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Estudio y evaluación de ingredientes desarrollados a partir de subproductos de uva con alto contenido de compuestos fenólicos y sus nuevos usos en la agricultura

Study and evaluation of ingredients based on grape wastes with high content of phenolic compounds and new uses in agriculture

Tesis para optar al grado de Doctor en Ciencias de la Agronomía

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Dedicatoria

A mis hijos y mi esposa que son el motor que me lleva a trabajar por ser mejor persona.

A mis padres, por entregarme educación y valores, por siempre creer en mí.

A mis amigos que sin nombrarlos saben quiénes son, por estar ahí en los buenos momentos pero sobre todo en los tiempos difíciles.

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En los últimos años ha aumentado la preocupación y la búsqueda de soluciones para el tratamiento de los materiales de desecho generados por la industria del vino. Estos residuos generados en la producción de vino se caracterizan por la alta concentración de antioxidantes naturales, donde, algunos de ellos ya se están reutilizando en las industria alimentaria y cosmética. En el ámbito agrícola, los subproductos del vino se están utilizando principalmente como acondicionadores del suelo, como adsorbentes para metales pesados y como fertilizantes.

El presente trabajo estudió y evaluó el potencial bioactivo de los residuos de orujos de uva, var. Tintorera, obtenidos como subproductos del proceso de vinificación, así como la formulación, encapsulación y estabilidad de un suplemento dietético para polinizadores. El estudio se focalizó en el potencial antiviral de este ingrediente, específicamente sobre virus de abejas. Para ello, el Capítulo II entrega una revisión bibliográfica de los trabajos actuales para las distintas aplicaciones de los orujos y su actividad antiviral, donde se documenta información actualizada de los conocimientos sobre la composición nutricional y fitoquímica de los residuos vitivinícolas, convirtiéndolos en una alternativa viable para un manejo sustentable y reducir el impacto medioambiental de la producción de vino en todo el mundo.

Una vez conocida las características de los distintos subproductos de la vinificación, en el Capítulo III se presenta un estudio sobre la formulación del suplemento dietético para polinizadores con alto valor bioactivo, empleando la técnica de *Spray Drying* (una de las técnicas de encapsulación más utilizadas en la industria alimentaria), la caracterización del suplemento obtenido y posteriormente el

establecimiento de un ensayo *in vivo*. El objetivo principal de este ensayo fue evaluar la capacidad antiviral del suplemento en particular, su efecto contra el virus de las alas deformes (DWV) en abejas melíferas (*Apis mellifera*) a través de la respuesta del sistema inmune de las abejas. Para ello, se aplicaron diferentes dosis del suplemento previamente formulado (0,5; 1; 2,5; 5%) de extracto de orujo de uva (*Vitis vinifera* L.) en la dieta de abejas. La detección del efecto antiviral se realizó mediante el método de cuantificación de PCR en tiempo real (qPCR). Entre los principales resultados obtenidos se pudo destacar que el suplemento dietético, después del tratamiento térmico, mantuvo un alto contenido de antocianos totales (1102,45 mg 100 g^{-1}) y logró disminuir la carga de DWV en las abejas melíferas a través del tiempo, concluyendo que el contenido bioactivos presentes en el suplemento desarrollado en este estudio tiene un efecto positivo en el sistema inmunitario de las abejas frente a la presencia del DWV.

ABSTRACT

In recent years, the concern and search for solutions for the treatment of waste materials generated by the wine industry have increased. These by-products generated in the production of wine are characterized by the high concentration of natural antioxidants, some of which are already being reused in the food and cosmetic industries. In the agricultural field, wine wastes are mainly being used as soil conditioners, adsorbents for heavy metals and fertilizers.

The present work studied and evaluated the bioactive potential of grape pomace wastes var. Tintorera, obtained as by-products of the vinification process, as well as the formulation, encapsulation and stability of a dietary supplement for pollinators. The antiviral potential of this ingredient, specifically on bee viruses was specifically studied. To carry out this, Chapter II provides a bibliographic review of the current works for the different applications of pomace and its antiviral activity, where updated information on the knowledge about the nutritional and phytochemical composition of wine wastes is documented, making them a viable alternative for sustainable management and reducing the environmental impact of wine production around the world.

After the characteristics of the different vinification by-products are known, Chapter III presents a study on the formulation of the dietary supplement with high bioactive value, using the Spray Drying technique (one of the most widely used encapsulation techniques in the food industry), a characterization of the supplement obtained and subsequently the establishment of an *in vivo* test. The main objective of this trial was to evaluate the antiviral capacity of the supplement in particular, its effect against deformed wing virus (DWV) in honeybees (*Apis mellifera*). For this, different doses of

the previously formulated supplement (0.5, 1, 2.5 and 5%) of grape pomace extract (*Vitis vinifera* L.) were applied to the diet of bees. The detection of the antiviral effect was performed using the real-time PCR quantification method (qPCR). Among the main results obtained, it can be highlighted that the dietary supplement, in spite of drying, reached a high content of total anthocyanins (1102.45 mg 100 g^{-1}) and managed to reduce the load of DWV in honey bees over time, concluding that the bioactive content present in the supplement developed in this study had a positive effect on the immune system of bees against the presence of DWV.

CAPÍTULO I

INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. Importancia del aprovechamiento de los residuos de la vinificación

La uva es uno de los frutos con una alta producción a lo largo del mundo, con valores de más de 75 millones de toneladas anuales, donde el 80% de la producción de la uva está destinada a la fabricación de vino (Fontana et al., 2017).

El proceso de vinificación se divide en diversas etapas como la cosecha, el triturado, maceración, la fermentación, maduración, clarificación y embotellado. En cada una de estas etapas se generan residuos particulares, los más destacados del proceso de vinificación son el orujo de uva, semillas, tallos, lías y aguas residuales (Jackson, 2020).

Gran parte de los residuos generados en la industria vinícola se caracterizan por poseer como metabolitos secundarios compuestos fenólicos, los cuales se extraen principalmente del hollejo y semillas. Estos compuestos fenólicos son de importancia por su impacto en la salud, ya sea como antioxidantes, o bien por sus propiedades antimicrobianas y antivirales (Souza et al., 2014).

El orujo de la uva es el residuo más abundante de la industria vitivinícola y está formado principalmente por material lignocelulósico (Mattos et al., 2017). Este subproducto se obtiene tras el prensado y la fermentación de la uva, y representa alrededor del 20% en peso del total de la uva procesada (Fontana et al., 2013). El

orujo de uva es nutricionalmente muy rico, teniendo aproximadamente un 40% de fibra dietética, 16% de aceite esencial, 11% de proteína, 7% de compuestos fenólicos, entre otras sustancias (Bordiga et al., 2019).

Las semillas representan entre el 2 y el 5% del peso de la uva y constituyen aproximadamente el 38-52% de los residuos sólidos generados por las industrias vitivinícolas (Beres et al., 2016). En general, contienen alrededor de un 40% de fibra, 10-20% de lípidos, 10% de proteínas, complejos fenólicos, así como azúcares y minerales (Ye et al., 2016). Las fracciones indigeribles, principalmente celulosa y pectinas, son los principales constituyentes de las semillas de uva, representando alrededor del 80% de la materia seca sin azúcar (Beres et al., 2017).

Las semillas de uva se valoran principalmente por las propiedades nutricionales del aceite, que es rico en ácidos grasos insaturados (oleico y linoleico) y compuestos fenólicos (Gupta et al., 2020).

Todos los residuos y subproductos generados tienen un gran potencial, sin embargo, en esta tesis, nos centramos en el estudio del orujo y en el desarrollo de nuevos productos de interés agrícola en base a orujo.

1.2. Compuestos fenólicos encontrados en residuos de uva

En la naturaleza existe una amplia variedad de compuestos que presentan una estructura molecular caracterizada por la presencia de uno o varios anillos fenólicos. Estos compuestos podemos denominarlos compuestos fenólicos. Se

originan principalmente en las plantas, que los sintetizan en gran cantidad, como producto de su metabolismo secundario. Algunos son indispensables para las funciones fisiológicas vegetales. Otros participan en funciones de defensa ante situaciones de estrés y estímulos diversos (hídrico, luminoso, etc.). Existen varias clases y subclases de compuestos fenólicos que se definen en función del número de anillos fenólicos que poseen y de los elementos estructurales que presentan estos anillos. Los principales grupos de compuestos fenólicos son: ácidos fenólicos (derivados del ácido hidroxibenzoico o del ácido hidroxicinámico), estilbenos, lignanos, alcoholes fenólicos y flavonoides (Vidal-Casanella et al., 2021).

Los polifenoles pertenecen al grupo de flavonoides, y se encuentran en una amplia variedad de fuentes de alimentos siendo los ingredientes activos más comunes de los constituyentes de plantas con base fenólica. Debido a sus característicos grupos fenólicos OH, tienen la capacidad de quelar iones metálicos altamente redox activos, lo que refuerza su efecto protector contra el daño oxidativo (Chaudhary et al., 2015).

Los compuestos fenólicos están presentes en diferentes partes de la planta y juegan un rol de protección contra enfermedades, plagas y condiciones ambientales adversas. Son generados como una respuesta al estrés. Mientras más estrés se genere en la planta, más compuestos fenólicos son biosintetizados. Siendo así, cada variedad o cultivar tiene una composición fenólica propia, que está fuertemente condicionada por factores agronómicos y ambientales, como el

ataque de hongos, restricción hídrica, radiación ultravioleta y variación de temperatura (Pascual et al., 2017).

La función protectora de los compuestos fenólicos en la planta puede ser extrapolada al ser humano. Se ha demostrado que la ingesta continua de estos compuestos previene el daño producido por especies reactivas del oxígeno (ROS). Un exceso de ROS en el organismo puede potenciar el desarrollo de enfermedades crónicas no transmisibles como cáncer, desórdenes cardiovasculares, daño neurodegenerativo, Alzheimer e inflamaciones en diferentes órganos (Ballance et al., 2019).

Dentro de los compuestos fenólicos se destacan los flavonoides. En la naturaleza, los flavonoides se pueden extraer de distintas partes de la planta y actualmente hay cerca de 6.000 flavonoides estudiados que contribuyen a los coloridos pigmentos de frutas, hierbas, vegetales y plantas medicinales (Manzoor et al., 2020). Constituyen, también, una de las clases más características de compuestos en plantas superiores y comprende subgrupos como las chalconas, antocianos, antocianidinas, flavonoles, flavanoles, flavonas e isoflavonas (Figura 1).

Los beneficios que estos compuestos derivados del metabolismo secundario tienen frente a la salud humana se han determinado en numerosos estudios. Se ha reportado actividad antinflamatoria (Read, 1995; Joseph *et al*.,2014), frente al síndrome metabólico en adultos mayores, específicamente en enfermedades

crónicas como diabetes y obesidad (Tsuda, 2016) y problemas cardiovasculares (Wightman y Heuberger, 2015).

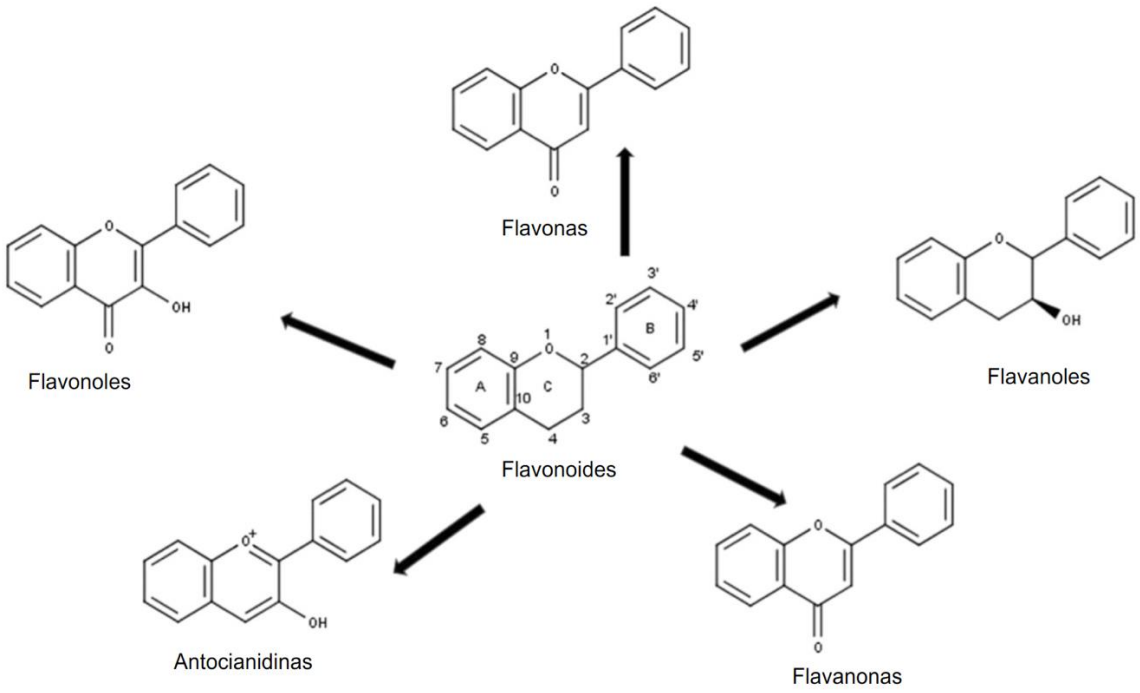

Figura 1. Estructura de los distintos flavonoides.

La clasificación de los flavonoides depende de las diferencias en la estructura del anillo C. Así, los flavonoides pueden clasificarse en flavonoles (miricetina, quercetina y kaempferol), flavonas (apigenina y luteolina), flavanoles (catequinas, epicatequina, epigalocatequina y galato de epicatequina), flavanonas (naringenina), antocianidinas o isoflavonoides (genisteína, daidzeína, dihidrodaidzeína y equol) (Garrido and Borges, 2013).

La capacidad antioxidante está determinada por la facilidad que tienen los compuestos descritos anteriormente (flavonoides y concretamente antocianinas)

para atrapar radicales libres o iones metálicos para evitar procesos oxidativos a nivel celular o de ADN y así aportar beneficios a la salud (Shen et al., 2022).

Los antocianos, antocianidinas unidas a un azúcar, son responsables de la coloración (naranjo, rosado, rojo, violeta y azul) de los pétalos de las flores y frutos de gran variedad de plantas (Lu et al., 2021). Existen numerosas fuentes de antocianos, siendo una de las principales fuentes el orujo del proceso de vinificación de vinos tinto. Después del primero aislamiento de la enocianina, que data de 1879, numerosos autores han desarrollado patentes para la producción de soluciones acuosas concentradas de antocianos para uso alimentario (Markakis, 2012). El contenido de polifenoles a partir de orujo puede alcanzar una alta capacidad antioxidante con concentraciones que van desde 11,7% hasta 86,3% (Castrica et al., 2019). Hoy en día, la Unión Europea permite el uso de los antocianos como colorante alimentario de bebidas, mermeladas, pastelería, helados y productos farmacéuticos (Commission, 2011).

Los antocianos son responsables del color y aroma de las flores y frutas y aportan en el desarrollo y buen funcionamiento de las plantas, ya que actúan como señales alelopáticas, atrayentes o repelentes de insectos, al igual que como agentes protectores contra la luz UV y otros factores abióticos y bióticos. En cuanto al color, confieren una gran diversidad de colores, tocando prácticamente todos los espectros visibles, desde naranja y rojo hasta tonos púrpura y azul (Fernandes, 2014). Adicionalmente presentan propiedades relacionadas con la salud humana basado en su actividad antioxidante.

Aunque se conocen más de 550 antocianos sólo unas pocas antocianidinas son encontradas en las flores, frutas y en las plantas en general. Estas son cianidina, delfinidina, pelargonidina, peonidina, malvidina y petunidina, que se enlazan con diferentes azúcares. Los antocianos como la malvidina 3-glucósido, la delfinidina 3-glucósido y el cumarato de malvidina 3-glucósido son los compuestos predominantes de todos los berries comestibles (Braga *et al.*, 2018)

Los compuestos fenólicos y la capacidad antioxidante contenida en un producto han tomado importancia en la elaboración de compuestos saludables como una forma de dar valor agregado a la materia prima utilizada, al producto final y a los residuos o subproductos.

1.3. Microencapsulación

La técnica de microencapsulación puede ser aplicada para mejorar la resistencia de los materiales alimenticios empleados a las condiciones de procesamiento y empacado, mejorando el sabor, aroma, estabilidad, valor nutritivo y apariencia de sus productos, y de esa manera proteger sustancias sensibles al ambiente. Las propiedades de liberación de los microencapsulados pueden depender del contenido de las partículas, el rompimiento, la solubilización, el calentamiento, el pH, o la acción enzimática. Por otro lado, ayudan a enmascarar sabores y olores desagradables de las sustancias (es decir, permiten controlar la liberación del material hasta el estímulo adecuado)(Choudhury et al., 2021). Las sustancias que se microencapsulan pueden ser vitaminas, minerales, colorantes, prebióticos, probióticos, sabores nutraceúticos, antioxidantes, olores, aceites, enzimas, bacterias, perfumes, drogas e incluso fertilizantes (Neekhra et al., 2022).

