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Strategies to increase sustainability of Rice processing: technological, microbial and nutritional approach

Estrategias innovadoras para revalorizar el arroz: aspectos
físicoquímicos, nutricionales y de seguridad alimentaria

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Doña **Eva Grau Fuentes** ha realizado bajo su dirección la Tesis Doctoral que lleva por título “**Strategies to increase sustainability of rice processing: technological, microbial and nutritional aproach**”, y autorizan su presentación para optar al título de Doctor por la Universitat de Valencia.

Y para que así conste, expiden y firman el siguiente certificado.
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“Progress is impossible without change, and those who cannot change their minds cannot change anything”

George Bernard Shaw

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Resumen global

Introducción

Sostenibilidad y seguridad alimentaria

Sostenibilidad, implica una evaluación holística de la existencia humana, considerando las dimensiones sociales, medioambientales y económicas, todo con una perspectiva de futuro para beneficiar a las generaciones venideras. En la actualidad, este concepto ocupa un lugar destacado en las políticas de las distintas instituciones gubernamentales, desde el nivel global al local. Las iniciativas internacionales comenzaron en la Cumbre de la Tierra de 1992 y continuaron con la Agenda 2030 y sus 17 Objetivos de Desarrollo Sostenible. La Comisión Europea también adoptó el "Pacto Verde Europeo" en 2019, con el objetivo de que la Unión Europea (UE) sea climáticamente neutra para 2050. En este contexto, en un sistema alimentario global interconectado, la seguridad y calidad de los alimentos son esenciales para garantizar la salud pública, la estabilidad económica y el comercio. Organizaciones como la Organización Mundial de la Salud (OMS), Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO) y el *Codex Alimentarius* son claves en la promoción de normas de seguridad alimentaria. La gestión efectiva de la seguridad alimentaria, a través de enfoques basados en riesgos y avances tecnológicos, mejora la trazabilidad y transparencia en la cadena de suministro. La transición hacia sistemas alimentarios sostenibles requiere investigación, innovación, servicios de asesoramiento y herramientas financieras, garantizando así un futuro saludable y sostenible para todos.

Arroz y seguridad alimentaria

El arroz (*Oryza*) es el cereal más consumido en el mundo, alimentando a casi la mitad de la población mundial y suministrando el 75% de las calorías diarias en países asiáticos. En 2023/24 se consumieron 522,1 millones de toneladas métricas de arroz. Su proceso de molienda da lugar a diversos subproductos ricos en nutrientes y compuestos bioactivos. Un proceso de molturación óptimo suele producir aproximadamente un 25% de cascarilla, un 10 % de salvado y germen y un 65% de endospermo (arroz blanco), dependiendo de la variedad. Compuesto principalmente por carbohidratos (79%), proteínas (7%) y grasas (2%), el arroz blanco es un sustrato óptimo

para el crecimiento de microorganismos como *Bacillus cereus*. Esta bacteria grampositiva y formadora de esporas puede sobrevivir en condiciones extremas y producir toxinas diarreicas y eméticas que resisten el almacenamiento prolongado en lugares frescos y secos sin pérdida de viabilidad situándose como un problema de salud pública. *B. cereus* es común en entornos como el polvo, el suelo, la superficie de las plantas y la rizosfera, y, por lo tanto, en situaciones de falta de higiene puede estar presente en el alimento final. En 2022, las toxinas de *B. cereus* representaron el 5,3% de los brotes alimentarios asociados a toxinas en la Unión Europea, con un aumento significativo respecto a los producidos en 2021. La refrigeración inadecuada y el almacenamiento prolongado del arroz cocido, sobre todo en establecimientos como restaurantes y comedores donde se preparan grandes cantidades, favorecen estos brotes. Las esporas de *B. cereus* son capaces de sobrevivir a las temperaturas de cocción del arroz, y la proliferación microbiana post-cocción depende del pH y la humedad del sustrato, destacando la importancia de prácticas de higiene adecuadas durante la manipulación y procesamiento.

Estrategias sostenibles para garantizar la seguridad alimentaria

En los últimos años, ha aumentado el interés por la extracción de compuestos naturales capaces de inhibir el crecimiento de microorganismos, especialmente los responsables del deterioro de los alimentos y de numerosas enfermedades. Dentro de ellos destacan los compuestos fenólicos por sus funciones antioxidantes y antimicrobianas. Presentes en el extracto de uva, un subproducto de la industria vitivinícola, pueden penetrar las membranas celulares y detener el crecimiento bacteriano. La valorización de este subproducto es económicamente relevante y reduce, al mismo tiempo, el impacto ambiental. Según la bibliografía estos subproductos tienen propiedades antimicrobianas, ofreciendo una alternativa a los conservantes artificiales. Además, la combinación de tratamientos térmicos, variaciones de pH y antimicrobianos naturales (aplicación de tecnología de barreras) puede ser eficaz para controlar patógenos en alimentos procesados, garantizando su seguridad y calidad. La realización de estudios microbiológicos para obtener estimaciones (microbiología predictiva) que determinen la supervivencia de los microorganismos tras aplicar diversas combinaciones de tratamientos son ampliamente utilizados. La microbiología predictiva cuantitativa puede evaluar cómo las operaciones de procesado, distribución y almacenamiento

afectan la seguridad y calidad de los alimentos, empleando modelos matemáticos para predecir el comportamiento de patógenos. A su vez desempeñan un papel crucial en los sistemas de análisis de peligros y puntos de control crítico (APPCC), el desarrollo de nuevos productos y la determinación de la vida útil dentro de la industria alimentaria. El Reglamento 178/2002 establece el análisis de riesgos como base de la seguridad alimentaria, comprende la evaluación, gestión y comunicación de riesgos. Este enfoque mejora los controles para asegurar alimentos seguros, reducir enfermedades y facilitar el comercio. La evaluación de la exposición es crucial y se realiza mediante modelos matemáticos probabilísticos o estocásticos, como la simulación Monte Carlo, que ayudan a evaluar múltiples escenarios proporcionando una representación completa y realista para la toma de decisiones en la industria alimentaria.

Subproductos de arroz: Salvado de arroz

La creciente demanda de arroz, impulsada por el aumento de la población y el cambio de las preferencias alimentarias, requiere mejoras en la su producción. La importancia de estas mejoras se pone de manifiesto ya que las predicciones apuntan a un crecimiento significativo de la producción mundial de arroz para 2030, llegando hasta 567 millones de toneladas, lo que conlleva la generación significativa de subproductos como el salvado de arroz. El salvado de arroz es rico en lípidos (15-20%) y valiosos compuestos bioactivos como γ -Orizanol, tocoferoles, tocotrienoles y esteroides. El γ -Orizanol, en particular, ha suscitado un alto interés en la industria cosmética por sus propiedades y atributos medicinales. Tras la extracción de grasa, el salvado desgrasado, que representa el 90% de la producción del salvado, es rico en fibra dietética y proteínas y se utiliza como residuo o en alimentación animal. Las proteínas del salvado de arroz (13,2 - 17,3%) contienen todos los aminoácidos esenciales, y poseen una alta digestibilidad y biodisponibilidad, superando en valor biológico al aislado de proteína de soja. Estas proteínas son hipoalergénicas y ofrecen beneficios para la salud, ya que poseen propiedades antioxidantes, inmunomoduladoras, antidiabéticas, anticancerígenas y reductoras del colesterol. Además, las proteínas de salvado de arroz pueden mejorar propiedades fisicoquímicas de los alimentos, como solubilidad y capacidad emulsionante. Por su parte, la fibra del salvado de arroz (27,6 - 33,3%) se compone de fibra dietética insoluble y soluble. La fibra insoluble, que incluye celulosa, lignina y parte de la hemicelulosa,

beneficia la flora intestinal, mejora el tránsito y puede inhibir la lipasa pancreática. A su vez, la fibra soluble, compuesta de hemicelulosa e hidrocoloides, que reduce la respuesta glucémica y el colesterol plasmático, tiene actividad inmunomoduladora y puede prevenir el cáncer colorrectal. Ambas fibras también afectan las propiedades fisicoquímicas de los alimentos, modulando la textura y las propiedades reológicas de las masas. Estos atributos hacen del salvado de arroz un subproducto valioso tanto desde el punto de vista nutritivo como tecnológico.

La mayor preferencia por el arroz blanco frente al arroz integral (que contiene endospermo, germen y salvado), da lugar a la generación de una gran producción de salvado de arroz que suele desperdiciarse o usarse para alimentación animal. Este rechazo se debe a su sabor astringente, textura firme y color oscuro, entre otros motivos. Para fomentar su consumo la industria alimentaria estudia su incorporación en diversos productos como noodles, pasta, pan o bebidas vegetales, entre otros. El arroz, solo o combinado con otros ingredientes, se utiliza cada vez más como materia prima para bebidas vegetales, además este cuenta con una larga tradición en Oriente y Occidente, destacando el *Sikhye* (una bebida típica en Corea del Sur). Sin embargo, el arroz enfrenta retos tecnológicos en su incorporación en bebidas, especialmente en términos de fibra y aceite. El salvado de arroz, rico en fibra y proteínas, puede mejorar el perfil nutricional y la formulación de estas bebidas, respondiendo a la demanda de productos nutritivos y sostenibles, y reduciendo subproductos en la industria arrocera. A pesar de las continuas mejoras en la investigación sobre bebidas, aún existen retos tecnológicos, como la incorporación de ingredientes ricos en fibra o la reducción del aceite en bebidas en las que ha sido necesario añadirlo. El uso del salvado de arroz como ingrediente rico en fibra y proteínas, podría mejorar el perfil nutricional y tecnológico de este tipo de bebidas fomentando además la sostenibilidad dentro de este sector.

Estrategias sostenibles para revalorizar el salvado de arroz

El desarrollo de nuevas estrategias para la recuperación y valorización de subproductos agroindustriales está emergiendo como un sector crucial para promover la sostenibilidad y mejorar los hábitos dentro del sistema alimentario. Revalorizar el salvado de arroz modificando sus propiedades fisicoquímicas y tecno-funcionales es crucial para maximizar su uso como ingrediente alimentario. Aunque varios estudios se han centrado en revalorizar

fracciones aisladas del salvado de arroz, como aislados proteicos o de fibra, es necesario un enfoque integral que considere una revalorización del salvado de arroz en su totalidad.

El uso de enzimas para la valorización de residuos agroindustriales es una estrategia muy prometedora ya que cuentan con una aplicación histórica en la mejora tanto de la calidad como de la eficiencia de la producción de alimentos, alineándose con la creciente necesidad de soluciones alimentarias sostenibles. En este sentido, se han empleado tratamientos enzimáticos para modificar las propiedades tecnológicas de varias fracciones de salvado de arroz (aislados proteicos o fracciones ricas en fibra) modificando las propiedades funcionales como la capacidad de hidratación, la mejora de la emulsificación y las propiedades espumantes. Sin embargo, hay poca información sobre el efecto de estos tratamientos sobre el salvado de arroz en su conjunto.

El procesado por altas presiones es una tecnología no térmica empleada en la industria alimentaria que ofrece ventajas en la conservación de nutrientes, mejora de la calidad sensorial y extensión de la vida útil de los alimentos, garantizando un tratamiento uniforme mediante una distribución isostática de la presión. Inicialmente usada para la conservación, demostrando afectar a los microorganismos disminuyendo la síntesis de ADN, aumentando la permeabilidad de las membranas y desnaturalizando las proteínas, se ha visto que las altas presiones también consiguen modificar las propiedades tecnológicas de los alimentos. El procesado por altas presiones ha sido aplicado a distintas fracciones proteicas procedentes de salvado de arroz mejorando atributos funcionales como solubilidad de proteínas, capacidad de hidratación, emulsificación y propiedades espumantes. Pero, es necesario llevar a cabo estudios sobre el efecto de esta tecnología en el salvado de arroz.

Objetivos

El objetivo general de esta tesis es potenciar el valor del arroz y sus subproductos en la industria alimentaria mediante el desarrollo de estrategias innovadoras, centrándose en dos áreas clave:

I. Mejorar la seguridad alimentaria del arroz cocido mediante el empleo de antimicrobianos naturales y técnicas avanzadas de modelización de riesgos.

II. Mejorar las propiedades tecnológicas, físico-químicas y tecno-funcionales del salvado de arroz utilizando una serie de tratamientos sostenibles.

Para lograrlo, se establecieron los siguientes objetivos específicos:

1. Evaluar la eficacia del extracto de uva como antimicrobiano natural frente a *B. cereus* en una matriz de arroz, combinado con diferentes tratamientos térmicos, temperaturas de almacenamiento y pH.

2. Desarrollar un modelo de evaluación de la exposición que estime la concentración de *B. cereus* tras la cocción térmica y el tiempo de almacenamiento.

3. Identificar tendencias y lagunas en la composición y el valor nutricional del mercado actual de bebidas de origen vegetal, que puedan ayudar al diseño de este tipo de bebidas basado en el conocimiento científico.

4. Evaluar el efecto de los tratamientos enzimáticos en la mejora de las propiedades tecno-funcionales del salvado de arroz.

5. Analizar el impacto de la presión hidrostática en la mejora de la seguridad, propiedades físico-químicas y tecno-funcionales del salvado de arroz.

Resultados

Bloque I: Estrategias para garantizar la seguridad alimentaria

Como se ha comentado anteriormente, *B. cereus* es una bacteria gran positiva común en brotes alimentarios asociados al arroz. Se evaluó la capacidad antimicrobiana del extracto de uva contra *B. cereus* en arroz cocido. Esta evaluación se llevó a cabo mediante una simulación del proceso de cocción (mediante un tratamiento de termorresistencia) y del almacenamiento. Durante la simulación el proceso de cocción (donde se usaron esporas de *B. cereus*), destacó que temperaturas altas y pH ácidos aumentaron la inactivación de esporas, y que en presencia de extracto uva se consiguió una reducción de la supervivencia de esporas a bajas temperaturas de cocción (90 y 95°C). Durante el almacenamiento se mostró que concentraciones altas de extracto de uva (0,5 y 1%) tienen efectos bactericidas

significativos, y que, a la menor concentración utilizada (0,1%), el pH y la temperatura jugaban un papel fundamental para conseguir un efecto bactericida o al menos bacteriostático. La efectividad del extracto de uva se asoció a su contenido en polifenoles.

Para facilitar la predicción del comportamiento del *B. cereus* en diferentes escenarios, se evaluó la eficacia del extracto de uva como antimicrobiano natural aplicando la función de supervivencia de Weibull para modelizar la inactivación. Se determinaron los parámetros de escala y forma para cada combinación de pH, temperatura y concentración de extracto. Durante la cocción, el extracto de uva redujo significativamente la resistencia de las esporas, especialmente en condiciones ácidas y altas temperaturas. En el almacenamiento, las concentraciones más altas de extracto y temperaturas redujeron el parámetro de escala. Se construyó un modelo secundario mediante regresión múltiple vinculando el logaritmo del factor de escala (a) del modelo de Weibull con la temperatura y el pH para concentraciones fijas de extracto de uva. Además, incluyendo los términos polinómicos más relevantes, se desarrolló un modelo global para mejorar la precisión de los coeficientes en el modelo secundario. De los resultados experimentales de la evaluación del extracto de uva como antimicrobiano natural frente al *B. cereus* se desarrolló un modelo de evaluación de la exposición industrial para estimar los niveles de contaminación en arroz cocido en distintos escenarios, utilizando ecuaciones matemáticas (Weibull y Gompertz modificado) para simular la inactivación y crecimiento microbianos bajo condiciones de tratamiento térmico (simulación del proceso de cocción) y almacenamiento. Este modelo permitió una estimación precisa de la concentración bacterianas con y sin extracto de uva, mejorando las decisiones de Análisis de Peligros y Puntos Críticos de Control (APPCC). Aunque la herramienta de evaluación de la exposición permite simular resultados para numerosos escenarios, se incluyeron los resultados de dos escenarios que reproducen condiciones realistas pero desfavorables durante la preparación y el almacenamiento del arroz, tanto con como sin extracto de uva. Estos escenarios consistieron en una baja temperatura de cocción (90 °C) durante un tiempo de cocción estándar para el arroz (20 minutos), seguida de un almacenamiento prolongado (20 horas) a 20 °C para simular una ruptura de la cadena de frío. Se observó que el extracto de uva reducía significativamente la tasa de crecimiento de *B. cereus*. Esta reducción del riesgo de ingerir una carga

infecciosa de *B. cereus* destaca el potencial del extracto de uva como antimicrobiano natural.

Bloque II: Estrategias de revalorización del salvado de arroz

El alto consumo de arroz y su producción genera grandes cantidades de subproductos debido a la baja aceptación del arroz integral. La industria alimentaria busca incorporar más arroz integral en productos como fideos, pan, pasta, pasteles, aperitivos, bollería y bebidas. Las bebidas de origen vegetal, en particular, es un segmento de crecimiento exponencial en todo el mundo dado que cada vez más individuos adoptan dietas basadas en vegetales y priorizan la sostenibilidad medioambiental. En este sentido, se realizó un estudio de mercado en los principales supermercados de Europa y Norteamérica, en el cual se analizaron 306 bebidas vegetales. Este estudio se basó en el análisis de las etiquetas, enfocándose en la lista de ingredientes y sus composiciones nutricionales. Con materias primas principalmente de cereales, semillas, frutos secos o legumbres, los ingredientes principales de las bebidas fueron agua, la materia prima específica de cada bebida, aceite, gomas y azúcar. El 14% de las bebidas contenían dos o más ingredientes principales, y el 32% contenían aceite, siendo las bebidas de cereales las más comunes en este grupo. El 60% contenían hidrocoloides para la emulsión estable, mientras que las bebidas de cereales, con alto contenido de almidón, no los necesitaban. Las bebidas consideradas en el estudio contenían generalmente bajas cantidades de calorías y grasas, con variaciones en carbohidratos y proteínas según las materias primas. Adicionalmente, se pueden considerar bajas en sal ya que su contenido era menor a 0,3 g/ 100 g y más del 50% de las bebidas estaban enriquecidas con vitaminas y calcio, aunque su biodisponibilidad sigue cuestionándose. En el estudio destacó la necesidad de reducir el contenido de aceite en aquellos casos a los que se le añadía y aumentar la fibra en este tipo de bebidas ya que generalmente tenían un contenido muy bajo y no declaraban la cantidad en el etiquetado.

Dada la alta generación de subproductos como el salvado de arroz por la industria arrocera, el estudio de estrategias innovadoras para valorizar estos subproductos agroindustriales representa un sector en auge. En este trabajo se realizaron tratamientos térmicos, de remojo y enzimáticos al salvado de arroz desgrasado para mejorar sus propiedades fisicoquímicas y tecno-funcionales. Se realizaron un total de seis tratamientos enzimáticos, cuatro con carbohidrasas (Novozym®, Celluclast®, Ultimase® y Shearzyme Plus® 2x)

y dos proteasas (Alcalase 2.4 L FG y Flavourzyme®). La cantidad de enzima utilizada en el tratamiento se ajustó al 1% de la cantidad de fibra del salvado de arroz, en el caso de las carbohidrasas y el 1% de la cantidad de proteína en el caso de las proteasas. El tratamiento se llevó a cabo a un pH óptimo para las enzimas durante 120 minutos a 50°C y posteriormente las enzimas se inactivaron a 90°C durante 10 min. Los resultados se compararon con una muestra control con remojo pero sin adición de enzima. Mientras que el tratamiento térmico se llevó a cabo a 130°C durante 30 minutos. El análisis de la composición proximal reveló cambios significativos. Se observó un aumento del contenido de proteínas en todos los tratamientos. Además, los tratamientos de remojo mejoraron eficazmente el contenido de fibra dietética del salvado de arroz desgrasado, aumentando notablemente la fracción de fibra soluble. El tratamiento con proteasa aumentó la hidrólisis de hemicelulosa, indicando posibles actividades enzimáticas laterales en los preparados comerciales. Estos cambios en el perfil de la fibra pueden mejorar las propiedades funcionales y nutricionales del salvado de arroz como ingrediente alimentario. Además, todos los tratamientos consiguieron reducir el tamaño de partícula de las muestras. Los tratamientos enzimáticos aumentaron significativamente las capacidades de hidratación, y se encontraron correlaciones positivas entre estas capacidades y el contenido de proteínas y la fibra insoluble. Se halló una fuerte correlación negativa entre el tamaño de partícula y la capacidad de unión del aceite ($r = -0,80$), la retención de agua desionizada ($r = -0,74$) y el carbonato sódico ($r = -0,71$). Estos resultados resaltaron la importancia del tamaño de partícula en las propiedades de hidratación, cruciales para la futura aplicación del salvado desgrasado tratado como ingrediente alimentario. Finalmente, el análisis de la fuerza de penetración mostró que los tratamientos reducían la resistencia necesaria para penetrar las muestras, correlacionándose positiva, aunque no significativamente, con el tamaño de las partículas.

Bloque III: Estrategias para garantizar la seguridad alimentaria revalorizando el salvado de arroz

Siguiendo la búsqueda de estrategias innovadoras y sostenibles para valorizar el salvado de arroz, se realizó un tratamiento por altas presiones (HPP) a 500 MPa durante 15 minutos con el salvado de arroz a diferentes niveles de hidratación correspondiéndose con el contenido de humedad operativa estándar de los cereales (15%) para la menor de las hidrataciones y a la capacidad de retención de agua (77%) para el más alto, mientras que los

niveles intermedios se eligieron en medio del intervalo anterior (30 y 60%). El tratamiento por HPP y la hidratación influyeron significativamente en las propiedades fisicoquímicas del salvado de arroz, especialmente en su composición de fibra dietética. Niveles bajos de hidratación aumentaron el contenido de fibra soluble con HPP. La solubilidad de glucosa y proteínas también se vio afectada, necesitándose una hidratación mínima del 30% para mejorarla. Los valores de peróxido se aumentaron tras el tratamiento por HPP a altas hidrataciones, mientras que a bajas hidrataciones el tratamiento por HPP disminuyó estos valores. Además, la hidratación moderada (30%) promovió la aglomeración de partículas resultando en un aumento del tamaño de partículas, mientras que las altas hidrataciones (60 - 77%) lo redujeron. El tratamiento por HPP indujo la formación de una estructura celular en panal, observable a nivel microestructural, a partir de una hidratación mínima del 30%. Incrementar la hidratación mejoró las capacidades de retención de agua y aceite, con la HPP potenciando la fijación del aceite a niveles altos de hidratación, además estas capacidades de fijación de solventes mostraron una correlación inversa con el tamaño de las partículas ($r = -0,71$ en el caso del agua y $r = -0,80$ con el aceite). Las propiedades espumantes se correlacionaron negativamente con la capacidad de unión al agua y al aceite ($r > -0,69$ en ambos casos), sugiriendo que una menor capacidad de unión al agua contribuye a una mejor estabilidad espumante. Los niveles de hidratación más altos disminuyeron tanto la capacidad espumante como la estabilidad de la espuma. A pesar de ello, el mayor impacto de las HPP se observó al 60% de hidratación, que mejoró tanto la capacidad como la estabilidad, lo que indica una posible interacción entre la presión y los niveles específicos de hidratación. A pesar de reducir la actividad emulsionante, las HPP mejoraron la estabilidad de la emulsión en todos los niveles de hidratación, probablemente aumentando la flexibilidad de las proteínas del salvado de arroz. El tratamiento por HPP también afectó la fuerza de penetración, aumentándola a niveles bajos de hidratación (30%) y disminuyéndola a niveles altos (60 y 77%). Además, el tratamiento por HPP es un método ampliamente utilizado en la industria alimentaria para garantizar la seguridad de los alimentos, por lo tanto, en el estudio también se evaluó su impacto en la carga microbiana del salvado de arroz. Las HPP redujeron significativamente las bacterias mesófilas y mohos/levaduras a niveles de hidratación del 30 - 77%, pero las bacterias formadoras de esporas no mostraron una reducción significativa. Adicionalmente, niveles moderados de

hidratación (30%) mostraron un impacto notable de las HPP en la inactivación de microorganismos, lo cual es destacable dado que la literatura sugiere que el nivel mínimo de agua libre necesario para que las HPP tengan efecto en la inactivación es del 40%. Estos resultados podrían optimizar el tratamiento por HPP sin requerir niveles elevados de hidratación ante la inactivación de microorganismos.

Conclusiones

1. La combinación de temperaturas de cocción suaves (90-95°C), pH ácido (4,5 - 5,5) y extracto de uva (0,1%) aumenta la inactivación de las esporas de *B. cereus* durante el proceso de cocción, resultando una estrategia eficaz para mejorar la seguridad alimentaria del arroz cocido.
2. Durante el almacenamiento, el extracto de uva al 0,1% inhibe el crecimiento de *B. cereus* a pH bajo (4,5) o temperatura (10°C), mientras que las concentraciones de 0,5 y 1% demuestran actividad bactericida independientemente de ambos factores ambientales. Con este método se consiguen reducciones de hasta 6 log de *B. cereus*, lo que proporciona pruebas sólidas que apoyan el extracto de uva como estrategia eficaz de conservación del arroz.
3. Los modelos deterministas desarrollados facilitaron la cuantificación de la actividad antimicrobiana y la optimización de los parámetros para un control microbiano eficaz. El software desarrollado podría utilizarse en la industria para ayudar en la toma de decisiones relativas al pH y la temperatura en varios niveles de concentración de extracto de uva, teniendo en cuenta la carga microbiana inicial.
4. El modelo de evaluación de la exposición industrial desarrollado proporciona una estimación probabilística de la concentración final de patógenos en diferentes condiciones de procesado y almacenamiento, teniendo en cuenta la incertidumbre y la variabilidad. Se trata de una herramienta valiosa para la industria alimentaria, que permite tomar decisiones basadas en datos y aplicar medidas de control eficaces para reducir el riesgo de *B. cereus* en los productos a base de arroz.

5. El análisis comparativo de las bebidas vegetales comerciales pone de manifiesto una tendencia hacia una mayor diversificación de las materias primas y un mayor enriquecimiento con vitaminas y minerales. Sin embargo, es necesario seguir mejorando en la reducción del contenido de sal y aceite y mejorar el enriquecimiento con fibra.

6. Los tratamientos enzimáticos del salvado de arroz desgrasado muestran un potencial considerable para modificar su composición y funcionalidad. Estos tratamientos duplican el contenido de fibra dietética soluble y reducen el tamaño de las partículas hasta un 50%, lo que mejora directamente las propiedades de hidratación y la capacidad de retención de disolventes. Estas mejoras podrían facilitar una mejor integración del salvado de arroz en matrices alimentarias.

7. El procesado por altas presiones (HPP) del salvado de arroz revela efectos dependientes de la hidratación sobre sus propiedades tecno-funcionales y su seguridad microbiológica, observándose un punto de inflexión significativo al 30% de humedad. Comprender cómo afectan los niveles de hidratación al tratamiento HPP es esencial para mejorar la eficacia antimicrobiana y las propiedades físico-químicas y tecno-funcionales.

La investigación pone de relieve la eficacia de integrar tecnologías innovadoras y sostenibles en la industria alimentaria, como los antimicrobianos naturales, las modificaciones enzimáticas y el tratamiento de altas presiones hidrostáticas. Estos enfoques mejoran la seguridad microbiológica, la calidad nutricional y las propiedades funcionales del arroz y sus subproductos.

Abstract

In today's food industry, sustainability has emerged as an essential component, alongside the equally critical aspect of food safety. This thesis addresses the intersection of these two principles, focusing on rice, the most widely consumed staple food globally. Improper storage and handling of rice present substantial public health risks, as it can serve as a vector for foodborne illnesses, particularly associated with *Bacillus cereus* outbreaks. Additionally, the large-scale production of rice generates significant by-products, including rice bran. Despite being rich in fiber, proteins, and bioactive compounds, rice bran is often discarded or relegated to animal feed, thereby underutilizing its valuable nutritional and technological potential.

The objective of this thesis was to increase the value of rice and its by-products in the food industry, focusing on two critical areas: food safety and sustainability. To this end, the antimicrobial efficacy of grape extract was assessed against *B. cereus* spores during the cooking process, as well as vegetative cells during the storage of cooked rice under different pHs and temperature conditions. Predictive microbiological models and exposure assessment were employed to evaluate the effectiveness of the proposed control strategies. Furthermore, sustainable processing techniques, including enzymatic treatments, soaking, thermal processing, and high hydrostatic pressure (HHP) under different hydration levels, were applied to defatted and non-defatted rice bran. The effects of these treatments on the physicochemical, techno-functional, and food safety properties of rice bran were thoroughly investigated.

The results indicated that grape extract, owing to its polyphenol content, effectively increased inactivated spores at 90–95°C and inhibited *B. cereus* growth at high concentrations (0.5 – 1%) under low pH (4.5–5.5) and temperature (10–20°C) conditions. Moreover, exposure assessment—a critical tool in food safety management—demonstrated a significant reduction in the risk of *B. cereus* ingestion in the presence of grape extract, highlighting its potential as a natural antimicrobial in the food industry.

In parallel, heat, soaking, and enzymatic treatments enhanced the nutritional and functional properties of rice bran by increasing its soluble fiber

content and improving hydration capacity, correlating with reduced particle size. Similarly, HHP treatments at different hydration levels improved hydration properties and increased the solubility of glucose and proteins, while significantly reducing microbial loads, particularly mesophilic bacteria, molds, and yeasts, with more pronounced effects at higher hydration levels. These advancements optimized the techno-functional properties of rice bran, positioning it as a versatile ingredient for food applications, particularly within the expanding plant-based beverage sector.

This thesis presents innovative solutions to improve both food safety and sustainability, addressing the increasing demand for healthier and safer food products. It contributes to global efforts to promote a more sustainable food system and advance the principles of a circular economy.

Resumen

En la actualidad, la sostenibilidad se ha convertido en un pilar clave de la industria alimentaria, sin desatender otro aspecto esencial: la seguridad alimentaria. Esta tesis aborda estos dos grandes desafíos, aplicándolos al alimento más consumido en el mundo, el arroz. Este cereal presenta riesgos microbiológicos significativos para la salud si se almacena o manipula incorrectamente, convirtiéndose en un vector potencial de brotes de intoxicación alimentaria comúnmente asociados al *Bacillus cereus*. Por otro lado, el elevado consumo de arroz y su consecuente producción masiva generan en la industria arrocería una gran cantidad de subproductos, entre los que se encuentra el salvado de arroz. Este subproducto con un alto contenido en fibra, proteínas y compuestos bioactivos, es normalmente descartado o utilizado para alimentación animal desaprovechando sus propiedades tanto nutricionales como tecnológicas.

El objetivo de esta tesis es valorizar el arroz y sus subproductos en la industria alimentaria mediante el desarrollo de estrategias innovadoras, centrándose en la mejora de dos áreas clave, la seguridad alimentaria y la sostenibilidad del arroz. Con este propósito se evaluó el uso de extracto de uva como agente antimicrobiano natural frente a esporas, en el proceso de cocción, y células vegetativas de *B. cereus*, en el almacenamiento del arroz cocido a distintos pH y temperaturas. Se utilizaron modelos predictivos microbiológicos y una evaluación de la exposición para analizar la eficacia de las estrategias de control propuestas. Por otro lado, se investigaron distintas estrategias sostenibles, como el tratamiento enzimático, de remojo y térmico, y el de altas presiones hidrostáticas a distintas hidrataciones, aplicadas al salvado de arroz desgrasado y sin desgrasar, respectivamente, para revalorizar este subproducto mediante el análisis del impacto de los tratamientos en las propiedades fisicoquímicas, tecno-funcionales y de seguridad alimentaria del salvado de arroz.

Los resultados demuestran la capacidad antimicrobiana del extracto de uva, atribuida a su contenido en polifenoles, frente a la inactivación de esporas a temperaturas de 90 y 95°C, inhibiendo además el crecimiento de *B. cereus* a altas concentraciones de extracto (0,5 – 1%) y a pH y temperaturas bajas (4,5 - 5,5, y 10 – 20°C) para la concentración más baja estudiada. Además, la

evaluación de la exposición, consolidada como una herramienta clave en la gestión de la seguridad alimentaria, demostró de manera convincente la reducción del riesgo de ingerir una carga infecciosa de *B. cereus* en presencia de extracto de uva, subrayando su potencial como antimicrobiano natural en la industria alimentaria.

Adicionalmente, la investigación reveló que los tratamientos, térmico, de remojo y enzimático, mejoraron las propiedades nutricionales y funcionales del salvado, aumentando su contenido de fibra soluble y mejorando sus propiedades de hidratación, las cuales estuvieron correlacionadas con una disminución del tamaño de partícula. De manera similar, en el tratamiento por altas presiones combinado con diferentes niveles de hidratación, también mejoró las propiedades de hidratación correlacionadas nuevamente con una disminución del tamaño de partícula, mientras que incrementó la solubilidad de glucosa y proteínas en todos los casos. Además, las altas presiones redujeron la carga microbiana de mesófilos, mohos y levaduras, siendo el efecto más pronunciado con mayores niveles de hidratación. Los tratamientos propuestos suponen una mejora en las propiedades tecno-funcionales del salvado, haciéndolo un ingrediente más versátil para aplicaciones alimentarias, especialmente en el creciente mercado de bebidas vegetales.

En conjunto, esta tesis ofrece soluciones innovadoras para mejorar la seguridad alimentaria, fomentar la sostenibilidad y satisfacer la creciente demanda de opciones alimentarias más seguras y nutritivas, alineándose con los esfuerzos globales para crear un sistema alimentario más sostenible y apoyar una economía circular.

Resum

En l'actualitat, la sostenibilitat s'ha convertit en un pilar clau de la indústria alimentària, sense desatendre un altre aspecte essencial: la seguretat alimentària. Aquesta tesi es desenvolupa en la intersecció d'aquests dos principis, aplicant-los a l'aliment més consumit en el món, l'arròs. Aquest cereal presenta riscos significatius per a la salut pública si s'emmagatzema o manipula incorrectament, convertint-se en un vector potencial de brots d'intoxicació alimentària normalment associats al *Bacillus cereus*. L'elevat consum d'arròs i la seua conseqüent producció masiva generen en la indústria arrosiera una gran quantitat de subproductes, entre els quals es troba el segó d'arròs. Aquest subproducte amb un alt contingut en fibra, proteïnes i compostos bioactius, és normalment descartat o utilitzat per a alimentació animal desaprofitant les seues propietats tant nutricionals com tecnològiques.

L'objectiu d'aquesta tesi és valorar l'arròs i els seus subproductes en la indústria alimentària mitjançant el desenvolupament d'estratègies innovadores, centrant-se en dos àrees clau, la seguretat alimentària i la sostenibilitat de l'arròs. Amb aquest propòsit es va avaluar l'ús d'extracte de raïm com a agent antimicrobià natural contra espores, en el procés de cocció, i de cèl·lules vegetatives de *B. cereus*, en l'emmagatzematge de l'arròs cuit a diferents pH i temperatures. Es va fer ús de models predictius microbiològics i una avaluació de l'exposició per a analitzar l'eficàcia de les estratègies de control proposades. A més, es van investigar diferents estratègies sostenibles, com el tractament enzimàtic, de remull i tèrmic, i el d'altres pressions hidrostàtiques a diferents hidratacions, aplicades al segó d'arròs desgreixat i sense desgreixar, respectivament, per a revalorar aquest subproducte mitjançant l'anàlisi de l'impacte dels tractaments en les propietats fisicoquímiques, tecno-funcionals i de seguretat alimentària del segó d'arròs.

Els resultats demostren que l'extracte de raïm, atribuït al seu contingut en polifenols, és rellevant en la inactivació d'espores a temperatures de 90 i 95 °C i a més inhibeix el creixement de *B. cereus* a altes concentracions d'extracte (0,5 - 1%) i a pH i temperatures baixes (4,5 - 5,5, i 10 - 20 °C) per a la concentració més baixa estudiada. A més, l'avaluació de l'exposició, consolidat com una ferramenta clau en la gestió de la seguretat alimentària, va demostrar de manera convincent la reducció del risc d'ingerir una càrrega

infecciosa de *B. cereus* en presència d'extracte de raïm, subratllant el seu potencial com a antimicrobià natural en la indústria alimentària.

La investigació va revelar que els tractaments, tèrmic, de remull i enzimàtic, van millorar les propietats nutricionals i funcionals del segó, augmentant el seu contingut de fibra soluble i millorant les seues propietats d'hidratació, les quals van estar correlacionades amb una disminució de la grandària de partícula. De manera similar, en el tractament per altes pressions combinat amb diferents nivells d'hidratació, també va millorar les propietats d'hidratació correlacionades novament amb una disminució de la grandària de partícula, mentre que va incrementar la solubilitat de glucosa i proteïnes en tots els casos. A més, les altes pressions van reduir la càrrega microbiana de mesòfils, fongs i llevats, sent l'efecte més pronunciat amb majors nivells d'hidratació. Estes millores van optimitzar les propietats tecno-funcionals del segó, fent-lo un ingredient més versàtil per a aplicacions alimentàries, especialment en el creixent mercat de begudes vegetals.

Aquesta tesi oferix solucions innovadores per a millorar la seguretat alimentària, fomentar la sostenibilitat i satisfer la creixent demanda d'opcions alimentàries més segures i nutritives, alineant-se amb els esforços globals per a crear un sistema alimentari més sostenible i donar suport a una economia circular.

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Chapter 5. Unlocking hidden potential of rice bran: enzymatic treatment for enhancing techno-functional properties

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Abbreviations

α or δ	Scale parameter
ADF	Acid detergent fiber
ADL	Acid detergent lignin
Af	Accuracy factor
AL	Alcalase
ANOVA	Analisis of variance
AU	Absorbance units
b or p	Shape parameter
CE	Celluclast
CFU	Colony forming units
DG18	Dichloran-glycerol agar
DRB	Defatted rice bran
DRBC	Cichloran rose bengal chloramphenicol
EA	Emulsifying activity
ES	Emulsifying stability
FC	Foaming capacity
FL	Flavourzyme
FS	Foaming stability
HACCP	Hazard analysis and critical control point
HPP	High pressure process
IDF	Insoluble dietary fiber
MANOVA	Multivariate analysis of variance
MSE	Mean square error
NBA	Nutrient broth agar
NDF	Neutral detergent fiber
NO	Novozym
OBC	Oil binding capacity
PBB	Plant based beverages
PCA	Principal componen analysis
PCA	Plate count agar
PV	Peroxide value
RB	Rice bran

SDF	Soluble dietary fiber
SEM	Scanning electron microscopy
SH	Shearzyme
SRC	Solvent retention capacity
UL	Ultimase
TDF	Total dietary fiber
TSA	Tryptone soy agar
WBC	Water binding capacity

Samples codes

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RB	Rice bran
DRB	Defatted rice bran
DRB-T	Defatted rice bran thermally treated
DRB-T-C	Defatted rice bran thermally treated and soaked
DRB-C	Defatted rice bran soaked
DRB-NO	Defatted rice bran treated with Novozym®
DRB-CE	Defatted rice bran treated with Celluclast®
DRB-UL	Defatted rice bran treated with Ultimase® BWL 40
DRB-SH	Defatted rice bran treated with Shearzyme® Plus 2x
DRB-AL	Defatted rice bran treated with Alcalase® 2.4 L FG
DRB-FL	Defatted rice bran treated with Flavourzyme®

CHAPTER

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RB	Rice bran
RB15	Rice bran tempering to hydration of 15%
RB15HPP	Rice bran tempering to hydration of 15% + high pressure treatment (500 MPa 15 minutes)
RB30	Rice bran tempering to hydration of 30%
RB30HPP	Rice bran tempering to hydration of 30% + high pressure treatment (500 MPa 15 minutes)
RB60	Rice bran tempering to hydration of 60%
RB60HPP	Rice bran tempering to hydration of 60% + high pressure treatment (500 MPa 15 minutes)
RB77	Rice bran tempering to hydration of 77%
RB77HPP	Rice bran tempering to hydration of 77% + high pressure treatment (500 MPa 15 minutes)



Introduction

1. Justification of the study

Rice, a staple food across many cultures, poses significant public health risks if improperly stored or handled, potentially becoming a vector for pathogen transmission. Outbreaks of food poisoning associated with cooked rice are alarmingly common, primarily due to insufficient cooking temperatures and inadequate refrigeration. These conditions allow for the germination of heat-resistant spores and the subsequent growth of *Bacillus cereus*. Given the global importance of rice consumption, developing effective strategies to control microbial growth in cooked rice is critical for ensuring food safety. One promising approach involves the use of natural antimicrobials derived from agro-industrial by-products, such as grape extract. This extract is rich in polyphenols, compounds known for their potent antimicrobial properties. Employing these by-products not only enhances food safety but also contributes to waste reduction in the agro-industrial sector. This dual benefit aligns with sustainable practices in food production and preservation. By harnessing the antimicrobial capacity of these natural compounds, we can potentially mitigate the risks associated with rice consumption while promoting resource efficiency.

As previously mentioned, rice, one of the most widely consumed cereals globally, produces significant waste during processing, particularly focused in rice bran. This by-product, often considered valueless by producers, has significant potential as a food ingredient due to its high fiber, protein, and bioactive compounds content. However, its use is limited by its physicochemical and techno-functional properties that may affect final product quality and acceptability. Modifying the properties of rice by clean processing technologies could expand its potential as a versatile ingredient in various food applications and overcome these limitations. Reevaluating rice bran as a natural ingredient with health benefits in new product formulations or reformulations of existing products addresses growing consumer demands. Today's consumers increasingly looking for functional products free from synthetic additives, that are fresh or minimally processed, and retain their organoleptic and nutritional characteristics. At the same time, achieving safety improvements alongside these modifications represents a fundamental advance in the rice industry's circular economy.

In this context, this thesis explores strategies to enhance sustainability in rice processing through technological, microbial, and nutritional approaches. It addresses the safety of cooked rice by utilizing natural antimicrobials and improves the properties of rice bran using clean technologies. The innovative solutions developed in this research contributes to environmental sustainability, aligning with current industry needs and consumer demands for safer and more nutritious food options.

2. State of the Art

2.1. Sustainability

Sustainability /sə,steɪ.nə'bɪl.ə.ti/ *n* [U] **1** the quality of being able to continue over a period of time **2** the quality of causing little or no damage to the environment and therefore able to continue for a long time (Cambridge University Press, n.d.).

In today's context, the concept of sustainability transcends its traditional lexical boundaries and extends well beyond the confines of conventional definitions, encompassing multifaceted implications. Sustainability, involves a holistic assessment of human existence, considering social, environmental, and economic dimensions, all with a forward-looking perspective to benefit future generations (Sandberg et al., 2023).

This concept plays a fundamental role of promoting policy frameworks across different government institutions, from the global to the local levels. International sustainability efforts were formally initiated at the 1992 Earth Summit in Rio de Janeiro, and more recently, in 2015, the United Nations General Assembly established the *2030 Agenda for Sustainable Development*, aiming to steer public policy and motivate social stakeholders towards global sustainable progress through 17 Sustainable Development Goals (SDG) and 169 detailed targets (Biermann et al., 2022; Zaharia et al., 2021). The SDG framework has also provided a foundation for other institutions to develop their sustainability policies. In this context, the European Commission adopted the "European Green Deal" in 2019. This initiative aims to make the European Union (EU) the first climate-neutral continent by 2050. The European Green Deal outlines a new, sustainable, and inclusive growth strategy designed to improve the economy, health and quality of life, and protect the environment, while ensuring that no European citizen is left behind (European Commission, 2019). In 2024, the European Commission selected the city of Valencia as the European Green Capital. This recognition is to cities that demonstrate an exemplary ecological transition and implement measures aligned with the SDG and the European Green Deal. This distinction underscores the importance of sustainability at both international and local levels, highlighting it as an essential component of the daily policies and practices required to ensure a sustainable future.

2.2. Food safety and sustainability

Leveraging new technologies and scientific breakthroughs, alongside heightened public awareness and the growing demand for sustainable food options, promises benefits for all involved parties. In this sense, food safety and security are integral components of a sustainable future, mutually reinforcing each other to ensure the well-being of both people and planet. To advance the transition towards an equitable, health-conscious, economic and environmentally sustainable food system, critical components like advisory services and financial instruments are indispensable. The World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the Codex Alimentarius Commission are crucial in promoting global food safety standards and policy harmonization (Schonrock, 2023). Their collective efforts safeguard public health, ensure the safety and integrity of the global food supply, and support the development of the agricultural sector, thereby paving the way for a more sustainable global food system.

Furthermore, research and innovation emerge as essential factors, significantly enhancing solution design and evaluation, overcoming obstacles, and exploring new market opportunities. Technological advances also play a crucial role in food safety. Innovative technologies such as blockchain, internet of things (IoT) sensors, and artificial intelligence (AI) are being integrated into the food safety framework. These technologies enhance traceability and transparency throughout the supply chain, facilitate real-time monitoring, enable early risk identification, and support the implementation of preventive measures. Additionally, they aid in informed decision-making (Arinze et al., 2024). These advancements not only optimize risk-based approach also improve the efficiency and sustainability of the food system, supporting data-driven decisions and promoting a safer, more resilient approach.

2.3. Rice

Rice (*Oryza*) is one of the major cereals and recognized as the most consumed food in the world and feeds almost half the world population due to its abundant nutrients and relatively low price (Wasaya et al., 2022; Wei & Huang, 2019). Rice is staple food for approximately 3.5 billion people

worldwide and supplies the 75% of the daily calories intake in Asian countries (Park et al., 2017). 522.1 million metric tons of rice were consumed worldwide in the 2023/24 crop year (US Department of Agriculture, 2024). Paddy rice (**Figure 1**) is the final product of the rice harvest, composed by husk, bran, endosperm and germ. The rice milling process comprises cleaning, husking, and post-husking treatments (bleaching, polishing, and classification), resulting in various rice by-products (Esa et al., 2013). The proportion of these by-products in rice is influenced by factors including the milling rate and rice type. An optimal milling process typically yields approximately 25% husk, 10% bran and germ, and 65% “white rice” (endosperm), contingent upon the variety (Muthayya et al., 2014). Layers surrounding the rice grain (husk and bran), similarly to other cereal species, have elevated content in bioactive compounds, minerals, vitamins, dietary fibers, proteins and lipids contrasting with the endosperm, which is characterized by simple carbohydrates and starch granules (Spaggiari et al., 2021).

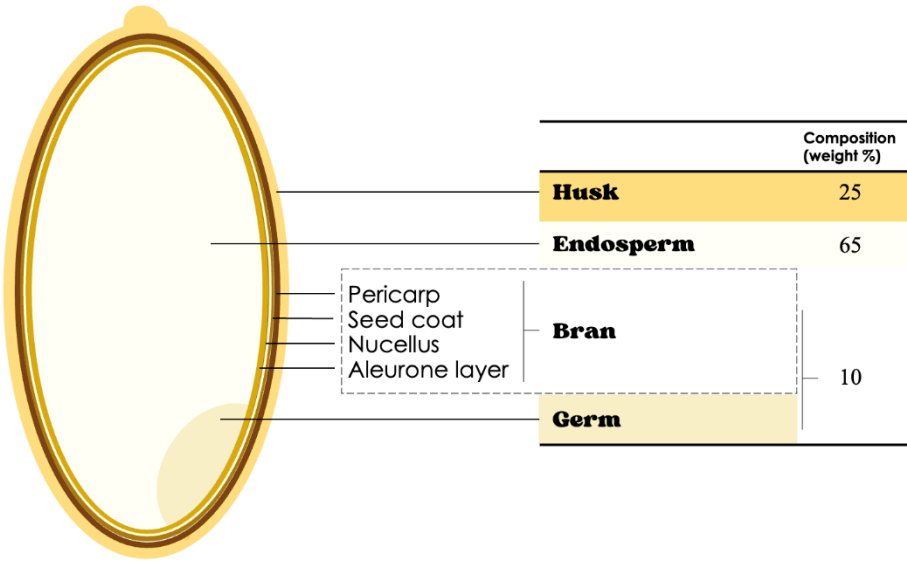


Figure 1. Schematic representation of rice grain, highlighting the bran fraction. Adapted from Muthayya et al., (2014) and Spaggiari et al., (2021).

2.3.1. Rice and food safety

The principal constituents of white rice (mainly endosperm) are carbohydrates (79%), mainly starch, 7% protein, and 2% fat, along with essential vitamins and minerals, and provide an optimal substrate for the growth and development of microorganisms (Borba et al., 2020; Rodrigo et al., 2021). Bacteria are one of the major microorganisms able to promoting rice foodborne diseases since some of these are capable to produce spores that can survive under extreme conditions (Borba et al., 2020). One of the main microorganism frequently involved in rice foodborne outbreaks is *Bacillus cereus* (Rodrigo et al., 2021).

B. cereus is a gram-positive, spore forming, facultative anaerobic bacterium (Jessberger et al., 2020). It is ubiquitously present in environments such as dust, soil, plant surfaces, and the rhizosphere (Vilain et al., 2006). From there, vegetative cells and especially spores can easily enter the food chain via crop plants. *B. cereus* is a habitual saprophyte, which is resistant to low humidity, high temperatures, dehydration, radiation, and acidity and has the ability to produce food toxins, posing significant public health concerns (Hendriksen et al., 2006; Rodrigo et al., 2021; Sánchez et al., 2016). *B. cereus* can produce two types of toxins, diarrheal and emetic. One of the main characteristics of those toxins are their resilience in storage, as they can remain viable for extended periods, up to 48 weeks, in cool dry storage without loss of viability (Gilbert et al., 1974). In 2022, foodborne outbreaks in the European Union attributed to bacterial toxins accounted for 19.8%, with *B. cereus* toxins ranking highest at 5.3% of reported cases. Notably, *B. cereus* was associated with 6.6% of reported human illness cases. Comparing 2022 to 2021, there was a significant surge in foodborne outbreaks attributable to *B. cereus* toxins, with 219 more incidents recorded, marking a relative increase of 251.7% (EFSA & ECDC, 2023). Outbreaks of *B. cereus* associated with cooked rice are frequently attributed to inadequate cooling or prolonged storage at room temperature, particularly in establishments such as restaurants and canteens where large quantities are prepared (Juneja et al., 2019). The main source of contamination is the presence of heat-resistant spores that survive conventional rice cooking temperatures (near to 100 °C); however, vegetative forms of *B. cereus* can be easily eliminated (Gilbert et al., 1974; Sarriás et al., 2002; Soni et al., 2018). Upon cooking, rice attains a pH close to 7, creating an optimal environment for microbial proliferation when

substrate humidity reaches appropriate water activity levels (Delbrassinne et al., 2015; Pao et al., 2006). Despite standard cooking procedures potentially reducing spore levels, the final risk is heavily influenced by initial microbial concentrations and adherence to proper hygiene practices during handling and processing (Rodrigo et al., 2021).

2.3.2. Sustainable strategies to ensure food safety

In recent years, there has been growing interest in extracting natural compounds capable of inhibiting the outgrowth of microorganisms, particularly those responsible for food spoilage and numerous diseases (Michielin et al., 2009; Pinelo et al., 2007). Phenolic compounds are phytochemicals with diverse functions including pigmentation, astringency, antioxidant and antimicrobial (Oliveira et al., 2003). Acting as antimicrobial agents, these phenolic compounds can penetrate the semipermeable cell membranes, interacting with cellular proteins or the cytoplasm. Their negative charge makes them effective in inhibiting the growth of pathogenic bacteria (Arts & Hollman, 1998; Cheng et al., 2012; Oki et al., 2002; Yadav et al., 2015). Grape extract is a by-product from the wine industry, and a rich source of phenolic compounds, as well as lipids, proteins and polysaccharides. Grape extract, consisting of skins, seeds, and stems and since it is an abundant by-product, its valorization holds significant economic interest (Deng et al., 2011; Schieber, 2017). According to Balaban et al., (2021), the concentration of polyphenols may vary and depends on their origin (grape seeds or skin), the extraction solvent employed and the analysis method utilized for polyphenol quantification. As a result, values may vary accordingly. Grapes (*Vitis vinifera* L.), one of the oldest and second-largest fruit crops in the world, are mainly (80%) used by the wine industry and approximately 25% of the grape weight is transformed into waste, posing challenges for effective management (Dwyer et al., 2014; Ferreira et al., 2014). Besides offering compounds with useful properties, revalorization, reduces the environmental impact by reusing these grape by-product which are generally not used by industry (Goncalves et al., 2021). Various studies have examined the antimicrobial properties of grape by-products as an alternative to artificial preservers (**Table 1**).

Ensuring the safety and quality of processed food products requires the effective control or elimination of foodborne pathogens post-processing and during storage (Boisrobert et al., 2010).

Table 1. Studies where have investigated the antimicrobial properties of grape by-products

Grape extract	Grape type	Phenol compounds extraction	Organisms	Effect	Reference
Grape seeds	Red	Acetone:water:acetic acid (90:9.5:0.5) Methanol:water:acetic acid (90:9.5:0.5)	<i>Bacillus cereus</i>	Antibacterial	Jayaprakasha et al., (2003)
			<i>Bacillus coagulans</i>		
			<i>Bacillus subtilis</i>		
			<i>Staphylococcus aureus</i>		
Grape seeds	-	Acetone:water:acetic acid (90:9.5:0.5)	<i>Escherichia coli</i>	Antibacterial	Baydar et al., (2006)
			<i>Pseudomonas aeruginosa</i>		
			<i>E. coli</i> O157:H7		
			<i>S. aureus</i>		
Grape extract	White	-	<i>Aeromonas hydrophila</i>	Antibacterial Antifungal	Serra et al., (2008)
			<i>E. coli</i>		
			<i>Salmonella poona</i>		
			<i>B. cereus</i>		
Grape seed extract	-	-	<i>Saccharomyces cerevisiae</i>	Antibacterial Antibiofilm	Furiga et al., (2009)
			<i>Candida albicans</i>		
			<i>Porphyromonas gingivalis</i>		
			<i>Fusobacterium nucleatum</i>		
Grape skins (14 grape varieties)	Red	-	<i>E. coli</i> O157:H7	Antibacterial	Katalinić et al., (2010)
			<i>Salmonella Infantis</i>		
			<i>Campylobacter coli</i>		
			<i>S. aureus</i>		
Grape pomace	Red	Supercritical fluid extraction (CO ₂)	<i>B. cereus</i>	Antibacterial	Oliveira et al., (2013)
			<i>S. aureus</i>		
			<i>B. cereus</i>		

Grape extract	Grape type	Phenol compounds extraction	Organisms	Effect	Reference
Grape seeds	-	-	<i>Campylobacter jejuni</i> (12 strains)	Antibacterial	Silván et al., (2013)
Grape stems (3 varieties)	Red	Methanol/distilled water (70:30)	<i>S. aureus</i> <i>Enterococcus faecalis</i> <i>E. coli</i> <i>Klebsiella pneumoniae</i> <i>P. aeruginosa</i> <i>Listeria monocytogenes</i>	Antibacterial	Gouveinhas et al., (2018)
Grape pomace (7 Grape varieties)	4 Red 3 White	Water:ethanol (50:50) Water:Methanol (50:50)	<i>E. coli</i> <i>C. albicans</i> <i>P. aeruginosa</i> <i>S. aureus</i>	Antibacterial Antifungal	Luchian et al., (2019)
Grape pomace	-	Methanol	<i>E. coli</i> <i>Streptococcus sobrinus</i> <i>Bifidobacterium spp.</i> <i>Lactobacillus spp.</i>	Antibacterial	Karamati Jabelhedar et al., (2019)
Grape marc extracts	Red	Ethanolic solutions with temperature from 30 to 65°C	<i>B. subtilis</i> <i>S. aureus</i> <i>E. coli</i> <i>K. pneumoniae</i>	Antibacterial	Ghendov-Mosanu et al., (2022)

To achieve this target, employing a combination of hurdles, such as heat treatments, pH variations, and natural antimicrobials, can effectively control or even prevent pathogen growth, thereby reducing associated risks (Juneja et al., 2019). Microbiological studies are usually conducted to derive estimations (predictive microbiology) that determine the survival of microorganisms after applying various combinations of these hurdles. Defined as a quantitative science, predictive microbiology rigorously evaluates the effect of processing, distribution, and storage operations on the microbiological safety and quality of food, with the behavior of foodborne pathogens being predicted through mathematical models in response to environmental conditions (Ross & McMeekin, 1994). Their premise lies in the reproducibility of microbial population responses to environmental factors. This enables the prediction of microbial behavior under similar conditions based on experimental observations (Ross et al., 2000). According to Whiting & Buchanan, (1997), these predictive models can be classified into three levels:

i) *Primary models*: that describe the variation in the number of microorganisms over time, based on specific environmental and culture conditions.

ii) *Secondary models*: These equations illustrate how the parameters of primary models (such as growth rate and adaptation phase duration) change in response to different environmental factors (such temperature or pH).

iii) *Tertiary models*: These are computer programs that integrate primary and secondary models to calculate microbial responses under various conditions.

Predictive models have evolved from a promising field in food microbiology to an essential tool for quality control and food safety in the industry. They play a crucial role in hazard analysis and critical control point (HACCP) systems, the development of new products and the determination of shelf life within the food industry. Regulation 178/2002 establishes the Risk Analysis process as the foundation for food safety measures. Risk analysis (**Figure 2**) comprises three phases: risk assessment, risk management, and risk communication. This systematic approach is designed to enhance food safety control systems, aiming to ensure their safety, diminish the incidence of associated diseases, and facilitating national and international in food trade. Once the stages of risk analysis have been defined, experts in microbial

science mainly focus on the risk assessment phase, particularly at a quantitative level (Quantitative Microbiological Risk Assessment, QMRA). This phase requires significant resources and a multidisciplinary approach and comprises and involves identifying microbiological hazards, characterizing their nature and potential adverse effects, assessing exposure, and characterizing the risk through models that estimate the probability of impact on the population (FAO/WHO, 2006). The most crucial step in industrial Risk Assessment for decision-making is the Exposure Assessment stage. According to Codex, Exposure Assessment is defined as the qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant (Codex Alimentarius Commission, 1999).

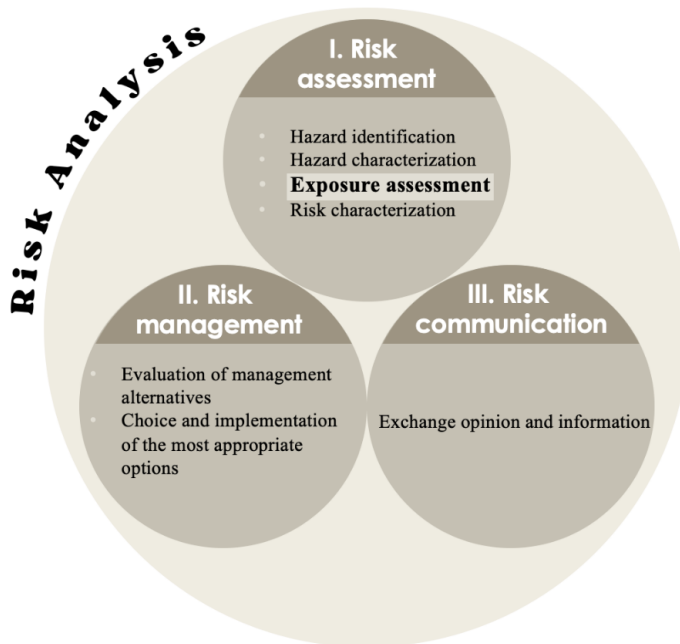


Figure 2 Components of Risk Analysis: assessment, management and communication and their stages.

QMRA can be either deterministic or stochastic. Deterministic assessments provide simple average values, offering a single-point estimate. In contrast, stochastic assessments capture the full range of variability and

uncertainty in each input variable through probability distributions, providing a more comprehensive representation of potential outcomes. Shifting from deterministic assessment to probabilistic or stochastic evaluation is a complex process. Computer simulation programs enable comprehensive exploration across diverse study domains. In stochastic analysis, Monte Carlo simulation stands out as a commonly employed tool for crafting probability distributions of output variables. These simulation tools utilize data entered into an Excel sheet, that are made using key input parameters defined by probability distributions and derived from experimentation in laboratory, industry, or public administration. This enables them to emit outputs that reproduce the real behavior of the complete system and allows for the evaluation of different hypothetical situations or scenarios by introducing desired changes in the selected inputs. The advantage of stochastic models over deterministic models lies in the volume of information they return to us, which closely resembles reality as it can be an estimate of the final load levels of microorganism and they are crucial for supporting decision-making processes.

2.3.3. Rice by products: Rice bran

The growing demand for rice, driven by increasing population growth and changing dietary preferences, requires enhancements in rice production. The importance is highlighted by predictions suggesting a significant growth in global rice production by 2030, with estimates indicating an increase to 567 million tons (Mohidem et al., 2022). This substantial growth in rice production also leads to an increase in the by-products produced by rice industries. On average, paddy rice produced 10% of bran and germ (Muthayya et al., 2014). In 2022, a total of 726 million tons of paddy rice were harvested, equivalent to 72.6 million tons of bran and germ (FAO, 2024; Muthayya et al., 2014). Just in the Valencian Community, a total of 115 thousand tons of rice were produced in 2021, equivalent amount of 11.5 thousand tons of rice bran as a by-product (Conselleria de Agricultura, Desarrollo Rural, Emergencia Climática y Transición Ecológica, 2021).

Rice bran (RB) comprises the pericarp, seed coat, nucellus and aleurone layer, as well as a small portion of endosperm (caused by the degree milling) (Sharif et al., 2014). Rich in lipids, comprising 15 to 20% of its composition, RB oil constitutes an important source of valuable bioactive compounds, such γ -oryzanol (1.1-2.6%), tocopherols (0.2%), tocotrienols (70%), and sterols (3-

5%) (Garba et al., 2019). γ -Oryzanol from rice bran oil has garnered considerable attention from the cosmetic industry owing to its distinctive properties and medicinal attributes, that renders interesting applications for the development of a variety of cosmetic formulations (Garba et al., 2019). Following fat extraction, the remaining secondary by-product, accounting for approximately 90% of the annual RB production, rich in dietary fiber and protein, often has two main destinies,; disposed of as waste or applied as an ingredient in animal feed formulations (Gul et al., 2015).

As said, the secondary by-product (defatted RB) is rich in fiber and protein. RB proteins (13.2 – 17.3% of RB) have an important nutritional value, due that it contains all essential amino acids for human nutrition, which includes lysine, methionine, threonine, tryptophan, histidine, leucine, isoleucine, valine, and phenylalanine, beside good digestibility and bioavailability of them (Dang & Vasanthan, 2019; Park et al., 2017; Zheng et al., 2019). Food and Agricultural Organization (FAO) and World Health Organization (WHO) recommend the protein quality evaluation using the Protein Digestibility-Corrected Amino Acid Score (PDCAAS) (WHO/FAO/UNU, 2007). In cereals, lysine is the primary limiting amino acid. Amagliani et al., (2017), Mota et al., (2016) and Wang et al., (2015) reported that RB protein has higher lysine content compared to other grains such oat, maize and wheat protein. Furthermore, the biological value of RB protein exceeds that of soy protein isolate, while its PDCAAS value is comparable (Han et al., 2015). Rice bran proteins are generally perceived as hypoallergenic and no rice protein related food allergies has been reported (Zheng et al., 2019). RB proteins and derivates have been shown as contributors of health advantages, comprising an essential group of bioactive molecules involved in physiological processes (Park et al., 2017; Zheng et al., 2019). Among these activities are their antioxidant capability, immunomodulatory activities, angiotensin-converting enzyme activities (which play an important role in the control of blood pressure), antidiabetic properties, anticancer potential, and cholesterol-lowering capabilities (Adebiyi et al., 2008; Boonla et al., 2015; Park et al., 2017; Uraipong & Zhao, 2016; Wang et al., 2015; Zheng et al., 2019). In addition, this RB protein and derivates (isolates and concentrates) play a fundamental role in physicochemical properties such as solubility, water and oil binding capacity, foaming and emulsifying properties, important for the application as ingredient in final product (Hamada, 2000; Phongthai et al., 2016).

On the other hand, RB fiber (27.6 – 33.3%), is an excellent source of insoluble dietary fiber (IDF) and there are, in a lower proportion, soluble dietary fiber (SDF), both being known for their good physical and bioactive properties (Dang & Vasanthan, 2019). IDF contains cellulose, lignin, and some hemicellulose, while SDF contains some hemicellulose and many hydrocolloids (Jia et al., 2019). Furthermore, IDF and SDF have distinct physiological functions (Jia et al., 2019). IDF is related with the beneficial function for intestinal flora and transit, increase stool volume, and can inhibit the pancreatic lipase (Jia et al., 2019; Mudgil & Barak, 2013; K. Zhu et al., 2010), and SDF benefits are related with reducing glycemic response and plasma cholesterol, as well as having immunomodulatory activity and can prevent the colorectal cancer (Anderson et al., 2009; Jia et al., 2019; Qi et al., 2015). Dietary fiber also exhibits physicochemical properties, as it can modulate texture, emulsifying and gelling abilities and usually alters the rheological properties of dough when is incorporated into a formulation (Abdul-Hamid & Luan, 2000; Jia et al., 2019; Park et al., 2017).

The substantial quantity of rice by-products is also the result of the low consumer acceptance of wholegrain or brown rice (containing endosperm, germ and bran), as polished white rice is generally preferred (Nakayama et al., 2017; Saleh et al., 2019; Zhang et al., 2010). The lower consumer acceptability can be attributed due to the presence of bran, which causes various reasons for rejection, such as: i) Its higher fiber content and phenolic compounds contribute to an astringent taste and a firmer texture, making it less palatable for chewing. Additionally, its darker color is often less appealing to consumers; ii) The higher oil content in the outer bran layer makes it more susceptible to oxidation and the development of off-flavors, resulting in a shorter shelf life when stored at room temperature. iii) The higher content of anti-nutritional compounds, such as phytic acid and tannins (Gujral & Kumar, 2003; Lanning & Siebenmorgen, 2011; Mohan et al., 2017; Saleh et al., 2019; Wang et al., 2011).

To increase the consumption of wholegrain, the food industry has conducted various studies to assess the possibility of incorporating a higher proportion of brown rice into common food products such as noodles, bread, pasta, cakes, snacks, pastries, and beverages. These efforts involve the developing of new recipes that use brown rice exclusively or in combination with white rice and/or other ingredients (Cornejo & Rosell, 2015; Saleh et al.,

2019). Focusing on beverages, an exponentially growing segment worldwide (Vaikma et al., 2021), there is a rising number of individuals following plant-based diets and those concerned about environmental sustainability (Penha et al., 2021; Sethi et al., 2016; Shori & Al Zahrani, 2022). This increasing interest in plant-based beverages represents a substantial opportunity for the development of them using diverse plant sources (Sethi et al., 2016) or by-products to fortify some nutritional aspects. The use of rice (either alone or in combination with other ingredients) is becoming increasingly common as a raw material and is one of the most frequently used cereals for this purpose (Munekata et al., 2020). Rice, as a raw material for plant-based beverages, have a long tradition in eastern and western world, being *Sikhye* one of the most traditionally beverages in South Korea (Bernat et al., 2014; H. Kim et al., 2012). Despite ongoing improvements in beverage research, there are still technological challenges, such as incorporating fiber-rich ingredients or reducing oil in beverages where it has been necessary to add it. In this sense, rice bran can play an important role in plant-based food due to its high fiber and protein content, which enhances their nutritional profile and formulation. This not only meets the growing consumer demand for nutritious and environmentally responsible products but also supports sustainability by adding value to rice bran as an ingredient, thereby reducing waste in the rice industry.

2.3.4. Sustainable strategies to rice bran's revalorization

The study of innovative recovery strategies focused on the valorization of agro-industrial by-products represents a growing sector concerning about sustainability and dietary habits within the food system (Spaggiari et al., 2021). As said before, RB is an important by-product that remains undervalued because of technological limitations. Improving the technological capabilities associated with RB is essential for fully utilizing its potential as a food ingredient and therefore, enhancing the sustainability of rice production. Many technologies have been employed to fully take advantage of the multiple benefits of RB and to reduce rancidity and enhance the quality of its components. However, these techniques are typically applied to individual isolate fractions of RB rather than utilizing it as a comprehensive set of components.

a. Enzymatic processing

Enzymes are proteins produced by living organisms that are synthesized as a cellular compound and may or may not possess a non-protein prosthetic group (Mishra et al., 2017). Their main function is as biological catalysts, accelerating biochemical reactions (Robinson, 2015). Microorganisms represent an important source of enzymes due to they could be cultured in large amount in short period being able to produce large quantities of enzymes (Barcelos et al., 2020). Enzymes, essentials in industrial bioprocesses, exhibit extensive utility across various industrial sectors, encompassing the detergent, paper, and textile industries, beside playing an important role within the food sector (Raveendran et al., 2018). With historical application in food preservation, these enzymes persist in enhancing both the quality and efficiency of food production, aligning with the mounting need for sustainable food solutions (Mishra et al., 2017). The use of enzymes for the valorization of agro-industrial wastes is very promising. Enzymatic treatments have been applied for enhancing the extraction of some compounds from RB. Kim & Lim, (2016) used different carbohydrases (Viscozyme[®], Termamyl[®], Celluclast[®], amyloglucosidase, Ultraflo[®] and Pentopan[®]) to improve the extraction of antioxidant compounds from RB. These enzymes enhanced the extraction of phenolic compounds and promoted their hydrolysis, resulting in modifications of the phenolic profile and increased antioxidant activity. Patindol et al., (2007) also applied enzymatic treatment (with cellulase) to improve the oligosaccharides extractables from RB. Enzymatic treatments have been employed to modify the technological properties of various rice bran (RB) fractions (**Table 2**). These changes encompass functional attributes such as hydration capacity, improved emulsification, and foaming properties.

b. Physics: High pressure processing

High pressure processing (HPP), stands out as the most employed non-thermal technology in food product treatment within the industry. Its application has proven to be a promising alternative to conventional thermal processing methods in the food industry, while offering substantial advantages in terms of nutrient preservation, sensory quality enhancement, and extension of shelf life of food products (Bello et al., 2014; Cappa et al., 2016; Roobab & Aadil, 2023). The application of this technology relies on two fundamental scientific principles: Le Chatelier's and Pascal's Principle. These principles ensure the uniform treatment of food, irrespective of its size,

shape, or volume, achieved through an isostatic distribution of applied pressure (Nabi et al., 2021; Oh et al., 2008). Therefore, HHP could be applied to liquid foods or to suitably packaged products immersed in a pressurizing liquid. The equipment consists in a pressurization chamber in which the applied pressures vary between 150 and 600 MPa (and time desired). The chamber is closed after filling with a fluid (usually water) and pressure transmission occurs quickly and fairly uniformly throughout the food, (Roobab et al., 2021). The volume of the pressurization chamber can range from less than one liter, for laboratory-scale applications, to approximately 400 liters for industrial food processing applications (applying pressures up to 900 MPa or 600 MPa respectively). The product's allowable volume within the chamber should account for between 50% and 75% of the internal space (Téllez-Luis et al., 2001).

Initially, HPP was primarily used for food preservation purposes. The impact of HPP on microorganisms has extensively documented, elucidating effects at the cellular level that undermine microbial viability. These effects include decreased DNA synthesis, heightened permeability in cell membranes, and denaturation of key proteins and enzymes. While some of these effects are reversible at low pressures (< 200 MPa), they become irreversible at higher pressures (> 300 MPa) (Huang et al., 2014). The efficacy of HHP in microbial inactivation is contingent upon treatment variables such as pressure, time, and temperature, as well as the food composition and the specific type of microorganism involved. Over the last years, it has been seen that HPP can also induce molecular-structural alterations in food components modifying their techno-functional properties (Kim & Lim, 2016; Liu et al., 2022). HPP has been applied to RB fraction to modify their technological properties (**Table 2**). As said in the enzymatic treatment, these changes involve functional attributes such as protein solubility, hydration capacity, improved emulsification, and foaming properties.

Table 2. Studies about rice bran treat with enzymes or HPP and their impact in the technological functions

Enzymatic treatment				
Enzyme	Type	RB fraction	Effect	Reference
Alcalase and Flavourzyme	Proteases	Proteins	Increasing solubility, emulsification activity and stability of protein hydrolysates	Hamada, (2000)
			Increasing soluble dietary fiber. Reduce the water and oil holding capacity, but increased the swelling capacity, cholesterol and sodium taurocholate absorption capacity of RBDF.	
Cellulase and xylanase	Carbohydrases	Fiber		Wen et al., (2017)
Papain	Protease	Proteins	Increasing protein solubility, change the foaming and emulsion properties,	Singh et al., (2021)
HPP				
Pressure (MPa)				
100 - 500		Proteins	Modifications in functional properties and establishing a correlation between surface hydrophobicity and these attributes	Zhu et al., (2017)
100 - 300		Proteins	Improvements in solubility, emulsifying properties, and foaming properties	Wang et al., (2022)

References

- Abdul-Hamid, A., & Luan, Y. S. (2000). Functional properties of dietary fibre prepared from defatted rice bran. *Food Chemistry*, 68(1), 15–19. [https://doi.org/10.1016/S0308-8146\(99\)00145-4](https://doi.org/10.1016/S0308-8146(99)00145-4)
- Adebiyi, A. P., Adebiyi, A. O., Ogawa, T., & Muramoto, K. (2008). Purification and characterisation of antioxidative peptides from unfractionated rice bran protein hydrolysates. *International Journal of Food Science & Technology*, 43(1), 35–43. <https://doi.org/10.1111/j.1365-2621.2006.01379.x>
- Amagliani, L., O'Regan, J., Kelly, A. L., & O'Mahony, J. A. (2017). Composition and protein profile analysis of rice protein ingredients. *Journal of Food Composition and Analysis*, 59, 18–26. <https://doi.org/10.1016/j.jfca.2016.12.026>
- Anderson, J. W., Baird, P., Davis, J., Richard H., Ferreri, S., Knudtson, M., Koraym, A., Waters, V., & Williams, C. L. (2009). Health benefits of dietary fiber. *Nutrition Reviews*, 67(4), 188–205. <https://doi.org/10.1111/j.1753-4887.2009.00189.x>
- Arinze, C. A., Ajala, O. A., Okoye, C. C., Ofodile, O. C., & Daraojimba, A. I. (2024). Evaluating the integration of advanced it solutions for emission reduction in the oil and gas sector. *Engineering Science & Technology Journal*, 5(3), 639–652. <https://doi.org/10.51594/estj.v5i3.862>
- Arts, I., & Hollman, P. (1998). Optimization of a quantitative method for the determination of catechins in fruits and legumes. *Journal of Agricultural and Food Chemistry*, 46(12), 5156–5162. <https://doi.org/10.1021/jf9805092>
- Balaban, M., Koc, C., Sar, T., & Akbas, M. Y. (2021). Antibiofilm effects of pomegranate peel extracts against *B. cereus*, *B. subtilis*, and *E. faecalis*. *International Journal of Food Science and Technology*, 56(10), 4915–4924. <https://doi.org/10.1111/ijfs.15221>
- Barcelos, M. C. S., Ramos, C. L., Kuddus, M., Rodriguez-Couto, S., Srivastava, N., Ramteke, P. W., Mishra, P. K., & Molina, G. (2020). Enzymatic potential for the valorization of agro-industrial by-products. *Biotechnology Letters*, 42(10), 1799–1827. <https://doi.org/10.1007/s10529-020-02957-3>
- Baydar, N. G., Sagdic, O., Ozkan, G., & Cetin, S. (2006). Determination of antibacterial effects and total phenolic contents of grape (*Vitis vinifera* L.) seed extracts. *International Journal of Food Science & Technology*, 41(7), 799–804. <https://doi.org/10.1111/j.1365-2621.2005.01095.x>
- Bello, E. F. T., Martínez, G. G., Ceberio, B. F. K., Rodrigo, D., & López, A. M. (2014). High pressure treatment in foods. *Foods*, 3(3), 476–490.

- <https://doi.org/10.3390/foods3030476>
- Bernat, N., Cháfer, M., Chiralt, A., & González-Martínez, C. (2014). Vegetable milks and their fermented derivative products. *International Journal of Food Studies*, 3, 93–124. <https://doi.org/10.7455/ijfs/3.1.2014.a9>
- Biermann, F., Hickmann, T., Sénit, C.-A., Beisheim, M., Bernstein, S., Chasek, P., Grob, L., Kim, R. E., Kotzé, L. J., Nilsson, M., Ordóñez Llanos, A., Okereke, C., Pradhan, P., Raven, R., Sun, Y., Vijge, M. J., van Vuuren, D., & Wicke, B. (2022). Scientific evidence on the political impact of the Sustainable Development Goals. *Nature Sustainability*, 5(9), 795–800. <https://doi.org/10.1038/s41893-022-00909-5>
- Boisrobert, C. E., Stjepanovic, A., Oh, S., & Lelieveld, H. L. M. (Eds.). (2010). Chapter 4—A simplified guide to understanding and using food safety objectives and performance objectives. In *Ensuring Global Food Safety* (pp. 91–98). Academic Press. <https://doi.org/10.1016/B978-0-12-374845-4.00004-7>
- Boonla, O., Kukongviriyapan, U., Pakdeechote, P., Kukongviriyapan, V., Pannangpetch, P., & Thawornchinsombut, S. (2015). Peptides-derived from thai rice bran improves endothelial function in 2K-1C renovascular hypertensive Rats. *Nutrients*, 7(7), 5783–5799. <https://doi.org/10.3390/nu7075252>
- Borba, V. S., Paiva Rodrigues, M. H., & Badiale-Furlong, E. (2020). Impact of biological contamination of rice on food safety. *Food Reviews International*, 36(8), 745–760. <https://doi.org/10.1080/87559129.2019.1683745>
- Cambridge University Press (n.d.). Sustainability. In Cambridge dictionary. Retrieved May 31, 2024 <https://dictionary.cambridge.org/dictionary/english/sustainability>
- Cappa, C., Lucisano, M., Barbosa-Cánovas, G. V., & Mariotti, M. (2016). Physical and structural changes induced by high pressure on corn starch, rice flour and waxy rice flour. *Food Research International*, 85, 95–103. <https://doi.org/10.1016/j.foodres.2016.04.018>
- Cheng, V. J., Bekhit, A. E. D. A., McConnell, M., Mros, S., & Zhao, J. (2012). Effect of extraction solvent, waste fraction and grape variety on the antimicrobial and antioxidant activities of extracts from wine residue from cool climate. *Food Chemistry*, 134(1), 474–482.
- Codex Alimentarius Commission. (1999). Principles and guidelines for the conduct of microbiological risk assessment. *CAC/GL-30*.
- Conselleria de Agricultura, Desarrollo Rural, Emergencia Climática y Transición Ecológica. (2021). Informe del sector agrario valenciano 2021.

