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

# EVALUACIÓN DEL EXTRACTO ACUOSO DE LA CORTEZA DEL ÁRBOL DE MANGO EN LA MODULACIÓN DE LA MICROBIOTA INTESTINAL

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## APROBACIÓN

Los miembros del comité designado para la revisión de la tesis de Gabriela Vazquez Olivo, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctora en Ciencias.

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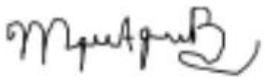

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# 1. SINOPSIS

## 1.1. Justificación

Las actividades agrícolas generan una gran cantidad de residuos. Estos residuos agrícolas son una fuente importante de compuestos bioactivos pero son poco utilizados. Entre estos compuestos bioactivos se encuentran los compuestos fenólicos. Los compuestos fenólicos tienen importancia debido a los diferentes efectos benéficos en la salud atribuidos. Por ejemplo, su actividad antioxidante, antimicrobiana y recientemente se ha descrito su actividad prebiótica. Por otra parte, México, es el sexto país productor de mango y cada año se generan toneladas de residuos agrícolas derivados de su cultivo. Por ejemplo, residuos de la cáscara del fruto, del hueso y de la poda del árbol. Los residuos de la poda del árbol; estos han sido utilizados para la obtención de corteza y su infusión es usada en la medicina tradicional para el tratamiento de diversas afecciones, por ejemplo problemas gastrointestinales. También se conoce que este material vegetal es rico en mangiferina, galotaninos y ácidos fenólicos. Estos compuestos podrían ser los responsables del efecto en la salud observado al consumir la infusión de corteza del árbol de mango, sin embargo, aún faltan estudios que demuestren el efecto de los compuestos fenólicos de la corteza del mango en la promoción de la salud gastrointestinal. Este efecto podría estar relacionado a su impacto en la microbiota intestinal. Por tal motivo, el interés de este trabajo fue conocer la cantidad de compuestos fenólicos de los residuos de la poda del árbol del mango producido en el estado de Sinaloa, el cual es uno de los estados con mayor producción nacional de mango, y evaluar el efecto biológico de esos compuestos fenólicos. Finalmente, esos resultados podrán ser utilizados en investigaciones posteriores que permitan dar un valor agregado a este residuo agrícola.

## 1.2 Antecedentes

### 1.2.1 Compuestos Fenólicos

Los compuestos fenólicos son un gran grupo de metabolitos secundarios presentes principalmente en las plantas actuando como mecanismo de protección de las plantas contra factores de estrés como la radiación UV y el ataque de patógenos. Los compuestos fenólicos forman una parte importante de la dieta humana, encontrándose en frutas, tés, especias y hierbas. En promedio, una persona consume un gramo por día de compuestos fenólicos. Dichos compuestos contribuyen a las propiedades organolépticas de los alimentos de origen vegetal, como su aroma, sabor y color (Cory *et al*., 2018; Kühnau, 1976). La estructura química de los compuestos fenólicos es diversa, y su biosíntesis proviene de dos rutas: la del ácido shiquímico y la ruta de los poliacetatos. La primera sintetiza aminoácidos aromáticos y ácidos cinámicos y sus derivados, mientras que la segunda proporciona las quinonas y xantonas (Bravo, 1998). Los compuestos fenólicos se caracterizan por la presencia de al menos un anillo aromático unido a uno o más grupos hidroxilo (grupo fenol). En la naturaleza, los compuestos fenólicos se pueden encontrar como derivados glicosilados o acilados. Los principales grupos de compuestos fenólicos son los ácidos fenólicos, flavonoides, estilbenos, lignanos y otros, y de estos derivan más clases de compuestos (Grosso *et al*., 2014; Manach *et al.*, 2004). Los ácidos fenólicos son los compuestos más simples que se encuentran en los alimentos de origen vegetal; ejemplos de estos son el ácido ferúlico, ácido gálico y el ácido benzoico. Se caracterizan por tener al menos un ácido carboxílico y se pueden dividir en ácidos hidroxibenzoicos e hidroxicinámicos. Los flavonoides son el grupo más común de compuestos fenólicos y su estructura básica son dos anillos de benceno unidos a una cadena de tres carbonos o un anillo heterocíclico. En la naturaleza se encuentran como β-glucósidos o como agliconas. Estos compuestos a su vez se categorizan en antocianinas, flavanones (catequinas), flavonas, flavanonas, y flavonoles. Los estilbenos y lignanos son raramente encontrados en la dieta humana. Los estilbenos consisten de un núcleo 1,2-difeniletileno, y un ejemplo común es el resveratrol. Por su parte, la estructura básica de los lignanos es un dibencil butano y están presentes principalmente en la linaza. (Manach *et al*., 2004; Rao *et al*., 2010). Las xantonas pertenecen a otros grupos de

compuestos fenólicos, y al igual que los flavonoides, tienen un esqueleto de carbono que consiste en dos anillos aromáticos (llamados anillo A y anillo B) unidos por un anillo heterocíclico. En los anillos A y B se localizan sustituyentes como: grupos hidroxilo, metoxilo, glicosilo, y prenilo. Las xantonas se clasifican en oxigenadas simples, glucósidos, preniladas y xantonas derivadas como xantonolignoides, y el grupo más común es el de las xantonas preniladas (El-Seedi *et al*., 2010). Por otro lado, es importante mencionar que el contenido y las estructuras químicas de los compuestos fenólicos de las plantas y por ende en los alimentos derivados de las mismas, es influenciado por diversos factores: por ejemplo, factores ambientales, procesamiento, grado de madurez, entre otros. Asimismo, la composición de compuestos fenólicos es diferente dependiendo de la parte de la planta que se analice (Palafox-Carlos *et al*., 2012a).

### 1.2.2 Corteza del Árbol de Mango como Fuente de Compuestos Fenólicos

La corteza del árbol de mango es un material vegetal que se puede obtener de la poda del árbol de mango. Su infusión se ha utilizado ampliamente en la medicina tradicional para el tratamiento de infecciones, anemia, diarrea y diabetes (Núñez-Sellés *et al*., 2002). Algunos estudios han elucidado que las propiedades biológicas de la infusión de la corteza del árbol de mango se atribuyen a los compuestos fenólicos que contiene. Actualmente, la corteza del árbol de mango es considerada como residuo, y la generación de los mismos es abundante, y continuará aumentando de acuerdo al incremento de la producción de mango, que, particularmente en Sinaloa es de 29 mil 506 ha (SIAP, 2018) y de esta producción, se ha estimado que se generan aproximadamente 3.5 toneladas de residuos de la poda del árbol por hectárea cultivada. Asimismo, estos residuos generan un impacto negativo al medio ambiente ya que son quemados o dejados en el campo (Velázquez-Martí *et al*., 2012).

En este contexto, una alternativa propuesta para el uso de la corteza del árbol de mango es aprovecharla como fuente de obtención de compuestos fenólicos. Algunos estudios han reportado que la corteza del árbol de mango contiene en su mayoría mangiferina, flavonoides, ácidos fenólicos y galotaninos (Mouho *et al*., 2018; Vazquez-Olivo *et al*., 2019). Los compuestos fenólicos de la corteza del árbol de mango han demostrado tener beneficios en la salud tanto en

ensayos *in vitro* como *in vivo*. Por ejemplo, el ácido gálico tiene actividad antioxidante, lo cual se relaciona con su estructura. Este compuesto tiene 3 grupos hidroxilo en su estructura, lo que le da estabilidad. Además, el grupo -OH en la posición "para" de esta molécula es muy eficiente en el secuestro de radicales. Lo anterior hace que el ácido gálico ayude a proteger a las membranas celulares mediante la prevención de la peroxidación de lípidos (Badhani *et al*., 2015).

Por su parte, mangiferina es un compuesto que posee cuatro grupos hidroxilo en su estructura, lo que le confiere una alta actividad antioxidante, así como capacidad como quelante de hierro (Imran *et al*., 2017; Núñez-Selles *et al*., 2016). Asimismo, esta molécula tiene propiedades antivirales, anticancerígenas, antidiabéticas, inmunomodulatorias, hepatoprotectoras y efectos analgésicos. En ratones, la administración intraperitoneal de mangiferina (100 mg/Kg) restaura la actividad de enzimas antioxidantes (Prabhu *et al*., 2006), y tiene un efecto protector en la hiperlipidemia inducida por una dieta alta en grasa, por lo que es una molécula que puede ayudar en el tratamiento en la prevención de padecimientos como diabetes y obesidad (Imran *et al*., 2017; Zhou *et al*., 2015).

Asimismo, se ha reportado la sinergia entre compuestos fenólicos del mango. Por ejemplo, se ha demostrado que la actividad antioxidante de pulpa de mango se debió al efecto aditivo de sus principales ácidos fenólicos: ácido gálico, clorogénico, vanílico y protocatecuico (Palafox-Carlos *et al*., 2012b).

Otra bioactividad importante de los compuestos fenólicos del mango es su actividad antimicrobiana. Por su parte, la semilla de mango tiene actividad antimicrobiana contra *Staphylococcus aureus* MTCC 737 con un halo de inhibición de 8.5 mm, *Escherichia coli* MTC 46 con un halo de inhibición de 8.2 mm y *Bacillus subtilis* subsp. Subtilis DSM 10 con un halo de inhibición de 6.6 mm (Raju *et al.*, 2019). Igualmente, Vega-Vega *et al.* 2013, reportaron que la cáscara del fruto y la semilla de mango tienen efecto antimicrobiano contra *E. coli* 0157:H7, *Salmonella choleraesuis*, *Listeria monocytogenes* y *Staphylococcus aureus*. En relación a la pulpa de mango, en dosis de 400 mg/mL se ha demostrado actividad antimicrobiana contra *Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* y *Salmonella* sp. Además, para *E. coli* y *Salmonella* sp, la concentración mínima inhibitoria (MIC) fué de > 400 mg/mL, mientras que para *P. aeruginosa* y *S. aureus* fue de 37.5, y la concentración mínima bactericida (MIB) fue de 50 mg/mL. Asimismo, para *L. monocytogenes*, la MIC fue de 25 mg/mL y la MBC de 37.5 mg/mL (Paz *et al*., 2015).

Por otro lado, los derivados glicosilados del ácido gálico han demostrado una relación actividad-

estructura entre la actividad antimicrobiana y el número de anillos pirogalol en su estructura. El ácido gálico tiene un anillo, por lo que tiene una actividad antimicrobiana moderada, además, éste ácido fenólico ha demostrado prevenir la formación de biopelículas por *E. coli* y *P. aeruginosa*, *S. aureus*, y *L. monocitogenes*, demostrando una reducción en la actividad de biopelículas mayor al 70%. Asimismo, el ácido gálico inhibe el crecimiento de bacterias orales de *Streptococcus mutants* e influencia las propiedades de adhesión de *S. aureus* (Marín *et al*., 2015).

Es importante señalar que el estudio de la bioactividad de los compuestos fenólicos *in vitro* nos ofrece un panorama general de su efecto potencial en la salud. Pero también es necesario evaluar el metabolismo de los compuestos fenólicos al pasar por el tracto gastrointestinal, ya que finalmente su efecto benéfico *in vivo* depende de qué tanto se absorben a lo largo del tracto gastrointestinal y qué compuestos logran llegar a un órgano diana. En este sentido, existe información limitada acerca del metabolismo de los compuestos fenólicos de la infusión de corteza del árbol de mango.

### 1.2.3 Metabolismo de Compuestos Fenólicos

1.2.3.1 Bioaccesibilidad y Biodisponibilidad. Desde el punto de vista de la nutrición, la biodisponibilidad de un componente bioactivo, se refiere a la fracción del mismo que después de ser ingerido llega a la circulación sanguínea y puede llegar a un órgano diana. Este término incluye, la digestión gastrointestinal, absorción, metabolismo, distribución en los tejidos y la bioactividad (Fernández-García *et al*., 2009). Asimismo, la biodisponibilidad incluye el término "bioaccesibilidad". La bioaccesibilidad se refiere a la cantidad de un compuesto bioactivo que es liberado de la matriz alimentaria y se vuelve disponible para su absorción. Éste término abarca las transformaciones de los alimentos en el tracto gastrointestinal, asimilación en las células del epitelio intestinal, así como el metabolismo hepático. Además, la bioaccesibilidad se ve influenciada por diversos factores y se absorben de manera diferente dependiendo de estos, por ejemplo, las interacciones entre los diferentes componentes de la matriz alimentaria, su estructura química, factores propios del individuo, su interacción con enzimas digestivas, proteínas, y el pH (Eran Nagar *et al*., 2020).

La digestión de algunos compuestos fenólicos como las antocianinas y ácidos fenólicos puede comenzar en el estómago, mientras que otros son hidrolizados por enzimas del borde de las células del epitelio del intestino delgado. En las células del epitelio, las agliconas pueden volver a metabolizarse mediante diferentes modificaciones, como metabolitos glucuronidos, metilados y/o sulfatados, y pasar a la circulación sistémica, o ser enviados de nuevo al lumen del intestino delgado. Los metabolitos que llegan a la circulación, son sometidos a metabolismo de fase II en el hígado y pueden también regresar al lumen del intestino delgado después del transporte entero hepático en la excreción biliar. Los compuestos fenólicos que no pueden hidrolizarse por enzimas humanas, son metabolizados en colon por enzimas bacterianas.

Para evaluar la bioaccesibilidad de un componente bioactivo se utilizan métodos de digestión *in vitro*, para simular los procesos digestivos en el estómago y el intestino delgado, y usualmente también se utilizan ensayos de absorción celular con células Caco-2 (Kosińska y Andlauer, 2012). La monocapa de células Caco-2 se deriva de células de adenocarcinoma de colon humano. Este cultivo, al someterse en cultivo a un proceso de diferenciación que dura 21 días, se forma una monocapa de células que expresan varias características funcionales y bioquímicas semejantes a las del epitelio del intestino delgado. La morfología de la monocapa es polarizada cilíndrica con microvellosidades en la parte apical y con uniones estrechas entre las células adyacentes, expresando actividades muchas de las enzimas hidrolasas intestinales (tales como la sacarasa-isomaltasa, lactasa, aminopeptidasa N y dipeptidil peptidasa IV) en la membrana apical. Además, expresa los sistemas de transporte activo tal y como sucede en la pared del intestino delgado humano, y la glicoproteína P e induce el eflujo basolateral a apical de xenobióticos específicos. La monocapa de células Caco-2 ha sido extensamente estudiada por años como una herramienta para evaluar la permeabilidad intestinal, evaluar el potencial de absorción oral y estudiar mecanismos de absorción de xenobióticos.

A pesar de que se han evaluado otras líneas celulares, las células Caco-2, son la línea celular mejor caracterizada, y más comúnmente utilizada para estudios de absorción de compuestos fenólicos (Hubatsch *et al*., 2007). Por otro lado, como se mencionó anteriormente, los compuestos fenólicos que llegan al colon, son sometidos a biotransformaciones por la microbiota intestinal.

### 1.2.4 Microbiota Intestinal Humana

El ser humano alberga una gran densidad de microorganismos que incluyen bacterias, hongos, arqueas, virus y protozoos. Al conjunto de estos microrganismos que colonizan un sitio anatómico específico en el cuerpo humano se le conoce como microbiota, y los genes que la codifican es el microbioma (Jandhyala *et al*., 2015). El microbioma es 100 veces mayor que los genes en el genoma humano, y la microbiota es 10 veces mayor que el número de células humanas (Qin *et al*., 2010). El tracto gastrointestinal está dominado por bacterias, dentro de este la cantidad de bacterias también es variable dependiendo del sitio, y el intestino grueso es especialmente rico en diversidad y densidad bacteriana, siendo el colon el sitio donde reside hasta el 70% del total de bacterias (Dieterich *et al*., 2017). Estudios metagenómicos han demostrado que la mayoría de estos microorganismos son bacterias, teniendo hasta $10^{14}$ células bacterianas, y de 500 a 1000 especies diferentes de bacterias (Sender *et al*., 2016). Las comunidades microbianas en un individuo adulto se establecen desde los tres años y cada sitio anatómico tiene un conjunto de taxones característicos. Una diversidad alta de microorganismos es asociada a una microbiota saludable. En un adulto saludable, los filos dominantes son los Firmicutes (60-80%) y Bacteroidetes (25%), y en menor proporción a los filos: Actinobacteria, Verrucomicrobia, Proteobacteria y Fusobacteria (Rinninella *et al*., 2019). Las anaerobias facultativas son las bacterias más abundantes, por ejemplo, bacterias pertenecientes a los grupos *Clostridium, Bifidobacterium* y *Bacteroides* (Delgado *et al*., 2006). Otras bacterias encontradas comúnmente en heces a nivel de familia, son: *Bacteroidaceae, Clostridiaceae, Prevotellaceae, Eubacteriaceae, Ruminococcaceae, Bifidobacteriaceae, Lactobacillaceae, Enterobacteriaceae, Saccharomycetaceae,* y *Methanobacteriaceae* (Lloyd-Price *et al*., 2016), y a nivel de género, las bacterias más abundantes son: *Bacteroides, Clostridium, Prevotella, Porphyromonas, Eubacterium, Ruminococcus, Streptococcus, Tenerobacterium, Enterococcus, Lactobaacillus, Peptostreptococcus* y *Fusobacteria* (Jandhyala *et al*., 2015).

Asimismo, en los plegamientos del intestino grueso, se diferencían dos tipos de microbiota dependiendo de si se trata de la parte luminar o mucosa, encontrándose los géneros predominantes en la parte luminar o luz intestinal: *Bacteroides, Bifidobacterium, Streptococcus, Enterococcus, Clostridium, Lactobacillus* y *Ruminococcus*, mientras que en la mucosa, se encuentran *Clostridium, Lactobacillus, Enterococcus* y *Akkermancia* (Jandhyala *et al*., 2015).

### 1.2.5 Adquisición de la Microbiota Intestinal

El tracto gastrointestinal del feto es estéril hasta después de su nacimiento. La colonización inicial de un bebé comienza con bacterias adquiridas por vía materna. Dependiendo del tipo de nacimiento, los bebés nacidos vaginalmente, adquieren microbiota similar a la microbiota vaginal de la madre, dominada por *Lactobacillus Prevotella* y *Sneathia* spp., mientras que los bebés nacidos por cesárea poseen la microbiota característica de la piel, por ejemplo, *Staphylococcus*, *Corynebacterium* y *Proponibacterium* spp. La microbiota intestinal durante la primera semana de vida de un ser humano, interactúa con el sistema inmune. El sistema inmune permite el establecimiento de bacterias comensales. Una explicación a esto es porque reconoce patrones moleculares asociados a patógenos, expresados por las bacterias, virus y hongos que componen la microbiota intestinal. Este reconocimiento lo realizan los receptores tipo Toll, los cuales a su vez se encargan de activar una serie de señales bioquímicas en el interior de las células dendríticas y de los macrófagos, lo cual resulta en una tolerancia inmunitaria frente a microorganismos comensales (Round *et al*. 2012). La microbiota intestinal de neonatos se caracteriza por una baja diversidad bacteriana y un dominio de bacterias pertenecientes principalmente a los filos Proteobacteria y Actinobacteria (Pannaraj *et al*. 2017). Con la introducción de alimentos sólidos, la diversidad bacteriana aumenta, hasta llegar a una comunidad parecida a la que tiene un adulto, donde dominan los filos Firmicutes y Bacteroidetes. Asimismo, durante este tiempo el sistema inmune comienza a diferenciar a las bacterias patógenas de las comensales. Algunos factores afectan la primera colonización de la microbiota intestinal del infante, como la dieta, por ejemplo, si es alimentado por la madre o con fórmula. Además, el uso de antibióticos causa el retardo o alteración de la colonización bacteriana con pérdida de algunas comunidades como *Enterocacteriaceae, Lachnospiraceae y Erysipelotricheae* (Dieterich *et al*., 2018). La colonización inicial es importante para el desarrollo de una interacción balanceada entre la microbiota y el hospedador, a los 3 años de edad, la microbiota se parece completamente a la de un adulto en términos de composición y diversidad y se mantiene estable (Bäckhed, 2011; Clemente *et al*., 2012; Rodríguez *et al*., 2015). Aunque la composición de la microbiota es relativamente estable en el individuo adulto sano, existen factores que también son constantes y tienen un impacto en la modificación de la microbiota intestinal, como el ambiente, enfermedades y la dieta.

