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REVIEW ARTICLE

Lactobacilli-Derived Extracellular Vesicles: A Focus on Isolation and Characterization Methods

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ABSTRACT

Lactobacilli-derived extracellular vesicles (Lb-EVs) are nanoscale lipid-bilayer structures that carry diverse bioactive cargo. They are increasingly recognized as important mediators of communication between bacteria and between bacteria and host cells and may contribute to some of the biological effects associated with probiotic lactobacilli. However, differences in bacterial strains, culture conditions, isolation procedures, sample handling, and analytical methods make it difficult to reproduce and compare findings across studies. This review summarizes current knowledge of Lb-EV biogenesis, physicochemical properties, and molecular cargo, with particular emphasis on the methods used for their isolation, purification, and characterization. Current evidence indicates that Lb-EV yield, size distribution, morphology, surface properties, stability, and cargo composition are influenced by strain-specific and environmental factors, as well as by the methods used throughout sample preparation and analysis. Because higher particle recovery does not necessarily indicate greater purity and no universal molecular marker is currently available for bacterial EVs, neither isolation alone nor a single analytical technique can reliably confirm EV identity, preparation purity, or the intravesicular localization of molecular cargo. Reliable evaluation of Lb-EVs therefore requires a combination of morphological, biophysical, and molecular analyses together with appropriate experimental controls. Greater standardization of culture and processing conditions, along with transparent methodological reporting, is needed to improve reproducibility, support meaningful comparisons across studies, and provide a stronger basis for future research on Lb-EVs.

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INTRODUCTION

Extracellular vesicles (EVs) are nanoscale membrane-enclosed particles naturally secreted by cells into the extracellular environment [1]. Although EVs research initially focused largely on eukaryotic cells, recent studies have demonstrated that bacteria also produce and release these vesicles [1]. Owing to their lipid bilayer, EVs can carry a wide range of molecules, including proteins, lipids, nucleic acids, and other bioactive compounds [1, 2]. Accordingly, these vesicles have attracted considerable attention as vehicles for the transfer of biological molecules and as mediators of intercellular communication [2]. Available evidence further indicates that EVs may participate in several biological processes, including cellular interactions, immune responses, and adaptation to environmental conditions [2, 3].

Among bacterial extracellular vesicles [B-EVs], increasing research has focused on those released by lactic acid bacteria [LAB], particularly members of the former broad genus *Lactobacillus*[4]. Lactobacilli represent an important group within LAB, characterized by biological diversity, adaptation to diverse ecological niches, and broad applications in the food, medical, and biotechnology sectors [5]. Like other bacterial EVs, vesicles produced by lactobacilli can contain selected bioactive components of their parental cells, including proteins, lipids, nucleic acids, and cell-envelope-associated molecules, thereby providing

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a potential means of intercellular communication and interaction with host cells [4]. Through the transfer or presentation of these biologically active components, Lactobacilli-derived extracellular vesicles (Lb-EVs) may contribute to some of the host-interacting and immunomodulatory impacts associated with probiotic bacteria [4].

The characteristics of Lb-EVs, including particle size, size distribution, morphology, surface properties, and molecular composition, may be influenced by the bacterial species and strain, growth conditions, and the methods used for production, isolation, and purification [4, 6]. Moreover, the presence of cellular debris, free proteins, and other non-vesicular particles in isolated preparations can affect measured characteristics and the interpretation of experimental findings [4, 7]. Therefore, the choice of an EVs isolation method should take into account not only recovery efficiency but also sample purity, preservation of vesicle integrity, molecular composition, and the specific objectives of the study [8, 9]. Accordingly, this review provides a broad overview of Lb-EVs, focusing on their biogenesis, physicochemical characteristics and molecular composition, isolation and purification methods, and the approaches used for their characterization [6]. The advantages and limitations of different isolation procedures and commonly applied characterization techniques are compared, with particular emphasis on the use of complementary methods to evaluate morphology, particle size and concentration, surface properties, and molecular composition [6]. Finally, current challenges in standardizing the production, isolation, and characterization of Lb-EVs and potential directions for future research are discussed [6, 8].

