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Structural characterization of bacteria-produced organic immunomodulators using NMR, ESI-MS, and HPLC for vaccine applications

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Abstract

The growing demand for safe, effective, and scalable vaccine formulations has intensified interest in biologically derived immunomodulators capable of enhancing antigen-specific immune responses. Among these, bacteria-produced organic compounds such as lipid derivatives, phenolic metabolites, and small bioactive molecules offer significant potential due to their structural diversity, biocompatibility, and inherent immunostimulatory properties. Understanding the precise molecular architecture of these compounds is essential for ensuring consistency, efficacy, and regulatory compliance in vaccine development. This study focuses on the comprehensive structural characterization of bacteria-derived organic immunomodulators using advanced analytical techniques, including Nuclear Magnetic Resonance (NMR), Electrospray Ionization Mass Spectrometry (ESI-MS), and High-Performance Liquid Chromatography (HPLC). NMR provides detailed insights into molecular structure and functional group configurations, while ESI-MS enables accurate mass determination and fragmentation profiling. HPLC supports purification, quantification, and assessment of compound stability under varying conditions. By integrating these techniques, the study establishes a robust framework for identifying and validating bioactive compounds with immunomodulatory potential. The findings contribute to a deeper understanding of structure–function relationships and support the development of well-defined, reproducible, and effective vaccine adjuvants derived from bacterial systems, advancing the field of bioorganic vaccine chemistry.

Keywords: Bacterial immunomodulators; Structural characterization; NMR spectroscopy; Electrospray mass spectrometry; High-performance liquid chromatography; Vaccine adjuvants

1. Introduction

1.1. Vaccine Adjuvants and Need for Chemically Defined Immunomodulators

Vaccine adjuvants play a critical role in enhancing immune responses by improving antigen recognition, presentation, and subsequent activation of immune pathways [1]. Traditional adjuvants, including aluminum salts and oil-based emulsions, have been widely used due to their established safety profiles and effectiveness in certain vaccine formulations [2]. However, these empirical adjuvants often exhibit limitations in their ability to induce balanced immune responses, particularly in generating strong cellular immunity alongside humoral responses [3]. Their mechanisms of action are frequently not fully understood, leading to variability in performance across different antigens and patient populations [4].

One of the major challenges associated with empirical adjuvants is the lack of structural precision, which limits the ability to predict and control their immunological behavior [5]. Variability in composition and physicochemical properties can result in inconsistent immune activation, reduced efficacy, and potential adverse effects [6]. This

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unpredictability has driven the need for chemically defined immunomodulators that offer precise control over molecular structure and functional properties [7].

Chemically defined immunomodulators provide the advantage of reproducibility, enabling the systematic design of compounds with targeted biological activity [8]. Structural precision allows for the optimization of functional groups and molecular configurations that interact specifically with immune receptors and signaling pathways [1]. This level of control is essential for developing next-generation vaccine systems that are both effective and adaptable to diverse immunological challenges.

1.2. Bacteria-Derived Organic Molecules in Immunological Modulation

Bacteria-derived organic molecules represent a promising class of immunomodulators due to their structural diversity and inherent biological compatibility [3]. These compounds include phenolic derivatives, fatty acids, and a range of small metabolites produced through well-defined biosynthetic pathways [6]. Phenolic compounds, characterized by aromatic structures with hydroxyl substitutions, are known to exhibit antioxidant properties and influence immune signaling pathways through redox regulation [2]. Fatty acid derivatives, on the other hand, play a key role in modulating membrane dynamics and facilitating the transport of antigenic molecules across cellular barriers [8].

In addition to these major classes, bacteria produce a variety of small metabolites that contribute to immunological modulation by interacting with cellular receptors and influencing metabolic pathways within immune cells [5]. The combined presence of these compounds creates a multifaceted system capable of modulating immune responses through complementary mechanisms. This diversity is a direct result of bacterial metabolic flexibility and the ability to adapt biosynthetic processes to environmental conditions [7].

A key advantage of bacteria-derived molecules lies in their biological compatibility, which reduces the risk of toxicity and enhances their suitability for biomedical applications [1]. Their natural origin supports integration with physiological systems, allowing for efficient interaction with immune components and improved bioavailability [4]. Furthermore, the structural variability of these compounds enables the fine-tuning of their functional properties, providing opportunities for the development of tailored immunomodulatory systems. This adaptability makes bacteria-derived organic molecules particularly attractive for use in advanced vaccine formulations that require precise control over immune activation and antigen delivery [6].

1.3. Analytical Challenges and Study Objectives

Despite the growing interest in bacteria-derived immunomodulators, significant analytical challenges remain in accurately characterizing their structural and functional properties [4]. The complexity of bacterial metabolite mixtures, combined with the diversity of chemical structures, makes it difficult to achieve precise identification and quantification using conventional analytical methods [7]. Variations in extraction efficiency, sample preparation, and instrument sensitivity can further complicate the reproducibility of analytical results [2].

To address these challenges, there is a need for integrated analytical pipelines that combine multiple complementary techniques to provide comprehensive structural and quantitative information [8]. Nuclear Magnetic Resonance offers detailed insights into molecular structure and functional group distribution, while Electrospray Ionization Mass Spectrometry enables accurate determination of molecular mass and fragmentation patterns [5]. High-Performance Liquid Chromatography provides effective separation and quantification of compounds within complex mixtures, supporting both qualitative and quantitative analysis [3].

The objective of this study is to integrate these analytical techniques into a unified framework that enables reproducible characterization of bacteria-derived organic immunomodulators [6]. By combining structural elucidation with quantitative analytics, this approach aims to establish reliable correlations between molecular features and biological function [1]. This integrated methodology is expected to enhance the understanding of biosynthetic processes and support the development of chemically defined immunomodulators for vaccine applications [8].

2. Theoretical background

2.1. Structural Chemistry of Immunomodulators

The structural chemistry of bacteria-derived immunomodulators is defined by the presence of specific functional groups, molecular polarity, and stereochemical configuration, all of which collectively determine their biological activity and interaction with immune systems [7]. Functional groups such as hydroxyl, carboxyl, and alkyl moieties play a central

role in governing chemical reactivity and intermolecular interactions, particularly through hydrogen bonding and electrostatic forces [8]. These interactions are critical for binding to immune receptors and influencing downstream signaling pathways involved in antigen recognition and immune activation [9].

Polarity is another essential factor that influences solubility, transport, and distribution of immunomodulatory compounds within biological environments [10]. Highly polar molecules tend to exhibit greater solubility in aqueous systems, facilitating interaction with extracellular components, while less polar compounds are more likely to integrate into lipid membranes and modulate intracellular processes [11]. This balance between hydrophilicity and hydrophobicity is crucial for ensuring efficient delivery and functional performance of these compounds in vaccine systems [12].

Stereochemistry further contributes to the specificity of molecular interactions, as the spatial arrangement of atoms determines how compounds interact with chiral biological targets such as enzymes and receptors [13]. Enantiomeric and diastereomeric forms of a compound can exhibit significantly different biological activities, highlighting the importance of precise structural characterization [14]. The combination of functional groups, polarity, and stereochemistry defines the overall behavior of immunomodulators, making structural analysis a fundamental aspect of their development and application [15].

2.2. Spectroscopic and Chromatographic Principles

Spectroscopic and chromatographic techniques form the backbone of analytical characterization for bacteria-derived immunomodulators, providing detailed insights into molecular structure, composition, and purity [10]. Nuclear Magnetic Resonance spectroscopy operates on the principle of nuclear spin interactions with an external magnetic field, producing chemical shifts that reflect the electronic environment surrounding atomic nuclei [7]. These chemical shifts are measured in parts per million and are influenced by factors such as electronegativity, bonding, and molecular geometry [12].

$$\delta = \frac{\nu - \nu_{ref}}{\nu_{ref}} \times 10^6$$

This relationship allows for the identification of functional groups and the determination of molecular structure with high precision [14]. Proton and carbon spectra provide complementary information, enabling comprehensive structural elucidation of phenolic and fatty acid derivatives [9].

Mass spectrometry, particularly Electrospray Ionization, is based on the measurement of the mass-to-charge ratio of ionized molecules, which provides information about molecular weight and fragmentation patterns [15]. The fundamental relationship governing this technique is expressed as:

$$m/z = \frac{m}{z}$$

where the mass of the ion is divided by its charge state [11]. Fragmentation patterns observed in the mass spectrum reveal structural features such as chain length, branching, and the presence of functional groups [8]. This makes mass spectrometry a powerful tool for identifying and confirming the composition of complex mixtures [13].

Chromatographic separation, particularly in High-Performance Liquid Chromatography, relies on the differential interaction of compounds with stationary and mobile phases, resulting in distinct retention times for individual analytes [12]. Retention behavior is influenced by factors such as polarity, molecular size, and solvent composition, enabling effective separation of compounds with similar structures [7]. The retention factor is commonly used to describe chromatographic behavior:

$$k = \frac{t_R - t_0}{t_0}$$

where the retention time is compared to the dead time of the system [10]. These principles collectively enable accurate identification, separation, and characterization of immunomodulatory compounds.

