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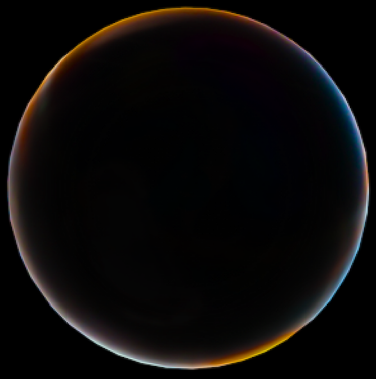
TESIS DOCTORAL

Development of food packaging films from residual proteins and evaluation of their physical and antimicrobial properties



OVIEDO SPAIN
PHD PROGRAMME IN
CHEMICAL, ENVIRONMENTAL
AND BIO-FOOD ENGINEERING

Shihan Weng

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DEPARTMENT OF
CHEMICAL AND
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ENGINEERING

Universidad de Oviedo

Department of Chemical and Environmental Engineering

“Development of food packaging films from residual proteins and evaluation of their physical and antimicrobial properties”

PhD Programme in

Chemical, Environmental and Bio-Food Engineering

PhD thesis by

Shihan Weng

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October-2023

Universidad de Oviedo

Ingeniería Química y Tecnología del Medio Ambiente

“Preparación de envases alimentarios a partir de proteínas residuales y evaluación de sus propiedades físicas y antimicrobianas”

Programa de Doctorado en

Ingeniería Química, Ambiental y Bioalimentaria

Tesis doctoral por

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Preparación de envases alimentarios a partir de proteínas residuales y evaluación de sus propiedades físicas y antimicrobianas	Inglés: Development of food packaging films from residual proteins and evaluation of their physical and antimicrobial properties

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RESUMEN (en español)

El desarrollo de envases para alimentos basados en biopolímeros (con características biodegradables, renovables y/o comestibles) es una forma de ayudar a reducir la presión de la protección del medio ambiente, mejorar el almacenamiento y el transporte de alimentos y promover el desarrollo sostenible de la sociedad humana. La contaminación causada por el deficiente reciclado de los residuos de envases de plástico amenaza el medio ambiente y la salud humana, mientras el mercado mundial de envases flexibles sigue creciendo año tras año.

Los bioplásticos se basan principalmente en biomacromoléculas como proteínas, polisacáridos y lípidos. Estos materiales tienen generalmente las ventajas de ser sustancias biológicamente activas no tóxicas y fáciles de polimerizar. Los bioplásticos basados en proteínas como matriz principal son una vía importante para el desarrollo de nuevos materiales de envasado de alimentos.

En este contexto, esta tesis explora y amplía el campo de investigación de los bioplásticos de envasado de alimentos a base de proteínas. Las proteínas plasmáticas animales se pretrataron mediante precipitación con etanol combinada con una acidificación. Las “películas” proteicos se caracterizaron y estudiaron en diferentes condiciones. La modificación de las proteínas permite una preparación mejorada de “películas”. Además, para superar la desventaja de la alta solubilidad de las “películas”. La celulosa nanofibrilada seincorporó al bioplástico, lo que no solo mejora la resistencia a la disolución, sino que también aumenta la resistencia estructural. Las “películas” que contienen un 10 % de celulosa nanofibrilada (NFC) presentan mejoras significativas en las propiedades mecánicas y las propiedades de barrera contra el vapor de agua.

En esta tesis, se utilizaron tres tipos diferentes de agentes antimicrobianos para conferir propiedades bioactivas a las “películas”. Se utilizaron péptidos antimicrobianos añadidos a los bioplásticos de proteínas plasmáticas, y se añadieron fagos y timol a los bioplásticos con base gelatina. Asi mismo se probaron por separado contra dos patógenos comunes transmitidos por los alimentos: Staphylococcus aureus (grampositivo) y Escherichia coli (gramnegativo). A partir de los resultados, el péptido antimicrobiano nisina es afectivo en la película de proteína plasmática. El fago pilIPLA-RODI no tuvo efecto sobre las propiedades físicas del “película” de gelatina independientemente de la concentración. Pero el rendimiento antimicrobiano cuando se preparó en forma de “Recubrimientos” fue mejor que en forma de “Películas”.

Se utilizaron nanopartículas de ácido poliláctico (PLA) para encapsular timol y mejorar su persistencia en las “películas” de gelatina. Exhiben alta transparencia, microestructura uniforme y propiedades antimicrobianas. Además, estas nanopartículas exhiben una alta estabilidad de almacenamiento a diferentes valores de pH.
Esta tesis también revisa el estado de la investigación de bioplásticos comestibles basados en proteínas y polisacáridos de insectos. Actualmente, los investigadores se están enfocando más en los materiales de quitosano que en las proteínas.

Finalmente se revisan diferentes tecnologías para la producción de bioplásticos en forma de “películas” y “recubrimientos”. Resulta de gran importancia elegir la tecnología de producción más adecuada teniendo tanto la producción como las propiedades intrínsecas del material.

Universidad de Oviedo

RESUMEN (en Inglés)

The development of biopolymer-based food packaging (with biodegradable, renewable, and/or edible characteristics) is a way to help reduce pressure from environmental protection, improve food storage and transportation, and promote the sustainable development of human society. Pollution caused by poorly recycled plastic packaging waste threatens the environment and human health, while the global market for flexible packaging continues to grow year by year.

Bioplastics are mainly based on biomacromolecules such as proteins, polysaccharides and lipids. These materials generally have the advantage of being non-toxic and readily incorporating active substances. Protein-based bioplastics as the main matrix are an important avenue for the development of new food packaging materials.

In this context, this thesis explores and broadens the research field of protein-based food packaging bioplastics. Animal plasma proteins were pretreated by ethanol precipitation combined with acidification. The protein films were characterized and studied under different conditions. Protein modification allows for improved film preparation. In addition, to overcome the disadvantage of the high solubility of the "films". Nanofibrillated cellulose was incorporated into the bioplastic, which not only improves dissolution resistance, but also increases structural strength. Films containing 10% nanofibrillated cellulose (NFC) show significant improvements in mechanical properties and water vapor barrier properties.

In this thesis, three different types of antimicrobial agents were used to confer bioactive properties to the "films". Antimicrobial peptides added to the plasma protein bioplastics were used, and phage and thymol were added to the gelatin-based bioplastics. They were tested separately against two common foodborne pathogens: Staphylococcus aureus (gram-positive) and Escherichia coli (gram-negative). From the results, the antimicrobial peptide nisin is affective on the plasma protein film. The pilIPLA-RODI phage had no effect on the physical properties of the gelatine film regardless of the concentration. But the antimicrobial performance when prepared in the form of "Coating" was better than in the form of "Film".

Polylactic acid (PLA) nanoparticles were used to encapsulate thymol and improve its persistence in gelatine films. They exhibit high transparency, uniform microstructure, and antimicrobial properties. Furthermore, these nanoparticles exhibit high storage stability at different pH values.

This thesis also reviews the state of research on edible bioplastics based on insect proteins and polysaccharides. Currently, researchers are focusing more on chitosan materials than on proteins.

Finally, different technologies for the production of bioplastics in the form of "films" and "coatings" are reviewed. It is importance to choose the most suitable production technology taking into account both the production and the intrinsic properties of the material.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN _ INGENIERIA QUÍMICA, AMBIENTAL Y BIOALIMENTARIA_

三十功名尘与土，八千里路云和月。
莫等闲、白了少年头，空悲切。

To dust is gone the fame achieved in thirty years; Like cloud-veiled moon the thousand-mile Plain disappears.
Should youthful heads in vain turn grey, We would regret for aye。

Se ha ido al polvo la fama alcanzada en treinta años; Como la luna velada por nubes, la Llanura de mil millas desaparece.
Si las cabezas jóvenes en vano se vuelven grises, nos arrepentiremos por sí.

--岳飞《满江红·怒发冲冠》

--Yue Fei's "Full River Red"

--"Río lleno Rojo" de Yue Fei

Acknowledgements

I googled the template, but most of them are too simple and pale. Thank God first, then thank parents, and everyone who appeared in my doctoral career, and thank people who have helped me with my thesis investigation.

So, I pondered for a long time, because I think acknowledgment will be a very important carrier, even if it is just a few pages of words. For 7 years, I stayed in a place 10,000+ kilometres away from my hometown. The precious 7 years of my life, from 22 to 29 years old, thanks to Oviedo city and the University of Oviedo. Let me meet a lot of good friends, let me enjoy the scenery of Spain, let me continue to climb higher in the ivory tower of knowledge. It is a particularly interesting experience to understand the differences between Chinese culture and Western European culture.

To whom I want to say thanks.

Part1:

The first one is my respected supervisor, Mario. My image of Mario is like the headmaster in Harry Potter, but without the beard, more handsome, kind-hearted and caring about the students. What impresses me is that Jefe Mario uses iPhone and MAC computer, and sometimes he has doubts about the operation of the software, so he will ask us for help to see how to operate it. What I want to say is that it is a very touching spirit. Live and learn. I have bookmarked this precious attitude.

The second is to pay tribute to my tutor Manuel. Because 5 years ago in 2018, when I was confused in my life and wanted to continue my studies and go further on the academic road, Jefe Manuel and Jefe Mario gave me a chance to come to Spain from China to study for a Ph.D. And Jefe Manuel is a lovely and responsible PhD advisor. On weekdays, Manuel spends his free time before and after café time, before and after lunch, and before and after get off work. He will go to the laboratory and the student office to see, ask us what is difficult in the experiment, and give us timely help. From this earnestness, I know that it is not easy to teach and educate people. I think this may be the brilliance of the five hundred years of teaching history in Europe since the Renaissance.

The third is Ismael, who was also my tutor of TFM. His affability is recognized. I am grateful for all the help he has given me over the years. I learned a lot about the importance of experimental understanding and experimental design, and sensitivity to new trends. The most important thing is his persistence in scientific research, which I admire. Always the first to arrive at the office and the last to go home. Every year, he patiently explains and guides the graduate work of master students and undergraduate students.

To Sergio and Paula, Sergio is the friendliest teacher I have ever met, and Paula often patiently helps me correct my Spanish. To Andriana and Amanda, their kindness and smiles often encourage me in the lab.

To my friends, we are the same age, wearing the same white coats, playing with the same beakers and spectrophotometers in the laboratory. I believe that many years later, I will miss the days when I came to the second floor of the chemistry building and saw everyone smiling, and a group of people went downstairs one by one to drink in the café. It was these ordinary but interesting days that you taught me a lot of Spanish vocabulary and gave me a different perspective on the life of Spanish youth. What better opportunity to learn than this? What could be a better day to enjoy yourself than this? You guys are the best.

I still remember the first year of my Ph.D., when Daniel, Silvia, Sara, Marta, Ana, Cristina, Manuel, Lucia and Victor were all there. Daniel is a good brother, he will invite me to listen to him playing drums, learn to speak Chinese from me, and invite me to play paddle tennis. From him, I have seen that his attitude towards life that accepts new things is very excellent. Willing to try new things and live with such an attitude, life will always be colourful. Sara's kindness is unmatched. She helps me in many experiments. She has good research habits. The steps and parameters of each experiment will be clearly recorded in the notebook. This serious attitude towards scientific research is worth learning. She is a good friend and I hope she is happy every day. Marta is one of the old classmates who have known each other for a long time, from the MBTA class in 2016 to the PhD graduation in 2023. Her Ph.D. dissertation precedes mine, so I wish her the best of luck. Ana has the best smile, her PhD defence

is the first I have ever witnessed, excellent and confident demeanour. The most regrettable thing is that I missed the defence of Cris and Manuel. This regret taught me to grasp the information. Silvia and I both love sidra., which makes me understand that life is a choice and it is important to be happy. There needs to be a proper balance between studies and life. Lucia's smartness and seriousness about her professionalism and Victor's easy going are all good qualities I remember, and they both tried to help me due to my limited Spanish. Danisol y Andrea are hilarious and easy going and kind sidekicks who are TBR's pistachios. Sara Alvarez also worked on TFG this year. Her serious experimental attitude and ingenuity are enviable, and based on her many works, we have this thesis.

In the second year, 2019-2020, I got to know Flor and Esther this year. It's a pity that I forgot how I met Flor for the first time, but she is just like her name Flor. Honored to have a seat next to Flor. Every day we imagine and plan the wonderful life after becoming a doctor and in a good mood. I admire her communication skills from the bottom of my heart. Moreover, she and her boyfriend Hugo often took me to go hiking, which moved a student who was alone in a foreign land. Just like the fragrance of flowers, it makes life full of beauty. Esther, you have to use the word “cute” to describe her. She accidentally broke the experimental glass, and would blame herself for a long time. This sense of responsibility taught me to cherish every experiment, every experimental step and result.

In the third year, Abel and Maria did TFM this year, and Juanfer and Luis came to study for Ph.D. Everyone was having a good time in the laboratory again. Especially Maria, I am so envious, she has all the qualities to be a good doctor, as well as young age, smart head, hardworking attitude, efficient action, and super amiable, perfect. Luis often taught me some slang and uncommon words, which allowed me to surpass the D1 level in certain Spanish vocabulary areas. Juanfer's calmness is another skill I need to learn. His eyes revealed the wisdom of being a big boss. They are also the partners who push me to play basketball and exercise, and maintain a healthy life attitude.

That summer, I went to Shanghai Jiao Tong University in China for an internship, nearly 3 months. I was very grateful to be able to study in the group of Tutor Yue Jin. This

Estancia has broadened my scientific horizons in different countries.

In the fourth year, wasted. But met Diego and Enrique who are kind and friendly. Should have gotten to know you earlier.

The final year, I want to say to you guys, really thank you, thank you to every lovely person I know. I hope everyone can live the life you want.

Part 2

Thanks to the Chinese students in Oviedo. Thank you for organizing interesting activities together and encouraging each other.

Thanks to the local Chinese Business Association in Oviedo for taking great care of Chinese students.

Thanks to Panda y tola, the best Chinese school in Oviedo, and the boss Susana, for giving me the opportunity to promote Chinese culture and language. I also thank each student who has taken my class, and I hope they can learn Chinese well and have a bright future.

Most of all, I want to say thanks to my parents for raising me up to 30 years old. They provided my financial support, so that I did not feel the pressure of living overseas. Even if I write another hundred thousand sentences, I can't express my gratitude to my parents.

Part3

If I've left out someone to thank, I must have been negligent. Please keep my most sincere wishes and accept my most sincere thanks!

In addition, I would like to thank myself, the decision to do a doctorate is correct, although the past five years have not worked hard, but it is still worth it! This is life!

Agradecimientos

Busqué en Google un modelo, pero la mayoría de ellos son demasiado simples y livianos. Agradecer primero a Dios, luego agradecer a mis padres, y a todos los que aparecieron en mi carrera doctoral, y agradecer a las personas que me han ayudado con la investigación de mi tesis.

Reflexioné durante mucho tiempo porque creo que el reconocimiento será un vehículo muy importante, incluso si son solo unas pocas páginas de palabras. Durante 7 años, me quedé en un lugar a más de 10 000 kilómetros de mi terra natal. Fueron 7 preciosos años de mi vida, de 22 a 29 años, gracias a la ciudad de Oviedo y a la Universidad de Oviedo. La tesis me permitió conocer muchos buenos amigos, dejarme disfrutar del paisaje de España, dejarme seguir escalando más alto en la torre de marfil del conocimiento. Ha sido una experiencia particularmente interesante para comprender las diferencias entre la cultura china y la cultura europea occidental.

Quiero agradecer especialmente a muchas personas:

Parte 1:

El primero es mi respetado supervisor, Mario. Mi imagen de Mario es como el director de Harry Potter, pero sin barba, más guapo, bondadoso y preocupado por los alumnos. Lo que me impresiona es que Jefe Mario usa iPhone y computadora MAC, y a veces tiene dudas sobre el funcionamiento del software, por lo que nos pide ayuda para ver cómo operarlo. Lo que quiero decir es que es un espíritu muy inquieto. Vive y aprende. Reconozco esta preciosa actitud.

El segundo es rendir homenaje a mi tutor Manuel. Porque hace 5 años en 2018, cuando estaba confundido en mi vida y quería continuar mis estudios y avanzar más en el camino académico, el Jefe Manuel y el Jefe Mario me dieron la oportunidad de venir a España desde China para realizar mi doctorado. . Y el Jefe Manuel es un asesor de doctorado encantador y responsable. Entre semana, Manuel pasa antes y después de la hora del café, antes y después del almuerzo, y antes y después de salir del trabajo por nuestra sala. Va al laboratorio y a la sala de estudiantes para vernos, preguntarnos sobre los experimentos y brindarnos ayuda oportuna. Por esta seriedad, sé que no es

fácil enseñar y educar a la gente. Creo que esto puede explicar la brillantez de los quinientos años de enseñanza de la historia en Europa desde el Renacimiento.

El tercero es Ismael, que también fue mi tutor de TFM. Reconozco su afabilidad. Estoy agradecido por toda la ayuda que me ha brindado a lo largo de los años. Aprendí mucho sobre la importancia de la comprensión experimental y el diseño experimental, y la sensibilidad a las nuevas tendencias. Lo más importante es su persistencia en la investigación científica, que admiro. Siempre el primero en llegar a la oficina y el último en irse a casa. Cada año, explica y guía pacientemente el trabajo de posgrado de estudiantes de maestría y estudiantes de pregrado.

Para Sergio y Paula, Sergio es el profesor más amigable que he conocido y Paula a menudo me ayuda pacientemente a corregir mi español. Para Andriana y Amanda, sus amabilidades y sonrisas muchas veces me alientan en el laboratorio.

Para mis compañeros y amigos, que son de misma edad, vestimos las mismas batas blancas, manejamos los mismos vasos de precipitados y espectrofotómetros en el laboratorio. Creo que muchos años después, voy a extrañar los días en que llegué al segundo piso del edificio de química y vi a todos sonriendo, y un grupo de personas bajaba una a una para tomar el café. Fueron estos días ordinarios pero interesantes que me enseñaron mucho vocabulario español y me dieron una perspectiva diferente sobre la vida de la juventud española. ¡Qué mejor oportunidad para aprender que esta! ¿Qué podría ser un mejor día para disfrutar que este?

Todavía recuerdo el primer año de mi doctorado, cuando estaban allí Daniel, Silvia, Sara, Marta, Ana, Cristina, Manuel, Lucía y Víctor. Daniel es un buen hermano, me invitará a escucharlo tocar la batería, aprenderá a hablar chino conmigo y me invitará a jugar al pádel. De él he visto que su actitud ante la vida, siempre con cosas nuevas es excelente. La amabilidad de Sara es inigualable. Ella me ayudó en muchos experimentos. Tiene buenos hábitos de investigación. Los pasos y parámetros de cada experimento se registraron claramente en el cuaderno. Es una buena amiga y espero que sea feliz todos los días. Marta es una de las antiguas compañeras de clase que conozco desde hace más tiempo. Desde la clase de MBTA 2016 hasta la graduación de doctorado de 2023. Su defensa de doctorado está por delante de la mía, así que le

deseo la mejor de las suertes. Ana tiene la mejor sonrisa, su defensa de doctorado es la primera que he presenciado, comportamiento excelente y confiado. Lo más lamentable es que me faltó la defensa de Cris y Manuel. Este arrepentimiento me enseñó a captar la información. A Silvia le gusta beber sidra al igual que a mí, lo que me hace entender que la vida es una elección y es importante ser feliz. Tiene que haber un equilibrio adecuado entre los estudios y la vida. La inteligencia y la seriedad de Lucía sobre su profesionalismo y la tranquilidad de Víctor son todas buenas cualidades que recuerdo, y ambos trataron de ayudarme debido a mi español limitado. Dani Sol y Andrea son compinches divertidos, tranquilos y amables que son los pistachos de TBR. Sara Álvarez también trabajó en TFG este año. Su seria actitud experimental e ingenio son envidiables.

En el segundo año, 2019-2020, conocí a Flor y Esther este año. Es una pena que me olvidé de cómo conocí a Flor por primera vez, pero ella es como su nombre Flor. Es un honor tener un asiento al lado de Flor. Todos los días imaginamos y planificamos la maravillosa vida después de convertirnos en médicos y de buen humor. Admiro sus habilidades de comunicación desde el fondo de mi corazón. Además, ella y su novio Hugo a menudo me llevaban a hacer caminatas, lo que conmovió a un estudiante que estaba solo en un país extranjero. Al igual que la fragancia de las flores, llena la vida de belleza. Esther, tienes que usar la palabra “linda” para describirla. Accidentalmente rompió el vidrio experimental y se culparía a sí misma durante mucho tiempo. Este sentido de responsabilidad me enseñó a apreciar cada experimento, cada paso y resultado experimental.

En tercer año, Abel y María hicieron TFM este año, y Juanfer y Luis vinieron a desarrollar el doctorado. Todo el mundo estaba pasando un buen rato en el laboratorio de nuevo. En especial a María quien tiene todas las cualidades para ser una buena doctora, además de joven, cabeza inteligente, actitud trabajadora, acción eficiente y súper amable, perfecta. Luis me enseñó a menudo algunas jergas y palabras poco comunes, lo que me permitió superar el nivel D1 en ciertas áreas del vocabulario en español. La tranquilidad de Juanfer es otra habilidad que necesito aprender. Sus ojos revelaron la sabiduría de ser un gran jefe. También son los compañeros que me

empujan a jugar baloncesto y hacer ejercicio, y mantener una actitud de vida saludable.

Ese verano, fui a la Universidad Jiao Tong de Shanghai en China para una pasantía de 3 meses. Estoy muy agradecido de poder estudiar en el grupo de Yue Jin. Esta Estancia amplió mis horizontes científicos.

En el cuarto año, perdido. Pero conocí a Diego y Enrique que son amables y amigables. Debería haberos conocido antes.

El último año, quiero decirles, muchas gracias, gracias a todas las personas encantadoras que conozco. Espero que todos puedan vivir la vida que quieren.

Parte 2

Gracias a los estudiantes chinos en Oviedo. Gracias por organizar actividades interesantes juntos y animarnos unos a otros.

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Gracias a Panda y Tola, la mejor escuela de chino de Oviedo, y sobre todo a la jefa Susana, por darme la oportunidad de promover la cultura y el idioma chino. También agradezco a cada estudiante que ha tomado mi clase. Espero que puedan aprender chino lo mejor posible y tener un futuro brillante.

Pero, sobre todo, quiero dar las gracias a mis padres por criarme hasta los 30 años. Me brindaron apoyo financiero para que no sintiera la presión de vivir en el extranjero. Incluso si escribo otras cien mil oraciones, no puedo expresar mi gratitud a mis padres.

Parte 3

Si he dejado a alguien a quien agradecer, debo pedir disculpas. ¡Mis más sinceros deseos y acepte mi más sincero agradecimiento!

Además, me gustaría agradecerme a mí mismo, la decisión de hacer un doctorado es correcta, aunque los últimos cinco años no he trabajado mucho, ¡pero aun así vale la pena! ¡Así es la vida!

Contents

Resumen

El desarrollo de envases para alimentos basados en biopolímeros (con características biodegradables, renovables y/o comestibles) es una forma de ayudar a reducir la presión de la protección del medio ambiente, mejorar el almacenamiento y el transporte de alimentos y promover el desarrollo sostenible de la sociedad humana. La contaminación causada por el deficiente reciclado de los residuos de envases de plástico amenaza el medio ambiente y la salud humana, mientras el mercado mundial de envases flexibles sigue creciendo año tras año.

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En este contexto, esta tesis explora y amplía el campo de investigación de los bioplásticos de envasado de alimentos a base de proteínas. Las proteínas plasmáticas animales se pretrataron mediante precipitación con etanol combinada con una acidificación. Las “películas” proteicos se caracterizaron y estudiaron en diferentes condiciones. La modificación de las proteínas permite una preparación mejorada de “películas”. Además, para superar la desventaja de la alta solubilidad de las “películas”. La celulosa nanofibrilada seincorporó al bioplástico, lo que no solo mejora la resistencia a la disolución, sino que también aumenta la resistencia estructural. Las “películas” que contienen un 10 % de celulosa nanofibrilada (NFC) presentan mejoras significativas en las propiedades mecánicas y las propiedades de barrera contra el vapor de agua.

En esta tesis, se utilizaron tres tipos diferentes de agentes antimicrobianos para conferir propiedades bioactivas a las “películas”. Se utilizaron péptidos antimicrobianos añadidos a los bioplásticos de proteínas plasmáticas, y se añadieron fagos y timol a los bioplásticos con base gelatina. Asi mismo se probaron por separado contra dos patógenos comunes transmitidos por los alimentos: *Staphylococcus aureus*

(grampositivo) y *Escherichia coli* (gramnegativo). A partir de los resultados, el péptido antimicrobiano nisina es afectivo en la película de proteína plasmática. El fago *pilIPLA-RODI* no tuvo efecto sobre las propiedades físicas del "película" de gelatina independientemente de la concentración. Pero el rendimiento antimicrobiano cuando se preparó en forma de "Recubrimientos" fue mejor que en forma de "Películas".

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Esta tesis también revisa el estado de la investigación de bioplásticos comestibles basados en proteínas y polisacáridos de insectos. Actualmente, los investigadores se están enfocando más en los materiales de quitosano que en las proteínas.

Finalmente se revisan diferentes tecnologías para la producción de bioplásticos en forma de "películas" y "recubrimientos". Resulta de gran importancia elegir la tecnología de producción más adecuada teniendo tanto la producción como las propiedades intrínsecas del material.

Abstract

The development of biopolymer-based food packaging (with biodegradable, renewable, and/or edible characteristics) is a way to help reduce pressure from environmental protection, improve food storage and transportation, and promote the sustainable development of human society. Pollution caused by poorly recycled plastic packaging waste threatens the environment and human health, while the global market for flexible packaging continues to grow year by year.

Bioplastics are mainly based on biomacromolecules such as proteins, polysaccharides and lipids. These materials generally have the advantage of being non-toxic and readily incorporating active substances. Protein-based bioplastics as the main matrix are an important avenue for the development of new food packaging materials.

In this context, this thesis explores and broadens the research field of protein-based food packaging bioplastics. Animal plasma proteins were pretreated by ethanol precipitation combined with acidification. The protein films were characterized and studied under different conditions. Protein modification allows for improved film preparation. In addition, to overcome the disadvantage of the high solubility of the "films". Nanofibrillated cellulose was incorporated into the bioplastic, which not only improves dissolution resistance, but also increases structural strength. Films containing 10% nanofibrillated cellulose (NFC) show significant improvements in mechanical properties and water vapor barrier properties.

In this thesis, three different types of antimicrobial agents were used to confer bioactive properties to the "films". Antimicrobial peptides added to the plasma protein bioplastics were used, and phage and thymol were added to the gelatin-based bioplastics. They were tested separately against two common foodborne pathogens: *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative). From the results, the antimicrobial peptide nisin is affective on the plasma protein film. The pilIPLA-RODI phage had no effect on the physical properties of the gelatine film regardless of the concentration. But the antimicrobial performance when prepared in the form of "Coating" was better than in the form of "Film".

Polylactic acid (PLA) nanoparticles were used to encapsulate thymol and improve its persistence in gelatine films. They exhibit high transparency, uniform microstructure, and antimicrobial properties. Furthermore, these nanoparticles exhibit high storage stability at different pH values.

This thesis also reviews the state of research on edible bioplastics based on insect proteins and polysaccharides. Currently, researchers are focusing more on chitosan materials than on proteins.

Finally, different technologies for the production of bioplastics in the form of "films" and "coatings" are reviewed. It is importance to choose the most suitable production technology taking into account both the production and the intrinsic properties of the material.

Chapter 1: Introduction to the thesis

1.1. Introduction

Even in the third decade of the 21st century, plastics still dominate the flexible packaging industry strongly. The poor disposal of plastic packaging waste has brought about serious white pollution and greenhouse gas problems, threatening our natural environment at all times. Some researchers have predicted that by 2050, from the plastic production stage to processing, 6500 Mt equivalent CO_2 will be emitted into the environment (Mazhandu et al., 2020). Furthermore, the growth of the global food delivery industry due to the impact of the COVID-19 pandemic has increased the consumption of plastic food containers such as food wrappers and grocery bags (Oliveira et al., 2021). These disposable plastic packages turn into plastic waste shortly after consumers use them. Promoting the difficulty of recycling and reprocessing waste plastics (Sundqvist-Andberg & Åkerman, 2021). Even in recent years, the accumulation of microplastics (MPs) and their contamination of food have become a global threat to the environment and human health. It is estimated that people ingest about 2977 microplastics per year through takeaway food (Jadhav et al., 2021). Plastic is threatening life and health. But according to the latest report by Grand View Research, Inc. ("Flexible Packaging Market Size, Share and Trend Analysis Report 2023 - 2030", n.d.), the global flexible packaging market size is expected to reach USD 373.3 billion by 2030. The market is still growing and is expected to grow at a CAGR of 4.7% from 2023 to 2030.

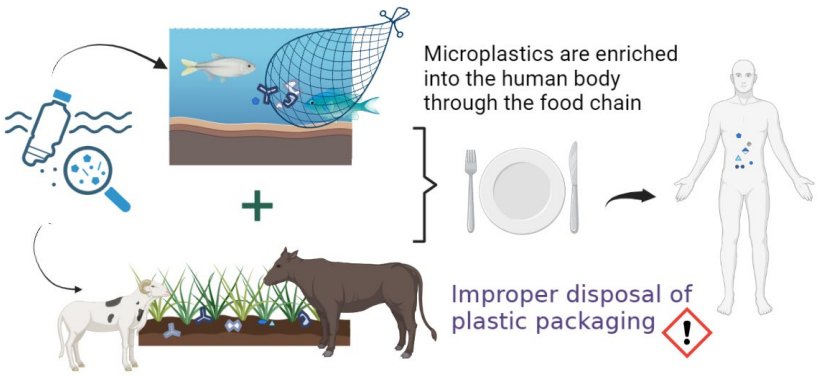

Figure 1.1: The minimalist process of enriching microplastics into the human body

In this context, the development of biopolymer-based food packaging materials will reduce environmental pressure, help food storage and transportation, and promote sustainable development of human society. Food packaging manufacturers and the food industry have been working to replace traditional, non-renewable petroleum resources with abundant, low-cost, renewable and biodegradable alternatives (Chaudhary et al., 2022) Biodegradable is generally defined as being able to decompose materials into natural elements such as carbon dioxide, methane, water, and inorganic compounds through microbial fermentation.

At present, the public's awareness of the environmental problems caused by traditional plastic packaging is increasing, and consumers' expectation of improving the concept of sustainable development is gradually strengthening. This is one of the reasons leading to the development of bio-based, biodegradable or edible food packaging materials. (Sundqvist-Andberg & Åkerman, 2021). Although some researchers currently predict that degradable or edible films will not completely replace traditional packaging materials. But they can prolong food stability and improve the efficiency and environment of food packaging by reducing the exchange of moisture, lipids, volatiles and gases between the food and the surrounding environment, reducing the need for petroleum-derived polymers (Anita Ivanković, Karlo Zeljko, Stanislava Talić, Anita Martinović Bevanda, 2017) Biopolymer-derived films can also be added with various active ingredients such as antimicrobials, antioxidants, nutrients and colours. Keep food hygienic and safe, extend the shelf life of products by preventing the growth of pathogenic microorganisms (such as *S. aureus*, *E. coli*, etc.)(Yuvaraj et al., 2021).

Renewable organic substances (plants, animals and microorganisms) and compounds are the main source of materials for biodegradable and even edible food packaging. Biodegradable polymers can be divided into two categories based on structure (S. Shaikh et al., 2021). One category is natural agricultural polymers, or those derived from biomass. The other category consists of biopolyesters (biopolyesters are derived from microorganisms, or synthesized by natural monomers or synthetic monomers by

chemical means).

Agricultural waste is an excellent source of natural biopolymer material collection. The by-products obtained in the production process of fruits, vegetables, meat and dairy products have great usability and potential economic value (Phiri et al., 2023; Visco et al., 2022). Cellulose, hemicellulose, chitin, lignin, starch, pectin, alginate, protein, etc. are common raw materials that can be extracted from agricultural waste. (Tajeddin & Abukhedri, 2020) Among them, proteins are composed of amino acids containing various functional groups. Amino acids are repolymerized to form polypeptides, and complex functional structures are formed through peptide bonds. These are considered by the researchers to have the potential to be translated into food packaging. And these materials generally have the advantages of being non-toxic and biodegradable. Polymers of carbohydrates and proteins and their derivatives are generally edible, and are considered to be important basic materials for sustainable development (Mkandawire & Aryee, 2018).

Polymers derived from biomass through biological, physical and chemical synthesis are mainly classified as biopolyester materials. Polylactic acid (PLA), which is polymerized with lactic acid produced by biofermentation as the main raw material, is one of the most widely used biopolyesters. Attempts by the manufacturing industry to partially replace petrochemical plastics with PLA are increasing every year. (Jariyasakoolroj et al., 2020; Khodaei et al., 2021; Pantani & Turng, 2015; Pinto et al., 2021; M. Zhang et al., 2022) Because these materials, known as “bioplastics”, have similar properties in terms of mechanical, physicochemical and thermal properties compared to petrochemical polymers. Even PLA is the first commercial polymer produced from renewable resources and is recognized by the US Food and Drug Administration (FDA) as "generally recognized as safe" (GRAS), suitable for packaging materials such as food and drugs (Swetha et al., 2023).

In addition to use biomass materials as food packaging, what can help environmental protection and sustainable development is the production method of food packaging. Appropriate technology can enhance the original properties of the material and exert

better performance. Research on how to implement reasonable and efficient coating and film production technologies such as Dipping ,Electrospinning, Calendering film and even emerging 3D printing technology is also summarized in this doctoral thesis.

1.2. Objectives

The main research objective of this doctoral thesis is the production and application of new food packaging films using natural biomass source materials.

From this general aim, the following specific objectives have been formulated.

1. To develop, synthetize and characterize edible food packaging films based on protein-based biomass materials (e.g., plasma proteins and gelatine).

2. To study the effect of modified polysaccharide biomass material cellulose on the structure of protein films prepared for food packaging.

3. To explore the antibacterial performance of protein active compounds such as phages and antimicrobial peptides in the food packaging films against *Staphylococcus aureus* commonly used in food hygiene. And also, the antibacterial performance of active substances such as thymol on *Escherichia coli*.

4. To Evaluate the encapsulation performance of biodegradable polymers containing PLA nanoparticles for active ingredient thymol in the food packaging films

5. Summarize the existing technologies for producing edible packaging coatings and films at different scales, as well as the latest developed from insects, one of the novel food resources for humans, as edible packaging films.

1.3 Thesis Structure Memory

This Ph.D. thesis document is presented in form of publications compilation. The content of this thesis belongs to the field of new food packaging materials. All content is divided into the traditional structure, namely background introduction, materials and methods, results, discussion, and conclusion. The content covered in this thesis involves 5 research articles and a review article that have all been published, and another review article submitted. There are a total of 8 chapters in this thesis, which are divided into sub-chapters and subsections. Here is a brief overview:

In the first chapter, the introduction (Section 1.1) briefly states the immediate reasons for making this doctoral thesis. Likewise, this chapter also indicates the goals that the research undertaken seeks to achieve (Section 1.2).

The second chapter is divided into four subsections. First, the demand for new biomass materials in the field of food packaging is expounded, namely subsection 1 (2.1). At the same time, it reveals the unfavourable factors of existing mainstream materials (2.1.1) And from 4 aspects, it expresses why new materials are needed. (2.1.2). And in the second subsection, all biomass materials involved in this thesis are introduced in detail, and are classified by material type. Nanofibrillated fibres (2.2.1), gelatine and plasma proteins (2.2.2), active antimicrobial substances (2.2.3), pathogenic species (2.2.4). And it involves the analysis of the advantages of these materials in the third subsection (2.3). Finally, in the fourth subsection, a brief description is given of the different levels of scale (2.4) currently produced for food packaging.

The third chapter introduces the experimental methods and analysis techniques used in this doctoral dissertation, as well as the sources of materials involved. Section 3.1 lists the specific information of the experimental materials. Section 3.2 involves the preparation methods and processes of all materials that need to be pretreated, including the preparation of films, screening and incubation of pathogenic bacteria, and other information. Section 3.3 is a summary of all experimental methods involved in film characterization.

In Chapter 4, the obtained research results are presented. Preparation of plasma protein films (4.1.1) and incorporation of nanofibrillated cellulose (4.1.2), followed by a review on food packaging technologies (4.1.3). Section 4.2 is based on gelatine film, and the additive phage (4.2.1) and PLA nanoparticle-encapsulated thymol (4.2.2/4.2.3) are studied. Finally, there is a review of research on insect-derived food packaging materials (4.3.1).

Chapter 5 provides a general discussion of the results obtained and Chapter 6 contains the most important conclusions drawn from this study.

Chapter 7 includes a bibliography common to this thesis (specific references related to Chapters 3 and 4 are omitted and can be found in the “References” section of the corresponding chapter or publication).

Finally, Chapter 8 is "Additional Documentation". Contains information on lists of figures (section A1), tables (section A2), equations (section A3), abbreviations (section A4) and symbols (section A5). In addition, publication information (section A6.1) and communications with national and international conferences (section A6.2) for doctoral dissertations are covered in this chapter.

Chapter 2: Foreground Knowledge

2.1. New packaging materials are required

Food packaging is considered an integral part of the food commodity. Usually, food spoilage is caused by oxidation, microbial growth and metabolism. It is also possible that food is affected by environmental pollution and other reasons, such as temperature, humidity, light, physical damage, microbes, dust entering and wasting food resources.(Morris, 2017) The food packaging can protect the food, so that the food can prevent the damage of biological, chemical, physical and other external factors during the circulation process from the factory to the consumer. It can also maintain the stable quality of the food itself, which is convenient for the food to be used by consumers.

At present, biodegradable or edible food packaging is very popular among scholars in the research of food packaging materials. These materials can ignore the cost of packaging recycling, and the organic components can naturally degrade. Although the suitable research results found in these studies have not yet effectively entered the consumer market, the real large-scale commercialization is still being explored.(Aguirre-Joya et al., 2018; Beikzadeh et al., 2020; Han, 2013; Mangaraj et al., 2019; Tassinari et al., 2023; Trajkovska Petkoska et al., 2021)

It is well known that there are many types of food packaging, and the packaging categories can be divided according to different functions, different attributes, and different packaging times. The focus of food packaging involved in this thesis is within the scope of flexible packaging and primary packaging. The so-called flexible packaging refers to the packaging in which the shape of the container can change after the contents are filled or taken out. Compared with rigid packaging, flexible packaging is lighter in weight and has certain folding resistance, elasticity, vibration resistance and deformation capacity.(*What Is Flexible Packaging?*, n.d.)

In addition, primary packaging refers to the layer of packaging closest to the product, which is the last protection and isolation between food and the outside world. This layer of packaging is in direct contact with food. (Yuvaraj et al., 2021)The subject of

this research is to use biomass-derived organic materials to make primary flexible packaging, which helps to achieve the existing environmental protection goals through the renewability of the materials themselves and the non-polluting properties after degradation. At the same time, the material's own structure improves the protection of environmental protection packaging to help food enhance the quality, and the addition of active substances helps food extend the shelf life. This chapter will sort out and explain in detail the significance and background of this thesis from the following perspectives.

2.1.1 Mainstream materials of food packaging and their disadvantages

There are three main types of flexible packaging materials that occupy the mainstream in the industry today: metal, paper, and plastic.

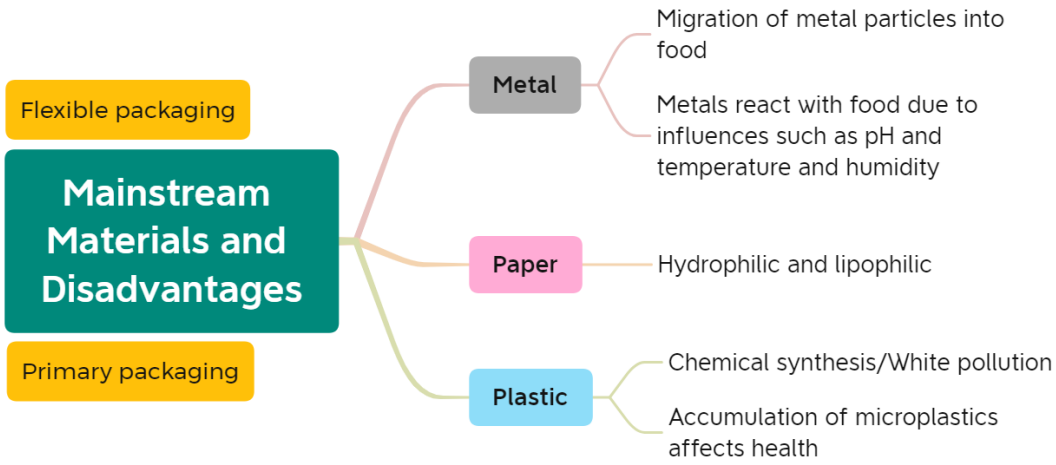

Figure 2.1: Three flexible packaging materials and their brief disadvantages

2.1.1.1 Metal Packaging

The mainstream materials in metal materials include Coated steel (Tin plate, Tin free steel (TFS), Polymer coated steel), Stainless steel, Aluminium. The adverse health effects of metal-packaged foods are mainly related to two main processes, one is migration and the other is interaction. Migration is the transfer of metal packaging components to the packaged food during storage or processing. Common migrants

include tin, lead, aluminium, chromium, bisphenol A (BPA), and others. Interactions are physical, chemical or microbiological reactions that occur at the food-packaging interface. (Deshwal & Panjagari, 2020)The compatibility of food with metal containers depends on its chemical composition, pH, storage temperature and humidity. Metal-food interactions can lead to corrosion, pitting, perforation, loss of coatings, and product deterioration and discoloration. Common catalysts that also exist to enhance the reaction between food and metals include nitrates, phosphates, plant pigments, synthetic pigments, copper and sulphur compounds. The well-known Alzheimer's disease has been proved by animal experiments that it may be related to excessive intake of aluminium (Deshwal & Panjagari, 2020; Gauba, Pammi,Reema Gabrani, 2020; Ribeiro et al., 2021), but we often use aluminium paper packaging food in our daily packaging. In addition, according to the World Food and Agriculture Organization (Howe, 2005), tin metal can cause gastrointestinal discomfort, such as nausea, vomiting, diarrhoea, abdominal cramps, bloating, fever and headache. The maximum permissible tin content in solid foods is therefore 250 mg/kg and the maximum permissible tin content in beverages is 150 mg/kg. In terms of environmental protection, aluminium beverage cans are often discarded in wastewater, causing blockage and pollution of water bodies. But if they can be recovered properly, they can be reused, which is more cost-effective than remelting directly from aluminium ore (Deshwal & Panjagari, 2020).

2.1.1.2 Paper Packaging

Paper packaging materials are generally sheets made of an intertwined network of cellulose fibres extracted from wood. Fibers are pulped and/or bleached with chemical oxidizing agents and reinforced with chemicals such as plasticizer emulsions. General paper packaging is not used to protect food for a long time, because it has poor barrier properties and cannot be heat-sealed. When used as primary packaging (i.e. in contact with food), paper is almost always surface treated with waxes, resins, etc. to improve protection (Asim et al., 2021; Kumar et al., 2022). This is because the fibre material has

good water absorption properties, in other words it is not waterproof. In addition, the extensive use of paper packaging is also a cause for concern for the consumption of forest resources (Hubbe, 2013). And a large amount of waste water needs to be treated in the process of chemical treatment such as fibre softening and pulp bleaching, which is also a pressure point for environmental protection (O, 2023).

2.1.1.3 Plastic Packaging

Plastic, mentioned briefly in the introduction, is a material widely used in flexible food packaging and production of plastic has grown faster than any other material since the 1970s. Due to its easy production, strong plasticity, low price, good stability, and excellent packaging capabilities such as visibility, it is estimated that the global primary plastic output will reach 1.1 billion tons by 2050. (Kibria et al., 2023; Ncube et al., 2021; *Our Planet Is Choking on Plastic*, n.d.; Watkins, 2022)About 36 % of this was used in packaging, including single-use plastic products used in food and drink containers. And about 85% of these plastic bags end up in landfill or as unregulated waste. Since plastic products are artificially synthesized chemicals, they have extremely poor degradation performance in nature. Several common materials used for packaging, such as polyethylene terephthalate (PET), low-density polyethylene (LDPE), high-density polyethylene (HDPE), polypropylene (PP) and polystyrene (PS) are high molecular weight polymers (Atiwesh et al., 2021; Narinder Singh, 2022). In the absence of reprocessing, the degradation of plastics in the natural environment can be understood as changes in the overall structure at the physical level, such as cracking, embrittlement and peeling, or at the chemical level, changes at the molecular level, such as bond breaking or Oxidizes long polymer chains to produce new molecules(Chamas et al., 2020). In addition, during the plastic degradation process, the microplastics produced will eventually accumulate in the food chain through factors such as soil and water flow, which is a threat to human health(Jadhav et al., 2021; Sewwandi et al., 2023).

2.1.2 Requirements for new Bio-materials

The widespread use and improper disposal of packaging made of traditional plastics and chemicals in today's market poses a growing threat to both the environment and biological health. The cost of metal food flexible packaging is high, and its chemical stability is poor, it is easy to rust, and its acid and alkali resistance is weak. Especially when packaging acidic food, metal ions will precipitate and affect product quality. Therefore, people need better food packaging materials, and they need to reduce the damage and threat of packaging materials to the food itself and the natural environment while ensuring the quality of the packaging. These requirements are divided into two perspectives, the objective perspective and the subjective perspective, based on the needs of consumers or users.

2.1.2.1 Functional requirements

The first requirement is the most basic packaging the functional requirements. Food packaging has been fulfilling this requirement since humans harvested food resources in ancient times. In order to store food and water, and to facilitate social activities such as transportation, carrying and trading, there is a need for food packaging. For example, using leaves, fruit shells, animal skins, shells, turtle shells and other items to hold and transport food and water can be considered as the original form of biomass packaging materials.(Teixeira-Costa & Andrade, 2021; Verma et al., 2021)

2.1.2.2 Social requirements

Human society needs sustainable development, needs to solve the problem of global warming, and needs to use the power of packaging to help food to be transported and stored for a long time, so as to help poor or disaster areas. The source of biomass material is not non-renewable petroleum resources. This helps reduce carbon emissions and mitigate climate warming.

2.1.2.3 Security requirements

The pursuit of safety is the basic instinct of human beings. In terms of food packaging materials, it is required that the materials can meet the hygienic quality and safety

requirements during packaging. Biomass materials have positive aspects in meeting food safety requirements. For example, the packaging of polysaccharide chitosan, which has been popular in recent years, itself has a certain antibacterial effect, which is an incomparable natural property of other flexible packaging materials.(Liu et al., 2022)

2.1.2.4 Aesthetic requirements

In a subjective cognition, human beings also need to meet their own aesthetic requirements when consuming food. Edible packaging films used as primary food packaging are very close to the food that needs to be packaged. They play a positive role in shaping the appearance of food, helping food to have a better external image and stimulating consumers' purchase needs (Azeredo et al., 2022; Kauppi & Van Der Schaar, 2020). In addition, there are different application requirements. Compared with plastics and metals, biomass materials can be applied to different scenarios according to the respective properties of biomass.

2.2 Bio-based materials from Proteins and Carbohydrates

In this thesis, according to the properties of bio-based materials, they are mainly classified into two categories, namely carbohydrates and proteins, which are two of the three mainstream biomacromolecules. Whether it is a prokaryote or a eukaryote, for a living individual, it is composed of biological macromolecules such as proteins, nucleic acids, sugars, and lipids, as well as some small molecular compounds and inorganic salts. Therefore, in terms of the source selection of food packaging materials, bio-based materials can be fully supplied and demanded, so the involvement of fossil resources will be reduced. Of course, what goes further is that these biomass materials can be found in the current industrial chain, and they can be by-products or wastes that are defined by traditional cognition as having no economic value. Therefore, if these studies are truly commercialized and applied on a large scale, they can further

help the protection of the natural environment.

In order to enhance the functionality of food packaging, the addition of natural active substances to packaging materials is also classified separately. The addition of active substances can provide additional attributes to food packaging (Kuai et al., 2021; H. Yong & Liu, 2021). Compared with the main bio-based materials that contribute to the physical structure, active substances tend to help food storage at the biological or chemical level.

In addition to natural biological macromolecules, some macromolecular organic materials indirectly formed through the intervention of certain artificial synthesis techniques or microbial fermentation methods are also the objects of this research, provided that the materials are biodegradable.

2.2.1. Carbohydrates/polysaccharides

Carbohydrates are also called sugars in the nutritional classification. They are all a class of compounds composed of three elements: carbon, hydrogen, and oxygen. Carbohydrates are a broad concept of sugars, including monosaccharides, disaccharides, sugar alcohols, oligosaccharides, polysaccharides and other types.(Jayasekara et al., 2022) Among the current food packaging and packaging materials, polysaccharide source materials are sought after. Because of its rich functional performance. Polysaccharides are classified according to their functions, and a storage polysaccharide usually exists in plants in the form of starch. Since both native starch and modified starch have completely biodegradable characteristics, edible characteristics and low cost. It has therefore also received considerable attention in the formulation of biodegradable films (Ceballos et al., 2020; Versino et al., 2016).

Several factors affect the properties of starch films. For example, the source of starch, and the proportion of starch components. Although starch film has the advantages of thin thickness, flexibility and transparency. There are some disadvantages that need to be mentioned, such as poor mechanical properties and water vapor permeability.

Therefore, the use of starch alone to produce films will lead to its limited use. To improve the mechanical properties of starch films and increase moisture resistance, various approaches can be used, including starch modification techniques such as starch crosslinking and combining starch with other natural polymers. Starch is important because of its low cost and abundance in nature.(Guz et al., 2021a; Pelissari et al., 2018; M. Shaikh et al., 2019)

The other type is structural polysaccharides, such as cellulose and hemicellulose that make up plant cell walls, chitin that exists in the exoskeleton of crustaceans and the cell walls of fungi. They can provide structural support for food packaging substrates and even have a strong structure as a food packaging substrate alone. Chitosan is deacetylated to obtain chitin, and both chitosan and chitin are biodegradable polymers with excellent film-forming ability and antibacterial activity. (Rinaudo & Goycoolea, n.d.)But it reflects poor water resistance, good mechanical strength and barrier properties to aroma, O_2 and CO_2. In addition, pectin is composed of a large amount of glucose-polymerized dextran and poly-α-(1-4)-D-galacturonic acid linear chains with different degrees of methylation. A gum made of galactose, arabinose, rhamnose, and glucuronic acid. and alginate composed of 1-4-linked α-d-mannuronic acid (M) and β-l-guluronic acid (G) monomers. They all have very strong gelling and film-forming capabilities, and are key research materials for food packaging.(Galus et al., 2020; Ribeiro et al., 2021; J. Wang et al., 2022)

The research material included in this thesis is nanocellulose derived from structural polysaccharide material cellulose. Nanocellulose material is a "cellulose nanofiber" made by nanofiberizing (ultrafine) the main component of cellulose, which constitutes the plant cell wall. It is considered to have the characteristics of "lightness, toughness, and environmental protection". attracted the attention of researchers. And cellulose itself (consisting of β-d-glucose monomers linked by 1,4-β-glycosidic bonds) is one of the most abundant polysaccharides in nature.(Kocira et al., 2021; Pirozzi et al., 2021; Zhong, 2020)

The cell walls of wood and plants, certain bacteria and algae, and tunicates (the only

known cellulose-containing animals) are all possible sources of cellulose. Cellulose materials inherently possess renewability, anisotropic shape, excellent mechanical properties, good biocompatibility, customizable surface chemical qualities, and interesting optical properties. And all of them are attracting more and more attention in a series of applications related to the fields of materials science and biomedical engineering. Cellulose macromolecules have four hydroxyl (OH) groups at one end and a carbonyl (aldehyde) group at the other end, usually in the form of a hemiacetal, the only carbonyl group present in cellulose. Although it has three hydroxyl groups per anhydroglucose unit (AGU), cellulose is insoluble in water. Because these groups participate in intramolecular and intermolecular hydrogen bonds, resulting in complex and highly ordered networks with high crystallinity. Nanocellulose can be divided into three types of materials: the first is cellulose nanocrystals (CNCs), also known as nanocrystalline cellulose (NCC) and cellulose nanowhiskers (CNWs), and the second is cellulose Nanofibrils (CNFs), also known as nano-fibrillated cellulose (NFC), and a third type is bacterial cellulose (BC). Due to the different extraction methods used, the nanoparticle crystallinity and surface chemical properties and mechanical properties are not the same.(Lavoine et al., 2012; Missoum et al., 2013; Pritchard et al., 2022; Vanitha & Kavitha, 2019)

In this PhD thesis, nanofibrillated cellulose (NFC) is one target in the study. NFCs are micrometre-long entangled fibrils containing amorphous and crystalline cellulose domains, and the entanglement of long particles produces highly viscous aqueous suspensions at relatively low concentrations (less than 1 wt.%). The nanofibrillated fibres have a uniform width of ≈3 nm and an average length of >500 nm, and a large number of charged groups exist on their surface. Cellulose fibres (mainly from wood) are mechanically broken down into micro and nano fibrils. It is prepared by applying shear force to "decompose" plant cellulose fibres in a water environment, that is, the water suspension of low-concentration wood or plant pulp is obtained by mechanical means such as a homogenizer. Of course, NFC can also be decomposed from sources that still contain a lot of hemicellulose and lignin. This category belongs to mechanical

processing (homogenization, grinding and milling) to obtain NFC. There is also a chemical treatment method (such as TEMPO oxidation) is also one of the means to obtain NFC.(Samarasekara et al., 2018; Weishaupt et al., 2015; Zaaba et al., 2021)

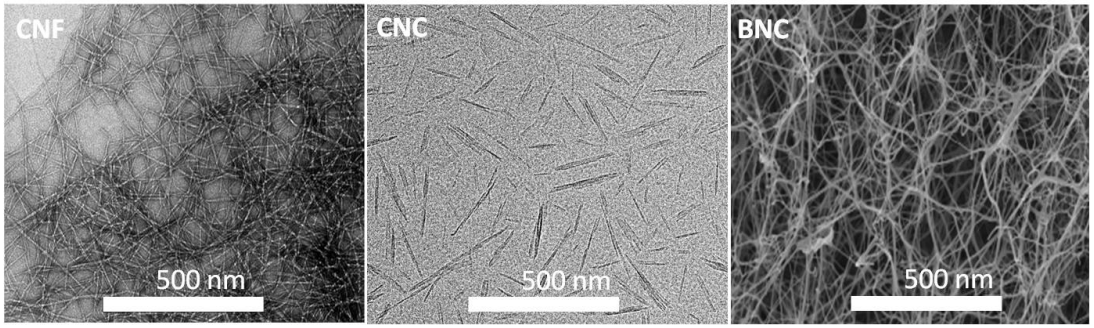

Figure 2.2: Scanning Electron Microscope Image of The Structures of Cellulose Nanofibers, Cellulose Nanocrystals and Bacterial Nanocellulose. Quoted from Thomas, S., & Pottathara, Y. B. (2020). Nanocellulose based composites for electronics. Elsevier, p.2 (Pottathara, 2020)

In addition to natural polysaccharides that are directly used in packaging materials, there are also polymer materials formed by processing natural biomass through synthetic techniques. PLA polylactic acid belongs to this type of material. High-performance lactic acid polymers are produced by fermenting carbohydrate-rich substances (such as long rice, sugar beets, cassava and other crops and organic waste) with certain strains of bacteria into lactic acid, and then undergoing cyclodimerization of monomeric lactic acid or direct polymerization of lactic acid. In addition, there are PHA, PBS/PBSA, etc., for example.(Alippilakkotte & Sreejith, 2018; Conn et al., 1995; Dong et al., 2012; Jem & Tan, 2020; Marichelvam et al., 2019) Compared with cellulose, the research and application of this type of product has only begun to take shape and become popular in recent years. In the current large-scale preparation of polylactic acid, there are many artificial synthesis methods. The more mature one is the direct polycondensation method of lactic acid, and the other is to synthesize lactide from lactic acid first, and then ring-opening polymerization under the action of a catalyst. There is also a method of solid-state polymerization.

