. cereus*, con la finalidad de obtener datos que sean de utilidad a la hora de establecer obstáculos que garanticen la seguridad de los productos vegetales refrigerados mínimamente procesados (RMPFs).

3.- PUBLICACIONES

3.- PUBLICACIONES.

3.1.- Valero, M., Hernández-Herrero, L. A., Fernández, P. S., Salmerón, M. C. 2002. Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. *Food Microbiology, 19:* 491-499.

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ORIGINAL ARTICLE

Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests

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Bacillus cereus *contamination has been evaluated in a total of 56 samples of fresh peppers, cucumbers, tomatoes, carrots, zucchini, garlic and onions, used commonly as ingredients to manufacture refrigerated minimally processed foods. It has been also evaluated in some of these products, such as gazpacho (Mediterranean vegetable cold soup made from fresh vegetables), salmorejo (vegetable cold purée containing bread and egg), ajoblanco (cold cream containing bread, almond, and olive oil as main ingredients) and zanaranja (carrot and orange juice mixture). The average counts did not exceed* 10^4 *cfu* g^{-1} *in any case and all the vegetables and processed products except garlic and zanaranja presented contamination of this foodborne pathogen. Cucumbers presented the highest count among all the vegetable products analysed, followed by carrots. According to the ISO 7932:1993(F) confirmation procedure, a total of 36 presumptive* B. cereus *strains were isolated from the 85 samples of various vegetable products analysed. Isolates were further characterized and identified by API 50CH/20E phenotypic system using APILAB Plus software, combined with additional tests of motility, oxidase activity and anaerobic growth. Of the 36 presumptive* B. cereus *isolates, 32 (88·9%) were confirmed to be* B. cereus*, two strains (5·5%) were identified as* B. licheniformis*, and other two strains (5·5%) were identified as* B. firmus*. About 81·3% (26 strains) of 32* B. cereus *tested isolates hydrolysed starch and 71·9% (23 strains) produced diarrheal enterotoxin in TSB culture as detected by the BCET-RPLA test. The heat resistance at 90°C for spores of the 32* B. cereus *strains ranged from 1·4 to 21·2 min. Strains unable to hydrolyse starch were the most heat-resistant, with* D_{90} *values higher than 10·8 min. All the enterotoxin-positive strains were able to hydrolyse starch.*

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Introduction

The development of refrigerated minimally processed foods (RMPFs) can allow diversification and promotion of the processed vegetable products industry, which is very important for the economy of the southeast of Spain. The quality and safety of a group of these products relies on a mild heat treatment in combination with refrigeration temperatures below 8°C during storage and distribution. Generally, the main microbiological hazard for minimally

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processed low-acid products is the presence of psychrotrophic sporulated bacteria that are able to survive mild heat treatments. When these foods are packed in modified atmospheres containing oxygen, the hazard consists in psychrotrophic strains of *Bacillus cereus*.

Bacillus cereus is frequently isolated from both the natural environment (soil and growing plants) and foods, including raw and pasteurized milk and milk products (Ahmed et al. 1983), dried products (Kim and Goepfert 1971), pulses and cereals (Blakey and Priest 1980), spices (Powers et al. 1976), meat products, raw meat and meat product additives (Konuma et al. 1988), fresh vegetables and ready-to-eat vegetable-based foods (Roberts et al. 1982, Harmon et al. 1987, Magnusson et al. 1990, King et al. 1991, Harmon and Kautter 1991, Kaneko et al. 1999). *B. cereus* can cause illness by one of two types of enterotoxin both of which can be produced in foods. Foods associated with illness caused by the emetic toxin include starchy foods, particularly fried and cooked rice, pasta, pastry and noodles. Foods implicated in diarrheal syndromes include puddings and sauces, soups, green vegetables, mashed potatoes, milk and dairy products, and proteinaceous foods such as cooked meats.

The possible presence of psychrotrophic *B. cereus* strains on fresh vegetables could be a microbial risk to refrigerated minimally processed foods containing vegetables as ingredients. We recently demonstrated *B. cereus* ability to grow in carrot substrate broth (Valero et al. 2000). The aim of this work was the detection, enumeration, isolation, biochemical identification and confirmation of *B. cereus* in a wide range of fresh vegetables and refrigerated minimally processed food samples.

Material and Methods

Vegetable material

Fresh peppers (PE), cucumbers (CU), tomatoes (TO), carrots (CA), zucchini (ZU), garlic (GA) and onions (ON), which are commonly used as ingredients in RMPFs were provided by a local factory of these products. Samples of refrigerated minimally processed foods based on vegetables were purchased from local supermarkets. RMPFs such as gazpacho (GZ), salmorejo (SA), ajoblanco (AB) and zanaranja (ZN) were used.

Isolation and enumeration

To enumerate *B. cereus* contamination in fresh vegetables, the method described by AFNOR (1996) was used. In brief, 10 g of vegetable material was homogenized in a Masticator with 90 ml of peptone-water. Next 1 ml of the suspension was transferred to 20 ml test tube containing 9 ml thinner, and three serial decimal dilutions were performed. Detection and enumeration were performed by spread plating on selective solid medium [*Bacillus cereus* CeNAN Agar Base (Scharlau Chemie, S.A., Barcelona) supplemented with 100 mg l^{-1} of polymyxin B sulfate and 100 ml l^{-1} of egg yolk sterile solution]. This medium (polymyxin mannitol egg yolk phenol red agar, PMYPA) was designed by Mossel et al. (1967) and has been adopted by the Centro Nacional de Alimentación y Nutrición (CeNAN 1982), since it permits a good differentiation of *B. cereus* colonies from all types of food samples. The cultures were incubated at 30°C for 24 h. Colonies presenting a pink or purple color with an irregular edge surrounded by a white area were considered as positives and enumerated. At least five colonies from each sample were then streaked (subcultured) in CeNAN selective agar and were confirmed subsequently as *B. cereus* by biochemical testing as specified in the ISO 7932:1993(F). They were then streaked in tryptone soya agar (TSA) slants and stored refrigerated as pure cultures.

For detecting and enumerating *B. cereus* in refrigerated minimally processed, ready-to-eat, vegetable products, the statistical estimate of the most probable number (MPN) procedure was used, since the initial numbers of *B. cereus* were very low. Three serial decimal dilutions from each sample were performed and then 1 ml from each dilution was used to inoculate three series of three tubes containing the selective enrichment broth base without mannitol or indicator, and supplemented with 100 mg l^{-1} of polymyxin B sulfate. The tubes were

incubated at 30°C for 24 h. The differential isolation of the strains detected was performed following the procedure outlined above. The addition of polymyxin to the base liquid medium inhibits most of the accompanying flora, while it does not affect the growth of *B. cereus*. The transfer of the culture to the selective solid medium restricts even further the growth of competitive flora, at the same time as stimulating the growth of *B. cereus* and making it possible to differentiate characteristics of the colonies.

Biochemical identification and confirmation of B. cereus

Bacillus cereus isolates from each sample were identified/confirmed with biochemical tests, and data were processed with APILAB Plus software V3.2.2 Version B 01.93 (bioMérieux, Marcy l'Etoile, France). For the biochemical tests, API 50CH gallery was used to investigate the fermentation of 49 sugars, and API 20E gallery was used for 12 additional tests (ONPG, ADH, LDC, ODC, CIT, H2S, URE, TDA, IND, VP, GEL and NIT). The oxidase activity on filter paper using a wooden applicator to make the extension, the motility in a semi-solid medium, and the anaerobic growth in an agarified medium for anaerobes with neither glucose nor indicator were also tested (Pascual Anderson 1992, Atlas and Parks 1993).

The inocula used for the biochemical tests were prepared using young cultures grown in tryptone soya broth (TSB) medium and incubated at 30°C. After 18 h of incubation, a decimal dilution was made of the different cultures and 0·1 ml aliquots were then distributed with a Drigalski spatula on the surface of Petri plates containing J-Agar (Gordon et al. 1973, Claus and Berkeley 1986). Once the inocula had been reabsorbed, the plates were incubated at 30°C for 16 18 h. Two milliliters of sterilized saline solution (NaCl 0·85%) were then added to each of the plates and the cultures were collected from the agar surface with sterile pipettes. Suspensions were prepared with the cultures with a turbidity equivalent to 2 MacFarland. The API 50CH and API 20E galleries were filled with the inoculum, incubated and interpreted according to the instructions specified for each test.

Spore heat resistance

Bacillus cereus strains were grown overnight in nutrient broth at 30°C to achieve the stationary phase. Culture volumes of 0·2 ml were dispensed on plates containing fortified nutrient agar (FNA) as sporulation medium (Johnson et al. 1982, Mazas et al. 1995), and incubated at 30°C during 4 days. Sporulation was verified daily by microscopical examination. When at least 90% sporulation was reached, spores were harvested by flooding the agar surface with sterile distilled water, centrifuged, resuspended and stored at 4°C until use.

Spore heat resistance was measured at 90°C in vaseline oil using 10 µl microhematocrit capillary tubes which ensure uniform heat transmission (Fernández et al. 1999). Capillary tubes were filled with bacterial spore suspensions in sterilized water containing approximately 10^5 10^6 spores ml^{-1}. Spore suspensions were centrally injected into glass capillary tubes with a syringe, and the capillaries were then flame-sealed. Three series of six capillary tubes were filled from each strain assayed. They were submerged in a stirred oil bath (HAAKE DC5) at 90°C constant temperature. Subsequently, tubes were removed at regular intervals and cooled in ice-water, washed in soap solution, rinsed in distilled water, and immersed in ethanol. Spore heat treatments were done in triplicate.

For each strain, the initial spore concentration was determined after heating at 80°C for 10 min. Plate count agar (PCA) was used to recover heated spores. The $\log_{10}$ numbers of surviving spores were plotted against time and D values were calculated from the slope of the linear phase of the spore destruction as the time in minutes needed to decrease the population one $\log_{10}$ cycle (ICMSF 1980). The analysis of variance for D values by strains heated at 90°C and a multiple comparison procedure (Duncan's multiple range test) were performed using SPSS 10.0.

Enterotoxin production

Enterotoxin production was tested in culture filtrates of isolates grown at 30°C in TSB using the *B. cereus* enterotoxin reverse passive latex agglutination (BCET-RPLA) test from Oxoid following the instructions of the manufacturer.

Hydrolysis of starch

To test starch hydrolysis, strains were grown on nutrient agar plates containing (0·2% w/v) soluble starch. After 2 days at 30°C, hydrolysis was revealed by floading plates with Lugol's liquor.

Results and Discussion

B. cereus *counts in fresh vegetables*

Table 1 shows the numbers of *B. cereus* enumerated in different fresh vegetables commonly used to manufacture refrigerated minimally processed foods. The average numbers of *B. cereus* in cucumbers, carrots and peppers ranged from 10^2 to $7{\cdot}8 \times 10^3$ cfu g^{-1}. These levels were sufficient to initiate growth of psychrotrophic *B. cereus* strains at 5 8°C in natural carrot (Valero et al. 2000) and zucchini (Valero et al. 2002) broths. These broths are considered as relatively poor growth media compared to synthetic media and other foods such as meat or milk. This outlines the risk that the *B. cereus* numbers found in the ingredients can pose for the final product. In zucchini and carrots, initial numbers of *B. cereus* were below 10 cfu g^{-1}.

Except in one sample, *B. cereus* could not be isolated from garlic and onions, which contain natural antimicrobial sulfur compounds [allicin (diallyl thiosulfinate; thio-2-propene-1-sulfinic acid-S-allyl ester) and thiopropanal-S-oxide, respectively]. These antimicrobial agents inhibited the growth and toxin production of *B. cereus* (Saleem and Al-Delaimy 1982) and other micro-organisms (Davidson 1997), being also responsible for the flavor of onions and garlic. In addition, onions contain the phenolic compounds, protocatechuic acid and catechol, which could contribute to their antimicrobial activities.

B. cereus *in refrigerated minimally processed vegetable products*

Bacillus cereus was not detected on PMYPA selective medium from samples of the RMPFs. Since the initial numbers of *B. cereus* were very low, the statistical estimate of the MPN procedure was then used for detecting and enumerating *B. cereus* in these products, using selective enrichment broth.

Table 2 shows an estimate of the MPN of *B. cereus* per gram of various RMPFs. All the tubes inoculated with samples of zanaranja did not present contamination and consequently growth of this foodborne pathogen (MPN < 3), while one or two tubes of the three series from each sample of gazpacho, ajoblanco and salmorejo showed *B. cereus* growth.

Biochemical identification and confirmation of B. cereus

Tables 3 and 4 present the parameters of identification obtained using the APILAB system (Choma et al. 2000; Guinebretière et al. 2001),

Table 1. Average *Bacillus cereus* counts obtained in fresh vegetables that are commonly used as ingredients in RMPFs of extended durability

Fresh vegetables	No. of samples	Average counts (cfu g^{-1}) ± s.d.	No. of samples containing *B. cereus*	% of samples containing *B. cereus*
Pepper	11	98·1 ± 25·4 c*	11	100
Cucumber	9	7866·7 ± 1315·3 a	3	33·3
Garlic	6	0	0	0
Tomato	10	8·0 ± 10·3 c	9	90
Onion	7	< 1	1	14·2
Carrot	7	2008·6 ± 902·9 b	3	42·8
Zucchini	6	6·7 ± 8·2 c	2	33·3

*Mean separation between vegetables by Fisher's least significant difference (LSD) procedure, $P \leq 0{\cdot}05$.

Table 2. Numbers of *Bacillus cereus* in RMPFs based on vegetables

RMPFs	No. of samples	MPN (g^{-1}) ±s.d.	No. of samples containing *B. cereus*	% of samples containing *B. cereus*
Gazpacho[a]	9	3·5±0·5	2	22·2
Salmorejo[b]	9	8·0±1·0	4	44·4
Ajoblanco[c]	4	3·5±0·5	1	25·0
Zanaranja[d]	7	<3	0	0

[a]Mediterranean vegetable cold soup made from fresh vegetables.
[b]Vegetable cold purée containing bread and egg.
[c]Cold cream containing bread, almond, and olive oil as main ingredients.
[d]Carrot and orange juice mixture.

combined with results of oxidase activity, motility and anaerobic growth.