El material o mezclas de materiales a encapsular puede ser cubierto o atrapado dentro de otro material o sistema. Una microcápsula consiste en una membrana semi-permeable, esférica, delgada y fuerte alrededor de un centro solido/líquido. Los materiales que se utilizan para el encapsulamiento pueden ser gelatina, grasas, aceites, goma arábiga, alginato de calcio, ceras, almidón de trigo, maíz, arroz, papa, nylon, ciclodextrina, maltodextrina, caseinato de sodio, proteína de lactosuero o proteína de soya. Las aplicaciones de la microencapsulación se dirigen a la industria, principalmente en la industria textil, metalúrgica, química, alimenticia, cosméticos, farmacéutica y medicina (Neekhra et al., 2022)

Existen varios procesos para producir microencapsulados, entre los que están el secado por aspersión, la aspersión con enfriamiento, el lecho fluidizado, la coacervación/separación de fase, la gelación, la evaporación de solvente, la expansión de fluido supercrítico, la polimerización interfacial (policondensación), la polimerización de la emulsión y la extrusión (Nesterenko et al., 2013). Elegir la técnica de microencapsulación para un proceso en particular depende del tamaño, la biocompatibilidad y la biodegradación que necesite la partícula. Actualmente el secado por aspersión es uno de los métodos más utilizados en la industria alimenticia (Eun et al., 2020).

Para lograr con éxito la liberación deben tenerse en cuenta los siguientes aspectos: selección de la membrana, naturaleza química, morfología, temperatura de transición. Los métodos de liberación de las cápsulas se pueden llevar a cabo por disolución normal reacciones químicas y enzimáticas o por cambios en la presión osmótica. La eficiencia de la liberación controlada, principalmente

depende de la composición y estructura de la pared, pero también de las condiciones de operación durante la producción y uso de estas partículas (temperatura, pH, presión, humedad) (Choudhury et al., 2021).

El secado por aspersión es el método de microencapsulación más utilizado en la industria alimentaria, ya que es económico y flexible. El consumo de energía del secado por pulverización es de 6 a 10 veces menor en comparación con el secado por congelación y produce un producto de buena calidad. El proceso implica la dispersión del material del núcleo, formando una emulsión o dispersión, seguida de la homogeneización del líquido, y luego la atomización de la mezcla en la cámara de secado (Figura 2). Esto conduce a la evaporación del disolvente. Es importante subrayar que, en esta técnica, la entrada del producto, la temperatura de entrada, el flujo de gas deben controlarse (Martins et al., 2016).

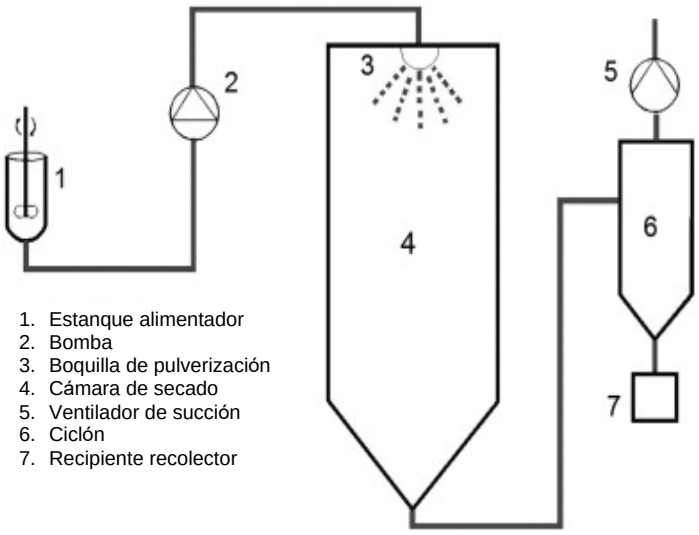

Figura 2. Esquema de las distintas etapas en el proceso de secado por aspersión Fuente: adaptado de (Martins et al., 2016).

La ventaja del proceso es que puede ser operado de manera continua. La desventaja es que la alta temperatura utilizada en el proceso puede no ser adecuada para encapsular ciertos compuestos. El daño por calor de la membrana celular es uno de los daños objetivo más susceptibles durante el secado por aspersión. Estas altas temperaturas durante el secado por aspersión hacen que los poros celulares filtren las sustancias intracelulares (Anekella and Orsat, 2013). Sin embargo, el ajuste y el control adecuados de las condiciones de procesamiento, como las temperaturas de entrada y salida, pueden lograr cultivos encapsulados viables con una distribución de tamaño de partícula deseada (Martins et al., 2016).

1.4. Potencial biológico de los residuos de uva

En los últimos años, el interés de los consumidores hacia las consecuencias para la salud de los alimentos ha aumentado progresivamente, y muchos alimentos funcionales han sido comercializados (Pasqualone et al., 2013). Estos alimentos funcionales son capaces de administrar de manera eficaz agentes beneficiosos que disminuyen el riesgo de enfermedades, además pueden entregar características nutricionales que promueven la salud y mejoran la calidad de vida. Para ello, debe considerarse la solubilidad y la interacción de los bioactivos con la matriz cuando se desarrolla una formulación (Sęczyk et al., 2017) ya que, en algunos casos, en lugar de aportar beneficios, la interacción podría degradar uno o varios compuestos de interés.

Dentro de la función biológica de los componentes del vino y de sus subproductos destacan por sus propiedades antivirales, anticancerígenas y antiinflamatorias (Kallo et al., 2020). En la actualidad, los brotes virales representan una amenaza crítica para la salud pública, especialmente cuando no se dispone de vacunas o terapias antivirales eficaces (Bavinger et al., 2020). Esto se apoya en diversos mecanismos de acción, como la inhibición de la adsorción, la entrada del virus, la unión del virus, la RTasa, la integrasa, la proteasa, la inhibición de la replicación de las ADN y ARN polimerasas, y la formación de complejos proteicos.

Los flavonoides en la actualidad se consideran un componente indispensable en una variedad de aplicaciones nutracéuticas, farmacéuticas, medicinales y cosméticas (Panche et al., 2016). A pesar de los beneficios que las bayas o sus subproductos ofrecen, posterior a la ingesta, no todos sus compuestos bioactivos son asimilados y depositados en el sitio “diana”. Esto puede ocurrir por factores como la baja estabilidad de ciertos compuestos como es el caso de los antocianos.

A pesar de todos estos beneficios que presentan en la salud, el uso más común de los orujos de uva y los residuos de poda es su distribución en las tierras agrícolas para aumentar la materia orgánica del suelo (Novara et al., 2020). Las aplicaciones como fuente nutricional para los viñedos de los residuos y/o subproductos está bien documentada. El uso de los residuos con un alto contenido en materia orgánica estabilizada se considera una estrategia eficaz para mitigar los procesos de erosión y degradación de los suelos (Sharma et al., 2019).

Los orujos y residuos de poda poseen una elevada relación C/N (entre 25 y 40) y un alto contenido en taninos (Paradelo et al., 2013), incidiendo en una deficiencia de N y estrés para la población microbiana del suelo. Los subproductos vitícolas retienen nutrientes inorgánicos, como Mg^{2+} y K^{+}, que pueden ser liberados al suelo tras su mineralización (Viel et al., 2018). Además, otra práctica habitual es enterrar los sarmientos de la poda también podría conducir a un aumento de los propágulos de patógenos fúngicos y bacterianos, aumentando así la incidencia de las enfermedades de las plantas (Han et al., 2021). Sin embargo, a pesar de su actual uso, y debido a su potencialidad biológica sobre otros seres vivos, explorar nuevos ingredientes a base de residuos o subproductos de uva es indispensable.

2. HIPÓTESIS

El uso de subproductos de la industria vitivinícola presenta potencial bioactivo que puede ser aprovechado utilizando técnicas de encapsulación para combatir virus de polinizadores.

3. OBJETIVO GENERAL

Estudiar y evaluar los compuestos bioactivos presentes en los subproductos de uva, con énfasis en el orujo, con el fin de conocer las ventajas de su encapsulación y desarrollo de ingredientes funcionales con potencial uso en la agricultura.

4. OBJETIVOS ESPECIFICOS

- Analizar antecedentes bibliográficos de las características particulares de los residuos de la vinificación con énfasis en los orujos y su potencial para contrarrestar enfermedades y virus.
- Formular y encapsular los compuestos bioactivos presentes en el orujo de uva var. Tintorera para el desarrollo de ingredientes de nueva generación para uso agrícola.
- Establecer ensayo *in vivo* en abejas melíferas *(Apis mellifera* L*.)* para determinar la capacidad antiviral del encapsulado de orujo de uva.

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CAPITULO II

Title: Next Generation Ingredients Based on Winemaking By-Products and an Approaching to Antiviral Properties.

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Abstract

Management of waste and use of winemaking by-products plays an important role in the development of new ingredients, especially with antiviral properties. Although the richness of bioactive compounds from wine waste is known, less is known about potential antiviral action. Bioactive compounds and health-enhancing effects of winery by-products make them potential candidates for use in antiviral ingredients. The design of new formulations by using nano-microencapsulation techniques will be necessary to successfully control diseases produced by viruses. Outcomes about the use of winery by-products, bioactive compounds found in winery wastes, green extraction techniques to concentrate these compounds, and development of formulations to obtain new ingredients were extracted from research around the world to be discussed and updated in this manuscript. The evidence collected in this review aims to encourage transfer of in vitro and in vivo knowledge to a new step for the development of antiviral and treatments.

Keywords: phenolic compounds; virus; wine waste; extraction technique

1. Introduction

Wine production is one of the world's oldest industries and one of the most important agricultural activities around the world, with an estimated surface area of 7.3 mha for the production of wine, table grapes, and raisins [1]. According to the International Wine Organization [2], 100 kg of grapes generate about 25 kg of waste, corresponding to skins (50%), stems, or rachis (25%) as well as seeds and liquids or semi-liquids (25%). In fact, the production of wine generates large amounts of solid and liquid waste by-products such as pomace, seeds, stems, waste from pruning, lees, and water, leading to a waste-management issue. The nature of the waste produced depends on the cultivar and specific vinification procedures used, which can also affect the properties of the residual material generated. Therefore, adequate waste treatment is needed to reduce the environmental impact of residues [3], and allow for a sustainable and environmentally friendly production. Currently, a large part of the waste management in the wineries is aimed at composting pomace to be reintroduced into vineyards to maintain organic matter levels and comply with the increasing consumer demands for natural products and sustainable practices [4]. Most of the by-products generated during winemaking are rich in bioactive compounds and their impact on human health cannot be underestimated. This high content of bioactives opens a wide range of possibilities for different applications, from colorants for beverages and functional food, a source of biofuel, animal feed, and a special focus on the generation of natural medicines (Table 1).

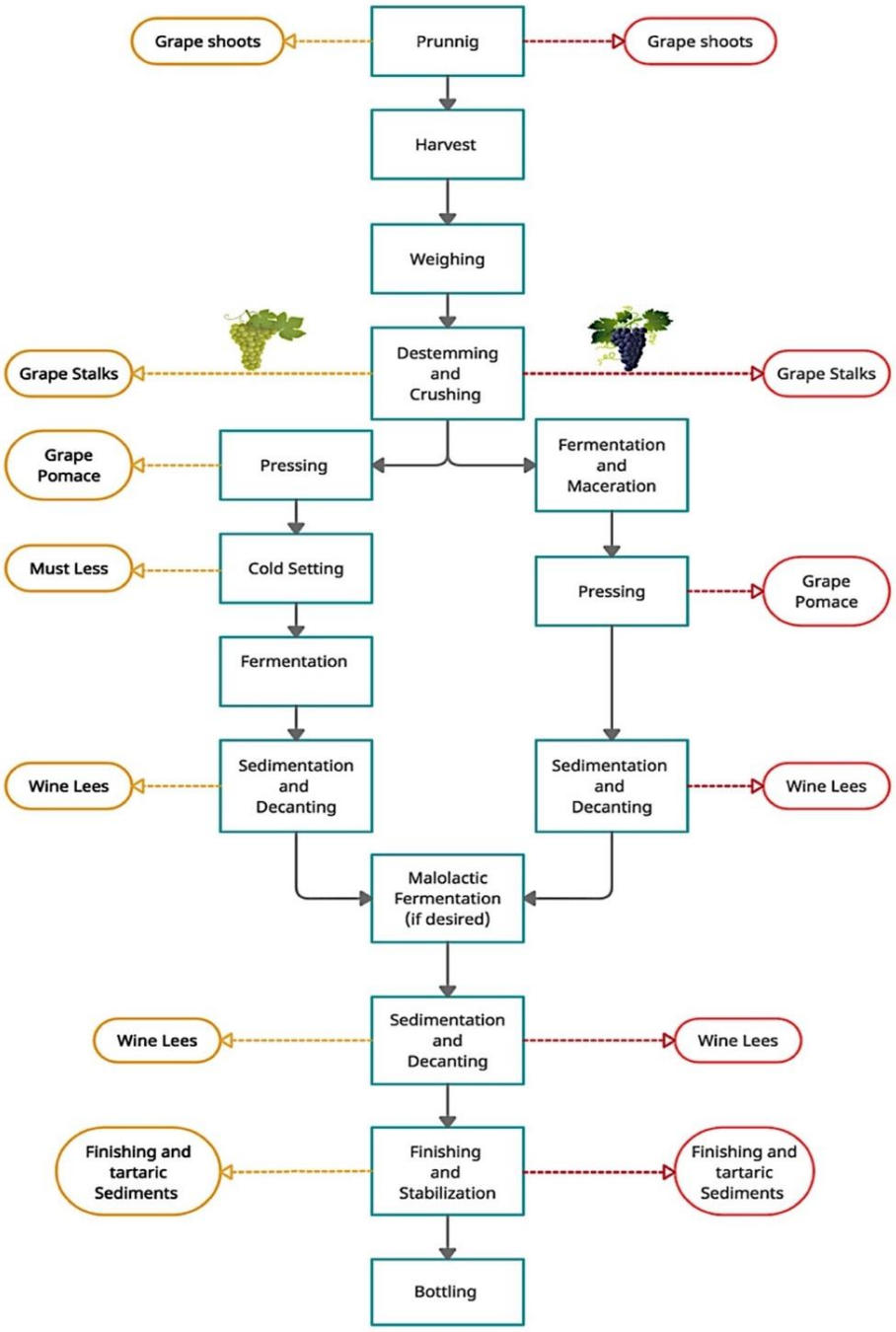

Figure 1. Shows the different wastes produced during wine production.

Grape seeds mainly contain water (25%–45%), glycidic compounds (34%–36%), tannins (4%–10%), nitrogenous compounds (4%–6.5%), minerals (2%–4%), lipids (13%–20%), and lower concentrations of other substances such as sugars [5].

Total polyphenols in seeds can reach up to 60%–70% of the extractable compounds, being a rich and natural source of antioxidants for the pharmaceutical, cosmetic, and food industries [6].

Table 1. Main by-products available in wine making and their uses.

By-products	Bioactive compounds	Current use	Reference
Grape pomace waste	Organic matter content, Polyphenols (anthocyanins and tannins),Flavanol content, Ethanol precipitate	Alternative source of antioxidant compounds and dietary fiber for yogurt	[7,8]
		Energy source	[9]
		To extend shelf life of lamb meat	[10]
		To reduce acrylamide formation	[11]
		To neutralise the production of reactive oxygen	[12]
		To reduce cholesterol level	[13]
		Stable delivery system, protecting resveratrol	[14]
		Biomethane	[15]
		Cosmetic formulation (skin aging)	[16]
		Dietary fiber supplement, human food supplement	[17]
Grape seed	Flavanol content. Lignocellulosic content	To modify the formulation of meat products	[18]
		Energy production, biodiesel	[19]
		Direct inclusion of natural antioxidants	[20]
		Skin moisturiser (gel formulation)	[21]
		Animal feed (rainbow trout)	[22]
		Extraction with supercritical CO2	[23,24]
Wastewater	Tartaric acid and malic acid content	Acidulant compound in soft drinks	[9]
vine shoot and stems	Phenolic compounds	Biodegradable packaging	[25]
		Energy production, biomethan	[26]

The variety of applications detailed in Table 1 shows the applicability of the by-products of the wine industry, maximizing efficiency and generating economically viable alternatives such as the generation of biofuel, a problem that is becoming important with respect to a non-renewable resource such as fossil fuels.

On the other hand, grape stems, which can be partially or totally part of the fermen- tation and/or pressing process depending on the vinification procedure used, are a good source of proanthocyanidins [27], providing astringency to the resulting wine. The com- mercial value of stems is low, and they are usually recycled as organic fertilizers. There is also evidence that stalks are a rich source of bioactive compounds such as trans-resvera- trol and derivatives, flavan-3-ols, and phenolic acid glycosides [28]. Vitis leaves are used in traditional medicine as laxatives, stomachics, diuretics, and refreshers, and also in palliative treatments of chronic bronchitis, heart disease, and gout [29].

Other wastes generated in the vineyard are after pruning (1 ton of biomass waste per hectare) [30], which are suitable for energy valorization, and mainly composed of cellulose and lignin with a low moisture content and high C/N ratio. Although biomass waste is an excellent source of bioenergy, its uses are still limited. In some areas, it is crushed and mixed with the soil as a fertilizer. Therefore, the roasting of these residues can be a profitable option to improve their fuel properties [31].

Finally, lees and winery wastewater are by-products that could be reused, although both present some inconveniences. The composition of wine lees is

highly variable and depends on the winery process, whereas winery wastewater presents low pH, but is high in both sulphides and sodium content, and it also presents a high organic matter content. Although the properties attributed to winemaking by-products as phenolic compounds have been extensively studied, specifically their antiviral capacity has not been developed significantly and there are limited antiviral products or ingredients made from wine industry residues. The scientific community has been concerned about the growing number of foodborne illnesses caused by some pathogens, which has led to the development of safe antimicrobial compounds derived from novel plants, including those present in grapes and grape products [32]. In this sense, winery waste by-products constitute a good source of natural polyphenols and antioxidants, which are considered completely safe compared with synthetic antioxidants. Therefore, this review summarized scientific information on the composition, richness, and functionality of mainly phenolic compounds present in waste by-products generated by the wine industry. It also focused on the development of some ingredients and advances on the preventive role that these compounds can play in the pathologies caused by viruses.

