TESIS DOCTORAL

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

ESTRATEGIAS TECNOLÓGICAS PARA MEJORAR LA DESINFECCIÓN Y PROCESADO DE DERIVADOS DE ARROZ

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Instituto de Agroquímica y Tecnología de Alimentos

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Valencia, julio 2024

EXCELENCIA SEVERO OCHOA

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Presentada por:

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Para optar el título de
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La Dra. Dña. María Dolores Rodrigo Aliaga, Investigadora Científica del Consejo Superior de Investigaciones Científicas (CSIC) y el Dr. D. Antonio Martínez López, Profesor de Investigación del Consejo Superior de Investigaciones Científicas (CSIC).

CERTIFICAN:

Que el trabajo que presenta María Inés Valdez Narváez para optar al grado de Doctor por la Universidad Politécnica de Valencia, con el título **Estrategias tecnológicas para mejorar la desinfección y procesado de derivados de arroz** ha sido realizado bajo nuestra dirección, en el Instituto de Agroquímica y Tecnología de Alimentos del Consejo Superior de Investigaciones Científicas (IATA-CSIC) Excelencia Severo Ochoa.

Y para que así conste a los efectos oportunos, firman este certificado en Paterna,

Dra. Dña. Mª Dolores Rodrigo Aliaga

Dr. D. Antonio Martínez López

La presente tesis doctoral se enmarca dentro del Programa de Doctorado Ciencia, Tecnología y Gestión Alimentaria de la Universidad Politécnica de Valencia.

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RESUMEN

El arroz, es uno de los cereales más consumidos a nivel mundial y debido a su alto valor nutricional, forma parte de la dieta básica de la población en general. Su elevado contenido de almidón, sin modificar o tras su modificación, lo convierte en un alimento atractivo para la industria alimentaria. No obstante, este mismo componente favorece el desarrollo del *Bacillus cereus* después de su cocción. Este microorganismo forma esporas resistentes al calor, lo que supone un riesgo para el consumidor, especialmente si el arroz cocinado no se almacena a temperatura adecuada. En la presente tesis doctoral, se han propuesto algunas estrategias tecnológicas para mejorar el procesado y garantizar la inocuidad alimentaria del arroz y sus derivados. Estas estrategias consisten en la aplicación de plasma frío y en el uso de quitosano de insecto (*Tenebrio molitor*) como antimicrobiano. Desde el punto de vista tecnológico, se ha visto que la tecnología de plasma es capaz de modificar determinadas propiedades tecnológicas del almidón que dependen de la variedad del arroz. Estos resultados abren la puerta a un uso más amplio de este componente en la industria alimentaria y en la formulación de alimentos a la carta.

En cuanto a la inocuidad del arroz, el plasma frío reduce los niveles de células vegetativas y de esporas de *B. cereus*, convirtiéndolo en una tecnología alternativa de desinfección de materias primas como el arroz, antes de su procesado posterior. El nivel de inactivación tanto de células vegetativas como de esporas, estuvo determinado por el tiempo y potencia de tratamiento, así como la matriz que lo contenía en el caso de las esporas. El quitosano de insectos redujo la termorresistencia del *B. cereus* y resultó ser bacteriostático o bactericida en función de la concentración y la temperatura de almacenamiento. Estos efectos lo convierten en una buena estrategia de control de dicho microorganismo en arroz y sus derivados. A través de un modelo de evaluación a la exposición se ha podido corroborar los resultados sobre el efecto del quitosano en el *B. cereus*, a la vez que se pone de manifiesto la importancia que tienen cambios accidentales en las condiciones de procesado sobre

el nivel final de microorganismos en el alimento y del número inicial de microorganismos sobre el porcentaje de unidades infectas después del tratamiento.

Los resultados de la presente tesis ponen de manifiesto estrategias más sostenibles que son capaces de mejorar el procesado y la inocuidad del arroz y productos derivados del mismo.

RESUM

L'arròs és un dels cereals més consumits a nivell mundial i, a causa del seu alt valor nutricional, forma part de la dieta bàsica de la població en general. El seu alt contingut d'almidó, sense modificar o després de la seva modificació, el converteix en un aliment atractiu per a la indústria alimentària. No obstant això, aquest mateix component afavoreix el desenvolupament del *Bacillus cereus* després de la seva cocció. Aquest microorganisme forma espores resistents al calor, la qual cosa suposa un risc per al consumidor, especialment si l'arròs cuinat no es conserva a una temperatura adequada. En la present tesi doctoral, s'han proposat algunes estratègies tecnològiques per millorar el processament i garantir la innocuïtat alimentària de l'arròs i els seus derivats. Aquestes estratègies consisteixen en l'aplicació de plasma fred i en l'ús de quitosà d'insecte (*Tenebrio molitor*) com a antimicrobià. Des del punt de vista tecnològic, s'ha vist que la tecnologia de plasma és capaç de modificar certes propietats tecnològiques de l'almidó que depenen de la varietat de l'arròs. Aquests resultats obren la porta a un ús més ampli d'aquest component en la indústria alimentària i en la formulació d'aliments a la carta.

Quant a la innocuïtat de l'arròs, el plasma fred redueix els nivells de cèl·lules vegetatives i d'espores de *B. cereus*, convertint-lo en una tecnologia alternativa de desinfecció de matèries primeres com l'arròs, abans del seu processament posterior. El nivell d'inactivació tant de cèl·lules vegetatives com d'espores va estar determinat pel temps i potència de tractament, així com la matriu que les contenia en el cas de les espores. El quitosà d'insectes va reduir la termoresistència del *B. cereus* i va resultar ser bacteriostàtic o bactericida en funció de la concentració i la temperatura d'emmagatzematge. Aquests efectes el converteixen en una bona estratègia de control d'aquest microorganisme en l'arròs i els seus derivats. A través d'un model d'avaluació a l'exposició s'ha pogut corroborar els resultats sobre l'efecte del quitosà en el *B. cereus*, alhora que es posa de manifest la importància que tenen accidentals canvis en les condicions de processat sobre el nivell final de microorganismes en l'aliment i del

nombre inicial de microorganismes sobre el percentatge d'unitats infectades després del tractament.

Els resultats de la present tesi posen de manifest estratègies més sostenibles que són capaces de millorar el processament i la innocuïtat de l'arròs i productes derivats del mateix.

ABSTRACT

Rice is one of the most widely consumed cereals worldwide, due to its high nutritional value, it is part of the population basic diet. Its high starch content, unmodified or modified, makes it an attractive ingredient for the food industry. However, this same component promotes the development of *Bacillus cereus* after cooking. This microorganism forms heat-resistant spores, which poses a risk to consumers, especially if the cooked rice is not stored at the correct temperature. In this doctoral thesis, some technological strategies have been proposed to improve the processing and ensure the food safety of rice and its derivatives. These strategies include the application of cold plasma and the use of insect chitosan from *Tenebrio molitor* as an antimicrobial. From a technological perspective, plasma technology has demonstrated its ability to modify technological properties of starch, which vary based on the type of rice. These results open the door to a broader use of this component in the food industry and in customized food formulation.

Regarding the safety of rice, cold plasma reduces the levels of vegetative cells and spores of *B. cereus*, making it an alternative technology for disinfecting raw materials such as rice before further processing. The level of inactivation of both vegetative cells and spores was determined by the time and power of the treatment, as well as the matrix containing them in the case of the spores. Insect chitosan reduced the thermo-resistance of *B. cereus* and was found to be bacteriostatic or bactericidal depending on the concentration and storage temperature. These effects make it a good control strategy for this microorganism in rice and its derivatives. Through an exposure assessment model, the results on the effect of chitosan on *B. cereus* have been corroborated, while also highlighting the importance of accidental changes in processing conditions on the final level of microorganisms in the food and the initial number of microorganisms on the percentage of infected units after treatment.

The results of this thesis demonstrate more sustainable strategies that are capable of improving the processing and safety of rice and its derivatives.

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1. JUSTIFICACIÓN DEL TEMA

1. Justificación del Tema

Los alimentos en grano o en polvo, como el arroz, se consideran alimentos no perecederos, ya que debido a su baja actividad de agua (a_w) son más estables durante el almacenamiento y tienen una vida útil más larga. Sin embargo, a pesar de esta baja actividad de agua, pueden estar expuestos durante su cultivo y procesado a la contaminación por microorganismos patógenos. Algunos de estos microrganismos tienen la capacidad de formar esporas y aunque se someten a estrictos procesos de fabricación para controlar a estos microorganismos, en determinadas circunstancias, las esporas microbianas no pueden eliminarse por completo, representando un riesgo para la salud del consumidor, especialmente en productos listos para el consumo.

Hasta ahora, son pocos los métodos de higienización que sean capaces de reducir la carga microbiana, especialmente de esporas, sin afectar las propiedades sensoriales y nutricionales de los alimentos. Esta limitación ha llevado a la necesidad de desarrollar nuevas tecnologías y métodos de control más efectivos de modo que se pueda garantizar la inocuidad alimentaria, sin comprometer de manera significativa las características nutricionales y organolépticas de los alimentos.

Los métodos de conservación tradicionales, pueden ser físicos, químicos y biológicos. Dentro de los métodos químicos, durante muchos años se han utilizado antimicrobianos de origen sintético para el control microbiológico de los alimentos. Actualmente, se plantea la posibilidad del uso de antimicrobianos de origen natural, ya sea de forma individual o combinada con otros tratamientos, como una estrategia para mejorar la inocuidad alimentaria siendo una alternativa más saludable y respetuosa con el medio ambiente.

Por otra parte, dentro de los métodos físicos de conservación, se han desarrollado nuevas tecnologías de conservación no térmicas, entre las cuales el plasma frío se plantea como una opción interesante y prometedora para la higienización de materias primas de baja actividad de agua. La principal ventaja del plasma frío frente a otros métodos no térmicos de conservación reside en su capacidad de eliminar formas esporuladas de microorganismos patógenos sin elevar significativamente la

temperatura del producto, lo que minimiza el riesgo de deterioro de la calidad nutricional y organoléptica.

Las matrices ricas en carbohidratos, como el arroz y en concreto el almidón, se emplean en la industria alimentaria no solo por su valor nutricional, sino también por su capacidad para modular las propiedades tecnológicas del alimento del que forma parte (por ejemplo, viscosidad, capacidad de gelificación, entre otras). Esas propiedades dependen principalmente de las interacciones entre la conformación, estructura y composición de las moléculas poliméricas del alimento al interaccionar con su entorno. El plasma generado en la superficie de un alimento, además de inactivar microorganismos, es capaz de reaccionar con dichas moléculas poliméricas dando lugar a procesos de de/polimerización, rotura de enlaces, oxidaciones e incluso a la formación de entrecruzamientos entre cadenas, produciendo cambios de estructura o de conformación. Por lo tanto, mediante un tratamiento de plasma se podría modificar de manera controlada las propiedades tecnológicas de determinados ingredientes, utilizándose como herramienta para la formulación de alimentos "a la carta".

En el contexto de la presente tesis doctoral se evaluó la aplicación de nuevas tecnologías no térmicas de procesado, como el plasma frío y/o el uso de antimicrobianos naturales, como alternativas interesantes tanto en el campo de la higienización y control microbiológico de alimentos de baja actividad de agua, como en el campo del procesado de alimentos, como herramienta para la modificación selectiva de determinadas propiedades tecnológicas y funcionales. Estas alternativas son interesantes para mejorar la inocuidad alimentaria y proteger la salud del consumidor, a la vez que se mantiene en mayor medida la calidad del alimento.

2. ANTECEDENTES BIBLIOGRÁFICOS

2. Antecedentes Bibliográficos

2.1. Arroz

El arroz es una semilla que proviene de una planta herbácea del género *Oryza*, y se ha cultivado durante más de 8 000 años. En la actualidad su producción principal es para consumo humano, pero también se utiliza como alimento para ganado en casos de cosechas de mala calidad. Es un alimento básico para más de la mitad de la población mundial, especialmente en países en vías de desarrollo (Juliano, 1993).

Después de la maduración del grano, se cosecha en forma de arroz con cáscara y posteriormente pasa por un proceso de secado, almacenamiento y molienda. Dependiendo del tipo de arroz que se desee obtener, o se mantiene toda la estructura del grano (arroz integral) y se desecha solo la cáscara, o se elimina todo y se mantiene solo el endospermo (arroz blanco). Este cereal se comercializa en diversas formas, incluyendo arroz integral, vaporizado, precocido y molido como harina (Ghanghas et al., 2022). Cada tipo de arroz presenta un riesgo diferente, dependiendo del tratamiento que haya recibido durante su procesamiento y del uso que le vaya a dar el consumidor, por ello, es importante tener en cuenta que el manejo y almacenamiento adecuado del arroz es fundamental para prevenir la contaminación bacteriana y asegurar su inocuidad alimentaria (EFSA, 2005).

Antes de su consumo, tanto el arroz blanco como el arroz integral, deben pasar por un proceso de cocción, siendo los métodos más comunes la cocción a presión, al vapor o la ebullición rápida, adaptados según el tipo de plato que se desee preparar (Ghanghas et al., 2022). En el caso del arroz, el crecimiento bacteriano solo puede ocurrir con el arroz cocido, ya que el arroz seco tiene una baja actividad de agua que inhibe el crecimiento de microorganismos. Una vez que se cocina el arroz y se introduce humedad, las bacterias presentes pueden comenzar a multiplicarse si el arroz no se manipula y almacena adecuadamente (Rodrigo et al., 2021). Por lo tanto, es crucial seguir medidas adecuadas de inocuidad alimentaria al cocinar y almacenar arroz para prevenir la contaminación bacteriana y las enfermedades transmitidas por los alimentos. Esto incluye cocinar el arroz completamente, almacenarlo a

temperaturas de refrigeración y consumirlo dentro de un tiempo razonable para minimizar el riesgo de crecimiento bacteriano (EFSA, 2005).

2.1.1. Propiedades del arroz

Desde el punto de vista nutricional, el arroz es una fuente rica en carbohidratos, más o menos el 75-80% de su composición son carbohidratos siendo el almidón su principal componente. El grano de arroz puede contener entre un 12-14% de agua y el porcentaje restante se dividen entre 7% de proteínas, 3% de fibra y un 2% de grasa (Juliano, 1993; Verma & Srivastav, 2020).

Además, también se ha visto que puede ser una fuente importante de algunas vitaminas del complejo B como la niacina, la riboflavina y la tiamina; así como fuente de algunos minerales como calcio (Ca), magnesio (Mg) y fósforo (P) y en pequeñas trazas cobre (Cu), hierro (Fe), manganeso (Mn) y zinc (Zn) (Verma & Srivastav, 2020).

A pesar de que el porcentaje de proteínas no es elevado, el perfil de aminoácidos del arroz es muy interesante, ya que aporta cantidades importantes de treonina, leucina y fenilalanina, junto con aminoácidos ricos en azufre como la metionina y cisteína, que son cruciales para el cuerpo humano. Esto hace que estas proteínas sean de fácil digestión y con alto poder biológico (Ghanghas et al., 2022). Sin embargo, la cantidad de los compuestos bioactivos presentes en el arroz, van a depender de los diversos cultivares, la fertilidad del suelo y de las condiciones ambientales en las que se cultive (Ghasemzadeh et al., 2018).

Por todos estos valores nutricionales, el arroz es considerado por algunos autores como el “rey de los cereales” y está ganando cada vez más atención tanto de los consumidores como de la industria alimentaria, ya que, debido a su importante actividad biológica, pueden tener un impacto positivo sobre la digestibilidad y en la salud en general (Verma & Srivastav, 2020).

2.1.2. Consumo del arroz

Debido a su perfil nutricional, el arroz es el tercer cereal más consumido a nivel mundial. Es un alimento que forma parte de la dieta básica de muchos países, abasteciendo a más de la mitad de la población mundial diariamente. China es el mayor consumidor de arroz, en el periodo 2022-2023, lideró el ranking de consumo de este cereal con cerca de 154 millones de toneladas, seguida de India con un consumo de 109 millones de toneladas (USDA, 2024).

En términos de producción, durante el año 2022, la producción de arroz a nivel global superó las 500 millones de toneladas, siendo India el mayor productor con 22,24 millones de kilogramos en el año 2022, le siguen países como Tailandia con casi 8 millones de kilogramos y Vietnam con 6,5 millones de kilogramos de producción para arroz en el mismo año (USDA, 2024).

Dentro de la Unión Europea (UE), el mayor productor de arroz es Italia, con una producción de 1 459 toneladas al año, seguido de Rusia con 1 076 toneladas y España con 617 toneladas (FAO, 2021). Ya específicamente en España, durante el año 2022, se consumieron 177 millones de kilogramos de arroz, siendo la Comunidad Valencia y Cataluña las principales productoras y consumidoras. Cataluña registró una producción de arroz de 127 000 toneladas, seguida de la Comunidad Valenciana con 118 000 toneladas. Además, durante el año 2022, también se observó un aumento del 13,3% en el consumo de platos preparados a base de arroz, lo que sugiere una tendencia creciente en esta forma de consumo (Mercasa, 2023; Ministerio de Agricultura,, Pesca y Alimentación, 2023).

Estos datos, ponen de manifiesto la importancia del arroz a nivel mundial, tanto en términos de producción como de consumo. Es importante entonces, una gestión eficiente en su producción y abastecimiento para garantizar la inocuidad alimentaria a nivel mundial.

2.1.3. Almidón de arroz: propiedades tecnológicas

El almidón, es el componente principal del arroz, está compuesto por amilosa y amilopectina y su proporción depende de la variedad de arroz (Kang et al., 2006). La amilosa es un polímero de cadena lineal de moléculas de glucosa unidas por enlaces α-1,4; mientras que, la amilopectina es un polímero con estructura ramificada de moléculas de glucosa con enlaces α-1,4 y aproximadamente entre un 5 y un 6 % de enlaces α-1,6 en los puntos de ramificación (Amagliani et al., 2016).

El comportamiento y la funcionalidad del almidón de arroz a nivel tecnológico está determinado por algunos factores, especialmente durante los procesos de cocción, ya que aquí se producen cambios como la transición vítrea, gelatinización y retrogradación del almidón que permiten obtener granos de arroz con características específicas (Amagliani et al., 2016).

En un proceso de cocción normal en agua, se da la gelatinización del almidón, esto produce algunos cambios como el hinchamiento del gránulo y la fusión del cristal nativo, provocados por un desorden de la estructura entre la amilosa y la amilopectina, siendo estos cambios reversibles o no dependiendo del grado de gelatinización y la presencia de mayor o menor amilosa y amilopectina (Amagliani et al., 2016; Verma & Srivastav, 2022).

La retrogradación del almidón ocurre durante el almacenamiento, es un proceso en el cual, la amilosa y amilopectina desordenadas durante la gelatinización se vuelven a unir para formas estructuras ordenadas y firmes, lo que va a determinar la textura del arroz cocido. La funcionalidad reológica del almidón de arroz, está influenciada por su contenido de amilosa y amilopectina. Por lo que, conocer su comportamiento es fundamental para comprender y controlar las propiedades del arroz durante la preparación y el almacenamiento (Amagliani et al., 2016; Verma & Srivastav, 2022).

2.1.4. Platos listos para el consumo

Hoy en día, los cambios en el estilo de vida de muchas personas han impulsado a un aumento importante en el consumo de platos preparados listos para consumir. El arroz, es un ingrediente muy habitual en este tipo de platos al ser un alimento básico en diversas culturas y con un buen perfil nutricional. Sin embargo, su preparación implica procesos cruciales que afectan su calidad e inocuidad alimentaria (Juliano, 1993).

Según algunas investigaciones, se ha visto que en general, los alimentos listos para el consumo pueden ser alimentos muy susceptibles a contaminación microbiana. Chen et al., (2022) vieron que un total de 91 de las 1 071 muestras que analizaron estaban contaminadas con *Bacillus cereus* y que, el nivel de contaminación de en el 0,5 % de las muestras de productos de arroz listos para usar superó los 10^3 UFC/g. En otro estudio realizado en Estados Unidos en productos listos para el consumo como fiambres, quesos y ensaladas frescas, se vio que el de 31 705 muestras analizadas, 577 estaban contaminadas con *L. monocytogenes*. De estas muestras, 402 presentaban niveles de $1{,}5x10^5$ UFC/g, 21 muestras con niveles $>10^2$ UFC/g, y el resto de muestras con niveles intermedios (Gombas et al., 2003).

En último informe de la Autoridad Europea de Seguridad alimentaria , EFSA, (EFSA & ECDC, 2023), sobre enfermedades zoonóticas trasmitidas por alimentos, los productos alimenticios pertenecientes al grupo de "alimentos compuestos, alimentos con ingredientes múltiples y otros alimentos" ocuparon el primer lugar entre los vehículos alimentarios implicados en intoxicaciones alimentarias. En la Unión Europea (UE), durante el año 2022, se observaron un total de 143 casos de intoxicación alimentaria, asociadas al consumo de alimentos procesados, multi-ingredientes. Esto representó un aumento del 34,9 % con respecto al año 2021 (EFSA & ECDC, 2023).

La EFSA indica que, los incidentes que conducen a la contaminación de este tipo de alimentos pueden originarse por el uso de ingredientes contaminados, contaminaciones cruzada entre alimentos, en el entorno y el equipo de procesamiento,

por lo que es importante una combinación y manipulación adecuada de los ingredientes, para garantizar la salud del consumidor (EFSA & ECDC, 2023).

2.2. *Bacillus cereus*

Un microorganismo relacionado con distintos brotes de enfermedad alimentaria en los que el arroz se ha identificado como vehículo, es el *Bacillus cereus*.

Bacillus cereus es una bacteria Gram-positiva, móvil (flagelada), formadora de esporas y con forma de bastón que pertenece al género *Bacillus* (Rajkowski & Bennett, 2003).

B. cereus está relacionado con dos tipos de enfermedades la diarreica y la emética. La enfermedad diarreica es una infección y se produce por el crecimiento del microorganismo en el intestino delgado, mientras que la enfermedad emética es una intoxicación alimentaria que ocurre al consumir alimentos que contienen la toxina cereulida, sintetizada por el microorganismo durante su crecimiento en el alimento contaminado con esporas (Webb et al., 2019).

El síndrome emético suele estar asociado con brotes donde los alimentos contaminados son platos de arroz o pasta, especialmente cuando el arroz se hierve en gran cantidad y se almacena para luego freírse o recalentarse antes de servirlo. Esta práctica permite que las esporas que queden viables tras la cocción se reproduzcan durante el almacenamiento y sinteticen la toxina cereulida, que al ser termoestable no se destruye durante la fritura o recalentamiento posterior del arroz (Webb et al., 2019)

Diferentes estudios han revelado que *B. cereus* presenta una notable variabilidad en la resistencia térmica de las esporas (Choma et al., 2000; Fernández et al., 1999; Wijnands et al., 2006). Además, un estudio comparativo que incluyó cien cepas de *B. cereus* de diferentes fuentes encontró que las cepas productoras de toxina emética mostraban una mayor resistencia al calor en comparación con las cepas diarreicas y ambientales (Carlin et al., 2000; Luu-Thi et al., 2014).

La variabilidad en la resistencia térmica de las esporas de *B. cereus* puede plantear desafíos en términos de garantizar la inactivación completa de este microorganismo durante los procesos de cocción habituales. Esta incertidumbre aumenta el riesgo de que algunas cepas resistentes puedan sobrevivir y, potencialmente, causar enfermedades transmitidas por alimentos si no se manejan adecuadamente (EFSA Panel on Biological Hazards (BIOHAZ), 2016).

Para abordar este riesgo, es importante que adicionalmente al manejo adecuado del arroz y sus derivados, incluyendo el almacenamiento adecuado y la higiene durante la preparación y manipulación de estos productos, se lleven a cabo procesos de higienización previos en la materia prima con objeto de reducir la carga microbiana, especialmente de las esporas, lo cual puede ayudar a reducir el riesgo por contaminación de *B. cereus* y otras bacterias patógenas (EFSA, 2005).

2.2.1. Antecedentes del impacto de *B. cereus* en la Inocuidad alimentaria

B. cereus se encuentra entre las diez principales combinaciones patógeno/alimento que causan el mayor número de brotes de origen alimentario con evidencia sólida. En 2022, la tasa de notificación de intoxicaciones alimentarias causadas por toxinas bacterianas fue de 0,25 por 100 000 habitantes. Esto representó un aumento relativo del 68,2 % en comparación con la tasa de 2021, debido principalmente al aumento de notificaciones de brote transmitido por alimentos asociados con toxinas *de B. cereus* (EFSA & ECDC, 2023).

B. cereus estuvo involucrado en 306 brotes con evidencia sólida, representando el 5,3% del total de brotes en la Unión Europea, con 3 192 casos en humanos, 66 hospitalizaciones y 2 muertes. Las toxinas *de B. cereus* ocuparon el primer lugar en cuanto al número de brotes trasmitidos por los alimentos. Lo comunicaron ocho estados miembros (Bélgica, Finlandia, Francia, Alemania, Hungría, Italia, Portugal y España) y otro no miembro (Suiza). En la UE, este aumento es atribuible

principalmente a Francia, que por sí sola registró el 90,8% de todos estos brotes (208 brotes más que en 2021, un aumento relativo del 297,1%) (EFSA & ECDC, 2023).

Algunos brotes relativamente recientes en otros países también se han asociado con este patógeno, como el caso de 45 personas afectadas por un brote en un restaurante en Camberra (Australia) en 2018 (Thirkell et al., 2019) y 200 estudiantes en un brote en una escuela en China en 2018 (D. Chen et al., 2019). Las exigencias de los consumidores de alimentos refrigerados, poco procesados, de vida útil limitada están llevando a un incremento de los brotes por *B. cereus* tal y como lo ha reconocido la EFSA y la ECDC en su reporte del año 2023 (EFSA & ECDC, 2023).

En muchas ocasiones *B. cereus* se ha asociado también con productos alimenticios mixtos; estos productos pueden incluir arroz como componente, sin embargo, otros productos a base de arroz y alimentos farináceos como pasta y fideos también pueden estar contaminados e implicados en intoxicaciones por *B. cereus* (Grande et al., 2006).

Las esporas de *B. cereus* pueden sobrevivir en productos de arroz secos. Jaquette y Beuchat (1998) indicaron que durante el almacenamiento en condiciones frescas y secas no se apreció una pérdida de viabilidad de las esporas en cereales para el destete a base de arroz durante 48 semanas de almacenamiento. No obstante, el almacenamiento en condiciones de temperatura alta (45ºC) y actividad de agua 0,78 resultó en alguna pérdida de viabilidad de las esporas a partir de la semana 16 en adelante.

El principal problema asociado con la contaminación por *B. cereus* radica en la presencia de esporas termorresistentes que pueden sobrevivir a las condiciones de cocción típicas, que suelen ser de alrededor de 15 minutos a 100ºC (Gilbert et al., 1974). Hay estudios que indican que durante el proceso de cocción del arroz se produce una reducción logarítmica significativa, entre 2 y 3 órdenes de magnitud, en la cantidad de esporas presentes. No obstante, el nivel de contaminación del producto cocinado depende en gran medida de la concentración inicial de esporas y de las

prácticas de higiene durante la manipulación del alimento (Vessoni-Penna & Moraes, 2002).

Después de la cocción, las esporas que quedan aún pueden germinar y crecer, alcanzando niveles de 10^7 a 10^9 UFC/g tras 24 horas a 26 o 32ºC respectivamente (Harmon & Kautter, 1991; Shelef & Liang, 1982); sin embargo, incluso a temperaturas más bajas, el crecimiento puede ocurrir, aunque a un ritmo más lento. Después de 10 días de almacenamiento a 8ºC, se observó un crecimiento considerable de *B. cereus*, que variaba entre 10^4 UFC/g y 10^8 UFC/g (Messelhäußer & Ehling-Schulz, 2018). Este comportamiento supone un riesgo importante cuando durante el almacenamiento se produce abuso de temperatura tanto en los lineales de supermercados como refrigeradores domésticos o en restaurantes.

2.3. Estrategias de tecnológicas para la desinfección y procesado alimentos con baja actividad de agua: Tecnología de plasma frío

Durante muchos años, se han investigado numerosos métodos para el procesado y descontaminación de alimentos con baja actividad de agua como son los granos, harinas y otros alimentos en polvo. En general, se han utilizado métodos químicos (soluciones a base de cloro, ozono, ácidos orgánicos, agua electrolizada), métodos biológicos (cultivos protectores, bacteriocinas, bacteriófagos, enzimáticos) y algunos métodos físicos (microondas, radiofrecuencia e irradiación gamma, luz ultravioleta, etc.) (Butscher et al., 2020). Sin embargo, los métodos químicos pueden a llegar a ser tóxicos y poco respetuosos con el medio ambiente, mientras que los enzimáticos son muy costosos y difíciles de controlar. Es por esta razón que las nuevas tecnologías físicas como altas presiones, pulsos eléctricos o plasma son las que están teniendo mucho interés en los últimos años. (Bourke et al., 2017; Laroque et al., 2022).

El plasma es considerado el cuarto estado de la materia debido a sus características únicas que lo hacen distinto de los tres estados básicos de la materia. En este contexto, se le define como un gas ionizado con buenas capacidades para conducir la electricidad (Bermúdez-Aguirre, 2020; Pankaj et al., 2018).

Se tienen registros de que el plasma fue utilizado en el año 1815 por Ernst Siemens, quien por primera vez lo había empleado para descontaminar el agua de contaminantes biológicos; sin embargo, no fue hasta el año 1928 en donde Langmuir utilizó por primera vez el término "plasma" para describir a un gas ionizado que contenía el mismo número de iones y electrones, que daba como resultado una región de cargas equilibradas (Bermudez-Aguirre, 2020; Misra et al., 2016).

Durante los años 60s, se hablaba del plasma como un buen método de higienización. Esta tecnología fue aplicada y patentada por primera vez en 1968 (Afshari & Hosseini, 2013). Sin embargo, no fue hasta los años 70s y 80s cuando se empezó a utilizar el plasma dentro de la industria informática y en los años 90s, gracias a los avances tecnológicos, se empieza a desarrollar un equipo capaz de trabajar en buenas condiciones para la desinfección y su posible aplicación en la industria alimentaria (Misra et al., 2016). Actualmente, se está estudiando como una alternativa prometedora para la modificación de componentes alimentarios y para la descontaminación de alimentos en general, ya que gracias tanto a las especies reactivas que genera y la radiación UV, ofrece una alta reactividad a temperaturas moderadas (<70ºC) importante para tratar alimentos sensibles a la temperatura (Bourke et al., 2018).

En los últimos años, cada vez hay más artículos sobre descontaminación por plasma para alimentos en general. Se han realizado estudios sobre alimentos con alta actividad de agua como carne y jamón, frutas y verduras, queso y huevos, zumos de frutas y en alimentos de baja actividad de agua como semillas y granos de cereales (Asl et al., 2022; Bourke et al., 2018; Butscher et al., 2020).

Se ha visto que, en los alimentos de baja actividad de agua como granos y semillas, la geometría del alimento puede afectar al campo eléctrico y como consecuencia a la eficacia de la inactivación por plasma. Así mismo, la superficie es un factor clave para la eficacia de la inactivación microbiológica, especialmente cuando se compara una superficie lisa con una superficie rugosa que puede tener pliegues profundos

pudiendo esto servir de protección a los microorganismos presentes en el alimento (Butscher et al., 2020).

La humedad desempeña un papel importante en los tratamientos por plasma, ya que tanto la humedad relativa del ambiente (Patil et al., 2014) como la del alimento pueden afectar a estos procesos. En el caso de los alimentos de baja actividad de agua, aunque inicialmente tienen un contenido de humedad reducido, debido a los cambios en la superficie del alimento durante el tratamiento, el agua residual contenida en el interior empieza a migrar a la superficie, ayudando a la generación de otras especies reactivas capaces de intensificar el efecto del plasma. Además, se ha visto que después del tratamiento por plasma, se produce un cambio notable en el contenido de materia seca, esto puede estar relacionado con la evaporación del agua debido a temperaturas más altas después del tratamiento con plasma (Alves Filho et al., 2020; Rao et al., 2023).

2.3.1. ¿Cómo se genera el plasma?

El plasma se genera gracias a la aplicación de un campo eléctrico o electromagnético a un gas, en ese momento, los electrones libres empiezan a captar la energía del medio y esta energía se eleva lo suficiente para que los átomos y moléculas con las que los electrones colisiona, empiecen a ionizarse y liberar más electrones, estos electrones liberados producen una disociación molecular que permite la formación de átomos y radicales libres que al retornar al estado estable, emiten un exceso de energía en forma de radiaciones electromagnéticas, incluyendo radiaciones UV (Laroque et al., 2022; Pedrow et al., 2020). En la Figura 2.1 se ilustran las interacciones entre el alimento y la nube de plasma en el interior de la cámara de tratamiento.

