

# Microbiota-derived extracellular vesicles in interkingdom communication in the gut

Natalia Díaz-Garrido<sup>1,2</sup>  | Josefa Badia<sup>1,2</sup>  | Laura Baldomà<sup>1,2</sup> 

<sup>1</sup> Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain

<sup>2</sup> Institut de Recerca Sant Joan de Déu (IRSJD), Institut de Biomedicina de la Universitat de Barcelona (IBUB), Barcelona, Spain

## Correspondence

Laura Baldomà, Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain.  
Email: lbaldoma@ub.edu

Josefa Badia and Laura Baldomà authors contributed equally to the supervision of the study

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## Abstract

The intestine is fundamental in controlling human health. Intestinal epithelial and immune cells are continuously exposed to millions of microbes that greatly impact on intestinal epithelial barrier and immune function. This microbial community, known as gut microbiota, is now recognized as an important partner of the human being that actively contribute to essential functions of the intestine but also of distal organs. In the gut ecosystem, bidirectional microbiota-host communication does not involve direct cell contacts. Both microbiota and host-derived extracellular vesicles (EVs) are key players of such interkingdom crosstalk. There is now accumulating body of evidence that bacterial secreted vesicles mediate microbiota functions by transporting and delivering into host cells effector molecules that modulate host signalling pathways and cell processes. Consequently, vesicles released by the gut microbiota may have great influence on health and disease. Here we review current knowledge on microbiota EVs and specifically highlight their role in controlling host metabolism, intestinal barrier integrity and immune training.

## KEYWORDS

bacterial membrane vesicles, gut microbiota, gut permeability, immune regulation, intestinal homeostasis, probiotics

## 1 | INTRODUCTION

The skin and mucosal surfaces of the human body are colonized by a huge number of microbes, collectively called the microbiome (“The Integrative Human Microbiome Project.” 2019). Colonization is especially relevant in the intestinal tract, where the microbial population reaches numbers that exceed  $10^{11}$  organisms per gram of wet weight of faecal content in the colon. The gut microbial community, known as the gut microbiota, mainly consist of bacteria, but also archaea, fungi and viruses. Collectively, the gut microbiota contributes with more than three million genes, which far surpass and complement the genetic information encoded by the human genome. In fact, the gut microbiota is considered a “hidden organ” since microbiota-encoded products actively contribute to numerous essential host functions. In addition to its role in nutrition, metabolism and energy production, the gut microbiota regulates immune homeostasis and inflammatory responses against pathogens and other injuries (Jandhyala et al., 2015). The pivotal role of the gut microbiota in the development of the gastrointestinal immune system has been evidenced in germ-free mice models. Among other alterations, gnotobiotic animals exhibit impaired immune function with atrophy of lymphoid organs and decreased production of immunoglobulin A (IgA). They are more susceptible to infections and allergies (Fiebigler et al., 2016; Kennedy et al., 2018).

**Abbreviations:** BEVs, bacterial extracellular vesicles; CD, Crohn’s disease; CMV, cytoplasmic membrane vesicles; DCs, dendritic cells; EVs, extracellular vesicles; IBD, inflammatory bowel disease; IECs, intestinal epithelial cells; InsP6, inositol polyphosphates; LPS, lipopolysaccharide; MAMPs, microbial associated molecular patterns; miRNAs, microRNAs; MMP-9, matrix metalloprotease 9; NOD, nucleotide-binding oligomerization domain; O-IMVs, outer-inner membrane vesicles; OMVs, outer membrane vesicles; PRRs, pattern recognition receptors; PSA, polysaccharide A; SCFAs, short-chain fatty acids; TFF3, trefoil factor 3; TLRs, toll-like receptors; UC, ulcerative colitis; ZO, zonula occludens

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The gut microbiota establishes a complex, dynamic, bidirectional relation with host cells of the intestinal mucosa (Kaparakis-Liaskos & Ferrero, 2015). Due to the anatomical structure of the gut barrier, interkingdom interactions do not involve direct cell contacts. The mucus layer that covers the intestinal epithelium creates a physical separation that prevents luminal bacteria from accessing the underlying epithelial cells (Donaldson et al., 2016). In the colon, the secreted mucus gel-forming mucin 2 creates a well-defined layer, stratified in two sections. The inner layer is highly compact and physically excludes luminal bacteria. In addition, the inner mucus layer contains antimicrobial peptides and immune effectors secreted by epithelial and immune cells that target luminal bacteria. This contributes to spatial host–bacterial segregation (Tropini et al., 2017). The outer mucus layer is loose and serves as an adhesion site for a great number of bacteria that colonize the intestinal tract. This external layer is degraded by specific bacteria of the gut microbiota. Therefore, it needs to be constantly renewed to preserve the inner mucus layer. Production of mucin 2 by goblet cells is upregulated by toll-like receptor (TLR) signalling in response to its degradation by commensals or mechanical sources.

Since access of the microbiota to the intestinal epithelium is restricted physically and chemically, communication with the host mainly depends on microbiota-secreted factors such as metabolites, proteins, and extracellular vesicles (EVs) that can go through the mucin layer and reach host cells at the intestinal mucosal surface. There is mounting evidence that interkingdom crosstalk is principally mediated by EVs released by gut microbiota or by host intestinal cells (Díaz-Garrido et al., 2021). This review is specifically focused on microbiota-secreted EVs and their role as key mediators of bacteria–host interplay in the gut. In the following sections, we discuss current knowledge of their contribution as signals that modulate functions that are crucial for intestinal homeostasis and human health.

## 2 | BACTERIAL EXTRACELLULAR MEMBRANE VESICLES

The production of EVs by bacteria was discovered in 1967 in *Vibrio cholerae*. Electron microscopy analyses of logarithmic growing bacterial cells showed the release of spherical membrane-bound structures produced by pinching off the bacterial outer membrane. These structures were postulated to serve as a mechanism for toxin secretion (Chatterjee & Das, 1967). Initially, these vesicle-type particles were considered needless, artefactual structures. However, as research advanced, they gained recognition as an active, regulated essential mechanism for bacterial cell physiology and communication. Due to their origin, these vesicles were named as membrane vesicles. In recent decades, intensive research has been carried out to establish their mechanisms of biogenesis, composition and functions (Bitto & Kaparakis-Liaskos, 2017; Haurat et al., 2015; Schwechheimer & Kuehn, 2015).

It is now well known that all bacteria release EVs as a mechanism of communication between species. Bacterial extracellular vesicles (BEVs) are spherical lipid bilayer nanostructures, ranging from 20 to 300 nm in size. They are produced by Gram-negative and Gram-positive bacteria (Kim et al., 2015; Kulp & Kuehn, 2010). They are natural carriers of bacterial molecules that include lipopolysaccharides (LPS), peptidoglycans, lipids, proteins, nucleic acids and in the case of pathogens, toxins and virulence factors. They act as a secretion mechanism that allows long-distance delivery of bacterial active compounds in a protected environment, avoiding direct intercellular contact (Gill et al., 2019). The composition and content of BEVs originate in the producer strain and differ depending on the growth phase and conditions (Dauros Singorenko et al., 2017). Currently, it is assumed that the vesicle cargo defines their function.

### 2.1 | Nomenclature and types of BEVs

Studies performed with large number of bacterial species have shown that Gram-negative bacteria generate different kinds of EVs (Avila-Calderón et al., 2015; Jan, 2017; Kulp & Kuehn, 2010). The main type derives from the outer membrane and are referred to as “outer membrane vesicles” (OMVs) (Jan, 2017; Schwechheimer & Kuehn, 2015). OMVs are spherical particles consisting of an outer leaflet of LPS and an inner leaflet of phospholipids. They originate by blebbing of the outer membrane and are therefore enriched in outer-membrane and periplasmic biomolecules (Kulkarni & Jagannadham, 2014; Volgers et al., 2018). In addition, vesicles known as “outer-inner membrane vesicles” (O-IMVs) have been observed in Gram-negative bacteria (Pérez-Cruz et al., 2013). These vesicles display both the outer and the cytoplasmic membrane. In addition to periplasmic components, they include inner membrane proteins and cytosolic molecules (Pérez-Cruz et al., 2015). It is assumed that most Gram-negative bacteria produce both kinds of vesicles, OMVs and O-IMVs. Recent studies have described a new type of Gram-negative bacteria derived vesicles called “explosive outer-membrane vesicles” (E-OMVs), in accordance with the biogenesis mechanism (Toyofuku et al., 2019). This model, which is based on cell lysis induced by phage-encoded endolysin, could explain why OMVs also contain DNA and cytosolic proteins.

In contrast to Gram-negative bacteria, the cell wall of Gram-positive bacteria consists of a thick, rigid external layer of peptidoglycan, which was initially thought to prevent the release of EVs (Gill et al., 2019). For many years, the study of EVs from Gram-positive bacteria was overlooked. This area has only received more attention in the last decade. Research developed in multiple Gram-positive species such as *Staphylococcus aureus*, *Bacillus anthracis*, *Listeria monocytogenes*, *Clostridium*

*perfringens*, or *Bacillus subtilis* has confirmed that Gram-positive-derived EVs are also spherical membrane particles of 20–100 nm in diameter (Brown et al., 2015; Kim et al., 2015; Lee et al., 2009; Palacios et al., 2020), although some species such as *Bacillus* sp., and *C. perfringens* produce larger vesicles of up to 400 nm (Brown et al., 2014; Jiang et al., 2014). Given the lack of an outer membrane, the name “cytoplasmic membrane vesicles” (CMVs) has been suggested for Gram-positive EVs (Toyofuku et al., 2019). EVs from Gram-positive bacteria lack LPS and periplasmic components but carry similar types of cargo molecules as Gram-negative EVs, including peptidoglycan, lipids, proteins, and nucleic acids (Brown et al., 2015).

To simplify nomenclature, we use the term “bacterial extracellular vesicle” (BEV) to refer to all types bacteria-derived vesicles, except when the information that applies to a specific BEV subset (e.g., OMV or CMV) is emphasized. BEV is consistent with the MISEV 2018 guidelines that recommend “extracellular vesicle” (EV) as the generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate (Théry et al., 2018).

## 2.2 | Biogenesis of BEVs

Despite the significant biological implication of BEVs, the mechanisms of biogenesis are still poorly understood. Several models have been proposed and reformulated in the light of technological advances and new findings (Schwechheimer & Kuehn, 2015; Zingl et al., 2020).

For Gram-negative bacteria, initial reports suggested that BEVs are produced under natural conditions as side products of cellular processes related with cell wall turnover. During cell wall recycling, loss of interaction between the outer membrane and peptidoglycan leads to outer membrane budding and the subsequent release of OMVs (Deatherage et al., 2009; Hoekstra et al., 1976; Wensink & Witholt, 1981). BEVs are also shed in response to stress conditions. One model is based on the physical stress caused by accumulation of peptidoglycan fragments or misfolded proteins in the periplasm, which results in increased turgor pressure that leads to the outer membrane bulging out (McBroom et al., 2006; McBroom & Kuehn, 2007). This model supports the idea that OMVs formation could be a helpful waste mechanism to get rid of an excess of harmful proteins (Schwechheimer et al., 2013; Schwechheimer & Kuehn, 2013). Moreover, stress induced in *Stenotrophomonas maltophilia* by antibiotic ciprofloxacin promotes increased release of a heterogeneous population of BEVs, including OMVs and O-IMV (Devos et al., 2017). A third model involves structural changes in LPS in response to external factors (Schwechheimer & Kuehn, 2015). Changes in the concentration of cations that cross-bridge the highly electronegative LPS may lead to accumulation of local negative charges and subsequent repulsion between LPS molecules, which results in local deformation of the bacterial cell membrane and vesicle shedding (Kadurugamuwa & Beveridge, 1995; Mashburn & Whiteley, 2005). Insertion of the *Pseudomonas* quinolone signal (PQS) into the outer leaflet of the outer membrane increases membrane curvature and OMVs formation through this mechanism. This quorum sensing molecule sequesters positive charged ions, triggering destabilization of  $Mg^{+2}$  and  $Ca^{+2}$  salt bridges that leads to anionic repulsion between LPS molecules (Mashburn-Warren et al., 2009; Schertzer & Whiteley, 2012). Other factors that influence LPS remodelling also result in modifications in the outer membrane curvature and OMVs formation (Volgers et al., 2018). LPS is anchored to the outer leaflet of the outer membrane through an acylated disaccharide named lipid A. In *Salmonella enterica* serovar thyphymurium, deacylation of lipid A results in hypervesiculation and OMVs production. In contrast, *pagL* mutants, which are defective in the enzyme that catalyses Lipid A deacylation, show significant reduction in OMV release relative to the wild-type strain (Elhenawy et al., 2016). Recent studies suggest that asymmetric distribution of phospholipids between the inner and outer leaflets of the outer membrane represent a general mechanism for OMV formation under all growth conditions (Roier, Zingl, Cakar, & Schild, 2016; Roier, Zingl, Cakar, Durakovic, et al., 2016). In fact, mutants of the phospholipid transporter VacJ/Yrb show an hypervesiculation phenotype. This is due to the accumulation of phospholipids in the outer leaflet of the outer membrane, which triggers asymmetric expansion, membrane bulging, and the subsequent OMVs release (Henderson et al., 2016; Roier, Zingl, Cakar, Durakovic, et al., 2016). This mechanism involving the VacJ/Yrb ABC transport system is regulated by iron availability in Gram-negative bacteria (Roier, Zingl, Cakar, Durakovic, et al., 2016). As stated above (Section 2.1), a new mechanism for OMVs formation based on explosive cell lysis has been described in *Pseudomonas aeruginosa* (Turnbull et al., 2016). In this model, cell lysis is triggered by a phage-encoded endolysin that degrades the peptidoglycan cell wall. Once the peptidoglycan is degraded, the cells round up and explode. The shattered membrane fragments round up and self-assemble into E-OMVs (Turnbull et al., 2016).