La dieta ha mostrado tener un impacto significativo en la modificación de la microbiota. Por ejemplo, en humanos se ha visto que al cambiar a una dieta alta en grasas-baja en fibra a una baja en grasas-rica en fibra, causa cambios significativos en la microbiota intestinal en 24 h (Wu *et al*., 2011). La microbiota se mantiene relativamente estable hasta la edad avanzada. Después de los 65 años de edad, la diversidad de bacterias se reduce, esto es debido principalmente a un deterioro de la salud conforme la edad aumenta así como el uso de medicamentos. En adultos mayores (>65 años) se reporta una mayor proporción de las especies: *Bifidobacterium longum, Lactobacillus reuteri, Lactobacillus johnsonii,* y *Lactobacillus rhamnosus* comparado con adultos (24-57 años) (Drago *et al*. 2012).

### 1.2.6 Métodos para Estudiar la Microbiota Intestinal

Actualmente, la información acerca de la clasificación taxonómica y funcional de bacterias intestinales se realiza mediante técnicas moleculares. Éstas tecnologías desarrolladas a finales del siglo 20, permiten el estudio del material genómico bacteriano, ya sea mediante el análisis del genoma de todos los microorganismos (metagenómica) o la secuenciación de amplicones (por ejemplo: secuencias del gen 16 S del ARN ribosomal). El gen 16S del ARNr bacteriano tiene un tamaño de 1.5 Kb con algunas variaciones entre especies. Este gen tiene nueve regiones hipervariables (V1-V9), y las variaciones en las secuencias nucleotídicas en estas regiones reflejan divergencia evolutiva, y por eso, estas secuencias proveen un método confiable para la identificación y clasificación filogenética de especies bacterianas.

Los métodos para la identificación bacteriana basada en secuencias de nucleótidos en estas regiones tienen la ventaja de que no necesitan un cultivo bacteriano previo y por eso pueden detectar bacterias tanto cultivables como aquellas que no crecen fácilmente en un medio de cultivo (Payne *et al*., 2012; Sarangi *et al*., 2019). La selección de métodos moleculares disponibles es relativamente amplia, pero en años recientes, las tecnologías de secuenciación de nueva generación son las más utilizadas en la investigación en la microbiota intestinal.

Actualmente, se emplean uno de los dos equipos de un proveedor (Ilumina), llamado MiSeq e Illumina HiSeq. Estas permiten la secuenciación de uno o dos regiones hipervariables del gen 16S

del ARNr, y esta información permite determinar la diversidad bacteriana a nivel de género y la abundancia relativa. Con estas tecnologías, diversos estudios han logrado establecer una relación entre varias especies de microorganismos y diferentes patologías que afectan al humano como la obesidad, la enfermedad de Crohn y el síndrome del intestino irritable. (Jandhyala *et al*., 2015; Sarangi *et al*., 2019).

Para estudiar la microbiota intestinal se requiere tener una muestra representativa de la misma. Una muestra representativa de la microbiota intestinal se puede obtener por colonoscopía o usando muestras fecales. En este sentido, las muestras fecales son una muestra que nos puede dar la mejor información acerca de las bacterias que están en el colon y son ampliamente utilizadas en la mayoría de los estudios sobre microbiota intestinal. Como todas las metodologías tiene sus ventajas y desventajas. Una desventaja de las muestras fecales es que no representan a la microbiota intestinal presente en la mucosa del intestino, pero sus ventajas son: la obtención de muestras no es invasiva, y pueden muestrearse repetidamente. Por eso estas muestras son las más recomendadas para analizar la microbiota intestinal (Tang *et al*., 2020).

### 1.2.7 Funciones de la Microbiota Intestinal y su Efecto en la Salud Humana.

La microbiota intestinal es considerada un órgano debido a la importancia y diversidad de las funciones que realiza, y mantiene una relación de simbiosis con el humano. Participa en diferentes procesos metabólicos e incluso tiene impacto en órganos distantes como el cerebro. Por lo anterior, la microbiota intestinal tiene un impacto importante en la salud, tanto positivo como negativo, por ejemplo, una disbiosis puede ocasionar diversos padecimientos como obesidad, cáncer, enfermedad del intestino irritable, entre otras. Las funciones de la microbiota intestinal incluyen funciones metabólicas, de protección y funciones relacionadas con el sistema inmune del huésped (Sekirov *et al*., 2010).

La microbiota intestinal hace una importante contribución al metabolismo del huésped mediante la contribución de enzimas que no son codificadas por el genoma humano (Rowland *et al*., 2018). Una de las funciones metabólicas más importantes es la digestión de componentes de la dieta. La fermentación de carbohidratos no digeribles es la principal fuente de energía de las bacterias del

colon. Los principales productos de esta fermentación son los ácidos grasos de cadena corta (AGCC). Los AGCC más abundantes son: el ácido acético, el ácido propiónico y el ácido butírico y tienen un impacto importante en la fisiología del huésped. Estos productos proveen aproximadamente el 10% de los requerimientos calóricos diarios del humano (Alexander *et al*., 2019). Además, los AGCC son fuente de energía para algunas bacterias, por ejemplo, el acetato y lactato producido por las bifidobacterias, pueden ser usados como alimento por *Anaerostipes* spp. y *Eubacterium hallii* y estas producen ácido butírico (Duncan *et al*., 2004; Flint *et al*., 2012). El acetato y butirato estimulan la producción y secreción de moco en el epitelio intestinal. También, los AGCC reducen el pH luminal, lo que ayuda a limitar el crecimiento de patógenos (Makki *et al*., 2018).

Además de ser fuente de energía para los colonocitos y algunas bacterias, los AGCC pueden ser absorbidos y liberados en la circulación para ser utilizados por el hígado y otros tejidos periféricos, donde actúan como moléculas de señalización mediante la activación de receptores acoplados a proteínas G (GPR41 y GPR43), y regulan la expresión de algunas hormonas y péptidos (Alexander *et al*., 2019; Lin *et al*., 2012). Las principales bacterias que producen AGCC pertenecen a filo Firmicutes (familias: *Streptococcaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Acidaminococcaceae*, y *Veillonellaceae*), seguido de Bacteroidetes (familias: *Bacteroidaceae*, *Prevotellaceae*) y algunas pertenecientes a Proteobacteria (familia: *Enterobacteriaceae*), Verrucomicrobia (*Verrucomicrobiaceae*) y Actinobacteria (familia: *Bifidobacteriaceae*) (Xu *et al*., 2020).

Por otro lado, el metabolismo de proteínas por parte de la microbiota también genera AGCC. La actividad proteolítica se ha atribuido a los géneros *Bacteroides*, *Clostridium*, *Propionibacterium*, *Fusobacterium*, *Streptococcus* y *Lactobacillus*. La fermentación de aminoácidos genera además metabolitos como compuestos fenólicos (por ejemplo, el *p*-cresol) e índoles, amoniaco, aminas, y sulfuro de hidrógeno. Es importante mencionar que la alta concentración de estos metabolitos se ha relacionado con la aparición de diferentes afecciones como colitis ulcerosa y la enfermedad intestinal inflamatoria (Scot *et al*., 2013). Asimismo, la capacidad metabólica de las bacterias del colon incluye también el metabolismo de xenobióticos como fármacos y fitoquímicos (Marín *et al*., 2015).

La microbiota intestinal participa en la síntesis de vitaminas. Algunas bifidobacterias pueden sintetizar vitaminas del complejo vitamínico B (p.e. vitamina $B_1$, vitamina $B_3$, vitamina $B_6$, ácido

fólico y vitamina $B_{12}$) (Deguchi *et al*., 1985), y la vitamina K, producida por *Enterobacter agglomerans, Serratia marcescens* y *Enterococcus faecium* y favorece la absorción de microelementos (Cooke *et al*., 2006). La microbiota intestinal también tiene una función protectora contra la colonización de patógenos. En este sentido, las bacterias comensales, ayudan en la inhibición de los patógenos, mediante la creación de un ambiente de competencia por espacio y nutrientes o mediante la producción de toxinas y antibióticos como las bacteriocinas, el peróxido de hidrógeno y el ácido láctico (Garcia-Gutierrez *et al*., 2019).

Otro efecto muy importante de la microbiota intestinal es su efecto en la estimulación del sistema inmune. El desarrollo y maduración del sistema inmune así como el establecimiento de la microbiota intestinal son dos procesos que ocurren en paralelo y sucede durante los primeros tres años de vida. Debido a lo anterior es que se menciona en la literatura que ambos procesos se ayudan uno del otro, y esto hace posible que ocurran (Dzidic *et al*., 2018). La interacción sistema inmune-microbiota intestinal mantienen la homeostasis del intestino. Se han estudiado tres mecanismos por los que la microbiota ayuda al desarrollo del sistema inmune. El primero es que la microbiota ayuda en el desarrollo del tejido linfoide asociado con el intestino, el cual tiene una función importante en desencadenar la respuesta inmune. En el segundo, la microbiota ayuda en el desarrollo y diferenciación de los linfocitos T cooperadores 17 (Th17), y los linfocitos T reguladores que controlan la actividad de las células Th. Por último, la microbiota regula la secreción de inmunoglobulina A (Zareef *et al*., 2020).

Además, la microbiota comensal inhibe la adhesión de patógenos a las células del epitelio intestinal mediante la generación de moco intestinal, síntesis de proteínas antimicrobianas, y ayudan en patologías relacionadas con procesos inflamatorios directamente influenciando la promoción o inhibición de citoquinas pro-inflamatorias y anti-inflamatorias (Thompson-Chagoyán *et al*., 2005). Una microbiota normal, mantiene un sistema inmune funcionando correctamente. El sistema inmune a su vez, ayuda a mantener la integridad del intestino mediante la inhibición de la adhesión de bacterias patógenas al epitelio intestinal. Esto lo logra mediante la secreción de mucina, que forma una doble capa de moco en la barrera intestinal. También secreta proteínas antimicrobianas que pueden destruir la pared de las bacterias, y por último, induce la secreción de inmunoglobulina A, que inhibe la adhesión bacteriana. Una alteración en la homeostasis intestinal resulta en una disbiosis. La disbiosis es una alteración de la microbiota normal del cuerpo y se relaciona con la aparición de diversas enfermedades; por ejemplo, alergias, la enfermedad inflamatoria intestinal,

la colitis ulcerosa, y la enfermedad de Crohn, las cuales son asociadas con una disminución en la diversidad bacteriana (Sha *et al*., 2013). Por ejemplo, en pacientes con síndrome del intestino irritable, las bacterias *Lactobacillus, Collinesella y Bifidobacterium,* están en menor proporción, mientras que incrementa la abundancia de bacterias de los géneros *Bacteroides, Allinsonella, Ruminococcus, Streptococcus* (Kassinen *et al*., 2007). En contraste, los géneros *Bifidobacterias, Faecalbacterium prausnitzii* y *Akkermansia muciniphila* se asocian con la prevención de esta patología (Cheng *et al*., 2017; John y Mullin, 2016).

Por otro lado, la microbiota intestinal tiene una interacción con el cerebro, ésta interacción se conoce como el eje intestino-cerebro. En esta vía de comunicación se involucran el sistema nervioso central, el tracto gastrointestinal y la microbiota intestinal. La microbiota se comunica con el sistema nervioso central mediante el nervio vago, el sistema inmune y la vía sistémica mediante la síntesis de metabolitos generados por bacterias, por ejemplo, los AGCC, los ácidos biliares secundarios, neurotransmisores, entre otros, los cuales ejercen su función en dos ámbitos, bien disparando señales ascendentes que se inician a nivel local, o bien atravesando la barrera intestinal para pasar a la circulación sistémica, e incluso actuando directamente en el sistema nervioso central tras atravesar la barrera hematoencefálica (Strandwitz, 2018).

### 1.2.8 Modulación de la Microbiota Intestinal

La composición y diversidad de bacterias depende de factores como: la cantidad de sustratos disponibles, el pH y compuestos antimicrobianos. Estos factores, a su vez, dependen de la fisiología del hospedador y de la dieta. En el colon ascendente es donde se realiza la mayor actividad fermentativa de sustratos de la dieta, que principalmente son carbohidratos. En este sitio, el pH es bajo (de 5.4-5.9) comparado con el resto del colon, y los AGCC producidos por la fermentación ayudan a la reducción del pH del medio. Por otro lado, en el colon descendente se realiza la digestión de proteínas que provienen tanto de la dieta como de enzimas secretadas en el intestino delgado. La digestión de proteínas favorece el crecimiento de bacterias sacarolíticas, ya que provee de nitrógeno para su crecimiento y de bacterias proteolíticas (Figura 1) (Duncan *et al*., 2009).

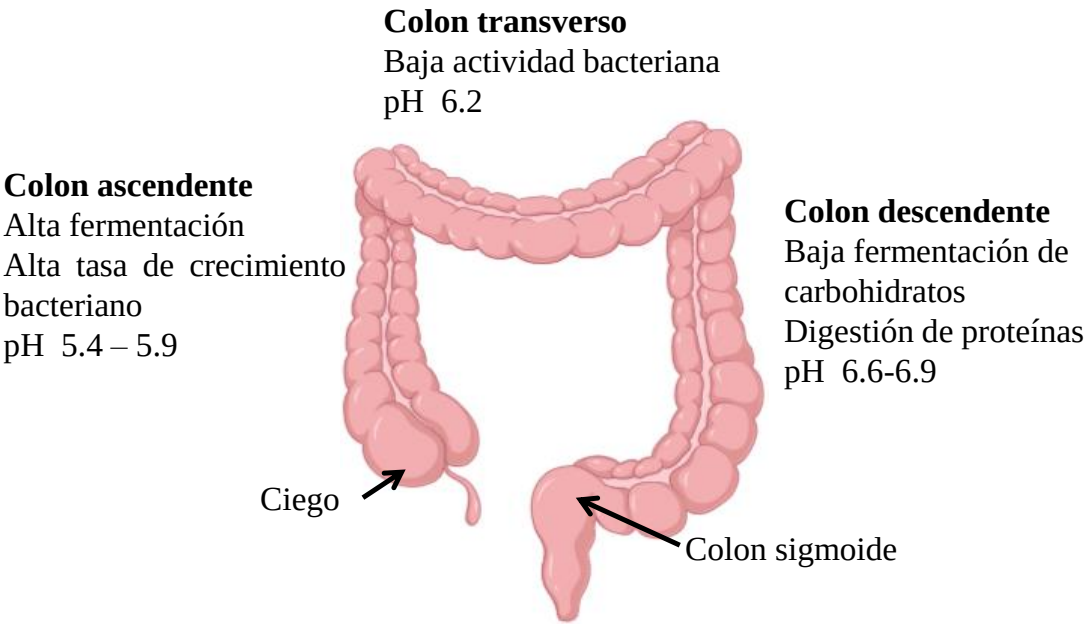


**Figura 1** Actividad fermentativa de las bacterias en diferentes regiones del colon.
Fuente: Elaboración propia con datos de: (Cummings y Macfarlane, 1991).

Las especies de bacterias que logran establecerse en el colon, son aquellas que tienen la capacidad de adaptarse a las condiciones intestinales y de utilizar los nutrientes disponibles. Algunos nutrientes provienen del hospedador, como las mucinas, sin embargo, la mayor fuente de nutrientes que llega al colon proviene de la dieta (Thursby y Juge, 2017).

Diversos estudios ponen en evidencia el efecto de la dieta en la modulación de la microbiota intestinal, además, como se ha mencionado anteriormente, la composición de la microbiota intestinal impacta en el estado salud del hospedador. Es por ello, que el interés en la mejora de estrategias que ayuden en el tratamiento de la disbiosis intestinal, ha ido en incremento (Riaz-Rajoka *et al*., 2017; Yang *et al*., 2020). Las estrategias terapéuticas hoy en día son el uso de prebióticos, probióticos y simbióticos, y se han utilizado como coadyuvantes en la restauración del equilibrio de la microbiota intestinal. Además, algunos estudios han evaluado a los compuestos fenólicos por su efecto en la modulación de la microbiota intestinal. Esto debido a sus propiedades como su actividad antimicrobiana (Cardona *et al*. 2013). Los estudios enfocados en la evaluación de compuestos fenólicos en la modificación de las bacterias del colon utilizan principalmente el té verde (Liu *et al*. 2016), vino tinto (Nash *et al*. 2018), frutos rojos (Li *et al*. 2019), granada (Mosele *et al*. 2015) y cocoa (Sorrenti *et al*. 2020).

1.2.7.1 Probióticos. El interés en el mantenimiento de la salud mediante el consumo de microorganismos benéficos ha evolucionado a lo largo del último siglo, con la observación de que los productos lácteos fermentados se asociaban con la longevidad, hasta que se llegó a la utilización de los probióticos como una terapia para promover la salud humana por Fuller, 1989. La definición actual propuesta por la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO, por sus siglas en inglés), es:

*"Microorganismos vivos, que al ser administrados en cantidades adecuadas confieren un beneficio para la salud del huésped" (FAO/WHO, 2001).*

A su vez, la Asociación Científica Internacional para Probióticos y Prebióticos (ISAPP, por sus siglas en inglés), estableció que, una actividad general de los probióticos es la modulación de la "microbiota perturbada" (Suez *et al*., 2020).

La mayoría de las bacterias utilizadas como probióticas pertenecen a los géneros *Lactobacillus* y *Bifidobacterium* (Altamura *et al*., 2020). Los mecanismos de acción asociados a los probióticos son: la prevención de la adhesión o colonización de patógenos, producción de metabolitos con actividad antimicrobiana, como las bacteriocinas, y la estimulación del sistema inmune (Diez-Gutiérrez *et al*., 2020). Los efectos de los probióticos en la salud son específicos de cada cepa bacteriana; la mitigación de la intolerancia a la lactosa y la prevención de diarrea provocada por rotavirus son los efectos mayormente esclarecidos científicamente. Asimismo, los efectos potenciales de los probióticos son: el tratamiento y la prevención de alergias, enfermedad inflamatoria intestinal, inhibición de patógenos intestinales, entre otros. Igualmente, se menciona que los efectos en la salud pueden ser mejorados por la adición de una mezcla de probióticos, o la adición de algún prebiótico, sin embargo, esto aún se encuentra en estudio (Kycia *et al*., 2020; Vasiljevic y Shah, 2008).

1.2.7.2 Prebióticos. La definición de prebiótico es:

*"Un sustrato que los microorganismos del huésped utilizan selectivamente para aportar un beneficio a la salud" (Gibson et al., 2017).*

Los criterios que debe cumplir un sustrato para ser considerado un prebiótico son: 1) el sustrato es un ingrediente no digerible por enzimas digestivas humanas, 2) debe ser fermentado en el colon, 3) debe promover el crecimiento y/o actividad de bacterias probióticas. Las fuentes de prebióticos

son: la leche materna, y las plantas. Los fructooligosacáridos (FOS) y los galactooligosacáridos, son los prebióticos más comercializados. El efecto en la microbiota intestinal de los FOS se ha reportado, por ejemplo, el consumo de FOS incrementa la abundancia de *Bifidobacterium* y *Lactobacillus*, los cuales son géneros de bacterias asociadas con efectos benéficos en la salud (Tandon *et al*. 2019). Además del balance de la microbiota intestinal, los prebióticos han demostrado diversos efectos al ser consumidos como alivio en la constipación, ayudan en la absorción de minerales, disminución en los niveles de colesterol, y efectos anticancerígenos. Recientemente, los prebióticos se han utilizado en combinación con probióticos para mejorar la sobrevivencia de las bacterias probióticas (Ashwini *et al*., 2019).

