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REVIEW

# Bacterial extracellular vesicles for gut microbiome−host communication and drug development

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**Abstract** As the intricate interplay between microbiota and the host garners increasing research attention, a significant parallel surge has emerged in the investigation of intestinal bacterial extracellular vesicles (BEVs). Most intestinal bacteria secrete BEVs, which harbor specific cargo molecules and exhibit diverse functions, encompassing interactions among bacteria themselves and between bacteria and the host. These interactions can either bolster host health or contribute to various pathologies. By integrating the characteristics of BEVs, we summarized the current research landscape, delving into the intricate interplay between BEVs and different diseases. Furthermore, we offer a succinct overview of the challenges faced in BEVs-based research, encompassing separation, detection, engineering for drug purposes, clinical diagnostics, safety, and future study. In essence, these summaries may serve as invaluable guides for BEVs as communication tools between the gut microbiome and host, ultimately propelling the discovery of novel studies and drug discovery.

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## 1. General introduction

As the research on the implications of microbiota−host interplay progressively intensifies, there has been a concomitant surge in the study of intestinal bacterial extracellular vesicles (BEVs). Serving as crucial endogenous nanoparticles, BEVs exhibit bilayer lipid vesicle structures, ranging in diameter from 20 to 400 nm. Subject to the type of parental bacteria, intestinal BEVs harbor a diverse array of bioactive components, encompassing proteins (a small amount of periplasmic protein and cytoplasmic protein), nucleic acids (RNA and DNA), lipopolysaccharides, peptidoglycans, and various metabolites[1]. In comparison to traditional bacterial secretory secondary metabolites (hydrophilic small molecules), BEVs possess numerous unique characteristics. Firstly, bacterial vesicles are particularly valuable for the export of lipids, hydrophobic molecules, and insoluble substances. Secondly, the concentration of effector molecules does not diminish with increasing distance from the initial secretion site. Additionally, BEVs enable the targeted delivery of multifunctional molecules to specific cells, potentially leading to synergistic effects[2]. Traditionally, BEVs were regarded as carrying virulence factors and metabolites that modulate the host immune system[1,3]. Nowadays, investigating their pivotal role in pathogenicity and treatment strategies constitutes a crucial research direction. Furthermore, the BEVs were regarded as a promising tool in the field of drug delivery systems and vaccines[4-6]. Due to its important biological function and medical value, scientists have gradually discovered that intestinal BEVs or engineering BEVs participate in various physiological and pathological processes of the host. However, this is still an emerging field, and there are still significant challenges that impede further study. There is an urgent need to better understand the effect of BEVs and be acquainted with the wealth of new studies in this field. In this review, in addition to briefly introducing the basic background of BEVs, we will describe in detail the relationship between BEVs and different tissues and diseases and also show the important future application directions of BEVs.

## 2. Origin and (sub)types

Currently, there is a dearth of targeted research investigating the origins of BEVs in the human body. Given that the gut harbors the majority of the organism's microbiota, it is plausible to postulate that the digestive tract serves as the primary source, albeit not exclusive, as the skin, mucosa, respiratory system, and urogenital system, which are in contact with the external environment, are also potential contributors[7]. Furthermore, in instances where the intestinal barrier is compromised by medications or illnesses, the influx of intestinal BEVs into the body may be exacerbated[8]. Based on their origin and synthesis pathway, BEVs can be categorized into various types, including outer membrane vesicles (OMVs), outer inner membrane vesicles (OIMVs), cytoplasmic membrane vesicles (CMVs), explosive outer membrane vesicles (EOMVs), and explosive outer inner membrane vesicles (EOIMVs)[2].

At least two models have been devised to illustrate the genesis of BEVs from bacteria. These models include outer membrane blisters, stemming from the dysregulation of the envelope due to imbalances in the biosynthesis of cell wall components or the insertion of hydrophobic molecules for vesicular secretion. The second model is explosive bacterial lysis, wherein lysed bacterial cell fragments coalesce and fuse to form vesicular structures[9]. It is postulated that outer membrane blisters can be triggered by changes in periplasmic pressure, anion charge repulsion among lipopolysaccharide (LPS) molecules, and the loss of peptidoglycan (PGN) and outer membrane connectivity[10]. The inner membrane of OMVs formed *via* these mechanisms remains intact, preventing direct access to cytoplasmic components. Conversely, the explosive bacterial lysis occurs through the degradation of PGN by phage-derived endolysins. Once PGN is degraded, the cells congregate and rupture, reorganizing into OIMVs and EOMVs[11,12]. Here, we delve into the types of OMVs secreted by gram-negative bacteria and CMVs excreted by Gram-positive bacteria.

### 2.1. OMVs

Initially documented in the 1960s[1,9], OMVs are currently the most extensively researched BEVs. Derived from the outer membrane (OM) of gram-negative bacteria, OMVs harbor periplasmic and cytoplasmic components, ranging in diameter from approximately 20 to 250 nm. Possessing internal phospholipid leaflets and external LPS leaflets, these vesicles activate immune cells *via* Toll-like receptors (TLRs) 2, 4, and 5. The production of membrane vesicles by gram-negative bacteria occurs through the blebbing of the OM or explosive cell lysis. The formation of OMVs is often attributed to disruptions in the cell membrane, such as imbalances in PGN accumulation, denatured protein biosynthesis, or the embedding of hydrophobic molecules within the OM. Consequently, OMVs typically lack cytoplasmic components but are rich in periplasmic proteins and OM components. Conversely, OIMVs contain cytosolic contents[2].

### 2.2. CMVs

In 2007, BEVs isolated from *Mycobacteria*, initially establishing that gram-positive bacteria are capable of secreting BEVs, subsequently referred to as CMVs. Derived from the cytoplasmic membrane, CMVs encompass substances from the cytosol and possess a diameter ranging from approximately 20 to 400 nm[10]. They exhibit surface lipoteichoic acid (LTA), which triggers immune cell activation *via* TLR2[12]. Studies have shown that CMVs originate from conservative blebbing mechanisms, independent of cell death, termed "bubbling cell death". This process can be triggered by exposure to DNA-damaging antibiotics like ciprofloxacin, phage-derived endolysins, and autolysins [13-15]. During this process, endolysins initially weaken the PGN, causing *Bacillus subtilis* (*B. subtilis*) cells to protrude their cytoplasmic membrane, forming membrane blebs, and ultimately releasing them as BEVs. In the case of *Lacticaseibacillus casei* (*L. casei*), no specific stimulation is necessary, as the spontaneous induction rate of its prophage suffices to produce significant quantities of explosive CMVs under standard culturing conditions.

Consequently, membrane lipoproteins, cytoplasmic proteins, and DNA or RNA are found in CMVs through cell lysis (Fig. 1)[1].

## 3. Composition and functions

BEVs convey a diverse array of components, including lipids, proteins, nucleic acids, metabolites, and toxins, originating from their parent bacteria. Proteomic investigations[16] have unveiled that the proteins harbored by BEVs are linked to crucial biological processes, encompassing structural proteins, porins, and transporters. Lipids, essential components of the bacterial cell membrane, remain a subject of ongoing research regarding their composition in BEVs. Prior studies[17] have indicated that BEVs derived from various bacteria possess distinct lipid profiles, potentially linked to bacterial adaptability and survival. Furthermore, advancements in metabolomics technology have revealed that BEVs loaded with metabolites and effector molecules can modulate the functionality of target cells, potentially playing a role in vital processes such as bacterial communication and biofilm formation[18]. As membrane structure complexes of all of these components above, BEVs play important roles in the physiological and pathogenic processes of their parent bacteria such as carriers in waste excretion and nutrient acquisition, and also facilities against adverse factors from the environment (phage or antibiotics), and as medium in bacterial communications or host-microbiota interactions (Fig. 2)[1,3,9].

### 3.1. *Disposal of wastes and nutrient acquisition*

Enhanced BEV formation is postulated as a mechanism to combat adverse environmental conditions. In scenarios where misfolded toxic products or overexpressed proteins accumulate in the periplasm, the generation of OMVs alleviates the membrane stress triggered by the accumulation of misfolded proteins, PGN fragments, or LPS in the periplasmic space[19-21]. Additionally, BEVs play a pivotal role in acquiring external nutritional support. Specifically, BEVs from certain bacteria harbor alkaline phosphatases that facilitate the fusion of prey bacteria, and the DNA and proteins associated with some BEVs serve as sources of nitrogen and phosphorus for bacterial growth[22-24]. Furthermore, BEVs can transport hydrolytic enzymes, such as hydrolases and polysaccharide lyases, essential for bacterial utilization of polysaccharides, as well as cytosolic metabolites comprising vitamins, amino acids, and carbon metabolic components, all contributing to bacterial growth[25,26].

### 3.2. *Competition and defense*

BEVs play a pivotal role in bacterial survival strategies and defense mechanisms. It has been documented that BEVs neutralize membrane-targeted antibiotics, including polymyxin, colistin, and daptomycin, thereby shielding bacteria from host defense factors such as antimicrobial peptides from mammalian tissues and factors from the blood complement system[14,27,28]. Furthermore, BEVs serve as a defensive barrier against phage invasion, acting akin to a "jamming bomb"[27]. Additionally, BEVs contribute to bacterial survival by competing with other bacteria or fungi and facilitating communication within bacterial communities, enabling the transfer of resistance plasmids[29]. Moreover, due to the nature of their membrane structure, BEVs can be absorbed by host cells, facilitating membrane exchange and subsequent adjustment of functional capabilities[30,31].

### 3.3. *Bacterial communications*

BEVs thus confer significant advantages in iron acquisition, interbacterial competition, and horizontal gene transfer (HGT)

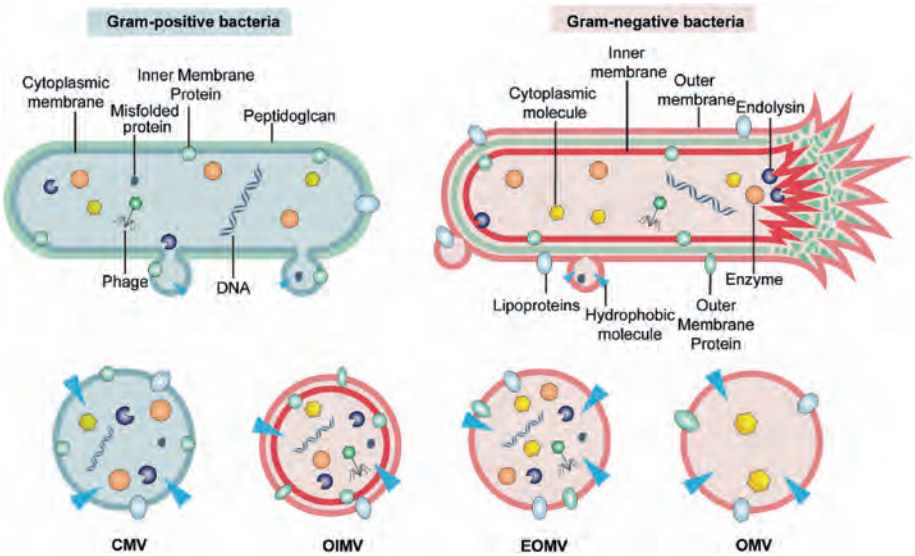



**Figure 1** Schematic illustration of the biogenesis of bacterial extracellular vesicles (BEVs) in both Gram-positive and Gram-negative bacteria is presented. In Gram-positive bacteria, BEVs arise as a consequence of bubbling cell death, a process triggered by endolysin-mediated degradation of the peptidoglycan layer. This degradation event culminates in the release of cytoplasmic membrane vesicles (CMVs) into the surrounding environment. Conversely, in Gram-negative bacteria, the genesis of outer membrane vesicles (OMVs) stems from imbalances in cell wall biosynthesis. Notably, the phage-derived endolysins capable of degrading the peptidoglycan cell wall, can induce an explosive form of cell lysis. During this process, membrane fragments coalesce and self-assemble, giving rise to two distinct types of vesicles: outer-inner membrane vesicles (OIMVs) and explosive outer-membrane vesicles (EOMVs). Reproduced with permission. Reprinted with the permission from Ref. 1. 