Los gases utilizados para generar plasma pueden ser: oxígeno (O_2), nitrógeno (N_2), aire atmosférico o gases nobles como el helio y el argón. La composición del gas define principalmente las especies reactivas formadas por ionización, influyendo en la eficiencia del plasma y su acción, siendo los gases nobles los más eficientes. También se ha visto que el aire atmosférico puede tener buenos efectos biocidas

gracias a la combinación de especies reactivas de oxígeno (ROS) y nitrógeno (RNS) que se generan (Bermudez-Aguirre, 2020; Pedrow et al., 2020).

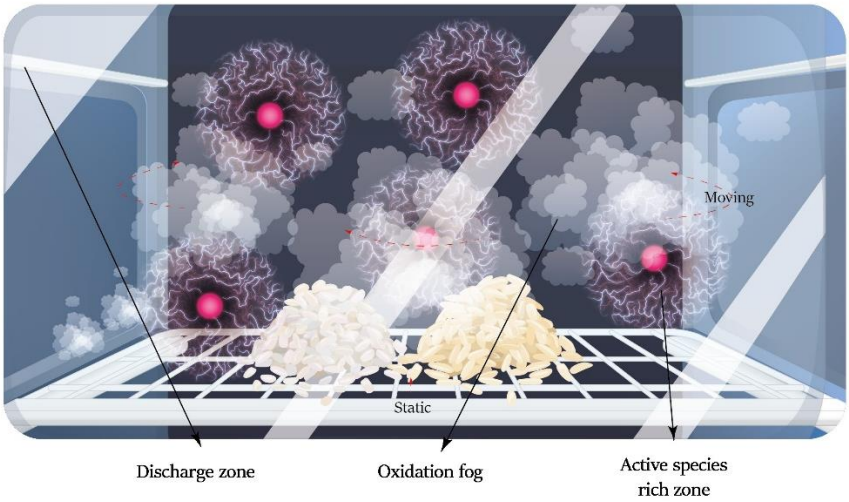


Figure 2.1. Interacción de las especies reactivas de plasma con la muestra de arroz.

Cuando se habla de tratamientos por plasma, es importante también tener en cuenta diversas condiciones de trabajo, como la presión y la temperatura. Hoy en día, existen equipos que permiten trabajar tanto en condiciones de vacío como a presión atmosférica. Además, el plasma puede generarse en diferentes estados térmicos, incluyendo altas temperaturas para el plasma en equilibrio térmico, temperaturas moderadas de alrededor de 100-105°C para el plasma en casi equilibrio, y temperaturas más bajas por debajo de los 70°C para el plasma en desequilibrio térmico (Misra et al., 2016).

En la actualidad, además existen distintas configuraciones de equipos para generar plasma. Los más utilizados son: descarga de barrera dieléctrica (DBD), descarga de corona (CD), plasma de radiofrecuencia (RF), plasma Jet (PJ) y microondas (MW) (Laroque et al., 2022; Misra et al., 2016).

A continuación, se describe con mayor detalle la configuración de los electrodos de los equipos de plasma DBD y plasma Jet, ya que actualmente son de los más

utilizados y han sido los equipos con los que se ha trabajado durante el desarrollo de esta tesis.

2.3.2. Equipos de plasma frío

- Descarga de barrera dieléctrica (DBD)

Es un equipo formado por dos electrodos separados por una barrera dieléctrica de cristal, cuarzo, cerámica o material polimérico que actúa como estabilizador y permite que se generen micro descargas y se obtenga un tratamiento más homogéneo (Laroque et al., 2022; Pankaj et al., 2018). Los electrodos están conectados a diferente voltaje y por lo tanto sometidos a una diferencia de potencial capaz de generar el plasma. El alimento se sitúa entre los dos electrodos y por lo tanto toda su superficie está expuesta a la acción del plasma generado como se muestra en la Figura 2.2.

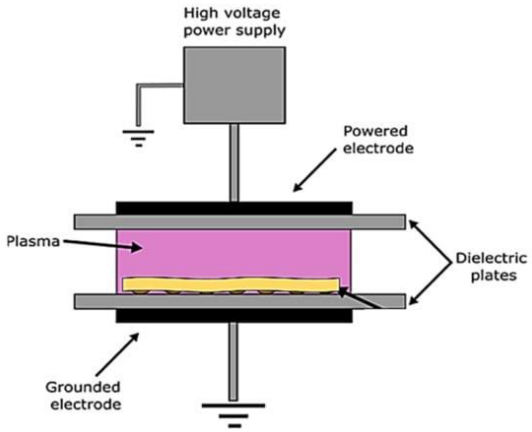


Figure 2.2. Esquema de un equipo de plasma DBD. Perera et al., (2022)

- Plasma Jet (PJ)

Está formado por dos electrodos entre los cuales fluye el plasma (Figura 2.3). El electrodo exterior está conectado a tierra y el electrodo central es excitado por RF; en este caso, el gas está fluyendo a una velocidad de flujo alta y va empujando al plasma formado fuera de la región del electrodo en forma de chorro, descargando especies de plasma en el ambiente abierto. Tiene la ventaja de producir una descarga estable,

homogénea y uniforme a presión atmosférica, aunque el alimento solo está sometido al tratamiento por la zona de exposición al jet de plasma (Laroque et al., 2022; Pankaj et al., 2018).

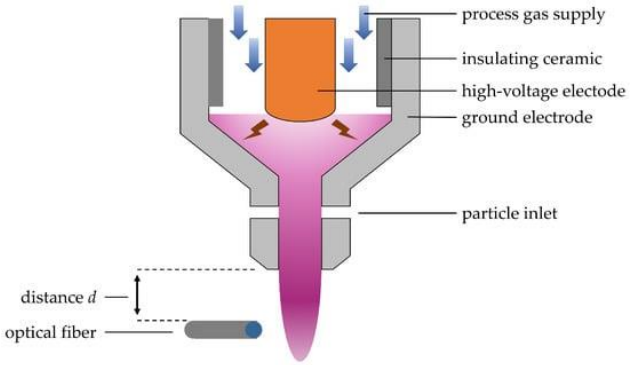


Figure 2.3. Esquema de un equipo de plasma JET. Mrotzek & viöl, (2022)

2.3.3. Efectos de la tecnología de plasma sobre la desinfección y control microbiológico de alimentos.

El efecto que tiene el plasma frío sobre las bacterias, se debe principalmente a la interacción entre los compuestos oxidantes del plasma y la radiación UV que también se genera con los componentes celulares de las bacterias, Figura 2.4. Las especies reactivas de oxígeno (ROS) como O, O_2, O_3, OH^- y las especies reactivas de nitrógeno (RNS) como NO y NO_2, que se forman al chocar las partículas cargadas con el gas o la humedad en el sustrato o microorganismo, provoca una desintegración de las paredes celulares y membranas mediante la oxidación, junto con la generación de componentes volátiles como CO, CO_2, H_2O (Bermudez-Aguirre, 2020; Bourke et al., 2017; Butscher et al., 2020; Laroque et al., 2022).

Todo ello, genera efectos en las células bacterianas que son capaces de provocar su muerte. En el caso de las bacterias y esporas, hay un daño a nivel de la membrana celular y de la capa más externa de la espora llamado exosporio y en el caso de virus hay un daño a nivel del ARN principalmente (Bermudez-Aguirre, 2020; Bourke et al., 2017).

Entre estos efectos están, la desnaturalización de proteínas, oxidación de compuestos peptídicos, formación de radicales de peróxido, oxidación de lípidos insaturados de la bicapa de la pared celular y cambios en la morfología celular. Todo ello provoca un estrés oxidativo y fallos en los mecanismos de defensa, osmorregulación y transporte, impidiendo la supervivencia y duplicación del microorganismo (Bermudez-Aguirre, 2020; Laroque, et al., 2022).

La efectividad del plasma sobre las esporas microbianas depende de la especie microbiana, de las condiciones de tratamiento y de las matrices empleadas (Bermudez-Aguirre, 2020; Bourke et al., 2017). Las esporas bacterianas poseen estructuras con múltiples capas que le da mayor resistencia a las condiciones ambientales y a diversas condiciones de procesado de alimentos; sin embargo, cuando se someten a tratamientos por plasma, las radiaciones UV que se generan producen una desnaturalización de proteínas, tanto de las que están presentes en la capa externa de la espora como las de tipo α/β (SASP), diseñadas específicamente para proteger el material genético contra el daño. En este caso, como consecuencia del tratamiento de la espora, el protoplasto experimenta una fuerte deshidratación, provocando una inmovilización de proteínas dentro de la espora. Así mismo, otro efecto sería el que causan los otros compuestos oxidantes del plasma, que reaccionan químicamente con los componentes celulares dando lugar a la formación de ácido nítrico y nitroso principalmente, que provocan fallos en sus funciones, destrucción de la estructura celular y por lo tanto su muerte (Wells-Bennik et al., 2016). En la Figura 2.4, se observa un esquema del efecto de las especies reactivas de plasma sobre los componentes celulares.

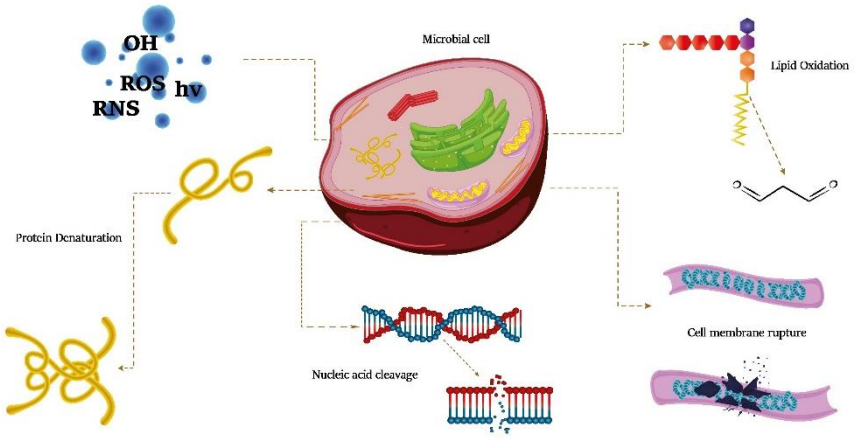


Figure 2.4. Efecto de las especies reactivas del plasma sobre los componentes celulares.

2.3.4. Efectos de la tecnología de plasma sobre los principales componentes de los alimentos.

A pesar de la extensa bibliografía existente sobre el efecto del plasma frío (CP) en las matrices alimentarias, tal efecto no está aun suficientemente descrito debido a la compleja composición de los alimentos y al efecto sinérgico que puede existir entre las especies reactivas del plasma y los componentes alimentarios. Por lo tanto, dilucidar el efecto del CP requiere de estudios específicos en cuanto a la calidad y composición de cada matriz después del tratamiento con plasma. En la Figura 2.5, se presenta un esquema detallado que ilustra los principales componentes de los alimentos afectados por el tratamiento con plasma.

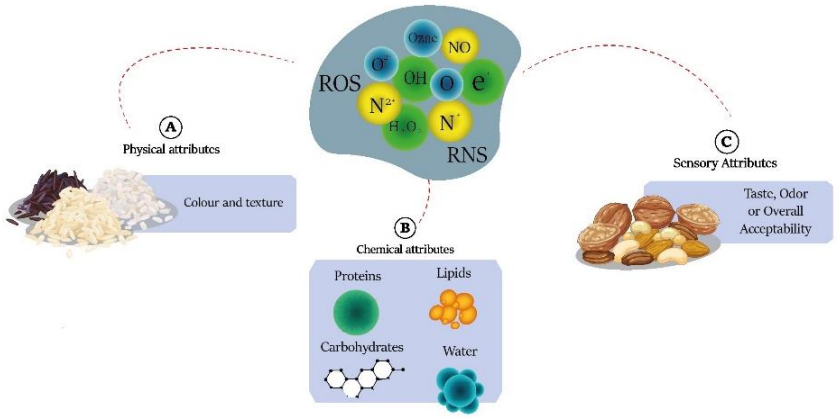


Figure 2.5. Efecto de las especies reactivas de plasma sobre los alimentos.

- Efecto sobre las proteínas

El tratamiento con CP ofrece un enfoque novedoso para mejorar las propiedades de las proteínas en general. Las especies reactivas de oxígeno (ROS) generadas por el plasma interactúan con las proteínas y modifican sus estructuras primarias, secundarias, terciarias y cuaternarias, lo que mejora sus propiedades funcionales y nutricionales (Rao et al., 2023). Los ROS empiezan a reaccionar con los aminoácidos de las proteínas, principalmente con los aminoácidos aromáticos y con grupos azufrados, se empiezan a dar reacciones de oxidación en la que algunos pueden oxidarse reversiblemente como el caso de la metionina que se oxida a sulfóxido de metionina, pudiendo actuar como un antioxidante endógeno, es decir protegiendo a los sitios sensibles de la proteína; algunos efectos similares se han encontrado en el triptófano. Además, se ha visto que al producirse una carbonilación de algunos aminoácidos como arginina, histidina, lisina, entre otros, se provoca la inactivación de algunas enzimas como la catalasa (Laroque et al., 2022; Misra et al., 2016).

Por otra parte, las proteínas también se pueden emplear en la industria como ingredientes, no sólo por sus altos valores nutricionales, sino también por sus propiedades tecnológicas y funcionales. Las principales propiedades tecno-funcionales de las proteínas, están relacionadas con la capacidad espumante, la

gelificación y la capacidad emulsificante, entre otras (Bußler et al., 2016; Pérez-Andrés et al., 2019) y una modificación de la composición o la estructura de las proteínas inducida por las especies reactivas del plasma, permitiría diseñar su funcionalidad para aplicaciones específicas (Tolouie et al., 2018).

- Efecto sobre lípidos

El efecto del CP sobre los lípidos de los alimentos depende de varios factores, entre los cuales está la composición grasa del alimento, así como de la potencia del tratamiento y del tipo de gas que se utilice (Gavahian et al., 2018; Rao et al., 2023). Las especies reactivas generadas por el plasma, pueden reaccionan con los grupos metilo de los alimentos, especialmente aquellos que contienen dobles y triples enlaces, que son más susceptibles a la oxidación y formación de radicales peróxido, afectando especialmente al ácido linoleico y el ácido α-linolénico, generando sabores y aromas desagradables en el producto (Alves Filho et al., 2020; Misra et al., 2016). La mayor parte de la oxidación de las grasas se debe al O_3, que reacciona directamente con los dobles enlaces de las cadenas de grasas insaturadas, formando productos de oxidación como ozónidos, aldehídos y ácidos carboxílicos (Laroque et al., 2022). A pesar de los efectos negativos que puede ocasionar el plasma en los alimentos, algunos autores sugieren que si se hace una optimización de los parámetros del proceso y ajustes en la formulación del producto (uso de antioxidantes), la aplicación de plasma en condiciones controladas puede ser interesante. Adicionalmente y en otro ámbito de trabajo puede ser una tecnología interesante para procesos de oxidación acelerada y como un proceso avanzado para tratar efluentes industriales (Gavahian et al., 2018; Laroque et al., 2022; Rao et al., 2023).

- Efecto sobre los carbohidratos

Como se ha comentado en apartados anteriores, el CP puede inducir una ozonólisis que causa una despolimerización y oxidación de macromoléculas. En concreto en lo que respecta a los carbohidratos, el efecto resulta en la descomposición de los oligosacáridos en moléculas de menor tamaño, aumentando del contenido de sacarosa (Pankaj et al., 2018).

Una especial atención merece el efecto del plasma sobre el almidón, ya que puede ser una técnica novedosa e interesante para su modificación. Gracias a las especies reactivas que se generan durante el tratamiento por plasma, se puede inducir modificaciones en la estructura del almidón, ya que los iones reactivos aumentan la energía superficial, incorporando grupos funcionales en su estructura e induciendo a la reticulación y despolimerización del almidón (Sun et al., 2022; Wongsagonsup et al., 2014), un efecto con mucho interés a nivel tecnológico.

Se ha visto que el CP puede modificar la estructura molecular, concretamente la de las moléculas de amilosa y amilopectina. Estas modificaciones pueden dar lugar a cambios en la estructura química de estos compuestos, que conlleven variaciones en sus propiedades tecnológicas. Así, características específicas como, las propiedades de hidratación, la capacidad hinchamiento, de absorción de agua (WAI) y su solubilidad (WSI), entre otras, se pueden ver afectadas como consecuencia de un tratamiento por CP, convirtiéndose en una estrategia sostenible para mejorar de manera selectiva determinadas propiedades tecnológicas de ingredientes de alimentos (Thirumdas, Trimukhe, et al., 2017).

- Efectos sobre vitaminas y antioxidantes

El efecto del plasma frío (CP) sobre las vitaminas y compuestos antioxidantes en los alimentos está influenciado por varios factores como el tiempo y potencia del tratamiento, así como el tipo de matriz alimentaria. Determinados estudios han demostrado que no hay una disminución significativa en vitaminas como la C, A y B con el tratamiento de CP. La cantidad de compuestos antioxidantes como flavonoides, polifenoles y vitamina C puede variar con el tratamiento de CP, siendo influenciada por los parámetros como la presión, potencia y tiempos de tratamiento (Pankaj et al., 2018; Sruthi et al., 2022a). El contenido de antioxidantes en el alimento puede ser muy controversial, ya que dependerá desde que punto de vista se analice, pues si bien, el plasma puede aumentar la biodisponibilidad de estos compuestos al descomponer moléculas, también puede provocar su disminución al reaccionar con especies reactivas. En alimentos con alto contenido de grasa, estos compuestos antioxidantes

pueden ayudar a reducir el enranciamiento, lo que puede ser beneficioso desde el punto de vista tecnológico.

- Efectos de CP sobre las propiedades físico-químicas y sensoriales

Así como se ha estudiado el efecto del plasma frío (CP) sobre los componentes nutricionales del alimento, su efecto sobre las propiedades sensoriales también es crucial en la industria alimentaria. Se ha observado que el tratamiento con CP puede influir en varios aspectos sensoriales y físico-químicos, como el color, el aroma, el sabor y la textura de los alimentos (Misra et al., 2016).

Se ha observado que el plasma puede ocasionar cambios en la coloración de los alimentos debido a la oxidación que provoca; así como también por causa de esta misma oxidación, provocar cambios en el sabor y el aroma, especialmente de aquellos alimentos ricos en grasas, lo que puede repercutir en la calidad del producto final. En cuanto a la textura, el CP puede influir en la firmeza y la jugosidad de los alimentos, aunque los efectos pueden variar según el tipo de alimento y las condiciones de tratamiento, por ejemplo, se ha visto que en productos como la carne, se obtuvo una mejor textura, por el cambio de la configuración proteica (Rao et al., 2023; Sruthi et al., 2022a). Por lo tanto, es posible la utilización del plasma frío para modificar de manera selectiva determinadas propiedades físico-químicas y sensoriales de los alimentos.

2.4. Estrategias para controlar el crecimiento de microorganismos: Antimicrobianos naturales.

Entre las estrategias para controlar el crecimiento de microorganismos, se encuentran algunos procedimientos físicos como la refrigeración y otros químicos como los antimicrobianos sintéticos y naturales.

Existe un interés creciente en el uso de compuestos naturales que permitan controlar el crecimiento microbiológico en los alimentos. Durante muchos años, se han empleado sustancias sintéticas como nitratos, benzoatos, sulfitos, sorbatos y

formaldehidos para este control. Sin embargo, estas sustancias, están asociadas a efectos secundarios sobre la salud, motivando esto a la búsqueda de alternativas naturales y más seguras (Ibáñez Peinado, 2022).

Los agentes antimicrobianos naturales pueden ser una alternativa adecuada, ya que se pueden obtener a partir de plantas, microorganismos, algas u hongos, y más sostenible desde el punto de vista medioambiental ya que muchos de ellos puede proceder de materias primas alternativas (insectos, algas, etc.) o incluso de subproductos industriales (Ibáñez Peinado, 2022). Entre los antimicrobianos naturales se ha prestado especial atención al quitosano dado que ha demostrado su capacidad para inhibir o inactivar a los microorganismos alteradores y patógenos durante el almacenamiento de los alimentos (Aranaz et al., 2021).

El quitosano es un biopolímero que ha ido ganando gran interés en los últimos años. Gracias a su biodegradabilidad, compatibilidad biológica, actividad antimicrobiana, antioxidante y alta seguridad (Aranaz et al., 2021; Avelelas et al., 2019), se ha visto que puede tener importantes aplicaciones en las industrias farmacéuticas, médicas y alimentarias (Aranaz et al., 2021; Rinaudo, 2006).

El quitosano fue descrito en el año 1859 y gracias a sus interesantes características, ha sido aprobado en Europa, Japón, Corea y Estados Unidos para utilizarlo como conservante alimentario y como antioxidante natural. Además, en los últimos años, ha ganado relevancia gracias a su potencial como antimicrobiano natural contra diversas bacterias Grampositivas y Gramnegativas (Abd El-Hack et al., 2020; Ke et al., 2021).

El quitosano, es un biopolímero de cadena lineal de glucosamina y N-acetilglucosamina. Se obtiene gracias a la desacetilación parcial de la quitina, un elemento presente en el exoesqueleto de los crustáceos e insectos (Shahidi et al., 1999). La quitina, es un compuesto blanco y rígido, es prácticamente insoluble en agua y en la mayoría de los disolventes orgánicos debido a sus fuertes enlaces de hidrógeno. Esta característica limita la aplicación industrial de la quitina; sin embargo, el grado de desacetilación de la quitina influye en la solubilidad del

quitosano obtenido, principalmente determinado por su peso molecular. Por lo general, el quitosano es soluble en soluciones ácidas con un pH entre 6,3 y 6,5 (Ibáñez Peinado, 2022), lo que amplía su potencial de uso industrial.

El efecto antimicrobiano del quitosano se atribuye a la interacción con la membrana y la pared celular de las bacterias, lo que altera la permeabilidad y causa daños estructurales que llevan a la muerte celular. Sin embargo, este efecto va a estar influenciado por algunos factores que se detallan a continuación (Ke et al., 2021).

- Naturaleza del microorganismo

En general, se ha visto que, las bacterias gramnegativas pueden ser más susceptibles debido a su alta carga negativa, que facilita la unión del quitosano catiónico con los fosfolípidos de la membrana celular especialmente en ambientes ácidos (Aranaz et al., 2021; Ke et al., 2021). Sin embargo, estudios también han demostrado la eficacia del quitosano contra bacterias grampositivas (Abd El-Hack et al., 2020).

Por otro lado, la fase de crecimiento en la que se encuentre el microorganismo también va a influenciar, ya que, debido a cambios en el número de cargas negativas en la superficie celular, las bacterias pueden ser más o menos sensibles al quitosano. Esta variación podría estar asociada con una mayor sensibilidad de las células en la fase exponencial final, seguida de las células en la fase estacionaria y en la fase exponencial intermedia (Ibáñez Peinado, 2022; Kong et al., 2008).

- Características físico-químicas del quitosano

La capacidad antimicrobiana del quitosano está determinada por su naturaleza y método de obtención, influenciada por factores como la densidad de carga, peso molecular, hidrofobicidad y capacidad quelante (Aranaz et al., 2021).

La densidad de carga positiva del quitosano está directamente relacionada a su grado de desacetilación, lo que afecta a su interacción con componentes celulares de carga opuesta; así mismo, el carácter hidrofílico/hidrofóbico del quitosano, también está determinado por la proporción de grupos acetilados y desacetilados, lo que influye en

sus interacciones y lo convierte en una molécula anfifílica (Ibáñez Peinado, 2022; Kumirska et al., 2011).

Por otra parte, el peso molecular y la capacidad quelante del quitosano están interrelacionados, ya que el quitosano de bajo peso molecular tiene un efecto tanto a nivel intracelular como extracelular, afectando funciones celulares, como la síntesis de ARN y proteínas por su capacidad de quelación y penetración. Mientras que el quitosano de alto peso molecular no tiene la capacidad de penetrar la pared celular, de tal manera que, su efecto se enfoca más en la quelación de metales que altera el paso de nutrientes a las células (Abd El-Hack et al., 2020; Aranaz et al., 2021; Ke et al., 2021).

- Factores medioambientales

Entre los factores medioambientales que pueden afectar a la capacidad antimicrobiana del quitosano está el pH, la fuerza iónica, la temperatura y el tiempo.

El pH del entorno influye en la capacidad antimicrobiana del quitosano, siendo más efectivo a valores bajos de pH, ya que a pH superiores a 6,5 hay mayor presencia de grupos amino no protonados y la solubilidad del quitosano disminuye a pH neutro. La fuerza iónica también afecta su capacidad, ya que un aumento de iones con carga positiva en el medio puede competir con los componentes negativos de la pared celular bacteriana. Además, la temperatura y el tiempo pueden alterar su capacidad antimicrobiana al afectar su peso molecular y otras características específicas como su densidad e hidrofobicidad (Ibáñez Peinado, 2022; Kumirska et al., 2011).

2.4.1. Quitosano de insecto

Actualmente la principal fuente de quitosano proviene del exoesqueleto de los crustáceos, sin embargo, debido a la sobreexplotación de recursos marinos, se están buscando alternativas que no tengan efectos adversos sobre la vida marina. El consumo de insectos tiene raíces milenarias en muchos países orientales y del sur de América, sin embargo, solo en los últimos años ha despertado gran interés industrial

a nivel de los países occidentales (Raheem et al., 2019). A partir de estos insectos comestibles como *Tenebrio molitor*, se pueden obtener productos innovadores y nutritivos como suplementos proteicos, snacks y alimentos para animales (Huis, 2013).

Un compuesto interesante presente en el exoesqueleto de los insectos es la quitina (Goy et al., 2009), que al igual que en los crustáceos, se puede procesar para obtener quitosano, como ya se mencionó en el apartado anterior.

En la actualidad, hay pocos estudios que se centran en determinar las propiedades del quitosano procedente de la quitina de insectos (Khayrova et al., 2021; Ma et al., 2022; Mohan et al., 2020). Hay que tener en cuenta que la FAO en el año 2013, realizó un informe que promueve el consumo de estos alimentos gracias a su alto valor proteico, fomentando su inclusión en la dieta humana y animal. Este incremento en el consumo de insectos puede llevar a un incremento de residuos, especialmente los exoesqueletos, cuya revalorización puede tener un gran impacto industrial como antimicrobiano, en cosmética, o complemento alimentario (Huis, 2013).

Por lo tanto, es necesario investigar, sobre las propiedades y efectos del quitosano de insecto para su posible aplicación industrial, como una fuente alternativa de quitina, especialmente para abordar desafíos relacionados con la inocuidad alimentaria.

2.4.2. Tecnología de barreras para el control de microorganismos patógenos: combinación de antimicrobianos naturales con tratamiento térmico

Dada la complejidad de los factores que determinan las propiedades de resistencia de determinados microorganismos, resulta necesario desarrollar estrategias efectivas que ayuden a su control, minimizando el impacto sobre las propiedades del alimento fresco. Una de estas estrategias es la tecnología de barreras que consiste en la aplicación de diferentes técnicas de conservación a baja intensidad y de manera consecutiva de modo que se alcance una inactivación microbiana suficiente para garantizar la inocuidad alimentaria, pero con el menor impacto posible sobre las

propiedades organolépticas, físico-químicas y nutricionales del alimento (Khan et al., 2017). Su objetivo último es producir alimentos de alta calidad. En este sentido, se ha visto que el tratamiento combinado de métodos físicos y químicos como el tratamiento térmico con antimicrobianos naturales puede ser sinérgico y eficaz para controlar el crecimiento de determinados microorganismos (Cho & Chung, 2020). Estudios recientes han demostrado la eficacia de estas combinaciones; por ejemplo, se ha visto que la combinación del uso de extracto del arbusto *Vitex-negundo* y un tratamiento de 60, 65 y 70ºC, ayudó a un control eficaz de *S. aureus* y *Shigella flexneri* en ensaladas de pollo (Jafarpour et al., 2022). Otro estudio sobre el efecto antimicrobiano sinérgico entre el extracto de *Dryopteris erythrosora* y un tratamiento térmico suave contra *Staphylococcus aureus*, se vio que se logró una reducción aproximada de 6 ciclos logarítmicos en matrices inertes utilizando el tratamiento combinado (Yun & Bai, 2023). Así mismo, en otro estudio demostraron que la combinación de nisina y carvacrol o timol era muy eficaz a pH neutro contra las células vegetativas de *B. cereus*, especialmente cuando las células estaban expuestas a un suave pretratamiento térmico (Periago et al., 2001).

Al igual que ocurre con los antimicrobianos naturales mencionados en los párrafos anteriores, el quitosano se puede combinar con el tratamiento térmico para contribuir a la estabilización de los alimentos cocidos durante el almacenamiento dado el efecto antimicrobiano que se ha observado en dicho producto. En el caso de productos derivados de arroz, esta podría ser una de las estrategias que se sugiere para el control del crecimiento de *B. cereus*, como una medida adicional al tratamiento térmico de productos listos para el consumo. La combinación de estas dos estrategias, posibilita el empleo de temperaturas de cocción más suaves, sin comprometer la calidad del producto final. Si bien, en este caso el tratamiento térmico es fundamental para su consumo, en el caso de microorganismos esporulados, puede no ser suficiente para garantizar la inocuidad alimentaria (Huertas et al., 2014; Khan et al., 2017).

Por lo tanto, el uso de quitosano de insecto procedente de insectos comestibles como las larvas de *Tenebrio molitor*, combinado con tratamientos térmicos suaves puede representar una estrategia efectiva para controlar bacterias patógenas como *B. cereus*

en productos alimenticios, garantizando la seguridad y conservando la calidad nutricional y sensorial.

2.5. Evaluación industrial del riesgo

La evaluación de riesgos es el proceso científico que permite determinar la relación entre la exposición a un peligro determinado, bajo un conjunto de condiciones, y la probabilidad de un efecto adverso para la salud o una enfermedad (Jouve, 2000), permitiendo evaluar de manera sistemática los riesgos potenciales asociados con los peligros biológicos en los alimentos.

La evaluación de riesgo microbiológico se subdivide en cuatro etapas principales:

1. Identificación del peligro
2. Caracterización del peligro
3. Evaluación de la exposición
4. Caracterización de riesgos

Aunque las evaluaciones de riesgos, bien cualitativas, semicuantitativas o cuantitativas tienen como fundamento la toma de decisión por parte de los organismos de salud pública, esta herramienta también se puede aplicar a nivel industrial. La introducción de la evaluación de riesgos a nivel industrial sirve de apoyo al Análisis de Peligros y Control de Puntos Críticos, ya que permite que esta herramienta se fundamente en datos objetivos cuantitativos (Membré & Boué, 2018).

A nivel industrial, la etapa más importante es la evaluación de la exposición ya que permitirá llevar a cabo medidas de gestión en la propia industria. La evaluación de la exposición, es una etapa que permite comprender y cuantificar la probabilidad de cómo y en qué medida los consumidores pueden estar expuestos a peligros microbiológicos presentes en los alimentos, considerando los cambios necesarios desde materias primas, procesos y nuevos métodos de conservación hasta el consumo (McLauchlin et al., 2004; Membré & Boué, 2018). Este proceso implica evaluar varios factores, como los métodos de procesado de alimentos, las condiciones de

almacenamiento, las prácticas de manipulación y los patrones de consumo, para estimar el nivel de contaminación microbiana que los consumidores probablemente encontrarán a lo largo de la cadena alimentaria (Membré & Boué, 2018).

Para llevar a cabo una evaluación de la exposición cuantitativa uno de los métodos más utilizados es la simulación Montecarlo. La simulación Montecarlo es un método no determinista utilizado para aproximar de una manera precisa y clara aquellas expresiones matemáticas que son difíciles y costosas de evaluar. Se dice que es un método no determinista porque permite resolver una expresión matemática obteniendo números aleatorios para conseguir resultados aproximativos sobre el comportamiento de un efecto en estudio. Todos los resultados obtenidos se promedian para obtener un valor aproximado al deseado. La simulación Montecarlo puede ser muy útil en el caso de que no se pueda resolver una expresión matemática de manera analítica y exacta o cuando hay muchas variables involucradas (Machain, 2015). Como resultado de la simulación se puede obtener un análisis de sensibilidad o un análisis de optimización.

El análisis de sensibilidad permite determinar la influencia que tienen las variables de entrada “inputs” sobre las variables de salida “outputs”, de tal manera que se puedan identificar las variables de entrada que mayor efecto tienen sobre las variables de salida con valores numéricos de los coeficientes de correlación (Machain, 2015; Pina Pérez, 2011). Una vez que conocemos cuales son las variables que más influyen en la salida, se puede llevar a cabo el análisis de optimización con objeto de maximizar el efecto de estas variables de entrada sobre la variable de salida.