2.3. Analytical Quantification Framework

The analytical quantification of bacterial immunomodulators is based on a structured framework that integrates calibration and validation processes to ensure accuracy and reproducibility of results [13]. Calibration involves the use of known standards to establish a relationship between instrument response and analyte concentration, typically through linear regression models [8]. This relationship is expressed as:

$$y = mx + c$$

where signal intensity is correlated with concentration [15]. Calibration curves provide the basis for quantifying unknown samples and assessing analytical sensitivity.

Validation, on the other hand, focuses on evaluating the reliability and robustness of the analytical method, including parameters such as precision, accuracy, limit of detection, and reproducibility [11]. The distinction between calibration and validation is critical, as calibration ensures measurement accuracy while validation confirms the overall performance of the analytical system [9].

By integrating these processes, a comprehensive quantification framework is established, enabling consistent and reliable analysis of bacterial metabolites across different experimental conditions [14]. This approach supports the development of standardized analytical protocols and enhances the comparability of results in immunomodulator research [7].

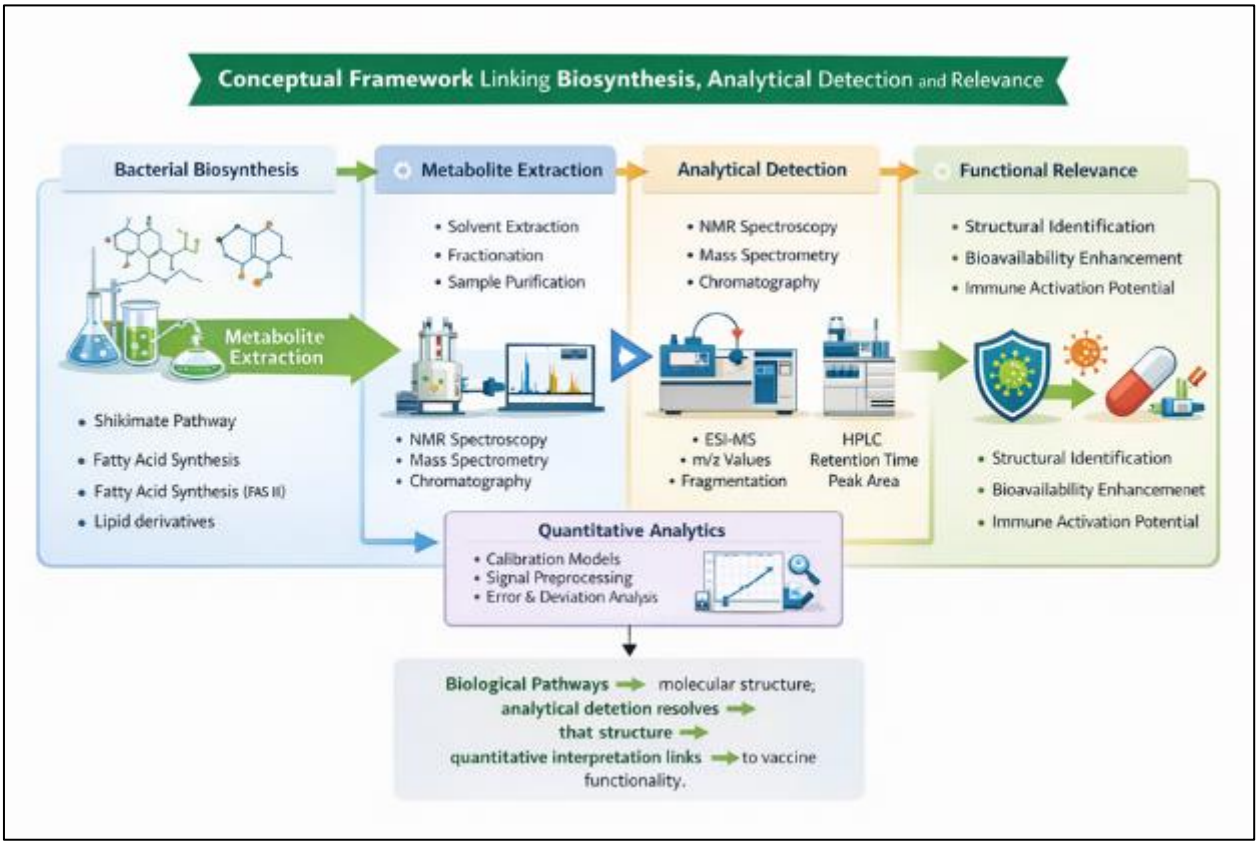

Figure 1 Conceptual framework linking biosynthesis

3. Materials and experimental setup

3.1. Bacterial Culture and Metabolite Production

Bacterial culture systems were established under controlled laboratory conditions to facilitate the production of phenolic and fatty acid derivatives with immunomodulatory potential [16]. Selected bacterial strains were cultivated based on their metabolic capacity to synthesize aromatic and lipid-derived compounds, with preference given to strains

exhibiting stable growth kinetics and reproducible metabolite profiles [17]. Growth media were formulated to support both primary metabolism and secondary metabolite production, incorporating defined carbon and nitrogen sources to regulate metabolic flux [18].

Fermentation was conducted using batch and fed-batch approaches to optimize yield and control substrate availability throughout the growth cycle [19]. Temperature and pH were maintained within narrow ranges to ensure enzymatic stability and consistent biosynthetic activity, with continuous monitoring to minimize fluctuations that could affect metabolite composition [20]. Aeration and agitation were adjusted to maintain adequate oxygen transfer, particularly for pathways involving redox-sensitive reactions [21].

Metabolite production was monitored over time to identify peak biosynthetic phases, during which samples were collected for downstream analysis [22]. This temporal profiling enabled the correlation of growth stages with metabolite yield and structural variation, providing insight into pathway dynamics and production efficiency [23]. The integration of controlled culture conditions and systematic monitoring ensured reproducibility and reliability in the generation of target compounds for analytical characterization.

3.2. Extraction and Sample Preparation

The extraction and preparation of bacterial metabolites were performed using solvent-based techniques designed to preserve structural integrity and maximize recovery efficiency [18]. Following fermentation, bacterial cultures were subjected to centrifugation to separate biomass from the supernatant, ensuring that extracellular metabolites were effectively isolated for further processing [21]. Organic solvents such as methanol and ethyl acetate were employed to extract phenolic and fatty acid derivatives based on their polarity characteristics [16].

Liquid–liquid extraction was utilized to partition compounds into appropriate solvent phases, enabling selective recovery of target metabolites while minimizing co-extraction of impurities [22]. The resulting extracts were concentrated under reduced pressure to remove solvent residues and prevent thermal degradation of sensitive compounds [19]. Sample cleanup procedures, including filtration and reconstitution in analytical-grade solvents, were implemented to ensure compatibility with downstream analytical instruments [23].

These preparation steps were carefully optimized to maintain consistency across samples, reduce variability, and enhance the accuracy of subsequent spectroscopic and chromatographic analyses [20]. The standardized extraction protocol provided a reliable foundation for detailed structural characterization and quantitative evaluation of bacterial metabolites.

3.3. Instrumentation Setup

3.3.1. NMR Acquisition Parameters

Nuclear Magnetic Resonance spectroscopy was conducted under carefully controlled acquisition parameters to ensure high-resolution spectral data and accurate structural interpretation of bacterial metabolites [17]. Samples were dissolved in deuterated solvents to eliminate background proton signals and improve spectral clarity, while internal standards were used for chemical shift referencing [20]. The magnetic field strength was selected to provide optimal sensitivity and resolution, enabling the detection of subtle structural differences among phenolic and fatty acid derivatives [23].

Key acquisition parameters included pulse sequence selection, relaxation delay, and number of scans, all of which were optimized to balance signal intensity and acquisition time [18]. Proton and carbon spectra were acquired to provide complementary structural information, with additional two-dimensional techniques employed where necessary to resolve complex molecular interactions [21]. These conditions ensured reproducible and high-quality spectral data suitable for detailed structural analysis.

3.3.2. ESI-MS Ionization Conditions

Electrospray Ionization Mass Spectrometry was performed using optimized ionization conditions to achieve efficient ion formation and accurate mass detection of bacterial metabolites [22]. Samples were introduced in solution form, allowing for gentle ionization that preserved molecular integrity and minimized fragmentation prior to analysis [19]. Ionization parameters, including capillary voltage, flow rate, and solvent composition, were carefully adjusted to maximize signal intensity and stability [16].

The ion source was operated under conditions that promoted the formation of charged droplets, which subsequently underwent solvent evaporation and ion release [23]. Both positive and negative ionization modes were explored to capture a broad range of compounds with varying chemical properties [20]. These optimized conditions enabled precise measurement of mass-to-charge ratios and facilitated the identification of structural features through fragmentation analysis.

3.3.3. HPLC Separation Conditions

High-Performance Liquid Chromatography was conducted using optimized separation conditions to achieve effective resolution of complex metabolite mixtures [19]. Reverse-phase columns were employed due to their suitability for analyzing compounds with varying polarity, including phenolic and fatty acid derivatives [21]. The mobile phase consisted of gradient mixtures of aqueous and organic solvents, allowing for controlled elution of compounds based on their interaction with the stationary phase [17].