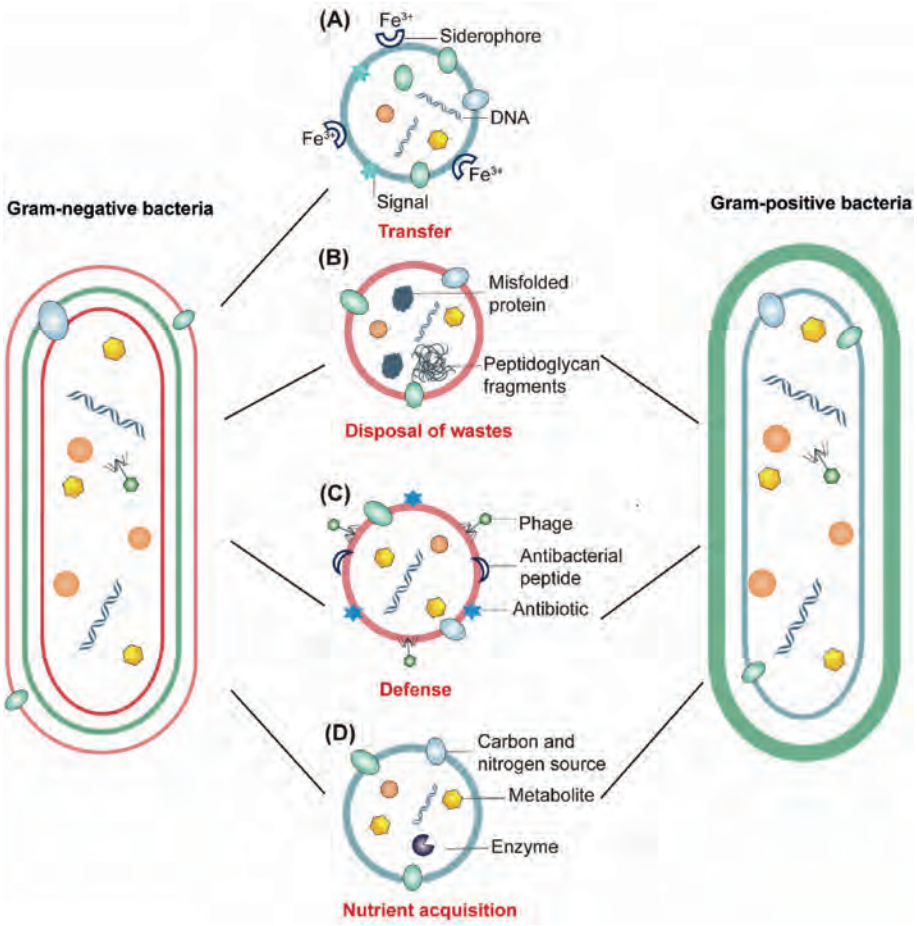



**Figure 2** The biological functions of BEVs. (A) BEVs confer significant advantages in iron acquisition, interbacterial signal, and horizontal gene transfer (HGT) between bacterial cells. (B) the generation of BEVs alleviates the membrane stress triggered by the accumulation of misfolded fragments, such as misfolded toxic products or overexpressed proteins accumulate in the periplasm. (C) BEVs can inactivate phages, neutralization of externally added antibiotics, defense antimicrobial peptides from mammalian tissues. (D) BEV serve as the tool for sources of nitrogen and phosphorus for bacterial growth.

between bacterial cells and their surrounding environment[32-34]. Research has demonstrated that the hydrophobic signal *N*-hexadecanoyl-L-homoserine lactone, produced by *Paracoccus* spp. (*Par*), with the aid of membrane vesicles, can not only solubilize in aqueous environments but also be transported to various bacteria, triggering quorum-sensing[35]. Furthermore, the fusion of BEVs with bacteria exhibits a degree of specificity, particularly ligand−receptor interactions between BEVs and target cells contribute to the specificity of delivery, thus establishing the pivotal role of BEVs in bridging bacteria and the microbial environment. Notably, BEVs possess transfer components that efficiently bind and deliver nutrients, such as iron and zinc, to target cells[35]. For instance, *Cupriavidus necator* (*C. necator*)'s type VI secretion system (T6SS) secretes TeoL, which interacts with its OMVs *via* LPS. This interaction facilitates OMVs' binding to outer membrane receptors CubA and CstR, ensuring the entry of OMV contents into the cell[36]. Gene transfer stands as one of the most traits of BEVs, as exemplified by numerous studies across diverse bacterial species[32,37-39]. Compelling evidence underscores the role of BEVs-mediated HGT in fostering long-term adaptive antibiotic resistance within and among bacterial populations. Specifically, BEVs from *Escherichia coli* (*E. coli*) have been shown to harbor transferable resistance genes that confer resistance to *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Deinococcus radiodurans* (*D. radiodurans*), leading to the acquisition of resistance against membrane-disrupting antibiotics like colistin and melittin[40]. Moreover, BEVs derived from an antibiotic-resistant strain of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) demonstrated the ability to transmit both plasmid-mediated penicillin resistance and chromosomally encoded streptomycin resistance to an antibiotic-sensitive *N. gonorrhoeae* strain[37]. Similarly, *Acinetobacter baumannii* (*A. baumannii*) can gain resistance to carbapenem antibiotics through OMVs that carry the plasmid-borne $bla_{OXA\text{-}24}$ gene[41]. Upon the aforementioned works, it becomes evident that BEVs hold a pivotal position in bacterial communication, thereby establishing a cornerstone for the modulation of intestinal microbe through its agency.

### 3.4. *Host immune activation and modulation*

Owing to their innate nano-scale dimensions and diverse biological attributes, BEVs possess the ability to traverse various physiological barriers, encompassing the intestinal epithelium, blood-brain barrier (BBB), and placental barrier[9,42,43]. These

vesicles harbor a plethora of microorganism-associated molecular patterns (MAMPs), comprising DNA, RNA, lipoproteins, LPS, and PGN, which trigger host pattern recognition receptors (PRRs), initiating a cascade of inflammatory signaling pathways that culminate in the generation of cytokines, chemokines, and antimicrobial peptides. After passing through the barriers and entering the circulatory system, BEVs further engage with immune cells. They modulate the function of immune cells in diverse manners, contingent upon their origin and virulence determinants. Specifically, the interaction between BEVs and dendritic cells (DCs) not only enhances the antigen-presenting capabilities of DCs, upregulates major histocompatibility complex II (MHC-II) and co-stimulatory molecules, but also boosts the production of proinflammatory cytokines and chemokines, thereby promoting protective B-cell and T-cell responses [44,45]. OMVs stimulate macrophages to produce proinflammatory cytokines, and they also induce human peripheral blood mononuclear cells to produce interleukin-6 (IL-6). Additionally, they elevate the expression of MHC-II, co-stimulatory molecules CD80, CD86, and intercellular adhesion molecule-1 (ICAM-1) in macrophages[46,47]. The bacterial DNA carried by BEVs can effectively activate the cyclic guanosine monophosphate−adenosine monophosphate synthase (cGAS)−stimulator of interferon genes (STING)−Type I Interferon (IFN-I) axis, safeguarding distant organs from viral threats[48]. Conversely, BEVs coupled with the host's bactericidal permeability-increasing protein (BPI) may bind and neutralize endotoxin or immunoglobulins, thereby contributing to bacterial survival. In diverse disease milieus, the specific mode of action of BEVs is intricately linked to the pathological environment of its constituent components. Subsequently, we will provide a detailed summary and illustration of the effects of BEVs in various diseases.

## 4. Effect of BEVs to the host

### *4.1. BEV and gastrointestinal microenvironment*

The intestinal tract functions as the important locale in the body where BEVs originate. These intestinal-derived BEVs are then expelled into the intestinal lumen, first engaging with the intestinal microenvironment. Notably, their diminutive size allows BEVs to effortlessly traverse the intestinal mucus layer, thereby facilitating subsequent interactions with immune cells, epithelial cells, or goblet cells within the intestinal mucosal layer. Here, we provide an overview of the effects of BEVs in gastrointestinal microenvironment.

#### *4.1.1. BEVs and IBD*

Inflammatory bowel disease (IBD) is a chronic intestinal condition characterized by microbiota dysbiosis, which plays a crucial role in both the onset and progression of the disease. Understanding the dysbiosis of the gut microbiota and the subsequent release of associated BEVs is essential in elucidating IBD processes. Firstly, BEVs-specific products exert a direct effect on epithelial cells, compromising the integrity of the intestinal epithelial barrier. Wang et al.[49] discovered that OMVs activate caspase-5, which subsequently triggers the phosphorylation of tyrosine kinases LYN, prompting nuclear translocation of Snail/Slug. This cascade ultimately results in the downregulation of E-cadherin, thereby disrupting the function of the intestinal epithelial barrier. Besides, high-temperature requirement A (HTRA) proteins, chaperones, and serine proteases, which are secreted into BEVs by various bacterial species, including *Helicobacter pylori* (*H. pylori*), *Bacillus anthracis* (*B. anthracis*), *Shigella flexneri* (*S. flexneri*), and *Campylobacter jejuni* (*C. jejuni*). Notably, HTRA enzymes cleave crucial components of tight junctions between epithelial cells, like occludin and E-cadherin, ultimately leading to the disruption of the epithelial barrier and subsequent extensive epithelial damage[50-52]. The BEVs derived from *Fusobacterium nucleatum* (*F. nucleatum*) significantly diminished the expression of tight junction proteins, including zona occludens (ZO-1), claudin-1, and occludin, as well as mucin-1/2, ultimately disrupting the epithelial barrier integrity[53]. Furthermore, mechanistically, *F. nucleatum*-BEVs promote autophagy through the miR-574-5p/CARD3 (caspase activation and recruitment domain 3) pathway, thereby increasing proinflammatory cytokines and exacerbating colitis[54]. Pathogen-associated molecular patterns (PAMPs), exemplified by LPS or peptidoglycan found within BEVs, activate caspase-11 (human caspase-4/5). The subsequent $K^+$ efflux, resulting from both apoptosis and pyroptosis, triggers the NLRP3 (nucleotide-binding and oligomerization domain, NOD-, leucine rich repeat, LRR- and pyrin domain-containing protein 3) inflammasome, ultimately facilitating caspase-1-mediated generation of the inflammatory signal IL-1$\beta$[4]. In *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) BEV-monocyte co-culture studies, it was established that the nuclear factor-kappa B (NF-$\kappa$B) pathway, stimulated by BEV, relies on TLR4 and the Toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP). Notably, inhibiting the TLR and TIRAP signaling pathways effectively abrogates BEV-induced monocyte activation[55]. The fulminant colitis in genetically susceptible mice, triggered by *B. thetaiotaomicron*, relies on the anaerobic sulfatase maturating enzyme (anSME), implying that the access of *B. thetaiotaomicron*-BMVs to macrophages prompts inflammatory immune stimulation in vulnerable hosts[56].

However, the microenvironment of IBD is profoundly complex. A clinical study revealed that BEVs from *B. thetaiotaomicron* induced IL-10 expression in healthy colonic DCs, whereas they failed to do so in IBD patients with reduced DC counts[57], suggesting that cell type and health status profoundly influence BEVs-host interactions. Additionally, BEVs secreted by some commensal bacteria (categorized as probiotics) significantly strengthen the mucosal barrier by reinforcing tight junction structures and modulating the immune environment in IBD. In terms of safeguarding the integrity of the intestinal epithelial barrier, TcpC secreted and packaged in *Escherichia coli Nissle* 1917 (*EcN*) derived OMVs can be internalized by target cells, activating the protein kinase C-$\zeta$ (PKC$\zeta$) and extracellular-signal-regulated kinase 1/2 (ERK1/2) signaling pathway to enhance the epithelial barrier[58]. In the progression of IBD, certain BEVs and their active components also contribute to the immunomodulatory effect. Wang et al.[59] discovered that BEVs derived from *Faecalibacterium prausnitzii* (*F. prausnitzii*) reprogramed metabolism of macrophages by inhibiting oxidative phosphorylation and glycolysis and diminish the expression of peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) and undergo an altered lipid processing, marked by reducing cholesterol efflux, promoted energy reprogramming. Then, *F. prausnitzii*-BEVs possess the capacity to steer the polarization of peripheral blood mononuclear cells (PBMCs) towards an M2b macrophage phenotype, which is linked with anti-fibrotic activities, highlighting their immunomodulatory potential. In experimental colitis, DCs detect *Bacteroides fragilis* (*B. fragilis*) OMV-associated polysaccharide A (PSA) *via* TLR2 in a growth arrest and DNA damage-inducible proteins

(GADD45A)-dependent manner, ultimately leading to an increase in regulatory T cells and an anti-inflammatory effect[60]. *B. fragilis*-OMVs also elicit regulatory T cells and safeguarding the intestinal tract from colitis related with IBD-associated genes, namely ATG16L1 and NOD2[61]. In addition to *B. fragilis*, BEVs derived from *Roseburia intestinalis* (*R. intestinalis*) have exhibited robust anti-inflammatory properties. The inclusion of Ile-Pro-Ile (isoleucine-proline-isoleucine) the structural makeup of these *R. intestinalis*-BEVs is instrumental in diminishing dipeptidyl peptidase-4 (DPP4) activity within inflamed colonic tissues and enhancing the bioavailability of active glucagon-like peptide-1 (GLP-1). Consequently, this leads to the downregulation of NF-$\kappa$B and signal transducer and activator of transcription 3 (STAT3) signaling cascades, mediated through the phosphatidylinositol 3-kinase (PI3K) pathway[62]. As a promising probiotic, *Akkermansia muciniphila* (*Akk*)'s OMVs are favored for IBD therapy. These OMVs alleviate colitis symptoms by upregulating tight junctions, including ZO-1, claudin-1, claudin-4, claudin-5, and occludin, and regulating T-cell differentiation. Notably, Amuc1100 and its phospholipid (a diacyl phosphatidylethanolamine (PE) with two branched chains, a15:0 - i15:0 PE) significantly contribute to this beneficial outcome[63,64].