3. OBJETIVOS

3. Objetivos

El objetivo general de la presente tesis doctoral es estudiar el efecto de diferentes estrategias tecnológicas tanto sobre el control microbiológico de materias primas de baja actividad de agua (arroz) y de productos elaborados (arroz cocido), así como sobre la modificación selectiva de determinadas propiedades tecnológicas de dichos alimentos.

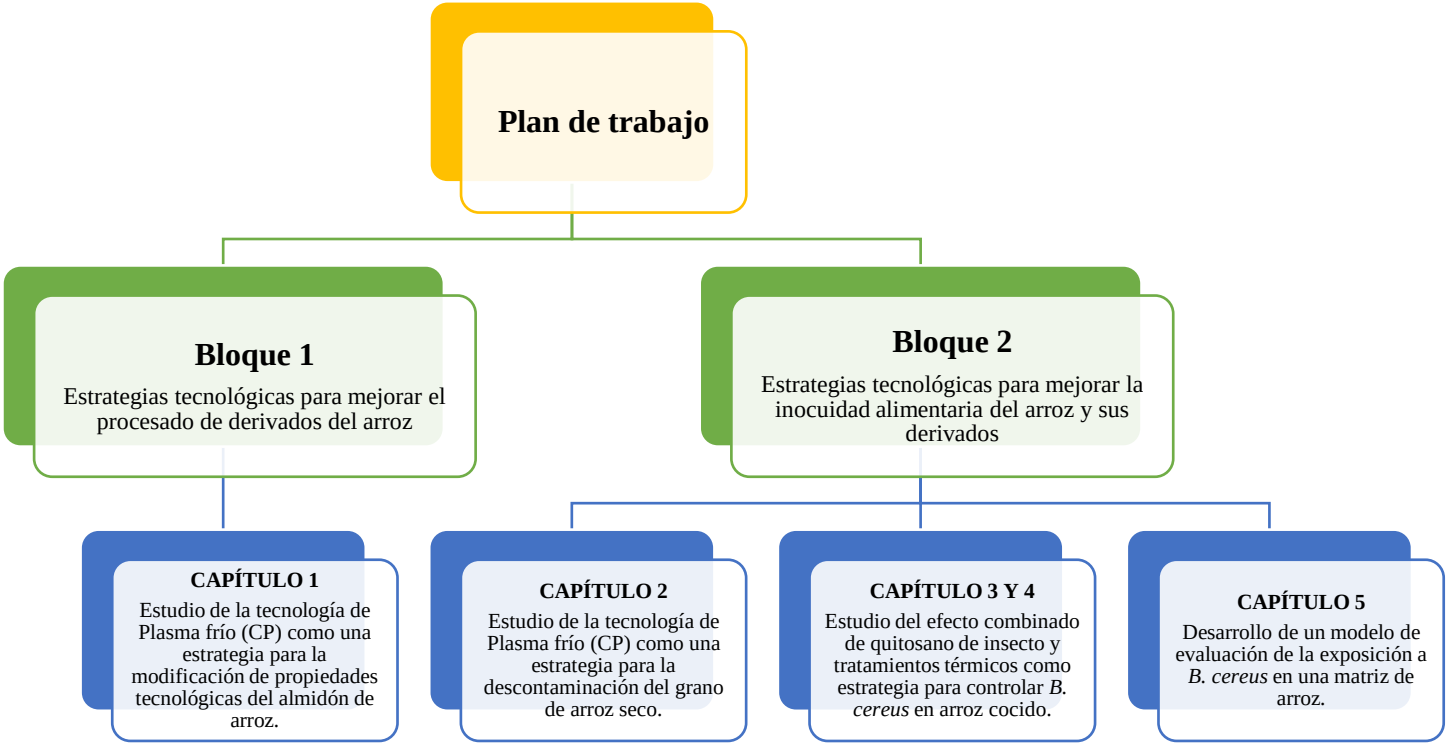
Este objetivo general se concreta en los siguientes objetivos particulares:

- Estudiar el efecto del uso de la tecnología de plasma frío como una estrategia para la modificación selectiva de propiedades tecnológicas de matrices alimentarias derivadas del arroz, como el almidón.
- Estudiar el efecto del uso de la tecnología de plasma frío como una estrategia para la descontaminación de materias primas de baja actividad de agua (arroz).
- Estudiar el efecto conjunto del uso de un antimicrobiano natural como el quitosano de insecto (*Tenebrio molitor*) con tratamientos térmicos, como una estrategia para controlar el crecimiento de *B. cereus* en una matriz de arroz cocido.
- Desarrollar un modelo de evaluación de la exposición a *B. cereus* en una matriz de arroz cocido, de manera que éste aporte información de interés industrial y facilite la gestión de la inocuidad alimentaria en la propia industria.

4. PLAN DE TRABAJO

4. Plan de trabajo

Para cumplir con los objetivos descritos se propone el siguiente plan de trabajo.



5. RESULTADOS

CAPÍTULO 5.1

ESTUDIO DE LA TECNOLOGÍA DE PLASMA FRÍO (CP) COMO UNA ESTRATEGIA PARA LA MODIFICACIÓN DE PROPIEDADES TECNOLÓGICAS DEL ALMIDÓN DE ARROZ.

Study of the Impact of Plasma Treatment on the Physicochemical Properties of Rice Starch from Different Varieties: Basmati and Japonica rice

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Abstract

Rice starch has a great interest in recent years, due to its techno-functional properties with a wide application at a technological level in the food industry. Plasma technology, as an emerging method of non-thermal food processing, offers an alternative to modify starch without resorting to chemical or enzymatic treatments, which can be costly and difficult to handle. In this study, starch samples from two rice varieties, Basmati and Japonica, as well as a commercial starch, were treated with Thermal Plasma Jet equipment and were analysed. The treatments were carried out for 20 and 40 minutes, using compressed air as ionization gas. Results indicated that moisture content decreased with increasing treatment time, and significant changes in colour and increase in colour difference (ΔE), as the treatment progressed. Viscosity properties increased in the Basmati starch (BS) and Japonica starch (JS), while they decreased evidently in the commercial starch, the latter being the most unstable under treatment, to the point of being unable to form a gel after 40 min of treatment. As for flour hydration properties, no significant changes were observed, but significant changes were observed in gel hydration properties, especially in the Basmati starch and commercial starch (CS). Therefore, it can be affirmed that plasma treatment induces significant changes in the techno functional and physicochemical properties, especially in the BS and CS samples. Therefore, plasma treatment causes interesting changes in the molecular structure of starches and offers an interesting alternative for their modification according to the properties desired for their industrial application.

Key words: Rice starch, Plasma Treatment, Starch modification, Basmati, Japonica

5.1.1. Introduction

Rice (*Oryza sativa*) is one of the most widely cultivated crops globally and nowadays different rice species have become more widely consumed. Rice is rich in starch and generally starch contains about 25% amylose and 75% amylopectin, being amylose a linear-chain polysaccharide and amylopectin a branched-chain polysaccharide (Huang et al., 2007). The amount of amylose or amylopectin can vary depending on the rice species. **Basmati** rice is long, slender grains and pleasant aroma which is highly valued for its aromatic qualities and is predominantly consumed in India and Pakistan. Its moderate amylose content contributes to its specific characteristics features (Bhattacharjee et al., 2002). On the other hand, **Japonica** rice is known for its oval and round grains, with higher yields compared to Indica rice. Japonica rice typically has a higher amylopectin content compared to other rice species, resulting in a stickier texture (Luo et al., 2021).Different rice varieties may have different amylose and amylopectin starch compositions, which can influence their techno-functional properties, so that a variety-dependent modification of these properties may positively affect the starch characteristics, which in turn could be relevant for specific culinary or industrial applications (Huang et al., 2007).

Starch is highly valued in various sectors such as food, textile, pharmaceutical, and cosmetic industries due to its versatile applications. However, it encounters challenges regarding temperature stability and industrial processes. As a result, significant research efforts have been directed towards modifying it through physical, chemical, and enzymatic methods, since modified starches are widely used in food processing because they have better functional qualities than their natural analogues. Therefore, physically modified starches are preferred over chemically and enzymatically modified ones because they can be improved without introducing foreign compounds, which aligns with customers' growing desire for safe, additive-free products (Gupta et al., 2023). Physical modifications, leveraging innovative technologies, emerge as a promising and less intrusive option to enhance its properties in the food industry (Verma & Srivastav, 2022).

Previous studies (Sun et al., 2022; Thirumdas, Trimukhe, et al., 2017), have noticed that plasma technology can be a novel and interesting technique for starch modification. **Plasma** is a non-thermal technology based on the ionization of a gas (such as air, O_2, N_2, Argon, or helium), allowing the generation of reactive species like O^-, O_2^-, O^+, N^+, N_2^-, NO^+, CO_2^+, which can induce modifications in the starch structure. These reactive ions increase the surface energy, incorporating functional groups into the starch structure, and inducing starch cross-linking and depolymerization (Sun et al., 2022; Wongsagonsup et al., 2014).

Therefore, the aim of this research was to study the effect of plasma treatment, using air as an ionization gas, on the physicochemical properties of rice starch obtained from different varieties, including Japonica and Basmati, as well as a commercial starch. This study sought to evaluate the effectiveness of starch modification, assess the stability of the induced changes, and explore how the treatment affects the final properties of the starch. These findings are intended to provide a broader understanding for potential industrial applications.

5.2.1. Materials and methods

5.2.1.1. Starch extraction

For this study, rice starch was extracted from two rice varieties, Basmati (long grain) and Japonica (short grain). These varieties were acquired from a local market and Sigma rice starch was used as commercial reference starch.

An alkaline extraction method based on Souza et al., (2016) method was employed to extract rice starch. This involved crushing rice to obtain rice flour as the raw material. The rice flour was immersed in a 0.18% NaOH solution at 30ºC for 30 min. Afterward, the solution was centrifuged at 3 380 x g for 5 min, and the extracted starch was washed and dried in an oven at 40ºC for 24h. The dry starch was milled and kept at room temperature until it was used. This method was chosen for its quicker process and shorter soaking time in NaOH to achieve adequate starch extraction. The aim was to ensure that the extraction process minimally altered the starch structure, making it as gentle as possible.

5.2.1.2. Starch plasma treatment

Once the rice starch was extracted, thermal plasma treatments were carried out using a plasma beam system (Diener electronic GmbH & Co. KG, Ebhausen, Germany) operating at 20 kHz. This operates at atmospheric pressure and is equipped with a condenser; cooling coil was used to maintain the temperature of the activated gas generated by the plasma at ambient temperature. Cold water (4 ± 0.5 °C) was circulated through the condenser using external refrigerating system (Lauda Ecoline, RE104). The high voltage generator produced a voltage up to 10 kV and compressed air was used as the ionization gas. Two treatments were chosen based on the preliminary experiments as 20 and 40 minutes. The starch sample was placed in a gas diffuser so that the ionized air passes through tubes, ensuring homogeneous diffusion of the gas throughout the treatment.

5.2.1.3. Starch moisture

The moisture content was determined following the AACC 44-15.02-Moisture-Air-Oven Method. Approximately 3 g of starch sample were weighed into metal containers and placed in an oven at 100°C for approximately 24 hours or until constant weight is achieved using an oven (Memmert GmbH+Co. KG, Schwabach, Germany).

5.2.1.4. Starch colour

Colour was measured with a HunterLab UltraScan Pro CIE Lab* colorimeter equipped with a dual-beam xenon flash spectrophotometer (Hunter Associates Laboratory, Inc., Reston, VA, USA). Calibration was performed using a light trap (L = 0) and a transparent adhesive film covering a standard white tile. Colour readings were recorded in the CIE (Commission Internationale de l'Éclairage) colour space format with outputs of L^* (lightness), a^* (redness), and b^* (yellowness). The samples were wrapped in transparent cling film. Total colour difference (ΔE) (Eq. 1) caused by sample oxidation during plasma treatment was calculated using the L^*, a^*, and b^*

readings of the untreated control sample as reference values and compared with the values obtained after each treatment as shown in Equation 1.

$$\Delta E = ((L^* - L^*_{r)} + (a^* - a^*_{r)} + (b^* - b^*_{r})^2)^{1/2} \quad (1)$$

Where L^* is lightness, a^* is redness and b^* is yellowness. The subscript r indicates the reference value.

5.1.2.5. Starch pasting properties

The pasting properties of rice starch were determined using a Rapid Visco Analyzer (RVA) (Newport Scientific Pty Ltd, Warriewood, Australia) according to the method of Thirumdas et al. (2017). Viscosity profiles of starches from various rice species were recorded using starch suspensions, with moisture weight correction to ensure uniform results. The solution relation was 3:25 w/w. Temperature and time parameters included a heating phase from 50 to 95 °C at a rate of 6 °C/min (after an equilibration period of 1 minute at 50 °C), followed by a holding period of 1.5 minutes at 95 °C, a cooling phase from 95 to 50 °C at 6 °C/min, and a holding period of 2 minutes at 50 °C, conditions set out in accordance with the equipment manual.

5.1.2.6. Texture gel properties

Starch gel texture was measured using a Texture Analyzer (TA-XT2i, Stable Microsystems, Surrey UK) by two-cycle compression of 40% sample initial height. A stainless probe with 36 mm diameter (P/36R) compressed the sample at a 5 mm s^{-1} compressive strength of 10 g. Hardness, springiness, and cohesiveness were derived from the texture profile analysis (TPA) graph, where hardness was the maximum force of the first peak, springiness was the ratio of distance to peak 2 to distance to peak 1, and cohesiveness was the ratio of the second positive area under the compression curve to that of the first positive area.

5.1.2.7. Gel hydration properties

The gel hydration properties of starch such as water absorption index (WAI), water solubility index (WSI) and swelling power (SP) were determined following the protocols of Rosell et al., (2011), Thirumdas, Deshmukh, et al., (2016) and Thirumdas, Kadam, et al., (2017) studies.

5.1.2.8. Flour hydration properties

The water holding capacity (WHC) of a sample was determined by soaking 1 g in 10 ml of distilled water for 24 hours and then measuring the water retained after discarding the supernatant. Water binding capacity (WBC) was measured by adding 10 mL of water to 1 g of sample, centrifuging at 2000 x g for 10 minutes, and then reweighing after discarding the supernatant to determine force-bound water. The Oil Holding Capacity (OHC) was determined by mixing 100 mg of sample with 1 ml of vegetable oil, followed by shaking, vortex and centrifugation at 3000 x g for 10 minutes at 4ºC and then, the sample was reweighed to measure oil retention. Gel hydration properties was determined heating 50 mg sample with 1 ml of distilled water at 90ºC for 10 min, followed by cooling in an ice water bath for 10 min and centrifuged at 3000 x g at 4ºC for 10 min, to evaluate water absorption under thermal conditions. The supernatant was decanted into small pre-weighed glass vials and the weight of dry solids was recovered by evaporating the supernatant at 105ºC to constant weight.

5.1.2.9. Statistical analysis

The statistical significance of the data obtained was determined by analysis of variance (ANOVA) (p-value <0.05), and the differences between groups were determined by the Fisher's Least Significant Difference (LSD) test, which identifies homogeneous subsets of means that do not differ from each other. This analysis was performed on the mean data obtained in each determination, analysing the differences between each plasma treatment. Statgraphics Centurion XIX software (version 19.6.03, copyright Statgraphics Technologies, Inc.) was used.

5.1.3. Results and discussion

5.1.3.1. Effect of plasma treatment on rice starch moisture

In Table 5.1.1, significant differences (p value <0.05) are observed between the control sample and the samples treated with plasma for 20 minutes and 40 minutes. For Basmati starch (BS), the moisture decreased from 9.27 ± 0.06 (g/100 g) to 7.37 ± 0.19 (g/100 g), while for Japonica starch (JS) it decreased from 7.97 ± 0.04 (g/100 g) to 4.77 ± 0.05 (g/100 g) and for Commercial starch (CS), there was a significant decrease from 11.29 ± 0.11 (g/100g) to 10.10 ± 0.07 (g/100g). However, no significant differences were observed when increasing the treatment time from 20 to 40 minutes for the BS and JS samples. On the contrary, for the CS, a decrease was recorded from 10.10 ± 0.07 (g/100 g) to 9.89 ± 0.03 (g/100 g) after 40 minutes of treatment.

Thirumdas, Trimukhe, et al., (2017) investigated the effect of low-pressure cold plasma on brown rice and observed a similar behaviour, as the treatment time and power increased, the moisture content of the samples decreased. The decrease in moisture content in the samples may be attributed to various processes occurring during plasma treatment, such as desorption, decomposition, and changes in the starch structure. These processes result from the interactions between the sample and the ions generated by the plasma, leading to the decomposition of some hydroxyl groups in the polymers. This damages the starch structure, causing it to lose its water retention capacity. Additionally, the water released from the surface also ionizes and forms ions that may enhance the treatment effect (Sun et al., 2022; Verma & Srivastav, 2022; Zou et al., 2004). Despite the significant decrease in moisture content in BS and JS samples after 20 minutes of treatment, no significant difference was observed between 20 and 40 minutes of treatment. This could be because the initial moisture content is already low, indicating that it is strongly bound water within the starch structure. Therefore, even with increasing the treatment time, the tightly bound water is more difficult to remove.

Table 5.1.1. Moisture and Colour content of rice starch after plasma treatment (g/100g)

Sample		Moisture (g/100g)	Colour			
			*L**	*a**	*b**	ΔE
Basmati starch	Control	9.27 ± 0.06 [b]	97.98 ± 0.01 [b]	-0.34 ± 0.01 [a]	2.75 ± 0.01 [a]	
	20 min	7.37 ± 0.19 [a]	97.28 ± 0.23 [a]	0.07 ± 0.04 [b]	3.50 ± 0.05 [b]	0.71 ± 0.11 [a]
	40 min	7.44 ± 0.17 [a]	97.25 ± 0.04 [a]	0.11 ± 0.02 [b]	3.70 ± 0.05 [c]	0.82 ± 0.05 [b]
Japonica starch	Control	7.97 ± 0.04 [b]	98.56 ± 0.01 [c]	-0.22 ± 0.02 [a]	1.98 ± 0.01 [a]	
	20 min	4.77 ± 0.05 [a]	98.17 ± 0.06 [b]	0.06 ± 0.04 [b]	3.02 ± 0.09 [b]	0.66 ± 0.12 [a]
	40 min	4.86 ± 0.05 [a]	97.70 ± 0.12 [a]	0.25 ± 0.01 [c]	3.15 ± 0.07 [b]	1.17 ± 0.18 [b]
Commercial Starch	Control	11.29 ± 0.11 [c]	99.99 ± 0.09 [b]	-0.33 ± 0.02 [b]	1.04 ± 0.02 [a]	
	20 min	10.10 ± 0.07 [b]	99.07 ± 0.22 [a, b]	-0.76 ± 0.07 [a]	3.26 ± 0.46 [b]	2.99 ± 0.11 [a]
	40 min	9.89 ± 0.03 [a]	98.26 ± 1.14 [a]	-0.67 ± 0.07 [a]	3.44 ± 0.09 [b]	4.43 ± 0.15 [b]

Mean data ± SD. Lower case letters (a, b and c) indicate statistically significant differences between the different study groups for each treatment. ANOVA (p-value<0.05)

On the other hand, when observing the moisture loss among the three samples it's noted that the starch of BS and CS exhibits a similar proportion of moisture loss, between 1 and 2%. However, the JS sample experienced a higher loss, nearly 3%. This could be attributed to the fact that this variety has a higher amount of amylopectin. With the plasma treatment, changes in its internal molecular structure, especially in the amylopectin, may have occurred. The breaking of amylopectin bonds, caused by the reactive species from the plasma, could have led to a decrease in water retention capacity (Lu et al., 2011). These findings will be further corroborated in the subsequent sections, where other results will be analysed in more detail.

5.1.3.2. Effect of plasma treatment on rice starch colour

As seen in Table 5.1.1, the colour variation (ΔE) increases as the treatment time increases for all three samples. The ΔE values for Basmati rice starch (BS) were 0.71 ± 0.11 at 20 minutes of treatment and 0.82 ± 0.05 at 40 minutes. For Japonica rice starch (JS), the ΔE values were 0.66 ± 0.12 at 20 minutes and 1.17 ± 0.18 at 40 minutes. On the other hand, for Commercial rice starch (CS), the ΔE values were higher, reaching 2.99 ± 0.11 at 20 minutes and 4.43 ± 0.15 at 40 minutes of treatment. The values of $\Delta E < 2$ for BS and JS suggest a small and imperceptible difference, according to the CIE classification criteria (Mokrzycki & Tatol, 2011). However, for CS samples, where $\Delta E > 2$, the change is recognizable to the observer. With these results, it can be observed that the starches extracted from BS and JS are more stable against colour changes due to plasma treatment, as evidenced by the significant variation observed in the CS starch.

After conducting an analysis of variance (ANOVA) to identify significant differences among the L^*, a^*, and b^* values of the treated samples (BS, JS, and CS), some observations were made. In the case of BS, both L^* and a^* values exhibit significant differences after 20 minutes of treatment, but this colour alteration does not increase significantly after 40 minutes. However, there is a noteworthy difference in the b^* value between the 20 and 40-minute treatment times. Similarly, for JS, both L^* and

*a** values demonstrate significant differences as the treatment duration increases, though there are no significant disparities in the *b** value between the 20 and 40-minute treatments, while for CS, the values of *a** and *b** exhibit significant variation with 20 minutes of plasma treatment, although the *L** value decreases, it does not do so significantly after 20 minutes of treatment, only there is a significant difference at 40 minutes of treatment.

Few studies (Beyrer et al., 2020; Sruthi et al., 2022) on plasma treatment and its effect on the coloration of foods in general, have indicated that changes in sample coloration depend on the reactive species formed, and hence on the ionization gas used. Generally, when air is used as the ionization gas, reactive oxygen species (ROS) and reactive nitrogen species (RNS) can be generated, leading to significant colour changes. Specifically, ROS have a greater capacity to modify colour compared to RNS. Additionally, another phenomenon that can also be associated with this change in coloration is the Maillard reaction. Thanks to the reactive species generated by the plasma, this reaction can oxidize reducing sugars, leading to the appearance of brown compounds and resulting in a more yellowish coloration, as observed in the *b** values of this study (Gupta et al., 2024; Liu et al., 2021).

On the other hand, Gupta et al., (2023) investigated the effect of atmospheric dielectric barrier discharge (DBD) plasma on the physicochemical properties of taro starch. They observed that luminosity increased as treatment time and power increased. With a maximum power of 34 kV and a maximum time of 8 minutes, they obtained *L** values of 97.52 ± 0.14, similar to those obtained in our study for the BS and JS samples after 40 minutes of treatment. However, in our study, the values decreased as treatment time increased, which could be explained by oxidation of rice components due to prolonged exposure to reactive plasma species.

5.1.3.3. Effect of plasma treatment on rice starch pasting properties

Based on the data from Table 5.1.2, it can be observed that in the BS starch sample, after 20 minutes of treatment, all values increased. Peak viscosity (PV) rose from 1821.6 ± 16.83 cP to 2118.67 ± 38.51 cP, breakdown (BD) increased from 405.33 ±

11.50 cP to 475.50 ± 23.07 cP, setback (SB) rose from 2034.50 ± 13.44 cP to 2069.00 ± 31.68 cP, and final viscosity (FV) increased from 3423.75 ± 20.73 cP to 3717.50 ± 49.67 cP. However, after 40 minutes of treatment, some values decreased, such as BD to 336.50 ± 36.54 cP, SB to 1664.33 ± 25.15 cP, and FV to 3426.67 ± 54.42 cP. Nevertheless, SB values remained higher than BD, and the final viscosity did not significantly differ from the control sample.

Regarding the JS samples, a modification is also evident after plasma treatment. After 20 minutes of treatment, the breakdown (BD) significantly decreased from 846.67 ± 9.29 cP to 377.75 ± 5.56 cP. However, the values of peak temperature (PT), peak viscosity (PV), setback (SB), and final viscosity (FV) increased significantly compared to the control sample. The PT increased from 75.08 ± 0.04 ºC to 78.77 ± 0.49 ºC, PV from 2255.67 ± 7.51 cP to 2517.67 ± 10.02 cP, SB from 1091.50 ± 2.12 cP to 1228.50 ± 7.68 cP, and FV from 2508.00 ± 2.65 cP to 3291.67 ± 17.62 cP.

Furthermore, although after 40 minutes of treatment all these values decreased significantly, with PV at 2057.75 ± 16.82 cP, SB at 1133.67 ± 13.20 cP, and FV at 2841.00 ± 7.94 cP, the SB and FV values remained higher than those of the control sample, and the BD value (342.25 ± 10.44 cP) remained lower than the control sample.

On the other hand, examining the data obtained for CS in Table 5.1.2, it is evident that this sample is the most susceptible to plasma treatment, as a significant change in the data can be observed after just 20 minutes of treatment. PT, PV, and BD increased significantly compared to the control sample, with PT increasing from 83.18 ± 0.25ºC to 90.08 ± 0.46ºC, PV from 2207.00 ± 12.73ºC to 2240.00 ± 3.61ºC, and BD from 675.00 ± 24.04ºC to 881.20 ± 10.33ºC, while the SB and FV values decreased significantly from 1363.50 ± 10.61 cP to 734.00 ± 23.02 cP and from 2895.50 ± 0.71 cP to 2051.50 ± 77.74 cP, respectively.

Table 5.1.2. Pasting properties of rice starch after plasma treatment.

Sample		Pasting temp (PT) ºC	Peak Viscosity (PV) cP	Breakdown (BD) cP	Setback (SB) cP	Final viscosity (FV) cP
Basmati starch	Control	83.67 ± 0.49 [a]	1821.6 ± 16.83 [a]	405.33 ± 11.50 [b]	2034.50 ± 13.44 [b]	3423.75 ± 20.73 [a]
	20 min	87.72 ± 0.45 [b]	2118.67 ± 38.51 [b]	475.50 ± 23.07 [c]	2069.00 ± 31.68 [b]	3717.50 ± 49.67 [b]
	40 min	88.76 ± 0.04 [c]	2177.00 ± 44.14 [c]	336.50 ± 36.54 [a]	1664.33 ± 25.15 [a]	3426.67 ± 54.42 [a]
Japonica starch	Control	75.08 ± 0.04 [a]	2255.67 ± 7.51 [b]	846.67 ± 9.29 [c]	1091.50 ± 2.12 [a]	2508.00 ± 2.65 [a]
	20 min	78.77 ± 0.49 [b]	2517.67 ± 10.02 [c]	377.75 ± 5.56 [b]	1228.50 ± 7.68 [c]	3291.67 ± 17.62 [c]
	40 min	83.60 ± 0.41 [c]	2057.75 ± 16.82 [a]	342.25 ± 10.44 [a]	1133.67 ± 13.20 [b]	2841.00 ± 7.94 [b]
Commercial Starch	Control	83.18 ± 0.25 [a]	2207.00 ± 12.73 [b]	675.00 ± 24.04 [b]	1363.50 ± 10.61 [c]	2895.50 ± 0.71 [c]
	20 min	90.08 ± 0.46 [b]	2240.00 ± 3.61 [c]	881.20 ± 10.33 [c]	734.00 ± 23.02 [b]	2051.50 ± 77.74 [b]
	40 min	92.70 ± 0.00 [c]	762.50 ± 18.27 [a]	565.67 ± 15.89 [a]	175.00 ± 10.03 [a]	362.50 ± 22.13 [a]

Mean data ± SD. Lower case letters (a. b and c) indicate statistically significant differences between the different study groups for each treatment. ANOVA (p-value<0.05)

After 40 minutes of treatment, all the values of the adhesive properties decreased except for the PT, which continued to increase to 92.70 ± 0.00ºC. PV decreased to 762.50 ± 18.27 cP, BD to 565.67 ± 15.89 cP, SB to 175.00 ± 10.03 cP, and FV to 362.50 ± 22.13 cP. These obtained values indicate that after 40 minutes of treatment, the starch structure can be completely modified, resulting in a more fluid product with low viscosity and without the ability to form gels.

These results indicate that the plasma treatment for 20 minutes significantly improved the viscoelastic properties of the BS and JS starches, providing them with greater water retention capacity and the formation of firmer and more stable gels. Although some values decreased after 40 minutes of treatment, the obtained viscoelastic properties could still be relevant for industrial applications. In contrast, the CS sample was the most susceptible to this modification, resulting in more significant changes in its adhesive properties. This suggests that plasma mediated modification could lead to products with lower viscosity and without the ability to form stable and firm gels.

Thirumdas, Trimukhe, et al., (2017) studied the modification of rice starch using DBD and found an increase in PV and FV after plasma treatment, which they attributed to starch cross-linking. The plasma induces depolymerization of starch molecules by breaking hydrogen bonds and allowing the incorporation of water molecules, resulting in increased viscosity, as observed in the results obtained for BS and JS. Also, Ge et al., (2022) investigated the effect of DBD plasma on water-soluble granular rice starch and observed a modification in this starch. They observed an increase in PV and FV after plasma treatment for short times of 3 and 6 minutes; however, these values began to decrease after 9 minutes. The increase in FV during the cooling process was associated with the onset of amylose retrogradation and the reformation of bonds between chain molecules. Although in this study, a similar behaviour was observed for the BS and JS sample with an increase in PV and FV as the treatment time extended, the periods used were longer. This suggests that the ability to observe changes over short periods of time in the Ge et al., (2022) study may be related to the specific type of matrix and pre-treatments that were applied.

Additionally, Sun et al., (2022) studied the modification of rice starch using plasma and microwaves and found that short treatments with cold plasma did not significantly reduce the maximum viscosity of rice starch, but longer treatments led to a notable reduction on this value. This pattern was also observed in our study with the CS sample, where the viscosity decreased as the treatment time increased.

Plasma treatment significantly affects the pasting properties of rice starch by altering its viscosity in relation to the duration of treatment. This indicates that plasma can be used strategically to adjust the stickiness of rice starch, allowing the creation of more or less viscous starch products depending on specific requirements. The treatment time can be adapted to achieve the desired starch consistency.

5.1.3.4. Effect of plasma treatment on rice starch texture

Table 5.1.3 shows that plasma treatment affected the textural properties of rice starch from the two varieties studied (BS and JS) and CS. It can be seen that hardness, springiness, gumminess, and chewiness increase in the BS sample as the treatment time increases, while adhesiveness and cohesiveness decrease, a similar behaviour is seen in the JS samples, there is an increase in all properties after 20 minutes of treatment, however, after 40 minutes of treatment there is a decrease in all the texture properties studied. On the contrary, in the case of CS, it is seen that with the 20-minute treatment, all the texture properties decrease significantly with respect to the control sample and after 40 minutes of treatment, the starch completely changed its configuration, without having the ability to form a gel.

Table 5.1.3. Texture properties of rice starch after plasma treatment.

Sample		Hardness (g)	Adhesiveness (g. sec)	Cohesiveness	Springiness	Gumminess	Chewiness
Basmati starch	Control	182.19 ± 6.95^{a}	-251.57 ± 10.87^{a}	0,645 ± 0,022^{b}	0.909 ± 0.002^{a}	114.13 ± 2.29^{a}	107.88 ± 3.62^{a}
	20 min	222.0.4 ± 5.18^{b}	-141.04 ± 3.14^{b}	0,638 ± 0,009^{b}	0.944 ± 0.003^{b}	142.89 ± 4.03^{b}	131.67 ± 5.19^{b}
	40 min	242.45 ± 4.14^{c}	-134.32 ± 6.15^{b}	0,615 ± 0,017^{a}	0.944 ± 0.015^{b}	148.62 ± 5.13^{c}	128.43 ± 5.71^{b}
Japonica starch	Control	82.32 ± 1.24^{a}	-124.59 ± 3.05^{c}	0,606 ± 0,010^{a}	0.864 ± 0.013^{a}	50.22 ± 1.25^{a}	42.12 ± 0.70^{a}
	20 min	125.72 ± 2.96^{c}	-200.14 ± 5.10^{a}	0,660 ± 0,006^{b}	0.860 ± 0.009^{a}	83.28 ± 0.51^{c}	71.66 ± 0.46^{c}
	40 min	104.70 ± 3.77^{b}	-135.42 ± 4.23^{b}	0,660 ± 0,006^{b}	0.885 ± 0.012^{b}	67.99 ± 3.50^{b}	61.66 ± 0.83^{b}
Commercial Starch	Control	87.57 ± 2.14^{b}	-123.60 ± 4.00^{a}	0,640 ± 0,004^{b}	0.867 ± 0.011^{a}	56.09 ± 1.65^{b}	49.30 ± 2.01^{b}
	20 min	78.26 ± 4.60^{a}	-92.85 ± 6.46^{b}	0,596 ± 0,012^{a}	0.864 ± 0.007^{a}	46.71 ± 1.49^{a}	42.80 ± 3.21^{a}
	40 min	---	---	---	---	---	---

Mean data ± SD. Lower case letters (a. b and c) indicate statistically significant differences between the different study groups for each treatment. ANOVA (p-value<0.05)

In general, it can be said that the varied behaviours between the studied samples is due to their molecular structures. Lu et al., (2011) suggested that the amylose and amylopectin content of the variety play an important role, especially the amylose content influencing the water mobility of starch gels, which affects the specific viscoelasticity and textural properties of starch gels. Therefore, the reactive species of the plasma will affect differently depending on the molecular structure of each variety. With these results, it can be seen that the reactive species in the plasma interfered with the molecular structures, especially amylopectin. In varieties with high amylopectin content, such as JS, these modifications make it easier for the molecules to align and change their configuration. This contrasts with the varieties that have a lower content of amylopectin, where the modifications induced by the plasma do not facilitate molecular reorganization to the same extent. This is evident in BS, which contains more amylose and has a more stable structure to the plasma treatment. Theoretically, it can also be said that CS may contain more amylopectin than amylose, to explain its instability when treated with plasma.

