UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
DOCTORADO EN CIENCIAS DE LA PRODUCCIÓN Y DE LA SALUD ANIMAL
FACULTAD DE MEDICINA VETERINARIA Y ZOOTECNIA

EXPRESIÓN DE PROTEÍNAS RECOMBINANTES DEL VIRUS DE INFLUENZA AVIAR EN MICROALGAS DE LA ESPECIE *DUNALIELLA SALINA* Y SU EVALUACIÓN ANTIGÉNICA EN MUCOSAS DE POLLO DE ENGORDE

TESIS
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RESUMEN

Esta tesis tuvo como objetivo la expresión de proteínas recombinantes, en partícular la hemaglutinina (HA) del virus de la influenza aviar (vIA) en el modelo experimental *Dunaliella salina*; así como los objetivos particulares: transformación de *D. salina* mediante el protocolo de agroinfiltración, y caracterización antigénica de la proteína recombinante H5 en mucosas en el modelo experimental animal aves de origen comercial. La primera fase del experimento se enfocó en el diseño del gen con base a la secuencia de la HA de la cepa vacunal mexicana Influenza A/chicken/Hidalgo/28159-232/1994 (H5N2) reportada en la base de datos Genbank del Centro Nacional de Información Biotecnológica (por su acrónimo en inglés NCBI). El gen sintetizado y adquirido de un proveedor comercial, contó con la secuencia completa del gen *H5* codón optimizado para *D. salina* dirigido por los promotores de la proteína de choque térmico 70 (Hsp70) y de la subunidad pequeña de RUBISCO, así como el terminador RBCS2 (estrategia promotor híbrido/terminador), clonado en el vector binario pCAMBIA-1301. El protocolo de agroinfiltración basado en *Agrobacterium tumefaciens* fue aplicado en cultivos de *D. salina* para el analísis de expresión y caracterización bioquímica por medio de la prueba de electroforesis en gel de poliacrilamida con dodecilsulfato sódico (por su acrónimo en inglés SDS-PAGE) al 12%, y su posterior análisis por inmunodetección por el ensayo de Western Blot (WB) con anticuerpos. La segunda fase del experimento constó de la evaluación antigénica de la proteína recombinante H5 en animales de origen comercial, mediante su aplicación en mucosas oculares, determinando los niveles de estimulación inmunológica local y sistémica mediante pruebas serológicas. Los resultados demostraron que el sistema de microalga de la especie *D. salina* tiene la capacidad de expresión de glicoproteínas de superficie del vIA similares a las virales nativas; la caracterización bioquímica permitió identificar una proteína recombinante con el peso molecular estimado para H5 del vIA; identificada posteriormente como H5 por anticuerpos monoclonales y policlonales. La expresión y la capacidad hemaglutinante de la proteína se confirmó similar a la de un virus nativo. Los resultados de la segunda fase experimental, demostraron que el sistema es capaz de expresar glicoproteínas con actividad antigénica similar a las virales nativas de la cepa vacunal influenza A/chicken/Hidalgo/28159-232/1994 (H5N2) procedentes de embriones de pollos inoculados con esta cepa; mediante la inducción de una respuesta inmune de manera local y sistémica medida por pruebas de ELISA de S-IgA total y de inhibición de la hemaglutinación (IH) respectivamente. Los datos obtenidos mediante este experimento, indicaron un aumento en la estimulación local de S-IgA en fluido lagrimal de animales inmunizados tanto con antígeno recombinante H5 como antígenos de origen viral, en comparación con grupo testigo negativo. Aunando a la evaluación local, de manera sistémica, la proteína recombinante H5 como el antígeno viral nativo fueron capaces de estimular una respuesta inmune sistémica específica medida por la prueba de IH en suero de estos animales de experimentación, comprobando la importancia de esta ruta de vacunación para antígenos virales de origen recombinante y nativos. En conjunto, este experimento demostró que un sistema de expresión alternativo, como lo es la microalga de la especie *D. salina*, es capaz de producir antígenos virales con la caraterísticas similares a proteínas virales nativas de un vIA; por lo que su uso puede ser considerado para futuros experimentos relacionados al desarrollo de sistemas de expresión basado en microalgas.

Palabras clave: *Dunaliella salina*, influenza aviar, proteína recombinante, hemaglutinina, mucosa

ABSTRACT

This thesis aimed at expression of virus recombinant proteins, in particular, the hemagglutinin (HA) of the avian influenza virus (AIV) in the experimental model *Dunaliella salina*; as well as the specific objectives: transformation of *D. salina* through the agroinfiltration protocol, and antigenic characterization of the recombinant protein H5 in mucous membranes in the experimental animal model (commercial birds). The first stage of the experiment was focused on designing the recombinant gene for the recombinant protein based on the sequence reported in the National Center for Biotechnology Information (NCBI) Genbank database coding for the HA protein of AIV; Corresponding to the Mexican vaccine strain Influenza A/chicken/Hidalgo/28159-232/1994 (H5N2). The gene synthesized and purchased from a commercial supplier presented the complete sequence of the codon-optimized *H5* gene for *D. salina* led by the heat shock protein 70 (Hsp70) and RUBISCO small subunit promoters, as well as the RBCS2 terminator, promoter hybrid strategy (Hybrid/terminator), cloned into the binary vector pCAMBIA-1301. The agroinfiltration protocol based on *Agrobacterium tumefaciens* was applied to cultures of *D. salina* for expression analysis and biochemical characterization. The second stage of the experiment consisted of the antigenic evaluation of the recombinant protein H5 in animals of commercial origin using its application in ocular mucous membranes, determining the levels of local and systemic immunological stimulation using serological tests. The results demonstrated the ability to express AIV surface glycoproteins similar to native viral in the microalgae system of the *D. salina* species based on nucleus transformation using the agroinfiltration protocol; according to the identification of a recombinant protein with the molecular weight estimated for the AIV H5 protein using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis test (SDS-PAGE) at 12%, and its subsequent analysis by immunodetection by the Western Blot (WB) assay with monoclonal and polyclonal antibodies, as well as by the characterization of its hemagglutinating biological activity. Subsequently, the results obtained in the second experimental stage showed that the system could express glycoproteins with antigenic activity similar to the native viral strains of the influenza A/chicken/Hidalgo/28159-232/1994 (H5N2) vaccine strain from embryos by inducing an immune response locally and systemically by means of total S-IgA ELISA and hemagglutination inhibition (HI) test, respectively. Data obtained through this experiment indicated an increase in the local stimulation of total S-IgA in the lacrimal fluid of animals immunized with recombinant H5 antigen and antigens of viral origin compared to the negative control group. Adding to the local evaluation, in the evaluation of systemic immune response, both H5 recombinant protein and native viral antigen were able to stimulate a specific systemic immune response measured by the HI test in the serum of these experimental animals, verifying the Importance of this vaccination route for viral antigens of recombinant and native origin. This experiment demonstrated that an alternative expression system, such as the microalgae of the species *D. salina*, is capable of producing viral antigens with characteristics similar to native viral proteins of AIV; Therefore, its use can be considered for future experiments related to the development of expression systems based on microalgae.

Keywords: *Dunaliella salina*, avian influenza, recombinant protein, hemagglutinin, mucosa

CONTENIDO

LISTA DE CUADROS

1. INTRODUCCIÓN

La tesis contiene los requisitos indispensables para la graduación doctoral con base en publicaciones científicas en revistas indexadas. Por lo que se estructuró con introducción, discusión y conclusiones; además, se anexaron tres publicaciones científicas que incluyen la caracterización bioquímica, la evaluación inmunológica *in vivo* en pollos de origen comercial inmunizados por vía ocular con la proteína recombinante H5 y la revisión bibliografía del sistema de microalga de la especie *D. salina* para su uso como sistema de producción de antígenos para su aplicación en vacunas en mucosas.

1.1. INFLUENZA AVIAR

La influenza aviar (IA) es una enfermedad sistémica, con diversas presentaciones clínicas que van de subclínicas a mortalidades del 100% en animales susceptibles, con una prevalencia a nivel mundial (1). Entre sus principales sinonimias se encuentran plaga aviar, peste aviar, gripe aviar, entre otros (1,2). El estudio de la IA se puede dividir en tres eventos principales, reportes de virus de IA (vIA) de alta virulencia (por su acrónimo en inglés HP), reconocimiento de los vIA de mediana o baja virulencia (por su acrónimo en inglés LP) en pollos domésticos y la identificación de los vIA asintomáticos en aves salvajes (3). La clasificación de virus de Influenza (vI) en los tipos A, B, C y D se basa en reacciones serológicas (4,5), donde vIA pertenece al tipo A (1,2), los tipos B, C y D han sido aislados en mamiferos (6). La subclasificación del vI basada en la tipificación de las proteínas hemaglutinina (HA) y neuroaminidasas (NA), describe 18 HA y 11 NA reconocidas (6,7), de las cuales la mayoría de las combinaciones de HA y NA se ha observado en los vIA en pollos y aves de vida silvestre (8). La tipificación de las proteínas HA y NA por pruebas serológicas en especies como aves, porcinos, caballos y aislamientos en seres humanos (9–11); así como pruebas de inmunoabsorvancia ligada a enzimas (por su acrónimo en inglés ELISA) y virus suero neutralización, se considera pruebas estándares para la identificación y establecimiento de relaciones inmunogénicas detalladas entre aislamientos (1), considerando tambien la secuenciación de los segmentos 4 y 6 condificantes para las proteínas HA y NA respectivamente (12). El conjunto de esta información permite el uso de una nomenclatura internacional estándar para los vI (9).

El vIA pertenece a la familia de los *Orthomyxoviridae* género *Influenzavirus* tipo A, con un virión esférico, pleomórfico o filamentoso y una nucleocápside con simetría helicoidal, con un diámetro de 80 a 120 nm, recubierto en su superficie exterior por dos glicoproteínas: trímericas alargadas con forma de vara denominadas HA y tetrameros con forma de hongo denominadas NA (3). En general el vIA es sensible a condiciones extremas de calor y pH, asi como a diversos detergentes y desinfectantes como aldehídos, hipoclorito de sodio, fenoles, sin embargo su resistencia puede ser mayor en presencia de material orgánico que lo recubran (13,14), permaneciendo activo desde 7 hasta 100 días (1). Su conservación se recomienda a temperaturas de -70ºC asi como en condiciones de liofilización, y con menor efectividad a 4ºC en líquido alantoideo una vez inactivado con formalina, etilenimina binaria y beta propiolactona sin alterar la actividad de HA y NA virales (15).

El genoma viral del vIA esta constituido por ácido ribonucleico (por su acrónimo en inglés RNA) de cadena sencilla de sentido negativo segmentado (vRNA (-) ss) con 8 segmentos, los cuales codifican para las proteínas virales polimerasa básica 2 (PB2) en el segmento 1, polimerasa básica 1 (PB1) en el segmento

2, polimerasa ácida (PA) en el segmento 3, HA en el segmento 4, nucleoproteína (NP) en el segmento 5, NA en el segmento 6, matriz 1 (M1) y matriz 2 (M2) en el segmento 7, y no estructural 1 (NS1) y no estructura 2 (NEP) anteriormente NS2 en el segmento 8 (5). Las proteínas de superficie virales HA y NA en conjunto con la M2, las proteínas internas M1, las polimerasas (PB1, PB2, y PA), la NP, y la NEP/ NS2 conforman la composición proteíca del virión, incluyendo otras proteínas no estructurales accesorias como PB1-N40 (16), PA-X (17), PB1F2, M42 (18), NS3 (19), PA-N155 y PA-N182 (20). El vIA posee un complejo ribonucleico-proteína (RNP) conformado por las proteínas PB1, PB2 y PA, la cual es una RNA polimerasa dependiente de RNA, asociada al vRNA(-)ss viral y la proteína NP (1). En conjunto, estas proteínas corresponden aproximadamente a un 70% de la partícula viral, junto con lípidos (20%), carbohidratos (5 al 8 %) y RNA (0.8 a 1%) (1). El período de incubación tiene un rango de horas por vía intravenosa, hasta 3 días en un ave y más de 14 días en una parvada en trasmisión por contacto con secreciones nasales o excretas, lo cual depende de la dosis viral, la ruta de exposición, la especie expuesta al virus y a la detección de signos clínicos (1). Su transmisión en poblaciones de aves silvestres es por via oral-fecal, atribuida a la contaminación de aguas por virus (21), observándose una primera replicación en tracto digestivo (22), con una eliminación del virus en concentraciones altas, llegando a un titulo de $1x10^{7.8}$ dosis letal a embrión de pollo (por su acrónimo en inglés ELD50) por gramo de heces (23).

El ciclo de replicación inicia al penetrar en la célula huésped por endocitosis mediada por la unión de HA con los receptores de superficie que presentan ácido sialico (por su acrónimo en inglés SA), como se le conoce al derivado del ácido neuramínico con funciones de adhesión y paso de fluidos a través de la membrana. El SA puede corresponder al ácido N-acetilneuramínico (NeuAC) ó al ácido N-glicolilneuramínico (NeuGC) unido a una galactosa, el tipo de ácido así como su distribución y sitio de unión del SA con el azúcar del receptor en los tejidos varia de especie a especie, dando especificidad a la unión de HA, debido a que los vI equinos y vIA presentan predilección por el SAα2-3 galactosa (SAα2-3Gal) en especial NeuGcα2-3Gal; mientras que en los vI humanos, la unión preferentemente se realiza en el SAα2-6Gal (24,25). Una vez dentro de los endosomas, se da la fusión de las membranas virales y celulares bajo condiciones de pH bajos, previa separación proteolítica de HA0 en HA1 y HA2 (26), para después liberar el complejo vRNA-proteína y ser transportado hacia el núcleo donde el complejo RNP comienza la síntesis de seis RNA mensajeros (por su acrónimo en inglés mRNA) virales monocistrónicos, los cuales son traducidos a las proteínas HA, NA, NP, PB1, PB2 y PA, en el caso de los mRNA correspondientes para los genes NS y M, estos son sometidos al proceso de "*splicing"* de donde surgen dos mRNA maduros de cada mRNA original, los cuales son traducidos a las proteínas NS1, NEP/NS2, M1 y M2. Las proteínas HA y NA son glucosiladas en el retículo endoplásmico, modificadas en el aparato de Golgi y transportadas a la superficie donde son embebidas en la membrana celular. Las proteínas internas se ensamblan para formar la RNP y migran hacia la membrana junto con las proteínas M2 y M1, donde la proteína M1 promueve una asociación con la membrana celular y constituyendo así a los viriones (1).

La participación de las características de una proteína de superficie HA, juega un papel decisivo de acuerdo a la Organización Mundial de Sanidad Animal (OMSA) en su clasificación basada en los signos clínicos asociados a su virulencia (8), debido al sitio de reconocimiento de las proteasas para la virulencia de los vIA de HP y LP (27) y al proceso de separación en HA1 y HA2, que resulta esencial para la replicación viral del vIA. En los vIA de LP se presenta una secuencia de aminoácidos con el sitio de separación proteolítica Arg (R) R-X-X-R reconocidos por proteasas tipo tripsina (28) y un sitio de glucosilación en el

residuo 13 que delimita el sitio de corte de la enzima proteolítica. La distribución de estas enzimas en diversos sitios anatómicos, como células epiteliales respiratorias y digestivas, así como secreciones respiratorias, hace de la replicación de los vIA de LP restringida a estos sitios, debido a esto, las afecciones se localizan principalmente en tracto respiratorio, digestivo, urinario o incluso reproductor (1). La morbilidad se considera elevada y la mortalidad baja, llegando esta hasta un 5% con complicaciones bacterianas (29). Entre las lesiones macroscópicas se encuentran principalmente en tracto respiratorio así como en cavidad peritoneal. Las lesiones microscópicas se observan como neumonías fibrino-celular, traqueitis, bronquitis, depleción linfoide en bolsa cloacal (BC) y otros tejidos linfoides (1). En el caso de los vIA de HP, el sitio de separación proteolítica polibásico Arg (R) y Lys (K) (R-R-R/K-R y R-X-R/K-R) reconocidos por enzimas intracelulares tipo furina, proteasas de serina MSPL y TMPRSS13 (enzima transmembranal) (28,30), aunado a la pérdida del sitio de glucosilación del residuo 13 (29,31–34), permiten un cambio drástico en la virulencia. Las enzimas tipo furina, endoproteasas localizadas en el aparato de Golgi altamente conservadas entre células eucariotas, son capaces de procesar precursores de una variedad de proteínas como factores de crecimiento, proteínas séricas, sistema del complemento, receptores entre otros (27,35–37); las cuales estan presentes en muchos tipos celulares de órganos internos, sistema nervioso, así como sistema cardíaco permitiendo la replicación del vIA de HP en estos tejidos (37,38), lo que permite que la capacidad de daño y afinidad por especie varié entre cepas vIA (23,24,27,37).