#### *4.1.2. BEV and gut microorganism*

As mentioned earlier, BEVs possess the capability to transport bacterial metabolites and substances, including amino acids, DNA, fatty acids, hydrolase enzymes, and numerous other components. These functionalities significantly contribute to the survival of bacteria within the gut's ecological niche, either through collaborative or competitive interactions. BEVs are paramount in facilitating horizontal gene transfer. For instance, vesicles from *Ruminococcus* spp. (*Rum.*) facilitate the transfer of cellulolytic genes, enabling the degradation of crystalline cellulose from other bacteria[29]. Additionally, *N*-acetylmuramoyl-L-alanine amidase present in *Staphylococcus aureus* (*S. aureus*) BEVs can eliminate competing bacteria through peptidoglycan degradation and cell lysis[65]. The *Vibrio cholerae* (*V. cholerae*) BEVs-associated protein, outer membrane-associated biofilm facilitating protein A (OBFA), exerts significant influence on biofilm formation and intestinal colonization by modulating pathways that encompass HapR, a pivotal transcriptional regulator within the *V. cholerae* quorum sensing (QS) cascade, which serves as a potent repressor, curbing both biofilm development and virulence[66]. The BEVs derived from *Clostridium butyricum* (*C. butyricum*) exhibit the potential to alleviate bacterial dysbiosis in colitis mice, significantly reducing the prevalence of prominent pathogens like *E. coli* and *S. flexneri*[67]. Furthermore, the adhesion to the intestinal mucosa and antimicrobial properties of probiotic BEVs effectively reduce pathogenic bacteria colonization and enhance probiotic colonization, thereby playing a crucial role in the therapeutic benefits of probiotics[68].

### *4.2. BEV and cancer*

#### *4.2.1. Oncogenicity*

The role of microorganisms in malignant tumor development, diagnosis, and treatment has long been debated. Studies reveal that microbes may rely on mediators to modulate tumor progression, both locally and remotely[69]. BEVs, as active bacterial-derived molecules, possess potential tumor-promoting mechanisms that influence genome stability, and tumor microenvironments, and suppress immune surveillance. Currently, the underlying mechanisms of BEVs pathogenesis in microbe-dense tissues of the digestive tract, encompassing the mouth, stomach, colon, and others, as well as the respiratory tract, specifically the lung, are partly well understood.

Recent research indicates that BEVs derived from *F. nucleatum* can trigger oral cancer metastasis, potentially linked to the activation of the intracellular autophagy pathway in cancer cells[70]. In colorectal cancer, the fusobacterium autotransporter protein 2 (FAP2), expressed on the outer membrane vesicles of *F. nucleatum*, specifically targets the overexpressed Gal-GalNAc glucose residues present on the exterior of colon tumor cells, thereby facilitating tumor growth and dissemination. Additionally, this protein binds to the T cell immunoreceptor with Ig and ITIM domains (TIGIT) molecule, suppressing T cell activity within the tumor microenvironment. Furthermore, *F. nucleatum*-OMV induces epithelial-mesenchymal transition (EMT)-related markers like N-cadherin, integrin-$\alpha$5, Snail, and fibronectin-1, contributing to tumor metastasis[71]. The *H. pylori* exerts toxic effects on host cells *via* the release of OMVs, containing cytotoxin associated gene A (CagA) and cytotoxin associated gene A (VacA) proteins, which significantly contribute to the progression of precancerous lesions and ultimately tumor formation[72,73]. OMVs have a heightened capacity to elicit inflammatory reactions compared to bacterial metabolites such as LPS alone[74]. Additionally, some studies associate OMVs with TLR-mediated inflammatory responses, which have been intimately tied to various malignancies, encompassing colorectal cancer, breast cancer, and liver cancer[75].

#### *4.2.2. Therapeutics*

Conversely, the utilization of bacteria in tumor therapy dates back to the dawn of the 1890s, pioneered by William Coley who administered attenuated *Streptococcus pyogenes* (*S. pyogenes*) to cancer patients[76]. Given the persistent risk of infection posed by bacteria, non-replicating BEVs emerge as a safer alternative. Notably, attenuated BEVs alone exhibit anti-tumor properties, as Kim et al.[77] initially reported in 2017, highlighting OMVs secreted by gram-negative bacteria as a tumor immunotherapy agent. Their findings revealed that OMVs specifically accumulate in tumor tissues, inducing anti-tumor immune responses in mice *via* the interferon-gamma (IFN-$\gamma$) signaling pathway. The activation of TLR responses by OMVs is crucial for fostering a tumor inflammatory microenvironment, enabling OMVs to present neoantigens as tumor vaccine carriers. Through bacterial genetic engineering, heterologous antigens and functional proteins can be affixed to the surface of OMVs *via* cytolysin A (ClyA), and outer surface protein A (OspA)[78]. These OMV-based vaccines have demonstrated efficacy in suppressing the metastasis and growth of melanoma and colorectal cancer tumors[5,79,80]. At present, vaccines based on OMVs are predominantly being researched for infectious diseases stemming from *Neisseria meningitidis* (*N. meningitidis*), with origins dating back decades[81]. However, their immense potential in clinical applications remains largely unexplored and underutilized.

Beyond immunostimulatory tumor treatments, the anti-tumor effects of bacteria have also garnered attention, exemplified by the star *Akk*. Jiang and coworkers[82] discovered that the Amuc_2172 protein found in *Akk*-derived OMVs enters colon cancer cells, functioning as an acetylation transferase on Lys14 of histone H3 (H3K14ac). This elevation of H3K14ac on *HSPA1* loci enhances the transcription and secretion of heat-shock protein 70 (HSP70), which triggers immune activation of T cells during colorectal tumor microenvironment reprogramming. Wang and coworkers[83]

further revealed that transplantation of *Akk*-associated OMVs into the intestine augments anti-programmed cell death protein 1 therapy against colorectal cancer by regulating intestinal homeostasis.

### 4.3. BEVs and central nervous system diseases

The intricate ecosystem of the human gut microbiota plays a pivotal role in maintaining homeostasis, extending its influence beyond the gastrointestinal tract to regulate nervous tissue function[84,85]. Among the emerging mediators along the gut-brain axis, BEVs have garnered significant attention as novel communicators, possessing the ability to traverse the host brain *via* circulatory or neural pathways[86]. Compelling evidence suggests that OMVs originating from intestinal microbiota can breach the BBB and gain entry into the central nervous system (CNS), potentially facilitating the conveyance of signals from the intestinal flora to central nervous cells [87].

#### 4.3.1. Alzheimer's disease and Parkinson's disease

Substantial disparities in gut microbiota are observed between patients suffering from neurodegenerative diseases and healthy counterparts, where disturbances in these microbiotas may potentially trigger or accelerate disease progression[88]. Beyond regulating the intestinal barrier and releasing metabolites into circulation, gut bacteria can directly transmit proteins, nucleic acids, and other components to the central nervous system *via* BEVs. Investigations have isolated bacterial BEVs from human cerebrospinal fluid, associating them with the initiation and progression of neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD)[89].

BEVs originating from the gut microbiota of AD patients have displayed the capability to stimulate tau phosphorylation and initiate AD pathology in murine models, emphasizing the potential threat posed by BEVs derived from intestinal microbiota entering the bloodstream[90,91]. Further exploration has identified specific bacterial species contributing to the formation of these disease-associated BEVs. For instance, chronic *H. pylori* infection has been associated with the pathogenesis of neurodegenerative diseases[92]. Research has shown that *H. pylori*-derived OMVs induce astrocyte reactivity *via* NF-$\kappa$B activation, resulting in neuronal damage in AD murine models[93,94]. Additionally, Xie's study[95] has demonstrated the capacity of *H. pylori*-derived OMVs to breach the BBB and be internalized by astrocytes, activating glial cells, impairing neuronal function, and promoting amyloid-$\beta$ aggregation *via* the complement component 3 (C3)a receptor (C3aR) signaling pathway. Similarly, OMVs from *Porphyromonas gingivalis* (*P. gingivalis*) have triggered NLRP3 inflammasome activation, facilitating the delivery of gingipains into cerebral microvascular endothelial cells. This sequence of events culminates in neuroinflammation, BBB disruption, ferroptosis, tau phosphorylation, and memory impairment in murine models[96-98].

Conversely, BEVs from certain probiotics have been proposed as modulators of neuroinflammation and neurodegeneration. *Lactobacillus* (*Lac*)-derived CMVs enhance brain-derived neurotrophic factor (BDNF) expression in hippocampal neurons and exhibit antidepressant-like effects in murine models[99]. These CMVs also inhibit amyloid-$\beta$ protein deposition by upregulating MECP2 (methyl CpG binding protein 2) and SIRT1 (silent information regulator sirtuin 1) in transgenic Tg-APP ($\beta$-amyloid precursor protein)/PS1 (presenilin) mice[100]. Additionally, OMVs from *Akk* ameliorate high-fat diet-induced brain inflammation and cognitive dysfunction by inhibiting microglia activation[101]. Intriguingly, *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*)-OMVs possess the potential for retrograde transfer from axon terminals to the cell bodies of trigeminal ganglion neurons[102], though further investigation is required to determine if similar properties exist in other bacterial OMVs and their potential for drug delivery.

#### 4.3.2. CNS infection

Owing to various pathogenic substances, BEVs have long been acknowledged as significant vectors in bacterial infections within the central nervous system. Notably, *Haemophilus influenzae type b* (*Hib*), for instance, triggers meningitis through the delivery of lipooligosaccharide (LOS) *via* OMVs[103]. However, recent research conducted by Liu et al.[104] offers a nuanced perspective on the role of BEVs in the dynamics of CNS infection. Their study revealed that OMVs derived from *E. coli K1*, a major cause of neonatal bacterial meningitis, can activate microglia and astrocytes during the initial stages of infection, ultimately aiding in the recruitment of neutrophils to the CNS to counter the infection. Besides, the multi-component meningococcal B vaccine, 4CMenB, comprises three surface-exposed recombinant proteins (fHbp, NadA, and NHBA), alongside OMVs derived from the New Zealand strain, which possess PorA 1.4 antigenicity. The comprehensive clinical development program for 4CMenB encompasses adults, adolescents, and infants, ensuring a broad spectrum of protection[105]. This vaccine has been endorsed by both the European Medicines Agency and the U.S. Food and Drug Administration for the prevention of meningococcal infections. These indicate that although BEVs have the potential to trigger infections in the central nervous system, they also exhibit the capacity to stimulate immune responses, thereby offering novel insights into the role of diverse bacterial BEVs in the gut and throughout the various stages of CNS infectious diseases.

### 4.4. BEVs and bone related diseases

Mounting evidence underscores the pivotal role of the gut-bone axis in orthopedic disorders. The gut microbiota exerts intricate yet significant influences on the skeletal system, modulating the host's nutritional status, endocrine functions, and metabolic pathways[106,107]. Notably, BEVs play a crucial role as messengers in this intricate interplay. Diseases related to bone aging have garnered substantial interest in BEVs research. Some groups are exploring the potential of BEVs in developing therapeutic and preventive strategies for osteoporosis, emphasizing their significant therapeutic potential in the realm of orthopedic diseases.

