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The biological activity and potential of probiotics-derived extracellular vesicles as postbiotics in modulating microbiota-host communication

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Abstract
Probiotics such as *Lactobacillus* and *Bifidobacterium* spp. have been shown to be critical for maintaining host homeostasis. In recent years, key compounds of postbiotics derived from probiotic metabolism and cellular secretion have been identified for their role in maintaining organ immunity and regulating intestinal inflammation. In particular, probiotic-derived extracellular vesicles (PEVs) can act as postbiotics, maintaining almost the same functional activity as probiotics. They also have strong biocompatibility and loading capacity to carry exogenous or parental active molecules to reach distal organs to play their roles. This provides a new direction for understanding the intrinsic microbiota-host communication mechanism. However, most current studies on PEVs are limited to their functional effects/benefits, and their specific physicochemical properties, composition, intrinsic mechanisms for maintaining host homeostasis, and possible threats remain to be explored. Here, we review and summarize the unique physicochemical properties of PEVs and their bioactivities and mechanisms in mediating microbiota-host communication, and elucidate the limitations of the current research on PEVs and their potential application as postbiotics.

Introduction

Since birth, the human body has come into contact with and acquired exogenous microorganisms that colonize the respiratory or intestinal mucous membranes, skin, etc., and evolve over time with environmental changes, ultimately forming a population of bacteria that sustain the ecological niche microbial balance and host health [1]. Numerous studies have shown that dysbiosis of the commensal microbiota is often associated with inflammatory diseases of the gut, type 2 diabetes, obesity, and other diseases [2]. Modulation of microcosmic homeostasis helps the host recover from disease states. Probiotics can be a powerful means of regulating the microecological balance of the host, and the concept of probiotics is of Greek origin, meaning "for life" [3]. In recent years, probiotics have been redefined as "live microorganisms that, when ingested in sufficient quantities, provide health benefits to the host" [4]. Currently, the common probiotics known to help maintain host homeostasis mainly include *Lactobacillus* and *Bifidobacterium* spp., etc. *Akkermansia* is poised to become the next generation of probiotics with its unique efficacy in the areas of host metabolic disorders, cancer and immunotherapy [5]. Many studies have confirmed the key role of these representative probiotics in resisting pathogen

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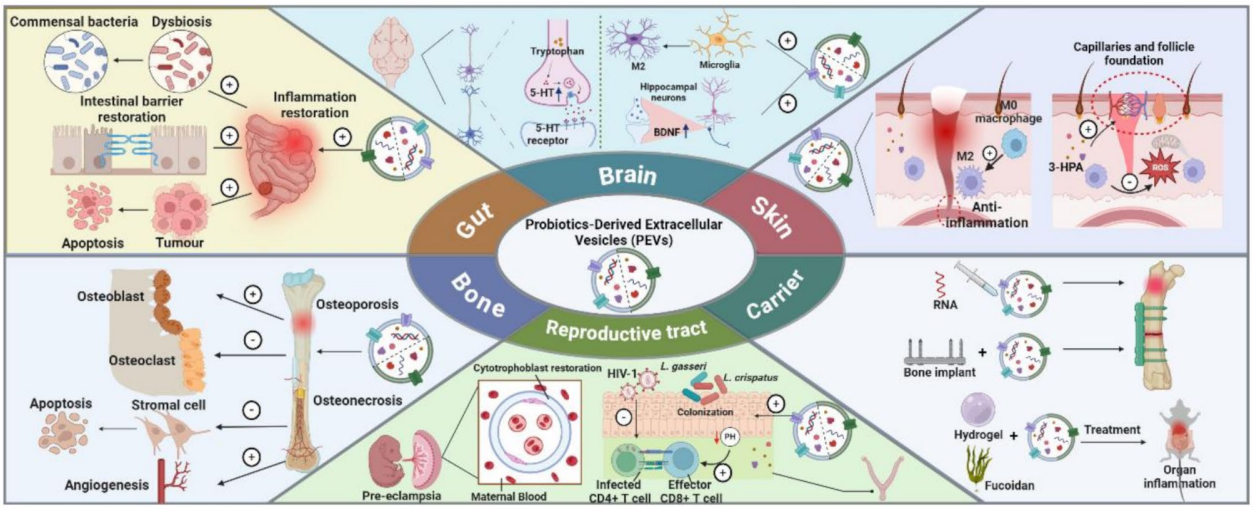
Graphical abstract



Keywords Probiotic-derived extracellular vesicles (PEVs), Extracellular vesicles (EVs), Postbiotics, Host homeostasis, Microbiota-host communication

colonization [6], modulating host metabolism [7] and inflammatory damage [8]. They can be considered a class of exogenous supplements, which are nowadays added to foods or nutraceuticals in appropriate doses [9, 10]. However, the intrinsic mechanisms by which probiotics regulate the host microenvironment and health have yet to be explored, their efficacy and safety in many special populations, especially in children [11], immunosuppressed individuals and critically ill patients [12], are still subject to debate and confusion.

In recent years the concept of postbiotics has been proposed and rapidly applied in nutritional research and food fields. Postbiotics are inactivated microorganisms and/or functionally active ingredient formulations, which mainly include bacterial supernatants, bacterial lysates and various metabolites [13]. Since postbiotics do not contain live bacteria, they have a significantly improved safety profile after host ingestion, and postbiotics can be considered as a suitable alternative to probiotics. Currently, probiotic-derived extracellular vesicles (PEVs) are emerging as postbiotics, and their powerful applications in health and disease treatment are being explored [14].

Extracellular vesicles (EVs) are a class of nanoscale vesicle-like particles secreted primarily by eukaryotes, bacteria, fungi, archaea, and parasites [15]. In recent years, PEVs have been found in the supernatant of probiotic cultures, secreted from the bacterial cell wall or outer membrane as the bacteria continue to proliferate, encapsulating an abundance of active macromolecules of parental components (e.g. nucleic acids, proteins, and lipopolysaccharides) [6, 16]. PEVs maintain biological activities similar to those of probiotics and play a crucial role in maintaining the microecological environment and health of the host [17, 18]. This study provides new ideas for investigating the intrinsic mechanisms of host commensal microbiota and gut homeostasis, as well as new insights to facilitate the development and application of PEVs as postbiotics.

Here, we summarize the unique physicochemical properties and bioactivities of PEVs, some of the regulatory mechanisms mediating microbiota-host communication; and highlight the potential for the development of PEVs as postbiotics for drug and vector delivery. In addition, we discuss the limitations of PEVs in maintaining host homeostatic functions and mechanisms, and highlight the potential threats to PEVs as well as the potential for future development as drug carriers, which provide a guiding direction for future research on PEVs.

Characterization of PEVs

Classification and physicochemical properties of PEVs

Figure 1 shows the differences in PEVs produced by Gram-negative and Gram-positive probiotics from assembly and secretion, physicochemical properties, and bioactive components.

Due to the thick and rigid cell wall of Gram-negative bacteria, the peptidoglycan and envelope are wrapped by a bilayer lipid membrane, forming a double membrane sandwich structure, thus there have been multiple hypotheses regarding how Gram-negative bacteria release EVs. It is currently believed that such EVs are released from the extracellular lipid membranes after

