Centro de Investigación en Alimentación y Desarrollo, A.C.

MICROESFERAS DE PECTINAS FERULADAS Y ARABINOXILANOS FERULADOS ACARREADORAS DE *Saccharomyces boulardii*: DISEÑO Y EVALUACIÓN *in vitro* DE UN SISTEMA DE LIBERACIÓN DIRIGIDO A COLON

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CONTENIDO

CONTENIDO (continuación)

RESUMEN

Las pectinas feruladas y los arabinoxilanos ferulados tienen la capacidad de formar geles covalentes por acoplamiento oxidativo del ácido ferúlico presente en estos polisacáridos. Sin embargo, hasta antes del desarrollo de la presente investigación, no existía información sobre la formación de geles mixtos de ambos polisacáridos, ni del potencial de estos geles mixtos como matriz acarreadora de microorganismos de interés terapéutico. En particular, *Saccharomyces boulardii* es un probiótico de interés tanto para la industria farmacéutica, como la alimentaria; cuya viabilidad se ve afectada bajo las condiciones del pH gástrico, lo cual resulta en que gran parte de estos probióticos mueren en el entorno gástrico antes de llegar al principal sitio de acción (colon). En este sentido, el presente trabajo propone el diseño de microesferas de pectinas feruladas y arabinoxilanos ferulados como acarreadores de *Saccharomyces boulardii*, así como su evaluación *in vitro* como sistemas de liberación dirigida a colon. Para ello, se extrajeron pectinas feruladas y se determinaron sus características químicas y fisicoquímicas. Posteriormente se prepararon y caracterizaron geles covalentes mixtos de pectinas feruladas + arabinoxilanos ferulados (relación 1:1 de ácido ferúlico) con y sin células células y sin *Saccharomyces boulardii*. Una vez estudiada la cinética de formación del gel mixto, se prepararon microesferas mixtas por la técnica de electroaspersión con arreglo coaxial, atrapando *Saccharomyces boulardii* en su interior (2.08×10^8 células/ mL). Estas microesferas presentaron un diámetro promedio de 344 ± 126 µm y una eficiencia de encapsulación de *Saccharomyces boulardii* de 94 ± 28 %. Se evaluó además la actividad antioxidante de las micropartículas, siendo de 20.66 ± 0.03 µmol TEAC/g por la técnica de $ABTS^+$ y 6.85 ± 0.58 µmol TEAC/g por la técnica de DPPH. Bajo condiciones simuladas del sistema gastrointestinal humano las microesferas mixtas permanecieron estables permitiendo la mayor liberación de *Saccharomyces boulardii* en la sección de colon (1.1×10^8 CFU/ml). Este trabajo demuestra la factibilidad de atrapar *Saccharomyces boulardii* en el interior de microesferas mixtas de pectinas feruladas + arabinoxilanos ferulados las cuales permanecen estables bajo las condiciones del pH gástrico y permiten la liberación dirigida a colon de estos probióticos.

Palabras clave: Pectinas feruladas, arabinoxilanos ferulados, micropartículas, *Saccharomyces boulardii*.

ABSTRACT

Ferulated pectins and ferulated arabinoxylans have the ability to form covalent gels by oxidative coupling reaction of the ferulic acid present in these polysaccharides. However, until the development of the present investigation, there was no evidence on the formation of mixed gels of both polysaccharides, nor on the potential of these mixed gels as carrier matrix of microorganisms of therapeutic interest. In particular, *Saccharomyces boulardii* is a probiotic of interest to both the pharmaceutical and food industries; whose viability is affected under gastric pH conditions, which results in a large part of these probiotics dying in the gastric environment before reaching the main site of action (colon). In this sense, the present work proposes the design of microspheres of splinted pectins and splinted arabinoxylans as carriers of *Saccharomyces boulardii*, as well as their *in vitro* evaluation as delivery systems directed to the colon. For this, ferulated pectins were extracted and their chemical and physicochemical characteristics were determined. Subsequently, mixed covalent gels of splinted pectins + splinted arabinoxylans (1:1 ratio of ferulic acid) with and without cells and without *Saccharomyces boulardii* were prepared and characterized. Once the kinetics of mixed gel formation had been studied, mixed microspheres were prepared by the electrospray technique with a coaxial arrangement, trapping *Saccharomyces boulardii* inside (2.08 x 10^8 cells/mL). These microspheres had an average diameter of 344 ± 126 µm and an encapsulation efficiency of *Saccharomyces boulardii* of 94 ± 28 %. The antioxidant activity of the microparticles was also evaluated, being 20.66 ± 0.03 µmol TEAC/g by the ABTS+ technique and 6.85 ± 0.58 µmol TEAC/g by the DPPH technique. Under conditions simulated in the human gastrointestinal system the mixed microspheres remained stable allowing the greatest release of *Saccharomyces boulardii* in the colon section (1.1 x10^8 CFU/ml). This work demonstrates the feasibility of trapping *Saccharomyces boulardii* inside mixed microspheres of splinted pectins + splinted arabinoxylans, which remain stable under gastric pH conditions and allow colon-directed release of these probiotics.

Keywords: Ferulated pectin, ferulated arabinoxylans, microparticles, *Saccharomyces boulardii*.

1. SINOPSIS

1.1. Justificación

Los probióticos se definen como "microorganismos vivos que, cuando se consumen en cantidades adecuadas, confieren un efecto benéfico sobre la salud del huésped" (Morelli y Capurso, 2012). Para que los probióticos brinden beneficios a la salud del huésped, una cantidad importante de ellos deben sobrevivir al recorrido por el sistema gástrico humano y llegar a la sección del colon para brindar beneficios a la salud. Sin embargo, estos microorganismos pueden perder viabilidad durante el tránsito gastrointestinal debido a la sensibilidad de las enzimas digestivas y al bajo pH (Martín *et al.,* 2015). *Saccharomyces boulardii* es una levadura probiótica reconocida como de uso seguro en humanos (GRAS, por sus siglas en inglés) (Douradinha *et al.,* 2014). Sin embargo, la viabilidad de *Saccharomyces boulardii* se ve afectada en condiciones ambientales estresantes y desfavorables, como lo es pH ácido (Kalkan *et al.,* 2018; Martinez-Flores *et al.,* 2017). En este sentido, se han desarrollado avances tecnológicos como la técnica de microencapsulación para proteger la viabilidad de los probióticos (Argin, 2007; Arslan-Tontul y Erbas, 2017; Champagne y Fustier, 2007). La microencapsulación permite que los materiales bioactivos se recubran con uno o una mezcla de materiales protectores, como lípidos, proteínas, polisacáridos, azúcares y sus combinaciones (Chávez y Ledeboer, 2007; Huq *et al.,* 2013; Lapsiri *et al.,* 2012; Rascón-Chu *et al.,* 2018). La electroaspersión es un método de microencapsulación adecuado que utiliza un campo eléctrico para generar gotas nanométricas y micrométricas. El proceso consiste en someter una solución a un campo eléctrico durante el flujo a través de un capilar mantenido a alto potencial; al alcanzar el valor crítico por un campo eléctrico, se forma un chorro (cono de Taylor). Posteriormente, el campo eléctrico provoca la deformación y la distribución del chorro, lo que da como resultado que las gotas finas formen micropartículas (Rehman *et al.,* 2019). Esta técnica facilita la preparación de micropartículas con composición y morfología especificas diseñadas en base a las necesidades y a las aplicaciones (Pawar *et al.,* 2018). Las pectinas feruladas y los arabinoxilanos ferulados son adecuados para uso en la técnica de microencapsulación porque son biodegradables, biocompatibles. y presentan un alto potencial de modificación química o física, lo

que favorece la preparación exitosa de nanopartículas y micropartículas (Lara-Espinoza *et al.*, 2018). Además, estos polisacáridos están contenidos en la fibra dietética de frutas y cereales presentes en la dieta humana. Ambos polisacáridos comparten la característica de tener ácido ferúlico esterificado en su estructura (Chasquibol Silva *et al.*, 2008). Tradicionalmente, las pectinas se obtienen a partir de cáscaras de cítricos y bagazo de manzana (Masmoudi *et al.*, 2012; Rascón-Chu *et al.,* 2009), aunque las pectinas feruladas se recupera principalmente de la remolacha azucarera. Así mismo, se han explorado fuentes de extracción alternas como lo es la pulpa de remolacha azucarera, un subproducto de la producción de azúcar de mesa. La creciente demanda de remolacha azucarera para la producción de azúcar refinada y bioetanol ha aumentado la disponibilidad de residuos sólidos de remolacha azucarera, lo que lleva a su uso como fuente alternativa de extracción de pectina (Almohammed *et al.,* 2017). Estructuralmente, las pectinas feruladas consisten en cadenas lineales formadas principalmente por unidades de ácido galacturónico unidas glicosídicamente por enlaces α-(1→4) (región de homogalacturonano, HG), con secciones ramificadas de azúcares neutros (ramnogalacturonanos I y II; RGI y RGII) y otros sustituyentes. El ácido ferúlico se encuentra unido a residuos de galactosa (*O-6*) y arabinosa (*O-2* y *O-5*) principalmente en la ramificación RG-I (Ralet *et al.*, 2005). Las pectinas feruladas pueden tener un grado de esterificación bajo o alto, y aquellas que registran un grado de esterificación bajo pueden mostrar dos mecanismos de gelificación, uno dado por las interacciones del ácido galacturónico con iones divalentes (por ejemplo, Ca^{2+}) siguiendo el modelo de caja de huevo (Braccini y Pérez, 2001), y el segundo mecanismo de gelificación ocasionado por el acoplamiento oxidativo de ácido ferúlico (Oosterveld *et al.*, 1997). Por otro lado, los arabinoxilanos son polisacáridos no amiláceos neutros obtenidos principalmente a partir de cereales y subproductos de su industrialización, como los granos de maíz secos con solubles destilados (DDGS) provenientes de la producción de bioetanol (Mendez-Encinas *et al.,* 2019). Estos polisacáridos están constituidos por un esqueleto lineal de unidades de xilosa unidas por β-(1→4) que pueden no estar sustituidos, estar monosustituidos con arabinosa a través de α-(1→3) y disustituidas con arabinosa a través de α -(1→3) y α -(1→2). El ácido ferúlico se encuentra unido a éster C(O)-5 de los residuos de arabinosa generalmente unidos a C(O)-3 de unidades de xilosa (Izydorczyk y Biliaderis, 1995).

Los geles de pectinas feruladas entrecruzados covalentemente con enzima lacasa son estables a los cambios de pH pero no son mecánicamente fuertes porque las cadenas laterales RGI y RGII

limitan el contacto intermolecular entre cadenas adyacentes (Oosterveld *et al.,* 1997). Por otra parte, los geles iónicos de pectina son fuertes, pero no estables a los cambios de pH, principalmente en condiciones ácidas (Zaidel *et al.,* 2012), ya que su red polimérica se basa en puentes de calcio (de Vries *et al.,* 1983). Los arabinoxilanos forman geles covalentes más fuertes y resistentes a los cambios de pH, temperatura y fuerza iónica (Mendez-Encinas *et al.,* 2019; Niño-Medina *et al.,* 2010; Saulnier y Jean-Francó, 1999). Sin embargo, la reticulación de arabinoxilanos puede presentar tiempos de curado relativamente prolongados. El presente trabajo tiene como finalidad demostrar la factibilidad de inmovilizar células en el interior de micropartículas mixtas de pectinas/ arabinoxilanos, estables a cambios de pH durante su paso por un sistema de simulación gástrico, capaces de brindar un efecto protector para la viabilidad celular a su paso por el sistema de simulación gástrico y permitir su liberación en colon por efecto de fermentación de la microbiota en el sistema *in vitro*.

1.2. Antecedentes

1.2.1 Sistemas de Liberación Controlada

Un sistema de liberación controlada consiste en una formulación o dispositivo que promueva y facilite la liberación de un compuesto químico terapéutico en el cuerpo humano, considerando una entrega predeterminada (constante o no) por periodos prolongados de tiempo. Para que un sistema de liberación controlada cumpla con su objetivo, deberá mejorar la distribución espacial y temporal del compuesto químico terapéutico dentro del cuerpo, para ello, el o los componentes estructurales del sistema, tendrán que proteger al compuesto activo de una degradación fisiológica, así como una subsecuente eliminación corpórea (Ronald y Rathbone, 2012).

Tradicionalmente, los tratamientos terapéuticos implementados para combatir un padecimiento fisiológico se basan en la administración de fármacos directamente en el tejido u órgano afectado o utilizando una ruta sistémica, como la oral o transdérmica; a pesar de que estos mecanismos

tradicionales de administración de fármacos son eficientes después de varias y repetidas dosis, es inevitable presenciar picos máximos en la concentración del compuesto activo al inicio de una dosis. Por lo anterior, los tratamientos tradicionales imposibilitan controlar los niveles de un compuesto activo por periodos prolongados de tiempo. En este sentido, los sistemas de liberación controlada no sólo son capaces de entregar compuestos activos de manera controlada; también de mantener los niveles del compuesto dentro de la concentración terapéutica deseada en el cuerpo (Coelho *et al.*, 2010).

A pesar de que los sistemas de liberación controlada poseen remarcadas ventajas asociadas principalmente al confort y recuperación oportuna de un paciente, en comparación de los sistemas tradicionales, existen algunas desventajas ligadas a los materiales constituyentes del sistema de liberación. Actualmente, los polímeros son los materiales con mayor interés de estudio para su aplicación como acarreadores de compuestos activos, ya sean polímeros de origen natural (biopolímeros) o sintéticos. De estos últimos, cabe la posibilidad de contener remanentes tóxicos y de formar productos nocivos después de su degradación (Gilhotra y Mishra, 2009; Kelner y Schacht, 2005). Contrariamente, los productos generados por la degradación de los biopolímeros dentro del cuerpo humano son metabolitos comunes que suelen metabolizarse de una manera sencilla y eficaz (Dan Mogoşanu *et al.*, 2016). Entre los biopolímeros más utilizados para la elaboración de sistemas de liberación controlada encontramos proteínas, polisacáridos y derivados de celulosa; cada grupo con características y cualidades únicas aplicables a diseños específicos de sistemas de liberación controlada (Coelho *et al.*, 2010).

1.2.2. Clasificación de los Sistemas de Liberación Controlada

Los sistemas de liberación de compuestos activos presentan una gran variedad de diseños y estructuras, seleccionados en base a las características del agente activo de interés con el objetivo de asegurar y controlar la entrega del compuesto en un periodo de tiempo establecido, directamente sobre una región deseada del cuerpo humano. Las liberaciones controladas de estos compuestos son llevadas a cabo por diversos mecanismos los cuales suelen activarse por diversos fenómenos

químicos, físicos o una mezcla de ambos. Algunos de los mecanismos más importantes de liberación son la disolución, difusión, ósmosis, partición, hinchamiento y erosión. A continuación, se discuten algunos aspectos importantes a considerar en cada uno de estos mecanismos (Ronald y Rathbone, 2012).

En el mecanismo de disolución las moléculas de los solutos, incluyendo el compuesto activo, se encuentran disueltos en un solvente que actúa como vehículo de transporte. En este mecanismo, la trasferencia del compuesto activo ocurre desde la fase sólida hacia el medio circundante, generalmente medios acuosos como tejidos. El objetivo del proceso es lograr la saturación del medio con el compuesto de interés (M. L. Bruschi, 2015).

La liberación basada en el mecanismo de partición se basa en la afinidad compartida que puede tener el compuesto activo con uno o más grupos de materiales que constituyen el sistema de liberación. Un ejemplo de estos sistemas son los liposomas (M. L. Bruschi, 2015; Florence y Attwood, 2006).

El mecanismo de liberación controlada por difusión se basa en un fenómeno conocido como transferencia de masa, en el cual, ciertas moléculas se mueven de un lugar por efecto de gradientes de concentración. En este mecanismo, la interface entre la matriz acarreadora y un medio acuoso es crucial para iniciar la difusión y, por lo tanto, para iniciar la entrega de un compuesto activo. En los sistemas de reservorio, que son un tipo de matriz para la entrega dosificada de compuestos activos, la membrana polimérica actúa como controlador del proceso de difusión y liberación de agentes activos. Los sistemas de reservorio controlan la liberación por diferencia de concentraciones, suponiendo un tiempo inicial de reacción igual a cero, la concentración del agente activo al interior de la matriz polimérica se encuentra saturada y la concentración de ese mismo agente en el medio exterior es cero; de tal forma, que el fenómeno de iniciación es estudiado y explicado por la primera ley de Fick (M. L. Bruschi, 2015; Crank, 1975; Ronald y Rathbone, 2012). En la dosificación de agentes activos controlada por osmosis, un disolvente se transfiere a través de una membrana semipermeable para diluir una solución conformada por un soluto y un solvente. Cuando dos soluciones de diferentes concentraciones están separadas por una membrana semipermeable que es permeable a las moléculas más pequeñas del solvente, este último tendrá

que fluir a través de la membrana semipermeable desde la solución menos concentrada a la más concentrada. Este mecanismo trata de igual la concentración de los solutos permeables en ambos lados de la membrana. En sistemas biológicos, el solvente en cuestión será agua y la presión osmótica será la encargada de dirigir la liberación de agentes activos (M. L. Bruschi, 2015; Ronald y Rathbone, 2012).

Durante el mecanismo de hinchamiento, los enlaces e interconexiones de las cadenas poliméricas que constituyen una matriz de liberación se encuentran rodeadas de agua, de tal manera que dichos enlaces suelen expandirse y formar nuevas interacciones químicas o físicas entre ellos. El mecanismo de hinchamiento es muy similar al de osmosis; las moléculas de agua interactúan relativamente rápido con el polímero, sin embargo, la disolución del polímero en agua (si ocurre) es comparativamente más lenta por el grado de entrecruzamiento. El proceso de expansión y el aumento de volumen disponible dentro de una matriz polimérica pueden ser utilizados para el diseño de sistemas de liberación de compuestos activos(M. L. Bruschi, 2015; Ronald y Rathbone, 2012).

El mecanismo de erosión es de especial relevancia para situaciones en que se desea una liberación rápida en el área de interés. Las matrices poliméricas diseñadas para cumplir con este proceso, por lo general son constituidas por más de una capa polimérica que resguardan el agente activo; es decir, son matrices multicapa. Los componentes que constituyen cada capa de matriz deberán desintegrarse casi inmediatamente después de haber sido insertados en un lugar específico del cuerpo humano (M. L. Bruschi, 2015; Ronald y Rathbone, 2012).

En general, las matrices de liberación de fármacos o compuestos activos desarrollan una combinación de mecanismos para completar su trabajo, dependiendo del diseño de la matriz en cuestión. Uno de los diseños para matrices de reservorio con mayor impacto de aplicación en el área de estudio son los hidrogeles, característicos por combinar varios mecanismos de liberación. Las cualidades de estos materiales para su aplicación en el área biomédica se discuten abiertamente en la siguiente sección.

1.2.3. Hidrogeles como Sistemas de Liberación Controlada

Los hidrogeles son redes poliméricas tridimensionales e hidrofílicas que poseen la capacidad de absorber grandes cantidades de agua o fluidos biológicos y, además mantenerla dentro de su estructura (Dragan, 2014; Memic *et al.,* 2015; Varaprasad *et al.,* 2017). Estos materiales han recibido considerable atención durante los últimos 50 años debido a las cualidades que poseen y que permiten su aplicación en diversas áreas. Se caracterizan por presentar una consistencia suave y con cierta elasticidad (Dragan, 2014), además poseen un grado de flexibilidad muy similar a la de los tejidos vivos debido a su alto contenido de agua (Ahmed, 2015; Dragan, 2014).

Los hidrogeles se pueden clasificar de múltiples maneras. Sin embargo, como los hidrogeles están constituidos básicamente mediante redes reticuladas, se clasifican en dos categorías de acuerdo a su entrecruzamiento: (a) hidrogel físico o autoensamblado e (b) hidrogel químico (Ahmed, 2015; Varaprasad *et al.,* 2017).

1.2.3.1. Hidrogeles Físicos. Los hidrogeles entrecruzados físicamente son geles reversibles que han sobresalido gracias a su producción relativamente sencilla y la ventaja que representa no utilizar ningún agente de reticulación durante el proceso de síntesis. La estructura de estos materiales se conserva debido a interacciones físicas que existen entre las cadenas poliméricas (Varaprasad *et al.,* 2017). Los métodos descritos en la literatura para obtener hidrogeles reticulados de forma física son: congelamiento-descongelamiento, formación de estereocomplejos, interacciones iónicas, enlaces de hidrógeno y maduración (agregación inducida por calor) (Varaprasad *et al.,* 2017).

1.2.3.2. Hidrogeles Químicos. En los hidrogeles entrecruzados químicamente, existen enlaces covalentes entre diferentes cadenas de polímeros. Por lo tanto, tienen alta estabilidad y no se disuelven en ningún solvente a menos que los puntos de reticulación estén escindidos. Con este tipo de reticulación se obtienen materiales con una resistencia mecánica relativamente alta y,

dependiendo del tipo de enlaces químicos presentes pueden ocurrir tiempos de degradación relativamente prolongados. Los diversos métodos descritos para obtener hidrogeles químicos: entrecruzamiento químico (reacción química), injerto, polimerización radical, reacciones de condensación y radiaciones de alta energía (Varaprasad *et al.,* 2017).

Otra vía potencial para la formación de estos hidrogeles es a través de la reticulación enzimática. El entrecruzamiento con enzimas resulta atractivo debido a que estas especies pueden formar enlaces covalentes entre sustratos y además exhiben un alto grado de especificidad hacía ellos. Debido a que la cantidad de enzima presente es uno de los factores determinantes en la velocidad de reticulación global, este tipo de entrecruzamiento ofrece un control cinético de la formación del gel y, por lo tanto, para la formación de hidrogeles homogéneos in situ a través del simple control de la concentración de enzima (Sperinde y Griffith, 1997).

1.2.4. Hidrogeles Constituidos de Polisacáridos

Los polisacáridos son un grupo de biopolímeros a los que pertenecen una gran diversidad de biopolímeros sintetizados naturalmente en plantas, algas, crustáceos marinos, bacterias y hongos. Se caracterizan por poseer gran cantidad de enlaces glucosídicos en su estructura, los cuales enlazan las unidades de repetición constituidas de monosacáridos. Algunas de sus ventajas frente a otros polímeros de origen natural es su estabilidad a diferentes pH y la factibilidad de modificar su estructura química gracias a la presencia de grupos funcionales reactivos a lo largo de la cadena principal. Adicionalmente, la mayoría de los polisacáridos son solubles en agua y por consecuencia, poseen la habilidad de formar hidrogeles, siendo una opción excelente para diseñar sistemas de deliberación controlada (Coelho *et al.,* 2010; Nair y Laurencin, 2006; Omidian y Park, 2012).

Los hidrogeles basados en polisacáridos han destacado considerablemente como componentes de sistemas de liberación sensibles a estímulos, particularmente porque las matrices pueden ser obtenidas de fuentes naturales, mediante procesos bien caracterizados y reproducibles. Los polisacáridos iónicos son relativamente fáciles de entrecruzar para formar redes sensibles a una

gran variedad de variables internas y externas, y de esta manera son aptos para liberar sustancias bioactivas mediante un sistema de encendido-apagado. Esta regulación en la liberación de alguna biomolécula se encuentra en función de un cambio de pH, concentración, intensidad de un campo magnético y eléctrico, longitud de onda, temperatura, potencial redox y ciertas moléculas (Alvarez-Lorenzo *et al.,* 2013).

Entre los polisacáridos más utilizados para el diseño de acarreadores de compuestos activos se encuentran la celulosa, almidón, ácido algínico y ácido hialurónico. De los anteriores, el almidón es el polisacárido más utilizado en la industria farmacéutica. En el año 2012 se registraron cerca de 130 medicamentos que utilizaron este biopolímero como excipiente, sólo en el mercado de Estados Unidos de América. Cabe destacar, que las pectinas y otros polisacáridos como el quitosano y los arabinoxilanos se han estudiado y propuesto como componentes de matrices de liberación controlada de fármacos, tal es caso de hidrogeles (Coelho *et al.,* 2010; Nair y Laurencin, 2006; Omidian y Park, 2012).

A la fecha se han propuesto hidrogeles mixtos de pectinas y acrilamida (Sutar *et al.,* 2008), pectinas y polivinilpirrolidona (Mishra *et al.,* 2008), así como pectinas y calcio para la liberación controlada de diversos compuestos activos (Munjeri *et al.,* 1997), destacando la ausencia de matrices a base de hidrogeles constituidos por dos polisacáridos en conjunto, específicamente pectinas y arabinoxilanos. Considerando el hecho anterior, se abre un área de oportunidad para el estudio de hidrogeles diseñados a partir de estos dos polisacáridos, así como su potencial como acarreador de compuestos activos.

1.2.5. Pectinas como Constituyentes de Hidrogeles

Las pectinas constituyen los componentes más complejos de la pared celular de frutos y vegetales. La función principal de este compuesto es regular la expansión celular, así como de funcionar como una especie de cemento intercelular para controlar la rigidez y elasticidad de la pared celular (Chasquibol Silva *et al.,* 2008; Ovodov, 2009).

Las pectinas son compuestos de gran variedad, estructura y peso molecular. Son heteropolisacáridos constituidos por residuos de ácido galacturónico unidos por enlaces α-(1,4) (GalA), con un grupo carboxilo en el C-6. La cadena principal de este polisacárido puede contener azúcares neutros laterales, por lo que se podría encontrar regiones ramificadas y regiones sin ramificar (Ovodov, 2009; Voragen *et al.*, 2009).

1.2.5.1. Tipos de Pectinas. Las pectinas constituyen los componentes más complejos de la pared celular de frutos y vegetales. La función principal de este compuesto es regular la expansión celular, así como de funcionar como una especie de cemento intercelular para controlar la rigidez y elasticidad de la pared celular (Chasquibol Silva *et al.*, 2008; Ovodov, 2009). Las pectinas son compuestos de gran variedad, estructura y peso molecular. Son heteropolisacáridos constituidos por residuos de ácido galacturónico unidos por enlaces α-(1,4) (GalA), con un grupo carboxilo en el C-6. La cadena principal de este polisacárido puede contener azúcares neutros laterales, por lo que se podría encontrar regiones ramificadas y regiones sin ramificar (Ovodov, 2009; Voragen *et al.*, 2009). De acuerdo al grado de esterificación (GE) de las pectinas, aquéllas con un GE mayor al 50% se denominan pectinas con alto grado de esterificación (AGE); mientras que por debajo de este valor, se consideran pectinas de bajo grado de esterificación (BGE).

1.2.5.2. Propiedades de Gelificación de Pectinas. Las pectinas son moléculas hidrofílicas que contienen grupos polares como lo son los hidroxilos y los carboxilos. Cuando se encuentran dispersas en agua, algunos de los grupos carboxilo se ionizan y se enlazan con agua para formar grupos polares en la molécula. Seguidamente, se da origen a la formación de sol estable, definido como un sólido disperso en agua como fase continua (Vaclavik y Christian, 2007).

Un aspecto crucial para la gelificación de pectinas es el GE, el cual se refiere al porcentaje de grupos carboxilo en los residuos de ácido galacturónico esterificados con grupos CH_3. La primera condición para obtener geles de pectinas con AGE es un ambiente de bajo pH para que los grupos ácidos, minoritarios, se encuentren en forma no ionizada y no exista repulsión entre cargas. Adicionalmente, se requiere la presencia de un co-soluto, usualmente sacarosa al 60%. La función

de la sacarosa es reducir la actividad del agua, aumentando la hidrofobicidad entre las cadenas que en conjunto con un bajo pH reduce la ionización de los grupos, provocando la formación de zonas de unión entre cadenas de pectina (Löfgren y Hermansson, 2007).

En el caso de las pectinas de BGE, el mecanismo de geles es totalmente distinto debido a que las uniones entre cadenas se producen a través de iones de calcio, que forman puentes entre cargas negativas. La concentración de calcio es importante hasta llegar a una cierta cantidad, que depende de cada tipo concreto de pectinas y que se le conoce como "saturación de calcio". Este valor suele encontrarse alrededor de 500 ppm, por encima de esta cantidad no se ha detectado efecto favorable. Complementariamente, se ha determinado que la presencia de azúcar reduce la cantidad de calcio necesaria para llevar a cabo la gelificación.

1.2.5.3. Fuentes de Obtención. En general, todas las plantas contienen pectinas como componente de la pared celular, sin embargo, no todas se consideran materia prima como su fuente de obtención debido a que hay varios factores que estudiar para una selección adecuada. El rendimiento, tiempo y costos de extracción, son considerados como los principales criterios a evaluar. Actualmente, se busca obtener pectinas a partir de residuos y desechos agroindustriales, así como de la industria de alimentos con el objetivo de darles un valor agregado. Unos de los vegetales con una amplia aplicación industrial principalmente en la alimenticia es la remolacha azucarera y cuyos contenidos de pectinas es alto, no sólo en el vegetal fresco también en la pulpa que es obtenida después de su procesamiento industrial (Fernández *et al.*, 2014).

Las aplicaciones industriales alimentarias de la remolacha azucarera son múltiples y diversas. Del procesamiento de la raíz de esta hortaliza se puede producir azúcar refinada gracias a que posee concentraciones elevadas de sacarosa; así mismo, es factible producir levadura, alcohol etílico, bebidas alcohólicas, producción de químicos y farmacéuticos. Algunos de los residuos obtenidos del procesamiento industrial de la remolacha azucarera, tales como hojas frescas, melaza y fibra (pulpa) de raíz, son fuentes ricas en minerales, proteínas, pectinas y azúcares, y han sido empleados como fuente alimenticia para ganado vacuno y ovino (*FAO. Food and Agriculture Organization of the United Nations*, 2018).

1.2.6. Arabinoxilanos como Constituyentes de Hidrogeles

Los arabinoxilanos (AX) son polisacáridos hemicelulósicos que se encuentran en la pared celular de gramíneas y algunos pastos. Su función principal es brindar soporte estructural a vegetales (Mazumder y York, 2010). Su estructura está constituida por moléculas de xilosa unidas por enlaces β-(1,4) y con ramificaciones de monómeros de α-L-arabinofuranosa en α-1,3 y α-(1,2). Algunas arabinosas se hayan enlazadas con ácido ferúlico por enlaces éster, macromoléculas conocidas como arabinoxilanos ferulados (AXF). El grado de ferulación es el índice que determina la relación promedio de moléculas de ácido ferúlico por cada 1000 residuos de xilosa, este indicador es de suma importancia debido a que el ácido ferúlico juega un rol importante en el mecanismo de gelificación de estos componentes (Carvajal-Millan *et al.,* 2005).

1.2.6.1 Propiedades de Gelificación. Los AX pueden ser extraíbles en agua (WEAX, wáter extractable arabinoxylans) o no extraíbles en agua (WUAX, wáter mextractable arabinoxylans). El proceso de extracción de los AX afecta principalmente al peso molecular de estos. En WEAX, los pesos moleculares varían de 10 a 10,000 kDa y para WUAX son superiores a 10,000 kDa. En general, los AX de mayor peso molecular y alto contenido de ácido ferúlico presentan mejores propiedades de gelificación (Carvajal-Millan *et al.,* 2005; Izydorczyk y Biliaderis, 1995).

Los AXF pueden formar geles químicos muy estables a cambios de temperatura y pH. El mecanismo para la formación de este tipo de geles está dirigido por el acoplamiento oxidativo del radical fenoxilo. Este radical puede ser formado por vía enzimática (lacasa o peroxidasa) o química ($FeCl_3$, $KMnNO_4$). Estos agentes oxidantes generan radicales libres sobre la estructura del ácido ferúlico, los cuales al acoplarse forman dímeros y trímeros de ácido ferúlico (Carvajal-Millan *et al.,* 2005). El mecanismo de gelificación seguido por lacasa y peroxidasa difieren en la peroxidasa requiere peróxido de hidrogeno, en cambio la lacasa oxida el sustrato directamente teniendo como aceptor al oxígeno (Zaidel *et al.,* 2012). Por tal motivo, el mecanismo de gelificación enzimática dirigido por lacasa es más conveniente para el diseño de matrices para aplicaciones biomédicas.

