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

ANÁLISIS GENÓMICO Y PROTEÓMICO DE SEROTIPOS AMBIENTALES DE *Salmonella enterica*

Por:

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RESUMEN

Salmonella es el agente causal de la salmonelosis tifoidea y no tifoidea, siendo la principal causa de enfermedad diarreica a nivel mundial. La transmisión de esta bacteria se produce principalmente a través del consumo de alimentos y/o agua contaminados. *Salmonella* cuenta con más de 2600 serotipos potencialmente patógenos y se encuentran ampliamente distribuidos en diversos ambientes, como los ambientes acuáticos. En el Valle de Culiacán, Sinaloa, se han reportado una gran variedad de serotipos no tifoideos de esta bacteria en los ríos Culiacán, Tamazula y Humaya, siendo los serotipos Oranienburg, Montevideo y Pomona los de mayor prevalencia. La permanencia y sobrevivencia de estos serotipos en estos ambientes es debido a su capacidad adaptativa que les permite responder ante las condiciones de estrés, lo cual puede conllevar a aumentar su patogenicidad. En este sentido es necesario entender los mecanismos genéticos y metabólicos que están involucrados en estos fenómenos, así como, desarrollar una estrategia para la prevención y el control de la enfermedad que pueden llegar a causar estos serotipos. Por lo tanto, el objetivo del presente estudio fue realizar un análisis genómico y proteómico para identificar genes relacionados con la patogenicidad y virulencia de serotipos ambientales y clínicos de *Salmonella* Montevideo y Pomona. Asimismo, el diseño *In silico* de una vacuna multi-epítopo candidata contra *Salmonella* no-tifoidea basada en proteínas de respuesta a estrés del serotipo Oranienburg. Se encontró que cepas ambientales presentaron mayor contenido genético relacionado a resistencia a antibióticos comparado con las clínicas. Adicionalmente se identificaron genes relacionados al sistema de secreción tipo cuatro exclusivamente en cepas ambientales. También, se identificaron genes relacionados a sistemas de transporte de fosfotransferasas para N-acetil-D-glucosamina, manitol, manosa, y galactosa, indicando versatilidad metabólica en cepas ambientales y clínicas. Para el diseño *In silico* de la vacuna se utilizaron epítopos provenientes de las proteínas del sistema de respuesta a estrés por falta de nutrientes (SSR), los cuales presentaron características óptimas como antigenicidad, no toxicidad, y no alergenicidad. Además, mediante análisis de acoplamiento molecular se determinó que la vacuna formó interacciones favorables y fuertes con receptores inmunológicos tipos Toll. La simulación inmunológica mostró una estimulación de células B y antígenos IgG e IgM por parte de la vacuna. La combinación de ambos análisis permite proponer y desarrollar nuevas estrategias

para control y/o prevención de la enfermedad que puede causar *Salmonella.*

Palabras clave: *Salmonella*; *In silico*; Genoma; Virulencia; Resistencia a antibióticos; Metabolismo; Vacuna multi-epítopo

ABSTRACT

Salmonella is the causative agent of typhoid and non-typhoid salmonellosis and is the leading cause of diarrhoea worldwide. The transmission of this bacterium occurs mainly through the consumption of contaminated food and/or water. *Salmonella* has more than 2600 potentially pathogenic serotypes and is widely distributed in various environments, such as aquatic environments. In the Culiacan Valley, Sinaloa, a wide variety of non-typhoidal serotypes of this bacterium have been identified in the Culiacán, Tamazula and Humaya rivers, with Oranienburg, Montevideo and Pomona being the most prevalent. The persistence and survival of these serotypes in these types of environments is due to their adaptive ability that allows them to respond to the stress conditions of these environments, which can also lead to increase their pathogenicity. In this sense, it is necessary to understand the genetic and metabolic mechanisms involved in these phenomena, as well as to develop a strategy for the prevention and control of the disease that these serotypes can cause. Therefore, the aim of the present study was to perform a genomic and proteomic analysis to identify genes related to the pathogenicity and virulence of environmental and clinical serotypes of *Salmonella* Montevideo and Pomona. Moreover, the *In silico* design of a multi-epitope candidate vaccine against non-typhoidal *Salmonella* based on Oranienburg serotype stress response proteins. It was found that environmental strains were found to present higher genetic content related to antibiotic resistance compared to clinics. Additionally, genes related to the type four secretion system were identified exclusively in environmental strains. Also, genes related to phosphotransferase transport systems for N-acetyl-D-glucosamine, mannitol, mannose, and galactose were identified, indicating metabolic versatility in environmental and clinical strains. For the *In silico* vaccine design, epitopes from the starvation stress response system (SSR) proteins were used, which presented optimum characteristics such as antigenicity, non-toxicity and non-allergicity. Furthermore, molecular docking analysis determined that the vaccine formed favorable and strong interactions with immune receptors type toll. Immunological simulation showed stimulation of B cells and IgG and IgM antigens by the vaccine. The combination of both analyses allows proposing and developing new strategies for the control and/or prevention of the disease that can cause *Salmonella*.

Key words: *Salmonella*; *In silico*; Genome; Virulence; Antibiotic resistance; Metabolism; multi-epitope vaccine

1. SINOPSIS

1.1. Justificación

Las enfermedades diarreicas constituyen una causa importante de morbilidad y mortalidad a nivel global. Se ha identificado que *Salmonella* es el agente patógeno predominante en la mayoría de los casos reportados de estas enfermedades anualmente, siendo responsable de aproximadamente el 41% de las defunciones asociadas a enfermedades diarreicas (Besser, 2018). En este sentido, el Centro para el Control y Prevención de Enfermedades (CDC, por sus siglas en inglés) en Estados Unidos reportan aproximadamente 1.35 millones de enfermedades y 420 muertes al año, causadas por esta bacteria (CDC, 2023). Estas enfermedades son provocadas por la ingestión de alimentos o agua contaminados con *Salmonella* (WHO, 2018). Por ejemplo, se han identificado alimentos procesados derivados de carne de res, cerdo, pollo, mariscos y huevo como fuentes de contagio. No obstante, también se ha documentado el riesgo asociado al consumo de productos frescos, como ciertas hortalizas (Pang *et. al.*, 1995; Scherer, 2001; CDC, 2023).

Salmonella puede dispersarse y ser transmitida a través de las heces de sus hospedadores, tanto humanos como animales, pudiendo alcanzar ambientes acuáticos como ríos, lagos y otros humedales. Estos ambientes representan el principal medio para la transmisión de este microorganismo. Además, el arrastre de materia orgánica debido a las precipitaciones, las actividades agrícolas y ganaderas, así como los desagües, son también factores que favorecen la introducción de la *Salmonella* en este tipo de entornos (Winfield y Groisman, 2003).

Sin embargo, cuando *Salmonella* se encuentra expuesta en este tipo de ambientes, su sobrevivencia se ve comprometida debido a que, al no estar en su habitat natural, enfrenta diversos factores como éstres ósmotico, variaciones de temperatura, variaciones de pH y principalmente la falta de nutrientes especificamente la falta de fuentes de carbono (C) (Winfield y Groisman, 2003). Para esto, *Salmonella* cuenta con diferentes estrategias adaptativas que permiten responder ante tales condiciones y sobrevivir por periodos largos de tiempo. Por ejemplo el sistema de respuesta a estrés por falta de nutrientes (“SSR” por sus siglas en ingles), el cual induce cambios a nivel genético y fisiológico (Spector, 1998). Este sistema inicia su expresión durante las primeras 2-5 horas de exposición a un ambiente con baja disponibilidad de nutrientes y es regulado mediante los factores

sigma *rpoS*, *rpoE* y los genes *crp* y *cyaA* que codifican para las proteínas señalizadoras cAMP y ppGpp (Spector y Kenyon, 2012). Uno de los efectos inducidos por el SSR es estimular la activación de genes asociados con rutas metabólicas alternativas, como la Entner-Doudoroff. Esta ruta es empleada en situaciones de estrés, presenta un menor requerimiento energético y enzimático para la bacteria, y posibilita la utilización de diferentes fuentes de carbono (Spector y Kenyon, 2012; Flamhölz *et. al.*, 2013). La expresión del SSR en *Salmonella* conlleva la activación de diferentes sistemas de transporte de fosfotransferasas, ("PTS" por sus siglas en inglés), a través de la regulación de genes específicos para cada sistema y de los genes centrales *pstI* y *pstH*. Estos codifican proteínas que facilitan la fosforilación de nutrientes (Postma *et. al.*, 1993). La capacidad de adaptación y supervivencia de *Salmonella* en diversos ambientes está estrechamente ligada al contenido genético presente, el cual puede variar entre serotipos. Se ha observado que *Salmonella* puede colonizar los sedimentos de ambientes acuáticos, donde se ha detectado la presencia de este microorganismo junto con otras enterobacterias. Este hallazgo sugiere que los mecanismos de adaptación y respuesta a condiciones adversas mencionados anteriormente influyen en el establecimiento de *Salmonella* en este nicho ecológico (Hassard *et. al.*, 2016).

La capacidad de *Salmonella* para adaptarse bajo condiciones de estrés está relacionada con su habilidad para llevar a cabo transferencia genética horizontal de genes (“HGT” por sus siglas en inglés), lo cual permite obtener material genético nuevo de otra bacteria, incluyendo genes de virulencia y de resistencia a antibióticos (Dos Santos *et. al.*, 2021). Este proceso es crucial para la evolución de *Salmonella*, y puede derivar en consecuencias severas en la salud del hospedador (Pradham y Negi, 2019; Li *et. al.*, 2021). Para desarrollar medidas efectivas que permitan controlar la dispersión de esta bacteria y reducir las enfermedades que puede ocasionar, es esencial comprender las variaciones genéticas e identificar los factores de virulencia asociados con diferentes serotipos. La capacidad de *Salmonella* para establecerse y sobrevivir en diversos ambientes incrementa su potencial para causar enfermedades severas especialmente cuando ésta pasa del ambiente a un hospedador (Chakroun *et. al.*, 2017; Ramírez *et. al.*, 2018; Dos Santos *et. al.*, 2021). En este sentido, el presente estudio tuvo como objetivo analizar genómica y proteínicamente serotipos ambientales de *Salmonella enterica*. El análisis genómico permitió identificar genes de virulencia, metabolismo y resistencia a antibióticos, mientras que el análisis proteómico permitió identificar proteínas esenciales para el desarrollo de una medida de prevención para la enfermedad causada por serotipos ambientales de *Salmonella* no tifoidea.

1.2. Antecedentes

1.2.1. Características Generales de *Salmonella*

El género *Salmonella* se encuentra dentro de la familia Enterobateriaceae, son bacilos Gram negativos, presentan respiración anaerobia facultativa, no desarrollan esporas y tienen movilidad debido a la presencia de flagelos de tipo perítrico (Pedraza *et. al.,* 2014). Este género se divide en dos especies: *bongori* y *enterica*, esta última está dividida en seis subespecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* e *indica* (Brenner *et. al.,* 2000), destacando la subespecie *enterica* ya que cuenta con más de 2,600 serotipos reportados (CDC, 2013). La mayoría de estos serotipos pueden crecer en temperaturas que oscilan entre los 5°C y 47°C, siendo el rango de temperatura óptima entre 35°C y 37°C, mientras que el pH óptimo para el crecimiento de este microorganismo oscila entre 6.5 y 7.5 aunque también puede crecer en un rango de entre 4 y 9 (Pedraza *et. al.,* 2014).

Salmonella es la principal causante de enfermedades transmitidas por los alimentos y agua a nivel mundial (WHO, 2018). El Centro de control y prevencion de enfermedades ("CDC" por sus siglas en inglés) en los Estados Unidos de América y la autoridad Europea de Seguridad Alimentaria ("EFSA" por sus siglas en inglés) reportan más de 1,000,000 y 100,000 casos de infecciones por *Salmonella* no tifoidea respectivamente (EFSA, 2015; CDC, 2023), mientras que en México se reportan aproximadamente 70,000 casos de infecciones por esta bacteria cada año (DGE, 2020). Esta bacteria se encuentra distribuida ampliamente en la naturaleza, en donde encuentra como principal reservorio a los animales de sangre caliente y fría como pueden ser aves de corral, cerdos, vacas, aves silvestres, perros, roedores, tortugas, gatos etc., y es mediante la excreta de estos hospederos, que puede llegar a diseminarse en diversos ambientes (Percival *et. al.,* 2004).

1.2.2. Adaptación y Respuesta de *Salmonella* al Medio Ambiente Externo

Solo en condiciones de laboratorio se puede lograr el crecimiento y desarrollo ideal de *Salmonella*, con una gran cantidad de nutrientes disponibles, temperatura, pH y oxígeno constante, sin factores de estrés que puedan afectar su sobrevivencia (Spector y Kenyon, 2012). Aunque en un hospedero también pueden existir condiciones favorables, esta bacteria requiere responder y evadir algunos factores de estrés durante su patogénesis. Estos factores estresantes incluyen la acidez del estómago, cantidades reducidas de oxígeno, sales biliares, péptidos antimicrobianos y competencia por la utilización de nutrientes con microorganismos residentes de la microbiota intestinal (Savageau, 1983; Rychlik y Barrow, 2005). Asimismo, uno de los principales factores de estrés que *Salmonella* puede enfrentar es la constante variación de temperatura, pH y concentraciones de nutrientes en un ambiente externo (Spector 1998). En condiciones ideales para su crecimiento, *Salmonella* consume principalmente nutrientes como glucosa, manosa y glucosa 6-fosfato. Sin embargo, en condiciones externas, la disponibilidad de estos nutrientes es limitada, lo que significa que la sobrevivencia de *Salmonella* dependerá de su capacidad para detectar y responder ante estas condiciones, asi como de utilizar sustratos alternativos (Winfield y Groisman, 2003; Götz y Goebel, 2010). Medrano-Félix y colaboradores (2017) reportaron que los serotipos ambientales de Oranienburg y Saintpaul, en condiciones de agua de río, consumieron altos niveles de fuentes de carbono alternas o ambientales, como N-acetil-D-glucosamina, ácido D-glucosaminico, ácido D-galactonico y manitol, en comparación con el serotipo control clínico Typhimurium. Esto demuestra la alta capacidad de adaptación y pre-acondicionamiento de los serotipos debido a que el SSR se activa cuando hay poca disponibilidad de nutrientes esenciales como la glucosa en el ambiente (Spector, 1998). La bacteria experimenta una serie de cambios genéticos y fisiológicos como resultado de la activación de este sistema. Estos incluyen (1) mayor afinidad para utilizar nuevos sistemas de transporte y nuevos sustratos, así como un proceso de "scavenging" para obtener nutrientes de otras bacterias presentes en el ambiente, (2) el uso de enzimas de "canibalismo" para consumir componentes celulares como proteínas de ribsomas y lipidos de la envoltura. (3) enzimas que metabolizan fuentes de carbono alternativas o inusuales, (4) proteínas que condensan el cromosoma para protegerlo de daños, (5) enzimas que modifican la membrana celular y (6) enzimas que evitan o reparan el daño celular causado por el estrés ambiental (Spector, 1998; Almirón *et. al.*, 1992; Kenyon *et. al.*, 2010; Spector y Kenyon, 2012).

Las moléculas señalizadoras de adenosin monofosfato 3',5' cíclico (cAMP) con su proteína receptora (CRP), guanosina 3',5' bifosfato [(p)ppGpp] y los factores sigma *rpoS* y *rpoE* regulan

estos cambios provocados por el SSR. La expresión de los genes *cyaA* y *crp* regula los niveles de cAMP y CRP en la célula. Estos genes actúan en respuesta a la falta de nutrientes como la glucosa bifosfato [(p)ppGpp], y los factores sigma *rpoS* y *rpoE* (Spector y Kenyon, 2012). Además, activan o reprimen la expresión de otros genes relacionados con el transporte y el uso de fuentes de carbono alternativas como el gluconato, el manitol o la N-acetil glucosamina (Spector, 1990; Görke y Stülke, 2008). Sawant y Shashidar (2020) demostraron la importancia de *crp*, ya que una mutación en este gen puede alterar de manera negativa la resistencia a condiciones de estrés como radiación, calor y estrés oxidativo en *Salmonella* Typhimurium, además de generar varios fenotípos relacionados con el metabolismo de fuentes de carbono como D-mannosa, D-glicerol y D-manitol, demostrando que este gen funciona como un regulador global de la respuesta a estrés. Por otro lado, se ha demostrado que la expresión de los genes *relA* y *spoT* propician el incremento en los niveles de la proteína (p)ppGpp, y que su función es unirse a un ARN polimerasa alterando la transcripción de sitios promotores relacionados a la respuesta a estrés (Sharma y Chatterji, 2010). Por otro lado, Kenyon y colaboradores (2007) mencionan que la escasez de glucosa, un sustrato común para la nutrición de *Salmonella*, puede propociar la activación del SSR durante las primeras 2-5 horas bajo esta condición, y es regulado por el operón *stiC* el cual esta compuesto por los genes *yohC* y *pbpG*.

1.2.3. Sistemas de Transporte de Fosfotransferasas (“PTS”)

Los sistemas de transporte de fosfotransferasas (PTS) están compuestos principalmente de una proteína transmembranal y 4 proteínas solubles. La enzima EI y la proteína HPr son los componentes citoplásmicos generales de los PTS, las cuales están relacionadas con la utilización de los carbohidratos tanto en *Salmonella* como en otros microorganismos y los genes encargados de su codificación son *ptsI* y *ptsH* respectivamente, ambos pertenecientes al operón *ptsHI* (Postma *et. al.*, 1993; Deutscher *et. al.*, 2014). Por otro lado las proteínas EIIA, EIIB,EIIC y EIID son específicas para un carbohidrato o en algunos casos para algún grupo de carbohidratos relacionados. El funcionamiento de los PTS inicia mediante la autofosforilación de la enzima EI con fosfoenolpiruvato (PEP), enseguida se transfiere el grupo fosforilo a su residuo en la proteína

HPr, después se dona el grupo fosforilo a un residuo HPr en el domino A de una enzima EII. Posteriormente el grupo fosforilo se transfiere a un residuo de EIIB y por último a los carbohidratos durante su traslocación a través de la enzima EIIC. Esta modulación de los estados de fosforilación de proteínas PTS en respuesta a las condiciones nutricionales y el estado metabólico de la célula son la base para la señalización y regulación de los sistemas PTS (Figura 1) (Postma *et. al.*, 1993). Como parte del proceso de respuesta a estrés por falta de nutrientes el aumento del uso de sistemas PTS es uno de los cambios favorecidos por la activación de esta respuesta. En general, estos sistemas facilitan el transporte y la fosforilación de los carbohidratos disponibles para el consumo de las bacterias (Postma *et. al.*, 1993; Spector, 1998).

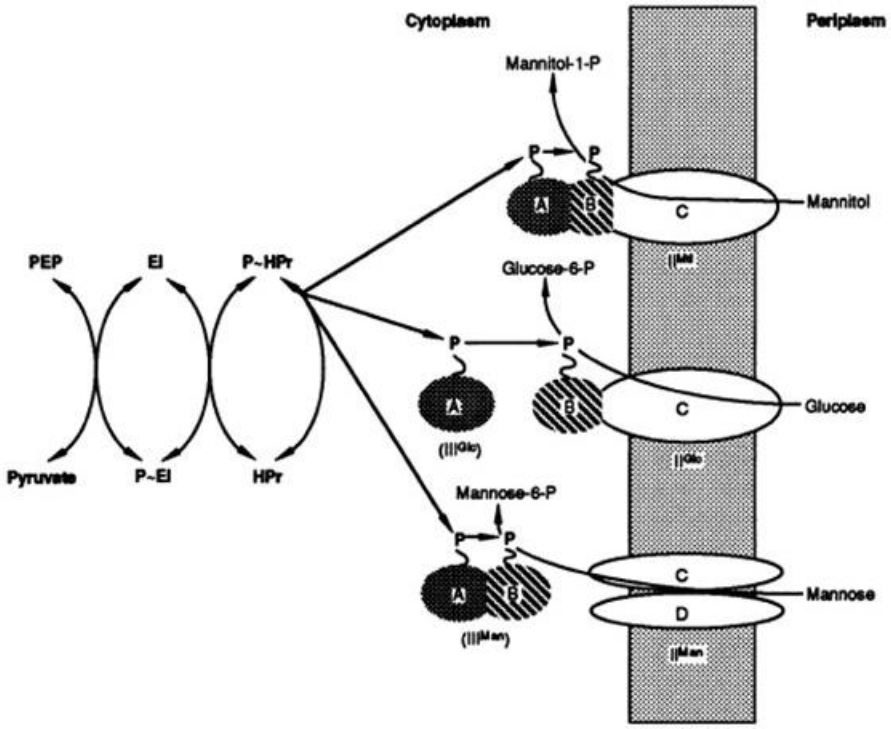

Figura 1. Organización del sistema de transporte PTS y sus componentes. EI y HPr son las proteínas generales para todos los sistemas PTS. En la figura se muestran tres EIIs: Específicas para manitol (Mtl), glucosa (Glc) y manosa (Man). Cada una contiene dos dominios hidrofílicos: IIA contiene el primer sitio de fosforilación (P-His) y IIB que contiene el segundo sitio de fosforilación. El dominio IIC se puede dividir en dos dominios (IIC y IID) IIMtl, IIGlc, IIGlc, IIMan son específicos para manitol, glucosa, y manosa respectivamente. P indica la forma fosforilada de las diversas proteínas (Postma *et. al*, 1993).

1.2.4. Ruta Alterna Entner-Doudoroff

La ruta Entner-Doudoroff (ED) fue descubierta en 1952 en la bacteria *Pseudomonas saccharophila*. Esta ruta se puede utilizar por diversas especies de bacterias Gram negativas y es una alternativa de la ruta Embden-Meyerhof-Parnas (Conway, 1992). *Salmonella* es capaz de utilizar esta ruta alterna, la cual está mediada por actividad enzimática que se encuentra en función de la fuente de carbono disponible y las necesidades de la célula bacteriana (Madigan *et. al.*, 1999). La ruta ED tiene dos enzimas exclusivas; la 6-fosfogluconato deshidrogenasa codificada por el gen *edd* y la KDPG aldolasa codificada por el gen *eda* (Figura 2). En esta ruta metabólica, el proceso comienza con la fosforilación de la glucosa para formar glucosa-6-fosfato (Moat *et. al.*, 2003). Posteriormente, la glucosa-6-fosfato se oxida a ácido-6-fosfoglucónico y, mediante deshidratación, se genera el 2-ceto-3-desoxi-6-fosfogluconato. Este compuesto se hidroliza, produciendo una molécula de ácido pirúvico y una de gliceraldehído-3-fosfato. La gliceraldehído-3-fosfato es metabolizada con enzimas compartidas con la glucólisis, lo que resulta en la formación de una segunda molécula de ácido pirúvico. Durante este proceso, se produce una molécula de ATP y una de NADPH como rendimiento energético (Götz y Goebel, 2010; Moat *et. al.*, 2003).

Enzima	Función
Gluconato-6-fosfato deshidrogenasa	Convertir gluconato-6-fosfato en 2-ceto-3-desoxi-6-fosfogluconato
KDPG aldolasa	Rompe el grupo aldol de 2-ceto-3-desoxi-6-fosfogluconato para formar piruvato y gliceraldehído-3-fosfato

Cuadro 1. Enzimas exclusivas de la ruta Entner-Doudoroff

Fuente: (Ortega y Rivas 2003)

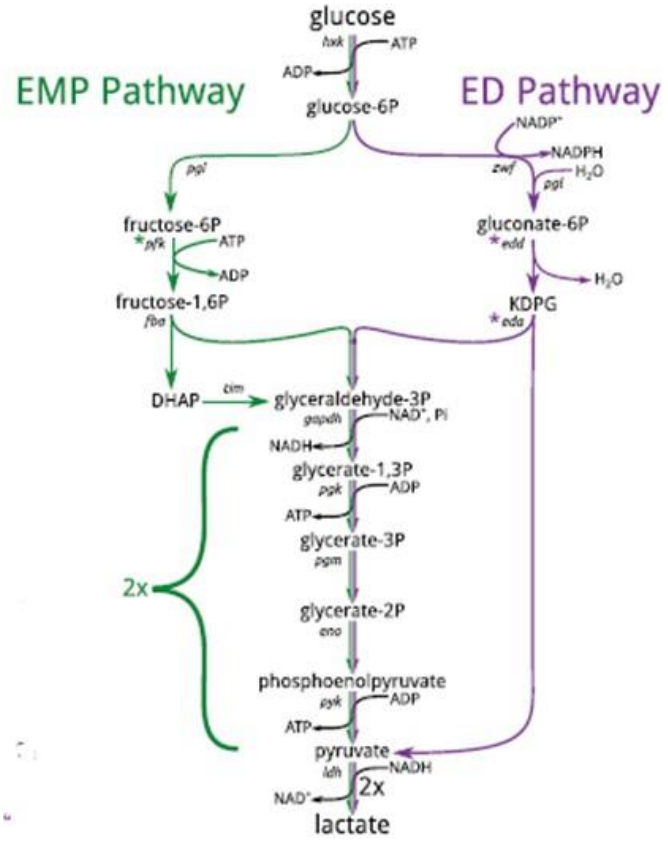

Figura 2. Esquema de las rutas metabólicas Embden-Meyerhof-Parnas y Entner-Doudoroff, (Flamhölz *et. al.*, 2013).

1.2.5. Ruta Central Embden Meyerhoff-Parnas

La ruta Embden-Meyerhoff-Parnas o también demonimada glucólisis (Figura 3), es considerada la ruta central en la mayoría de los seres vivos (Moat *et. al.*, 2003). Durante este proceso, una molécula de glucosa es descompuesta a través de una secuencia de reacciones catabólicas. Estas reacciones conducen a la formación de dos moléculas de piruvato, utilizando un intermediario conocido como fructosa 1,6 bifosfato. Además, se producen dos moles de ATP a partir de la glucosa, lo cual es fundamental para la producción de energía (Koneman y Allen, 2008).

En esta ruta metabólica, el grupo carbono 6 (C-6) de la glucosa se fosforila, dando lugar a la formación de D-glucosa-6-fosfato, que posteriormente se convierte en D-fructosa-6-fosfato. Esta última se fosforila en el carbono 1 (C-1), resultando en la formación de D-fructosa-6-bifosfato, con

el consumo de una molécula de ATP en cada reacción de fosforilación. A partir de la molécula D-fructosa-1,6-bifosfato se generan dos moléculas de tres carbonos: dihidroxiacetona fosfato y gliceraldehído-3-fosfato. En esta etapa se lleva a cabo la lisis que caracteriza a esta vía metabólica. La molécula de dihidroxiacetona fosfato experimenta una isomerización, dando lugar a la formación de una segunda molécula de gliceraldehído-3-fosfato (Voet y Voet, 2006). Cada molécula de gliceraldehído-3-fosfato se oxida y se fosforila por un fosfato inorgánico formando 1-3-bifosfoglicerato, y durante esta conversión a piruvato, se lleva a cabo la liberación de energía y esta se conserva mediante la fosforilación de dos moléculas de ADP a ATP. Se obtiene un rendimiento final de dos moléculas de ATP, ya que en la primera etapa se invirtieron dos ATP, además se conserva energía en esta etapa mediante la formación de dos moléculas de NADH por molécula de glucosa (Figura 3) (Voet *et. al.,* 2014). La ruta Entner-Doudoroff se considera como una alternativa a la ruta EMP. A pesar de que los esquemas de ambas rutas son similares, la diferencia principal radica en el intermediario metabólico de carbono 6 que sirve como sustrato para el anclaje del aldol. En la ruta EMP, el anclaje de la fructosa-1,6,-bifosfato se realiza a través de la fructosa bifosfato aldolasa para obtener una molécula de gliceraldehído-3-fosfato y dihidroxiacetona fosfato. Por otro lado, en la ruta ED, se lleva a cabo el anclaje del 2-ceto-3-desoxi-6-fosfogluconato (KDPG) mediante la enzima KDPG aldolasa para formar gliceraldehído-3-fosfato y piruvato (Conway, 1992).

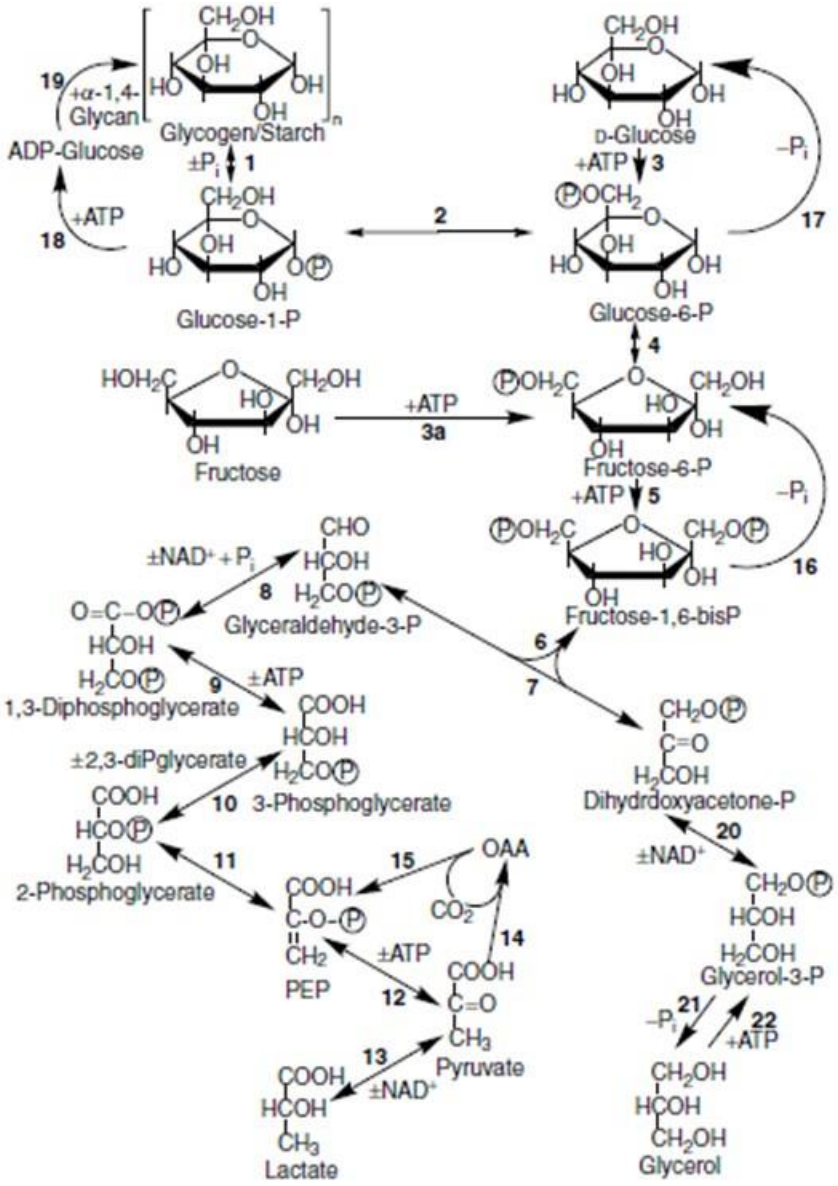

Figura 3. Esquema general de la ruta Embden-Meyerhoff-Parnas o Glucólisis (Moat *et. al.*, 2003)

1.2.6 Características del Genoma de *Salmonella*

El genoma de *Salmonella* tiene un tamaño aproximado de 4.8 a 5.2 millones de pares de bases (pb), conteniendo alrededor de 4,500 a 5,000 genes (Estrada-Acosta *et. al.,* 2013; Medrano-Félix *et. al.*, 2013; Casteñeda-Ruelas *et. al.,* 2017). Esta bacteria posee una amplia variedad de factores de

virulencia, como adhesinas, toxinas y sistemas de secreción, que le permiten colonizar e infectar células hospederas (Wagner y Hensel, 2011; Jajere, 2019). Además, *Salmonella* puede experimentar mutaciones genéticas y procesos de recombinación, las cuales favorecen la diversidad genética entre las especies (Didelot *et. al.,* 2011; Sun *et. al.,* 2014). Esta variabilidad genética contribuye a la adaptabilidad y a la persistencia de esta bacteria en varios ambientes además de a la virulencia, lo cual la hace un importante problema de salud pública.

La secuenciación del genoma completo ("WGS" por sus siglas en inglés) de *Salmonella* se ha convertido en una herramienta importante, ya que permite un mejor rastreo de los brotes epidemiológicos e identificación de fuentes de contaminación (Ibrahim y Morin, 2018; Koutsoumanis *et. al.,* 2019). A partir de la información contenida en el genoma de los diferentes serotipos de *Salmonella*, es posible identificar genes especificos responsables de virulencia y de resistencia a antibióticos, lo cual puede ayudar a desarrollar estrategias más específicas y eficaces para el control de este microorganismo (Brown *et. al.,* 2021).

1.2.6.1 Pan-genoma. *Salmonella* cuenta con un pan-genoma, el cual está compuesto por todos los genes compartidos por todas las cepas o serotipos (Chand *et. al.,* 2020) (Figura 4) . Este pan-genoma permite a la bacteria evolucionar rapidamente y adquirir nuevas caracteristicas, como resistencia a nuevos antibióticos o capacidades de sobrevivencia en condiciones de estrés (Laing *et. al.,* 2017). El pan-genoma también puede revelar la diversidad y evolución de *Salmonella*, así como su adaptación a diferentes entornos y hospedadores (Seif *et. al.,* 2018). En este sentido, Aguirre-Sánchez y colaboradores (2021) llevaron a cabo un estudio comparativo del pan-genoma de los serotipos Saintpaul, Thompson y Weltevreden de origen ambiental y clínico. Se logró identificar genes específicos vinculados al metabolismo y sistemas de secreción, los cuales variaban dependiendo del origen del aislamiento. Por otro lado, González-Torres y colaboradores (2023) analizaron el pan-genoma de 5 cepas de *S.* Oranienburg en donde se reportó un pan-genoma abierto con 5,594 familias de genes y 3,303 genes pertenecientes al genoma "*core*", además mediante este análisis fue posible detectar genes relacionados a ressitencia a antibióticos, metabolismo y virulencia.