Cambios en la secuencia de la proteína HA permiten establecer relaciones filogenéticas, clasificación, así como las posibles especies susceptibles (12). Su adaptación a especies como pollos y pavos parece estar relacionado a un evento asociado a la aparición de los vIA de HP en aves silvestres (24,31,36,39), siendo las HA tipo H5 y H7, las que muestran mayor probabilidad de presentarse en los vIA de HP (31,40). Se considera que las cepas de vIA de HP son capaces de inducir una mortalidad superior a 75% de los animales afectados de una edad de 4 a 6 semanas de edad dentro de los primeros 10 dias post-inoculación (DPI) intravenosa, incluso hasta un 100 % en las primeras 36 a 48 horas post-inoculación (PI), fuera de este rango, las cepas se consideran de LP (41). En aves infectadas por vIA de HP, los signos clínicos van desde muerte súbita sin la presencia de signología clínica por lesiones severas en órganos internos, sistema cardiovascular hasta infecciones con presentación clínica nerviosa (1). En aves productoras de huevo y reproductoras, la postura baja hasta llegar al cese a los 6 días aproximadamente, siendo la mortalidad lo más característico de un brote, la cual se encuentra en un rango de 50% a 89% llegando hasta el 100% (1). Entre las lesiones macroscópicas en aves que no mueren en los primeros días del brote, se presentan lesiones hemorrágicas a necróticas de vísceras y piel, edema subcutáneo con presencia de petequias, necrosis focal en su superficie y parénquima del órgano, necrosis multifocal en placas de Peyer (PP), páncreas, bazo, corazón, hígado, riñones, órganos linfoides primarios (OLP) y órganos linfoides secundarios (OLS), por lo que BC y timos se encuentran atróficos, en cerebro se observa meningoencefalítis linfocítica con focos de glíosis, necrosis neuronal, neurofagia, edema y hemorragía. Se observa daño en tejido muscular, células endoteliales y renales por necrosis, así como microtrombos en capilares y pequeños vasos sanguíneos de la piel, vasculitis, perivasculitis, edema subcutáneo y necrosis de endotelio de capilares (1). Los mecanismos por medio de los cuales el vIA ejerce su efecto patológico son dos, por necrosis debido a la intensa replicación (42,43) en células de tubulos renales, miocitos cardiacos, células cortico adrenales, células epiteliales pulmonares (42,44,45), linfocitos y neuronas (46–48), y apoptosis con la participación de citosinas como el interferón-β (INF-β) y el factor de crecimiento-β

(49–51). Aundo a la capacidad de replicación sistémica de los virus de HP (26), estos mecanismos inducen la presentación de las lesiones características de esta patología.

Para la detección y caracterización del vIA, es posible el uso de diversas técnicas de laboratorio, entre las que se encuentran la reacción en cadena de la polimerasa en tiempo real de transcripion reversa (por su acrónimo en inglés RT-qPCR), aislamiento viral e inmunoensayos de antígenos disponibles de manera comercial, siendo el aislamiento viral uno de los más realizados debido a que por medio de éste es posible la obtención, almacenaje y caracterización más detallada del vIA (3). Entre las más usadas en campo se encuentran la detección de anticuerpos contra vIA, como pruebas de ELISA comerciales, ensayos de precipitación en gel de agar (por su acrónimo en inglés PAG) y pruebas de inhibición de la hemaglutinación (IH), sin embargo su empleo es en base al propósito del estudio así como su reconocimiento ante las autoridades locales (3).

En México, la IA se encuentra bajo control epidemiológico por parte del gobierno mexicano, bajo el seguimiento de instituciones como la Secretaría de Agricultura y Desarrollo Rural (SADER) y la Comisión México-Estados Unidos para la Prevención de la Fiebre Aftosa y otras Enfermedades Exóticas en los Animales (CPA). El texto de referencia en el territorio nacional se considera la Norma Oficial Mexicana (NOM), NOM-044-ZOO-1995, Campaña Nacional contra la Influenza Aviar así como su modificación (11-17-98 Modificación a la Norma Oficial Mexicana NOM-044-ZOO-1995, Campaña Nacional contra La Influenza Aviar (52). Un gran número de aves domesticas y de vida libre son susceptibles a la infección con el vIA (1), siendo las acuáticas como las *Anseriformes* (patos y gansos) y *Charadriiformes* (gaviotas y aves costeras), de mayor relevancia para su transmisión y reservorio (53). En el caso de México, se considera presente la enfermedad en parte del territorio nacional, reportando brotes de IA de HP en territorio nacional, siendo estas zonas en control (52).

La vacunación y medidas de bioseguridad son la principal estrategia para el control de IA. En la actualidad las vacunas no pueden proteger contra muchos serotipos debido a que los pollos son susceptibles a la mayoria de los serotipos de HA conocidos (6). El empleo de vacunas basadas en virus inactivados han sido una herramienta útil para prevenir signología clínica y mortalidad en diversas especies, pero esta es solo para la HA y NA en la cual se basa esta vacuna, sin embargo, al darse un brote y caracterizar al vIA, es conveniente la vacunación contra ese subtipo presente en la granja (1). Debido a la capacidad de adaptacion del vI mediante dos eventos conocidos como "*drift*" y "*shift*", mutaciones puntuales acumuladas (39,40) ó el intercambio de genes entre cepas al momento de la replicación respectivamente (53), su capacidad de evadir el sistema inmune por los cambios antigénicos resultan de relevancia para su control (3). El empleo de estas vacunas de virus inactivado es aprobada solo en algunos estados de la republica, así como también bajo la autorización y vigilancia de la SADER. El empleo de vacunas recombinantes que expresan HA, así como de vacunas de ácido desoxirribonucleico (por su acrónimo en inglés DNA), son otro enfoque ante el problema de brotes de IA de HP (1). El uso de la técnica de genética reversa (54), permite la generación de vI bajo el esquema de diseño de HA de LP para su uso de manera comercial como antígenos vacunales (55), sin embargo estas requiere de un previo estudio del virus prevalente en la zona, así como el uso de la adyuvantes más eficientes, debido a que los resultados indican una baja inmunogenicidad por la estrategia de arreglo 6+2 para candidatos para vacunas (55).

Actualmente la investigación con el vIA ha llevado al desarrollo de antígenos basado en proteínas subunitarias de la HA (56); las cuales han probado reactividad a su administración en modelos experimentales. La HA es una de las principales glicoproteínas de superficie del vI (57), dado que puede presentarse hasta 10 veces más abundante en comparación con la NA (58). La HA es una glicoproteína con forma de bara alargada conformada por un trímero (225 kDa) de monómeros (75 kDa), los cuales poseen dos cadenas polipeptídicas (HA1 y HA2) con una longitud de 10–14 nm y un diametro de 4–6 nm. Los dos polipéptidos (HA1 y HA2) se organizan en una estructura helicoidal (dominio del tallo) con cadenas laterales de N-oligosacáridos y tres cabezas globulares en la estructura monomérica de HA (HA0) (36). Su función principal como receptor viral permite que el virus se una e infecte a las células huésped (59), así como la fusión de membranas (60). Debido a su participación en el proceso de infección y su abundancia en los viriones, es considerada como principal inductor de la inmunidad del huésped (57); por lo que la vacunación con esta proteína, es la táctica más eficiente para combatir el vIA en el campo, enfocadose principalmente en el subtipo HA en circulación en la población suceptible (8). En la actualidad, la expresión de la HA en sistemas de expresión heterologos ha demostrado su viabilidad como estrategia alterna para la producción de antígenos del vI (56), por lo que su estudio a generado interés a nivel industrial.

1.2. EXPRESIÓN DE PROTEÍNAS RECOMBINANTES EN MICROALGAS DE LA ESPECIE *D. salina*

Entre los sistemas de expresión de proteínas recombinantes se pueden describir sistemas con diferentes características particulares. Estos ejemplos incluyen organismos procariotas y eucariotas con diversos parámetros que los diferencian como: costos de producción, modificaciones post-traduccionales, y tiempos de cultivo, entre otros (61) (Cuadro 1). Entre estos podemos mencionar a un sistema alternativo, las microalgas, en cuyo caso, su uso no ha sido completamente explorado.

Cuadro 1. Comparación de diferentes sistemas de expresión de proteínas recombinantes, actualización de Potvin *et al.*, 2010 (61).

Sistema			Características de los sistemas de expresión de proteínas										
			Molecular					Operacional					
			Glicosilación	Tamaño del gen	Sensibilidad al esfuerzo constante	Rendimiento de producto	Vehículo de administración	Tiempo de producción	Costo de cultivo	Costo de escalonamiento	Costo de almacenamiento	Seguridad	Rendimiento de proteína total
Procariota	Bacteria		Ninguna	Desconocido	Medio	Alto	No	Corto	Medio	Alto	Bajo (–20 °C)	Bajo	Medio
Eucariota	Levadura		Aceptable	Desconocido	Medio	Medio	No	Medio	Medio	Alto	Bajo (–20 °C)	Bajo	Alto
	Insecto		Aceptable	Desconocido	Alto	Alto	No	Largo	Alto	Alto	Alto (N2 líquido)	Medio	Medio / alto
	Mamífero	Cultivo de células	Aceptable	Desconocido	Alto	Medio / alto	No	Largo	Alto	Alto	Alto (N2 líquido)	Medio	Medio / alto
		Animales	Aceptable	Limitado	N/A*	Medio / alto	Si	Largo	Alto	Alto	Alto (N2 líquido)	Alto	Alto
	Planta	Nuclear	Aceptable	No limitado	N/A*	Medio / Alto	Si	Largo	Bajo	Muy bajo	Barato TA+	Alto	Alto
		Cloroplasto	Aceptable	No limitado	N/A*	Alto	Si	Largo	Bajo	Muy bajo	Barato TA+	Alto	Alto
	Microalgas		Aceptable	No limitado	Bajo	Alto	Si	Corto	Muy bajo	Bajo	Barato TA+	Alto	Alto

*N/A (no aplica), + TA (Temperatura ambiental)

En la actualidad, la denominación microalga abarca una amplia gama de organismos unicelulates con capacidad fotosintética (62), las cuales han sidio aprovechadas por la humanidad por un amplio lapso de tiempo (63), para aplicaciones en áreas de la medicina, cosmética y alimenticia (62–64). Entre las principales microalgas conocidas para su uso industrial podemos encontrar los géneros *Chlamydomonas*, *Botryococcus*, *Chlorella*, *Haematococcus*, *Spirulina* y especificamente *Dunaliella* (62). Estos grupos de organismos presentan diversas ventajas para su uso como sistema de expresión de proteínas recombinantes, entre las que se pueden mencionar: su alta tasa de crecimiento, condiciones de cultivo en sistemas confinados, disponibilidad de herramientas de ingeniería genética, ausencia de compuestos tóxicos, generalmente reconocidos como seguros (por su acrónimo en inglés GRAS), capacidad de realizar modificaciones post-traduccionales, así como una alta capacidad biosintética en términos de rendimiento de biomasa (61,65). Una de las características principales a considerar durante la selección de un sistema previo a su uso para la expresión de una proteína, en especial un antígeno, es su capacidad de glicosilación, responsable directamente de la inmunogenicidad (66).

En general, los sistemas de expresión de proteínas poseen N-glucanos específicos de la especie. En el caso de las microalgas, se reportan dos vías de glicosilación diferentes: (i) independiente de la enzima GnT I consiste en 5 Man y 2 GlcNAc N-linked procesadas por xilosiltransferasas (XyT) y metiltransferasas (MeT), lo que lleva a estructuras únicas N-linked que contienen manosas metiladas unidas a una o dos xilosas con estructuras que varían ligeramente entre especies de microalgas, y (ii) enzima dependiente GnT I que transfiere un residuo de N-acetilglucosamina a la proteína ligada a N5 manosa y 2 GlcNAc, sometida a α-manosidasa II (α-Man II) y fucosiltransferasa (FuT), lo que da como resultado N-glicanos fucosilados paucimanosídicos (Man 3–4GlcNAc 2). Sin embargo, en estos procesos, las especies de microalgas muestran patrones más similares a los humanos, en comparación con otros sistemas (67).

El potencial de producción de proteínas recombinantes en especies de microalga como *C. reinhardtii, C. vulgaris, C. ellipsoidea, D. salina, P. tricornutum y N. oculata* (61,67,68) han sido explorado ampliamente en el caso de anticuerpos, nanocuerpos, citocinas, péptidos antimicrobianos, vacunas, hormonas y enzimas (67,69,70) (Cuadro 2), así como su producción a nivel industrial mediante sistemas en confinamiento altamente especializados (71,72), por lo que producir biomasa para proteínas recombinantes es un paso lógico en su uso.

Cuadro 2. Proteínas recombinantes expresadas en sistemas de microalgas, actualización de Barolo *et al.*, 2020 (67).

Organismo	Sitio de inserción del gen recombinante	Proteína recombinante	Referencia
Chlamydomonas. reinhardtii	Cloroplasto	E7 of HPV-16	Demurtas *et al.*, 2013 (73)
		D2-CTB	Dreesen *et al.*, 2010 (74)
		α-galactosidasa, fitasa, xilanasa	Georgianna *et al.*, 2013 (75)
		Pfs25, Pfs28	Gregory *et al.*, 2012 (76)
		Pfs25-CTB	Gregory *et al.*, 2013 (77)
		E2	He *et al.*, 2007 (78)
		Pfs48/45	Jones *et al.*, 2013 (79)
		M-SAA	Manuell *et al.*, 2007 (80)
		Anti-HSV glicoproteína D Isc	Mayfield *et al.*, 2003 (81)
		12FN3, eritropoyetina, HMGB1, interferón β, proinsulina, SAA-10FN3, VEGF	Rasala *et al.*, 2010 (82)
		Aloficocianina	SU *et al.*, 2005 (83)
		VP1-CTB	M. Sun *et al.*, 2003 (84)
		V28	Surzycki *et al.*, 2009 (85)
		Anti-PA 83 ántrax IgG1	Tran *et al.*, 2009 (86)
		Anti-CD22-gelonina sc	Tran, Henry, *et al.*, 2013 (87)
		Anti-CD22-ETA sc	Tran, Van, *et al.*, 2013 (88)
		GAD65	Wang *et al.*, 2008 (89)
		TRAIL	Yang *et al.*, 2006 (90)
		Fitasa (AppA)	Yoon *et al.*, 2011 (91)
		Metalotioneína -2	Zhang *et al.*, 2006 (92)
		Hemaglutinina truncada H5 (vIA)	Castellanos-Huerta *et al.*, 2016 (93)
	Núcleo	Factor de crecimiento epidérmico humano	Baier *et al.*, 2018 (94)
		VEGF-165	Chávez *et al.*, 2016 (95)
		GBSS-AMA1, GBSS-MSP1	Dauvillée *et al.*, 2010 (96)
		Eritropoyetina	Eichler-Stahlberg *et al.*, 2009 (97)
		Sep-15	Hou *et al.*, 2013 (98)
		Lolium Perenme IBP	Lauersen *et al.*, 2013 (99)
		β-1,4-endoxilanasa	Rasala *et al.*, 2012 (68)
Chlorella vulgaris, C. sorokiniana	Núcleo	Hormona del crecimiento humano	Hawkins *et al.*, 1999 (100)
Chlorella ellipsoidea	Núcleo	mNP-1	Bai *et al.*, 2013 (101)
		NP-1	Y. Chen *et al.*, 2001 (102)
		Hormona del crecimiento de la platija	Kim *et al.*, 2002 (103)
Dunaliella tertiolecta	Cloroplasto	α-galactosidasa, fitasa, xilanasa	Georgianna *et al.*, 2013 (75)
Dunaliella salina	Núcleo	V28	Feng, *et al.*, 2014 (104)
		HBsAg	Geng *et al.*, 2003 (105)
		Hemaglutinina H5 (vIA)	Castellanos-Huerta *et al.*, 2022 (106)
Phaeodactylum tricornutum	Núcleo	Anti-Hepatitis B IgG	Hempel *et al.*, 2012 (107)
		Anti-MARV NP IgG	Hempel *et al.*, 2017 (108)
Nannochloropsis oculata	Núcleo	Lactoferricina bovina (LFB)	S.-S. Li *et al.*,, 2009 (109)
		Hormona del crecimiento de la platija	H. L. Chen *et al.*, 2008 (110)

Recientemente *Dunaliella* sp., una alga verde unicelular, halófila, biflagelada, *Phylum Chlorophyta*, Clase *Chlorophyceae*, orden *Volvocales*, familia *Polyblepharidaceae* (67,111), ha sido propuesta como uno de los modelos para el estudio de la expresión de proteínas, en partícular por su capacidad de producción de proteína (112), su capacidad de crecimiento en condiciones extremas (sal del 3 a 31%) (113), temperatura (<0 °C a >38 °C) (114), reducida contaminación en su cultivo (115), presencia de modificaciones transcripcionales (70) y falta de una pared celular rígida, lo que facilita los procesos de transformación genética (116). *Dunaliella* sp. contienen un solo cloroplasto, clorofilas (a y b), y orgánulos observados en algas verdes: núcleo, mitocondrias, vacuolas, aparato de Golgi y una membrana plasmática elástica cubierta por una capa superficial de moco con la capacidad de contraerse o hincharse según las condiciones hipertónicas e hipotónicas (116,117). *D. salina*, al igual que otras microalgas, posee un ciclo de vida complejo, divisiones celulares longitudinales en el estado móvil (células vegetativas), y reproducción sexual (formación de cigoto sexual) (118). En las mejores condiciones de crecimiento, la tasa de división puede ir de 0,5 a 1,22 divisiones por 24 horas (118). Una concentración promedio de sal en los medios de cultivo de *D. salina* (12 %) y *D. viridis* (6 %) se considera óptima para el crecimiento (119). Sin embargo, otras cepas presentan diferentes condiciones de crecimiento (120). En general, las condiciones de cultivo son a una temperatura de 25±2°C bajo luz fluorescente blanca de 52.84 µmol fotones m^{-2} s^{-1} sin aireación, en agitación a 110 rpm/min (agitador orbital) (114,121). Los medios de cultivo para el producción a nivel experimental incluyen: medio de Johnson modificado, medio de Erdschreiber, medio F/2 de Guillard, medio ASP modificado y agua de mar enriquecida (121,122). Entre las técnicas de manipulación genética aplicadas en *Dunaliella* sp. podemos incluir electroporación (105,123), bombardeo de partículas (124), perlas de vidrio (125), método mediado por acetato de litio/polietilenglicol (PEG) (126) y método mediado por *Agrobacterium tumefaciens* (agroinfiltración) (127). En general, todas las técnicas presentan una serie de ventajas y desventajas para su uso en microalgas (70), sin embargo la eficacia en los niveles de expresión depende de factores como optimización del codones, actividad de la proteasa, toxicidad de la proteína y modificación genotípica asociada a la transformación (85). Uno de los métodos de transformación nuclear más prometedores para la expresión de proteínas recombinantes en *D. salina* es el protocolo de agroinfiltración (127,128). Este protocolo se basa en la capacidad de *A. tumefaciens*, un método indirecto, para transferir DNA exógeno a las células vegetales a través de un sistema de conjugación bacteriano (sistema de secreción tipo IV (T4SS) y complejos proteína-DNA) (129). Las plantas se ven naturalmente afectadas por *A. tumefaciens*, incluidas las angiospermas y las gimnospermas (130). En resumen, *A. tumefaciens* al detectar compuestos fenólicos de una planta herida, se adhiere y comienza a transformar las células vegetales al inducir la transcripción de genes de virulencia presentes en un plásmido llamado Tumor-inductor (Ti-DNA). El Ti-DNA, junto con las proteínas de virulencia bacteriana (VirD1, VirD2, VirE2), inducen la transcripción, el procesamiento del DNA de transferencia (T-DNA) y su integración en el genoma de la planta. Por lo que el T-DNA al ser manipulado con un gen de interés permite su inserción en el genoma del núcleo de las células mediante este protocolo (130,131). Debido la naturaleza del proceso de inserción aleatoria observada en este método, se sugiere un mecanismo de recombinación no homólogo (132). El uso de un promotores eficientes es fundamental para lograr la expresión del gen de interés, por lo que entre los principales usados para *D. salina* se encuentran: el virus del mosaico de la coliflor 35, CaMV35S (123,124), Ubiquitina (Ubil), Ubil-Ω, CaMV35S-Ubil, CaMV35S-Ubil-Ω, promotores endógenos del gen de actina (133) y gliceraldehído-3-fosfato deshidrogenasa (134), con alta actividad en la expresión génica. En el caso de los potenciadores, una correcta selección podría evitar el efecto de silenciamiento génico debido al efecto de posición (135). Los potenciadores reportados en *D. salina* es encuentran: las regiones de unión

a la matriz (MAR) (105) y la secuencia líder 5' del RNA del virus del mosaico del tabaco (elemento Ω) combinado con los promotores Ubil y CaMV35S-Ubil (105). En general, la expresión en el núcleo de *D. salina* se centra actualmente en genes reporteros como el gen de la β-glucuronidasa (136), proteína fluorescente verde (134) y marcadores de selección como la fosfinotricina acetiltransferasa bajo el promotor DCA1 (135), la cloramfenicol acetiltransferasa (115), y proteína de resistencia a la zeocina (133). A pesar de esto, la expresión de proteínas de valor comercial es reducida en este modelo (137,138), incluidos los inmunógenos (136). Sin embargo, de acuerdo a los hallazgos reportados, el sistema *Dunaliella* resulta ser atractivo para su estudio en un enfoque para aplicación industrial, en partícular en la producción de antígenos virales.