1.2.6.2. Fuentes de Obtención. El endospermo y el pericarpio de los cereales son la principal fuente de AX. Los cereales más estudiados como fuentes de AX son trigo, centeno, cebada, avena, arroz, sorgo, maíz y mijo. Es importante mencionar que la estructura molecular de los AX y el grado de ferulación, varían en base a su fuente y proceso de extracción (Izydorczyk y Biliaderis, 1995).

Al igual que en el caso de las pectinas, los AX se pueden extraer de residuos de diversas industrias, principalmente de la alimenticia. Un ejemplo de ello es la recuperación a partir de pericarpio de maíz, en donde se han obtenido hasta un 20% de AX (Carvajal-Millán *et al.*, 2007).

1.2.7. Hidrogeles en la Liberación Controlada Dirigida a Colon

En la actualidad, existe un incremento en la demanda de sistemas de entrega inteligentes para compuestos encapsulados y/o inmovilización que sean liberados en el lugar y el momento apropiado. Al mismo tiempo, la biodisponibilidad oral puede mejorarse eficazmente. De acuerdo con el entorno de cada porción del tracto gastrointestinal, los acarreadores inteligentes como los hidrogeles pueden diseñarse para responder a cambios ambientales como variación de pH y presencia de ciertas enzimas (Chai *et al.*, 2018). Los acarreadores específicos para el colón son diseñados para liberar su contenido cuando su estructura se ha degradado por enzimas de microorganismo colónicos, como es la dextranasa (Garinot *et al.*, 2007).

Una variedad de proteínas, péptidos, polifenoles, vitaminas y probióticos son fácilmente degradados en ambientes altamente ácidos que contienen numerosas enzimas digestivas, como la pepsina en el tracto gastrointestinal. Estas condiciones provocan grandes pérdidas antes de que lleguen al intestino delgado. Con una inmovilización o encapsulación adecuada se obtiene una mejor protección contra la degradación por ácidos y enzimas. De esta forma, los compuestos encapsulados se retienen en el estómago, pero su liberación ocurre en el intestino delgado o en el colón, lo que es esencial para aumentar su bioaccesibilidad (Chai *et al.*, 2018). Un probiótico de gran demanda para la industria alimentaria y que aporta granes beneficios a la salud, es *Saccharomyces boulardii*. Esta levadura a pesar de poseer una resistencia natural a la acides

gástrica del intestino, su grado de supervivencia o viabilidad se ve disminuida hasta el 1- 3 %, después de haber cruzado el tracto gastrointestinal. En base a lo anterior, la inmovilización de esta levadura en una matriz tridimensional a base de polisacáridos, podría mejorar su índice de supervivencia durante su paso por el tracto gastrointestinal.

1.2.8. *Saccharomyces boulardii*

Es una levadura considerada probiótico. Un probiótico es un microorganismo vivo que al ser administrado o ingerido en una concentración adecuada, confiere beneficios a la salud (Partlow *et al.,* 2016). Algunas de las cualidades y aportes benéficos de este agente probiótico son: regulación de la homeostasis microbiana intestinal, interferir en la colonización e infección de agentes patógenos en la mucosa intestinal, modulación local y sistemática de las respuestas inmunitarias, estabilizar las barreras gastrointestinales, inhibir el desarrollo de enzimas procarcinogénicas e inducir la actividad enzimática para promover la absorción de nutrientes (Qin *et al.,* 2018; Thomas *et al.,* 2014; Tranquilino-Rodriguez *et al.,* 2017). Para que este agente probiótico entregue sus beneficios, será necesario que sobreviva al paso y tránsito del todo el sistema gastrointestinal y llegue vivo a su lugar de acción. Sin embargo, la eficiencia también dependerá de la concentración de microorganismos vivos que llegan al colon. Considerando como una barrera limitante el ambiente ácido del estómago, a pesar de que *Saccharomyces boulardii* posee resistencia genética a la acides gástrica, su exposición a este ambiente por un periodo de tiempo mayor a una hora, mata a aproximadamente al 25% de su población. Adicionalmente, estudios in *vivo* en ratas y humanos, han demostrado que menos de 1% de la población total de *Saccharomyces boulardii* administradas puede ser encontrada con viabilidad en heces. Por tal motivo, recurrir a agentes de protección y de soporte para mejor la viabilidad de estos probióticos son un reto importante que pueden impactar en aplicaciones para la industria alimentaria, así como la industria biomédica (Arslan *et al.,* 2015; Graff, Chaumeil, *et al.,* 2008; Graff, Hussain, *et al.,* 2008; Tranquilino-Rodriguez *et al.,* 2017).

1.3. Hipótesis

El atrapamiento de *Saccharomyces boulardii* en microesferas de pectinas feruladas y arabinoxilanos ferulados entrecruzados covalentemente, permite la liberación dirigida de estos probióticos en colon, bajo condiciones *in vitro*.

1.4. Objetivo General

Diseñar microesferas de pectinas feruladas y arabinoxilanos acarreadores de *Saccharomyces boulardii* y evaluar *in vitro* como un sistema de liberación dirigida a colon.

1.5. Objetivos Específicos

1. Extraer pectinas feruladas de un residuo industrial de remolacha azucarera (*Beta vulgaris*) y determinar la composición química y características fisicoquímicas
2. Evaluar la capacidad de gelificación de las pectinas feruladas extraídas, así como caracterizar los geles obtenidos.
3. Diseñar y caracterizar un gel covalente constituido de pectinas feruladas y arabinoxilanos ferulados.
4. Fabricar y caracterizar microesferas de pectinas feruladas y arabinoxilanos ferulados como acarreadores de *Saccharomyces boulardii*.
5. Evaluar *in vitro* la liberación de *Saccharomyces boulardii* contenida en microesferas de pectinas feruladas y arabinoxilanos ferulados como un sistema de liberación dirigida a colon.

1.6. Sección Integradora del Trabajo

La presente tesis de investigación está integrada por 1 capítulo de libro y 3 artículos de investigación originales, que se presentan en los capítulos 2, 3, 4 y 5 del manuscrito. En el capítulo 2 se presenta un capítulo de libro titulado "Pectin nanoparticles: Fabrication and uses" donde se discute ampliamente las características químicas y fisicoquímicas de las pectinas, sus funciones estructurales como parte importante del tejido vegetal, sus fuentes de obtención y extracción; algunas aplicaciones consideradas como "tradicionales" y una sección exclusiva en donde se exploran las aplicaciones más novedosas de nanopartículas de pectinas en áreas de tecnologías emergentes como lo son la medicina, alimentación, construcción y desarrollo urbano, ingeniería ambiental y electrónica. Adicionalmente, el capítulo cuenta con una sección en donde se desglosan las características y fundamentos de las técnicas más novedosas e importantes para la preparación y elaboración de nanopartículas de pectinas. Entre las técnicas presentadas para la preparación de nanopartículas se encuentra la técnica de electroaspersión, técnica utilizada en el presente trabajo de tesis para la obtención de microesferas inmovilizando células de *Saccharomyces boulardii* en su interior. La relevancia de este capítulo radica en presentar un estudio profundo sobre la diversidad de tecnológica que existe para la preparación de nanopartículas, las cuales pueden ser utilizadas para preparar nanopartículas de pectinas u cualquier otro polisacárido o polímero. Finalmente, se presentan una amplia revisión bibliográfica enfocada a las aplicaciones presentes y futuras que tienen las nanopartículas de pectinas, no solo en áreas de conocimiento tradicionales, también en áreas tecnológicas emergentes como la ingeniería electrónica, construcción y ambiental.

El capítulo 3 consiste en un primer artículo de investigación original que da cumplimiento tanto al objetivo general, como a los objetivos particulares 1 y 2. En este primer trabajo de investigación se lograron extraer y caracterizar pectinas feruladas de un residuo agroindustrial de remolacha azucarera proveniente de una industria regional de producción de bioetanol. En breve se describen algunos de los resultados más sobresalientes y las conclusiones más importantes de esta sección. Las pectinas feruladas extraídas del bagazo de remolacha azucarera se obtuvieron con un rendimiento del 4.4% (p/p). Algunas de las características químicas y fisicoquímicas más sobresalientes de las pectinas feruladas fueron las siguientes: un peso molecular de 459 kDa, un

contenido de ácido galacturónico de 52.2%, un bajo grado de esterificación (30%), y un contenido de ácido ferúlico de 2.1 µg/mg de polisacárido. Partiendo del conocimiento de este último dato, se realizaron los cálculos pertinentes para determinar la cantidad de agente entrecruzante (enzima lacasa) a utilizar para preparar geles de pectina ferulada al 4% p/v. A los geles covalentes de pectinas feruladas se les estudió su contenido de ácido ferúlico, dimero y trímeros de ácido ferúlico, su cinética de gelificación y su microestructura. Por su parte, la cinética de gelificación expresó que el tiempo de gelificación (G´=G´´) fue de aproximadamente 5 min, con un módulo de almacenamiento máximo (G´) de 44 Pa y un módulo de pérdida máximo de 0.6 Pa. Finalmente, la microestructura de los geles de pectina ferulada se encontró semejante a un panal de abeja imperfecto con diámetro promedio en sus cavidades de 4.5 ± 1.4 µm. El primer trabajo de investigación que conforma esta tesis fue base para el desarrollo consecutivo del resto de objetivos particulares, principalmente porque la caracterización de las pectinas feruladas demostró que estas contenían ácido ferúlico esterificado, y que presentaban un bajo grado de esterificación. Como resultado las pectinas feruladas extraídas presentaron dos mecanismos de gelificación, uno por interacciones iónicas y el segundo por acoplamiento oxidativo. Ambas cualidades utilizadas en los trabajos de investigación posteriores para el diseño y la preparación de microesferas por la técnica de electroaspersión.

El capítulo 4 consiste en un segundo artículo de investigación original con el cual se da cumplimiento al objetivo general y a los objetivos particulares 3 y 4. El capítulo 4 lleva por nombre "Ferulated Pectins and Ferulated Arabinoxylans Mixed Gel for *Saccharomyces boulardii* Entrapment in Electrosprayed Microbeads" cuyo tema principal es demostrar la formación de un nuevo material constituido por enlaces covalentes entre pectinas y arabinoxilanos por medio dímeros y trímeros de ácido ferúlico, generado por el acoplamiento oxidativo entre sus residuos de ácido ferúlico, ocasionado por enzima lacasa como agente entrecruzante. Adicionalmente, la preparación y caracterización de microesferas mixtas constituidas por pectinas + arabinoxilanos con y sin células de *Saccharomyces boulardii* en su interior, por la técnica de electroaspersión fue uno de los temas centrales. En breve se resumen algunos de los principales hallazgos en este trabajo de investigación. La formación del nuevo gel mixto constituido de pectinas + arabinoxilanos fue demostrado por la formación de nuevos enlaces covalentes conocidos como dímeros y trímeros de ácido ferúlico, así como la disminución del contenido de ácido ferúlico libre en una dispersión constituida de ambos polisacáridos. Los dímeros mayoritarios encontrados en el nuevo gel mixto

fueron el 8-5´, 8-*O*-4´ y 5-5´ con 79%, 18% y 3%, respectivamente. Una vez demostrada la formación del nuevo gel mixto se procedió a preparar microesferas de pectinas + arabinoxilanos con y sin células de *Saccharomyces boulardii* en su interior por la técnica de electroaspersión. Para ello se utilizó la combinación de los dos mecanismos de gelificación presentado por las pectinas feruladas, es decir, interacciones iónicas y entrecruzamiento covalente por acoplamiento oxidativo presentado por los polisacáridos utilizados en este trabajo de investigación. La técnica de electroaspersión para la preparación de las microesferas se ajustó a la necesidad de garantizar la gelificación por ambos mecanismos de gelificación. Como resultado, se logró inmovilizar en el interior de las microesferas mixtas una cantidad de 2.08 x 10^8 células/ mL de *Saccharomyces boulardii*, con morfología estable y bien definida, con un diámetro promedio de 344 µm. Las conclusiones principales de este segundo trabajo de investigación original fueron la factibilidad de preparar microesferas mixtas sin presencia de coalescencia y agregados por la técnica de electroaspersión, así como la opción de inmovilizar células en el interior de las microesferas sin afectar de manera negativa la viabilidad celular durante su procesamiento por la técnica de electroaspersión.

El capítulo 5 consiste en un tercer artículo de investigación original con el cual se da cumplimiento al objetivo general y a los objetivos particulares 4 y 5; así como afirmar la hipótesis del tema de investigación. El capítulo 5 lleva por nombre "Entrapment of *Saccharomyces boulardii* in Ferulated Pectins and Ferulated Arabinoxylans Mixed Gels and Microbeads: Rheological properties, Antioxidant Activity and *in vitro* Fermentation" cuyo tema principal fue dar respuesta a la hipótesis del tema de investigación, para ello se realizó un ensayo *in vitro* de simulación gastrointestinal contemplando la zona de colon como parte final del ensayo. Una muestra de microesferas mixtas con y sin células de *Saccharomyces boulardii* fue sometida al ensayo *in vitro*, encontrando que las microesferas no liberan las células de su interior a su paso por las secciones de boca, estomago, intestino delgado e intenstino grueso. En otras palabras, las microesferas mixtas al estar formadas por enlaces covalentes no sucumben a los cambios de pH, ni enzimas encontradas en dichas secciones. De forma contraria, la liberación de las células de *Saccharomyces boulardii* se presenta en la sección de colon debido a la fermentación de bacterias presentes en la microbiota humana, que tiene la capacidad de utilizar polisacáridos como lo son pectinas y arabinoxilanos como fuente de energía. La liberación de células de Saccharomyces boulardii se demostró con el aumento de CFU comparando una muestra control (microesferas mixtas sin células) y la muestra

problema, en donde las CFU aumentaron de 4.4 x 10^7 CFU/ml a 1.1 x 10^8 CFU/ml en la zona de colon. Adicionalmente, un aspecto destacable observado en las microesferas mixtas fue su capacidad antioxidante aun cuando estas presentas células de *Saccharomyces boulardii* en su interior. Lo anterior les da un plus a estos nuevos sistemas de inmovilización y transporte de fármacos.

2. PECTIN NANOPARTICLES: FABRICATION AND USES.

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CHAPTER SEVEN

Pectin nanoparticles: Fabrication and uses

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7.1 Introduction

Pectins are structural polysaccharides present in the cell wall of fruits and vegetables, with remarkable structural variety caused by the presence of neutral sugars as branches and, consequently, having high molecular weights (Coelho et al., 2010; Omidian and Park, 2012). Pectins are deposit naturally during the early stages of primary cell wall development and can form up to a third of the dry matter of it in plants such as dicotyledons and monocotyledons. The main function of pectins is to provide structural support to plant tissue; additionally, to provide mechanical support and cell turgor, to promote resistance to temperature changes; especially at low temperatures and drought (Parre and Geitmann, 2005). The concentration and structure changes of pectins is largely related to the characteristics of the vegetable and fruits, throughout growth, maturation, senescence and for commercial fruit and produce, storage, and shelf life (Mellerowicz and Sundberg, 2008).

Pectins have been widely used in the food industry as a gelling, thickening, stabilizing, and emulsifying agent (Müller-Maatsch et al., 2016; Rascon-Chu et al., 2016). In addition, this polysaccharide has been noted for its health benefits for its high content of soluble fiber and prebiotic qualities (Gómez et al., 2016; Yang and Xu, 2018). However, the versatility of this polysaccharide emerges from its physicochemical properties, finding great potential to develop in other areas, such as biomedical, environmental technology, even construction and emerging areas such as electronics (Lara-Espinoza et al., 2018).

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In the next sections we will discuss the physicochemical properties, the fabrication techniques, and relevant applications of pectin based nanoparticles. The present review is not an extensive compilation of all fabrication methods and applications of pectin based nanoparticles, but highlights the potential for new knowledge that will impact from short to middle term in the design of pectin nanoparticles for food and nonfood products. Firstly, a little review on the chemical structure of pectins is described. Second, the most reported and representative fabrication methods for pectin based nanoparticles, in the last few years are described. Finally, a discussion of current and potential uses of pectin based nanoparticles is presented.

7.1.1 Chemical structure

Pectins are characterized for having a complex chemical structure, containing considerable neutral sugars. The chemical composition of this polysaccharide is affected by several factors, such as vegetable or fruit type that synthesizes them that is, the source of extraction, extraction conditions, and environmental factors such as geographic location and climate. Likewise, its chemical composition can change due to the action of the harvesting and processing of vegetables and/or fruits (Geerkens et al., 2015; Liu et al., 2015). By definition, pectins are heteropolysaccharides consisting of galacturonic acid residues linked by α- (1,4) (GalA) bonds, with a carboxyl group at C-6. The main chain of this polysaccharide may contain neutral side sugars, so it is possible to find branched and unbranched regions (Ovodov, 2009; Voragen et al., 2009). Considering the above, the chemical structure of pectins has been broadly classified mainly into three regions, homogalacturonan, rhamnogalacturonan I (RG- I), and rhamnogalacturonan II (RG-II) (Mohnen, 1999).

7.1.1.1 Homogalacturonan

Linear region (Fig. 7.1A), consisting mainly of D-galacturonic acid units, linked by α- (1-4) glycosidic bonds. The *O*-2 or *O*-3 positions may be acetylated, and the carboxylic group found at the C-6 position may be partially esterified by methyl groups. On the other hand, free acid groups can be partially or completely neutralized with sodium, potassium or ammonium ions (Liu et al., 2015; Xu et al., 2015).

Based on the proportion of esterified groups distributed along the main chain, an index known as degree of esterification (DE) has been established. Additionally, the degree of amidation has been established to denote the reaction of the GalA group with ammonia, producing carboxamides. Based

on the percentage of DE, pectins are classified as high degree of esterification (HDE) and low degree of esterification (LDE); HDE refers to the ratio where more than 50% of the carboxyl groups must be esterified, while less than 50% of the carboxyl groups esterified corresponds to LDE (Löfgren and Hermansson, 2007). The DE of pectins largely determines the physicochemical properties, such as viscosity, as well as the demands for gel formation. In this sense, it is of utmost importance to consider the type of pectins for the design and preparation of these materials.

7.1.1.2 Rhamnogalacturonan I

It is a family of peptide polysaccharides consisting of repeating units (α-D-GalA-1,2-α-L-rhamnose-1,4) n. This region contains a significant amount of neutral sugars, such as arabinose and galactose, which together form arabinan, galactan, and arabinogalactan attached in position O-4 to rhamnose, which are considered ramifications (Fig. 7.1B) (Yapo, 2011; Palin and Geitmann, 2012). Besides neutral sugars, hydroxycinnamic acids, mainly ferulic acid, may be esterified to arabinans and galactans in the RG-I of some Chenopodiaceae species. The most reported is Sugar beet *(Beta vulgaris)*, and ferulic acid confers pectins the capacity to gel covalently (Renard et al., 1995; Levigne et al., 2004; Ralet et al., 2005).

7.1.1.3 Rhamnogalacturonan II

The RG-II region is characterized by being a compound of repeating D-galacturonic acid units linked by α- (1-4) glycosidic bonds, as the base

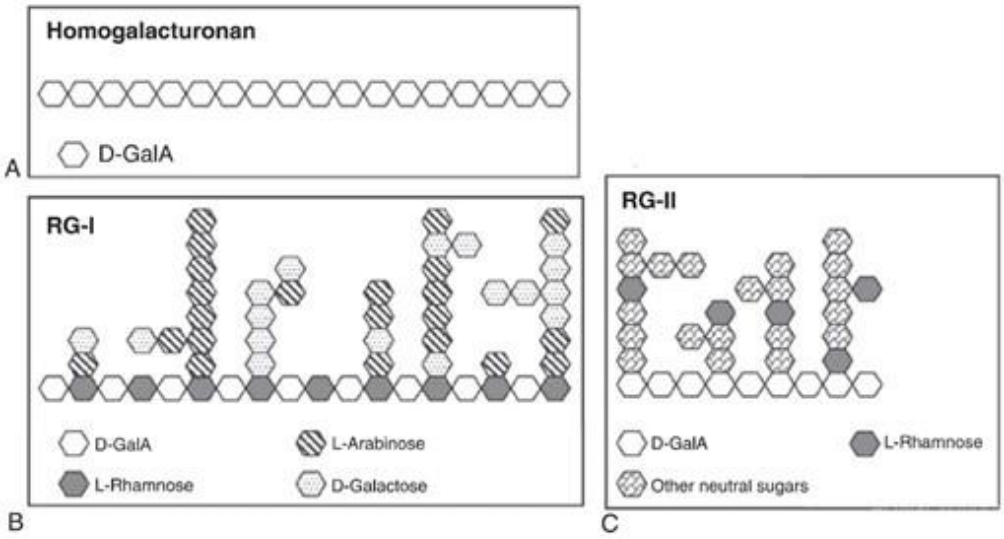

Figure 7.1 General chemical structure of pectin scheme (Modified from Voragen et al., 2009).

structure. This structure is replaced by L-rhamnose, D-galactose, and other neutral sugars rarely found in nature, such as apiosa, 3-*O*-methyl-L-fucosa and 2-O-methyl-D-xilosa (Fig. 7.1C). The RG-II region is the most complex of the three structural regions that make up the pectins, but is also the region with the highest degree of structural conservation among plant species (Voragen et al., 2009; Yapo, 2011; Palin and Geitmann, 2012).

7.1.2 Sources of pectin extraction

Pectins are part of the extracellular wall of almost all plants; however, commercial pectins are extracted mainly from citrus fruits such as lemon, oranges, as well as apples and grapefruit. Commercial pectins are mainly extracted from industrial waste generated by obtaining concentrated juices. Currently, the aim is to obtain pectins from agroindustrial residues and wastes, as well as from the food industry with the aim of giving them added value. Though citric and apple peels are largely the most important raw material, an emerging source with a wide industrial application, mainly in foodstuffs, is sugar beet and whose pectin content is high, not only in the fresh vegetable but also in the pulp that is obtained after its industrial processing (Fernández et al., 2014).

7.1.3 Pectin types

GalA carboxyl groups can be esterified with alkyl, methyl, and acetyl groups (Vaclavik and Christian, 2007). Based on the proportion of esterified groups distributed along the main chain, an index known as degree of esterification has been established. Additionally, the degree of amidation has been established to denote the reaction of the GalA group with ammonia, producing carboxamides (Thirawong et al., 2008). Based on the percentage of DE, pectins are classified as HDE and LDE; to be classified as HDE, more than 50% of the carboxyl groups must be esterified, when less than 50% of the carboxyl groups are esterified are called LDE (Löfgren and Hermansson, 2007). The DE of pectins largely determines the physicochemical properties, such as viscosity, as well as the mechanisms for gel formation.

Pectins can also be classified into splinted pectins that is, ferulic acid-containing pectins forming part of the ramifications of the RG-I region. In this region, ferulic acid is linked to arabinose residues by ester bonds in the *O*-2 position, as well as to galactose residues in the *O*-6 position (Colquhoun et al., 1994). Some vegetables that contain splinting pectins are sugar beet (*B. vulgaris*) (Levigne et al., 2004; Ralet et al., 2005), spinach

(Fry, 1982; Ishii, 1994), amaranth (Bunzel et al., 2005), and quinoa (Renard et al., 1999). The presence of structural ferulic acid in pectins confers an additional gelling mechanism. Esterified ferulic acid can form ferulic acid dimers by oxidative coupling, generating covalent cross-links between RG-I regions of pectins (Figueroa-Espinoza and Rouau, 1998; Ralet et al., 2005).

7.1.4 Gelling properties

Pectins are hydrophilic molecules that contain polar groups such as hydroxyl and carboxyl. When dispersed in water, some of the carboxyl groups ionize and interact with water due to polarity. Subsequently, the formation of stable sol takes place, defined as a solid dispersed in water as a continuous phase (Vaclavik and Christian, 2007). Three mechanisms are currently accepted for pectins gelling. The first two are the most referred and dependent on DE. Thirdly, covalent crosslinking by oxidative coupling is dependent on ferulic acid residue presence in the RG-I region.

As mentioned above, a crucial aspect for pectin gelation is DE. The first condition for obtaining pectin gels with HDE is a low pH environment so the minor acid groups are in nonionized form and there is no repulsion between charges. Additionally, the presence of a cosolute, usually 60% sucrose, is required. The function of sucrose is to reduce the activity of water, increasing hydrophobicity between the chains which together with a low pH reduces the ionization of the groups, causing the formation of binding zones between pectin chains (Löfgren and Hermansson, 2007).

On counterpart, LDE pectins gelling mechanism is totally different for bonds between chains are produced through calcium ions, which form bridges between negative charges. Calcium concentration is important until a certain amount -threshold-is reached, which depends on each specific type of pectin and is known as "calcium saturation." This value is usually around 500 ppm, above this amount no favorable effect has been detected. In addition, it has been determined that the presence of sugar reduces the amount of calcium necessary to carry out gelation.

Finally, ferulated pectins can form chemical gels by the formation of C-C covalent bonds between two or three phenyl rings of ferulic acid. The mechanism for the formation of this type of gels is driven by an enzymatic reaction, where laccase and peroxidase act as oxidizing agents that generate free radicals on the ferulic acid structure, generating the formation of dimers and trimers (Carvajal-Millan et al., 2005). The gelation mechanism generated by both enzymes differ by the oxidizing agent that initiates the reaction. Peroxidase requires hydrogen peroxide, whereas laccase oxidizes the

substrate directly having oxygen as its acceptor (Zaidel et al., 2012). This type of gelation can be controlled based on the concentration of pectins and the amount of ferulic acid residues (by weight) per unit weight of pectins.

7.2 Pectin nanoparticles fabrication

Pectin nanoparticles synthesis involves multidisciplinary strategies and processes from different fields of pharmaceutical, such as biomedical and chemical areas of expertise. Nanoparticle research is therefore an area of great scientific interest due to its potential applications. Methodologies for fabrication like electrospraying, spray drying, nanoemulsions, and others are presented below.

7.2.1 Electrospraying

Electrospraying is a method that uses an electric field for the generation of nanometric droplets. This process (Fig. 7.2) involves subjection of a polymeric solution to an electric field during airflow capillary maintained

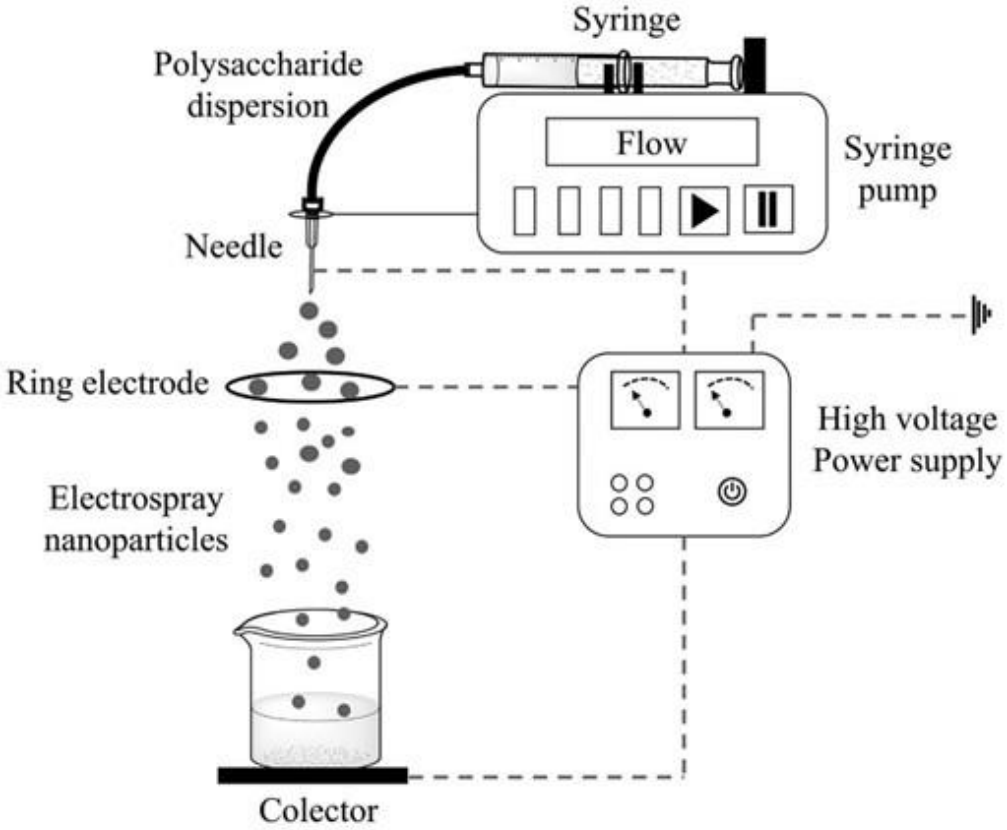

Figure 7.2 Schematic diagram of a basic electrospraying process for nanoparticle fabrication (Alehosseini et al., 2017).

at high potential, upon achieving critical value by an electric field, a jet is formed (Taylor cone). Afterwards, the electric field causes deformation and distribution of jet resulting in fine droplets forming nanoparticles (Rehman et al., 2019). This methodology has many advantages, such as obtention of smaller droplet sizes with a narrow size distribution, less agglomeration and coagulation since charged droplets are self-dispersing in the space, and improved control of motion and deposition efficiency of charged droplets (Faridi-Esfanjani and Jafari, 2016). Disruption of a charged liquid droplet was described by Rayleigh (1882) and showed that the high charge potential forces results in dividing charged droplets into smaller droplets. It was defined as Coulomb fission of the droplets which causes originally dispersed droplets forming many smaller and more stable droplets. Therefore, the main physical forces that govern electrospraying include electrodynamic forces, inertia, gravity, and drag forces. The application of these principles in the last two decades has given rise to many innovative devices and equipment accessories.

New process discoveries have expanded applications known as simple electrospraying (Jahangiri and Adibkia, 2016) use of single capillary (Zamani et al., 2014), double capillary or coaxial (Davoodi et al., 2015) and tricapillary or triaxial (Cao et al., 2014). This technique offers ease in preparation of micro and nanoparticles with tumble compositions, tailored properties of morphology, shape, and size with customized microstructure for a variety of applications (Pawar et al., 2018). Various natural polymer-based nanoparticles have been fabricated successfully. Pectins are suitable for encapsulation because they are biodegradable, biocompatible, and possess high potentials for their modification to achieve the required properties (Lara-Espinoza et al., 2018).

Diaz-Baca et al. (2014) reported the fabrication by coaxial electrospray technique and physicochemical characterization of core-shell microspheres composed of pectin and arabinoxylans as insulin carriers. Afterwards, Rascon-Chu et al. (2018) development core-shell microbeads based on pectin-arabinoxylans as a controlled drug release system for insulin, in this study showed that the beads under optimal conditions were symmetrical and stable without aggregation, with an average particle size of 1.02 ± 0.24 μm. On other field of application, Jayakumar et al. (2014) generated collagen-pectin microparticles in a size range less than 500 nm, by an electrospray forming, where cell viability of fibroblast proliferation was found to be significantly increased for collagen–pectin microparticles compared to control. Also, Hsu et al. (2012) developed Eudragit-S100 coated pectin-alginate microspheres with cisplatin for colon drug delivery, they showed markedly reduced drug release in simulated gastric conditions compared to

the non-coated. Similar strategies were reported by Subudhi et al. (2015). In brief, the different advances made in the fabrication of pectin nanoparticles, microparticles, microbeads, and nano and microspheres generate enough support for the future development of innovative matrices and delivery vehicles.