Comprender el pan-genoma es fundamental para rastrear la aparición de cepas de *Salmonella* nuevas y más peligrosas (Lefébure *et. al.,* 2010; Brown *et. al.,* 2021). El análisis del pan-genoma

además puede permitir el desarrollo de nuevas vacunas y tratamientos, ya que provee informacion sobre factores genéticos que contribuyen a la virulencia y resistencia a antibióticos (Muzzi, 2007). Esta información se puede utilizar para identificar genes o proteínas específicos que son necesarios para la sobrevivencia de *Salmonella*, lo que resulta en una terapia más efectiva (Barh *et. al.*, 2020). Un pan-genoma se considera cerrado cuando contiene todos los genes presentes en una especie, sin que se añadan nuevos genes a medida que se secuencian más genomas (Vernikos *et. al.*, 2015). Este concepto de pan-genoma cerrado permite analizar y comparar con precisión la composición genética de diferentes cepas de *Salmonella*, lo que conduce a una comprensión más completa de su evolución y patogenicidad (Laing *et. al.*, 2017). Al estudiar un pan-genoma cerrado, es posible identificar marcadores genéticos clave que permite distinguir entre diferentes cepas, lo que permite intervenciones dirigidas para controlar y prevenir brotes de infecciones (Yap *et. al.*, 2014).

Además, el estudio del pan-genoma cerrado también proporciona información valiosa sobre los mecanismos de resistencia a los antibióticos en *Salmonella*, permitiendo explicar como estas bacterias se adaptan y evolucionan en respuesta a las presiones selectivas (Yuan *et. al.*, 2022). La comprensión de la base genética de la resistencia a los antibióticos es crucial para desarrollar estrategias eficaces de tratamiento y combatir la propagación de cepas multidrogo resistentes (Terreni *et. al.*, 2021). El concepto de pan-genoma cerrado tiene repercusiones en el ámbito del desarrollo de vacunas, dado que facilita la identificación de antígenos conservados que podrían ser considerados como blancos para la formulación de éstas (Muzzi *et. al.*, 2007).

La naturaleza dinámica de los genomas bacterianos, según Guimaraes *et al.*, (2015), se caracteriza por un flujo continuo de nuevos genes en la población bacteriana, lo que resulta en un conjunto de genes diversos y en constante evolución. Esta perspectiva del pan-genoma abierto resalta la importancia de la vigilancia y la investigación constantes para abordar las posibles amenazas emergentes, como señalan Gardy y Loman (2017). Este enfoque no solo puede facilitar la identificación de posibles blancos para nuevas vacunas y agentes antimicrobianos, contribuyendo a combatir la propagación de bacterias resistentes a los antibióticos, como indican Naz *et al.*, (2020), sino que también puede desempeñar un papel crucial en la detección y supervisión de nuevos mecanismos de resistencia a los antibióticos. Esto permite la detección temprana de posibles amenazas y la implementación oportuna de intervenciones adecuadas.

En general, el estudio del pan-genoma de *Salmonella* es esencial para combatir este patógeno versátil y reducir su impacto en las poblaciones humanas y animales (Park y Andam, 2020). Para

desarrollar intervenciones dirigidas y gestionar eficazmente los brotes, es esencial comprender su composición genética (Ricke, 2014). De esta manera las instituciones de salud pública pueden identificar rápidamente cepas emergentes, rastrear su movimiento y tomar medidas de control adecuadas para prevenir la contaminación generalizada al incorporar datos pan-genómicos en los programas de vigilancia.

1.2.6.2 Genoma "*core*". Representa el material genético conservado y presente en todas las cepas de una especie en particular, brindando información necesaria que permite el entendimiento de la biología básica del patógeno (Le Breton *et. al.*, 2015). Este genoma "core" sirve como punto de referencia para el estudio de la evolución y diversidad genetica de *Salmonella*, permitiendo identificar genes esenciales asociados a virulencia y resistencia a antibióticos (Valot *et. al.*, 2015). Los genes pertenecientes a este grupo pueden servir para el desarrollo de nuevas terapias enfocadas o basadas en estos, como el desarrollo de nuevos antibióticos o vacunas (Seib *et. al.*, 2009)

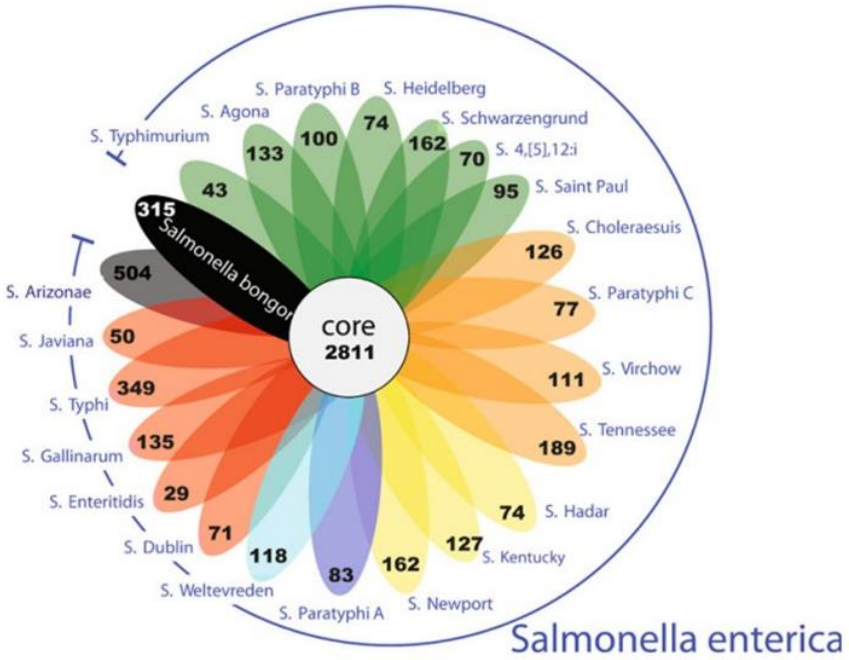

Figura 4. Gráfica de flor que representa las familias de genes únicos en diferentes serotipos de *Salmonella*. La figura representa el promedio del número de familias de genes encontradas en cada genoma y que son unicos para cada serotipo. Además se muestra el tamaño del genoma "core". El color de cada petalo del gráfico representa un serotipo de *Salmonella* (Jacobsen et. al., 2011).

1.2.7. Patogenicidad y Virulencia de *Salmonella*

Salmonella spp. es reconocida por su capacidad de adaptación a diferentes ambientes y por su potencial para provocar infecciones en seres humanos y animales (Andino y Hanning, 2015). Los factores de virulencia desempeñan un papel fundamental en la patogenicidad y en la capacidad de esta bacteria para causar enfermedades (Kaur y Jain, 2012). Algunos de estos factores de virulencia incluyen adhesinas, que permiten a *Salmonella* adherirse a las células del hospedador, y toxinas, que causan daño a las células y a los tejidos (Kline *et. al.*, 2009). Además, *Salmonella* puede invadir las células hospedadoras y replicarse dentro de estas, haciendo que la infección se propague más rapido (Li, 2022). Otros factores de virulencia significativos en *Salmonella* son los flagelos, que facilitan el movimiento y penetracion en los tejidos del hospedador (Duan *et. al.*, 2012), mientras que los sistemas de secreción permiten la liberación de toxinas directamente en las células (Rapisarda y Fronzes, 2018).

Salmonella, además, presenta lipopolisacáridos (LPS) en su membrana, los cuales actúan como toxinas desencadenando una intensa respuesta inflamatoria en el huésped (Giordano *et. al.*, 2020). Esta reacción inflamatoria puede provocar síntomas como fiebre, diarrea y dolor abdominal (Domínguez-Medina *et. al.*, 2020).

La relevancia de otro factor de virulencia es el sistema de secreción tipo III, el cual posibilita la transferencia de proteínas efectoras por parte de la bacteria a las células que está infectando (Mota y Cornelis, 2005). Estas proteínas tienen la capacidad de interferir con las vías de señalización del huésped, lo que favorece la supervivencia y replicación prolongada de *Salmonella* (Schlumberger y Hardt, 2006). Además, *Salmonella* produce enzimas que pueden degradar los tejidos, provocando mayor daño (Bhunia, 2018). Estas enzimas también pueden modificar la respuesta inflamatoria del huésped, generando un entorno propicio para el crecimiento bacteriano y facilitando su diseminación a otros órganos y tejidos (Gast y Porter, 2019). Esta situación puede desencadenar complicaciones graves como sepsis o disfunción de órganos (Liaudet *et. al.*, 2002).

Según King y Roberts (2016), las fimbrias, que son proteínas filamentosas encontradas en la superficie de la *Salmonella*, representan un factor de virulencia que favorece su capacidad de adherirse a las células hospedadoras y de eludir la respuesta del sistema inmunitario.

La importancia de las fimbrias en la capacidad de invasión y colonización de *Salmonella* ha sido

destacada por Tribble y Lamont (2010). Estas estructuras también contribuyen a la formación de biopelículas, las cuales mejoran la supervivencia de *Salmonella,* como señalan Jahan *et al.* (2022). La formación de biopelículas puede conferir resistencia a antibióticos y a respuestas inmunitarias, lo que complica el tratamiento de las infecciones causadas por *Salmonella* (Singh *et al*. 2022). Además las biopelículas contribuyen a la persistencia de *Salmonella* en el ambiente, incrementando el riesgo de contaminación y transmisión (Kumar, 2012). Es fundamental comprender el papel de las fimbrias y otros factores de virulencia de *Salmonella* en el contexto del diseño de estrategias innovadoras para prevenir y tratar infecciones provocadas por esta bacteria (Jahan *et. al.*, 2022). Esto implica la posibilidad de crear vacunas dirigidas a estos factores de virulencia o inspiradas en ellos, así como de idear inhibidores para las funciones de proteínas asociadas con la virulencia (Krachler y Orth, 2013).

Estudios de análisis genómico han posibilitado la identificación de numerosos genes vinculados a factores de virulencia (den Bakker *et. al.,* 2011; Rakov *et. al.,* 2019; de Melo *et. al.,* 2021). Por ejemplo, el gen *invA*, responsable de codificar una proteína crucial en la invasión de *Salmonella* en las células hospedadoras (Galán *et. al.,* 1992). La importancia de este gen radica en su función patogénica para desencadenar infecciones (Galán y Curtiss, 1991). Asimismo, el gen *sipA* se ha asociado con la capacidad de manipular las funciones celulares del hospedador, lo que facilita la supervivencia y reproducción dentro de este (Marcus *et. al.,* 2000). Se ha observado que mutaciones en genes de virulencia pueden tener un impacto negativo en la capacidad de *Salmonella* para replicarse y sobrevivir en el hospedador (Collazo y Galán, 1997; Jajere, 2019). Como evidencia de la diversidad genética relacionada con factores de virulencia en *Salmonella*, Aguirre-Sánchez y colaboradores (2021), mediante un análisis genómico comparativo entre cepas ambientales y clínicas, encontraron la presencia de genes relacionados a fimbrias, inductores de macrofagos, consumo de magnesio, adhesinas no relacionadas a fimbrias, sistemas de secreción y adaptación al estrés en todos los genomas analizados. Por su parte, González-Torres y colaboradores (2023), llevarón a cabo un análisis genómico de cepas ambientales de *S.* Oranienburg, en donde se identificaron genes de virulencia particularmente para adherencia, obtención de magnesio y hierro, biosintesis de enterobactina, y sistemas de secreción. Un mejor entendimiento de la función de estos genes puede permitir el desarrollo de nuevos tratamientos para controlar o prevenir las infecciones causadas por *Salmonella.*

1.2.7.1 Proceso de adherencia de *Salmonella*. El proceso de adhesión de *Salmonella* implica interacciones específicas entre la bacteria y las células del hospedador, lo que le permite adherirse e invadir los tejidos (Brett y Brumell, 2000). Diversas adhesinas y fimbrias presentes en la superficie bacteriana facilitan este proceso al unirse a receptores en la superficie de la célula hospedadora, que suelen ser carbohidratos y proteínas como la fibronectina e integrinas (Solanki *et. al.*, 2018) (Figura 5). Las adhesinas tienen una función fundamental en las fases tempranas de la infección al facilitar la colonización de *Salmonella* en el huésped y eludir la respuesta inmunitaria (Worley, 2023).

La fimbria tipo 1 es una de las adhesinas fundamentales en el proceso de adherencia de *Salmonella*. Estas estructuras, similares a pelos largos, son responsables de mediar la unión a receptores de carbohidratos específicos en las células del hospedero (Lindhorst *et. al.*, 2009). Por otro lado, adhesinas como la fimbria curli y el inyectisoma del sistema de secreción tipo III (T3SS) de la isla de patogenicidad tipo 1 de *Salmonella* (SPI-1) también juegan un papel crucial en la promoción de la adherencia e invasión de *Salmonella* (Hajra *et. al.*, 2021) (Figura 6).

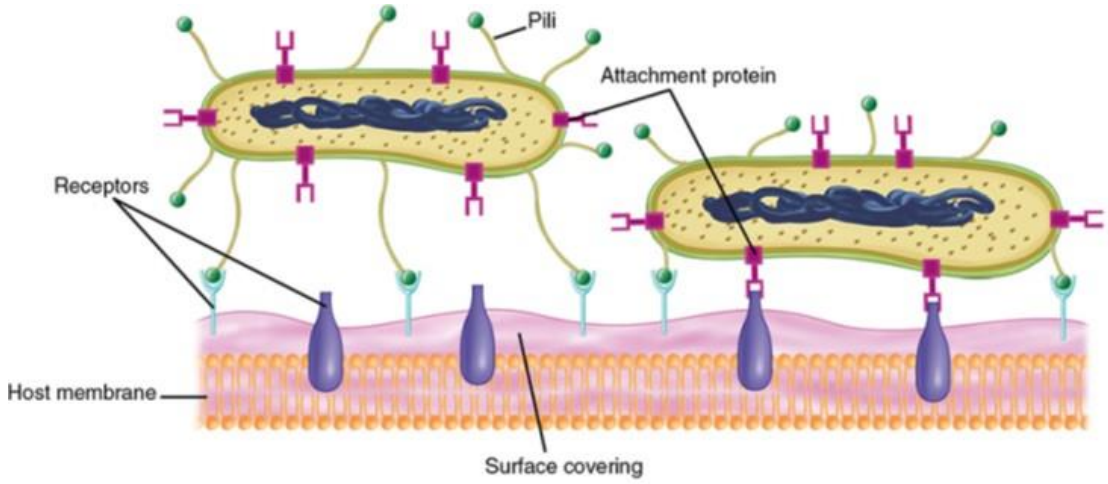

Figura 5. Proceso de adherencia bacteriana (Ryan *et. al.*, 2022).

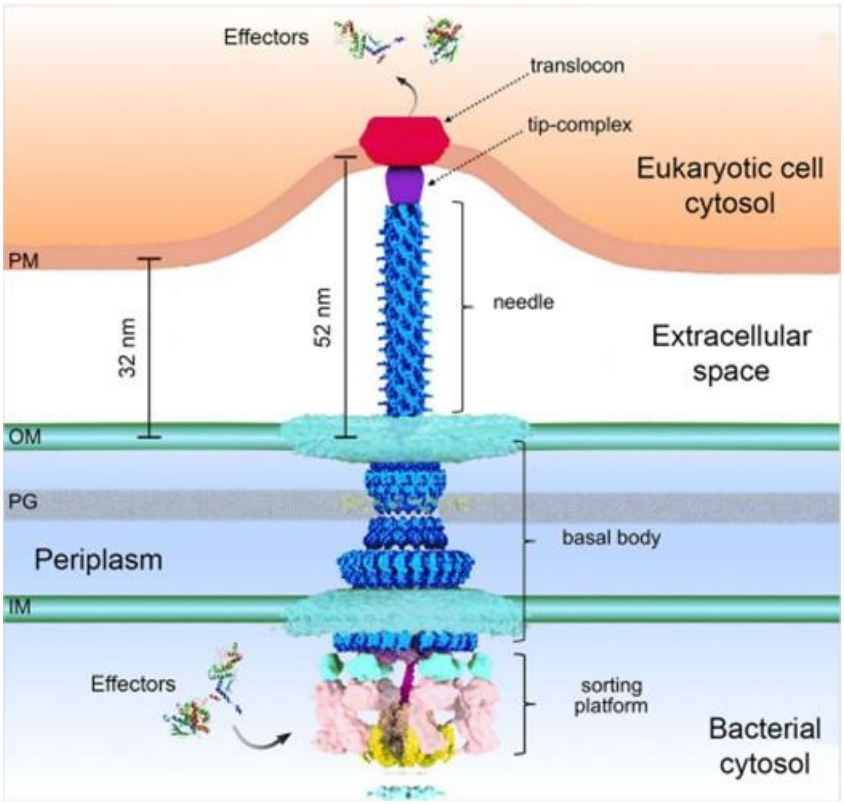

Figura 6. Representación gráfica del inyectisoma formado por el T3SS y la secreción de proteínas efectoras hacia la célula hospedadora (Lara-Tejero y Galán, 2019).

Estas adhesinas trabajan en conjunto para asegurar que *Salmonella* sea capaz de colonizar con éxito al hospedador y causar la infección. La fimbria tipo 1, por ejemplo, ayuda a la bacteria a adherirse a la superficie de la célula, mientras que la fumbria curli participa en la formación de biopelículas, proporcionando protección y promoviendo el establecimiento de la infección (Barnhart y Chapman, 2006; Tursi y Tükel, 2018). El inyectisoma SPI-1 T3SS, por otro lado, permite a *Salmonella* inyectar proteínas efectoras directamente en la célula anfitriona, manipulando su entorno interno y promoviendo la invasión (Moest y Méresse, 2013; Gaytán *et. al.*, 2016).

Además, el SPI-1 T3SS también desempeña un papel crucial en el desencadenamiento de las vías de señalización de las células del hospedero que conducen a rearreglos citoesqueléticos, permitiendo que *Salmonella* logre invadir (Srikanth *et. al.*, 2011). Este esfuerzo coordinado de las adhesinas y el inyectisoma destaca los complejos mecanismos que utiliza *Salmonella* para asegurar su sobrevivencia y proliferación dentro del hospedador (Saleh *et. al.*, 2023). Además, la regulación de estos factores de virulencia está estrechamente controlada por factores ambientales, como la

presencia de sales biliares y otros factores dentro del hospededor, lo que demuestra la adaptabilidad de *Salmonella* en respuesta a su entorno (de Pina *et. al.,* 2021). En general, la compleja interacción entre las adhesinas y el SPI-1 T3SS es esencial para la patogenesis de esta bacteria y representa un objetivo prometedor para el desarrollo de nuevas estrategias terapéuticas contra este patógeno humano.

1.2.7.2 Proceso de invasión de *Salmonella.* Los mecanismos de invasión de *Salmonella* implican una serie de interacciones complejas entre el patógeno y las células del hospedador. Un aspecto clave de la invasión de *Salmonella* es su capacidad para adherirse e invadir las células epiteliales intestinales, lo que le permite establecer la infección (Ly y Casanova, 2007; Malik-Kale *et. al.,* 2011). Este proceso es facilitado por la expresión de adhesinas que promueven la unión a los receptores de las células huesped, así como la secreción de proteínas efectoras a través del SPI-1 T3SS que permiten a las bacterias invadir a las células del hospedador (Gaytán *et. al.,* 2016; dos Santos *et. al.,* 2018).

Además, *Salmonella* también es capaz de inducir la ruptura de la membrana celular del hospedador permitiendo asi su internalización (Goosney *et. al.,* 1999; Galán, 2001). Una vez dentro, la bacteria puede sobrevivir y replicarse dentro de una vacuola especializada conocida como vacuola que contiene a *Salmonella* (“SCV” por sus siglas en inglés) (Bakowski *et. al.,* 2008). Este nicho intracelular proporciona protección contra las defensas inmunes del hospedador y permite a las bacterias establecer una infección persistente (Diacovich *et. al.,* 2016). Además, *Salmonella* también puede emerger de la VSC y propagarse a las células vecinas a través de mecanismos como la propagación de célula a célula y la formación de protrusiones de membrana (Wang *et. al.,* 2020). Estos complejos mecanismos de invasión y sobrevivencia intracelular contribuyen a la patogenicidad de *Salmonella* y su capacidad para causar enfermedad en humanos y animales (Li, 2022). Además de su capacidad para evadir las respuestas inmunes del hospedador, esta bacteria también posee una variedad de factores de virulencia que le permiten manipular los procesos de las células huesped y promover su propia sobrevivencia y replicación (Viana *et. al.,* 2021). Uno de esos factores de virulencia es el T3SS, que le permite inyectar proteínas efectoras en las células anfitrionas para modular las vías de señalización celular y subvertir las defensas del hospedador (Raymond *et. al.,* 2013).

Este sistema desempeña un papel crucial en el establecimiento de la infección y la formación de SVC. Al manipular los procesos de las células anfitrionas, *Salmonella* es capaz de crear un nicho para sí misma donde puede evitar la detección y destrucción por el sistema inmunológico (Behnsen *et. al.,* 2015). Además, la capacidad de replicarse dentro de las células del hospedador permite la diseminación del patógeno en todo el cuerpo, lo que conduce a una infección sistémica y a complicaciones potencialmente mortales (Eng *et. al.,* 2015). La comprensión de la compleja interacción entre *Salmonella* y las células anfitrionas es esencial para desarrollar estrategias eficaces para combatir este patógeno versátil y resistente.

1.2.7.3 Islas de patogenicidad. Las islas de patogenicidad son grandes cúmulos de genes que pueden ser adquiridos mediante transferencia horizontal y desempeñan un papel clave en la virulencia de *Salmonella* (Marcus *et. al.,* 2000). Estas islas contienen genes que codifican diversos factores de virulencia, como toxinas, adhesinas y sistemas de secreción, que permiten a *Salmonella* adherirse, invadir y manipular las células hospedadoras (Kombade y Kaur, 2021). Estas se encuentran a menudo en el cromosoma bacteriano o en los plásmidos, y pueden variar en tamaño y composición entre diferentes cepas de *Salmonella* (Marcus *et. al.,* 2000; Schmidt y Hensel, 2004). Se cree que estas islas han evolucionado como resultado de la presión selectiva del sistema inmunológico del hospedador, permitiendo a las bacterias adaptarse y sobrevivir en diversos ambientes (Sousa, 2003; Bliven y Maurelli, 2016). La presencia de islas de patogenicidad en *Salmonella* se ha relacionado con un aumento de la patogenicidad y la gravedad de la enfermedad en los individuos infectados (Jones *et. al.,* 2007). Además, las islas de patogenicidad también pueden contener genes que confieren resistencia a los antibióticos, complicando aún más las opciones de tratamiento para las infecciones causadas por estas cepas de *Salmonella* (Gal-Mor y Finlay, 2006; Jajere, 2019*)*. Asimismo, estudios han demostrado que algunas islas de patogenicidad identificadas en *Salmonella* pueden haber sido adquiridas mediante transferencia horizontal de genes, lo que conduce a la propagación de factores de virulencia y genes de resistencia a los antibióticos (Mirold *et. al.,* 2001; Gyles y Boerlin, 2013; Salazar-Echegarai *et. al.,* 2014).

Algunas islas de patogenicidad más estudiadas en *Salmonella* son SPI-1 y SPI-2, conocidas por desempeñar papeles clave en la invasión de las células huéspedes y la sobrevivencia dentro del entorno del huésped (Zhang *et. al.,* 2016; Lou *et. al.,* 2019). Estas islas de patogenicidad se

encuentran típicamente en grandes plásmidos o integradas en el cromosoma bacteriano, lo que permite su herencia estable y expresión en diferentes poblaciones bacterianas (Gal-Mor y Finlay, 2006).

Adicionalmente, la isla de patogenicidad 3 de *Salmonella* (SPI-3), también ha sido identificada y se ha reportado que está involucrada en la adquisición de hierro y la sobrevivencia intracelular (Bhunia, 2018). Además, se ha encontrado que la expresión de SPI-3 es influenciada por señales ambientales, como los bajos niveles de hierro, lo cual demuestra la adaptabilidad y versatilidad de estas islas de patogenicidad en *Salmonella* (Moreira *et. al.,* 2010). SPI-3 también está relacionada con otros factores de virulencia, como sistemas de secreción de tipo III, que ayudan a *Salmonella* a evadir la respuesta inmune del hospedador (Wang *et. al.,* 2020). Esta compleja red de islas de patogenicidad y factores de virulencia permite a *Salmonella* colonizar e infectar eficazmente a los organismos huesped, causando desde gastroenteritis hasta infecciones sistémicas.

Además, la presencia de múltiples islas de patogenicidad en *Salmonella* sugiere que estas bacterias han evolucionado mecanismos sofisticados para la sobrevivencia y la persistencia en diversos entornos. La capacidad de detectar y responder a las señales ambientales, como los cambios en la disponibilidad de nutrientes o las defensas del hospedador, permite a *Salmonella* adaptarse rápidamente y establecer infecciones (Schmidt y Hensel, 2004).

1.2.8. Mecanismos De Resistencia a Antibióticos En *Salmonella*

Además de los genes de virulencia, la investigación enfocada en los mecanismos de resistencia a antibioticos es de suma importancia. Este fenómeno es de gran preocupación para el tratamiento de las infecciones bacterianas, ya que limita la efectividad de antibióticos comunmente utilizados (Mancuso *et. al.,* 2021). El entendimiento de cómo *Salmonella* puede desarrollar resistencia a los antibióticos es crucial para el desarrollo de nuevas estrategias de tratamiento (Eng *et. al.,* 2015). Un mecanismo común de resistencia en esta bacteria es la adquisición de genes de resistencia través de procesos de transferencia genética horizontal (“HGT” por sus siglas en inglés) (Bello-López *et. al.,* 2019; Lerminiaux y Cameron, 2019). Este proceso permite a la bacteria adaptarse rápidamente a nuevos antibióticos y evadir los efectos del tratamiento tradicional (Arnold *et. al.,*

2021). La transferencia de material genético entre bacterias puede ocurrir de diversas maneras, ya sea a través del intercambio de material genético en el ambiente o por contacto directo entre bacterias (Heuer y Smalla 2007; Aminov 2011). Uno de los mecanismos de transferencia genética horizontal es la conjugación, en la cual la bacteria transfiere su ADN mediante el contacto directo célula-célula utilizando el pili bacteriano(Zaneveld *et. al.*, 2008). Esta forma de transferencia facilita la diseminación rápida de genes de resistencia a antibióticos en las poblaciones bacterianas, lo que representa un desafío para el tratamiento de infecciones causadas por cepas bacterianas resistentes, como indican Villa y colegas (2019).

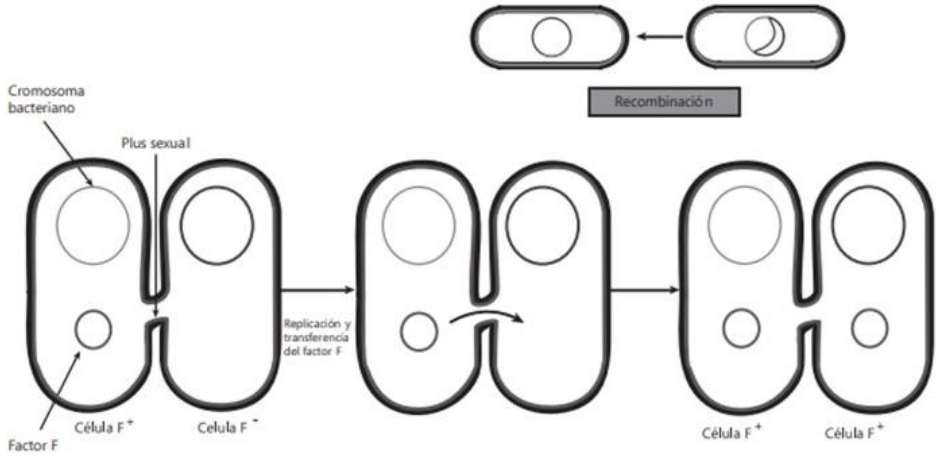

Figura 7. Mecansimo de conjugación bacteriana (Sánchez *et. al.*, 2012)

Adicionalmente a la conjugación, *Salmonella* puede adquirir material genético de resistencia a antibióticos mediante transformación, en donde se toma el ADN libre del ambiente (Figura 8), o por transducción, donde el ADN es transferido mediante bacteriófagos (Figura 9) (Lorenz y Wackernagel, 1994; Muniesa *et. al.*, 2013). Algunos estudios se ha encontrado que el rango de transferencia de genes de resistencia a antibióticos es mayormente por conjugación (Chen *et. al.*, 2019; Zhang *et. al.*, 2024). Esto sugiere la importancia de enfocarse en el estudio de los mecanismos que regulan este proceso para encontrar estrategias de mitigación (Schillaci *et. al.*, 2017; Vrancianu *et. al.*, 2020). Álvarez-Rodríguez y colaboradores (2020) mencionan que proteínas involucradas en el proceso de conjugación que pueden ser blanco de nuevos agentes antimicrobianos. Inhibir estos mecanismos cruciales de transferencia genética, puede permitir una

posible reducción del rango de resistencia a antibióticos.

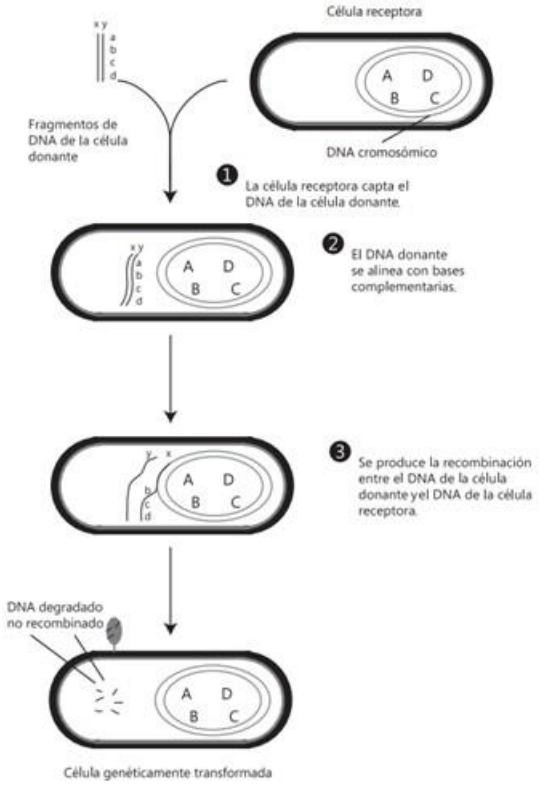

Figura 8. Mecanismo de transformación bacteriana (Sánchez et. al., 2012)

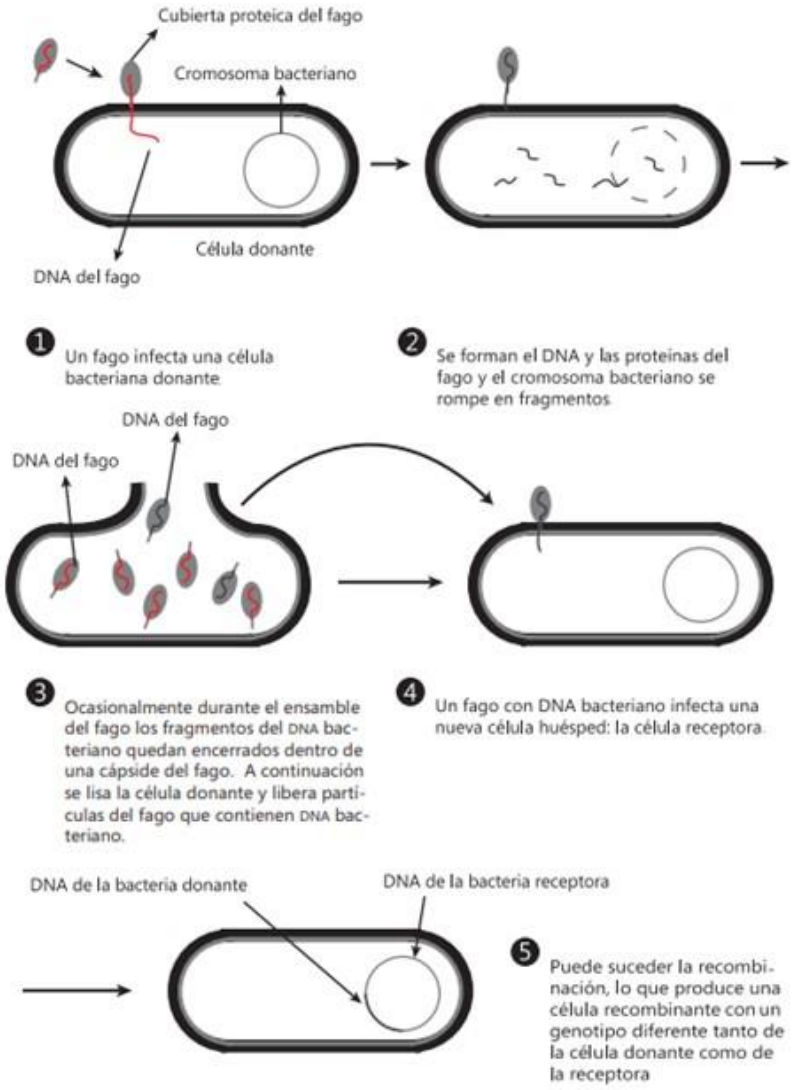

Figura 9. Mecanismo de transducción bacteriana (Sánchez *et. al.*, 2012).