And the PLA material itself is considered to be comparable to other traditional plastics such as PP and PET in terms of mechanical, physical, biocompatibility and processing properties, which are suitable for industrial use. Polylactic acid also has subtle

differences between PLLA and PDLA due to the asymmetric carbon atoms in the lactic acid molecule.(Jem & Tan, 2020)

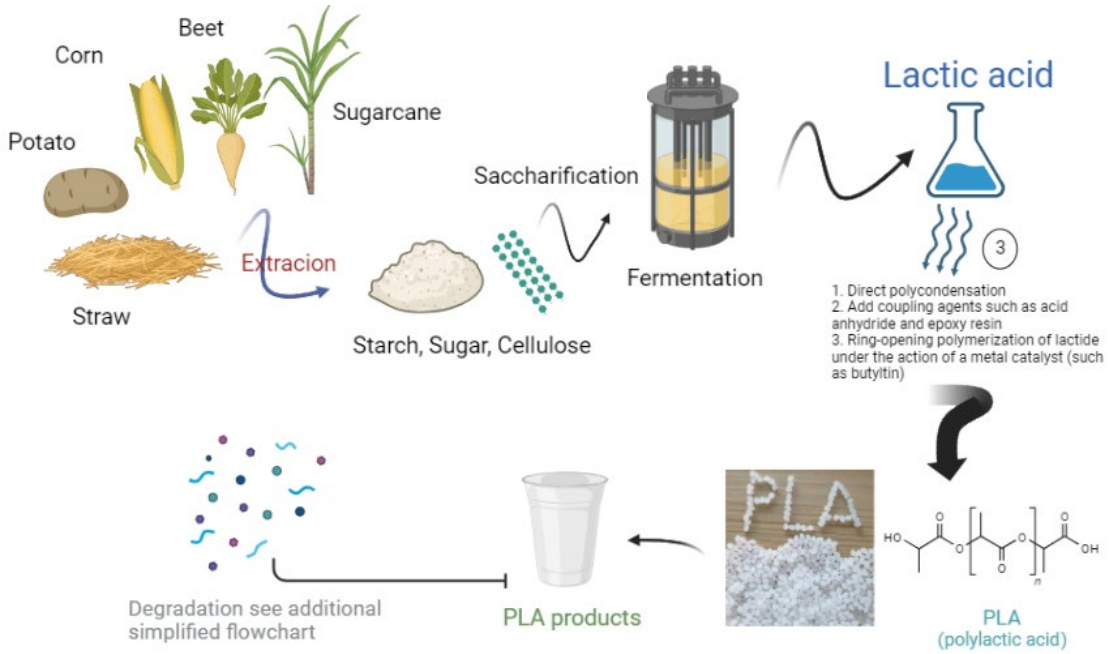

Figure 2.3: Simple production process of PLA polylactic acid

2.2.2 Proteins

Proteins are macromolecular biopolymers due to the complexity of their composition and structure. They have diverse functional properties attributed to their high intermolecular binding capabilities through different bond types. The secondary, tertiary, and quaternary structures of proteins lead to distinct interactions and bindings that vary in location, type, and energy. Protein-based edible films have better mechanical properties than other sources. At the same time, the protein structure can be changed by various chemical and physical treatments and reagents. The film-forming ability of protein molecular materials depends largely on their molecular properties, such as molecular weight, conformation, charge, flexibility, and thermal stability. The degree of elongation, the nature of the amino acid sequence, and the distribution and sum of interactions between protein chains are closely related to the mechanical properties of the film. In addition, packaging films based on protein materials can serve as carriers for active compounds due to their amphiphilic nature.

Several studies focused on controlling the release of active mass transfer across membranes have also demonstrated their effectiveness. Raw materials for the development of protein-based films can be extracted from animal and plant sources, by-products, and wastes, which, in addition to increasing the wide range of sources of protein materials, also help to increase the economic value of by-products and reduce the pressure on the environment.(Gómez-Estaca et al., 2016; Lacroix & Vu, 2013; Mihalca et al., 2021; Wihodo & Moraru, 2013; Y. Zhao et al., 2021; Zink et al., 2016)

Materials being investigated for the development of protein-based packaging films and coatings can be divided into two categories: vegetable proteins and animal proteins.

Soy protein, wheat gluten, zein, leguminous plant seed protein, etc. are commonly used in vegetable protein, and there are many sources. At present, research on the film-forming properties of these vegetable proteins is also extensive. For example, soybean protein is often highly refined into soy protein isolate (SPI) for edible packaging. Although the soy protein films produced from different soy protein fractions exhibited different properties, the increase in the molecular weight of soy protein resulted in an increase in tensile strength and elongation, but no change in the water vapor barrier properties. In addition to soybeans, gluten present in wheat, barley, and rye is also a common research material. According to the solubility of gluten in aqueous alcohol solution, gluten protein is mainly divided into two groups: gliadin and glutenin. The former is soluble and the latter is insoluble. Previous studies have shown that the uniformity of gluten films can be controlled by adjusting the alkaline or acidic conditions of the film-forming solution. The tensile strength of films prepared from alkaline solutions was significantly higher than that of films prepared from acidic solutions. Although from the perspective of the demand for source materials, plant protein is an important source for the total protein demand of the whole human being, and high-quality plant protein has shown that it can reduce various health problems caused by animal protein. But most of the vegetable proteins currently in the food industry are isolated from wheat and soy which belong to the eight major allergen groups. Vacuole protein (P34), beta-conglycinin, glycinin, omega-

5 prolamin, alpha-amylase inhibitor prolamin, etc. are contraindicated in soy sensitive patients and gluten sensitive patients. Therefore, this thesis ultimately locks the source of protein in animal sources.(Estell et al., 2021; Hadidi et al., 2022)

As for materials of animal origin, collagen and gelatine are extracted from waste from the meat and fish processing and poultry industries. Casein is mainly extracted from milk, along with by-product whey protein. In addition, egg white protein and keratin are currently research hotspot materials in food packaging. Milk protein (approximately 80% casein and 20% whey protein (WP)) is considered an essential protein source for humans. Casein micelles are porous enough to hold large amounts of water and their surface contains Several active sites to react with other functional groups. Casein films have been studied as anticoagulant and antidenaturing biomaterials. It is one of the candidates for good stability under various parameters such as pH, temperature and salt/sugar content. Whey protein has been used as a food supplement, or for drug delivery, biopolymer construction, etc. Whey protein-based films also exhibit excellent film formation and excellent barrier properties against aroma, oxygen/gas and oil at low or moderate relative humidity. The main disadvantages of WP-based films are their high water vapor permeability (WVP) and low mechanical strength compared to casein films and some other vegetable protein-based biopolymers.(Antoniak et al., 2022; Chen et al., 2019; Gorbunova & Zakharov, 2021; Schmid & Müller, 2018)

On the other hand, egg white protein (EWP) is widely used as an ingredient in food formulations due to its numerous nutritional and functional properties as gelling, emulsifying and foaming agents. When used as a packaging film, it is less brittle and flexible, and although less water resistant, it has higher rigidity, heat resistance and oxygen barrier properties. More by-products of animal origin mainly come from the by-products of industrial intensive processing of meat, fish and poultry such as bones, skin and offal. These meats are not considered edible in typical Western food culture. It is estimated that over 20 million tons of by-products are produced annually in Europe alone. These by-products are often used as low-value feed, fertilizer, pet food, or

discarded, which is not only a waste of resources, but also causes environmental pollution. So it makes sense to recycle it and use it in the creation of high-value food packaging.(Fuertes et al., 2017; Umaraw et al., 2020)

In terms of spatial structure, collagen shows a special three-strand helical winding structure. The three independent collagen peptide chains rely on the hydrogen bonds formed between glycine to maintain the triple helix intertwined structure. The general molecular weight is around 300 kDa, and 29 types have been identified so far. Among them, type I collagen is an important one, having a fibrous structure with a high aspect ratio. Through additional cross-linking methods or high-pressure homogenization, some researchers have found that films prepared with smaller-sized collagen fibres have a more uniform and denser structure. The mechanical properties and waterproof performance of the collagen film are significantly improved with the reduction of the fibre size.

Smaller molecular weight products such as gelatine and collagen peptides can also be derived from collagen. Gelatine, heat-denatured collagen with a molecular weight (MW) of 15 to 250 kDa, exhibits unique rheological properties and is commonly used in the food industry as a food additive, microencapsulation agent, and biodegradable packaging material.(Etxabide et al., 2017; Luo et al., 2022; Nur Hanani et al., 2014; Said et al., 2021)

Collagen peptides belong to the group of collagen hydrolysates representing a mixture of various amino acids and peptides of different sizes, obtained by chemical or enzymatic hydrolysis of collagen or gelatine. Due to its excellent biological activity, current collagen peptide products are more inclined to be used as synthetic materials in composite packaging. In this PhD thesis gelatine is used as the base material for food packaging film.

From the chemical structure of gelatine, it is composed of different polypeptide chains, such as α-chain (one polymer/single chain), β-chain (two covalently cross-linked α-chains) and γ-chain (three covalently cross-linked α-chain). During processing, gelatine exhibits its cold-setting and heat-reversible properties. At temperatures below 35 to

40°C, it becomes gel-like, and above its critical gel temperature, it can transform into a sol state. However, the long-time boiling state will cause its molecules to decompose and produce property changes, which cannot be restored after cooling.(Y. Cheng et al., 2023; Luo et al., 2022; C. Tang et al., 2022; Toniciolli Rigueto et al., 2022; Tyuftin & Kerry, 2021; L. Z. Wang et al., 2007)

In general, two types of gelatine, type A (pI ≈ 8-9) and type B (pI ≈ 4-5), can be obtained according to two different acidic or alkaline pretreatment conditions. When collagen is hydrolysed into gelatine with alkaline, glutamine may be deamidated to glutamic acid and asparagine may be deamidated to aspartic acid.(Zink et al., 2016) Although collagen denatures and loses its native structure during gelatine manufacturing, the resulting fragments can still form a partially ordered collagen-like triple helix structure during cooling. In terms of structure, gelatine has excellent physical properties, such as jelly force, affinity, high dispersibility, low viscosity characteristics, dispersion stability and water retention. Of course, different sources will also cause different physical and chemical properties of gelatine. The current production of gelatine raw materials mainly comes from different animal by-products. The three main sources are bovine (ox bone and hide) and pig (pork bone and hide) and fish (fish bones and scales). Among them, the gelatine source material from pigs is a more acceptable material in the commercial market due to factors such as low production costs and relatively fast reproductive speed of pigs themselves. Of course, in the field of gelatine products, fish-derived gelatine has become one of the most promising substitutes for mammalian gelatine due to its fast dissolution rate and low melting point.(Kchaou et al., 2018; Park et al., 2021)

Except for gelatine, which is relatively mature in commercial production and consumption, plasma protein is very small as a protein source for food packaging substrates. But it does also help to increase the value and environmental protection of the by-product, which can be considered one of the most problematic products in the food industry due to the large volumes produced and the high polluting capacity of blood. Plasma is widely used in the food industry as a binder for meat products, a pasta

fortifier, a fat substitute, etc. The research on using plasma protein as a biodegradable or edible film material is also one of the few attempts in recent years. (Del Hoyo et al., 2008; Nuthong et al., 2009)Plasma protein refers to the protein in plasma except haemoglobin, accounting for 7-8% of the total plasma. Plasma proteins can be divided into several components such as albumin, globulin and fibrin. At present, the main sources of research involving plasma proteins as thin film materials focus on pig blood and bovine blood. In addition, unlike blood protein, red blood cells (containing 35% of the key component haemoglobin) are removed, and plasma protein has no home iron, so the colour depth and metallic taste brought by haemoglobin will have less impact on food packaging.(Bah et al., 2013)

2.2.3 Active substances and additives

Factors such as the economic development of the current society and the requirements for environmental protection require food packaging to be continuously updated to meet the continuously developing market demand. The demand of the market and consumers for food packaging has gradually shifted from traditional distribution, product protection and convenience to higher requirements such as improving food quality and extending the shelf life of packaged food. Therefore, in the case of determining degradable or edible packaging source materials, in addition to helping food storage and protection from the physical level, researchers also hope to help improve the safety and quality of packaged food from the biological or chemical level. The incorporation of active substances will help realize this idea. Generally speaking, the functions of active substances are mainly divided into the following categories: deoxidation, anti-oxidation, antibacterial activity, deodorant and fragrance-preserving activity, etc.(Dainelli et al., 2008; Peidaei et al., 2021)

Its principle of action can be mainly divided into two types: absorption line and release type. The type of active substance is generally determined based on the type of product that needs to be packaged. Based on this thesis, the protein-based packaging film is mainly used as a packaging film. Due to the closeness of properties, the food

suitable for preservation as a primary packaging is more inclined to meat products rather than fruits and vegetables. However, meat is prone to the growth of microorganisms. The growth of microorganisms will lead to product spoilage and shorten the shelf life. If there is contamination by pathogenic bacteria, it will also bring health risks to consumers. Therefore, how to help inhibit bacteria has become a problem that needs to be solved in meat product packaging. Five common antibacterial active substances include essential oils, enzymes/bacteriocins, antibacterial polymers, organic acids and other organic compounds, and antibacterial nanoparticles (mainly metal ions). They act on bacteria, molds and fungi commonly found in microbial contamination of meat products, such as Gram-negative bacteria *Escherichia coli*, Gram-positive bacteria *Staphylococcus aureus*, etc.(Peidaei et al., 2021)

In this work, three different types of antibacterial substances such as essential oil thymol, bacteriocin Nisin, and bacteriophage *phiIPLA-RODI* are used to help food storage prolong.

Thymol (2-isopropyl-5-methylphenol) got its name from being found in thyme. A phenolic derivative of p-cymene, a major monoterpene phenolic genus isolated from essential oils of plants belonging to the Labiatae family (thyme, basil, oregano, and mint). Its molecular formula is $C_{10}H_{14}O$, which is isomer with carvacrol, soluble in organic solvents such as ethanol, slightly soluble in water and glycerin. Due to their antibacterial and antioxidant properties, essential oils extracted from the above species have been used since ancient times as flavoring agents and preservatives in the food industry. And it has been certified by the European Union and the US Food and Drug Administration as a safe additive that will not cause health risks to consumers.(Heckler et al., 2020; Pivetta et al., 2018)

Nisin is a bacteriocin produced by a group of Gram-positive bacteria belonging to Lactococcus and Streptococcus. Nisin is classified as a type A (I) lantibiotic and is synthesized from mRNA. The translated peptide contains several unusual amino acids due to post-translational modifications, namely anhydrous amino acid residues (serine

and threonine) and thioether amino acids forming five lanthionine rings. Over the past few decades, nisin has been widely used as a food biopreservative. In 1969, Nisin was approved as a safe food additive by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO). As a broad-spectrum bacteriocin, nisin is mainly effective against Gram-positive foodborne bacteria.(de Souza de Azevedo et al., 2019; Field et al., 2015; Gharsallaoui et al., 2016; Shin et al., 2016)

Phages are viruses that can infect and kill bacteria, but are harmless to humans, animals and plants. This makes them a safe alternative to traditional antimicrobials. And since some bacteria are increasingly resistant to inhibitors such as antibiotics, using phages to combat them constitutes an effective alternative. Phage *phiIPLA-RODI* is a newly isolated virus species in 2015 and belongs to the family Myoviridae. Gene organization is typical for members of the Spounavirinae, with long immediate terminal repeats (LTRs), gene grouping into modules that are not clearly separated from each other, and several group I introns. Furthermore, four genes encoding tRNAs were identified in *phiIPLA-RODI*. Comparative DNA sequence analysis revealed high similarity to two bacteriophages, GH15 and 676Z, belonging to the genus Twort-like viruses (>84% nucleotide identity)(González-Menéndez et al., 2018)

A plasticizer is a class of substances that are added to a material to make it softer, more flexible, and increase the plasticity of the material. When making bio-based packaging materials, most polysaccharide and protein-based films and coatings are inherently fragile. To solve this problem, plasticizers are added to enhance the flexibility and elasticity of the film. It is generally accepted that the correct choice of plasticizer for a given polymer can optimize the mechanical properties of the film while minimizing the film's permeability. Due to the need to be biodegradable or edible, the selected types of plasticizers mainly include monosaccharides, oligosaccharides, polyols, lipids and derivatives. And the choice of plasticizer needs to consider issues such as plasticizer compatibility, efficiency, durability and economy. Glycerine is a simple polyol compound. Due to its three hydroxyl groups, glycerine is miscible with water and has

hygroscopicity. As an additive (sweetener, humectant, etc.) widely used in food and drug fields, food-grade glycerine is usually Recognized as safe (GRAS), the use of glycerol as a plasticizer for packaging films in this paper helps the packaging film to have better structure.(Azelee et al., 2019; Tarique et al., 2021)

2.2.4 Common infectious bacteria in food packaging

Foodborne illnesses are usually contagious or toxic and are caused by bacteria, viruses, parasites, or chemicals entering the body through contaminated food or water. *Salmonella, Campylobacter*, and *E. coli* are among the most common foodborne pathogens, affecting millions of people each year. Foodborne cases of *Campylobacter* infection are mainly caused by raw milk, raw or undercooked poultry, and drinking water. EHEC has been associated with unpasteurized milk, undercooked meats, and fresh fruits and vegetables. Listeria infection, which can cause miscarriage in pregnant women or death of newborn babies, is commonly found in unpasteurized dairy products and a variety of ready-to-eat foods and may grow at refrigerated temperatures. Orphan cholera infects people through contaminated water or food and can cause severe dehydration and death. Rice, vegetables, millet porridge, and various seafood have been implicated in cholera outbreaks. *Staphylococcus aureus* can produce enterotoxin and cause food poisoning. It is the third largest microbial pathogen after *Salmonella* and *parahaemolyticus*. In this thesis, the simulated confrontation is *Escherichia coli* and *Staphylococcus aureus*.(Todd, 2014)

Escherichia coli belongs to the category of coliform bacteria, and the coliform bacteria in general food exceeds the standard, which means that the food has been contaminated by feces. *Escherichia coli (E. coli)* normally lives in the gut of healthy humans and animals. Most types of *E. coli* are harmless or cause relatively short-lived diarrhoea. But a few strains, such as E. coli O157:H7, can cause severe stomach cramps, bloody diarrhoea, and vomiting.(Rani et al., 2021) The skins of raw fruits and vegetables and undercooked meats are possible sources of *E. coli* infection.

Staphylococcus aureus often exists in mucous membranes and feces such as the skin,

hair, nasal cavity and suction mouth of the human body, especially in suppurative wounds, so it is very easy to contaminate food from the human body. Some strains have the ability to produce heat-stable enterotoxins that persist in food even when it is cooked, causing staphylococcal food poisoning. *S. aureus* can easily multiply on foods that have been manually processed but not reheated. Improper storage of food can also cause the bacteria to multiply rapidly and produce toxins on foods, such as salads, sandwiches, ham, etc.(Y. Y. Yong et al., 2019)

2.3 Advantages and disadvantages of the biomaterials

The main advantage of using biomass materials is environmental protection. By replacing plastic and metal food packaging, reducing recycling costs and pollution, it reflects the purpose of sustainable development. The reduction in the use of petrochemical packaging materials and the reuse of agricultural organic waste are complementary and synergistic. In addition, the use of bio-mass materials undoubtedly expands material selectivity, enriches diversity, and reduces dependence on plastics and metals. As for the market, it gives consumers more choices and will promote the upgrading of the industrial chain. At the same time, it can also give scientific researchers the opportunity to develop new materials. Several characteristics and feasible applications of materials studied in this work are commented.

2.3.1 Applications and advantages

a) Nanofibillated cellulose

Nanofibrillated cellulose (NFC) has the advantages of degradability, pollution-free, light weight, high strength, high stability, fine nanostructure, good mechanical strength and low thermal expansion coefficient. In the field of food packaging, based on the high crystallinity, specific surface area and mechanical strength of nanocellulose, it can be used as a nanofiller mixed with other biomass materials to help enhance the mechanical properties. Based on the advantages of stable structure, surface

modification diversity and good biocompatibility, it can be used as a good carrier material and skeleton material to help enhance the functionality of packaging materials. In this PhD thesis, the high toughness, stability, and mechanical structure of NFC are utilized to help enhance the structural strength of biomatrix membranes.

b) Polylactic acid

PLA can be produced from renewable resources such as cornstarch or other carbohydrate sources. Its renewable, recyclable, biodegradable and compostable properties are considered to be able to replace traditional petroleum-based polymers. PLA has excellent transformation ability and is suitable for processing by various methods. Injection molding, film extrusion, blow molding, thermoforming, fibre spinning, and tape casting are all techniques used to process PLA. Good odor and flavour barrier properties, good twist rate or folding performance, and heat shrinkable packaging quality are the basic guarantees for this material to become a food packaging material.(Conn et al., 1995; Jama, 2017; Mulla et al., 2021)In this thesis, PLA is used to make nanoparticles for slow release of active substances. Used to help increase the bioavailability of active substances in food packaging, minimize volatility and extend the shelf life of bioactive molecules.

c) Gelatine

Gelatine has been widely used in various industries. It has a stable and mature business development foundation. It has long been used as a food ingredient (such as gelling agent and foaming agent), preparation of pharmaceutical products (such as soft and hard capsules, microspheres) and biomedical fields (wound dressing and three-dimensional tissue regeneration), and even in many non-food applications (e.g. photography) (Baziwane & He, 2003).The advantages of gelatine materials as packaging are also reflected in the gelatinity and flexibility of the material itself. Some scholars have found that compared with polysaccharides (SA, CMC and PS), films made of proteins (WPI and gelatine) are more resistant to solvents. While gelatine films have the greatest flexibility.(L. Z. Wang et al., 2007) In addition, because gelatine film generally has relatively high light transmittance, it can better maintain the appearance

of food as food packaging.

d) Blood plasma

Plasma proteins obtained from porcine and bovine plasma consist of albumin, globulin and fibrinogen. It has the characteristics of gelation, foaming and film formation. The combination of protein substrates with polysaccharides or hydrophobic materials or other polymers may provide a simple alternative to improve the water resistance and mechanical properties of composite films. At present, the research on using animal plasma protein as food packaging is a relatively new direction.

2.3.2 Disadvantage

Compared with fossil-based materials, bio-based and biodegradable materials usually have poor water vapor barrier properties and mechanical properties, thermal stability, and processability. Therefore, it is challenging to improve the barrier and mechanical properties of packaging without compromising biodegradability. The use of commercially available biopolymer films is mainly for products with a short shelf life or perishable products, such as fruit and vegetable products that need to breathe and retain humidity.

In addition, the consideration of the safety of bio-based packaging is also essential. Those food packaging materials that may involve toxic solvents or processes, and the possible migration of harmful substances into food during the packaging process, need to be further quantitatively evaluated and should meet the requirements of specific food safety regulations.

2.4 Production technologies for Bioplastics

At present, in addition to the large-scale production of bioplastics such as PLA, more bio-based materials in food packaging have mechanical problems such as insufficient elongation, poor safety and sanitation, high cost, and difficult processing. So, they are generally still in the stage of laboratory research.

Usually, the technology of producing packaging materials is manifested as casting

method at the laboratory stage. The bio-based material is typically dissolved in a solvent and poured into a casting mold to create a film ready for packaging. During the production of casting film, a large amount of solvent needs to be evaporated, so a long evaporation time is required. Therefore, this process is generally considered to be energy-intensive. At the laboratory level, both 3D printing and Electrospinning are representatives of new technologies and new development trends.

Continuous production, control of drying time, thickness control, reduction of energy consumption, and cost reduction all need to be considered on the industrial scale. "Dry methods" such as Extrusion, Blown film, and Hot pressing are the underlying technologies for large-scale industrial film production. Of course, unlike the laboratory stage, which is more inclined to develop new materials, industrial production is more inclined to mature technology, reasonable manufacturing procedures, and large-scale economic considerations. Pilot-scale studies are also necessary.

Chapter 3: Materials and methods

3.1. General Materials

3.1.1 Polysaccharides

- Nanofibrillated cellulose (NFC): Cellulose was bleached by the ECF (European Climate Foundation) industrial process from eucalyptus provided by the company ENCE (Navia, Asturias, Spain). Bleached cellulose was subjected to chemical and mechanical treatment to obtain nanofibrillated cellulose (NFC).

3.1.2 Proteins

- Gelatine: Purchased from Sigma-Aldrich, G1890, pigskin gelatine powder, gel strength ~300 g Bloom, Type A.
- Plasma proteins: Porcine and bovine blood were collected from local abattoirs (Asturias, Spain) immediately after slaughter. Add 2% (w/v) sodium citrate as an anticoagulant. Separate the plasma from the cellular fraction by centrifugation at 10,000 g and 10°C for 10 min. (Kubota Model 6500) Plasma, the supernatant from centrifugation, is decanted and stored at -20°C until use.

3.1.3 Additives and active substances

- Glycerine: Purchased from Sigma-Aldrich, ≥99.0% (GC), as a plasticizer
- Thymol: Purchased from Sigma-Aldrich, ≥98.5%, ref. T0501.
- Antimicrobial Peptides (Nisin): Purchased from Sigma-Aldrich, potency: ≥900 IU/mg
- Phage: Stock solution of bacteriophage *phiIPLA-RODI* (Gutíerrez et al., 2015) in TSB (Tryptic Soy Broth, ref. 22,902, Sigma-Aldrich, Germany) at a titer of 7×10^8 PFU/mL, donated by the Dairy Institute of Asturias IPLA-CSIC (Asturias, Spain).

3.1.4 Other reagents

- Polylactic acid (PLA): Purchased from NATUREWORKS Company, Ingeo™ Biopolymer 4032D.
- Dichloromethane (DCM, Ref. 270,997), Polyvinyl Alcohol (PVA, Ref. P8136), Buffer Trizma® pH 7.0 (Ref. T1819), 2,2-Diphenyl-1-picrylhydrazine (DPPH, Ref. D9132) and Nutrient Broth (Ref. 70149NB), Agar (Ref. 20767.298), Magnesium Nitrate (Ref. 237175), Ethanol 96% (Ref. 83804.360).

3.1.5 Common Foodborne Bacteria

- *Escherichia coli*: Non-pathogenic strain of *Escherichia coli CECT 101* (CECT, Colección Española de Cultivos Tipo, Spanish Type Culture Collection).
- *Staphylococcus aureus*: Staphylococcus aureus IPLA1 isolated from dairy industry contact surfaces (Gutíerrez et al., 2012) by Asturias IPLA-CSIC (Asturias, Spain)

3.2 Methods

3.2.1 Preparations of Bio-material ingredients

3.2.1.1 Nanofibrillated Cellulose (NFC)

NFC is obtained through two steps, step A "chemical pretreatment" and step B "physical pretreatment". Before processing, the cellulose raw material is as shown in the Figure 3.1

Figure 3.1: Bleached eucalyptus cellulose from the company ENCE (Navia, Asturias, Spain)

Step A: "Tempo" pretreatment

In the pretreatment stage, the use of "TEMPO" (2,2,6,6-tetramethylpiperidin-1-oxyl) reagent to oxidize cellulose fibres can reduce the energy consumption in the mechanical treatment stage. Currently, "TEMPO" oxidation process is one of the most studied cellulose pretreatment processes.

5 g of cellulose, soaked in 400 ml of distilled water for 12 hours to soften. Before processing, the cellulose was stirred for 4 hours to form a slurry. Magnetic stirring was used to mix the slurry in real time, and a pH meter was used to detect pH changes at 25 °C. Then 0.06 g TEMPO and 0.6 g NaBr were added and after complete dissolution, 100 mL of NaClO (6-14% active chlorine EMPLURA® Sigma-Aldrich, Germany) was slowly added. The mixture was kept in contact for 3 hours while the pH was not lower than 10.0 and readjusted with 1.0M NaOH.

After 3 hours the reaction was stopped by lowering the pH to 7.0 with 1.0 M HCl. Centrifuged at 8000 rpm for 1 hour, removed the supernatant, and washed the cellulose with 1 L of distilled water, twice. The centrifuge temperature was maintained at 8°C.

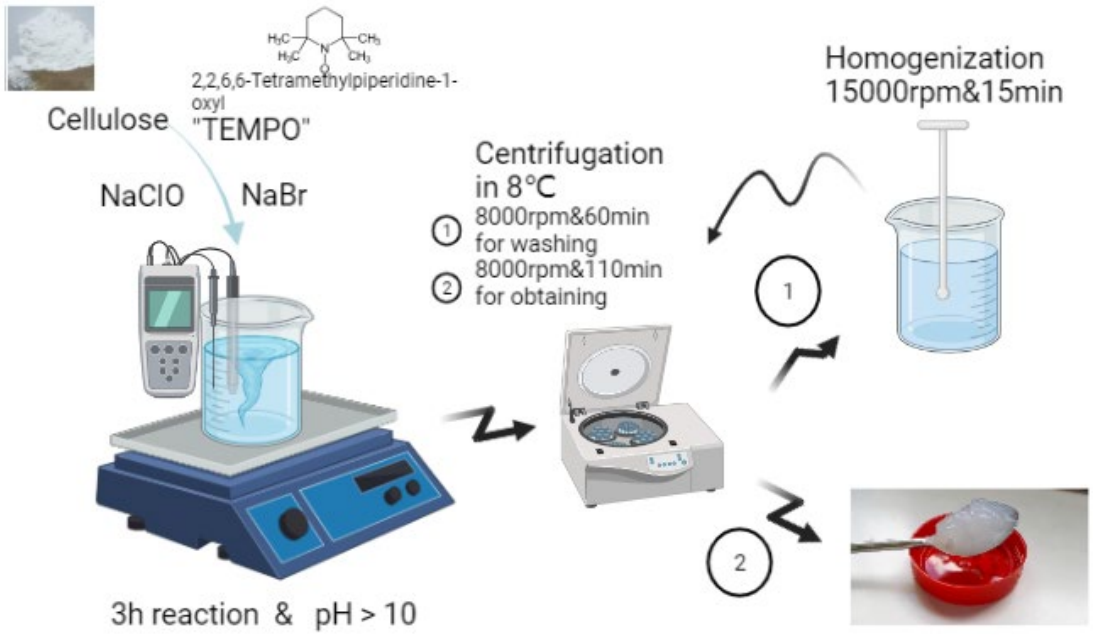

Figure 3.2: Simple illustration process for making NFC gel

Step B: Homogenization

The first step was to redisperse the cellulose in 400 mL of distilled water using a homogenizer (SilentCrusher M, Heidolph, EE. UU.) at 15,000 rpm for 15 min. Then the slurry was filtered through a 250 µm pore size sieve to remove unbroken coarse fibre clumps that may be present. Finally, the liquid was centrifuged at 8000 rpm for 110 min to increase its concentration removing the excess of water. The centrifuge temperature was maintained at 8°C. The resulting gel was stored in a 4°C refrigerator until use.

3.2.1.2 Preparation of thymol-loaded PLA nanoparticles

PLA nanoparticles were prepared following the single emulsion preparation technique (Lee et al., 2016) with slight modifications.

In the first step, a 1% PVA solution was prepared in a 90 °C water bath. After the solution had cooled to room temperature, 2 mg/mL thymol was added and the mixture was stirred overnight. N°1 Whatman filter paper was used for filtration to

remove undissolved thymol. Filtration was repeated twice.

After that the PLA pellets were dissolved in 7.5 mL of Dichloromethane (DCM) and mixed the DCM solution with 30 mL of the previously prepared PVA solution. After that the mixture for 2.5 min (Sonopuls HD 2070 system, Bandelin, Germany) was sonicate with an MS 73 probe at a frequency of 20 kHz and an ultrasound amplitude of 90% (100% corresponds to 212 µm). This sonication amplitude corresponds to a sonication intensity of 80 W/cm2. During sonication, samples were kept on ice to avoid temperature rise. The emulsified solution was kept in a low-pressure medium at 40 °C for 40 min using a rotary evaporator (Büchi R-205, Büchi Labortechnik, Essen, Germany) to remove the DCM.

Finally, samples were centrifuged at 13,000 rpm for 20 minutes. Removing the supernatant, adding the same volume of distilled water to the pellet, resuspending the nanoparticles and centrifuging again at 4000 rpm for 5 min. Finally, the sediment was discarded to remove PLA aggregates to obtain a suspension with PLA nanoparticles encapsulated thymol.

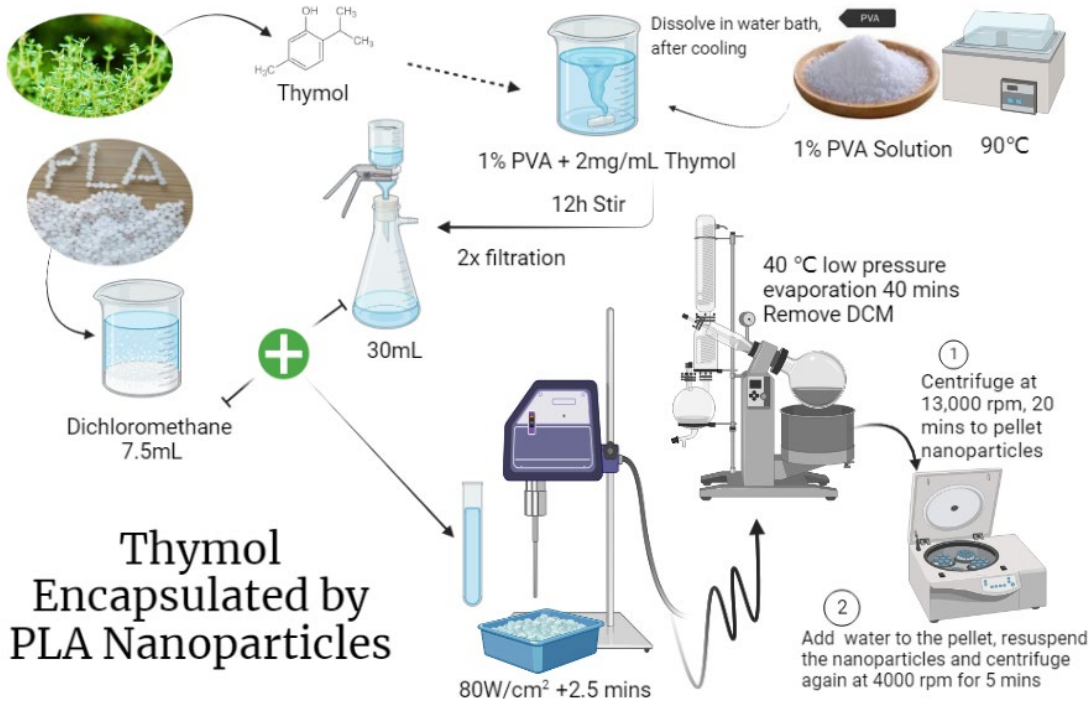

Figure 3.3: A brief process of encapsulating thymol into PLA nanoparticles

3.2.1.3 Obtaining lyophilized plasma protein and modifying the protein by ethanol acidification

- Freeze-dried plasma:

Sodium citrate (2% (w/v) (Sigma-Aldrich) was added to fresh blood provided by a local slaughterhouse (Macelo de Asturias, S.A., Asturias, Spain) as an anticoagulant. Passed at 10 Centrifuge at 10,000 × g for 10 minutes at °C to separate the plasma from the cell fraction. Use a 14 kDa cellulose membrane (Dialysis Membrane, Sigma-Aldrich) to remove residual salt components in the plasma. Freeze the plasma at -80°C for 12 hours, and then lyophilized (Telstar Cryodos, 0.1 mBar, -70°C, 24 hours) into powder.

- "Acidified and alcoholized" precipitated plasma:

1.5 g of freeze-dried plasma powder was dissolved in 50 mL of distilled water. The pH was adjusted to 2.5 with HCl 3.0 M. The plasma was acidified plasma adding 400 mL of 96% ethanol and the pH adjusted to 1.5 with 3.0 M HCl. The mixture was centrifuge at 10,000 × g for 30 min at 10 °C to collect the precipitate.

3.2.2 Methods of film preparation

3.2.2.1 PLA nanoparticle gelatin film encapsulating thymol

150 mg thymol and 150 mg polylactic acid were dissolved in 7.5 mL dichloromethane. The solution was emulsified in 30 mL of polyvinyl alcohol solution by sonication to prepare encapsulated nanoparticles.

A stock solution of 6% (w/v) pigskin gelatine was prepared in a 65 °C water bath. Glycerine was added in an amount corresponding to 30% (w/w) of the gelatine powder. 1%, 2% and 3% (based on the weight of gelatine powder) PLA nanoparticles and thymol film-forming solutions were prepared. And the film-forming solution was dried at room temperature for 2 days in a petri dish with a diameter of 4 cm (each piece contained raw gelatine 216mg). Finally, the obtained films were stored at room temperature in a desiccator containing $Mg(NO_3)_2$.

3.2.2.2 Fabrication of bovine and porcine plasma protein films

Alcoholised and acidified plasma proteins (bovine/porcine) were formulated into a 0.3 g/mL (w/v) film-forming solution. Add 65% (w/w) glycerol as a plasticizer. 8.85 mg protein/cm^2 of this film-forming solution was poured into a Petri dish. Films were dried overnight in an oven at 40 °C.

The control film was prepared with 30 g of freeze-dried plasma protein powder dissolved in 100 mL of distilled water. Then, it was prepared using the same glycerol ratio and drying procedure. Protein content in pellets was determined by the Dumas combustion method using a CNHS/O Elementar Vario EL analyzer (Elementar, Germany).

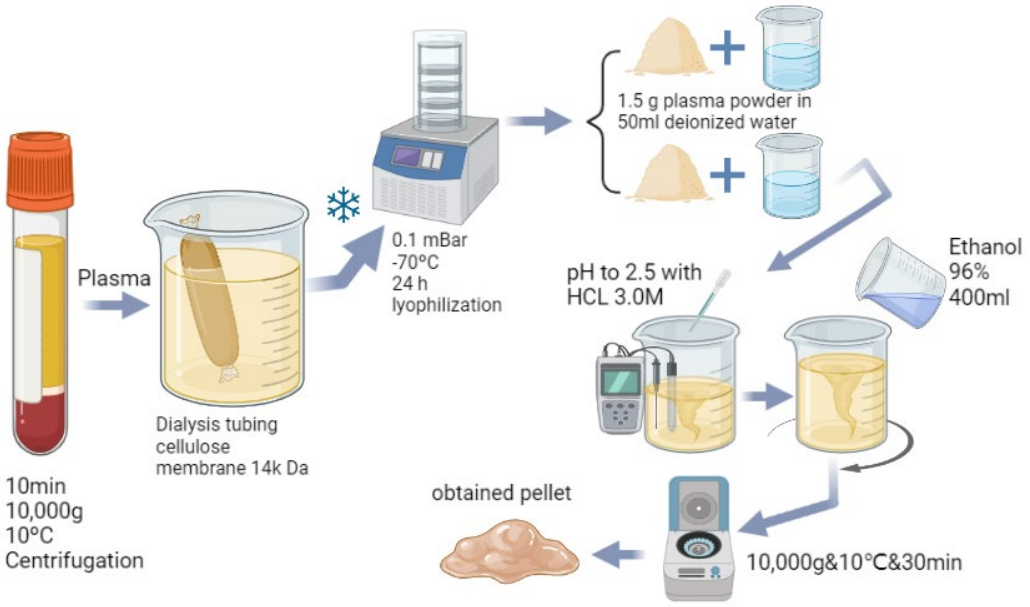

Figure 3.4: A Simple Graphical Workflow for Plasma Protein Production

3.2.2.3 Bovine plasma protein film mixed with NFC

The film group obtained from bovine plasma protein prepared by acidification and aldolization precipitation is called “Group FA” for short. The abbreviation of directly using freeze-dried plasma protein composition is called “Group FL”. And the control group with only NFC is the “Group FC”. Determination of protein concentration

followed the preparation mentioned in section 3.2.2.2.

Both proteins were dissolved in distilled water and stirred at room temperature for 30 minutes. Three different percentages of NFC (10%, 30%, and 50%), by weight, were added to different proportions prepared from FA plasma proteins (FA90FC10, FA70FC30, and FA50FC50) and FL plasma (FL90FC10, FL70FC30, and FL50FC50). The NFC film is 100% nanofibrillated cellulose (FC). In the film solution, the solid concentration of the film forming solution was kept at 0.030 g/mL. In all cases, glycerol (Sigma-Aldrich) was used as a plasticizer and it was added to the solution at 70% (w/w solids). All film-forming solutions were cast on silicone molds and dried at 37°C for 24 hours.

3.2.2.4 Gelatine film containing bacteriophage

Stock solution of phage *phiIPLA-RODI* in TSB (Tryptic Soy Broth) medium with a titer of 7×10^8 PFU/mL, 10% gelatine and 2% glycerol (w/v) were stirred for 20 min at 50°C. The solution was cooled at 40 °C and filtered under sterile conditions using a 0.45 µm pore size syringe filter. The phage stock solution was added and stirred gently for 5 minutes to obtain a film-forming solution containing phage. The final concentrations of phage tested in the film-forming solution were 1.75×10^8 PFU/mL (GF1), 1.16×10^8 PFU/mL (GF2), and 6.35×10^7 PFU/mL (GF3). Finally, each aliquot of the film-forming solution was poured into a petri dish, and 0.11 mL of the film-forming solution was cast on the surface of the petri dish per cm^2. Film formation required 2 days of drying at room temperature in a sterile fume hood.

3.2.3 Antibacterial films

3.2.3.1 *Escherichia coli*

- PLA nanoparticles (+Thymol):

  Non-pathogenic *E. coli strain CECT 101* was incubated in NB medium containing 2% agar at 30°C for 48 hours. After, the bacteria were transferred to NB broth,

they were incubated on a shaker (250 rpm) at 30°C for 10 hours. Each 1 g apple slice was inoculated with 100 µL of *E. coli* and allowed to stand for 2 minutes. The concentration of *E. coli* used in this experiment was 10^5 UFC/mL. Apple slices were immersed in 5 mL of a solution containing 2 mg/mL thymol or thymol-loaded nanoparticles for 1.5 min. (Concentration of dissolved thymol was determined by mixing 0.1 mL of thymol filtered solution with 9.9 mL of ethanol and measuring the absorbance at 275 nm.). Apple slices were sealed in polyethylene boxes and stored at 5 °C until analysis. The collected samples were mixed with 9 mL of 0.7% NaCl. The samples were then homogenized by a blender (Stomacher 80 Biomaster, Seward Ltd, Worthing, UK) and cultured in NB-agar medium. After being cultured at 30°C for 24 hours, the *E. coli* colonies were counted, and the obtained results were expressed in log10 UFC/g.

- Gelatine films (+Thymol):

  Similar to the procedure above, 1 g apple slices were inoculated with non-pathogenic *Escherichia coli CECT 101* (CECT, Spanish Collection of Type Cultures). The strain was grown in NB (Nutrient Broth) medium supplemented with 2% agar at 30°C for 48 hours. Then, the bacteria were incubated in NB broth for 10 h at 30°C with orbital agitation at 26.17 rad/s. Apple slices were inoculated with 0.1 mL of this solution with an *E. coli* concentration of 10^5 CFU/mL. These apple slices are covered with gelatine film and sealed using a heat sealer. Each sealed apple slice was stored in a Petri dish at 5°C for 14 days. After the film was removed, the apple slices were mixed with 9 mL of 0.7% NaCl and the mixture was ground to 120 µL using a Stomacher (IUL Instruments). The obtained liquid samples were diluted and inoculated in NB medium containing 2% agar. After 24 hours of incubation at 30°C, colonies were counted and expressed as log10 CFU/g.

3.2.3.2 *Staphylococcus aureus*

- Plasma+NFC film (+Nisin):

  Staphylococcus aureus CECT 240 (from the Spanish Type Culture Collection,

Valencia, Spain) was grown in 100 mL of TSB (Tryptone Soy Broth, Sigma-Aldrich) in an orbital shaker at 200 rpm and 37 °C for 24 h. Then, 50 g of meat (purchased at a local market) was cut into squares. Each piece was infected with 100 µL of 105 CFU/mL of *S. aureus* in a 0.7% NaCl solution (Sigma-Aldrich). After the liquid had dried, the pieces of meat were coated with films loaded with 3 mg/mL of nisin (1000 IU/mg; Sigma-Aldrich) (FA90FC10 films). All pieces were stored in a fridge and the growth evolution of *S. aureus* was analysed at different times (0, 1, 3, 6, 10, and 15 days). For this purpose, the pieces of meat to be sampled were placed in a Stomacher™ bag (Seward, West Sussex, UK) with 10 mL of NaCl 0.7% (w/v) and were homogenized with a Stomacher™ device (Seward, UK) at maximum speed for 120 s. Microbial growth was analysed by preparing serial dilutions (1:10) and incubating on Baird-Parker medium enriched with egg yolk tellurite emulsion (both from Sigma-Aldrich) with 2% of agar (VWR) plates for 48 h at 37 °C. Each sample was carried out in triplicate and results were expressed in log10 CFU/g of meat.

- Gelatine film and coating (+*phiIPLA-RODI* phage):

  1. In vitro antimicrobial activity

  0.4 g of each film as described in section 3.2.2.4. Immerse in 100 mL of TSB liquid medium. with an initial concentration of *S. aureus IPLA1* of 10^6 CFU/mL. Once the film pieces were dissolved in TSB, the concentrations of phage in the liquid medium were 5.25×10^6 PFU/mL (GF1-TSB), 3.48×10^6 PFU/mL (GF2-TSB), and 1.90×10^6 PFU/mL (GF3-TSB). Two control groups, one with films made exclusively of gelatine (G) and one with TSB infected with *S. aureus* only (WB) were tested under the same conditions. These samples were incubated for 17 hours at 37°C with agitation at 250 rpm. The broth was diluted with 0.7% NaCl and inoculated on Baird-Parker agar medium (Sigma-Aldrich, USA) enriched with egg yolk tellurite emulsion. After culturing at 37°C for 48 hours, the colonies were counted, and the results were expressed in log10 CFU/mL.

  2. On cheese pieces contaminated with *S. aureus*

Fresh cheese is divided into cylindrical pieces weighing about one gram with a hollow punch. Each piece of cheese was infected with 100 µL of 10^5 CFU/mL of *S. aureus* in 0.7% NaCl solution. Contaminated cheese is processed in one of the following ways:

A. The phage stock solution in TSB was diluted to the same concentration of GF1, GF2, and GF3 in the film-forming solution as described in section 3.2.2.4. For each concentration, three tainted cheeses were tested. The cheese slices are submerged in one of the solutions and stirred gently for 3 minutes. Recovered and stored in airtight polypropylene tubes under refrigeration (4°C). A control sample containing contaminated cheese soaked in TSB but without phage was also tested.

B. Contaminated cheese slices were immersed in gelatine film-forming solutions containing different concentrations of bacteriophage. After 3 min, the fragments were recovered, dried at room temperature for 10 min, and stored in polypropylene tubes at 4°C. A control sample containing contaminated cheese (without phage) soaked in gelatine was also evaluated.

C. Gelatine films loaded with different proportions of phage were prepared as described in section 3.2.2.4, then sealed and stored at 4°C together with contaminated cheese blocks.

In all cases, samples were taken at time 0 and after 3 and 6 days. Blocks of cheese to be sampled were placed in sterilized plastic bags filled with 10 mL of 0.7% NaCl and ground for 120 s using a Stomacher (IUL Instruments, Barcelona, Spain). Finally, liquid samples were diluted and inoculated into Baird-Parker medium containing 2% agar. After culturing at 37°C for 48 hours, count the colonies, and the results are expressed in log10 CFU/m

3.3 Physico-chemical characterization of the Bioplastics

3.3.1 Light transmission and transparency

The barrier properties of the films against UV and visible light were evaluated according to the method of Dick et al. (2015). Briefly, films were cut into rectangular pieces and placed in spectrophotometer test cells. The transmittance of the samples was tested in the range of 280 to 800 nm using a Helios gamma spectrophotometer (Thermo Fisher Scientific, USA) with an empty test cell as a reference.

The transparency of the film is calculated according to equation:

$$Transparency = A_{600}/x \qquad \text{Equation 3-1}$$

where A600 is the absorbance of the film sample at 600 nm and x is the film thickness (mm). The thickness of the film was measured using a digital micrometre (Mitutoyo C., Japan) with an accuracy of ±1 µm. The thickness is measured at five different areas, one at the centre of the film and four at the film perimeter.

3.3.2 Mechanical properties

In order to know the two mechanical studied parameters of the film, namely the puncture strength (PS) and puncture deformation (PD). Performed using a texture analyser TA.XT.plus (Stable Microsystems, Surrey, UK) equipped with a 5 kg load cell and a 5 mm diameter probe (P/5S). For this, the films were cut into 40 × 20 mm strips and attached to the detection platform between two plates. Through the two plates, a 1 cm hole allows contact between the probe and the film sample so that the probe can stretch the film until it ruptures. The probe velocity was 1 mm/s. PS and PD parameters were calculated according to the following equations (Péerez-Mateos et al., 2009):

$$PS = Fm/Th \qquad \text{Equation 3-2}$$

$$PD = (\sqrt{D^2 + R^2} - R)/R \quad \text{Equation 3-3}$$

where Fm is the maximum force applied before the membrane ruptures, Th is the membrane thickness, D is the distance covered by the probe in contact with the membrane until the membrane ruptures, and R is the radius of the hole in the plate.

3.3.3 Water vapor permeability and solubility

3.3.3.1 Water vapor permeability

Undamaged, non-porous films were cut into disc shape. These discs (1 cm diameter) should have the same diameter as the mouth of the PVC cups filled with distilled water. A gap of about 1 cm should be left between the water surface and the film. The film and the mouth of the cup need to fit snugly without leaks. Cups were stored in a desiccator filled with silica gel and passed by weighing during the first 7-10 hours, recording weight loss hourly. The weight loss was plotted against time and the water vapour transmission rate (WVTR) was calculated according to equation 3-4:

$$WVTR=G/(t*A) \quad \text{Equation 3-4}$$

Among that, G/t is the weight change of the cup per unit time (g/h), and A(m2) is the area of the film covering the mouth of the cup.

These WVTR values can be used to calculate water vapor.

Water Vapor Permeability (WVP) is calculated by equation 3-5:

$$WVP = (WVTR * Th)/\Delta P \quad \text{Equation 3-5}$$

where Th (mm) is the film thickness and ΔP (kPa) is the partial pressure difference between the two faces of the film.

3.3.3.2 Water solubility

To measure film solubility, films are cut into small pieces. A portion was obtained by drying in an oven at 105 °C for 24 h to obtain their dry weight. Others were immersed in a solution containing 20% Trizma 0.1 M pH 7.0 buffer. After standing at room temperature for 24 hours, undissolved film residues were recovered by vacuum

filtration using previously weighed Whatman nº 1 paper. Finally, the paper was dried with the residue at 95-105°C for 24 h and weighed. The following equation was used to calculate the percent undissolved:

$$WS(\%) = (m1 - m2)/m1 \times 100 \qquad \text{Equation 3-6}$$

where WS (%) is the percentage of dissolved membrane, m1 is the initial dry weight of the membrane, and m2 is the remaining dry weight of the undissolved membrane.

3.3.4 Scanning electron microscopy (SEM)

Micrographs were taken using a JSM-6610LV (JEOL, USA) scanning electron microscope with the aim of studying the microstructure of the film cross-section. For this, 1 × 1 cm square film sample fragments were cut with a scalpel blade. These films were attached vertically around the stubs using double-sided carbon-based tape as an adhesive, and then the films were gold-plated in an argon atmosphere for 5 min. The magnification and voltage settings used to observe the cross-section of the film are adjusted according to the specific imaging results.

3.3.5 Transmission Electron Microscopy (TEM)

Thymol-encapsulated PLA nanoparticles were optically observed using a transmission electron microscope (TEM, JEOL-2000 EX-II, Tokyo, Japan). A drop of nanoparticle solution was placed on the copper grid and negatively stained with a drop of 2% (w/v) phosphotungstic acid (PTA) solution. TEM was operated at 200 kV.

3.3.6 Nanoparticle size measurement

The average size of the nanoparticles and their polydispersity index (PDI) were measured using dynamic light scattering (DLS, Nanosizer ZS, Malvern Instruments, Malvern, UK).

3.3.7 Thermogravimetric analysis (TGA)

The tested samples were lyophilized for 24 hours while applying a pressure of 0.33 mbar. TGA curves were performed in an SDTA851e TGA analyzer (Mettler-Toledo, Columbus, USA) under nitrogen atmosphere from 30 °C to 700 °C. The heating rate is 10 °C/min.

3.3.8 Nanoparticle Encapsulation Efficiency

The nanoparticle suspension was centrifuged at 13,000 rpm (Thermo Scientific Multifuge X1R) for 20 min and the supernatant was replaced with the same volume of ethanol (96°). A sonication system disperses the nanoparticle-containing sediment in ethanol. The nanoparticle-containing sediment was dispersed in ethanol by sonication. The solution was centrifuged again at 13,000 rpm for 20 minutes. 0.1 mL of this ethanol solution was diluted with 9.9 mL of new ethanol. The absorbance of the resulting solution was measured by a spectrophotometer (Spekol 1500, Analytik Jena AG, Jena, Alemania) at 275 nm. The concentration of thymol in ethanol was measured at this wavelength, and the calibration was calculated.

3.3.9 Data analysis

All experiments were performed in triplicate and results are shown as the mean value. To analyze differences between the groups tested, analysis of variance (ANOVA) was carried out. Fischer's Least Significant Difference (LSD) was used to determine significant differences between the groups. A level of $p < 0.05$ was considered significant. Analyses were performed using IBM® SPSS® Statistics V25 statistical software and Statgraphics® V.15.2.06 statistical software.

Chapter 4: Experimental results

In this chapter, the main content is divided into three parts to describe the research of bio-based materials used as food packaging film materials. The main purpose is to explore the properties of biodegradable films developed from animal-derived plasma proteins and gelatine proteins as substrates, which are helpful for food storage and enhance antibacterial performance. In addition, the research results of PLA nanoparticles encapsulating active substances are also used as supplements. Then, a review involving the discussion of laboratory-level edible film research compared with large-scale commercial production technology research will be provided in this thesis. It mainly involves edible bio-matrix film coating packaging technology and film production technology in the past five years. Finally, recent research results on the use of insect-derived biomass materials as edible food packaging films are reviewed.