7.2.2 Spray drying

Spray drying is a technique that has been used to obtain dry powders for a wide variety of industries (pharmaceutical, cosmetic and food), due to being highly reproducible and scalable (Miranda-Linares et al., 2020). It is a technique frequently applied to increase the stability of the emulsification of food ingredients such as flavonoids or nutritional oils (Gharsallaoui et al., 2007). Above all, in the food industry, it has been mainly used to obtain functional re dispersible powders.

Locali et al. (2019) encapsulated essential oil of pink pepper (*Schinus terbinbinifolia Raddi*), using pectin and soy protein. Since the presence of pectin is important to help proteins in the emulsification and stabilization of oil during spray drying, avoiding oil losses during its microencapsulation. In the same way, they mention that the addition of pectin allowed a more homogeneous distribution of particle sizes, in addition to the fact that the double-layer powders had higher oil retention and better shelf life properties. Moser et al. (2020) used spray drying technology to encapsulate buriti oil in microcapsules of chickpea protein and high methoxyl pectin (CP-HMP). However, the encapsulation efficiency (EE) of the CP-HMP microcapsules was higher than that of the CP microcapsules, suggesting a synergistic effect. It should be noted that the spray drying temperature did not affect the EE.

On the other hand, the technique has been improved during recent years, developing new alternatives for nanoparticle manufacturing, such as nano spray drying or nano spray. Using equipment that uses a vibration mesh spray technology, creating small droplets (before evaporation) in one order of magnitude smaller size than conventional spray dryers (Wang et al., 2016a). This technology has a simple and efficient process for preparing hollow polymer particles. Due to the diffusion rate of the polymeric solute being slower than the solvent during the process, it leads to an accumulation of it on the surface of the spray droplet, resulting in the formation of a solid cover (Vehring, 2008).

In the pharmaceutical industry, spray drying plays a key role in converting pharmaceutical formulations to liquid powder form. Studies carried out by

Wang et al. (2016a) developed solid lipid nanoparticles (SLNs) with soy lecithin as a natural emulsifier, followed by the subsequent coating with sodium caseinate and pectin, using Spray-dried Nano Spray Buchi B-90 to obtain ultrafine powders of SLN. From which particles were obtained in a spherical shape and with a smooth surface, which was shown to be redispersible in water without variation in dimension, shape, and morphology. In addition to preparing SLN with double-layer biopolymer coating (DL) caseinate (NaCas) and pectin, to encapsulate and administer curcumin, the DL coating between NaCas and covalently bound pectin, using two crosslinkers: glutaraldehyde (GA) and 1-ethyl-3- (3-dimethyl aminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS). This cross-linking process further assisted in the spray drying of SLN by the formation of homogeneous dust particles (Wang et al., 2016b).

Together with the above, Wang et al. (2017) designed a series of formulations for solid lipid nanoparticles (SLN) and nanostructured lipid transporters (NLC) with sodium caseinate (NaCas) as emulsifier and pectin as coating, with minimal addition of a synthetic surfactant, Tween 80. Their results demonstrated a novel strategy for preparing small, homogeneous SLNs with exceptional gastrointestinal stability and high load capacity as a possible oral delivery system. Next, Wang et al. (2016c), also carried out a work of SLN and NLC (nanostructure) with five polysaccharides, (pectin, gum Arabic, alginate, carboxymethyl cellulose (CMC, 90,000 Da), and carrageenan). Therefore, solid lipid nanoparticles and the nanostructures that were coated with pectin and gum arabic showed a smaller size and a more homogeneous size distribution of approximately 200 and 0.25 nm. In this paper, they similarly evaluated nano-spray drying technology for its suitability to produce ultrafine, stable powders with minimal toxicity.

It should be noted that pectin as a vehicle (wall material) for nanoencapsulation is useful for targeted administration and protection of bioactive ingredients. Besides, it is beneficial to control the rate of release and the targeted administration of therapeutic agents (Rehman et al., 2019). Moreover, in the biomedical area, Cicco et al. (2014) used nano spray drying technology to produce a gentamicin sulfate (GS)-based nanoparticle powder loaded with an alginate-pectin mixture, with the ability to gel quickly into the cavity of a wound. This to achieve the specific controlled release of antibiotics. Similarly, nano spray drying technology has been successfully used to produce stable submicron powders, loaded with a peptide derived from ANXA1 AC2-26. Del Gaudio et al. (2020) conducted wound healing tests *in vitro* studies on HaCaT cells, a study that showed a potential

application of alginate-pectin particles loaded with Ac2-26, as a dressing for wound healing processes. In studies by Serfert et al. (2013) they mention that there is a synergistic effect of low methoxyl pectin and β-lactoglobulin on the oxidative stability of lipophilic ingredients in the liquid state, in the same way in spray drying state, in addition to showing high efficiency of microencapsulation in all particles dried by spray.

Spray drying has also been applied to develop powder formulations of functional instant drinks from pectin-casein with built-in mint oil as a possible antimicrobial agent (Wang et al., 2016d). Similarly, the feasibility of spray drying technology to produce nanoscale powders from the dispersion of low-density pectin-lipoprotein nanogels has been demonstrated, avoiding irreversible gelling (Miranda-Linares et al., 2020).

Xue et al. (2017) developed a novel method of preparation of SLN without organic solvents and synthetic surfactants, from natural ingredients, using stearic acid as a solid lipid matrix and sodium caseinate with pectin as an emulsifier and natural stabilizers, SLNs prepared without organic solvent exhibited better physicochemical properties than SLNs prepared with an organic solvent. Ultrafine powders showed excellent dispersibility when produced by nano spray drying. In addition, microwave-treated pectin has been used for the formulation of nanoparticles. Pectin was dried with nanospray. The nanoparticles were characterized by an average size of less than 600 nm, considered appropriate for use as nanocarriers of cancer therapies concerning the permeation and retention attributes of the tumor vasculature (Zainudin et al., 2018). However, despite the uses and advantages of the spray drying technique, electrospray could produce smaller particles compared to nano spray drying (Pérez-Masiá et al., 2015).

7.2.3 Nanoemulsions

Nanoemulsions are a mix of two or more immiscible liquids, they can be oil in water (O/W), water in oil (W/O), both liquids can act as stabilizers for the use as an appropriate surfactant. Surfactants are amphiphilic molecules that confer stability to nanoemulsions by reducing interfacial tension and prevent droplet aggregation. They tend to quickly absorb in the interface water-oil providing double or steric electrostatic or electrostatic stabilization (Mason et al., 2006).

Nanoemulsions are defined as colloidal dispersions of two immiscible liquids that are thermodynamically unstable. One of the liquids forms the dispersed phase and the other the dispersion medium (McClements, 2012).

Nanoemulsions are considered when the drops formed have a diameter in a radius of 10–200 nm or up to 500 nm in each drop (Kumar et al., 2019). The main components for a nanoemulsion system are oils, lipids, surfactants, water-soluble cosolvents, and water. The oils used in nanoemulsions can include triglycerides, tri-, di-, or mono-acylglycerols, vegetable oils, mineral oils, and free fatty acids (Gonçalves et al., 2018). Surfactants are used to decrease or have a negative interfacial tension, as well as co-solvents. The nanoemulsions have the characteristic of having long-term physical stability, being a consequence of the small size of the drop, which reduces the occurrence of conventional destabilization phenomena such as cream formation, sedimentation, and coalescence (Singh et al., 2017).

Nanoemulsions can be made by different methods of which high energy methods are widely used since they carry out the application of strong disruptive forces that break with large droplets, reducing their size and producing nanoemulsions with high kinetic energy, besides that, the generation of these provides greater control of the stability, rheology, and the emulsion color. These disruptive forces are created by mechanical devices such as ultrasound, microfluidizer, and high-pressure homogenizers, which exert a homogeneous high-energy flow. In turn, the formation of nanoemulsions depends on their composition, type of homogenization, and the conditions of homogenization such as energy intensity, time, temperature, and in some cases pressure (Singh et al., 2017; Rai et al., 2017; Mungure et al., 2018; Kumar et al., 2019).

7.2.3.1 High-pressure homogenizers

High-pressure homogenizers supply high energy and provide homogeneous flow to generate the smallest particle sizes (Kumar et al., 2019). They are used to create intensely disruptive forces that form extremely low particle size nanoemulsions (up to 1 nm) (Rai et al., 2017). The coarse emulsion is then passed through a small hole with high pressure (500 to 5000 psi) (Kumar et al., 2019).

7.2.3.2 Microfluidizer

A microfluidizer, as shown in Fig. 7.3, is a technology that mixes at the micron size level, using hydraulic shear, impact, wear, intense turbulence, and cavitation, to achieve drop size reduction. Furthermore, fluids are forced to pass through the microchannels under the influence of a high-pressure displacement pump in an interaction chamber (500–50,000 psi), resulting in very fine droplets. In the interaction chamber, two macroemulsion streams

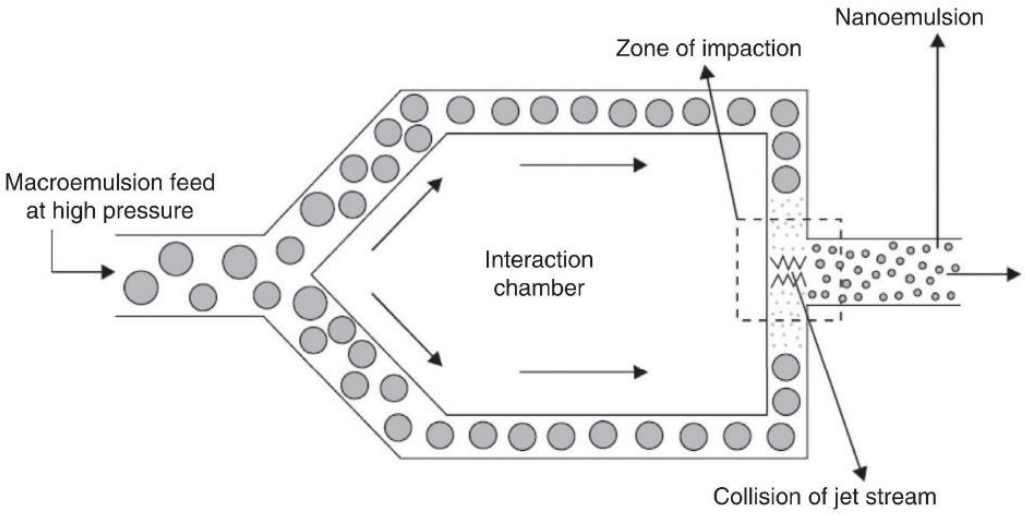

Figure 7.3 Microfluidization nanoparticle fabrication technique (Singh et al., 2017).

hit each other at high speed due to a collision that generates forces such as shear, cavitation, and impact, which produce stable nanoemulsions with a low concentration of surfactant (Singh et al., 2017; Kumar et al., 2019).

7.2.3.3 Ultrasound

Ultrasound methods depend on the emission of high-frequency sound waves (20 kHz and higher), the physical shear is mainly provided by the acoustic cavitation process that breaks the macroemulsion down to nanoemulsion by intense disruptive force from the tip of the sonicator immersed in the sample. The implosion creates shock waves, which in turn create a jet stream of surrounding liquid, pressurizing the dispersed droplets, and effecting their size reduction. It has been observed that by varying the ultrasonic energy input and time, the particle size is reduced and the stability of the nanoemulsion is increased (Fig. 7.4) (Singh et al., 2017; Kumar et al., 2019).

7.2.3.4 Low energy emulsion methods

Low energy emulsion techniques produce thermodynamically stable nanoemulsion (Shakeel et al., 2012), using the stored energy of the system to form small droplets. Emulsification using these approaches occurs by changing the parameters (temperature or composition) that affect the hydrophilic-lipophilic balance (HLB) of the systems (Rai et al., 2017).

7.2.3.5 Phase inversion emulsion method

In this method, the spontaneous curvature of the surfactant causes the phase transition during the emulsion process. Changes in the spontaneous

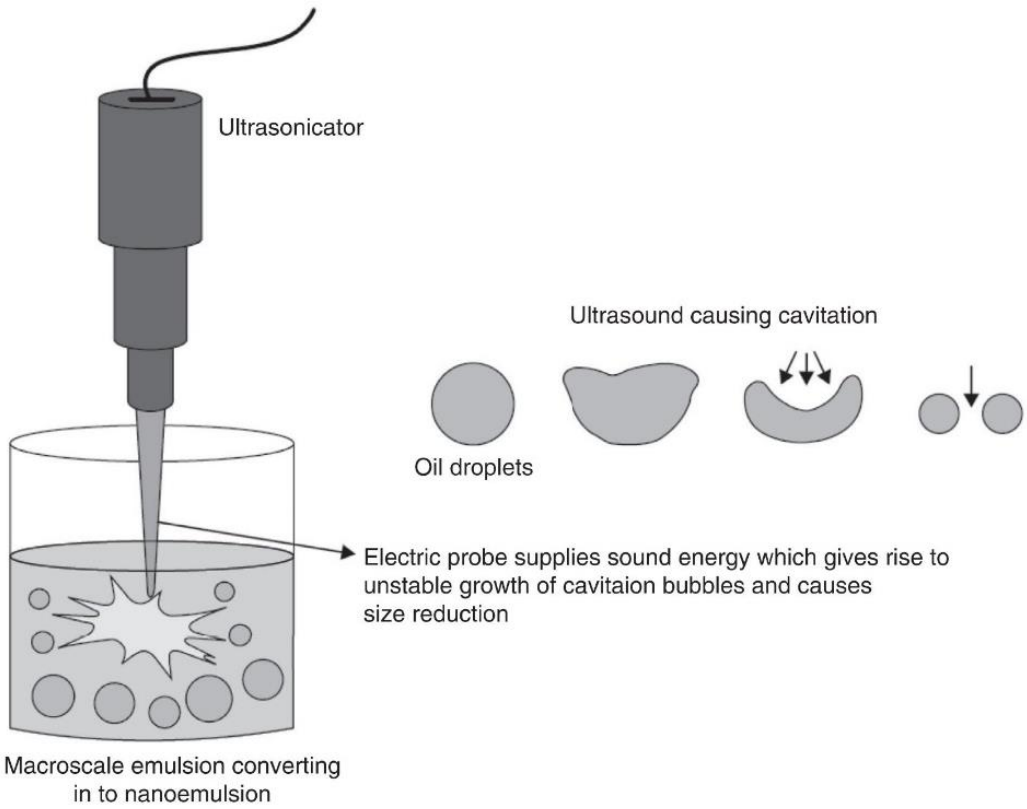

Figure 7.4 Scheme for ultrasonication nanoparticle fabrication technique (Singh et al., 2017).

curvature of the surfactant occur due to changes in parameters such as temperature, composition (Solé et al., 2010). It is based on the specific property of temperature-sensitive emulsifiers, such as the ability to change the distribution affinity for water and oil depending on the progressive change in system temperature (Rai et al., 2017). Rapid cooling of the system produces kinetically stable nanoemulsions with a narrow droplet size distribution (Aveyard et al., 1986).

7.2.3.6 Auto nanoemulsification method

In the auto-emulsification method, nanoemulsion formation is achieved without changing the spontaneous curvature of the surfactant. Surfactant and/or cosolvent molecules rapidly diffuse from the dispersed phase to the continuous phase, causing turbulence and creating nano-sized emulsion droplets (Fig. 7.5) (Solans and Solé, 2012).

7.2.3.7 Pectin nanoemulsions

Some studies have implemented the use of pectin as a surfactant for its stabilizing properties in emulsions, as it is an amphiphilic molecule, reducing

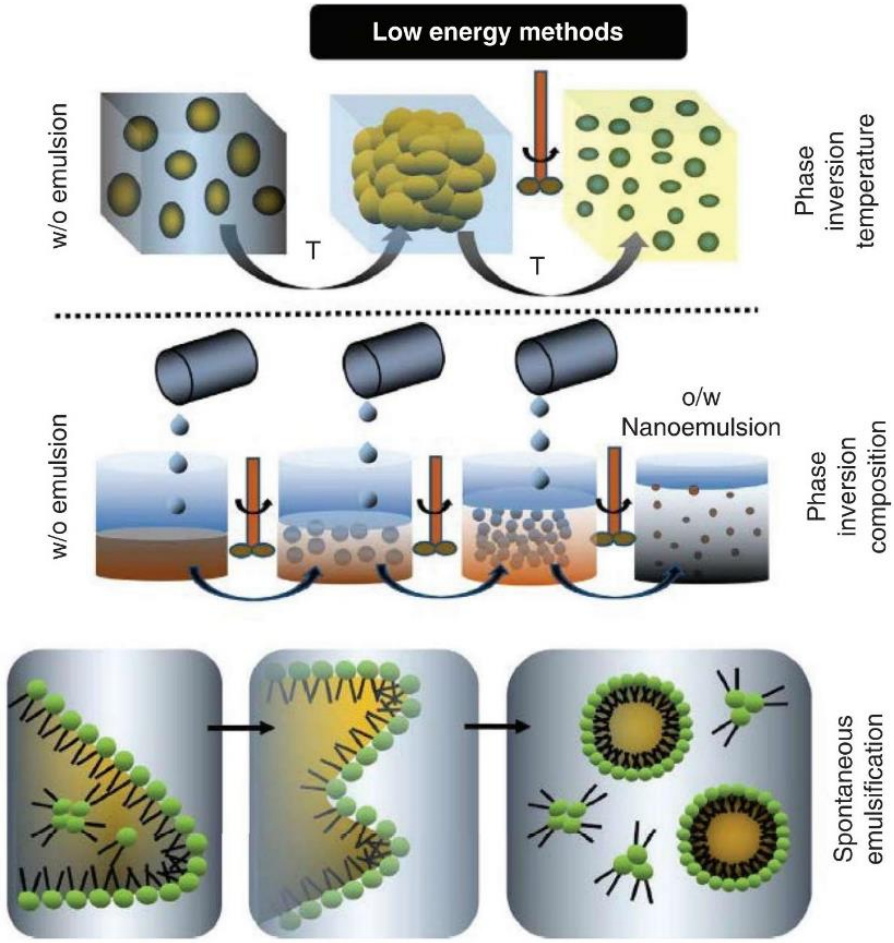

Figure 7.5 Schematic representation of the method of preparation of nanoemulsion by low energy methods (Rai et al., 2017).

the interfacial tension between oil and water (Burapapadh et al., 2012). High methoxyl pectin molecules are more hydrophobic nature providing an active surface and enhancing their activity as an interfacial agent. Beet pectin has excellent emulsifying properties due to the ramifications like protein residues and acetyl groups that it presents (Burapapadh et al., 2012; Guerra-Rosas et al., 2016).

In a study by Norcino et al. (2020), they prepared oil-in-water (O/W) emulsions by adding citric pectin (CP) at 1%, 3%, and 6% (weight of water) and Tween 80 (1% by weight of CP) to ultrapure water. Both phases were mixed by magnetic stirring at 3400 rpm for 1 min at room temperature to obtain a thick emulsion. To obtain the nanoemulsion, the ultrasonic emulsion by the sonicator probe at 20 kHz and 150 W was used. The probe of the apparatus with a diameter of 15 mm was immersed in the thick emulsion

with a depth of 25 mm for 5 min. In this study, they observed that the increase in the concentration of CP favors the reduction in the size of the drops.

In another study by Guerra-Rosas et al. (2016), they formulated emulsions with essential oils of oregano, thyme, lemongrass, and mandarin. They dissolved high methoxyl pectin (1% w/v) in the water at 80°C, with continuous stirring. Then performed primary emulsions by mixing the aqueous solution of pectin and the essential oil (2% v/v) and Tween 80 (5% w/v) employing an Ultraturrax digital T-25 at 9500 rpm for 2 min. They obtained the nanoemulsions by microfluidization at 150 MPa for five cycles. The nanoemulsions were cooled at the outlet of the microfluidization unit through an external coil immersed in an ice-water bath, maintaining a temperature of 10°C. In this work, the results showed stable long-term nanoemulsions.

In a study by Celli et al. (2019), they carried out the preparation of nanoemulsions (O/W) with hydrocolloids: low methoxyl pectin, high methoxyl pectin, λ-carrageenan, and κ-carrageenan with concentrations of 0.025 and 0.05% (w/w) with 10% corn oil, 1% (w/w) fractionated lecithin, and Tween 80 0.5% (w/w) in the oil phase, providing a slight excess of surfactant required to emulsify the corn oil. The aqueous phase consisted of a 10 mM citrate buffer with or without the hydrocolloids (pH 3). Both phases were equilibrated at 60°C in a water bath for 1 h to solubilize the lecithin and hydrate the hydrocolloids, after which they were combined immediately before coarse homogenization at 10,000 rpm for 2 min with an Ultra Turrax T25 digital. They carried out the size reduction bypassing the emulsions five times through a Nano DeBEE benchtop electric laboratory homogenizer at a pressure of 35,000 psi.

Several studies have demonstrated the potential of using pectin to improve the stability of nanoemulsions against environmental and process stresses, high temperatures, and different pHs. The stability of the nanoemulsions prepared by pectin depends on the type of pectin, the degree of esterification (DE), and the concentration of pectin in the nanoemulsion formulation (Mungure et al., 2018).

7.3 Applications and perspectives of pectin nanoparticles

The physicochemical characteristics of pectins make it a versatile polysaccharide with diverse and broad technological applications in diverse fields. A traditional area of application for pectins is in the food industry,

where is used mainly as an emulsifying, thickening, stabilizing, and gelling agent (Vaclavik and Christian, 2007; Yapo, 2009; Gómez et al., 2016; Yang and Xu, 2018). Despite its wide use in this area, this polysaccharide continues to be studied for the development of new food products.

Additionally, pectins are viewed as a promising biopolymer for product development in emerging areas of science, such as biomedical, building materials, environmental technology, and electronics. For this, the following sections describe some of the most prominent uses and applications in each of the aforementioned areas; highlighting trends and areas of opportunity proposed by the authors.

7.3.1 Trends in food applications

In the food industry, pectin continues being used for its excellent texturizing, thickening, emulsifying, and gelling properties. The future of this polysaccharide in this field continues to be promising due to its health benefits, as a prebiotic (Gómez et al., 2016; George Kerry et al., 2018; Yang and Xu, 2018), antiobesogenic (Gómez et al., 2016), and antioxidant (Zhang et al., 2016a; Zhang et al., 2016b; Lara-Espinoza et al., 2018; Merheb et al., 2019); thus, the development of new food products and manufacturing strategies are increasing in number.

Following this context, in a study conducted by Namir et al. (2015) the addition of pectin extracted from tomato pomace to fresh beef hamburgers was evaluated. The main purposes of pectin addition were to act as a texturizer and decrease the fat load in the meat used for the preparation of hamburgers. The authors reported that a 25% substitution (in weight) with pectin did not negatively affect the sensorial properties of the product, reducing the fat load by adding fiber with beneficial contributions to health.

Several studies have shown that pectin can form nanoparticles suitable for the encapsulation and controlled delivery of bioactive compounds like vitamins, folic acid, hesperetin or food preservatives like nisin, and this trend has been increasing within the food industry (Madziva et al., 2006; Fathi and Varshosaz, 2013; Zhao and Zhou, 2016). Nanoencapsulation presents several advantages over microencapsulation and other systems. Said advantages includes (1) faster dissociation, (2) higher surface area compared to mass proportion, (3) higher intracellular uptake, (4) pass along the smallest body fenestrations, among others. Nanoparticles loaded with vitamins are being studied recently in many natural polymers, since vitamins are bioactive

molecules very sensitive to degradation. In this regard, the bioavailability of these compounds can be compromised during the process of absorption. Currently, nanoencapsulation is a promising method for adequate delivery of vitamins protecting them from harmful agents like heat and oxidants (Katouzian and Jafari, 2016). Nevertheless, Chau et al. (2007) pointed out some recent food applications of nanotechnology, safety and risk problems of nanomaterials, routes for nanoparticles entering the body, existing regulations of nanotechnology in several countries, and certification system of nanoproducts. In this regard, pectin is present in solutions used to prevent hypovolemic shock due to blood loss during World War II (Hartman et al., 1941; Popper et al., 1945). At the time, the studies showed pectin was excreted in urine. Research should be undertaken to fully determine the fate of pectin and other molecules that enter the body as nanoparticles, especially if nanostructured pectins are still excreted in urine would ease side effects in human health concerns. Consumers are growing skeptical when "nano-something" is to appear in food labels.

Following this context, nisin, a small cationic peptide, is largely employed in the food industry as a food preservative for its wide-spectrum antimicrobial activity. Nevertheless, the antimicrobial activity in some food products can be reduced by interference with some food components such as lipids or enzymatic degradation of this peptide. To improve the stability and extend the efficacy of antimicrobial activity of nisin in food products, the encapsulation in food-grade biopolymers and delivery of this peptide has been proposed and studied. Khaksar et al. (2014) evaluated the release of nisin encapsulated in microparticles of alginate-high methoxylation pectin. The particles showed a spherical structure and the nisin content influenced the microparticles size and the encapsulation efficiency and loading capacity. The nisin release showed a sustained release profile following a Fickian diffusion mechanism. The authors concluded that the pectin-alginate complex can act as a carrier for the controlled release of nisin acting as an antimicrobial agent in food products. Pectin nanoparticles loaded with nisin were also studied by Krivorotova et al. (2016). The authors established that methoxylation degree had a great influence on the nisin loading capacity and particle size. Also, the antimicrobial activity was dependent on the type of biopolymer used. The authors inferred that pectin nanoparticles are suitable candidates to be used as an antimicrobial agent in food products.

Moreover, hesperetin, a natural potent antioxidant, shows low solubility in water and has a bitter taste that makes impossible its use for food fortification. In order to counter this, the encapsulation of this antioxidant

in nanoparticles of LDE pectin and other biopolymers was evaluated. The nanoparticles were able to incorporate said antioxidant into polysaccharides matrix and results showed that nanoparticles presented high zeta potential and excellent stability against aggregation. Also, the sensorial analysis showed that nanoparticles loaded with hesperetin can be used for milk fortification, improving its physicochemical characteristics (Fathi and Varshosaz, 2013). Furthermore, Zimet and Livney (2009) evaluated the incorporation of an essential oil on a beta-lactoglobulin-low methoxyl pectin nanocomplex to protect it from oxidation and improve its water solubility. The authors found that the lactoglobulin-pectin nanocomplex provided good protection against the degradation of the fatty oil during a shelf-life stress test and also presented good colloidal stability with a mean particle size of 100 nm. The nanoparticles showed the ability to encapsulate essential fatty acids of long chain and can be useful to enrich acid nonfat drinks.

The trend for the use and applications of pectins in the food area is visualized in two aspects, the first includes the addition of pectins to food matrices and finished products to decrease the content of fats and sugars, without modifying the sensory qualities of the product. The second aspect is the design of new biodegradable vehicles of molecules of interest for the industry, like food packaging, containing micro or nanoparticles of pectin to provide protection. An area of opportunity for the generation of scientific knowledge is proposed to study the effects of using pectins for the design of the aforementioned materials.

7.3.2 Trends in pharmaceutical applications

The ability of pectin to form hydrogels and capability to interact with diverse molecules has allowed it to have a wide field of study in the design of systems for the delivery of drugs and bioactive compounds. Pectin has shown some advantage concerning other natural polymers mainly due to its stability to pH changes, feasibility to structural modifications, and also, the capacity to gel under three different gelling mechanisms, mentioned above (Nair and Laurencin, 2006; Coelho et al., 2010; Omidian and Park, 2012). Some of the most relevant applications for this versatile polysaccharide are shown in Table 7.1. Based on the scientific evidence presented here, the current trend for the use of pectins in the biomedical/pharmaceutical field is focused mainly on the design of systems for the detection/immobilization of biomolecules of interest (Lin et al., 2013; Devasenathipathy et al., 2014; Zhang et al., 2015a; Tummalapalli et al., 2016; Markov et al., 2017; Kokulnathan et al., 2018).

Table 7.1 Relevant application of pectin nanoparticles/nanocomposites in pharmaceutical/biomedical industries.

Application	Source/material	Reference
Dopamine determination	Commercial pectin (HDM)	Devasenathipathy et al., 2014
Dopamine/paracetamol determination	Commercial pectin	Kokulnathan et al., 2018
Drug release system	Pectin-lysozyme	Lin et al., 2015
	Pectin-dihydroartemisinin/ hydroxycamptothecin	Liu et al., 2017
	Pectin-doxorubicin	Ye et al., 2018; Tian et al., 2016
	Protein-pectin	Kaushik et al., 2019
	Silver-pectin	Kodoth et al., 2019
Type 2 diabetes mellitus	Pectin	Chinnaiyan et al., 2018a
Glucose adsorption	Pectin	Chinnaiyan et al., 2018b
Insulin release	Pectin-chitosan	Maciel et al., 2017
Fat substitute	β-Lactoglobulin-pectin	Noreen et al., 2017
Iron supply system	Magnetite-pectin	Ghibaudo et al., 2019
Skin tissue scaffold	Pectin- chitosan-PVA	Lin, H. Y. et al., 2013
Wound dressing composite	Pectin (LDM)-gelatin	Tummalapalli et al., 2016
Bone tissue engineering	Calcium phosphate-pectin	Zhao et al., 2016
Bone reconstruction	Banana peel pectin	Gopi et al., 2014
Bone regeneration:	Pectin-poly(lactide-co-glycolide)	Liu et al., 2004
	Pectin-coated titanium	Kokkonen et al., 2008
Antibacterial activity:	Banana peel pectin-hydroxypatite	Gopi et al., 2014
	Opuntia ficus-indica peel pectin-hydroxypatite	Gopi et al., 2015
Tissue engineering	Pectin-silver	Vedhanayagam et al., 2017

HDM, high methoxylation pectin; LDM, low methoxylation pectin; PVA, polyvinyl alcohol.

Moreover, pectins are considered the most promising components for colon-directed drug delivery, because pectin can be used as a mucoadhesive excipient, combined with resistance to upper gastrointestinal media and easily degraded by pectinases produced by colonic microflora (Villanova et al., 2015; Khotimchenko, 2020). For instance, Thirawong et al. (2008) used self-assembling pectin-liposome nanocomplexes to improve intestinal absorption of calcitonin; they attributed the success to the ability of pectin to adhere to the mucus layer and prolong retention in the intestinal mucosa.

On the other hand, the molecular weight of pectins is related to different benefits. Pectic oligosaccharides (POS), produced by pectin depolymerization (Holck et al., 2014) have prebiotic, antibacterial, anticancer, and antioxidant activities (Zhu et al., 2019). Specifically, cancer has gained much attention and reviewed extensively (Leclere et al., 2013; Zhang et al., 2015b). Therefore, within the pharmaceutical industry, pectins have great advantages and the application of nanoparticles in the drug release system exhibits better absorption by cells than larger macromolecules (Thankappan et al., 2020).

Lin et al. (2015) developed nanoparticles from lysozyme-pectin through self-assembly, as a methotrexate encapsulator (MTX). The nanoparticles underwent an MTT assay (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide), which indicated that encapsulated MTX exhibited higher anticancer activity than free MTX. Also, Liu et al. (2017), developed by self-assembly nanoparticles based on pectin-dihydroartemisinin conjugates for the administration of anticancer drugs for release targeted to tumor sites. Producing pectin-dihydroartemisinin/hydroxycamptothecin (PDC-H NPs) nanoparticles with a particle size of 70 nm.

Besides, in recent years, pectin has been used as carrier material for doxorubicin (DOX) as a model drug. DOX-loaded pectin nanoparticles have been shown to have a stronger inhibitory rate in test cancer cells than the DOX solution (Tian et al., 2016). Apparently, there was a synergistic effect. In other studies, self-assembled hybrid nanoparticles based on pectin-doxorubicin conjugates (PDC-NP) were evaluated on hepatocellular carcinoma. Remarkably, PDC-NP showed to reduce the lethal side effect of DOX in athymic BALB/C nude mice. H&E staining and serum biochemistry further confirmed the excellent biological safety of PDC-NP (Ye et al., 2018).