The changes generated in the textural properties of rice starch, especially the JS and in (CS), were corroborated with the results obtained by Laricheh et al., (2022). They observed that when corn starch was subjected to plasma treatment for a long time, the strength of the gel could decrease, as well as the hardness, adhesiveness and cohesiveness followed a similar pattern. However, also these authors suggested that, under some conditions, the stiffness of the gel can increase, which can be attributed to reactive species that can create cross-relationships between amylose chains as is the case of the BS.

Thirumdas, Saragapani, et al., (2016) also reported a similar decrease in hardness in brown rice samples, especially when there is an increase in the time and potency of plasma treatment. Likewise, they noticed that with a decrease in hardness, there was always a reduction in cohesiveness, but they did not find significant differences in terms of cohesion and elasticity after the treatment.

5.1.3.5. Effect of plasma on flour and gel hydration properties

Studies on plasma application have shown that plasma can alter the molecular structure of starch, significantly improving its water absorption-related properties, including swelling capacity and gel characteristics (Thirumdas, Saragapani, et al., 2016).

Regarding hydration properties, such as Water Holding Capacity (WHC), Oil Holding Capacity (OHC) and Water Binding Capacity (WBC), the results are presented in Table 5.1.4 showing differentiated behaviours according to the starch variety analysed. For the BS, no significant changes in these properties were observed after two plasma treatments. On the other hand, for the JS, an increase in WBC was recorded as the treatment time elapses; OHC remains constant, while WHC showed a significant increase after 20 minutes of treatment. As for the CS sample, WBC increases only after 20 minutes and decreases after 40 minutes of treatment. OHC shows a slight increase over the treatment time, but WHC shows no significant change.

In Thirumdas, Trimukhe, et al., (2017) study, rice starch samples showed an increased in WHC and OHC after plasma treatment. This change was attributed to the increased hydrophilicity to the incorporation of hydroxyl (OH) groups from the plasma, a conclusion that was supported by Fourier transform infrared spectroscopy (FTIR) analysis.

The gel hydration properties, including Water Absorption Index (WAI), Water Solubility Index (WSI) and Swelling Power (SP), are detailed in Table 5.1.4. Significant differences in WAI, WSI and SP are observed between treated and untreated samples of the BS. After 20 minutes of treatment, there is a significant increase in WAI, WSI and SP. However, after 40 minutes, the differences in WAI and SP are not significant, while WSI shows a slight decrease. While no significant differences in WAI and SP were observed in the JS samples, the WSI shows a slight decrease after 20 minutes of treatment, but a slight increase after 40 minutes.

Table 5.1.4. Flour and gel hydration properties of rice starch after plasma treatment

Sample		WBC	OHC	WHC	WAI	WSI	SP
Basmati starch	Control	14.062 ± 0.368[a]	7.642 ± 0.465[a]	15.131 ± 0.071[a]	6.490 ± 0.144[a]	1.696 ± 0.136[a]	6.602 ± 0.137[a]
	20 min	14.365 ± 0.440[a]	7.056 ± 0.695[a]	15.333 ± 0.943[a]	7.114 ± 0.326[b]	2.800 ± 0.000[c]	7.299 ± 0.329[b]
	40 min	13.996 ± 0.617[a]	6.863 ± 0.314[a]	15.256 ± 0.693[a]	7.177 ± 0.067[b]	2.130 ± 0.126[b]	7.333 ± 0.068[b]
Japonica starch	Control	10.622 ± 0.173[a]	7.352 ± 0.019[a]	12.068 ± 0.255[a]	7.021 ± 1.074[a]	2.398 ± 0.286[b]	7.195 ± 1.121[a]
	20 min	12.290 ± 0.543[b]	8.255 ± 0.292[a]	13.286 ± 0.490[b]	7.187 ± 0.037[a]	1.399 ± 0.344[a]	7.289 ± 0.054[a]
	40 min	13.389 ± 0.183[c]	8.106 ± 0.453[a]	13.303 ± 0.541[b]	6.819 ± 0.048[a]	2.207 ± 0.003[b]	6.949 ± 0.058[a]
Commercial Starch	Control	8.706 ± 0.038[a]	10.623 ± 0.334[a]	8.379 ± 0.039[a]	7.228 ± 0.038[c]	3.112 ± 0.142[a]	7.460 ± 0.029[c]
	20 min	10.048 ± 0.172[b]	11.560 ± 0.394[a, b]	8.537 ± 0.120[a]	5.551 ± 0.584[b]	9.688 ± 2.671[a]	5.129 ± 0.363[b]
	40 min	8.562 ± 0.211[a]	10.981 ± 0.030[b]	8.302 ± 0.302[a]	1.804 ± 0.278[a]	24.747 ± 2.471[b]	2.468 ± 0.238[a]

Mean data ± SD. Lower case letters (a. b and c) indicate statistically significant differences between the different study groups for each treatment. ANOVA (p-value<0.05)

On the contrary, samples of the CS show an opposite behaviour, as the treatment time increase, WAI and SP decrease, but with considerable increase in WSI. This can be explained by a higher damage and disintegration of the starch granules, resulting in higher solubility but lower swelling capacity. These results agree with data obtained on the pasting and textural properties of this sample.

Thirumdas, Trimukhe, et al., (2017) observed that the WAI, WSI and SP of plasma-treated rice starch increased with increasing plasma power, although not necessarily with increasing treatment time, as shown by the results for the BS. In another study, Taslikh et al., (2022) applied DBD cold plasma to corn starch and observed a significant increase in the SP of the starches evaluated. They explained that different types of starch react differently due to variations in the molecular structure of the granules, influenced by the way amylose and amylopectin are integrated into the starch paste. This affects the ability of the granules to absorb and retain water, resulting in an increase in SP after plasma treatment, as observed in the BS, but not in the JS and CS samples due to their different amylose and amylopectin compositions.

Furthermore, Kusumayanti et al., (2015) reported that starches with higher amylose content tend to show lower SP. However, this effect seems to be counteracted in the BS samples, despite its higher amylose content, a significant increase in SP was recorded after plasma treatment. This discrepancy may suggest that the impact of plasma treatment may overcome the inherent structural limitations of amylose and amylopectin composition, potentially offering advantages in specific applications where improved starch swelling and solubility are desired.

In general, the observed changes in WAI, WSI and SP have been associated by several authors (Banura et al., 2018; Carvalho et al., 2021; Taslikh et al., 2022; Thirumdas, Trimukhe, et al., 2017) to the depolymerization of the branched chains of amylose and amylopectin, which facilitates the formation of simple sugars. These simple sugars retain more water, thus increasing water holding capacity. Besides, plasma reactive species modify the surface of starch granules, increasing their surface

area and hydrophilicity. This improves the ability of the granules to absorb water, an effect particularly evident in the BS samples.

However, the same effect is not observed in the JS and CS starch samples. In these cases, the gel hydration properties decrease, resulting in less stable starches. This behaviour may be associated with the different responses of amylopectin to plasma treatment. The variability in the molecular structure of each type of starch may influence how they react to treatment conditions, thus altering their physicochemical properties.

5.1.4. Conclusion

Plasma is an emerging non-thermal technique that shows great potential for starch modification. In our study, we observed that an increase in treatment time significantly affected the moisture content, colour and viscosity of starch samples, although hydration properties showed a less marked response to treatment time. These results underline the importance of optimising plasma treatment parameters, such as exposure time and intensity, to maximise its benefits for starch modification. This is especially relevant for the use of plasma to different types of starch, since as seen in this research, starches extracted from different rice varieties behave differently and therefore specific characteristics can be obtained. Exploring how plasma interacts with the different molecular structures of starch can open new avenues for process design and product formulation to better meet market needs and quality requirements. In addition, further investigation of the mechanisms underlying the observed changes may facilitate the development of innovative applications of plasma in the food industry, improving the texture, stability and functionality of processed foods.

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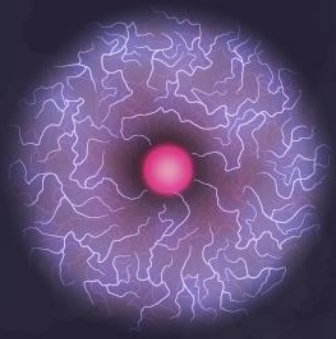
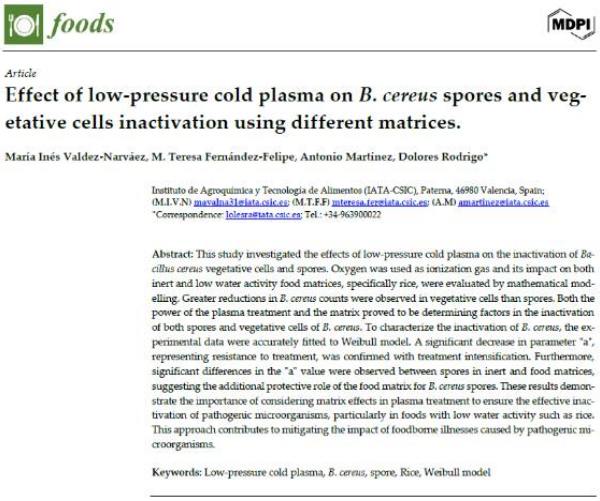
CAPÍTULO 5.2



ESTUDIO DE LA TECNOLOGÍA DE PLASMA FRÍO (CP) COMO UNA ESTRATEGIA PARA LA DESCONTAMINACIÓN DEL GRANO DE ARROZ SECO

ENVIADO A LA REVISTA FOODS



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Article

Effect of low-pressure cold plasma on *B. cereus* spores and vegetative cells inactivation using different matrices.

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Abstract: This study investigated the effects of low-pressure cold plasma on the inactivation of *Bacillus cereus* vegetative cells and spores. Oxygen was used as ionization gas and its impact on both inert and low water activity food matrices, specifically rice, were evaluated by mathematical modelling. Greater reductions in *B. cereus* counts were observed in vegetative cells than spores. Both the power of the plasma treatment and the matrix proved to be determining factors in the inactivation of both spores and vegetative cells of *B. cereus*. To characterize the inactivation of *B. cereus*, the experimental data were accurately fitted to Weibull model. A significant decrease in parameter "a", representing resistance to treatment, was confirmed with treatment intensification. Furthermore, significant differences in the "a" value were observed between spores in inert and food matrices, suggesting the additional protective role of the food matrix for *B. cereus* spores. These results demonstrate the importance of considering matrix effects in plasma treatment to ensure the effective inactivation of pathogenic microorganisms, particularly in foods with low water activity such as rice. This approach contributes to mitigating the impact of foodborne illnesses caused by pathogenic microorganisms.

Keywords: Low-pressure cold plasma, *B. cereus*, spore, Rice, Weibull model

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Journal	*Foods*
Section	Food Packaging and Preservation
Special Issue	Emerging Technologies In Food Safety Intervention
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Keywords	Low-pressure cold plasma, B. cereus, spore, Rice, Weibull model
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CONGRESOS

Póster: Effect of Cold Plasma on the survival of *B. cereus* in a rice matrix.
Evento: I Congreso Innovación alimentaria
Lugar: Universidad de Valencia (Valencia-España)
Fechas: 13 de marzo de 2023

Effect of low-pressure cold plasma on *B. cereus* spores and vegetative cells inactivation using different matrices.

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Abstract

This study investigated the effects of low-pressure cold plasma on the inactivation of *Bacillus cereus* vegetative cells and spores, in an inert matrix (borosilicate glass slide) and in rice grains, using oxygen as ionization gas. Greater reductions in *B. cereus* counts were observed in vegetative cells than spores. The experimental date obtained, showed that both the power of the plasma treatment and the matrix proved to be determining factors in the inactivation of both spores and vegetative cells of *B. cereus*. To characterize the inactivation of *B. cereus*, the experimental data were accurately fitted to Weibull model. A significant decrease in parameter "a", representing resistance to treatment, was confirmed with treatment intensification. Furthermore, significant differences in the "a" value were observed between spores in inert and food matrices, suggesting the additional protective role of the food matrix for *B. cereus* spores. These results demonstrate the importance of considering matrix effects in plasma treatment to ensure the effective inactivation of pathogenic microorganisms, particularly in foods with low water activity such as rice. This approach contributes to mitigating the impact of foodborne illnesses caused by pathogenic microorganisms.

Keywords: Low-pressure cold plasma, *B. cereus*, spore, Rice, Weibull model

5.2.1. Introduction

The cultivation of rice (*Oryza sativa* L.) holds a historical significance as one of the oldest practices, considering it as a staple food for humans and its global prominence as one of the most extensively cultivated crops. In recent decades, rice production has witnessed nearly a twofold increase, facing threats from various challenges such as limited agricultural land, water scarcity, soil fertility concerns, climate change, insect infestations, and diseases [1,2]. Owing to its nutritional composition, particularly its elevated starch content, rice is susceptible to a spectrum of infections caused by bacteria, viruses and fungi, with *Bacillus cereus* as a principal contaminant [3].

Bacillus cereus is a Gram-positive bacterium known for its ability to form spores, which allows it to survive in diverse environmental conditions. This bacterium is commonly found in soil and can contaminate various types of food, causing foodborne illnesses [4,5]. Diarrhoeal syndrome is an infection caused by the growth of the micro-organism in the small intestine, resulting in diarrhoea, while emetic syndrome is a syndrome that results in vomiting when food containing the previously formed cereulide toxin is ingested. *B. cereus* can be present in a wide range of foods, including rice, pasta, meat, vegetables, and dairy products. The spores of this bacterium are resistant to heat, allowing them to survive cooking processes [6]. Therefore, proper food handling, cooking, and storage are essential to prevent the spores' germination, growth of *B. cereus* and the production of toxins [4,5]. In the European Union, the One Health 2022 zoonoses report [7] indicated that *B. cereus* toxins are ranked as the leading cause of reported Foodborne Outbreaks (FBOs) attributed to bacterial toxins. Specifically, there was a significant surge in the number of Adverse Events (AEs) caused by *Bacillus cereus* toxins in 2022 compared to 2021 (an increase of 219 AEs in 2022, representing a relative rise of 251.7%). *B. cereus* toxins were identified as the causative agent in five highly significant Adverse Event Outcomes (AEOs), defined as having 100 or more reported cases. In this same report, the EFSA associates 8.33% of outbreaks in non-animal origin processed foods, including rice.

In the quest to address foodborne illnesses, heat sterilization has been employed as the primary choice in the food industry. Nevertheless, traditional heat processes can result in a decline in the quality of food, particularly in cases involving heat-sensitive products. In recent times, non-thermal technologies have been implemented for the microbial management of food, focusing notably on spore inactivation [8] and this is where the plasma, as a non-thermal technology that has the ability to inactivate microbial spores [8], plays a crucial role in this study. Plasma is considered the fourth state of matter, it has unique properties that distinguish it from the three basic states: solid, liquid and gas. It is generated thanks to the atoms have undergone ionization, meaning they have lost or gained electrons, resulting in a mixture of free electrons and ions [9,10]. This characteristic makes plasma an excellent electrical conductor and gives its distinctive properties that make it valuable in various applications. The disinfection effect of plasma technology is due to the quantity of reactive species it can generate, mainly reactive oxygen (ROS), nitrogen (RNS) species and UV radiation. Reactive oxygen and nitrogen substances include specifically O_3, H_2O_2, OH, O_2-, and NO_2, which can cause significant oxidative damage to bacterial cells, resulting in oxidative stress and ultimately leading to microbial death [11]. Various types of plasma can be generated depending on temperature and pressure conditions [9]. This study focuses on low-temperature (<70°C) and low-pressure plasma which is characterized because it is easier to control the working conditions [12,13].

Researches have been carried out to understand how different parameters, such as power, time, frequency, voltage, plasma composition, temperature, or plasma electrode configuration affect plasma generation and therefore the efficiency of microbial inactivation [14–16]. However, in addition to these electrical parameters, food matrices have also been found to play a crucial role in the efficiency of microbial inactivation. For example, recent research has shown that food composition and moisture can significantly influence the response to plasma treatment [16,17]. Another relevant aspect is the variability in the microorganism sensibility to plasma depending on the microbial species but also on the vegetative forms (vegetative cells

or spores) underscoring the need to investigate and understand the specific interactions between plasma and microorganisms of interest [17–19].

Another aspect to consider in cold plasma technology is the transition of this technology from the laboratory to the food industry, since this also represents a challenge. Therefore, modelling the outcomes of microbial inactivation becomes essential. Mathematical modelling allows plasma treatment processes to be optimized according to the specific processing parameters, food matrix and target microorganism. By understanding how these factors interact, more effective strategies can be developed to ensure food safety [14].

In this context, the aim of this study was to evaluate the impact of low-pressure cold plasma on *B. cereus* vegetative cells and spores in a rice matrix and in an inert matrix. Furthermore, data were fitted to a mathematical model to improve the understanding of the potential applications of cold plasma treatment, particularly in the realms of microbial control and food safety on an industrial scale.

5.2.2. Materials and Methods

5.2.2.1. Matrix.

Two types of matrices were employed in this study. A borosilicate glass slide (24 x 50 mm), being an inert and non-porous surface, as a model for food contact surface that enables the observation of the direct impact of cold plasma on the microorganism without external influences, and a round grain rice (*Oryza sativa* L.) (5 g) bought from a local supermarket, as a model for low water activity food, that allows the evaluation of cold plasma in real food environments. Prior to the plasma treatment, the moisture content of the rice was determined and it was dried at 80ºC for 24 h to a constant weight, reaching a moisture content of 9%.

5.2.2.2. Test microorganism

The tests were conducted using a pure lyophilized culture of *B. cereus* obtained from the Spanish Type Culture Collection (CECT 148).

The lyophilized culture was reconstituted with 0.2 ml of sterile Nutrient Broth (NB) liquid medium (Scharlab Chemie S.A., Barcelona, Spain). After a 30 minutes incubation period at 30ºC, the entire suspension was inoculated into an Erlenmeyer flask containing 500 ml of NB medium. The flask was incubated at 30°C in a thermostatic bath with continuous shaking for 14 h to achieve cells in a stationary growth phase. Subsequently, the cells underwent two centrifugation steps at 3750 x g at 4°C for 15 minutes, using a Beckman centrifuge (JLA-16,250 rotor). After decanting the supernatant, the cells were resuspended in 50 ml of NB medium. After the second centrifugation, the cells were again resuspended in NB and distributed into 2 ml cryovials, with 1 ml per cryovial. To each cryovial, 1 ml of 20% glycerol in NB, serving as a cryoprotectant, was added. The 2 ml samples were promptly frozen and stored at -80°C until needed. The concentration of *B. cereus* in the cryovials was determined by plate count, revealing a concentration of 10^8 CFU/ml.

5.2.2.3. Sporulation procedure

One of the *B. cereus* in cryovials from section 5.2.2.2 was used for the bacterial sporulation. The strain was reactivated in nutrient broth undergoing shaking for 24 hours at 32˚C. Subsequently, 20 Roux flasks (Fisher Scientific SL, Madrid, Spain) containing Fortified Nutrient Agar (Scharlab Barcelona, Spain) were prepared, each flask with 0.5 ml inoculum of the *B. cereus* culture and they were incubated at 30˚C.

Once the sporulation level reached approximately 90%, spores were collected using a modified Digralsky metal loop (Deltalab, Barcelona, Spain). This involved gently sweeping the agar surface and washing with double-distilled water. The collected solution was centrifuged at 2500g for 15 minutes at 5˚C, and the supernatant was removed. The spores were then re-suspended in 5 ml of double-distilled water and subjected to centrifugation under the same conditions. This process was repeated four times. Finally, the spores from the pellet were stored at 4˚C in distilled water.

5.2.2.4. Plasma equipment

A low-pressure cold plasma system based on a Dielectric Barrier Discharge (DBD) design from Electronic Diener Plasma Surface Technology PCCE, model Pico-AR-200-PCCE7, was used. This system generates plasma through two circular plate electrodes and operates under low-pressure conditions (0.35 mbar), facilitated by a vacuum pump (Leybold Trivac D16T). This equipment operates at a frequency of 13.56 MHz and a power range of 0 to 300 W.

In this study, 100% pure oxygen gas (Alphagaz) was employed as the ionization gas, and the system operated at a pressure of 0.35 mbar. Power levels of 100, 200, and 300 W were applied, with treatment durations ranging from 5 to 60 minutes as a variable parameter in the process.

5.2.2.5. Plasma treatment

Before each plasma treatment, different samples were prepared. For spores, borosilicate glass slides, previously disinfected and degreased, were inoculated with 100 µl of *B. cereus* spores at a concentration of 10^7 CFU/ml. Similarly, 5 g of rice were weighed separately, introduced in a borosilicate crystallization capsule (diameter 6cm) and inoculated with 100 µl of *B. cereus* spores at a concentration of 10^7 CFU/g. In both cases, before plasma treatment, samples were dried in a biosafety cabinet at room temperature for 20 hours. For vegetative cells, 5 g of rice were weighed separately in borosilicate crystallization capsule (diameter 6 cm) and they were inoculated with 1 ml of *B. cereus* vegetative cells at a concentration of 10^7 CFU/g. The control samples were prepared exactly as treatment samples for both, spores and vegetative cells in stationary phase, but they were directly plated without undergoing any plasma treatment.

After each plasma treatment, the samples were diluted with 10 ml of peptone water for vegetative cells and distilled water for spores. To prevent spore aggregation, vigorous shaking with glass beads was performed before taking each sample for plating. Through agitation, the *B. cereus* vegetative cells or spores that remained after

treatment were extracted. Following the recovery of the solution, two sets of serial decimal dilutions (Series A and B) were prepared in duplicate. From each decimal solution, 100 µl were plated in duplicate on nutrient agar (Scharlab, Barcelona, Spain) enriched with 1g/l starch (Scharlab, Barcelona, Spain) and incubated for 18–20 hours at 30°C. After the incubation period, a manual count of *B. cereus* colonies was conducted.

5.2.2.6. Modelling

Experiments were performed in triplicates with two replicas per count. The experimental results were computed in Microsoft Excel by applying the Log_{10} of the survival fraction (LogS) as calculated by Equation 1.

$$Log(S) = N / N_0 \quad (1)$$

where N is the bacterial concentration (CFU/ml) at time t (min) and N_0 is the initial bacterial concentration (CFU/ml) (t_0). These data were plotted using the OriginPro software (Version 2023b. OriginLab Corporation, Northampton, MA, USA).

The obtained mean data were fitted to the Weibull Survival Function (2) using GInaFiT (Version 1.8-Microsoft Office 365 copyrighted by the Katholieke Universiteit Leuven KU Leuven, Belgium) program. This non-linear regression is a powerful technique for modelling microbial inactivation, as it allows for the simultaneous obtention of a and b parameters from survival curves.

$$Log10(N)=Log10(N_0) - ((t/a) \wedge b) \quad (2)$$

where N is the microbial concentration after treatment, N_0 is the initial microbial concentration before treatment, t is the treatment time (min), “a” is the scale parameter, and “b” is the shape parameter.

5.2.2.7. Accuracy Factor

The model has been validated by calculating the Accuracy Factor (AF) of the experimental data with respect to that predicted by the model. This factor provides a

measure of the average precision of the estimates and is given by equation 3. The accuracy factor must always be greater than or equal to one, and is one if there is perfect agreement between all predictions and observed values [20].

$$AF = 10\text{^} (\Sigma \mid \log (predicted/observed) \mid)/n \quad (3)$$

where n is the number of observations used in the calculation

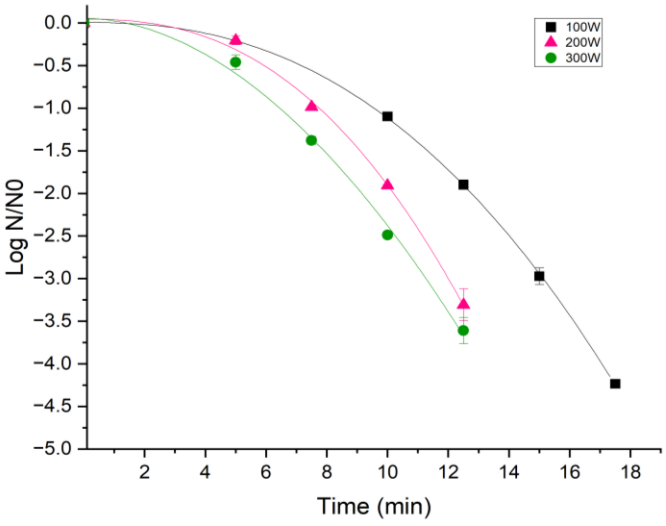
5.2.2.8. Statistical analysis

Statistical differences of the parameters obtained were determined with an analysis of variance (ANOVA) (p-value <0.05) and intergroup differences were determined using Fisher's test (LSD), which identifies homogeneous subsets of means that do not differ from each other. This analysis was carried out on the mean data of parameter for each power and matrix, using Statgraphics Centurion XIX Software (version 19.6.03 copyrighted by Statgraphics Technologies, Inc).

5.2.3. Results and discussion

The variation in *B. cereus* response is shown in Figure 5.2.1 (A, B) and Figure 5.2.2. A comparison has been conducted among different treatment powers in various scenarios: Figure 5.2.1A, shows *B. cereus* inactivation of vegetative cells inoculated in a rice matrix; Figure 5.2.1B, shows inactivation of *B. cereus* spores inoculated in a rice matrix and Figure 5.2.2, shows inactivation of *B. cereus* spores spread on a borosilicate glass slide. These distinct scenarios provide insights into the varied effects of treatment powers on different microbial forms and matrices, contributing to a comprehensive understanding of the cold plasma treatment outcomes.

(A)



(B)

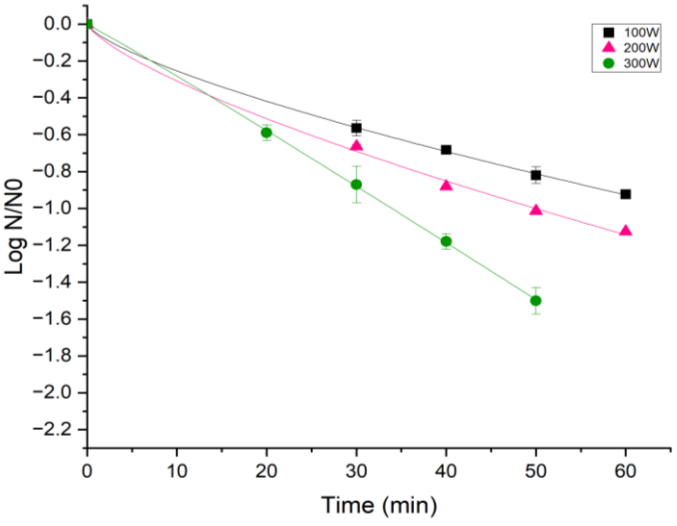


Figure 5.2.1. Weibull distribution function fit for *B. cereus* vegetative cells (A) and *B. cereus* spores (B) within rice grain. Inactivation as a function of power (100W (■), 200W(▲), 300W(●). The icons show the experimental values and the line the predictions obtained by the model.

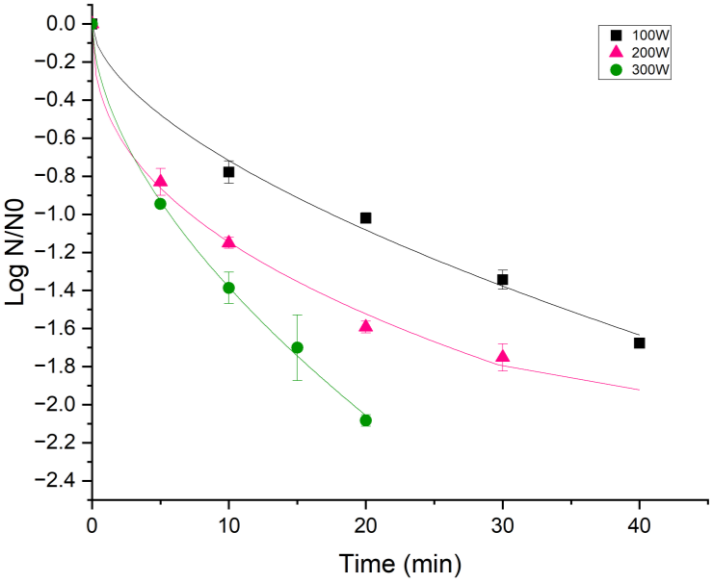


Figure 5.2.2. Weibull distribution function fit for *B. cereus* spores within an inert matrix (borosilicate glass slides). Inactivation as a function of power (100W (■), 200W (▲), 300W (●). The icons show the experimental values and the line the predictions obtained by the model.

5.2.3.1. Effect of low-pressure cold plasma on *B. cereus* vegetative cells inoculated in a rice matrix.

Figure 5.2.1A illustrates the effect of plasma technology on *B. cereus* vegetative cells within a rice matrix after plasma treatment with different times and powers, using O_2 as ionization gas. As treatment duration and intensity increase a corresponding decrease in *B. cereus* vegetative cell counts is observed. For all power levels, the bacterial reductions achieved were close to 4 logarithmic cycles, reaching the detection limit of the method. Specifically, they ranged from 1.0 to 4.23 log CFU/ml with treatment times of 10 to 17.5 minutes at 100 W, from 0.2 to 3.3 log CFU/ml with times of 5 to 12.5 minutes at 200 W and from 0.46 to 3.61 log CFU/ml with times of 5 to 12.5 minutes at 300 W.

These results can be attributed to the effect of reactive species generated by the plasma, such as reactive oxygen and nitrogen species identify as O_2, O_3, O_2-, N, N_2, and as a consequence of the moisture present OH and H_2O_2 (spectra not shown in this document). These reactive species are formed upon collision of charged particles with the background gas or moisture in the substrate or microorganism, leading to disintegration of cell walls and membranes through oxidation, along with the generation of volatile components like CO, CO_2, H_2O, and deep etching channels, as a consequence, direct permeabilization of the membrane cell wall, causing the leakage of cellular components (potassium, nucleic acid and proteins), the denaturation of proteins and DNA damage. Therefore, higher plasma exposure and higher treatment potency correlated with higher decontamination [9,21–25].

Choi et al. [18] studied the effect of plasma on dried black-mouth angler, observing a slightly slower decline in *B. cereus* vegetative cells when applying atmospheric DBD plasma for 30 minutes at 120W, resulting in a reduction of 1.06 log cycles. Jeon et al. [26] reported a decrease of only 0.96 log CFU/g in *B. cereus* vegetative cells in red pepper powder after treatment with atmospheric DBD plasma for 20 min at 120 W. In contrast, results found in the present study were similar to those reported by Lee et al. [27] in brown rice. They investigated the impact of atmospheric plasma DBD on *B. cereus* vegetative cells in cooked white and brown rice and found that when the treatment time was increased to 20 min at 250W it led to a reduction of nearly 5 log cycles in cooked white rice. However, smaller reductions (1.9 log cycles) were found in white rice showing variations in the plasma effectiveness depending on the matrix. In the same sense, Y. H. Kim et al. [17] found that treatment with atmospheric DBD plasma had different effects on *B. cereus* and *E. coli* depending on the matrix. They found after a 15-minute treatment at 31 kW a reduction of 1 log CFU/ml in *B. cereus*, while *E. coli* showed a reduction of 2 log CFU/ml when contained in culture medium, but when *B. cereus* and *E. coli* were within red pepper powder, only 1 log CFU/ml reduction of *E. coli* were observed and no significant reduction of *B. cereus* was observed even with a longer treatment duration.

This variability in the results obtained by different studies, could be attributed to the fact that the efficacy of plasma is significantly impacted by the nature of the matrix. Additionally, other factors such as the microbial strain or type of microorganism and the distribution of cells within the product play key roles. These factors can significantly influence the outcome affecting the observed resistance or susceptibility of microorganisms to different treatments or conditions. Understanding and controlling these variables is crucial for ensuring the accuracy and reliability of microbial assays in various applications, including food safety, pharmaceuticals, and environmental monitoring [17,18, 26, 27].