#### 4.4.1. Osteoporosis

Multiple studies have clarified the crucial role of BEVs in the development of osteoporosis. For instance, Kim et al.[108,109] revealed that CMVs, released by *Filifactor alocis* (*F. alocis*), a Gram-positive anaerobic periodontal pathogen, trigger proinflammatory cytokine production, osteoclastogenesis, and bone resorption through TLR2 activation by lipoproteins, thus contributing to systemic bone loss. Conversely, BEVs originating from other probiotics exhibit protective effects on bone health. Specifically, OMVs from children's gut microbiota or *Akk* can accumulate in bone tissues, mitigating osteoporotic phenotypes induced by ovariectomy (OVX) by enhancing osteogenic activity and inhibiting osteoclast formation[110]. Despite significant advancements, several gaps continue to exist in the research

surrounding BEVs in osteoporosis. The precise components of BEVs that regulate pathogenic or therapeutic effects remain unclear, thus necessitating further investigation into their mechanisms of action on bone tissue cells. Furthermore, the merits of BEVs as vehicles for osteoporosis treatment in systemic delivery deserve thorough examination[111,112], particularly in evaluating the comparative advantages and limitations of probiotic OMVs against live bacteria in managing osteoporosis. Additionally, it is crucial to undertake further research into how the engineered BEVs modulate the intestinal microenvironment in an osteoporotic state, along with the specific microenvironment characteristic of osteoporosis.

#### *4.4.2. Arthritis and osteonecrosis*

Acknowledging the pivotal role of gut microbiota in orchestrating both intestinal and systemic immune responses, investigations into the implications of BEVs in rheumatoid arthritis (RA) and osteoarthritis (OA) have attracted the attention of researchers. *P. gingivalis,* a prominent periodontal pathogen, has been implicated in the development of RA through its peptidylarginine deiminase (PPAD) enzyme. Notably, OMVs of this bacterium harbor PPAD, protecting it from degradation and effectively mediating its translocation to joint locations, ultimately instigating an autoimmune reaction[113,114]. Furthermore, *F. nucleatum* intensifies RA *via* FadA (*F. n* secrete adhesin)-bearing on OMVs by upregulating the *Rab5a* (RAS oncogene family member)-YB-1(Y-box binding protein 1) pathway[115]. Intriguingly, this study proposes that the RNA and DNA components of OMVs may not significantly affect host cells, contradicting previous discoveries[48] and highlighting potential variations in OMVs mechanisms depending on bacterial origin. Nonetheless, these studies have yet to delve into the direct impact of OMVs on chondrocytes, osteoblasts, and osteocytes.

Osteonecrosis of the femoral head poses formidable challenges in joint surgery, with its treatment being particularly challenging[116]. Recent advancements suggest a potential involvement of BEVs in glucocorticoid-induced osteonecrosis. Specifically, CMVs derived from *Lac* exhibit proangiogenic, pro-osteogenic, and antiapoptotic properties within the femoral head, albeit with a decrease in their abundance following glucocorticoid exposure[117]. However, this study remains confined to animal and cell characterization, necessitating further exploration of the underlying mechanisms governing BEV actions.

### *4.5. BEV and metabolic related diseases*

Gastrointestinal bacteria significantly modulate glucose metabolism and tissue inflammation, thereby exerting a profound influence on diabetes and its associated complications. Consequently, it is undeniable that BEVs play a pivotal role in this intricate process. Specifically, *P. gingivalis* derived OMVs translocate to the liver, attenuating insulin-mediated protein kinase B (Akt)/glycogen synthase kinase-3 (GSK-3$\beta$) signaling, thereby disrupting glucose homeostasis and accelerating diabetes progression in mice[118]. Furthermore, these OMVs exacerbate retinal microvascular endothelial cell dysfunction in diabetic retinopathy *via* protease-activated receptor-2 (PAR-2) activation[119]. In addition, dysbiosis-induced OMVs traverse the leaky gut barrier, infiltrating distant tubulointerstitium and eliciting cellular inflammation, ultimately contributing to renal tubulointerstitial injury in diabetic kidney disease[120]. Notably, Importantly, liver CRIg$^+$ (complement receptor of the immunoglobulin superfamily) macrophages effectively clear DNA-laden BEVs from circulation *via* a C3-mediated opsonization mechanism, yet obesity is notably correlated with a significant reduction in CRIg$^+$ macrophage counts. This depletion exacerbates the dissemination of BEVs to distant metabolic tissues, amplifying tissue inflammation and metabolic dysfunctions[121]. Intriguingly, Gao's research[122] underscores a parallel finding: microbial DNA-bearing BEVs traverse the compromised gut barrier in obesity, delivering microbial DNAs directly to $\beta$-cells. This triggers the activation of the cGAS/STING pathway, resulting in augmented inflammation and impaired insulin secretion. Conversely, Vsig4$^+$ (V-set and immunoglobulin domain containing 4) macrophages emerge as a vital protective tool, thwarting BEVs infiltration into $\beta$-cells through C3-dependent opsonization. This underscores the intricate and dynamic interplay among gut microbiota, immune system components, and metabolic homeostasis.

Conversely, probiotic *EcN*-derived OMVs exhibit the capacity to diminish blood glucose levels and augment plasma insulin concentrations in obese mice[123]. Nevertheless, the direct implications of *EcN*-OMVs on glucose metabolism within host cells and pancreatic endocrine function necessitate further exhaustive investigation. However, the investigation into *Akk* and its OMVs in the realm of metabolic regulation has progressed to a more profound level. Notably, *Akk* is renowned for its remarkable ability to mitigate fat mass accumulation, insulin resistance, and dyslipidemia in mice [124-126]. Remarkably, Amuc_1100, a distinct protein isolated from the outer membrane of *Akk* and also present in *Akk*'s OMVs, has been shown to interact with TLR2. This interaction fortifies the gut barrier and partially recapitulates the salutary effects observed in the context of obesity and related disorders[127,128].

### *4.6. BEVs and other diseases*

BEVs in the digestive tract, serving as pivotal mediators of bacterial signals disseminated throughout the body, have garnered significant attention across a broad spectrum of diseases[129]. Bittel et al.[87] utilized the *Cre-LoxP* system to introduce *E. coli* expressing *Cre* recombinase, subsequently tracking the dissemination of OMVs harboring *Cre* mRNA in tdTomato mice. Their findings reveal that the OMVs secreted by the modified *E. coli* are capable of traversing the intestinal barrier, accumulating in vital organs such as the liver, spleen, heart, and kidney, and interestingly, emitting a red fluorescent signal in neuronal cells within the brain. Beyond traversing the intestinal and blood-brain barriers, BEVs are also capable of crossing the placental barrier, highlighting their pervasive nature. In the context of placental injury, maternal bacteria exert a profound influence on embryonic development, emphasizing their role in early life stages[130]. Remarkably, OMVs derived from *P. gingivalis* have been implicated in early pregnancy and placentation, disrupting trophoblast contributions to vascular remodeling and immune homeostasis[131]. In contrast, OMVs from *Akk*, transferred from the gut to the placenta, alleviate preeclampsia-like symptoms in mice by facilitating trophoblast invasion and spiral artery remodeling, processes mediated through the epidermal growth factor receptor (EGFR)−phosphatidylinositol-3-kinase (PI3K)−AKT signaling cascade[132]. Regarding cardiovascular diseases, *P. gingivalis*-derived OMVs elevate vascular permeability by proteolytic degradation of endothelial cell−cell adhesins such as platelet-endothelial cell adhesion molecule-1 (PECAM-1), thereby promoting the pathogenesis of these conditions[133]. Additionally, *Helicobacter pylori*-derived OMVs deliver LPS and CagA protein,

which elevate ROS levels and augment NF-κB activation in human umbilical vein endothelial cells (HUVECs), accelerating atherosclerosis plaque formation *via* endothelial damage[134]. Another prime illustration is diseases in the liver. Fizanne et al.[135] discovered that fecal extracellular vesicles (fEVs) isolated from patients with non-alcoholic steatohepatitis (NASH) not only augment intestinal permeability, an effect mitigated by the inhibition of non-muscular myosin light chain kinase (nmMLCK). Notably, NASH-derived fEVs are capable of activating profibrotic and proinflammatory pathways in hepatic stellate cells, ultimately contributing to liver injury. Natsui et al.[136] discovered the presence of OMVs derived from intestinal bacteria (*E. coli*) in the ascites of patients with decompensated cirrhosis. Furthermore, they validated that oral administration of these OMVs exacerbated liver inflammation, predominantly by modulating macrophage activation, exacerbating fibrosis, and decreasing albumin levels in a mouse model of cirrhosis. In patients afflicted with primary sclerosing cholangitis (PSC) in conjunction with IBD, OMVs are detected within the systemic circulation and liver. These OMVs initiate the TLR4 and NLRP3-GSDMD (Gasdermin D) signaling cascades, ultimately stimulating the activation of both hepatocytes and stellate cells. This activation process subsequently intensifies liver inflammation and fibrosis, thereby presenting a potential novel therapeutic target for fibrosis intervention[137].

Here we summarized the current landscape of research investigating the intricate relationship between BEVs and various diseases, offering valuable insights for future endeavors (Fig. 3, Table 1). Virtually, various tissues are intricately interconnected with the intestinal system *via* BEVs, emphasizing the need for a comprehensive understanding of their roles. However, it is imperative to acknowledge the existing research gaps, particularly in expanding the scope of bacterial species studied and delving deeper into the effector molecules within BEVs. Furthermore, the underutilization of germ-free mice or genetically modified animal models poses a significant hindrance to the advancement of this field.

## 5. The application of BEVs

### *5.1. The separation and detection of BEVs*

The isolation and detection of BEVs have emerged as a crucial technical hurdle in the realm of this research. Drawing upon the unique biophysical characteristics of BEVs, such as particle size, density, charge, and specific surface molecules, diverse methodologies have been formulated, which often draw parallels with strategies employed for the separation of EVs in general. A comprehensive summary of the frequently utilized techniques for isolating BEVs is introduced and outlined in Table 2.

Ultracentrifugation, the prevalent technique utilized in laboratories, stands as a paramount illustration in this context[138]. Prior to isolation, a filtration step is pivotal in eliminating contaminants. Subsequently, bacterial cells are eliminated through low-speed centrifugation, operating within a range of 2000 to $10{,}000\times g$, which is followed by the meticulous filtration of the supernatant through 0.45 and 0.22 μm filters, respectively[138,139]. However, there exists a degree of disagreement concerning the employment of filters, as while the 0.22 μm filter may elevate purity standards, it could inadvertently diminish the overall yield[140]. Subsequent to filtration, the supernatant undergoes ultracentrifugation at exceptionally high speeds, exceeding $100{,}000\times g$. Although ultracentrifugation is frequently regarded as the gold standard owing to its accessibility and cost-effectiveness, there are drawbacks, including the potential for structural disruption of BEVs, coprecipitation of impurities, and comparatively lengthy processing times[141,142].

Density gradient centrifugation emerges as a frequently employed technique for the isolation of biofluids. This methodology capitalizes on the disparate sedimentation velocities of cells or vesicles within a gradient medium under centrifugal forces, yielding distinct, separable layers[143,144]. It excels in eliminating impurities, particularly from intricate fluid matrices like urine and fecal solutions[145,146]. Nevertheless, it necessitates sophisticated instrumentation and is time-consuming, often requiring 18 h or longer to complete[147]. Furthermore, researchers must undertake preliminary studies and experiments to predetermine crucial physical parameters, such as the density of BEVs, adding an additional layer of complexity to the process.

Ultrafiltration entails the passage of the supernatant from the bacterial culture medium through an ultrafiltration membrane that possesses a defined molecular weight cutoff, typically spanning 50−100 kDa, aimed at eliminating impurities[138,148]. This technique boasts advantages like swiftness. Nevertheless, its limitation lies in inadequately removing proteins whose molecular weights are either comparable to or larger than the cutoff threshold, potentially leading to the contamination of the resultant BEVs with supernatant proteins, thereby jeopardizing experimental reliability[149,150]. Consequently, ultrafiltration is frequently employed in conjunction with additional separation methodologies to enhance the purity of BEVs.