1.2.7.3 Compuestos fenólicos. Los compuestos fenólicos comprenden un gran grupo de fitoquímicos, cuya biosíntesis tiene lugar a través de dos rutas primarias: la del ácido shiquímico y la ruta de los poliacetatos. La primera sintetiza aminoácidos aromáticos y ácidos cinámicos y sus derivados y la segunda proporciona las quinonas y xantonas (Bravo, 1998). La estructura molecular de los compuestos fenólicos se caracteriza por la presencia de al menos un anillo aromático unido con uno o más grupos hidroxilo (grupo fenol). Los compuestos fenólicos son de interés debido a sus propiedades biológicas relacionadas a su capacidad para quelar metales y captar radicales libres, que les confieren propiedades como la actividad antioxidante y la actividad antimicrobiana. Estos compuestos orgánicos se encuentran naturalmente en una variedad de alimentos, como el cacao, vegetales, granos de cereal, los frutos rojos, el té verde, entre otros.

Los compuestos fenólicos se clasifican de acuerdo a su estructura química en flavonoides y no flavonoides (Tsao, 2010). Algunos estudios han reportado la interacción bi-direccional de los compuestos fenólicos con la microbiota intestinal. Los compuestos fenólicos pueden inhibir o promover el crecimiento de algunas bacterias del colon, mientras que los compuestos fenólicos pueden ser transformados a monómeros por algunas especies de bacterias del colon (Gowd *et al*., 2019).

Algunos autores han demostrado que los monómeros generados a partir de compuestos fenólicos por la microbiota intestinal, pueden tener una mayor bioactividad que el compuesto del cual se derivan (compuesto parental), ya que se vuelven más absorbibles (Wang *et al*., 2019). Aproximadamente, el 5 - 10% de los compuestos fenólicos que se ingieren en la dieta se absorben

en el intestino delgado, mientras que el 90 – 95% se metabolizan por la microbiota residente del colon (Cardona *et al*., 2013). Por lo tanto, es mediante la biotransformación por parte de la microbiota intestinal que los compuestos fenólicos llegan a los órganos diana. En consecuencia, las bacterias que residen en el colon son en gran parte responsables del efecto en la salud que ejercen los compuestos fenólicos presentes en los alimentos de origen vegetal como tés, frutas, y vegetales, al transformarlos en moléculas con mayor biodisponibilidad.

Existe evidencia científica de que la actividad biológica que tiene el consumo de la infusión de corteza de mango es debido a sus compuestos fenólicos (García-Rivera *et al*., 2011; Rymbai *et al*., 2013). Estudios tanto *in vitro* como *in vivo* han demostrado la actividad biológica de los compuestos fenólicos de la corteza de mango y de la infusión de corteza de mango. Por ejemplo, se ha demostrado que tanto mangiferina como la corteza de mango evitan el daño en el ADN de linfocitos humanos (Rodeiro *et al*., 2012), y que tienen efecto anticancerígeno en células de cáncer de mama MDA-MB231 (García-Rivera *et al*., 2011). En ratas, mangiferina ha demostrado efecto anti-inflamatorio y atenuación de los síntomas de colitis, mediante la inhibición de la señalización del factor nuclear NF-κB y la vía vía MAPK (Das *et al*., 2012; Dou *et al*., 2014), y efectos neuro-protectores (Kasbe *et al*., 2015) y antidepresivos mediante la atenuación del estrés oxidativo y la interleucina 1 beta (IL-1β) (Jangra *et al*.,2014). Además, los compuestos fenólicos presentes en la infusión de la corteza de mango poseen importante actividad antibacteriana (Pacheco-Ordaz *et al*., 2018; Singh *et al*., 2012; Vega-Vega *et al*., 2013).

Recientemente el interés en el estudio de la interacción de los compuestos fenólicos con las bacterias del colon ha incrementado. La corteza del árbol de mango es una infusión ampliamente utilizada en la medicina tradicional, y es rica en compuestos bioactivos con importantes efectos en la salud. Sin embargo, aún es necesario elucidar mecanismos y efectos en la salud intestinal, particularmente, en las bacterias del colon. Por lo tanto, la evidencia de la calidad de los compuestos fenólicos de la corteza de mango, la hace un material valioso para su estudio. Es importante mencionar también que el entendimiento del efecto en la salud de este material vegetal, potenciará su uso, lo cual puede ayudar a reducir los desperdicios generados de corteza de mango a través de la poda del árbol.

Los compuestos fenólicos del extracto acuoso de la corteza del árbol de mango son los componentes mayoritarios (aproximadamente 45%), y principalmente consiste de una mezcla compleja de compuestos fenólicos con diferentes estructuras, que son: los ácidos fenólicos, flavan-

3-oles, galotaninos y la xantona polifenólica mangiferina (Figura 2) (Abdel-Mageed *et al*., 2014; Barreto *et al*., 2008; Núñez-Sellés *et al*., 2002).

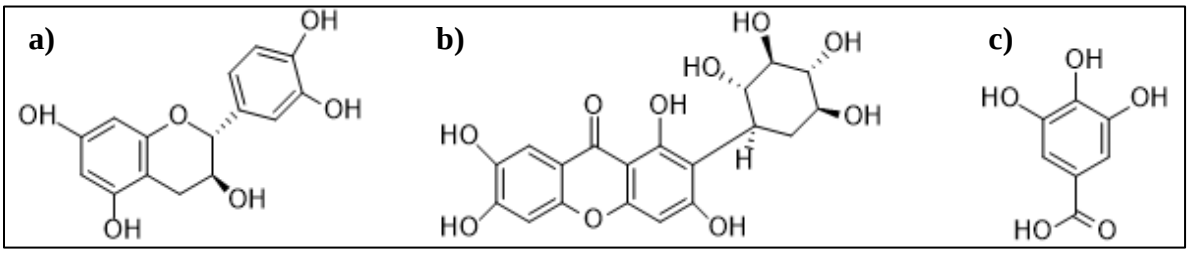


**Figura 2** Estructura de los principales polifenoles reportados en la corteza del árbol de mango: a) Catequina, b) Mangiferina, c) Ácido gálico. Fuente: Elaboración propia (ChemDraw 12.0).

Los compuestos fenólicos no glicosilados se absorben en el intestino delgado, mientras que los compuestos glicosilados, como los flavan-3-ol, galotaninos y xantonas, necesitan ser hidrolizados. Esta hidrólisis puede llevarse a cabo por células de la mucosa intestinal y enzimas bacterianas (β-glicosidasas, lactasa-floricina hidrolasa) en el intestino delgado (Marín *et al*., 2015). Pero otros compuestos pueden pasar intactos al intestino grueso y ser metabolizados por la microbiota del colon. Por ejemplo, mangiferina es metabolizada en el intestino grueso por la bacteria intestinal humana *Bacteroides* sp. MANG, gracias a la enzima que escinde el enlace C-glucósido de la molécula, generando noratiriol, a su vez, este metabolito puede seguirse modificando hasta ser absorbido (Liu *et al*., 2011; Sanugul *et al*., 2005). Por su parte, los galotaninos, para ser absorbidos, deben ser hidrolizados en pequeñas moléculas (tri, di y monómeros) y el principal sitio para su metabolismo es en el colon por la acción de la microbiota colónica. Por lo tanto, los compuestos fenólicos que pueden alcanzar los órganos diana, son distintos en cuanto a estructura y funcionalidad, de las formas originales (Kroon *et al*., 2004).

Estudios *in vitro* se han llevado a cabo con el propósito de evaluar la acción selectiva de diferentes compuestos fenólicos a diferentes concentraciones. Por ejemplo, Tzounis *et al*., 2008, incubaron microbiota de heces humanas con compuestos fenólicos del té (catequina y epicatequina) a diferentes concentraciones. Los resultados mostraron un incremento de *Lactobacillus* y *Bifidobacterium* a la concentración más baja, en cambio, con la concentración más alta, el crecimiento de *Clostridium histolyticum* se disminuyó. En otro estudio, mediante un modelo de

digestión *in vitro* utilizando microbiota de heces humanas, y el análisis de secuencias del gen 16S ARNr, se observó que la cáscara del fruto de mango favoreció la abundancia relativa de *Bifidobacterium* hasta en un 83% al tiempo 24 h, comparado con el control (40% aproximadamente), mientras que la abundancia del género *Bacteoides* disminuyó significativamente. Además, la cáscara de mango aumentó la abundancia de bacterias gram-positivas del género *Dorea* y un género no identificado del orden *Lactobacillales*. Por otro lado, se redujo la abundancia de un género de la familia *Ruminococcacear* y de *Lactococcus* y de la familia *Lachnospiraceae* (Sáyago-Ayerdi *et al*., 2019).

Por su parte, Kim *et al*., 2018, demostraron que los compuestos fenólicos del mango mejoran los síntomas de colitis en ratas, mediante la modulación de la microbiota intestinal y la producción de AGCC. En cuanto a las bacterias del colon, se mejoró el crecimiento de bacterias productoras de tanasa: *Lactobacillus plantarum* y *Lactococcus lactis* significativamente, además de la bacteria productora de butirato *Clostridium butyrium*. Además, incrementó la concentración de ácido butírico.

En un estudio en humanos con enfermedad inflamatoria intestinal, el consumo de pulpa de mango, se observó una modulación en la microbiota intestinal, según el análisis de las heces. El consumo de mango durante 8 semanas, incrementó significativamente la abundancia relativa de *Lactobacillus* spp., además, promovió el crecimiento de *Lactobacillus reuteri* y de dos especies de bacterias productoras de tanasas (*Lactobacillus plantarum* y *Lactobacillus lactis*), que producen pirogalol y ácido gálico a partir de galotaninos, los cuales se sugiere son los responsables de la reducción en la inflamación en este estudio. Asimismo, se incrementó la producción de ácido butírico, el cual es un AGCC asociado con mejorar la función de la barrera intestinal (Kim *et al*., 2020).

Existen interesantes e importantes resultados sobre los compuestos fenólicos del mango, y su efecto selectivo en algunos grupos de bacterias intestinales, sin embargo, la corteza del árbol de mango, necesita ser estudiada en este sentido, para elucidar los mecanismos de acción asociados a sus efectos en la salud humana.

1.2.7.4 Modelos *in vitro* para el estudio de la microbiota intestinal. Los métodos para evaluar los cambios de la microbiota intestinal humana en respuesta a una intervención por la dieta o

componentes de la dieta, consisten en métodos cultivables o tecnologías de secuenciación de alto rendimiento (no cultivables), y generalmente, se analizan muestras de heces humanas (Williams *et al*., 2015). El estudio de estas muestras, como cualquier estudio, tiene sus ventajas y limitantes. En cuanto a sus ventajas, estas muestras se utilizan por ser de bajo costo, no invasivas, sin restricciones éticas, y se pueden obtener fácilmente. Por otro lado, las limitantes incluyen: 1) no se toman en cuenta los procesos de interacción microbiota-hospedador que suceden *in vivo*, por ejemplo, con células del epitelio y del sistema inmune. 2), existe la variabilidad entre individuos, la cual se puede minimizar al utilizar mezcla de heces de diferentes voluntarios, y 3) la microbiota no está distribuida uniformemente en las heces, lo cual también se ha minimizado mediante homogenización de las muestras (Tang *et al*., 2020; Venema y van den Abbeele, 2013). A pesar de estas limitantes, el uso de heces humanas es ampliamente utilizado para elucidar el efecto potencial de componentes de la dieta en las bacterias del colon (Gong *et al*., 2019; Pérez-Burillo *et al*., 2019).

El modelo *in vitro* más simple para estudiar la microbiota intestinal es la fermentación por lote. En este modelo se utiliza un inóculo fecal o poblaciones microbianas específicas que son derivadas de las heces y se llevan a cabo en recipientes cerrados. Estos modelos han sido usados para evaluar la capacidad de las bacterias intestinales para metabolizar diferentes sustratos y evaluar su crecimiento. Estos sistemas tienen la limitante de ser de corta duración, debido a la acumulación de AGCC, y metabolitos, así como cambios de pH, que podrían inhibir el crecimiento bacteriano, y no reflejan el ambiente real del colon. A pesar de sus limitantes, estos modelos han demostrado ser útiles para evaluar la eficacia de los probióticos, prebióticos (por ejemplo, fibras y compuestos fenólicos), y evaluar los metabolitos producidos como los AGCC. Además, son importantes para tener un primer acercamiento sobre el metabolismo intestinal de los compuestos fenólicos, o para comparar diferentes fuentes o dosis de los mismos, antes de utilizar un modelo *in vivo* (Macfarlane y Macfarlane, 2007; Tsitko *et al*., 2019; Venema y van den Abbeele, 2013).

Por otro lado, la capacidad de los modelos *in vitro* para simular condiciones reales que suceden en el tracto gastrointestinal no muestran la interacción del sustrato evaluado con el epitelio intestinal y el moco intestinal. Por ello, se han implementado experimentos utilizando cultivos celulares. Una de las líneas celulares más usadas para el estudio del metabolismo de compuestos fenólicos y su interacción con células del epitelio, son las células Caco-2, las cuales tienen la capacidad de formar una monocapa de células con una funcionalidad similar a las células que revisten el epitelio

intestinal.

La creciente evidencia que resalta la importancia de la microbiota humana en la salud, lleva al desarrollo y evaluación de nuevos ingredientes de la dieta para modular la microbiota intestinal. Es por ello que es necesario llevar a cabo diferentes tipos de experimentos, como celulares e in vitro, para entender con mayor claridad las interacciones de los compuestos fenólicos dentro del cuerpo humano.

## 1.3 Hipótesis

1. El extracto acuoso de la corteza del árbol de mango de cuatro cultivares (Keitt, Ataulfo, Kent y Tommy Atkins) contienen ácidos fenólicos, xantonas, taninos y flavonoides.
2. La capacidad antioxidante es diferente para cada cultivar de mango (Keitt, Ataulfo, Kent y Tommy Atkins) teniendo mayor capacidad antioxidante en la variedad con mayor contenido fenólico.
3. Los flavonoides y ácidos fenólicos presentes en el extracto acuoso de la corteza del árbol de mango (cv. Keitt, Ataulfo, Kent y Tommy Atkins) son absorbidos en un modelo de epitelio intestinal humano (Caco-2).
4. Los compuestos fenólicos presentes en el extracto acuoso de la corteza de mango (cv. Keitt) modifican la composición de las bacterias del colon beneficiando la proporción de bacterias probióticas.

## 1.4 Objetivo General

Determinar la absorción *in vitro* del extracto acuoso de la corteza del árbol de mango y su efecto en la microbiota intestinal *in vitro.*

## 1.5 Objetivos Específicos

1. Caracterizar y cuantificar los compuestos fenólicos presentes en el extracto acuoso de la corteza del árbol de mango (cv. Keitt, Ataulfo, Kent y Tommy Atkins).
2. Determinar y comparar la actividad antioxidante del extracto acuoso de la corteza del árbol de mango en las variedades Keitt, Ataulfo, Kent y Tommy Atkins.
3. Determinar y comparar el transporte de los compuestos mayoritarios del extracto acuoso de la corteza del árbol de mango (cv. Keitt, Ataulfo, Kent y Tommy Atkins) a través de un modelo de células Caco-2.
4. Evaluar el efecto del extracto acuoso de la corteza del árbol de mango cv. Keitt sobre la microbiota presente en heces humanas.

## 1.6 Sección Integradora del Trabajo

En esta tesis se incluyen tres artículos, dos artículos originales y uno de revisión. El primer artículo fue un artículo de revisión y se encuentra publicado en el *Journal of Food Biochemistry*. En éste artículo de revisión se incluye la información reciente sobre las propiedades prebióticas de compuestos obtenidos a partir de sub-productos agrícolas y los estudios recientes enfocados en su efecto en la microbiota intestinal humana. Como resultados, los oligosacáridos tienen efecto en la promoción del crecimiento de bacterias probióticas principalmente del género *Bifidobacterium* y *Lactobacillus* y no estimulan el crecimiento de *Escherichia coli*. En conclusión, es necesario realizar más estudios usando modelos animales y ensayos clínicos. Igualmente, se necesita evaluar la modificación de los oligosacáridos al someterse a las condiciones del tracto gastrointestinal humano, por ejemplo, cambios de pH y enzimas digestivas. Lo anterior será de ayuda para la producción de alimentos funcionales y el desarrollo de formulaciones simbióticas usando prebióticos en combinación con bacterias probióticas. Éste artículo de revisión se relaciona con el proyecto de doctorado debido a que el objetivo general fué la evaluación del efecto en la microbiota intestinal humana de un extracto obtenido a partir de un sub-producto agrícola y en esta revisión

se resumen los principales compuestos estudiados, en este sentido, nos sirve de base para comparar el efecto observado con el material vegetal que utilizamos.

El segundo artículo, fue un artículo original y se encuentra publicado en el *Journal of the Science of Food and Agriculture*. Con este artículo se cumplió con los primeros tres objetivos específicos de la tesis. Como resultados, los principales fitoquímicos encontrados fueron: mangiferina y un derivado de ácido gálico. Estos compuestos fenólicos estuvieron en mayor concentración en la variedad Keitt. Todas las variedades presentaron actividad antioxidante y no fueron citotóxicas en un modelo de células Caco-2 a la concentración máxima de 100 µg/mL. La permeabilidad intestinal de mangiferina presente en el extracto acuoso de la corteza del árbol de mango fue de 3 a 4.8 veces mayor que mangiferina como estándar, mientras que la permeabilidad intestinal in vitro para ácido gálico fue variable entre las variedades. Como conclusión, el extracto acuoso de la corteza del árbol de mango tiene actividad antioxidante a nivel celular y es una fuente abundante y de bajo costo para la extracción de mangiferina. A partir de esta investigación se logró decidir sobre una variedad de corteza de mango con mayor potencial para actuar como prebiótico debido a su concentración de compuestos fenólicos y su actividad biológica.

En el tercer artículo se logró cumplir con el último objetivo de investigación y así concluir con el objetivo general del proyecto. En este observamos que la corteza de mango puede tener un efecto en la modulación de la microbiota intestinal dado su comportamiento en la inhibición o promoción de algunos filos, familias, géneros y especies. Gracias al uso de tecnologías de secuenciación de última generación, logramos obtener la agrupación de 1687 Unidades Taxonómicas Operacionales (OTUs, por sus siglas en inglés). Aunque a no encontramos diferencias estadísticas, existe una relevancia biológica en los hallazgos encontrados, lo que permite realizar investigaciones futuras. Asimismo, observamos un efecto en la generación de ácidos grasos de cadena corta, lo cual se comparó con un prebiótico comercial (inulina) y observamos resultados similares, lo que ayuda a elucidar que el efecto de la corteza del árbol de mango en su uso etnobotánico para el tratamiento de diarreas y afecciones gastrointestinales puede relacionarse a su efecto en la microbiota y particularmente a su efecto en la generación de ácidos grasos de cadena corta, así como a su potencial antioxidante y su riqueza en compuestos bioactivos, que son tanto fibra como polifenoles. Este artículo original se encuentra en preparación y se pretende enviar al *Food Research International*.

## 2. PREBIOTIC COMPOUNDS FROM AGRO-INDUSTRIAL BY-PRODUCTS

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REVIEW

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# Prebiotic compounds from agro-industrial by-products

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## Abstract

Prebiotics are nondigestible food components that have an impact on gut microbiota composition and activity, which in turn results in the improvement of health conditions. Nowadays, the production of prebiotics from agro-industrial by-products is under investigation. In this regard, polysaccharides are usually found in these sources and their potential use as prebiotics has been studied recently since these compounds act as substrates for the human gut microbiota, and they have the potential to modulate its composition through many mechanisms. Additionally, the use of agricultural by-products is advantageous because it is a cheap and abundantly available material. This review focuses on the recent scientific literature regarding the prebiotic properties of polysaccharides from agro-industrial by-products.