1.3. SISTEMA INMUNE AVIAR EN MUCOSAS

El sistema inmune en aves en especial en mucosas, juega un papel muy importante para la defensa contra agentes patógenos cuya entrada o presencia en mucosas es un problema constante en los animales (139). Su conocimiento resulta útil para el desarrollo de vacunas, debido a que existen una gran variedad de vacunas empleadas en la producción animal para la prevención y control de enfermedades propias de la especie, con diversos niveles de eficiencia de acuerdo a la intensidad con la cual el sistema inmune reacciona ante sus componentes (1). Al igual que en los mamíferos, el sistema inmune depende de una compleja relación entre células especializadas y factores solubles, que al trabajar en conjunto confieren protección contra agentes patógenos. En general, el sistema inmune de las aves presenta similitudes con el de los mamíferos, con algunas excepciones (140). Los tejidos linfoides aviares como bolsa cloacal (BC) y timo, organos linfoides primarios (OLP), los cuales liberan a circulación células diferenciadas que colonizan organos linfoides secundarios (OLS) como el bazo, medula ósea y agregados linfoides como la glándula de Harder (GH), tejidos linfoides asociados a mucosas (por su acrónimo en inglés MALT) como el tejidos linfoide asociado al intestino (por su acrónimo en inglés GALT), tejido linfoide asociado a bronquios (por su acrónimo en inglés BALT), tejido linfoide asociado a la cavidad nasal (por su acrónimo en inglés NALT), tejido linfoide asociado a piel (por su acrónimo en inglés SALT) y tracto genital, tonsilas esofágica (TE), tonsilas pilóricas (TP), placas de Peyer (PP), tonsilas cecales (TC), divertículo de Meckel (MD) y tejido linfoide asociado a la glándula pineal, sitios donde se presenta la principal respuesta inmune antígeno dependiente. En estos órganos los linfocitos T (LT) y linfocitos B (LB) ocupan zonas T-dependientes y zonas B-dependientes o centros germinales (CG); en los CG maduros es posible encontrar proliferación de LB, LT CD4+, además de células dendríticas foliculares (por su acrónimo en inglés FDC) y difusos LT CD8+ (140). El componente celular del sistema inmune aviar se localiza en su mayoría en los OLP y en OLS (1), así como células no linfoides entre las que se encuentran las celulas presentadoras de antígeno (por su acrónimo en inglés APC), como las celulas dendrítica (por su acrónimo en inglés DC) (140,141). Los órganos linfoides tienen su propio arreglo entre células linfoides y células no linfoides, donde las zonas T-dependientes y B-dependientes bien delimitadas dan lugar a la presentación de antígenos a los LT por medio de las APC y su posterior interacción con los LB para la producción de inmunoglobulinas (Ig) (140,142). La descripción del tejido linfoide en el caso de mucosas en las aves resulta de interés dado sus particulares características aplicables para la vacunación por esta vía (140). A diferencia de los mamíferos, las aves carecen de nodos linfáticos encapsulados bien definidos, salvo un tejido linfoide rudimentario no encapsulado carente de fibras reticulares, macrófagos y rodeado por tejido adiposo, denominado como nodo linfoide mural (por su acrónimo en inglés MLN) característico en aves asociado con las venas

femorales, popitleas y tibiales, así como un tejido linfoide descrito en aves acuáticas como estructuras parecidas a nodos cervicotoracios. Por lo que la presencia de un repertorio de MALT resulta ser la respuesta a la necesidad de protección en el caso de las aves (140,143,144). El MALT puede presentar diferencias entre aves libres de patógenos específicos (ALPES) y pollo comercial, sin embargo el escaso desarrollo del MALT en ALPES son una de las marcas más características (141). Entre estos agregados linfoides en mucosas, podemos destacar GH, presente en todos los vertebrados con la excepción de los peces, algunos anfibios y primates, mientras que en las aves de corral, GH es el principal contribuyente a la producción de Ig ocular (145). GH es una glándula orbital dominante en las aves ubicada anatómicamente ventral y posteromedial al globo ocular conectada al ángulo medial de la membrana nictitante (145). GH es responsable de la producción del isotipo IgA secretora (S-IgA) (146,147), como resultado de la estimulación local (148); siendo una parte esencial en la respuesta inmune en mucosas, principalmente en los ojos y el tracto respiratorio superior (148). S-IgA, polipéptido de dos monómeros IgA, es el isotipo de Ig predominante en la mayoría de las superficies mucosas; debido a su estabilidad molecular y fuertes propiedades antiinflamatorias, es un componente de inmunidad protector ideal de las superficies mucosas (149). La S-IgA representan una parte importante de las defensas a nivel de mucosas debido a su función de prevenir la adhesión de patógenos como bacterias y virus, limitando su efecto patógeno, y neutralizando y facilitando su excreción (150). Aproximadamente 40 mg de S-IgA por kg del peso corporal son secretados diariamente, especialmente en el tracto digestivo, casi lo doble del inmunoglobulina G (IgG) producido al día, sin embargo parte de esta producción de S-IgA no es liberada a mucosas (151,152). De acuerdo a esto, se esperaría una respuesta inmune de S-IgA a la administración oral de antígeno, lo cual en muchos casos no ocurre, ya que la presencia de S-IgA es mínima o nula, aunado a que al aumentar la dosis de este antígeno vía oral es posible inducir una respuesta nula ante una administración parenteral, contraria a la deseada, ante el mismo antígeno, denominada como tolerancia oral (152–155), por lo que una correcta presentación del antígeno en cuestión es crucial para lograr una producción de S-IAg capaz de proteger al organismo.

1.4. OBJETIVO GENERAL

Expresión de proteínas recombinantes, en partícular la hemaglutinina (HA) del virus de la influenza aviar (vIA) en el modelo experimental *Dunaliella salina*.

1.4.1. OBJETIVOS PARTICULARES

1.4.1.1. Transformación de *Dunaliella salina* mediante el protocolo de agroinfiltración.

1.4.1.2. Caracterización antigénica la proteína recombinante H5 en mucosas en el modelo experimental animal aves de origen comercial.

2. PUBLICACIONES GENERADAS

Se elaboraron dos experimentos. El primero fue para probar la capacidad de expresión de proteínas, en particular la proteína HA del vIA en microalgas de la especie *D. salina* mediante el protocolo de transformación nuclear mediada por *A. tumefaciens* (agroinfiltración) y el segundo experimento fue realizado para evaluar la capacidad antigénica de la proteína recombinante H5 expresada en *D. salina* mediante su aplicación vía ocular en pollos de origen comercial. Como resultado de los experimentos se publicaron en revistas científicas con reconocimiento internacional tres artículos científicos.

2.1. DOS EXPERIMENTOS REALIZADOS Y TRES PUBLICACIONES GENERADAS

2.1.1. EXPERIMENTO 1. EXPRESIÓN DE LA PROTEÍNA RECOMBINANTE H5 DEL VIA EN MICROALGAS DE LA ESPECIE *Dunaliela salina* MEDIANTE EL PROTOCOLO DE AGROINFILTRACIÓN

2.1.1.1. Publicación 1. Transformation of *Dunaliella salina* by *Agrobacterium tumefaciens* for the expression of the hemagglutinin of avian influenza virus H5. (Pag. 34) (ANEXO 1)

Microorganisms. 2022 Feb 4;10(2):361.

Factor de impacto: 4.926

Editorial: **MDPI**

DOI: https://doi.org/10.3390/microorganisms10020361

2.1.2. EXPERIMENTO 2. EVALUACIÓN DE LA CAPACIDAD ANTIGÉNICA DE LA PROTEÍNA RECOMBINANTE H5 EXPRESADA EN *Dunaliela salina* MEDIANTE SU APLICACIÓN VÍA OCULAR EN POLLOS DE ORIGEN COMERCIAL

2.1.2.1. Publicación 2. Immune evaluation of avian influenza virus HAr protein expressed in *Dunaliella salina* in the mucosa of chicken. (Pag. 45) (ANEXO 2)

Vaccines 2022, 10(9), 1418

Factor de impacto: 4.961

Editorial: **MDPI**

DOI: https://doi.org/10.3390/vaccines10091418

2.1.2.2. Publicación 3. *Dunaliella* salina as a potential biofactory for antigens and vehicle for mucosal application. (Pag. 55) (ANEXO 3)

Processes. 2022; 10(9):1776.

Factor de impacto: 3.352

Editorial: **MDPI**

DOI: https://doi.org/10.3390/pr10091776

3. DISCUSIÓN

3.1. EXPERIMENTO 1. EXPRESIÓN DE LA PROTEÍNA RECOMBINANTE H5 DEL VIA EN MICROALGAS DE LA ESPECIE *Dunaliella salina* MEDIANTE EL PROTOCOLO DE AGROINFILTRACIÓN

Publicación 1

Transformation of *Dunaliella salina* by *Agrobacterium tumefaciens* for the Expression of the Hemagglutinin of Avian Influenza Virus H5. (Pag. 34) (ANEXO 1).

En la actualidad, los sistemas de producción de antígenos recombinantes resultan de interés para su uso a nivel industrial (156). Debido a esto, es una necesidad la busqueda constante de plataformas alternativas innovadoras para la generación de antígenos para la investigación y el control de enfermedades en todo el mundo (157). En el caso de la IA, la producción de virus en modelos biológicos como embriones es una parte esencial del proceso de desarrollo de vacunas (158,159), no obstante los embriones representan un alto costo y deben ser utilizados animales en la etapa de producción (160). Como resultado, existe un creciente interés a nivel farmacéutico y de laboratorio en el desarrollo de métodos alternativos de fabricación de antígenos de este tipo para su posible uso para el control de enfermedades a nivel industrial. La expresión de la proteína HA ha sido explorada en diversos sistemas alternos como bacterias, plantas, células de insectos, cultivos de células de mamíferos, e incluso en microalgas, con diferentes niveles de expresión y eficiencia de glicosilación (56,161,162), sin embargo, el uso de las microalgas de la especie *D. salina*, una de las especies actualmente con aplicación industrial, ha demostrado en el presente experimento la expresión de la proteína H5 del subtipo H5N2 del vIA en un sistema basado en microalgas.

Previo a la exploración de un sistema biológico para la expresión de proteínas recombinantes, es necesaria la consideración de propiedades como los niveles de expresión, estructura de las proteínas y procesos de glicosilación (61,163), los cuales son requeridos para el plegamiento y la funcionalidad de las proteínas (61,163). Investigaciones recientes con microalgas han demostrado que estos organismos unicelulares tienen beneficios sobre otros sistemas de expresion de proteínas recombinantes (164). Entre las diferentes ventajas en comparación con otros sistemas se incluyen: duplicación de la biomasa en 24 horas, tiempos de crecimiento relativamente cortos, la expresión de proteínas en el núcleo, cloroplasto y mitocondria, modificaciones post-traduccionales, crecimiento fototrófico o heterótrofo, condiciones de temperatura controlada, luz y nutrientes, riesgo reducido de escape de transgenes al medio ambiente; clasificacion GRAS y finalmente, la posibilidad de que su liofilización para su almacenamiento ó para administración oral (61,165,166).

De acuerdo a los resultados obtenidos en el ensayo de WB, la reactividad a anticuerpos monoclonales y policlonales dirigidos contra la proteína recombinante H5 obtenida en cultivos de *D.salina* transformados, indican la expresión de la proteína H5 mediante el sistema propuesto. Mediante este sistema, es posible observar en un lapso de 30 días aproximadamente, hasta 225 μg de proteína soluble recombinante H5 a partir de 2 g de peso húmedo de un cultivo de *D. salina*, congruente a lo observado en el previos experimentos en este modelo biológico (167). Por lo que los resultados indican una posible ventaja, al considerar que el proceso actual para obtener una cepa vacunal toma no menos de seis meses

y requiere un promedio de 100 huevos embrionados para 10 mg de proteína viral total (159), además de la posible contaminación con vIA en el parvadas productoras de embriones, poniendo en riesgo la disponibilidad de estas parvadas para la producción de vacunas (168). En modelos de expresión de la proteína HA en tejido vegetal reportados previamente, se pueden alcanzar hasta el 9,7% de la proteína total soluble (PTS) (169) y al ser secretados al medio pueden alcanzar hasta 1 mg/50 mL de HA en el caso de *Schizochytrium* sp. (170), sin embargo, debido a que una proporción considerable de la proteína HA recombinante en el tejido vegetal se encuentra en la proteína insoluble, se estima que la cantidad de la proteina H5 recombinante en *D. saline*, sea mayor que la que se observa en PTS (170). El tiempo de cultivo, el costo medio y el manejo de la contaminación que se establecen en el presente estudio subrayan las ventajas de *D. salina*. En el caso de modificaciones post-traduccionales como la glicosilación en proteínas, el sistema de microalgas mostró la capacidad de realizar estas modificaciones, permitiendo la funcionalidad de proteínas complejas con actividad biológica, como es el caso de la proteína en modelos como *C. reinhardtii* y *Schizochytrium* sp., entre otros (170,171). Estos sistemas han demostrado cumplir con las características necesarias para lograr la expresión de proteínas recombinantes con requerimientos de glicosilación muy específicos (172). Estas características, junto con estudios de las rutas metabólicas y genes asociados en procesos como la glicosilación, demuestran que estos organismos pueden generar modificaciones post-traduccionales para lograr el plegamiento, la funcionalidad y la antigenicidad requerida en cada caso (173). Sin embargo, se requieren más estudios en modelos como *D. salina* para determinar estas modificaciones, sus posibles efectos sobre las características de las proteínas expresadas en este sistema y su interacción en un modelo animal.

Entre las principales características de la proteína HA necesarias para su correcto plegamiento y la presencia de actividad hemaglutinante (receptor viral) están las modificaciones post-traduccionales como la glicosilación (172). En el caso de la HA, los patrones de glicosilación varían entre las diferentes cepas y tipos de HA, incluso la presencia de secuencias de N-glicosilación no es suficiente para estar glicosilada; debido a que el tipo de célula donde se replica el virión (embrión de pollo, células humanas, etc.), afecta la presencia de estas modificaciones post-traduccionales (174). Entre algunas de las glicosilaciones constantemente observadas entre los diferentes tipos de HA se denominan los glucósidos 27 N, 40 N, 49 N, 176 N y 303 N, sin embargo, el 27 N es necesaria para adquirir su función biológica de unión a receptores celulares (172). Dado que la actividad receptor-ligando de la proteína HA requiere una modificación post-traduccional mínima como la glicosilación con 27 N, la determinación de la actividad biológica de la proteína H5 recombinante mediante el ensayo de hemaglutinación realizado, permite demostrar una proteína funcional que requiere la 27 N modificación post-traduccional. Este trabajo muestra la producción de la proteína H5 con actividad biológica con título a la prueba de hemaglutinación comparables al virus utilizado como control. La capacidad de unión al receptor de la proteína H5 recombinante, similar a la reportada previamente en modelos de microalgas (169), permite considerar este sistema de expresión viable para su posterior investigación. En esta fase del presente estudio se observa la capacidad de expresión de proteínas del vIA, en partícular H5 en el sistema de microalga de la especie *D. salina*, sin embargo se observan limitaciones relacionadas a la evaluación de la proteína H5 recombinante como inductor de una respuesta inmune en un modelo animal. Aunado a que son necesarios más estudios para confirmar la presencia de modificaciones posteriores a la traducción como un factor que también podría afectar la antigenicidad y el plegamiento de proteínas (175,176).

3.2. EXPERIMENTO 2. EVALUACIÓN DE LA CAPACIDAD ANTIGÉNICA DE LA PROTEÍNA RECOMBINANTE H5 EXPRESADA EN *Dunaliella salina* MEDIANTE SU APLICACIÓN VÍA OCULAR EN POLLOS DE ORIGEN COMERCIAL

Publicación 2

Immune Evaluation of Avian Influenza Virus HAr Protein Expressed in *Dunaliella salina* in the Mucosa of Chicken. (Pag. 45) (ANEXO 2).