Three types of identification results were obtained after performing the strain profiles on APILAB. A good identification was obtained when one species was proposed with a percentage of identification (proximity to the different taxa of the data base, % Id) superior to 80%, which was the case of strains EPSO (Escuela Politécnica Superior de Orihuela) 1PE, 2PE, 3PE, 4PE, 5PE, 6PE, 7PE, 8PE, 9PE, 10PE, 11CU, 12CU, 13CA, 16GZ, 17SA, 18SA, 19SA, 20TO, 21TO, 22TO, 24TO, 25TO, 26TO, 27TO, 28PE, 30CU, and 37SA. Most of these strains were clearly confirmed to be *B. cereus*, while strain EPSO-13CA was identified as *B. firmus*, and strains EPSO-28PE and 30CU were *B. licheniformis*.

For those profiles where the APILAB system proposed more than one species, usually belonging to one subgroup of *Bacillus*, for example, *B. cereus* group, the identification % is often distributed between those species. This was the case of strains EPSO-23TO, 29ON, 31ZU, 32TO, 33AB and 34ZU. Generally, for *B. cereus* group (*B. cereus*, *B. thuringiensis*, *B. mycoides* and *B. anthracis*) APILAB is not sufficiently discriminative. The four members are closely related, in fact the latter three species should be considered subspecies of *B. cereus* (Ash et al. 1991). Additional tests should be performed to discriminate those species. Motility (Granum 1997) was a fine test to distinguish *B. cereus* from *B. mycoides* and *B. anthracis*.

When the APILAB system proposed few species which are not very similar, the strain profiles remain unidentified. That is the case of strains EPSO-14CA, 15CA and 36GZ. Species of isolates were then determined by additional tests. Anaerobic growth on agarified medium for anaerobes was a fine test to discriminate *B. firmus* from *B. cereus* and a presumptive *B. anthracis* isolate.

In any case, it was useful to confirm the results by the production of mucus and pellicles, and observation of the morphology of endospores. *B. cereus* has large cells and oval endospores which do not swell the sporangium. The production of mucus and pellicles in their cultures and the results of API tests show that the strains EPSO-28PE and 30CU belong to *B. licheniformis* species. In addition to endospore observation, *B. cereus* strains were oxidase negative confirming the accurate API identification of isolates and ruled out the possibility of *B. thuringiensis*.

API system tests have been used to distinguish between *B. anthracis* strains and strains of closely related *Bacillus* species (Logan and Berkeley 1984, Logan et al. 1985), to determine species of different strains of *Bacillus* spp. isolates from clinical blood cultures (Weber et al. 1988), and to confirm presumptive *B. cereus* colonies isolated from pasteurized, low-fat milk, in household refrigerators (Te-Giffel et al. 1997). Also, isolates obtained from broccoli, zucchini, carrot, leek, potato, and split pea purées and confirmed as *B. cereus* by ISO 7932:1993(F) have been characterized by API 50CH and API 20E strips (Carlin et al. 2000, Choma et al. 2000, Guinebretière et al. 2001).

Table 3. Characterization of *Bacillus cereus* isolates from fresh vegetables (PE: pepper, CU: cucumber, CA: carrot, TO: tomato, ON: onion, ZU: zucchini) and RMPFs (GA: gazpacho, SA: salmorejo, AB: ajoblanco) on the basis of 67 both physiological and biochemical properties

Strains isolated from	% Id	API name	Motility	Anaerobic growth	Name	BCET-RPLA test	Starch hydrolysis	D_{90}
Vegetables								
EPSO-1PE	99·7	*B. cereus*				+	+	4·3±0·1 jkl*
2PE	99·7	*B. cereus*				–	–	15·7±0·6 b
3PE	95·0	*B. cereus*				–	–	13·2±0·6 c
4PE	99·7	*B. cereus*				–	–	12·3±0·6 c
5PE	99·8	*B. cereus*				–	–	21·2±2·1 a
6PE	99·7	*B. cereus*				+	+	3·6±0·1 klmn
7PE	99·4	*B. cereus*				+	+	6·9±0·2 f
8PE	99·4	*B. cereus*				+	+	4·5±0·2 ijk
9PE	99·4	*B. cereus*				+	+	2·9±0·1 mno
10PE	99·4	*B. cereus*				+	+	4·4±0·1 ijk
11CU	99·4	*B. cereus*				+	+	4·3±0·2 jkl
12CU	99·3	*B. cereus*				+	+	5·4±0·3 hij
13CA	96·5	*B. firmus*						
14CA	95·0/2·4	*B. firmus/B. cereus*		+	*B. cereus*	–	–	10·8±0·3 d
15CA	95·0/2·4	*B. firmus/B. cereus*		+	*B. cereus*	–	–	12·3±0·4 c
20TO	99·1	*B. cereus*				+	+	3·8±0·1 klm
21TO	93·7	*B. cereus*				+	+	5·6±0·2 ghi
22TO	98·9	*B. cereus*				+	+	2·9±0·3 mno
23TO	99·8	*B. cereus/B. mycoides*	+		*B. cereus*	+	+	3·2±0·1 lmno
24TO	99·1	*B. cereus*				+	+	2·5±0·1 nop
25TO	99·1	*B. cereus*				+	+	1·5±0·1 p
26TO	99·8	*B. cereus*				+	+	2·8±0·1 mno
27TO	99·1	*B. cereus*				+	+	2·1±0·1 op
28PE	99·8	*B. licheniformis*						
29ON	99·8	*B. cereus/B. mycoides*	+		*B. cereus*	+	+	6·6±0·1 fg
30CU	99·8	*B. licheniformis*						
31ZU	98·0	*B. cereus/B. mycoides*	+		*B. cereus*	–	+	3·5±0·1 klmn
32TO	99·7	*B. anthracis / B. cereus*	+		*B. cereus*	–	+	9·0±0·9 e
34ZU	99·8	*B. mycoides/B. cereus*	+		*B. cereus*	+	+	5·1±0·3 ij
RMP foods								
EPSO-16GA	91·9	*B. cereus*				+	+	1·4±0·1 p
17SA	99·8	*B. cereus*				+	+	2·8±0·3 mno
18SA	95·7	*B. cereus*				+	+	5·2±0·3 ij
19SA	98·3	*B. cereus*				+	+	6·4±0·7 fgh
33AB	99·8	*B. cereus/B. mycoides*	+		*B. cereus*	–	+	5·1±0·2 ij
36GA	94·7/3·6	*B. anthracis/B. firmus*		–	*B. firmus*			
37SA	93·4	B. cereus				+	+	5·5±0·2 ghij

*Mean separation between strains by Duncan's multiple range test, $P \leq 0{\cdot}05$.

Heat resistance of B.cereus *spores*

Thirty-two strains of *B. cereus* isolated from the two kinds of vegetable products used were analysed for spore heat resistance. The D_{90} values ranged from 1·4 to 21·2 min (Table 3). D_{90} values ranged from 3 to 6 min were the most common range (14 strains) of heat resistance found. Eight strains had D_{90} values lower than 3 min, three strains showed D_{90} values from 6 to 9 min, and seven were more heat-resistant, with D_{90} values higher than 9 min.

Enterotoxin production and hydrolysis of starch

The 32 isolates characterized as *B. cereus* were tested for diarrheal enterotoxin production: 23 strains (71·9%) were positive and nine strains

(28·1%) were negative with the BCET-RPLA test (Table 3).

About 81·3% of the 32 *B. cereus* strains hydrolysed starch (Table 3). Hundred percent of the 23 enterotoxin-positive strains were able to hydrolyse starch, while only 33·3% of the nine enterotoxin-negative strains hydrolysed starch.

Conclusions and Recommendations

The *B. cereus* counts (10^2 $7{\cdot}8 \times 10^3$ cfu g^{-1}) estimated in some fresh vegetables may indicate a problem for the processed vegetable products industry. This ought to be evaluated and prevented, since *B. cereus* is a food pathogen whose capacity for growth in vegetable broths and purées has been clearly shown. In this sense, the low initial levels of *B. cereus* contamination found in the refrigerated minimally processed products pointed out to us that the industrial procedures applied in order to reduce significantly the presence of this micro-organism in the final product are adequate.

The percentage of presumptive *B. cereus* isolates obtained from different samples analysed varies considerably for each of the fresh vegetables and refrigerated minimally processed products assayed. However, it is worth pointing out the isolation of nine strains of *B. cereus* from 10 samples of tomato (90%) and 11 strains from 11 samples of pepper (100%) (Table 1). A total of 36 presumptive *B. cereus* strains were isolated from the 85 samples of various vegetable products analysed (Tables 1 and 2). According to the API 50CH, API 20E and the supplementary tests (Table 3), 32 of these isolates (88·9%) were biochemically and physiologically confirmed as *B. cereus*, two strains (5·5%) were identified as *B. licheniformis*, and other two strains (5·5%) were identified as *B. firmus*.

A large diversity in heat resistance was observed among the *B. cereus* isolates. The range of heat resistance found here (Table 3) was higher than that reported by Shehata and Collins (1972), Bassen et al. (1989), Arinder et al. (1999) and Choma et al. (2000), but very similar to that found by Dufrenne et al. (1994, 1995). Dufrenne et al. (1994) published D_{90} values ranging from 4·6 to 14 min for *B. cereus* strains isolated fron different sources, and Dufrenne et al. (1995) reported D_{90} values from 2·2 to 9·2 min for spores of 11 strains of *B. cereus* isolated from different food products and food-borne disease outbreaks.

Strains unable to hydrolyse starch were the most heat-resistant, with D_{90} values higher than 10·8 min. It is in agreement with the results obtained by Choma et al. (2000). These strains could be *B. cereus* strains producing emetic toxin which are usually unable to degrade starch (Kramer and Gilbert 1989, Agata et al. 1996). Diarrheal enterotoxin was detected in the culture supernatant fluid of 71·9% (23 strains) of the isolates, using immunochemical assays. All the enterotoxin-positive strains were able to hydrolyse starch. The ability of *B. cereus* strains to grow in vegetable broths over the temperature range of 5 16°C, as well as the influence of factors such as sodium chloride (NaCl), pH, and naturally occurring antimicrobials on bacterial growth is currently being determined.

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Survival, isolation and characterization of a psychrotrophic *Bacillus cereus* strain from a mayonnaise-based ready-to-eat vegetable salad

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Abstract

Incidence and population levels of *Bacillus cereus* in American salad, an industrially manufactured, packaged and refrigerated deli salad containing vegetables and mustard, were determined. Of 12 ready-to-eat samples examined, one (8.3%) was positive for *B. cereus* at less than 5×10^3 cfu g^{-1}. According to the ISO confirmation procedure, a strain was isolated and further characterized and identified as *B. cereus* EPSO-35AS by API 50CH/20E phenotypic system, combined with additional tests of motility, oxidase activity and anaerobic growth. This strain produced diarrhoeal enterotoxin in tryptic soy broth culture as detected by BCET-RPLA test, hydrolysed starch and had a low D_{90}-value (2.1 min), with an estimated z-value of 6.79 °C. After a lengthy lag phase (9–12 days of incubation), the strain was able to grow at 8 °C in both nutrient broth and tyndallized carrot broth with specific growth rates from 0.009 to 0.037 h^{-1}, respectively. In the vegetable substrate, lag time was approximately 3 days (66 h) shorter than in laboratory medium. The effect of temperature abuses on the safety of the product during the time of use or consumption is discussed.

Keywords: Psychrotrophic *Bacillus cereus*; Enterotoxin; American salad; Deli salads; Ready-to-eat salads; API system tests

1. Introduction

Raw vegetables have become very popular among health-conscious consumers in recent years. They provide a great variety of fiber contents, vitamins, minerals and others phytochemicals, which are important in human health. Demand for fresh, minimally processed vegetables has led to an increase in the quantity and variety of ready-to-eat or ready-to-use products available to the consumer. These products constitute a suitable meal for today's lifestyles because they need no preparation. Minimally processed fresh (MPF) vegetables may be simply trimmed vegetables or may consist of trimmed, peeled, sliced/shredded and washed and/or disinfected vegetables (Francis et al., 1999). Modified atmosphere packaging (MAP), in combination with refrigeration, is employed as a mild preservation technique to ensure quality and storage-life. Moreover, products based on vegetables account for an important part of Refrigerated Processed Foods of Extended Durability, REPFEDs (also known as cooked chilled foods; Choma et al., 2000), which are thermally processed products of high organoleptic quality. The vegetables may be also combined with cooked vegetables, meats, sea-foods and pasta, and kept in dressing or preserved with the use of chemical additives (stabilizers and preservatives). The products are packaged and stored at refrigeration temperatures.

Bacillus cereus is a spore-forming pathogenic bacterium widespread in nature because of the resistance of its endospores to various stresses and their long-term survival. From this natural environment, it is easily spread to many types of foods, especially of plant origin (Roberts et al., 1982). It has been frequently isolated from vegetable sprouts (Harmon et al., 1987; Kim et al., 2004), intact vegetables (Kaneko et al., 1999; Valero et al., 2002), MPF vegetables (King et al., 1991; Kaneko et al., 1999) and REPFEDs based on vegetables (Carlin et al., 2000; Choma

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et al., 2000; Valero et al., 2002; Guinebretière et al., 2003). Nowadays, heat-resistant psychrotrophs are considered a recurring problem in the refrigeration storage and distribution of foods. This group of microorganisms include psychrotrophic spore-forming *B. cereus* strains which are common contaminants of raw and pasteurized milk and dairy products, infant foods and dried milk products, pasteurized dried/liquid egg yolk, cooked rice, and vegetable REPFEDs (van Netten et al., 1990; Baker and Griffiths, 1993; Becker et al., 1994; Dufrenne et al., 1995; Jaquette and Beuchat, 1998; Carlin et al., 2000; Choma et al., 2000).

The psychrotrophic *B. cereus* strain INRA TZ415, isolated from REPFEDs containing vegetables in the Station de Technologie des Produits Végétaux (Institut Nacional de la Recherche Agronomique, Avignon, France) is able to grow at 7 °C in J-broth (Carlin, personal communication) as well as at 5 and 8 °C in nutrient broth (Valero et al., 2000). Its spores have the ability to germinate and grow at refrigeration temperatures (≤8 °C) in different vegetable substrates, such as tyndallized carrot broth, tyndallized zucchini broth, and cooked carrot purée (Valero et al., 2000, 2003). In these and subsequent studies (Valero and Salmerón, 2003; Valero and Francés, 2006), we also reported the influence of pH and natural antimicrobial compounds (plant essential oils and some of their isolated constituents) in combination with refrigeration temperatures on its kinetics of growth from activated spores in order to develop hurdle technology for use in vegetable-based REPFEDs preservation. This communication documents the detection, enumeration, isolation, confirmation, biochemical and physiological identification and characterization of other psychrotrophic *B. cereus* strain from a vegetable deli salad.