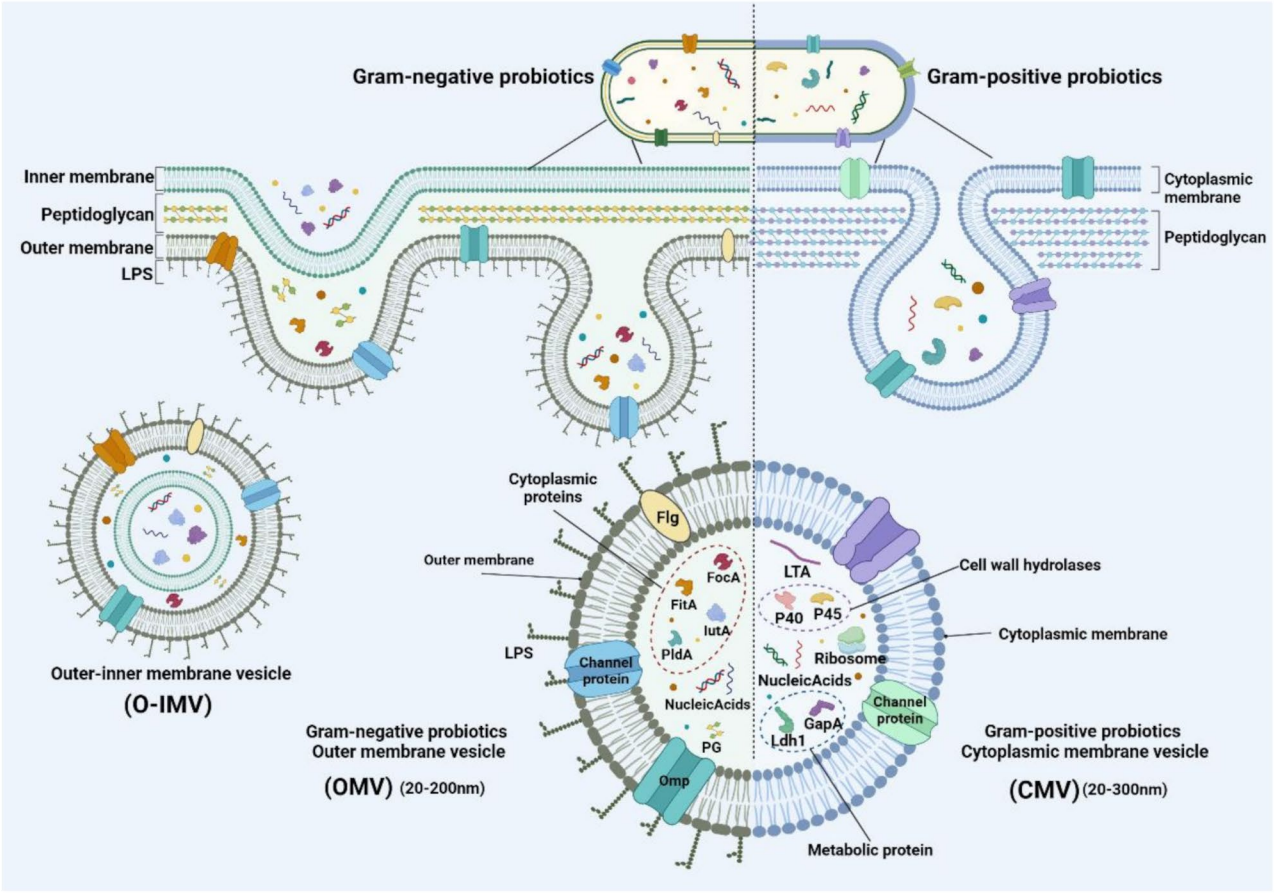


Fig. 1 Schematic representation of probiotics-derived Extracellular Vesicles (PEVs). Figure 1 depicts the cell wall structure of Gram-negative and Gram-positive probiotics and the process of extrusion of Outer membrane vesicle (OMV) and Cytoplasmic membrane vesicle (CMV) from the cell wall, and also includes the morphology of the bilayer lipid membrane structure of OMV and CMV as well as the internal structure of the membrane proteins, cytoplasmic It also includes the bilayer lipid membrane structure of OMV and CMV, as well as their internal structures including membrane proteins, cytoplasmic proteins, nucleic acids and bacterial cell wall components, which visualize the differences in the production, secretion and composition of OMV and CMV

foaming and extrusion by swelling, which are called outer membrane vesicles (OMVs) [19, 20]. In addition, outer-inner membrane vesicles (O-IMVs), which originate from the inner layer of the membrane, carry a large number of cytoplasm, as well as other types of vesicles have been reported in Gram-negative bacteria [21, 22]. The most reported Gram-negative probiotics in the published studies are *E. coli* Nissle1917 (*ECN*) and *Akkermansia muciniphila* (*A. muciniphila*). Both of these OMVs showed classical lipid bilayer vesicle-like structures under electron microscopy, and their particle sizes were in the range of 20–200 nm, based on the differences in bacterial strains, species, genera, as well as on the diverse procedures for characterisation and isolation of the OMVs [23–32].

In contrast, the cell wall structure of Gram-positive bacteria lacks an outer membrane but contains a thick and rigid peptidoglycan structure, which was thought to be incapable of secreting EVs in earlier studies until 1990 when it was first reported [33]. Unlike the OMVs produced by Gram-negative bacteria, EVs from Gram-positive bacteria do not contain an outer membrane, originate from cell membrane outgrowth and secreted across the thick cell wall and contain a large amount of cytoplasmic proteins. These EVs are often referred to as cytoplasmic membrane vesicles (CMVs) [34, 35]. The most reported CMVs are mainly derived from *Bifidobacterium* and *Lactobacillus* spp. Their appearance under electron microscopy is not significantly different from that of OMVs, but the particle size is much larger, ranging from approximately 20–300 nm [36–50], whereas probiotics such as *Ligilactobacillus salivarius* (*Lactobacillus salivarius)* produce CMVs with sizes up to 400 nm [51, 52].

Here, when we refer to different *Lactobacillus*, we will use the new *genus* designation. When an older *genus* is mentioned in the literature, we will indicate the older *genus* in parentheses next to it.

Physicochemical properties of PEVs based on different detection techniques and separation methods

Currently, various methods for characterizing the basic physicochemical properties of PEVs have been published, and there are also differences in the characterization of the same PEVs. Table 1: shows a summarizes the methods for the isolation and detection of PEVs, as well as the size and number of PEVs. Interestingly, differences in the size and morphology of the detected PEVs based on different bacterial cultures, methods of PEVs isolation and purification, and detection techniques led to differences in the size and morphology of the detected PEVs, despite they were isolated and detected from the same probiotic strain. This findings highlights the importance of isolation and detection methods for PEVs.

The most commonly used separation technique for PEVs reported in the literature to date is ultracentrifugation, which separates particles by solute precipitation, primarily based on particle density and size. However, the inevitable co-precipitation of other free protein contaminants in the bacterium complicates exploration for downstream applications [53, 54]. To further remove impurity contamination, Sects [23, 38]. and ultrafiltration [55, 56] techniques are often coupled with ultracentrifugation. However, the specific filtration devices used in these methods may affect the final particle size and yield, although they isolate purer PEVs. In addition, density gradient centrifugation has been applied in some studies [25, 57]. Nevertheless there is a lack of standards and limitations in PEVs separation techniques, which may have implications for the investigation of the biological functions of different and the same PEVs.

Common techniques used to analyze PEVs dimensions are nanoparticle tracking analysis (NTA), dynamic light scattering (DLS) and transmission electron microscopy (TEM). The TEM is more damaging to the sample than the latter two, with limited sensitivity and resolution, and an overly broad detection range [51, 58]. In contrast, the combined application of TEM and NTA can further refine the average size of PEVs [59, 60]. In addition to size, quantity has also been the focus of studies on exocyst parameter. NTA is the most commonly used quantification technique, and the number of PEVs in all studies was in the range of 10^8-10^{12} particles/mL [23, 38, 61, 62], in addition to spectrophotometry [21, 22], and flow cytometry [63]. However, there is a lack of uniformity and standardized procedures for research techniques on the physicochemical parameters of PEVs. Objective comparison and reasonable recognition of the physicochemical properties of multiple PEVs are still needed.

The composition of PEVs

PEVs harbour a variety of active substances, including various membrane structures and secreted proteins, lipids, nucleic acids and bacterial metabolites. Most studies on the components of OMVs have mainly focused on *ECN*, which has been found to possess a strong protein reservoir [23, 64]. Especially, the content of proteins embedded in the outer lipid membranes is as high as 60%, which mainly includes flagellin proteins directly encoded by bacterial genes such as *flgK*, *flgE* and *flgA*. Outer membrane proteins such as OmpA, OmpC, and OmpF, are involved in peptidoglycan rearrangement and bacterial outer membrane construction, assisting in the adhesion of bacteria to epithelial tissues [65] and facilitating survival in the host ecological niche [66]. Interestingly, OMVs also carry a large number of protein molecules encoded by genes linked to symbiotic or pathogenic Gram-negative bacterial chains [67, 68], among which, FocA and FocH are involved in bacterial adhesion, and PldA and FitA are involved in metabolism, which are internalised by the intestinal epithelial cells and act as mild antigens to stimulate the host cells, assist in mucus production, help resist pathogen adhesion, and inflammatory damage [69, 70]. In addition to these abundant proteins, a study by Laura Baldomà' et al. [71] found that the peptidoglycan (PG) structure of *ECN*, when encapsulated by OMVs and endocytosed by the intestinal epithelium, maintains the innate immune response through activation of the cytoplasmic receptor NOD1 signaling and through a cascade reaction. In addition, outer membrane proteins from *A. muciniphila* OMVs are also a current research hotspot, and the critical role of live or pasteurized bacterial Amuc_1100 proteins in the treatment of metabolic disorders, such as obesity [72], and the maintenance of intestinal epithelial integrity [73] has been demonstrated in the past. Recently, Zhang et al. revealed that Amuc_1100, produced by OMVs secreted by trained *A. muciniphila*, reversed type 2 diabetes by reversing insulin resistance through the GLP-1 signaling pathway [74]. In addition, another outer membrane protein, Amuc_2172, gradually comes into view, which not only induces macrophage polarization toward M1 type [75], but also promotes $CD8^+$ T cells signaling to reprogram the tumor microenvironment via OMVs delivery [76]. However, more evidence of the presence of outer membrane proteins on *A. muciniphila* OMVs is still needed to demonstrate the advantages of OMVs delivery.