2. Updating of Bioactive Compounds Extracted from Winemaking By-Products

Most of the residues from winemaking are rich in phenolic compounds. Phenolic compounds in red wine pomaces comprise a diversity of chemical structures involved in the formation of the structure, color, transparency, and stability of the wine [7]. However, the composition of each pomace can vary depending on the

grape variety of origin or growing conditions [8] and also the percentage of phenolic compounds that remains in the residues. Therefore, it is necessary to utilize an adequate extraction system where the losses are reduced, and it is more environmentally sustainable. The extraction of bioactive compounds presents some challenges because they differ in recovery yield and solubility. Recently, new extractive technologies have been applied to grape pomace in accordance with the principles of green chemistry such as supercritical fluid extraction (SFE), micro- wave-assisted extraction (MAE), microwave hydrodiffusion and gravity (MHG), ultra- sound-assisted extraction (UAE), pulsed electric field (PEF), and ohmic heating (OH) since they are economic, innovative, and environmental-friendly processes [9] (Figure 2).

The use of conventional extraction technology such as solid–liquid extraction, heating, or grinding produces greater losses of bioactive compounds and environmental damage. Nevertheless, non-conventional technology such as pulsed electric fields, high voltage electrical discharges, pulsed ohmic heating, ultrasounds, microwave-assisted extractions, sub- and supercritical fluid extractions, or pressurized liquid extraction methods have already been applied for the extraction of high-added-value compounds from winery-processed samples [10].

Despite these techniques exemplifying a promising tool to recover high-added-value compounds from winery wastes and by-products, several considerations must be taken into account before choosing the technology, such as the matrix to

be processed, the selectivity, the energy consumption, the equipment cost, and the value of the extract [10].

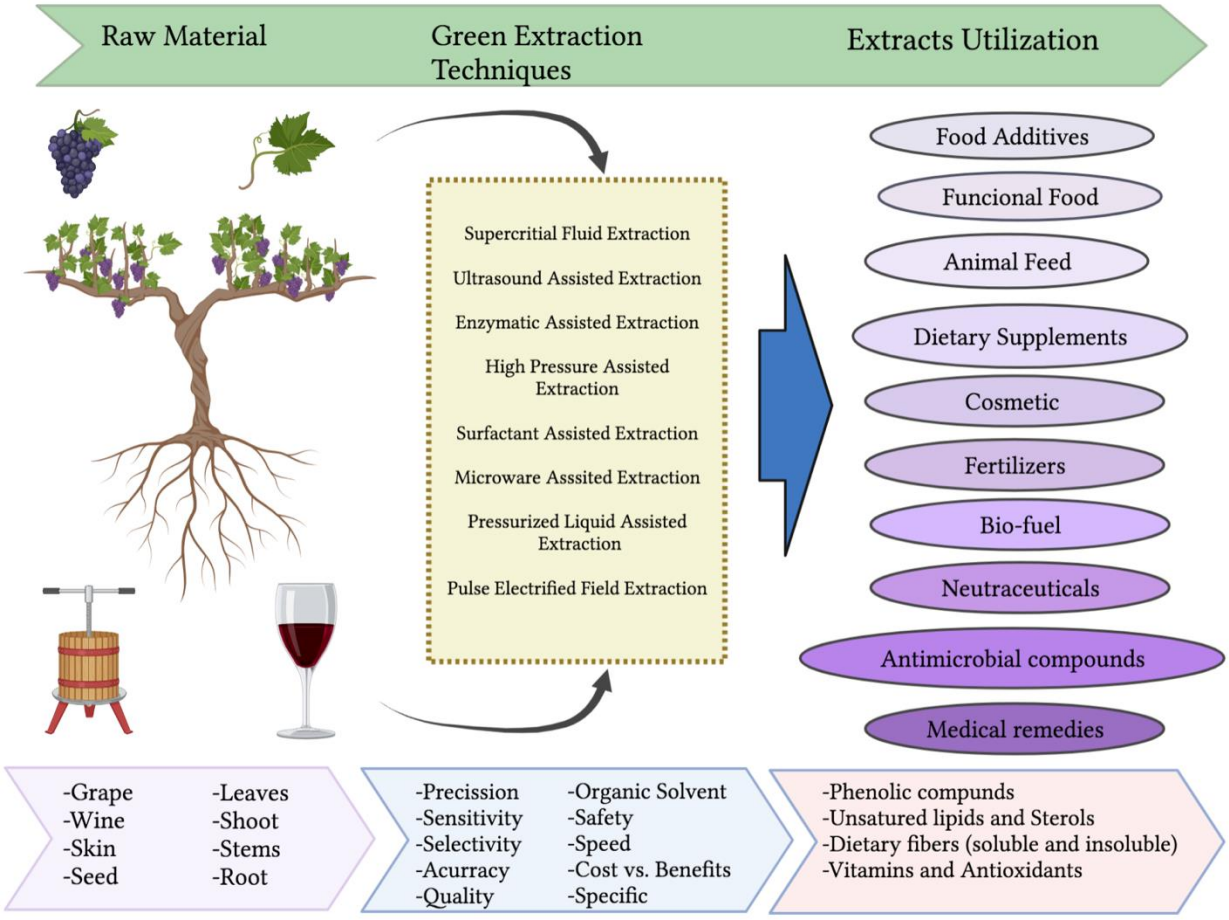

Figure 2**.** Green extraction techniques applied to by-products from the wine industry.

A study comparing the use of Pulsed Electric Fields (PEF) during alcoholic fermentation in the recovery of phenols compared with a thermal treatment for the Cabernet Franc variety showed a significant increase in the content of anthocyanins and tannins (approximately 51%–62% compared with the thermal treatment) [11]. Additionally, the microwave-assisted extraction of grape pomace showed advantages as a remarkable decrease in extraction times, from 5 h to

5min, compared with a solid–liquid extraction method, increasing, as well, the content of acyl derivates not detected in the conventional method [10].

The use of the potential antioxidant capacity present in the by-products produced in the wine industry has been widely studied and information is available regarding the content of phytochemicals that promote human health. The phytochemicals present in the wine wastes are presented in Table A1.

Phenolic acids are the most prominent class of bioactive chemicals grouped under phenolic compounds present in various plant sources such as fruits, vegetables, spices grains, and beverages [12,13]. These compounds frequently appear in a conjugated form, namely as glycosylated derivatives or esters of quinic acid, shikimic acid, and tartaric acid [13,14]. The genotype appears to be the major factor influencing the relative concentrations of the different phenolic compounds [33]. Flavonoids belonging to this group, such as flavan-3-ols, have a nuclear molecular structure of C6-C3-C6 and differ in the degree of oxidation of the central pyran ring [15]. They are abundantly found in seed and skin residues and play a very important role in the organoleptic properties of wines [34].

Anthocyanins, malvidin, petunidin, cyanidin, peonidinm and delphinidin (in the form of 3-*O*-glycosides) are the flavonoids responsible for the characteristic red color pig- mentation. They are produced during ripening and are mainly found in grape skins. They are susceptible to light, temperature, oxygen, and pH [35]. Kaempferol, quercetin, myricetin, and isorhamnetin are the most abundant flavonols found in grapes, wine, and in the main by-products.

Hydroxybenzoic acids are derivatives of benzoic acid with a framework of seven car- bon atoms of C6–C1 structure. Gallic acid can be found abundantly in grape stems, skins, and seeds, followed by syringic acid in grape stems, and protocatechic acid in grape seeds and skins [16].

Hydroxycinnamic acids are the derivatives of cinnamic acids having the framework of the C6–C3 structure. The most common hydroxycinnamic acid and its derivatives are p-coumaric acid, cinnamic acid, caffeic acid, ferulic acid, sinapic acids, isoferulic acid, and p-hydroxycinnamic [13]. The trans conformation of some phenolic acids (e.g., resveratrol) is naturally occurring, while the cis conformation is induced by UV exposure [36]. All these bioactive compounds must be protected and transformed into new ingredients; consequently, the use of encapsulation techniques is necessary.

Nevertheless, it is important to point out that the content of these molecules afore- mentioned vary depending on the starting material and the extraction technique used.

Therefore, current trends in extraction procedures from winery wastes and by-products will keep developing to replace conventional technologies with non-conventional ones since they present clear advantages including handling, reduction of the processing time, energy, the reduction of harmful and expensive solvents, and the increase of the extraction yields.

Among the many applications that have been described to wine by-products, development of antiviral ingredients has been hardly established. Hence, green

extraction techniques mentioned above, where the content of the bioactive compound of interest is enhanced, with greater safety and innocuousness, open great possibilities of work on the line of constituents with antiviral potential.

3. Development of Ingredients of Products Based on Winemaking Products

Encapsulation is the technology used to safeguard sensitive materials by packaging materials in the form of micro- or nanoparticles. Encapsulation efficiency and stability of the capsules is closely related to the selection of the wall material [37]. Maltodextrin (MD) is the most commonly used encapsulating agent due to its high water solubility, low viscosity, and low sugar content [17]. There are also other encapsulating agents that offer a viable alternative such as gum arabic, skim milk powder, ascorbic acid, among others, which are generally used in mixture with MD. It is important to note that the success of the encapsulation will depend directly on the encapsulating agent or mixture of these, the working conditions of the equipment (air inlet temperature, pump power, feed flow, etc.), and the material to be encapsulated. Some examples are shown in Table 2.

The technology involved in encapsulation is effective in masking the unpleasant odor of the extracts and the product is rapidly soluble in water, releasing about 100% of the bioactive extracts within a few minutes. Therefore, the final products show improved technological characteristics suitable for the manufacture of functional foods and food supplements [18]. The current extraction techniques aforementioned show benefits like reduction of the extraction time, number of unit operations, energy consumption, environmental impacts, economical costs, quantity of solvent, and waste production, aiming to guarantee safe and quality

extracts and/or products, and being more efficient to recover the phytochemicals present in wine by-products [19] as well as to enhance the formulation and microencapsulation techniques to develop an ingredient to be used in the industry (Table 2).The use of mixtures of encapsulating agents increases the efficiency and prolongs the durability of the encapsulation, as detailed in the works in the table above. One of the main advantages of encapsulation is to maintain and/or improve the shelf life and stability of bioactive compounds. In summary, encapsulation can generate a strategy to improve the stability of these compounds, and therefore the development of value-added food- stuffs to meet the growing consumer demand in the food, agricultural, and pharmaceutical industries [24].

One investigation carried out in grape pomace compared different types of maltodextrins and concluded that the choice of agent plays a crucial role in the storage stability of polyphenols, showing maltodextrin DE4-7 as significantly better in protection than maltodextrin DE17-20 in all conditions. Moreover, under identical experimental conditions, the stability of microencapsulated polyphenols was much higher at a relative humidity of 33% than at 52% [22].

The mixture of encapsulate material represents an alternative to improve the compound stability. Another study used protein concentrate (WPC), maltodextrin (MD), and gum arabic (GA) as encapsulating materials in differences preparation, WPC:MD/GA (5:0, 4:1, 3:2, and 0:5) followed by freeze drying.

Table 2. Main technologies for the encapsulation of winemaking wastes.

Raw Material	Technology	Process Variable/Formulation	Encapsulation Agent	Main result	References
Dry grape residue pressed	Microcapsulation.Buchi B-290 spray drying (Buchi Labortechnic AG, Switzerland).	Spray drying with the main chamber of 165 mm diameter, 600 mm cylindrical height, and 1.5mm nozzle diameter at four air inlet temperatures (120, 140, 160, 180°C). The pump power was kept at 40% to maintain feed flow rate as 12mLmin^{-1}, and air flow rate as 35m^3 h^{-1}. During drying processes, the temperature of the feed mixture was 25 °C	Maltodextrin and gum arabic as a coating material. Two different core: coating material ratios (1:1 and 1:2), three different maltodextrin: gum arabic ratios (10:0, 8:2 and 6:4)	Encapsulation efficiency 98.8% and 99.1% for core: coating ratios of 1:1 and 1:2. Highest yield (64.9%) MD: GA ratio 10:0, temperature 180ºC	[20]
Agiorgitiko (*Vitis vinifera*) grape pomace	Spray drying (Buchi, B-191, Buchi Laboratoriums-Technik, Flawil, Switzerland)	Ratio of wall-to-core material of 8.8, an inlet air temperature of 189 °C, a drying air flow rate of 65%	Maltodextrin:skim milk powder (50:50)	Optimum values of encapsulation efficiency (92.49%) and yield (37.28%)	[21]
Dry grape residue pressed	Spray drying process Buchi B-290 equipped with a 1.5mm nozzle diameter and 600 mm × 165 mm main spray chamber	Peristaltic pump set to 40% power, 12 mL min^{-1} feed flow rate, and 35 m3h^{-1} air flow rate. The temperature of the feed mixture kept constant at 25 °C during drying process.	Maltodextrin dextrose equivalents (MDDE4-7 and MDDE17- 20) and gum Arabic (G9752)	The microcapsules obtained under optimal conditions were stored at two different relative humidities (33% and 52%) during 75 days.	[22]
Byproducts (seeds and peels) of Bordo red grapes (*V. labrusca*)	Pilot spray drying model MSD 5.0; freeze-drying in the proper equipment model LC 1500	Used a 2mm nozzle and air flow of 40L/min. The compressor air pressure was 0.2 MPa and the feed rate of the mixture 44 mL/min, performed by a peristaltic pump. Variables tested were inlet air temperature (130, 150, and 170 ºC)	The carrier agent used in the atomization process was maltodextrin MOR-REX® 1910	Bordo grape extracts using maltodextrin produces powders with low moisture content, low hygroscopicity, high solubility, and stable color.	[23]

The grape seed extract [38] microcapsules coated with a WPC:MD/GA ratio of 4:1 and 3:2 with core-to-coat ratio of 1:5 were found to have the highest encapsulation efficiency (87.90%–91.13%) and the smallest particle size with the maximum retention of antioxidant activity [39].

Hence, these are a source of functional compounds that can be exploited in the pro- duction of innovative foods and packaging, cosmetics, and also of new ingredients of the next-generation focus on viruses.

In fact, encapsulation of antivirals for food applications has been little explored. The lack of absorption of the bioactive compounds extracted from by-products of the wine industry limits their health benefits or their pharmaceutical use [25]. Their low stability is mainly attributed to poor absorption from the human gastrointestinal tract. Phenolic com- pounds found in wastes have to pass through the human gastrointestinal tract and be absorbed by enteric epithelial cells as they are administered orally. Furthermore, the extremely low pH (approximately 2.0) of gastric fluid and digestive enzyme can degrade these bioactive compounds in the human stomach [25]. Consequently, they have very low bioavailability. Hence, nanoparticle-based carriers present a great promise for control release since the passive transcellular pathway, the paracellular pathway, and endocytosis may be able to absorb nanoparticles loaded with bioactive compounds extracted from the wine industry.

Recent developments in the encapsulation of antiviral compounds include the use of chitosan to enhance the protection of epigallocatechin (-) gallate (a green tea

polyphenol) [26]. This compound, microencapsulated, showed the potential to prolong antiviral activity against murine norovirus through gradual bioactive release combined with its protection against degradation under simulated physiological conditions. Therefore, these results highlight the potential of encapsulated natural antiviral compounds for use in food applications. Encapsulation of these antiviral compounds may provide enhanced and prolonged antiviral activity thanks to biopolymeric encapsulation matrices. Moreover, different studies demonstrated the efficacy of alginate-based release particles [40], suggesting that encapsulation could represent a viable tool for the transport and delivery of antiviral compounds.

4. New Insights Against Disease and Viruses

Phenolic compounds from wine by-products play a protective role in plants that can be extrapolated to other living organisms. For humans, an excess of Reactive Oxygen Species (ROS) in the body can enhance the development of chronic non-communicable diseases such as cancer, cardiovascular disorders, neurodegenerative damage, Alzheimer's disease, and inflammation in different organs [41]. Special attention is given to anthocyanins that contribute 90% of the antioxidant capacity of fruits, whereas the remaining 10% is attributed to flavonols, flavan-3-ols, and phenolic acids [42]. In addition, phenols exhibit chelating, anticancer, antimicrobial, and anti-inflammatory activities [43]. These capacities allow these phenols to react in biological systems, decreasing the occurrence of degenerative diseases associated with oxidative stress in tissues and organ systems [44]. This con- firms that the continuous intake of food products with a

high antioxidant content is associated with a lower incidence/severity of developing pathophysiological problems [41]. The target for valorization of these is not only limited to remediating environ-mental problems, but also to utilize them as a source of functional ingredients. Valorization of waste from wineries provides commercialization of phenolic extracts, dietary fibers, and oil derived from grape pomace. Some of the residues and/or by-products generated in the production of wine have compounds with health-promoting properties, e.g., anthocyanins, which are highly concentrated in the pomace and have anti-inflammatory properties and antioxidant activity in human low-density lipoproteins [45], as well as positive effects on microcirculation diseases and ocular function [46]. Some studies have reported antiviral activity of phenolic compounds from grapes and grape products as well as from wine by-products (Table 3) [47].

Animal models are the main focus of studies to evaluate the in vivo antioxidant activity of phenolic matrices from winemaking wastes. Nowadays, there are biomarkers in urine and blood that are evaluated after the supply of the functional ingredient in rats, and even the intestinal microbiota, can be studied in relation to bioassimilation and/or bioavailability [57].

A study in rats showed that a high-cholesterol diet with 15% pomace incorporated halved liver and serum cholesterol levels, increased high-density lipoprotein by up to 26%, and had positive effects on microcirculatory disease and ocular function [58].

