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SISTEMA DE ESTUDIOS DE POSGRADO

DESARROLLO DE UNA BEBIDA LÁCTEA A BASE DE SUERO DE LECHE Y CAS CON ACTIVIDAD PROBIÓTICA Y PREBIÓTICA

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Ciencia de Alimentos para optar al grado y título de Maestría Académica en Ciencia de Alimentos

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Tabla de contenidos

Resumen

El hallazgo de soluciones simple y económicas para añadir valor al suero de leche aún es un reto para la industria láctea con el fin de alcanzar una bioeconomía circular. El resultado de esta investigación brinda información práctica acerca del potencial industrial de una bebida probiótica, destacando la valorización del suero y la pulpa de cas (*Psidium friedrichsthalianum* -Nied.). Primero, un medio de cultivo a base de suero de leche fue desarrollado para la fermentación del *Lactobacillus rhamnosus* GG (LGG). El medio de cultivo a base de suero fue suplementado con extracto de levadura y fermentado en biorreactor con una tasa máxima de crecimiento de 0.32 h^{-1} luego de 48 horas de tiempo de fermentación. La biomasa del LGG producida fue utilizada como probiótico en una bebida formulada a base de suero de leche con pulpa de cas. La supervivencia del LGG, así como las propiedades fisicoquímicas y fitoquímicas fueron estudiadas durante 56 días en almacenamiento a 4 °C. LGG sobrevivió en la bebida formulada y su cinética de supervivencia no fue afectada de forma significativa por la adición del cas ($P>0.05$). En general, la vida útil de la bebida inoculada supera los 40 días con una población de al menos 10^6 CFU/mL, cumpliendo con los requisitos legales para el etiquetado de un producto probiótico. Otras propiedades como: pH, contenido de fructosa, glucosa, sacarosa y proantocianidinas (PACs) mostraron diferencias significativas luego del tiempo de almacenaje ($P<0.05$). Finalmente, tres formulaciones distintas de la bebida se compararon con el fin de estudiar la influencia de la concentración de suero sobre la percepción sensorial. El prototipo de la bebida con la mayor aceptación fue del 50% suero en relación con el prototipo original. Adicionalmente, resultados preliminares muestran que la bebida formulada tiene la capacidad de modificar la respuesta inmune en ensayos *in vivo,* lo que a su vez se puede relacionar con un potencial efecto fotoprotector. En conclusión, esta investigación muestra la posibilidad de diseñar una bebida probiótica a base de suero y cas. Según el estado del arte a la fecha, este es el primer reporte acerca de bebidas de cas como vectores de probióticos y de un producto alimenticio orientado a la fotoprotección.

Índice de cuadros

Marco teórico

Artículos

Índice de figuras

Marco teórico

Artículos

Lista de abreviaturas

Abreviatura	Definición
ADN	Ácido desoxirribonucleico
ATCC	American Type Culture Collection
BAL	Bacterias ácido lácticas
CENIBiot	Centro Nacional de Innovaciones Biotecnológicas
CITA	Centro Nacional de Ciencia y Tecnología de Alimentos
CRG	Costa Rican guava fruit pulp
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GOS	Galacto-oligosacáridos
GRAS	Generalmente reconocido como seguro
IL-10	Interleucina 10
LGG	*Lactobacillus rhamnosus* GG
LTA	Ácido lipoteicoico
MRS	de Man, Rogosa & Sharpe
MWM	Medio de cultivo suero de leche
PACs	Proantocianidinas
PBS	Buffer salino de fosfatos
RTCR	Reglamento Técnico de Costa Rica
PGE2	Prostaglandina E2
UFC	Unidades formadoras de colonias
USDA	Departamento de Agricultura de los Estados Unidos
UVR	Radiación ultravioleta
XOS	Xilo-oligosacáridos
WHO	World Health Organization

“To those that devote their lives to science nothing can give more happiness than making discoveries, but their cups of joy are full only when the results of their studies find practical application.”

Louis Pasteur

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Capítulo 1 **Introducción**

En la actualidad, la incidencia de males cutáneos, incluyendo melanoma y cáncer de piel no melanoma, es equivalente a la incidencia de tumores malignos en todos los otros órganos combinada (Katiyar et al., 2017; Narayanan et al., 2010a). En Costa Rica, el registro Nacional de Tumores a cargo de la Dirección de Vigilancia de la Salud, reporta que el cáncer de piel es el de mayor incidencia en el país, tanto en hombres como mujeres.

Desde la década de 1930, existe evidencia científica que relaciona la exposición prolongada a la radiación ultravioleta (UVR) con la aparición de cáncer de piel (Findlay, 1928). La energía contenida en la UVR puede transferirse a diferentes moléculas dentro de las células de la piel; esta transferencia de energía puede modificar moléculas como el ADN. Los queratinocitos, las células más abundantes en la epidermis (95% de la epidermis), son el objetivo más frecuente de la UVR.

Uno de los efectos más importantes de la exposición cutánea a UVR es la respuesta pro-inflamatoria. Debido a lo anterior, una forma análoga de estudiar el efecto que tiene la UVR sobre un sistema *in vivo* sin utilizar modelos de irradiación, es mediante la exposición a alérgenos fuertes, estudiando la respuesta inflamatoria así como el monitoreo de la interleucina IL-10 (Bindels et al., 2015).

Tanto probióticos como prebióticos se han propuesto como inmunomoduladores capaces de revertir en cierta medida la inmunosupresión ocasionada por la exposición a la radiación ultravioleta (UVR). El papel que desempeña la microbiota intestinal en la salud ha generado un enorme interés en modificar su composición y comprender su función metabólica. Durante los últimos años, los probióticos han surgido como una nueva estrategia en la fotoprotección sistémica (Bouilly-Gauthier et al., 2010). En 2013, un grupo de investigación de la Universidad de Buenos Aires (Argentina) determinó que el ácido lipoteicoico (LTA, componente estructural de la pared celular) del *Lactobacillus rhamnosus* GG (LGG) puede restaurar la homeostasis de la piel afectada por la UVR (Weill, Cela, Paz, Ferrari, Leoni, & González Maglio, 2013).
El LTA representa cerca del 50% de la masa total de membrana bacteriana (Claes et al., 2010; Weill, Cela, Paz, Ferrari, Leoni, & Maglio, 2013) y es una molécula macro anfifílica anclada en

la membrana citoplásmica a través de su extremo glicolípido (Morath et al., 2001). Por otra parte, el LGG es una de las bacterias probióticas con más información reportada desde su descubrimiento por Goldin & Gorbach y es una de las pocas bacterias que cuenta con sello

GRAS (Generally Recognized As Safe) de la FDA (U.S. Food & Drug Administration). Si bien el estudio realizado por Weill et al. (2013) sugiere la incorporación del LTA en matrices alimenticias, actualmente no hay reportes de productos alimenticios orientados a la fotoprotección atribuida a la presencia de LTA en LGG.

Además de la fotoprotección a partir de componentes biológicos, existe evidencia científica del efecto preventivo que poseen algunas moléculas (prebióticos) frente al daño ocasionado por la UVR en la piel. Los mecanismos de acción son muy variados incluyendo: actividad antioxidante, antiinflamatoria e inmunomoduladora (Gilaberte & González, 2010). Dentro de este último grupo, se incluyen las proantocianidinas (PACs), cuya actividad relacionada con la fotoprotección fue reportada por primera vez en 2003 (Mittal et al., 2003). Katiyar *et al.* utilizaron un extracto de PACs de la semilla de uva, y demostraron su capacidad anticarcinogénica en cáncer de piel tipo no melanoma.

Estudios preliminares muestran que el cas (*Psidium friedrichsthalianum*) es fuente de PACs con una importante actividad antioxidante *in vivo* (Rojas-Garbanzo et al., 2019). No existen reportes que asocien las PACs del cas con actividad inmunomodulatoria; sin embargo, dada que la actividad se le atribuye a la molécula y no a la matriz alimenticia, la hipótesis es que la administración de una bebida formulada con el probiótico LGG y pulpa de cas tendrá efectos de inmunomodulación y se espera una actividad potenciada debido a la sinergia de ambos componentes.

La bebida planteada en este proyecto entra dentro de la categoría de alimentos funcionales, los cuales continúan aumentando su popularidad entre los consumidores que buscan obtener beneficios adicionales, más allá de la nutrición básica. Adicionalmente, se busca ofrecer una solución práctica para la utilización y revalorización del suero de leche como subproducto de la producción de queso, mediante herramientas biotecnológicas.El presente proyecto se realizó de manera conjunta entre el Centro Nacional de Ciencia y Tecnología de Alimentos (CITA-UCR) y el Centro Nacional de Innovaciones Biotecnológicas (CENIBiot-CeNAT).

Capítulo 2 **Marco teórico**

2.1 Probióticos

El concepto de probiótico fue desarrollado cerca del año 1900 por Elie Metchnikoff, quien observó que una bacteria viable (*Lactobacillus bulgaricus*) en la leche fermentada confería beneficios a la salud de quienes la consumían. Actualmente, los probióticos son definidos como microorganismos vivos, los cuales administrados en cantidades adecuadas confieren beneficios a la salud (FAO/WHO, 2002). Distintas cepas de bacterias e incluso levaduras han sido identificadas como probióticos, siendo las cepas del género *Lactobacillus* las más estudiadas. La evidencia científica sugiere que los efectos sobre la salud son específicos según la cepa; por tanto, es necesario conocer la identidad del microorganismo de interés, como también, debe cumplir una serie de requerimientos para poder ser declarada como probiótico (Kleerebezem et al., 2010; Weill, Cela, Paz, Ferrari, Leoni, & Maglio, 2013). Las principales pruebas *in vitro* para el estudio de las potenciales cepas probióticas se incluyen en el apartado 2.1.1 (de Melo Pereira et al., 2018; Vinderola et al., 2017). Un resumen de la información de muestra en la Figura 2.1.

2.1.1 Cepas con actividad probiótica: requerimientos de eficacia y seguridad

- ***Tolerancia a condiciones de estrés***

La tolerancia al tránsito gastrointestinal es un criterio de selección, comúnmente utilizado en la investigación de probióticos. Al ser ingeridos, los microorganismos deben ser resistentes a las enzimas presentes en la cavidad oral, tales como amilasa y lisozima. Luego de la ingestión, los microorganismos enfrentan una serie de factores antimicrobianos en el estómago (bajo pH, jugos gástricos y pepsina) y en los intestinos (pancreatina y sales biliares). La resistencia a las condiciones gastrointestinales varía de acuerdo con la especie. Por ejemplo, mientras los *Lactobacillus* son ampliamente resistentes al pH bajo, las *Bifidobacterias* son extremadamente sensibles, con poca o nula supervivencia en pH entre 2 - 3. Las pruebas a nivel *in vitro* incluyen la exposición de la cepa de interés, a diferentes grados de acidez con la presencia de enzimas y otras sustancias que simulan las condiciones gastrointestinales. La resistencia se determina mediante el conteo de unidades formadoras de colonia o absorbancia a través del tiempo.

- ***Adherencia a la mucosa***

La adherencia de los probióticos al epitelio intestinal puede contribuir a su permanencia en la superficie de la mucosa. Sin embargo, los estudios indican que la colonización del intestino por probióticos administrados vía oral parece ser temporal. Al interrumpir el consumo de probióticos prosigue la eliminación. A nivel *in vitro* la hidrofobicidad y la autoagregación del probiótico se utilizan como indicadores de la interacción entre microorganismos y las células epiteliales del huésped. La autoagregación se puede estudiar mediante la absorbancia de la cepa en suspensión de buffer salino de fosfatos (PBS) medida a lo largo del tiempo. Por otro lado, la hidrofobicidad se puede determinar de forma directa utilizando líneas celulares Caco-2, HT-29 e I-407.

- ***Actividad anti-patogénica***

Luego de la adhesión al intestino, los microrganismos probióticos producen componentes extracelulares con capacidad antimicrobiana para eliminar bacterias patógenas. Dentro de estos componentes extracelulares se incluyen ácidos orgánicos, enzimas, peróxido de hidrógeno, bacteriocinas y péptidos de baja masa molecular. Otro mecanismo antagónico es la competencia por nutrientes y estimulación del sistema inmune. Estos mecanismos varían de acuerdo con la cepa. La producción de metabolitos antimicrobianos se puede evaluar a nivel *in vitro* mediante ensayos de zonas de inhibición en placas con agar.

- ***Evaluación de la seguridad***

Se debe evaluar el riesgo de consumir microorganismos vivos. Algunas iniciativas de la comunidad europea (la regulación de los alimentos novedosos de la Unión Europea, QPS y PROSAFE), los Estados Unidos (FDA y OMS) y Canadá (Departamentos de Salud: NHPR) se han dedicado a establecer criterios para la evaluación de seguridad de los probióticos para uso humano. Las recomendaciones comunes incluyen registros de los aislamientos, taxonomía, ausencia de virulencia y genes transferibles de resistencia a antibióticos. Actualmente, la mayoría de los probióticos disponibles han sido aislados de seres humanos sanos para aumentar la compatibilidad con otros probióticos y garantizar la supervivencia en el tracto gastrointestinal (Wang et al., 2020).

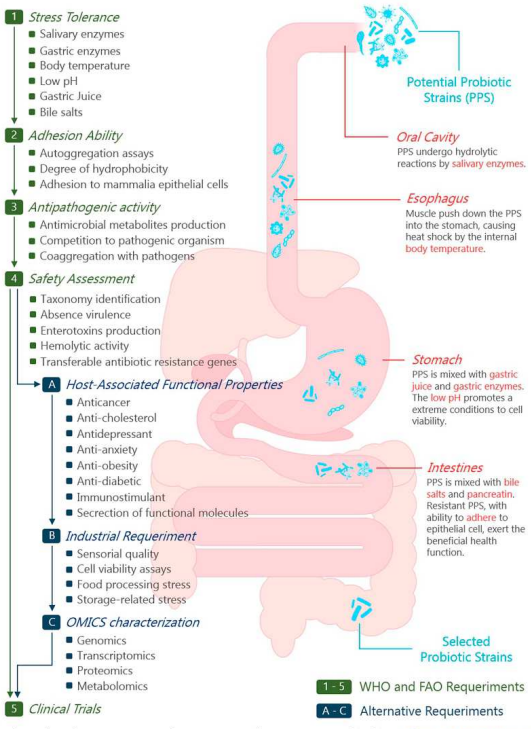

Figura 2.1 Caracterización de cepas probióticas de acuerdo con WHO/FAO (de Melo Pereira et al., 2018).