Generalitat Valenciana. <https://agroambient.gva.es/es/informes-del-sector-agrario-valenciano>

- Cornejo, F., & Rosell, C. M. (2015). Physicochemical properties of long rice grain varieties in relation to gluten free bread quality. *LWT - Food Science and Technology*, 62(2), 1203–1210. <https://doi.org/10.1016/j.lwt.2015.01.050>
- Dang, T. T., & Vasanthan, T. (2019). Modification of rice bran dietary fiber concentrates using enzyme and extrusion cooking. *Food Hydrocolloids*, 89, 773–782. <https://doi.org/10.1016/j.foodhyd.2018.11.024>
- Delbrassinne, L., Botteldoorn, N., Andjelkovic, M., Dierick, K., & Denayer, S. (2015). An emetic *Bacillus cereus* outbreak in a kindergarten: detection and quantification of critical levels of cereulide toxin. *Foodborne Pathogens and Disease*, 12(1), 84–87. <https://doi.org/10.1089/fpd.2014.1788>
- Deng, Q., Penner, M. H., & Zhao, Y. (2011). Chemical composition of dietary fiber and polyphenols of five different varieties of wine grape pomace skins. *Food Research International*, 44(9), 2712–2720. <https://doi.org/10.1016/j.foodres.2011.05.026>
- Dwyer, K., Hosseinian, F., & Rod, M. (2014). The market potential of grape waste alternatives. *Journal of Food Research*, 3(2), 91.
- EFSA & ECDC, E. F. S. and E. C. for D. P. and C. (2023). The European Union One Health 2022 zoonoses report. *EFSA Journal*, 21(12), e8442. <https://doi.org/10.2903/j.efsa.2023.8442>
- Esa, N. M., Ling, T. B., & Peng, L. S. (2013). By-products of rice processing: an overview of health benefits and applications. *Journal of Rice Research*, 1(107). <https://doi.org/10.4172/jrr.1000107>
- European Commission. (2019). *The European Green Deal*, COM/2019/640 Final. https://eur-lex.europa.eu/resource.html?uri=cellar:b828d165-1c22-11ea-8c1f-01aa75ed71a1.0004.02/DOC_1&format=PDF
- FAO. (2024, February 7). Paddy rice production worldwide in 2022, by country (in million metric tons). *Statista*. <https://www.statista.com/statistics/255937/leading-rice-producers-worldwide/#statisticContainer>
- Ferreira, A. S., Nunes, C., Castro, A., Ferreira, P., & Coimbra, M. A. (2014). Influence of grape pomace extract incorporation on chitosan films properties. *Carbohydrate Polymers*, 113, 490–499. <https://doi.org/10.1016/j.carbpol.2014.07.032>
- Furiga, A., Lonvaud-Funel, A., & Badet, C. (2009). In vitro study of antioxidant capacity and antibacterial activity on oral anaerobes of a grape seed extract. *Food Chemistry*, 113(4), 1037–1040.

- <https://doi.org/10.1016/j.foodchem.2008.08.059>
- Garba, U., Singanusong, R., Jiamyangyeun, S., & Thongsook, T. (2019). Extraction and utilisation of rice bran oil. A review. *La Rivista Italiana delle Sostanze Grasse*, 96(3), 161–170.
- Ghendov-Mosanu, A., Cojocari, D., Balan, G., Patras, A., Lung, I., Soran, M.-L., Opriş, O., Cristea, E., & Sturza, R. (2022). Chemometric optimization of biologically active compounds extraction from grape marc: composition and antimicrobial activity. *Molecules*, 27(5). <https://doi.org/10.3390/molecules27051610>
- Gilbert, R., Stringer, M., & Peace, T. (1974). Survival and growth of *Bacillus cereus* in boiled and fried rice in relation to outbreaks of food poisoning. *Journal of Hygiene*, 73(3), 433–444. <https://doi.org/10.1017/S0022172400042790>
- Goncalves, L. A., Lorenzo, J. M., & Trindade, M. A. (2021). Fruit and agro-industrial waste extracts as potential antimicrobials in meat products: a brief review. *Foods*, 10(7). <https://doi.org/10.3390/foods10071469>
- Gouvinhas, I., Santos, R. A., Queiroz, M., Leal, C., Saavedra, M. J., Domínguez-Perles, R., Rodrigues, M., & Barros, A. I. R. N. A. (2018). Monitoring the antioxidant and antimicrobial power of grape (*Vitis vinifera* L.) stems phenolics over long-term storage. *Industrial Crops and Products*, 126, 83–91. <https://doi.org/10.1016/j.indcrop.2018.10.006>
- Gujral, H. S., & Kumar, V. (2003). Effect of accelerated aging on the physicochemical and textural properties of brown and milled rice. *Journal of Food Engineering*, 59(2), 117–121. [https://doi.org/10.1016/S0260-8774\(02\)00438-7](https://doi.org/10.1016/S0260-8774(02)00438-7)
- Gul, K., Yousuf, B., Singh, A. K., Singh, P., & Wani, A. A. (2015). Rice bran: Nutritional values and its emerging potential for development of functional food—A review. *Bioactive Carbohydrates and Dietary Fibre*, 6(1), 24–30. <https://doi.org/10.1016/j.bcdf.2015.06.002>
- Hamada, J. S. (2000). Characterization and functional properties of rice bran proteins modified by commercial exoproteases and endoproteases. *Journal of Food Science*, 65(2), 305–310. <https://doi.org/10.1111/j.1365-2621.2000.tb15998.x>
- Han, S.-W., Chee, K.-M., & Cho, S.-J. (2015). Nutritional quality of rice bran protein in comparison to animal and vegetable protein. *Food Chemistry*, 172, 766–769. <https://doi.org/10.1016/j.foodchem.2014.09.127>
- Hendriksen, N. B., Hansen, B. M., & Johansen, J. E. (2006). Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in a sandy loam. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 89(2), 239–249. <https://doi.org/10.1007/s10482->

- Huang, H.-W., Lung, H.-M., Yang, B. B., & Wang, C.-Y. (2014). Responses of microorganisms to high hydrostatic pressure processing. *Food Control*, 40, 250–259. <https://doi.org/10.1016/j.foodcont.2013.12.007>
- Jayaprakasha, G. K., Selvi, T., & Sakariah, K. K. (2003). Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. *Food Research International*, 36(2), 117–122. [https://doi.org/10.1016/S0963-9969\(02\)00116-3](https://doi.org/10.1016/S0963-9969(02)00116-3)
- Jessberger, N., Dietrich, R., Granum, P. E., & Märtlbauer, E. (2020). The *Bacillus cereus* food infection as multifactorial process. *Toxins*, 12(11). <https://doi.org/10.3390/toxins12110701>
- Jia, M., Chen, J., Liu, X., Xie, M., Nie, S., Chen, Y., Xie, J., & Yu, Q. (2019). Structural characteristics and functional properties of soluble dietary fiber from defatted rice bran obtained through *Trichoderma viride* fermentation. *Food Hydrocolloids*, 94, 468–474. <https://doi.org/10.1016/j.foodhyd.2019.03.047>
- Juneja, V. K., Golden, C. E., Abhinav Mishra, Harrison, M. A., Mohr, T., & Silverman, M. (2019). Predictive model for growth of *Bacillus cereus* during cooling of cooked rice. *International Journal of Food Microbiology*, 290, 49–58. <https://doi.org/10.1016/j.ijfoodmicro.2018.09.023>
- Karamati Jabehdar, S., Mirzaei Aghjehgheshlagh, F., Navidshad, B., Mahdavi, A., & Staji, H. (2019). In vitro antimicrobial effect of phenolic extracts and resistant starch on *Escherichia coli*, *Streptococcus* spp., *Bifidobacterium* and *Lactobacillus* spp. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 25(2). <https://doi.org/10.9775/kvfd.2018.20290>
- Katalinić, V., Možina, S. S., Skroza, D., Generalić, I., Abramović, H., Miloš, M., Ljubenković, I., Piskernik, S., Pezo, I., Terpinc, P., & Boban, M. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 *Vitis vinifera* varieties grown in Dalmatia (Croatia). *Food Chemistry*, 119(2), 715–723. <https://doi.org/10.1016/j.foodchem.2009.07.019>
- Kim, H., Kim, H., Bang, J., Kim, Y., Beuchat, L. R., & Ryu, J.-H. (2012). Reduction of *Bacillus cereus* spores in sikhye, a traditional Korean rice beverage, by modified tyndallization processes with and without carbon dioxide injection. *Letters in Applied Microbiology*, 55(3), 218–223. <https://doi.org/10.1111/j.1472-765X.2012.03278.x>
- Kim, S.-M., & Lim, S.-T. (2016). Enhanced antioxidant activity of rice bran extract by carbohydrase treatment. *Journal of Cereal Science*, 68, 116–121. <https://doi.org/10.1016/j.jcs.2016.01.006>

- Lanning, S., & Siebenmorgen, T. (2011). Comparison of milling characteristics of hybrid and pureline rice cultivars. *Applied Engineering in Agriculture*, 27(5), 787–795.
- Liu, N., Lin, P., Zhang, K., Yao, X., Li, D., Yang, L., & Zhao, M. (2022). Combined effects of limited enzymatic hydrolysis and high hydrostatic pressure on the structural and emulsifying properties of rice proteins. *Innovative Food Science & Emerging Technologies*, 77, 102975. <https://doi.org/10.1016/j.ifset.2022.102975>
- Luchian, C.E., Cotea, V.V., Vlase, L., Toiu, A.M., Colibaba, L.C., Răschip, I.E., Nadăș, G., Gheldiu, A.M., Tuchiluş, C., & Rotaru, L. (2019). Antioxidant and antimicrobial effects of grape pomace extracts. *BIO Web Conf.*, 15, 04006. <https://doi.org/10.1051/bioconf/20191504006>
- Michielin, E. M. Z., Salvador, A. A., Riehl, C. A. S., Smânia, A., Smânia, E. F. A., & Ferreira, S. R. S. (2009). Chemical composition and antibacterial activity of *Cordia verbenacea* extracts obtained by different methods. *Bioresource Technology*, 100(24), 6615–6623. <https://doi.org/10.1016/j.biortech.2009.07.061>
- Mishra, S. S., Ray, R. C., Rosell, C. M., & Panda, B. (2017). Microbial Enzymes in Food Applications. *Microbial Enzyme Technology in Food Applications*, 3–18.
- Mohan, V., Ruchi, V., Gayathri, R., Ramya Bai, M., Shobana, S., Anjana, R. M., Unnikrishnan, R., & Sudha, V. (2017). Hurdles in Brown Rice Consumption. In A. Manickavasagan, C. Santhakumar, & N. Venkatachalapathy (Eds.), *Brown Rice* (pp. 255–269). Springer International Publishing. https://doi.org/10.1007/978-3-319-59011-0_15
- Mohidem, N. A., Hashim, N., Shamsudin, R., & Che Man, H. (2022). Rice for food security: revisiting its production, diversity, rice milling process and nutrient content. *Agriculture*, 12(6). <https://doi.org/10.3390/agriculture12060741>
- Mota, C., Santos, M., Mauro, R., Samman, N., Matos, A. S., Torres, D., & Castanheira, I. (2016). Protein content and amino acids profile of pseudocereals. *Food Chemistry*, 193, 55–61. <https://doi.org/10.1016/j.foodchem.2014.11.043>
- Mudgil, D., & Barak, S. (2013). Composition, properties and health benefits of indigestible carbohydrate polymers as dietary fiber: A review. *International Journal of Biological Macromolecules*, 61, 1–6. <https://doi.org/10.1016/j.ijbiomac.2013.06.044>
- Munekata, P. E. S., Domínguez, R., Budaraju, S., Roselló-Soto, E., Barba, F. J., Mallikarjunan, K., Roohinejad, S., & Lorenzo, J. M. (2020). Effect of innovative food processing technologies on the physicochemical and nutritional properties and quality of non-dairy plant-based beverages.

- Foods*, 9(3). <https://doi.org/10.3390/foods9030288>
- Muthayya, S., Sugimoto, J. D., Montgomery, S., & Maberly, G. F. (2014). An overview of global rice production, supply, trade, and consumption. *Annals of the New York Academy of Sciences*, 1324(1), 7–14. <https://doi.org/10.1111/nyas.12540>
- Nabi, B. G., Mukhtar, K., Arshad, R. N., Radicetti, E., Tedeschi, P., Shahbaz, M. U., Walayat, N., Nawaz, A., Inam-Ur-Raheem, M., & Aadil, R. M. (2021). High-pressure processing for sustainable food supply. *Sustainability*, 13(24). <https://doi.org/10.3390/su132413908>
- Nakayama, T., Nagai, Y., Uehara, Y., Nakamura, Y., Ishii, S., Kato, H., & Tanaka, Y. (2017). Eating glutinous brown rice twice a day for 8 weeks improves glycemic control in Japanese patients with diabetes mellitus. *Nutrition & Diabetes*, 7(5), e273. <https://doi.org/10.1038/nutd.2017.26>
- Oh, H. E., Hemar, Y., Anema, S. G., Wong, M., & Pinder, D. N. (2008). Effect of high-pressure treatment on normal rice and waxy rice starch-in-water suspensions. *Carbohydrate Polymers*, 73(2), 332–343. <https://doi.org/10.1016/j.carbpol.2007.11.038>
- Oki, T., Masuda, M., Kobayashi, M., Nishiba, Y., Furuta, S., Suda, I., & Sato, T. (2002). Polymeric procyanidins as radical-scavenging components in red-hulled rice. *Journal of Agricultural and Food Chemistry*, 50(26), 7524–7529. <https://doi.org/10.1021/jf025841z>
- Oliveira, D. A., Salvador, A. A., Smania, A., Smania, E. F. A., Maraschin, M., & Ferreira, S. R. S. (2013). Antimicrobial activity and composition profile of grape (*Vitis vinifera*) pomace extracts obtained by supercritical fluids. *Journal of Biotechnology*, 164(3), 423–432.
- Oliveira, A. L. de, Brunini, M. A., Salandini, C. A. R., & Bazzo, F. R. (2003). Caracterização tecnológica de jabuticabas ‘Sabará’ provenientes de diferentes regiões de cultivo. *Revista Brasileira de Fruticultura*, 25(3), 397–400.
- Pao, S., Khalid, F., & Kalantari, A. (2006). Inhibiting the growth of *Bacillus cereus* in raw sprouts and cooked rice using red clover seeds. *Internet Journal of Food Safety*, 8.
- Park, H.-Y., Lee, K.-W., & Choi, H.-D. (2017). Rice bran constituents: immunomodulatory and therapeutic activities. *Food Function*, 8(3), 935–994. <https://doi.org/10.1039/c6fo01763k>
- Patindol, J., Wang, L., & Wang, Y.-J. (2007). Cellulase-Assisted Extraction of oligosaccharides from defatted rice bran. *Journal of Food Science*, 72(9), C516–C521. <https://doi.org/10.1111/j.1750-3841.2007.00551.x>
- Penha, C. B., Santos, V. D. P., Speranza, P., & Kurozawa, L. E. (2021). Plant-based beverages: Ecofriendly technologies in the production process.

- Innovative Food Science & Emerging Technologies*, 72, 102760. <https://doi.org/10.1016/j.ifset.2021.102760>
- Phongthai, S., D'Amico, S., Schoenlechner, R., & Rawdkuen, S. (2016). Comparative study of rice bran protein concentrate and egg albumin on gluten-free bread properties. *Journal of Cereal Science*, 72, 38–45. <https://doi.org/10.1016/j.jcs.2016.09.015>
- Pinelo, M., Ruiz-Rodríguez, A., Sineiro, J., Señoráns, F. J., Reglero, G., & Núñez, M. J. (2007). Supercritical fluid and solid–liquid extraction of phenolic antioxidants from grape pomace: A comparative study. *European Food Research and Technology*, 226(1), 199–205. <https://doi.org/10.1007/s00217-006-0526-3>
- Qi, J., Li, Y., Yokoyama, W., Majeed, H., Masamba, K. G., Zhong, F., & Ma, J. (2015). Cellulosic fraction of rice bran fibre alters the conformation and inhibits the activity of porcine pancreatic lipase. *Journal of Functional Foods*, 19, 39–48. <https://doi.org/10.1016/j.jff.2015.09.012>
- Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A., Rebello, S., & Pandey, A. (2018). Applications of microbial enzymes in food industry. *Food Technology and Biotechnology*, 56(1), 16–30. <https://doi.org/10.17113/ftb.56.01.18.5491>
- Robinson, P. K. (2015). Enzymes: Principles and biotechnological applications. *Essays in Biochemistry*, 59, 1–41. <https://doi.org/10.1042/bse0590001>
- Rodrigo, D., Rosell, C. M., & Martinez, A. (2021). Risk of *Bacillus cereus* in relation to rice and derivatives. *Foods*, 10(2). <https://doi.org/10.3390/foods10020302>
- Roobab, U., Shabbir, M. A., Khan, A. W., Arshad, R. N., Bekhit, A. E.-D., Zeng, X.-A., Inam-Ur-Raheem, M., & Aadil, R. M. (2021). High-pressure treatments for better quality clean-label juices and beverages: overview and advances. *LWT - Food Science and Technology*, 149, 111828. <https://doi.org/10.1016/j.lwt.2021.111828>
- Ross, T., Dalgaard, P., & Tienungoon, S. (2000). Predictive modelling of the growth and survival of *Listeria* in fishery products. *International Journal of Food Microbiology*, 62(3), 231–245. [https://doi.org/10.1016/S0168-1605\(00\)00340-8](https://doi.org/10.1016/S0168-1605(00)00340-8)
- Ross, T., & McMeekin, T. A. (1994). Predictive microbiology. *International Journal of Food Microbiology*, 23(3), 241–264. [https://doi.org/10.1016/0168-1605\(94\)90155-4](https://doi.org/10.1016/0168-1605(94)90155-4)
- Saleh, A. S. M., Wang, P., Wang, N., Yang, L., & Xiao, Z. (2019). Brown rice versus white rice: nutritional quality, potential health benefits, development of food products, and preservation technologies.

-
- Comprehensive Reviews in Food Science and Food Safety*, 18(4), 1070–1096. <https://doi.org/10.1111/1541-4337.12449>
- Sánchez, J., Correa, M., & Castañeda, L. (2016). *Bacillus cereus* an important pathogen the microbiological control of food. *Revista Facultad Nacional de Salud Pública*, 34(2), 230–242. <https://doi.org/10.17533/udea.rfnsp.v34n2a12>
- Sandberg, H., Alnoor, A., & Tiberius, V. (2023). Environmental, social, and governance ratings and financial performance: Evidence from the European food industry. *Business Strategy and the Environment*, 32(4), 2471–2489. <https://doi.org/10.1002/bse.3259>
- Sarrias, J. A., Valero, M., & Salmerón, M. C. (2002). Enumeration, isolation and characterization of *Bacillus cereus* strains from Spanish raw rice. *Food Microbiology*, 19(6), 589–595. <https://doi.org/10.1006/fmic.2002.0514>
- Schieber, A. (2017). Side streams of plant food processing as a source of valuable compounds: selected examples. In *Annual Review of Food Science and Technology* (Vol. 8, Issue Volume 8, 2017, pp. 97–112). Annual Reviews. <https://doi.org/10.1146/annurev-food-030216-030135>
- Schonrock, F. T. (2023). Chapter 48—The role of international, regional, and national organizations in the development of standards. In V. Andersen, H. Lelieveld, & Y. Motarjemi (Eds.), *Food Safety Management (Second Edition)* (Second Edition, pp. 1005–1017). Academic Press. <https://doi.org/10.1016/B978-0-12-820013-1.00050-4>
- Serra, A. T., Matias, A. A., Nunes, A. V. M., Leitão, M. C., Brito, D., Bronze, R., Silva, S., Pires, A., Crespo, M. T., Romão, M. V. S., & Duarte, C. M. (2008). In vitro evaluation of olive- and grape-based natural extracts as potential preservatives for food. *Innovative Food Science & Emerging Technologies*, 9(3), 311–319. <https://doi.org/10.1016/j.ifset.2007.07.011>
- Sethi, S., Tyagi, S., & Anurag, R. (2016). Plant-based milk alternatives an emerging segment of functional beverages: A review. *Journal of Food Science and Technology-Mysore*, 53(9), 3408–3423. <https://doi.org/10.1007/s13197-016-2328-3>
- Sharif, M. K., Butt, M. S., Anjum, F. M., & Khan, S. H. (2014). Rice bran: a novel functional ingredient. *Critical Reviews in Food Science and Nutrition*, 54(6), 807–816. <https://doi.org/10.1080/10408398.2011.608586>
- Shori, A. B., & Al Zahrani, A. J. (2022). Non-dairy plant-based milk products as alternatives to conventional dairy products for delivering probiotics. *Food Science and Technology*, 42, e101321. <https://doi.org/10.1590/fst.101321>
- Silván, J. M., Mingo, E., Hidalgo, M., Pascual-Teresa, S. de, Carrascosa, A.

- V., & Martinez-Rodriguez, A. J. (2013). Antibacterial activity of a grape seed extract and its fractions against *Campylobacter* spp. *Food Control*, 29(1), 25–31. <https://doi.org/10.1016/j.foodcont.2012.05.063>
- Singh, T. P., Siddiqi, R. A., & Sogi, D. S. (2021). Enzymatic modification of rice bran protein: Impact on structural, antioxidant and functional properties. *LWT*, 138, 110648. <https://doi.org/10.1016/j.lwt.2020.110648>
- Soni, A., Oey, I., Silcock, P., & Bremer, P. J. (2018). Impact of temperature, nutrients, pH and cold storage on the germination, growth and resistance of *Bacillus cereus* spores in egg white. *Food Research International*, 106, 394–403. <https://doi.org/10.1016/j.foodres.2018.01.006>
- Spaggiari, M., Dall'Asta, C., Galaverna, G., & del Castillo Bilbao, M. D. (2021). Rice bran by-product: from valorization strategies to nutritional perspectives. *Foods*, 10(1). <https://doi.org/10.3390/foods10010085>
- Téllez-Luis, S. J., Ramírez, A. A., Pérez-Lamela, C., Vázquez, M., & Simal-Gándara, J. (2001). Application of high hydrostatic pressure in the food preservation. *Ciencia y Tecnología Alimentaria*, 3(2), 66–80.
- Ume Roobab, X. Z., Muhammad Inam-Ur-Raheem, Abdul Waheed Khan, Rai Naveed Arshad, & Aadil, R. M. (2023). Innovations in high-pressure technologies for the development of clean label dairy products: A Review. *Food Reviews International*, 39(2), 970–991. <https://doi.org/10.1080/87559129.2021.1928690>
- Uraipong, C., & Zhao, J. (2016). Rice bran protein hydrolysates exhibit strong in vitro α -amylase, β -glucosidase and ACE-inhibition activities. *Journal of the Science of Food and Agriculture*, 96(4), 1101–1110. <https://doi.org/10.1002/jsfa.7182>
- US Department of Agriculture. (2024, January 12). Total rice consumption worldwide from 2008/2009 to 2023/2024 (in 1,000 metric tons)* Graph. *Statista*. <https://www.statista.com/statistics/255977/total-global-rice-consumption/>
- Vaikma, H., Kaleda, A., Rosend, J., & Rosenvald, S. (2021). Market mapping of plant-based milk alternatives by using sensory (RATA) and GC analysis. *Future Foods*, 4, 100049. <https://doi.org/10.1016/j.fufo.2021.100049>
- Vilain, S., Luo, Y., Hildreth, M. B., & Brözel, V. S. (2006). Analysis of the life cycle of the coil caprophyte *Bacillus cereus* in liquid soil extract and in soil. *Applied and Environmental Microbiology*, 72(7), 4970–4977. <https://doi.org/10.1128/AEM.03076-05>
- Wang, J., Shimada, M., Kato, Y., Kusada, M., & Nagaoka, S. (2015). Cholesterol-lowering effect of rice bran protein containing bile acid-binding proteins. *Bioscience, Biotechnology, and Biochemistry*, 79(3),

- 456–461. <https://doi.org/10.1080/09168451.2014.978260>
- Wang, K. M., Wu, J. G., Li, G., Zhang, D. P., Yang, Z. W., & Shi, C. H. (2011). Distribution of phytic acid and mineral elements in three indica rice (*Oryza sativa* L.) cultivars. *Journal of Cereal Science*, 54(1), 116–121. <https://doi.org/10.1016/j.jcs.2011.03.002>
- Wang, S., Wang, T., Sun, Y., Cui, Y., Yu, G., & Jiang, L. (2022). Effects of high hydrostatic pressure pretreatment on the functional and structural properties of rice bran protein hydrolysates. *Foods*, 11(1). <https://doi.org/10.3390/foods11010029>
- Wasaya, A., Yasir, T. A., Sarwar, N., Atique-ur-Rehman, Mubeen, K., Rajendran, K., Hadifa, A., & Sabagh, A. E. L. (2022). Climate change and global rice security. In N. Sarwar, Atique-ur-Rehman, S. Ahmad, & M. Hasanuzzaman (Eds.), *Modern Techniques of Rice Crop Production* (pp. 13–26). Springer Singapore. https://doi.org/10.1007/978-981-16-4955-4_2
- Wei, X., & Huang, X. (2019). 1—Origin, taxonomy, and phylogenetics of rice. In J. Bao (Ed.), *Rice (Fourth Edition)* (pp. 1–29). AACC International Press. <https://doi.org/10.1016/B978-0-12-811508-4.00001-0>
- Wen, Y., Niu, M., Zhang, B., Zhao, S., & Xiong, S. (2017). Structural characteristics and functional properties of rice bran dietary fiber modified by enzymatic and enzyme-micronization treatments. *LWT - Food Science and Technology*, 75, 344–351. <https://doi.org/10.1016/j.lwt.2016.09.012>
- Whiting, R. C., & Buchanan, R. L. (1997). Development of a quantitative risk assessment model for *Salmonella enteritidis* in pasteurized liquid eggs. *International Journal of Food Microbiology*, 36(2), 111–125. [https://doi.org/10.1016/S0168-1605\(97\)01262-2](https://doi.org/10.1016/S0168-1605(97)01262-2)
- WHO/FAO/UNU. (2007). Protein and amino acid requirements in human nutrition. In *World Health Organization technical report series* (Vol. 935).
- Yadav, D., Kumar, A., Kumar, P., & Mishra, D. (2015). Antimicrobial properties of black grape (*Vitis vinifera* L.) peel extracts against antibiotic-resistant pathogenic bacteria and toxin producing molds. *Indian Journal of Pharmacology*, 47(6), 663–667. <https://doi.org/10.4103/0253-7613.169591>
- Zaharia, A., Diaconeasa, M.-C., Maehle, N., Szolnoki, G., & Capitello, R. (2021). Developing sustainable food systems in Europe: national policies and stakeholder perspectives in a four-country analysis. *International Journal of Environmental Research and Public Health*, 18(14). <https://doi.org/10.3390/ijerph18147701>
- Zhang, G., Malik, V. S., Pan, A., Kumar, S., Holmes, M. D., Spiegelman, D., Lin, X., & Hu, F. B. (2010). Substituting brown rice for white rice to lower

- diabetes risk: a focus-group study in chinese adults. *Journal of the American Dietetic Association*, 110(8), 1216–1221. <https://doi.org/10.1016/j.jada.2010.05.004>
- Zheng, Y., Gao, N., Wu, J., & Yin, B. (2019). Chapter 11—Rice bran protein: extraction, nutraceutical properties, and potential applications. In L.-Z. Cheong & X. Xu (Eds.), *Rice Bran and Rice Bran Oil* (pp. 271–293). AOCS Press. <https://doi.org/10.1016/B978-0-12-812828-2.00011-1>
- Zhu, K., Huang, S., Peng, W., Qian, H., & Zhou, H. (2010). Effect of ultrafine grinding on hydration and antioxidant properties of wheat bran dietary fiber. *Food Research International*, 43(4), 943–948. <https://doi.org/10.1016/j.foodres.2010.01.005>
- Zhu, S. M., Lin, S. L., Ramaswamy, H. S., Yu, Y., & Zhang, Q. T. (2017). Enhancement of functional properties of rice bran proteins by high pressure treatment and their correlation with surface hydrophobicity. *Food and Bioprocess Technology*, 10(2), 317–327. <https://doi.org/10.1007/s11947-016-1818-7>



Objectives

The **general objective** of this thesis is to enhance the value of rice and its by-products in the food industry through the development of innovative strategies, focusing on two key areas:

- I. Increasing the food safety of cooked rice by employing natural antimicrobials and advanced risk modeling techniques.
- II. Enhancing the technological and functional properties of rice bran using a range of sustainable treatments.

To achieve this, the following **specific objectives** were established:

I 1. To assess the effectiveness of grape extract as a natural antimicrobial against *Bacillus cereus* in a rice matrix, under different heat treatments, storage temperatures and pHs.

2. To develop an exposure assessment model that estimates the concentration of *B. cereus* after thermal cooking and storage time.

II 3. To identify trends and gaps in the composition and nutritional value of the current plant-based beverages market, which could help in the design of these types of beverages based on scientific knowledge.

4. To evaluate the effect of enzymatic treatments on enhancing rice bran techno-functional properties

5. To analyze the impact of hydrostatic pressure on enhancing the safety, physical-chemical and techno-functional properties of rice bran.

Working plan

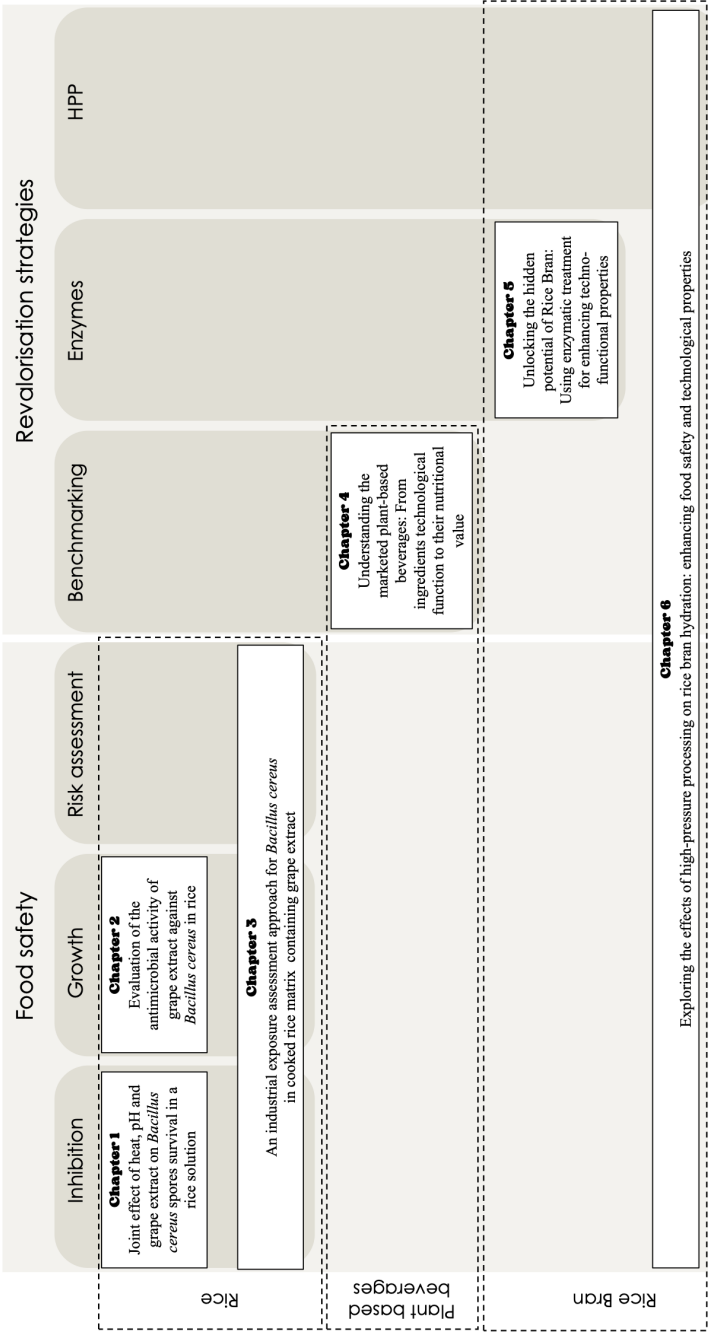


Figure 1. Overview of the different chapters of the results section

This doctoral thesis has been organized into six different chapters:

Chapter 1: Heat treatment at different temperatures of a rice matrix at various pH levels was conducted to assess the antimicrobial efficacy of 0.1% grape extract against *B. cereus*. The results were subsequently fitted to the Weibull model (**Figure 2**).

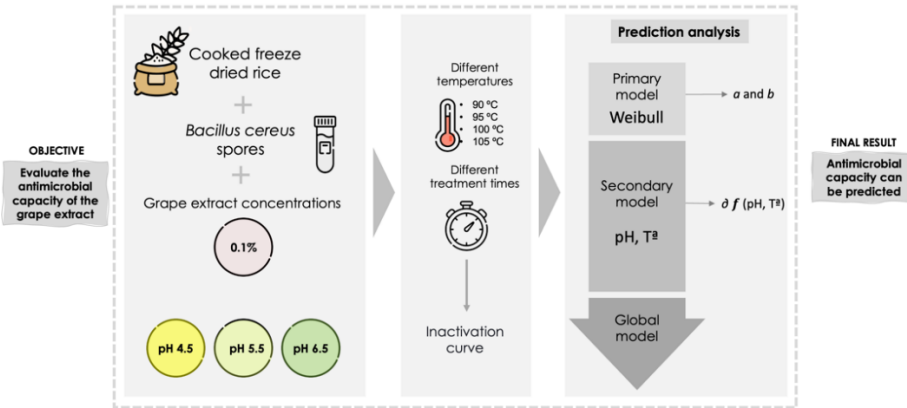


Figure 2. Graphical abstract of chapter one

Chapter 2: Storage process at different temperatures of a rice matrix at various pH levels was conducted to assess the antimicrobial efficacy of different concentrations of grape extract against *B. cereus*. The results were subsequently fitted to the Weibull model (**Figure 3**).

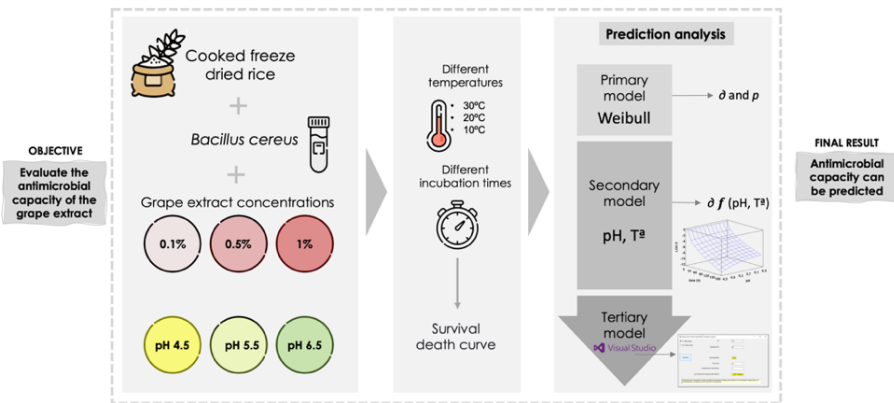


Figure 3. Graphical abstract of chapter two

Chapter 3: Heat treatment and storage process results were used to develop an industrial exposure assessment that estimates the level of *B. cereus* in cooked rice using 0.1% of grape extract as a natural antimicrobial (**Figure 4**).

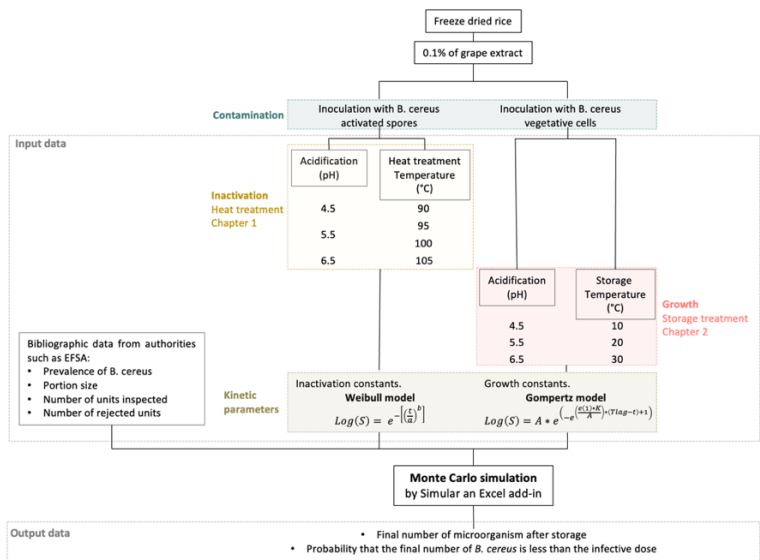


Figure 4. Graphical abstract of chapter three

Chapter 4: Extensive benchmarking analysis of the European and North American plant-based beverage markets was performed by studying the commercial labels of 306 beverages to compare their compositions and nutritional information (**Figure 5**).

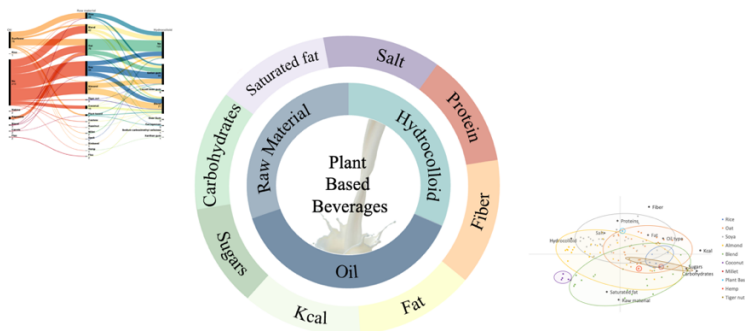


Figure 5. Graphical abstract of chapter 4

Chapter 5: Enzymatic, with six enzymes, and thermal treatment were applied to defatted rice bran to enhance its technological and functional properties (**Figure 6**).

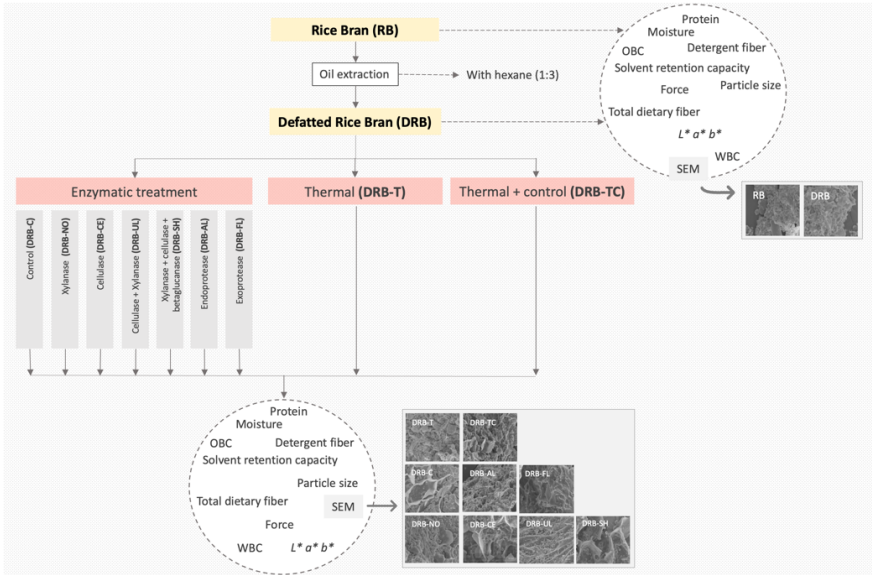


Figure 6. Graphical abstract of chapter 5

Chapter 6: Four different hydration levels and high-pressure treatment of 500 MPa during 15 minutes were applied on rice bran to improve its food safety and to modify its physico-chemical and techno-functional properties (**Figure 7**).

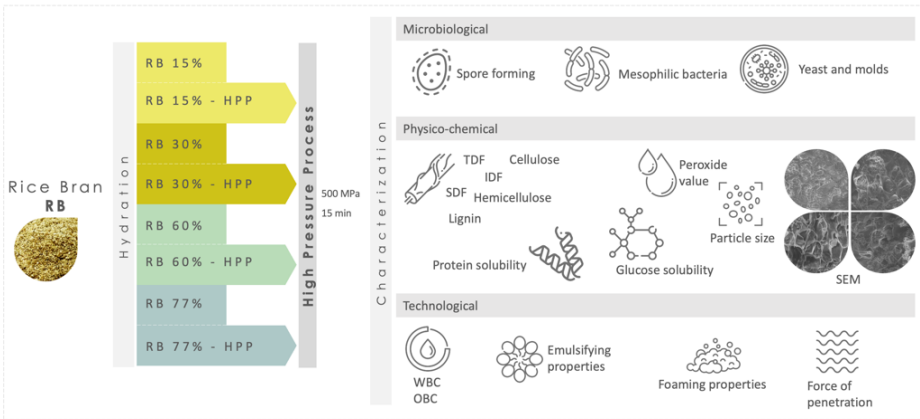


Figure 7. Graphical abstract of chapter 6



Block I

Enhancing strategies for ensuring food safety

Joint effect of heat, pH and grape extract on *Bacillus cereus* spores survival in a rice solution

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Natalia Morató, Raquel Garzón-Lloría and Dolores Rodrigo

*contributed equally

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10820132231216770.

***Bacillus cereus* spores**

Grape extract

Rice

Hurdle technology

Antimicrobial effect

Thermal treatment

Rice due to its high carbohydrate content, is an ideal medium for *Bacillus cereus* growth, a spore-producing microorganism. The objective of this study was to determine the antimicrobial activity of a grape extract in combination with heat treatments and different pH against *B. cereus* spores in a rice solution. The survivor data obtained were fitted to the Weibull survival function, and the values of parameters a and b (scale and shape indexes, respectively) were determined. Results showed that the grape extract affected the survival of *B. cereus* spores at 90°C and 95°C, reaching greater logarithmic reductions in acidic pH values. This behaviour was reflected in a parameter of the Weibull survival function which decreased as the temperature increased and at acidic pH values. In addition, a secondary model was developed by relating the logarithm of a to the independent variables (temperature and pH). A global model relating *B. cereus* inactivation with temperature and pH was developed, and validated by calculating the accuracy factor. The results demonstrate the usefulness of grape extract as a by-product, which can be used as an additional control measure for rice, especially when combined with mild heat treatments and acidic pH values.

1.1 Introduction

Bacillus cereus is a Gram-positive, ubiquitous, facultative anaerobic and spore-forming bacterium. It can grow in foods with a water activity > 0.93 (Batt, 2014) and a wide pH range (4.9–9.3) (Jessberger et al., 2020). Its spores can resist even stomach pH (1–5.2) and are resistant to adverse conditions such as high temperatures, dehydration, and radiation; however, vegetative forms of *B. cereus* can be easily eliminated when foods are subjected to conventional heat treatments (cooking) (Coroller et al., 2001; Sarriás et al., 2002; Soni et al., 2016). *B. cereus* can produce two types of toxins (diarrheal and emetic). Diarrheal disease is usually associated with protein-rich foods, such as meat, vegetables, puddings and dairy products and it is caused by vegetative cells producing enterotoxins in the small intestine. *B. cereus* spores can resist traditional cooking treatments allowing them to grow and produce the emetic toxin in the food before consumption. This toxin is heat-stable (up to 121 °C) and commonly associated with starchy foods such as fried and cooked rice, pasta and noodles (Abee et al., 2011; Tirloni et al., 2022). The growth temperature range is wide, ranging from 4 °C to 48 °C (Drobniewski, 1993; Miliotis & Bier, 2003). If the food is stored at inadequate temperatures (> 5 °C) for several hours, concentrations equal to or greater than the infective dose of *B. cereus* (10^5 UFC/g) can be achieved. In these cases, the microorganism can infect the intestine causing food poisoning in the consumer (diarrhea) (Hazards (Biohaz), 2016). Also, it can be seen that modifying the pH of food could help to reduce the heat resistance of *B. cereus* spores (Authority (EFSA), 2005; Soni et al., 2016). *B. cereus* is responsible for many foodborne health issues each year. EFSA has reported that, in 2021, 679 cases of *B. cereus* food poisoning were identified within the European Union, ranking *B. cereus* as the pathogen with the highest number of food outbreaks by toxin-producing bacteria mainly in foods such as rice, seeds, nuts and almonds (Authority & European Centre for Disease Prevention and Control, 2022).

Due to its high starch content, rice may be an ideal substrate for *B. cereus* growth. Rice, *Oryza sativa*, is one of the most important cereals worldwide, together with corn and wheat. It is widely consumed and represents the basis of the diet of almost half the world's population (Bhattacharya, 2011; Wei & Huang, 2019). It has been shown that the main habitat of emetic strains is

related to rice roots, tubers and mycorrhizae, which could explain its high prevalence in this food (Rodrigo et al., 2021; Sarriás et al., 2002). Rice is a frequent ingredient (in varying percentages) in complex, multi-ingredient, ready-to-eat foods (i.e. pure rice and lentils). In most cases, these products involve long production processes and multi-actor supply chains, where the probability of microbial contamination is very high. It is important to consider their properties and the type of processing, as the type of food consumed has a considerable effect on the survival of bacteria/spores in the stomach transit. In addition to the starch content, the pH and the process temperatures can have an important role in the evolution of the remaining microorganisms during the storage period (Jessberger et al., 2020).

Globalization of the food chain, together with the pressure to reduce waste and improve sustainability, is driving the reuse and revaluation of waste generated during food production processes. Grapes are one of the oldest and most widely cultivated fruit crops in the world. The wine industry generates large quantities of waste as a consequence of the pressing and fermentation processes, about 25% of the weight of grapes becomes waste which is difficult to manage (Dwyer et al., 2014). Grape pomace consists of ~ 10%–30% of the crushed grape mass and other value-added products, they contain high amounts of phenolic compounds such as flavonoids, catechins and anthocyanins, which have beneficial properties for human health (Garavaglia et al., 2016; Grases et al., 2015; Poudel et al., 2008) and antimicrobial properties, which could be useful in the food industry (Baydar et al., 2004; Katalinić et al., 2010; Serra et al., 2008). In addition, several studies (Hassan et al., 2019; Jayaprakasha et al., 2003; Katalinić et al., 2010; Oliveira et al., 2013) have focused on characterizing the antimicrobial effect of grape residues as an alternative to the use of artificial preservatives or as an additional control measure in food preservation processes, showing that polyphenolic compounds with a very high negative charge have the strongest antimicrobial effect. A recent study by Grau-Fuentes et al., (2023) demonstrated the antimicrobial effect of grape extract on the growth of *B. cereus* vegetative cells during storage.

In this context, the main objective of this study was to evaluate the effect of a grape extract on *B. cereus* spores survival in a rice substrate at different pH levels (4.5 – 5.5 – 6.5), during a heat treatment at different temperatures,

including those used in the cooking process of rice and dishes containing rice in the recipe (90, 95, 100, and 105 °C) and to develop an experimental model that relates *B. cereus* inactivation with exposure time, pH and temperature.

1.2. Material and methods

1.2.1. Bacterial strain and sporulation

A pure freeze-dried culture of *B. cereus* provided by the Spanish Type Culture Collection (CECT 148), which is equivalent to ATCC 13061, was used for the experiment. The culture was rehydrated with Nutrient Broth (NB) liquid medium (Scharlab Chemie S.A., Barcelona, Spain). The suspension was incubated overnight at 32 °C in a thermostatic bath with continuous stirring to obtain vegetative cells of *B. cereus* in the stationary growth phase.

For the sporulation, 20 roux bottles were prepared with modified Fortified Nutrient Agar (FNA) (Fernández et al., 1999). Roux bottles were surface inoculated with 0.5 mL of vegetative cells of *B. cereus*. The bottles were incubated at 32 °C for 24 h in an upward position, after which they were turned over. After the third day, sporulation was monitored by phase contrast microscopy, until 90 % of sporulation was reached in about 5 – 7 days.

Then, spores were collected with a bent glass rod in sterile distilled water. Spores were washed with sterile distilled water four times and concentrated by centrifugation at 2500 g at 4 °C and 15 min in a Beckman centrifuge (rotor JLA-16.250). They were stored in sterile distilled water at 4 °C until use. Before use, *B. cereus* spores were shaken for 10 min, in the presence of glass beads to prevent aggregation. Following protocols on heat resistance studies (Condon & Sala, 1992; Stumbo, 1973; Zuijlen et al., 2010), the spores were activated in a water bath at 80°C for 10 min before proceeding to heat resistance studies to avoid over-estimation of survivors and the presence of curves with non-activation shoulders.

1.2.2. Grape solution

The grape extract EV-3 was provided by the Sociedad Española de Colorantes Naturales y Afines SA-SECNA (Chiva, Valencia, Spain). This extract is a natural red-coloured liquid extracted from red grapes with a pH of 3.30 ± 0.05 . The grape extract was frozen in Eppendorf tubes at –80 °C until use and directly inoculated in the rice solution for heat resistance studies.

1.2.3. Sample preparation: rice solution and *B. cereus* spore inoculation

White rice obtained from a local retailer was cooked, freeze-dried (moisture content 8.66%) and ground. The rice solution was prepared with the rice powder (2% w/v) in distilled water. The solutions were autoclaved before pH adjustment, the addition of grape extract and inoculation of spores.

For the study, two experimental sets were considered: the control sample without grape extract and the grape sample. In both cases, they were adjusted to pH 4.5, 5.5 and 6.5. The pH of the control sample was adjusted with citric acid 1% (v/v) whereas the pH of the sample with grape extract was adjusted with NaOH 1% (v/v). After adjusting the pH of the samples, activated *B. cereus* spores were inoculated into the rice solutions at a concentration of 10^8 CFU/mL and, in the case of grape samples, 0.1% of grape extract was also added.

1.2.4. Heat treatment

For efficient and homogeneous heat treatment, an adaptation of the capillary tube method was used (Fernández et al., 1999). Specifically, Vitrex capillaries (reference 217913) measuring $1.50 \times 2.00 \times 100$ mm sealed at one end were used. The capillaries were filled with the samples using a sterile chamber, fitted with a vacuum pump; once the capillaries were filled, they were sealed at the other end with silicone (Quiadsa, Madrid, Spain). The capillaries were placed on racks and heated in a silicone oil bath with shaking. Ten capillaries were prepared as replicates for each treatment time (0 – 50 min depending on the temperature) and temperature (90 – 105 °C). After each heating period, the capillaries were immediately cooled in an ice water bath and immersed in ethanol 70% to avoid external contamination. For sample plating, the capillaries were broken and serial dilutions were made. Samples were plated on starch-enriched nutrient agar and viable counts were based on duplicate counts of the dilutions. Plates were incubated at 30 °C for 24 h.

1.2.5. Mathematical model

Primary model. The mean value of survival data (N , CFU/ mL) was fitted by non-linear regression to the survival function of the Weibull distribution (**equation (1)**). Fernández et al., (1999) and Peleg & Cole, (1998) demonstrate the usefulness of this function in fitting survival data of *B. cereus* spores and *Clostridium botulinum* spores.

$$S_t = e^{-(t/a)^b} \quad (1)$$

where S_t is the survival function, t is the treatment time, a is the scale parameter and b is the shape parameter.

The parameter a is the scale, considered as a non-biological kinetic parameter representing the change of the microbial load along the treatment time at an isothermal temperature of heating, and b is the shape parameter. The b parameter describes the shape of the survival curve so, when $b < 1$ the survival curve is concave, when $b > 1$ the survival curve is convex, and when $b = 1$ the survival curve is a straight line on a log scale (equal to the Bigelow model). To assess the goodness of fit R^2_{adj} and MSE parameters were calculated (Sampedro et al., 2006).

Secondary model. A secondary model was developed by relating the logarithm of primary parameter a to the independent variables, temperature and pH (**equation (2)**). For this, a forward stepwise multiple regression was carried out to detect relevant terms in the polynomial.

$$\text{Log } a = a + \beta * pH + \gamma * T + \delta * (pH * T) + \varepsilon * pH^2 + \zeta * T^2 \quad (2)$$

where a is the scale parameter, pH is the pH of the sample and T is the treatment temperature.

Global model. Considering the more relevant terms of the polynomial, a global model that related $\text{Log } S$ with pH and T was derived based on the Weibull equation (**equation (3)**) (Fernández et al., 2002).

$$\text{Log } S = - \left(\left(\frac{t}{10^{(\alpha + \beta * pH + \gamma * T^2)}} \right)^b \right) \quad (3)$$

where S is the survival fraction, t is the treatment time, pH is the pH of the sample, T is the treatment temperature and b is the shape parameter.

Accuracy factor. A new set of experimental data was used to validate the global model by calculating the accuracy factor (AF) according to Ross (1996).

Results

This factor provides a measure of the average precision of the estimates and is given by **equation (4)**.

$$AF = 10^{\left(\frac{\sum \log(\text{predicted}/\text{observed})}{n}\right)} \quad (4)$$

where n is the number of observations used in the calculation.

The accuracy factor should always be greater than or equal to one, and it is one if there is a perfect agreement between all predictions and observed values.

1.2.6. Statistical analysis

Experiments were performed in triplicates with two replicas per count. The data set was divided into two groups, and one was used for validation purposes. The experimental results were shown as Log_{10} of the survival fraction ($\text{Log } S$) calculated by **(equation (5))**:

$$\text{Log } (S) = \frac{N}{N_0} \quad (5)$$

where N is the bacterial concentration (CFU/mL) at time t (min) and N_0 is the initial bacterial concentration (CFU/ mL) (t_0). Statistical analysis was carried out with Statgraphics Centurion 18 version 18.1.13 by Statgraphics Technologies, Inc. Identified outliers were previously eliminated. S the statistical significance of the data was determined with an analysis of variance (ANOVA) (p -value < 0.05) and intergroup differences were determined using (LSD) of the Fisher test, which identifies homogeneous subsets of averages that do not differ from each other. Data modelling was performed with the mean data for each specific pH and temperature with Statgraphics Centurion 18 version 18.1.13 by applying non-linear regression.

1.3. Results and discussion

The present work has studied the antimicrobial effect of a grape extract against *B. cereus* spores in a rice solution subjected to four temperatures (90 °C, 95 °C, 100 °C and 105 °C), which include those simulating traditional cooking treatments. Antimicrobial effects were compared between control samples (2 % rice solution without grape extract) and 2 % rice solution with

grape extract, adjusted in all cases to different pH values (4.5 – 5.5 – 6.5) simulating the pH of different dishes in which rice could be present.

The logarithm of the survival fraction was plotted as a function of treatment time for each temperature and pH studied. **Figure 1.1(A) to (D)** shows that, as time and temperature increase and pH decreases, the *B. cereus* inactivation increases. **Figure 1.1(A) and (B)** shows a reduction in *B. cereus* spores of more than three logarithmic cycles, while in **Figure 1.1(C) and (D)**, there is a reduction of more than four logarithmic cycles. These results resemble the survival behaviour of *B. cereus* spores, previously reported by Fernandez et al., (1996); Sarrias et al., (2002) and Soni et al., (2016).

In **Figure 1.1(A) and (B)** (90 °C and 95 °C), it is shown the antimicrobial effect of grape extract, since the logarithmic reduction of *B. cereus* spores was significantly higher ($p \leq 0.05$) in the samples with grape extract compared to the control samples. On the other hand, in **Figure 1.1(C) and (D)** no significant differences ($p > 0.05$) between control samples and those with grape extract can be observed, attributing the reduction of *B. cereus* spores only to temperature, since it has been seen that at temperatures above 95 °C, the resistance of *B. cereus* spores decreases and therefore there are no significant differences ($p > 0.05$) if grape extract is added or not.

Regarding the pH, there is a significant antimicrobial effect ($p \leq 0.05$) of grape extract in combination with pH (4.5 and 5.5) and temperatures lower than 100 °C on the inactivation of *B. cereus* as it can also be shown in **Figure 1.1(A) and (B)**.

The mean experimental data were fitted to the Weibull survival function (**equation (1)**) by using non-linear regression, obtaining parameters that can be used to compare the effect of pH, temperature and grape extract on the *B. cereus* spores survival.

Results

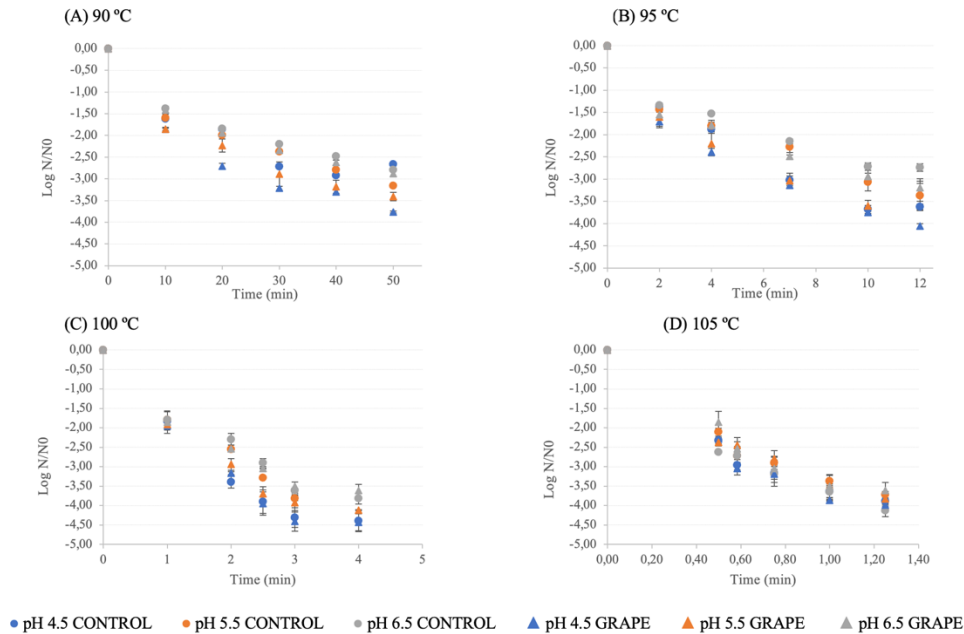


Figure 1.1. Survival of *B. cereus* spores in rice solution at different pH and treated at 90 °C (A), 95 °C (B), 100 °C (C) and 105 °C (D). Circles data represent values observed in control samples (without extract), while triangles represent the values obtained in samples with grape extract.

Table 1.1 shows the values of the Weibull survival function parameters and the fitting parameters R_2^{adj} and MSE. An ANOVA was performed with the results obtained for parameter a , to determine if there were significant differences in terms of exposure to grape extract. The results show that parameter a represents a measure of resistance to the treatment, showing that there is a significant decrease ($p \leq 0.05$) at acidic pH values and at 90 °C and 95 °C, at 100 °C and 105 °C and when the pH is 6.5 there are no significant differences ($p > 0.05$). This could indicate that at a high-temperature range and a pH close to neutrality, the antimicrobial effect of the grape extract is affected. Additionally, the shape parameter follows a normal distribution of 0.48 ± 0.06 and 0.52 ± 0.07 , for grape extract and the control, respectively (**Table 1.1**). In previous studies grapes have been found to have an antimicrobial effect, especially against Gram (+) bacteria (Ghendov-Mosan et al., 2022; Jayaprakasha et al., 2003; Oliveira et al., 2013). It is important to note that these previous studies have been carried out on the basis of

vegetative cell growth of *B. cereus*, without using other preservation treatments in food matrices. Serra et al., (2008) demonstrated that the natural grape extract drastically inhibited the growth of three bacterial strains in a dose-dependent manner, and the inhibitory effect was also most effective on vegetative cells of the Gram (+) strain *B. cereus*. On the other hand, Katalinić et al., (2010) studied the antimicrobial effect of the skins of 14 grape varieties (white and red), their results showed that there was a significant effect of grape extracts on both Gram (+) and Gram (–) bacteria; however, red grape extracts had the greatest antimicrobial effect on *B. cereus*. Another study that investigated the antimicrobial effect of grape seeds Jayaprakasha et al., (2003) showed a higher antimicrobial effect of seeds, as also reported by Baydar et al., (2004), who studied the antimicrobial effect of seeds and bagasse separately. However, Oliveira et al., (2013) studied the antimicrobial effect of grape bagasse and found it displayed good antimicrobial activity when it is extracted by supercritical fluids.

Regarding the modelling of survival curves, it can be highlighted that at temperatures of 90 °C and 95 °C, *a* value of the samples with grape extract were significantly lower than *a* values in the control samples for all pH values studied (4.5 – 5.5 – 6.5); except when the temperature was 90 °C and pH 6.5. This indicates that, at a pH closer to neutrality, the *B. cereus* spores are more resistant to treatment and grape extract can no longer exert an effect.

The comparison of results, presented in rows with lowercase letters (**Table. 1.1**), shows that at a temperature of 90 °C, the *a* value of control sample decreases significantly as the pH decreases, a behavior that has also been mentioned by other authors (Leguerinel & Mafart, 2001; Mazas et al., 1998). With respect to the grape samples, similar behavior can be seen, the value of *a* decreases significantly when pH decreases from 6.5 to 5.5; however, when the pH decreases from 5.5 to 4.5, there are no significant differences. Rhodes et al., (2006), studied the antilisterial activity of grape juice and grape extract. They found that this activity was pH-dependent. At low pH, the antilisterial activity was clearly seen, explaining this could be due to the fact that part of the anthocyanin is in the form of a stabilized flavin cation and can interact with the negatively charged bacterial cell wall.

Table 1.1. Weibull model parameters obtained by fitting experimental data to equation 2

T (°C)	pH	CONTROL				GRAPE			
		a	b	R ² _{adj}	MSE	a	b	R ² _{adj}	MSE
90	4.5	2.62 ± 0.08 ^{*a,A}	0.36 ± 0.00	0.945	0.171	1.87 ± 0.17 ^{*a,A}	0.40 ± 0.01	0.991	0.086
	5.5	4.02 ± 0.34 ^{*b,A}	0.40 ± 0.02	0.995	0.051	2.43 ± 0.35 ^{*a,A}	0.40 ± 0.00	0.995	0.058
	6.5	4.96 ± 0.16 ^{c,A}	0.44 ± 0.01	0.999	0.016	4.38 ± 0.23 ^{b,A}	0.44 ± 0.01	1.000	0.011
95	4.5	1.14 ± 0.10 ^{*a,B}	0.57 ± 0.01	0.980	0.136	0.66 ± 0.11 ^{*a,B}	0.48 ± 0.02	1.000	0.009
	5.5	1.24 ± 0.01 ^{*a,B}	0.52 ± 0.05	0.987	0.091	0.58 ± 0.15 ^{*a,B}	0.42 ± 0.05	0.972	0.149
	6.5	1.30 ± 0.02 ^{*a,B}	0.46 ± 0.01	0.986	0.083	0.89 ± 0.01 ^{*a,B}	0.44 ± 0.03	0.993	0.054
100	4.5	0.26 ± 0.11 ^{a,C}	0.56 ± 0.06	0.965	0.234	0.25 ± 0.02 ^{a,C}	0.56 ± 0.03	0.968	0.214
	5.5	0.34 ± 0.08 ^{a,C}	0.53 ± 0.00	0.974	0.161	0.25 ± 0.01 ^{a,B}	0.53 ± 0.00	0.979	0.161
	6.5	0.43 ± 0.18 ^{a,C}	0.61 ± 0.13	0.974	0.142	0.28 ± 0.02 ^{a,C}	0.50 ± 0.02	0.986	0.103
105	4.5	0.07 ± 0.01 ^{a,C}	0.48 ± 0.01	0.986	0.108	0.08 ± 0.01 ^{a,C}	0.51 ± 0.02	0.977	0.149
	5.5	0.10 ± 0.02 ^{a,C}	0.53 ± 0.04	0.987	0.085	0.12 ± 0.05 ^{a,B}	0.57 ± 0.12	0.996	0.060
	6.5	0.08 ± 0.01 ^{a,C}	0.51 ± 0.01	0.999	0.030	0.06 ± 0.01 ^{a,C}	0.46 ± 0.09	0.997	0.052

T, Temperature; a, scale parameter; b, shape parameter; R²_{adj}, r² adjusted; MSE, mean square error
The asterisk indicates significant differences between the values per column. Lower case letters compare values per row between pH at a specific temperature; while upper case letters compare values per row but between temperatures at a specific pH. Different letters indicate significant differences (p <0.05).

By contrast, when the samples were adjusted to pH 7.0, the antilisterial activity was lost, which may be due to the fact that most of the remaining anthocyanin in the polymeric structure is in the form of an uncharged quinonoidal base. Likewise, in another investigation by Hsieh et al., (2001) studying the antimicrobial effect of various plant extracts (Corni fructus, cinnamon and Chinese chive), the authors found that at lower pH values the antimicrobial effect against all bacteria studied, including *B. subtilis*, increased when the treatment temperature increased. Another study by Melis et al., (2014) and Xu et al., (2021) reported that when *B. cereus* spores were subjected to acidic pH values, additional stresses (low water activity) and mild heat treatment, their ability to germinate and grow decreased. This would explain the possible synergistic effect of grape extract; pH and heat treatment observed in this study and the importance of investigating the combination of a heat treatment with the use of a natural antimicrobial on *B. cereus* spores, which is more resistant and it would require stronger cooking treatments that affect the quality of the food.

On the other hand, when the temperature was 95 °C, 100 °C and 105 °C, *a* values in the control and in the grape extract samples did not decrease significantly when pH decreased. This would indicate that at these temperatures the antimicrobial effect is due to the temperature; since at these temperatures there is significant damage to the cellular structure of the spore, the exosporium is damaged and some proteins suffer denaturalization, thereby losing resistance to heat treatment and preventing germination and growth (Luu-Thi et al., 2014; Lv et al., 2019).

Finally, the comparison of the results presented in the rows with capital letters (**Table 1.1**), corroborates the effect that pH and temperature have on the thermo-resistance of *B. cereus* spores. At pH 6.5 there were significant differences at all temperatures (90 °C, 95 °C, 100 °C, and 105 °C), when the pH value was very close to neutrality, showing the effect of temperature on *B. cereus* spores alone. Meanwhile, at pH 4.5 or 5.5, there were significant differences only between the temperatures 90 °C, 95 °C and 100°C; in this case, *B. cereus* spore resistance decreased due to the effect of more acidic pH values, and therefore the decimal reduction was higher. Therefore, we can state that *B. cereus* spore development can be inhibited by combining factors such as pH, a natural antimicrobial and a mild heat treatment (Mafart et al., 2010; Soni et al., 2018; Xu et al., 2021). These results highlight the need to

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combine heat treatment with other factors such as low pH or natural antimicrobials, in order to control microorganisms without adversely impacting food quality, as occurs at elevated temperatures.

The relationship between parameter a , pH and temperature (secondary model) was determined by means of a multiple linear regression. **Equation (6)** was obtained for control samples and **equation (7)** for the samples with grape extract. Variables were delimited considering statistical significance with a confidence level of 95.0%:

$$\text{Log}(a) = 4.77 + 0.0763 * pH - 0.00057 * T^2 \quad (6)$$

$$\text{Log}(a) = 9.341 - 0.0994 * T^2 \quad (7)$$

Subsequently, to improve the estimation of the parameters all the data points ($\text{Log } S$) were fitted to the global model developed, **equation (8)** for the control samples and **equation (9)** for the samples with grape extract. The fitting results of these equations can be seen in **Table 1.2**.

$$\text{Log } S = - \left(\left(\frac{t}{10^{(\alpha + \beta * pH + \gamma * T^2)}} \right)^{0.52} \right) \quad (8)$$

$$\text{Log } S = - \left(\left(\frac{t}{10^{(\alpha + \beta * T)}} \right)^{0.48} \right) \quad (9)$$

Table 1.2. Coefficients of the polynomial were obtained by fitting the global model (equations (8) and (9)) to the total experimental data.

Control			Grape extract		
Coefficient	Estimated	Standard error	Coefficient	Estimated	Standard error
α	5.401	0.143	α	9.685	0.628
β	0.051	0.016	β	-0.1030	0.0054
Γ	-0.000617	0.000011			

Finally, the global model has been validated by calculating the AF (equation (4)) with an extra set of experimental data not used for the model fitting. For the control sample, it was 1.003, indicating that the percentage error is 0.3% for the predictions; while in the case of grape samples, the AF for the global model was 1.028 indicating a percentage error of 2.8%. According to the AF, the global model can be used to predict the remaining *B. cereus* spores in rice solution, after being subjected to a preservation process, for both the control and samples containing grape extract, in which the pH and temperature are the independent environmental variables.

1.4 Conclusions

The hurdle concept is getting very popular as a preservation process. Results of the present work, show that the combined use of mild cooking temperatures, an acidic pH and grape extract, could be considered as a hurdle preservation process for acidic ready-to-eat dishes containing rice processed at 90 °C – 95 °C that are very common mild pasteurization temperatures. The advantage of combining heat and the use of grape extract could be an extension of the shelf life of the product and an improvement of the safety against *B. cereus* spores present in rice. The effect of the grape extract takes place at the thermal process level in the product inoculated with *B. cereus* spores. Additional studies could indicate if the effect goes beyond the thermal process during storage.

A global model has been developed that can lead to a tertiary model to be used by the industry in the development of pasteurization processes for ready-to-eat foods.

Although the results are interesting, further studies are needed, such as the characterization of the grape extract, as well as the maximum concentration of grape extract that can be added to obtain a hurdle effect at low acid conditions without negatively influencing the sensory characteristics of the product.

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References

- Abee, T., Groot, M. N., Tempelaars, M., Zwietering, M., Moezelaar, R., & Voort, M. van der. (2011). Germination and outgrowth of spores of *Bacillus cereus* group members: Diversity and role of germinant receptors. *Food Microbiology*, 28(2), 199–208. <https://doi.org/10.1016/j.fm.2010.03.015>
- Authority, E. F. S. & European Centre for Disease Prevention and Control. (2022). The European Union One Health 2021 Zoonoses Report. *EFSA Journal*, 20(12), e07666. <https://doi.org/10.2903/j.efsa.2022.7666>
- Authority (EFSA), E. F. S. (2005). Opinion of the Scientific Panel on biological hazards (BIOHAZ) on *Bacillus cereus* and other *Bacillus* spp in foodstuffs. *EFSA Journal*, 3(4), 175. <https://doi.org/10.2903/j.efsa.2005.175>
- Batt, C. (2014). *BACILLUS Bacillus cereus*. In *Encyclopedia of Food Microbiology* (2nd ed., pp. 124–128). MA: Academic Press.
- Baydar, N. G., Özkan, G., & Sağdıç, O. (2004). Total phenolic contents and antibacterial activities of grape (*Vitis vinifera* L.) extracts. *Food Control*, 15(5), 335–339. [https://doi.org/10.1016/S0956-7135\(03\)00083-5](https://doi.org/10.1016/S0956-7135(03)00083-5)
- Bhattacharya, K. (2011). Rice quality: A guide to rice properties and analysis. In *Woodhead Publishing Series in Food Science, Technology and Nutrition* (p. 219). Oxford: WP, Woodhead Publ.
- Condon, S., & Sala, F. J. (1992). Heat Resistance of *Bacillus subtilis* in Buffer and Foods of Different pH. *Journal of Food Protection*, 55(8), 605–608. <https://doi.org/10.4315/0362-028X-55.8.605>
- Coroller, L., Leguérinel, I., & Mafart, P. (2001). Effect of Water Activities of Heating and Recovery Media on Apparent Heat Resistance of *Bacillus cereus* Spores. *Applied and Environmental Microbiology*, 67(1), 317–322. <https://doi.org/10.1128/AEM.67.1.317-322.2001>
- Drobniewski, F. A. (1993). *Bacillus cereus* and related species. *Clinical Microbiology Reviews*, 6(4), 324–338. <https://doi.org/10.1128/cmr.6.4.324>
- Dwyer, K., Hosseinian, F., & Rod, M. (2014). The market potential of grape waste alternatives. *Journal of Food Research*, 3(2), 91.