LACTOBACILLI AS A SOURCE OF EVS

Taxonomic Overview and General Characteristics of Lactobacilli

Lactobacilli comprise a diverse and important group of LAB with extensive applications across the food, medical, and biotechnology fields [5]. They are generally Gram-positive, rod-shaped, non-spore-forming, and predominantly catalase-negative bacteria that obtain energy through carbohydrate fermentation [10]. The production of lactic acid as a major metabolic end product is a defining characteristic that contributes to their biological properties and industrial applications [11]. Lactobacilli occur in diverse habitats, including the gastrointestinal tract of humans and animals, mucosal surfaces, human milk, fermented foods, and other nutrient-rich environments [5, 12]. Their environmental adaptability and metabolic diversity have made them an important group in applied microbiology research [11, 13].

A major phylogenomic revision introduced in 2020 reclassified numerous species previously assigned to the genus Lactobacillus into newly defined genera, including Lacticaseibacillus, Lactiplantibacillus, and Limosilactobacillus. In this review, the term "lactobacilli" is used collectively for members of the former broad *Lactobacillus* group [5, 14].

Probiotic Roles and Biological Significance of the Lactobacilli

Probiotics are viable microorganisms that can exert beneficial effects on host physiological functions when administered in sufficient amounts [15]. Many Lactobacilli have been used as probiotic strains because of characteristics such as tolerance to the acidic conditions of the gastrointestinal tract, adhesion to epithelial surfaces, production of antimicrobial compounds, and interactions with the immune system [16].

One of the major biological roles of lactobacilli is their contribution to maintaining host microbial homeostasis and limiting the growth of pathogenic microorganisms. Through mechanisms including the production of organic acids and bacteriocins, competition for adhesion sites, and modulation of immune responses, these bacteria can influence the microbial ecosystem and host–microbe interactions [16].

Although the probiotic effects of lactobacilli were historically attributed to them while they are viable, more recent studies have demonstrated that bacterial-derived components can also contribute to biological responses [17]. In this context, Lb-EVs have emerged as a potential mechanism through which these bacteria exert biological effects [4]. By transporting a range of biomolecules, these vesicles may contribute to communication between bacteria and host cells and represent a promising avenue for understanding probiotic functions [4]. Moreover, probiotic-derived EVs have attracted attention as potential cell-free immunomodulatory agents, with possible applications in the management of inflammatory and autoimmune diseases. [18].

Reported EV-Producing Species and Strains

With the expansion of research on BEVs, several Lactobacilli have been identified as EVs producers [4]. Although EVs yield, physicochemical properties, and molecular composition vary among species and strains, current evidence indicates that numerous probiotic bacteria can release vesicles containing biologically active cargo (Table 1) [16]. Such differences may be influenced by strain-specific characteristics, growth conditions, and the methods used for EVs isolation and

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Table 1. Reported EVs-producing species within the Lactobacilli and their characteristics.

Species	Representative EV-producing strain(s)	Main reported EV characteristics or biological finding	Ref.
Lacticaseibacillus rhamnosus	GG, JB-1, PCM489	EVs from different strains have been associated with immunomodulatory activity, modulation of the gut microbiota, host-cell interactions, and carriage of bioactive molecular cargo	(20, 22)
Lactiplantibacillus plantarum	WCFS1, KCTC 11401BP, BGAN8, APsulloc 331261, PCM 2675	Reported EVs contain diverse protein, lipid, metabolite, and RNA cargo and have been associated with immune regulation, protection of epithelial cells, and cellular uptake by intestinal epithelial cells	(23, 24)
Lactobacillus acidophilus	ATCC 53544,	EVs carry a strain-specific protein cargo and can facilitate the delivery of bacteriocin-associated peptides to susceptible bacterial cells.	(25)
Lacticaseibacillus casei	BL23	EVs carry the surface-associated proteins p40 and p75 and can activate EGFR-dependent signaling associated with epithelial-barrier maintenance.	(26)
Limosilactobacillus reuteri	DSM 17938, ATCC 23272	EVs are released under both planktonic and biofilm growth conditions and contain strain- and growth-condition-dependent protein and lipid components, including lipoteichoic acid.	(27)

characterization [6].