2.1.2 Bacterias ácido-lácticas

Las bacterias ácido-lácticas (BAL) son un grupo diverso que produce ácido láctico como principal producto del metabolismo. Son bacterias Gram positivas, catalasa y oxidasa negativas, no forman esporas, además crecen anaeróbicamente y son aerotolerantes (Bovo et al., 2014). Pueden ser homofermentativas, producen principalmente ácido láctico al fermentar glucosa, o heterofermentativas productoras de ácido láctico, CO_2, etanol y/o ácido acético (Gao et al., 2017).

La principal fuente de carbono para las BAL es la lactosa. Las BAL tienden a ser nutricionalmente exigentes, requiriendo aminoácidos específicos y vitamina B como factores de crecimiento. La literatura reporta que, al aumentar la concentración de ácido láctico, el pH desciende, pasando de un pH típico de fermentación ~6,3 a uno de ~4,5; lo cual puede llevar a la utilización incompleta de la lactosa. Estas bacterias son usualmente cultivadas en un medio específico y estandarizado como el caldo MRS (de Man, Rogosa & Sharpe). Sin embargo, estos medios de cultivo con alta especificidad tienen un alto valor económico y son utilizados a escala laboratorio. En caso de producción a gran escala es necesario buscar medios de cultivo de bajo costo. Dado lo anterior, ha habido un incremento en la demanda de medios de cultivo a partir de los subproductos de la industria alimentaria, como el suero de leche (Bovo et al., 2014).

2.1.3 Lactobacillus rhamnosus GG

Previo al aislamiento de la cepa *Lactobacillus rhamnosus* GG (LGG), los investigadores Gorbach y Goldin (1985) concluyeron que las cepas comúnmente utilizadas en la industria de lácteos (*L. casei, L.bulgaricus y L. acidophilus*) no poseían las características biológicas necesarias para colonizar el intestino humano y así, es poco probable que tengan un efecto beneficioso sobre la salud. Por este motivo, enlistaron las propiedades críticas que debía tener una cepa de *Lactobacillus* y se propusieron aislarla. Para comenzar aislaron cepas de *Lactobacillus* de las heces de humanos sanos y las cepas potenciales se sometieron a pruebas como las descritas en la sección 2.1.1. El microorganismo con las mejores propiedades fue denominado *Lactobacillus rhamnosus* GG en honor a sus descubridores (Doron et al., 2005; Gorbach et al., 2017).

A nivel bacteriológico, LGG es una bacteria bacilar, Gram positiva, que forma una colonia cremosa cuando se cultiva en placa Petri y presenta un característico olor a mantequilla (ver Figura 2.2). Fermenta xilosa, trehalosa, sorbitol, salicina, ribosa, ramnosa, melezitosa, manosa, manitol, glucosa, fructosa y celobiosa.

Es una de las cepas probióticas más estudiadas, se reporta el uso de esta cepa en productos lácteos como yogurt, leche fermentada, queso semiduro y algunos productos no lácteos como jugos y suplementos alimenticios. A nivel industrial, una de las características del LGG es su

buena estabilidad al ser refrigerada, con poca o nula pérdida en la viabilidad durante meses (Lee, Y.K. & Salminen, 2008).

Figura 2.2 Cultivo de LGG en placa Petri (Papizadeh et al., 2016).

Dentro de la funcionalidad de este probiótico se encuentran los siguientes beneficios a la salud: reducción del periodo de diarrea aguda (Szajewska et al., 2007), alivio de ciertas alergias ocasionadas por alimentos (dermatitis en niños) (Rautava et al., 2002), reducción de infecciones en niños (Hatakka et al., 2001), alivio en afecciones pulmonares e inflamación intestinal (Bruzzese et al., 2004) y mejoramiento de la formación de anticuerpos y respuesta inmune (Schultz et al., 2003).

2.1.4 Ácido lipoteicoico

Las células de los *lactobacilli* incluyen peptidoglicanos y ácidos teicoicos. Un gran número de funciones biológicas se han descrito para el ácido teicoico como, por ejemplo, adhesión celular e interacción con el sistema inmune. Hay dos tipos de ácido teicoico en los lactobacilli: ácidos teicoicos de la pared, los cuales se enlazan a los peptidoglicanos y los LTA anclados a la pared de la membrana citoplasmática a través de un glicolípido. El LTA representa cerca del 50% de la masa total de membrana bacteriana (Claes et al., 2010; Weill, Cela, Paz, Ferrari, Leoni, & Maglio, 2013). En términos de estructura (ver Figura 2.3 **Representación esquemática de la estructura del LTA aislado del LGG,**) una molécula macro anfifílica anclada en la membrana citoplásmica a través de su extremo glicolípido. Consiste en una cadena de glicerol-fosfato o ribitol-fosfato con sustituciones de éster de d-alanina o glicosilo (Morath et al., 2001).

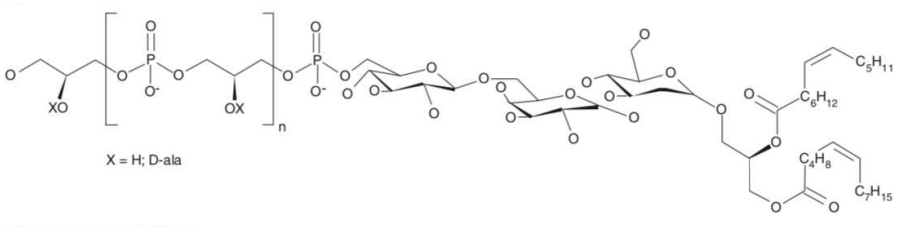

Figura 2.3 Representación esquemática de la estructura del LTA aislado del LGG, X = 74% D-ala, n = 50 (en promedio) (Lebeer et al., 2010).

2.1.5 Efecto fotoprotector asociado al ácido lipoteicoico

La relación entre la salud intestinal y el sistema inmune cutáneo no es clara; sin embargo, hay evidencia de la existencia de una relación cruzada entre ellos. La fotoprotección inducida por nutrientes específicos ha demostrado ser exitosa en la prevención de los efectos dañinos de la UVR (Weill, Cela, Paz, Ferrari, Leoni, & Maglio, 2013)

La UVR es indispensable para la vida en la Tierra; sin embargo, una exposición prolongada puede causar daños en la salud humana. La incidencia de los rayos solares induce múltiples respuestas perjudiciales como la inmunosupresión, edema, quemaduras solares, eritema, hiperpigmentación, envejecimiento prematuro y cáncer de piel (Pérez-Sánchez et al., 2014). La UVR causa daño directo sobre el material genético celular (ADN), inflamación de tejidos, supresión de la respuesta inmune y la formación de radicales libres causando oxidación de proteínas, lípidos y ADN (Svobodova et al., 2006). Se ha demostrado que la radiación crónica causa hiperplasia, un factor clave en el carcinoma de piel, resultado de la proliferación de las células epiteliales y la supresión de la apoptosis (Weill, Cela, Paz, Ferrari, Leoni, & Maglio, 2013).

Durante los últimos años, los probióticos han surgido como una nueva estrategia en la fotoprotección sistémica (Bouilly-Gauthier et al., 2010). En su estudio, Weill *et al.* (2013) investigaron si la administración del LTA purificado proveniente del LGG puede modular la inmunosupresión de los efectos de la radiación ultravioleta y prevenir la formación de tumores en piel en ratones sin pelo (SKH:hr1). La hipótesis planteada por el grupo fue que la

administración oral de LTA podría modular el tejido linfoide asociado al intestino y que a través del sistema inmune intestinal se podría restaurar la homeostasis de la piel afectada por la UVR, reduciendo la producción de tumores inducidos (Weill, Cela, Paz, Ferrari, Leoni, & Maglio, 2013).

2.1 Prebióticos

El concepto de prebiótico fue presentado por Gibson & Roberfroid por primera vez en 1995, describe aquellas sustancias que no se absorben en la parte superior del tracto gastrointestinal y estimulan el crecimiento de microorganismos que colonizan el intestino grueso (Blaut, 2002; Gibson et al., 2004). El prebiótico es un sustrato selectivo para uno o un número limitado de probióticos. Los probióticos son estimulados para crecer y producir ácidos grasos de cadena corta por los prebióticos. Por consiguiente, el prebiótico es capaz de alterar la microbiota del colón del huésped hacia una composición más sana. Para confirmar la selectividad de un prebiótico es importante monitorear con precisión los cambios en la microbiota tanto con ensayos *in vitro* como *in vivo* (Al-Sheraji et al., 2013). Dentro de los principales grupos de moléculas prebióticas se encuentran los polifenoles; para fines de este proyecto se hace especial énfasis en las proantocianidinas, proveniente de frutas y para este caso en particular, los provenientes del cas (*Psidium friedrichsthalianum* Ndz).

2.2.1 Cas

Pertenece a la familia *Myrtaceae* que contiene a los géneros *Myrciaria* y *Psidium.* El género *Psidium* consiste en 150 especies aproximadamente, pero solo 20 de ellas producen frutos comestibles. La especie cultivable más común es la guayaba (*Psidium guajava* L.), seguido de la guayaba fresa (*P. cattleianum* Sabine), guayaba del Brasil (*P. guineense* Sw.) y la guayaba costarricense (P. *friedrichsthalianum* Ndz.) (Mani et al., 2011). La guayaba costarricense, de aquí en adelante, cas, proviene de un árbol de mediana altura, que se encuentra especialmente en América Central, sur de México y al Norte de Sudamérica.

Estudios fitoquímicos demuestran que es una fruta con alta diversidad de compuestos polifenólicos, reconocidos por su gran potencial antioxidante. Los estudios epidemiológicos han demostrado una relación beneficiosa entre la ingesta de compuestos polifenólicos y la

reducción del riesgo de algunas enfermedades, atribuyéndolo tanto a las propiedades antioxidantes, como antiinflamatorias de dichos compuestos (Arts & Hollman, 2005; Flores et al., 2013; Pino et al., 2002).

2.2.2 Polifenoles

El término polifenol aparece en los años cincuenta para describir una estructura química de múltiples anillos de fenol que poseen otros compuestos (Sharma, 2014). Los polifenoles provenientes de los alimentos son compuestos naturales que se encuentran en las plantas. Se les puede encontrar en frutas, vegetales, té, vino, café y otros. Los compuestos fenólicos incluyen tanto moléculas simples como polímeros de alta masa molecular (Jiménez et al., 2014). Se clasifican basados en estructura química y complejidad en flavonoides y no flavonoides. Los flavonoides se clasifican en otras subclases de acuerdo con sus diferentes estructuras en flavononas, flavonas, dihidroflavonoles, flavonoles, flavan-3-ol o flavonoles, antocianidinas, isoflavonas y proantocianidinas (Cardona et al., 2013; de la Rosa et al., 2009).

Durante la digestión los polifenoles son reconocidos por el cuerpo humano como xenobióticos con una biodisponibilidad más baja que otros micronutrientes. Estudios previos con animales y humanos han mostrado cómo la complejidad de la estructura y la polimerización se relaciona con la biodisponibilidad; por ejemplo, compuestos polifenólicos de bajo peso molecular pueden ser absorbidos en el intestino delgado. Por otro lado, compuestos de mayor tamaño como los taninos condensados o hidrolizados alcanzan el colon casi sin ningún cambio (Cardona et al., 2013; Visioli, 2015; Walle, 2004).

Se estima que tan solo un 5-10 % del consumo total de polifenoles se absorbe en el intestino delgado. El otro 90-95% puede acumularse en el lumen del intestino grueso donde se someten a actividades enzimáticas por parte de la población microbiana intestinal (Cardona et al., 2013). El metabolismo intestinal de polifenoles como parte de alimentos y bebidas ha sido poco estudiado y es escaza la información respecto a la biotransformación intestinal de compuestos polifenólicos puros sin una matriz alimenticia (Sadeghi Ekbatan et al., 2016). Algunos investigadores han demostrado que los ciertos polifenoles pueden modificar la composición o inhibir el microorganismo que vive en este ecosistema (Stoupi et al., 2010).

2.2.3 Proantocianidinas

Las proantocianidinas también conocidas como taninos condensados son oligómeros o polímeros de estructuras de flavan-3-ol vinculados por enlaces entre flavonoides. En el trabajo presentado por Ou & Gu (2014) se definen las proantocianidinas con grado de polimerización 2-4 como oligómeros, >4 polímeros y >10 polímeros de alto peso. Las proantocianidinas tipo B presentan un enlace C4 – C8 y/o enlace C4 – C6. El tipo A presentan un enlace adicional entre el C2 – O7. El tamaño molecular de las proantocianidinas está descrito como el grado de polimerización. Aquellos compuestos con un grado de polimerización de 1, 2, 3 ó 4 son llamados monómeros, dímeros, trímeros o tetrámeros, respectivamente. Las unidades del flavan-3-ol al final de la molécula son unidades terminales. Todas las unidades encima de la unidad terminal son unidades de extensión. Los flavan-3-ol tienen dos anillos aromáticos (A y B) y un heterociclo (Ou & Gu, 2014).

Cerca del 40% de la ingesta total de proantocianidinas son monómeros u oligómeros absorbibles. El porcentaje restante son polímeros no absorbibles con un grado de polimerización superior a 4. Las proantocianidinas están compuestas por monómeros de catequina y epicatequina, la Figura 2.4 muestra una estructura representativa de una antocianina vinculada a un dímero c-PAC.