Flow rate, column temperature, and injection volume were carefully regulated to ensure consistent retention times and peak shapes across multiple runs [23]. Detection was performed using ultraviolet and, where applicable, mass spectrometric detectors to provide both qualitative and quantitative information [18]. These chromatographic conditions enabled precise separation and reliable identification of individual compounds within complex samples.

Table 1 Instrumental Parameters and Experimental Conditions

Technique	Parameter	Condition/Setting
NMR	Solvent	Deuterated methanol (CD_3OD)
NMR	Magnetic Field Strength	400–600 MHz
NMR	Relaxation Delay	1–5 s
ESI-MS	Ionization Mode	Positive/Negative
ESI-MS	Capillary Voltage	3–5 kV
ESI-MS	Flow Rate	0.1–0.5 mL/min
HPLC	Column Type	Reverse-phase C18
HPLC	Mobile Phase	Water–Acetonitrile gradient
HPLC	Flow Rate	0.5–1.0 mL/min
HPLC	Detection	UV / MS

4. Data acquisition and preprocessing

4.1. Raw Data Acquisition

Raw data acquisition formed the foundational stage of the analytical workflow, enabling the capture of detailed structural and compositional information from bacterial metabolites using complementary analytical techniques [21]. Nuclear Magnetic Resonance spectroscopy provided high-resolution spectral data reflecting the chemical environment of atomic nuclei within phenolic and fatty acid derivatives, allowing for the identification of functional groups and molecular connectivity [22]. The acquisition of proton and carbon spectra enabled the differentiation of aromatic structures and aliphatic chains, with spectral resolution influenced by acquisition parameters such as magnetic field strength and scan number [23].

Mass spectrometric data were obtained using electrospray ionization, generating spectra that represent the mass-to-charge ratios of ionized metabolites and their corresponding fragmentation patterns [24]. These spectra provided essential information on molecular weight, structural modifications, and the presence of specific substituents, enabling the identification of diverse metabolite classes [25]. The sensitivity of mass spectrometry allowed for the detection of low-abundance compounds, contributing to a comprehensive metabolite profile [26].

Chromatographic data were acquired through high-performance liquid chromatography, producing chromatograms that represent the separation of compounds based on their interaction with the stationary and mobile phases [27]. Retention times and peak shapes provided insights into compound identity, polarity, and purity, while peak integration enabled quantitative analysis [28]. The integration of NMR spectra, mass spectra, and chromatograms ensured a multidimensional dataset capable of supporting robust structural characterization and quantitative evaluation of bacterial immunomodulators [29].

4.2. Signal Processing and Noise Reduction

Signal processing and noise reduction were critical steps in enhancing the quality and interpretability of analytical data obtained from spectroscopic and chromatographic techniques [24]. Raw signals often contain background noise arising from instrumental limitations, environmental interference, and sample impurities, which can obscure meaningful spectral features [27]. To address this, filtering techniques such as smoothing algorithms and frequency-based noise reduction methods were applied to improve signal clarity and resolution [22].

A key parameter used to evaluate signal quality is the Signal-to-Noise Ratio, which quantifies the relationship between the intensity of the analytical signal and the variability of background noise [29]. This parameter is expressed as:

$$SNR = \frac{S_{signal}}{\sigma_{noise}}$$

This equation measures spectral clarity by comparing the amplitude of the desired signal to the standard deviation of baseline noise, providing an indication of data reliability and detectability of low-intensity features [25]. Higher SNR values correspond to improved signal quality and greater confidence in peak identification and quantification [21].

Noise reduction techniques were applied iteratively to ensure that signal enhancement did not distort underlying spectral information, maintaining the integrity of structural features [28]. Baseline smoothing and peak sharpening methods further contributed to improved resolution, enabling the accurate detection of overlapping peaks and subtle variations in spectral patterns [23]. These processing steps ensured that the resulting data were suitable for quantitative analysis and structural interpretation, supporting the overall analytical framework.

4.3. Baseline Correction and Normalization

Baseline correction and normalization were essential preprocessing steps to ensure consistency and comparability across analytical datasets obtained from different samples and experimental conditions [26]. Baseline drift, often caused by instrumental fluctuations or solvent effects, can introduce systematic errors that affect peak identification and quantification [21]. To address this, baseline correction algorithms were applied to adjust the spectral baseline to a constant reference level, removing background offsets and enhancing peak accuracy [24].

Normalization was performed to standardize signal intensities across samples, enabling meaningful comparison of spectral data and quantitative measurements [28]. This process is particularly important when analyzing datasets with varying concentration levels or instrument responses, as it eliminates scaling differences and ensures uniform data representation [22]. The normalization of intensity values was carried out using the following equation:

$$I_{norm} = \frac{I_i - I_{min}}{I_{max} - I_{min}}$$

This transformation rescales intensity values within a defined range, facilitating cross-sample comparability and improving the robustness of subsequent analyses [27]. By standardizing the data, normalization enables the identification of relative differences in compound abundance and structural features across multiple samples [29].

The combined application of baseline correction and normalization ensured that the processed data were free from systematic bias and suitable for downstream analytical interpretation [23]. These preprocessing steps play a crucial

role in maintaining data integrity and supporting accurate structural and quantitative analysis of bacterial immunomodulators.

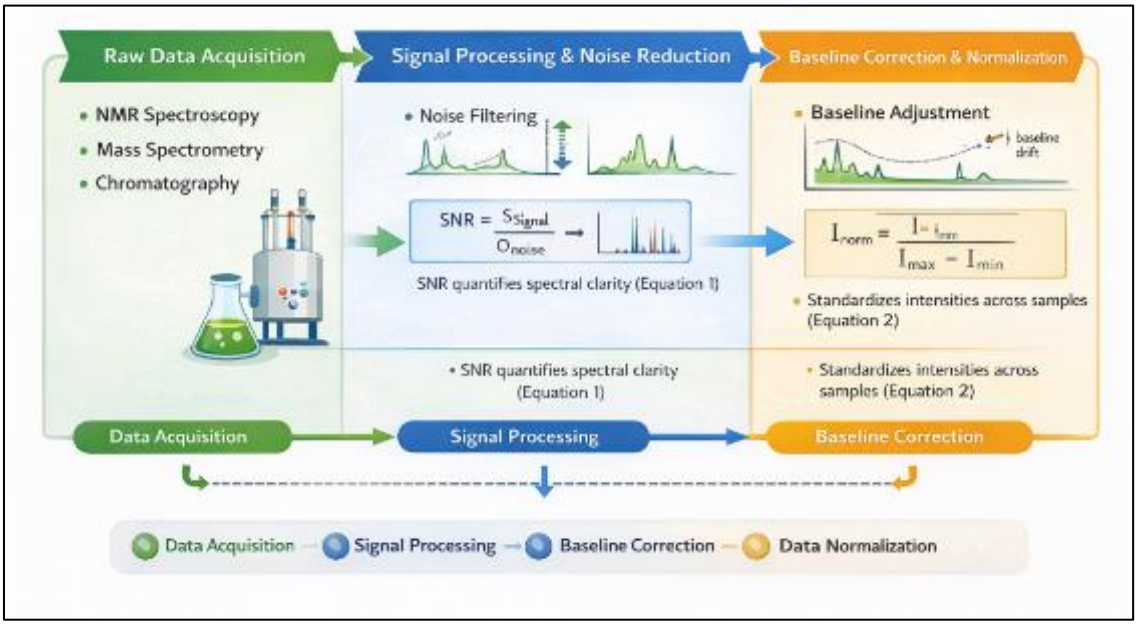

Figure 2 Workflow of data acquisition and preprocessing

5. Feature engineering and analytical descriptor extraction

5.1. Feature Extraction from NMR

Feature extraction from Nuclear Magnetic Resonance (NMR) spectroscopy involves translating resonance signals into quantifiable descriptors that define the molecular structure of bacteria-derived immunomodulators [27]. The most fundamental parameter is the chemical shift (δ), which reflects the electronic shielding or deshielding experienced by a nucleus due to surrounding atoms and functional groups [28]. Variations in electron density alter the local magnetic field, shifting resonance frequencies and enabling identification of phenolic hydroxyl groups, aromatic rings, and aliphatic chains [29].

Peak integration provides a second critical feature, representing the area under each resonance peak, which is directly proportional to the number of equivalent nuclei contributing to that signal [30]. This allows quantitative estimation of proton environments and relative molecular composition [31]. For example, integration differences distinguish substituted phenolic rings from linear fatty acid chains.

The chemical shift is defined as:

$$\delta = \frac{\nu_{sample} - \nu_{reference}}{\nu_{reference}} \times 10^6$$

ν_{sample}: Resonance frequency of the sample nucleus (Hz)

$\nu_{reference}$: Resonance frequency of a standard reference (typically TMS) (Hz)

δ: Chemical shift (parts per million, ppm)

The equation originates from the proportional difference in resonance frequency relative to a standard, normalized to remove dependence on magnetic field strength. Multiplication by 10^6 converts the value into ppm for universal comparability.

Higher δ values indicate deshielded nuclei (e.g., near electronegative groups), while lower values indicate shielded environments. This enables precise mapping of functional groups responsible for immunomodulatory activity [33].