In a different approach, Kaushik et al. (2019) reported the use of pH-responsive hydrogels of hierarchically self-assembled protein (zein, in the form of its nanoparticles of size 80–120 nm) and polysaccharide (pectin),

for the encapsulation and release of anticancer drug doxorubicin (DOX) in the cell nucleus. Doxorubicin drug loaded hydrogels exhibited superior cytotoxicity toward cervical cancer cell lines by inducing intracellular-antioxidative stress-based apoptosis.

Moreover, studies conducted by Chinnaiyan et al. (2018a) produced pectin nanoparticles loaded with metformin to evaluate their sustained action for the management of type 2 diabetes mellitus. Obtaining 482.7 nm nanoparticles with spherical shape and zeta potential of +38.85 mV, the results demonstrated a possible advantage in prolonged release to achieve a reduced dose frequency. Subsequently, the same authors (Chinnaiyan et al., 2018b) evaluated the synergistic effect of pectin-chitosan nanoparticles with metformin, with special emphasis on the effect of the drug with biopolymers. This showed a higher Metformin content 92.1% to 3.3%. The pectin-coated nanoparticles had a smooth spherical morphology with a size of 581.8 nm. Nanoparticles showed healthier biodistribution at the same time the ability to penetrate vital organs. The synergistic effects of the drug and biopolymers were due to their corresponding mechanism to improve glucose absorption, minimizing adverse effects during diabetic therapy.

In the same, nano and microparticles of pectin-chitosan have been manufactured as an insulin encapsulator. Insulin release was evaluated in vitro in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Showing controlled release for 2 hours in pH 6.8 (SIF) and less than 13.0% was released in SGF (pH 1.2, 2 h). It is therefore offered as an alternative route for oral administration of insulin with good stability under acidic (stomach) and basic (intestinal) conditions (Maciel et al., 2017). It should be mentioned that pectin has been used as drug vehicles for indomethacin, penicillin, acetaminophen, ketoprofen, ambroxol, 5-fluorouracil, sulfamethoxazole, in addition to drugs for the treatment of Alzheimer's as donepezil (Wong et al., 2011; Kodoth et al., 2019; Khotimchenko, 2020).

As additional information, Noreen et al. (2017) collected information, mentioning the use of pectin to prepare nanoparticles of β-lactoglobulin-pectin (d = 200–300 nm) to be useful in drug delivery systems or fat substitutes in the food, pharmaceutical, cosmetic and other industries. Also, molecular interactions between lactoglobulin (LG) and sugar beet pectin (SBP) have also been investigated using high-performance size exclusion chromatography (HPSEC). The soluble complexes between LG and SBP were formed when combined under relevant physiological conditions, resulting in an increase in molecular weights and altered forms compared

to LG and SBP. This study hypothesized that approximately half of the interacting LG-molecules (about 3.5%) they bind to a small number of non-covalently joined ferulic groups, possibly present in SBP. These ferulic remains in SBP were released after their strong interaction with LG, both in native oligomeric/polymeric states and heat-induced (Qi et al., 2014).

Thankappan et al. (2020) investigated the potential of the biopolymeric nanoparticles of corn Zeina as a nucleus and pectin of the ivy pumpkin fruit as a shell, and a fraction of ethyl acetate from the *Mimosa pubic* plant as a drug. SEM research of developed nanoparticles revealed a size ranging from 150 to 200 nm in diameter. The FT-IR characterization study confirmed the incorporation of all components into nanoparticles. While the sustained release of the drug was observed *in vitro* release studies of the drug at intestinal pH. Moreover, Ghibaudo et al. (2019) manufactured magnetite nanoparticles covered with pectin as iron supply systems and protective matrices for the probiotic strain *Lactobacillus Plantarum* CIDCA 83114. The results confirmed the absorption of pectin on the surface of magnetite nanoparticles, and these compounds provided adequate supplies for probiotic bacteria, targeting the gut.

That is why the application of pectin as a hauling matrix for controlled or targeted release, is useful both in the pharmaceutical and nutraceutical and cosmetic industries, among others.

7.3.3 Trends in tissue engineering application

Pectin based nanoparticles have various applications in the biomedical field. One of these areas is tissue engineering. Every year millions of patients suffering from bone defects die due to an insufficient and nonideal bone substitute (Zhao et al., 2016). The application of autografts and allografts is limited due to the donor shortages or the potential risk of inducing adverse immunological responses and transmission diseases, although they have been widely used for bone reconstruction (Gopi et al., 2014). Polymeric biomaterials have been extensively used over the past few years in tissue engineering. Among them, pectin is a gifted biodegradable, biocompatible, and eco-friendly polysaccharide recognized for his antimicrobial and anti-inflammatory properties. Since pectin improves the proliferation of osteoblast, it has been recently attracting much attention for his use in bone regeneration (Liu et al., 2004; Kokkonen et al., 2008).

Hydroxyapatite has been widely used for several decades due to its chemical composition which is remarkably close to that of human hard

tissues, such as living bone and teeth (Cao et al., 2010). In this sense, Gopi et al. (2014) synthetized a banana peel pectin-hydroxyapatite nanoparticle that showed strong antibacterial activity against both, gram positive and negative bacteria like *Staphylococcus aureus* and *Escherichia coli*, respectively. The SEM images demonstrated that the concentration plays a major role in controlling as well as reducing the size of nanoparticle and hence the optimum concentration for obtaining the uniformly distributed spherical nanoparticles. Similarly, Gopi et al., (2015) developed an *Opuntia ficus-indica* peel derived pectin-hydroxyapatite nanoparticle, this study showed that the pectin concentration has an obvious influence on the purity, crystallinity, particle size as well as the morphology of the particles. The antimicrobial activity showed improvement toward the tested pathogenic *S. aureus, E. coli,* and *Candida albicans* than the nanoparticles derived without pectin. The latter probably due to the presence of trace minerals. Hopefully, it could be considered as a promising bioactive antimicrobial coating material for orthopedic and dental implants. Furthermore, Begum and Deka (2017) formulated banana bract pectin-hydroxyapatite nanoparticles and confirmed that concentration of pectin is crucial in the synthesis of nanoparticles.

On the other hand, Vedanayagam et al. (2017) studied the role of size nanoparticle in self assembling processes of collagen for tissue engineering application. They synthesized different sizes of pectin caged silver nanoparticles and subsequently introduced the self-assembly process of collagen leads to provide a collagen-Ag-pectin-based scaffold. These results suggested that 10 nm sized Ag-Pectin nanoparticles significantly increased the denaturation temperature and mechanical strength in comparison with native collagen. In addition, the *in vitro* biocompatibility assay revealed that collagen-Ag-pectin nanoparticle-based scaffold provided higher antibacterial activity against gram positive and gram negative as well as enhanced cell viability towards keratinocytes. This study opens the possibility of employing the pectin caged silver nanoparticles to develop collagen based nano constructs for biomedical applications. In conclusion, the study of pectin nanoparticles in tissue engineering still has a long but promising way to go to reach its application in bone and other tissue regeneration.

7.3.4 Trends in construction materials

An emerging area of pectin applications is its use as an anticorrosive material in construction. Pectin structure allows it to interact with divalent elements and metallic surfaces. Some applications of pectin in this area are shown in Table 7.2. Diverse authors suggest that pectin application in this field

Table 7.2 Relevant applications of pectins in the design of construction materials.

Source	Application	Reference
Not available	Corrosion inhibitor	Geethanjali et al., 2014
Commercial pectin (HDM)	Corrosion inhibitor	Umoren et al., 2015
Nopal (prickly pear)	Corrosion inhibitor	Saidi et al., 2015
Tomato peel	Corrosion inhibitor	Grassino et al., 2016

HDM, high methoxylation pectin.

is promising due to the emerging studies in other aspects different to the surface coatings. The design of blocks, added concrete and thermal insulators, using pectins can be an interesting area for the development of new products (Geethanjali, et al., 2014; Saidi et al., 2015; Umoren et al., 2015). The emerging applications in construction materials still face the controversy of biodegradation of pectin by environmental and biological agents. Nevertheless, plant fibers have been part of bricks since biblical times. "You are no longer to supply the people with straw for making bricks; let them go and gather their own straw" (Ex. 5, 7). Interesting enough for a rediscovery.

7.3.5 Trends in environment technology

The studies of pectins focused on the solution of environmental problems, generally are applied specifically to the removal of heavy metals from water. In this context, an emerging field of study of pectin is the removal of heavy metals from air and soil. Additionally, this polysaccharide can interact with some oxides with photocatalytic capacity, which could be used as a means for the removal of other contaminants different from heavy metals, such as dyes and pesticides (Mata et al., 2009; Seenuvasan et al., 2013; Zhang and Liu, 2016; Kollarigowda, 2017). Some of the most important applications in this field are summarized in Table 7.3.

The pesticide residues can be found at different levels of soil and water, which can cause severe damages and consecutively induce health and environmental problems. Even so, the use of pesticides has been forced to increase to improve agricultural productivity. Amitrole is a nonselective herbicide that is widely used combined with other active agents at an industrial scale to control a wide range of weeds in agriculture areas. Evidently, the compound is a pollutant to natural water resources due to its high solubility, polarity, and low volatility in water. For this reason, it is difficult to determine amitrole from water sources. Electrochemical methods were proved to be reliable and cost-effective for its determination; nevertheless, the use of

Table 7.3 Application of pectin gels and pectin nanoparticles on environmental technology.

Source/material	Application	Reference
Sugar beet pectin	Heavy metal removal	Mata et al., 2009
Sugar beet pectin	Mercury removal	Zhang and Liu, 2016
Calcium cross-linked pectin	Amitrole (herbicide) detection	Mani et al., 2015
Pectin-chitosan-sodium tripolyphosphate	Paraquat (herbicide) encapsulation	Rashidipour et al., 2019
Chitosan-pectin	Carbendazim (fungicide) encapsulation	Kumar et al., 2016
Starch-pectin	Phosphate ions release (fertilizer)	Sciena et al., 2019
Seagrass pectin	Metal-binding	Khozhaenko et al., 2016
Pectin-nano-titanium dioxide	Heavy metal ions sorption	Bok-Badura et al., 2017
Gluten/pectin/Fe_3O_4	Pollutants removal	Pirsa et al., 2020
Calcium-crosslinked pectin	Water reservoir	Sharma et al., 2017
Wastes by-products pectin	Dyes adsorption	Attallah et al., 2016a
Magnetite/silica/pectin	Fluoroquinolones adsorption	Attallah et al., 2016b
Pectin-silver	Dyes adsorption	Babaladimath and Badalamoole, 2018

pectin nanoparticles as a detector for amitrole in water has been proposed. Mani et al. (2015) developed a sensing approach based on calcium cross-linked pectin stabilized gold nanoparticles. Their results showed that the sensor was feasible to use in water by detecting amitrole at different ranges of molecular weights with an outstanding sensing ability.

Another approach to the use of pectin nanoparticles is as a delivery system, as described in a study realized by Rashidipour et al. (2019) where they synthesized novel nanoparticles composed of pectin, chitosan, and sodium tripolyphosphate (PEC/CS/TPP) loaded with paraquat (an herbicide). The results showed that these nanoparticles have a highly efficient encapsulation, and a delayed release, also significantly improving its herbicide activity. Within this result, the nanocomposite was less toxic to the human environment and cells, had less soil sorption, cytotoxicity, and mutagenicity compared to the pure or commercial forms.

Similarly, Kumar et al. (2016) evaluated the encapsulation of the fungicide carbendazim in chitosan-pectin nanocapsules by the ionic gelation method, to evaluate its potential to reduce its phytotoxicity and application as a carrier. The authors results showed that the nanoformulation prolonged the release of carbendazim, being less harmful to seeds, and safer for germination and root growth, and exhibited higher antifungal activity than pure carbendazim and commercial formulation. Also, the nanocapsules showed greater efficacy at a lower concentration. The nanoformulation obtained can be explored for the target delivery of carbendazim for disease control.

Furthermore, the application of fertilizing substances has been explored too. For instance, Sciena et al. (2019) implemented the use of pectin to improve the phosphate ions release used as a fertilizer. In fact, hydroxyapatite nanoparticles were stored in biodegradable sachets composed of thermoplastic starch/pectin blends with different polymer ratios. The results showed that the smallest and less crystalline hydroxyapatite nanoparticles presented the highest solubility and that after being stored with starch/pectin sachets, solubility greatly improved, enhancing the phosphorus and other macronutrients released from fertilizers. The authors concluded that the polymer sachets composition can be modulated according to the type of fertilizer to control its solubility, making them potential systems for rational nutrient release in agriculture.

Pectins features are varied, but its interaction with metals is one more commonly used to improve a variety of products. Natural biopolymers have been used and studied for their binding activity and have been proposed to be applied for the elimination of metal ions and reduction of

pollution in wastewater. For their three-dimensional structure, pectins are more effective to interact with metals. The industrial wastewater generally contains substantial amounts of heavy metal ions such as Hg, Pb, Cr, Ni, Cu, Cd, Zn, and others. These metals are considered dangerous for animals and humans due to their toxicity, incremental accumulation in the food chain, and persistence in the ecosystem. Therefore, the efficient removal of these ions from wastewater is a widely studied research area with several technologies proposed including chemical precipitation, electrodeposition, membrane systems, ion exchange, and adsorption (Akunwa et al., 2014).

Khozhaenko et al. (2016) evaluated a natural pectin obtained from seagrass *P. iwatensis* that possess significant metal-binding activity. The partial degradation of pectin and chemical modification in its structure showed an increment in the metal-binding activity to Cd^{2+} and Pb^{2+} ions. The proposed pectin can be effective for the removal of metal ions from water solutions to achieve a higher water purity degree. The authors related the results obtained to the increment of the D-galacturonic acid content in pectin structure due to the easy formation of junction zones between biopolymer and metal ions. Moreover, Bok-Badura et al. (2017) prepared a hybrid pectin-nano-titanium dioxide composite for heavy metal ions sorption. Due that the titanium dioxide has many industrial applications, its incorporation to nanoparticles for the removal of various metal ions was evaluated. The combination of the unique properties of titanium dioxide nanoparticles and pectin led to a new material with good mechanical and sorption properties. The results showed that the adsorbent capacity of the hybrid pectin-nano-titanium dioxide on Cu(II), Cd(II), Zn(II), and Pb(II), was effective. The titanium dioxide contributed to the removal and adsorption of metal ions, being enhanced with the hybrid beads of pectin.

In a study from Pirsa et al. (2020), Fe_3O_4 nanoparticles and gluten/pectin/Fe_3O_4 composite hydrogels were synthesized to evaluate its potential to remove pollutants from lake sediments. The results showed that the removal efficiency of total heavy metals was 62% and the removal efficiency of total organic compounds was 42%. The gluten/pectin/ Fe_3O_4 hydrogel had better performance in reducing environmental pollutants of sediment than wheat gluten/ Fe_3O_4 hydrogels, also, the authors inferred that factors like time and temperature had a significant effect on the results. On the other hand, Sharma´s research group synthesized and characterized calcium-crosslinked nanocarriers of pectin and examined their possible application in controlled irrigation in agriculture. They focused on the chemically

designed nanostructure system that is capable of releasing water for longer periods in agricultural fields, particularly in those areas where rainfall is quite scanty. They obtained calcium pectinate nanoparticles that functioned as a water reservoir and, therefore, proved to be an excellent means to provide sustained irrigation in areas where water is scarce. They also observed that the plants that were exposed with nanoparticle-mixed soil exhibited a better growth, concluding that there is a promising use of these nanocomposites as an efficient water reservoir (Sharma et al., 2017).

Dyes are one of the most hazardous materials in industrial effluents. Their structure and degradation products can cause severe health problems in humans since they exhibit high toxicity and potential mutagenic and carcinogenic effects. Attallah et al. (2016a) evaluated pectin from wastes by-products as the adsorption agent on cationic and anionic dyes. They synthesize three magnetite nanoparticles, one magnetite-pectin nanoparticles (MP-NPs), a magnetite silica nanoparticle (MS-NPs), and a magnetite/silica/pectin nanoparticle (MSP-NPs). By comparing their absorption behaviors, the MSP-NPs showed higher adsorption capacity on cationic dyes while the MP-NPs favored the adsorption of anionic dyes. The authors concluded that these pectin nanoparticles could be a promising alternative for cationic and anionic dyes removal from wastewater for their high adsorption capacities and separation convenience. The same authors evaluated the efficiency of adsorption of two photosensitive fluoroquinolones, ciprofloxacin (CIP), and moxifloxacin (MOX), on the surface of synthesized MP-NPs and MSP-NPs in aqueous solutions. Fluoroquinolone metabolites from drugs can reach the environment and represent a threat to the safety of aquatic ecosystems or even for the quality of drinking water. The results showed that the adsorption of CIP and MOX together with their photodegraded molecules occurred and was enhanced by increasing the pH, NPs loading, and contact time while decreasing the initial drug concentration (Attallah et al., 2016b).

Diversity of pollutants include dyes from industrial effluents. Babaladimath and Badalamoole (2018) investigated the influence of a system formed with silver nanoparticles embedded with a pectin–based hydrogel on the adsorption capacity over cationic dyes, in aqueous systems, like wastewater. The incorporation of pectin hydrogel gave stability and anchor to the silver-nanoparticles and the swelling and the dye (methylene blue) adsorption was enhanced as well. The author also inferred that this composite can be regenerated for pectin can be used in different areas due its outstanding properties such as biocompatibility and biodegradability. In this regard, there are still many possibilities to investigate the pectin-based

nanomaterials including the synthesis methods and applications in other fields (Kollarigowda, 2017).

7.3.6 Trends in electronic field

The incursion of pectins in this technological area has been little detected, so, the perspectives and application options are broad. Pectins can be used for their affinity and capacity to interact with metals, oxides, and electrically conductive polymers. With the continuous development of the media, pectins could be a candidate for the design and production of flexible biodegradable electrically conductive films, an aspect that has been neglected in recent decades.

Xu et al. (2019) developed a flexible electroconductive film based on pectins extracted from citrus fruit residues. The film of this polysaccharide served as the basis for synthesizing and depositing silver nanoparticles "*in situ*," to later coat the film of pectin plus silver nanoparticles with a layer of indium tin oxide. These authors state that the presence of the pectin film provides an adequate flexibility for its application in electronic devices; likewise, the composite film can withstand several bending cycles and can be degraded effectively in water after its useful life has ended. On the other hand, Zhao et al. (2017) synthesized a copolymer consisting of pectins and polyaniline, through an additional polymerization reaction. The synthesized polymer presented good electrically conductive properties and additionally, the authors elaborated aerogels of the synthesized polymer to evaluate its thermal properties, finding that the material is a good thermal insulator.

Also, these authors propose the application of pectins in the production of flexible films capable of conducting electrical energy. To achieve this, pectins can be structurally modified to add resonating rings and alternating C-C double bonds to promote electron movement. Additionally, photocatalytic oxide precursors such as titanium dioxide and silicon dioxide can be included during the chemical modification reaction and in this way incorporate a section that generates free electrons to promote the said application. The proposed material is envisioned as flexible films applicable to electronic devices and solar cells.

7.4 Conclusion

Pectin nanoparticles are an emerging technology and an area of opportunity in diverse and broad fields of knowledge and technology. Besides the

food industry, pectin nanoparticles are being tested for composition and rheology in products, packaging, and even effluents treatment. New applications have appeared in the realm of food and nonfood technologies. The most promising include biomedical and environmental pollution removal, while some emerging applications as construction and electronics are surprisingly taking its place in the realm of dietary fiber applications.

Pectin interaction with a wide variety of biomolecules, metallic and nonmetallic oxides, and synthetic polymers is an advantage that science has considered for the development of new materials for food, health, and the environment. Time has brought new challenges to normal life, and science is developing new solutions for an outstanding new normal.

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3. FERULATED PECTINS FROM SUGAR BEET BIOETHANOL SOLIDS: EXTRACTION, MACROMOLECULAR CHARACTERISTICS, AND ENZYMATIC GELLING PROPERTIES.

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Ferulated Pectins from Sugar Beet Bioethanol Solids: Extraction, Macromolecular Characteristics, and Enzymatic Gelling Properties

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Abstract: Pectin from sugar beet (*Beta vulgaris* L.) (SBP) was extracted from a sugar beet waste (SBW) registering a 4.4% (*w/w*) yield. SBP presented a weight-average molar mass of 459 kDa, galacturonic acid content of 52.2%, and a low esterification degree (30%). The macromolecular characteristics of SBP revealed a flexible and extended coil chain conformation. The main neutral sugars in SBP were galactose (20.7%), mannose (5.0%), and arabinose (3.60%) while ferulic acid (FA) content was 2.1 $\mu g \cdot mg^{-1}$ sample. FA remained in the SBP chain mainly in RG I region even after suffering both, industrial processing and harsh weathering conditions. Consequently, SBP formed covalent gels induced by laccase. Covalent cross-linking content (dimers and trimer of FA) was 0.97 $mg \cdot g^{-1}$ SBP. The 8-5′, 5-5′, and 8-*O*-4′ dimers of FA isomers proportions were 75, 17, and 8%, respectively. SBP gels at 4% (*w/v*) registered storage (G′) and loss (G″) moduli final values of 44 and 0.66 Pa, respectively. SBP gels were soft and adhesive according to texture profile analysis. Scanning electron microscopy analysis of SBP lyophilized gels revealed an imperfect honeycomb-like structure with 4.5 ± 1.4 µm average cavities diameter.

Keywords: agro-industrial waste; ferulated polysaccharide; oxidative crosslinking; sugar beet waste; microstructure

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1. Introduction

Sugar beet (*Beta vulgaris* L.) is a resource highly used in industrial processes for sugar production and other products [1]. The beet is known for having a high content of sucrose; as a result, refined sugar, alcoholic beverages, and ethyl alcohol can be produced from it [2]. Some of the sugar beet agro-industrial processing waste products are rich in fiber, protein, and minerals; thus, these are used primarily as cattle and ovine feed [2,3]. Besides this, sugar beet waste (SBW) is a residue with high pectin content (15–30% dry weight basis) [1,4]. The European Union is the world´s leading sugar beet-based sugar producer. In America, sugar beet is not only used for sugar production but also for bioethanol. The United States leads the sugar beet and bioethanol production in America [2,3]. Currently, Mexico is exploring and starting to produce bioethanol from this plant. It is considered an agricultural species capable of adapting to warm areas with high yield potential during winter [5]. As a result, in the last decade, the potential to grow and harvest sugar beet for bioethanol production in the northwest semi-desert regions of Mexico has been explored, generating SBW. For instance, Jimenez-Leon et al. [6] evaluated the productive potential of three sugar beet cultivars; Cadet, Coronado large, and SV MEI in two sowing dates in the experimental field of the University of Sonora (Coordinates: 29°_00′48″ NL, 111°08′07″ WL and 151 MASL (meters above sea level)), under split-plot design. Authors reported

that Coronado's large cultivar sowing in November had the highest tubers production (109.2 t·ha^{-1}) and highest total soluble solids content (19.3 °Brix), while the highest foliage production (25.4 t·ha^{-1}) in dry weight was obtained in Coronado's large cultivar sowing in October. Jimenez-Leon et al. concluded that tuber yields and total soluble solid content obtained under the agroecological conditions of their study are similar to the highest values reported in the literature as a consequence compared to commercial exploitations. Based on the above-mentioned report and a previous one [5], a bioethanol production facility was set in Sonora state, Mexico (Coordinates: 30°42′59″ NL, 112°08′60″ WL and 275 MASL (meters above sea level)). After harvest, beets were milled and fermented; then, filtered and the solids were piled under the sun to dry. The SBW had a granulate appearance and a dark brown-purple color.

In general, SBW contains ferulated pectin (SBP), as previously reported [7–9]. Pectins are structural polysaccharides found in the plant cell wall composed mainly of galacturonic acid (GA) units with high variations in structure, composition, and molecular weight [10]. They contribute to the firmness and structure of plant tissue, bringing mechanical resistance to cell walls. During plant and fruit growth, pectins are important in texture quality [11]. Structurally, this polysaccharide has three main domains as presented in Figure 1: homogalacturonan (HG), rhamnogalacturonan I (RG I), and rhamnogalacturonan II (RG II) [12,13].

HG is a linear region, consisting mainly of D-galacturonic acid units, linked by α-(1-4) glycosidic bonds. The *O*-2 or *O*-3 positions may be acetylated, and the carboxylic group found at the C-6 position may be partially esterified by methyl groups [13]. Based on the proportion of esterified groups distributed along the main chain, an index known as the degree of esterification (DE) has been established. Based on the percentage of DE, pectins are classified as having a high degree of esterification (HDE) and low degree of esterification (LDE); HDE refers to the ratio where more than 50% of the carboxyl groups are esterified, while less than 50% of the carboxyl groups esterified corresponds to LDE [14]. The DE of pectins largely determines the physicochemical and gelling properties of pectins.

RG I is a branched region of the polysaccharides consisting of repeating units [α-D-GalA-1,2-α-L-rhamnose-1,4] n. This region contains a significant amount of neutral sugars, such as arabinose and galactose, which together form arabinan, galactan, and arabinogalactan; attached in position *O*-4 to rhamnose, they are considered ramifications [15,16]. Besides neutral sugars, hydroxycinnamic acids, mainly ferulic acid, may be esterified to arabinans and galactans in RG I of some species from *Chenopodiaceae* [9,17]. The most-reported species containing ferulated pectin is sugar beet (*Beta vulgaris* L.) referred to as SBP. In fact, SBP has ferulic acid (FA) associated with its chemical structure [18–20]. FA groups are ester-linked with pectins mainly on the *O*-2 and *O*-5 position of arabinose residue and *O*-6 of galactose residues in side chains of RG I [9,18,19]. This compound is distributed almost equally between the arabinan and galactan components of the pectin side chains [21]. SBP can form gels driven through an oxidative coupling reaction, mediated by chemical or enzymatic oxidation, and the cross-linking is formed by a carbon- carbon covalent bond between two ferulated phenyl rings given the formation of phenoxy radicals [22]. In general, the gelling ability of pectins enables the use of pectin in the food industry as gelling agents, stabilizers, thickening agents, and emulsifiers [23,24].

RG II region is characterized by being a compound of repeating D-galacturonic acid units linked by α-(1-4) glycosidic bonds, as the base structure. This structure is replaced by L-rhamnose, D-galactose, and other neutral sugars rarely found in nature, such as apiose, 3-*O*-methyl-L-fucose, and 2-*O*-methyl-D-xilose. The RG II region is the most complex of the three structural regions that make up the pectins but is also the region with the highest degree of structural conservation among plant species [15,16,25]

In the present study, an added value product was extracted from an agro-industrial waste (SBW) subject to harsh weathering conditions. Authors hypothesize that the gelling property of SBP will not be adversely affected by the industrial pretreatment and extraction process, and ferulic acid will remain in the RG I region to allow covalent gel-forming

through an oxidative coupling reaction. In particular, FA, dimers of FA (di-FA), and trimers of FA (tri-FA) content before and after SBP gelation using laccase as a crosslinking agent were determined by high-performance liquid chromatography. Then, SBP gelation kinetics was followed by the storage (G′) and loss (G″) modulus obtained from rheological measurements. Finally, texture profile analysis and microstructural characterization of SBP gels were studied by Texture Analyzer and scanning electron microscope, respectively. Here the authors report the potential for SBW, considered regional industrial waste generated in the northwest of Mexico, specifically under Sonoran Desert conditions, as a source of ferulated pectin for the food and non-food industries.

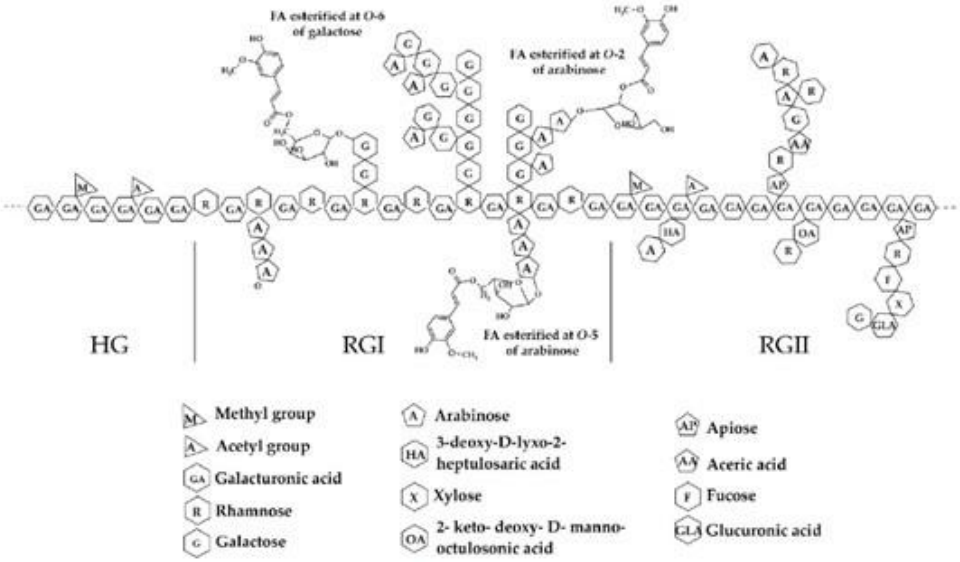

Figure 1. Schematic structure of SBP. Adapted from [25]. Reproduced with permission from Ohlmaier-Delgadillo F. et al., Molecules; published by MDPI, 2021.

2. Materials and Methods

2.1. Industrial Waste

Solid, brown powdered SBW was bought as cattle feed at an ethanol production facility located in Sonora, Mexico (Coordinates: 30°42′59″ NL, 112°08′60″ WL and 275 MASL (meters above sea level)). The sample was used directly without extra processing for pectin extraction. Extraction experiments were carried out between January and March 2019.

2.2. SBW Characterization

Ash content was determined according to the AOAC 4.1.03 (1934) method 934.01. [26]. The Kjeldahl method was used to characterize the protein content, according to the AOAC method 2.057 (1984) [27]. Fat content was determined by the Soxhlet AOAC method 996.06, and total fiber content was determined according to AOAC (1995) method [28].

2.3. Pectin Extraction and Purification

Pectin was extracted from SBW, based on the methodology reported by Li et al. [29] with some modifications. Briefly, 150 g of dried SBW was dispersed in 1.5 L of 0.1 M hydrochloric acid (1:10/*w:v*) and pH was adjusted to 1.5. The mixture was homogenized and heated on a plate with stirring at 85 °C for 2 h and allowed to cool at room temperature. Then, the mixture was centrifuged at 10,000× *g* for 15 min and 25 °C in a centrifuge (Thermo Scientific, USA) to keep the supernatant. SBP was precipitated with ethanol (96%) in a 1:2 (*v/v*) ratio. Next, pectins were centrifuged (10,000× *g* for 10 min and 25 °C) and

then dried overnight by solvent exchange (acetone). SBP was milled in a porcelain mortar until a fine powder was obtained.

The extracted SBP was purified following the procedure described by Yapo et al. [30] with some modifications. Dried pectin was dispersed in Milli-Q water (1:200 *w/w*) for 24 h with stirring; afterward, centrifuged at 10,000× *g* for 15 min and filtered through 3, 1.2, 0.8, and 0.45 µm membrane filters, sequentially. SBP was precipitated by ethanol (96%) under 1:2 (*v/v*) liquid relation and stored at 4 °C. Then, SBP was concentrated by centrifugation and dried by solvent exchange with acetone. Finally, SBP was stored in the dark at room temperature until use [30].

2.4. Pectin Yield

SBP yield was determined by the proportion of the weight of the extracted pectin after drying concerning the original weight of SBW ($g \times 100\ g^{-1}$). Recovery was calculated as follows:

$$\text{SBP yield } (\%\ w \times w^{-1}) = (m_0 \times m^{-1}) \cdot 100 \quad (1)$$

where m_0: dry SBP weight in grams; m: dry SBW weight in grams.