Size-exclusion chromatography (SEC) serves as another technique for segregating BEVs by introducing the supernatant onto a column packed with porous beads, which gradually filter the sample based on the molecular size of its components[151]. Specifically, larger entities, including bacterial debris, are initially eluted, followed by smaller vesicles akin to BEVs[152]. The gentleness of the SEC process is noteworthy, as it abstains from vigorous agitation or homogenization that could potentially compromise the integrity of delicate biological molecules such as nucleic acids, thereby enhancing their structural preservation and functional activity[153]. Nonetheless, SEC encounters a similar limitation to ultrafiltration in that it struggles to discern between impurities and BEVs with comparable molecular weights. Furthermore, the reliance on specialized chromatographic columns adds to operational expenses, while the technique's finite loading capacity can lead to a diminished final yield of BEVs, thereby constraining its scalability.

Precipitation techniques entail the strategic incorporation of specialized reagents into the supernatant, subsequent to the elimination of bacteria and bacterial debris. This addition disrupts the delicate balance between surface charges and hydrogen bonding, thereby fostering the agglomeration of both BEVs and protein constituents[154]. For example, Shin et al.[155] devised a novel aqueous two-phase system leveraging polyethylene glycol (PEG) and dextran. This innovative system effectively segregates aggregated BEVs through conventional centrifugation at approximately $10{,}000\times g$, achieving a remarkable recovery rate that surpasses ultracentrifugation by nearly fourfold, with yields approximating 70%. Critically, this approach drastically minimizes the reliance on ultracentrifugation. Nevertheless, the introduction of precipitating agents may potentially hinder downstream experimental workflows and complicate subsequent modifications to BEVs.

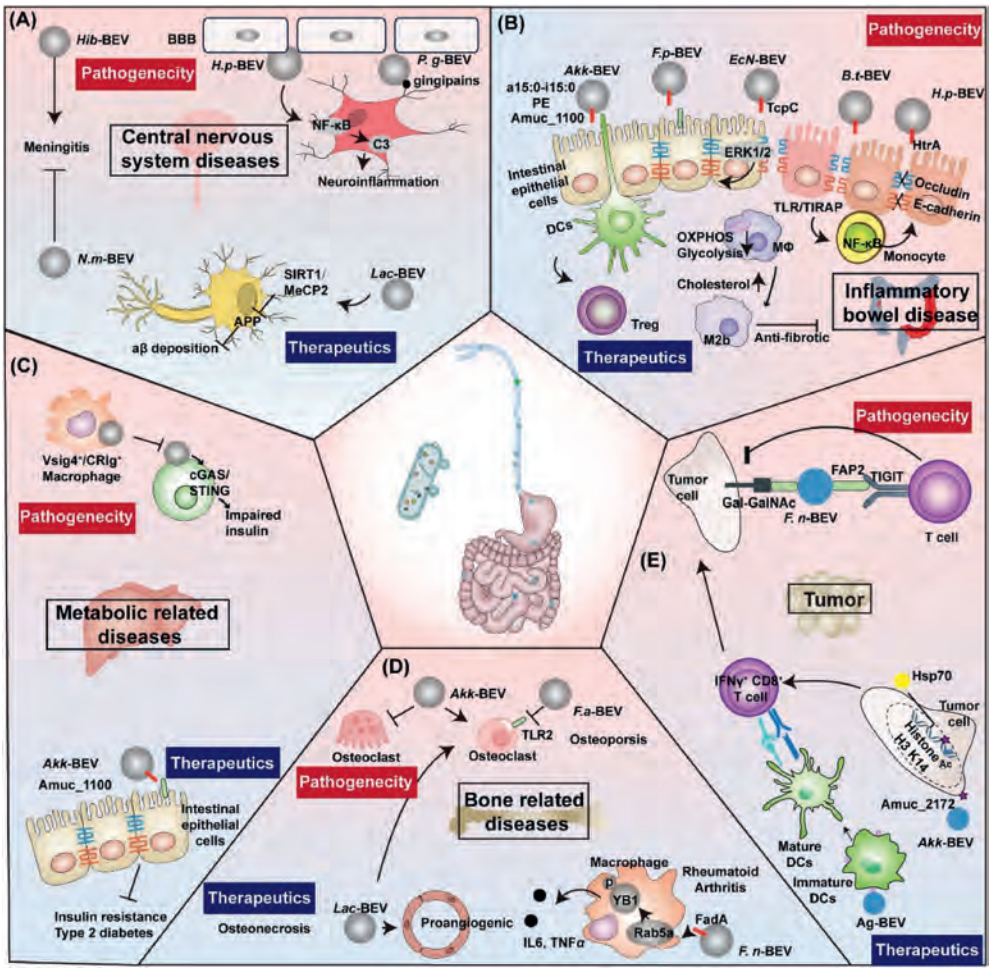



**Figure 3** A schematic depiction of the contemporary research landscape, exploring the effect of exemplary BEVs and its biologically active molecules in a diverse array of diseases, encompassing central nervous system(A), inflammatory bowel disease (B), metabolic diseases (C), bone-related disorders (D), tumors (E) from both pathogenic and therapeutic perspectives. *F. n*, *Fusobacterium nucleatum*; *B. t*, *Bacteroides thetaiotaomicron*; *H. p*, *Helicobacter pylori*; *Akk*, *Akkermansia muciniphila*; *EcN*, *Escherichia. coli Nissle 1917*; *F. p*, *Faecalibacterium prausnitzii*; Ag, antigen; *P. g*, *Porphyromonas gingivalis*; *Hib*, *Hemophilus influenzae type b*; *N. m*, *Neisseria Meningitidis*; *F. a, Filifactor alocis*; *Lac*, *Lactobacillus*; BBB, blood-brain barrier; NF-κB, nuclear factor-kappa B; C3, complement component 3; SIRT1, silent information regulator sirtuin 1; MeCP2, methyl CpG binding protein 2; APP, β-amyloid precursor protein; PE, phosphatidylethanolamine; HtrA, high-temperature requirement A; Treg, regulatory T cells; TIGIT, T cell immunoreceptor with Ig and ITIM domains; FAP2, fusobacterium autotransporter protein 2; TLR, toll-like receptors; TIRAP, toll-interleukin-1 receptor domain-containing adaptor protein; OXPHOS, oxidative phosphorylation; YB1, Y-box binding protein 1; Rab5a, RAS oncogene family member; FadA, *F. n* secrete adhesin; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; Vsig4, V-set and immunoglobulin domain containing 4; CRIg, complement receptor of the immunoglobulin superfamily; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase; STING, stimulator of interferon genes.

An even more promising avenue is immune-affinity-based separation, such as immuno-precipitation (IP) harnessing the exquisite antigen-antibody interactions to selectively bind to the surface proteins of BEVs with remarkable affinity[156]. This methodology is tailor-made for isolating tagged BEVs, as exemplified by Alves et al.[157], who adeptly utilized affinity separation to purify OMVs containing OmpA-his6 from *E. coli* cultures. Theoretically, this technique offers unparalleled specificity, enabling the isolation of BEVs from designated bacterial strains, even amidst a complex mix of bacterial cultures[156,158]. Nevertheless, a formidable obstacle lies in pinpointing specific target proteins unique to BEVs across diverse bacterial origins, and the costs incurred in developing and deploying tailored antibodies may significantly escalate the overall cost of extraction.

Microfluidics introduces a groundbreaking approach that integrates sample preparation, reaction, separation, and detection seamlessly within a microscale chip, automating the comprehensive analytical workflow for biological, chemical, and medical endeavors[159]. In the context of BEVs isolation, microfluidic devices harness the power of customizable chips to isolate BEVs with precision, leveraging their unique attributes such as size, immunoreactivity, or dynamic behavior[160]. This strategy revolutionizes the automation of BEVs separation, ensuring a high degree of purity. Nonetheless, its efficacy is intricately tied to the design and utilization of tailored microfluidic chips, which subsequently escalates both the financial investment and the intricacy associated with experimental reproducibility. Additionally, the preliminary sample handling stage poses further obstacles, as

**Table 1** Representative studies investigating the relationship between gut associated-BEVs and various diseases.

| BEVs origin | Active substance | Intervention mechanism | Pathological change |
|---|---|---|---|
| IBD | | | |
| *E. coli* | ____ | OMVs activate caspase-5, then triggers pLYN, prompt nuclear translocation of Snail/Slug, down-regulate of E-cadherin | Damage epithelial barrier |
| *H. pylori*, *B. anthracis*, *S. flexneri*, *C. jejuni* | HTRA | Cleave junction factors including E-cadherin, occludin, and claudin-8, as well as extracellular matrix proteins such as fibronectin, aggrecan, and proteoglycans | Disruption of the epithelial barrier and substantial host cell damage |
| *F. nucleatum* | ____ | BEVs activated and promoted the migration of RIPK1 and RIPK3 into necrosome in Caco2 cells through FADD-RIPK1-caspase-3 signaling | Epithelial necroptosis and gut barrier disruption |
| *F. nucleatum* | ____ | BEVs mediate experimental colitis severity through miR-574-5p/CARD3-dependent autophagy activation | Regulate cytokines and epithelial barrier dysfunction |
| *B. thetaiotaomicron* | ____ | Stimulate TLR4 and TIRAP | Induce monocyte activation |
| *B. thetaiotaomicron* | anSME | Prompt macrophage inflammatory immune stimulation | Development of spontaneous, fulminant colitis in genetically susceptible mice |
| *B. thetaiotaomicron* | ____ | Induced IL-10 expression in healthy colonic DCs but not in DCs from IBD patients | Immune response to constituents of the microbiota locally and systemically |
| *EcN* | TcpC protein | Induced PKC$\zeta$ and ERK1/2 phosphorylation, then upregulation of the barrier-forming tight junction protein claudin-14 | Improvement of intestinal barrier function and remission in ulcerative colitis |
| *F. prausnitzii* | ____ | Diminish expression of PPAR$\gamma$ and undergo an altered lipid processing, marked by reducing cholesterol efflux, promoted energy reprogramming | Polarize of PBMCs towards an M2b macrophage phenotype, which linked with anti-fibrotic activities in IBD |
| *B. fragilis* | Polysaccharide A (PSA) | Activate TLR2 of DCs in a GADD45A-dependent manner | Increase in Tregs and inhibition of inflammation |
| *R. intestinalis* | Ile-pro-Ile | Downregulate of NF$\kappa$B and STAT3 signaling cascades, mediated through the PI3K pathway | Anti-inflammatory properties |
| *Akk* | Amuc1100; a15:0-i15:0 PE | Non-canonical TLR2−TLR1 signaling pathway | Upregulation of tight junctions, increase in Tregs, remission of intestinal inflammation |
| Tumor | | | |
| *F. nucleatum* | FAP2 | Targets Gal-GalNAc glucose residues present on colon tumor cells; bind TIGIT molecule | Facilitate tumor growth and dissemination; suppressing T cell activity |
| *H. pylori* | CagA and VacA | Endolysosomal vesicular trafficking and impairs the autophagy pathway | Contribute to the progression of precancerous lesions and ultimately tumor formation |
| Genetically modified *E. coli* | Antigen | Activation adaptive immunity of $CD8^+$T cells as a vaccine | Prevention of tumor proliferation and metastasis in CT26, B16BL6, 4T1 and MC38 cell line. |
| *E. coli* | LPS | Stimulate to secrete significant amounts of inflammatory cytokine IL-1$\beta$. IL-1$\beta$ reaches the bone marrow through systemic circulation, causing lineage shifts and epigenetic remodelling of haematopoietic progenitor cells | Induce trained immunity to enhance tumour vaccinations |
| *Akk* | Amuc_2172 | As an acetyltransferase of Lys14 on histone H3 (H3K14ac) to promote the transcription and secretion of HSP70 in cancer cells | High level of HSP70 promoted $CD8^+$ cytotoxic T lymphocytes-related immune response in the process of tumor-genesis |