5.2.3.2. Effect of cold plasma at low pressure on *B. cereus* spores inoculated in an inert matrix and in a rice matrix.

As mentioned above, *B. cereus* is a bacterium with the ability to form spores, which represents the real threat of this bacteria. As it is a bacterium found in the soil, it can become the main contaminant of rice crops. *B. cereus* spores present in rice grains can resist traditional cooking methods and pose a health risk to consumers [28]. Hence, this study also aimed to examine the effect of plasma on *B. cereus* spores, both when they are spread in an inert matrix (borosilicate glass slides) and when they are inoculated in a food matrix, such as rice.

Figure 5.2.2 shows *B. cereus* spores' inactivation in an inert matrix. It is observed that as the treatment time and power increase, the inactivation of *B. cereus* spores increases, thus with a treatment time of 20 minutes and 300W there is a reduction of 2 logarithmic cycles. J. E. Kim et al. [29] found similar results, since they observed that increasing the duration of the treatment produced greater inactivation of *B. cereus* spores. A significant increase in the mortality rate was observed, ranging from 1.5 ± 0.1 to 2.1 ± 0.2 log spores/cm² as the treatment time increased from 30 to 40 minutes. Liu et al. [19] also observed a similar behaviour, noting that with an atmospheric Jet plasma, inactivation increased as treatment power increased when the matrix containing the spores was inert. But the effectiveness of their treatment was lower than the one shown in the present study since they only managed to inactivate 0.99

log CFU/ml with 600 W, 2.17 log CFU/ml with 800 W, and 2.37 log CFU/ml with 1000 W when *B. cereus* spores were in petri dishes. This could be attributed to the low-pressure conditions used in this study, where the mean free path between gas particles is extended. As a result, electron acceleration dominates in the electric field over elastic collisions with heavy particles, which would otherwise heat the background gas. Furthermore, the use of dielectric barrier discharge (DBD) equipment guarantees a more uniform treatment throughout the sample [22].

In general, the inactivation of *B. cereus* spores by plasma may be related to the effect of reactive species generated by plasma. Bacterial endospores, with their multiple layers of resistance, are difficult to remove. However, plasma power can increase electron density and the concentration of reactive species, such as atomic oxygen and hydroxyl radicals, causing damage to the cell wall, inducing electrostatic stress and morphological changes, while UV radiation contributes to cell nucleus damage, specifically causes the formation of thymine dimers in DNA [11,19,21]. While these potential effects of plasma on spores have not been fully confirmed and require further studies, some of these effects have been supported by Van Bokhorst-van De Veen et al. [30]. They morphologically analysed *B. cereus* spores after exposure to atmospheric plasma. In this case, they observed severe physical damage to the spores, evident in irregular surfaces. Spores treated with plasma showed a reduction in viable counts of 0.8 $\log_{10}$ after 20 minutes. An interesting finding was that, when examined under a microscope, the spores transitioned from the bright to the grey phase, indicating limited water and nutrient entry. These plasma-induced grey-phase spores were unable to grow into vegetative cells in BHI media.

Figure 5.2.1B shows *B. cereus* spores' inactivation in a rice matrix. In this case, with a treatment time of 60 minutes and a power of 100 W, a reduction of almost 1 logarithmic cycle is achieved. Similarly, at 60 minutes with 200 W power, a reduction of 1.1 logarithmic cycles is attained, and with a power of 300W for 50 minutes, there is a reduction of 1.4 logarithmic cycles. The results show that the inactivation reached in a food matrix is lower than the one obtained in the inert surface. It could be due to diverse reasons. The rice grain is a porous surface, and this porosity may have even

increased as a consequence of the plasma's effect on the rice grain's surface. It is well documented that plasma treatment has the capability to modify surfaces [24,31]. Consequently, the microorganism may have become trapped in these pores, which could be another reason why plasma is less effective on rice grains compared to inert surfaces. Beyrer et al [32] used *B. coagulans* spores to investigate how spore loading on flat glass carriers or mixed in the aqueous phase with a non-soluble powder affects the efficiency of inactivation by a DBD CAP. The inactivation effect for spores directly exposed to DBD CAP on flat glass carriers (reference value) was clearly reduced by native rice starch granules (non-porous powder model system) and shells of diatoms (highly porous dust model system). The granules are associated with significant protective effects, probably due to shading UV light, structural modification of starch and zein powder. An alternative explanation is based on the erosion effect on particles in general and spores specifically, less exposed to UV effects than on non-inert surfaces. In conclusion, pores in shells of diatoms would protect microorganisms a magnitude better than the starch particles with more smooth surfaces, being the less protecting for spores against the Cold Plasma, the inert surface. The matrix effect on the spore's inactivation was noted also by Liu et al. [19]. When the spores were inoculated on dry pepper grains, only a reduction of 1.37 log CFU/g was obtained using a power of 800 W for 20 minutes. The matrix effect can be associated as in rice medium, cells can elongate and form non-spherical microcolonies that resemble structured spaghetti strands, as observed in the study by Warda et al. [33]. These structures pose challenges for removal from adhered surfaces due to their viscous nature. Further-more, the abundant nutrients and other organic components of foods contribute to the formation of these *B. cereus* spore aggregates. As a result, when exposed to plasma treatment, the outer layer of the spore becomes more resistant to the treatment.

Therefore, the results of this study agree with the findings of other researchers, suggesting that the matrix effect plays a relevant role in the response of *B. cereus* spores to plasma treatment. It can be said that, as in other food processes, the food matrix, considering the pH, a_w, composition, etc., play an important role in the

inactivation of microorganisms or bacterial spores due to its complex structure and the formation of aggregates. This poses additional challenges for effective spore removal, especially because rice is a highly porous granulated matrix that could have two effects, on the one hand by shielding ultraviolet light and on the other by protecting bacterial spores in its pores, so the net effect of the cold plasma would be less than on the inert surface where the spores are arranged in a monolayer. Although more research is required to fully understand these effects of plasma, preliminary studies have shown significant physical damage to the spores, suggesting a reduction in their viability and ability to develop into vegetative cells. These findings underscore the importance of considering the matrix effect when designing plasma treatment strategies to ensure effective decontamination in various food matrices.

5.2.3.3. Low-pressure cold plasma power effect on the inactivation of *B. cereus* at specific time.

To further investigate the effect of plasma as a function of treatment intensity (treatment power), a specific treatment time was established to evaluate the inactivation of *B. cereus* spores dispersed on an inert matrix (borosilicate glass slides) and inoculated in a rice matrix, as well as vegetative cells of *B. cereus* inoculated in a rice matrix. The chosen treatment times were 30 minutes for the spores and 12.5 minutes for the vegetative cells. These durations were selected based on an analysis of variance (ANOVA) conducted with the experimental data obtained, which indicated that these treatment durations were able to reveal the impact of power on the inhibition of *B. cereus*.

Treating vegetative cells of *B. cereus* in a rice matrix for 12.5 min results in a reduction of 1.9 to 3.31 CFU/ml when increasing the power from 100W to 200W. However, no further significant logarithmic reduction ($p>0.05$) is observed when increasing the power from 200W to 300W (3.31 to 3.61 CFU/ml, respectively). On the other hand, as far as spores in a rice matrix is concerned, it is necessary to increase the treatment power up to 300 W to obtain significant differences in spores' inactivation (0.87 CFU/ml) while in an inert matrix (borosilicate glass slides) there is

a significant logarithmic reduction increase with power (1.34, 1.75 and 2.59 CFU/ml for 100W, 200W and 300W, respectively). This effect on bacterial spores could be attributed to the fact that the increase in treatment power leads to a higher density of reactive species. The high-power density can also contribute to the breakage of disulphide bonds in the protein coat of spore cells [12,34]. These chemical and structural changes may make the spores more susceptible to the attack of reactive species or excited molecules [19,21].

Based on the results obtained in this research, it can be observed that the use of low-pressure cold plasma has a significant effect on the reduction of both vegetative cells and spores of *B. cereus*. It was observed that the power intensity and the type of matrix are critical variables that affect the effectiveness of the treatment, more than time. These observations suggest that higher power densities may improve treatment efficacy by promoting chemical and structural changes in spores, making them more susceptible to reactive species. This highlights the importance of optimizing the energy settings to achieve effective microbial inhibition in different matrices. Despite less effective spore inactivation compared to vegetative cells when present in a rice matrix, it suggests the possibility of using this technology as a non-thermal disinfection method before rice processing. By reducing the initial *B. cereus* spore load in the dry rice grain, it could help make subsequent rice cooking procedures more efficient. For example, if combined with other technologies such as microwaves [29], as explored in previous studies, or with natural antimicrobials, as investigated in other works [6,35], it is possible to obtain ready-to-eat food products that meet safety standards.

5.2.3.4. Modelling the impact of low-pressure cold plasma on *B. cereus*.

In this study, the Weibull model was employed to characterize inactivation parameters using experimental data from both vegetative cells and spores of *B. cereus* treated with low-pressure cold plasma sterilization. While initially proposed by Mafart et al. [36] and Peleg & Cole, [37] for thermal sterilization, this model has also been successfully applied by Valdez-Narváez et al., [38] in previous research that

investigated thermal treatment combined with natural antimicrobials for *B. cereus* inactivation and it has demonstrated good performance when microbial inactivation is modelled by non-thermal treatments such as high hydrostatic pressures and pulsed electric fields. Therefore, it is interesting to determine if this model is also a good candidate for rep-resenting inactivation with other non-thermal technologies, such as cold plasma.

For a specific condition, the average experimental inactivation data were fitted to Weibull model (equation 2) to obtain the scale parameter "a" and shape parameter "b". The "a" parameter serves as an indicator of treatment resistance, representing *B. cereus*' survival capability under various treatment conditions. On the other hand, the "b" parameter reflects the curve's shape, indicating the relationship between treatment resistance and time. A "b" value >1 suggests a concave (upward) curve, a value equal to 1 indicates a linear curve, and a value <1 represents a convex (downward) curve.

Results (Table 5.2.1) showed that the Weibull model fitted the experimental data favourably, providing insights into the effect of cold plasma in different study scenarios. The survival curves exhibited different shapes, as depicted in Figure 5.2.1 (A, B). When dealing with *B. cereus* vegetative cells in a rice matrix (Figure 5.2.1A), the curves displayed a shoulder region (gentle initiation). The minimum time required to cause damage to bacterial cells is reflected in the shoulder region, and it can be observed that the mortality rate is time-dependent with “b” values greater than 1. On the other hand, *B. cereus* spores’ inactivation Figure 5.2.1B and Figure 5.2.2 was characterized by inactivation curves with convex shapes (“b” values <1), suggesting the existence of a residual spore fraction resistant to plasma treatment. However, in figure 5.2.1B, it can be seen that for 300W the inactivation of *B. cereus* spores is linear.

After statistical analysis for all the samples and combinations studied, as plasma power increases a significantly decrease (p-value < 0.05) in the parameter "a" was confirmed, meaning that the *B. cereus* resistance decreases as treatment intensity increases. However, there are significant differences between “a” values for

vegetative cells and spores, being the later more resistant to treatment as previously mentioned by Liu et al., [19] and Bourke et al., [21]. At the same time, it can also be found significant differences between “a” values for spores depending on the matrix, denoting a higher spore’s resistance in rice than in the inert surface.

As indicated before, the food matrix acts as an additional protective barrier for spores. Comparing the "a" value from Table 5.2.1, it is evident that it significantly decreases when spores are contained in an inert surface. For instance, at 100W power, the "a" value is 17.72 for spores on slides and 68.17 for spores on rice. At 200W, the "a" value for spores on slides is 7.57, and for spores on rice, it is 49.69. When treated with 300W, the "a" value is 5.90 for spores on slides and 34.05 for spores on rice. This significant difference in "a" value and the shape of the curve detailed above, confirms what was previously observed with the inactivation data that the matrix has a substantial impact on the treatment; when the spores are contained in the food matrix, adhere to rice grains, making it more challenging for plasma to penetrate the matrices and have a reduced effect on the spores.

Regarding methods for fitting cold plasma effects, several authors have found that the Weibull model effectively represents the impact of plasma on bacterial inactivation. Qian et al., [39], studied the effect of plasma on *Listeria monocytogenes* and *Salmonella enteritidis* with three kinetic models in which they found that the Weibull model had a good fit, demonstrating its suitability for describing the inactivation kinetics of these pathogens under plasma treatment conditions. Kim et al., [29] also used the Weibull model to study the effect of plasma on *B. cereus*, obtaining values of “a” 20.66 and “b” 0.41 after 40 minutes of treatment at 400W, while Hertwig et al., [40] also used a Weibull model to study the effect of plasma, obtaining values of “a” 2.35 and “b” 1.10 for *B. atrofaeus* and for *B. subtilis* values “a” 7.40 and “b” 4.27. These results differ from those in this study, which could be attributed to variations in the matrix or treatment equipment.

Table 5.2.1. Weibull survival function parameters obtained by fitting experimental mean data.

	100W				200W				300W			
	a	*b*	R^2_{adj}	MSE	*a*	*b*	R^2_{adj}	MSE	*a*	*b*	R^2_{adj}	MSE
RV	9.54 ± 0.26 $^{*, a}$	2.39 ± 0.10	0.9997	0.0008	7.59 ± 0.10 $^{*, b}$	2.43 ± 0.13	0.9951	0.0091	6.28 ± 0.20 $^{*, c}$	1.91 ± 0.15	0.9914	0.0188
RS	68.17 ± 0.04 $^{*, A, a}$	0.71 ± 0.07	0.9994	0.0001	49.69 ± 0.04 $^{*, A, b}$	0.73 ± 0.03	0.9950	0.0010	34.05 ± 1.50 $^{*, A, c}$	1.04 ± 0.04	0.9995	0.0002
BS	17.72 ± 0.10 $^{B, a}$	0.60 ± 0.02	0.9870	0.0053	7.57 ± 0.07 $^{B, b}$	0.41 ± 0.02	0.9917	0.0040	5.90 ± 0.90 $^{B, c}$	0.58 ± 0.02	0.9978	0.0014

RV: Rice and vegetative cells RS: Rice and spores BS: Borosilicate slide glass and spores. The asterisk denotes statistically significant differences between vegetative cells and spores of *B. cereus* within a rice matrix. while capital letters (A and B) denote statistically significant differences between *B. cereus* spores within distinct matrices. Lowercase letters (a, b, and c) denote statistically significant differences between the various study groups for each power level. ANOVA ($p<0.05$)

Finally, the model was validated by calculating the accuracy factor (AF) using equation 3. For *B. cereus* vegetative cells within a rice matrix, the AF value was 1.033, indicating a prediction error rate of 3.3%. For *B. cereus* spores within the same matrix, the AF value was 1.021, corresponding to a 2.1% prediction error rate. For *B. cereus* spores on a borosilicate slide, the AF value was 1.009, reflecting a prediction error rate of 0.9%. This model effectively predicts the resistance of *B. cereus* vegetative cells and spores on both rice and borosilicate glass slides after exposure to various plasma treatments.

These data provide a comprehensive understanding of the effects of cold plasma application at different stages of the *B. cereus* life cycle and within diverse matrices. Modelling the kinetics of microbial inactivation is crucial to predict how the microbial population changes with time and treatment conditions, allowing scale-up at an industrial level to optimize disinfection processes, validate food preservation methods, design control strategies of contamination and comply with food safety standards.

5.2.4. Conclusions

This study demonstrated that using a low-pressure cold plasma system with O_2 as the ionization gas, can reduce both, vegetative cells and spores of *B. cereus*, in both a rice matrix and inert surface However, despite achieving less spore inactivation compared to vegetative cells when contained in a rice matrix, this could be promising for the use of this technology as a non-thermal disinfection technique prior to processing/cooking low water activity matrices such as rice. Plasma treatment has shown its ability to reduce the initial *B. cereus* spore's load, so the knowledge gained in this study highlights the potential industrial applications of cold plasma technology in food processing. By integrating cold plasma treatments as a pre-processing step, food manufacturers can improve the microbial safety of products, extending shelf life and ensuring consumer health and safety. This non-thermal disinfection method offers a viable alternative, however, seeing the impact that the matrix has on the

treatment, further research is necessary to optimize and industrially scale this technology in various food matrices.

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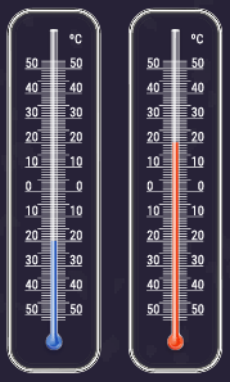
CAPÍTULO 5.3



EFECTO CONJUNTO DE LA TEMPERATURA Y EL QUITOSANO DE INSECTO SOBRE LA RESISTENCIA AL CALOR DE LAS ESPORAS DE *BACILLUS CEREUS* EN DERIVADOS DEL ARROZ

PUBLICACIONES

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RESEARCH ARTICLE

Joint effect of temperature and insect chitosan on the heat resistance of *Bacillus cereus* spores in rice derivatives

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Abstract

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CONGRESOS

Presentación oral: Antimicrobial effect of insect chitosan against *B. cereus* spores in rice
Evento: 19th European Young Cereal Scientists and Technologists Workshop
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Abstract

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Keywords: Spores, *Bacillus cereus*, thermal resistance, antimicrobial additive effect, food safety

5.3.1. Introduction

Bacillus cereus is present in many foods due to its ubiquitous nature. This microorganism is one of the top ten pathogens responsible for many foodborne diseases in humans [1]. According to the latest EFSA and ECDC report [2] there is strong evidence for *B. cereus* was involvement in 38 outbreaks and weak evidence of involvement in 117 outbreaks out of a total of 155 outbreaks reported in 2019. Some recent outbreaks in non-EU countries have also been associated with this pathogen; 45 people were affected in an outbreak in a restaurant in Canberra (Australia) in 2018 [3] and 200 students in an outbreak in a school in China in 2018 [4]. *Bacillus cereus* causes two types of food poisoning one of an emetic nature and the other of a diarrheal nature [5]. On the one hand diarrheal syndrome is caused by a gastrointestinal disorder due to the ingestion of *B. cereus* spores present in food and at a dose given, an appreciable probability that cells cross the stomach barrier and implanting themselves in the small intestine is possible. Once they germinate in the small intestine they produce enterotoxins that cause disease. On the other hand, emetic syndrome is associated with the production of cereulide toxin in the food contaminated with spores that germinate and produce the toxin resulting in foodborne poisoning [6]. In general, this microorganism is associated with complex food products that may include rice as a component; however, other rice-based products and farinaceous foods such as pasta and noodles are also frequently contaminated and involved in cases of *B. cereus* poisoning [7]. The ability of *B. cereus* to form spores and biofilms enables its persistence in various ecological niches and food products resulting in its presence in processed foods such as cooked rice [8]. Furthermore, it is the bacteria most commonly present in rice and rice-based products [9]. Rice is a basic cereal in many diets and is widely consumed by the general population given its ample supply of nutrients and its relatively low cost. This cereal is one of the most important staple crops feeding almost half of the world's population [10]. Starch is the most abundant component of a rice grain constituting about 80% of the dry weight of a brown rice grain and approximately 90% of a milled rice grain [11]. Rice also provides an important variety of micronutrients including vitamins such as niacin, thiamine, pyridoxine or vitamin E, and minerals such as potassium, phosphorus,

magnesium and calcium [12]. These conditions provide a very good substrate for *B. cereus* growth and subsequent toxin production. This cereal is habitually contaminated by *B. cereus* spores throughout all production stages from cultivation to the later stages of processing and consumption. It is believed that the primary habitat of emetic strains could be related to roots tubers and mycorrhizae of some plants such as rice which could explain the generally higher prevalence of these strains in carbohydrate-rich foods. In fact, starch has been shown to promote *B. cereus* growth and emetic toxin production. This would explain why most outbreaks of emetic disease are associated with starch-rich farinaceous foods [13]. Some works pointed out that the current cooking processes for rice and rice derivatives do not inactivate *B. cereus* spores and consequently they can germinate and grow in food if it is not stored properly [1]. Different control measures have been proposed to control *Bacillus cereus* in foods. As an additional strategy, heat treatment can be combined with other control measures (hurdle technology). In this respect, chitosan from different sources (crustacean or fungi) has received attention as an antimicrobial. It is a polysaccharide with a well-documented antibacterial activity towards vegetative cells which has already been effectively applied as edible chitosan films [14] and in food packaging applications [15, 16]. According to Van Huis et al. [17] rearing insects is a sustainable activity more friendly with the environment than fishing or traditional farming. Besides, as indicated by Mohan et al. [18], the extraction of chitin and chitosan from insects is more advantageous in terms of extraction methods, chemical consumption, time and yield compared to existing sources. Existing chitin resources have some natural challenges, including insufficient supplies, seasonal availability, and environmental pollution. As an alternative, insects could be utilized as unconventional but feasible sources of chitin and chitosan. According to previous in vitro studies [19], insect chitosan could be used as antimicrobial instead of chitosan from other sources. Based on those results it could be also applied as an additional control measure during heat processing of rice thus favouring the destruction of *B. cereus* spores by affecting their heat resistance. Currently there are no data on the joint effect of insect chitosan and heat on the heat resistance of *B. cereus* spores since chitosan from other sources is used as a natural antimicrobial in the preservation

processes. The purpose of this study is to determine how *B. cereus* spore inactivation is affected by the presence of insect chitosan during the heat treatment. This knowledge can pave the way to a better control of *B. cereus* during and after the cooking processes of rice and its derivatives.

5.3.2. Material and methods

5.3.2.1. Microorganisms and sporulation procedure

The *Bacillus cereus* CECT 148 strain used in this study was obtained from the Spanish Type Culture Collection (CECT), (Valencia, Spain). The strain was reactivated in nutrient broth by shaking for 24 hours at 32˚C and subsequently 0.5 ml of the *B. cereus* culture was inoculated in 20 Roux flasks (Fisher Scientific SL, Madrid, Spain) with Fortified Nutritive Agar (Scharlab. Barcelona, Spain) and incubated at 30˚C. When the sporulation level reached approximately 90% the spores were collected. Spore harvesting was performed using a modified metal Digralsky loop (Deltalab, Barcelona, Spain) gently sweeping the agar surface and washing it with double distilled water. The collected solution was centrifuged at 2500g for 15 minutes at 5˚C the supernatant was removed suspended again in 5 ml of double distilled water and was centrifuged under the same previously described conditions this process was repeated 4 times. Finally, the spores from the pellet were stored at 4˚C in distilled water.

5.3.2.2. Substrate preparation

The rice solutions from cooked and lyophilized rice supplied for a local company were prepared by dissolving 0.4 g in 19 ml H_2O. All solutions were heat sterilized. After sterilizing the rice solution, 1 ml of the spore solution was added and homogenous distribution was guaranteed by a vortex. Two solutions of rice with insect chitosan from *Tenebrio molitor* (150 and 250 μg/ml chitosan) (ecoProten, Cordoba, Spain) were used for the heat resistance studies on the food matrix. Those concentrations were chosen because in previous studies carried out by Valdez et al. [20] 250 μg/ml showed a higher antimicrobial effect than the 150 μg/ml concentration. The pH was adjusted to between 6.8 and 6.9 by using NaOH. Finally, 1 ml of the spore suspension was added and homogenous distribution was guaranteed

by a vortex. The resulting 20 ml of solution containing spores and chitosan were poured into a 50 ml sterile beaker. In all cases the spore concentration in the resulting rice solution was 10^8 spores/ml.

5.3.2.3. Capillary filling and heat treatment

The capillary tubes with one end closed were supplied by Vitrex, reference 217913 (1.50 x 2.00 x 100 mm). For the heat resistance study capillaries were filled using a drying chamber with a vacuum pump. Once the vacuum was achieved, it was broken and the rice solution rose through the capillaries, which were filled to a volume of 2/3 of their capacity. After that, the solution column was centred in the capillaries they were removed from the chamber and the open end was closed with a quick-drying silicone. Before the heat resistance study spores were heat activated in order to create the conditions for them to germinate and grow in the culture medium. For the activation of *B. cereus* spores the capillaries were placed in hooked racks designed for this type of study. The racks with the capillaries were immersed in a water bath (HAAKE N3) at 80˚ C ± 0.5 for 10 minutes. Both the rice solution alone and the rice solution containing chitosan were heat treated at 90, 95, 100 and 105˚C for different exposure times from 0 to 50 min depending on treatment temperature. A silicone oil bath (HAAKE DC5) was used for this treatment. For time zero (0) and for each treatment temperature a capillary rack was removed after spore activation and was not heat-treated thus considered as control. The rest of the racks were withdrawn from the activation bath and immediately immersed in the oil bath at the selected temperature. A rack was removed at each time interval and immersed in ice water to stop the treatment.

Before the solution was plated the capillaries were cleaned with 96% ethanol and using forceps the ends were split to extract the solution. The content of eight capillaries was deposited into sterile Eppendorf tubes. With the solution recovered from the capillaries two series of serial decimal dilutions (series A and B) were made up to 10^{-6} in duplicate. From each decimal solution 100 µl was plated in duplicate on nutrient agar (Scharlab, Barcelona, Spain) enriched with 1g/l starch (Scharlab, Barcelona, Spain) and incubated for 18–20 hours at 30˚C. After the incubation time,

a manual count of *B. cereus* colonies was carried out. Spore aggregation was prevented by vigorous shaking with glass beads before taking each sample for plating.

5.3.2.3.1. Statistical analysis

All statistical analyses including the one step nonlinear regression were performed using Statgraphics Centurion XVI Software (Addinsoft SARL. New York. NY. USA). Non-linear regression is a powerful technique for standardizing data analysis [21], it allows obtaining the D and z values from survival curves at once

5.3.3. Results

In the present work, the heat resistance of *Bacillus cereus* was studied in a rice substrate without insect chitosan and with insect chitosan at two concentrations. The survival curves at each temperature tested in the study can be seen in Fig 5.3.1A–D. In general, at all temperatures studied *B. cereus* spore's inactivation in the rice substrate was lower than in the substrate without chitosan. Regarding chitosan concentrations, we also observed that for all temperatures the heat resistance of *B. cereus* spores was quite similar, so the chitosan concentration in the heating medium did not affect the survival of these spores. The parameters defining the spore's inactivation were derived by a non-linear one-step fitting of the survival data. Nonlinear models often capture the relationships in a data set better than linear models. Perrin [22] described the disadvantages of the usual linear least squares analysis of first- and second-order kinetic data and nonlinear least squares fitting was recommended as an alternative. In our study the value of the studentized residuals was in all cases two or less than two in any case three as absolute value this means that in no case the residuals exceed two standard deviations. Table 5.3.1 shows the estimation of the parameters that define the heat resistance of *B. cereus* spores D_T for each of the substrates and temperatures studied. Table 5.3.2 shows the z value for each of the studied substrate.

Table 5.3.1. Estimation of thermal resistance parameters by a nonlinear regression in the different substrates.

Temperature (ºC)	Estimated D value (min)		
	Without chitosan	Chitosan 150 µg/ml	Chitosan 250 µg/ml
90	18.90 ± 1.25	15.47 ± 0.94	14.17 ± 1.09
95	5.87 ± 0.21	4.27 ± 0.06	4.83 ± 0.06
100	1.82 ± 0.06	1.18 ± 0.06	1.64 ± 0.04
105	0.56 ± 0.05	0.32 ± 0.02	0.56 ± 0.03

Table 5.3.2. Estimated z values (ºC) in different substrates.

Substrate	Estimated z value (ºC)	Standard Error Asymptotic
without chitosan	9.84	0.30
chitosan 150 µg/ml	8.95	0.20
chitosan 250 µg/ml	10.70	0.32

The value of the parameter D_T estimated by the model is clearly higher in the substrate without chitosan than in the substrate containing chitosan which is related with the lower spore inactivation as previously shown by the survival curves. Regarding the value of the parameter, D_T estimated by the model when chitosan is present little difference was found between the two chitosan concentrations. It seems that the effect of chitosan on the inactivation of *B. cereus* spores does not depend on the concentration of chitosan between 150 and 250 µg/ml during heating. With respect to the value of the z parameter estimated by the model varied between 8.9 and 10.7, those are quite common values for this microorganism [23, 24].

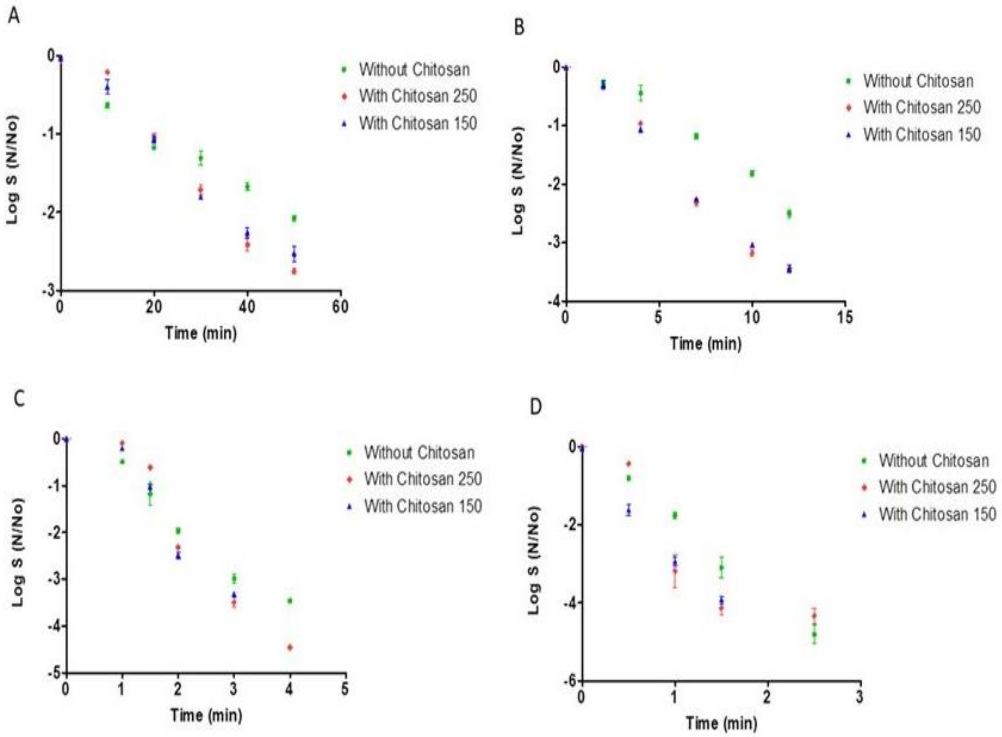

Figure 5.3.1. Survival curves for *Bacillus cereus* (A) heated at 90˚C, (B) heated at 95˚C, (C) heated at 100˚C, (D) heated at 105˚C.

5.3.4. Discussion

Bacillus cereus is a ubiquitous microorganism that can cause serious food safety issues especially in rice products and their derivatives. Proper characterization of its thermal resistance is essential for the design and development of suitable cooking processes. Likewise, the prospect of using combined processes in this case with natural antimicrobials can pave the way to improving the safety of these widely consumed products around the world. Currently there is information on the effect of temperature and on the effect of chitosan separately on *B. cereus* spores. Several works have reported the variation in the D_T and z values of the microorganism in different heating substrates. Pendurka and Kulkarni [25] studied the heat resistance of the spores of five *Bacillus* species including *B. cereus* in distilled water and

pasteurized skim milk. The authors found that in all cases the spores survived the cooking conditions applied to the rice. At 100˚C a D_T value of 19 min was shown by *B. cereus* in distilled water while *B. cereus* spores were completely inactivated in skim milk at the same temperature (100˚C). This result indicates low levels of heat resistance. In the present work at 100˚C a D_T value of 1.82 min was recorded when the spores were heated in a rice solution. However, the great variability that exists between *B. cereus* spores in relation to heat resistance is well known. Fernandez et al. [23] studied the heat resistance of two *Bacillus cereus* strains isolated from cooked chilled foods containing vegetables and found D_T values between 0.22 and 2.5 min at 100˚C.

More recently Salwa Abu El-Nour Ali Hammad [26] found D85-values of *B. cereus* spores ranging from 24.9 to 35.2 min. D90-values ranging from 7.6 to 11.6 min. whereas D95-values ranged from 2.4 to 4.7 min. depending on the type of substrate. The values obtained in the present work are slightly higher probably due to the strain and substrate differences.

Regarding the z value Fernandez et al. [23] reported values of 8.1 and 8.4˚C depending on the strain considered obtained on a reference substrate. Salwa Abu El-Nour Ali Hammad [26] reported z values of *B. cereus* spores suspended in different media ranging from 9.81 to 11.24˚C. In the present work, the z value ranged from 8.9˚C to 10.7˚C depending on the substrate used. The z values obtained in the present work are in accordance with previously reported results; therefore, these results can be considered a suitable reference to develop suitable cooking conditions for rice.