Además de la HGT, otros mecansimos de resistencia a antibióticos en *Salmonella* incluyen las bombas de eflujo (Figura 10) (Alenazy, 2022). Estas son proteínas de transporte que expulsan a los antibióticos fuera de la célula bacteriana, reduciendo la efectividad del medicamento (Van Bambeke *et. al.*, 2000). Las bombas de eflujo funcionan reconociendo y uniendose a los antibióticos, para luego expulsarlos de la célula (Fernández y Hancock, 2012). Este proceso permite a la bacteria sobrevivir y multiplicarse en presencia de los antibióticos, lo cual conlleva el desarrollo de resistencia. Las bombas de eflujo han sido identificadas como componentes importantes en la resistencia a múltiples antibióticos en *Salmonella* haciéndolos blancos

potenciales para el desarrollo de nuevos agentes antimicrobianos que puedan inhibir su función y restaurar la efectividad de los antibióticos contra este patógeno (Shriram *et. al.,* 2018; Gaurav *et. al.*, 2023). Algunos genes identificados relacionados a estas bombas de eflujo han sido *acrAB, tolC* y *mdrABC* (Li y Nikaido, 2016; Alenazy, 2022). Estos genes codifican proteínas que forman parte de la maquinaria de estas bombas, y que son responsables del transporte de los antibióticos fuera de la célula (Blair *et. al.,* 2014). En un estudio realizado por Chen y colaboradores (2007) se encontraron mutaciones en estos genes que pueden incrementar su eficiencia, contribuyendo al aumento de la resistencia en *Salmonella.* Más estudios son necesarios para entender como estos genes pueden contribuir con la resistencia a antibióticos.

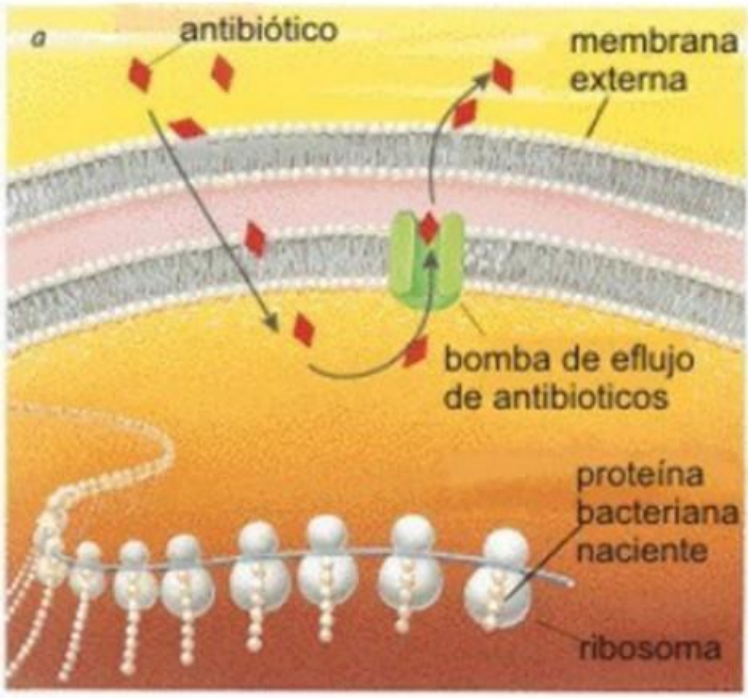

Figura 10. Mecanismo de bombas de eflujo bacterianas (de Rivas, 2006).

Otros mecanismos relacionados a la resistencia a antibióticos en *Salmonella* incluyen mutaciones en proteínas diana y la producción de enzimas que pueden degradar antibióticos (Martins *et. al.,* 2011; Chaudhari *et. al.,* 2023). Algunas de estas enzimas, son las beta-lactamasas, las cuales son de gran importancia debido a que pueden inactivar los antibióticos rompiendo su estructura química

(Bush, 2009). Adicionalmente las mutaciones en proteínas diana pueden alterar los sitios de unión de los compuestos antibióticos, haciéndolos menos efectivos (Lambert, 2005). Entender estos diversos mecanismos de resistencia puede llevar a desarrollar nuevas estrategias para combatir la creciente resistencia a antibióticos en *Salmonella*.

1.2.9. Vacunas Contra *Salmonella*

Una vacuna puede definirse como una preparación biológica que ayuda a aumentar la inmunidad de humanos o animales contra una enfermedad específica (Pollard y Bijker, 2020). En el caso de *Salmonella*, se pueden desarrollar vacunas destinadas a cepas específicas y proporcionar protección contra la infección (Garmory *et. al.,* 2002; Barrow, 2007; Sears *et. al.,* 2021). Las vacunas funcionan estimulando el sistema inmune para producir anticuerpos que pueden reconocer y atacar al patógeno, y así prevenir la infección o reducir la gravedad de los síntomas (Sell, 2019). Para *Salmonella*, se pueden administrar vacunas que permitan prevenir la infección en poblaciones de alto riesgo, como individuos con sistemas inmunes comprometidos o aquellos que trabajan en estrecho contacto con animales (Christenson, 2013; Heithoff *et. al.*, 2015). Además, las vacunas también pueden utilizarse en situaciones de brotes epidemiológicos para ayudar a controlar la propagación de las bacterias y proteger a los individuos vulnerables (Jansen *et. al.,* 2018; Chumakov *et. al.,* 2021).

Las vacunas contra *Salmonella* suelen administrarse por inyección, aunque algunas se pueden administrar por vía oral. Estas vacunas están diseñadas para dirigirse a cepas específicas que se sabe que causan enfermedades en humanos (Cárdenas y Clements, 1992; Garmory *et. al.*, 2002; Spreng *et. al.*, 2006).

El primer paso en el diseño de una vacuna es identificar las cepas o serotipos más comunes de *Salmonella* que representan una amenaza para la salud pública (MacLennan *et. al.*, 2014). Una vez identificadas estas cepas, los científicos pueden comenzar a desarrollar una vacuna que estimule el sistema inmune para reconocer y atacar estas bacterias específicas. Este proceso a menudo implica el uso de formas debilitadas o inactivadas de las bacterias para desencadenar una respuesta inmune sin causar enfermedad (Curtiss *et. al.,* 2010; Tennant y Levine, 2015). Existen diversos tipos de

vacunas, incluyendo vacunas vivas atenuadas, vacunas inactivadas, vacunas de subunidades y vacunas conjugadas, cada una de las cuales ofrece diferentes ventajas y desventajas en términos de eficacia y seguridad. El objetivo de todas estas vacunas es proporcionar inmunidad contra las cepas dañinas de *Salmonella*, reduciendo en última instancia el riesgo de infección y previniendo la propagación de la enfermedad en las comunidades (Kallerup y Foged, 2014; Yadav *et. al.*, 2020). La eficacia de las vacunas en la disminución y erradicación de las infecciones causadas por microorganismos patógenos ha sido ampliamente demostrada tanto en seres humanos como en animales (Sell, 2019; Wagner y Weinberger, 2020). Junto con las vacunas tradicionales, es fundamental explorar nuevas estrategias de vacunación, como las vacunas orales y las basadas en ADN, que puedan garantizar altos niveles de eficacia y una administración sencilla (Simerska *et. al.*, 2009; Vela Ramirez *et. al.*, 2017; Wallis *et. al.*, 2019). Además, los avances en la ingenieria genética han permitido la elaboración de vacunas que se enfocan en cepas específicas de bacterias patógenas, presentando un método aun más preciso de prevención (Galen y Curtiss, 2014; Sears *et. al.*, 2021). Una de las estrategías más recientes, son el desarrollo de vacunas multi-epítopo, las cuales utilizan múltiples epítopos de varias proteínas de un patógeno para producir una respuesta inmune fuerte (Goumari *et. al.*, 2020). Estas vacunas que utilizan múltiples epítopos pueden proveer una amplia protección contra varias cepas de *Salmonella*, haciendolas muy efectivas (Chand y Singh, 2021; Zafar *et. al.*, 2022). Las herramientas computacionales han sido beneficiosas para el diseño y evaluación de este tipo de vacunas, permitiendo predecir y seleccionar varios epítopos con propiedades inmunogénicas para incluirlos en la construcción de la vacuna (Bahrami *et. al.*, 2019; Parvizpour *et. al.*, 2020). Este enfoque permite tener una vacuna eficaz, mientras el riesgo de efectos secundarios es menor (Salaikumaran *et. al.*, 2022). Este tipo de vacunas son más seguras debido a que están diseñadas para el reconocimiento de epítopos inmunogénicos específicos de un patógeno, reduciendo la posibilidad de una respuesta inmune no deseada (Naveed *et. al.*, 2022). Además, tienen la capacidad de estimular una fuerte y específica respuesta inmunitaria sin el riesgo de causar daño al hospedador (Goumari *et. al.*, 2020). Algunas proteínas que se utilizan comunmente para el diseño de estas vacunas incluyen flagelinas, proteínas de membrana, y porinas, las cuales son altamente inmunogénicas y conservadas en diferentes cepas (Sirajee y Ahsan, 2022; Jafari Najaf Abadi *et. al.*, 2023). Sin embargo, proteínas como lipopolisacáridos, fimbriales y proteínas relacionadas al metabolismo, que son proteínas esenciales para la patogénesis de la bacteria, también son exploradas para su potencial de poder diseñar una

vacuna multi-epítopo (Dennehy y McClean, 2012; Grover *et. al.*, 2014). Adicionalmente a estas proteínas antigénicas, se pueden añadir adyuvantes y sistemas de entrega usados en las vacunas para potenciar su efectividad (Lei *et. al.*, 2019). Por otro lado, la secuenciación genómica puede proveer hallazgos importantes en la diversidad genética bacteriana, permitiendo la identificación de antigenos comunes que pueden ser utilizados para el diseño de una vacuna (Scarselli *et. al.*, 2005; Muzzi *et. al.*, 2007). Combinando la información obtenida de la secuenciación con el conocimiento de proteínas esenciales, es posible desarrollar estrategias que mejoren la proteccion contra distintos serotipos de *Salmonella*. En un estudio llevado a cabo por Beizadeh (2023), se diseñó y evaluó una vacuna multi-epítopo para salmonelosis no tifoidea mediante el uso de herramientas bioinformáticas, utilizando proteínas de membrana OmpA, OmpD y la enterotoxina Stn, obteniendo resultados favorables en cuanto a las propiedades inmunogénicas de la vacuna, además la vacuna tuvo fuerte afinidad por receptores tipo Toll que puede estimular la respuesta inmune ante la presencia de una infección bacteriana. De manera similar, Zafar y colaboradores (2022), diseñaron y evaluaron una vacuna multi-epítopo *In silico* utilizando la proteína TolA de *Salmonella* la cual es altamente antigénnica y está relacionada con procesos de entrada de la bacteria al hospedador. Los resultados indicaron que la vacuna fue capaz de estimular una respuesta inmune favorable.

1.3. Hipótesis

1.- Los serotipos ambientales de *Salmonella enterica* contienen genes relacionados a adherencia, invasión y sobrevivencia.

2.- Los serotipos ambientales de *Salmonella enterica* contienen genes relacionados a resistencia a al menos un antibiótico.

3.- Los serotipos ambientales de *Salmonella enterica* contienen los genes necesarios para la regulación y activación de la respuesta a estrés por falta de nutrientes.

4.- Las proteínas de respuesta a estrés por falta de nutrientes son genes esenciales para la sobrevivencia de serotipos ambientales de *Salmonella enterica.*

5.- Las proteínas de respuesta a estrés por falta de nutrientes son candidatas para el diseño *In silico*

de una vacuna multi-epítopo contra salmonelosis no tifoidea.

1.4 Objetivo General

Analizar genotípicamente y proteínicamente serotipos ambientales de *Salmonella enterica*

1.5 Objetivos Específicos

- Identificar genes de virulencia presentes en serotipos ambientales de *Salmonella enterica*
- Identificar genes de resitencia a antimicrobianos presentes en serotipos ambientales de *Salmonella enterica*
- Identificar genes relacionados a la activación y regulación de la respuesta a estrés por falta de nutrientes presentes en serotipos ambientales de *Salmonella enterica*
- Identificar y caracterizar proteínas esenciales para sobrevivencia de serotipos ambientales de *Salmonella enterica*
- Diseñar *In silico* una vacuna multi-epítopo basada en proteíans esenciales para sobrevivencia de *Salmonella enterica*

1.6 Sección Integradora del Trabajo

Como productos generados a partir del presente trabajo se desarrollaron dos artículos científicos. El primer artículo, que lleva por título *"Genomic characteristics of Salmonella Montevideo and Pomona: impact of isolation source on antibiotic resistance, virulence and metabolic capacity",* y comprendió los objetivos uno, dos, tres y cuatro. En este artículo se realizó un análisis genómico comparativo de los serotipos no tifoideos Montevideo y Pomona de *Salmonella*, los cuales han sido poco estudiados a nivel genómico y ha su vez relacionados con infecciones y brotes epidemiolgícos

a nivel mundial. Se utilizaron genomas de cepas secuenciadas y proporcionadas por el Laboratorio Nacional para la Investigación en Inocuidad Alimentaria (LANIIA), además de genomas reportados en la base de datos del Centro Nacional para la Información Biotecnológica ("NCBI" por sus siglas en inglés). Las cepas analizadas provinieron de diversas fuentes de aislamiento, como sedimentos, agua de río y alimentos, y fueron comparadas con cepas de origen clínico. Durante el análisis, se logró la identificación de genes de virulencia, tales como aquellos asociados a bombas de eflujo, adquisición de hierro, adhesinas, porinas, islas de patogenicidad, efectores y sistemas de secreción. Asimismo, se detectaron genes vinculados a la resistencia a antibióticos, como los de resistencia a aminoglucósidos, beta lactamasas, fluoroquinolonas, sulfonamidas y bombas de eflujo. Además, se identificaron genes relacionados con diversos sistemas de transporte de fosfotransferasas para la utilización de diferentes sustratos, como glucosa, n-acetil-D-glucosamina, manitol, manosa y galactosa, entre otros. Uno de los hallazgos principales fue la alta similitud genética entre las cepas ambientales y clínicas, lo que sugiere una notable capacidad de adaptabilidad. Además, se observó que la fuente de aislamiento no parece influir significativamente en la diversidad genética en términos de potencial patogénico entre las cepas ambientales y clínicas. No obstante, se identificó como una característica distintiva la presencia de genes asociados al sistema de secreción tipo IV únicamente en cepas ambientales pero no en las clínicas. Este sistema está relacionado con procesos de intercambio genético como la conjugación bacteriana, lo cual sugiere que este proceso predomina mayormente en cepas de origen ambiental, y quiza esto puede contribuir al intercambio de genes de virulencia y resistencia a antibióticos. En conclusión, se logró identificar la presencia de diversos genes relacionados con virulencia, resistencia a antibióticos y metabolismo en cepas de *S.* Pomona y *S.* Montevideo, tanto ambientales como clínicas. Se observaron similitudes y diferencias en el contenido genético de ambos serotipos, sugiriendo la posible transferencia horizontal de genes. Además, se plantea que las cepas ambientales podrían tener una mayor capacidad de llevar a cabo procesos de conjugación, atribuible a la presencia exclusiva del sistema de secreción tipo IV en estas cepas. Se recomienda la realización de estudios a nivel de laboratorio para analizar la expresión de los genes identificados, así como la búsqueda de estrategias y medidas preventivas contra las enfermedades asociadas a estos serotipos ambientales.

El segundo artículo generado a partir de la presente tesis de investigación, lleva por título *"Immunoinformatic Approach for Designing a Multi-epitope Vaccine Against Non-Typhoidal*

Salmonellosis Using Starvation-stress Response Proteins from Salmonella Oranienburg". Este artículo cumplió con el objetivo cuatro. En el presente estudio se realizó el diseño y la evaluación *In silico* de una vacuna multi-epítopo utilizando proteínas del sistema de respuesta al estrés por falta de nutrientes ("SSR", por sus siglas en inglés) de *Salmonella* Oranienburg, un serotipo no tifoideo que, a pesar de su alta prevalencia ambiental, ha sido asociado con brotes epidemiológicos e infecciones a nivel global debido a su potencial patogénico. Para ello, se extrajeron las secuencias aminoacídicas de las proteínas SSR de cinco genomas de *S.* Oranienburg aislados de sedimentos de río. Estas secuencias están altamente conservadas en los serotipos no tifoideos de *Salmonella* además de ser esenciales para su sobrevivencia dentro y fuera de un hospedero. Las secuencias fueron analizadas mediante herramientas inmunoinformáticas para predecir epítopos inmunogénicos. Posteriormente, se realizaron análisis de acoplamiento molecular con proteínas receptoras HLA para identificar los mejores epítopos. Estos epítopos seleccionados se utilizaron para diseñar una vacuna multi-epítopo, la cual fue evaluada para determinar su potencial respuesta inmunológica en el hospedero y su capacidad de unirse a receptores tipo Toll. Los resultados del estudio revelaron que la vacuna fue capaz de generar una respuesta inmunológica sólida, demostrada por la producción de anticuerpos igG e igM, así como la activación de células B, tal como se observó en el análisis bioinformático predictivo. Asimismo, se observaron interacciones beneficiosas, principalmente mediante la formación de puentes de hidrógeno con receptores tipo Toll 1, 2 y 4, los cuales están asociados con la detección de infecciones bacterianas, como las causadas por bacterias gram-negativas como *Salmonella*. La evaluación de la cobertura poblacional de la vacuna indicó que esta podría proteger a más del 50% de la población mundial, considerando los alelos HLA tipos I y II. En resumen, la vacuna multi-epítopo basada en proteínas SSR arrojó resultados prometedores en los diversos análisis realizados. Por lo tanto se sugiere la realización de estudios adicionales para la producción y evaluación de esta proteína a nivel experimental.

2. GENOMIC CHARACTERISTICS OF *Salmonella* MONTEVIDEO AND POMONA: IMPACT OF ISOLATION SOURCE ON ANTIBIOTIC RESISTANCE, VIRULENCE AND METABOLIC CAPACITY

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Genomic characteristics of *Salmonella* Montevideo and Pomona: Impact of isolation source on antibiotic resistance, virulence and metabolic capacity

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ABSTRACT

Salmonella enterica is known for its disease-causing serotypes, including Montevideo and Pomona. These serotypes have been found in various environments, including river water, sediments, food, and animals. However, the global spread of these serotypes has increased, leading to many reported infections and outbreaks. The goal of this study was the genomic analysis of 48 strains of *S.* Montevideo and *S.* Pomona isolated from different sources, including clinical. Results showed that environmental strains carried more antibiotic resistance genes than the clinical strains, such as genes for resistance to aminoglycosides, chloramphenicol, and sulfonamides. Additionally, the type 4 secretion system, was only found in environmental strains. Also many phosphotransferase transport systems were identified and the presence of genes for the alternative pathway Entner-Doudoroff. The origin of isolation may have a significant impact on the ability of *Salmonella* isolates to adapt and survive in different environments, leading to genomic flexibility and a selection advantage.

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KEYWORDS
Salmonella; in silico; antimicrobial resistance; virulence; metabolism

Introduction

Salmonella is a Gram-negative bacterium that causes a wide range of diseases in humans and animals. The diversity within the *Salmonella* genus is vast, with over 2,600 serotypes identified to date (Wray and Wray 2000; Kim and Kim 2021). The Centers for Disease Control and Prevention (CDC) reported that *Salmonella* infections cause approximately 1.35 million illnesses and 420 deaths in the United States (CDC Center for Disease Control and Prevention 2018). This bacterial pathogen is primarily transmitted through consumption of contaminated food, particularly raw or undercooked eggs, poultry, and meat. Other sources of transmission include contaminated water, contact with infected animals, and poor hygiene. *Salmonella* infection, also known as salmonellosis, can cause symptoms, such as fever, diarrhea, abdominal cramps, and vomiting (Coburn et al. 2006; Carrasco et al. 2012; Eng et al. 2015). Surface waters are a common habitat for *Salmonella*, despite

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the fluctuating and potentially unsuitable conditions for its survival (Moore et al. 2003; Medrano-Félix et al. 2017). *Salmonella* encounters stress factors like temperature, pH, and nutrient changes, which can decrease its viability and ability to cause infection (Winfield and Groisman 2003). However, *Salmonella* employs survival strategies, such as the starvation stress response (SSR), to adapt and survive in nutrient-deprived environments by altering its metabolism and gene expression and triggering protective mechanisms like biofilm formation and stress protein production (Kenyon et al. 2002; Spector and Kenyon 2012). The ability of *Salmonella* to adapt under stress conditions is highlighted by its capacity for horizontal gene transfer (HGT), which enables the bacterium to obtain new genetic material from other bacteria, including antibiotic-resistance genes (dos Santos et al. 2021). This process is crucial in the evolution of *Salmonella* (Li et al. 2021), and it can lead to severe health consequences for the host (Pradhan and Devi Negi 2019).

Salmonella enterica, a pathogen with a diverse range of serotypes, has caused numerous outbreaks and incidents of foodborne illnesses worldwide, including Montevideo and Pomona (Jeong et al. 2017). These serotypes have been linked to numerous outbreaks and incidents of foodborne illnesses across the globe, demonstrating the worldwide impact of *Salmonella* infections (Gieraltowski et al. 2012; Harris et al. 2016; Paradis et al. 2023). Additionally, they exhibit high adaptability, making them challenging to control and prevent. To develop effective measures to curb the spread of this bacterium and reduce the burden of foodborne illnesses, it is essential to comprehend the genetic variations and virulence factors associated with different serotypes. *S.* Montevideo and *S.* Pomona are two serotypes that exhibit resistance to various antibiotics and have been associated with human salmonellosis infections (CDC 2012, 2018; Punchihewage-Don et al., 2022). *S.* Montevideo possesses genes that confer resistance to multiple antibiotics, increasing its virulence, while *S.* Pomona has been linked to 18% of human salmonellosis infections in the United States due to interactions with reptile pets (Bosch et al. 2015). Genomic studies of *S.* Pomona have identified genes related to dynamic metabolism, resistance to aminoglycoside antibiotics, and iron acquisition, contributing to its virulence (Burgueño-Roman et al. 2019). The ability of *Salmonella* to thrive in various environments increases its potential to cause severe disease, especially when it returns to a host (Chakroun et al. 2017; Ramírez et al. 2018; dos Santos et al. 2021). To develop better diagnostic tools and targeted therapies, it is crucial to study the genetic diversity of *Salmonella* serotypes isolated from different sources (Aguirre-Sanchez et al. 2021; Achtman et al. 2012; Page et al. 2017). In this sense, this study aimed to analyze the genetic content between clinical and environmental strains of *S.* Montevideo and Pomona, comparing virulence, metabolic capacities, and antibiotic resistance. Understanding the differences and similarities between these strains is essential for improving public health outcomes and addressing knowledge gaps in the epidemiology and pathogenicity of this bacteria.

Materials and methods

Salmonella *Montevideo and Pomona isolates and sequencing*

A total of 24 genomes each of *S.* Montevideo and 24 genomes of *S.* Pomona were analyzed. This included 10 clinical and 14 environmental strains of *S.* Montevideo, as well as 10 clinical and 14 environmental strains of *S.* Pomona. The dataset was retrieved from the National Center for Biotechnology Information (NCBI) (Agarwala et al. 2017). The accession numbers and corresponding information for these genomes are listed in Supplementary Table S1. In addition, the Laboratorio Nacional para la Investigación en Inocuidad Alimentaria (LANIIA) provided a total of four genomes of *S.* Pomona (JCS-04, JCS-07, JCS-08, and JCS-25) and four genomes of *S.* Montevideo (JCS-06, JCS-27, JCS-28, and JCS-34). The genomes were acquired from strains isolated from river sediment in previous studies. The Whole Genome Shotgun project of the previous *S.* Pomona and Montevideo strains mentioned has been deposited at DDBJ/ENA/GenBank under the accession JAOBPZ000000000, JAOBPY000000000, JAOBPX000000000, JAOBPW000000000, JAOBQD000000000, JAOBQC000000000, JAOBQB00 0000000, and JAOBQA00000000.

Assembling and annotation of Salmonella *Montevideo and Pomona genomes*

Reads quality of the genomes provided by the LANIIA was enhanced using Trimmomatic v0.32, as described by Bolger et al. (2014). The initial 20 bases of each sequence were excluded, and a sliding window of four bases was employed to identify segments with an average Phred quality score of 15 or less. Reads with fewer than 50 bases were excluded. In accordance with the methodology described by Coil et al. (2015), we employed A5-miseq v20160825 to perform the de novo assembly of draft genomes for each river sediment strain.

The amino acid sequences of all genomes in FASTA format were acquired from the RAST seed server and annotated using BlastKOALA (Kanehisa et al. 2016) to examine the metabolic pathways, transport systems, and secretion systems of *Salmonella* in clinical and environmental strains. The metabolic pathways were subsequently recreated with KEGG Mapper (Kanehisa and Sato 2020) based on prior genome annotation.

Identification of antimicrobial resistance and virulence genes

To identify antimicrobial resistance genes in the genomes of *S.* Pomona and *S.* Montevideo strains, ResFinder v3.2 program was used (Zankari et al. 2012) to conduct a comprehensive search for both antimicrobial resistance genes and chromosomal mutations. A criterion was created wherein mutations exhibiting a minimum alignment of 70% and identity of 90% or higher were considered. In addition, we utilized the ABRicate software v0.8.13 (https://github.com/tseemann/abricate). This software was used to do a comparative assessment of resistance gene detection using the Comprehensive Antimicrobial Resistance Database (CARD), which can be accessed at: https://card.mcmaster.ca/home. Additionally, this software was utilized to identify virulence genes in the genomes of *S.* Montevideo and *S.* Pomona strains. The identified genes were compared with the virulence factor database VFDB, as described by Liu et al. (2019). The criteria employed were the existence of genes with a similarity level above 90% and a minimum alignment threshold of 70%.

Phylogenetic Inference

Phylogenetic relationships were established using a core alignment-based phylogenetic tree. The harvest suite alignment and visualization tool were utilized for this purpose (Treangen et al. 2014). The core genome was aligned using Parsnp (Treangen et al. 2014), considering a randomly selected reference. The output obtained was used as an input to create a multi-FASTA file using the HarvestTool software (Pisarenko et al. 2019). A Maximum Likelihood (ML) inference by RAxML was used to construct a phylogenetic tree considering the general time reversible model of nucleotide under the Gamma model of rate heterogeneity (GTRGAMMA) with a statistical support of 100 bootstraps replicates (Stamatakis 2015). Visualization and editing of the resulting tree were performed using the online application iTOL (Letunic and Bork 2021). The analysis was conducted for both the clinical and environmental strains.

Results

Prediction of Metabolic pathways and capabilities using KEGG mapper

The Montevideo and Pomona strains exhibited various metabolic pathways, including carbohydrate, energy, lipid, nucleotide, amino acid, glycan, cofactor and vitamin, and terpenoid and polyketide metabolism. The Embden Meyerhof-Parnas (EMP) pathway was present and complete in both environmental and clinical strains, while the Entner-Doudoroff (ED) pathway, an alternative to the EMP pathway, was also present in all strains (Figure 1). It is suggested that these strains may be capable to utilize various carbon substrates, such as d-glucuronate, galactose, d-galactate, ascorbate, glycogen, trehalose, N-acetyl-D-glucosamine, and glyoxylate, showing similar nutrient

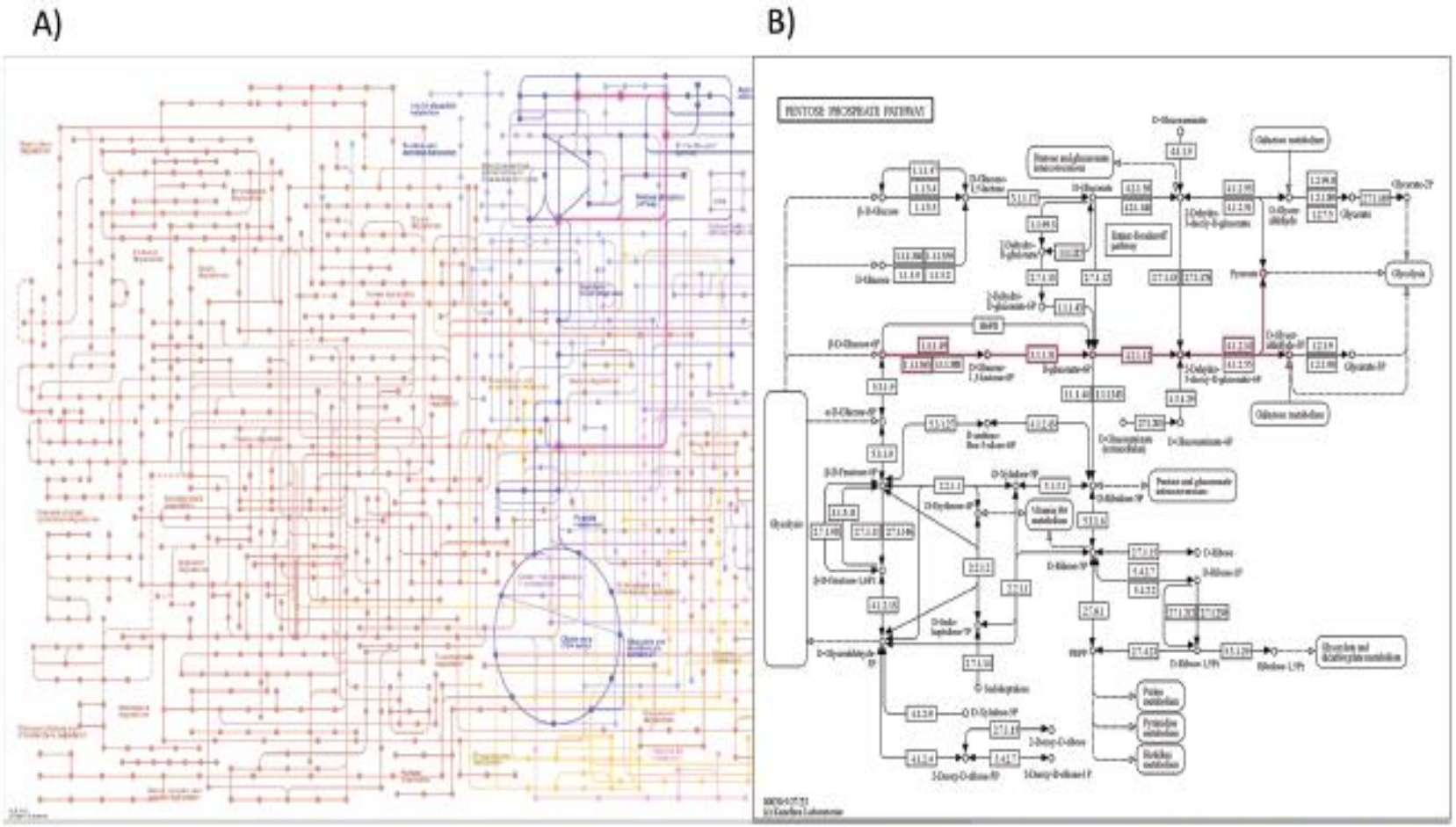

Figure 1. (a) Graphical illustration of the global metabolic pathways of *Salmonella*. Notably, the Entner-Doudoroff pathway is highlighted in pink, denoting its inclusion within this category of metabolic pathways. (b) A schematic representation of the Entner-Doudoroff pathway, a metabolic pathway for all strains of *S.* Pomona and *S.* Montevideo. The pink hue serves as an indicator of the presence of enzymes involved in the process, hence signifying the completion of the pathway.

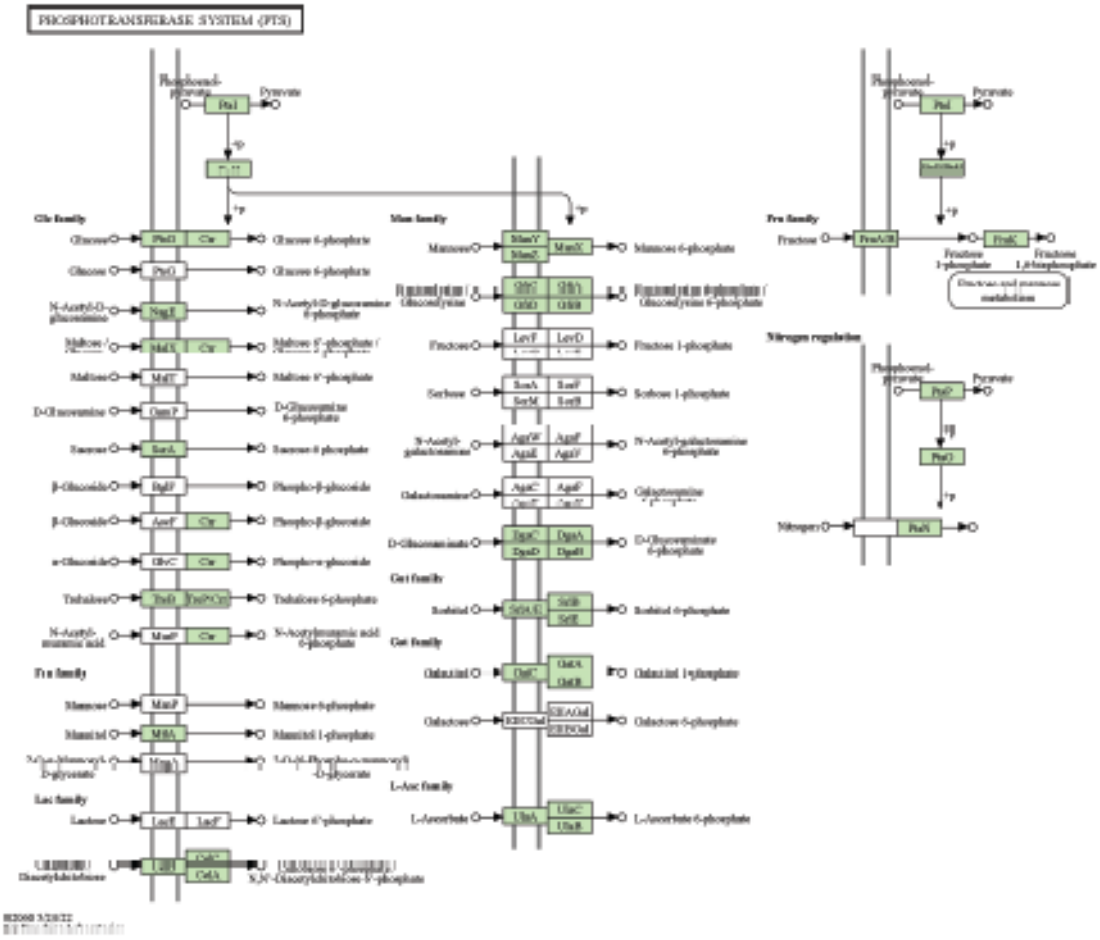

Figure 2. Phosphotransferase systems (PTS) for both environmental and clinical strains of S. Pomona and S. Montevideo. The green-colored blocks serve as an indication of the existence of the gene associated with the corresponding PTS.

acquisition features regardless of isolation source. Additionally, phosphotransferase systems (PTS) for glucose, fructose, lactose, mannose, glucitol, galactitol, l-ascorbate, and nitrogen regulation were detected in both environmental and clinical strains, indicating high metabolic adaptability advantageous for *Salmonella* survival, both outside and inside the host (Figure 2). Interestingly, the presence of genes associated with T1SS, T3SS, T6SS, secretory proteins, and twin arginine targeting proteins was detected in both environmental and clinical strains (Figure 3). Most environmental strains of *S.* Montevideo and Pomona possessed T4SS genes, which are related to membrane proteins and bacterial conjugation. These results indicate that these strains have the potential to adapt to different environments and suggest a high capacity for exchanging genetic material. In contrast, the clinical strains only displayed the presence of these genes in CFSAN023348, CFSAN034931 of *S.* Montevideo, and PNUSAS005642 of *S.* Pomona with only *VirB5* and *VirB6* genes associated with this particular secretion system.