In contrast, the components of CMVs are significantly different. Based on different cell wall compositions, LPS and periplasm are completely lacking in CMVs [77], but some Gram-positive bacteria-specific lipophosphatidic wall acids (LTA) are encapsulated. LTA in *Lactobacillus*-derived CMVs [78, 79] exhibit potent host immunomodulatory activity with LPS-like bacterial active functions. In contrast, the proteins of CMVs are distinguished from the large number of membrane proteins of OMVs, most of which are the result of passive packaging of a large

Table 1 Characterisation of PEVs and its detection

Bacterial producer	Separation and purification	Size	Amount	Reference
Gram-negative bacteria				
Escherichia coli Nissle 1917(ECN)serotype O6:K5:H1	Ultracentrifugation	20–60 nm (TEM)	ND	[21, 27]
		28.2 ± 9.54 nm (TEM)	ND	[24]
	Ultracentrifugation + SEC	149–189 nm (TEM)	7.8×10^{10} – 1.9×10^{12} particles/mL (NTA)	[23]
	Ultracentrifugation + Density gradient ultracentrifugation	50–150 nm (TEM) 99.2 nm (NTA)	ND	[28]
Akkermansia muciniphila ATCC BAA-835	Density gradient ultracentrifugation	181.9 ± 42.4 nm (DLS)	ND	[25]
	Ultracentrifugation	40–150 nm (SEM)	ND	[29, 30]
		40–60 nm (DLS)	ND	[31]
		20–200 nm (TEM)	ND	[32]
Gram-positive bacteria				
Lacticaseibacillus rhamnosus GG PTCC1637	Ultracentrifugation	30–100 nm (TEM)	ND	[39]
		50–150 nm (TEM)	ND	[44]
Bifidobacterium longum ATCC 15,707	Ultracentrifugation	50–150 nm (NTA)	ND	[40]
*Bifidobacterium longum**	Ultracentrifugation	126 ± 3.78 nm (NTA)	ND	[41]
Bifidobacterium longum AO044	Ultracentrifugation	100–150 nm (cyo-TEM)	ND	[42]
Bifidobacterium longum NCC2705	Ultracentrifugation + Ultrafiltration	50–80 nm (NTA)	ND	[56]
Lactiplantibacillus plantarum Q7 GenBank: CP019712-16	Ultracentrifugation + Ultrafiltration	185.5 ± 65.4 nm (DLS)	ND	[39]
Lactiplantibacillus plantarum NCIMB 8826	Ultracentrifugation + SEC	117 ± 24 nm (NTA)	6×10^{11} particles/mL (NTA)	[38]
	Ultracentrifugation	50–200 nm (cryo-TEM) 116 ± 9 nm (NTA)	7.2×10^{10} – 1.7×10^{12} particles/mL (NTA)	[60]
Lactiplantibacillus plantarum BCRC 10,357	Ultracentrifugation	124–130 nm (NTA)	3.8×10^{8} particles/mL (NTA)	[61]
Lactiplantibacillus plantarum APsulloc 331,261	Ultracentrifugation + Density gradient ultracentrifugation	72–84 nm (DLS)	$2.95–5.75\times10^{10}$ particles/mL (TRPS)	[43]
	Ultracentrifugation	104 ± 42.4 nm (DLS)	$2.45–3.06\times10^{10}$ particles/mL (TRPS)	[78]
Lactiplantibacillus plantarum BGAN8	Ultracentrifugation	20–140 nm (cryo-TEM)	ND	[80]
Lacticaseibacillus paracasei *	Ultracentrifugation	20–100 nm (DLS)	ND	[48]
Lactococcus lactis FM-YL11	Ultracentrifugation	50–300 nm (TEM)	ND	[49]
*Lactococcus lactis**	Ultracentrifugation	60–100 nm (DLS)	ND	[50]
Ligilactobacillus salivarius *	Ultracentrifugation	325–425 nm (DLS)	ND	[52]
Lactobacillus animalis ATCC 35,046	Density gradient ultracentrifugation	118.8 ± 49.5 nm (NTA)	$3.7–4.6\times10^{10}$ particles/mL (NTA)	[57]
Lacticaseibacillus casei ATCC 393	Ultracentrifugation	40.7 ± 20.3 nm (SEM) ~ 227.3 nm (DLS)	ND	[45]
		10–300 nm (AFM) 143 ± 52 nm (NTA) ~70 nm and ~250 nm (DLS)	3×10^{9} – 1×10^{10} particles/mL (NTA)	[46]

Table 1 (continued)

Bacterial producer	Separation and purification	Size	Amount	Reference
Lacticaseibacillus casei BL23	Ultracentrifugation	13–28 nm (TEM) ~24 nm (DLS)	ND	[47]
		33 ± 3 nm (AFM) 37 ± 3 nm (DLS) 48 ± 3 nm (SEM)	ND	[82]
Lacticaseibacillus casei DSMZ 20,011	Ultracentrifugation + SEC	50–200 nm (cryo-TEM) 116 ± 5 nm (NTA)	$3.3\times10^{11}-4.4\times10^{12}$ particles/mL (NTA)	[38]
	Ultracentrifugation	113 ± 12 nm (NTA)	ND	[60]
Lacticaseibacillus paracasei PC-H1	Ultracentrifugation	~200 nm (NTA)	ND	[104]
CGMCC 22,285				
Lacticaseibacillus rhamnosus GG ATCC 53,103	Ultracentrifugation	161.9 ± 54.8 nm (DLS)	ND	[105]
Lactobacillus gasseri	Ultracentrifugation	133.14 ± 2.90 nm 141.26 ± 9.78 nm (NTA)	$5.87\times10^{10}-1.32\times10^{11}$ particles/mL (NTA)	[150]
BC12 and BC13*				
Lacticaseibacillus rhamnosus JB-1*	Ultracentrifugation	~130 nm (NTA)	3.1×10^{11} particles/mL (NTA)	[79]
		~134 nm (NTA)	$2.3–2.6\times10^{10}$ particles/mL (NTA)	[116]
Lactobacillus crispatus	Ultracentrifugation	130–140 nm (NTA)	$1.18–3.26\times10^{10}$ particles/mL (NTA)	[150]
BC3 and BC5				

Abbreviations: ND, no data; SEC, Size exclusion chromatography; TEM, Transmission electron microscopy; cryo-TEM, Cryogenic transmission electron microscopy; SEM, Scanning electron microscope; AFM, Atomic force microscopy; DLS, Dynamic light scattering; NTA, Nanoparticle tracking analysis; TRPS, Tunable resistive pulse sensing; * Bacterial strain was not reported

number of intracellular non-membrane-associated functional proteins by the bacterial cytoplasm during vesicle formation, including cell wall hydrolase-like enzymes (P40, P45), metabolism proteins (Ldh1, GapA, Pgi), and ribosomal structural proteins (30 S and 50 S) [56, 80–82] that predict biological functions such as metabolic regulation, translation and transcription. However, the specific functions of these enzymes and proteins have not been fully elucidated in actual studies and have only been mentioned in component studies. In addition to protein components, nucleic acids in CMVs play an important role. Small RNAs carried by *Lactiplantibacillus plantarum* CMVs inhibit LPS-induced cellular inflammation in a concentration-dependent manner [37], in contrast, RNAs from *Lactobacillus gasseri* CMVs partially inhibited the proliferation of $CD4^+$ T cells and attenuated the delayed-acting hypersensitive response mediated by these cells in a Myd88-dependent manner [83]. This also suggests that when referring to the anti-inflammatory function of PEVs, in addition to various types of active proteins, RNAs may also be one of the anti-inflammatory mechanisms, although the current study work has not yet fully revealed the complete anti-inflammatory signaling mechanism of RNAs and proteins.

Currently, most studies on PEVs are at the functional level, and little is known about component studies. Further investigation of the components of PEVs will help to elucidate the intrinsic mechanisms by which PEVs mediate the effects of beneficial microorganisms on host homeostasis and will also contribute to the development and translation of functional components of bacteria in the future.

PEVs-mediated Microbiota-Host communication

Role of PEVs in intestinal homeostasis

Numerous studies have demonstrated the role of PEVs in maintaining host intestinal health. Figure 2 demonstrates the potential of PEVs in maintaining microbiota balance and their intestinal immune and anti-colonic tumour immunological applications.