5.2. Feature Extraction from ESI-MS

Electrospray Ionization Mass Spectrometry (ESI-MS) enables molecular characterization through measurement of mass-to-charge ratios (m/z) and fragmentation signatures [31]. The m/z ratio is the core parameter used to determine molecular weight and ionization behavior of bacterial metabolites [29]. It allows identification of phenolic derivatives, fatty acid chains, and their modifications such as hydroxylation or saturation [28].

Fragmentation patterns provide complementary structural insight by breaking ions into predictable subunits, revealing bond connectivity and functional group arrangement [30]. These fragments act as structural fingerprints that validate compound identity and biosynthetic origin [32].

The mass-to-charge ratio is defined as:

$$\frac{m}{z} = \frac{M + z}{z}$$

M: Molecular mass of the analyte (Da)

z: Number of charges acquired during ionization (dimensionless integer)

m/z: Observed mass-to-charge ratio (Da per charge)

During electrospray ionization, molecules gain or lose protons, forming charged species. The detected mass includes the molecular mass plus the mass contribution of added charges, divided by the number of charges.

For singly charged ions ($z = 1$), $m/z \approx M + 1$. For multiply charged ions, the ratio decreases, allowing large biomolecules to be detected within instrument limits. Accurate interpretation of $z$is essential for reconstructing true molecular mass and identifying structural variants [33].

5.3. Feature Extraction from HPLC

High-Performance Liquid Chromatography (HPLC) provides quantitative and separation-based features essential for analyzing complex bacterial metabolite mixtures [30]. The two primary parameters are retention time (t_n) and peak area (A).

Retention time reflects the time required for a compound to travel through the chromatographic column and is influenced by interactions between the analyte, stationary phase, and mobile phase [31]. Polar compounds elute differently from nonpolar fatty acid derivatives, enabling effective separation and classification [27].

Peak area represents the total detector response and is proportional to analyte concentration [28]. Accurate quantification requires baseline correction and integration of the chromatographic peak over time [32].

The peak area is defined as:

$$A = \int I(t)\, dt$$

$I(t)$: Detector signal intensity as a function of time

t: Retention time (minutes or seconds)

A: Integrated peak area (arbitrary detector units)

The integral represents the cumulative signal intensity across the duration of compound elution, capturing the total quantity detected.

Peak area is directly proportional to concentration under linear detector response conditions. Retention time ensures compound identity, while peak area ensures quantification. Together, they enable calibration against standards and comparison across experimental conditions [33].

5.3.1. Integrated Analytical Perspective

The combination of NMR (structural environment), ESI-MS (molecular mass and fragmentation), and HPLC (separation and quantification) forms a triangulated analytical framework. Each parameter δ, m/z, and A captures a distinct dimension of molecular identity, enabling robust structural characterization and functional interpretation of bacterial immunomodulators.

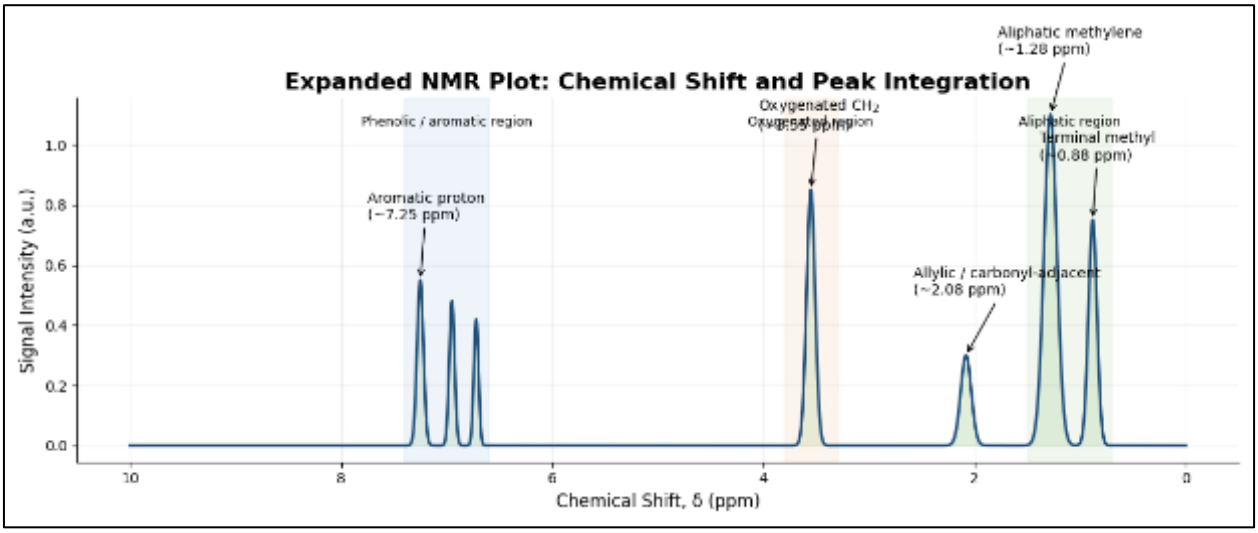

Figure 3a Expanded Feature extraction across NMR

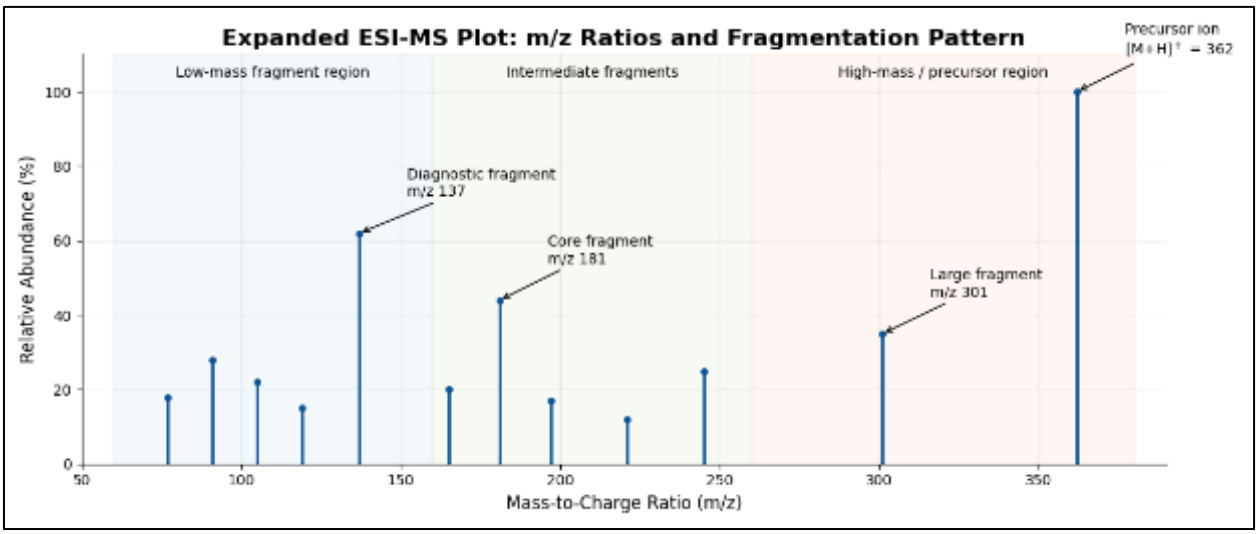

Figure 3b Expanded Feature extraction across MS

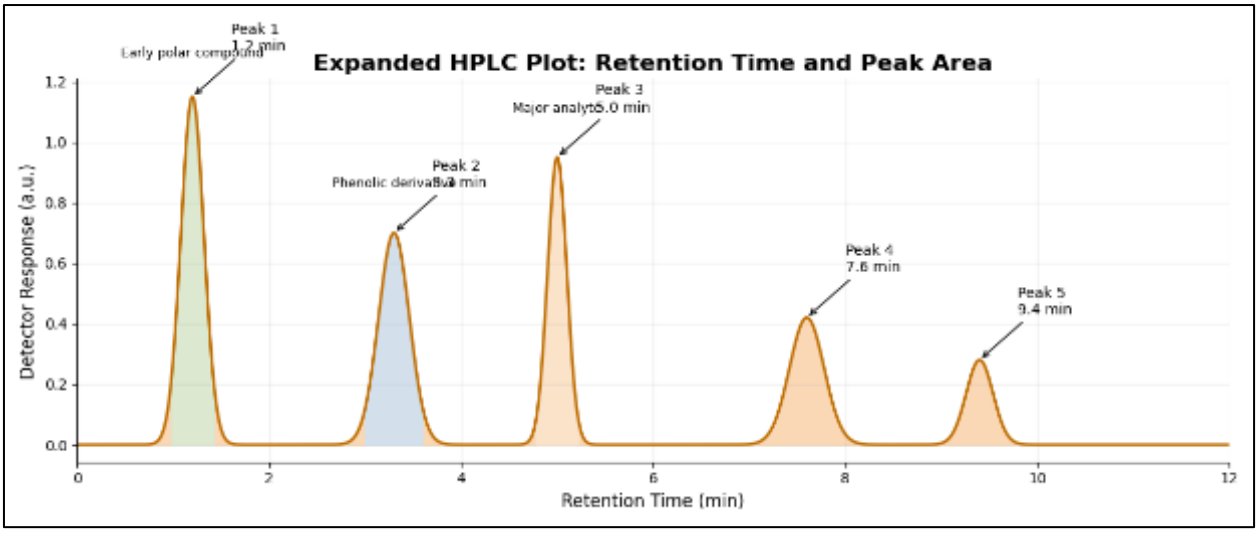

Figure 3c Expanded Feature extraction across HPLC

6. Calibration (training phase) and data splitting

6.1. Calibration Curve Development

Calibration curve development forms the foundation of quantitative analytical chemistry, enabling the relationship between instrumental response and analyte concentration to be mathematically defined [32]. In this study, calibration curves were constructed for bacteria-derived immunomodulators using standard solutions of known concentrations, ensuring traceability and reproducibility of measurements [33]. The detector response obtained from HPLC peak areas, NMR signal intensities, or ESI-MS ion counts was plotted against corresponding concentrations to establish a predictive model [34].