Antimicrobial Resistance (AMR) genes present in environmental and clinical strains of S. Pomona and S. Montevideo

Figure 4 shows the presence of AMR genes in the S. Montevideo strains, which contain *AAC(6′)-ly*, *APH(3″)-lb*, and *APH(6)-ld* genes, known for their role in aminoglycoside resistance. The

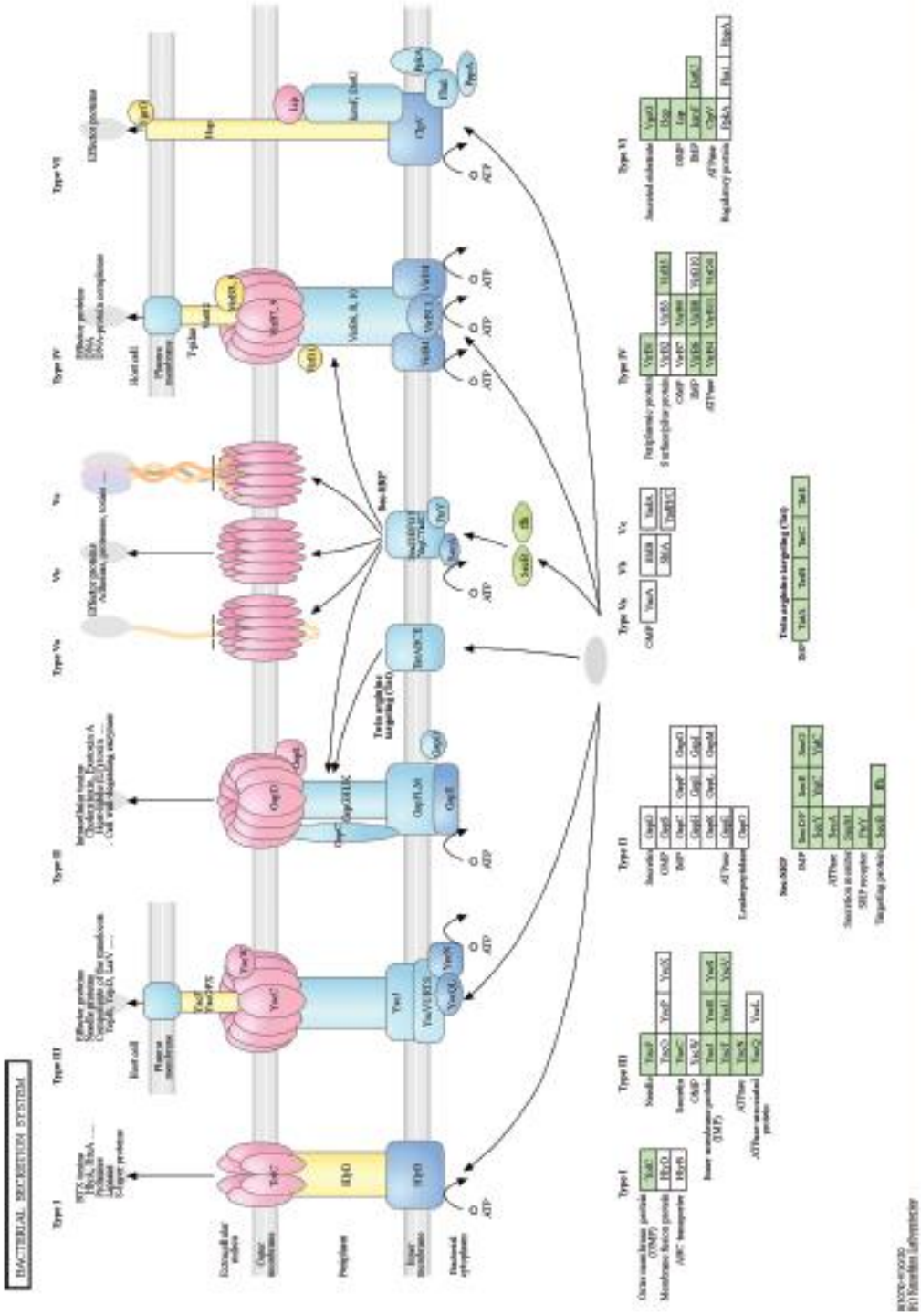

Figure 3. Secretion systems identified in environmental strains of *S. Montevideo* and *S. Pomona*. The green blocks serve as indicators for the existence of genes associated with the respective secretion system. In this case, the occurrence of genes associated with the T4SS were identified mostly in environmental strains.

AAC(6′)-Iy gene was found in both environmental and clinical strains, whereas the other two genes, *APH(3″)-Ib* and *APH(6)-Id*, were exclusive to four environmental strains. The presence of *ampH* in clinical and environmental *S.* Montevideo strains suggest possible resistance to β-lactams. Furthermore, clinical and environmental strains of *S.* Montevideo possess *acrA*, *acrB*, and *acrD* genes, which are associated with efflux pumps, as well as *emrA*, *emrB*, and *emrR* genes, which encode drug-binding proteins. The four environmental strains exhibited the presence of the *floR* gene, which is related to resistance to florfenicol. The *golS* and *kdpE* genes, which are associated with metal ion resistance and K+ transport, respectively, were detected in all strains of *S.* Montevideo, including clinical and environmental strains. The *marA* gene, which regulates an efflux pump, was absent in only three environmental strains. The *msbA* gene, which is involved in lipopolysaccharide biosynthesis, was present in all strains except one environmental strain. Notably, three environmental strains did not contain the *ramA* gene which suggest susceptibility to fluoroquinolone antibiotics. All clinical and environmental *S.* Montevideo genomes contained the *sdiA* gene, which is related to bacterial quorum sensing, suggesting the potential for this process to occur, which may lead to increased bacterial virulence. Only four environmental strains possessed the *sul2* and *tet(A)* genes, which are related resistance to sulfonamide and tetracycline, respectively. The *tolC* gene, which is involved in efflux pumps, was found in clinical and environmental *S.* Montevideo strains, suggesting the potential to efflux a wide range of antimicrobial compounds and toxins. Finally, the *yojI* gene, which is related to resistance against antimicrobial peptides, was absent in only two environmental strains.

The present study found that all clinical and environmental strains of *S.* Pomona had the genes *AAC(6′)-Iy*, *Escherichia coli-ampH*, and *FosA7*. Additionally, the strains had genes related to transcriptional regulation, efflux pumps, and different types of antibiotic resistance. The study also revealed that the environmental and clinical strains shared the *sdiA* and *tolC* genes but lacked the *yojI* gene. These results suggest that there is a similar potential for pathogenicity in both environmental and clinical strains of *S.* Pomona, regardless of the isolation source.

Virulence genes present in environmental and clinical strains of S. Pomona and S. Montevideo

The virulence gene profiles of the *S.* Montevideo strains are illustrated in Figure 4. All environmental and clinical strains of *S.* Montevideo were found to possess *acrB*, *espO*, *fepG*, *misL*, *ompA*, *sipD*, *slrp*, *sopA*, *sopB*, *sopD*, and *tae4*. These genes are involved in various functions related to pathogenicity, such as efflux pumps, T3SS, iron acquisition, adhesins, porins, translocations associated with pathogenicity islands, T4SS secretion effectors, and antibacterial amidases. In contrast, *mrkA*, *mrkB*, and *mrkC*, which play a role in fimbriae production, were only detected in three clinical strains of *S.* Montevideo. The allantoinase gene *allB* was found in only five clinical strains, whereas it was present in six environmental strains. Furthermore, *avrA*, which is associated with the T3SS effector, was detected in only three clinical strains and two environmental strains. The *entA* gene, which is involved in siderophore production, was absent in both environmental strains. Similarly, *entB* was absent only in one clinical strain. The two environmental strains lacked the *fepC* gene, which is associated with an inner membrane transporter protein. The *pipB2* gene, which is related to a secretion effector protein, was found in three clinical strains and two environmental strains. On the other hand, the *ratB* gene, which plays a role in the colonization of the human gut, was detected in eight clinical strains and seven environmental strains. Interestingly, the *sopD2* gene, which is responsible for encoding the secretory protein of the T3SS, was absent in three clinical strains. Moreover, *sopE2* was not detected in any clinical strain. Additionally, one clinical strain and two environmental strains lacked the protein effector-related gene, *sseL*. Furthermore, the *steC* gene, which is also associated with an effector protein, was absent in two environmental strains. Lastly, the *tlde1* gene, which is linked to T6SS, was identified in one clinical strain and five environmental strains. Figure 5 depicts the existence of several virulence

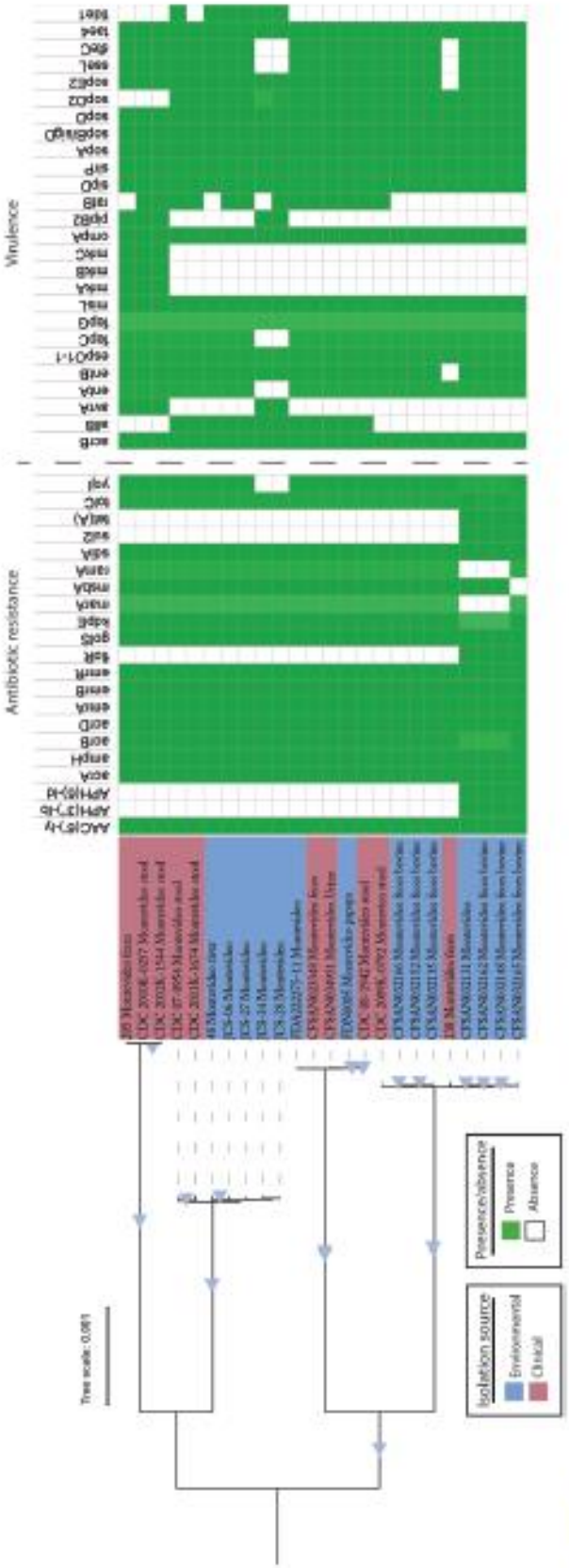

Figure 4. Phylogenetic tree representing the relationship between environmental and clinical strains of *S. Montevideo*, together with the presence of antibiotic resistance and virulence genes. Bootstrap values greater than 85% are shown in blue triangles.

genes in *S.* Pomona strains, both clinical and environmental. These genes include *acrB, fimA, fimC, fimD, fimF, fimW, fimY, fimZ, invA, invB, invC, ompA, orgA, rcsB, rpoS, sicA, sicP, sifA, ssax, sscA, sseK1, steB,* and *steC*. These genes are responsible for various virulence factors such as efflux pumps, fimbriae production, invasion regulation, porins, oxygen control, response regulation to biofilm development, sigma factors, T3SS, and secretion effector proteins associated with pathogenicity islands. Only two environmental strains had the *allB* gene, which is associated with allantoinase, while one clinical strain had the east1 gene, linked to enteroaggregative heat-stable toxin 1. Four environmental strains had genes such as *galf, hcp2, ipfE, sspH1, tlde1,* and *tssL*, and one environmental strain was missing *avrA* and *misL* genes, linked to an effector protein and T6SS. Only three clinical strains had an absence of *sopD2* and *steA* genes, while one environmental strain lacked the *tae4* gene, linked to antibacterial amidase activity.

Phylogenetic analysis

Phylogenetic analysis showed that the clinical and environmental strains of *S.* Montevideo and *S.* Pomona were genetically similar, with their core genomes exhibiting > 80% coverage for both species (Figures 4 and 5). This suggests a close evolutionary relationship between the clinical and environmental isolates of the *Salmonella* serotype. The constructed phylogenetic tree revealed distinct clustering of the clinical and environmental strains into four major clades within each serotype, with a combination of clinical and environmental isolates present in each clade. The arrangement of genomes in the clades was not influenced by the source of isolation, suggesting that there is no discernible difference between clinical and environmental strains solely based on their genetic relationship.

Discussion

The Montevideo and Pomona serotypes are known for their capacity to cause illnesses through contaminated food, water or contact with reptiles and have been implicated in recent outbreaks in the United States, Europe, Australia, and Asia. *S.* Montevideo has been associated with a growing number of cases of illness and outbreaks, while highly pathogenic *S.* Pomona strains are frequently isolated from reptilian species like snakes, lizards, and turtles, which could pose a risk to human health as reptiles are popular pets (Lalsiamthara and Lee 2017; Haendiges et al. 2021; Colon et al. 2022; Lee et al. 2022). This suggests that reptiles may serve as reservoirs for *S.* Pomona and contribute to the spread of the bacteria to humans and other animals (Song et al. 2023). These serotypes can also be found in environments such as in river water and sediments, which indicates a high risk for individuals to be infected by these serotypes via various routes.

In the present study the analysis 24 genomes of clinical and environmental *S.* Montevideo and *S.* Pomona strains revealed the presence of the alternative metabolic pathway Entner-Doudoroff in environmental and clinical strains of both serotypes, indicating its possible role in the survival and persistence of these strains. This pathway allows for the utilization of a broader range of carbon sources, enhancing the bacterium's ability to thrive in diverse environments (Patra et al. 2012; Flamholz et al. 2013). Understanding this metabolic pathway could aid in the development of targeted interventions to control the spread of these strains and alleviate their impact on public health. The versatility in sugar utilization, enabled by the presence of multiple PTS systems, allows these strains to adapt to different environments and exploit a wide range of ecological niches, enhancing their survival and persistence in different environments (Barabote and Saier 2005; Comas et al. 2008; Lim et al. 2019; Jeckelmann and Erni 2019). The PTS system involving N-acetyl-D-glucosamine was detected in all strains. Previous research has shown that environmental *Salmonella* strains in aquatic environments often use this alternative carbon source (Medrano-Félix et al. 2017; Gonzáles-López et al., 2021; Chaidez et al. 2020). In addition, the N-acetyl-D-glucosamine PTS has been associated with the induction of the *mdtEF* genes, which

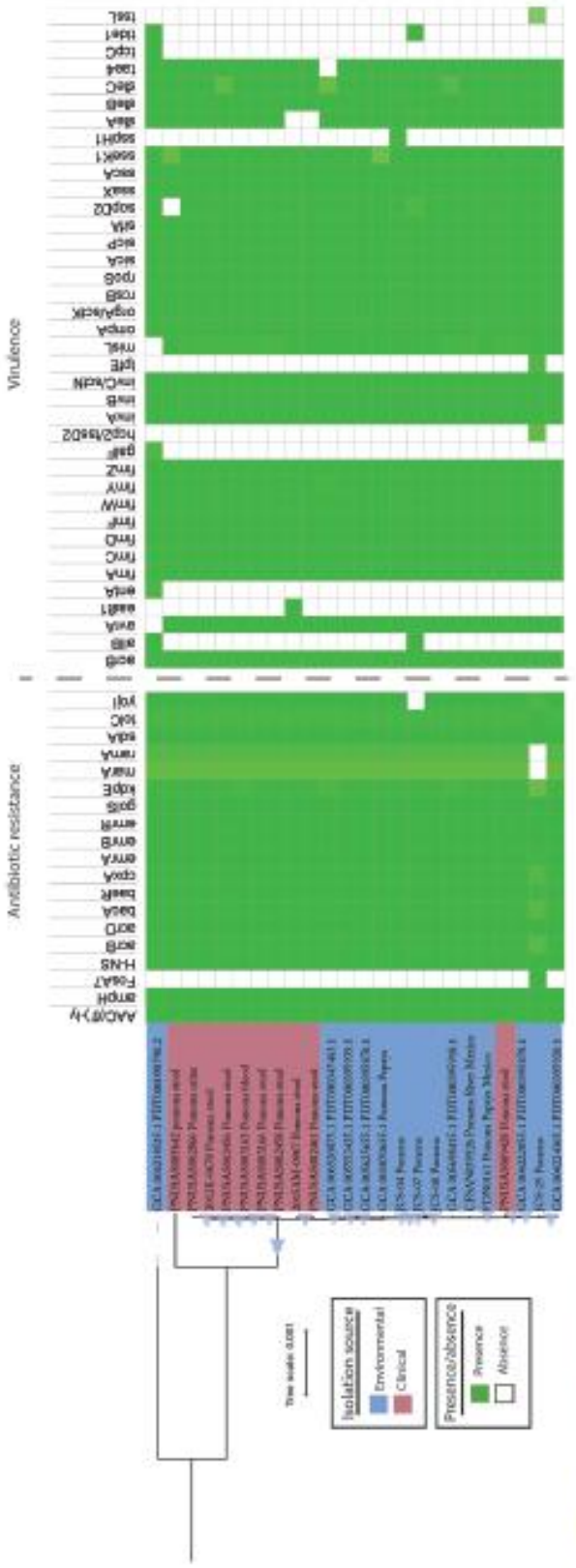

Figure 5. Phylogenetic tree representing the relationship between environmental and clinical strains of *S. Poromona*, together with the presence of antibiotic resistance and virulence genes. Bootstrap values greater than 85% are shown in blue triangles.

provide resistance to many antibiotics (Hirakawa et al. 2006). This finding highlights the potential of targeting this specific system to develop targeted therapies against these strains. Further investigation into the regulation of these PTS systems could provide valuable insights into how their expression can be manipulated to limit their ability to use various carbon sources and ultimately hinder their survival. Overall, studying these PTS systems has the potential to contribute to understanding their ecological adaptation and support the development of effective intervention strategies.

Several AMR genes were identified in both environmental and clinical *S.* Montevideo and Pomona strains. Notably, only the environmental strains of *S.* Montevideo contained the *APH(3")-lb* and *APH(6)-ld* genes, which are responsible for aminoglycoside antibiotic resistance (de Melo et al. 2021)). Additionally, the environmental strains of Montevideo were the only ones with the *floR* gene, which confers resistance to florfenicol and chloramphenicol. The presence of this gene in environmental strains indicates that antibiotic resistance can be transmitted between environmental and clinical settings (Cloeckaert et al. 2000; Nasim et al. 2015; Mei et al. 2021). The presence of specific genes such as *sul2* and *tet(A)* was exclusively observed in the environmental strains of *S.* Montevideo. These genes confer resistance to sulfonamide and tetracycline, two widely used antibiotics in human and animal medicine, respectively, because of their effectiveness against various types of bacteria (Pavelquesi et al. 2021). Multiple factors contribute to the acquisition of tetracycline resistance, such as mobile genetic elements, ribosome-binding site modifications, and chromosomal mutations (Adesoji et al. 2015; Sheykhsaran et al. 2019). These mechanisms lead to the spread and persistence of multidrug-resistant strains, which are highly adaptable and challenging to control.

The findings in the present study showed that environmental and clinical strains of *S.* Pomona shared similar AMR gene profiles, suggesting the possibility of resistance gene transmission between them. However, the environmental strain JCS-25, obtained from river sediments, exclusively displayed *FosA7*, a gene providing resistance to fosfomycin. Acquiring this gene is linked to the transmission of plasmids, implying the potential transfer of mobile elements to the environment where the strains were isolated. This environment may contribute to the spread of antibiotic resistance, and the potential transfer of plasmids carrying the gene implies that horizontal gene transfer is a crucial factor in the dissemination of antibiotic resistance in the environment (Rehman et al. 2017; Wang et al. 2021).

The analysis of virulence genes in *S.* Montevideo strains revealed that only clinical strains isolated from human feces contained *mrkA*, *mrkB*, and *mrkC* genes. The identification of these genes as components of the *mrk* operon and their association with type 3 fimbriae, which promote biofilm formation, suggests that clinical strains possess a high potential for persistence and colonization (Ong et al. 2008). On the other hand, environmental strains of *S.* Montevideo mostly carry the *allB* gene, which is involved in allantoinase synthesis and helps the bacterium adapt and survive in diverse environments using allantoin as a nitrogen source (Cusa et al. 1999; Hafez et al. 2017). The presence of these virulence and adaptive genes indicates that both clinical and environmental strains of *S.* Pomona have the potential to cause various illnesses in humans.

Both environmental and clinical strains of *Salmonella* possess T1SS, T3SS, and T6SS, which are critical for the delivery of virulence factors into host cells and contribute to the pathogenicity of *Salmonella*. The T4SS, which is found in many bacteria including *Salmonella*, was only observed in the environmental strains of *S.* Montevideo and Pomona. It plays a role in the direct delivery of proteins into host cells, promoting infection and survival (Backert and Meyer 2006; Galán and Waksman 2018; Bao et al. 2020). The presence of T4SS in environmental strains of *Salmonella* indicates their ability to infect both humans and other organisms. T4SS is also involved in the process of conjugation, a crucial mechanism for bacterial gene transfer. The presence of T4SS in these strains enhances their capacity to exchange genetic material with other bacteria, which may lead to the acquisition of antibiotic resistance genes (Alvarez-Martinez and Christie 2009; Christie et al. 2016; Bao et al. 2020). Additionally, T4SS plays a crucial role in *Salmonella's* ability to persist

within macrophages and epithelial cells by inhibiting the host's innate immune response (Khajanchi and Foley 2022). Understanding the mechanisms of T4SS in *Salmonella* pathogenesis can provide valuable insights for developing targeted therapies against this persistent drug-resistant pathogen. Phylogenetic analysis demonstrated that both clinical and environmental strains exhibited genetic similarities. This suggests that genetic factors contributing to *Salmonella* virulence, antibiotic resistance and metabolism are likely to be conserved across various environments (Zakaria et al. 2021). Furthermore, the phylogenetic tree revealed that certain clinical isolates were closely related to environmental isolates, suggesting the potential transmission of the pathogen between different reservoirs (Pornsukarom et al. 2018). Additionally, the short branches in the tree indicate a high degree of genetic relatedness between the strains, suggesting a recent common ancestor (Zhang et al. 2006). Elucidating the mechanisms by which *Salmonella* adapts to different environments can provide valuable insights into its evolutionary history and potential future threats. By examining the genetic similarities and differences between clinical and environmental strains, it is possible to identify the key genetic determinants that drive *Salmonella* pathogenicity and drug resistance.

Conclusion

In summary, analysis of the genomes of *S.* Montevideo and Pomona strains from various sources demonstrated substantial dissimilarities in their resistance to antibiotics and their capacity to transfer genetic material, emphasizing the need to comprehend the genomic attributes of these serotypes to control and prevent infections. Future research should focus on elucidating the mechanisms underlying the genomic versatility and selection advantage of *S.* Montevideo and *S.* Pomona strains in different environments, as well as exploring potential interventions to restrict the propagation of antibiotic-resistant strains.

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author, JAMF. The data are not publicly available due to restrictions of the repository.

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Supplementary Material

Supplementary table 1. Genome data of the strains of *S.* Montevideo and *S.* Pomona

Strain	Serotype	Source	Country	Bioproject	Biosample	Accession
48	Montevideo	River	Mexico	PRJNA313928	SAMN10261302	AADUWH000000000
205	Montevideo	Feces	USA	PRJNA275961	SAMN08514419	AAGFNU000000000
238	Montevideo	Feces	USA	PRJNA433689	SAMN08514452	QDLN00000000
CDC_07-0954	Montevideo	Stool	USA	PRJNA236677	SAMN02716884	CP017974
CDC_08-1942	Montevideo	Stool	USA	PRJNA236678	SAMN02716885	CP017975
CDC_2009K-0792	Montevideo	Stool	USA	PRJNA236679	SAMN02716886	CP020752
CDC_2010K-0257	Montevideo	Stool	USA	PRJNA236683	SAMN02716890	CP020912
CDC_2011K1674	Montevideo	Stool	USA	PRJNA236680	SAMN02716887	CP017976
CDC_2012K-1544	Montevideo	Stool	USA	PRJNA236681	SAMN02716888	CP017977
CFSAN023348	Montevideo	Feces	USA	PRJNA183847	SAMN02989064	AAFZIX000000000
CFSAN032131	Montevideo	Primal cut	Mexico	PRJNA275961	SAMN03577664	AALLXZ000000000
CFSAN032135	Montevideo	Feces bovine	Mexico	PRJNA275961	SAMN03577668	AALLXW000000000
CFSAN032148	Montevideo	Feces bovine	Mexico	PRJNA275961	SAMN03577681	AALLYB000000000
CFSAN032152	Montevideo	Feces bovine	Mexico	PRJNA275961	SAMN03577685	AALNLL000000000
CFSAN032162	Montevideo	Feces bovine	Mexico	PRJNA275961	SAMN03577695	AALLYA000000000
CFSAN032165	Montevideo	Feces bovine	Mexico	PRJNA275961	SAMN03577698	AALLYC000000000
CFSAN032166	Montevideo	Feces bovine	Mexico	PRJNA275961	SAMN03577699	AAGFNU000000000
CFSAN034931	Montevideo	Urine	USA	PRJNA183850	SAMN03795320	AAFWNP000000000
FDA222275-11	Montevideo	Ground shrimp	Mexico	PRJNA186035	SAMN02845266	AAIYIR000000000
FDN0005	Montevideo	Papaya	Mexico	PRJNA186035	SAMN02345540	AAFZGL000000000
JCS-06	Montevideo	River sediment	Mexico	PRJNA878598	SAMN30733580	JAOBQD000000000
JCS-27	Montevideo	River sediment	Mexico	PRJNA878598	SAMN30733581	JAOBQC000000000
JCS-28	Montevideo	River sediment	Mexico	PRJNA878598	SAMN30733582	JAOBQB000000000
JCS34	Montevideo	River sediment	Mexico	PRJNA878598	SAMN30733583	JAOBQA000000000
2012K-0678	Pomona	Stool	USA	PRJNA380913	SAMN06651530	CP020718
2015AM-0907	Pomona	Stool	USA	PRJNA230403	SAMN07409617	AANZQD000000000
CFSAN039526	Pomona	River water	Mexico	PRJNA186035	SAMN04155448	MEKB00000000

FDN0161	Pomona	Papaya	Mexico	PRJNA186035	SAMN02678682	RSHC00000000
GCA003870655.1	Pomona	Papaya	Mexico	PRJNA186035	SAMN02847577	RSKM00000000
GCA004218525.1	Pomona	Papaya	Mexico	PRJNA186035	SAMN02847574	AAACNW000000000
GCA004222855.1	Pomona	River water	Mexico	PRJNA313928	SAMN10261323	AAACWE000000000
GCA004224365.1	Pomona	River water	Mexico	PRJNA313928	SAMN10261336	AAACXQ000000000
GCA005553435.1	Pomona	River water	Mexico	PRJNA313928	SAMN10261330	AADEMF000000000
GCA005625655.1	Pomona	River water	Mexico	PRJNA313928	SAMN10261321	AADJRT000000000
GCA005698415.1	Pomona	River water	Mexico	PRJNA313928	SAMN10261342	AADOZR000000000
GCA006520875.1	Pomona	River water	Mexico	PRJNA480281	SAMN09638229	AAFNNW000000000
JCS-04	Pomona	River sediment	Mexico	PRJNA878598	SAMN30733584	JAOBPZ000000000
JCS-07	Pomona	River sediment	Mexico	PRJNA878598	SAMN30733585	JAOBPY000000000
JCS-08	Pomona	River sediment	Mexico	PRJNA878598	SAMN30733586	JAOBPX000000000
JCS-25	Pomona	River sediment	Mexico	PRJNA878598	SAMN30733587	JAOBPW000000000
PNUSAS002458	Pomona	Stool	USA	PRJNA230403	SAMN05232281	AAKJVP000000000
PNUSAS002461	Pomona	Stool	USA	PRJNA230403	SAMN05263637	AANQUJ000000000
PNUSAS002866	Pomona	Urine	USA	PRJNA230403	SAMN05390826	AANSYM000000000
PNUSAS003163	Pomona	Blood	USA	PRJNA230403	SAMN05558814	AAOIBV000000000
PNUSAS003166	Pomona	Stool	USA	PRJNA230403	SAMN05570514	AAOHWP000000000
PNUSAS005642	Pomona	Stool	USA	PRJNA230403	SAMN06045438	AAOINX000000000
PNUSAS009428	Pomona	Stool	USA	PRJNA230403	SAMN06556552	AAKRFW000000000

3. IMMUNOINFORMATIC APPROACH FOR DESIGNING A MULTI-EPITOPE VACCINE AGAINST NON-TYPHOIDAL SALMONELLOSIS USING STARVATION-STRESS RESPONSE PROTEINS FROM *Salmonella* ORANIENBURG

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Immunoinformatic Approach for Designing a Multi-epitope Vaccine Against Non-Typhoidal Salmonellosis Using Starvation-stress Response Proteins from *Salmonella* Oranienburg

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ABSTRACT:

Non-typhoidal *Salmonella* is responsible for gastrointestinal illnesses worldwide. Therefore, it is important to implement effective therapeutic interventions for preventing these diseases. Vaccines have proven highly efficacious in the treatment and prevention of several illnesses. Nevertheless, there is currently no authorized vaccine available for non-typhoidal salmonellosis. This study aimed to employ *In silico* techniques to develop a multi-epitope vaccine targeting non-typhoidal salmonellosis. Specifically, we focused on proteins associated with the starvation stress response (SSR) in *Salmonella* Oranienburg. The presence of these proteins is essential for the survival and disease of the host organism. The vaccine sequence was constructed utilizing B-cell and T-cell epitopes. Linkers, adjuvants, and PADRE sequences were used to establish connections between epitopes. The vaccine exhibited no allergenicity, toxigenicity, and a significantly high antigenicity score. Docking analysis conducted between the designed vaccine and the TLR-1, TLR-2, and TLR-4 receptors demonstrated favorable interactions and the potential to activate these receptors. In addition, it was found through immunological simulation testing that the vaccine elicits a robust immune response. The use of these proteins in the construction of a multi-epitope vaccine shows potential in terms of both safety and immunogenicity.

Key words: *Salmonella*, *In silico*, starvation-stress response, multi-epitope vaccine, molecular docking

1. Introduction

Non-typhoidal *Salmonella* (NTS) infections are a major cause of diarrheal diseases, resulting in approximately 93 million cases and 155,000 fatalities annually (Balasubramanian et. al., 2019). These infections can occur when contaminated food and water containing fecal matter are ingested (WHO, 2018). Serotype Oranienburg is a common cause of NTS infection, leading to significant illness and death worldwide. The Centers for Disease Control and Prevention (CDC) have reported several outbreaks caused by S. Oranienburg in relation to contaminated food consumption (CDC, 2006; CDC, 2014; CDC, 2021). In addition to foodborne transmission, NTS infections can be acquired through contact with infected animals or their environment (Mukherjee *et. al.*, 2019).

Natural environments such as water sources and soil can also harbor NTS serotypes such as Oranienburg, thereby increasing the risk of contamination and transmission to humans (Winfield & Groisman, 2003; Contreras-Soto et. al., 2019). When *Salmonella* is expelled and introduced into these habitats, it is exposed to a range of stress factors, such as temperature changes, pH fluctuations, and nutritional deprivation (Winfield & Groisman, 2003; Spector & Kenyon, 2012). The ability of *Salmonella* to survive in harsh conditions is facilitated by its starvation stress response (SSR), which allows it to adapt and endure (Spector, 1990; Kenyon et. al., 2002; Spector & Kenyon, 2012). This response includes alterations in gene expression, metabolism, and cellular structure to ensure the survival of the bacterium (Humphreys et. al., 2003; Kenyon et. al., 2007). The regulation and activation of this response are mediated by the cAMP receptor protein (CRP), the alarmone molecule (p)ppGpp, and sigma factors rpoS and rpoE (Spector & Kenyon, 2012).These regulatory proteins work together to coordinate the bacterial response to stress, ensuring that *Salmonella* can effectively adapt to its environment and continue to thrive (Spector & Kenyon, 2012; Kalia et. al., 2013; Franchini et. al., 2015). The activation of the SSR is crucial for the ability to persist in diverse habitats (Schofield et. al., 2018; Zhang et. al., 2020). This adaptation mechanism can also be related to survival within a host immune system, allowing *Salmonella* to evade detection and continue to grow and replicate causing severe infections (Monack et. al., 2004; Bernal-Bayard & Ramos-Morales, 2018). In addition to aiding in survival within a host organism, the activation of stress response proteins in *Salmonella* can also enhance virulence factors, increasing the ability of the bacterium to cause disease (Pradhan & Devi Negi, 2019).