- Fernández, A., Collado, J., Cunha, L. M., Ocio, M. J., & Martínez, A. (2002). Empirical model building based on Weibull distribution to describe the joint effect of pH and temperature on the thermal resistance of *Bacillus cereus* in vegetable substrate. *International Journal of Food Microbiology*, 77(1), 147–153. [https://doi.org/10.1016/S0168-1605\(02\)00046-6](https://doi.org/10.1016/S0168-1605(02)00046-6)
- Fernández, A., Salmerón, C., Fernández, P. S., & Martínez, A. (1999). Application of a frequency distribution model to describe the thermal inactivation of two strains of *Bacillus cereus*. *Trends in Food Science & Technology*, 10(4), 158–162. [https://doi.org/10.1016/S0924-2244\(99\)00037-0](https://doi.org/10.1016/S0924-2244(99)00037-0)
- Fernandez, P., Ocio, M., Rodrigo, F., Rodrigo, M., & Martinez, A. (1996). Mathematical model for the combined effect of temperature and pH on the thermal resistance of *Bacillus stearothermophilus* and *Clostridium sporogenes* spores. 1996, 33, 225–233. [https://doi.org/0.1016/0168-1605\(96\)01118-X](https://doi.org/0.1016/0168-1605(96)01118-X)
- Garavaglia, J., Markoski, M. M., Oliveira, A., & Marcadenti, A. (2016). Grape Seed Oil Compounds: Biological and Chemical Actions for Health. *Nutrition and Metabolic Insights*, 9, NMI.S32910. <https://doi.org/10.4137/NMI.S32910>
- Ghendov-Mosanu, A., Cojocari, D., Balan, G., Patras, A., Lung, I., Soran, M.-L., Oprea, O., Cristea, E., & Sturza, R. (2022). Chemometric Optimization of Biologically Active Compounds Extraction from Grape Marc: Composition and Antimicrobial Activity. *Molecules*, 27(5). <https://doi.org/10.3390/molecules27051610>
- Grases, F., Prieto, R. M., Fernández-Cabot, R. A., Costa-Bauzá, A., Sánchez, A. M., & Prodanov, M. (2015). Effect of consuming a grape seed supplement with abundant phenolic compounds on the oxidative status of healthy human volunteers. *Nutrition Journal*, 14(1), 94. <https://doi.org/10.1186/s12937-015-0083-3>
- Grau-Fuentes, E., Úbeda-Manzanaro, M., Martínez, A., Garzón, R., Rosell, C. M., & Rodrigo, D. (2023). Evaluation of the antimicrobial activity of grape extract against *Bacillus cereus* in rice. *LWT*, 175, 114481. <https://doi.org/10.1016/j.lwt.2023.114481>
- Hassan, Y. I., Kosir, V., Yin, X., Ross, K., & Diarra, M. S. (2019). Grape Pomace as a Promising Antimicrobial Alternative in Feed: A Critical Review. *Journal of Agricultural and Food Chemistry*, 67(35), 9705–9718. <https://doi.org/10.1021/acs.jafc.9b02861>
- Hazards (BIOHAZ), E. P. on B. (2016). Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. Including *Bacillus thuringiensis* in foodstuffs. *EFSA Journal*, 14(7), e04524. <https://doi.org/10.2903/j.efsa.2016.4524>

- Hsieh, P.-C., Mau, J.-L., & Huang, S.-H. (2001). Antimicrobial effect of various combinations of plant extracts. *Food Microbiology*, 18(1), 35–43. <https://doi.org/10.1006/fmic.2000.0376>
- Jayaprakasha, G. K., Selvi, T., & Sakariah, K. K. (2003). Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. *Food Research International*, 36(2), 117–122. [https://doi.org/10.1016/S0963-9969\(02\)00116-3](https://doi.org/10.1016/S0963-9969(02)00116-3)
- Jessberger, N., Dietrich, R., Granum, P. E., & Märtlbauer, E. (2020). The *Bacillus cereus* Food Infection as Multifactorial Process. *Toxins*, 12(11). <https://doi.org/10.3390/toxins12110701>
- Katalinić, V., Možina, S. S., Skroza, D., Generalić, I., Abramović, H., Miloš, M., Ljubenković, I., Piskernik, S., Pezo, I., Terpinč, P., & Boban, M. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 *Vitis vinifera* varieties grown in Dalmatia (Croatia). *Food Chemistry*, 119(2), 715–723. <https://doi.org/10.1016/j.foodchem.2009.07.019>
- Leguerinel, I., & Mafart, P. (2001). Modelling the influence of pH and organic acid types on thermal inactivation of *Bacillus cereus* spores. *International Journal of Food Microbiology*, 63(1), 29–34. [https://doi.org/10.1016/S0168-1605\(00\)00394-9](https://doi.org/10.1016/S0168-1605(00)00394-9)
- Luu-Thi, H., Grauwet, T., Vervoort, L., Hendrickx, M., & Michiels, C. W. (2014). Kinetic study of *Bacillus cereus* spore inactivation by high pressure high temperature treatment. *Innovative Food Science & Emerging Technologies*, 26, 12–17. <https://doi.org/10.1016/j.ifset.2014.07.005>
- Lv, R., Zou, M., Chantapakul, T., Chen, W., Muhammad, A. I., Zhou, J., Ding, T., Ye, X., & Liu, D. (2019). Effect of ultrasonication and thermal and pressure treatments, individually and combined, on inactivation of *Bacillus cereus* spores. *Applied Microbiology and Biotechnology*, 103(5), 2329–2338. <https://doi.org/10.1007/s00253-018-9559-3>
- Mafart, P., Leguérinel, I., Couvert, O., & Coroller, L. (2010). Quantification of spore resistance for assessment and optimization of heating processes: A never-ending story. *Food Microbiology*, 27(5), 568–572. <https://doi.org/10.1016/j.fm.2010.03.002>
- Mazas, M., López, M., González, I., González, J., Bernardo, A., & Martín, R. (1998). Effects of The Heating Medium pH on Heat Resistance of *Bacillus Cereus* Spores. *Journal of Food Safety*, 18(1), 25–36. <https://doi.org/10.1111/j.1745-4565.1998.tb00199.x>
- Melis, C. C. J. van, Besten, H. M. W. den, Groot, M. N. N., & Abee, T. (2014). Quantification of the impact of single and multiple mild stresses on outgrowth heterogeneity of *Bacillus cereus* spores. *International Journal*

- p>of Food Microbiology, 177, 57–62.
-
- <https://doi.org/10.1016/j.ijfoodmicro.2014.02.015>
- Miliotis, M., & Bier, J. (2003). International handbook of food- borne pathogens. In *Food Science and Technology* (p. 125). New York: M. Dekker.
- Oliveira, D. A., Salvador, A. A., Smania, A., Smania, E. F. A., Maraschin, M., & Ferreira, S. R. S. (2013). Antimicrobial activity and composition profile of grape (*Vitis vinifera*) pomace extracts obtained by supercritical fluids. *Journal of Biotechnology*, 164(3), 423–432.
- Peleg, M., & Cole, M. B. (1998). Reinterpretation of Microbial Survival Curves. *Critical Reviews in Food Science and Nutrition*, 38(5), 353–380.
<https://doi.org/10.1080/10408699891274246>
- Poudel, P. R., Tamura, H., Kataoka, I., & Mochioka, R. (2008). Phenolic compounds and antioxidant activities of skins and seeds of five wild grapes and two hybrids native to Japan. *Journal of Food Composition and Analysis*, 21(8), 622–625. <https://doi.org/10.1016/j.jfca.2008.07.003>
- Rhodes, P. L., Mitchell, J. W., Wilson, M. W., & Melton, L. D. (2006). Antilisterial activity of grape juice and grape extracts derived from *Vitis vinifera* variety Ribier. *International Journal of Food Microbiology*, 107(3), 281–286. <https://doi.org/10.1016/j.ijfoodmicro.2005.10.022>
- Rodrigo, D., Rosell, C. M., & Martínez, A. (2021). Risk of *Bacillus cereus* in Relation to Rice and Derivatives. *FOODS*, 10(2).
<https://doi.org/10.3390/foods10020302>
- Ross, T. (1996). Indices for performance evaluation of predictive models in food microbiology. *Journal of Applied Bacteriology*, 81(5), 501–508.
<https://doi.org/10.1111/j.1365-2672.1996.tb03539.x>
- Sampedro, F., Rivas, A., Rodrigo, D., Martínez, A., & Rodrigo, M. (2006). Effect of temperature and substrate on Pef inactivation of *Lactobacillus plantarum* in an orange juice–milk beverage. *European Food Research and Technology*, 223(1), 30–34. <https://doi.org/10.1007/s00217-005-0096-9>
- Sarrias, J. A., Valero, M., & Salmerón, M. C. (2002). Enumeration, isolation and characterization of *Bacillus cereus* strains from Spanish raw rice. *Food Microbiology*, 19(6), 589–595. <https://doi.org/10.1006/fmic.2002.0514>
- Serra, A. T., Matias, A. A., Nunes, A. V. M., Leitão, M. C., Brito, D., Bronze, R., Silva, S., Pires, A., Crespo, M. T., Romão, M. V. S., & Duarte, C. M. (2008). In vitro evaluation of olive- and grape-based natural extracts as potential preservatives for food. *Innovative Food Science & Emerging Technologies*, 9(3), 311–319. <https://doi.org/10.1016/j.ifset.2007.07.011>
- Soni, A., Oey, I., Silcock, P., & Bremer, P. (2016). *Bacillus* Spores in the

- Food Industry: A Review on Resistance and Response to Novel Inactivation Technologies. *Comprehensive Reviews in Food Science and Food Safety*, 15(6), 1139–1148. <https://doi.org/10.1111/1541-4337.12231>
- Soni, A., Oey, I., Silcock, P., & Bremer, P. J. (2018). Impact of temperature, nutrients, pH and cold storage on the germination, growth and resistance of *Bacillus cereus* spores in egg white. *Food Research International*, 106, 394–403. <https://doi.org/10.1016/j.foodres.2018.01.006>
- Stumbo, C. (1973). Thermobacteriology in food processing. In *Food science and technology* (2nd ed., pp. 1–336). New York: Academic Press.
- Tirloni, E., Stella, S., Celandroni, F., Mazzantini, D., Bernardi, C., & Ghelardi, E. (2022). *Bacillus cereus* in Dairy Products and Production Plants. *Foods*, 11(17). <https://doi.org/10.3390/foods11172572>
- Wei, X., & Huang, X. (2019). 1—Origin, taxonomy, and phylogenetics of rice. In J. Bao (Ed.), *Rice (Fourth Edition)* (pp. 1–29). AACC International Press. <https://doi.org/10.1016/B978-0-12-811508-4.00001-0>
- Xu, J., Janahar, J. J., Park, H. W., Balasubramaniam, V. M., & Yousef, A. E. (2021). Influence of water activity and acidity on *Bacillus cereus* spore inactivation during combined high pressure-thermal treatment. *LWT*, 146, 111465. <https://doi.org/10.1016/j.lwt.2021.111465>
- Zuijlen, A. van, Periago, P. M., Amézquita, A., Palop, A., Brul, S., & Fernández, P. S. (2010). Characterization of *Bacillus sporothermodurans* IC4 spores; putative indicator microorganism for optimisation of thermal processes in food sterilisation. *Food Research International*, 43(7), 1895–1901. <https://doi.org/10.1016/j.foodres.2009.11.011>

**Evaluation of the
antimicrobial activity of
grape extract against
Bacillus cereus in rice**

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By-products Natural antimicrobials Survival Predictive microbiological Modelling

The antimicrobial potential of grape extract was assessed in cooked rice against *Bacillus cereus*. Grape extract efficacy was tested at 1, 5 and 10 mL/L, at pH 4.5, 5.5 and 6.5; and at incubation temperatures simulating different storage scenarios, specifically temperature abuse (10 °C), cool chain break (20 °C) and optimal *B. cereus* growth temperature (30 °C). Survival curves for grape extract concentration versus time were obtained. The results indicate that antimicrobial activity of grape extract was dependent on temperature, pH and grape extract concentration. A bactericidal effect of the grape extract was shown at concentration levels ≥ 5 mL/L at all temperatures and pHs studied. Inactivation curves of *B. cereus* under grape extract exposure were fitted to a Weibull distribution function for 5–10 mL/L grape extract concentration. Observations showed that the higher the incubation temperature and grape extract concentration, the lower the kinetic rate value. In other words, lower resistance of the microorganism to environmental conditions. The maximum inactivation level was 6 log₁₀ cycles after 24 h of exposure at 10 mL/L of grape extract concentration and pH 4.5. Results indicate that the grape extract could be a good additional control measure for preventing *Bacillus cereus* growth in cooked rice during storage.

2.1 Introduction

Rice (*Oryza sativa* L.) is a basic cereal, widely consumed by the general population due to its abundant nutrients and relatively low price. It is one of the most important staple crops on the planet and feeds almost half the world population (Wei & Huang, 2019). However, it is also frequently involved in foodborne *B. cereus* outbreaks (Rodrigo et al., 2021). Once cooked, it has a pH close to 7, being an excellent growth medium when the humidity of the substrate reaches adequate water activity values for the growth of the microorganism (Delbrassinne et al., 2015; Pao et al., 2006; Rodrigo et al., 2021). *B. cereus* is a gram-positive spore forming bacterium, which is a habitual saprophyte, resistant to high temperatures and low humidity (Hendriksen et al., 2006; Rodrigo et al., 2021). It is a public health issue because produces food toxins and can survive 48 weeks in rice in cool dry storage without loss of viability (Gilbert et al., 1974; Sánchez et al., 2016).

One of the main factors contributing to *B. cereus* outbreaks related to cooked rice is poor cooling, or storage at room temperature for a prolonged period, particularly when cooking large quantities in restaurants and/or canteens (Juneja et al., 2019). The main source of this contamination is the presence of heat-resistant spores that survive normal rice cooking temperatures (close to 100 °C) (Gilbert et al., 1974). During standard cooking conditions, 2-3 decimal log reductions in the initial spore load can be achieved, therefore final product risk levels depend largely on the initial concentration of microorganisms and hygienic measures during handling, cooking or processing (Rodrigo et al., 2021). After being cooked, the spores that have survived the process can germinate and grow up to 10^7 or 10^9 CFU/g after 24 h at 26 or 32 °C, respectively (Lake et al., 2004; S. Yu et al., 2020) and produce a heat-stable (emetic) toxin (Little et al., 2002). Around 95% of outbreaks of an emetic syndrome are mainly caused by the consumption of cooked or fried rice (Juneja et al., 2019). Controlling the storage temperature (lower than 4 °C or higher than 55 °C) is the procedure commonly accepted by food safety authorities. Consequently, it is of great interest to have an additional control measure, other than post-cooking storage temperature, in these products, especially if they are not going to be consumed immediately after preparation (Juneja et al., 2019).

Grape (*Vitis vinifera* L.), represents the second-largest crop in the world. Its skin and seeds, are also rich in phenolic compounds, as well as lipids, proteins and polysaccharides (Ferreira et al., 2014; Gokturk Baydar et al., 2007). Phenolic compounds are phytochemicals with functions related to pigmentation, astringency, protection against ultraviolet rays, as well as antioxidant and antimicrobial activity (Oliveira et al., 2003). As antimicrobial agents, these polyphenols can penetrate the semipermeable cell membrane where they react with the cytoplasm or cellular proteins. Therefore, these polyphenolic compounds with a highly negative charge can be used to prevent the growth of pathogenic bacteria (Arts & Hollman, 1998; Cheng et al., 2012; Oki et al., 2002). Grape extract is one of the ingredients that has received attention in recent years due to its natural antimicrobial capacity. One advantage of these extracts is that they are derived from industrial waste products, such as grape juice/wine production (Shi et al., 2003; J. Yu et al., 2005, 2010). In addition to providing compounds with functional properties, revalorization reduces the environmental impact by reusing part of the grape, such as seeds and skins, which are generally not used by industry (Goncalves et al., 2021). Identifying the value of these products will result in reducing waste, reusing raw products, and providing an all-natural alternative to synthetic preservatives (Levy et al., 2017), which will improve food-industry sustainability and positively impact on the UN's Sustainable Development Goals. Therefore, the use of this raw material as a natural substitute for synthetic antimicrobial additives represents a positive alternative to prevent foodborne outbreaks (Goncalves et al., 2021; Prado Martin et al., 2012).

In this context, the main objective of this work was to evaluate the antimicrobial capability of the grape extract against vegetative cells of *B. cereus* (germinated spores) in a rice matrix, using different storage conditions resembling real ones, at different storage temperatures (10, 20 and 30 °C), different grape extract concentrations (1, 5 and 10 mL/L) and different pH values (4.5, 5.5 and 6.5).

2.2. Material and methods

2.2.1. Microbial strains

Pure culture of *B. cereus* (Spanish Type Culture Collection (CECT) 148) were provided by the Spanish Type Culture Collection and, following their

procedure, lyophilized samples were rehydrated with 0.2 mL of sterile Nutrient Broth (NB) (Scharlab S.A., Barcelona, Spain). After 30 min, rehydrated cultures were transferred to 500 mL of NB medium, and incubated in a water bath shaker for 14 – 18 h at 30 °C, to obtain cells in a stationary growth phase. The *B. cereus* cells were centrifuged twice at 5000 revolutions per minute (rpm), 4 °C and 15 min, in a Beckman centrifuge (JLA-16,250 rotor), the supernatant was decanted and resuspended in 50 mL of NB. Process was repeated twice, and then cells were resuspended in 50 mL of NB and distributed in cryovials (1 mL); 1 mL of 200 mL/L glycerol in NB was added as protectant and kept at – 80 °C until further use. The final inoculant concentration was determined by plate count and was of 10⁸ colony forming units/mL (CFU/mL).

2.2.2. Rice matrix

Commercial rice acquired in the market was used. Rice was cooked into an electric cooker, and after cooling down cooked rice was freeze-dried. Rice powder had moisture content of 8.66 g water/100 g of product. Three different batches were prepared. The rice matrix was prepared by diluting 20 g of powder rice/L, and it was sterilized in an autoclave.

2.2.3. Grape extract

Grape color liquid EV-3 is a natural red colorant extracted from red grapes (EEC code: E-163). The grape color extract was provided by Sociedad Española de Colorantes Naturales y Afines SA - SECNA (Chiva, Valencia, Spain). This extract, with a pH of 2.5, was frozen in Eppendorf at – 80 °C for later use. 1, 5 and 10 mL/L grape extract concentrations were added to the rice matrix for testing the antimicrobial activity against *B. cereus*.

2.2.4. Evaluation of antimicrobial activity of grape extract

The grape extract antimicrobial activity was tested at pH 4.5; 5.5 and 6.5, by using a sodium hydroxide solution (1 mL/100 mL) to modify the pH value of samples. Microbial growth at these conditions was compared with control samples at the same pHs but without grape extract. In order to carry out the experiment, 10⁷ UFC/mL of *B. cereus* was inoculated to each solution of rice. The inoculated media were incubated under shaking at 10 °C, 20 °C and 30 °C for 168 h (10 °C) and 24 h (20 and 30 °C). For each media, samples were

Results

taken at different time intervals. Two series of each sample (A and B) were serially diluted in peptone water (0.1 mg/100 mL), plated in duplicate and incubated in nutritive broth agar (NBA) (Scharlab S.A., Barcelona, Spain) at 37 °C for 24 h. After incubation, colonies were counted (CFU/mL). Experimental results are shown as log₁₀ of the survival fraction (log S) calculated as **equation (1)**:

$$\text{Log } S = \text{Log}_{10} \left(\frac{N}{N_0} \right) \quad (1)$$

Where N is the bacterial concentration (CFU/mL) at time t (h) and N_0 initial bacterial concentration (CFU/mL) (t_0). Therefore, positive values indicate microbial growth while negative values show microbial inactivation compared to the initial inoculation value (N_0).

2.2.5. Mathematical modelling of *B. cereus* inactivation

For the concentrations of 5 and 10 mL/L grape extract, the survival curves were fitted with the model proposed by (Mafart, Couvert, Gaillard, & Leguerinel, 2002) based on the Weibull distribution function. The Weibull model is a simple non-linear model, which has been successfully used to fit the nonlinear inactivation curves of many microorganisms under different conditions.

$$\text{Log}_{10} (N) = \text{Log}_{10} (N_0) - \left(\left(\frac{t}{\delta} \right)^p \right) \quad (2)$$

Where N is the population concentration at time t (CFU/mL); N_0 is the initial population concentration (CFU/mL), δ is the first 10-fold reduction (kinetic parameter) and p is the shape parameter. For the case $p < 1$, the equation allows the tailing portion fitting, inward concavity, of the inactivation curve, for $p = 1$ linear survival curves. For $p > 1$, the shoulder portion, outward convexity, can be predicted (Albert & Mafart, 2005; Marugan, van Grieken, Sordo, & Cruz, 2009). Values of p and δ were determined by using GInaFIT add-in for Microsoft® Excel solver function (Version 1.7) (Geeraerd, Valdramidis, & Van Impe, 2006).

A secondary model was built by means of a forward stepwise multiple regression, relating the logarithm of the scale factor of the Weibull model (δ)

with temperature and pH. This is intended to limit the number of terms of the secondary model. Likewise, a global model is built in order to improve the value of the coefficients of the secondary model. A tertiary model is developed using the coefficients deduced by the global model in C# (Microsoft Visual Studio Community 2022 (64 bits) Version 17.0.2)

2.2.6. Statistical analysis

The fit of the models to the polynomials (secondary model) was performed with STATGRAPHICS Centurion XVIII (STATGRAPHICS, Warrenton, VA), including an ANOVA analysis to test significant differences in estimated model parameters depending on pH and temperature in the same grape extract concentration. The goodness of fit of the model was assessed using adjusted the regression coefficient (adjusted- R^2) and root mean square error (RMSE). To assess predictions made by the model, the Accuracy Factor parameter (Af) (Ross, 1996) was used:

$$Af = 10^{\left(\frac{\sum \left(\log \left(\frac{\text{predicted}}{\text{observed}} \right) \right)}{n} \right)} \quad (3)$$

The predicted/observed ratio refers to the relationship between the survival fraction predicted by the model and the one obtained experimentally and n is the number of observations used to make the calculations.

2.3. Results and discussion

2.3.1. Effect of grape extract concentration, pH and incubation temperature on *B. cereus* growth

In the present work, the effect of different grape extract concentrations on vegetative cells of *B. cereus*, stored at three temperatures and three pH values, has been studied and compared with those obtained in controls. Vegetative cells were used because during storage spores germinate to vegetative cells, producing toxins during growth, so this is the bacterial stage that should be controlled.

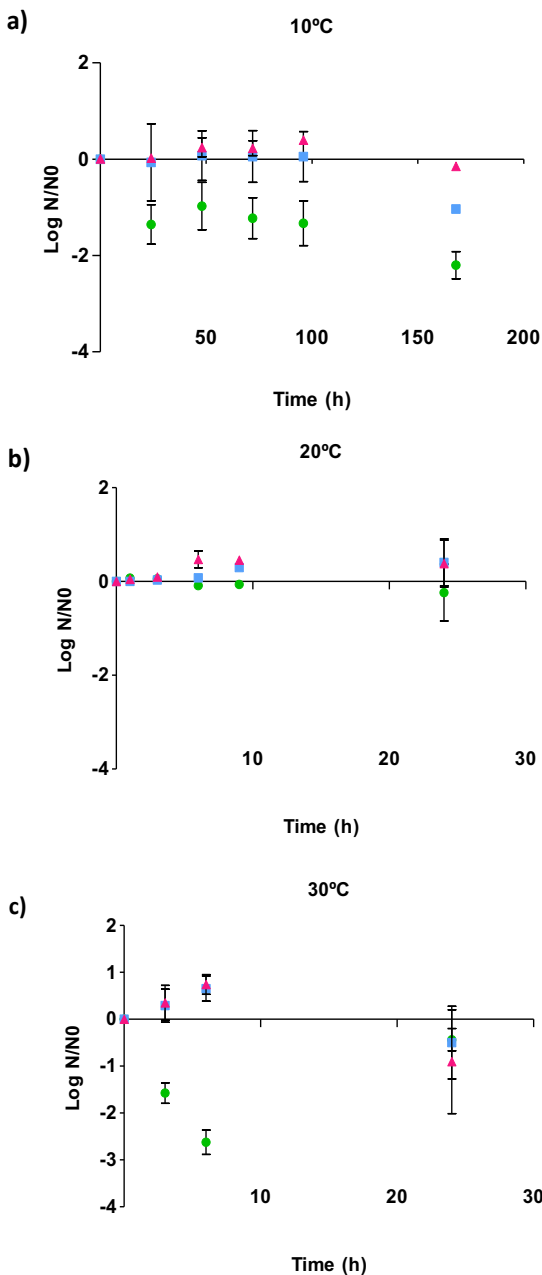
Results

T ^a	pH	CA	1 mL/L grape	5 mL/L grape	10 mL/L grape
30°C	4.5				
	5.5				
	6.5				
20°C	4.5				
	5.5				
	6.5				
10°C	4.5				
	5.5				
	6.5				

Figure 2.1 Schematic representation of *B. cereus* growth/inhibition depending on temperature (10°C, 20°C and 30°C), pH (4.5, 5.5 and 6.5) and grape extract concentration (0 mL/L (CA), 1 mL/L, 5 mL/L and 10 mL/L). Colors code: **Red** represents unsafe conditions, where the microorganism grows; **yellow**, bacteriostatic conditions; and **green** are microbiologically safe conditions, where the antimicrobial acts as a bactericide.

Figure 2.1 represents the behavior of *B. cereus* qualitatively and schematically under all study conditions. In the control (CA), *B. cereus* grew at all pH values at a temperature of 30 and 20°C, while the temperature of 10°C had a bacteriostatic effect with long lag phases, independently of pH, and only in the case of pH 6.5 some significant growth could be observed after 96 hours of incubation time. Therefore, neither the pH nor the temperature above 10°C proved sufficient barriers to prevent *B. cereus* growth. This finding justifies the need to introduce an antimicrobial to prevent microbial growth in the event of cold chain breach or temperature abuse.

Figure 2.2 shows the behavior of *B. cereus* for the lowest grape extract concentration assayed: 1 mL/L. Positive log values (N/N_0) indicate microbial growth, whereas negative values indicate microbial inactivation compared to the initial inoculation (N_0). At 10°C and all pH values, the final concentration of *B. cereus* (after 168 hours of storage) was lower, or slightly lower (for pH 6.5) than the initial concentration of the microorganism, showing a bactericidal (pH 4.5 and 5.5) or bacteriostatic effect (for pH 6.5).



For the same grape extract concentration but at a temperature of 20°C, a lag phase was observed for all pH values studied. At pH 4.5 and 5.5 the grape extract acted as a bacteriostatic, since *B. cereus* did not grow, while at pH 6.5 the lag phase lasted approximately 6 hours, which was longer than the result with acidic control, thus the addition of grape extract delayed microorganism growth. Finally, at 30°C there was no lag phase for any of the pH values studied. Therefore, at 1 mL/L an increase in storage temperature implies a decrease in lag phase duration.

Figure 2.2
Growth/inhibition of *B. cereus* with 1 mL/L grape extract concentration depending on pH (4.5 (●), 5.5 (■) and 6.5 (▲)) and temperature (10°C (a), 20°C (b) and 30°C (c)).

2.3.2. Effect of grape extract concentration, pH and incubation temperature on *B. cereus* inactivation

As mentioned above, under the pH and temperature conditions studied, grape extract exerted a bactericidal effect for concentrations equal to or greater than 5 mL/L. Those conditions were selected to quantitatively characterize the bactericidal effect of grape extract. The results showed that the grape extract can be bacteriostatic or bactericidal depending on concentration, storage temperature and pH of the medium. The antimicrobial capacity of grape extract has been attributed to its polyphenol content (Fontana et al., 2013; D. A. Oliveira et al., 2013; Yadav et al., 2015). Various authors have reported total polyphenol content in grape extract between 128.22 and 215.93 mg Gallic Acid Equivalents/Dry Weight (mg GAE/DW) (Ky & Teissedre, 2015); 36.6–88.7 mg GAE/DW (Ky et al., 2014); 212–279 mg GAE/DW (Brezoiu et al., 2019); 24.5–60.1 mg GAE/DW (Bosso et al., 2020); 33.2–37.5 mg GAE/DW (Guaita & Bosso, 2019). The quantity of polyphenols depends on the origin (grape seeds or skin), as well as the extraction solvent used for polyphenol quantification and the analysis method; therefore values could differ, accordingly (Balaban et al., 2021)). Many classes of negatively charged polyphenols have been identified in grapes, such as phenolic acids (benzoic and hydroxycinnamic acids), stilbene derivatives (resveratrol), flavan-3-ols (catechin, epicatechin), flavanols (kaempferol, quercetin, myricetin), anthocyanins, etc. (D. A. Oliveira et al., 2013; Yadav et al., 2015). Gram-positive bacteria were more sensitive to grape extract than Gram-negative bacteria. These differences could be explained by the presence of the lipopolysaccharide cell wall in Gram-negative bacteria, which can limit the penetration of polyphenols (Gerardi et al., 2021). This potential is greater in grape-skin extract because phenolic acids are present in undissociated forms (Yadav et al., 2015). In this frame, Katalinić et al., (2010) studied the antimicrobial activity of 14 phenolic extracts, both from white grape and red grape (seven and seven), utilizing a broth microdilution test with Gram-negative (*E. coli* O157: H7, *Salmonella Infantis*, *C. coli*) and Gram-positive (*S. aureus*, *B. cereus*) bacteria. Antimicrobial activity was confirmed against all gram-negative and gram-positive bacteria for all extracts. Differences in phenolic efficiency of white and red grape cultivars have been observed for different test organisms but no significant differences were found in the susceptibility of Gram-positive and Gram-negative bacteria (Katalinić et al., 2010). In the present work, results shown that grape extract exerted an

antimicrobial activity against *B. cereus*, in accordance with Katalinić et al., (2010). Furthermore, as occurs in the present study with grape extract concentrations, in a study of the antimicrobial effect of pomegranate peel extracts at different dilution ratios against *B. subtilis*, *B. cereus*, and *E. faecalis* strains, Balaban et al., (2021) observed that the antibacterial activity of the extracts decreased when the extract dilution increased. In addition, in most cases, all the extracts and their dilutions were found to be more effective against *B. cereus* growth.

In this study, a 24 h period was considered a good control point for comparison between different pH values and temperatures since it is the time taken for control samples to reach the stationary phase at 20 °C. This temperature is important as it is considered as a cold chain breach. **Figure 2.3** shows that the bactericidal effect of the grape extract against *B. cereus* varied depending on the incubation temperature, the grape extract concentration and the pH of the medium. Considering the effect of pH, the greatest bactericidal effect occurred at pH 4.5, which differed with statistical significance to effects at pH 5.5 and 6.5 at 10 and 20 °C, and reaching up to 6 logarithmic reductions at 30 °C. Similar effect occurs in the study of Mau et al., (2001) where studied the antimicrobial effect of mixed different natural extracts (Chinese achieve +cinnamon +corni fructus (1:1:1)) in other food matrices (orange juice, pork and milk) against 15 microbiological strains, at different pH (original, 4.5, 5.5 and 6.5) and storage temperature (4 and 25 °C). Higher inhibitory effect was obtained at more acidic pH values and in their study, it was independent by the storage temperature. Regarding temperature effects in the present work, the greatest inactivation occurred at 30 °C (**Figure 2.3**), regardless of pH values and the grape extract concentration studied. This may be because these are optimal conditions for microorganism growth and thus defense mechanism against stress are not activated (Rodrigo et al., 2003). For pH 4.5, there were no significant differences between the inactivation levels reached at 10 or 20 °C, while for pH 5.5 and 6.5, (less stressful conditions) a greater inactivation was achieved at 20 °C. Likewise, Periago & Moezelaar, (2001), observed that nisin on its own, showed a small effect at 8 °C against *B. cereus* strain, needing to be combined with lower pH, carvacrol, or both of them for maximum bactericidal action. The combined effect of nisin and carvacrol was significant at pH 6.3 and 7 and was significantly greater at 30 °C than at 8 °C.

Results

A similar effect can be observed in the present study since the combination of different factors enhances the effect of the natural antimicrobial.

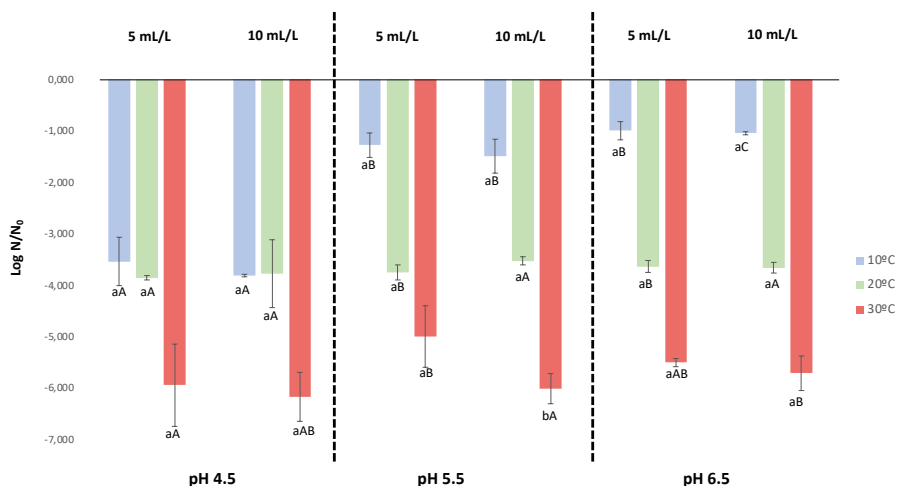


Figure 2.3. *B. cereus* inactivation levels at 24 hours storage time under exposure to concentrations of 5 and 10 mL/L of grape extract, at incubation temperatures of 10°C, 20°C and 30°C, and pH 4.5, 5.5 and 6.5. Letters on the bars indicate significant differences ($p \leq 0.05$) between concentrations of 5 and 10 mL/L for the same pH and temperature (lowercase letters) and between pH (4.5 - 5.5 - 6.5) for the same temperature and concentration (capital letters).

Regarding the effect of grape extract concentration on inactivation, in general, there were no significant differences between the values reached with 5 or 10 mL/L. Only at a pH of 5.5 and a temperature of 30 °C did the grape concentration produce a significant increase in inactivation (**Figure 2.3**).

2.3.3. Kinetics of *B. cereus* inactivation by grape extract

In order to complete the evaluation of the grape extract, quantify its natural antimicrobial activity in cooked rice products, and make predictions for different scenarios, the experimental results were adjusted for concentrations of 5 and 10 mL/L of grape extract (conditions in which the expected result was achieved) to the Weibull distribution function, using the GInaFIT excel add-in (Version 1.7). Each individual experimental survival curve obtained was fitted separately and the estimated parameters (scale parameter δ and shape parameter p) were derived for each pH, temperature, and grape extract concentration (**Table 2.1**).

Table 2.1. Weibull parameters (δ and p) and model fit (Adjusted R^2 and RMSE) for *B. cereus* inactivation under exposure to 5 and 10 mL/L grape extract concentration at 10°C, 20°C and 30°C.

Grape extract concentration	Temperature	pH	Weibull parameters		Accuracy fit	
			δ	p	R^2_{adj}	RSME
5 mL/L	10 °C	4.5	3.070 ± 0.868 ^{aA}	0.34 ± 0.005	0.916	0.691
		5.5	5.681 ± 0.949 ^{bA}	0.42 ± 0.018	0.965	0.347
		6.5	6.952 ± 0.689 ^{bA}	0.40 ± 0.043	0.946	0.458
	20 °C	4.5	*2.256 ± 0.510 ^{aB}	0.572 ± 0.052	0.971	0.294
		5.5	4.224 ± 0.691 ^{bB}	0.840 ± 0.059	0.972	0.308
		6.5	5.649 ± 1.150 ^{cA}	0.905 ± 0.135	0.993	0.134
	30 °C	4.5	0.655 ± 0.283 ^{aC}	0.500 ± 0.061	0.948	0.707
		5.5	0.499 ± 0.119 ^{abC}	0.439 ± 0.051	0.887	1.032
		6.5	0.296 ± 0.168 ^{bB}	0.283 ± 0.043	0.765	1.148
10 mL/L	10 °C	4.5	2.510 ± 0.422 ^{aA}	0.26 ± 0.006	0.837	0.985
		5.5	6.724 ± 0.368 ^{bA}	0.45 ± 0.012	0.95	0.457
		6.5	7.052 ± 0.200 ^{bA}	0.46 ± 0.006	0.929	0.524
	20 °C	4.5	*0.526 ± 0.129 ^{aB}	0.359 ± 0.049	0.927	0.523
		5.5	3.209 ± 0.219 ^{bB}	0.583 ± 0.015	0.998	0.07
		6.5	2.160 ± 0.252 ^{cB}	0.559 ± 0.024	0.98	0.246
	30 °C	4.5	0.363 ± 0.282 ^{aB}	0.420 ± 0.054	0.977	0.46
		5.5	0.394 ± 0.059 ^{aC}	0.462 ± 0.025	0.983	0.442
		6.5	0.322 ± 0.134 ^{aC}	0.389 ± 0.042	0.969	0.62

Values followed by different letters within the same pH (small letters) and within the same temperature (capital letters) are significantly different ($p \leq 0.05$). Values with (*) indicate significant differences ($p \leq 0.05$) for the same pH and temperature between concentrations.

Results

The scale parameter δ is the first 10-fold reduction, related to the microorganism's resistance to the inactivation treatment, as the higher the treatment intensity, the lower the resistance. In general, as shown in **Table 2.1**, increasing extract concentrations and temperatures was associated with a δ parameter reduction although it was not always statistically significant. Additionally, there was a significant effect of pH (4.5 versus 6.5) for resistance to microorganism death for any concentration and temperature, except 20 °C – 10 mL/L.

A secondary model was developed by a forward stepwise multiple regression to define the dependence of $\log \delta$ with temperature and pH for fixed concentrations of grape extract (5 or 10 mL/L), **equations 3 and 4**. The p value is less than 0.05, thus there is a significant relationship between the dependent variable ($\log \delta$) and independent variables at the 95% of confidence level. Kwon et al., (2020) obtained shape p values lower than 1 at all temperatures studied, indicating rapid decrease of the microorganisms, which agrees with our results as shown in **Table 2.1**.

$$\text{Log } \delta = -2.28 + 0.418 * \text{pH} \pm 0.0175 * \text{pH} * T + 0.204 * T - 0.0040 * T^2 \quad R^2 = 97\% \quad (3)$$

$$\text{Log } \delta = -8.11 + 3.10 * \text{pH} - 0.248 * \text{pH}^2 - 0.0104 * \text{pH} * T \quad R^2 = 94\% \quad (4)$$

To improve the value of coefficients of the secondary model (polynomial model) obtained, a global model was built using all data points ($\text{Log } S$) and fitting them to **equations 5 and 6**, using one-step nonlinear regression (Statgraphics Centurion XVIII). The estimated coefficients and the confidence intervals obtained are shown in **Table 2.2**.

$$\text{Log } S = - \left[\left(\frac{t}{10^{(0.780 + 0.165 * \text{pH} - 0.00095 * \text{pH} * T - 0.0746 T + 0.00077 * T^2)}} \right)^p \right] \quad (5)$$

$$\text{Log } S = - \left[\left(\frac{t}{10^{(-7.29 + 2.84 * \text{pH} - 0.231 * \text{pH}^2 - 0.0101 * \text{pH} * T)}} \right)^p \right] \quad (6)$$

Table 2.2. Coefficient estimates obtained by fitting the global model (equation 5 to 5 mL/L grape extract concentration and equation 6 to 10 mL/L) to the total experimental data.

5 mL/L grape extract			10 mL/L grape extract		
Parameter	Estimated	Standard error	Parameter	Estimated	Standard error
a	0.7797	0.4372	a	-7.2862	1.4601
b	0.1648	0.0752	b	2.8449	0.5529
c	-0.0009	0.0032	c	-0.2308	0.0390
d	-0.0746	0.0310	d	-0.0101	0.0001
e	0.0007	0.0006			

The performance of the global inactivation model was validated by the Accuracy factor (*Af*). *Af* is a measure of how close the predicted value is to the experimental value (the further from 1, the more inaccurate) (Oscar, 2005). In the present study, *Af* for 5 and 10 mL/L of grape concentration were 1.26 and 1.15, respectively, which indicates an error rate of 26% and 15% for these predictions. Therefore, the models developed can accurately predict *B. cereus* inactivation in a rice matrix, at different pH values and in a temperature range between 10 and 30 °C, which is considered critical from the food safety point of view. Similar to this study, Rodrigo et al., (2003) analyzed *Escherichia coli* inactivation by pulsed electric fields and the experimental data were fitted to the Weibull distribution function using a one-step non lineal regression and scale and shape parameters were deduced. A secondary model for the scale parameter was also built. To improve the precision of the coefficients of the secondary model, a global model using all experimental data was constructed similar to **equations (5) and (6)** of the present study, with very good results. A problem that could arise in applying the kinetic parameter obtained by a secondary model to estimate the residual microbiological load is related with the shape parameter in the primary models. Couvert et al., (2005) solved this problem using a single *p* value, estimated from the entire data set. Despite a slight loss of fit goodness, this modification improved the robustness of the model, a fact that also occurred in the present work, in which a single *p* value has been used within the data set of each concentration of grape extract. This method was also suggested by other authors (Corradini et al., 2008; Mafart et al., 2002).

Results

Figure 2.4 illustrates the response surface of the global model with the relationship between the influence of pH and incubation time on *B. cereus* inhibition for concentrations 5 mL/L (a) and 10 mL/L (b) of grape extract. The figure reveals that inactivation increased with treatment time and pH decreased. In addition, for the 10 mL/L concentration, a slight decrease in inactivation was observed for pH 5.5.

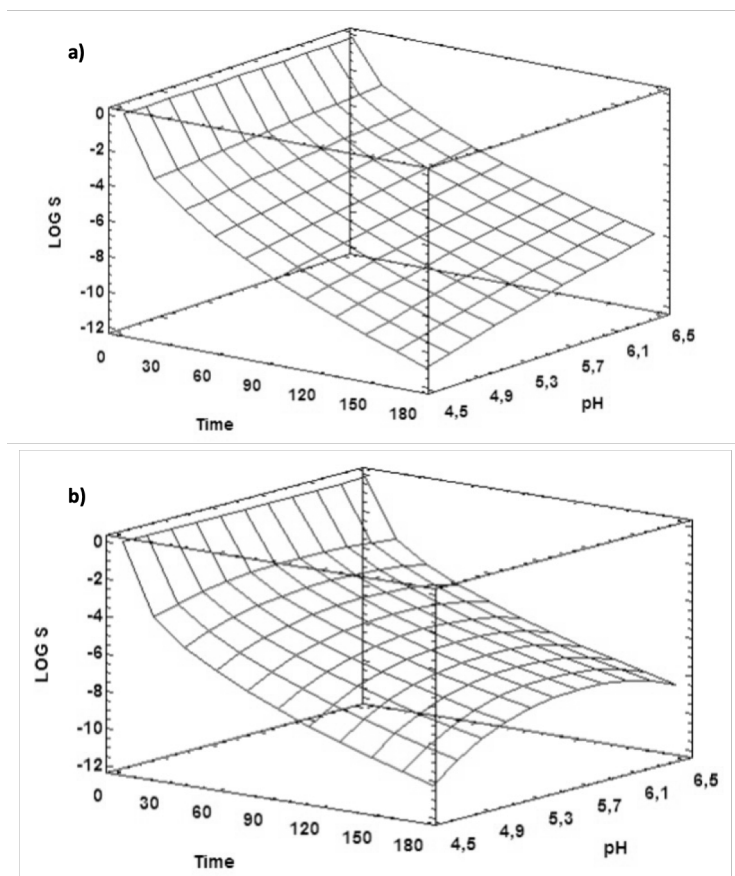


Figure 2.4. Three-dimensional relationship between the influence of pH and incubation time at a constant temperature of 20°C on *B. cereus* inactivation for a) 5 mL/L and b) at 10 mL/L concentration of grape extract.

Considering the global model obtained (equations (5) and (6)), a tertiary model was constructed using Microsoft Visual Studio C++. This developed software can be implemented in industry to help in the decision-making regarding pH and temperature at each concentration level with reference to

the initial microbial load. It will allow an industrial business operator to adjust the storage conditions (time and temperature) to achieve the microbiological stability of the food based on its initial contamination. Besides, it can be chosen between two concentrations of grape extract, according to the food matrix properties. At the same time, for specific pH and storage temperature conditions it can also be adjusted the maximum incubation time that the food matrix can be stored without causing any food safety issue (final microbial concentration remains below a specific value) the software will. **Figure 2.5** shows a screenshot of the software.

Figure 2.5. Screenshot of the software developed (tertiary model) to calculate the microorganism concentration after grape extract treatment combined with pH and temperature.

2.4. Conclusions

The present study proposes a quantitative approach to evaluate the antimicrobial potential of a natural coloring agent coming from an industrial by-product, namely grape extract, against *B. cereus*. Grape extract, at a concentration of 5 mL/L, has proven value as a way to control the bacterial load in a potential cold chain breach. In addition, this food ingredient, used in combination with other preservation treatments such as lowering the pH of the medium and the temperature, can attain maximum levels of *B. cereus* inactivation (6 log reductions) at grape extract concentrations of 5–10 mL/L.

Furthermore, software developed from the global model can help in selecting the combination of grape extract concentration, incubation time and temperature, as well as the pH of the medium in order to attain the desired log reduction of *B. cereus* loads. Although the study indeed has some limitations that are indicated by the limits of the parameters that have been studied, temperatures between 10 and 30 °C, pH between 4.5 and 6.5 and grape extract concentrations 1, 5 and 10 mL/L.

The results reported in this manuscript provide evidence for the positive effects of adding food ingredients such as grape extract, which have bacteriostatic or bactericidal properties. Further investigation is needed to evaluate the sensory acceptability of such products to consumers.

Declaration of conflicting interests: None.

CRedit authorship contribution statement: Eva Grau-Fuentes: Investigation, Writing – original draft. María Úbeda-Manzanaro: Investigation. Antonio Martínez: Data curation, Writing – review & editing. Raquel Garzón: Formal analysis. Cristina M. Rosell: Funding acquisition, Writing – review & editing. Dolores Rodrigo: Supervision, Conceptualization, Writing – review & editing.

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References

- Albert, I., & Mafart, P. (2005). A modified Weibull model for bacterial inactivation. *International Journal of Food Microbiology*, 100(1–3), 197–211. <https://doi.org/10.1016/j.ijfoodmicro.2004.10.016>
- Arts, I., & Hollman, P. (1998). Optimization of a quantitative method for the determination of catechins in fruits and legumes. *Journal of Agricultural and Food Chemistry*, 46(12), 5156–5162. <https://doi.org/10.1021/jf9805092>
- Balaban, M., Koc, C., Sar, T., & Akbas, M. Y. (2021). Antibiofilm effects of pomegranate peel extracts against *B. cereus*, *B. subtilis*, and *E. faecalis*. *International Journal of Food Science and Technology*, 56(10), 4915–

4924. <https://doi.org/10.1111/ijfs.15221>
- Bosso, A., Cassino, C., Motta, S., Panero, L., Tsolakis, C., & Guaita, M. (2020). Polyphenolic Composition and In Vitro Antioxidant Activity of Red Grape Seeds as Byproducts of Short and Medium-Long Fermentative Macerations. *Foods*, 9(10). <https://doi.org/10.3390/foods9101451>
- Brezoiu, A., Matei, C., Deaconu, M., Stanciuc, A., Trifan, A., Gaspar-Pintiliescu, A., & Berger, D. (2019). Polyphenols extract from grape pomace. Characterization and valorisation through encapsulation into mesoporous silica-type matrices. *Food and Chemical Toxicology*, 133. <https://doi.org/10.1016/j.fct.2019.110787>
- Cheng, V. J., Bekhit, A. E. D. A., McConnell, M., Mros, S., & Zhao, J. (2012). Effect of extraction solvent, waste fraction and grape variety on the antimicrobial and antioxidant activities of extracts from wine residue from cool climate. *Food Chemistry*, 134(1), 474–482.
- Corradini, M., Normand, M., & Peleg, M. (2008). *Nonlinear Kinetics: Principles and Potential Food Applications* (G. GutierrezLopez, G. BarbosaCanovas, J. WelteChanes, & E. ParadaArias, Eds.; p. 71). https://doi.org/10.1007/978-0-387-75430-7_3
- Couvert, O., Gaillard, S., Savy, N., Mafart, P., & Leguerinel, I. (2005). Survival curves of heated bacterial spores: Effect of environmental factors on Weibull parameters. *International Journal of Food Microbiology*, 101(1), 73–81. <https://doi.org/10.1016/j.ijfoodmicro.2004.10.048>
- Delbrassinne, L., Botteldoorn, N., Andjelkovic, M., Dierick, K., & Denayer, S. (2015). An Emetic *Bacillus cereus* Outbreak in a Kindergarten: Detection and Quantification of Critical Levels of Cereulide Toxin. *Foodborne Pathogens and Disease*, 12(1), 84–87. <https://doi.org/10.1089/fpd.2014.1788>
- Ferreira, A. S., Nunes, C., Castro, A., Ferreira, P., & Coimbra, M. A. (2014). Influence of grape pomace extract incorporation on chitosan films properties. *Carbohydrate Polymers*, 113, 490–499. <https://doi.org/10.1016/j.carbpol.2014.07.032>
- Fontana, A. R., Antonioli, A., & Bottini, R. (2013). Grape Pomace as a Sustainable Source of Bioactive Compounds: Extraction, Characterization, and Biotechnological Applications of Phenolics. *Journal Of Agricultural And Food Chemistry*, 61(38), 8987–9003. <https://doi.org/10.1021/jf402586f>
- Geeraerd, A., Valdramidis, V., & Van Impe, J. (2006). GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves (vol 102, pg 95, 2005). *International Journal of Food Microbiology*, 110(3), 297–297. <https://doi.org/10.1016/j.ijfoodmicro.2006.04.002>

- Gerardi, C., Pinto, L., Baruzzi, F., & Giovinazzo, G. (2021). Comparison of Antibacterial and Antioxidant Properties of Red (cv. Negramaro) and White (cv. Fiano) Skin Pomace Extracts. *Molecules*, 26(19). <https://doi.org/10.3390/molecules26195918>
- Gilbert, R., Stringer, M., & Peace, T. (1974). Survival and Growth of *Bacillus Cereus* in boiled and fried rice in relation to outbreaks of food poisoning. *Journal of Hygiene*, 73(3), 433–444. <https://doi.org/10.1017/S0022172400042790>
- Gokturk Baydar, N., Ozkan, G., & Yasar, S. (2007). Evaluation of the antiradical and antioxidant potential of grape extracts. *Food Control*, 18(9), 1131–1136. <https://doi.org/10.1016/j.foodcont.2006.06.011>
- Goncalves, L. A., Lorenzo, J. M., & Trindade, M. A. (2021). Fruit and Agro-Industrial Waste Extracts as Potential Antimicrobials in Meat Products: A Brief Review. *Foods*, 10(7). <https://doi.org/10.3390/foods10071469>
- Guaita, M., & Bosso, A. (2019). Polyphenolic Characterization of Grape Skins and Seeds of Four Italian Red Cultivars at Harvest and after Fermentative Maceration. *Foods*, 8(9). <https://doi.org/10.3390/foods8090395>
- Hendriksen, N. B., Hansen, B. M., & Johansen, J. E. (2006). Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in a sandy loam. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 89(2), 239–249. <https://doi.org/10.1007/s10482-005-9025-y>
- Juneja, V. K., Golden, C. E., Abhinav Mishra, Harrison, M. A., Mohr, T., & Silverman, M. (2019). Predictive model for growth of *Bacillus cereus* during cooling of cooked rice. *International Journal of Food Microbiology*, 290, 49–58. <https://doi.org/10.1016/j.ijfoodmicro.2018.09.023>
- Katalinić, V., Možina, S. S., Skroza, D., Generalić, I., Abramović, H., Miloš, M., Ljubenković, I., Piskernik, S., Pezo, I., Terpinč, P., & Boban, M. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 *Vitis vinifera* varieties grown in Dalmatia (Croatia). *Food Chemistry*, 119(2), 715–723. <https://doi.org/10.1016/j.foodchem.2009.07.019>
- Kwon, M. J., Rhee, M. S., & Yoon, K. S. (2020). A risk assessment study of *Bacillus cereus* in packaged tofu at a retail market in Korea. *Food Science and Biotechnology*, 29(3), 339–350. <https://doi.org/10.1007/s10068-019-00670-0>
- Ky, I., Lorrain, B., Kolbas, N., Crozier, A., & Teissedre, P.-L. (2014). Wine by-Products: Phenolic Characterization and Antioxidant Activity Evaluation of Grapes and Grape Pomaces from Six Different French Grape

- Varieties. *Molecules*, 19(1), 482–506.
<https://doi.org/10.3390/molecules19010482>
- Ky, I., & Teissedre, P.-L. (2015). Characterisation of Mediterranean Grape Pomace Seed and Skin Extracts: Polyphenolic Content and Antioxidant Activity. *Molecules*, 20(2), 2190–2207.
<https://doi.org/10.3390/molecules20022190>
- Lake, R., Hudson, A., & Cressey, P. (2004). *Risk profile of Bacillus spp. In rice*. <https://www.mpi.govt.nz/dmsdocument/26138/direct>
- Levy, J., Boyer, R. R., Neilson, A. P., O’Keefe, S. F., Chu, H. S. S., Williams, R. C., Dorenkott, M. R., & Goodrich, K. M. (2017). Evaluation of peanut skin and grape seed extracts to inhibit growth of foodborne pathogens. *Food Science & Nutrition*, 5(6), 1130–1138.
<https://doi.org/10.1002/fsn3.503>
- Little, C. L., Barnes, J., Mitchell, R. T., & Food Standards Agency (FSA) and Public Health Laboratory Service (PHLS). (2002). Microbiological quality of take-away cooked rice and chicken sandwiches: Effectiveness of food hygiene training of the management. *Communicable Disease and Public Health*, 5(4), 289–298.
- Mafart, P., Couvert, O., Gaillard, S., & Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: Application of the Weibull frequency distribution model. *International Journal of Food Microbiology*, 72(1–2), 107–113. [https://doi.org/10.1016/S0168-1605\(01\)00624-9](https://doi.org/10.1016/S0168-1605(01)00624-9)
- Marugan, J., van Grieken, R., Sordo, C., & Cruz, C. (2009). Kinetics of the photocatalytic disinfection of Escherichia coli suspensions (vol 82, pg 27, 2008). *Applied Catalysis B-Environmental*, 88(3–4), 582–583.
<https://doi.org/10.1016/j.apcatb.2008.11.006>
- Mau, J.-L., Chen, C.-P., & Hsieh, P.-C. (2001). Antimicrobial Effect of Extracts from Chinese Chive, Cinnamon, and Corni Fructus. *Journal of Agricultural and Food Chemistry*, 49(1), 183–188.
<https://doi.org/10.1021/jf000263c>
- Oki, T., Masuda, M., Kobayashi, M., Nishiba, Y., Furuta, S., Suda, I., & Sato, T. (2002). Polymeric procyanidins as radical-scavenging components in red-hulled rice. *Journal of Agricultural and Food Chemistry*, 50(26), 7524–7529. <https://doi.org/10.1021/jf025841z>
- Oliveira, D. A., Salvador, A. A., Smania, A., Smania, E. F. A., Maraschin, M., & Ferreira, S. R. S. (2013). Antimicrobial activity and composition profile of grape (*Vitis vinifera*) pomace extracts obtained by supercritical fluids. *Journal of Biotechnology*, 164(3), 423–432.
- Oliveira, A. L. de, Brunini, M. A., Salandini, C. A. R., & Bazzo, F. R. (2003).

- Caracterização tecnológica de jabuticabas ‘Sabará’ provenientes de diferentes regiões de cultivo. *Revista Brasileira de Fruticultura*, 25(3), 397–400.
- Oscar, T. (2005). Development and validation of primary, secondary, and tertiary models for growth of *Salmonella typhimurium* on sterile chicken. *Journal of Food Protection*, 68(12), 2606–2613. <https://doi.org/10.4315/0362-028X-68.12.2606>
- Pao, S., Khalid, F., & Kalantari, A. (2006). Inhibiting the growth of *Bacillus cereus* in raw sprouts and cooked rice using red clover seeds. *Internet Journal of Food Safety*, 8.
- Periago, P. M., & Moezelaar, R. (2001). Combined effect of nisin and carvacrol at different pH and temperature levels on the viability of different strains of *Bacillus cereus*. *International Journal of Food Microbiology*, 68(1), 141–148. [https://doi.org/10.1016/S0168-1605\(01\)00461-5](https://doi.org/10.1016/S0168-1605(01)00461-5)
- Prado Martin, J., Porto, E., Corrêa, C., Alencar, S., da Gloria, E., Simone, I., Cabral, R., & Aquino, L. (2012). *Antimicrobial potential and chemical composition of agro-industrial wastes*.
- Rodrigo, D., Barbosa-Canovas, G., Martinez, A., & Rodrigo, M. (2003). Weibull distribution function based on an empirical mathematical model for inactivation of *Escherichia coli* by pulsed electric fields. *Journal of Food Protection*, 66(6), 1007–1012. <https://doi.org/10.4315/0362-028X-66.6.1007>
- Rodrigo, D., Rosell, C. M., & Martinez, A. (2021). Risk of *Bacillus cereus* in Relation to Rice and Derivatives. *Foods*, 10(2). <https://doi.org/10.3390/foods10020302>
- Ross, T. (1996). Indices for performance evaluation of predictive models in food microbiology. *Journal of Applied Bacteriology*, 81(5), 501–508. <https://doi.org/10.1111/j.1365-2672.1996.tb03539.x>
- Sánchez, J., Correa, M., & Castañeda, L. (2016). *Bacillus cereus* an important pathogen the microbiological control of food. *Revista Facultad Nacional de Salud Pública*, 34(2), 230–242. <https://doi.org/10.17533/udea.rfnsp.v34n2a12>
- Shi, J., Yu, J., Pohorly, J. E., & Kakuda, Y. (2003). Polyphenolics in grape seeds-biochemistry and functionality. *Journal of Medicinal Food*, 6(4), 291–299. <https://doi.org/10.1089/109662003772519831>
- Wei, X., & Huang, X. (2019). 1—Origin, taxonomy, and phylogenetics of rice. In J. Bao (Ed.), *Rice (Fourth Edition)* (pp. 1–29). AACC International Press. <https://doi.org/10.1016/B978-0-12-811508-4.00001-0>
- Yadav, D., Kumar, A., Kumar, P., & Mishra, D. (2015). Antimicrobial properties of black grape (*Vitis vinifera* L.) peel extracts against antibiotic-

- resistant pathogenic bacteria and toxin producing molds. *Indian Journal of Pharmacology*, 47(6), 663–667. <https://doi.org/10.4103/0253-7613.169591>
- Yu, J., Ahmedna, M., & Goktepe, I. (2010). Potential of peanut skin phenolic extract as antioxidative and antibacterial agent in cooked and raw ground beef. *International Journal of Food Science & Technology*, 45(7), 1337–1344.
- Yu, J., Ahmedna, M., & Goktepe, P. (2005). Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chemistry*, 90(1–2), 199–206. <https://doi.org/10.1016/j.foodchem.2004.03.048>
- Yu, S., Yu, P., Wang, J., Lie, C., Guo, H., Liu, C., Kong, L., Yu, L., Wu, S., Lei, T., Chen, M., Zeng, H., Pang, R., Zhang, Y., Wei, X., Zhang, J., Wu, Q., & Ding, Y. (2020). A Study on Prevalence and Characterization of *Bacillus cereus* in Ready-to-Eat Foods in China. *Frontiers in Microbiology*, 10. <https://doi.org/10.3389/fmicb.2019.03043>



**An industrial exposure
assessment approach for
Bacillus cereus in cooked
rice matrix containing
grape extract**

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and Dolores Rodrigo.

Monte Carlo simulation
Risk
Estimate final load
HAACCP
Modular modelling

Cooked rice is a commonly eaten staple food in many countries. Its high carbohydrate content creates a favorable environment for the growth of *Bacillus cereus*. Despite traditional cooking methods, the spores of *B. cereus* are resistant and can still become active and multiply under certain storage conditions after cooking. This poses a significant health risk to consumers. To address this, natural antimicrobials have gained popularity as effective methods of preventing bacterial growth. This study aimed to evaluate the presence of *B. cereus* in cooked rice, both with and without the addition of 0.1% grape extract. The objective was to develop a modular Industrial Exposure Assessment model to obtain an estimate of the final load level of *B. cereus* after undergoing combined treatments of different control measures in a rice matrix. This information can assist in decision-making and recommend appropriate control measures to reduce the risks associated with this bacteria on rice derivative foodstuffs. The Exposure Assessment model developed incorporates mathematical models obtained on previous chapters and has a heating module and a storage module into a Excell (Microsoft Corporation) spreadsheet. By utilizing Monte Carlo simulation, the level of *B. cereus* at the time of consuming in the rice matrix can be estimated.

3.1. Introduction

As discussed in previous chapters, *Bacillus cereus* is an important spore-forming pathogen that can cause food poisoning in humans. In 2021, EFSA reported that 679 cases of *B. cereus* food poisoning were identified in the European Union, which places *B. cereus* as the pathogen with the highest number of food outbreaks by toxin-producing bacteria mainly in rice (Authority & European Centre for Disease Prevention and Control, 2022). As mentioned in previous chapters, the main source of contamination is the heat-resistant spores that can survive standard cooking conditions (Gilbert et al., 1974; Rodrigo et al., 2021). Following cooking, any surviving spores can germinate and grow rapidly because poor cooling or prolonged storage at room temperature, particularly in restaurants and canteens where large quantities of rice are cooked, contributes to *B. cereus* outbreaks (Juneja et al., 2019; Lake et al., 2004; Yu et al., 2020). Taking control of the growth of *B. cereus* in rice-based products is necessary to improve public health. Therefore, to ensure the safety and quality of processed products, it is essential to ensure that the growth of foodborne pathogens is eliminated or reduced after processing and during storage (Boisrobert et al., 2010). In this sense, the combination of hurdles (such as the combination of heat treatments, pH variations and the use of natural antimicrobials) can control or even prevent the growth of pathogens, resulting in a reduction of risk (Juneja et al., 2019). Microbiological studies are usually performed from which approximations (microbiological predictive models) are obtained to determine the survival of the microorganism after the application of different treatments. Those models are used to calculate the kinetic parameters, with the handicap that these models do not take into account real variations of the input variables and provide fixed output values predicting how bacterial proliferation will be under given particular conditions. Considering the above, and using deterministic models as a baseline, industry need to assess risk based on probability functions to help in setting up a Hazard Analysis and Critical Control Point (HACCP) systems in the processing line, and in making decision on the most appropriate preservation or cooking process. A process for making decisions is the Risk Assessment and has four components: Hazard Identification, Hazard Characterization, Exposure Assessment and Risk Characterization. It is currently used by public health authorities in decision

making. The tool can be also used at the industrial level providing beside the HACCP tool the highest levels of food safety.

The most important step of the industrial Risk Assessment to make decisions is the Exposure Assessment component. Codex defines Exposure Assessment as “the qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant.” (CAC, 1999). Exposure assessment may be undertaken as part of a risk assessment, or it can be a stand-alone process, Exposure Assessment component in a quantitative Risk Assessment will often rely on a model, encompassing knowledge of the factors and their interactions that affect the number and distribution of the hazard in foods, to estimate the microbial level in the finished product or during refrigeration storage during transport and distribution. Those models can include key input parameters defined by probability distributions, and deterministic data. The output of the model can be an estimate of the final load levels of *B. cereus* after undergo combined treatments of different control measures. Therefore, the aim of this study was to develop an Industrial Exposure Assessment model for the estimation of *Bacillus cereus* level after heat treatment (rice cooking stage) and storage treatment (storage of cooked rice) in the presence of 0.1% of grape extract as a natural antimicrobial in a cooked rice matrix.

3.2. Material and methods

3.2.1. Exposure Assessment model

The Exposure Assessment model was developed considering the parameters that define the inactivation and growth curves of *Bacillus cereus* in a rice derivative matrix.

The experimental results used for the development of this exposure assessment correspond to those obtained in **Chapters 1 and 2 (Figure 3.1)**. As seen in **Chapter 1**, the results of the heat treatment or inactivation were fitted to the Weibull distribution function as a predictive model to determine the final number of *B. cereus* after treatment. Ongoing through the growth part, or storage treatment, which corresponds to **Chapter 2**, data from the 0.1 % grape extract concentration were modeled for the present study. These data during the storage period were fitted to the modified Gompertz equation (**Equation 1**). (Zwietering et al., 1990).

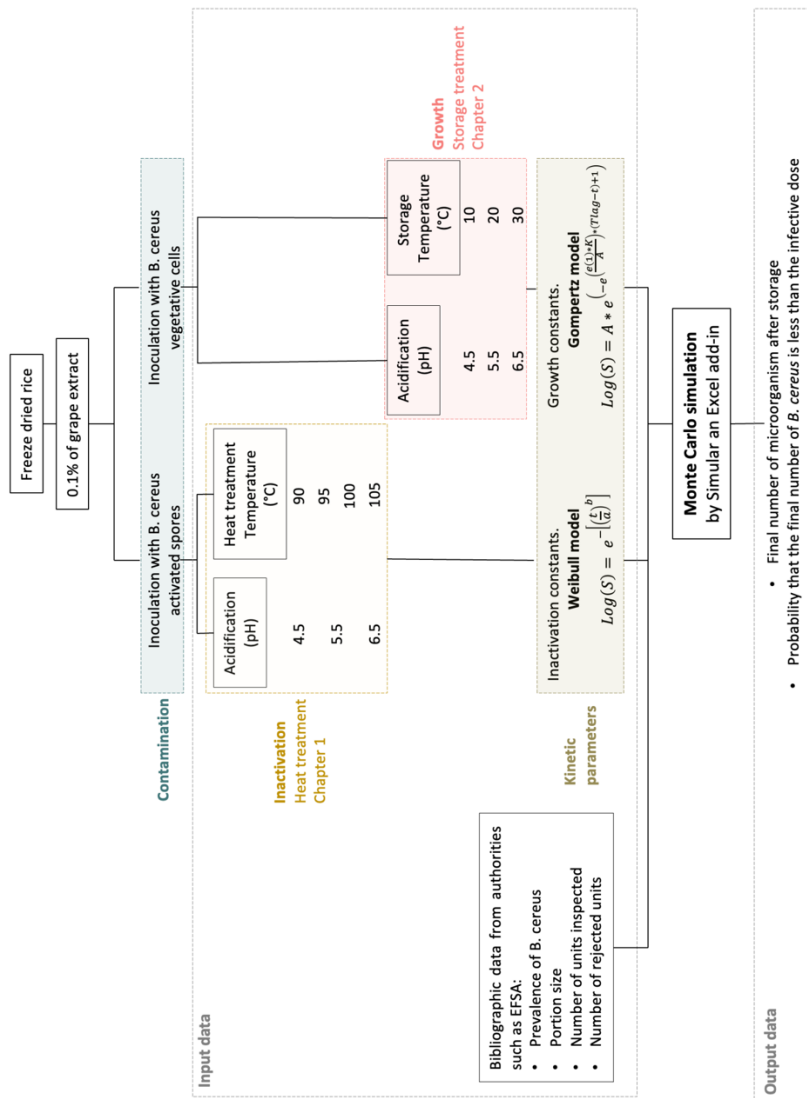


Figure 3.1. Schematic representation of the development of the exposure assessment carried out, where the input data and output data can be observed.

$$\text{Log}(S) = A * e^{\left(e^{\left(\frac{e(1)*K}{A} \right) * (T \text{lag} - t) + 1} \right)} \quad \text{Equation 1}$$

Where Log (S) is the log of the survival fraction; A asymptote estimation; K is the growth rate; T lag is the duration of the latency phase; t is the storage time.

Therefore, kinetic parameters were obtained to determine the inactivation constants of the Weibull model and the growth constants of the Gompertz equation. In the stochastic model, probability distributions affects to the survival and storage parameters were used and analyzed by Monte Carlo simulation (Vose, 1998). The overall inactivation/growth model can be viewed as a computation of three blocks. The input parameters, the stochastic model itself and the output parameters:

1. Input parameters:

- a. Conditions under the food has been treated. Conditions that are chosen within the model such as temperature, pH and cooking time of the rice, and temperature, pH and storage time.
- b. Experimental data: Initial contamination of *B. cereus*.
- c. Bibliographic data from authorities, such as EFSA. Those used in this model are the prevalence of *B. cereus*, serving size, per capita rice consumption, and number of units tested and rejected.
- d. Kinetic constants. As described above, the kinetic constants for exposure assessment are calculated from fits to the Weibull and Gompertz models.

2. The model itself. Where the Monte Carlo simulation was used.

3. The output data. Which gives the estimated number and the probability of *B. cereus* cells ingested after specified cooking and storage conditions.

3.2.2. Simulation

Monte Carlo simulation was carried out by using the *Simular* excel add-in (Version 26e), (<https://www.simularsoft.com.ar/>), wich uses Monte Carlo simulation to estimate the level of *B. cereus* after storage time of cooked rice

with or without 0.1% grape extract. The simulation was conducted using a predetermined number of iterations. An iteration in Monte Carlo simulation involves a complete cycle of generating random input values, executing the model, and obtaining a result (**Figure 3.2**). This process is repeated multiple times to accurately capture the variability inherent in the model's outcomes.

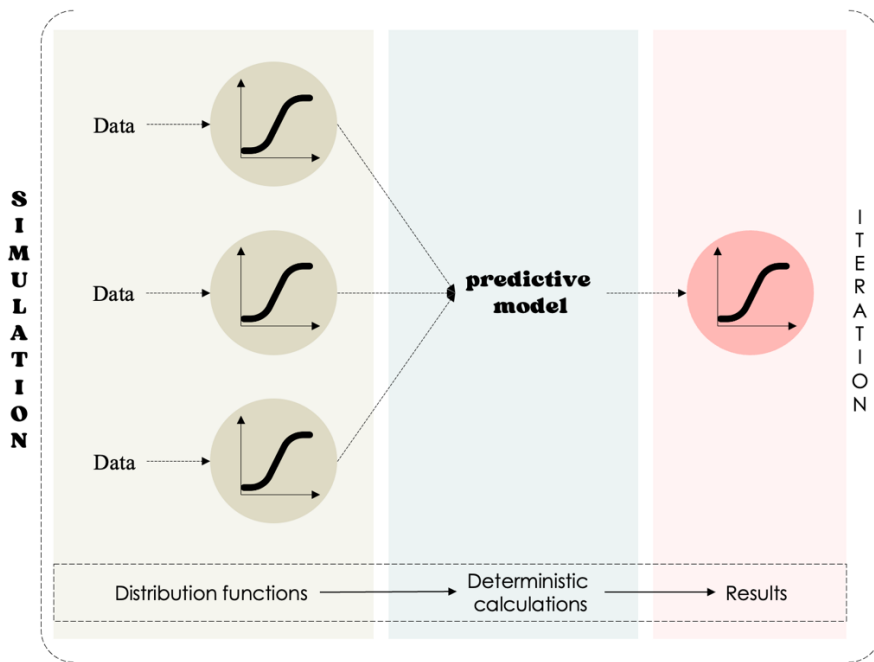


Figure 3.2. Monte Carlo simulation process

3.3. Results and discussion

The modular inactivation/growth exposure assessment model is a stochastic model in which the final *B. cereus* load and probability that the final number of the microorganism is lower than the infective dose is deduced. To see the applicability of the Exposure Assessment model it was applied in two specific scenarios (**Table 3.1**), with the presence (scenario I) and absence (scenario II) of grape extract. In those scenarios unfavorable and real conditions are presented, such as a low cooking temperature (90 °C) at a pH close to neutrality (6.5) for a more usual rice cooking time (20 min), and storage in which the cold chain has been broken for several hours (20 °C for 20h).

Table 3.1. Conditions for the two specific scenarios used to explain the application of the exposure assessment model.

	Scenario I	Scenario II
Grape Extract	0.1%	-
Cooking temperature (°C)	90	
pH	6.5	
Cooking time (min)	20	
Storage temperature (°C)	20	
Storage time (h)	20	

Input kinetic values mentioned above were calculated by using the Weibull distribution function (rice inactivation or cooking module) and the Gompertz model (rice growth or storage module), (Table 3.2) for both scenarios. Observing the values shown in Table 3.2, it can be seen, as expected, that the value of the growth rate is lower in scenario I (presence of grape extract) than in scenario II (absence of grape extract), and it can also be seen that the parameter scale parameter “*b*” follows the same trend as the growth rate. Those parameters were used as input values on the Exposure Assessment model, along to the initial contamination of the raw material that follows a normal distribution. The shape parameter “*a*” of the Weibull distribution function followed also a normal distribution and was also considered as input data. Intermedial calculations, running Monte Carlo simulation, of the remaining cells after cooking or the microbial load after storage were obtained by the Weibull distribution function and the Gompertz equation respectively.

Table 3.2. Input data result for the two example scenarios.

	Scenario I	Scenario II
scale parameter	3,8926	3,8926
shape parameter	0,3595	0,4467
Initial Contamination	5,3log	5,4log
growth rate	0,06	0,31
Lag phase	0.13	11.34

Table 3.3. Output results obtained after running the Monte Carlo simulation for the two scenarios used in the current chapter.

	Scenario I	Scenario II
Iterations N°	1000	
Minimum	0	0
Average	80068.070	283896.356
Maximum	18633109.812	66041662.322
Median	2259.967	7931.553
Standard deviation	684712.587	2427323.796
Kurtosis	552.740	552.351
Skewness	21.599	21.591
Coef. of variation	8.552	8.550
Percentile 5%	21.53	81.51
Percentile 24%	354.08	1266.38
Percentile 25%	380.12	1380.0
Percentile 50%	2259.97	7931.55
Percentile 75%	13824.04	48726.42
Percentile 95%	186319.66	662255.11
Less than infective dose	71.50%	53.90%

Table 3.3 shows the Monte Carlo simulation output values for scenario I and II. A total of 1000 iterations, were performed. These values were between -1.670 and 18633109cfu/ g for scenario I and 0.010 and 66041662cfu/ g for scenario II. As can be seen in the same table, the Skewness values for scenario I and scenario II are positive, which indicates that we are dealing with an asymmetric distribution with a long tail to the right (Brys et al., 2004). This fact is related to the median value since it is observed that 50% of the values are below 2259 and 7931 cfu/ g for the cases with grape extract and without, respectively. These values are closer to the minimum values for *B. cereus*. Therefore, the Skewness values are positive and the tail is on the right. In the literature, a value of 10^5 - 10^8 cfu/ g is usually considered to be the infective dose for *B. cereus*, but lower bacterial counts have also been reported in cases of human poisoning; therefore, no food containing more than 10^4 cfu/ g can be considered completely safe for consumption (Hazards (BIOHAZ), 2016). Therefore, regarding the probability that the *B. cereus* load is lower than the infective dose (10^4 cfu/ g) the results obtained for scenario I are 71.50% and

53.90% for scenario II. These probability data show that assuming the conditions of these scenarios, out of 100 rice portions cooked and stored under the conditions of scenario I, 72 of them will have a final *B. cereus* value $\leq 10^4$ and in the case that the conditions of scenario II are met, 54 portions will have a *B. cereus* load lower than the infective dose. Within all the output values provided by the Monte Carlo simulation, it is worth noting that the addition of grapes reduces the probability of risk of ingestion of a rice ration with a *B. cereus* load above the infective dose. Specifically, for the scenarios used in this chapter, this reduction in probability between adding grape extract and not adding grape extract is 18%.

As seen in the results presented in **Chapter 2** (Grau-Fuentes et al., 2023), grape extract has bactericidal capacity at concentrations of 0.5 and 1%, and bacteriostatic or bactericidal at concentrations of 0.1% depending on pH and storage temperature, a fact that is corroborated in the present chapter since the probability that the final load of *B. cereus* is lower than the infective dose is considerably higher in the presence of grape extract. The study by (Katalinić et al., 2010) corroborates the antimicrobial capacity of grape extracts, in this case of white and red grapes, achieving this capacity against large positive and large negative bacteria in both cases. This chapter has worked on the exposure assessment process for *B. cereus* in rice by performing a heat treatment and a storage treatment, using a holistic strategy in the presence and absence of grape extract and varying the pH of the medium and temperature. Risk assessments have also been performed for *B. cereus* in different food matrices such as in water-based buffalo mozzarella cheese (Montone et al., 2020), couscous semolina (Ziane et al., 2019), milk-based beverages (Pina-Pérez et al., 2012), the latter in combination with different strategies such as the use of high hydrostatic pressures and the use of cocoa as an antimicrobial intervention. The use of the hurdles combination is very usable in the preservation of foodstuffs as it provides a reduction in the risk of microorganism growth, obtaining products with greater food safety.

3.4. Conclusions

In this chapter, we evaluate the exposure to *Bacillus cereus* in cooked rice during both the cooking process and storage, considering the presence and absence of grape extract as a natural antimicrobial. Predictive mathematical

models were employed to describe the microorganism's inactivation during cooking and its growth during storage, utilizing Monte Carlo simulations. This methodology provides a stochastic estimate of the final *B. cereus* count at the end of storage under specific conditions. The exposure assessment offers a comprehensive overview of how various stages of the production chain (such as processing, distribution, handling, and preparation) affect the level and severity of the hazard at the point of consumption. Additionally, it helps correlate the probability of achieving a specific final concentration of *B. cereus* with the existing controls (such as antimicrobial use, pH, and temperature regulation), aiming to enhance the microbiological safety of rice. This assessment is a valuable tool for the industry, as it delivered precise estimates of pathogen levels in the final product, while clearly addressing uncertainty and variability. Consequently, it supports predictive decision-making and the implementation of necessary control measures to reduce the risk associated with *B. cereus*.

References

- Authority, E. F. S. & European Centre for Disease Prevention and Control. (2022). The European Union One Health 2021 Zoonoses Report. *EFSA Journal*, 20(12), e07666. <https://doi.org/10.2903/j.efsa.2022.7666>
- Boisrobert, C. E., Stjepanovic, A., Oh, S., & Lelieveld, H. L. M. (Eds.). (2010). Chapter 4—A Simplified Guide to Understanding and Using Food Safety Objectives and Performance Objectives. In *Ensuring Global Food Safety* (pp. 91–98). Academic Press. <https://doi.org/10.1016/B978-0-12-374845-4.00004-7>
- Brys, G., Hubert, M., & Struyf, A. (2004). A Robust Measure of Skewness. *Journal of Computational and Graphical Statistics*, 13(4), 996–1017. <https://doi.org/10.1198/106186004X12632>
- Gilbert, R., Stringer, M., & Peace, T. (1974). Survival and growth of *Bacillus cereus* in boiled and fried rice in relation to outbreaks of food poisoning. *Journal of Hygiene*, 73(3), 433–444. <https://doi.org/10.1017/S0022172400042790>
- Grau-Fuentes, E., Úbeda-Manzanaro, M., Martínez, A., Garzón, R., Rosell, C. M., & Rodrigo, D. (2023). Evaluation of the antimicrobial activity of grape extract against *Bacillus cereus* in rice. *LWT*, 175, 114481. <https://doi.org/10.1016/j.lwt.2023.114481>
- Hazards (BIOHAZ), E. P. on B. (2016). Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. Including *Bacillus thuringiensis* in foodstuffs. *EFSA Journal*, 14(7), e04524. <https://doi.org/10.2903/j.efsa.2016.4524>

- Juneja, V. K., Golden, C. E., Abhinav Mishra, Harrison, M. A., Mohr, T., & Silverman, M. (2019). Predictive model for growth of *Bacillus cereus* during cooling of cooked rice. *International Journal of Food Microbiology*, 290, 49–58. <https://doi.org/10.1016/j.ijfoodmicro.2018.09.023>
- Katalinić, V., Možina, S. S., Skroza, D., Generalić, I., Abramović, H., Miloš, M., Ljubenković, I., Piskernik, S., Pezo, I., Terpinč, P., & Boban, M. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 *Vitis vinifera* varieties grown in Dalmatia (Croatia). *Food Chemistry*, 119(2), 715–723. <https://doi.org/10.1016/j.foodchem.2009.07.019>
- Lake, R., Hudson, A., & Cressey, P. (2004). *Risk profile of Bacillus spp. In rice*. <https://www.mpi.govt.nz/dmsdocument/26138/direct>
- Montone, A. M. I., Capuano, F., Mancusi, A., Di Maro, O., Peruzy, M. F., Proroga, Y. T. R., & Cristiano, D. (2020). Exposure to *Bacillus cereus* in Water Buffalo Mozzarella Cheese. *Foods*, 9(12). <https://doi.org/10.3390/foods9121899>
- Pina-Pérez, M. C., Silva-Angulo, A. B., Rodrigo, D., & López, A. M. (2012). A preliminary exposure assessment model for *Bacillus cereus* cells in a milk based beverage: Evaluating High Pressure Processing and antimicrobial interventions. *Food Control*, 26(2), 610–613. <https://doi.org/10.1016/j.foodcont.2012.01.063>
- Rodrigo, D., Rosell, C. M., & Martinez, A. (2021). Risk of *Bacillus cereus* in Relation to Rice and Derivatives. *FOODS*, 10(2). <https://doi.org/10.3390/foods10020302>
- Vose, D. J. (1998). The Application of Quantitative Risk Assessment to Microbial Food Safety. *Journal of Food Protection*, 61(5), 640–648. <https://doi.org/10.4315/0362-028X-61.5.640>
- Yu, S., Yu, P., Wang, J., Lie, C., Guo, H., Liu, C., Kong, L., Yu, L., Wu, S., Lei, T., Chen, M., Zeng, H., Pang, R., Zhang, Y., Wei, X., Zhang, J., Wu, Q., & Ding, Y. (2020). A Study on Prevalence and Characterization of *Bacillus cereus* in Ready-to-Eat Foods in China. *Frontiers in Microbiology*, 10. <https://doi.org/10.3389/fmicb.2019.03043>
- Ziane, M., Leguerinel, I., & Membré, J.-M. (2019). A quantitative microbiological exposure assessment of *Bacillus cereus* group IV in couscous semolina, Algeria. *Microbial Risk Analysis*, 11, 11–22. <https://doi.org/10.1016/j.mran.2018.07.001>
- Zwietering, M. H., Jongenburger, I., Rombouts, F. M., & Riet, K. van 't. (1990). Modeling of the Bacterial Growth Curve. *Applied and Environmental Microbiology*, 56(6), 1875–1881. <https://doi.org/10.1128/aem.56.6.1875-1881.1990>



Block II

Enhancing strategies for the reevaluation of rice bran



**Understanding the marketed
plant-based beverages: From
ingredients technological
function to their
nutritional value**

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Benchmarking
Non-dairy
Drinks
Nutritional composition
Ingredients
Food labelling

Despite the market expansion of plant-based beverages (PBB) there is limited information about what is driven the market and the nutritional status of the existing beverages. The objective was to identify the existing gaps in the PBB market with particular emphasis on their composition and nutritional value. PBB are mainly based on individual flour/powder and blends and sunflower oil is frequently present, besides gellan gum to stabilize the emulsion. In general, PBB are low calorie drinks (10–84 Kcal/ 100 mL), with low amount of saturated fat (0.1–1.90 g/ 100 mL) and fibers, and large variation in proteins (0.1–12 g/ 100 mL). The calcium fortification of PBB is comparable to the calcium levels of whole cow's milk, although the vitamin fortification is low. Analysis reveals that salt and oil reduction, as well as fibers enrichment might drive future innovations.