The origin of Gram-positive BEVs remains unclear and is still not fully understood. Earlier studies had suggested that vesicles may be forced through pores in the cell wall by turgor pressure after budding from the cell membrane (Brown et al., 2015). However, the current model involves peptidoglycan-degrading enzymes in a mechanism that resembles the explosive cell lysis described in *P. aeruginosa* (Brown et al., 2015; Toyofuku et al., 2017; Wang et al., 2018). This mechanism was evidenced in *B. subtilis* treated with mitomycin C. This drug induces genotoxic stress that leads to activation of endolysins encoded by prophages integrated in the bacterial chromosome. The subsequent degradation of peptidoglycan causes cells to protrude cytoplasmic material through holes in the peptidoglycan layer, allowing CMV release (Toyofuku et al., 2017). Whereas *P. aeruginosa* cells round up and explode, *B. subtilis* derived CMVs are released by pores formed in the wall. Additionally, recent studies in *S. aureus* have indicated that this bacterium produces proteins such as phenol-soluble modulins and autolysins that increase cell membrane

fluidity, facilitating CMV formation (Schlatterer et al., 2018). Likewise, oxidative stress induced by antibiotics in *S. aureus* triggers CMVs production by increasing the permeability of the peptidoglycan layer in a phage-independent manner (Andreoni et al., 2019). In addition to enzymatic control of CMV production in Gram-positive bacteria, genetic regulation of vesiculation has been investigated. Disruption of genes encoding factor  $\sigma^B$  (*sigB*) in *L. monocytogenes* or the two component system CovRS in *Streptococcus pyogenes* resulted in altered CMV production, thus pointing to a regulatory role in vesicle biogenesis (Lee et al., 2013; Resch et al., 2016).

## 2.3 | Composition of BEVs

Once released, BEVs mediate cell-to-cell communication. The effects exerted by BEVs depend on their cargo. Cargo composition is influenced by the producer bacteria, growth and environmental conditions, and the mechanism of biogenesis. Diversity in cargo molecules confer BEVs crucial roles in bacteria–bacteria and bacteria–host interactions. Many studies have revealed that certain proteins and lipids are selectively incorporated into BEVs. However, how these molecules are picked up remains largely unknown. In this context, mechanisms based on preformed outer membrane microdomains could be proposed to explain the differential enrichment of certain proteins in BEVs (Bonnington & Kuehn, 2014). In this section, we recapitulate the general information about the composition of BEVs in terms of proteins, lipids, nucleic acids and small molecules.

**Proteins.** The protein content of BEVs is not universal and depends on the taxonomic group of the parental bacteria (genus, species, strain) and specific features such as whether they are symbiotic or pathogenic. Studies based on proteomics approaches have identified proteins connected with many biological processes that fit in six categories according to their function as structural proteins, porins, ion channels, transporters, enzymes and proteins related to stress responses (Lee et al., 2008). From these studies we have learned that BEVs released by Gram-negative bacteria are enriched in outer membrane proteins such as OmpA, OmpC and OmpF, and periplasmic proteins such as AcrA and alkaline phosphatase. Proteomic analysis of BEVs from diverse Gram-negative pathogens revealed the presence of virulence factors and a large number of proteins involved in biofilm formation, pathogenicity and host–cell interaction (Altindis et al., 2014; Choi et al., 2011; Jang et al., 2014). Proteins involved in vesicle biogenesis or in the modulation of vesicle size, yield and cargo have also been identified in *S. enterica* Serovar Typhi OMVs (Nevermann et al., 2019). Proteomic studies of vesicles released by Gram-negative probiotic and commensal strains have shown that they contain proteins that contribute to colonization, competition and bacterial survival in the intestinal tract, and modulation of host immune and defence responses. Some of the identified proteins are strain-specific (Aguilera et al., 2014; Elhenawy et al., 2014; Hong et al., 2019; Lee et al., 2007; Zakhazhevskaya, Vanyushkina, et al., 2017). Interestingly, the proteomic analysis of BEVs released by the probiotic *Escherichia coli* Nissle 1917 (EcN) identified a set of proteins that are common to BEVs secreted by Gram-negative pathogens. These are moonlighting proteins that play different functions depending on the cell location. This group of bacterial proteins is mainly composed by chaperones (GroEL, DnaK) and metabolic enzymes (glyceraldehyde-3-phosphate dehydrogenase, enolase, phosphoglycerate kinase). Besides their intracellular activity, these proteins have functions related with host interaction and tissue colonization when located extracellularly (Jeffery, 2018). In addition, the study revealed proteins that are encoded by strain-specific genes. Most of them are adhesins and fitness factors that help bacterial growth and survival in the harmful gut environment (Aguilera et al., 2014; Hong et al., 2019). Reviews with specific information on proteomic analyses of BEVs are available (Kroniger et al., 2018; Lee et al., 2016). Regarding BEVs from Gram-positive bacteria, proteomic analyses in different species (*S. aureus*, *S. pyogenes*, *S. pneumoniae*, and *S. agalactiae*, *B. anthracis*) identified membrane and cytoplasmic proteins related with several biological processes. As in Gram-negative bacteria, pathogen-derived BEVs are loaded with virulence factors and toxins that contribute to pathogenicity (Dean et al., 2019; Dean et al., 2020; Jeon et al., 2016; Lee et al., 2009; Olaya-Abril et al., 2014; Resch et al., 2016; Rivera et al., 2010; Surve et al., 2016; West et al., 2020). Recent research shows that CMVs isolated from several Gram-positive probiotics transport proteins that are responsible for their beneficial effects in the context of immune modulation and host interaction (Behzadi et al., 2017; Domínguez Rubio et al., 2017; Lee et al., 2016; Li et al., 2017).

**Lipids.** Lipids are important structural components of the bacterial cell envelop, and the corresponding BEVs from Gram-negative and Gram-positive bacteria; the OMVs and CMVs, respectively. Consistently, outer membrane phospholipids are present in OMVs. However, there is evidence that OMVs contain some lipids that are not found in the bacterial outer membrane (Chowdhury & Jagannadham, 2013; Kulkarni & Jagannadham, 2014; Tashiro et al., 2011). Studies in *E. coli* have revealed that OMVs comprise lipids involved in membrane curvature, such as glycerophospholipids, phosphatidylglycerol, phosphatidylethanolamine and cardiolipin (Barák & Muchová, 2013; Horstman & Kuehn, 2000). The structures of the fatty acid moieties affect the fluidity and rigidity of the lipid membranes. In *P. aeruginosa* OMVs, the relative amount of saturated fatty acids was around 1.5-fold higher than in the cellular outer membrane, a fact that correlates with membrane rigidity (Li et al., 2018; Tashiro et al., 2012). In contrast, in Gram-positive group A Streptococcus strains, overall levels of saturated fatty acids (C16 and C19) were not significantly different between BEVs and bacterial cell membrane. However, distinctive differences in their distribution in cardiolipin and phosphatidylglycerol were apparent (Resch et al., 2016). LPS is a general component of bacterial OMVs, although differences in LPS composition may exist between vesicles and the bacterial outer membrane. In this context, it

has been reported that *Porphyromonas gingivalis* OMVs carry LPS molecules with long sugar chains and deacylated lipid A compared to the cell wall (Haurat et al., 2011). Likewise, deacylated lipid A accumulates in Salmonella OMVs (Elhenawy et al., 2014). In Gram-positive bacteria, lipidomic studies have revealed that CMVs and the plasma membrane conserve essential aspects but show some differences in certain lipids and lipoproteins. In *B. anthracis* and *S. pneumoniae*, vesicles are enriched in C12-C16 chain saturated fatty acids (Olaya-Abril et al., 2014; Rivera et al., 2010). Conversely, lipids containing unsaturated fatty acids are more abundant in *L. monocytogenes*-derived vesicles than in bacterial cells. In addition, CMVs from this pathogen are enriched in phosphatidylethanolamine, sphingolipids and triacylglycerols, but glycolipids are underrepresented (Coelho et al., 2019). Overall, these studies show that the lipid composition of CMVs may vary with the bacterial species. These differences may be related with bacterial adaptation and survival in certain ecological niches (Coelho et al., 2019).

**Nucleic acids.** Many studies show that Gram-negative and Gram-positive-derived BEVs are loaded with nucleic acids, including DNA and RNA. Therefore, BEVs allow delivery of DNA into other bacterial cells, which mediates horizontal gene transfer (Domingues & Nielsen, 2017). In addition, BEVs facilitate the interaction of transported nucleic acids with their specific intracellular receptors in host cells, which triggers modulation of host immune responses (Gilmore et al., 2021). BEVs contain luminal and surface associated DNA (Bitto & Kaparakis-Liaskos, 2017; Jiang et al., 2014; Liao et al., 2014; Renelli et al., 2004). In BEVs from *P. aeruginosa*, most of the luminal DNA correspond to genes involved in specific functions related with antibiotic resistance, virulence and stress response (Bitto & Kaparakis-Liaskos, 2017). In the Gram-positive *C. perfringens*, BEVs carry genes encoding bacterial toxins such as  $\alpha$ -toxin (*plc*) and perfringolysin O (*pfoA*) (Jiang et al., 2014). External DNA in *Streptococcus mutans*-derived BEVs was mainly related with biofilm formation and maturation. These are essential processes during bacterial adhesion (Liao et al., 2014).

Besides DNA, some studies have shown the presence of RNA in BEVs, with important implications for the regulation of the host immune system and other cellular processes (Lee, 2019). RNA associated with BEVs is protected from degradation, which facilitates its delivery to eukaryotic target cells. A compilation of RNAs associated with BEVs has been published (Dauros-Singorenko et al., 2018). These studies revealed that the RNA included in BEVs is enriched in small non-coding RNAs, which are mainly involved in the regulation of gene expression post-transcriptionally. In some cases, the RNA cargo was shown to induce phenotypic changes in the target cells. In certain pathogenic bacteria, small RNAs secreted through BEVs mediate dysregulation of host immune responses (Choi et al., 2017; Koeppen et al., 2016). Moreover, many sequences of the transported small non-coding RNAs found in *E. coli* BEVs align to human genome regions involved in the regulation of gene expression associated with epigenetic mechanisms (chromatin remodelling, histone modifications) or cell-specific transcriptional control (Celluzzi & Masotti, 2016). It has been suggested that BEV-associated small RNAs have a function similar to the regulatory role of eukaryotic miRNAs (Dauros-Singorenko et al., 2018).

**Small molecules.** BEVs are also loaded with metabolites and effector molecules that modulate functions of target cells. Metabolomic approaches have revealed that BEVs contain metabolites, which were selectively packaged depending on the producer strains. In *B. thetaiotaomicron*, the varying metabolite content between BEVs isolated from pathogenic or commensal strains was consistent with the ability of each strain to colonize and survive in the intestinal tract (Bryant et al., 2017; Zakharzhenskaya, Vanyushkina, et al., 2017). Other effectors packaged into BEVs also act as fitness factors, helping bacterial survival in specific niches. For instance, BEVs from *B. fragilis* are loaded with the antimicrobial peptide BSAP, which displays inhibitory activity against other Bacteroidales of the human gut (Chatzidaki-Livanis et al., 2014). BEVs also serve as vehicles to secrete hydrophobic quorum sensing molecules that mediate communication in bacterial communities, controlling important processes such as virulence or biofilm formation (Brameyer et al., 2018).

## 2.4 | Functions of BEVs

Secretion of BEVs is a costly process that requires energy expenditure. This secretion mechanism was selected by evolution because BEVs play essential roles that give bacteria self-protection or promote interaction and communication with other members of the microbial community or the host. BEVs are involved in numerous biological functions, which may differ depending on their specific cargo. Functions of BEVs can be classified into two groups: those linked to bacteria-bacteria interaction and those related with bacteria-host communication. Specific reviews are available on this topic (Caruana & Walper, 2020; Jan, 2017; Schwechheimer & Kuehn, 2015).

Regarding bacteria-bacteria interactions, BEVs allow producer bacteria to persist in the ecological niche by helping, competing with, or killing other bacteria. In this context, BEVs may serve as a microbial defence mechanism by providing protective functions against harmful agents that include phages, antibiotics, reactive oxygen species and antimicrobial peptides. Released BEVs can sequester phages, so that they do not directly interact with bacterial cells, preventing deleterious effects (Manning & Kuehn, 2011). BEVs can also serve as decoys for contaminants or antibiotics that target the bacterial membrane (Manning & Kuehn, 2011). Besides their passive role as antibiotic targets, BEVs also release enzymes that confer resistance to antibiotics, which benefits the producer strain and other susceptible bacteria in the microbial community. In this context, beta-lactamases have been found in BEVs from *S. aureus* and *Bacteroides sp* (Lee et al., 2013; Régis Stentz et al., 2015) and enzymes

that hydrolyse carbapenem in *Acinetobacter baumannii* BEVs (Liao et al., 2015). Genes encoding  $\beta$ -lactamases have been found in BEVs. In this case, BEVs allow intra- and interspecies horizontal transfer of genes encoding antibiotic resistance (Chatterjee et al., 2017). In addition, BEVs can confer metabolic advantages. The presence of transport systems for siderophores, amino acids or fatty acids facilitates nutrient acquisition and bacterial survival and may serve as a competing mechanism against other bacteria. BEVs can also harbour hydrolase-type enzymes that catalyse degradation of the proteins and complex polysaccharides present in the environment. This may assist the microbial community in nutrient acquisition (Elhenawy et al., 2014).

Besides beneficial effects for the bacterial community, BEVs can mediate offensive functions. In this sense, they carry degradative enzymes such as murein hydrolases, peptidoglycan hydrolases or endopeptidases for killing competing bacteria (Jan, 2017; Lee et al., 2008). As an example, BEVs from some *Myxococcus* sp. species have been shown to contain factors with antimicrobial activity and a great variety of hydrolytic enzymes that cause cell lysis of target bacteria, thus conferring predatory activity (Evans et al., 2012; Marshall & Whitworth, 2019).