4.1 Development of New Protein Matrix Films and Summary of Food Packaging Production Technology

The results of this chapter can be divided into three parts. The first part is to use pig blood and cow blood from slaughterhouses to extract plasma proteins and make pure plasma protein films. The difference lies in the technical changes of protein extraction. The study compared the characterization changes of modified proteins and directly extracted proteins when they were used as packaging films. The second part is based on the previous period, supplemented with nano-fibrillated cellulose that helps the pure protein film improve its structure and improve its hydrophilic performance. The third part is to summarize different scales and types of food packaging technologies, and to broaden the knowledge and understanding of making coatings and films.

4.1.1 New preparation procedure of plasma protein edible film

Due to the high solubility of plasma proteins in water, cross-linking agents are usually required to improve the resistance of the film to dissolution. But some cross-linking agents are toxic. Several attempts were made by Nuthong et al. to reduce the solubility of these films by adding cross-linking agents such as caffeic acid and glyoxal. However, glyoxal is a highly toxic compound and caffeic acid can negatively affect the appearance of the produced films. Therefore, this PhD thesis describes for the first time the preparation of water-insoluble edible films from bovine and porcine blood plasma without the use of cross-linking agents, aiming to use livestock blood to produce environmentally friendly, healthy and safe edible packaging materials. For this, plasma is acidified and treated with ethanol to precipitate proteins, which are dissolved in water and mixed with glycerol to produce a film-forming solution. The resulting films were studied to determine absorbance, transparency, microstructure, mechanical

properties, and solubility at several different pH values and compared to control films prepared with untreated plasma. In contrast to the yellow-orange colour of the control film, the new film demonstrated in this thesis was completely transparent and colourless upon visual inspection. In addition, the microstructure of these new films was more uniform, while they showed better mechanical properties than the control films. And it was found that these films were highly insoluble in buffer solutions near neutral pH, while the control films were almost completely soluble in the same buffer. Finally, despite the high consumption of ethanol, there is no doubt that it can be easily recycled to minimize the use of reagents, thus making the process more sustainable.

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A new procedure to prepare transparent, colourless and low-water-soluble edible films using blood plasma from slaughterhouses

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ABSTRACT:

Some attempts to produce films using blood plasma proteins can be found in the literature; however, due to their high solubility in water, it is usually necessary to use crosslinkers, which may entail some disadvantages. In this work, a procedure to prepare water-insoluble edible films from bovine and porcine blood plasma without using crosslinkers is described for the first time, with the objective of producing sustainable packaging materials from livestock blood. For this purpose, the blood plasma fraction was acidified and treated with ethanol to precipitate the proteins, which were solubilised in water and mixed with glycerol in order to produce a film-forming solution. The generated films were investigated to determine light absorbance,

transparency, microstructure, mechanical properties and solubility at several pHs and compared with a control film prepared with untreated plasma. The new films presented in this work were completely transparent and colorless on visual inspection, in contrast to the yellowish-orange colour of the control films. Furthermore, the microstructure of these new films was more homogeneous, and therefore they showed better mechanical properties than the control film. Finally, these films were found to be highly insoluble in buffer solutions of close to neutral pH, whereas the control film was almost completely solubilized in the same buffers.

1. Introduction

The most common proteins that have been typically used in film and coating formulations are collagen, gelatine, corn zein, casein, whey protein, wheat gluten and soy protein (Hassan, Chatha, Hussain, Zia, & Akhtar, 2018). Protein-based films exhibit poor resistance to water, i.e., are prone to dissolve if the humidity of the medium is high enough; however, they are superior to polysaccharides in their capacity to form films with good mechanical and barrier properties (Mellinas et al., 2016). The revalorization of blood, or its fractions as rich protein sources, is strongly encouraged, since this co-product can be considered as one of the most problematic in the food industry due to the large amount that is generated and its high polluting power. In this sense, the use of blood plasma as a raw material for films potentially offers a way to minimize the environmental impact of blood generation while increasing the added value of blood proteins. Elaboration and characterization of plasma protein-based films have been reported by several authors; Nuthong, Benjakul, and Prodpran (2009b) prepared films using porcine plasma previously dialyzed and lyophilized with glycerol as plasticizer. However, the films obtained showed a water solubility higher than 96 %, which is highly undesirable for applications such as food coating or packaging. The same authors made several attempts to decrease the solubility of these films by adding crosslinkers, such as caffeic acid and glyoxal; however, glyoxal is a highly toxic compound and caffeic acid could exert a negative effect on the appearance of the films produced (Nuthong, Benjakul, & Prodpran, 2009a). Taking all this into consideration

and with the intention of overcoming such problems, in this study a new procedure, in which cross-linkers or other non-food-grade chemicals are not involved, is presented for the first time. This procedure is capable of preparing totally transparent and highly water-insoluble films from both bovine and porcine plasma proteins obtained from blood generated in a local slaughterhouse. In a preliminary analysis of the physical and functional properties of these films, they were tested and compared with those of a control film prepared by the traditional method.

2. Material and methods

2.1. Blood plasma collection

Porcine and bovine blood was collected immediately after slaughtering from a local slaughterhouse (Asturias, Spain) and poured into 3 L plastic containers. Sodium citrate, previously added, at 2 % (w/v) was used as an anticoagulant. Plasma was separated from the cell fraction by centrifugation for 10 min at 10,000 g and 10°C. The plasma, which is the supernatant resulting from centrifugation, was decanted and stored at − 20 °C.

2.2. Film preparation

The procedure for the preparation of the new films from bovine and porcine plasma was the same in both cases. First, plasma was dialyzed, employing 14 kDa cellulose membranes (Dialysis tubing cellulose membrane, Sigma- Aldrich, United States) and then lyophilized and stored at a temperature of − 20 °C until used.

Afterwards, 1.5 g of lyophilized plasma was dissolved in 50 mL of distilled water and the pH adjusted to 2.5, employing a solution of HCl 3.0 M. This acidified plasma solution was added dropwise to 400 mL of 96 % ethanol (VWR, United States) and the pH adjusted to 1.5, once more employing HCl 3.0 M. The resulting mixture was centrifuged at 10,000 g, at 10 °C for 30 min.

After centrifugation, the supernatant was discarded, and the pellet recovered. This pellet was dissolved in distilled water to concentration of 0.3 g/mL (w/v) by stirring at 600 rpm. Finally, 65 g glycerol/100 g of protein was added as plasticizer and 8.85 mg of protein/cm2 of this film forming solution was poured into Petri dishes. The films

were dried in an oven at 40 °C overnight.

The control film was prepared according to Nuthong et al. (2009a) with slight modifications. Firstly, 30 g of lyophilized porcine plasma was dissolved in 100 mL of distilled water. Afterwards, the same glycerol ratio and drying procedure, previously reported, was used to prepare the control films.

The protein content in both the lyophilized untreated plasma and the pellet after the ethanol treatment, was determined by the Dumas combustion method using a CNHS/O Elementar Vario EL analyzer (Elementar, Germany).

2.3. Film characterization

Prior to testing, films were equilibrated for at least 1 day in a desiccator at room temperature and with a controlled relative humidity of 54 ± 2 %. To maintain this relative humidity, a saturated solution of $Mg(NO_3)_2$ was placed at the bottom of the desiccator.

2.3.1. Light transmission and transparency

The light barrier properties of the films to visible and ultraviolet light were tested at different wavelengths following the method proposed by Saricaoglu, Tural, Gul, and Turhan (2018). The absorbance of the films was measured with an Analytik Jena Spekol® 1500 (LabWrench, Canada) from 200 nm to 600 nm. An empty quartz cuvette was used as a blank. Thickness of film samples was determined using a micrometer.

The transparency of the films is calculated according to the following equation:

$$Transparency = A_{600}/x$$

where A_{600} is the absorbance of the films at 600 nm and χ is the thickness of the film in mm. The higher the transparency value obtained with this equation, the lower was the transparency of the film, and vice versa, the lower the transparency value calculated with this equation, the higher the transparency of the film.

2.3.2. Scanning electron microscope (SEM)

The microstructures of the film cross sections were analysed using a scanning electron microscope (SEM) (JSM-6610LV, JEOL, USA) according to the method described by Galus and Kadzínska (2016), with some modifications.

Firstly, the film samples were lyophilized and then cut into squares of approximately 1 cm^2 employing a surgical blade. These pieces were fixed to metal supports and gold coated in order to observe the crosss sectional microstructure of the films. The magnification used was x350 and the voltage was set at 20 kV.

2.3.3. Mechanical properties

Mechanical properties of films were analysed employing a TA.XT. plus Texture Analyser (Stable Microsystems, United Kingdom), using a 50 N load cell and a 5 mm diameter probe (P/5S). To carry out the test, the films were cut into strips of 15 × 20 mm and placed between two plates which form part of the analysis device. These plates, firmly attached to the analyser, have an orifice of 10 mm that allows the probe to enter in contact with the film at a velocity 1 mm/s, stretching the film until it breaks.

The mechanical properties measured with this test were the puncture strength (PS) and the puncture deformation (PD). These properties were calculated according to the following equations:

$$PS = Fm/Th$$

$$PD = (\sqrt{D^2 + R^2} - R)/R$$

where *Fm* (N) is the maximum force applied before the film rupture, and *Th* (mm) is the film thickness; *D* (mm) is the distance reached by the probe before the film is broken; and *R* (mm) is the radius of the orifice in the plates.

2.3.4. Film solubility

Solubility was determined following the method proposed by Gontard, Duchez, Cuq, and Guilbert (1994) with slight modifications. Circular pieces of 1.9 cm were excised from the film and immersed in 20 mL of three different buffer solutions: Trizma® hydrochloride solution 0.1 M at pH 7.0 (Sigma-Aldrich, Estados Unidos), a carbonate-bicarbonate 0.1 M buffer solution at pH 9.0, and an acetic-acetate 0.1 M buffer solution at pH 5.0.

After 24 h of immersion, the circular pieces were recovered by employing a vacuum filtration system and Whatman Nº1 paper. The filter paper was weighed before proceeding with the filtration step. The filter paper and the film pieces were dried in

an oven at 97 ˚C for 8 h.

On the other hand, intact pieces of film not previously immersed in the buffer solutions were directly dried at 97 ◦C for 8 h to determine the dry matter contained in the films. The amount of dry matter in the films recovered after the immersion in the buffers was compared with the same value of dry matter for the intact films to calculate the amount of film solubilized during the experiment.

2.4. Statistical analysis

For data processing, an ANOVA test was used for variance analysis, and least significant differences (LSD) were calculated by Fisher's test to determine significant differences between the tested samples. The analyses were performed using Statgraphics® V.15.2.06 statistical software.

3. Results

3.1. Preparation of films, visual appearance, light transmission and transparency

Fig. 1 illustrates the process of fabrication of the new blood plasma based films. It was expected that the combination of both low pH and excess of ethanol would produce conformational changes in the plasma protein, and therefore lead to its aggregation and precipitation. In fact, after the centrifugation step, the supernatant had a clear, yellowish visual appearance, while the sediment showed a whitish aspect, which suggests that the plasma pigments and lipids were solubilised in the ethanol solution. The ethanol in the supernatant can be recovered with a rotary evaporator and reused. Electrophoresis analysis confirmed that all the main proteins of blood plasma were present in the sediment in the same proportion as they are found in the untreated blood plasma (data not shown). The dispersion of the recovered proteins in water resulted in a slightly viscous solution, which was then mixed with glycerol and dried to prepare the films.

The visual appearance of the films is also shown in Fig. 4.1. After drying, the films were peeled easily from the Petri dishes, showed a homogeneous appearance and were sufficiently flexible to wrap a piece of food without breaking. Films prepared according

to the novel method described in this paper (Fig. 4.1B and C) were completely transparent and with no colour, whereas the control film had a yellowish-orange appearance, most likely due to the presence of bilirubin, carotenoids and hemoglobin, since all these compounds give the blood plasma its characteristic colour. The light transmission and transparency of the films tested are shown in Table 4.1. As was expected, the three films tested exhibited a high absorbance at 200 and 280 nm, mainly due to the absorption of light by carbonyl groups within the peptide bonds, the presence of aromatic amino acids that form part of the primary structure of the proteins, and desulphated bonds (Banga, 2015). This property is common to any protein-based film, and it is very desirable for packaging applications, since it can act as a barrier to UV radiation, hindering the UV-mediated oxidative degradation of the lipids that can occur in many food items (Cooper, Suman, Wiegand, Schumacher, & Lorenzen, 2018). When analysing the absorbance in the visible range, this was significantly higher for the control films in the entire range. This effect may be explained because the pigments and compounds that are found in the untreated plasma, and which subsequently constitute part of the control film, exhibit absorbance and light scattering at those wavelengths. Such compounds were removed by the ethanol in the new film making process. Finally, although both treated and untreated films were found to be highly transparent, the least transparent was the control, with a transparency value of 0.50, in contrast with transparency values of 0.10 and 0.21 for the porcine and bovine films, which clearly shows that this characteristic of the films was improved by the new process.

3.2. Microstructure and mechanical properties

The microphotographs of the films' transversal sections are shown in Fig. 4.2. In this case, the new films (Fig. 4.2B and C) showed a more homogeneous and compact microstructure than the control films (Fig. 4.2A). When the film-forming solution is drying, the protein chains are approaching each other and forming, mainly, inter- and intramolecular non-covalent bonds, namely hydrophobic interactions, hydrogen bonds and ionic interactions. In any case, the treatment applied to the plasma to prepare the

new films produces a partial denaturalization of the plasma proteins, leading to the exposure of their hydrophobic cores. Hence, when the solvent of the film-forming solution is evaporating, these proteins can interact through a higher number of hydrophobic bonds and thereby increase the degree of packing of the film matrix.

Furthermore, it must be remembered that most of the blood-plasma compounds remain in the control film, where some of these compounds may hamper the approximation of the protein chains, decreasing the number of non-covalent bonds.

3.3. Mechanical properties

The mechanical properties (Table 4.1) seem to be closely related to the microstructure of the films. In this sense, the more homogeneous the film microstructure is, the better are the mechanical properties. In this case, the values of the PS and PD parameters were significantly higher for the new films when compared to the control. Among the new films, the porcine based ones showed the best PS values, while bovine based ones showed the highest PD readings, although the differences detected could be considered minor considering the mean values obtained for these parameters. These small differences may be produced due to slight changes in the amino acid sequence between bovine and porcine plasma proteins, which mainly involve some amino acid substitutions in the main protein fraction (immunoglobulin). Such small differences may lead to different final properties of the films, as shown in this work.

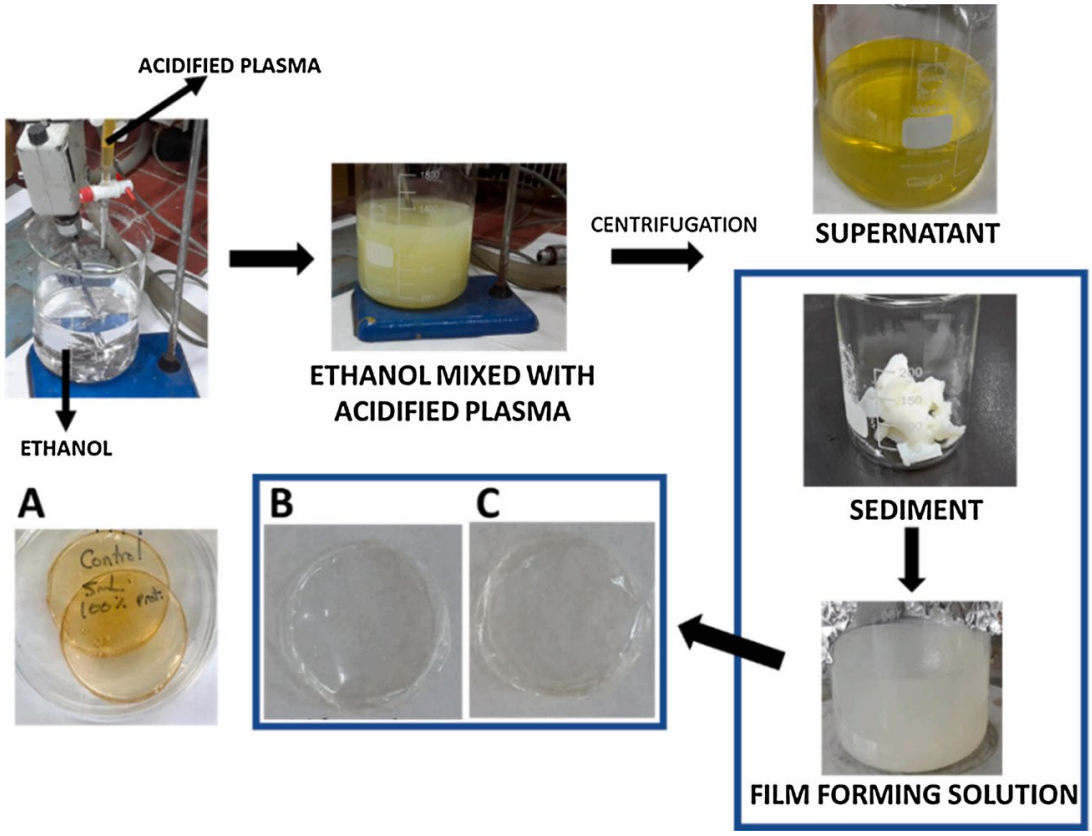

Figure.4.1. Fabrication process of the new plasma films and their visual appearance. visual appearance of the control film (A), and the new films prepared from bovine (B) and porcine (C) plasma.

Table 4.1: Thickness, puncture strength (PS), puncture deformation (PD), water solubility (WS), absorbance and transparency of the tested films.

	Thickness (mm)	*PS* (N/mm)	*PD* (%)	WS (%)			Absorbance (Wavelength, nm)							*Transparency*
				5.0	7.0	9.0	200	280	300	350	400	500	600	
Control	0.179 ± 0.004^{a}	23.4 ± 5.1^{a}	12.3 ± 2.5^{a}	96.0 ± 1.1^{a}	95.2 ± 3.5^{a}	97.1 ± 2.1^{a}	3.00	3.00	2.10	0.36	0.30	0.23	0.09	0.50
New porcine film	0.168 ± 0.010^{ab}	47.0 ± 1.9^{b}	22.4 ± 3.8^{b}	21.2 ± 2.9^{b}	8.8 ± 3.1^{b}	15.0 ± 0.3^{b}	2.53	2.70	1.62	0.06	0.06	0.02	0.01	0.10
New bovine film	0.160 ± 0.012^{b}	42. 2 ± 1.6^{c}	30.3 ± 1.2^{c}	26.1 ± 3.4^{b}	11.6 ± 5.5^{b}	17.0 ± 2.7^{b}	2.20	2.45	1.58	0.08	0.05	0.04	0.03	0.21

Different letters in the same columns indicate significant differences ($P < 0.05$).

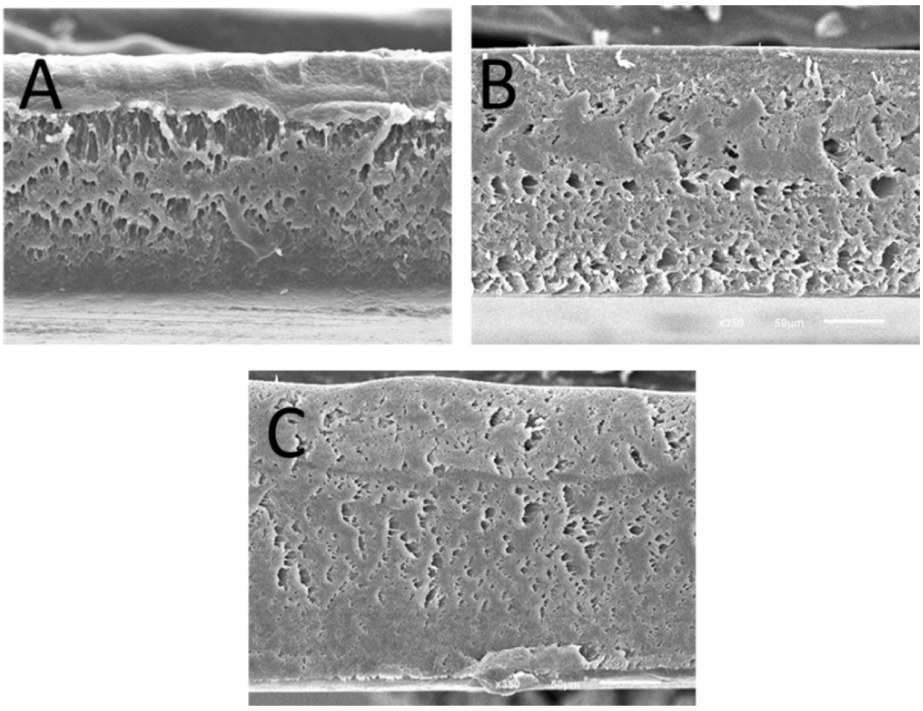

Figure. 4.2: Microphotographs of the transverse section of the control film (A), the new plasma film from bovine blood (B) and the new plasma film from porcine blood(C).

3.4. Water solubility (WS)

The WS values of the tested films are shown in Table 4.1. WS is an important assessment parameter for novel films prepared using natural biopolymers. It must be borne in mind that many food products have relatively high moisture levels, and this water content might damage the integrity of the film, which would diminish film performance, therefore, its range of applications. Because of the biological function and biochemical properties of plasma proteins, they have high water solubility at physiological pH, so the films prepared using untreated plasma are expected to be solubilised to a large extent when they are immersed in the buffer solution at pH 7.0. In this case, the control sample was almost completely solubilised after 24 h of exposure at this pH, which is in agreement with the findings of other authors (Nuthong et al., 2009b). In addition, the control film was also almost completely solubilised at pHs 5.0 and 9.0. On the other hand, the new films prepared here showed a significantly lower water solubility at the same testing conditions. In fact, the lowest WS values

were obtained at pH 7.0, the solubility of the new porcine plasma film being 8.8 ± 3.1 % of its total dry matter, and that of the new bovine plasma film 11.6 ± 5.5 % of its total dry matter. However, the amount of film solubilised increased noticeably at pH 5.0, reaching WS values of 21.2 ± 2.9 % and 26.1 ± 3.4 % for the new porcine and bovine plasma films respectively; and to a lesser extent at pH 9.0, showing in this case WS values of 15.0 ± 0.3 and 17.0 ± 2.7 for the new porcine and bovine plasma films respectively. These findings suggest that the acidification of the plasma protein and its precipitation in ethanol produced a variation in the solubility profile of these proteins, causing these films to become mostly insoluble at pH levels close to neutrality. In regard to this, it has to be said that most food products have a pH that is slightly acid or close to neutral, so it is expected that these new films will be suitable for wrapping a wide range of foodstuffs.

4. Conclusions

A successful new procedure to produce films using bovine and porcine blood plasma collected from slaughterhouses was described. These new films performed significantly better in key parameters such as colour, transparency and mechanical strength when compared to other methods described previously. They also showed low solubility in buffer solutions at different pH values relevant for most food products. Furthermore, it was proven that if the plasma is treated in this way, the films produced show a more homogeneous and compact matrix microstructure. Finally, in spite of the high consumption of ethanol, there is no doubt that it could be easily recovered in order to minimize reagent usage, thus making the process more sustainable.

CRediT authorship contribution statement

Sara Álvarez: Investigation, Writing - original draft. Shihan Weng: Investigation. Carlos Álvarez: Writing - review & editing. Ismael Marcet: Conceptualization, Methodology, Writing - original draft. Manuel Rendueles: Supervision, Funding acquisition. Mario Díaz: Supervision, Funding acquisition.

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4.1.2 NFC Enhanced Bovine Plasma Protein Edible Packaging Film

Based on previous research on films made from porcine and bovine plasma proteins, it was found that films made entirely of protein have low strength and high-water solubility. This makes them difficult to use in the food industry. Therefore, in this chapter, the concept of using cellulose materials to make composite films is introduced. For example, nanofibrillated cellulose (NFC), used as a structural material, can improve the properties of these films. Bovine plasma was acidified and treated with ethanol to precipitate its proteins, which were used to prepare films enhanced with several concentrations of NFC. In addition, control membranes with untreated bovine plasma membranes and NFC alone were prepared. The best characterized films were selected for nisin addition and their antimicrobial properties were tested by wrapping meat previously contaminated with *S. aureus*. Characterization results showed that reinforcing the films with 10% (w/w) NFC reduced their water solubility and improved their puncture strength and water vapor barrier properties. Films prepared with ethanol-extracted proteins after acidifying bovine plasma and enhancing with NFC showed better properties than those observed in control films prepared with untreated plasma. However, when NFC is incorporated into these films at high concentrations, it deteriorates their mechanical properties, among other things. Therefore, the enhancement capability of NFC in such films is limited. The best formulation was found to be FA90FC10. Films formulated with 10% NFC showed significant improvements in mechanical and water vapor barrier properties, while their water solubility was reduced without affecting their optical properties, such as light transmittance and clarity index. Furthermore, it was found that these films could be easily formulated with antimicrobial compounds such as nisin.

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Novel Bovine Plasma Protein Film Reinforced with Nanofifibrillated Cellulose Fiber as Edible Food Packaging Material

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Abstract

Proteins, such as those in blood from slaughterhouses, are a good option for developing edible films. However, films made exclusively from proteins have low strength and high water solubility, which makes them difficult to use in the food industry. The use of cellulosic material, such as nanofibrillated cellulose (NFC), can improve the properties of these films. In the present work, bovine plasma was acidified and treated with ethanol to precipitate its proteins, and these proteins were used to prepare films reinforced with several concentrations of NFC. In addition, control films prepared with untreated bovine plasma and reinforced with NFC were prepared as well. These new edible films were characterized according to their mechanical properties, water vapor permeability, light transmittance, and microstructure. Furthermore, the film with the best properties was selected to be additivated with nisin to test its antimicrobial properties by wrapping meat previously contaminated

with Staphylococcus aureus. In this sense, films prepared with the extracted proteins showed better properties than the films prepared with untreated plasma. In addition, the results showed that the reinforcement of the films with a 10% (w/w) of NFC decreased their water solubility and improved their puncture strength and water vapor barrier properties. Finally, the addition of nisin to the films prepared with extracted protein from bovine plasma and NFC gave them antimicrobial properties against S. aureus.

Keywords:

Blood, Plasma, Protein, Nanofibrillated Cellulose Fiber, Packaging

1. Introduction

Nowadays, the use of traditional plastics as packaging material is being replaced by new biodegradable materials, as they are an environmentally friendly alternative [1]. As an advantage, these new materials can be designed to extend the shelf life and improve the quality of foodstuff by controlling different aspects such as water transfer and lipid oxidation [2]. These new edible films and coatings will meet the present and future needs and demands of the food sector.

Of all the possible options for the development of these materials, polysaccharides (as starch or alginate) and proteins (as gelatine and casein) stand out [2,3]. One of the advantages of these materials over synthetic materials is that they can be obtained from co- and by-products from the food industry itself. One of the most problematic co-products of the food industry is blood since it is a major by-product of slaughterhouses and it has a high pollutant capacity [4,5,6]. A total amount of worldwide blood produced from livestock slaughtering of around 4.56×10^9 L [7] has been estimated, considering that 15 L of blood would be obtained from each cattle and 2 to 3 L from each pig [8]. The European Community report calculates similar numbers since they estimate that from each bovine between 10 and 20 L of blood are obtained and from each pig between 2 and 4 L [9]. Therefore, the volumes obtained are remarkably high.

Blood itself has an excellent nutritive value due its high protein content, the rich content of iron, and the bioavailability of its nutrients [5,6,7]. Although there may be some objection to its consumption, it is a good source of nutrients that has been used since ancient times [7]. In addition, in spite of the fact that blood can be processed to generate high-added value food ingredients due to its outstanding functional properties, it is estimated that only 30% of the blood produced in slaughterhouses is employed as food ingredients, mostly as black pudding and similar food products [5].

In this sense, blood can be fractionated into plasma, which represents 65–70% of its content. Blood plasma is rich in proteins (7.9% of protein content [10]), composed mainly of albumin, globulins, and fibrinogen [7]. Some researchers have used plasma proteins as substitutes for other food components. For example, in the production of gluten-free bread (using bovine plasma to improve textural properties [11]), surimi (using plasma proteins as protease inhibitors [12]), or in ham pate (bovine plasma as fat replacer [13,14]). Furthermore, these proteins can used as a matrix for the development of edible films, considerably reducing the environmental and economic impact of this by-product [4,15].

Although films made from proteins generally have good characteristics, they have some limitations, particularly in terms of mechanical strength and hydrophilic characteristics [1,2,3,16]. To address this problem, there are different alternatives to improve their characteristics. Among the most common strategies are chemical and enzymatic modifications and the use of different crosslinking agents [2]. In this sense, nanocellulose and its derivates have recently attracted attention as they can be used as a reinforcement agent in protein films [17,18]. Nanofibrillated cellulose (NFC) is a promising renewable and environmentally friendly material [17,18], which can improve film properties due its characteristics, such as low density and high strength [19]. Thus, the addition of this material to protein films has the potential to develop novel packaging that meets the requirements for a wide range of different food products. In addition, these new types of films can be additivated with different compounds, such as antioxidants or antimicrobials. Among the antimicrobial compounds, nisin stands

out. It is a bacteriocin that has antimicrobial activity against a wide range of Gram-positive foodborne pathogens [20]. Nisin affects both cell wall synthesis and the formation of membrane pores [21]. Furthermore, it is the only bacteriocin that is widely employed in the preservation of commercial foodstuff [22].

Therefore, the aim of this research is to develop blood plasma protein-based films with improved properties by adding NFC in their formulation. Firstly, bovine plasma was acidified and treated with ethanol to precipitate its proteins [4], and these proteins were used to prepare films reinforced with several concentrations of NFC. In addition, control films prepared with untreated bovine plasma and reinforced with NFC were prepared. The effect of the protein extraction and the different NFC ratios on the mechanical and physical properties of the films prepared was studied. Finally, the films that showed the best properties were chosen to be additivated with nisin and their antimicrobial properties were tested by wrapping a piece of meat previously contaminated with a common food-borne pathogen such as *Staphylococcus aureus*.

2. Materials and Methods

2.1. Obtaining Lyophilized Plasma

Blood was collected from a local slaughterhouse (Macelo de Asturias, S.A., Asturias, Spain). As anticoagulant, sodium citrate (2% (w/v) (Sigma-Aldrich, Steinheim, Germany)) was used. Plasma was separated from the cell fraction by centrifugation at 10,000×g at 10 °C for 10 min. Residual salt components in the plasma were removed using 14 kDa cellulose membranes (Dialysis tubing cellulose membrane, Sigma-Aldrich). Finally, the plasma was frozen at −80 °C for 12 h and then lyophilized (Telstar Cryodos, 0.1 mBar, −70 °C for 24 h).

2.2. Acidizing Treatment and Ethanol Extraction of Plasma Protein

In order to obtain acidified plasma protein, the protocol of Álvarez et al. [4] was followed. Briefly, 1.5 g of lyophilized plasma powder were dissolved in 50 mL of distilled water. The pH was adjusted to 2.5 with HCl 3.0 M (Sigma-Aldrich). The acidified plasma was added into 400 mL of 96% ethanol (VWR, Radnor, PA, USA) and pH was adjusted to 1.5 with HCl 3.0 M. The mixture was centrifuged at 10,000× g at 10 °C for 30 min and the

pellet was stored for further use in film preparation. As stated in the previous work, the main proteins found in the untreated blood plasma were present in the sediment in the same proportion. As control, lyophilized non-acidified plasma was used to develop films as described by Nuthong, Benjakul, and Prodpran [15]. In both cases, the protein content was determined by the Dumas combustion method using a CNHS/O elementar vario EL analyzer (Elementar, Germany).

2.3. Preparation of Cellulose Nanofibrillated Fiber (NFC)

Cellulose was bleached by the ECF industrial process from eucalyptus provided by the company ENCE (Navia, Asturias, Spain). Bleached cellulose was subjected to chemical and mechanical treatment to obtain nanofibrillated cellulose fiber (NFC).

To this end, 5 g of compressed cellulose were soaked and softened by adding 400 mL of deionized water and stirring gently for 12 h. In order to oxidize the cellulose, it was treated with TEMPO® (BioMérieux, France). For that purpose, 0.06 g TEMPO® (BioMérieux, France) and 0.6 g NaBr (Sigma-Aldrich) were added to the soaked cellulose pulp. Once they were dissolved, 100 mL of NaClO (6–14% aqueous solution, Merck) was added and the pH was adjusted to 10.0 to start the chemical reaction. The mixture was left 3 h at room temperature and the reaction was stopped by raising the pH to 7.00. Then, the mixture was centrifuged at 10,000× g at 8 °C for 1 h and the pellet was washed once with distilled water. Finally, in order to obtain the NFC, the oxidized cellulose was dissolved in 400 mL of distilled water and was homogenized at 15,000 rpm for 15 min employing a Silent Crusher M homogenizer (Hidford, CT, USA). The mixture was centrifugated at 10,000× g at 8 °C for 110 min and NFC was obtained in the pellet. The solid content was analysed using an HR73 Halogen Moisture analyser (Mettler Toledo, Columbus, OH, USA).

2.4. Film Preparation

Films were prepared using protein extracted from acidified plasma treated with ethanol (FA) or lyophilized plasma (FL) in combination with NFC. The film-forming solutions were prepared considering the protein concentration in the lyophilized plasma and in the pellet obtained after treating the bovine plasma, as was described I

Section 2.2. The detailed composition of the film-forming solutions is shown in Table 4.2.

Table 4.2: Composition of the film-forming solutions prepared using acidified plasma protein, lyophilized plasma, and nanofibrillated cellulose (NFC). FA refers to films prepared with acidified plasma protein, FL to films prepared with lyophilized plasma, and FC to films prepared with NFC.

Film	Acidified Plasma Protein (g/mL)	Lyophilized Plasma Protein (g/mL)	NFC (g/mL)
FA100	0.030	-	-
FA90FC10	0.027	-	0.003
FA70FC30	0.021	-	0.009
FA50FC50	0.015	-	0.015
FL100	-	0.030	-
FL90FC10	-	0.027	0.003
FL70FC30	-	0.021	0.009
FL50FC50	-	0.015	0.015
FC100	-	-	0.030

Acidified plasma protein and lyophilized plasma protein were dissolved in distilled water by stirring at 600 rpm for 30 min at room temperature. NFC films (FC) were prepared by adding NFC to the film-forming solution and stirring at 5000 rpm for 5 min. In this case, three different percentages of NFC (10, 30, and 50%) were added to the film-forming solutions prepared with both acidified plasma protein (FA90FC10, FA70FC30, and FA50FC50) and lyophilized plasma (FL90FC10, FL70FC30, and FL50FC50), maintaining the solids' concentration at 0.030 g/mL for every film-forming solution prepared. In all cases, glycerol (Sigma-Aldrich) was employed as plasticizer and added to the solutions at 70% (w/w of solids). All film-forming solutions were cast on silicone molds and were dried at 37 °C for 24 h.

2.5. Film Characterization

Prior to testing, all films were placed in a desiccator at room temperature for 24 h. The desiccators maintained a relative humidity of 54 ± 2% and had a saturated solution of $Mg(NO_3)_2$ (Sigma-Aldrich) placed at the bottom.

2.5.1. Thickness and Mechanical Properties

The thickness of the films was measured at five different points, both inside and outside the films. For this purpose, a digital micrometer (Mitutoyo, Kawasaki, Japan) was employed. The film's thickness reported was the average of these values.

The mechanical properties were tested according to the method described by [14]

using TA.XT plus Texture Analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell. Films were cut into squares and placed on the test platform. Samples were subjected to a penetration test at room temperature using a P/5 S probe (5 mm of diameter) and a test speed of 1 mm/s. Puncture strength (PS) and puncture deformation (PD) values were obtained according to the following equations [23]

$$PS = Fm/Th$$

$$PD = (\sqrt{D^2 + R^2} - R)/R$$

where Fm is the maximum force applied before the film was broken (N), Th is the thickness of the film (mm), D is the distance covered by the probe in contact with the film until it is broken (mm), and R is the radius of the hole in the plates (mm). Experiments were carried out in triplicate and reported results correspond to the mean value.

2.5.2. Light Transmission and Transparency

Visible and ultraviolet (UV) light barrier properties of the films were tested at different wavelengths in the range of 200 to 600 nm [24] using a spectrophotometer (Helios gamma, Thermo Fischer Scientific, Waltham, MA, USA). An empty quartz cuvette was employed as blank, and samples were measured as rectangular pieces of film. The transparency of the film was calculated according to following equation

$$Transparency = A_{600}/x$$

where A600 is the absorbance of the film sample at 600 nm and X is the film thickness (mm). Experiments were carried out in triplicate and reported results correspond to the mean value.

2.5.3. Water Vapor Permeability (WVP)

Undamaged films with no holes were cut into circles with the same diameter as PVC cups filled with distilled water. The films stuck to the glasses, leaving a gap of 1 cm between the water surface and the films. The mounted cups were placed in a room at 20 °C and the weight loss was recorded every hour for the first 10 h and finally after 24 h. The weight loss was plotted against time and the water vapor transmission rate (WVTR) was calculated according to the following equation

$$WVTR=G/(t*A)$$

Where G/t is the change in the weight of the cup per unit of time (g/h) and A is the area of the cup covered by the film (m2).

These WVTR values were used to calculate the water vapor permeability (WVP) using the following equation

$$WVP = (WVTR * Th)/\Delta P$$

Where Th is the thickness of the film (mm) and ΔP is the water vapor difference across the film (kPa). All experiments were carried out in triplicate and reported results correspond to the mean value.

2.5.4. Water Solubility (WS)

In order to test WS, circular pieces of the films were immersed in 20 mL of distilled water with 2% (w/w) of HCl at pH 7.0 (Sigma-Aldrich) and were kept at room temperature for 24 h. After that time, the film pieces were recovered by filtering using a vacuum pump and Whatman N °1 paper, and then dried in an oven at 105 °C for 24 h. Furthermore, pieces of the film were dried at the same conditions without first being dissolved. The solubility was calculated as follows [25]

$$WS(\%) = (m1 - m2)/m1 \times 100$$

where m1 is the weight (g) of the film pieces dried in an oven at 105 °C for 24 h, and m2 is the weight (g) of the undissolved films pieces once they have been dried. Experiments were performed in triplicate and reported results correspond to the mean value.

2.5.5. Scanning Electron Microscopy (SEM)

The cross-section microstructure of the films was observed by using a scanning electron microscope (SEM) (JSM-6610LV, JEOL, Pleasanton, CA, USA). Lyophilized films were cut into 1 cm2 square pieces employing a surgical blade. These samples were mounted on stubs and coated with gold for 5 min in an argon atmosphere. The morphology of the films was observed at magnifications of between 370 and 500× with a voltage of 20 kV.

2.6. Antimicrobial Properties of Films Additivated with Nisin

To test the antimicrobial capacity against *Staphylococcus aureus*, nisin was added to FA90FC10 films. *S. aureus CECT 240* (from the Spanish Type Culture Collection, Valencia, Spain) was grown in 100 mL of TSB (Tryptone Soy Broth, Sigma-Aldrich) in an orbital shaker at 200 rpm and 37 °C for 24 h. Then, 50 g of meat (purchased at a local market) was cut into squares. Each piece was infected with 100 µL of 105 CFU/mL of *S. aureus* in a 0.7% NaCl solution (Sigma-Aldrich). After the liquid had dried, the pieces of meat were coated with films loaded with 3 mg/mL of nisin (1000 IU/mg; Sigma-Aldrich). All pieces were stored in a fridge and the growth evolution of *S. aureus* was analyzed at different times (0, 1, 3, 6, 10, and 15 days). For this purpose, the pieces of meat to be sampled were placed in a Stomacher™ bag (Seward, West Sussex, UK) with 10 mL of NaCl 0.7% (w/v) and were homogenized with a Stomacher™ device (Seward, UK) at maximum speed for 120 s. Microbial growth was analyzed by preparing serial dilutions (1:10) and incubating on Baird-Parker medium enriched with egg yolk tellurite emulsion (both from Sigma-Aldrich) with 2% of agar (VWR) plates for 48 h at 37 °C. Each sample was carried out in triplicate and results were expressed in log10 CFU/g of meat.

2.7. Statistical Analysis

All experiments were performed in triplicate and results are shown as the mean value. To analyse differences between the groups tested, analysis of variance (ANOVA) was carried out. Fischer's Least Significant Difference (LSD) was used to determine significant differences between the groups. A level of $p < 0.05$ was considered significant. Analyses were performed using IBM® SPSS® Statistics V25 statistical software.

3. Results and Discussion

3.1. Film Characterization

3.1.1. Thickness and Mechanical Properties

The thickness and mechanical properties of the films were analyzed, and the results are shown in Table 2. Regarding thickness, the films ranged from 123 to 176 µm. Significant differences ($p < 0.05$) were detected between the films according to their

composition, with the mixture of FLFC films being the thinnest.

Table 4.3: Puncture strength (PS), puncture deformation (PD), and thickness of the films. ANOVA and LSD were performed. Different letters in the same column indicate significant differences ($p < 0.05$).

Film	PS (N/mm)	PD (%)	Thickness (μm)	Transparency
FA100	68.3 ± 4.4^{a}	34.8 ± 5.0^{a}	167 ± 12^{a}	0.45 ± 0.06^{de}
FA90FC10	75.6 ± 3.0^{b}	25.4 ± 6.1^{b}	172 ± 20^{a}	1.25 ± 0.17^{cd}
FA70FC30	55.4 ± 0.7^{c}	13.1 ± 1.6^{cd}	161 ± 24^{ab}	2.11 ± 0.24^{b}
FA50FC50	21.0 ± 2.2^{d}	10.1 ± 1.5^{d}	158 ± 19^{ab}	5.32 ± 0.20^{a}
FL100	23.4 ± 2.1^{d}	15.7 ± 5.3^{bc}	176 ± 47^{a}	0.64 ± 0.10^{de}
FL90FC10	46.3 ± 6.2^{c}	17.6 ± 6.0^{bcd}	140 ± 28^{bc}	1.19 ± 0.28^{cd}
FL70FC30	32.5 ± 2.7^{e}	17.7 ± 6.6^{bc}	144 ± 10^{bc}	1.91 ± 0.10^{bc}
FL50FC50	29.6 ± 6.3^{e}	12.4 ± 1.2^{cd}	123 ± 4^{c}	5.65 ± 0.17^{a}
FC100	30.5 ± 1.1^{e}	8.8 ± 3.7^{d}	159 ± 20^{a}	5.84 ± 0.14^{a}

FA refers to films prepared with acidified plasma protein, FL to films prepared with lyophilized plasma, and FC to films prepared with nanofibrillated cellulose. The number refers to the percentage of each component added to prepare the film.

Concerning the mechanical properties, significant differences were found between the FA and FL films (Table 4.3). The PS values were notably different, with a mean value of 68.3 N/mm for FA100 films and 23.4 N/mm for FL100 films. Therefore, the treatment to which the bovine plasma protein was subjected affected the mechanical properties of the films. This treatment causes an exposure of the hydrophobic cores of the plasma proteins by their partial denaturalization [4]. In this way, treated proteins have a high capacity for forming hydrophobic bonds, increasing the PS values of these films

Regarding the addition of NFC, the best PS result was obtained for the FA90FC10 films, where the value of this parameter noticeably increased. With the addition of a higher amount of NFC, the PS values started to decrease in all cases. The use of NFC as a reinforcement in protein films is being widely studied and similar results were obtained when “faba” bean protein [3] and fish myofibrillar protein [26] were employed. Hydroxyl groups of NFCs have the capacity to interact with the hydrophilic groups of the proteins in the film matrix by forming hydrogen bonds [26,27], which increases the cohesion of the biopolymers and improves the mechanical properties of the materials prepared [3,17]. However, an excessive addition of NFC could lead to its uneven distribution, resulting in an inhomogeneous film due to the agglomeration of the modified cellulose, and, hence, in the production of films with poor mechanical

properties [17].

In the case of the elasticity of the films, the addition of NFC decreased the values of the PD parameter (Table 4.3). This decrease in the PD values is usual in protein-based films reinforced with cellulose fibres, and the same behaviour has been reported by several authors [27,28]. The presence of cellulose derivatives reduces the mobility of the polymer due to an increase in interactions with the proteins in the matrix, leading to a loss of elasticity and ductility of the films [26].

3.1.2. Light Transmittance and Transparency

The light transmittance of the films ranged between 200 and 600 nm (Figure 4.3). For UVC and UVB regions (200–280 nm and 280–315 nm, respectively), the transmittance of all of the samples was very low. In the UVA range (315–400 nm), there was an increase in the optical transmittance in the samples that continued in the visible region (400–600 nm). Low transmittance in the UV range is a desirable property, as this radiation is one of those responsible for the formation of free radicals in lipid-rich foodstuff and its degradation by oxidation processes [29,30]. Thus, films developed using proteins contain aromatic amino acids in their composition, such as tyrosine and tryptophan, that are capable of absorbing the UV light [31]. Significant differences ($p < 0.05$) were found between the samples in the visible region. As the proportion of NFC added increased, the values of transmittance were lower. Therefore, the lowest transmittance values in the visible region were obtained for FC100 films (7.16%) and FA50FC50-FL50FC50 films (15.23% and 19.07%, respectively). Other authors obtained similar results in terms of light transmittance by adding NFC to protein films [3,26,32]. This low transmittance could be attributed to the presence of Xylan since it is supposed to interfere partially with the complete dispersion of the nanofibrils in water [19] and to the distribution of the nanofibers in the polymer substrate as they form their own network [26]. For films developed with protein extracted from acidified plasma and untreated lyophilized plasma, no significant differences were observed ($p > 0.05$). Therefore, this treatment did not affect the values of light transmittance.

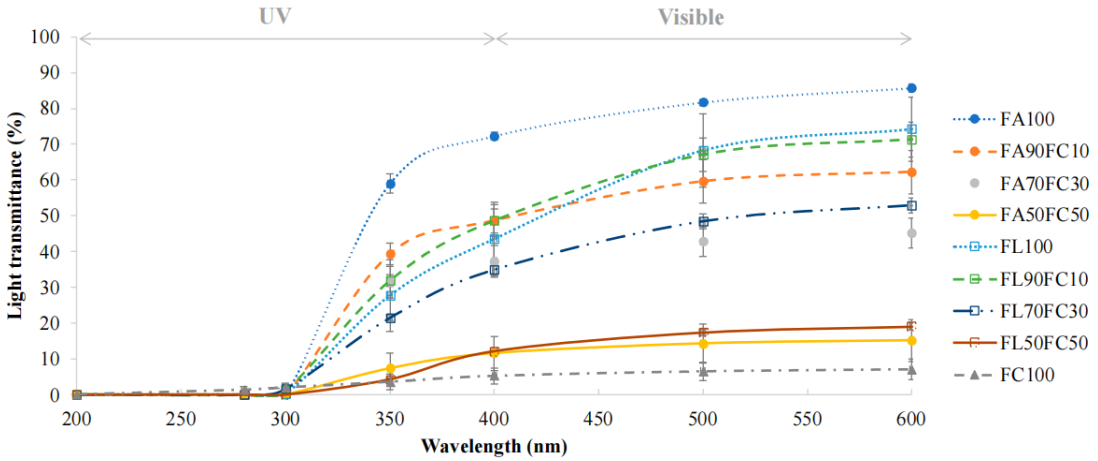

Figure 4.3: Light transmittance (%) of the films at different wavelengths (200–600 nm). FA refers to acidified plasma protein, FL to lyophilized plasma protein and FC to NFC. The number refers to the percentage of each component added to prepare the film. ANOVA and LSD were performed. Significant differences ($p < 0.05$) were found between four different groups (FA100, FL100-FA90FC10- FL90FL10, FA70FC30-FL70FC30, and FC100-FA50FC50-FL50FC50).

Regarding the transparency index of the films, results are shown in Figure 4.3. According to the results obtained when the light transmittance was measured in the visible region, the most transparent films were those made exclusively from plasma protein (FA100 and FL100). As the concentration of NFC added to the films increased, the transparency index values also increased, so the films became opaquer. This opacity is caused by the light scattering by the cellulose nanofibers, causing a reduction in the light passing through [26], and this has also been observed by other authors [3,19,33]. In this case, the reinforcement of 10% NFC was the most adequate, as a high degree of transparency was not lost.

3.1.3. Water Vapor Permeability (WVP) and Water Solubility (WS)

WVP results are reported in Table 4.4. Significant differences were found between FA and FL samples, so the treatment improved the water vapor barrier properties. In addition, the increase in hydrophobic and hydrophilic interactions between the NFC and the proteins promoted a decrease in the permeability of the water vapor across the films tested [26]. Furthermore, the low permeability of these films added with NFC can be enhanced by the high degree of crystallization of cellulose and its ability to form a dense percolating network [19]. The results obtained are similar to those of other

authors who have reinforced films with CNF in alginate [17] and sodium caseinate matrices [28]. However, other researchers have found that an excess of cellulosic material can lead to increased WPV values due to an accumulation of NFC and the loss of a well compacted structure [17].

Table 4.4: Water vapor permeability (WVP) and water solubility (WS) of the films. ANOVA and LSD were performed. Different letters in the same column indicate significant differences ($p < 0.05$).

Film	WVP ($g \times mm/m^2h \times kPa$)	WS (%)
FA100	3.81 ± 0.10^{a}	14.40 ± 2.31^{a}
FA90FC10	2.92 ± 0.11^{b}	11.32 ± 0.72^{b}
FA70FC30	2.87 ± 0.10^{b}	10.50 ± 1.60^{b}
FA50FC50	2.72 ± 0.12^{b}	7.10 ± 1.00^{c}
FL100	4.10 ± 0.16^{c}	99.60 ± 0.20^{d}
FL90FC10	3.80 ± 0.53^{a}	88.70 ± 3.23^{e}
FL70FC30	3.23 ± 0.15^{d}	75.25 ± 0.41^{f}
FL50FC50	2.58 ± 0.17^{e}	64.03 ± 2.36^{g}
FC100	2.75 ± 0.18^{e}	14.40 ± 2.31^{a}

FA refers to films prepared with acidified plasma protein, FL to films prepared with lyophilized plasma, and FC to films prepared with nanofibrillated cellulose. The number refers to the percentage of each component added to prepare the film.

Regarding the WS of the films, results are shown in Table 4.4. Films made exclusively with lyophilized bovine plasma showed a high degree of solubility (FL100), but films made with extracted proteins from acidified plasma were highly insoluble (FA100). Acid treatment and ethanol precipitation could be responsible for the variation in solubility [4]. This would be a key factor for their use in the food field as most foodstuffs have a high moisture content and the films would be water resistant [2]. The addition of NFC allowed improvements to be made to the characteristics of the films as the solubility values decreased as the percentage of NFC added was increased. The strong interactions of hydrophobic and hydrogen bonds between the NFC surface and proteins make the films more consistent and, therefore, reduce the sensitivity to water molecules [26,34]. Other authors have explained this lower water solubility due to the high level of crystallization of NFC [35]. As for films with other CNF-reinforced protein matrices [3,26], acidified plasma protein films showed a similar WS. Furthermore, the addition of NFC made it possible to reduce further the solubility of the films produced, which improves their performance and range of applications as they can be used in a

greater number of foodstuffs.

3.1.4. Visual Appearance and Scanning Electron Microscopy (SEM)

The visual appearance of the films is shown in (Figure 4.4). They were all homogeneous although films made directly from lyophilized bovine plasma (FL) showed a more yellowish colour than those made with protein extracted from acidifiied plasma protein (FA). This is due to the presence of yellowish compounds in the bovine plasma, such as bilirubin [36], carotenoids [37], and haemoglobin [38]. These compounds were removed by the acid-ethanol treatment [4] and, hence, the FA films obtained were colourless.

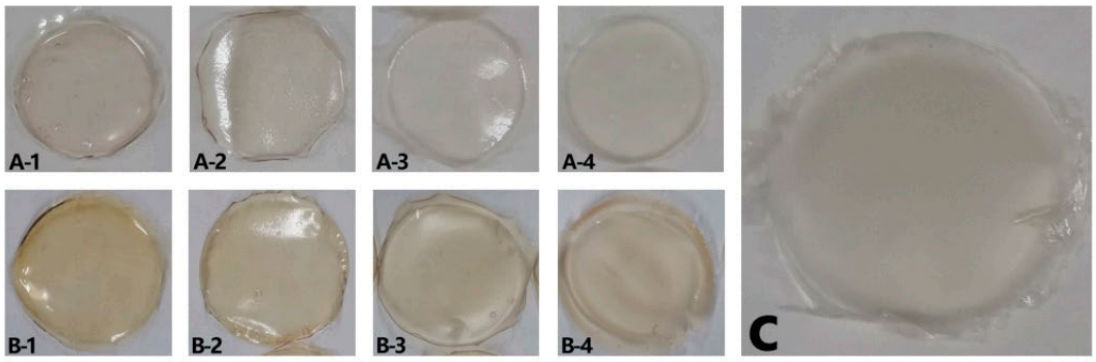

Figure 4.4: Visual appearance of the films. (A-1) FA100, (A-2) FA90FC10, (A-3) FA70FC30, (A-4) FA50FC50, (B-1) FL100, (B-2) FL90FC10, (B-3) FL70FC30, (B-4) FL50FC50, and (C) FC100. FA refers to acidified plasma protein, FL to lyophilized plasma protein, and FC to NFC. The number refers to the percentage of each component added to prepare the film.

In addition, as the proportion of NFC in the film composition increased, the films became opaquer, as was observed when the transparency index values were calculated (Table 4.3). Therefore, the most transparent films were those that did not contain NFC in their composition (FA100 and FL100). In addition, transparency may be considered good even for films containing 30% NFC, as they were not so opaque that foodstuff could not be inspected.

Regarding microstructure, micrographs of the cross-section are shown in Figure 4.5. FA100 and FL100 films showed a homogeneous and uniform appearance. The addition of a high percentage of NFC (30% or above) led to some changes in the microstructure, becoming less homogeneous, with agglomerations inside the matrix. This may be due to the natural tendency of cellulosic fillers to self-associate via hydrogen bonds as their

concentration increases [39]. In addition, it should be noted that there was a decrease in the amount of protein that could interact with the film itself, which make these materials less compact and weaker. Moreover, at an NFC concentration of 10%, no changes in microstructure were observed, suggesting the optimal interfacial adhesion between the protein matrix and the cellulose reinforcement. This result agreed with the mechanical properties of the films (Section 3.1) as FA90FC10 and FL90FC10 showed the best strength results in their group.