**Table 1** (*continued*)

| BEVs origin | Active substance | Intervention mechanism | Pathological change |
|---|---|---|---|
| *Akk* | ____ | Augment anti-programmed cell death protein 1 | Inhibit of CT26 colorectal cancer |
| Central nervous system | | | |
| Gut microbiota of AD patients | ____ | Increase of tau phosphorylation | Initiate of AD pathology in murine models. |
| *H. pylori* | ____ | Astrocyte reactivity *via* NF-$\kappa$B activation | Neuronal damage in AD murine models |
| *H. pylori* | ____ | Activate glial cells and promote amyloid-$\beta$ aggregation *via* the C3aR signaling pathway | Impair neuronal function |
| *P. gingivalis* | Gingipains | Activate of NLRP3 inflammasome through delivery of gingipains into cerebral microvascular endothelial cells, initiation of AD pathology | Neuroinflammation, BBB disruption, ferroptosis, tau phosphorylation, and memory impairment in murine models |
| *Lactobacillus* | ____ | Increase BDNF expression in hippocampal neurons | Exhibit antidepressant-like effects in AD murine models |
| *Lactobacillus* | ____ | Upregulate MeCP2 and Sirt1 | Inhibit amyloid-$\beta$ protein deposition in transgenic Tg-APP/PS1 mice |
| *Hemophilus influenzae type b (Hib)* | Lipooligosaccharide (LOS) | − | Triggers meningitis |
| *Escherichia coli K1* | ____ | Activate microglia and astrocytes; recruit of neutrophils to the CNS | Neonatal bacterial meningitis |
| Meningococcal B vaccine, 4CMenB | fHbp, NadA, and NHBA and OMV | PorA 1.4 antigenicity | Prevent of meningococcal infections |
| Bone-related disorder | | | |
| *F. alocis* | Lipoproteins | TLR2 activation | Proinflammatory cytokine production, osteoclastogenesis, and bone resorption |
| *Akk* | ____ | ____ | Mitigate osteoporosis through enhancing osteogenic activity and inhibiting osteoclast formation |
| *P. gingivalis* | Peptidylarginine deiminase (PPAD) | ____ | Instigate an autoimmune reaction |
| *F. nucleatum* | FadA | Activate Rab5a-YB-1 pathway to induce RA | Induce rheumatoid arthritis |
| *Lactobacillus (Lac)* | ____ | ____ | Exhibit proangiogenic, pro-osteogenic, and antiapoptotic properties within the femoral head |
| Metabolic disease | | | |
| *P. gingivalis* | ____ | Attenuate insulin-mediated Akt/GSK-3$\beta$ signaling | Disrupt glucose homeostasis and accelerate diabetes progression in mice |
| *P. gingivalis* | ____ | PAR-2 activation | Retinal microvascular endothelial cell dysfunction in diabetic retinopathy |
| Fecal bacteria from diabetic rat | DNA | Delivered microbial DNAs into $\beta$ cells by triggering cGAS/STING activation | Result in elevating inflammation and impaired insulin secretion |
| *EcN* | Obesity | ____ | Diminish blood glucose levels and increase plasma insulin concentrations |
| *Akk* | Amuc_1100 | Activate TLR receptors | Improve the intestinal barrier, reduce blood glucose and limited fat accumulation |
| Other disease | | | |
| *Akk* | ____ | Through the EGFR−PI3K−AKT signal | Alleviate preeclampsia-like symptoms in mice by facilitating trophoblast invasion and spiral artery remodeling |
| *P. gingivalis* | ____ | Degradation of endothelial cell−cell adhesins (such as PECAM-1) | Elevate vascular permeability |

(*continued on next page*)

**Table 1** (*continued*)

| BEVs origin | Active substance | Intervention mechanism | Pathological change |
|---|---|---|---|
| *H. pylori* | LPS and CagA protein | Elevate ROS levels and augment NF-κB activation in HUVECs cell | Atherosclerosis plaque formation *via* endothelial damage |
| BEVs from non-alcoholic steatohepatitis patients | ____ | Through nmMLCK pathway | Activate profibrotic and proinflammatory pathways in hepatic stellate cells, contribute to liver injury |
| BEVs from decompensated cirrhosis patients | *E. coli* OMV | ____ | Activate macrophage, exacerbate fibrosis, decrease albumin levels in a mouse model of cirrhosis |
| BEVs from primary sclerosing cholangitis in conjunction with IBD | ____ | Initiate the TLR4 and NLRP3-Gasdermin D signaling cascades | Activate of both hepatocytes and stellate cells, then intensify liver inflammation and fibrosis |
| BEVs from gut microbiota | DNA | Activate the cGAS-STING-IFN-I axis | Safeguard from viral threats. |

BEVs, bacterial extracellular vesicles; IBD, inflammatory bowel disease; OMV, outer membrane vesicles; pLYN, phosphorylation of tyrosine kinases LYN; HTRA, high-temperature requirement A; RIPK1/RIPK3, receptor-interacting protein kinase 1/3; FADD, Fas-associated death domain; CARD3, caspase activation and recruitment domain 3; TLR, toll-like receptors; TIRAP, toll-interleukin-1 receptor domain-containing adaptor protein; IL-10, interleukin-10; DC, dendritic cells; PKCζ, protein kinase C-ζ; ERK1/2, extracellular-signal-regulated kinase 1/2; anSME, anaerobic sulfatase maturating enzyme; PPARγ, peroxisome proliferator-activated receptor γ; PBMCs, peripheral blood mononuclear cells; GADD45A, growth arrest and DNA damage-inducible proteins; NFκB, nuclear factor-kappa B; STAT3, signal transducer and activator of transcription 3; PI3K, phosphatidylinositol 3-kinase; FAP2, fusobacterium autotransporter protein 2; TIGIT, T cell immunoreceptor with Ig and ITIM domains; HSP70, heat-shock protein 70; C3aR, complement component 3 (C3)-C3a receptor; NLRP3, nucleotide-binding and oligomerization domain, NOD-, leucine rich repeat, LRR- and pyrin domain-containing protein 3; AD, Alzheimer's disease; BBB, blood-brain barrier; BDNF, brain-derived neurotrophic factor; MECP2, methyl CpG binding protein 2; SIRT1, silent information regulator sirtuin 1; Tg-APP/PS1, Tg-APP(β-amyloid precursor protein)/PS1(presenilin); CNS, central nervous system; Rab5a-YB-1, RAS oncogene family member-Y-box binding protein 1; RA, rheumatoid arthritis; Akt, attenuating insulin-mediated protein kinase B. GSK-3β, glycogen synthase kinase-3; PAR-2, protease-activated receptor-2; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase; STING, stimulator of interferon genes; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol-3-kinase; PECAM-1, platelet-endothelial cell adhesion molecule-1; nmMLCK, non-muscular myosin light chain kinase; ROS, reactive oxygen species; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase; STING, stimulator of interferon genes; IFN-I, type I interferon.

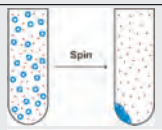
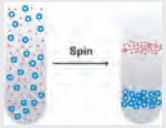
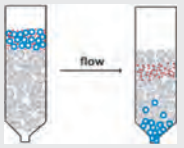
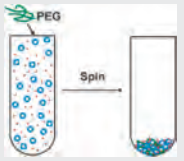
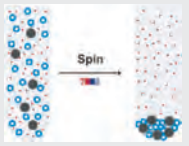
bacterial debris and viscous secretions from specific bacteria can potentially obstruct the microfluidic channels, necessitating meticulous attention during the pretreatment process[159].

In recent times, novel separation strategies have emerged, harnessing the prowess of novel materials and integrating the strengths of diverse methodologies. A prime illustration is EV-FISHER, a platform grounded in a metal-organic framework, adorned with cleavable lipid probes. This innovative tool adeptly extracts EVs from plasma through conventional centrifugation. Subsequently, the entrapped EVs are liberated and collected *via* the targeted cleavage of PSDC by deoxyribonuclease I, a streamlined process that necessitates approximately 40 min and boasts an impressive isolation efficiency of 74.2%, as reported[161]. Another noteworthy commercial advancement in EVs detection is the EXODUS ultrafast-isolation system, which ingeniously exploits negative pressure oscillations and a double-coupled harmonic oscillator-enhanced membrane to achieve exosome purification[162].

It is important to note that isolated BEVs represent a collection of EVs within a specific diameter range[163]. However, the development of techniques for the specific separation of BEVs particularly within complex biological samples remains a formidable challenge. As we know, apart from a small amount of BEVs, eukaryotic cells secrete a substantial proportion of exosomes into blood and body fluids. The current separation methodologies, however, are plagued by lengthy and intricate procedures. Magnetic beads tailored specifically for unique proteins of BEVs like LPS and LTA may possibly address this challenge[164]. Alternatively, it is possible to label the outer membrane components of BEVs, notably lipid proteins, and subsequently harness techniques, *e.g.*, click chemistry, to couple these labeled BEVs with readily separable nanoparticles, ultimately facilitating the rapid and efficient isolation of BEVs from biological samples. Furthermore, bacterial culture conditions, sample handling procedures, and isolation methodologies affect the separation of BEVs[138]. This inherent variability poses obstacles to the reproducibility of BEVs separation, thereby emphasizing the paramount importance of rigorous characterization and identification protocols to guarantee consistent quality standards. Additionally, it is imperative for researchers to furnish exhaustive details of preconditioning factors, including bacterial culture concentration and incubation period, aspects that are frequently overlooked. Establishing a comprehensive reference database dedicated to characterizing BEVs derived from diverse bacterial origins and purification strategies is indispensable for standardizing research endeavors within this domain. Furthermore, contemporary isolation techniques, barring immune-affinity-based approaches, struggle to differentiate among various BEVs subtypes, thereby restricting the scope of further research subdivisions within this field[165].

BEVs encompass a diverse array of biomolecules, including bacterial DNA and RNA, proteins, lipids, and metabolites. To gain insights into their unique attributes, such as biological heterogeneity, structure, and molecular cargo, various techniques

**Table 2** The summarize of different isolation methods for BEVs.

| Methods | Scheme | Strengths | Issues |
|---|---|---|---|
| Ultracentrifugation |   | Gold standard; stable | Long time |
| Density gradient centrifugation |   | High purity | Long time |
| Ultrafiltration |   | Simple operation; time saving | Membrane life; low-purity |
| Size-exclusion chromatography (SEC) |   | High purity; fast simple | Packing materials; specialized equipment |
| PEG precipitation |   | Fast and easy; high recovery | Low-purity |
| Immuno-precipitation (IP) |   | High specificity and simple operation | Low efficiency; multiple influencing factors; complex operation |
| Microfluidic technology |   | Easy to operate; enlarge | Lack of standardized operation |

have been devised. Initially, morphological characteristics are analyzed using methods like transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and dynamic light scattering (DLS). To delve into specific proteins presented in BEVs, techniques like Western blotting, (Immuno)electron microscopy, and flow NanoAnalyzer are employed[147,166]. Moreover, 16S rRNA-based nucleic acid detection methods are frequently utilized to identify specific BEV species[48]. Beyond these established methods, there is a growing interest in developing rapid and sensitive detection strategies for EVs, which can be adapted for BEVs detection as well. For instance, Zheng[167] at Tulane University utilized nanoparticle-enhanced immunoassay read by dark-field microscopy (NEI image) to detect lipoarabinomannan (LAM) and membrane protein LprG, two virulence factors co-existing on EVs secreted by *Mycobacterium tuberculosis* (*M. tuberculosis*)-infected macrophages. Here they report a nanoparticle-enhanced immunoassay read by dark-field microscopy that detects two *M. tuberculosis* virulence factors (the glycolipid lipoarabinomannan and its carrier protein) on the surface of circulating extracellular vesicles. Additionally, aggregation-induced emission (AIE) has been applied to label BEVs, achieving a remarkable fluorescence signal-to-background ratio[168]. Collectively, these advancements provide practical tools that enable the investigation of BEVs functionalities in both physiological and pathological contexts (Fig. 4).

### 5.2. *BEVs serve as novel diagnostic biomarkers*

As the digestive tract harbors an abundant array of commensal bacteria, these microorganisms secrete substantial BEVs that traverse the intestinal epithelium, infiltrating the circulatory system and even reaching distal organs. In instances of intestinal barrier dysfunction, damage, disease, aging, or drug exposure, an increased influx of BEVs into the bloodstream is observed[8,169]. Remarkably, in conditions including human immunodeficiency virus (HIV), IBD, and the presence of tumors, where there is an impairment in intestinal barrier function, the concentration of BEVs in human peripheral venous blood has been documented to surge significantly, reaching levels as high as $10^5$–$10^6$ particles per milliliter[147]. These BEVs encapsulate a diverse array of biological information molecules and metabolic products derived from their parental bacteria. Disseminated across various biological fluids like blood, urine, and saliva, they serve as mirrors of the

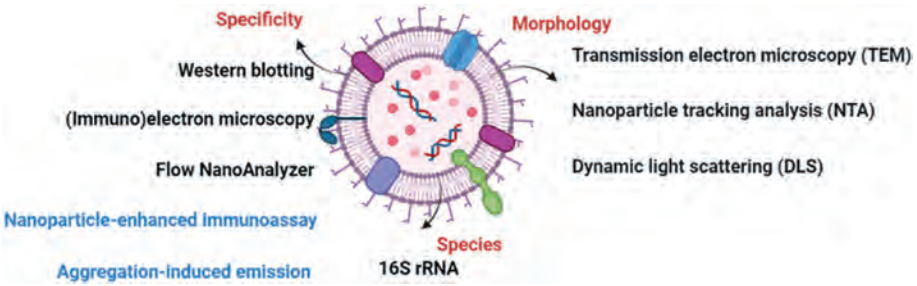



**Figure 4** Schematic depiction of the BEVs planform for detection. To scrutinize the morphology and size of BEVs, various techniques are routinely employed, including transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and dynamic light scattering (DLS). For the examination of specialized structures like proteins adorning BEVs, Western blotting, (immuno)electron microscopy, and the utilization of a Flow NanoAnalyzer are highly recommended approaches. Furthermore, 16S rRNA-based nucleic acid detection methodologies are frequently leveraged to identify specific BEVs species with precision. Additionally, innovative methods involving nanoparticle-enhanced immunoassay and aggregation-induced emission have been devised, expanding the toolbox for BEVs analysis. The figure was created with https://app.biorender.com/.

host's microbial composition, actively engaging in bacterial-bacterial and host-bacterial crosstalk. They reflect the physiological and pathological states of the host, offering insights into its well-being. Through the systemic circulation of BEVs, the intestinal microbiome exerts immunomodulatory effects on recipient cells located in distant organs, underscoring its pivotal role in maintaining host health and homeostasis.