La descripción de la respuesta del sistema inmune de las mucosas es necesaria para la comprensión de varias patologías de origen infeccioso, particularmente en el caso de IA (177,178). Como es sabido, la principal vía de entrada de patógenos en un organismo es a través de las mucosas (179). Por lo tanto, este tejido representa la primera barrera que hay que superar para lograr la infección y la generación de enfermedades infecciosas. Por este motivo, las mucosas requiere de un sistema inmunitario altamente especializado para la correcta respuesta y protección frente a diversos patógenos (177,178). En el caso de las aves, estos órganos linfoides presentan algunas variaciones con respecto a otras especies (177,180); sin embargo, el tejido especializado juega un papel vital en la homeóstasis, como en otras especies.

La aplicación de antígenos en mucosas para la vacunación es ampliamente utilizada en la industria avícola como una opción para enfermedades como la enfermedad de Newcastle y la bronquitis aviar (146,148), debido a su practicidad, así como a la relevancia y participación de agregados linfocíticos en mucosas, por ejemplo, GH (145–147). La participación activa de GH en la producción de una respuesta inmune adaptativa luego de la administración local representa una herramienta para la aplicación de antígenos en mucosas, en este caso oculares (56,181) debido a la relevancia en la participación de S-IgA en la protección frente a infecciones, así como en la inducción de inmunidad tras la vacunación (182). Sin embargo, la medición de este subtipo de Ig en muestras de líquido lagrimal (183), no es una práctica habitual. El objetivo principal de este fase del experimento fue determinar la antigenicidad de la proteína recombinante H5 en comparación con los antígenos virales nativos de vIA.

La proteína recombinante H5 previamente expresada fue administrada vía mucosas oculares en un modelo animal (aves de corral), debido a la importancia de la inmunidad local de las mucosas contra enfermedades causadas por virus respiratorios como IA (184). Un kit ELISA de S-IgA total de origen comercial se utilizó para determinar la antigenicidad a la aplicación en mucosa ocular de antígenos mediante la estimulación del tejido linfoide asociado al ojo (148). Debido a la disponibilidad de animales donados para este experimento, se emplearon aves de origen comercial con proposito de postura, bajo el mismo protocolo propuesto para animales de engorda. A pesar de la detección no específica de S-IgA frente a AI, fue posible determinar diferentes niveles de estimulación de S-IgA durante la aplicación tanto de los antígenos virales como de la proteína recombinante como se describe a continuación: A los 7 DPI, se detectó un aumento en los niveles de S-IgA total en las muestras de líquido lagrimal después de la aplicación de proteína de origen viral y proteína recombinante H5, en comparación con el grupo de control negativo, debido a la estimulación local del sistema inmune a los antígenos administrados (185). Para el segundo muestreo a los 14 DPI, el antígeno de origen viral mostró un aumento significativo en comparación con el antígeno recombinante y el grupo de control negativo en los niveles totales de S-IgA, debido a los componentes antigénicos de vIA

(proteínas NA, M) así como proteínas virales internas (186). Los valores de S-IgA totales inducidos a los 14 DPI por la proteína recombinante H5 mantuvieron el mismo nivel en comparación con el muestreo de 7 DPI; sin embargo, los componentes bacterianos (lipo-polisacaridos) empleados como adyuvante fueron capaces de estimular una respuesta inmune adaptativa contra estos componentes bacterianos (187), detectable por el kit ELISA S-IgA total en el grupo de control negativo. Los resultados de S-IgA total a los 7 DPI mostraron la capacidad de las proteínas virales y la proteína recombinante H5 para estimular la inmunidad local. Sin embargo, a los 14 DPI, debido al uso de un adyuvante a base de componentes bacterianos y la ausencia componentes virales presentes en el vIA, los resultados observados reflejan una respuesta inmune local con menor actividad de la proteína recombinante H5 en comparación con el antígeno viral completo (186), por lo que hace necesaria más investigación en el caso de antígenos subunitarios, asi como la caracterización de la respuesta de S-IgA especificas contra antígenos vIA para una evaluación precisa de esta respuesta inmune.

El ensayo IH se propuso para determinar la capacidad de estimular una respuesta inmunitaria sistémica mediante la administración en mucosas oculares de proteínas virales y la proteína recombinante H5, como previamente se ha demostrado como vía de estimulación del sistema inmune (56,188). Los resultados observados en la inmunización a los 14 DPI con proteínas virales, proteína recombinante H5 y la aplicación de un control negativo, no mostraron diferencia significativa entre ellos. Sin embargo, a los 21 DPI, se observó un aumento en los títulos de IH en los animales sometidos a tratamientos con la proteína viral y la proteína recombinante H5, en comparación con el grupo de control negativo, lo que demuestra que la estimulación inmune por mucosas oculares es relevante para la respuesta inmune local y sistémica (189). Esto se debió a la presencia de agregados de linfocitarios en particular, la GH (177), así como su participación en la presentación de antígenos al sistema inmune para la generación de la respuesta humoral a nivel sistémico (190). Como se observó en la medición de S-IgA total en líquido lagrimal, la generación de una respuesta inmune sistémica inducida por el estímulo también estuvo influenciada por la participación de otros antígenos virales presentes en la muestra de proteína viral (190); por lo tanto, la presencia de un solo antígeno de subunidad presenta una capacidad antigénica menor a un antígeno nativo viral completo.

El aumento de los títulos expresados en media geometrica (TMG) en el ensayo IH tras la aplicación del antígeno recombinante H5 demuestran la estimulación inmunitaria sistémica específica al vIA mediante la administración en mucosas oculares de los animales de experimentación (189), debido a que el ensayo IH mide la reactividad de los anticuerpos estimulados frente a la inmunización específica de IA (8). Debido al desempeño inmunológico de la proteína recombinante H5 en comparación con proteínas virales administradas bajo el mismo adyuvante y vía de aplicación, es necesario continuar su estudio como posible antígeno recombinante para IA antes de considerar experimentos de desafío viral en animales. Los títulos obtenidos del ensayo IH, inducido por la aplicación de antígenos virales y proteína recombinante H5, sugieren los títulos mínimos de protección (TMG $\geq$ 32) (191). Sin embargo, se requieren varios experimentos para determinar el nivel mínimo de protección en condiciones de laboratorio y en campo.

Publicación 3

***Dunaliella salina* as a Potential Biofactory for Antigens and Vehicle for Mucosal Application. (Pag. 55) (ANEXO 3).**

La mejora en los sistemas de expresión de proteínas recombinantes de alto valor fomenta la investigación de diversos modelos biológicos con diversas características, tales como costo de escalamiento y características en las modificaciones post-traduccionales (61,192,193). Estos aspectos resultan a consideración ante los retos en la producción de proteínas recombinantes. En el caso de *D. salina*, la investigación muestra a este sistema de expresión como una solución práctica para la producción de diferentes tipos de proteínas recombinantes y promete ser un método de producción basado en las ventajas del cultivo, método de transformación, compuestos inmunomoduladores, patrones de glicosilación y encapsulación natural.

El estado de la investigación sobre la manipulación genética de *D. salina* se encuentra en etapas iniciales; sin embargo, los datos sugieren que puede ser una opción práctica y tangible para la industria de las vacunas. La administración mucosal de antígenos (oral, ocular o intranasal) presenta requisitos para una correcta aplicación, que no todos los sistemas de expresión de proteínas recombinantes pueden cumplir (61). Por lo tanto, las microalgas tienen ventajas sobre otros sistemas. Además de las ventajas descritas anteriormente, las microalgas son organismos capaces de realizar la fotosíntesis mediante la captura de carbono inorgánico ambiental (CO2) (194) de manera muy eficiente (195). Por lo tanto, es necesario considerar no solo las ventajas de un sistema, si no tambien factores sociales y ambientales para desarrollar una plataforma de expresión de proteínas a nivel industrial. En la actualidad es requerida más investigación sobre el modelo de microalgas para la expresión de proteínas heterólogas, las cuales incluyen las secuencias reguladoras, la optimización de codones (196,197) y vectores de expresión más eficientes (85,198,199). Sin embargo, los datos disponibles permiten considerar a *D. salina* como un sistema de expresión de proteínas con potencial para la producción de antígenos y su administración mucosal a futuro.

4. CONCLUSIONES

El presente estudio, mediante la aplicación del protocolo de transformación nuclear mediada por la bacteria *A. tumefaciens* logró la inserción del gen recombinante de la proteína viral H5 en el núcleo de las microalgas de la especie *D. salina*, detectable por pruebas de reacción en cadena de la polimerasa con oligos específicos a partir de material genómico de las microalgas transformadas y a su vez la expresión de este gen heterólogo dirigido por los promotores: proteína de choque térmico 70 (Hsp70) y subunidad pequeña de RUBISCO, así como el terminador RBCS2 (estrategia promotor híbrido/terminador), clonados en el cassette de expresión diseñado para este experimento, demostrando tanto la capacidad de transformación como la capacidad de expresión de una proteína heteróloga en un sistema basado en cultivos de microalgas. Las pruebas para la detección y caracterización de esta proteína recombinante en partícular, mostraron una relación conservada con las proteínas virales provenientes de muestras obtenidas por protocolos de producción viral estándares para su formulación como antígenos vacunales; por lo que esto resulta de intéres para la demostración de la capacidad de síntesis de proteínas con características requeridas para la formulación de antígenos, en especial del vIA, los cuales demuestran incluso la actividad de hemaglutinación, la cual requiere de modificaciones post-traduccionales mínimas para realizar esta función.

El presente experimento tuvo como principal enfoque, el demostrar la capacidad de expresión de proteínas virales, en partícular una tan compleja como la HA del vIA, bajo un sistemas novedoso y nunca antes evaluado con este propósito; a pesar de estos resultados, debido a los rendimientos obtenidos hasta el momento, es requerido un mayor análisis de alternativas de expresión dentro de este sistema, dado que el sistema en si sustenta la sintésis de la proteína H5, el protocolo de expresión debe de explorar otras posibles mejoras del sistemas disponibles a nivel experimental para lograr rendimientos que puedan ser considerados como factibles para su uso dentro un enfoque industrial a futuro.

Con respecto a la evaluación antigénica de la proteína recombinante H5 obtenida por este método de expresión en las microalgas de la especie *D. salina*; los resultados demuestran que este tipo de proteína recombinante presenta caraterísticas similares a un antígeno viral obtenido por metodos estándares al ser evaluados por su administración en animales de experimentación de origen comercial. Los resultados aportan información tanto de la capacidad antigénica de la proteína recombinante H5, así como el uso de la vía ocular para la inducción de una respuesta inmune local y sistémica para una enfermedad de ínteres internacional, como lo es IA; así como el uso de técnicas de valoración de esta respuesta inmune, tanto por ELISA S-IgA total, como por la prueba de IH. Esto orienta la investigación hacia el estudio de antígenos recombinantes para la enfermedad de IA, las diversas rutas de aplicación para su posible uso para el estudio de la vacunacion en campo, así como la necesidad de la evaluación de antígenos subunitarios para su uso en vacunas, debido a que a pesar de ser capaz de inducir una respuesta inmune medible por métodos estándares para la evaluación de IA, estos resultaron inferiores a los obtenidos por un antígeno completo del vIA; por lo que su uso require tanto de mejora en sistema de expresión, como en el diseño de antígenos compuestos por diversas proteínas para una mejor estimulación, vía de aplicación, así como adyuvantes que permitan un mejor desempeño de este tipo de inmunógenos.

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ANEXO 1. Artículo científico publicado

PUBLICACIÓN 1. TRANSFORMATION OF *Dunaliella salina* BY *Agrobacterium tumefaciens* FOR THE EXPRESSION OF THE HEMAGGLUTININ OF AVIAN INFLUENZA VIRUS H5

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Transformation of *Dunaliella salina* by *Agrobacterium tumefaciens* for the Expression of the Hemagglutinin of Avian Influenza Virus H5

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Abstract: Avian influenza (AI) is one of the main threats to the poultry industry worldwide. Vaccination efforts are based on inactivated, live attenuated, and recombinant vaccines, where the virus hemagglutinin (HA) is the main component of any vaccine formulation. This study uses *Dunaliella salina* to express the AIV HA protein of an H5 virus. *D. salina* offers a system of feasible culture properties, generally recognized as safe for humans (GRAS), with N-glycosylation and nuclear transformation by *Agrobacterium tumefaciens*. The cloning and transformation of *D. salina* cells with the H5HA gene was confirmed by polymerase chain reaction (PCR). SDS-PAGE and Western blot confirmed HA5r protein expression, and the correct expression and biological activity of the HA5r protein were confirmed by a hemagglutination assay (HA). This study proves the feasibility of using a different biological system for expressing complex antigens from viruses. These findings suggest that a complex protein such as HA5r from AIV (H5N2) can be successfully expressed in *D. salina*.

Keywords: avian influenza; *Dunaliella salina*; *Agrobacterium tumefaciens*; hemagglutinin; recombinant

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1. Introduction

Avian influenza (AI) is one of the most critical illnesses in the poultry industry worldwide [1]. AI is defined as a systemic disease ranging from clinically undetected to severe with a high mortality depending on the virus subtype [2]. The AI virus (AIV), a member of the *Orthomyxoviridae* family, is the etiologic agent of AI [3]. Hemagglutinin (HA) and neuraminidase (NA) are the major glycoproteins found on the surface of the influenza virus [4], though HA is the most abundant (up to 10-fold more than NA) [5]. HA is a trimeric elongated rod-shaped protein with three monomers (75 kDa) connected in a trimer (225 kDa) with a length of 10–14 nm and a 4–6 nm diameter. Two polypeptides (HA1

and HA2) are organized into a core-helical coiled-coil (stem-like domain) with N-linked oligosaccharide side chains and three globular heads in the monomeric structure of HA (HA0) [6]. The primary role of HA is to act as a viral receptor by interacting with sialic acid (SA) to allow the virus to bind and attach to the host cells [6,7]. Influenza virus HA is the primary inducer of host immunity; thus, vaccination is the most crucial tactic for combating AIV on the ground and is mostly focused on the HA subtype in circulation [8,9]. Currently, the expression of HA in heterologous systems demonstrates the feasibility of its use as a strategy against AIV [6,8–12]. Among the recombinant protein expression systems, particular characteristics can be described. These examples include high production costs in the case of animal and insect cells, undesirable post-translational modifications observed in yeasts, prolonged cultivation times as in plants, and the lack of glycosylation in proteins as in the case of bacteria models [13]. However, there are other systems, such as microalgae, where their use has not been fully explored. A potential system of expression proposed is the eukaryotic green alga, *Dunaliella salina* [14].

Dunaliella sp. is a unicellular, halophilic, bi-flagellate, and naked green alga, Phylum *Chlorophyta*, Class *Chlorophyceae*, order *Volvocales*, family *Polyblepharidaceae*, and genus *Dunaliella* with a total of 29 species [15]. It was first described in 1905 [16] and named in honor of Michel Felix Dunal [17]. Currently, the genetic manipulation of *Dunaliella sp.* includes electroporation [18], particle bombardment [19], glass beads [20], lithium acetate/polyethylene glycol (PEG)-mediated [21], and genetic nuclear transformation by *Agrobacterium tumefaciens* [22,23], which present different degrees of effectiveness for the expression of heterologous genes [24]. The *A. tumefaciens*-mediated nuclear transformation system has demonstrated the stable integration of recombinant genes into host cells [25], taking advantage of the capacity of *A. tumefaciens* to transfer foreign genes to the host genome using the T- DNA region from a binary vector [26,27].

The *D. salina* system for antigen production offers industrial advantages such as low production costs, low risk of biological contamination with animal pathogens, the capacity for post-translational modification such as N-glycosylation in proteins similar to human glycoproteins, and the possible oral administration of the antigen [28]. This study aimed to establish the expression of the recombinant H5rD protein of AIV in the nucleus of *D. salina* and examine its bioactivity in vitro.

2. Materials and Methods

2.1. Synthetic Design of the H5rD Gene

The synthetic design of the *H5rD* gene was based on the whole sequence of the HA gene of the reference strain A/chicken/Hidalgo/28159-232/1994 (H5N2) Genbank # CY006040.1, which consists of 1695 bp and presents a low-pathogenicity cleavage site [29]. The gene *H5rD* was synthesized and codon-optimized for *D. salina* by GenScript Inc. (GenScript, Piscataway, NJ, USA).

2.2. D. salina Strain and Culture Conditions

The UTEX-1644 strain of *D. salina* from the Culture Collection of Algae at the University of Texas (Austin, TX, USA) was purchased and used for all the experiments. *D. salina* was cultured in PKS (phosphate-potassium-sodium) modified medium at 26 °C over 12-h night–day cycles under a constant light intensity (30 µmol photons m^{-2} s^{-1}) provided by fluorescent lamps [30]. At the logarithmic growth phase (10^5 cells mL^{-1}), cells were harvested, centrifuged, and analyzed for their size uniformity, shape, and movement during the complete experiment.

2.3. DNA Cloning Vector pH5HPDS

A DNA cassette of expression (2493 bp) contained two promoters upstream: heat shock protein 70 (Hsp70) (275 bp) and RUBISCO small subunit (199 bp). Downstream, it contained an RBCS2 terminator (hybrid promoter/terminator sequences strategy) (234 bp) [24,31] of the cloning site for the *H5rD* exogenous gene. The complete cassette was synthesized

by GenScript Inc. (GenScript, Piscataway, NJ, USA) and subcloned into the binary vector pCAMBIA-1301 (Genbank No.AF234297) at the *EcoRI-PstI* sites by standard cloning methods [32]. The resulting vector named pH5HPDS was confirmed by restriction analysis to verify the correct integrity. The vector pH5HPDS was transferred into *Agrobacterium tumefaciens* (LBA4404 strain) by electroporation [33]. The sequence and map of the constructed vector pH5HPDS are available upon request.