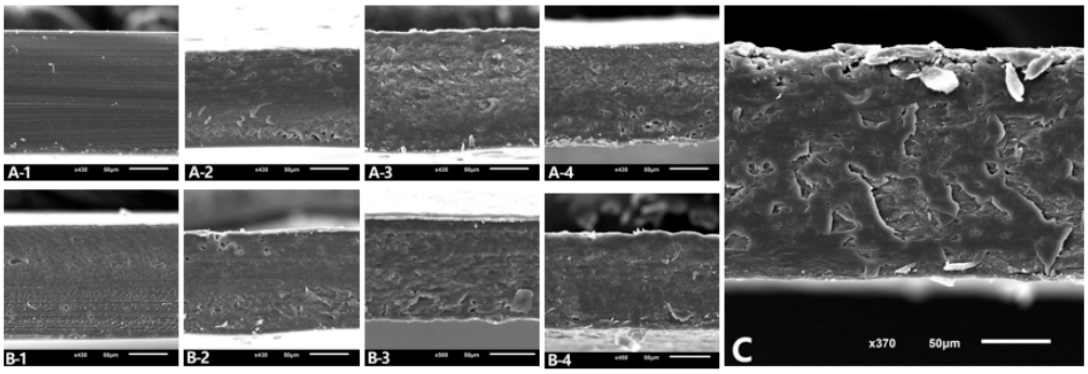

Figure 4.5: SEM images of the cross-section of the films. Scale bars correspond to 50 µm. (A-1) FA100, (A-2) FA90FC10, (A-3) FA70FC30, (A-4) FA50FC50, (B-1) FL100, (B-2) FL90FC10, (B-3) FL70FC30, (B-4) FL50FC50, and (C) FC100. FA refers to acidified plasma protein, FL to lyophilized plasma protein, and FC to NFC. The number refers to the percentage of each component added to prepare the film.

3.2. Antimicrobial Properties of Films Additivated with Nisin

For the antimicrobial tests, FA90FC10 films were selected as they showed the best properties in the previous analyses. To test the inhibitory effect on a real food model, pieces of meat were wrapped with these dried films additivated with nisin. As can be shown in Figure 4.6 A, there were no breaks, and the films were in contact with the whole surface of the previously contaminated piece of meat. The effect in the growth of *S. aureus* was analysed over time and results are shown in Figure 4B.

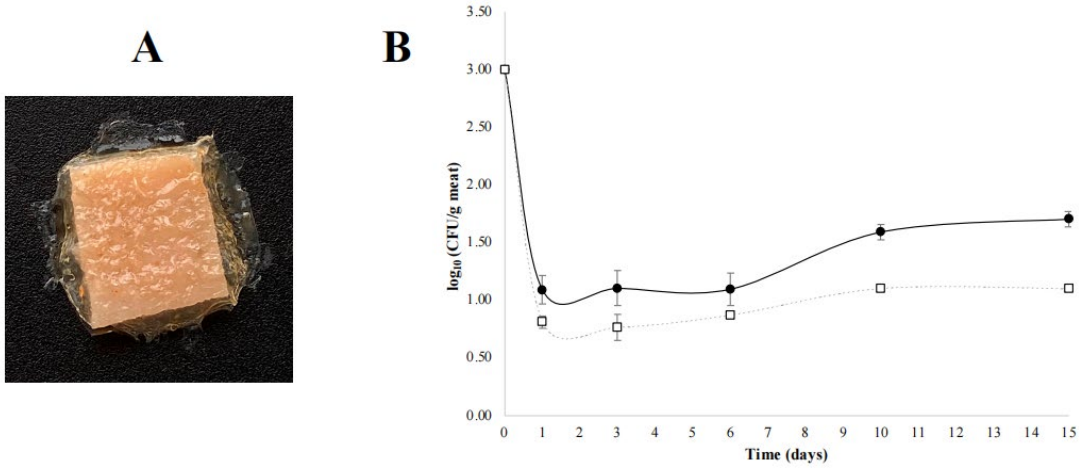

Figure 4.6: (A) Visual appearance of pieces of meat wrapped with FA90FC10 films additivated with nisin. (B) Evolution of S. aureus growth in meat for 15 days storage at 4 °C; (□) FA90FC10 films additivated with nisin and (●) FA90FC10 control films without nisin. ANOVA and LSD were performed. Significant differences between samples were found from day 6.

In both cases, there was a rapid decrease in *S. aureus* concentration (of at least two logarithmic units) during the first day of storage (Figure 4.6 B). This could be explained by the sudden change in environmental conditions and temperature as the bacteria were transferred from 30 °C in a laminar flow hood to 4 °C in a fridge. From day one onwards, the concentration of *S. aureus* began to grow, and significant differences were observed between the control and nisin-additivated films from day 6. After 15 days, the final concentration of *S. aureus* was 1.70 log10 CFU/g of meat for the control films and 1.00 log10 CFU/g of meat for the nisin-additivated FA90FC10 films. In both cases, the concentrations were below the recommended limit in meat products (10^3 CFU/g or mL of food product) [40,41].

Therefore, the films developed in this research showed good characteristics and can be additivated with different antimicrobial compounds. This would make it possible to extend the shelf life of perishable food products or foodstuff susceptible to contamination during the production and transport process. For the specific case of *S. aureus*, it is commonly detected in raw meat, but the main problem is that it can be transmitted most often via food handlers and in the food production chain [42,43]. Thus, the development of films that are able to control its growth and proliferation in foodstuffs is a key factor.

4. Conclusions

Films prepared with ethanol-extracted proteins after acidifying bovine plasma and being reinforced with NFC showed better characteristics than those observed in the control films prepared with untreated plasma. However, when NFC was incorporated into these films in high concentrations, it worsened certain characteristics, such as their mechanical properties. Therefore, NFC has limited capacity as a reinforcement in this type of film. After the characterization of all of the films developed, it was found that the optimum formulation was FA90FC10. These films, formulated with 10% NFC, showed a significant improvement in their mechanical and water vapor barrier properties, whilst their water solubility decreased, without compromising their optical properties such as the light transmittance and the transparency index. In addition, it was found that these films can be easily formulated with antimicrobial compounds such as nisin. A more detailed study of the packaging possibilities of these materials is needed to check other aspects, such as which type of application is the most suitable in foodstuffs (coatings or films) or their possible antioxidant capacity.

Author Contributions

Conceptualization, I.M.; methodology, I.M.; validation, I.M.; formal analysis, S.W., S.S.-O.; investigation, S.W., S.S.-O.; data curation, S.W., S.S.-O.; writing—original draft preparation, S.W., S.S.-O.; writing—review and editing, S.W., S.S.-O.; supervision, M.R., M.D.; project administration, M.R., M.D.; funding acquisition, M.R., M.D. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data are contained within the article.

Conflicts of Interest

The authors declare no conflict of interest

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4.1.3 Small-scale to large-scale edible film and coating production technology

A Review--Comparison of technologies for edible food packaging from laboratory to factory production scale

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Abstract:

Edible packaging materials play an important role in the field of food packaging, and innovations in the production and preparation technologies of edible films or coatings have been lacking in recent years. This review provides a brief summary of the last five years of research on the production of edible films and coatings using different scale technologies. In the form of "Coating", dipping, panning, spraying, brushing, vacuum impregnation, etc. are coating methods with high commercial maturity, while electrospray and electrospinning are currently more inclined to use laboratory scale. Due to the variety of shapes, fruits and vegetables are often coated to achieve maximum protection. And the physical properties of the food surface are an important coating decision parameter. In "film" form, efficiency is a key determinant in how the film is produced. The solution casting method is often used to develop new materials in the laboratory. At the industrial level, in addition to continuous solvent casting, "dry methods" with pressure and temperature changes are used (such as hot pressing, blown film, calendering, etc.). The type of food packaging processes need to be determined according to the actual packaging equipment and production purposes.

Laboratory-scale research focuses more on developing and exploring properties of materials. The technology used for industrial-scale production needs to consider various factors such as cost and efficiency.

Introduction:

The global food packaging market size was valued at USD 380.8 billion in 2022 and is projected to grow at a compound annual growth rate (CAGR) of 5.7% from 2023 to 2030.(Grand View Research, 2022) Plastic, the dominant material in food packaging, is being called upon by the United Nations Environment Program, and it is time to change the way we produce, consume and dispose of the plastic we use(O, 2023). While plastic-based packaging plays an important role in the safety, shelf life and affordability of many foods, petrochemical plastics(Phelan et al., 2022). For example, their good barrier properties to oxygen, as well as tensile and tear strength.(Alamri et al., 2021; Sina Ebnesajjad, 2013) But they are poorly managed as waste, bringing serious white pollution and greenhouse gas emissions that threaten our natural environment.(Alamri et al., 2021; Beltran et al., 2021; Ncube et al., 2021; Phelan et al., 2022; Sina Ebnesajjad, 2013) Because most of the plastic packaging used today is produced from fossil resources and chemically synthesized, almost half of the plastic waste consists of five types of plastic packaging: polyethylene terephthalate (PET), low density polyethylene (LDPE), high density polyethylene (HDPE), polypropylene (PP) and polystyrene (PS).(Organisation for Economic Co-operation and Development, 2018; Peidaei et al., 2021) In the absence of recycling (global recycling rates of 14% to 18%), natural degradation produces substances such as microplastics (1 to 5,000 particles), which threaten global environmental and biological health, among other issues.(Hale et al., 2020; Jadhav et al., 2021; Sewwandi et al., 2023) Even in the case of bioplastics, which can be defined as plastics based on renewable resources (bio-based) or biodegradable and/or compostable plastics. They also have disadvantages such as the thermal instability, brittleness, low melt strength, high water vapour and oxygen permeability of PLA(Jama, 2017; Swetha et al., 2023) and the low water vapour barrier

of starch and cellulose packaging materials due to their hydrophilic nature, etc.(Azeredo et al., 2019; Díaz-Montes & Castro-Muñoz, 2021; Salwa et al., 2019; Versino et al., 2016; X. Zhao et al., 2020) Also bioplastics such as PLA need to be recycled or composted due to their slow natural degradation, and these subsequent economic costs need to be taken into account.(Nilsen-Nygaard et al., 2021; X. Zhao et al., 2020) In this context, the development of edible food packaging materials is one of the advantageous means to help reduce the pressure on the environment and to promote sustainable development of human society. Bio-based packaging materials are now being used as green alternatives in many areas, and biodegradable and edible films are becoming the preferred choice when it comes to packaging food products.(Jayan et al., 2018; Reichert et al., 2020; Salwa et al., 2019) This kind of packaging plays an important role in the food supply chain. At the same time, edible films and coatings are also considered as one of the emerging strategies for food quality optimization.(Bhardwaj & Thakur, 2020; Bizymis & Tzia, 2021; Díaz-Montes & Castro-Muñoz, 2021) According to globenews wire, the global edible films and coatings market is forecast to reach US$4.2 billion by 2028, growing at a CAGR of 7.5% during the forecast period.(Globenewswire, 2022) Edible food packaging is simply defined as food that can be consumed with or without further removal and is considered an "edible coating or film". They should also be pleasant to taste, free from toxic, allergenic and indigestible ingredients. (Bhardwaj & Thakur, 2020)It is even now thought that in some cases these packages can be considered as part of the food product and not just part of the packaging system, even though they often perform packaging-related functions.(Azeredo et al., 2022) This is not difficult to understand, since most of the source materials used as edible packaging materials are prepared from naturally occurring renewable resources (polysaccharides, proteins, lipids and composites). The use of edible films and coatings also has the significant advantage that a variety of active chemicals can be included in the polymer matrix and taken with the food, thereby improving the safety as well as the nutritional and organoleptic properties of the food. For example, the inclusion of antimicrobial peptides or

nanoparticles in the packaging.(Trajkovska Petkoska et al., 2021; Xie et al., 2022)

Research into edible film materials is popular and researchers usually make the choice of the most suitable composition depending on the characteristics required in relation to each food product to be preserved. In contrast, innovation and adaptation of production techniques for edible films or coatings has been lacking in recent years, and generally researchers conclude their articles by stating that their findings are a good direction for commercial production if scaling up production is interesting. However, they basically ignored the fact that differences in production scale may prevent research results from being used for reference, and that the consistency of research results also needs to be verified under different platforms or scale conditions. In other words, if the laboratory-level research methods are scaled up to the scale of industrial application. From a technical point of view, the product will face new challenges in terms of its barrier properties, thermal properties, and mechanical properties. From a commercial point of view, producers have to consider the associated high manufacturing and processing costs. (Jeya Jeevahan et al., 2020)These two conditions alone make it very valuable to have edible films or coatings that are right on the ground and can reach the secondary consumer market. Some researchers (Jeya Jeevahan et al., 2020)mentioned that the current laboratory-scale production of edible films has problems such as inability to make continuous films, long drying time, and inaccurate thickness control, which must be resolved before industrial scale-up production. And the current lack of evidence on edibility, toxicology and health effects, insufficient commercial marketing, lack of awareness among consumers, and problems with different cultural backgrounds will all affect food safety and consumer acceptance.

So, the purpose of writing this review is to summarize the past 5 years, as of the first quarter of 2023, the published performance of edible food packaging under different manufacturing methods. Try to summarize the film-making process suitable for industrial production, classify it into appropriate production specifications according to different production methods, and understand the problems that food packaging

films produced in each mode need to face. At the same time, it is also hoped that this review can help readers understand the current status of materials and technologies that are currently advantageously invested in edible food packaging films. And through small-scale innovation experiments to large-scale production, discover the comparison of different properties of edible raw materials in practice and practical difficulties under different specifications, and look for possible improvements.

1: A Simple Understanding of Edible Packaging Materials

"Edible" is a specific property of the materials described in this review. Currently in this research field, the definition of edible generally refers to something that is suitable for human consumption, not indigestible, not containing toxic substances, and preferably not containing allergenic ingredients.(Bizymis & Tzia, 2021) Therefore, within the scope of researchers' understanding, choose the main sources of basic substances as packaging materials: proteins, polysaccharides, and lipids, which are directly extracted from biomass, such as cellulose derivatives, starch, and alginate, pectin, chitosan, and carrageenan are commonly used polysaccharides, and the most commonly used proteins are soybean protein, wheat gluten, zein, gelatine, whey protein, casein and keratin, etc. Commonly used in lipids are beeswax, palm wax, free fatty acids, oils, etc. The composite material is to combine more than one edible packaging material to improve the overall performance of the packaging. (Galus et al., 2020; Nilsen‐Nygaard et al., 2021) Since both polysaccharide and protein materials are hydrophilic, the integrity of the package will be reduced under high humidity conditions. Therefore, lipophilic substances can be added to help improve the anti-dissolution performance, or methods such as protein modification and cross-linking can be added to help improve the barrier performance and mechanical strength of the film as a packaging material.(Y. Cheng et al., 2023) In addition to the above-mentioned popular materials, researchers are still keen to explore more usable materials from traditional crops or livestock or seafood products or by-products or catering waste(Gupta et al., 2022).

Since most films and coatings based purely on polysaccharides and proteins are inherently brittle, it is advisable to make them more resilient with the help of

additives.(Morris, 2017) For example, glycerin, polyethylene glycol, and sorbitol are relatively common types of plasticizers on the market, which can enhance the flexibility and elasticity of the film.(Hassan et al., 2018; Y. Zhang et al., 2018) The choice of plasticizer needs to consider the compatibility, efficiency, durability and economy of the plasticizer. Also, active substances and nanomaterials are popular research hotspots. Various bioactive substances, both synthetic and natural, such as essential oils, antimicrobial peptides and enzymes, have been investigated and applied to antimicrobial packaging systems.(Álvarez et al., 2021; Ribeiro-Santos et al., 2017; Umaraw et al., 2020) And nanomaterials are having a positive impact on the performance and sustainability of packaging by preparing nanoencapsulated bioactive agents for incorporation into edible packaging materials. (Chawla et al., 2021; Jai et al., 2021; Sáez-Orviz et al., 2020) Not only does it help prevent unwanted changes in food such as off-flavours, colour changes or any food-borne consequences, but it also ensures controlled release of active substances over a longer period of time to maintain food quality. In addition to a greater variety of sources of edible films and the active substances added to them, scientists are also advancing in the methods and technologies used to process the materials. In contrast to the traditional classification of preparation methods as simply wet or dry, based on the degree of moisture content of the main material used in the preparation. (Lisitsyn et al., 2021) This review prefers to classify the different production methods according to the ease and practicability of scaling up conditions, based on whether the method of making food packaging is more suitable for large-scale commercial production.

2: Two forms of food packaging “Film and coating”

2.1: Coating technology

Edible packaging should be classified as flexible packaging, because the shape of the packaging container can change after filling or removing the food inside. (*What Is Flexible Packaging?*, n.d.) Of course, edible packaging also undoubtedly belongs to the Primary packaging. The packaging closest to the product is an important line of defense between the product and the outside world.(Ahari & Soufiani, 2021) There

are two types of finished products, namely film and coating, and of course there are two types mixed. There is a difference between the form of film and Coating. In theory, the edible film is essentially a self-supporting structure, while the edible coating needs to adhere to the surface of the food. In the fabrication process, the "film" form can be interpreted as first giving the base material a directional structural support. After the deformation of the material is completed, replace it with the food that needs to be wrapped. There are differences in the two forms of packaging production, or in the steps of wrapping food objects. However, the process of "coating" is to use edible raw materials through non-directional methods such as spraying and dipping, and directly use the final food itself as a structural support. Finally, after drying or cooling, a protective structure is formed.

Coating materials are often in liquid form during the preparation process of coating (including direct packaging of food or coating other films), and then through dipping, scraping, spraying, layer-by-layer coating, etc. are applied to food, supplemented by drying or cooling methods. Coating materials in gaseous, solid, and gel states are applied in fewer ways than liquid ones. For example, edible aloe vera gel coating is mainly applied by dipping or coating.(Sarker & Grift, 2021) As for the solid material presenting in powder form that meets the definition of coating, fluidized bed coating technology is a good choice. (Dehghani & Farhadian, 2023; Foroughi-Dahr et al., 2017) In addition, since a single layer of edible coatings often cannot provide effective protective barrier capabilities, the combination of multiple layers of edible coatings is the main object of consideration.(Morris, 2017)

Furthermore, the choice of a specific coating method depends on the food surface and the rheological and adhesion properties of the coating material chosen. Stability between the target food and the coating material is critical to forming a good coating on the food. (María Pilar Montero García M. Carmen Gómez-Guillén M. Elvira López-Caballero Gustavo V. Barbosa-Cánovas, 2017) Before making edible coatings, it may be worthwhile to consider and think about design experiments from three perspectives. That is, from the perspective of surface tension, density, solvent and volatility of added

active ingredients of coating materials. And the shape, size, type and expected extended shelf life of the packaged food. As well as the thickness, hardness and appearance of the formed coating, the decision of these three dimensions will affect the choice of coating method.

2.1.1 Dipping

Dipping is one of the most common as well as straightforward and convenient techniques for creating coatings. A simple narrative of the process is that it consists of four stages: soaking, remain, evacuation and drying. (Ribeiro et al., 2021; Senturk Parreidt et al., 2018; Suhag et al., 2020) Put food into the prepared coating solution and keep it for a certain period of time. After the food surface has fully contacted with the solution, remove the food with appropriate tools and dry it. The moisture in the coating solution evaporates, forming a protective layer for the food. Simplicity, cost-effectiveness, and the ability to create coverage on both regular and irregular foods are advantages for dipping method compared to other available technologies (ex: irregularly shaped fruits such as dragon fruit and durian. Because we learned that coated packaging is possible to successfully limit moisture loss from stored fruit. Standard packaging may not be suitable for certain fruits or environments, durian's sharp spines can easily pierce the film shape or the headspace inside the film packaging can accumulate condensation and cause the fruit to rot).(Md Nor & Ding, 2020; Prashanth & Aparna, 2022) The dipping technique critically depends on the process parameters. For example, material factors such as solvent type, temperature and viscosity of the coating solution, and surface tension (rheological information, etc.). Process factors such as the speed of immersion and removal, number of immersion repetitions, and immersion time also need to be considered (for example, (Hamzah et al., 2022) used the corresponding surface method to optimize the effect of silk protein coating on eggplant). Finally, whether the properties of the coating material itself match the characteristics of the protected food is also the key to be confirmed ((Y.-L. Cheng et al., 2016) mentioned the properties of the protected food in the research on fruit coating, such as structure, type, maturity stage and

physiological metabolism and other different attribute information are crucial for formulating coating raw materials). Edible solution viscosity is an important process parameter when dipping food products. In the study of sodium caseinate-based reactive biopolymer coatings, researchers(Valentino et al., 2020) found that viscosity affects the thickness of both liquid and dry coatings if the coating material follows Newtonian behaviour. If the edible coating solution has high viscosity, dipping method is suitable; if the viscosity is low, methods such as spraying may be a better choice. In addition, the impregnation technique also has disadvantages. Due to factors such as drying method and gravity, the thickness of the coating after forming is uneven.(Mannozzi et al., 2021) Due to the popularity of fresh-cut fruit sales in the market, after coating with dipping technology, the appearance has become a factor that needs to be considered more. However, when it comes to protein foods such as meat and fish, the requirements are more stringent due to the basic limitation that they are more perishable than fruit.(de Souza de Azevedo et al., 2019) Especially in the production phase of large-scale operations, for example, the control of temperature, the problem of microbial growth needs to be controlled.

2.1.2 Vacuum impregnation

Vacuum impregnation (VI), as an extension of conventional dipping, is also considered by some scholars as a non-destructive technique based on hydrodynamic mechanism (HDM). (Soares et al., 2018)A solution with a specific composition can be introduced into the intercellular spaces in the food matrix and thus produce foods that meet specific nutritional and functional requirements while achieving a surface coating effect. Compared with impregnation under normal pressure, vacuum impregnation means that when the food contacts the coating material, the air in the impregnation tank is removed by an air pump to form a vacuum environment. After the immersion time is reached, repress to normal.(Senturk Parreidt et al., 2018) Because of this, compared with dipping, vacuum impregnation technology often promotes a better combination of food and coating liquid, and the coating tends to be thicker and more uniform, which makes up for the shortcomings of insufficient impregnation under

normal pressure and poor permeability. Due to the vacuum environment in the impregnation stage, this technology still has objective requirements for the food to be protected. For example, the porosity of food, impregnation coating can quickly, uniformly and thoroughly penetrate into the pores of food. Or the degree of deformation of the food, if the texture of the food changes greatly due to the vacuum environment, this method is not suitable.(Panayampadan et al., 2022; Soares et al., 2018) Therefore, before the vacuum impregnation technology can be applied to the factory line, laboratory-scale experiments are essential. It is necessary to determine the pressure level at which the vacuum will cause the least change in the composition of the food and coating solution and its physical properties, and to process the food at a minimum. The author even thinks that the vacuum impregnation cannot ignore the repressing stage, and the formation of the real coating is in the repressing stage. The vacuum stage expands the contact between the food and the coating material, while the repressing stage brings the two into tight fit, but this research area has not been deeply studied.

Table4.5 briefly summarizes the performance of dipping and vacuum impregnation techniques in edible coatings that have appeared in research articles since 2018.

At present, in the research of dipping and vacuum impregnation coating at the laboratory level, only fruits and vegetables are designed to be protected foods, and there are no protein foods such as fish. This may be due to the fact that the physical state of the fruit is more suitable for the later coating forming to facilitate the research, while the texture of meat and fish is not as hard as fruits and vegetables. So, the researchers turned to study the performance of foods that are easily protected by coatings. As far as the coating itself is concerned, vacuum dipping is superior to single dipping in terms of coating thickness, uniformity, and coverage, while single dipping is better than hand-held spraying. In addition, when the introduction of suitable intermediary coating materials may be a solution to help the main dipping coating and the protected food more harmonious in hydrophilic and hydrophobic properties, multi-layer dipping is a practical way. (Parreidt et al., 2019)

Table 4.5: Performance of Dipping and Vacuum impregnation techniques in edible coatings

Coating material	Protected food	Dipping*Vacuum impregnation condition	Special feature	Characterizing properties: Coating itself	References
Chitosan + Lauric Acid	Microprocessed Pumpkin	4 mins + 500 mmHg vacuum pressure	Compared with conventional immersion (1atm 8min)	Coating Thickness: the "VI" is thicker than the "Dipping". lauric acid reduces surface tension leading to higher wettability Coating Surface Microstructure: Vacuum dipping coated samples show a more uniform coating with less uncoated area (pore)	(Soares et al., 2018)
Alginate	Diced Cantaloupe & Whole Strawberries	Regular soaking 2 minutes, draining 1 min, 1 atm	Adding an impregnation step with calcium lactate solution before the regular impregnation	Additional soaking in calcium lactate solution promotes gel formation on the fruit surface and improves coating uniformity. The new coating process strengthens the adhesion of the alginate coating. Hydrophilic saggravate water loss from porous foods.	(Parreidt et al., 2019)
Carvacrol/Alginate	Andean blueberries	Regular Dipping in 1% w/v calcium chloride solution 30 mins	/	Surface solid density (SSD) was estimated as an indicator of average thickness of the coating, and the reduction of SSD by carvacrol may indicate good uniformity and compatibility of the coating.	(Medina-Jaramillo et al., 2020)
Cornmeal pectin, corn flour and beetroot powder	Tomato	/	Dip coatings, but first make thin films when studying coating characterization	Compositional changes were not significant for thickness, but had an effect on WVP, with water-soluble pigments or beetroot powder resulting in higher WVP	(Sucheta et al., 2019)
Ascorbic Acid & Citric Acid & Alginate	Fresh cut watermelon	Regular Dipping at room temperature (23 ± 1 °C) for 30 s	Comparison with Spray Technology (Handheld Sprayers)	During storage, the coating thickness remained almost constant, with good stability and adhesion, and the dipping had a lower standard deviation and was more uniform and homogeneous compared to the sprayed samples.	(Mannozzi et al., 2021)

2.1.3 Spraying

As a coating technology applicable to large-scale production and continuous production, the simple description of spraying is to apply a certain pressure to the coating material (usually a liquid material) through the hole of the nozzle or by means of fast-flowing air and coating solution. It is atomized to form small droplets, which can reach the surface of the food to be protected within a certain range. After the coating solvent evaporates, a protective coating is formed.(Ribeiro et al., 2021) Air spray atomization, airless atomization, and air-assisted airless atomization combining the two. These three basic forms of spray atomization have been summarized by .(Suhag et al., 2020). The main difference lies in the formation method of mist liquid. For example, the coating material required by spraying technology is less than that of dipping, and the material utilization rate is higher. It is a widely applicable method in the industry. In terms of materials, the viscosity, surface tension, rheology, etc. of the coating solution, and in terms of equipment, the shape of the nozzle and the size of the outlet hole, as well as the temperature and pressure during manufacturing. These factors will have an impact on the efficiency of spraying. (Andrade et al., 2012; de Souza de Azevedo et al., 2019; G. Lara et al., 2020; Silva-Vera et al., 2018) Parameters such as the flow rate of the coating material, the diameter of the liquid droplet, the spray distance, the angle and the overlap rate are also the key factors that affect the quality of the package. Spraying is applicable to more types of food than dipping, because coating materials are first transported by pipelines, and there is a gap before food is attached. No need to worry about contamination of edible coating materials by foods, especially fresh-cut fruits.(Aayush et al., 2022; Filho et al., 2021) Dipping may dilute the coating solution and cause a large amount of residual coating material. In addition, dipping cannot easily control the appropriateness of the coating solution and requires a relatively longer time to dry the excess solution, which may hinder its industrial application. (Pham et al., 2023) Both panning and fluidized bed coating systems require intense tumbling operations, which risk damaging the food. These are the disadvantages that can be avoided by using the spray mode.

Table 4.6 makes statistics on the research articles that use spraying technology or electrospray technology to make edible films in recent years but involve coating performance.

As can be seen from Table 4.6, there are few studies on the performance of coating solutions prepared by spraying or electrostatic spraying methods or the coating behaviour after spraying. Researchers are more concerned with the direct protective effect of edible coatings on food (e.g., food weight loss, oxidation, bacterial infection, etc.), rather than the performance of the coating itself. The author expects future researchers to strengthen research in this area. From limited articles, we found that the actual performance of edible coatings depends on two aspects, the way of spraying and the coating material itself (for example, in the experiment of (G. Lara et al., 2020), coating viscosity is an important factor affecting the specific thickness, while.(Jiang et al., 2019) shows that electrospray can form a more uniform and continuous coating structure).

Table 4.6: Research on the properties of edible coatings involving spraying, electrostatic spraying and electrospinning: 2018-2023

Conventional (CS) /Electrostatic (ES)	Main coating content	Food	Spraying process	Behaviour of the coating solution	Coating Properties Results	Reference
CS	0.1%, 0.3%, or 0.5% xanthan gum solution, 2% (w/w) citric acid as anti-browning agent and 1% (w/w) glycerin as plasticizer	Fresh cut lotus root	The coating was applied to the surface of fresh-cut lotus root (5 mm) using a pilot spray system.	Increasing the xanthan gum concentration from 0.1% to 0.5% (w/w) resulted in an increase in the viscosity of the solution from 6.04 to 75.00 mPa.s The average droplet size of the sprayed coatings was between 5.14 and 5.61 µm	The increase in viscosity resulted in an increase in coating thickness from 17.03 to 66.67 µm, and the smoothness seen by SEM increased with increasing viscosity	(G. Lara et al., 2020)
CS、ES	Chitosan with different degrees of deacetylation (81.0, 88.1 and 95.2%). 30% glycerol (w/w CH) as plasticizer and 5% Tween 80 (w/w CH) as surfactant	Strawberry	Spray the chitosan solution onto the surface for 1 min + electrostatic sprayer with a nozzle diameter of 3 mm. The spray rate is 3.8 l/h. Conventional spraying (CS) with the same equipment and settings, except the voltage was zero.	/	Fluorescence micrographs of conventionally sprayed strawberries showed large bright spots and distinct areas of discontinuity on the surface, poor coating coverage, negatively impacting barrier and mechanical properties. The electrostatically sprayed ones formed a more uniform and continuous structure on the strawberries with smaller droplet sizes.	(Jiang et al., 2019)
CS handheld	Bagasse cellulose nanocrystals (BCNCs), chitosan nanofibers (ChNFs) and sodium alginate (SA) mixed with oregano essential oil (OEO)	Strawberry	50 ml fine mist sprayer bottle to apply approximately 350 µl of suspension (spray four times) to each strawberry surface	The SA/BCNC/ChNF coating suspension inhibits the spread of Botrytis cinerea) mycelia, and BCNC as a filler reduces the fluidity of the coating suspension. In addition, OEO helps to improve the antifungal properties of coating suspensions.	The viscosity increased slightly with the incorporation of OEO, and surfactants in OEO droplets and suspensions may affect the formation of more complex networks. Low viscosity and low solids suspension can provide consistent spray volume and desired film thickness by spray method	(Lee et al., 2022)
CS	Salmon Gelatin SG 10% w/v + Riboflavin 0.025% w/v hydrogels by exposing SG-Rf suspensions (pH 5.0 or 8.5) to UV or visible light	Salmon fillet	Spray the suspension onto the salmon fillets using an air compressor (Huracan 1520, Indura, Santiago, Chile) with a 1.5 mm diameter nozzle. Expose the coated fillets to VL (4 cm from the surface) for 2 min	The cross-linking reaction between gelatin and riboflavin can be formed by UV and visible light polymerization, resulting in heat-resistant gels, which are more pronounced at pH 8.5.	/	(Char et al., 2019)

2.1.4 Electrospraying & Electrospinning

The words "Electrospinning" and "Electrospraying" are very similar and both are used as techniques for making coating materials. But one of them shows people in a fibrous form, and the other produces a granular form. Logically, the final product of electrospinning or electrostatic spraying is not a "coating" technology, but a "film" technology. After the material has been ejected from the nozzle in a high voltage electric field, a receiver plate is needed to form the film before wrapping the foodstuff, which is not a direct coating.(Anu Bhushani & Anandharamakrishnan, 2014; Castro Coelho et al., 2021; Jayaprakash et al., 2023)

Anu Bhushani et al.(Anu Bhushani & Anandharamakrishnan, 2014) also elaborated the electrohydrodynamic process of both techniques. In electrospinning, a coating solution is placed in a high-voltage electrostatic field, causing it to become charged and deformed. A Taylor cone is formed at the tip of the nozzle, and a charged coating jet is ejected from the tip of the Taylor cone when the charge repulsion on the droplet surface exceeds the surface tension. These ejected fluids undergo high-speed stretching by electric field force, solvent volatilization and solidification, and finally deposit on the receiving plate to form fibrous materials. In electrostatic spraying, there is a high-voltage electrostatic field between the nozzle and the receiving device, and the sprayed liquid has an electrostatic field force. At the same time, the liquid also has a surface tension that interacts with the electric field force. When the electric field force overcomes the surface tension, the droplet bursts into nanoscale or micron scale, and solidifies into micro/nano particles as the solvent volatilizes. The main difference between the two technologies is also due to the inconsistent concentration of the edible coating solution. The viscosity of the electrospinning solution is relatively high, and the entanglements between the molecular chains are many and tight, and the product is a nanofibrous film.(Dede et al., 2022; Ebrahimi et al., 2019; Rodríguez-Sánchez et al., 2021; Topuz & Uyar, 2020) The electrostatic spray precursor solution has less viscosity, less entanglement between molecular chains, and the product is micro/nano particles.(Cakmak et al., 2018, 2019; Gaona-Sánchez et al., 2021; Y. Wang

et al., 2020) From the application point of view, electrospinning and electrospray are not only popular technologies in the fields of nanoelectronic equipment, medical treatment (drug delivery system), biological tissue engineering, textiles, etc., but also have advantages in the field of food packaging. Structurally it can modify the size and morphology of the package, providing a highly porous structure, large surface area and interentangled fibrous structure. In terms of food packaging materials, the packaging made by electrospinning and electrostatic spraying generally has slow-release characteristics, high encapsulation efficiency, and can enhance the stability of encapsulated bioactive compounds.(Castro Coelho et al., 2021; Jayaprakash et al., 2023; Topuz & Uyar, 2020)

Figure4.7 is used to count the patents and articles using these two technologies for edible food packaging in the past five years. The relevant patents are collected by using the Espacenet website, and the statistics of the articles use the Scopus platform. We set up two technologies on two platforms at the same time, each set of three keywords or roots: "edible"—"package (packaging)" and "Electrospray (Electrospraying) or Electrospin (Electrospinning)" set all text in patent search fields or names, the date start and end is set to 2018.01.01-2023.12.31:

Manual screening criteria are based on whether the patent involves the production of edible coatings or films, and the same criteria apply to articles.

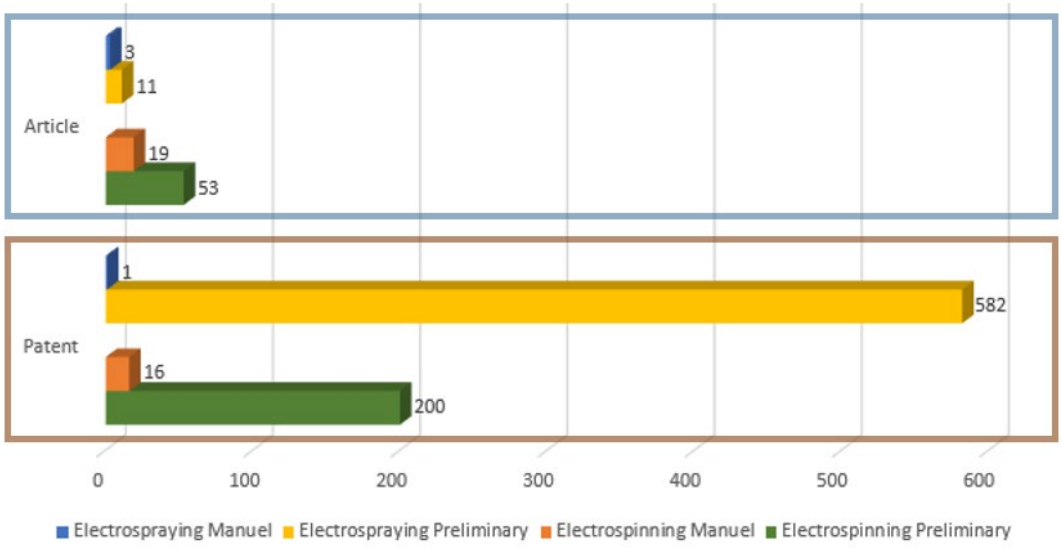

Figure 4.7: Comparison of Patents and Articles of Electrospraying & Electrospinning in 2018-2023

The final screening collected 22 articles and 17 patents that were close to the topic of edible packaging. Specifically, in the search results of Scopus, articles accounted for 100%, and there were no relevant review articles or conference proceedings. In terms of patents, it focuses on the research and development of coatings and films materials, and there are no process technology patents.

By comparing patent inventors and article writers, only one research team (LANYUE, 2019; Y. Tang et al., 2019) converted all of his team's research into patents. And most of the remaining patent holders are institutions. Compared with Electrospraying & Electrospinning, there is an obvious gap between the two in terms of the subject of edible food packaging. The number of research papers and patents on electrospinning technology for edible packaging on the market is more than that of electrospraying technology.

Selected papers and patents duly demonstrate that electrospinning is a technology that can help nanomaterials achieve spatial fulfilment, although industrial-scale fabrication of electrospun fibres and electrospray particles for food preservation is hoped. However, at the current stage, it is mainly on the laboratory scale, and it is difficult to commercialize it. It is recommended to conduct advanced research on electrospinning and electrospray technology in the field of food processing and preservation to make the application feasible on an industrial scale.

2.1.5 Panning

The process of panning is simply to put the food into a rotatable container, and then scoop or spray the coating solution (there is no high technical requirement like spraying) into the rotating plate or pot.(Andrade et al., 2012) The food is tumbled in the pan to evenly distribute the edible coating solution over the surface of the food. Compared with the dipping method, panning requires the food to be able to be turned, so it also has certain requirements for the physical properties of the food, so that it is currently a method commonly used in the pharmaceutical, confectionary, nut and chocolate industries. (Gauba, Pammi,Reema Gabrani, 2020) Because candy, nuts and

other ingredients are relatively hard. Edible coatings or films can be classified as food, and coating technology can also explain how to simply use the food itself as a packaged part. Similar to traditional Chinese glutinous rice balls or chocolate peanut beans in the world, it is a typical example of the main production method using the coating technology, with a high degree of industrialization and mature technology. Jeff Bogusz (Bogusz, 2017) summarized the use of panning equipment, from small shops equipped with countertop translation discs to large factories equipped with groups of automatic panning discs. As for the shape of the pan, the user is more concerned about the shape of the final product and tends to choose a known shape. Pans are typically mounted at an angle of 15 to 25 degrees for increased capacity and uniformity of centre motion. When going from small-scale to large-scale production, the main challenge is to eliminate the deformation of food caused by the increase in pot size, capacity, and pressure on the product itself. Surface treatments on the inside of the pan and operations such as adding assist balls can help achieve more efficient coatings. In addition, the temperature control and duration control of the pot are considered critical, and air handling equipment is essential. In addition to attaching the coating, panning's pans can also polish the product. Soft belt coating machines are more suitable for light or delicate foods such as wafers, etc. These are also often called soft coating methods.(Suhag et al., 2020) In addition, (nelma aghazadeh , Mohsen Esmaiili, 2021) have modelled the panning process in the production of hazelnut dragees to improve the modeling of hard candy panning. And this technology is also commonly used in the encapsulation of food colouring. This is also in line with the patent search, if only panning, edible and coating are used as keywords, the results are generally patents about candy and food colouring.

2.1.6 Fluidized bed

Fluidized bed coating technology can solve the dilemma of food clusters that occur during the coating process.(Foroughi-Dahr et al., 2017) In simple terms, a fluidized bed is a reactor that uses gas or liquid to pass through the granular solid layer to keep the

solid particles in a state of suspension and movement, and to perform a gas-solid phase reaction process or a liquid-solid phase reaction process. In the field of food coating packaging, we can also regard it as a fluidized coating technology suitable for micro food (granule) packaging.(Dehghani & Farhadian, 2023) To make the driving gas can stratify the food, in addition to increasing the air pressure and water pressure, the food is also required to be as light or small as possible.(Morris, 2017) For example, the edible coating solution is sprayed with a nozzle, and the solution flows over the surface of small-volume foods such as wheat, puffed food, and nuts, and an edible coating is formed as the solvent dries. Fluid bed requires more coating solution than pan coating because it is lost to the column walls during spray coating. Fluid bed coating can also be used to "enhance" or "tune" the impact of functional ingredients. (Dehghani & Farhadian, 2023) still mentioned that the fluidized bed process is often classified into three categories due to the relationship between the spraying direction of the coating solution and the fluidization agitation direction, namely top spray, bottom spray and side spray. A spiral pattern in which the three directions are combined is considered the best. The top-spray method is considered more suitable for scale-up and industrial production due to its high versatility, relatively high batch size, and relative simplicity. However, due to the high cost compared with other coating methods, large consumption of coating solution, premature evaporation and other factors are its inevitable defects. (Müller et al., 2018) have studied the performance and application of beeswax, carnauba wax and palm oil mixture in hot melt coating through Worst fluidized bed. Discover the fundamental difference between solvent-free or hot-melt coatings made of natural or food-grade materials and solvent-based coatings. Crucial to the coating process is an understanding of thermal behaviour, especially the crystallization temperature range. (Rocha et al., 2018) also came to a similar conclusion in the study of the spouted bed coating process of pearl grass granules. Atomization pressure or droplet size have little effect on process kinetics, while the combination of high inlet temperature and high coating flow rate, or low inlet temperature and low coating flow rate is the best combination for coating efficiency and particle growth. In

addition, fluidized bed technology coating is usually a display platform technology of microcapsule technology and nano coating technology, and double fortification of salt is the most direct proof.(Modupe et al., 2022)

2.1.7 Brushing approach

A method of coating in which an edible coating is applied to the surface of a food using a brush. It is mainly related to the problem of continuous production, and it is a simple and reliable coating method. The length, hardness and thickness of the bristles may affect the brushing effect. (Azevedo et al., 2022) For the food to be painted, if it is a complex surface, the effect of brushing is not as good as that of a smooth surface. If done manually, while avoiding the use of huge dipping tanks or expensive initial purchases of machinery, industrial-scale production can be labour intensive and inefficient. It is also not suitable for quick-drying edible coating materials because of the occurrence of brush marks, sags and uneven brushing defects. From 2018 to 2023Q1, there are 2,300+ patents related to food brushing device patents involved at the industrial level (search keyword food coating Brushing device). The main ones involved are cakes, and the surface of bread is coated with liquid.

2.1.8 Layer by layer

Layer-by-layer coatings can improve food protection by combining the properties of different edible coatings. This is also due to the fact that a single material often cannot meet the requirements of many applications. Since the different layers have specific properties, such as moisture resistance and mechanical stability, or gas barrier properties. (María Pilar Montero García M. Carmen Gómez-Guillén M. Elvira López-Caballero Gustavo V. Barbosa-Cánovas, 2017) The superposition of composite layers can be adjusted according to the properties of the food itself, the number of layers to be coated, the coating technology used, the diversity of coating materials, and the order of adding coatings.(Flores-López et al., 2016; Trajkovska Petkoska et al., 2021). (Arnon-Rips & Poverenov, 2018) have summarized the LbL method and believe that

taking such an approach can produce more advanced edible coatings. Currently, LbL edible coatings are mainly applied to fresh fruits and vegetables. But it can also be applied to other foods, with polysaccharides being the most common LbL coating material. (Bilbao-Sainz et al., 2018b; Saberi et al., 2018; Yan et al., 2019) The electrostatic interaction during the formation of LbL coatings is the most common chemical force in this structure. The overall electrical neutrality of the LbL structure is achieved through a charge overcompensation mechanism. The mechanism is based on intrinsic and extrinsic charge compensation and competing ion pairs. In addition to electrostatic interactions, hydrogen bonding and hydrophobic interaction forces may also drive the LbL assembly process.(Arnon-Rips and Poverenov 2018; Jin et al. 2021; H. Wang, Qian, and Ding 2018; G. R. Lara et al. 2020)

Table 4.7 focuses on the coating research content of the LBL process in recent 5 years. As can be seen from Table 4.7, the research and application of LBL is mainly concentrated on fruits and vegetables. The stacking method is more concentrated in two types of layer-by-layer impregnation and electrostatic deposition. This provides a basis for the industrial scale-up of the process.

Table 4.7: Experimentation with edible coatings by layer-by-layer stacking

Main laminated materials	Laminated technology	Number of layers	Protected Food	Results	Reference
Chitosan and Carboxymethyl Cellulose	Electrostatic deposition	3	Strawberry	Application of LBL significantly reduced the content of primary metabolites involved in carbohydrate, fatty acid, and amino acid metabolism, and secondary metabolites involved in terpenoid, carotenoid, phenylpropanoid, and flavonoid metabolism after storage.	(Yan et al., 2019)
Linseed Gum and Chitosan	Electrostatic deposition	2	Mongolian Cheese	Polysaccharide layer-by-layer electrostatic self-assembly technology improves the quality of Mongolian cheese. Chitosan is antibacterial, and linseed gum can be used as an emulsifier to reduce the formation of fat grooves.	(Jin et al., 2021)
Chitosan-gelatin complex and pomegranate peel extract	Dipping	2	Yellow croaker	There is a synergy between chitosan-gelatin coating and PPE. Bilayer coatings outperform composite coatings combined with PPE in reducing lipid oxidation in fish fillets	(Saki et al., 2018)
Flourensia cernua antibacterial extract, Alginate, Chitosan	Dipping	5	Tomato	The nanolaminate coating results in reduced permeability to water and oxygen. Extends shelf life of fruit by reducing microbial growth, reducing gas exchange, maintaining firmness and color	(Salas-méndez et al., 2019)
Lipid blend of pea starch and guar gum with shellac and oleic acid	Spraying	2	Orange	LBL coating reduces weight loss and respiration rate and improves firmness retention, but double coating also results in higher levels of ethanol which increases odor perception	(Saberi et al., 2018)
Sodium Alginate (SA) and l-menthol-β-cyclodextrin-graft-chitosan	Dipping	18	Apple	The addition of l-menthol produces a clear, smooth, dense coat. It has better mechanical properties and self-healing properties. And thanks to the release of l-menthol bound in beta-cyclodextrin, the coating achieves a controlled release profile.	(Yifan et al., 2020)
Chitosan/Pectin	Dipping	2	Okara Insoluble	LBL-coated particles are negatively charged and exhibit significantly increased	(Wei et al.,

			Dietary Fiber Granules	hydrodynamic radius, higher water absorption capacity, and water suspension. Tofu with modified O-IDF had better texture properties, stronger resistance to mechanical damage, and higher sensory scores.	2018)
Cross-Linked Sesame Protein/Guar Gum	Dipping	2	Mango	There were significant differences in the effect of coating and storage temperature on fruit quality and shelf life. Sesame protein isolate cross-linked with organic acids is a better and more cost-effective alternative	(Sharma et al., 2019)
κ-Carrageenan and Quercetin	Dipping	5	Assessing antioxidant capacity and potential cytotoxicity in vitro	Good antioxidant activity without cytotoxicity (alveolar macrophage culture)	(Souza et al., 2018)
Chitosan (CS) and Sodium Alginate (SA) and Cinnamon Essential Oil (CEO)	Electrostatic deposition	1-2-3	Apple	Thermogravimetric analysis (TGA) and Fourier transform infrared (FT-IR) analysis revealed that the layer-by-layer approach altered intermolecular interactions and thermal stability. Multilayer films exhibited longer-lasting CEO release and higher retention.	(W. Zhang et al., 2019)
Alginate and Mushroom Chitosan	Electrostatic deposition	2	Fruit bar	LbL-coated fruit bars containing alginate-chitosan showed increased ascorbic acid content, antioxidant capacity, firmness, and fungal growth prevention during storage.	(Bilbao-Sainz et al., 2018a)
Alginate and Chitosan with Grapefruit Seed Extract	Dipping	2	Vannamei shrimp	The antimicrobial effect of edible coatings was greatly influenced by GSE and partially by chitosan. Chi-Al coating has growth inhibitory effect on mesophilic and psychrophilic bacteria.	(J.-H. Kim et al., 2018)
Main laminated materials	**Laminated technology**	**Number of layers**	**Protected Food**	**Results**	**Reference**

2.1.9 Short summary about "Coating":

Coating, as a non-thermal processing-based technology, has excellent performance in the manufacture of food packaging containing heat-sensitive antimicrobial agents. In addition, it has advantages over direct incorporation of active materials by maintaining the bulk properties of packaging materials (e.g., mechanical and physical properties) and minimizing the amount of antimicrobial agents to achieve sufficient efficacy.

When converting from laboratory scale to industrial mass production, more changes and difficulties are involved in continuous production, and the use of different scales of production tools requires different process parameters.

Coating is often used to protect the food mainly fruits and vegetables, because the appearance conditions of these substrates are relatively complex, and the use of film packaging may not provide maximum protection. Dipping, Panning, Spraying, Brushing, Vacuum impregnation etc. are coating methods with high commercial maturity, and Electrospraying & Electrospinning are currently more inclined to use the laboratory scale.

The deposition method depends on the nature of the food to be coated, the supporting surface being the main parameter for a successful coating. In addition, the surface tension, density and viscosity of the coating material influence the choice of application method and equipment used. Dip and spray techniques are commonly used as application methods for fresh product coating materials to ensure uniformity over rough and complex shapes. In the case of spraying, several disadvantages are mainly related to the operating parameters of the equipment and must be combined with the physicochemical properties of the coating. For low-density coated materials, fluidized bed processing is the method of choice, while in the confectionery industry gold panning is used to apply thick layers to hard materials.

3: Film in all scales

Different from the "coating" form, the "film" form is to make a packaging material with a specific shape before packaging. And the tasks undertaken by "coating" and "film"

are also different. "Film" is more focused on controlling or isolating the external influence of gas or light on food quality, and even helping food to improve storage quality. However, "coating" is more commonly used to improve the physical integrity of the product to be coated, enhancing the appearance and structure of the product. Their different physical characteristics will also provide more development options in the field of edible packaging, more exploration directions based on scientific research, and more practical mode choices for producers.(Galus et al., 2020; Gupta et al., 2022; Jeevahan & Chandrasekaran, 2019; Suhag et al., 2020)

This section summarizes the mainstream "film" production technologies currently involved, in the field of edible films, the technologies currently suitable for laboratory scale and the technologies supporting the industrial scale production of edible films, and their advantages and disadvantages.

3.1 Laboratory scale

3.1.1 Solution casting

The Solution Casting method (or solvent casting) is a popular preparation method for researchers in the laboratory or pilot scale because of its simple operation steps and drying process. Generally, researchers will first disperse or dissolve the edible biopolymer to be studied in a selected solvent (heating and stirring are commonly used to promote dissolution). The addition of natural plasticizers (polyols, mono-, di-, and oligosaccharides, etc.) aids in film formation.(Sothornvit & Krochta, 2005) After complete dissolution, the solution is cast in a mold (debubbling procedure is also a step to improve film quality, vacuum defoaming or ultrasonic defoaming), usually plastic or glass Petri dishes (also common acrylic, silicone or PTFE plates wait).(Asim et al., 2021; Otoni et al., 2017) Then volatilize the excess solvent under the action of normal temperature or oven heating. After demoulding, it is generally stored in a space with a moderate temperature that can control the humidity and avoid light to ensure the stability of the material for subsequent research. (Galus et al., 2020; Marcet et al., 2018; Maurizzi et al., 2022; Peidaei et al., 2021)This method is also known as a "wet

method"(Galus et al., 2020). With regard to making multilayer films in this way, they can be formed by sequentially applying solutions to the film and then allowing it to dry. In general, laboratory-scale experiments are necessary to determine acceptable formulation and process conditions before scaling up a production process in a pilot line or commercial production facility. In the process of making edible films under laboratory conditions, the optimization of film materials may be more research hot and trendy than the optimization of the technology itself.(Khalid & Arif, 2022; Martins et al., 2021; Santhosh et al., 2021) When the Solution Casting method is extended to industrial scale production, the main improvement is the efficiency issue, that is, the rapid molding of the cast film, which involves the drying technology of the casting solvent, and the continuous casting is the combination of rapid drying and assembly line layout production.(Jeya Jeevahan et al., 2020)

3.1.2 3D printing

The concept that needs to be distinguished first in this chapter is that 3D technology is used to print food packaging films rather than food. It is undeniable that their boundaries are blurred in many aspects, but we try to focus on the production of edible films. 3D printing technology is simply a rapid prototyping manufacturing technology based on 3D mathematical model data. It involves building objects through sequential physical stacking and is widely used in several industries, including construction, aerospace, medical, biological, and food industries.(Nachal et al., 2019; Nida et al., 2021) This technology can also be called Rapid Prototyping Manufacturing (RPM), and it is also called Additive Manufacturing (AM) from the technical division of the manufacturing process. Unlike several other 3D printing processes (Stereolithography (SL), Powder Bed Fusion (PBF), Laminated Object Manufacturing (LOM), Binder Jetting (BJ) (suitable for confectionary), in the field of food Fused Deposition Manufacturing (FDM) is the mainstream production method (of course limited by the thermoplastic properties of raw materials). Candy, chocolate, pancakes, and vegetable protein are ideal materials of choice for fused deposition

modeling.(Nachal et al., 2019) The printing process in the simple (FDM) mode can be divided into the following steps: 1. The computer aided design(CAD) model determines the printing trajectory. 2. Preparation of an edible material that can be melted into a fluid. 3. The printer heating chamber heats the edible material to a critical state (semi-fluid state). After that, it moves along the two-dimensional geometric trajectory confirmed by CAD, and at the same time, the nozzle extrudes the material in a semi-fluid state. The material forms a base plane as the temperature decreases (the production of edible films can be done horizontally again in 2 dimensions). Then the nozzle moves vertically, printing and accumulating edible materials layer by layer, and edible finished products can be obtained after curing. (Nachal et al., 2019) As for the production details, in the article by (Rowat et al., 2021), the feasibility of using vegetable protein as a source material is comprehensively revealed. They believe that the plasticizing temperature and glass transition temperature of the material are the most important. Making edible packaging films can be different than overall food production. As early as 2021, (Leaw et al., 2021) used cornstarch-gelatin film, and added glycerin and hawthorn berry (Crataegus pinnatifida) extract as active substances as the printing source material. The study showed that 3D printed CSG-HBE edible films containing 4% (w/v) glycerol exhibited higher elongation at break compared to the control. Meanwhile, the films containing 6% (wt/vol) HBE had elongation at break and TS that met conventional standards. It also effectively inhibits the growth of Pseudomonas aeruginosa. In 2022, (Yap et al., 2022) in the same laboratory as the former incorporate garcinia cambogia extract on the basis of gelatin as a printing source material. As the glycerol concentration increased from 0% to 20% per gram of gelatine, the hardness, thickness, water solubility, total colour difference, and elongation at break of the 3D printed films all increased. However, both tensile strength and Young's modulus decreased with increasing glycerol concentration. The optimal concentration of glycerol was detected to be 20% per gram of gelatin. Whereas, as GAE was increased from 1% to 4% (w/w), a decrease in the hardness and thickness of the 3D printed films was observed. Different from the former two using

gelatin as the base material, (Dey et al., 2022) use soybean functional edible protein as the base material. Before printing, the concentration range of soybean protein isolate was set at a concentration of 5%-15%, the pH was adjusted to 8.5, glycerol (2, 3.5 and 4% w/w) was used as a plasticizer, and a water bath at 85°C for 30 minutes was used to promote protein denaturation. After cooling, the film-making solution was printed onto a silicone-coated mylar plastic sheet using a consumer-grade 3D food Foodini (Natural machines, Spain). Finally, through the response surface experiment, it was determined that the optimal film-forming conditions were soybean protein concentration: 8.91%, plasticizer concentration: 3.00%, drying time: 3.98 h, and the ideal value was 0.7428. The films were able to achieve a thickness of 0.108–0.114 mm; a tensile strength of 14.79–16.07 MPa; a puncture strength of 6.97–8.20 N, and an elongation at break of 104.4–105.7%.