The typical approach to managing *Salmonella* infections commonly involves antibiotic therapy to target and eliminate the bacterium from the host (Gut et. al., 2018). However, due to the increasing prevalence of antibiotic resistance in *Salmonella* strains, alternative treatment options such as vaccines are being explored and developed (Eng et. al., 2015; Micoli et. al., 2021). While there are several vaccines accessible for the treatment of typhoid *Salmonella* infections that demonstrate a certain degree of efficacy in pediatric populations, the existing literature and scientific research on vaccine designs for preventing non-typhoid salmonellosis are constrained in terms of its extent and comprehensiveness (Syed et. al., 2020). Multi- epitope vaccines that target multiple antigens of *Salmonella* are being studied as a potential solution to combat antibiotic resistance (Chand & Singh, 2021; Micoli et. al., 2021). These vaccines aim to induce a broader immune response and

provide protection against a variety of *Salmonella* strains and serotypes (Mahapatra et. al., 2021). Research in this area is ongoing, with the hope of developing effective vaccines that can prevent non-typhoid salmonellosis in both pediatric and adult populations. Additional studies are needed to further explore the potential of multi-epitope vaccines in addressing the growing threat of antibiotic-resistant *Salmonella* infections. One approach being explored is the use of immunoinformatics to identify conserved epitopes that are shared among different *Salmonella* serotypes (Tanu et. al., 2014; Shams et. al., 2019). In this regard the aim of the present study was to design and evaluate a novel multi-epitope vaccine against non-typhoidal salmonellosis based on the SSR related proteins from *S.* Oranienburg, using immunoinformatics tools.

2. Materials and Methods

2.1 Retrieval of amino acid sequences associated with the SSR in *Salmonella* Oranienburg

The present study utilized five distinct strains of *Salmonella* Oranienburg genomes sourced from the National Laboratory for Research in Food Safety (LANIIA) collection. These strains were obtained from river sediments in Culiacán, Sinaloa, México, and subsequently subjected to sequencing protocols, as previously documented by González-Torres *et al.*, (2023). The sequences can be found in GenBank and are associated with accession numbers JALPLR000000000, JALPLS000000000, JALPLT000000000, JALPLU000000000, and JALPLV000000000. These sequences are part of the BioProject PRNJNA831307. The genomes were annotated using the RAST seed server (https://rast.nmpdr.org/) (Overbeek et. al., 2013), which facilitated the identification of amino acid sequences associated with SSR-related genes, including *crp*, *cyaA*, *spoT*, *relA*, *rpoS*, and *rpoE*. These six amino acid sequences were further used to predict B-cell and T-Cell epitopes and to design the multi-epitope vaccine.

2. 2 Prediction and visualization of the SSR protein structures

The SWISS-MODEL server (https://swissmodel.expasy.org/) (Schwede, 2003) was used to estimate the structure of each protein. The evaluation of the models was conducted by employing the Qualitative Model Energy Analysis (QMEAN) score function, Global Model Quality Estimation (GMQE), and Ramachandran plots (Biasini et. al., 2014). For the CRP protein, the 4hzf.1.A template was utilized, which demonstrated 99.52% identity. Similarly, for the cyaA protein, the 5a61.1.A template was employed, showing 84.99% identity. For the spoT protein, the Q8ZL41.1.A template was used, exhibiting 100% identity. In the case of the relA protein, the 5kpw.1.w template was selected, displaying 96.10% identity. For the rpoS protein, the F5ZTT9.1.A template was selected, demonstrating 100% identity. Lastly, for the rpoE protein, the 1or7.1.A template was employed, exhibiting 99.48% identity. The 3D structures of the proteins were visualized using Discovery Studio Visualizer (Adhikari et. al., 2018).

2.3. Physicochemical characterization and secondary structure prediction of the SSR proteins and the designed vaccine

The ProtParam online server (https://web.expasy.org/protparam) (Gasteiger, 2003) was employed to predict the physicochemical characteristics of the SSR proteins and the designed vaccine, which includes the theoretical isoelectric point (pI), half-life, molecular weight (MW), instability, aliphatic index, and grand average of hydropathicity (GRAVY). The SOPMA Secondary Structure Prediction Method server (https://npsa-prabi.ibcp.fr/) (Geourjon & Deleage, 1995) was utilized to predict the secondary structures of the SSR proteins and the proposed vaccine, including the alpha helix, beta sheets, and random coils. In addition, the Protein Sol database (https:// prote in sol. manchester.ac.uk) (Hebditch et. al., 2017) was utilized to evaluate the vaccine's solubility. The scaled solubility value (QuerySol) indicates the expected solubility. Because the population average of the experimental dataset (PopAvrSol) is 0.45, any value greater than this is expected to have a higher solubility than the average soluble *E. coli* protein.

2.4 B-Cell Epitopes prediction and assesment

The IEDB (https://tools.iedb.org/main/) (Dhanda et. al., 2019) and ABCpred (https://webs.iiitd.edu.in/raghava/abcpred/) (Malik et. al., 2021) servers were utilized to identify potential B-cell epitopes from SSR-related proteins. The graphical representation of B-cell epitope positions was achieved using Kolaskar and Tongaonkar antigenicity, Emini surface accessibility, Karplus and Schulz flexibility prediction, Chou-Fesman beta-turn, and Parker hydrophilicity tests. Each SSR protein was predicted to possess 16 residue-long linear epitopes. The start and end positions of the B-cell epitopes predicted is shown in supplementary table 1.

2.5 Prediction and assessment of CTL and HTL epitopes

The IEDB web server (http://tools.iedb.org/main/) was used to predict the Cytotoxic T lymphocytes (CTLs) epitopes (Dhanda et. al., 2019). This tool to predicts epitopes for major histocompatibility complex class I (MHC-I) utilizing the entire set of human leukocyte antigen (HLA) alleles. Additionally, the tool was used to predict Helper T lymphocyte (HTL) epitopes. The consensus approach of 2.22, was employed to determine the HLA allele reference sets and MHC-II-binding epitopes. The IL4pred (https://webs.iiitd.edu.in/raghava/il4pred/index.php) (Dhanda et. al., 2013), IL10pred (https://webs.iiitd.edu.in/raghava/il10pred/) (Singh et. al., 2021), and IFNepitope (http://crdd.osdd.net/raghava/ifnepitope/) (Ali et. al., 2021) servers were used to evaluate the potential for interleukin-4 (IL-4), interleukin-10 (IL-10), and gamma interferon (IFN-γ) induction. Furthermore, the toxicity, allergenicity, and antigenicity profiles were assessed using the ToxinPred server (https://webs.iiitd.edu.in/raghava/toxinpred/index.html) (Gupta et. al., 2013), AllergenFP 1.0 (https://www.ddg-pharmfac.net/AllergenFP/index.html) (Dimitrov et. al., 2013),

and Vaxijen 2.0 (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) (Doytchinova & Flower, 2007), respectively. An antigenicity index threshold of 0.4 was utilized for prediction. Epitopes that met all the specified criteria were selected for further study.

2.6 Structure prediction of the epitopes and HLA Molecules

In this study, the TrRosetta prediction server (https://yanglab.qd.sdu.edu.cn/trRosetta/) (Du et. al., 2021) was used to predict the 3D structure of the selected epitopes derived from the SSR proteins. Additionally, refinement was conducted using the GalaxyRefine Web server (https://galaxy.seoklab.org/) (Ko et. al., 2012). The models that exhibited the highest level of confidence were selected. The 3D structures of HLA molecules were obtained from the RCSB protein databank (https://www.rcsb.org/).

2.7 Molecular docking

The GalaxyPepDock web server (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=PEPDOCK) (Lee et. al., 2015) was used for the docking analysis between T-cell epitopes and their associated HLA alleles. This tool utilizes algorithms to predict the interactions between proteins and peptides. Ten conformational structures were created by evaluating their docking and interaction similarity scores with the alleles. Moreover, this server is can predict the essential amino acid residues involved in the binding of HLA molecules to epitopes (Morsy & Morsy, 2021; Mukhtar et. al., 2022). The dock models with better scores were selected for visualization using Discovery Studio Visualizer. The Cluspro 2.0 server (https://cluspro.bu.edu/login.php?redir=/home.php) (Kozakov et. al., 2017) was used for molecular docking between MHC-II epitopes and their corresponding alleles. Furthermore, this tool was also utilized for docking between the designed multi-epitope vaccine and Toll-like receptors (TLR) 1, 2, and 4. The 3D structures of the TLR receptors were obtained from the RCSB Protein Data Bank service (https://www.rcsb.org/). The interactions between the vaccine and TLR docked complexes was analyzed using the PDBsum Database (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/).

2.8 *In silico* multi-epitope vaccine design and assesment

To design a multi-epitope vaccine utilizing SSR proteins, an AAY linker was employed to establish connections between the selected B-cell and HTL epitopes. Similarly, the CTL epitopes were connected using a GPGPG linker. Furthermore, to increase immunogenicity, the ribosomal protein L7/L12, known to activate the TLR-4, was fused to the N-terminus of the vaccine sequence using an EAAAK linker. Additionally, a 13-amino acid sequence, AKVAAWTLKAAAG, with pan HLA DR-binding epitope (PADRE) properties, was linked to the aforementioned fusion construct using

the HEYGAEALERAG linker (Solanki et. al., 2019; Chand & Singh, 2021). The BLASTp tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) was utilized to evaluate the similarity between the designed vaccine and the human proteome, with the aim of mitigating the risk of an autoimmune response. Tertiary structure of the vaccine construct was predicted using the GalaxyWeb server and the TrRosetta server. The toxicity, allergenicity, antigenicity, and immunogenicity of the vaccine were assessed using the ToxinPRed server, AllergenFP 1.0, Vaxijen 2.0, and IEDB server, respectively.

2.9 Population coverage of the vaccine construct

The selected epitopes used for the vaccine construct, along with the corresponding HLA alleles (Class I and Class II), were entered into the population coverage analysis tool of the IEDB database (http://tools.iedb.org/population/) (Dhanda et. al., 2019). The default parameters were used, which include 109 countries spanning 16 distinct geographical regions. The population coverage analysis tool determines the proportion of individuals who are expected to have a response to a specific set of epitopes, taking into account their known MHC constraints (Bui et. al., 2006). The program calculates the following for each individual epitope: (i) the predicted population coverage, (ii) the average number of epitope hits per HLA combination recognized by the populations, and (iii) the minimum number of epitope hits per HLA combination recognized by 90% of the population (PC90). These computations are performed using HLA genotypic frequencies, assuming that there is no association between HLA loci.

2.10 Modeling of the multi-epitope vaccine

The structure of the multi-epitope vaccine was predicted using TrRosetta web server. The protein structure was constructed with minimal direct energy. A common constraint is the interresidual distance distribution and orientation predicted by a deep residual neural network. Homogeneous models have been used in the domain of network prediction to enhancing precision in relation to fundamental aims (Zafar et. al., 2022).

2.11 Tertiary structure refinement, and validation

The vaccine was improved using the GalaxyRefine web server to enhance structural integrity. Additionally, the verified structure was assessed using the PROCHECK server (https://saves.mbi.ucla.edu.) (Laskowski et. al., 1993; Rathore & Shakya, 2014). A Ramachandran plot was constructed to assess the proportion of residues located inside areas that were considered favorable. In addition, ERRAT scores were used to validate the structures.

2.12 Immune simulation

To evaluate the immune response profile in the *In silico* model, the amino acid sequence of the vaccine was uploaded to the C-ImmSim server (https://kraken.iac.rm.cnr.it/C-IMMSIM/) (Rapin et. al., 2011). The C-ImmSim service employs a position-specific scoring matrix (PSSM) and machine learning methodologies to evaluate the humoral and cellular reactions to vaccine models (Castiglione et. al., 2021). The activation of the mammalian immune response in real-life is determined by three distinct anatomical components: the bone marrow, thymus, and tertiary lymphatic organs, as facilitated by the PSSM mechanism. To induce immunological stimulation, all simulation parameters were configured according to default defaults. A series of three injections were administered at time steps of 1, 84, and 168 h, with a four-week interval between each injection. The total number of simulation steps conducted was 1050 (Beikzadeh, 2023).

2.13 Molecular Dynamics Simulation

The Amber22 software (Shah et. al., 2022) was used to conduct MD simulations of the vaccine construct and the complexes with TLR-1, TLR-2, and TLR-4 receptors. Topological parameters of the vaccine were generated using the Tleap module with the ff19SB force field (Tian et. al., 2019). The system was subsequently solvated and neutralize, using the TIP3P water model and the addition of counter ions, respectively (Grotz et. al., 2021). The prepared system of vaccine was minimized using the steepest descent algorithm, followed by the conjugate gradient algorithm. The minimized system was then gradually heated from 0 to 300 K in NVT and NPT equilibration. The Particle Mesh Ewald method was employed for computing long-range electrostatic interactions with the cutoff of 8 Å and the SHAKE algorithm was used to maintain the hydrogen bonds constraints. The final production of 100 ns was carried out with an integration time of 2.0 fs and the coordinates were saved after every 1 ps. The trajectories were analyzed by plotting the root mean square deviation (RMSD), root mean square fluctuation (RMSF) using CPPTRAJ module (Srivastava et. al., 2021).

2.14 Binding Free Energy

In order to evaluate the relative binding energies of the vaccine in complex with immune receptors, MM/PBSA method was utilized (Genheden & Ryde, 2015). In this connection, the MMPBSA.py python script implemented in the Amber software was utilized, and the calculation relied on 1000 snapshots extracted from the last 10 ns of the trajectories, employing the following equation.

$$\Delta G(bind) = \Delta G(complex)-[\Delta G(receptor)+ \Delta G(ligand)]$$

2.15 Codon optimization and *In silico* cloning

The essential significance of the vaccine model's success in the cloning and expression cannot be overstated, particularly in the context of in vitro manufacture. To start the optimization of vaccine manufacture, the Java Codon Adaptation Tool (JCat) (https://www.jcat.de/) (Grote et. al., 2005) was employed to ascertain the most favorable protein expression in *E. coli* strain K12. The determination of protein expression levels relies on the evaluation of codon adaptation index (CAI) values that exceed 0.8, as well as the consideration of GC contents within the range of 30% to 70%. The enhanced vaccine sequence was subsequently inserted into the *E. coli* plasmid vector pET-30a (+) at the *XhoI* and *BssHII* restriction sites, using the SnapGene software (Beikzadeh, 2023; Priyadarsini et. al., 2021).

3. Results

3.1 Prediction and quality assessment of 3D SSR protein structure

Protein structure prediction was carried out using SWISS-MODEL through sequence homology analysis (Figure 1). Due to the presence of significant similarity in the amino acid sequences of the SSR-related proteins across all strains of *S.* Oranienburg, a representative strain was selected at random to obtain the necessary sequences for subsequent prediction analysis. The predictive accuracy of the models for CRP, cyaA, relA, rpoE, rpoS, and spoT proteins was assessed using of the QMEAN, GMQE, and Ramachandran plot scores. Hence, the findings indicate satisfactory quality for the proteins that were modeled in the present investigation (Table 1).

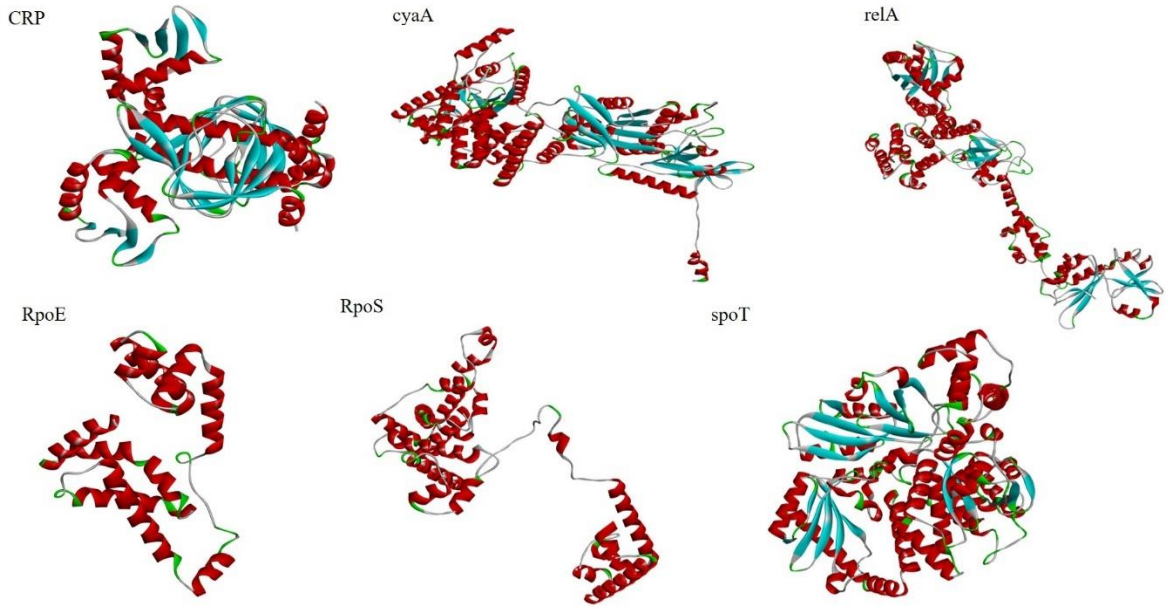

Figure 1. Protein structures related to the regulation and activation of the starvation stress sesponse (SSR) of *Salmonella* Oranienburg. Prediction was made with SWISS-Model Server and vizualized in Discovery Studio.

Table 1. Quality values of SSR proteins. Showing the QMEAN, GMQE and Ramachandran preferred region scores.

Protein	QMEAN	GMQE	Ramachadran Favored regions
CRP	0.85	0.89	98.74%
cyaA	0.9	0.95	99.53%
relA	0.7	0.76	86.40%
rpoE	0.78	0.85	98.92%
rpoS	0.78	0.8	93.45%
spoT	0.64	0.38	94.71%

3.2 Prediction of secondary structures and physicochemical characteristics of SSR-related proteins

Table 2 displays the physicochemical attributes of the SSR proteins. The proteins used are composed of amino acids ranging from 191 to 744 in length, with molecular weights spanning from 7915.66 g/mol to 84098.88 g/mol. Additionally, their isoelectric points ranged from 4.85 to 8.81, with positively charged residues varying from 25 to 103, and negatively charged residues ranging from 24 to 94. Analysis of secondary structures indicates that the proteins under investigation

consisted of many elements, including alpha helices, extended strands, beta sheets, and random coils. Among these structures, the alpha helix is shown to be the most prevalent in proteins. The results are shown in Supplementary Figure 1.

Table 2. Physicochemical characteristics of the SSR proteins and secondary structures

Protein	No. of Amino acids	MW	pI	PR	NR	Alpha Helix	Extended Strand	Beta sheet	Random Coils
relA	744	84098.88 g/mol	6.31	103	93	56.45%	11.56%	5.78%	26.21%
spoT	703	7915.66 g/mol	8.81	84	94	51.78%	14.94%	51.69%	27.60%
CRP	210	23656.43 g/mol	8.38	26	24	43.81%	23.33%	7.02%	25.24%
rpoS	330	37932.74 g/mol	4.85	46	65	65.45%	7.27%	6.97%	20.30%
rpoE	191	21711.74 g/mol	5.38	25	28	68.06%	5.24%	4.71%	21.99%
cyaA	433	48511.35 g/mol	6.19	47	54	59.82%	9.24%	4.62%	26.33%

3.3 B-Cell epitopes identification and structure visualization

B-cell epitopes were predicted and visually illustrated using several prediction techniques from the IEDB service in the ElliPro program (Supplementary Figure 2-7). Following the assessment of antigenicity, toxicity, allergenicity, and immunogenicity, three 16-mer epitopes were selected for each of the SSR-proteins to be utilized in further vaccine development (Table 3). Figure 2 displays the 3D structure of the epitopes inside their respective SSR-associated protein.

Table 3. Predicted B-Cell epitopes from the SSR related proteins.

SSR Protein	B-Cell epitopes	Antigenicity	Allergenicity	Toxicity	Immunogenicity
CRP	DGMQIKITRQEIGQIV	0.7427	Probable Non-Allergen	Non-Toxin	0.09004
	TLEWFLSHCHIHKYPS	0.7173	Probable Non-Allergen	Non-Toxin	0.15649
	AKQPDAMTHPDGMQIK	1.1063	Probable Non-Allergen	Non-Toxin	-0.23226
cyaA	RVSGQTWGLFFERLNV	0.9049	Probable Non-Allergen	Non-Toxin	0.50438
	FPLRLPAPTPKALYSP	1.267	Probable Non-	Non-Toxin	0.16764

			Allergen		
	HDRFTYGSSFINFNLP	0.7327	Probable Non-Allergen	Non-Toxin	0.21516
relA	EELLAAIGGGDIRLNQ	1.0513	Probable Non-Allergen	Non-Toxin	0.47682
	AYHIHSDVGHRCIGAK	1.2225	Probable Non-Allergen	Non-Toxin	0.31576
	LGPGGKTVEIQIRTKQ	1.4861	Probable Non-Allergen	Non-Toxin	0.13088
rpoE	GDSAFYTWLYRIAVNT	0.9779	Probable Non-Allergen	Non-Toxin	0.58306
	VDAIEAENFESGGALK	0.9916	Probable Non-Allergen	Non-Toxin	0.51526
	WLYRIAVNTAKNYLVA	0.6086	Probable Non-Allergen	Non-Toxin	0.12336
rpoS	KFDPERGFRFSTYATW	0.4818	Probable Non-Allergen	Non-Toxin	0.38426
	TAEEEVYFARRALRGD	0.4248	Probable Non-Allergen	Non-Toxin	0.61139
	VHDLNEDAEFDENGVE	0.6804	Probable Non-Allergen	Non-Toxin	0.52417
spoT	KEHGETSTTAQIRAQR	1.6647	Probable Non-Allergen	Non-Toxin	0.16336
	TVEIITAPGARPNAAW	0.412	Probable Non-Allergen	Non-Toxin	0.52161
	HGHLPIKGADGVLITF	1.0964	Probable Non-Allergen	Non-Toxin	0.1986

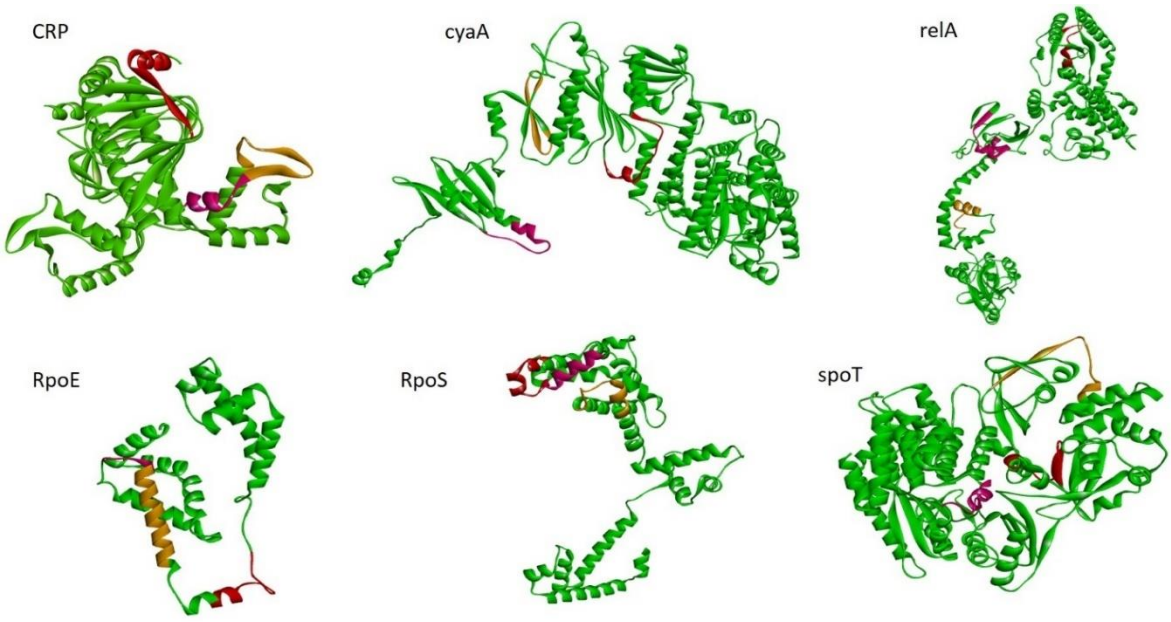

Figure 2. Visualization of B-Cell epitopes in the SSR proteins. (A) CRP protein with their 3 epitopes, DGMQIKITRQEIGQIV (pink), TLEWFLSHCHIHKYPS (red), and AKQPDAMTHPDGMQIK (orange), (B) cyaA protein with their 3 epitopes, RVSGQTWGLFFERLNV (orange), FPLRLPAPTPKALYSP (red), and HDRFTYGSSFINFNLP (pink), relA protein with their 3 epitopes EELLAAIGGGDIRLNQ (orange), AYHIHSDVGHRCIGAK (pink), and LGPGGKTVEIQIRTKQ (red), rpoE protein with their 3 epitopes GDSAFYTWLYRIAVNT (pink), VDAIEAENFESGGALK (red), and WLYRIAVNTAKNYLVA (orange), rpoS protein with their 3 epitopes KFDPERGFRFSTYATW (orange), TAEEEVYFARRALRGD (pink), and VHDLNEDAEFDENGVE (red), and the spoT protein with their 3 epitopes KEHGETSTTAQIRAQR (pink), TVEIITAPGARPNAAW (orange), and HGHLPIKGADGVLITF (red).

3.4 T-Cell epitopes prediction

The IEDB MHC-I allele binding prediction tool was used to predict T cell epitopes. A comprehensive collection of HLA allele references was used to identify MHC-class I epitopes derived from SSR proteins. The epitopes were assessed for allergenicity, antigenicity, and toxicity. The results of the predicted epitopes are presented in Table 4. Three epitopes were selected for each of the SSR proteins, except for rpoE, in which two epitopes were selected. The IEDB tool was also utilized to predict MHC-II epitopes, and epitopes with lower scores were selected for evaluating antigenicity, allergenicity, toxicity and the capacity to induce IFN-γ, IL-4, and IL-10. Table 5 shows the results of the predicted MHC-II epitopes and their assessment.

Table 4. Selected MHC-I CTL epitopes with their corresponding HLA molecule from the SSR related proteins

SSR Proteins	Epitopes	Alleles	Antigenicity	Allergenicity	Toxicity
CRP	EVAEISYKKF	HLA-A*68:01	0.6021	Probable Non-Allergen	Non-Toxin
	CEVAEISYKK	HLA-A*11:01	0.6007	Probable Non-Allergen	Non-Toxin
	SAHGKTIVVY	HLA-B*15:01	0.9057	Probable Non-Allergen	Non-Toxin
relA	RSGHEDRIAW	HLA-B*57:01	1.6784	Probable Non-Allergen	Non-Toxin
	EVYGRPKHIY	HLA-A*68:01	0.8085	Probable Non-Allergen	Non-Toxin
	KTVEIQIRTK	HLA-A*11:01	1.5877	Probable Non-Allergen	Non-Toxin
spoT	EIYVFTPEGR	HLA-A*68:01	0.5131	Probable Non-Allergen	Non-Toxin
	HSIMDIYAFR	HLA-A*68:01	0.7749	Probable Non-Allergen	Non-Toxin
	GQTRSSGEPY	HLA-B*15:01	0.9953	Probable Non-Allergen	Non-Toxin
rpoS	LRLNERITSV	HLA-A*02:03	0.9914	Probable Non-Allergen	Non-Toxin
	ELLSQGATQR	HLA-A*68:01	0.7059	Probable Non-Allergen	Non-Toxin
	RMIESNLRLV	HLA-A*02:06	0.664	Probable Non-Allergen	Non-Toxin
rpoE	AENFESGGAL	HLA-A*68:01	0.5435	Probable Non-Allergen	Non-Toxin
	RVQKGDQKAF	HLA-B*15:01	1.0473	Probable Non-Allergen	Non-Toxin
cyaA	YSLLPTLLHY	HLA-B*57:01	0.7515	Probable Non-Allergen	Non-Toxin
	RLHDGEIVSF	HLA-B*15:01	0.5945	Probable Non-Allergen	Non-Toxin
	SFINFNLPQF	HLA-A*24:02	0.6954	Probable Non-Allergen	Non-Toxin

Table 5. Selected MHC-II HTL epitopes with their corresponding HLA molecule, from the SSR related proteins

SSR Proteins	Epitopes	Alleles	Antigenicity	Allergenicity	Toxicity	IFN-γ	IL-4	IL-10
CRP	PDILMRLSSQMARRL	HLA-DRB1*04:01	0.7939	Probable Non-Allergen	Non-Toxin	Negative	Inducer	Inducer
	DILMRLSSQMARRLQ	HLA-DRB1*04:01	0. 7019	Probable Non-Allergen	Non-Toxin	Negative	Inducer	Inducer
relA	ELEDYCFRYLHPAEY	HLA-DRB5*01:01	0.8278	Probable Non-Allergen	Non-Toxin	Positive	Inducer	Inducer
	EILSTLSMDIDTLRA	HLA-DRB3*01:01	0.7427	Probable Non-Allergen	Non-Toxin	Positive	Inducer	Non-Inducer
	EDYCFRYLHPAEYKR	HLA-DRB5*01:01	0.628	Probable Non-Allergen	Non-Toxin	Negative	Inducer	Inducer
spoT	WLNFVVSSKARAKIR	HLA-DRB5*01:01	0.6629	Probable Non-Allergen	Non-Toxin	Positive	Inducer	Inducer
	AWLNFVVSSKARAKI	HLA-DRB5*01:01	0.4336	Probable Non-Allergen	Non-Toxin	Positive	Inducer	Inducer
	NFVVSSKARAKIRQL	HLA-DRB5*01:01	0.4986	Probable Non-Allergen	Non-Toxin	Positive	Inducer	Non-Inducer
	AAWLNFVVSSKARAK	HLA-DRB5*01:01	0.4931	Probable Non-Allergen	Non-Toxin	Negative	Inducer	Inducer
rpoS	ERVRQIQVEGLRRLR	HLA-DRB5*01:01	0.431	Probable Non-Allergen	Non-Toxin	Positive	Inducer	Inducer
	LPIHIVKELNVYLRT	HLA-DRB1*15:01	0.726	Probable Non-Allergen	Non-Toxin	Positive	Inducer	Inducer
rpoE	YRIAVNTAKNYLVAQ	HLA-	0.4827	Probable	Non-	Positive	Non-	Non-

		DRB1*04:01		Non-Allergen	Toxin		Inducer	Inducer
	KAFNLLVVRYQHKV A	HLA-DRB1*11:01	0.7229	Probable Non-Allergen	Non-Toxin	Negative	Non-Inducer	Inducer
	QKAFNLLVVRYQHK V	HLA-DRB5*01:01	0.6717	Probable Non-Allergen	Non-Toxin	Positive	Non-Inducer	Non-Inducer
cyaA	EVSFFLIDENRFRHN	HLA-DRB3*01:01	0.4281	Probable Non-Allergen	Non-Toxin	Negative	Non-Inducer	Inducer
	VEVSFFLIDENRFRH	HLA-DRB3*01:01	0.5669	Probable Non-Allergen	Non-Toxin	Negative	Non-Inducer	Inducer
	IVNLEYDPTAAFRNK	HLA-DRB3*01:01	0.4705	Probable Non-Allergen	Non-Toxin	Negative	Inducer	Inducer

3.5 Molecular Docking between predicted CTL and HTL epitopes with MHC alleles

Molecular docking analysis was conducted to investigate the interaction between the proposed vaccine and toll-like receptors (TLR-1, TLR-2, and TLR-4), as well as between the cytotoxic T lymphocyte (CTL) and helper T lymphocyte (HTL) epitopes and their respective related alleles. The data obtained from the docking of CTL epitopes are presented in supplementary table 2. For protein-peptide interactions, it is necessary for the similarity score to be approximately 0.96, likewise, the interaction similarity score should be approximately 50, while the accuracy must exceed 0.70. The docking models of the selected HTL epitopes with their corresponding MHC-II alleles, which exhibited the lowest binding energy as assessed by the weighted score, were selected (Supplementary Table 3).

Discovery Studio Visualizer was used to perform epitope docking. The results were evaluated using this tool to establish exactly how the contact between the epitopes and their corresponding MHC alleles occurred. CTL epitopes from six SSR-related proteins developed persistent connections with their corresponding MHC-I alleles. The CRP protein exhibited binding to HLA-A*68:01 through the EVAEISYKKF epitope, HLA-A*11:01 through the CEVAEISYKK epitope, and HLA-B*15:01 through the SAHGKTIVVY epitope (see Supplementary Figure 8). The relA protein was shown to have the RSGHEDRIAW epitope bound to HLA-B*57:01, EVYGRPKHIY epitope bound to HLA-A*68:01, and KTVEIQIRTK epitope bound to HLA-A*11:01, as shown in Supplementary Figure 9. For the spoT protein, the epitopes EIYVFTPEGR and HSIMDIYAFR were found to bind HLA-A*68:01, whereas the epitope GQTRSSGEPY was observed to bind HLA-B*15:01(Supplementary Figure 10). The rpoS protein was shown to have binding affinity with LRLNERITSV epitope HLA-A*02:03, ELLSQGATQR epitope to HLA-A*68:01, and RMIESNLRLV epitope HLA-A*02:06 (Supplementary Figure 11). For the rpoE protein, AENFESGGAL and RVQKGDQKAF epitopes bounded HLA-A*68:01 and HLA-B*15:01, respectively (Supplementary Figure 12). The cyaA protein exhibited binding to HLA-B*57:01 through the YSLLPTLLHY epitope, to HLA-B*15:01 through the RLHDGEIVSF epitope, and to HLA-A*24:02 through the SFINFNLPQF epitope (Supplementary Figure 13).