Table 3. Studies on the health/biochemical properties of different bioactive extracts against some diseases and viruses.

Bioactive ingredient extract	Mechanism of Action	Reference
Grape seed and grape marc meal extract	Gut morphology, apparent digestibility of nutrients, microbial composition in faeces, and the expression of pro-inflammatory genes in the intestine of pigs.	[48]
Extraction from wine production residue (seeds, skin and pomace) from Pinot noir and Pinot meunier	anti-influenza activity	[49]
Polyphenols extraction from Cabernet Sauvignon grape pomace	Effect of different classes of antibiotics against *Staphylococcus aureus* and *Escherichia coli*, especially against multi-drug resistant clinical isolates	[50]
Oligostilbenoids isolated from extracts of Vitis vinifera L. Pinot Noir grape canes	Antiproliferative activity on four different cell lines (MCR-5, AGS, SK-MES-1, and J82) determined by means of the MTT reduction assay.	[51]
Leaf extract *Vitis vinifera* var. Paulsen 1103	Antiviral activity against two human viruses: the Herpes simplex virus type 1 (HSV-1) widespread severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).	[52]
Phenolic extract from grape stems (Vitis vinifera var. Red Globe)	Inhibit the growth of *Listeria monocytogenes, Staphylococcus aureus, Salmonella enterica* subsp. enterica serovar *Typhimurium*, and *Escherichia coli* O157	[53]
Hydroalcoholic extract from grape pomace var. Máximo IAC 138-22	Ovicidal and larvicidal activity against gastrointestinal nematodes of sheep.	[54]
Grape seed extract	Antiviral activities against hepatitis A virus (HAV) and human norovirus surrogates (feline calicivirus (FCV-F9) and murine norovirus (MNV-1)).	[55]
Grape seed-extracted proanthocyanidin	Inhibition of porcine reproductive and respiratory syndrome virus (PRRSV)	[56]

There is also evidence that grape seed extracts are a rich source of polyphenols, reducing the risk of heart disease by inhibiting Low Density Lipoprotein (LDL) oxidation, improving endothelial function, lowering blood pressure, preventing platelet aggregation, reducing inflammation, and activating proteins that prevent cellular senescence [59]. In addition, polyphenols from grape pomace increased the biodiversity degree of intestinal microbiota in broiler chicks [60], and improved the gain-to-feed ratio and overall performance in pigs [38,48]. Furthermore, grape pomace altered the nitrogen metabolism and decreased the ruminal ammonia production in male sheep [61] and modified the rumen microbial population involved in methane metabolism [62], enhancing the growth of facultative probiotic bacteria, and inhibited the growth of pathogenic ones in lambs [63].

Recent studies have shown that flavonoids exhibit antiviral activity against HIV, HSV, influenza virus (IV), RSV, severe acute respiratory syndrome coronavirus (SARS-CoV), measles, and rotavirus [64]. Resveratrol was also recently reported as inhibiting MERS-CoV infection by extending cell survival after virus infection and decreasing the expression of the nucleocapsid (N) protein, essential for MERS-CoV replication. This is supported by varied mechanisms of action, such as inhibition of adsorption, virus entry, virus binding, RTase, integrase, protease, inhibition of replication of DNA and RNA polymerases, and formation of protein complexes [65].

Grape extracts (skin and whole red grapes), grape juice, and wine were reported to inactivate various enteric viruses and herpes simplex virus (HSV) type 1 [66,67].

Bioactive compounds isolated from winemaking wastes, mainly flavonoids, may represent an interesting option against viral diseases. Indeed, flavonoids lack systemic toxicity, while their ability to synergize with conventional drugs has been widely demonstrated. Furthermore, they are considered as pleiotropic compounds, which indicates that multiple pathways where intercepted and also different cellular targets [68]. These characteristics make flavonoids potential candidates for interfering with the life cycle of coronaviruses [69]. To achieve maximum benefit from wine production waste extracts as an antiviral natural compound, more information is needed on the active compounds present in these extracts or isolated, the effect of the extraction method and the green extraction system used to obtain the bioactive compounds [67].

5. Future Perspective

The worldwide demand for wine has been growing in recent decades, which has led to an increase in the use of winemaking by-products. The studies discussed in this review point out the potential of phenolic compounds present in wine and winemaking waste. Bioactivity, bioavailability, and toxicology of phytochemicals are based on the knowledge studied and provide a possibility of use as promoters of human health, dietary enhancers in animals, and other uses such as those exploited by the cosmetic industry. It is essential to perform tests in in vitro and in vivo conditions and to focus the work on the processes of green extraction, isolation, purification, and recovery to obtain greater quantities of healthy bioactive phytochemicals to discern the interaction within the food matrix. Now-adays, there is a clear need for potentially efficient natural products against

COVID-19, which requires an in-depth study of the phytochemical potentials found in residues. Special attention has been given to flavonoids as natural substances that promote the prevention or recovery from SARS-CoV-2 infection due to the wide range of their biological effects, including the modulation of inflammatory processes and immune responses. At pre- sent, the fight against coronavirus has been conducted based on treatment with drugs traditionally used against other pathologies generated by viruses (HIV protease inhibitors, such as ritonavir and lopinavir, and anti-inflammatory agents, such as tocilizumab or dexamethasone); the bioactive compounds mentioned in this review, such as flavonoids, are a viable alternative since they lack systemic toxicity, generate a synergy with traditional drugs, and present a pleiotropic effect, since their functional groups can inter- act with different cellular targets and intercept multiple pathways. It is also important to point out that the use of these bioactive compounds as antivirals can have not only a curative but also a preventive effect since, they can inhibit the proteases of the viruses, blocking their propagation.

With the current knowledge regarding the nutritional and phytochemical composition of winemaking residues, more and better research is needed to understand the composition and deliver accurate results for use and application of innovative products that contribute not only to the nutritional or medical field but are a viable alternative for saving time and reducing the environmental impact of wine production worldwide. Finally, it is important to point out that the characteristics of the by-products will depend on a variety of factors, mainly regarding grape production, agronomic management (irrigation, fertilization, etc.), the geographical

place of origin, and the management inherent to wine pro- duction within the winery.

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6. Supplementary Material

Table S1. Main bioactive compounds found in pomace by-products.

Compounds of interest	Grape pomace (Skin and seed)	Grape skin	Grape seed
Gallic	1090.1 µg g-1 of extract (RP-HPLC) [70]	122 µg g-1 of extract (HPLC–UV) [71]	9.8 mg kg-1 of fresh grape (HPLC-DAD-FLV) [72]
	397.67 µg mL-1 of extract (HPLC-DAD) [73]	8.76 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]	30.3 mg kg-1 dw (RP-HPLC/UV) [75]
	252.8 µg g-1 of extract (HPLC-MWD) [8]	1.19 mg kg-1 of grape (HPLC-DAD) [76]	136.74 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]
	95.36 mg kg-1 dw (HPLC-ESI/MS/MS) [77]		1.92 mg kg-1 of grape (HPLC-DAD) [76]
	260.92 mg L-1 of extract (HPLC-PDA-MS) [78]		
Syringic acid	1731.7 µg g-1 of extract (HPLC-MWD) [8]		
Caffeic acid	16.0 µg g-1 of extract (HPLC-MWD) [8]	0.54 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]	1.06 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]
	438.43 mg kg-1 dw (HPLC-ESI/MS/MS) [77]		
p-Coumaric acid	64.6 µg g-1 of extract (HPLC-MWD) [8]	1.96 mg kg-1 of grape (HPLC-DAD) [76]	
	214.55 mg kg-1 dw (HPLC-ESI/MS/MS) [77]		
Ferulic acid	24.1 µg g-1 of extract (HPLC-MWD) [8]	2.12 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]	2.17 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]
	1.33 mg kg-1 dw (HPLC-ESI/MS/MS) [77]		
Caftaric acid	1.80 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]		
(+)-Catechin	5083 µg g-1 of extract (RP-HPLC) [70]	13.20 mg kg-1 dw (HPLC-DAD) [84]	117 mg kg-1 dw (HPLC-DAD) [84]
	89.73 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]	628 µg g-1 of extract (HPLC–UV [71]	270 mg kg-1 of fresh grape (HPLC-DAD-FLV) [75]
	275.09 µg mL-1 of extract (HPLC-DAD) [73]	49.38 mg kg-1 of grape (HPLC–DAD–ESI-MS/MS) [85]	21.1 mg kg-1 dw (RP-HPLC/UV) [75]
	3387.5 µg g-1 of extract (HPLC-MWD) [8]	7.47 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]	86.73 mg kg-1 of grape (HPLC–DAD–ESI-MS/MS) [85]
		11.45 mg kg-1 of grape (HPLC-DAD) [76]	270.26 mg kg-1 dw (UHPLC-DAD-MS/MS) [72]
		25 mg kg-1 of fresh grape (HPLC-DAD-FLV) [72]	106.5 mg kg-1 of grape (HPLC-DAD) [76]

Table A1. *Cont.*

Compounds of interest	Grape pomace (Skin and seed)	Grape skin	Grape seed
(-)-Epicatechin	192.8 µg g-1 of extrac (RP-HPLC) [70]	323 µg g-1 of extract (HPLC–UV) [71]	210 mg kg-1 of fresh grape (HPLC-DAD-FLV) [72]
	1763.4 µg g-1 of extract (HPLC-MWD) [8]	13.55 mg kg-1 of grape (HPLC–DAD–ESI-MS/MS) [85]	38.1 mg kg-1 dw (RP-HPLC/UV) [75]
	112.72 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]	3.56 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]	6.81 mg kg-1 of grape (HPLC–DAD–ESI-MS/MS) [85]
		2.67 mg kg-1 of grape (HPLC-DAD) [76]	223.08 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]
		13 mg kg-1 of fresh grape (HPLC-DAD-FLV) [72]	77.51 mg kg-1 of grape (HPLC-DAD) [76]
		47.50 mg kg-1 dw (HPLC-DAD) [84]	
Quercetin	650.2 µg g-1 of extract (RP-HPLC) [70]	316 µg g-1 of extract (HPLC–UV) [71]	1009.4 mg kg-1 dw (RP-HPLC/UV) [75]
	159.60 µg mL-1 of extract (HPLC-DAD) [73]	40.03 mg kg-1 dw (HPLC-DAD) [84]	11.72 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]
	557.3 µg g-1 of extract (HPLC-MWD) [8]	0.53 µmol kg-1 of grape (HPLC-DAD/FLD) [82]	
	26.25 mg kg-1 dw (HPLC-ESI/MS/MS) [77]	121.94 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]	
	382.93 mg L-1 of extract (HPLC-PDA-MS) [78]	1043 mg kg-1 dw (UPLC-DAD-MS) [80]	
	0.54 mg g-1 of extract (HPLC-UV-DAD) [86]	3.68 mg kg-1 dw (HPLC-DAD) [84]	
	392 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83]		
	15.30 mg kg-1 dw (HPLC-UV-DAD) [81]		
	251.06 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]		
Myricetin	36.77 mg kg-1 dw (HPLC-ESI/MS/MS) [77]	1.8 µmol kg-1 of grape (HPLC-DAD/FLD) [82]	2.42 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]
	452 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83]	2.1 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]	
	2.45 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]		
Rutin	998.5 µg g-1 of extract (RP-HPLC) [70]	57.04 mg kg-1 dw (HPLC-DAD) [84]	9.05 mg kg-1 dw (HPLC-DAD) [84]
	112.96 µg mL-1 of extract (HPLC-DAD) [73	223 µg g-1 of extract (HPLC–UV) [71]	30.7 mg kg-1 dw (RP-HPLC/UV) [75]

Table S1. *Cont.*

Compounds of interest	Grape pomace (Skin and seed)	Grape skin	Grape seed
Kaempferol	346.8 µg g-1 of extract (RP-HPLC) [70]	34.2 mg kg-1 dw (UPLC-DAD-MS) [80]	
	28.53 mg kg-1 dw (HPLC-ESI/MS/MS) [77]	0.41 µmol kg-1 of grape (HPLC-PDA-ESI-MS/MS) [79]	
	2.37 mg kg-1 dw (HPLC-UV-DAD) [81]	8.93 mg kg-1 dw (HPLC-DAD/FLD) [82]	
	34.23 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]	14.89 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]	
		1.53 mg kg-1 dw (UPLC-DAD-MS) [80]	
Quercetin 3-glucuronide	130 mg kg-1 dw (HPLC-UV-DAD) [81]	22 mg kg-1 dw (UPLC-DAD-MS) [80]	
	81.42 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]	0.98 mg 100g-1 (HPLC-DAD) [76]	
	990 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83]		
Trans- resveratrol	36.0 µg g-1 of extract (HPLC-MWD) [8]	5.64 mg kg-1 dw (UHPLC-DAD-MS/MS) [74]	
	20.66 mg kg-1 dw (HPLC-ESI/MS/MS) [77]	1.43 mg kg-1 of grape (HPLC-DAD) [74]	
Delphinidin 3-*O*-glucoside	0.16 mg g-1 of extract (HPLC-UV-DAD) [86]	870 mg kg-1 dw (UPLC-DAD-MS) [80]	
	4581 µg g-1 of extract (HPLC-MWD) [8]		
	4.47 mg L-1 of extract (HPLC-PDA-MS) [78]		
	775 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83]		
	3.73 mg kg-1 dw (HPLC-UV-DAD) [81]		
Cyanidin 3-*O*-glucoside	870 µg g-1 of extract (HPLC-MWD) [8]	528 mg kg-1 dw (UPLC-DAD-MS) [80]	
	6.99 mg kg-1 dw (HPLC-UV-DAD) [81]		

Table S1. *Cont.*

Compounds of interest	Grape pomace (Skin and seed)	Grape skin	Grape seed
Peonidin 3-*O*-glucoside	0.15 mg g-1 of extract (HPLC-UV-DAD) [86]	551 mg kg-1 dw (UPLC-DAD-MS) [80]	
	2460 µg g-1 of extract (HPLC-MWD) [8]		
	18.31 mg L-1 of extract (HPLC-PDA-MS) [78]		
	1591 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83]		
	18.70 mg kg-1 dw (HPLC-UV-DAD) [81]		
	0.97 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]		
Malvidin 3-*O*-glucoside	5,70 mg g-1 of extract (HPLC-UV-DAD) [86]	2489 mg kg-1 dw (UPLC-DAD-MS) [80]	
	26,658 µg g-1 of extract (HPLC-MWD) [8]		
	955.85 mg L-1 of extract (HPLC-PDA-MS) [78]		
	12182 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83]		
	64.6 mg kg-1 dw (HPLC-UV-DAD) [81]		
	142.22 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]		
Delphinidin 3-*O*-acetylglucoside	1043 µg g-1 of extract (HPLC-MWD) [8]		
	9.79 mg L-1 of extract (HPLC-PDA-MS) [78]		
Petunidin 3-*O*-acetylglucoside	1424 µg g-1 of extract (HPLC-MWD) [8]		
	72.13 mg L-1 of extract (HPLC-PDA-MS) [78]		
	0.86 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]		
Peonidin 3-*O*-acetylglucoside	1902 µg g-1 of extract (HPLC-MWD) [8]		
	32.64 mg L-1 of extract (HPLC-PDA-MS) [78]		
	1.83 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]		

Table S1. *Cont.*

Compounds of interest	Grape pomace (Skin and seed)	Grape skin	Grape seed
Malvidin 3-*O*-acetylglucoside	2,02 mg g-1 of extract (HPLC-UV-DAD) [86] 4021 µg g-1 of extract (HPLC-MWD) [8] 1718.92 mg L-1 of extract (HPLC-PDA-MS) [78] 937 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83] 0.96 mg kg-1 dw (HPLC-UV-DAD) [81] 195.01 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]	486 mg kg-1 dw (UPLC-DAD-MS) [80]	
Cyanidin 3-*O*-p-coumaroylglucoside	1886 µg g-1 of extract (HPLC-MWD) [8] 3.99 mg L-1 of extract (HPLC-PDA-MS)[78]	327 mg kg-1 dw (UPLC-DAD-MS) [80]	
Petunidin 3-*O*-p-coumaroylglucoside	2481 µg g-1 of extract (HPLC-MWD) [8] 29.95 mg L-1 of extract (HPLC-PDA-MS) [78] 765 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83] 72.95 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]	339 mg kg-1 dw (UPLC-DAD-MS) [80]	
Peonidin 3-*O*-p-coumaroylglucoside	1854 µg g-1 of extract (HPLC-MWD) [8] 41.46 mg L-1 of extract (HPLC-PDA-MS) [78] 796 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83] 1.18 mg kg-1 dw (HPLC-UV-DAD) [81] 42.71 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]	294 mg kg-1 dw (UPLC-DAD-MS) [80]	
Malvidin 3-*O*-p-coumaroylglucoside	0.99 mg g-1 of extract (HPLC-UV-DAD) [86] 12864 µg g-1 of extract (HPLC-MWD) [8] 764.20 mg L-1 of extract (HPLC-PDA-MS) [78] 4700 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83] 2.52 mg kg-1 dw (HPLC-UV-DAD) [79]	758 mg kg-1 dw (UPLC-DAD-MS) [80]	

Table S1. *Cont.*

Compounds of interest	Grape pomace (Skin and seed)	Grape skin	Grape seed
Petunidin 3-*O*-glucoside	0,32 mg g-1 of extract (HPLC-UV-DAD) [86]	1109 mg kg-1 dw (UPLC-DAD-MS) [80]	
	6880 µg g-1 of extract (HPLC-MWD) [8]		
	18.98 mg L-1 of extract (HPLC-PDA-MS) [78]		
	1295 ppm of dry extract (HPLC-DAD-ESI-MS/MS) [83]		
	10.70 mg kg-1 dw (HPLC-UV-DAD) [81]		
	0.87 mg kg-1 dw (HPLC-PDA-ESI-MS/MS) [79]		

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CAPITULO III

Title: Dietary supplement of grape wastes enhances honeybee immune system and reduces deformed wing virus (DWV) load.