Lacticaseibacillus rhamnosus, formerly reported as *Lactobacillus rhamnosus*, is among the most extensively studied species in the field of Lb-EVs [19]. Owing to its well-established probiotic properties and ability to interact with host cells, this species has been widely used as a model for investigating the production, composition, and functions of B-EVs [20].

Lactiplantibacillus plantarum is another important EV-producing species and has received considerable research attention because of its high metabolic diversity and widespread occurrence in fermented food environments [21]. In addition, species such as *Limosilactobacillus reuteri*, *Lacticaseibacillus paracasei*, and *Lactobacillus acidophilus* have been reported to produce EVs, and several studies have investigated the potential roles of these vesicles in microbial and biological interactions [4].

BIOGENESIS AND BIOLOGICAL CHARACTERISTICS OF LB-EVS

Mechanisms of EV Biogenesis and Formation in Gram-Positive Bacteria

EVs are naturally produced and released into the extracellular environment by a broad range of bacteria, including both Gram-negative and Gram-positive species [1]. Although EVs production by Gram-negative bacteria has been known for years, their subsequent identification in Gram-positive bacteria challenged the earlier view that vesicle formation was limited to bacteria with an outer membrane [1].

EVs production in Gram-positive bacteria is more complex than in Gram-negative bacteria because of the presence of a thick peptidoglycan cell wall [1, 3]. Unlike Gram-negative vesicles, which originate from the outer membrane, Gram-positive EVs derive from the cytoplasmic membrane and must cross the peptidoglycan layer to be released [3]. Thereafter, their biogenesis is not yet fully understood, and several mechanisms have been proposed to account for EV formation [1].

According to the currently accepted model, EVs formation begins with localized protrusion of the cytoplasmic membrane [28]. Selective accumulation of certain lipids, membrane proteins, and other biomolecules within specific membrane regions induces membrane curvature and the formation of vesicular buds [1]. These buds subsequently separate from the cell membrane and form independent vesicular structures within the space associated with the peptidoglycan layer [28]. Passage through the thick cell wall, however, requires the formation of pores or regions of reduced density within the peptidoglycan network [28].

Recent evidence suggests that cell-wall hydrolytic enzymes, especially autolysins and other enzymes involved in peptidoglycan degradation, may have an important role in this stage of EV formation [29]. The activity of these enzymes can modify the peptidoglycan barrier and facilitate EVs passage through the cell wall [29]. In addition, intracellular turgor pressure has been proposed as a driving force for vesicle release, whereby pressure generated by cytoplasmic contents may facilitate the outward movement of newly formed vesicles [28].

Growing evidence indicates that EV biogenesis

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is a regulated process that can be influenced by the physiological state of bacteria and their environmental conditions. EVs contain a diverse molecular cargo, including proteins, metabolites, and nucleic acids, with specific molecular components enriched within these structures [21, 30]. Although the mechanisms governing selective cargo incorporation remain incompletely understood, this process likely contributes to the biological functions of EVs [30].

Microscopic observations and proteomic analyses have confirmed EVs production in multiple members of the *Lactobacilli* [20, 21, 30]. Although the precise mechanisms of EVs biogenesis in this group remain unresolved, mechanisms described for other Gram-positive bacteria provide a useful framework for understanding their formation [1, 28]. Species- and strain-specific differences in EVs yield, size, and molecular composition have also been reported, indicating that EVs characteristics can vary considerably among *Lactobacillus* producers [22]. Additional studies are therefore required to better define the mechanisms that regulate EV biogenesis in this bacterial group.

Factors Affecting EVs Production, Biogenesis, and Release in Lactobacillus

Bacterial growth conditions are among the major determinants of EVs production [19]. Growth time, culture-medium composition, and pH can alter bacterial physiological conditions, thereby affecting both the extent of EVs production and the physicochemical and molecular properties of the vesicles produced [19]. Environmental stresses induced by changes in culture conditions may also influence EVs production and cargo composition [19]. In addition, species- and strain-specific genetic and physiological differences may contribute to variations in EVs yield, size, molecular composition, and functional characteristics [31].