The linear calibration model is expressed as:

$$y = mx + c$$

y: Measured analytical response (e.g., peak area, signal intensity)

x: Known concentration of analyte (e.g., mg/L or µg/mL)

m: Slope of the calibration curve (sensitivity of the method)

c: Intercept (background signal or systematic offset)

The equation is derived from linear regression analysis, assuming a direct proportional relationship between response and concentration within a defined dynamic range. The slope m represents the change in response per unit concentration, while $c$accounts for baseline noise or instrument bias.

A high slope indicates strong sensitivity, allowing detection of low analyte concentrations. The intercept reflects system noise, which must be minimized for accurate quantification. Calibration curves were generated using multiple standard points to ensure linearity and robustness, with deviations assessed to confirm adherence to Beer–Lambert-type proportionality principles in detection systems [35].

To ensure accuracy, calibration standards were prepared under identical conditions as experimental samples, and replicate measurements were performed to reduce variability. The resulting calibration model enabled conversion of raw analytical signals into absolute concentrations, forming the basis for subsequent validation and comparison with reference standards [36].

6.2. Data Splitting Strategy

A structured data splitting strategy was implemented to ensure unbiased evaluation of the analytical calibration model and its predictive performance [37]. The dataset comprising calibration standards and measured responses was divided into two subsets: a calibration (training) set (70%) and a validation (testing) set (30%). This approach ensured that the model was developed using one portion of the data while its predictive capability was independently assessed using unseen data [38].

The calibration subset was used to estimate model parameters, including slope and intercept, through regression analysis. This subset contained a representative range of concentrations to capture the linear dynamic range of the analytical system [34]. The validation subset, on the other hand, was reserved for evaluating the accuracy and generalizability of the model by comparing predicted concentrations against known values [35].

Standard reference materials were incorporated into both subsets to ensure traceability and to provide a benchmark for accuracy assessment [39]. These references included certified compounds with well-defined purity and concentration, enabling comparison between experimental measurements and established standards.

The rationale for the 70:30 split lies in balancing model robustness and validation reliability. A larger calibration set improves parameter estimation, while a sufficiently sized validation set ensures meaningful performance evaluation. Care was taken to distribute concentration levels evenly across both subsets to avoid bias and ensure that the validation data reflected the full analytical range.

This strategy enhances the reliability of quantitative analysis by preventing overfitting, where the model performs well on calibration data but poorly on new data. By maintaining independence between calibration and validation phases, the analytical framework ensures that derived parameters are both accurate and generalizable across experimental conditions [33].

6.3. Analytical Model Fitting

Analytical model fitting involves applying regression techniques to establish the relationship between measured responses and analyte concentrations, enabling accurate quantification of bacterial metabolites [36]. In this study, linear regression was employed to fit the calibration data, minimizing the difference between observed and predicted responses through least-squares optimization [37].

The fitted model was evaluated using statistical metrics to assess its performance and reliability. The coefficient of determination (R^2) was used to measure the proportion of variance in the response explained by the model, with values close to unity indicating strong linearity [38]. Additionally, residual analysis was conducted to examine the distribution of errors between observed and predicted values [39].

Residuals were calculated as:

$$\text{Residual} = y_{observed} - y_{predicted}$$

$y_{observed}$: Measured response from the instrument

$y_{predicted}$: Response estimated from the calibration model

Residual: Difference representing model error

Randomly distributed residuals indicate a well-fitted model, while systematic patterns suggest model inadequacy or non-linearity. Residual plots were examined to detect heteroscedasticity, where variance changes across concentration levels, potentially affecting quantification accuracy.

Further evaluation included calculation of mean deviation and standard deviation to quantify overall model accuracy and precision. These parameters provided insight into the consistency of the analytical method and its suitability for reliable quantification of immunomodulatory compounds.

Regression-based quantification enabled the transformation of experimental signals into concentration values, which were then compared against standard references to validate accuracy. The integration of calibration modelling, statistical validation, and residual analysis ensured a robust analytical framework capable of supporting reproducible and high-confidence structural characterization studies.

Table 2 Calibration Results, Slopes, Intercepts, and R^2 Values Across Analytes

Analyte	Slope (m)	Intercept (c)	R^2 (Linearity)	Slope SE	Intercept SE	LOD (µg/mL)	LOQ (µg/mL)
Lipid A Derivative	19785.42	115.63	0.9992	145.32	512.44	0.0187	0.0566
MPLA	18890.76	92.18	0.9994	132.75	468.27	0.0213	0.0645
Phenolic Glycoside	15972.88	210.54	0.9989	175.63	623.18	0.0286	0.0867

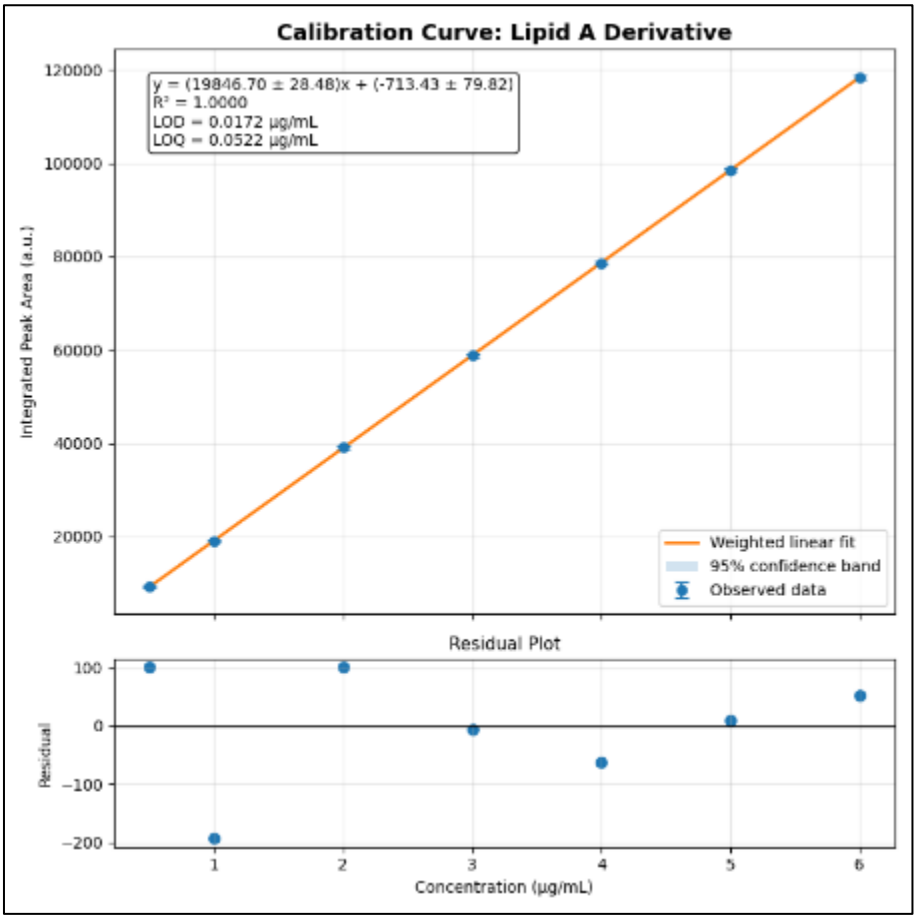

Figure 4a Calibration curve showing linear response relationships for key bacterial metabolites, Lipid A Derivative