Today, chitosan is extensively studied given the multiple applications that it can have in both the food and the pharmaceutical industries. One of these applications is its use as a natural antimicrobial in food preservation. Ke et al. [27] indicated that the broad-spectrum antimicrobial activity of chitosan offers great commercial potential for this product. Some studies have been published in which the effectiveness of chitosan against *B. cereus* has been demonstrated. Fernandes et al. [28] found a relationship between the molecular weight of chitosan and its antimicrobial activity for both vegetative cells and spores of *B. cereus*. Mellegård et al. [29] studied the

inhibition of *B. cereus* spore outgrowth and multiplication by chitosan; they found chitosan exerts antimicrobial activity that appears to be concentration-dependent and related to the average molecular weight and fraction of acetylation of the chitosan used as antimicrobial.

Currently the industry is looking into combined treatments in which the different control measures are administered with lower intensities than when applied individually. In this way, pathogenic microorganisms are inactivated in a way that improves both the nutritional and sensory quality of food. In some cases, this combination is interesting because it can provide greater inactivation by heat than when heat is administered alone. There are no studies in the literature reporting the combination of heat treatment and chitosan to achieve control and inactivation of *B. cereus* in rice-based substrates. However, the effect of combining heat treatment or other control measures with natural antimicrobials has been reported in the scientific literature. Ueckert et al. [30] reported that exposure to heat and nisin caused synergistic reductions of *Lactobacillus plantarum* viability. Huertas et al. [31] studied the combined effect of natural antimicrobials (nisin. citral and limonene) and thermal treatments on *Alicyclobacillus acidoterrestris* spores. Authors concluded that the antimicrobial agents tested did not affect the heat resistance of the spores; however, the antimicrobials were effective in controlling the growth of the microorganisms after the heat treatment. Kamdem et al. [32] studied the effect of mild heat treatments on the antimicrobial activity of some essential oils. Authors indicated that the combination of temperature and those essential oils reduced the treatment time needed to inactivate 7 log CFU/ml of *Salmonella enteritidis*. In the present work a joint effect of heat and chitosan on *B. cereus* spore's inactivation was found, D_T values were in general lower on samples containing chitosan than in the sample without chitosan. The decrease in the D_T values may be due to a reduction in the spores' resistance caused by the joint effect of the thermal treatment and the chitosan, as it has been observed in vegetative cells of *E. coli*, *L. mocytogenes* and *S. Typhimurium* [19]. Probably, the additive effect during heat treatment depends on the type of microorganism or the type of antimicrobial. It is also possible that the reduction in D_T could be also due to chitosan is blocking outgrowth of *Bacillus* spores

that have been damaged by wet heat. In any case, the presence of chitosan increases the inactivation or prevents the development of spores of *B. cereus*, significantly improving the food safety of the food. Besides, in the present work, we found that the effect on D_T values was not dependent on chitosan concentration. It is possible that at this level the chitosan concentration does not play an important role but rather it is the molecular structure of the chitosan that facilitates the action of heat on the bacterial spores thus reducing the number of spores capable of germinating and growing.

5.3.5. Conclusions

This study investigated the nature of the inactivation of *Bacillus cereus* spores by combining insect chitosan with heat treatment. The results indicated that the presence of chitosan regardless of its concentration produced reductions in the D_T value of *B. cereus* spores in a rice substrate. These findings pave the way to a better control of *B. cereus* during and after the cooking processes of rice and its derivatives making the combination of chitosan with heat treatment feasible in order to improve the safety of these types of products. These results also indicate that insect chitosan could be also used as chitosan from other sources, in combination with heat treatment as an additional control measure.

5.3.6. Acknowledgements

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CAPÍTULO 5.4

QUITOSANO DE INSECTO COMO ANTIMICROBIANO NATURAL CONTRA CÉLULAS VEGETATIVAS DE *BACILLUS CEREUS* EN UNA MATRIZ DE ARROZ COCIDO

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Insect chitosan as a natural antimicrobial against vegetative cells of *Bacillus cereus* in a cooked rice matrix

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ABSTRACT

This study investigates the antimicrobial activity of insect chitosan against vegetative cells of *Bacillus cereus* in a rice matrix. Sample culture solutions were prepared with different concentrations of insect chitosan (150, 180, 220 and 250 μg/mL) and tested at three temperatures (30 °C, 20 °C and 10 °C), which simulate different storage temperature scenarios of precooked rice. The results indicate that insect chitosan has antimicrobial activity that depends on temperature and chitosan concentration. For the assays with chitosan at 10 °C, all concentrations were bactericidal during the study time, reaching a maximum inactivation of 6 log cycles for 250 μg/mL. At 20 °C and at 30 °C a bacteriostatic activity was observed for concentrations of 150 μg/mL and 180 μg/mL. Results also showed that concentrations of 220 μg/mL and 250 μg/mL were bactericidal for all the temperatures tested during the storage time. When rice is cooked and not stored at an appropriate temperature, below 10 °C, the consumer's health is at risk. In these cases, insect chitosan could be a good additional control measure to control *B. cereus* growth and toxin formation in cooked rice.

CONGRESOS

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Abstract

This study investigates the antimicrobial activity of insect chitosan against vegetative cells of *Bacillus cereus* in a rice matrix. Sample culture solutions were prepared with different concentrations of insect chitosan (150, 180, 220 and 250 µg/ml) and tested at three temperatures (30ºC, 20ºC and 10ºC), which simulate different storage temperature scenarios of precooked rice. The results indicate that insect chitosan has antimicrobial activity that depends on temperature and chitosan concentration. For the assays with chitosan at 10ºC, all concentrations were bactericidal during the study time, reaching a maximum inactivation of 6 log cycles for 250 µg/ml. At 20ºC and at 30ºC a bacteriostatic activity was observed for concentrations of 150 µg / ml and 180 µg/ml. Results also showed that concentrations of 220 µg/ml and 250 µg/ml were bactericidal for all the temperatures tested during the storage time. When rice is cooked and not stored at an appropriate temperature, below 10ºC, the consumer's health is at risk. In these cases, insect chitosan could be a good additional control measure to control *B. cereus* growth and toxin formation in cooked rice.

Keywords: Insect chitosan, *Bacillus cereus*, growth, cooked rice

5.4.1. Introduction

Bacillus cereus is present in many foods due to its ubiquitous nature and has become one of the top ten pathogens responsible for many foodborne cases of infection (Rodrigo et al 2021). In 2018, *B. cereus* was involved in 31 strong evidence outbreaks and 67 weak evidence outbreaks, with a total of 98 reported among EU member states, representing1.9% of the total outbreaks in the EU, with 1539 human cases accounting for 111 hospitalizations and 1 death (EFSA and ECDC 2019). In general, *B. cereus* toxic-infection episodes have been associated with complex, mixed food products that may include rice as a component (Little et al., 2002); however, other rice-based products such as pasta and noodles are also frequently contaminated and involved in *B. cereus* toxic-infection (Grande et al., 2006). It has also been found in a wide variety of non-cereals including milk and dairy products, meat products, pasteurized liquid eggs, ready-to-eat vegetables, fruits, and spices (Yu et al., 2020). Due to the extensive distribution of strains in the environment, it is practically impossible to obtain raw materials or food that is free of *B. cereus* spores (Ehling-Schulz et al., 2019; Enosi Tuipulotu et al., 2020; Griffiths and Schraft, 2017). This implies that food contamination can occur during any stage of production, including primary production, harvesting or in slaughterhouses, processing, storage, preparation and consumption of food (Enosi Tuipulotu et al., 2020). *Bacillus cereus* strains can vary with respect to their growth and survival characteristics, with growth limits that are not absolute and depend on the strain and environmental factors, such as the composition of the medium, temperature, pH and water activity (Enosi Tuipulotu et al., 2020). *B. cereus* can produce two types of toxi-infections: diarrheal and emetic syndromes. The first one occurs when a high number of *B. cereus* cells are consumed, the microorganism implants and grows in the small intestine producing the enterotoxin, while emetic syndrome occurs when a food containing preformed cereulide toxin is consumed produced during the growth of *B. cereus* (Kramer et al. 1989).

Rice is a basic cereal in the diet, widely consumed by the general population due to its ample supply of nutrients and its relatively low price. Generally referred to as

Asian grown rice (*Oryza sativa* L.), it is one of the most important staple crops and feeds almost half the world population (Wei and Huang, 2019). This cereal, however, is frequently contaminated by *B. cereus* spores, from cultivation to the later stages of processing and consumption (Kramer et al., 1989). The primary habitat of emetic strains could be related to roots, tubers, and mycorrhizae of rice, which could explain their generally high prevalence in carbohydrate-rich foods (Navaneethan et al., 2021). In fact, starch has been shown to promote *B. cereus* growth and emetic toxin production. This would explain why most outbreaks of emetic disease are associated with starch-rich farinaceous foods (Ehling-Schulz et al., 2015). *B. cereus* is able to generate spores that are resistant to the typical rice cooking or pasteurization processes (Fernández et al., 1999), making it the main opportunistic pathogen found in this substrate (Rodrigo et al., 2021). The main safety issue arises when *B. cereus* spores, once activated by heat, germinate spontaneously in the cooked rice and grow producing the toxin if the cooked rice is stored at an inappropriate temperature, between 5 ° C to 50 ° C (Griffiths and Schraft, 2017). Consequently, having an additional control measure other than temperature alone is highly recommendable in these types of products, especially if they are not being consumed immediately after preparation. In this respect, natural antimicrobials can play an essential role.

Chitosan is a polymer of animal origin with a great interest at the moment. It has gained considerable attention in recent decades, due to its biodegradability, biological compatibility, antimicrobial activity, antioxidant and high safety (Jadhav et al., 2018; Avelelas et al., 2019; Aranaz et al., 2021), which is why it is already used in a large number of pharmaceutical, medical and food applications, among others (Rinaudo et al., 2006; Aranaz et al., 2021). Chitosan is an approved food ingredient in Europe, Japan, Korea, and the United States, and has already been used as a food preservative to prevent spoilage and act as a natural antioxidant (Abd El-Hack et al., 2020). Furthermore, its potential as an antimicrobial has continued to gain ground as a natural food preservative against diverse Gram + and Gram – bacteria (Abd El-Hack et al., 2020; El-Saber Batiha et al., 2021). Insect exoskeleton is also rich in chitin that could be transformed into chitosan. Due to the increase in western countries for insect

farming and consumption promoted by FAO recommendations (FAO 2013), chitosan could be easily obtained from insect's by-product. However, the vast majority of studies have been carried out with chitosan from crustaceans while very little information can be found on the properties of chitosan from insects. In this sense, it would be necessary to investigate whether insect chitosan has antimicrobial capacity and whether this, as described by Kumirska et al., (2011) and Kong, Chen, Liu, et al., (2008) for crustacean chitosan is dependent on the environmental conditions of application, such as the temperature and the concentration applied.

Consequently, the main objective of this work is to evaluate the antimicrobial capacity of insect chitosan against vegetative cells of *B. cereus* in a cooked rice substrate, considering different storage temperatures and chitosan concentrations.

5.4.2. Material and methods

5.4.2.1. Test microorganism

The tests were carried out with a pure lyophilized culture of *B. cereus* provided by the Spanish Type Culture Collection (CECT 148) that is equivalent to ATCC 13061.

The culture was rehydrated with 0.2 ml of sterile Nutrient Broth (NB) liquid medium (Scharlab Chemie S.A., Barcelona, Spain). After 30 minutes, the entire suspension was inoculated in an Erlenmeyer flask with 500 ml of NB medium. This was incubated at a temperature of 30 °C in a thermostatic bath with continuous shaking for 14 h, to obtain cells in a stationary growth phase.

The cells were centrifuged twice at 5000 revolutions per minute (rpm), 4 ° C and 15 minutes, in a Beckman centrifuge (JLA-16,250 rotor). After decanting the supernatant, the cells were resuspended in 50 ml of NB medium. After the second centrifugation the cells were resuspended in NB and then distributed in 2 ml cryovials, adding 1 ml per cryovial. To each cryovial, 1 ml of 20% glycerol in NB was also added, which acts as a cryoprotectant. The 2 ml samples were immediately frozen and stored at -80 ° C until use. The concentration of *B. cereus* in the cryovials was determined by plate count having a concentration of 10^8 CFU/ml.

5.4.2.2. Insect chitosan

The antimicrobial used for the tests was an insect chitosan from the *Tenebrio molitor* (MealFood Europe S.L, Salamanca, Spain; reference 6101. Currently TEBRIO, Salamanca, Spain) purity 90-95%, deacetylation degree >85%.

Chitosan stock solutions were prepared at a concentration of 1% (w/v) of insect chitosan, diluted in a 1% (v/v) acetic acid stock solution (Scharlab Chemie S.A., Barcelona, Spain). This organic acid is used as a diluent to solubilize the chitosan. To improve chitosan solubilization, the solutions were left under continuous stirring for 48 h. Subsequently, and before use, the chitosan solutions were filtered with a sterile 0.45 µm membrane filter for sterilization (MF-Millipore® Membrane Filters)

5.4.2.3. Rice substrate

For the growth of *B. cereus*, powdered freeze-dried cooked rice was used, with a moisture content of 8.66%. This substrate was prepared in the laboratory.

The lyophilized rice was used as a growth matrix at a concentration of 2% (w/v). For this, 1 g of lyophilized rice was diluted in 50 ml distilled water in a bottle with a magnet and a screw cap. Before being used, the rice solutions were sterilized in an autoclave.

5.4.2.4. *Bacillus cereus* growth studies

Sample culture solutions were prepared with different concentrations of insect chitosan (150, 180, 220 and 250 µg/ml), which were tested at pH 6.25 ± 0.2. The tests also included two *B. cereus* controls (rice substrate without chitosan). The first one at the natural pH of the rice substrate (6.85 ± 0.2), and a second one, acidified (acetic acid 0.025% v/v), to reach the same pH (6.25 ± 0.2) that have the chitosan solutions. This second control was considered to evaluate a possible antimicrobial effect of acetic acid under the study conditions.

The different sample solutions were inoculated with the content of a previously thawed and resuscitated (overnight growth in Nutrient Broth, NB, Scharlab Chemie, Barcelona, Spain) vial from the stock, up to a final cell concentration of approximately 10^7 CFU/ml. Subsequently, the solutions were kept in an incubator with continuous shaking at 350 rpm, at temperatures of 30 ± 0.5ºC; 20 ± 0.5°C; and 10 ± 0.5 ºC, simulating different storage temperatures of precooked rice. Being 30ºC a temperature within the optimal range for the growth of *B. cereus*; 20ºC, a temperature that would represent a cold-chain breakdown in the storage process; and 10ºC as an example of refrigeration temperature abuse.

The antimicrobial effect of chitosan was evaluated by taking sampling points at different incubation times (between 0 and 170 hours), depending on the storage temperature. The samples at each control time were diluted by serial decimal dilutions in 0.1% (w / v) peptone water, and plated in NB agar culture medium (Scharlab Chemie, Barcelona, Spain). Plates were incubated at 30ºC for 24 hours before counting.

The experimental results were shown as log10 of the survival fraction (log S) calculated by (Equation 1).

$$\log S = Log\ 10\ (\frac{N}{N_0}) \qquad \text{Equation 1}$$

Where N is the bacterial concentration (CFU/ml) at time t (h) and N_0 the initial bacterial concentration (CFU/ml) (t_0).

5.4.2.5. Statistical analysis

The experimental results were processed in the Microsoft Excel 365 program and the statistical analysis of the experimental data was carried out using the SPSS Statistics V27.0.1.0 program. Outliers were identified and removed prior to data analysis. The statistical significance of the data was determined by an analysis of variance (ANOVA) (p-value <0.05) and inter-group differences were determined by Tukey's

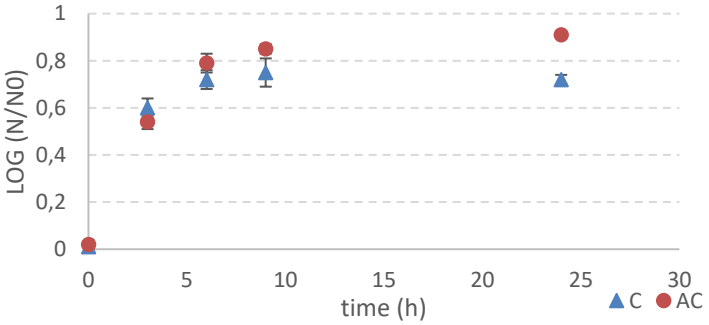
post hoc test, which identifies homogeneous subsets of means that do not differ from each other.

5.4.3. Results

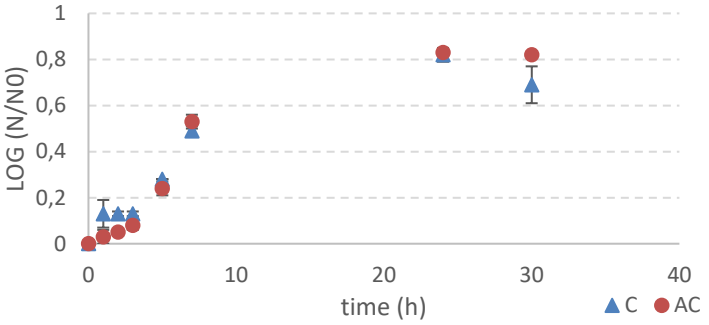
The present work has studied the effect of different concentrations of insect chitosan on vegetative cells of *Bacillus cereus*, stored at three temperatures, comparing the results with those of growth in a control at pH 6.8 and an acid control at pH 6.25.

Figure 5.4.1 (A, B and C) shows the behaviour of the microorganism in the control without acidification (pH 6.8) and in the control acidified with acetic acid (pH 6.25) at the three storage temperatures. Results show that 10ºC is a limiting temperature for the growth of the microorganism used in this study (Figure 5.4.1C). After 200 hours of storage, the microorganism concentration decreased with respect to the initial concentration in both control media, acid control and control without acidification. However, at 20ºC, which is a temperature, considered as a cold-chain breakdown, the microorganism exponentially grows after a short lag phase (Figure 5.4.1B). No significant differences were observed in the final *B. cereus* concentration (30 hours of storage) between acidified and non-acidified control. At 30ºC, which is the optimal growth temperature for this microorganism, no lag phase was identify and the stationary phase was reached at around 10 hours of storage. The final concentration of *B. cereus* cells at 24 hours was significantly higher in the acidified medium than in the non-acidified one, probably due to the fact that the cells in the acid control have some stress resistance mechanism active that makes the chitosan less effective.

(A)



(B)



(C)

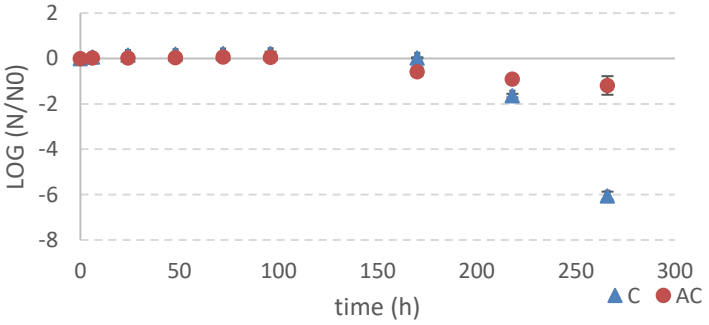


Figure 5.4.1. *B. cereus* behaviour (growth/inactivation) in control samples (C) and acid control samples (AC) at 30ºC (A), 20ºC (B) and 10ºC (C)

The results for *B. cereus* growth as a function of the storage temperature and the concentration of insect chitosan can be seen in Figure 5.4.2 (A, B, C and D). The most outstanding result is the effect of the presence of chitosan on the bacterial counts, that induces a decrease in *B. cereus* concentration compared to the initial one at all storage temperatures and concentrations considered in this study.

On analysing the results by temperature (Figure 5.4.2) we observe that at 10ºC there is a decrease in the concentration of *B. cereus* as the storage time advances, for all the chitosan concentrations in the study. This decrease in *B. cereus* counts was greater as the concentration of chitosan in the medium increased. If we compare these results with those obtained in the controls, acidic and non-acidified media (Figure 5.4.1), we can see that the presence of chitosan exerted an additive effect to the temperature in controlling microorganism growth. Thus, a bactericidal effect for chitosan was observed.

At temperatures of 30ºC and 20ºC, we observed that the cultures treated with the lowest concentrations of chitosan, 150 µg/ml and 180 µg/ml, have a greater recovery capacity during the storage period as compared to the initial inoculation value (N_0). In the cultures treated with chitosan concentrations of 220 µg/ml and 250 µg/ml, the antimicrobial effect of chitosan was higher and, consequently, the recovery capacity of the *B. cereus* cells was reduced, without achieving the levels observed at the lower insect chitosan concentrations during the storage period. Therefore, at these concentrations' chitosan exerted a marked bactericidal effect.

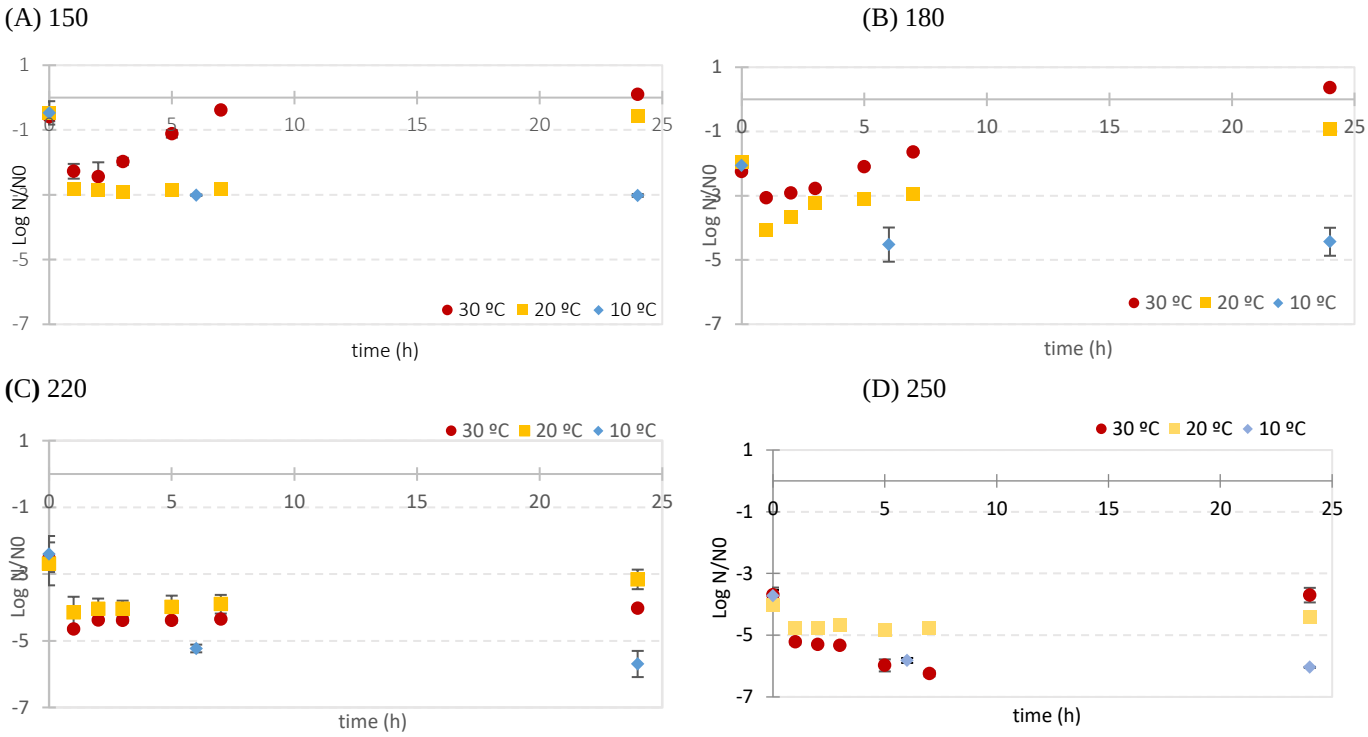


Figure 5.4.2. Effect of insect chitosan concentration 150 µg/ml (A), 180 µg/ml (B), 220 µg/ml (C) and 250 µg/ml (D) on the inactivation of *Bacillus cereus* at 10, 20 and 30ºC.

Table 5.4.1 shows the survival of *B. cereus* after 24 h of incubation for the controls (C) and Acid Control (AC) and the studies with insect chitosan (ICH). The 24 h incubation time was considered a good control point for comparison, since it is the moment when all controls (C and AC) reach the stationary phase.

Table 5.4.1. Survival of *B. cereus* after 24 hours of incubation, represented as the mean of log N/N_0 (CFU/ml) ± SD (C: control; AC: acid control; ICH: assay with insect chitosan at concentrations of 150, 180, 220 and 250 µg/ml).

Substrate	Log N/N_0 (CFU/ml)		
	30ºC	20ºC	10ºC
	Time = 24 h	Time = 24 h	Time = 24 h
C	0.72 ± 0.02 (a, A)	0.82 ± 0.03 (a, B)	0.15 ± 0.01 (a, C)
AC	0.91 ± 0.01 (b, A)	0.91 ± 0.02 (a, A)	0.07 ± 0.01 (a, B)
ICH 150	0.1 ± 0.01 (c, A)	-0.57 ± 0.04 (b, B)	-3.02 ± 0.6 (b, C)
ICH 180	0.36 ± 0.04 (d, A)	-0.92 ± 0.06 (c, B)	-4.43 ± 0.62 (c, C)
ICH 220	-4.03 ± 0.03 (e, A)	-3.16 ± 0.29 (d, B)	-5.7 ± 0.39 (d, C)
ICH 250	-3.7 ± 0.24 (f, A)	-4.43 ± 0.04 (e, B)	-6.04 ± 0.01 (d, C)

Values with the same lowercase letter do not differ significantly by column, and values with the same uppercase letter do not differ significantly by row. Different letters indicate significant differences (p-value <0.05). Positive values indicate growth and negative values mean microbial inactivation with respect to the initial inoculation value (N_0).

According to the table, the statistical analysis only showed significant differences (p-value <0.05) after 24 h of incubation between the C and AC samples for the study at a temperature of 30ºC, where the acid control (AC) grew above the non-acidified control (C), although these differences are not significant in terms of growth. According to these results, the addition of acetic acid at a concentration of 0.025% does not seem to adversely affect microbial growth under these conditions.

Regarding the chitosan exposure studies, the statistical analysis showed significant differences between the different ICH concentrations and between the ICH with the two controls (C and AC), for the different temperatures studied. With the exception of exposures at concentrations of 220 µg/ml and 250 µg/ml at a temperature of 10ºC, where there were no significant differences. The effect of the concentration of insect chitosan was significant in terms of the capacity that the recovery culture had after 24 h of incubation.

Comparison of the results presented in rows (Table 5.4.1) indicates that temperature also exerted an important effect on trends in *B. cereus* counts. In this respect, statistically significant differences were recorded depending on temperature for all controls and exposures to chitosan, with the exception of AC at 30ºC and 20ºC, where there were no differences.

5.4.4. Discussion

Crustacean chitosan exhibits antibacterial activity on vegetative cells, including *Bacillus cereus* cells (Gerasimenko et al., 2004; No et al., 2002; Park et al., 2004; Tsai et al., 2002). Nevertheless, its antimicrobial activity varies as a function of its physicochemical characteristics and depends on the type of microorganism (Ke et al., 2021). In this sense, the present work has investigated the effect of different concentrations of insect chitosan on vegetative cells of *B. cereus* stored at different temperatures. The results have shown that insect chitosan can exert bactericidal or bacteriostatic effects depending on the concentration and the storage temperature, being bactericidal for 220 and 250 µg/ml at all temperatures tested and for 180 and 150 µg/ml at 10ºC while at low concentrations (180 and 150) and 20 and 30ºC it was bacteriostatic (growth inhibition but not death of microorganisms). Mellegård et al (2011) studied the ability of chitosan to inhibit *B. cereus* spore outgrowth and multiplication. They used six different chitosans with defined macromolecular properties. Results of their studies indicated that growth was inhibited by chitosan, but germination was not. Chitosan action was concentration-dependent and also closely related to the molecular weight and fraction of acetylation of the biopolymer. According to these findings, chitosan concentration may play an important role in its antimicrobial capacity, as also observed in the present study, in which the greatest antimicrobial effect of all concentration tested was observed when *B. cereus* vegetative cells were in contact with 250 µg/ml of insect chitosan. Fernandes et al (2009) studied the antimicrobial effect of chitosans with different molecular weight on vegetative cells of *B. cereus*; however, they did not consider different storage temperatures or diverse concentrations of chitosan. These authors used atomic force microscopy imaging to reveal how chitosans with different molecular weight behave

differently against *B. cereus* cells in terms of their antimicrobial effect. Low molecular weight chitosan caused more visible damage in *B. cereus* vegetative cells than high molecular weight chitosan; this was most probably due to cell penetration.

Insect chitosan as an antimicrobial has also been studied for *E coli*, *Salmonella* and *Listeria monocytogenes* cells and compared with crustacean chitosan (Ibañez-Peinado et al 2020). The authors found a very rapid effect of both chitosans during the first hours of storage. This effect was maintained for 24 hours, after which an adaptation of the microbial cells took place, evidenced by an increase in their concentration until reaching the initial levels of inoculation and even exceeding them in some cases. They recorded differences in activity between insect or crustacean chitosan that depended on the microorganism, the initial inoculum concentration and pH of the media, probably due to its different origin, which would affect its physicochemical properties. In this study something similar occurred with *B. cereus* vegetative cells, with a rapid decrease in their concentration followed by a more or less stable period in terms of the number of cells until 24 hours, after which these was an increase in their concentration. The population dynamics of *B. cereus* depended on the incubation temperature and the chitosan concentration. The increase at 24 hours was greater at lower chitosan concentrations (150-180 µg/ml) than at higher concentrations (220 and 250 µg/ml) while at 10ºC the bactericidal effect was observed throughout the incubation time.

Regarding the antimicrobial effects of insect chitosan, these results will provide new opportunities for the use of natural antimicrobials, fostering the reuse of waste from the livestock and food industry and reducing impact on the environment. We should consider that the rearing of insects in mini-farms has a lower impact on the environment than the use of conventional livestock (FAO 2013), with lower greenhouse gas production, water and food consumption, and land use. It should also be pointed out that overexploitation of the seas will lead to a shortage of crustacean skeletons from which to extract chitin in order to produce chitosan.

5.4.5. Conclusions

The use of insect chitosan as an antimicrobial against *B. cereus* may be useful as an additional control measure in ready-to-eat dishes based on precooked rice and its derivatives. Its use together with an adequate storage temperature can reduce the microbial load of *B. cereus* to levels well below the infective dose; therefore, it is applicable to improved food safety of this type of food.

A concentration of insect chitosan of 150 µg/ml and a storage temperature of 10ºC may be ample to guarantee the microbiological stability of precooked rice and its derivatives.

5.4.6. Acknowledgements

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CAPÍTULO 5.5

DESARROLLO DE UN MODELO DE EVALUACIÓN DE LA EXPOSICIÓN A *B. CEREUS* EN UNA MATRIZ DE ARROZ

EVNVIADO A FOOD SCIENCE AND TECHNOLOGY INTERNATIONAL

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Development of an Exposure Assessment industrial model for *Bacillus cereus* in rice matrix containing insect Chitosan.

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Keywords:	Antimicrobial activity, Antimicrobials, Bacterial spores, Foodborne microorganisms, Risk assessment
Abstract:	An exposure assessment model for industrial use has been developed by using kinetic data from inactivation and growth of *Bacillus cereus* spores. It can provide a valuable tool for estimating the concentration of *B. cereus* after a storage period of 24 hours at a specified temperature (20ºC) and for an estimation of the percentage of contaminated portions according to the input data of the model. This model considers a rice-derived product that has undergone a standard cooking process at 95ºC for 20 minutes. According to the results, the presence of chitosan affects the final microbial load after storage, potentially serving as an additional control measure in the event of cold chain abuse or break. Chitosan's antimicrobial properties likely play a role in reducing microbial growth during storage, thereby contributing to enhanced food safety. In practical terms, this suggests that incorporating chitosan into food products, especially those susceptible to microbial contamination like rice derivatives, could help mitigate risks associated with temperature abuse or cold chain disruptions. By acting as a protective barrier against microbial proliferation, chitosan offers a preventive measure to maintain product quality and safety throughout the supply chain. Considering two scenarios, 10^4 or 10^7 as initial contamination the model estimated that the 55 and 100 % of portions would be respectively contaminated,

CONGRESOS

Póster: An exposure assessment model for *Bacillus cereus* in a rice solution, evaluating the intervention of insect chitosan as a natural antimicrobial.
Evento: New advances from PRIMA projects for improving Mediterranean Agro-Food value chains.
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Fechas: 18 y 19 de mayo 2023

Development of an Exposure Assessment industrial model for *Bacillus cereus* in rice matrix containing insect Chitosan.