Physicochemical Characteristics of Lb-EVs

Particle size and size distribution are important characteristics of EVs preparations because they reflect the vesicle population present in a sample and its degree of homogeneity [21, 22]. Lb-EVs have generally been reported within the nanometre range; however, their size distribution can vary among species and strains [21, 22]. In addition to intrinsic bacterial characteristics, growth conditions and isolation procedures can affect the final measured size of EVs and the occurrence of aggregated particles or non-vesicular structures [19, 32].

Morphology and membrane organization are additional key features of B-EVs [21]. These vesicles are generally spherical structures enclosed by a lipid membrane that surrounds and contains their molecular cargo [21]. The EVs membrane contains lipids, proteins, and other surface-associated molecules that can influence vesicle interactions with target cells and contribute to their functional properties [22].

Surface characteristics, including surface electrical charge, play an important role in EVs stability and interactions with surrounding molecules and cells [33]. EVs surface properties are associated with their molecular composition, particularly membrane lipids and surface-associated molecules, while environmental parameters such as pH and ionic strength can influence vesicle stability [33].

The physical and chemical stability of EVs is also critical for maintaining their structure and function [33]. Temperature, pH, storage conditions, freeze–thaw cycles, and the composition of the storage medium can influence membrane integrity, particle aggregation, and preservation of biological cargo [33-35]. Careful control of sample storage and processing conditions is therefore essential for maintaining the native characteristics of Lb-EVs [33].

Molecular Composition and Biological Cargo of Lb-EVs

The biological activity of Lb-EVs is largely determined by their molecular cargo [21]. Although many of these molecules originate from the parent bacterial cell, they are not necessarily incorporated into EVs in the same proportions as they occur within the cell. The enrichment of certain components in EVs suggests that cargo incorporation may be selective to some extent [24]. Proteins represent a major functional component of Lb-EVs and include surface-associated, cytoplasmic, and membrane-embedded proteins [30, 36]. Proteomic analyses have revealed that these vesicles carry various functionally active proteins, including metabolic enzymes, chaperones, moonlighting proteins such as GAPDH and enolase, and mucus-binding factors involved in host–microbe interactions and immunomodulatory effects [30, 36]. The enrichment of specific proteins in EVs relative to the corresponding bacterial cell pellets, together with differences in vesicular protein cargo across bacterial growth stages, suggests that protein incorporation into Lb-EVs is not simply a passive reflection of the cellular proteome but may involve selective cargo loading [36, 37].

Lipids form the EV membrane and are essential for maintaining vesicle structure and stability [21, 24]. The lipid composition of EVs can differ from that of the bacterial membrane from which they originate, with certain lipids being more abundant in EVs than in the parent cell [21, 24]. These

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differences may alter the physical properties of the vesicle membrane and thereby affect EV stability and interactions with recipient cells [38, 39].

DNA, RNA, and metabolites constitute additional, although less extensively studied, components of Lb-EV cargo. DNA has been detected as a component of EV cargo in several lactobacilli; however, considerably less is known about DNA associated with Lb-EVs than about their protein cargo [40]. Different RNA species, particularly small RNAs, have also been detected in Lb-EVs and may contribute to communication with recipient cells. Nevertheless, their biological roles remain poorly understood, particularly whether specific RNAs are selectively packaged and can influence host-cell responses [23]. Metabolomic analyses have detected a wide range of small molecules in these vesicles, including several lipid-related compounds, although the contribution of individual metabolites to EV-mediated biological effects remains poorly understood .[21] Overall, differences in cargo composition among species and strains may partly explain the diverse biological effects reported for Lb-EVs [31].

ISOLATION AND PURIFICATION OF EVS

Isolation and purification are fundamental steps in EVs research because bacterial suspensions contain bacterial cells, cellular debris, free proteins, and other particles that may be co-isolated with EVs [6]. Consequently, the isolation method affects not only recovery yield but also sample purity, molecular composition, and EVs size distribution [6]. Recent studies on B-EVs, particularly those derived from lactic acid bacteria, indicate that no universally accepted standardized protocol is currently available [6]. The most appropriate method should therefore be selected according to the objective of the study and the desired characteristics of the final preparation [6].