HTL epitopes from the six SSR related proteins also showed stable interactions with their corresponding MHC-II alleles. The CRP protein bounded to HLA-DRB1*04:01 through the epitopes PDILMRLSSQMARRL and DILMRLSSQMARRLQ (Supplementary Figure 14). The relA protein bounded to the HLA-DRB5*01:01 molecule through the ELEDYCFRYLHPAEY

epitope, to the HLA-DRB3*01:01 molecule through the EILSTLSMDIDTLRA epitope, and to the HLA-DRB5*01:01 molecule through the EDYCFRYLHPAEYKR epitope (Supplementary Figure 15). The spoT protein was shown to have a binding affinity to HLA-DRB5*01:01 for the following epitopes: WLNFVVSSKARAKIR, AWLNFVVSSKARAKI, NFVVSSKARAKIRQL, and AAWLNFVVSSKARAK (Supplementary Figure 16). The rpoS protein was shown to bind to the HLA-DRB5*01:01 allele through the ERVRQIQVEGLRRLR epitope, whereas the HLA-DRB1*15:01 allele was observed to bind to the LPIHIVKELNVYLRT epitope (Supplementary Figure 17). The rpoE protein bounded to different epitopes when interacting with different HLA molecules. Specifically, the YRIAVNTAKNYLVAQ epitope bounded to HLA-DRB1*04:01, the KAFNLLVVRYQHKVA epitope bounded to HLA-DRB1*11:01, and the QKAFNLLVVRYQHKV epitope bounded to HLA-DRB5*01:01 (Supplementary Figure 18). Finally, the cyaA protein bounded to the HLA-DRB3*01:01 molecule with the EVSFFLIDENRFRHN, VEVSFFLIDENRFRH, and IVNLEYDPTAAFRNK epitopes, as shown in Supplementary Figure 19.

3.6 SSR multi-epitope vaccine construct

The multi-epitope vaccine construct was based on the six *Salmonella*-specific SSR-related proteins. The selection of B-cell, CTL, and HTL epitopes for each protein was based on their optimal properties. The B-cell epitopes employed in this study consisted of the following sequences: AKQPDAMTHPDGMQIK, FPLRLPAPTPKALYSP, LGPGGKTVEIQIRTKQ, VDAIEAENFESGGALK, VHDLNEDAEFDENGVE, and KEHGETSTTAQIRAQR. The CTL epitopes used consisted of the following sequences: SAHGKTIVVY, RSGHEDRIAW, GQTRSSGEPY, LRLNERITSV, RQKGDQKAF, and YSLLPTLLHY. The HTL epitopes used consisted of the following sequences: PDILMRLSSQMARRL, ELEDYCFRYLHPAEY, WLNFVVSSKARAKIR, LPIHIVKELNVYLRT, KAFNLLVVRYQHKVA, and VEVSFFLIDENRFRH. To enhance immunogenicity, adjuvant 50 ribosomal protein L7/L12 was included at the start of the vaccine sequence, then followed by a PADRE sequence. The epitopes, adjuvant, and PADRE sequences were joined using the following linkers: AAY, GPGPG, EAAK, and HEYGAEALERAG, as shown in Figure 3A. Furthermore, BLASTp analysis revealed a lack of similarity between the vaccine and the human proteome.

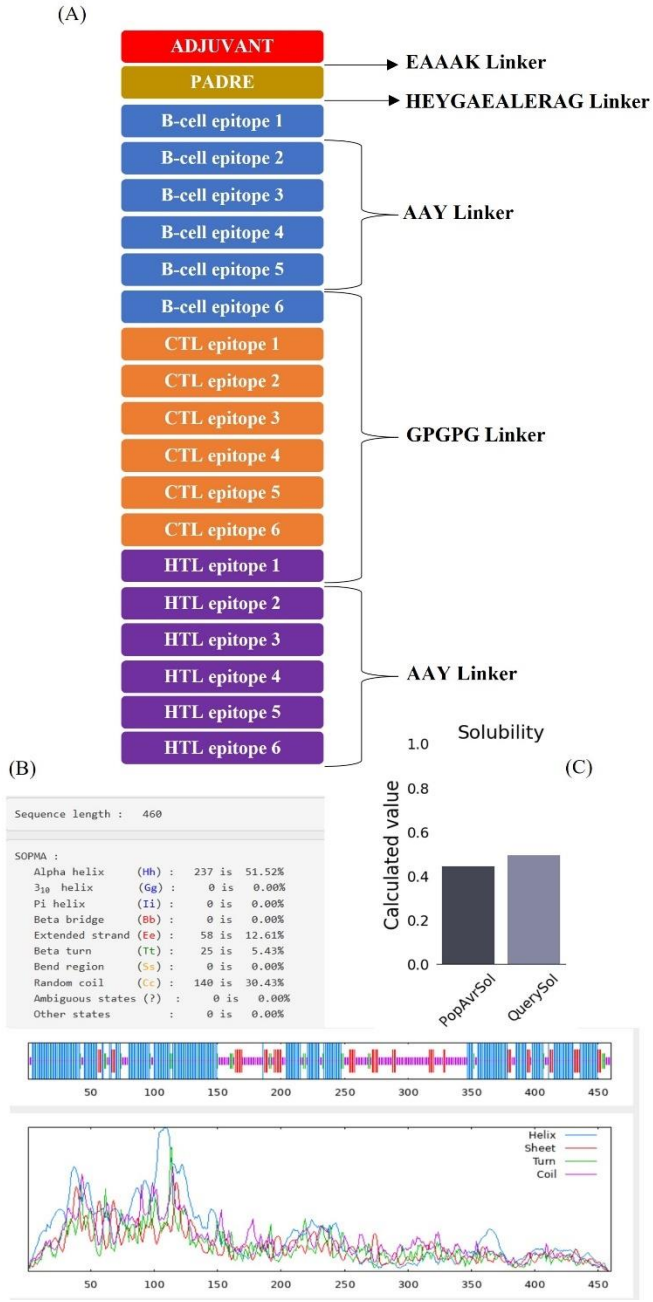

Figure 3. A schematic diagram depicting the structure of the multi-epitope vaccine that has been created. The 50 ribosomal protein L7/L12 adjuvant is connected to a PADRE sequence by an EAAAK linker. Similarly, the PADRE sequence is connected to the first B-cell epitope through a HEYGAEALERAG linker, and the other B-cell epitopes are connected through AAY linkers. The CTL epitopes are connected via GPGPG linkers, and the HTL epitopes are connected through AAY linkers. The vaccine's secondary structure prediction is visually depicted, revealing the existence of many structural elements like alpha helix, extended strand, beta-turn, and random coil formations. The Solubility index was found to have an estimated value of 0.49.

3.7 Population coverage analysis of the vaccine construct

The results of the population coverage analysis carried out using the IEDB tool server, showed the constructed vaccine covered 68% of the world population (Supplementary Table 4). The 16 region included East Asia, Northeast Asia, South Asia, Southest Asia, Euripe, East Africa, West Africa, Central Africa, South Africa, West Indies, North America, Central America, South America and Oceania. The regions where the vaccine had more coverage were East Asia, Northeast Asia, Sourhest Asia, North America and Oceania (Figure 4).

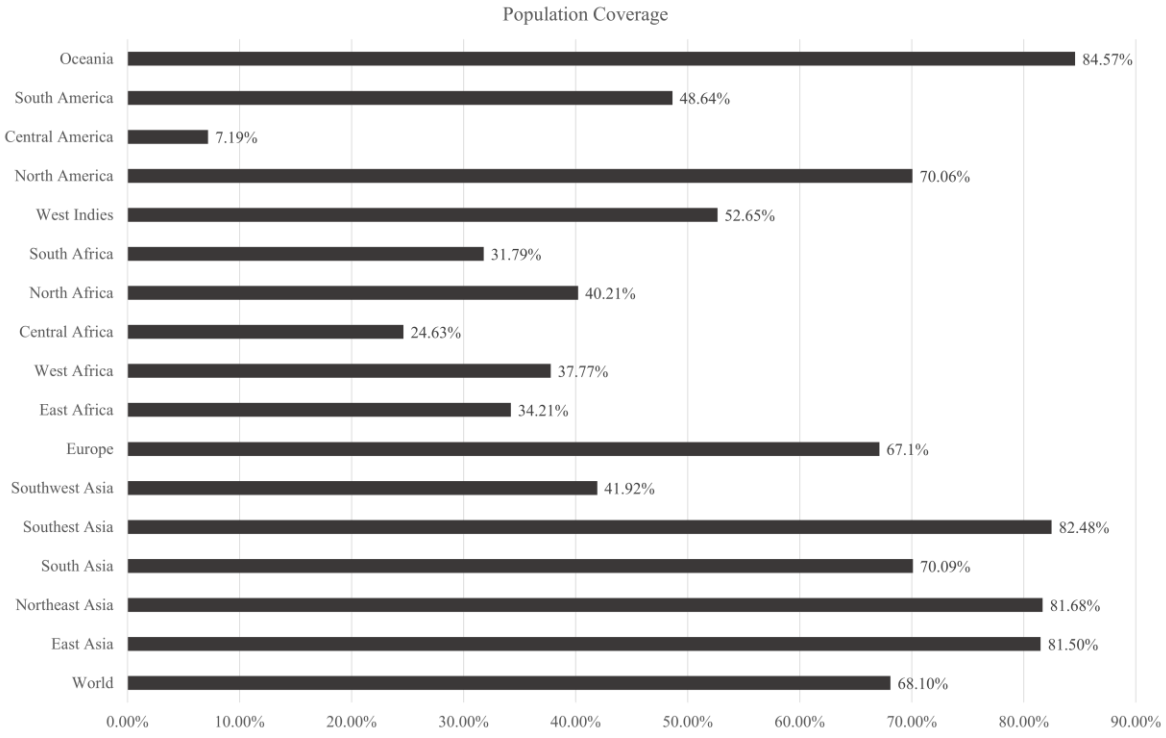

Figure 4. Population coverage analysis results for the SSR vaccine construct

3.8 Physicochemical characteristics and safety assessment of the multi-epitope SSR vaccine design

Based on the results obtained from the ProtParam online service, the multi-epitope vaccine has a molecular weight of 49,108.92 kilodaltons (kDa) and consists of a sequence including 460 amino acids. Additionally, it possesses a theoretical isoelectric point (pI) of 6.45. The vaccine contained a total of 56 residues with a negative charge and 53 residues with a positive charge. Its estimated half-life was approximately 30 h when tested in vitro using mammalian reticulocytes. In vivo experiments conducted with yeast shown a half-life above 20 hours, while *in vivo* experiments with *Escherichia coli* showed a half-life exceeding 10 hours. Stability of the protein was indicated by

an instability score of 30.62. The aliphatic index, as determined by (Ikai,1980), was 83.98, suggesting a high level of thermostability in the protein. Additionally, the grand average of hydropathicity (GRAVY) was calculated to be -0.186. Furthermore, the solubility score derived from the Protein Sol database online service was 0.498, suggesting that the protein had a high likelihood of becoming soluble upon expression (Figure 3C). The analysis conducted using SOPMA revealed that the developed vaccine consisted of 51.52% alpha helices, 12.61% extended strands, 5.43% beta sheets, and 30.43% random coils (Figure 3B). The antigenicity findings revealed a value of 0.84, suggesting that the protein exhibits a high level of antigenicity. Furthermore, the vaccine was non-allergenic and non-toxigenic. Finally, the immunogenicity score of the vaccine ultimately measured at 3.29.

3.9 3D structure modeling, refinement, and validation of multi-epitope vaccine structure

The multi-epitope vaccine was modeled using the TrRosetta algorithm, resulting in a model with poor confidence. Nevertheless, the vaccine structure was refined by submission to the GalaxyRefine Web server. After the enhancement of the first vaccine model, five additional models were developed. Model 1 was selected based on its structural features, including GDT-HA (0.9587), RMSD (0.401), MolProbity (1.317), Clash score (5.8), Poor rotamers (0.3), and Rama preferred (98.5). The PROCHECK server was used to construct a Ramachandran plot to assess the validity of the model. The plot revealed that the vaccine exhibited 94.4% of residues in areas that were highly preferred, while just 0.5% of residues were found in regions that are considered prohibited. These findings suggest that the model possesses a favorable quality. The ERRAT tool yields an aggregate quality factor of 91.66. Figure 5 displays the 3D structure of the vaccine, together with the Ramachandran plot and ERRAT graph results.

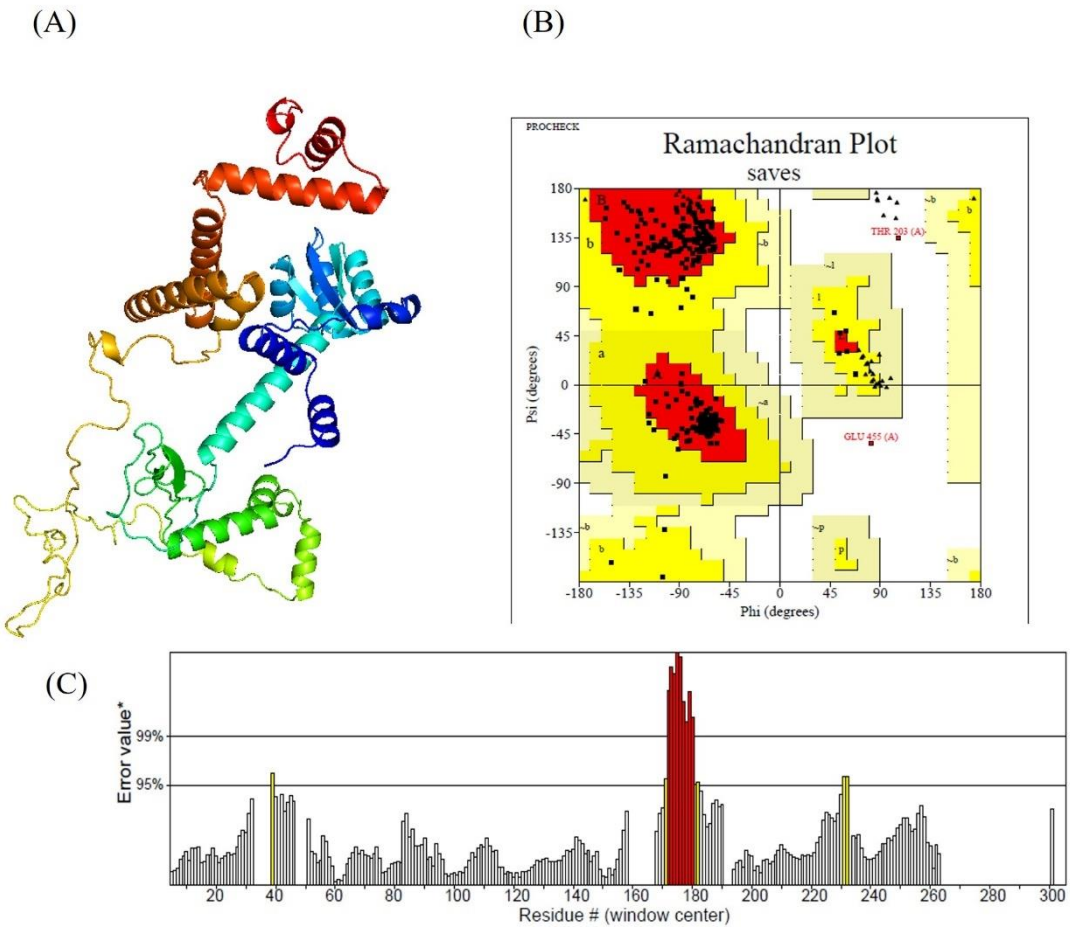

Figure 5. (A) The 3D structure of the multi-epitope SSR vaccine. (B) The Ramachandran plot indicates that 94.4% of the residues are in the most preferred areas, whereas 0.5% are in the prohibited regions. (C) ERRAT graph displaying a 91.66 overall quality factor.

3.10 SSR vaccine docking with TLR´s

Molecular docking was conducted to examine the interaction between the designed vaccine and Toll-like receptors (TLR-1, TLR-2, and TLR-4). The models that exhibited the lowest weighted score were selected as the most optimal ones (Table 6). Visualization of the docking results was visualized using Discovery Studio software. Figures 6-8 illustrate the visual representation of the docking process between the SSR vaccine and TLR receptors. The interaction analysis conducted using PDBsum revealed that the docking of the vaccine and TLR-1 exhibited 36 interface residues, five salt bridges, nine hydrogen bonds, and 212 non-bonded contacts (Figure 6). The analysis of the interaction between the vaccine and TLR-2 revealed the presence of 35 interface residues, 7 salt bridges, 22 hydrogen bonds, and 246 non-bonded contacts (Figure 7). Docking analysis of the vaccine and TLR-4 revealed the presence of 39 interface residues, 6 salt bridges, 32 hydrogen bonds, and 302 non-bonded contacts (Figure 8). These findings indicate that the vaccine can elicit

an immunological response through the activation of TLR receptors.

Table 6. Values of the molecular docking between the designed vaccine and the TLR-1, TLR-2 and TLR-4 receptors

Vaccine receptor	**Members**	**Representative**	**Weighted Score**
TLR-1	14	Center	-1138.2 kcal/mol
		Lowest Energy	-1138.2 kcal/mol
TLR-2	41	Center	-1144 kcal/mol
		Lowest Energy	-1492.1 kcal/mol
TLR-4	13	Center	-1257.5 kcal/mol
		Lowest Energy	-1257.5 kcal/mol

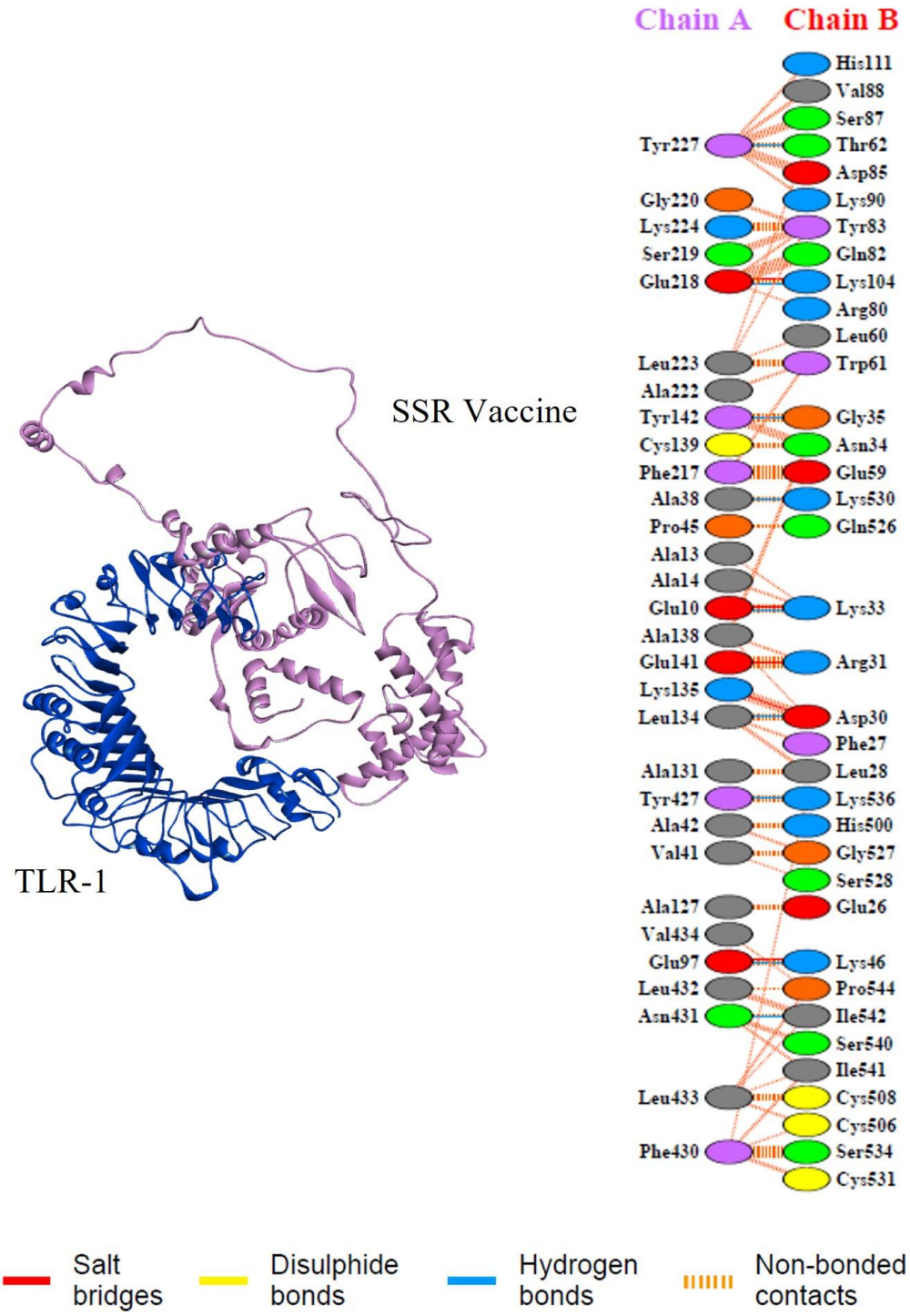

Figure 6. Docking of the designed SSR vaccine with the TLR-1 receptor (left) and interactions created with the PDBsum server (right).

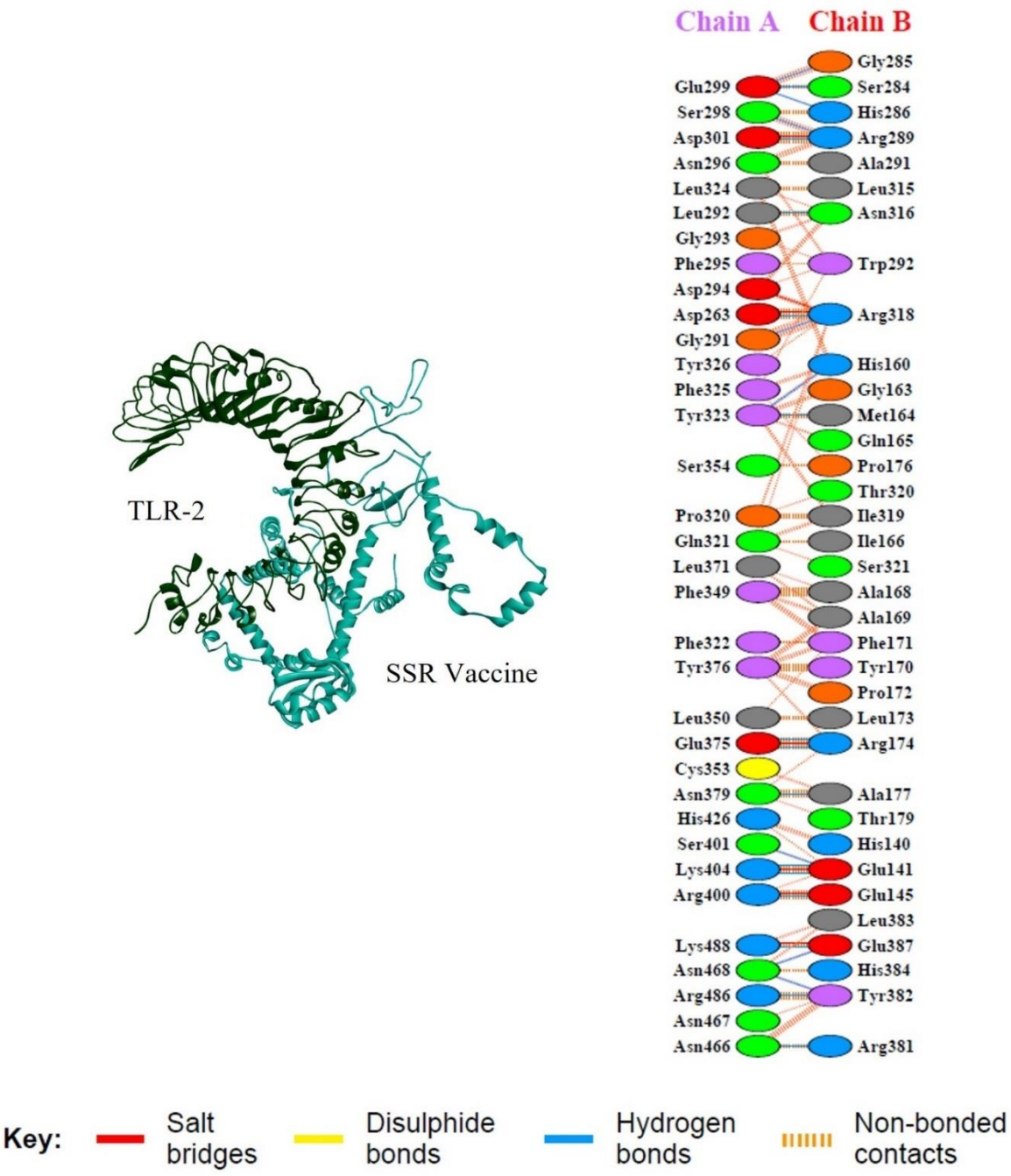

Figure 7. Docking of the designed SSR vaccine with the TLR-2 receptor (left) and interactions created with the PDBsum server (right).

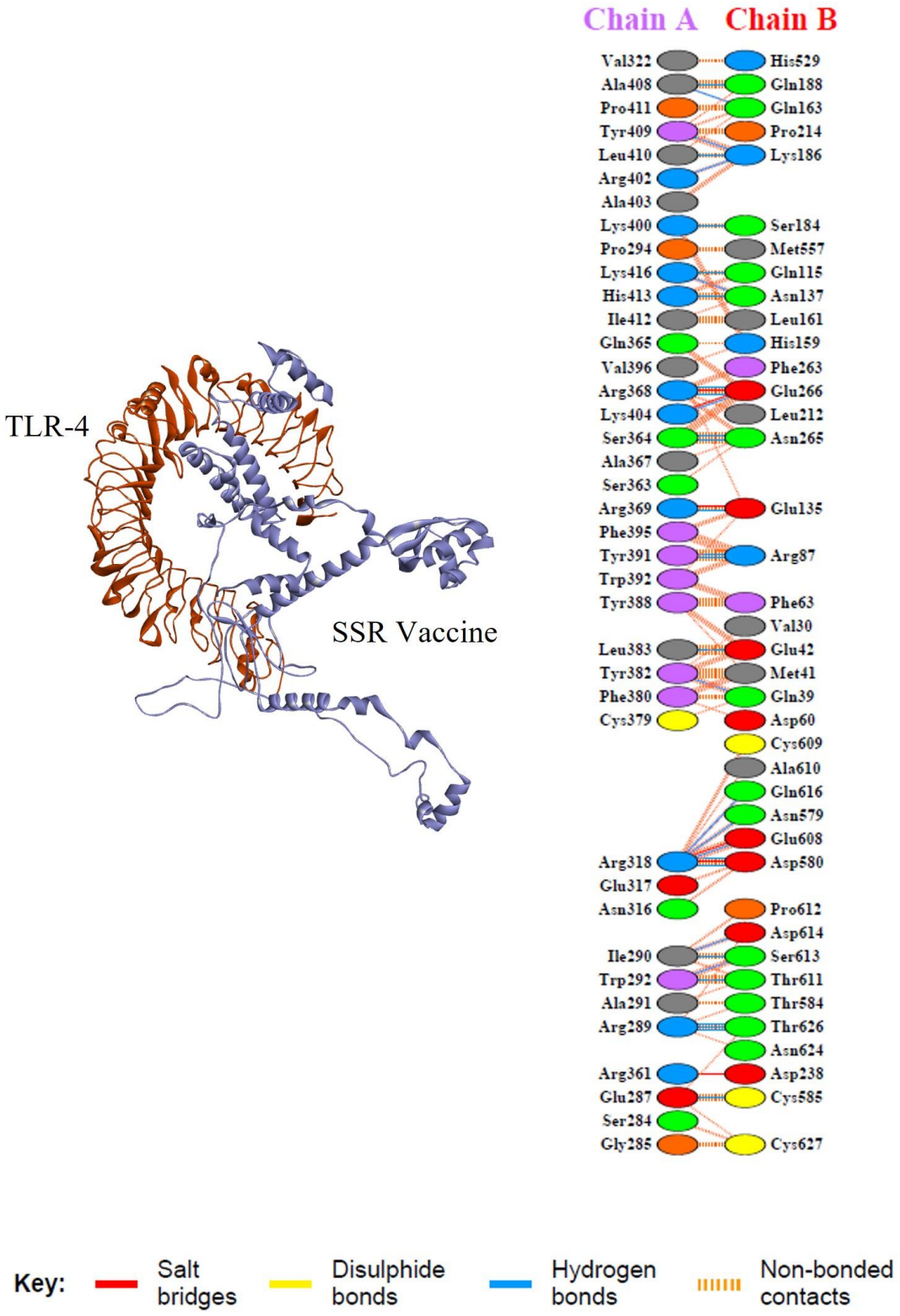

Figure 8. Docking of the designed SSR vaccine with the TLR-4 receptor (left) and interactions created with the PDBsum server (right).

3.11 Molecular Dynamics Simulation

To evaluate the dynamic behavior of the predicted vaccine construct in complex with immune receptors, MD simulation was carried out. The stability of the complexes was assessed by plotting RMSD of backbone carbon atom (Figure 9). The RMSD plot of apo vaccine and vaccine-TLR-1 complex showed a deviation up to 6 Å with variable deviation. Whereas the RMSD of vaccine-TLR-2 and vaccine-TLR-4 complexes was less than 5 Å with few deviations. Notably, no major fluctuations were observed throughout the simulation which suggested that the binding of vaccine stabilized the immune receptors specifically TLR-2 and TLR-4. Similarly, to evaluate the intrinsic flexibility of the vaccine residues, RMSF was calculated (Figure 9). All systems exhibited deviations of up to 12 Å, with the apo vaccine demonstrating lower fluctuations compared to the complexes. This increased fluctuation in the complexes is likely attributed to the binding of TLR receptors, indicating their significant influence on the overall structural dynamics. Collectively, the results of MD simulation suggested the significant stability and potential of the vaccine construct.

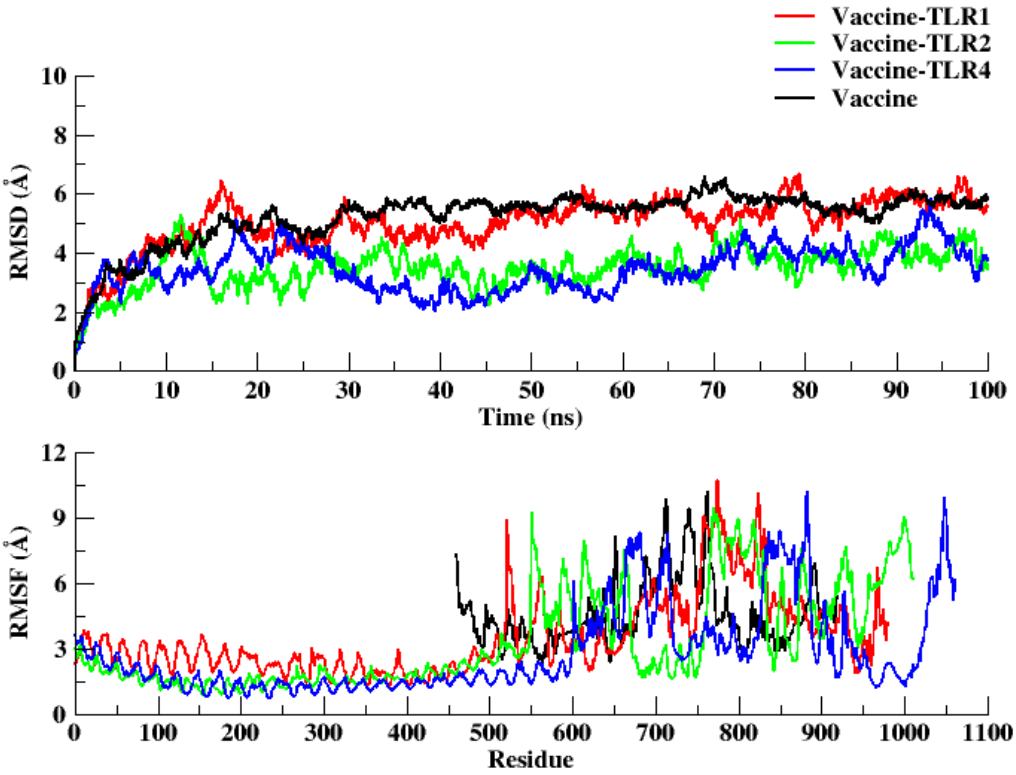

Figure 9. Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) analysis depicting the dynamic behavior of the vaccine and its complexes with TLR-1, TLR-2, and TLR-4. In RMSF plot, the terminal residues align with the vaccine correspond to vaccine, while the initial residues correspond to the TLR receptors.

3.11 Binding Free Energy

The relative binding energy for the vaccine complexes with TLR-1, TLR-2, and TLR-4 was calculated using the MM/PBSA method and the results are presented in Table 7. The TLR-4 complex showed the most negative ΔG value, -40.80 kcal/mol followed by TLR-2 and TLR-1 complex with the value of -33.29 kcal/mol and -31.22 kcal/mol, respectively. Among the individual energy components, the van der Waals (vdW) interactions contribute significantly with values of -85.21 kcal/mol, -89.54 kcal/mol, and -105.59 kcal/mol, for TLR-1, TLR-2 and TLR-4 complex, respectively. Electrostatic interactions play a dominant role, with values of -1335.29 kcal/mol, -1673.65 kcal/mol, and -1983.17 kcal/mol for TLR-1, TLR-2, and TLR-4 complexes, respectively. The high negative values indicate strong electrostatic attractions between the vaccine and TLR receptors, highlighting their significance in the overall binding. The Generalized Born (GB) term, which accounts for solvation effects, is positive in all complexes, contributing 1399.76 kcal/mol, 1742.87 kcal/mol, and 2047.10 kcal/mol, respectively. Moreover, surface area (SURF) interactions contribute negatively to the total energy, with values of -20.38 kcal/mol, -16.45 kcal/mol, and -17.30 kcal/mol for TLR-1, TLR-2, and TLR-4 complexes, respectively and this suggested that a reduction in the surface area might contribute to the stability of the complexes. Overall, the results inferred those electrostatic interactions played a more significant role in stabilizing the complexes compared to other energy terms.