## Practical applications

Currently, the maintenance of gut homeostasis is a target for the improvement of human health. This review can broaden the perspective on the utilization of agro-industrial by-products that can compete in the market with the commercial ones or act as a source for new food ingredients.

**KEYWORDS**
Agro-industrial, By-products, gut microbiota, polysaccharides, prebiotics

## 1 | INTRODUCTION

Prebiotics are defined as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves the host's health" (Gibson & Roberfroid, 1995).

Through resistance to digestion, nondigestible carbohydrates pass through the upper digestive tract almost intact and are hence fermented in the colon by the resident microbiota, here; the colon microbiota produce metabolites such as short-chain fatty acids which in turn act to lower the pH enhancing the growth of bifidobacteria and lactobacilli, therefore reducing the pathogens (Lamsal, 2012; Slavin, 2013).

Some carbohydrates such as fructo-oligosaccharides (FOS) are well known for their prebiotic effects. Nowadays, the production and evaluation of the prebiotic activity of other carbohydrates such as xylo-oligosaccharides (XOS), pectic-oligosaccharides (POS), arabinoxylo-oligosaccharides (AXOS), and isomaltooligosaccharides (IMOS) have been reported (Lomax & Calder, 2008). These carbohydrates can be obtained from agro-industrial by-products as they are a low-cost and abundant raw material of these polymers. Moreover, the concern of the consumers nowadays has increased in preferring supplements with health claims of natural origin, where polysaccharides from agro-industrial by-products could be a promising source (Kaprelyants, Zhurlova, Shpyrko, & Pozhitkova, 2017).

## 2 | OLIGOSACCHARIDES AS PREBIOTIC COMPOUNDS

Oligosaccharides (OS) are carbohydrates that have been defined by some authors as polymers with 2 to 10 monosaccharide residues (Mano et al., 2018), while others agree that OS are carbohydrates composed of 3–10 monosaccharide units linked by *O*-glycosidic bonds. OS can be found in by-products from the agricultural processing industry, regularly treated as waste (Martínez-Villaluenga

  

& Frias, 2014). Naturally, OS can be found throughout the plant kingdom (i.e., in roots or seeds these polymers function as energy storage), and can be classified based on their chemical composition as POS, XOS, AXOS, FOS, and IMOS. Additionally, regarding their digestibility, OS can be classified into digestible and nondigestible OS, the latter being constituted by monosaccharides through non-hydrolyzable glycosidic bonds.

Different studies have recognized nondigestible OS for its prebiotic activity (Chung et al., 2017; Moura, Macagnan, & Silva, 2015; Zhang, Hu, Wang, Liu, & Pan, 2018). The main beneficial effect of OS is focused on its effect on gut bacteria modulation. Nondigestible OS are not metabolized by the human gut as humans do not to produce hydrolases that metabolize OS, thus they travel undigested to the colon where they are used by the gut microbiota as carbon a source, and as a product of the metabolism, the microbiota produce short-chain fatty acids such as acetate, lactate, propionate, and butyrate (Erejuwa, Sulaiman, & Wahab, 2012). As aforementioned, there is a myriad of OS that may vary on its sugar moieties composition and degree of polymerization (DP), all of which directly have an effect on the potential prebiotic properties of OS (Blaut, 2002). Therefore, this review covers the latest works (2012–2018) regarding the prebiotic effects of nondigestible OS produced from agro-industrial by-products. Table 1 summarizes the prebiotic activity reported in various types of OS extracted from agro-industrial by-products.

## 2.1 | Pectic-oligosaccharides

POS are obtained by the depolymerization of pectin; this polymer is the main polysaccharide of the primary cell wall and middle lamella. It has been reported that the main function of pectin is to provide cell adhesion and to confer textural characteristics of plant organs. Currently, eight pectic domains are recognized: homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II, xylogalacturonan, apiogalacturonan, galacturonagalacturonan, galactogalacturonan, and arabinogalacturonan (Figure 1) (McCartney, Ormerod, Gidley, & Knox, 2000; Yapo, 2011).

Although the most commonly studied OS are FOS, galacto-oligosaccharides, and gluco-oligosaccharides, the study of POS is increasing due to its bioactive properties in the modulation of gut microbiota, and is often described as a better prebiotic than pectin alone and FOS (Zhang et al., 2018). Hereof, agro-industrial by-products have been studied as a rich source of POS. For instance, sugar beet pulp has been reported to contain galacturonic acid (16.5%), arabinose (16.0%), galactose (5.6%), and rhamnose (1.1%) (Babbar, Dejonghe, Sforza, & Elst, 2017; Prandi et al., 2018). Also, POS from citrus peel contain l-rhamnose, D-galacturonic acid, D-glucose, and D-galactose (Zhang et al., 2018). Additionally, orange peel by-products have been reported to contain arabinose, glucose, and galacturonic acid as the main components (Di et al., 2017; Li, Xia, Nie, & Shan, 2016).

As previously mentioned, pectin includes many polysaccharides with homogalacturonan, xylogalacturonan, rhamnogalacturonan I with arabinan, galactan, and arabinogalactan side chains, and rhamnogalacturonan II. The different compositions of the structure of the pectin in a sample are responsible for its prebiotic potential. Hence, the study of the composition of pectin as an agro-industrial by-product is pivotal (Hotchkiss et al., 2009; Manderson et al., 2005). The structural elucidation of POS is often troublesome since their distribution is dependent on the agricultural source and the method of extraction; for this reason, the evaluation of the prebiotic activity of POS from different sources is often accompanied by structural studies (Prandi et al., 2018). In this sense, Zhang et al. (2018) assessed various POS fractions obtained from citrus peel pectin by chemical degradation with trifluoroacetic acid (TFA) using hydrogen peroxide. The results showed that hydrolysis by TFA at different concentrations yielded three OS, ranging from in molecular weights (MW) of 3,000–4,000 Da, 2000–3,000 Da, and lower than 2000 Da. On the other hand, hydrogen peroxide at distinct concentrations yielded OS of 3,000–4,000 Da and 2000–3,000 Da. Even though the MW of the fractions was similar, fractions obtained from hydrogen peroxide degradation exhibit the highest prebiotic score for the probiotic bacteria *Lactobacillus paracasei* LPC-37 (0.41) and *Bifidobacterium bifidum* ATCC 29521 (0.92). The differences in the prebiotic effect with the same MW OS may be attributed to the linkage type between the monosaccharide residues, the degree of esterification, and the differences in the composition of the OS structure. One of the factors that affects the gut modulation of POS is the degree of methylation. POS derived from low-methoxy (8%) or high-methoxy (66%) citrus pectin had more prebiotic activity than the whole pectin molecule (Hotchkiss et al., 2009). In another study, Prandi et al. (2018) evaluated the combination of hydrolysis and fractionation of pectin from sugar beet pulp. The results showed a potential prebiotic activity for the different fractions; furthermore, enzymatic extraction and POS with low DP arabinans and small amount or no free galacturonic acid resulted in a more efficient growth stimulation of lactic acid bacteria; moreover, the growth of pathogenic *Escherichia coli* strains was not stimulated.

Chung et al. (2017) confirmed that homogalacturonan OS from sugar beet pectin (DP 4-5) were used as a carbon source by *Faecalibacterium prausnitzii* strains. Interestingly, homogalacturonan OS promoted the production of IL-10, an antiinflammatory cytokine, showing the antiinflammatory potential of these oligosaccharides.

Most studies aim to obtain POS by a combination of enzymatic/acidic hydrolysis and further fractionation. However, Babbar et al., (2017) by means of response surface methodology successfully produced POS using a one-step enzymatic approach. Their model was able to obtain a hydrolysate rich in POS composed of rhamnose, arabinose, galactose, xylose, and galacturonic acid, at 0.9, 15.2, 5.1, 1.4, and 13.2 g/L, respectively. Studies on POS have shown prebiotic effect, and in some instances they have demonstrated to reduce pathogenic bacteria. For instance, different POS from orange peel have enhanced lactobacilli growth, while POS from orange peel with low MW and low deesterification showed the most anti-adhesive activity for *E. coli* O157:H7 (Di et al., 2017).

Due to the potential use of POS in the food industry, the current demand of these compounds is on the rise; therefore, new

**TABLE 1** Prebiotic effect of different polysaccharides extracted from agro-industrial wastes

| Prebiotic extracted | Type of agro-industrial by-products | Effect on probiotic bacteria | References |
|---|---|---|---|
| POS | Orange peel | Modulation of *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Clostridium perfringens*, and *Bacteroides fragilis* | Li et al. (2016) |
| POS | Sugar beet | Modulation of *Faecalibacterium prausnitzii* | Chung et al. (2017) |
| POS | Orange peel | Stimulatory effect on the growth of lactobacilli | Di et al. (2017) |
| POS | Sugar beet | Non-stimulation of pathogenic *Escherichia coli* | Prandi et al. (2018) |
| POS | Citrus peel pectin | Modulation of *Lactobacillus paracasei* LPC-37 and ATCC 29,521 | Zhang et al. (2018) |
| AXOS | Wheat bran | Increased bifidobacteria population | Gullón, Gullon, Tavaria et al. (2014) |
| AXOS | Brewer's spent grain | Stimulatory effect on the growth of bifidobacteria | Gómez et al. (2015) |
| AXOS | Brewer's spent grain | Stimulatory effect on the growth of *L. brevis* and *B. adolescentis* | Sajib et al. (2018) |
| (A)XOS | Wheat bran | Stimulatory effect on the growth of *L. brevis* and *B. adolescentis*. No stimulation of the growth of pathogenic *E. coli* | Mathew et al. (2018) |
| IMO | Rice starch | Increasing of bifidobacteria and lactobacilli, and reduction of pathogenic bacteria | Plongbunjong et al. (2017a) |
| IMO | Rice starch | Stimulatory effect on the growth bifidogenic bacteria | Plongbunjong et al. (2017b) |
| XOS | Corncob | Stimulatory effect on the growth of *Bifidobacteria* spp. and no effect on *Lactobacillus* spp. | Chapla et al. (2012) |
| XOS | Corncob | Stimulatory effect on the growth of *Enterococcus fecalis* CCD10, *Enterococcus faecium* TCD3, *L. maltromicus* MTCC108, and *L. viridiscens* NCIM2167 | Samanta et al. (2012) |
| XOS | Wheat straw | Stimulatory effect on the growth of *B. adolescentis* and *B. bifidum* | Chapla et al. (2013) |
| XOS | Corncob | Stimulation of *Lactobacillus* spp. growth | Boonchuay et al. (2014) |
| XOS | Corncob | Increase in relative abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. | Christensen et al. (2014) |
| XOS | Corncob | Stimulatory effect on the growth of *B. adolescentis* and *L. acidophilus* | Driss et al. (2014) |
| XOS | Wheat bran | Stimulatory effect on the growth of *L. brevis*, *B. adolescentis*, and *Weissella* spp. | Immerzeel et al. (2014) |
| XOS | Barley by-products | Stimulatory effect on the growth of *Lactobacillus* strains | Gullón, Gullon, Cardelle-Cobas et al. (2014) |
| XOS | Rice husks and barley by-products | Stimulatory effect on the growth of *Lactobacillus* strains | Gullón, Gullon, Cardelle-Cobas et al. (2014) |
| XOS | Wheat straw | Stimulatory effect on the growth of *L. brevis* DSM 1,269 | Faryar et al. (2015) |
| XOS | Garlic straw | Stimulatory effect on the growth of *B. adolescentis* and *L. acidophilus* | Kallel et al. (2015) |
| XOS | Corncob | Stimulatory effect on the growth of *L. plantarum* strains | Yu et al. (2015) |
| XOS | Corn stover | Stimulation of the growth of bifidobacteria, and *Lactobacillus-Enterococcus* and *Bacteroides-Prevotella* groups | Burulana et al. (2017) |
| XOS | Wheat bran | Stimulatory effect on the growth of *L. brevis* and *Bacillus clausii* | Geetha and Gunasekaran (2017) |
| XOS | Beechwood | Stimulatory effect on the growth of Prebiotic XOS | Ma et al. (2017) |
| XOS | Birchwood | Inhibition of the growth of enterobacteria and stimulation of probiotic bacteria | Nieto-Domínguez et al. (2017) |
| XOS | Finger millet seed coat | Stimulatory effect on the growth of *L. acidophilus L. casei*, *L. lactis* and *L. plantarum* | Palaniappan et al. (2017) |

*Note*. POS, pectic-oligosaccharides; AXOS, arabinoxylo-oligosaccharides; (A) AXOS, (a mixture of XOS and AXOS); IMO, isomaltooligosaccharides; XOS, xylo-oligosaccharides.

methodologies to obtain them are needed. Embaby, Melika, Hussein, El-Kamel, and Marey (2016) took a biotechnological approach by growing *Aspergillus* sp. section Flavi strain EGY1 DSM 101520 on citrus pectin-based medium to produce POS. This study allowed the authors to determine that citrus pectin (2.28% w/v), peptone (0.026% w/v), and $NaH_2PO_4$ (0.28%) achieved a net amount of 1.3 g POS/2.28 g citrus pectin. Nonetheless, further studies are needed before moving the process to a pilot plant scale-up. In this sense,

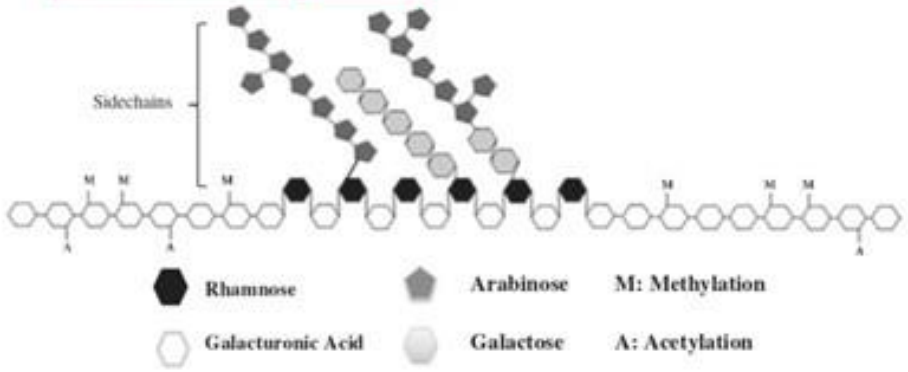


**FIGURE 1** Example of the pectin structural elements extracted from sugar beet (Holck et al., 2011)

a multi-enzymatic complex from *Aspergillus japonicus* PJ01 was used on orange peel by-products to obtain POS, which resulted in the fractionation of three major OS with different MW and composed mainly of glucose, arabinose, and galacturonic acid. The fractions were reported with better prebiotic properties than FOS on *Bifidobacterium infantis*, *L. acidophilus*, *Clostridium perfringens*, and *Bacteroides fragilis* (Li et al., 2016). From the above-mentioned studies, it can be stated that POS with better prebiotic activity are those with low DP of arabinans, almost no free galacturonic acid, low MW, and low deesterification in their structures.

## 2.2 | Arabinoxylooligosaccharides

AXOS are short arabinoxylans composed of a xylose backbone and only one arabinose moiety (Figure 2).

Some researchers have assessed the content of this OS in agro-industrial by-products; for example, barley flour contains 5.02% arabinoxylan and brewer's spent grain 14.75% arabinoxylan (Sajib et al., 2018). Also, Gomez, Miguez, Veiga, Parajo, and Alonso (2015) reported that brewer's spent grain is composed of glucan (24.91%), xylan (13.94%), arabinan (6.68%), and acid insoluble residue (22.82%). Nowadays, AXOS are considered as prospective functional ingredients due to their prebiotic effect (Yamada et al., 1994). For instance, *L. brevis* and *B. adolescentis* have shown to be able to utilize arabinoxylans and AXOS from brewer's spent grain; however, the results showed a similar or slightly less efficiency compared to commercial XOS, FOS, and galactooligosaccharides (Sajib et al., 2018). Furthermore, AXOS from brewer's spent grain showed a higher number of bifidobacteria compared to commercial FOS; however, lactobacilli growth was lower than FOS. The same authors evaluated the clostridia population and found the same number observed as the control, which suggests that the growth of this population may be due to the components of the basal medium. Besides, this AXOS were not selectively used by bacteroides (Gómez et al., 2015). In another study, the fermentation of AXOS from wheat bran with fecal inoculum demonstrated an increased bifidobacteria population in comparison with FOs (Gullón, Gullon, Tavaria et al., 2014). It has been reported that the consumption of AXOS by bacteria might be due to the presence of high-DP linear chains in its structure since smaller and highly ramified AXOS are preferred than linear fragments (Gómez et al., 2015).

## 2.3 | Isomaltooligosaccharides

IMO are another type of nondigestible OS which can be metabolized by colonic microbiota. This type of OS is made up of α-D-O-glucose residues that are linked by α-(1→6) glycosidic bonds (Figure 3).

IMO has been produced in Asia from corn starch; however, recent studies have attempted to find more sources of these polymers due to their beneficial effects on human health. For instance, rice starch has been reported to contain isomaltohexaose (26.7%),

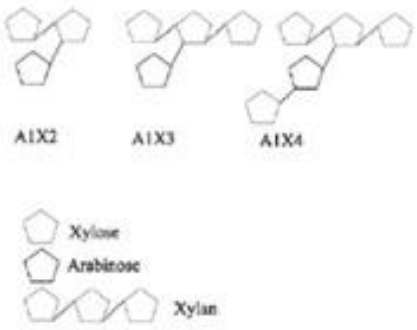


**FIGURE 2** Chemical structures of arabinoxylooligosaccharides (Kaneko et al., 1998)

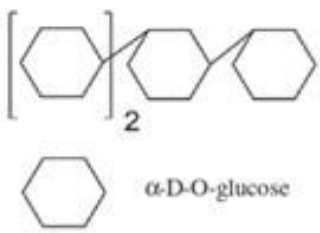


**FIGURE 3** Chemical structure of isomaltooligosaccharide (Xiao et al., 2011)

isomaltotetraose (16.2%), and glucose (16.1%) (Plongbunjong, Graidist, Knudsen, & Wichienchot, 2017a). Few studies have attempted to research the prebiotic activity of IMO extracted from agricultural by-products, where rice starch has been considered as a by-product (Mitmesser & Combs, 2017). In this regard, Plongbunjong et al., (2017a) showed the prebiotic effect of IMO from rice starch. The authors showed that the mixture of instant rice porridge with IMO increased the growth of prebiotic bacteria such as bifidobacteria and lactobacilli. Moreover, pathogenic bacteria were reduced. In another study, IMO from native rice starch showed a comparable prebiotic effect as commercial IMO; furthermore, the growth of bifidogenic bacteria was enhanced; however, the mechanisms of prebiotic activity related to the structure of IMO are still unclear (Plongbunjong, Graidist, Knudsen, & Wichienchot, 2017b).

## 2.4 | Xylooligosaccharides

XOS are sugar oligomers formed by xylose residues linked through β-(1→4) linkages. The xylose residues vary from 2 to 10 and usually side groups are found like acetyl groups, α-D-glucopyranosyl uronic acid or its 4-O-methyl derivative, and arabinofuranosyl residues (Figure 4) (Aachary Ayyappan & Prapulla Siddalingaiya, 2010; Vázquez, Alonso, Domínguez, & Parajó, 2000).