Previous studies have indicated that the size and number of serum OMVs could serve as valuable tools in diagnosing potential infections, with the OMV-bacteria aggregation assay emerging as a rapid diagnostic test for identifying infectious microorganisms in osteomyelitis patients[113]. Ou et al.[164] made an interesting discovery, observing a marked elevation in circulating BEVs in human blood, which they attributed to an age-related intensification of intestinal permeability. Moreover, they established a positive correlation between the presence of BEVs in the bloodstream and gut-related diseases, including colorectal cancer and colitis. Further research has revealed that $LPS^+$ OMVs, along with global 5 mC methylation and OMVs secreted by four key periodontal pathogens, are significantly elevated in the saliva of periodontitis patients compared to healthy individuals[170]. A prospective study involving 2222 adults contributed urine samples for the analysis of genomic DNA from BEVs, revealing that the composition of *Proteobacteria* is associated with an increased risk of abdominal obesity, while that of *Firmicutes* positively correlates with it. This suggests that the composition of BEVs in urine may serve as a predictor for the 10-year risk of abdominal obesity[171]. Additionally, the presence of OMVs in bodily fluids holds immense potential as diagnostic biomarkers for psychiatric disorders. Research has shown a reduced concentration of blood-borne bacterial extracellular vesicles among individuals with psychosis[172], while Lee et al.[173] have validated the significance of urine-derived bacterial extracellular vesicles in swiftly assessing microbiota alterations in patients with autism spectrum disorder (ASD), highlighting their potential as non-invasive diagnostic tools. Circumstantial evidence underscores that the tissues and blood of cancer patients harbor unique microbial fingerprints, capable of distinguishing between a broad spectrum of cancer types, even those distant from the digestive tract[174]. Acknowledging this intricate connection, it becomes increasingly apparent that the intestinal microbiome is intricately interlinked with virtually all aspects of cancer pathology. The proteomes of BEVs sourced from solid tumor patients exhibited greater diversity than those from the control group. Notably, these proteins were enriched in processes involving amino acid and carbohydrate metabolism, nucleotide binding capabilities, and oxidoreductase activities. Additionally, utilizing BEVs for metadata classification of samples achieved a higher accuracy rate of 100%, surpassing the 93% accuracy achieved with fecal samples[175]. Notably, while the precise role of BEVs in neoplastic diseases is yet to be fully elucidated, their presence has been consistently detected in diverse bodily fluids associated with cancer patients, as consolidated in Table 3 [177-182].

Drawing upon the available evidence, BEVs in the digestive tract possess the capability to traverse the barrier and infiltrate into the internal environment. The isolation of BEVs from human body fluids, coupled with metagenomic sequencing technology, offers fresh biological perspectives into the intricate relationship between microbiota and diseases. Furthermore, BEVs hold promising potential to emerge as a novel class of biomarkers for diagnosing and understanding various disease states[176].

### *5.3. BEVs as drug cargo from gut to other issues*

As previously stated, in contrast to conventional bacterial metabolite molecules, BEVs hold exceptional significance for the production of hydrophobic molecules or insoluble substances, facilitating long-distance transport and enabling synergistic therapeutic approaches. Given their pivotal biological roles and immense medical potential, BEVs in the digestive tract are being actively explored as therapeutic carriers for various diseases. Our research group[79] has garnered experience in harnessing BEVs in the digestive tract to modulate host immunity. Specifically, we employed *E. coli* OMVs to express tumor antigens (Ag) and the Fc fragment of mouse IgG (mFc), fused to the C-terminus of ClyA, a surface protein of OMVs (ClyA-Ag-mFc). This fusion protein was recognized by DCs *via* the neonatal Fc receptor (FcRn), thereby specifically activating intestinal immunity-the largest immune system in the body-to develop oral tumor vaccines. Remarkably, these OMV-Ag-mFc constructs efficiently traversed intestinal epithelial barriers and were subsequently internalized by DCs located in the lamina propria. This led to their transport to lymph nodes, where tumor antigen presentation occurred. Consequently, tumor antigen-specific immune activation

**Table 3** The latest research of BEVs as potential biomarkers in cancer diseases.

| Source of BEVs | Disease | Methods | Application | Ref. |
|---|---|---|---|---|
| Serum and brain tissue | Brain tumor | Metagenomic analysis of BEVs in serum and brain tissue samples based on the V3−V4 regions of 16S rDNA, logistic regression and machine learning algorithms | Developed diagnostic models to assess the ability of various dietary supplements to reduce brain tumors risk *in vivo* | 177 |
| Serum | Ovarian cancer | Obtained serum samples from patients with pathologically confirmed ovarian cancer and patients with benign ovarian tumors, isolation of BEVs from serum samples of the patients and 16S rDNA amplicon sequencing | Developed a diagnostic model identifying ovarian cancer from benign ovarian tumor | 178 |
| Stool | Ovarian cancer | Metagenomic profiling by sequencing 16S rDNA, gas chromatography-time-of-flight mass spectrometry | Determined the association of metagenomics and metabolomics in gut BEVs of CRC and healthy subjects | 179 |
| Serum | Lung cancer | Microbiome analysis of indoor dust EVs isolated from mattresses in apartments and hospitals, ELISA | Developed diagnostic models based on the anti-BEVs antibodies detected in serum samples in lung disease | 180 |
| Blood | Pancreatic cancer | 16S rRNA gene analysis to identify compositional differences of microbiome between patients with pancreatic cancer and healthy controls, acquired BEVs from blood samples, 16S rRNA gene analysis | Constructed a pancreatic cancer prediction model | 181 |
| Urine | Colorectal cancer | BEV isolation from the urine of patients with CRC and healthy controls, DNA extraction, next-generation sequencing of the 16S rRNA | BEVs as a potential biomarker for the diagnosis of CRC | 182 |

BEVs, bacterial extracellular vesicles; CRC, colorectal cancer; ELISA, enzyme-linked immuno sorbent assay.

was achieved, resulting in a pronounced inhibition of tumor growth and enhanced resistance to tumor challenges across multiple murine cancer models. Coincidentally, Tomasi et al.[80] also strengthen the novel function of OMVs in host-pathogen interaction and exploited of engineered probiotics and OMVs with tumor specific-antigens as personalized mucosal cancer vaccines. Additionally, Huang et al.[183] have validated the biofilm-integrated nano-drug delivery system, comprising OMVs, mesoporous silica nanoparticle (MSN), and 5-Fluorouracil, for its potent anti-tumor effect on lymph node metastasis arising from oral squamous cell carcinoma. In addition to tumor treatment, our team has genetically engineered *EcN* to secrete OMVs, whose surfaces have been modified with nanobodies (anti-TNF$\alpha$) for the treatment of IBD. These genetically modified OMVs not only modulate the immune response within the colonic lamina propria to alleviate colitis but also serve as efficient vehicles for delivering anti-TNF$\alpha$ nanobodies to repair the intestinal barrier. Notably, this OMV-based delivery approach demonstrates superior therapeutic efficacy compared to secretory nanoantibodies, as evidenced by our unpublished data. Apart from its application in the gut, BEVs has garnered widespread adoption as drug vehicles in various therapeutic avenues. We summarized several engineered strategies tailored for BEV's utilization as a drug cargo, which could also be used in digestive tract (Fig. 5).

**Expression**. Heterologous peptides, nanobodies, or even entire proteins can be displayed on outer membranes, inner membranes, or within the lumen *via* genetic engineering techniques[79,184,185]. It contingent upon the type or orientation (N- and C-terminals) of the fusion protein utilized. **Insertion**. Given that BEVs are primarily composed of lipids, phospholipids, or hydrophobic molecules can be incorporated into BEVs *via* extrusion or ultrasound techniques, leveraging principles of similarity and dissimilarity[186,187]. Moreover, during the secretion phase of BEVs from bacteria, the incorporation of artificial polysaccharides and phospholipids onto these vesicles becomes feasible through the addition of nutrient substitutes[188], thereby enhancing their potential for further engineering and diverse applications. **Encapsulation.** Owing to the presence of the lumen in BEVs, the interior space can load nanoparticles, nucleic acids, drugs, *via* extrusion, ultrasound and electroporation methods[183,189,190]. **Absorption**. Due to the BEVs surface's negative and hydrophilic characteristics, it possesses the capability to adsorb positively charged antigens, nucleic acids, or hydrophilic drugs when mixed in precisely controlled proportions[191]. **Biomineralization**. BEVs possess the capability to fabricate functional inorganic metal materials or ion endowed with a multi-level ordered composite structure *via* biomineralization processes, enabling the attainment of advanced therapeutic functionalities[192]. **Conjugation**. By incorporating innovative components into the BEVs using the aforementioned methodologies, we can procure an enhanced refinement system *via* biological conjugation or chemical conjugation processes, thereby augmenting their functionality[5,6,188,193,194].

We firmly believe that BEVs, owing to their nanosized structures, low toxicity, and exceptional biocompatibility within the gut,

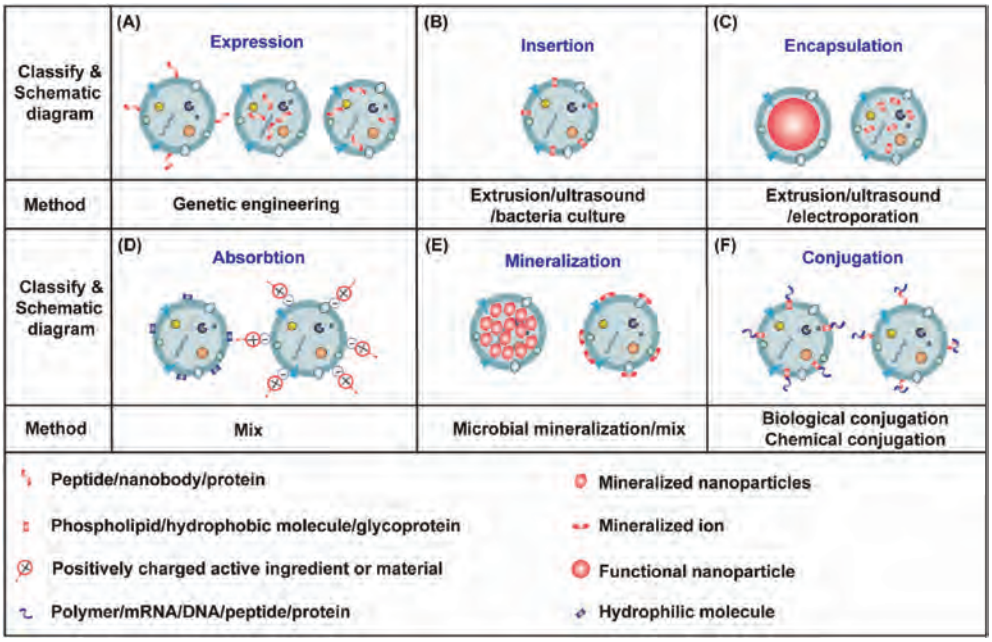



**Figure 5** Methods of engineering BEVs. (A) Expression: the target molecules are genetically engineered to be expressed on the outer membrane, inner membrane, or lumen of BEVs, optimizing their localization for specific applications (B) Insertion: hydrophobic molecules are incorporated into the membrane of BEVs utilizing extrusion or ultrasound. (C) Encapsulation: nanoparticles, nucleic acids, and drugs are encapsulated within the lumen of BEVs through extrusion, ultrasound, and electroporation methods. (D) Adsorption: positively charged or hydrophilic molecules are adsorbed onto the surface of BEVs *via* a simple mixing process. (E) Biomineralization: inorganic metal materials or ions are integrated with BEVs through biomineralization processes, creating composite structures. (F) Conjugation: target molecules are coupled to BEVs *via* biological or chemical conjugation processes, synergistically combining the aforementioned methodologies.

hold immense potential as a promising platform for the treatment of a wide array of diseases. It demonstrated the remarkable ability to locally access distant tissues *via* oral administration, underscoring its potential as an efficacious and minimally invasive drug delivery platform, extending to liver, bone, and various other tissues. These engineered BEVs regulate the gut environment through direct and indirect effects that ultimately affect the body.