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Abstract

An exposure assessment model for industrial use has been developed by using kinetic data from inactivation and growth of *Bacillus cereus* spores. It can provide a valuable tool for estimating the concentration of *B. cereus* after a storage period of 24 hours at a specified temperature (20°C) and for an estimation of the percentage of contaminated portions according to the input data of the model. This model considers a rice-derived product that has undergone a standard cooking process at 95°C for 20 minutes. According to the results, the presence of chitosan affects the final microbial load after storage, potentially serving as an additional control measure in the event of cold chain abuse or break. Chitosan's antimicrobial properties likely play a role in reducing microbial growth during storage, thereby contributing to enhanced food safety. In practical terms, this suggests that incorporating chitosan into food products, especially those susceptible to microbial contamination like rice derivatives, could help mitigate risks associated with temperature abuse or cold chain disruptions. By acting as a protective barrier against microbial proliferation, chitosan offers a preventive measure to maintain product quality and safety throughout the supply chain. Considering two scenarios, 10^4 or 10^7 as initial contamination the model estimated that the 55 and 100 % of portions would be respectively contaminated, according to a Performance Criteria of 4 log reductions.

Keywords: *Bacillus cereus*, Insect chitosan, Exposure Assessment, Rice, Monte Carlo simulation, Food safety

5.5.1. Introduction

Rice is undeniably one of the most consumed foods globally, serving as a staple in the diet of many countries, particularly in Asia, with Bangladesh standing out as the world's largest producer and consumer of rice (USDA, 2024). In the European Union (EU), rice production in 2022 reached 1.1 million tons, with Italy accounting for 50% and Spain for 30% of the production area dedicated to rice cultivation (European Comission, 2023). It is an important source of energy in human nutrition due to its high carbohydrate content (Juliano, 1993). However, its composition, particularly the fact that almost 90% of its dry matter is starch, makes it an ideal substrate for the growth of certain pathogenic microorganisms such as *B. cereus*, as indicated by Rodrigo et al., (2021). Therefore, it is crucial to implement proper handling and processing procedures to inactivate pathogenic and spoilage microorganisms as well as incorporate storage conditions to prevent microbiological growth and ensure food safety when dealing with rice products.

Bacillus cereus is one of the microorganisms that can cause foodborne illness when consuming rice or its derivatives. The European Food Safety Authority (EFSA) report for 2022 is alarming because *B. cereus* toxins were the leading cause of foodborne outbreaks attributed to bacterial toxins and were associated with two deaths. There was a significant increase in the number of adverse events (AEs) caused by *B. cereus* toxins in 2022 compared to 2021 (an increase of 219 AEs in 2022, representing a relative increase of 251.7% (EFSA & ECDC, 2023). *B. cereus* is a Gram-positive, ubiquitous, facultative anaerobic, spore-forming bacterium commonly found in soil. It is a mesophilic bacterium capable of growing over a wide range of pH (4.9 to 9.3), temperature (4-48°C), and in foods rich in starch or with a water activity greater than 0.93 (Batt, 2014; Ehling-Schulz et al., 2014). This bacterium is known to produce two types of toxins: one that causes diarrheal illness and another that causes emetic illness. The diarrheal illness is caused by vegetative cells that produce enterotoxins in the small intestine and is often associated with protein-rich foods such as meat, vegetables, puddings, and dairy products. However, *B. cereus* spores can withstand traditional cooking treatments and remain stable up to 121°C (Valdez, Úbeda-

Manzanaro, et al., 2022). They can grow and produce the emetic toxin in foods before consumption. This toxin is commonly associated with starch-rich foods such as fried and cooked rice, pasta, and noodles. The emetic toxin is particularly concerning in the context of rice, as it can withstand high cooking temperatures and remain active in the food, potentially leading to food poisoning if contaminated or mishandled rice is consumed (Rodrigo et al., 2021).

The issue with *B. cereus* in rice lies in the fact that, with a traditional cooking process at 100°C for 20 minutes, only 2-3 logarithmic reductions of vegetative cells can be achieved, but its spores can withstand these treatments (Valdez, Úbeda-Manzanaro, et al., 2022). This poses a problem for ready-to-eat products, which, after receiving the thermal treatment, must be stored under refrigeration. A commercial sterilization treatment could help eliminate the spores; however, excessive heat treatment can significantly reduce the commercial value of the product (Webb et al., 2019). Therefore, it is necessary to seek additional control alternatives for such products. Natural antimicrobials, along with new non-thermal preservation technologies, could be interesting alternatives. They are more environmentally sustainable and can help maintain the nutritional and sensory characteristics of the food (Khan et al., 2017).

Chitosan has developed as a natural antimicrobial with various potential applications in the agri-food industry. Chitosan has been obtained from the exoskeletons and shells of crustaceans and has demonstrated excellent antimicrobial and antioxidant properties (Goy et al., 2009). Based on those previous studies with crustacean chitosan, Valdez, Garcia, et al., (2022) and Valdez, Úbeda-Manzanaro, et al., (2022) carried out studies to assess whether chitosan obtained from insects exhibits the same antimicrobial properties as chitosan derived from crustaceans. The results suggested that insect-derived chitosan could be an interesting alternative as a natural antimicrobial, while also offering opportunities for the valorisation of insect-based industry by-products. These findings are significant as they imply that chitosan derived from insects might be equally effective in inhibiting the growth of pathogenic microorganisms, thereby expanding the sources of this important antimicrobial substance. Furthermore, the repurposing of industry by-products, such as insect

exoskeletons, for chitosan production, can promote sustainability and the circular economy within the food chain and other industries (Valdez, Garcia, et al., 2022).

When dealing with a new product and a novel control measure, it is crucial to conduct an exposure assessment at an industrial level to complement measures such as HACCP. This assessment would enable the implementation of safety management actions for final products before they are released to the market. Additionally, it is essential to evaluate the impact that the refrigerated storage process may have on the final microbial load of the product before consumption (EFSA Panel on Biological Hazards (BIOHAZ), 2016). By conducting an exposure assessment, potential risks associated with the new product and control measure can be identified and addressed proactively. This helps ensure that the final products meet safety standards and are safe for consumption. Moreover, assessing the impact of refrigerated storage on microbial loads can provide valuable insights into the effectiveness of this storage method in controlling microbial growth and ensuring product safety throughout the supply chain (Membré & Boué, 2018). Overall, a comprehensive evaluation of exposure and risk management measures is essential for safeguarding the safety of new products and maintaining consumer confidence in the food supply.

Microbiological Risk Assessment (MRA) is indeed a systematic approach used to evaluate the potential risks associated with biological hazards in food. While its primary focus is on food safety, it is important to recognize that in an industrial level, the concept of "risk" can extend beyond just safety concerns to include aspects related to food quality as well. Microbial spoilage, for instance, can compromise the quality of food products, and depending on the microbial ecology promote the growth of pathogenic microorganisms. Therefore, when conducting MRA in an industrial context, it is essential to consider both safety and quality aspects to ensure the overall integrity of the food supply chain (EFSA, 2005)

When transferring risk assessment concept to the industry, exposure assessment becomes the main component. Exposure assessment involves understanding and quantifying the likelihood of consumers being exposed to microbiological hazards

present in food. This step is essential because it provides crucial information about how and to what extent consumers may be exposed to microbiological risks. It considers changes from raw materials, processes or the use of new preservation methods through to consumption. This process involves evaluating various factors such as food processing methods, storage conditions, handling practices, and consumption patterns to estimate the level of microbial contamination that consumers are likely to encounter during the entire food chain (Membré & Boué, 2018).

In this context, the main objective of this study is to conduct a risk analysis by developing a modular model for assessing exposure to *B. cereus* spores in a rice matrix when it contains insect-derived chitosan as a natural antimicrobial considering environmental factors as the cooking temperature, chitosan concentration and storage temperature.

5.5.2. Material and methods

5.5.2.1. Building the model: structure, equations, inputs, outputs

The model structure plays a critical role in understanding how the inputs of the model influence the response, or the main output. Figure 5.5.1, shows a scheme that illustrates the relationships between the various components of the exposure assessment model.

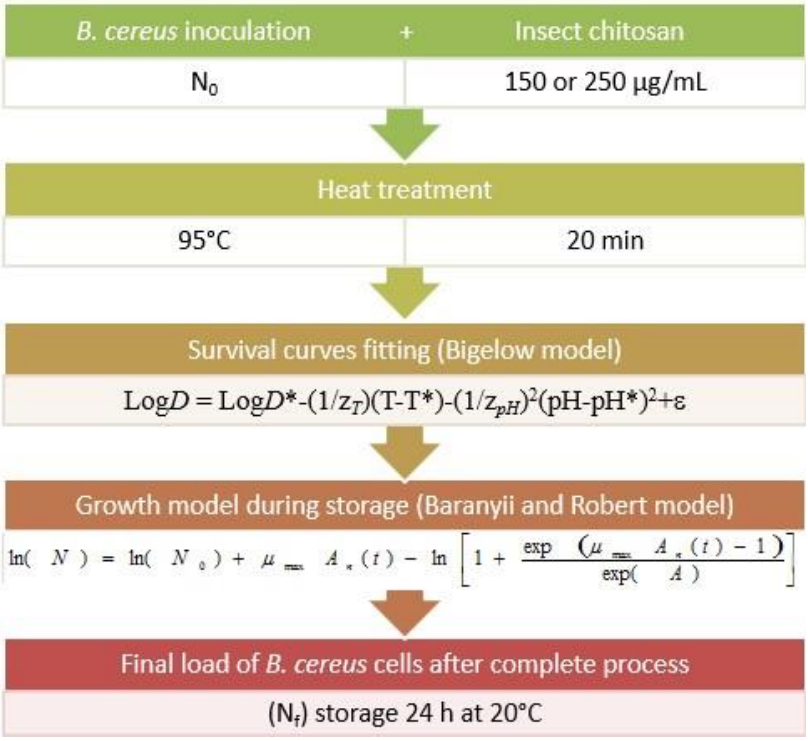


Figure 5.5.1. Flowchart for risk assessment model developed based on the final load of *B. cereus* in rice matrix, using insect chitosan as a natural antimicrobial and stored for 24h at 20ºC.

A modular exposure assessment model was constructed, considering the kinetic parameters obtained from mathematical models of inactivation and growth. This modular approach integrates the results of microbial inactivation and growth models into a coherent and understandable exposure assessment framework. By combining these models into a modular approach, the exposure to pathogenic or spoilage microorganisms in foods can be comprehensively assessed throughout the entire process, from production to consumption. This provides a powerful tool for food safety professionals and regulators to understand and manage microbiological risks in the food chain

The selection of equations depends on the specific aspects of the system being modelled. For example, in a model assessing microbial growth, equations describing

microbial growth kinetics such as the Baranyi & Roberts, (1994) model and modified Gompertz equation (Bhaduri et al., 1991) may be used to predict population dynamics over time. Similarly, equations describing microbial inactivation kinetics such as Weibull (Weibull, 1951) or first-order Bigelow models (Bigelow, 1921) may be employed to predict the reduction of microbial populations under different processing conditions.

5.5.2.2. Model equations

5.5.2.2.1. Inactivation model

In general, when a heat treatment step is involved in microbial inactivation, the resulting reduction in microbial populations is often described using a first-order kinetic model. In this case, the Bigelow model was used (equation 1).

$$\log N = \log N_0 - \frac{1}{D_R * 10^{\left(\frac{T_R - T}{z}\right)}} * t \quad (1)$$

Where N_0 is the initial concentration of microorganisms (CFU/ml) at time (t) zero, N is the concentration of microorganisms (CFU/ml) at time t, D_R is the primary kinetic parameter, often referred to as the D-value (minutes) at reference temperature, which represents the time required to reduce the microbial population by one log cycle at a specific temperature and Z is the secondary kinetic parameter (degrees Celsius), which represents the temperature change required to alter the D-value by a factor of 10.

5.5.2.2.2. Growth model

In the context of a model for microbial growth, variables may include initial microbial concentration, temperature, time, pH, antimicrobial concentration and other factors that affect microbial of *Bacillus cereus*. In this case, the Baranyi and Roberts growth model was used (Baranyi & Roberts, 1994) to obtain the specific growth rate and the lag phase duration (equation 2).

$$\ln(N) = \ln(N_0) + \mu_{max} A_n(t) - \ln\left[1 + \frac{\exp(\mu_{max} A_n(t) - 1)}{\exp(A)}\right] \quad (2)$$

Where $N(t)$ represents the microbial population at time t, N_0 is the initial microbial population. A is the maximum microbial population, μ is the specific growth rate (h^-1), λ is the duration of the lag phase (h).

5.5.2.3. Industrial Exposure Assessment model input

The Industrial exposure assessment model developed has two types of inputs: variables and settings.

Variables: These are quantities or parameters that can vary and directly influence the behaviour of the model or system being modelled.

Settings: These are parameters or conditions that are set by the operator or user and remain constant throughout the simulation or analysis. Settings are often used to represent operating conditions, equipment specifications, or environmental constraints that are fixed and do not change during the simulation.

Table 5.5.1 shows the model inputs considered in the industrial exposure assessment model.

Table 5.5.1. Model inputs considered in the industrial exposure assessment model.

Inputs	Values	Units
Initial Contamination	$5.4 \times 10^7 \pm 0.37$	CFU/portion
Cooking Temperature	95	ºC
Treatment Time	20	minutes
Chitosan Concentration	0, 150, 250	μg/ml
Storage temperature	20	ºC
Storage time	24	hours

5.5.2.4. Industrial Exposure Assessment model output

The output obtained from an Industrial exposure assessment model, typically provides a quantity or concentration of microorganisms in a food product and/or in a consumer's portion. This output essentially represents an exposure assessment, as it

quantifies the level of microbial contamination that consumers may be exposed to throughout the food chain. Table 5.5.2 shows the outputs considered in the model

Table 5.5.2. Model outputs considered in the industrial exposure assessment model.

Outputs	Values	Units
Final number of microorganisms after cooking	Normal (Average; Standard Deviation)	CFU/portion
Final number of microorganisms after storage	Normal (Average; Standard Deviation)	CFU/portion
Number of contaminated units	Binomial (Initial contamination level; probability a microorganism survives the process)	#

5.5.2.5. Simulation

Monte Carlo simulation was carried out by using the *Simular* excel add-in (Version 26e), (https://www.simularsoft.com.ar/), to estimate the level of *B. cereus* after storage time of cooked rice with or without chitosan.

5.5.2.6. Interpretation of Industrial Exposure Assessment model output, what-if scenarios.

An Industrial Quantitative Microbial Risk Assessment (IQMRA) model aims to assess the risk associated with the transmission of microorganisms from their source (such as raw material for industry) to the final consumption (fork). The output of such a model typically includes the estimation of the quantity or concentration of microorganisms present in the food product or the consumer's portion. By quantifying this microbial load, it helps in understanding the potential health risks associated with consuming the food product. Therefore, in essence, a processing-to-fork model is an exposure assessment model, as it evaluates the potential exposure of consumers to microbial hazards throughout until consumption.

What if scenarios are indeed powerful tools in risk assessment, offering insights into how different variables or parameters can affect outcomes. They allow analysts to

explore a range of possibilities and make informed decisions. In the context of food safety, these scenarios can take various formats, such as:

Sensitivity Analysis: The goal of sensitivity analysis is to identify which inputs have the most significant impact on the outputs, allowing decision-makers to focus their attention and resources on those variables that matter most. It helps in understanding the uncertainty and risks associated with the model or decision and can inform strategies to mitigate those risks.

Process Optimization: By varying parameters like temperature, pressure, or time in a thermal process, one can assess their impact on microbial reduction or product quality. This helps in finding the most effective and efficient processing conditions (Membré & Boué, 2018).

5.5.3. Results

5.5.3.1. Industrial Exposure Assessment model

Table 5.5.3 shows the output data from the exposure assessment model after the complete process. The final microbial load ($N_{fHC\ +\ STORAGE}$) without using insect chitosan as an antimicrobial was estimated to be 1.6 x10^5 CFU/g. This value exceeds the infective dose of 10^5 CFU/ml (EFSA Panel on Biological Hazards (BIOHAZ), 2016) with only a 7.2% probability of this load being lower than this concentration.

However, by adding insect chitosan at different concentrations, this risk significantly decreases. For instance, at an insect chitosan concentration of 150 µg/ml, the final *B. cereus* concentration in a rice solution was estimated to be 6.42x10^3 CFU/g, with a 67.8% probability for $N_{fHC\ +\ STORAGE}$ to be lower than 10^4 CFU/ml, a value below the infective dose (EFSA Panel on Biological Hazards (BIOHAZ), 2016), as shown in Table 5.5.3.

Similarly, in Table 5.5.3, it can be seen that if the insect chitosan concentration increases to 250 µg/ml, the $N_{fHC\ +\ STORAGE}$ decreases even further, estimated at

$5.87x10^2$ CFU/g, with a 99% probability for the final *B. cereus* load to be lower than 10^4 CFU/ml.

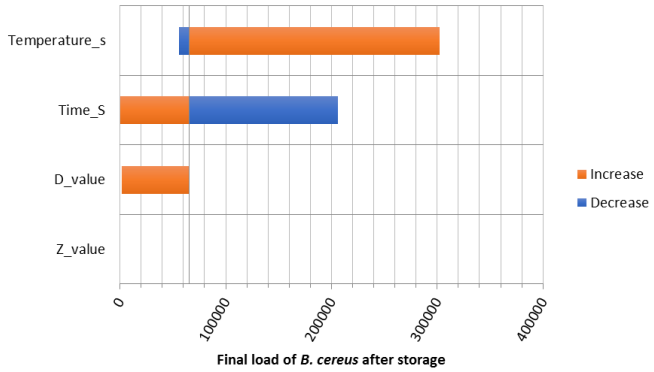
Table 5.5.3. Summary of the input and output parameter values of the exposure assessment model for a cooking process of 95ºC for 20 min and storage temperature of 20ºC for 24 h.

Input/output data	Insect Chitosan concentration (µg/ml)	Results	Units
D values estimated by the model at different chitosan concentration	0	5.87 ± 0.3	minutes
	150	4.27 ± 0.18	
	250	4.83 ± 0.26	
Z values estimated by the model at different chitosan concentration	0	9.84 ± 0.30	ºC
	150	8.95 ± 0.20	
	250	10.7 ± 0.31	
Final load after the cooking process at different chitosan concentrations (N_{fHC})	0	$2.95x10^4 \pm 9.2\ x10^1$	CFU/g
	150	$4.15x10^3 \pm 2.5x10^1$	
	250	$2.02x10^1 \pm 7.00$	
Final load of microorganisms after storage at different chitosan concentrations ($N_{fHC + storage}$)	0	$1.6x10^5 \pm 5.82\ x10^2$	CFU/g
	150	$6.42x10^3 \pm 2.31\ x10^2$	
	250	$5.87x10^2 \pm 6.65$	

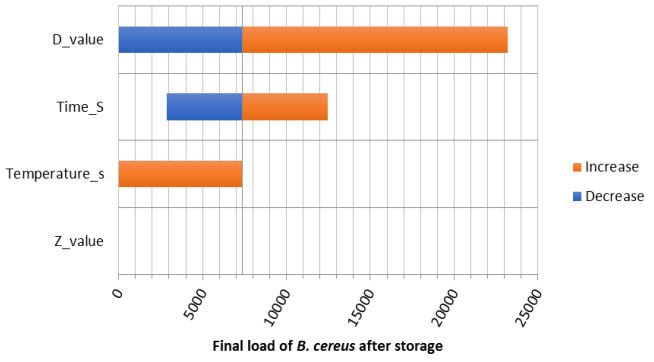
5.5.3.2. Sensitivity analysis

Additionally, a sensitivity analysis was conducted, revealing that the variable that most affects the final number of microorganisms in the event of a cold chain breach ($N_{fHC\ +\ STORAGE}$) is temperature, especially if it increases (Figure 5.5.2A). This behaviour is typical for *B. cereus*, as explained in previous studies (Valdez, Úbeda-Manzanaro, et al., 2022; Wang et al., 2014). However, when insect chitosan is utilized, the variable that most influences the final *B. cereus* load is the value of *D* (Figure 5.5.2B, C). In this case, insect chitosan affects the microorganism through the growth rate constant. Chitosan interacts with the plasma membrane of the thermally treated bacteria, causing permeabilization and intracellular potassium loss (Ke et al., 2021; Li & Zhuang, 2020). This affected the thermo-resistance of *B. cereus* and induces its death.

(A)



(B)



(C)

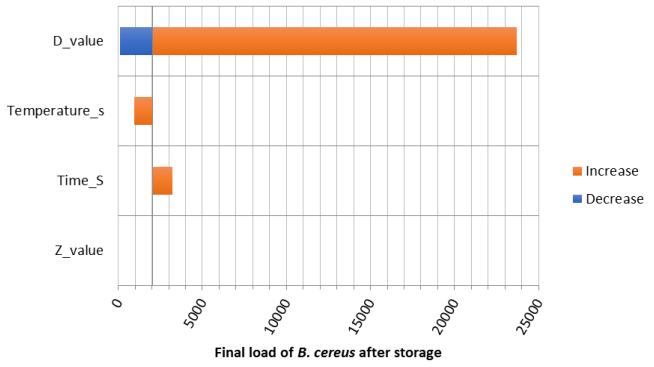


Figure 5.5.2. Tornado plot of the sensitivity analysis performed when control samples and chitosan samples are stored at 20°C for 24h. A (control), B (insect chitosan concentration 150 µg/ml), C (insect chitosan concentration 250 µg/ml).

On the other hand, the Figure 5.5.3 shows the effect of a 2% deviation in cooking temperature on the residual number of microorganisms after the process, when the rice matrix contains 250 µg/ml of insect chitosan as a natural antimicrobial. As can be observed in the figure, a 2% decrease in temperature (93ºC) impacts on the number of residual microorganisms increase them on more than 4 log, unless it does not reach the infective dose of 10^5 CFU (EFSA Panel on Biological Hazards (BIOHAZ), 2016).

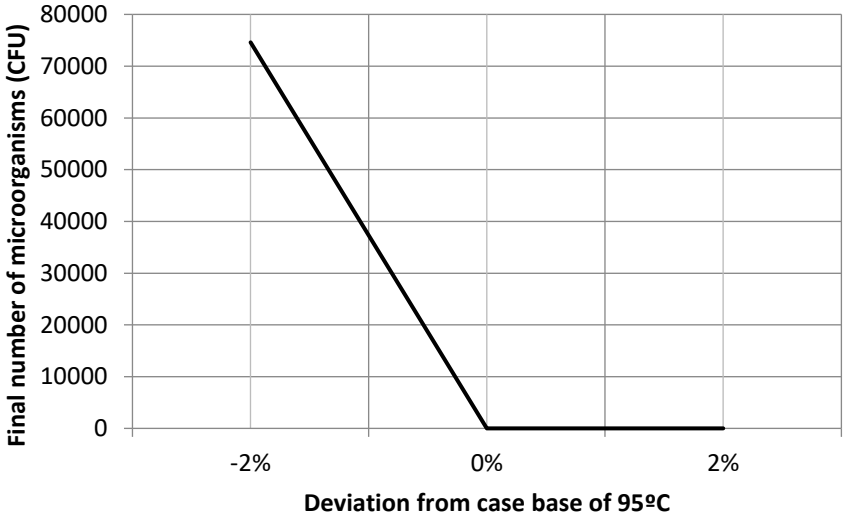


Figure 5.5.3. Effect of a deviation of the 2% on temperature in relation to the case base of 95ºC.

While, Figure 5.5.4 illustrates the effect of a 10% deviation in cooking time on the residual number of microorganisms after the process when the rice matrix contains 250 µg/ml of insect chitosan as a natural antimicrobial. As depicted in the figure, a 10% decrease in time (18 minutes) barely affects the number of residual microorganisms, failing to reach the infective dose of 10^5 CFU (EFSA Panel on Biological Hazards (BIOHAZ), 2016).

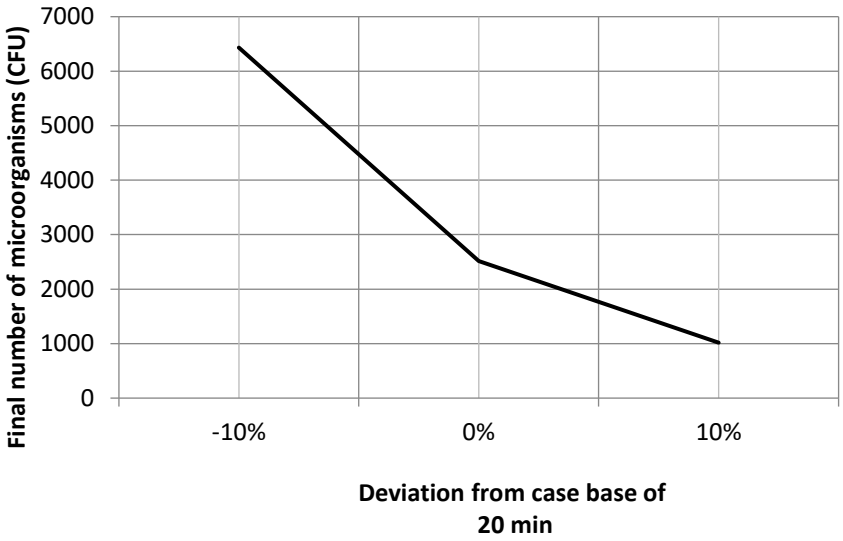


Figure 5.5.4. Effect of a deviation of the 10% on the cooking time in relation to the case base of 20 min.

5.5.3.3. Prediction of the number of contaminated units

The exposure assessment model has an additional module that allows for determining the percentage of units contaminated considering a performance criterion (PC) defined by the ratio of treatment time to the *D*-value at the treatment temperature (Nauta, 2002). The probability that a bacterium survives the thermal treatment (*Pone*) would be equal to 10^{-PC}. The number of bacteria surviving the thermal treatment would be given by a binomial distribution (*No*; *Pone*) (Membré & Boué, 2018). As an example, three scenarios were considered.

5.5.3.3.1. First scenery: Initial contamination of 10^4 cells/portion

The fist scenery was considered the control samples. The simulation results can be seen in Table 5.5.4 and Figure 5.5.5. If the initial contamination is 10^4 cells/portion using the described model, the simulation suggests that when the rice matrix does not contain insect chitosan and then applied a heat treatment with the given quantity

parameters (D value, treatment temperature and treatment time), approximately 96% of the units would be contaminated after treatment.

Table 5.5.4. Results of simulation scenery 1

		Percentiles	
Mean	5th	50th	95th
3.95	1	4	7

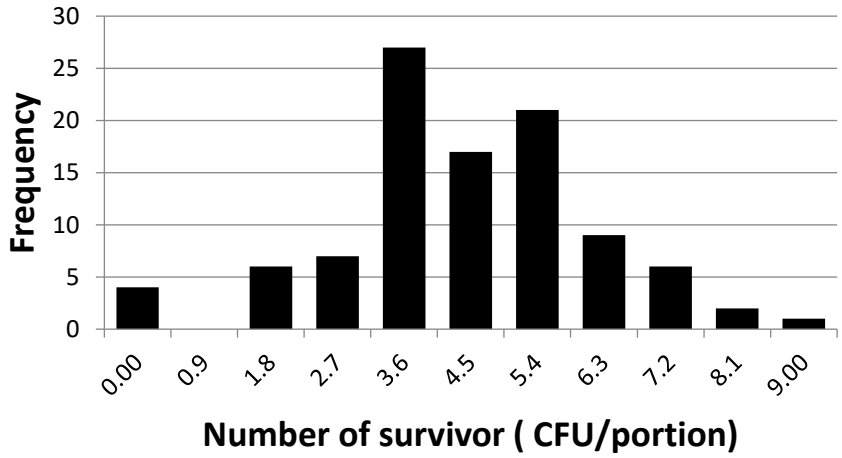


Figure 5.5.5. Frequency chart for an initial contamination of 10^4 cells/portion.

5.5.3.3.2. Second scenery: Initial contamination of 10^4 cells/portion

However, in the second scenery if the initial contamination is 10^4 cells/portion by using the described model, the simulation suggests that when the rice matrix contains 250 µg/ml of insect chitosan and then the thermal treatment is applied with the given parameters (D-value, treatment temperature, and treatment time), approximately 55 % of the units would be contaminated after treatment. This decrease in the number of contaminated units can be attributed to the effect of insect chitosan as an antimicrobial used for additional control of *B. cereus*. Results of simulation for second scenery can be seen in Table 5.5.5 and Figure 5.5.6.

Table 5.5.5. Results of simulation scenery 2

		Percentiles	
Mean	5th	50th	95th
0.71	0	1	2

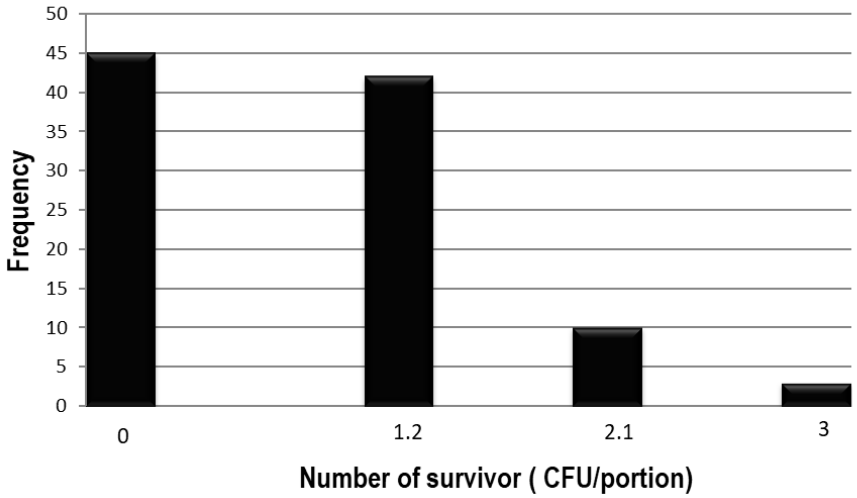


Figure 5.5.6. Frequency chart for an initial contamination of 10^4 cells/portion

5.5.3.3.3. Three scenery: Initial contamination of 10^7 cell/portion

Results of simulation for three scenery can be seen in Table 5.5.6 and Figure 5.5.7. In the case of an initial contamination of 10^7 microorganisms, the simulation indicates that 100% of the units would be contaminated after treatment, even if the rice matrix contains 250 µg/ml of insect chitosan. This could be due to bacteria's resistance to thermal treatment and the survival probability established by the performance criterion.

Table 5.5.6 Results of simulation scenery 3

		Percentiles	
Mean	5th	50th	95th
729.48	677.9	730	770.05

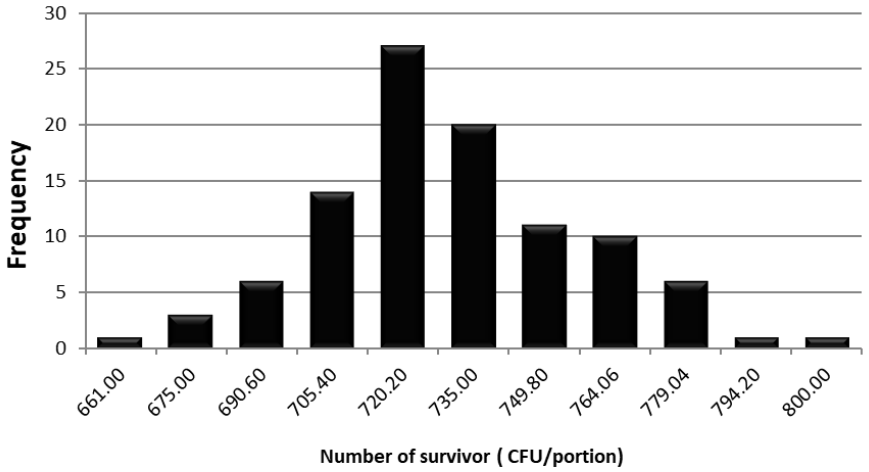


Figure 5.5.7. Frequency chart for an initial contamination of 10^7 cells/portion

5.5.4. Conclusions

The exposure assessment model built in this work allows obtaining an estimate of the final load levels of *B. cereus* after the combination of different control measures: heat treatment, the addition of insect chitosan and storage temperatures. It also allows predicting the concentration level of the microorganism in different process scenarios of a rice-based food.

Using a minimum concentration of insect chitosan (150 µg/ml) that allows controlling the final load of *B. cereus* below 10^5 CFU/ml could increase the level of food safety in rice derivatives and potentially prevent food poisoning issues by reducing the final *B. cereus* load, suggesting it is effective in mitigating microbiological contamination risks.