Many agricultural by-products have been used for the extraction of XOS. The quantity of XOS present in these sources varies, depending on the source of xylan, and on the way of extraction (Aachary Ayyappan & Prapulla Siddalingaiya, 2010). In this sense, XOS from wheat straw xylan yielded 36% of XOS, and the ratio of X3/X3 increased with higher temperatures (Faryar et al., 2015). XOS from wheat bran xylan yielded 59% at 185°C and 10 min of residence time (Immerzeel et al., 2014). Xylan extracted from wheat straw and rice straw by dilute alkali extraction method yielded up to 7.06 and 3.45 mg/ml of XOS from wheat straw and rice straw, respectively. Rice straw contains less hemicellulose than wheat straw. As the incubation time increased, there was rise in the production of XOS with lower chain length, for example, xylobiose and xylotriose, and in turn, a decrease in the production of XOS with higher chain length (Chapla, Dholakiya, Madamwar, & Shah, 2013). Likewise, XOS from corncob, garlic straw, and wheat bran xylan was composed mainly of xylobiose and xylotriose (Chapla, Pandit, & Shah, 2012; Kallel et al., 2015; Mathew, Aronsson, Karlsson, & Adlercreutz, 2018).

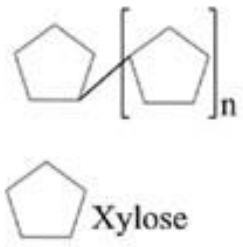


**FIGURE 4** Schematic structure of a xylooligosaccharide, where n is a variable number of xylose units (Vázquez et al., 2000)

Xylan extracted from finger millet CO9 seed coat yielded 72% of XOS (Palaniappan, Balasubramaniam, & Antony, 2017). XOS from *Eucalyptus globulus* wood, rice husks, wheat bran, and barley wastes contained 0.65, 0.682, 0.362, and 0.513 g of XOS/g of nonvolatile compounds (Gullón, Gullon, Cardelle-Cobas et al., 2014). Buruiana, Gómez, Vizireanu, and Garrote (2017) reported 11.7 g/L of XOS from corn stover. The extraction of XOS from birchwood xylan yielded 28.8% and was composed mainly of xylobiose, xylotriose, and xylotetraose (Nieto-Domínguez et al., 2017).

On the other hand, agricultural by-products have been evaluated for their prebiotic effects. The way to evaluate this activity is by fermenting XOS with probiotic bacteria or with pathogenic bacteria, and this activity is usually compared to the one exerted by FOS as it is the gold standard of a prebiotic (Gullón, Gullon, Tavaria et al., 2014) and/or with glucose (Chapla et al., 2012). Due to their importance, the main bacteria assessed are bacillus and lactobacilli; nonetheless, comparisons of the modulatory activity of XOS in different bacterial groups have been done. In this regard, XOS produced from corncob positively enhanced the growth of probiotic bacteria (*Enterococcus fecalis* CCD10, *Enterococcus faecium* TCD3, *L. maltromicus* MTCC108, and *L. viridiscens* NCIM2167) and this growth was higher than glucose or control but lower than FOS (Samanta et al., 2012). Contrary to these results, Faryar et al. (2015) showed that *L. brevis* DSM 1269 could use XOS from wheat straw xylan; however, the difference in the growth of this strain in XOS and in the control was negligible. In another study, XOS from heat-pretreated wheat bran and hydrolyzed with endo-xylanase were assessed as a carbon source for probiotic bacteria. XOS from wheat bran showed an uptake by *L. brevis*, *B. adolescentis*, and *Weissella* spp. (Immerzeel et al., 2014). Likewise, another work showed that *Bacillus adolescentis* and *B. bifidum* can grow well on XOS obtained from wheat straw and rice straw (10 g/L). Moreover, the growth was higher than glucose. The authors stated that these results were due to the ability of *Bifidobacteria* spp. to produce xylanase and β-xylosidase which are necessary for the degradation of XOS (Chapla et al., 2013). Similarly, Chapla et al. (2012) showed a good uptake of XOS from corncob by *Bifidobacteria* spp. Additionally, these authors reported that *Lactobacillus* spp. could not use XOS as efficiently as glucose because of the lack of β-xylosidase activity in *Lactobacillus* spp. Likewise, Driss et al. (2014) reported that XOS from the hydrolysis of corn cob xylan as a carbon source was utilized by *B. adolescentis* and *L. acidophilus* with dissimilar efficiency because of the lack of *Lactobacillus* spp. to produce β-xylosidase (Driss et al., 2014). In a similar study, *B. adolescentis* and *L. acidophilus* showed significant growth in XOS obtained from garlic straw xylan, although they showed different efficiencies (Kallel et al., 2015). XOS extracted from corncobs were fermented with the known probiotic lactic acid bacteria *L. casei* TISTR1463, *L. lactis* TISTR1464, and *L. plantarum* TISTR1465. From these bacteria, *L. lactis* TISTR1464 showed the highest viable cell counts and the highest maximum specific growth rates. The authors suggested that the differences among strains in XOS utilization might be due to specific mechanisms of each strain to

use OS (Boonchuay, Techapun, Seesuriyachan, & Chaiyaso, 2014). The same results have been shown by Yu et al. (2015) who evaluated 10 *L. plantarum* strains. The results showed differences in their efficacy to utilize XOS from corncob. XOS from finger millet seed coat were utilized as efficient as commercial XOS by four strains of lactic acid bacteria (*L. acidophilus L. casei, L. lactis*, and *L. plantarum*); however, *L. plantarum* showed the highest growth with all substrates (Palaniappan et al., 2017). Wheat bran XOS's syrup with 0.5% sugar concentration was tested against several probiotic strains of which *L. brevis* showed the maximum growth followed by *Bacillus clausii* (Geetha & Gunasekaran, 2017). Moreover, *Lactobacillus* strains have shown more ability to grow on XOS composed of OS with low DP and fewer branching, as described by Gullón, Gullon, Cardelle-Cobas et al. (2014) who reported that *Lactobacillus* grew better using XOS from rice husks and barley by-products than using *Eucalyptus* wood and wheat bran.

On the other hand, Buruiana et al. (2017) studied purified XOS streams obtained from corn stover. The authors reported that the media containing stream with 21.94 g/L of XOS showed the maximum growth of bifidobacteria after 10 hr of incubation. Additionally, the stream with 15.53 g/L of XOS showed a higher growth of the *Lactobacillus-Enterococcus* group after 24 hr of fermentation, and also both streams showed similar stimulatory effect on the growth of the *Bacteroides-Prevotella* group.

Moreover, the modulatory effect of XOS on pathogenic bacteria has been demonstrated. In a study performed with fecal microbiota of breast-fed children, 200 g/L of XOS from birchwood xylan inhibited the increase of the growth of enterobacteria, while 400 g/L lowered their concentration. Additionally, XOS stimulated the growth of probiotic bacteria (Nieto-Dominguez et al., 2017). Furthermore, a recent study demonstrated that the pathogenic bacteria *Escherichia coli* cannot grow in (A)XOS (the mixture of XOS and AXOS) from wheat bran, while *L. brevis* and *B. adolescentis* were able to grow in this carbon source (Mathew et al., 2018).

Additionally, in vivo assays have been done. For instance, differences in the overall microbiota composition between rats fed with XOS from corncob, *B. pseudolungum* TR2_39, or water (control) were not found. Nonetheless, XOS-fed rats showed an increase in relative abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. compared to the control (Christensen, Rask Licht, Dyrmann Leser, & Iain Bahl, 2014). Besides, the administration of these XOS combined with *L. plantarum* in mice showed an increase in the growth of lactobacilli and in bifidobacterias while the same treatment showed a reduction in the growth of *Enterococcus*, *Clostridia* spp., and *Enterobacter* (Yu et al., 2015).

Many studies showed promising results regarding the prebiotic activity of XOS, and have stated that it is affected by the way of extraction, the natural origin of XOS, and their DP. In this sense, the preference of XOS by bifidobacteria bacteria has been related to the substituents of OS, as less substituted XOS seems to be preferred by this prebiotic bacteria; furthermore, the DP, the type of linkage, and the monosaccharide composition are the structural features associated with the biological activity of XOS; regarding the chain length, it has been reported that longer molecules (with DP > 20) can reach the distal colon and produce higher concentrations of short chain fatty acids. It is also important to mention that the fermentation of XOS depends highly on the composition of the initial microbiota of the individual (Chapla et al., 2012; Gullón, Gullon, Cardelle-Cobas et al., 2014). Finally, the validation of the prebiotic properties needs further investigations to be carried out in animals and humans to assure their health claims (Palaniappan et al., 2017).

## 3 | CONCLUSION

Although many studies have shown the prebiotic effect of oligosaccharides, there is a need for further in vivo feeding studies using animal models and clinical evaluations with human pathogens. Also, the assessment of the interaction between OS and the human gastrointestinal tract conditions such as the different pH values and enzymes is desirable. All this may be useful to allow for the production of functional foods, and the development of symbiotic formulations using prebiotics in combination with probiotic bacteria.

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## CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

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# 3. CELLULAR ANTIOXIDANT ACTIVITY AND *in vitro* INTESTINAL PERMEABILITY OF PHENOLIC COMPOUNDS FROM FOUR VARIETIES OF MANGO BARK (*Mangifera indica* L.)

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# Cellular antioxidant activity and *in vitro* intestinal permeability of phenolic compounds from four varieties of mango bark (*Mangifera indica* L.)

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## Abstract

**BACKGROUND: Mango bark is an important agro-industrial residue from mango pruning. In traditional medicine, the aqueous extract from mango bark (MBE) has been used in ethnomedicine for the treatment of many diseases. However, there is scarce information using cellular models to evaluate the potential use of this plant material for human consumption. In this study, the phenolic content from the MBE from four varieties (Kent, Keitt, Ataulfo and Tommy Atkins) was analyzed by high-performance liquid chromatography coupled to photodiode array detector (HPLC-DAD) and liquid chromatography coupled with time-of-flight mass spectrometry (LC/MS-TOF). Additionally, the cellular antioxidant activity of the MBE from the four mango varieties were compared. Finally, the intestinal permeability of the main polyphenols found in the MBE (mangiferin and gallic acid) was evaluated.**

**RESULTS: Mangiferin and gallic acid were the main constituents in the MBE from the four mango varieties. Furthermore, the Ataulfo variety showed the highest cellular antioxidant activity (67%) at the concentration of 100 μg mL$^{-1}$. The intestinal permeability of mangiferin present in the bark extracts was 3- to 4.8-fold higher than those of mangiferin as standard, whereas the intestinal permeability of gallic acid varied among the tested extracts.**

**CONCLUSION: MBE has the potential to exert antioxidant activity at the cellular level and can have an impact on human health. It may also be a good source for the extraction of polyphenols mainly mangiferin.**


**Keywords:** mango bark; mangiferin; gallic acid; intestinal permeability; cellular antioxidant activity; Caco-2 cells

## INTRODUCTION

*Mangifera indica* L. is one of the main tropical fruits produced in the world. The aqueous extract of the bark from mango (mango bark extract, MBE) has been widely used for several years in ethnomedicine to treat pathological conditions including diabetes, anemia and diarrhea.[1] The MBE contain a high concentration of phenolic compounds including phenolic acids, xanthones, flavonoids, benzophenones and gallotannins. Furthermore, marked inter-varietal differences in the quantitative composition of these compounds have been observed.[2,3]

The existence of phenolic constituents in the human diet is correlated with protective effects against some chronic-degenerative diseases related to oxidative stress. Some reports show the biological activities of the MBE and its main phenolic constituents such antioxidant activity.[2] Likewise, mangiferin (MG), a polyphenol of C-glycosylxanthone structure has been reported to possess pharmacological properties.[4] Nonetheless, the extent to which polyphenol-rich extracts are effective in the human body depends on their bioavailability and metabolism *in vivo*.[5] In this regard, it has been stated that intestinal permeability is a key factor that impacts the bioavailability of drugs. Hence, to take advantage of phenolic compounds it is necessary to examine their intestinal permeability and toxicity to understand their potential bioactivity. Furthermore, the plant matrix and the other components present in the extracts can modify the pharmacokinetics of the active components.[6] Since MBE is rich in polyphenol compounds, studying its intestinal permeability is important to find new applications for this extract. The aim of this study was to compare the

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antioxidant potential of MBE from four varieties of mango (Tommy Atkins, Ataulfo, Keitt and Kent) and make a judgment about their intestinal permeability using the Caco-2 human colon cell line as a model of human intestinal absorption.

## MATERIALS AND METHODS

### Chemical supplies

MG, epicatechin, catechin and gallic acid (GA) standards, lucifer yellow, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2,2′-azino-bis (3-ethylb enzothiazoline-6-sulphonic acid) (ABTS), 4-dimethylaminocinnam aldehyde (DMCA) Trolox, fluorescein, water, formic acid, acetonitrile, HPLC-grade methanol, and Folin–Ciocalteu's phenol reagent, were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM-F12) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Phosphate buffered saline (PBS) solution pH 7.4 (1x), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), trypsin–EDTA 0.25% w/v, penicillin (10 000 Unit $mL^{-1}$), and streptomycin (10 000 µg $mL^{-1}$) were obtained from GIBCO (Grand Island, NY, USA). The CellTiter 96 ® Aqueous One Solution Cell Proliferation Assay was acquired from Promega Corporation (Madison, WI, USA).

### Plant material

The bark of four varieties (Ataulfo, Tommy Atkins, Keitt and Kent) of mango was collected in September 2016 in the region of El Rosario (Tommy Atkins, Keitt and Kent, 22° 55′ 22″ north, 105° 50′ 14″ west; Ataulfo, 22° 53′ 34″ north, 105° 49′ 13″ west), Sinaloa, México. The barks were chopped and oven-dried at 40 °C for 24 h before milling on an IKA M20 universal mill (Wilmington, NC, USA). After that, the samples were sieved (0.297-mm mesh), and stored at −20 °C until use.

### Preparation of *Mangifera indica* L. bark extracts

The samples (1 g) were extracted as previously described[7] using distilled water (40 mL) at 75 °C in a heating bath (B-491, Büchi, Switzerland) for 45 min. The solution was centrifuged (11 180×*g*, 10 min) with a Thermo Scientific Legend XTR Centrifuge (Thermo Fisher Scientific). The supernatant was lyophilized using a freeze dryer and stored at −20 °C until use. The weight was registered to obtain the extraction yield.

### Total flavonoid content (TFC)

An aluminum chloride ($AlCl_3$) colorimetric method reported previously[8,9] with modifications was used. Briefly, 30 µL, of each MBE was mixed with 250 µL of distilled water, 10 µL of $AlCl_3$ (10%) and 10 µL of 1 M potassium acetate in a 96-well plate. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm using a microplate reader (Synergy HT, Bio-Tek, Winooski, VT, USA). The total flavonoid content (TFC) was calculated as quercetin equivalents (QE) based on a calibration curve from 0 to 400 µg $mL^{-1}$, and expressed as milligrams of quercetin equivalents per gram of dry weight (DW) of the sample (mg QE $g^{-1}$ DW).

### Total phenolic content (TPC) and tannin content

The total phenolic content (TPC) was determined according to the Folin–Ciocalteau method[10,11] with modifications. Briefly, 15 µL of the MBE was mixed with 240 µL of distilled water and 15 µL of 2 N Folin–Ciocalteau reagent. After incubation for 3 min, 30 µL of 4 N sodium carbonate ($Na_2CO_3$) was added to neutralize the reaction mixture, and the mixture was incubated in the dark for 2 h at room temperature. The absorbance was measured at 725 nm using a microplate reader (Synergy HT, Bio-Tek, Winooski, VT, USA). A standard calibration curve was prepared using catechin. TPC and tannin content in each extract were calculated and expressed as milligrams of catechin equivalents (CE) per gram of DW of the bark extract (mg CE $g^{-1}$ DW). The non-tannin phenol content was calculated as previously described.[12] The protocol consisted of precipitating tannins with polyvinyl polypyrrolidone (PVPP), which binds tannins, and the Folin–Ciocalteu method was used to determine the content of non-tannin phenols. The total tannins (TT) were calculated by subtracting the non-tannin phenol content from the total phenol content (Eqn (1)).

$$TT = \text{Phenolic compounds} - \text{Non} - \text{tannin phenols} \quad (1)$$

### Determination of condensed tannin (CT) and hydrolyzable tannin (HT)

The content of condensed tannin (CT) was estimated according to the method reported by Heil *et al.*[13] Briefly, 100 µL of each of the diluted MBE was thoroughly mixed with 1 mL of 4-dimethylaminocinnamaldehyde (DMCA) solution (0.1% DMCA in methanol–hydrochloric acid 9:1 *v/v*). The absorption at 640 nm was measured after 5 min of color development at room temperature the CT concentration was calculated and expressed as milligrams of CE per gram of DW of the sample (mg CE $g^{-1}$ DW). The hydrolyzable tannin (HT) were calculated by subtracting the CT from the TT and expressed as milligrams of CE per gram of DW of the sample (mg CE $g^{-1}$ DW) (Eqn (2)). All measurements were performed in triplicate.

$$HT = TT - CT \quad (2)$$

### Quantification of phenolic compounds by high-performance liquid chromatography coupled to photodiode array detector (HPLC-DAD)

Lyophilized bark powders were dissolved in methanol and used to quantify the phenolic compounds. Briefly, 20 µL of each of the extracts was separated by high-performance liquid chromatography coupled to photodiode array detector (HPLC-DAD) (Agilent 1100 Santa Clara, CA, USA) using a Zorbax Eclipse XDB-C18, 4.6 mm ID × 150 mm (5 µm) reverse column. Gradient elution was conducted with (A) water and 0.1% formic acid and (B) acetonitrile at a flow rate of 0.5 mL $min^{-1}$. The gradient used was as follows: 0 → 8 min, 18% B; 8 → 16 min, 22% B; 16 → 30 min, 35% B; and 30 → 35 min, 100% B. Chromatograms were acquired at 280 nm for phenolic acids and at 320 and 365 nm for MG derivatives and integrated by HP-Agilent Software (Chemstation for LC, Agilent Technologies, Santa Clara, CA, USA, 1990–2003). All compounds were quantified using the following standard curves: MG standard curve from 6.25 to 200 µg $mL^{-1}$, GA standard curve from 2 to 200 µg $mL^{-1}$ and epicatechin standard curve from 6.25 to 100 µg $mL^{-1}$. Results were expressed as micrograms of MG equivalents (µg MG $mg^{-1}$), micrograms of GA equivalents (µg GAE $mg^{-1}$); or epicatechin equivalents (µg ECE $mg^{-1}$) per milligram of dry extract.

### Liquid chromatography coupled with time-of-flight mass spectrometry (LC/MS-TOF)

Tentative identifications of the phenolic compounds in MBE from different varieties were made by comparing their UV spectra

  

and characteristic ions *m/z* with those previously reported in the literature.[3] Mass spectrometric analyses were performed by liquid chromatography coupled with time-of-flight mass spectrometry (LC/MS-TOF) (Agilent Technologies) according to the chromatographic conditions described earlier and using the Analyst QS 1.1 software (Applied Biosystems, Carlsbad, CA, USA). Mass spectra were collected using an electrospray ionisation source in negative mode (ESI−) under the following conditions: *m/z* range, 150–1500; nitrogen gas; gas temperature, 300 °C; drying gas flow rate, 13 L $min^{-1}$; nebulizer pressure, 40 psig; capillary voltage, 4000 V; and fragmentor voltage, 40 V.

### Antioxidant capacity

The free radical scavenging activity of the extracts was measured using the stable DPPH· radical as previously described.[14] A standard calibration curve of Trolox was used, and the results were expressed as millimole of Trolox equivalents (TE) per gram of DW (mmol TE $g^{-1}$ DW). An oxygen radical absorbance capacity (ORAC) assay was performed by the method previously described[15] and the ORAC values were expressed as micromole of Trolox equivalents (TE) per gram of DW (μmol TE $g^{-1}$ DW). The $ABTS^{+}$ radical scavenging activity was measured according to a method reported earlier.[9] The values were expressed as micromole TE per 100 g of DW (μmol TE $g^{-1}$ DW).