2. Materials and methods

2.1. Commercial sources and composition of deli salad

Twelve tamper containers (transparent polypropylene containers with a tamper-proof lid) containing 250 g of American salad (AS) were purchased from three retail supermarkets located in Orihuela (four from each one). American salad was industrially manufactured in a processing factory located in Navarra, Spain. This product consisted of vegetables (40% white cabbage, 15% carrot, 12% onion), condiments (mayonnaise and mustard), stabilizers (guar and xanthan gums), and preservatives (potassium sorbate and sodium benzoate). The ingredients of mayonnaise included pasteurized egg yolk, vegetable oil, water, vinegar, sugar and salt. The salads have a minimal shelf life under refrigeration temperatures (2 5 °C) of 30 days. Five-days-old products were transported to the laboratory immediately in isothermal polystyrene containers and samples were then analysed. The pH of the 12 samples was determined by duplicate measurements using a puncture Xerolyt electrode and a micropH 2001 pH meter (Crison Instruments S.A., Alella, Barcelona, Spain).

2.2. Enumeration and isolation

The Most Probable Number (MPN) technique was used for the determination of the number of viable *B. cereus* in the salad because it is the best way to enumerate samples with small populations (less than 10 cells per gram or millilitre). Ten grams of salad were homogenized for 1 min with 90 ml of sterile 0.1% (w/v) buffered peptone water (PW; Scharlau Chemie, S.A., Barcelona, Spain) in a stomacher '400' bag (Seward Medical, London, UK), using a Masticator Silver (IUL, S.A., Barcelona, Spain), and then two serial decimal dilutions 1:100 and 1:1000 were performed from the resulting 1:10 diluted homogenate. Three series of three tubes containing 9 ml of modified PMYP (polymyxin-mannitol-egg yolk-phenol red) selective enrichment broth without mannitol, egg yolk and indicator (Guinebretière et al., 2003) were prepared. Each series was inoculated with 1 ml per tube from the respective dilution. The tubes were incubated at 30 °C for 24 h. The transfer of the positive tube-cultures to selective solid medium PMYPA (Mossel et al., 1967; CeNAN, 1982) from Scharlau Chemie made possible to differentiate characteristics of the *B. cereus* colonies.

When the MPN of microorganisms estimated per gram of sample was high, new dilutions were prepared from the original sample stored at 2 °C and enumeration was performed again by spread plating of 0.1 ml portions on duplicate PMYPA plates. The cultures were incubated at 30 °C for 24 h. Colonies presenting a pink or purple colour with an irregular edge surrounded by a white area were considered as positives and enumerated as described by Valero et al. (2002).

From each positive sample, at least five colonies grown on PMYPA were subcultured on the same medium and confirmed subsequently as *B. cereus* by glucose fermentation, Voges Proskauer (VP) reaction and nitrate reduction, as specified in the international norm ISO 7932:1993(F) (AFNOR, 1996). They were then streaked in tryptone soya agar (TSA; Oxoid Ltd., Basingstoke, Hampshire, England) slants and stored at 4 °C as pure cultures.

2.3. Spore production and storage of isolated strain

Volumes of 0.2 ml from stationary *B. cereus* cultures grown overnight at 30 °C in nutrient broth (NB; pH: 7.4 approximately; Scharlau Chemie, S.A.) were dispensed on plates containing fortified nutrient agar (FNA) slightly modified as described by Fernández et al. (1999) and incubated at 30 °C for 4 days. Spores were harvested by flooding the agar surface with 10 ml of sterile distilled water and removing them with a sterile bent glass rod, decanting the spore suspension in sterile tubes and later centrifugation at 2000 *g* for 30 min. The supernatant fluid was discarded and the resulting pellet was resuspended in 5 ml

of sterile distilled water, stored at 4 °C and used as inoculum in subsequent experiments.

2.4. Identification and characterization of B. cereus

Computer-assisted bacterial identification with APILAB Plus software V3.2.2 Version B 01.93 (bioMérieux, Marcy l'Etoile, France) was used to identify isolates as *B. cereus*. Identification was based on the analysis of 61 biochemical tests of the API 50CH and API 20E strips (bioMérieux). Inocula were prepared as we described in an earlier study (Valero et al., 2002), using 18-h-old cultures grown on J-agar (Gordon et al., 1973; Claus and Berkeley, 1986) and incubated at 30 °C. The API 50CH and API 20E galleries were filled, incubated and interpreted according to the instructions specified for each test.

Oxidase activity on filter paper, motility in a semi-solid medium and anaerobic growth in an agarified medium for anaerobes with neither glucose nor indicator was also tested as described by Sarrías et al. (2002). Enterotoxin production was tested in culture filtrates of isolates grown in tryptic soy broth (TSB; Scharlau Chemie, S.A.) at 30 °C using the *B. cereus* enterotoxin reverse passive latex agglutination (BCET-RPLA) test from Oxoid, following the instructions of the manufacturer. To test starch hydrolysis, isolates were grown on nutrient agar (NA; Scharlau Chemie, S.A.) containing 0.2% (w/v) soluble starch, and after incubation for 2 days at 30 °C, hydrolysis was revealed by depositing 5 ml of iodine solution on the surface of plates bearing 5 20 colonies.

2.5. Growth temperatures

Thirty-three *B. cereus* strains were grown at 5, 8, 10, 12, 30, 37, and 42 °C in 20-ml tubes containing 10 ml TSB. Thirty-two strains belong to our own collection isolated from fresh vegetables and REPFEDs (Valero et al., 2002), and the remaining strain was isolated from a sample of American salad during this study. Cultures were observed for visible growth after 1, 3, and 5 days at temperatures of 30, 37, and 42 °C, and after 3, 5, 7, 14, 21, 28, and 56 days at temperatures between 5 and 12 °C. Positive cultures were checked for purity by streaking with an inoculating loop on PMYPA plates and microscopic examination of spore production and morphology after incubation at 30 °C for 4 days.

2.6. Behaviour of isolated B. cereus strain from American salad in this food

Inoculation experiments were performed in the vegetable deli salad. Eighteen tamper containers containing 250 g of American salad were aseptically injected using Myjector sterile syringes (Terumo Europe N.V., Leuven, Belgium). For inoculation, 0.1 ml of the aqueous spore suspension stored at 4 °C was diluted with 9.9 ml of sterile distilled water and heated at 80 °C for 10 min. The heat-activated spore suspension was then diluted in sterile distilled water to a concentration of 10^6 spores ml^{-1}, and 1 ml of this dilution was inoculated into each tamper container to obtain a contamination level of 4×10^3 spores g^{-1}, approximately. Inoculated tamper containers were incubated at abuse temperature (12, 16, and 25 °C). Two containers were sampled at regular intervals (7, 14, and 21 days) to enumerate *B. cereus* on PMYPA plates. Viability of the heat-shocked spore suspension was estimated by spread-plating on plate count agar (PCA; Scharlau Chemie, S.A.).

2.7. Growth kinetics of B. cereus strain isolated from American salad

Growth curves were obtained in both NB and tyndallized carrot broth (TCB, pH: 6.2) prepared as described by Valero and Giner (2006). The broths were dispensed in 100 ml volumes into 250-ml Erlenmeyer flask before steam sterilization with an autoclave or tyndallization by heating at 80 °C for 1 h on 3 consecutive days. Once sterilized, the flasks were stored overnight at 8 °C. Inoculation was by depositing 1 ml of the heat-activated spore suspension diluted previously in sterile distilled water to a concentration of 10^4 spores ml^{-1} into 100 ml NB and TCB to obtain final numbers of 10^2 spores ml^{-1}, approximately. Inoculated broths were incubated at 8 °C in a refrigerated incubator without shaking.

Initial samples were taken at 0 h, and subsequent samples (1 ml) were removed from each culture at appropriate time intervals, using a sterile sampling system similar to that described by Sutherland et al. (1996). Dilutions were made in PW and 0.1 ml samples were spread on PCA for enumeration of *B. cereus*. Duplicate growth curves for each substrate were obtained. Before inoculation, and at the end of the exponential phase of growth, 5 10 ml portions of broth from each flask were taken and their pHs were determined.

2.8. Modelling of growth curves and kinetic parameters

A program implemented in Microsoft Excel (D-model; Institute of Food Research, Norwich, UK) was used to fit the Baranyi equation (Baranyi et al., 1993) to the growth data. Kinetic parameters, including specific growth rate, generation time or doubling time, lag time, $\log_{10}$ maximum population density, and the coefficient of determination (R^2) for each fitted growth curve were calculated. All growth curves were constructed from at least 10 data points.

2.9. Spore heat resistance: D_T and z-values

Heat treatments of bacterial spore suspensions were performed in 10 µl disposable glass micro-pipettes with ring mark (Hirschmann Laborgerate, Eberstadt, Germany) which ensure uniform heat transmission. Micro-pipettes were filled with bacterial spore suspensions in sterile

distilled water containing approximately 10^5 10^6 spores ml^{-1}. These were centrally injected into the capillaries as described by Valero et al. (2002). Three series of 6 10 capillaries were filled for each treatment temperature. They were submerged in a stirred oil bath with immersion circulator HAAKE DC5 (Gebrüder HAAKE GmbH, Karlsruhe, Germany) at 80, 85, 87.5, 90, or 95 °C constant temperature. Capillaries were removed at intervals and cooled in ice-water, washed in soap solution, rinsed in distilled water, and immersed in ethanol. Spores surviving heat treatments were recovered by incubation on PCA at 30 °C for 24 h. All experiments were performed in triplicate.

The $\log_{10}$ numbers of surviving spores were plotted against time and the decimal reduction times (D_T-values) were calculated from the slope of the linear phase of the spore destruction as the time in minutes needed to decrease the population one $\log_{10}$ cycle (ICMSF, 1980). z-Values were determined as the increase in temperature required to reduce the D_T-value by one $\log_{10}$ cycle when $\log_{10}$ D_T was plotted against temperature (Valero et al., 2006). Correlation coefficients (r_o) were calculated by a program implemented in Microsoft Excel [TerModel, Escuela Politécnica Superior de Orihuela (EPSO), Alicante, Spain].

3. Results

3.1. Enumeration of B. cereus in samples of American salad

Except in the tubes inoculated from a specific food sample, *B. cereus* growth was not detected in modified PMYP selective enrichment broth. Taking as positive all the tubes where there was turbidity, a 3:3:3 combination of positive tubes was observed and a MPN > 2400 of viable microorganisms per gram contained in that original sample was calculated, according to the probability table (Pascual Anderson, 1992) with a 95% confidence interval. *B. cereus* counts, ranging from 2.2 to 4.5×10^3 cfu g^{-1}, were found when the spread-plating technique on PMYPA was applied.

3.2. Identification and characterization of isolates

Five separate colonies with the mannitol negative and lecithinase positive characteristics, shape and appearance of *B. cereus* were randomly selected and isolated from PMYPA plates. They were all Gram-positive, catalase positive, produced oval spores located centrally in a non-swollen sporangium and grew under anaerobic conditions. Since all isolates were further confirmed as *B. cereus* by ISO 7932 procedure, only one were selected and identified by API 50CH/20E phenotypic system using APILAB Plus software. It belonged (98.7% Id) to the API-profile intermediate between *B. cereus* 1 and *B. mycoides* that belongs to the *B. cereus* group. The isolates were all motile; therefore, 100% of the isolates was regarded as *B. cereus* because motility is a test to discriminate *B. mycoides* and *B. anthracis* from *B. cereus*. Also, all isolates were oxidase negative confirming the identification of isolates as *B. cereus* and ruled out the possibility of *B. thuringiensis*.

Subsequently, the isolate characterized by API System tests labelled as *B. cereus* EPSO (Escuela Politécnica Superior de Orihuela)-35AS strain, was tested for its ability to hydrolyse starch and to produce diarrhoeal enterotoxin. The strain hydrolysed starch and produced enterotoxin in TSB culture at 30 °C as detected by BCET-RPLA test from Oxoid.

3.3. Growth temperatures

None of the 33 *B. cereus* strains isolated from different kinds of food products was able to grow at 5 °C; 3% (1) grew at 8 °C and 87.9% (29) at 10 °C. All strains grew at 12, 30, and 37 °C; 57.6% (19) grew at 42 °C (Table 1). Most of the strains belonged to this latter group was unable to grow at 10 °C or to grow rapidly at this temperature (4 + 8 = 12 strains), developing a turbid culture within 3 or 7 days. The strain EPSO-35AS isolated and characterized throughout this study was able to grow in TSB after 21 28 days at 8 °C but did not grow at 42 °C.

3.4. B. cereus growth in American salad: inoculation study

Although the level of contamination (ca. 4×10^3 spores g^{-1}) was similar to that found in an anomalous sample, no growth occurred in the food within 21 days at all of the tested abuse temperatures of storage. Inability of American salad to support the growth of *B. cereus* strain EPSO-35AS may be attributed to either the acid pH (4.42 ± 0.06) of the product or the action of preservatives used in the formulation.

Table 1
B. cereus strains isolated from fresh vegetables, REPFEDs, and American salad (AS), which grew at temperatures ranging from 8 to 42 °C

Growth observed at temperatures of (°C)	Strains	Total
8, 10, 12, 30, 37	EPSO-35AS	1
10, 12, 30, 37	EPSO-1PE[a], 9PE, 16GZ[b], 17SA, 18SA, 20TO, 21TO, 22TO, 24TO, 25TO, 26TO, 29ON, and 34ZU	13
10, 12, 30, 37, 42	EPSO-2PE, 5PE, 6PE, 7PE, 8PE, 10PE, 11CU, 12CU, 14CA, 15CA, 19SA, 31ZU, 32TO, 33AB, and 37SA	15
12, 30, 37, 42	EPSO-3PE, 4PE, 23TO, and 27TO	4

[a] Strains isolated from fresh peppers (PE), tomatoes (TO), onions (ON), zucchinis (ZU), cucumbers (CU), and carrots (CA) by Valero et al. (2002).
[b] Strains isolated from REPFEDs such as gazpacho (GZ), salmorejo (SA), and ajoblanco (AB) by Valero et al. (2002).

3.5. Growth of B. cereus at low temperature

The growth kinetics of germinated cells from activated spores of the *B. cereus* strain EPSO-35AS in both NB and TCB were evaluated at 8 °C. After a lengthy lag phase (9 12 days of incubation), the strain was able to grow in both media with specific growth rates from 0.009 to 0.037 h^{-1}, equivalent to generation times of 19 77 h. Nevertheless, germinated spores of *B. cereus* in NB exhibited a longer lag phase (290 vs. 224 h) and a slower generation time than in TCB, reaching a lower final population density (6.59 vs. 7.47 $\log_{10}$ cfu ml^{-1}). The high values calculated for R^2 (0.993 and 0.996) suggest a good fit of the Baranyi equation to the growth data in both media. Fig. 1 shows the means of the population values obtained in duplicate broths prepared for each experiment and the fitted growth curves of the *B. cereus* strain EPSO-35AS.