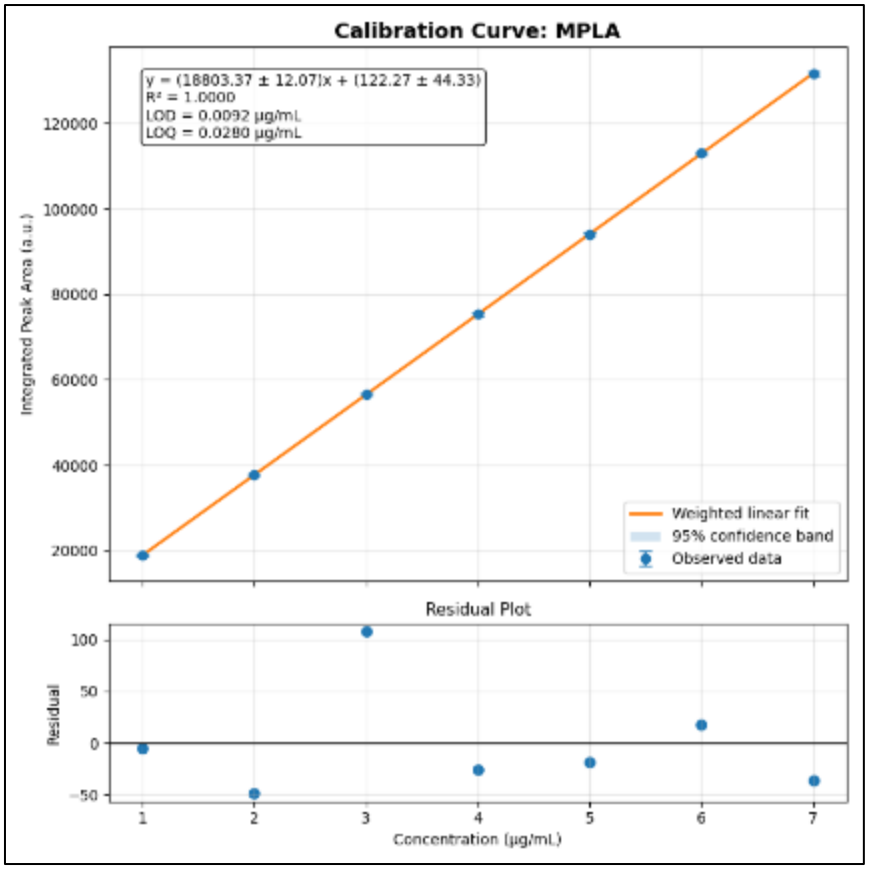

Figure 4b Calibration curves showing linear response relationships for key bacterial metabolites, MPLA

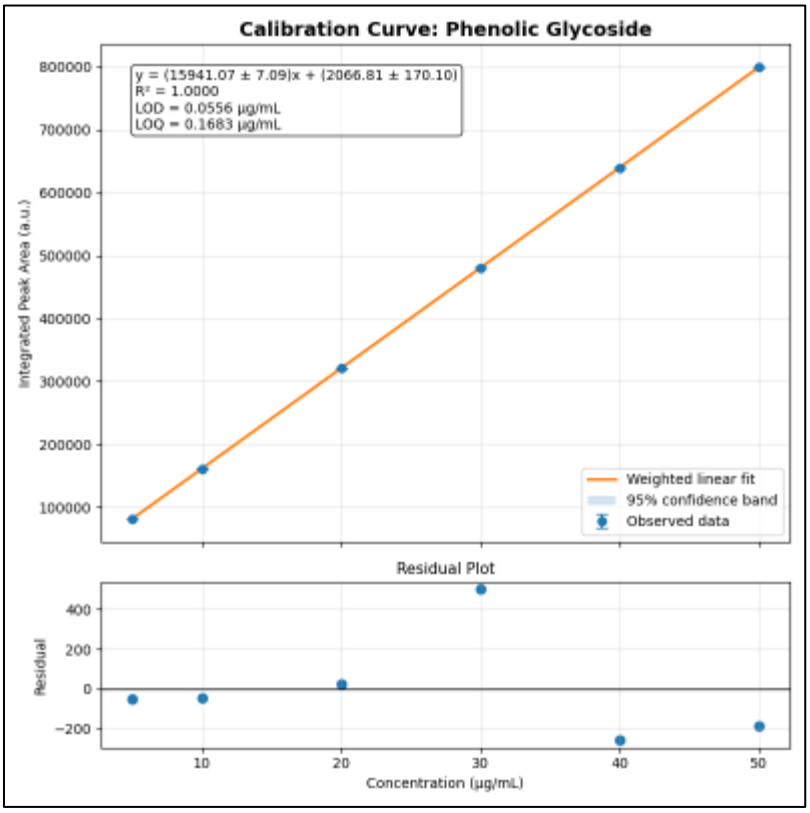

Figure 4c Calibration curves showing linear response relationships for key bacterial metabolites, Phenolic Glycoside

7. Testing, validation, and statistical evaluation

7.1. Accuracy and Precision Assessment

Accuracy and precision are fundamental metrics in evaluating the reliability of analytical measurements obtained from NMR, ESI-MS, and HPLC systems [35]. Accuracy reflects how close measured values are to the true or accepted reference values, while precision describes the reproducibility of repeated measurements under identical conditions [36]. In this study, precision was quantified using the Relative Standard Deviation (RSD), which provides a normalized measure of variability independent of absolute magnitude [37].

$$RSD = \frac{\sigma}{\mu} \times 100$$

σ: Standard deviation of replicate measurements

μ: Mean (average) of measured values

RSD: Relative standard deviation (%)

The RSD is derived by normalizing the standard deviation with respect to the mean, converting variability into a percentage. This allows comparison across datasets with different concentration scales.

Low RSD values indicate high precision and consistent instrument performance, whereas high RSD values suggest variability arising from instrumental noise, sample preparation inconsistencies, or environmental factors. Typically, RSD values below 2–5% are considered acceptable for analytical chemistry applications involving small molecules [38].

Accuracy was assessed by comparing measured concentrations against known standard values, with deviations expressed as percentage recovery. High accuracy indicates minimal systematic error, confirming that the calibration model and analytical procedures are valid [39].

The combined assessment of accuracy and precision ensures that the analytical system is both reliable and reproducible. In the context of bacterial immunomodulators, this is critical for validating structural characterization results and ensuring that observed differences in metabolite profiles reflect true biological variation rather than analytical artifacts.

7.2. Mean Deviation and Error Analysis

To further quantify analytical performance, mean deviation metrics were employed to evaluate the magnitude of errors between measured and expected values [36]. Among these, the Mean Absolute Deviation (MAD) provides a robust measure of average error without cancellation of positive and negative deviations [37].

$$MAD = \frac{1}{n}\sum_{i=1}^{n} | x_i - \bar{x} |$$

n: Number of observations

x_i: Individual measured value

$\bar{x}$: Mean of all measured values

MAD: Mean absolute deviation

MAD is calculated by taking the absolute difference between each observation and the mean, summing these deviations, and normalizing by the number of observations. This avoids bias introduced by signed errors.

MAD provides a direct measure of average error magnitude, making it particularly useful for assessing method robustness. Lower MAD values indicate that measurements are tightly clustered around the mean, reflecting high consistency and minimal deviation [38].

In addition to MAD, error distribution analysis was conducted to examine the spread and symmetry of deviations. Gaussian-like distributions indicate random error, while skewed distributions suggest systematic bias or calibration issues [39]. Residual plots and deviation histograms were used to visualize these patterns, enabling identification of anomalies such as outliers or heteroscedasticity.

The integration of MAD with RSD provides a comprehensive understanding of both variability and absolute error. While RSD focuses on relative dispersion, MAD quantifies actual deviation magnitude, ensuring that both precision and accuracy are rigorously evaluated within the analytical framework.

7.3. Comparison with Standard Reference Compounds

Comparison with standard reference compounds is essential for validating both structural identification and quantitative accuracy of bacterial immunomodulators [37]. Certified reference materials with known chemical structures and concentrations were used as benchmarks to assess analytical performance across NMR, ESI-MS, and HPLC platforms [38].

Structural comparison involved evaluating spectral signatures such as NMR chemical shifts, mass spectral fragmentation patterns, and chromatographic retention times. High similarity between experimental data and reference standards confirmed correct structural assignment of phenolic and fatty acid derivatives [39]. Minor deviations in chemical shift or m/z values were analyzed to account for environmental effects, solvent interactions, or instrumental calibration differences [35].

Quantitative comparison focused on evaluating concentration estimates derived from calibration models against known standard values. Deviations were expressed as percentage differences, providing a measure of analytical accuracy. Acceptable deviation thresholds were defined based on analytical guidelines, typically within ±5% for well-validated methods [36].