In general, 3D printing edible films is considered to have obvious advantages in terms of sustainable development. Less material waste, less post-processing. In addition, compared with other film production technologies, 3D printing films can achieve customized film shapes is also a feature. Today, there are no printers on the market that specialize in edible films, and they are still based on food 3D printers. They did not involve printers for industrial mass production.

3.1.3 2D/4D printing

Two-dimensional printing belongs to the traditional printing technology, which is used to make two-dimensional structures with decoration or information transmission function on food.(Pallottino et al., 2016) Some researchers used 2D printing technology to make films, they develop an edible oral film containing the non-steroidal anti-inflammatory drug diclofenac sodium. Based on solubility studies and rheological property evaluations, the preparation of drug-loaded inks was carried out in combination with ethanol and propylene glycol as optimal solvents. Drug deposition is achieved by increasing the number of printed layers on the edible substrate. They also created inkjet-printed edible films for oral delivery of B-complex vitamins.(G.

Eleftheriadis et al., 2020; G. K. Eleftheriadis et al., 2018)

4D printing technology is an extension of 3D printing. The colour, shape and taste of a product can change physically or chemically when consumed and used by the user. At present, it is more inclined to the production of food as a whole, and packaging film is a possible new research direction.(Pulatsu et al., 2022; Teng et al., 2021; Yang et al., 2022)

3.2: Pilot plant scale

The Pilot plant scale stage is the necessary transition link from small scale experiment to industrial production, focusing on "verification and application". The purpose is to verify, review and improve the synthetic process route determined by the laboratory process, whether it is mature and reasonable and whether the main economic and technical indicators are close to the production requirements.(Sarghini et al., 2013) This scale of research is mainly for the structural design, material information, installation process and workshop layout of the selected industrial production equipment. Provide reference data and material information for formal production. It is an effective measure to reduce the risk of industrialization.(Franco et al., 2020; Munhoz et al., 2018a)

3.2.1 Continuous casting

Continuity means the transformation of thin film production from small-scale, single-batch inefficient mode to high-efficiency mode pursued by industrialization, and it is also a difficult point in the process of scaling up. Continuous casting refers to the mixing and casting of film materials without interruption. This is done in "solution casting" and the film is shaped by a drying machine. All steps are connected in one process. It's like a production line.(Cai et al., 2020; Hamed et al., 2022; Leite et al., 2020) The advantages of the general solution casting continuous casting mode mainly come from the carrying of the conveyor belt and drying technology. Some scientists have conducted relevant research on edible film conveyor belt materials.(Gamboni et al.,

2021) They found that metal belts, in addition to being expensive, required more demanding construction details than those corresponding to flexible belts. The thin thickness of the TAC 36 (PTFE) support material makes it difficult and expensive to obtain an annulus without discontinuities at the joints. According to the calculation of the adhesion value and spreading coefficient, the film-forming solution can be easily spread on metal and hygienic rubber, followed by silicone and polyurethane belts. However, it may be difficult to peel off the film with a rubber conveyor belt.

Table 4.8 summarizes all the researches on edible packaging films made of biomass-derived materials by continuous casting in the past five years. Critical casting details and subsequent drying methods are the focus of attention.

Table 4.8 shows that Machine KTF is the most popular continuous casting equipment for researchers, which may also be related to the fact that research in this field is mainly concentrated in European and American countries. The homogenization and degassing of the edible coating solution in the experimental casting is worth considering, and the drying of the film mainly uses a high-temperature oven with air convection, and some use infrared preheating. The selection of the production process under the pilot scale is subject to the consideration of the producers from the Laboratory scale and industrial scale.

Table 4.8: Partially published articles on continuous casting aspects since last 5 years

Base material	Continuous casting equipment	Casting details	Drying method	Reference
Passion fruit marc	KTF-B laboratory coater (Werner Mathis AG, Zürich, Switzerland)	Pectin, rind and solvent are vigorously homogenized in a polyacetal vessel at 25 °C and 20 000 rpm. The suspension was degassed by applying vacuum at −400 mmHg and mechanical agitation, and immediately cast as a monolayer film on a KTF-B laboratory coater using a spatula to adjust the wet layer thickness to 1.7 mm.	Pass through an IR pre-dryer with an emission efficiency of 40-55% at 12 cm min-1. Subsequent drying through an air-circulating oven (total length 80 cm) equilibrated at 120°	(Munhoz et al., 2018b)
Peach pulp, hydroxypropyl methylcellulose and nanocellulose	KTF-B Lamination System (Werner Mathis AG, Switzerland)	1. Feeding: LFF is poured on a polyester film (DuPont Teijin Films America, Inc., USA) conveyor belt; 2. Lamination: LFF is forced through the gap between the polyester substrate and the knife to form a 1.50-mm thick , 26-cm wide wet layer;	The wet LFF layer was passed through an infrared pre-drying stage (approximately 45 °C and 0.10 m min -1 for 30 cm) and two convective drying stages (at 120 °C and 0.10 m min -1 for 92 cm each	(Franco et al., 2020)
Bacterial Cellulose/Tomato Paste	KTF-B Lamination System (Werner Mathis AG, Switzerland)	Adjust film-forming viscosity (500 g solids for 2500 mL dispersion). Continuously (manually) spread the dispersion onto a polyester film substrate moving through a conveyor belt, adjusting the wet layer thickness to 0.8 mm with a Type B spatula	The wet layer is passed through two consecutive 80 cm long drying chambers with a controlled temperature (80 °C) at a speed of 10 cm min	(Freitas et al., 2022)
Mango Pulp + Pectin	KTF-B Lamination System (Werner Mathis AG, Switzerland)	The film-forming solution was added to a polyacetal vessel and homogenized vigorously using an ultraturrax at 25 °C and 7168 g for 10 min. The solution was degassed by applying a vacuum at -400 mmHg with mechanical agitation and immediately became a monolayer film on a KTF-B laboratory coater. On the coating device of type B doctor blade, a wet solution layer with a thickness of 1.5mm is formed using a pair of contrast dials (±0.001mm).	The wet layer is passed through an IR pre-dryer at 40–55% emission power at 12 cm min and then through an air-circulating oven at 100 °C (total length 80 cm)	(Oldoni et al., 2021)
Tapioca Starch, Cocoa Butter, Lemongrass Essential Oil Nanoemulsions	KTFS-B Lamination System (Werner Mathis AG, Switzerland)	TPS (8% w/v), glycerol (20 wt.%), CB (30 wt.%), and BSG (5 or 10 wt.%)—weight percentages are relative to starch dry mass—was combined at 1:1 (weight ratio) with the prepared emulsion. The emulsions LEO (droplet size [z-average]: 372 ± 5 nm, polydispersity index [PdI]: 0.29 ± 0.01) The suspensions were poured onto a MylarR conveyor belt and forced through a gap between the polyester	Perform in two ovens at 90 °C at a speed of 0.10 m/min.	(Juliana Farinassi Mendes et al., 2021)

and Cereal Fibers		substrate and a knife in a 1.50-mm-thick wet layer.		
Apple and Carvacrol	laboratory coater (Werner Mathis AG, Zürich, Switzerland)	0.041 in. thickness apple solution on a Mylar sheet conveyor moving at 0.11 m/min.	The film was first partially dried by an infrared heater adjusted to 0 and 90% energy emission at the bottom and top, respectively. Next, the convective heating stage was controlled at 132 °C and 1500 m/min air velocity.	(Du et al., 2008)
Cocoa Butter Bound Pectin	Mathis KTF-SB, Germany	Shake at 7000 rpm for 45 min and degas under vacuum for about 1 h. Then dry the PEC and PEC/CB films in a continuous caster. The coating knife adjusts the thickness of the sample and spreads the solution on the substrate. The film-forming formulation was deposited onto a polyester conveyor belt moving at 0.10 mm/min	The slurry is forced through the gap and spread into a uniform 1.5 mm thick (distance between coating knife and substrate) wet layer which passes through two oven drying stages at 80 °C	(J. F. Mendes et al., 2020)
Oregano Essential Oil Loaded Zein/Gelatin	DL-LYJ-QE150 Casting Machine China Delong Company	On PET tape, blade height 0.75 mm, for multilayer films FFS is cast on the previous cast layer, running two or three consecutive casting cycles to form double or triple layer films	Dry in an oven at 60 °C at a rolling speed of 0.02 m/min	(Cai et al., 2020)
Active starch of lemongrass essential oil	Mathis KTF-SB, Germany	Homogenize at 15,500 rpm for 15 min, and vacuum degas for 20 min. TPS and TPS/emulsion formulations are dried into films in a continuous mode on the KTF-SB lamination system.	The film-forming formulation was deposited onto a polyester conveyor belt moving at 0.10 mm/min. The slurry is forced through a gap and spread in a uniform 1.5 mm thick wet layer. This wet layer is passed through two oven drying stages at 90 °C.	(J.F. Mendes et al., 2020)
Base material	**Continuous casting equipment**	**Casting details**	**Drying method**	**Reference**

3.3: Industrial production scale

The disadvantages of film production on a laboratory scale include problems such as inability to continuously form films, long drying time, inaccurate thickness control, high energy consumption, and high cost. This needs to be solved on an industrial scale, and it is also a challenge for industrial-scale production.(Jeya Jeevahan et al., 2020) This chapter will mainly summarize the film production technologies called "dry method", which is because the source material involved has less moisture.(Galus et al., 2020; Kumar et al., 2022) Including Extrusion molding, Pressing, Calendering, etc. Due to less moisture, the drying step don’t cost a lot of time and is a good choice for large-scale production.

3.3.1 Extrusion molding

Extrusion molding can be simply described as the edible material that passes through the mutual extrusion between the extruder barrel and the screw and the thermal gelatinization, and then outputs the mixture through the nozzle of the extruder. (V et al., 2022) In the extrusion equipment, the screw extruder is the most common production equipment. And according to the number of screws, it is divided into single-screw type, twin-screw type and multi-screw type (the production of edible materials is concentrated in the first two methods). Relying on the pressure and shear force generated by the rotation of the screw, the material can be fully mixed and the pressure increased, and then extruded through the die.(Chevalier, Chaabani, et al., 2018; Huntrakul et al., 2020a; Vedove et al., 2021) There is also ram extrusion, which is achieved in a piston extruder, where instead of an extrusion screw, a plunger or piston is used. The relationship between this extrusion technique and 3D printed films was also verified by Musazzi et al., who demonstrated the feasibility of extemporaneous preparation of maltodextrin orodispersible film (ODF) by hot-melt plunger extrusion 3D printing.(Gutierrez et al., 2019; Musazzi et al., 2018) Extrusion as a way of mixing edible materials is the basis of industrialized efficient production, and it also needs to be combined with other sheeting processes to make the material into a film form.

Table 4.9 counts the research articles related to the production of edible films by screw extrusion as a substrate mixing process in the past 5 years. Process variables during film extrusion, such as screw speed, temperature, feed rate, and moisture content, have a strong impact on film properties. Extruded edible coatings mainly depend on the thermoplasticity or heat sensitivity of the material.

From Table 4.9, it can be basically understood that the twin-screw extrusion method is more popular than the single-screw extrusion method, and blown film is also mostly selected for making films. In terms of materials, the most popular should be starchy biological substrates.

In addition, co-extrusion film is also a film production method that is tended to be used in industry. Two or more sets of extruders lead different edible materials into the extrusion port, merge them side by side, and finally form a double-layer or multi-layer co-extruded edible package, which can increase the composite properties of the film and make up for the shortcomings of the material itself.

Table 4.9: 2018-2023 Edible Film Articles Based on Extrusion Process

Extrusion type	Film forming type	Edible material	Extrusion Process Parameters	Reference
Twin Screw Extrusion	Pressing	Tapioca Starch with Wheat or Oat Bran	Co-rotating twin screws, D (screw diameter) is 16 mm, L (length)/D = 40, die is 3-5 mm at 80 rpm screw speed and 90~140 °C. Place the resulting silk thread in a desiccator at 56.7% RH for 72 hours	(Ochoa-Yepes et al., 2023)
Twin Screw Extrusion	Blown	Starch/gelatin (S/G) of natural waxes (beeswax (BW), candelilla (CL) and carnauba (CB))	The screw diameter (D) was 21.7 mm and the length was 40 D. The barrel temperature settings from the feed zone to the die zone were 85~105°C. The screw speed was set at 130 rpm. Air-cool the extrudate and cut into pellets	(Y. Cheng et al., 2023)
Twin Screw Extrusion	Calenderin g	Native tapioca starch (NS) vs. commercially hydrolyzed (HS) or carboxymethyl (CMS) starches	A co-rotating twin-screw extruder with a screw diameter of 16 mm and an L/D ratio of 40, 10 was used for processing. A flat film forming die with a width of 80 mm is attached to a home-made two-roll calender. The extruder temperature profile and screw speed are 90-120 °C and 120 rpm (feed rate: 12 g/min), respectively, and set the calender at 120 °C.	(Guz et al., 2021b)
Twin Screw Extrusion	Casting	Hydroxypropyl tapioca starch, agar and maltodextrin	Mix the dry powder with glycerin for 5 minutes at a weight ratio of powder to glycerin of 100:35. Pass through a slot die (100 mm × 2 mm) at a screw speed of 50–80 rpm using an extrusion temperature profile of 90–165 °C	(Wongphan & Harnkarnsujarit, 2021)
Twin Screw Extrusion	Blown	Starch/Pea Protein	Dried AS:PI was mixed in 95:5 (5%PI), 90:10 (10%PI) and 80:20 (20%PI). Glycerin and distilled water Add plasticizer at 35% (w/w) and 20% (w/w) of total solids, L/D ratio 40, screw diameter 20 mm, barrel temperature control curve 85-153 ° C (from hopper to die) and a screw speed of 180 rpm.	(Huntrakul et al., 2020b)
Twin Screw Extrusion	Blown	ε-polylysine hydrochloride (ε-PL), starch/gelatin (S/G)	Starch (2000 g), gelatin (1000 g), glycerol (900 g), deionized water (250 g) and ε-PL were mixed with a screw speed of 150 rpm. The temperature curve of the extruder is set at 85~105°C from the feed area to the die head area, the screw specification is 20mm in diameter, and the aspect ratio is 40D	(Y. Cheng et al., 2022)
Twin Screw Extrusion	Hot pressing	Rennet casein, acid casein and sodium caseinate	Casein powder (rennet or acid casein or sodium caseinate) is introduced into the first zone of the extruder. Add glycerol (13.2 or 24.2% (w/w) of protein powder) to the second area. Temperature range from 10 °C to 75 °C from hopper to mold (5 cm wide and 1 mm thick). Sheet thickness is approximately	(Chevalier, Assezat, et al., 2018)

			500 µm ± 50 µm, depending on the formulation. The residence time in a twin-screw extruder with a powder feed rate of 2 kg/h and a screw speed of 170 rpm was determined to be between 2 and 6 min.	
Twin&Single Screw Extrusion	Blown	natural tapioca starch	Native tapioca starch was physically mixed with three different types of plasticizers, glycerol (G), an equal amount of glycerol/xylitol mixture (GX) and an equal amount of glycerol/sorbitol mixture (GS) using L/D Ratio is 40, screw diameter is 20 mm, barrel temperature curve 80~145 °C (from hopper to mold) and a screw speed of 180 rpm.	(Dang & Yoksan, 2021)
Single Screw Extrusion	Extruding	Hydroxypropylated normal corn starch (HP) and native normal corn starch (NS)	Single-screw extrusion with a screw diameter of 11 mm and a length of 500 mm was used. In the first stage, mixing was carried out from the feed zone to the die zone at a screw speed of 75 rpm at a temperature of 75/130/140 C, respectively. 3 mm mold. Again, a sheet-shaped film of 150-mm width and 600-µm thickness was obtained. Temperature Profile During sheet extrusion, the temperature inside the barrel from the feed to the die was 75~160 C, and the extruder was run at a screw speed of 75 rpm.	(H. Y. Kim et al., 2020)
Single Screw Extrusion	Casting	High Amylose Corn Starch and Glycerin	The length-to-diameter ratio is 20:1, the compression ratio is 1:1, and a circular die with a diameter of 2.8 mm is used. The feed rate was kept constant at 35 g/min, while the moisture content was 20 ± 1%. Dissolve the sample obtained by the extrusion process in distilled water (1:4)	(Calderón-Castro et al., 2018)
Extrusion type	**Film forming type**	**Edible material**	**Extrusion Process Parameters**	**Reference**

3.3.1.1 Injection molding

Injection molding is the process of injecting edible materials prefabricated by an injection machine into a specific mold under the thrust of a plunger or screw, and cooling to obtain a film. It is often understood as a mode independent of extrusion, but it is closely related to extrusion.(Jariyasakoolroj et al., 2020) "Extrusion" can be understood as part of the process of "injection" film formation. "Injection" film formation requires specific molds to hold the edible material into shape.(Desai et al., 2018). developed and evaluated a solvent-free injection molding (IM) coating technology. Coating formulations were prepared by hot-melt extrusion using a vertical co-rotating conical miniature twin-screw extruder.

3.3.2 Blown film

Blown film forming is also a film-making process, which usually refers to blowing the film material to the required thickness by high-pressure and high-speed flowing air after the edible material is extruded, but still has a good flow state. Then it becomes a film after cooling. (Huntrakul et al., 2020a; Khumkomgool et al., 2020) In other words, the edible substrate is extruded from the extruder into a cylindrical film tube with a certain amount of compressed air during gelatinization. Make it inflate like a bubble and blow it into a tube with a larger diameter. The cooled film tube is stacked into a double layer by a pair of traction rollers and flattened, then sent to the coiler through the guide roller, and continuously coiled into a ready-to-use film. The film blowing methods that are often introduced include up blowing, down blowing and flat blowing, and the above blowing methods are the most widely used. (Aguirre-Joya et al., 2018; Y. Cheng et al., 2021, 2022; Huntrakul et al., 2020a) From the studies mentioned in Table 6 it can be seen in detail how the blown film process is currently performed in the production of edible films. In experiments to modify edible films of acetylated tapioca starch (AS) with pea protein isolate (PI). (Huntrakul et al., 2020a)dried the edible mixed granules in a hot-air oven at 60°C overnight. Films with an aspect ratio of 30 were then produced using a single-screw blown film extruder (LE-25-30/C; Labtech

Engineering Co., Ltd., Thailand). Similarly, (Y. Cheng et al., 2023) prepared starch/gelatin and natural wax films using an SCM-50 laboratory blown film extruder (Lianjiang Machinery, Zhangjiagang, China). In addition, there is also ε-polylysine hydrochloride loaded starch/gelatin edible antibacterial film, which is also studied by them.(Y. Cheng et al., 2021). (Dang & Yoksan, 2021) used a single-screw extruder (LE-25-30/C; Labtech Engineering) to blow thermoplastic starch TPS (tapioca starch) into a film to investigate the effect of mixing polyol plasticizers and their concentrations on the film.

In only four studies, a single-screw extruder was used as the extrusion device prior to film blowing. However, there is no mention of traction speed, film blowing wind speed, take-up speed and other information, and there is no perfect production process data. The temperature control of the material during the film blowing process and the flow and pressure control of the film blowing air flow are two key parameters, because the final thickness and uniformity of the finished film are affected by the former.

3.3.3 Pressing molding

The simple description of Pressing molding is that at the molding temperature, the edible material is placed on the platen in the mold cavity, and then the mold is closed and pressed to solidify.(María Pilar Montero García M. Carmen Gómez-Guillén M. Elvira López-Caballero Gustavo V. Barbosa-Cánovas, 2017) Temperature and pressing pressure are key to the process. The difference between this process and extrusion molding is that the pretreatment of the material and the force it receives are different. In other words, Pressing molding is simpler and does not involve material flow and shear forces. In extrusion molding, edible materials can be formed into a thin film by mixing in a screw or a plunger, or even through a film-forming nozzle, but film-forming is in a limited number of stamping processes, with the effect of temperature changes (The difference between hot pressing and cold pressing lies in the definition of the process temperature), and the edible material is stamped into the desired shape. Heat pressing is a relatively common pressing technique.(Ceballos et al., 2020;

Khumkomgool et al., 2020) In a recent study of films made from fish and mammalian gelatin, (E. H. Kim et al., 2022) compressed the two gelatin film materials (mixing glycerol and distilled water in different ratios).They found that the light transmittance and tensile strength of the hot-pressed FG film were lower than those of the hot-pressed MG film, and there was no significant difference. However, compared with casting technology, hot-pressed FG film has lower tensile strength and higher waterproof ability. The hot-pressed FG film has a smoother and more uniform surface microstructure than the cast FG film and the transparency is also higher than that of the cast FG film. Interestingly, in 2018, there were also studies by (Chuaynukul et al., 2018) on bovine and fish gelatin films prepared by compression molding and solution casting. Instead, they concluded that compression-molded films generally had lower gloss and slightly higher WVP than cast films, and that heat-pressed films exhibited lower stress and tear behaviour than casting films. In addition to protein-based compression, (Weligama Thuppahige et al., 2023) uses cassava (Monihot esculanta) bagasse to synthesize biodegradable food packaging films. Although starch-based substrates were successfully synthesized by hot pressing into films, unfortunately they were not considered viable, only biodegradable. In addition, (Pasquier et al., 2021) are studying the by-products of insect farming (fly larvae and mealworms) when upcycling into chitin nanofibers and films. Chitin nanofibers were dried by hot pressing (HP) at 70 bar and 100 °C for 30 min and cold pressing (CP) at room temperature (0.012 bar pressure) overnight to form thin films. The results of the study show that the products provided by cold pressing have more excellent mechanical properties, and the hot-pressing process greatly reduces the maximum tensile strain and toughness.

3.3.3.1 Calendering

Based on the ductility of the material, calendering film formation is also an advantageous process for efficient film formation on an industrial scale. It is a processing method that uses the strong shear force between the rollers and the corresponding processing temperature to make the viscous edible material repeatedly

squeezed and stretched to become a thin sheet product with a certain width and thickness.(Pranata et al., 2019) Calendering process conditions, including roll temperature, roll speed, speed ratio, stock volume, roll distance, etc., are the key factors affecting the quality of calendared products.(Singh et al., 2021) However, there are few clear literature surveys on the application of calendering technology to edible films on food packaging. The film prepared from the modified tapioca starch mentioned in Table 6 above(Guz et al., 2021b), the twin-screw extruder is combined with a domestic 80mm wide calender. But did not collect more rolling data, such as roll speed and so on. Other studies have involved biomass edible material substrates, but did not clarify the edible properties of the flakes. For example, when (Pranata et al., 2019) produced egg white protein films, calendering with heating at 115–120 °C and a rotational speed of 0.111 rpm yields a film with the desired properties from the extrudate.

In addition, there is also Lamination method for making edible films by compression molding. That is, in the case of using or not using an adhesive, by means of temperature changes or pressure, two or more layers of different materials are laminated and combined into a whole. It is an effective way to produce composite films. At present, in the research field, the solution casting method is basically used to prepare multilayer films.(Ahari & Soufiani, 2021; María Pilar Montero García M. Carmen Gómez-Guillén M. Elvira López-Caballero Gustavo V. Barbosa-Cánovas, 2017)

In general, due to the "dry process", the source material moisture is less than that of the casting method. Although film formation time is saved, the high temperature (thermoregulation) generated during the extrusion process leads to changes in the biopolymer, which can lead to nutritional and sensory loss of the edible film or to the incapacitation of the active substance. Therefore, these technologies have stricter requirements on the properties of edible materials. More content support for research on the influence of temperature or pressure on materials is needed.

3.4： A short summary of the three scales in “film”

In addition, looking at the research on the production of films that are suitable for three different production scales, the research on industrial scale methods is also miniaturized, and no researchers directly use large machines. This may be due to research cost and purposes, and the miniaturization is more flexible and has the advantage of more convenient adjustment of process parameters. In film fabrication, efficiency is a key determinant in determining how thin films are produced. The Solution casting method is more uniform in the mixing of raw materials and requires less equipment. Although it is more time-consuming, it is acceptable for laboratory research. And it is also the basis of continuous solution casting. Its main adjustments are conveying and drying to help improve production efficiency. Temperature and pressure are the two dimensions of industrial-scale film production. Using pressure and temperature instead of solvent in casting directly helps material plasticity and improves production efficiency. However, because of the high temperature and pressure, the addition of active substances has become an industrial-scale difficulty, and there is currently no research involving this field. However, technologies like emerging 3D printing require longer-term considerations and commercial R&D investment in terms of actual cost and production efficiency.

4: Conclusion

Edible packaging with flexible packaging and primary packaging attributes can give food protection through two forms of "Coating" and "Film". The production technology of packaging is based on objective or subjective factors such as operational difficulty/application purpose/instrument and equipment/process requirements, and different coating technologies are applicable at different scales of production levels. In actual operation, the matching degree of attributes of packaging materials, production methods and food must be considered. For example, coating is often used to protect fruits and vegetables because the shape conditions are relatively complex, and "coating" provides more complete protection than "film". In addition, as a non-thermal processing-based technology, "coating" can help heat-sensitive antimicrobial

agents work in food packaging. In terms of specifications, except for electrospinning and electrostatic spraying, which are currently high-cost and low-efficiency technologies, the rest of Dipping, Vi, Spraying, Panning, Brushing, Fluidized Bed, etc. have the potential and ability to expand production. At the same time, properties such as surface tension, density and viscosity of coating materials are also another major factor affecting the choice of application methods and equipment. Among the technologies involved in the production of packaging films, solution casting is the most convenient, and it is also adopted by most research articles at the laboratory level. However, in industrial-scale manufacturing, it is more preferred to use efficient injection molding, blowing, calendering and other methods to reduce the film formation time. But there are also requirements for material properties involving high temperature and high pressure. Each type of technology for making food packaging needs to be determined according to the actual packaging equipment and production purposes. Laboratory-scale research is essential for the development of materials and for exploring material characterization. The technology used in industrial-scale production needs to consider various factors such as cost and efficiency, and the intention is to bring the best new and environmentally friendly products from the laboratory to consumers as much as possible. We also look forward to scientific and technological progress in the mechanical field to help realize more "coating" and "film" production methods.

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4.2 Performance and optimization of antibacterial agents in protein matrix films

This chapter will also be divided into three parts. Different types of active substances were added to gelatin films against two common foodborne pathogenic bacteria, *Staphylococcus aureus* and *Escherichia coli* (CECT 101). The two materials represented by bacteriophage phiIPLA-RODI and terpene substance thymol transformed this antibacterial effect into reality. This chapter focuses more on the study of the synergy between the active substances in the material protein film and the film substrate itself, or in other words, after adding natural active substances to the protein-based film, the change of the protein film itself and the performance of the antibacterial effect. Also supplements the encapsulation technology and characterization of active substances in PLA nanoparticles.

4.2.1 Gelatin film and phage phiIPLA-RODI

The use of antibiotics in the food industry is being challenged due to the emergence of resistant strains. For the use of phages, it may be a suitable choice, because phages may allow selective elimination of microorganisms. After characterization studies, in terms of physical properties, the film of protein matrix was not affected by the addition of phage or the change of concentration. In terms of antibacterial activity, the phage concentration phiIPLA-RODI and the antibacterial Staphylococcus aureu showed a positive correlation.

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Effectiveness of bacteriophages incorporated in gelatine films against *Staphylococcus aureus*

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Abstract

The use of antibiotics in the food industry is declining due to the emergence of resistant bacteria. Thus, the use of bacteriophages may provide a suitable alternative, since it allows the selective elimination of microorganisms. Another issue that faces the industry is concern about the difficulty of removing petroleum-derived plastics from the environment. This problem makes the search for new food packaging materials with extended properties a necessity. In this study, edible gelatine films containing bacteriophages were produced, and the effects of increasing concentrations of bacteriophages on the light transmission, water vapour permeability, solubility and mechanical properties of the films were characterised; in addition, micrographs of transverse film sections were made and analysed. Finally, with the purpose of assessing the influence of the manner of application and the antimicrobial properties of the prepared packaging materials, pieces of cheese previously

contaminated with *Staphylococcus aureus* were either coated by immersion in the film-forming solution or wrapped directly with gelatine films loaded with bacteriophages. According to the results obtained, the physical properties of the films remained unaltered, irrespective of the bacteriophage concentration. The pieces of cheese immersed in the film-forming solution showed higher microbial reduction than the pieces of cheese wrapped with previously dried films. Overall, the packaging materials prepared possessed concentration-dependant antimicrobial properties, but the results obtained underline the importance of the manner of application of the bacteriophages on the foodstuff in maximizing their antimicrobial properties.

Keywords

Packaging Edible films, antimicrobial properties, Bacteriophages, *Staphylococcus aureus*, Cottage cheese

1. Introduction

Edible films in the food industry must provide a barrier against humidity and oxygen, and prevent the movement of solids out of the food matrix (Guilbert, Gontard, & Cuq, 1995). These edible films are usually prepared with natural, totally biodegradable polymers, and they must meet certain requirements to be useful in this role. Thus, in addition to their barrier properties, edible films must maintain the colour and appearance of the food they wrap, they must have the mechanical strength to be handled and to support the pieces of food they contain, and they must be harmless when they are consumed together with their contents. These properties depend on the raw materials selected to prepare the films, their fabrication process and finally, the mode of application (Guilbert, Gontard, & Gorris, 1996). Among the biopolymers that have been considered for preparing these films, polysaccharides and proteins must be highlighted, and among them, edible films based on gelatine occupy an important position, as is clear from the extensive research reported in the literature on this subject (Etxabide, Uranga, Guerrero, & de la Caba, 2017).

Gelatine is produced by the thermal hydrolysis or physical and chemical degradation of collagen, a protein widely present in the bones and skin of animals (Avila-Rodríguez, Rodriguez-Barroso, & Sánchez, 2018). Gelatine possesses a high degree of biocompatibility and biodegradability, and it has been widely used in the food industry as a gelling agent and as an ingredient that stabilises foams and emulsions. Furthermore, films prepared using gelatine are transparent and mechanically strong enough to coat pieces of food effectively; moreover, the properties of these films can be easily improved by introducing bioactive compounds in their formulation. To this end, Sáez-Orviz, Marcet, Weng, Rendueles, and Díaz (2020) prepared gelatine films loaded with polylactic acid nanoparticles with antimicrobial properties, Kanmani et al. (2014) blended gelatine with silver nanoparticles to prepare antimicrobial composite films, and Neira, Agustinelli, Ruseckaite, and Martucci (2019) prepared edible fish gelatine films with added carvacrol, increasing the storage period of breaded hake medallions. Other authors have added red cabbage extracts (Musso, Salgado, & Mauri, 2019), eugenol (Dammak & Sobral, 2019), pomegranate peel powder (Hanani, Yee, & Nor-Khaizura, 2019) or tea extracts (Wu et al., 2013) to gelatine films to give them different functional properties. In this regard, bacteriophages are an antimicrobial agent gaining interest among the scientific community. They are a group of viruses that are able to infect and to lyse bacteria with high specificity, so they are harmless to animals, plants or any bacteria other than the specific bacterial strain recognised by each particular species of bacteriophage. Because of this, bacteriophages have been used in a wide range of biotechnology applications since they were discovered in 1915 (Sillankorva, Oliveira, & Azeredo, 2012). Furthermore, bacteriophages occur naturally on the surface of many foodstuffs, so they are frequently consumed by humans with no health risk, and moreover, there are already several commercial phage cocktails such as EcoShield™, Salmo Fresh™, and ListShield™ approved by the FDA for their application directly on foodstuffs (Sadekuzzaman, Yang, Mizan, Kim, & Ha, 2017). In this sense, several authors have taken advantage of the antimicrobial properties of bacteriophages to prepare different films and coatings to cover foodstuffs. The

biopolymers that have been used for this purpose are sodium alginate (Alves, Cerqueira, Pastrana, & Sillankorva, 2020; Alves et al., 2019), xanthan gum coating a polylactic acid film (Radford et al., 2017), chitosan (Amarillas et al., 2018; Cui, Yuan, & Lin, 2017), whey protein (Vonasek, Le, & Nitin, 2014), methylcellulose (Kalkan, 2018), and acetat cellulose (Gouvêa, Mendonça, Soto, & Cruz, 2015). Although all these papers are relevant to the food technology field, none of them assess how an increasing concentration of bacteriophages might affect the film matrix, and furthermore, regarding the antimicrobial properties of the packaging materials, neither do they seek to investigate whether wrapping food with a previously dried film is more or less effective than a coating prepared by submerging the piece of food in the same film-forming solution used to create the dried film.

In this study several concentrations of the bacteriophage pilIPLA-RODI, which was discovered in a sewage treatment plant in Asturias, Spain, and is able to lyse the food-poisoning bacteria *S. aureus* (Gutiérrez et al., 2015), were mixed with gelatine and glycerol to prepare film-forming solutions. These film-forming solutions were used to prepare edible films, in which the effect of the bacteriophages on the film matrix was assessed; furthermore, in order to test the influence of the method of application of the bacteriophages on their antimicrobial activities in a real-case scenario, previously contaminated pieces of cheese were alternatively coated by immersion in the film-forming solution or wrapped with previously dried films.

2. Materials and methods

2.1. Preparation of gelatine films with phiIPLA-RODI bacteriophage

The stock of bacteriophage phiIPLA-RODI (Gutiérrez et al., 2015) in TSB (Tryptic Soy Broth, ref. 22,902, Sigma-Aldrich, Germany) medium with a titre of 7×10^8 PFU/mL and the strain *S. aureus IPLA1* isolated from contact surfaces of the dairy industry (Gutiérrez et al., 2012) were kindly donated by the Dairy Research Institute of Asturias IPLA-CSIC (Asturias, Spain).

To prepare the film-forming solutions, gelatine (gelatine from porcine skin, G1890,

Sigma-Aldrich) and glycerol in water were solubilised by heating the mixture at 50 °C for 20 min under continuous stirring. Then, this solution was cooled to 40 °C and filtered using a 0.45 µm pore size syringe filter under aseptic conditions. Afterwards, different volumes of the bacteriophage stock were added and gently stirred for 5 min, so as to obtain final concentrations of gelatine and glycerol for every film-forming solution with bacteriophages of 10% and 2% (w/v) respectively. The final concentrations of bacteriophages tested in the film-forming solution were 1.75×10^8 PFU/mL (GF1), 1.16×10^8 PFU/mL (GF2) and 6.35×10^7 PFU/mL (GF3). A control sample with no bacteriophages was also assessed. Finally, every film-forming solution was poured into a Petri dish in such a way that 0.11 mL was cast per cm^2 of Petri dish surface. The films, still in their Petri dish moulds, were dried in a laminar flow chamber for 2 days at room temperature and then completely removed from the dishes as single intact discs.

2.2. Physical properties of gelatine films loaded with increasing concentrations of bacteriophages

2.2.1. Light transmission and transparency

The barrier properties of the films against ultraviolet and visible light were assessed according to Dick et al. (2015). Briefly, films were cut into rectangular pieces and placed in a spectrophotometer test cell. The light transmission of the samples was tested using a Helios gamma spectrophotometer (Thermo Fisher Scientific, USA), from 280 to 800 nm, with an empty test cell as reference. The transparency of the films was calculated according to equation:

$$Transparency = A_{600}/x$$

Where A600 is the absorbance of the film sample at 600 nm and x is the film thickness (mm).

A digital micrometre (Mitutoyo C., Japan), with a precision of ±1 µm, was used to

measure the thickness of the films. This thickness was measured in five different areas, one of them in the centre of the film and the other four around the film perimeter.

2.2.2. Mechanical properties

The mechanical properties of the gelatine films loaded with bacteriophages were tested by means of a puncture test according to the methodology described by Sobral, Menegalli, Hubinger, and Roques (2001), and using a TA.XT. plus Texture Analyser (Stable Microsystems, UK) equipped with a 5 kg load cell and a 5mm diameter probe (P/5S). For this purpose, the films were cut into 4×2 cm strips and placed in the texture analyser between two plates. These plates have a hole, allowing contact between the film sample and the probe, which can stretch the film to breaking. In this case, the probe speed was 1 mm/s and the puncture strength (PS) and puncture deformation (PD) values were obtained according to equations (Otero-Pazos et al., 2016):

$$PS = Fm/Th$$

$$PD = (\sqrt{D^2 + R^2} - R)/R$$

Where Fm is the maximum force applied before the film breaks, Th is the film thickness, D is the distance covered by the probe while it is in contact with the film until the film is broken, and R is the radius of the orifice in the plates.

2.2.3. Water vapour permeability and solubility

Polyvinyl chloride cups were filled with distilled water and sealed with films that had been cut into circles with the same diameter as the cup mouth. A height of 1 cm was left between the water surface and the gelatine films, and the thickness of the film samples was measured. The samples used in this experiment were visually checked and films with pinholes or breakages were discarded. Finally, the containers were weighed, placed in desiccators with silica gel, and the change in their weights was registered every hour for 7 h. The weight loss was plotted against time and the water vapour transmission rate (WVTR) was calculated according to equation:

$$WVTR=G/(t*A)$$

Where G/t is the change in the weight of the cup per unit of time (g/h) and A (m2) is the area of the cup mouth covered by the film.

These WVTR values can be used to calculate the water vapour permeability (WVP) by means of equation:

$$WVP = (WVTR * Th)/ \Delta P$$

Where Th (mm) is the film thickness and ΔP (kPa) is the difference in partial pressure between the two faces of the film.

The solubility measurement was conducted according to Marcet, Sáez, Rendueles, and Díaz (2017), with some minor modifications. Briefly, gelatine films loaded with bacteriophages were cut into circles of 2 cm diameter, and their dry weight was obtained by drying them at 80 °C in an oven for 24 h.

Other intact film circles were immersed in a buffered solution of Trizma 0.1 M pH 7.0 at room temperature and, after 24 h, the undissolved film remains were recovered by vacuum filtration using Whatman nº 1 paper that had been weighed previously. Finally, the paper, together with the remains, was dried at 80 °C for 24 h and weighed. The following equation was used to calculate the percentage of undissolved film:

$$WS(\%) = (m1 - m2)/m1 \times 100$$

Where S (%) is the percentage of solubilised film, m1 is the initial dry weight of the film and m2 is the dry weight of the non-solubilised film remains.

2.2.4. Scanning electron microscopy (SEM)

Micrographs were taken with a JSM-6610LV (JEOL, USA) scanning electron microscope with the aim of studying the microstructure of the transverse section of the gelatine films loaded with bacteriophages. For that purpose, film samples were cut into square pieces of 1×1 cm using a surgical blade. These films were attached perpendicularly around stubs using double-sided carbon-based tape as adhesive and then the films were gold-sputter-coated for 5min in an argon atmosphere. The magnification used to observe the transverse section of the films was ×900, and the voltage was set at 20 kV.

2.3. Antimicrobial activity of films and coatings

2.3.1. In vitro antimicrobial activity of films

In order to determine the inhibitory capacity of *phiIPLA-RODI* in the films after the

drying step, a test was performed in TSB liquid medium. For that purpose, a 0.4 g piece of each of the films prepared with the different bacteriophage concentrations described in section 2.1. was immersed in 100 mL of TSB medium with an initial concentration of 10^6 CFU/mL of *S. aureus IPLA1*. Therefore, once the pieces of film were dissolved in TSB, the concentrations of bacteriophages in the liquid medium were 5.25×10^6 PFU/mL (GF1-TSB), 3.48×10^6 PFU/mL (GF2-TSB) and 1.90×10^6 PFU/mL (GF3-TSB). Furthermore, a film made exclusively from gelatine was tested in the same conditions (G), and another control sample with just TSB infected with *S. aureus* was also assessed (WB). These samples were incubated for 17 h at 37 °C, under orbital stirring at 250 rpm. Afterwards, the liquid media were diluted with 0.7% NaCl and seeded in Baird-Parker agar medium enriched with egg yolk tellurite emulsion (Sigma-Aldrich, USA), a Staphylococcus-selective agar medium. After 48 h of incubation at 37 °C, the colonies were counted, and the results expressed as log10 CFU/mL.

2.3.2. Antimicrobial properties of films and coatings on cheese pieces contaminated with *S. aureus*

To investigate the antimicrobial activity of the films and coatings, 100 g of fresh cheese was purchased in a local market and divided into several cylindrical pieces of 1 g using a hollow punch. Afterwards, every piece of cheese was infected with 100 µL of 105 CFU/mL of *S. aureus* in 0.7% NaCl. These contaminated pieces of cheese were then each submitted to one of the following treatments:

a) The bacteriophage stock solution was diluted in TSB to the same concentration as GF1, GF2 and GF3 for the film-forming solutions described in section 2.1. (1.75×10^8 PFU/mL (GF1), 1.16×10^8 PFU/mL (GF2) and 6.35×10^7 PFU/mL (GF3)) and, for every concentration prepared, three contaminated pieces of cheese were tested. For that purpose, the pieces of cheese were immersed in one of these solutions, stirred gently by hand for 3 min, recovered and saved in tightly closed polypropylene tubes under refrigeration (4 °C). A control sample with contaminated cheese and immersed in TSB but without bacteriophages was also tested.

b) Contaminated pieces of cheese were immersed in gelatine film-forming solutions

with bacteriophages at the GF1, GF2 and GF3 concentrations (as described in section 2.1.). After 3 min, the pieces were recovered, dried at room temperature for 10 min, and saved in polypropylene tubes at 4 °C. A control sample with contaminated cheese immersed in gelatine with no bacteriophages was also assessed.

c) Gelatine films loaded with different proportions of bacteriophages were prepared as was described in section 2.1. These films were used to wrap the contaminated pieces of cheese, thermosealed, and stored at 4 °C.

In all cases, samples were taken at time 0 and after 3 and 6 days, a characteristic sampling time for this type of food product (Amatiste et al., 2014). The pieces of cheese to be sampled were placed in sterilised plastic bags with 10 mL of 0.7% NaCl and triturated using a Stomacher (IUL Instruments, Barcelona, Spain) for 120 s. Finally, the liquid sample was diluted and seeded in Baird-Parker medium with 2% agar. After 48 h of incubation at 37 °C the colonies were counted, and the results expressed as log10 CFU/mL.

2.4. Statistical analysis

Analysis of variance (ANOVA) was applied. Least significant differences (LSD) were calculated by Fisher's test to determine significant differences between the tested samples. These analyses were performed using Statgraphics® V.15.2.06 statistical software.

3. Results and discussion

3.1. Physical properties of gelatine films loaded with increasing concentrations of bacteriophages

3.1.1. Visual aspect of the films, light transmission and transparency

The prepared films were easily peeled from the Petri dishes in one piece and were homogeneous and easy-to-handle in every case tested; moreover, they were found to be neither brittle nor sticky. Their visual appearance is shown in Fig. 4.13, and it was noticeable that all of them were completely transparent, with no visual difference between the control and the samples loaded with bacteriophages, even at the highest

bacteriophage concentration tested.

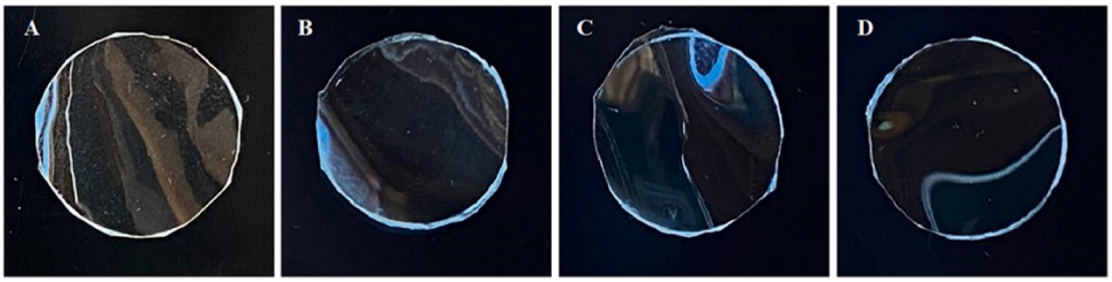

Figure 4.8: Visual aspect of the films. A) Gelatine film without bacteriophages. B) GF1: Gelatine film prepared with a film-forming solution with a bacteriophage concentration of 1.75 × 10^8 (GF1), C) 1.16 × 10^8 PFU/mL (GF2), D) 6.35 × 10^7 PFU/mL (GF3).

The transmittance values for the films at 280 nm were lower than at the other wavelengths tested (Table 4.7), which could be explained by the presence of aromatic amino acids in the gelatine's composition. However, in comparison with other edible protein-based films, such as those prepared using delipidated egg yolk proteins (Marcet et al., 2017), these light transmission values at 280 nm could be considered high. This is because, although these aromatic amino acids are present, their contribution to the primary structure of the gelatine peptides is low in comparison to that found in the components of other protein-based films. To be more specific, pork skin gelatine protein contains just 3 amino acid residues of tyrosine, 14 of phenylalanine, and no tryptophan per 1000 amino acids. (Zhou, Mulvaney, & Regenstein, 2006). In fact, a slight difference can be observed between the light transmission properties of the films tested; the film with the highest concentration of bacteriophages in its composition showing the lowest values at every wavelength tested. These differences can also be observed in the transparency value, for which the higher the concentration of bacteriophages, the lower was the transparency value. However, although these differences were measurable, they were not great enough to be appreciated by visual inspection.

Table 4.10: Light transmission and transparency of gelatine films prepared with film-forming solutions with bacteriophage concentrations of 1.75 × 10^8 PFU/mL (GF1), 1.16 × 10^8 PFU/mL (GF2) and 6.35 × 10^7 PFU/mL (GF3). Control is a phage-free gelatine film.

Film	Light Transmission (%)						Transparency
	280 nm	300 nm	350 nm	400 nm	500 nm	600 nm	
Control	6.58	61.16	82.92	89.25	91.70	92.30	0.39
GF1	6.52	39.59	63.54	76.23	82.98	85.33	1.04
GF2	6.69	44.36	67.99	79.55	85.87	88.21	0.70
GF3	10.33	55.14	75.97	85.49	89.46	90.89	0.61

3.1.2. Mechanical properties

The mechanical properties of the gelatine films loaded with bacteriophages are shown in Table 4.8. In this case, no statistical difference was detected between any of the films tested. They all showed a statistically similar thickness, PS and PD value. The bacteriophage phiIPLA-RODI is a Myoviridae, which belongs to the Spounavirinae subfamily (Gutiérrez et al., 2015); the members of this family possess heads of 87–94 nm diameter and tails that are 140–219 nm long (Lavigne et al., 2009). So, taking into consideration the thickness of the gelatine film, the dimensions of one bacteriophage are small enough not to produce any disruption in the protein packaging film; however, a high bacteriophage concentration may lead to structural problems in the film matrix, since the protein chain packaging involves forces such as disulphide bonds, hydrophobic interaction, electrostatic forces and hydrogen bonds (Wihodo & Moraru, 2013). These compacting forces can be weakened if the bacteriophages introduce sufficient heterogeneity into the film matrix to hinder the physical approach of the protein chains. In this study, a stock with a bacteriophage concentration of 7×10^8 PFU/mL was used, but this number cannot be related to a particular number of bacteriophage particles, and in any case, the results obtained indicate that the phage concentrations used in these films were too low to produce any effect on either the strength or the flexibility of the gelatine films prepared, even for the films with the highest concentration of bacteriophages. Similar findings were reported by other authors, and in this regard, Gouvêa et al. (2015) did not find any statistical difference in the values of the PS parameter between control films prepared using acetate cellulose and those prepared with acetate cellulose and bacteriophages.

Table 4.11: Thickness, puncture strength (PS) and puncture deformation (PD) of gelatine films prepared with

film-forming solutions with bacteriophage concentrations of 1.75 × 10^8 PFU/mL (GF1), 1.16 × 10^8 PFU/mL (GF2) and 6.35 × 10^7 PFU/mL (GF3). Control is a phage-free gelatine film.

Film	Thickness (mm)	PS (N/mm)	PD (%)
Control	0.070 ± 0.003 [a]	823.01 ± 46.54 [a]	17.96 ± 1.51 [a]
GF1	0.075 ± 0.007 [a]	803.37 ± 86.21 [a]	20.85 ± 2.41 [a]
GF2	0.073 ± 0.01 [a]	725.45 ± 77.61 [a]	24.12 ± 8.28 [a]
GF3	0.076 ± 0.005 [a]	754.83 ± 51.90 [a]	16.56 ± 4.76 [a]

Different letters in the same column indicate significant differences (P < 0.05).

3.1.3. Water vapour permeability (WVP) and solubility

The WVP of biopolymer-based films depends on several factors, such as the kind and concentration of biopolymer used, the kind of plasticiser, and the amount and nature of the additives included in the film matrix to extend their functional properties. Furthermore, there are two models to explain the water barrier properties of a protein-based film, one of which refers to the formation of voids in the internal structure of the film matrix during the drying step (Ukai, Ishibashi, Tsutsumi, & Marakami, 1976), while the other involves the formation of water micropathways, which are a result of the hydrophilic nature of the polymer matrix itself (Krochta, 1990). Therefore, it is a film property that is closely related to the microstructure of a protein-based film. In these experiments, as is shown in Table 4.9, the incorporation of bacteriophages in the film matrix did not produce any effect on the WVP of the gelatine films tested, which suggests that there was no alteration of the film microstructure caused by the presence of bacteriophages at the concentrations tested. Similar results were obtained by Alves et al. (2020), who found no statistically significant differences in this parameter between sodium alginate films with bacteriophages and those prepared without bacteriophages used as control.

Table 4.12: Water vapour permeability (WVP) and solubility of gelatine films prepared with film-forming solutions with bacteriophage concentrations of 1.75 × 10^8 PFU/mL (GF1), 1.16 × 10^8 PFU/mL (GF2), and 6.35 × 10^7 PFU/mL (GF3). Control is a phage-free gelatine film.

Film	WVP (g × mm/m^2*h × kPa)	Solubility (%)
Control	1.61 ± 0.17 ^{a}	21.33 ± 3.32^a
GF1	1.41 ± 0.38 ^{a}	27.79 ± 4.95^a
GF2	1.49 ± 0.21 ^{a}	28.50 ± 6.24^a
GF3	1.83 ± 0.11 ^{a}	24.22 ± 0.31^a

Different letters in the same column indicate significant differences (P < 0.05).

The solubility values of the films are also shown in Table 4.9. In this case as well, the incorporation of the bacteriophage did not produce any statistically significant change in the parameter assessed, which supports the previous suggestion that the addition of bacteriophages to the film-forming solution did not change the overall hydrophilicity of the films produced.

3.1.4. Scanning electron microscopy (SEM)

Micrographs of the gelatine films loaded with bacteriophages are shown in Fig. 4.14. Their microstructure was seen to be similar for every sample tested, showing a smooth, compact, continuous film matrix, similar to other gelatine film micrographs found in the literature (Ge, Wang, Shi, & Yin, 2012). As was suggested in the tests performed before, the incorporation of bacteriophages did not produce any noticeable change in the film microstructure.

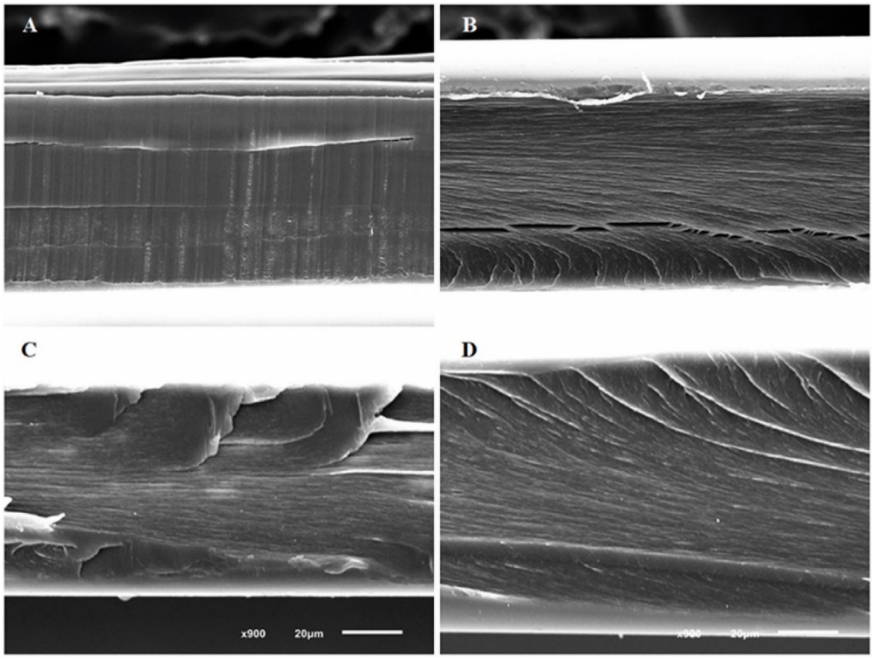

Figure 4.9: Micrographs of the gelatine films loaded with bacteriophages. A) Gelatine film without bacteriophages. B) Gelatine films prepared with film-forming solutions with bacteriophage concentrations of 1.75 × 10^8 PFU/mL (GF1), C) 1.16 × 10^{8}PFU/mL (GF2), D) 6.35 × 10^7 PFU/mL (GF3).

3.2. Antimicrobial activity of films and coatings

3.2.1. In vitro antimicrobial activity of films

S. aureus IPLA1 growing free in a TSB liquid medium reached a mean concentration of 3.85×10^9 CFU/mL after 17 h of incubation at 37 °C (Fig.4.15, sample WB), while a similar value to that was obtained for the contaminated TSB liquid medium treated with gelatine film without bacteriophages (G), confirming that gelatine proteins do not have any growth-inhibiting effect on *S. aureus IPLA1*. On the other hand, every microbial assay performed with gelatine films loaded with *phiIPLA-RODI* at any of the concentrations tested showed a decrease in the microbial load at the end of the test. In this case, samples GF2-TSB and GF3-TSB exerted a similar effect on the microbial population, producing a reduction of five log units in the microbial load. The best result was achieved with the GF1-TSB sample, which contained the highest concentration of bacteriophages tested and consequently, reduced the viable counts to 70 CFU/mL. These results show that the antimicrobial activity of the bacteriophage *phiIPLA-RODI* remains after the film-forming solution drying step and therefore, that the incorporation of bacteriophages in films allows them to conserve their infective capacity. It is also shown that the activity is dependent on the concentration of bacteriophages included in the film matrix. Similar antimicrobial properties and results were also observed by other authors employing other bacteriophages in different types of films, such as whey-protein (Vonasek et al., 2014), sodium alginate (Alves et al., 2019) and acetate cellulose (Gouvêa et al., 2015).