No significant changes in pH were observed as a result of *B. cereus* growth; the pH values of broths remained close to 7.3 and 6.1, respectively.

3.6. Heat resistance of spores

Spores of the *B. cereus* strain EPSO-35AS suspended in distilled water were tested for heat resistance over the temperature range 80 95 °C. Fig. 2 shows survival curves of spores heated at 80, 85, 87.5, 90, or 95 °C. The plots of log survivors vs. heating time at each temperature yielded straight lines, none of them presented shoulders and tails. The graphs were defined by no less than six points, which are the means of the surviving spore counts, expressed as log cfu ml^{-1}, obtained in three separate experiments. They covered at least three log reduction cycles, except for the lower heating temperature (1.5 log cycles), with correlation coefficients $r_o > 0.99$. The mean D_T-values ± S.D. determined for spores are listed in Table 2. D_T-values markedly diminished with the heating temperature.

Fig. 3 illustrates the linear regression of thermal death time curve for spores of the *B. cereus* strain EPSO-35AS.

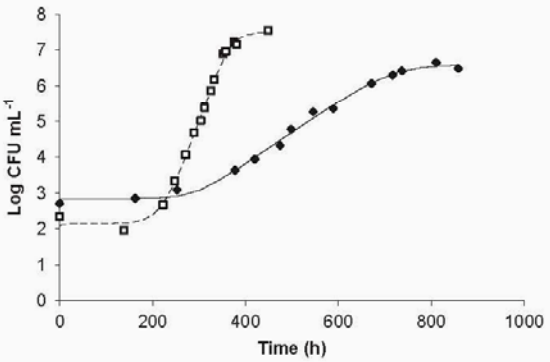

Fig. 1. Means of the population values obtained in duplicate broths and fitted growth curves of *B. cereus* strain EPSO-35AS at 8 °C in both (♦) NB and (□) TCB.

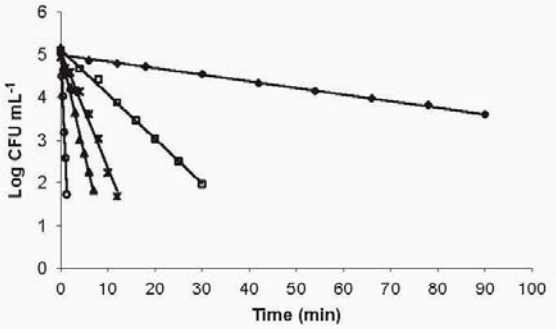

Fig. 2. Survival curves of *B. cereus* EPSO-35AS spores suspended in distilled water and heated at: (♦) 80, (□) 85, (*) 87.5, (▲) 90, and (○) 95 °C. Each point is the mean of the surviving spore counts obtained in three separate experiments.

Table 2
Mean *D*-values (min) of three experiments ± S.D. obtained for spores of *B. cereus* EPSO-35AS strain suspended in distilled water and heated at different temperatures

Temperature (°C)	*D*-value (min)	Correlation coefficient (r_o)
80.0	64.848 ± 0.879	0.999
85.0	9.529 ± 0.239	0.991
87.5	3.696 ± 0.066	0.993
90.0	2.115 ± 0.060	0.986
95.0	0.375 ± 0.002	0.996

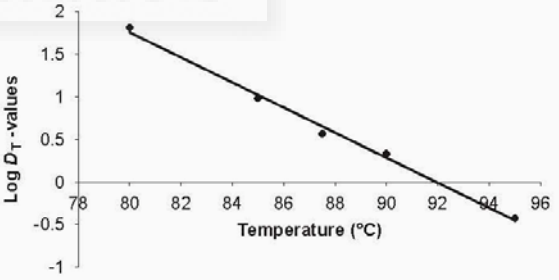

Fig. 3. Least-squares regression analysis of the thermal death time curve obtained for *B. cereus* EPSO-35AS spores suspended in distilled water.

The *z*-value obtained in the temperature range studied was 6.79 °C, with a high regression coefficient (0.997).

4. Discussion

B. cereus was detected in 8.3% (1 of 12) of packaged and refrigerated American salad samples at less than 5×10^3 cfu g^{-1}. This contamination level is between 20- and 500-fold higher than those reported by Carlin et al. (2000), Choma et al. (2000), Valero et al. (2002) and Guinebretière et al. (2003) in REPFEDs containing

vegetables. Numbers of *B. cereus* were also superior to those found by Kaneko et al. (1999) and Edgar and Aidoo (2001) in MPF vegetables, but very similar to those published by Valero et al. (2002) in intact carrots and cucumbers.

Possible primary sources of *B. cereus* contamination are the vegetables used for processing and the ingredients blended with them to obtain the final product. High numbers (3×10^3 cfu g^{-1}) of *B. cereus* have been found in intact carrots whereas its populations ranged from undetectable in most samples of onions to low (10^2 cfu g^{-1}) in marketed cabbages (Ueda et al., 1991; Valero et al., 2002; Hendriksen and Hansen, 2006). On the other hand, among the foods most frequently found positive for *B. cereus* are spices and pasteurized egg yolk (van Netten et al., 1990). Since mustard and pasteurized egg yolk are components of the American sauce, they may significantly contribute to the final contamination of the deli salad; however, undetectable *B. cereus* populations in mustard have been reported (Andress et al., 2001). Carrots and pasteurized egg yolk would therefore be the most probable sources of contamination for American salad because secondary sources could be effectively avoided by the processors, as observed by Guinebretière et al. (2003).

There are two types of *B. cereus* foodborne illness. The first type is caused by an emetic toxin that causes vomiting, while the second type that can be caused by more than one enterotoxin, causes diarrhoea. In Europe and North America, the diarrhoeal type is most frequently reported, with infective doses ranging from about 10^5 10^8 viable cells or spores per gram in part because of the large differences in the amounts of enterotoxin produced by different strains (Granum, 1994). The isolated *B. cereus* EPSO-35AS strain has the ability to produce enterotoxin detected by the Oxoid BCET-RPLA assay kit and it was present in the vegetable deli salad at numbers 10-fold lower than the calculated infective doses (10^4 10^5 cells g^{-1}) for two outbreaks which took place in the 1990s in Norway associated with eating stew (Granum, 1997).

In this study, survival but not growth of *B. cereus* was detected in all of the inoculated salad samples stored at mild, moderate and severe abuse temperatures (12, 16, and 25 °C) during the shelf-life of the product. These findings show that the isolated strain is not able to grow in the food within this period and exclude the hypothetical bacterial proliferation derived from a possible interruption somewhere in the chilled supply chain. Hence, the counts of *B. cereus* found in a specific non-inoculated food sample may suggest an unusually and unknown contamination source.

American salad contains in her formulation E202 as preservative. Potassium sorbate (0.2%, w/v) has been found to be inhibitory to growth of *B. cereus* in association with pH values <6.7 by Thomas et al. (1993). Del Torre et al. (2001) also reported that the use of sorbic acid combined with acidulants (citric or lactic acid) to pH 5.0 was effective in inhibiting the outgrowth of *B. cereus* spores in gnocchi even when temperature abuse occurs (12 and 20 °C). In agreement with these reports and since the pH of American salad is more stressful than both aforementioned pH-values, the absence of bacterial growth in the temperature-abused samples could confirm the effectiveness of potassium sorbate to prevent growth of *B. cereus* under acidic conditions. It is worth noting that in the light of our results American salad could not be considered as potential vehicle for *B. cereus* foodborne disease.

Moreover, in laboratory conditions, we showed that the isolated *B. cereus* EPSO-35AS strain was able to grow at 8 °C but did not grow at refrigeration temperature (5 °C). At 8 °C, the strain grew in TCB in shorter time than in NB. By comparison with the reference medium, the lag time decreased by 3 days (66 h) approximately. This evidence, together with others obtained in earlier studies (Valero et al., 2000, 2003), appears to indicate a protecting and stimulating effect of vegetable substrate on *B. cereus* growth, when the broths were incubated at low temperature.

Although the relation between heat resistance of spores and minimum growth temperature is not certain, it has been reported that psychrotrophic *B. cereus* strains are more sensitive to heat than mesophilic strains (Choma et al., 2000). D_{90}-values reported for psychrotrophic strains have ranged from 0.8 to >100 min, the strain LWL90 being the most heat-resistant (Dufrenne et al., 1995; Choma et al., 2000). The D_{90}-value found for *B. cereus* EPSO-35AS strain (2.1 min) is in the low extreme of this range, however, it is close to values reported for mesophilic strains (Choma et al., 2000; Valero et al., 2002), providing information about the apparent nature of the differences in heat sensitivity among both mesophilic and psychrotrophic strains.

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Effective chemical control of psychrotrophic *Bacillus cereus* EPSO-35AS and INRA TZ415 spore outgrowth in carrot broth

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Abstract

The growth kinetic parameters of germinated cells from heat-activated spores of the psychrotrophic *Bacillus cereus* EPSO-35AS strain in nutrient broth (NB) and in tyndallized carrot broth (TCB) were evaluated at different temperatures (8, 12, and 16 °C) for control samples and for samples acidified with citric acid or lemon juice at pH values between 4.7 and 5.5. Lowering the pH from 7.4 or 6.2 to 5.2 inhibited bacterial growth in both tested media after 60 days at 12 °C and lower temperatures, confirming the effectiveness of acidification in association with refrigeration to control *B. cereus* proliferation in minimally processed foods (MPFs) based on carrot. The activities of selected concentrations of cinnamon essential oil, cinnamaldehyde, carvacrol, and eugenol against *B. cereus* EPSO-35AS and INRA TZ415 strains in both media over the same temperature range were also studied. Addition of either cinnamon essential oil or cinnamaldehyde at concentrations of 5 and 2 μL 100 mL^{-1}, respectively, caused complete inhibition of the growth of both psychrotrophic strains even if mild temperature abuse occurred (12 °C). Hence, a combination of one of these compounds and refrigerated storage may be useful for preservation of MPFs in which major ingredient was carrot. On the contrary, carvacrol and eugenol were not able to prevent *B. cereus* growth in TCB during storage at 8 °C. Their effects on the organoleptic characteristics of TCB are discussed.

Keywords: *Bacillus cereus*; Hurdle technology; Food preservatives; Essential oil components; Minimally processed foods

1. Introductioqn

Foods often provide an ideal environment for microbial survival and growth. So, the development of food preservation processes has been driven by the need to extend their shelf life. Several food preservation systems can be used to reduce the risk of outbreaks of food poisoning; however, these techniques can negatively modify some food properties such as flavor, color, or nutritional value (Kimbal, 1999; van Calenberg et al., 1999; Zenker et al., 2003; Valero and Giner, 2006).

'Minimal processing' is a concept describing approaches to food preservation and safety that are designed to retain the natural and as-fresh properties of foods (Manvell, 1997). The 'hurdle technology' is a minimal processing technique that exploits interactions between traditional preservation systems like heat treatments, low temperature, acidification, oxidation–reduction potential, water activity or availability (a_w), and the addition of preservatives. The antimicrobial effect of preservation methods can be optimized by combining them as 'hurdles' in an overall preservation strategy. According to the hurdle concept, the combination of two or more preservation treatments at lower individual intensities minimizes their impact on sensory and nutritive properties of the food (Leistner, 1999, 2000).

Bacillus cereus and *Clostridium botulinum* have been identified as one of the main potential hazards in vegetable-based cooked chilled foods (Carlin et al., 2000), which account for an important part of Refrigerated Processed Foods of Extended Durability (REPFEDs). As carrot is a low-acid vegetable widely used as ingredient in these foods, a tyndallized carrot broth (TCB) is used for us in a routine way as a model for minimally processed foods (MPFs) based on vegetables because it supports good microbial

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proliferation, and no inhibitory factors influencing growth of *B. cereus* have been detected (Valero et al., 2000). Spores of the psychrotrophic *B. cereus* strain INRA TZ415 are able to germinate and grow at 5 °C in this vegetable substrate (Valero et al., 2000). We also reported either the influence of pH or natural antimicrobial compounds in combination with low temperatures on its kinetics of growth in order to develop hurdle technology for use in vegetable-based REPFEDs preservation (Valero et al., 2000, 2003; Valero and Salmerón, 2003; Valero and Francés, 2006).

Recently, other psychrotrophic *B. cereus* strain EPSO-35AS was isolated from a mayonnaise-based ready-to-eat vegetable deli salad and further characterized on the basis of 66 biochemical and physiological tests (Valero et al., 2007). The aim of this study was to evaluate the ability of heat-activated spores of strain EPSO-35AS to grow over the temperature range of 8 16 °C in nutrient broth (NB) and TCB at natural and acidified pH. It was also conducted to determine the activities of four naturally occurring antimicrobial compounds (carvacrol, eugenol, cinnamaldehyde, or essential oil of cinnamon) on the kinetics of growth of germinated cells from spores in both media. Finally, findings were compared with those obtained previously for the strain INRA TZ415.

2. Material and methods

2.1. Bacterial strains and spore production

The *B. cereus* strain EPSO-35AS used was isolated from American salad, an industrially manufactured, packaged, and refrigerated deli salad containing vegetables and mustard obtained from a retail supermarket located in Orihuela (Valero et al., 2007). Psychrotrophic, enterotoxigenic *B. cereus* INRA TZ415, isolated from REPFEDs based on vegetables, was kindly provided by Dr. Frédéric Carlin (Station de Technologie des Produits Végétaux, Institut National de la Recherche Agronomique, Avignon, France).

Aliquots of 0.2 mL from stationary *B. cereus* cultures grown overnight at 30 °C in NB (Scharlau Chemie, S.A., Barcelona, Spain) were dispensed on plates containing fortified nutrient agar (FNA) slightly modified as described by Fernández et al. (1999). Spores were harvested, centrifuged, resuspended, and stored until used according to the method described by Valero et al. (2007).

2.2. Preparation of natural carrot broth

After washing and peeling, 500 g lots of carrots (*Daucus carota* L. var. Sativa) were homogenized with 850 mL of distilled water using a Tecator 1094 Homogenizer (Foss Tecator, Höganäs, Sweden). The homogenate was filtered and dispensed in 100 mL volumes into sterile 250-mL Erlenmeyer flasks before tyndallization as described by Valero and Giner (2006). Once tyndallized, the flasks were stored overnight at 8, 12, and 16 °C. The efficiency of heat treatment to eliminate natural microbiota of homogenate was evaluated by spread plating on plate count agar (PCA; Scharlau Chemie, S.A.) at 30 °C for 24 h and *B. cereus* polymyxin manitol egg yolk phenol red selective agar (PMYPA; Scharlau Chemie, S.A.).