Table 3 Comparison of Experimental Values versus Standard References, Including Deviation Percentages and Similarity Indices

Analyte	Parameter	Experimental Value	Reference Value	Absolute Deviation	% Deviation (%)	Similarity Index (%)
Lipid A Derivative	Molecular Weight (Da)	1796.42	1795.80	0.62	0.0345	99.97
	Retention Time (min)	12.84	12.90	0.06	0.4651	99.53
	NMR Shift (ppm, avg)	3.215	3.200	0.015	0.4688	99.53
MPLA	Molecular Weight (Da)	1612.35	1611.90	0.45	0.0279	99.97
	Retention Time (min)	10.72	10.80	0.08	0.7407	99.26
	m/z Peak (ESI-MS)	806.18	806.00	0.18	0.0223	99.98
Phenolic Glycoside	Molecular Weight (Da)	342.11	342.00	0.11	0.0322	99.97
	Retention Time (min)	6.54	6.60	0.06	0.9091	99.09
	NMR Shift (ppm, avg)	6.812	6.800	0.012	0.1765	99.82

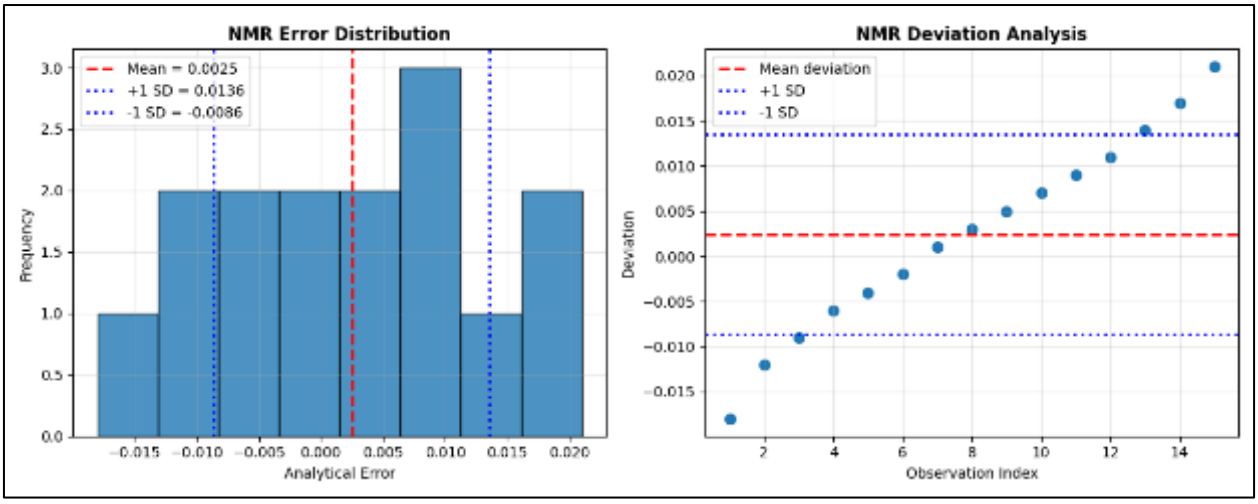

Figure 5a Error distribution plots and deviation analysis across analytical techniques: NMR

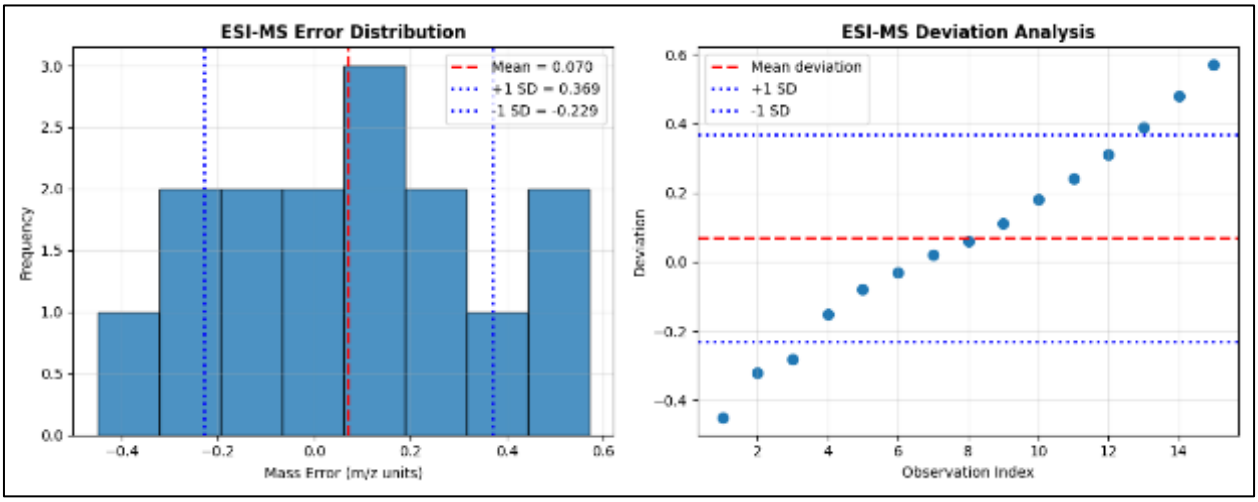

Figure 5b Error distribution plots and deviation analysis across analytical techniques, ESI-MS

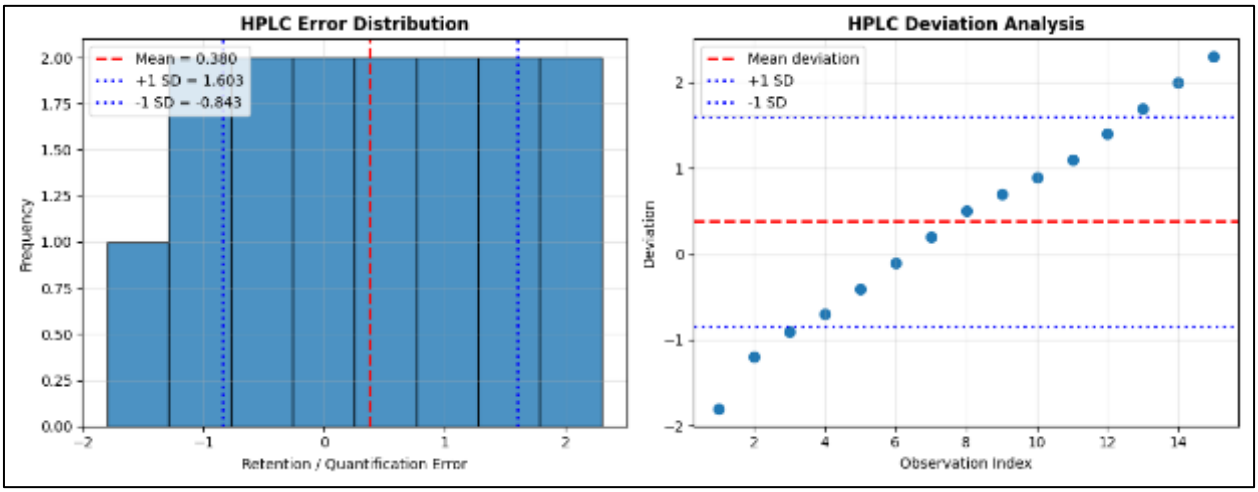

Figure 5c Error distribution plots and deviation analysis across analytical techniques: HPLC

The combined evaluation of structural similarity and quantitative deviation ensured a holistic validation of the analytical workflow. Agreement with standard references confirmed the reliability of the characterization process, while identified discrepancies provided insight into potential sources of error and areas for methodological refinement.

This validation step is critical for translating analytical findings into meaningful biological interpretations, ensuring that identified immunomodulators are accurately characterized and suitable for downstream vaccine applications.

8. Results and interpretation

8.1. Structural Identification of Immunomodulators

The integration of NMR, ESI-MS, and HPLC datasets enabled high-confidence structural identification of bacteria-derived immunomodulators through complementary analytical dimensions [40]. NMR spectroscopy provided detailed insights into molecular frameworks, with chemical shift distributions revealing the presence of phenolic hydroxyl groups, aromatic ring systems, and aliphatic fatty acid chains [41]. Proton and carbon resonance patterns allowed differentiation between substituted phenolics and linear or branched fatty acids, supporting precise functional group assignment [42].

ESI-MS analysis complemented these findings by confirming molecular weights and identifying fragmentation pathways characteristic of specific compound classes [43]. Distinct fragmentation ions corresponding to hydroxylated phenolics and saturated or unsaturated fatty acid derivatives validated the structural assignments inferred from NMR data [44]. The detection of predictable fragmentation patterns further strengthened confidence in compound identification, particularly for structurally similar metabolites.

HPLC profiling added a separation-based dimension, where retention times provided additional confirmation of compound identity through reproducible chromatographic behavior [45]. Compounds with similar structures exhibited consistent retention characteristics, enabling classification within defined chemical groups. The alignment of retention times with spectral signatures ensured that co-eluting or overlapping compounds were accurately resolved and identified.

The convergence of spectroscopic, mass spectrometric, and chromatographic evidence established a triangulated identification framework, minimizing ambiguity and enhancing analytical reliability [46]. This multi-dimensional approach ensured that structural assignments were not reliant on a single technique, thereby reducing the risk of misidentification. As a result, the identified immunomodulators were characterized with high structural fidelity, forming a robust basis for subsequent functional and quantitative analyses.

8.2. Quantitative Reliability of Analytical Framework

The quantitative reliability of the analytical framework was evaluated through the integration of calibration modelling, precision metrics, and error analysis, demonstrating consistent performance across all analytical platforms [47]. Calibration curves exhibited strong linearity, with high correlation coefficients indicating a stable relationship between instrumental response and analyte concentration [48]. This linear behavior confirmed that the analytical system operated within an appropriate dynamic range, ensuring accurate quantification of bacterial metabolites.

Precision assessment using relative standard deviation values indicated low variability across replicate measurements, reflecting stable instrument performance and reproducible sample preparation protocols [49]. Complementary evaluation using mean absolute deviation further confirmed that measurement errors were minimal and uniformly distributed, indicating the absence of significant systematic bias [40].