In microbial communities, BEVs serve as key players in cell-to-cell communication. As stated above, hydrophobic quorum sensing molecules that coordinate bacterial growth and behaviour depending on population density are secreted into BEVs. Quorum sensing molecules also drive the microbial community to grow in biofilms. There is evidence that BEVs are important components of the biofilm matrix, which consists of polysaccharides, lipids, nucleic acids and proteins. Formation of biofilms protects multispecies bacterial communities and has benefits for nutrient acquisition and survival. In this context, BEVs can contribute to these functions (Flemming et al., 2016).

In the context of bacteria–host interactions, BEVs are well adapted and become essential for interkingdom communication. There is evidence that they control host cell pathways and processes by delivering bacterial effectors into target cells. BEVs are equipped with adhesin-type molecules that help their adhesion to host cells, and microbial molecules recognized by immune receptors involved in the activation of inflammatory and defence responses (see Section 3). In pathogens, BEVs carry virulence factors, toxins and immunomodulatory effectors that help bacteria to attack host cells and subvert the host machinery to escape the immune system (Jan, 2017; Kaparakis-Liaskos & Ferrero, 2015; Lee et al., 2008). For strains that establish a symbiotic relation with the host, released BEVs serve as a mechanism for the delivery of bacterial mediators that modulate important host functions (Badia & Baldomà, 2020).

In addition, based on their properties as long-distance delivery vehicles and their intrinsic versatility and biocompatibility, BEVs are being explored as promising novel therapies. Engineered BEVs are being developed as new vaccines and adjuvants or specialized drug delivery vehicles for the treatment of emerging and new diseases. Specific reviews are available on this topic (Bitto & Kaparakis-Liaskos, 2017; Cai et al., 2018).

### 3 | INTERACTION OF BEVS WITH MAMMALIAN HOST CELLS

#### 3.1 | Internalization of BEVs by host cells

BEVs from Gram-negative and Gram-positive bacteria can deliver their content into the host cell cytoplasm. The uptake of BEVs by mammalian host cells is mainly mediated by endocytosis. This process occurs through different pathways depending on the surface and cargo of the vesicles (O'Donoghue et al., 2017; O'Donoghue & Krachler, 2016). Phagocytosis is the main route used by phagocytic cells of the immune system (neutrophils, macrophages, dendritic cells) to internalize BEVs. In non-phagocytic cells, such as epithelial cells, other endocytic routes allow the entry of BEVs. These include macropinocytosis, clathrin-mediated endocytosis and lipid raft-mediated processes that may depend on caveolin. Each pathway allows internalized vesicle cargo to be sorted to specific subcellular locations. Actin-dependent macropinocytosis is driven by the polymerization of an actin ring below the cell membrane. This causes a circular lump that closes at the top to encircle a portion of the extracellular space. The process mostly allows internalization of very large cargos, up to 1  $\mu$ m. Clathrin-mediated endocytosis is triggered by ligand binding to a cell surface receptor located in clathrin-coated pits, which mature into vesicles inside the cells in a process that requires the GTPase dynamin. During intracellular trafficking, bacterial vesicles are sorted to endosomes and lysosomal compartments. Lipid rafts are dynamic membrane microdomains rich in cholesterol, sphingolipids and certain proteins that are associated with distinct endocytic pathways. One such pathway is caveolin-mediated endocytosis, which involves membrane lipid raft domains that are enriched in caveolin, a protein that triggers membrane invaginations called caveolae and the subsequent formation of endocytic vesicles. Fission from the plasma membrane also requires dynamin, although trafficking of caveolar vesicles does not involve the same endosomal compartments as clathrin-mediated endocytosis. Finally, BEVs can enter host cells through lipid-raft domains in a caveolin-independent manner. These routes are not mutually exclusive, since certain BEVs have been shown to use more than one pathway to enter epithelial cells depending on their size and cargo (O'Donoghue & Krachler, 2016). More details about this topic can be found in specific reviews (Anand & Chaudhuri, 2016; Bitto & Kaparakis-Liaskos, 2017; Kaparakis-Liaskos & Ferrero, 2015; O'Donoghue & Krachler, 2016).

Most of the available information on the mechanisms for BEV uptake in mammalian host cells comes from studies dealing with Gram-negative pathogens. The first study on microbiota-derived BEVs focused on the probiotic *E. coli* Nissle 1917 (EcN) and commensal *E. coli* strains (Cañas et al., 2016). The authors showed that BEVs from beneficial gut resident *E. coli* strains are internalized by several epithelial cell lines mainly by clathrin-mediated endocytosis. Consistently, internalized BEVs colocalized with clathrin and specific markers of endosomes and lysosomes. Disruption of lipid rafts and caveolae domains with specific inhibitors did not affect vesicle uptake (Cañas et al., 2016). Furthermore, cryo-transmission electron microscopy analyses of BEVs isolated from an hypervesiculating EcN *tolR* mutant evidenced highly structural heterogeneity in the isolated vesicles, with the presence of abundant aberrant structures that did not correspond with common OMVs or O-IMVs (Pérez-Cruz et al., 2016). This study revealed that *tolR* mutant-derived BEVs displayed diminished cell binding capacity, which in turn, negatively influence the uptake levels in Caco-2 cells. This indicates that only conventional BEVs efficiently enter epithelial cells through clathrin-mediated endocytosis (Pérez-Cruz et al., 2016). A recent study revealed the mechanism of uptake and traffic of BEVs from the resident gut microbiota *B. thetaiotaomicron* (Jones et al., 2020). By means of in vitro cultures, authors showed that *B. thetaiotaomicron* BEVs are internalized by intestinal epithelial cells (IECs) principally by dynamin-dependent endocytosis and are sorted to endo-lysosomal vesicles. Moreover, in vivo imaging studies showed that a proportion of *B. thetaiotaomicron* BEVs cross the intestinal epithelium by paracellular transmigration and reach systemic circulation. This demonstrates that BEVs mediate long-distance communication with extra-intestinal tissues (Jones et al., 2020).

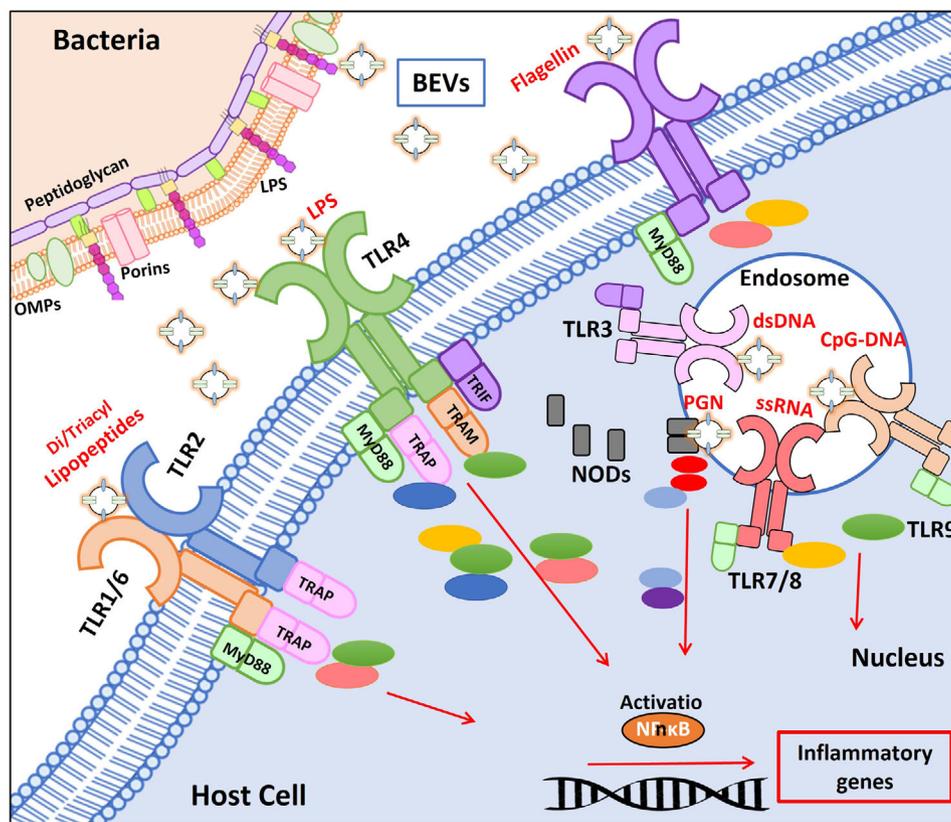
There are few studies on the internalization pathways used by BEVs from Gram-positives. In the probiotic *Lactiplantibacillus plantarum* BGAN8, time-course internalization experiments performed in the presence of specific inhibitors of endocytosis pathways evidenced that the main entry route for secreted vesicles in IECs is the clathrin-mediated pathway, although the uptake kinetics was slower than that reported for BEVs of Gram-negative probiotics. Whereas internalized *E. coli* BEVs were apparent at 15–30 min incubation, more than 1 h was required to detect intracellular *L. plantarum* BEVs (Bajic et al., 2020). Authors suggested that the abundant exopolysaccharide material secreted by this probiotic that accompanied isolated BEVs could cause a trapping effect and delay the interaction of BEVs with epithelial cell membrane structures needed for endocytosis. Other internalization mechanisms may operate in parallel, depending on the vesicle size. The exopolysaccharide network that entraps BEVs creates a large volume coating the BEVs isolated from cultures of this probiotic that could be taken up by micropinocytosis (Bajic et al., 2020).

### 3.2 | Recognition of BEVs by immune receptors

BEVs contain biological components produced by the parental strain. In this context, surface cargo molecules that mediate adhesion to host extracellular proteins or specific cell receptors are determinant for the primary interaction with target cells, and consequently with the derived effects (Kulp & Kuehn, 2010). BEVs also carry a set of molecules known as microbe-associated molecular patterns (MAMPs) that are recognized by specific receptors expressed by host epithelial and immune cells. These pattern recognition receptors (PRRs) are key components of innate immunity as they sense gut microbes and mediate appropriate responses (Figure 1). Mammalian cells express different types of PRRs according to their location and association with cell membranes (Mu et al., 2015; Zhou et al., 2020). TLRs are transmembrane proteins that can be located at the plasma membrane (TLR2, TLR4, TLR5) or at intracellular endosomal membranes (TLR3, TLR7, TLR9). MAMPs present in the surface of BEVs (extracellular polysaccharides, LPS and lipoproteins) can bind extracellular TLRs to activate cell signalling pathways involved in the regulation of immune and defence responses. In addition, BEVs are internalized by mammalian host cells through endocytic pathways. By this mechanism, BEVs allow intracellular delivery of other enclosed MAMPs such as DNA or RNA that are recognized by specific TLR receptors in the membrane of endocytic compartments. At intracellular level, there are cytosolic immune receptors (not associated with organelle membranes), such as nucleotide-binding oligomerization domain protein 1 (NOD1) and NOD2 that are activated by bacterial peptidoglycan fragments. In non-invasive Gram-negative pathogens, internalized BEVs are a mechanism for the delivery of peptidoglycan inside the host cell and subsequent activation of NOD receptors (Chatterjee & Chaudhuri, 2013; Kaparakis et al., 2010; Thay et al., 2014). Studies performed with *H. pylori* revealed that interaction of NOD1 with peptidoglycan included in the endocytosed vesicles takes place at early endosomes, where NOD1 is recruited during intracellular trafficking of BEVs (Irving et al., 2014). Following peptidoglycan binding, these receptors initiate the inflammatory response through activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) or the mitogen-activated protein kinase (MAPK) pathways, which ultimately upregulate transcription of inflammatory genes (Figure 1). This mechanism is also activated by BEVs released by microbiota *E. coli* strains (Section 4.3.1).

## 4 | CONTRIBUTION OF MICROBIOTA BEVS TO INTESTINAL HOMEOSTASIS

In the last 20 years, numerous studies have focused on BEVs from Gram-negative pathogens, due to their role as vehicles for the delivery of virulence factors and molecules that inhibit and escape host immune responses (Karakakis-Liaskos & Ferrero, 2015).



**FIGURE 1** Recognition of BEV-associated molecular patterns by host immune receptors. The drawing schematically shows toll-like receptors (TLRs) located at the host cell membrane (TLR1/6, TLR2, TLR4, and TLR5), TLRs located at the endosomal membranes (TLR3, TLR7/8, TLR9) and cytosolic NODs (NOD1/NOD2), and the downstream signalling pathways that lead to activation of NF- $\kappa$ B. Proteins of the different signalling pathways are drawn as coloured ellipses. The molecular associated pattern that specifically interacts with each receptor is indicated in red. LPS: lipopolysaccharide, PGN: peptidoglycan

The study of probiotic and gut microbiota-derived vesicles started in the early 2010s (Lopez et al., 2012; Shen et al., 2012). Since then, most studies have focused on Gram-negative commensals, specifically *Bacteroides fragilis*, *B. thetaiotaomicron*, *Akkermansia muciniphila*, and intestinal *E. coli* isolates, including the probiotic EcN. Studies on BEVs from Gram-positive gut microbes started later and were mostly focused on probiotics of the genus *Lactobacillus* and *Bifidobacterium* (Liu et al., 2018). In this section, we review current knowledge of the role of BEVs as important players in the gut microbiota effects on intestinal homeostasis. We focus on how microbiota BEVs control food metabolism, epithelial barrier integrity and immune responses. The information provided in this section is summarized in Table 1.