2.5. SBP Characterization

2.5.1. Chemical Composition

Galacturonic acid content was determined following the procedure established by Urias-Orona et al. [31] where 10 mg of SBP were hydrolyzed with 2 N trifluoroacetic acid at 120 °C for 2 h. The hydrolysis reaction was stopped on ice, and the extracts were evaporated under airflow at 40 °C, rinsed twice with 200 µL of water. The evaporated extract was solubilized in 500 µL of water. Mannitol was used as an internal standard. Samples were filtered through 0.45 µm (Whatman) and analyzed by high-performance liquid chromatography (HPLC, Varian Prostar 210 within a Refractive Index Detector Prostar 350) using a MetaCarb H Plus column (Agilent, Santa Clara, CA, USA; 7.8 × 300 mm). The mobile phase was 0.001 N sulfuric acid (H_2SO_4), and the elution rate was 0.4 $mL \cdot min^{-1}$ at 65 °C.

Sugar composition was determined according to Blakeney et al. [32] with some modifications. Accordingly, 20 mg of SBP were hydrolyzed with 3 N H_2SO_4 for 2 h at 100 °C, and derivatives were separated and quantified by gas chromatography (PerkinElmer, Clarus 580) using a high-performance capillary column (Elite 225, PerkinElmer, 30 mL × 0.32 mm ID × 0.15 µm film thickness). Neutral sugar standards were L-rhamnose (99%, Sigma-Aldrich, St. Louis, MO, USA), L-arabinose (99%, Sigma-Aldrich, St. Louis, MO, USA), D-xylose (99%, Sigma-Aldrich, St. Louis, MO, USA), D-mannose (99%, Sigma-Aldrich, St. Louis, MO, USA), D-galactose (99%, Sigma-Aldrich, St. Louis, MO, USA), and D-glucose (99%, Sigma-Aldrich, St. Louis, MO, USA). Inositol (99%, Sigma-Aldrich, St. Louis, MO, USA) was used as an internal standard. Samples were filtered through a 0.45 µm membrane prior to injection.

FA, dimers of FA (di-FA), and trimers of FA (tri-FA) content were analyzed using high-performance liquid chromatography. First, 100 mg of SBP were saponified using 2 N NaOH, later acidified with 4N HCl. Finally, they were extracted with diethyl ether. Samples were injected in an HPLC (Waters, e2695), equipped with an Alltima C18 column (Alltech, Deerfield, IL, USA; 250 × 2.6 mm), previously filtered through 0.45 µm membrane [33].

Residual soluble protein content in SBP was determined according to the Bradford method [34]; and ash, according to the AOAC 4.1.03 (2000) method 934.01 [26].

2.5.2. Macromolecular Characteristics

The weight-average molar mass (Mw), number-average molar mass (Mn), polydispersity index (PDI, $Mw \cdot Mn^{-1}$), radius of gyration (RG), hydrodynamic radius (Rh), intrinsic viscosity ([*n*], $mL \cdot g^{-1}$), and the Mark-Houwink-Saturada constants (α, K) were determined by the combination of multi-angle laser light scattering and size- exclusion chromatography using SEC- MALLS equipment (Wyatt Technology, Santa Barbara, CA, USA), following the methodology described by Yang et al. [35] with some modifications. The ASTRA 6.1 software was used. The mobile phase was sodium azide 0.02% ($w \cdot v^{-1}$) and sodium

nitrate 50 mM. The sample was prepared as follows, 5 mg of SBP were dissolved in 1 mL of the mobile phase, to ensure the dispersion, the sample was vortexed for 10 s and then, set at 80 °C for 1 h. After, the sample was tempered to room temperature and centrifuged at 15,000×·*g* for 10 min. Finally, the sample was filtered through a 0.45 µm membrane before injection. The specific refractive index increment ($dn{\cdot}dc^{-1}$) value of 0.148 $mL{\cdot}g^{-1}$ was used [36]. The characteristic ratio (C∞) and persistence length (q) were calculated as previously described [37] using the following equations:

$$q = (C\infty + 1) \times (I_0 \times 2^{-1}) \quad (2)$$

$$C\infty = 6 \times RG^2 \times (M_0 \times {I_0}^{-2}) \times Mw \quad (3)$$

where C∞ is the characteristic ratio, I_0 = 0.45 nm (length of α-D-galacturonic acid residue) [38], M_0 = 194 $g{\cdot}mol^{-1}$ (molar mass of anhydro-galacturonic acid residue) [39], and Mw the molar mass of SBP.

The degree of methylation (DM) and acetylation (DA) of SBP were determined following the technique proposed by Levigne et al. [40]. Briefly, methanol and acetic acid were produced by saponification of pectin with 1 M NaOH at 4 °C for 2 h. Isopropanol was used as an internal standard. Samples were centrifuged for 10 min at 8000× *g* and 25 °C. Also, supernatants were neutralized through a Maxiclean IC-H device (S*Pure, Singapore) before injection. Methanol, acetic acid, and isopropanol were quantified by HPLC Varian 500 within a Refractive Index Detector on a C18 column (Superspher 100 RP-18 endcapped, Merk KGaA, 250 × 4 mm). Elution was carried out with 4 mM H_2SO_4 at 0.7 $mL{\cdot}min^{-1}$ and 25 °C [40].

2.5.3. Infrared Spectroscopic Analysis

Fourier Transform-Infrared Spectroscopy (FTIR) analysis was performed on a NICOLET IS-50 spectrometer (ThermoScientific ™, Waltham, MA, USA) with an attenuated refractive detector. Spectrum scan was achieved from 4000 cm^{-1} to 400 cm^{-1}. Samples were dried before being processed and absorbance vs. wavelength was reported in graphs [41]. FTIR spectrums of SBP and low methylated commercial citrus pectin (Grinsted® pectin LC950 Danisco) were analyzed and used as the control.

2.6. SBP Gel Preparation

SBP gels were prepared using SBP dispersion at 4% (*w/v*) in 0.1 M sodium acetate buffer, pH = 5.5, at 25 °C, as previously described by Ohlmaier-Delgadillo F. et al. [25]. Laccase from *Trametes versicolor* (Sigma-Aldrich, St. Louis, MO, USA) 24 units·mg^{-1} FA was dispersed in sodium acetate buffer (pH = 5.5) and added to SBP as cross-linking agent. The gelling process was set to 80 min at 25 °C.

2.7. FA, di-FA, and tri-FA Content of SBP Gel

Ferulic acid (FA), di-FA, and tri-FA contents of SBP gels were analyzed using high-performance liquid chromatography after (80 min) laccase exposure as previously described. In the saponification step, 50 mg of SBP gel were used [42].

2.8. Rheological Measurements

The formation of SBP gel was followed using a low deformation strain-controlled rheometer (Discovery HR-2 rheometer, TA instruments) in oscillatory mode, as reported previously [43]. Cold (4 °C) solutions of 4% (*w/v*) SBP were mixed with laccase (24 units·mg^{-1} FA) and immediately placed in the cone and plate geometry (5.0 cm in diameter, 0.04 rad in cone angle) maintained at 4 °C. SBP gelation kinetics were monitored at 25 °C for 80 min by following the storage (G′) and loss (G″) modulus. All measurements were carried out at a frequency of 0.25 Hz and 5% strain (linearity range of viscoelastic behavior). The mechanical behavior of SBP gel was obtained by frequency (0.01 Hz to 10 Hz, 5% strain) and strain (1% to 10%, 0.25 Hz) sweep at the end of the network formation at 25 °C.

2.9. Texture Profile Analysis

Additionally, 6 mL of 4% (*w/v*) SBP gels were fresh and directly prepared in a 50 mL glass flask with 55 mm height and 35 mm of internal diameter. Texture profile analysis (TPA) was made in a TA.XTA Texture Analyzer (Texture Analyzer Stable Micro Systems, Surrey, UK) within a cylindrical plunger (diameter 25 mm). SBP gels were deformed by compression at a constant speed of 1.0 $mm \cdot s^{-1}$ to 4 mm from the gel surface. Eight parameters were used to describe texture: hardness, fracturability, adhesiveness, springiness, cohesiveness, gumminess, chewiness, and resilience [31,43].

2.10. Scanning Electron Microscopy

Surface characterization of SBP gels was performed using a scanning electron microscope (SEM) JEOL54 10LV equipped with an INCA dispersive X-ray detector system (Oxford Instruments) and operated at a voltage of 20 kV. Samples were flash-frozen with liquid nitrogen and then lyophilized, later coated with gold before being observed under a high vacuum using the secondary electron detector according to Morales-Burgos et al. [44]. The average porous diameter was determined from SEM images using ImageJ software.

2.11. Statistical Analysis

All measurements were performed by triplicate, and the coefficients of variation were lower than 5%. Texture measurements were done six times, and the coefficients of variation were lower than 10%. All results were expressed as mean values and standard deviation, expressing descriptive statistics. To determine significant differences between the respective values, a one-way analysis of variance (ANOVA) was performed, and averages were compared using the Tuckey–Kramer test with a $p \leq 0.05$. The statistic software NCSS 12 was used.

3. Results and Discussion

3.1. SBW Characterization

Table 1 shows the constituents of SBW on a dry matter basis. The recovery percentage of SBP from waste was 4.4 ± 0.1% (w SBP· w^{-1} SBPW). This value is not only higher than those reported by Müller et al. [45] for two different SBWs (2.8–3.2%); but also lower than those reported by Phatak et al. [46] for SBP (19.3–24.7%), who studied the effect of changing extraction parameters conditions, as hydrolysis solution, pH, temperature and hydrolysis time [46]. Several authors agree that the extraction conditions have important effects on the quantity, as well as the quality of the extracted pectins. Among the main parameters, we find the temperature, pH, extraction time, and the nature of the acid [47]. Additionally, SBW studied in this work is stored outdoor with no protection against weather conditions, reaching up to 45 °C and 10 UV index in summertime. Plus, previous industrial processes of SBW might affect pectin content and extraction yield as well. SBW was found with low fiber content, representing 44 ± 3%. Low fiber content is desired to get higher yields of pectin extraction [45]. As revealed by Zieminski et al. [48], SBW with high amounts of fiber (close to 85%) affects biogas yield production in an industrial approach. Protein content in SBW was 9.5 ± 0.3% dw (dried weight), a value comparable to some previously reported for similar materials in research papers, 8.2% [49], 11.3% [50], and 10.3% [48]. SBW is usually used as an ovine feed due to its great amount of proteins, and in Mexico, this represents the main economical usage of SBW [2,3]. Fat content for SBW was 0.67%, and this result is also compared to 0.9% reported for Turquois et al. [49], who defined the waste as a low-fat waste. Ash content represents 12.2% of the total weight; this value is quite comparable to the value reported by Turquois et al. [41], for a raw SBW, corresponding to 13.3%, but highly contrasting to 1.2% reported by Ziemiński et al. [48] for a raw SBW used for biogas production. It is well documented that the physical and chemical properties of SBW present variations, among others, caused mainly by industrial processing conditions [46,49,50]. As early discussed, in Mexico, SBW is used for ovine feed with no extra usage; however, pectin extraction from SBW represented an alternative usage of industrial solids with important

pectin yield (4.4%); furthermore, SBW recovered after pectin extraction could be used as ovine feed with some process adaptations, but further research is needed to test such an argument.

Table 1. Pectin yield and SBW composition (dry matter basis).

Content	% (*w*/*w*)
Pectin yield	4.4 ± 0.1
Fiber	44 ± 3
Protein	9.5 ± 0.3
Fat	0.67 ± 0.02
Ash	12.2 ± 0.2

Values are presented as means ± standard deviations of triplicates ($n = 3$).

3.2. SBP Characterization

3.2.1. Chemical Composition

The chemical composition of SBP is presented in Table 2. Galacturonic acid content was found to be 52.2% of SBP´s total monomers. Our value is higher than 46.5% reported by Turquois et al. [49] who obtained pectins from a similar industrial waste and under comparable extraction conditions. Furthermore, Pi et al. [51] extracted SBP with 66.2% of GalA content, higher than GalA content found in this work. It seems that not only industrial process conditions but also sugar yield (in our case) are relevant to determine pectin characteristics. As mentioned above, differences in industrial processes might induce changes in the chemical structure of pectins extracted, such as galacturonic acid content.

Table 2. Chemical composition of SBP.

Component	Value
Galacturonic acid §	52.2 ± 1.6
Rhamnose §	1.50 ± 0.02
Arabinose §	3.60 ± 0.04
Xylose §	1.20 ± 0.02
Mannose §	5.0 ± 0.04
Galactose §	20.7 ± 0.4
Glucose §	12.3 ± 0.2
Ferulic acid ¶	2.1 ± 0.1
Ferulic acid dimers ¶	0.22 ± 0.02
8-5′ ¶	0.060 ± 0.003
8-5′benzo ¶	0.030 ± 0.003
8-*O*-4′ ¶	0.09 ± 0.01
5-5′ ¶	0.040 ± 0.004
Protein §	2.4 ± 0.1
Ash §	1.0 ± 0.1

Values are presented as means ± standard deviations ($n = 3$). § Results are expressed g·100 g^{-1} SBP dry matter. ¶ Phenolics are expressed in mg·g^{-1} SBP dry matter.

The total sugar content of SBP is also shown in Table 2. As presented, arabinose, mannose, and galactose are the main neutral sugars found in SBP. Additionally, some glucose was found in the sample. The more representative neutral sugar amount was galactose with 20.7%, followed by mannose (5%), arabinose (3.6%), rhamnose (1.5%), and xylose (1.2%). As discussed previously, arabinose, and galactose are crucial neutral sugars where FA is commonly found esterified to rhamnose in RG I structural region for SBP. Neutral sugar content for our SBP differed from some SBP reported previously, Phatak et al. [46] found lesser amounts of arabinose, galactose, and rhamnose; 1.6, 5.7, and 0.94%, respectively. In contrast, Turquois et al. [49] found a higher percentage of arabinose (9.6%), less galactose (3.1%), and less rhamnose (0.7%) for their SBP. In recent work, Pi et al. [51] found less content of galactose (7.1%), and a higher amount of arabinose (5%)

and rhamnose (3.6%). Based on information previously presented authors aimed that neutral sugar content in SBP is affected not only by industrial processing and the pectin extraction process but also by waste storage. Particularly, the SBW used in this study is stored outdoors without extra protection from the climate and temperature changes, as a result, this could deteriorate polysaccharide chemical structure, obtaining less neutral sugar content as observed in rhamnose content, which is related to RG I degradation. As a counterpart, we can infer that the glucose content of SBP can be related to galacturonic acid percentage. While low glucose content is related to high industrial yield and high glucose content, to low industrial yield; sugar beet undergoing aggressive chemical treatment for high glucose yield can result in low glucose SBW with low galacturonic acid percentage SBP content as observed by Chen et al. [4] and Turquois et al. [49]. In contrast, SBP in this study showed both high glucose and high galacturonic acid percentage content, which suggests the industrial ethanol process is an opportunity for optimization.

FA content is also shown in Table 2. As presented, our SBP had an FA content of 2.1 $mg \cdot g^{-1}$ of pectin. Certainly, the FA content is higher than some values reported previously for a comparable SBP: 1.9 $mg \cdot g^{-1}$ of pectin reported by Chen et al. [4], 0.68 $mg \cdot g^{-1}$ of pectin reported by Phatak et al. [46], 0.28 $mg \cdot g^{-1}$ of pectin reported by Pi et al. [51], and 0.38 $mg \cdot g^{-1}$ of pectin reported by Pacheco et al. [52]. This fact may be correlated with the low content of neutral sugars in those studies, which correlated with the lower amount of FA in SBP. All these references, determined less galactose and arabinose neutral sugar, as compared to SBP reported in this work. As previously described, FA groups are ester-linked with pectins mainly on the *O*-2 and *O*-5 position of arabinose residue and *O*-6 of galactose residues in side chains of RG I [9,18,19]. Furthermore, our research group in a recent study [41] observed that FA content in pectin extracted from three fresh sugar beet (*Beta vulgaris* L.) cultivars grown under similar desertic conditions, geographic location, and extraction conditions to the SBW used in this study, were higher than 2.1 $mg \cdot g^{-1}$ of pectin (FA_1 = 3.5, FA_2 = 4.7, and FA_3 = 5.5 $mg \cdot g^{-1}$ of pectin); thus, the chemical composition of the vegetable matrix was modified during the bioethanol production process and possibly due to the outdoor storage conditions of SBW. By changing the storage conditions of SBW to indoor and more controlled conditions, the FA content of SBP could be made higher, but more studies to confirm this idea must be conducted.

The total feruloyl dimers content is also presented in Table 2. The values range between 0.03 to 0.09 $mg \cdot g^{-1}$ of pectin. From the total dimers of FA studied, the 8-5´, 8-5´benzo, 8-*O*-4´, and 5-5´ dimers were detected in SBP, with the dimers 8-*O*-4´ and 8-5´ being most prevalent with about 40.9% and 27.3% of the total dimers of FA, respectively. In addition, minor percentages of 5-5´and 8-5´benzo dimers of FA were also detected, with approximately 18.2% and 13.6%, respectively. It is well documented that dimers 8-5´ and 8-*O*-4´ are the major dimers formed after oxidation reactions of FA monomers, in sugar beet pectins [21,53].

Protein content in SBP was found lower than various values found in the literature, 6% [49], 10.4% [52], and 6.7% [51]. Certainly, protein can enhance the emulsifier properties of pectins.

3.2.2. Macromolecular Characteristics

The macromolecular characteristics of SBP are presented in Table 3. The Mw of SBP was 459 kDa; which is superior to other values reported in the literature. Phatak et al. [46] found Mw values of 35 kDa, 44.7 kDa, and 39.8 kDa for pectins extracted from a similar industrial waste sample. Also, Yapo et al. [30] reported an Mw rounding of 90 kDa for a similar pectin. The higher Mw registered for SBP in the present study can be related not only to SBW characteristics but also to extraction conditions. The Mn of SBP was 94 kDa, the relationship between Mw and Mn brings up the PDI value, which is a measure of the width of molecular weight distribution. The PDI value for SBP was 4.9. A high PDI value indicates the presence of high Mw fractions; the larger the PDI, the broader the molecular weight [54]. In addition, the RG, Rh, and [η] of SBP were 45 nm, 14.3 nm, and

77.6 mL·g^{-1}, respectively. Fishman et al. [55] studied the conformational structure of SBP extracted from fresh sugar beet root by microwave-assisted flash-extraction under various conditions (temperature, pressure, and time). In their work, RG and [η] values ranged from 35 nm to 51 nm and 300 mL·g^{-1} to 430 mL·g^{-1}, respectively. The RG value obtained in the present study, corresponding to 45 nm, matched in the range reported by Fishman et al., however [η] is lower. A low [η] value can describe fairly rigid molecular chains as in this case [56]. Some other polysaccharides have presented a low [η] caused by its highly branched structure, similarly to SBPs, which are known for having highly branched structural regions (RG I and RG II) [57]. In a recent study, Hotchkiss et al. [36] reported the macromolecular characteristics of blueberry pomace pectin obtained by microwave-assisted extraction under two process conditions (condition 1: pH = 2, 3 min, 80 °C; condition 2: pH= 1, 10 min, 120 °C). In their study, RG, Rh, and [η] values ranged from 40 nm to 48.9 nm, 52 nm to 63.3 nm, and 37 mL·g^{-1} to 280 mL·g^{-1}, respectively. As observed, RG and [η] found in the present study are similar to those reported by Hotchkiss et al. however, Rh is lower. From the previous information, we can infer that macromolecular characteristics of pectins will change based on vegetable or row material source and extraction process and conditions. For instance, pH (acid medium) and temperature (80 °C) of extraction conditions reported by Hotchkiss et al. were similar to our research work, yet extraction time and extraction process were distinct. As a consequence of the above-mentioned similarities in extraction conditions, RG and [η] values could be comparable. Furthermore, C∞ and q values were calculated for SBP. The persistence length represents the average projection of the end-to-end distance vector and indicates the distance through which the longitudinal axis of a chain can be considered as linear, therefore giving information on the chain stiffness described by characteristic ratio [58]. The values calculated for q and C∞ were 5.9 nm and 25.4 (dimensionless unit), respectively. These values are similar to those reported by Cros et el. [58], q = 6 nm and C∞ = 57 (dimensionless unit) for citric pectin with a similar degree of methylation (40%) to the SBP studied in the present work. In particular, both q values are too close, indicating that pectin chain flexibility is similar and higher than other citrus pectins with a lower degree of methylation (28%) with q = 7.5 nm, as studied by Cross et al. In addition, Cros et al. expressed that C∞ = 57 (dimensionless unit) describes an extended polysaccharide chain, this value is quite a bit higher than C∞ = 25.4 (dimensionless unit) as determined in the present work, consequently, we can assume that SBP chains are extended. High C∞ values indicate "unperturbed" chains; therefore, HG chains adopt extended conformations having a low probabilities of folding, a feature that may be largely attributed to the axial-axial glycosidic linkage [58]. SBP Mark–Houwink–Sakurada constants were obtained, α = 0.45 and K = 0.32. Values of α and K can be used to study the molecular conformation of SBP in sodium nitrate (50 mM) dispersion. It has been established that the exponent α usually lies in the range of 0.5–0.8 for linear random coil polysaccharides and increased with increasing chain stiffness [59]. The constant α is an indicator of interaction between polysaccharide and solvent, a low α value indicates a poor solvent, and a high α value indicates a good solvent. In our case, SBP presented a value lower than 0.5, indicating that sodium nitrate was probably not the best solvent option for SBP, and a sodium chloride solution can be a good alternative to increase the α value as observed in previous works [59–62]. In contrast, the K value obtained in this study was higher compared to some others obtained using a sodium chloride solution as pectin solvent such as K = 0.0234 [59]. High K values describe a polysaccharide with expanded coil conformation [56].

Table 3. Macromolecular characteristics of SBP.

Component	Value
Mw (kDa)	459 ± 3
Mn (kDa)	94 ± 6
PDI ($Mw \cdot Mn^{-1}$)	4.9 ± 0.3
RG (nm)	45 ± 5
Rh (nm)	14.3 ± 1.5
$[\eta]$ ($mL \cdot g^{-1}$)	77.6 ± 0.1
C∞	25.4
q (nm)	5.9
Mark-Houwink-Sakurada α	0.45 ± 0.01
Mark-Houwink-Sakurada K	0.32 ± 0.04

Mw, weight-average molar mass. Mn, number-average molar mass. PDI, polydispersity index. RG, radius of gyration. Rh, hydrodynamic radius. $[\eta]$, intrinsic viscosity. C∞, characteristic ratio. q, persistence length.

As a counterpart, the degrees of methylation and acetylation of SBP were 30% and 13%, respectively. Therefore, SBP is classified as a low methylated and low acetylated pectin. In a previous report, Turquois et al. [49] stated that extraction conditions were the main aspects leading to the chemical composition of extracted pectins. Accordingly, the authors, low methylated and acetylated pectins are obtained only after alkaline extraction. Hence, a high degree of methylation and acetylation is caused by acidic conditions. Interestingly, this work shows some other factors affecting the chemical composition of extracted pectins, mainly obtained from the industrial waste matrix, as previous treatments during industrial process and storage conditions (indoor, outdoor). As revealed by Yapo et al. [30], it is possible to extract low methylated (14.4%) and acetylated (3.1%) SBP from SBW under acidic conditions, under similar conditions. Also, Gómez et al. [63], and Pi et al. [51] recovered low methylation and low acetylation degree pectins from similar SBW. In recent work, Lara-Espinoza et al. [41] observed that pectin extracted from three fresh sugar beet (*Beta vulgaris* L.) cultivars grown under similar desertic conditions, geographic location, and extraction conditions than SBW used in this study have a high degree of methylation (up to 50%), supporting the fact that previous industrial processes suffering through vegetal matrix and storage condition will affect pectin characteristics extracted from SBW. The degree of methylation was also confirmed by estimation, analyzing the FT-IR spectrums of SBP based on Urias-Orona et al. [31]. The degree of methylation was 37%, supporting the result obtained by HPLC. On the other hand, low methylated and ferulated pectin may combine two gelation mechanisms enabling interesting new applications.

3.3. Infrared Spectroscopic Analysis

Figure 2 shows the FT-IR spectrum of (a) SBP and (b) low methylated commercial citrus pectin (CCP). Chemical and structural information was collected from spectra and some differences were highlighted. Wide absorption bands at 3343 cm^{-1} and 3245 cm^{-1}, corresponding to stretching vibration of hydrogen-bonded O-H groups of galacturonic acid, are presented for SBP and CCP, respectively. In addition, a characteristic stretching signal of O-CH_3 from methyl esters of galacturonic acids is found to be proximally 2920 cm^{-1} for SBP and 2946 cm^{-1} for CCP. Other common signals found in pectin spectra are those related to the degree of methylation; regularly, both signals are located side by side proximally to 1700 and 1600 cm^{-1}. In Figure 2, bands at 1721 cm^{-1} (Figure 2a) and 1735 cm^{-1} (Figure 2b) are associated with the vibrations of esterified carboxyl groups or ester carbonyl (C=O). Besides this, bands located at 1600 cm^{-1} (Figure 2a) and (Figure 2b) 1598 cm^{-1} correspond to free carboxyl groups or carboxylate ion (COO^-) [3,64]. Stretching vibrations commonly observed on second derivative spectra (inserted in (Figure 2a) at 1520 cm^{-1} (C-C aromatic ring) and 1049 cm^{-1} (-OCH_3) are assigned to FA content. The region from 700 to 1500 cm^{-1} is the fingerprinting region for pectins [1,65]. Within this region, a small signal at 1410 cm^{-1} (Figures 2a and 3b) can be attributed to the symmetric stretching vibrations of the COO^- functional group of amino acid side chains, free fatty acids, or other derivative

compounds carried as an effect of the extraction process [1]. An absorption band located at 1232 cm^{-1} is assigned to the C-CO bending vibration of pectin polysaccharides. C-O-C vibration of glycosidic linkages of the pectin backbone structure is observed at 1136 cm^{-1} (Figure 2a) and 1151 (Figure 2b) cm^{-1}. An important band located at 1001 cm^{-1} (Figure 2a) and 1014 cm^{-1} (Figure 2b) are attributable to C-O stretching of side groups in C-O-C glycosidic linkages. In addition, an absorption band characteristic of α-linkage in pectin polysaccharides is identified by the weak peak at 811 cm^{-1} in the anomeric region of the spectra (950–750 cm^{-1}) [1,3,66].

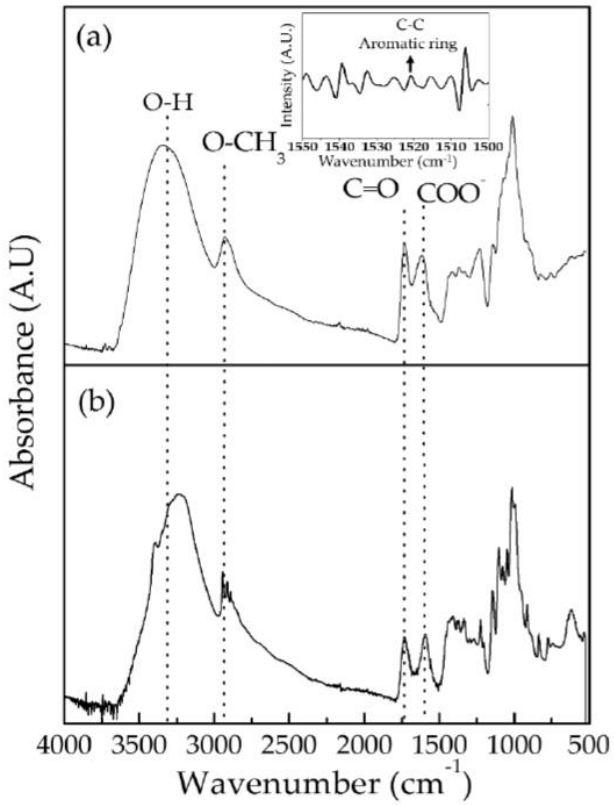

Figure 2. FT–IR spectra for SBP (**a**) and commercial citrus pectin (**b**).

3.4. SBP Gels

3.4.1. Covalent Cross-Linking

FA, di-FA, and tri-FA were detected in SBP gels induced by laccase as a cross-linking agent. FA dimerization promoted the formation of three di-FA isomers (8,5′, 8-*O*-4′ and 5,5′) and one tri-FA (4-*O*-8′,5′-5″-dehydrotriferulic acid) in SBP gels, commonly found in pectin gels [25,42]. Table 4 shows FA, di-FA, and tri-FA contents in SBP dispersion at 0 and 80 min of laccase action. After SBP gelation, 53% of the initial FA content was oxidized resulting in the formation of di-FA and tri-FA participating in the 3D network development. The 8-5′, 5-5′, and 8-*O*-4′ structures represented 75, 17, and 8% of the total di-FA in the SBP gel, respectively. The same FA dehydrodimers were found in SBP before gelation, however, the major dehydrodimers found in SBP before gelation were 8-*O*-4′ with approximately 40.9%, 8,5′ with about 27.3%, and 5-5′with 18.2% of the total di-FA. Notably, the thermodynamic stability of 8-5′ dehydrodimer contributed to increasing this di-FA after the gelation step using laccase as an enzymatic free radical generating agent [66]. The increase in the 8-5′di-FA isoform is quite relevant for the potential application of SBP gels in the food industry since scientific evidence has demonstrated the contribution

of this isoform to major elasticity of ferulated polysaccharide hydrogels as pectins and arabinoxylans, caused by crosslinking between polysaccharide chains [9,41,44,66,67].

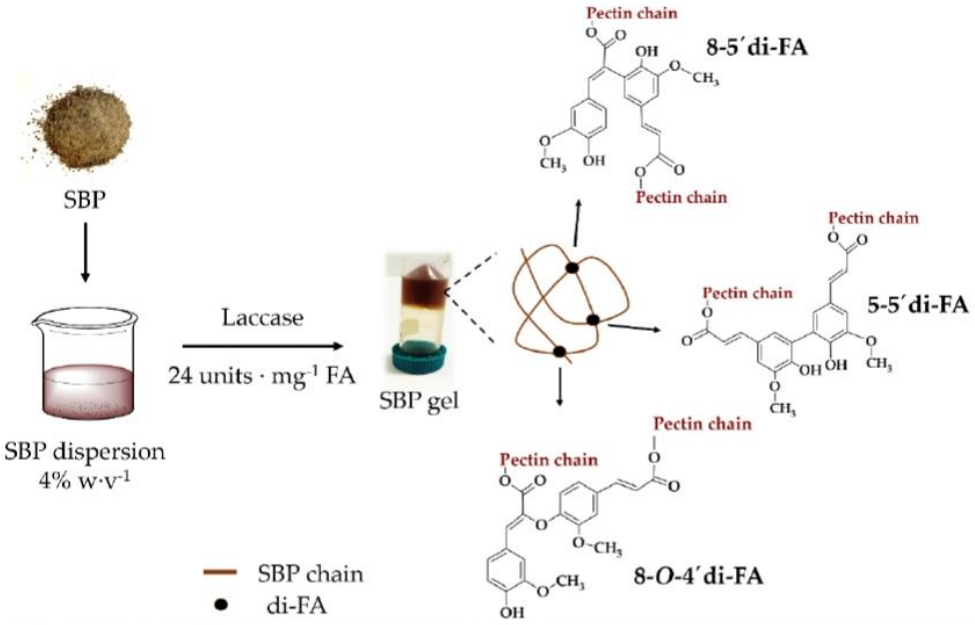

Figure 3. Schematic steps to SBP gel preparation and di-FA isomers found in SBP gel.

Table 4. Ferulic acid (FA), dimers of FA (di-FA), and trimer of FA (tri-FA) contents in SBP dispersion before (0 min) and after (80 min) enzymatic gelation.