Table 7. Energetic contributions of various components, including van der Waals (vdW), electrostatic, generalized born (GB), surface area (SURF), and the total free energy (ΔG) in kcal/mol.

Complex	**vdW kcal/mol**	**Electrostatic kcal/mol**	**GB kcal/mol**	**SURF kcal/mol**	**ΔG kcal/mol**
Vaccine-TLR-1	-85.21	-1335.29	1399.76	-20.38	-31.22
Vaccine-TLR-2	-89.54	-1673.65	1742.87	-16.45	-33.29
Vaccine-TLR-4	-105.39	-1983.17	2047.10	-17.30	-40.80

3.12 *In silico* simulation of immune responses

The C-ImmSim server was used to conduct simulations related to the reaction of the immune system. The findings demonstrated a statistically significant augmentation in immune responses that corresponded to actual immunological responses (Figure 10). The prevailing reaction showed was a significant elevation in IgM levels. The administration of secondary and tertiary injections resulted in an augmentation of B-cell population, as well as an elevation in the levels of IgG1 + IgG2 antibodies and IgG + IgM antibodies. However, antigen levels were reduced. Furthermore, the vaccine demonstrated the capacity to enhance the proliferation of memory and cytotoxic T cells within the T helper cell subset. The production of IFN-γ and IL-2 was observed to be heightened with repeated exposure. The findings of this study provide evidence of the antigenic and immunogenic characteristics of SSR vaccine.

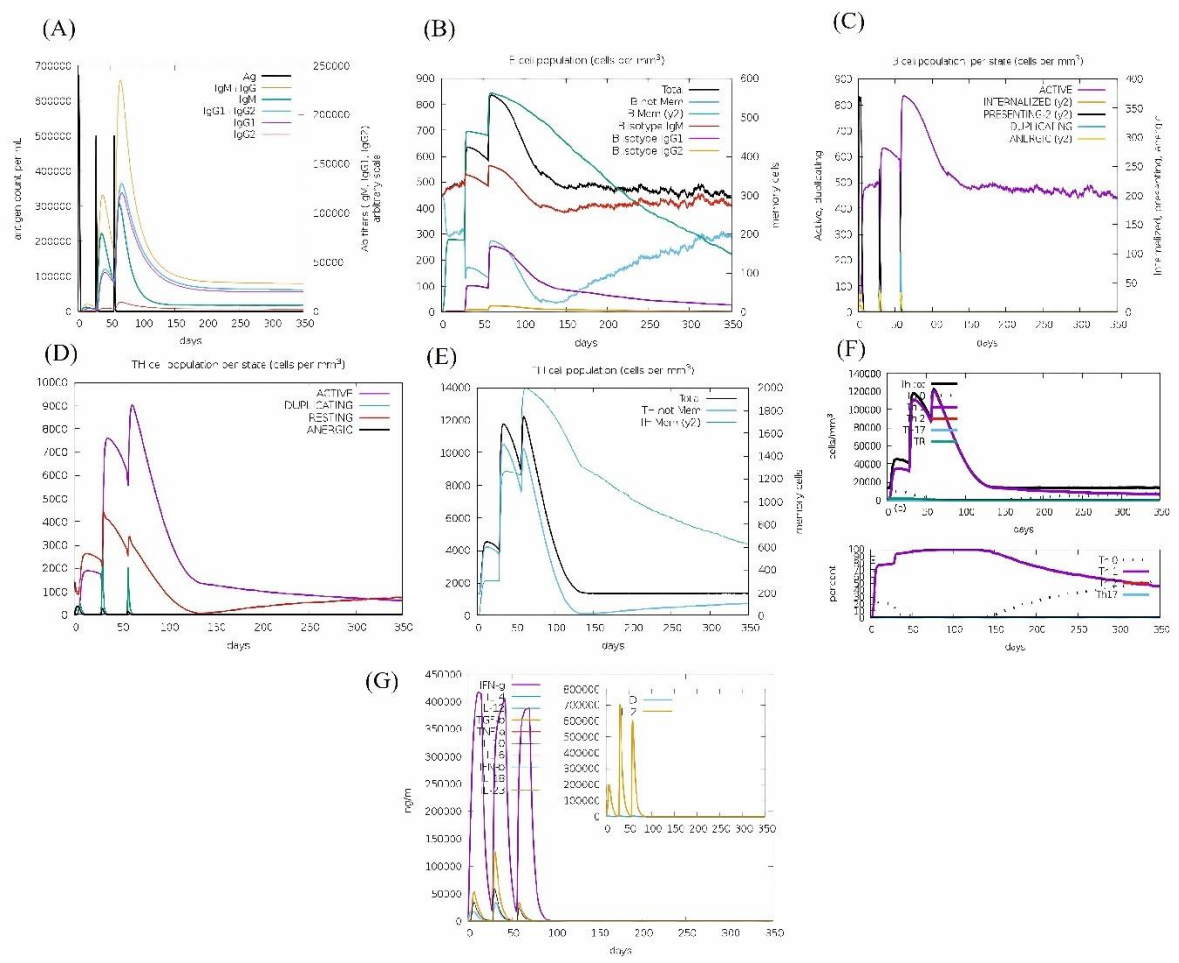

Figure 10. The immune response to the SSR vaccine was simulated using the C-ImmSim server. The yellow hue represents an elevated activity of IgM and IgG response, whereas the black color indicates a reduced activity of the antigen. The graph displays the B-cell population, with green representing heightened activity of memory B-cells. The activity of the B-cell population is shown by the purple hue, indicating heightened levels of activity. The activity of TH cells is shown by purple, which indicates an elevated level of activity. The activity of TH cells is shown by green, which indicates an increase in activity. The polarization of T-cells in response to Th1 is observed in purple color. The activity of IFN-γ was found to be raised in the purple hue, whereas the activity of IL-2 was observed to be elevated in the yellow color.

3.13 Optimization of the codon and *In silico* cloning of the SSR vaccine.

The JCat server was employed to enhance the codon utilization of the SSR vaccine in *Escherichia coli*, specifically the K12 strain. The length of the optimized sequence was 1350 nucleotides. The Codon Adaptation Index (CAI) value of the optimized nucleotide was determined to be 0.95, falling within the ideal range of 0.8-1.0 (Ali et. al., 2017; Ikram et. al., 2018). These findings indicate the favorable expression of the vaccine within the *E. coli* host. The optimized SSR vaccine sequence was successfully replicated *In silico* in the pET30a (+) plasmid. The resulting construct exhibited a total length of 5367 bp, as shown in Figure 11.

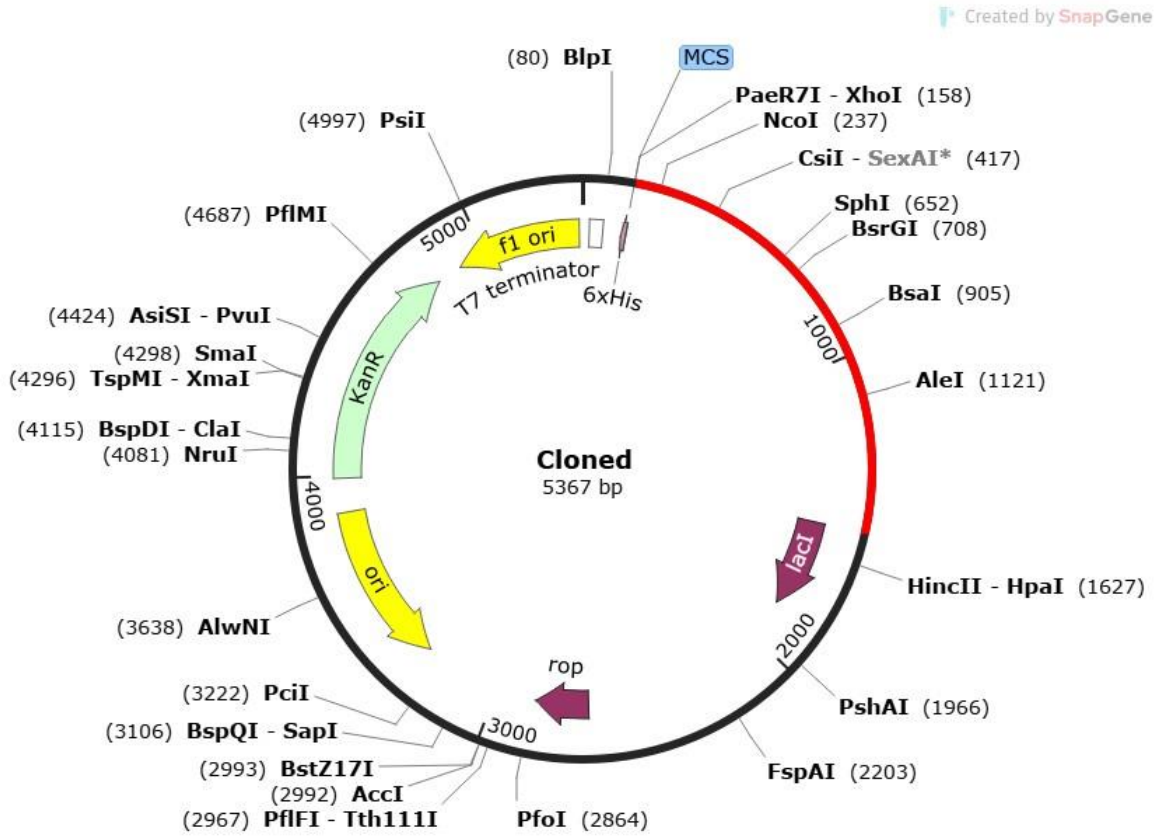

Figure 11. SSR vaccine sequence computationally cloned into the plasmid pET30a(+). The vaccine, denoted by its red hue, was introduced into the genetic sequence between the XhoI and BssH2 restriction sites. The overall length of the clone was determined to be 5367 base pairs (bp).

4. Discussion

Non-typhoidal *Salmonella* (NTS) is responsible for a significant number of bacterial enteritis cases, amounting to approximately 93.8 million occurrences annually (Gong et. al., 2022). *Salmonella*e NTS is often self-limiting, meaning that it can resolve on its own without treatment. However, it is important to note that it can also be harmful and even fatal, depending on the specific host and serotype characteristics (Levantesi et. al., 2012). *Salmonella enterica* serotype Oranienburg poses a significant concern in terms of public health and food safety due to its recent association with foodborne outbreaks. Furthermore, this serotype has the ability to endure water and sediment even under unfavorable environmental conditions, thereby enhancing its potential as a pathogenic strain (González-Torres et. al., 2023).

For this study, we selected several proteins that are associated with SSR in *Salmonella* for the development of a multi-epitope vaccine. These proteins include the cAMP receptor protein, adenylate cyclase, sigma factor rpoS, sigma factor rpoE, (p)ppGpp synthetase I, and bi-functional (p)ppGpp synthetase II/guanosine -3',5'-bis pyrophosphate 3'-pyrophosphohydrolase. Utilizing multiple protein sequences to develop a multi-epitope vaccine enables a more comprehensive immune response against various aspects of the pathogen, thereby enhancing the prospect of protection (Sanches et. al., 2021; Tahir ul Qamar et. al., 2021; Tarrahimofrad et. al., 2021). Commonly multi-epitope vaccines are constructed by selecting specific epitopes from different proteins that are essential for the survival and virulence of the pathogen (Goumari et. al., 2020). Additionally, the use of multiple epitopes reduces the chance of immune evasion by the pathogen, as targeting multiple essential proteins makes it harder for the pathogen to develop resistance (Dar et. al., 2019; Oli et. al., 2020; Mushtaq et. al., 2022). These proteins are critical for the SSR activation and regulation, allowing *Salmonella* to survive and adapt to adverse conditions both within the host and in the external environment. This results in significant changes in the physiology of the bacterium, enhancing its virulence and pathogenicity (Spector & Kenyon, 2012; Chakroun et. al., 2017; Gong et. al., 2022). SSR-related genes are essential for bacterial survival and perform a variety of physiological activities, making them attractive targets for vaccine development (Spector & Kenyon, 2012; El Mouali et. al., 2018; Chand & Singh, 2021).

To help prevent *Salmonella* infection, it is crucial to administer a highly effective and all-encompassing vaccine that covers a broad spectrum of serovars (Tosta et. al., 2020). Despite being widely recognized as the most efficacious and economically viable approach to prevent certain illnesses, there is now a lack of authorized vaccine options (Chiu et. al., 2020). The conventional methods employed for vaccine manufacture are characterized by a prolonged duration, and there exists a potential hazard of the weakened virus regaining its original virulence (Kumar et. al., 2019). In recent years, there has been a marked increase in the utilization of bioinformatics methods for the analysis of various species, such as viruses, bacteria, and parasites (Maljkovic Berry et. al., 2019). This trend can be attributed to the numerous advantages associated with these approaches, including their cost-effectiveness, safety, and shorter time length. Multi-epitope-based subunit

vaccines (MESVs) can be anticipated and formulated utilizing various proteomics and immunoinformatic methodologies (Suleman et. al., 2023). These vaccines exhibit remarkable stability, non-toxicity, profitability, and ease of construction. Additionally, MESVs offer the advantage of safeguarding the host against excessive antigenic burden and allergic reactions (Khan et. al., 2020; Zhang, 2018). In a study conducted by Beikzadeh, (2023), immunoinformatic methodologies were employed to develop a multi-epitope vaccine targeting non-typhoidal salmonellosis. The vaccine design incorporated the outer membrane proteins OmpA, OmpD, and Stn (enterotoxin), resulting in favorable properties in terms of vaccine antigenicity and safety. Furthermore, the vaccine demonstrated a strong affinity for interaction with Toll-like receptor (TLR) receptors.

In a similar study, Zafar et. al., (2022), designed and evaluated a multi-epitope vaccine targeting *Salmonella* Typhimurium focused on the TolA protein, which plays a crucial role in antigenicity and the bacterial entry process into the host. The findings indicated that the vaccine exhibited favorable antigenic and immunogenic properties. Previous research has focused on utilizing outer membrane proteins as potential vaccine candidates due to their ability to be readily recognized by the immune system (Ain et. al., 2018). However, the present study has developed a vaccine that incorporates B-cell, HTL, and CTL epitopes derived from SSR proteins, which has shown promising prospects for immunization of the host.

The vaccine was constructed by including a total of six B-cell epitopes, six HTL epitopes, and six CTL epitopes derived from the SSR proteins. The accurate identification and prediction of epitopes play a crucial role in the development and progress of preventive and immunotherapeutic vaccines. T cell cytokines have a crucial role in regulating many adaptive immunological responses, making them essential components of the adaptive immune system (Tian et. al., 2018). B-cell activation, B-cell class switching, macrophage activation for microbial destruction, and cytotoxic T lymphocyte (CTL) activation for the elimination of infected target cells are all functions performed by B-cells (Zouali & Richard, 2011). Hematopoietic T lymphocytes (HTLs) initiate the activation of B-cells by initially recognizing the peptide-major histocompatibility complex (MHC) complex (Buteau et. al., 2002). Additionally, they play a role in the secretion of lymphokines, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IFN-γ, therefore providing support for both cytotoxic T lymphocyte (CTL) and humoral immune responses (Chand & Singh, 2021; Kalita et. al., 2019).

In addition, the incorporation of the ribosomal protein L7/L12 in the development of the vaccine was intended to improve its overall effectiveness. This compound acts as a TLR-4 agonist and has been found to be an effective activator of both the innate and adaptive immune responses (Kumar et. al., 2019). A HEYGAEALERAG linker was also used to connect the 13-amino acid synthetic sequence PADRE to the vaccine design. The sequence exhibits an affinity for MHC class II molecules, hence inducing the activation of CD4+ helper T lymphocytes (HTL) (Sanches et. al., 2021). The administration of a vaccine incorporating the PADRE gene resulted in more robust cytotoxic T lymphocyte (CTL) responses compared to a vaccine lacking this genetic component

(Solanki et. al., 2019; Chand & Singh, 2021). Furthermore, the utilization of EAAK, AAY, and GPGPG linkers was applied to establish connections between all the epitopes. This approach contributes to the facilitation of antigen processing and presentation, as well as enhancing the structural flexibility of the vaccine (Athanasiou et. al., 2017).

The ultimate design of the SSR vaccine had a molecular weight of 49,108 kDa and consisted of a total of 460 amino acids. According to existing literature, it has been suggested that proteins with a molecular weight below 110,000 kDA are considered advantageous due to their ease of extraction and suitability for incorporation into vaccine formulations (Dey et. al., 2022; Beikzadeh, 2023). Additional physicochemical parameters were assessed to further evaluate the vaccine design's characteristics. These parameters encompassed the isoelectric point, instability index, solubility, aliphatic index, and Grand average of hydropathicity. The results obtained from these evaluations indicated that the vaccine design exhibits stability, solubility, and thermostability (Guruprasad et. al., 1990; Priyadarsini et. al., 2021). The vaccine sequence was assessed for its antigenicity, allergenicity, and toxicity, and the results were positive in all three areas.

The 3D structure of the SSR vaccine was computationally predicted using the trRosetta method. Analysis of the Ramachandran Plot demonstrated that 94.4% of amino acid residues occupied the most favorable areas, suggesting a very stable conformation (Ramakrishnan & Ramachandran, 1965; Priyadarsini et. al., 2021).

The protein derived from the SSR vaccine was further subjected to docking simulations with the TLR-1, TLR-2, and TLR-4 receptors, which have been identified as crucial components in the immune response against non-typhoidal salmonellosis (Arpaia et. al., 2011; Beikzadeh, 2023). TLRs are pattern-recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) carried by bacterial outer membrane proteins (OMPs) and can activate the transcription factor NF-kB, resulting in the production of pro-inflammatory cytokines that initiate and influence the adaptive immune response (Maeshima & Fernandez, 2013; Chand & Singh, 2021).

The findings from the docking interactions demonstrated a persistent binding of the vaccine to Toll-like receptor (TLR) receptors, suggesting a robust stability in the docking process (Nain et. al., 2020). Moreover, the presence of hydrogen bonds within the interactions suggests a robust affinity (Ali et. al., 2022). In this context, the vaccine establishes a total of 33 hydrogen bonds with the TLR-4 receptor, signifying a more substantial docking activity The TLR-4 receptor is implicated in the detection of lipopolysaccharides (LPS) derived from gram-negative bacteria, such as *Salmonella* and the activation of this receptor leads to the production of proinflammatory mediators (Tötemeyer et. al., 2003). Based on the previous information, along with the results derived from molecular dynamics simulations, it can be inferred that the interaction between the vaccine and TLR receptors exhibits a high degree of stability.

The C-ImmSim server was used to assess immunological stimulation, which revealed the activation of humoral response along with T-cell and Th1 activation. The results of this study indicate that the administration of the SSR vaccine can induce an immune response characterized by the production of antibodies and the development of long-lasting T and B-cell memory. Moreover,

subsequent to repetitive stimulation, the levels of IFN-γ and IL-2 increased , therefore establishing a positive correlation with the amount of T-helper cells. In the context of *S.* Typhimurium infection, it is worth noting that TH cells are not the sole contributors to the production of IFN-γ. Other cell types, such as neutrophils and NK cells, also play a role in the generation of IFN-γ (Kurtz et. al., 2017; Rahman et. al., 2020; Priyadarsini et. al., 2021; Beikzadeh, 2023). To facilitate efficient production of SSR vaccine, codon optimization was conducted for *E. coli* str. K12, which is a highly favored host for recombinant protein synthesis (Beikzadeh, 2023). The findings revealed that the GC content of the sample was 71.7%, which was within the desirable range. Additionally, CAI score of 0.95 suggested a strong expression of the vaccine (Khatoon et. al., 2017).

5. Conclusion

This study proposes the use of immunoinformatic techniques to develop a multi-epitope vaccine focused on proteins involved in the regulation and stimulation of the SSR. The selected proteins do not belong to the category of membrane proteins but are essential for the survival, colonization, and nutritional well-being of *Salmonella*. The results obtained suggest the vaccine construct is able to stimulate TLR-1, TLR-2, and TLR-4 receptors. Additionally, immunological simulations showed that the proposed vaccine can elicit both humoral and cellular responses. Furthermore, the vaccine has demonstrated no allergenic or toxigenic properties while exhibiting a high level of antigenicity. However, further research and testing are needed to fully assess the effectiveness and immunogenicity of this multi-epitope vaccine.

CRediT author statement

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Disclosure statement

The authors report there are no competing interests to declare.

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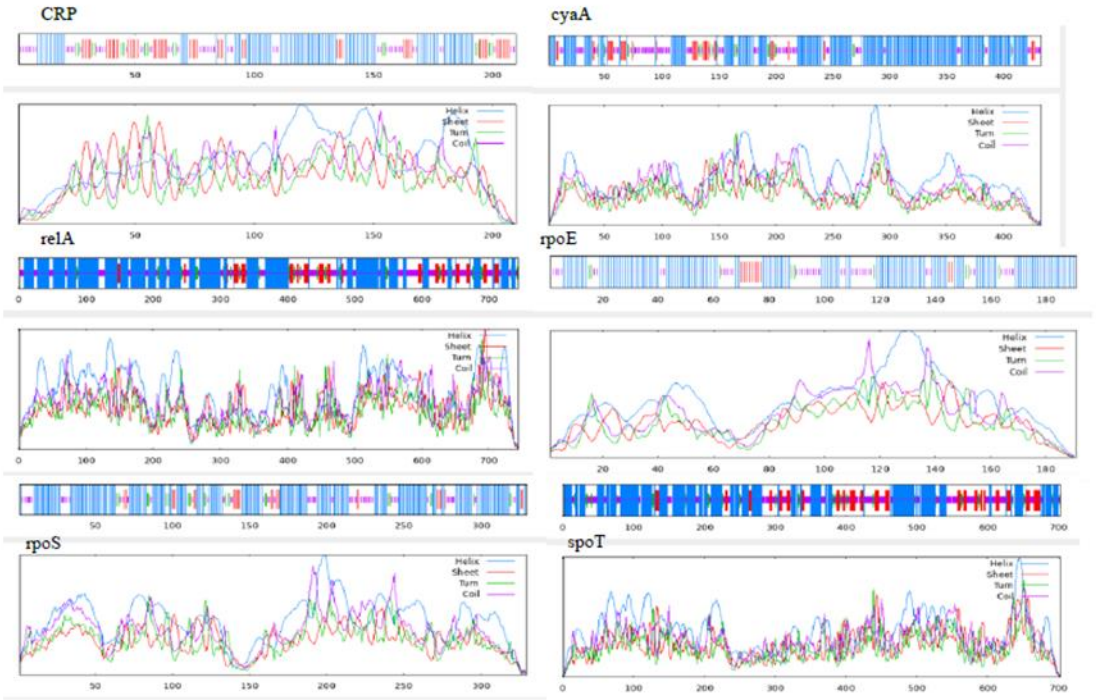
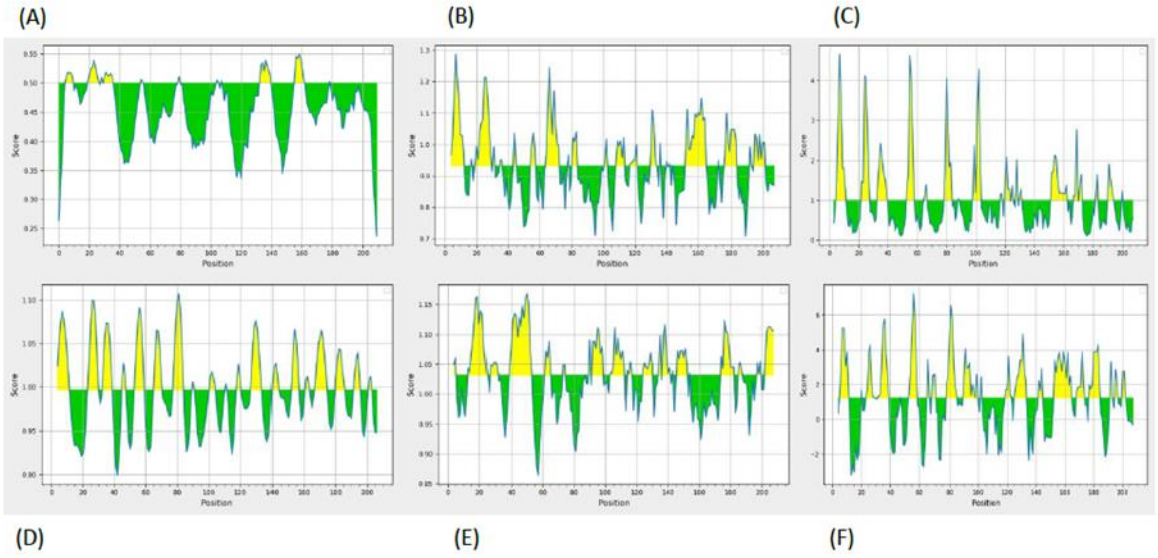
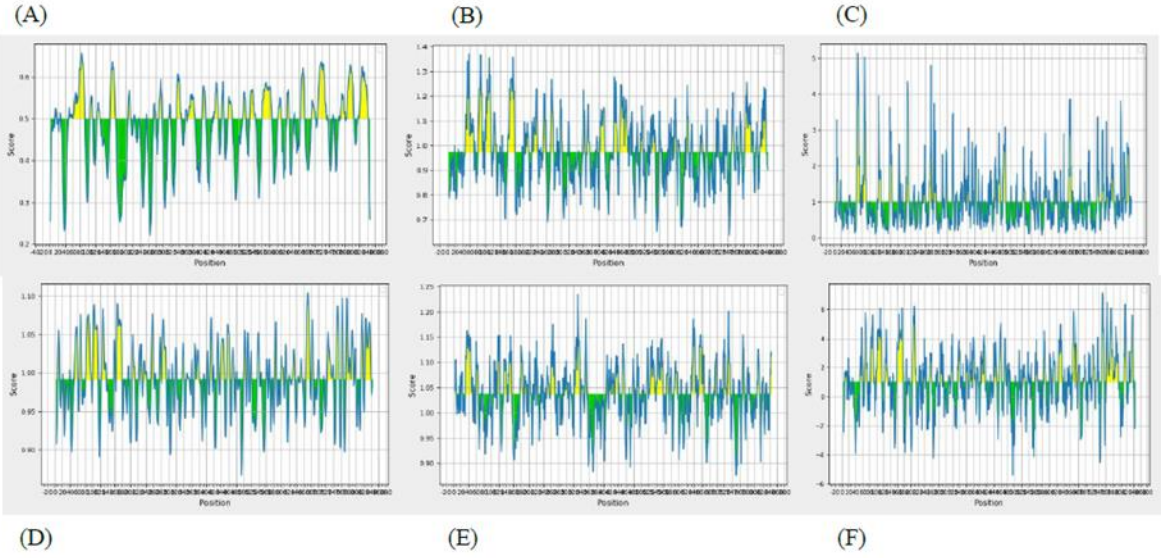
Supplementary Materials



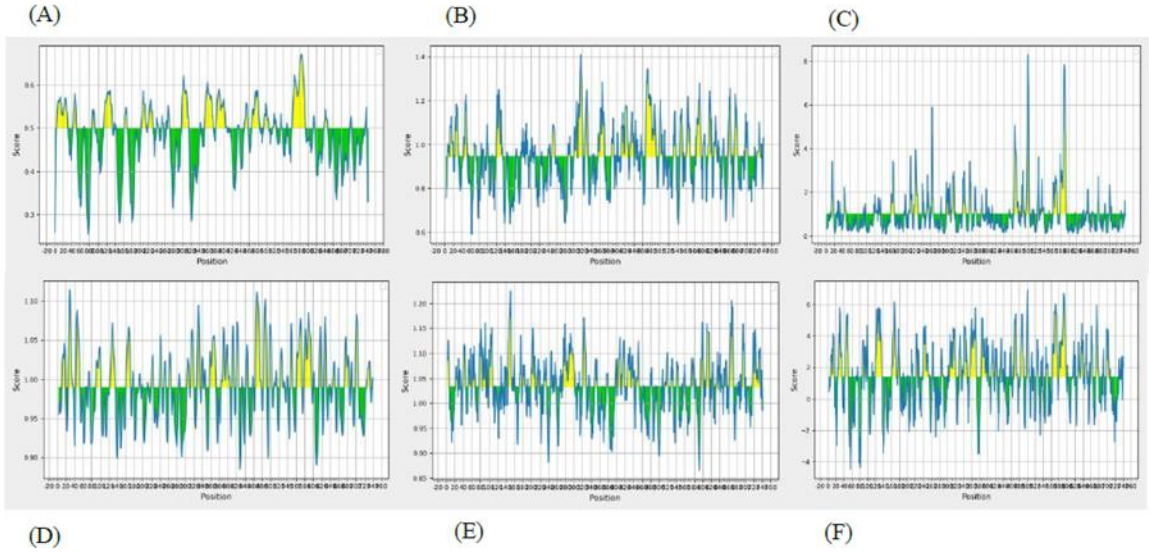
Supplementary Figure 1. Secondary structures of *Salmonella* Oranienburg SSR-related proteins are shown graphically. The presence of Alpha Helix structures is represented by blue. The presence of Beta Sheet structures is shown by the color red. Turns are represented by green. The presence of random coils is shown in purple.



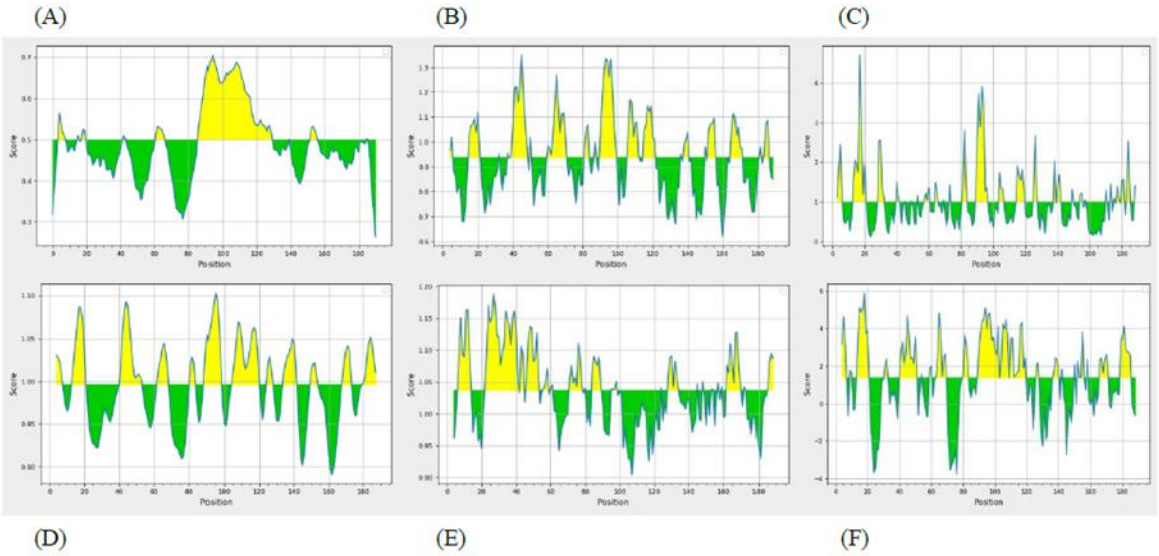
Supplementary Figure 2. B-cell epitope prediction using the CRP protein of the *Salmonella* Oranienburg SSR. (A) Prediction of bepriped linear epitopes. (B) Antigenicity graph of Kolaskar and Tongaonkar. (C) Emini surface accessibility graph. (D) The flexibility prediction graph of Karplus and Schulz. (E) Chou-Fasman beta turn graph. (F) Parker hydrophilicity graph.



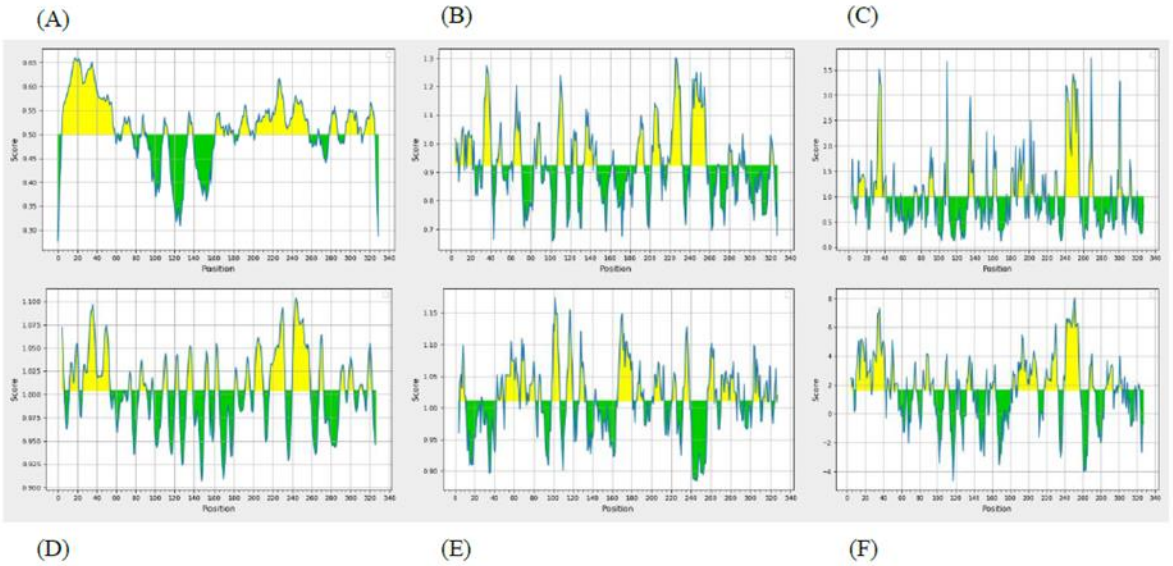
Supplementary Figure 3. B-cell epitope prediction using the cyaA protein of the *Salmonella* Oranienburg SSR. (A) Prediction of bepriped linear epitopes. (B) Antigenicity graph of Kolaskar and Tongaonkar. (C) Emini surface accessibility graph. (D) The flexibility prediction graph of Karplus and Schulz. (E) Chou-Fasman beta turn graph. (F) Parker hydrophilicity graph.