Conventional Methods

Differential Centrifugation

Differential centrifugation is a fundamental step in EVs isolation and is commonly used for the sequential removal of bacterial cells and cellular debris by applying progressively increasing centrifugal forces [41]. The bacterial suspension is initially centrifuged at relatively low speed to remove intact cells and large particles, after which the remaining supernatant is used for subsequent isolation steps [41]. In some protocols, filtration is performed after the initial centrifugation to remove residual cells [41].

The principal advantage of differential centrifugation is its relative simplicity and suitability as a preliminary step in multistage isolation workflows [6]. However, when used alone, it does not yield a highly purified EVs population because particles with similar sedimentation properties, cellular debris, and aggregates may remain and subsequently co-isolate with EVs [6]. Differential centrifugation is therefore generally used as part of a multistep procedure rather than as a complete purification method [6].

Ultracentrifugation

Ultracentrifugation is one of the most widely used methods for isolating B-EVs [6]. Following removal of bacterial cells and large particles, the supernatant is subjected to very high centrifugal forces, causing EVs to sediment as a pellet [42]. In some protocols, the resulting pellet is washed and resuspended in an appropriate buffer [42].

The widespread use of ultracentrifugation is largely related to its simplicity, accessibility, and ability to concentrate EVs from bacterial culture supernatants [6]. Nevertheless, high-speed pelleting can promote particle aggregation, and the resulting pellet does not necessarily represent a completely pure EVs population [6]. Moreover, this method requires specialized equipment and is generally more time-consuming than several alternative approaches [43].

Density-Gradient Centrifugation

Density-gradient centrifugation is commonly used as an additional purification step for preparations obtained through primary isolation methods [44]. In this approach, particles are distributed within a gradient medium according to their density, and EVs accumulate within fractions corresponding to their characteristic density [44]. This strategy can facilitate separation of EVs from contaminants and particles that remain after preliminary isolation [6].

In reported B-EVs protocols, density-gradient centrifugation is typically performed after an initial step such as ultracentrifugation [44]. Accordingly, it is more appropriately regarded as a complementary purification step than as an independent method for recovering EVs directly from the starting sample [6]. Its limitations include increased procedural complexity and the requirement for additional fractionation and collection steps [45].

Ultrafiltration

Ultrafiltration is a membrane-based approach used to concentrate and separate EVs according to particle size and membrane pore characteristics [6]. Following removal of cells and large debris, it can be used to reduce sample volume and increase EVs concentration [42]. In B-EVs studies, membranes with defined molecular-weight cut-offs have been

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used to retain EVs while allowing a proportion of smaller components to pass through [42].

A major advantage of ultrafiltration is its ability to efficiently concentrate EV-containing samples and reduce sample volume before subsequent purification steps. However, its performance depends on membrane characteristics and filtration conditions, and interactions between EVs and the membrane surface may affect recovery. Moreover, ultrafiltration alone does not necessarily ensure complete removal of contaminants with overlapping size ranges and is therefore frequently combined with additional isolation or purification procedures [42].

Emerging Methods

Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) is a method used to separate EVs based on differences in particle size. Samples pass through a column containing a porous stationary phase, and molecules and particles elute at different times according to the extent to which they enter the pores. In BEVs studies, SEC has been used to improve sample purification and separate EVs from certain soluble components [33].

Compared with precipitation-based approaches, SEC is a relatively gentle method and has increasingly been incorporated into recent protocols as either a primary or complementary EVs purification strategy [6, 33]. Nevertheless, SEC does not guarantee complete sample purity, as its size-based separation may result in the co-isolation of non-vesicular particles with dimensions similar to EVs [46].

Polymer-Based Precipitation

Precipitation-based methods provide an alternative approach for EVs isolation, in which agents such as polyethylene glycol [PEG] are added to the cell-free supernatant to promote EVs precipitation and subsequent recovery by centrifugation [41].

This method has been used in some studies because it does not require specialized equipment and is comparatively inexpensive [6]. However, the non-specific nature of precipitation allows non-vesicular components to be recovered together with EVs. Accordingly, preparations obtained by polymer precipitation may differ in purity from those generated through multistep purification procedures [6].

A recent comparative study of EVs from *Lactobacillus acidophilus* and *Escherichia coli Nissle* also indicated that ultracentrifugation yielded a higher EVs recovery than precipitation[47]. Thus, the performance of these approaches should be evaluated according to the bacterial strain, sample characteristics, and intended application.