Intestinal immunity and microbial homeostasis

Strong scientific evidence has shown that colonization of the gut by beneficial microorganisms can facilitate intestinal innate immune regulation and help maintain intestinal homeostasis. As PEVs are gradually coming into the limelight, they will assume the role of key messengers in functional interactions between bacteria and the host gut.

Despite differences in animal models and administered doses, PEVs such as *Lacticaseibacillus rhamnosus* (*Lactobacillus rhamnosus)* GG CMVs [39], *Lactiplantibacillus plantarum* (*Lactobacillus plantarum)* Q7 CMVs [55], *Bacteroides acidifaciens* CMVs [84], and A.*muciniphila*

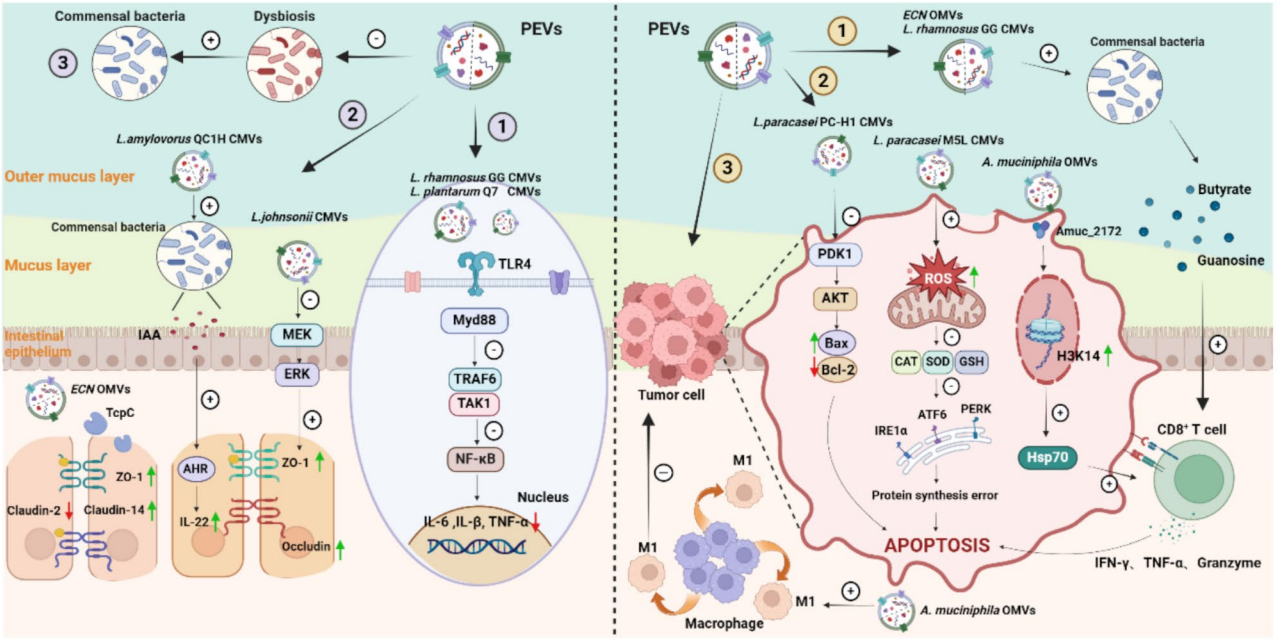


Fig. 2 Schematic representation of the role of PEVs in the maintenance of host intestinal homeostasis. Figure 2 firstly demonstrates the functions of various PEVs in maintaining intestinal homeostasis by down-regulating inflammatory signaling pathways to alleviate inflammation; regulating the levels of intestinal epithelial cell tight junction proteins to maintain the integrity of the intestinal barrier and improving the intestinal microbiota. In addition, PEVs enhanced the tumor-killing effect of $CD8^+$ T cells by regulating intestinal microbial metabolites; induced tumor cells apoptosis by regulating the expression of mitochondrial and endoplasmic reticulum stress and apoptosis proteins; and induced the polarization of macrophages toward M1-type killing phenotypes, which emphasized the anticolonic tumor effect of PEVs

OMVs [85] successfully enter the colon after oral administration and inhibit the production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) by inhibiting the phosphorylation of host cytokine/chemokine signaling pathway, which in turn significantly reverses dextrose sodium sulfate (DSS)-induced colonic inflammation. Among them, *Lacticaseibacillus rhamnosus* GG CMVs reversed colonic inflammation by inhibiting the gene expression of TLR4/Myd88/NF-κB-related signaling pathway factors (TLR4, Myd88, P53, and NF-κB) [39]. This is the same therapeutic effect that *Lacticaseibacillus rhamnosus* GG organisms have had in the past for treating colonic inflammation [86]. Furthermore, recent studies have shown that *Lactiplantibacillus plantarum* CMVs exhibit the same anti-inflammatory signaling network gene expression and inhibit inflammation-induced apoptosis of intestinal epithelial cells through the mPTP-CytC-Caspase pathway [55, 87]. *Lactiplantibacillus plantarum (Lactobacillus plantarum)* UJS001 [88], *Bacteroides acidifaciens* [84], and *Clostridium butyricum* [89] CMVs similarly attenuate pro-inflammatory cytokine expression; however, the specific anti-inflammatory signaling pathways and components remain unknown. Taken together, we found that there are few mechanistic studies on the components of PEVs that play a role in the specific signaling pathways that exert anti-inflammatory activity. In the future, studies on the anti-inflammatory activity of the components and mechanisms of PEVs could be linked to probiotics, which could also help identify anti-inflammatory active components that are similar to both. Finally, it is worth emphasizing that LPS, an outer membrane component of Gram-negative bacteria, promotes inflammation mainly through activation of the TLR4/NF-κB signaling pathway [90]. However, *ECN* [27] and *A. muciniphila* [85] OMVs did not cause activate inflammatory pathway via the TLR4 pathway, but instead inhibited the colonic inflammation induced by DSS, which may be based on the fact that these PEVs carry certain secretory active proteins that play a more potent anti-inflammatory role, although the specific components are still unknown. This is an interesting finding regarding on the role of PEVs in maintaining intestinal immunity. In addition to the effects of these bacterial immunoreactive components, the content of certain bacterial proteins carried by *ECN* OMVs, especially transfer and cleavage enzymes such as MtlA, MtlB and EmtA [64], and cell wall degrading enzymes related to Prostaglandin (PG) metabolism, were also found to affect the rate of internalisation and host ligand activation process of PEVs. Polyamine metabolism and synthesis in eukaryotes regulate the normal development of host cells, and disorders in the host intestinal microenvironment are usually accompanied by abnormalities in polyamine synthesis, regulating polyamine synthesis and metabolism

may be an effective way to maintain intestinal homeostasis [91, 92]. Recently, Fan et al. [93] also found that small RNAs (*sR-182871, sR-242825*) derived from *Lactobacillus murinus* CMVs could reduce the intestinal polyamine level and alleviate the inflammation of the mouse colon by regulating the expression of key enzymes for polyamine synthesis, such as ornithine decarboxylase-1 and thiamine decarboxylase-1.

As the first line of defence against exogenous damage and infection, the integrity of the physical and biochemical barriers formed by the intestinal epithelium is essential for host homeostasis [94, 95]. The ability of AMPK phosphorylation to upregulate the $Ca2^{+}$-induced assembly of tight junction proteins has been studied previously [96]. Recent studies have found that *A. muciniphila* OMVs have been shown to be potent activators of AMPK phosphorylation, restoring tight junction function in LPS-induced Caco-2 cells through the upregulation of tight junction proteins (Occludin), which may be related to their surface outer membrane proteins [31]. Recently, Liu et al. not only discovered the critical role of *Lactobacillus amylovorus* QC1H CMVs in attenuating inflammatory injury and intestinal microbial imbalance induced by exogenous inflammatory mediator aflatoxin B1 (AFB1), but also further elucidated in their experiments that *Lactobacillus amylovorus* QC1H CMVs could improve the expression level of tight junction proteins by activating AHR/IL-22 signaling related to tryptophan metabolism in gut microorganisms. expression levels and reverse-validated their conclusions by means of AHR pathway inhibitors [97]. Laura Baldomà et al. found that *ECN* OMVs containing the secreted protein TcpC promoted the expression of intact membrane functional proteins such as Claudin-14 and ZO-1 and down-regulated the expression of Claudin-2 permeability proteins, which enhanced the intestinal barrier function and restored inflammation-induced intestinal epithelial damage [98]. In addition to being induced by inflammatory mediators, another study by Laura Baldomà's team showed that *ECN* OMVs also reversed the intestinal barrier damage induced by the pathogenic bacterium *enteropathogenic Escherichia coli* (*EPEC*) [99]. Recently, Wei et al. [100] found that *Lactobacillus johnsonii* CMVs repaired *enterotoxigenic Escherichia coli* (*ETEC*)-induced intestinal barrier damage by inhibiting the activation of ERK protein, which is a core component of MAPK, inducing the polarization of M2 macrophage, and blocking the NLRP3 signaling pathway. However, the specific components and mechanisms by which various PEVs exert their protective effects on the host intestinal barrier are still unknown and need to be further investigated.