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Abstract

The winemaking industry generates tons of waste that should be treated and reintroduced in a frame of sustainable production. In this sense, new alternatives and uses of wine wastes that allow the reconversion into ingredients rich in bioactive compounds, such as phenolic compounds, which play an important role in the prevention of various diseases and the control of viruses, are being analyzed. Population loss in honeybee (*Apis mellifera* L.) colonies is a matter of increasing concern, being deformed wing virus (DWV) one of the most common viruses infecting apiaries worldwide. The objective of this study was to evaluate the effect of grape pomace powder (GPP) as a dietary supplement to enhance the immune system of honeybees affected by DWV. The characteristics of the grape pomace supplement obtained by spray-dry technology revealed a high anthocyanin content (1102.45 mg 100 g^{-1}). The powder was applied at doses of 0.5, 1, 2.5 and 5% of GPP as a dietary supplement for bees infected by DWV. The results showed that the GPP treatments strengthened the immune response of honeybee against DWV. Moreover, the expression of Relish gene was significantly higher in bees fed with GPP compared to the infected control. This study, framed in the search of food waste valorization for environmental sustainability, proves the feasibility of using grape wastes as dietary supplements for pollinators and provides knowledge of the influence of the polyphenols from grape pomace on the expression profiles of immune-related genes in honeybees.

Keywords: winemaking industry, anthocyanins, *Apis mellifera*, antiviral activity, agro-sustainability, food waste valorization.

1. Introduction

Integrated solutions to climate change, resource scarcity, environmental degradation, food waste management, and an increasing demand for food are essential for the future of food and agriculture. In this sense, new products and strategies based on the reuse of environmentally sustainable materials are increasingly emerging, while technologies can broaden the scope of intervention, facilitating and accelerating the development of waste-free food systems.

Wine production reached between 253.9 and 262.2 mhL (excluding juices and musts) in 2020, generating large number of wastes. Traditionally, grape by-products have been used as fertilizers and/or biomass. In recent years, however, they have been proposed as an interesting source of bioactive compounds that could be used for several applications (Pintać et al., 2018).

Pomace from red grapes, one of the main winemaking waste, contains phenolic compounds such as anthocyanidins, catechins, proanthocyanidins, tannins and organic acids with proven antiviral activity (Antonic et al., 2020). For instance, it has been reported that compounds of wine and its by-products can inactivate various enteric viruses and herpes simplex virus (HSV) type 1 (Bekhit et al., 2019; Konowalchuk and Speirs, 1976; Pascual et al., 2022). In addition, several studies have focused on the mechanisms of action of these bioactive compounds, demonstrating that these molecules act on the inhibition of adsorption, virus entry, virus binding, RTase, integrase, protease, inhibition of replication of DNA and RNA polymerases, and formation of protein complexes (Loaiza-Cano et al., 2021).

Hence, opportunities to reduce food waste and integrate approach to the entire supply chain and profit the beneficial properties of these bioactive compounds are required. In this sense, the use of new ingredients for pollinators, as proposed in this study, could be a potential alternative for food waste reduction (Lipinski et al., 2013).

Honeybee (*Apis mellifera* L.) is an important species with a key role in agriculture as pollinator of crops and plant species, thus enhancing biodiversity of both agricultural and non-agricultural landscapes (Brutscher et al., 2015). Therefore, honeybee losses have become a matter of increasing concern worldwide. Deformed wing virus (DWV), which is a widespread virus associated with the spread of the *Varroa destructor* mite, is considered as one of the most important factors contributing to bee losses at the global level (Loope et al., 2019). The virus causes physical malformations in wings, abdominal swelling, paralysis, and behavioral disturbances in bees. In addition, studies have reported that DWV infection can alter the molecular mechanisms of learning, affecting the memorization process and disrupting the central and peripheral nervous systems (Iqbal and Mueller, 2007). DWV transmission follows several routes with varying pathogen abundance and virulence. In *A. mellifera* colonies, DWV can be transmitted vertically (i.e., from queen or drone to offspring) and horizontally via trophallaxis and shared food resources (Grozinger and Flenniken, 2019). Consequently, viral diseases have presented a negative impact on food security and wildlife as well as global economic stability (Hall and Steiner, 2019).

Currently, beekeepers have several tools to control the vector of DWV, Varroa mite. Among the measures, the use of synthetic chemical acaricides or natural repellents, such as organic acids and essential oils, combined with the use of management practices are included, though, they are not being effective enough (van der Steen and

Vejsnæs, 2021). Moreover, treatments for DWV are focused on vector control but there is not curative treatment for colonies with high viral loads.

There are several biotic and abiotic factors that contribute to colony health and survival (e.g., the diet of the colony), constituting alternatives to control DWV. Consumption of a poor-quality diet (e.g., sugar water) can result in greater DWV levels compared to bees fed higher-quality, pollen-containing diets (Tritschler et al., 2017). Phytochemicals, particularly phenolics, terpenoids and alkaloids, present antimicrobial activity (Berenbaum and Calla, 2021; Nicolson et al., 2007) and even protect against microbial alterations in bees. Recently, researchers have described antiviral activity in extracts from the mycelium of multiple polypore fungal species. In fact, (Stamets et al., 2018) found that honeybees may gain health benefits from fungi and their antimicrobial compounds after observing the bees feeding with water from the fungal sources, which may be considered as a case of self-medication. In addition, it has been reported that levels of DWV decreased up to 500-fold when young bees were fed thymol (isolated from thyme) in sugar syrup and released into field colonies (Palmer-Young et al., 2017). Respect to phenolic compounds, a study conducted by Arismendi et al. (2018) evaluated the effects of methanolic extracts of Chilean native plant leaves (*Aristotelia chilensis, Ugni molinae* and *Gevuina avellana*) and propolis on the load of *N. ceranae*. The authors concluded that leaf extracts, with high concentrations of rutin and myricetin, and propolis with high concentrations of galangin and pinocembrin, presented antiparasitic and antimicrobial activity. In addition, they proved an improved survival of bees and a decreased load of *N. ceranae* in infected bees and described some of the mechanisms involved in the enhanced immune system response of honeybees by the action of the phytochemicals.

Honey bees are endowed with an RNA interference (RNAi) molecular machinery, a eukaryotic antiviral immune system (Brutscher and Flenniken, 2015). RNAi is a post-transcriptional, sequence-specific, gene silencing mechanism and the small interfering RNA (siRNA)-mediated pathway is the main antiviral defense mechanism in insects (Chejanovsky et al., 2014). This RNAi could be induced by feeding or injecting dsRNA as an alternative to prevent gene expression and**,** consequently**,** block the replication of RNA viruses, including DWV (Brutscher et al., 2017; Leonard et al., 2020). As other insects, honeybee antiviral responses also include autophagy, apoptosis, eicosanoid biosynthesis, endocytosis, melanization, and JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription), Toll, NF-κB (Nuclear Factor κB), JNK (c-Jun N-terminal kinase), and MAPK (Mitogen-Activated Protein Kinases) pathways (Ryabov et al., 2016).

Consequently, grape wastes, specifically grape pomace, rich in phenolic compounds, could be formulated as supplement enhancing honeybee immune system. In order to protect bioactive compounds obtained from grape waste and avoid their degradation over time, encapsulation techniques have been developed to ensure their stability. Among of them, spray drying has been widely used in the large-scale production of encapsulated formulations since it is economical and adaptable, while it makes a product of excellent quality. The process generates protection and stability to the bioactive compounds by acting as a physical barrier and allowing for slow releases. In terms of the loss of bioactive content, high temperature in the process is the main inconvenient, and thus the process needs to be optimized, while the properties and bioactivity of the final ingredient should be studied (Akhavan Mahdavi et al., 2016). Studies carried out with Tintorera have shown that the use of this technique preserves

anthocyanin content by maintaining the formulation for four weeks (López-Belchí et al., 2021). The authors demonstrated that after spray drying, the ingredient had an adequate anthocyanin encapsulation efficiency, resulting in increased stability and bioaccessibility.

Hence, the use of environmentally friendly alternatives to help mitigate phytosanitary problems in apicultural systems and reuse winemaking wastes is worthy for environmental sustainability. Therefore, the objective of this study was to evaluate the effects of grape pomace powder as a dietary supplement to enhance the immune system of honeybees affected by DWV.

2. Materials and Methods

2.1. Material

Grape pomace var. Tintorera was collected in the Itata Valley, Ñuble Region, (36°37'6.96"S, 72°19'30.81"O) in the 2021 season. The pomace was frozen and transported to the BIOINVE (Bioactive Compounds and Ingredients from Plants Laboratory, Faculty of Agronomy, University of Concepción, Concepción, Chile) and kept at −80 °C until processed and analyzed.

2.2. Preparation of the microencapsulated supplement by spray drying and characterization of the grape pomace powder (GPP)

Batchs of 500 g of grape pomace was mixed with ethanol 96% (w/v) following the methodology described by Romero Román (2021). The resulting solutions were homogenized by constant agitation while they were fed into a mini spray-dryer B-290 (Büchi, Flawil, Switzerland) at room temperature and at a flow rate of 4 mL min^{-1}. The inlet temperature was maintained at 120 °C, whereas the outlet air temperature was

80 °C (Table S1). The dried powder was collected and stored in an opaque, air-tight container at 4 °C for further analysis. Morphological characterization of the grape pomace powder (GPP) was carried out by SEM, Scanning Electron Microscopy (FEI-Inspect S50, FEI Company, Oregon, USA). GPP samples were attached to double-sided adhesive tape mounted on SEM stubs, covered with a gold layer (9 nm) under vacuum SEM working at 5 kV, with 10,003 and 50,003 magnification. Finally, powder recovery (ratio between the quantities of powder versus the initial mass solids), loading capacity (quantity of total phenolic compounds per 100 grams of powder) and entrapment efficiency (g total phenolic compounds encapsulated 100 g^{-1} phenolic compounds from pomace added) were calculated. Total phenolic compounds (g) were obtained by HPLC-DAD at 280, 320, 360 and 520 nm similar to described in 2.3.

2.3. Characterization and quantification of anthocyanins and antioxidant properties from grape pomace powder

The extraction and chemical characterization of the GPP was performed according to the methodology described by López-Belchí et al. (2021). Anthocyanin analysis was performed by using the extracted samples and quantified by a Hitachi HPLC-DAD system (Hitachi technologies, MERCK, Darmstadt, Germany) using the same chromatographic conditions described by López-Belchí et al. (2021), recording chromatograms at 520 nm and using cyanidin 3-*O*-glucoside as external standard (Sigma-Aldrich, St. Louis, MO,USA). All the solvents used in the extractions were of analytical grade and obtained from Merck (Darmstadt, Germany). The results were expressed in mg 100 g^{-1} DW.

The antioxidant activity was determined by spectrophotometry 2,2-diphenil-1-picrilhidracilo (DPPH*) and oxygen radical absorbance capacity assays (Pantelić et al.,

2016) (López-Belchí et al., 2021; Mena et al., 2011). Briefly, the antioxidant capacity for ORAC-FL was evaluated by measuring the variation in fluorescence after 120 min of reaction with the radical. DPPH was determined by measuring the variation in absorbance at 515 nm after 30 min of reaction with the radical. The assays were performed using 96-well micro in a Synergy H1 hybrid multi-mode microplate reader (Biotek, Winooski, VT, US). The results were expressed as µmol Trolox 100 g^{-1} DW. Six replicates were performed for each analysis.

2.4. Inoculum DWV-A Preparation

An isolate of the deformed wing virus variant-A (DWV-A) was obtained from infected colonies according to Gusachenko et al. (2020). A group of 20 bees was homogenized in a Stomacher bag with phosphate-buffered saline (1X PBS) for 90 s at high speed in a Stomacher 80 Lab Blender (Seward, London, UK). The samples were centrifuged twice at 1500 × *g* for 10 min at 4 °C, followed by 10000 × *g* for 10 min at the same temperature. The supernatant was purified by filtration with a 0.22 µM filter (PES, Merck Millipore, Darmstadt, Germany). Subsequently, 200 µL of the supernatant were used for RNA extraction, cDNA synthesis, and the subsequent viral load quantification of the inoculum according to the methodology described in the section described below 2.6. The rest of the extracted inoculum was stored at −80 °C until further use.

2.5. Bee Inoculation and experimental design

Adults of *A. mellifera* were obtained from the experimental apiary located in El Nogal Experimental Station (36°35′58.25” S–72°04′51.77” W), University of Concepción, Chillán, Chile. Prior to the assay, the health status of the apiary was determined by identifying the viral level in each colony (*n* = 54 beehives). Pathogen level was determined by molecular techniques in accordance with Vargas et al. (2017). Brood

combs with capped worker bees were then removed from colonies with low DWV load (1.0×10^2 copy number per bee); no colonies were detected DWV-free. The brood combs were maintained under controlled conditions in a rearing room (30 °C ± 1; 60 % ± 3 RH). Afterwards, newly emerged worker bees (24 h old) were carefully collected from the brood combs and randomly confined in plastic cages (base = 8 cm diameter, mouth = 10 cm diameter, and height = 15 cm). Bees were inoculated orally with 5 µL of a viral suspension (1.0×10^9 copy number per bee) in a 50% sucrose solution according to the methodology described by Porrini et al. (2013). The bees that did not consume the total amount of viral inoculum were discarded from the assay. Then, four treatments consisting of different doses of GPP (0.5%, 1%, 2.5% and 5%) dissolved in sugar syrup at 50% weight/volume ratio ad libitum were used. Two control treatments without GPP were also included, a non-inoculated (N – DWV) control and an inoculated (I – DWV) control (see Klapan-Meiners survival curves in supplementary material Figure S1). For all the GPP treatments and controls, 4 replicates were carried out with 80 worker bees each. A dietary regimen was established for the treatments with the GPP. The regimen consisted of 24 h with the bioactive supplement and 48 h with sugar syrup, both *ad libitum*, starting on day 0 and alternating until day 14 of the experiment, where the maximum viral load occurs because of viral replication (Silva et al., 2021). In addition, all cages contained 3 g of pollen substitute. Five bees were collected per cage, starting on the day of virus inoculation according to the dietary regimen described. Finally, 11 collections were obtained during the whole assay, 17 days post-inoculation (20-day-old bees).

2.6. RNA Extraction and cDNA Synthesis

Total RNA extraction was performed according to the methodology described by Riveros et al. (2019). Five bees were collected by sampling time, treatment, and replicate. These were ground in 5 mL of PBS buffered saline. From the macerate, 200 µL were collected and 500 µL of TrizolTM (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) were added. RNA extraction was carried out following using E.Z.N.A. ®Total RNA kit I (Omega Bio-Tek, Norcross, GA, USA). RNA quality and yield were determined using a spectrometer (Infinite 200 PRO NanoQuant, Tecan Group, Männedorf, Switzerland). The extracted RNA was subsequently used for first-strand cDNA synthesis by using the enzyme reverse transcriptase M-MLV (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.7. Real-Time PCR Quantification (qPCR).

To quantify the viral load and expression of defense genes, specific primers were used (Table 1). The PCR reaction was carried out using 1× of KAPA SYBR FAST Universal 2× qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA), following the manufacturer's instructions. Samples were brought to a reaction volume of 15 µL, including 20 ng of cDNA, 530 nM of each primer, and sterile filtered molecular-grade water to reach 15 µL. The thermal reaction conditions used were 96 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Real-time PCR assays were performed on a Stratagene Mx3000P thermal cycler (Agilent Technologies, Santa Clara, CA, USA), and the data were analyzed using MxPro software (Stratagene, Agilent Technologies, Santa Clara, CA, USA). The relative expression of each gene was calculated after normalization with an endogen gene (β-actin) as described by Pfaffl (2001). Viral load was determined by absolute

quantification of DWV-A. A standard curve using purified PCR product Wizard®VR SV gel and PCR clean-up system (Promega, Madison, WI, USA), belonging to the viral target sequence, was used (Riveros et al., 2019). The purified amplicon was then quantified by spectrophotometry (EpochTM Microplate Spectrophotometer, BioTek, Winooski, VT, USA) to calculate the copy number according to Wu et al. (2017). Thus, linear standard curves (95–100% efficiency) were then generated using a serial dilution (1.0×10^1 to 1.0×10^9) of viral copy numbers of purified cDNA. Afterwards, the cycle treshold (Ct) values were plotted against copy number values ($\log_{10}$). Thus, the sample copy numbers were estimated using the Ct values and comparisons with the linear equation of the standard curve and normalizing values of the housekeeping β-actin gene (Yang and Cox-Foster, 2005). Data were then expressed as the copy number of DWV per bee by considering the dilutions that were performed in the cDNA synthesis and qPCR reaction.

Table 1. Nucleotide sequence of defense gene primers from *Apis mellifera* and deformed wing virus (DWV-A) used in qRT-PCR.