4.1. Introduction

Plant-based beverages (PBB) is an exponentially growing segment all over the world (Vaikma et al., 2021). The global PBB market was valued at approximately US\$9.8 billion in 2017 and it is expected to reach US\$19.7 billion by 2023, growing US\$10 million in 6 years (Statista, 2022). However, PBB cannot be considered new, as there is a long tradition in Eastern and Western cultures for this type of beverages that are commonly present in the market (Bernat et al., 2014). Among them, *horchata* (Spain, from tiger nuts); *Masvuvu* (Zimbabwean, malted millet); *Boza* (Turkish, fermented millet); *Sikhye* also called *dansul* or *gamju* (South Korea, cooked rice and malt extract); and the most well-known is the *soy milk* from China (Arici & Daglioglu, 2002; Codina et al., 2016; Kim et al., 2012; Mäkinen et al., 2016; Sethi et al., 2016; Zvauya et al., 1997). Initially marketed as an alternative to cow's milk for people with lactose intolerance, the consumption of the soy beverage jumped 50 years ago from the local to global market (Mäkinen et al., 2016). Currently, followers of plant based diets and people concerned about the environment are increasing, and with them, the interest for these PBB (Penha et al., 2021; Sethi et al., 2016; Shori & Al Zahrani, 2022), turning this segment as a great opportunity to create PBB using different plant sources (Sethi et al., 2016).

Plants from different sources have been used for making PBB, including the following groups: i) cereals (rice, oat, corn, spelt, rye, quinoa, kamut); ii) legumes (soy, peanut, lupin, cowpea), iii) nuts (almond, coconut, hazelnut, pistachio, walnut); iv) seeds (sesame, flax, sunflower, pumpkin, hemp); and v) pseudocereals (quinoa, teff, amaranth) (Munekata et al., 2020). Whatever the origin, common processing stages are: wet milling, filtration, sterilization, homogenization, aseptic packaging, and storage (Aydar et al., 2020).

Although dietary restrictions or nutrition are the driving forces of this market, the individuals that enjoy sensory experiences cannot be neglected. Recent scientific literature reports that consumer preferences are dominated by the texture, viscosity, and mouthfeel of PBB (Jaeger & Giacalone, 2021; Moss et al., 2022; Vaikma et al., 2021). To achieve these characteristics is important to form an oil-in-water emulsion, using oils and emulsifiers to modify their stability (McClements, 2015). Nevertheless, much more interest has been focused on the nutritional component and healthy benefits,

specifically micronutrient fortification (calcium and vitamins), and bioactive compounds (Ahmad & Ahmed, 2019; Aydar et al., 2020; Silva et al., 2022), although observing high variability in their nutritional composition. In fact, Chalupa-Krebzdak et al., (2018) analyzed 18 beverages based on soy, coconut, rice, hemp, almond and cashew, observing that the protein content ranged between 0.42-3.16 g/ 100 mL, while fats varied from 0.83 g/100 mL to 6 g/100 mL. Likewise, Vanga & Raghavan, (2018) analyzed 23 beverages made from almond, soy, rice or coconut, obtaining similar variability, even in saturated fats (0-5 g/ 100 mL), sugar (0-15.6 g/ 100 mL), and salt (till 0.48 g/ 100 mL). Recently, with the increasing popularity of PPBs, Craig & Fresán, (2021) were able to analyze 148 beverages with new main ingredients (almond, cashew, coconut, hazelnut, macadamia, oat, pea, rice, soy and other -not specify), and authors confirmed previous findings, stating the high variability, and their high content in salt, sugar and saturated fat (composition range in g/ 100 mL: protein 0-8.9, saturated fats 0-4.8; sugar 0-14). However, the market is rapidly growing and with it, the search for alternative plant sources. Therefore, the purpose of the research was to conduct an extensive analysis (benchmarking) of the current market, to identify trends in the composition and nutritional value of PBB, which could help in designing PBB based on scientific knowledge.

4.2. Material and methods

Data gathering was conducted from May 2022 to August 2022. PBB from the major groceries stores in Europe (El Corte Inglés, Consum, Mercadona, Herbolario Navarro, Eroski in Spain; Tesco in United Kingdom; Edeka in Germany; Auchan, Carrefour, Dia present across Europe) and North America (Walmart in USA and Canada) were included, having samples from private and commercial brands. Data collected included PBB as non-dairy alternatives that are obtained from an aqueous extract of the raw materials and not as a squeezing of the raw material itself, like occurs in juices from vegetables or fruits. Different data processing programs were used throughout the study: Microsoft® Excel 2019 (Version 16.67), Stata/SE (Version 17.0) (College Station, TX, US), and ggplot2 package (Version 3.3.2) (Wickham, 2016) for R (Version 4.0.3) (R core team, 2020). Labels were analyzed for the PBB composition and nutritional facts information. Commercial PBB made from nuts, cereals, seeds, pseudocereal and legumes were considered

(including those with cocoa, coffee, fruit, and other flavors), those PBB with missing nutritional information were excluded. Ingredients were recorded and classified into raw materials, oils, gums, salt and minor ingredients (added as a fortifiers). A nutritional role was assigned to the minor ingredients without technological contribution. Afterward, a frequency analysis and an alluvial plot were carried out with the ingredients to identify processing trends. A box-and-whisker plot compared the macronutrients composition of these beverages, classifying them by raw material. Salt was plotted considering both raw material and its presence or absence in the list of ingredients. Principal component analysis (PCA) was carried out to discriminate beverages according their nutritional pattern and recipe (type of oil, raw material and hydrocolloid).

4.3. Results and discussion

4.3.1. Plant based beverages composition

Plant based beverages present in groceries stores were analyzed, which accounted up to 306. Labels were analyzed in relation to their nutritional composition and the ingredients listed in the package. On average the beverages analyzed contained eight ingredients in their formulation, including vitamins and minerals added with the purpose of fortification. Ingredients used for processing were water, raw plant-based powders, and in some cases, oil, gums, and sugar. Water was the main ingredient, which allows soaking and softening the rest of ingredients, making them ready for grinding. Water is also the basis for preparing the emulsion that will result in the beverage.

The second ingredient in PBB were the vegetable powders or flours, such as almonds, coconut, rice, oats, soybeans, etc. The type of consumers of this type of beverage has changed and with that their preferences. PBB were sought primarily as dairy substitutes produced from soybeans, oats or coconut (Paul et al., 2020). Currently, a wide variety of grains, and even blends, are used to prepare PBB. In fact, 84% of the analyzed beverages contained a unique powder, 14% of them were formulated with two or more main ingredient, and 2% were produced from protein isolates (manly pea protein). An alluvial plot was used to show the relationships and frequencies of the different ingredients that were categorized as raw materials, oils and hydrocolloids (**Figure 3.4.1**). Principal raw materials or sources of powder for PBB included, oat (76), soy (68), almond (57), rice (28), coconut (15), plant-

based (7) (no defined) or tiger nut (3). To a lesser extent cashew (2), millet (2), flax (2), hazelnut (1), spelt (1), birdseed (1) and hemp (1) were used. A group of beverages contained blends (42), that were a variety of binary combinations of powders, mainly rice, oat and soy combined with different types of nuts, coconut or seeds. The analysis revealed that the PBB market is not expanded based in a unique source of vegetables, but it is rapidly growing to offer a great variety of flavors and textures from a range of vegetable sources. Therefore, technological properties and nutritional composition will be rather different, which force to adapt PBB processing to the type of raw material used (nuts, pulses, cereals, etc.) (Sethi et al., 2016). Therefore, the concentration of the other ingredients was variable since they were accommodated to the need and application of the ingredients. The other important aspect is that this trend in raw materials confirms the interest in bioactive compounds enrichment in PBB, as has been reported for other foods (Betoret et al., 2020).

Vegetable oils are a common ingredient in PBB, because some consumers of this type of beverage often seek dairy substitutes resembling the texture of the cow milk. The system oil-in-water emulsion allows imitating many sensory characteristics of cow's milk, such as appearance, viscosity, stability, mouthfeel and taste (Aydar et al., 2020; Martínez-Padilla et al., 2020; McClements, 2015; Pineli et al., 2015). For this reason, among the 306 beverages gathered for this study, 31.37% of them contained vegetable oil as an ingredient in their labelling (**Figure 3.4.1**). Within the group of beverages containing vegetable oils as part of their ingredients, sunflower oil was the most frequent one (78.13%) and particularly in PBB containing either rice or oat, which suggested those beverages were marketed as alternatives to dairy milk for those with dietary restrictions. Other oils present in the PBB were rapeseed oil in 11.46% of PBB containing oils, followed by oil blends (rapeseed + palm; sunflower + shea; canola + sunflower) in 3.12% of them, or rapeseed, canola, and flax in a minority (2.08%). The presence or absence of vegetable oils in the formulation could be related to the type of the vegetable powder or raw material. In fact, beverages made with high-fat raw materials like almond, coconut, soy, cashew, hazelnut, or tiger nut, rarely (< 3%) contained other oil source in their label. Conversely, the presence of vegetable oil was higher in beverages based on cereal grains (rice, millet, spelt) or seeds (flax, birdseed). Surprisingly, oat based PBB showed added

vegetable oil, despite of being a cereal with high fat content. In the case of blends, they were majorly combining a cereal with a high-fat commodity that might provide oil functionality in the emulsion. Because of that, more than 80% of the beverages made with blended raw materials did not require in their formulation the presence of oil to obtain the silky appearance and palatability characteristics. This is an interesting technological and nutritional advance, that attends one of the most recent demands of consumers, such as avoiding the consumption of added fats (Asioli et al., 2017).

To obtain stable oil-in-water emulsion in the production of beverages, it is necessary the use of stabilizers that can reduce the droplet size of the oil used, thus keeping the aqueous and oil phases homogenized during the storage time (Krempel et al., 2019). **Figure 3.4.1** shows that 184 PBB (60% of total PBB analyzed) do contain hydrocolloids in their formulation. Within those PBB, the most used hydrocolloid was the gellan gum (51.11%), followed by a mixture of different sources (41.85%), and then locust bean gum (2.71%), guar gum (1.63%), carrageenan (1.63%), sodium carboxymethyl cellulose (0.54%) and xanthan gum (0.54%). When a mixture of different hydrocolloids was incorporated, 74% of those PBB had gellan gum within the mixture or gellan gum + locust bean gum. Hydrocolloids functionality in this type of emulsions stabilize the oil droplet, and reduce the surface tension of the emulsion interface, without increasing the viscosity during storage (Krempel et al., 2019). Fallourd & Viscione, (2009) pointed out that the selection of the hydrocolloid depends on many factors like the amount of protein, the pH and the amount of oil and particles in suspension, and those authors identified gellan gum as efficient suspending agent to prepare structured liquids and low viscosity gels.

Despite the stabilizing functionality of the hydrocolloids, some PBB did not contain any, and those were mainly composed of cereals. Specifically, from the cereal-based beverages (107), only 34 contained hydrocolloids. Considering that PBB processing comprised a heat treatment and homogenization stage (Penha et al., 2021; Qamar et al., 2020), presumably the starch content of cereals provides the stabilizing functionality in the absence of hydrocolloids. In fact, Boulemkahel et al., (2021) reported that low pressure homogenization modifies the rice flour properties increasing the emulsifying properties.

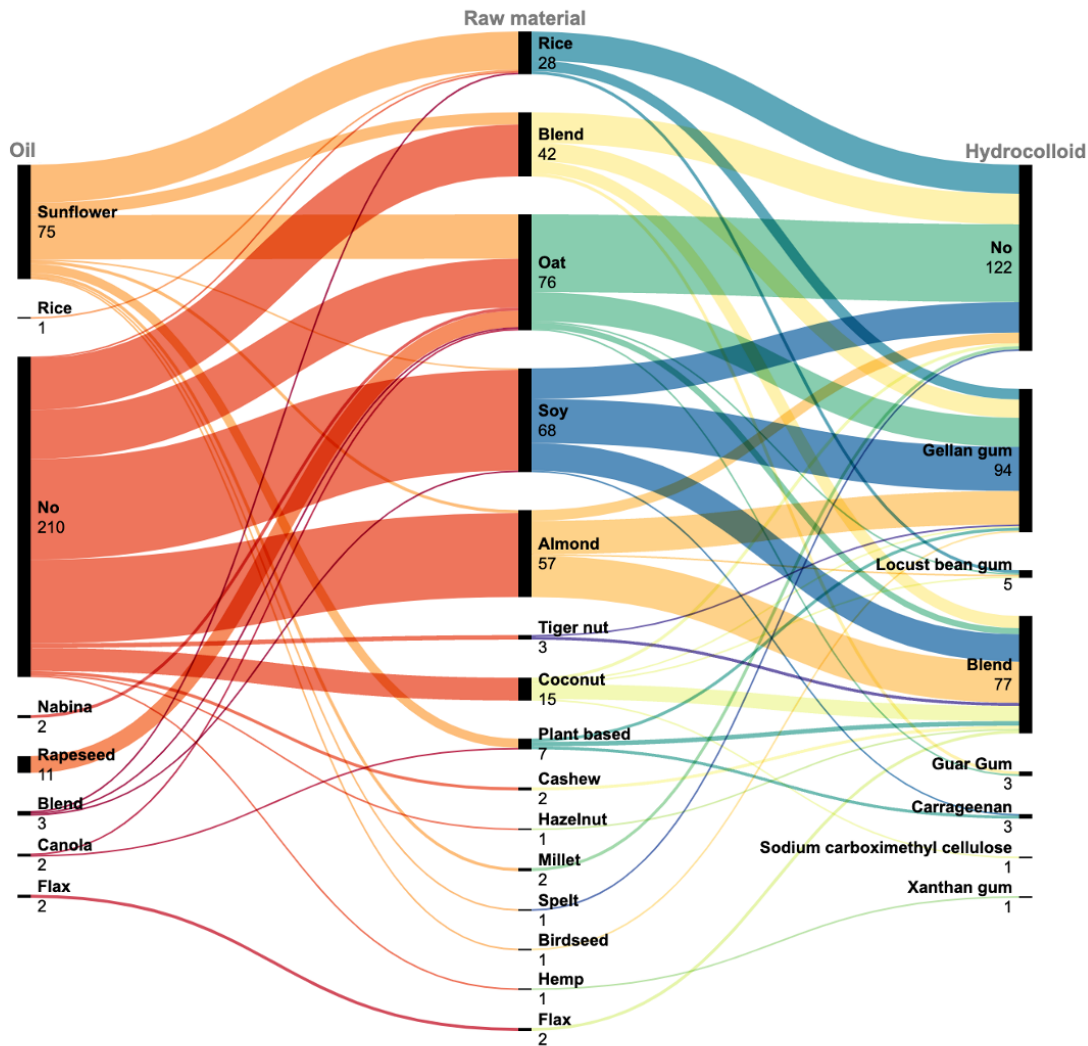


Figure 4.1. Alluvial plot to the main ingredients of PBB. Relationship and frequencies between the type of oil, raw materials and hydrocolloids included in the labels of the marketed PBB.

4.3.2. Analysis of the nutritional facts of the currently marketed plant-based beverages

As the market of PBB is expanded, a special attention must be paid to their nutritional profile. Energy is one of the main indicators that consumers consider and affects the purchasing decision. Typically, health-conscious consumers relate high calories content to less healthy products, associated to obesity among other diseases (Charbonnier et al., 2015). As shown in **Figure 4.2 A**, the energy content of the different PBB analyzed was rather variable.

In general, cereal-based beverages provide higher energy than the PBB average, and the least energy dense PBB were those made from coconut, nuts and seeds. Hemp based beverages provides similar energy than millet-based ones, 55 kcal/100 mL and 58.33 kcal/100 mL, respectively; nonetheless, the presence in the market is still incipient. The tiger nut-based drinks were the most caloric ones, ranging from 52 to 84 kcal/ 100 mL, all of them over the average value, 41.73 kcal/ 100 mL. Tiger nut has been used from ancient times to obtain a sweet and cold drink consumed mainly during hot periods (Martín-Esparza & González-Martínez, 2016). Traditionally, this drink is prepared adding sugar, which results in a high caloric drink (Corrales et al., 2012). Vegetable drinks made of powders blends showed the highest deviation in the energy content (4.17-74 kcal/ 100 mL), related to the variability of raw materials. In contrast, rice-based beverages had the lowest variation of all plant-based drinks (45-57 kcal/ 100 mL). Chalupa-Krebzdak et al., (2018) also reported great variability in the energy content of marketed PBB from different brands, being energy dense PBB those with higher content in carbohydrates and sugars, which also raises the glycemic index of these beverages (Walter et al., 2022).

PBB have low total fat content, with an average value of 1.56 g/ 100 mL (**Figure 4.2 B**), which is far lower than the fat content of whole cow's milk (3.2 g/ 100 mL, being 1.86 g/ 100 mL coming from saturated fat) reported by the USDA (2022). Likewise, the fat content in the present analysis was lower than that described by Vanga & Raghavan, (2018), who reported values that were 40-50% higher for PBB made of almond, coconut, rice, or soybean. This divergence confirms that the PBB segment is shifting towards low fat content beverages. Nevertheless, the present analysis found some exceptions, like

PBB made with almond, blended powders, flax, oat or soy, that exceeded the amount of fat in whole cow's milk, particularly those containing almond (4.7 g fat/ 100 mL). In general, results agree with those described by Chalupa-Krebzdak et al., (2018), except for soy beverages that presently contain low fat values (**Figure 4.2 B**), confirming the nutritional improvement of this type of beverages. Regarding the type of fats, saturated and unsaturated, the information was available in some PBB, which, although no general statement could be done, but the analysis revealed that PBB have low content of saturated fats, and more monounsaturated and polyunsaturated fats. Recently, Craig & Fresán, (2021) described that in soy, flax and hemp-based beverages, polyunsaturated fats were predominant, whereas rice and almond based beverages, contained mainly monounsaturated fats, which are associated with cardiovascular health because of their action controlling the levels of glucose in blood and reducing total cholesterol (Jenkins et al., 2006; Sabaté et al., 2003). The average content of saturated fat in those beverages was 0.34 g/ 100 mL; only coconut-based beverages showed a completely different profile, with a much higher saturated fat content (1.90 g/ 100 mL). Although saturated fat contents are related to unhealthy diet, because they raise LDL cholesterol levels, coconut oil increases HDL cholesterol, being able to compensate for its high saturated fat composition (Eyres et al., 2016). It must also consider that coconut oil contains lauric acid, as saturated fat, which has been associated to brain development and boosting the immune system (Sethi et al., 2016). As expected, PBB do not declare cholesterol content, whereas whole cow's milk contains 12 mg/ serving (USDA, 2022). Therefore, except for coconut-based beverages, analyzed PBB show healthier lipid profile than cow's milk for people suffering diseases related to high blood lipid levels.

Carbohydrates and sugars content in the marketed PBB were different, which was largely dependent on the raw material of the beverages (**Figure 4.2 C**). Drinks based on almond, cashew, coconut, flax, undefined plants, or soy, had lower carbohydrate content than the average (5.33 g/ 100 mL). Tiger nut-based drinks had the highest carbohydrate content (11.27 g/ 100 mL), despite the low carbohydrate content reported for tiger nuts (Sánchez et al., 2016), but they usually content high amounts of added sugar (70% of carbohydrates were sugars). Rice based beverages, in general those containing cereals (millet and oat) showed high carbohydrate content, which was expected considering their starch content (Sultana et al., 2022).

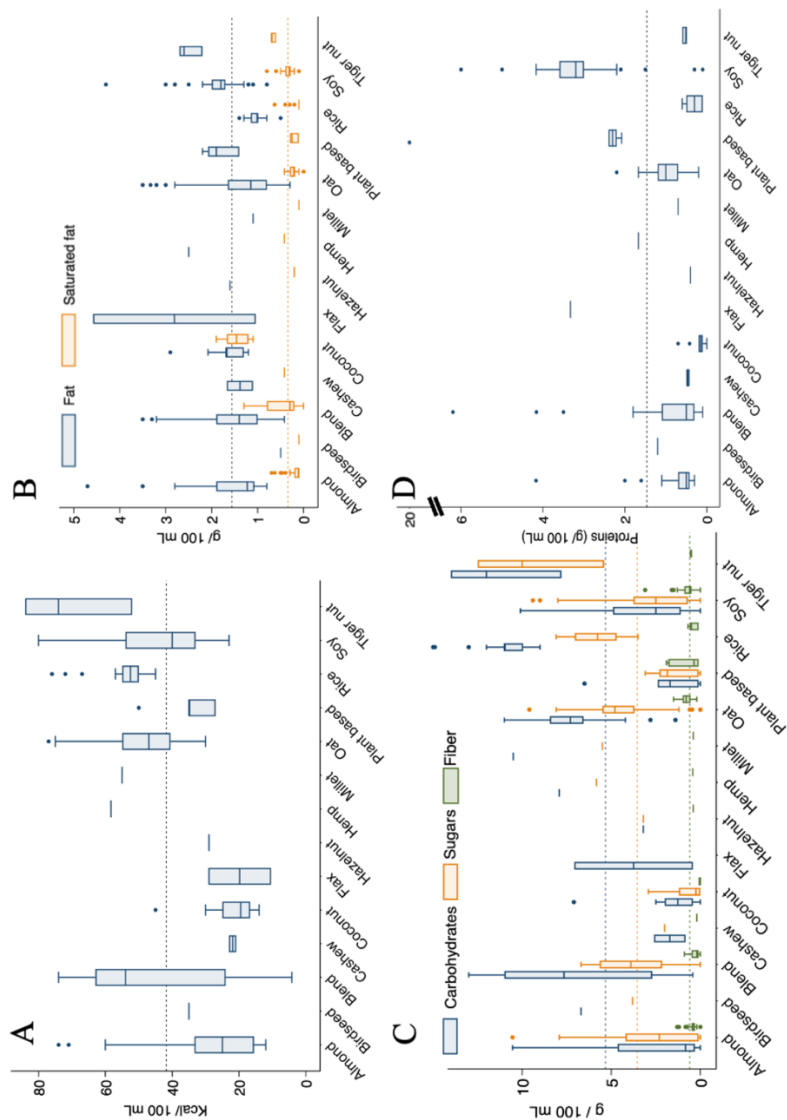


Figure 3.4.2. Analysis of nutritional facts of PBB. A: energy (kcal); B: Total Fats (blue) and saturated fats (orange); C: carbohydrate (blue), sugars (orange) and fiber (green); D: proteins. Horizontal dotted lines represent the mean of all values obtained for that compound

Therefore, in general, PBB have higher amount of carbohydrates than whole cow's milk (4.67 g/ 100 mL) (USDA, 2022).

The content of sugars (**Figure 4.2 C**) in the analyzed beverages followed the same trend described for total carbohydrates, although with lower levels. However, it must be stressed that the great difference between total carbohydrates and sugars content in the cereal-based drinks occurred in those containing coconut or blends. The level of carbohydrate and sugars in beverages made from nuts (almonds, cashews, hazelnuts), seeds (flax, birdseed, hemp) and legumes (soy) was rather similar, and most of the carbohydrates content were due to the sugar content. The highest average was found in the tiger nut-based beverages (9.30 g/ 100 mL), as mentioned before, and the lowest in the coconut-based beverages (0.82 g/ 100 mL). Only 78 from 306 beverages had sugar in their list of ingredients, so most of the sugars content was from the raw materials used in the formulation of the beverages.

Although the fiber content (**Figure 4.2 C**) was not always declared, these beverages did not have a notable fiber content (mean value was 0.59 g/ 100 mL), with oat, soy, and plant blend based showing 0.79, 0.71 and 0.77 g/ 100 mL, respectively. The fiber content was mainly determined by the type of raw material, but in some cases the incorporation of fiber was remarkable, i.e., a beverage from the group of plant-based containing chicory root reached 1.9 g/ 100 mL of fiber. In addition, it should be considered that hydrocolloids presence could provide an extra contribution of fiber, although no direct relationship was found between the beverages with added hydrocolloids and fiber content. The production of fiber-rich foods have been challenging for research and industry, especially in beverage segment, where consumer perception is largely affected by the appearance and texture of the liquid (Moss et al., 2022).

Just like the rest of macronutrients, the market study revealed large differences in the protein content (**Figure 4.2 D**). The highest content was observed in beverage group made with undefined plants, particularly a PBB with 20 g protein/ 100 mL, which was enriched with 2.5% pea protein isolate (**Figure 4.2 D**). Apart from those, the PBB with higher amount of proteins were those containing flax (3.3 g/ 100 mL) or soy (3.15 g/ 100 mL). The rest of beverages had lower protein content than the overall average (1.46 g/ 100 mL). Protein content of these PBB was below the protein contribution of

whole cow's milk (3.18 g/ 100 mL) (USDA, 2022). An alternative to increase the amount of proteins is enhancing the level of seeds or blending different protein rich raw materials, like tiger-nut (Sethi et al., 2016), which could also improve the quality of the amino acids profile (Qamar et al., 2020).

The salt content was not correlated with its presence or absence as ingredient in the manufacturer's label (**Figure 4.3**). From the 306 PBB, 263 of them declared salt as an ingredient (blue shadowed in Figure 4.3) and 37 did not (pink shadowed in Figure 4.3), whereas 6 of them did not mention the salt neither as ingredient nor in the nutritional facts. Overall, the average salt content was 0.1 g/ 100 mL. In general, salt content of the beverages without salt added was lower than the content in PBB containing salt as ingredient. Undefined plant-based beverage was the group with the highest salt content in both PBB clusters, with or without salt as ingredient. Nevertheless, within non-added salt PBB it should be stressed that the group of plant-based beverages displayed the highest salt content (mean of 0.16 g/ 100 mL). Salt is commonly used to enhance flavor, presumably salt is masking off-flavors in the group of undefined plants, being added with that purpose or through plants that have high salt content. Within the group of PBB that declared salt as an ingredient, two outliers were detected with 0.33 g salt/100 mL, which contained oat or rice-coconut blend. Health worldwide authorities advise low salt intake (<5 grams per day in adults) to reduce blood pressure and the risk of cardiovascular disease, stroke and myocardial infarction (*World Health Organization*, 2023). According to the European Commission in its “REGULATION (EC) No 1924/2006 on nutrition and health claims” PBB could be considered low salt when its content is less than 0.3 g/ 100 mL, or very low salt when its content is less than 0.1 g/ 100 mL.

Dairy substitute beverages have been used as vehicles to introduce a greater number of vitamins, minerals and even probiotics to achieve balanced diets. In the analyzed beverages, more than 50% of them were fortified with either vitamins, a calcium salt or a combination of several salts and vitamins. Calcium is an essential nutrient necessary for human growth and development and it is a limiting nutrient in cereals such as rice and oats (Sethi et al., 2016).

Results

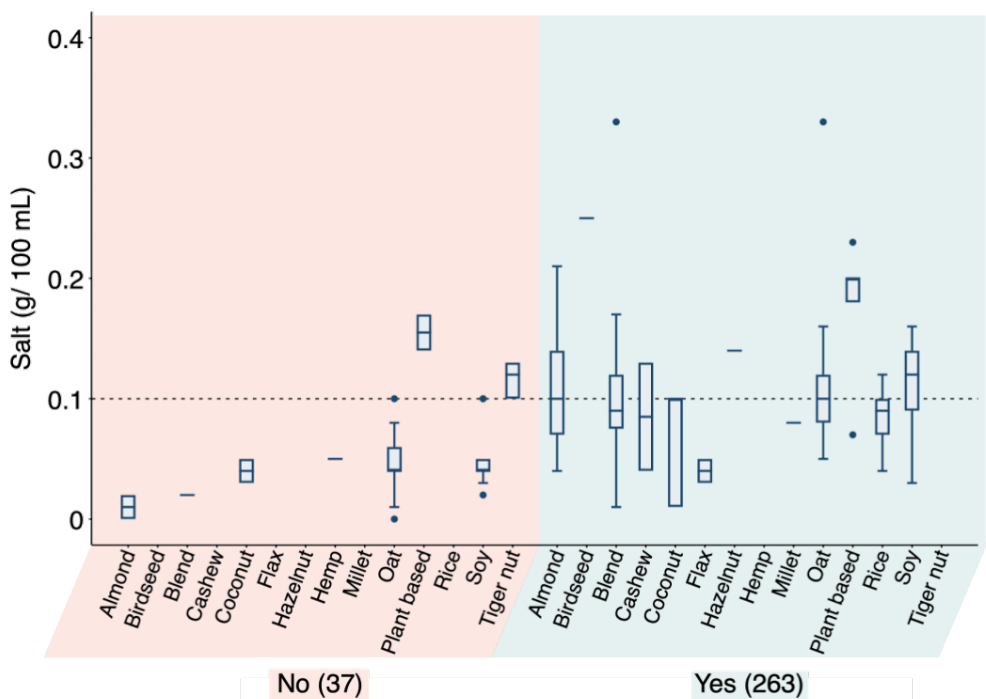


Figure 4.3. Analysis of salt content in the plant-based beverages (PBB) gathered from the market. PBB containing salt as an ingredient (blue bottom) or without salt (red bottom) were split in the plot. Horizontal dotted line represents the mean salt content of all beverages analyzed.

From those, 159 were fortified with calcium but only 136 beverages declared the calcium content in their nutritional information, with an average of 122.32 mg calcium/ 100 mL, comparable to 123 g of calcium/ 100 mL of whole cow's milk (*USDA*, 2022), although it is still questioned if they have similar bioavailability (*Chalupa-Krebzdak et al.*, 2018). Calcium carbonate was the most used salt (46%), followed by tricalcium phosphate (37%) and a mixture of both (7%). Nowadays, calcium carbonate is the most common calcium salt and its absorption is similar to calcium from bovine milk (*Kruger et al.*, 2003; *Zhao et al.*, 2005). Other salts such as tricalcium citrate, dicalcium phosphate or calcium salts of orthophosphoric acid were also used, in lesser extent (10% among the three salts). Regarding vitamins, the number of fortified beverages was lower (141) than that of minerals, and vitamin D was the most frequent added vitamin (in 136 PBB). This vitamin D has been

targeted because it is one of the most deficient vitamins worldwide, related to many diseases such as cardiovascular diseases, hypertension or problems in calcium homeostasis (Müller et al., 2011). Other vitamins widely used for the fortification of PBB are vitamins B12, B2, A and E, always in combination with vitamin D. Only 6 beverages were fortified with vitamin B6, B9, B3 or C. However, there is very limited information about the bioavailability of vitamins and minerals after processing, which is particularly important considering the presence of anti-nutrients in the raw materials (Aydar et al., 2020). In addition, it is important to consider that some of the raw ingredients currently present in the PBB are increasing their vitamins composition. For instance, almonds are an excellent source of vitamin E, which cannot be synthesized by the body and its antioxidant action is required for protecting against free-radical reactions (Sethi et al., 2016).

4.3.3. Overall analysis of marketed plant-based beverages using a principal component analysis

A principal component analysis was built up to identify potential PBB clusters based on the nutritional composition, type of raw material, oil and hydrocolloid, and the presence or absence of salt. Those variables could explain 46.6% of the differences among the commercial beverages analyzed (**Figure 4.4**). Principal component 1 (PC1) explained 27.9% of the variability, based on ingredients, specifically hydrocolloids and salt (in the negative x-axis) and oil type (in the positive x-axis). Conversely, raw materials and macronutrients composition allowed differentiate PBB along principal component 2 (PC2), with the raw material in the negative y-axis and fiber, proteins and fat content in the positive y-axis. Soybeans, almonds, oats, and blends based PBB were scattered in all quartiles, indicating their large variability. Soy and almond based beverages were characterized by the presence of hydrocolloids and salt. The other extensive cluster included the PBB made from powder or raw material blends, that were extended along x-axis, confirming the variability in their formulation and they contained higher levels of saturated fats. Tiger-nut-based beverages were grouped because of their high sugar and carbohydrates content. As it was explained above, this type of beverages are traditionally sweet drinks with high sugar content (Sánchez et al., 2016). Rice drinks clustered on the x- axis and was associated with a high caloric content and a high presence of carbohydrates, as shown during the study.

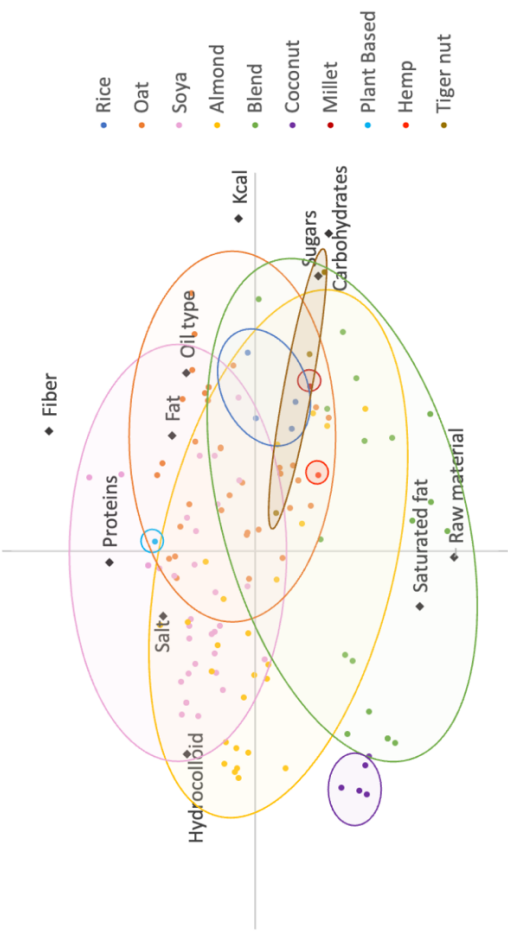


Figure 3.4.4. Principal component analysis of PBB that included all the variables analyzed (ingredients in the formulation and nutritional facts). Identified clusters based on their raw materials, appeared circled in different colors.

It is noteworthy to mention that oat-based beverages were grouped because their high fat content, that was associated to the addition of oil, which resulted in energy dense drinks. Coconut drinks formed a well-defined cluster distinguished by their content in saturated fat, mainly due to the lipid profile of coconut (Sethi et al., 2016).

4.4. Conclusions

Plant based beverages existing in the market are rapidly changing, incorporating a variety of vegetable powders or raw materials, that directly affect their final composition. The raw materials used for making PBB reflects the trend towards healthy foods and beverages. Specifically, beverages were mainly based on cereal grains (rice, millet, oats and spelt), seeds (birdseed and flax) and nuts or legumes. Regarding their nutritional composition, it was largely dependent on the raw material. Despite the improvement in the type of ingredients used in their formulations, the salt and oil reduction in PBBs, as well as fiber enrichment, would be advisable. It should be noted that more than 50% of the beverages analyzed were fortified with vitamins and/or minerals, which is desirable for people concerned about a balanced diet and health. Regarding nutritional differences either within the same raw materials or commercial brands, it would be important to develop a public education initiative on labelling reading.

Declaration of conflicting interests: Authors declare that there are not ethical issues associated with the research carried out in the manuscript entitled Understanding the functionality of the marketed plant-based beverages.

CRedit authorship contribution statement: **Eva Grau-Fuentes:** Formal analysis, Investigation, Writing – original draft. **Dolores Rodrigo:** Supervision, Writing – review & editing. **Raquel Garzón:** Conceptualization, Supervision, Formal analysis, Writing – review & editing. **Cristina M. Rosell:** Funding acquisition, Conceptualization, Writing – review & editing.

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References

- Ahmad, A., & Ahmed, Z. (2019). 3—Fortification in Beverages. In A. M. Grumezescu & A. M. Holban (Eds.), *Production and Management of Beverages* (pp. 85–122). Woodhead Publishing. <https://doi.org/10.1016/B978-0-12-815260-7.00003-1>
- Arici, M., & Daglioglu, O. (2002). Boza: A lactic acid fermented cereal beverage as a traditional Turkish food. *Food Reviews International*, 18(1), 39–48. <https://doi.org/10.1081/FRI-120003416>
- Asioli, D., Aschemann-Witzel, J., Caputo, V., Vecchio, R., Annunziata, A., Næs, T., & Varela, P. (2017). Making sense of the “clean label” trends: A review of consumer food choice behavior and discussion of industry implications. *Food Research International*, 99, 58–71. <https://doi.org/10.1016/j.foodres.2017.07.022>
- Aydar, E. F., Tutuncu, S., & Ozcelik, B. (2020). Plant-based milk substitutes: Bioactive compounds, conventional and novel processes, bioavailability studies, and health effects. *Journal of Functional Foods*, 70, 103975. <https://doi.org/10.1016/j.jff.2020.103975>
- Bernat, N., Cháfer, M., Chiralt, A., & González-Martínez, C. (2014). Vegetable milks and their fermented derivative products. *International Journal of Food Studies*, 3, 93–124. <https://doi.org/10.7455/ijfs/3.1.2014.a9>
- Boulemkahel, S., Betoret, E., Benatallah, L., & Rosell, C. M. (2021). Effect of low pressures homogenization on the physico-chemical and functional properties of rice flour. *Food Hydrocolloids*, 112, 106373. <https://doi.org/10.1016/j.foodhyd.2020.106373>
- Chalupa-Krebzdak, S., Long, C. J., & Bohrer, B. M. (2018). Nutrient density and nutritional value of milk and plant-based milk alternatives. *International Dairy Journal*, 87, 84–92. <https://doi.org/10.1016/j.idairyj.2018.07.018>
- Charbonnier, L., van Meer, F., van der Laan, L. N., Viergever, M. A., & Smeets, P. A. M. (2015). Standardized food images: A photographing protocol and image database. *Appetite*, 96, 166–173. Scopus. <https://doi.org/10.1016/j.appet.2015.08.041>
- Codina, I., Trujillo, A. J., & Ferragut, V. (2016). Horchata. In K. Kristbergsson & J. Oliveira (Eds.), *Traditional Foods: General and Consumer Aspects* (pp. 345–356). Springer US. https://doi.org/10.1007/978-1-4899-7648-2_28
- Corrales, M., de Souza, P. M., Stahl, M. R., & Fernández, A. (2012). Effects of the decontamination of a fresh tiger nuts’ milk beverage (horchata) with short wave ultraviolet treatments (UV-C) on quality attributes. *Innovative Food Science & Emerging Technologies*, 13, 163–168. <https://doi.org/10.1016/j.ifset.2011.07.015>

- Craig, W. J., & Fresán, U. (2021). International Analysis of the Nutritional Content and a Review of Health Benefits of Non-Dairy Plant-Based Beverages. *Nutrients*, 13(3). <https://doi.org/10.3390/nu13030842>
- Eyres, L., Eyres, M. F., Chisholm, A., & Brown, R. C. (2016). Coconut oil consumption and cardiovascular risk factors in humans. *Nutrition Reviews*, 74(4), 267–280. <https://doi.org/10.1093/nutrit/nuw002>
- Fallourd, M. J., & Viscione, L. (2009). 1—Ingredient selection for stabilisation and texture optimisation of functional beverages and the inclusion of dietary fibre. In P. Paquin (Ed.), *Functional and Speciality Beverage Technology* (pp. 3–38). Woodhead Publishing. <https://doi.org/10.1533/9781845695569.1.3>
- Jaeger, S. R., & Giacalone, D. (2021). Barriers to consumption of plant-based beverages: A comparison of product users and non-users on emotional, conceptual, situational, conative and psychographic variables. *Food Research International*, 144, 110363. <https://doi.org/10.1016/j.foodres.2021.110363>
- Jenkins, D. J. A., Kendall, C. W. C., Josse, A. R., Salvatore, S., Brighenti, F., Augustin, L. S. A., Ellis, P. R., Vidgen, E., & Rao, A. V. (2006). Almonds Decrease Postprandial Glycemia, Insulinemia, and Oxidative Damage in Healthy Individuals. *The Journal of Nutrition*, 136(12), 2987–2992. <https://doi.org/10.1093/jn/136.12.2987>
- Kim, H., Kim, H., Bang, J., Kim, Y., Beuchat, L. R., & Ryu, J.-H. (2012). Reduction of *Bacillus cereus* spores in sikhye, a traditional Korean rice beverage, by modified tyndallization processes with and without carbon dioxide injection. *Letters in Applied Microbiology*, 55(3), 218–223. <https://doi.org/10.1111/j.1472-765X.2012.03278.x>
- Krempel, M., Griffin, K., & Khouryieh, H. (2019). 13—Hydrocolloids as Emulsifiers and Stabilizers in Beverage Preservation. In A. M. Grumezescu & A. M. Holban (Eds.), *Preservatives and Preservation Approaches in Beverages* (pp. 427–465). Academic Press. <https://doi.org/10.1016/B978-0-12-816685-7.00013-6>
- Kruger, M. C., Gallaher, B. W., & Schollum, L. M. (2003). Bioavailability of calcium is equivalent from milk fortified with either calcium carbonate or milk calcium in growing male rats. *Nutrition Research*, 23(9), 1229–1237. [https://doi.org/10.1016/S0271-5317\(03\)00100-3](https://doi.org/10.1016/S0271-5317(03)00100-3)
- Mäkinen, O. E., Wanhälina, V., Zannini, E., & Arendt, E. K. (2016). Foods for Special Dietary Needs: Non-dairy Plant-based Milk Substitutes and Fermented Dairy-type Products. *Critical Reviews in Food Science and Nutrition*, 56(3), 339–349. <https://doi.org/10.1080/10408398.2012.761950>
- Martín-Esparza, E., & González-Martínez, C. (2016). Horchata de Chufa: A Traditional Spanish Beverage with Exceptional Organoleptic, Nutritive,

- and Functional Attributes. In K. Kristbergsson & S. Ötles (Eds.), *Functional Properties of Traditional Foods* (pp. 371–375). Springer US. https://doi.org/10.1007/978-1-4899-7662-8_26
- Martínez-Padilla, E., Li, K., Blok Frandsen, H., Skejovic Joehnke, M., Vargas-Bello-Pérez, E., & Lykke Petersen, I. (2020). In Vitro Protein Digestibility and Fatty Acid Profile of Commercial Plant-Based Milk Alternatives. *Foods*, 9(12). <https://doi.org/10.3390/foods9121784>
- McClements, D. J. (2015). *Food Emulsions: Principles, Practices, and Techniques* (3rd ed.). CRC press. <https://doi.org/10.1201/b18868>
- Moss, R., Barker, S., Falkeisen, A., Gorman, M., Knowles, S., & McSweeney, M. B. (2022). An investigation into consumer perception and attitudes towards plant-based alternatives to milk. *Food Research International*, 159, 111648. <https://doi.org/10.1016/j.foodres.2022.111648>
- Müller, D. N., Kleinewietfeld, M., & Kvakan, H. (2011). Vitamin D review. *Journal of the Renin-Angiotensin-Aldosterone System*, 12(2), 125–128. <https://doi.org/10.1177/1470320311410924>
- Munekata, P. E. S., Domínguez, R., Budaraju, S., Roselló-Soto, E., Barba, F. J., Mallikarjunan, K., Roohinejad, S., & Lorenzo, J. M. (2020). Effect of Innovative Food Processing Technologies on the Physicochemical and Nutritional Properties and Quality of Non-Dairy Plant-Based Beverages. *Foods*, 9(3). <https://doi.org/10.3390/foods9030288>
- Paul, A. A., Kumar, S., Kumar, V., & Sharma, R. (2020). Milk Analog: Plant based alternatives to conventional milk, production, potential and health concerns. *Critical Reviews in Food Science and Nutrition*, 60(18), 3005–3023. <https://doi.org/10.1080/10408398.2019.1674243>
- Penha, C. B., Santos, V. D. P., Speranza, P., & Kurozawa, L. E. (2021). Plant-based beverages: Ecofriendly technologies in the production process. *Innovative Food Science & Emerging Technologies*, 72, 102760. <https://doi.org/10.1016/j.ifset.2021.102760>
- Pineli, L. de L. de O., Botelho, R. B. A., Zandonadi, R. P., Solorzano, J. L., de Oliveira, G. T., Reis, C. E. G., & Teixeira, D. da S. (2015). Low glycemic index and increased protein content in a novel quinoa milk. *LWT - Food Science and Technology*, 63(2), 1261–1267. <https://doi.org/10.1016/j.lwt.2015.03.094>
- Qamar, S., Manrique, Y. J., Parekh, H., & Falconer, J. R. (2020). Nuts, cereals, seeds and legumes proteins derived emulsifiers as a source of plant protein beverages: A review. *Critical Reviews in Food Science and Nutrition*, 60(16), 2742–2762. <https://doi.org/10.1080/10408398.2019.1657062>
- Sabaté, J., Haddad, E., Tanzman, J. S., Jambazian, P., & Rajaram, S. (2003). Serum lipid response to the graduated enrichment of a Step I diet with almonds: A randomized feeding trial. *The American Journal of Clinical*

- Nutrition*, 77(6), 1379–1384. <https://doi.org/10.1093/ajcn/77.6.1379>
- Sánchez, J., Correa, M., & Castañeda, L. (2016). *Revista Facultad Nacional de Salud Pública*, 34(2), 230–242. <https://doi.org/10.17533/udea.rfnsp.v34n2a12>
- Sethi, S., Tyagi, S., & Anurag, R. (2016). Plant-based milk alternatives an emerging segment of functional beverages: A review. *Journal of Food Science and Technology-Mysore*, 53(9), 3408–3423. <https://doi.org/10.1007/s13197-016-2328-3>
- Shori, A. B., & Al Zahrani, A. J. (2022). Non-dairy plant-based milk products as alternatives to conventional dairy products for delivering probiotics. *Food Science and Technology*, 42, e101321. <https://doi.org/10.1590/fst.101321>
- Silva, J. G. S., Rebellato, A. P., Abreu, J. S. de, Greiner, R., & Pallone, J. A. L. (2022). Impact of the fortification of a rice beverage with different calcium and iron sources on calcium and iron bioaccessibility. *Food Research International*, 161, 111830. <https://doi.org/10.1016/j.foodres.2022.111830>
- Statista. (2022). <https://www.statista.com/statistics/948450/plant-based-beverages-market-value-worldwide/>
- Sultana, S., Faruque, M., & Islam, M. R. (2022). Rice grain quality parameters and determination tools: A review on the current developments and future prospects. *International Journal of Food Properties*, 25(1), 1063–1078. <https://doi.org/10.1080/10942912.2022.2071295>
- USDA. (2022). <https://fdc.nal.usda.gov/fdc-app.html#/food-search>
- Vaikma, H., Kaleda, A., Rosend, J., & Rosenvald, S. (2021). Market mapping of plant-based milk alternatives by using sensory (RATA) and GC analysis. *Future Foods*, 4, 100049. <https://doi.org/10.1016/j.fufo.2021.100049>
- Vanga, S. K., & Raghavan, V. (2018). How well do plant based alternatives fare nutritionally compared to cow's milk? *Journal of Food Science and Technology*, 55(1), 10–20. <https://doi.org/10.1007/s13197-017-2915-y>
- World Health Organization. (2023). <https://www.who.int/news-room/fact-sheets/detail/salt-reduction>
- Zhao, Y., Martin, B. R., & Weaver, C. M. (2005). Calcium Bioavailability of Calcium Carbonate Fortified Soymilk Is Equivalent to Cow's Milk in Young Women. *The Journal of Nutrition*, 135(10), 2379–2382. <https://doi.org/10.1093/jn/135.10.2379>
- Zvaunya, R., Mygochi, T., & Parawira, W. (1997). Microbial and Biochemical Changes Occurring During Production of masvusvu and mangisi, traditional Zimbabwean beverages. *Plant Foods for Human Nutrition*, 51(1), 43–51. <https://doi.org/10.1023/A:1007972428849>

Unlocking hidden potential of rice bran: enzymatic treatment for enhancing techno-functional properties

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In LWT - Food Science and Technology, 207, 116673

Carbohydrases

Proteases

Thermal treatment

Hydration properties

Microstructure

Rice bran (RB) is a by-product with limited application due to technological constraints. Enhancing its technological functionality as potential food ingredient will improve the sustainability of rice production. The aim was to study the impact of enzymatic and thermal treatments on defatted rice bran using six distinct commercial enzymes (carbohydrases and proteases) and dry heating by evaluating its technological, nutritional and functional properties. Enzymatic treatment increased up to 208% the soluble dietary fiber content (8.19 g/100 g) of defatted RB. Moreover, the solvent retention capacity, including water, oil, sodium carbonate, and sucrose, exhibited a noteworthy increase across all treatments ($p < 0.05$). Bran color changed after treatments, increasing its luminosity (L^*) and decreasing the value of a^* in all cases, but b^* decreased when treated with protein-acting enzymes while increased with carbohydrate-acting enzymes. Proteases played a pivotal role in reducing particle size and forming gels requiring minimal force for application. Microscopic analysis revealed that carbohydrases-treated samples exhibited prominent cell wall breakage, while protease-treated ones showed a gel-like surface with less distinct protein bodies and layered walls. These comprehensive study sheds new transformations brought about by these enzymatic interventions, offering valuable insights into the optimization of rice bran functionality.

5.1. Introduction

In 2022, a total of 726 million metric tons of unprocessed paddy rice was harvested, which generate 72.6 million metric tons of rice bran (RB) (FAO, 2024). Rice bran has attracted the interest of the cosmetic industry due to its high oil content, rich in bioactive compounds like γ -oryzanol (Garba, Singanusong, Jiamyangyeun & Thongsook, 2019). However, after fat extraction the remaining secondary by-product is often employed in animal feed, resulting in an underutilization of its nutritional potential (Gul, Yousuf, Singh, Singh & Wani 2015; Spaggiari, Dall'Asta, Galaverna & del Castillo Bilbao, 2021).

To fully harness the many benefits of defatted RB, some research has been dedicated to enhancing the extraction of its components, protein and fibers, or modifying their properties. Physical treatments such as dry heat, parboiling, and microwave treatment have been applied to modify the properties of the protein isolates derived from RB for enhancing oil absorption capacity, emulsifying properties, and gelling abilities of (Khan et al., 2011). Freeze-thaw, sonication or high hydrostatic pressure have been applied to increase the extractability of proteins from defatted RB, either individually or combining sonication with amylase and protease treatment (Tang, Hettiarachchy & Shellhammer, 2002). Other authors opted for using different carbohydrases, singly or combined, to improve the protein extraction (Scarabattoli et al., 2023), and the emulsifying and foaming properties of the protein concentrates improved when cellulase was added to extruded defatted RB (Leal, Senna, Kupski, Mendes & Badiale-Furlong 2021). Even those protein concentrates have been further hydrolyzed with proteases to improve their physico-chemical properties (Tang, Hettiarachchy, Horax & Eswaranandam, 2003). Likewise, Hamada, (2000) successfully created a highly soluble protein hydrolysate with superior emulsifying activity and stability using commercial proteases.

Lately, more attention has been paid to the dietary fraction that could be extracted from defatted RB. With that purpose, dietary fiber extracted from defatted RB, with the aid of amylase, alcalase and glucoamylase, was treated with cellulase to modify its properties, particularly the water binding capacity (Liu, Zhang, Yi, Quan & Lin, 2021). Similarly, the combination of cellulase and xylanase has been very effective to hydrolyze the intramolecular

hydrogen bonds in the hemicellulose and cellulose, although water and oil binding capacities were reduced likely due to the low molecular weight of the hydrolysis products (Wen, Niu, Zhang, Zhao & Xiong, 2017).

Previous studies have highlighted the interest on proteins and dietary fiber extracted from rice bran. However, using one or the other, results in a by-product, without fully utilizing all bran constituents. Nevertheless, the inclusion of RB as ingredient has been challenging owing to the structural disruption and the impact on hydration due to the cellulose content, particularly in bakery products (Doan, Lai, Vo & Nguyen, 2021; Majzoobi, Sharifi, Imani & Farahnaky, 2013). Enzymatic treatments have proven effective in improving proteins or dietary fiber fractions, but there has been less exploration of modifying rice bran as a food ingredient. Hence, the aim of this study is to evaluate the effect of six different enzymatic treatments, including carbohydrate or protein acting hydrolases, on technological and functional attributes. In addition, thermal treatment was employed for comparison purposes. This approach will unlock the full nutritional and functional potential of RB in a more efficient and sustainable manner.

5.2. Material and methods

Rice bran from Japonica rice type was procured from Arrocería Pons (Valencia, Spain). The bran was sieved, using a 1000 µm sieve, to obtain a homogenous sample. Enzymes were donated by Novozymes (Bagsvaerd, Denmark). Enzymes selected were: Novozym® (NO) (xylanase, EC 3.2.1.8), Celluclast® (CE) (cellulase, EC 3.2.1.4; 700 U/g), Ultimase® BWL 40 (UL) (cellulase and xylanase; 300 AGU/mL), Shearzyme® Plus 2x (SH) (xylanase, cellulase and β-glucanase, EC 3.2.1.6; 280 U/mL) Alcalase® 2.4 L FG (AL) (endoprotease, EC 3.4.21.62; 2.4 AU-A/g) and Flavourzyme® (FL) (peptidase preparation containing EC 3.2.1.1, EC 3.4.11, EC 3.4.14, EC 3.4.21.63, EC 3.4.24, EC 3.4.24.39; ≥500 U/g).

5.2.1. Defatting process

RB was defatted following Mohammadi et al., (2021) methodology with some modifications. RB was suspended in hexane (1:3, w:v) and shake for 2 h. The mixture was centrifugated at 4000 x g, 20°C for 10 min, in a Beckman centrifuge (Beckman Instruments, USA). Supernatant was decanted and

sediment was resuspended in the same amount of hexane. To increase efficiency this step was repeated three times leading to the defatted rice bran (DRB). The supernatants were pooled together and evaporated using a rotary evaporator (Heidolph, Afora, Barcelona, Spain) to get crude RB oil. DRB were kept frozen (-20°C) till further analysis.

5.2.2. Enzymatic and thermal treatment

DRB was subjected to six different enzymatic treatments was carried out for the treatment, two of them mainly protein-acting enzymes and the other four carbohydrate-acting enzymes. RB was suspended in water keeping the ratio 1: 6.5 (w:v). Proteases were added at 1% of the protein content of DRB (Vallabha, Indira, Jyothi Lakshmi & Tikku, 2015), and treatment occurred at optimal pH (6.1) and temperature (50°C) for 120 min. Carbohydrases were incorporated at a concentration of 1% relative to the DRB fiber content, under identical pH, temperature, and time conditions as the proteases. Then, enzymes were inactivated by raising temperature to 90°C for 10 min.

The heat treatment (dry) consisted of heating the RB placed in a stainless-steel tray for 30 min at 130°C using a lab oven (J.P Selecta S.A., Barcelona, Spain).

After enzymatic and thermal treatments, samples were kept at -80°C and then were freeze-dried and the obtained powders were collected for further analysis.

5.2.3. Proximate composition

Standard methods were used to determine the proximate composition of the samples. Moisture (ISO 712:2009) and total nitrogen (ISO 16634-2:2016), applying 6.25 as a nitrogen to protein conversion factor, were quantified following ISO methods. Total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF) contents were estimated following the method 37-02 (AACC, 2000). Neutral detergent fiber (NDF) content was analyzed following the method proposed by Van Soest, Robertson & Lewis, (1991). Acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined following the procedures outlined in AOAC (973.18). For ADL analysis, samples were treated with 72% sulfuric acid for 3 hours. The hemicellulose fraction was calculated by ADF - NDF, and cellulose was calculated by subtracting ADL from ADF.

5.2.4. Physical properties

5.2.4.1. Particle size distribution

The particle size distribution was evaluated with a Malvern Mastersizer equipment (Mastersizer Scirocco 2000; Malvern Instruments Ltd., Worcestershire, U.K.). The parameter recorded was volume-weighted mean diameter ($d(4.3)$). The determination was done three times for each sample.

5.2.4.2. Scanning Electron Microscopy

The structures of all different samples were examine using scanning electron microscopy (SEM) (Hitachi S-4800, Tokyo, Japan). All RB samples were coated with gold using a vacuum evaporator (JEE 400; JEOL, Tokyo, Japan). Observation was done an accelerating voltage of 10 kW and 2000x magnification.

5.2.4.3. Color

The color of the all-study samples was evaluated utilizing a Minolta colorimeter (Chroma Meter CR-400/410, Konica Minolta, Tokyo, Japan). The CIE- $L^*a^*b^*$ values of the samples were captured, including L^* (representing [+] lightness/ [-] darkness), a^* (indicating [+] redness/ [-] greenness), and b^* (denoting [+] yellowness/ [-] blueness). The measurements were conducted at three distinct points on the sample's surface and in different replicates. The ΔE was calculated by using the following equation 1 (Islam et al., 2019):

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

where L^*_{DRB} , a^*_{DRB} , b^*_{DRB} , are the values of L^* , a^* , b^* of DRB.

5.2.4.4. Water Binding Capacity

Water binding capacity (WBC) was analyzed following the method described by Cornejo & Rosell, (2015). Briefly, distilled water (1 mL) was added to the DRB ($100.0 \text{ mg} \pm 0.5 \text{ mg}$), vortexed for 5 min and centrifuged in Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany) at $2000 \times g$ for 10 min at room temperature (25°C). WBC was expressed following the formula given by Cornejo & Rosell, (2015).

5.2.4.5. Oil Binding Capacity

Oil binding capacity (OBC) was quantified following the method described by Boulemkahel, Betoret, Nenatallah & Rosell, (2021) with some modification. DRB ($100.0 \text{ mg} \pm 0.5 \text{ mg}$) and vegetal oil (1.0 mL) was

vortexed for 5 min and then centrifuged (Eppendorf centrifuge 5430 R, Hamburg, Germany) at 3000 x g, 4°C for 10 min. After removing the supernatant, the tubes were inverted for 25 min to drain oil residues. OBC was calculated by the equation explain by Boulemkahel et al., (2021).

5.2.4.6. Solvent Retention Capacity

The Solvent Retention Capacity (SRC) for all samples was determined by adapting the AACC 56-11 method. The SRC values were determined using the following solvents: deionized water, 50% (w/w) sucrose solution, and 5% (w/w) sodium carbonate solution as solvents. SRC was assessed by quantifying the solvent retained by the samples following a 20-minute immersion in the solvent, subsequent centrifugation, and a 10-minute gel drainage period. The SRC value was expressed as percent of bran weight, on a 14% moisture basis.

5.2.4.7. Force for penetration

To evaluate the force of penetration of the samples, a 1:5 ratio of bran to water (w:v) was used to ensure optimal hydration of all components, accounting for the moisture content of each sample. Each sample was prepared and analyzed three times. Suspensions and subsequent measurements were performed in 50 mL beakers. Compression was applied at a 10 mm distance from the probe's contact with the sample. A TA-XT plus texture analyzer (Stable Microsystems, Godalming, UK), equipped with a 5 kg load cell and a 25 mm diameter cylindrical aluminum probe was used. The test speed was set at 1.0 mm/s, trigger type auto and the trigger force was 5.0 g. The maximum force required to penetrate the suspensions was recorded. The analysis was conducted in triplicate.

5.2.5. Statistical analysis

A descriptive statistical analysis (mean $\pm$ standard deviation) was applied to the data for the physico-chemical properties. The significant differences associated with the different treatments were evaluated by an analysis of the variance (ANOVA). Fisher's least significant differences test was the method used to present the results. Significant differences were considered as $p < 0.05$. Moreover, Pearson correlation analysis was applied to experimental values obtained from enzymatically treated samples (including the soaked control) to discern potential relationships within analytical parameters.

All analyses were run with the OriginPro versión 2022b, (Origin Lab Corporation, Northampton, MA, USA). All experiments were carried out in triplicate. Principal Component Analysis (PCA) was performed to discern differences among samples. The input matrix comprised 7 rows and 18 columns. PCA analysis was automatically scaled using the statistical program OriginPro.

5.3. Results and discussion

To better understand the impact of enzymatic and thermal treatments on the rice bran features, rice bran and defatted rice bran were evaluated, although those are not usually included in most of the reported studies. Likewise, to give more forceful results that can be attributed to the treatments or the process itself, a control sample exposed to soaking without enzyme addition (DRB-C) has been considered. Also, a sample subjected to the thermic treatment and subsequently to soaking without enzyme (DRB T-C) was carried out to explore potential synergistic effects arising from these two treatments.

5.3.1. Proximate composition

The results of the proximate composition and the different dietary fiber fractions of RB, DRB, different enzymatic and heat treatments, and their respective control, are listed in **Table 5.1**. As expected, freeze-dried samples showed significant lower moisture content than non-freeze-dried samples. All treatments promoted a significant increase in protein content compared to the control sample RB. Among the enzymatically treated samples, the most substantial increase occurred in the DRB-FL sample, reaching 21.11 g protein/100 g, although no significant differences were detected. The highest protein content was recorded in the defatted, heat-treated and soaked sample (DRB-T-C), with 21.91 g protein/100 g. As expected, defatting increase the TDF, particularly the IDF that was the predominant fraction. Furthermore, heat and enzymatic treatments had a minimal impact on TDF content of the DRB, but there was a significant increase trend in SDF content. Although significant differences were found in the SDF content of the enzymatically treated samples, soaking had the greatest impact and no trend regarding the type of enzymatic activities could be envisaged based on dietary fibers solubility. A deeper analysis of the TDF was performed quantifying its composition (**Table 5.1**).

Table 5.1. Proximate composition of RB with different treatments. Means with different letters within a column were significantly different ($p < 0.05$).

Proximate composition					
Treatment	Moisture (%)	Protein (%) db	TDF (%) db	IDF (%) db	SDF (%) db
RB	11.48 ± 0.04 ^b	16.06 ± 0.14 ^d	33.52 ± 3.53 ^c	30.30 ± 4.53 ^b	3.22 ^k
DRB	11.97 ± 0.06 ^a	20.79 ± 0.43 ^{bc}	39.75 ± 0.39 ^{ab}	35.82 ± 2.06 ^a	3.94 ^j
DRB-T	11.31 ± 0.35 ^b	20.82 ± 0.03 ^{bc}	38.19 ± 1.70 ^b	32.84 ± 0.82 ^{ab}	5.34 ⁱ
DRB-T-C	6.89 ± 0.09 ^g	21.91 ± 0.47 ^a	39.20 ± 1.70 ^{ab}	31.99 ± 1.45 ^{ab}	7.21 ^d
DRB-C	7.65 ± 0.14 ^f	20.55 ± 0.01 ^c	42.36 ± 3.34 ^a	32.79 ± 4.77 ^{ab}	9.57 ^a
DRB-NO	8.00 ± 0.09 ^e	20.74 ± 0.16 ^{bc}	39.40 ± 0.64 ^{ab}	32.98 ± 0.56 ^{ab}	6.43 ^g
DRB-CE	9.05 ± 0.04 ^d	21.10 ± 0.05 ^{bc}	39.74 ± 0.42 ^{ab}	33.53 ± 1.62 ^{ab}	6.21 ^h
DRB-UL	7.59 ± 0.05 ^f	20.84 ± 0.22 ^{bc}	39.13 ± 1.54 ^{ab}	32.69 ± 0.89 ^{ab}	6.45 ^f
DRB-SH	9.47 ± 0.12 ^c	20.71 ± 0.27 ^{bc}	42.29 ± 1.02 ^{ab}	34.88 ± 0.18 ^{ab}	7.41 ^c
DRB-AL	9.52 ± 0.33 ^c	21.00 ± 0.34 ^{bc}	41.36 ± 0.17 ^{ab}	34.00 ± 1.18 ^{ab}	8.19 ^b
DRB-FL	9.04 ± 0.28 ^d	21.11 ± 0 ^b	41.03 ± 1.65 ^{ab}	37.58 ± 0.44 ^a	7.16 ^e

TDF (% of db TDF)					Fibers (% of TDF)	
Treatment	IDF (%)	SDF (%)	Hemicellulose (%)	Cellulose (%)	Lignin (%)	
RB	82.73 ± 3.56 ^b	9.61 ^k	44.41 ^c	27.56 ± 2.93 ^{ab}	15.12 ± 0.99 ^{ab}	
DRB	90.09 ± 5.18 ^a	9.91 ^j	46.30 ^b	27.99 ± 1.02 ^a	14.35 ± 1.40 ^{ab}	
DRB-T	86.01 ± 2.15 ^{ab}	13.98 ⁱ	59.55 ^a	28.30 ± 1.87 ^a	14.30 ± 0.51 ^{ab}	
DRB-T-C	81.60 ± 3.71 ^b	18.39 ^c	37.16 ^g	27.40 ± 0.89 ^{abcd}	14.31 ± 0.54 ^b	
DRB-C	72.03 ± 4.13 ^c	22.59 ^a	23.59 ^j	25.53 ± 1.87 ^{ab}	11.30 ± 1.21 ^{ab}	
DRB-NO	83.69 ± 1.42 ^b	16.32 ^g	43.47 ^d	20.77 ± 1.98 ^d	15.73 ± 2.47 ^a	
DRB-CE	84.38 ± 4.07 ^{ab}	15.63 ^h	41.29 ^e	26.27 ± 2.83 ^{abc}	12.69 ± 0.39 ^{ab}	
DRB-UL	83.52 ± 2.27 ^b	16.48 ^f	30.58 ⁱ	27.37 ± 3.72 ^{ab}	13.49 ± 0.36 ^{ab}	
DRB-SH	82.48 ± 0.42 ^b	17.52 ^d	37.85 ^f	23.10 ± 1.90 ^{bcd}	12.91 ± 2.81 ^{ab}	
DRB-AL	79.81 ± 2.75 ^b	19.80 ^b	11.04 ^k	21.77 ± 3.74 ^{cd}	14.10 ± 2.40 ^{ab}	
DRB-FL	82.56 ± 2.60 ^b	17.45 ^e	31.54 ^h	26.63 ± 3.36 ^{ab}	14.32 ± 0.86 ^{ab}	

Abbreviations: RB: rice bran; DRB: defatted rice bran; DRB-T: defatted rice bran thermally treated; DRB-T-C: defatted rice bran thermally treated and soaked; DRB-C: defatted rice bran soaked; DRB-NO: defatted rice bran treated with Novozym® (xylanase); DRB-CE: defatted rice bran treated with Celluclast® (cellulase); DRB-UL: defatted rice bran treated with Ultimase® BWL 40 (cellulase and xylanase); DRB-SH: defatted rice bran treated with Shearzyme® Plus 2x (xylanase, cellulase and β-glucanase); DRB-AL: defatted rice bran treated with Alcalase® 2.4 L FG (endoprotease); DRB-FL: defatted rice bran treated with Flavourzyme® (peptidase preparation).

The fiber of RB was composed of hemicellulose (44.41%), followed by cellulose (27.56%) and lignin (15.12%), being 82.7% IDF and approx. 10% SDF. Thermal treatment significantly increased the amount of hemicellulose, likely water-soluble, based on the enhancement of SDF observed in that sample. Again, soaking affected the fiber distribution. In the samples that underwent soaking (DRB-T-C, DRB-C, and enzymatically-treated samples) SDF were significantly higher. After soaking, enzymatic treatments increased the amount of hemicellulose when treated with xylanase (DRB-NO) or cellulase (DRB-CE) but the combination of those (DRB-UL) did not intensify the effect. This observation could be attributed to the higher enzymatic activity of CE (700 U/g) compared to the enzymatic preparation UL (300 AGU/g). Likewise, cellulose was significantly reduced after treating with xylanase (DRB-NO) or xylanase, cellulase and β -glucanase mixture (DRB-SH), but no after cellulase treatment (DRB-CE). Those results agree with the research conducted by Coda, Rizzello, Curiel, Poutanen & Katina, (2014) in wheat bran treated with xylanase, obtaining an enhancement of the water extractable arabinoxylans and SDF content. It must highlight that the sample treated with endoprotease (DRB-AL) displayed the highest hydrolysis of hemicellulose and extensive hydrolysis of cellulose. These results suggest the existence of side-enzymatic activities in the commercial preparations.

Enzymatic treatment of rice bran has been previously reported and even combined with other physical treatments like micronization (Wen et al. (2017) or high-pressure homogenization (Xie et al. (2019)). Those studies reported a substantial increase of SDF content in rice bran and provide significant physicochemical data. However, present study provides information about the various fiber fractions and how enzymatic treatments specifically impact the amounts of each fraction.

5.3.2. Particle size distribution and microstructure of treated rice bran

Figure 5.1 displays the particle size distribution obtained for the different samples, indicating that all treatments reduced the particle size compared to RB. This reduction was cumulative, as it is evident that particle size decreases progressively after each treatment (DRB > DRB-T > DRB-T-C > DRB-C > all enzymatically treated samples). The mean diameter ($d_{(4,3)}$) ranged from 237-434 μm (**Table 5.2**), showing significant differences among the samples.

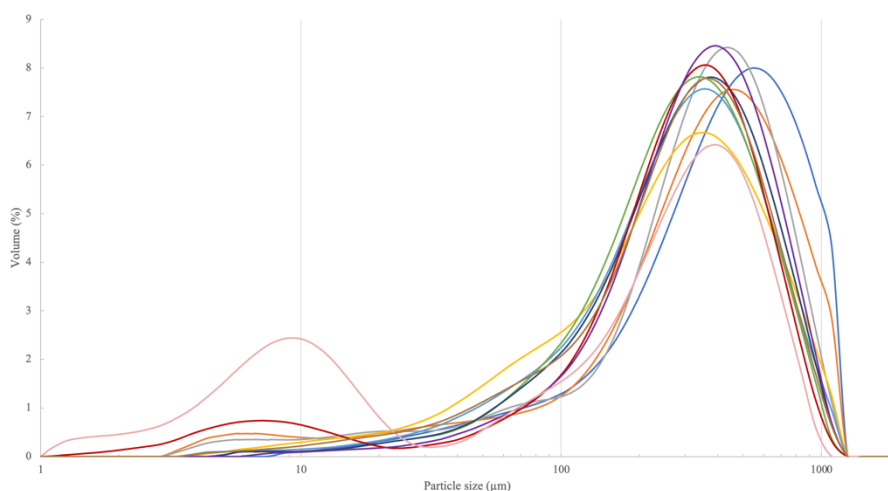


Figure 5.1. Particle size distribution of rice bran subjected to different treatments. Abbreviations: RB: rice bran (●); DRB: defatted rice bran (●); DRB-T: defatted rice bran thermally treated (●); DRB-T-C: defatted rice bran thermally treated and soaked (●); DRB-C: defatted rice bran soaked (●); DRB-NO: defatted rice bran treated with Novozym® (xylanase) (●); DRB-CE: defatted rice bran treated with Celluclast® (cellulase) (●); DRB-UL: defatted rice bran treated with Ultimase® BWL 40 (cellulase and xylanase) (●); DRB-SH: defatted rice bran treated with Shearzyme® Plus 2x (xylanase, cellulase and β -glucanase) (●); DRB-AL: defatted rice bran treated with Alcalase® 2.4 L FG (endoprotease) (●); DRB FL: defatted rice bran treated with Flavourzyme® (peptidase preparation) (●).

As noted by Guillon & Champ, (2000), the reduction in particle size in samples without enzymatic treatment may be due to the solubilization of some RB components, such as fiber, during processing, or resulting from aggregates dispersion, as mentioned by Rosell et al., (2009). The initial monomodal distribution shifted to bimodal one, particularly after treatment with DRB-AL or DRB-UL. In addition, the sample treated with endoprotease (DRB-AL) has the smallest mean particle size of all the samples. In contrast, the DRB-SH sample, among the group of enzymatically-treated samples, exhibited the largest mean particle size. Therefore, changes in the fiber composition promoted differences in the particle size distribution. Chen, Gao, Yang & Gao, (2013) arrived to similar conclusion when oat samples were subjected to microfluidization process for reducing their particle size, observing a redistribution of IDF and SDF.

Table 5.2. Technological characteristics of RB with different treatments. Means with different letters within a column were significantly different ($p < 0.05$).

Technological properties						
Treatment	$d_{(43)}$ (μm)	L^*	a^*	b^*	ΔE	
RB	434.06 $\pm$ 2.48 ^a	67.79 $\pm$ 0.02 ^a	1.78 $\pm$ 0.05 ^a	18.88 $\pm$ 0.01 ^e	13.59 $\pm$ 0.01	
DRB	379.33 $\pm$ 2.57 ^b	80.32 $\pm$ 0.01 ^k	0.49 $\pm$ 0.01 ^h	13.79 $\pm$ 0.01 ⁱ	4.12 $\pm$ 0.33 ^h	
DRB-T	358.89 $\pm$ 15.19 ^{bc}	77.13 $\pm$ 0.02 ^b	1.00 $\pm$ 0.02 ^f	16.31 $\pm$ 0.60 ^h	8.36 $\pm$ 0.06 ^f	
DRB-T-C	306.33 $\pm$ 10.44 ^{ef}	74.87 $\pm$ 0.05 ^c	1.35 $\pm$ 0.01 ^d	20.07 $\pm$ 0.03 ^b	10.72 $\pm$ 0.06 ^c	
DRB-C	316.03 $\pm$ 11.16 ^{ef}	71.28 $\pm$ 0.06 ⁱ	1.51 $\pm$ 0.01 ^b	19.45 $\pm$ 0.01 ^{cd}	9.68 $\pm$ 0.04 ^{de}	
DRB-NO	311.62 $\pm$ 6.67 ^{ef}	72.40 $\pm$ 0.02 ^f	1.19 $\pm$ 0.03 ^e	19.30 $\pm$ 0.03 ^d	9.77 $\pm$ 0.02 ^d	
DRB-CE	328.35 $\pm$ 8.99 ^{de}	72.59 $\pm$ 0.03 ^e	1.23 $\pm$ 0.03 ^e	19.72 $\pm$ 0.02 ^c	11.46 $\pm$ 0.04 ^a	
DRB-UL	301.56 $\pm$ 33.08 ^f	70.90 $\pm$ 0.05 ^j	1.43 $\pm$ 0.01 ^c	20.24 $\pm$ 0.02 ^b	11.08 $\pm$ 0.02 ^b	
DRB-SH	349.19 $\pm$ 8.67 ^{cd}	71.63 $\pm$ 0.01 ^h	1.48 $\pm$ 0.02 ^b	20.59 $\pm$ 0.04 ^a	7.95 $\pm$ 0.02 ^g	
DRB-AL	237.42 $\pm$ 12.30 ^g	73.57 $\pm$ 0.02 ^a	0.66 $\pm$ 0.07 ^g	17.98 $\pm$ 0.01 ^g	9.53 $\pm$ 0.05 ^e	
DRB-FL	314.28 $\pm$ 9.23 ^{ef}	72.07 $\pm$ 0.08 ^g	0.96 $\pm$ 0.01 ^f	18.54 $\pm$ 0.04 ^f		

Treatment	WBC (g water/g) db	OBC (g oil/g) db	Deionized Water (%) db	Sucrose (%) db	Sodium Carbonate (%) db	Force (g)
RB	2.93 $\pm$ 0.10 ^e	1.93 $\pm$ 0.04 ^g	258.02 $\pm$ 3.85 ^g	511.81 $\pm$ 16.44 ^d	263.02 $\pm$ 1.93 ^f	4131.22 $\pm$ 224.41 ^a
DRB	3.22 $\pm$ 0.11 ^d	2.62 $\pm$ 0.11 ^f	339.16 $\pm$ 11.91 ^e	591.05 $\pm$ 16.17 ^a	361.58 $\pm$ 11.25 ^{de}	742.34 $\pm$ 166.00 ^{fg}
DRB-T	3.13 $\pm$ 0.21 ^{de}	2.91 $\pm$ 0.07 ^f	337.27 $\pm$ 8.16 ^e	591.23 $\pm$ 18.61 ^a	354.97 $\pm$ 8.51 ^{de}	1022.21 $\pm$ 445.96 ^{ef}
DRB-T-C	2.96 $\pm$ 0.31 ^{de}	4.69 $\pm$ 0.07 ^{cd}	360.53 $\pm$ 9.52 ^{cd}	586.96 $\pm$ 11.71 ^a	383.47 $\pm$ 3.62 ^{bc}	2524.01 $\pm$ 254.73 ^b
DRB-C	3.54 $\pm$ 0.07 ^c	4.32 $\pm$ 0.36 ^e	312.09 $\pm$ 8.02 ^f	563.74 $\pm$ 7.80 ^{bc}	345.54 $\pm$ 2.51 ^e	2737.27 $\pm$ 266.81 ^b
DRB-NO	4.02 $\pm$ 0.12 ^b	4.55 $\pm$ 0.15 ^{de}	345.07 $\pm$ 8.05 ^{de}	551.54 $\pm$ 16.16 ^c	354.44 $\pm$ 17.05 ^{de}	1667.24 $\pm$ 26.22 ^e
DRB-CE	3.67 $\pm$ 0.17 ^c	5.30 $\pm$ 0.45 ^b	343.69 $\pm$ 12.77 ^{de}	585.05 $\pm$ 13.95 ^{ab}	372.01 $\pm$ 18.22 ^{cd}	1109.70 $\pm$ 111.83 ^{def}
DRB-UL	3.69 $\pm$ 0.26 ^c	5.03 $\pm$ 0.22 ^{bc}	340.46 $\pm$ 10.39 ^e	506.96 $\pm$ 7.09 ^{de}	393.32 $\pm$ 13.59 ^{ab}	1435.32 $\pm$ 130.86 ^c
DRB-SH	4.00 $\pm$ 0.14 ^b	4.48 $\pm$ 0.16 ^{de}	364.45 $\pm$ 17.83 ^c	508.19 $\pm$ 5.01 ^{de}	400.45 $\pm$ 7.22 ^{ab}	1180.05 $\pm$ 57.07 ^{de}
DRB-AL	3.77 $\pm$ 0.16 ^{bc}	4.91 $\pm$ 0.16 ^c	383.19 $\pm$ 15.38 ^b	486.16 $\pm$ 15.13 ^{ef}	390.42 $\pm$ 6.60 ^b	255.42 $\pm$ 38.45 ^h
DRB-FL	4.54 $\pm$ 0.15 ^a	5.72 $\pm$ 0.11 ^a	415.98 $\pm$ 3.69 ^a	483.21 $\pm$ 13.95 ^f	409.02 $\pm$ 11.48 ^a	375.70 $\pm$ 84.82 ^{gh}

Abbreviations: RB: rice bran; DRB: defatted rice bran; DRB-T: defatted rice bran thermally treated; DRB-T-C: defatted rice bran thermally treated and soaked; DRB-C: defatted rice bran soaked; DRB-NO: defatted rice bran treated with Novozym® (xylanase); DRB-CE: defatted rice bran treated with Celluclast® (cellulase); DRB-UL: defatted rice bran treated with Ultimase® BWL 40 (cellulase and xylanase); DRB-SH: defatted rice bran treated with Shearzyme® Plus 2x (xylanase, cellulase and β -glucanase); DRB-AL: defatted rice bran treated with Alcalase® 2.4 L FG (endoprotease); DRB_FL: defatted rice bran treated with Flavourzyme® (peptidase preparation).