## 6. The challenge and further study of BEVs

### *6.1. The fate of BEVs in vivo*

Despite the presence of robust physical and chemical barriers in the intestine, including the mucous layer lining the intestinal lumen, tight junction proteins, lysosomal degradation mechanisms, immunoglobulins, and digestive enzymes, BEVs inevitably gain access to the bloodstream and subsequently distribute to various organs. This prompts the inquiry: what mechanisms enable these BEVs to overcome a multitude of biological obstacles, including the blood-brain barrier, placental barrier, and phagocytic clearance within the intestinal tract, to successfully enter and circulate within the host's systemic circulation?

A potential route for traversing these barriers is through receptor-mediated transcytosis. Studies have documented that the outer membrane protein A (OmpA) present on *E. coli K1* facilitates invasion of the BBB by binding to gp96 on BBB endothelial cells[195]. Chen et al.[196] capitalized on this mechanism, utilizing LPS-free *E. coli K1*-derived BEVs combined with nanoparticles to achieve efficient intracerebral drug accumulation. However, it is imperative to acknowledge that the disparities between BEVs in different species could potentially influence the transcytosis process. In addition to receptor-mediated transcytosis, Liam-Or[197] delved into the influence of the protein corona-extracellular vesicles (EVs derived from mesenchymal stem cells) complex under both *in vitro* and *in vivo* conditions. They found that the binding of albumin to these EVs creates a unique signature that redirects EVs away from hepatic macrophages and enhances cellular uptake by hepatocytes, liver sinusoidal endothelial cells, and hepatic stellate cells. Kumari[198] additionally discovered that EVs derived from both human, murine plasma cell culture, have an intrinsic capacity to bind bacterial LPS, which is also abundant in OMVs. These researches underscore the potential of a camouflage strategy, whereby BEVs might form a corona-BEVs complex. This approach could significantly impact *in vivo* transport and fate, offering exciting avenues for further exploration.

### *6.2. Ethical implications and safety of BEVs*

The clinical utilization of novel therapeutic technologies underscores the paramount importance of meticulously weighing safety and efficacy, adhering to fundamental ethical principles, notably the principle of informed consent[199]. This scrutiny is particularly evident in preceding treatment modalities, such as fecal microbiota transplantation (FMT), which has encountered obstacles pertaining to safety and standardization[200]. Given the individual variations and the likelihood of concealed underlying ailments, ensuring the health of donors poses a significant challenge, thereby complicating the management of FMT's composition and quality[199,201]. Long-term employment of FMT carries the peril of transmitting chronic illnesses from donors to recipients, while the colonization of viable bacteria heightens the risk of infection, particularly among vulnerable populations[202].

Currently, bioactive constituents sourced from live bacteria, notably probiotics like BEVs and short-chain fatty acids, are being scrutinized as viable alternatives[203]. These "postbiotics" are hypothesized to recapitulate the salutary effects of live bacteria while mitigating certain associated hazards[204,205]. For patients with compromised intestinal barriers, immunodeficiency, or weakened immune systems, BEVs-devoid of proliferative activity-represent a potentially safer therapeutic avenue than live bacterial interventions. Additionally, BEVs may boast enhanced quality control through strategic selection of source strains, refined extraction techniques, and theoretically superior stability during frozen storage and transportation compared to fecal microbiota[2,206,207]. Furthermore, BEVs from diverse bacterial origins can be amalgamated in a more controlled fashion, empowering physicians to personalize treatment doses according to individual patient requirements.

Nonetheless, despite these promising attributes, the clinical safety of BEVs necessitates further exploration. While numerous preclinical studies have successfully mitigated BEV toxicity by reducing surface LPS and other immunogenic moieties or employing specific surface modifications and engineered remodeling, prudence is warranted in their application[208,209]. As bacterial derivatives, BEVs possess the capability for systemic dissemination *via* the bloodstream and exhibit robust penetration across various physiological barriers, including the blood−brain barrier and placental barrier[132,195]. Accumulation of BEVs in non-target organs may elicit unforeseen adverse effects. Moreover, the potential for BEVs to traverse critical barriers evokes concerns regarding the induction of autoimmune responses, particularly considering the association of BEVs from certain pathogenic bacteria with autoimmune disease pathogenesis[115].

Beyond the approved meningococcal B vaccine, numerous research groups have been diligently engaged in the development of vaccines or drugs utilizing BEVs over an extended period. Here we summarized diverse research teams regarding the *in vivo* safety evaluation of BEV pre-clinical animal models. Within our group[184] in Li Yao's study, vesicles harboring 50 μg of protein, equivalent to approximately 5−6 ng of LPS, significantly below the LPS $LD_{50}$ documented in literature[210] (15 mg/kg, translating to 300 μg per mouse). The limulus amebocyte lysate (LAL) test confirmed that the administered OMV dosage through vein injection (20 μg/mL of vesicle protein per mouse) fell within a highly secure threshold. Ping's group[186] observed little hemolysis effects upon achieving a total protein concentration of 50 μg/mL in the final BEV-based formulation. The optimized dosing protocol for OMV administration, established as 20 μg/mL per mouse with a total of three administrations, ensured both efficacy and safety were meticulously balanced. However, in Qing's study[211], regarding the various doses administered through intravenous injection, specifically low-dose BEV (5 μg × 1), high-dose BEV (25 μg × 1), and multiple-dose BEV (administered every other day, totaling 5 μg × 5), it was noted that by the experiment's endpoint on Day 14, 50% of the mice in the single high-dose group had succumbed. Notably, fatalities were also recorded in both the single low-dose and multiple-dose groups. However, in sharp contrast to the naked BEVs, none of the biomineralized BEV-treated mice died at the endpoint. This detoxifying effect of engineered BEVs, similarly observed in our research utilizing OMV-PEG, underscores the potential of such modifications[188]. Therefore, while BEVs present a promising alternative to live bacterial therapies, their clinical translation must proceed cautiously and be underpinned by exhaustive research to guarantee patient safety. To ensure the safe and efficacious use of BEVs, it is imperative to regulate dosage, mode of administration, as well as the specific therapeutic context in which they are employed.

### 6.3. Future prospects of BEVs-related research

From a biological perspective, research delving into the inherent biological implications of gut BEVs remains inadequate. A deeper understanding of the specific pathogenic or therapeutic constituents residing on the membrane surfaces and within the vesicles of BEVs, particularly those originating from diverse bacterial sources in gut microbiome, is crucial. The potential consequences of prokaryote-derived enzymes, peptides, nucleic acids, and other molecular cargo on eukaryotic cells are still largely unexplored. Besides, once we have identified the gut-associated BEVs and the pivotal molecules that modulate the diverse physiological and pathological pathways of the host, we can devise a strategy for this process, grounded firmly in our foundational research. For instance, the secretion of intestinal bacterial vesicles can be intricately modulated, either augmenting or diminishing it, *via* targeted methods like genetic engineering and molecular interventions[212,213]. Additionally, the advantageous components of BEVs hold immense potential for further development into therapeutic agents, innovative applications, and clinical interventions.

The current methodologies employed for the extraction and purification of BEVs are still in need of comprehensive optimization. Despite recent breakthroughs, including the EV-Fisher and Exodus technologies, which have significantly hastened the process and enhanced the purity of BEVs extraction, their substantial costs continue to pose an obstacle, hindering their potential to supersede traditional techniques like ultracentrifugation[161,162]. Consequently, relentless endeavors directed towards refining and devising more economical purification approaches for large scale are imperative. Additionally, given that the characterization of BEVs is influenced by numerous factors, including growth stage and extraction procedures[165,214], establishing a unified set of BEVs characterization standards that encompass various strains and extraction methodologies is vital for ensuring reproducibility across diverse research laboratories. This endeavor is fundamental to advancing the scientific understanding and application of BEVs. Furthermore, the gradual deterioration of active components within BEVs over time and their structural vulnerability during freeze-thaw cycles present another challenge[215]. The development of efficacious stabilizers is paramount to preserving the integrity and functionality of BEVs throughout storage and transportation.

As previously elaborated, the inherent immunogenicity of BEVs renders them ideally suited for various applications in vaccines or tumor immunotherapy, encompassing immune induction and potentiation. Nevertheless, the market remains scarce in terms of vaccine availability, and the prokaryotic bacteria inherent to BEV lack the glycosylation modification, rendering them more compatible with bacterial vaccine formulations. Besides, despite extensive research endeavors delving into the pivotal functions of BEVs in stimulating immune cells, facilitating tumor cell apoptosis, immune priming, and enhancing chemosensitivity, the aspect of safety necessitates continued attention and consideration. Anticipating future advancements, conducting rigorous Phase I and II clinical trials is indispensable for comprehensively evaluating the safety profile and therapeutic efficacy of BEVs within the realm of clinical tumor immunotherapy or drug

delivery. These trials will yield invaluable insights into the transformative potential of BEVs as novel therapeutic modalities, thereby guiding their future clinical utilization.

## 7. Summary

The research on BEVs has made tremendous progress recently. Overall, our review summarized the origin of BEVs, the function in bacteria-bacteria interactions and bacteria-host communication. Different from the conventional perception of BEVs as a pathogenic agent, our summary delved into the effect of BEVs derived from digestive tract bacteria, focusing on their dualistic (bolster host health or contribute to pathologies) impacts on a spectrum of conditions, encompassing IBD, tumors, central nervous system disorders, bone related diseases, and metabolic related diseases. Intestinal permeability is significantly influenced by both external and internal factors, including the aforementioned dietary habits and medications, as well as hormonal fluctuations in disease states, and aging. Consequently, the influence of circulatory BEVs in various disease states deserve further study. We believe that our current summation represents merely the surface of the influence that BEVs have on human health. As deeper exploration into the realm of digestive tract microbiota and increase investments in this pivotal area, we anticipate uncovering a wealth of additional evidence elucidating the effects of BEVs on overall well-being, ultimately informing and guiding the application of clinical interventions for disease management.

Simultaneously, as our understanding of BEVs expands, several engineering and technological challenges pertinent to them necessitate further scrutiny. As a result, we also provide a summary of the current research endeavors focused on BEVs, encompassing their separation, detection, and innovative drug cargo and diagnostic applications. To elaborate, current methodologies for isolating BEVs often mirror those used for EVs. Given the diverse composition of biomolecules within BEVs, the development of tailored separation techniques is both feasible and imperative for further exploration. Furthermore, BEVs offer advantages over their bacterial progenitors, such as enhanced diffusivity, superior biocompatibility, and reduced infection risks, making them highly attractive candidates for novel therapeutic and diagnostic avenues. Thus, there is a significant interest in leveraging these unique properties for various medical applications, though the safety aspects remain worthy of discussion. Lastly, we highlight the fundamental challenges surrounding the *in vivo* fate of BEVs, ethical implications and safety and the future BEV-related study. We eagerly anticipate that greater emphasis will be placed on this research trajectory, thereby furnishing a robust theoretical foundation for elucidating the intricate interplay between BEVs and the host, as well as advancing the development of BEVs-inspired engineering and bionic drug technologies in clinic. Furthermore, we aspire for this review to act as an invaluable compass for BEVs investigations, ultimately propelling the exploration of groundbreaking studies pertaining to gut microbiome-host communication.

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## Author contributions

Dingfei Qian, Peijun Xu, Xinwei Wang, Chong Du wrote the original draft. Xiao Zhao conceptualized and supervised the article. Jiaqi Xu conceptualized, supervised, wrote, revised the article, and visualized the figures.

## Conflicts of interest

All authors declare that they have no competing interests.

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