In addition, PEVs are again involved in gut bacteria-bacteria communication, restoring inflammation- and infection-induced imbalances in the intestinal microbiota by fusing with *Firmicutes* and reducing the relative abundance of *Proteobacteria* [39, 89]. Regarding how they affect bacterial growth and colonization, Wang et al. [101] found differences in the binding ability of different bacteria EVs by fluorescently labelling *A. muciniphila* OMVs and incubating them with representative intestinal bacteria in vitro, and affecting bacterial proliferation to some extent. This selective fusion of PEVs with bacteria also explains the extent of the similarity of material transfer between bacteria and bacterial components.

Colorectal Cancer

Due to their strong non-specific physiological and immune functions, PEVs have great potential in colon tumour therapy. A large body of evidence now shows a twofold role of PEVs. During the proliferation and apoptosis of tumour cells, *Lacticaseibacillus paracasei (Lactobacillus paracasei)* components: extracellular polysaccharides [102] and cell wall membrane-conjugated proteins [103] have been shown to reach the colonic tumour microenvironment and be taken up by cells through the packaging of PEVs. On the one hand, *Lacticaseibacillus paracasei* M5L CMVs [102] induces the production of large amounts of reactive oxygen species (ROS) radicals in a concentration-dependent manner in mitochondria and inhibit antioxidants (catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH)) in tumor cells, and further activates the endoplasmic reticulum leading to protein misfolding and phosphorylation of the endoplasmic reticulum receptor/transmembrane proteins (IRE1α, PERK and ATF6) phosphorylation causing apoptosis in colon tumor cells. Another study showed that *Bifidobacterium longum* CMVs also prevented liver fibrosis and carcinogenesis in a manner that prevented oxidative stress in hepatocytes [40]. However, studies on ROS and endoplasmic reticulum stress produced by PEVs in tumor cells are only at the cellular level and require further animal experiments and repeated validation of inhibitors, as well as further elucidation of the specific components of PEVs induced stress. On the other hand, *Lacticaseibacillus paracasei* PC-H1 [104] and *Lacticaseibacillus rhamnosus(Lactobacillus rhamnosus)* GG CMVs [105] up-regulated the expression of apoptotic protein Bax by down-regulating the phosphorylation of key apoptotic pathway proteins, inositol-3-phosphate-dependent protein kinase-1 (PDK1) and AKT, and decreased the expression of the anti-apoptotic protein Bcl-2, which ultimately induced apoptosis in tumor cells. Previous studies hanve shown that colon cancer cells prefer the glycolytic pathway to generate energy under hypoxic conditions [106]. Shi et al. first found that *Lacticaseibacillus paracasei* CMVs were able to inhibit the expression of hypoxia-inducible transcription factor-1α (HIF-1α)

[107], which led to the down-regulation of the expression of glucose transporter 1 (GLUT1) and other glycolytic proteins, thus failing to meet the energy metabolic demands of tumour cell growth, which then inhibited the proliferation of tumour cells. However, the specific components of glycolysis inhibition-associated PEVs are not yet known and need to be further explored.

In anti-tumour immunity, cytotoxic $CD8^+$ T lymphocytes (CTLs) are important effector cells in the tumour immune response. These cells bind to ligands on the surface of tumour cells and secrete IFN-γ, granzyme and TNF-α, thereby inducing cell death [108]. Past studies have confirmed the toxic effect of Amuc_1100, a bacterial membrane protein of *A. muciniphila*, in tumour cells. In recent years, Jiang et al. [76] found for the first time that Amuc_2172, an outer membrane protein of *A. muciniphila* OMVs, acted as a GCN5-associated acetyltransferase, causing acetylation of the H3 histone in tumour cells and thus induce the transcription and secretion of HSP70 to inhibit tumour development. In addition to the activation of CTLs and host inflammation by PEVs and their components, Luo et al. noted that a large number of macrophages were also recruited at the tumour cells under the intervention of *A. muciniphila* OMVs and polarized towards M1-like tumour-killing phenotypes to limit cancer cell proliferation and invasion [25]. Given the previously mentioned critical role of polyamine metabolism in the maintenance of intestinal homeostasis, Fan et al. [93] also found that mice and populations with colon cancer had significantly elevated levels of polyamines in the intestines and significantly decreased expression of genes related to key enzymes for polyamine synthesis. However, the authors did not further validate the role of *Lactobacillus murinus* CMVs, and the specific mechanism of their role in the tumor microenvironment remains to be further elucidated.

Conventional anti-programmed cell death 1 (anti-PD-1) immunotherapy is associated with multiple drug resistance and adverse effects and is usually supplemented with other targeted drugs in combination. Administration of *Lacticaseibacillus rhamnosus* (*Lactobacillus rhamnosus)* CMVs [109] and *ECN* OMVs [110] in addition to anti-PD-1 therapy was found to improve intestinal dysbiosis and increase bacterial anti-tumour metabolites such as guanosine and butyrate, which further increased the proliferation and activity of $CD8^+$ T cells in mesenteric lymph nodes and tumour tissue, resulting in enhanced anti-tumour activity of anti-PD-1 therapies. Based on this immune synergy, PEVs could also become cancer immunotherapy adjuvants in the future.

The role of PEVs in protecting inflammation and damage in peripheral organs through Gut-X axis

In addition to their role in the gut, PEVs can exert functional activity by translocating through the blood circulation or by direct action on distal organs. Figure 3 demonstrates the application of PEVs to protect for

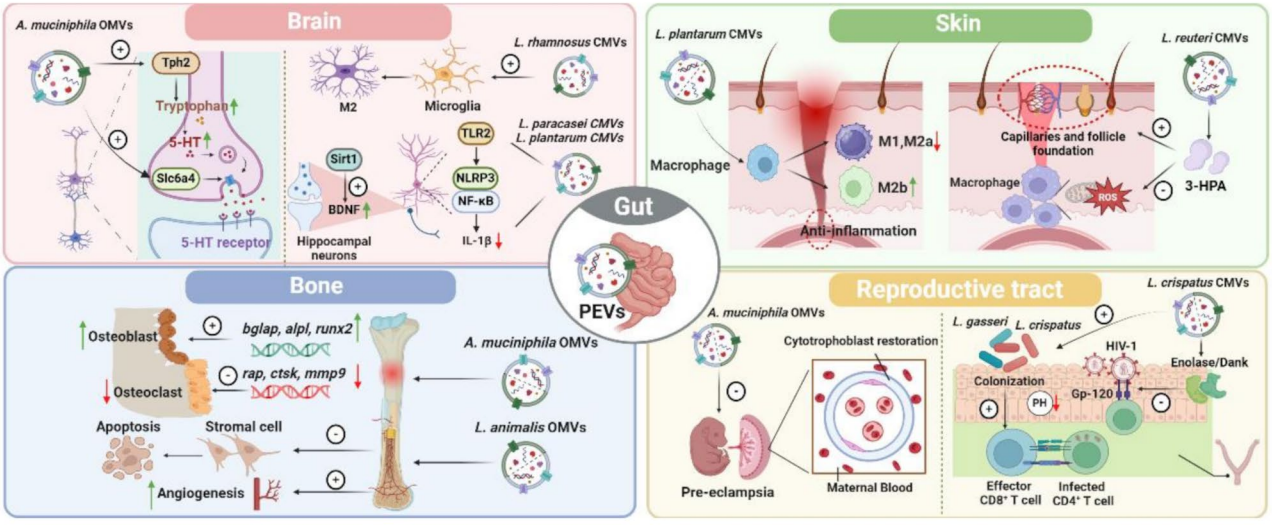


Fig. 3 Schematic representation of PEVs in regulating the health of host distal organs. Figure 3 is a schematic illustration of PEVs regulating host distal organ health, mainly demonstrating the biological activities of PEVs in the brain, bone, skin and reproductive tract. In the brain, PEVs ameliorate neuroinflammation by inhibiting microglia activation, reverse neuronal damage and improve learning memory by promoting brain-derived neurotrophic factor (BDNF) expression and serotonin signaling. In bone, PEVs promote bone healing by inhibiting osteoclasts, promoting osteoblast formation, and facilitating revascularization. In the skin, PEVs induced macrophage polarization to reduce skin inflammation and promoted wound healing through local revascularization and hair follicle establishment. In the reproductive tract, PEVs resisted HIV-1 infection by both interfering with HIV-1 binding to $CD4^+$ T cells and promoting colonization by *Lactobacillus*; they also rescued pregnancy outcome by ameliorating placental trophoblast cell stress and fetal injury in disease states

protecting inflammation and damage in peripheral organs, such as the brain, bone, skin, vagina and pregnancy health through the gut-X axis.