#### 4.1 | Gut ecology and food metabolism

The gut microbiota maintains a symbiotic association with the host at the intestinal mucosa and provides important metabolic, immunological and gut protective functions in the healthy individual. One important function of the microbiota is to generate metabolites that influence both microbes and host. One of the most abundant phyla present in the intestinal microbiota is *Bacteroides* spp, which exploit diverse biochemical mechanisms to colonize the harsh environment of the intestine. These commensal bacteria are well studied for their role in carbohydrate metabolism and production of short-chain fatty acids (SCFAs) that provide daily energy requirements for the host (Wexler & Goodman, 2017; Wexler, 2007). In particular, *B. thetaiotaomicron* is a good model for investigating commensal bacteria–host interaction, since it has a well-defined role in complex carbohydrate metabolism and its genome is fully sequenced (Eckburg et al., 2005). In this context, *B. thetaiotaomicron* codifies several mucin-degrading enzymes such as glycosyl hydrolases and sulfatases that allow assimilation of mucin glycans and dietary nutrients, thus conferring an adaptive advantage over other bacteria. Some of the breakdown products can be used as a source of nutrients by other components of the gut microbiota. This favours the establishment of a balanced microbial symbiotic community (Benjdia et al., 2011; Martens et al., 2009). Importantly, bacterial metabolic activity on complex carbohydrates generates a pool of SCFAs that are reabsorbed through the large intestine and used by host as an energy source, which provides a significant amount of daily energy requirements (Singh, 2019).

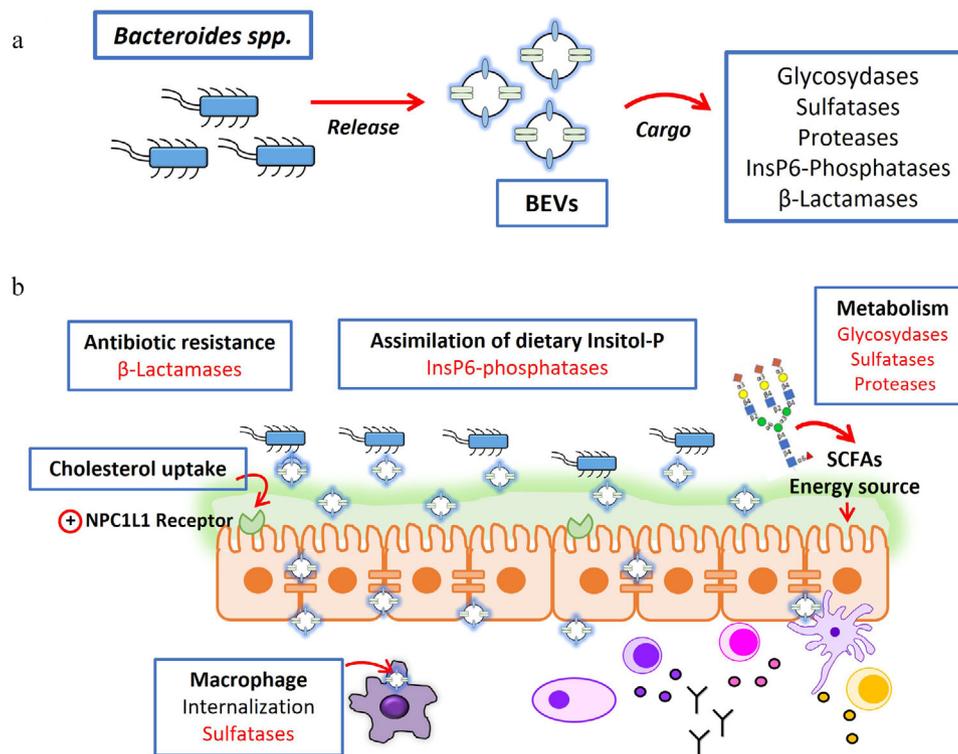
**TABLE 1** Studies evaluating the role of microbiota derived BEVs as modulators of intestinal homeostasis-related processes

Bacteria	Mechanism	Experimental approach	References
Gut ecology and food metabolism			
<i>Bacterioides fragilis</i>	Metabolism of complex carbohydrates to produce SFCAs: Expression of glycosyl-hydrolases, sulfatases, proteases	Proteomics of BEVs by mass spectrometry	Valguarnera et al. (2018)
<i>B. thetaiotaomicron</i>	Cholesterol uptake: upregulation NPC1L1 receptor Metabolites in BEVs that facilitate intestinal colonization	In vitro Caco-2 cell culture In silico, proteomic and metabolomic analysis	Elhenawy et al. (2014) Ahmadi Badi et al. (2020) Bryant et al. (2017)
<i>B. thetaiotaomicron</i>	Assimilation of dietary Inositol-P Macrophage internalization (Sulfatases)	Biochemical characterization of InsP6-phosphatase Experimental model of colitis using genetically modified mice	Zakharzhevskaya, Tsvetkov, et al. (2017) Zakharzhevskaya, Vanyushkina, et al. (2017) Stentz et al. (2014) Hickey et al. (2015)
<i>Bacteroides fragilis</i>	Antibiotic resistance ( $\beta$ -lactamases)	Knockout mutant of putative $\beta$ -lactamase gene	Stentz et al. (2015)
Epithelial barrier integrity			
<i>E. coli</i> Nissle 1917 ECOR63 strain	Upregulation of TJ proteins ZO-1 and claudin-14, downregulation of claudin-2 Protection against EPEC-induced damage: preservation of occludin and claudin-14 mRNA levels, redistribution of ZO1, amelioration of F-actin disorganization	In vitro Caco-2 and T-84 cell cultures: RT-qPCR, confocal microscopy In vitro Caco-2 and T-84 cell cultures infected with EPEC: RT-qPCR, confocal microscopy, paracellular permeability assays	Alvarez et al. (2016) Alvarez et al. (2019)
<i>E. coli</i> Nissle 1917	Upregulation TFF3 and MMP-9 mRNA	In vivo mice model of DSS-induced colitis	Fabrega et al. (2017)
<i>Akkermansia muciniphila</i>	Upregulation of ZO-1, claudin 5 Upregulation of ZO-1, occludin, claudin-1 Upregulation of occludin, ZO-1/2, claudin-4	In vivo high-fat diet (HFD)-induced diabetic mice model, and Caco-2 cell culture In vivo HFD-induced obesity mice model In vitro Caco-2 cells challenged with LPS	Chelakkot et al. (2018) Ashrafian, Shahriary, et al. (2019) Ashrafian, Behrouzi, et al. (2019)
Gut immunity: modulation of inflammatory responses through the intestinal epithelium			
<i>E. coli</i> Nissle 1917 ECOR12 strain	Upregulation of IL-6, IL-8, TNF- $\alpha$ , IL-10, MIP1 $\alpha$ Upregulation of IL-22 and $\beta$ -defensin Downregulation of IL-12 Activation of NOD-1 / NF- $\kappa$ B pathway	In vitro Caco-2/PBMCs cell coculture model Ex vivo model of colonic explants Caco-2 cells: NOD1 silencing - RIP2 kinase inhibition	Fabrega et al. (2016) Fabrega et al. (2016) Cañas et al. (2018)
<i>E. coli</i> Nissle 1917	Upregulation of IL-10; downregulation of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-12, IL-17, iNOS and COX-2 in colonic tissue	In vivo mice model of DSS-induced colitis	Fabrega et al. (2017)
<i>Lactobacillus kefir</i> <i>L. kefirifaciens</i> <i>L. kefirgranum</i>	Downregulation of IL-8 Counteract oxidative stress by decreasing myeloperoxidase serum levels	Caco-2 cells challenged with TNF- $\alpha$ In vivo mice model of TNBS-induced IBD	Seo et al. (2018)

(Continues)

TABLE 1 (Continued)

Bacteria	Mechanism	Experimental approach	References
Gut immunity: modulation of DCs and derived T cell responses			
<i>Bacteroides fragilis</i>	Induction of Treg cells (CD4+CD25+FOXP3+) and IL-10 production through a mechanism that involves TLR2 Activation of autophagy. Induction of Treg cells and IL-10 production depends on functional ATG16L1 and NOD2.	In vivo mice model of TNBS-induced colitis In vitro bone marrow-derived DCs culture BEVs from wild-type and PSA deficient strains Bone-marrow derived DCs from wild type, ATG16L1- and NOD2 deficient mice. In vitro cocultures of BMDCs with CD4+T cells In vivo mice model DNBS-induced colitis	Shen et al. (2012) Chu et al. (2016)
<i>Bacteroides vulgatus</i> mpk	Induction of DC tolerance via TLR2 and TLR4 Upregulation of co-stimulatory molecules including MHC-II, CD40, CD80 and CD86 in CD11c+ cells	In vitro bone marrow-derived DCs culture TLR4/TLR2 knockout mice model	Maerz et al. (2018)
<i>Lactobacillus rhamnosus</i> JB-1	Increased production of IL-10 and regulatory (CD4+CD25+FOXP3+) T cells	In vitro bone marrow-derived DCs culture In vivo mice model	Al-Nedawi et al. (2015)
<i>Lactobacillus sakei</i>	Enhance IgA expression	Ex vivo model of murine Peyer's patches	Yamasaki-Yashiki et al. (2019)
<i>Bifidobacterium bifidum</i> LMG13195	Promote differentiation to regulatory T cells (CD4+CD25+FOXP3+) and IL-10 secretion	In vitro model of monocyte-derived DCs co-cultivated with CD4+ T cells	Lopez et al. (2012)
<i>Bifidobacterium longum</i>	Apoptosis of bone-marrow-derived mast cells through ESBP vesicular protein	In vivo mouse model of allergen-induced food allergy	Kim et al. (2016)
<i>E. coli</i> Nissle 1917	Upregulation of driver Th cytokines by DCs in a strain-specific manner	In vitro model of monocyte-derived DCs co-cultivated with CD4+ T cells	Diaz-Garrido et al. (2019)
Commensal <i>E. coli</i>	Differential induction of Th1, Th2, Th17/Th22 and T regulatory responses Regulation of key miRNAs in immunity (miR-155, miR-146a/b and miR-let7i) Differential modulation of miRNAs involved in tolerogenic responses (miR-125a/99b/let7e, miR-125b, miR-24)	In vitro model of monocyte-derived DCs: RNA seq approaches to identify differentially expressed miRNAs	Diaz-Garrido et al. (2020)



**FIGURE 2** Metabolic activities associated with *Bacteroides*-derived BEVs known to contribute to gut ecology and food metabolism. (a) The enzymes included as cargo in *B. thetaiotaomicron* and *B. fragilis* BEVs responsible for metabolic effects are indicated. (b) The drawing shows the intestinal epithelial barrier and the underlying immune system. The mucin layer (in green) maintains segregation between luminal microbes and the intestinal epithelium. Blue boxes connect the vesicular enzyme (in red) with each specific function. SCFAs: short-chain fatty acids, InsP6: inositol phosphates

BEVs produced by *Bacteroides* species are a source of such enzymatic activities and have a great impact on host metabolism (Figure 2). Most studies have been carried out on *B. thetaiotaomicron* and *B. fragilis* (Ahmadi Badi et al., 2020). *B. fragilis* is a good colonizer of the intestinal tract. In fact, it is commonly isolated from healthy human stools (Huang et al., 2011). *B. thetaiotaomicron*-derived BEVs contain enzymes that help polysaccharide digestion in the gut. Dietary inositol polyphosphates (InsP6) present in vegetables interfere with polysaccharide digestion by chelating divalent cations. Since human IECs do not express enzymes that can dephosphorylate InsP6, this activity depends on enzymes encoded by gut microbiota. In this context, *B. thetaiotaomicron*-derived BEVs are a luminal source of InsP6 phosphatases in the gut (Stentz et al., 2014). In addition, diverse hydrolytic enzymes such as glycosidases, sulfatases and proteases have been found as cargo in *Bacteroides*-derived BEVs that participate in the breakdown of host-indigestible glycans and host mucins (Elhenawy et al., 2014; Valguarnera et al., 2018). There is evidence that *B. thetaiotaomicron* BEVs are internalized in mucosa resident macrophages in a sulfatase-dependent manner. By this mechanism, BEVs could cross the mucosal barrier and facilitate the delivery of bacterial antigens and factors to immune cells (Hickey et al., 2015).

Various studies focus on the association between *Bacteroides* BEVs and cholesterol metabolism (Figure 2). Production of BEVs from *B. thetaiotaomicron* and *B. fragilis* is stimulated by saturated fatty acids, such as palmitate (Mirjafari Tafti et al., 2019). Moreover, BEVs from both bacteria upregulate expression of the apical surface-expressed transporter Niemann-Pick C1-like (NPC1L1) that mediates cholesterol uptake by enterocytes (Ahmadi Badi et al., 2020).

Metabolomic analyses has revealed that BEVs from *Bacteroides* species are loaded with metabolites with a relevant role in interaction with the host (Bryant et al., 2017; Zakhazhevskaya, Vanyushkina, et al., 2017). Concerning *B. thetaiotaomicron* BEVs, the putative role of the metabolites was predicted by *in silico* analysis. This showed that the vesicles are enriched with metabolites known to facilitate intestinal colonization and metabolites that could be incorporated into mouse metabolic pathways (Bryant et al., 2017). Studies performed in *B. fragilis* revealed significant differences in the metabolomic and proteomic content of BEVs depending on the producer strain (Zakhazhevskaya, Vanyushkina, et al., 2017). Strains of *B. fragilis* can be distinguished by their ability to produce a zinc-dependent metalloprotease toxin (BTF). They are classified accordingly as nontoxicogenic *B. fragilis* (NTBF) and enterotoxigenic *B. fragilis* (ETBF). ETBF strains cause intestinal inflammation and, in some cases, can contribute to colon cancer (Sears et al., 2014). The toxin BTF is secreted through BEVs as a surface exposed protein. In this location, BTF interacts with phospholipids and polysaccharides of the host cell membrane through hydrophobic and electrostatic interactions (Zakhazhevskaya, Tsvetkov, et al., 2017). Besides virulence factors, proteomic and metabolomic characterization of BEVs from

NTBF and ETBF strains showed that BEVs from the non-pathogenic strain are enriched in enzymes required for polysaccharide utilization, a metabolic activity that positively contributes to intestinal ecology. Conversely, BEVs from ETBF contain a high number of catabolic enzymes that belong to energy-producing pathways, which allow long persistence in the gut. Fluxomic experiments with isotope-labelled glucose confirmed that these pathways are active in pathogen-derived BEVs (Zakharzhevskaya, Vanyushkina, et al., 2017).