2.4. D. salina UTEX-1644 Strain Transformation

A single colony of *D. salina*, strain UTEX-1644, was inoculated in 150 mL of PKS-modified medium and incubated in a shaker at 150 rpm with a constant light intensity (30 µmol photons m^{-2} s^{-1}) provided by fluorescent lamps in a 12/12 light–dark cycle and a temperature of 24 ± 1 °C [30]. Once the culture reached optical density$_{600nm}$ (OD) values of ~0.7 (7 days), 100 mL of the culture was inoculated in 500 mL of PKS-modified medium with magnetic agitation (Corning Pyrex glass Proculture® spinner flasks, Corning, NY, USA). The culture was incubated until it reached OD_{600nm} values of ~0.7 (21 days). Genetic transformation of *D. salina* was performed by co-culture of *D. salina* cells with *A. tumefaciens* as follows: 5 mL of liquid LB culture of *A. tumefaciens* at an OD_{600nm} = 1.0 (20 mg/L rifampicin, 50 mg/L kanamycin) was supplemented with 100 µM acetosyringone and incubated for 4 h; then, the culture was centrifuged at 6000 rpm for 5 min, and the bacterial pellet was resuspended in 5 mL of PKS-modified medium and added to a culture of *D. salina* (500 mL, OD_{600nm} ~0.7). The co-culture was incubated at 25 °C without light for 48 h [34]. The cultures were centrifuged at 1000 rpm for 2 min and washed three times with PKS-modified medium containing 500 mg/L cefotaxime. Subsequently, *D. salina* cells were cultured in PKS-modified medium (50 mg/L hygromycin) and incubated at 25 °C in continuous light for 48 h. To determine the expression of recombinant protein H5rD in *D. salina* cultures, samples were harvested, centrifuged at 13,000 rpm for 15 min, and stored at −80 °C until further analysis.

2.5. DNA Extraction and PCR Analysis

To confirm the integration of the H5HA gene named *H5rD*, three samples of the transfected culture of *D. salina* were harvested and analyzed by PCR. Briefly, 10 mL of microalgae cells were centrifuged at 3500 rpm for 5 min, and genomic DNA extraction was performed by the CTAB method [32]. A PCR with specific oligonucleotides targeting the transgene *H5rD* (forward 5′ATGGAAAGAATAGTGATTGCCTTTG3′, reverse 5′TTAGATGCAAATTCTGCACTGC3′) was used to amplify a fragment de 1695 bp. The plasmid pH5HPDS was used as a positive control and DNA of *D. salina* wild type (WT) as a negative control. The cycling conditions were 94 °C for 5 min, 35 cycles at 94 °C for 20 s, 52 °C for 30 s, 72 °C for 180 s, and a final extension at 72 °C for 8 min. PCR products were analyzed by electrophoresis on 1% agarose gel stained with ethidium bromide.

2.6. SDS-PAGE of Total Soluble Protein

Samples from total soluble protein (TSP) were obtained from a pellet of 2 g wet weight (WW) of the transfected *D. salina* or from wild-type (WT) *D. salina* cells. To detect H5rD expression, samples were processed as follows: Pellets were resuspended in 10 mL of lysis buffer (1% SDS, 10 mM Tris-MOPS, 2 mM MgCl2, 10 mM KCl pH 7.5, and 2 mM PMSF, added before use). Cell disruption was carried out by sonication at 30% amplitude (10 cycles of 30 s each). Samples were centrifuged at 13,000 rpm for 15 min at 4 °C, and the supernatants were filtered through a polyvinylidene difluoride (PVDF) membrane (pore diameter 0.22 µm, Millex-GV; Millipore, Billerica, MA, USA). Then, samples of each TSP extraction were analyzed with Quick Start Bradford protein assay–Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA), homogenized TSP concentration 3.5 µg/µL. Finally, samples were analyzed in a 12% SDS-polyacrylamide gel (PAGE): the gel was stained with Coomassie blue, and the level of expression of the H5rD protein was determined using the

Quantity One software (Bio-Rad). Each TSP sample was maintained at −80 °C for later use in Western blot (WB) testing and hemagglutinin (HA) assay.

2.7. Western Blot (WB) Analysis of H5rD Protein

WB was performed as previously reported [8]. Briefly, 20 µL of each TSP lysates was mixed with protein-loading buffer (10% sodium dodecyl sulfate [SDS], 0.25 M Tris [pH 6.8], 0.1% Bromophenol blue, 7.73% dithiothreitol, and 50% glycerol) in a 1:1 volume and boiled for 5 min, run in a 12% SDS-PAGE gel. Nitrocellulose membranes were incubated with either anti–avian influenza A hemagglutinin antibody (Abcam AB135382, Cambridge, MA, USA, 1:1000 dilution) or anti–H5 AIV antiserum (1:1000 dilution), with a titer using hemagglutination inhibition (HI) test assay of 128 geometric mean titer (GMT) obtained from birds vaccinated with the influenza A-VIREN commercial vaccine (lot 21-078, AIV vaccine; Viren SA de CV, Queretaro, Qro, Mexico). After 12 h of incubation, membranes were washed 2X with TBS buffer, and incubated with either a rabbit anti-mouse IgG H&L (HRP) secondary antibody (Abcam AB97046, Cambridge, MA, USA, 1:2000 dilution) or a rabbit anti-chicken IgY H&L (HRP) antibody (Abcam AB6753, Cambridge, MA, USA, 1:2000 dilution), respectively. Low-pathogenicity AIV strain A/chicken/Hidalgo/28159-232/1994(H5N2) at a dose of 10^3 50% chicken embryo infectious dose/0.1 mL was used as a positive control. The virus was treated with NP-40 buffer for protein extraction [35]. A second positive control of the anti–avian influenza A hemagglutinin antibody consisted of a standard protein hemagglutinin HA (Influenza A virus (A/Vietnam/1203/2004(H5N1)) (Abcam AB190125, Cambridge, MA, USA). WB was revealed by incubation for 5 min with a solution of DAB (HRP Color Development Reagent, DAB (3,3′-diaminobenzidine), Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Hemagglutination Assay (HA)

According to Killian [36], TSP was evaluated for hemagglutination activity. Briefly, TSP (H5rD protein) in triplicate at an initial concentration of 12.5 µg was two-fold diluted in PBS (pH 7.4) and incubated at 4 °C for two h with 25 µL of 1% chicken erythrocytes in U-bottom 96-well microtiter plates. The highest dilution where complete hemagglutination was observed was considered as one HA unit (HAU). Bovine serum albumin (BSA) with a concentration of 10 µg/mL was used as a negative control. The AIV strain A/chicken/Hidalgo/28159-232/1994(H5N2) was used as a positive control [37].

3. Results

3.1. Cloning of H5HA and Expression of Recombinant Protein H5rD in D. salina

The correct integration of the H5HA gene was confirmed by three randomly selected samples of the purified genomic DNA from transfected cultures of *D. salina*. A 1695 bp amplicon was observed in all three samples corresponding to the transgene *H5rD*. No amplification was observed in the genomic DNA from *D. salina* WT (Figure 1a).

The expression of a protein from TSP was confirmed in *D. salina* pellets from the transfected cells (Figure 1b). Results from a single sample showed the presence of a protein of approximately molecular weight (MW) 69 kDa, the putative recombinant protein H5rD. According to the quantitative densitometry of proteins stained with Coomassie blue, approximately 255.5 µg of recombinant protein was recovered from 2 g WW of *D. salina*. The expression of H5rD was confirmed by the TSP from *D. salina* either with monoclonal antibodies (Figure 1c) or with the anti-H5 AIV serum (Figure 1d). Samples from *D. salina* WT showed no antibody reactivity. The recombinant protein H5rD has a length of 564 corresponding to the amino acid sequence reported in GenBank # ABB88379.1 for the strain A/chicken/Hidalgo/28159-232/1994(H5N2).

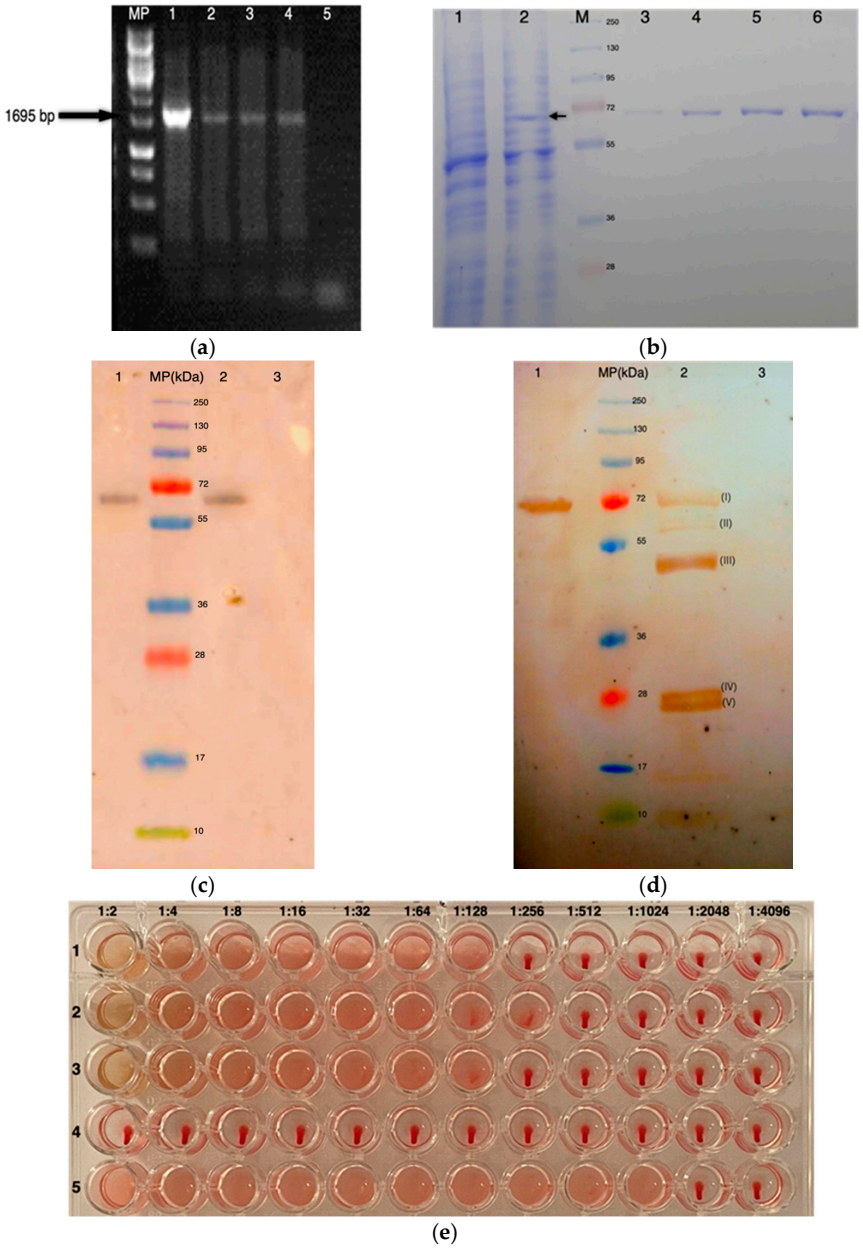

Figure 1. (**a**) One percent agarose gel to show PCR products corresponding to the *H5HA* gene of 1695 bp: lane 1, positive control (plasmid DNA with the gene *H5rD*); lanes 2–4, PCR product from 3 samples from transformed *D. salina* culture; lane 5, negative control (DNA from 1 culture sample of untransformed *D. salina*); MP, molecular weight marker (GeneRuler 1 kb DNA Ladder SM0311, Thermo Fisher, Waltham, MA, USA). (**b**) Coomassie blue-stained 12% SDS-PAGE gel showing the expression of a putative protein in transformed *D. salina* cultures: lane 1, TSP of WT *D. salina* cultures; lane 2, TSP of transformed *D. salina* cultures (adjusted to 70 µg); lane MP, molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa 26619, Thermo Fisher, Waltham, MA, USA); lanes 3–6, BSA at total concentration of 0.1, 0.5, 1, and 1.5 µg, respectively. (**c**) Western blot of

the H5rD protein with monoclonal antibodies: lane 1, H5rD protein detected with anti–avian influenza A hemagglutinin antibody (Abcam AB135382, Cambridge, MA, USA); lane 2, H5 standard protein as positive control (Abcam AB190125, Cambridge, MA, USA) molecular weight ~64 kDa without post-translational modification; lane 3, TSP sample from *D. salina* WT; MP, molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa 26619, Thermo Fisher, Waltham, MA, USA). (**d**) Western blot detection of H5rD protein with polyclonal antibodies: lane 1, H5rD protein from TSP detected with a chicken serum (IgY anti-H5); lane 2, viral proteins from low pathogenic virus A/Chicken/México/232/94/CPA; lane 3, TSP sample from *D. salina* WT; lane MP, molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa 26619, Thermo Fisher, Waltham, MA, USA); protein bands identified in lane 2 correspond to (I) HA, (II) NA / NP, (III) HA1, (IV) M1 and (V) HA2. (**e**) HA assay of ultrafiltered H5rD protein incubated with 1% suspension of chicken erythrocytes: row 1–3, Microalgae-produced hemagglutinin; row 4, BSA negative control serially diluted, initial concentration of 10 µg/mL; row 5, allantoic fluid from chicken embryos infected with low pathogenic virus A/Chicken/México/232/94/CPA as a positive control.

3.2. Bioactivity Analysis

The activity of the protein H5rD as a native H5HA was determined by HA assay. To increase the concentration of recombinant protein H5rD, a sample of 1 mL of TSP from transfected *D. salina* was concentrated by ultra-filtering, and upon 10-fold increase in the sample, a sufficient concentration was achieved. The hemagglutination results show that for all three samples of protein H5rD, complete hemagglutination was observed at a 1:128 dilution, corresponding to 1 HAU (Figure 1e, rows 1–3). H5rD protein concentrations of $\geq$0.09 µg were unable to induce hemagglutination. No hemagglutination activity was observed in wells with a negative control (Figure 1e, row 4); the positive control was a HA titer of 1:1024.

4. Discussion

Nowadays, recombinant antigen production systems are more economically viable [38]. Innovative alternative platforms for the generation of antigens for disease research and control are required around the world [39]. In the case of AI, producing viruses in biological models such as embryos is an essential part of the vaccine development process [40,41]. However, embryos represent a high cost, and they must be used on animals in the manufacturing stage [42]. As a result, there is growing interest at the laboratory and pharmaceutical levels in developing alternative antigen manufacturing methods.

In this study, we have shown the successful expression of soluble H5HA protein from avian influenza subtype H5N2 in a microalgae system using *D. salina* as a host species. HA protein expression has previously been reported in bacteria, plants, insect cells, mammalian cell cultures, and microalgae, referring to different levels of expression and glycosylation efficiency [8,12,43], but no advantages of using *D. salina* as a protein expression system for IAV have been identified.

Employing biological systems to express recombinant proteins is required to fulfil several properties, including levels of expression, protein complexity, and glycosylation processes [13]. Proper protein folding and functionality, along with glycosylation, are the most critical characteristics to achieve [44]. Therefore, based on the results of WB and reactivity to monoclonal and polyclonal antibodies directed against the H5HA, the findings suggest the proper expression of the H5rD protein.

Recent research on microalgae has shown that these unicellular organisms have benefits over other systems [45]. There are several distinct advantages compared to other bioreactor systems, including: biomass doubling in 24 h; relatively short growth times; the expression of proteins in the nucleus, chloroplast, and mitochondria; post-translational modifications; phototrophic or heterotrophic growth; temperature-controlled conditions, light, and nutrients; reduced risk of escape of transgenes into the environment; some species being recognized as GRAS (Generally Recognized as Safe for humans); and, finally, the possibility of their lyophilization being used for storage or for oral administration [13,46,47].

Using *D. salina*, we report that in a timeframe of 30 days, it was possible to recover up to 225 µg of soluble protein from 2 g of WW of a culture of *D. salina*. These results are encouraging considering that the actual process followed to obtain a vaccine strain takes no less than six months and requires an average of 100 embryonated eggs for 10 mg of total viral protein [40], as well as causes possible contamination with AIV in the embryo-producing flocks, putting at risk the availability of these flocks for the production of vaccines [48].

In prior research using *D. salina*, the expression levels of recombinant proteins were observed at similar levels [49]. In models of transitory expression of HA protein in plant tissue, they can reach up to 9.7% of the TPS [50], and when excreted into the medium they can reach up to 1 mg/50 mL of HAr in the case of *Schizochytrium sp.* [51]. However, because a considerable proportion of recombinant HA protein in plant tissue is found in the insoluble protein, the amount of H5rD in *D. saline* is likely to be greater than that which is observed in TPS [51]. The cultivation time, medium cost, and contamination management we established in our study underline *D. salina* advantages.

In the case of post-translational modifications such as glycosylation in proteins, the microalgae system showed the capacity to carry out these modifications, allowing the functionality of complex proteins with biological activity, as is the case for the HA protein [51]; models such as *Chlamydomona reinhardtii* and *Schizochytrium sp.*, among others [51,52], have been shown to fulfil the characteristics necessary to achieve the expression of recombinant proteins with very specific glycosylation requirements [53]. These characteristics, together with studies of the metabolic pathways and associated genes in processes such as glycosylation, demonstrate that these organisms can generate post-translational modifications to achieve the folding, functionality, and antigenicity required in each case [54]. However, more studies are required in models such as *D. salina* to determine these modifications, their possible effects on the characteristics of the proteins expressed in this system, and their interaction in an animal model.