Role of PEVs in the Gut-Brain Axis

In addition to improving the intestinal ecosystem, PEVs and their beneficial constituents can be internalised by intestinal epithelial cells, and become active in various tissues and organs through the blood circulation. They can even cross the placental barrier [111] and blood-brain barriers [74] to accumulate in the brain and the foetus as a means to reduce inflammation and damage. Numerous rodent studies have shown that dysregulation of the gut-brain axis mediated by dysbiosis usually induces stress-related neurological disorders such as stress [112] and anxiety [113]. It has been found that increasing the population of beneficial bacteria such as *Lacticaseibacillus rhamnosus* (*Lactobacillus rhamnosus)* can have a beneficial effect on the central nervous system (CNS) via the vagus nerve [114] and regulatory T cell (Treg)-mediated immune responses [115]. However, the intrinsic mechanisms underlying the production and transport of neurotransmitters and neuropeptides associated with neurological disorders, and the translation of intestinal immunity into the immune response of the CNS have not been elucidated. Recent studies have found that PEVs can act as crucial messengers for microbial immune response messaging in the gut-brain axis.

First, PEVs mediate material and signaling between the brain and gut, and the primary condition is to allow PEVs to be absorbed by intestinal epithelial cells and enter the bloodstream, and to be able to reach the brain tissue through the blood-brain barrier with the blood circulation. In the past, Kevin et al. [116] detected *Lacticaseibacillus rhamnosus* JB-1 CMVs in mouse plasma after feeding with *lacticaseibacillus rhamnosus* JB-1 for a period of time, with intact morphology and retaining immunoreactivity of bacterial fractions of the bacterium (e.g., LTA), and also showed accumulation of CMVs in the brain in vitro fluorescence imaging. Recently, Pei et al. demonstrated the accumulation of CMVs across the blood-brain barrier in different brain regions by injecting mice with fecal-derived EVs from mice raised with a fluorescently labeled probiotic blend [117]. Numerous evidences suggest that PEVs are indeed able to translocate from the gut to brain tissue and function. However, there is currently no evidence showing the details of the process by which PEVs cross the blood-brain barrier in vitro and in vivo, although there is now evidence that other types of bacterial ectodermal vesicles can cross artificially simulated blood-brain barrier structures [118]. Therefore, a future comprehensive characterization of the dynamic process of PEVs circulating in vivo is necessary to study PEVs bioactivity and carrier function.

PEVs substitute parent bacteria in the production of specific brain anti-inflammatory activities. Microglia, as nervous system-specific cells, are involved in neurodevelopment and repair responses to nerve injury, and are involved in neural signaling and cytokine release [119]. There is evidence that *Lacticaseibacillus rhamnosus* (*Lactobacillus rhamnosus)* CMVs, when administered at appropriate doses, stimulate the polarization of microglia towards the M2 anti-inflammatory cell type, which in turn attenuates LPS-induced neuroinflammation [120]. After injection of fecal-derived EVs containing a probiotic complex in gavaged mice, ethanol-induced astrocyte and microglial cell damage and activity were significantly reduced, and neuroinflammatory TLR2/NLRP3/NF-κB protein and gene expression were significantly reduced in glial cells [117]. However, it is not known which components of PEVs are involved in the anti-inflammatory response of glial cells.

PEVs may ameliorate neuronal pathological damage and cognitive deficits through epigenetic modifications. In physiological states, active expression of brain-derived neurotrophic factor (BDNF) modulates synaptic plasticity and contributes to hippocampus-dependent learning and memory [121]. Recent studies have found that *Lacticaseibacillus paracasei* (*Lactobacillus paracasei)* and *Lactiplantibacillus plantarum* (*Lactobacillus plantarum)* CMVs [122, 123] can be directly taken up by neurons and upregulate the BDNF pro-expression-associated epigenetic factor Sirt1, which induces BDNF expression in the hippocampus of HT-22 and mice, which in turn increases dendritic formation and neurogenesis, and reverses Alzheimer's disease (AD)-like pathologic impairments and depressive-like behavior. In addition, CMVs from *Lacticaseibacillus paracasei* effectively reversed the activation of the TNFα-TNFR1-S1PR2-IL-1β-CCL2-BDNF-TrkB pathway in rat hippocampus [124], which in turn improved the learning memory function in hyperammonemic brain-damaged rats. Furthermore, the authors attempted to assess the potentially beneficial roles of the P40 and P75 proteins in the CMVs from *Lacticaseibacillus paracasei*, although the results were not due to this protein performing the above-mentioned functions, but the exploration of the role of bioactive components of PEVs is necessary. Recent studies have found that treatment with *Lacticaseibacillus paracasei (Lactobacillus paracasei)* CMVs significantly ameliorated dysregulated genes (*drd2*, *oxtr*, *adcy5*) in brain regions associated with Autism spectrum disorder (ASD) behaviors in mice and identified Oxtr and Oxtr receptors as potential therapeutic targets [125]. Finally, we also need to pay attention to the indirect modulatory effects of PEVs on AD injury. Chin et al. [126] demonstrated that alterations in the gut microbiota in AD patients caused significant dysregulation of bile acid metabolism, whereas treatment

with *Lactobacillus johnsonii* CMVs significantly inhibited the production of toxic secondary bile acids produced by high-abundance *Clostridium scindens* by reducing the intestinal Aβ accumulation in mice to alleviate AD progression.

In addition, PEVs can induce changes in bidirectional neural signaling between the host gut and the brain. Serotonin (5-HT) is a neurotransmitter that is mainly involved in the functional homeostasis of the gastrointestinal tract, vagus nerve and central nervous system, and regulates physiological functions such as mood and sleep [127]. The gut is the main reservoir of 5-HT, and most of the 5-HT from the gut can be distributed through the bloodstream and involved in the control of gastrointestinal motility, vagal reflex activity, etc., which are mainly regulated by metabolites of the intestinal microbiota (e.g., short-chain fatty acids) [128]. Recently, Rezvan et al. [129] found that feeding *A. muciniphila* OMVs affected the expression of both intestinal and hippocampal serotonin-related genes in mice, mainly in the form of upregulation of the expression of Tph1, which is involved in serotonin biosynthesis, as well as of Slc6a4, which is involved in serotonin transport. This study reveals for the first time the impact of PEVs co-regulating serotonin signaling between the gut and the brain, and also provides new insights into revealing the connections between the enteric nervous system (ENS) and CNS, even though 5-HT produced by the gut and the brain is not subject to direct transmission and translation [130].

In conclusion, there is now evidence that PEVs are able to cross the blood-brain barrier and mediate probiotics' ability to achieve gut-brain information exchange by affecting microglia activation, ameliorating neuronal damage, and influencing serotonin signaling in three ways. However, more evidence is still needed to supplement the whole process of PEVs dynamic transport, and the intrinsic mechanisms of PEVs components and improvement of brain microenvironment need to be further explored.

Role of PEVs in the Gut-Bone Axis

In the past, it has been found that there is a close link between the gut and the presence of the skeletal system, which is not only limited to intestinal absorption of minerals and bone turnover but also intestinal transgenes, cytokines, serotonin [131] as well as the numerous *Bifidobacterium* and *Lactobacillus* spp. in the gut and their production of growth factors, and metabolites that are beneficial for bone development and health [132–134]. Studies on PEVs in the treatment of osteoporosis and osteonecrosis are being reported in recent years.