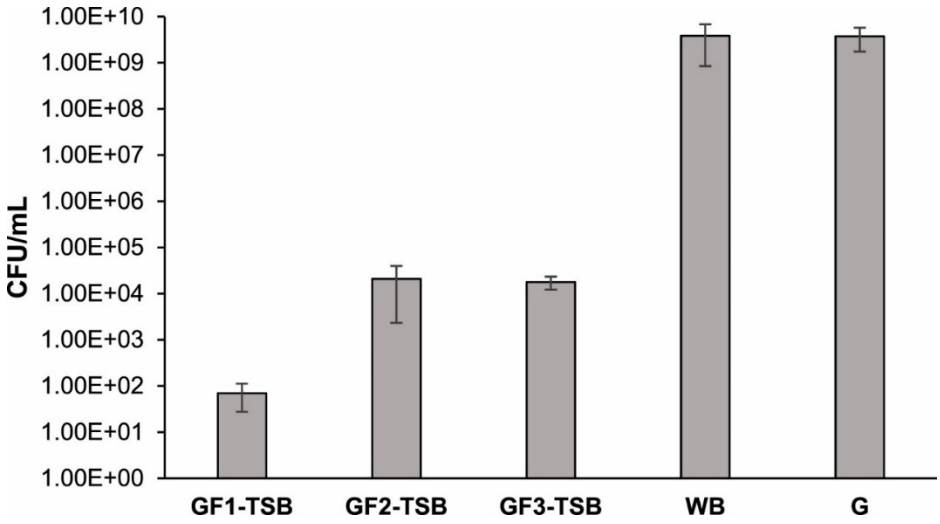

Figure 4.10: Bacteriophage inhibitory capacity of films in TSB medium previously contaminated with S. aureus IPLA1 (106 CFU/mL). Bacteriophage concentration of the films was GF1-TSB: 5.25 × 10^6 PFU/mL. GF2-TSB: 3.48 × 10^6 PFU/mL. GF3-TSB 1.90 × 10^6 PFU/mL. WB: pure culture of S. aureus IPLA1 without phages or films. G: assay with a gelatine film

3.2.2. Antimicrobial properties of films and coatings on cheese pieces contaminated with *S. aureus*

The films analysed in the previous section were used to wrap contaminated pieces of cheese and then thermosealed. However, it is also possible to prepare an edible coating with the same film-forming solution by immersing the foodstuff pieces in it, and recovering them in such a way that a thin layer of edible film is formed on their surface (Lacroix & Vu, 2014). Therefore, to test the inhibitory effect of the films on a real food model and to investigate the repercussions of the way these gelatine-based packaging materials are applied, three different tests were carried out, using previously contaminated pieces of cheese.

In the first experiment, contaminated pieces of cheese were directly immersed in TSB liquid medium with the same bacteriophage concentrations as the film-forming solutions prepared according to section 2.1 (Fig. 4.16A). In the case of the GF1 and GF2 bacteriophage concentrations, on day 3 a slight decrease in the concentration of *S. aureus* was observed, and the populations remained almost constant until day 6. The final concentration of *S. aureus* for the cheese pieces immersed in the liquid with the highest concentration of bacteriophage (GF1) was 407 CFU/g, which was similar to the

value for sample GF2 (524 CFU/g). The lowest bacteriophage concentration tested (GF3) resulted in a microbial load at the end of the test similar to that for the control sample.

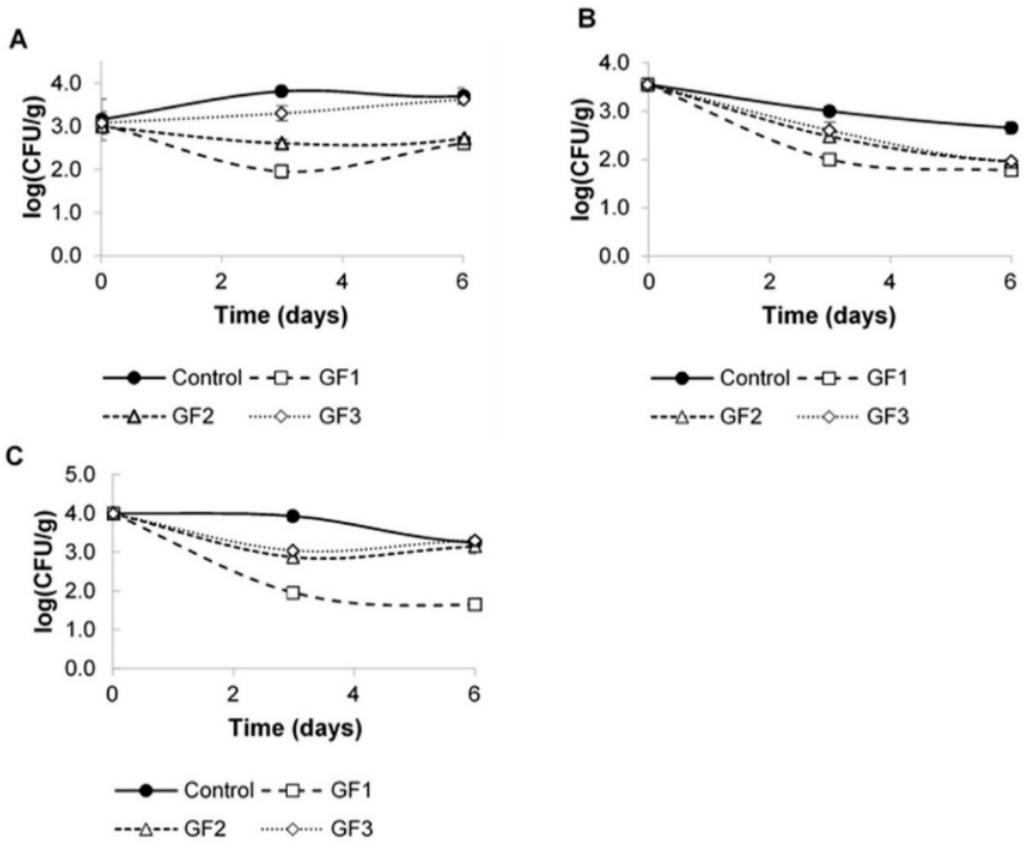

Figure 4.11: Evolution of *S. aureus* growth in cheeses treated with: (A) bacteriophages in TSB liquid medium at three different concentrations (GF1, GF2 and GF3); (B) bacteriophages dissolved in the film-forming solution at three different concentrations (GF1, GF2 and GF3); (C) gelatine films prepared with film-forming solutions containing bacteriophages at three different concentrations (GF1, GF2 and GF3). In every case, the bacteriophage concentrations used were either 1.75×10^8 PFU/mL (GF1), 1.16×10^8 PFU/mL (GF2) or 6.35×10^7 PFU/mL (GF3).

In the second experiment, the cheese pieces were coated with a gelatine solution containing different concentrations of bacteriophages (GF1, GF2 and GF3) (Fig. 4.16B). The gelatine coating formed around the cheese pieces is distributed evenly over the entire surface with a similar appearance in all cases (Fig. 4.17A). As regards the inhibitory effect, a decrease in microbial load was observed in all cases, but it was slightly more pronounced for GF1, with a final value of 60 CFU/g.

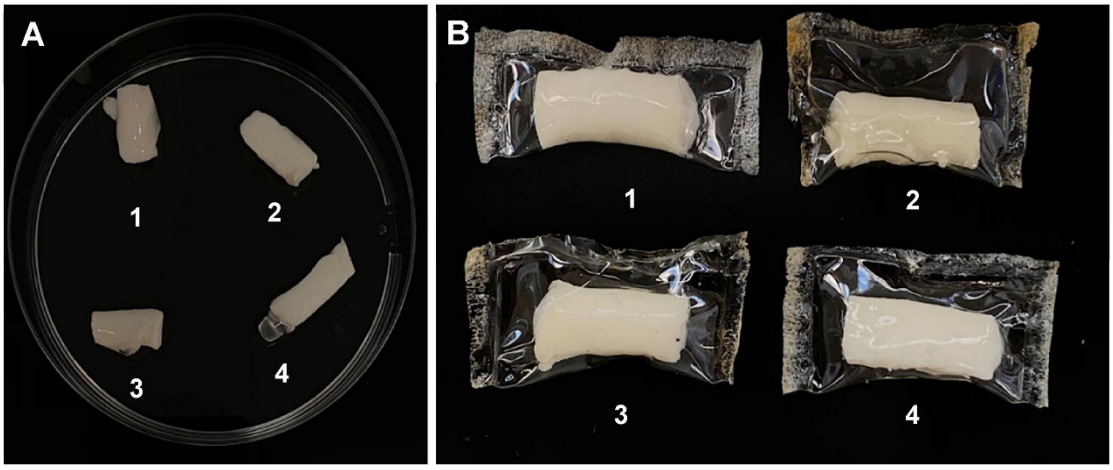

Figure 4.12: Visual appearance of coated cheeses. Gelatine-coated cheeses (A) and cheeses wrapped with films (B). 1. Control (gelatine coating or film); 2. Gelatine coating or film prepared with a film-forming solution with a bacteriophage concentration of 1.75 ×10^8 PFU/mL (GF1); 3.-1.16 ×10^8 PFU/mL (GF2); 4.- 6.35 ×10^7 PFU/mL (GF3).

In the last experiment, the cheese pieces were coated with previously dried films containing the three concentrations of bacteriophages (GF1, GF2 and GF3) (Fig. 4.16C). The appearance obtained was similar in every case tested. There were no breaks and the films were in contact with the entire surface of the cheeses (Fig. 4.17B). In this case, a great reduction in the number of *S. aureus* was observed for the samples wrapped using the film with the highest concentration of bacteriophages (GF1), with a mean final value of 44 CFU/g. Cheese samples wrapped with GF2 and GF3 showed a decrease in the microbial load until day 3, but then the microorganism proliferated again, reaching a microbial concentration similar to that for the control sample. Both in Fig. 4.16 B and C, there is a noticeable decrease in the number of viable bacteria in the control sample, which suggests a slight inhibitory effect produced only by the gelatine material covering the cheese samples. This decrease in the number of viable bacteria may have occurred due to the physical presence of the gelatine surrounding the piece of cheese, which may affect the growth of the bacteria, possibly by hindering their nutrient intake or limiting their growing space. Other authors, studying chitosan films loaded with phages and *Escherichia coli*, another anaerobic facultative bacteria, noted the same inhibitory effect produced by the unloaded films covering infected pieces of meat (Cui et al., 2017).

When analysing the results of these experiments, it should be borne in mind that it is easier for bacteriophages to infect bacteria in a liquid medium (Gutiérrez et al., 2015) or in an environment with a high level of humidity (Götz, 2002). In this case, the poorest antimicrobial results were obtained for those pieces of contaminated cheese that were directly immersed in a liquid medium with no gelatine. This may be explained because over time, the surface of the cheese dried faster than when a gelatine coating or film was used, since these protein-based packaging materials have the ability to preserve the water near the food surface for a longer period of time (Lin & Zhao, 2007). In addition, during the experimental time span, the cheese begins to ripen, with a high loss of moisture (Everett & Auty, 2008), making it more difficult for the phage to infect *S. aureus*. This may explain why the highest antimicrobial effect was observed during the first three days. Furthermore, the coatings formed by the immersion of the pieces of cheese in film-forming solutions GF2 and GF3 showed better antimicrobial performance after six days of storage than the films that were made with the same film-forming solutions and then used to wrap the cheese. These results suggest that the coatings were better at retaining the moisture on the surface of the cheese, but further investigation is required to corroborate this assumption. However, this difference was diminished at the highest bacteriophage concentration tested (GF1), so increasing the phiIPLA-RODI concentration in coatings and films could lead to a reduction in the relative importance of the manner of application of these materials on the foodstuff.

4. Conclusions

The bacteriophage *phiIPLA-RODI* was introduced successfully into gelatine-based films, and the physical and antimicrobial properties of these films were assessed. It was found that the prepared films were not physically affected by the bacteriophages in the film-forming solution, even at the highest bacteriophage concentration assessed. The antimicrobial properties of the prepared packaging materials were tested using pieces of cheese previously contaminated with *S. aureus*, and except at the highest

concentration of bacteriophages tested, the best results were obtained when the cheese was immersed in the film-forming solution mixed with bacteriophages and the coating was directly formed on the surface of the cheese. Taking all this into consideration, a liquid solution of gelatine, glycerol and bacteriophages could be sprayed on the surface of foodstuffs that are commonly contaminated with *S. aureus*, such as fresh cheese, fruits and vegetables, to protect the consumers from this pathogenic bacteria, although further investigation into the performance of this coating on fruits and vegetables has to be conducted, as well as studies into the optimization of the concentrations of bacteriophages, protein and glycerol in order to maximize the antimicrobial properties of the coatings.

CRediT authorship contribution statement

Shihan Weng: Investigation, Methodology. Abel López: Investigation, Methodology. Sara Sáez-Orviz: Methodology, Formal analysis. Ismael Marcet: Conceptualization, Writing-original draft,Writing-review&editing. Pilar García: Conceptualization. Manuel Rendueles: Supervision, Funding acquisition. Mario Díaz: Supervision, Funding acquisition.

Declarations of competing interest

None

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4.2.2 Thymol encapsulated in gelatin film and PLA nanoparticles

Thymol, an active agent with significant antimicrobial properties but low solubility in water, is thought to be able to negatively affect the mechanical properties of protein-based films. Furthermore, it is highly volatile and may be lost to evaporation during the drying of the film-forming solution. Therefore, thymol was optimally encapsulated using PLA nanoparticles. The thymol partially encapsulated in the PLA nanoparticles is retained in the gelatin film, which can act as a sustained release and prolong the antibacterial effect of thymol. However, the influence of residual free thymol on the mechanical properties of the produced material can also be considered slight.

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PLA nanoparticles loaded with thymol to improve its incorporation into gelatine films

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Abstract

Thymol is an active agent with remarkable antimicrobial properties but with low solubility in water and is able to exert a negative effect on the mechanical properties of protein-based films. Furthermore, it is highly volatile and during the drying of the film-forming solution it may be lost through evaporation, a question that has barely been studied.

In a previous investigation, the encapsulation of thymol using PLA was optimized, and, in the present study, these PLA nanoparticles are incorporated into gelatine films to assess their effect on the film properties and thymol evaporation during the drying of the film-forming solution.

At the thymol concentrations tested, all the free thymol was completely evaporated during the drying of the film-forming solution, while a part of the thymol encapsulated in PLA nanoparticles remained in the gelatine film matrix. The gelatine films with PLA-thymol nanoparticles showed high transparency, a homogeneous microstructure and antimicrobial properties.

Keywords

Gelatine film, Thymol, Nanoparticle Polylactic acid, Antimicrobial properties, Mechanical strength

1. Introduction

Films made using natural biopolymers can be used directly on food and they have several functions, such as forming a physical separation between food and the environment (avoiding food spoilage due to pollution or the presence of microorganisms in the media), decreasing food dryness due to water evaporation, maintaining the organoleptic characteristics of the food and, in short, increasing food shelf-life. In order to fulfil these objectives, films have to possess certain characteristics: they have to be water-resistant, improve food appearance, be easily manipulated, have low viscosity, resist mechanical pressure to some extent, and be able to carry active agents (Dhall, 2013). These types of films are usually made up of a plasticizer and a biopolymer solubilised in a common solvent. This solution is cast in a mould and the solvent is evaporated with, or sometimes without, the application of heat. Among the biopolymers seen as candidates for preparing these films are some polysaccharides, such as starch (Bonilla et al., 2013; Dang and Yoksan, 2016; Nouri and Nafchi, 2014), and proteins, such as the gelatine (Arfat et al., 2014; Etxabide et al., 2017; Tongnuanchan et al., 2014). Gelatine is produced by the hydrolysis of animal collagen, so it can be obtained inexpensively from a wide variety of sources. Furthermore, gelatine shows excellent biocompatibility, biodegradability and non-toxicity and it can be used to prepare edible films capable of carrying different active agents (Etxabide et al., 2017).

Among the active agents that have received the attention of the research community, thymol can be highlighted due to its major antimicrobial properties. Thymol is obtained from essential oils of plants of the family Lamiaceae, such as the genus Thymus, Ocimum, Origanum, Satureja, Thymbra and Monarda. When pure, it is a crystalline substance with a characteristic scent. Thymol and other compounds that

are present in essential oils have been registered by the Food and Drug Administration (FDA) as Generally Recognised as Safe (GRAS), and due to their harmlessness and to their medicinal properties, they have also attracted the interest of the food industry. However, thymol has some disadvantages that hinder its direct use in foods, since it is a highly volatile compound, with low solubility in water and high solubility in ethanol and other organic solvents and highly degraded by the light. Principally as a result of these inconveniences, the incorporation of this active agent into edible films could be considered problematic. If thymol is added to the film-forming solution, its low solubility in water could lead to its aggregation during the solvent evaporation stage, producing a deterioration of the film matrix, and therefore, a decrease in the mechanical properties of the films obtained. This problem can be solved by solubilizing the thymol in a solvent of high-ethanolic content, such as that required to solubilize the protein zein. Films made with this protein and thymol have been widely reported by several authors (Del Nobile et al., 2008; Li et al., 2012; Mastromatteo et al., 2009; Park et al., 2012). Furthermore, during the solvent evaporation stage, the high volatility of thymol could lead to its partial or total evaporation, a question that has scarcely been investigated and which is of great interest when considering the possibility of introducing thymol as an additive in protein-based films in order to take advantage of its desirable properties. Kavoosi et al. (2013) produced gelatine films with thymol and in their study the mechanical properties of the film were reinforced using glutaraldehyde, a toxic crosslinker that cannot be allowed to come into contact with food. Despite the use of this crosslinker, the addition of thymol produced a noticeable decrease in the mechanical properties of the film.

In recent research, Marcet et al. (2018)described the preparation and characterization of polylactic acid (PLA) nanoparticles loaded with thymol. PLA is a biodegradable and biocompatible polymer that has been used by several authors to encapsulate other natural antioxidants from plants, such as vanillin (Dalmolin et al., 2016) or aureusidin (Roussaki et al., 2014). The addition of these nanoparticles loaded with thymol to the film-forming solution of a protein-based film could solve two problems: first of all, it

could potentially hinder the loss of thymol during the evaporation step and furthermore, it might avoid the structural changes in the film matrix caused by the active agent when it is free in the solution.

So, in this investigation, the PLA nanoparticles loaded with thymol that were reported in previous work (Marcet et al., 2018) were incorporated into gelatine films. The amount of thymol that remained in the film matrix after the film-forming solution drying step, the mechanical properties, microstructure, thymol release behaviour and antioxidant properties of these gelatine films were evaluated. Furthermore, the antimicrobial properties of the films obtained were tested using apple pieces previously inoculated with *Escherichia coli*.

2. Materials and methods

2.1. Materials

The following reagents were acquired from Sigma-Aldrich (St Louis, USA): thymol (ref. T0501), gelatine from porcine skin (ref. G1890), glycerol (ref. G7893), magnesium nitrate (ref. 237175). The Nutrient Broth (NB, ref. 70149NB) and ethanol 96% (ref.83804.360) were acquired from VWR (Pennsylvania, USA).

2.2. Preparation of PLA nanoparticles loaded with thymol

The PLA nanoparticles loaded with thymol were prepared using the single emulsion preparation technique, a procedure reported in previous work (Marcet et al., 2018). This previous study showed that the nanoparticles able to encapsulate the highest amount of active agent were those prepared by dissolving 150 mg of thymol and 150 mg of PLA in 7.5 mL of dichloromethane. This solution was then emulsified by ultrasound in 30 mL of a polyvinyl alcohol solution. The nanoparticles thus prepared were spherical in shape and had an average size of 244.6 ± 4.5 nm.

2.3. Film preparation

A stock solution of gelatine from porcine skin 6% (w/v) in distilled water was prepared. For that purpose, the gelatine-water mixture was heated in a water bath at 65 °C for 25 min. Then, an amount of glycerol equivalent to 30% (w/w) of the gelatine powder

previously dissolved was added, and the resulting solution was cooled at room temperature to 35 °C. With this stock were prepared several film-forming solutions that contained 1%, 2% and 3% (w/w of gelatine) of free thymol or alternatively of encapsulated thymol. These film-forming solutions were poured into Petri dishes of 4 cm diameter in such a way that every film was made up of 216 mg of gelatine, and then dried at room temperature for 2 days. Finally, the films were manually peeled from the dishes. The films obtained were conditioned for 1 day at room temperature (21 °C) in a closed chamber that contained a saturated solution of Mg(NO3)2.

2.4. Light absorbance, transparency, thickness and thymol content

Light absorbance and transparency were determined according to Dick et al. (2015) using a spectrophotometer (Spekol 1500, Analytik Jena AG, Jena, Germany) at selected wavelengths between 200 and 800 nm. For this purpose, rectangular pieces of film were placed in the spectrophotometer test cell, while an empty cell was used as reference. The transparency of the films was calculated according to the following equation:

$$Transparency = A_{600}/T_h$$

Where A_{600} is the absorbance of the film sample at 600 nm and Th is the film thickness (mm).

The film thickness was measured using a digital micrometre (Model MDC-25PX, Mitutoyo C., Kanagawa, Japan), with a measuring range of 0–25 mm and a precision of ±1 µm. The film thickness was measured in five different areas, one of them in the centre of the film and the other four around the film perimeter.

To check the amount of thymol that remained in the films after the solvent evaporation of the film-forming solution, a preliminary thymol release assay was performed to determine the time required for all the thymol contained in the films to be released into an ethanol solution (96%). Ethanol was selected for this experiment to ensure the diffusion of thymol, which is highly soluble in this solvent. For that purpose, every type of film produced was placed in an amber vial, filled with ethanol at 40 °C under orbital stirring for 72 h. To calculate the amount of thymol in the ethanol, an aliquot of 2 mL

for each vial was taken at several times and the absorbance at 275 nm was measured. The thymol concentration in the sample was determined by preparing a calibration curve with known concentrations of thymol in ethanol. The possible interferences produced by the film matrix were corrected using films without thymol as blank. The amount of thymol released at 40 °C after 24 h was considered to be all the thymol contained in the films. The amount of thymol released into the ethanol was compared with the amount of thymol incorporated into the film-forming solution to calculate the thymol loss during the drying step.

2.5. Mechanical properties of the gelatine films

The measurement of the puncture strength (PS) and puncture deformation (PD) of the gelatine films was carried out using a Texture Analyser TA.XT. plus (Stable Microsystems, Surrey, UK) equipped with a load cell of 5 kg and a probe of 5 mm diameter (P/5S). For this purpose, the films were cut into strips of 40 × 20 mm and attached in the assay platform between two plates. Through the two plates a hole of 1 cm allows contact between the probe and the film sample, so that the probe can stretch the film until it breaks. The probe velocity was 1 mm s−1 and the PS and PD parameters were calculated according to the following equations (Pérez-Mateos et al., 2009):

$$PS = Fm/Th$$

$$PD = (\sqrt{D^2 + R^2} - R)/R$$

Where Fm is the maximum force applied before the film breaks, Th is the film thickness, D is the distance covered by the probe while it is in contact with the film until the film is broken and R is the radius of the orifice in the plates.

2.6. Thymol release from gelatine films loaded with PLA nanoparticles

The thymol released from the gelatine films into the liquid medium was evaluated in accordance with ASTM D4754-98 (ASTM, 2006). In this case only the films loaded with PLA-thymol nanoparticles were evaluated, and the release of the thymol was carried out at three different temperatures (5 °C, 20 °C and 40 °C), using ethanol (96%) as the food simulant. Ethanol has been commonly used as a fatty food simulant by other

authors to study the release of different non-water soluble active agents from films (Manzanarez-López et al., 2011; Ortiz-Vazquez et al., 2011; Rodríguez-Martínez et al., 2016). For this purpose, each film tested was cut into four round discs (2 cm of diameter). These four discs were immobilized using a stainless-steel wire and separated one from another using glass beads of 5 mm diameter. Then, the film pieces were placed in amber vials that were filled with 20 mL of ethanol. The ethanol volume/film area was 0.8 mL cm^{-2}, which is within the volume-to-surface area range, from 155 to 0.31 mL cm^{-2}, recommended by the ASTM D4754-98. The release of thymol from the films to the liquid medium was measured at several times, using the technique explained in section 2.4. After every measurement, the ethanol sample was returned to the vials.

To calculate de diffusion coefficient of the thymol (D), the analytical solution of Crank (1979) (equation (4-1)) to study the diffusion phenomena in a plane sheet was used. To apply this equation, it was assumed there was no reaction between thymol and the film matrix, a homogeneous concentration distribution of thymol, that the amount of solvent can be considered infinite, negligible edge effect, and therefore a unidirectional diffusion of the active agent from the surface of the film into the liquid).

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2\pi^2} e^{\{-D(2n+1)^2\pi^2 t/4L^2\}} \qquad \text{Equation 4-1}$$

where Mt is the amount of thymol released at each time, M∞ is the amount of thymol released after infinite time and L is the half-thickness of the films tested. If instead of considering all the data collected during this experiment, only the values of Mt/M∞ > 0.4 are taken, equation (4-2) can be rewritten as the following linear regression model (Han et al., 2000):

$$\ln\left(1 - \frac{M_t}{M_\infty}\right) = \ln\frac{8}{\pi^2} - \frac{D\pi^2 t}{4L^2} \qquad \text{Equation 4-2}$$

When the first term of this equation is plotted vs time, the D parameter can be calculated from the slope.

To assess the dependence of the thymol diffusion on temperature, the activation energy (Ea) was calculated. For that purpose, the Arrhenius model was applied using the D values obtained from equation (4-3) (Limm and Hollifield, 1996):

$$\ln D = \ln D_0 - \frac{E_a}{RT}$$ Equation 4-3

Where D_0 is a constant, T is the temperature tested (K) and R is the universal gas constant.

2.7. Scanning electron microscopy

The micrographs of the transversal section of the films were performed according to Kadam et al. (2015) and using a scanning electron microscope (JSM-6610LV, JEOL, Tokyo, Japan). To obtain these micrographs, the films were lyophilized and cut into 1 × 1 cm squares. These samples were mounted perpendicularly on aluminium stubs and covered with gold. The microscope was operated with a voltage of 20 kV.

2.8. Antimicrobial properties of the gelatine films

To test the antimicrobial properties of the gelatine films with thymol and the nanoparticles loaded with thymol, 1 g pieces of apple (Royal Gala apple variety) were inoculated with the non-pathogenic strain of *E. coli CECT 101* (CECT, Colección Española de Cultivos Tipo, Spanish Type Culture Collection). This strain was cultured using NB (Nutrient Broth) medium, supplemented with 2% agar and incubated at 30 °C for 48 h. It was then incubated in NB liquid for 10 h at 30 °C under orbital stirring at 26.17 rad s^{-1}.

The apple pieces were inoculated with 0.1 mL of this solution, containing an *E. coli* concentration of 105 CFU mL^{-1}. These apple pieces were then covered with the gelatine films and sealed using a heat-sealing machine. Every piece of apple sealed was placed in a Petri dish and stored at 5 °C for 14 days. To follow the growth of *E. coli* with each type of gelatine film tested, after 3 or 4 days of storage time a sample was taken, the film removed, the apple mixed with 9 mL of NaCl 0.7% and the mixture triturated using a Stomacher (IUL Instruments, Barcelona, Spain) for 120 s. Then, the liquid sample obtained was diluted and seeded in NB medium with 2% agar. After 24 h of incubation at 30 °C the colonies were counted and expressed as log10 CFU mL^{-1}.

2.9. Statistical analysis

Experiments were performed in triplicate and are shown as the mean value ± standard deviation of three independent experiments (n = 3). Least significant differences (LSD)

were calculated by Fisher's test to determine significant differences between the tested samples. These analyses were performed using the statistical software Statgraphics® V.15.2.06.

3. Results

3.1. Light absorbance, transparency, thickness and thymol content

After the solvent evaporation of the film-forming solution for 2 days, the films were peeled easily and entirely, not showing a sticky or brittle appearance. The visual aspect of the films is shown in Fig. 4.7. The gelatine films with free thymol are not shown because their appearance was identical to that of the control films. In this Figure it can be observed how the increment in the amount of PLA nanoparticles produced a decrease in the film transparency. This variation in the transparency parameter was also noticed when the film strips were analysed using the spectrophotometer (Table 4.5), a rise from 0.48 in the control films to 1.04 in the films with 3% thymol encapsulated in PLA nanoparticles being detected. It is understood that a rise in this value corresponds to a decrease in the film transparency.

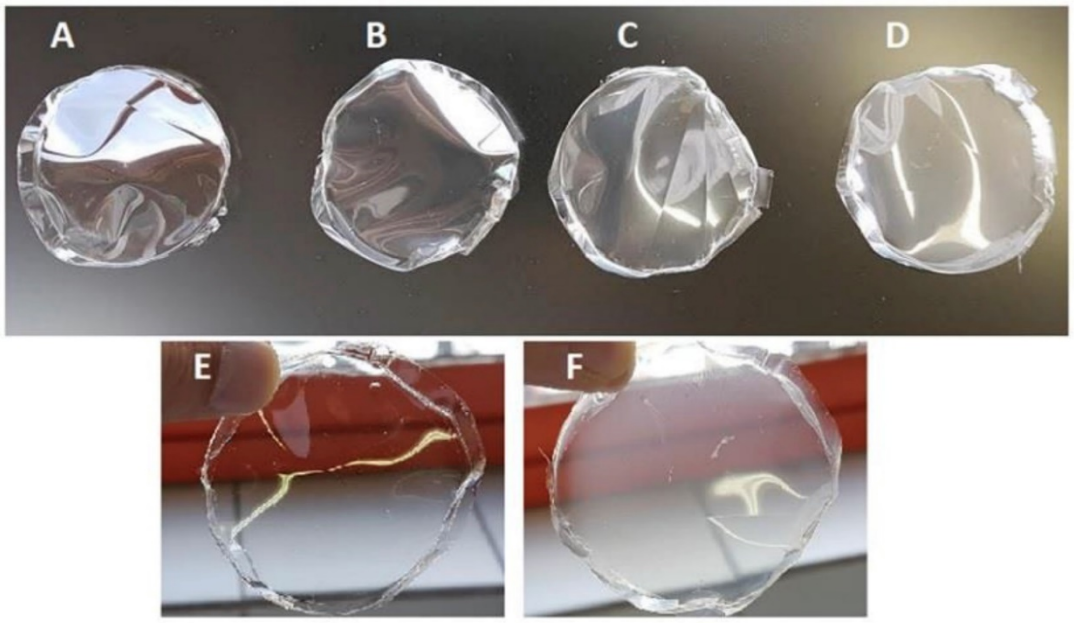

Figure 4.13: Visual appearance of the films obtained. A and E. Gelatine films control, without the addition of thymol. B. Gelatine film with 1% of thymol encapsulated in nanoparticles. C. Gelatine film with 2% of thymol encapsulated in nanoparticles. D and F. Gelatine film with 3% thymol encapsulated in nanoparticles.

Table 4.13: Film thickness, transparency, absorbance at different wavelengths and amounts of thymol remaining after the drying of the film-forming solution.

	Thickness (mm)	Absorbance									Transparency	Thymol that remains in the film (%)
		200 nm	250 nm	280 nm	300 nm	400 nm	500 nm	600 nm	700 nm	800 nm		
Control	0.094 ± 0.007^{a}	2.407	0.894	0.808	0.181	0.056	0.046	0.045	0.044	0.043	0.48	–
NP 1%	0.102 ± 0.002^{ab}	>3.0	1.147	1.059	0.289	0.112	0.085	0.072	0.065	0.064	0.71	11.6 ± 0.7^{a}
NP 2%	0.102 ± 0.003^{ab}	>3.0	1.198	1.189	0.323	0.132	0.099	0.083	0.075	0.069	0.81	14.3 ± 1.0^{b}
NP 3%	0.110 ± 0.003^{b}	2.846	1.496	1.521	0.452	0.196	0.142	0.115	0.102	0.09	1.04	18.5 ± 1.2^{c}
Thymol free 3%	0.096 ± 0.005^{a}	2.455	0.880	0.838	0.191	0.058	0.045	0.045	0.044	0.043	0.46	0

Different letters in the same column indicate significant differences ($P < 0.05$).

Regarding the absorbance values at different wavelengths, is also desirable that a good film can act as a barrier to ultraviolet light, since this is an important starter for the lipid oxidation process (Coupland and McClements, 1996). Most protein-based films have a high capacity to absorb ultraviolet light, due to the presence of amino acids with aromatic side chains, such as tyrosine, tryptophan and phenylalanine. However, the gelatine protein lacks tryptophan and it has a fairly low amount of tyrosine and phenylalanine (Nhari et al., 2011). This results in a relatively low barrier capacity at these wavelengths for the gelatine films in comparison with other protein-based films. Table 4.13 shows that the control films present an absorbance at 280 nm of 0.808, whereas the gelatine film with 3% thymol in nanoparticles has an absorbance of 1.521, which is a rise of 0.71. Taking into account that PLA does not show particularly high absorbance at this wavelength, this increase could be produced by the addition of thymol, which exhibits an absorption spectrum with a major peak at 274 nm (Hajimehdipoor et al., 2010). Furthermore, the gelatine films with 3% free thymol showed an absorption profile similar to that found for the control film, which suggests the total evaporation of the thymol during the film-forming solvent evaporation step. The gelatine films with 2% and 1% free thymol showed the same absorbance value as that found for the gelatine film with 3% free thymol.

With respect to the thickness, a slight increase was detected when the nanoparticles were added, in particular, when the thickness value for the control films is compared with the value for the 3% nanoparticles gelatine films (Table 4.13). The addition of free thymol to the film-forming solution produced films with the same thickness value as that of the control films.

The film transparency, absorbance at 280 nm and thickness values obtained suggest that all the free thymol incorporated into the gelatine films was evaporated during the drying of the film-forming solution. This supposition was confirmed when the amount of thymol in these films was tested (Table 4.13). Films with 3% free thymol lost all their active agent content during the solvent evaporation step. This could be explained by both the high volatility of thymol and the low capacity of gelatine to retain the thymol molecules. In the case of proteins, molecular binding studies carried out by Pan et al. (2014) showed that thymol is able to bind to tyrosine and tryptophan residues in a protein, but as was mentioned previously, the former amino acid is found at a very low proportion in gelatine, and the latter is not present at all, due to it is being degraded during the production of the gelatine (Hafidz et al., 2011). In addition, when thymol encapsulated in PLA nanoparticles was incorporated into the film-forming solution, the thymol loss was also noticeable, but a proportion of the added thymol remained in the dried film (Table 4.5). It is to be expected that during the drying of the film-forming solution a part of the thymol in the nanoparticles is continuously diffusing into the aqueous medium and is then evaporated together with the solvent. This effect is likely to be enhanced at the beginning of the drying step, when the amount of water in the film-forming solution is large enough to produce a major release of thymol from the nanoparticles to the water.

Taking into account the total evaporation of the free thymol in the gelatine films during the film-forming solution drying step, in the following experiments only the gelatine films loaded with PLA-thymol nanoparticles were considered.

3.2. Gelatine films: mechanical properties

The PS and PD values of the gelatine films are shown in Fig. 4.8. The PS parameter indicates the mechanical resistance of a film, and in this case, the addition of nanoparticles loaded with thymol did not produce any statistically significant variation ($p < 0.05$). Furthermore, the PD parameter, which is a measurement of the film's elasticity, was influenced by the addition of PLA nanoparticles, a statistically significant difference appearing between the control film and those with 2% and 3% thymol in

PLA nanoparticles. During the film drying, the protein chains come closer and establish non-covalent interactions with one another. In this type of film, the glycerol and water avoid the excessive structuration of the film matrix, allowing some degree of flexibility. In this case, nanoparticles could be occupying places within the film matrix where the glycerol and the water had previously accumulated, replacing them and leading to a decrease in the flexibility of the material. In any case, the decrease in the PD parameter due to the addition of the nanoparticles could be considered slight, from 37.2 ± 7.0% in the control film to 26.5 ± 6.2% in the case of the gelatine film with 3% thymol in nanoparticles.

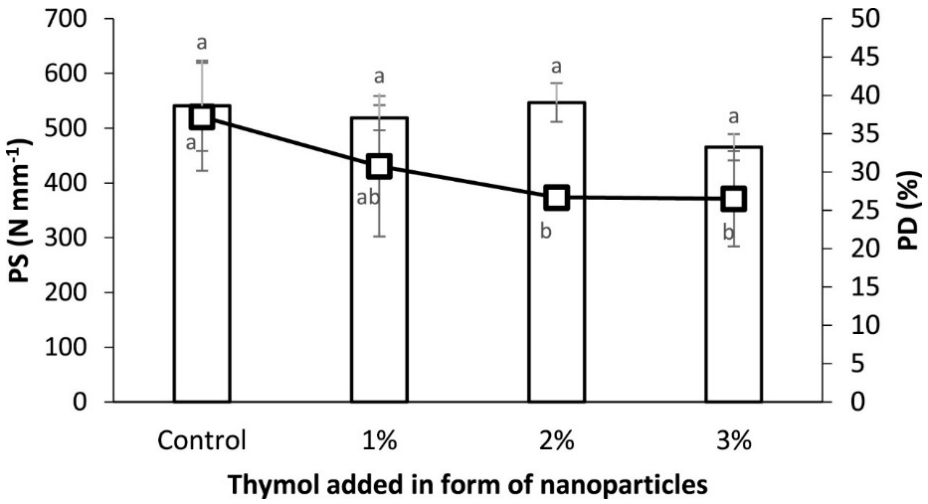

Figure 4.14: Mechanical properties of the tested films. PS parameter is represented with bars, while PD is represented with squares. For each parameter, different letters indicate statistically significant difference ($p < 0.05$).

3.3. Scanning electron microscopy

The micrographs of the film matrix are shown in Fig. 4.15. In these micrographs it can be observed how the films showed a highly homogeneous transverse section, even in the case of the gelatine films with 3% thymol encapsulated in nanoparticles. However, the smoothest cross section area was that found for the gelatine control film, and a slight decrease in this homogeneity can be observed as the amount of encapsulated thymol increased. This slight difference between the micrographs shown in Fig. 4.15 A and D could explain the loss of elasticity that was appreciated when the mechanical

properties of the films were tested.

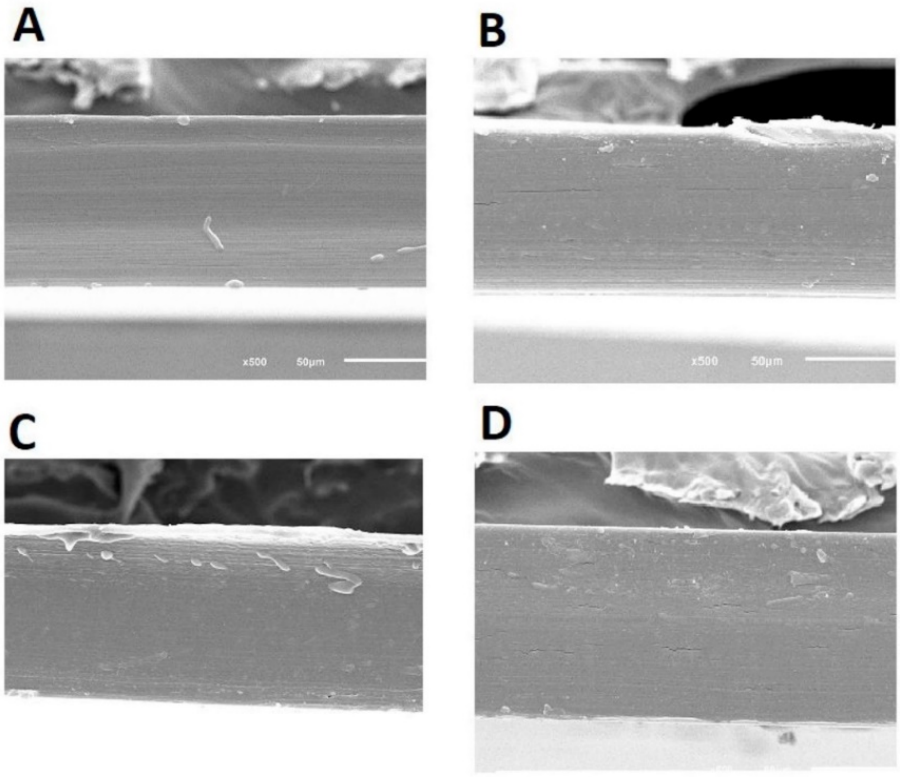

Figure 4.15: Micrographs of the transverse section of the gelatine films loaded with PLA-thymol nanoparticles at 500x. A. Control film. B. Gelatine film with 1% thymol. C. Gelatine film with 2% thymol. D. Gelatine film with 3% thymol.

3.4. Thymol release from gelatine films loaded with PLA-thymol nanoparticles

The thymol release profiles for the gelatine films with different thymol-loaded nanoparticle concentrations were very similar to each other at every temperature tested, regardless of the amount of thymol incorporated into the films (Fig. 4.16). Furthermore, changes in temperature produced variations in the shape of the curves for the three thymol concentrations tested, as was expected. In particular, at 40 °C, all the thymol contained in the nanoparticles was released in 2 h independently of the concentration of thymol. At 20 °C, almost all the thymol was released in 23 h, and at 5 °C all the thymol incorporated was released in 71 h. The differences in the shape of the thymol release profiles at each temperature tested could be mainly due to the effect of the temperature on thymol solubility. So, at 40.4 °C the thymol solubility value is 946 mg/mL, while at 30.9 °C this value decreases to 744 mg/mL (Villanueva Bermejo et al., 2015). The diffusion coefficients calculated using equation (4-3) for these release

curves are shown in Table 4.6. The experimental data obtained fitted to this equation with a high coefficient of determination ($R^2 > 0.99$). As is shown in this Table, the value of these coefficients decreased by a similar degree from 40 °C to 20 °C as from 20 °C to 5 °C, which suggests that the temperature does not affect the thymol release mechanism, and that the drop in the thymol release rate is caused mainly by a decrease in thymol solubility. Furthermore, the diffusion coefficient decreased by one order of magnitude when the temperature fell from 40 °C to 5 °C, which was also seen by other authors working with thymol and zein films (Kashiri et al., 2017). In addition, similar diffusion values to those shown in Table 4.14 were reported by Ramos et al. (2014), studying thymol release from polypropylene films using ethanol 95% as food simulant and at 40 °C. In this case, these authors reported a diffusion coefficient of $1.01 \times 10{-}10\ cm^2\ s^{-1}$.

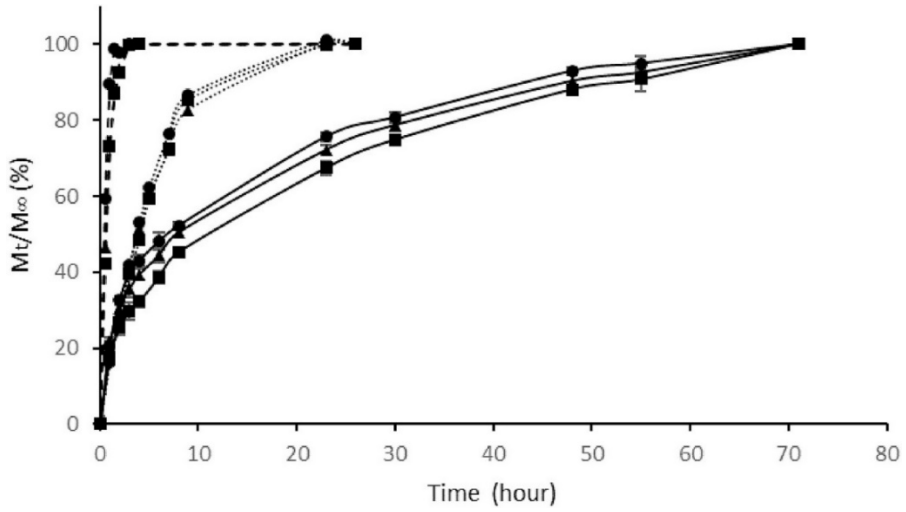

Figure 4.16: Diffusion kinetics of thymol in gelatine films loaded with PLA-thymol nanoparticles. Broken line, 40 °C. Dotted line, 20 °C. Continuous line, 5 °C. Circles, gelatine film with 1% thymol. Triangle shape, gelatine film with 2% thymol. Squares, gelatine film with 3% thymol.

Table 4.14: Diffusion coefficients of thymol released from gelatine films loaded with PLA-thymol nanoparticles into ethanol at 5, 20 and 40 °C, and activation energies for the diffusion of thymol from the gelatine films.

Films with nanoparticles loaded with thymol	Diffusion coefficients ($cm^2\ s^{-1}$)			Activation Energies (KJ mol^{-1})
	5 °C	20 °C	40 °C	
3%	1.10 (x 10^{-10})	8.60 (x 10^{-10})	4.90 (x 10^{-9})	78.13
2%	1.05 (x 10^{-10})	6.33 (x 10^{-10})	4.22 (x 10^{-9})	76.20
1%	1.58 (x 10^{-10})	7.38 (x 10^{-10})	5.27 (x 10^{-9})	72.62

To study the temperature dependence of the diffusion values obtained, an Arrhenius plot was carried out (Fig.4.17). The diffusion values shown in Table 4.14 were fitted to the Arrhenius equation with a high coefficient of determination ($R^2 > 0.99$), which suggest that the diffusion of encapsulated thymol into the gelatine films can be explained by the Arrhenius activation model. Therefore, the diffusion process is driven mainly by the amount of energy provided to the medium, with no structural modification in the film matrix due to the temperature involved. The activation energies calculated from Fig. 4.17 are shown in Table 4.14, and as was expected, variations in the concentration of encapsulated thymol added to the films did not produce a great variation in the value of this parameter.

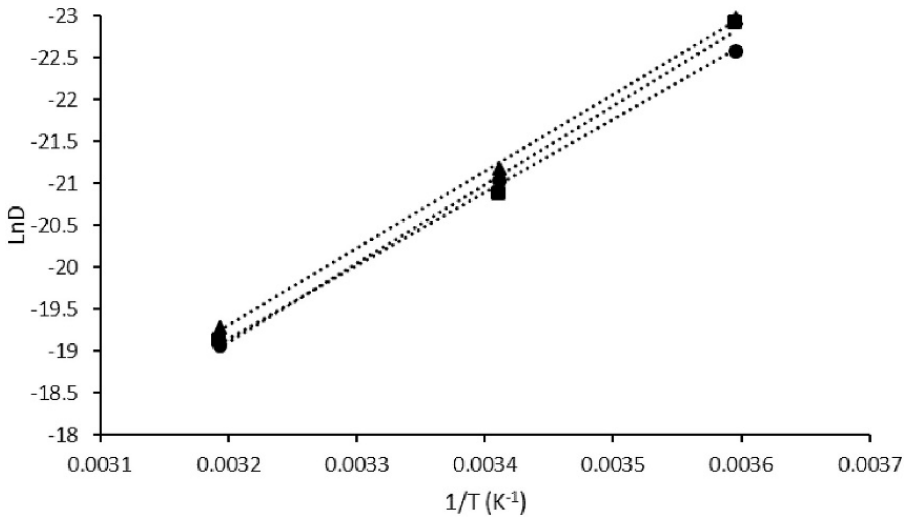

Figure 4.17: Arrhenius plot for the release of thymol from the gelatine films with thymol encapsulated in nanoparticles. Circles, gelatine film with 1% thymol. Triangle shape, gelatine film with 2% thymol. Squares, gelatine film with 3% thymol.

3.5. Antimicrobial capacity of the gelatine films loaded with thymol

The effect of the gelatine films with PLA nanoparticles loaded with thymol is shown in Fig. 4.18.

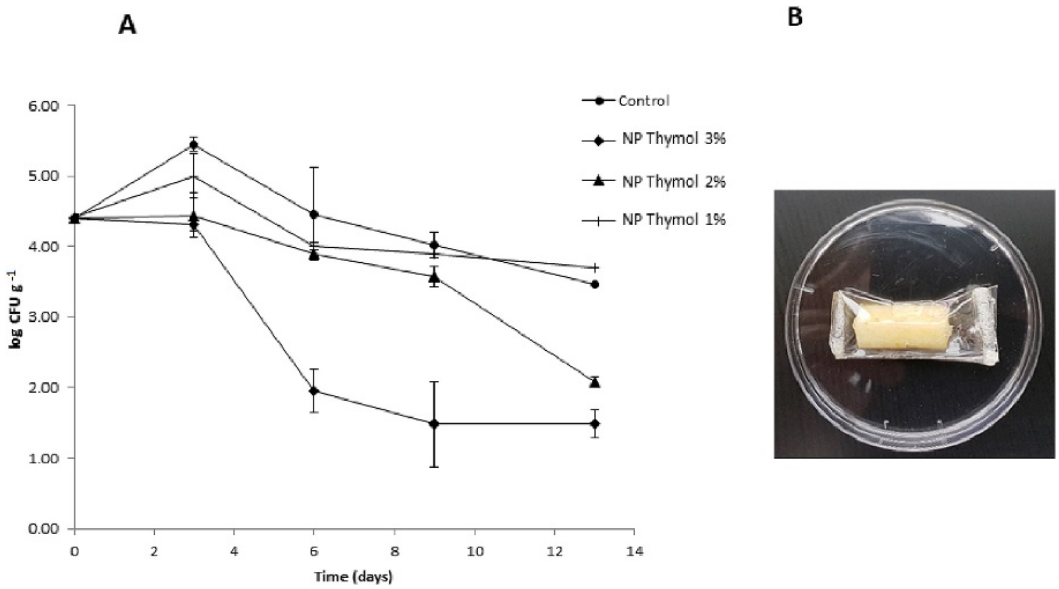

Figure 4.18: A. Growth of E. coli CECT 101 on apples covered with gelatine films that have PLA-thymol nanoparticles incorporated. B. Sample of apple piece covered with a gelatine film that has a 3% PLA-thymol nanoparticle content.

Films loaded with encapsulated thymol at 3% and 2% exhibited a similar, low antimicrobial capacity during the first 3 days of storage, but at the sixth day the difference between these two samples was noticeable: the gelatine films loaded with 3% encapsulated thymol decreased the CFU g^{-1} value to 1.95, while in the case of the films loaded with 2% encapsulated thymol, the CFU g^{-1} value decreased to 3.9. During the following days, the decrease in the CFU value was more pronounced in the 2% films, whilst it remained relatively constant for the 3% film. At the end of the storage time, a CFU g^{-1} value of 2.0 was found for the 2% films, while a decrease to 1.5 CFU g^{-1} was measured for the 3% films. It should be expected that the release of thymol from the film to the surface of a food with a relatively high-water content, such as a piece of apple, will be slower than the relatively fast release of thymol to ethanol shown in section 3.4. The reason for this difference in diffusivity is mainly due to the low solubility of thymol in water. This long-term effect is desirable and could explain the reduction in CFUs after 6 days of storage for the pieces of apple covered with 3% and

2% PLA-thymol nanoparticles gelatine films. The antimicrobial effect of the PLA-thymol nanoparticles on *E. coli*-inoculated pieces of apple was tested in a recent study (Marcet et al., 2018), and these antimicrobial properties were found to be slightly worse than those shown by the gelatine films loaded with the same nanoparticles. In the case of the PLA-thymol nanoparticles without film, the amount of thymol encapsulated was 0.5 mg mL^{-1} in the case of the best result obtained, while the concentration of thymol in the PLA-thymol nanoparticles included in the gelatine films was higher than that measured in the PLA-thymol nanoparticles without film, even taking into consideration the evaporation of thymol during the film-forming solution drying step. This could explain the better antimicrobial capacity observed when the PLA-thymol nanoparticles were incorporated into films.

4. Conclusions

The amount of thymol evaporated during the production of protein-based films is a subject that has barely been addressed in this type of packaging materials, and it could have a major impact on the costs associated with their production, especially considering that this problem is likely to be common to the use of other volatile essential oils. In this study, the total evaporation of non-encapsulated thymol from the gelatine films during the drying step could be due to both to the relatively low amount of thymol added to the film-forming solution and also to the particular amino acid composition of the gelatine proteins; the former is recommendable, since it reduces the weakening effect exerted by the thymol on the mechanical properties of the films, and the latter is very difficult to avoid. In any case, the incorporation of thymol in the form of PLA-thymol nanoparticles in the film-forming solution resulted in the production of solid gelatine films with the active agent present in the film matrix. This thymol that remains in the film matrix showed antimicrobial properties, and its impact on the mechanical properties of the materials produced can be considered slight.

Acknowledgements

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4.2.3 Polylactic acid nanoparticles encapsulated thymol (supplement)

The incorporation of slow-release thymol in the gelatine film mentioned in Section 4.2.1 is to be encapsulated in PLA nanoparticles to help the active substance stay in the biomass packaging film for a longer time to exert its antibacterial effect. This section is based on the supplement of the previous research results, on the characterization of the effect of encapsulating thymol with PLA nanoparticles, a degradable material. Since the high volatility, photosensitivity and low water solubility of thymol hinder its free application in the field of food technology, the average size and thymol encapsulation of nanoparticles were investigated using different concentrations of PLA and thymol. Efficiency parameters have become a means to help the material expand its application direction. Indeed, by studying the morphology, storage stability, and in vitro thymol release profiles of nanoparticles at several pH values, as well as their thermal degradation profiles, it was found that these nanoparticles were more effective than unencapsulated thymol. It showed high storage stability at various pH values and improved antibacterial properties to a certain extent.

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Production and characterisation of biodegradable PLA nanoparticles loaded with thymol to improve its antimicrobial effect

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Abstract

Thymol is widely recognised as an antibacterial compound. However, its use in food technology is hindered by its high volatility, its light sensitivity and its low solubility in water. To overcome these drawbacks, the preparation of nanoparticles using polylactic acid (PLA) is proposed in this study.

The average size of the nanoparticles and thymol encapsulation efficiency parameters were studied using different PLA and thymol concentrations. Furthermore, the morphology, the storage stability of the nanoparticles at several pHs and the in vitro thymol release profile were also studied, as well as their thermal degradation profile. Finally, their antimicrobial activity on a real food model was measured, using for this purpose apple pieces previously inoculated with *E. coli*.

The PLA was found to be the key variable in optimizing the nanoparticle preparation, producing spherical nanoparticles with a thymol encapsulation efficiency of 60.3 ± 8%. These nanoparticles showed a high storage stability at several pHs and improved

antimicrobial properties in comparison with the non-encapsulated thymol.

Keywords

PLA, Thymol, Encapsulation, Nanoparticles, Antimicrobial properties, Storage stability, Thermal degradation, Release profile

1. Introduction

The aromatic and medicinal properties of the plants of the genus Thymus have been widely recognised. These plants are usually used to make tea, as a flavouring agent and for medicinal purposes (Stahl-Biskup and Sáez, 2003). The results reported reveal that the main constituent obtained from the aerial parts of the plant are geraniol, linalool, gamma-terpineol, carvacrol and thymol (Piccaglia et al., 1993). Thymol (2-isopropyl-5-methylphenol) is a volatile compound which is a phenolic derivate of the terpene 3-hidroxy-p-cymene and its production by the plant is associated with part of its defensive strategy against phytopathogenic microorganisms. Numerous studies have reported the antibacterial and fungicidal properties of thymol (Andrade-Ochoa et al., 2015, Džamić et al., 2015, Hernández-Hernández et al., 2014). For this reason, this compound has received the attention of the food industry, thymol having been used as an antimicrobial agent and authorized by the Food and Drugs Administration (FDA) of the USA as generally recognised as safe (GRAS). However, there are technical limitations which hinder its use, such as the high volatility of thymol at room temperature, its high light sensibility and its low solubility in water (Mastelić et al., 2004).