Among the main characteristics of the HA protein needed for its correct folding and the presence of hemagglutinating activity (viral receptor) is post-translational modifications glycosylation [53]. In the case of HA, glycosylation patterns vary between the different strains and types of HA [53]. Even the presence of N-glycosylation sequences is not sufficient to be glycosylated; in addition to this, the type of cell where the virion replicates affects these post-translational modifications (chicken embryo, human cells, etc.) [55]. Glycosylation is constantly present where different types of HA (glycosides 27, 40, 176, 303, and 497) are recognized. However, 27 is necessary to acquire biological function [53]. Since the receptor-ligand activity of the HA protein requires post-translational modification such as 27 N-glycosylation, the determination of biological activity of the H5rD protein using hemagglutination assay allows the demonstration of a functional protein that requires post-translational modification. This work shows accurate production of the H5rD protein and biological activity, where HA titers were comparable to the wild type of virus used as a control. The receptor binding capacity of the H5rD protein is similar to that which was previously reported in microalgae models [50]; hence, its expression in this biological model can be regarded as viable.

Our study has some limitations; further studies are needed to evaluate if the H5rD protein is expressed as native HA protein, affecting the antigenicity [56] and the possibility of induction of an immune response in an animal model. Moreover, further studies are needed to confirm the presence of post-translation modification as a factor that could also affect antigenicity and protein folding [57,58].

5. Conclusions

According to the current findings, the *D. salina* system can carry out the expression and accurate folding of HA protein, allowing the biochemical features and biological activity of binding to chicken erythrocytes to be preserved. As a result, the *D. salina* expression system is a viable option for the creation of novel recombinant protein production methodologies.

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ANEXO 2. Artículo científico publicado

PUBLICACIÓN 2. IMMUNE EVALUATION OF AVIAN INFLUENZA VIRUS HAR PROTEIN EXPRESSED IN *Dunaliella salina* IN THE MUCOSA OF CHICKEN

vaccines

MDPI

Communication

Immune Evaluation of Avian Influenza Virus HAr Protein Expressed in *Dunaliella salina* in the Mucosa of Chicken

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Abstract: Avian influenza (AI) is a serious threat to the poultry industry worldwide. Currently, vaccination efforts are based on inactivated, live attenuated, and recombinant vaccines, where the principal focus is on the type of virus hemagglutinin (HA), and the proposed use of recombinant proteins of AI virus (AIV). The use of antigens produced in microalgae is a novel strategy for the induction of an immune response in the mucosal tissue. The capacity of the immune system in poultry, particularly in mucosa, plays an important role in the defense against pathogens. This system depends on a complex relationship between specialized cells and soluble factors, which confer protection against pathogens. Primary lymphoid organs (PLO), as well as lymphocytic aggregates (LA) such as the Harderian gland (HG) and mucosa-associated lymphoid tissue (MALT), actively participate in a local immune response which is mainly secretory IgA (S-IgA). This study demonstrates the usefulness of subunit antigens for the induction of a local and systemic immune response in poultry via ocular application. These findings suggest that a complex protein such as HAr from AIV (H5N2) can successfully induce increased local production of S-IgA and a specific systemic immune response in chickens.

Keywords: avian influenza; S-IgA; immunoglobulin; mucosal; hemagglutinin; recombinant protein

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1. Introduction

Avian influenza (AI) is classified from a respiratory to a systemic disease, with a high economic and health impact on the poultry industry worldwide [1]. Clinical presentations of AI range from undetected to serious illness with high mortality depending on the virus subtype and factors such as age, sex, and the commercial purpose of the poultry [2]. The AI virus (AIV), a member of the *Orthomyxoviridae* family [3], presents the following two glycoproteins on its surface: hemagglutinin (HA) and neuraminidase (NA) [4]. HA, the principal surface antigen, and the most abundant protein on the surface of viruses [5], is a trimer (≈225 kDa) made up of identical monomers (≈75 kDa) that forms a trimeric, elongated,

rod-shaped protein organized into two polypeptides—HA1 and HA2 polypeptides—and intertwined in a core-helical coiled-coil (stem-like domain) with N-linked oligosaccharide side chains and three globular heads in the monomeric structure of HA0 [6]. The globular head of HA is the viral receptor, which interacts with a cellular receptor (sialic acid) [6,7]. The vaccination strategy against AI is largely based on the use of the HA subtype circulating in the susceptible population [8] through the use of inactivated virus vaccines and recombinant virus vaccines [9,10]. Another strategy proposed is the expression of recombinant antigenic proteins from AI [10]. In particular, the expression of HA in heterologous systems demonstrates the ability of these systems to produce recombinant proteins as possible antigens against AI with different approaches [6,8,11–14] including a heterologous protein expression system with high potential in various applications such as microalgae [15]. Traditional systems present particularly undesirable characteristics including high production costs, undesirable post-translational modifications, prolonged cultivation times, and the lack of glycosylation in proteins in some cases [16]; however, microalgae present several advantages for the expression of proteins with particular characteristics, such as the HA protein [17].

The immune system of poultry, especially in the mucosal tissue, plays a very important role in the defense against pathogens, whose entry or presence in the mucosa is a constant problem in animals [18]. In general, the immune system depends on a complex relationship between specialized cells and soluble factors, working together to confer protection against pathogens [1,19,20]. In particular, the mucosal immune system of birds has similarities with mammals, such as the presence of mucosa-associated lymphoid tissue (MALT), with some exceptions [19]. Among these, we can highlight the presence of primary lymphoid organs, such as the bursa of Fabricius (FB) and lymphocytic aggregates such as the Harderian gland (HG). The HG is observed in all vertebrates with the exception of fishes, some amphibians and primates, whereas in poultry HG is the major contributor to ocular Ig production [20]. HG is a dominant orbital gland in birds, with a strap-like structure, located anatomically ventral and posteromedial to the eyeball, and connected to the medial angle of the nictitating membrane [21]. HG is responsible for the production of secretory IgA (S-IgA) [22,23], mainly as a result of local stimulation [24]. Therefore, HG participates in the immune response in mucous membranes, mostly in the eyes and upper respiratory tract [24]. S-IgA, a polypeptide of two monomers IgA, is the predominant Ig isotype on most mucosal surfaces; due to its molecular stability and strong anti-inflammatory properties, S-IgA is an ideal protective immunity component of mucosal surfaces [25]. S-IgA and immunoglobulin M (IgM) represent an important part of the defenses at the mucosal level due to their function of preventing the adhesion of pathogens such as bacteria and viruses, limiting their pathogenic effect, and neutralizing and facilitating their excretion [26]. However, the parenteral administration of antigens in many cases is inefficient for its induction, hence the mucosal immune system is considered as separate from the systemic immune system [27]. The administration of antigens via mucosa led to the stimulation of a systemic response [28]; however, the main focus is the local stimulation of an immune response due to the need for protection in these tissues.

In the case of recombinant antigens from microalgae, due to all advantages over other antigen-production systems [29], it is possible to explore their use as mucosal vaccines, as a complementary stimulation pathway for parenteral immunization [30]. This study aimed to examine the local and systemic immune response of the recombinant HAr protein of AIV expressed in microalgae (*Dunaliella salina*) in chickens via mucosal administration.

2. Materials and Methods

2.1. Expression of HAr Recombinant Protein

HAr expression was performed in a previously reported study. Briefly, a sequence of the HA gene of the reference strain A/chicken/Hidalgo/28159-232/1994 (H5N2) Genbank # CY006040.1) was used for the agroinfiltration protocol for expression in *D. salina* according to the method of Castellanos-Huerta et al. [15].

2.2. Preparation of Antigens for Mucosa Application

2.2.1. Preparation of Virus-Inactivated Antigens

Virus-inactivated antigens for mucosa application were prepared as follows: allantoic fluid from 13-day-old embryos was inoculated with AIV H5N2 A/chicken/Hidalgo/28159-232/1994 (H5N2), and low pathogenicity AIV (LPAIV) with a minimum viral titer of $10^{9.1}$ 50% chicken embryo infectious dose (CEID)/1 mL was harvested and inactivated with 0.2% formaldehyde for 1 h under constant stirring at room temperature and stored for 24 h at 4 °C, with a minimum titer of hemagglutination test of 512 hemagglutination units (HAU)/mL, then it was mixed with the immunomodulator A1 at a ratio of 98:2 (v/v, allantoic fluid: A-1), according to the method of Fernandez-Siurob et al. [31]. Immunomodulator A1 was prepared from the lipopolysaccharide mixture as described by Westphal et al. [32], which was extracted from *Escherichia coli* strains characterized on an outbreak field [33].

2.2.2. Preparation of HAr Antigen Produced in *Dunaliella salina*

The production of microalgae biomass was carried out in 15 L of PKS (phosphate–potassium–sodium) modified medium in flasks with magnetic agitation (Corning PYREX glass Proculture® Spinner Flasks 15,000 mL. (New York City, NY, USA). The biomass from the culture was harvested by centrifugation at 13,000 rpm for 15 min at 4 °C and stored at −80 °C until ready for use. For antigen HAr's preparation protocol, biomass was ground using a mortar and pestle in liquid nitrogen, suspended in lysis buffer (1% SDS, 10 mM Tris-MOPS, 2 mM MgCl2, 10 mM KCl pH 7.5, and 2 mM PMSF, added before use), and treated to obtain total protein soluble (TPS) according to the methods of a previous report [15]. According to the quantitative densitometry of proteins stained with Coomassie blue, approximately 1.277 mg of recombinant protein was recovered from 10 g wet weight (WW) of *D. salina*. TPS from *D. salina* culture extracts was filtered through a PVDF membrane pore diameter of 0.22 µm and verified by centrifugation. Recombinant protein HAr was concentrated by performing ultra-filtering; upon the 10-fold increase in the sample a sufficient concentration was achieved. The samples were analyzed using a Quick Start™ Bradford Protein assay (Bio-Rad, Hercules, CA, USA) mixed with the designated immunomodulator A1, and homogenized to a final concentration of ~25 µg of recombinant protein HAr, each in 50 µL. The antigen and immunomodulator A1 were combined at a ratio of 98:2 (v/v, recombinant protein: A1), mixed for 10 min, and maintained at 4°C until use. One dose consisted of 100 µL of the antigen preparation (HAr), with 50 µL in each eye. A total of 50 µg of recombinant protein HAr was considered as a dose per animal, as previously observed [11].

2.3. Animal Experiment

The trial was performed to determine the antigenicity of the recombinant antigen HAr compared with a virus-inactivated antigen on mucosal application, as previously reported [31]. The animal experimentation consisted of 60 laying hen chickens divided into groups of 20 for treatments A, B, and C. In all of the experiments, animals were housed in poultry coops and divided according to the applied treatments. Treatment A consisted of the positive control via the ocular route with 100 µL (50 µL in each eye) of virus-inactivated antigen/adjuvant prepared as described above. Treatment B consisted of the application of 100 µL (total of 50 µg of recombinant protein HAr) protein/adjuvant (50 µL in each eye) via the ocular route. Treatment C consisted of the negative control including a treatment of PBS/A1 combined at a ratio of 98:2 (v/v, PBS/A1), mixed for 10 min, and maintained at 4 °C until its use via the ocular route (total of 100 µL of PBS, 50 µL in each eye). For this experiment, a calibrated dropper was used to deposit a 50 µL drop in each eye according to the manufacturer's instructions. In all of the experiments, environmental and animal management conditions were upheld according to the standards of laying chicken breeding. Feed and water were supplied ad libitum throughout the entire experiment. For the trial, treatment A (virus-inactivated antigen), treatment B (HAr), and treatment C (PBS/A1) were applied at 14 and 21 days of age. For each treatment, 10 birds were sampled for total S-IgA

quantitation in lachrymal fluid at 7 and 14 days post-treatment (DPT) (28 and 36 days of age); the samples were analyzed by performing an ELISA test according to the method of Merino-Guzman et al. [34]. A total of 12 birds were bled for serum at 14 and 21 DPT (36 and 42 days of age) for the inhibition of the hemagglutination (HI) assay to determine the serum immunoglobulin levels against AIV H5N2 [35]. All samples were stored at −80 °C until use. During the experiment, the experimental animals were handled according to ethical protocols for animal welfare [36].

2.4. ELISA Test of S-IgA from Lachrymal Fluid

Quantification of total S-IgA in lachrymal fluid samples was carried out using a commercial ELISA kit (Catalog #: E33-103, Bethyl Laboratories, Inc., Montgomery, TX, USA) following the manufacturer's guidelines. Samples were diluted at 1:1000 (v/v lachrymal fluid: dilution buffer), prior to their use in the ELISA test. Concentrations of S-IgA were expressed as nanograms of total S-IgA per ml (ng/mL).

2.5. Inhibition of Hemagglutination (HI) Assay

The stimulation of the systemic immune response of treatments A, B, and C was evaluated using the HI assay expressed as the geometric mean titer (GMT). All measurements of serum AI-specific antibody levels were analyzed with 4 HAU of the AIV A/chicken/Hidalgo/28159-232/1994 (H5N2) strain, according to the WHO Manual on Avian Influenza Diagnosis and Surveillance [37].

2.6. Statistical Analyses

Absorbance results in the ELISA test (for total S-IgA in the lachrymal fluid) were extrapolated onto a polynomial calibration chart according to the supplier's instructions. The data confirmed normal distribution (Shapiro–Wilk test) and homoscedasticity (Levene test). Consequently, the data were subjected to a parametric test and one-way ANOVA, followed by Tukey's multiple comparison test with the level of statistical significance set at $p < 0.05$.

3. Results

3.1. ELISA Test of S-IgA from Lachrymal Fluid

The quantification of total S-IgA determined by performing the ELISA test at 7 DPT in the lachrymal fluid of animals on treatments A (48,334 ng/mL ± 4196 ng/mL) and B (44,759 ng/mL ± 8002 ng/mL), showed a statistical difference ($p > 0.05$) compared with treatment C (24,224 ng/mL ± 3496 ng/mL) (Figure 1A). Samples obtained at 14 DPT showed an increase in the local immune response for the concentration of total S-IgA in treatment A (57,486 ng/mL ± 7192 ng/mL), with a statistical difference ($p > 0.05$) compared with treatment B (43,043 ng/mL ± 5733 ng/mL) and treatment C (35,633 ng/mL± 6195 ng/mL) (Figure 1B). No statistical difference was observed between treatments B and C.

3.2. HI Assay from Serum

According to the results, for treatment A (GMT = 8.97 ± 1.30), treatment B (GMT = 12.70 ± 1.98), and treatment C (GMT = 10.67 ± 1.59) at 14 DPT no statistical difference was observed ($p > 0.05$) (Figure 2A). The systemic immune response observed at 21 DPT presents a statistical difference ($p > 0.05$) by comparing treatment A (GMT = 38.05 ± 1.54), treatment B (GMT = 33.90 ± 1.86), and treatment C (GMT = 6.72 ± 1.54) (Figure 2B).

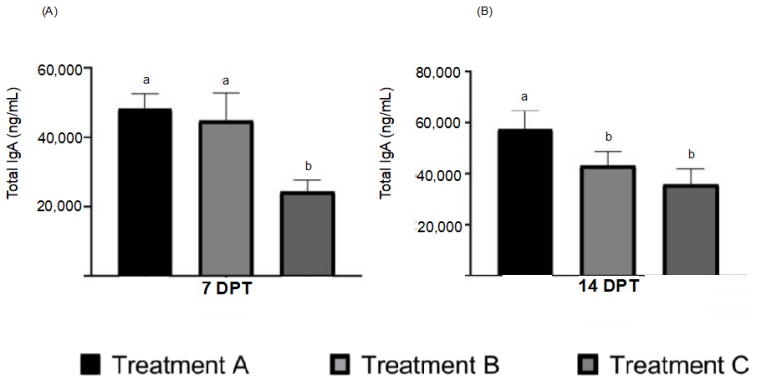

Figure 1. ELISA test of total S-IgA from lachrymal fluid of treatments A, B, and C. The concentration of total S-IgA from lachrymal fluid samples was determined using a commercial ELISA kit. (**A**). ELISA test results of total S-IgA in the lacrimal fluid were observed at 7 days post-treatment (DPT) on treatment A (virus-inactivated antigen), treatment B (HAr), and treatment C (PBS/A1). The results at 7 DPT showed a statistical difference ($p < 0.05$) in total S-IgA concentration in treatment A (48,334 ng/mL ± 4196 ng/mL) and B (44,759 ng/mL ± 8002 ng/mL) compared to treatment C (24,224 ng/mL ± 3496 ng/mL). (**B**). ELISA test results at 14 DPT showed an increase in concentration of S-IgA on treatment A (57,486 ng/mL ± 7192 ng/mL) with a statistical difference ($p < 0.05$) compared with treatment B (43,043 ng/mL ± 5733 ng/mL) and treatment C (35,633 ng/mL ± 6195 ng/mL). No statistical difference was observed in the cases of treatments B and C at 14 DPT. Literals indicate a statistical difference ($p < 0.05$).

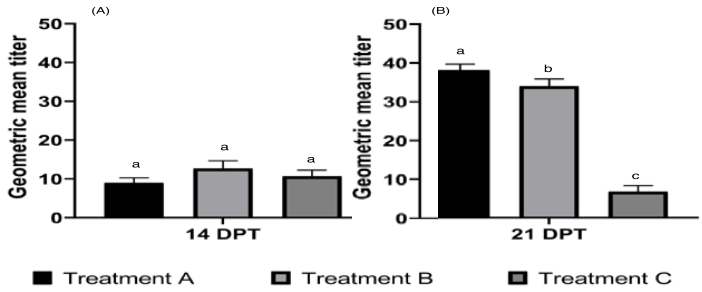

Figure 2. Inhibition of hemagglutination assay (HI) from serum of treatments A, B, and C. Systemic immune response of treatment A (virus-inactivated antigen), treatment B (HAr), and treatment C (PBS/A1) via ocular application at 14 and 21 days post-treatment (DPT) were evaluated by the HI assay expressed in geometric mean titer (GMT). (**A**). Treatment A (GMT = 8.97 ± 1.30), treatment B (GMT = 12.70 ± 1.98), and treatment C (GMT = 10.67 ± 1.59) indicated that no statistical difference was observed ($p > 0.05$) at 14 DPT. (**B**). Immune response at 21 DPT was observed in treatment A (GMT = 38.05 ± 1.54), treatment B (GMT = 33.90 ± 1.86) and treatment C (GMT = 6.72 ± 1.54) with a statistical difference ($p > 0.05$) in comparison to each other. Literals indicate a statistical difference (p-value < 0.05).