2.3. Acidification

Batches of flasks containing 100 mL of NB (pH 7.4) or TCB (pH 6.2) were acidified with either citric acid anhydrous (Panreac Química, S.A., Barcelona, Spain) or natural lemon juice, reaching pH values close to those desired. After autoclaving (121 °C for 15 min) and tyndallization of NB and TCB, respectively, the pH values were tested and adjusted to the following final pH levels: 5.5, 5.4, 5.3, 5.2, 5.1, 5.0, 4.9, 4.8 and 4.7 using 0.1 N HCl and 0.1 N NaOH (Panreac Química, S.A.). The pH of the culture substrates was determined using a micropH 2001 pHmeter (Crison Instruments S.A., Barcelona, Spain).

2.4. Natural antimicrobials

Three isolated oil constituents and one essential oil were used in this study. The chemicals were carvacrol, cinnamaldehyde, and eugenol at concentrations of 5, 2, and 35 μL 100 mL^{-1}, respectively. The essential oil of cinnamon was added to the media at 5 μL 100 mL^{-1}. The antimicrobials were all obtained from Destilerías Muñoz Gálvez, S.A. (Murcia, Spain). The essential oil was considered 100% pure. The purity levels of chemicals were higher than 92% according to the manufacturer. The stated concentrations of the compounds tested were chosen as a result of studies of the influence of naturally occurring compounds on the kinetics of growth of activated *B. cereus* INRA L2104 spores in TCB stored at 16 °C (Valero and Salmerón, 2003; Valero and Giner, 2006). They were added to single flasks containing 100 mL of culture substrate (NB and TCB) that had been stored overnight at the selected (8, 12, and 16 °C) incubation temperature. After addition of the antimicrobial, the flasks were shaken by hand for approximately 10 s.

2.5. Inoculation of culture media

Single flasks were inoculated with 1 mL of a water-diluted heat-activated (80 °C for 10 min) spore suspension to obtain final numbers of 10^2 spores mL^{-1} approximately (Valero and Giner, 2006). Viability of spores was estimated by spread plating on PCA at 30 °C for 24 h.

2.6. Growth kinetics

Growth curves of *B. cereus* EPSO-35AS were obtained in non-acidified and acidified NB and TCB. The broths were incubated in a cooled incubator MIR-153 (Sanyo Electric Co., Ltd., Gunma, Japan) without shaking at 8, 12, and

16 °C. The temperature was recorded and was kept within ±1 °C of the target temperature. At appropriate time intervals, samples (1 mL) of the cultures were removed from each flask representing a particular condition and decimally diluted in 0.1% (w/v) peptone water (Scharlau Chemie, S.A.). Adequate dilutions were spread in duplicate on PCA for enumeration of *B. cereus* colonies after incubation at 30 °C for 24 h (Valero and Giner, 2006). Antimicrobial supplemented flasks containing either EPSO-35AS or INRA TZ415 cultures in NB and TCB were also sampled and enumerated at selected incubation times. At the end of the exponential phase of growth, 5 10 mL aliquots of the cultures were taken for pH testing. Flask were kept incubating for up to 60 days if growth was not detected. The experiments were repeated twice using spores from the same crop stored at 4 °C.

2.7. Modeling of growth curves

The estimated mean $\log_{10}$ CFU mL^{-1} for each condition and interval was plotted against time to generated growth curves. A program implemented in Microsoft Excel [DMFit (DM: dynamic modeling); Institute of Food Research, Norwich, UK] was used to fit the equation of Baranyi et al. (1993) to the mean values of the bacterial numbers obtained from two separate experiments. Kinetic parameters, including specific growth rate (SGR, $\log_{10}$ CFU mL^{-1} h^{-1}), generation time (GT, h), lag time (LT, h), $\log_{10}$ maximum population density ($\log_{10}$ MPD, $\log_{10}$ CFU mL^{-1}), and the coefficient of determination of fit (R^2) for each growth curve were calculated.

3. Results

3.1. Growth of B. cereus EPSO-35AS in NB and in natural carrot-based substrate

The growth kinetics of germinated cells from heat-activated spores of the *B. cereus* strain EPSO-35AS in NB and TCB were evaluated at 8, 12, and 16 °C. Table 1 shows the main growth parameters estimated for this strain under every condition. Growth curves obtained fitted well to the Baranyi equation with R^2 values greater than 0.99. At 16 °C, fast growing was recorded and lag phases shorter than 10 h was observed in NB and TCB. At mild abuse temperature (12 °C), strain EPSO-35AS exhibited a lag phase about 1 day long. Lengthy lag phases as far as 9 and 12 days at refrigerated storage (8 °C) have already been reported by Valero et al. (2007) as a particular characteristic of the strain.

Strain EPSO-35AS grew fast at 16 °C, reaching the stationary phase after 2 3 days of incubation, approximately. The generation time was doubled from 4.81 to 9.36 h in NB and from 3.92 to 8.15 h in TCB when the temperature was reduced at 12 °C. This decrease in temperature also reduced the maximum *B. cereus* populations obtained.

Tyndallization provided a good level of decontamination with <1 CFU mL^{-1} background microorganisms remaining in the carrot broth. No significant changes in pH were observed as result of *B. cereus* growth, being determined reductions of 0.1 0.3 pH units from the initial values adjusted.

3.2. Growth of B. cereus EPSO-35AS in acidified NB and carrot-based substrate (citric acid or lemon juice)

The combined effect of pH (4.7 5.5) and incubation temperature (8, 12, and 16 °C) on the growth of germinated cells from heat-activated spores of the *B. cereus* strain EPSO-35AS was evaluated. At 8 °C, growth was not observed at any pH value tested during at least 60 days of incubation. Nevertheless, bacterial growth was observed at 12 °C when media were acidified at pH 5.3 or higher values with citric acid, although differences between media were found (Table 2). The lowest pH for growth in NB and TCB at 16 °C was 5.0. For this combination of environmental conditions, the lag phase was increased 8.6 9.6-fold and the generation time 2.4 3.1-fold in NB and TCB, respectively, when compared with the same growth kinetic parameters estimated for cultures in unacidified broths (Table 1). In general, it was evident that an increase in pH always produced a reduction in the lag phase duration, as

Table 1
Growth kinetic parameters calculated for germinated cells from heat-activated spores of *B. cereus* EPSO-35AS incubated at different temperatures in NB and TCB

Medium	Temperature (°C)	$\log_{10} N_0$	LT	SGR	GT	$\log_{10}$ MPD	R^2
NB	16	2.697	9.562	0.144	4.81	7.296	0.998
	12	2.392	22.339	0.074	9.36	6.631	0.990
	8	2.845	290.113	0.009	77.00	6.591	0.993
TCB	16	2.984	9.793	0.177	3.92	7.543	0.991
	12	2.884	24.215	0.085	8.15	6.897	0.995
	8	2.140	224.078	0.037	18.73	7.472	0.996

N_0, initial bacterial number (CFU mL^{-1}); LT, lag time (h); SGR, specific growth rate ($\log_{10}$ CFU mL^{-1} h^{-1}); GT, generation time (h); MPD, maximum population density (CFU mL^{-1}); R^2, coefficient of determination for fit of the D-model equation (Baranyi et al., 1993) to the mean values of growth data obtained from two separate experiments.

Table 2
Variable conditions and growth parameters estimated using the Baranyi et al. (1993) function for cultures of *B. cereus* EPSO-35AS grown from heat-activated spores incubated in NB and TCB acidified with citric acid

Medium	Temperature (°C)	pH	$\log_{10} N_0$	LT	SGR	GT	$\log_{10}$ MPD	R^2
NB	16	5.2	2.580	78.350	0.074	9.36	7.128	0.999
		5.1	2.763	80.446	0.065	10.66	7.255	0.994
		5.0	2.592	82.938	0.059	11.74	6.838	0.996
		4.9	2.954	NG	–	–	–	–
		4.8	1.967	NG	–	–	–	–
	12	5.5	2.123	173.488	0.028	24.75	7.539	0.995
		5.4	2.216	343.986	0.014	49.50	7.340	0.992
		5.3	2.125	NG	–	–	–	–
		5.2	2.237	NG	–	–	–	–
	8	5.5	2.422	NG	–	–	–	–
TCB	16	5.2	2.683	70.842	0.065	10.66	7.042	0.992
		5.1	2.650	84.452	0.060	11.55	6.977	0.991
		5.0	2.442	94.058	0.057	12.16	6.663	0.995
		4.9	2.380	NG	–	–	–	–
		4.8	2.320	NG	–	–	–	–
	12	5.5	2.506	76.881	0.044	15.75	6.820	0.997
		5.4	2.377	112.763	0.047	14.74	6.892	0.992
		5.3	2.338	223.725	0.045	15.40	6.904	0.995
		5.2	2.444	NG	–	–	–	–
		5.1	2.101	NG	–	–	–	–
	8	5.5	2.223	NG	–	–	–	–

N_0, initial bacterial number (CFU mL^{-1}); LT, lag time (h); SGR, specific growth rate ($\log_{10}$ CFU mL^{-1} h^{-1}; GT, generation time (h); MPD, maximum population density (CFU mL^{-1}); R^2, coefficient of determination for fit; NG, no growth within 60 days.

well as increases in the calculated SGRs except for TCB cultures at 12 °C, which showed closely values.

When lemon juice was used as acidulant, *B. cereus* EPSO-35AS growth was observed at the same pH values and temperatures with minor differences in the kinetic parameters (Table 3). The fit of the Baranyi function to the growth data was excellent ($R^2 > 0.99$) in all trials and no changes in pH derived from *B. cereus* growth were observed.

3.3. *Effects of antimicrobials on growth of psychrotrophic B. cereus strains EPSO-35AS and INRA TZ415 in NB and in natural carrot-based substrate over the temperature range of 8–16 °C*

3.3.1. *Influence on the kinetics of growth from activated B. cereus EPSO-35AS spores*

The influence of single natural antimicrobials on the kinetics of growth from activated *B. cereus* EPSO-35AS spores in NB and TCB were evaluated at different temperatures. The Baranyi growth parameters derived from the fitted growth curves for this strain in supplemented broths are shown in Table 4. All growth curves fitted well to this function with R^2 values higher than 0.99. At 16 °C, strain EPSO-35AS was able to grow in presence of all antimicrobials, but with delays regarding its behavior in absence of them (Table 1). Increases in lag times ranged from 22% to >800% with 35 μL of eugenol and 5 μL of carvacrol per 100 mL NB, respectively. It implies delays of approximately 2 and 83 h before bacterial multiplication starts. The addition of eugenol to TCB had no effect on lag phase and the generation time was only slightly increased. At 12 °C, no growth occurred in presence of cinnamon essential oil and cinnamaldehyde within 60 days of incubation and inability of carvacrol and eugenol to inhibit the growth of *B. cereus* at the tested concentrations was observed. Nevertheless, the addition of 5 μL of carvacrol per 100 mL of broth prolonged the lag phases by 25 and 96 h in TCB and NB, with generation times of 15.4 and 38.5 h, respectively. Full suppression of growth and a reduction of the viable counts were found after 60 days at 8 °C for all combinations tested.

3.3.2. *Influence on the kinetics of growth from activated B. cereus INRA TZ415 spores*

The antibacterial activity of the aforementioned antimicrobials against the strain INRA TZ415 was studied in order to make a comparison in sensitivity between both psychrotrophic *B. cereus* strains. Growth parameters of INRA TZ415 in NB supplemented with carvacrol, eugenol, cinnamaldehyde or cinnamon oil, as well as in TCB added with eugenol, are shown in Table 5. INRA TZ415 growth data at 8, 12, and 16 °C in TCB added with cinnamon oil (Valero and Salmerón, 2003), cinnamaldehyde or carvacrol (Valero and Francés, 2006) have been published previously. At 16 °C, strain INRA TZ415 was able to grow in

Table 3
Variable conditions and growth parameters estimated using the Baranyi et al. (1993) function for cultures of *B. cereus* EPSO-35AS grown from heat-activated spores incubated in NB and TCB acidified with lemon juice

Medium	Temperature (°C)	pH	$\log_{10} N_0$	LT	SGR	GT	$\log_{10}$ MPD	R^2
NB	16	5.2	2.540	64.653	0.077	9.00	7.127	0.991
		5.1	2.123	72.554	0.063	11.00	6.964	0.993
		5.0	2.664	84.346	0.059	11.74	6.836	0.997
		4.9	2.065	NG	–	–	–	–
		4.8	2.693	NG	–	–	–	–
	12	5.5	2.142	195.834	0.028	24.75	6.442	0.997
		5.4	2.127	341.483	0.016	43.31	6.528	0.998
		5.3	2.598	NG	–	–	–	–
		5.2	2.124	NG	–	–	–	–
	8	5.5	2.441	NG	–	–	–	–
TCB	16	5.2	2.714	69.345	0.055	12.60	7.069	0.996
		5.1	2.137	81.432	0.056	12.37	6.987	0.995
		5.0	2.444	89.110	0.051	13.59	7.188	0.994
		4.9	2.103	NG	–	–	–	–
		4.8	2.049	NG	–	–	–	–
	12	5.5	2.518	83.273	0.044	15.75	6.885	0.998
		5.4	2.428	112.064	0.042	16.50	7.007	0.997
		5.3	2.342	229.282	0.041	16.90	6.903	0.996
		5.2	2.763	NG	–	–	–	–
		5.1	1.967	NG	–	–	–	–
	8	5.5	2.503	NG	–	–	–	–

N_0, initial bacterial number (CFU mL^{-1}); LT, lag time (h); SGR, specific growth rate ($\log_{10}$ CFU mL^{-1} h^{-1}); GT, generation time (h); MPD, maximum population density (CFU mL^{-1}); R^2, coefficient of determination for fit; NG, no growth within 60 days.

presence of all antimicrobials, but with prolonged lag periods compared with those estimated for unsupplemented NB (Valero et al., 2000) and TCB (Valero and Salmerón, 2003) cultures. The addition of antimicrobials induced a generalized increase in the lag phases between 15% and 290%, which implies delays between 1 and 42 h. At 12 °C, no growth after 60 days was observed in presence of cinnamon oil and cinnamaldehyde. On the contrary, carvacrol and eugenol, at the tested concentrations, were not effective in inhibiting the *B. cereus* spore outgrowth at any temperature assayed. The addition of 5 μL of carvacrol per 100 mL of broth prolonged the lag phase by 65 h in NB, while it did by 9 h only in TCB (Valero and Francés, 2006). Eugenol caused slight changes on the kinetic parameters of growth from heat-activated *B. cereus* INRA TZ415 spores even with a decrease in temperature from 12 to 8 °C. Growth of *B. cereus* at refrigeration temperature was also observed in the presence of carvacrol, but it increased the lag phase from 43.6 to 396.7 h in NB and from 109.7 to 123 h in TCB (Valero and Francés, 2006).