Time	FA	di-FA	tri-FA
(min)	(mg·g^{-1} SBP Dry Matter)		
0	2.1 ± 0.1	0.22 ± 0.02	nd
80	0.97 ± 0.02	0.36 ± 0.03	0.13 ± 0.01

Values are presented as means ± standard deviations (n = 3). nd = non detected.

SBP gel preparation steps and di-FA isomers found in SBP gels after laccase action are schematized in Figure 3.

3.4.2. Rheology

The gelation of SBP was studied by dynamic mode rheological analysis. Figure 4a shows the evolution of storage (G′) and loss (G″) moduli as a function of time for 4% (*w*/*v*) SBP exposed to laccase. As observed, values increased rapidly in the first 10 min for both moduli, G′ and G″, indicating the ability of SBP gel to resist deformation during measurement evolution, in other words, the increase of elasticity. The elasticity of SBP gel increased over time caused by the formation of new covalent bonds between FA residues found in SBP chemical structure and laccase action. In fact, FA dehydrodimers are formed between contiguous polysaccharide chains leading to gel formation.

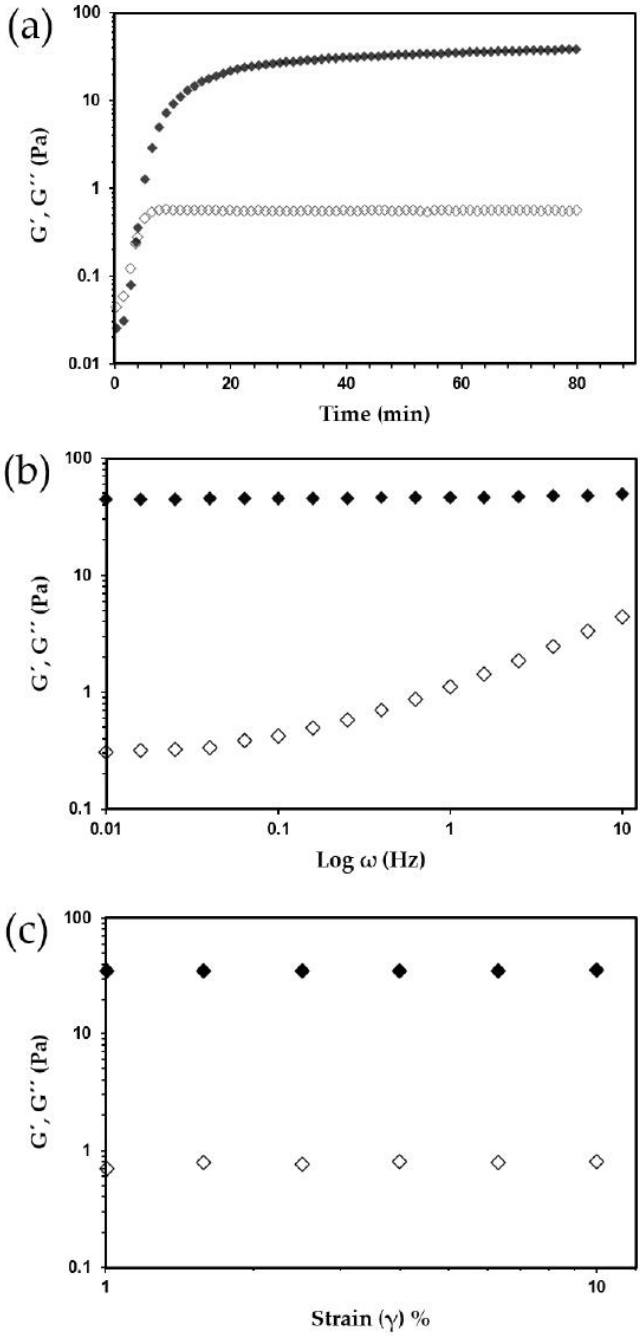

Figure 4. (**a**) Rheological kinetics of 4% (*w/v*) SBP during laccase induced cross-linking; (**b**) Mechanical spectrum and (**c**) Strain sweep of 4% (*w/v*) SBP gel. Gelation was induced with laccase (24 units·mg^{-1} FA), G′♦, G″ ◊.

After the first 10 min, the evolution process of G′ and G″ becomes slower. As a result, asymptotic behavior was reached and SBP gel was completely formed. G′ and G″ values were 44 and 0.6 Pa, respectively. In addition, gelation time was determined by the cross-over among G′ and G″ (G′ = G″), where G′ reaches higher values than G″; in this study, it was observed at ~5 min.

Tan δ = 0.014 ($G'' \cdot G'^{-1}$) [66] defines the overall behavior of SBP gel, predominating the elasticity than viscosity in the new covalently tridimensional network This behavior has also been observed in arabinoxylans gels [66,68] and previous ferulated SBP gels [69].

The mechanical spectrum (Figure 4b) and strain sweep (Figure 4c) of 4% (*w/v*) SBP gel after 80 min of laccase exposure were typical of solid-like materials with a linear G′ independent of frequency and strain, also G″ smaller than G′ and dependent of frequency [70].

The rheological analysis demonstrates gel formation, because of dehydrodimer formation caused by the oxidative coupling of FA triggered by laccase action, the was affected SBP while being extracted. No pretreatment was required to gel.

3.4.3. Texture Profile Analysis

Table 5 presents the texture profile analysis (TPA) for SBP gels formed by laccase action as an oxidative crosslinking agent. TPA results are correlated with previous texture profiles retrieved from SBP hydrogels constituted by pectins directly collected from the vegetable (sugar beet) due to limited information found in the literature regarding SBP extracted from SBW.

Table 5. Texture profile analysis of SBP gels.

Index	Value
Hardness (N)	3.0 ± 0.1
Fracturability (N)	1.6 ± 0.2
Adhesiveness (N)	1.3 ± 0.2
Springiness (mm)	0.98 ± 0.02
Cohesiveness (%)	0.32 ± 0.04
Gumminess (N)	1.0 ± 0.2
Chewiness (mJ)	1.0 ± 0.2

Values are presented as means ± standard deviations (n = 3).

Hardness is defined as the force required to compress 10% of gel. Then, for SBP gels hardness was 3.0 N. Norsker et al. [71] reported 11.2 and 10 N as hardness values for sugar beet pectin gels, meaning that our SBP gel is more sensitive to compression, thus softer. Additionally, Chen et al. [72] reported 1.99 N for hardness; actually, this parameter is highly related to morphological aspects; as a result, the three-dimensional morphology of SBP gels had a higher value of hardness compared to non-three-dimensional structures [72].

As mentioned by Norsker et al. [71] fracturability is highly related to hardness value, and even though neither Norsker et al. [71] nor Chen et al. [72] reported this value. In this study, fracturability was 1.6 N representing the force needed for the gel to crack, related to SBP chain-breaking caused by the applied force.

Our adhesiveness values (1.3 N) were also higher than those reported by Chen et al. [72], probably porosity contributes to this parameter to some extent. On the other hand, springiness for our gel was lower than the reported value by Chen et al. [72]. Springiness is the distance recording to compress 10% of the total mass, therefore the higher the hardness, the less the springiness.

Cohesiveness (0.32%), gumminess (1.0 N), and chewiness (1.0 mJ) values were similar comparing SBP gels and gels reported on Chen et al. [72]. On the other hand, gumminess is a property to be cohesive and sticky, defined as the energy required to disintegrate a semisolid food to a state of readiness for swallowing [73]. On the other hand, chewiness is defined as the energy required to masticate a solid food product [72,73]. At this point, morphological characteristics of the gel appear not to affect either of these textural parameters drastically.

3.4.4. Morphological Analysis

A three-dimensional network of SBP gels (4% *w/v*) were prepared through covalent cross-linking of FA upon oxidation by enzymatic free radical-generating agents. Therefore, the microstructure and network structure of gels are studied in various magnifications and

multiple images. Figure 5a shows a 200 magnification image of SBP gel and evidence of the presence of cavities and irregular surfaces. Also, at this magnification, we can assume that cavity shapes are well defined. In the same way, from Figure 5b we can ensure the presence of cavities in SBP gels. At 500 magnification, there is evidence for the presence of thin walls. This fact implies that the oxidative gelation process initiated by laccase promotes an organized cross-linking process. Figure 5c shows the organization of cavities, in an imperfect honeycomb-like structure.

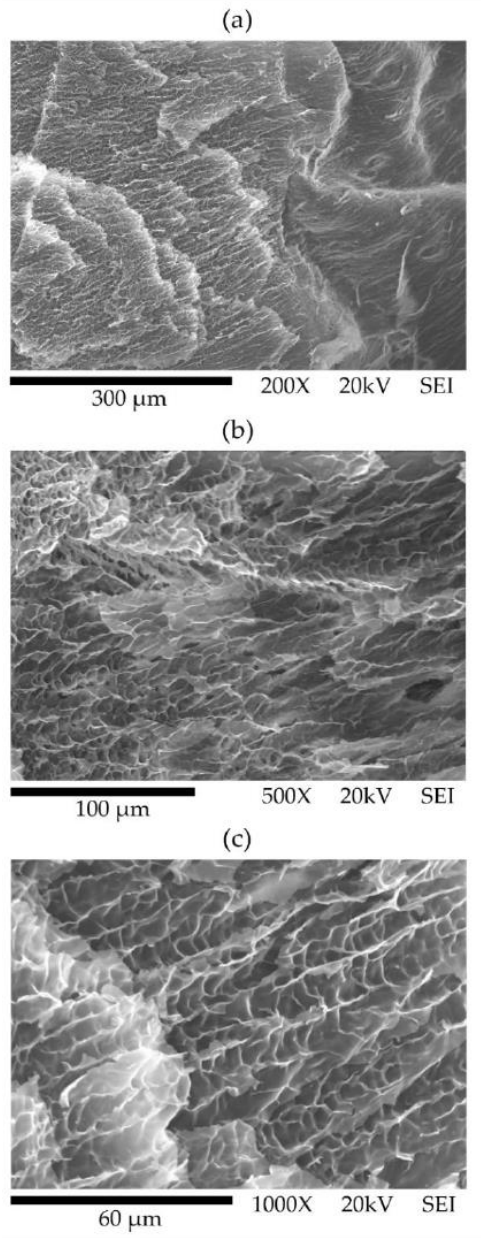

Figure 5. SEM micrographs of SBP gel at various magnifications (**a**) 200X magnification, (**b**) 500X magnification, and (**c**) 1000X magnification.

As far as the authors reviewed, this is the first report on morphological analysis for hydrogels composed of SBP extracted from SBW. However, recently some studies presented

SEM micrographs of hydrogels composed of pectins extracted directly from whole sugar beet vegetables. Certainly, the microstructure observed for SBP gels in this work contrasts those previously reported. To show that, Chen et al. [72] described their sugar beet pectin gels as non-three-dimensional structures, almost a solid filled gel with sphere-like pores. Considering the chemical composition of pectins and some critical aspects for gelling properties like FA content, polysaccharide molecular weight, and enzyme as a gelling agent are different, morphological characteristics of the resulting gel reflect them accurately.

As expected, the industrial processes used in the food industry modify the chemical and physicochemical composition of the organic matter used to produce food products. However, the foregoing does not limit the possibility of finding a utility for agro-industrial waste obtained after industrial processes, as has been shown in this work. The chemical and physicochemical characteristics of SBP reported in our research are comparable to SBPs extracted directly from the plant, which have been previously reported. Therefore, we have demonstrated that SBPs extracted from an agro-industrial residue have not lost the ability to form covalent gels by oxidative coupling reaction of FA triggered by laccase action. Even more important, the previous industrial processes undergone by sugar beet waste did not alter the typical imperfect honeycomb microstructure of SBP covalent gels due to laccase action. Hence, a comparable texture profile of SBP gels is maintained according to SBP gels previously reported in the literature. The fact that the SBP gels reported in this research maintained a three-dimensional structure opens a range of possibilities of potential applications for this polysaccharide, including use in the food industry as a protection and entrapment matrix for nutraceutical products, as well in the design of controlled release systems for drugs and biomolecules in the biomedicine and pharmaceutical areas.

4. Conclusions

SBP from the industrial waste of sugar beet bioethanol production was extracted successfully. SBP compositional and macromolecular characteristics were in the range reported for other ferulated pectins in the literature. Particularly, macromolecular characterization of SBP described a flexible and extended coil conformation with low probabilities of folding. SBP form 4% (*w/v*) covalent gels with G′ and G″ values of 44 and 0.66 Pa, respectively. As described above, SBPs extracted from an agro-industrial residue have not lost the ability to form covalent gels by oxidative coupling reaction of ferulic acid triggered by laccase action. Texture profile analysis exhibits a soft texture for SBP gels. Lyophilized SBP gels present an imperfect honeycomb-like structure with an average cavity diameter of 4.5 μm. SBP could be applied as a food additive to texturize or stabilize food products, such as jams and gummies, where soft food products are desired. Covalently cross-linked SBP gel could be an attractive food additive due to its stability to changes in temperature and pH.

Author Contributions: F.O.-D. performed the experiments and writing the paper. A.R.-C. conceived and designed the experiments, contributed to the discussion of the results, and edited the paper. E.C.-M. help in the design of the experiments and contributed to the discussion of the results. Y.L.L.-F. and M.A.I.-O. analyzed the data and contributed to the discussion of the results. C.L.-E. contributed to the discussion of results. J.A.M.-E. and J.A.S.-V. help in the design of experiments. All authors have read and agreed to the published version of the manuscript.

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4. FERULATED PECTINS AND FERULATED ARABINOXYLANS MIXED GEL FOR *Saccharomyces boulardii* ENTRAPMENT IN ELECTROSPRAYED MICROBEADS.

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Ferulated Pectins and Ferulated Arabinoxylans Mixed Gel for *Saccharomyces boulardii* Entrapment in Electrosprayed Microbeads

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Abstract: Ferulated polysaccharides such as pectin and arabinoxylan form covalent gels which are attractive for drug delivery or cell immobilization. *Saccharomyces boulardii* is a probiotic yeast known for providing humans with health benefits; however, its application is limited by viability loss under environmental stress. In this study, ferulated pectin from sugar beet solid waste (SBWP) and ferulated arabinoxylan from maize bioethanol waste (AX) were used to form a covalent mixed gel, which was in turn used to entrap *S. boulardii* (2.08×10^8 cells/mL) in microbeads using electrospray. SBWP presented a low degree of esterification (30%), which allowed gelation through Ca^{2+}, making it possible to reduce microbead aggregation and coalescence by curing the particles in a 2% $CaCl_2$ cross-linking solution. SBWP/AX and SBWP/AX+ *S. boulardii* microbeads presented a diameter of 214 and 344 µm, respectively, and a covalent cross-linking content (dimers di-FA and trimer tri-FA of ferulic acid) of 1.15 mg/g polysaccharide. The 8-5′, 8-*O*-4′and 5-5′di-FA isomers proportions were 79%, 18%, and 3%, respectively. Confocal laser scanning microscopy images of propidium iodide-stained yeasts confirmed cell viability before and after microbeads preparation by electrospray. SBWP/AX capability to entrap *S. boulardii* would represent an alternative for probiotic immobilization in tailored biomaterials and an opportunity for sustainable waste upcycling to value-added products.

Keywords: ferulated polysaccharide; oxidative cross-linking; laccase; yeast entrapment

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1. Introduction

Probiotic microorganisms are defined as "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host" [1]. Many probiotics must reach the colon section to provide health benefits, mainly to the human gastric system. However, these microorganisms can lose viability during gastrointestinal transit due to digestive enzyme sensitiveness and low pH [2]. *Saccharomyces boulardii* (*S. boulardii*) is a probiotic yeast acknowledged and generally recognized as safe (GRAS) [3]. However, *S. boulardii* viability is damaged under stressful environmental conditions [4,5]. In this regard, technological advances such as microencapsulation have been developed to protect probiotics' viability [6–8]. Microencapsulation allows bioactive materials to be coated with a single or a mixture of protective materials such as lipids, proteins, polysaccharides, sugars, and their combinations [9–12]. Electrospraying is a suitable microencapsulation method that uses an electric field to generate nano and micrometric droplets. The process involves subjecting a solution to an electric field during flow through capillary maintained at high potential; upon achieving critical value by an electric field, a jet (Taylor cone) is formed. Afterwards, the electric field causes deformation and jet distribution, resulting

in fine droplets forming microparticles [13]. This technique facilitates the preparation of microparticles with tailored composition and morphology, as well as customized microstructures, for various applications [14]. Ferulated pectins and ferulated arabinoxylans are suitable for microencapsulation because they are biodegradable and biocompatible. These polysaccharides present a high potential for chemical or physical modification, which favors the achievement of suitable properties [15]. Additionally, these polysaccharides are contained in the dietary fiber of fruits and cereals already present in the human diet. They have gained great relevance due to their wide applications and health benefits. Both polysaccharides share the characteristic of having ferulic acid (FA) in their structure [16]. Traditionally, pectins are obtained from citrus peels and apple bagasse [17,18], although ferulated pectin is mainly recovered from sugar beet. Sources like sugar beet pulp, a byproduct from sugar production, have been explored. The increasing demand for sugar beet for refined sugar and bioethanol production has increased the availability of sugar beet solid waste, leading to its use as an alternative pectin extraction source [19]. Structurally, ferulated pectins consist of linear chains formed mainly by galacturonic acid units linked glycosidically by α-(1→4) bonds (homogalacturonan region, HG), with branched sections of neutral sugars (rhamnogalacturonans I and II; RGI and RGII) and other substituents. These pectins have FA bound to galactose (*O*-6) and arabinose (*O*-2 and *O*-5) residues primarily in the RG-I branching [20] (Figure 1a). Ferulated pectins may have a low or high degree of esterification, and those registering low esterification can show two gelling mechanisms, one given from galacturonic acid interactions with divalent ions (i.e., Ca^{2+}) following the egg-box model [21], and the second driven by oxidative coupling of FA [22]. On the other hand, arabinoxylans are neutral nonstarch polysaccharides mainly obtained from cereals and byproducts of their industrialization, e.g., dried distillers' grains with solubles (DDGS) from bioethanol production [23]. These polysaccharides are constituted by a linear backbone of β-(1→4)-linked xylose units which may be unsubstituted, monosubstituted with arabinose via α-(1 →3), and disubstituted with arabinose via α-(1 →3) and α-(1 → 2). FA is ester-linked to C(*O*)-5 of the arabinose residues generally attached to C(*O*)-3 of xylose units [24] (Figure 1b). Ferulated pectins' gels induced by laccase are stable to pH changes but are not mechanically strong because RGI and RGII side chains limit the intermolecular contact [22]. Ionic pectin gels are strong but not stable to pH changes, particularly under acidic conditions [25], as their polymeric network is based on calcium bridges [26]. Arabinoxylans form stronger covalent gels resistant to changes in pH, temperature, and ionic strength [23,27,28]. However, arabinoxylans' cross-linking can present relatively extended curing times. The formation of a mixed ferulated pectin/ferulated arabinoxylan gel has not been yet reported, despite the high application potential and attractive characteristics that could be generated in the resulting biomaterial (especially for the design of carrying matrices). Combining the two-gelling mechanism of ferulated low-esterified pectin could be a strategy to avoid the coalescence and aggregation phenomena commonly observed during the arabinoxylan microparticle preparation electrospray technique [12]. Additionally, the presence of arabinoxylan in a pectin/arabinoxylan mixed gel could bring mechanical stability to the new biomaterial. In the present work, ferulated low-esterified pectin from sugar beet solid waste (SBWP) and ferulated arabinoxylan from maize bioethanol waste (AX) were used to form a mixed gel involving two-gelling mechanisms (ionic and covalent) to entrap *S. boulardii* using electrospray, maintaining cellular viability after the microencapsulation process.

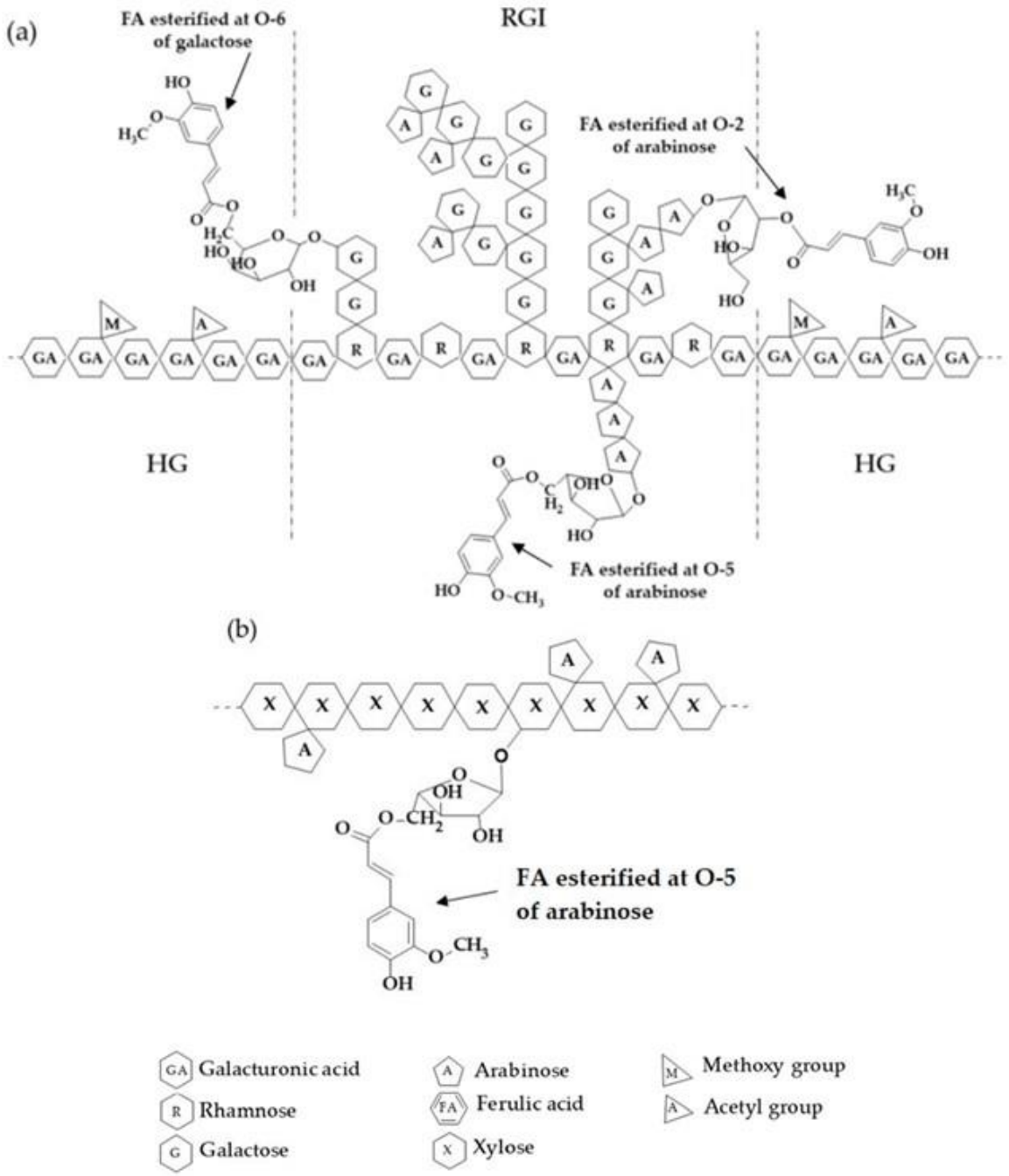

Figure 1. Schematic chemical structure for ferulated pectins (**a**) and ferulated arabinoxylans (**b**). HG = Homogalacturonan region, RGI = Rhamnogalacturonan I region.

2. Results and Discussion

2.1. SBWP Characterization

The chemical composition of SBWP is presented in Table 1. The galacturonic acid content in SBWP in the present study was 52.2% (*w*/*w*), which is higher than the value

(46.5% *w*/*w*) reported in a previous study for pectin from a similar industrial waste under comparable extraction conditions [29]. The galacturonic acid content in commercial pectin is approximately 60% (*w*/*w*); however, it is well documented in the literature that previous industrial processing of plant biomass might induce changes in the chemical structure of plant cell wall components, including the galacturonic acid content of pectin [25,30,31]. The neutral sugar content of SBWP is also shown in Table 1, with arabinose, mannose, and galactose being the main components. Additionally, some glucose was found in the sample. The FA content registered in SBWP (2.1 mg/g polysaccharide) was higher than some values reported in the literature for comparable pectins (0.28–1.9 mg/g) [32,33]. The FA content in SBWP was required for the oxidative coupling cross-linking mechanism in this polysaccharide.

Table 1. Chemical composition of sugar beet solid waste (SBWP).

Component	Value
Galacturonic acid [1]	52.2 ± 1.6
Rhamnose [1]	1.50 ± 0.02
Arabinose [1]	3.60 ± 0.04
Xylose [1]	1.20 ± 0.02
Mannose [1]	5.00 ± 0.04
Galactose [1]	20.7 ± 0.4
Glucose [1]	12.3 ± 0.2
Ferulic acid [2]	2.1 ± 0.1

[1] Results are expressed g/100 g SBWP dry matter. [2] Phenolics are expressed in mg/g SBWP dry matter. Values are presented as means ± standard deviations ($n = 3$).

The physicochemical characteristics of SBWP are presented in Table 2. The average molecular weight of SBWP was 468 kDa; this value is superior to some expressed in the literature (35–90 kDa), probably due to aggressive extraction conditions used in those studies [24,34]. The degree of methoxylation and acetylation of SBWP were 30% and 13%, respectively. Therefore, SBWP was classified as low-esterified and low-acetylated pectin, capable of forming calcium-induced gels. The latter is a handy feature for the fabrication of microbeads by electrospray [12]. The combination of two gelling mechanisms in this pectin allowed for particle surface stabilization by fast ionic gelation with Ca^{2+} ions and a covalent cross-linking from the inside by laccase-triggered oxidative coupling. AX used in the present study was extracted and characterized as previously described [23]. It presented a FA content of 6.46 mg/g polysaccharide, an arabinose to xylose ratio of 1:1, and a molecular weight of 200 kDa.

Table 2. Physicochemical characteristics of SBWP.

Component	Value
Molecular weight (kDa)	468 ± 8
Degree of esterification (%)	30 ± 2
Degree of acetylation (%)	13 ± 2

Values are presented as means ± standard deviations ($n = 3$).

2.2. Biomass Production and Cells Viability

After 6 h of biomass production, 2.08×10^8 cells/ mL of *S. boulardii* were recovered. A fresh sample of probiotic cells was stained with propidium iodide and analyzed in a confocal laser scanning microscope, leading to viability confirmation (Figure 2). It has been reported that viable cells do not take in propidium iodide; conversely, unviable cells take this stain inside. Therefore, only unviable cells show the propidium iodide fluorescence [34].

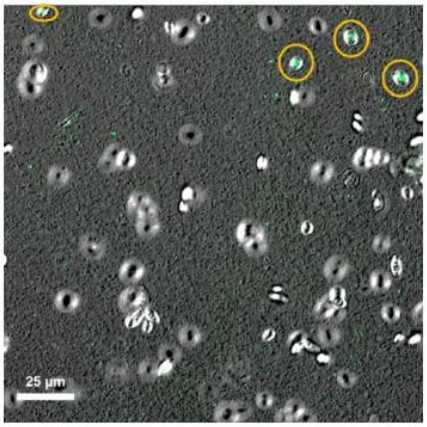

Figure 2. Confocal laser scanning microscopy image of *S. boulardii* fresh cells stained with propidium iodide, observed to 20× magnification applying a monoparametric detection.

As observed from Figure 2, cellular viability was maintained after biomass production. The color selected to note fluorescence was green. Additionally, as no viability loss was observed from several fields, an exhaustive search for some extracellular damaged cells was carried out. The only observed cells with extracellular damage are shown in Figure 2. These results confirmed that *S. boulardii* harvested after 6 h of culture under our biomass production conditions presented cellular viability.

2.3. Polysaccharides Gelation and Microbeads Preparation

The gelation of ferulated polysaccharides has been investigated via FA dimerisation using oxidative conditions; for instance, enzymatic free radical generating agents (i.e., laccase) [24]. Four di-FA isomers (8-5′, 8-*O*-4′, 5-5′ and 8-8′) and one tri-FA (4-*O*-8′, 5′-5″-dehydrotriferulic acid) structure have been reported in the gels formed. The 8-5′and 8-O-4′ di-FA forms are commonly preponderant in these gels [28]. In the present study, the covalent cross-linking structures (di-FA and tri-FA) were measured in SBWP/AX microbeads before and after laccase exposure (Table 3). After 2 h of polysaccharides gelation, 54% of the initial FA content in the mixture was oxidized, resulting in the formation of di-FA and tri-FA participating in the polymer´s network development. The 8-5′, 8-*O*-4′, and 5-5′ structures represented 79%, 18%, and 3% of the total di-FA in the mixed gel, respectively. It is well documented that 8-5′ and 8-*O*-4′ di-FA are the major isomers formed after oxidation reactions of FA monomers in ferulated polysaccharides [35]. Indeed, in nature, the major dehydrodimers are 8-*O*-4′ and 8-5′ for most angiosperm tissues, but abundant dimer isomers are also found in ferulated polysaccharide gels prepared under controlled conditions [36,37]. Just a small amount of tri-AF was formed in the microbeads after 120 min of laccase exposure. The di-FA + tri-FA (covalent cross-linking content) in SBWP/AX microbeads was 1.15 mg/g polysaccharide, which is high in relation to other gels based on ferulated polysaccharides recovered from agroindustrial byproducts [23,37].

Table 3. Ferulic acid (FA), dimers of FA (di-FA) and trimer of FA (tri-FA) contents in SBWP/AX microbeads before (0 min) and after (120 min) laccase exposure.

Time	FA	di-FA	tri-FA
(min)		(mg/g polysaccharides)	
0	3.58 ± 0.04	0.27 ± 0.04	nd
120	1.64 ± 0.02	1.01 ± 0.20	0.14 ± 0.02

Values are presented as means ± standard deviations (n = 3). nd = non detected.

SBWP/AX and SBWP/AX+ *S. boulardii* microbeads presented an average diameter of 214 ± 66 µm and 344 ± 126 µm, respectively (Figure 3). SBWP/AX+ *S. boulardii* microbeads showed a more spherical and regular shape in relation to SBWP/AX, which could be related to the yeast's presence inside the structure. It has been reported that other microorganisms (such as *Bifidobacterium* and *Debaryomyces*) improved the AX gel morphology [37,38]. Still, this kind of behavior has not previously been reported for mixed gel or *S. boulardii*. Complementary research is needed to elucidate the mechanism by which this yeast improves the microbead structure stabilization and shape. It has been suggested that *Bifidobacterium* interacts with polysaccharides such as AX, forming biofilms via bacteria surface protein complexes [39]. An interaction *S. boulardii*–AX/pectin could be present in the present study, contributing to preserving the microbead structure, but further research is needed to test such an argument.

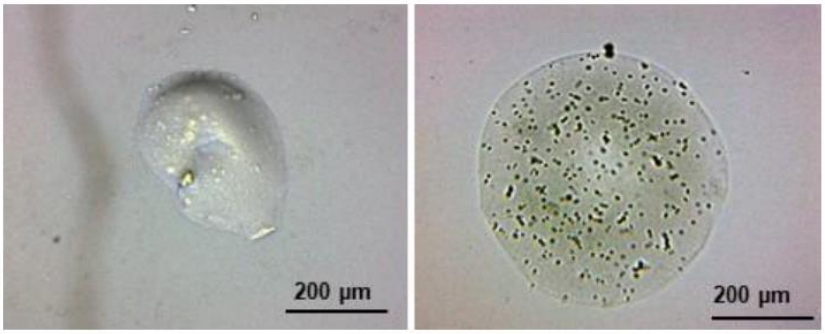

Figure 3. SBWP/AX (**a**) and SBWP/AX+ *S. boulardii* (**b**) microbeads images. Optical microscopy observation at 10× magnification.