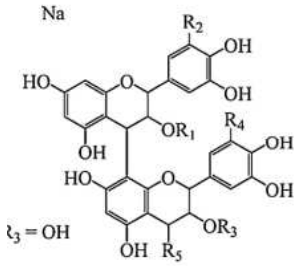

Figura 2.4 Estructura química de la proantocianidina encontrada en el cas, donde los grupos sustituyentes varían entre H, OH y catequina, dependiendo de la molécula específica (Rojas-Garbanzo et al., 2019).

La evidencia científica muestra que las proantocianidinas tienen el potencial de brindar salud modulando la microbiota del tracto gastrointestinal con un efecto prebiótico. En modelo animal

las proantocianidinas en la dieta variaron la población bacteriana hacia cepas más resistentes, reportando un incremento de bacterias Gram negativas (Smith & Mackie, 2004).

2.2.4 Efecto fotoprotector asociado a las proantocianidinas

Existe evidencia científica del efecto preventivo que poseen algunas moléculas frente al daño ocasionado por la UVR en la piel. Los mecanismos de acción son muy variados incluyendo actividad antioxidante, antiinflamatoria e inmunomoduladora (Gilaberte & González, 2010). El efecto fotoprotector de las proantocianidinas fue reportado por primera vez en 2003 por Mittal *et al.*, quienes demostraron que la administración de extractos de proantocianidinas de uva (0,2 y 0,5% w/w) durante 24 semanas previene la aparición de tumores inducidos por la UVR en ratones sin pelo SKH-1; además, previene la multiplicidad, crecimiento y tamaño de los tumores. Éstas y otras observaciones reportadas en la literatura sugieren que los polifenoles poseen la habilidad de proteger la piel de los efectos adversos de la UVR *in vivo* en modelo animal (Katiyar et al., 2017).

2.2 Alimentos funcionales

El departamento de Agricultura de los Estados Unidos (USDA) define los alimentos funcionales como “alimentos procesados o naturales que contiene compuestos, conocidos o desconocidos, biológicamente activos los cuales en cantidades no tóxicas proveen un beneficio a la salud clínicamente probado y documentado para la prevención, manejo o tratamiento de enfermedades crónicas” (Hunter & Hegele, 2017).

2.3.1 Definición

Una de las características de un alimento funcional es la presencia de compuestos con actividad biológica e implica el estudio del papel de estos compuestos cuando se combinan en un producto alimenticio terminado. Los alimentos funcionales continúan aumentando su popularidad entre los consumidores que buscan beneficios más allá de la nutrición básica. Uno de los grupos más populares dentro de los alimentos funcionales son los alimentos que contienen probióticos. Se proyecta que el mercado mundial de probióticos alcanzará $77.09 mil millones (USD) impulsado principalmente por el aumento de la demanda en los mercados

asiáticos y europeos (de Simone, 2019; Foligné et al., 2013). En términos de tipo de producto, las bebidas probióticas surgieron como el segmento más grande en 2018 con $39.56 mil millones (USD) en ventas (Grand View Research, 2019)

2.3.2 Probióticos como alimentos funcionales

Un importante número de microorganismos han sido propuestos como potencialmente probióticos de acuerdo con su resultados *in vitro*; sin embargo, sólo aquellas cepas suficientemente documentadas y robustas durante su procesamiento industrial logran incursionar al mercado, el cual es predominado por los géneros *Lactobacillus* y *Bifidobacterium* (Ashaolu, 2020).

El desarrollo de productos que incluyan probióticos debe considerar la viabilidad celular, el control de calidad de la fermentación (medio de cultivo o matriz alimenticia, pH, fuente de carbono, temperatura y tiempo de fermentación, entre otros) y los procesos luego de la fermentación (secado por aspersión, liofilización, homogenización, mezclado, empaque, etc.) (Meybodi et al., 2020).

2.3.3 Regulación en materia de probióticos

A diferencia de los fármacos, los alimentos funcionales o suplementos alimenticios no cuentan con un marco regulatorio estandarizado; es por esto, que la regulación varía en distintas partes del mundo. En 2010, representantes de EFSA (European Food Safety Authority) rechazaron cerca de 300 de las alegaciones de salud (*'claims'*) atribuidas a las bacterias probióticas; como consecuencia en el mercado europeo ningún producto probiótico o suplemento puede incluir dentro de su etiqueta el beneficio a la salud que promueve. Esta decisión del bloque europeo preocupó a la comunidad científica e industrial, quienes manifestaron que algunas de las declaraciones de salud contaban con respaldo científico suficiente (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2016; Foligné et al., 2013).

En Japón, país donde se origina el concepto de alimento funcional, la agencia FOSHU (Foods for Specific Health Use) estableció una regulación desde 1993, donde se aprueban aquellas declaraciones de salud asociadas a alimentos o ingredientes con suficiente evidencia

científica; una vez aprobada puede aparecer en la etiqueta. Aunque Japón cuenta con uno de los mayores mercados de alimentos funcionales del mundo, existen relativamente pocos alimentos aprobados por FOSHU (Iwatani & Yamamoto, 2019).

En Costa Rica, el Reglamento Técnico RTCR 414:2008 regula las características del yogurt para consumo directo. Uno de los aspectos más relevantes dentro del Reglamento es el establecimiento de un contenido mínimo de 10^6 UFC/g (unidades formadoras de colonias por gramo) de bacterias probióticas para denominarlo yogurt (Ministerio de economía, industria y comercio et al., 2008).

Es importante recordar que la dosis y el beneficio a la salud deben corroborarse para las distintas cepas. En la revisión bibliográfica presentada por Hunter & Hegele (2017) se recomienda una dosis diaria de 10^7 UFC en 175-350 g de yogurt con probióticos (*Lactobacillus acidophilus* LA5 y *Bifidobacterium animalis* BB12) donde el beneficio sobre la salud esperado es la reducción del colesterol total, con un incremento de las lipoproteínas de alta densidad (Asemi et al., 2012; Hunter & Hegele, 2017; Sadrzadeh-Yeganeh et al., 2010).

2.3.4 Prebióticos como alimentos funcionales

La demanda global de prebióticos ha crecido en los últimos 10 años hasta cerca de 500 000 toneladas por año (Mohanty et al., 2018). El tamaño del mercado mundial de prebióticos se valoró en $3,34 millones (USD) en 2016 y se estima un crecimiento del 10% hasta 2024, especialmente debido a la demanda de la industria láctea. Los prebióticos de mayor venta son los fructo-oligosacáridos (FOS), inulina, galacto-oligosacáridos (GOS) y manano-oligosacáridos (MOS). Además, se estima que los avances tecnológicos en el desarrollo de inulina y oligosacáridos reemplacen gran parte del azúcar añadido en alimentos (Grand View Research, 2018).

2.2.5 Regulación en materia de prebióticos

En general, se asume que los prebióticos son seguros ya que provienen de fuentes naturales; sin embargo, es importante estudiar *in vivo* las dosis, posibles efectos nocivos que la administración de estas moléculas pueda ocasionar y los beneficios a la salud que confieren

de forma específica. Por ejemplo, Manning & Gibson reportan que el consumo de 2 g/día de xilo-oligosacáridos (XOS), 10 g/días GOS y al menos 4 g/día FOS incrementa el nivel de bifidobacterias en el tracto gastrointestinal (Manning & Gibson, 2004; Mohanty et al., 2018).

2.3.5 Bebidas a base de suero de leche

Los lácteos fermentados se obtienen a través de la reducción del pH y la coagulación de la leche por microorganismos lácticos específicos. Estos productos pueden tener otros componentes alimenticios incluyendo suero y mantequilla (Farah et al., 2017). Las bebidas a base de suero de leche son una importante opción para revalorizar este subproducto el cual se obtiene como resultado de la producción del queso. El suero de leche es rico en nutrientes, por ejemplo, lactosa, proteínas solubles, lípidos y minerales, reteniendo cerca del 55% del total de las proteínas solubles de la leche. Además, como beneficio paralelo está la reducción de la contaminación ocasionada por el suero de leche en los mantos acuíferos y/o de los costos de operación asociados a su tratamiento biológico (Prazeres et al., 2012).

En el Cuadro 2.1 se observa la composición de dos tipos de suero, diferenciados entre sí por su valor de pH y en los sólidos totales se presenta una alta concentración de lactosa, fuente de carbohidratos para el desarrollo de las bacterias ácido-lácticas (BAL). El suero es un medio de cultivo incompleto para los *lactobacilli* y requiere la adición de micronutrientes para su desarrollo (Jelen, 2009).

2.3.6 Alimentos funcionales orientados a la fotoprotección

En el Cuadro 2.2 se muestra un resumen de las investigaciones acerca del uso de probióticos y prebióticos para el desarrollo de productos alimenticios orientados a la fotoprotección.

Cuadro 2.1 Composición característica de dos distintos tipos de suero de acuerdo con su pH (Jelen, 2009).

Componente	Suero de leche dulce (g/L)[a]	Suero de leche ácido (g/L)[b]
Sólidos totales	63,0-70,0	60,0-68,0
Lactosa	46,0-52,0	43,0-50,0
Proteína	6,0-10,0	6,0-8,0
Ceniza	5,0-5,7	4,8-7,0
Calcio	0,4-0,6	1,2-1,6
Fosfato	1,0-3,0	2,0-4,5
Lactato	2,0	6,4
Cloruro	1,1	1,1

[a] Con un pH neutro entre 6,2-6,5 | [b] Con pH ácido entre 4,8-4,5

2.3.7 Ensayos in vivo

Tal como se menciona en secciones anteriores, se requiere de una fuerte evidencia científica para poder afirmar que un producto sea un alimento funcional, es decir, un alimento capaz de aportar beneficios a la salud más allá de la nutrición básica. El estudio de dicha funcionalidad incluye complejas relaciones entre varios sistemas del organismo, por ejemplo, la interacción entre el sistema gastrointestinal y el sistema inmune, relación que no es reproducible de forma *in vitro* con células o tejidos cultivados en el laboratorio. Es por lo anterior, que se recurre a los ensayos *in vivo*, es decir, modelos que involucran un organismo vivo como los animales de laboratorio o pacientes y voluntarios. Debido a la naturaleza de dichos ensayos la aplicación de estos experimentos es regulada por comités de ética (Granato et al., 2017).

Uno de los animales de laboratorio más utilizado son los ratones, ya que tienen características únicas que contribuyen al estudio de la biología humana, debido a que estos roedores poseen muchas similitudes fisiológicas, genéticas e inmunológicas con los seres humanos. La estructura general del sistema inmunológico de ratones y humanos es muy similar, a pesar de la consideración necesaria de las discrepancias entre el sistema inmunitario y adaptativo del ratón y el humano (Haley, 2003; Mestas & Hughes, 2004).

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Cuadro 2.2 Investigaciones acerca del uso de probióticos y prebióticos orientados a la fotoprotección

Principio activo	Dosis	Ensayo *in vivo*	Resultados promisorios	Efectos adversos	Referencia
Modelo murino					
LTA aislado de LGG	100 μL, 1 mg/mL diario	Ratones hembra de 8 semanas, irradiados de manera crónica y a largo plazo según su grupo experimental.	Administración con LTA retrasó la aparición de tumores cutáneos en ratones con irradiación crónica.	No reporta	(Weill, Cela, Paz, Ferrari, Leoni, & Maglio, 2013)
Lactobacillus johnsonii NCC 533 (La1)	100 μL, $1x10^8$ UFC diario	Ratones hembra de 8 - 10 semanas de edad, suplementación durante 10 días. Se combina el modelo de irradiación con UVR con la sensibilización utilizando un agente químico.	El consumo del formulado tuvo un efecto protector contra la inmunosupresión que ocasiona la exposición a UVR al desafiar la piel con la prueba de sensibilización. Disminuyen la densidad de células de Langerhans y aumentan los niveles de IL-10.	No reporta	(Guéniche et al., 2006)
Modelo humano					
Lactobacillus johnsonii NCC 533 (La1) y carotenoides	$5x10^8$ UFC de La1 y 7,2 mg de carotenoides (diario)	Suplementación durante 10 semanas, en mujeres de más de 18 años.	Reducción del daño de la piel causado por exposición natural o artificial a la radiación UV en una amplia población de sujetos experimentales.	No reporta	(Bouilly-Gauthier et al., 2010)
Lactobacillus johnsonii NCC 533 (La1)	$1x10^{10}$ UFC diario	Suplementación de 8 semanas, en 54 voluntarios adultos que fueron irradiados durante el periodo de experimentación.	Recuperación temprana de las células epidérmicas. Por lo tanto, estos datos clínicos refuerzan la suposición de que ciertos probióticos pueden contribuir a modular el sistema inmunitario de la piel.	No reporta	(Guéniche et al., 2009)

2.3.8 Correlación entre inmunomodulación, fotoprotección y ensayos con alérgenos

La energía contenida en la UVR puede transferirse a diferentes moléculas dentro de las células de la piel. Esta transferencia de energía puede modificar moléculas como el ADN, lo que lleva a cambios moleculares y desata una compleja serie de respuestas celulares. Los queratinocitos, las células más abundantes en la epidermis (95% de la epidermis), son el objetivo más frecuente de la UVR. Estas células son capaces de detectar y reaccionar ante un estimulo, incluyendo la UVR, uno de los efectos más importantes de la exposición cutánea a UVR es la respuesta proinflamatoria produciendo citoquinas proinflamatorias (TNF-α, IL-1α y IL-1β, IL-6, IL-18, INF-γ), quimioquinas, factores de crecimiento y péptidos antimicrobianos. Además, de estos mediadores proinflamatorios, cuando la piel se expone a UVR, los queratinocitos y células del sistema inmune producen mediadores como las interleucinas IL-10, IL-4 y prostaglandina E2 (PGE2). A su vez la presencia de estos mediadores conlleva una serie de reacciones bioquímicas descritas por Cela *et al.* que convergen en la producción de IL-10, molécula que ha sido reconocida por tener una potente actividad antiinflamatoria de amplio espectro (Cela et al., 2018; Mosser & Zhang, 2008). Una forma análoga de estudiar el efecto que tiene la UVR sobre un sistema *in vivo* sin utilizar modelos de irradiación, es mediante la exposición a alérgenos fuertes y el estudio de la respuesta inflamatoria y el monitoreo de la interleucina IL-10.