Microfluidic Technologies

In recent years, microstructured and microfluidic technologies have attracted attention as alternative approaches for EVs isolation and processing. These platforms enable controlled sample processing at the microscale and can exploit different mechanisms for EV capture and release [43]. In recent BEVs research, microstructured particle-control strategies have been investigated as potential approaches for achieving faster and more selective EVs isolation [43].

Nevertheless, these technologies remain less established for BEVs isolation, and further methodological development and standardization are required before their widespread implementation [6, 43]. At present, microfluidic and related microscale technologies represent emerging approaches for BEVs isolation, whereas ultracentrifugation and SEC remain commonly used conventional methods [6, 43].

Comparison of Isolation Methods

Isolation methods for Lb-EVs differ in yield, purity, procedural complexity, cost, and equipment requirements (Table 2) [6]. Ultracentrifugation remains one of the most commonly used approaches for B-EV recovery, whereas SEC and ultrafiltration can serve as alternative or complementary methods [6].

Importantly, higher recovery does not necessarily correspond to greater purity [47]. Precipitation methods may recover larger quantities of particles but may also increase the likelihood of co-isolating non-vesicular components [47]. Conversely, density-gradient centrifugation and SEC can improve sample purity, although they may require additional purification or fractionation steps [6].

The choice of isolation strategy should therefore reflect the specific objective of the study and the relative priority assigned to yield and purity [6]. No single isolation method is universally superior for Lb-EVs, and combined strategies are often used for impurity removal, EVs concentration, and further purification [5, 6]. The lack of standardized workflows, together with variation in culture and isolation conditions, remains a major challenge for reproducibility and comparison across studies [6].

CHARACTERIZATION OF EVS

EVs characterization is an essential step for assessing the identity, physical properties, and molecular composition of isolated vesicles [48]. Because limitations inherent to isolation procedures may permit the presence of non-vesicular particles and components in EVs preparations, no single

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Table 2. Comparison of the principal isolation methods used for Lb-EVs.

Method	Separation principle	Main advantage	Main limitation	Typical application	Ref.
Differential centrifugation	sequential sedimentation based on particle size and density	simple and suitable for initial processing	Limited purity when used alone	Removal of bacterial cells and large debris	(6, 41)
Ultracentrifugation	High-speed sedimentation based on particle size and density	Widely used and provides relatively high EV recovery	Requires specialized equipment and may cause EV aggregation and co-pelleting of contamination	EV recovery and concentration	(6, 42, 47)
Density-gradient centrifugation	Separation according to Buoyant density in a gradient medium	Improved purity and separation from contaminants	Time-consuming and technically complex	Additional purification of crude EV preparations	(6, 44, 45)
Ultrafiltration	Size-based retention using a membrane with a defined molecular-weight cut-off	Rapid concentration and volume reduction	Potential EV loss through membrane adsorption and co-retention of contaminants	EV concentration and sample pretreatment	(6, 42)
SEC	Separation according to hydrodynamic size	Gentle purification with minimal impact on EV integrity	Limited sample capacity and possible co-elution of similarly sized particles	EV purification after an initial concentration step	(6, 33)
Polymer precipitation	Polymer-induced reduction of EV solubility followed by low-speed centrifugation	Simple; does not require ultracentrifugation	Co-precipitation of proteins and other non-vesicular components	EVs recovery and concentration	(6, 41, 47)
Microfluidics	Microscale affinity capture and controlled release of EVs	Rapid and selective isolation using small sample volume	Requires specialized devices and further standardization	Rapid isolation of EVs from culture supernatants and biological samples	(43)

analytical method is sufficient to confirm EVs identity [48]. Recent studies therefore emphasize the use of complementary techniques to evaluate the physical and biochemical properties of EVs [49]. In BEVs research, assessment of morphology, particle size and concentration, protein composition, and other molecular features can collectively provide a more robust evaluation of sample quality [32, 48]. Unlike eukaryotic EVs, however, B-EVs currently lack a universally accepted set of specific molecular markers [48].