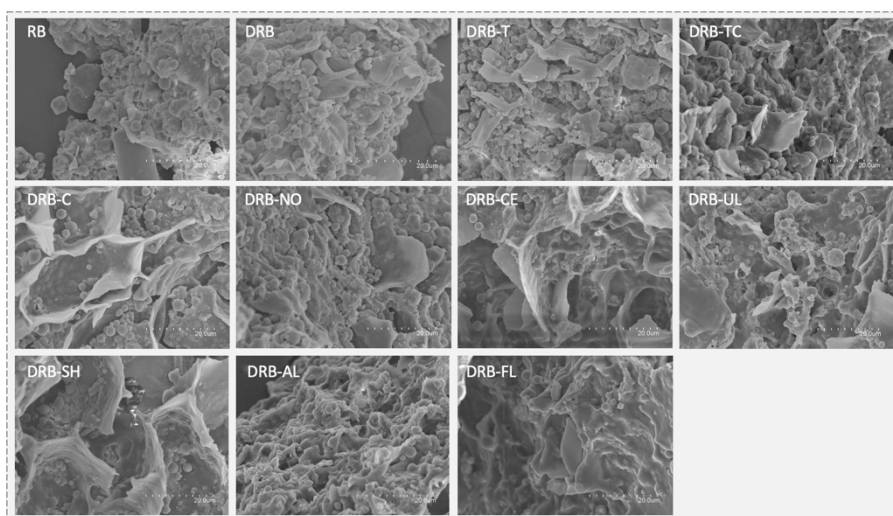


Figure 5.2. Scanning electron microscopy analysis of rice bran subjected to different treatments. Abbreviations: RB: rice bran; DRB: defatted rice bran; DRB-T: defatted rice bran thermally treated; DRB-T-C: defatted rice bran thermally treated and soaked; DRB-C: defatted rice bran soaked; DRB-NO: defatted rice bran treated with Novozym® (xylanase); DRB-CE: defatted rice bran treated with Celluclast® (cellulase); DRB-UL: defatted rice bran treated with Ultimase® BWL 40 (cellulase and xylanase); DRB-SH: defatted rice bran treated with Shearzyme® Plus 2x (xylanase, cellulase and β -glucanase); DRB-AL: defatted rice bran treated with Alcalase® 2.4 L FG (endoprotease); DRB-FL: defatted rice bran treated with Flavourzyme® (peptidase preparation).

At microstructure level, differences associated to the treatments could be envisaged (**Figure 5.2**). Depending on the treatments applied, different proportions of small spherical substances were observed. Starch was predominantly associated with more polygonal spheres, while proteins corresponded to more rounded spheres (Chittapalo & Noomhorm, 2009). Both structures were observable in RB and DRB. In the treated samples, only proteins were observed. The treatment involving cellulases, xylanases, and β -glucanases (DRB-NO, DRB-CE, DRB-UL, and DRB-SH) effectively led to the breakdown of cell walls, as observed in **Figure 5.2**. This finding aligns with Mishra, Ray, Rosell & Panda, (2017) compilation of cell wall degrading enzymes, which includes the aforementioned enzymes. Likewise, Chittapalo & Noomhorm, (2009) identified starch and proteins on the surface of the DRB and observed changes in the cell wall structure when applied physical treatments for the DRB proteins extraction. Visibly, samples without enzymatic treatment (RB, DRB, DRB-T, DRB-T-C, DRB-C) exhibited greater

disaggregation of all compounds, in contrast to enzyme-treated samples, which displayed a more gel-like structure in their surface. The protease-treated samples displayed noticeable structural changes compared to other enzymatically treated samples. Firstly, these samples exhibited tighter binding, resulting in less distinct differentiation of protein bodies and the layered walls. Moreover, in the sample treated with the endoprotease (DRB-AL), the protein bodies still maintained a spherical shape, while in the sample treated with the peptidase preparation (DRB-FL) the protein bodies acquired a film shape structure, likely due to small peptides fragments released.

5.3.3. Impact of rice bran treatment on color

Although only visual differences were perceived after defatting, the results of the instrumental color characterization showed slight but statistically significant changes ($p < 0.05$) for all the treatments performed (**Table 5.2**). The most notable change was in the DRB sample. The L^* parameter significantly increase, up to 15.60% when comparing RB with DRB, while the a^* parameter showed a reduction of 72.47% in those samples, and the b^* parameter varied by 33.03% compared DRB sample to DRB-SH. Defatting increases the L^* indicating the lightening of the bran when removing the fat. Regarding the a^* parameter (representing red-green tones), all treatments decreased it, shifting to more greenish. The b^* parameter (representing yellow-blue tones) increased in the samples treated with carbohydrate-acting enzymes, compared to RB, while samples treated with proteins-acting enzymes showed a decrease in this parameter. The overall color change (ΔE) was determined as the disparity between each treatment and the DRB. The most significant color change was observed in samples subjected to soaking, particularly those treated with carbohydrases, especially Ultimase treatment (DRB-UL), which exhibited the highest difference compared to the DRB, at 11.46. Samples treated with proteases displayed lower and more homogeneous values, akin to the sample subjected to heat treatment and soaking, while the heat-treated sample (DRB-T) showed the least overall color variation relative to the DRB.

5.3.4. Hydration properties of treated rice bran

The enzymatic treatments yielded noticeable increases in both WBC (Water Binding Capacity) and OBC (Oil Binding Capacity) values when compared to the non-enzymatically treated sample (**Table 5.2**). The most

remarkable enhancement was observed in the case of DRB-FL, where the WBC and the OBC showed an increase of 1.55 and 2.96 times, respectively, in comparison to the untreated RB. Likely, the action of the peptidase preparation modified the proteins' structure and their affinity for water and oil. Notably, the OBC exhibited a more pronounced increase than the WBC, a trend that was consistently observed in all the samples that had soaking in the presence and absence of enzymes, but no other distinguishable pattern was envisaged due to the type of enzyme added.

However, it is worth noting that water binding exhibited a significant positive correlation ($r = 0.69$) with insoluble fiber content, and oil binding capacity was related ($p < 0.05$) with SDF. These results align with findings from Zhu, Huang, Peng, Qian & Zhou, (2010), where insoluble fiber led to higher WBC, but unlike them OBC was positively correlated to soluble fiber. Similarly, a negative relationship has been reported between particle size and hydration capacity (Chau, Wang & Wen, 2007; Zhao, Yang, Gai & Yang, 2009), because smaller particles expose more surface area, facilitating greater interaction with surrounding solvents. This concept is consistent with results in the current study, where all treatments resulted in a particle size reduction, with a simultaneous increase in the WBC and OBC values. This accentuates a robust and statistically significant negative correlation between particle size and OBC ($r = -0.80$). This observation underscores the relevance of particle size in influencing hydration properties.

Similarly, SRC in three distinct solvents was conducted (Table 5.2). Specifically, the SRC of sodium carbonate correlates with the levels of damaged starch, while that of sucrose is associated with pentosan characteristics. Deionized water, on the other hand, serves as a control solvent in this context. This method has been adopted to provide a comprehensive understanding of how the various compounds present in rice bran influence its behavior when employed as an ingredient in a food matrix. The treatments significantly increased SRC, except SRC in sucrose for the samples treated with protein-acting enzymes (DRB-AL and DRB-FL), that decreased those values. All SRC values obtained in the analysis ranged from 100 to nearly 400 times higher than those observed in wheat flour, confirming the remarkable solvent retention capacity of rice bran. The starch content in the RB used in this study was 12.99%, falling within the reported range of 10-20% (Sharma, Chauhan & Agrawal, 2004). The increase observed in the SRC in sodium

carbonate, was indicative of enhanced amount of damaged starch. The highest value observed at 409.02%, corresponded to DRB-FL, which also had the highest SRC in deionized water, while yielding the lowest value in sucrose. Therefore, defatted rice bran with carbohydrate-acting enzymes increased SRC values in sucrose, water, and carbonate solvents. DRB-SH had the most pronounced effect on water and carbonate, while DRB-CE on the sucrose. Again, a notable negative correlation was evident between particle size and the retention of deionized water ($r = -0.74$) as well as the retention of sodium carbonate ($r = -0.71$). Therefore, the SRC in different solvents could be used to discriminate among enzymatic treatments performed on rice bran.

5.3.5 Textural properties of treated rice bran

The texture of the rice bran samples, after hydrating them by adding five times water, was evaluated (**Table 5.2**). Treatments applied to RB led to a notable reduction in the force needed to penetrate the sample. A positive correlation with the particle size of each sample was observed, although it did not reach statistical significance. RB without any treatment exhibited the highest penetration force, 4131.22 g. Within the group treated with enzymes targeting carbohydrates, no statistically significant differences were observed neither between the DRB-NO and DRB-UL, or the pair DRB-CE and DRB-SH. In contrast, defatted bran treated with proteases, particularly DRB-AL, gave the softest samples.

3.5.6. Overall features of treated rice bran

A principal components analysis (PCA) (**Figure 5.3**) was conducted to discern potential clusters based on enzymatic treatment (carbohydrate-acting enzymes, and protein-acting enzymes), using the proximate composition and technological properties as studied factors. This analysis accounts for 63.39% of the variances observed among the treated samples. Principal component 1 (PC1) accounted for 43.70% of the variability, while principal component 2 (PC2) explained 19.69%. In **Figure 5.3**, variables that are closely located indicate stronger positive correlations, whereas variables positioned in opposite locations demonstrate inverse correlations. In this sense, PC1 is primarily influenced by the solvent retention capacity in deionized water, proteins, and insoluble fiber, while penetration force and a^* value have an opposite influence. In PC2, hemicellulose, d (4.3), and the b^* represent the driving variables while and an opposite effect was observed for soluble fiber.



Figure 5.3. Graph showing the set of defatted rice bran samples treated by soaking (DRB-C) and with enzymatic treatment, and the variables studied by principal component analysis (PCA). The three different circles group the samples depending on the treatment to which they have been subjected, (•) sample treated by soaking, (•) samples treated with carbohydrate-acting enzymes and (•) samples treated with protein-acting enzymes.

Figure 5.3 clearly showed that PC1 discriminated between samples treated with protein-acting enzymes, positioning them on the positive axis, and the DRB-C sample (as control sample) was placed on the negative axis, confirming the efficiency of the enzymatic treatment in modifying the physico-chemical properties of the DRB. Meanwhile, PC2 discriminated samples treated with carbohydrate-acting enzymes, situating them on the positive axis, with the exception of the DRB-NO sample, which was positioned on the negative axis. This placement of DRB-NO was associated with its high TDF and SDF content. The PCA validates the findings presented in **Tables 5.1 and 5.2** by grouping the control sample (DRB-C) with penetration force, soluble fiber, and total fiber (the latter being shared with DRB-NO). Hemicellulose and cellulose content, particle size, and color, effectively clustered the samples treated with carbohydrate-acting enzymes.

The majority of technological properties successfully clustered and differentiated the DRB-FL sample, while the DRB-AI sample exhibited an opposite trend in relation to particle size, which stands out as one of its most distinct characteristics.

5.4. Conclusions

Enzymatic treatments can effectively modify the defatted rice bran composition and functionality. Nevertheless, the extension of the impact was highly dependent on the enzymes used for the treatment. Carbohydrate and protein degrading enzymes augmented the proportion of SDF, which was not obtained with the heat treatment. Those variations in the proportion of the fiber composition allowed reducing the particle size distribution with the subsequent impact on the solvent retention capacity of the defatted rice bran, and on the texture of the hydrated bran. The analysis of the solvent retention ability of the rice in different solvents gave indications about the constituents that were modified, starch and proteins, which were also confirmed by SEM micrographs. Modifications were also observed in the chromatic parameters, particularly in brightness, which increased by 100% in all instances. Given the interest in increasing the fiber content of foods and beverages, enzymatically treated rice bran shows promise for better integration into food matrices due to its smaller particle size and improved hydration performance. Nevertheless, considering the complexity of the bran matrix, additional studies will be needed to explain the mechanisms behind each enzymatic treatment. Thermal and mechanical analysis of the isolated constituents will be undertaken in future studies.

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References

- Boulemkahel, S., Betoret, E., Benatallah, L., & Rosell, C. M. (2021). Effect of low pressures homogenization on the physico-chemical and functional properties of rice flour. *Food Hydrocolloids*, 112, 106373. <https://doi.org/10.1016/j.foodhyd.2020.106373>
- Chau, C.-F., Wang, Y.-T., & Wen, Y.-L. (2007). Different micronization methods significantly improve the functionality of carrot insoluble fibre. *Food Chemistry*, 100(4), 1402–1408. <https://doi.org/10.1016/j.foodchem.2005.11.034>
- Chen, J., Gao, D., Yang, L., & Gao, Y. (2013). Effect of microfluidization process on the functional properties of insoluble dietary fiber. *Food Research International*, 54(2), 1821–1827. <https://doi.org/10.1016/j.foodres.2013.09.025>
- Chittapalo, T., & Noomhorm, A. (2009). Ultrasonic assisted alkali extraction of protein from defatted rice bran and properties of the protein concentrates. *International Journal of Food Science & Technology*, 44(9), 1843–1849. <https://doi.org/10.1111/j.1365-2621.2009.02009.x>
- Coda, R., Rizzello, C. G., Curiel, J. A., Poutanen, K., & Katina, K. (2014). Effect of bioprocessing and particle size on the nutritional properties of wheat bran fractions. *Innovative Food Science & Emerging Technologies*, 25, 19–27. <https://doi.org/10.1016/j.ifset.2013.11.012>
- Cornejo, F., & Rosell, C. M. (2015). Physicochemical properties of long rice grain varieties in relation to gluten free bread quality. *LWT - Food Science and Technology*, 62(2), 1203–1210. <https://doi.org/10.1016/j.lwt.2015.01.050>
- Doan, N. T. T., Lai, Q. D., Vo, H. V., & Nguyen, H. D. (2021). Influence of adding rice bran on physio-chemical and sensory properties of bread. *Journal of Food Measurement and Characterization*, 15(6), 5369–5378. <https://doi.org/10.1007/s11694-021-01111-5>
- FAO. (2024, February 7). Paddy rice production worldwide in 2022, by country (in million metric tons). *Statista*. <https://www.statista.com/statistics/255937/leading-rice-producers-worldwide/#statisticContainer>
- Garba, U., Singanusong, R., Jiamyangyeun, S., & Thongsook, T. (2019). Extraction and utilisation of rice bran oil. A review. *LA RIVISTA ITALIANA DELLE SOSTANZE GRASSE*, 96(3), 161–170.

- Guillon, F., & Champ, M. (2000). Structural and physical properties of dietary fibres, and consequences of processing on human physiology. *Food Research International*, 33(3), 233–245. [https://doi.org/10.1016/S0963-9969\(00\)00038-7](https://doi.org/10.1016/S0963-9969(00)00038-7)
- Gul, K., Yousuf, B., Singh, A. K., Singh, P., & Wani, A. A. (2015). Rice bran: Nutritional values and its emerging potential for development of functional food—A review. *Bioactive Carbohydrates and Dietary Fibre*, 6(1), 24–30. <https://doi.org/10.1016/j.bcdf.2015.06.002>
- Hamada, J. S. (2000). Characterization and Functional Properties of Rice Bran Proteins Modified by Commercial Exoproteases and Endoproteases. *Journal of Food Science*, 65(2), 305–310. <https://doi.org/10.1111/j.1365-2621.2000.tb15998.x>
- Islam, M. Z., Saha, T., Monalisa, K., & Hoque, M. M. (2019). Effect of starch edible coating on drying characteristics and antioxidant properties of papaya. *Journal of Food Measurement and Characterization*, 13(4), 2951–2960. <https://doi.org/10.1007/s11694-019-00215-3>
- Khan, S. H., Butt, M. S., Sharif, M. K., Sameen, A., Mumtaz, S., & Sultan, M. T. (2011). Functional Properties of Protein Isolates Extracted from Stabilized Rice Bran by Microwave, Dry Heat, and Parboiling. *Journal of Agricultural and Food Chemistry*, 59(6), 2416–2420. <https://doi.org/10.1021/jf104177x>
- Leal, F. H. P. N., Senna, C. de A., Kupski, L., Mendes, G. da R. L., & Badiale-Furlong, E. (2021). Enzymatic and extrusion pretreatments of defatted rice bran to improve functional properties of protein concentrates. *International Journal of Food Science & Technology*, 56(11), 5445–5451. <https://doi.org/10.1111/ijfs.15017>
- Liu, Y., Zhang, H., Yi, C., Quan, K., & Lin, B. (2021). Chemical composition, structure, physicochemical and functional properties of rice bran dietary fiber modified by cellulase treatment. *Food Chemistry*, 342, 128352. <https://doi.org/10.1016/j.foodchem.2020.128352>
- Majzoobi, M., Sharifi, S., Imani, B., & Farahnaky, A. (2013). The Effect of Particle Size and Level of Rice Bran on the Batter and Sponge Cake Properties. *Journal of Agricultural Science and Technology*, 15, 1175–1184.
- Mishra, S. S., Ray, R. C., Rosell, C. M., & Panda, B. (2017). Microbial Enzymes in Food Applications. *Microbial Enzyme Technology in Food Applications*, 3–18.
- Mohammadi, F., Marti, A., Nayebzadeh, K., Hosseini, S. M., Tajdar-oranj, B., & Jazaeri, S. (2021). Effect of washing, soaking and pH in combination with ultrasound on enzymatic rancidity, phytic acid, heavy metals and coliforms of rice bran. *Food Chemistry*, 334, 127583.

<https://doi.org/10.1016/j.foodchem.2020.127583>

- Muthayya, S., Sugimoto, J. D., Montgomery, S., & Maberly, G. F. (2014). An overview of global rice production, supply, trade, and consumption. *Annals of the New York Academy of Sciences*, 1324(1), 7–14. <https://doi.org/10.1111/nyas.12540>
- Rosell, C. M., Santos, E., & Collar, C. (2009). Physico-chemical properties of commercial fibres from different sources: A comparative approach. *Food Research International*, 42(1), 176–184. <https://doi.org/10.1016/j.foodres.2008.10.003>
- Scarabattoli, L., Sangiorgio, S., Romagnuolo, F., Gelati, L., Cavuoto, D., Rabuffetti, M., Morelli, C. F., Lupinelli, S., & Speranza, G. (2023). Use of carbohydrases to promote protein extraction from rice bran and soybean meal: A comparative study. *LWT*, 184, 115060. <https://doi.org/10.1016/j.lwt.2023.115060>
- Sharma, H. R., Chauhan, G. S., & Agrawal, K. (2004). Physico-Chemical Characteristics of Rice Bran Processed by Dry Heating and Extrusion Cooking. *International Journal of Food Properties*, 7(3), 603–614. <https://doi.org/10.1081/JFP-200033047>
- Spaggiari, M., Dall'Asta, C., Galaverna, G., & del Castillo Bilbao, M. D. (2021). Rice Bran By-Product: From Valorization Strategies to Nutritional Perspectives. *Foods*, 10(1). <https://doi.org/10.3390/foods10010085>
- Tang, S., Hettiarachchy, N. S., Horax, R., & Eswaranandam, S. (2003). Physicochemical Properties and Functionality of Rice Bran Protein Hydrolyzate Prepared from Heat-stabilized Defatted Rice Bran with the Aid of Enzymes. *Journal of Food Science*, 68(1), 152–157. <https://doi.org/10.1111/j.1365-2621.2003.tb14132.x>
- Tang, S., Hettiarachchy, N. S., & Shellhammer, T. H. (2002). Protein Extraction from Heat-Stabilized Defatted Rice Bran. 1. Physical Processing and Enzyme Treatments. *Journal of Agricultural and Food Chemistry*, 50(25), 7444–7448. <https://doi.org/10.1021/jf025771w>
- Vallabha, V. S., Indira, T. N., Jyothi Lakshmi, A., Radha, C., & Tikku, P. K. (2015). Enzymatic process of rice bran: A stabilized functional food with nutraceuticals and nutrients. *Journal of Food Science and Technology*, 52(12), 8252–8259. <https://doi.org/10.1007/s13197-015-1926-9>
- Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science*, 74(10), 3583–3597. [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2)
- Wen, Y., Niu, M., Zhang, B., Zhao, S., & Xiong, S. (2017). Structural characteristics and functional properties of rice bran dietary fiber modified

- by enzymatic and enzyme-micronization treatments. *LWT*, 75, 344–351. <https://doi.org/10.1016/j.lwt.2016.09.012>
- Xie, F., Zhao, T., Wan, H., Li, M., Sun, L., Wang, Z., & Zhang, S. (2019). Structural and Physicochemical Characteristics of Rice Bran Dietary Fiber by Cellulase and High-Pressure Homogenization. *Applied Sciences*, 9(7). <https://doi.org/10.3390/app9071270>
- Zhao, X., Yang, Z., Gai, G., & Yang, Y. (2009). Effect of superfine grinding on properties of ginger powder. *Journal of Food Engineering*, 91(2), 217–222. <https://doi.org/10.1016/j.jfoodeng.2008.08.024>
- Zhu, K., Huang, S., Peng, W., Qian, H., & Zhou, H. (2010). Effect of ultrafine grinding on hydration and antioxidant properties of wheat bran dietary fiber. *Food Research International*, 43(4), 943–948. <https://doi.org/10.1016/j.foodres.2010.01.005>



**Enhancing strategies for
ensuring food safety while
revaluing rice bran.**

**High pressure processing at
different hydration levels as
a tool to enhance rice bran
stability and techno-
functionality**

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Cristina M. Rosell

Submitted to journal.

Microbiology counts
Technological properties
Fibers
Peroxide content
Particle size
Food safety
By-product

High-pressure processing (HPP) enhances food safety and shelf life by inactivating microorganisms and preserving food quality, yet its effectiveness in low-humidity environments has not been evaluated. This study investigated the effects of HPP at 500 MPa for 15 minutes across varying hydration levels (15, 30, 60, 77%) on rice bran (RB), aiming to identify microbial effectiveness, techno-functional and physicochemical properties for reaching more sustainable food industry practices. HPP effectively reduced mesophilic bacteria, molds and yeast of RB at >15% hydration level, though it did not significantly affect spore inactivation. HPP treatment of $\geq 30\%$ hydrated RB induced particles aggregation and a honeycomb formation. The interaction between hydration and HPP treatment significantly affected the distribution of total dietary fibers, and the tendency was dependent on the hydration condition. Protein solubility was enhanced by hydration (15, 30 and 60%), and peroxide values decreased after HPP treatment at low hydration ($\leq 30\%$) but increased when applied to high hydrated ($>30\%$) RB. Emulsifying activity decreased upon HPP treatment of highly hydrated RB ($\geq 60\%$), but more stable emulsions were achieved after HPP, regardless of the hydration level. Therefore, this study highlights the potential of HPP as a sustainable approach to enhance the utilization of rice bran in food applications, addressing existing knowledge gaps regarding its processing under different moisture conditions.

6.1 Introduction

Rice bran (RB), still considered a by-product of rice milling processes, has substantial potential for its content in lipids, proteins and dietary fiber (Sapwarobol et al., 2021; Wang et al., 2022). Actually, fiber content has been related to water and oil-binding capacities, and the protein fraction demonstrates promising emulsifying and foam stabilizing abilities (Liu et al., 2022; Spaggiari et al., 2021). However, the high microbial load of the rice bran, besides its tendency to rancidity due to the high fat content, limits the use of RB in food applications, opting for utilising defatted bran as ingredient.

High-pressure processing (HPP) is widely employed as non-thermal technology for enhancing food safety (Allai et al., 2023). In fact, it has been applied for reducing the viable counts in milk, cold brew tea, sauces, meat and seafood (Considine et al., 2008; Hurtado et al., 2019; Song et al., 2021; Stratakos et al., 2019). Those applications require the optimization of HPP conditions because microbial load reduction varies depending on the target microorganism and food matrix, being the most commonly used 600 MPa for 5-6.5 minutes (Daryaei & Balasubramaniam, 2012; Hiperbaric, 2022). This preservation technique aligns with nowadays consumer preferences for fresh and safe products, extending shelf life by inactivating enzymes as well as microorganisms, and preserving the sensory and nutritional integrity of foods (Bello et al., 2014; Cappa et al., 2016; Roobab & Aadil, 2023).

Although the initial applications of HPP were as preservative non-thermal treatment, lately, HPP has been applied to modify the physicochemical properties of raw materials (Liu et al., 2022). For instance, Cappa et al., (2016) subjected corn starch, rice flour, and waxy rice flour suspensions (40%) to HPP treatments at 400 and 600 MPa (5 and 10 min) enhancing their paste behavior and solvent retention capacity. Likewise, Ahmed et al., (2007) studied the impact of HPP (350 – 650 MPa; 7.5 – 15 min) on rice slurries containing 16.5%, 22.9%, or 33% rice flour to understand the pressure susceptibility of the main rice components. Authors reported lower starch gelatinization temperature and modifications in the secondary structure of the proteins after treatment. Similarly, changes in proteins have been observed when applying HPP (from 100 to 500 MPa) to 1% (w/v) rice bran (RB) protein fractions (Zhu et al., 2017). In addition, Wang et al., (2022) reported significant improvements in solubility, emulsifying properties, and foaming

properties in 1% (w/v) RB protein dispersions after HPP treatment at 100, 200 and 300 MPa for 30 min. However, those treatments were applied to very diluted systems that require posterior dehydration for powder applications.

HPP treatments are typically conducted under high moisture conditions, particularly for microbial inactivation, that requires at least 40% of free water (Muntean et al., 2016), but its effectiveness in low-humidity environments has been scarcely studied. Recently, Seo et al. (2023) indicated that HPP (400-600 MPa for 10 minutes) were effective enhancing the water absorption, solubility, swelling power of rice flour using hydrations as low as 35, 45 or 55%. Therefore, an understanding of the impact of HPP across different moisture levels as the possibility of successfully processing RB samples with a very high sample-to-water concentration level is important, given its crucial role in the food industry, closely linked with the transformation and formulation processes of food products.

This research aims to study the impact of high-pressure treatment on different hydration levels of rice bran, with a dual focus on improving food safety and modifying its technological and functional properties, addressing the knowledge gaps concerning the application of high pressures under conditions of low hydration. By adopting a comprehensive approach that considers rice bran as a whole, rather than focusing solely on isolated fractions as commonly observed in the literature, this research was made to promote more sustainable practices within the food industry.

6.2. Material and methods

6.2.1. Rice bran pretreatment

The RB from Arrocería Pons (Valencia, Spain) was sieved to eliminate foreign matter (> 1 mm). Moisture content was determined using the standard method ISO 712:2009. Rice bran (150 g) was adjusted to different moisture levels (15%, 30%, 60% and 77%) following the ISO procedure (ISO 27971:2023) designed for tempering cereals, obtaining values of water activity (a_w) of 0.772, 0.951, 0.977, and 0.993, respectively. Samples were coded using RB for rice bran followed with the moisture levels used for pretreatment (RB-15, RB-30, RB-60, RB-77).

6.2.2. High-pressure treatment

The tempered samples were sealed in polyethylene bags (MULTIVAC Thermosealer, Switzerland) and placed into the HPP unit (High Pressure Food Processor; EPSI NV, Belgium). HPP samples were subjected at 500 MPa for 15 min, excluding rise and fall times. Unpressurized samples for each hydration level were kept as references. 10 g of each sample was reserved for microbiology analysis and the remaining portion was frozen at -80 °C for subsequent freeze-drying. HPP treated samples were coded with HPP.

6.2.3. Microbiology

Samples for microbiological analysis were collected immediately post HPP and from those without treatment. Uninoculated bran samples (10 g) were homogenized in 0.1% (w/v) sterile peptone water (Scharlab Chemie S. A., Barcelona, Spain) using a Stomacher (Scharlab, S.L, Barcelona, Spain) before culturing. Total mesophilic bacteria were enumerated following ISO 4833-1:2013 on plate count agar (PCA, Scharlab Chemie S. A., Barcelona, Spain) under aerobic conditions at 30°C for 48 h. To analyze molds and yeasts, a_w of each sample were taken into account. Since the 15% a_w was lower than 0.95, the dichloran-glycerol agar (DG18) (Scharlab S.A., Barcelona, Spain) culture medium was used, as detailed in the ISO 21527-2:2008. Following the same protocol but part 1 (ISO 21527-1:2008), samples RB-30, RB-60, and RB-77, were cultured using the dichloran rose bengal chloramphenicol (DRBC) (Scharlab S.A., Barcelona, Spain) medium due to their a_w exceeding 0.95. The samples were incubated under aerobic conditions at 25°C for five days. Bacterial spore counts were determined following a Katina et al. (2012) protocol with some modifications. Five mL of the homogenized samples were heated at 80°C in a water bath for 15 minutes to inactivate vegetative cells. Samples were decimally diluted in 0.1% (w/v) sterile peptone water and aerobic spore-forming bacteria were quantified on tryptone soy agar (TSA) (Scharlab S.A., Barcelona, Spain) plates, which were incubated at 30°C for 48 h. All microbiological counts were reported as colony-forming units per gram (CFU/g).

6.2.4. Physical characterization

Particle size distribution and microstructure of the RB were evaluated in the freeze-dried samples. The Malvern Mastersizer equipment (Mastersizer Scirocco 2000; Malvern Instruments Ltd., Worcestershire, U.K.) was used for

assessing particle size distribution. The volume-weighted mean diameter ($d_{(4.3)}$) was recorded from three replicates for each sample.

Scanning electron microscopy (SEM) was used to examine the microstructure. RB samples were coated during 300 s with gold using a vacuum evaporator (JEE 400; JEOL, Tokyo, Japan). Observation was done in SEM (Hitachi S-4800, Tokyo, Japan) at an accelerating voltage of 10 kV at low (600x) and high (1000x) magnification.

6.2.5. Chemical composition

Moisture was quantified following ISO methodology (ISO 712:2009). The estimation of Total Dietary Fiber (TDF), Insoluble Dietary Fiber (IDF), and Soluble Dietary Fiber (SDF) contents was carried out by following method 32-07 (AACC, 1999). The Neutral Detergent Fiber (NDF) content was assessed following the methodology proposed by Van Soest et al., (1991). For Acid Detergent Fiber (ADF) and Acid Detergent Lignin (ADL) determination the procedures outlined in ISO 13906:2008 were used. The hemicellulose and cellulose fractions were calculated as Equation 1 and 2, respectively.

$$\text{Hemicellulose} = \text{NDF} - \text{ADF} \quad \text{Equation 1}$$

$$\text{Cellulose} = \text{ADF} - \text{ADL} \quad \text{Equation 2}$$

Glucose and soluble protein content were also evaluated. Samples (0.2 g) were suspended in 20 mL of deionized water, mixed vigorously for 3 hours using a magnetic stirrer and centrifuged at 16,000 $\times g$ for 15 min. The supernatant was used to quantify glucose and soluble protein. The glucose content was quantified using a glucose oxidase–peroxidase (GOPOD) kit (Megazyme, Dublin, Ireland). The absorbance was measured at 510 nm using an SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany). Protein content was quantified with a PierceTM bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, USA). Bovine serum albumin was used as the standard protein, and absorbance was measured at 562 nm. All experiments were conducted in quadruplicate.

Peroxide values were determined using AOCS Cd 8-53 method (AOCS 2004). The sample was solubilized in a solution composed of acetic acid and chloroform in a 3:2 ratio, followed by treatment with potassium iodide

saturated solution. The resulting iodine liberated during the reaction was subsequently titrated using a 0.01 N solution of sodium thiosulfate, in the presence of starch solution as an indicator for endpoint determination. Results were expressed as the average of at least three replicates in meq O₂ / kg fat.

6.2.6. Hydration, foaming and emulsifying properties

Water binding capacity (WBC) analysis was conducted according to the protocol described by Cornejo & Rosell, (2015). Samples (100.0 mg ± 0.5 mg) were suspended in one mL of distilled water, vortexed for 5 min, and then centrifuged at 2,000 x g for 10 min at room temperature. WBC was calculated using the formula provided by Cornejo & Rosell, (2015).

Oil binding capacity (OBC) determination was analyzed using the procedure defined by Boulemkahel et al., (2021) with slight modifications. Sample (100.0 ± 0.5 mg) was mixed with one mL of vegetable oil, followed by vortex for 5 min and centrifugation at 3,000 x g, 4°C for 10 min. Drained samples were used to calculate OBC as reported (Boulemkahel et al., (2021).

Foaming properties were determined following Martínez et al., (2014) method with slight modifications. RB suspension (4 g/100 mL) was whipped in an Ultra Turrax (IKA T18 basic, Wilmington, NC) at 14,000 rpm for one min at room temperature. The foam volume at 30 s and 20 min was recorded and used to calculate the foam capacity (FC) and foam stability (FS) as follows:

$$FC = \left(\frac{ifv}{tsv} \right) * 100 \quad \text{Equation 3}$$

$$FS = \left(\frac{ffv}{tsv} \right) * 100 \quad \text{Equation 4}$$

Where *ifv* is the initial foam volume at 30 s, *ffv* is the foam volume after 20 min and *tsv* is the total suspension volume. Results were the average of three determinations.

Emulsifying properties were assessed using the method reported by Rios et al., (2018). The absorbance of the emulsions was measured at 500 nm in a spectrophotometer (UV mini-1240, Shimadzu Corporation, Kyoto, Japan). Emulsifying activity was expressed as the initial absorbance, expressed as

Results

absorbance units (AU), and emulsion stability (EA) was calculated using the equation 5 explain by Rios et al., (2018). Values being the average of four replicates.

$$ES (\%) = \left(\frac{Abs_{60 \text{ min}}}{Abs_0 \text{ min}} \right) * 100 \quad \text{Equation 5}$$

6.2.7. Force of penetration

The force of penetration was evaluated following the methodology described by Grau-Fuentes et al., (2024). A TA-XT plus texture analyzer (Stable Microsystems, Godalming, UK) equipped with a 5 kg load cell and a 25 mm diameter cylindrical aluminum probe was used to carry out the analysis. A 1:5 bran-to-water ratio (w:v) was used to ensure optimal hydration of all components, accounting for each sample's moisture content. A compression at a 10 mm distance from the probe's contact was used and the maximum force required to penetrate the rice bran suspensions was recorded. Analysis was performed in triplicate.

6.2.8. Statistical analysis

The proximate composition, physic-chemical properties and microbiology data were analyzed using OriginPro version 2022b (Origin Lab Corporation, Northampton, MA, USA), and each experiment was conducted in triplicate. Distinctions among the multivariate analysis of variance (MANOVA), and Fisher's least significant differences test was employed to present the results, expressed as mean $\pm$ standard deviation. Differences of *p value* < 0.05 were considered significant. Additionally, Pearson correlation analysis was conducted on the results to identify probable relationships among experimental parameters. Principal Component Analysis (PCA) was conducted to discriminate between samples.

5.3. Results and discussion

RB was tempered to four different hydration levels before being subjected to HPP. The lowest hydration level (15%) is aligned with the standard cereal operational moisture content and the highest (77%) was the water binding capacity of the RB. The intermediate levels were chosen in between of the above range.

5.3.1. Microbiology

To explain the impact of hydration levels and the efficacy of HPP on microbial inactivation all samples were analyzed (**Figure 6.1**). Mesophilic bacteria, spore-forming bacteria and yeast and molds were analyzed in the original RB (non-hydrated, non-HPP treated) obtaining a 7.11, 2.99 and 5.71 Log N (CFU/g), respectively. At 15 and 30% hydration, a higher concentration of mesophilic bacteria, helped by the RB conditioning process over a 24 h period at room temperature, was observed. However, at hydration levels of 60 and 77%, the sample's dilution resulted in a lower bacterial concentration. Remarkably, HPP treatment significantly reduced mesophilic bacteria load by 1 to 2 logarithmic cycles at hydration levels of 30, 60 and 77%, whereas at 15% hydration, the effect was minimal due to the low a_w of the sample. Spore-forming bacteria exhibits significant resistance to HPP when in the spore form (Muntean et al., 2016). T. In fact, in the present study the HPP treatment does not notably decrease spore concentration in samples compared to untreated hydrations, only at 30% was observed a significative reduction comparing treated and untreated sample, as know bacterial spores are much more resistant to pressure than vegetative cells (Zhang & Mittal, 2008). These results are consistent with those obtained by Roberts & Hoover, (1996), who employed a pressure of 400 MPa at room temperature, obtaining no significant impact on the reduction of viable spores of *Bacillus coagulans*. Molds and yeasts to grow typically require a longer incubation period compared to bacteria, which explains the lack of growth observed during the RB conditioning phase at desired hydration levels. Furthermore, the progressive dilution of samples leads to a decrease in the initial mold and yeast load across untreated samples as hydration levels rise. Molds and yeasts display a high degree of vulnerability to HPP, leading to significant modifications in cell morphology. Particularly, subjecting them to high pressures such as 500 MPa, as the present study pressure, results in notable alterations and harm to their cell wall integrity (Ogawa et al., 1990; Shimada et al., 1993). In samples with a moisture content of 15%, no discernible difference between the analogs can be observed due to the low a_w decreasing the effectiveness of HPP treatment. However, for hydrations of 30, 60 and 77%, the HPP process proves highly effective, achieving a notable reduction of 2 to 4 logarithmic cycles in mold and yeast load across samples. Notably, the magnitude of reduction increases proportionally with higher hydration levels.

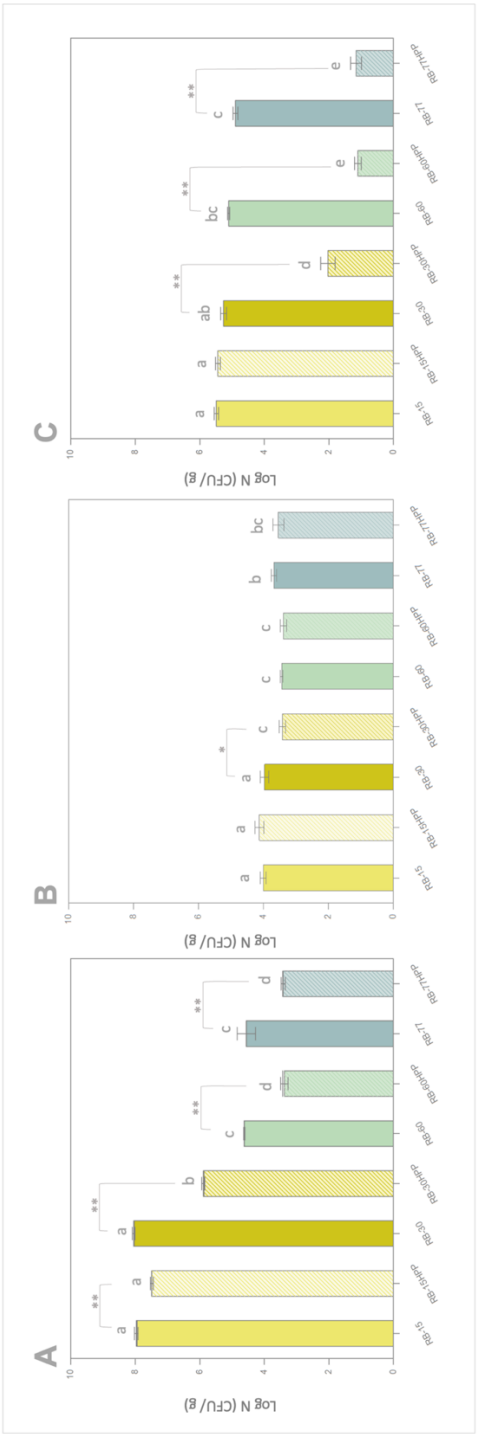


Figure 6.1. Graph showing the effect of HPP and hydration level on different microbiological analysis: (A) mesophilic bacteria; (B) spore-forming; (C) molds and yeast. Letters on the bars indicate significant differences ($p \leq 0.05$) between all samples. “Asterisk” indicates significant differences between pair with the same hydrations, * indicates p value between 0.05 – 0.01, and ** indicates $p \leq 0.01$.

Table 6.1. Technological characteristics of RB with different hydrations and treatments.

Techno-functional characteristics				
	$d_{(4.3)}$ (μm)	WBC (g water/g db)	OBC (g oil/g db)	
RB-15	361 $\pm$ 2 ^b	2.65 $\pm$ 0.23 ^{cd}	2.08 $\pm$ 0.13 ^{cd}	
RB-15HPP	376 $\pm$ 9 ^b	2.82 $\pm$ 0.08 ^{bc}	1.91 $\pm$ 0.12 ^d	
RB-30	426 $\pm$ 10 ^a	2.67 $\pm$ 0.11 ^{cd}	2.12 $\pm$ 0.11 ^c	
RB-30HPP	415 $\pm$ 17 ^a	2.53 $\pm$ 0.18 ^d	1.99 $\pm$ 0.04 ^{cd}	
RB-60	309 $\pm$ 9 ^{cd}	2.87 $\pm$ 0.20 ^{abc}	2.69 $\pm$ 0.09 ^b	
RB-60HPP	318 $\pm$ 7 ^c	2.68 $\pm$ 0.21 ^{cd}	2.95 $\pm$ 0.16 ^a	
RB-77	308 $\pm$ 5 ^{cd}	3.14 $\pm$ 0.03 ^a	3.08 $\pm$ 0.05 ^a	
RB-77HPP	299 $\pm$ 5 ^d	2.96 $\pm$ 0.08 ^{ab}	3.11 $\pm$ 0.10 ^a	
Hydration <i>p</i> -value	0.0000	0.0012	0.0000	
HPP <i>p</i> -value	0.7859	0.1977	0.9547	
Interaction between hydration and HPP <i>p</i> -value	0.0875	0.1867	0.0108	
	Foaming capacity (%) db	Foaming Stability (%) db	Emulsifying Activity (AU)	Emulsifying Stability (%)
RB-15	23.59 $\pm$ 2.24 ^a	22.54 $\pm$ 1.88 ^a	0.240 $\pm$ 0.06 ^{ab}	42.85 $\pm$ 3.30 ^b
RB-15HPP	23.21 $\pm$ 0.22 ^a	22.10 $\pm$ 1.08 ^a	0.232 $\pm$ 0.05 ^{ab}	60.50 $\pm$ 4.13 ^a
RB-30	20.51 $\pm$ 0.18 ^b	18.80 $\pm$ 0.16 ^b	0.210 $\pm$ 0.02 ^{bc}	48.30 $\pm$ 3.42 ^b
RB-30HPP	18.97 $\pm$ 0.00 ^c	16.95 $\pm$ 1.78 ^c	0.168 $\pm$ 0.02 ^{cd}	67.47 $\pm$ 0.80 ^a
RB-60	0 $\pm$ 0 ^e	0 $\pm$ 0 ^e	0.279 $\pm$ 0.07 ^a	48.32 $\pm$ 5.40 ^b
RB-60HPP	13.34 $\pm$ 1.07 ^d	10.91 $\pm$ 0.20 ^d	0.159 $\pm$ 0.02 ^{cd}	60.51 $\pm$ 7.99 ^a
RB-77	0 $\pm$ 0 ^e	0 $\pm$ 0 ^e	0.275 $\pm$ 0.04 ^a	44.02 $\pm$ 1.82 ^b
RB-77HPP	0 $\pm$ 0 ^e	0 $\pm$ 0 ^e	0.147 $\pm$ 0.02 ^d	60.26 $\pm$ 4.48 ^a
Hydration <i>p</i> -value	0.0000	0.0000	0.1520	0.1001
HPP <i>p</i> -value	0.0001	0.0001	0.0000	0.0000
Interaction between Hydration and HPP <i>p</i> -value	0.0000	0.0000	0.0128	0.5719

Means with different letters within a column were significantly different ($p < 0.05$)

Abbreviations: RB-15; RB-30; RB-60; RB-77: rice bran tempered at 15, 30, 60 and 77% moisture levels used for pretreatment; RB-15HPP; RB-30HPP; RB-60HPP; RB-77HPP: rice bran tempered at 15, 30, 60 and 77% moisture levels and treated with HPP.

6.3.2. Physical analysis

Particle size distribution of the RB was evaluated to identify possible particles' aggregation due to the treatments (Grau-Fuentes et al., 2024). The mean diameter ($d_{(4.3)}$) of the RB samples ranged from 299 μm to 426 μm . There was significant impact of the different hydration levels applied for tempering on the particle size distribution (**Table 6.1**). Nevertheless, hydration higher than 15% was required to modify the particles size. Tempering at 30% hydration led to certain agglomeration increasing the $d_{(4.3)}$ (426 μm for RB-30), but the opposite effect was observed beyond that hydration.

At the microstructural level (**Figure 6.2**), noticeable distinctions associated with the treatments were observed, validating the outcomes of particle size analysis. Micrographs at low magnification (600x) readily show the impact of hydration on the samples surface. At 15% hydration, HPP treated and untreated samples showed similar size. In contrast, particle aggregation was observed in samples with 30% hydration, resulting in large particles with a spongelike structure on the surface. This spongelike with deep groves formation was also discernible on the surfaces of samples with higher hydration, besides some fragmentation into smaller particles. Wu et al. (2021) noted that treating insoluble rice bran fiber with HPP resulted in the formation of a rough and porous structure within the fiber. Furthermore, as pressure increased (up to 150 MPa), the fiber microstructure exhibited increased looseness. The HPP treatment employs the hydration present in the samples as a pressure transmission medium, while its powerful mechanical force disrupts the regular fiber structure and creates deep grooves (Floury et al., 2002). For this reason, a more pronounced alteration in the surfaces of the samples could occur at higher hydration levels. The observation at higher magnification (1000x) revealed a cell structure, like a honeycomb, at high hydration levels (60 and 70%), but also it could be envisaged at lower hydration (30%). Presumably, the carbohydrates present in the samples, particularly starch, might be responsible for the formation of those structures.

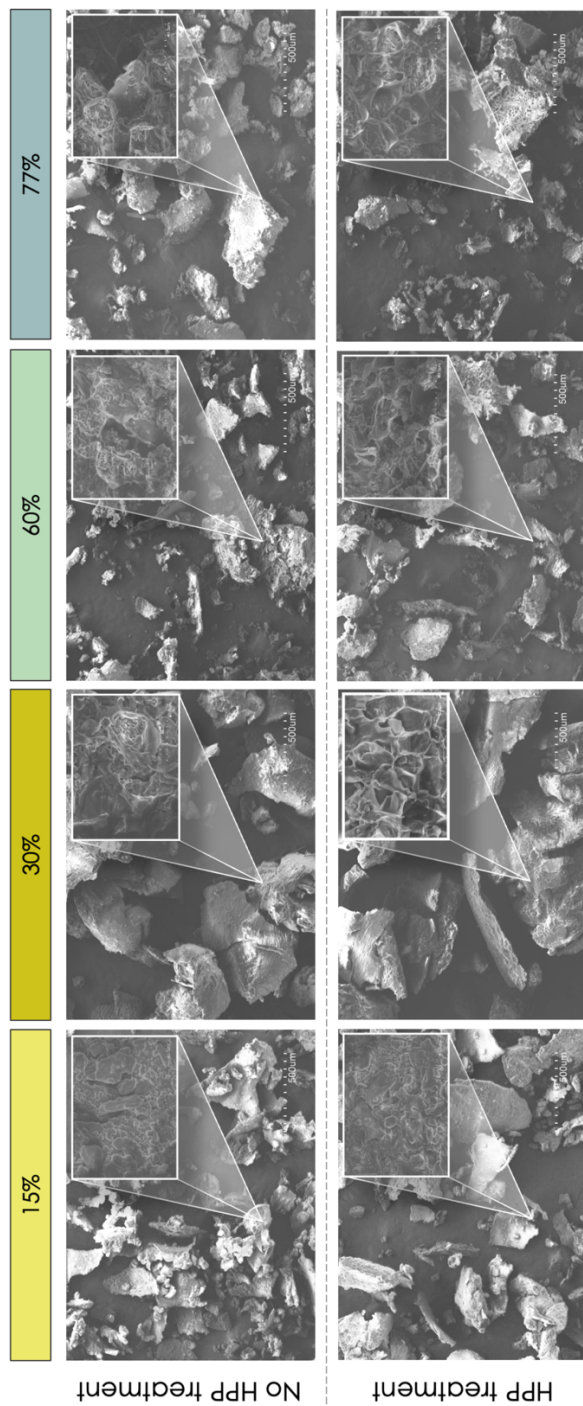


Figure 6.2. Scanning electron microscopy analysis of rice bran subjected to different hydrations and HPP treatment. Micrographs of larger scale correspond to lower magnification levels (600x), whereas smaller micrographs had higher magnification (1000x).

6.3.3. Chemical composition

The total (TDF) and soluble (SDF) dietary fibers content was significantly affected by the hydration level during tempering, but HPP did not have a statistical effect in TDF (**Table 6.2**). However, the interaction between both factors induced a significant impact, depending on the hydration condition. The HPP treated sample containing 15% hydration (RB-15HPP) showed the highest TDF (37.95 g/100 g) and SDF (11.03 g/100 g) contents, with lower values obtained when hydrating at 60% (both with and without HPP treatment). The changes observed in IDF were not associated with either hydration or treatment. SDF levels hardly decreased with the hydration level till 30% but greatly drop at 60% hydration in the HPP treated and untreated RB, and augmented at the highest hydration (77%). Similarly, Li et al. (2024) reported a fiber redistribution on insoluble fiber isolates from highland barley bran. They observed a reduction of 4.77% in IDF and a 68% increase in SDF after treating twice at 120 MPa. Dietary fiber is commonly categorized into soluble and insoluble fiber, but based on its technological attributes, it can be subdivided into hemicellulose, cellulose, and lignin. Hemicellulose was the main fraction in the RB samples, followed by cellulose and lignin (ADL). Hydration level and the interaction between hydration and HPP treatment significantly affected the cellulose content in the RB samples, and the ADL was affected due to the interaction between hydration and HPP treatment. Nevertheless, hemicellulose was significantly affected by both factors and through their interaction. However, no consistent trend in the effect of HPP treatment related with the hydration ratio was detected. The compressibility of air and water might explain the incongruity, resulting in different behaviors among samples with higher hydration due to the greater compressibility of water in those samples. Indeed, the significance of the fluid-sample ratio has been highlighted, affecting the effect of the treatment and the heating behavior (Balasubramaniam et al., 2015).

To provide additional understanding of the changes occurring during tempering and HPP treatments of RB, the glucose content and protein solubility were evaluated (**Table 6.2**). The glucose content in the untreated RB, significantly increased when tempering up to 30% hydration levels, whereas lower values were obtained beyond that hydration. Interestingly, it was observed that HPP played a crucial role in the glucose content of the samples but was significantly dependent on the hydration level.

Table 6.2. Chemical composition of RB with different hydrations and treatments.

Proximate composition					
	TDF (g/100 g) db	IDF (g/100 g) db	SDF (g/100 g) db	Glucose (g/100 g) db	Protein solubility (g/100 g) db
RB-15	31.64 ± 0.23 ^{cd}	22.92 ± 1.44 ^b	8.73 ^b	1.35 ± 0.08 ^e	11.89 ± 4.54 ^{abc}
RB-15HPP	37.95 ± 1.56 ^a	26.92 ± 0.53 ^a	11.03 ^a	1.03 ± 0.02 ^f	9.33 ± 0.25 ^c
RB-30	34.12 ± 1.52 ^b	25.95 ± 2.19 ^{ab}	8.17 ^c	1.68 ± 0.15 ^d	13.58 ± 0.57 ^a
RB-30HPP	29.84 ± 0.95 ^{de}	24.57 ± 1.74 ^{ab}	5.27 ^f	2.61 ± 0.10 ^b	12.11 ± 0.38 ^{ab}
RB-60	30.76 ± 0.52 ^{de}	26.31 ± 2.13 ^a	4.45 ^g	0.68 ± 0.02 ^g	11.26 ± 1.47 ^{abc}
RB-60HPP	29.01 ± 0.34 ^e	26.09 ± 0.77 ^{ab}	2.93 ^h	3.10 ± 0.13 ^a	11.36 ± 0.28 ^{abc}
RB-77	31.90 ± 0.40 ^{bcd}	25.16 ± 1.75 ^{ab}	6.73 ^e	0.48 ± 0.07 ^h	9.61 ± 1.86 ^{bc}
RB-77HPP	33.66 ± 0.79 ^{bc}	26.62 ± 0.76 ^a	7.04 ^d	2.32 ± 0.07 ^c	10.01 ± 0.32 ^{bc}
Hydration <i>p</i> -value	0.0004	0.5846	0.0000	0.0000	0.0199
HPP <i>p</i> -value	0.3297	0.2100	0.0000	0.0000	0.1858
Interaction between hydration and HPP <i>p</i> -value	0.0002	0.0988	0.0000	0.0000	0.3519
Fibers (% of TDF)					
	ADL (g/100 g) db	Cellulose (g/100 g) db	Hemicellulose (g/100 g) db		
RB-15	13.09 ± 2.81 ^b	26.16 ± 3.17 ^a	50.86 ^e		
RB-15HPP	10.83 ± 1.34 ^b	18.65 ± 0.36 ^{bc}	40.62 ^h		
RB-30	10.87 ± 0.86 ^b	22.80 ± 2.91 ^{ab}	45.4 ^g		
RB-30HPP	11.09 ± 0.04 ^b	26.64 ± 0.35 ^a	56.58 ^b		
RB-60	11.53 ± 0.27 ^b	22.21 ± 2.24 ^{ab}	57.11 ^a		
RB-60HPP	17.46 ± 0.78 ^a	20.20 ± 0.02 ^{bc}	55.29 ^c		
RB-77	11.98 ± 2.39 ^b	19.46 ± 2.06 ^{bc}	51.84 ^d		
RB-77HPP	13.21 ± 1.59 ^b	15.98 ± 2.77 ^c	46.83 ^f		
Hydration <i>p</i> -value	0.0537	0.0056	0.0000		
HPP <i>p</i> -value	0.1220	0.0624	0.0000		
Interaction between hydration and HPP <i>p</i> -value	0.0252	0.0328	0.0000		

Means with different letters within a column were significantly different ($p < 0.05$)

Abbreviations: RB-15; RB-30; RB-60; RB-77: rice bran tempered at 15, 30, 60 and 77% moisture levels used for pretreatment; RB-15HPP; RB-30HPP; RB-60HPP; RB-77HPP: rice bran tempered at 15, 30, 60 and 77% moisture levels and treated with HPP.

A minimum hydration of 30% was required to enhance the glucose content after applying HPP. The most substantial increase was observed at the 77% hydration level, where HPP demonstrated the capability to enhance glucose content by 383%. Therefore, HPP did induce a significant effect when combined with the right level of hydration, being able to release glucose molecules. Consequently, it can be inferred that HPP influences glucose content by breaking down larger carbohydrates, thereby facilitating their conversion into monomeric glucose forms or facilitating its extraction. The samples subjected to hydration or HPP treatment, particularly those with increased soluble fiber content (such as RB-15 treated and untreated), exhibited reduced glucose solubility. This might be related to possible interactions between glucose and soluble fiber fractions. This finding aligns with existing research indicating that elevated soluble fiber intake correlates with reduced glucose absorption, although those physiological studies attributed the effect to the fiber's influence on enzymatic activity or intestinal transit duration (Perry & Ying, 2016; Tan et al., 2020).

RB contained 16.06 ± 0.14 g/100 g protein and to identify possible impact of the treatments, the protein solubility was evaluated. Protein solubility exhibits an overall tendency to increase across all samples, compared to the original RB (9.58 g/100 g). The highest concentration of soluble protein (13.58 g/100 g) was recorded for sample RB-30. The impact of HPP treatment varies depending on hydration levels, although the interactions between those factors was not significant. At lower hydration (15%), the capacity for protein solubility in the HPP treated samples declines compared to untreated sample, reaching a minimum concentration of 9.33 g/100 g in sample RB-15HPP. However, at higher hydration, the concentration of soluble proteins in HPP-treated samples did not show a significant change. Conversely, Cao et al., (2018) observed an increment in protein solubility after treating a suspension of 10% pine nuts protein isolate at 200 MPa.

To identify the impact of HPP on the fat present in RB the peroxide value (PV) was selected to quantify the concentration of peroxides and hydroperoxides formed in the first's stages of lipid oxidation. As displayed in **Figure 6.3**, HPP significantly influences the PV of RB. It must be stressed that at lower hydration levels (15%), HPP decreased the PV value up to 32%. Similarly, HPP reduced the PV value of RB-30, although in lower extent. Conversely, opposite effect was induced by HPP at higher hydration levels,

resulting in a 38% increase in the PV for higher hydration levels (60 and 77%). Hendrickx et al., (1998) reviewed the effect of high pressures on the lipoxygenase enzyme, responsible for increasing PV, concluding that only pressures greater than 700 MPa were able to reduce its activity in fresh vegetables at room temperature. Nevertheless, the present study conducted at 500 MPa reveals for the first time that HPP impact is dependent on the hydration of the material. In low hydrated (15 and 30%) materials, HPP is effective in reducing PV and decreasing enzyme activity. Conversely, at higher hydration levels, enzyme activity is activated by HPP.

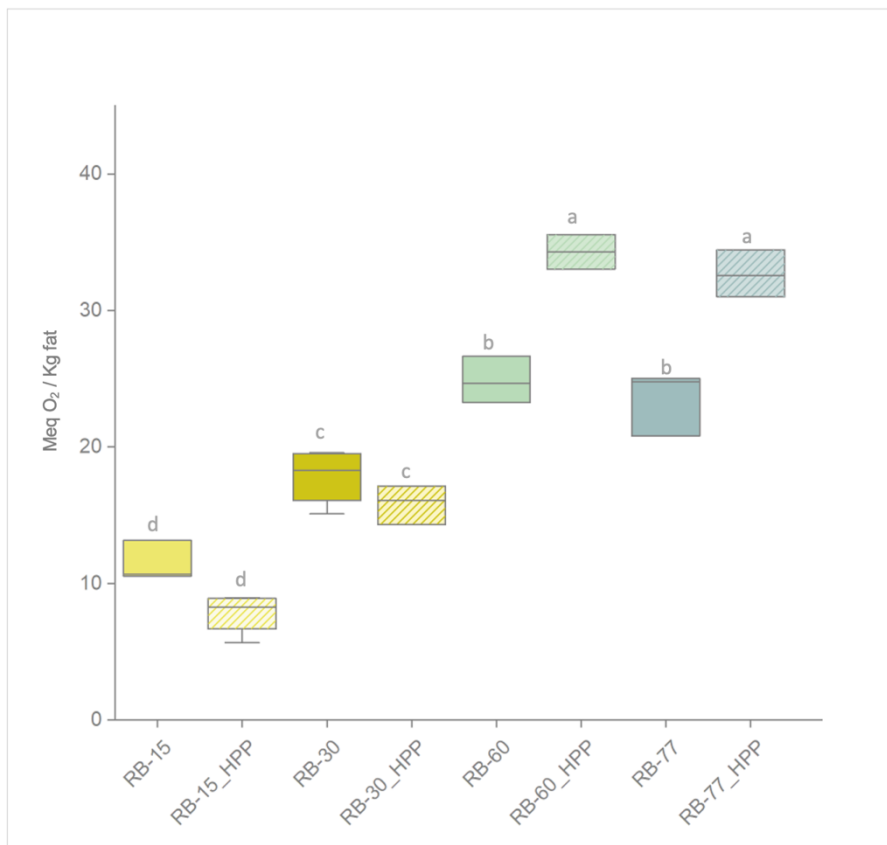


Figure 6.3. Analysis of peroxide value in the rice bran (RB) samples subjected to HPP after being tempered at different hydration levels (15%, 30%, 60%, 77%). Numbers following RB indicated the hydration level of untreated and treated (HPP) samples. Letters on the bars indicate significant differences ($p \leq 0.05$) between samples.

6.3.4. Technological characteristics

Impact of hydration and HPP on the techno-functional properties was assessed (**Table 6.1**). WBC capacity and OBC were significantly influenced by hydration, being OBC also affected by the interaction of both parameters (hydration and HPP). The most remarkable change was observed in RB-77, increasing by 12% and 48% the WBC and OBC, respectively, compared to RB-15. Regarding OBC, tempering using different hydration levels induced a steady increase of the OBC, which confirmed structural changes that modify the hydrophobicity of the RB. However, HPP treatment was only able to enhance that effect at high ($\geq 60\%$) hydration. Presumably, those results might be related to the different particle size, in fact a negative correlation was found with WBC and OBC ($r = -0.71$ and $r = -0.80$, respectively). Xie et al., (2019) related the higher hydration capacity of RB dietary fiber with the reduction in their particle size, which was induced by the combination of enzymatic and HPP treatment. In the present study, no significant impact on the particle size distribution was observed after HPP treatment.

The foaming properties of the RB were significantly affected by HPP and the tempering hydration levels (**Table 6.1**). Foaming properties include foaming capacity (FC) and stability (FS). At low hydration level (15%), the FC increased and also the FS with or without HPP treatment. However, FC and FS decreased when increasing the tempering hydration, being negligible at maximum hydration (77%). Surprisingly, HPP increased the FC and FS of RB tempered at 60% hydration level (RB-60HPP). The 60% hydrated sample initially lacked foaming abilities; nevertheless, following HPP treatment, a notable improvement was observed, resulting in 13.34% and 10.91%, FC and FS, respectively. According to the findings of Zhang et al., (2022), who investigated the impact of humidity on the structural and functional properties of pea protein isolates during extrusion process, FC decreases at moistures higher than 30%. The foaming properties exhibited a strong negative correlation with WBC and OBC ($r > -0.69$ in all cases). Considering that FC refers to the volume of foam generated under specific conditions (Raikos et al., 2007) and it is related to the sample hydrophobicity, the foaming capacity increases when enhancing the material hydrophobicity (Sun et al., 2022). However, in the present study, no correlation was observed with OBC, thus other interactions seem to affect the foaming properties.

Concerning the emulsifying properties of the samples, HPP treatment using different tempering hydration levels significantly affected both, emulsifying stability (ES) and activity (EA) (**Table 6.1**). The EA was reduced in all cases, with HPP stressing that reduction, although the reduction was only significant with hydration levels beyond 30%. Moreover, HPP enhances the ES independently of the hydration level of the RB. Similar impact was reported for RB protein hydrolysates when 1% dispersion was subjected to 200 MPa (Wang et al., 2022). An increase in emulsion stability and activity was observed, but authors reported a decrease when HPP was 300 MPa. In contrast, in the present study conducted at a significantly higher working pressure (500 MPa), consistently higher stability values were observed. No significant differences among all the HPP treated samples were found. The sample RB-30HPP showcases the highest stability a 67.47% value (representing an increment of 40% compared to its untreated counterpart). Molecular flexibility of proteins is a prerequisite for emulsion stability (Kato & Nakai, 1980), thus, it seems that HPP treatment confer greater flexibility to protein molecules, resulting in enhanced emulsion stability. Hence, HPP treatment of RB decreases emulsifying activity but they are more stable.

RB pastes were prepared by hydrating samples with five volumes of water (1:5 (w/v)). The force required to penetrate the hydrated bran was used to indicate its ability to expand, with lower force indicating higher swelling. In **Figure 6.4** could be observed that at 30% hydration level, there was an increase in penetration force observed in both HPP-treated (97 N) and untreated (53 N) samples. Conversely, samples with higher hydration levels displayed a decrease in the penetration force, and that was independent of the HPP treatment. Hydration exerted a considerable effect on this analysis ($p < 0.05$). These findings agree with previous results reported by (Grau-Fuentes et al., 2024), who explored different treatments (thermal and enzymatic) of RB and identified a positive correlation between penetration force and particle size. Results obtained applying tempering with or without HPP treatments also confirmed this significantly positive correlation ($r = 0.80$).

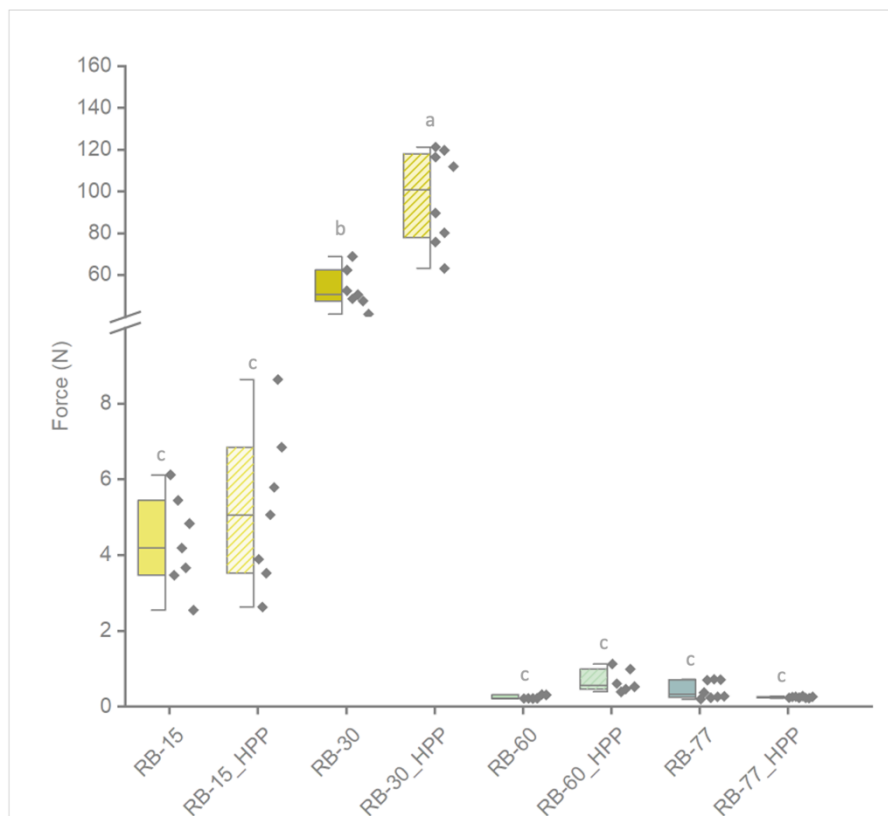


Figure 6.4. Analysis of force of penetration in all of samples studied. Rhombuses represents all the determinations considered for the results. Letters on the bars indicate significant differences ($p \leq 0.05$) between samples.

6.3.5. Principal component analysis

A principal component analysis (PCA) (**Figure 6.5**) was conducted to discern potential groupings among the various hydration levels and HPP treatments applied to the RB, using proximal composition, techno-functional properties, and microbiological analysis. Within the graph, the closer the variables are to each other, the stronger their positive correlation, while variables positioned on opposite sides indicate inverse correlations. This PCA explained 69.1% of the observed variance among the treated samples, with Principal Component 1 (PC1) explaining 39.6% of the variability and Principal Component 2 (PC2) explaining 29.5%. PC1 indicated positive associations with low hydrations (15% and 30%) and negative associations with high hydration levels (60%, 77%).

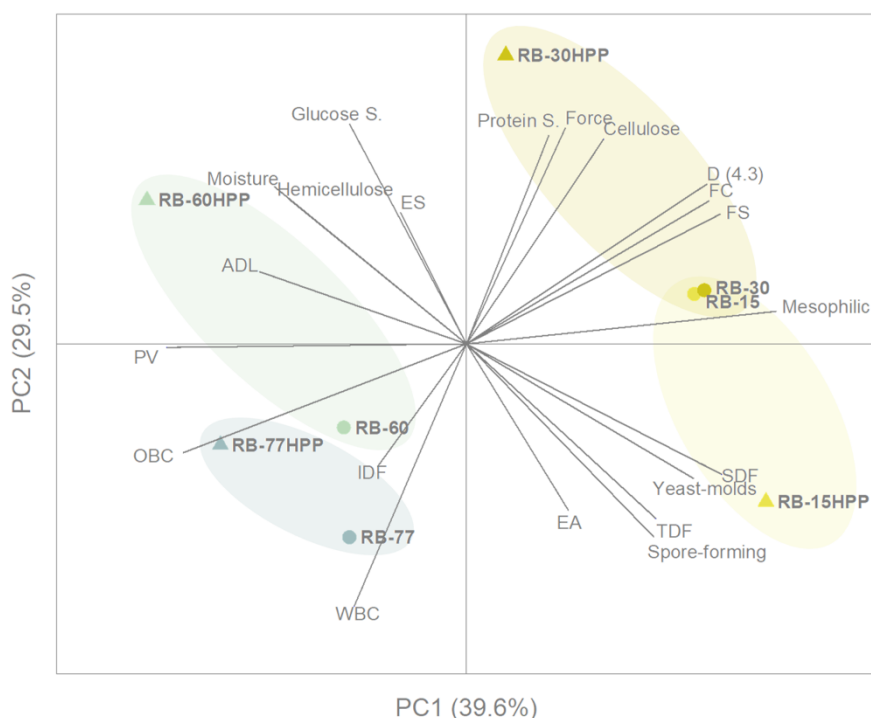


Figure 6.5. Principal component analysis the samples hydrated and hydrated + treated with HPP that included all the variables analyzed (proximate composition, techno functional properties and microbiology analysis). Identified clusters based on their hydration levels appeared circled in different colors: 15 % (■), 30 % (■), 60 % (■) and 77 % (■). (●) Indicates samples without HPP treatment and (▲) indicates samples underwent HPP treatment.

Specifically, samples with 15% and 30% hydration levels were positioned positively along PC1, closely correlated with mesophilic bacteria analysis, yeast-molds and spore-forming, foaming properties, particle size distribution, total and soluble dietary fiber, cellulose content, penetration force, and protein solubility. Conversely, high hydration levels were linked with hydration properties, insoluble dietary fiber, particularly lignin, peroxide value and glucose solubility. HPP discriminated the samples along the y-axis, with RB-30HPP showing the strongest impact followed by RB-60HPP. Conversely, samples treated at 77% and 15% hydration were positioned on the negative axis. The least impact of HPP treatment was observed with the highest tested hydration (77%) compared to its counterpart without treatment; however, samples with 15% and 60% hydration were the most significantly affected by HPP treatment positioned in different quartiles than their corresponding

untreated samples. In the case of 15% hydration, as evidenced in the microbiological section, this differentiation was determined by its limited efficacy in reducing microorganisms' post-treatment and its higher content of soluble fiber. For the RB-60HPP sample, the differences compared to the untreated sample were less pronounced but still present. This distinction was particularly evident when comparing the lignin content (ADL) and IDF for RB-60HPP and RB-60, respectively. The results obtained in the PCA demonstrate how the effect of high pressure varied depending on the hydration used in all samples, and the efficiency of using high hydration levels for microbiological purposes, but low hydration when looking for modifying physic-chemical features.

5.4. Conclusions

HPP treatment represents a versatile approach capable of enhancing its microbiological safety while concurrently changing the composition and functionality of rice bran. Although usually HPP are carried out at high hydration levels, the present study reveals that even at low hydration levels HPP can be effective promoting microstructure and chemical changes. The efficacy of HPP treatment is significantly influenced by the hydration level of the sample. Low hydration levels helped fiber redistribution, resulting in increased SDF content, reduced peroxide value, and enhanced foam capacity and stability. Conversely, samples with higher moisture content were microbiologically safe and exhibit increased protein solubility post-HPP treatment, besides a reduction in particle size, and improvement in WBC and OBC. Nonetheless, the observed changes in the samples did not demonstrate a linear relationship with the moisture levels utilized during treatment. However, the greater the hydration, the greater the antimicrobial effect of the HPP treatment for molds and yeasts, with 30% humidity again affecting a greater reduction in mesophilic bacteria. These findings underscore the importance of understanding hydration-dependent effects in optimizing HPP treatment strategies for rice bran, contributing valuable insights for enhancing its functional attributes in various food applications. For the first time HPP is applied at low hydration levels using RB as a model, opening the possibility of fostering technological modifications in raw materials by modulating their hydration.