Tanto probióticos como prebióticos se han propuesto como inmunomoduladores capaces de revertir en cierta medida la inmunosupresión ocasionada por la exposición a UVR. En el estudio realizado por Guéniche y colaboradores, se investigó si el *Lactobacillus johnsonii* (La1) tenía el potencial de modular los efectos de la UVR sobre el sistema inmune mediante la evaluación de los efectos de la hipersensibilidad, densidad de células epidérmicas Langerhans y niveles de IL-10. Este grupo de investigación encontró que al suministrar una población de 10^8 UFC/día del La1 durante 10 días los animales (Skh:hr1) conservaban la capacidad inmunitaria cutánea después de la exposición UVR.

Otro estudio, realizado por Zachariassen *et al.* (2017) bajo la hipótesis de que al inocular microorganismos probióticos provenientes de ratones con alta y baja respuesta ante un

alérgeno fuerte como el 4-etoximetilen-2-feniloxazol-5-ona (Figura 2.5 Estructura de la Oxazolon) en ratones libres de microorganismos, estos ratones inoculados tendrían una respuesta diferente a la Oxazolona en comparación con los ratones convencionales. Dicho estudio se realiza en animales de linaje C57BL/6Ntac y dentro de sus resultados destaca el impacto que tiene la microbiota sobre afecciones de la piel, en este caso, la dermatitis atópica. Este grupo de investigación utiliza un modelo de sensibilidad en oreja el cual consiste en una inducción de la inflamación de la piel utilizando Oxazolona disuelta en acetona y aceite de oliva (4:1) en ambos lados de la oreja derecha.

La prueba de sensibilidad en oreja mediante la exposición a un alérgeno fuerte ha sido utilizada en el laboratorio del Dr. Daniel González Maglio en Buenos Aires, Argentina, a manera de prueba preliminar que indique si un tratamiento es capaz de modular la respuesta inmune. De esta manera sólo aquellos tratamientos que tengan capacidad de inmunomodulación son llevados a la etapa de irradiación, la cual consiste en inducir tumores en el sujeto experimental mediante la exposición crónica o aguda (según el estudio) a UVR. Con la implementación de esta prueba preliminar se busca reducir el número de animales que son llevados al modelo de irradiación y, especialmente, no exponer de forma innecesaria a los roedores a modelos de irradiación cuando el tratamiento no tiene la capacidad de restaurar la homeostasis de la piel.

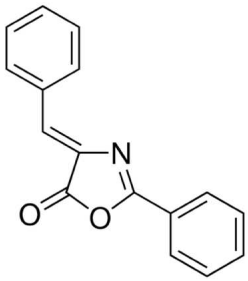

Figura 2.5 Estructura de la Oxazolona

Capítulo 3 **Hipótesis y objetivos**

3.1 Hipótesis

La administración de la bebida láctea formulada con el microorganismo probiótico *Lactobacillus rhamnosus GG* y pulpa de cas (*Psidium friedrichsthalianum*) modula la respuesta inflamatoria en un modelo *in vivo.*

3.2 Objetivo general

Desarrollar una bebida láctea a base de suero de leche utilizando el microorganismo probiótico *Lactobacillus rhamnosus GG* y cas (*Psidium friedrichsthalianum*) como componente prebiótico.

3.3 Objetivos específicos

- Diseñar e implementar el proceso de fermentación del LGG en un medio preparado a partir de suero de leche y azúcar.
- Comparar el contenido de proantocianinas de la bebida funcional con lo reportado en la literatura para fotoprotección por agentes prebióticos.
- Evaluar los productos obtenidos de las fermentaciones por medio de un análisis sensorial para seleccionar la formulación de mayor agrado ante el público.
- Determinar la viabilidad del cultivo probiótico a través del tiempo almacenamiento.
- Medir la respuesta antiinflamatoria para determinar la eficacia de la bebida.

Artículo 1. Condiciones de crecimiento y cinética de supervivencia durante el almacenamiento de Lactobacillus rhamnosus GG para el diseño de una bebida probiótica sostenible a base de suero de leche que contiene pulpa de cas

Growth conditions and survival kinetics during storage of *Lactobacillus rhamnosus* GG for the design of a sustainable probiotic whey-based beverage containing Costa Rican guava fruit pulp

Resumen

Encontrar aplicaciones económicas y prácticas para el suero de leche sigue siendo un desafío para las industrias lácteas. Este artículo presenta información sobre el desarrollo de una bebida probiótica-prebiótica a base de *Lactobacillus rhamnosus* GG (LGG) y pulpa de cas (CRG, por sus siglas en inglés) con potencial industrial. Primero, se desarrolló un medio de suero suplementado para el crecimiento de LGG, y el medio de suero suplementado se usó para la fermentación en biorreactores. Después de 48 horas de fermentación el LGG alcanzó una tasa de crecimiento máxima de 0,32 h^{-1}. Los probióticos cultivados en suero se mezclaron con pulpa CRG para producir la bebida probiótica-prebiótica. La cinética de supervivencia de LGG en la bebida formulada no se vio afectada por la adición de pulpa CRG ($P > 0.05$), y la vida útil de la bebida inoculada superó los 40 días con una población mínima de 10^6 UFC/mL. Las propiedades fisicoquímicas como el contenido de pH, fructosa, glucosa, sacarosa y proantocianidinas (PAC) exhibieron una diferencia significativa después del tiempo de almacenamiento ($P < 0.05$). Este es el primer reporte sobre las bebidas CRG como un vector probiótico.

Abstract

The finding of economical and practical applications for milk whey is still a challenge for dairy industries. This paper presents information about the development of a probiotic-prebiotic beverage based on *Lactobacillus rhamnosus* GG (LGG) and Costa Rican guava (CRG) fruit pulp with industrial potential. First, a supplemented whey media was developed for LGG growth, and the whey-supplemented media was used for fermentation in bioreactors. LGG reached a maximum growth rate of 0.32 h^{-1} after 48 hours of fermentation. The whey-grown probiotics were then mixed with CRG pulp to produce the probiotic-prebiotic beverage. The survival kinetics of LGG in the formulated drink was not affected by the addition of CRG pulp ($P>0.05$), and the shelf-life of the inoculated beverage surpassed 40 days with a minimum population of 10^6 CFU/mL. Properties as pH, fructose, glucose, sucrose, and proanthocyanidins (PACs) content exhibited a significant difference after storage time ($P<0.05$). Finally, three different formulas of the beverage with different whey content were compared through sensory evaluation. The highest acceptability was achieved with 50% whey, which remarks about the possibility of developing a probiotic whey-based beverage containing CRG. Furthermore, this is the first report about CRG beverages as a probiotic vector.

Keywords

Lactobacillus rhamnosus GG, fermentation, Costa Rican guava fruit, microbial growth, microbial survival.

Practical Application

This research focuses on the evaluation of the properties of a probiotic beverage, with a promissory industrial application using whey, as a dairy industry by-product, combined with the pulp of the highly nutritious and sub-utilized Costa Rican guava (CRG) fruit.

Introduction

Functional foods are dietary supplements that, in addition to their nutritional value, can positively modulate health on the human body (Granato et al., 2020; Guimarães et al., 2020; Yadav et al., 2019). Dairy products comprise almost 40% of the functional food market, most of them, fermented products. Some examples of successful fermented products are yogurt and kefir, besides a wide range of products supplemented with additional features, such as probiotics and prebiotics (Turkmen et al., 2019). Within this sector, the formulation of probiotic beverages using by-products and non-exploited fruits is remarkably growing research field worldwide (Mantzourani et al., 2020; Mituniewicz–Małek et al., 2019; Nazir et al., 2019; J. Ryan et al., 2020; Schoina et al., 2019).

Practical applications for the utilization of valuable food processing by-products are still a challenge for dairy industries aiming towards a circular bioeconomy (Mak et al., 2020). Whey is a yellow-green liquid rich in lactose, proteins, vitamins, and minerals. Due to its nutritional value, whey could be utilized for human consumption and microbial growth (Ahmad et al., 2019; Carlozzi et al., 2019; Cordeiro et al., 2019). About 90% of the milk volume used worldwide for cheese production is transformed into whey, generating an estimated 190 x 10^6 ton/year of this by-product. The development of whey-based biotechnological processes looks towards offering a solution for some of the whey generated (Lopes et al., 2019; M. P. Ryan & Walsh, 2016).

On the other hand, Costa Rican guava (CRG) fruit (*Psidium friedrichsthalianum* -Nied.) is a tropical fruit with a unique flavor and high vitamin C and polyphenols content (Flores et al., 2013; Gill, 2016). As Rojas-Garbanzo et al. (2019) noted, CRG could be considered as a good source of condensed and hydrolyzable tannins such as proanthocyanidins (PACs) and ellagitannins, respectively (Rojas-Garbanzo et al., 2019). PACs are highly associated with significant human health benefits such as antioxidant activity, anticancer, photoprotection, and antimicrobial properties (Katiyar et al., 2017; Kawahara et al., 2019; Rauf et al., 2019). Currently, even though CRG fruit is a known source of PACs, derived products such as beverages have not been studied as vectors for probiotics. The combination of CRG fruit pulp and whey could confer the beverage a prebiotic activity.

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Roobab et al., 2020; Zucko et al., 2020). Lactic acid bacteria (LAB) and *Bifidobacterium* represent broadly studied species of probiotics (Champagne et al., 2018; de Melo Pereira et al., 2018). Nowadays, one of the most studied LAB strains is *Lactobacillus rhamnosus* GG (LGG), isolated in 1985 from fecal samples of a healthy human adult (Gorbach et al., 2017). LGG is a gram-positive bacteria strain that does not form spores; it may grow in a temperature range between 15 °C or below 40 °C (Assaf et al., 2019; Mitra & Ghosh, 2019). LGG was isolated as part of a project to find a *Lactobacillus* strain with the requirements of an ideal probiotic (de Melo Pereira et al., 2018): resistant to stomach acid and bile (Liu et al., 2020), with the ability to adhere on human intestinal epithelial cells and to colonize the intestine (Vargas García et al., 2015), antipathogenic activity (Kamal et al., 2018; Lin et al., 2020), safety for human consumption (Scalabrin et al., 2017), and beneficial effects on human health. As Yahfoufi (2018) noted, the positive effect of probiotics and prebiotics on human health has been frequently attributed to their immunomodulatory capacity (Yahfoufi et al., 2018). There is extensive scientific evidence supporting the use of LGG in the food industry for more than 30 years (Li et al., 2016; Mitra & Ghosh, 2019).

Prebiotics are food ingredients or substances that are not digested in the upper part of the gastrointestinal tract and stimulate the bacterial growth that colonizes the large bowel (Farias et al., 2019; Gibson et al., 2017). Some prebiotics as dietary polyphenols have a close relationship with the intestinal microbiota, influencing the microbiome population (dos Santos et al., 2017; Garcia et al., 2020). It has been estimated that only 5-10% of the total polyphenols intake is absorbed in the small intestine; the other 90-95% may accumulate in the large intestinal lumen where it is subjected to enzymatic degradation by the gut's microbial population (Yadav et al., 2019).

However, polyphenols are not the only compounds with prebiotic potential in CRG fruit. Previous research demonstrated that the addition of *Psidium guajava* L. had a prebiotic effect, increasing the growth of *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* spp. *lactis* BB-12, and *Escherichia coli* (ATCC 29922) attributed to dietary fiber (Thuaytong & Anprung, 2011). Besides health benefits, it has been demonstrated that the addition of prebiotic ingredients has a positive impact on rheological, physicochemical properties and sensory characteristics (Esmaeilnejad Moghadam et al., 2019; Ferrão et al., 2018; Heydari et al., 2018).

This research focuses on the development of a probiotic whey-based beverage containing CRG pulp. In the last years, the development of innovative food products that contribute to the utilization of by-products with environmental impact reduction and considering low-cost alternatives is gaining attention in the food industry (Bigliardi & Galanakis, 2021).

This paper contributes to the development of a probiotic-prebiotic whey-based beverage containing CRG pulp. It presents practical information about the industrial potential of a new beverage, highlighting the valorization potential of whey to produce LGG and its further mix with CRG.

Materials and Methods

Costa Rican guava pulp processing

CRG fruits at maturity stage were harvested in Alajuela, Costa Rica in August 2017. The fruits were processed, as shown in Figure 1. The whole fruits were manually cut into small pieces and processed in a fruit pulper. The fruit pulp was pasteurized at 90 °C for 30 s in a cooking pot (Groen Division Corp., IL, USA). The pasteurized pulp was stored in 800 g DoyPack bags, cooled to room temperature, and stored at - 30 °C.

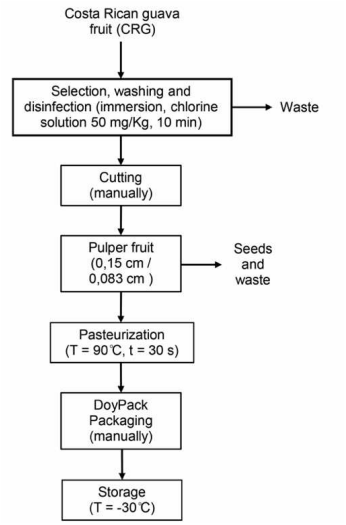

Figure 1 Process flow for producing CRG fruit pulp.

Milk whey processing

Fresh sweet whey from fresh cheese production with bright yellow-green color at 4 °C and pH 5.98 was processed in a milk skimmer (Westfalia-GEAS, Bonn, Germany) and pasteurized at 75 °C for 5 min in a cooking pot. The pasteurized milk whey was packed manually in DoyPack bags, cooled to room temperature, and stored in a freezer at -30 °C. Milk whey was used as a growth medium and later as part of the CRG beverage.