### Cellular antioxidant activity (CAA) assay

Before performing the cellular antioxidant activity (CAA) and permeability assays, the cytotoxicity of MBE from different varieties (at a concentration of 100 μg $mL^{-1}$) on Caco-2 culture (passage number 37) was tested with a CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), to ensure that the viability of the cells was over 90%. To assess CAA, the method reported earlier using the Caco-2 cell line was used.[11] The Caco-2 cell line was obtained from ATCC (American Tissue Culture Collection, Rockville, MD, USA). Briefly, MBE was tested at different concentrations (12.5, 25, 50 and 100 μg $mL^{-1}$). A day before the experiment, Caco-2 cells were cultured in a black-walled, clear-bottomed 96-well microplate (Costar, Corning, NY, USA) at a density of $5 \times 10^5$ $mL^{-1}$. After 24 h, the medium was removed, and cells were washed with 100 μL of PBS solution. Subsequently, cells were treated with 100 μL of the MBE containing DCFH-DA (60 μM), and the cells were then incubated at 37 °C for 20 min. Following incubation, the treatment solutions were removed and the cells were washed twice with PBS solution. Finally, 100 μL of 500 μM AAPH solution was added to each well, except for the blank and negative control wells. Fluorescence emitted at 538 nm with excitation at 485 nm was measured every 2 min for 90 min at 37 °C. The CAA values were calculated using Eqn (3).

$$(\%)\ \mathrm{CAA} = 1 - \left( \int \mathrm{SA} / \int \mathrm{CA} \right) \quad (3)$$

where $\int$ SA is the integrated area under the sample fluorescence versus time curve and $\int$ CA is the integrated area of the control curve.

### Intestinal permeability assay

Caco-2 cells were cultured in DMEM-F12 supplemented with 10% FBS and maintained at 37 °C in 5% carbon dioxide ($CO_2$). Caco-2 cells (passage number 37) were seeded onto 6-well transwell plates containing inserts with a polycarbonate membrane with a surface area of 4.71 $cm^2$ and a pore diameter of 0.4 μm (Costar) at a final seeding density of $1 \times 10^6$ cells/insert, and the plates were cultured for 21 days. Permeability assays were performed according to a previous report.[16] Briefly, the Caco-2 cell monolayers were washed with warm HBSS at pH 7.4, and then MBEs from different varieties, MG or GA were added to the apical (AP) side (AP: 1.5 mL), while the receiving compartment or basolateral (BL) side (BL: 2.5 mL) contained the corresponding volume of HBSS. The plates were incubated at 37 °C in an orbital shaking incubator (100 rpm), and aliquots of 1 mL were withdrawn from both compartments at 15, 30, 60 and 120 min. The samples were analyzed by HPLC. All experiments were performed in triplicate. The apparent permeability coefficient (Papp) was calculated from the cumulative amount of permeated cells versus the time profile according to Eqn (4).

$$\mathrm{Papp} = (dQ/dt) \times \left( V / A \times C_0 \right) \quad (4)$$

where $dQ/dt$ is the change in drug concentration in the receiver solution (in μM $s^{-1}$), $V$ represents the volume of the solution in the receiving compartment (in milliliters), $A$ denotes the membrane surface area (in $cm^2$), and $C_0$ is the initial concentration in the donor solution in the BL compartment (in μM).

Integrity of cell monolayers was assessed by monitoring the lucifer yellow (LY) rejection (Fig. 3(c)), a paracellular marker across a cell monolayer, according to the report by Desrosiers and Weathers.[17] The percentage of LY rejection value was calculated according to Eqn (5).

$$\text{LY rejection } (\%) = 100 \left( 1 - \mathrm{RFU_{BL}} / \mathrm{RFU_{AP}} \right) \quad (5)$$

where RFU is the relative fluorescent units (excitation 485 nm; emission 530 nm) measured using a microplate reader (Synergy HT, BioTek, Winooski, VM, USA). Membranes displaying a LY rejection (%) value below 95% were excluded. The LY concentration was calculated on the basis of a standard curve (1.0–100 μM).

### Statistical analysis

The statistical analysis was carried out using JMP 13.0 software (SAS Institute Inc., Cary, NC, USA). Experiments were performed at least in triplicate. Analysis of variance (ANOVA) was performed followed by Tukey's HSD (honest significant difference) tests ($P$ values $< 0.05$ were considered statistically significant).

## RESULTS AND DISCUSSION

### Total phenolic content (TPC)

The TPC, TFC and TT of four varieties of MBE are presented in Table 1. The TFC was lower than the values reported for MBE from varieties grown in Pakistan (56.87–65.45 mg CE $g^{-1}$ DW).[2] In this study, it was found that the range of flavonoids was from 11.3 to 20.5 mg QE $g^{-1}$ DW. MBE from Cuba contains 226.2 mg of catechin, a flavan-3-ol,[1] on the contrary, another study did not report any flavonoid in MBE from Brazilian cultivars.[3] The highest concentration of TFC was observed in the Tommy Atkins variety, and it was different from that of the other varieties ($P < 0.05$). Differences in the TPC among varieties were expected and have been reported in the literature; the reasons for these variations may be the genotype of the plant, which influences the accumulation and structure of phenolic compounds.[18] The TPC was measured by a Folin–Ciocalteu assay, and the results indicated differences among the studied varieties (30.50–232.50 mg CE $g^{-1}$ DW) ($P < 0.05$); Keitt variety had the highest TPC (232.50 mg CE $g^{-1}$ DW), which was

  

**Table 2.** Tentative identification of phenolic compounds determined by HPLC-TOF in extracts from the bark of *Mangifera indica* L. varieties

| Peak | Retention time (min) | Molecular formula | Tentative assignment | $\lambda_{max}$ (nm) | $[M-H]^-$ (m/z) | Accurate mass | Variety | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | KT | KN | A | TA | |
| 1 | 2.9 | $C_{38}H_{34}O_{22}$ | Gallic acid derivative | 256 316 369 | 841 | 842.06 | X | x | x | x | 20 |
| 2 | 3.9 | $C_{14}H_{16}O_{10}$ | 3-Galloyl quinic acid | 273 | 343 | 344.04 | X | | | | 21,22 |
| 3 | 4.4 | $C_7H_6O_5$ | Gallic acid | 270 | 169 | 170.00 | X | x | x | x | 21 |
| 4 | 8.7 | $C_{19}H_{19}O_{10}$ | Iriflophenone-3-*C*-glucoside | 295 | 407 | 408.06 | X | x | | x | 4,23 |
| 5 | 11.4 | $C_{30}H_{23}O_{11}$ | Epiafzelechin-epicatechin dimer | 296 | 559 | 560.05 | X | x | | x | 24 |
| 6 | 11.7 | $C_{19}H_{18}O_{11}$ | Mangiferin | 256 316 369 | 421 | 422.04 | X | x | x | x | 4 |
| 7 | 11.7 | $C_{19}H_{18}O_{11}$ | Isomangiferin | 256 316 369 | 421 | 422.04 | X | | | | 25 |
| 8 | 13.0 | $C_{20}H_{19}O_{11}$ | Homomangiferin | 256 316 369 | 435 | 436.05 | X | x | x | x | 4,24,26 |
| 9 | 17.5 | $C_{26}H_{24}O_{12}$ | Iriflophenone 3-*C*-(2-*O*-galloyl)-*β*-D-glucoside | 266 | 527 | 528.07 | | | | x | 4 |
| 10 | 22.7 | $C_8H_8O_5$ | Methyl gallate | 265 | 183 | 184.06 | | x | | x | 27 |

Note: KT, Keitt; KN, Kent; A, Ataulfo; TA, Tommy Atkins.

($\lambda_{max}$ 265), and it was identified as methyl gallate according to a previous report.[27]

The concentrations of the phenolic constituents in the MBE are shown in Table 3. MBE from the Keitt variety presented the highest concentration of phenolic compounds (169.65 μg mg$^{-1}$ DW), followed by Tommy Atkins (56.73 μg mg$^{-1}$ DW), Ataulfo (43.41 μg mg$^{-1}$ DW) and Kent (40.89 μg mg$^{-1}$ DW). MG, a polyphenolic xanthone, was the most abundant compound in almost all extracts, followed by the GA derivative. The concentration of MG was significantly different among the mango varieties, and its amount was approximately 2- to 6-fold higher in the Keitt variety than it was in the others. Barreto *et al.*[3] reported differences in the concentration of MG in the bark of 16 Brazilian varieties, with values ranging from 4.7 to 107.18 μg mg$^{-1}$. Núñez Sellés *et al.*[1] reported 71.40 μg mg$^{-1}$ of MG in the aqueous extract from MBE grown in Cuba. Furthermore, this molecule is present in greater quantities in the bark than in other tissues of the mango tree; therefore, mango bark is a good source of MG.[4] By contrast, the concentration of the GA derivative was significantly different among the varieties, and Keitt had the highest value (56.14 μg mg$^{-1}$ DW). Additionally, GA was found to be abundant in all varieties (from 2.10 to 10.93 μg mg$^{-1}$ DW). These results are in accordance with the data reported earlier[1] that reported *M. indica* L. cultivars grown in Cuba contained 2.8 μg mg$^{-1}$ DW of GA, and Barreto *et al.*[3] reported 0.24 μg mg$^{-1}$ dry material of GA in methanol extracts from the bark of mango cultivar Van Dyke from Brazil. The concentration of iriflophenone-3-*C*-glucoside was significantly different among the varieties, and it was found at concentrations ranging from not detected to 6.81 μg mg$^{-1}$ DW, with the Keitt variety showing the highest value. In the present work, the concentration of iriflophenone-3-*C*-glucoside was higher than those reported in other studies.[3]

### Chemical antioxidant capacity

Chemical assays were conducted to measure the antioxidant capacity based on different mechanisms. The ORAC assay is based on hydrogen atom transfer (HAT) and measures the ability of phenolic compounds (antioxidants) to inhibit the decline in fluorescence induced by a peroxyl radical, whereas ABTS and DPPH assays measure the ability to scavenge the cationic radical $ABTS^{+}$ and the DPPH radical, respectively. Both methods are classified by some authors as mixed-mode (having both electron transfer and HAT mechanisms).[29] Table 4 shows the antioxidant capacity of MBE from different varieties in terms of ABTS, DPPH and ORAC values. The ABTS scavenging capacity of MBE from Tommy Atkins variety was significantly higher ($P < 0.05$), being from 2.3 to 3.2 times more powerful than that of the other varieties. Joo *et al.*[30] reported an ABTS value of 0.1773 μmol TE g$^{-1}$ in the crude hot water extract from *Ulmus pumila* stem bark. In the present work, the ABTS value was similar to those reported by the earlier authors (0.116 μmol TE g$^{-1}$ DW). To date, no comparative reports have been found regarding analysis of the antioxidant capacity of MBE by means of the ABTS method. In the DPPH assay, Tommy Atkins showed the higher antioxidant capacity, and the results were in the range from 289.36 to 882.55 mmol TE g$^{-1}$ DW. In the ORAC assay, Tommy Atkins produced the highest value of 1110.76 μmol TE g$^{-1}$ DW, followed by Keitt, Kent and Ataulfo, with values of 866.45, 383.02 and 372.50 μmol TE g$^{-1}$ DW, respectively. The antioxidant capacity of MBE could be related to its flavonoid content, as determined spectrophotometrically. Additionally, the phenolic content evaluated by HPLC showed the presence of potent antioxidant molecules such as MG and GA. The MG concentration was high in the Keitt variety (Table 3); however, this extract did not show the highest antioxidant capacity. This may have been due to the hydroxyl groups of MG, which have been reported to be hindered by the steric effect that made MG difficult to access to the radical site of the DPPH. Therefore, MG showed a lower antioxidant value, as measured by this chemical assay.[31] Moreover, the antioxidant potential of an extract has been related to the presence of specific phenolic structures and the synergy among all of the components present in the plant extract, which can enhance or reduce the antioxidant activity.[32] Furthermore, to obtain a better understanding of the main contributors to the antioxidant capacity of the MBE, antioxidant measurements of the purified compounds present in the extract should be evaluated.

### Cellular antioxidant activity (CAA)

Prior to running the cellular experiments, the cytotoxicity of MBE from different varieties was tested on Caco-2 cells at the maximum

  

**Table 3.** Concentration[†] of phenolic[‡] compounds in the extracts from the bark of *Mangifera indica* L. varieties[§]

| Peak | Compound | Concentration (μg mg$^{-1}$ DW) | | | |
|---|---|---|---|---|---|
| | | Variety | | | |
| | | Keitt | Kent | Ataulfo | Tommy Atkins |
| 1 | Gallic acid derivative | 56.14 ± 0.47$^{1,a}$ | 12.86 ± 0.30$^{1,b}$ | 13.44 ± 3.58$^{1,b}$ | 10.45 ± 1.68$^{1,b}$ |
| 2 | 3-Galloyl quinic acid | 3.05 ± 0.29$^{2,a}$ | *n.d.*$^{b}$ | *n.d.*$^{b}$ | *n.d.*$^{b}$ |
| 3 | Gallic acid | 10.93 ± 0.49$^{2,a}$ | 3.55 ± 0.86$^{2,b}$ | 3.79 ± 0.07$^{1,b}$ | 2.10 ± 0.02$^{2,c}$ |
| 4 | Iriflophenone-3-C-glucoside | 6.81 ± 0.33$^{1,a}$ | 3.98 ± 0.27$^{1,c}$ | *n.d.*$^{d}$ | 5.10 ± 0.07$^{1,b}$ |
| 5 | Epiafzelechin-epicatechin dimer | 13.92 ± 0.18$^{3,a}$ | 3.87 ± 0.11$^{3,b}$ | *n.d.*$^{d}$ | 3.22 ± 0.28$^{3,c}$ |
| 6 | Mangiferin | 67.50 ± 11.16$^{1,a}$ | 10.5 ± 0.75$^{1,c}$ | 21.33 ± 0.87$^{1,b}$ | 24.94 ± 1.95$^{1,b}$ |
| 7 | Isomangiferin | 3.73 ± 1.45$^{1,d}$ | *n.d.*$^{b}$ | *n.d.*$^{b}$ | *n.d.*$^{b}$ |
| 8 | Homomangiferin | 7.57 ± 0.12$^{1,a}$ | 5.37 ± 0.48$^{1,b}$ | 4.89 ± 0.19$^{1,b}$ | 5.32 ± 0.10$^{1,b}$ |
| 9 | Iriflophenone 3-C-(2-O-galloyl)-β-D-glucoside | *n.d.*$^{b}$ | *n.d.*$^{b}$ | *n.d.*$^{b}$ | 4.22 ± 0.11$^{1,a}$ |
| 10 | Methyl gallate | *n.d.*$^{c}$ | 0.76 ± 0.07$^{2,b}$ | *n.d.*$^{c}$ | 1.38 ± 0.12$^{a}$ |

[†]Microgram mangiferin equivalents (MG) per milligram of dry weight (DW).
[‡]Microgram gallic acid equivalents (GAE) per milligram of dry weight (DW).
[§]Microgram epicatechin equivalents (ECE) per milligram of dry weight (DW).
Letters in the same row show statistic difference ($P < 0.05$).
n.d., not detected.

**Table 4.** Antioxidant capacity of mango bark extracts of four varieties

| Antioxidant assay | Variety | | | |
|---|---|---|---|---|
| | Keitt | Kent | Ataulfo | Tommy Atkins |
| ABTS (μmol TE g$^{-1}$ DW) | 0.0491 ± 0.00$^{b}$ | 0.0360 ± 0.00$^{b}$ | 0.0456 ± 0.00$^{b}$ | 0.116 ± 0.01$^{a}$ |
| DPPH (mmol TE g$^{-1}$ DW) | 727.12 ± 57.00$^{b}$ | 289.36 ± 68.35$^{d}$ | 495.51 ± 70.34$^{c}$ | 882.55 ± 111.27$^{a}$ |
| ORAC (μmol TE g$^{-1}$ DW) | 866.45 ± 30.62$^{b}$ | 383.02 ± 33.16$^{c}$ | 372.50 ± 57.29$^{c}$ | 1110.76 ± 156.81$^{a}$ |

Note: μmol TE g$^{-1}$ DW, micromole Trolox equivalents per gram of dry weight; mmol TE g$^{-1}$ DW, millimole Trolox equivalents per gram of dry weight. ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH, 2,2-diphenyl-1-picryl-hydrazyl; ORAC, oxygen radical absorbance capacity.
Letters in the same row show statistic difference ($P < 0.05$).

concentration used in the experiments (100 μg mL$^{-1}$) to ensure that the viability of the cells would not be affected. All of the extracts showed a cellular viability over 90%. The CAA from the MBE of four varieties is shown in Fig. 2. Similar to MG, the extracts at different concentrations showed the ability to quench peroxyl radical-induced oxidation; furthermore, a concentration-dependent trend was shown by Kent and Ataulfo varieties ($P < 0.001$). Similar to our study, Wen *et al.*[33] reported a concentration-dependent response. Differences between this study and ours could be due to the different phenolic composition of the plant material. Among all tested concentrations, 100 μg mL$^{-1}$ showed the highest antioxidant potential. The MBE from the Ataulfo variety at the maximum concentration (100 μg mL$^{-1}$) showed the highest CAA value (67%), and the lowest CAA value was shown by the Keitt variety at a concentration of 12.5 μg mL$^{-1}$ (19.5%). Additionally, analysis of the main effects showed differences between the varieties ($P < 0.001$). Differences in the CAA value among the varieties might have been due to the permeability of the phenolic constituents of each extract.[34] Furthermore, contrary to the expected results, the Keitt variety showed a greater content of phenolic compounds, but its CAA was not significantly different from that of the other varieties. An explanation could be that this variety had the highest concentration of MG and its derivatives, which have been reported to be effluxed across the AP membrane by the action of enzymes in the cells, thereby, the CAA value could be diminished.[35] However, the major compounds present in the MBE were MG and GA, which have been reported to have high antioxidant potential.[36] GA may be responsible for the CAA values, as it has been reported to possess antioxidant activity due to its hydroxyl groups.[37] GA has been reported to possesses higher antioxidant activity than MG [half maximal effective concentration ($EC_{50}$) = 1.2 μg mL$^{-1}$ versus $EC_{50}$ = 32.9 μg mL$^{-1}$, respectively]. Additionally, in the same study a synergistic antioxidant effect was reported in the mixture of curcumin and GA, whereas the mixture of curcumin and MG did not display this synergistic effect.[31] Therefore, the antioxidant power of the MBE may be ascribed to the synergistic effects of their phenolic content with other compounds present in the extract. In the present study, the CAA values of MBE were lower than those of the standard (MG). These results are similar to the ones reported for *Garcinia lucida Vesque* and *Hymenocardia lyrata* bark extracts. These extracts showed a lower hydroxyl scavenging activity than did the standard (catechin).[38] Differences between the chemical antioxidant assays may be ascribed to the bioavailability, stability and/or mechanism of action of the phenolic compounds present in the MBE. Moreover, these differences may be due to the mechanisms of each method, as the CAA assay measures the activity of antioxidant molecules within cells. Unlike the ORAC assay, the fluorescent probe and the stressor molecule that generates the peroxyl radical passes through the cellular membrane by

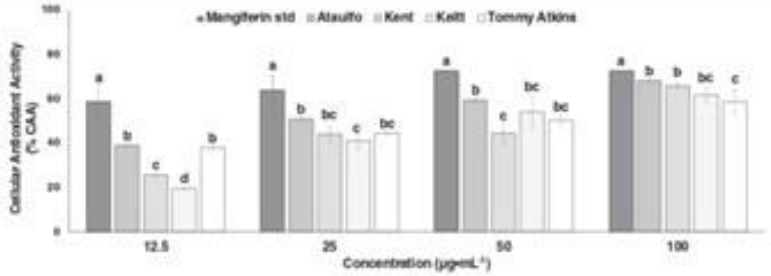


**Figure 2.** Cellular antioxidant activity (CAA) of the extracts from the bark of mango varieties: Ataulfo, Kent, Keitt and Tommy Atkins. Caco-2 cells were treated with 12.5, 25, 50 and 100 μg mL$^{-1}$ of each extract for 20 min. Values were expressed as %CAA. Different letters indicate significant differences among extracts from the same concentration ($P < 0.05$).

passive diffusion, and this probe-precursor is oxidized inside the cell. The antioxidants that pass through the membrane can prevent this oxidation, which results in an inhibition of fluorescence.[39] Our results showed that MBE has the potential to prevent intracellular reactive oxygen species accumulation and, consequently, the oxidative damage of cell components.