Primers	Sequences	Reference
DWV-A	F TATCTTCATTAAAGCCACCTGGAA R TTTCCTCATTAACTGTGTCGTTGAT	(Yang and Cox-Foster, 2005)
β-Actin	F ATGCCAACACTGTCCTTTCTGG R GACCCACCAATCCATACGGA	(Yang and Cox-Foster, 2005)
Relish	F GCAGTGTTGAAGGAGCTGAA R CCAATTCTGAAAAGCGTCCA	(Evans, 2006)
Cactus	F CACAAGATCTGGAGCAACGA R GCATTCTTGAAGGAGGAACG	(Evans, 2006)
Argo-2	F ACCTGCTGAGTTATGCACAGT R AGCCTTTAGAACTCTTGCTGGT	(Zhao et al., 2019)
Dicer	F AGCAGTAGCTGATTGTGTGGA R TGAAGGATGTGTAAACGCCTGT	(Zhao et al., 2019)
Dorsal	F CTCATCGGAAGACATGACAGTGA R TGAATTCAAAGCCAGTTCGAAAA	(Zhao et al., 2019)
Amel102	F CAACTCCAGAATTGGAAATAGCA R TTTGCAATAGGAAAAGCAGTTG	(Di Prisco et al., 2016)

2.8 Data analysis

The effectiveness and achievability of the ingredient based on grape pomace was established on the response of honeybee immune system. Gene expression level were analyzed using an one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for significance using R software version 4.0.3. Viral load was analyzed using Tukey's test. A $P < 0.05$ indicated a statistically significant result.

3. Results and Discussion

3.1 Characterization of the grape pomace dietary supplement.

Diet plays a fundamental role in maintaining strong and healthy honeybee colonies (Frizzera, 2020). In fact, there is evidence that the capacity of the colony to face both biotic and abiotic stressors can be enhanced by maintaining strong bees through a convenient supply of nutrients (Annoscia et al., 2017), activating the most important protective physiological system of all organisms, the immune system. The workflow presented in this study follows the scheme in Fig 1.

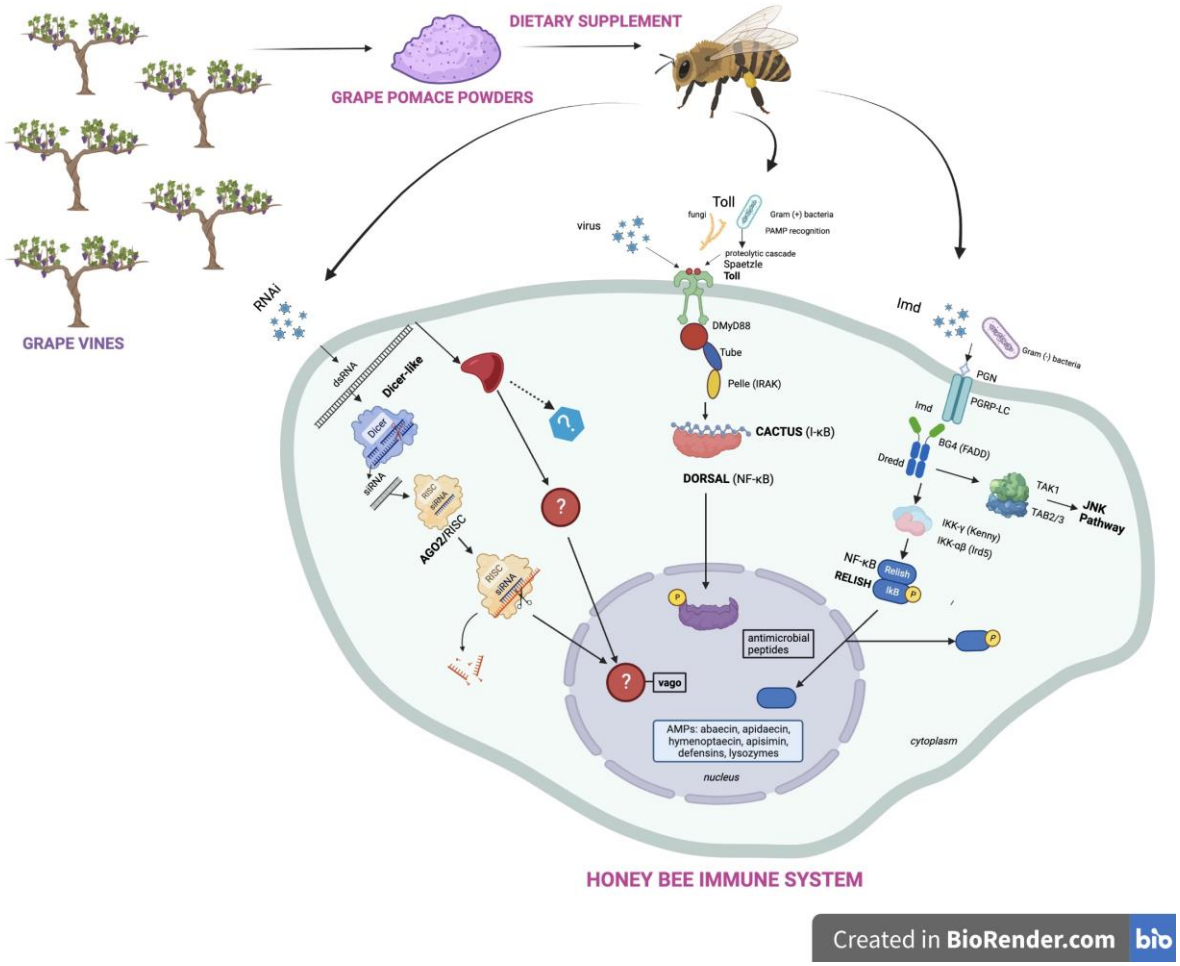

Fig 1. Workflow of the production of GPP to apply as dietary supplement strengthening honeybee immune system.

Grape pomace (Wu et al., 2021) and GPP obtained by spray drying were chromatographically analyzed. Ten anthocyanins, as main phenolic compounds, were quantified by HPLC-DAD (Table 2). Regarding concentration of phenolic compounds, the obtained values agree with those reported by Wu et al. (2021). The main anthocyanins quantified from GP raw material and GPP were malvidin 3-*O*-hexoside and petunidin 3-*O*-hexoside, while content losses of the compounds ranged between 36 and 53 %, with an average of 41% of the GPP with respect to the GP raw material. It is important to point out that despite the loss of anthocyanins due to drying process,

the final content was still high, making this ingredient a source of bioactive compounds to supplement the diet of bees.

Table 2. Anthocyanin content (mg 100 g^{-1} DW) in grape pomace and grape pomace powder (GPP) from Tintorera grapes.

	GP	GPP	Anthocyanin content Losses (%)
Cyanidin 3-*O*-hexoside	175.79 a	96.25 b	45
Peonidin 3-*O*-hexoside	40.34 a	18.87 b	53
Delphinidin 3-*O*-hexoside	180.90 a	108.03 b	40
Petunidin 3-*O*-hexoside	403.70 a	227.93 b	43
Malvidin 3-*O*-hexoside	682.34 a	420.44 b	38
Malvidin 3-*O*-(6-acetil)-glicoside	42.09 a	26.35 b	37
Delphinidin 3,5-*O*-di-hexoside	38.26 a	18.42 b	51
Malvidin 3,5-*O*-di-hexoside	189.39 a	111.56 b	41
Petunidin 3,5-*O*-di-hexoside	34.21 a	21.65 b	36
Malvidin derivatives	86.26 a	53.14 b	38
Total Anthocyanins	1873.29 a	1102.65 b	41

Different letters within the same row indicate significant differences at $p < 0.05$ in drying treatments according to Tukey's test.

The antioxidant activity was determined by using two assays, 2,2-diphenyl-1picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (Pantelić et al., 2016). Assays were carried out and measured for the raw material, GP, and for ingredient obtained after the spray-drying process (GPP), obtaining high antioxidant activity for GPP (2988 µmol Trolox g^{-1} of sample DW in DPPH assay and 4921 µmol Trolox g^{-1} of sample DW in ORAC assay) (see Table S3).

After encapsulation, micrographs of the formulation were visualized and analyzed (Fig 2). SEM images showed spherical shape though most of its surfaces were depressed. The photos also confirmed that the maltodextrin used as a matrix to encapsulate the

compound, worked effectively and efficiently (Fredes et al., 2018). It was also possible to estimate the particle size of the anthocyanin-rich grape pomace coatings by this visualization. Due to agglomeration, some particles showed a slightly rough surface. Particle sizes were stable (average 7 µm).

Grape pomace and ingredients based on this has a high-added value due to its high content of bioactive compounds and its wide variety of applications. Therefore, given the great number of wastes generated by the wine industry, the reintroduction of new high-value ingredients would represent a good alternative to address food waste management.

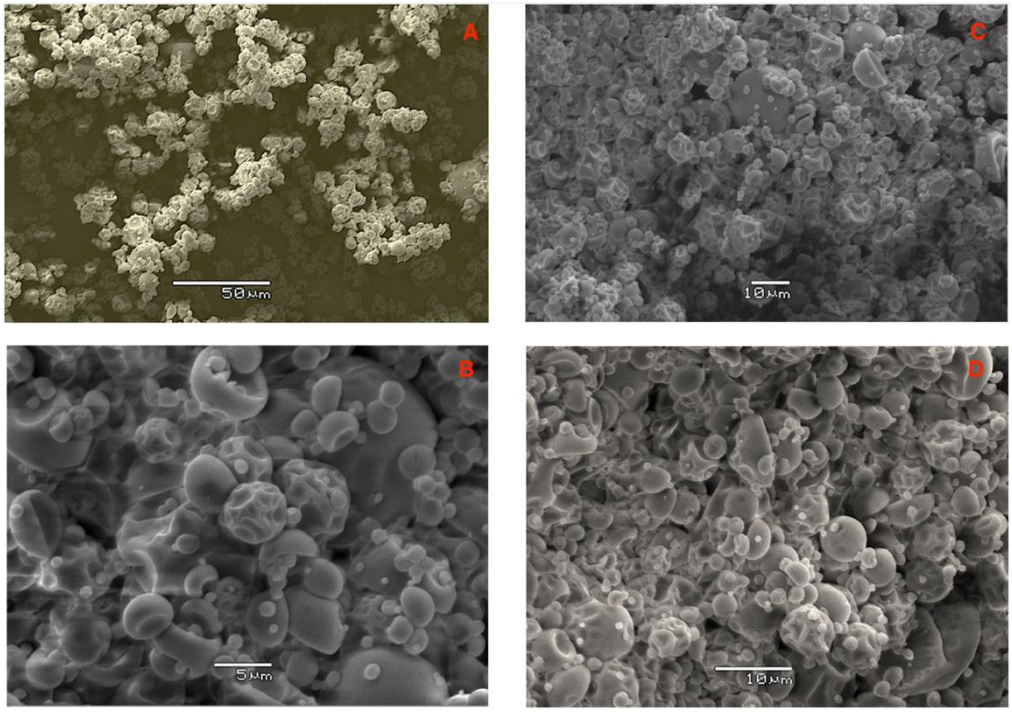

Figure 2. SEM images of the spray-dried grape pomace powder encapsulated with 20% maltodextrin for the spray-drying condition of 120°C inlet air temperature. Different scales are shown: 50µm (A); 5 µm (B); and 10µm (C and D).

3.2 DWV load after feeding with a grape pomace supplement

The relationship between honeybee viral load and the provision of a supplementary diet rich in bioactive compounds was studied, while the transcriptional profiles of

multiple separate (though inextricably coupled) immune pathways were determined and analyzed with respect to DWV.

Respect to the honeybee viral loads, I – DWV presented similar viral loads to the GPP treatments on 0,5 day post-inoculation, while N - DWV showed significantly lower differences that did not exceed 6.0 viral load (1.0×10^6 copy number per bee) (Figure 3).

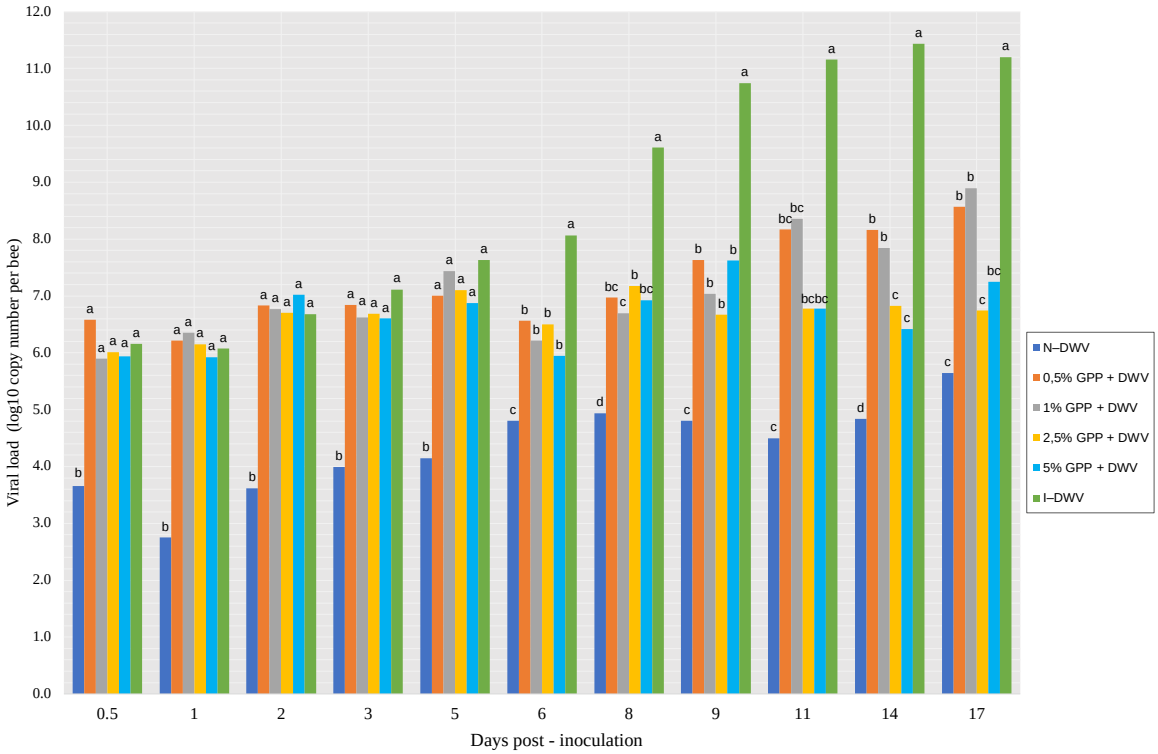

Figure 3. DWV loads in worker honeybees recorded days post-inoculation throughout the assay. Grape pomace powder (GPP) concentrations (0.5, 1, 2.5 and 5%), inoculated control (I - DWV)) and non-inoculated control (N - DWV) are shown in different colors. Different letters indicate significant differences according to Tukey's test ($p<0.005$).

Viral load varied significantly between bees that were inoculated with DWV and supplemented with GPP (doses of 0.5, 1, 2.5 and 5%) and those inoculated (I-DWV) (Figure 3). These differences were observed for each collection day, from day 6 post-inoculation and until the end of the assay. All the treatments containing GPP decreased viral load, showing significant differences with respect to I-DWV and N-

DWV. For example, the viral load recorded on day 6 post-inoculation was 30% lower in bees fed with GPP supplement at 5% (1.0×10^6 copy number per bee) compared to the I-DWV control (1.0×10^{11} copy number per bee), with significant differences between the treatments. On day 11 post-inoculation, viral load decreased by 30% with the 0.5 and 1% GPP treatments, while the 2.5 and 5% GPP treatments resulted in reductions of 40% with respect to I-DWV. These results are in agreement with Felicioli et al. (2020), who tested an artificial diet capable of stimulating the immune system in bees, and reported that the provision of 1,3-1,6 β-glucans to honeybees infected by DWV reduced or maintained DWV viral load compared with the control group.

3.3 Response in honeybee gene expression after feeding with a grape pomace supplement.

Studies on transcriptional level in honeybees involve uncharacterized genes/pathways in antiviral responses (Brutscher et al., 2017). In this sense, the roles of genes in the Toll, Imd, Jak-STAT, JNK, and RNAi pathways are the best characterized. In the present study colonies with very low levels of DWV were used, in absence of *Varroa* or other pathogens according to the strategy described in other comparable studies (Ryabov et al., 2019; Silva et al., 2021; Tesovnik et al., 2020).

RNAi is the main antiviral mechanism in insects (Gammon and Mello, 2015). Therefore, honeybees were expected to mount an RNAi response when inoculated with DWV. The RNAi-pathway is initiated by Dm Dicer-2 cleavage of viral dsRNA into 21–22 bp siRNAs; Am Dicer-like share 30% aa identity with Dm Dicer-2. The siRNAs are then loaded into AGO2 (Argonaute-2), the catalytic component of the RISC (RNA Induced Silencing Complex) (McMenamin et al., 2018). A single strand of the siRNA is retained in the RISC and used to specifically target cognate viral genome sequences

for cleavage. In addition, Dm Dicer-2 serves as a dsRNA sensor that mediates a signal transduction cascade resulting in increased expression of Dm Vago and suppression of viral replication. Am Dicer-like may serve as a dsRNA sensor, and honeybees have a Vago orthologue, which is up-regulated in DWV-infected honeybees (Brutscher et al., 2017).

Even though no significant differences in Dicer and Argo-2 expression were found between the treatments, peaks of expression were observed on collection days 1 and 8 for low and medium doses (0.5;1; 2.5%), which showed higher gene expression (Fig 4).

A study of the relationship between the *Varroa* parasite and DWV showed that expression of both Dicer and Argonaute-2 increased in parallel with DWV viral loads in pupae over time, while Dicer was the only gene that was up-regulated in DWV-injected pupae compared to the two control groups at all time points (Norton, 2021). In this study Dicer showed jumps in expression throughout the assay (Fig 4A).