Bacterial strain and growth kinetics fermentation

The strain used was *Lactobacillus rhamnosus* GG (LGG; ATCC 53103) obtained from the American Type Culture Collection (Rockville, MD, USA). A bacterial suspension was prepared by growing LGG strain in tubes containing 10 mL of MRS broth overnight (De Man et al., 1960). Growth kinetics were studied using a whey (50% v/v), and glucose (20% w/v) medium (WGM) supplemented with different components, one per each treatment. Sterile 96-well flat-bottomed plates were filled with 250 μL per well of WGM supplemented with either (g per liter): 10 peptone, 8 meat extract, 4 yeast extract, 20 glucose, 5 K_2HPO_4, 0.2 $MnSO_4 \cdot 4H_2O$ or 0.05 $MgSO_4 \bullet 7H_2O$. MRS was used as positive control and the WGM without supplementation as the negative control.

The bacterial suspension was diluted to an initial absorbance of 0.05 at 600 nm (OD_{600}). Growth kinetics were determined by monitoring OD_{600} at 37 °C for 24 h in a microplate reader (Biotek, Winooski, VT, USA).

The best treatment was selected and the fermentation process was scaled to 7-liter in a stirred-tank batch (Applikon, Schiedam, Netherlands) with an operating volume of 4 liters, 0.5 vvm, pH 6.8, and 37 °C, in order to obtain a final population between 10^7-10^8 CFU/mL. For LGG viability cell count, samples were plated in MRS agar and incubated in aerobic conditions (37 °C for 24 h) (Brahma et al., 2019). Lactic acid production and sugars consumption were analyzed using a biochemistry analyzer (YSI Incorporated, OH, USA).

Beverage processing

The beverage was formulated in a laminar flow cabinet under aseptic conditions as following: 40 mL whey, 15 g CRG pulp, 10 mL LGG (8 log CFU/mL) for a final probiotic concentration of 7 log CFU/mL, 8 g sugar, and adjusted with water up to 100 mL (see Figure 2). Also, beverages without fruit pulp were formulated. The beverages samples were packed in glass bottles and, stored at 4±1 °C until viability cell count of LGG and physicochemical analysis were done.

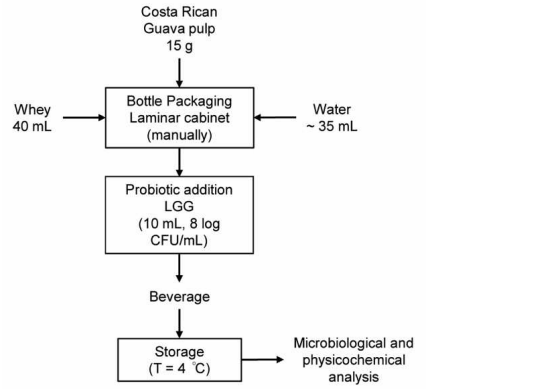

Figure 2 Process flow for CRG pulp beverage manufacturing

Survival kinetics and physicochemical characteristics

LGG viability cell count was achieved every 7 days until 56 days of storage by making serial dilutions of the beverage in sterile saline solution (0.9% NaCl) by the pour plating method in MRS agar after incubation under aerobic conditions at 37 °C for 48 h. The colony counts were expressed as log CFU/mL.

The following physicochemical properties were monitored in the beverage samples: pH, organic acids, carbohydrates, titratable acidity (942.15 AOAC), protein (920.152 AOAC), ashes (950.14 AOAC), fat and PACs by 4-Dimethylamino-cinnamaldehyde assay following the method described previously using (-)-epicatechin as a standard for the calibration curve (Sigma-Aldrich, ON, Canada) (Prior et al., 2010).

Physicochemical properties of samples without fruit pulp and CRG beverage were analyzed on day 1 and after 56 days of storage except for pH, which was measured every 7 days until 56 days of storage. The measurements were performed in triplicate.

Organic acid quantification was performed using a high-performance liquid chromatography (HPLC) system (Shimadzu, MD, USA) equipped with an autosampler, and a photodiode array detector (Shimadzu, SPD-M20AV, 210 nm). Carbohydrates were performed in a 1260 infinity Series HPLC system (Agilent, CA, USA) equipped with an autosampler, and a refractive index detector (Agilent, G1362 A). For organic acids analysis, 1 mL of sample was cleaned up using Oasis HLB cartridges (Waters, MA, USA) and filtered through a 0.45 µm membrane filter before an injection using a Hi-Plex H column (Agilent, 8 μm, 300 x 7,8 mm). The column temperature was 60°C, and the mobile phase, 2.25 mM sulfuric acid, had a flow rate of 0.5 mL/min; the injection volume was 5 µL (Toldrá & Marshall, 2018). For carbohydrate analysis, the sample was extracted using the method described by the ISO 11868:2007 (Gambelli, 2017; Ohlsson et al., 2017). The final mixture was filtered through a 0.45 µm membrane filter and analyzed using a Zorbax Carbohydrate column (Agilent, 5 μm, 4,6 x 150 mm), the column temperature was 30°C and the mobile phase, 75:25::acetonitrile:water, had a flow rate of 1.2 mL/min; the injection volume was 5 µL. The quantification of organic acids and carbohydrates was performed using a standard curve with known concentration solutions of standards, and the integration of chromatographic peaks was carried out using the LabSolutions and OpenLab CDS software, respectively. Beverage without CRG pulp was also evaluated.

Statistical analysis

The JMP software package (version 15) was used to perform a one-way analysis of variance followed by Dunnett's and Tukey as post-hoc tests to determine significant differences ($P<0.05$) on the measured parameters among samples (SAS Institute Inc. 2019).

Results and Discussion

Whey-based media optimization for LGG fermentation

A whey-based culture media was optimized to design a green and sustainable biotechnological process for LGG fermentation. Growth kinetics curves and growth parameters are shown in Figure 3 and Table 1, respectively. WGM shows a reduced growth rate compared to MRS media. After 24 hours, WGM had an OD_{600} of 1.05, whereas the culture in MRS reached an OD_{600} of 1.89. The nitrogen:carbon ratio of 1:14 was previously reported for MRS, while for WGM, a ratio of 1:6 was described (Zadow, 2003). This suggests that the WGM is a poor substrate for LGG growth, perhaps due to the lack of salts. LAB are recognized by their strict nutritional requirements, which vary among strains. Therefore, to make suitable the use of an agro-industrial by-product such as whey, it is necessary to supplement the substrates with salts as well as other components (Hayek & Ibrahim, 2013). The use of WGM without supplementation prevents the microorganism from growing adequately due to the lack of nutrients. The supplemented profile and concentrations used for the WGM in this study were derived from the ones used for the MRS medium.

The addition of glucose to WGM (up to 40%) did not improve the LGG growth, which suggests that the low growth, when compared to MRS, is not associated with the lack of a carbon source in the media. LGG growth rate in WGM was not improved by the addition of K_2HPO_4 or $MnSO_4 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$ either. In other substrates like banana purée, the addition of salts improved the *Lactobacillus casei* growth, expressed as productivity of lactic acid, with no significant differences between the supplemented media and positive control (Chan-Blanco et al., 2003; Velázquez et al., 2001).

In contrast to the addition of salts, the LGG growth was much higher in WGM after its supplementation with meat extract, peptone or yeast extract (0.29 h^{-1}, 0.32 h^{-1}, 0.32 h^{-1}, respectively) and did not show significative differences with the MRS media (0.33 h^{-1}), indicating that nitrogen WGM may be suitable for LGG growth after nitrogen supplementation.

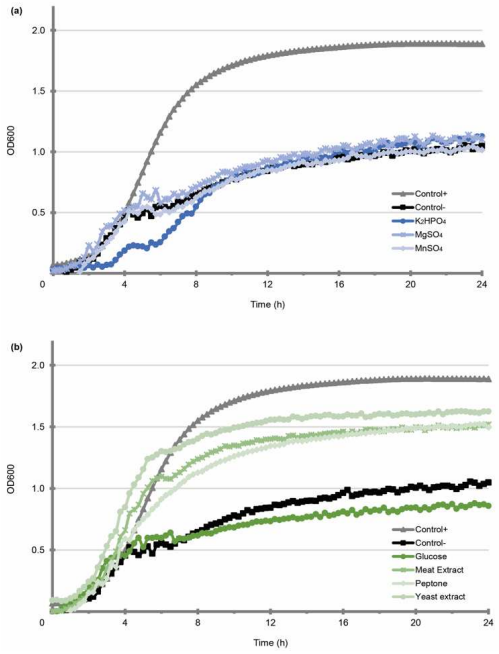

Figure 3 Kinetics of LGG with WGM supplemented with (a) different salts and (b) other components at 30 °C for 24 hours. MRS as positive control and the WGM without supplementation as the negative control. The data was expressed as a mean ± standard deviation of values obtained from triplicates of the experiments.

This is consistent with previous studies where the supplementation of glucose, yeast extract, and $(NH_4)_2SO_4$ improved in a 1.5 factor the production of lactic acid in the fermentation of *Lactobacillus delbrueckii* (Arasaratnam et al., 1996). The addition of complex organic nitrogen sources as meat extract, peptone, and yeast, provides a relatively large proportion of free amino acids, short peptides, and growth factors as manganese and magnesium, which are critical factors to the lactate dehydrogenase and protein synthesis, respectively (Abbasiliasi et al., 2011). Supplemented WGM, besides being an alternative for LAB growth, may also be used for industrial lactic acid production (López-Gómez et al., 2019). Besides the enrichment of the culture media with nutrients, the fermentation process time must be considered, which is an essential factor for industrial applications. According to Sun (2019), the use of supplemented milk with amino acids and purine reduces fermentation times by 5 h for LGG (Sun et al., 2019).

Table 1 Maximal optical density, specific growth rate and concentrations for LGG growth kinetics in medium WGM medium supplemented with different salts and other components. MRS as positive control and the WGM without supplementation as the negative control.

Media/Supplement	Maximal OD600	Growth rate (h^{-1})	Concentration ($g\ L^{-1}$)
Control+	1.891±0.005[a]	0.33±0.01[a]	-
Control-	1.05±0.04[c]	0.18±0.05[b]	-
K_2HPO_4	1.13±0.06[c]	0.12±0.02[b]	5.0
$MgSO_4 \bullet 7H_2O$	1.14±0.06[c]	0.14±0.03[b]	0.05
$MnSO_4 \bullet 4H_2O$	1.04±0.08[c,d]	0.18±0.03[b]	0.20
Glucose	0.88±0.05[d]	0.17±0.03[b]	20
Meat extract	1.52±0.07[b]	0.29±0.06[a]	8.0
Peptone	1.52±0.04[b]	0.32±0.01[a]	10
Yeast extract	1.62±0.08[b]	0.32±0.01[a]	4.0

[a]The data was expressed as a mean ± standard deviation of values obtained from triplicates of the experiments. Dunnett's as post-hoc tests to determine significant differences ($P<0.05$) using Control +. Different letters in a column values means significative differences.

LGG growth kinetics and bioreactor fermentation on supplemented MWM

The WGM supplemented with yeast extract was selected for bioreactor fermentation. Figure 4 shows the kinetics of LGG during a 48 hours fermentation process. Bioprocess parameters such as lactic acid yield and productivity were calculated to compare different endpoints of the LGG fermentation process (Chan-Blanco et al., 2003). After 48 h fermentation the process reached a lactic acid yield of 38,2%, with productivity of 0.493 g h^{-1} and an OD_{600} of 5.31. In comparison, after 28 hours, the microorganism had already consumed the total amount of glucose and sucrose and reached a lactic acid yield of 37,3% with productivity of 0.827 g h^{-1} and an OD_{600} of 5.08. The fermentation process was controlled for 48 hours; however, for an industrial application, a 28-hour would suffice considering the experimental results shown in Figure 4.

Survival kinetics, physicochemical characteristics and stability of phytochemicals during storage

In general, the critical concentration of viable probiotic cells required to meet content criteria for probiotics products in a food product matrix is about 10^6-10^7 CFU/mL (Mantzourani et al., 2020). The shelf-life of the CRG beverage inoculated with LGG surpassed 40 days with a

minimum population of 10^6 CFU/mL. The survival kinetics of LGG in the CRG beverage were not affected by the addition of CRG pulp ($P>0.05$) as compared with the no fruit added beverage. The viable count of probiotic bacteria is shown in Figure 5a.

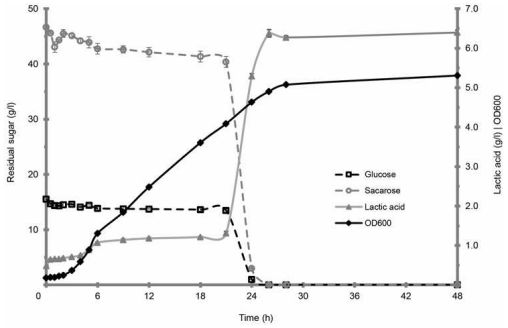

Figure 4 Kinetics of LGG in WGM medium supplemented with yeast extract in 7-liter stirred-tank batch bioreactor with an operating volume of 4 liters, 0.5 vvm, pH of 6.8 and 37 °C. The data was expressed as a mean ± standard deviation of values obtained from triplicates of the experiments.

The loss of viability of the LGG increased over time for the CRG beverage ($P<0.05$). Similar behavior was reported by Fan (2017) for *Lactobacillus acidophilus* in cucumber juice stored at 4 °C (Fan et al., 2017). Previous research demonstrated that *Lactobacillus rhamnosus* Lr-32 survival in a whey-based beverage formulated with *Psidium guajava* pulp did not show significant differences from the values determined at 7 and 14 days (Buriti et al., 2014).

As Bedani et al. demonstrated, the addition of mango and guava pulp on soy yogurt did not affect the viability of *Lactobacillus acidophilus and Bifidobacterium animalis;* nevertheless, the strains decreased its survival at *in vitro* conditions (Bedani et al., 2014). Similar results were reported by Casarotti, where the inclusion of *Psidium guajava* by-products on fermented beverages decreased in probiotic survival during the *in vitro* gastrointestinal simulation test (Casarotti et al., 2018). Conducting *in vivo* tests would be recommended to validate the synergic effect of LGG and PAC's in CRG beverage (Hill et al., 2017).