Morphological Assessment

Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) provides high-resolution imaging and is widely applied to assess EV morphology [49]. TEM enables direct visualization of nanoscale vesicles and provides information on their morphology and approximate size [21, 32]. TEM has been applied to Lb-EVs and has demonstrated vesicular structures with spherical morphology and a lipid bilayer [50]. Despite its widespread application, sample preparation and imaging conditions can influence the apparent morphology of observed particles [49]. TEM should therefore not be used as the sole method for comprehensive EVs characterization and should be complemented by quantitative approaches for assessing particle size and concentration [49].

Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryogenic transmission electron microscopy (cryo-TEM) enables visualization of EVs under conditions that better preserve their native morphology [49]. In cryo-TEM, samples are vitrified under cryogenic conditions, avoiding dehydration and reducing structural alterations associated with conventional TEM preparation [51]. By minimizing preparation-induced deformation, cryo-TEM can provide detailed information on EVs morphology, lipid bilayers, and internal vesicular structures [51]. Nevertheless, cryo-TEM requires specialized instrumentation and considerable expertise in sample preparation, imaging, and data analysis [52].

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM} is a high-resolution imaging technique used to assess the morphology of EVs [49]. It enables direct morphological and size-based profiling of EVs preparations and may also help distinguish EVs

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from non-vesicular extracellular particles based on their size and morphological features [53]. However, sample preparation—particularly dehydration—may alter the apparent morphology of EVs. Moreover, SEM imaging is generally limited by low throughput and potential bias in field-of-view selection; therefore, its findings should be interpreted alongside complementary characterization methods [49].

Field-emission scanning electron microscopy, a higher-resolution variant of conventional SEM, has also been applied to examine EVs surface morphology and assess the presence of co-isolated particles in EVs preparations [54].

Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) allows individual EVs to be visualized without labeling or staining and provides information about vesicle size, surface ultrastructure, and nanomechanical properties [49]. AFM measurements can be performed on dried samples or in liquid environments and have been applied to characterize BEVs by evaluating particle dimensions, morphology, and nanomechanical properties [32, 55].

Particle Size and Size-Distribution Analysis

Dynamic Light Scattering

Dynamic light scattering (DLS) determines particle hydrodynamic diameter in dilute aqueous dispersions by analyzing time-dependent variations in scattered laser-light intensity arising from Brownian motion [49]. Because EVs preparations are typically polydisperse, DLS should not be used to quantitatively determine their average hydrodynamic diameter; rather, it is more appropriate for qualitatively confirming the presence of submicrometer particles or aggregates and should be interpreted alongside complementary characterization methods [49].

Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis (NTA) estimates particle concentration and hydrodynamic diameter in suspension by evaluating the Brownian movement of individual particles and calculating their corresponding diffusion coefficients [49]. This technique has also been applied to BEVs, including EVs derived from probiotic bacteria, for particle size and concentration measurements [55]. Because NTA provides particle concentration and hydrodynamic-size estimates but does not establish particle identity or morphology, it should be combined with imaging techniques such as TEM, cryo-TEM, or AFM to obtain complementary structural information [32, 55].

Zeta potential

Zeta potential, calculated from electrophoretic mobility, provides an indirect estimate of EVs surface charge and can be used to compare the electrokinetic and colloidal behaviour of EVs preparations, provided that measurement conditions such as pH and ionic composition are standardized [56]. Zeta potential should be interpreted as a complementary physicochemical parameter rather than as evidence of EVs identity; therefore, it should be combined with orthogonal measurements of particle size, concentration, morphology, and molecular composition [49].

Protein Analysis

BCA Assay

The bicinchoninic acid [BCA] assay provides a colorimetric approach for estimating the total protein content of EV preparations and can support relative comparisons or sample standardization when experimental conditions are kept consistent [57]. However, because the BCA assay measures total protein—including proteins associated with co-isolated non-vesicular material—protein concentration is neither specific for EVs identity nor generally recommended as a surrogate for EVs concentration and should therefore be interpreted alongside complementary characterization methods [49].

SDS-PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE] resolves EV-associated proteins mainly on the basis of their apparent molecular mass, generating qualitative banding patterns that can be compared across EV preparations from different strains or experimental conditions[24] . SDS-PAGE has been applied to profile proteins in LAB-derived EVs and can be followed by mass spectrometry to identify proteins contained within selected gel bands [24].