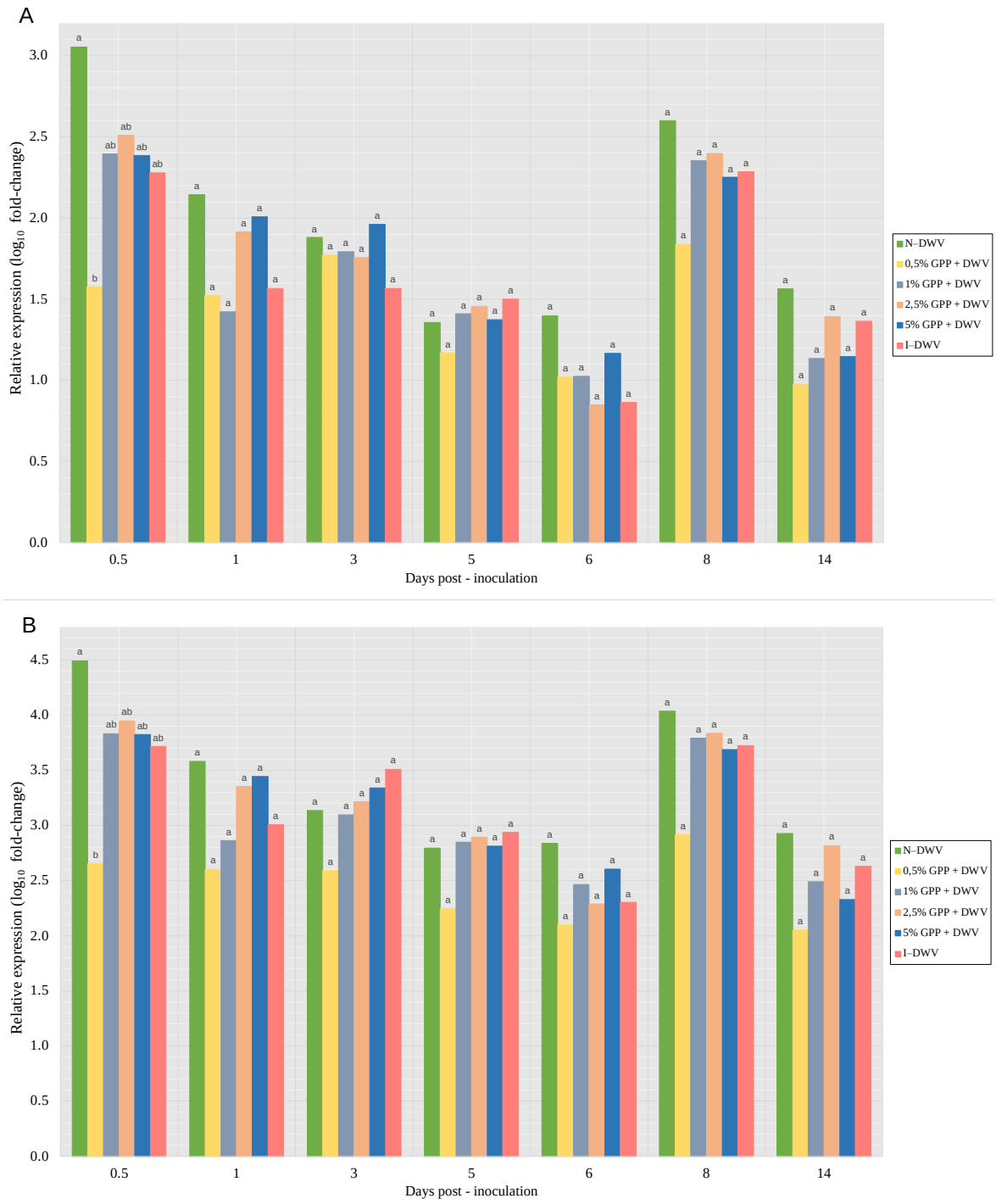

Figure 4. Expression level of *Dicer* (A) and *Argo-2* (B) genes involved in RNAi-pathway in worker honeybees per collection throughout the assay. Grape pomace powder (GPP) concentrations (0.5, 1, 2.5 and 5%), inoculated control (I - DWV) and non-inoculated control (N - DWV) are shown in different colors. Different letters indicate significant differences according to Tukey's test ($p<0.005$).

Although the quantification of the transcript expression of *Vago* gene revealed some significant differences, it is important to highlight the low expression observed for the lowest concentration (0.5%) throughout the assay compared to the controls and the other GPP treatments (Fig 5). A direct relationship between the expression of these two genes and the expression of Vago was also found. In *B. terrestris*, Vago limits viral infection in fat bodies in a Dicer-dependent manner (McMenamin et al., 2018).

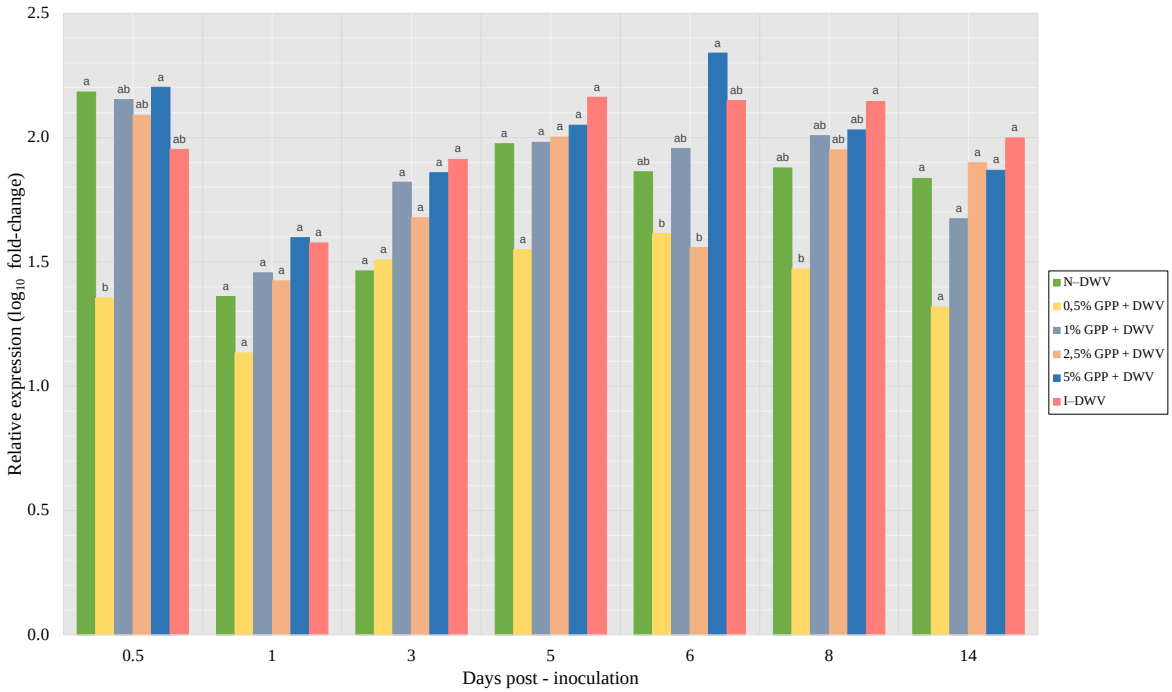

Figure 5. Expression level of *Vago* gene in worker honeybees per collection throughout the assay. Grape pomace powder (GPP) concentrations (0.5, 1, 2.5 and 5%), inoculated control (I - DWV) and non-inoculated control (N - DWV) are shown in different colors. Different letters indicate significant differences according to Tukey's test ($p<0.005$).

RNA interference (RNAi) via double stranded RNA (dsRNA) is a sequence specific post-transcriptional gene-regulatory and the principal insect innate immune response for the detecting and inhibiting virus replication in insects (Gammon and Mello, 2015). Activation of this pathway in bees results in increased expression of the Vago gene, an

orthologue found in *Drosophila*, resulting in suppression of viral replication (Larsen et al., 2019).

Recent data suggest that the Toll and Imd pathways also contribute to defense against viral pathogens (Felicioli et al., 2020). The Toll pathway is generally associated with fungi and gram-positive bacteria, and the Imd pathway with gram-negative bacteria (Yang et al., 2018). *Dorsal* (Toll), and *Relish* (Imd) are both NF-κB family transcription factors involved in the production of antimicrobial peptides (AMPs) in fat bodies, a tissue analogous to the mammalian liver (Norton, 2021). It has been previously suggested that the Toll pathway, particularly the expression of NF-κB factor *Dorsal-1A,* is a key element in regulating the immune response of honeybees against DWV (Nazzi et al., 2012; Nazzi and Pennacchio, 2018).

Cactus is a NF-κB inhibitor, which must be degraded to allow nuclear translocation of Dorsal (Zuo et al., 2016). Increased cactus expression in DWV infected adult bees parasitized by *V. destructor* has been associated with the down-regulation of Dorsal (Norton, 2021; Zanni et al., 2017). This is consistent with the results of the present study since significant differences were observed for Cactus and Dorsal in both control and GPP treatments (Fig 6), while the expression of Cactus silenced the expression of Dorsal mainly in the non-inoculated control (N - DWV).

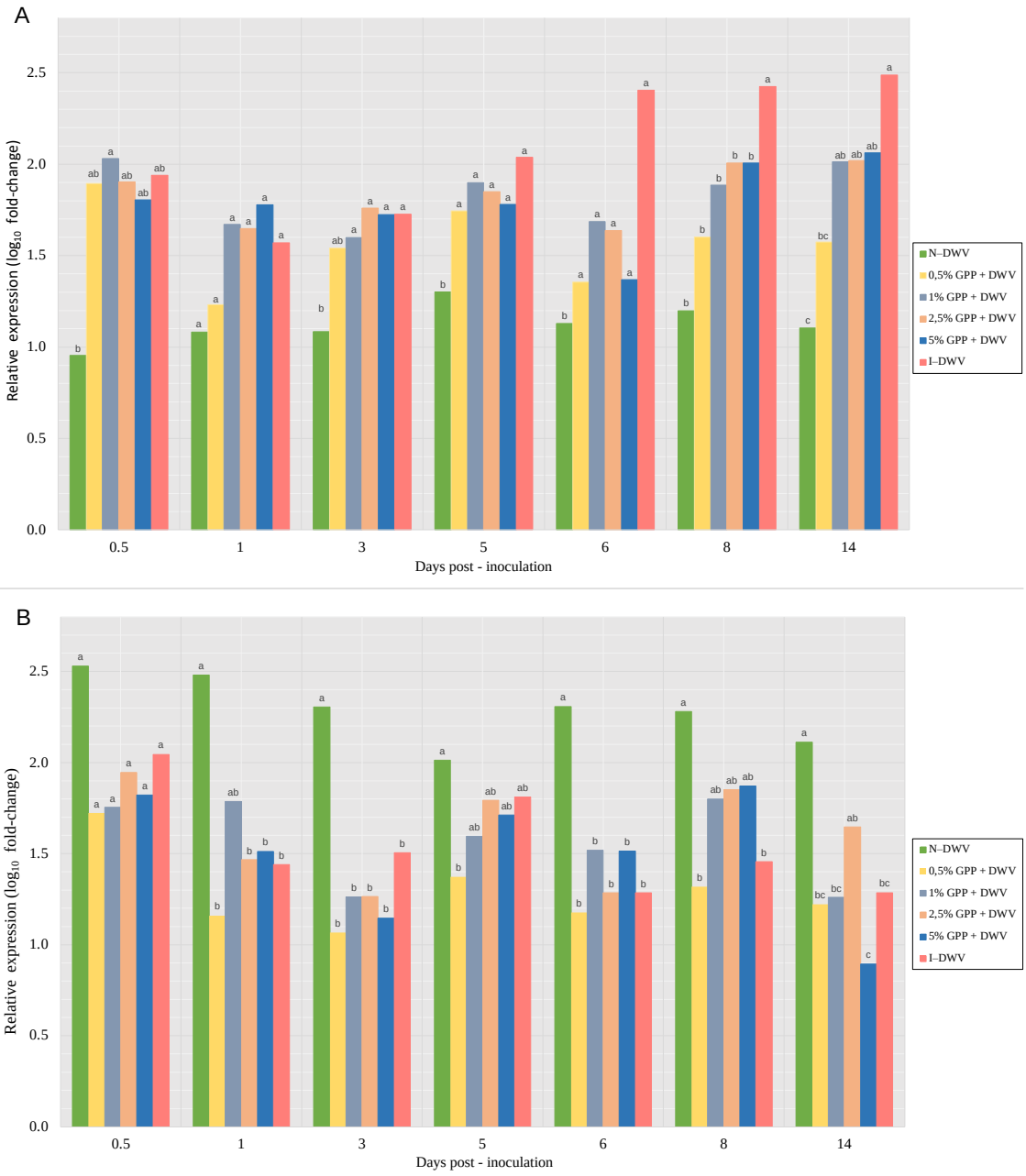

Figure 6. Expression level of *Cactus* (A) and *Dorsal* (B) genes involved in Toll pathway, worker honeybees per collection throughout the assay. Grape pomace powder (GPP) concentrations (0.5, 1, 2.5 and 5%), inoculated control (I - DWV) and non-inoculated control (N - DWV). Different letters indicate significant differences according to Tukey's test ($p<0.005$).

In addition, all the GPP treatments at different concentrations showed an intermediate expression with respect to N - DWV and I – DWV control treatments, with some exceptions. This clearly indicates that the dietary supplementation based on GPP influences the expression of Toll pathway genes.

In bees and flies, the immune-deficiency signaling pathway (Imd) activates the Relish transcription factor (homologue to NF-κB transcription factor) (Larsen et al., 2019).

In Drosophila, immune pathways are tightly regulated in order to balance immune responses, and thus increased expression of pathway inhibitors, like Cactus, does not necessarily indicate complete or continuous repression of the pathway (e.g., Toll) (Brutscher et al., 2017). Relish is a transcription factor in the Imd pathway, which upon cleavage, leads to the production of antimicrobial peptides (AMPs) abaecin and hymenoptaecin (Norton, 2021).

In the present study, the quantification of Relish gene (Fig 7) showed significant differences between the treatments with respect to the inoculated control (I - DWV) throughout the assay, showing a higher expression than I - DWV and maintaining gene expression similar to N – DWV (before 14 days). There is evidence that both the Toll and Imd pathways defend against viral invasion in insects (Ferreira et al., 2014; Lu et al., 2020).

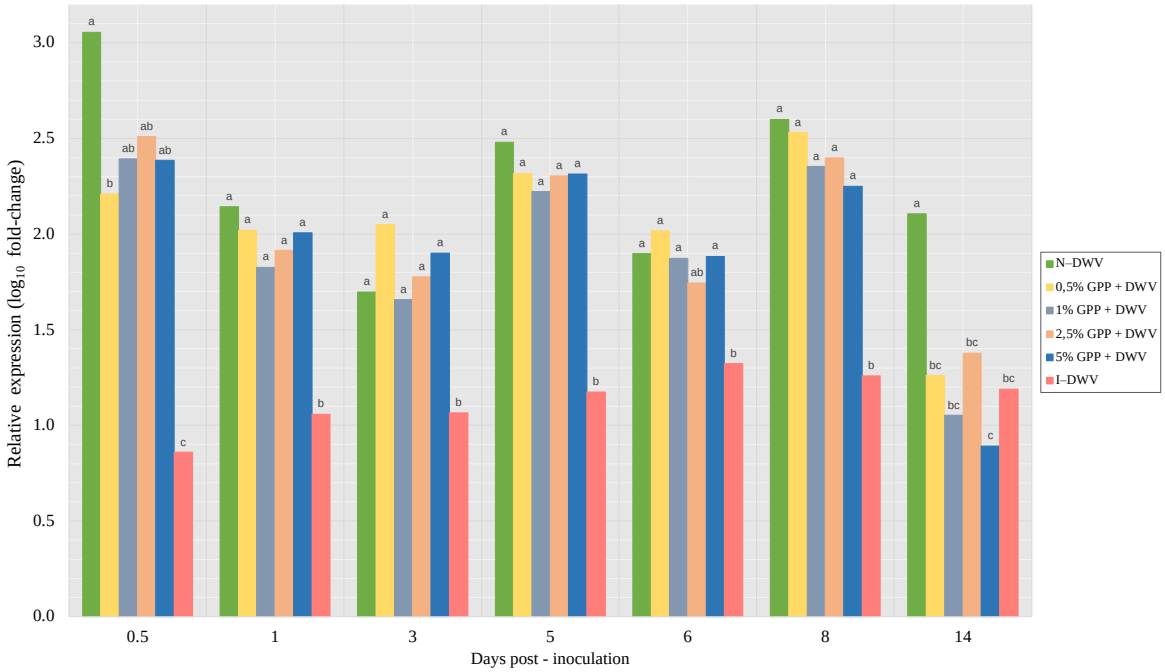

Figure 7. Expression level of *Relish* gene involved in Imd-pathway in worker honeybees per collection throughout the assay. Grape pomace powder (GPP) concentrations (0.5, 1, 2.5 and 5%), inoculated control (I - DWV) and non-inoculated control (N - DWV). Different letters indicate significant differences according to Tukey's test ($p<0.005$).

The results showed that the GPP dietary supplement inhibited DWV infection in DWV-infected bees. This indicates that the bioactive compounds found in grape pomace strengthen the immune system by up-regulating the expression of genes involved in pathways known to influence immune responses (RNAi, Toll and Imd), protecting honeybees from external stress caused by viral infections. A study on bees infected with DWV conducted by Lu et al. (2020), with the aim to investigate how caffeine affects bee response against pathogens and the expression profiles of genes involved in the immune response, showed that caffeine can increase the expression of genes involved in immunity and reduce the copy number of the virus, indicating that it has the potential to enhance to capacity of bees to fight against viral infection.

Although the results are shown separately for the viral load and the expression of each gene (with some significant differences), an overall view shows that each gene is

reflected in the DWV viral load (Fig 3), being consistent with the gene expression and representing a lower viral load, which suggests that the supplement had an effect by reducing viral load.