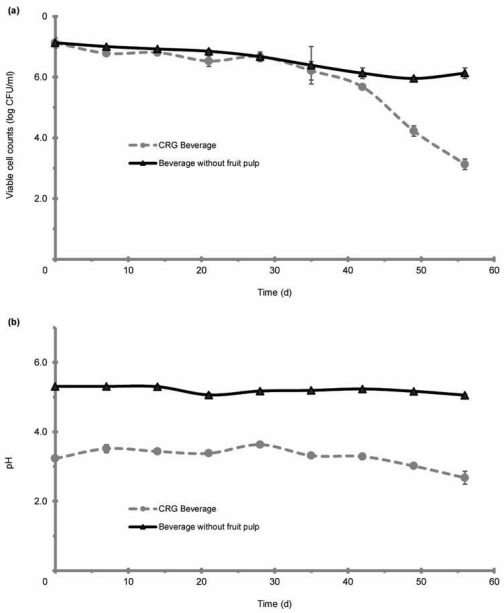

Figure 5 (a) Viability and (b) pH of LGG population of beverage without fruit pulp and CRG beverage determined weekly up to 56 days of storage. All trials were performed in triplicate.

The survival of probiotic strains in fruit matrices is still a challenge due to the adverse effects of pH and organic acid content on probiotic culture viability (Vinderola et al., 2017). Changes in pH values after beverage formulation are shown in Figure 5b. After the addition of the CRG fruit pulp, pH values decreased from 5.31 to 3.23 on the final product. Consistently, the acidity increased approximately five times, with the addition of the pulp, which is attributed to the low pH of CRG pulp (2.76) (Rojas-Garbanzo et al., 2019). The addition of inulin as a prebiotic has been studied to counteract this effect, obtaining promising results on the viability of probiotics and sensory acceptance (Buriti et al., 2007, 2010; Xavier-Santos et al., 2019). Table 2 shows the composition and physicochemical properties of the formulated beverage pH, fructose, glucose, sucrose, and proanthocyanidins (PACs) exhibit a significant difference after storage time ($P<0.05$). On our CRG beverage, the PACs content was initially 0.174 mg/100 g, and after storage study, it was around twice higher, which could be attributed to the acid hydrolysis of PACs into the monomers, especially epicatechin (Luo et al., 2018). Some characteristics as humidity, fat, galactose, lactose, and ashes did not exhibit differences among the control and the formulated CRG beverage.

Table 2 Composition and physicochemical properties of formulated beverage over time, control samples (without fruit pulp) were also evaluated. All trials were performed in triplicate.

Properties (g/100 g)	Control*	Beverage*	
		Initial	Final
pH	5.31±0.01[a]	3.23±0.03[b]	2.7±0.2[c]
Humidity	91±13[a]	91±13[a]	88±12[a]
Protein	< 0.20[a]	0.286±0.017[b]	0.264±0.016[b]
Fat	< 0.20[a]	< 0.20[a]	< 0.20[a]
Acidity	0.108±0.003[a]	0.540±0.014[b]	0.565±0.015[b]
Carbohydrates	8.6±1.4[a]	7.9±1.3[a]	10.9±1.7[a]
Fructose	< 0.13[a]	< 0.46[b]	0.91±0.13[c]
Glucose	< 0.16[a]	< 0.16[a]	0.74±0.12[b]
Galactose	< 0.21[a]	< 0.21[a]	< 0.21[a]
Sucrose	6.12±0.72[a, b]	4.73±0.56[b]	6.86±0.81[a]
Lactose	2.14±0.38[a]	1.76±0.31[a]	1.96±0.35[a]
Citric acid	0.102±0.010[a]	0.538±0.054[b]	0.564±0.056[b]
Lactic acid	8.22±0.82[a]	1.60±0.16[b]	1.49±0.15[b]
PACs** (mg/ 100 g)	n.r.	0.174±0.002[a]	0.377±0.002[b]
Ash	0.280±0.021[a]	0.294±0.022[a]	0.295±0.022[a]

*The data were expressed as a mean ± standard deviation of values obtained from triplicates of the experiments. Different letters in row values mean significant differences in comparison with the beverage without fruit pulp.
**PACs: proanthocyanidins

The formulation of fermented milk with strawberry juice and banana pulp did not favor the growth of *Lactobacillus acidophilus* and *Bifidobacterium longum* but improved *L.casei*'s growth (de Oliveira Ribeiro et al., 2020). According to De Almeida et al. and do Espíritu Santo et al., adding açai pulp in fermented milk promoted the growth of *L. acidophilus, B. longum,* and *B. lactis* (De Almeida et al., 2008; do Espírito Santo et al., 2011). Also, Oliveira Ribeiro et al. studied the interaction of different fruit matrices and probiotic strains; and suggested that the phenolic content in a juçara beverage may influence the viability of those strains, which could also be a critical factor in the survival kinetics of LGG in the formulated beverage with CRG pulp (de Oliveira Ribeiro et al., 2020).

These results bring practical information to the industrial area, highlighting both the valorization potential of cheese production by-products and sub-utilized CRG fruit pulp. The combination of whey, CRG guava pulp, and LGG could confer to the beverage prebiotic and probiotic activity.

This is especially promissory for the food industry of tropical countries where *Psidium friedrichsthalianum* -Nied. is an underutilized crop.

Conclusion

The findings of this research highlighted the possibility of combining the nutritional value of an industrial by-product such as whey and the beneficial properties of CRG to develop novel value-added products. To the best of our knowledge, there are no previous studies reporting survival kinetics of LGG in a beverage formulated with CRG pulp during storage conditions. Data shows that LGG survived in the whey-based formulated beverage, and its survival kinetics were not affected by the addition of CRG fruit pulp ($P>0.05$). The shelf-life of the inoculated whey-based CRG beverage surpassed 40 days with a minimum population of 10^6 CFU/mL, which is required to meet content criteria for probiotic products.

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Artículo 2. Recopilación de opiniones tempranas de consumidores para el proceso de creación de prototipos de una bebida probiótica con potencial fotoprotector

Collecting consumer early opinion to inform the prototyping process of a probiotic beverage with photo-protective potential

Resumen

Se desarrolló una bebida a base de suero con pulpa de cas (CRG) para obtener un alimento funcional con beneficios para la salud, como la actividad probiótica y prebiótica, centrada principalmente en una potencial actividad de fotoprotección e inmunomodulación. Con el fin de comprender las opiniones del consumidor en una fase temprana del proceso de desarrollo del producto, se recopilaron datos utilizando la técnica cualitativa "*mini focus group".* Durante la sesión, el concepto del producto fue evaluado positivamente pero el prototipo del producto tuvo un desempeño pobre en comparación con otros productos comerciales similares. En consecuencia, utilizando los comentarios de los consumidores, dos nuevas formulaciones de bebidas fueron prototipadas y probadas con un nuevo grupo de 12 panelistas que completaron una prueba hedónica informal. Un menor contenido de suero demostró generar opiniones positivas de los consumidores hacia el prototipo. La recopilación temprana de opiniones acerca del producto mediante métodos cualitativos simples o pruebas hedónicas informales hace posible incorporar ajustes clave durante las fases de creación de prototipos del producto sin incurrir en importantes inversiones de dinero. Una vez identificados e incorporados los ajustes, se deben realizar estudios formales de aceptación para completar el experimento. Por ejemplo, los estudios futuros para complementar este trabajo pueden incluir pruebas formales de aceptación sensorial del consumidor (al menos 100 consumidores) y un análisis descriptivo (panel entrenado) del producto antes de su lanzamiento.

Abstract

A probiotic whey-based beverage with Costa Rican guava (CRG) pulp was developed to offer a functional food with health benefits such as probiotic and prebiotic activity, mainly focus on potential photoprotection and immunomodulation. Data was collected using the qualitative technique “mini focus group” to understand the consumer’s opinions in an early phase of the product development process. During the session, the product concept was assessed positively, but the product prototype performed poorly in comparison with other similar commercial products. Consequently, using the feedback given by the consumers, two new beverage formulations were prototyped and tested with a new group of 12 consumers who completed an informal hedonic test. Lower contents of whey, up to 50% less, conducted to obtain more positive consumer opinions for the beverage prototype. Collecting early feedback through qualitative methods and informal hedonic tests make possible to incorporate critical adjustments during the early product prototyping phases without incurring significant money and time investments. After the essential adjustments are identified and incorporated, formal acceptance, and sensory studies need to be undertaken to complete the experiment.

Keywords

Whey-based beverage, *Lactobacillus rhamnosus* GG, Costa Rican guava fruit, general liking

Introduction

Previous research findings highlighted the possibility of an industrial application for a probiotic-prebiotic Costa Rican guava whey-based beverage. Data showed that *Lactobacillus rhamnosus* GG (LGG) survived in the whey-based beverage, and its survival kinetics were not affected by the addition of fruit pulp ($P>0.05$). The shelf-life of the inoculated beverage surpassed 40 days with a minimum population of 10^6 CFU/mL, required to meet content criteria for probiotics products.

Costa Rican guava (*Psidium friedrichsthalianum* -Nied) is one of the most cultivated species of *Psidium* (Flores et al., 2013). Its consumption has been associated with health benefits because of the content of vitamin C and polyphenols. Polyphenols compounds have become increasingly popular, the claiming of health benefits include anti-inflammatory, anti-cancer, photoprotection, and other immune-stimulating activities; for this reason, there is a growing number of functional foods that have included these compounds in different formulations (Katiyar et al., 2017; Mittal et al., 2003; Unusan, 2020). According to Rojas-Garbanzo et al., the content of proanthocyanidins in Costa Rican guava flesh is 318 mg/g dry weight approximately (Rojas-Garbanzo et al., 2019). Considering that Costa Rican guava fruit is a known source of PACs, a whey-based beverage was developed as vector for probiotics.

The insertion of new food products oriented to reduce costs, eliminate by-products, and reduced environmental impact has been gaining more attention in the business field during the last years (Bigliardi & Galanakis, 2021). Traditionally, the well-established food companies were responsible for the new trends in food products; after that, the role of consumers in market innovation has received more interest in consumer culture studies (Branstad & Solem, 2020). This research contributes to collecting early feedback through simple qualitative methods or hedonic tests to improve the prototype of a probiotic-prebiotic whey-based beverage containing Costa Rican Guava pulp. First, a prototype was compared to different commercial products with at least one ingredient in common to evaluate the general liking. After the focus group assessment, three different formulations of the prototype were designed for the taste test with potential consumers.

Materials and methods

Product prototyping

Briefly, Costa Rican guava fruits were collected at the maturity stage. Whole fruits were manually cut into small pieces and processed in a fruit pulper. The pasteurized pulp was stored at - 30 °C. Fresh milk whey from cheese production with bright yellow-green color at 4 °C and a pH 4.6 was processed in a milk skimmer. The pasteurized milk whey was packed manually in DoyPack bags, cooled to room temperature, and stored in a freezer at -30 °C. Milk whey was used as part of the Costa Rican guava beverage.

On the other hand, an optimized whey media was supplemented with yeast extract and fermented in a bioreactor for the fermentation of *Lactobacillus rhamnosus* GG (LGG). The biomass produced was used as a probiotic in the beverage. The beverage was formulated in a laminar cabinet under aseptic conditions using (per 100 mL): 40 mL whey, 15 g Costa Rican guava pulp, $1x10^7$ LGG, 8 g sugar, and water up to 100 mL. The bottles were stored at 4±1 °C. From mini focus group results, new prototypes were developed with different whey content (see Table 4).

Mini Focus Group Session

A mini focus group is a qualitative research technique that is generally used in the early stages of product development and marketing research to discuss a set of new product concepts (Barlagne et al., 2017). This experiment was conducted in San Pedro de Montes de Oca (San José, Costa Rica) at the National Center for Food Science and Technology (CITA) facilities. Four participants were recruited by a professional marketing agency (Qualimark), considering the following aspects for the screening: a) balanced gender proportion, two women and two men b) ages between 28 and 40 year old, c) living in the big metropolitan area, d) daily physical activity, e) high sun exposure, e) currently full time employed. The panelists were compensated for their participation with $40. The test objective is to compare the formulate beverage with three commercial beverages to develop a new prototype driven by potential consumer opinions.

Furthermore, to collect the early opinions of the potential consumer about the concept of the formulate beverage.

The mini focus group session was realized in a Gessel chamber, equipped with an 8-angles and audio-video recording. The moderator introduced herself, a note-taker, and a laboratory assistant. Focus group panelist was introduced to the ground rules, which included a) the freedom to participate, b) the allowance of one person talking at a time and c) the need to respect others' opinion (Lee, C. M. & Lee, 2007). The moderator facilitated the discussion according to a previously planned guideline (see Appendix 1), developed based on previous research (Chung et al., 2011; Guerrero & Xicola, 2018). The discussion lasted approximately 2 hours. First, to get everyone acquainted with one another and to get the participants were asked to think about the topic of interest, the participants were asked to state their information over a drawing of their silhouette (name, interest, likes, dislikes, healthy lifestyle definition, sun exposure hours). Then, to evaluate their response to the product concept, an activity similar to a reality show where participants can decide if they want to invest or not in a product was performed. Finally, a testing test with three commercial beverages was used for sensory evaluation to compare the liking with the whey-based beverage. A list of the products, along with a brief description, is shown in Table 3. All beverages were taken out from individual packaging and were presented in 100 mL glass cups, were labeled with letters (A, B, C, or D).

Data analysis

An unequivocal transcription was performed using the session written notes, audio, and video recordings. The transcripts were reviewed by two people, edited for clarity, and summarized by removing comments unrelated to the discussion. A grid for data analysis was built, and a thematic analysis was done to answer the next questions: Who is the target consumer? What is their concept of a healthy lifestyle? What are their reality and issues concerning solar exposure? What is their perception of functional ingredients? How do they react in response to the product idea? How do they react in response to the product prototype? How do they image the product? Image, charts, and pictures were used to support and illustrate the discussion results.