4. Discussion

Microbial growth is greatly affected by environmental factors and an understanding of their influences can aid in the control of foodborne microorganisms, providing the development of food preservation strategies (Martínez et al., 2007). For conventional preservation treatments, optimal microbial control is achieved through the hurdle concept, with synergistic effects resulting from different components of the microbial cell being targeted simultaneously (Ross et al., 2003).

This study describes the combined effects of low temperature (16, 12, and 8 °C) and pH on the growth kinetics from heat-activated spores of the psychrotrophic *B. cereus* EPSO-35AS strain in NB and TCB. At low temperatures, a temperature rise increases the growth rate because metabolism is more active at higher temperatures (Prescott et al., 1999). Hence, the microorganism grows faster when the temperature is raised, although beyond a certain point, further increases actually slow growth. The growth parameters estimated for the strain EPSO-35AS in both media (Table 1) were shown to be consistent with this characteristic temperature dependence of microbial growth. Choma et al. (2000) studied a rage of temperature from 5 to 42 °C for the psychrotrophic *B. cereus* strain INRA TZ415. They found that temperature increases slow growth above the optimal growth temperature of 31 °C and complete inhibition of growth at 40 °C was observed.

The internal pH of most microorganisms is close to neutrality. It is not surprising that a drastic variation in environmental pH affects microbial growth promoting stressful changes in the cytoplasmic pH. Although microorganisms often grow over wide ranges of pH, each species has a definite pH growth range and pH growth optimum. *B. cereus* is able to grow at a pH range of 4.3 9.0 (Lund,

Table 4
Variable combinations and kinetic values estimated using the Baranyi et al. (1993) function for cultures of *B. cereus* EPSO-35AS grown from heat-activated spores incubated in NB and TCB containing natural antimicrobials

Medium	Antimicrobial	Concentration (μL 100 mL^{-1})	Temperature (°C)	$\log_{10} N_0$	LT	SGR	GT	$\log_{10}$ MPD	R^2
NB	Cinnamon oil	5	16	2.445	37.864	0.090	7.70	6.962	0.995
			12	2.122	NG	–	–	–	–
			8	2.584	NG	–	–	–	–
	Cinnamaldehyde	2	16	2.180	32.759	0.112	6.19	7.452	0.996
			12	2.103	NG	–	–	–	–
			8	2.503	NG	–	–	–	–
	Carvacrol	5	16	3.687	92.927	0.035	19.8	7.363	0.997
			12	3.650	118.221	0.018	38.5	6.558	0.998
			8	2.441	NG	–	–	–	–
	Eugenol	35	16	3.123	11.668	0.122	5.68	7.260	0.991
			12	3.071	32.718	0.057	12.16	7.121	0.997
			8	2.693	NG	–	–	–	–
TCB	Cinnamon oil	5	16	2.102	14.263	0.080	8.66	7.193	0.994
			12	2.124	NG	–	–	–	–
			8	2.600	NG	–	–	–	–
	Cinnamaldehyde	2	16	2.401	16.189	0.087	7.96	7.265	0.990
			12	2.137	NG	–	–	–	–
			8	2.065	NG	–	–	–	–
	Carvacrol	5	16	2.478	13.504	0.105	6.6	7.304	0.997
			12	2.397	49.168	0.045	15.4	6.332	0.992
			8	1.938	NG	–	–	–	–
	Eugenol	35	16	2.559	9.969	0.135	5.13	7.792	0.997
			12	2.433	24.330	0.055	12.6	6.855	0.991
			8	2.142	NG	–	–	–	–

N_0, initial bacterial number (CFU mL^{-1}); LT, lag time (h); SGR, specific growth rate ($\log_{10}$ CFU mL^{-1} h^{-1}); GT, generation time (h); MPD, maximum population density (CFU mL^{-1}); R^2, coefficient of determination for fit; NG, no growth within 60 days.

Table 5
Variable combinations and kinetic values estimated using the Baranyi et al. (1993) function for cultures of *B. cereus* INRA TZ415 grown from heat-activated spores incubated in NB and TCB containing natural antimicrobials

Medium	Antimicrobial	Concentration (μL 100 mL^{-1})	Temperature (°C)	$\log_{10} N_0$	LT	SGR	GT	$\log_{10}$ MPD	R^2
NB	Cinnamon oil	5	16	2.275	28.634	0.101	6.86	6.777	0.997
			12	2.417	NG	–	–	–	–
			8	2.333	NG	–	–	–	–
	Cinnamaldehyde	2	16	2.172	24.383	0.117	5.92	7.400	0.995
			12	2.319	NG	–	–	–	–
			8	2.428	NG	–	–	–	–
	Carvacrol	5	16	3.701	55.922	0.041	16.90	7.761	0.999
			12	3.673	94.322	0.025	27.72	7.597	0.998
			8	3.614	396.661	0.013	53.31	6.308	0.997
	Eugenol	35	16	2.800	18.257	0.139	4.98	7.437	0.995
			12	2.996	33.630	0.083	8.35	7.174	0.993
			8	2.882	47.561	0.029	23.90	6.801	0.996
TCB	Eugenol	35	16	2.434	10.104	0.160	4.33	7.774	0.999
			12	2.505	26.781	0.137	5.06	7.716	0.992
			8	2.441	112.793	0.033	21.00	7.463	0.999

N_0, initial bacterial number (CFU mL^{-1}); LT, lag time (h); SGR, specific growth rate ($\log_{10}$ CFU mL^{-1} h^{-1}); GT, generation time (h); MPD, maximum population density (CFU mL^{-1}); R^2, coefficient of determination for fit; NG, no growth within 60 days.

1990); however, the minimal pH for its growth varies among strains and also depends on the acidulants because organic acids are more able to permeate the cell membrane than inorganic ones. Despite their acid tolerance, bacterial death occurred by inhibiting the activity of enzymes and membrane transport proteins if the internal pH of vegetative cells drops much below 5.0 5.5 (Prescott et al., 1999).

When combined with low temperature, lowered pH may result in greater inactivation of vegetative bacteria than at neutral pH (Peck, 1997; Valero et al., 2000). Compared to the high population densities reached at the end of the exponential phase of growth in NB and TCB, at pH 7.4 and 6.2 (Table 1), respectively, the simultaneous use of refrigerated storage (8 °C) and mild acidification with citric acid or lemon juice to pH 5.5 inhibited the growth from heat-activated spores (Tables 2 and 3). The minimum pH for initiation of growth of the *B. cereus* strain EPSO-35AS was reduced to pH 5.3 with an increase in temperature from 8 to 12 °C, a temperature slightly outside the guideline conditions recommended for the preservation of cooked chilled foods (ECFF, 1996). In short, acidification at pH 5.2 resulted in no growth of *B. cereus* EPSO-35AS strain in NB and TCB at 12 °C or lower temperatures. This acidic condition is less stressful than that earlier reported for cultures of the psychrotrophic INRA TZ415 strain in TCB and tyndallized zucchini broth (Valero et al., 2000; Valero et al., 2003) acidified to pII 5.0 with lemon juice, but similar to that found for a mixture of *B. cereus* strains INRA TZ415 and INRA L2104 inoculated in carrot purée at pH levels between 5.0 and 5.5 (Valero et al., 2003). Our results as a whole showed that citric acid and lemon juice were effective in inhibiting *B. cereus* growth. In agreement with these findings, Hsiao and Siebert (1999) reported that citric acid inhibited *B. cereus* at pH 5.25 with a 3.68 $g\,L^{-1}$ minimum inhibitory concentration. By contrast, Del Torre et al. (2001) noted that citric acid was not able to prevent growth of *B. cereus* in gnocchi samples acidified to pH 5.0. In this study, only the pH 5.0 was not effective in inhibiting the *B. cereus* EPSO-35AS spore outgrowth when moderate temperature abuse occurred (16 °C).

Since at 12 °C or lower temperatures, equivalent levels of citric acid and lemon juice inhibited *B. cereus* EPSO-35AS growth in both media studied (NB and TCB) at pH 5.2, it is evident that lemon juice can be considered as a more natural alternative to citric acid for food acidification. The combination of refrigeration and mild acidification with lemon juice could be an effective way to control *B. cereus* growth in MPFs based on carrot. In fact, a refrigerated carrot juice with a twist of lemon (pH 5.0) is currently marketable in the European Community (EC). Peck (1997) also indicated that low temperature associated with lowering pH to 5.0 was advisable to assure the safety of REPFEDs regarding non-proteolytic *C. botulinum*. Acidification at pH lower than 5.0 is not recommended, as it could confer a very sour flavor to the food, which could be unacceptable to the consumers (Liem and de Graaf, 2004).

On the other hand, the antibacterial activities of four naturally occurring compounds (carvacrol, eugenol, cinnamaldehyde, or essential oil of cinnamon) on the kinetics of growth of heat-activated spores from the psychrotrophic *B. cereus* strains EPSO-35AS and INRA TZ415, in NB and TCB over the temperature range 8 16 °C, were investigated to provide information on their potential use as an alternative to 'traditional food preservatives'. Addition of cinnamon essential oil or its major component cinnamaldehyde, at concentrations of 5 and 2 $\mu L\,100\,mL^{-1}$ of broth, respectively, caused complete inhibition of the growth of both strains (Tables 4 and 5 and data reported before in Valero and Salmerón, 2003 and in Valero and Francés, 2006) even if mild temperature abuse occurred (12 °C). In agreement with these findings, some essential oils have been found to be inhibitory to germination and vegetative growth of *B. cereus* T spores (Chaibi et al., 1997).

Carvacrol and eugenol were less effective than cinnamon oil and cinnamaldehyde in preventing the *Bacillus* spore outgrowth. These antimicrobials were not able to prevent *B. cereus* INRA TZ415 growth even if refrigeration (8 °C) was used. For these combinations of variables studied, the prolonged lag periods and reduced growth rates estimated were shown to be consistent with the temperature and antimicrobial dependencies. Nevertheless, no growth was observed when carvacrol and eugenol were tested against *B. cereus* EPSO-35AS at low temperature. This result could be attributed to a synergistic effect between refrigeration and antimicrobial on germinated cells from heat-activated spores of *B. cereus* EPSO-35AS. At 8 °C, we have reported differences in the INRA TZ415 and EPSO-35AS behaviors associated with the lag phases and SGRs estimated for NB and TCB cultures (Valero et al., 2000, 2007). Outgrowth of *B. cereus* spores was influenced by carvacrol and eugenol because they might induce cytoplasm membrane damage on vegetative cells (Helander et al., 1998; Ultee et al., 2002; Walsh et al., 2003).

Antimicrobials affected the sensory characteristics of TCB in a different way. At the concentrations used, cinnamon oil and cinnamaldehyde had no negative effect on the taste and smell of TCB, in fact, they enhanced them (Valero and Salmerón, 2003; Valero and Giner, 2006). Hence, a combination of one of these compounds and refrigerated storage may be useful for preservation of MPFs based on carrot. In contrast, the negative sensory effects derived from the addition of carvacrol (Valero and Giner, 2006) and eugenol to TCB together with their poor antibacterial efficiency at the concentrations tested limit their application as preservatives in this type of products. Eugenol, at concentrations $\geqslant 35\,\mu L\,100\,mL^{-1}$, conferred acrid and hot flavor on TCB that was unacceptable by panelists. Therefore, it is important to add concentrations of carvacrol and eugenol as low as possible to minimize the influence on the sensory qualities of TCB. More research is needed to elucidate their antimicrobial activity and sensory effects in combination with other natural antimicrobial agents (Ultee et al., 2000; Pol and Smid, 1999; Periago and

Moezelaar, 2001) and non-thermal technologies (Karatzas et al., 2001; Ross et al., 2003).

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4.- RESULTADOS Y DISCUSIÓN

4.- RESULTADOS y DISCUSIÓN.

4.1.- Aislamiento y caracterización de *Bacillus cereus*.

4.1.1.- Recuentos de B. cereus en vegetales frescos.

La media del número de *B. cereus* en pepino, zanahoria y pimiento osciló desde 10^2 hasta 7,8 x 10^3 UFC/g (Tabla 2). En ajo no se detectó *B. cereus*, mientras que en cebolla el recuento también fue casi nulo, ello podría ser debido a los compuestos naturales sulfurados con propiedades antimicrobianas (allicin (dialil tiosulfinato, tio-2-propanol-1-sulfínico ácido-S-alil éster) y tiopropanol-S-óxido) que contienen estos materiales vegetales. Estos agentes antimicrobianos además inhiben el crecimiento y producción de toxinas de *B. cereus* y otros microorganismos.

Tabla 2. Conteos de *Bacillus cereus* aislados de vegetales frescos comúnmente usados como ingredientes de alimentos mínimamente procesados (RMPFs). Para una misma columna, los valores seguidos de una misma letra no son significativamente diferentes (P ≤ 0,05). SD: desviación estándar.

Vegetales frescos	Nº de muestras	Conteos medios (ufc g^{-1}) ± SD	Nº de muestras que contienen *B. cereus*	% de muestras que contienen *B. cereus*
Pimiento	11	98 ± 25 c	11	100
Pepino	9	7867 ± 1315 a	3	33,3
Ajo	6	0	0	0
Tomate	10	8 ± 10 c	9	90
Cebolla	7	< 1	1	14,2
Zanahoria	7	2009 ± 903 b	3	42,8
Calabacín	6	7 ± 8 c	2	33,3

4.1.2.- B. cereus en productos vegetales refrigerados mínimamente procesados (RMPFs).

Las muestras de zanaranja fueron las únicas en mostrar ausencia de contaminación (Tabla 3). La muestra de ensalada americana mostró la mayor contaminación por *B. cereus*, mayor 2400 microorganismos/g, de acuerdo con las tablas de probabilidad (Pascual, 1992). Estos resultados son similares a los encontrados en los posteriores conteos en cultivo agar fenol rojo de polimixina-manitol-yema de huevo (PMYPA) que oscilaron entre 2,2 y 4,5 x 10^3 UFC/g.