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References

- AACC International. (1999). Method 32-07: Determination of soluble, insoluble, and total dietary fiber in foods and food products. In *Approved Methods of the AACC* (10th ed.). AACC International.
- Ahmed, J., Ramaswamy, H. S., Ayad, A., Alli, I., & Alvarez, P. (2007). Effect of high-pressure treatment on rheological, thermal and structural changes in Basmati rice flour slurry. *Journal of Cereal Science*, 46(2), 148–156. <https://doi.org/10.1016/j.jcs.2007.01.006>
- Allai, F. M., Azad, Z. R. A. A., Mir, N. A., & Gul, K. (2023). Recent advances in non-thermal processing technologies for enhancing shelf life and improving food safety. *Applied Food Research*, 3(1), 100258. <https://doi.org/10.1016/j.afres.2022.100258>
- Balasubramaniam, V. M. (Bala), Martínez-Monteagudo, S. I., & Gupta, R. (2015). Principles and Application of High Pressure–Based Technologies in the Food Industry. In *Annual Review of Food Science and Technology* (Vol. 6, Issue Volume 6, 2015, pp. 435–462). Annual Reviews. <https://doi.org/10.1146/annurev-food-022814-015539>
- Bello, E. F. T., Martínez, G. G., Ceberio, B. F. K., Rodrigo, D., & López, A. M. (2014). High Pressure Treatment in Foods. *Foods*, 3(3), 476–490. <https://doi.org/10.3390/foods3030476>
- Boulemkahel, S., Betoret, E., Benatallah, L., & Rosell, C. M. (2021). Effect of low pressures homogenization on the physico-chemical and functional properties of rice flour. *Food Hydrocolloids*, 112, 106373. <https://doi.org/10.1016/j.foodhyd.2020.106373>
- Cao, B., Fang, L., Liu, C., Min, W., & Liu, J. (2018). Effects of high hydrostatic pressure on the functional and rheological properties of the

- protein fraction extracted from pine nuts. *Food Science and Technology International*, 24(1), 53–66. <https://doi.org/10.1177/1082013217726883>
- Cappa, C., Lucisano, M., Barbosa-Cánovas, G. V., & Mariotti, M. (2016). Physical and structural changes induced by high pressure on corn starch, rice flour and waxy rice flour. *Food Research International*, 85, 95–103. <https://doi.org/10.1016/j.foodres.2016.04.018>
- Considine, K. M., Kelly, A. L., Fitzgerald, G. F., Hill, C., & Sleator, R. D. (2008). High-pressure processing – effects on microbial food safety and food quality. *FEMS Microbiology Letters*, 281(1), 1–9. <https://doi.org/10.1111/j.1574-6968.2008.01084.x>
- Cornejo, F., & Rosell, C. M. (2015). Physicochemical properties of long rice grain varieties in relation to gluten free bread quality. *LWT - Food Science and Technology*, 62(2), 1203–1210. <https://doi.org/10.1016/j.lwt.2015.01.050>
- Daryaei, H., & Balasubramaniam, V. M. (2012). 13—Microbial decontamination of food by high pressure processing. In A. Demirci & M. O. Ngadi (Eds.), *Microbial Decontamination in the Food Industry* (pp. 370–406). Woodhead Publishing. <https://doi.org/10.1533/9780857095756.2.370>
- Floury, J., Desrumaux, A., & Legrand, J. (2002). Effect of Ultra-high-pressure Homogenization on Structure and on Rheological Properties of Soy Protein-stabilized Emulsions. *Journal of Food Science*, 67(9), 3388–3395. <https://doi.org/10.1111/j.1365-2621.2002.tb09595.x>
- Grau-Fuentes, E., Garzón, R., Rodrigo, D., & Rosell, C. M. (2024). Unlocking hidden potential of rice bran: Enzymatic treatment for enhancing techno-functional properties. *LWT*, 207, 116673. <https://doi.org/10.1016/j.lwt.2024.116673>
- Hendrickx, M., Ludikhuyze, L., Broeck, I. V. den, & Weemaes, C. (1998). Effects of high pressure on enzymes related to food quality. *Trends in Food Science & Technology*, 9(5), 197–203. [https://doi.org/10.1016/S0924-2244\(98\)00039-9](https://doi.org/10.1016/S0924-2244(98)00039-9)
- Hiperbaric. (2022). *Hiperbaric. High Pressure Technologies*. <https://www.hiperbaric.com/es/tecnologia-hpp/equipos/hpp-in-pack/>
- Hurtado, A., Dolors Guàrdia, M., Picouet, P., Jofré, A., Bañón, S., & Ros, J. M. (2019). Shelf-life extension of multi-vegetables smoothies by high-pressure processing compared with thermal treatment. Part I: Microbial and enzyme inhibition, antioxidant status, and physical stability. *Journal of Food Processing and Preservation*, 43(10), e14139. <https://doi.org/10.1111/jfpp.14139>
- International Organization for Standardization. (2013). *ISO 4833-1:2013*

- Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 1: Colony count at 30 degrees C by the pour plate technique.* Geneva, Switzerland: ISO.
- International Organization for Standardization. (2009). *ISO 712:2009. Cereals and cereal products — Determination of moisture content — Reference method.* Geneva, Switzerland: ISO.
- International Organization for Standardization. (2008). *ISO 21527-1:2008 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds — Part 1: Colony count technique in products with water activity greater than 0.95.* Geneva, Switzerland: ISO.
- International Organization for Standardization. (2008). *ISO 21527-2:2008 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of yeasts and moulds - Part 2: Colony count technique in products with water activity less than or equal to 0.95.* Geneva, Switzerland: ISO.
- International Organization for Standardization. (2008). *ISO 13906:2008 Animal feeding stuffs - Determination of acid detergent fibre (ADF) and acid detergent lignin (ADL) contents.* Geneva, Switzerland: ISO.
- Katina, K., Juvonen, R., Laitila, A., Flander, L., Nordlund, E., Kariluoto, S., Piironen, V., & Poutanen, K. (2012). Fermented Wheat Bran as a Functional Ingredient in Baking. *Cereal Chemistry*, 89(2), 126–134. <https://doi.org/10.1094/CCEM-08-11-0106>
- Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta (BBA) - Protein Structure*, 624(1), 13–20. [https://doi.org/10.1016/0005-2795\(80\)90220-2](https://doi.org/10.1016/0005-2795(80)90220-2)
- Li, J., Xi, H., Wang, A., Nie, M., Gong, X., Lin, R., Zhang, X., Tian, Y., Wang, F., & Tong, L.-T. (2024). Effects of high-pressure microfluidization treatment on the structural, physiochemical properties of insoluble dietary fiber in highland barley bran. *International Journal of Biological Macromolecules*, 262, 129743. <https://doi.org/10.1016/j.ijbiomac.2024.129743>
- Liu, N., Lin, P., Zhang, K., Yao, X., Li, D., Yang, L., & Zhao, M. (2022). Combined effects of limited enzymatic hydrolysis and high hydrostatic pressure on the structural and emulsifying properties of rice proteins. *Innovative Food Science & Emerging Technologies*, 77, 102975. <https://doi.org/10.1016/j.ifset.2022.102975>
- Martínez, M. M., Rosell, C. M., & Gómez, M. (2014). Modification of wheat flour functionality and digestibility through different extrusion conditions. *Journal of Food Engineering*, 143, 74–79.

- <https://doi.org/10.1016/j.jfoodeng.2014.06.035>
- Muntean, M.-V., Marian, O., Barbieru, V., Cătușescu, G. M., Ranta, O., Drocas, I., & Terhes, S. (2016). High Pressure Processing in Food Industry – Characteristics and Applications. *Agriculture and Agricultural Science Procedia*, 10, 377–383. <https://doi.org/10.1016/j.aaspro.2016.09.077>
- Ogawa, H., Fukuhisa, K., Kubo, Y., & Fukumoto, H. (1990). Pressure Inactivation of Yeasts, Molds, and Pectinesterase in Satsuma Mandarin Juice: Effects of Juice Concentration, pH, and Organic Acids, and Comparison with Heat Sanitation. *Agricultural and Biological Chemistry*, 54(5), 1219–1225. <https://doi.org/10.1080/00021369.1990.10870118>
- Perry, J., & Ying, W. (2016). A Review of Physiological Effects of Soluble and Insoluble Dietary Fibers. *Journal of Nutrition & Food Sciences*, 06(02). <https://doi.org/doi:10.4172/2155-9600.1000476>
- Raikos, V., Campbell, L., & Euston, S. R. (2007). Effects of sucrose and sodium chloride on foaming properties of egg white proteins. *Food Research International*, 40(3), 347–355. <https://doi.org/10.1016/j.foodres.2006.10.008>
- Rios, R. V., Garzón, R., Lannes, S. C. S., & Rosell, C. M. (2018). Use of succinyl chitosan as fat replacer on cake formulations. *LWT*, 96, 260–265. <https://doi.org/10.1016/j.lwt.2018.05.041>
- Roberts, C. M., & Hoover, D. G. (1996). Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity and nisin. *JOURNAL OF APPLIED BACTERIOLOGY*, 81, 363–368.
- Sapwarobol, S., Saphyakhajorn, W., & Astina, J. (2021). Biological Functions and Activities of Rice Bran as a Functional Ingredient: A Review. *Nutrition and Metabolic Insights*, 14, 11786388211058559. <https://doi.org/10.1177/11786388211058559>
- Shimada, S., Andou, M., Naito, N., Yamada, N., Osumi, M., & Hayashi, R. (1993). Effects of hydrostatic pressure on the ultrastructure and leakage of internal substances in the yeast *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, 40(1), 123–131. <https://doi.org/10.1007/BF00170440>
- Song, Y., Bi, X., Zhou, M., Zhou, Z., Chen, L., Wang, X., & Ma, Y. (2021). Effect of combined treatments of ultrasound and high hydrostatic pressure processing on the physicochemical properties, microbial quality and shelf-life of cold brew tea. *International Journal of Food Science & Technology*, 56(11), 5977–5988. <https://doi.org/10.1111/ijfs.15245>
- Spaggiari, M., Dall'Asta, C., Galaverna, G., & del Castillo Bilbao, M. D. (2021). Rice Bran By-Product: From Valorization Strategies to Nutritional Perspectives. *Foods*, 10(1). <https://doi.org/10.3390/foods10010085>

- Stratakos, A. C., Inguglia, E. S., Linton, M., Tollerton, J., Murphy, L., Corcionivoschi, N., Koidis, A., & Tiwari, B. K. (2019). Effect of high pressure processing on the safety, shelf life and quality of raw milk. *Innovative Food Science & Emerging Technologies*, 52, 325–333. <https://doi.org/10.1016/j.ifset.2019.01.009>
- Sun, J., Chang, C., Su, Y., Gu, L., Yang, Y., & Li, J. (2022). Impact of saccharides on the foam properties of egg white: Correlation between rheological, interfacial properties and foam properties. *Food Hydrocolloids*, 122, 107088. <https://doi.org/10.1016/j.foodhyd.2021.107088>
- Tan, W. S. K., Chia, P. F. W., Ponnalagu, S., Karnik, K., & Henry, C. J. (2020). The Role of Soluble Corn Fiber on Glycemic and Insulin Response. *Nutrients*, 12(4). <https://doi.org/10.3390/nu12040961>
- Ume Roobab, X. Z., Muhammad Inam-Ur-Raheem, Abdul Waheed Khan, Rai Naveed Arshad, & Aadil, R. M. (2023). Innovations in High-pressure Technologies for the Development of Clean Label Dairy Products: A Review. *Food Reviews International*, 39(2), 970–991. <https://doi.org/10.1080/87559129.2021.1928690>
- Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science*, 74(10), 3583–3597. [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2)
- Wang, S., Wang, T., Sun, Y., Cui, Y., Yu, G., & Jiang, L. (2022). Effects of High Hydrostatic Pressure Pretreatment on the Functional and Structural Properties of Rice Bran Protein Hydrolysates. *Foods*, 11(1). <https://doi.org/10.3390/foods11010029>
- Wu, Q., Wu, J., Ren, M., Zhang, X., & Wang, L. (2021). Modification of insoluble dietary fiber from rice bran with dynamic high pressure microfluidization: Cd(II) adsorption capacity and behavior. *Innovative Food Science & Emerging Technologies*, 73, 102765. <https://doi.org/10.1016/j.ifset.2021.102765>
- Xie, F., Zhao, T., Wan, H., Li, M., Sun, L., Wang, Z., & Zhang, S. (2019). Structural and Physicochemical Characteristics of Rice Bran Dietary Fiber by Cellulase and High-Pressure Homogenization. *Applied Sciences*, 9(7). <https://doi.org/10.3390/app9071270>
- Zhang, B., Kang, X., Cheng, Y., Cui, B., & El-Aty, A. M. A. (2022). Impact of high moisture contents on the structure and functional properties of pea protein isolate during extrusion. *Food Hydrocolloids*, 127, 107508. <https://doi.org/10.1016/j.foodhyd.2022.107508>
- Zhang, H., & Mittal, G. S. (2008). Effects of High-Pressure Processing (HPP) on Bacterial Spores: An Overview. *Food Reviews International*, 24(3),

330–351. <https://doi.org/10.1080/87559120802089290>

Zhu, S. M., Lin, S. L., Ramaswamy, H. S., Yu, Y., & Zhang, Q. T. (2017). Enhancement of Functional Properties of Rice Bran Proteins by High Pressure Treatment and Their Correlation with Surface Hydrophobicity. *Food and Bioprocess Technology*, 10(2), 317–327. <https://doi.org/10.1007/s11947-016-1818-7>



General discussion

General discussion

Rice is one of the most consumed foods worldwide. Its importance in global nutrition is undeniable, but its production and processing present several challenges, both in terms of food safety and the management of by-products derived from its cultivation, with rice bran being the most important due to its high nutritional value and potential in the food industry.

Rice processing is crucial for both safe consumption and the effective reuse of bran. From one side, rice in its raw form, is not suitable for human consumption, making processing (cooking) essential to transform the grain into a safe and nutritious food. However, this same cooking, if not accompanied by proper storage, can lead to food safety issues. From the other, rice bran, despite its nutritional value, requires specific processing to modify its properties and make it more useful in the formulation of food products.

This doctoral thesis focuses on investigating more sustainable processes to address these issues, aiming to improve food safety and the efficient management of resources in rice production.

Sustainable strategies to ensure rice safety

Bacillus cereus is one of the main microorganisms frequently involved in foodborne outbreaks in rice, one of the foods most consumed worldwide (Rodrigo et al., 2021; Wasaya et al., 2022). The results of this PhD thesis showed and quantified the antimicrobial activity of grape extract against *B. cereus* in cooked rice. This natural antimicrobial was evaluated using a combination of hurdles involving pH and temperature, applied at different stages of rice preparation for consumption. **Chapter 1** addressed the heat or cooking process, while **Chapter 2** focused on the storage stage. The pH range used (4.5, 5.5, and 6.5) simulated the pH levels of various meals in which rice might be present.

For the cooking process, **Chapter 1**, *B. cereus* spores were used because they are ubiquitous in rice, can survive cooking and eventually lead to the formation of the emetic toxin responsible for 95% of outbreaks linked to the consumption of cooked or fried rice, as documented in the literature (Juneja et al., 2019; Lake et al., 2004; Little et al., 2002; Yu et al., 2020). *B. cereus* spores' inactivation increased with higher temperatures and more acidic pH levels. For lower or gentle cooking temperatures (90-95°C) the presence of

grape extract was remarkably relevant due to the greater reduction in spore survival compared to samples without the grape extract. Melis et al., (2014) and Xu et al., (2021) reported that when *B. cereus* spores were exposed to acidic pH, additional stress (low water activity), and mild heat treatment, their ability to germinate and grow decreases, similar to the results explained. However, at temperatures above 95 °C, the antimicrobial effect of the grape extract was less pronounced, indicating that the temperature alone was sufficient to inactivate the spores.

As described in **Chapter 2**, the storage process was conducted at temperatures representative of mean domestic refrigeration (10 °C), cold chain breakage (20 °C), and optimal grow temperature as control (30 °C). Vegetative cells were used to simulate the germinated spores that resist cooking temperatures. Depending on the concentration, grape extract exhibited bacteriostatic or bactericidal activity, or no effect on *B. cereus*, particularly at the highest pH and temperatures and with the lowest antimicrobial concentration. Higher concentrations of grape extract (0.5 and 1%) demonstrated a bactericidal effect across all temperatures and pH levels, achieving up to a 6-log reduction at 30 °C and pH 6.5. This was the optimal temperature for *B. cereus* growth likely prevented the activation of stress defense mechanisms, making them more susceptible to the antimicrobial effect (Rodrigo, Barbosa-Canovas, et al., 2003; Rodrigo, Ruiz, et al., 2003).

The antimicrobial capacity of grape extract is primarily attributed to its polyphenol content, which can differ based on factors such as the grape origin (seeds or skin), the extraction solvent, and the analytical methods applied (Balaban et al., 2021). Gram-positive bacteria exhibit higher sensitivity to grape extract compared to gram-negative bacteria. This differential sensitivity is likely due to structural differences in bacterial cell walls, with the lipopolysaccharide layer in gram-negative bacteria potentially impeding polyphenol penetration (Gerardi et al., 2021; Ghendov-Mosanu et al., 2022; Jayaprakasha et al., 2003; Oliveira et al., 2013). This suggests that grape extracts were particularly effective in controlling gram-positive bacterial contaminants in food matrices. The findings in **Chapters 1 and 2** corroborate the results of Katalinić et al., (2010) and Serra et al., (2008), supporting the potential of grape extracts as natural antimicrobials in food preservation.

Kinetics of *B. cereus* inactivation by grape extract

In order to assess the effectiveness of grape extract as a natural antimicrobial and to facilitate predictions for different scenarios, the studies utilized the Weibull survival function to fit the inactivation of *B. cereus*. The scale and shape parameters were determined for each pH, temperature, and grape extract concentration, 0 and 0.1% for the heat treatment, and, 0.5 and 1% for the storage process.

The fit for the cooking process showed that the scale parameter decreased with increasing temperature and acidity, and this decrease was greater in the presence of antimicrobial. This indicated that the spores' resistance to heat treatment was significantly reduced in the presence of grape extract, especially under acidic conditions and high temperatures. This suggested that heat directly affected the spore structure, damaging the exosporium and denaturing certain proteins related to its resistance to heat treatment (Luu-Thi et al., 2014; Lv et al., 2019). For the storage process, increasing extract concentrations and temperatures were linked to a decrease in the scale parameter, although this decrease was not always statistically significant.

A secondary model was built using a stepwise multiple regression, linking the logarithm of the scale factor of the Weibull model to temperature and pH for fixed concentrations of grape extract. Additionally, including the most relevant polynomial terms, a global model was developed to improve the accuracy of the coefficients in the secondary model. Similar to the approach used in **Chapters 1 and 2**, Rodrigo, Barbosa-Canovas, et al., (2003) fitted their data to the Weibull distribution and developed a global model to enhance coefficient accuracy. A potential challenge arises when applying the kinetic parameter from secondary models to estimate the residual microbial concentration, particularly for the shape parameter in primary models. To mitigate this, Couvert et al., (2005) used a single value of the shape parameter derived from the entire dataset, a strategy employed in **Chapters 1 and 2** of this thesis. Despite a slight reduction in goodness-of-fit, this adjustment improved the model robustness. Similar fitting methodologies have been recommended by other researchers, such as Corradini et al., (2008) and Mafart et al., (2002).

Based on the global model obtained, a tertiary model was developed (Microsoft Visual Studio C++). This software represents a practical

application of the modelling underwent in this research and can be utilized in the industry to assist with decision-making regarding pH and temperature at various grape extract concentration levels, considering the initial microbial load. This software enables an industrial operator to adjust storage conditions (time and temperature) to ensure the microbiological stability of food based on its initial contamination levels, or in other words, to establish the shelf life of this product.

Exposure assessment

To conclude the food safety block, in **Chapter 3** an industrial exposure assessment model was developed to estimate *B. cereus* concentration in cooked rice. By using probability distributions, the model enhances Hazard Analysis and Critical Control Point (HACCP) decisions, thereby ensuring the microbiological safety of rice products. The model developed incorporated mathematical equations (Weibull and modified Gompertz) to simulate microbial inactivation and growth under heat treatment and storage conditions, allowing for a more accurate estimation of bacterial concentration with (0.1%) and without grape extract.

The scenarios simulated in **Chapter 3** replicated realistic yet unfavorable conditions that could occur during rice preparation and storage: low cooking temperature (90 °C) combined with a near-neutral pH (6.5) and a typical cooking time of 20 minutes, followed by long storage (20 hours) at 20 °C to simulate a break in the cold chain. The presence of grape extract was found to significantly reduce the growth rate of *B. cereus*, suggesting that grape extract could serve as an additional barrier against pathogen growth under suboptimal conditions. This reduction in the risk of ingesting an infectious load of *B. cereus* underscores the potential of grape extract as a natural antimicrobial in the food industry.

This type of exposure assessment models represented a significant advancement in food safety management, providing a robust tool for industry stakeholders. Integrating this model into food safety systems can lead to more effective monitoring and control of pathogens, ultimately enhancing public health protection.

Sustainable strategies to rice bran's revalorization

As a result of the high rice consumption and, therefore, production, large quantities of by-products are generated by the rice industry. The amount of rice by-products is also due to the low consumer acceptance of brown rice. The food industry has conducted several studies to evaluate the possibility of incorporating brown rice in common food products such as noodles, bread, pasta, cakes, snacks, pastries, and beverages (Cornejo & Rosell, 2015; Saleh et al., 2019). The last category, plant-based beverages, is a segment globally growing as more individuals adopt plant-based diets and prioritize environmental sustainability (Penha et al., 2021; Sethi et al., 2016; Shori & Al Zahrani, 2022; Vaikma et al., 2021). To understand the current state of the market for plant-based beverages, a market study was conducted in groceries located in Europe and North America (**Chapter 4**), considering 306 plant-based beverages. The raw materials used to make these beverages reflect the trend towards healthy foods and beverages, predominantly based on cereal grains (rice, millet, oats, and spelt), seeds (birdseed and flax), and nuts or legumes. The main ingredients in plant-based beverages were water, raw plant-based powders, and sometimes oil, gums, and sugar, varying with the primary raw material. 14% of beverages contained two or more main ingredients, complementing each other nutritionally and technologically. About 32% included oil, mainly in cereal-based drinks, and 60% used hydrocolloids for stable emulsions, while 60% of those without hydrocolloids were also cereal-based. It is assumed that the starch content in cereals provided the necessary stabilizing functionality in the absence of hydrocolloids, considering that the processing of the beverages included heat treatment and homogenization steps (Penha et al., 2021; Qamar et al., 2020). These beverages were generally low in calories and fat, with carbohydrate content higher in cereal-based drinks due to starch (Sultana et al., 2022). Fiber content was not reported for all beverages, and in those that did report it, the fiber content was very low. Protein content varied, generally lower than in cow's milk (USDA, 2022). The study highlighted a gap in plant-based beverages, suggesting that, despite improvements in ingredient formulations, reducing oil content and enriching fiber in these beverages would be beneficial. In this sense, rice bran, with its rich nutritional properties, could present a promising solution for enhancing the quality of plant-based beverages, effectively addressing the gaps identified in **Chapter 4**. Including rice bran into these formulations would not only increase the fiber and protein

content but also, through the modification of its functional properties, offer an alternative to the use of hydrocolloids and fats. Integrating rice bran into these beverages could provide stability through modification of emulsifying capacity and particle size reduction, closely linked to hydration properties of this by-product. This approach would contribute to a more comprehensive nutritional profile and a cleaner label by reducing the need for additional ingredients.

The study of innovative recovery strategies for the valorization of agro-industrial by-products represents a growing sector, enhancing sustainability and transforming eating habits within the food system (Spaggiari et al., 2021). This thesis also focused on revalorizing one of the rice industry's by-products: rice bran. The study assessed the effects of various sustainable treatments, specifically enzymatic and thermal methods, as well as hydration and high-pressure processing (HPP), on the properties of defatted and non-defatted rice bran (**Chapters 5 and 6**). Analyzing the physicochemical and technological properties of treated rice bran, provided valuable insights into how these treatments influence the composition and functionality of rice bran, highlighting their potential for enhancing the value of this by-product, and also addressing technological challenges that include exploring the application of HPP under low hydration conditions.

Impact on physicochemical properties

Analysis of the proximate composition revealed significant changes following the various treatments. Across all treatments conducted in **Chapter 5** (thermal, soaking, and enzymatic), an increase in protein content was observed. Among the proximate analyses, soaking treatments effectively enhanced the dietary fiber content of defatted rice bran, causing significant modifications in the distribution between insoluble and soluble fiber fractions, notably increasing the latter. Thermal treatment also influenced fiber composition, primarily by increasing hemicellulose content. Specifically, the hemicellulose fraction in the endoprotease treated sample showed the highest hydrolysis, suggesting side-enzymatic activities in the commercial preparations. These modifications in the fiber profile could potentially impact the functional and nutritional properties of rice bran as a food ingredient. Particle size reduction was observed across all thermal, soaking, and enzymatic treatments. Enzymatic treatments were expected to reduce particle size due to their lytic activities, while reductions in samples without

enzymatic treatment was attributed to the solubilization of components like fiber during processing or dispersion of aggregates (Guillon & Champ, 2000; Rosell et al., 2009).

The HPP treatment of rice bran at different hydration levels (**Chapter 6**) significantly influenced the physicochemical properties of rice bran, particularly modifying its dietary fiber composition. Lower hydration levels promoted fiber redistribution and increased soluble fiber content when combined with HPP treatment. Adequate hydration (minimum 30%) was essential for HPP to enhance glucose and protein solubility in rice bran, with water compressibility likely affecting these compounds response to HPP across different hydration levels, as noted by researchers emphasizing the fluid-sample ratio's impact on treatment efficacy and behavior (Balasubramaniam et al., 2015). HPP's ability to reduce peroxide values in rice bran reduced at higher hydration levels, whereas improvements in oxidation were observed at lower hydrations. Hydration levels also influenced particle size, promoting agglomeration at moderate hydration (30%) and reducing particle size at higher hydration levels (60 – 77%). Although HPP did not directly alter particle size, structural changes were evident in micrographs, particularly as of a minimum of 30% hydration, HPP induced the formation of the honeycomb cell structure, indicating that pressure altered the regular structure of the fibers and created these microstructural changes (Floury et al., 2002).

Impact on technological properties

The enzymatic treatments conducted in **Chapter 5** significantly influenced the hydration capacities of the samples, increasing the water and oil binding capacity compared to the non-enzymatically treated samples. Notable correlations were observed with other properties, such as protein content, which enhanced its affinity for water ($r = 0.67$) and oil ($r = 0.68$) binding capacity. Additionally, water-binding capacity showed a significant positive correlation ($r = 0.69$) with insoluble fiber, consistent with findings from the literature (Zhu et al., 2010). A reduction in particle size and an increase in the hydration properties for various solvents were noted in the treated samples. A strong negative correlation was found between particle size and the binding capacity of oil ($r = -0.80$), retention of deionized water ($r = -0.74$), and sodium carbonate ($r = -0.71$). These findings underscore the impact of particle size on hydration properties, which is crucial for the future application of treated

defatted bran as a food ingredient, corroborated by previous studies (Chau et al., 2007; Zhao et al., 2009). Penetration force analysis revealed that the treatments reduced the force required to penetrate the samples. Samples treated with protease, particularly DRB-AL, displayed the lowest penetration resistance. This decrease in force showed a positive correlation, though not significant, with particle size ($r=0.56$).

Increasing the hydration level, particularly at higher levels, enhanced the water and oil binding capacities of rice bran (**Chapter 6**). Similar to **Chapter 5**, hydration properties of HPP RB exhibited an inverse relationship with particle size ($r=-0.71$ for water and $r=-0.80$ for oil). Additionally, foaming properties showed a strong negative correlation with water and oil binding capacity ($r > -0.69$ in both cases), suggesting that lower water binding capacity or higher hydrophobicity contributes to better foaming stability (Raikos et al., 2007; Sun et al., 2022). Higher hydration levels decreased both foaming capacity and stability. Despite this, the greatest impact of HPP was observed at 60% hydration, improving both foaming capacity and stability, indicating a potential interaction between pressure and specific hydration levels. While HPP reduced the emulsifying activity of rice bran, as reported in previous studies (Wang et al., 2022), it significantly enhanced emulsion stability across all hydrations levels. Literature suggests that molecular flexibility of proteins is crucial for emulsion stability (Kato & Nakai, 1980). Thus, it was inferred that HPP likely enhances the flexibility of rice bran proteins, contributing to improved emulsifying stability across different hydration levels. HPP increased penetration force at lower hydration levels (specially at 30%), whereas at higher hydration levels (60 and 77%), it decreased penetration force. This aligns with findings from **Chapter 5**, where penetration force was also positively correlated with particle size, but in **Chapter 6** was significantly correlated ($r=0.80$).

Impact of high-pressure processing on rice bran microbial load

As explained in **Chapter 6**, HPP is a widely used method in the food industry to ensure food safety, so it was decided to conduct a study to see how it also affected the microbial load of rice bran. HPP significantly reduced mesophilic bacteria and molds/yeasts, particularly at hydration levels of 30 - 77%. However, spore-forming bacteria showed resistance to the treatment (Muntean et al., 2016). At lower hydration (15%), the antimicrobial effectiveness of HPP was limited. It was noted that even at moderate hydration

levels (30%), HPP had a noticeable impact on rice bran load, though literature typically suggests a minimum of 40% free water is required for HPP to affect microbial load (Muntean et al., 2016). However, those high hydration percentages suppose a barrier for powder applications systems that require posterior dehydration. Understanding these hydration-dependent effects is crucial for optimizing HPP without requiring high levels of hydration during treatment.

The significance of modifying the physicochemical and techno-functional properties of both defatted and non-defatted rice bran extend its potential to become a versatile and valuable ingredient in a wide range of food applications. Achieving improvements in safety alongside these properties represents a pivotal advancement within the circular economy of the rice industry. By enhancing these characteristics, rice bran can better integrate with other food components, offering nutritional enhancements and technological advantages to a broad spectrum of products.

The research conducted in this doctoral thesis highlights the relevance of adopting a holistic and interdisciplinary approach to enhancing rice quality and safety. This approach not only drives future innovations but also promotes sustainability, benefiting both consumers and the environment.

References

- Balaban, M., Koc, C., Sar, T., & Akbas, M. Y. (2021). Antibiofilm effects of pomegranate peel extracts against *B. cereus*, *B. subtilis*, and *E. faecalis*. *International Journal of Food Science and Technology*, 56(10), 4915–4924. <https://doi.org/10.1111/ijfs.15221>
- Balasubramaniam, V. M. (Bala), Martínez-Monteagudo, S. I., & Gupta, R. (2015). Principles and application of high pressure-based technologies in the food industry. In *Annual Review of Food Science and Technology* (Vol. 6, Issue Volume 6, 2015, pp. 435–462). Annual Reviews. <https://doi.org/10.1146/annurev-food-022814-015539>
- Chalupa-Krebzdak, S., Long, C. J., & Bohrer, B. M. (2018). Nutrient density and nutritional value of milk and plant-based milk alternatives. *International Dairy Journal*, 87, 84–92. <https://doi.org/10.1016/j.idairyj.2018.07.018>
- Chau, C.-F., Wang, Y.-T., & Wen, Y.-L. (2007). Different micronization methods significantly improve the functionality of carrot insoluble fibre.

- Food Chemistry*, 100(4), 1402–1408.
<https://doi.org/10.1016/j.foodchem.2005.11.034>
- Cornejo, F., & Rosell, C. M. (2015). Physicochemical properties of long rice grain varieties in relation to gluten free bread quality. *LWT - Food Science and Technology*, 62(2), 1203–1210.
<https://doi.org/10.1016/j.lwt.2015.01.050>
- Corradini, M., Normand, M., & Peleg, M. (2008). *Nonlinear Kinetics: Principles and Potential Food Applications* (G. GutierrezLopez, G. BarbosaCanovas, J. WelitiChanes, & E. ParadaArias, Eds.; p. 71).
https://doi.org/10.1007/978-0-387-75430-7_3
- Couvert, O., Gaillard, S., Savy, N., Mafart, P., & Leguerinel, I. (2005). Survival curves of heated bacterial spores: effect of environmental factors on Weibull parameters. *International Journal of Food Microbiology*, 101(1), 73–81. <https://doi.org/10.1016/j.ijfoodmicro.2004.10.048>
- Floury, J., Desrumaux, A., & Legrand, J. (2002). Effect of ultra-high-pressure homogenization on structure and on rheological properties of soy protein-stabilized emulsions. *Journal of Food Science*, 67(9), 3388–3395.
<https://doi.org/10.1111/j.1365-2621.2002.tb09595.x>
- Gerardi, C., Pinto, L., Baruzzi, F., & Giovinazzo, G. (2021). Comparison of antibacterial and antioxidant properties of red (cv. Negramaro) and white (cv. Fiano) skin pomace extracts. *Molecules*, 26(19).
<https://doi.org/10.3390/molecules26195918>
- Ghendov-Mosanu, A., Cojocari, D., Balan, G., Patras, A., Lung, I., Soran, M.-L., Oprea, O., Cristea, E., & Sturza, R. (2022). Chemometric optimization of biologically active compounds extraction from grape marc: composition and antimicrobial activity. *Molecules*, 27(5).
<https://doi.org/10.3390/molecules27051610>
- Guillon, F., & Champ, M. (2000). Structural and physical properties of dietary fibres, and consequences of processing on human physiology. *Food Research International*, 33(3), 233–245. [https://doi.org/10.1016/S0963-9969\(00\)00038-7](https://doi.org/10.1016/S0963-9969(00)00038-7)
- Jayaprakasha, G. K., Selvi, T., & Sakariah, K. K. (2003). Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. *Food Research International*, 36(2), 117–122. [https://doi.org/10.1016/S0963-9969\(02\)00116-3](https://doi.org/10.1016/S0963-9969(02)00116-3)
- Juneja, V. K., Golden, C. E., Abhinav Mishra, Harrison, M. A., Mohr, T., & Silverman, M. (2019). Predictive model for growth of *Bacillus cereus* during cooling of cooked rice. *International Journal of Food Microbiology*, 290, 49–58.
<https://doi.org/10.1016/j.ijfoodmicro.2018.09.023>

- Katalinić, V., Možina, S. S., Skroza, D., Generalić, I., Abramović, H., Miloš, M., Ljubenković, I., Piskernik, S., Pezo, I., Terpinč, P., & Boban, M. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 *Vitis vinifera* varieties grown in Dalmatia (Croatia). *Food Chemistry*, 119(2), 715–723. <https://doi.org/10.1016/j.foodchem.2009.07.019>
- Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta (BBA) - Protein Structure*, 624(1), 13–20. [https://doi.org/10.1016/0005-2795\(80\)90220-2](https://doi.org/10.1016/0005-2795(80)90220-2)
- Lake, R., Hudson, A., & Cressey, P. (2004). *Risk profile of Bacillus spp. In rice*. <https://www.mpi.govt.nz/dmsdocument/26138/direct>
- Little, C. L., Barnes, J., Mitchell, R. T., & Food Standards Agency (FSA) and Public Health Laboratory Service (PHLS). (2002). Microbiological quality of take-away cooked rice and chicken sandwiches: Effectiveness of food hygiene training of the management. *Communicable Disease and Public Health*, 5(4), 289–298.
- Luu-Thi, H., Grauwet, T., Vervoort, L., Hendrickx, M., & Michiels, C. W. (2014). Kinetic study of *Bacillus cereus* spore inactivation by high pressure high temperature treatment. *Innovative Food Science & Emerging Technologies*, 26, 12–17. <https://doi.org/10.1016/j.ifset.2014.07.005>
- Lv, R., Zou, M., Chantapakul, T., Chen, W., Muhammad, A. I., Zhou, J., Ding, T., Ye, X., & Liu, D. (2019). Effect of ultrasonication and thermal and pressure treatments, individually and combined, on inactivation of *Bacillus cereus* spores. *Applied Microbiology and Biotechnology*, 103(5), 2329–2338. <https://doi.org/10.1007/s00253-018-9559-3>
- Mafart, P., Couvert, O., Gaillard, S., & Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: Application of the Weibull frequency distribution model. *International Journal of Food Microbiology*, 72(1–2), 107–113. [https://doi.org/10.1016/S0168-1605\(01\)00624-9](https://doi.org/10.1016/S0168-1605(01)00624-9)
- Melis, C. C. J. van, Besten, H. M. W. den, Groot, M. N. N., & Abee, T. (2014). Quantification of the impact of single and multiple mild stresses on outgrowth heterogeneity of *Bacillus cereus* spores. *International Journal of Food Microbiology*, 177, 57–62. <https://doi.org/10.1016/j.ijfoodmicro.2014.02.015>
- Muntean, M.-V., Marian, O., Barbieru, V., Cătușescu, G. M., Ranta, O., Drocas, I., & Terhes, S. (2016). High pressure processing in food industry – characteristics and applications. *Agriculture and Agricultural Science Procedia*, 10, 377–383. <https://doi.org/10.1016/j.aaspro.2016.09.077>
- Oliveira, D. A., Salvador, A. A., Smania, A., Smania, E. F. A., Maraschin,

- M., & Ferreira, S. R. S. (2013). Antimicrobial activity and composition profile of grape (*Vitis vinifera*) pomace extracts obtained by supercritical fluids. *Journal of Biotechnology*, 164(3), 423–432.
- Penha, C. B., Santos, V. D. P., Speranza, P., & Kurozawa, L. E. (2021). Plant-based beverages: ecofriendly technologies in the production process. *Innovative Food Science & Emerging Technologies*, 72, 102760. <https://doi.org/10.1016/j.ifset.2021.102760>
- Qamar, S., Manrique, Y. J., Parekh, H., & Falconer, J. R. (2020). Nuts, cereals, seeds and legumes proteins derived emulsifiers as a source of plant protein beverages: A review. *Critical Reviews in Food Science and Nutrition*, 60(16), 2742–2762. <https://doi.org/10.1080/10408398.2019.1657062>
- Raikos, V., Campbell, L., & Euston, S. R. (2007). Effects of sucrose and sodium chloride on foaming properties of egg white proteins. *Food Research International*, 40(3), 347–355. <https://doi.org/10.1016/j.foodres.2006.10.008>
- Rodrigo, D., Barbosa-Canovas, G., Martinez, A., & Rodrigo, M. (2003). Weibull distribution function based on an empirical mathematical model for inactivation of *Escherichia coli* by pulsed electric fields. *Journal of Food Protection*, 66(6), 1007–1012. <https://doi.org/10.4315/0362-028X-66.6.1007>
- Rodrigo, D., Rosell, C. M., & Martinez, A. (2021). Risk of *Bacillus cereus* in relation to rice and derivatives. *Foods*, 10(2). <https://doi.org/10.3390/foods10020302>
- Rodrigo, D., Ruiz, P., Barbosa-Canovas, G., Martinez, A., & Rodrigo, M. (2003). Kinetic model for the inactivation of *Lactobacillus plantarum* by pulsed electric fields. *International Journal of Food Microbiology*, 81(3), 223–229. [https://doi.org/10.1016/S0168-1605\(02\)00247-7](https://doi.org/10.1016/S0168-1605(02)00247-7)
- Rosell, C. M., Santos, E., & Collar, C. (2009). Physico-chemical properties of commercial fibres from different sources: A comparative approach. *Food Research International*, 42(1), 176–184. <https://doi.org/10.1016/j.foodres.2008.10.003>
- Saleh, A. S. M., Wang, P., Wang, N., Yang, L., & Xiao, Z. (2019). Brown rice versus white rice: nutritional quality, potential health benefits, development of food products, and preservation technologies. *Comprehensive Reviews in Food Science and Food Safety*, 18(4), 1070–1096. <https://doi.org/10.1111/1541-4337.12449>
- Serra, A. T., Matias, A. A., Nunes, A. V. M., Leitão, M. C., Brito, D., Bronze, R., Silva, S., Pires, A., Crespo, M. T., Romão, M. V. S., & Duarte, C. M. (2008). In vitro evaluation of olive- and grape-based natural extracts as potential preservatives for food. *Innovative Food Science & Emerging*

- Technologies*, 9(3), 311–319. <https://doi.org/10.1016/j.ifset.2007.07.011>
- Sethi, S., Tyagi, S., & Anurag, R. (2016). Plant-based milk alternatives an emerging segment of functional beverages: a review. *Journal of Food Science And Technology-Mysore*, 53(9), 3408–3423. <https://doi.org/10.1007/s13197-016-2328-3>
- Shori, A. B., & Al Zahrani, A. J. (2022). Non-dairy plant-based milk products as alternatives to conventional dairy products for delivering probiotics. *Food Science and Technology*, 42, e101321. <https://doi.org/10.1590/fst.101321>
- Spaggiari, M., Dall'Asta, C., Galaverna, G., & del Castillo Bilbao, M. D. (2021). Rice bran by-product: from valorization strategies to nutritional perspectives. *Foods*, 10(1). <https://doi.org/10.3390/foods10010085>
- Sultana, S., Faruque, M., & Islam, M. R. (2022). Rice grain quality parameters and determination tools: a review on the current developments and future prospects. *International Journal of Food Properties*, 25(1), 1063–1078. <https://doi.org/10.1080/10942912.2022.2071295>
- Sun, J., Chang, C., Su, Y., Gu, L., Yang, Y., & Li, J. (2022). Impact of saccharides on the foam properties of egg white: Correlation between rheological, interfacial properties and foam properties. *Food Hydrocolloids*, 122, 107088. <https://doi.org/10.1016/j.foodhyd.2021.107088>
- USDA. (2022). <https://fdc.nal.usda.gov/fdc-app.html#/food-search>
- Vaikma, H., Kaleda, A., Rosend, J., & Rosenvald, S. (2021). Market mapping of plant-based milk alternatives by using sensory (RATA) and GC analysis. *Future Foods*, 4, 100049. <https://doi.org/10.1016/j.fufo.2021.100049>
- Wang, S., Wang, T., Sun, Y., Cui, Y., Yu, G., & Jiang, L. (2022). Effects of high hydrostatic pressure pretreatment on the functional and structural properties of rice bran protein hydrolysates. *Foods*, 11(1). <https://doi.org/10.3390/foods11010029>
- Wasaya, A., Yasir, T. A., Sarwar, N., Atique-ur-Rehman, Mubeen, K., Rajendran, K., Hadifa, A., & Sabagh, A. E. L. (2022). Climate change and global rice security. In N. Sarwar, Atique-ur-Rehman, S. Ahmad, & M. Hasanuzzaman (Eds.), *Modern Techniques of Rice Crop Production* (pp. 13–26). Springer Singapore. https://doi.org/10.1007/978-981-16-4955-4_2
- Xu, J., Janahar, J. J., Park, H. W., Balasubramaniam, V. M., & Yousef, A. E. (2021). Influence of water activity and acidity on *Bacillus cereus* spore inactivation during combined high pressure-thermal treatment. *LWT*, 146, 111465. <https://doi.org/10.1016/j.lwt.2021.111465>

- Yu, S., Yu, P., Wang, J., Lie, C., Guo, H., Liu, C., Kong, L., Yu, L., Wu, S., Lei, T., Chen, M., Zeng, H., Pang, R., Zhang, Y., Wei, X., Zhang, J., Wu, Q., & Ding, Y. (2020). A study on prevalence and characterization of *Bacillus cereus* in ready-to-eat foods in China. *Frontiers in Microbiology*, 10. <https://doi.org/10.3389/fmicb.2019.03043>
- Zhao, X., Yang, Z., Gai, G., & Yang, Y. (2009). Effect of superfine grinding on properties of ginger powder. *Journal of Food Engineering*, 91(2), 217–222. <https://doi.org/10.1016/j.jfoodeng.2008.08.024>
- Zhu, K., Huang, S., Peng, W., Qian, H., & Zhou, H. (2010). Effect of ultrafine grinding on hydration and antioxidant properties of wheat bran dietary fiber. *Food Research International*, 43(4), 943–948. <https://doi.org/10.1016/j.foodres.2010.01.005>



Conclusions

1. The combination of mild cooking temperatures (90-95°C), acidic pH (4.5 – 5.5), and grape extract (0.1%) increases *B. cereus* spores' inactivation during cooking process, offering an effective preservation strategy.
2. During storage, grape extract at 0.1% inhibits *B. cereus* growth at low pH (4.5) or temperature (10°C), while concentrations of 0.5 and 1% demonstrates bactericidal activity independently of both environmental factors. This method achieves up to 6 log reductions of *B. cereus*, providing strong evidence supporting grape extract as an effective preservation strategy for rice.
3. The deterministic models developed facilitated antimicrobial activity quantification and the optimization of parameters for effective microbial control. The software developed could be utilized in the industry to assist with decision-making regarding pH and temperature at various grape extract concentration levels, considering the initial microbial load.
4. The industrial exposure assessment model developed provides a probabilistic estimate of the final pathogen concentration under different processing and storage conditions taking into account uncertainty and variability. It is a valuable tool for the food industry, enabling data-driven decision-making and the implementation of effective control measures to reduce the risk of *B. cereus* in rice products.
5. The comparative analysis of commercial plant-based beverages highlights a trend toward greater diversification of raw materials and increased fortification with vitamins and minerals. However, there is a need for further improvements in reducing salt and oil content and enhancing fiber fortification.
6. Enzymatic treatments of defatted rice bran show considerable potential for modifying its composition and functionality. These treatments double soluble dietary fiber content and reduce particle size up to 50%, which directly enhances hydration properties and solvent retention capacity. These improvements could facilitate better integration of rice bran into food matrices.

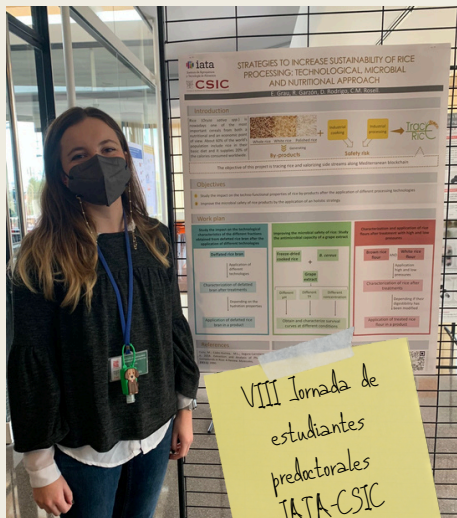
7. High pressure processing (HPP) of rice bran reveals hydration-dependent effects on its techno-functional properties and microbiological safety, with a significant tipping point observed at 30% moisture. Understanding how hydration levels affect HPP treatment is essential for enhancing antimicrobial efficacy, physical-chemical and techno-functional properties.

The research highlights the efficacy of integrating innovative and sustainable technologies in the food industry, including natural antimicrobials, enzymatic modifications, and high pressure processing. These approaches enhance the microbiological safety, nutritional quality, and functional properties of rice and rice by-products.

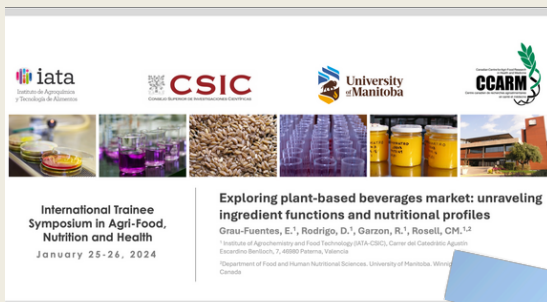


Appendix

Science communications



19th European Young Cereal Scientists and Technologists Workshop
2022



1. 10th Food science, Biotechnology & Safety Congress. Latin Food 2022
- 2 & 3. 1st (2023) and 2nd (2024) International trainee symposium in agri food, nutrition and health CCARM² Rapid Fire Research Symposium



TRACE-RICE
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We are pleased to share the technical poster "Understanding the marketed plant based beverages from ingredients technological function to their nutritional value" which focuses on vegetable beverages, featuring a large group of rice beverages.

This poster was presented at the "X International PhD Student Symposium IATA," which took place on the 20th of October at the Institute of Agrochemistry and Food Technology in Paterna, Valencia, Spain.

Do you want to know more about this poster? Please click the following link.
https://lnkd.in/dAF_W495

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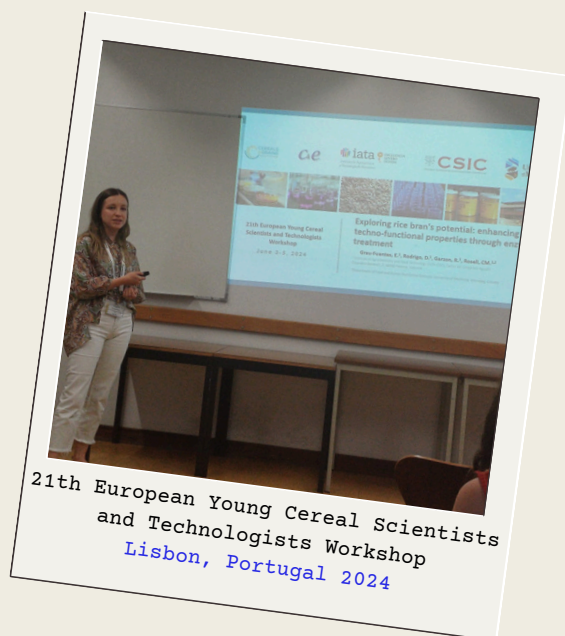
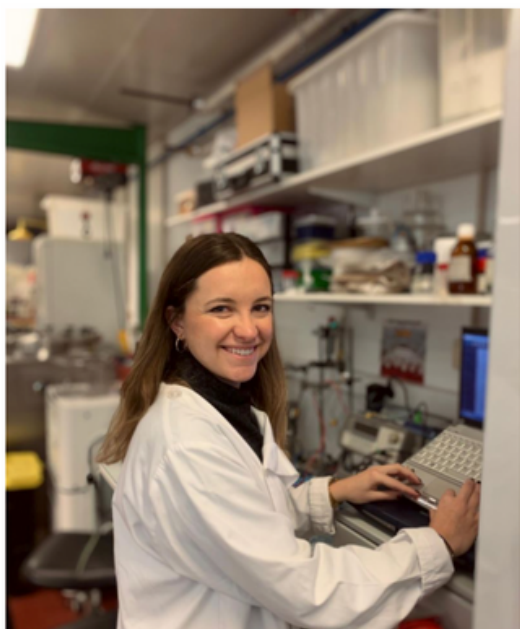
TRACE-RICE - Understanding the marketed plant-based beverages: from ingredients technological function to...
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We are proud to share that our colleague, **Eva Grau Fuentes**, is currently undertaking a PhD research stay at the Industries and Processes Laboratory in the **CEB - Centre of Biological Engineering** under the supervisors of Professor **Antonio Vicente** and **Ricardo Pereira** in Braga, Portugal 🇵🇹

We are confident that this experience will not only contribute significantly to her academic and professional growth but will also bring valuable insights and innovations back to our team 🌱🔬

#Research #ProfessionalDevelopment #TeamAchievements
 Ver traducción



the 1990s, the number of people in the UK who are aged 65 and over has increased by 1.5 million (1990–1999) and is projected to increase by a further 1.5 million by 2020 (Office of National Statistics 2000).

There is a growing awareness of the need to address the health care needs of the ageing population. The Department of Health (2000) has set out a strategy for the future of health care, which includes a commitment to 'improve the health and quality of life of older people'. The strategy also states that 'the health care system must be able to meet the needs of older people, both in terms of the range of services and the way in which they are delivered'. The strategy also states that 'the health care system must be able to meet the needs of older people, both in terms of the range of services and the way in which they are delivered'.

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Publications

Valdez-Narváez, M. I.*, **Grau-Fuentes, E.***, Morató, N., Garzón-Lloría, R., & Rodrigo, D. (2023). Joint effect of heat, pH and grape extract on *Bacillus cereus* spores survival in a rice solution. *Food Science and Technology International*, 0(0), 10820132231216770.

Grau-Fuentes, E., Úbeda-Manzanaro, M., Martínez, A., Garzón, R., Rosell, C. M., & Rodrigo, D. (2023). Evaluation of the antimicrobial activity of grape extract against *Bacillus cereus* in rice. *LWT-Food Science and Technology*, 175, 114481.

Grau-Fuentes, E., Rodrigo, D., Garzón, R., & Rosell, C. M. (2023). Understanding the marketed plant-based beverages: From ingredients technological function to their nutritional value. *Journal of Functional Foods*, 106, 105609.

Grau-Fuentes, E., Rodrigo, D., Garzón, R., & Rosell, C. M. (2024). Unlocking hidden potential of rice bran: Enzymatic treatment for enhancing techno-functional properties. *LWT - Food Science and Technology*, 207, 116673.

Grau-Fuentes, E., Rodrigo, D., Garzón, R., & Rosell, C. M. (2024). Rice bran hydration as a tool to modulate the impact of high-pressure processing. *Submitted to journal*.

Research fellowship

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Joint effect of heat, pH and grape extract on *Bacillus cereus* spores survival in a rice solution

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Abstract

Rice due to its high carbohydrate content, is an ideal medium for *Bacillus cereus* growth, a spore-producing microorganism. The objective of this study was to determine the antimicrobial activity of a grape extract in combination with heat treatments and different pH against *B. cereus* spores in a rice solution. The survivor data obtained were fitted to the Weibull survival function, and the values of parameters *a* and *b* (scale and shape indexes, respectively) were determined. Results showed that the grape extract affected the survival of *B. cereus* spores at 90 °C and 95 °C, reaching greater logarithmic reductions in acidic pH values. This behaviour was reflected in a parameter of the Weibull survival function which decreased as the temperature increased and at acidic pH values. In addition, a secondary model was developed by relating the logarithm of *a* to the independent variables (temperature and pH). A global model relating *B. cereus* inactivation with temperature and pH was developed, and validated by calculating the accuracy factor. The results demonstrate the usefulness of grape extract as a by-product, which can be used as an additional control measure for rice, especially when combined with mild heat treatments and acidic pH values.

Keywords

Bacillus cereus spores, grape extract, rice, hurdle technology, antimicrobial effect, thermal treatment

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INTRODUCTION

Bacillus cereus is a Gram-positive, ubiquitous, facultative anaerobic and spore-forming bacterium. It can grow in foods with a water activity > 0.93 (Batt, 2014) and a wide pH range (4.9–9.3) (Jessberger et al., 2020). Its spores can resist even stomach pH (1–5.2) and are resistant to adverse conditions such as high temperatures, dehydration, and radiation; however, vegetative forms of *B. cereus* can be easily eliminated when foods are subjected to conventional heat treatments (cooking) (Coroller et al., 2001; Sarriás et al., 2002; Soni et al., 2016).

B. cereus can produce two types of toxins (diarrheal and emetic). Diarrhoeal disease is usually associated with

protein-rich foods, such as meat, vegetables, puddings and dairy products and it is caused by vegetative cells producing enterotoxins in the small intestine. *B. cereus* spores can resist traditional cooking treatments allowing them to grow and produce the emetic toxin in the food before consumption. This toxin is heat-stable (up to 121 °C) and commonly associated with starchy foods such as fried and cooked rice, pasta and noodles (Abee et al., 2011; Tirloni et al., 2022). The growth temperature range is wide, ranging from 4 °C to 48 °C (Drobniewski, 1993; Miliotis and Bier, 2003). If the food is stored at inadequate temperatures (> 5 °C) for

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several hours, concentrations equal to or greater than the infective dose of *B. cereus* (10^5 UFC/g) can be achieved. In these cases, the microorganism can infect the intestine causing food poisoning in the consumer (diarrhoea) (EFSA Panel on Biological Hazards (BIOHAZ), 2016). Also, it can be seen that modifying the pH of food could help to reduce the heat resistance of *B. cereus* spores (European Food Safety Authority (EFSA), 2005; Soni et al., 2018). *B. cereus* is responsible for many foodborne health issues each year. EFSA has reported that, in 2021, 679 cases of *B. cereus* food poisoning were identified within the EU, ranking *B. cereus* as the pathogen with the highest number of food outbreaks by toxin-producing bacteria mainly in foods such as rice, seeds, nuts and almonds (European Food Safety Authority and European Centre for Disease Prevention and Control, 2022).

Due to its high starch content, rice may be an ideal substrate for *B. cereus* growth. Rice, *Oryza sativa*, is one of the most important cereals worldwide, together with corn and wheat. It is widely consumed and represents the basis of the diet of almost half the world's population (Bhattacharya, 2011; Wei and Huang, 2019). It has been shown that the main habitat of emetic strains is related to rice roots, tubers and mycorrhizae, which could explain its high prevalence in this food (Rodrigo et al., 2021; Sarriás et al., 2002). Rice is a frequent ingredient (in varying percentages) in complex, multi-ingredient, ready-to-eat foods (i.e. pure rice and lentils). In most cases, these products involve long production processes and multi-actor supply chains, where the probability of microbial contamination is very high. It is important to consider their properties and the type of processing, as the type of food consumed has a considerable effect on the survival of bacteria/spores in the stomach transit. In addition to the starch content, the pH and the process temperatures can have an important role in the evolution of the remaining microorganisms during the storage period (Jessberger et al., 2020).

Globalization of the food chain, together with the pressure to reduce waste and improve sustainability, is driving the reuse and revaluation of waste generated during food production processes. Grapes are one of the oldest and most widely cultivated fruit crops in the world. The wine industry generates large quantities of waste as a consequence of the pressing and fermentation processes, about 25% of the weight of grapes becomes waste which is difficult to manage (Dwyer et al., 2014). Grape pomace consists of ~ 10%–30% of the crushed grape mass and other value-added products, they contain high amounts of phenolic compounds such as flavonoids, catechins and anthocyanins, which have beneficial properties for human health (Garavaglia et al., 2016; Grases et al., 2015; Poudel et al., 2008) and antimicrobial properties, which could be useful in the food industry (Baydar et al., 2004; Katalinić et al., 2010; Serra et al., 2008). In addition, several studies (Hassan et al., 2019; Jayaprakasha et al., 2003; Katalinić et al., 2010; Oliveira et al., 2013) have focused on characterizing the antimicrobial

effect of grape residues as an alternative to the use of artificial preservatives or as an additional control measure in food preservation processes, showing that polyphenolic compounds with a very high negative charge have the strongest antimicrobial effect. A recent study by Grau-Fuentes et al. (Grau-Fuentes et al., 2023) has demonstrated the antimicrobial effect of grape extract on the growth of *B. cereus* vegetative cells during storage.

In this context, the main objective of this study was to evaluate the effect of a grape extract on *B. cereus* spores survival in a rice substrate at different pH levels (4.5–5.5–6.5), during a heat treatment at different temperatures, including those used in the cooking process of rice and dishes containing rice in the recipe (90 °C, 95 °C, 100 °C, and 105 °C) and to develop an experimental model that relates *B. cereus* inactivation with exposure time, pH and temperature.

MATERIALS AND METHODS

Bacterial strain and sporulation

A pure freeze-dried culture of *B. cereus* provided by the Spanish Type Culture Collection (CECT 148), which is equivalent to ATCC 13061, was used for the experiment. The culture was rehydrated with Nutrient Broth (NB) liquid medium (Scharlab Chemie S.A., Barcelona, Spain). The suspension was incubated overnight at 32 °C in a thermostatic bath with continuous stirring to obtain vegetative cells of *B. cereus* in the stationary growth phase.

For the sporulation, 20 roux bottles were prepared with modified Fortified Nutrient Agar (FNA) (Fernández et al., 1999). Roux bottles were surface inoculated with 0.5 mL of vegetative cells of *B. cereus*. The bottles were incubated at 32 °C for 24 h in an upward position, after which they were turned over. After the third day, sporulation was monitored by phase contrast microscopy, until 90% of sporulation was reached in about 5–7 days.

Then, spores were collected with a bent glass rod in sterile distilled water. Spores were washed with sterile distilled water four times and concentrated by centrifugation at 2500 g at 4 °C and 15 min in a Beckman centrifuge (rotor JLA-16.250). They were stored in sterile distilled water at 4 °C until use. Before use, *B. cereus* spores were shaken for 10 min, in the presence of glass beads to prevent aggregation. Following protocols on heat resistance studies (Condon and Sala, 1992; Stumbo, 1973; Van Zuijlen et al., 2010), the spores were activated in a water bath at 80 °C for 10 min before proceeding to heat resistance studies to avoid over-estimation of survivors and the presence of curves with non-activation shoulders.

Grape solution

The grape extract EV-3 was provided by the Sociedad Española de Colorantes Naturales y Afines SA-SECNA

(Chiva, Valencia, Spain). This extract is a natural red-coloured liquid extracted from red grapes with a pH of 3.30 ± 0.05 . The grape extract was frozen in Eppendorf tubes at -80°C until use and directly inoculated in the rice solution for heat resistance studies.

Sample preparation: rice solution and *B. cereus* spore inoculation

White rice obtained from a local retailer was cooked, freeze-dried (moisture content 8.66%) and ground. The rice solution was prepared with the rice powder (2% w/v) in distilled water. The solutions were autoclaved before pH adjustment, the addition of grape extract and inoculation of spores.

For the study, two experimental sets were considered: the control sample without grape extract and the grape sample. In both cases, they were adjusted to pH 4.5, 5.5 and 6.5. The pH of the control sample was adjusted with citric acid 1% (v/v) whereas the pH of the sample with grape extract was adjusted with NaOH 1% (v/v). After adjusting the pH of the samples, activated *B. cereus* spores were inoculated into the rice solutions at a concentration of 10^8 CFU/mL and, in the case of grape samples, 0.1% of grape extract was also added.

Heat treatment

For efficient and homogeneous heat treatment, an adaptation of the capillary tube method was used (Fernández et al., 1999). Specifically, Vitrex capillaries (reference 217913) measuring $1.50 \times 2.00 \times 100$ mm sealed at one end were used. The capillaries were filled with the samples using a sterile chamber, fitted with a vacuum pump; once the capillaries were filled, they were sealed at the other end with silicone (Quiadsa, Madrid, Spain). The capillaries were placed on racks and heated in a silicone oil bath with shaking. Ten capillaries were prepared as replicates for each treatment time (0–50 min depending on the temperature) and temperature (90°C – 105°C). After each heating period, the capillaries were immediately cooled in an ice water bath and immersed in ethanol 70% to avoid external contamination. For sample plating, the capillaries were broken and serial dilutions were made. Samples were plated on starch-enriched nutrient agar and viable counts were based on duplicate counts of the dilutions. Plates were incubated at 30°C for 24 h.

Mathematical models

Primary model. The mean value of survival data (N , CFU/mL) was fitted by non-linear regression to the survival function of the Weibull distribution (equation (1)). Fernández et al. (Fernández et al., 1999) and Peleg et al. (Peleg and Cole, 1998) demonstrate the usefulness of this function in

fitting survival data of *B. cereus* spores and *Clostridium botulinum* spores.

$$S_t = e^{-(t/a)^b} \quad (1)$$

where S_t is the survival function, t is the treatment time, a is the scale parameter and b is the shape parameter.

The parameter a is the scale, considered as a non-biological kinetic parameter representing the change of the microbial load along the treatment time at an isothermal temperature of heating, and b is the shape parameter. The b parameter describes the shape of the survival curve so, when $b < 1$ the survival curve is concave, when $b > 1$ the survival curve is convex, and when $b = 1$ the survival curve is a straight line on a log scale (equal to the Bigelow model). To assess the goodness of fit R^2_{adj} and MSE parameters were calculated (Sampedro et al., 2006).

Secondary model. A secondary model was developed by relating the logarithm of primary parameter a to the independent variables, temperature and pH (equation (2)). For this, a forward stepwise multiple regression was carried out to detect relevant terms in the polynomial.

$$\begin{aligned} \text{Log}(a) = & \alpha + \beta * \text{pH} + \gamma * T + \delta * (\text{pH} * T) + \varepsilon \\ & * p H^2 + \zeta * T^2 \end{aligned} \quad (2)$$

where a is the scale parameter, pH is the pH of the sample and T is the treatment temperature.

Global model. Considering the more relevant terms of the polynomial, a global model that related Log S with pH and T was derived based on the Weibull equation (equation (3)) (Fernández et al., 2002).

$$\text{Log} S = - \left(\left(\frac{t}{10^{(\alpha + \beta * \text{pH} + \gamma * T)}} \right)^b \right) \quad (3)$$

where S is the survival fraction, t is the treatment time, pH is the pH of the sample, T is the treatment temperature and b is the shape parameter.

Accuracy factor. A new set of experimental data was used to validate the global model by calculating the accuracy factor (AF) according to Ross (Ross, 1996).

This factor provides a measure of the average precision of the estimates and is given by equation (4).

$$AF = 10^{\left(\frac{\sum \log(\text{predicted}/\text{observed})}{n} \right)} \quad (4)$$

where n is the number of observations used in the calculation.

The accuracy factor should always be greater than or equal to one, and it is one if there is a perfect agreement between all predictions and observed values.

Statistical analysis

Experiments were performed in triplicates with two replicas per count. The data set was divided into two groups, and one was used for validation purposes. The experimental results were shown as Log_{10} of the survival fraction ($\text{Log}(S)$) calculated by (equation (5)):

$$\text{Log}(S) = \frac{N}{N_0} \quad (5)$$

where N is the bacterial concentration (CFU/mL) at time t (min) and N_0 is the initial bacterial concentration (CFU/mL) (t_0). Statistical analysis was carried out with Statgraphics Centurion 18 version 18.1.13 by Statgraphics Technologies, Inc. Identified outliers were previously eliminated. S the statistical significance of the data was determined with an analysis of variance (ANOVA) (p -value < 0.05) and inter-group differences were determined using (LSD) of the Fisher test, which identifies homogeneous subsets of averages that do not differ from each other. Data modelling was performed with the mean data for each specific pH and temperature with Statgraphics Centurion 18 version 18.1.13 by applying non-linear regression.

RESULTS AND DISCUSSIONS

The present work has studied the antimicrobial effect of a grape extract against *B. cereus* spores in a rice solution subjected to four temperatures (90 °C, 95 °C, 100 °C and 105 °C), which include those simulating traditional cooking treatments. Antimicrobial effects were compared between control samples (2% rice solution without grape extract) and 2% rice solution with grape extract, adjusted in all cases to different pH values (4.5–5.5–6.5) simulating the pH of different dishes in which rice could be present.

The logarithm of the survival fraction was plotted as a function of treatment time for each temperature and pH studied. Figure 1(A) to (D) shows that, as time and temperature increase and pH decreases, the *B. cereus* inactivation increases. Figure 1(A) and (B) shows a reduction in *B. cereus* spores of more than three logarithmic cycles, while in Figure 1(C) and (D), there is a reduction of more than four logarithmic cycles. These results resemble the survival behaviour of *B. cereus* spores, previously reported by Sarrias et al. (Sarrias et al., 2002; Fernández et al., 1996, 2002; Soni et al., 2018).

In Figure 1(A) and (B) (90 °C and 95 °C), it is shown the antimicrobial effect of grape extract, since the logarithmic reduction of *B. cereus* spores was significantly higher ($p \leq 0.05$) in the samples with grape extract compared to the control samples. On the other hand, in Figure 1(C) and (D) no significant differences ($p > 0.05$) between control samples and those with grape extract can be observed, attributing the reduction of *B. cereus* spores only to temperature, since it has been seen that at temperatures above

95 °C, the resistance of *B. cereus* spores decreases and therefore there are no significant differences ($p > 0.05$) if grape extract is added or not.

Regarding the pH, there is a significant antimicrobial effect ($p \leq 0.05$) of grape extract in combination with pH (4.5 and 5.5) and temperatures lower than 100 °C on the inactivation of *B. cereus* as it can also be shown in Figure 1(A) and (B).

The mean experimental data were fitted to the Weibull survival function (equation (1)) by using non-linear regression, obtaining parameters that can be used to compare the effect of pH, temperature and grape extract on the *B. cereus* spores survival.

Table 1 shows the values of the Weibull survival function parameters and the fitting parameters R^2_{adj} and MSE. An ANOVA was performed with the results obtained for parameter a , to determine if there were significant differences in terms of exposure to grape extract. The results show that parameter a represents a measure of resistance to the treatment, showing that there is a significant decrease ($p \leq 0.05$) at acidic pH values and at 90 °C and 95 °C, at 100 °C and 105 °C and when the pH is 6.5 there are no significant differences ($p > 0.05$). This could indicate that at a high-temperature range and a pH close to neutrality, the antimicrobial effect of the grape extract is affected. Additionally, the shape parameter follows a normal distribution of 0.48 ± 0.06 and 0.52 ± 0.07 , for grape extract and the control, respectively (Table 1).

In previous studies grapes have been found to have an antimicrobial effect, especially against Gram (+) bacteria (Ghendov-Mosan et al., 2022; Jayaprakasha et al., 2003; Oliveira et al., 2013). It is important to note that these previous studies have been carried out on the basis of vegetative cell growth of *B. cereus*, without using other preservation treatments in food matrices. Serra et al. (Serra et al., 2008) demonstrated that the natural grape extract drastically inhibited the growth of three bacterial strains in a dose-dependent manner, and the inhibitory effect was also most effective on vegetative cells of the Gram (+) strain *B. cereus*. On the other hand, Katalinić et al. (Katalinić et al., 2010) studied the antimicrobial effect of the skins of 14 grape varieties (white and red), their results showed that there was a significant effect of grape extracts on both Gram (+) and Gram (–) bacteria; however, red grape extracts had the greatest antimicrobial effect on *B. cereus*. Another study that investigated the antimicrobial effect of grape seeds (Jayaprakasha et al., 2003) showed a higher antimicrobial effect of seeds, as also reported by Baydar et al. (Baydar et al., 2004), who studied the antimicrobial effect of seeds and bagasse separately. However, Oliveira et al. (Oliveira et al., 2013) studied the antimicrobial effect of grape bagasse and found it displayed good antimicrobial activity when it is extracted by supercritical fluids.

Regarding the modelling of survival curves, it can be highlighted that at temperatures of 90 °C and 95 °C, a

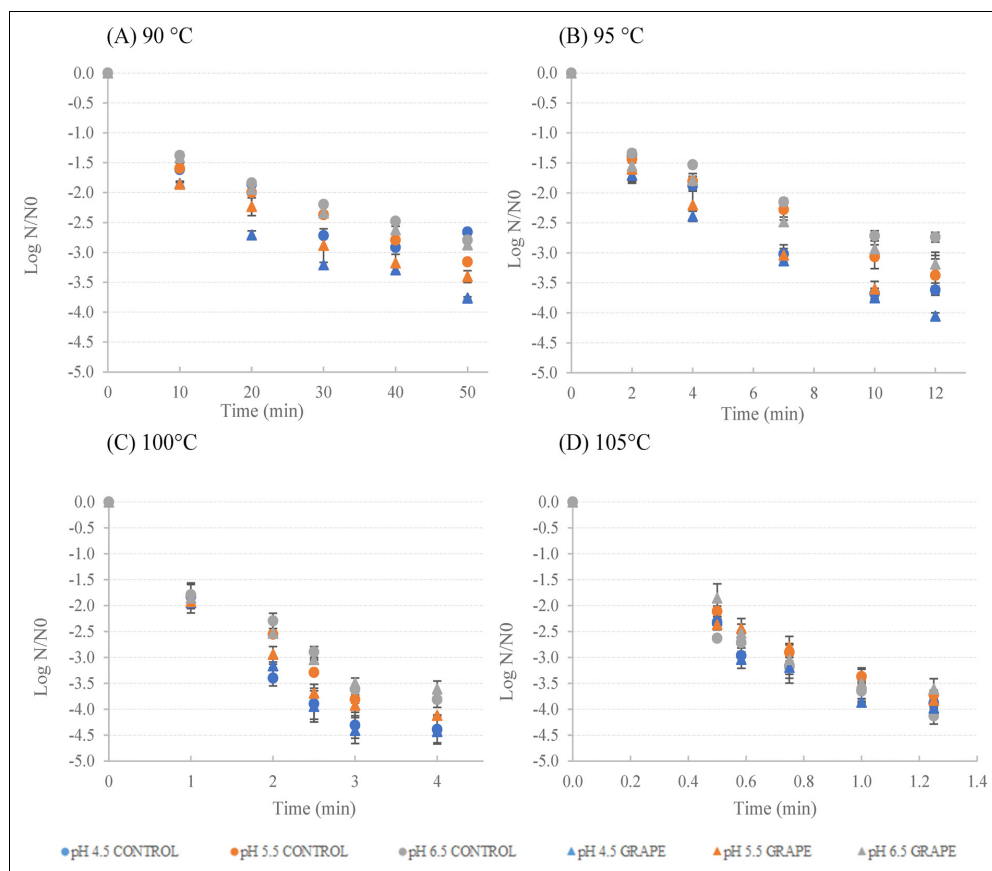


Figure 1. Survival of *B. cereus* spores in rice solution at different pH and treated at 90 °C (A), 95 °C (B), 100 °C (C) and 105 °C (D). Circles data represent values observed in control samples (without extract), while triangles represent the values obtained in samples with grape extract.

values of the samples with grape extract were significantly lower than *a* values in the control samples for all pH values studied (4.5–5.5–6.5); except when the temperature was 90 °C and pH 6.5. This indicates that, at a pH closer to neutrality, the *B. cereus* spores are more resistant to treatment and grape extract can no longer exert an effect.

The comparison of results, presented in rows with lowercase letters (Table 1), shows that at a temperature of 90 °C, the *a* value of control sample decreases significantly as the pH decreases, a behaviour that has also been mentioned by other authors (Leguerinel and Mafart, 2001; Mazas et al., 1998). With respect to the grape samples, similar behaviour can be seen, the value of *a* decreases significantly when pH decreases from 6.5 to 5.5; however, when the pH decreases from 5.5 to 4.5, there are no significant differences. Rhodes et al. (Rhodes et al., 2006), studied the antilisterial activity of grape juice and grape extract. They found that this activity was pH-dependent. At low

pH, the antilisterial activity was clearly seen, explaining this could be due to the fact that part of the anthocyanin is in the form of a stabilised flavin cation and can interact with the negatively charged bacterial cell wall. By contrast, when the samples were adjusted to pH 7.0, the antilisterial activity was lost, which may be due to the fact that most of the remaining anthocyanin in the polymeric structure is in the form of an uncharged quinonoidal base. Likewise, in another investigation by Hsieh et al. (2001) studying the antimicrobial effect of various plant extracts (*Corni fructus*, cinnamon and Chinese chive), the authors found that at lower pH values the antimicrobial effect against all bacteria studied, including *B. subtilis*, increased when the treatment temperature increased. Another study by van Melis et al. (2014) and Xu et al. (2021) reported that when *B. cereus* spores were subjected to acidic pH values, additional stresses (low water activity) and mild heat treatment, their ability to germinate and grow

Table 1. Weibull survival function parameters were obtained by fitting experimental mean data to equation (1).

T (°C)	pH	Control				Grape extract			
		a	b	R ² _{adj}	MSE	a	b	R ² _{adj}	MSE
90	4.5	2.62 ± 0.08 ^{* a. A}	0.36 ± 0.01	0.945	0.171	1.87 ± 0.17 ^{* a. A}	0.40 ± 0.01	0.991	0.086
	5.5	4.02 ± 0.34 ^{* b. A}	0.40 ± 0.02	0.995	0.051	2.43 ± 0.35 ^{* a. A}	0.40 ± 0.01	0.995	0.058
	6.5	4.96 ± 0.16 ^{c. A}	0.44 ± 0.01	0.999	0.016	4.38 ± 0.23 ^{b. A}	0.44 ± 0.01	1.000	0.011
95	4.5	1.14 ± 0.10 ^{* a. B}	0.57 ± 0.01	0.980	0.136	0.66 ± 0.11 ^{* a. B}	0.48 ± 0.02	1.000	0.009
	5.5	1.24 ± 0.01 ^{* a. B}	0.52 ± 0.05	0.987	0.091	0.58 ± 0.15 ^{* a. B}	0.42 ± 0.05	0.972	0.149
	6.5	1.30 ± 0.02 ^{* a. B}	0.46 ± 0.01	0.986	0.083	0.89 ± 0.01 ^{* a. B}	0.44 ± 0.03	0.993	0.054
100	4.5	0.26 ± 0.11 ^{a. C}	0.56 ± 0.06	0.965	0.234	0.25 ± 0.02 ^{a. C}	0.56 ± 0.03	0.968	0.214
	5.5	0.34 ± 0.08 ^{a. C}	0.53 ± 0.01	0.974	0.161	0.25 ± 0.01 ^{a. B}	0.53 ± 0.01	0.979	0.161
	6.5	0.43 ± 0.18 ^{a. C}	0.61 ± 0.13	0.974	0.142	0.28 ± 0.02 ^{a. C}	0.50 ± 0.02	0.986	0.103
105	4.5	0.07 ± 0.01 ^{a. C}	0.48 ± 0.01	0.986	0.108	0.08 ± 0.01 ^{a. C}	0.51 ± 0.02	0.977	0.149
	5.5	0.10 ± 0.02 ^{a. C}	0.53 ± 0.04	0.987	0.085	0.12 ± 0.05 ^{a. B}	0.57 ± 0.12	0.996	0.060
	6.5	0.08 ± 0.01 ^{a. D}	0.51 ± 0.01	0.999	0.030	0.06 ± 0.01 ^{a. C}	0.46 ± 0.09	0.997	0.052

T: temperature; a: scale parameter; b: shape parameter; R²_{adj}: R² adjusted; MSE: mean square error. The asterisk indicates significant differences in a parameter between control samples and those with grape extract. Lower case letters compare a values between pHs at a specific temperature; while upper case letters compare a values between temperatures at a specific pH. Different letters indicate significant differences (p ≤ 0.05).

decreased. This would explain the possible synergistic effect of grape extract; pH and heat treatment observed in this study and the importance of investigating the combination of a heat treatment with the use of a natural antimicrobial on *B. cereus* spores, which is more resistant and it would require stronger cooking treatments that affect the quality of the food.

On the other hand, when the temperature was 95 °C, 100 °C and 105 °C, *a* values in the control and in the grape extract samples did not decrease significantly when pH decreased. This would indicate that at these temperatures the antimicrobial effect is due to the temperature; since at these temperatures there is significant damage to the cellular structure of the spore, the exosporium is damaged and some proteins suffer denaturalization, thereby losing resistance to heat treatment and preventing germination and growth (Luu-Thi et al., 2014; Lv et al., 2019).

Finally, the comparison of the results presented in the rows with capital letters (Table 1), corroborates the effect that pH and temperature have on the thermo-resistance of *B. cereus* spores. At pH 6.5 there were significant differences at all temperatures (90 °C, 95 °C, 100 °C, and 105 °C), when the pH value was very close to neutrality, showing the effect of temperature on *B. cereus* spores alone. Meanwhile, at pH 4.5 or 5.5, there were significant differences only between the temperatures 90 °C, 95 °C and 100 °C; in this case, *B. cereus* spore resistance decreased due to the effect of more acidic pH values, and therefore the decimal reduction was higher.

Therefore, we can state that *B. cereus* spore development can be inhibited by combining factors such as pH, a natural antimicrobial and a mild heat treatment (Mafart et al., 2010;

Soni et al., 2018; Xu et al., 2021). These results highlight the need to combine heat treatment with other factors such as low pH or natural antimicrobials, in order to control microorganisms without adversely impacting food quality, as occurs at elevated temperatures.

The relationship between parameter *a*, pH and temperature (secondary model) was determined by means of a multiple linear regression. Equation (6) was obtained for control samples and equation (7) for the samples with grape extract. Variables were delimited considering statistical significance with a confidence level of 95.0%:

$$\text{Log}(a) = 4.77 + 0.0763 * \text{pH} - 0.00057 * T^2 \quad (6)$$

$$\text{Log}(a) = 9.341 - 0.0994 * T \quad (7)$$

Subsequently, to improve the estimation of the parameters all the data points (Log *S*) were fitted to the global model developed, equation (8) for the control samples and equation (9) for the samples with grape extract. The fitting results of these equations can be seen in Table 2.

$$\text{Log } S = - \left(\left(\frac{t}{10^{(\alpha + \beta * \text{pH} + \gamma * T^2)}} \right)^{0.52} \right) \quad (8)$$

$$\text{Log } S = - \left(\left(\frac{t}{10^{(\alpha + \beta * T)}} \right)^{0.48} \right) \quad (9)$$

Finally, the global model has been validated by calculating the AF (equation (4)) with an extra set of experimental data not used for the model fitting. For the control sample, it was 1.003, indicating that the percentage error is 0.3% for the predictions; while in the case of grape samples, the AF for the global model

Table 2. Coefficients of the polynomial were obtained by fitting the global model (equations (8) and (9)) to the total experimental data.

Control			Grape extract		
Coefficient	Estimated	Standard error	Coefficient	Estimated	Standard error
α	5.40	0.14	α	9.68	0.63
β	0.051	0.016	β	−0.1030	0.0054
Γ	−0.000617	0.000011			

was 1.028 indicating a percentage error of 2.8%. According to the AF, the global model can be used to predict the remaining *B. cereus* spores in rice solution, after being subjected to a preservation process, for both the control and samples containing grape extract, in which the pH and temperature are the independent environmental variables.