Table 3 Products used for sensory evaluation in the focus group†

Product Code	Product type	Major ingredients†
A	Smoothie	Milk, water, passion fruit powder, sugar, passion fruit essence, probiotic cultures
B	Nectar	Water, pear pulp, sugar, vitamin C
C	Home-made juice	Costa Rican guava pulp, water, sugar
D	Costa Rican guava beverage prototype	Costa Rican guava pulp, whey, water, sugar and probiotic culture

† From ingredients list shown on product packaging.

Informal hedonic test

A group of evaluators familiar with the sensory panel technique was recruited from the staff at CITA. Evaluators were recruited based on their interest in whey-based beverages and healthy product consumption habits, with a proportion of 50% male and 50% female. All evaluators participated in the test voluntarily. To ensure the evaluations were independent, each participant did the test alone.

The tasting booth was installed in a room where the ambient conditions for general illumination, temperature, and relative humidity were constant during the experiment according to international standards (ISO 8589:2007).

Three formulas were prepared (see Table 4) following the prototyping procedure explained above, the serving temperature was 5 ± 1 °C. The samples were placed into glass containers of equivalent amounts (50 mL), labeled with letters (A: original prototype, B: 50% whey or C: 0% whey).

The evaluators were welcomed and informed they were going to participate in the tasting experiment of a newly developed beverage with three different formulas. Beverages were assessed for their sensory characteristics regarding acidity, sweetness, flavor, fluidity (Schoina et al., 2019). The intensity of the studied attributes was evaluated using a 5-point just about

right scale (JAR) (1 = much too low, 2 = a little too low, 3 = just about right, 4 = a little too much, and 5 = much too much). The overall acceptability was rated on a 7-point scale for overall liking (1 = dislike very much, 2 = dislike moderately, 3 = dislike slightly, 4 = neither like nor a dislike, 5 = like slightly, 6 = like moderately, and 7 = like very much) (Narayanan et al., 2014b). The evaluators could interact freely with the sample and drink the quantity of beverage they desired. Evaluators used water to neutralize and clean their mouths between sample testing. There was no time limit for the tasting session. Thus, when evaluators decided they had finished, they filled in a form with their impression of each beverage sample.

Table 4 Products used for sensory evaluation in the hedonic test†

Ingredients (per 100 mL)	**D**	**E**	**F**
Whey	40,0	20,0	0,0
Costa Rican guava pulp (g)	15,0	15,0	15,0
Sugar (g)	8,0	8,0	8,0
Probiotic solution	10,0	10,0	10,0
Water*	27,0	47,0	67,0

† Quantity sufficient for 100 mL.

Data analyses

Information from general liking hedonic test and sensory evaluation from evaluators forms were tabulated.

Results and discussion

Mini Focus group discussion

In general, we cannot extrapolate the results obtained using this mini focus technique to a whole population of consumers. However, the attitudes and opinions of the potential consumers making up the different groups reflect trends that coincide with those obtained in similar studies carried out in more significant samples of participants (Barrios et al., 2008).

Mini focus group discussion began with a healthy lifestyle and functional food topics. Panelists recognized themselves as healthy people with regular physical activity such as soccer, golf, walking, and CrossFit. Further, panelists associated proper nutrition with home-made food and poor nutrition with fast food. They also relate sugar intake reduction, avoiding soda beverages, and whole-food ingredients with a healthy lifestyle.

Because of its probiotic and prebiotic beverage components, *Lactobacillus rhamnosus* GG and proanthocyanidins, respectively, the formulated whey-based beverage could be associated with photoprotection and inmuno-modulation effects. One of the topics discussed during the mini focus group was sun exposure experiences; each participant indicated having suffered sunburns.

Most panelists were aware that probiotics are usually used for 'stomach disease', 'diarrhea' and, 'to recover microbiota after antibiotics ingestion'. However, these asseverations did not come from panelist's actual experiences but directly from hearsay knowledge. Participants claimed to possess knowledge of antioxidants. They associated the antioxidant concept with 'to purify the blood' and 'to increase body oxygenation'. The fact that the majority of participants recognized some health benefits of antioxidants consumption seems to be influenced by the growth in sales of dark chocolate, blueberries, and cranberries products (Katz, 2012).

A consumer acceptance test and a descriptive analysis with other commercial food products were conducted. The products used for sensory evaluation in the focus group are shown in Table 3. The home-made Costa Rican guava beverage (C) was the best-received product by the panelists. On the other hand, the whey-based beverage (D) was poorly accepted. Fortification of foods with bioactive compounds occasionally generates undesirable flavors and eventually decreases the sensory acceptance of the food (Chung et al., 2011; Siró et al., 2008). Findings from this study supported the previous report because panelists expressed that the whey-based beverage was 'too fermented'. This expression was associated with probiotic addition. Three prototypes were compared to study the effect of whey content over the sensorial characteristic in the beverage of interest.

Effect of whey content on sensory characteristics of Costa Rican guava beverage

The Costa Rican guava beverage (D: 100% whey) was evaluated compared with two more new formulations of the beverage (E: 50% and F: 0% whey) to study the sensorial characteristics in function of the whey content. It is essential to mention that this sensorial evaluation was developed with descriptive purposes. To obtain quantitative results is necessary to validate these findings with a bigger sample of potential consumers (*i.e.,* 100-120 evaluators). The overall acceptability of the beverage is shown in Figure 6, the prototype with highest sensory acceptability was prototype E. The overall acceptability of the CRG beverage of this study was comparable with previous research in a probiotic beverage using different concentrations of fermented whey where beverage formulation with the best sensory quality was around 50% of whey content (Castro et al., 2013; Turkmen et al., 2019).

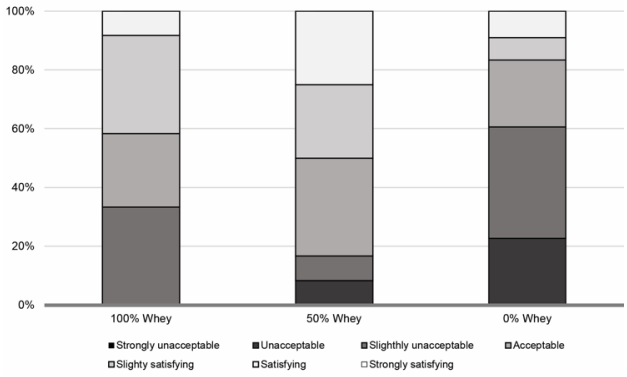

Figure 6 General liking of the prototypes in the hedonic test (D: original prototype 100% whey, E: 50% whey or F: 0% whey content).

Flavor and texture are important factors influencing the purchase decisions of health-targeted food products. Consumers may not buy and consume functional food products unacceptable in taste (Chung et al., 2011).

According to Figure 7, the organoleptic parameters of acidity, sweetness, flavor, and fluidity did not show significant differences (P>0.05) among prototypes E and F.

These results bring practical information to the industrial area, highlighting the valorization potential of cheese production by-product and sub-utilized Costa Rican guava fruit pulp. The

combination of whey, Costa Rican guava pulp, and LGG could confer to the beverage with prebiotic and probiotic activity. This result is especially promissory for the food industry of tropical countries where *Psidium friedrichsthalianum* -Nied. is an underutilized crop.

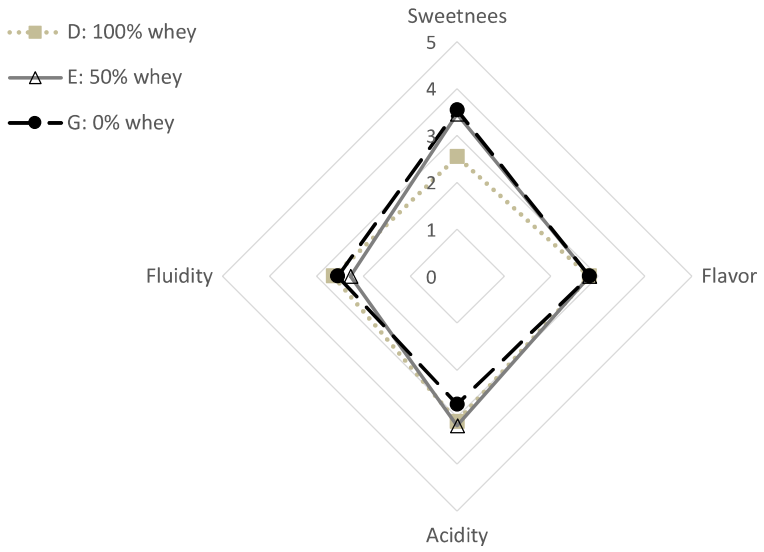

Figure 7 Sensory evaluation by descriptive analysis of CRG Beverage containing three different whey concentrations.

Conclusion

A new probiotic whey-based beverage with Costa Rican guava pulp has been developed and re-formulated based on mini focus group results. Overall sensory quality of Costa Rican guava beverage shows it possess satisfactory sensory properties when evaluated by potential consumers. The most valuable beverage prototype with the highest sensorial acceptability was the one with 50% whey content. Future studies may include a consumer acceptance test and descriptive analysis for the newly formulated beverage to identify which sensory attributes draw consumers to these products so that the results may be used to confirm the acceptance or develop a new and more palatable version of the beverage.

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Artículo 3. Ensayos in vivo: resultados preliminares

***In vivo* test: preliminary results**

Resumen

En Costa Rica, el Centro Nacional de Tumores informa que el cáncer de piel es el que tiene la mayor incidencia en el país. En los últimos años, los probióticos han surgido como una nueva estrategia en fotoprotección sistémica. El objetivo de las pruebas *in vivo* fue determinar si el consumo de la bebida formulada puede asociarse con la inmunomodulación de los sujetos experimentales. La prueba preliminar *in vivo* realizada proporcionó información para futuros experimentos. Los principales resultados fueron el establecimiento de una formulación semisólida de la bebida para una mayor facilidad de consumo por parte de los animales, la observación preliminar de un efecto sobre la inflamación asociado con el consumo de la bebida formulada y, finalmente, el establecimiento de la desviación estándar de la medición de grosor de oreja para calcular la población requerida para estudios posteriores.

Abstract

In Costa Rica, the National Tumor Center reports that skin cancer has the highest incidence in the country. In recent years, probiotics have emerged as a new strategy in systemic photoprotection. The *in vivo* tests' objective was to determine if the consumption of the formulated drink can be associated with the immunomodulation of the experimental subjects. The *in vivo* test carried out provided information for future experiments. The main results were establishing of a semi-solid formulation of the beverage for greater ease of consumption by animals. Also, the preliminary observation of an effect on inflammation associated with consumption of the formulated beverage, and finally, the determination of the standard deviation of the ear thickness measurement to calculate the population required for further studies.

Keywords

Whey-based beverage, *Lactobacillus rhamnosus* GG, Costa Rican guava fruit and, photoprotection, *in vivo* test

Introduction

The incidence of non-melanoma and melanoma skin cancer is equivalent to the sum of all other organs combined (Cleaver, 2002; Narayanan et al., 2010). In Costa Rica, the National Tumor Center reports that skin cancer has the highest incidence in the country, in men and women. Ultraviolet radiation (UVR) prolonged exposure has been presented as the leading cause of skin cancer. Probiotics and prebiotics have been demonstrated to successfully prevent some of the harmful effects of UVR on skin health.

In recent years, probiotics have emerged as a new strategy in systemic photoprotection (Bouilly-Gauthier et al., 2010). In 2013, a research group from the University of Buenos Aires (Argentina) determined that lipoteichoic acid (LTA, a structural component of the cell wall) from *Lactobacillus rhamnosus* GG (LGG) can restore homeostasis of the skin affected by UVR (Weill, Cela, Paz, Ferrari, Leoni, & Maglio, 2013). LTA is a teichoic acid that is part of the cytoplasmic membrane of Gram-positive bacteria as lactobacilli and is well known for its immunostimulant activity. LTA molecule consists of poly-glycerol-phosphate units with D-alanyl esters. Weill et al. (2013) report that oral administration of LTA in mice restores skin homeostasis after UVR exposure, reducing the tumor appearance in LTA-treated animals (Weill et al., 2013). As Klaenhammer (2012) noted, the positive effect of probiotics and prebiotics on human health has been frequently attributed to their capacity for immunomodulation (Klaenhammer et al., 2012).

In addition to photoprotection from biological components, there is scientific evidence of the preventive effect of some molecules (prebiotics) against the damage caused by UVR on the skin. The mechanisms of action vary, including anti-inflammatory, antioxidant, and immunomodulatory activity (Gilaberte & González, 2010). PACs, a type of polyphenols, are highly associated with significant human health benefits such as antioxidant activity, anticancer, photoprotection, and antimicrobial properties. Katiyar *et al.* used an extract of PACs from the grape seed and demonstrated their anticarcinogenic capacity in non-melanoma skin cancer. Preliminary studies show that Costa Rica guava (*Psidium friedrichsthalianum)* (CRG) pulp is a PACs source with significant antioxidant activity in vivo (González et al., 2012; Rojas-Garbanzo et al., 2019). No reports are associated with PACs from CRG with immunomodulatory activity. However, since the activity is attributed to the molecule and not the food matrix, we hypothesize

that the administration of a drink formulated with the probiotic LGG and CRG pulp will have immunomodulating effects. An enhanced activity is expected due to the synergy of both components.

In a study carried out by Zachariassen *et al.* (2017) under the hypothesis that by inoculating probiotic microorganisms from mice with a high and low response to a potent allergen such as 4-ethoxymethylene-2-phenyloxazol-5-one (Oxazolone) in mice free of microorganisms, a different response to Oxazolone compared to conventional mice would be observed. This study was carried out on animals of the C57BL/6Ntac lineage, and its results highlight the impact that the microbiota has on skin conditions, in this case, topical dermatitis. This research group uses an ear sensitivity model, which consists of an induction of skin inflammation using Oxazolone dissolved in acetone and olive oil (4: 1) on both sides of the right ear. This protocol is described in detail below.

The ear sensitivity test using a potent allergen has been used in the laboratory of Dr. González Maglio (Argentina) as a preliminary test indicating whether a treatment can modulate the immune response (Weill, Cela, Paz, Ferrari, Leoni, & Maglio, 2013).