Tabla 3. Conteos de *Bacillus cereus* en productos vegetales refrigerados mínimamente procesados (RMPFs). [a] Sopa refrigerada de vegetales mediterráneos elaborada con vegetales frescos. [b] Puré refrigerado de vegetales que contiene pan y huevos. [c] Crema refrigerada que contiene pan, almendras y aceite de oliva como principales ingredientes. [d] Mezcla de zumos de zanahoria y naranja. [e] E: ensalada, col blanca, remolacha, cebolla, mayonesa y mostaza. SD: desviación estándar.

RMPFs	Nº de muestras	NMP (g^{-1}) ± SD	Nº de muestras que contienen *B. cereus*	% de muestras que contienen *B. cereus*
Gazpacho[a]	9	3,5 ± 0,5	2	22,2
Salmorejo[b]	9	8,0 ± 1,0	4	44,4
Ajoblanco[c]	4	3,5 ± 0,5	1	25,0
Zanaranja[d]	7	< 3,0	0	0
E. Americana[e]	12	> 2400	1	8,3

4.1.3.- Identificación bioquímica y confirmación de Bacillus cereus.

La identificación bioquímica de los microorganismos aislados se realizó mediante el sistema APILAB. En los casos donde el sistema APILAB propuso más de una especie, se realizaron pruebas adicionales (actividad oxidasa, movilidad y crecimiento anaeróbico) para determinar los resultados. En cuanto al sistema APILAB, se consideró que una buena identificación era obtenida cuando una especie era propuesta con un porcentaje de identificación (Id %) superior al 80%, el cual fue el caso de las siguientes cepas de la EPSO (Escuela Politécnica Superior de Orihuela): 1PE, 2PE, 3PE, 4PE, 5PE, 6PE, 7PE, 8PE, 9PE, 10PE, 11CU, 12CU, 13CA, 16GZ, 17SA, 18SA, 19SA, 20TO, 21TO, 22TO, 24TO, 25TO, 26TO, 27TO, 28PE, 30CU, 35AS y 37SA. Los resultados finales mostraron que la mayoría de las cepas aisladas fueron claramente confirmadas como *B. cereus*, mientras que la cepa EPSO-13CA fue identificada como *B. firmus*, y las cepas EPSO-28PE y EPSO-30CU como *B. licheniformis* (Tabla 4).

Tabla 4. Caracterización de *B. cereus* aislado de vegetales frescos (PE: pimiento, CU: pepino, CA: TO: tomate, ON: cebolla, ZU: calabacín) y alimentos vegetales mínimamente procesados (RMPFs) (GA: gazpacho, SA: salmorejo, AB: ajoblanco, AS: ensalada americana) en función de sus propiedades fisiológicas y bioquímicas. Para una misma columna, los valores seguidos de una misma letra no son significativamente diferentes (P ≤ 0,05).

Cepas aisladas de:	% Id	Nombre API	Movili-dad	Creci-miento anaeróbico	Nombre	Test BCET-RPLA	Hidrólisis de almidón	Valor D_{90}
Vegetales								
EPSO-1PE	99,7	*B. cereus*				+	+	4,3 ± 0,1 jkl
2PE	99,7	*B. cereus*				-	-	15,7 ± 0,6 b
3PE	95,0	*B. cereus*				-	-	13,2 ± 0,6 c
4PE	99,7	*B. cereus*				-	-	12,3 ± 0,6 c
5PE	99,8	*B. cereus*				-	-	21,2 ± 2,1 a
6PE	99,7	*B. cereus*				+	+	3,6 ± 0,1 klmn
7PE	99,4	*B. cereus*				+	+	6,9 ± 0,2 f
8PE	99,4	*B. cereus*				+	+	4,5 ± 0,2 ijk
9PE	99,4	*B. cereus*				+	+	2,9 ± 0,1 mno
10PE	99,4	*B. cereus*				+	+	4,4 ± 0,1 ijk
11CU	99,4	*B. cereus*				+	+	4,3 ± 0,2 jkl
12CU	99,3	*B. cereus*				+	+	5,4 ± 0,3 hij
13CA	96,5	*B. firmus*						
14CA	95,0/2,4	*B. firmus / B. cereus*		+	*B. cereus*	-	-	10,8 ± 0,3 d
15CA	95,0/2,4	*B. firmus / B. cereus*		+	*B. cereus*	-	-	12,3 ± 0,4 c
20TO	99,1	*B. cereus*				+	+	3,8 ± 0,1 klm
21TO	93,7	*B. cereus*				+	+	5,6 ± 0,2 ghi
22TO	98,9	*B. cereus*				+	+	2,9 ± 0,3 mno
23TO	99,8	*B. cereus / B. mycoides*	+		*B. cereus*	+	+	3,2 ± 0,1 lmno
24TO	99,1	*B. cereus*				+	+	2,5 ± 0,1 nop
25TO	99,1	*B. cereus*				+	+	1,5 ± 0,1 p
26TO	99,8	*B. cereus*				+	+	2,8 ± 0,1 mno
27TO	99,1	*B. cereus*				+	+	2,1 ± 0,1 op
28PE	99,8	*B. licheniformis*						
29ON	99,8	*B. cereus / B. mycoides*	+		*B. cereus*	+	+	6,6 ± 0,1 fg
30CU	99,8	*B. licheniformis*						
31ZU	98,0	*B. cereus / B. mycoides*	+		*B. cereus*	-	+	3,5 ± 0,1 klmn
32TO	99,7	*B. anthracis / B. cereus*	+		*B. cereus*	-	+	9,0 ± 0,9 e
34ZU	99,8	*B. mycoides / B. cereus*	+		*B. cereus*	+	+	5,1 ± 0,3 ij
RMPFs								
EPSO-16GA	91,9	*B. cereus*				+	+	1,4 ± 0,1 p
17SA	99,8	*B. cereus*				+	+	2,8 ± 0,3 mno
18SA	95,7	*B. cereus*				+	+	5,2 ± 0,3 ij
19SA	98,3	*B. cereus*				+	+	6,4 ± 0,7 fgh
33AB	99,8	*B. cereus / B. mycoides*	+		*B. cereus*	-	+	5,1 ± 0,2 ij
35AS	98,7	*B. cereus / B. mycoides*	+	+	*B. cereus*	+	+	
36GA	94,7/3,6	*B. anthracis / B. firmus*		-	*B. firmus*			
37SA	93,4	*B. cereus*				+	+	5,5 ± 0,2 ghij

4.1.4.- Resistencia térmica de las esporas de B. cereus.

Los valores D_{90} de los 33 aislados caracterizados como *B. cereus* oscilaron entre 1,4 y 21,2 min (Tabla 4). Los valores D_{90} mayoritarios (14 de las 33 cepas) oscilaron ente 3 y 6 min de resistencia térmica. Ocho cepas tuvieron valores D_{90} inferiores a 3 min, 3 cepas mostraron valores entre 6 y 9 min, y 7 cepas mostraron resistencias térmicas superiores a 9 min.

4.1.5.- Producción de enterotoxinas e hidrólisis de almidón.

A los 33 aislados caracterizados como *B. cereus* se les realizó el test BCET-RPLA de producción de enterotoxina diarreica, resultando 24 cepas (72,7%) positivas y 9 cepas (27,3%) negativas (Tabla 4). Por otro lado, el 81,8% de las 33 cepas de *B. cereus* hidrolizaron el almidón. El 100% de las 24 cepas enterotoxinas-positivo fueron capaces de hidrolizar el almidón, mientras que solamente el 33,3% de las 9 cepas enterotoxinas-negativo lo hidrolizaron.

4.1.6.- Temperaturas de crecimiento.

Ninguna de las 33 cepas fue capaz de crecer a 5 ºC. Sólo la cepa EPSO-35AS (el 3%) creció a 8 ºC, mientras que 29 cepas (el 87,9%) crecieron a 10 ºC. Todas las cepas fueron capaces de crecer a temperaturas de 12, 30 y 37 ºC, mientras que a 42 ºC sólo lo hicieron 19 cepas (el 57,6%) (Tabla 5).

Tabla 5. Cepas de *B. cereus* aisladas de vegetales frescos, alimentos vegetales mínimamente procesados (RMPFs) y ensalada americana que fueron capaces de crecer a temperaturas que oscilaron entre los 8 y 42 ºC. [a] Cepas aisladas de pimiento (PE), tomate (TO), cebolla (ON), calabacín (ZU), pepino (CU) y zanahoria (CA). [b] Cepas aisladas de RMPFs tales como gazpacho (GZ), salmorejo (SA) y ajoblanco (AB).

Crecimiento observado a temperatura (ºC) de:	**Cepas**	**Total**
8, 10, 12 , 30, 37	EPSO-35AS	1
10, 12, 30, 37	EPSO-1PE[a], 9PE, 16GZ[b], 17SA, 18SA, 20TO, 21TO, 22TO, 24TO, 25TO, 26TO, 29ON y 34ZU	13
10, 12, 30, 37, 42	EPSO-2PE, 5PE, 6PE, 7PE, 8PE, 10PE, 11CU, 12CU, 14CA, 15CA, 19SA, 31ZU, 32TO, 33AB y 37SA	15
12, 30, 37, 42	EPSO-3PE, 4PE, 23TO y 27TO	4

4.2.- Supervivencia de la cepa psicrótrofa de *B. cereus* EPSO-35AS.

4.2.1.- Resistencia térmica.

La resistencia térmica de las esporas de la cepa de *B. cereus* EPSO-35AS resuspendidas en agua destilada se determinó en un rango de temperaturas que osciló entre 80 y 95 ºC, mostrando la tabla 6 sus resultados. A partir de estos resultados se obtuvo un valor Z de 6,79 ºC.

Tabla 6. Valor D (min) obtenido por las esporas de *B. cereus* EPSO-35AS suspendidas en agua destilada y calentadas a diferentes temperaturas. Los datos expresan el valor medio ± la desviación estándar de tres repeticiones.

Temperatura (ºC)	Valor D (min)	Coeficiente de correlación (r_o)
80,0	64,9 ± 0,9	0,999
85,0	9,5 ± 0,2	0,991
87,5	3,7 ± 0,1	0,993
90,0	2,1 ± 0,1	0,986
95,0	0,4 ± 0,0	0,996

Aunque la relación entre la resistencia térmica de las esporas y la temperatura mínima de crecimiento no esta clara, se ha descrito que las cepas psicrótrofas de *B. cereus* son más sensibles al calor que las cepas mesófilas (Choma *et al*., 2000). Los valores D_{90} descritos para cepas psicrótrofas de *B. cereus* se encuentran en un rango comprendido entre 0,8 y 100 min (Dufrenne *et al*., 1995; Choma *et al*., 2000). Como ya hemos comentado en el apartado "*4.1.4.- Resistencia térmica de las esporas de B. cereus*", los valores D_{90} de las cepas de *B. cereus* aisladas oscilaron entre 1,4 y 21,2 min (Tabla 4), situándose por tanto los 2,1 min de la cepa *B. cereus* EPSO-35AS en el extremo inferior de ambos rangos. Solamente 3 cepas de *B. cereus* han mostrado un valor D_{90} igual o inferior al de la cepa EPSO-35AS (Tabla 4), y ninguna de ellas ha sido capaz de crecer a 42 ºC.

4.2.2.- Crecimiento en caldo nutritivo (NB) y caldo de tindalizado (TCB).

Las cinéticas de crecimiento en NB y TCB de las células germinadas procedentes de las esporas térmicamente activadas fueron evaluadas a 8, 12 y 16

ºC. En la tabla 7 podemos observar como a medida que aumenta la temperatura se incrementa el crecimiento, todos los parámetros de crecimiento. Las esporas germinadas de *B. cereus* en NB frente a las germinadas en TCB muestran a 8 ºC una mayor fase de latencia (290 frente a 224 h) y tiempo de generación (77,00 frente a 18,73 h), con lo que la densidad de población final es de 6,59 frente a 7,47 Log_{10} (UFC mL^{-1}).

Tabla 7. Parámetros de cinética de crecimiento calculados para la germinación de células procedentes de esporas activadas térmicamente de *B. cereus* EPSO-35AS incubadas a diferentes temperaturas en caldo nutritivo (NB) y caldo de tindalizado (TCB). N_0, número inicial de bacterias (UFC mL^{-1}). LT, fase de latencia (h). SGR, ratio de crecimiento específico (Log_{10} UFC mL^{-1} h^{-1}). GT, tiempo de generación (h). MPD, densidad máxima de población (UFC mL^{-1}). R^2, coeficiente de determinación para la ecuación de Baranyi *et al.* (1993) para la media de valores de los datos de crecimiento obtenidos por los dos ensayos.

Medium	Temperature (ºC)	Log_{10} N_o	LT (h)	SGR	GT (h)	Log_{10} MPD	R^2
NB	16	2,697	9,56	0,144	4,81	7,296	0,998
	12	2,392	22,34	0,074	9,36	6,631	0,990
	8	2,845	290,11	0,009	77,00	6,591	0,993
TCB	16	2.984	9,79	0,177	3,92	7,543	0,991
	12	2,884	24,22	0,085	8,15	6,897	0,995
	8	2,140	224,08	0,037	18,73	7,472	0,996

4.3.- Control químico del crecimiento de la cepa psicrótrofa de *B. cereus* EPSO-35AS.

4.3.1.- Crecimiento en caldo nutritivo (NB) y caldo de tindalizado (TCB) acidificado con ácido cítrico o zumo de limón.

Los parámetros cinéticos de crecimiento de la cepa psicrótrofa EPSO-35AS, en NB y en TCB fueron evaluados a diferentes temperaturas (8, 12 y 16 ºC) en combinación con la acidificación con ácido cítrico o zumo de limón a valores de pH comprendidos entre 4,8 y 5,5. Bajando el pH desde 7,4 en NB o 6,2 en TCB hasta 5,2 se inhibe el crecimiento bacteriano en ambos medios testados después de 60 días a 12 ºC o temperaturas inferiores (Tablas 8 y 9). La muerte bacteriana debida al bajo pH del medio se debe tanto a la inhibición de la actividad de las enzimas como de las proteínas transportadoras de la membrana. El menor pH para el crecimiento en NB y TCB a la temperatura más alta (16 ºC) fue 5,0. En general, es evidente que un incremento en el pH siempre produce una reducción en la duración en la fase de latencia e incrementos en el ratio de crecimiento específico, excepto para los cultivos en TCB a 12 ºC, donde los valores de ratio de crecimiento específico son similares.