4. Discussion

A description of the response of the mucosal immune system is necessary for the understanding of several pathologies of infectious origin, particularly in the case of AI [38,39]. As is known, the main route of entry for pathogens into an organism is via the mucosa tissue [40]. Therefore, this tissue represents the first barrier that needs to be overcome in order to achieve infection and the generation of infectious diseases. For this reason, the mucosa requires a highly specialized immune system for the correct response and protection against various pathogens [38,39]. In the case of birds, these lymphoid organs present some variations with respect to other species [38,41]; however, the specialized

tissue plays a vital role in homeostasis, as in other species. The ocular route for vaccination is widely used in the poultry industry as an option for diseases such as Newcastle disease and avian bronchitis [23,24] due to its practicality, as well as the relevance and participation of lymphocytic aggregates, e.g., HG [20,22,23] The active participation of HG in the production of an adaptive immune response after ocular administration represents a tool for the local application of antigens [11,42] due to the relevance in the participation of S-IgA in the protection against infections, as well as the induction of immunity after vaccination [43]. Nevertheless, the measurement of this Ig subtype in lachrymal fluid samples [34] is not a regular practice.

The main objective of this study was to determine the antigenicity of a recombinant protein compared with native viral antigens of AIV. The recombinant protein (HAr) was previously produced in a protein-recombinant expression model [15] with advantages in terms of production costs, expression levels, as well as its possible use in the oral vaccination of animals [29]. Therefore, mucosal vaccination was demonstrated in an animal model (poultry) due to the importance of local mucosal immunity against diseases caused by respiratory viruses such as AI [44].

The total S-IgA ELISA kit was utilized to determine the reactivity of the ocular application of antigens by stimulating eye-associated lymphoid tissue [24]. Despite the non-specific detection of S-IgA against AI, it was possible to determine different levels of S-IgA stimulation during the application of both viral antigens and the recombinant protein. At 7 DPT, an increase in the levels of total S-IgA in the lachrymal fluid samples was detected following the application of the viral protein and recombinant protein, compared to the negative control group, thereby demonstrating HG stimulation in the presence of the administered antigens [45]. For the second sampling at 14 DPT, an antigen of viral origin showed a significant increase compared to the recombinant antigen and the negative control group in the total S-IgA levels, due to antigenic components of AIV (proteins NA, M) as well as internal viral proteins [46]. The total S-IgA values induced at 14 DPT by the recombinant HAr protein maintained the same level compared to 7 DPT sampling; however, bacterial components in the immunomodulator used called A1 were able to stimulate an adaptive immune response against these bacterial components [47], detectable by the total S-IgA ELISA kit in the negative control group. The results of total S-IgA at 7 DPT showed the ability of viral proteins and the HAr protein to stimulate local immunity. Nevertheless, at 14 DPT, due to the use of an adjuvant based on bacterial components and the absence of viral components, the results observed reflect a local immune response with less activity from the HAr protein compared to the complete viral antigen [46], thus reflecting the necessity to improve HAr subunit antigens, including the determination of SIgA-specific antigens against AIV antigens for an accurate evaluation.

The HI assay was proposed to determine the ability to stimulate a systemic immune response through ocular administration of viral proteins and a recombinant protein HAr. [11]. The results observed at immunization at 14 days DPT with viral proteins, recombinant protein and the application of PBS/A1, showed no significant difference. However, at 21 days DPT, an increase in HI titers was observed in animals subjected to treatments with the virus protein and recombinant HAr protein, compared to the negative control group, demonstrating that immune stimulation via the ocular route is relevant for the local immune response [48]. This was due to the presence of a lymphocyte aggregate HG, [38] as well as its participation in the presentation of antigens to the immune system for the generation of the humoral response at the systemic level [49]. As observed in the measurement of total S-IgA in lachrymal fluid, the stimulus-induced generation of a systemic immune response was also influenced by the participation of other viral antigens present in the sample of AI [50]; therefore, the presence of a single subunit antigen presents an antigenic capacity not similar to a complete viral native antigen.

The increase in GMT titers in the HI assay upon the application of recombinant antigens demonstrates AI-specific systemic immune stimulation by the ocular administration of a recombinant protein [49] since the HI assay measures the reactivity of stimulated

antibodies to AI-specific immunization [37]. Due to the immunological performance of HAr compared to viral proteins administered under the same adjuvant and application route, it is necessary to continue its study as a possible recombinant antigen for AI before considering viral challenge experiments in animals. The titers obtained from the HI assay, induced by the application of viral antigens and recombinant protein HAr, are suggestive of the minimum protection titers (GMT $\geq$ 32) [51]. However, various experiments are required to determine the minimum protection level.

5. Conclusions

The present study described the antigenic capacity of the recombinant protein HAr, applied by the ocular route in experimental animals, in comparison with a complete viral antigen. The results indicate the capacity of protein HAr for stimulation of the local and systemic immune system in an ocular application, for its recognition as a viral protein by HG, as well as its processing and induction of an antibody-specific immune response. The antibodies generated at the serum level proved to be reactive with a wild-type viral antigen, used in the HI assay, thus demonstrating that protein HAr presents characteristics of an antigen of AI. Nevertheless, viral antigens present more antigenic activity according to the ELISA test S-IgA results and HI assay; therefore, further studies are required to develop a recombinant antigen capable of inducing an immune response similar to that observed in antigens of viral origin.

The results of total measured S-IgA in this experiment demonstrate the efficiency of a recombinant protein to achieve antigenic stimulation in experimental animals; however, these results require a more specific evaluation, because total quantification is not able to determine the specific stimulation against protein HAr and its relationship with viral antigens, describing no specific local immune response. Therefore, its evaluation requires further study.

In this experiment, the AIV H5N2 A/chicken/Hidalgo/28159-232/1994 (H5N2) strain complied with the stipulations of the Mexican authority for use in the production of vaccines against AI and experimentation in vitro. Nevertheless, the absence of evidence of viral challenges in experimental animals leaves the capacity of these types of antigens unevaluated in the presence of pathogenic viruses, so their efficacy as vaccine antigens requires permission from the local authority to carry out their evaluation in viral challenges in order to understand its full scope as a possible vaccine antigen.

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ANEXO 3. Artículo científico publicado

PUBLICACIÓN 3. *Dunaliella salina* AS A POTENTIAL BIOFACTORY FOR ANTIGENS AND VEHICLE FOR MUCOSAL APPLICATION

processes

MDPI

Review

Dunaliella salina as a Potential Biofactory for Antigens and Vehicle for Mucosal Application

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Abstract: The demand for effective, low-cost vaccines increases research in next-generation biomanufacturing platforms and the study of new vaccine delivery systems (e.g., mucosal vaccines). Applied biotechnology in antigen production guides research toward developing genetic modification techniques in different biological models to achieve the expression of heterologous proteins. These studies are based on various transformation protocols, applied in prokaryotic systems such as *Escherichia coli* to eukaryotic models such as yeasts, insect cell cultures, animals, and plants, including a particular type of photosynthetic organisms: microalgae, demonstrating the feasibility of recombinant protein expression in these biological models. Microalgae are one of the recombinant protein expression models with the most significant potential and studies in the last decade. Unicellular photosynthetic organisms are widely diverse with biological and growth-specific characteristics. Some examples of the species with commercial interest are *Chlamydomonas*, *Botryococcus*, *Chlorella*, *Dunaliella*, *Haematococcus*, and *Spirulina*. The production of microalgae species at an industrial level through specialized equipment for this purpose allows for proposing microalgae as a basis for producing recombinant proteins at a commercial level. A specie with a particular interest in biotechnology application due to growth characteristics, composition, and protein production capacity is *D. salina*, which can be cultivated under industrial standards to obtain βcarotene of high interest to humans. *D saline* currently has advantages over other microalgae species, such as its growth in culture media with a high salt concentration which reduces the risk of contamination, rapid growth, generally considered safe (GRAS), recombinant protein biofactory, and a possible delivery vehicle for mucosal application. This review discusses the status of microalgae *D. salina* as a platform of expression of recombinant production for its potential mucosal application as a vaccine delivery system, taking an advance on the technology for its production and cultivation at an industrial level.

Keywords: *Dunaliella salina*; vaccines; recombinant protein; mucosal

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1. Introduction

Nowadays, biotechnology applied to developing products in the health industry is highly diversified worldwide [1,2]; this interdisciplinary branch of biological sciences presents greater participation in the market of pharmaceuticals and vaccines each day [3,4]. Therefore, searching for new scientific developments with practical applications is a priority in the industry. The approaches of biotechnology applications on microalgae range from metabolic modification [5,6], to phytochemicals production (lipids, carbohydrates, terpenoids, phenolics, and alkaloids) [7], to the expression of recombinant proteins [8]. In this area, efforts are focused primarily on developing expression systems capable of achieving both the industrial aspects of production costs and the quality of the recombinant proteins expressed [8]. Through the development of protein expression systems, the study of biological models begins with prokaryotic models (*Streptomyces* spp., *Bacillus* spp., *Lactococcus lactis*, *Escherichia coli*, and *Corynebacterium glutamicum*) [9], in conjunction with different plasmid-based gene expression strategies, with advantages such as high level of protein expression, rapid cell division, low cost for the production of raw biomass.

Nevertheless, the prokaryotic system presents limitations such as forming insoluble inclusion bodies, purification process requirements due to the presence of endotoxins, and limited post-translational [10,11], which limit its use in the expression of high-quality proteins. Unlike prokaryotic systems, eukaryotic systems allow the possible design of complex proteins with post-translational characteristics [12,13], which in many cases compromise folding and function. Model eukaryotic expression platforms include yeast, animal, plant, mammalian/insect cells, and microalgae. Potvin et al., 2010 describe differences between these expression platforms, including (i) size of the heterologous gene, (ii) sensitivity to shear stress, (iii) recombinant product yield, (iv) production time, (v) cost of production, (vi) scale-up and storage cost. A complete comparison with several other production systems could be visualized in this review [14].

Due to its characteristics, the eukaryotic organism yeast is one of the expression systems widely used in the industry [15]. This organism presents advantages in its use, range of reproduction, culture in confinement (biological reactors), and an average cost of production; however, it has disadvantages such as post-translational modifications being significantly different from humans, and the high price of scale-up costs [14], require preliminary analysis to consider this system for the production of antigens. In the case of eukaryotic cell systems, including invertebrate and mammalian cell lines, advantages include the ability to achieve post-translational modifications resulting in high-quality products and efficient protein secretion to the medium, which facilitates purification and the availability of standard methods for genetic manipulation [16,17]. Nonetheless, its production cost, the high nutrient requirements, cell growth rate, and the risk of contamination by pathogens (viruses, bacteria, prions), in some cases, limit their use in vaccine production models [17]. A different approach to producing recombinant proteins is the generation of genetically modified organisms, e.g., plants and animals. The main advantage of using transgenic animals to produce recombinant proteins is the high yields of a high-quality product. Despite its benefits, the process of generating transgenic animals implies a long time between the genetic engineering phase and the start of production, a low rate of gene integration, and unpredictable behavior of the transgenes [18,19]. In particular, plants such as cereals, tobacco, legumes, fruits, and vegetables [19], present attractive advantages for their use in the production of recombinant proteins due to their production cost, the capacity of post-translational modifications, cultivation cost advantages, and low scale-up cost [8], but disadvantages such as production time, lacks regulatory approval [19], as well as the possible genetic contamination in populations of non-genetically modified plants [20], hinder its mass production. Plant cell culture in vitro represents an essential role as a new expression platform at the industrial level [21]. Plant cells, as they are not vectors of animal pathogens, viruses, prions, or bacteria, carry out complex post-translational modifications [22], as well as cultivation in closed systems, in addition to storing recombinant proteins at adequate levels stabilized by a simply freeze-drying process, show attractive advantages for its use

in mucosal vaccination. Nonetheless, this expression system is limited by the long period between the production of transgenic plants and recombinant proteins [21,23].

This outlook reflects the need for next-generation platforms to overcome some limitations in conventional systems. During the last two decades, microalgae have also emerged in this field as a potential new platform for the production of biopharmaceuticals [7,22] due to various advantages that are mentioned below.

2. Approaches for Mucosal Vaccine Delivery

The mucosal surface is a specialized tissue with the function of a selective barrier of the internal and external environment of organisms, capable of exchange of nutrients and oxygen and preventing the passage of foreign objects and pathogens. The protection of this particular tissue is mainly based on the participation of mucosa-associated lymphoid tissues (MALT), distributed along the mucosal surface [24], which are responsible for the production and secretion of a particular type of secretory immunoglobulin A (S-IgA). The predominant isotype in the local immune response in mucous membranes [25], S-IgA is an essential part of defenses at the local level, preventing the entry of pathogens [26]. Parenteral administration of antigens is not practical for their induction, so the mucosal immune system is considered separate from systemic immunity [27].

Interestingly, despite the advantages of mucosal vaccination such as non-invasiveness, mucosal solid immune response to prevent the entry of most infectious agents, ability to avoid the previous immune response by parenteral vaccination, local immune stimulation as systemic, as well as its easy application [25], mucosal vaccines approved for use are limited [26]. The design of a mucosal vaccine requires the correct selection of the following components: (1) an antigen capable of inducing an efficient immune response, (2) an adjuvant capable of stimulating the adaptive immune response, and (3) a suitable administration system. In the case of oral and nasal mucosal administration systems include viral vectors, virus-like particles, emulsions, immune-stimulating complexes, monophosphoryl lipid A, calcium phosphate nanoparticles, polymeric nanoparticles, liposomes, proteasomes, cholesterol-bearing pullulan nanoparticles, self-assembled peptides, nanogels, chitosan [25], plant tissue [27], and microalgae [28]. Microalgae models in recent years have reflected a development in the production of recombinant proteins, so its use as a vehicle for vaccine administration is interesting for research.

3. Microalgae as a Biofactory for Proteins

The denomination microalgae include all unicellular organisms with a photosynthetic capacity [29]. Therefore, this denomination comprises a broad polyphylogenic group, including species from cyanobacteria to eukaryotes [30]. The production of microalgae for human benefit is a practice known for over 2000 years [31]. Currently, the cultivation of microalgae has applications in the area of food, cosmetic, and pharmaceutical industries [32] because it constitutes a natural source of lipids, vitamins, pigments (zeaxanthin, lutein, astaxanthin, and phycocyanin), antimicrobials [33,34], and antioxidants [35]. Species with industrial interest include *Chlamydomonas*, *Botryococcus*, *Chlorella*, *Dunaliella*, *Haematococcus,* and *Spirulina* [29]. Another approach is protein production for human, and animal nutrition [35,36], due to high protein content [37,38]. In particular, the protein synthesis capacity of some microalgae species supports a possible use for the industrial production of recombinant proteins. The advantages of microalgae compared to other systems include a high growth rate, culture conditions in confined systems, availability of genetic engineering tools, absence of toxic compounds (generally recognized as safe GRAS classification), post-translational modifications, and high biosynthetic capacity in terms of biomass yield [14,33].

Regarding post-translational modifications, glycosylation is directly responsible for the immunogenicity of an antigen [39]. Therefore, a previous glycosylation patterns analysis of a system is necessary before selecting it for vaccine production. In general, protein expression systems possess species-specific N-glycans, with differences from human post-

translation modification including "hyper-mannosidosis" structures (excess of mannose residues assembled on yeast), absence of essential human residues (α (2,6)-sialic acid and α(1,4)-fucose), undesired non-human residues (N-glycolylneuraminic acid (Neu5Gc) and galactose-α (1,3)-galactose (α-Gal)) on CHO cells, glycans containing immunogenic residues (β(1,2)-xylose and core α(1,3)-fucose) on plant cells. In the particular case of microalgae, two different glycosylation pathways are reported: GnT I enzyme-independent consists of 5 Man and 2 GlcNAc N-linked protein subjected to xylosyltransferases (XyT) and methyltransferases (MeT), leading to unique N-linked structures containing methylated mannoses linked to one or two xyloses with structures vary slightly among microalgae species, and GnT I enzyme-dependent transfers an N-acetylglucosamine residue to the 5 Mannose and 2 GlcNAc N-linked protein, subjected to α-mannosidase II (α-Man II) and fucosyltransferase (FuT), resulting in paucimannosidic (Man 3–4GlcNAc 2) fucosylated N-glycans. However, in these processes, microalgal species showed patterns more similar to humans [40].

In biopharmaceutical production, microalgae are a potential biofactory for antibodies, nanobodies, cytokines, antimicrobial peptides, vaccines, hormones, and enzymes [41,42]. A comprehensive list of proteins expressed in microalgae with industrial and biopharmaceutical applications in animals and humans has been reported in different species. Barolo et al., 2020 extensively describe proteins produced and the microalgae used in their essays [40].

Due to the industrial interest in microalgae cultivation, developing highly specialized systems focused on efficient and low-cost cultivation showed progress over the last decade [43–47]. Microalgae culture systems are classified into two types: open-type photobioreactors (raceways) and closed-type photobioreactors (PBR) [48]. These present systems differ in their design; however, their main concerns are the correct exposure to light, nutrients, temperature, and proper O_2 and CO_2 management [48]. Both designs are widely used. Therefore, producing biomass for recombinant proteins is a logical step. Some species considered possible expression platforms are *C. reinhardtii*, *C. vulgaris*, *C. ellipsoidea*, *D. salina*, *P. tricornutum*, and *N. oculata* [14,40,49].

Genetic engineering on microalgae proved potential for protein expression at the industrial level [18,40,50,51]. In addition, assays with microalgae species led to new protein expression platforms with distinct and innovative characteristics. Recently, *Dunaliella* sp. is one of the models proposed for study in protein expression [28,52–55]. The following subheadings describe the biological and industrial characteristics of *D. salina*.