Western Blotting

Western blotting enables the antibody-based detection of selected proteins in EVs preparations and has been used to examine specific proteins, such as P40, in *Lactobacillus paracasei*-derived EVs [57]. However, candidate marker proteins should be selected based on species- or strain-specific evidence because no single protein is currently recognized as a universal marker for all BEVs, and the detection of one protein alone is insufficient to establish EVs identity [49].

Proteomic Analysis by LC–MS/MS

Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) enables broad

identification and relative comparison of proteins in EVs preparations and can reveal proteins that are differentially represented between EVs and their parental cell membranes [58]. Proteomic analysis has been applied to LAB-derived EVs to characterize their protein cargo, identify proteins enriched in the vesicular fraction, and select candidate proteins for further investigation as potential EV-associated biomarkers [58].

Nucleic Acid Analysis

DNA and RNA Extraction

Extraction and analysis of DNA and RNA from purified EV preparations can provide information on their potential nucleic-acid cargo. However, extracellular or particle-associated nucleic acids may be recovered together with EVs; therefore, nuclease treatment performed both before and after membrane disruption can help distinguish protected intravesicular cargo from extravesicular nucleic acids [32, 59].

PCR and RT-PCR

PCR can be used to detect selected DNA sequences in EVs preparations, whereas reverse-transcription PCR (RT-PCR) detects specific RNA sequences following their conversion to cDNA; quantitative RT-PCR has been applied to validate selected small RNAs identified in *Lactobacillus plantarum*-derived EVs preparations [23]. However, detection of a DNA or RNA sequence in an EVs preparation does not establish its intravesicular localization; therefore, nuclease treatment in the absence and presence of membrane-disrupting detergents is required to distinguish free or surface-associated nucleic acids from protected intravesicular cargo [32, 59].

RNA Sequencing

RNA sequencing enables broad profiling of RNA populations in EVs preparations and can be used to identify and compare different RNA classes or individual RNA sequences across samples; this approach has been applied to characterize small RNAs in *Lactobacillus plantarum*-derived EVs preparations [23]. Combining RNA sequencing with targeted RT-PCR validation and nuclease-protection controls can provide more reliable evidence regarding the identity, relative abundance, and localization of RNA associated with LAB-derived EVs preparations [23, 59].

CONCLUSION

Lb-EVs are nanoscale, membrane-enclosed carriers of biomolecules that may participate in microbe–microbe and microbe–host communication. Their physicochemical and molecular properties are shaped not only by the characteristics of the producing strain but also by culture conditions, EVs production and isolation methods, and sample handling and storage. Therefore, comparisons across studies should take into account methodological differences throughout the EV production, isolation, and characterization workflow [49].

No single isolation method can be considered universally optimal for all studies of Lb-EVs. Differential centrifugation and ultracentrifugation can facilitate cell removal, EVs concentration, and recovery, whereas density-gradient centrifugation and size-exclusion chromatography may improve separation from non-vesicular material. Conversely, precipitation-based and some concentration methods may increase EVs recovery but can also promote the co-isolation of soluble proteins, aggregates, and other non-vesicular components. The isolation strategy should therefore be selected according to the biological question and the required balance among recovery, purity, scalability, and preservation of EVs characteristics. In many cases, combining concentration and purification steps may provide a more appropriate workflow than relying on a single method.

Characterization of Lb-EVs preparations should likewise integrate complementary analytical methods. Imaging techniques such as TEM, cryo-TEM, SEM, and AFM provide information on morphology and structure; DLS and NTA estimate particle-size distributions, while NTA can additionally estimate particle concentration; zeta-potential measurements provide information on electrokinetic and colloidal behaviour; and biochemical and molecular methods—including BCA assays, SDS-PAGE, Western blotting, LC–MS/MS, PCR, RT-PCR, and RNA sequencing provide complementary information about the molecular composition of EV preparations. However, no single measurement is sufficient to establish EV identity, purity, or intravesicular cargo localization. Standardized protocols, appropriate controls, multimodal characterization, and transparent reporting of experimental conditions are therefore essential for improving reproducibility and facilitating meaningful comparisons among studies [32].

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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