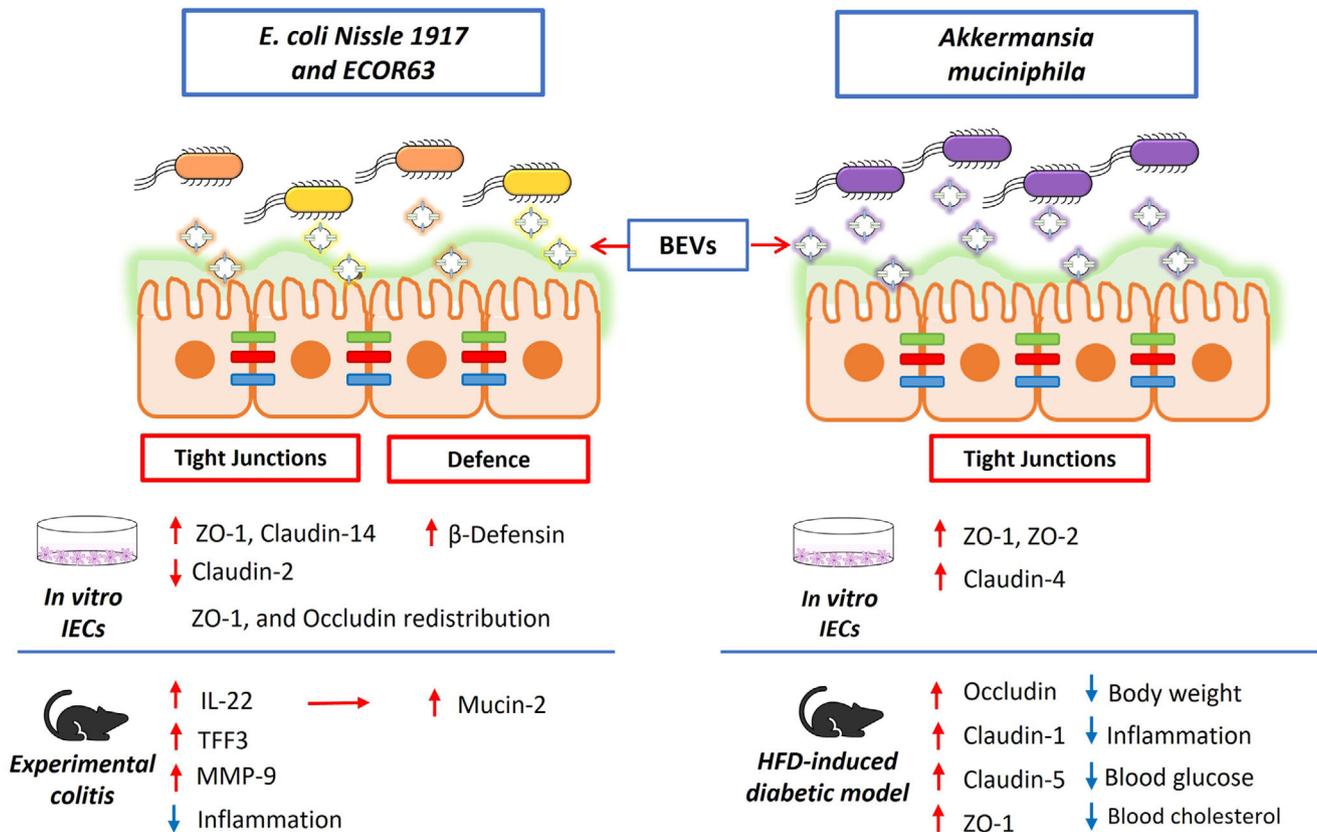
## 4.2 | Modulation of intestinal epithelial barrier integrity

In the gut, besides host-derived EVs (Díaz-Garrido et al., 2021), microbiota BEVs act as regulators of epithelial barrier integrity. This regulation is critical to gut homeostasis, as disruption of this barrier leads to enhanced intestinal permeability, a condition that causes inflammatory, allergic and metabolic diseases (Suzuki, 2013).

The intestinal epithelium serves as a physical barrier that restricts translocation of luminal microbes and antigens into host tissues without interfering with the passage of ions through the paracellular space. An important mechanism governing epithelial barrier function is the formation of tight junctions (TJs) between adjacent IECs. TJs establish intercellular connections in the apical part of the polarized epithelium through transmembrane proteins that include occludin, claudins, tricellulin and junctional adhesion molecules. These proteins are anchored to the actin cytoskeleton by the cytosolic proteins zonula occludens (ZO)-1 and ZO-2. TJs are dynamic structures that modulate epithelial barrier integrity and paracellular permeability. Therefore, the expression and subcellular location of TJ proteins are controlled by signalling pathways and mechanisms in response to external stimuli (Shen, 2012). Enteric pathogens cause TJ disruption as part of their infective mechanisms, whereas certain microbiota strains reinforce TJ structures as a mechanism to maintain gut barrier integrity (Hiippala et al., 2018). Besides TJs, other structures maintain connectivity between IECs, namely adherens junctions, gap junctions and desmosomes (Luissint et al., 2016).

Several studies in gut microbiota species show the role of microbiota BEVs as modulators of epithelial barrier integrity (Badia & Baldomà, 2020). Concerning intestinal *E. coli* isolates, the probiotic EcN has been extensively studied due to its recognized immunomodulatory and anti-inflammatory properties that positively contribute to microbiota balance (Sonnenborn, 2016; Sonnenborn & Schulze, 2009; Toloza et al., 2015). The probiotic EcN reinforces the epithelial barrier through regulation of TJ proteins ZO-1 (Ukena et al., 2007), ZO-2 (Zyrek et al., 2007), claudin-14 (Hering et al., 2014) and the desmosome protein pinin (Veltman et al., 2012). Hering and co-workers showed that the probiotic factor that triggers upregulation of claudin-14 is the secreted protein TcpC, which activates the ERK1/2 signalling pathway (Hering et al., 2014). Further studies in our group with EcN cell-free supernatants revealed that in addition to TcpC, secreted BEVs have a strengthening barrier function (Alvarez et al., 2016). Our studies included other *E. coli* strains of intestinal origin, such as ECOR12 (group A; TcpC negative) and ECOR63 (group B2; TcpC positive). We have shown that BEVs released by *E. coli* strains modulate epithelial barrier integrity in a strain-specific manner through direct and indirect mechanisms. In monolayers of colonic cell lines, BEVs from EcN and ECOR63 reinforce the epithelial barrier through upregulation of TJ proteins ZO-1 and claudin-14. Moreover, BEVs from both strains modulate barrier permeability through downregulation of claudin-2, a leaky protein that facilitates barrier permeability (Figure 3). These regulatory activities do not depend on TcpC, which is secreted as free soluble protein. Conversely, ECOR12 BEVs have no effect on epithelial barrier integrity (Alvarez et al., 2016). The protective role of EcN and ECOR63 BEVs was studied in a cellular model of epithelial barrier dysfunction induced by infection with enteropathogenic *E. coli* (EPEC) (Alvarez et al., 2019). In this model, BEVs from both *E. coli* strains were able to protect the epithelial barrier integrity against EPEC infection by activating compensatory regulatory mechanisms at transcriptional and post-transcriptional levels that affect TJ proteins. Specifically, BEVs counteracted altered mRNA expression of occludin and claudin-14, retained ZO-1 and occludin at TJs in the cell boundaries and ameliorated F-actin cytoskeleton disorganization (Alvarez et al., 2019). In the human gut, crosstalk between epithelial and immune cells is crucial for maintaining intestinal homeostasis. In this context, we have shown that BEVs released by intestinal *E. coli* strains activate other mechanisms in the gut mucosa that can synergistically reinforce the epithelial barrier (Figure 3). In human colonic explants, EcN BEVs activate IL-22 expression. This cytokine helps to preserve the integrity of the epithelial barrier by inducing mucin production by goblet cells (Fabrega et al., 2016). In the experimental mouse model of colitis, oral administration of EcN BEVs preserves the colonic cytoarchitecture of the mucosa, an effect that may be attributed to upregulation of trefoil factor 3 (TFF3), a bioactive peptide involved in epithelial repair. Since TFF3 promotes redistribution of ZO-1 from the cytoplasmic compartment to the intercellular junctions in IEC monolayers, activation of TFF3 by EcN BEVs indirectly reinforces TJs in colitic mice and counteracts increased barrier permeability (Fabrega et al., 2017). Another barrier protective mechanism exerted by EcN BEVs is downregulation of matrix metalloprotease-9 (MMP-9) expression, an enzyme that contributes to intestinal inflammation by disrupting TJs between IECs, which in turn leads to increased intestinal permeability and the subsequent pro-inflammatory effects (Fabrega et al., 2017).

Reduced microbiota diversity, and especially low numbers of the Gram-negative bacterium *A. muciniphila*, has been linked with the development of inflammatory bowel disease (IBD) (Png et al., 2010; Vignsnes et al., 2012), and metabolic alterations such as diabetes and obesity (Zhou & Zhang, 2019). This microorganism is an abundant inhabitant of the human intestinal tract that provides metabolic benefits to the host by degrading intestinal mucin into SCFAs (de Vos, 2017). Butyrate is used as an



**FIGURE 3** Modulation of the gut epithelial barrier by microbiota BEVs. Schematic representation of the intestinal epithelium, where tight junction (TJ) proteins are indicated by coloured bars connecting adjacent intestinal epithelial cells (IECs). BEVs released by *E. coli* Nissle 1917 (EcN) and ECOR63 (left panel), or *A. muciniphila* (right panel) migrate through the inner mucus layer and reach the epithelium. Regulatory mechanisms influencing the integrity of the intestinal barrier known to be activated by BEVs are indicated below the drawing, specifying whether evidences were obtained from in vitro assays (culture of IECs) or in vivo models of increased intestinal permeability (experimental colitis and high fat diet (HFD)-induced diabetic model). Upregulation/downregulation of gene transcription is indicated by red arrows. For each experimental model, the beneficial effects of BEVs counteracting disease alterations are shown by blue arrows. Overall, the regulatory effects mediated by microbiota BEVs result in gut epithelial barrier reinforcement and the subsequent reduction of intestinal permeability

energy source by colonic epithelial cells and propionate can signal to the host through GPR41 and GPR43 receptors (Clausen & Mortensen, 1995; Le Poul et al., 2003). Interestingly, SCFAs are also important molecules involved in appetite control and food intake (Flint et al., 2012). In addition, *A. muciniphila* has other beneficial properties associated with gut barrier strengthening and immune regulation (Ottman et al., 2017). Consistently, pretreatment of mice with *A. muciniphila* prior to exposure to dextran sulphate sodium (DSS) resulted in a reduction of colitis severity (Bian et al., 2019; Gobert et al., 2016; Zhai et al., 2019). Studies based on the same experimental model of colitis revealed that BEVs released by *A. muciniphila* mediate the protective effects observed. Oral administration of isolated BEVs ameliorated colitis symptoms and disease scores by reducing infiltration of immune cells in the colonic tissue and production of pro-inflammatory cytokines (Kang et al., 2013).

As stated above, the increased gut permeability that follows epithelial barrier disruption allows the passage of endotoxins and luminal antigens into the intestinal lamina propria. This trafficking initiates a mucosal immune response that causes chronic low-grade inflammation, prompting metabolic disorders characterized by non-alcoholic fatty liver disease and insulin resistance that lead to type 2 diabetes and eventually obesity. Metagenomic analyses revealed diminished abundance of *A. muciniphila* in patients with obesity and type 2 diabetes, and their stool samples also contain fewer *A. muciniphila*-derived MVs than healthy controls (Chelakkot et al., 2018).

The role of *A. muciniphila* BEVs in the modulation of gut permeability was studied in a high-fat diet (HFD)-induced diabetic mice model compared with control mice fed a normal diet (Chelakkot et al., 2018). Oral administration of *A. muciniphila* BEVs improved all the alterations caused by HFD, affecting gut permeability and metabolic function (Figure 3). Specifically, BEVs restored the intestinal barrier, reduced the subsequent recruitment of immune cells, and upregulated the altered TJ-proteins occludin, ZO-1 and claudin-5. Transcriptional activation of occludin by *A. muciniphila* BEVs was verified by immunoblotting in Caco-2 cell monolayers challenged with LPS (Chelakkot et al., 2018). Regarding metabolic effects, the treated mice had decreased body weight and improved glucose tolerance. These metabolic outcomes were attributed to restored barrier integrity, since at six

hours post-administration, BEVs were found in colon, but not in liver, muscle, fat tissue, pancreas or spleen (Chelakkot et al., 2018). Other studies also evidenced the beneficial effects of *A. muciniphila*-derived BEVs when given to mice fed an HFD. They could reduce weight gain, adipose tissue inflammation, blood glucose and cholesterol levels (Ashrafian, Shahriary, et al., 2019). Moreover, animals that received *A. muciniphila* BEVs showed upregulated expression of ZO-1, occludin and claudin-1. Interestingly, *A. muciniphila* BEVs elicited stronger effects than live bacteria (Ashrafian, Shahriary, et al., 2019). Authors confirmed upregulation of ZO-1, ZO-2 and claudin-4 by *A. muciniphila* BEVs in the human epithelial colorectal adenocarcinoma cell line Caco-2 (Ashrafian, Behrouzi, et al., 2019). Overall, these studies point to the therapeutic potential of *A. muciniphila* BEVs against inflammatory diseases, acting as positive regulators of intestinal barrier integrity and thus reducing inflammation (Ashrafian, Behrouzi, et al., 2019).