One way to overcome these problems is the preparation of nanoparticles which contain thymol. In the form of nanoparticles, the amount of thymol introduced into the food matrix could be increased above its solubility limit, since the Brownian movement of the nanoparticles prevents their sedimentation in the lower layers of the food (Huang, 2012). Furthermore, the high surface-area ratio of nanoparticles enhances their contact with the microorganisms, and the protective environment

provided could limit the volatility of the thymol and its light-induced degradation. For this reason, several authors have prepared protein-based nanoparticles which incorporate thymol. To prepare these nanoparticles, the main protein used was zein, since both zein and thymol are soluble in ethanol and the particles can be prepared by antisolvent precipitation (da Rosa et al., 2015, Li et al., 2012, Zhang et al., 2014 and Chen et al. 2015). However, the use of biodegradable synthetic polymers instead of proteins to encapsulate thymol remains unstudied, although their use to prepare nanoparticles is a topic that has received a lot of attention from the scientific community. These synthetic polymers have some advantages with respect to proteins: they are produced with a high level of purity, the experiments investigating their use have excellent reproducibility and they do not produce any antigenic effect (Nair and Laurencin, 2005). In particular, polylactic acid (PLA) has been widely used in biomedicine and it is considered a good material to prepare micro and nanoparticles, since the particle size and shape can be controlled so as to satisfy the preparation requirements. Furthermore, in this type of nanoparticle, the active agent is usually homogeneously dispersed within the PLA matrix, which is positively related to the appropriate liberation of the active agent (Lee et al., 2016).

To the best of our knowledge, this study documents the first preparation of PLA nanoparticles containing thymol. The production of these nanoparticles was evaluated using different PLA and thymol concentrations, and then they were characterised by evaluating their morphology, encapsulation efficiency, stability at several pHs and thermal degradation profile. The antimicrobial properties of the nanoparticles obtained were tested on apple pieces previously inoculated with *E. coli*.

2. Materials and methods

2.1. Materials

The PLA (180 kDa) was produced by NatureWorks (4032D). The following reagents were acquired in Sigma-Aldrich (St Louis, USA): thymol (ref. T0501), dichloromethane (DCM, ref 270,997), polyvinyl alcohol (PVA, ref P8136), buffer Trizma® pH 7.0 (ref.

T1819), 2,2-diphenyl-1-picrylhydrazyl (DPPH, ref. D9132) and Nutrient Broth (NB, ref. 70149NB). The agar was acquired in VWR (Pensilvania, USA).

2.2. Preparation of PLA nanoparticles

Nanoparticles were prepared following the single emulsion preparation technique (Lee et al., 2016) with slight modifications: a 1% PVA solution was prepared and saturated with thymol. In order to prepare this solution, the PVA at the required concentration was heated in a water bath at 90 °C until it was completely dissolved. This solution was cooled to room temperature, 2 mg/mL of thymol was added and the mixture was stirred overnight. Then, the solution was filtered to remove the non-solubilised thymol using a vacuum pump and Nº1 Whatman paper. The filtration process was repeated twice.

Three different amounts of PLA were dissolved in 7.5 mL of DCM, and for each of these PLA concentrations, three different amounts of thymol were dissolved in the same DCM volume. These percentages of thymol with respect to the weight of PLA, as well as the precise amounts of thymol and PLA tested in each case are shown in Table 4.15.

Table 4.15: Amounts of PLA and thymol dissolved in the organic phase and tested to optimize the nanoparticle preparation.

PLA	Thymol		
	66%	100%	133%
100 mg	66 mg	100 mg	133 mg
150 mg	100 mg	150 mg	200 mg
200 mg	133 mg	200 mg	266 mg

This DCM solution was carefully poured into 30 mL of the previously prepared PVA solution. The mixture was ultrasonicated for 2.5 min using the Sonopuls HD 2070 system (Bandelin, Germany) and the MS 73 probe, at a frequency of 20 kHz and applying a sonication amplitude of 90% (100% corresponds to 212 µm). This sonication amplitude corresponds to an ultrasonic intensity of 80 W/cm2. During the sonication process, the sample was kept in ice to avoid temperature increase. To remove the DCM contained in the preparation, the emulsified solution was kept in a low-pressure medium at 40 °C for 40 min using a rotavapor (Büchi R-205, Büchi Labortechnik, Essen,

Germany). During the DCM evaporation, part of the thymol was able to pass from the nanoparticles to the PVA medium. It was precisely to avert this problem that the PVA solution was saturated with thymol, as was described previously.

After the DCM evaporation, the sample was centrifuged at 13,000 rpm for 20 min to precipitate the nanoparticles. The supernatant, which contains the PVA and is saturated with thymol, was removed by decantation and it could be reused to prepare new nanoparticles. The same volume of distilled water was added to the sediment, and the nanoparticles were resuspended and centrifuged again at 4000 rpm for 5 min. In this case, the sediment was discarded to remove PLA aggregates, the nanoparticles remaining in suspension.

2.3. Nanoparticle size measurement and thymol content

The average size of the nanoparticles and their polydispersion index (PDI) were measured using dynamic light scattering (DLS, Nanosizer ZS, Malvern Instruments, Malvern, UK).

The amount of thymol encapsulated was measured as follows: the nanoparticle suspension was centrifuged at 13,000 rpm for 20 min and the supernatant was removed and replaced with the same volume of ethanol (96°). The sediment, which contains the nanoparticles, was dispersed in the ethanol with the aid of the sonicator system. Then, the sample was centrifuged again at 13,000 rpm for 20 min. During this process and due to the high solubility of thymol in ethanol, the active agent was extracted from the nanoparticles. Finally, 0.1 mL of this ethanolic solution was diluted with 9.9 mL of fresh ethanol. The absorbance of the resulting solution was measured at 275 nm using a spectrophotometer (Spekol 1500, Analytik Jena AG, Jena, Alemania). Previously, known concentrations of thymol in ethanol were measured at this wavelength to calculate a calibration curve. The encapsulation efficiency (EE%) was calculated according to the following equation.

$$EE\% = \frac{thymol\ escapsulated}{thymol\ added\ in\ the\ DCM\ solution} * 100 \qquad \text{Equation 4.4}$$

2.4. Nanoparticle morphology

A drop of the nanoparticle solution was placed on a copper grid and negatively stained with a drop of phosphotungstic acid solution 2% (w/v). The micrographs were obtained using a transmission electron microscope (TEM, JEOL-2000 EX-II, Tokyo, Japan) operated at 200 kV. Furthermore, micrographs obtained using a scanning electron microscopy (SEM, JSM-6610LV, JEOL, USA) were also performed. In this case, a drop of the sample was dried on a glass microscope slide and coated with gold. The surface morphology of the nanoparticles was observed at 20 kV.

2.5. Nanoparticle storage stability at various pHs

A freshly prepared solution of nanoparticles was centrifuged at 13,000 rpm for 20 min and the supernatant was discarded. The sediment, which contains the nanoparticles, was resuspended in a buffer solution at pH 4.0 (phosphate-citrate), or at pH 7.0 (Trizma®), or at pH 9.0 (sodium carbonate-bicarbonate) and stored at 5 °C. The buffer concentration was 0.01 M in all cases. Periodically, the average size of the nanoparticles and the thymol content were measured.

2.6. Thermogravimetric analysis (TGA)

The samples tested were lyophilized for 24 h whilst applying a pressure of 0.33 mbar. The TGA curves were carried out in an SDTA851e TGA analyser (Mettler-Toledo, Columbus, USA) from 30 °C to 700 °C, under a nitrogen atmosphere. The heating rate was 10 °C/min. In this case, four samples were tested: the nanoparticles loaded with thymol; the nanoparticles prepared as described in section 2.2, but without the addition of thymol (unloaded nanoparticles); the thymol and the PLA.

2.7. In vitro thymol release profile

To evaluate the thymol release profile, 10 mL of a freshly prepared solution of nanoparticles was placed in a dialysis tube of 10 kDa MWCO, which was placed in a thermostatically controlled, 15 L capacity water bath (ref. 3001373, J. P. Selecta, Barcelona, Spain). The water in the bath was under agitation due to a water stream provided by a pump. Several temperatures were tested, and periodically one aliquot of 0.5 mL was taken from the dialysis tube and the thymol content in the nanoparticles

measured. To characterise the thymol release profile, the data obtained were modelled using a first order equation (eq. (4.5)) and the Korsmeyer-Peppas equation (eq. (4.6)) (Siepmann and Peppas, 2001).

$$\frac{M_t}{M_\infty} = 1 - e^{-kt} \quad \text{Equation 4.5}$$

$$\frac{M_t}{M_\infty} = k - t^n \quad \text{Equation 4.6}$$

Where $\frac{M_t}{M_\infty}$ is the accumulated percentage of thymol released at each time (t), k is the release constant of thymol and n is the diffusion exponent that characterises the release mechanism of the thymol.

2.8. Nanoparticle antimicrobial properties

To compare the antimicrobial properties of thymol and the nanoparticles loaded with thymol, apple pieces weighing 1 g were inoculated with the non-pathogenic *E. coli* CECT 101 strain. This strain was incubated at 30 °C for 48 h using NB medium supplemented with 2% agar. To prepare the liquid inoculum, *E. coli* was incubated in NB liquid medium for 10 h, at 30 °C with orbital stirring (250 rpm).

For all these treatments, each piece of apple was inoculated with 100 µL of *E. coli* and left for 2 min. The *E. coli* concentration used for this experiment was 10^5 UFC/mL, which might be considered high, but the strong antimicrobial properties of thymol made it necessary so as to be able to detect variation in the ability to decrease *E. coli* growth on the apple pieces between different preparations. After the inoculation, the apple pieces were submerged for 1.5 min in 5 mL of an aqueous solution that contained thymol or nanoparticles loaded with thymol. To solubilize the thymol, crystals of this active agent were added to distilled water at a concentration of 2 mg/mL, stirred overnight and filtered using n°1 Whatman paper. The concentration of dissolved thymol was determined by mixing 0.1 mL of this filtered solution with 9.9 mL of ethanol and measuring the absorbance at 275 nm, in accordance with the methodology described in section 2.3. To investigate the antimicrobial effect of this solution, three thymol concentrations were tested: 0.94 mg/mL, which corresponds to the maximum concentration of thymol dissolved in water, 0.5 mg/mL and 0.1 mg/mL.

The same concentrations of thymol were used for the nanoparticle samples. After the treatment, the apple pieces were hermetically closed in polyethylene boxes and stored at 5 °C until their analysis. To follow the growth of the *E. coli* on the apple surface, samples were taken at different times and mixed with 9 mL of NaCl 0.7%. Then, the samples were homogenised using a stomacher (Stomacher 80 biomaster, Seward Ltd, Worthing, UK) and cultured in NB-agar medium. After 24 h of incubation at 30 °C the *E. coli* colonies were counted and the results obtained expressed as log10 UFC/g.

2.9. Statistical analysis

Experiments were performed in triplicate and are shown as the mean value ± standard deviation of three independent experiments (n = 3). Least significant differences (LSD) were calculated by Fisher's test to determine significant differences between the tested samples. These analyses were performed using the statistical software Statgraphics® V.15.2.06.

3. Results

3.1. Size, polydispersity index, thymol recovered and encapsulation efficacy of the nanoparticles prepared

There are several parameters that could have a significant effect on the preparation of these nanoparticles and have the capacity to modify their average size and the efficiency with which they encapsulate thymol. In this sense, the variables which should be considered would include the amount of DCM and water, the time and type of stirring used to obtain the emulsion (ultrasounds or mechanical stirring), the amount of energy applied during the process, the type and concentration of surfactant used to stabilise the emulsion, and the amounts of PLA and of thymol. In our previous tests it was observed that, among all these parameters, variations in the PLA and thymol concentrations produced changes in the properties of the nanoparticles in a meaningful and straightforward way, and for this reason they were considered to be key variables for this process.

Fig. 4.18A indicates the average size of the prepared nanoparticles and their PDI, whilst

in Fig. 4.18B the amount of thymol encapsulated and the encapsulation efficiency are shown. According to Fig. 4.18A, and at each thymol/PLA proportion tested (66%, 100% and 133%), the higher the concentration of PLA in the organic phase, the greater is the average size of the nanoparticles obtained. This effect may be due to the increase in viscosity of the organic phase produced by the increase in the amount of PLA, and therefore, at the maximum PLA concentrations tested (solid bars), a higher ultrasound intensity may be necessary to produce a higher dispersion of the more viscous organic phase.

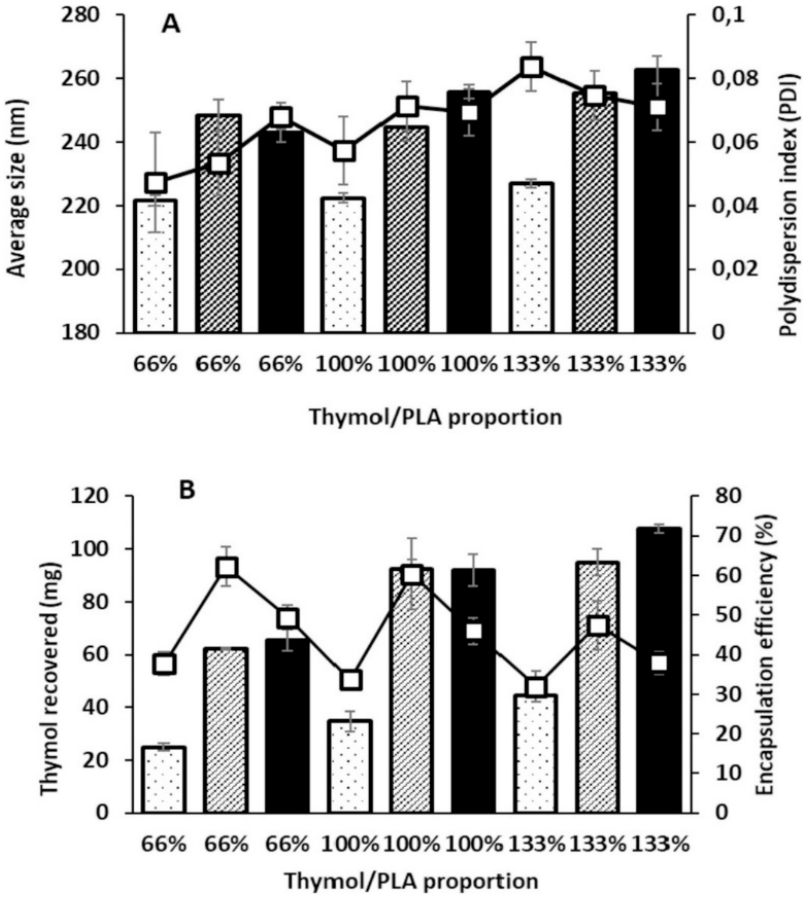

Figure 4.19: Parameters of interest for the characterisation of the nanoparticle preparations with respect to the PLA and thymol concentrations. Dotted bars correspond to the nanoparticles prepared using 100 mg de PLA, striped bars 150 mg of PLA and solid bars 200 mg of PLA. At each amount of PLA, three different thymol/PLA proportions were tested (66%, 100% and 133%). A: Average size (bars) and polydispersity index (line) of the nanoparticles prepared. B: Thymol recovered (bars) and encapsulation efficiency (line) of the nanoparticles prepared.

A similar but not so evident trend was observed when the amount of PLA was kept constant and the concentration of thymol was increased. The increase in the average

size of the nanoparticles was particularly pronounced in the 200 mg PLA samples (solid bars), being negligible at the other amounts of PLA tested. In broad terms, for these parameters, the increase in the amount of thymol did not produce such an evident effect on the average nanoparticle size as the amount of PLA, probably because thymol is a small molecule in comparison with the PLA, and its effect on the viscosity of the organic phase was limited.

Furthermore, in all cases the PDI values obtained were low enough to be considered satisfactory, being even lower than those obtained by other authors in the preparation of similar PLA nanoparticles (Roussaki et al., 2014, Wrona et al., 2017).

As regards Fig. 4.18B, the thymol recovered from the nanoparticles followed a similar trend to the average size, in that increasing the amount of PLA and thymol led to an increase in the amount of thymol recovered. However, the encapsulation efficiency showed a different trend to the recovered thymol parameter, and the highest values obtained were from the 150 mg PLA preparations. In this case, the 100 mg PLA preparations showed the lowest encapsulation efficiency values, perhaps because the amount of PLA was low enough to produce nanoparticles with low internal density and high porosity, which were incapable of retaining the thymol. In the case of the 200 mg PLA nanoparticles, their higher size may facilitate their precipitation during the last centrifugation (5000 rpm, 4 min), which was carried out in accordance with the methodology described in the materials and method section, and therefore producing a decrease in the thymol encapsulation efficiency parameter.

Taking into account all these results, and although the concentration of thymol and PLA produced variations in the size of the nanoparticles, in all the tested preparations this parameter was maintained within the 220–260 nm range. Since this could be considered a narrow range, it might be more effective to optimize the encapsulation efficiency in order to prevent loss of reagent and to increase the amount of thymol recovered. From the results shown in Fig. 4.18B, the best preparation was seen to be that obtained using PLA and thymol concentrations of 150 mg. Furthermore, the PLA concentration resulted more relevant than the thymol concentration to maximize the

efficacy of encapsulation parameter.

3.2. Nanoparticle morphology

The TEM and SEM micrographs of the nanoparticles prepared with 150 mg of PLA and several concentrations of thymol are shown in Fig. 4.19. The SEM micrographs are generated from the secondary electron dislodged from the surface of the nanoparticles, providing high quality 3D images that are very useful for appreciating the superficial topography of the nanoparticles. As can be appreciated in Fig. 4.19A–C, the nanoparticles showed a spherical morphology in all the cases tested, with a smooth surface and with a size variation between 240 and 260 nm, corroborating the values obtained by DLS provided in section 3.1. The morphology of the nanoparticles prepared using proteins or polysaccharides is not usually so regular, and they may have a rougher appearance, which could have some influence on the release profile of the active agent. In this sense, Wattanasatcha et al. (2012) produced nanoparticles of ethylcellulose/methylcellulose loaded with thymol and with an almost spherical morphology. Similar results were obtained by Xue and Zhong (2014) preparing nanodispersions of lecithin, gelatin and thymol.

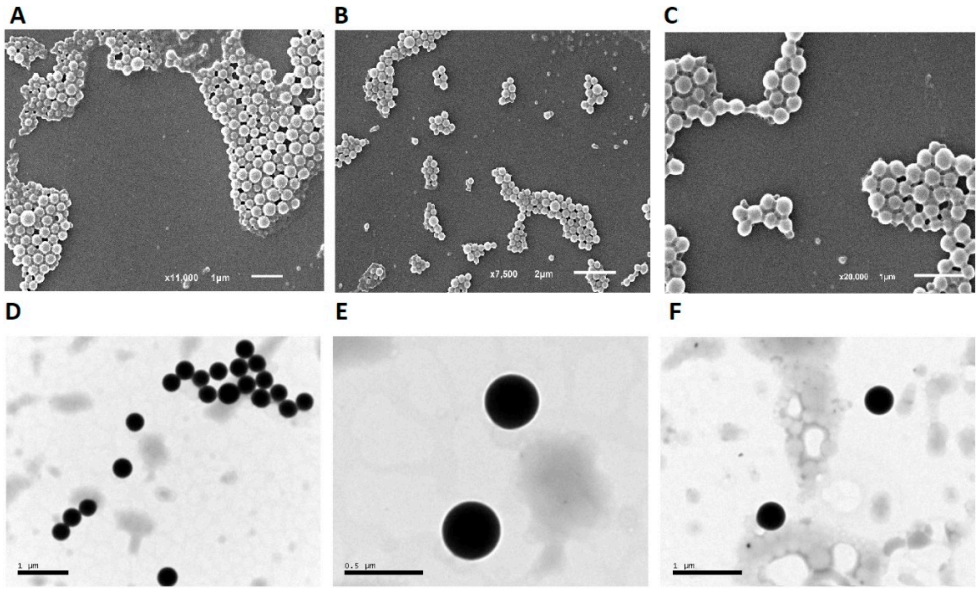

Figure 4.20: SEM (A, B, C) and TEM (D, E, F) micrographs of the nanoparticles prepared using 150 mg of PLA and 100 mg (A, D), 150 mg (B, E) and 200 mg (C, F) of thymol.

Furthermore, in Fig. 4.20D–F the TEM micrographs of the nanoparticles are shown.

TEM produces micrographs due to the transmission of electrons through the sample, so it can provide useful information about the internal state of the nanoparticles. In this case, as was expected, the nanoparticles were observed as black spots due to the formation of a solid PLA-based nanoparticle matrix, which prevents the passage of the electron beam through the sample, and where the active agent is usually homogeneously dispersed (Lee et al., 2016).

3.3. Nanoparticle storage stability at several pHs

The storage stability of the nanoparticles was tested at several pHs. For this purpose, the average size and the thymol encapsulated were evaluated every 10 days for 40 days. The results obtained are shown in Table 4.16. In all the tested samples, during a period of 40 days no changes were detected in the size of the nanoparticles at any pH. As regards the thymol encapsulated, at basic pH a slight decrease in this parameter was detected. In the case of the nanoparticles stored at pH 7.0 and 4.0, this decrease was even less pronounced. This could be due to the deprotonation of the phenolic hydroxyl group of the thymol at basic pH (Wu et al., 2012), which would increase its solubility in water, therefore enhancing the movement of the active agent from the nanoparticle to the aqueous medium. In any case, the decrease detected in the thymol loaded within the nanoparticles in 40 days can be considered low at all the pHs tested. Finally, it should be remembered that the encapsulation of thymol in protein-based nanoparticles is usually pH-dependent, since the proteins tend to aggregate or solubilize at a specific range of pH. In this case, the PLA nanoparticles do not exhibit this inconvenience and can be used over a wide range of pHs.

Table 4.16: Storage stability of the nanoparticles loaded with thymol at several pHs for 40 days.

	Average size (nm)					Thymol encapsulated (%)				
	Day 1	Day 10	Day 20	Day 30	Day 40	Day 1	Day 10	Day 20	Day 30	Day 40
pH **4.0**	241.3 ± 1.3^{a}	242.7 ± 2.7^{a}	240.15 ± 0.2^{a}	239.1 ± 2.2^{a}	238.0 ± 2.5^{a}	100^{a}	95.8 ± 0.6^{b}	96.4 ± 0.6^{b}	96.0 ± 1.0^{b}	96.5 ± 1.0^{b}
pH **7.0**	238.7 ± 1.3^{a}	241.0 ± 0.5^{a}	238.9 ± 1.8^{a}	238.1 ± 1.5^{a}	239.5 ± 1.2^{a}	100^{a}	98.2 ± 0.7^{b}	98.9 ± 0.8^{b}	98.0 ± 1.2^{b}	97.3 ± 0.6^{b}
pH **9.0**	238.6 ± 0.1^{a}	240.8 ± 1.5^{a}	238.8 ± 1.8^{a}	240.4 ± 2.0^{a}	239.0 ± 1.0^{a}	100^{a}	96.7 ± 0.8^{b}	95.5 ± 1.3^{b}	96.2 ± 0.9^{b}	92.2 ± 1.4^{c}

Different letters in the same row indicate significant differences ($P < 0.05$).

3.4. TGA analysis

The TGA analysis of the lyophilized nanoparticles prepared using 150 mg of PLA and the same amount of thymol, the nanoparticles control without thymol, the reactive thymol and the PLA are shown in Fig. 4.20A. The derivative of the TGA curves (DTG) obtained are shown in Fig. 4.20B. In these Figures it can be observed that the pure thymol is degraded completely in one single stage and over the temperature range 65–190 °C, showing a maximum rate of degradation at 158 °C. The PLA was degraded in a similar way as the thymol but at temperatures between 280 and 370 °C. The nanoparticles control, without thymol, showed a degradation curve similar to that found for the pure PLA, but divided into two stages. The first of them corresponds to PLA degradation, from 280 °C to 370 °C and the second one, from 390 °C to 470 °C, corresponds to PVA degradation (Yu et al., 2003). The degradation of the nanoparticles loaded with thymol was produced in three stages (Fig. 4.20B). While the second and third stages are similar to those found for the control nanoparticles, the first stage could be attributed to the degradation of thymol, but it is offset to the right in comparison with that found for the pure thymol. In fact, this first degradation stage takes place from 120 °C to 250 °C, with a maximum degradation rate at 182 °C. This increase in the temperature of degradation of the encapsulated thymol could occur due to its inclusion in the nanoparticles, which provide a protective environment. Furthermore, it is significant that the amount of thymol in these nanoparticles is only around 10% of the weight of the lyophilized nanoparticles. This decrease in the thymol content likely was due to the evaporation of thymol during the lyophilization process, since this active agent is a highly volatile essential oil and the drying process involves the application of low pressures for a relatively long time.

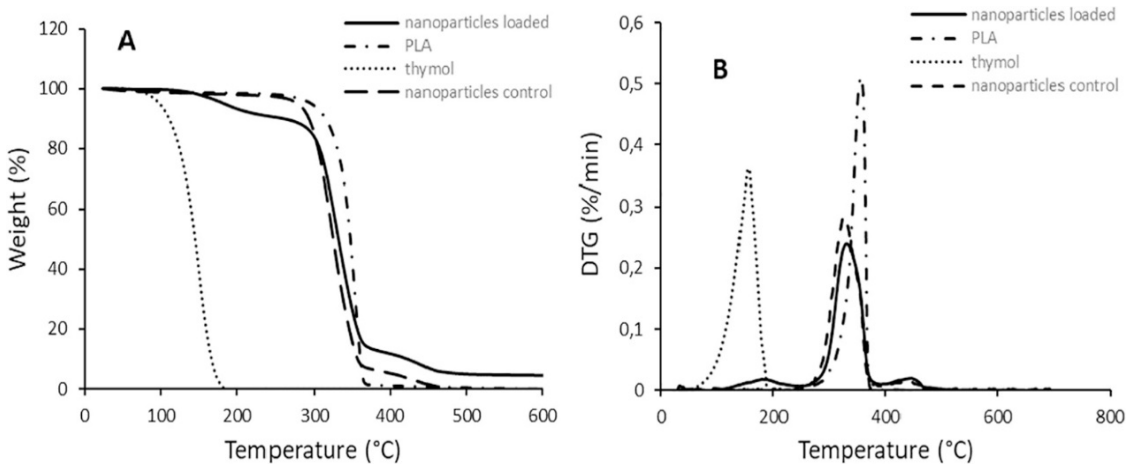

Figure 4.21: TGA (A) and DTG (B) curves of the nanoparticles loaded with thymol, the nanoparticles control without thymol, the chemical thymol and the PLA.

3.5. In vitro thymol release profile

The thymol release profiles of the loaded (150 mg of PLA and of thymol) nanoparticles at different temperatures were determined and are shown in Fig. 4.21. Although this type of experiment has been designed principally to investigate the behaviour of injectable encapsulated drugs in the bloodstream, it can also provide valuable information about the state of the active agent inside the nanoparticle matrix and could provide evidence about possible interactions between the nanoparticle polymers and the active agent. According to this Figure, the thymol diffuses rapidly from the nanoparticles into the aqueous medium at 35 °C, releasing 93% of the active agent in 6 h. In the same time, if the temperature is lowered to 22 °C, 80% of the thymol is released. However, at 15 and 5 °C, the release of thymol over 6 h decreases to 60%. As was to be expected, the rate of thymol release is temperature-dependent. Both at 35 and 22 °C the release of thymol for the first 2 h is fast, and then a slight decrease in this release rate was detected. This first burst effect in 2 h could be due to the release of the thymol adhered to the surface of the nanoparticles and to the thymol that is within the nanoparticles, but in the more external layers (Yadav and Sawant, 2010). The geometry of the nanoparticles and their high surface/volume ratio could be the reason for this first fast release. From this point, the thymol released came from the inner layers of the nanoparticles.

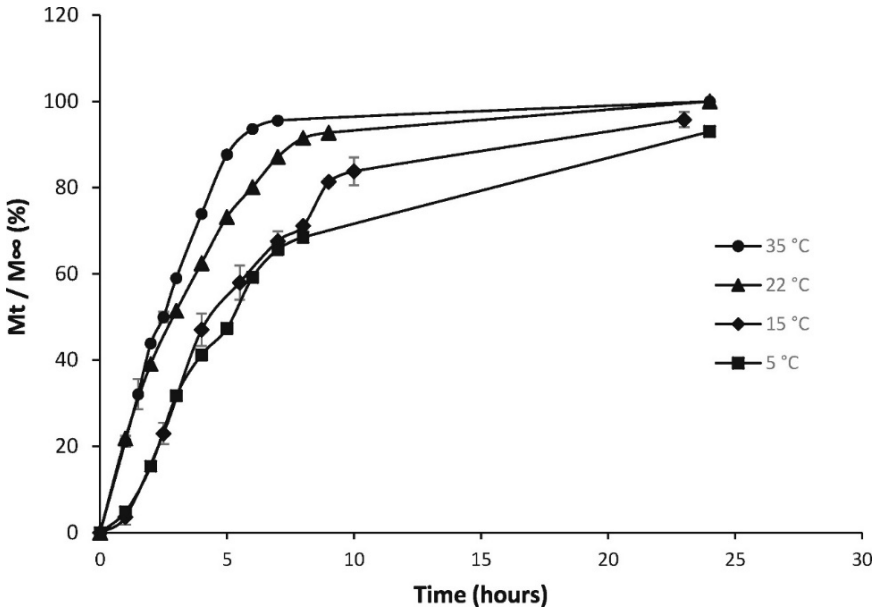

Figure 4.22: Thymol release from the nanoparticles prepared using 150 mg of PLA and 150 mg of thymol at several temperatures.

Furthermore, during the first hour at 15 and 5 °C there is a slight delay in the thymol release. This slow release could be due to the presence of free thymol remaining in the solution containing the nanoparticles. At the beginning of the test this thymol would be in the process of diffusing out of the dialysis tube, whereas at the highest temperatures tested, the thymol cleared quickly from the dialysis tube. Once the thymol excess disappears from the dialysis tube, the active agent is released quickly between the first and the fourth hour, in the case of the 15 °C experiment, and between the first and third hour when the temperature is lowered to 5 °C. Then, the thymol in the inner layers of the nanoparticles is also released, but at a lower speed. In any case, and bearing in mind that the antimicrobial and antioxidant properties of the thymol are concentration-dependent, this initial burst effect is desirable, since it allows an initial increase in the amount of free thymol in the medium.

There are several empirical models that can easily be applied to release profiles similar to those shown in Fig. 4.21 and that have been widely reported in bibliography. In this case, the Korsmeyer-Peppas model was used, and the results obtained are shown in Table 4.17. To apply this model, only the data from the release curve up to 60% thymol

release were considered (Siepmann and Peppas, 2012). Furthermore, neither the first hour in the experiments at 35 and 22 °C, nor the first 2 h in those at 15 and 5 °C were considered. This omission was due to the distortion produced in the model by the burst effect at the two highest temperatures tested, and the delay detected in the thymol release at the two lowest temperatures. In this model, the value of the exponent "n" could be used to interpret, to some extent, the process of the mobilization of the thymol from the interior of the nanoparticle to the aqueous medium. Thus, if "n" is equal to 0.43, the diffusion of the active agent follows Fick's law. On the other hand, if the "n" value varies between 0.43 and 0.85, then the transport mechanism is catalogued as anomalous, being a mixture of Fickian diffusion and the movement of thymol produced by swelling of the nanoparticle. If alternatively, the value of "n" is equal to 0.85, then the transport is caused mainly by the nanoparticle swelling (Ritger and Peppas, 1987). Bearing this in mind and referring to Table 4.17, in all the cases in this experiment the mechanism of transport is anomalous, except at 5 °C, where due to the decrease in the temperature, the diffusion mechanism was hindered and the transport produced by the nanoparticle swelling becoming more important.

Table 4.17: Parameters of interest obtained from Fig. 4.21.

	First order		Korsmeyer-Peppas	
	R^2	k	R^2	n
35 °C	0.94	0.40	0.99	0.77
22 °C	0.98	0.28	0.99	0.67
15 °C	0.97	0.17	0.99	0.60
5 °C	0.97	0.14	0.98	0.85

In Table 4.17 are also shown the first order kinetic equation parameters obtained from the experimental data. These results showed that in all the cases the thymol release can be adjusted to this type of kinetic with a high coefficient of determination (R2), which suggests that this release is dependent on the thymol concentration.

Furthermore, and as was expected, the “k” value decreased when the temperature also decreased, showing that the release of this active agent at low temperatures is slower in comparison with the same release at high temperatures.

3.6. Antimicrobial properties of the nanoparticles

As previously shown in the TGA analysis (section 3.4), the lyophilization of the nanoparticles produced a strong decrease in the amount of thymol encapsulated, what hinders their use as a dry powder. It should be considered that during the lyophilization process pressures of 0.1 mBar were applied to the nanoparticles solutions for 24 h. These pressure conditions could be considered as extremely low, enhancing thymol evaporation. Furthermore, and as was shown in the storage stability experiments, in an aqueous solution the nanoparticles were able to maintain approximately 97% of the thymol encapsulated after 40 days of storage. Therefore, it seems that the use indicated for these nanoparticles is to be applied in the form of a suspension on the surface of food. For these experiments, apple slices were used since apple is a cheap fruit that can be purchased throughout the year.

In Fig. 4.22 is shown the effect of the aqueous solution with free thymol and the thymol encapsulated in nanoparticles on *E. coli* growth. In the apple samples treated with free and encapsulated thymol at 0.94 mg/mL, it was not to possible detect *E. coli* growth during the 14 days of treatment, so the thymol at this concentration was able to kill all the bacterial population inoculated. On the other hand, the solutions with a thymol concentration of 0.1 mg/mL showed a behaviour similar to that found in the control sample, it being impossible to detect differences between the encapsulated and non-encapsulated thymol. Therefore, the lowest thymol concentration tested did not have any effect on the growth of *E. coli*. However, in the samples treated with 0.5 mg/mL of thymol it was possible to detect a difference between the nanoparticles and the free thymol. While the free thymol was seen to be more effective than the nanoparticles in the initial days of the experiment, the encapsulated thymol produced a continuous decrease in *E. coli* growth during the 14 days of the experiment, proving

to be more effective than the free thymol in the long-term. This could be because in the experimental conditions, the nanoparticles provide an environment that protects the thymol from evaporation, maintaining its effect for longer. Although in agreement with the in vitro thymol release profile experiments performed previously, almost all the thymol was released in a non-saturated and stirred aqueous medium in less than 24 h at 5 °C (15 L of distilled water for 10 mL of nanoparticles solution) and this behaviour cannot be extrapolated to the experiment with apples. In this case, the apple slices were completely covered by the nanoparticles loaded with thymol, and the thymol can migrate from the nanoparticles to the apple matrix, the external layers of apple being quickly saturated with thymol, part of the active agent remaining inside the nanoparticles. Probably, in the case of the experiments performed using only free thymol, part of this excess of active agent will be evaporated and part will be slowly and progressively disseminated into the apple matrix as the thymol concentration decreases in the external layers of the apple. In the case of the PLA nanoparticles loaded with thymol, the PLA matrix could act as a protector to hinder the evaporation phenomenon, maintaining the active agent effect for longer.

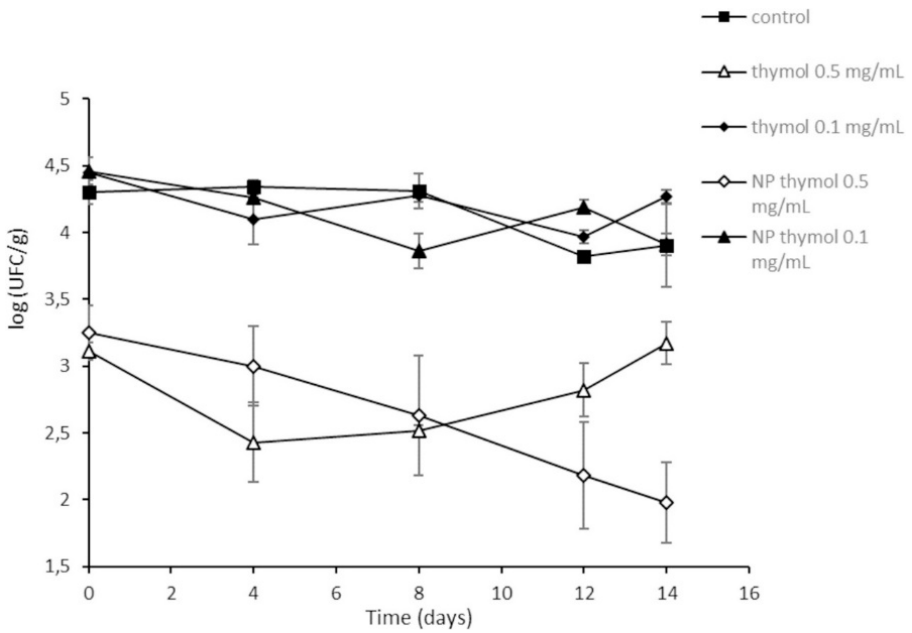

Figure 4.23: Growth of *E. coli CECT 101* on apples treated with solutions containing free thymol and nanoparticles loaded with thymol.

Furthermore, The superficial application of the nanoparticles loaded with thymol on the surface of a solid food could be a more realistic use for them than the typical applications found in the bibliography, which are mainly focused on the antibacterial properties of nanoparticles in liquid foods (Chen et al., 2014, Chen et al., 2015, Pan et al., 2014, Shah et al., 2012). Although from a scientific point of view, the use of liquid food to study the antibacterial properties of thymol-loaded nanoparticles is very interesting, in a practical way it requires a homogeneous dispersion of the thymol throughout the volume of the liquid, and in this case the flavour of the thymol could affect the organoleptic properties of the product, even though this active agent is encapsulated. However, the use of these nanoparticles to protect the surface of solid food requires only its immersion in a nanoparticle suspension, which limits the contact of the product with the active agent while the nanoparticles maintain their capacity to protect the thymol and release it over time.

4. Conclusions

In the tests performed using different PLA and thymol concentrations, the amount of PLA was found to be a key parameter to optimize the preparation of the thymol-loaded nanoparticles. These nanoparticles exhibited a spherical morphology, with a smooth surface and high storage stability over a wide range of pHs. However, as can be seen from the TGA curves of the lyophilized PLA nanoparticles shown previously, a large proportion of the thymol evaporated during the lyophilization process, although this inconvenience could be compensated by the great stability of the nanoparticles in an aqueous suspension. Finally, using apple pieces inoculated with *E. coli* as a real food model, the PLA nanoparticles showed the capacity to maintain the thymol activity for 14 days, probably limiting its evaporation from the wet surface of the apple and favouring its release over time. The use of thymol-loaded nanoparticles to protect the surface of solid foods could be a more realistic use for them than their incorporation in liquid foods, since the first use limits the amount of thymol necessary to produce its antibacterial effect, minimising the strong flavour of the thymol.

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4.3 A review on the expansion of protein materials for food packaging--insect sources

Chapter 4.1/4.2 mainly involves the research and development of food packaging films made of residual protein matrix. And in order to expand more biological materials, such as protein resources. In this chapter, the research status of insect-derived edible film materials in recent years is summarized.

4.3.1 Edible film packaging materials for protein sources in another direction

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Insect-Derived Materials For Food Packaging-A Review

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Abstract

Due to the increasing global population and the serious environmental pollution, human beings face increased demand for high-quality nutritious food and are showing

increased willingness to adopt green materials. Edible insects are considered to be a core source of nutrition for the future. Insect ingredients as edible food packaging will be a help to solve human nutritional needs and augment green environmental protection. Insect-derived films are basically at the level of small-scale research in the laboratory, and the order Orthoptera are the current research focus. Chitin extracted from insects to make packaging films has natural antibacterial advantages. In terms of mechanical properties, the chitin films can have a tensile stress of up to 89.6MPa. In terms of light transmittance, insect protein films have a greater shading rate, 6.168%. Generally, insect-derived food packaging films still need more exploration and research to enrich the basis of practical operation and theoretical research.

Key words: Insects, Edible, Food Packaging Film, Protein, Chitosan

1. INTRODUCTION

It is undeniable that traditional plastic packaging is functionally successful; however, the production of petroleum-based plastics and the incineration degradation process both increase carbon emissions due to a lack of collection or proper disposal (Ncube et al. 2021). Plastic is usually buried in landfills and geological processes can lead to it eventually polluting the oceans. At least 14 million tons of plastic finds its way into the ocean every year. Plastic debris is currently the most abundant type of litter in the ocean, making up 80% of all marine debris found from surface waters to deep-sea sediments (IUCN 2021), and one of the main sources of this class of pollution has been identified as food and beverage packaging (Ncube et al. 2021). The manufacture of traditional plastic packaging is mainly based on the use of fossil fuels as the raw material, and in 2018, 4-8% of global oil production was used for plastic production (Organisation for Economic Co-operation and Development 2018). 40% of this is used to make single-use plastics, whose use is primarily driven by increasing food and beverage consumption. Most food packaging plastics create a waste stream shortly after purchase, especially in single-use packaging applications for short-lived goods

(Sundqvist-Andberg and Åkerman 2021). In addition, due to the impact of the COVID-19 epidemic, the growth of the global takeaway industry has increased the consumption of foam takeaway containers such as food wrapping paper and food bags (Oliveira et al. 2021). The accumulation of microplastics (MPs) and their contamination of food has become a global threat to the environment and human health. It is estimated that human consumption of microplastics through takeaway food is about 2977 microplastics per person per year (Jadhav et al. 2021). Food packaging manufacturers and the food industry have been working to replace traditional, non-renewable petroleum sources with abundant, low-cost, renewable and biodegradable alternatives (Chaudhary et al. 2022). Growing public awareness of the environmental challenges associated with traditional plastic materials, and consumer pressure to improve sustainability, has led to the development of bio-based, biodegradable, edible food packaging materials (Sundqvist-Andberg and Åkerman 2021). Although some researchers currently predict that biodegradable or edible films will not completely replace traditional packaging materials, they can prolong the stability of food and improve the efficiency of food packaging by reducing the exchange of moisture, lipids, volatiles and gases between the food and the surrounding environment (Zeljko 2017), which reduces the need for petroleum-derived polymers.

These bioplastics are commonly considered for use as primary packaging materials, some of which may be consumed together with the food they contain. In addition to increasing the shelf life of food by physically preventing contact between the food and environmental microorganisms and contaminants, these films can play an active role, since many are capable of carrying antimicrobial agents, nutrients, anti-browning compounds, and colouring agents, which can prevent pathogenic microorganisms from growing on food surfaces (Das et al. 2022). Although some of these bioplastics can be prepared using non-edible polyesters, such as polylactic acid or polyhydroxyalkanoates, others can be prepared using edible biopolymers, such as carbohydrates, lipids and proteins (Han 2013), and these compounds can be extracted from many different natural sources. One of these possible sources of edible

biopolymers, insects, have attracted the interest of the research community in recent years, since insects can be eaten directly, which is very convenient considering the growing world population. Moreover, their biopolymers can be extracted and used to prepare bioplastics that may also be edible.

1.1. Insects as food

At present, the most common system of protein production for human consumption is the intensive raising of livestock, although protein production from animal husbandry generates important ecological concerns because of its environmental impact, as it requires large quantities of water, energy and land (Godfray et al. 2018). As human food production becomes a major driver of global environmental change, there is growing recognition of the importance of shifting to more sustainable dietary patterns (Cottrell et al. 2021). At the same time, combined with the current population pressure and socio-economic growth trends, it is necessary to introduce alternatives to traditional animal protein (Fasolin et al. 2019).

In this sense, insects have received attention as an alternative protein source due to their favourable environmental impact (Churchward-Venne et al. 2017). Insects are considered one of the most environmentally friendly sources of animal protein because of their very low carbon footprint and high protein content (van Huis and Oonincx 2017; Ros-Baró et al. 2022). Edible insects are still a novelty in Western culture, and they also represent a challenging concept for many people around the world. Regulations allowing insects to be sold commercially for use and consumption in the EU only came into effect in 2018 (Halloran et al. 2018), but in some African, Asian and South America countries edible insects already have a high market value, sometimes similar to that of traditional livestock, or even higher (Abril et al. 2022; Raheem et al. 2019). Therefore, insects are a commodity with an existing market. The global edible insect market is expected to grow to USD 9.6 billion by 2030 (Wood 2022). In addition to the consumption of insects as potential providers of protein nutrients, natural active products derived from insects for antimicrobial, antifungal, antiviral, anticancer, antioxidant, anti-inflammatory and immunomodulatory effects (e.g., chitin,

antimicrobial peptides or specific fatty acids) also have excellent prospects (Wang, Qian, and Ding 2018; Kaya et al. 2019).

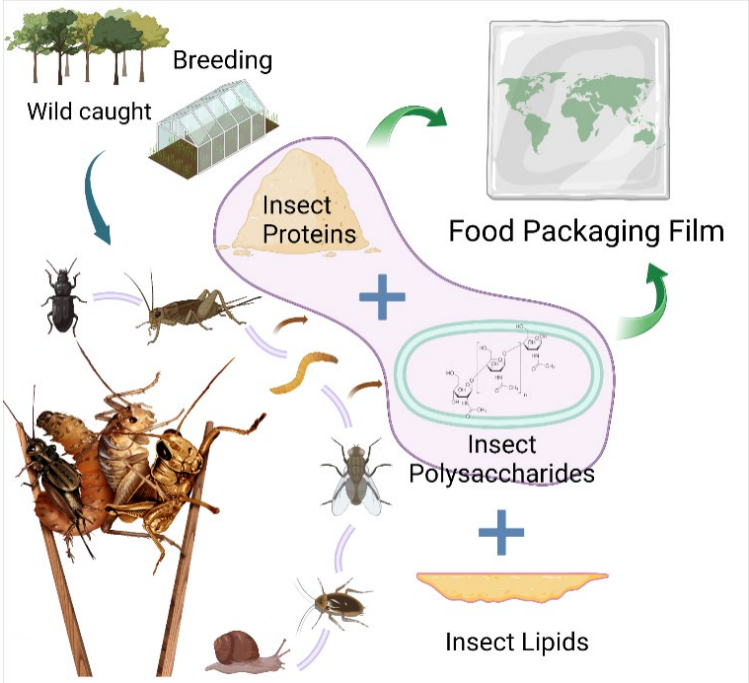

Figure.4.24: Scheme of the preparation of edible insects packaging films

1.2. Insects as a source of biopolymers for preparing edible films and coatings

As insects are a source of valuable biopolymers, such as proteins and carbohydrates, they can be processed to produce not only foodstuffs but also bioplastics (Barbi et al. 2019). The use of insects or their derivatives as raw materials to allow the substitution of petroleum packaging materials, whilst simultaneously taking advantage of their protein content as a nutrient source, is a biotechnological solution presented a few years ago. Several insect species feed naturally on organic waste, and by digesting organic matter within their bodies, they make it possible to reduce the amount of waste while producing more homogeneous and valuable biomass for a variety of purposes (e.g., for feed/food ingredients, cosmetics, pharmaceuticals, bioplastics, etc.) (Franco et al. 2022). Caligiani et al. (2018) were the first to propose the potential feasibility of producing bioplastics from insect proteins. In particular, the protein obtained by alkaline extraction from *Hermetia illucens* has been proposed for the production of bioplastics because the protein is of low quality and is not suitable for nutritional requirements (Leni, Caligiani, and Sforza 2021). These insect-derived films

are a promising source for some types of biocompostable plastics, with the added value of using proteins produced by insects digesting waste, and thus contributing to the prospect of a circular economy. Sanandiya et al. (2018) extracted chitin from *Hermetia illucens*, deacetylated it and mixed cellulose to produce packaging materials. The introduction of insect-based materials for the production of bioplastics not only represents a way to add value to food waste and by-products that are used as growth substrates for insects, but is also an effective alternative to unsustainable plastic packaging. Figure 4.24 shows an abstracted process of forming a simple edible insect-derived food packaging film. The first step is to obtain insects by natural capture or artificial breeding. Then, the edible insects with high nutritional value undergo an extraction process by means of physical separation and chemical extraction to obtain biological organic macromolecular materials such as proteins, polysaccharides, and lipids. Among these, the first two are the most widely used to prepare environmentally friendly food packaging materials.

Therefore, considering that the use of biopolymers extracted from insects to create edible films and coatings for food packaging is a topic of increasing interest, this review aims to summarize recent scientific research on this subject, describing the current state of development and future trends in this field.

2, INSECTS AS A SOURCE OF BIOPOLYMERS TO PREPARE BIOPLASTICS

The main biopolymers found in insects that can be used to prepare edible films and coatings are proteins, carbohydrates and lipids. Table 1 shows the basic nutritional composition of some edible insects that can be consumed by humans or livestock as food or feed, including crude protein, fat, carbohydrates and other nutrients found in relevant studies in recent years.

Protein is the main macromolecule found in insects and the reason why these insects have attracted the attention of the international community of food researchers. Protein content in insects has been reported to be as high as 21-76% of dry biomass in different insect types (Kouřimská and Adámková 2016). Because insects have considerable differences in the expression of their internal structures between species,

generally related to their behaviour and ecology, the sources and structures of insect proteins vary widely. There are also differences between species in terms of the distribution of proteins of different molecular weights, and of many other components such as fats and carbohydrates (Mishyna, Keppler, and Chen 2021). Furthermore, production techniques and feed composition in larval rearing also affect protein quality (Tschirner and Simon 2015). In terms of amino acid content, Coleoptera, Hymenoptera, Lepidoptera and Orthoptera averaged or exceeded the IAA (indispensable amino acid) requirement for human adults (Churchward-Venne et al. 2017). The first limiting amino acid, methionine, is present in relatively low quantities in insects in general. In addition, in the study of Shikha Ojha et al. (2021), the BV value is the biological value of the protein, which is used to indicate the degree to which the protein is utilized by the organism. Insects such as crickets (*G. assimilis*) have higher BV values (85.49–93.02%) than casein (73.45%). In a nutritional study of the epidermis and meat of *Clanis bilineata tsingtauica* larvae by Ying Su et al. (2021), four proteins were identified, including albumin, globulin, glutenin, and prolamin, which had different concentrations in larval flesh and epidermis. In Table 4.18, the proportion of protein to total mass of Lepidoptera and Orthoptera is seen to be higher than that of Hemiptera and Blattella and, furthermore, in the column of Coleoptera, we find that the protein ratio of aquatic insects is nearly half that of terrestrial species.

After proteins, the second bulk component of insects is lipids. The lipid content of insects varies with individual species, but also with their growth stage, environmental conditions and dietary specifications (Lorrette and Sanchez 2022). Lipids can typically represent 10% to 25% of insect dry matter, similar to the data presented in Table 1. Some species such as Odontotermes and *Ruspolia differens* shown in Table 1 can even approach 50%. Therefore, lipids have also become one of the main by-products of the insect industry. The composition of insect fat may vary in the quantity and composition of the fatty acid distribution (Lorrette and Sanchez 2022). Insect species, growth stage and extraction technique are some of the parameters that affect fat quality. Insect lipids are mainly composed of triacylglycerols. Other types of lipids present in small

amounts include cholesterol, partial glycerides, free fatty acids (FFAs), phospholipids and wax esters. (D.A. Tzompa-Sosa, et al., 2019) As insect lipids for human consumption, this raw material may be an alternative to the resource-intensive and more expensive soybean oil, palm kernel oil, coconut oil, and fish oil (Franco et al. 2021). Although some studies suggest that insect lipids function well in food, crude fats and oils have an aftertaste that requires refining to improve their organoleptic properties (Lorrette and Sanchez 2022).

In addition, in the data collected in Table 4.13, the proportion of fat in cockroaches is significantly better than that of insects of other orders, while the proportion of fat in Odontotermes even accounts for half of the dry weight.

Regarding insect carbohydrates, the insect exoskeleton contains a lot of fibre. For example, in *Oecophylla smaragdina* (Kim et al. 2019), the research data showed that about 19.84% of the dry matter is fibre, which contains functional dietary fibre and this has effects related to regulating host intestinal health and improving glucose and lipid metabolism disorders (Cronin et al. 2021). Measured as crude fibre, acid detergent fibre, or neutral detergent fibre, the composition of these fibre fractions is unknown (Finke 2007), but includes hardened proteins, minerals and other compounds that bind to chitin. Chitin, an N-acetyl-β-D-glucosamine polymer, provides rigidity to the insect's exoskeleton (Doucet and Retnakaran 2012). It is present in the epidermis of the insect exoskeleton, the two innermost layers of the epidermis, and its content depends on the insect species and developmental stage (Oonincx and Finke 2021). The chitin content of some insects listed in Table 4.13 ranges from the 4.72% of *Tenebrio molitor* to 7.34% of Crickets to the highest content, 14.1%, of black soldier fly larvae. Another polysaccharide substance is called chitosan, with the chemical name polyglucosamine (1-4)-2-amino-B-D glucose, which can be obtained by deacetylation of chitin. The extracted chitosan has antibacterial, antioxidant, anti-cancer, anti-inflammatory properties and wastewater treatment capacity (Cronin et al. 2021), and is a valuable research direction. It is a new research trend to develop high value-added products such as chitosan by means of comprehensive utilization of insect resources.