El mínimo pH para el crecimiento bacteriano varía con las cepas y también depende de los acidulantes, por ejemplo, los ácidos orgánicos son más capaces de penetrar en la membrana celular que los inorgánicos. En nuestro ensayo, el crecimiento de *B. cereus* EPSO-35AS se produjo para ambos acidulantes, ácido cítrico y zumo de limón, a los mismos valores de pH y temperatura.

Tabla 8. Parámetros de crecimiento estimados usando la ecuación de Baranyi *et al*. (1993) para cultivos de *B. cereus* EPSO-35AS procedentes de esporas activadas térmicamente e incubadas en caldo nutritivo (NB) y caldo de zanahoria tindalizado (TCB) acidificado con ácido cítrico. N_0, número inicial de bacterias (UFC mL^{-1}). LT, fase de latencia (h). SGR, ratio de crecimiento específico (Log_{10} UFC mL^{-1} h^{-1}). GT, tiempo de generación (h). MPD, densidad máxima de población (UFC mL^{-1}). R^2, coeficiente de determinación para la ecuación de Baranyi *et al.* (1993) para la media de valores de los datos de crecimiento obtenidos por los dos ensayos. NG, no crecimiento durante 60 días.

Medio	Temperatura (ºC)	pH	Log_{10} *No*	LT (h)	SGR	GT (h)	Log_{10} MPD	R^2
NB	16	5,2	2,580	78,35	0,074	9,36	7,128	0,999
		5,1	2,763	80,45	0,065	10,66	7,255	0,994
		5,0	2,592	82,94	0,059	11,74	6,838	0,996
		4,9	2,954	NG	-	-	-	-
		4,8	1,967	NG	-	-	-	-
	12	5,5	2,123	173,49	0,028	24,75	7,539	0,995
		5,4	2,216	343,99	0,014	49,50	7,340	0,992
		5,3	2,125	NG	-	-	-	-
		5,2	2,237	NG	-	-	-	-
	8	5,5	2,422	NG	-	-	-	-
TCB	16	5,2	2,683	70,84	0,065	10,66	7,042	0,992
		5,1	2,650	84,45	0,060	11,55	6,977	0,991
		5,0	2,442	94,06	0,057	12,16	6,663	0,995
		4,9	2,380	NG	-	-	-	-
		4,8	2,320	NG	-	-	-	-
	12	5,5	2,506	76,88	0,044	15,75	6,820	0,997
		5,4	2,377	112,76	0,047	14,74	6,892	0,992
		5,3	2,338	223,73	0,045	15,40	6,904	0,995
		5,2	2,444	NG	-	-	-	-
		5,1	2,101	NG	-	-	-	-
	8	5,5	2,223	NG	-	-	-	-

Tabla 9. Parámetros de crecimiento estimados usando la ecuación de Baranyi *et al*. (1993) para cultivos de *B. cereus* EPSO-35AS procedentes de esporas activadas térmicamente e incubadas en caldo nutritivo (NB) y caldo de zanahoria tindalizado (TCB) acidificado con zumo de limón. N_0, número inicial de bacterias (UFC mL^{-1}). LT, fase de latencia (h). SGR, ratio de crecimiento específico (Log_{10} UFC mL^{-1} h^{-1}). GT, tiempo de generación (h). MPD, densidad máxima de población (UFC mL^{-1}). R^2, coeficiente de determinación para la ecuación de Baranyi *et al.* (1993) para la media de valores de los datos de crecimiento obtenidos por los dos ensayos. NG, no crecimiento durante 60 días.

Medio	Temperatura (ºC)	pH	Log_{10} *No*	LT (h)	SGR	GT (h)	Log_{10} MPD	R^2
NB	16	5,2	2,540	64,65	0,077	9,00	7,127	0,991
		5,1	2,123	72,55	0,063	11,00	6,964	0,993
		5,0	2,664	84,35	0,059	11,74	6,836	0,997
		4,9	2,065	NG	-	-	-	-
		4,8	2,693	NG	-	-	-	-
	12	5,5	2,142	195,83	0,028	24,75	6,442	0,997
		5,4	2,127	341,48	0,016	43,31	6,528	0,998
		5,3	2,598	NG	-	-	-	-
		5,2	2,124	NG	-	-	-	-
	8	5,5	2,441	NG	-	-	-	-
TCB	16	5,2	2,714	69,35	0,055	12,60	7,069	0,996
		5,1	2,137	81,43	0,056	12,37	6,987	0,995
		5,0	2,444	89,11	0,051	13,59	7,188	0,994
		4,9	2,103	NG	-	-	-	-
		4,8	2,049	NG	-	-	-	-
	12	5,5	2,518	83,27	0,044	15,75	6,885	0,998
		5,4	2,428	112,06	0,042	16,50	7,007	0,997
		5,3	2,342	229,28	0,041	16,90	6,903	0,996
		5,2	2,763	NG	-	-	-	-
		5,1	1,967	NG	-	-	-	-
	8	5,5	2,503	NG	-	-	-	-

4.3.2.- Efectos de los antimicrobianos sobre el crecimiento de B. cereus EPSO-35AS e INRA TZ415 en caldo nutritivo (NB) y caldo de zanahoria tindalizado (TCB).

La actividad antibacteriana de 4 compuestos naturales (aceite esencial de cinnamon, cinamaldehído, carvacrol y eugenol) fue evaluada para proporcionar información sobre el uso potencial como una alternativa a los conservantes sintéticos tradicionales de alimentos, debido al daño que pueden inducir sobre la membrana citoplasmática de la célula vegetativa (Helander *et al*., 1998; Ultee *et al.*, 2002; Walsh *et al*., 2003). Estos antimicrobianos fueron ensayados en las cepas de *B. cereus* EPSO-35AS e INRA TZ415 en ambos medios (NB y TCB) y al mismo rango de temperaturas (8, 12 y 16 ºC). La adición de aceite esencial de cinnamon o de cinamaldehído a concentraciones de 5 y 2 µL/100mL respectivamente, causaron la completa inhibición del crecimiento de ambas cepas psicrótrofas a 12 ºC (Tablas 10 y 11). Así pues, la combinación de uno de estos compuestos y la temperatura de refrigeración podría ser usada para la conservación de los RMPFs, cuyo ingrediente principal sea la zanahoria. Por el contrario, el carvacrol y eugenol fueron menos efectivos, a concentraciones de 5 y 35 µL/100mL respectivamente, sólo fueron capaces de prevenir el crecimiento de *B. cereus* EPSO-35AS durante el almacenamiento a 8 ºC. Esta acción inhibitoria pudo ser debida a un efecto sinérgico entre la refrigeración y el carvacrol o eugenol, sobre la germinación de células de las esporas de *B. cereus* EPSO-35AS activadas térmicamente.

Además de resultar poco eficaces como antimicrobianos, carvacrol y eugenol afectaron negativamente a las características sensoriales del TCB a las concentraciones ensayadas. Sin embargo, el aceite de cinnamon y cinamaldehído influenciaron positivamente al gusto y olor del TCB. Por tanto, una combinación de uno de estos componentes y el almacenamiento refrigerado podría ser útil para la conservación de RMPFs basados en zanahoria. El eugenol, a concentraciones superiores o iguales a 35 µL/100 mL, confirió un flavor agrio al TCB que fue inaceptable por los catadores. Por tanto, para la posible utilización de estos dos compuestos habría que determinar con más exactitud las concentraciones a utilizar para lograr un efecto antimicrobiano minimizando los efectos sobre la calidad sensorial del TCB.

Tabla 10. Parámetros de crecimiento estimados usando la ecuación de Baranyi *et al*. (1993) para cultivos de *B. cereus* EPSO-35AS procedentes de esporas activadas térmicamente e incubadas en caldo nutritivo (NB) y caldo de zanahoria tindalizado (TCB) que contenían antimicrobianos naturales. N_0, número inicial de bacterias (UFC mL^{-1}). LT, fase de latencia (h). SGR, ratio de crecimiento específico (Log_{10} UFC mL^{-1} h^{-1}). GT, tiempo de generación (h). MPD, densidad máxima de población (UFC mL^{-1}). R^2, coeficiente de determinación para la ecuación de Baranyi *et al.* (1993) para la media de valores de los datos de crecimiento obtenidos por los dos ensayos. NG, no crecimiento durante 60 días.

Medio	Antimicrobiano	Concentración (µL 100 mL^{-1})	Temperatura (ºC)	Log_{10} *No*	LT (h)	SGR	GT (h)	Log_{10} MPD	R^2
NB	Cinnamon	5	16	2,445	37,86	0,090	7,70	6,962	0,995
			12	2,122	NG	-	-	-	-
			8	2,584	NG	-	-	-	-
	Cinamaldehído	2	16	2,180	32,76	0,112	6,19	7,452	0,996
			12	2,103	NG	-	-	-	-
			8	2,503	NG	-	-	-	-
	Carvacrol	5	16	3,687	92,93	0,035	19,8	7,363	0,997
			12	3,650	118,22	0,018	38,5	6,558	0,998
			8	2,441	NG	-	-	-	-
	Eugenol	35	16	3,123	11,67	0,122	5,68	7,260	0,991
			12	3,071	32,72	0,057	12,16	7,121	0,997
			8	2,693	NG	-	-	-	-
TCB	Cinnamon	5	16	2,102	14,26	0,080	8,66	7,193	0,994
			12	2,124	NG	-	-	-	-
			8	2,600	NG	-	-	-	-
	Cinamaldehído	2	16	2,401	16,19	0,087	7,96	7,265	0,990
			12	2,137	NG	-	-	-	-
			8	2,065	NG	-	-	-	-
	Carvacrol	5	16	2,478	13,50	0,105	6,60	7,304	0,997
			12	2,397	49,17	0,045	15,4	6,332	0,992
			8	1,938	NG	-	-	-	-
	Eugenol	35	16	2,559	9,97	0,135	5,13	7,792	0,997
			12	2,433	24,33	0,055	12,6	6,855	0,991
			8	2,142	NG	-	-	-	-

Tabla 11. Parámetros de crecimiento estimados usando la ecuación de Baranyi *et al*. (1993) para cultivos de *B. cereus* INRA TZ415 procedentes de esporas activadas térmicamente e incubadas en caldo nutritivo (NB) y caldo de zanahoria tindalizado (TCB) que contenían antimicrobianos naturales. N_0, número inicial de bacterias (UFC mL^{-1}). LT, fase de latencia (h). SGR, ratio de crecimiento específico (Log_{10} UFC mL^{-1} h^{-1}). GT, tiempo de generación (h). MPD, densidad máxima de población (UFC mL^{-1}). R^2, coeficiente de determinación para la ecuación de Baranyi *et al.* (1993) para la media de valores de los datos de crecimiento obtenidos por los dos ensayos. NG, no crecimiento durante 60 días.

Medio	Antimicrobiano	Concentración (µL 100 mL^{-1})	Temperatura (ºC)	Log_{10} *No*	LT (h)	SGR	GT (h)	Log_{10} MPD	R^2
NB	Cinnamon	5	16	2,275	28,63	0,101	6,86	6,777	0,997
			12	2,417	NG	-	-	-	-
			8	2,333	NG	-	-	-	-
	Cinamaldehído	2	16	2,172	24,38	0,117	5,92	7,400	0,995
			12	2,319	NG	-	-	-	-
			8	2,428	NG	-	-	-	-
	Carvacrol	5	16	3,701	55,92	0,041	16,90	7,761	0,999
			12	3,673	94,32	0,025	27,72	7,597	0,998
			8	3,614	396,66	0,013	53,31	6,308	0,997
	Eugenol	35	16	2,800	18,26	0,139	4,98	7,437	0,995
			12	2,996	33,63	0,083	8,35	7,174	0,993
			8	2,882	47,56	0,029	23,90	6,801	0,996
TCB	Eugenol	35	16	2,434	10,10	0,160	4,33	7,774	0,999
			12	2,505	26,78	0,137	5,06	7,716	0,992
			8	2,441	112,79	0,033	21,00	7,463	0,999

5.- CONCLUSIONES

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Primera. Todos los vegetales y productos mínimamente procesados presentaron contaminación por *Bacillus cereus* excepto el ajo y la zanaranja. Los pepinos, zanahorias y ensalada americana presentaron los mayores recuentos de todos los alimentos ensayados, que en ningún caso superaron los 10^4 UFC/g.

Segunda. Un total de 37 presuntas cepas de *B. cereus* fueron aisladas de 97 muestras. De las cuales, 33 (el 89,2%) fueron confirmadas como *B. cereus*, 2 (el 5,4%) como *B. licheniformis*, y las 2 (el 5,4%) cepas restantes fueron identificadas como *B. firmus*.

Tercera. De las 33 cepas de *B. cereus* aisladas, 27 (81,8%) son capaces de hidrolizar el almidón y 24 (72,7%) son productoras de la enterotoxina diarreica en cultivos de TSB.

Cuarta. La resistencia térmica a 90 ºC de las esporas de las 33 cepas de *B. cereus* varió entre 1,4 y 21,2 min. Las cepas incapaces de hidrolizar el almidón mostraron mayor resistencia térmica, con valores D_{90} superiores a 10,8 min. Todas las cepas productoras de enterotoxinas fueron capaces de hidrolizar el almidón.

Quinta. Solamente se aisló una cepa de *B. cereus* psicrótrofa, EPSO-35AS, capaz de crecer a 8 ºC.

Sexta. La cinética de crecimiento de la cepa EPSO-35AS a 8 ºC mostró un crecimiento más rápido y una población final más elevada en caldo zanahoria tindalizado que en caldo nutritivo.

Séptima. La acidificación con ácido cítrico o zumo de limón a pH 5,2 en caldo nutritivo o caldo zanahoria tindalizado inhibe el crecimiento bacteriano en ambos medios durante al menos 60 días a 12 ºC o temperaturas inferiores.

Octava. La adición de aceite esencial de cinnamon o de cinamaldehído a concentraciones de 5 y 2 µL/100mL respectivamente, causan la completa inhibición del crecimiento de las cepas de *B. cereus* EPSO-35AS e INRA TZ415 en caldo nutritivo y caldo zanahoria tindalizado a 12 ºC.

Novena. Carvacrol y eugenol, a concentraciones de 5 y 35 µL/100mL respectivamente, previenen el crecimiento durante 60 días de *B. cereus* EPSO-35AS solamente a la temperatura de 8 ºC.

Décima. Carvacrol y eugenol, a concentraciones de 5 y 35 µL/100mL respectivamente, afectan negativamente a las características sensoriales del caldo zanahoria tindalizado, aportando un flavor agrio. Sin embargo, el aceite de cinnamon y cinamaldehído influyen positivamente sobre el gusto y olor de éste.

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