Briefly, the energy contained in ultraviolet radiation (UVR) can be transferred to different molecules within skin cells. This energy transfer can modify molecules such as DNA, leading to molecular changes and triggering a complex series of cellular responses. Keratinocytes, the most abundant cells in the epidermis (95% of the epidermis), are the most frequent target of UVR. These cells are capable of detecting and reacting to a stimulus, including UVR, one of the most important effects of skin exposure to UVR is the pro-inflammatory response producing pro-inflammatory cytokines (TNF-α, IL-1α and IL -1β, IL-6, IL-18, INF-γ), chemokines, growth factors, and antimicrobial peptides. In addition to these pro-inflammatory mediators, when the skin is exposed to UVR, keratinocytes, and cells of the immune system produce mediators such as interleukins IL-10, IL-4, and prostaglandin E2 (PGE2). The presence of these mediators leads to a series of biochemical reactions described by Cela *et al.* that converge in the production of IL-10, a molecule that has been recognized for having a potent broad-spectrum anti-inflammatory activity. An analogous way of studying the effect that UVR has on an in vivo system without using irradiation models, It is by exposure to strong allergens and the study of the inflammatory response and monitoring of interleukin IL-10.

Within the framework of this general project, the *in vivo* tests' objective was to determine if the formulated drink's consumption can be associated with the immunomodulation of the experimental subjects. The presence of positive results can be extrapolated, with the drink's ability to restore homeostasis of cells exposed to UVR, that is, attribute a photoprotective effect to it. As Cela *et al*. noted, keratinocytes are the most significant component of the skin. Therefore, keratinocytes' response to UVR is also the response of the skin (Cela et al., 2015). Keratinocytes produce a series of pro-inflammatory cytokines that converge in the production of cytokine IL-10. The results obtained in these *in vivo* tests will allow concluding whether or not the formulated drink has immunomodulating properties. The pilot test's objective is to determine the standard deviation of the response variable: ear thickness measurement with digital Vernier (mm).

Materials and methods

The study was approved by the Comité Institucional para el Cuidado y Uso de los Animales (CICUA-UCR) and Comité Interno para el Cuido y Uso de Animales de Laboratorio (CICUA CENIBiot-Speratum). The study lasted three days in mice of the *Mus musculus* lineage species C57BL/6Cr with eight weeks of age, so that the immune system is fully developed. A sample of 6 mice is proposed, located in three boxes. This experiment was coordinated with the person in charge of the animal facility by CENIBiot.

In this first trial, the animals were divided into two experimental groups:

- Group A: Negative control: whey
- Group B: Treatment: drink with probiotic and Costa Rican guava pulp

Stage 1

Animals started with the diet according to their experimental group (A, B). The pilot trial conditioned animals to consume a feed supplement formulation. The beverage was formulated a semi-solid version using jelly to facilitate the consumption of mice. Stage 1 lasts 2 days.

Stage 2

All mice were be sensitized once with 0.8% (m/v) (Oxazolone) (Sigma-Aldrich) dissolved 4:1 in acetone and olive oil with a micropipette on both sides of the ear. Drink administration and

control are continued, respectively. After one day, the animals are challenged with a new 0.4% (m/v) solution of Oxazolone dissolved in acetone and 4:1 olive oil, on both sides of the ear, placing a drop (25 μL). All mice were sacrificed after experimentation.

Results and discussion

The *in vivo* test carried out provided preliminary information for subsequent tests. The main achievements were the establishment of a semi-solid formulation of the drink for greater ease of consumption by animals, the preliminary observation of an effect on inflammation associated with consumption of the formulated drink, and finally the establishment of the standard deviation of the ear measurement to calculate the population required for further studies.

Among the recommendations for subsequent studies, a more compact formulation is included to avoid reducing the animal's consumption time and avoiding waste. The interaction of the mouse with the drink in its semi-solid formulation is shown in Figure 8. This process was essential because there was the possibility of rejection by the animal towards the sample; however, after approximately 10-15 minutes, the animal begins to interact with the product and consume it.

a b

Figure 8 Mouse interaction with semi-solid formulation of the beverage.

A minor increase in the thickness of the ear was observed in the mice that received the drink (see Figure 9). The coefficient of variation of this measurement was 8.6%, which, according to area researchers, is acceptable in biological tests. Given this information, it is estimated that subsequent trials will require at least 7 mice per treatment to obtain a statistically significant result.

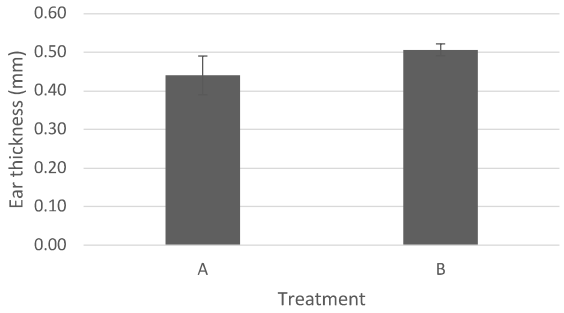

Figure 9 Mean and standard deviation for ear thickness measure.

Conclusions

The *in vivo* test carried out provides essential information for subsequent tests. The main achievements are the establishment of a semi-solid formulation of the beverage for greater ease of consumption by animals, the preliminary observation of an effect on inflammation associated with consumption of the formulated beverage, and finally the establishment of the standard deviation of the ear thickness measurement in order to calculate the population required for further studies.

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Capítulo 4 **Observaciones generales**

Este apartado incluye comentarios adicionales que se consideran de valor para el lector de este trabajo.

Artículo 2

Debido a que en el mercado nacional no existe un producto que incluya pulpa de fruta y suero de leche, los productos utilizados en el mini *focus group* se basan en la similitud con alguno de los elementos de la bebida formulada.

Artículo 3

Tal como se mencionó en capítulos anteriores el uso de ratones en experimentación provee información valiosa y escalable para aplicaciones humanas debido a las similitudes fisiológicas, genéticas e inmunológicas entre ambos seres vivos. Sin embargo, los experimentos con animales tienen implicaciones bioéticas que deben ser consideradas en los experimentos realizados y siempre que sea posible debe preferirse el estudio en líneas celulares.

En este estudio uno de los principales retos fue la administración de la bebida. La literatura reporta que una de las técnicas más utilizadas es la canulación de traquestomía, sin embargo, esto requiere un amplío entrenamiento y aún así se corre el riesgo de lesionar al ratón e incluso causarle la muerte. Una opción alterna y que genera menos estrés en el animal es lograr que lo consuma como parte de la dieta. Sustituir el agua del bebedero del animal por el producto de interés no es una opción viable, debido a que si al animal no le gusta el producto no lo va a ingerir y podría caer en estado de deshidratación. Por este motivo se recurre a una formulación alterna de la bebida, en una presentación semi-sólida en donde se sustituye la cantidad de agua (35 mL de cada 100 mL de bebida) por gelatina sin sabor (ver Figura 2, artículo 1).

Esta formulación semi-sólida, que consiste en incorporar gelatina sin sabor a la bebida, también puede ser un producto alternativo interesante, por ejemplo, para personas que practican deportes.

Publicación

Los resultados de los artículos 1 y 2 de este documento fueron publicados en la revista Journal of Food Science de Wiley Online Library, el documento se encuentra disponible en el siguiente enlace: https://onlinelibrary.wiley.com/doi/10.1111/1750-3841.15430. Debido a lo anterior, en el Anexo 3 se incluye la licencia por parte de Wiley para incluir la publicación dentro de esta tesis.

Capítulo 5 **Conclusiones y recomendaciones**

5.1 Conclusiones generales

Se presenta en este capítulo las conclusiones obtenidas a partir de la investigación y las recomendaciones en caso de trabajos posteriores en este tema.

Los hallazgos en esta investigación señalan la posibilidad de combinar el valor nutricional de un subproducto como el suero y las propiedades del cas para desarrollar novedosos productos con valor agregado. Al momento de la redacción de este documento, no existen otros estudios acerca de la supervivencia de LGG en una bebida formulada con pulpa de cas en condiciones de almacenamiento. Los datos muestran que LGG sobrevive en la bebida formulada a base de suero y su cinética de supervivencia no se afectó negativamente por la adición de la pulpa de cas ($P>0.05$). La vida útil de la bebida inoculada a base de suero y cas sobrepasó 40 días con una población mínima de 10^6 CFU/mL, lo cual se requiere para alcanzar los criterios de productos probióticos.

La calidad sensorial general de la bebida formulada muestra que posee propiedades sensoriales satisfactorias cuando es evaluada por consumidores potenciales. El prototipo de bebida más valioso con la mayor aceptabilidad sensorial fue el que contenía 20% de suero (v/v). Un ensayo preliminar indica que la ingesta de la bebida podría modificar la forma en que el sistema inmunológico de ratones de laboratorio responden frente a un alérgeno, es decir, la bebida logra modificar la respuesta inmune, lo que a su vez se puede relacionar mediante un serie de reacciones bioquímicas con el concepto de fotoprotección.

5.2 Recomendaciones generales

Los estudios de análisis sensorial deben ser validados con una muestra mayor de potenciales consumidores, por ejemplo, 100-120 evaluadores. Los estudios posteriores deben incluir un análisis descriptivo de los atributos sensoriales de la bebida. Adicionalmente, se requiere realizar ensayos *in vivo* para corroborar la relación entre la ingesta de la bebida, inmunomodulación y fotoprotección.

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Anexo 1. Guía de sesión grupal

0. OBJETIVOS
-Explorar el concepto de producto propuesto junto con el target
-Identificar posibles mejoras en el producto desarrollado

1. REQUERIMIENTOS

Recurso humano

1 moderador
1 asistente
1 documentador
4 potenciales consumidores (deportistas alta exposición al sol)

Materiales

1 plantilla para el ejercicio de presentación de los participantes
post its
marcadores
board de concepto de productos
formulario de evaluación de prototipos
plantilla para imaginario de producto

Espacio y equipamiento

1 sala de trabajo grupal con ventilación y luz adecuada
1 proyector
1 grabadora de sonido
1 cámara fotográfica

3. CRONOGRAMA

Tiempo	Actividad	Datos
00:05	Presentación y bienvenida	Notas
00:15	Presentación de los participantes	Recolección de material visual
00:25	Estilo de vida saludable	Post its / Grabación
00:35	Exposición solar	Post its / Grabación
00:45	Presentación del concepto	Grabación
00:55	Degustación de prototipos	Hoja de evaluación / Grabación
01:10	Cómo se imagina este producto	Hoja de trabajo / Grabación
01:15	Cierre de las actividades y agradecimiento	Notas

4. DESCRIPCIÓN DE LAS ACTIVIDADES

Presentación y bienvenida
-Los participantes se reciben en la sala de trabajo en grupo.
-Se da una bienvenida y un agradecimiento por venir.
-Se explica brevemente de qué se trata la invesigación.
-Se explica brevemente el cronograma.
-Se inicia con el primer ejercicio

Presentación de los participantes
- Se reparte una hoja con un diagrama de una persona en el que se resaltan su cerebro, sus manos y su su corazón. Los participantes deben completar cada sección: manos-actividades que practican con frecuencia, cerebro-cosas en las que piensan constantemente cómo metas, proyectos en ejecución, entre otros , corazón: cosas que aman u odian.

Estilo de vida saludable
-Todos ustedes tienen en común una gran pasión por mantener un estilo de vida saludable, pueden contarme un poco de sus rutinas diarias y sus hábitos para lograr este objetivo. Escribir en post its y colocar en el board.
-Cuáles son los 5 alimentos que a usted no le pueden faltar con el fin de lograr sus objetivos?
-Qué ha escuchado con respecto a los probióticos y los antioxidantes?

Exposición solar
-Entiendo que la mayoría de ustedes se exponen al sol por muchas horas y que esto les preocupa. Pueden contarme sus experiencias al respecto?
-Cuales son las medidas que de prevención que han tomado hasta el momento?
-Ha escuchado algo con respecto a la fotoprotección oral? Han intentado con estos productos anteriormente?

Presentación del concepto
-Hagamos de cuenta que ustedes son inversionistas y yo les vengo a hacer una propuesta de producto al estilo de “shark tanks”:

-Estamos desarrollando una bebida para ayudar a personas como ustedes. La bebida esta diseñada para que tenga un alto aporte protético y antioxidante; pero al mismo tiempo contiene una gran cantidad de probióticos fotoprotectores.

Quiénes están convencidos a un 100% y quieren invertir?
Quiénes están convencidos a un 50% y quieren invertir?
Quiénes no están convencidos y no quieren invertir?

Degustación de prototipos
-Vamos a probar algunos prototipos del producto que estamos desarrollando para que ustedes nos digan que piensan al respecto. Van a llenar individualmente la hoja de evaluación y cuando todos estemos listos la vamos a discutir.

Cómo se imagina este producto
-Ya que hemos hablado tanto de este producto queremos saber cómo usted se lo imagina. Para eso les vamos a dar esta plantilla la cual vamos a completar de manera individual.

Cierre de la actividad
-Se indica que la actividad a finalizado
-Se realiza un agradecimiento nuevamente

Anexo 2. Guía de sesión grupal versión en inglés

1. **INTRODUCTION (10 min)**

1.1. Moderator's introduction

a. General nature and purpose of a focus group

b. Role of the moderator

1.2. Objectives of this focus group

1.3. Group rules

a. Free to participate or not participate at any time

b. One person talking at a time

c. Respect other's opinions

1.4. Mention incentive and taping of the focus group

2. **WARM-UP (20 min)**

2.1. Self-introduction

a. State your name

2.2. Interest on the topic

a. Briefly describe the functional foods that you have consumed recently

b. What is the expectation when you consume and purchase those functional foods besides regular meals?

3. PROBING QUESTIONS (10 min)

3.1. Prototypes tasting

3.2. Packaging proposal for the product

4. CLOSE (5min)

4.1. Thank you for your time.

4.3. Distribute incentive.

Anexo 3. Términos y condiciones de la revista Journal of Food Science – Wiley: aprobación de inclusión de artículo publicado en documento de tesis

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