Table 4.18: Major nutrient composition of Insects (g/100g of dried sample)

INSECT		PROTEIN	FATS	CARBOHYDRATE	REFERENCE
Orthoptera	Grasshopper (*Arphia fallax* S., *Sphenarium histrio* G., *Sphenarium purpurascens* Ch. *Ruspolia differens*. etc.)	43.9±1.5 to 77.1±2.8	4.22±0.5 to 34.2±1.9	0.001±0.100 to 22.64±2.90 Fibre:3.00±0.90 to 12.17±2.80	(Siulapwa et al., 2012; Blásquez, (Moreno and Camacho, 2012; Paul et al., 2016; Montowska et al., 2019; Rodríguez-Miranda et al., 2019)
	Cricket (*Acheta domesticus*, *Teleogryllus emma*, *Gryllus bimaculatus*, *Gryllodes sigillatus*.etc.)	55.65±0.28 to 72.45±1.30	11.88±0.21 to 25.14±0.11	0.1±0.0 to 6.64±0.15 Chitin:7.34±0.73	(Blásquez, Moreno and Camacho, 2012; Brogan et al., 2021; Psarianos et al., 2022)
	Locust (*Locusta migratoria*, *Schistocerca gregaria* etc.)	53.80±0.50 to 76.0 ± 0.9	11.42±1.11 to 35.66±2.15	0.018±0.004 to 2.08±0.31	(Reem Mohamed Khalil and Prof Dr Ahmed El-awad El-faki, 2013; Ochiai, Inada and Horiguchi, 2020; Brogan et al., 2021)
Diptera	Black Soldier Fly （*Hermetia illucens*）	29.9±0.75 to 45.7±0.07	9.50±0.36 to 49.0±0.22	Chitin: 2.9 to 14.1	(Caligiani et al., 2018; Huang et al., 2019; Abd El-Hack et al., 2020; Bessa et al., 2020)
Coleoptera	Scarabs (*Allomyrina dichotoma* larvae, *Protaetia brevitarsis* larvae, *Zophobas morio* larvae)	44.23±0.25 to 54.18±1.50	15.36±0.40 to 20.24±0.25	Chitin:4.60±0.05 to 10.53±0.75	(Ghosh et al., 2017; Kim et al., 2019; Shin, Kim and Shin, 2019; Hahn et al., 2020; Kulma et al., 2020; Meyer-Rochow et al., 2021; Oonincx and Finke, 2021)
	Tenebrio molitor larvae & adult	52.35±1.1 to 53.22±0.32	24.7±1.5 to 34.54±0.87	2.2±0.3 to 11.45±0.38 Chitin:4.72±0.21	(Zielińska et al., 2015; Shin, Kim and Shin, 2019; Son et al., 2021)
	Water beetle (*Hydrophilus olilivaceous*, *Cybister tripunctatus*)	22.64±0.17 to 25.08±0.09	6.94±0.70 to 21.57±1.61	1.67±0.33 to 2.39±0.38 Fibre:14.25±0.46 to 15.13±0.57	(Shantibala, Lokeshwari and Debaraj, 2014)
Hemiptera	*Lethocerus indicus*	22.67±0.36	13.75±0.09	15.40±0.20	(Shantibala, Lokeshwari and Debaraj, 2014; Meyer-

				Fibre: 11.71±0.25	Rochow et al., 2021)
Lepidoptera	Caterpillars (*Gonimbrasia belina*, *Gynanisa maja* and *Clanis bilineata tsingtauica*)	55.92±0.04 to 71.85±3.18	10.0±0.2 to 12.1±0.2	8.4±0.4 to 10.7±0.3	(Siulapwa et al., 2012; Churchward-Venne et al., 2017; Hahn et al., 2020; Gao et al., 2021; Meyer-Rochow et al., 2021; Oonincx and Finke, 2021; Su et al., 2021)
	Silkworm (*Bombyx mori, pupae of muga and eri silkworm)*	53.07±0.10 to 72.48	16.66 to 33.30±16.00	1.24 to 1.62 Fibre: 3.06 to 3.25	(Churchward-Venne et al., 2017; Brogan et al., 2021; Sharma, 2022)
Blattaria	Odontotermes	33.672±0.329	50.930±1.097	Fibre: 6.298±0.088	(Chakravorty et al., 2016)
	Macrotermes falciger	43.26±0.03	43.0±0.2	32.8±0.6	(Siulapwa et al., 2012)
Hymenoptera	*Oecophylla smaragdina*	55.279±1.024	14.993±0.136	Fibre: 19.840±0.259	(Chakravorty et al., 2016)
	Laccotrephes maculatus	41.56±0.52	5.16±0.57	0.06±0.01 Fibre: 7.31±0.28	(Shantibala, Lokeshwari and Debaraj, 2014)
Odonata	*Crocothemis servilia*	70.48±0.43	4.93±0.17	1.18±0.09 Fibre: 9.62±0.24	(Shantibala, Lokeshwari and Debaraj, 2014)
INSECT		**PROTEIN**	**FATS**	**CARBOHYDRATE**	**REFERENCE**

3, CHARACTERIZATION OF INSECT-DERIVED PACKAGING FILMS

It can be seen from Table 4.19 that most of the studies are in the early stages of research, using laboratory-scale fabrication and solvent casting methods, with only one case involving hot-press and cold-press fabrication. The main reason is most likely that insect-derived materials are currently a new direction in edible packaging. Researchers mainly focus on research into the properties of the materials themselves and have not yet become involved in the development of industrial production and large-scale manufacturing processes. Therefore, solution casting is the most popular low-cost film-forming method in the laboratory. In recent years, researchers have been investigating the extraction of protein and chitin from insects to make edible films. The chitin or chitosan materials derived from insects have been reported to have advantages over those from marine crustaceans (Triunfo et al. 2022). For example, chitosan extracted from cicada slough, silkworm pupae, mealworms and grasshopper species showed higher potential water-holding capacity (594-795%) and fat-binding capacity (275-645%) compared to shrimp shell chitosan (Mohan et al. 2020). This is a promising property for food applications. Scientists today generally regard environmental protection as a major consideration and one of the main purposes of making biopolymer materials is to reduce the carbon footprint (Chandran et al. 2021). For example, *Hermetia illucens* is used as a farmed insect which can use organic waste (food waste) to complete low-carbon consumption. This concept unites the issues of organic waste management and insect rearing as well as alternative sources of protein and chitin.

Table 4.19: Extraction and preparation of packaging films from insects

SPECIES OF INSECTS	MATERIAL EXTRACTED	EXTRACTION METHOD	PREPARATION METHOD	FILM FORMATION & PH SETTING	REFERENCE
Grasshoppers	Protein (gelatin)	Freeze-dried, degreased by hexane, alkaline dispersion and acid precipitation	5%, 10%, 15%, 20% (w/v) gelatin powder was stirred at 50°C for 0.5H, + 10% glycerol	Casting 45℃	(Qoirinisa et al., 2022)
	Protein	/	+ Glycerol 45% (w/weight of protein powders) + Xylose (5, 10, and 15% (w/w)	Casting 40℃ pH 10	(Zhang, Zhou, et al., 2022)
Migratory Locust	Protein		~ 6% (w/v) GP/SPI blends (Proportion 8/2, 7/3, 6/4) were dissolved in deionized water + Glycerol 45% (w/w) of protein powders + Xylose 5, 10, 15% (w/w) of protein powders.	Casting 50℃ pH 9/10/11	(Zhang, Fang, et al., 2022)
Cricket *(Acheta domesticus*, *Gryllodes sigillatus* and *Gryllus bimaculatus)*	Chitin and Chitosan	At 90°C remove endogenous enzymes, at 55°C protein was hydrolysed, centrifuge at 4°C to obtain chitin, add 67% w/v NaOH to vary the degree of deacetylation	Different degrees of deacetylation: 72%, 76% and 80%. Chitosan solution (1% w/v) by dissolving cricket chitosan in 1% acetic acid (v/v) solution +glycerol 37.5% (w/w chitosan) as plasticizer	Casting 50℃	(Malm, 2021)
	Chitosan	Sodium hydroxide to remove protein, sodium hypochlorite to decolorize, oxalic acid to demineralize	8 – 12 ml of film-forming solution containing 1:2 glycerol: chitosan (w/w) per petri dish, 8.5 cm diameter	Casting Room temperature	(Jarolimkova, 2015)
	Protein	Full-fat or low-fat conditioning of insect powder with ethanol	SPI and full/low CF were formulated in mass ratios of 100:0 (standard), 85:15, 70:30, and 55:45 on dry matter basis.	HMEC Fed rate at 0.4 kg/hr and screw speed at 150 rpm	(Kiiru et al., 2020)
Maggot (Fly Larvae and Mealworm)	Chitin	Purification by Soxhlet extraction with hexane, positive pressure filtration with PVDF membrane. Sodium chloride	Films (30 g/m2) were produced using a positive pressure filtration unit with a 0.45µm PVDF membrane.	Hot-pressed at 100 °C + 70 bar for 30 mins or	(Pasquier et al., 2021)

		removes protein, sodium hypochlorite decolorizes		cold-pressed under a load of 0.012 bar and dried at room temperature. pH 3 or 7	
Black Soldier Fly (*Hermetia Illucens*)	Protein	Freeze-dried and milled, defatted with n-hexane.	Insect powder solution 4% (w/w) and chitosan solution 1% (w/w) were prepared in the ratio of (0:100, 70:30, 50:50 and 30:70) to film-forming solution, + a constant amount of Glycerin (0.5ml)	Casting 25℃	(Chandran et al., 2021)
Large pine weevil (*Hylobius abietis L.*)	Chitosan	Oven dried and milled, 0.5% NaOCl bleached, 2M HCL demineralized, 2M NaOH demineralized, deacetylated in hot NaOH	10 mg chitosan and 50 µl glycerol were mixed in 1% acetic acid solution (10 ml) for 2 days, the experimental group was supplemented with 0.1 mg β-carotene	Casting 35℃	(Kaya et al., 2019)
Tenebrio molitor	Chitosan	Freeze-dried and milled, demineralization and deproteinization, and deacetylation to obtain chitin.	Chitosan (1%, w/w) dissolved in acetic acid solution (1%; v/v), +30% glycerol (w/w; based on chitosan content)	Casting Room temperature	(Saenz-Mendoza et al., 2020)
Periplaneta americana	Chitosan	Degreased with n-hexane, deproteinized in a hot alkaline bath, and desalted, bleached and deacetylated	Chitosan (1 g) was dissolved in 50 mL of 1% acetic acid solution at room temperature, +1 g glycerol	Casting 65 ◦C	(Chen et al., 2021a)
Snails	Chitosan	Ozone disinfection and citric acid stimulating solution, stored at 4°C	1 g of chitosan was added to 70 mL of A (1% v/v) or L (1% v/v) followed by 30 mL of snail solution to give 100 mL of chitosan-S mixture in a ratio of 70:30.	Casting Room temperature	(Di Filippo et al., 2020)
SPECIES OF INSECTS	**MATERIAL EXTRACTED**	**EXTRACTION METHOD**	**PREPARATION METHOD**	**FILM FORMATION & PH SETTING**	**REFERENCE**

The studies in Table 4.19 suggest that insects of the order Orthoptera are popular experimental subjects, probably because Orthoptera insects, such as grasshoppers, crickets, and locusts, are widely distributed around the world, most of them being considered as herbivorous agricultural pests. These insects are eaten in many parts of the world. They have a short life cycle (4-8 weeks for adulthood), a fast reproduction rate, a protein content of up to 76%, and the shells of Orthoptera are also a good source of chitin (Blásquez *et al*, 2012; Paul *et al.*, 2016). On the other hand, in order to develop treatment methods for pests, the harm done by the American cockroach (Chen et al. 2021a) to the public environment and the destruction of forests by weevil (Kaya et al. 2019) are the main reasons why scientists use them as research subjects. Table 4.19 also shows that the current research into insect resources for food packaging is more directly using insects to produce source materials, and there is less research conducted on insect metabolites as source materials. In the preparation process, in addition to the addition of active substances with functional purposes, the film-forming liquid is additivated with plasticizers such as glycerine to help the film attain better structural characteristics. Unfortunately, researchers are more inclined to study the proportion of insect extracts contained in the film-forming fluid, and there is not much research into the impact of additives such as plasticizers. After the film form is made, the physical performance properties of the film, such as the optical properties, solubility, mechanical strength, and other properties of the film, are measured to provide the numerical data necessary for an objective evaluation of the degree of excellence of the film, and to judge whether it is suitable for food packaging.

3.1 Optical and Colour Properties

The optical properties (colour and clarity) of food packaging play a crucial role in the appearance and acceptance of packaging by consumers (Khalid and Arif 2022). Sensory evaluation of biopolymer-based films is critical to success in the market and it is well known that the addition of bioactive compounds to foods can significantly alter sensory acceptance (Trajkovska Petkoska et al. 2021). The colorimeter test is the most

common test method in the laboratory and the test gives the results "L*" for brightness, "a*" and "b*" for: "red/green" and "blue/ yellow" respectively. Table 4.15 below summarizes the results of the CIELAB colour space test and the light transmittance of the food packaging film made from insect-derived materials.

Table 4.20: Optical L*/a*/b* values and light transmittance of edible insect films.

INSECT & MATERIAL	COMPOSITION	THICKNESS (mm)	L*	a*	b*	TRANSPARENCY (%)	REFERENCE
Large pine weevil (*Hylobius abietis* L.)/Chitosan	10 mg chitosan and 50 µl glycerol in 1% acetic acid (10 ml)	0.044±0.001	/	/	/	70.1 at 600 nm	(Kaya et al., 2019)
Cricket/Chitosan	1:2 glycerol: chitosan (w/w) (8~12ml)	0.061±0.006	89.22±0.67	-0.25±0.04	12.07±0.87	/	(Jarolimkova, 2015)
	cricket chitosan in 1% acetic acid (v/v) +37.5% (w/w) glycerol	/	/	/	/	~70 at 600 nm	(Malm, 2021)
Black Soldier Fly (*Hermetia illucens*)/Protein	4% insect protein & 1% Chitosan (ratio 70:30) + 0.5ml glycerol	0.12±0.02	32.46±0.11	5.04±0.04	11.39±0.04	6.168 at 600 nm	(Chandran et al., 2021)
Maggot (Fly Larvae and Mealworm)/Chitin	cold-pressed (0.012 bar) Fly Larvae chitin	/	/	/	/	21 ± 2 (550nm)	(Pasquier et al., 2021)
Cockroach (*Periplaneta americana*)/Chitosan	Chitosan (0.5 g) +25 mL of 1% acetic acid + 0.5 g glycerol	0.080±0.004	/	/	/	65.39 at 600 nm	(Chen et al., 2021a)
Migratory Locust/Protein	7% Locust protein + 40% glycerol + pH 10	0.376±0.042	11.542 ±3.125	0.059±0.087	0.535±0.292	/	(Zhang, Fang, et al., 2022)

In a study using locust protein powder as film material, researchers (Zhang, Fang, et al. 2022) found that the pigmentation increased with increasing protein content, giving the film a dark brown colour, and that the increase in glycerol content resulted in an increase in the a* and b* values. Interactions between plasticizer molecules and water molecules can alter the refractive index of the LP (locust protein) component and thus the transparency of the resulting film. Similarly, in research on insect protein mixed with chitin, as the mixing ratio of Black Soldier Fly protein powder was reduced, the

degree of browning of the film lessened (Chandran et al. 2021). Two research articles using chitin extracted from crickets as food packaging films showed that these films were relatively dark. Researchers (Malm et al. 2021) found that the brightness values of cricket chitin films were lower than those of conventional commercial shrimp chitin films at the same degree of deacetylation, as shown by the L* values in Table 3. This is attributed to the absence of dark (brown/yellow) pigments in shrimp chitin/chitosan, which is different from that of crickets in Orthoptera, a result of the different mechanisms that crustaceans use during the production of their exoskeletons. In addition, some researchers have observed that the optical properties of the film are not only related to the source of the material, but also to the molecular weight and the degree of deacetylation of the chitosan (Jarolimkova 2015).

In respect to the light transmittance of insect-derived films, the chitin film from *Hylobius abietis* has a transmittance of 70.1% at 600 nm (Kaya et al. 2019), while chitin films from *Periplaneta americana* have a transmittance of 80.316% at 600 nm (converted from the provided opacity and thickness values) (Chen et al. 2021b). Chitosan films derived from crickets also have a light transmittance of about 70%, even when different cricket species are used (Jarolimkova 2015; Malm et al. 2021). Another study on *Hermetia illucens* (Chandran et al. 2021) tested a mixture of protein and chitosan films, giving opacity values from a 600 nm UV spectroscopy test. The opacity of the 100% chitosan film as a control was 4.853±0.40(λ/mm), while the mix with 70% protein showed 10.082±0.22(λ/mm). The films with half protein and half chitosan exhibited an opacity of 6.087±0.48(λ/mm) and the opacity of the films with 70% chitosan was 5.558±0.44(λ/mm). Also, based on the film thickness and opacity equations during the test, the absorbance of each mix at 600 nm was calculated as 0.33971, 1.20984, 0.54783 and 0.50022. The transmittance (%) was 45.739%, 6.168%, 28.325% and 31.607% respectively. The opacity of the films increased with increasing protein composition. In addition to films prepared by ordinary casting methods, hot-pressing and cold-pressing methods to fabricate chitin nanofiber films (Fly Larvae and Mealworm) have also been used by some researchers (Pasquier et al. 2021). The

transmittance of the Fly Larvae chitin fibre film when cold-pressed was 21±2%, while that of the hot-pressed film was only 1.8±0.2% (UV wavelength was 550mm). Researchers in the study suggested that the impermeability of the films may be due to the high porosity. When the film is hot pressed, the rate of drying and moisture evaporation is much higher than that of overnight cold drying (Pasquier et al. 2021). Thus, the chitin nanofibers in hot pressing had little time to rearrange and entangle, leaving porous or defective structures in the stacked flakes. In addition, the films made of chitosan extracted from *Periplaneta americana* (Chen et al. 2021c) exhibited excellent UVC (200-300 nm) light resistance, and the light transmittance was significantly lower than that of commercial shrimp chitosan films. That is considered to be because a large amount of the pigment deposited in the stratum corneum is bound to chitin (Chen et al. 2021c).

3.2 Barrier properties

One of the primary functions of food packaging is to prevent or reduce moisture transfer between the food and the surrounding environment (Alamri et al. 2021), although barrier requirements depend on end use. In general, biodegradable polymers have some disadvantages relative to conventional packaging in terms of moisture resistance (Shaikh, Yaqoob, and Aggarwal 2021; Wu, Misra, and Mohanty 2021).

In ideal food packaging, a barrier to maintain a low level of oxygen and a controlled degree of moisture is essential in order to conserve dry, moist or textured products (Trajkovska Petkoska et al. 2021). These traditional packaging attributes also apply to packaging films using insect-derived materials.

Food packaging films are often tested for contact angle, which is one of the methods commonly used to measure the wettability of surfaces or materials (Huhtamäki et al. 2018). Wetting refers to the study of the behaviour of liquids on substrates, whether it is diffusion or the ability of liquids and solids to form boundary surfaces. It was found in one study (Zhang, Zhou, et al. 2022) that the hydrophobicity of the film was increased by the soy protein isolate in the mixture material, and xylose as a cross-linking agent did not restrict access to the hydrophilic groups in the protein mixture.

When 30% cinnamaldehyde was added, the WCA (water contact angle) of the composite film was further increased to 38°, because CIN (cinnamaldehyde) conjugated with free amine groups to replace the hydrophilic groups with hydrophobic aromatic groups, thereby improving the hydrophobicity of the films. Another film, extracted from *Locusta migratoria* and also composed of protein, is hydrophilic (Zhang, Fang, et al. 2022). In general, WCA > 65° can be regarded as a hydrophobic surface, and < 65° is regarded as a hydrophilic surface (Feng et al. 2018). A rise in protein content increased the hydrophobicity of the surface, but when the glycerol content increased from 35% to 45%, the WCA decreased from 45.9° to 38.6°. Some researchers (Zhang, Fang, et al. 2022) believe that the added plasticizer glycerol tends to reduce the surface tension of the film, which is beneficial to the wettability of the film surface; the increased mobility of the polypeptide chain can promote water absorption and transport within the film. In addition, after the pH of the film-forming solution was adjusted from 9 to 11 in this study, the WCA decreased from 49.4° to 42.7°. The effect of pH on protein structure altered the WCA performance of the final films. These researchers (Zhang, Fang, et al. 2022) also mentioned that *L. migratoria* protein contains very low levels of cystine. The density of disulphide bonds in proteins is influenced by cystine. A low concentration of cystine leads to a low density of disulphide bonds in proteins, resulting in low-energy disulphide bonds within and between molecules that are more soluble, thus making the film itself more hydrophilic and soluble.

Unlike the cricket protein blend film, the shellac produced by lac bugs helped the original glycan film to have a higher WCA (Du et al. 2019), and this improvement may be attributed to the intrachain and interchain interactions between KGM (Konjac glucomannan) and SHL (shellac) molecules. The interaction between them forms a rigid structure. However, in Cricket chitosan films, the increased water contact angle may be a result of the possible presence of melanin (hydrophobicity) and possibly other residual components (Malm et al. 2021). Therefore, if melanin crosslinks are present, intramolecular interactions increase and lead to a decrease in the ability of

the film surface to interact with water. The increased complexity of cricket chitosan may be an advantage of chitosan biobased food packaging. In the study mentioned above, using hot-pressing and cold-pressing techniques (Pasquier et al. 2021), the contact angles of the hot-pressed shrimp chitin nanofiber film and the cold-pressed fly nano-chitin film ranged from 29.0±0.3° to 40.0±2.0°, respectively, which were highly hydrophilic. Compared with cold pressing, hot pressing gives a smoother film surface, which tends to lower contact angles. The films made from worms had slightly higher contact angles than the former. Due to the presence of a charge opposite to that of chitin, there are fewer water-interacting groups. Varying the pH between 3 and 7 did not affect the contact angle of the films.

The “water vapour barrier” property is important in a food packaging film, since the ability to block the passage of water vapour directly affects the shelf life of food. To delay food spoilage, the water vapor permeability value should be kept at a reasonably achievable low level (Bourlieu et al. 2009).

Table 4.21 lists the water evaporation rate performance of insect-derived films in the current research field, where molecular weight (Mw), cross-linking agent, film-forming material bulk and pH are all factors that affect water vapour permeability (WVP). Among the film-forming materials seen, the combination of glucomannan and shellac exhibited levels that were significantly different, by 10^{-7} to 10^{-6} orders of magnitude, compared to the other two materials (protein and chitin).

Table 4.21: WVP properties of insect derived food packaging films

Top Performing Group	WVP (g/m·s·Pa)	CHARACTERISTICS OF THE FILMS	REFERENCE
Grasshopper chitosan	1×10^{-10}	The microstructure of the CCF films appears to be rougher and more aggregated, leading to an increase in the length of the tortuous path for the diffusion of water vapour across the film. Ultimately, this will lead to a decrease in the water vapour permeability of the CCF, while the microstructure of the chitosan film is smoother and more compact, and the propagation path length of the water vapour will be shorter.	(Malm et al., 2021)
KSH5 (10ml SHL solution mixed)	~1.134×10^{-4}	One characteristic is the presence of intermolecular hydrogen bonds between KGM and SHL, which may reduce the number of hydrophilic groups. Furthermore, the presence of ester bonds in the blended films contributes to the formation of compact structures. Also, the decreased WVP of the hybrid film with increasing SHL content is due to the presence of uniformly dispersed SHL in the composite structure, which may force water vapour to overcome the tortuous path through the polymer matrix, resulting in an effective path with increased diffusion length.	(Du et al., 2019)
T. molitor	4.77×10^{-11}	As the melanin concentration increased, the WVP value increased. The WVP value of *T. molitor* chitosan film was 13% lower than that of *B. magna* chitosan film. These differences may be due to the Mw, as the Mw of *T. molitor* chitosan is higher than that of *B. magna* chitosan.	(Saenz-Mendoza et al., 2020)
"6-40-10"	~1.3×10^{-11}	Glycerol: Tends to aggregate with itself at high concentrations to increase chain spacing, thereby promoting the diffusivity of water vapour through the membrane and accelerating its transport. Alkaline pH changes protein structure. Growth of protein content increased WVP due to increased film thickness.	(Zhang, Fang, et al., 2022)
PaCSF	$(64.85\pm4.82)\times10^{-11}$	Higher Mv (viscosity) may contribute to better water repellency. And since many water molecules escaped from the chitosan film after drying and storage, many binding sites for water could freely accept water molecules again.	(Chen et al., 2021b)
70%GP+30%SPI	2.0×10^{-9}	The formation of the cross-linked network contributes to the dense structure of the film and reduces the permeability, but after adding xylose and CIN, there is no significant change in WVP compared with the 30% SPI group. This suggests that the influence of the substrate on the WVP is more important than that of the crosslinker.	(Zhang, Zhou, et al., 2022)

The last test related to water is the solubility or water absorption capacity of the packaging material in the water environment. A total of four groups of researchers investigated the water solubility of *Hylobius abietis L* chitosan films, *Tenebrio* and *Brachystola magna* insect chitosan films, Schisandra-containing cricket chitosan, and grasshopper protein films mixed with soybean protein (Saenz-Mendoza et al. 2020; Kaya et al. 2019; Kiiru et al. 2020; Zhang, Zhou, et al. 2022).

The results showed that the water solubility decreased after adding β-carotene to *Hylobius abietis L* chitosan films. The water solubility values of the chitosan control and chitosan-β-carotene films were recorded as 28.4 ± 1.21% and 31.1 ± 1.34%, respectively. For soil solubility, the weight loss values for chitosan control and chitosan-β-carotene films were 30.4% and 23.5%, respectively (Kaya et al. 2019). Chitosan films of *B. magna* (≈41.2%) showed higher solubility (≈35.4%) than those of *T. molitor*. However, these values did not show significant differences ($P < 0.05$), and insect chitosan films were more soluble than commercial chitosan films. The lower solubility of films of *T. molitor* chitosan can be explained by its higher Mw than *B. magna* chitosan. In addition to the above, the higher solubility in insect chitosan films may be due to the smaller intermolecular forces between chitosan chains. This is a result of ingredient residues (probably melanin) and the moisture content of the film. (Saenz-Mendoza et al. 2020) Some researchers believe that the water solubility and degree of swelling of cricket chitosan films are lower (WS 13.12 ± 3.42%; SD swelling degree 1.15 ± 0.43) compared with commercial shrimp chitosan, but adding *Schisandra chinensis* extract slightly increases the degree of swelling (Jarolimkova 2015). A possible explanation for the difference between shrimp and cricket chitosan is the lower degree of deacetylation and very low molecular weight of chitosan films, but there are no studies on the use of insect-derived chitosan or very low molecular weight/low deacetylation chitosan films. In the latest grasshopper protein film study, the authors found that WS with 10% xylose was significantly reduced from 91% to 46%. But when the dosage reaches 15%, the saturation effect occurs (Jarolimkova 2015).

The choice of protein/chitosan concentration, pH, temperature and plasticizer

determines solubility and water absorption capacity (Mihalca et al. 2021). WVP is directly affected by many factors (e.g., polymer chain mobility, thickness, film integrity). The main influence tending to increase water vapour permeability is the increased solubility of protein-based films and chitosan-based films.

3.3 Mechanical properties

Mechanical strength depends on the composition and process conditions when making biopolymer films (Gheribi et al. 2019). It is responsible for maintaining the integrity of the packaging during handling and storage. Mechanical properties include tensile strength, elongation, elasticity and Young's modulus (Assad et al. 2020). These can be improved and enhanced by different moulding conditions, processing parameters and the addition of plasticizers depending on the source and application. Table 4.22 summarizes the mechanical properties of existing films derived from insect components, including the tensile stress and elongation at break of the films.

Table 4.22: Tensile stress and elongation of several film materials prepared from insects

INSECT	MATERIAL	TENSILE STRESS (MPa)	ELONGATION AT BREAK (%)	REFERENCE
Fly Larvae and Mealworm	Chitin	89.6	10.6	(Pasquier et al., 2021)
Crickets	Chitosan	27.5±3.3	49.6±8.7	(Malm et al., 2021)
Laccifer lacca	Shellac	13.8	20.6	(Du et al., 2019)
Snail	Chitosan and Mucus	15±4	13±6	(Di Filippo et al., 2020)
Tenebrio molitor and *Brachystola magna*	Chitosan	43.51±0.91	66.28±2.48	(Saenz-Mendoza et al., 2020)
Migratory locust	Protein	2.51	13.16	(Zhang, Fang, et al., 2022)
Grasshopper	Protein/Soy Protein Isolate/Cinnamaldehyde	3.38±0.23	38.1±3.1	(Zhang, Zhou, et al., 2022)

Some researchers (Zhang, Zhou, et al. 2022) found that films with xylose glycosylation were more likely to form dense structures with high molecular weights. When xylose was increased from 10% to 15%, TS (tensile stress) and EAB (elongation at break) decreased due to saturation effects. CIN has a negative effect on TS and EAB, especially when added at levels above 10%. The effect of CIN on polymer mechanical properties depends on the substrate, and there is no particularly consistent trend. Some researchers (Zhang, Fang, et al. 2022) used the response surface design in their migratory locust study to obtain the TS and EB values of the films through the response surface formula. The plasticiser used has an increasing and then decreasing effect on TS, as the plasticising capacity of glycerine exceeds the threshold. For proteins, the difference in content affects the degree of aggregation between molecules. Of course, pH may lead to different degrees of accumulation by adjusting the charge properties of the protein network.

In an insect chitosan film study (Saenz-Mendoza et al. 2020), TS (≈42–44 MPa) and EM (≈737–1060 MPa) values in insect chitosan films were lower than those obtained in commercial chitosan films. In contrast, the E% (Elongation at break) in insect chitosan films (≈56–66%) was higher than in commercial chitosan films (≈38–41%). Insect chitosan films are more flexible than commercial chitosan films because insect films are about 15% to 25% more ductile. The mechanical properties of the film may be due to residual melanin in the insect chitosan, as well as higher film moisture content.

When a group of researchers (Di Filippo et al. 2020) studied the mechanical properties of chitosan films with added snail mucus, they found that the effect of adding snail mucus was similar to that of a plasticizer. At higher snail mucus concentrations, the increased ductility of the films can be attributed to the solution-polymer interaction. This reduces intermolecular interactions between polymer chains, promoting their sliding and fluidity, and improving overall ductility.

Additionally, the shellac-containing films were more stretchable in the tensile stress test. Possibly because of the low molecular weight of shellac, it can occupy the space between the macromolecules of the film, resulting in weaker connections between

the film macromolecules and thus increasing the flexibility of the food packaging film (Du et al. 2019).

And in another study, Malm et al. (2021) found that compared with commercial shrimp chitosan films, cricket chitosan had similar or better tensile strength (TS), and the degree of deacetylation had no significant effect on the films. The elongation rate is basically not as good as that of commercial chitosan films. The author believes that because chitosan is covalently cross-linked with the residual melanin of insects, it can be compared with commercial shrimp shell chitosan in terms of TS. The molecular weight of chitosan prepared from crickets is smaller, so the elongation rate is basically inferior to that of commercial chitosan films.

Using a hot-pressing process, some researchers (Pasquier et al. 2021) found that the strength of the chitin nanofiber network is mainly affected by the assembly conditions (CP or HP) rather than by the properties of the chitin precursors. The hot press process greatly reduces the maximum tensile strain and toughness. S-ChNF decreased from 5.5 ± 0.7 to 1.9 ± 0.2% and F-ChNF decreased from 5.0 ± 0.6 to 1.4 ± 0.1%. The reason is that after hot pressing, the moisture in the film is reduced. Cold-pressed products have better mechanical properties. In addition, it was found that the cellulose composition in W-NF was different from that of conventional CNF. Possibly W-NF molecules are smaller or highly impregnated with pigments or are entangled.

3.4 Antibacterial properties

In a study using cinnamaldehyde as an antibacterial agent in cricket protein films (Zhang, Zhou, et al. 2022), due to high volatility, the composite film has antibacterial activity only when the amount of cinnamaldehyde reaches 20%. In a study that also used crickets as research material, the experimenters compared the antibacterial properties of chitosan films and films to which *Schisandra chinensis* extract had been added (Jarolimkova 2015). This author shows that the antibacterial activity of the pure chitosan film itself depends on a complex combination of different factors, including molecular weight, degree of deacetylation, pH, film moisture content and bacterial strain species. The experimenter designed the agar diffusion experiment because it

was thought to more realistic for bacterial growth unless it was necessary to package beverages. And tests were performed on a variety of common strains (e.g., *Bacillus cereus, Escherichia coli, Listeria monocytogenes*). The combined effects of *S. chinensis* extract and cricket chitosan were found. Diffusion of the extract likely inhibited the initial growth, an effect supported by the slow diffusion of cricket chitosan after several days.

In contrast to adding antibacterial agents, some researchers (Kaya et al. 2019) (Chen et al. 2021b) have conducted experiments on the inherent antibacterial properties of the film made from insect components themselves. The performance of the *Periplaneta americana* chitosan film against common food-borne pathogens *Serratia marcescens* and *Escherichia coli* was significantly better than that of the commercial chitosan film. The *H. abietis* chitosan film has been studied with 28 different bacterial species (against 18 of which it had antibacterial properties) It has the highest activity against *Vibrio parahaemolyticus*, followed by *Acinetobacter baumannii* and *Streptococcus pneumoniae*, and relatively low activity against *Klebsiella pneumoniae* subsp.

4. PERSPECTIVES

Insects are already being commercialized as food in Western markets but they are not mainstream (Reverberi 2021). They face many commercial challenges, including production costs, certifications and regulations, marketing communications, and retail distribution and consumer positioning. In the current situation, most countries or regions, because of dietary habits, cultural background and religious beliefs, do not accept insect products to a high degree. For example, this review finds that because the image of *Orthoptera* insects may be more acceptable to humans than other insects, mainly because they are mostly herbivorous and widely distributed around the world, this group of insects is the most frequently studied. However, the use of packaging derived from insects such as *Periplaneta americana* and fly maggots will be limited by people's traditional consumption concepts. This type of perception about a food

product is very visceral and difficult to change and may require a large investment in marketing or a drastic change in public awareness, triggered by an external event, which in this case may be climate change.

Compared with the accumulated information that is available with respect to the safety of conventional animal and plant-derived materials, there are very few studies on insects as raw materials. Marshall et al. (2016) described in detail the types of infectious and intoxicating bacteria, viruses and parasites related to edible insects. Their paper also provides examples of careful insect food processing and preparation methods that ensure a safe, wholesome and nutritious product for consumers. Vandeweyer et al. (2021) identify the top three bacterial pathogens associated with insects for food as *Staphylococcus aureus*, pathogenic *Clostridium spp*. and pathogenic species of the *Bacillus cereus* group. Contamination risk assessments for the insect species employed will need to be carried out in the future. Edible insect supply chains may require the implementation of detailed sampling plans and, in each chain, the prediction of potential hazardous microorganisms to gain insight into the quality of the overall supply chain. Otherwise, there may be a risk of contamination of insect-derived film-forming materials.

In addition, Alok Bang et al. (2021) have warned that, in general, industrial farming of insects for feeding purposes is based on production models that use large numbers of non-native insect species. These insect species have considerable invasive potential. If there are loopholes in the implementation of transportation and breeding, or a lack of adequate policies and production standards, there may be threats to regional or even global biodiversity (Bang and Courchamp 2021). This issue must be taken into account when planning and instituting future developments in the farming of insects.

5. CONCLUSIONS

This review summarizes the results of the main scientific studies on the use of insects to prepare edible packaging that have been published over the past five years. The insect species currently receiving more in-depth study are crickets / grasshoppers /

locusts in the order Orthoptera. This is likely because the image of Orthoptera is more acceptable to humans than that of other insects, because most of them are herbivorous and widely distributed worldwide. Rather than extracting proteins from insects as a substrate for making packaging films, researchers have preferred to extract chitosan from insects. Chitosan films generally have higher tensile stress than protein films. With respect to antibacterial properties, protein-based films can carry antibacterial agents in combination to achieve antibacterial effects, while chitosan films themselves have certain antibacterial activity.

The currently available insect-derived food packaging films still have much room for improvement with respect to various attributes. In particular, insect pigments have a positive effect on the light tolerance of packaging films and the protection of the wrapped food from light, but more research and design work are needed to make them more aesthetically acceptable. The hydrophilic, hydrophobic and solubility characteristics of the films are generally not as good as those of traditional plastic packaging. In addition, the protein concentration, pH, temperature and choice of plasticizer of the insect-derived material all determine the final solubility and hydrophobicity of the film. In terms of mechanical properties, differences in the film fabrication processes used will have a huge impact.

Ethics statement file

This research did not include any human subjects and/or animal experiments.

CRediT authorship contribution statement

S.Weng: Conceptualization, Methodology, Investigation, Writing - Original Draft. I.Marcet: Methodology, Writing – review & editing, Supervision M. Rendueles: Methodology, Writing – review & editing, Supervision M. Díaz: Supervision

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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.

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Chapter 5: General Discussion

5. General discussion

In this PhD thesis, characterization studies and method innovations were made on the preparation of new biomaterials using biopolymers from natural sources. Protein materials such as gelatin and plasma protein are used as packaging film substrates. To enhance the structural properties, polysaccharide-based nanocellulose was introduced. In order to enhance the functionality, different types of active substances such as thymol, antimicrobial peptides, and bacteriophages are introduced to fight against common food-borne contamination bacteria, *Escherichia coli* and *Staphylococcus aureus*. In addition, PLA encapsulates active substances to achieve sustained release. Furthermore, a summary of the production technology status of food packaging materials and sources of new materials was made. By preparing biomacromolecule materials into food packaging opportunities, it helps to reduce the waste of biological resources and environmental pollution. Overall, the experimental results reflect an attempt and expansion of the research on the application of biopolymers in the field of food packaging materials. Successfully produced films complement the diversity in the field of food packaging. In addition, the thesis also pays attention to the importance of strengthening both laboratory research and industrial scale-up production to truly popularize biomaterial food packaging.

In terms of experimental results, in section 4.1.1, plasma proteins from porcine and bovine sources changed the original protein configuration through two steps of "acidification" and "alcohol modification". Under the superposition of the two precipitation methods, the puncture strength and puncture deformation of the plasma film were strengthened by about 1.8~2 or 1.8~2.4 times, respectively. We know that the main protein types in plasma proteins are albumin and globulin, and their isoelectric points (pI) are in the range of 4.7–4.9 and 5.3–7.3, respectively(Poehler et al., 2015). Lowering the pH allows the amino groups to capture electrons and the protein to become positively charged. The hydration shell around the protein is

destroyed, and the protein structure is deformed. Adding alcohol solvent at this time will reduce the dielectric constant in the solvent. Increases the attraction of opposite charges while altering the hydrogen and ionic bonds in the protein structure. The protein structure is strongly folded and there is no hydration shell (Yoshikawa et al., 2012). This also explains the change in solubility of the films in different pH water environments in Section 4.1.1. Even in the study of plant proteins, Zhang et al.(2019) found that the concentration of ethanol has an impact on the characterization of the final film.

In addition, in the content mentioned in the entire section 4.1, the third protein precipitation method, that is salting out precipitation (J. Zhang, 2012), is not involved. At present, there is, no research related to the film made of protein after salting out and precipitation. This may be attributed to the time-consuming and water resource consumption of salt out and salt in protein salting out and precipitation, as well as dialysis desalination, which does not have practical significance for subsequent large-scale development.

In Section 4.1.2, nanofibrillated cellulose (NFC) was designed as a structural reinforcement material. The results showed that the addition of 10% (w/w) NFC enhanced the characterization of plasma protein films. For water solubility, the modification of the protein itself is more decisive than the addition of NFC. There are two explanations for NFC as a filler in proteinaceous materials here. (1) As a cheaper nanofiller, reducing protein content without sacrificing mechanical properties. (2) The length-to-diameter ratio and crystallinity properties of NFC can improve structural strength (Samarasekara et al., 2018). Future studies on the carbohydrate-binding domain (CBM) of NFC and plasma proteins may provide a better explanation for the present evidence. In addition, cellulose nanocrystals (CNC), as one of the types of nanocellulose, has a high degree of crystallinity, which makes it have strong rigid mechanical properties. CNC generally contains many hydroxyl groups, which are easy

to form hydrogen bonds with itself or with other molecules. This also provides more possibilities for combining with plasma proteins to make high-performance films. At the same time, the edibility and toxicity of nanocellulose still need more research and regulatory support.(Zaaba et al., 2021; W. Zhang et al., 2021)

In Section 4.1.2, nisin is used as an antibiotic against the gram-positive bacterium *Staphylococcus aureus*. This is due to the fact that currently nisin is the only bacteriocin that is available commercially and industrially. (Lakshmayya et al., 2022) And it has affinity with protein matrix film. If other antimicrobial peptides are added, such as antimicrobial peptides isolated from housefly edible insects as active substances, it may also be a good research direction.(Hadj Saadoun et al., 2022; Qian et al., 2022)

Section 4.1.3 focuses on technology, reviewing the current research status of edible coatings and films made in different ways. In this section, it is found that there are a lot of scientific research results at the laboratory level. As far as large-scale production methods are concerned, very few research results have been obtained. This is understandable, since large-scale studies require higher investment and more researchers. And the research results of large-scale production methods are mainly designed for the complex business factors of privacy. The author hopes that there will be more efficient edible packaging production methods and the birth of new technologies in the future.

Based on the antibacterial performance of antimicrobial peptides and the strengthening of food-borne bacteria's resistance to antibiotics and other comprehensive factors. In section 4.2.1, phiIPLA-RODI phages were incorporated into protein films against Staphylococcus aureus. Restricted by the packaged food being fresh cheese, the entire experimental process was designed for six days, and the highest antibacterial effect was observed in the first three days. The duration of the antibacterial effect of phage in actual storage has not yet been clearly verified. In this

regard, researchers from IPLA-CSIC, the research institute that contributed *phiIPLA-RODI* phage, have done experiments on encapsulating *Staphylococcus aureus* phage *phiIPLA-RODI*.(González-Menéndez et al., 2018) They found nanovesicle technology to be a suitable candidate for the production of *phiIPLA-RODI*-based formulations. In addition, based on the research conclusion of this article, the way of coating can carry a relatively large amount of water in the initial stage of packaging, which is conducive to the reproduction and duration of bacteriophage. So, the cooperation between edible food packaging coatings and nanovesicles can be an interesting direction to try in the future.

Unlike phages and antimicrobial peptides against Gram-positive bacilli, this PhD thesis uses thymol (a natural volatile monoterpene phenol) against Gram-negative bacilli represented by *Escherichia coli*. In section 4.2.3, PLA is used as the material for making nanoparticles, and it is re-examined from the perspective of environmental protection. It may be more environmentally friendly to use completely edible nanoparticles. Heckler et al.(2020) loaded thymol and carvacrol into nanoliposomes to characterize their inhibitory effect on Salmonella. Although liposome-encapsulated antimicrobials showed reduced activity compared to their free counterparts, there was sufficient uniformity and high encapsulation efficiency to remain stable for 28 days under refrigerated conditions. If these research results are combined with protein-based food packaging films, based on the improvement of edibility, it may also be a good research direction. The advantage of the storage stability of PLA nanoparticles is that the change of pH has slight effect on it. Although PLA will undergo more intense intramolecular transesterification and depolymerization in an alkaline environment, (Xu et al., 2011) it was not clearly observed in this experiment. This is also understandable, because PLA is a polyester material with poor hydrophilicity and biological inertness. In Table 4.12, the "n" value shows the process of in vitro release of thymol-loaded PLA particles, which can be simply understood as the process of erosion and diffusion. (Ritger & Peppas, 1987)

According to the performance research results of thymol encapsulated by PLA nanoparticles, the nanoparticles were incorporated into gelatine films in Section 4.2.2, and the characterization properties and antibacterial effects of the films were discussed. Thymol-loaded PLA nanoparticles had a negative effect on the film on puncture deformation. In terms of transparency, the presence of thymol improves the resistance of the film to ultraviolet rays, but also increases the opacity. In contrast, the antibacterial performance of the film is more interesting. Thymol is slightly soluble in water and double-wrapped by gelatine film and PLA nanoparticles. The advantage is that the volatilization rate is reduced, but the time-consuming and path of exposure to pathogenic bacteria is prolonged, and the onset time is also prolonged. The target food for infection in this chapter is apple slices, and no water content analysis was done. Although 0.1ml of 10^5CFU mL^{-1} *E. coli* culture solution was inoculated, the effect of water content on the antibacterial process is not clear. At this point, it may be an interesting topic to continue to delve into the impact of food surface moisture on this antimicrobial effect.

The review of research on insect-derived food packaging in Section 4.3 can be likened to a summary of the results of human efforts to find and develop biological resources. Compared with proteins, chitosan has received more research attention, but all studies are limited to laboratory level. Commercialization, compliance, and industrialization have never been implemented. Among many reasons, the author believes that the competitiveness of insect materials is not strong due to the lack of urgent consumer demand and the unreasonable development of the market.

Furthermore, the study of packaging films developed based on protein matrices in this thesis has a lot to be extended in characterization studies. For example, mechanical properties such as tensile strength and flexibility are important attributes for maintaining the integrity of a material, with the most common parameters being

tensile strength, elongation at break, and Young's modulus. Among the barrier properties, the detection of oxygen permeability is also a key food packaging attribute that needs to be understood. In addition, in the study of hydrophilicity and hydrophobicity, the analysis of the contact angle between the film material and water is also a useful test to help us understand the properties of the film. As for the detection at the chemical level, the presence of functional groups in the film structure can also be analysed by Fourier transform infrared spectroscopy (FTIR). X-ray diffraction can help analyse changes in the material's crystal structure due to additives added to the film. As for thermogravimetric analysis, we can understand the ability of materials to resist degradation at high temperature and understand the thermal stability of thin films. The above characterization tests can enrich the characterization of packaging films based on plasma protein and gelatine in this thesis. In addition, the developed edible film needs to be further confirmed in terms of toxicity testing and compliance.

Chapter 6: Conclusions

6.Conclusions

The following conclusions can be drawn in detail from this doctoral thesis:

- A method for obtaining food packaging bioplastics from porcine and bovine plasma protein has been designed. The best results have been obtained with bioplastics based on modifying the pre-treatment method by acidification following alcohol precipitation.

- Nanofibrillated cellulose (NFC) has been obtained from paper pulp as a reinforcing material in protein bioplastics. This nanofibrillated cellulose blended with pre-treated plasma proteins precipitated at a concentration of 10% (w/w) was the optimal formulation for the development of bioplastics. When more than 10% (w/w) NFC is incorporated into the film, puncture resistance and film deformation gradually weaken. The acidification and alcohol precipitation pretreatment plays a key role in improving the water solubility and light transmission of the plasma protein film.

- The incorporation of the antimicrobial peptide Nisin did not adversely affect the mechanical properties of the plasma protein films, and an antimicrobial effect of Nisin was observed as it successfully inhibited *Staphylococcus aureus* for 14 days.

- The bacteriophage *phiIPLA-RODI* was successfully introduced into the gelatine bioplastic without negative effects on the mechanical properties of the film even at the highest experimental concentration of 1.75×10^8 CFU/mL used. The antimicrobial properties are improved when the bioplastic with the incorporated bacteriophage is applied as "Coating".

- In the preparation of PLA nanoparticles used to encapsulate thymol, it has been determined that the amount of PLA is a key factor to optimize the preparation of the nanoparticles. The encapsulation efficiency of thymol in spherical PLA nanoparticles reached 60.3 ± 8%. These thymol-encapsulating nanoparticles

showed high stability at the three different pH values (4/7/9) tested. In *E. coli* inhibition tests, PLA nanoparticles were shown to be able to maintain thymol activity for 14 days.

- PLA nanoparticles with thymol were successfully incorporated into gelatine films, exhibiting anti-*Escherichia coli* properties through the thymol retained in the film matrix. On the other hand, the effect of nanoparticles on the mechanical properties of the film can be considered insignificant.

- Compared to protein extraction, chitosan extracted from insects may be more suitable as a matrix for the preparation of bioplastics, because chitosan-made films show better tensile strength and antibacterial properties than proteins. Orthoptera (crickets, grasshoppers, locusts...) are the most studied species as a source of chitosan and protein. The insect's own pigments have a positive effect as light protection agents for foods covered with these films.

- The combination between the characteristics of the packaging material, the characteristics of the food and the method of production of the packaging is important to achieve a commercially usable packaging product. The "Coating" method is suitable when you want to use heat-sensitive antibacterial substances. The "Solution casting" method is the easiest way to form "films" and is currently the most widely used in laboratory studies. However, this method is not scalable at an industrial level, "Electrospinning", "Electrospraying" and “3D printing” are currently expensive technologies and difficult to bring to productive scales. "Dipping", "Vacuum impregnation", "Spraying", and "Fluidized beds" and other thermal methods have the potential to be scaled up to the industrial level in the production of bioplastics for commercial purposes.

6.Conclusiones

Las siguientes conclusiones pueden extraerse en detalle de esta tesis doctoral:

- Se ha diseñado un método de obtención de bioplásticos de envasado de alimentos a partir de proteína de plasma porcino y bovino. Los mejores resultados se han obtenido con los bioplásticos basados en modificar el método de pretratamiento mediante una acidificación previa a una precipitación alcohólica.

- Se ha obtenido celulosa nanofibrilada (NFC) a partir de pasta de papel como material de refuerzo en los bioplásticos de proteína. Esta celulosa nanofibrilada mezclada con proteínas plasmáticas pretratadas precipitadas a una concentración del 10% (w/w) fue la formulación óptima para el desarrollo de los bioplásticos. Cuando se incorpora más del 10% (en peso) de NFC en la película, la resistencia a la punción y la deformación de la película se debilitan gradualmente. El pretratamiento de acidificación y precipitación con alcohol juega un papel clave en la mejora de la solubilidad en agua y la transmisión de luz de la película de proteína plasmática.

- La incorporación del péptido antimicrobiano Nisina no afectó negativamente a las propiedades mecánicas de los "films" de proteínas plasmáticas, observándose un efecto antimicrobiano de la Nisina ya que logró inhibir con éxito *Staphylococcus aureus* durante 14 días.

- El bacteriófago *phiIPLA-RODI* fue introducido con éxito en el bioplástico de gelatina sin efectos negativos en las propiedades mecánicas del "film" incluso a la concentración experimental más alta utilizada de 1.75×10^8 UFC/mL. Las propiedades antimicrobianas se ven mejoradas cuando el bioplástico con el bacteriófago incorporado se aplica en forma de "Coating".

- En la preparación de nanopartículas de PLA utilizadas para encapsular timol se ha determinado que la cantidad de PLA es un factor clave para optimizar la

preparación de las nanopartículas. La eficiencia de encapsulación del timol en nanopartículas esféricas de PLA alcanzó el 60,3 ± 8%. Estas nanopartículas que encapsulan timol mostraron una alta estabilidad en los tres valores diferentes de pH (4/7/9) probados. En las pruebas de inhibición de *E. coli*, las nanopartículas de PLA demostraron ser capaces de mantener la actividad del timol durante 14 días.

- Las nanopartículas de PLA con timol se incorporaron con éxito en “films” de gelatina, exhibiendo propiedades anti-*Escherichia coli* a través del timol retenido en la matriz del “film”. Por otro lado, el efecto de las nanopartículas en las propiedades mecánicas de los “films” se puede considerar poco significativo.

- En comparación con la extracción de proteínas, el quitosano extraído de los insectos puede ser más adecuado como matriz para la preparación de bioplásticos, porque los “films” hechos de quitosano muestran una mejor resistencia a la tracción y mejores propiedades antibacterianas que las proteínas. Los ortópteros (grillos, saltamontes, langostas…) son las especies más estudiadas como fuente de quitosano y proteínas. Los pigmentos propios del insecto tienen un efecto positivo como agentes de protección contra la luz de los alimentos cubiertos con estos “films”.

- Es importante la combinación entre las características del material de empaque, las características del alimento y el método de producción del empaque para lograr un producto de envasado utilizable comercialmente. El método de "Coating" es adecuado cuando se quieren utilizar sustancias antibacterianas sensibles al calor. El método de " Solution casting" es la manera más fácil de formar “films” y actualmente es la utilizada mayoritariamente en los estudios a nivel de laboratorio. Sin embargo, esté método no es escalable a nivel industrial, "Electrospinning”, “Electrospraying” e “impresión 3D” son actualmente tecnologías costosas y difíciles de llevar a escalas productivas. "Dipping”, “Impregnación al vacío”, “Spraying”, y “los lechos fluidizados” y otros métodos

térmicos tienen potencial para ser escalados a nivel industrial en la producción de bioplásticos con fines comerciales.

Chapter 7: Bibliography

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Chapter 8: Appendix

App.1: Figure list

App.2: Table list

App.3: Formula list

App.4: Abbreviation list

AGU	Anhydroglucose Unit
AM	Additive Manufacturing
ANOVA	Analysis Of Variance
BC	Bacterial Cellulose
BCNCs	Bagasse Cellulose Nanocrystals
BPA	Bisphenol A
CAD	Computer Aided Design
CAGR	Compound Annual Growth Rate
CAS	Chemical Abstracts Service
CBM	Carbohydrate-Binding Domain
CFU	Colony Forming Units
ChNFs	Chitosan Nanofibers
CIN	Cinnamaldehyde
CMC	Carboxymethylcellulose
CNC	Cellulose Nanocrystals
CNF	Cellulose Nanofibrils
CNW	Cellulose Nanowhiskers
CP	Cold Pressing
DCM	Dichloromethane
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl-1-Picrylhydrazine
E. coli	Escherichia Coli
EAB/EB	Elongation At Break
ECF	European Climate Foundation
EHEC	Enterohemorrhagic Escherichia Coli
EWP	Egg White Protein
FAO	Food And Agriculture Organization Of The United Nations
FDA	Us Food And Drug Administration
FDM	Fused Deposition Manufacturing
FFAs	Free Fatty Acids
FTIR	Fourier Transform Infrared Spectroscopy
GRAS	Generally Recognized As Safe
HDPE	High-Density Polyethylene
HP	Hot Pressing
IM	Injection Molding
KGM	Konjac Glucomannan

LbL/LBL	Layer-By-Layer
LDPE	Low-Density Polyethylene
LSD	Least Significant Difference
LTRs	Long Immediate Terminal Repeats
MPs	Microplastics
mRNA	Messenger Ribonucleic Acid
NA	Not Available
NB	Nutrient Broth
NCC	Nanocrystalline Cellulose
NFC	Nano-Fibrillated Cellulose
OEO	Oregano Essential Oil
PBS/PBSA	Polybutylene Succinate
PD	Puncture Deformation
PDI	Polydispersity Index
PDLA	Poly D-Lactic Acid
PET	Polyethylene Terephthalate
PFU	Phage Forming Units
PHA	Polyhydroxyalkanoates
pI	Isoelectric Points
PLA	Polylactic Acid
PLLA	Poly L-Lactic Acid
PP	Polypropylene
PS	Polystyrene
PS	Polysacharides
PS	Puncture Strength
PTA	Phosphotungstic Acid
PTFE	Polytetrafluoroethylene
PVA	Polyvinyl Alcohol
R&D	Research And Development
S. aureus	Staphylococcus Aureus
SA	Sodium Alginate
SEM	Scanning Electron Microscopy
SHL	Shellac
SPI	Soy Protein Isolate
TEM	Transmission Electron Microscopy
TEMPO	2,2,6,6-Tetramethylpiperidine
TFS	Tin Free Steel
TGA	Thermogravimetric Analysis
TS	Tensile Stress/ Strength
TSB	Tryptic Soy Broth

UV	Ultraviolet
WCA	Water Contact Angle
WHO	World Health Organization
WP	Whey Protein
WPI	Whey Protein Isolate
WVP	Water Vapor Permeability

App.5: Common unit list

%	percent sign
°C	degree Celsius
atm	atmospheric pressure
g	gram
H/h	Hours
kDa	kilo Dalton
kg	kilogram
kV	kilovolt
L	Litre
mbar	millibar
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mmHg	millimetre mercury
Mt	megatons
N	newton
nm	nanometres
rpm	revolutions per minute
s	second
w/v	weight/volume
w/w	weight/weight

App.6: Propagation list

6.1-Articles covered in Thesis

- Álvarez, S., Weng, S., Álvarez, C., Marcet, I., Rendueles, M., & Díaz, M. (2021). A new procedure to prepare transparent, colourless and low-water-soluble edible films using blood plasma from slaughterhouses. *Food Packaging and Shelf Life*, *28*(January). https://doi.org/10.1016/j.fpsl.2021.100639
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- Weng, Shihan, López, A., Sáez-Orviz, S., Marcet, I., García, P., Rendueles, M., & Díaz, M. (2021). Effectiveness of bacteriophages incorporated in gelatine films against Staphylococcus aureus. *Food Control*, *121*(June 2020), 1–8. https://doi.org/10.1016/j.foodcont.2020.107666
- Weng, Shihan, Sáez-Orviz, S., Marcet, I., Rendueles, M., & Díaz, M. (2022). Novel bovine plasma protein film reinforced with nanofibrillated cellulose fiber as edible food packaging material. *Membranes*, *12*(1). https://doi.org/10.3390/membranes12010031

6.2-International Conference

- Weng, Shihan, Sáez-Orviz, S., Marcet, I., Rendueles, M., & Díaz, M. (2022). Novel bovine plasma protein film reinforced with nanofibrillated cellulose fiber as edible food packaging material. BioIberoamérica 2022: III Congreso Iberoamericano en Biotecnología 07-09/Abril/2022 Braga (Portugal) Report speech
- Weng, Shihan, Marcet, I., Rendueles, M., & Díaz, M. (2022).Black Biodegradable Mulching Film based on Gelatin and Humic Acid BioIberoamérica 2022: III Congreso Iberoamericano en Biotecnología 07-09/Abril/2022 Braga (Portugal) Poster display