### Permeability experiments

The membrane permeability of MBE from different varieties was tested in Caco-2 cells. This cell model simulated transport mechanisms such as paracellular transport through tight junctions, active transport via transporters, and the efflux phenomenon induced by P-glycoproteins (P-gp).[40] The apparent permeability coefficients from the AP to BL direction ($Papp_{(AP-BL)}$) of the MG present in MBE are shown in Fig. 3(a). The values of $Papp_{(AP-BL)}$ of the MG present in MBE from Keitt, Ataulfo and Tommy Atkins varieties were 4.66, 6.64 and $7.47 \times 10^{-5}$ cm s$^{-1}$, respectively. Unlike the extracts in other varieties, MG in the bark extracts from the Kent variety did not show permeability at any of the times assayed. These results were compared to the MG standard, which showed a value of $1.54 \times 10^{-5}$ cm s$^{-1}$ (Fig. 3(a)). Interestingly, the values of $Papp_{(AP-BL)}$ of the MG present in the different extracts were 3- to 4.8-fold higher than that of the standard (Fig. 3(a)). This may be ascribable to the chemical complexity of the extracts, which may positively or negatively influence membrane permeability.[41] Additionally, previous studies have reported that the solubility in water of MG alone is poor, which results in poor intestinal membrane permeability.[42] Tian et al.[43] reported that MG had higher absorption *in vivo* after the administration of the extract from Zhi-mu compared to that of the standard. Furthermore, the MG present in Zhi-mu extract showed a maximum plasma concentration ($C_{max}$) that was 14-fold higher than the standard when it was orally administered in Wistar rats. These findings indicate that the presence of other compounds in the extract increased the absorption of MG. Furthermore, it has been reported that MG is mainly metabolized by the gut microbiota;[44] therefore, it is important to perform further studies that include the involvement of gut microbiota in the metabolism of MBE as well as *in vitro* studies of its bioavailability and metabolism.

The $Papp_{(AP-BL)}$ of the GA present in MBE is shown in Fig. 3(b). The $Papp_{(AP-BL)}$ of GA as a standard ($4.16 \times 10^{-6}$ cm s) was significantly higher than the values of Tommy Atkins and Ataulfo bark extracts, with values of $Papp_{(AP-BL)}$ of 2.19 and $1.76 \times 10^{-6}$ cm s$^{-1}$, respectively, but lower than the $Papp_{(AP-BL)}$ of the Keitt and Kent varieties (11.92 and $7.24 \times 10^{-6}$ cm s$^{-1}$, respectively). The absorption of isolated or pure compounds from the plant extracts showed differences, which has been well reported previously,

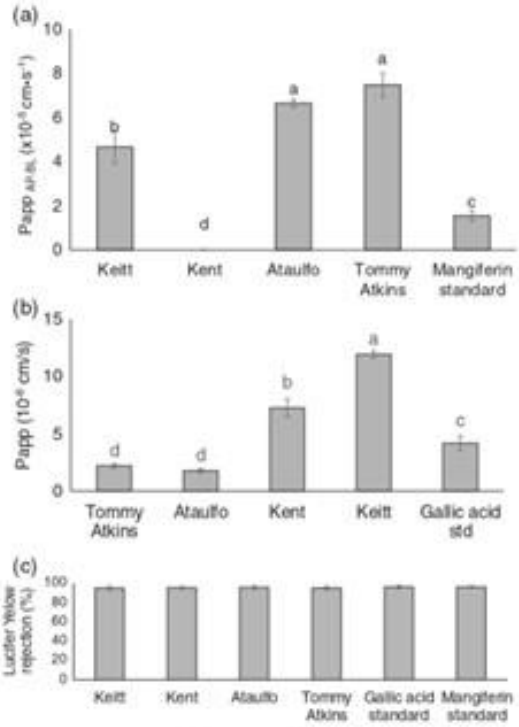


**Figure 3.** Apparent permeability (Papp) of (a) mangiferin and (b) gallic acid present in extracts from the bark of mango varieties and their respective standards from apical (AP) to basolateral (BL) compartments (AP-BL) (c) Lucifer yellow rejection (%) on the Caco-2 cells monolayers after permeability assay during 120 min with the extracts from the bark of mango varieties: Ataulfo, Kent, Keitt and Tommy Atkins, gallic acid standard and mangiferin standard at 100 μg mL$^{-1}$.

and could be attributed to some components of the extracts hindering the absorption of others across the membrane of the Caco-2 cells.[34] In contrast, the Papp of GA (5 μM) was reported to have a low-permeability value ($Papp_{(AP-BL)}$ of $1.80 \times 10^{-6}$ cm s$^{-1}$) in a Caco-2 cell model.[40] Additionally, this phenolic acid is transported across the membrane of Caco-2 cells through passive diffusion. Moreover, the same authors have reported that the degree of Papp ($\times 10^{-6}$ cm s$^{-1}$) in Caco-2 can be categorized as high-permeability (Papp >10), moderate-permeability ($5 \leq$ Papp $\leq 10$), and low-permeability (Papp <5).[40] Hence, the Papp reported

  

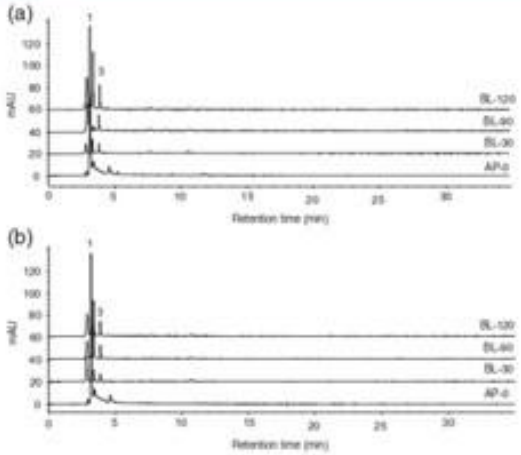


**Figure 4.** Chromatograms showing permeability experiments from mango bark extracts of (a) Keitt and (b) Kent varieties, from apical (AP) to basolateral (BL) compartments (AP-BL). Peak: 1, gallic acid derivative; Peak 3, gallic acid.

for MBE showed a moderate permeability. Chromatograms of the permeability experiments from MBE from the Keitt and Kent varieties from AP to BL compartments are shown in Fig. 4(a) and (b), respectively. Peak 1 shows the GA derivative compound with high concentrations in the AP compartment in the first 30 min. The concentration of this compound decreases over time, with an increase of the concentration of GA (Peak 3) which can be observed from 30 to 120 min. These results are similar to those reported earlier.[45] In this study, the authors observed at the same physiological conditions (pH 7.4, 37 °C) the hydrolysis of gallotannins following the release of free GA. It is worth mentioning that many studies regarding the absorption of compounds present in mango have been performed using isolated forms, which does not represent the delivery/absorption of phytochemicals from a complex food matrix such as MBE, during gastrointestinal digestion. For instance, it has been reported that the food matrix has an effect on the bioaccesibility of polyphenols, specifically GA was reported to be highly permeable from the food matrix compared to its isolation form.[6] Therefore, further studies including *in vivo* experiments and *in vitro* simulated digestion are needed to complement the understanding on the effect of MBE on the human body.

Overall, there is a limited understanding of the relationship between the intestinal absorption of phenolic compounds and their molecular properties, hence, further studies should be focused on the elucidation of the contribution of the molecular properties of phenolic compounds to the intestinal transport and the mechanisms implicated.

## CONCLUSIONS

The main phytochemicals present in MBE in the Tommy Atkins, Ataulfo, Keitt and Kent varieties were MG and a GA derivative that could not be identified. MBE presented promising antioxidant activities, and no toxicity towards the Caco-2 cell line was detected at the studied concentrations (from 12.5 to 100 μg $mL^{-1}$). The $Papp_{(AP-BL)}$ value of MG present in Tommy Atkins, Ataulfo and Keitt varieties was significantly higher than MG as standard, and in the Kent variety the $Papp_{(AP-BL)}$ value was lower than MG as standard. However, the $Papp_{(AP-BL)}$ value of GA present in Keitt and Kent varieties was significantly higher than GA as standard and in the Ataulfo and Tommy Atkins varieties, the $Papp_{(AP-BL)}$ value was lower than GA as standard. Besides the permeability that MG and GA present in MBE from the different varieties, the MBE also showed an important CAA, being slightly lower than MG as standard, which shows the potential of these compounds to permeate through biological membranes and exert an effect in them. Furthermore, the mango bark could be an excellent source of bioactive compounds since it is a renewable and an underutilized material. Further research evaluating the role of these bioactive compounds in the gut microbiota should be considered, in order to have a better understanding on the potential effect of mango bark extracts in human health.

## ACKNOWLEDGEMENTS

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# 4. *In vitro* FERMENTATION OF MANGO BARK POLYPHENOLS BY HUMAN COLONIC MICROBIOTA

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Artículo en preparación

Se enviará a la revista. Food Research International

## 4.1 Abstract

The aqueous extract of mango stem bark has been used in ethnomedicine for the treatment of gut related disorders. This plant material is rich in polyphenols which are related to the different bioactivities reported for mango stem bark, with mangiferin as a main component. Mangiferin is known to be metabolized by gut bacteria; furthermore, polyphenols have been reported to have prebiotic-like activity that could be related to the effect on the inhibition or enhancement of the growth on gut bacteria and/or the promotion of short chain fatty acids which are known by its different effects on the human health status. Therefore, the bioactivity of the aqueous extract of mango stem bark could be related to the effect of its polyphenols. In this study, the aqueous extract of mango bark was submitted to *in vitro* digestion and *in vitro* fermentation with human gut microbiota, and the effect on gut bacteria was evaluated through shotgun metagenomic sequencing. Phenolic compounds were evaluated with high performance liquid chromatography system coupled with a photodiode array detector and short chain fatty acids were evaluated through gas chromatography. Shotgun metagenomic sequencing showed a total of 1678 OTUs. At phylum level, mango bark increased the Bacteroidetes phylum compared to the standard prebiotic inulin, although, no statistical differences were detected. Besides, mango stem bark can modulate the production of short chain fatty acids, and these results are comparable to inulin. In conclusion, the aqueous extract of mango stem bark showed a positive effect on the growth of Bacteroidetes, although, no statistical differences were shown comparing to the commercial prebiotic (inulin), results regarding the concentration of SCFA were similar, which could be related to the mechanism of action for the potential effect of mango bark.

**Keywords**

*Gut microbiota, short chain fatty acids, in vitro digestion, in vitro fermentation, mango, mangiferin, phenolic compounds*

## 4.2. Introduction

In the colon resides the largest bacteria accumulation with up to $10^{14}$ bacterial cells (Sender *et al*., 2016). Many studies have reported the importance of colonic microbiota for the maintenance of the human health through many ways; for example, defense against pathogens, the production of short chain fatty acids, and generation of metabolites from the non-digested food components (Li *et al*., 2019). The composition of gut bacteria can be modified by several factors such age, mode of birth, antibiotics, lifestyle and diet. Every year, studies regarding the relation between diet, gut microbiota and human health increase, and some studies suggest the use of bioactive constituents of foods as a novel therapeutic approach to modulate the gut microbiota.

Polyphenols are bioactive compounds, present in the diet with many health promoting properties. These molecules have a bi-directional relationship with the colonic bacteria in the colon, since they serve as a substrate for the bacteria and help to proliferate some bacterial groups, and in turn, bacteria generate metabolites from polyphenols which can be more bioactive than the parental molecule (Cardona *et al*., 2013; Ozdal *et al.*, 2016). Therefore, polyphenol rich extracts could modulate the gut microbiota composition.

The aqueous extract from the mango stem bark has been used for several years in traditional medicine for the treatment of diabetes, anemia and gastric disorders (Rymbai, Srivastav, Sharma, Patel, & Singh, 2013). The mango stem bark is rich in phenolic acids, gallotannins, benzophenones, flavonoids and xanthones. The most abundant polyphenol in mango stem bark is mangiferin. Scientific reports have stated that mango stem bark and its main bioactive (mangiferin) possesses antioxidant activity in Caco-2 cells, anti-inflammatory activity, and antibacterial activity against gram negative (*Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa*) and gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) bacteria (Dou *et al.*, 2014; Ediriweera *et al*., 2016; Singh *et al*., 2015; Vazquez-Olivo *et al.*, 2019). Furthermore, the mango stem bark has shown no cytotoxic effects up to 1000 µg/mL (Rodeiro *et al*., 2007).

On the other hand, mangiferin has shown a bioavailability of 1.2% giving orally to rats and generally tolerates both acid and enzymatic hydrolysis. Besides, mangiferin its transformed into its aglycone norathyriol by human intestinal bacteria (Han *et al*., 2010; Sanugul *et al.*, 2005). Also, gallotannins have shown to be metabolized by the bacteria resident in the colon (Kawabata *et al*.,

2019). Therefore, we hypothesize that mango stem bark could have a potential effect on the bacterial communities of the colon. The aim of this study was to assess the potential impact of digested and undigested mango stem bark on the growth of bacterial groups as well as the short chain fatty acids formation by means of an *in vitro* fermentation approach with human feces.

## 4.3 Materials and Methods

### 4.3.1. Chemicals and Reagents

All reagents were HPLC-grade. Mangiferin and gallic acid standards were purchased from Sigma-Aldrich (St Louis, MO, USA). Volatile free fatty acid standard mix (CRM46975, Supelco) was purchased in Sigma-Aldrich (St Louis, MO, USA).

Plant material and sample preparation. Mango bark (cv. Keitt) was submitted to aqueous extraction as previously reported (Vazquez-Olivo *et al.*, 2019). Briefly, 1 g of mango bark was mixed with 40 mL of distilled water at 75 °C during 45 min. After that, the mango bark extract was centrifuged at 11180 *xg*, 10 min, and the supernatant was lyophilized. The dry sample was mixed in distilled water to make a solution of mango bark extract of 50 mg/mL.

### 4.3.2. *In vitro* Digestion

*In vitro* digestions were performed according to the previously reported method with modifications (Flores, Singh, Kerr, Pegg, & Kong, 2014). The solutions used for oral, gastric and intestinal phases are shown in Table 1. The samples submitted to *in vitro* digestion were: mango bark extract (50 mg/mL) and inulin (50 mg/mL) as a control. The experiment was performed in constant agitation in a shaker at 200 rpm, 37 °C. For the oral phase, 5 mL of each sample was mixed with 3.75 mL of the saliva juice during 5 min. Then, 5 mL of gastric juices was added to the mixture and after 1

h, 8.4 mL of the intestinal juices was added. This mixture was allowed to rest during 2 h. The pH was adjusted before each gastric phase was added. From each digestion phase (oral, gastric and intestinal), aliquots of 1.5 mL were taken and stored at -80°C until use. The intestinal fraction (IF) was lyophilized and the dry sample was used for the *in vitro* fermentation experiments. All assays were performed by triplicate.
Table 1.

### 4.3.3. *In vitro* Fermentation with Human Feces

The *in vitro* fermentation procedure was adapted from the method previously reported by (Gao *et al.*, 2019; Pérez-Burillo *et al.*, 2019). For the experiment, a pool of fresh fecal samples from three adult healthy donors (25-30 years old) was used. From this pool, 24 g were taken and thoroughly homogenized with 75 mL of phosphate buffer (pH 6.5). After that, the nutritive media brain-heart infusion was prepared. Then, 50 mg of each the IF samples and the undigested samples (50 mg of lyophilized mango bark and inulin), were added to centrifuge tubes, then, 7.5 mL of the nutritive media (brain-heart infusion) and 2 mL of the fecal slurry were added to each tube. In the same experiment, negative controls with fecal slurry and nutritive media without samples, and samples without fecal slurry were also included. The centrifuge tubes were incubated at 37 °C in a $CO_2$ incubator during 48 h. Aliquots of 1.5 mL from each sample were collected at 0, 2, 8, and 24 h. All samples were analyzed by triplicate and stored at – 20 °C for further analysis.

### 4.3.4. Chromatographic Analysis

Quantification of phenolic compounds present in the samples submitted to *in vitro* digestion and *in vitro* fermentation was performed as previously described (Vazquez-Olivo *et al.*, 2019). By using a high performance liquid chromatography system coupled with a photodiode array detector (Agilent 110 Santa Clara, CA, USA). The method used a Zorbax Eclipse XDB-C18, 4.6 mm ID x

150 mm (5 µm) reverse column. For the analysis, the eluent A was water/formic acid (90:10, v/v) and eluent B was acetonitrile. The gradient used was the following: 0-8, 18% B; 8-16 min, 22% B; 16-30 min, 35% B; and 30-35 min, 100% B. The flow rate used was 0.5 mL/min, and an injection volume of 20 µL. Chromatographs were acquired at 280, 320 and 365 nm. The standards used for quantification were mangiferin, gallic acid and epicatechin. The results were reported as micrograms of mangiferin, gallic acid or epicatechin equivalents per milliliter of sample. For the identification, liquid chromatography coupled with time-of-flight mass spectrometry (LC/MS-TOF, Agilent, Santa Clara, CA) was used. The analysis were done in negative mode with the following parameters: m/z range, 150-1500; nitrogen gas, gas temperature, 300 °C; drying gas flow rate, 13 L/min; nebulizer pressure, 40 psig; fragmentor voltage, 40 V and capillary voltage, 4000 V. The mass spectrometric analysis was performed using Analyst QS 1.1 software (Applied Biosystems, Carlsbad, CA).

#### 4.3.5. Short Chain Fatty Acids Analysis by Gas Chromatography (GC)

The samples from *in vitro* fermentation were centrifuged at 3000 rpm, 10 min. Supernatant was filtered through 0.2 µm filter. 1 mL of each sample was injected into a gas chromatograph Agilent Technologies, 6850 GC System (Santa Clara, CA, USA), equipped with a flame ionization detector. The column was from Agilent with dimensions of 24.1m length, 320 µm diameter, 0.50 µm thickness. Oven temperature was 240 °C; detector temperature was 300 °C; injector temperature was 240 °C; carrier gas was helium; split ratio was 5:1; injection volume was 1.0 µL. The column flow rate was 8.7 mL/min.

#### 4.3.6. Shotgun Metagenomic Analysis

Genomic DNA was extracted from the 10 fecal samples by using the cetyl trimethylammonium bromide (CTAB) method, as previously reported (J. Li *et al*., 2019). The genomic DNA was

prepared for Illumina pair-end sequencing using the Illumina Miniseq (Illumina, Inc., San Diego, CA) NexteraXT1® Guide following the protocol recommended for Illumina: (http://support.illumina.com/downloads/ nextera_xt_sample_preparation_guide_15031942.html). Concentration of DNA was quantified in a Qubit® dsDNA HS Assay Kit, and an equimolar pooled was prepared to be sequenced in a Illimuna Miniseq in the following conditions of 300 cycles, 2x150 pair-end using a standard flow cell.