### 4.3 | Modulation of gut immunity

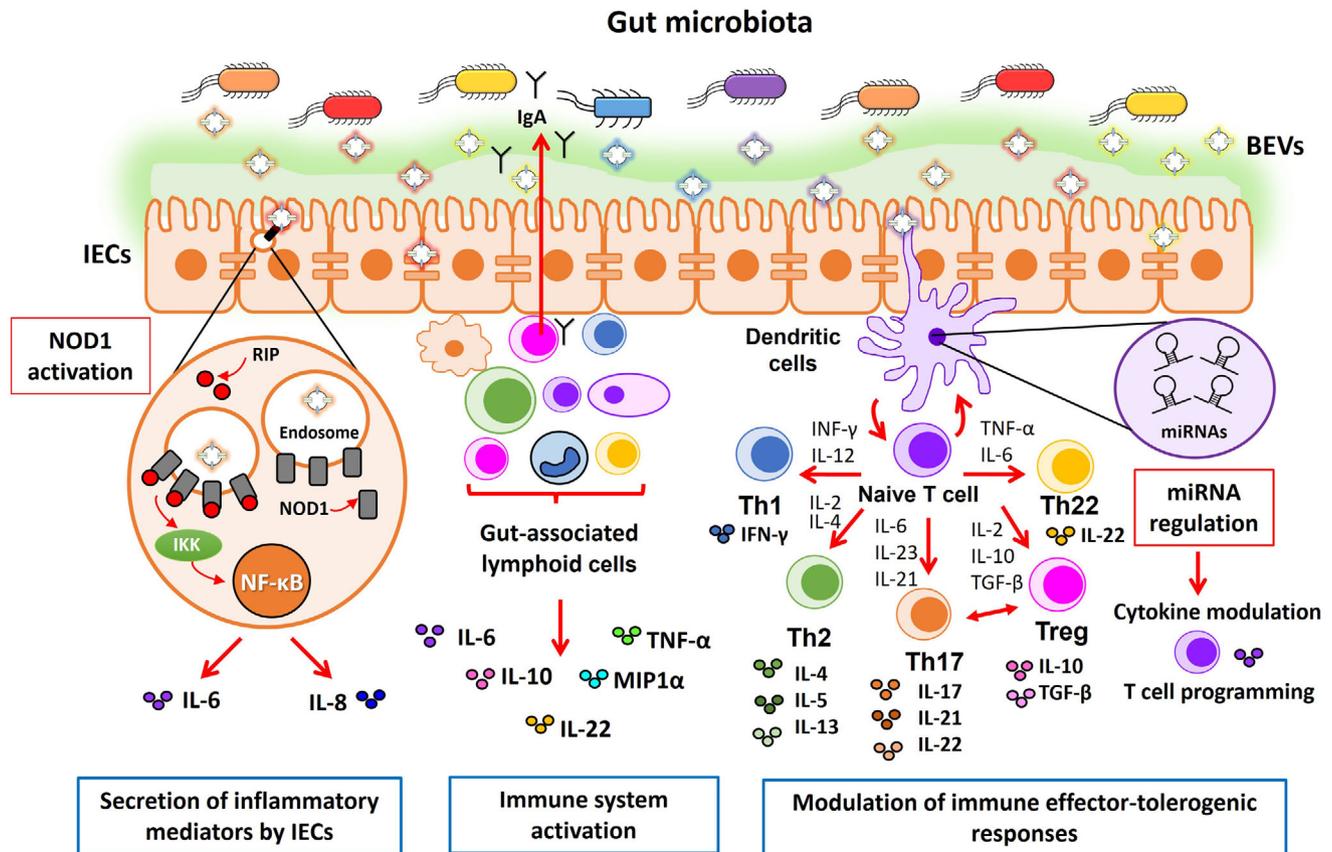
In this section, we discuss the role of commensal and probiotic-derived BEVs on the regulation of host immune responses. We consider mechanisms that involve indirect activation of immune cells through the intestinal epithelium and mechanisms that result from direct interaction of microbiota BEVs with immune cells. The main mechanisms described in this section are summarized in Figure 4.

#### 4.3.1 | Activation of immune responses by microbiota BEVs through the intestinal epithelium

Modulation of the immune system by commensal bacteria has been investigated in *in vitro* cellular models designed to mimic what occurs *in vivo* in the intestine. These models aimed to reproduce the crosstalk between the microbiota, the intestinal epithelium and the immune system, and consisted of co-cultures of epithelial and immune cells in Transwell devices. A monolayer of human IECs is grown in the upper chamber simulating the intestinal epithelial barrier, and immature dendritic cells (or other immune cells) are grown in the basolateral chamber mimicking the underlying immune system of the lamina propria (Bermudez-Brito et al., 2013). Several studies to evaluate the probiotic properties of *Lactobacillus* spp strains were performed in this model following apical stimulation with live bacteria (Bermudez-Brito et al., 2012, 2014).

Subsequently, similar models were used to study microbiota BEVs as modulators of immune responses. In this context, our group investigated the immunomodulatory role of BEVs from the probiotic EcN and commensal *E. coli* strains following apical stimulation of Caco-2/ peripheral blood mononuclear cells (PBMCs) co-cultures as a model to mimic intact intestinal mucosa. For comparison, direct stimulation of PBMCs was approached as a model of intestinal barrier disruption. Secretion of several cytokines and chemokines was analysed in the basolateral compartment, and their expression was assessed in Caco-2 cells and PBMCs. Whereas both BEVs and bacterial lysates activated the expression of IL-6, IL-8, TNF- $\alpha$ , IL-10, and MIP1 $\alpha$  in PBMCs treated following the barrier disruption model, only BEVs elicited immunomodulatory effects in the co-culture model (Fabrega et al., 2016). This proved that the epithelial barrier formed by Caco-2 cells prevented direct access of bacterial effector molecules to underlying PBMCs, and that PBMCs were activated by factors secreted by the epithelium in response to BEVs. This study showed the ability of microbiota-derived BEVs to trigger signalling events through the intestinal epithelial barrier (Fabrega et al., 2016). Upregulation of these cytokines by BEVs was verified in human colonic explants. Expression analysis of other immune mediators was also approached in this *ex vivo* model. As stated above (Section 4.2), BEVs from EcN and the commensal ECOR12 activated the expression of intestinal barrier effectors such as IL-22, and the antimicrobial peptide  $\beta$ -defensin-2 in this *ex vivo* model. In contrast, BEVs from these strains triggered downregulation of TGF- $\beta$  and the pro-inflammatory cytokine IL-12 (Fabrega et al., 2016). TGF- $\beta$  is a pleiotropic cytokine with relevant regulatory roles in inflammation. This cytokine triggers differentiation of Treg cells with anti-inflammatory properties. However, in the presence of IL-6, TGF- $\beta$  promotes differentiation of pro-inflammatory T helper 17 (Th17) cells. The balanced activity of Treg and Th17 cells in the intestinal mucosal surface is important to preserve intestinal homeostasis (Cheng et al., 2019). Imbalance in these phenotypes towards excessive production of Th17 cells can lead to development of inflammatory pathologies including IBD. In this context, reduction in TGF- $\beta$  levels caused by BEVs from these microbiota strains may help restore the Th17/Treg balance under inflammatory conditions. Th17 cells also influence production of mucin-1 by colonic cells. We showed that BEVs from EcN and ECOR12 triggered downregulation of this membrane-associated mucin in colonic explants. Mucin-1 and TGF- $\beta$  are overexpressed in several cancer types. Regulation of these markers by certain gut beneficial bacteria may reflect the influence of microbiota BEVs on cancer progression or treatment effectiveness (Gopalakrishnan et al., 2018). Overall, these findings support the role of microbiota-derived BEVs in the activation of immune and defence responses through the intestinal epithelial barrier. This proves that BEVs are an effective strategy used by gut bacteria to communicate with intestinal mucosa cells and regulate intestinal homeostasis.

In addition to *in vitro* models, the beneficial properties of the probiotic EcN have been shown in mouse models of colitis (Rodríguez-Nogales et al., 2018; Souza et al., 2016). Oral administration of EcN BEVs to DSS-treated mice ameliorated colitis symptoms, histological scores, intestinal permeability and inflammatory responses in the same way as the live probiotic (Fabrega



**FIGURE 4** Schematic picture summarizing the immunomodulatory effects elicited by microbiota-derived BEVs in the gut. The drawing shows the intestinal epithelium covered with the mucin layer that prevents access of luminal microbes while allowing passage of BEVs. Immune cells (lymphocytes, macrophages and dendritic cells) in the lamina propria are shown below the epithelial monolayer. Microbiota derived BEVs exert immune modulation by two main mechanisms. (i) Undirect activation of immune cells through the intestinal epithelium (left scheme). Internalized EVs by intestinal epithelial cells (IECs) activate the cytosolic receptor NOD1 that triggers secretion of immune effectors, which in turn stimulate gut-associated lymphoid cells to produce a wide range of cytokines. Activation of the NOD1 signalling pathway by microbiota BEVs is shown encircled in more detail. BEVs are internalized through clathrin-mediated endocytosis and recruit NOD1 (grey cylinders) to early endosomes. Activated NOD1 interacts with the specific kinase RIP2 (red circles), which leads to NF- $\kappa$ B activation and the subsequent upregulation of host genes involved in the inflammatory response (IL-6, IL-8). (ii) Direct activation of gut resident immune cells by microbiota BEVs that leads to secretion of immune mediators and secretory IgA (middle scheme). In addition to direct interaction with microbiota BEVs that reach the gut-associated lymphoid tissue via transcytosis across M cells, dendritic cells (DCs) also interact with luminal BEVs by extending pseudopodia across the epithelial cell layer (right scheme). Studies with several gut microbiota species revealed that BEVs activate DCs in a strain-specific manner. Differential regulation of miRNAs in DCs is one of the regulatory mechanisms involved in the specific immunomodulatory effects of BEVs isolated from probiotic and commensal *E. coli* (highlighted in the adjacent violet circle). In conclusion, DCs integrate incoming signals delivered by microbiota BEVs and set up specific programs that promote differentiation of naïve T cells into effector T cells (Th1, Th2, Th17, Th22) or regulatory T cells (Treg), thus allowing coordination of suitable T cell responses

et al., 2017). Besides counteracting the altered expression of tissue repair and wound healing markers (Section 4.2), EcN MVs mediated anti-inflammatory effects. Treatment with BEVs reduced the increased expression of pro-inflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-12, IL-17, and INF- $\gamma$  and increased expression of the anti-inflammatory cytokine IL-10. The beneficial effects were also associated with downregulation of well-known enzymes involved in injury and inflammation, such as cyclooxygenase-2 and inducible nitric oxide synthase. These outcomes correlated with lesser infiltration of inflammatory cells in EV-treated mice (Fabrega et al., 2017). Like *A. muciniphila* BEVs, vesicles from the probiotic EcN could be a safe probiotic-derived strategy free of bacteria to preserve gastrointestinal health by targeting intestinal inflammatory processes.

There is evidence that BEVs from Gram-positive bacteria elicit immunomodulatory effects in IECs. Kefir-derived strains *Lactobacillus kefir*, *Lactobacillus kefirifaciens*, and *Lactobacillus kefirgranum* alleviate the production of inflammatory cytokines under in vitro and in vivo inflammatory conditions (Seo et al., 2018). Treatment of TNF- $\alpha$  stimulated Caco-2 cells with BEVs isolated from these strains significantly diminished production of the proinflammatory cytokine IL-8 at mRNA and protein-secreted levels. The mechanism involved inhibition of TNF- $\alpha$  signalling by reducing phosphorylation of the NF- $\kappa$ B p65 subunit (Seo et al., 2018). In the mouse model of experimental colitis, administration of kefir *Lactobacillus*-derived BEVs alleviated disease symptoms and tissue damage scores. These vesicles also reduced the oxidative stress associated with inflammatory diseases, since serum levels of myeloperoxidase were significantly reduced in mice treated with BEVs (Seo et al., 2018).

### Activation of NOD signalling pathways in IECs

There is evidence on the involvement of NOD receptors in the molecular mechanisms and signalling pathways activated by microbiota BEVs in IECs that initiate the innate immune response. These cytosolic receptors are crucial for maintaining intestinal homeostasis. They play relevant roles in host defence responses against bacterial pathogens and regulation of the inflammatory response to microbiota (Caruso et al., 2014; Kaparakis-Liaskos & Ferrero, 2015). NOD1 detects D-glutamyl-meso-diaminopimelic acid that is mainly present in the peptidoglycan of Gram-negative bacteria (Chamaillard et al., 2003; Girardin et al., 2003). NOD2 detects muramyl dipeptide that is ubiquitously present in all bacterial peptidoglycan molecules (Girardin et al., 2003). Considering that gut microbiota is mainly composed of non-invasive bacteria, several pathways have been proposed to mediate intracellular delivery of peptidoglycan into IECs. Peptidoglycan fragments released into the gut lumen during bacterial division can be internalized in IECs through endocytosis or by specific membrane-associated transporters (Philpott et al., 2014; Swaan et al., 2008). The other pathway, mostly studied in Gram-negative pathogens, involves peptidoglycan delivery through BEVs (Section 3.2).