4. *Dunaliella* sp. as a Production and Delivery Vehicle for Antigens

4.1. General Features

Dunaliella sp. is a unicellular, halophilic, biflagellate, naked green alga *Phylum Chlorophyta*, Class *Chlorophyceae*, order *Volvocales*, family *Polyblepharidaceae* with a total of 29 species, as well as several varieties and forms [56,57]. *D. salina* was first described in 1905 [58]. The genus *Dunaliella* sp., named in honor of Michel Felix Dunal [59] is the richest natural source of βcarotene, violaxanthin, neoxanthin, zeaxanthin, and lutein with the function of photoprotective to the high irradiance [60,61] and vitamins, antioxidants, polyunsaturated fatty acids, minerals, and enzymes [62]. Recently, the study of this species raised interest in its protein content, which ranges from 50 to 80% (dry weight), also for the content of essential amino acids, which is higher than recommended by the Food and Agriculture Organization of the United Nations (FAO) [36]. *Dunaliella* sp. presents different forms (spherical, pyriform, fusiform, ellipsoid), sizes from 5 to 25 μm in length and from 3 to 13 μm in width, also contain a single chloroplast, chlorophylls a and b, and organelles observed in green algae: membrane-bound nucleus, mitochondria, vacuoles, Golgi apparatus, and an eyespot and elastic plasma membrane covered by a mucus surface coat with the capacity of shrinks or swells according with the hypertonic and hypotonic conditions [62,63]. *D. salina*, similar to other microalgae, undergoes a complex life cycle, cellular divisions by lengthwise division in the motile state (vegetative cells), but also presents sexual reproduction (sexual zygote formation) [56].

Several species of *Dunaliella* sp. are observed in high salt concentrations, classifying them as halophilic organisms. However, some species thrive in freshwater [64,65] as well as over a wide pH range, from pH1 (*D. acidophila*) to pH11 (*D. salina*) [66]. The high capacity to adapt to different concentrations of salinity (3 to 31%) and temperature range (<0 °C to >38 °C) make *Dunaliella* sp. a unique and highly resistant eukaryotic organism [67]. Because of these characteristics, various species of *Dunaliella* sp. have been isolated in diverse ecosystems over the world [68]. Because of all high-value features, *Dunaliella* sp. can be considered a promisor recombinant expression system [49,63,69,70]; between these features are included: the capacity to grow in a wide range of salt concentrations which can prevent contamination of the culture [66], transcriptional modifications [42,50,71], and lacking a rigid cell wall, facilitating genetic transformation procedures as well as the extraction during downstream processing [63].

4.2. Production Aspects

D. salina culture media have wide ranges in salts and pH (6 to 23 % of NaCl, and pH 6 to 9) [56,72]. Optimal grown conditions are between 2 and 8% salt; a high salt concentration affects the growth rate in some cases. Under the best conditions for growth, the division rate can go from 0.5 to 1.22 divisions per 24 h [56]. Based on several studies, an average concentration of salt in the culture media of *D. salina* (12%) and *D. viridis* (6%) are the optimal salt concentration for growing [73,74]. However, other strains present different growth conditions [75]. In general, the culture conditions are a temperature of 25 ± 2 °C under the white fluorescent light of 52.84 µmol photons m^{-2} s^{-1} without aeration in stirring at 110 rpm/min in the orbital shaker [67,76]. The efforts focus on developing an efficient condition for growing under laboratory and industrial requirements [60,77–79]. Media for growth of *D. salina* suggested include: modified Johnson's medium, Erdschreiber's medium, Guillard's F/2 medium, modified ASP medium, and enriched seawater [76,80].

4.3. Culture Systems of D. salina

Mass culture of microalgae is reported in systems such as open ponds, circular ponds, raceway ponds, cascade ponds, large bags, tanks, heterotrophic fermenters, and several kinds of closed PBR [81,82]. In the case of *D. salina*, it can be grown under controlled conditions in selective media and biological contamination-free [83]. Currently, PBR implementation for *Dunaliella's* intensive culture is widely reported [63,84]. PBR has several advantages compared with other culture systems, such as higher yield, cleaner product, and concentration of secondary metabolites. In general, there are three types of PBR: flat plate bioreactors, tubular PBR, and ultrathin immobilized configurations [81,82,85]. The use of PBR for *Dunaliella* sp. culture has been focused on secondary metabolite production; however, their possible use as a PBR system for recombinant protein production is also feasible [86–89].

4.4. Genetic Engineering Tools Applied to D. salina

Among the genetic manipulation techniques reported for *Dunaliella* sp. include electroporation [90,91], particle bombardment [92], glass beads [93], lithium acetate/polyethylene glycol (PEG)-mediated method [55], and *Agrobacterium*-mediated method (agroinfiltration) [52]. In general, all techniques present a range of advantages and disadvantages for their use in microalgae [42]. Expression-efficacy depends on codon optimization, protease activity, protein toxicity, and transformation-associated genotypic modification [94]. In the case of *D. salina*, some of the technical approaches reported for nuclear transformation include LiAc/PEG-mediated method, glass bead method, and agroinfiltration protocol. In the case of chloroplast transformation, the most recommended method is particle bombardment. The possible use of other techniques, ultrasonic delivery [95], ultraviolet laser microbeam [96], and aerosol gene delivery [97], allows the opportunity to explore new approaches to achieve the best form of genetic manipulation in *Dunaliella* sp. These methods present a relatively low level of transformation and differences in their practicality and

repeatability; however, most of these are focused on the expression of reporters, selecting genes, therapeutic application, and production of viral proteins. Viral antigens, including hepatitis B surface antigen (HBsAg), yielding 3.11 ng/mg of total soluble protein by transforming electroporation protocol, white spot syndrome virus (WSSV) VP28, yielding 3.04 ng/mg of soluble protein by gene glass beads transformation [42], and hemagglutinin influenza virus yielding 255.5 µg/2 g wet weight by agroinfiltration protocol [52]. Despite the low expression levels [98], these assays are focused on determining the ability of this system to express viral proteins, so yields require other approaches.

One of the most promising systems for expressing recombinant proteins in *D. salina* is the agroinfiltration protocol mediated by *Agrobacterium tumefaciens* [52,99,100]. This protocol is based on the ability of *A. tumefaciens*, an indirect method, to transfer exogenous desoxyribonucleic acid (DNA) to plant cells through a bacterial conjugation system (Type IV secretion system (T4SS) and protein-DNA complexes) [101]. Plants are naturally affected by *A. tumefaciens*, including angiosperms and gymnosperms [102]. Briefly, *A. tumefaciens*, a bacterium present in the soil, moves towards the wound upon detecting phenolic compounds from a wounded plant, adheres, and begins to transform plant cells by inducing the transcription of virulence genes present in a plasmid called Tumor-inducer (Ti-DNA). Ti-DNA, together with the bacterial virulence proteins (VirD1, VirD2, VirE2), induces the transcription, processing of transfer DNA (T-DNA), and integration into the plant genome. Transferential DNA with *A. tumefaciens* requires the insertion of a gene of interest in T-DNA present in Ti-DNA for its insertion into the genome of the nucleus of the cells [102,103]. The random insertion observed in this method suggests a non-homologous recombination mechanism [104]. Since the first experiments for the elaboration of transgenic plants using *A. tumefaciens* in 1983 [102], significant advances in understanding the T-DNA insertion process, protocols, and experimentation in model plants, including in *D. salina* have been achieved.

4.4.1. Selection Markers and Reporter Genes

In the case of *D. salina*, selection markers, similar to antibiotics, require a different approach [92], because this microalga presents inherent resistance to a variety of antibiotics streptomycin, kanamycin, hygromycin (600 µg mL^{-1}) [90], spectinomycin (1200 mg L^{-1}) [100]. The use of chloramphenicol (60 µg mL^{-1}) [90], and zeocin 5 mg L^{-1} [91] are feasible for the selection of transformed cells. Another selection gen for *D. salina* reported is herbicide phosphinothricin (PTT) (0.5 µg mL^{-1}) [105]. As reporter genes, the *gus* reporter gene [92], and the enhanced green fluorescent protein (EGFP), are applied [106].

4.4.2. Promoters and Enhancers for *D. salina*

The use of an efficient promoter is fundamental for the selection of any host. Among the principal exogenous promoters developed for *D. salina* are cauliflower mosaic virus 35, CaMV35S [55,91,92], Ubiquitin (Ubil), Ubil-Ω, CaMV35S-Ubil, CaMV35S-Ubil-Ω, endogenous promoters of actin gene [105], and glyceraldehyde-3-phosphate dehydrogenase [107], with high driving activity for gene expression. Inducible promoters include driving expression under a variety of sodium chloride concentrations (duplicated carbonic anhydrase 1 (DCA1)) [108], driving in the presence of nitrate, and inhibiting gene expression in the presence of ammonium ions (promoter *NR* gene) [109]. In the case of enhancers, a correct selection could prevent the gene silencing effect due to the position effect [110]. Enhancers reported on *D. salina* are the matrix attachment regions (MARs) [71] and 5′ leader sequence of tobacco mosaic virus RNA (Ω element) combined with promoters Ubil and CaMV35S-Ubil [90]. Studies suggest by MARs as a regulatory sequence increase expression levels [111,112], as well as stabilize their transcription processes [113,114] in transgenic offspring. Wang et al., 2005 demonstrated an increase in *CAT* gene expression 4.5-fold compared with other regulatory sequences through MARs in transformed *D. salina* [115,116]. Enhancers from other systems and genetic screening by selected UV-induced mutations with highly expressed nuclear transgenes open new possibilities [42,117]. In the case of

nuclear protein expression, random integration sites, RNA silencing, a compact chromatin structure, and non-conventional epigenetic effects [28,90,110], are possible factors affecting protein yield. Strategies to address these issues include surrounding insertion-site sequences analysis [118–120] and further study of regulatory sequences [111–114,121,122].

Currently, chloroplast transformation protocols in *D. salina* require a new approach due to potential observed in other organisms [42,123–126]; evaluation of regulatory sequences [42] and chloroplast transformation strategies [51,95,127–131] are possible improvement solutions. The publication of the complete sequence of the *D. salina* chloroplast genome (ptDNA) [132] encourages the development of more efficient transformation methods.

4.5. Advances in Dunaliella Transformation for Recombinant Biopharmaceutical Production

In general, expression in nucleus *D. salina* cells is focused mainly on reporter genes such as *β-glucuronidase* gene [50], enhanced green fluorescent protein [107], and selection markers such as phosphinothricin acetyltransferase under promoter DCA1 [110], chloramphenicol acetyltransferase [66], and zeocin resistance protein [105]. However, the expression of commercial value proteins is reduced [108,109], including immunogens [50]. Although several results [28,42,52,90], none of these proteins has led to the generation of products at the industrial level. According to findings, the *Dunaliella* system can be used in an approach for industrial applications, in particular in antigen production. The chloroplast is also an attractive expression protein system in microalgae due to advantages such as directed integration of genes via homologous recombination [133], high-level expression, organization of transgenes into operons, and no epigenetic interference [134,135], as previously reported [123,126]. Although there are few reports of expression in the chloroplast of *D. salina* [136], other systems such as *Chlamydomonas* [131], demonstrated that the use of chloroplast for the expression of recombinant proteins could be a proposal for proteins of commercial value. The purpose of new promoters and construction of expression vectors for *D. salina* chloroplast transformation is the following step [42] (Figure 1).

4.6. Immunological Aspects in Mucosal Vaccination with D. salina

Vaccination is one of the leading practices in medicine to control and prevent the vast majority of infectious-contagious diseases [137], based on the correct presentation of an antigen to the immune system. For this, it is necessary to determine the route of application, components of the formulation (adjuvant), type of immune responses, the dose required, and type of vaccine, either first group: (i) live attenuated vaccines (e.g., smallpox, yellow fever, measles, mumps, rubella, and chicken pox), or second group: (i) subunit vaccines (e.g., a vaccine against recombinant hepatitis B), (ii) toxoid vaccines (e.g., vaccines against diphtheria and tetanus), (iii) carbohydrate vaccines (e.g., vaccines against pneumococcus), and (iv) conjugate vaccines (e.g., vaccines against *Haemophilus influenzae* type B) [138].

Vaccination protocol and the immune system play a decisive role in correct immune response [139], particularly the immune system on the mucosal surface [140]. In general, the mucosal immune system presents highly specialized MALT, responsible for antigen presentation for the generation of an efficient mucosal immune response [24]. Due to the presence of these specialized tissues, mucosal administration of antigens demonstrated efficiency in wide pathologies [141], including influenza virus [142].

The expression of subunit vaccines in microalgae presents a convenient mucosal administration option with advantages including minimum processing before application [143] relatively low cost (<$1mg protein) in contrast to synthetic peptide antigen range between $35 and $95/mg peptide [143], algal cell wall appears sufficient to reduce antigen degradation by digestive system (bio-encapsulated) [98], subsequently broken down by digestive enzymes and commensal bacteria, and consequently, the recombinant proteins are released to be in contact with MALT [144,145]. Therefore, these drawbacks in consideration for oral vaccination are overcome by this expression model, as previously reported [28,52]. Considering that more than ten milligrams of an average subunit vaccine are required for oral administration (1000 times more than is necessary for an injected

route) [27], it is estimated that hundreds of grams of recombinant tissue are needed to stimulate an immune response. Nevertheless, increasing the concentration of the antigen by freeze-dried microalgae without losing antigenic capacity [27], antimicrobial activity [146], and immunomodulatory compounds naturally present in certain species of microalgae (*Dunaliella* sp.) exert synergistic effects with the vaccine formulation [147,148].

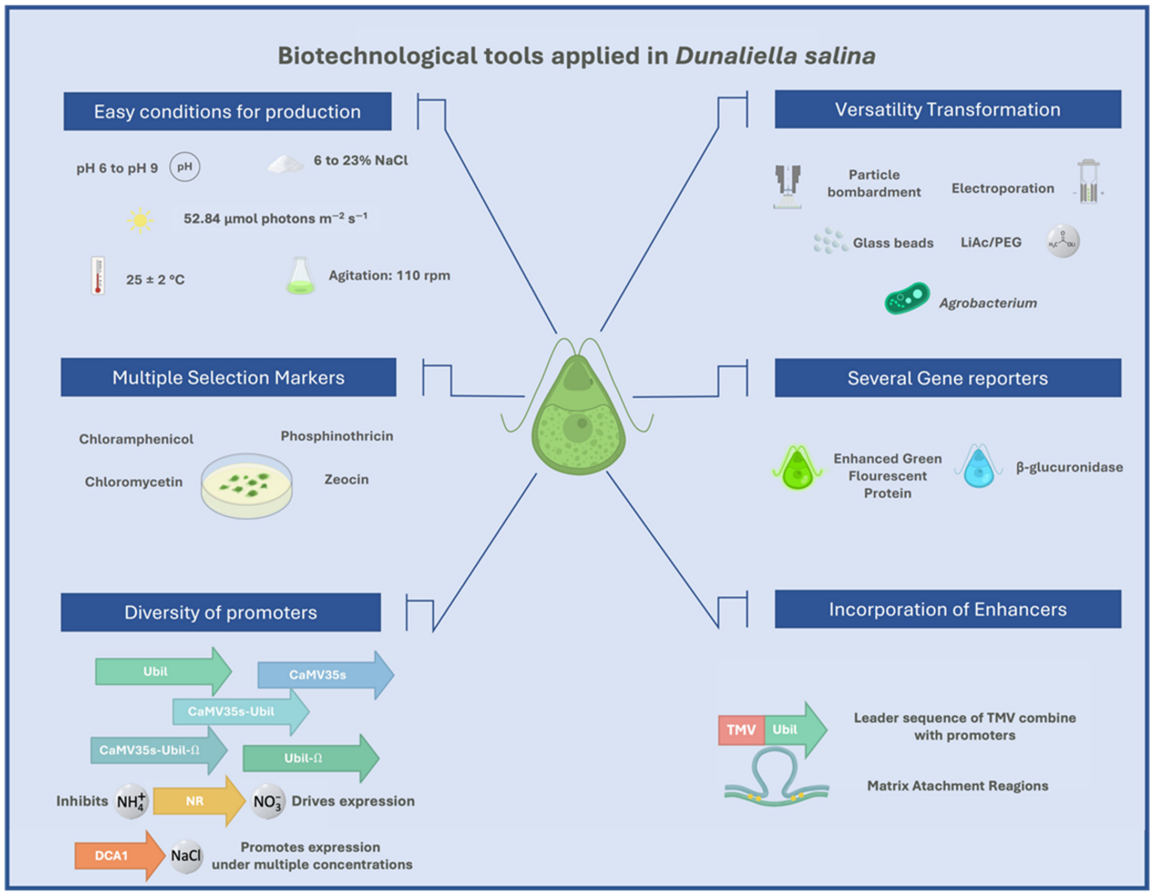

Figure 1. Biotechnological tools applied in *Dunaliella salina*.

5. Prospective View

Improvement in high-value recombinant protein expression systems encourages the research of several models with different advantages. Added to this, the cost of escalation and characteristics of post-translational modifications [14,149,150] are also aspects to consider.

In the case of *D. salina*, research showed this expression system as a practical solution for the production of different types of recombinant proteins and promises to be a production method based on the advantages of culture, transformation method, immunomodulatory compounds, glycosylation patterns, and natural encapsulation. The status of the investigation on the genetic manipulation of *D. salina* is in the early stages; however, the data suggest it can be a practical, tangible option for the vaccine industry. The mucosal administration of antigens (oral, ocular, or intranasal) presents requirements for a correct application, which not all recombinant protein expression systems can meet [14]. Hence, microalgae have advantages over other systems.

In addition to previously described advantages, microalgae are organisms capable of photosynthesizing by capturing environmental inorganic carbon (CO_2) [151]. As is known, microalgae are efficiently photosynthetic microorganisms [152]. Therefore, it is not only necessary to consider the advantages of a system such as microalgae without considering other social and environmental factors for developing a protein expression platform at an industrial level.

More research is needed on several details of the heterologous protein expression microalgae model, including regulatory sequences, codon-optimization [51,153], and efficient expression vectors [94,154,155]. Nevertheless, data available allows for considering *D. salina* as a protein expression system with potential for antigen production and its mucosal administration.

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