### 4.3.7. Bioinformatics Analysis

Raw samples were cleaned in order to remove low quality sequences and short reads as previously reported. FASTQ files were processed with the script multiple_fastq_cleaner usng pair-end sequences. This script removes the first 15 bases of every pair-end file, which usually are adaptors, and removes sequences below Q30, and finally it creates a folder with the clean sequences named as clean_fastq (Soriano *et al*., 2018). The assembly was performed using SPAdes assembler, and the taxonomic classification was performed in Kaiju (Menzel *et al*., 2016), using the Progenome database, and the clustering of the sequences with >97% similarity was performed with vsearch v2.5.0. Operational Taxonomic Units (OTUs) and relative abundance were obtained using mg_classifier v.1.7.0 (https://github.com/GenomicaMicrob/mg_classifier), sequences were clustered to 97% similarity with vsearch v2.7.1 and compared against SILVA database. Graphics were done with R software version 4.0.

### 4.3.8. Statistical Analysis

Experiments were performed at least in triplicate and were analyzed with Minitab 17. Analysis of variance was performed followed by Tukey's HSD test were $p < 0.05$ was statistically significant.

## 4.4. Results and Discussion

The analysis of the proximal composition of the aqueous extract of mango stem bark is shown in Table 2. The extract was composed of 31% crude fiber, 2% protein, 7 ash, and 1.6 % fat. The composition of phenolic compounds was previously reported by Vazquez-Olivo *et al.* (2019). Mango bark was submitted to *in vitro* digestion and fermentation with human gut microbiota and compared with the control which consisted of the inoculum without prebiotic and a commercial prebiotic (inulin) summited to the same conditions. Digested and undigested samples were assessed for its effect on the structure of human gut microbiota. A total of 11,247,262 pair-end sequences were obtained from shotgun metagenomic sequencing, with a quality of Q 36.2. After quality filtering of raw data, a total of 11,115,070 reads were obtained with a minimum sequence length of 20 bases and the maximum of 136 bases and the average of every read was of 555,754.

The evaluation of bacteria was performed extracting the 16 S rRNA amplicon and the sequences were clustered in 1687 Operational Taxonomic Units (OTUs). Rarefaction curves are plot in order to assess the distribution of bacteria. Rarefaction curves didn't reached to the asymptote, however, it can be observed that curves tended to reach to the asymptote which may indicate that the number of sequences for the analyses was appropriate. A rarefaction curve obtained from the sequencing data of all the samples are shown in Figure 1.

Changes on gut microbiota were detected at phylum, family, genus and species level. In this study, the core phyla detected were: Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes (Table 4). These phyla are important to human health. Firmicutes and Bacteroidetes are the most abundant phyla in the human gut. In this study, Firmicutes more abundant than Bacteroidetes, and their relative abundance in the control with inoculum at 24 h (HP24) was of 18.1 and 4.3% respectively (Table 4). In this sense, mango bark digested (CDH24) showed a reduction on Bacteroidetes compared to the standard prebiotic inulin (IDH24 and IH24). A higher proportion of Bacteroidetes is related to the increase of metabolic diseases (Kasai *et al.*, 2015). However, mango bark undigested (CMH24) slightly increased the relative abundance of Firmicutes and Bacteroidetes compared to the control HP24, but, the increase was statistically insignificant. Overall, at phylum level, there were no statistical differences regarding mango bark and control.

At family level, the heat map shows the abundance of the main families according to the OTUS generated (Figure 2). In the Table 5, it is shown the relative abundance (%) of the most important families for the human health found in this study. For instance, Prevotellaceae and *Bacteroidaceae* were the two families representatives of the Bacteroidetes phyla, and *Lachnospiraceae, Ruminococcaceae, Streptococcaceae, Veillonellaceae, Lactobacillaceae* were the representative families of the Firmicutes phyla, and for *Proteobacteria*, *Enterobacteriaceae* was the most abundant family. Compared to the control (HP24), CMH24 increased the relative abundance of *Prevotellaceae*. Also, this treatment, reduced the *Enterobacteriaceae* family, from 34% to 30.4 %. At genus level, *Prevotella*, *Faecalibacterium*, *Bacteroides*, *Escherichia* and *Sutterella* were the most abundant genus. *Prevotella* was reduced from 0 h to 24 h in all treatments. At 24 h, all treatments showed a higher percentage of *Prevotella* genus compared to the control (HP24) being higher in CMH24 and IH24. *Prevotella* predominant profile in the gut is associated with rapid gut transit (Vandeputte *et al.*, 2016). Besides, *Prevotella* spp. has been found to be predominant over *Bacteroides* spp. since they compete for fiber substrates (Chung *et al.*, 2020). In a study, *Prevotella* spp. was associated with weight loss in overweight individuals (Hjorth *et al.*, 2019). *Prevotella* is known to be one of the main producers of short chain fatty acids, specifically acetate producer (Ohira, Tsutsui, & Fujioka, 2017). Also, there was no effect on the abundance of *Faecalibacterium* genus. *Bacteroides* was slightly increased in CMH24 compared to the control HP24. *Bacteroides* and *Prevotella* also showed beneficial effects for weight loss. A high fat diet reduced the phyla Bacteroidetes and its genus *Bacteroides* and *Prevotella*, however, Chokeberry polyphenols changed these trends in rats. Therefore, mango bark may have anti-obesity effect through the modulation of *Bacteroides* and *Prevotella* genus (Zhu *et al.*, 2020)

On the other hand, *in vitro* and *in vivo* studies regarding polyphenols effects on *Bacteroides* spp. have shown mixed results. For instance, *in vitro* supplementation of polyphenols showed to increase the abundance of *Bacteroides*, however, *in vivo* studies showed no influence on the abundance of Bacteroides at doses lower than 540 mg/d or higher than 750 mg/d, but increased the abundance of Bacteroides at doses of 554 and 593 mg/d (Ma & Chen, 2020).

There was no significant effect on the abundance of *Escherichia*, however, CMH24 and digested inulin at 24 h (IDH24) slightly reduced its abundance compared to the control (HP24). There was a significantly reduction on the *Sutterella* genus, in CMH24, digested mango bark (CDH24) and IDH24.

Regarding species level, *Sutterella_wadsworthensis* was present only at 24h. Besides, it was reduced in all treatments compared to the control HP24. Also, there was no effect on *Escherichia_coli*. Furthermore, there was a slightly increasing on *Faecalibacterium_prauznitzii* compared to the control HP24 for CDH24 and CMH24. For *Prevotella_corpi* species, all the treatments reduced its abundance from 0h to 24, however, CDH24, CMH24 and IDH24 showed the greatest reduction compared to undigested inulin (IH24).

Regarding the effect of mango bark extract on short chain fatty acids (SCFA) production, SCFA production by inulin, mango bark, and mangiferin is shown in Figure 3. As the most important SCFA are butyric, propionic and acetic acids, this three SCFA were produced at all fermentation times, and were analyzed for statistical differences among inulin, mango bark and mangiferin. Butyric acid production was higher in inulin at 24 h. For propionic acid, mangiferin and inulin at 24 h showed the highest production; furthermore, Tukey test showed no statistical difference with inulin, mangiferin and mango bark at 24 h. Likewise, inulin, mango bark and mangiferin at 24 h showed no statistical difference for the production of acetic acid, being the highest values of this SCFA.

## 4.5. Conclusions

Mango stem bark showed to increase the content of Bacteroidetes compared to inulin, however, no statistical differences were shown. Besides, mango bark was compared to inulin in the production of short chain fatty acids after *in vitro* fermentation with human gut microbiota, and the results were comparable, showing the potential effect of mango bark compared to a commercial prebiotic.

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**Table 1. Composition of salival, gastric, intestinal, and bile juices.**

| | Saliva juice | Gastric juice | Intestinal juice | Bile juice |
|---|---|---|---|---|
| Stock solution components | 100 ml deionized water<br>11.7 mg NaCl<br>14.9 mg KCl<br>210 mg $NaHCO_3$<br>40 mg Urea | 100 ml deionized water<br>550 mg NaCl<br>164.8 mg KCl<br>53.2 mg $NaH_2PO_4$<br>79.8 mg $CaCl_2 2H_2O$<br>61.2 mg $NH_4Cl$<br>17 mg urea<br>1.3 ml concentrated HCl | 100 ml deionized water<br>1.402 g NaCl<br>112.8 mg KCl<br>677.6 mg $NaHCO_3$<br>16 mg $KH_2PO_4$<br>10 mg $MgCl_2$<br>20 mg urea<br>36 ml concentrated HCl | 100 ml deionized water<br>1.05 g NaCl<br>75.2 mg KCl<br>1.157 g $NaHCO_3$<br>50 mg urea<br>0.03 ml concentrated HCl |
| Add to the mixture | 100 mg mucin<br>200 mg α-amylase | 500 mg pepsin<br>600 mg mucin | 1.8 g pancreatin<br>300 mg lipase | 6 g bile salts |
| Adjust pH | 6.8 ± 0.2 | 1.30 ± 0.02 | 8.1 ± 0.2 | 8.2 ± 0.2 |

Reference: (Flores *et al.*, 2014)

**Table 2. Proximate analysis of mango bark cv. Keitt**

| **Proximate analysis (%)** | **Mango bark** |
|---|---|
| Moisture | 3.63 ± 0.17 |
| Ash | 7.86 ± 0.15 |
| Protein | 2.38 ± 0.12 |
| Crude fiber | 31.58 ± 0.80 |
| Fat | 1.60 ± 0.02 |

**Table 3. Relative abundance (%) of the major phylum.**

| **Major Phylum** | **Relative abundance (%)** | | | | |
|---|---|---|---|---|---|
| | Control (HP24) | CDH24 | CMH24 | IDH24 | IH24 |
| Firmicutes | 18.1 ± 1.7 | 16.9 ± 1.2 | 16.2 ± 0.6 | 26.0 ± 0.4 | 28.6 ± 0.9 |
| Bacteroidetes | 4.3 ± 0.3 | 4.9 ± 0.5 | 6.3 ± 0.6 | 6.3 ± 0.9 | 10.4 ±0.3 |
| Proteobacteria | 21.7 ± 0.6 | 21.4 ± 0.8 | 20.5 ± 0.2 | 10.4 ± 0.1 | 3.6 ± 0.1 |
| Actinobacteria | 0.3 ± 0.2 | 0.4 ± 0.1 | 0.2 ± 0 | 0.7 ± 0.1 | 0.4 ± 0 |

HP24: Inoculum + phosphate buffer at 24 h of fermentation, CDH24: Digested aqueous extract of mango bark at 24 h of fermentation, CMH24: Undigested aqueous extract of mango bark at 24 h of fermentation, IDH24: Digested inulin at 24 h of fermentation, IH24: Undigested inulin at 24 h of fermentation.

**Table 4. Relative abundance (%) of the major families.**

| Phyla | Major Families | Relative abundance (%) | | | | |
|---|---|---|---|---|---|---|
| | | Control (HP24) | CDH24 | CMH24 | IDH24 | IH24 |
| Bacteroidetes | *Prevotellaceae* | 3.8 ± 0.3 | 4.0 ± 0.5 | 7.5 ± 0.6 | 7.1 ± 1.2 | 16.9 ± 2.2 |
| Firmicutes | *Lachnospiraceae* | 8.1 ± 0.7 | 9.1 ± 1.4 | 9.9 ± 0.7 | 13.6 ± 1.6 | 6.5 ± 1.0 |
| Proteobacteria | *Enterobacteriaceae* | 34.0 ± 02.5 | 39.6 ± 2.7 | 30.4 ± 1.1 | 14.1 ± 1.7 | 4.5 ± 0.3 |
| Firmicutes | *Ruminococcaceae* | 8.7 ± 1.1 | 5.4 ± 0.9 | 7.0 ± 0.9 | 9.0 ± 0.4 | 8.7 ± 0.2 |
| Firmicutes | *Streptococcaceae* | 4.2 ± 0.5 | 5.3 ± 2.4 | 5.2 ± 0.3 | 5.9 ± 0.4 | 8.2 ± 1.5 |
| Firmicutes | *Veillonellaceae* | 1.6 ± 0.4 | 1.5 ± 0.4 | 1.6 ± 0.3 | 5.9 ± 0.6 | 9.0 ± 0.7 |
| Firmicutes | *Lactobacillaceae* | 1.4 ± 0.4 | 1.8 ± 0.6 | 1.8 ± 0.6 | 5.7 ± 0.5 | 10.9 ± 1.5 |
| Bacteroidetes | *Bacteroidaceae* | 1.0 ± 0.4 | 1.4 ± 0.9 | 0.9 ± 0.3 | 1.3 ± 0.1 | 1.7 ± 1.5 |

HP24: Inoculum + phosphate buffer at 24 h of fermentation, CDH24: Digested aqueous extract of mango bark at 24 h of fermentation, CMH24: Undigested aqueous extract of mango bark at 24 h of fermentation, IDH24: Digested inulin at 24 h of fermentation, IH24: Undigested inulin at 24 h of fermentation.

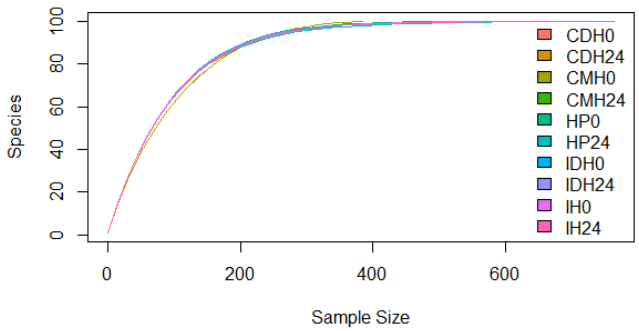


**Figure 1. Rarefaction curve of all samples**. CDH0: Digested aqueous extract of mango bark at 0 h of fermentation, CDH24: Digested aqueous extract of mango bark at 24 h of fermentation, CMH0: Undigested aqueous extract of mango bark at 0 h of fermentation, CMH24: Undigested aqueous extract of mango bark at 24 h of fermentation, HP0: Inoculum + phosphate buffer at 0 h of fermentation, HP24: Inoculum + phosphate buffer at 24 h of fermentation, IDH0: Digested inulin at 0 h of fermentation, IDH24: Digested inulin at 24 h of fermentation, IH0: Undigested inulin at 24 h of fermentation, IH24: Undigested inulin at 24 h of fermentation.

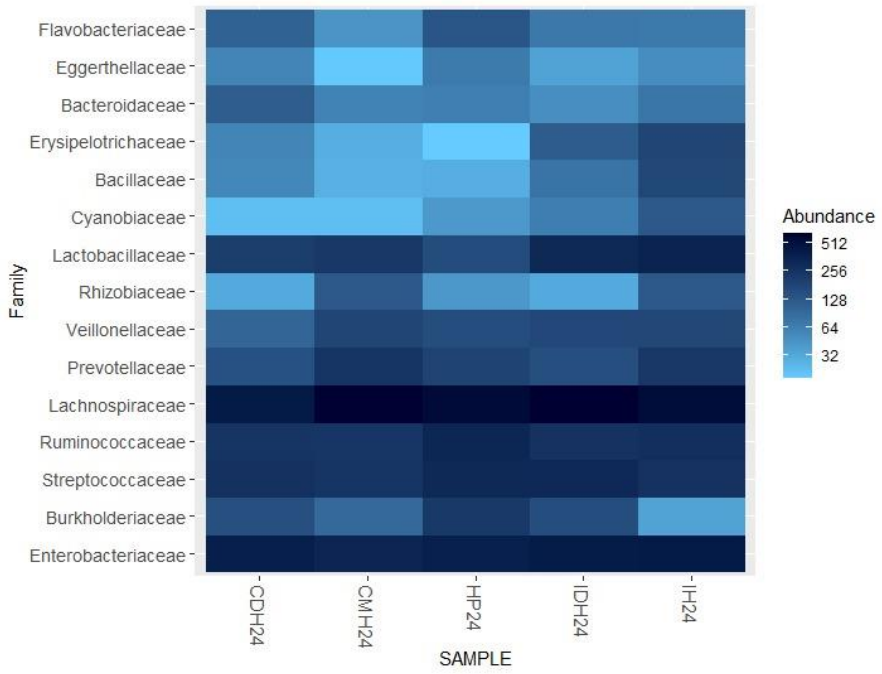


**Figure 2. Heat map of the abundance of the 15 main families in all samples at 24 h.**

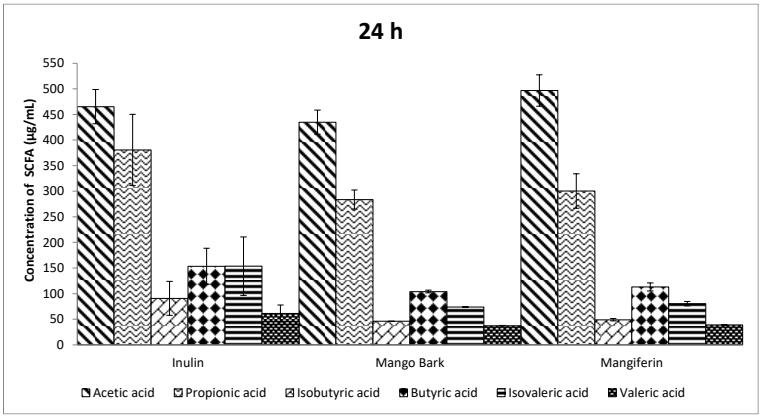


**Figure 3. SCFA production of inulin, mango bark and mangiferin at 24 h.**

# 5. CONCLUSIONES GENERALES

El extracto acuoso de la corteza del árbol de mango ha sido ampliamente utilizado en la medicina tradicional presentando diferentes actividades biológicas. Esta corteza del árbol de mango puede ser obtenida a partir de residuos de la poda del árbol, los cuales van en aumento con la producción de mango y actualmente son descartados por los productores o quemados, generando una preocupación por su impacto negativo al medio ambiente. La utilización de residuos agrícolas es de importancia debido al aprovechamiento integral de una biomasa y reducción de desperdicios y del impacto negativo al ambiente. En este sentido, se utilizaron residuos de la poda del árbol de variedades de importancia en nuestra región para evaluar la potencia del extracto acuoso de la corteza del árbol de mango y darle así un valor agregado al residuo de poda generado. De este proyecto se logró elucidar los compuestos fenólicos de cuatro variedades de mango, que son las de mayor producción en nuestra región. Como resultados se observó que la variedad Keitt presentó la mayor potencia por su contenido de compuestos fenólicos y su bioactividad. Las actividades biológicas evaluadas fue su capacidad antioxidante tanto por ensayos celulares como ensayos químicos y posteriormente se evaluó su metabolismo por medio de un ensayo *in vitro*. En este sentido se obtuvo que la mangiferina es el compuesto mayoritario en la corteza del árbol de mango, y que su permeabilidad intestinal es mayor cuando se encuentra en el extracto acuoso de corteza de mango que cuando se evalúa aislada como estándar. Posteriormente, se evaluó el potencial prebiótico del extracto de corteza del árbol de mango, variedad Keitt comparándolo con un prebiótico comercial. De los resultados obtenidos se tiene que se logró modificar el contenido de ácidos grasos de cadena corta, así como una modificación en el contenido de bacterias propias del colon humano, aunque estas modificaciones no fueron estadísticamente significativas. No obstante, la corteza del árbol de mango presenta gran potencial para futuras investigaciones dado que no mostró citotoxicidad y se logró tener un efecto relevante biológicamente en cuanto a la modulación de los ácidos grasos de cadena corta y los filos de bacterias relevantes para la salud humana.

# 6. RECOMENDACIONES

Para futuros proyectos se recomienda realizar una evaluación integral de la biomasa generada por la poda del árbol de mango, además de la optimización de la extracción de diferentes compuestos bioactivos, tanto fibra como polifenoles, y su aislamiento para observar qué compuestos pueden ser los más relevantes para su bioactividad.

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