CONCLUSIONS

The hurdle concept is getting very popular as a preservation process. Results of the present work, show that the combined use of mild cooking temperatures, an acidic pH and grape extract, could be considered as a hurdle preservation process for acidic ready-to-eat dishes containing rice processed at 90 °C–95 °C that are very common mild pasteurization temperatures. The advantage of combining heat and the use of grape extract could be an extension of the shelf life of the product and an improvement of the safety against *B. cereus* spores present in rice. The effect of the grape extract takes place at the thermal process level in the product inoculated with *B. cereus* spores. Additional studies could indicate if the effect goes beyond the thermal process during storage.

A global model has been developed that can lead to a tertiary model to be used by the industry in the development of pasteurization processes for ready-to-eat foods.

Although the results are interesting, further studies are needed, such as the characterization of the grape extract, as well as the maximum concentration of grape extract that can be added to obtain a hurdle effect at low acid conditions without negatively influencing the sensory characteristics of the product.

DECLARATION OF CONFLICTING INTERESTS

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REFERENCES

- Abee T, Groot MN, Tempelaars M, et al. (2011). Germination and outgrowth of spores of *Bacillus cereus* group members: Diversity and role of germinant receptors. *Food Microbiology* 28(2): 199–208.
- Batt CA (2014). BACILLUS *Bacillus cereus*. In: *Encyclopedia of Food Microbiology*. 2nd ed. Cambridge, MA: Academic Press, 124–128.
- Baydar NG, Özkan G and Sağdıç O (2004). Total phenolic contents and antibacterial activities of grape (*Vitis vinifera* L.) extracts. *Food Control* 15(5): 335–339.
- Bhattacharya KR (2011). Rice quality: A guide to rice properties and analysis. In: *Woodhead Publishing Series in Food Science, Technology and Nutrition*. Oxford: WP, Woodhead Publ, 219.
- Condon S and Sala FJ (1992). Heat resistance of *Bacillus subtilis* in buffer and foods of different pH. *Journal of Food Protection* 55(8): 605–608.
- Coroller L, Leguérinel I and Mafart P (2001). Effect of water activities of heating and recovery media on apparent heat resistance of *Bacillus cereus* spores. *Applied and Environmental Microbiology* 67(1): 317–322.
- Drobniński FA (1993). *Bacillus cereus* and related species. *Clinical Microbiology Reviews* 6: 324–338.
- Dwyer K, Hosseini F and Rod M (2014). The market potential of grape waste alternatives. *Journal of Food Research* 3(2): 91.
- EFSA Panel on Biological Hazards (BIOHAZ) (2016). Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs. *EFSA Journal* 14(7): e04524.
- European Food Safety Authority and European Centre for Disease Prevention and Control (2022). The European Union One Health 2021 zoonoses report. *EFSA Journal* 20(12): e07666.
- European Food Safety Authority (EFSA) (2005). Opinion of the Scientific Panel on biological hazards (BIOHAZ) on *Bacillus cereus* and other *Bacillus* spp in foodstuffs. *EFSA Journal* 3(4): 175.
- Fernández A, Collado J, Cunha LM, et al. (2002). Empirical model building based on Weibull distribution to describe the joint effect of pH and temperature on the thermal resistance of *Bacillus cereus* in vegetable substrate. *International Journal of Food Microbiology* 77(1–2): 147–153.
- Fernández A, Salmerón C, Fernández PS, et al. (1999). Application of a frequency distribution model to describe the

- thermal inactivation of two strains of *Bacillus cereus*. *Trends in Food Science & Technology* 10(4–5): 158–162.
- Fernández PS, Ocio MJ, Rodrigo F, et al. (1996). Mathematical model for the combined effect of temperature and pH on the thermal resistance of *Bacillus stearothermophilus* and *Clostridium sporogenes* spores. *International Journal of Food Microbiology* 32(1–2): 225–233.
- Garavaglia J, Markoski MM, Oliveira A, et al. (2016). Grape seed oil compounds: Biological and chemical actions for health. *Nutrition and Metabolic Insights* 9: NMI.S32910.
- Ghendov-Mosanu A, Cojocari D, Balan G, et al. (2022). Chemometric optimization of biologically active compounds extraction from grape marc: Composition and antimicrobial activity. *Molecules* 27(5): 1610.
- Grases F, Prieto RM, Fernández-Cabot RA, et al. (2015). Effect of consuming a grape seed supplement with abundant phenolic compounds on the oxidative status of healthy human volunteers. *Nutrition Journal* 14(1): 94.
- Grau-Fuentes E, Úbeda-Manzanaro M, Martínez A, et al. (2023). Evaluation of the antimicrobial activity of grape extract against *Bacillus cereus* in rice. *LWT* 175: 114481.
- Hassan YI, Kosir V, Yin X, et al. (2019). Grape pomace as a promising antimicrobial alternative in feed: A critical review. *Journal of Agricultural and Food Chemistry* 67(35): 9705–9718.
- Hsieh P-C, Mau J-L and Huang S-H (2001). Antimicrobial effect of various combinations of plant extracts. *Food Microbiology* 18(1): 35–43.
- Jayaprakasha GK, Selvi T and Sakariah KK (2003). Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. *Food Research International* 36(2): 117–122.
- Jessberger N, Dietrich R, Granum PE, et al. (2020). The *Bacillus cereus* food infection as multifactorial process. *Toxins* 12(11): 701.
- Katalinić V, Možina SS, Skroza D, et al. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 *Vitis vinifera* varieties grown in Dalmatia (Croatia). *Food Chemistry* 119(2): 715–723.
- Leguerinel I and Mafart P (2001). Modelling the influence of pH and organic acid types on thermal inactivation of *Bacillus cereus* spores. *International Journal of Food Microbiology* 63(1–2): 29–34.
- Luu-Thi H, Grauwet T, Vervoort L, et al. (2014). Kinetic study of *Bacillus cereus* spore inactivation by high pressure high temperature treatment. *Innovative Food Science & Emerging Technologies* 26: 12–17.
- Lv R, Zou M, Chantapakul T, et al. (2019). Effect of ultrasonication and thermal and pressure treatments, individually and combined, on inactivation of *Bacillus cereus* spores. *Applied Microbiology and Biotechnology* 103(5): 2329–2338.
- Mafart P, Leguérinel I, Couvert O, et al. (2010). Quantification of spore resistance for assessment and optimization of heating processes: A never-ending story. *Food Microbiology* 27(5): 568–572.
- Mazas M, López M, González I, et al. (1998). Effects of the heating medium pH on heat resistance of *Bacillus cereus* spores. *Journal of Food Safety* 18(1): 25–36.
- Miliotis MD and Bier JW (2003). International handbook of food-borne pathogens. In: *Food Science and Technology*. New York: M. Dekker, 125.
- Oliveira DA, Salvador AA, Smânia A, et al. (2013). Antimicrobial activity and composition profile of grape (*Vitis vinifera*) pomace extracts obtained by supercritical fluids. *Journal of Biotechnology* 164(3): 423–432.
- Peleg M and Cole MB (1998). Reinterpretation of microbial survival curves. *Critical Reviews in Food Science and Nutrition* 38(5): 353–380.
- Poudel PR, Tamura H, Kataoka I, et al. (2008). Phenolic compounds and antioxidant activities of skins and seeds of five wild grapes and two hybrids native to Japan. *Journal of Food Composition and Analysis* 21(8): 622–625.
- Rhodes P, Mitchell J, Wilson M, et al. (2006). Antilisterial activity of grape juice and grape extracts derived from *Vitis vinifera* variety Ribier. *International Journal of Food Microbiology* 107(3): 281–286.
- Rodrigo D, Rosell CM and Martínez A (2021). Risk of *Bacillus cereus* in relation to rice and derivatives. *Foods (Basel, Switzerland)* 10(2): 302.
- Ross T (1996). Indices for performance evaluation of predictive models in food microbiology. *Journal of Applied Bacteriology* 81(5): 501–508.
- Sampedro F, Rivas A, Rodrigo D, et al. (2006). Effect of temperature and substrate on Pef inactivation of *Lactobacillus plantarum* in an orange juice–milk beverage. *European Food Research and Technology* 223(1): 30–34.
- Sarríás JA, Valero M and Salmerón MC (2002). Enumeration, isolation and characterization of *Bacillus cereus* strains from Spanish raw rice. *Food Microbiology* 19(6): 589–595.
- Serra AT, Matias AA, Nunes AVM, et al. (2008). In vitro evaluation of olive- and grape-based natural extracts as potential preservatives for food. *Innovative Food Science & Emerging Technologies* 9(3): 311–319.
- Soni A, Oey I, Silcock P, et al. (2016). *Bacillus* spores in the food industry: A review on resistance and response to novel inactivation technologies: *Bacillus* spores in response to technologies. *Comprehensive Reviews in Food Science and Food Safety* 15(6): 1139–1148.
- Soni A, Oey I, Silcock P, et al. (2018). Impact of temperature, nutrients, pH and cold storage on the germination, growth and resistance of *Bacillus cereus* spores in egg white. *Food Research International* 106: 394–403.
- Stumbo CR (1973). Thermobacteriology in food processing. In: *Food science and technology*, 2nd ed. New York: Academic Press, 1–336.
- Tirloni E, Stella S, Celandroni F, et al. (2022). *Bacillus cereus* in dairy products and production plants. *Foods (Basel, Switzerland)* 11(17): 2572.
- van Melis CCJ, den Besten HMW, Nierop Groot MN, et al. (2014). Quantification of the impact of single and multiple mild stresses on outgrowth heterogeneity of *Bacillus cereus* spores. *International Journal of Food Microbiology* 177: 57–62.
- Van Zuijlen A, Periago PM, Amézquita A, et al. (2010). Characterization of *Bacillus sporothermodurans* IC4 spores; putative indicator microorganism for optimisation of thermal processes in food sterilisation. *Food Research International* 43(7): 1895–1901.
- Wei X and Huang X (2019). Origin, taxonomy, and phylogenetics of rice. In: *Rice*, 4th ed. AACC International Press, 1–29. Available at: <https://linkinghub.elsevier.com/retrieve/pii/B9780128115084000010> (accessed 12 March 2023).
- Xu J, Janahar JJ, Park HW, et al. (2021). Influence of water activity and acidity on *Bacillus cereus* spore inactivation during combined high pressure-thermal treatment. *LWT* 146: 111465.



Evaluation of the antimicrobial activity of grape extract against *Bacillus cereus* in rice

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ABSTRACT

The antimicrobial potential of grape extract was assessed in cooked rice against *Bacillus cereus*. Grape extract efficacy was tested at 1, 5 and 10 mL/L, at pH 4.5, 5.5 and 6.5; and at incubation temperatures simulating different storage scenarios, specifically temperature abuse (10 °C), cool chain break (20 °C) and optimal *B. cereus* growth temperature (30 °C). Survival curves for grape extract concentration versus time were obtained. The results indicate that antimicrobial activity of grape extract was dependent on temperature, pH and grape extract concentration. A bactericidal effect of the grape extract was shown at concentration levels ≥ 5 mL/L at all temperatures and pHs studied. Inactivation curves of *B. cereus* under grape extract exposure were fitted to a Weibull distribution function for 5–10 mL/L grape extract concentration. Observations showed that the higher the incubation temperature and grape extract concentration, the lower the kinetic rate value. In other words, lower resistance of the microorganism to environmental conditions. The maximum inactivation level was 6 log₁₀ cycles after 24 h of exposure at 10 mL/L of grape extract concentration and pH 4.5. Results indicate that the grape extract could be a good additional control measure for preventing *Bacillus cereus* growth in cooked rice during storage.

1. Introduction

Rice (*Oryza sativa* L.) is a basic cereal, widely consumed by the general population due to its abundant nutrients and relatively low price. It is one of the most important staple crops on the planet and feeds almost half the world population (Wei & Huang, 2019). However, it is also frequently involved in foodborne *B. cereus* outbreaks (Rodrigo, Rosell, & Martínez, 2021). Once cooked, it has a pH close to 7, being an excellent growth medium when the humidity of the substrate reaches adequate water activity values for the growth of the microorganism (Delbrassinne, Botteldoorn, Andjelkovic, Dierick, & Denayer, 2015; Pao, Khalid, & Kalantari, 2006; Rodrigo et al., 2021). *B. cereus* is a gram-positive spore forming bacterium, which is a habitual saprophyte, resistant to high temperatures and low humidity (Hendriksen, Hansen, & Johansen, 2006; Rodrigo et al., 2021). It is a public health issue because produces food toxins and can survive 48 weeks in rice in cool dry storage without loss of viability (Gilbert, Stringer, & Peace, 1974; Sánchez, Correa, & Castañeda, 2016).

One of the main factors contributing to *B. cereus* outbreaks related to cooked rice is poor cooling, or storage at room temperature for a prolonged period, particularly when cooking large quantities in restaurants and/or canteens (Juneja et al., 2019). The main source of this contamination is the presence of heat-resistant spores that survive normal rice cooking temperatures (close to 100 °C) (Gilbert et al., 1974). During standard cooking conditions, 2–3 decimal log reductions in the initial spore load can be achieved, therefore final product risk levels depend largely on the initial concentration of microorganisms and hygienic measures during handling, cooking or processing (Rodrigo et al., 2021). After being cooked, the spores that have survived the process can germinate and grow up to 10⁷ or 10⁹ CFU/g after 24 h at 26 or 32 °C, respectively (Lake, Hudson, & Cressey, 2004; Yu et al., 2020) and produce a heat-stable (emetic) toxin (Little, Barnes, Mitchell, FSA, & PHLS, 2002). Around 95% of outbreaks of an emetic syndrome are mainly caused by the consumption of cooked or fried rice (Juneja et al., 2019). Controlling the storage temperature (lower than 4 °C or higher than 55 °C) is the procedure commonly accepted by food safety authorities.

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Consequently, it is of great interest to have an additional control measure, other than post-cooking storage temperature, in these products, especially if they are not going to be consumed immediately after preparation (Juneja et al., 2019).

Grape (*Vitis vinifera* L.), represents the second-largest crop in the world. Its skin and seeds, are also rich in phenolic compounds, as well as lipids, proteins and polysaccharides (Ferreira, Nunes, Castro, Ferreira, & Coimbra, 2014; Gokturk Baydar, Ozkan, & Yasar, 2007). Phenolic compounds are phytochemicals with functions related to pigmentation, astringency, protection against ultraviolet rays, as well as antioxidant and antimicrobial activity (Oliveira, Brunini, Salandini, & Bazzo, 2003). As antimicrobial agents, these polyphenols can penetrate the semi-permeable cell membrane where they react with the cytoplasm or cellular proteins. Therefore, these polyphenolic compounds with a highly negative charge can be used to prevent the growth of pathogenic bacteria (Arts & Hollman, 1998; Cheng, Bekhit, McConnell, Mros, & Zhao, 2012; Oki et al., 2002; Yadav, Kumar, Kumar, & Mishra, 2015). Grape extract is one of the ingredients that has received attention in recent years due to its natural antimicrobial capacity. One advantage of these extracts is that they are derived from industrial waste products, such as grape juice/wine production (Shi, Yu, Pohorly, & Kakuda, 2003; Yu, Ahmedna & Goktepe, 2005, 2010). In addition to providing compounds with functional properties, revalorization reduces the environmental impact by reusing part of the grape, such as seeds and skins, which are generally not used by industry (Goncalves, Lorenzo, & Trindade, 2021). Identifying the value of these products will result in reducing waste, reusing raw products, and providing an all-natural alternative to synthetic preservatives (Levy et al., 2017), which will improve food-industry sustainability and positively impact on the UN's Sustainable Development Goals. Therefore, the use of this raw material as a natural substitute for synthetic antimicrobial additives represents a positive alternative to prevent foodborne outbreaks (Goncalves et al., 2021; Prado Martin et al., 2012).

In this context, the main objective of this work was to evaluate the antimicrobial capability of the grape extract against vegetative cells of *B. cereus* (germinated spores) in a rice matrix, using different storage conditions resembling real ones, at different storage temperatures (10, 20 and 30 °C), different grape extract concentrations (1, 5 and 10 mL/L) and different pH values (4.5, 5.5 and 6.5).

2. Materials and methods

2.1. Microbial strains

Pure culture of *B. cereus* (Spanish Type Culture Collection (CECT) 148) were provided by the Spanish Type Culture Collection and, following their procedure, lyophilized samples were rehydrated with 0.2 mL of sterile Nutrient Broth (NB) (Scharlab S.A., Barcelona, Spain). After 30 min, rehydrated cultures were transferred to 500 mL of NB medium, and incubated in a water bath shaker for 14–18 h at 30 °C, to obtain cells in a stationary growth phase. The *B. cereus* cells were centrifuged twice at 5000 revolutions per minute (rpm), 4 °C and 15 min, in a Beckman centrifuge (JLA-16,250 rotor), the supernatant was decanted and resuspended in 50 mL of NB. Process was repeated twice, and then cells were resuspended in 50 mL of NB and distributed in cryovials (1 mL); 1 mL of 200 mL/L glycerol in NB was added as protectant and kept at –80 °C until further use. The final inoculant concentration was determined by plate count and was of 10⁸ colony forming units/mL (CFU/mL).

2.2. Rice matrix

Commercial rice acquired in the market was used. Rice was cooked into an electric cooker, and after cooling down cooked rice was freeze-dried. Rice powder had moisture content of 8.66 g water/100 g of product. Three different batches were prepared. The rice matrix was

prepared by diluting 20 g of powder rice/L, and it was sterilized in an autoclave.

2.3. Grape extract

Grape color liquid EV-3 is a natural red colorant extracted from red grapes (EEC code: E-163). The grape color extract was provided by Sociedad Española de Colorantes Naturales y Afines SA - SECNA (Chiva, Valencia, Spain). This extract, with a pH of 2.5, was frozen in Eppendorf at –80 °C for later use. 1, 5 and 10 mL/L grape extract concentrations were added to the rice matrix for testing the antimicrobial activity against *B. cereus*.

2.4. Evaluation of antimicrobial activity of grape extract

The grape extract antimicrobial activity was tested at pH 4.5; 5.5 and 6.5, by using a sodium hydroxide solution (1 mL/100 mL) to modify the pH value of samples. Microbial growth at these conditions was compared with control samples at the same pHs but without grape extract. In order to carry out the experiment, 10⁷ UFC/mL of *B. cereus* was inoculated to each solution of rice. The inoculated media were incubated under shaking at 10 °C, 20 °C and 30 °C for 168 h (10 °C) and 24 h (20 and 30 °C). For each media, samples were taken at different time intervals. Two series of each sample (A and B) were serially diluted in peptone water (0.1 mg/100 mL), plated in duplicate and incubated in nutritive broth agar (NBA) (Scharlab S.A., Barcelona, Spain) at 37 °C for 24 h. After incubation, colonies were counted (CFU/mL). Experimental results are shown as log10 of the survival fraction (log S) calculated as equation (1):

$$\log S = \log_{10} \left(\frac{N}{N_0} \right) \quad (1)$$

Where N is the bacterial concentration (CFU/mL) at time t (h) and N₀ initial bacterial concentration (CFU/mL) (t₀). Therefore, positive values indicate microbial growth while negative values show microbial inactivation compared to the initial inoculation value (N₀).

2.5. Mathematical modelling of *B. cereus* inactivation

For the concentrations of 5 and 10 mL/L grape extract, the survival curves were fitted with the model proposed by (Mafart, Couvert, Gailard, & Leguerinel, 2002) based on the Weibull distribution function. The Weibull model is a simple non-linear model, which has been successfully used to fit the nonlinear inactivation curves of many microorganisms under different conditions.

$$\log_{10} (N) = \log_{10} (N_0) - \left(\frac{t}{\delta} \right)^p \quad (2)$$

Where N is the population concentration at time t (CFU/mL); N₀ is the initial population concentration (CFU/mL), δ is the first 10-fold reduction (kinetic parameter) and p is the shape parameter. For the case $p < 1$, the equation allows the tailing portion fitting, inward concavity, of the inactivation curve, for $p = 1$ linear survival curves. For $p > 1$, the shoulder portion, outward convexity, can be predicted (Albert & Mafart, 2005; Marugan, van Grieken, Sordo, & Cruz, 2009). Values of p and δ were determined by using GlnaFIT add-in for Microsoft® Excel solver function (Version 1.7) (Geeraerd, Valramidis, & Van Impe, 2006).

A secondary model was built by means of a forward stepwise multiple regression, relating the logarithm of the scale factor of the Weibull model (δ) with temperature and pH. This is intended to limit the number of terms of the secondary model. Likewise, a global model is built in order to improve the value of the coefficients of the secondary model. A tertiary model is developed using the coefficients deduced by the global model in C# (Microsoft Visual Studio Community 2022 (64 bits) Version 17.0.2).

2.6. Statistical analysis

The fit of the models to the polynomials (secondary model) was performed with STATGRAPHICS Centurion XVIII (STATGRAPHICS, Warrenton, VA), including an ANOVA analysis to test significant differences in estimated model parameters depending on pH and temperature in the same grape extract concentration. The goodness of fit of the model was assessed using adjusted the regression coefficient (adjusted- R^2) and root mean square error (RMSE). To assess predictions made by the model, the Accuracy Factor parameter (A_f) (Ross, 1996) was used:

$$A_f = 10 \left(\sum \left(\frac{\log \left(\frac{\text{predicted}}{\text{observed}} \right)}{n} \right)^2 \right) \tag{3}$$

The predicted/observed ratio refers to the relationship between the survival fraction predicted by de model and the one obtained experimentally and n is the number of observations used to make the calculations.

3. Results and discussion

3.1. Effect of grape extract concentration, pH and incubation temperature on *B. cereus* growth

In the present work, the effect of different grape extract concentrations on vegetative cells of *B. cereus*, stored at three temperatures and three pH values, has been studied and compared with those obtained in controls. Vegetative cells were used because during storage spores germinate to vegetative cells, producing toxins during growth, so this is the bacterial stage that should be controlled.

Fig. 1 represents the behavior of *B. cereus* qualitatively and schematically under all study conditions. In the control (CA), *B. cereus* grew at all pH values at a temperature of 30 and 20 °C, while the temperature of 10 °C had a bacteriostatic effect with long lag phases, independently of pH, and only in the case of pH 6.5 some significant growth could be observed after 96 h of incubation time. Therefore, neither the pH nor the temperature above 10 °C proved sufficient barriers to prevent *B. cereus* growth. This finding justifies the need to introduce an antimicrobial to prevent microbial growth in the event of cold chain breach or temperature abuse.

By contrast, at grape concentrations of 5 and 10 mL/L grape extract acted as a bactericide for all conditions considered in the study,

independently of pH or temperature. From a food safety point of view, it is worth pointing out that 1 mL/L grape extract concentration is the scenario in which temperature and pH are more critical. This is because depending on temperature and pH, grape extract concentration will act as a bactericidal, bacteriostatic or have no effect on *B. cereus* growth.

Fig. 2 shows the behavior of *B. cereus* for the lowest grape extract concentration assayed: 1 mL/L. Positive log values (N/N_0) indicate microbial growth, whereas negative values indicate microbial inactivation compared to the initial inoculation (N_0). At 10 °C and all pH values, the final concentration of *B. cereus* (after 168 h of storage) was lower, or slightly lower (for pH 6.5) than the initial concentration of the microorganism, showing a bactericidal (pH 4.5 and 5.5) or bacteriostatic effect (for pH 6.5). For the same grape extract concentration but at a temperature of 20 °C, a lag phase was observed for all pH values studied. At pH 4.5 and 5.5 the grape extract acted as a bacteriostatic, since *B. cereus* did not grow, while at pH 6.5 the lag phase lasted approximately 6 h, which was longer than the result with acidic control, thus the addition of grape extract delayed microorganism growth. Finally, at 30 °C there was no lag phase for any of the pH values studied. Therefore, at 1 mL/L an increase in storage temperature implies a decrease in lag phase duration.

3.2. Effect of grape extract concentration, pH and incubation temperature on *B. cereus* inactivation

As mentioned above, under the pH and temperature conditions studied, grape extract exerted a bactericidal effect for concentrations equal to or greater than 5 mL/L. Those conditions were selected to quantitatively characterize the bactericidal effect of grape extract. The results showed that the grape extract can be bacteriostatic or bactericidal depending on concentration, storage temperature and pH of the medium. The antimicrobial capacity of grape extract has been attributed to its polyphenol content (Fontana, Antoniolli, & Bottini, 2013; Oliveira et al., 2013; Yadav et al., 2015). Various authors have reported Total Polyphenol Content in grape extract between 128.22 and 215.93 mg Gallic Acid Equivalents/Dry Weight (mg GAE/DW) (Ky & Teissedre, 2015); 36.6–88.7 mg GAE/DW (Ky, Lorrain, Kolbas, Crozier, & Teissedre, 2014); 212–279 mg GAE/DW (Brezoiu et al., 2019); 24.5–60.1 mg GAE/DW (Bosso et al., 2020); 33.2–37.5 mg GAE/DW (Guaita & Bosso, 2019). The quantity of polyphenols depends on the origin (grape seeds or skin), as well as the extraction solvent used for polyphenol quantification and the analysis method; therefore values could differ, accordingly (Balaban, Koc, Sar, & Akbas, 2021). Many classes of negatively

T [°]	pH	CA	1 mL/L grape	5 mL/L grape	10 mL/L grape
30 °C	4.5				
	5.5				
	6.5				
20 °C	4.5				
	5.5				
	6.5				
10 °C	4.5				
	5.5				
	6.5				

Fig. 1. Schematic representation of *B. cereus* growth/inhibition depending on temperature (10 °C, 20 °C and 30 °C), pH (4.5, 5.5 and 6.5) and grape extract concentration (0 mL/L (CA), 1 mL/L, 5 mL/L and 10 mL/L). Colors code: Red represents unsafe conditions, where the microorganism grows; yellow, bacteriostatic conditions; and green are microbiologically safe conditions, where the antimicrobial acts as a bactericide. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

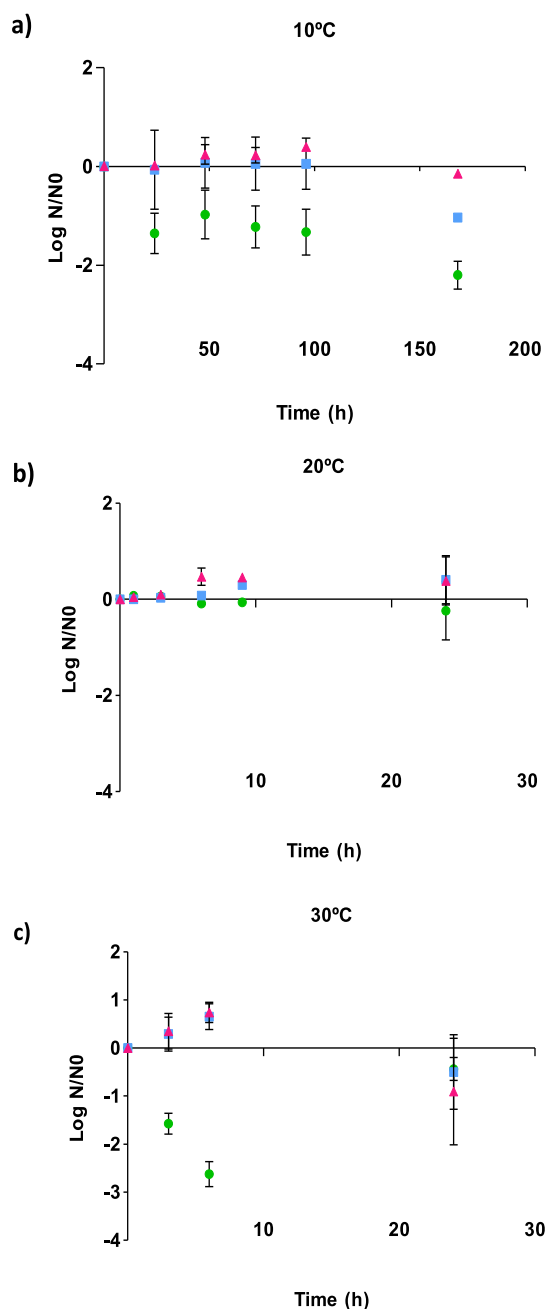


Fig. 2. Growth/inhibition of *B. cereus* with 1 mL/L grape extract concentration depending on pH (4.5 (●), 5.5 (■) and 6.5 (▲)) and temperature (10 °C (a), 20 °C (b) and 30 °C (c)).

charged polyphenols have been identified in grapes, such as phenolic acids (benzoic and hydroxycinnamic acids), stilbene derivatives (resveratrol), flavan-3-ols (catechin, epicatechin), flavanols (kaempferol, quercetin, myricetin), anthocyanins, etc. (Oliveira et al., 2013; Yadav et al., 2015). Gram-positive bacteria were more sensitive to grape extract than Gram-negative bacteria. These differences could be

explained by the presence of the lipopolysaccharide cell wall in Gram-negative bacteria, which can limit the penetration of polyphenols (Gerardi, Pinto, Baruzzi, & Giovino, 2021). This potential is greater in grape-skin extract because phenolic acids are present in undissociated forms (Yadav et al., 2015). In this frame, Katalinić et al. (2010) studied the antimicrobial activity of 14 phenolic extracts, both from white grape and red grape (seven and seven), utilizing a broth microdilution test with Gram-negative (*E. coli* O157: H7, *Salmonella Infantis*, *C. coli*) and Gram-positive (*S. aureus*, *B. cereus*) bacteria. Antimicrobial activity was confirmed against all gram-negative and gram-positive bacteria for all extracts. Differences in phenolic efficiency of white and red grape cultivars have been observed for different test organisms but no significant differences were found in the susceptibility of Gram-positive and Gram-negative bacteria (Katalinić et al., 2010). In the present work, results shown that grape extract exerted an antimicrobial activity against *B. cereus*, in accordance with Katalinić et al. (2010). Furthermore, as occurs in the present study with grape extract concentrations, in a study of the antimicrobial effect of pomegranate peel extracts at different dilution ratios against *B. subtilis*, *B. cereus*, and *E. faecalis* strains, Balaban et al. (2021) observed that the antibacterial activity of the extracts decreased when the extract dilution increased. In addition, in most cases, all the extracts and their dilutions were found to be more effective against *B. cereus* growth.

In this study, a 24 h period was considered a good control point for comparison between different pH values and temperatures since it is the time taken for control samples to reach the stationary phase at 20 °C. This temperature is important as it is considered as a cold chain breach. Fig. 3 shows that the bactericidal effect of the grape extract against *B. cereus* varied depending on the incubation temperature, the grape extract concentration and the pH of the medium. Considering the effect of pH, the greatest bactericidal effect occurred at pH 4.5, which differed with statistical significance to effects at pH 5.5 and 6.5 at 10 and 20 °C, and reaching up to 6 logarithmic reductions at 30 °C. Similar effect occurs in the study of Mau, Chen, and Hsieh (2001) where studied the antimicrobial effect of mixed different natural extracts (Chinese achieve + cinnamon + corni fructus (1:1:1)) in other food matrices (orange juice, pork and milk) against 15 microbiological strains, at different pH (original, 4.5, 5.5 and 6.5) and storage temperature (4 and 25 °C). Higher inhibitory effect was obtained at more acidic pH values and in their study it was independent by the storage temperature. Regarding temperature effects in the present work, the greatest inactivation occurred at 30 °C (Fig. 3), regardless of pH values and the grape extract concentration studied. This may be because these are optimal conditions for microorganism growth and thus defense mechanism against stress are not activated (Rodrigo, Ruiz, Barbosa-Canovas, Martinez, & Rodrigo, 2003). For pH 4.5, there were no significant differences between the inactivation levels reached at 10 or 20 °C, while for pH 5.5 and 6.5, (less stressful conditions) a greater inactivation was achieved at 20 °C. Likewise, Periago and Moezelaar (2001), observed that nisin on its own, showed a small effect at 8 °C against *B. cereus* strain, needing to be combined with lower pH, carvacrol, or both of them for maximum bactericidal action. The combined effect of nisin and carvacrol was significant at pH 6.3 and 7 and was significantly greater at 30 °C than at 8 °C. A similar effect can be observed in the present study since the combination of different factors enhances the effect of the natural antimicrobial.

Regarding the effect of grape extract concentration on inactivation, in general, there were no significant differences between the values reached with 5 or 10 mL/L. Only at a pH of 5.5 and a temperature of 30 °C did the grape concentration produce a significant increase in inactivation (Fig. 3).

3.3. Kinetics of *B. cereus* inactivation by grape extract

In order to complete the evaluation of the grape extract, quantify its natural antimicrobial activity in cooked rice products, and make

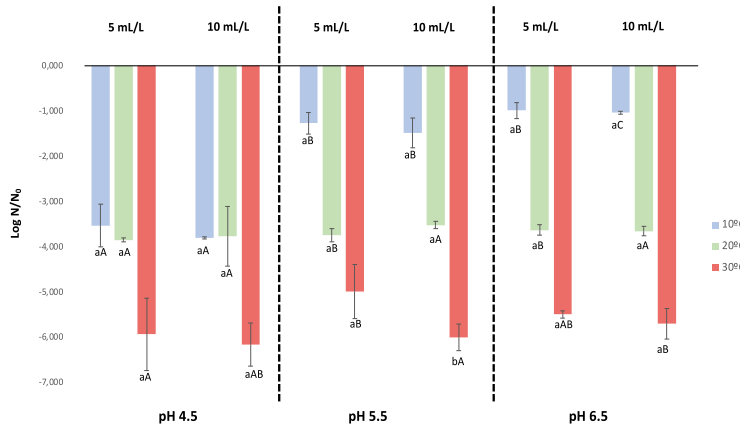


Fig. 3. *B. cereus* inactivation levels at 24 h storage time under exposure to concentrations of 5 and 10 mL/L of grape extract, at incubation temperatures of 10 °C, 20 °C and 30 °C, and pH 4.5, 5.5 and 6.5. Letters on the bars indicate significant differences ($P \leq 0.05$) between concentrations of 5 and 10 mL/L for the same pH and temperature (lowercase letters) and between pH (4.5–5.5–6.5) for the same temperature and concentration (capital letters).

predictions for different scenarios, the experimental results were adjusted for concentrations of 5 and 10 mL/L of grape extract (conditions in which the expected result was achieved) to the Weibull distribution function, using the GlnaFIT excel add-in (Version 1.7). Each individual experimental survival curve obtained was fitted separately and the estimated parameters (scale parameter δ and shape parameter p) were derived for each pH, temperature, and grape extract concentration (Table 1). The scale parameter δ is the first 10-fold reduction, related to the microorganism's resistance to the inactivation treatment, as the higher the treatment intensity, the lower the resistance. In general, as shown in Table 1, increasing extract concentrations and temperatures was associated with a δ parameter reduction although it was not always statistically significant. Additionally, there was a significant effect of pH (4.5 versus 6.5) for resistance to microorganism death for any concentration and temperature, except 20 °C – 10 mL/L.

A secondary model was developed by a forward stepwise multiple regression to define the dependence of $\log \delta$ with temperature and pH for fixed concentrations of grape extract (5 or 10 mL/L), equations 3 and 4. The p value is less than 0.05, thus there is a significant relationship between the dependent variable ($\log \delta$) and independent variables at the 95% of confidence level. Kwon, Rhee, and Yoon (2020) obtained shape p values lower than 1 at all temperatures studied, indicating rapid decrease of the microorganisms, which agrees with our results as shown in Table 1.

$$\begin{aligned} \log \delta &= -2.28 + 0.418 * pH \pm 0.0175 * pH * T + 0.204 * T - 0.0040 \\ &\quad * T^2 \\ &= 97\% \end{aligned} \quad (3)$$

$$\log \delta = -8.11 + 3.10 * pH - 0.248 * pH^2 - 0.0104 * pH * T \quad R^2 = 94\% \quad (4)$$

To improve the value of coefficients of the secondary model (polynomial model) obtained, a global model was built using all data points ($\log S$) and fitting them to equations 5 and 6, using one-step nonlinear regression (Statgraphics Centurion XVIII). The estimated coefficients and the confidence intervals obtained are shown in Table 2.

$$\log S = - \left[\left(\frac{t}{10^{(0.780 + 0.165 * pH - 0.00095 * pH * T - 0.0746 * T + 0.00077 * T^2)}} \right)^p \right] \quad (5)$$

$$\log S = - \left[\left(\frac{t}{10^{(-7.29 + 2.84 * pH - 0.231 * pH^2 - 0.0101 * pH * T)}} \right)^p \right] \quad (6)$$

The performance of the global inactivation model was validated by the Accuracy factor (A_f). A_f is a measure of how close the predicted value is to the experimental value (the further from 1, the more inaccurate) (Oscar, 2005). In the present study, A_f for 5 and 10 mL/L of grape concentration were 1.26 and 1.15, respectively, which indicates an error rate of 26% and 15% for these predictions. Therefore, the models developed can accurately predict *B. cereus* inactivation in a rice matrix, at different pH values and in a temperature range between 10 and 30 °C, which is considered critical from the food safety point of view. Similar to this study, Rodrigo, Barbosa-Canovas, Martinez, and Rodrigo (2003) analyzed *Escherichia coli* inactivation by pulsed electric fields and the experimental data were fitted to the Weibull distribution function using a one-step non linear regression and scale and shape parameters were deduced. A secondary model for the scale parameter was also built. To improve the precision of the coefficients of the secondary model, a global model using all experimental data was constructed similar to equations (5) and (6) of the present study, with very good results. A problem that could arise in applying the kinetic parameter obtained by a secondary model to estimate the residual microbiological load is related with the shape parameter in the primary models. Couvert, Gaillard, Savy, Mafart, and Leguerinel (2005) solved this problem using a single p value, estimated from the entire data set. Despite a slight loss of fit goodness, this modification improved the robustness of the model, a fact that also occurred in the present work, in which a single p value has been used within the data set of each concentration of grape extract. This method was also suggested by other authors (Corradini, Normand, & Peleg, 2008; Mafart et al., 2002).

Fig. 4 illustrates the response surface of the global model with the relationship between the influence of pH and incubation time on *B. cereus* inhibition for concentrations 5 mL/L (a) and 10 mL/L (b) of grape extract. The figure reveals that inactivation increased with treatment time and pH decreased. In addition, for the 10 mL/L concentration, a slight decrease in inactivation was observed for pH 5.5.

Considering the global model obtained (equations (5) and (6)), a tertiary model was constructed using Microsoft Visual Studio C++. This developed software can be implemented in industry to help in the decision making regarding pH and temperature at each concentration level with reference to the initial microbial load. It will allow an industrial business operator to adjust the storage conditions (time and temperature) to achieve the microbiological stability of the food based on its initial contamination. Besides, it can be chosen between two concentrations of grape extract, according to the food matrix properties. At the

Table 1
Weibull parameters (δ and p) and model fit (Adjusted R^2 and RMSE) for *B. cereus* inactivation under exposure to 5 and 10 mL/L grape extract concentration at 10 °C, 20 °C and 30 °C.

Grape extract concentration	Temperature	pH	Weibull parameters		Accuracy fit	
			δ	p	R^2_{adj}	RSME
5 mL/L	10 °C	4.5	3.07 ± 0.868^{aA}	0.34 ± 0.005	0.916	0.691
		5.5	5.68 ± 0.949^{bA}	0.42 ± 0.018	0.965	0.347
		6.5	6.95 ± 0.689^{bA}	0.40 ± 0.043	0.946	0.458
	20 °C	4.5	$*2.256 \pm 0.510^{aB}$	0.572 ± 0.052	0.971	0.294
		5.5	4.224 ± 0.691^{bB}	0.840 ± 0.059	0.972	0.308
		6.5	5.649 ± 1.150^{cA}	0.905 ± 0.135	0.993	0.134
	30 °C	4.5	0.655 ± 0.283^{aC}	0.500 ± 0.061	0.948	0.707
		5.5	0.499 ± 0.119^{abC}	0.439 ± 0.051	0.887	1.032
		6.5	0.296 ± 0.168^{bB}	0.283 ± 0.043	0.765	1.148
10 mL/L	10 °C	4.5	2.51 ± 0.422^{aA}	0.26 ± 0.006	0.837	0.985
		5.5	6.72 ± 0.368^{bA}	0.45 ± 0.012	0.95	0.457
		6.5	7.05 ± 0.200^{bA}	0.46 ± 0.006	0.929	0.524
	20 °C	4.5	$*0.526 \pm 0.129^{aB}$	0.359 ± 0.049	0.927	0.523
		5.5	3.209 ± 0.219^{bB}	0.583 ± 0.015	0.998	0.07
		6.5	2.160 ± 0.252^{cB}	0.559 ± 0.024	0.98	0.246
	30 °C	4.5	0.363 ± 0.282^{aB}	0.420 ± 0.054	0.977	0.46
		5.5	0.394 ± 0.059^{aC}	0.462 ± 0.025	0.983	0.442
		6.5	0.322 ± 0.134^{aC}	0.389 ± 0.042	0.969	0.62

Values followed by different letters within the same pH (small letters) and within the same temperature (capital letters) are significantly different ($p \leq 0.05$). Values with (*) indicate significant differences ($p \leq 0.05$) for the same pH and temperature between concentrations.

Table 2
Coefficient estimates obtained by fitting the global model (equations (5)–(5) mL/L grape extract concentration and equation 6–10 mL/L) to the total experimental data.

5 mL/L grape extract			10 mL/L grape extract		
Parameter	Estimated	Standard error	Parameter	Estimated	Standard error
a	0.7797	0.4372	a	−7.2862	1.4601
b	0.1648	0.0752	b	2.8449	0.5529
c	−0.0009	0.0032	c	−0.2308	0.0390
d	−0.0746	0.0310	d	−0.0101	0.0001
e	0.0007	0.0006			

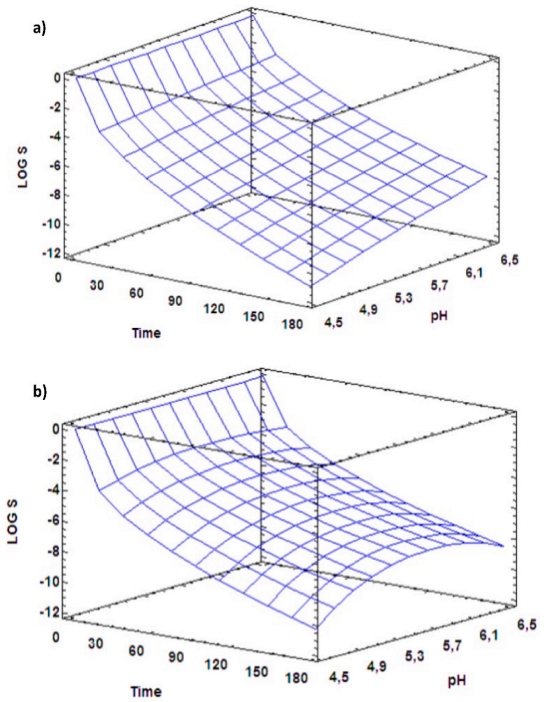


Fig. 4. Three-dimensional relationship between the influence of pH and incubation time at a constant temperature of 20 °C on *B. cereus* inactivation for a) 5 mL/L and b) at 10 mL/L concentration of grape extract.

same time, for specific pH and storage temperature conditions it can also be adjusted the maximum incubation time that the food matrix can be stored without causing any food safety issue (final microbial concentration remains below a specific value).he software will. Fig. 5 shows a screenshot of the software.

4. Conclusions

The present study proposes a quantitative approach to evaluate the antimicrobial potential of a natural coloring agent coming from an industrial by-product, namely grape extract, against *B. cereus*. Grape extract, at a concentration of 5 mL/L, has proven value as a way to control the bacterial load in a potential cold chain breach. In addition, this food ingredient, used in combination with other preservation treatments such as lowering the pH of the medium and the temperature, can attain maximum levels of *B. cereus* inactivation (6 log reductions) at grape extract concentrations of 5–10 mL/L. Furthermore, software developed from the global model can help in selecting the combination of grape extract concentration, incubation time and temperature, as well as the pH of the medium in order to attain the desired log reduction of *B. cereus* loads. Although the study indeed has some limitations that are indicated by the limits of the parameters that have been studied, temperatures between 10 and 30 °C, pH between 4.5 and 6.5 and grape extract concentrations 1, 5 and 10 mL/L.

The results reported in this manuscript provide evidence for the positive effects of adding food ingredients such as grape extract, which have bacteriostatic or bactericidal properties. Further investigation is needed to evaluate the sensory acceptability of such products to consumers.

Fig. 5. Screenshot of the software developed (tertiary model) to calculate the microorganism concentration after grape extract treatment combined with pH and temperature. Fig. 1.

CRediT authorship contribution statement

Eva Grau-Fuentes: Investigation, Writing – original draft. **María Úbeda-Manzanaro:** Investigation. **Antonio Martínez:** Data curation, Writing – review & editing. **Raquel Garzón:** Formal analysis. **Cristina M. Rosell:** Funding acquisition, Writing – review & editing. **Dolores Rodrigo:** Supervision, Conceptualization, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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References

- Albert, I., & Mafart, P. (2005). A modified Weibull model for bacterial inactivation. *International Journal of Food Microbiology*, 100(1–3), 197–211. <https://doi.org/10.1016/j.ijfoodmicro.2004.10.016>
- Arts, I., & Hollman, P. (1998). Optimization of a quantitative method for the determination of catechins in fruits and legumes. *Journal of Agricultural and Food Chemistry*, 46(12), 5156–5162. <https://doi.org/10.1021/jf9805092>
- Balaban, M., Koc, C., Sar, T., & Akbas, M. Y. (2021). Antibiofilm effects of pomegranate peel extracts against *B. cereus*, *B. subtilis*, and *E. faecalis*. *International Journal of Food Science and Technology*, 56(10), 4915–4924. <https://doi.org/10.1111/ijfs.15221>
- Bosso, A., Cassino, C., Motta, S., Panero, L., Tsolakis, C., & Gualita, M. (2020). Polyphenolic composition and in vitro antioxidant activity of red grape seeds as byproducts of short and medium-long fermentative macerations. *Foods*, 9(10). <https://doi.org/10.3390/foods9101451>
- Brezioiu, A., Matei, C., Deaconu, M., Stanciu, A., Trifan, A., Gaspar-Pintilieșcu, A., et al. (2019). Polyphenols extract from grape pomace. Characterization and valorisation through encapsulation into mesoporous silica-type matrices. *Food and Chemical Toxicology*, 133. <https://doi.org/10.1016/j.fct.2019.110787>
- Cheng, V. J., Bekhit, A. E. D. A., McConnell, M., Mros, S., & Zhao, J. (2012). Effect of extraction solvent, waste fraction and grape variety on the antimicrobial and antioxidant activities of extracts from wine residue from cool climate. *Food Chemistry*, 134(1), 474–482.
- Corradini, M., Normand, M., & Peleg, M. (2008). In G. GutierrezLopez, G. BarbosaCanovas, J. WeltiChanes, & E. ParadaArias (Eds.), *Nonlinear kinetics: Principles and potential food applications* (p. 71). https://doi.org/10.1007/978-0-387-75430-7_3
- Covert, O., Gaillard, S., Savy, N., Mafart, P., & Leguerinel, I. (2005). Survival curves of heated bacterial spores: Effect of environmental factors on Weibull parameters. *International Journal of Food Microbiology*, 101(1), 73–81. <https://doi.org/10.1016/j.ijfoodmicro.2004.10.048>
- Delbrassinne, L., Botteldoorn, N., Andjelkovic, M., Dierick, K., & Denayer, S. (2015). An emetic *Bacillus cereus* outbreak in a kindergarten: Detection and quantification of critical levels of cereulide toxin. *Foodborne Pathogens and Disease*, 12(1), 84–87. <https://doi.org/10.1089/fpd.2014.1788>
- Ferreira, A. S., Nunes, C., Castro, A., Ferreira, P., & Coimbra, M. A. (2014). Influence of grape pomace extract incorporation on chitosan films properties. *Carbohydrate Polymers*, 113, 490–499. <https://doi.org/10.1016/j.carbpol.2014.07.032>
- Fontana, A. R., Antonioli, A., & Bottini, R. (2013). Grape pomace as a sustainable source of bioactive compounds: Extraction, characterization, and biotechnological applications of phenolics. *Journal of Agricultural and Food Chemistry*, 61(38), 8987–9003. <https://doi.org/10.1021/jf402586f>
- Geraerd, A., Valdramidis, V., & Van Impe, J. (2006). GlnaFIT, a freeware tool to assess non-log-linear microbial survival curves (vol 102, pg 95, 2005). *International Journal of Food Microbiology*, 110(3). <https://doi.org/10.1016/j.ijfoodmicro.2006.04.002>, 297–297.
- Gerardi, C., Pinto, L., Baruzzi, F., & Giovino, G. (2021). Comparison of antibacterial and antioxidant properties of red (cv. Negramaro) and white (cv. Fiano) skin pomace extracts. *Molecules*, 26(19). <https://doi.org/10.3390/molecules26195918>
- Gilbert, R., Stringer, M., & Pearce, T. (1974). Survival and growth of *Bacillus cereus* in boiled and fried rice in relation to outbreaks of food poisoning. *Journal of Hygiene*, 73(3), 433–444. <https://doi.org/10.1017/S00222172400042790>
- Gokturk Baydar, N., Ozkan, G., & Yasar, S. (2007). Evaluation of the antiradical and antioxidant potential of grape extracts. *Food Control*, 18(9), 1131–1136. <https://doi.org/10.1016/j.foodcont.2006.06.011>
- Goncalves, L. A., Lorenzo, J. M., & Trindade, M. A. (2021). Fruit and agro-industrial waste extracts as potential antimicrobials in meat products: A brief review. *Foods*, 10(7). <https://doi.org/10.3390/foods10071469>
- Gualita, M., & Bosso, A. (2019). Polyphenolic characterization of grape skins and seeds of four Italian red cultivars at harvest and after fermentative maceration. *Foods*, 8(9). <https://doi.org/10.3390/foods8090395>
- Hendriksen, N. B., Hansen, B. M., & Johansen, J. E. (2006). Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in a sandy loam. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 89(2), 239–249. <https://doi.org/10.1007/s10482-005-9025-y>
- Juneja, V. K., Golden, C. E., Mishra, A., Harrison, M. A., Mohr, T., & Silverman, M. (2019). Predictive model for growth of *Bacillus cereus* during cooling of cooked rice. *International Journal of Food Microbiology*, 290, 49–58. <https://doi.org/10.1016/j.ijfoodmicro.2018.09.023>
- Katalinić, V., Možina, S. S., Skroza, D., Generalić, I., Abramović, H., Miloš, M., et al. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 *Vitis vinifera* varieties grown in Dalmatia (Croatia). *Food Chemistry*, 119(2), 715–723. <https://doi.org/10.1016/j.foodchem.2009.07.019>
- Kwon, M. J., Rhee, M. S., & Yoon, K. S. (2020). A risk assessment study of *Bacillus cereus* in packaged tofu at a retail market in Korea. *Food Science and Biotechnology*, 29(3), 339–350. <https://doi.org/10.1007/s10068-019-00670-0>
- Ky, I., Lorrain, B., Kolbas, N., Crozier, A., & Teissedre, P.-L. (2014). Wine by-products: Phenolic characterization and antioxidant activity evaluation of grapes and grape

- pomaces from six different French grape varieties. *Molecules*, 19(1), 482–506. <https://doi.org/10.3390/molecules19010482>
- Ky, I., & Teissedre, P.-L. (2015). Characterisation of mediterranean grape pomace seed and skin extracts: Polyphenolic content and antioxidant activity. *Molecules*, 20(2), 2190–2207. <https://doi.org/10.3390/molecules20022190>
- Lake, R., Hudson, A., & Cressey, P. (2004). Risk profile of *Bacillus* spp. In rice <https://www.mpi.govt.nz/dmsdocument/26138/direct>.
- Levy, J., Boyer, R. R., Neilson, A. P., O'Keefe, S. F., Chu, H. S. S., Williams, R. C., et al. (2017). Evaluation of peanut skin and grape seed extracts to inhibit growth of foodborne pathogens. *Food Science & Nutrition*, 5(6), 1130–1138. <https://doi.org/10.1002/fsn3.503>
- Little, C. L., Barnes, J., Mitchell, R. T., & Food Standards Agency (FSA) and Public Health Laboratory Service (PHLS). (2002). Microbiological quality of take-away cooked rice and chicken sandwiches: Effectiveness of food hygiene training of the management. *Communicable Disease and Public Health*, 5(4), 289–298.
- Mafart, P., Couvert, O., Gaillard, S., & Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: Application of the Weibull frequency distribution model. *International Journal of Food Microbiology*, 72(1–2), 107–113. [https://doi.org/10.1016/S0168-1605\(01\)00624-9](https://doi.org/10.1016/S0168-1605(01)00624-9)
- Marugan, J., van Grieken, R., Sordo, C., & Cruz, C. (2009). Kinetics of the photocatalytic disinfection of *Escherichia coli* suspensions (vol 82, pg 27, 2008). *Applied Catalysis B: Environmental*, 88(3–4), 582–583. <https://doi.org/10.1016/j.apcatb.2008.11.006>
- Mau, J.-L., Chen, C.-P., & Hsieh, P.-C. (2001). Antimicrobial effect of extracts from Chinese chive, cinnamon, and corni fructus. *Journal of Agricultural and Food Chemistry*, 49(1), 183–188. <https://doi.org/10.1021/jf000263c>
- Oki, T., Masuda, M., Kobayashi, M., Nishiba, Y., Furuta, S., Suda, I., et al. (2002). Polymeric procyanidins as radical-scavenging components in red-hulled rice. *Journal of Agricultural and Food Chemistry*, 50(26), 7524–7529. <https://doi.org/10.1021/jf025841z>
- Oliveira de, A. L., Brunini, M. A., Salandini, C. A. R., & Bazzo, F. R. (2003). Caracterização tecnológica de jabuticabas 'Sabará' provenientes de diferentes regiões de cultivo. *Revista Brasileira de Fruticultura*, 25(3), 397–400.
- Oliveira, D. A., Salvador, A. A., Smania, A., Smania, E. F. A., Maraschin, M., & Ferreira, S. R. S. (2013). Antimicrobial activity and composition profile of grape (*Vitis vinifera*) pomace extracts obtained by supercritical fluids. *Journal of Biotechnology*, 164(3), 423–432.
- Oscar, T. (2005). Development and validation of primary, secondary, and tertiary models for growth of *Salmonella typhimurium* on sterile chicken. *Journal of Food Protection*, 68(12), 2606–2613. <https://doi.org/10.4315/0362-028X-68.12.2606>
- Pao, S., Khalid, F., & Kalantari, A. (2006). Inhibiting the growth of *Bacillus cereus* in raw sprouts and cooked rice using red clover seeds. *Internet Journal Of Food Safety*, 8.
- Periago, P. M., & Moezelaar, R. (2001). Combined effect of nisin and carvacrol at different pH and temperature levels on the viability of different strains of *Bacillus cereus*. *International Journal of Food Microbiology*, 68(1), 141–148. [https://doi.org/10.1016/S0168-1605\(01\)00461-5](https://doi.org/10.1016/S0168-1605(01)00461-5)
- Prado Martin, J., Porto, E., Corrêa, C., Alencar, S., da Gloria, E., Simone, I., et al. (2012). *Antimicrobial potential and chemical composition of agro-industrial wastes*.
- Rodrigo, D., Barbosa-Canovas, G., Martinez, A., & Rodrigo, M. (2003). Weibull distribution function based on an empirical mathematical model for inactivation of *Escherichia coli* by pulsed electric fields. *Journal of Food Protection*, 66(6), 1007–1012. <https://doi.org/10.4315/0362-028X-66.6.1007>
- Rodrigo, D., Rosell, C. M., & Martinez, A. (2021). Risk of *Bacillus cereus* in relation to rice and derivatives. *Foods*, 10(2). <https://doi.org/10.3390/foods10020302>
- Rodrigo, D., Ruiz, P., Barbosa-Canovas, G., Martinez, A., & Rodrigo, M. (2003). Kinetic model for the inactivation of *Lactobacillus plantarum* by pulsed electric fields. *International Journal of Food Microbiology*, 81(3), 223–229. [https://doi.org/10.1016/S0168-1605\(02\)00247-7](https://doi.org/10.1016/S0168-1605(02)00247-7)
- Ross, T. (1996). Indices for performance evaluation of predictive models in food microbiology. *Journal of Applied Bacteriology*, 81(5), 501–508. <https://doi.org/10.1111/j.1365-2672.1996.tb03539.x>
- Sánchez, J., Correa, M., & Castañeda, L. (2016). *Bacillus cereus* an important pathogen the microbiological control of food. *Revista Facultad Nacional De Salud Pública*, 34(2), 230–242. <https://doi.org/10.17533/udea.rfnsp.v34n2a12>
- Shi, J., Yu, J., Pohorly, J. E., & Kakuda, Y. (2003). Polyphenolics in grape seeds—biochemistry and functionality. *Journal of Medicinal Food*, 6(4), 291–299. <https://doi.org/10.1089/109662003772519831>
- Wei, X., & Huang, X. (2019). 1—origin, taxonomy, and phylogenetics of rice. In J. Bao (Ed.), *Rice* (4th ed., pp. 1–29). AACC International Press. <https://doi.org/10.1016/B978-0-12-811508-4.00001-0>
- Yadav, D., Kumar, A., Kumar, P., & Mishra, D. (2015). Antimicrobial properties of black grape (*Vitis vinifera* L.) peel extracts against antibiotic-resistant pathogenic bacteria and toxin producing molds. *Indian Journal of Pharmacology*, 47(6), 663–667. <https://doi.org/10.4103/0253-7613.169591>
- Yu, J., Ahmedna, M., & Goktepe, P. (2005). Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chemistry*, 90(1–2), 199–206. <https://doi.org/10.1016/j.foodchem.2004.03.048>
- Yu, J., Ahmedna, M., & Goktepe, I. (2010). Potential of peanut skin phenolic extract as antioxidative and antibacterial agent in cooked and raw ground beef. *International Journal of Food Science and Technology*, 45(7), 1337–1344.
- Yu, S., Yu, P., Wang, J., Lie, C., Guo, H., Liu, C., et al. (2020). A study on prevalence and characterization of *Bacillus cereus* in ready-to-eat foods in China. *Frontiers in Microbiology*, 10. <https://doi.org/10.3389/fmicb.2019.03043>



Understanding the marketed plant-based beverages: From ingredients technological function to their nutritional value

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ABSTRACT

Despite the market expansion of plant-based beverages (PBB) there is limited information about what is driven the market and the nutritional status of the existing beverages. The objective was to identify the existing gaps in the PBB market with particular emphasis on their composition and nutritional value. PBB are mainly based on individual flour/powder and blends and sunflower oil is frequently present, besides gellan gum to stabilize the emulsion. In general, PBB are low calorie drinks (10–84 Kcal/ 100 mL), with low amount of saturated fat (0.1–1.90 g/ 100 mL) and fibers, and large variation in proteins (0.1–12 g/ 100 mL). The calcium fortification of PBB is comparable to the calcium levels of whole cow's milk, although the vitamin fortification is low. Analysis reveals that salt and oil reduction, as well as fibers enrichment might drive future innovations.

1. Introduction

Plant-based beverages (PBB) is an exponentially growing segment all over the world (Vaikma et al., 2021). The global PBB market was valued at approximately US\$9.8 billion in 2017 and it is expected to reach US \$19.7 billion by 2023, growing US\$10 million in 6 years (Statista, 2022). However, PBB cannot be considered new, as there is a long tradition in Eastern and Western cultures for this type of beverages that are commonly present in the market (Bernat et al., 2014). Among them, *horchata* (Spain, from tiger nuts); *Masvusu* (Zimbabwean, malted millet); *Boza* (Turkish, fermented millet); *Sikhye* also called *dansul* or *gamju* (South Korea, cooked rice and malt extract); and the most well-known is the *soy milk* from China (Arici & Daglioglu, 2002; Codina et al., 2016; Kim et al., 2012; Mäkinen et al., 2016; Sethi et al., 2016; Zvauya et al., 1997). Initially marketed as an alternative to cow's milk for people with lactose intolerance, the consumption of the soy beverage jumped 50 years ago from the local to global market (Mäkinen et al., 2016). Currently, followers of plant based diets and people concerned about the environment are increasing, and with them, the interest for these PBB (Penha et al., 2021; Sethi et al., 2016; Shori & Al Zahrani, 2022), turning this segment as a great opportunity to create PBB using different plant sources (Sethi et al., 2016).

Plants from different sources have been used for making PBB,

including the following groups: i) cereals (rice, oat, corn, spelt, rye, quinoa, kamut); ii) legumes (soy, peanut, lupin, cowpea); iii) nuts (almond, coconut, hazelnut, pistachio, walnut); iv) seeds (sesame, flax, sunflower, pumpkin, hemp); and v) pseudocereals (quinoa, teff, amaranth) (Munekata et al., 2020). Whatever the origin, common processing stages are: wet milling, filtration, sterilization, homogenization, aseptic packaging, and storage (Aydar et al., 2020).

Although dietary restrictions or nutrition are the driving forces of this market, the individuals that enjoy sensory experiences cannot be neglected. Recent scientific literature reports that consumer preferences are dominated by the texture, viscosity, and mouthfeel of PBB (Jaeger & Giacalone, 2021; Moss et al., 2022; Vaikma et al., 2021). To achieve these characteristics is important to form an oil-in-water emulsion, using oils and emulsifiers to modify their stability (McClements, 2015). Nevertheless, much more interest has been focused on the nutritional component and healthy benefits, specifically micronutrient fortification (calcium and vitamins), and bioactive compounds (Ahmad & Ahmed, 2019; Aydar et al., 2020; Silva et al., 2022), although observing high variability in their nutritional composition. In fact, Chalupa-Krebzdak et al. (2018) analyzed 18 beverages based on soy, coconut, rice, hemp, almond and cashew, observing that the protein content ranged between 0.42 and 3.16 g/ 100 mL, while fats varied from 0.83 g/100 mL to 6 g/ 100 mL. Likewise, Vanga & Raghavan, (2018) analyzed 23 beverages

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made from almond, soy, rice or coconut, obtaining similar variability, even in saturated fats (0–5 g/100 mL), sugar (0–15.6 g/100 mL), and salt (till 0.48 g/100 mL). Recently, with the increasing popularity of PBBs, [Craig & Fresán, \(2021\)](#) were able to analyze 148 beverages with new main ingredients (almond, cashew, coconut, hazelnut, macadamia, oat, pea, rice, soy and other -not specify), and authors confirmed previous findings, stating the high variability, and their high content in salt, sugar and saturated fat (composition range in g/100 mL: protein 0–8.9, saturated fats 0–4.8; sugar 0–14). However, the market is rapidly growing and with it, the search for alternative plant sources. Therefore, the purpose of the research was to conduct an extensive analysis (benchmarking) of the current market, to identify trends in the composition and nutritional value of PBB, which could help in designing PBB based on scientific knowledge.

2. Materials and methods

Data gathering was conducted from May 2022 to August 2022. PBB from the major groceries stores in Europe (El Corte Inglés, Consum, Mercadona, Herbolario Navarro, Eroski in Spain; Tesco in United Kingdom; Edeka in Germany; Auchan, Carrefour, Dia present across Europe) and North America (Walmart in USA and Canada) were included, having samples from private and commercial brands. Data collected included PBB as non-dairy alternatives that are obtained from an aqueous extract of the raw materials and not as a squeezing of the raw material itself, like occurs in juices from vegetables or fruits. Different data processing programs were used throughout the study: Microsoft® Excel 2019 (Version 16.67), Stata/SE (Version 17.0) (College Station, TX, US), and ggplot2 package (Version 3.3.2) (Wickham, 2016) for R (Version 4.0.3) (R core team, 2020). Labels were analyzed for the PBB composition and nutritional facts information. Commercial PBB made from nuts, cereals, seeds, pseudocereal and legumes were considered (including those with cocoa, coffee, fruit, and other flavors), those PBB with missing nutritional information were excluded. Ingredients were recorded and classified into raw materials, oils, gums, salt and minor ingredients (added as a fortifiers). A nutritional role was assigned to the minor ingredients without technological contribution. Afterward, a frequency analysis and an alluvial plot were carried out with the ingredients to identify processing trends. A box-and-whisker plot compared the macronutrients composition of these beverages, classifying them by raw material. Salt was plotted considering both raw material and its presence or absence in the list of ingredients. Principal component analysis (PCA) was carried out to discriminate beverages according their nutritional pattern and recipe (type of oil, raw material and hydrocolloid).

3. Results and discussion

3.1. Plant based beverages composition

Plant based beverages present in groceries stores were analyzed, which accounted up to 306. Labels were analyzed in relation to their nutritional composition and the ingredients listed in the package. On average the beverages analyzed contained eight ingredients in their formulation, including vitamins and minerals added with the purpose of fortification. Ingredients used for processing were water, raw plant-based powders, and in some cases, oil, gums, and sugar. Water was the main ingredient, which allows soaking and softening the rest of ingredients, making them ready for grinding. Water is also the basis for preparing the emulsion that will result in the beverage.

The second ingredient in PBB were the vegetable powders or flours, such as almonds, coconut, rice, oats, soybeans, etc. The type of consumers of this type of beverage has changed and with that their preferences. PBB were sought primarily as dairy substitutes produced from soybeans, oats or coconut ([Paul et al., 2020](#)). Currently, a wide variety of grains, and even blends, are used to prepare PBB. In fact, 84% of the

analyzed beverages contained a unique powder, 14% of them were formulated with two or more main ingredient, and 2% were produced from protein isolates (mainly pea protein). An alluvial plot was used to show the relationships and frequencies of the different ingredients that were categorized as raw materials, oils and hydrocolloids ([Fig. 1](#)). Principal raw materials or sources of powder for PBB included, oat (76), soy (68), almond (57), rice (28), coconut (15), plant-based (7) (no defined) or tiger nut (3). To a lesser extent cashew (2), millet (2), flax (2), hazelnut (1), spelt (1), birdseed (1) and hemp (1) were used. A group of beverages contained blends (42), that were a variety of binary combinations of powders, mainly rice, oat and soy combined with different types of nuts, coconut or seeds. The analysis revealed that the PBB market is not expanded based in a unique source of vegetables, but it is rapidly growing to offer a great variety of flavors and textures from a range of vegetable sources. Therefore, technological properties and nutritional composition will be rather different, which force to adapt PBB processing to the type of raw material used (nuts, pulses, cereals, etc.) ([Sethi et al., 2016](#)). Therefore, the concentration of the other ingredients was variable since they were accommodated to the need and application of the ingredients. The other important aspect is that this trend in raw materials confirms the interest in bioactive compounds enrichment in PBB, as has been reported for other foods ([Betoret and Rosell, 2020](#)).

Vegetable oils are a common ingredient in PBB, because some consumers of this type of beverage often seek dairy substitutes resembling the texture of the cow milk. The system oil-in-water emulsion allows imitating many sensory characteristics of cow's milk, such as appearance, viscosity, stability, mouthfeel and taste ([Aydar et al., 2020; Martínez-Padilla et al., 2020; McClements, 2015; Pineli et al., 2015](#)). For this reason, among the 306 beverages gathered for this study, 31.37% of them contained vegetable oil as an ingredient in their labelling ([Fig. 1](#)). Within the group of beverages containing vegetable oils as part of their ingredients, sunflower oil was the most frequent one (78.13%) and particularly in PBB containing either rice or oat, which suggested those beverages were marketed as alternatives to dairy milk for those with dietary restrictions. Other oils present in the PBB were rapeseed oil in 11.46% of PBB containing oils, followed by oil blends (rapeseed + palm; sunflower + shea; canola + sunflower) in 3.12% of them, or rapeseed, canola, and flax in a minority (2.08%). The presence or absence of vegetable oils in the formulation could be related to the type of the vegetable powder or raw material. In fact, beverages made with high-fat raw materials like almond, coconut, soy, cashew, hazelnut, or tiger nut, rarely (<3%) contained other oil source in their label. Conversely, the presence of vegetable oil was higher in beverages based on cereal grains (rice, millet, spelt) or seeds (flax, birdseed). Surprisingly, oat based PBB showed added vegetable oil, despite of being a cereal with high fat content. In the case of blends, they were majorly combining a cereal with a high-fat commodity that might provide oil functionality in the emulsion. Because of that, more than 80% of the beverages made with blended raw materials did not require in their formulation the presence of oil to obtain the silky appearance and palatability characteristics. This is an interesting technological and nutritional advance, that attends one of the most recent demands of consumers, such as avoiding the consumption of added fats ([Asioli et al., 2017](#)).

To obtain stable oil-in-water emulsion in the production of beverages, it is necessary the use of stabilizers that can reduce the droplet size of the oil used, thus keeping the aqueous and oil phases homogenized during the storage time ([Krempel et al., 2019](#)). [Fig. 1](#) shows that 184 PBB (60% of total PBB analyzed) do contain hydrocolloids in their formulation. Within those PBB, the most used hydrocolloid was the gellan gum (51.11%), followed by a mixture of different sources (41.85%), and then locust bean gum (2.71%), guar gum (1.63%), carrageenan (1.63%), sodium carboxymethyl cellulose (0.54%) and xanthan gum (0.54%). When a mixture of different hydrocolloids was incorporated, 74% of those PBB had gellan gum within the mixture or gellan gum + locust bean gum. Hydrocolloids functionality in this type of emulsions stabilize

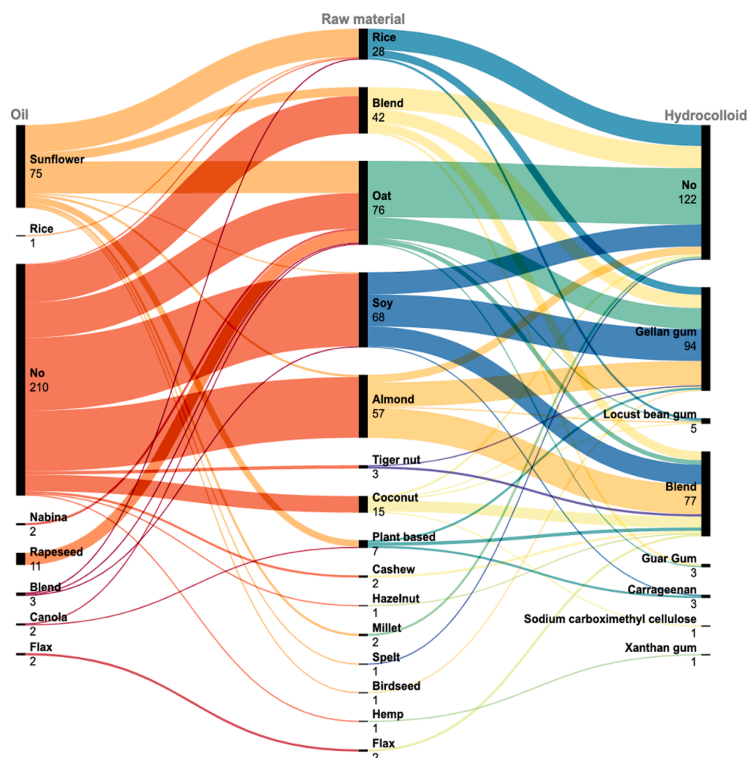


Fig. 1. Alluvial plot to the main ingredients of PBB. Relationship and frequencies between the type of oil, raw materials and hydrocolloids included in the labels of the marketed PBB.

the oil droplet, and reduce the surface tension of the emulsion interface, without increasing the viscosity during storage (Krempel et al., 2019). Fallour & Viscione, (2009) pointed out that the selection of the hydrocolloid depends on many factors like the amount of protein, the pH and the amount of oil and particles in suspension, and those authors identified gellan gum as efficient suspending agent to prepare structured liquids and low viscosity gels.

Despite the stabilizing functionality of the hydrocolloids, some PBB did not contain any, and those were mainly composed of cereals. Specifically, from the cereal-based beverages (107), only 34 contained hydrocolloids. Considering that PBB processing comprised a heat treatment and homogenization stage (Penha et al., 2021; Qamar et al., 2020), presumably the starch content of cereals provides the stabilizing functionality in the absence of hydrocolloids. In fact, Boulemkahel et al., (2021) reported that low pressure homogenization modifies the rice flour properties increasing the emulsifying properties.

3.2. Analysis of the nutritional facts of the currently marketed plant-based beverages

As the market of PBB is expanded, a special attention must be paid to their nutritional profile. Energy is one of the main indicators that consumers consider and affects the purchasing decision. Typically, health-conscious consumers relate high calories content to less healthy products, associated to obesity among other diseases (Charbonnier et al., 2015). As shown in Fig. 2 A, the energy content of the different PBB analyzed was rather variable. In general, cereal-based beverages provide higher energy than the PBB average, and the least energy dense PBB were those made from coconut, nuts and seeds. Hemp based beverages provides similar energy than millet-based ones, 55 kcal/100 mL and

58.33 kcal/100 mL, respectively; nonetheless, the presence in the market is still incipient. The tiger nut-based drinks were the most caloric ones, ranging from 52 to 84 kcal/ 100 mL, all of them over the average value, 41.73 kcal/ 100 mL. Tiger nut has been used from ancient times to obtain a sweet and cold drink consumed mainly during hot periods (Martín-Esparza & González-Martínez, 2016). Traditionally, this drink is prepared adding sugar, which results in a high caloric drink (Corrales et al., 2012). Vegetable drinks made of powders blends showed the highest deviation in the energy content (4.17–74 kcal/ 100 mL), related to the variability of raw materials. In contrast, rice-based beverages had the lowest variation of all plant-based drinks (45–57 kcal/ 100 mL). Chalupa-Krebdzak et al., (2018) also reported great variability in the energy content of marketed PBB from different brands, being energy dense PBB those with higher content in carbohydrates and sugars, which also raises the glycemic index of these beverages (Walther et al., 2022).

PBB have low total fat content, with an average value of 1.56 g/ 100 mL (Fig. 2B), which is far lower than the fat content of whole cow's milk (3.2 g/ 100 mL, being 1.86 g/ 100 mL coming from saturated fat) reported by the USDA (2022). Likewise, the fat content in the present analysis was lower than that described by Vanga & Raghavan, (2018), who reported values that were 40–50% higher for PBB made of almond, coconut, rice, or soybean. This divergence confirms that the PBB segment is shifting towards low fat content beverages. Nevertheless, the present analysis found some exceptions, like PBB made with almond, blended powders, flax, oat or soy, that exceeded the amount of fat in whole cow's milk, particularly those containing almond (4.7 g fat/ 100 mL). In general, results agree with those described by Chalupa-Krebdzak et al., (2018), except for soy beverages that presently contain low fat values (Fig. 2B), confirming the nutritional improvement of this type of beverages. Regarding the type of fats, saturated and unsaturated, the

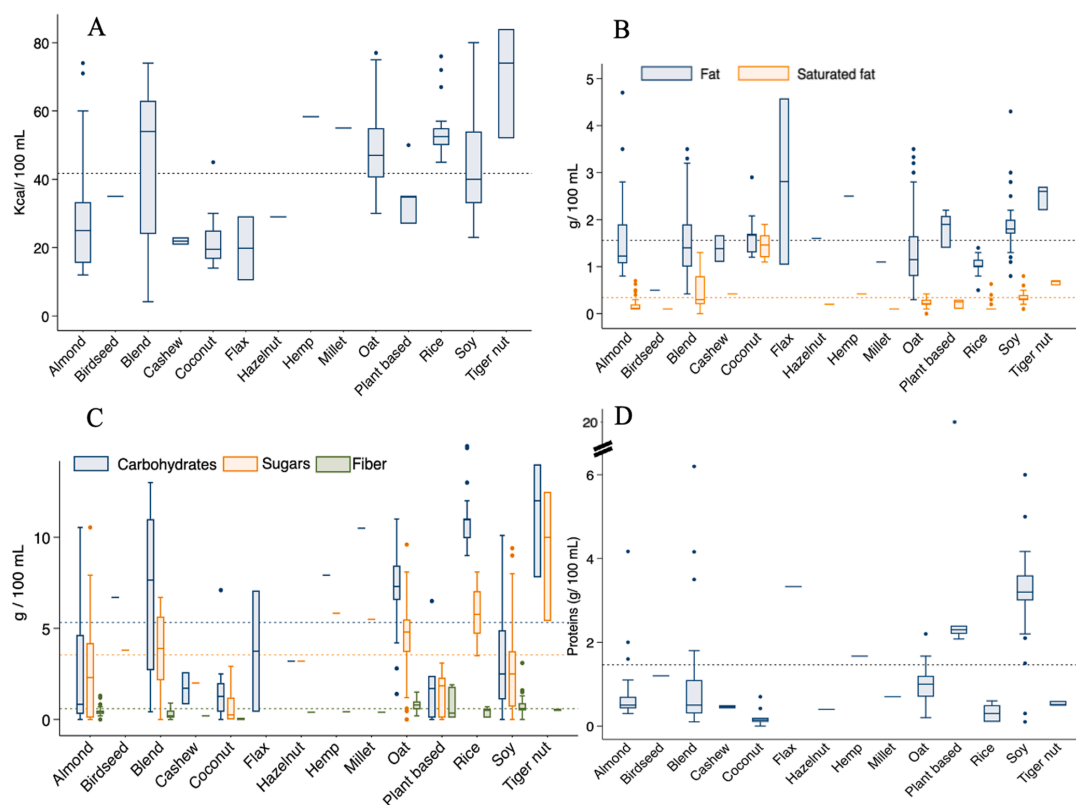


Fig. 2. Analysis of nutritional facts of pbb. a: energy (kcal); b: total fats (blue) and saturated fats (orange); c: carbohydrate (blue), sugars (orange) and fiber (green); d: proteins. horizontal dotted lines represent the mean of all values obtained for that compound. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

information was available in some PBB, which, although no general statement could be done, but the analysis revealed that PBB have low content of saturated fats, and more monounsaturated and polyunsaturated fats. Recently, [Craig & Fresán, \(2021\)](#) described that in soy, flax and hemp-based beverages, polyunsaturated fats were predominant, whereas rice and almond based beverages, contained mainly monounsaturated fats, which are associated with cardiovascular health because of their action controlling the levels of glucose in blood and reducing total cholesterol ([Jenkins et al., 2006](#); [Sabaté et al., 2003](#)). The average content of saturated fat in those beverages was 0.34 g/100 mL; only coconut-based beverages showed a completely different profile, with a much higher saturated fat content (1.90 g/100 mL). Although saturated fat contents are related to unhealthy diet, because they raise LDL cholesterol levels, coconut oil increases HDL cholesterol, being able to compensate for its high saturated fat composition ([Eyes et al., 2016](#)). It must also consider that coconut oil contains lauric acid, as saturated fat, which has been associated to brain development and boosting the immune system ([Sethi et al., 2016](#)). As expected, PBB do not declare cholesterol content, whereas whole cow's milk contains 12 mg/ serving ([USDA, 2022](#)). Therefore, except for coconut-based beverages, analyzed PBB show healthier lipid profile than cow's milk for people suffering diseases related to high blood lipid levels.