4. Conclusions

Given the great amount of waste and by-products generated by food processing (e.g., wine-making industry), waste management valorization is essential to ensure environmental sustainability. The present study evaluated the effect of different doses (0.5. 1. 1. 2.5. 5%) of grape pomace powder (GPP) supplement on the immune system of honeybees, specifically its capacity to control deformed wing virus (DWV). Loads of DWV were lower after feeding with the supplement (in the GPP treatments) respect to the DWV inoculated control (I – DWV). Gene expression determined for the Relish gene showed significant differences for all the GPP doses used with respect to I - DWV, while the treatments showed no significant differences compared to the non-inoculated control (N – DWV), except for the lowest dose (0.5%) on collection day 1. The results allow concluding that the inclusion of encapsulated GPP in the diet of honeybees is a promising alternative to improve bee health because it decreases DWV loads and influences the expression of genes involved in their immune system pathways. This study provides knowledge of the influence of the polyphenols of grape pomace on the expression profiles of immune-related genes in honeybees, offering an alternative to waste management from wine production.

Declaration of competing interest: The authors declare that they have no conflict of interest.

Ethics approval: Not applicable.

Consent to participate: All participants gave their informed consent to participate in the study.

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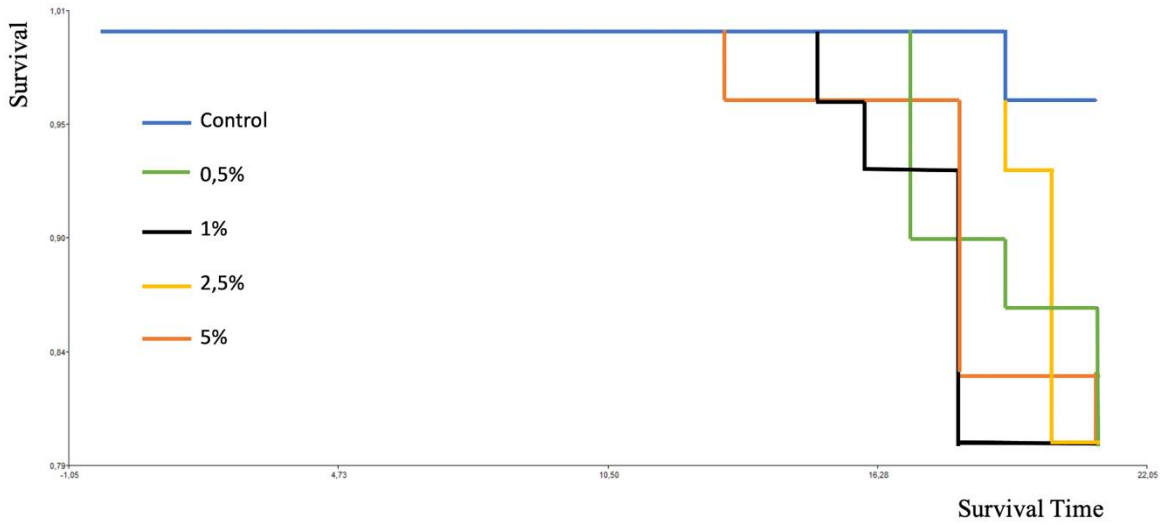
Supplementary Material

Table S1. Summary of conditions of the preparation of spray-drying

Conditions used	SD
Inlet air Temperature (°C)	120
Feed flow rate (mL min^{-1})	4
Wall material content (% w v^{-1})	20

SD: Spray-Drying (powder)

Figure S1. Klapan-Meiners survival curves for 4 doses of GPP (0.5 %; 1 %; 2.5 %; 5 %) in honeybees.



24 hours extract/48 hours 30 bees per cage, with 4 different doses of GPP (0.5; 1; 2.5 and 5%) plus a control without GPP, 4 repetitions per dose were made and they were fed 24 hours with extract, then 48 hours only syrup, repeating until day 12 (complete 4 times per 24 hours with extract) and then feeding with syrup. All treatments and their repetitions with protein cake.

Chi-square treatment for log rank test=4.515 p=0.340820

Treatment.	case	Time	Exposed.	Deaths	Survival	E.E
0,50%	1	17	30	1	0,97	0,03
0,50%	6	18	29	5	0,80	0,07
0,50%	30	21	24	0	0,80	0,07
1,00%	1	15	30	1	0,97	0,03
1,00%	2	16	29	1	0,93	0,04
1,00%	3	17	28	1	0,90	0,05
1,00%	4	19	27	1	0,87	0,06
1,00%	6	20	26	2	0,80	0,07
1,00%	30	21	24	0	0,80	0,07
2,50%	3	19	30	2	0,93	0,04
2,50%	5	20	28	2	0,87	0,06
2,50%	30	21	26	1	0,83	0,07
5,00%	1	13	30	1	0,97	0,03
5,00%	3	17	29	1	0,93	0,04
5,00%	8	18	28	3	0,83	0,06
5,00%	30	21	25	1	0,80	0,07
control	1	19	30	1	0,97	0,03
control	30	21	29	0	0,97	0,03

Table S2. Mean values for loading capacity, powder recovery and entrapment efficiency of powder based on grape pomace obtained by spray-drying.

Formulation Method[1]	Loading capacity[2]	Powder Recovery[3]	Entrapment Efficiency[4]
SD	77.06	42.04	41.10

[1]SD: Spray-Drying (powder); [2]Loading capacity was expressed as g *total phenolic compounds* per 100 g^{-1} powder; [3]Powder Recovery was expressed as g powder per 100 g^{-1} initial solids; [4]Entrapment efficiency was expressed as g *total phenolic compounds* encapsulated per 100 g^{-1} *phenolic compounds from pomace* added.

Table S3. Antioxidant capacity (DPPH• and ORAC) of grape pomace and grape pomace powder (GPP) from Tintorera grapes.

Sample	DPPH•	ORAC
GP	9340 a	8153 a
GPP	2988 b	4921 b

DPPH• (μmol Trolox g^{-1} of sample DW; ORAC (μmol Trolox g^{-1} of sample DW),). Different letters in the same column indicate significant differences at ($P \leq 0.05$).

CONCLUSIONES GENERALES

La extracción y el aprovechamiento de los subproductos de la industria vitivinícola abre un abanico de posibilidades para generar nuevos productos, ayuda a mitigar el impacto ambiental y mejorar la sustentabilidad en la producción agroindustrial vitícola. Es importante destacar que la calidad de los subproductos está condicionada a la variedad de uva (perfil fenólico), el manejo agronómico del viñedo, las técnicas enológicas aplicadas durante la vinificación, pero sobre todo a las tecnologías utilizadas para extraer y lograr estabilidad del material bioactivo.

La técnica de encapsulación de secado por atomización (spray-drying), mostró una alta eficiencia en la obtención de microcápsulas de orujo de uva logrando la conservación del 59 % de los compuestos fenólicos respecto a la materia prima, además de poseer y mantener una alta capacidad antioxidante, lo que hace que la encapsulación de compuestos fenólicos sea una alternativa con potencial biológico para mitigar enfermedades y virus. Por lo tanto, la utilización de extractos encapsulados de orujo de uva es una propuesta viable con el fin de escalar y producir nuevas formulaciones de alimentos y/o suplementos de alto valor agregado.

Finalmente, el trabajo investigativo realizado concluye que el suplemento dietético para abejas, a base de orujo de uva, obtenido de los subproductos generados por la vinificación y que fue encapsulado mediante la técnica de secado por atomización, tuvo un efecto positivo en moderar significativamente la carga del virus de las alas deformes (DWV) en abejas melíferas respecto al control infectado y mostró niveles similares o levemente mayores que el control no infectado con el virus en todas las dosis utilizadas. A su vez, logró regular la expresión de genes implicados en las rutas inmunitarias de las abejas, mostrando resultados prometedores, principalmente en el gen Relish donde el control no infectado mostró expresión génica sin diferencias respecto a tratamientos que recibieron el suplemento de orujo. Además, el control infectado presentó una menor expresión génica con diferencias significativas respecto a los tratamientos en sus distintas dosis y el control no infectado.

A futuro, se espera un aumento de nuevos ingredientes de alto valor añadido, generados a partir de los subproductos de la producción vitícola. Las investigaciones deberán estar enfocadas en la gestión de los residuos de las bodegas, los beneficios socioeconómicos y la seguridad medioambiental, pero generando innovación gracias a nuevos ingredientes a base de residuos de la vinificación con potencial bioactivo.

Divulgación de Resultados

Artículos

Pascual, G., Lopez, M.D., Vargas, M., Aranda, M., Canumir, J.A., 2022. Next Generation Ingredients Based on Winemaking By-Products and an Approaching to Antiviral Properties. Foods 11(11), 1604. doi:10.3390/foods11111604.

Pascual, G., Silva, D., Vargas, M., Aranda, M., Canumir, J.A., López, M.D. 2022. Dietary supplement of grape wastes enhances honey bee immune system and reduces deformed wing virus (DWV) load. Enviado a Environmental Research.

Otros Artículos

Pascual, G., Ide, W., Sabando, C., Castaño, J., Pettinelli, N., Bustos, R., Linares, A., Mora, L., Müller, N., Rodríguez-Llamazares, S., 2021. Grape (Vitis vinifera L. cv. País) Juices Obtained by Steam Extraction. Processes 9(9), 1670

López-Belchí, M.D., Caamaño, E.F., **Pascual, G**., Noriega, F., Fierro-Morales, P., Romero-Román, M.E., Jara, P., Schoebitz, M., Serra, I., Moreno, D.A., 2021. Spray-Dried Formulations Rich in Malvidin from Tintorera Grape Wastes: Characterization, Stability, and Storage. Processes 9(3), 518. doi:https://doi.org/10.3390/pr9030518.

Congresos, seminarios y conferencias

Comunicaciones orales

G. Pascual, M.D. López, P. Jara, A. Ruiz, J. Cañumin, M. Aranda. Variación del contenido fenólico en bayas de *Vitis vinifera* L. var. Tintorera durante dos temporadas. WJICA 2020.

G. Pascual, M. Vargas, M.D. López. Nuevo enfoque de ingredientes de nueva generación basado en los subproductos del vino para sistemas agroecológicos. WJICA 2021.

Becas y pasantías

Beca convocatoria Erasmus+ para la movilidad entre países asociados y el campus de excelencia internacional agroalimentario (CeiA3) proyecto ka107.

Pasantía de investigación en el Instituto de Investigación Vitivinícola y Agroalimentaria IVAGRO, Universidad de Cádiz, España II-2019.

ANEXO

Valle del Itata y Características de la Variedad Tintorera

El valle del Itata, se caracteriza por el cultivo más antiguo y tradicional de la vid en Chile, el cual se realiza en valles intermontanos de la cordillera de la Costa con una fuerte influencia del litoral en algunas áreas (Prieto, 2021). Actualmente su paisaje está fragmentado en pequeños predios, con un total de 4.815 propietarios (SAG, 2018). Sus viñedos, mayoritariamente, tienen un manejo tradicional, con vides plantadas sin ningún tipo de soporte, sistema conocido como "gobelet" y que en la zona es conocido como plantación "en cabeza", en general de bajo crecimiento vegetativo y alta densidad (Pszczolkowski et al., 2021). El proceso de vinificación destaca porque todavía se puede ver a los productores vinificando mediante antiguos métodos muy artesanales (Díaz y Muñoz, 2011). Se cultivan las variedades tradicionales del valle: Moscatel de Alejandría, Cinsault, y la cepa País, que destaca siendo la variedad más antigua cultivada en Chile (Prieto, 2021) y algunas variedades de pulpa coloreada que se ocupan para mejorar el color de los vinos tintos.

La falta de tecnología e industrialización durante la reconversión vitivinícola de Chile en la década de los 80, llevo a la zona a un estancamiento producto tanto en el proceso como con los manejos de los residuos los cuales actualmente se pierden en los campos contaminando los suelos y las aguas de los propios productores.

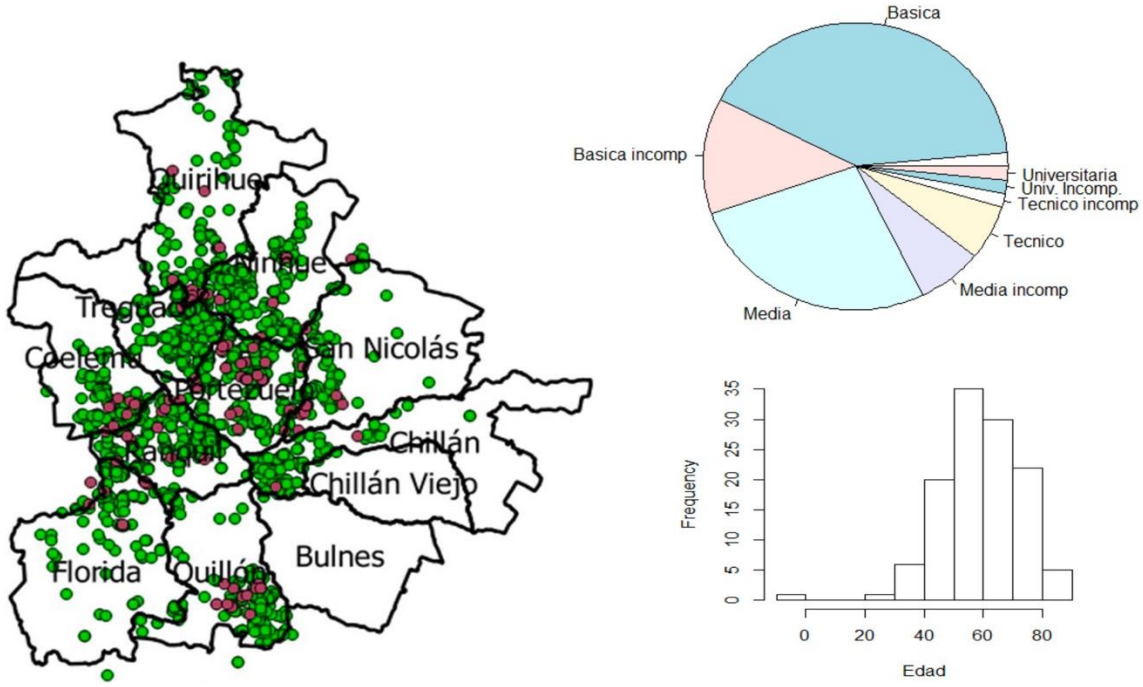


Figura 1. Características de los pequeños productores en el valle del Itata, ubicación, rango etario y nivel de educación (INDAP, 2016).

Geomorfológicamente, el Valle del Itata presenta un llano central fluvio-glacio-volcanico, donde algunas comunas presentas zonas de cordillera de la costa y de cuenca granítica marginal. El rio Itata presenta una longitud de 180 km con una superficie de 11.090 km. La fitogeografía de la zona está compuesta por matorral claro desértico siempre verde, vegetación de lomas, y bosque esclerófilo.

El cultivar Alicante Bouschet, cultivado en el valle del Itata, es uno de los pocos cultivares de uva *V. vinifera* con una pulpa de bayas de color rojo, por lo que también se lo conoce como cultivar Tintorera. La mayoría de las variedades tintoreras ahora cultivadas en todo el mundo fueron desarrolladas en el siglo XIX por Louis y Henri Bouschet. Son híbridos derivados de cruces los cuales fueron diseñados para aumentar la intensidad del color de las variedades más conocidas de vino tinto cultivadas en el pasado (Stefanello et al., 2020). En Chile existen 7.134 hectáreas de variedades tintoreras (SAG, 2018). En Particular estas variedades típicamente

contienen antocianinas monoglucosiladas, de las cuales la malvidina 3-glucósido es la más común (Zhu *et al.,* 2012).

Esta variedad es también conocida como Garnacha Tintorera (Lévate), Tinta Velasco (España), Alicante y Sumo Tinto (Rebello et al., 2013; Galet, 1990; Robinson, 1996). Entre todas las variedades Tintoreras, ésta es la que se ha cultivado más extensivamente en el mundo (Winkler, 1974).

Su descripción ampelográfica se basa en Galet (Galet, 1990) y Anónimos: El brote es asurcado, lanoso y con entrenudos de color verde con estrías longitudinales pardo rojizas; los zarcillos son trífidos y largos. El ápice es algodonoso, con una alta densidad de pelos tumbados, blancos con bordes intensamente carmín; el eje es velloso rosado. Las hojuelas basales son vellosas, ampolladas, de color verde bronceado sobre las depresiones; el envés es algodonoso, blanco rosáceo. Las apicales son brillantes, verdes bronceadas sobre las depresiones, el envés es lanoso a telarañoso, blanco rosáceo. Las hojas son orbiculares, enteras o débilmente trilobuladas, el seno peciolar es lira más o menos cerrada, si hay senos laterales ellos son poco profundos; los dientes son ojivales, medianos, poco visibles en el borde del limbo; la nervadura presenta pigmentación antociánica, el limbo es liso, grueso, brillante, ampollado, con bordes fuertemente doblados hacia el envés; el envés es velloso blanco, de intensidad media a alta de pelos tumbados, con la nervadura principal pubescente y con pigmentación antociánica. El follaje enrójese totalmente en otoño.

Los racimos son troncocónicos, de tamaño mediano a grande, alados, compactos a sueltos. Las bayas son esféricas, medianas, de color negro, con abundante pruina; la pulpa es coloreada y jugosa.

La alta concentración de ancianos en toda la baya genera mostos muy coloreados, los vino son astringentes y de intensidad aromática baja, en general son utilizados para mejorar el color de otras cepas tintas en proporciones que no superan el 10 % del volumen total.

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