The activation of NOD receptors by BEVs from gut microbiota has been studied in probiotic and commensal *E. coli* strains (Cañas et al., 2018). This study showed that BEVs isolated from the probiotic EcN and the commensal ECOR12 activate NOD1 signalling pathways in IECs. Several experimental approaches were used to confirm involvement of this cytosolic receptor in the cell response to internalized BEVs. These included NOD1 silencing with specific siRNA and the use of inhibitors of the specific kinase of the NOD1 pathway. Both strategies significantly abolished vesicle-mediated activation of NF- $\kappa$ B and consequently reduced IL-6 and IL-8 expression. Furthermore, BEVs from both microbiota strains colocalized with NOD1 in early endosomes. Interaction with NOD1 led to the formation of NOD aggregates in the cell cytoplasm and the subsequent recruitment of RIP2 kinase that initiates the phosphorylation cascade, resulting in NF- $\kappa$ B activation. This transcription factor activates genes involved in the inflammatory response that is transmitted to gut resident immune cells through the release of chemokines and other immune effectors (Cañas et al., 2018). This study proved that EVs from beneficial gut bacteria serve as a mechanism for peptidoglycan delivery into the host cytosol. In IECs, continuous stimulation of NOD receptors by microbiota BEVs steadily primes the innate immune system, leading to controlled inflammatory responses that assist pathogen eradication and intestinal homeostasis.

### 4.3.2 | Direct activation of immune cells by microbiota BEVs

To maintain intestinal balance, the immune system must respond to external antigens and trigger immune responses to protect the host against pathogens or injuries. The gut microbiota and the host mucosal immune system interact strongly, with important implications for human health. As stated above, the intestinal epithelium acts as an indirect transmitter of microbiota signals to immune cells. However, specialized cells of the gut-associated lymphoid tissue such as dendritic cells (DCs) can directly interact with gut microbes and direct appropriate immune responses. DCs detect bacteria or derived BEVs that reach the immune cells underneath the intestinal epithelium by transcytosis across M cells. However, DCs can also sample gut luminal content by extending pseudopodia across the epithelial cell layer (Rescigno et al., 2001). On recognition of MAMPs by PRRs, DCs activate the expression of cytokines and specific surface molecules that promote differentiation of T cells to specific functional subsets (Hooper et al., 2012). Therefore, DCs play a relevant role in connecting innate and adaptive responses, ensuring intestinal homeostasis by orchestrating the host immune responses to gut microbes.

*Bacteroides sp.* As mentioned in Section 4.1, *B. fragilis* is a gut resident bacterium with well-known beneficial properties in food metabolism and gut ecology. The immunomodulatory effects of this commensal have been proved in experimental models of colitis. Administration of *B. fragilis* protected against colitis development by suppressing activity of inflammatory molecules and inducing production of anti-inflammatory cytokines (Chang et al., 2017; Chiu et al., 2014; Mazmanian et al., 2008; Round & Mazmanian, 2010).

The first study proving the beneficial effects of BEVs from a commensal bacterium was focused on *B. fragilis* (Shen et al., 2012). Authors showed that BEVs from *B. fragilis* contain capsular polysaccharide A (PSA), an immunomodulatory molecule that induces regulatory T cells (Treg) and mucosal tolerance (Mazmanian et al., 2005). In a mouse colitis model, animals treated with BEVs from wild type *B. fragilis* showed improved disease scores and symptoms compared to animals treated with BEVs derived from a PSA-deficient mutant (Shen et al., 2012). Regarding molecular mechanisms, there was a strong association between the PSA content in BEVs and the activation of Treg cells. This was confirmed in an in vitro model of bone marrow-derived DCs pulsed with BEVs from *B. fragilis* and then co-cultured with naïve T cells. Increased numbers of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> T cells and IL-10 production were induced by DCs stimulated with wild-type BEVs but not by DCs treated with BEVs from the  $\Delta$ PSA mutant. These immunomodulatory effects were mediated by activation of the TLR2 pathway by PSA in DCs. A microarray analysis comparing mRNA levels induced by wild-type BEVs or  $\Delta$ PSA BEVs in DCs allowed identification of genes regulated by PSA in a TLR2-dependent manner (Shen et al., 2012). Another study showed that immune receptors other than TLR2 contributed to the signalling events activated by *B. fragilis* BEVs in DCs. This study focused on risk genes proposed for Crohn's disease (CD)

development, particularly ATG16L1 and the immune receptor NOD2. These proteins are involved in autophagy regulation and there is evidence that immune cells impaired in autophagy elicit exacerbated inflammatory responses (Chu et al., 2016). Results from co-cultures of CD4<sup>+</sup> T cells with bone marrow-derived DCs differentiated from wild type, ATG16L1-deficient or NOD2-/- knockout mice stimulated with *B. fragilis* BEVs revealed that the immunomodulatory effects of BEVs depend on functional ATG16L1 and NOD2 to induce a non-canonical autophagy pathway that is essential for colitis protection. In fact, ATG16L1-deficient DCs did not induce Tregs in response to BEVs from this commensal. Further analysis revealed that *B. fragilis* BEVs use the noncanonical autophagy pathway LAP (LC3-associated phagocytosis) to mediate tolerogenic responses (Chu et al., 2016). Likewise, NOD2 knockout bone marrow-derived DCs failed to induce Treg responses with reduced IL-10 production, which in turn compromises suppression of mucosal inflammation. This study also showed that immune cells from human subjects with the T300A variant of ATG16L1 linked with a high risk of developing CD are defective in Treg responses to *B. fragilis* BEVs (Chu et al., 2016).

The immunomodulatory properties of *B. thetaiotaomicron* have been proved in preclinical models of CD treated with live bacterial suspensions (Delday et al., 2019). The role of BEVs in mediating immunomodulatory effects has been recently evidenced in a study performed in DCs derived from healthy individuals and from patients with CD or ulcerative colitis (UC) stimulated with *B. thetaiotaomicron* BEVs (Durant et al., 2020). BEVs from this commensal induced IL-10 expression from healthy colonic DCs, which is an indicator of health state. The most abundant population were myeloid CD11<sup>+</sup> DCs. In DCs derived from PBMCs isolated from healthy donors, BEVs also activated expression of the co-stimulatory marker CD80 and IL-6. In contrast, *B. thetaiotaomicron* BEVs failed to induce IL-10 in colonic DCs from IBD patients. This correlated with reduced numbers of CD103<sup>+</sup> DCs, a population involved in T regulatory responses and immune balance (Durant et al., 2020). The activity of disease also influences the regulatory responses in IBD. In this context, this study showed that blood-derived DCs from patients with inactive and active UC showed variations in IL-10 levels. DCs from patients with inactive disease had decreased levels of IL-10. Conversely, DCs from patients with active disease showed IL-10 levels comparable to those of healthy controls. This suggests that intrinsic alterations in the immune responses to this commensal are important in IBD (Durant et al., 2020). This study indicates that BEVs from *B. thetaiotaomicron* could drive a balanced immune response to microbiota in healthy individuals. However, this response is altered in IBD states, which contributes to an inflammatory environment in the intestine.

*B. vulgatus* mpk is another gut symbiont of the Bacteroides family with relevant features that confer immunomodulatory properties. In fact, this commensal ameliorates colitis development in several IBD mouse models (Müller et al., 2008; Perez-Lopez et al., 2016). The mechanism by which *B. vulgatus* mpk elicits beneficial immunomodulatory effects is not well-established but a recent study has shown that secreted BEVs induce a tolerant semi-mature phenotype in bone marrow-derived DCs (Maerz et al., 2018). Notably, *B. vulgatus* BEVs elicited the same immunomodulatory response as the live commensal on CD11c<sup>+</sup> DCs, since no differences in the expression levels of MHC-II, CD40, CD80 and CD86 markers were observed between DCs stimulated with either *B. vulgatus* mpk or isolated BEVs. To decipher the mechanisms involved in the actions mediated by BEVs, DCs derived from mice deficient in toll-like receptor 2 (*Tlr2*<sup>-/-</sup>), TLR-4 (*Tlr4*<sup>-/-</sup>) or both receptors (*Tlr2*<sup>-/-</sup> and *Tlr4*<sup>-/-</sup>) were used as a model. The absence of both receptors impaired vesicle antigen recognition and the subsequent conversion of immature DCs into a semi-mature state, which correlated with reduced expression of MHC-II and TNF- $\alpha$ . The authors conclude that the presence of TLR-2 and TLR-4-ligands in *B. vulgatus* mpk BEVs could facilitate induction of tolerance in DCs by desensitizing the host to subsequent stimulation with endotoxin. However, the nature of the TLR2 ligand included in BEVs from this commensal is still unknown (Maerz et al., 2018).

*Escherichia coli*. As mentioned, the probiotic EcN has many beneficial effects that contribute to intestinal homeostasis and microbiota balance. Many studies have been conducted to elucidate the mechanisms and molecular factors that mediate the immunomodulatory and barrier strengthening properties of this probiotic. In the preceding sections, we reviewed the mechanisms activated by EcN BEVs that confer protection against IBD and highlighted their role as regulators of intestinal barrier integrity and immune responses in IECs. Here, we describe current knowledge on the immunomodulatory effects elicited by EcN and other gut resident *E. coli* on DCs and derived immune responses.

Our group showed that BEVs from EcN and commensal *E. coli* strains activate DCs to promote differentiation of T cell subsets in a strain-specific manner. Immature DCs were derived from CD14<sup>+</sup> monocytes isolated from buffy coats collected from healthy donors and stimulated with BEVs from EcN and ECOR strains of intestinal origin (Diaz-Garrido et al., 2019). Maturation of DCs was assessed by increased expression of CD83 and reduced expression of CD209 (DC-SIGN) markers compared to untreated DCs. For all strains, BEVs significantly increased secretion of the cytokines that drive the Th responses: INF- $\gamma$ , IL-12, IL-18 (Th1), IL-6, IL-23, TNF- $\alpha$ , and IL-1 $\beta$  (Th17/Th22) and IL-10 and TGF- $\beta$  (Treg). Vesicles from EcN and ECOR63 (both belonging to the phylogenetic group B2) elicited greater secretion of Th1 polarizing cytokines than BEVs from ECOR12 (phylogenetic group A) or the cytotoxic ECOR53 strain. Conversely, ECOR12 BEVs induced high levels of Treg-related cytokines (Diaz-Garrido et al., 2019). The ability of activated-DCs to differentially modulate activation of CD4<sup>+</sup> T cells towards specific effector subsets was evaluated in the in vitro co-culture DCs/CD4<sup>+</sup> T cell model. Cytokine quantification and cytometry analyses of specific T cell markers evidenced that BEVs from all strains activated the Th2 response with release of IL-4, IL-5, and IL-13. BEVs from ECOR12 elicited a strong Treg response with higher levels of TGF- $\beta$  and lower expression of proinflammatory cytokines than BEVs from the other strains. Accordingly, the highest number of CD4<sup>+</sup> CD25<sup>high</sup><sup>+</sup> FOXP3<sup>+</sup> T cells were obtained in the presence of DCs

stimulated with ECOR12 BEVs, and in a smaller proportion with EcN BEVs (Díaz-Garrido et al., 2019). This study indicated that in the gut, BEVs released by microbiota strains steadily prime the innate immune system through specific activation of DCs, which in turn orchestrate balanced immune tolerance and immune inflammatory responses that are essential to preserve intestinal homeostasis. Consistent with the beneficial effects of EcN, BEVs from this probiotic drive complex Th responses: Th2 (immunity to extracellular parasites and allergic inflammatory responses), Th22 (tissue protection), Th1 (pro-inflammatory, protective immunity against pathogens), Th17 (pro-inflammatory, antimicrobial response) and Treg (immune tolerance). Other commensal *B. coli* strains trigger similar Th responses but with a stronger pro-inflammatory profile. In contrast, ECOR12 BEVs mainly promote host tolerance against gut microbiota by increasing the Treg/Th17 balance and they protect gut integrity by activating the Th22 response. However, they fail to trigger proper Th1 responses against pathogens (Díaz-Garrido et al., 2019).

The regulatory mechanisms exploited by microbiota-derived BEVs to specifically activate DCs to prime appropriate T cell responses are complex and not fully elucidated. Several molecules linked to the signalling pathways activated by BEVs are involved in modulating cytokine and co-stimulatory molecules that lead to DCs with different activation profiles. In this context, microRNAs (miRNAs) have a relevant role as post-transcriptional regulators of the immune system that allow fine-tuning of signalling pathways. We have recently reported that differential immunomodulatory effects mediated by BEVs from probiotic and commensal *E. coli* in DCs are in part mediated through regulation of miRNAs (Díaz-Garrido et al., 2020). In this study, deep RNA sequencing was approached to identify miRNAs that are differentially expressed in DCs challenged with BEVs from EcN or ECOR12. This study revealed a common set of miRNAs modulated by BEVs from both strains. In addition, BEVs elicited differential expression of specific miRNAs depending on the producer strain (21 downregulated and 21 upregulated by EcN BEVs; 45 downregulated and 41 upregulated by ECOR12 BEVs). The differential expression of the miRNAs and their targets was consistent with the effects triggered by these BEVs on DCs. These effects are compatible with the ability of ECOR12 BEVs to drive DCs that orchestrate T regulatory responses that are critical for immune tolerance in the gut, and why EcN BEVs programme DCs to mount the Th1 responses required for pathogen eradication (Díaz-Garrido et al., 2020). Hence, differential regulation of miRNAs by microbiota BEVs exerts immune training to preserve intestinal homeostasis.