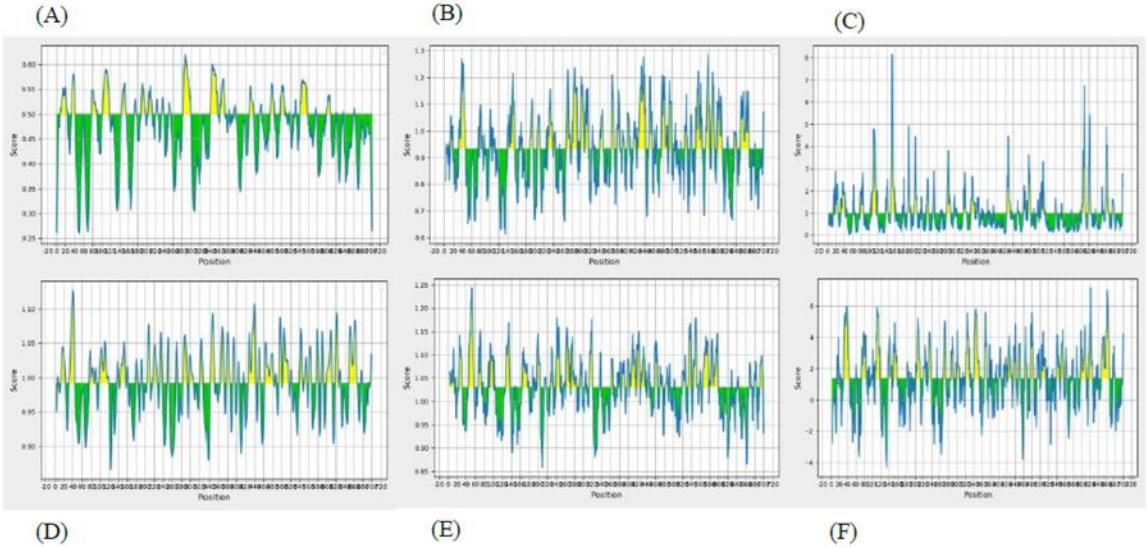
Supplementary Figure 4. B-cell epitope prediction using the relA protein of the *Salmonella* Oranienburg SSR. (A) Prediction of bepriped linear epitopes. (B) Antigenicity graph of Kolaskar and Tongaonkar. (C) Emini surface accessibility graph. (D) The flexibility prediction graph of Karplus and Schulz. (E) Chou-Fasman beta turn graph. (F) Parker hydrophilicity graph.



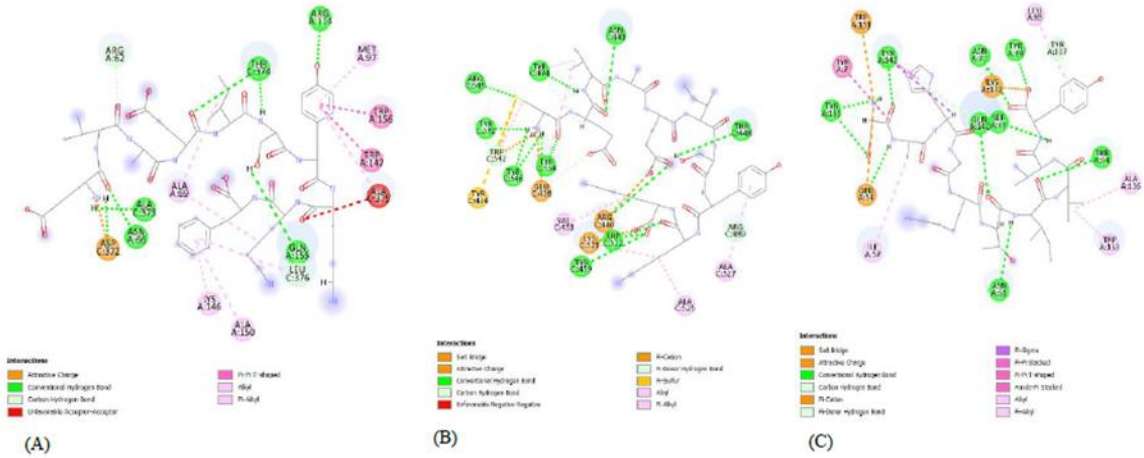
Supplementary Figure 5. B-cell epitope prediction using the rpoE protein of the *Salmonella* Oranienburg SSR. (A) Prediction of bepriped linear epitopes. (B) Antigenicity graph of Kolaskar and Tongaonkar. (C) Emini surface accessibility graph. (D) The flexibility prediction graph of Karplus and Schulz. (E) Chou-Fasman beta turn graph. (F) Parker hydrophilicity graph.



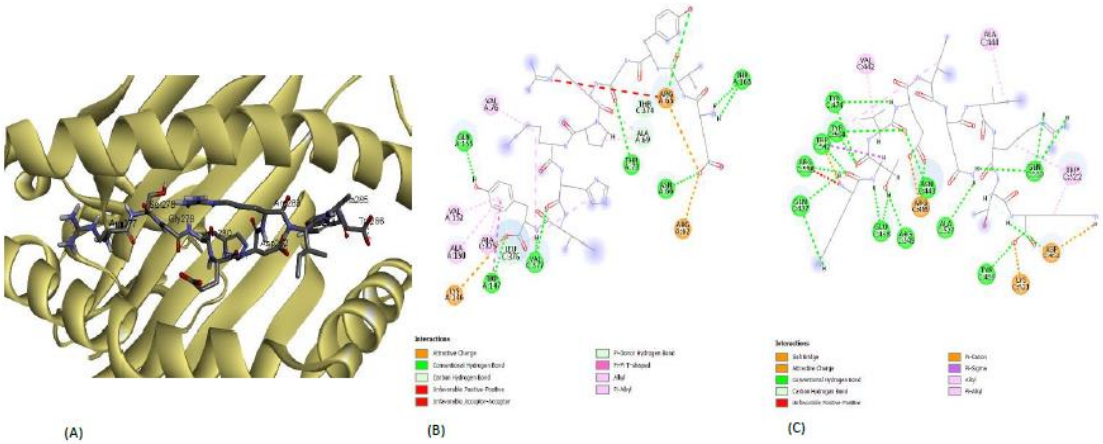
Supplementary Figure 6. B-cell epitope prediction using the rpoS protein of the *Salmonella* Oranienburg SSR. (A) Prediction of bepriped linear epitopes. (B) Antigenicity graph of Kolaskar and Tongaonkar. (C) Emini surface accessibility graph. (D) The flexibility prediction graph of Karplus and Schulz. (E) Chou-Fasman beta turn graph. (F) Parker hydrophilicity graph.



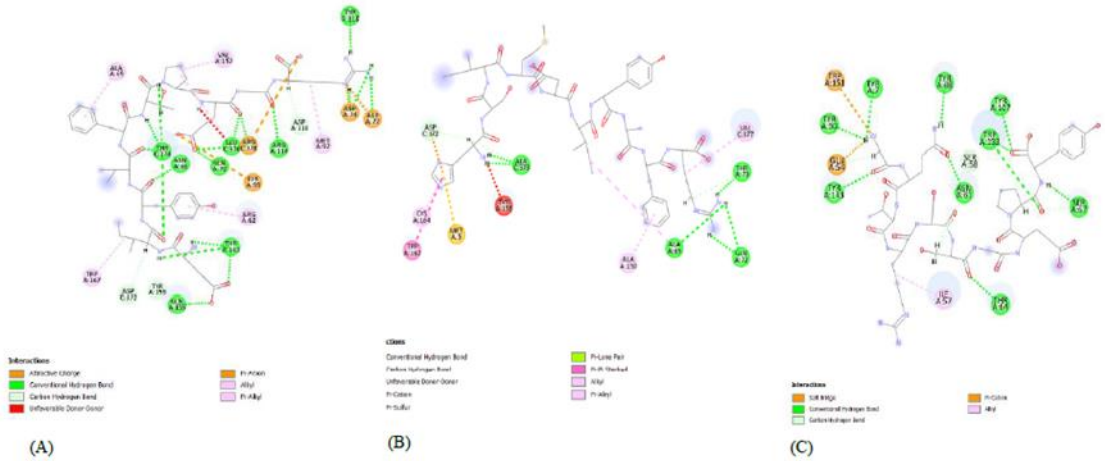
Supplementary Figure 7. B-cell epitope prediction using the spoT protein of the *Salmonella* Oranienburg SSR. (A) Prediction of bepriped linear epitopes. (B) Antigenicity graph of Kolaskar and Tongaonkar. (C) Emini surface accessibility graph. (D) The flexibility prediction graph of Karplus and Schulz. (E) Chou-Fasman beta turn graph. (F) Parker hydrophilicity graph.



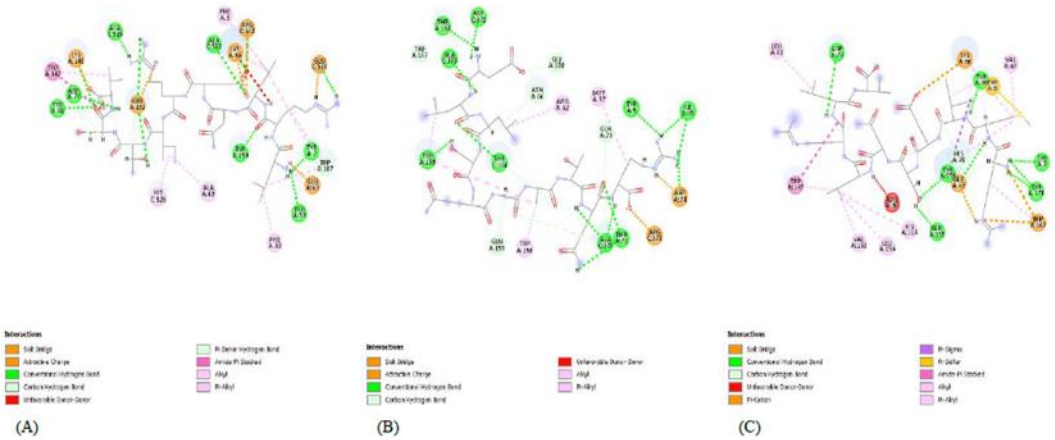
Supplementary Figure 8. 2D interaction between CTL epitopes from the CRP protein with their corresponding MHC-I alleles. (A) EVAEISYKKF epitope bound to HLA-A*68:01 with six favorable interactions, which included bonds of attractive charge, conventional hydrogen bonds, carbon-hydrogen bonds, pi-pi T-shaped bonds, alkyl bonds, and pi-alkyl, as well as one unfavorable acceptor-acceptor interaction, which is illustrated in red. (B) CEVAEISYKK epitope bound to HLA-A*11:01 with nine favorable interactions including salt bridge, attractive charge, conventional hydrogen bonds, carbon-hydrogen bonds, pi-cation bonds, pi-donor hydrogen bonds, pi-sulfur bonds, alkyl bonds, and pi-alkyl, as well as one unfavorable negative-negative interaction, which is illustrated in red. (C) SAHGKTIVVY epitope bound to HLA-B*15:01 with twelve favorable interactions including salt bridge, attractive charge, conventional hydrogen, carbon-hydrogen, pi-cation, pi-donor hydrogen, pi-sigma, pi-pi stacked, pi-pi T-shaped, amide-pi stacked, alkyl, and pi-alkyl bonds.



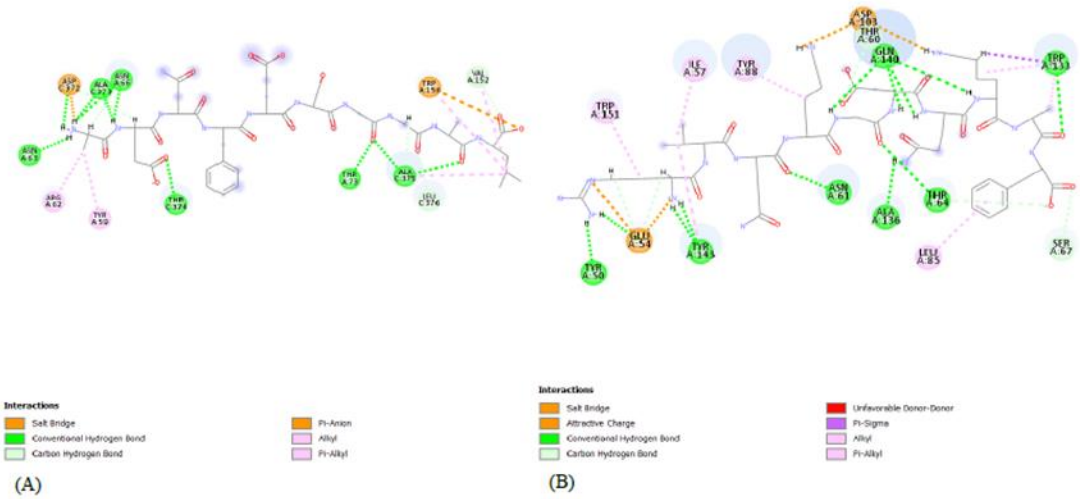
Supplementary Figure 9. Visualization of the interactions between CTL epitopes from the relA protein with their corresponding MHC-I alleles. (A) 3D interaction of the RSGHEDRIAW epitope bound to HLA-B*57:01. Seven favorable interactions were formed, including conventional hydrogen, attractive charge, salt bridge, pi-hydrophobic, alky, pi-alkyl, and pi-sulfur bonds (Figure not shown). (B) EVYGRPKHIY epitope bound to HLA-A*68:01 with seven favorable interactions including attractive charge, conventional hydrogen, carbon-hydrogen, pi-donor hydrogen, pi-pi T-shaped, alkyl, and pi-alkyl, as well as two unfavorable positive-positive and acceptor-acceptor interactions, which are illustrated in red. (B) KTVEIQIRTK epitope bound to HLA-A*11:01 with eight favorable interactions, including salt bridge, attractive charge, conventional hydrogen, carbon hydrogen, pi-cation, pi-sigma, alkyl, and pi-alkyl, as well as one unfavorable positive-positive interaction, which is illustrated in red.



Supplementary Figure 10. 2D interaction between CTL epitopes from the spoT protein with their corresponding MHC-I alleles. (A) EIYVFTPEGR epitope bound to HLA-A*68:01 with six favorable interactions including attractive charge, conventional hydrogen, carbon-hydrogen, pi-anion, alkyl, and pi-alkyl, as well as one unfavorable donor-donor interaction, which is illustrated in red. (B) HSIMDIYAFR epitope also bound to HLA-A*68:01 with eight favorable interactions including conventional hydrogen, carbon-hydrogen, pi-cation, pi-sulfur, pi-lone pair, pi-pi stacked, alkyl, and pi-alkyl, as well as one unfavorable donor-donor interaction, which is illustrated in red. (C) GQTRSSGEPY epitope bound to HLA-B*15:01 with five favorable interactions including salt bridge, conventional hydrogen, carbon-hydrogen, pi-cation, and alkyl bonds.

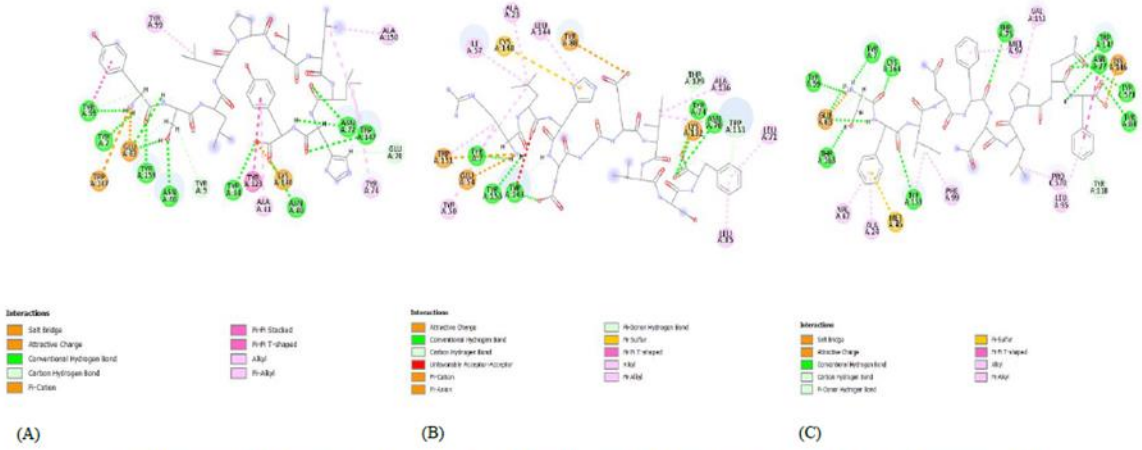


Supplementary Figure 11. 2D interaction between CTL epitopes from the rpoS protein with their corresponding MHC-I alleles. (A) LRLNERITSV bound to HLA-A*02:03 with eight favorable interactions including salt bridge, attractive charge, conventional hydrogen, carbon-hydrogen, pi-donor hydrogen, amide-pi stacked, alkyl, and pi-alkyl, as well as one unfavorable donor-donor interaction, which is illustrated in red. (B) ELLSQGATQR bound to HLA-A*68:01 with six favorable interactions including salt bridge, attractive charge, conventional hydrogen, carbon-hydrogen, alkyl, and pi-alkyl, as well as one unfavorable donor-donor interaction, which is illustrated in red. (C) RMIESNLRLV epitope bound to HLA-A*02:06 with nine favorable interactions including salt bridge, conventional hydrogen, carbon-hydrogen, pi-cation, pi-sigma, pi-sulfur, amide-pi stacked, alkyl and pi-alkyl, as well as one unfavorable donor-donor interaction, which is illustrated in red.



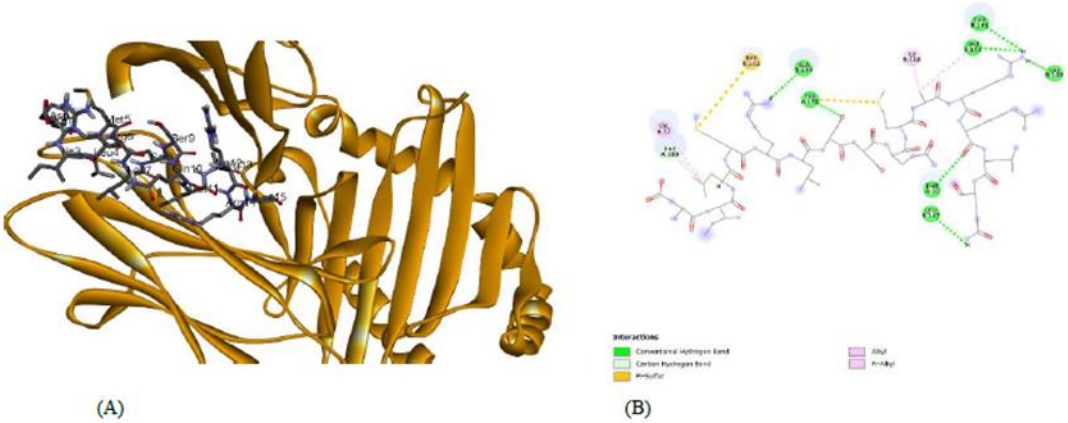
(A) (B)

Supplementary Figure 12. 2D interaction between CTL epitopes from the rpoE protein with their corresponding MHC-I alleles. (A) AENFESGGAL epitope bound to HLA-A*68:01 with six favorable interactions including salt bridge, conventional hydrogen, carbon-hydrogen, pi-anion, alkyl, and pi-alkyl bonds. (B) RVQKGDQKAF epitope bound to HLA-B*15:01 with seven favorable interactions including salt bridge, attractive charge, conventional hydrogen, carbon-hydrogen, pi-sigma, alkyl, and pi-alkyl, as well as one unfavorable donor-donor interaction, which is illustrated in red.



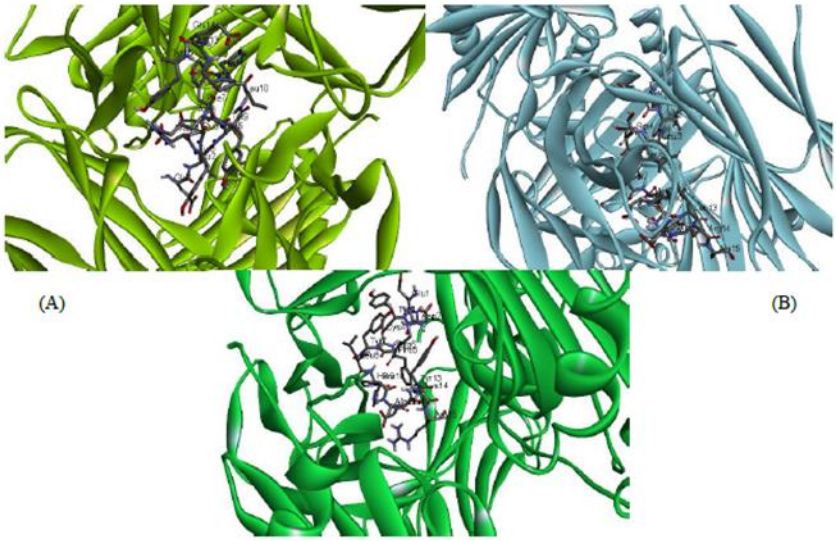
(A) (B) (C)

Supplementary Figure 13. 2D interaction between CTL epitopes from the cyaA protein with their corresponding MHC-I alleles. (A) YSLLPTLLHY epitope bound to HLA-B*57:01 with nine interactions including salt bridge, attractive charge, conventional hydrogen, carbon-hydrogen, pi-cation, pi-pi stacked, pi-pi T-shaped, alkyl, and pi-alkyl bonds. (B) RLHDGEIVSF epitope bound to HLA-B*15:01 with ten favorable interactions, including attractive charge, conventional hydrogen, carbon-hydrogen, pi-cation, pi-anion, pi-donor hydrogen, pi-sulfur, pi-pi T-shaped, alkyl, pi-alkyl, as well as one unfavorable acceptor-acceptor interaction, which is illustrated in red. (C) SFINFNLPQF epitope bound to HLA-A*24:02 with nine favorable interactions including salt bridge, attractive charge, conventional hydrogen, carbon-hydrogen, pi-donor hydrogen, pi-sulfur, pi-pi T-Shaped, alkyl and pi-alkyl bonds.



(A) (B)

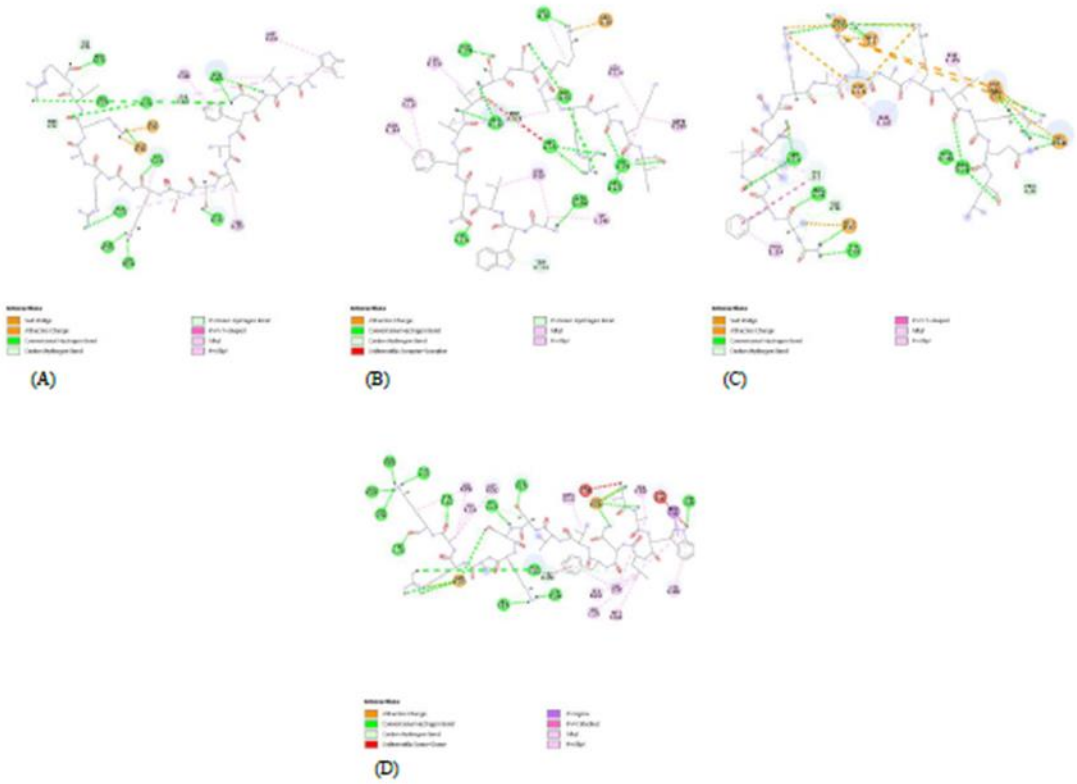
Supplementary Figure 14. Visualization of the interactions between HTL epitopes from the CRP protein with their corresponding MHC-II alleles. (A) 3D visualization of the PDILMRLSSQMARRL epitope bound to HLA-DRB1*04:01. Five favorable interactions, including conventional hydrogen, attractive charge, alkyl, pi-alkyl, and pi-sulfur bonds were formed (Figure not shown). (B) 2D visualization of the DILMRLSSQMARRLQ epitope bound to HLA-DRB1*04:01 with five favorable interactions, including conventional hydrogen, carbon-hydrogen, pi-sulfur, alkyl, and pi-alkyl bonds.



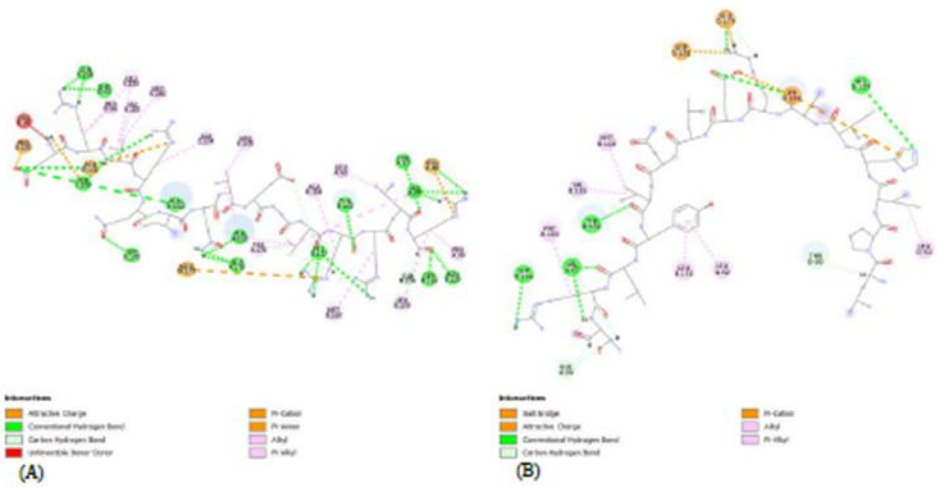
(A) (B)

(C)

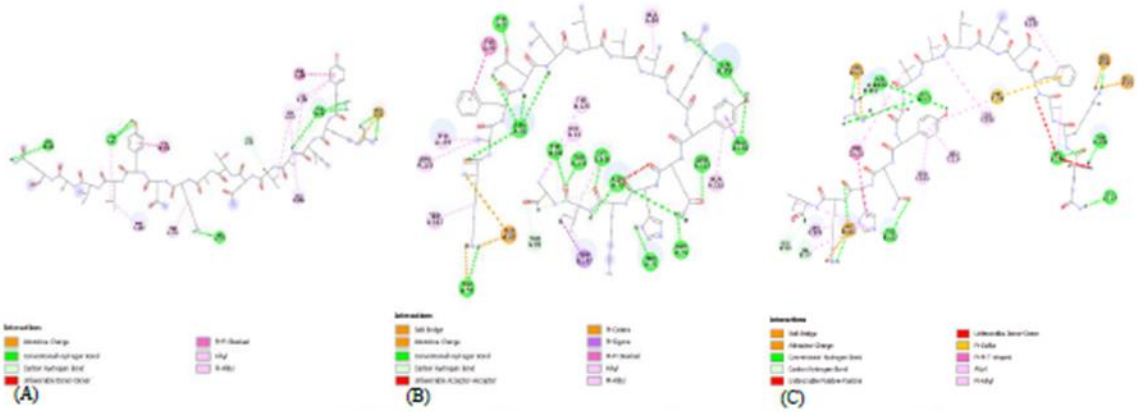
Supplementary Figure 15. 3D visualization of the docking between HTL epitopes from the relA protein, and their corresponding MHC-II alleles. (A) ELEDYCFRYLHPAEY epitope bound to HLA-DRB5*01:01 with six favorable interactions including conventional hydrogen, carbon hydrogen, salt bridge, attractive charge, pi-cation, alkyl, and pi-alkyl bonds (Figure not shown). (B) EILSTLSMDIDTLRA epitope bound to HLA-DRB3*01:01 with five favorable interactions including, conventional hydrogen, non-conventional hydrogen, attractive charge, pi-pi, and alkyl bonds (Figure not shown). (C) EDYCFRYLHPAEYKR epitope bound to HLA-DRB5*01:01 with seven favorable interactions including conventional hydrogen, carbon-hydrogen, attractive charge, pi-cation, alkyl, pi-alkyl, and pi-sulfur, as well as one unfavorable acceptor-donor interaction (Figure not shown).



Supplementary Figure 16. 2D interaction between HTL epitopes from the spoT protein with their corresponding MHC-II alleles. The four epitopes predicted, bound to HLA-DRB5*01:01. (A) WLNFVVSSKARAKIR epitope formed eight favorable interactions, including salt bridge, attractive charge, conventional hydrogen, carbon-hydrogen, pi-donor hydrogen, pi-pi T-shaped, alkyl, and pi-alkyl bonds. (B) AWLNFVVSSKARAKI epitope formed six favorable contacts, including attractive charge, conventional hydrogen, carbon-hydrogen, pi-donor hydrogen, alkyl, and pi-alkyl, as well as one unfavorable acceptor-acceptor interaction, which is illustrated in red. (C) NFVVSSKARAKIRQL epitope formed seven favorable interactions, including salt bridge, attractive charge, conventional hydrogen, carbon-hydrogen, pi-pi T shaped, alkyl and pi-alkyl bonds. (D) AAWLNFVVSSKARAK epitope formed seven favorable interactions, including attractive charge, conventional hydrogen, carbon-hydrogen, pi-sigma, pi-pi stacked, alkyl, and pi-alkyl, as well as one unfavorable donor-donor interaction, which is illustrated in red.



Supplementary Figure 17. 2D interaction between HTL epitopes from the rpoS protein with their corresponding MHC-II alleles. (A) ERVRQIQVEGLRRLR epitope bound to HLA-DRB5*01:01 with seven favorable interactions, including attractive charge, conventional hydrogen, carbon-hydrogen, pi-cation, pi-anion, alkyl, and pi-alkyl, as well as one unfavorable donor-donor interaction, which is illustrated in red. (B) LPIHIVKELNVYLRT epitope bound to HLA-DRB1*15:01 with seven favorable interactions, including, salt bridge, attractive charge, conventional hydrogen, carbon-hydrogen, pi-cation, alkyl, and pi-alkyl bonds.



Supplementary Figure 18. 2D interaction between HTL epitopes from the rpoE protein with their corresponding MHC-II alleles. (A) YRIAVNTAKNYLVAQ epitope bound to HLA-DRB1*04:01 with six favorable interactions, including attractive charge, conventional hydrogen, carbon-hydrogen, pi-pi stacked, alkyl and pi-alkyl, as well as one unfavorable donor-donor interaction, which is illustrated in red. (B) KAFNLLVVRYQHKVA epitope bound with HLA-DRB1*11:01 with nine favorable interactions, including salt bridge, attractive charge, conventional hydrogen, carbon-hydrogen, pi-cation, pi-sigma, pi-pi stacked, alkyl, and pi-alkyl, as well as one unfavorable acceptor-acceptor interaction, which is illustrated in red. (C) QKAFNLLVVRYQHKV epitope bound with HLA-DRB5*01:01 with eight favorable interactions including salt bridge, attractive charge, conventional hydrogen, carbon hydrogen, po-sulfur, pi-pi T-shaped, alkyl, and pi-alkyl, as well as two unfavorable positive-positive and donor-donor interactions, which are illustrated in red.

4. CONCLUSIONES GENERALES

En el presente estudio se estudiaron los serotipos Montevideo, Pomona y Oranienburg de *Salmonella*, los cuales son prevalentes en el ambiente y de gran importancia en salud pública. El análisis genómico realizado permitio identificar la diversidad genética en términos de virulencia, resistencia a antibióticos, respuesta a estrés y metabolismo presentes en genomas ambientales y su comparación con genomas clínicos, encontrando similitudes y algunas diferencias puntuales en cuanto a contenido genético relacionado con resistencia a antibióticos y presencia de sistemas de secreción. Asimismo, el análisis proteómico permitió encontrar proteínas esenciales para estos serotipos, que fueron utilizadas para el desarrollo *In silico* de una vacuna multi-epítopo que sea capaz de brindar protección ante salmonelosis no-tifoidea. La vacuna diseñada en este estudio, a partir de proteínas SSR, demostró alto potencial inmunogénico y seguridad para el hospedador. La combinación de ambos análisis es de suma importancia ya que no solo se limita a la identificación de genes esenciales en la sobrevivencia de la bacteria si no también proponer y desarrollar nuevas estrategias para control y/o prevención de la enfermedad que puede causar *Salmonella*, utilizando dichos genes como blanco.

5. RECOMENDACIONES

Se sugiere llevar a cabo más análisis genómicos con enfoque en otro tipo de factores relacionados a patogenicidad y sobrevivencia de serotipos ambientales de *Salmonella*. Asi como también llevar a cabo estudios a nivel laboratorio para evaluar la expresión genética de genes de relevancia. Por otro lado tambien se recomienda realizar estudios *In vitro* e *In vivo* para el desarrollo de la vacuna multi-epítopo diseñada en el presente estudio. Por último, es de gran importancia continuar este tipo de trabajos en donde se combinen distintos análisis, que permitan no solo identificar presencia de *Salmonella* y de genes o proteínas de importancia, si no también, empezar a utilizar la información generada para el desarrollo de estrategias para evitar la propagación de este microorganismo y prevenir la enfermedad que puede causar.

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