Liu et al. found for the first time that the gut microbiota, represented by *A. muciniphila* during the juvenile period, could reduce bone loss and improve bone metabolism, and by evaluating the oral, intravenous injection with fluorescent *A. muciniphila* OMVs method, they verified that OMVs can be rapidly entered into the bloodstream and translocated to the bone tissue accumulation, and verified in vitro experiments that *A. muciniphila* OMVs can lead to the suppression of osteoclasts, the formation of osteoblasts, and the amelioration of the osteoporosis symptoms in mice [135], although the functional molecules associated with OMVs are not yet known. In addition, Chen et al. found accumulation of *Lactobacillus animalis* CMVs in the femoral head by oral administration, and verified in *vitro* that *Lactobacillus animalis* CMVs could be absorbed and utilized by osteoblasts and vascular endothelial cells, and promoted femoral head microvascular remodeling in mice. The shortcoming is that the authors did not emphasize what role the specific proteins of *Lactobacillus animalis* CMVs played in this, although they pointed out that *Lactobacillus animalis* CMVs are enriched in a variety of substance transport and metabolism proteins [57]. Similar findings were recently reported by Zhang et al. in their study of *Ligilactobacillus salivarius* (*Lactobacillus salivarius)* CMVs, which significantly alleviated osteoporosis induced by physiological and pathological conditions in rats after oral administration of *Ligilactobacillus salivarius* CMVs by promoting osteoblast formation to increase bone density [136]. However, in the therapeutic regimen of orally administered PEVs, the accumulation of PEVs in the targeted tissues usually requires high doses of PEVs to achieve the desired effect. In order to improve the utilization and therapeutic effect of PEVs, Liu et al. in their recent study loaded *ECN* OMVs with the bone-targeting gene CXCR4 and bone growth factor BMP-2 genes based on biosynthesis and modification techniques, targeting the promotion of osteoblast differentiation and maturation to improve osteoporosis [137].

In conclusion, a variety of PEVs have been shown to accumulate in bone tissues through blood circulation and restore osteoporosis and osteonecrosis under pathological conditions by promoting osteoblast formation, inhibiting osteoclasts, and facilitating local microvascular reconstruction; however, the specific components of PEVs and their mechanisms still need to be explored. In addition, PEVs based on gene synthesis and modification may be able to achieve the therapeutic effect more efficiently, however, their efficacy, safety and production efficiency need to be further investigated.

The role of PEVs in skin wound healing

The communication between probiotics and host-mediated by PEVs also affects the damage repair process of peripheral tissues and organs. The skin is the defence organ with the largest surface area of the body [138], When exposed to adverse factors such as radiation,

pathogens and other physiological stresses, Keratinocytes and Langerhans cells can release inflammatory cytokines, triggering a cascade of reactions from the epidermis to the dermis, resulting in local inflammation and damage [139, 140]. As natural messengers carrying RNA, metabolites and other active substances, PEVs have been shown in several studies to efficiently and safely restore microbial homeostasis and reduce inflammation on the skin surface and may serve as a novel agent for the future treatment of wound healing and skin inflammation.

Skin anti-inflammatory activity of PEVs.The anti-inflammatory effect of *Lactiplantibacillus plantarum* (*Lactobacillus plantarum)* CMVs on chronic skin inflammation was first revealed by Kim et al. They found that long-term oral administration of *Lactiplantibacillus plantarum* CMVs attenuated chronic inflammation of the skin of mice in a dose-dependent manner and showed similar therapeutic effects to those of hormonal drugs [141]. A later study showed that the therapeutic potential of *Lactiplantibacillus plantarum (Lactobacillus plantarum)* CMVs was mainly based on inducing an anti-inflammatory response in macrophages transitioning to M2b macrophages. In addition, considering the yield and isolation difficulty of PEVs, the authors mass-produced vesicles similar to *Lactiplantibacillus plantarum* CMVs by high-pressure and continuous filtration and ultrafiltration of *Lactiplantibacillus plantarum* with similar cutaneous anti-inflammatory effects as those of *Lactiplantibacillus plantarum* CMVs [78], which provides a new direction for the subsequent mass production and isolation and purification of PEVs. In recent years, studies on *Lacticaseibacillus paracasei (Lactobacillus paracasei)* [142] and *Bifidobacterium longum* [143] CMVs have also revealed similar macrophage transformation processes, which highlight the anti-inflammatory effects of PEVs in dermatological diseases reached by inducing macrophage polarization. However, the question of which bacterial component exerts the anti-inflammatory effect remains largely unexplored.

PEVs promote wound healing through tissue repair. Recently, Chen et al. demonstrated that 3-hydroxypropionaldehyde (3-HPA) carried by *Limosilactobacillus reuteri* (*Lactobacillus reuteri)* CMVs alleviated oxidative stress in macrophages by alleviating LPS-induced macrophage mitochondrial permeability, reduced ROS production alleviated oxidative stress in macrophages and promoted macrophage M2-type polarization, which was ultimately manifested as wound follicle reconstruction and collagen fiber deposition, accelerating wound healing [36]. In addition to the role of PEVs-specific proteins, Wang et al. found that *Lacticaseibacillus rhamnosus* GG CMVs carrying *miR-21-5p* molecules accelerated wound healing in mice by activating endothelial cell and keratinocyte proliferation and migration as well as revascularization at the wound site through the PI3K-AKT/HIF1α pathway [144]. In addition, Fu et al. found that *Lactobacillus delbrueckii* CMVs not only promoted rapid tissue reconstruction by inducing the establishment of hair follicles and capillary reconstruction, but also inhibited the expression of fibroblast genes and proteins, which prevented wound hyperfibrosis and reduced the formation of keloid scars [145].

In conclusion, PEVs reduce the inflammatory response at the wound site mainly through macrophage polarization; and promote the establishment of hair follicles, revascularization, and wound healing through the pulling of keratinocytes at the wound site mediated by PEVs bioactives such as protein (3-HPA) and *miRNA* molecules. It is worth noting that, given the very limited production and bioavailability of PEVs at present, for inflammation and injury of peripheral organs such as skin, most studies tend to experiment with topical application or topical injection, which may be different from oral administration, and in addition to considering the difference in dosage, the potential risks (e.g., allergy) posed by topical or topical injections need to be critically considered.

Role of PEVs in vaginal health and pregnancy outcomes

The vagina of a healthy woman is characterized by a high colonization rate of *Lactobacillus* species, which are extremely abundant and responsible for maintaining the normal physiological and microbiological balance of the vagina. When the vaginal microecological environment is disturbed, in addition to treatment with antibiotics and other medications, additional probiotics can also quickly remodel their vaginal micro-environment [146]. In recent years, studies on the role of PEVs in maintaining vaginal health and pregnancy outcomes are being reported.

PEVs remodel the vaginal microenvironment. A recent study verified that CMVs derived from two vaginally abundant *Lactobacillus* species, *Lactobacillus crispatus* and *Lactobacillus gasseri*, promote bacterial adhesion to HeLa cells and resistance to pathogenic bacterial adhesion [147], demonstrating the unique appeal of PEVs in maintaining vaginal homeostasis. *Lactobacillus vaginalis* has been shown in the past to protect the vagina from HIV-1 infection by acidifying the vaginal ecotone and stimulating anti-HIV-1 immune responses [148, 149]. Recently, Palomino et al. identified for the first time the ability of certain specific strains of *Lactobacillus crispatus* CMVs to inhibit HIV-1-infected cells in vitro and affect the viral survival milieu to a certain extent, which may be due to the role of the enolase, chaperonin DnaK (Hsp70)-related proteins in CMVs [150]. Interestingly, in a follow-up study by the same authors, vaginal pathogenic bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis* also showed similar anti-HIV-1 infectivity as *Lactobacillus*, and this anti-infective potential did not depend on the cytotoxicity of the PEVs, but rather on

bacterial proteins shared on the surface of the PEVs that blocked the interaction of the Gp120 subunit of the viral envelope with $CD4^+$ T cells interaction [151]. Further characterization of this bacterial protein component will also provide new ideas for anti-HIV-1 infection. In addition, numerous scholars have verified the multiple roles of sterile supernatants of *Lactobacillus* of various vaginal origins in improving the inflammatory immune homeostasis of vaginal epithelial cells [152], the vaginal epithelial barrier function [153], and the resistance to pathogen colonization [154, 155], and even though these studies did not clearly identify the contribution of *Lactobacillus* CMVs to the maintenance of host homeostasis, the supernatant always contained CMVs. These results provide a basis for subsequent studies of the functions associated with different *Lactobacillus* CMVs.

PEVs crossing the blood-placental barrier affects adverse pregnancy outcomes for mother and child. Preeclampsia (PE) is a pregnancy-specific syndrome usually associated with cardiovascular disease, miscarriage, and adverse pregnancies [156]. Chen et al. [111] found that the gut microbiota of patients with clinical PE had low abundance of *A. muciniphila*. Subsequently, oral administration of *A. muciniphila* OMVs to PE mice improved maternal vasoconstriction and renal function while fluorescence showed that OMVs were also taken up by placental trophoblast cells and successfully crossed the placental barrier, eventually accumulating in the fetus; the study also found that OMVs increased epidermal growth factor receptor (EGFR) expression in placental trophoblast cells and activated the PI3K-AKT signaling pathway, which attenuated placental cell injury and improved adverse pregnancy outcomes. Meanwhile, a concurrent study by Kaisanlahti et al. again demonstrated that bacterial EVs can cross the placental barrier into the fetus, although they used total maternal fecal EVs [157]. Wang et al. [158] also demonstrated that *Lactobacillus crispatus* CMVs could help to alleviate the response of trophoblast cells to exogenous-induced oxidative stress by restoring the phosphorylation of mitochondrial Akt and inhibiting the fragmentation of mitochondria, thus further maintaining the balance of the fetal survival environment.