On the other hand, SBWP/AX and SBWP/AX+ *S. boulardii* microbeads showed no aggregation or coalescence. The fast ionic gelation of the low methoxy SBWP used in the present study stabilized the particle surface, minimizing the interlinking of polymers from one droplet to another, as previously reported for other low methoxy pectins [12]. These results indicate that combining the two gelling mechanisms present in the mixed gel system can be a strategy to avoid the coalescence and aggregation phenomena during microparticle preparation using the electrospray technique unlike previous reports where using AX with long gelation times showed high aggregation [12,37]. The microbeads surface of AX alone does not stabilize soon enough on the particle surface and may join the surrounding particles into one big bead or pearl, or into extended aggregates of particles. On the contrary, SBWP ionic interaction will stabilize the droplets' surface. SBWP has a low degree of esterification, which allows galacturonic acid interactions with divalent ions such as Ca^{2+} to form the egg-box model [21]. SBWP and AX (in the presence of free radical-generating agents such as laccase) undergo oxidative gelation through the coupling of FA residues resulting in the formation of di-FA and tri-FA. It has been reported that the FA aromatic ring operates as a reactive site in the cross-inking mechanism of ferulated polysaccharides [24]. The FA oxidative coupling gave rise to covalent cross-linking, allowing for a stable and robust SBWP-AX covalent polymeric network inside the particle. This combination has proven adequate for electrospray fabrication of particles with neither aggregation nor coalescence, a desirable characteristic for further standardized target delivery of the immobilized cells.

Figure 4 shows a confocal laser scanning microscopy image of a SBWP/AX+ *S. boulardii* microbead with yeast cells stained with propidium iodide. It can be observed that microbeads fabrication by electrospray did not considerably affect the viability of *S. boulardii* as only two nonviable cells showed the propidium iodide fluorescence (circled in red). Most particles showed fully viable cells after the electrospray conditions, exerting high voltage

on the whole system. To the authors' knowledge, this is the first report on ferulated-mixed polysaccharide gels and their application in microbeads design for immobilization of a probiotic cell.

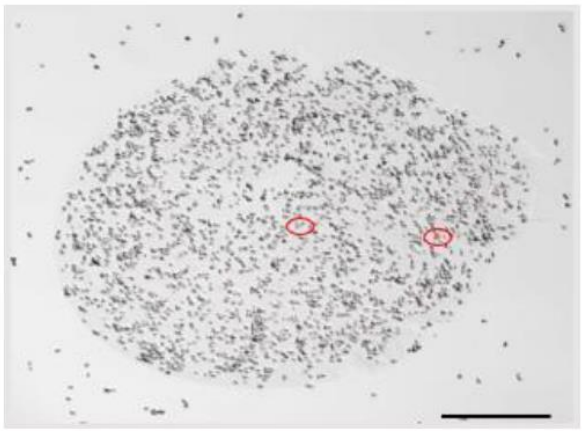

Figure 4. Confocal laser scanning microscopy image of SBWP/AX+ *S. boulardii* stained with propidium iodide. Circled in red two non-viable cells of *S. boulardii*. 10× magnification applying a monoparametric detection.

The results presented here show this mixed gel-based material's potential to design systems focused on carrying probiotics for food and nonfood applications. Furthermore, therapeutic compounds and functional molecules may also be trapped and carried by this system. The fine details of structural interaction between both ferulated polysaccharides is an opportunity for further research, to investigate whether their structural differences would partly restrict the oxidative coupling between pectins and arabinoxylans. Nevertheless, the microbeads fabrication method reported herein takes advantage of two gelling mechanisms, where fast ionic gelation of low methoxy SBWP with Ca^{2+} in the particle surface is combined with the strength and stability of covalent bonding inside the SBWP/AX microbead, resulting in an innovative fabrication technique. Furthermore, agrifood byproducts such as DDGS and sugar beet pulp may well constitute a further resource for gelling ferulated polysaccharides with exceptional characteristics for tailored value-added biomaterials.

3. Materials and Methods

3.1. Materials

Sugar beet solid waste (SBW) was kindly provided by a bioethanol production facility located in Sonora, Mexico. AX from maize dried distillers' grains with solubles (DDGS) was extracted and characterized as previously described [23]. Laccase (benzenediol:oxygen oxidoreductase, E.C.1.10.3.2) from *Trametes versicolor*, and all the chemical reagents used were purchased from Sigma Aldrich (St. Louis, MO, USA). Commercial *S. boulardii* (CNCM I-745) was used as a probiotic cell.

3.2. SBWP Extraction and Characterization

Pectin was extracted from SBW, based on a methodology previously reported [40], with some modifications. Briefly, 150 g of dried SBW was dispersed in 1.5 L of 0.1 M HCl, and pH was adjusted to 1.5. The mixture was homogenized and heated at 85 °C for 2 h. Then the mixture was centrifuged (10,000× *g*, 15 min, 25 °C) (Thermo Scientific, Waltham, MA, USA). The supernatant was treated with ethanol in a 1:2 (*v*/*v*) ratio and the precipitate formed was dried by solvent exchange. Dried precipitate was dispersed in Milli-Q water (1:200 *w*/*w*) for 24 h with stirring, centrifuged (10,000× *g* for 15 min), filtered through 3.0,

1.2, 0.8, and 0.45 µm and precipitated by ethanol:water 1:2 (*v*/*v*) at 4 °C to obtain SBWP which was dried by solvent exchange.

The galacturonic acid content in SBWP was determined following a procedure reported [41] using high-performance liquid chromatography (HPLC) (Varian Prostar 210, Refractive Index Detector Prostar 350, Varian, Palo Alto, CA, USA), a MetaCarb H Plus column (Agilent, Santa Clara, CA, USA; 7.8 × 300 mm) and 0.001 N H_2SO_4 at 0.4 mL/min and 65 °C as mobile phase. Neutral sugar composition was performed by gas chromatography [42] (PerkinElmer, Clarus 580) using a high-performance capillary column (Elite 225, PerkinElmer, 30 mL × 0.32 mm ID × 0.15 µm film thickness). FA and its dimers and trimer content were analyzed by HPLC [36] using an Alltima C18 column (250 × 4.6 mm; Alltech Associates, Deerfield, IL, USA) and a photodiode array detector, Waters 996 (Millipore, Milford, MA, USA). The degree of methoxylation and acetylation was determined as previously reported [43]. Methanol and acetic acid were produced during SBWP saponification with 1 M NaOH at 4 °C for 2 h. Isopropanol was used as an internal standard. Samples were centrifuged for 10 min at 8000× *g* and 25 °C. Supernatants were neutralized before injection. Methanol, acetic acid, and isopropanol were quantified by HPLC VARIAN 500 (Varian, St. Helens, Australia) within a Refractive Index Detector on a C18 column (Supersper 100 RP-18 endcapped, Merk KGaA, 250 × 4 mm). Elution was carried out with 4 mM H_2SO_4 at 0.7 mL/min and 25 °C. Molecular weight (Mw) was determined using a size exclusion chromatography system coupled to a DAWN HELOS-II 8 multi-angle laser light scattering (MALLS) detector, a refractive index Optilab T-rex detector (Wyatt Technology Corp., Santa Barbara, CA, USA), and an Agilent HPLC System (G1310B Iso-Pump, G1329B autosampler, and G1314F variable wavelength detector, Agilent Technologies, Inc., Santa Clara, CA, USA). Shodex OH-pak SBH-Q-804 and 805 (Shodex Showa Denco K.K., Tokyo, Japan) columns were utilized. The software ASTRA 6.1 was used [44].

3.3. Biomass Production

S. boulardii cells were cultured in 100 mL of commercial malt extract broth (Difco, TM, pH, 5.4 at 25 °C). Cells were incubated at 31 °C and 150 rpm for 20 h (Lab-line 3540 brand). Cell counts were conducted manually every 2 h using a Neubauer Chamber in optical microscopy (Zeiss Axio Vert. A1, Carl Zeiss Microscopy, Jena, Germany) equipped with a digital camera (Axio Cam ERC 5s, Jena, Germany). Fresh cells were recuperated by centrifugation at 6000 rpm for 5 min and 4 °C [30].

3.4. Microbeads Preparation

Immobilization of *S. boulardii* inside SBWP/AX microbeads was carried out by coaxial electrospray using SpraybaseTM system (ProfectorTM, Dublin, Ireland) and two programmable syringe pumps (worldPrecision Instruments, AL-1000, Sarasota FL, USA), independently feeding a coaxial needle, as previously reported [12]. Technical electrospray conditions used were 9 kV, 0.7 mL/h for the inner needle, and 0.3 mL/h for the outer needle. SBWP/AX microspheres were prepared with and without *S. boulardii*. The outer needle conducted the mixture SBWP/AX at an overall biopolymer concentration of 53 mg/mL (40 mg/mL SBWP and 13 mg/mL AX in a 3.1 *w*/*w* biopolymer ratio) dispersed in 0.1 M sodium acetate buffer pH = 5.5. This SBWP/AX ratio ensured a 1:1 (*w*/*w*) FA contribution from each polysaccharide. SBWP/AX mixture in the outer needle was used alone or with *S. boulardii* cells (amount of cell entrapped in microspheres, 2.08 × 10^8 cells/ mL). The inner needle conducted laccase in 0.1 M sodium acetate buffer pH = 5.5 as the cross-linking agent. Laccase dispersion contained 24 units/mg FA to ensure SBWP/AX gel-forming. The spray was received in $CaCl_2$ at 2% (*w*/*v*) in ethanol:water 1:2 (*v*/*v*) fixing a distance of 7 cm from coaxial needle. It has been reported that laccase activity shows significant stability in the presence of organic solvents like ethanol, even in 50:50 *v*/*v* proportion [45]. Microbeads were stored at 4 °C. Scheme depicting coaxial electrospray process used to produce SBWP/AX and SBWP/AX+ *S. boulardii* microbeads is presented in Figure 5.

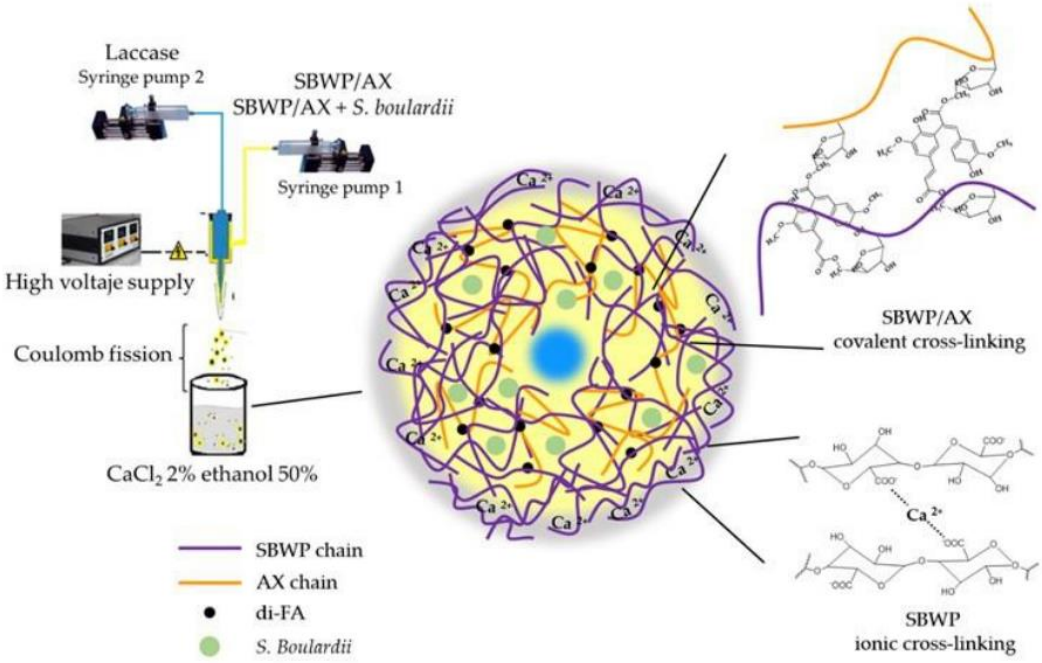

Figure 5. Scheme of coaxial electrospray process used to produce SBWP/AX and SBWP/AX+ *S. boulardii* microbeads.

SBWP/AX and SBWP/AX+ *S. boulardii* microbeads' morphological characteristics were investigated by optical microscopy using an inverted optical microscope (Zeiss Axio Vert. A1, Carl Zeiss Microscopy, Jena, Germany) equipped with a digital camera (Axio Cam ERC 5s, Jena, Germany). The microbeads' average diameter was determined by using ImageJ software. FA, di-FA, and tri-FA content in microbeads were determined by high-performance liquid chromatography as described in Section 3.2 [35].

3.5. S. boulardii Viability

Propidium iodide was used as staining for fresh and microencapsulated cells. Cells were incubated (5 µL/mL of propidium iodide) for 10 min at room temperature. Staining was also carried out after microencapsulation to evaluate the electrospraying effect on cell viability. Monoparametric detection of propidium iodide fluorescence was performed using FL-3 (488/620 nm) confocal laser scanning microscopy (Zeiss Airyscan, Carl Zeiss Microscopy, Jena, Germany), as previously reported [34]. Briefly, viable cells do not take in the stain; conversely, unviable cells take propidium iodide inside, which reacts to DNA and stains them. Only unviable cells show the fluorescence of propidium iodide.

3.6. Statistical Analysis

Results are expressed as means ± standard deviation (S.D) from triplicates.

4. Conclusions

Microbeads based on a mixed gel of ferulated pectins and arabinoxylans were prepared by electrospray. These microbeads did not present coalescence or aggregation under the coaxial electrospray technique arrangement used. Additionally, the electrospraying conditions allowed the encapsulation of *S. boulardii* cells without losing their viability in the process. These results suggest a high potential for probiotic-loaded SBWP/AX microbeads designed for a variety of applications. Additionally, extraction of SBWP and AX from bioethanol wastes could represent a chance for sustainable byproducts' use through upcycling to value-added products. Further studies will investigate the

protection of *S. boulardii* in SBWP/AX microbeads against environmental stress such as gastric conditions.

Author Contributions: Investigation, writing—original draft preparation, F.O.-D.; writing—review and editing, Y.L.L.-F. and M.A.I.-O.; formal analysis, supervision, writing—review and editing E.C.-M.; investigation, writing—review and editing, V.M. and C.A.-A.; formal analysis, supervision, writing—review and editing; funding acquisition, project administration, A.R.-C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to patent claim in process.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of *Saccharomyces boulardii* and SBWP are not available from the authors.

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5. ENTRAPMENT OF *Saccharomyces boulardii* IN FERULATED PECTINS AND FERULATED ARABINOXYLANS MIXED GELS AND MICROBEADS: RHEOLOGICAL PROPERTIES, ANTIOXIDANT ACTIVITY AND *in vitro* FERMENTATION.

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GELS

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Article

Entrapment of *Saccharomyces boulardii* in Ferulated Pectins and Ferulated Arabinoxylans Mixed Gels and Microbeads: Rheological properties, Antioxidant Activity and *in vitro* Fermentation.

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Abstract: Ferulated polysaccharides such as pectin and arabinoxylan form covalent gels attractive for drug delivery or cells immobilization. *Saccharomyces boulardii* is a probiotic yeast known for providing human health benefits; however, its application is limited by viability loss under environmental stress. In this study, ferulated pectin from sugar beet solid wastes (SBP) and ferulated arabinoxylan from maize bioethanol waste (AX) were used to form a covalent mixed gel and to entrap S. *boulardii* (2.08 x10^8 cells/ mL) in mixed gels and mixed microbeads by using electrospray. The antioxidant activity of gels and microbeads were determined by ABTS$^{\cdot}$ and DPPH methods. Antioxidant activity of SBP/AX macro gel was 19.02 ± 0.63 TEAC and 4.2 ± 0.3 TEAC for ABTS$^{\cdot}$ and DPPH methods, respectively. Antioxidant activity of SBP/AX + *S. boulardii* microbeads was 20.66 ± 0.03 TEAC and 6.85 ± 0.29 TEAC for ABTS$^{\cdot}$ and DPPH methods, respectively. Additionally, a molecular identification was performed by Infrared Spectroscopy of SBP/AX dispersion before gelation and SBP/AX microspheres after gelation, demonstrating the presence of the two individual components in the microbeads prepared by the electrospray technique. Additionally, the microstructure of SBP/AX gels and SBP/AX + *S. boulardii* microbeads was studied by scanning electron microscopy, observing an imperfect honeycomb-like microstructure in SBP/AX gels with an average cavity diameter of 19 ± 12 µm. SBP/AX + *S. boulardii* microbeads exhibited a wrinkled, rough surface, with average diameter of 344 ± 126 µm. Finally, the release of *S. boulardii* cells during colonic *in vitro* simulation demonstrate the capability of SBP/AX system to entrap *S. boulardii* and deliver in colon.

Keywords: Ferulated polysaccharide, oxidative cross-linking, laccase, yeast entrapment

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1. Introduction

Probiotics microorganisms are defined as "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host" [1]. However, these microorganisms can lose viability during gastrointestinal transit due to digestive enzyme sensitiveness, such as trypsin and pepsin; bile salt action and/ or low pH [2]. *Saccharomyces boulardii* (*S. boulardii*) is a probiotic yeast acknowledged as generally regarded as safe (GRAS), widely use in pharmaceutical industry for its efficacious to treat diarrheal associated diseases However, *S. boulardii* viability is damaged under stressful environmental conditions [4,5]. In this regard, technological advances such as microencapsulation have been developed to protect probiotics viability [6–8]. A promising technology that has gained rele-

vance in recent years for increasing the technological properties of probiotics is microencapsulation [3,4]. Microencapsulation is defined as a process by which bioactive materials are coated with single or mixture of protective materials [5]. Microencapsulation has been used for the preservation of probiotic microorganism during storage and food processing [6]. Some of the materials used for protective core designing have been lipids, proteins, polysaccharides, sugars, polymers, and their combinations [7,8]. Previously, Ohlmaier-Delgadillo et al. [9] microencapsulated *S. boulardii* in a covalent gel of ferulated pectins and arabinoxylans by electrospray. Electrospraying is a relevant microencapsulation process used in both, food, and pharmaceutical industry. This method uses an electric field for the generation of nano and/ or micrometric droplets [10]. This technique offers ease in preparation of microbeads with tumble compositions, tailored properties of morphology, shape, and size with customized microstructure for a variety of applications [11]. Various natural polymer-based microbeads have been fabricated successfully. Pectins and arabinoxylans are suitable for microencapsulation, because they are biodegradable, biocompatible, and possess high potentials for their modification to achieve the required properties [12]. Both polysaccharides may present ferulic acid (FA) in their structure. In nature, pectins contribute to the firmness and structure of plant tissue, bringing mechanical resistance to cell walls [13]. Structurally, pectins are composed by three main domains[14], homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II [15,16]. Besides neutral sugars, hydroxycinnamic acids mainly FA may be esterified to arabinans and galactans in rhamnogalacturonan I region of some chenopodiaceae species like, sugar beet *(Beta vulgaris)* [17,18]. The latter has been widely studied as an important source of pectins, not only due to its high pectin content, but also for actual growth in sugar beet deman for refined sugar and bioethanol industries, increasing the availability of sugar beet solid wastes (SBW) in the market leading its use as an alternative extraction source [19–21]. Pectins from SBW (SBP) have FA bound to galactose (*O*-6) and arabinose (*O*-2) residues in the RG-I chains, additionally, some SBP have low degree of esterification. Consequently these pectins, show two gelling mechanism, one given from ion interaction with galacturonic acid (GalA) groups and divalent ions (i.e, Ca^{2+}) [22]; and the second gelling mechanism is driven through oxidative coupling reaction, mediated by chemical or enzymatic oxidation, and the cross-linking is formed by a carbon- carbon covalent bond between two ferulated phenyl rings given the formation of phenoxy radical [23]. The gelling ability of SBP has become relevant to design controlled released vehicles to drugs and biomolecules of therapeutic interest [5,6]. Although, ferulated pectin gels tend to have low mechanical properties for its high acetyl groups content and presence of numerous side chains [24]. On counterpart, ferulated arabinoxylans (AX) are obtained from cereals and industrial solid wastes obtained from cereal industrial processing such as corn, wheat, barley and oats [25–27]. Structurally, AX consists of a linear backbone chain of xylose units containing arabinose substituents attached through *O*-2 and/or *O*-3 [28]. Some of the arabinose residues are ester-linked to FA through *O*-5 giving the ability to form covalent gels resistant to changes in pH, temperature and ionic strength [29]. The strengths of both polysaccharides to form gels can be combined to design controlled release matrices. For instance, the combination of the two gelling mechanism is an strategy to avoid coalescence and aggregation phenomena during microparticle preparation using electrospray technique, as previously reported on oxidative coupling of polysaccharides with long gelling time [8]. Apparently micro and nanoparticle surface of the latter do not stabilize soon enough and join the particles next to them into one big bead or pearl. In theory, ionic interaction stabilizes the surface of the droplets while oxidative coupling takes place and gives rise to covalent crosslinking within the inside of the particle.

In the present work, SBP/AX gels and microbeads were prepared by lacasse action as a crosslinking agent with and without *S. boulardii* cell entrapment. Additionally, SBP/AX microbeads with spherical shape were prepared combining two gelling mechanism using

the electrospraying technique. The effect on rheological properties and antioxidant activity of gels and microbeads systems by addition of *S. boulardii* cells was evaluated. The effect on *S. boulardii* cell survival after microencapsulation *in vitro* digestion tract simulation was also addressed.

2. Results and Discussion

2.1. Fourier Transform Infrared Spectroscopy

The infrared analyses were performed in order to compare the structural characteristics of SBP, AX, SBP/AX dispersion before gelation (Dis) and SBP/AX + *S. boulardii* microbeads after gelation. Initially, the AX and SBP spectra were found to be similar to those previously reported for other AX and SBP polysaccharides [30,31]. Wide absorption bands at approximately 3400 cm^{-1} corresponding to stretching vibration of hydrogen-bonded O-H groups are present for all the samples tested. In addition, a characteristic stretching signal of O-CH_3 from methyl esters of galacturonic acids is found in proximally 2900 cm^{-1} for SBP, SBP/AX dispersion and and SBP/AX + *S. boulardii* microbeads. The signal observed at approximately 2900 cm^{-1} presented in AX spectrum can be assigned to the CH_2 groups in AX chains. A particular signal located at approximately 1740 cm^{-1} is observed in PF, SBP/AX dispersion and SBP/AX + *S. boulardii* microbeads. These are a common signals found in pectin spectra and are related to the degree of methylation, these bands are associated with the vibrations of esterified carboxyl groups or ester carbonyl (C=O) [21,32]. As observed in Figure 1, the signal located at 1740 cm^{-1} is missing in AX spectrum, however was maintained in SBP/AX + *S. boulardii* microbeads indicating the presence of SBP in SBP/AX dispersion and SBP/AX + *S. boulardii* microbeads. In addition, bands located at approximately 1600 cm^{-1} for all spectra correspond to free carboxyl groups or carboxylate ion (COO^-) [21,32]. Besides, the intensity increase for bands located in approximately 1600 cm^{-1} and 1400 cm^{-1} observed in SBP/AX + *S. boulardii* microbeads spectrum can be related to the formation of new monoesters groups, attributed to the crosslinking of SBP and AX to gel in presence of laccase as crosslinking agent. In addition, the region from 700 to 1500 cm^{-1} is the fingerprinting region for polysaccharides [19,33]. Within this region, a signal at approximately 1400 cm^{-1} can be attributed to the symmetric stretching vibrations of COO^- functional group of amino acid side chains, free fatty acids or other derivative compounds carried as an effect of the extraction process [19]. C-O-C vibration of glycosidic linkages of polysaccharide's backbone structure is observed at approximately 1120 cm^{-1}. An important band located at 1070 cm^{-1} is attributable to C-O stretching of side groups in C-O-C glycosidic linkages [19,21,33].

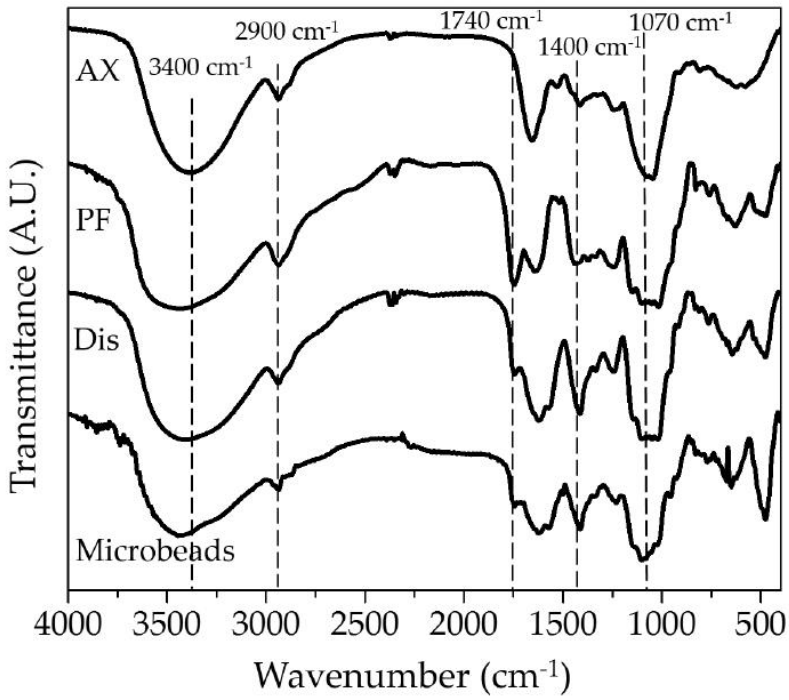

Figure 1. Fourier Transformed Infrared Spectroscopy (FT-IR) spectra for AX (polysaccharide), SBP (polysaccharide), SBP/AX dispersion (Dis), and SBP/AX + *S. boulardii* microbeads.

2.2. FA, di-FA and tri-FA Content of Pristine Polysaccharides, and gels

FA, dimers of FA (di-FA), and trimers of FA (tri-FA) content of pristine polysaccharides before gelation and gels after gelation by laccase action are presented in Table 1. As we previously described [31] SBP had a FA content of 2.1 μg/mg polysaccharide and its prevalent di-FA are 8-*O*-4′ and 8-5′ with about 40.9 % and 27.3% of total dimers of FA, respectively. After gelation of SBP caused by laccase action, 53% of the initial FA content was oxidized resulting in the formation of di-FA and tri-FA participating in the 3D network development. The 8-5′, 5-5′, and 8-*O*-4′structures represented 75, 17, and 8% of the total di-FA in the SBP gel, respectively. In addition, pristine AX had a FA content of 6.5 μg/mg polysaccharide. The 8-5′ (mainly in benzofuran form), 5-5′and 8-*O*-4′ structures represented about 45, 39 and 15% of the total di-FA in AX. After 2 h of gelation, FA content decreased from 6.5 to 2.62 μg/mg polysaccharide in AX gel due to covalently crosslinking promoted by laccase. The 8-5′ (mainly in benzofuran form), 5-5′and 8-*O*-4′ structures represented about 85, 12 and 1% of the total di-FA in AX gel. Moreover, the FA content decreased from 3.6 to 1.64 μg/mg polysaccharide when SBP/AX dispersion formed SBP/AX gel after 2 h of crosslinking step promoted by laccase. However, when adding *S. boulardii* cells to the dispersion in order to prepare the SBP/AX + *S. boulardii* gel, the FA content decreased from 3.6 to 1.94 μg/mg polysaccharide, quite higher than 1.64 μg/mg polysaccharide measured in SBP/AX gel without cells. These result could be caused by a physical phenomenom generated by the presence of cells in the dispersion when laccase started the crosslinking process. The cells could negative affect the laccase action during gelation process. As observed in table 1, the presence of cells during gelation of SBP/AX promoted

positively the formation of 8-5′ (mainly in benzofuran form) di-FA with approximatively 78% of the total di-FA isomers, compared to 54% of 8-5′ di-FA when SBP/AX gel lacked of cells. As we previously describe [31], the increase in the 8-5′ di-FA isoform is quite relevant for the potential application of these gels since scientific evidence has demonstrated the contribution of this isoform to major elasticity of ferulated polysaccharide hydrogels as pectins and arabinoxylans, caused by crosslinking between polysaccharides chains.

Table 1. FA, di-FA, and tri-FA contents in pristine polysaccharides and gels.

Sample	FA	di-FA	Tri-FA	di-FA isomers			
	μg/mg polysaccharide			%			
				8-5′	8-5′benzo	8-*O*-4′	5-5′
SBP	2.1 ± 0.1	0.22 ± 0.02	nd	27.3	13.6	40.9	18.2
AX	6.50 ± 0.01	0.46 ± 0.03	0.070 ± 0.001	12.8	32.6	15.2	39.1
SBP/AX dispersion	3.60 ± 0.04	0.27 ± 0.04	0.02 ± 0.01	37.0	7.4	40.7	22.2
SBP gel	0.97 ± 0.02	0.36 ± 0.03	0.13 ± 0.01	8.3	66.7	8.3	16.7
AX gel	2.62 ± 0.06	1.91 ± 0.19	0.27 ± 0.03	2.6	83.8	1.7	12.1
SBP/ AX gel	1.64 ± 0.02	1.0 ± 0.2	0.14 ± 0.02	21.0	33.0	18.0	29.0
SBP/AX + *S. boulardii* gel	1.94 ± 0.01	0.86 ± 0.02	0.024 ± 0.001	37.6	40.7	8.8	12.8

Values are presented as means ± standard deviations from triplicates. nd= non detected.

2.2. In vitro Antioxidant Activity

The *in vitro* antioxidant capacity of pristine polysaccharides (SBP and AX), gels (SBP, AX and mixed SBP/AX with or without *S. boulardii* cells), and SBP/AX microbeads with and without *S. boulardii* were measure using $ABTS^{+}$ and DPPH methods. The Table 2 shows the TEAC values of pristine polysaccharides (SBP and AX), gels (SBP, AX and mixed SBP/AX with or without *S. boulardii* cells), and SBP/AX microbeads with and without *S. boulardii* obtained after the *in vitro* essay. As presented in Table 2, antioxidant activity of pristine polysaccharides (SBP, AX and SBP/AX dispersion) decreased when each sample suffered the crosslinking process caused by laccase as crosslinking agent. As a result, FA is first oxidized, then coupling reaction takes place by the formation of di-FA and tri-FA. This behavior has been previously observed for AX gels and AX microbeads [27,30,34]. Additionally, some physical aspects could affect the antioxidant ability of samples. For instance, Mendez-Encinas et al., [30] described that gelation process increased the rigidity of the polysaccharide chains leading to decrease in antioxidant activity, observed in AX gels; due to restriction in physical availability and distribution. Similarly, the size of SBP/AX gels have an effect in antioxidant activity. On counterpart, microbeads presented higher antioxidant activity than macro gel, this behavior could be attributed to higher surface area in microbeads. As shown in Table 2, SBP/AX + *S. boulardii* gel, and SBP/AX + *S. boulardii* microbeads, kept their antioxidant activity after the crosslinking process, a relevant characteristic for this new entrapment matrix. Though lower total FA content, the dimers formed contribute highly to antioxidant capacity, consistent to Ohlmaier-Delgadillo et al. [9].

Table 2. Antioxidant activity of SBP, AX, SBP/AX dispersion, SBP gel, AX gel, SBP/AX gel, SBP/AX + *S. boulardii* gel, SBP/AX microbeads, and SBP/AX + *S. boulardii* microbeads.