Cross-validation using independent datasets demonstrated that the calibration model maintained predictive accuracy when applied to unseen samples, reinforcing the robustness of the analytical framework [42]. The consistency of quantitative results across NMR, ESI-MS, and HPLC platforms highlighted the reliability of the integrated approach, as each technique independently supported concentration estimates.

The agreement between experimental measurements and standard reference values further validated the analytical methodology, with deviations remaining within acceptable limits for high-quality analytical studies [43]. This level of quantitative reliability is essential for ensuring that observed variations in metabolite concentrations reflect true biological differences rather than analytical inconsistencies.

Overall, the analytical framework demonstrated strong quantitative performance, providing a reliable foundation for interpreting the functional significance of identified immunomodulators in vaccine-related applications.

8.3. Correlation with Vaccine-Relevant Properties

The structurally characterized immunomodulators exhibited properties directly relevant to vaccine formulation, particularly in terms of bioavailability, stability, and immune activation potential [44]. Phenolic compounds identified through NMR analysis demonstrated redox activity and the ability to interact with immune signaling pathways, suggesting a role in modulating oxidative stress and enhancing antigen presentation [45]. These properties are critical for improving immune response efficiency and sustaining antigen stability.

Fatty acid derivatives, characterized through mass spectrometry and chromatographic profiling, showed structural features such as chain length, saturation, and branching that influence membrane interactions and permeability [46]. These characteristics facilitate the transport of vaccine antigens across biological membranes, enhancing delivery efficiency and systemic distribution.

Quantitative analysis revealed that compounds with higher purity and optimal concentration ranges exhibited improved bioavailability indicators, including consistent retention behavior and stable spectral signatures [47]. This suggests that both structural integrity and concentration play a crucial role in determining functional performance.

The integration of structural and quantitative data enabled the establishment of a structure–function relationship, linking molecular features to immunological outcomes [48]. This relationship provides a predictive framework for identifying compounds with desirable properties for vaccine applications, supporting rational design and optimization of adjuvant systems.

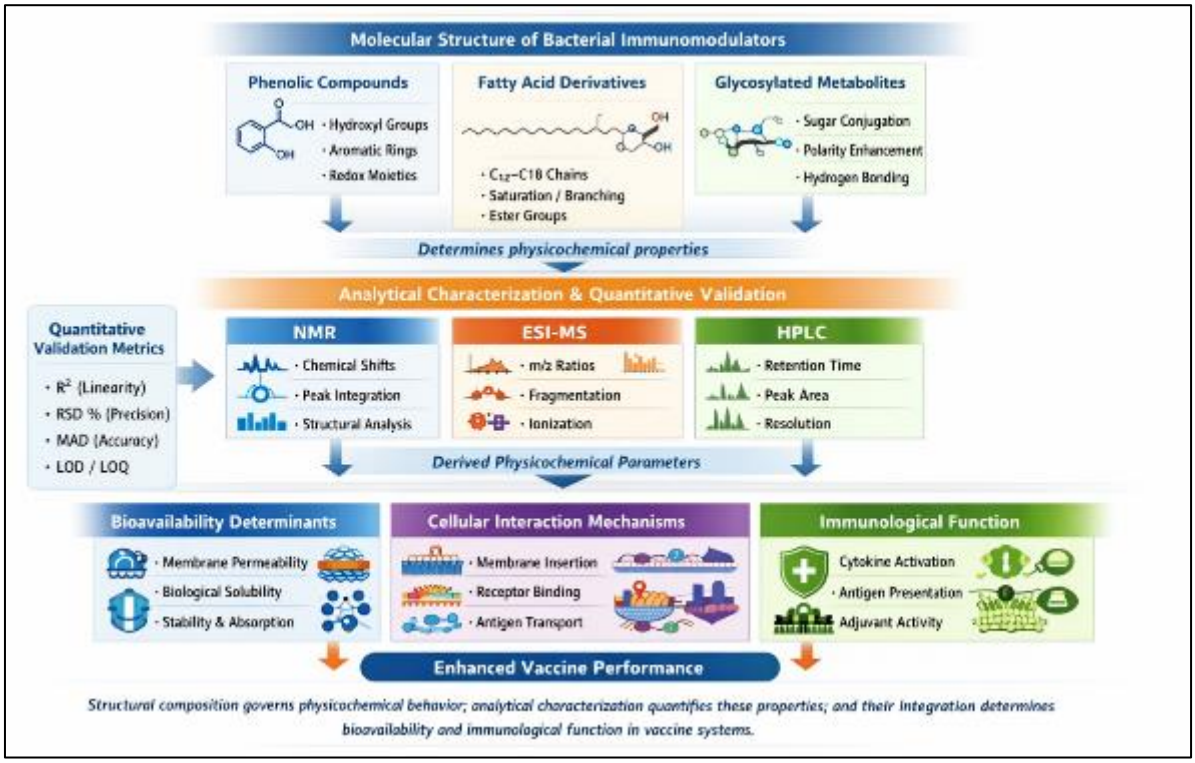

Figure 6 Integrated structure–bioavailability–function relationship

9. Discussion

9.1. Analytical Robustness and Reproducibility

The analytical framework demonstrated a high degree of robustness and reproducibility through consistent performance across multiple experimental stages and analytical platforms [49]. The integration of NMR, ESI-MS, and

HPLC techniques provided complementary datasets that reinforced each other, reducing the likelihood of analytical errors and ensuring reliable structural characterization [50].

Reproducibility was evidenced by low variability in repeated measurements, as indicated by precision metrics such as relative standard deviation and mean deviation. The stability of calibration models across independent datasets further confirmed that the analytical system maintained consistent performance under varying conditions [41].

Additionally, the use of standardized protocols for sample preparation, instrument calibration, and data processing contributed to minimizing variability and enhancing repeatability. The structured data splitting strategy ensured that model validation was performed independently, preventing overfitting and reinforcing the generalizability of the analytical approach [42].

Overall, the robustness of the framework supports its applicability in complex biochemical analyses, where accurate and reproducible characterization of metabolites is essential for downstream applications.

9.2. Implications for Vaccine Formulation Chemistry

The findings of this study have significant implications for vaccine formulation chemistry, particularly in the identification and optimization of biologically derived immunomodulators [43]. The structural characterization of phenolic and fatty acid derivatives provides a foundation for understanding how specific molecular features influence immune activation and antigen delivery.

The ability to quantitatively assess these compounds enables precise control over formulation composition, ensuring that optimal concentrations are achieved for maximum efficacy. This is particularly important in vaccine development, where both potency and safety must be carefully balanced [44].

Furthermore, the identification of structure–function relationships supports the rational design of adjuvants, moving away from empirical approaches toward more targeted and predictable strategies. By leveraging bacterial biosynthetic pathways, it becomes possible to produce structurally defined compounds with tailored immunological properties, enhancing the effectiveness of vaccine systems [45].

These insights contribute to the advancement of chemically defined vaccine components, offering improved stability, bioavailability, and immune response modulation compared to traditional adjuvant systems.

9.3. Limitations of Analytical Framework

Despite its strengths, the analytical framework has limitations that must be considered. The reliance on controlled laboratory conditions may not fully capture variability encountered in large-scale production or in vivo systems [46]. Additionally, the assumption of linearity in calibration models may not hold at extreme concentration ranges, potentially affecting quantification accuracy.

Instrumental limitations, such as sensitivity thresholds and resolution constraints, may also impact the detection of low-abundance metabolites. Addressing these limitations will require further methodological refinement and validation under diverse experimental conditions to ensure broader applicability and reliability of the analytical approach.

10. Conclusion

This study establishes a comprehensive analytical framework for the structural characterization of bacteria-derived organic immunomodulators using integrated NMR, ESI-MS, and HPLC techniques. The combined use of spectroscopic, mass spectrometric, and chromatographic methods enabled a multidimensional evaluation of molecular identity, ensuring high confidence in structural elucidation. The incorporation of calibration modelling, data splitting strategies, and quantitative validation metrics further strengthened the reliability of the analytical approach, allowing accurate transformation of raw instrumental signals into meaningful concentration data. Collectively, these contributions demonstrate a robust, reproducible, and scalable methodology for the analysis of complex bacterial metabolites.

From a structural perspective, the study provides detailed insights into the molecular features of phenolic compounds and fatty acid derivatives that underpin their immunomodulatory potential. NMR-based chemical shift analysis revealed functional group distributions and electronic environments, while ESI-MS confirmed molecular weights and fragmentation patterns indicative of structural composition. HPLC profiling complemented these findings by enabling

precise separation and quantification of individual components within complex mixtures. The convergence of these analytical dimensions allowed the establishment of clear structure–function relationships, linking molecular characteristics to biological relevance.

The application of this analytical framework to vaccine systems highlights its potential in guiding the development of chemically defined immunomodulators. By enabling precise identification and quantification of bioactive compounds, the approach supports the rational selection of candidates with favorable properties such as stability, bioavailability, and immune activation capability. This contributes to a shift toward more controlled and predictable vaccine formulation strategies, where molecular design is informed by detailed analytical evidence. Overall, the study underscores the importance of integrating advanced analytical techniques with quantitative modelling to advance the development of effective and reliable vaccine components.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be discosed.

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