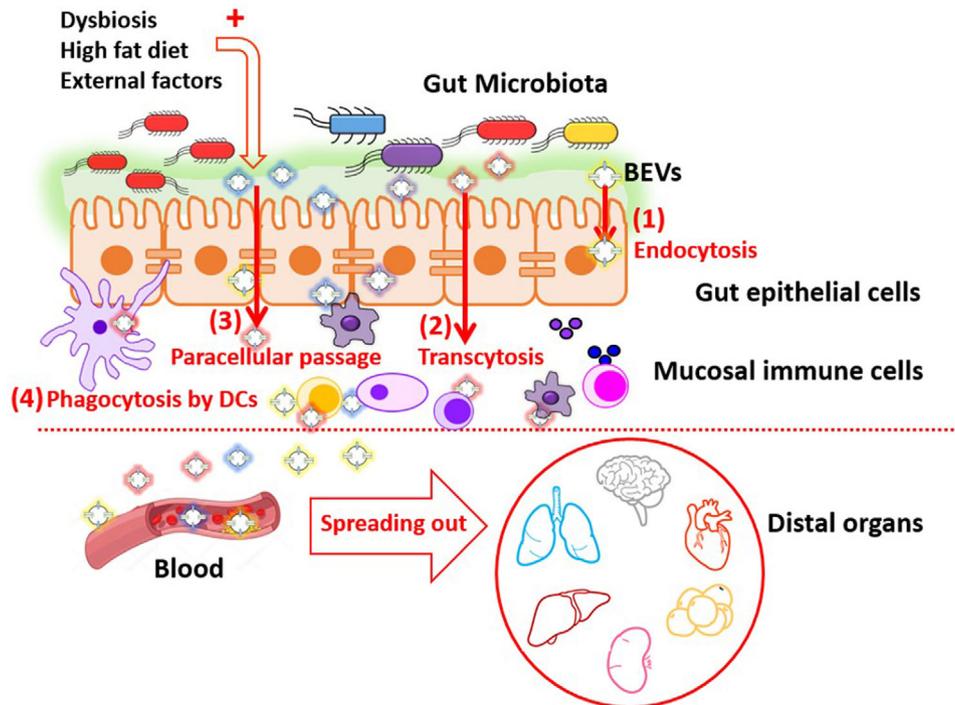
**Gram-positive beneficial bacteria: *Lactobacillus* sp and *Bifidobacterium* sp.** Most microbes documented to date as probiotics are Gram-positive bacteria. Among them, the genera *Lactobacillus* and *Bifidobacterium* are the most widely used and studied due to their health-promoting and immunomodulatory effects (Grimm et al., 2014; Wells, 2011). The effects of live probiotic suspensions on the modulation of immune responses have been extensively studied. However, few studies have been focused on their BEVs.

The immunomodulatory effects of BEVs from *Lactobacillus rhamnosus* JB-1 have been studied in mice models. Besides immunomodulatory effects, this probiotic exerts functional effects on peristalsis by activating the host enteric nervous system. Oral administered BEVs from *L. rhamnosus* JB-1 were shown to cross the epithelial layer and reach DCs located at Peyer's patches at early times post-treatment. After three days, the phenotype of DCs changed to a regulatory profile with increased levels of IL-10 production and polarization towards functional regulatory T cells CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> (Al-Nedawi et al., 2015). This study evidenced that *L. rhamnosus* JB-1-derived BEVs reproduced the same immunomodulatory and neuron-stimulating effects as the live probiotic. Moreover, ex vivo experiments revealed that modulation of the neuronal response depended on signals released by the gut epithelium in response to BEVs. These results confirm the key role of IECs as mediators of indirect communication between microbiota and cells of the intestinal submucosa (Al-Nedawi et al., 2015).

Secretion of IgA by gut mucosal tissue is known to play an important role in modulating microbiota composition and preventing invasion of enteric pathogens. Production of IgA by gut immune cells is activated by probiotic *Lactobacillus* strains such as *L. sakei* subsp. *sakei* NBRC15893. Experiments performed in cells isolated from murine Peyer's patches proved that the probiotic factor that mediates this effect was associated with purified BEVs (Yamasaki-Yashiki et al., 2019). Thus, by enhancing IgA expression, BEVs also serve as a mechanism for gut beneficial bacteria to reinforce the intestinal barrier and modulate gut microbiome composition.

There is evidence that in addition to protective properties against colitis, probiotics of the genus *Bifidobacterium* can alleviate allergic responses. Activation of DCs by strains of the genus *Bifidobacterium* and the subsequent polarization of naïve CD4<sup>+</sup> T cells into effector responses differs between strains. Specifically, the probiotic *Bifidobacterium bifidum* LMG13195 triggers a tolerogenic response (López et al., 2011). Analysis of several probiotic subcellular fractions on DCs proved that the effects were specifically mediated by BEVs. Similar to live *Bifidobacterium bifidum* LMG13195, BEVs activated DCs to promote differentiation of Treg cells (FOXP3<sup>+</sup>) and induce higher levels of IL-10 than Th1/Th17 proinflammatory cytokines. Based on the suppressor effects, the authors suggested a potential application of BEVs from this probiotic as adjuvants for allergen-specific immunotherapy (Lopez et al., 2012).

Food allergy results from an abnormal immune response to food components, and some probiotics can alleviate symptoms and associated inflammation. In this context, the healing potential of *Bifidobacterium longum* was assessed in a mouse model of allergen-induced food allergy with acute diarrhoea (Kim et al., 2016). This probiotic ameliorated inflammation by decreasing the number of mast cells in the small intestine, without counteracting the Th2 response. Authors showed that this activity was mediated by *B. longum*-derived BEVs, and specifically by the vesicular protein ESBP (family 5 extracellular solute-binding protein), which triggered apoptosis of bone marrow-derived mast cells (Kim et al., 2016).



**FIGURE 5** Mechanisms that mediate translocation of microbiota-derived BEVs across the intestinal epithelium. The processes are numbered and indicated in red. BEVs released by gut microbes are taken up by intestinal epithelial cells (IECs) via endocytosis (1). Internalized BEVs can reach the basolateral membrane of IECs and be translocated into the lamina propria by transcytosis (2). In addition, the paracellular route (3) also allows passage of luminal BEVs to the underlying submucosa. This route is favoured under conditions of gut dysbiosis. Once in the lamina propria, translocated BEVs directly interact with resident gut immune cells, triggering suitable immune responses. Direct sampling and phagocytosis of luminal BEVs by DCs also mediate passage of luminal BEVs into the internal milieu (4). There is evidence that BEVs cross endothelial barriers and reach blood vessels, thus being distributed to distal organs

## 5 | TRANSMIGRATION OF BEVS THROUGH THE GUT EPITHELIUM

There is considerable evidence that gut microbiota-derived compounds are found in the bloodstream and can be distributed through the body, reaching distal organs and tissues (Arentsen et al., 2017; Cani et al., 2007; Zhan et al., 2016). BEVs are one of the factors released by resident gut microbiota. These vesicles can cross the epithelial barrier to gain access to the underlying submucosa and interact with immune cells such as DCs and macrophages (Kaparakis-Liaskos & Ferrero, 2015). Besides, microbiota BEVs can be disseminated through the body by systemic or lymphatic circulation, reaching extraintestinal tissues (Stentz et al., 2018). The question of how these gut microbiota-derived BEVs can reach distant organs remains poorly explored. However, this is now an emerging research area in the field of the gut microbiome and its impact on health and disease.

The main theory about how BEVs reach systemic circulation is based on the leaky gut condition. Many factors that disturb the intestinal environment trigger gut microbiota imbalance. This condition, known as gut dysbiosis, leads to epithelial barrier disruption and a subsequent increase in paracellular permeability that allows translocation of BEVs into the bloodstream (Figure 5) (Chakaroun et al., 2020). Tulkens et al. investigated the presence of BEVs from gut microbes in the systemic circulation of patients with intestinal barrier dysfunction (Tulkens, Vergauwen, et al., 2020). They analysed plasma samples from healthy donors without barrier dysfunction and patients with well-defined intestinal barrier dysfunction (diagnosed with IBD, HIV and intestinal mucositis). After plasma fractionation, functional assays were performed to quantify BEV-associated LPS. The results showed increased levels of LPS-positive BEVs in plasma from patients suffering IBD, HIV and intestinal mucositis. Patients also presented higher plasma levels of zonulin, a marker of impaired barrier integrity. This protein causes TJ disassembly by phosphorylating ZO proteins (Tulkens, Vergauwen, et al., 2020). In this study, the paracellular translocation of BEVs was also confirmed in the *in vitro* model of Caco-2 cells challenged with DSS, which mimics barrier disruption. The same group developed an exhaustive protocol to separate BEVs from eukaryotic-derived EVs in stool and blood samples consisting of orthogonal separation that includes size-exclusion chromatography and density gradient-centrifugation. Specific features of BEVs, such as their ultrastructure, proteomic content, and presence of MAMPs were analysed in the isolated samples. This purification procedure preserves the integrity of BEVs and is suitable for further proteomic and biochemical characterization. However, specific settings should be considered to standardize protocols for isolation and handling according to each body fluid. Therefore, its application would be of great help to study BEVs in body fluids and their use as diagnostic tools (Tulkens, De Wever, et al., 2020). The existence of circulating BEVs can explain the presence of bacterial genetic material in body fluids. Several studies have reported the presence

of genomic DNA in blood samples from healthy donors (Païssé et al., 2016; Whittle et al., 2019). It is now accepted that blood is not a sterile fluid, and there is a human blood microbiome that is altered under pathological conditions (Castillo et al., 2019). In fact, analysis of the blood microbiome has been suggested as a potential method for cancer diagnosis (Poore et al., 2020). Nevertheless, when sequence-based techniques are used to study the microbiome in samples with low biomass, it is essential to avoid contamination during sample processing (Salter et al., 2014).

The idea that translocation of BEVs occurs only and exclusively when barrier disruption exists has been rejected. Recent evidence indicates that BEVs from the commensal bacterium *B. thetaiotaomicron* migrate through the intestinal epithelium via a paracellular route and enter the circulatory or lymphatic system in healthy conditions (Jones et al., 2020). Following oral administration of *B. thetaiotaomicron* EVs, in vivo imaging proved that small amounts of BEVs reach distal organs such as the liver, where vesicle passage could be through the portal vein. BEVs were also found in lungs and heart, which suggests that they can cross several cellular barriers, enter the bloodstream, and spread through the body (Jones et al., 2020). The way BEVs cross healthy epithelial barriers to reach the bloodstream remains unknown. However, the most plausible route involves active transcellular transport mediated by either active transporters or endocytosis-transcytosis-exocytosis processes (Figure 5). In fact, there is evidence that eukaryotic EVs called exosomes can enter the bloodstream and migrate through the blood-brain barrier by transcellular transport (Chen et al., 2016). Likewise, tumour-derived exosomes can cross the blood-brain barrier during the course of brain metastasis via transcytosis (Morad et al., 2019). Thus, it can be speculated that a similar process occurs with BEVs. In addition, other mechanisms that involve phagocytic cells of the intestinal mucosa facilitate the entry of gut BEVs into the circulation. Specifically, intestinal DCs are specialized in sampling and transporting luminal antigens and BEVs across the gut epithelium (Figure 5). The phagocytic epithelial M cells also contribute to this function. Once activated, DCs from the mucosal surfaces migrate to lymphoid organs to induce coordinated effector T cell responses. From these sites, DCs can spread through distal locations.

There is now a large body of evidence that BEVs cross cellular barriers and reach every tissue in the body (Figure 5). As an example, BEVs from gut-associated bacteria have been identified in the human milk of healthy lactating mothers (Kim & Yi, 2020). Microbiota BEVs can also reach the brain. This finding could explain the presence of bacterial DNA in the brain, despite the blood-brain barrier's strict permeability (Zhan et al., 2016). Recent studies focused on the microbiota-brain axis indicate that BEVs are involved in several brain disorders such as depression, anxiety, stress, autism and Alzheimer's (Haas-Neill & Forsythe, 2020). In an Alzheimer's disease mouse model, 16S rRNA metagenomic analyses of BEVs in blood allowed identification of more than 3000 taxonomic units that correlated with the gut microbiota profile. Interestingly, important alterations in certain taxonomic phyla were found in mutant mice with an Alzheimer disease phenotype compared with control wild type mice (Park et al., 2017). Based on significant statistical variations between experimental groups, the authors suggested that metagenomic analyses of blood BEVs could be used to identify gut microbiota imbalance associated with specific diseases (Park et al., 2017). Similarly, in humans with autism disorder, great differences in the genomic profile of BEVs isolated from urine samples have been found compared to healthy controls. These differences also reflected the changes in gut microbiome previously described in autism, thus supporting the potential use of BEVs in body fluids for diagnostic purposes (Lee & Kim, 2017).

## 6 | CONCLUDING REMARKS

The relevance of microbiota research is emphasised by the large number of publications, specific meetings and forums dedicated to this topic. Despite intense research in this area, there is still much to learn about the intrinsic mechanisms of microbiota action, especially those concerning the delivery of effectors to target cells. This knowledge is essential to understanding disease and developing translation strategies of microbiota or microbiota-derived products to human health. In this context, the study of microbiota BEVs is currently receiving growing interest. Evidence from preclinical assays in animal models point to potential applications of microbiota BEVs as therapeutic/nutritional strategies to prevent intestinal infections or inflammatory and immune-related disorders. Administration of isolated BEVs would overcome the potential risk of the use of live probiotics in immunocompromised individuals.

Imbalances in gut microbiota composition and diversity have been associated with a great variety of diseases including cancer, neurological, metabolic and immune disorders. In this context, gut microbiome profiling is envisaged as a potentially useful tool for diagnosis. The discovery of gut microbiota-derived BEVs in blood and urine samples of patients and healthy individuals with a profile that highly correlates with the gut microbiome opens novel strategies for diagnostic purposes.

The study of microbiota-derived EVs is quickly evolving. In the near future, it is expected that research efforts in this area will contribute to understanding the complex microbiota-host communication network, which is essential to preserve human health, and provide solid basis for propelling new therapeutic strategies based on EVs from gut beneficial bacteria as postbiotics.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

Conceptualization, J.B and L.B; investigation, N.D.G, writing—original draft preparation, N.D.G.; writing—review and editing, L.B and J.B; supervision L.B; project administration, J.B; funding acquisition, L.B and J.B.

## ORCID

Natalia Díaz-Garrido  <https://orcid.org/0000-0003-1220-015X>

Josefa Badia  <https://orcid.org/0000-0003-0768-5033>

Laura Baldomà  <https://orcid.org/0000-0002-7678-269X>

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