	Antioxidant Activity [a] (μmol TEAC/g)	
Sample	$ABTS^{+}$	DPPH
SBP	21 ± 3	13.4 ± 0.5
AX	50.9 ± 0.2	22.0 ± 0.1
SBP/AX dispersion	41.36 ± 0.05	20.7 ± 0.8
SBP gel	17.1 ± 0.3	3.7 ± 0.5
AX gel	20.68 ± 0.13	9.1 ± 0.9
SBP/AX gel	19.0 ± 1.6	4.2 ± 0.6
SBP/AX + *S. boulardii* gel	20.2 ± 0.4	5.8 ± 0.2
SBP/AX microbeads	19 ± 1	6.1 ± 0.4
SBP/AX + *S. boulardii* microbeads	20.66 ± 0.03	6.9 ± 0.6

[a] TEAC, in μmol/g sample. All values are mean ± standard deviation of triplicate.
Values are presented as means ± standard deviations of triplicates (n = 3)

2.3. Gelation and Covalent Crosslinking

The kinetics of gelation of SBP, SBP+ *S. boulardii* cells, AX, AX+ *S. boulardii* cells, mixed SBP/AX, and mixed SBP/AX+ *S. boulardii* cells were measured using a small deformation rheology, as previously reported by Ohlmaier-Delgadillo et al. [31]. Figure 2 (a) shows the evolution of storage (G′) and loss (G′′) moduli as a function of time for 4% (w/v) SBP, 4% (w/v) SBP+ *S boulardii*, 1.3% (w/v) AX, 1.3% (w/v) AX+ *S. boulardii*, SBP/AX (1:1 FA ration) mixed gel, and SBP/AX+ *S. boulardii* (1:1 FA ration) mixed gel. The gelation profiles presented in figure 2 (a) followed a characteristic kinetics steps observer previously for SBP and AX gels [30,31,35,36]. For instance, an initial increase in G′ and G′′, followed by a plateau region. This pattern indicates an initial formation of covalent linkages between FA of adjacent polysaccharide molecules generating a three dimensional network. After 10 min, the evolution process of G′ and G′′ becomes slower. As a result, asymptotic behavior was reached and gels were completely formed.

Figure 2 (b) shows the mechanical spectrum for 4% (w/v) SBP, 4% (w/v) SBP+ *S boulardii*, 1.3% (w/v) AX, 1.3% (w/v) AX+ *S. boulardii*, SBP/AX (1:1 FA ration) mixed gel, and SBP/AX+ *S. boulardii* (1:1 FA ration) mixed gel. Figure 2 (b) described a typical solid-like material with a linear G′ independent of frequency, and G′′ smaller than G′ and dependent on frequency. Similar behavior has been reported previously for maize AX gels, and SBP gels [26,31,34]. Figure 2 (b) expresses the mechanical stability of samples studied from 0.01 to 10 Hz, indicating the property of hydrogels to keep their mechanical strength. Stability of gels is a relevant feature when considering its application in pharmaceutical industry as these materials must keep their properties throughout their shelf life [26].

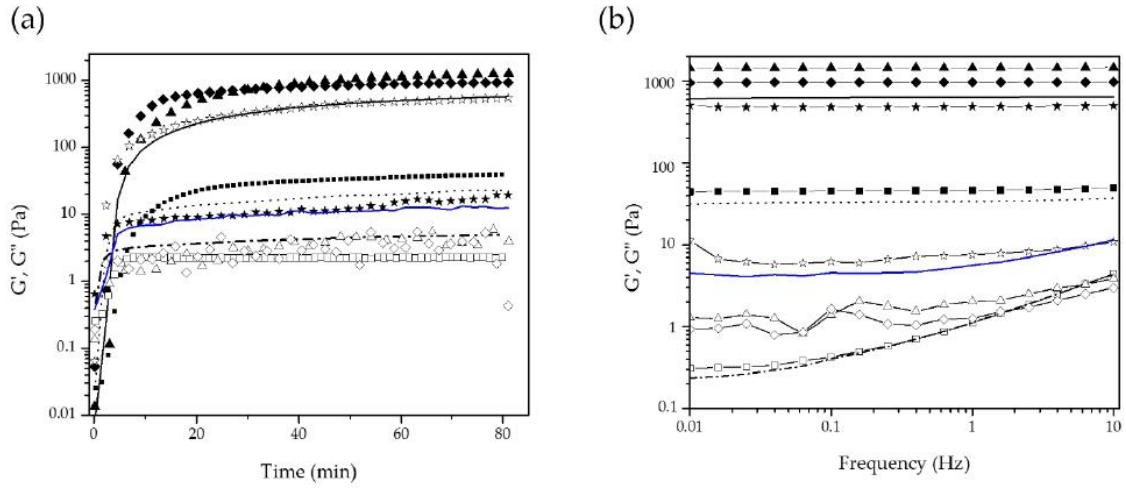

Figure 2. (a) Gelation kinetics; and (b) mechanical spectrum of gels. SBP (G′ (▪), G′′ (□)), SBP+ *S. boulardii* (G′ (···), G′′ (-··-)), AX (G′ (▲), G′′ (Δ)), AX+ *S. boulardii* (G′(♦), G′′ (◊)), mixed SBP/AX (G′(★), G′′ (☆)), and mixed SBP/AX+ *S. boulardii* (G′(—), G′′ (—)).

Table 3 shows G′, G′′, gelation time, and Tan δ values of all measured samples. As observed all rheological parameters were slightly affected by presence of *S. boulardii* cells. G′ value of SBP decreased from 39.13 Pa to 35.23 Pa when adding *S. boulardii* cells, and from 0.65 to 0.51 for G′′ values. The gelation time (tg, sol/gel transition point) decreased as well, from 5.05 to 4.33 min, it seems that addition of cells benefited to decrease tg. Somehow *S. boulardii* cells promoted stabilization during gelation step, as a result less tg was reach. The highest G′ value was presented by AX gel and AX+ *S. boulardii* cells with 1356.44 and 958.29 Pa, respectively. Their tg were 4.87 min for AX and 5.05 min to AX+ S. *boulardii cells*. The system formed by the combination of SBP and AX with and without cells, presented G′ values of 608.60 and 595.40 Pa, respectively. The addition of cells did not alter G′ values. Nevertheless, cell presence in SBP/AX mixed gel contributed to less tg, a positive effect when applying new polysaccharide systems to pharmaceutical applications. For instance, less tg value allows effective entrapment of molecules with biological importance. Above all, the rheological parameters presented for SBP/AX mixed gel were a combination of those values presented by its individual polysaccharides conformation, as reveled G′ value of SBP gel was only 39.13 Pa, but by adding AX to the system, the G′ value increased to 608.60 Pa for the mixed gel. In other words, the combination of the two polysaccharides affect positively the rheological parameters of the mixed system, towards the final application intended.

Table 3. Gelling Capability of SBP, SBP+ *S. boulardii*, AX, AX+ *S. boulardii*, mixed SBP/AX, and mixed SBP/AX+ *S. boulardii*.

Sample	Storage moduli (G′)	Loss moduli (G′′)	Gelation time (G′′/G′=1)	Tan δ (G′′/G′)
	Pa	Pa	min	
SBP	39.1 ± 2.5	0.65 ± 0.10	5.1 ± 2	0.017 ± 0.004
SBP+ *S. boulardii*	35 ± 11	0.51 ± 0.04	4.3 ± 0.7	0.046 ± 0.016
AX	1356 ± 205	2.4 ± 0.7	4.9 ± 1.7	0.0018 ± 0.0003
AX+ *S. boulardii*	958 ± 13	1.5 ± 0.1	5.05 ± 0.01	0.0016 ± 0.0001
SBP/ AX	608 ± 75	6.5 ± 2.5	4.4 ± 1.6	0.0109 ± 0.0054
SBP/ AX + *S. boulardii*	595 ± 4	4.64 ± 0.02	3.9 ± 0.1	0.0078 ± 0.0001

Values are presented as means ± standard deviations of triplicates.

2.4. Morphology and Microstructure of SBP/AX + S. boulardii Macro Gel and SBP/AX + S. boulardii Microbeads

Figure 3 presents the three-dimensional network of (a) SBP/AX gel and (b) SBP/AX + *S. boulardii* microbeads, prepared through covalent cross-linking of FA upon oxidation by enzymatic free radical-generating agents. Additionally, microstructure of (c) lyophilized SBP/AX + *S. boulardii* microbeads and (d) optical micrograph of microbeads are presented. The scanning electron microscopy (SEM) image of SBP/AX macro gel (Figure 3a) at 150 magnification shows the presence of well-defined cavities with irregular surface and thin walls. This fact implies that the oxidative gelation process initiated by laccase promotes an organized cross-linking process. In addition, lyophilized SBP/AX gels presented a sphere-like cavities with an average cavity diameter of 19 ± 12 μm. Sphere-like cavities have been previously observed in sugar beet pectin hydrogels prepared with pectin direct extracted from vegetable as described by Chen et al. [37]. From Figure 3b we can observe the *S. boulardii* cells addition to SBP/AX gel effect on the microstructure of the particle. Evidently, the sphere-like shape of the cavity was lost, and a coralline shape was observed when cellular presence was included. In this regard, we can infer that *S. boulardii* cells tend to occupy the gel cavities and act as a plasticizer to some extent. Similar behavior has been previously described by Paz-Samaniego et al. [25] for AX + *Bifidobacterium* macro gels.

Microstructure of SBP/AX + *S. boulardii* microbeads were observed by SEM (Figure 3c) and optical microscopy (Figure 3d). SBP/AX + *S. boulardii* microbead presented in Figure 3c exhibited a wrinkled, rough surface, with average diameter of 344 ± 126 μm. Some *S. boulardii* cells are observed within the microbead surface. The optical micrographs of SBP/AX + *S. boulardii* microbeads showed a sphere-like shape with no aggregation and homogeneous cell distribution. In this last image a difference in cell content for a third particles influence somewhat the shape on the overall. The authors hypothesize that a range for cell encapsulation must exist, for the hydrogel scaffold is disrupted by excessive cell content. In addition, void particles show instability in their structure as previously observed by Ohlmaier-Delgadillo et al. [31]. In summary, *S. boulardii* cells not only are carried by the particle but are part of the structure. Interestingly, the presence of *S. boulardii* does not affect much the onset as reflected by gelation time, in comparison to the nature of polysaccharides present in the gel.

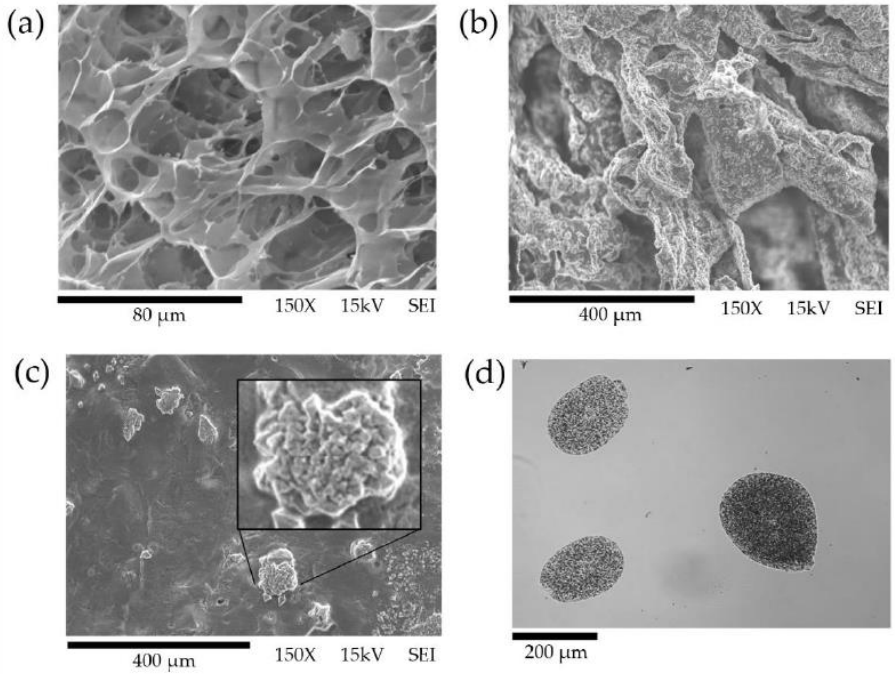

Figure 3. Scanning electron microscopy of lyophilized (a) SBP/AX macro gel at 150X magnification, (b) SBP/AX + *S. boulardii* macro gel at 150X magnification, (c) SBP/AX + *S. boulardii* dried microbeads at 150X magnification. (d) Optical microscopy observation of SBP/AX + *S. boulardii* hydrogel microbeads at 50X magnification.

2.4. Encapsulation Efficiency of S. boulardii cells in SBP/AX microbeads.

The *S. bouladii* encapsulation efficiency percentage in SBP/AX microbeads was 94 ± 28 %. A similar encapsulation efficiency percentages have been reported previously for *Bifidobacterium* in AX microbeads, with 90 ± 37 % of encapsulation efficiency [35]. In these previous work electrospray was used as a technical microbeads preparation, with tetraaxial arrangement, differing from the coaxial arrangement used in the present work. In addition, our encapsulation efficiency is higher than that found for microencapsulation of *Bifidobacterium* in alginate microbeads (43 – 50 %) [38]. These results indicate that SBP/AX mixed microbeads prepared by electrospray are good entrapment matrix to cells due to its high encapsulation efficiency.

2.5. In vitro Saccharomyces boulardii colon release.

S. boulardii cells release was studied after SBP/AX+ *S. boulardii* microbeads submitted to simulated gastro-intestinal digestion and colon fermentation. One control sample composed only by SBP/AX microbeads with no cells was also study. Our study demonstrated the mechanical stability of SBP/AX+ *S. boulardii* microbeads during its transit to simulated gastro-intestinal digestion, mainly through oral, gastric, and intestinal phases. No evidences of *S. boulardii* cells release in those phases were observed. It is well documented that gels formed by covalent bonds are no affected neither by changes in pH, nor temperature changes. As a result, SBP/AX+ *S. boulardii* microbeads were stable during its transit across the simulated gastro-intestinal digestion, because they are covalent bonded[29,39]. The *S. boulardii* release was observed after 24 h of colonic *in vitro* simulation with SBP/AX+ *S. boulardii* microbeads, compared to control sample composed by SBP/AX microbeads without *S. boulardii* cells. After 24 h of colonic *in vitro* simulation the CFU number increased from 4.4 x 10^7 CFU/ml to 1.1 x10^8 CFU/ml. Therefore, the increase in CFU can be

attributed to the degradation of SBP/AX+ *S. boulardii* microbeads by *Bifidobacterium* action that fermented polysaccharides as natural mechanism, and are presented in colon *in vitro* fermentation step, as observed in previous works [35].

3. Conclusions

The combination of two gelling mechanism for SBP and AX enabled the preparation of SBP/AX + *S. boulardii* microbeads with neither coalescence nor aggregation using the electrospraying technique with coaxial arrangement. ABTS and DPPH activity essays demonstrated that SBP/AX + *S. boulardii* macro gels and microbeads show antioxidant activity after gelation process and cellular presence did not negatively affect this feature nor the onset of the gel. SEM and optical images of SBP/AX + *S. boulardii* macro gels and microbeads demonstrated the homogeneous cell distribution. The presence of *S. boulardii* cells affects the sphere like cavities of SBP/AX macrogels. Finally, the electrospraying technique allowed the encapsulation of *S. boulardii* cells keeping their viability in the process as observed in a previous work [9]. The *in vitro S. boulardii* release during colonic simulation suggest a high potential for the design of new products with antioxidant activity to further industrial applications of probiotics and ferulated pectin/AX hydrogels.

4. Materials and Methods

4.1. Materials

Sugar beet solid waste (SBW) was kindly provided by a bioethanol production facility located in Sonora, Mexico as previously described [31]. SBP was extracted, purified and characterized as previously described by Ohlmaier-Delgadillo F. et al. [31]. In brief, SBP presented a weight-average molar mass of 459 kDa, a galacturonic acid content of 52.2%, a low esterification degree (30%) and ferulic acid content of 2.1 μg/ mg polysaccharide. AX from maize dried distillers' grains with solubles (DDGS) was extracted and characterized as previously described [27]. The AX presented 64% dry basis (d.b.) of pure AX (sum arabinose + xylose), an A/X ratio of 1.1, weight-average molar mass of 209 kDa and ferulic acid content of 6.5 mg/g polysaccharide. Laccase (benzenediol:oxygen oxidoreductase, E.C.1.10.3.2) from *Trametes versicolor*, and all the chemical reagents used were purchased from Sigma Aldrich Co. (St. Louis, Missouri). Commercial *S. boulardii* (CNCM I-745) was used as a probiotic cell.

4.2. Biomass Production

S. boulardii cells were cultured as previously described [9]. In brief, 100 mL of commercial Malt Extract Broth (Difco, TM, pH, 5.4 at 25 °C) was used to culture pro. Cells were incubated at 31 °C and 150 rpm during 20 h (Lab-line 3540 brand). Cells counts were conducted manually every 2 h using a Neubauer Chamber in an optical microscopy (Zeiss Axio Vert. A1, Carl Zeiss Microscopy, Jena, Germany) equipped with a digital camera (Axio Cam ERC 5s, Jena, Germany). Fresh cells were recuperated by centrifugation at 6,000 rpm for 5 min and 4 °C [40].

4.3. Gel Preparation

Gels with and without *S. boulardii* cells were prepared as previously described by Ohlmaier-Delgadillo et al [9,31]. Briefly, SBP gels with and without cells were prepared using SBP dispersion at 4% (w·v-1) in 0.1 M sodium acetate buffer, pH= 5.5, at 25 °C. AX gels with and without cells were prepared using AX dispersion at 1.3% (w·v-1) in 0.1 M sodium acetate buffer, pH= 5.5, at 25 °C. SBP/AX dispersions with or without *S. boulardii* cells were prepared at an overall biopolymer concentration of 53 mg/mL (40 mg/mL SBP and 13 mg/mL AX in a 3.1 w/w biopolymer ratio) dispersed in 0.1 M sodium acetate buffer

pH = 5.5. This SBP/AX ratio ensured a 1:1 (w/w) FA contribution from each polysaccharide. Laccase was used as a cross-linking agent (24 units/mg FA).

4.4 Microbeads Preparation: SBP/AX + S. boulardii Microbeads

SBP/AX microbeads with or without *S. boulardii* cells were prepared as previously described [9]. Briefly, immobilization of *S. boulardii* inside SBP/AX microbeads was carried out by coaxial electrospray using SpraybaseTM system (ProfectorTM, Dublin, Ireland) and two programmable syringe pumps (worldPrecision Instruments, AL-1000, Sarasota FL, USA), independently feeding a coaxial needle as previously reported [8]. Technical electrospray conditions used were 9 kV, 0.7 mL/h for the inner needle, and 0.3 mL/h for the outer needle. SBP/AX microspheres were prepared with and without *S. boulardii*. The outer needle conducted the mixture SBP/AX at an overall biopolymer concentration of 53 mg/mL (40 mg/mL SBP and 13 mg/mL AX in a 3.1 *w/w* biopolymer ratio) dispersed in 0.1 M sodium acetate buffer pH = 5.5. SBP/AX mixture in the outer needle was used alone or with *S. boulardii* cells (amount of cell entrapped in microspheres, 2.08×10^8 cells/ mL). The inner needle conducted laccase in 0.1 M sodium acetate buffer pH = 5.5 as the cross-linking agent. Laccase dispersion contain 24 units/mg FA to ensure SBP/AX gel-forming. The spray was received in $CaCl_2$ at 2% (*w/v*) in ethanol:water 1:2 (*v/v*) fixing a distance of 7 cm from coaxial needle. Microbeads were stored at 4 °C. Scheme depicting coaxial electrospray process used to produce SBP/AX and SBP/AX+ *S. boulardii* microbeads is presented in Figure 3.

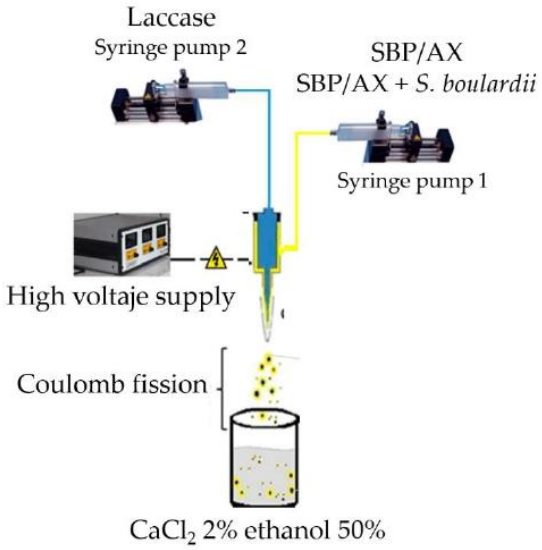

Figure 3. Scheme of coaxial electrospray process used to produce SBP/AX and SBP/AX+ *S. boulardii* microbeads.

4.5. Infrared Spectroscopic Analysis

Fourier Transform Infrared Spectroscopy (FTIR) analysis of SPB, AX, SBP/AX dispersion before gelation and microbeads of SBP/AX were performed on a NICOLET IS-50 spectrometer (ThermoScientific ™, USA) with an attenuated refractive detector. Spectrum scan was achieved from 4000 cm-1 to 400 cm-1. Samples were dried before processed and transmittance vs. wavelength is reported on graphs [41].

4.6. Optical Microscopy of Microbeads

Microspheres were observed using an optical microscopy (Zeiss Axio Vert. A1, Carl Zeiss Microscopy, Jena, Germany) equipped with a digital camera (Axio Cam ERC 5s, Jena, Germany), morphology and size distribution were evaluated. The average diameter and polydispersity index were determined by images obtained from optical microscopy using ImageJ software, at least 120 microbeads of SBP/AX + *S. boulardii* were counted. The average particle size was expressed as volume mean diameter (μm) ± SD values related to the mean [42].

4.7. Scanning Electron Microscopy

Surface characterization of AX, SBP and SBP/AX with and without *S. boulardii* cells gels and SBP/AX + *S. boulardii* microbeads were studied using a scanning electron microscope (SEM) JEOL54 10LV equipped with an INCA dispersive X-ray detector system (Oxford Instruments) and operated at a voltage of 20 kV. Samples were flash freezing with liquid nitrogen and then lyophilized, later coated with gold before being observed under a high vacuum using the secondary electron detector according to Morales-Burgos et al. [43]. The average porous diameter was determined from SEM images using ImageJ software.

4.8. FA content of SBP, AX, PF+AX dispersion, SBP gel, AX gel, SBP/AX gel, SBP/AX+ SB gel, and SBP/AX+ SB microbeads

FA, dimers of FA (di-FA), and trimers of FA (tri-FA) were analyzed by high performance liquid chromatography. 100 mg of each sample were first saponified using 2 N NaOH. Later, acidified with 4N HCl. Finally, extracted with diethyl ether. Samples were injected in an HPLC (Waters, e2695), equipped with an Alltima C18 column (Alltech, Deerfield, IL; 250 x 2.6 mm), previously filtered through 0.45 μm membrane [44].

4.9. Rheological Measurments

The kinetics of gelation of SBP, SBP+ *S. boulardii* cells, AX, AX+ *S. boulardii* cells, mixed SBP/AX, and mixed SBP/AX+ *S. boulardii* cells were followed using a low deformation strain-controlled rheometer (Discovery HR-2 rheometer, TA instruments) in oscillatory mode, as reported previously [45]. Cold (4 °C) solutions of each sample were mixed with laccase (24 units/mg FA) and immediately placed in the cone and plate geometry (5.0 cm in diameter, 0.04 rad in cone angle) maintained at 4 °C. Gelation kinetics was monitored at 25 °C for 80 min by following the storage (G′) and loss (G′′) modulus. All measurements were carried out at a frequency of 0.25 Hz and 5% strain (linearity range of viscoelastic behavior). The mechanical behavior of each sample was obtained by frequency (0.01 to 10 Hz, 5% strain) and strain (1 to 10%, 0.25 Hz) sweep at the end of the network formation at 25 °C.

4.10. Antioxidant Activity

The *in vitro* antioxidant capacity SBP, AX, SBP/AX dispersion, SBP gel, AX gel, SBP/AX gel, SBP/AX + *S. boulardii* gel, SBP/AX microbeads, and SBP/AX + *S. boulardii* microbeads were measure using $ABTS^+$ and DPPH methods.

The $ABTS^+$ scavenging capacity was performed as previously described [27,46,47]. The absorbance of the sample´s supernatant and $ABTS^+$ reagent mixture was measure at 734 nm. The antioxidant activity was measure by absorbance readings after 7, 15 and 21 min of reaction. All the results were expressed as μmol of Trolox (6-hydroxyl-2,5,7,8-tetramethylchoman-2-carboxylic acid) equivalent capacity per gram of sample (μmol TEAC/g). A dose- response curve of different concentration of Trolox was performed in order to determine the antioxidant capacity of the samples.

The antioxidant activity measured by DPPH method was followed as previously described [48]. In brief, stock dispersions were prepared by dissolving SBP, AX, SBP/AX

dispersion, SBP gel, AX gel, SBP/AX gel, SBP/AX + *S. boulardii* gel, SBP/AX microbeads, and SBP/AX + *S. boulardii* microbeads; in water (5 mg/mL). All samples were homogenized using an ultrasonic homogenizer (OMNI Sonic Ruptor 400, Kennesaw, GA, USA) at 50 pulse and 30% power for 5 min at 25°C. Diverse concentrations (0- 2000 μg/mL) of stock dispersions were prepared using ultrapure water. Standard solutions of Trolox (0- 15.0 μg/mL) in ultrapure water were also prepared. A 45 μM DPPH work solution was prepared by mixing 1.8 mg of DPPH and 30 mL of methanol until dissolved. Subsequently, 20 mL of water was added and finally stored in the dark until used. Aliquots of 400 μL of all sample solutions were mixed with 350 μL of absolute methanol. Then 750 μL of DPPH solution was added to the mixture, vortexed and left in the dark. The measurements were taken at 40 and 60 min at absorbance of 515 nm using a microplate spectrophotometer (Thermo Scientific MultiSkan Go, Madrid, Spain). Antioxidant activity was determined by means of dose-response curve of Trolox and results were expressed as μmol TEAC/g sample.

4.11. Encapsulation Efficiency

The *S. boulardii* entrapment efficiency of the particles was calculated using the following equation:

$$S.boulardii \;\; entrapment \;\; efficiency \;\; \% = \left(\frac{log_{10}N}{log_{10}N_0}\right) x100$$

Where N is the number of colonies formed from the particles, and N0 is the number of free cells added to SBP/AX microbeads and number of colonies formed. The entrapment efficiency experiments were performed as previously reported [49,50].

4.12. In vitro Gastro-Intestinal Digestion and Colon Fermentation

The in vitro gastro-intestinal digestion test was performed as previously described by Minekus et al. [51]. First, different standard solutions (0.5 M KCl, 0.5 M KH_2PO_4, 2 M $NaHCO_3$, 2 M NaCl, 0.15 M $MgCl_2$ and 0.5 M $(NH_4)2CO_3$) were prepared from which the salivary fluid conditions (SSF) were simulated, the gastric fluid (SGF) and intestinal fluid (SIF). For SSF, 6.04 mL of KCl, 1.48 mL of KH_2PO_4, 2.72 mL of $NaHCO_3$, 0.2 mL of $MgCl_2$, and 0.024 mL of $(NH_4)2CO_3$ were taken, added to a volumetric flask and brought to 200 mL. For SGF, 2.76 mL of KCl, 0.36 mL of KH_2PO_4, 5 mL of $NaHCO_3$, 4.72 mL of NaCl, 0.16 mL of $MgCl_2$ and 0.2 mL of $(NH_4)2CO_3$ were taken, volumetric to 200 mL, and for SIF 2.72 mL of KCl, 0.32 mL of KH_2PO_4, and 0.32 mL of KH_2PO_4 were used. 17 mL $NaHCO_3$, 3.84 mL NaCl, and 0.44 mL $MgCl_2$, calibrated to 200 mL. Second, 1 g of SBP/AX microbeads with and without *S. boulardii* cells were placed in a 50 mL Falcon tube, 3.5 mL SSF were added, 0.5 mL of α-amylase solution (150 U/mL) previously diluted in SSF solution, 25 μL 0.3 M $CaCl_2$ and 975 μL of miliQ water, shake gently, adjust the pH to 7 and incubate at 37 °C for 2 min at 100 rpm (IKA KS 3000 IC CONTROL, Staufen, Germany), Germany). Once the time was riched, an aliquot (1 mL) of the sample was taken and stored at -20 °C. In the same tube, the sample was conditioned to gastric simulation (SGF) adjusting the 1:1 ratio. Starting from the final volume, 3.7 mL of SGF, 0.8 mL of porcine pepsin (25000 U/mL of SGF solution), 2.5 μL of 0.3 M $CaCl_2$, 0.1 mL of 1 M HCl and 0.347 mL of water were added, the pH was adjusted to 3 and kept under the same shaking conditions for 2 h, taking an aliquot (1 mL) at the end of the incubation. In the same tube the next solutions were added: 4.95 mL of SIF, 2.25 mL of pancreatin (800 U/mL) and 1.125 mL of bile (75 mg/mL) diluted in SIF solution, 18 μL of 0.3 M CaCl2, 67.5 μL of NaOH and 589.5 μL of milliQ water. The pH was adjusted to 7 and incubated for 6 h under the same conditions, taking an aliquot (1 mL) after 1, 4 and 6 h. All samples were stored frozen at -20 °C until cell release quantification. Finally, the colonic simulation was performed following the protocol described by Pérez-Burillo et al. [52], with some modifications. First, human fecal

material provided by two healthy volunteers was collected, without consumption of antibiotics in the last three months, then a 0.1 M PBS (Phosphate Saline Buffer) buffer at pH 6.8 was prepared, of which 100 mL were taken and mixed with 10 g of feces to immediately filter through organza cloth. 10 mL of the mixture were taken and added to the tubes used in the previous simulation (SIF). It should be mentioned that 1 g of the sediment of the SIF solution was left. It was then left to incubate at 37 °C/30 rpm for 24 h and an aliquot (1 mL) was taken at the end of the incubation in order to measure the cell release.

4.13. Statistical Analysis

Results are expressed as means ± standard deviation (S.D) from triplicates.

Author Contributions: Federico Ohlmaier-Delgadillo performed the experiments and writing the paper. Agustín Rascón-Chu conceived and designed the experiments, contributed to the discussion of the results, and edited the paper. Elizabeth Carvajal-Millan help in the design of the experiments and contributed to the discussion of the results. Yolanda L. López-Franco and Maria A. Islas-Osuna analyzed the data and contributed to the discussion of the results. Micard, V. design of experiments and FA, di FA, tri FA determinations and analysis, Antoine-Assor, C., FA, di FA, tri FA determinations and analysis.

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6. CONCLUSIONES GENERALES

Las pectinas feruladas extraídas de los residuos agroindustriales de remolacha azucarera presentaron dos mecanismos de gelificación. Lo anterior abre una gran oportunidad para el uso de este polisacárido como matriz acarreadora de medicamentos, células, y biomoléculas; así como la factibilidad de diseñar y preparar partículas con diversidad en tamaños por la técnica de electroaspersión. Por otra parte, la combinación de pectinas feruladas y arabinoxilanos ferulados resultó en la obtención de un material totalmente nuevo, con características únicas nunca reportadas. Estas características se utilizaron exitosamente para el atrapamiento de *Saccharomyces boulardii* y su liberación dirigida específicamente a colon. La aplicación del nuevo sistema mixto conformado por pectinas y arabinoxilanos tiene un gran potencial debido a su estabilidad y sus propiedades mecánicas.

7. RECOMENDACIONES

Estudiar el potencial del nuevo sistema mixto para preparar nanopartículas por la técnica de electroaspersión. Adicionalmente, se recomienda realizar un ensayo de liberación de *Saccharomyces boulardii* en un sistema *in vivo*, por ejemplo, un ensayo murino.

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