Carbohydrates and sugars content in the marketed PBB were different, which was largely dependent on the raw material of the beverages ([Fig. 2C](#)). Drinks based on almond, cashew, coconut, flax, undefined plants, or soy, had lower carbohydrate content than the average (5.33 g/100 mL). Tiger nut-based drinks had the highest carbohydrate

content (11.27 g/100 mL), despite the low carbohydrate content reported for tiger nuts ([Sánchez et al., 2016](#)), but they usually content high amounts of added sugar (70% of carbohydrates were sugars). Rice based beverages, and in general those containing cereals (millet and oat) showed high carbohydrate content, which was expected considering their starch content ([Sultana et al., 2022](#)). Therefore, in general, PBB have higher amount of carbohydrates than whole cow's milk (4.67 g/100 mL) ([USDA, 2022](#)).

The content of sugars ([Fig. 2C](#)) in the analyzed beverages followed the same trend described for total carbohydrates, although with lower levels. However, it must be stressed that the great difference between total carbohydrates and sugars content in the cereal-based drinks occurred in those containing coconut or blends. The level of carbohydrate and sugars in beverages made from nuts (almonds, cashews, hazelnuts), seeds (flax, birdseed, hemp) and legumes (soy) was rather similar, and most of the carbohydrates content were due to the sugar content. The highest average was found in the tiger nut-based beverages (9.30 g/100 mL), as mentioned before, and the lowest in the coconut-based beverages (0.82 g/100 mL). Only 78 from 306 beverages had sugar in their list of ingredients, so most of the sugars content was from the raw materials used in the formulation of the beverages.

Although the fiber content ([Fig. 2C](#)) was not always declared, these beverages did not have a notable fiber content (mean value was 0.59 g/100 mL), with oat, soy, and plant blend based showing 0.79, 0.71 and 0.77 g/100 mL, respectively. The fiber content was mainly determined by the type of raw material, but in some cases the incorporation of fiber was remarkable, i.e., a beverage from the group of plant-based

containing chicory root reached 1.9 g/ 100 mL of fiber. In addition, it should be considered that hydrocolloids presence could provide an extra contribution of fiber, although no direct relationship was found between the beverages with added hydrocolloids and fiber content. The production of fiber-rich foods have been challenging for research and industry, especially in beverage segment, where consumer perception is largely affected by the appearance and texture of the liquid (Moss et al., 2022).

Just like the rest of macronutrients, the market study revealed large differences in the protein content (Fig. 2D). The highest content was observed in beverage group made with undefined plants, particularly a PBB with 20 g protein/ 100 mL, which was enriched with 2.5% pea protein isolate (Fig. 2D). Apart from those, the PBB with higher amount of proteins were those containing flax (3.3 g/ 100 mL) or soy (3.15 g/ 100 mL). The rest of beverages had lower protein content than the overall average (1.46 g/ 100 mL). Protein content of these PBB was below the protein contribution of whole cow's milk (3.18 g/ 100 mL) (USDA, 2022). An alternative to increase the amount of proteins is enhancing the level of seeds or blending different protein rich raw materials, like tiger-nut (Sethi et al., 2016), which could also improve the quality of the amino acids profile (Qamar et al., 2020).

The salt content was not correlated with its presence or absence as ingredient in the manufacturer's label (Fig. 3). From the 306 PBB, 263 of them declared salt as an ingredient (blue shadowed in Fig. 3) and 37 did not (pink shadowed in Fig. 3), whereas 6 of them did not mention the salt neither as ingredient nor in the nutritional facts. Overall, the average salt content was 0.1 g/ 100 mL. In general, salt content of the beverages without salt added was lower than the content in PBB containing salt as ingredient. Undefined plant-based beverage was the group with the highest salt content in both PBB clusters, with or without salt as ingredient. Nevertheless, within non-added salt PBB it should be stressed that the group of plant-based beverages displayed the highest salt content (mean of 0.16 g/ 100 mL). Salt is commonly used to enhance flavor, presumably salt is masking off-flavors in the group of undefined plants, being added with that purpose or through plants that have high salt content. Within the group of PBB that declared salt as an ingredient, two outliers were detected with 0.33 g salt/100 mL, which contained oat or rice-coconut blend. Health worldwide authorities advise low salt intake (less than 5 g per day in adults) to reduce blood pressure and the risk of cardiovascular disease, stroke and myocardial infarction (World Health Organization, 2023). According to the European Commission in its "REGULATION (EC) No 1924/2006 on nutrition and health claims"

PBB could be considered low salt when its content is less than 0.3 g/ 100 mL, or very low salt when its content is less than 0.1 g/ 100 mL.

Dairy substitute beverages have been used as vehicles to introduce a greater number of vitamins, minerals and even probiotics to achieve balanced diets. In the analyzed beverages, more than 50% of them were fortified with either vitamins, a calcium salt or a combination of several salts and vitamins. Calcium is an essential nutrient necessary for human growth and development and it is a limiting nutrient in cereals such as rice and oats (Sethi et al., 2016). From those, 159 were fortified with calcium but only 136 beverages declared the calcium content in their nutritional information, with an average of 122.32 mg calcium/ 100 mL, comparable to 123 g of calcium/ 100 mL of whole cow's milk (USDA, 2022), although it is still questioned if they have similar bioavailability (Chalupa-Krebzdak et al., 2018). Calcium carbonate was the most used salt (46%), followed by tricalcium phosphate (37%) and a mixture of both (7%). Nowadays, calcium carbonate is the most common calcium salt and its absorption is similar to calcium from bovine milk (Kruger et al., 2003; Zhao et al., 2005). Other salts such as tricalcium citrate, dicalcium phosphate or calcium salts of orthophosphoric acid were also used, in lesser extent (10% among the three salts). Regarding vitamins, the number of fortified beverages was lower (141) than that of minerals, and vitamin D was the most frequent added vitamin (in 136 PBB). This vitamin D has been targeted because it is one of the most deficient vitamins worldwide, related to many diseases such as cardiovascular diseases, hypertension or problems in calcium homeostasis (Müller et al., 2011). Other vitamins widely used for the fortification of PBB are vitamins B12, B2, A and E, always in combination with vitamin D. Only 6 beverages were fortified with vitamin B6, B9, B3 or C. However, there is very limited information about the bioavailability of vitamins and minerals after processing, which is particularly important considering the presence of anti-nutrients in the raw materials (Aydar et al., 2020). In addition, it is important to consider that some of the raw ingredients currently present in the PBB are increasing their vitamins composition. For instance, almonds are an excellent source of vitamin E, which cannot be synthesized by the body and its antioxidant action is required for protecting against free-radical reactions (Sethi et al., 2016).

3.3. Overall analysis of marketed plant-based beverages using a principal component analysis

A principal component analysis was built up to identify potential PBB clusters based on the nutritional composition, type of raw material, oil and hydrocolloid, and the presence or absence of salt. Those variables could explain 46.6% of the differences among the commercial beverages analyzed (Fig. 4). Principal component 1 (PC1) explained 27.9% of the variability, based on ingredients, specifically hydrocolloids and salt (in the negative x-axis) and oil type (in the positive x-axis). Conversely, raw materials and macronutrients composition allowed differentiate PBB along principal component 2 (PC2), with the raw material in the negative y-axis and fiber, proteins and fat content in the positive y-axis. Soybeans, almonds, oats, and blends based PBB were scattered in all quartiles, indicating their large variability. Soy and almond based beverages were characterized by the presence of hydrocolloids and salt. The other extensive cluster included the PBB made from powder or raw material blends, that were extended along x-axis, confirming the variability in their formulation and they contained higher levels of saturated fats. Tiger-nut-based beverages were grouped because of their high sugar and carbohydrates content. As it was explained above, this type of beverages are traditionally sweet drinks with high sugar content (Sánchez et al., 2016). Rice drinks clustered on the x-axis and was associated with a high caloric content and a high presence of carbohydrates, as shown during the study. It is noteworthy to mention that oat-based beverages were grouped because their high fat content, that was associated to the addition of oil, which resulted in energy dense drinks. Coconut drinks formed a well-defined cluster distinguished by their content in saturated fat, mainly due to the lipid profile of coconut (Sethi

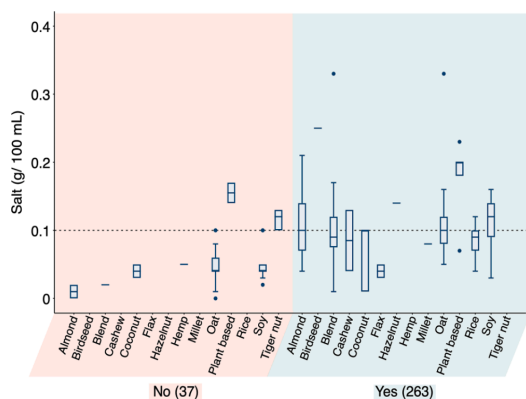


Fig. 3. Analysis of salt content in the plant-based beverages (PBB) gathered from the market. PBB containing salt as an ingredient (blue bottom) or without salt (red bottom) of all beverages analyzed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

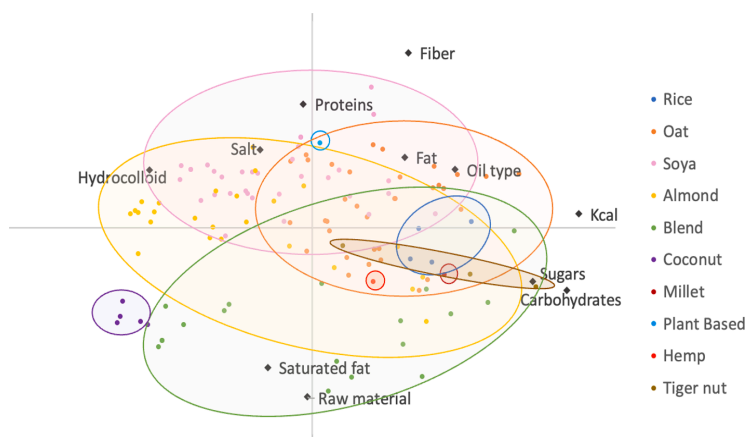


Fig. 4. Principal component analysis of PBB that included all the variables analyzed (ingredients in the formulation and nutritional facts). Identified clusters based on their raw materials, appeared circled in different colors.

et al., 2016).

4. Conclusions

Plant based beverages existing in the market are rapidly changing, incorporating a variety of vegetable powders or raw materials, that directly affect their final composition. The raw materials used for making PBB reflects the trend towards healthy foods and beverages. Specifically, beverages were mainly based on cereal grains (rice, millet, oats and spelt), seeds (birdseed and flax) and nuts or legumes. Regarding their nutritional composition, it was largely dependent on the raw material. Despite the improvement in the type of ingredients used in their formulations, the salt and oil reduction in PBBs, as well as fiber enrichment, would be advisable. It should be noted that more than 50% of the beverages analyzed were fortified with vitamins and/or minerals, which is desirable for people concerned about a balanced diet and health. Regarding nutritional differences either within the same raw materials or commercial brands, it would be important to develop a public education initiative on labelling reading.

5. Ethics statement

Authors declare that there are not ethical issues associated with the research carried out in the manuscript entitled Understanding the functionality of the marketed plant-based beverages.

CRediT authorship contribution statement

Eva Grau-Fuentes: Formal analysis, Investigation, Writing – original draft. **Dolores Rodrigo:** Supervision, Writing – review & editing. **Raquel Garzón:** Conceptualization, Supervision, Formal analysis, Writing – review & editing. **Cristina M. Rosell:** Funding acquisition, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Reference

- Ahmad, A., & Ahmed, Z. (2019). 3—Fortification in Beverages. In A. M. Grumezescu, & A. M. Holban (Eds.), *Production and Management of Beverages* (pp. 85–122). Woodhead Publishing. <https://doi.org/10.1016/B978-0-12-815260-7.00003-1>.
- Arici, M., & Daglioglu, O. (2002). Boza: A lactic acid fermented cereal beverage as a traditional Turkish food. *Food Reviews International*, 18(1), 39–48. <https://doi.org/10.1081/FRI-120003416>
- Asioli, D., Aschemann-Witzel, J., Caputo, V., Vecchio, R., Annunziata, A., Næs, T., & Varela, P. (2017). Making sense of the “clean label” trends: A review of consumer food choice behavior and discussion of industry implications. *Food Research International*, 99, 58–71. <https://doi.org/10.1016/j.foodres.2017.07.022>
- Aydar, E. F., Tutuncu, S., & Ozelcik, B. (2020). Plant-based milk substitutes: Bioactive compounds, conventional and novel processes, bioavailability studies, and health effects. *Journal of Functional Foods*, 70, Article 103975. <https://doi.org/10.1016/j.jff.2020.103975>
- Bernat, N., Cháfer, M., Chiralt, A., & González-Martínez, C. (2014). Vegetable milks and their fermented derivative products. *International Journal of Food Studies*, 3, 93–124. <https://doi.org/10.7455/ijfs/3.1.2014.a9>
- Betoret, E., & Rosell, C. M. (2020). Enrichment of bread with fruits and vegetables: Trends and strategies to increase functionality. *Cereal Chemistry*, 97(1), 9–19.
- Boulemlakel, S., Betoret, E., Benatallah, L., & Rosell, C. M. (2021). Effect of low pressures homogenization on the physico-chemical and functional properties of rice flour. *Food Hydrocolloids*, 112, Article 106373. <https://doi.org/10.1016/j.foodhyd.2020.106373>
- Chalupa-Krebsak, S., Long, C. J., & Bohrer, B. M. (2018). Nutrient density and nutritional value of milk and plant-based milk alternatives. *International Dairy Journal*, 87, 84–92. <https://doi.org/10.1016/j.idairy.2018.07.018>
- Charbonnier, L., van Meer, F., van der Laan, L. N., Viergever, M. A., & Smeets, P. A. M. (2015). Standardized food images: A photographing protocol and image database. *Appetite*, 96, 166–173. Scopus. <https://doi.org/10.1016/j.appet.2015.08.041>
- Codina, I., Trujillo, A. J., & Ferragut, V. (2016). Horchata. In K. Kristbergsson, & J. Oliveira (Eds.), *Traditional Foods: General and Consumer Aspects* (pp. 345–356). US: Springer. https://doi.org/10.1007/978-1-4899-7648-2_28
- Corrales, M., de Souza, P. M., Stahl, M. R., & Fernández, A. (2012). Effects of the decontamination of a fresh tiger nuts’ milk beverage (horchata) with short wave ultraviolet treatments (UV-C) on quality attributes. *Innovative Food Science & Emerging Technologies*, 13, 163–168. <https://doi.org/10.1016/j.ifset.2011.07.015>
- Craig, W. J., & Fresan, U. (2021). International analysis of the nutritional content and a review of health benefits of non-dairy plant-based beverages. *Nutrients*, 13(3). <https://doi.org/10.3390/nu13030842>
- Eyres, L., Eyres, M. F., Chisholm, A., & Brown, R. C. (2016). Coconut oil consumption and cardiovascular risk factors in humans. *Nutrition Reviews*, 74(4), 267–280. <https://doi.org/10.1093/nutrit/nuw002>
- Fallourd, M. J., & Viscione, L. (2009). 1—Ingredient selection for stabilisation and texture optimisation of functional beverages and the inclusion of dietary fibre. In P. Paquin (Ed.), *Functional and Speciality Beverage Technology* (pp. 3–38). Woodhead Publishing. <https://doi.org/10.1533/9781845695569.1.3>.

- Jaeger, S. R., & Giacalone, D. (2021). Barriers to consumption of plant-based beverages: A comparison of product users and non-users on emotional, conceptual, situational, conative and psychographic variables. *Food Research International*, 144, Article 110363. <https://doi.org/10.1016/j.foodres.2021.110363>
- Jenkins, D. J. A., Kendall, C. W. C., Josse, A. R., Salvatore, S., Brighenti, F., Augustin, L. S. A., ... Rao, A. V. (2006). Almonds decrease postprandial glycemia, insulinemia, and oxidative damage in healthy individuals. *The Journal of Nutrition*, 136(12), 2987–2992. <https://doi.org/10.1093/jn/136.12.2987>
- Kim, H., Kim, H., Bang, J., Kim, Y., Beuchat, L. R., & Ryu, J.-H. (2012). Reduction of *Bacillus cereus* spores in sikhye, a traditional Korean rice beverage, by modified tyndallization processes with and without carbon dioxide injection. *Letters in Applied Microbiology*, 55(3), 218–223. <https://doi.org/10.1111/j.1472-765X.2012.03278.x>
- Krempel, M., Griffin, K., & Khouryeh, H. (2019). 13—Hydrocolloids as emulsifiers and stabilizers in beverage preservation. In A. M. Grumezescu, & A. M. Holban (Eds.), *Preservatives and Preservation Approaches in Beverages* (pp. 427–465). Academic Press. <https://doi.org/10.1016/B978-0-12-816685-7.00013-6>
- Kruger, M. C., Gallaher, B. W., & Schollum, L. M. (2003). Bioavailability of calcium is equivalent from milk fortified with either calcium carbonate or milk calcium in growing male rats. *Nutrition Research*, 23(9), 1229–1237. [https://doi.org/10.1016/S0271-5317\(03\)00100-3](https://doi.org/10.1016/S0271-5317(03)00100-3)
- Mäkinen, O. E., Wanhälina, V., Zannini, E., & Arendt, E. K. (2016). Foods for special dietary needs: Non-dairy plant-based milk substitutes and fermented dairy-type products. *Critical Reviews in Food Science and Nutrition*, 56(3), 339–349. <https://doi.org/10.1080/10408398.2012.761950>
- Martín-Esparza, E., & González-Martínez, C. (2016). Horchata de chufa: A traditional Spanish beverage with exceptional organoleptic, nutritive, and functional attributes. In K. Kristbergsson, & S. Ödes (Eds.), *Functional Properties of Traditional Foods* (pp. 371–375). US: Springer. https://doi.org/10.1007/978-1-4899-7662-8_26
- Martínez-Padilla, E., Li, K., Blok Frandsen, H., Skejovic Joehne, M., Vargas-Bello-Pérez, E., & Lykke Petersen, I. (2020). *In vitro* protein digestibility and fatty acid profile of commercial plant-based milk alternatives. *Foods*, 9(12). <https://doi.org/10.3390/foods9121784>
- McClements, D. J. (2015). *Food Emulsions: Principles, Practices, and Techniques* (3rd ed.). CRC press. <https://doi.org/10.1201/b18868>
- Moss, R., Barker, S., Falkeisen, A., Gorman, M., Knowles, S., & McSweeney, M. B. (2022). An investigation into consumer perception and attitudes towards plant-based alternatives to milk. *Food Research International*, 159, Article 111648. <https://doi.org/10.1016/j.foodres.2022.111648>
- Müller, D. N., Kleiweitfeld, M., & Kvakan, H. (2011). Vitamin D review. *Journal of the Renin-Angiotensin-Aldosterone System*, 12(2), 125–128. <https://doi.org/10.1177/147032031140924>
- Munekata, P. E. S., Domínguez, R., Budaraju, S., Roselló-Soto, E., Barba, F. J., Mallikarjunan, K., ... Lorenzo, J. M. (2020). Effect of innovative food processing technologies on the physicochemical and nutritional properties and quality of non-dairy plant-based beverages. *Foods*, 9(3). <https://doi.org/10.3390/foods9030288>
- Paul, A. A., Kumar, S., Kumar, V., & Sharma, R. (2020). Milk Analog: Plant based alternatives to conventional milk, production, potential and health concerns. *Critical Reviews in Food Science and Nutrition*, 60(18), 3005–3023. <https://doi.org/10.1080/10408398.2019.1674243>
- Penha, C. B., Santos, V. D. P., Speranza, P., & Kurozawa, L. E. (2021). Plant-based beverages: Ecofriendly technologies in the production process. *Innovative Food Science & Emerging Technologies*, 72, Article 102760. <https://doi.org/10.1016/j.ifset.2021.102760>
- Pineli, L. de L. de O., Botelho, R. B. A., Zandonadi, R. P., Solorzano, J. L., de Oliveira, G. T., Reis, C. E. G., & Teixeira, D. da S. (2015). Low glycemic index and increased protein content in a novel quinoa milk. *LWT - Food Science and Technology*, 63(2), 1261–1267. <https://doi.org/10.1016/j.lwt.2015.03.094>
- Qamar, S., Manrique, Y. J., Parekh, H., & Falconer, J. R. (2020). Nuts, cereals, seeds and legumes proteins derived emulsifiers as a source of plant protein beverages: A review. *Critical Reviews in Food Science and Nutrition*, 60(16), 2742–2762. <https://doi.org/10.1080/10408398.2019.1657062>
- Sabaté, J., Haddad, E., Tanzman, J. S., Jambazian, P., & Rajaram, S. (2003). Serum lipid response to the graduated enrichment of a Step I diet with almonds: A randomized feeding trial. *The American Journal of Clinical Nutrition*, 77(6), 1379–1384. <https://doi.org/10.1093/ajcn/77.6.1379>
- J. Sánchez M. Correa L. Castañeda Revista Facultad Nacional de Salud Pública, 34(2), 230–242 2016 <https://doi.org/10.17533/udea.rfnsp.v34n2a12>
- Sethi, S., Tyagi, S., & Anurag, R. (2016). Plant-based milk alternatives an emerging segment of functional beverages: A review. *Journal of Food Science and Technology-Mysore*, 53(9), 3408–3423. <https://doi.org/10.1007/s13197-016-2328-3>
- Shori, A. B., & Al Zahran, A. J. (2022). Non-dairy plant-based milk products as alternatives to conventional dairy products for delivering probiotics. *Food Science and Technology*, 42, e101321.
- Silva, J. G. S., Rebellato, A. P., de Abreu, J. S., Greiner, R., & Pallone, J. A. L. (2022). Impact of the fortification of a rice beverage with different calcium and iron sources on calcium and iron bioaccessibility. *Food Research International*, 161, Article 111830. <https://doi.org/10.1016/j.foodres.2022.111830>
- Statista. (2022). <https://www.statista.com/statistics/948450/plant-based-beverages-market-value-worldwide/>
- Sultana, S., Faruque, M., & Islam, M. R. (2022). Rice grain quality parameters and determination tools: A review on the current developments and future prospects. *International Journal of Food Properties*, 25(1), 1063–1078. <https://doi.org/10.1080/10942912.2022.2071295>
- USDA. (2022). <https://fdc.nal.usda.gov/fdc-app.html#/food-search>
- Vaikka, H., Kaleda, A., Rosend, J., & Rosenvald, S. (2021). Market mapping of plant-based milk alternatives by using sensory (RATA) and GC analysis. *Future Foods*, 4, Article 100049. <https://doi.org/10.1016/j.fufo.2021.100049>
- Vanga, S. K., & Raghavan, V. (2018). How well do plant based alternatives fare nutritionally compared to cow's milk? *Journal of Food Science and Technology*, 55(1), 10–20. <https://doi.org/10.1007/s13197-017-2915-y>
- World Health Organization. (2023). <https://www.who.int/news-room/fact-sheets/detail/salt-reduction>
- Walther, B., Guggisberg, D., Badertscher, R., Egger, L., Portmann, R., Dubois, S., Haldimann, M., Kop-Bolanz, K., Rhy, P., Zoller, O., Veraguth, R., & Rezzi, S. (2022). Comparison of nutritional composition between plant-based drinks and cow's milk. *Frontiers in Nutrition*, 9, 2645. <https://doi.org/10.3389/fnut.2022.988707>
- Zhao, Y., Martin, B. R., & Weaver, C. M. (2005). Calcium bioavailability of calcium carbonate fortified soymilk is equivalent to cow's milk in young women. *The Journal of Nutrition*, 135(10), 2379–2382. <https://doi.org/10.1093/jn/135.10.2379>
- Zvaunya, R., Myguchi, T., & Parawira, W. (1997). Microbial and biochemical changes occurring during production of masvuvu and mangisi, traditional Zimbabwean beverages. *Plant Foods for Human Nutrition*, 51(1), 43–51. <https://doi.org/10.1023/A:1007972428849>



Unlocking hidden potential of rice bran: Enzymatic treatment for enhancing techno-functional properties

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ABSTRACT

Rice bran (RB) is a by-product with limited application due to technological constraints. Enhancing its technological functionality as potential food ingredient will improve the sustainability of rice production. The aim was to study the impact of enzymatic and thermal treatments on defatted rice bran using six distinct commercial enzymes (carbohydrases and proteases) and dry heating by evaluating its technological, nutritional and functional properties. Enzymatic treatment increased up to 208% the soluble dietary fiber content (8.19 g/100 g) of defatted RB. Moreover, the solvent retention capacity, including water, oil, sodium carbonate, and sucrose, exhibited a noteworthy increase across all treatments ($p < 0.05$). Bran color changed after treatments, increasing its luminosity (L^*) and decreasing the value of a^* in all cases, but b^* decreased when treated with protein-acting enzymes while increased with carbohydrate-acting enzymes. Proteases played a pivotal role in reducing particle size and forming gels requiring minimal force for application. Microscopic analysis revealed that carbohydrases-treated samples exhibited prominent cell wall breakage, while protease-treated ones showed a gel-like surface with less distinct protein bodies and layered walls. These comprehensive study sheds new transformations brought about by these enzymatic interventions, offering valuable insights into the optimization of rice bran functionality.

1. Introduction

In 2022, a total of 726 million metric tons of unprocessed paddy rice was harvested, which generate 72.6 million metric tons of rice bran (RB) (FAO, 2024). Rice bran has attracted the interest of the cosmetic industry due to its high oil content, rich in bioactive compounds like γ -oryzanol (Garba, Singanusong, Jiamyangyeun, & Thongsook, 2019). However, after fat extraction the remaining secondary by-product is often employed in animal feed, resulting in an underutilization of its nutritional potential (Gul, Yousuf, Singh, & Wani, 2015; Spaggiari, Dall'Asta, Galaverna, & delCastillo Bilbao, 2021).

To fully harness the many benefits of defatted RB, some research has been dedicated to enhancing the extraction of its components, protein and fibers, or modifying their properties. Physical treatments such as dry heat, parboiling, and microwave treatment have been applied to modify the properties of the protein isolates derived from RB for enhancing oil absorption capacity, emulsifying properties, and gelling abilities of (Khan et al., 2011). Freeze-thaw, sonication or high hydrostatic pressure

have been applied to increase the extractability of proteins from defatted RB, either individually or combining sonication with amylase and protease treatment (Tang, Hettiarachchy & Shellhammer, 2002). Other authors opted for using different carbohydrases, singly or combined, to improve the protein extraction (Scarabattoli et al., 2023), and the emulsifying and foaming properties of the protein concentrates improved when cellulase was added to extruded defatted RB (Leal, Senna, Kupski, Mendes, & Badiale-Furlong, 2021). Even those protein concentrates have been further hydrolyzed with proteases to improve their physico-chemical properties (Tang, Hettiarachchy, Horax, & Eswaranandam, 2003). Likewise, Hamada, (2000) successfully created a highly soluble protein hydrolysate with superior emulsifying activity and stability using commercial proteases.

Lately, more attention has been paid to the dietary fraction that could be extracted from defatted RB. With that purpose, dietary fiber extracted from defatted RB, with the aid of amylase, alcalase and glucoamylase, was treated with cellulase to modify its properties, particularly the water binding capacity (Liu, Zhang, Yi, Quan, & Lin, 2021).

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Similarly, the combination of cellulase and xylanase has been very effective to hydrolyze the intramolecular hydrogen bonds in the hemicellulose and cellulose, although water and oil binding capacities were reduced likely due to the low molecular weight of the hydrolysis products (Wen, Niu, Zhang, Zhao, & Xiong, 2017).

Previous studies have highlighted the interest on proteins and dietary fiber extracted from rice bran. However, using one or the other, results in a by-product, without fully utilizing all bran constituents. Nevertheless, the inclusion of RB as ingredient has been challenging owing to the structural disruption and the impact on hydration due to the cellulose content, particularly in bakery products (Doan, Lai, Vo & Nguyen, 2021; Majzoobi, Sharifi, Imani, & Farahnaky, 2013). Enzymatic treatments have proven effective in improving proteins or dietary fiber fractions, but there has been less exploration of modifying rice bran as a food ingredient. Hence, the aim of this study is to evaluate the effect of six different enzymatic treatments, including carbohydrate or protein acting hydrolases, on technological and functional attributes. Since the thermal treatment of bran rice has been the most reported one (Khan et al., 2011; Sharma, Chauhan & Agrawal, 2004; Tang, Hettiarachchy & Shellhammer, 2002), thermal treatment was employed in the present study for comparison purposes. This approach will unlock the full nutritional and functional potential of RB in a more efficient and sustainable manner.

2. Materials and methods

Rice bran from Japonica rice type was procured from Arroceria Pons (Valencia, Spain). The bran was sieved, using a 1000 µm sieve, to obtain a homogenous sample. Enzymes were donated by Novozymes (Bagsvaerd, Denmark). Enzymes selected were: Novozym® (NO) (xylanase, EC 3.2.1.8), Celluclast® (CE) (cellulase, EC 3.2.1.4; 700 U/g), Ultimease® BWL 40 (UL) (cellulase and xylanase; 300 AGU/mL), Shearzyme® Plus 2x (SH) (xylanase, cellulase and β-glucanase, EC 3.2.1.6; 280 U/mL), Alcalase® 2.4 L FG (AL) (endoprotease, EC 3.4.21.62; 2.4 AU-A/g) and Flavourzyme® (FL) (peptidase preparation containing EC 3.2.1.1, EC 3.4.11, EC 3.4.14, EC 3.4.21.63, EC 3.4.24, EC 3.4.24.39; ≥500 U/g).

2.1. Defatting process

RB was defatted following Mohammadi et al. (2021) methodology with some modifications. RB was suspended in hexane (1:3, w/v) and shake for 2 h. The mixture was centrifuged at 4000×g, 20 °C for 10 min, in a Beckman centrifuge (Beckman Instruments, USA). Supernatant was decanted and sediment was resuspended in the same amount of hexane. To increase efficiency this step was repeated three times leading to the defatted rice bran (DRB). The supernatants were pooled together and evaporated using a rotary evaporator (Heidolph, Afora, Barcelona, Spain) to get crude RB oil. DRB were kept frozen (−20 °C) till further analysis.

2.2. Enzymatic and thermal treatment

DRB was subjected to six different enzymatic treatments, two of the enzymes mainly protein-acting enzymes and the other four carbohydrate-acting enzymes. RB was suspended in water keeping the ratio 1: 6.5 (w/v). Proteases were added at 1 g/100 mL of the protein content of DRB (Vallabha, Indira, Jyothi Lakshmi & Tikku, 2015), and treatment occurred at optimal pH (6.1) and temperature (50 °C) for 120 min. Carbohydrases were incorporated at a concentration of 1% relative to the DRB fiber content, under identical pH, temperature, and time conditions as the proteases. Then, enzymes were inactivated by raising temperature to 90 °C for 10 min.

The heat treatment (dry) consisted of heating the RB placed in a stainless-steel tray for 30 min at 130 °C using a lab oven (J.P Selecta S. A., Barcelona, Spain).

After enzymatic and thermal treatments, samples were kept at −80 °C and then were freeze-dried and the obtained powders were collected for further analysis.

2.3. Proximate composition

Standard methods were used to determine the proximate composition of the samples. Moisture (ISO 712:2009) and total nitrogen (ISO 16634–2:2016), applying 6.25 as a nitrogen to protein conversion factor, were quantified following ISO methods. Total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF) contents were estimated following the method 37–02 (AACC, 2000). Neutral detergent fiber (NDF) content was analyzed following the method proposed by Van Soest, Robertson & Lewis, (1991). Acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined following the procedures outlined in AOAC (973.18). For ADL analysis, samples were treated with 72% sulfuric acid for 3 h. The hemicellulose fraction was calculated by ADF - NDF, and cellulose was calculated by subtracting ADL from ADF.

2.4. Physical properties

2.4.1. Particle size distribution

The particle size distribution was evaluated with a Malvern Mastersizer equipment (Mastersizer Scirocco 2000; Malvern Instruments Ltd., Worcestershire, U.K.). The parameter recorded was volume-weighted mean diameter ($d_{(4,3)}$). The determination was done three times for each sample.

2.4.2. Scanning electron microscopy

The structures of all different samples were examine using scanning electron microscopy (SEM) (Hitachi S-4800, Tokyo, Japan). All RB samples were coated with gold using a vacuum evaporator (JEE 400; JEOL, Tokyo, Japan). Observation was done an accelerating voltage of 10 kW and 2000x magnification.

2.4.3. Color

The color of the all-study samples was evaluated utilizing a Minolta colorimeter (Chroma Meter CR-400/410, Konica Minolta, Tokyo, Japan). The CIE- $L^*a^*b^*$ values of the samples were captured, including L^* (representing [−] lightness/[−] darkness), a^* (indicating [−] redness/[−] greenness), and b^* (denoting [−] yellowness/[−] blueness). The measurements were conducted at three distinct points on the sample's surface and in different replicates. The ΔE was calculated by using the following equation (1) (Islam, Saha, Monalisa, & Hoque, 2019):

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

where L^*_{DRB} , a^*_{DRB} , b^*_{DRB} , are the values of L^* , a^* , b^* of DRB.

2.4.4. Water binding capacity

Water binding capacity (WBC) was analyzed following the method described by Comejo and Rosell (2015). Briefly, distilled water (1 mL) was added to the DRB (100.0 mg ± 0.5 mg), vortexed for 5 min and centrifuged in Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany) at 2000×g for 10 min at room temperature (25 °C). WBC was expressed following the formula given by Comejo and Rosell (2015).

2.4.5. Oil binding capacity

Oil binding capacity (OBC) was quantified following the method described by Boulemkahel, Betoret, Benatallah, and Rosell (2021) with some modification. DRB (100.0 mg ± 0.5 mg) and vegetal oil (1.0 mL) was vortexed for 5 min and then centrifuged (Eppendorf centrifuge 5430 R, Hamburg, Germany) at 3000×g, 4 °C for 10 min. After removing the supernatant, the tubes were inverted for 25 min to drain oil residues.

OBC was calculated by the equation explain by Boulemkahel et al. (2021).

2.4.6. Solvent retention capacity

The Solvent Retention Capacity (SRC) for all samples was determined by adapting the AACC 56-11 method. The SRC values were determined using the following solvents: deionized water, 50 g/100 g sucrose solution, and 5 g/100 g sodium carbonate solution as solvents. SRC was assessed by quantifying the solvent retained by the samples following a 20-min immersion in the solvent, subsequent centrifugation, and a 10-min gel drainage period. The SRC value was expressed as percent of bran weight, on a 14% moisture basis.

2.4.7. Force for penetration

To evaluate the force of penetration of the samples, a 1:5 ratio of bran to water (w:v) was used to ensure optimal hydration of all components, accounting for the moisture content of each sample. Each sample was prepared and analyzed three times. Suspensions and subsequent measurements were performed in 50 mL beakers. Compression was applied at a 10 mm distance from the probe's contact with the sample. A TA-XT plus texture analyzer (Stable Microsystems, Godalming, UK), equipped with a 5 kg load cell and a 25 mm diameter cylindrical aluminum probe was used. The test speed was set at 1.0 mm/s, trigger type auto and the trigger force was 5.0 g. The maximum force required to penetrate the suspensions was recorded. The analysis was conducted in triplicate.

2.5. Statistical analysis

A descriptive statistical analysis (mean $\pm$ standard deviation) was applied to the data for the physico-chemical properties. The significant differences associated with the different treatments were evaluated by an analysis of the variance (ANOVA). Fisher's least significant differences test was the method used to present the results. Significant differences were considered as $p < 0.05$. Moreover, Pearson correlation analysis was applied to experimental values obtained from enzymatically treated samples (including the soaked control) to discern potential relationships within analytical parameters.

All analyses were run with the OriginPro versión 2022b, (Origin Lab Corporation, Northampton, MA, USA). All experiments were carried out in triplicate. Principal Component Analysis (PCA) was performed to discern differences among samples. The input matrix comprised 7 rows and 18 columns. PCA analysis was automatically scaled using the statistical program OriginPro.

3. Results and discussion

To better understand the impact of enzymatic and thermal treatments on the rice bran features, rice bran and defatted rice bran were evaluated, although those are not usually included in most of the reported studies. Likewise, to give more forceful results that can be attributed to the treatments or the process itself, a control sample exposed to soaking without enzyme addition (DRB-C) has been considered. Also, a sample subjected to the thermic treatment and subsequently to soaking without enzyme (DRB T-C) was carried out to explore potential synergistic effects arising from these two treatments.

3.1. Proximate composition

The results of the proximate composition and the different dietary fiber fractions of RB, DRB, different enzymatic and heat treatments, and their respective control, are listed in Table 1. As expected, freeze-dried samples showed significant lower moisture content than non-freeze-dried samples. All treatments promoted a significant increase in protein content compared to the control sample RB. Among the enzymatically treated samples, the most substantial increase occurred in the DRB-

Table 1
Effect of treatment on proximate composition (g/100 g, D.M.) of RB and composition of TDF (%).

Treatment	Proximate composition				
	Moisture	Protein	TDF	IDF	SDF (%) db
RB	11.48 $\pm$ 0.04 ^b	16.06 $\pm$ 0.14 ^d	33.52 $\pm$ 3.53 ^c	30.30 $\pm$ 4.53 ^b	3.22 ^k
DRB	11.97 $\pm$ 0.06 ^a	20.79 $\pm$ 0.43 ^{bc}	39.75 $\pm$ 0.39 ^{ab}	35.82 $\pm$ 2.06 ^a	3.94 ^j
DRB-T	11.31 $\pm$ 0.35 ^b	20.82 $\pm$ 0.03 ^{bc}	38.19 $\pm$ 1.70 ^b	32.84 $\pm$ 0.82 ^{ab}	5.34 ⁱ
DRB-T-C	6.89 $\pm$ 0.09 ^g	21.91 $\pm$ 0.47 ^a	39.20 $\pm$ 1.70 ^{ab}	31.99 $\pm$ 1.45 ^{ab}	7.21 ^d
DRB-C	7.65 $\pm$ 0.14 ^f	20.55 $\pm$ 0.01 ^c	42.36 $\pm$ 3.34 ^a	32.79 $\pm$ 4.77 ^{ab}	9.57 ^a
DRB-NO	8.00 $\pm$ 0.09 ^e	20.74 $\pm$ 0.16 ^{bc}	39.40 $\pm$ 0.64 ^{ab}	32.98 $\pm$ 0.56 ^{ab}	6.43 ^g
DRB-CE	9.05 $\pm$ 0.04 ^d	21.10 $\pm$ 0.05 ^{bc}	39.74 $\pm$ 0.42 ^{ab}	33.53 $\pm$ 1.62 ^{ab}	6.21 ^h
DRB-UL	7.59 $\pm$ 0.05 ^f	20.84 $\pm$ 0.22 ^{bc}	39.13 $\pm$ 1.54 ^{ab}	32.69 $\pm$ 0.89 ^{ab}	6.45 ^f
DRB-SH	9.47 $\pm$ 0.12 ^c	20.71 $\pm$ 0.27 ^{bc}	42.29 $\pm$ 1.02 ^{ab}	34.88 $\pm$ 0.18 ^{ab}	7.41 ^c
DRB-AL	9.52 $\pm$ 0.33 ^c	21.00 $\pm$ 0.34 ^{bc}	41.36 $\pm$ 0.17 ^{ab}	34.00 $\pm$ 1.18 ^{ab}	8.19 ^b
DRB-FL	9.04 $\pm$ 0.28 ^d	21.11 $\pm$ 0 ^b	41.03 $\pm$ 1.65 ^{ab}	37.58 $\pm$ 0.44 ^a	7.16 ^e
Treatment	TDF		Fibers		
	IDF (%)	SDF (%)	Hemicellulose (%)	Cellulose (%)	Lignin (%)
RB	82.73 $\pm$ 3.56 ^b	9.61 ^k	44.41 ^c	27.56 $\pm$ 2.93 ^{ab}	15.12 $\pm$ 0.99 ^{ab}
DRB	90.09 $\pm$ 5.18 ^a	9.91 ^j	46.30 ^b	27.99 $\pm$ 1.02 ^a	14.35 $\pm$ 1.40 ^{ab}
DRB-T	86.01 $\pm$ 2.15 ^{ab}	13.98 ⁱ	59.55 ^a	28.30 $\pm$ 1.87 ^a	14.30 $\pm$ 0.51 ^{ab}
DRB-T-C	81.60 $\pm$ 3.71 ^b	18.39 ^c	37.16 ^g	27.40 $\pm$ 0.89 ^{abcd}	14.31 $\pm$ 0.54 ^b
DRB-C	72.03 $\pm$ 4.13 ^c	22.59 ^a	23.59 ^j	25.53 $\pm$ 1.87 ^{ab}	11.30 $\pm$ 1.21 ^{ab}
DRB-NO	83.69 $\pm$ 1.42 ^b	16.32 ^s	43.47 ^d	20.77 $\pm$ 1.98 ^d	15.73 $\pm$ 2.47 ^a
DRB-CE	84.38 $\pm$ 4.07 ^{ab}	15.63 ^h	41.29 ^e	26.27 $\pm$ 2.83 ^{abc}	12.69 $\pm$ 0.39 ^{ab}
DRB-UL	83.52 $\pm$ 2.27 ^b	16.48 ^f	30.58 ⁱ	27.37 $\pm$ 3.72 ^{ab}	13.49 $\pm$ 0.36 ^{ab}
DRB-SH	82.48 $\pm$ 0.42 ^b	17.52 ^d	37.85 ^f	23.10 $\pm$ 1.90 ^{bcd}	12.91 $\pm$ 2.81 ^{ab}
DRB-AL	79.81 $\pm$ 2.75 ^b	19.80 ^b	11.04 ^k	21.77 $\pm$ 3.74 ^{cd}	14.10 $\pm$ 2.40 ^{ab}
DRB-FL	82.56 $\pm$ 2.60 ^b	17.45 ^e	31.54 ^h	26.63 $\pm$ 3.36 ^{ab}	14.32 $\pm$ 0.86 ^{ab}

Means with different letters within a column were significantly different ($p < 0.05$).

Abbreviations: RB: rice bran; DRB: defatted rice bran; DRB-T: defatted rice bran thermally treated; DRB-T-C: defatted rice bran thermally treated and soaked; DRB-C: defatted rice bran soaked; DRB-NO: defatted rice bran treated with Novozym® (xylanase); DRB-CE: defatted rice bran treated with Celluclast® (cellulase); DRB-UL: defatted rice bran treated with Ultimase® BWL 40 (cellulase and xylanase); DRB-SH: defatted rice bran treated with Shearzyme® Plus 2x (xylanase, cellulase and β -glucanase); DRB-AL: defatted rice bran treated with Alcalase® 2.4 L FG (endoprotease); DRB-FL: defatted rice bran treated with Flavourzyme® (peptidase preparation).

FL sample, reaching 21.11 g protein/100 g, although no significant differences were detected. The highest protein content was recorded in the defatted, heat-treated and soaked sample (DRB-T-C), with 21.91 g protein/100 g. As expected, defatting increase the TDF, particularly the IDF that was the predominant fraction. Furthermore, heat and enzymatic treatments had a minimal impact on TDF content of the DRB, but

there was a significant increase trend in SDF content. Although significant differences were found in the SDF content of the enzymatically treated samples, soaking had the greatest impact and no trend regarding the type of enzymatic activities could be envisaged based on dietary fibers solubility. A deeper analysis of the TDF was performed quantifying its composition (Table 1). The fiber of RB was composed of hemicellulose (44.41%), followed by cellulose (27.56%) and lignin (15.12%), being 82.7% IDF and approx. 10% SDF. Thermal treatment significantly increased the amount of hemicellulose, likely water-soluble, based on the enhancement of SDF observed in that sample. Again, soaking affected the fiber distribution. In the samples that underwent soaking (DRB-T-C, DRB-C, and enzymatically-treated samples) SDF were significantly higher. After soaking, enzymatic treatments increased the amount of hemicellulose when treated with xylanase (DRB-NO) or cellulase (DRB-CE) but the combination of those (DRB-UL) did not intensify the effect. This observation could be attributed to the higher enzymatic activity of CE (700 U/g) compared to the enzymatic preparation UL (300 AGU/g). Likewise, cellulose was significantly reduced after treating with xylanase (DRB-NO) or xylanase, cellulase and β -glucanase mixture (DRB-SH), but no after cellulase treatment (DRB-CE). Those results agree with the research conducted by Coda, Rizzello, Curiel, Poutanen, and Katina (2014) in wheat bran treated with xylanase, obtaining an enhancement of the water extractable arabinoxylans and SDF content. It must highlight that the sample treated with endoprotease (DRB-AL) displayed the highest hydrolysis of hemicellulose and extensive hydrolysis of cellulose. These results suggest the existence of side-enzymatic activities in the commercial preparations.

Enzymatic treatment of rice bran has been previously reported and even combined with other physical treatments like micronization (Wen et al. (2017) or high-pressure homogenization (Xie et al. (2019)). Those studies reported a substantial increase of SDF content in rice bran and provide significant physicochemical data. However, present study provides information about the various fiber fractions and how enzymatic treatments specifically impact the amounts of each fraction.

3.2. Particle size distribution and microstructure of treated rice bran

Fig. 1 displays the particle size distribution obtained for the different samples, indicating that all treatments reduced the particle size compared to RB. This reduction was cumulative, as it is evident that particle size decreases progressively after each treatment (DRB > DRB-T

> DRB-T-C > DRB-C > all enzymatically treated samples). The mean diameter ($d_{(4,3)}$) ranged from 237 to 434 μm (Table 2), showing significant differences among the samples. As noted by Guillon and Champ (2000), the reduction in particle size in samples without enzymatic treatment may be due to the solubilization of some RB components, such as fiber, during processing, or resulting from aggregates dispersion, as mentioned by Rosell, Santos, and Collar (2009). The initial monomodal distribution shifted to bimodal one, particularly after treatment with DRB-AL or DRB-UL. In addition, the sample treated with endoprotease (DRB-AL) has the smallest mean particle size of all the samples. In contrast, the DRB-SH sample, among the group of enzymatically-treated samples, exhibited the largest mean particle size. Therefore, changes in the fiber composition promoted differences in the particle size distribution. Chen, Gao, Yang, and Gao (2013) arrived to similar conclusion when oat samples were subjected to microfluidization process for reducing their particle size, observing a redistribution of IDF and SDF.

At microstructure level, differences associated to the treatments could be envisaged (Fig. 2). Depending on the treatments applied, different proportions of small spherical substances were observed. Starch was predominantly associated with more polygonal spheres, while proteins corresponded to more rounded spheres (Chittapalo & Nookhorm, 2009). Both structures were observable in RB and DRB. In the treated samples, only proteins were observed. The treatment involving cellulases, xylanases, and β -glucanases (DRB-NO, DRB-CE, DRB-UL, and DRB-SH) effectively led to the breakdown of cell walls, as observed in Fig. 2. This finding aligns with Mishra, Ray, Rosell, and Panda (2017) compilation of cell wall degrading enzymes, which includes the aforementioned enzymes. Likewise, Chittapalo and Nookhorm (2009) identified starch and proteins on the surface of the DRB and observed changes in the cell wall structure when applied physical treatments for the DRB proteins extraction. Visibly, samples without enzymatic treatment (RB, DRB, DRB-T, DRB-T-C, DRB-C) exhibited greater disaggregation of all compounds, in contrast to enzyme-treated samples, which displayed a more gel-like structure in their surface. The protease-treated samples displayed noticeable structural changes compared to other enzymatically treated samples. Firstly, these samples exhibited tighter binding, resulting in less distinct differentiation of protein bodies and the layered walls. Moreover, in the sample treated with the endoprotease (DRB-AL), the protein bodies still maintained a spherical shape, while in the sample treated with the peptidase preparation (DRB-FL) the protein bodies acquired a film shape structure,

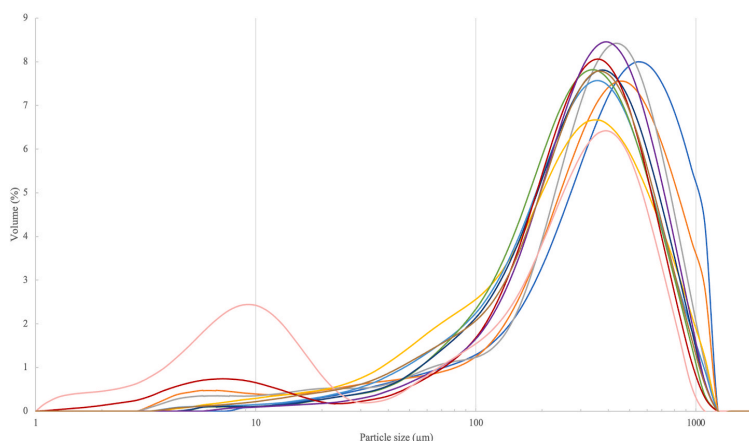


Fig. 1. Particle size distribution of rice bran subjected to different treatments. Abbreviations: RB: rice bran (●); DRB: defatted rice bran (●); DRB-T: defatted rice bran thermally treated (●); DRB-T-C: defatted rice bran thermally treated and soaked (●); DRB-C: defatted rice bran soaked (●); DRB-NO: defatted rice bran treated with Novozym® (xylanase) (●); DRB-CE: defatted rice bran treated with Celluclast® (cellulase) (●); DRB-UL: defatted rice bran treated with Ultimase® BWL 40 (cellulase and xylanase) (●); DRB-SH: defatted rice bran treated with Shearzyme® Plus 2x (xylanase, cellulase and β -glucanase) (●); DRB-AL: defatted rice bran treated with Alcalase® 2.4 L FG (endoprotease) (●); DRB-FL: defatted rice bran treated with Flavourzyme® (peptidase preparation) (●).

Table 2
Technological characteristics of RB with different treatments.

Technological properties						
Treatment	d (4,3) (μm)	L*	a*	b*	ΔE	
RB	434.06 ± 2.48 ^a	67.79 ± 0.02 ^a	1.78 ± 0.05 ^a	18.88 ± 0.01 ^e		
DRB	379.33 ± 2.57 ^b	80.32 ± 0.01 ^k	0.49 ± 0.01 ^h	13.79 ± 0.01 ⁱ	13.59 ± 0.01	
DRB-T	358.89 ± 15.19 ^{bc}	77.13 ± 0.02 ^b	1.00 ± 0.02 ^f	16.31 ± 0.60 ^h	4.12 ± 0.33 ^h	
DRB-T-C	306.33 ± 10.44 ^{ef}	74.87 ± 0.05 ^c	1.35 ± 0.01 ^d	20.07 ± 0.03 ^b	8.36 ± 0.06 ^f	
DRB-C	316.03 ± 11.16 ^{ef}	71.28 ± 0.06 ⁱ	1.51 ± 0.01 ^b	19.45 ± 0.01 ^{cd}	10.72 ± 0.06 ^c	
DRB-NO	311.62 ± 6.67 ^{ef}	72.40 ± 0.02 ^f	1.19 ± 0.03 ^e	19.30 ± 0.03 ^d	9.68 ± 0.04 ^{de}	
DRB-CE	328.35 ± 8.99 ^{de}	72.59 ± 0.03 ^e	1.23 ± 0.03 ^e	19.72 ± 0.02 ^c	9.77 ± 0.02 ^d	
DRB-UL	301.56 ± 33.08 ^f	70.90 ± 0.05 ^j	1.43 ± 0.01 ^c	20.24 ± 0.02 ^b	11.46 ± 0.04 ^a	
DRB-SH	349.19 ± 8.67 ^{cd}	71.63 ± 0.01 ^h	1.48 ± 0.02 ^b	20.59 ± 0.04 ^a	11.08 ± 0.02 ^b	
DRB-AL	237.42 ± 12.30 ^g	73.57 ± 0.02 ^d	0.66 ± 0.07 ^g	17.98 ± 0.01 ^g	7.95 ± 0.02 ^g	
DRB-FL	314.28 ± 9.23 ^{ef}	72.07 ± 0.08 ^g	0.96 ± 0.01 ^f	18.54 ± 0.04 ^f	9.53 ± 0.05 ^e	
Treatment	WBC (g water/g DW)	OBC (g oil/g DW)	Deionized Water (g/100 g DW)	Sucrose (g/100 g DW)	Sodium Carbonate (g/100 g DW)	Force (g)
RB	2.93 ± 0.10 ^e	1.93 ± 0.04 ^g	258 ± 4 ^g	512 ± 16 ^d	263 ± 2 ^f	4131 ± 224 ^a
DRB	3.22 ± 0.11 ^d	2.62 ± 0.11 ^f	339 ± 12 ^c	591 ± 16 ^a	362 ± 11 ^{de}	742 ± 166 ^{fg}
DRB-T	3.13 ± 0.21 ^{de}	2.91 ± 0.07 ^f	337.27 ± 8.16 ^c	591 ± 19 ^a	355 ± 9 ^{de}	1022 ± 446 ^{ef}
DRB-T-C	2.96 ± 0.31 ^{de}	4.69 ± 0.07 ^{cd}	361 ± 10 ^{cd}	587 ± 12 ^a	384 ± 4 ^{bc}	2524 ± 255 ^b
DRB-C	3.54 ± 0.07 ^c	4.32 ± 0.36 ^e	312 ± 8 ^f	564 ± 8 ^{bc}	346 ± 3 ^e	2737 ± 267 ^b
DRB-NO	4.02 ± 0.12 ^b	4.55 ± 0.15 ^{de}	345 ± 8 ^{de}	552 ± 16 ^c	354 ± 17 ^{de}	1667 ± 26 ^c
DRB-CE	3.67 ± 0.17 ^c	5.30 ± 0.45 ^b	344 ± 13 ^{de}	585 ± 14 ^{ab}	372 ± 18 ^{cd}	1110 ± 112 ^{def}
DRB-UL	3.69 ± 0.26 ^c	5.03 ± 0.22 ^{bc}	341 ± 10 ^e	507 ± 7 ^{de}	393 ± 14 ^{ab}	1435 ± 131 ^c
DRB-SH	4.00 ± 0.14 ^b	4.48 ± 0.16 ^{de}	365 ± 18 ^c	508 ± 5 ^{de}	401 ± 7 ^{ab}	1180 ± 57 ^{de}
DRB-AL	3.77 ± 0.16 ^{bc}	4.91 ± 0.16 ^c	383 ± 15 ^b	486 ± 15 ^{ef}	390 ± 7 ^b	255 ± 39 ^h
DRB-FL	4.54 ± 0.15 ^a	5.72 ± 0.11 ^a	416 ± 4 ^a	483 ± 14 ^f	409 ± 12 ^a	376 ± 85 ^{gh}

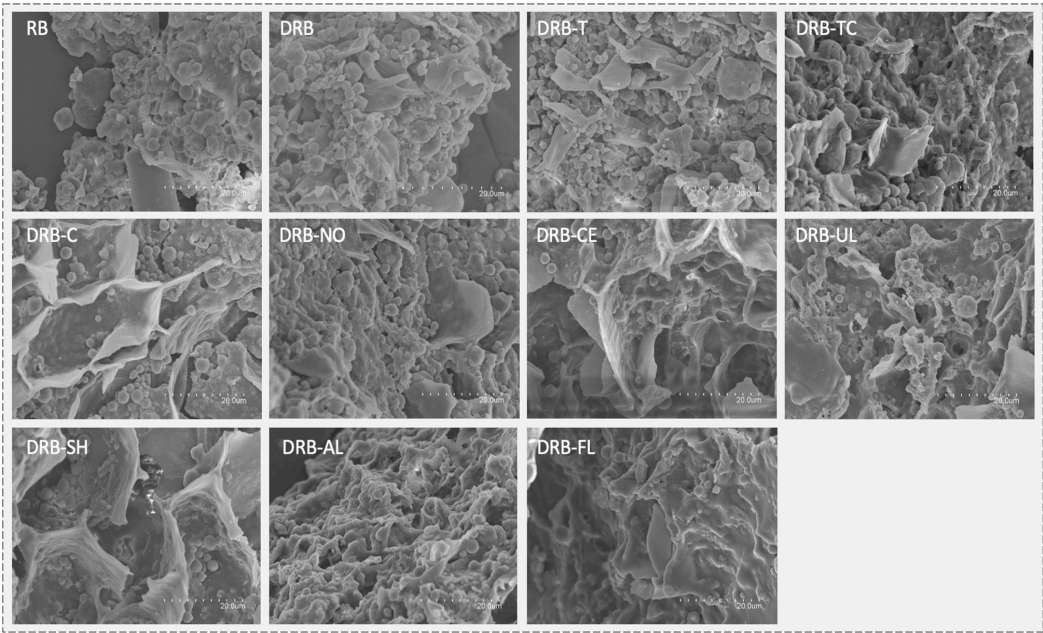


Fig. 2. Scanning electron microscopy analysis of rice bran subjected to different treatments. Abbreviations: RB: rice bran; DRB: defatted rice bran; DRB-T: defatted rice bran thermally treated; DRB-T-C: defatted rice bran thermally treated and soaked; DRB-C: defatted rice bran soaked; DRB-NO: defatted rice bran treated with Novozym® (xylanase); DRB-CE: defatted rice bran treated with Celluclast® (cellulase); DRB-UL: defatted rice bran treated with Ultimase® BWL 40 (cellulase and xylanase); DRB-SH: defatted rice bran treated with Shearzyme® Plus 2x (xylanase, cellulase and β-glucanase); DRB-AL: defatted rice bran treated with Alcalase® 2.4 L FG (endoprotease); DRB-FL: defatted rice bran treated with Flavourzyme® (peptidase preparation).

likely due to small peptides fragments released.

3.3. Impact of rice bran treatment on color

Although only visual differences were perceived after defatting, the results of the instrumental color characterization showed slight but

statistically significant changes ($p < 0.05$) for all the treatments performed. The most notable change was in the DRB sample. The L^* parameter significantly increase, up to 15.60% when comparing RB with DRB, while the a^* parameter showed a reduction of 72.47% in those samples, and the b^* parameter varied by 33.03% compared DRB sample to DRB-SH. Defatting increases the L^* indicating the lightening of the

bran when removing the fat. Regarding the a^* parameter (representing red-green tones), all treatments decreased it, shifting to more greenish. The b^* parameter (representing yellow-blue tones) increased in the samples treated with carbohydrate-acting enzymes, compared to RB, while samples treated with proteins-acting enzymes showed a decrease in this parameter. The overall color change was determined as the disparity between each treatment and the DRB. The most significant color change was observed in samples subjected to soaking, particularly those treated with carbohydrases, especially Ultimase treatment (DRB-UL), which exhibited the highest difference compared to the DRB, at 11.46. Samples treated with proteases displayed lower and more homogeneous values, akin to the sample subjected to heat treatment and soaking, while the heat-treated sample (DRB-T) showed the least overall color variation relative to the DRB.

3.4. Hydration properties of treated rice bran

The enzymatic treatments yielded noticeable increases in both WBC (Water Binding Capacity) and OBC (Oil Binding Capacity) values when compared to the non-enzymatically treated sample (Table 2). The most remarkable enhancement was observed in the case of DRB-FL, where the WBC and the OBC showed an increase of 1.55 and 2.96 times, respectively, in comparison to the untreated RB. Likely, the action of the peptidase preparation modified the proteins' structure and their affinity for water and oil. Notably, the OBC exhibited a more pronounced increase than the WBC, a trend that was consistently observed in all the samples that had soaking in the presence and absence of enzymes, but no other distinguishable pattern was envisaged due to the type of enzyme added.

However, it is worth noting that water binding exhibited a significant positive correlation ($r = 0.69$) with insoluble fiber content, and oil binding capacity was related ($p < 0.05$) with SDF. These results align with findings from Zhu, Huang, Peng, Qian, and Zhou (2010), where insoluble fiber led to higher WBC, but unlike them OBC was positively correlated to soluble fiber. Similarly, a negative relationship has been reported between particle size and hydration capacity (Chau, Wang & Wen, 2007; Zhao, Yang, Gai, & Yang, 2009), because smaller particles expose more surface area, facilitating greater interaction with surrounding solvents. This concept is consistent with results in the current study, where all treatments resulted in a particle size reduction, with a simultaneous increase in the WBC and OBC values. This accentuates a robust and statistically significant negative correlation between particle size and OBC ($r = -0.80$). This observation underscores the relevance of particle size in influencing hydration properties.

Similarly, SRC in three distinct solvents was conducted (Table 2). Specifically, the SRC of sodium carbonate correlates with the levels of damaged starch, while that of sucrose is associated with pentosan characteristics. Deionized water, on the other hand, serves as a control solvent in this context. This method has been adopted to provide a comprehensive understanding of how the various compounds present in rice bran influence its behavior when employed as an ingredient in a food matrix. The treatments significantly increased SRC, except SRC in sucrose for the samples treated with protein-acting enzymes (DRB-AL and DRB-FL), that decreased those values. All SRC values obtained in the analysis ranged from 100 to nearly 400 times higher than those observed in wheat flour, confirming the remarkable solvent retention capacity of rice bran. The starch content in the RB used in this study was 12.99%, falling within the reported range of 10–20% (Sharma, Chauhan & Agrawal, 2004). The increase observed in the SRC in sodium carbonate, was indicative of enhanced amount of damaged starch. The highest value observed at 409%, corresponded to DRB-FL, which also had the highest SRC in deionized water, while yielding the lowest value in sucrose. Therefore, defatted rice bran with carbohydrate-acting enzymes increased SRC values in sucrose, water, and carbonate solvents. DRB-SH had the most pronounced effect on water and carbonate, while DRB-CE on the sucrose. Again, a notable negative correlation was evident

between particle size and the retention of deionized water ($r = -0.74$) as well as the retention of sodium carbonate ($r = -0.71$). Therefore, the SRC in different solvents could be used to discriminate among enzymatic treatments performed on rice bran.

3.5. Textural properties of treated rice bran

The texture of the rice bran samples, after hydrating them by adding five times water, was evaluated (Table 2). Treatments applied to RB led to a notable reduction in the force needed to penetrate the sample. A positive correlation with the particle size of each sample was observed, although it did not reach statistical significance. RB without any treatment exhibited the highest penetration force, 4131 g. Within the group treated with enzymes targeting carbohydrates, no statistically significant differences were observed neither between the DRB-NO and DRB-UL, or the pair DRB-CE and DRB-SH. In contrast, defatted bran treated with proteases, particularly DRB-AL, gave the softest samples.

3.6. Overall features of treated rice bran

A principal components analysis (PCA) (Fig. 3) was conducted to discern potential clusters based on enzymatic treatment (carbohydrate-acting enzymes, and protein-acting enzymes), using the proximate composition and technological properties as studied factors. This analysis accounts for 63.39% of the variances observed among the treated samples. Principal component 1 (PC1) accounted for 43.70% of the variability, while principal component 2 (PC2) explained 19.69%. In Fig. 3, variables that are closely located indicate stronger positive correlations, whereas variables positioned in opposite locations demonstrate inverse correlations. In this sense, PC1 is primarily influenced by the solvent retention capacity in deionized water, proteins, and insoluble fiber, while penetration force and a^* value have an opposite influence. In PC2, hemicellulose, d (4,3), and the b^* represent the driving variables while and an opposite effect was observed for soluble fiber. Fig. 3 clearly showed that PC1 discriminated between samples treated with protein-acting enzymes, positioning them on the positive axis, and the DRB-C sample (as control sample) was placed on the negative axis, confirming the efficiency of the enzymatic treatment in modifying the physico-chemical properties of the DRB. Meanwhile, PC2 discriminated samples treated with carbohydrate-acting enzymes, situating them on the positive axis, with the exception of the DRB-NO sample, which was positioned on the negative axis. This placement of DRB-NO was associated with its high TDF and SDF content. The PCA validates the findings presented in Tables 1 and 2 by grouping the control sample (DRB-C) with penetration force, soluble fiber, and total fiber (the latter being shared with DRB-NO). Hemicellulose and cellulose content, particle size, and color, effectively clustered the samples treated with carbohydrate-acting enzymes. The majority of technological properties successfully clustered and differentiated the DRB-FL sample, while the DRB-AL sample exhibited an opposite trend in relation to particle size, which stands out as one of its most distinct characteristics.

4. Conclusions

Enzymatic treatments can effectively modify the defatted rice bran composition and functionality. Nevertheless, the extension of the impact was highly dependent on the enzymes used for the treatment. Carbohydrate and protein degrading enzymes augmented the proportion of SDF, which was not obtained with the heat treatment. Those variations in the proportion of the fiber composition allowed reducing the particle size distribution with the subsequent impact on the solvent retention capacity of the defatted rice bran, and on the texture of the hydrated bran. The analysis of the solvent retention ability of the rice in different solvents gave indications about the constituents that were modified, starch and proteins, which were also confirmed by SEM micrographs. Modifications were also observed in the chromatic parameters,

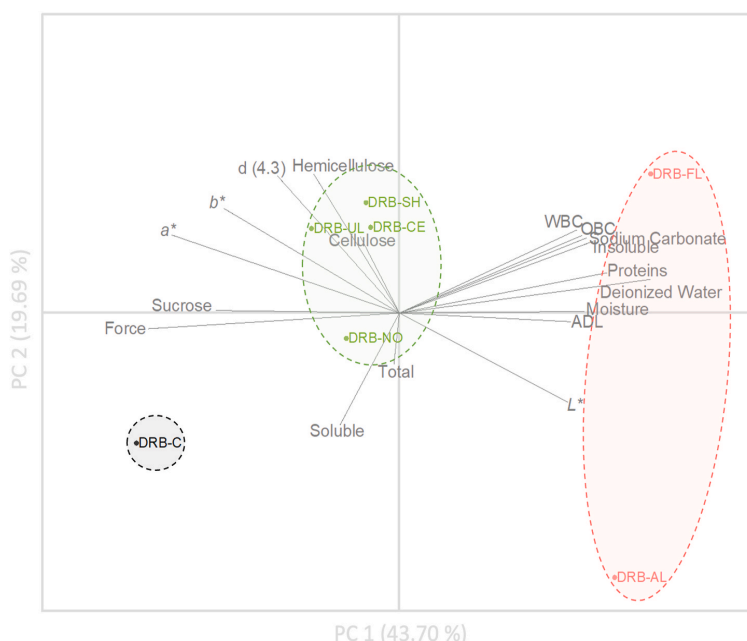


Fig. 3. Graph showing the set of defatted rice bran samples treated by soaking (DRB-C) and with enzymatic treatment, and the variables studied by principal component analysis (PCA). The three different circles group the samples depending on the treatment to which they have been subjected, (●) sample treated by soaking, (●) samples treated with carbohydrate-acting enzymes and (●) samples treated with protein-acting enzymes.

particularly in brightness, which increased by 100% in all instances. Given the interest in increasing the fiber content of foods and beverages, enzymatically treated rice bran shows promise for better integration into food matrices due to its smaller particle size and improved hydration performance. Nevertheless, considering the complexity of the bran matrix, additional studies will be needed to explain the mechanisms behind each enzymatic treatment. Thermal and mechanical analysis of the isolated constituents will be undertaken in future studies.

CRediT authorship contribution statement

Eva Grau-Fuentes: Writing – original draft, Investigation, Formal analysis. **Raquel Garzón:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Dolores Rodrigo:** Writing – review & editing, Supervision. **Cristina M. Rosell:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

On behalf of all the authors I declare that none have conflict of interest.

Data availability

Data will be made available on request.

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References

- Boulemlkahel, S., Betoret, E., Benatallah, L., & Rosell, C. M. (2021). Effect of low pressures homogenization on the physico-chemical and functional properties of rice flour. *Food Hydrocolloids*, 112, Article 106373. <https://doi.org/10.1016/j.foodhyd.2020.106373>
- Chau, C.-F., Wang, Y.-T., & Wen, Y.-L. (2007). Different micronization methods significantly improve the functionality of carrot insoluble fibre. *Food Chemistry*, 100(4), 1402–1408. <https://doi.org/10.1016/j.foodchem.2005.11.034>
- Chen, J., Gao, D., Yang, L., & Gao, Y. (2013). Effect of microfluidization process on the functional properties of insoluble dietary fiber. *Food Research International*, 54(2), 1821–1827. <https://doi.org/10.1016/j.foodres.2013.09.025>
- Chittapalo, T., & Noomhorm, A. (2009). Ultrasonic assisted alkali extraction of protein from defatted rice bran and properties of the protein concentrates. *International Journal of Food Science and Technology*, 44(9), 1843–1849. <https://doi.org/10.1111/j.1365-2621.2009.02009.x>
- Coda, R., Rizzello, C. G., Curiel, J. A., Poutanen, K., & Katina, K. (2014). Effect of bioprocessing and particle size on the nutritional properties of wheat bran fractions. *Innovative Food Science & Emerging Technologies*, 25, 19–27. <https://doi.org/10.1016/j.ifset.2013.11.012>
- Cornejo, F., & Rosell, C. M. (2015). Physicochemical properties of long rice grain varieties in relation to gluten free bread quality. *LWT - Food Science and Technology*, 62(2), 1203–1210. <https://doi.org/10.1016/j.lwt.2015.01.050>
- Doan, N. T. T., Lai, Q. D., Vo, H. V., & Nguyen, H. D. (2021). Influence of adding rice bran on physico-chemical and sensory properties of bread. *Journal of Food Measurement and Characterization*, 15(6), 5369–5378. <https://doi.org/10.1007/s11694-021-01111-5>
- FAO. (2024). *Paddy rice production worldwide in 2022, by country (in million metric tons)*. Statista. <https://www.statista.com/statistics/255937/leading-rice-producers-worldwide/#statisticContainer>
- Garba, U., Singanusong, R., Jiamyangyeun, S., & Thongsook, T. (2019). Extraction and utilisation of rice bran oil. A review. *La Rivista Italiana Delle Sostanze Grasse*, 96(3), 161–170.
- Guillon, F., & Champ, M. (2000). Structural and physical properties of dietary fibres, and consequences of processing on human physiology. *Food Research International*, 33(3), 233–245. [https://doi.org/10.1016/S0963-9969\(00\)00038-7](https://doi.org/10.1016/S0963-9969(00)00038-7)
- Gul, K., Yousuf, B., Singh, A. K., Singh, P., & Wani, A. A. (2015). Rice bran: Nutritional values and its emerging potential for development of functional food—a review. *Bioactive Carbohydrates and Dietary Fibre*, 6(1), 24–30. <https://doi.org/10.1016/j.bcdf.2015.06.002>
- Hamada, J. S. (2000). Characterization and functional properties of rice bran proteins modified by commercial exoproteases and endoproteases. *Journal of Food Science*, 65(2), 305–310. <https://doi.org/10.1111/j.1365-2621.2000.tb15998.x>

- Islam, M. Z., Saha, T., Monalisa, K., & Hoque, M. M. (2019). Effect of starch edible coating on drying characteristics and antioxidant properties of papaya. *Journal of Food Measurement and Characterization*, 13(4), 2951–2960. <https://doi.org/10.1007/s11694-019-00215-3>
- Khan, S. H., Butt, M. S., Sharif, M. K., Sameen, A., Mumtaz, S., & Sultan, M. T. (2011). Functional properties of protein isolates extracted from stabilized rice bran by microwave, dry heat, and parboiling. *Journal of Agricultural and Food Chemistry*, 59(6), 2416–2420. <https://doi.org/10.1021/jf104177x>
- Leal, F. H. P. N., Senna, C. de A., Kupski, L., Mendes, G. da R. L., & Badiale-Furlong, E. (2021). Enzymatic and extrusion pretreatments of defatted rice bran to improve functional properties of protein concentrates. *International Journal of Food Science and Technology*, 56(11), 5445–5451. <https://doi.org/10.1111/ijfs.15017>
- Liu, Y., Zhang, H., Yi, C., Quan, K., & Lin, B. (2021). Chemical composition, structure, physicochemical and functional properties of rice bran dietary fiber modified by cellulase treatment. *Food Chemistry*, 342, Article 128352. <https://doi.org/10.1016/j.foodchem.2020.128352>
- Majzoobi, M., Sharifi, S., Imani, B., & Farahnaky, A. (2013). The effect of particle size and level of rice bran on the batter and sponge cake properties. *Journal of Agricultural Science and Technology A*, 15, 1175–1184.
- Mishra, S. S., Ray, R. C., Rosell, C. M., & Panda, B. (2017). Microbial enzymes in food applications. *Microbial Enzyme Technology in Food Applications*, 3–18.
- Mohammadi, F., Marti, A., Nayeazadeh, K., Hosseini, S. M., Tajdar-oranj, B., & Jazaeri, S. (2021). Effect of washing, soaking and pH in combination with ultrasound on enzymatic rancidity, phytic acid, heavy metals and coliforms of rice bran. *Food Chemistry*, 334, Article 127583. <https://doi.org/10.1016/j.foodchem.2020.127583>
- Rosell, C. M., Santos, E., & Collar, C. (2009). Physico-chemical properties of commercial fibres from different sources: A comparative approach. *Food Research International*, 42(1), 176–184. <https://doi.org/10.1016/j.foodres.2008.10.003>
- Scarabattoli, L., Sangiorgio, S., Romagnuolo, F., Gelati, L., Cavuoto, D., Rabuffetti, M., et al. (2023). Use of carbohydrases to promote protein extraction from rice bran and soybean meal: A comparative study. *Lebensmittel-Wissenschaft und -Technologie*, 184, Article 115060. <https://doi.org/10.1016/j.lwt.2023.115060>
- Sharma, H. R., Chauhan, G. S., & Agrawal, K. (2004). Physico-chemical characteristics of rice bran processed by dry heating and extrusion cooking. *International Journal of Food Properties*, 7(3), 603–614. <https://doi.org/10.1081/JFP-200033047>
- Spaggiari, M., Dall'Asta, C., Galaverna, G., & del Castillo Bilbao, M. D. (2021). Rice bran by-product: From valorization strategies to nutritional perspectives. *Foods*, 10(1). <https://doi.org/10.3390/foods10010085>
- Tang, S., Hettiarachchy, N. S., Horax, R., & Eswaranandam, S. (2003). Physicochemical properties and functionality of rice bran protein hydrolyzate prepared from heat-stabilized defatted rice bran with the aid of enzymes. *Journal of Food Science*, 68(1), 152–157. <https://doi.org/10.1111/j.1365-2621.2003.tb14132.x>
- Tang, S., Hettiarachchy, N. S., & Shellhammer, T. H. (2002). Protein extraction from heat-stabilized defatted rice bran. 1. Physical processing and enzyme treatments. *Journal of Agricultural and Food Chemistry*, 50(25), 7444–7448. <https://doi.org/10.1021/jf025771w>
- Vallabha, V. S., Indira, T. N., Jyothi Lakshmi, A., Radha, C., & Tiku, P. K. (2015). Enzymatic process of rice bran: A stabilized functional food with nutraceuticals and nutrients. *Journal of Food Science and Technology*, 52(12), 8252–8259. <https://doi.org/10.1007/s13197-015-1926-9>
- Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74(10), 3583–3597. [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2)
- Wen, Y., Niu, M., Zhang, B., Zhao, S., & Xiong, S. (2017). Structural characteristics and functional properties of rice bran dietary fiber modified by enzymatic and enzyme-micronization treatments. *LWT - Food Science and Technology*, 75, 344–351. <https://doi.org/10.1016/j.lwt.2016.09.012>
- Xie, F., Zhao, T., Wan, H., Li, M., Sun, L., Wang, Z., et al. (2019). Structural and physicochemical characteristics of rice bran dietary fiber by cellulase and high-pressure homogenization. *Applied Sciences*, 9(7). <https://doi.org/10.3390/app9071270>
- Zhao, X., Yang, Z., Gai, G., & Yang, Y. (2009). Effect of superfine grinding on properties of ginger powder. *Journal of Food Engineering*, 91(2), 217–222. <https://doi.org/10.1016/j.jfoodeng.2008.08.024>
- Zhu, K., Huang, S., Peng, W., Qian, H., & Zhou, H. (2010). Effect of ultrafine grinding on hydration and antioxidant properties of wheat bran dietary fiber. *Food Research International*, 43(4), 943–948. <https://doi.org/10.1016/j.foodres.2010.01.005>