It is essential to consider that food safety is a paramount concern in the food industry, and any measure that reduces microbial burden and minimizes the risk of food poisoning is beneficial. The utilization of insect chitosan as an antimicrobial agent

can be an effective and natural strategy to enhance food safety, provided it is applied appropriately and complies with food safety regulations and standards.

5.5.5. Acknowledgement

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6. DISCUSIÓN GENERAL

6. Discusión general

En la presente tesis se han explorado diversas estrategias para mejorar la desinfección y el procesado del arroz y sus derivados. Mediante estas estrategias, se pretende obtener productos que no solo sean atractivos para los consumidores por sus atributos específicos, sino que también garanticen la inocuidad alimentaria. Este aspecto es fundamental para asegurar que los alimentos no representen riesgos para la salud pública.

Como se ha mencionado a lo largo del manuscrito, el arroz es uno de los alimentos más consumidos a nivel mundial y juega un papel crucial en el desarrollo de muchas sociedades por su influencia en la alimentación, cultura y economía de las mismas. Su procesado es fundamental para el consumo, con un marcado impacto tanto sobre la inocuidad como sobre la sostenibilidad de la cadena alimentaria. Es por ello que se han querido abordar dos aspectos fundamentales del procesado del arroz que son críticos para comprender y mejorar la utilización de este alimento esencial. Por un lado, el efecto de tecnologías de procesado innovadoras sobre derivados del arroz, con el objetivo de conseguir modificaciones en sus propiedades que puedan mejorar aplicaciones en la industria alimentaria. En segundo lugar, el efecto de diferentes tecnologías de procesado para mejorar la inocuidad alimentaria del arroz y alimentos derivados del mismo. De esta manera, la tesis abarca aspectos de calidad e inocuidad del arroz, no solo como un alimento básico en dietas alrededor del mundo, sino también en su potencial para el desarrollo de productos a la carta.

La tesis aporta información básica y aplicada de indudable interés industrial como se podrá apreciar en la discusión general que se hace a continuación. Esta información, adaptable a cada industria permitirá desarrollar estrategias de control más allá de los alimentos basados en arroz donde *B. cereus* pueda ser una amenaza para la inocuidad alimentaria; así mismo, abre la puerta a otros estudios donde se necesite modificar macromoléculas y desarrollar alimentos específicos para poblaciones de interés.

6.1. Estrategias tecnológicas para el procesado de derivados de arroz.

Una de las posibles aplicaciones del plasma frío está relacionada con la modificación de macromoléculas. Estas modificaciones permiten el desarrollo de alimentos con un interés estratégico pues pueden ir dirigidos a determinados segmentos de la población con necesidades específicas. Con esta idea, dentro de este primer bloque se ha abordado el tema de la modificación del almidón de arroz. Este compuesto, utilizado como ingrediente en la formulación de muchos alimentos complejos, tiene una función tecnológica esencial por su capacidad para modificar propiedades tan importantes de los alimentos, como la textura, viscosidad, la capacidad de retención de agua, la estabilización de emulsiones, entre otras.

Para su uso, en muchas ocasiones es necesario modificar el almidón en su forma nativa para adaptar sus propiedades a las necesidades específicas de diversas aplicaciones y así mejorar su funcionalidad, ampliando su rango de aplicación (Bemiller, 1997; Maniglia et al., 2021). Los tratamientos más comunes para la modificación del almidón son los químicos y en menor medida los enzimáticos. Aunque los químicos son los más utilizados, en una situación de cambio climático, en la que se prima la sostenibilidad de la cadena alimentaria, pueden suponer una amenaza para el medio ambiente e incluso, en alguno de los casos, un riesgo para la salud humana, limitando, por ello, su aplicación. Por otro lado, los tratamientos enzimáticos son costosos, específicos y más difíciles de controlar. En consecuencia, se han buscado técnicas que sean eficientes y a la vez respetuosas con el medio ambiente, aportando nuevas funcionalidades deseadas a los almidones y otras macromoléculas. Es aquí donde el plasma frío (CP), puede jugar un papel importante a nivel industrial con objeto de producir modificaciones a nivel estructural de determinados componentes alimentarios, en este caso del almidón.

En el capítulo I de esta tesis, se estudió el efecto de la tecnología de plasma frío sobre las propiedades físico-químicas y tecno-funcionales del almidón de arroz en función de la variedad del mismo en relación con su composición y estructura. Los resultados muestran cambios significativos en propiedades físico-químicas, tales como la

disminución de la humedad o el incremento de la diferencia de color ΔE (Tabla 5.1.1) al aumentar el tiempo de tratamiento. Adicionalmente, el tratamiento produjo un aumento en las propiedades viscoelásticas (viscosidad máxima, estabilidad y retrogradación) resultados que se observan en la tabla 5.1.2, y en la textura de los geles formados con almidones de las variedades Basmati y Japónica (Tabla 5.1.3) tras 20 min de exposición al tratamiento, siendo el primero más estable al tratamiento. Por el contrario, el tratamiento en el almidón comercial dio lugar a un producto más fluido, con baja viscosidad e incapaz de formar un gel después de 40 minutos de exposición al plasma. Del mismo modo, se observaron cambios significativos en las propiedades de hidratación del gel (Tabla 5.1.4), especialmente en el almidón Basmati.

Por lo tanto, se puede afirmar que el tratamiento con plasma induce cambios significativos en las propiedades tecnológicas y fisicoquímicas, que son dependientes de la estructura y composición del almidón. En el caso del Basmati al tener mayor cantidad de amilosa, una cadena lineal, ayuda a dar una mayor estabilidad ante las especies reactivas que se generan durante el tratamiento por plasma, en comparación con la variedad Japónica, cuyo componente principal es la amilopectina, polímero ramificado y más susceptible a las especies reactivas de plasma por un tiempo prolongado. Esto puede ampliar la capacidad de uso de estos almidones, permitiendo su incorporación como ingredientes en una gama más amplia de productos alimenticios.

En resumen y a la luz de los resultados obtenidos se puede decir que el plasma puede ser una alternativa interesante frente a la modificación del almidón por métodos químicos o enzimáticos. Ofreciendo a la industria alimentaria no solamente ingredientes con características atractivas, sino también la ventaja de tener un menor impacto ambiental y económico.

6.2. Estrategias tecnológicas para mejorar la inocuidad alimentaria del arroz y sus derivados.

La higienización de materias primas con baja actividad de agua, bien en grano, como el arroz, o en polvo, es un tema que, bajo el punto de vista de la inocuidad alimentaria, sobre todo en el caso de microorganismos esporulados, representa un reto industrial. Los procedimientos de cultivo y riego pueden contaminar estos productos con microorganismos patógenos capaces de formar esporas, como *B. cereus* y, aunque el arroz usualmente se somete a cocción a 100°C antes de su consumo, las esporas de este microrganismo pueden sobrevivir, representando un riesgo para la salud del consumidor si el alimento no se almacena en las condiciones adecuadas para evitar la proliferación de este microorganismo y la formación de toxinas.

En lo que se refiere a *B. cereus*, hasta la fecha, no se han encontrado métodos de higienización de alimentos en grano o en polvo eficaces que puedan reducir la carga microbiana, particularmente sus esporas, sin impactar negativamente en las propiedades sensoriales y nutricionales de los alimentos. Esta dificultad pone de manifiesto la necesidad de investigar estrategias innovadoras que puedan tener un impacto positivo sobre la inocuidad alimentaria.

Tradicionalmente, se ha considerado que los tratamientos de cocción usualmente empleados en los cereales (por debajo de 100ºC) inactivan las células vegetativas de los microorganismos prevalentes en este tipo de alimentos, siendo *B. cereus* el principal patógeno. En el caso de las formas esporuladas de estos microorganismos, su inactivación no es total, y depende en gran medida de las condiciones de tiempo y temperatura del tratamiento y de la contaminación inicial presente en el arroz, entre las más importantes (Rodrigo et al., 2021).

Como punto de partida, en la presente tesis doctoral se llevó a cabo un estudio de termorresistencia de *B. cereus* en una matriz de arroz (Figura 5.3.1). Los resultados muestran que con una temperatura de cocción de 90ºC, se necesitaron tiempos elevados (40 minutos) para alcanzar una inactivación de tan solo 2 ciclos logarítmicos (D_{90} = 18,90 min). Por otro lado, se observó que al incrementar la temperatura del

tratamiento hasta 105ºC, se logró una reducción de más de 3 ciclos logarítmicos en tiempos cortos, que sigue por debajo de lo recomendado por las autoridades sanitarias de alcanzar al menos 5 reducciones logarítmicas.

Este comportamiento es debido a que el *B. cereus* tiene valores de D_T a las temperaturas que habitualmente se utilizan en la cocción de arroz o pasterización de platos preparados. Es más, se ha observado una gran variabilidad en el valor D_T entre las cepas y sustratos (Choma et al., 2000; Fernández et al., 1999; Wijnands et al., 2006). Esto hace que siempre exista un riesgo para la salud del consumidor en el caso de que el alimento tenga un exceso de contaminación inicial o no se almacene refrigerado en las condiciones adecuadas.

Aumentar la temperatura de tratamiento tiene consecuencias que pueden ser importantes bajo el punto de vista de la calidad sensorial de los alimentos procesados por lo que es necesario apuntar hacia técnicas de higienización de las materias primas en forma de grano con baja actividad de agua de tal manera que, la carga microbiana de esporulados se reduzca hasta unos niveles que no supongan un peligro posterior a la preparación del alimento.

6.2.1. Tecnología de plasma frío como una estrategia para la desinfección de materias primas de baja actividad de agua (arroz).

En este contexto, una de las estrategias propuestas ha sido el uso de la tecnología de plasma frío (CP) para la higienización de materias primas de baja actividad de agua. Al ser una tecnología no térmica, puede reducir la carga microbiana patógenos como *B. cereus* (incluidas sus esporas) sin elevar significativamente la temperatura del producto y deteriorar su calidad. Este método tiene la ventaja de que, al disminuir la carga microbiana inicial, los tratamientos térmicos subsiguientes pueden ser más suaves y de menor duración, optimizando así el proceso de conservación sin comprometer la inocuidad alimentaria.

Los resultados han puesto de manifiesto el impacto diferencial que tiene el plasma frío en células vegetativas y esporas bacterianas, así como se ha revelado el efecto

que puede tener una matriz alimentaria en relación a un sistema inerte (vidrio de borosilicato) en la inactivación de esporas bacterianas. Al igual que otras tecnologías de procesado, las células vegetativas son más sensibles al tratamiento con plasma que las esporas bacterianas, aunque a diferencia con la alta presión hidrostática o los pulsos eléctricos de alta intensidad, el plasma sí que produce un nivel de reducción en las esporas bacterianas que se puede aprovechar para incrementar el nivel de inocuidad de los alimentos preparados, usando materias primas con bajo contenido en agua que han sido sometidas a la acción del plasma frío.

En relación al efecto de la matriz, queda claro en esta tesis que el alimento forma parte del sistema de inocuidad alimentaria. Es decir, al hablar de inocuidad hay que hablar tanto del microorganismo como del factor huésped y de la matriz alimentaria. No es raro que esta última juegue un papel importante en la inactivación de los microorganismos, ya que este hecho se ha observado para otras tecnologías que van desde el tratamiento térmico, a las más novedosas como las altas presiones hidrostáticas y los pulsos eléctricos de alta intensidad. La matriz bien sea por su pH elevado, su actividad de agua, contenido en grasa o simplemente que sea más o menos porosa, ha tenido un papel protector frente a la inactivación microbiana. En el caso del plasma frío, debido a su baja capacidad de penetración en las muestras, una mayor porosidad de la misma es posible que disminuya la efectividad del tratamiento debido a que los microorganismos dispuestos en estos poros estén menos expuestos a la acción de las especies reactivas. El otro resultado importante es la alta efectividad del plasma sobre una superficie lisa e inerte, como el vidrio de borosilicato, lo que abre las puertas al uso de esta tecnología en la desinfección de superficies en las que se preparan los alimentos en la industria, incluidas las superficies de corte de las máquinas usadas para el loncheado, aplicaciones que podrían ser objeto de estudio. Esto incluiría la posibilidad de tratar biofilms adheridos a esas superficies.

Cuando se aplica una nueva tecnología, bien para higienizar como para inactivar microorganismos en una operación básica, es importante interpretar de forma matemática el comportamiento del microorganismo. Para ello existe en la bibliografía científica numerosos modelos matemáticos predictivos entre los cuales se puede

seleccionar el más adecuado. Como ocurre con numerosas tecnologías de control novedosas, los microorganismos no siempre mueren según una relación lineal de primer orden. En la presente tesis se ha observado que cuando se aplica plasma frío, la inactivación con el tiempo a una potencia determinada siguió diferentes patrones dependiendo de si se trataba de esporas, células vegetativas o el tipo de superficie en las que estaban inoculadas, arroz o vidrio. La consecuencia inmediata es que no se puede aplicar la ecuación que describe las reacciones de primer orden, concretamente el modelo de Bigelow, en todos los casos. En la presente tesis se ha explorado el modelo de Weibull que ha dado muy buenos resultados en la explicación matemática de las curvas de supervivencia usando otras tecnologías de procesado, cuando resultan tener una cola de inactivación, un hombro o bien una relación lineal. El resultado de la aplicación del modelo de Weibull a los procesos de plasma frío ha sido satisfactorio y se han podido obtener los parámetros de escala ("a") y de forma ("b") que describen este tipo de comportamiento (Tabla 5.2.1). Aunque estos parámetros no tienen un sentido biológico, como el valor $D_{T,}$ sí que tienen un valor práctico pues permiten desarrollar procesos de inactivación adecuados cuando se producen cambios en la intensidad del tratamiento por razones estratégicas o de calidad del alimento.

Los resultados de este estudio permiten concluir que el uso de un sistema de plasma frío de baja presión, puede reducir tanto las células vegetativas como las esporas de *B. cereus*, tanto en una matriz de arroz como en una superficie inerte, siendo una técnica interesante para la desinfección no térmica antes del procesamiento de matrices de baja actividad de agua como el arroz, ya que al reducir la carga inicial de esporas de *B. cereus*, con un proceso posterior de cocción leve del arroz, puede proporcionar al consumidor productos listos para el consumo que cumplan las normas de seguridad, pudiendo esta estrategia ayudar a minimizar los riesgos de contaminación microbiológica en la cadena alimentaria.

6.2.2. Combinación de tratamiento térmico y antimicrobianos para el control de *B. cereus* en platos preparados listos para el consumo a base de arroz.

Un punto a tener en cuenta en la estrategia de control del *B. cereus* es el almacenamiento a temperatura adecuada (T<4ºC o T>55ºC) de los alimentos procesados, si estos no van a ser consumidos inmediatamente después del cocinado, como es el caso frecuente en comedores colectivos o en restauración. Adicionalmente, la demanda por parte del consumidor de alimentos listos para el consumo, ha incrementado en los últimos años el desarrollo de una gran variedad de alimentos de 4ª y 5ª gama, para los que, de nuevo, la temperatura de almacenamiento es un factor clave para garantizar su inocuidad. Ello hace que siempre exista un riesgo latente en el consumo de estos productos en relación al *B. cereus*. Frente a este desafío, se ha considerado la utilización de un antimicrobiano natural, solo o en combinación con calor, como estrategia para reforzar la inocuidad alimentaria.

El quitosano de crustáceo, es un antimicrobiano natural que ha demostrado ser efectivo en el control de microorganismos. Sin embargo, en un planeta donde los recursos empiezan a escasear debido al cambio climático y a la sobre explotación, parece que va a ser difícil alimentar a 9.000 millones de personas que se estima poblaran la tierra en 2050 y a la vez que alimentar a los animales de granja necesarios para suministrar proteína a ese número de personas. Los insectos en este contexto pueden ser la llave que mejore la situación. Los insectos, al igual que los crustáceos, tienen un exoesqueleto de quitina que será un residuo del procesado de alimentos donde la proteína de insecto tenga un papel complementario a la proteína tradicional. Una forma de aprovechar este residuo es su transformación en quitosano. Sin embargo, apenas existe documentación en relación a las propiedades antimicrobianas de este quitosano. Las propiedades antimicrobianas del quitosano se han relacionado con el grado de acetilación, por lo que es importante evaluar sus propiedades antimicrobianas y arrojar información básica que pueda ser de utilidad industrial al respecto.

En la presente tesis doctoral, se ha demostrado que el quitosano de insecto procedente de larvas de *Tenebrio molitor* como subproducto de la industria alimentaria, también es efectivo no solo en la reducción de la termorresistencia de *B. cereus* durante el proceso de cocción, sino también como un agente de control durante el

almacenamiento del arroz en refrigeración. Los datos obtenidos en esta tesis doctoral revelan que el quitosano de insecto puede ejercer efectos bactericidas o bacteriostáticos dependiendo de la concentración y de la temperatura de almacenamiento. En cualquier caso, queda demostrado que esta medida de control adicional es muy útil en casos de abuso de temperatura o de rotura de la cadena de frio en relación al peligro que supone el *B. cereus*. Este resultado añade una dimensión extra al uso de insectos en alimentación animal o humana.

Otro aspecto importante a destacar en la presente tesis doctoral está relacionado con el efecto conjunto del quitosano durante el tratamiento térmico, y que en consecuencia va a condicionar el comportamiento del microorganismo durante el almacenamiento en refrigeración. Como ocurre con otros factores como el pH acido, la concentración de sal u otros conservantes, el quitosano afecta a la resistencia térmica de la espora haciendo que los valores D_T sean ligeramente inferiores a los obtenidos en un medio sin quitosano (Tabla 5.3.1). Esto añade una ligera ventaja tecnológica a la hora de diseñar los procesos de cocción y contribuye a que estos os sean más eficientes y por ende el alimento procesado tenga un mayor nivel de inocuidad. El mecanismo por el cual el quitosano ejerce dicha acción no está claro pudiendo ser objeto de estudios más básicos que se escapan al objetivo de esta tesis.

6.2.3. Desarrollo de un modelo de evaluación de la exposición a *B. cereus* contenido en platos listos para el consumo a base de arroz.

Una vez que se dispuso de la información necesaria obtenida en los distintos capítulos que componen esta tesis doctoral, se procedió a explorar la posibilidad de llevar la evaluación de riesgos a la industria como una herramienta complementaria al análisis de peligros y puntos críticos de control APCC. Este ejercicio puede dotar al sistema de información numérica objetiva que permita discriminar entre los puntos críticos a controlar de los que no necesitarían un control. El resultado es un modelo modular de evaluación de la exposición materializado en una hoja de cálculo Excel sobre la que se lleva a cabo un proceso de simulación Montecarlo. Esta hoja de cálculo, es fácilmente exportable a la industria y con pocos cambios puede aplicarse a industrias

de distinto tipo donde el *B. cereus* pueda ser un peligro y en consecuencia un riesgo para la salud del consumidor. El resultado de la simulación permite llevar a cabo acciones de gestión en el interior de la industria para garantizar la inocuidad de los alimentos allí procesados antes de su puesta en circulación.

El modelo ha puesto de manifiesto la importancia relativa que puede tener una pequeña fluctuación en la temperatura o tiempo de tratamiento sobre el número más probable de microorganismos al final del tratamiento por calor. También pone de manifiesto la importancia que tiene reducir la carga inicial de un microorganismo patógeno en relación a un criterio de rendimiento (PC), reflejándose en el número de unidades que pueden estar contaminadas con una determinada cantidad de dicho patógeno. Toda esta información permite gestionar convenientemente las buenas prácticas de higiene y las buenas prácticas de manufactura en el entorno industrial.

Como epílogo a esta discusión general, se puede decir que la incorporación del plasma frío y el uso de quitosano de insectos solo o combinados con calor, representan alternativas prometedoras para la higienización y el control microbiológico, tanto para alimentos de baja actividad de agua como para los alimentos listos para el consumo, en los que intervienen estas materias primas.

Adicionalmente, se puede decir que con estas estrategias podrían reducir los costos de producción al requerir menos energía y tiempo de procesamiento, así como promover la revalorización de subproductos de la industria, posicionándose como opciones más sostenibles y ecológicas.

7. CONCLUSIONES

7. Conclusions

1. Cold plasma technology is an effective processing strategy to modify the technological properties of rice starch. The most influential variables are treatment time and variety. This opens up a new wide spectrum of industrial application possibilities.
2. Cold plasma technology is an effective disinfection strategy for low-moisture foods, such as rice, inactivating vegetative cells and spores of *B. cereus*. This reduces the risk of this microorganism being present in foodstuffs.
3. Resistance of vegetative cells and *B. cereus* spores to cold plasma decreases with increasing time and power of treatment, being bacterial spores more resistant.
4. The food matrix has a significant effect on spore inactivation by cold plasma. This variable should be considered when designing a disinfection process.
5. The Weibull frequency distribution model is suitable for mathematically interpreting the survival curves of vegetative cells and spores of *B. cereus* when cold plasma is used.
6. The presence of insect chitosan during cooking, reduces the heat resistance of *B. cereus* spores. This effect was found to be independent of chitosan concentration.
7. The presence of insect chitosan during storage has a bacteriostatic or bactericidal effect against *B. cereus* depending on chitosan concentration and storage temperature.
8. The exposure assessment model developed corroborates the findings regarding the effect of chitosan on *B. cereus*, both during cooking and storage.
9. This model allows to assess the impact of accidental changes in temperature or treatment time on the survival level of *B. cereus* after cooking. It also

allows to evaluate the impact of the initial contamination level on the number of units that may remain contaminated after the cooking treatment.

10. Additionally, the model predicts the microbial load that may occur during refrigerated storage under conditions of temperature abuse or cold chain disruption.

As a general conclusion, we can say that cold plasma is a useful technology to modify the technological properties and to increase the safety of rice-derived foods; likewise, insect chitosan is a suitable strategy in scenarios of temperature abuse or breakage of the cold chain, keeping *B. cereus* contamination levels below the infective dose.

8. REFERENCIAS

8. Referencias

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9. ANEXOS

Study of the Impact of Plasma Treatment on the Physicochemical Properties of Rice Starch from Different Varieties: Basmati and Japonica rice

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Abstract

Rice starch has a great interest in recent years, due to its techno-functional properties with a wide application at a technological level in the food industry. Plasma technology, as an emerging method of non-thermal food processing, offers an alternative to modify starch without resorting to chemical or enzymatic treatments, which can be costly and difficult to handle. In this study, starch samples from two rice varieties, Basmati and Japonica, as well as a commercial starch, were treated with Thermal Plasma Jet equipment and were analysed. The treatments were carried out for 20 and 40 minutes, using compressed air as ionization gas. Results indicated that moisture content decreased with increasing treatment time, and significant changes in colour and increase in colour difference (ΔE), as the treatment progressed. Viscosity properties increased in the Basmati starch (BS) and Japonica starch (JS), while they decreased evidently in the commercial starch, the latter being the most unstable under treatment, to the point of being unable to form a gel after 40 min of treatment. As for flour hydration properties, no significant changes were observed, but significant changes were observed in gel hydration properties, especially in the Basmati starch and commercial starch (CS). Therefore, it can be affirmed that plasma treatment induces significant changes in the techno functional and physicochemical properties, especially in the BS and CS samples. Therefore, plasma treatment causes interesting changes in the molecular structure of starches and offers an interesting alternative for their modification according to the properties desired for their industrial application.

Key words: Rice starch, Plasma Treatment, Starch modification, Basmati, Japonica

foods

MDPI

Article

Effect of low-pressure cold plasma on *B. cereus* spores and vegetative cells inactivation using different matrices.

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Abstract: This study investigated the effects of low-pressure cold plasma on the inactivation of *Bacillus cereus* vegetative cells and spores. Oxygen was used as ionization gas and its impact on both inert and low water activity food matrices, specifically rice, were evaluated by mathematical modelling. Greater reductions in *B. cereus* counts were observed in vegetative cells than spores. Both the power of the plasma treatment and the matrix proved to be determining factors in the inactivation of both spores and vegetative cells of *B. cereus*. To characterize the inactivation of *B. cereus*, the experimental data were accurately fitted to Weibull model. A significant decrease in parameter "a", representing resistance to treatment, was confirmed with treatment intensification. Furthermore, significant differences in the "a" value were observed between spores in inert and food matrices, suggesting the additional protective role of the food matrix for *B. cereus* spores. These results demonstrate the importance of considering matrix effects in plasma treatment to ensure the effective inactivation of pathogenic microorganisms, particularly in foods with low water activity such as rice. This approach contributes to mitigating the impact of foodborne illnesses caused by pathogenic microorganisms.

Keywords: Low-pressure cold plasma, *B. cereus*, spore, Rice, Weibull model

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1. Introduction

The cultivation of **rice** (*Oryza sativa L.*) holds a historical significance as one of the oldest practices, considering it as a staple food for humans and its global prominence as one of the most extensively cultivated crops. In recent decades, rice production has witnessed nearly a twofold increase, facing threats from various challenges such as limited agricultural land, water scarcity, soil fertility concerns, climate change, insect infestations, and diseases [1,2]. Owing to its nutritional composition, particularly its elevated starch content, rice is susceptible to a spectrum of infections caused by bacteria, viruses and fungi, with *Bacillus cereus* as a principal contaminant [3].

Bacillus cereus is a Gram-positive bacterium known for its ability to form spores, which allows it to survive in diverse environmental conditions. This bacterium is commonly found in soil and can contaminate various types of food, causing foodborne illnesses [4,5]. There are two main types of illnesses associated with *B. cereus*; emetic syndrome which is characterized by the production in the small intestine of a toxin that causes vomiting, and the diarrheal syndrome which is associated with the consumption of toxins that cause diarrheal illness. *B. cereus* can be present in a wide range of foods, including rice, pasta, meat, vegetables, and dairy products. The spores of this bacterium are resistant to heat, allowing them to survive cooking processes [6]. Therefore, proper food handling, cooking, and storage are essential to prevent the spores' germination, growth of *B. cereus* and the production of toxins [4,5]. In the European Union, the One Health 2022 zoonoses report [7] indicated that *B. cereus* toxins are ranked as the leading

PLOS ONE

RESEARCH ARTICLE

Joint effect of temperature and insect chitosan on the heat resistance of *Bacillus cereus* spores in rice derivatives

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Abstract

The heat resistance of *Bacillus cereus* spores inoculated in a rice substrate supplemented with insect chitosan as an alternative antimicrobial was studied. Two concentrations of insect chitosan were considered in order to assess the role of the insect chitosan concentration during the heat process. Results of the study indicated that the D_T values were higher in the substrate without chitosan than in the substrate containing chitosan thus indicating a greater heat resistance to heat treatment of the microorganism inoculated in the substrate without chitosan. This behaviour was also evidenced in the survival curves. There were no great differences between either of the insect chitosan concentrations tested regarding the D_T values. The z values were 9.8˚C on rice substrate and8.9˚C on rice substrate supplemented with insect chitosan at 150 μg/mL and 10.7˚C on rice substrate supplemented with 250 μg/mL of insect chitosan. The chitosan concentration appears to affect the z value of the microorganism. Our results indicate that the combination of heat with insect chitosan as an antimicrobial on foodstuffs subjected to cooking is feasible and can improve the safety of rice derivatives.

Introduction

Bacillus cereus is present in many foods due to its ubiquitous nature. This microorganism is one of the top ten pathogens responsible for many foodborne diseases in humans [1]. According to the latest EFSA and ECDC report [2] there is strong evidence for *B. cereus* was involvement in 38 outbreaks and weak evidence of involvement in 117 outbreaks out of a total of 155 outbreaks reported in 2019. Some recent outbreaks in non-EU countries have also been associated with this pathogen; 45 people were affected in an outbreak in a restaurant in Canberra (Australia) in 2018 [3] and 200 students in an outbreak in a school in China in 2018 [4].

Bacillus cereus causes two types of food poisoning one of an emetic nature and the other of a diarrheal nature [5]. On the one hand diarrheal syndrome is caused by a gastrointestinal disorder due to the ingestion of *B. cereus* spores present in food and at a dose given, an appreciable probability that cells cross the stomach barrier and implanting themselves in the small

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Insect chitosan as a natural antimicrobial against vegetative cells of *Bacillus cereus* in a cooked rice matrix

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ABSTRACT

This study investigates the antimicrobial activity of insect chitosan against vegetative cells of *Bacillus cereus* in a rice matrix. Sample culture solutions were prepared with different concentrations of insect chitosan (150, 180, 220 and 250 μg/mL) and tested at three temperatures (30 °C, 20 °C and 10 °C), which simulate different storage temperature scenarios of precooked rice. The results indicate that insect chitosan has antimicrobial activity that depends on temperature and chitosan concentration. For the assays with chitosan at 10 °C, all concentrations were bactericidal during the study time, reaching a maximum inactivation of 6 log cycles for 250 μg/mL. At 20 °C and at 30 °C a bacteriostatic activity was observed for concentrations of 150 μg/mL and 180 μg/mL. Results also showed that concentrations of 220 μg/mL and 250 μg/mL were bactericidal for all the temperatures tested during the storage time. When rice is cooked and not stored at an appropriate temperature, below 10 °C, the consumer's health is at risk. In these cases, insect chitosan could be a good additional control measure to control *B. cereus* growth and toxin formation in cooked rice.

1. Introduction

Bacillus cereus is present in many foods due to its ubiquitous nature and has become one of the top ten pathogens responsible for many foodborne cases of infection (Rodrigo et al., 2021). In 2018, *B. cereus* was involved in 31 strong evidence outbreaks and 67 weak evidence outbreaks, with a total of 98 reported among EU member states, representing 1.9% of the total outbreaks in the EU, with 1539 human cases accounting for 111 hospitalizations and 1 death (EFSA and ECDC 2019). In general, *B. cereus* toxico-infection episodes have been associated with complex, mixed food products that may include rice as a component (Little et al., 2002); however, other rice-based products such as pasta and noodles are also frequently contaminated and involved in *B. cereus* toxico-infection (Grande et al., 2006). It has also been found in a wide variety of non-cereals including milk and dairy products, meat products, pasteurized liquid eggs, ready-to-eat vegetables, fruits, and spices (Yu et al., 2020). Due to the extensive distribution of strains in the environment, it is practically impossible to obtain raw materials or food that is free of *B. cereus* spores (Ehling-Schulz et al., 2015; Enosi Tuipulotu et al., 2020; Griffiths and Schraft, 2017). This implies that food contamination can occur during any stage of production, including primary production, harvesting or in slaughterhouses, processing, storage, preparation and consumption of food (Enosi Tuipulotu et al., 2020). *Bacillus cereus* strains can vary with respect to their growth and survival characteristics, with growth limits that are not absolute and depend on the strain and environmental factors, such as the composition of the medium, temperature, pH and water activity (Enosi Tuipulotu et al., 2020). *B. cereus* can produce two types of toxi-infections: diarrheal and emetic syndromes. The first one occurs when a high number of *B. cereus* cells are consumed, the microorganism implants and grows in the small intestine producing the enterotoxin, while emetic syndrome occurs when a food containing preformed cerulide toxin is consumed produced during the growth of *B. cereus* (Kramer and Gilbert, 1989).

Rice is a basic cereal in the diet, widely consumed by the general population due to its ample supply of nutrients and its relatively low price. Generally referred to as Asian grown rice (*Oryza sativa* L.), it is one of the most important staple crops and feeds almost half the world population (Wei and Huang, 2019). This cereal, however, is frequently contaminated by *B. cereus* spores, from cultivation to the later stages of processing and consumption (Kramer and Gilbert, 1989). The primary habitat of emetic strains could be related to roots, tubers, and mycorrhizae of rice, which could explain their generally high prevalence in

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Development of an Exposure Assessment industrial model for *Bacillus cereus* in rice matrix containing insect Chitosan.

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Keywords:	Antimicrobial activity, Antimicrobials, Bacterial spores, Foodborne microorganisms, Risk assessment
Abstract:	An exposure assessment model for industrial use has been developed by using kinetic data from inactivation and growth of *Bacillus cereus* spores. It can provide a valuable tool for estimating the concentration of *B. cereus* after a storage period of 24 hours at a specified temperature (20ºC) and for an estimation of the percentage of contaminated portions according to the input data of the model. This model considers a rice-derived product that has undergone a standard cooking process at 95ºC for 20 minutes. According to the results, the presence of chitosan affects the final microbial load after storage, potentially serving as an additional control measure in the event of cold chain abuse or break. Chitosan's antimicrobial properties likely play a role in reducing microbial growth during storage, thereby contributing to enhanced food safety. In practical terms, this suggests that incorporating chitosan into food products, especially those susceptible to microbial contamination like rice derivatives, could help mitigate risks associated with temperature abuse or cold chain disruptions. By acting as a protective barrier against microbial proliferation, chitosan offers a preventive measure to maintain product quality and safety throughout the supply chain. Considering two scenarios, 10^4 or 10^7 as initial contamination the model estimated that the 55 and 100 % of portions would be respectively contaminated,