In conclusion, PEVs alleviated vaginal bacterial and HIV-1 infections to some extent and alleviated oxidative stress damage to trophoblast cells through the placental barrier. However, there is a lack of clarity regarding whether PEVs can function by reaching the vaginal environment through oral administration, as well as a need for elucidation regarding the function of various types of *Lactobacillus* CMVs in the vaginal environment.

Stability and biocompatibility of PEVs

The first half of this review focused on the excellent therapeutic potential of PEVs in maintaining the homeostasis of various organs of the host due to their own properties of carrying a large number of active substances (e.g., outer membrane proteins, nucleic acids, etc.) from the parent probiotic, even though the active components of most of them and their mechanisms of action have not been elucidated so far. It is noteworthy that most of these functions are based on PEVs biological stability and biohistocompatibility. Specifically, PEVs are mainly bilayer lipid-membrane vesicles synthesized and secreted by host cells in vitro, and the outermost bilayer lipid-membrane structure has always nurtured the strong stability of PEVs, which serves as a natural barrier to protect the internal active components. For example, the ability of *ECN* OMVs to successfully survive the acidic and enzymatic environment of simulated digestive fluids in vitro ensures that the OMVs can reach the gut to function [17, 109]. In the past, many evidences have verified the uptake of PEVs into host cells by labeling fluorescence methods [136, 144, 156], however, these fluorescence are usually non-specific membrane-labeled binding dyes (e.g., PKH26, DiR), which can only verify the accumulation of fluorescence in the cell, while the specific morphology and destination of PEVs in the host cells have not been elucidated and need to be further explored. Regarding how PEVs are specifically taken up by host cells, Laura Baldomà et al. [159] demonstrated in several intestinal epithelial cell models that *ECN* OMVs are endocytosed by host cells through a lattice protein-dependent pathway using inhibitors, without affecting cell viability and function. This is consistent with studies of EVs from other sources being taken up by host cells [160, 161]. In addition, based on their biocompatibility, PEVs are also capable of ingesting other active substances through drug delivery methods or obtaining certain traits through gene editing techniques, etc., as evidenced by the vector potential of PEVs specifically described below.

Carriers and application potential of PEVs

Compared with synthetic nanoparticles and other cell-derived EVs, naturally occurring PEVs are easy and efficient to obtain, less cytotoxic, and have the advantages of novel therapeutic carriers due to the strong biocompatibility, cargo loading capacity and tissue permeability of PEVs. With this carrier potential and its own biological activity, several studies are now beginning to apply PEVs to clinical drug development. Table 2 summarizes practical application attempts based on the immunological activity and carrier properties of PEVs.

Utilizing the immunomodulatory activity of PEVs, Tuhn et al. [162] combined *Lacticaseibacillus casei (Lactobacillus casei*) and *Lactiplantibacillus plantarum*

Table 2 Application of PEVs based on immunological activity and carrier properties

Bacterial producer	Support material	Activity and Usage	Reference
*Lactobacillus druckerii**	TA-Mg	Improve wound microenvironment and promote wound tissue healing	[164]
Lactiplantibacillus plantarum *	Fucoxanthin	Improves gut microbiota and reduces colitis response	[165]
		Reducing hepatic lipid accumulation and disorders of lipid metabolism	[166]
E. coli Nissle 1917 serotype O6:K5:H1	SOST siRNA	Induce osteogenic differentiation of BMSCs and ameliorate OP	[175]
Saccharomyces boulardii CNCM I-745	Adriamycin	Adriamycin is internalised and absorbed by intestinal epithelial cells and transported to selected cells and tissues in the host	[167]
*Lactobacillus animalis**	Cu-TCPP (tetrakis(4-carboxyphenyl) porphyrin) nanosheets	Enhances antimicrobial activity and enhances implant osteogenicity	[163]
Lacticaseibacillus casei DSM20011 & *Lactiplantibacillus plantarum* NCIBM 8826	Hydrogel	Improves wound healing and reduces scar formation	[162]

Abbreviations: BMSCs, Bone marrow mesenchymal stem cells; OP, Osteoporosis; Cu-TCPP, Tetrakis(4-carboxyphenyl) porphyrin; * Bacterial strain was not reported

(Lactobacillus plantarum) CMVs with aqueous particles to make a hydrogel formulation, which was used to improve the microenvironment of the wounds and to accelerate wound healing in mice. In this author's study, the optimal culture pH and concentration of CMVs-producing bacteria were screened by testing protein concentration and cytotoxicity to ensure the maximization of CMVs production as well as the strongest immunomodulatory effect, however, the authors' assessment of the effect of animal experiments on the hydrogel preparations was limited and the appropriate concentration of CMVs bound to water particles was not screened. To attenuate the stimulation of host immune responses by exogenous grafts, Li et al. [163] utilized the ability of *Lactobacillus animalis* CMVs to induce macrophage anti-inflammatory immunoreactivity, and applied CMVs together with the hypoxia-triggered antibiotic tinidazole (TNZ) and copper-tetrakis (4-carboxyphenyl) porphyrin (Cu-TCPP) together for coating of bone implants attenuated biofilm-induced inflammation in vitro and promoted the expression of osteogenic factors, further enhancing the efficiency of new bone deposition and osteogenic activity in mice.

Based on the carrier potential of PEVs, Qi et al. introduced magnesium ellagic acid (TA-Mg) into *Lactobacillus druckerii* CMVs via core-shell microneedles and further evaluated the cellular uptake and cellular activity. Subsequently, the good antimicrobial activity of the combination was verified from cellular and animal experiments, respectively, which promoted the proliferation and angiogenesis of fibroblasts and accelerated wound healing, however, the information on the TA-Mg to CMVs optimal ratio, the loading efficiency has not been evaluated [164]. Liang et al. [165] prepared fucoxanthin-encapsulated *Lactiplantibacillus plantarum* CMVs using a combination of ultrasonic treatment and ultracentrifugation, and affirmed the ability and cytotoxicity of fucoxanthin loading of CMVs by protein assay, and reversed inflammation and dysbiosis of the colon in mice after cellular intervention and oral supplementation with CMVs. In a recent study, Wu et al. validated the therapeutic potential of *Lacticaseibacillus paracasei (Lactobacillus paracasei)* CMVs loaded fucoxanthin in NAFLD with the same approach [166]. Recently, Liu et al. [137] loaded the bone-targeting gene CXCR4 and bone growth factor BMP-2 genes into *ECN* based on biosynthesis and modification techniques to secrete OMVs with CXCR4 and BMP-2 genes, and constructed a small-dose, and highly effective anti-osteoporosis drug by evaluating the cytotoxicity, and the maturation of osteoblasts differentiated from the mouse and the expression of the related proteins and genes. In addition, the study by Jolanta et al. [167] showed the first evidence of *Saccharomyces boulardii* EVs being absorbed into the intestinal epithelium by incubation with adriamycin, and also showed good loading potential, although the reproduction efficiency and intervention effects were not explored in depth.

Compared to eukaryotic organisms, probiotics, in addition to their own strong biological activity, rapid reproductive ability, simple culture conditions [168], and easy-to-use gene editing methods usually result in greater yields of PEVs and complete inheritance of the genetically modified biological patterns of the parent bacteria. In the past, it was commonly used to apply an electric field to EVs by electroporation to permeabilize the EVs membrane, thus enabling the direct introduction of *siRNA* into EVs [169]. Although this method is time-saving and convenient, it risks damaging the integrity of EVs and consuming a large amount of expensive *siRNA* due to the low efficiency of transfection. Therefore, the current strategies for vector loading of EVs usually precede or during the formation and loading. For example, Liu et al. introduced a recombinant plasmid into *ECN*, which enabled *ECN*-derived OMVs to carry bone-targeting and bone-growth-promoting factors and thus possessed efficient anti-osteoporotic activity [137]. However, there is no evidence to support whether the stability of PEVs as carriers is altered after being genetically introduced,

including their own morphology and size, bioactivity and anti-physical-chemical properties. There are also no studies showing the delivery efficiency of PEVs in vivo, including the uptake efficiency of PEVs by different delivery routes, their clearance in the liver and kidney, as well as their uptake and metabolism in different tissues and organs, which still need to be explored in the future.

In addition to loading nucleic acids, modified or engineered PEVs have excellent targeting and adjuvant functions for the treatment of various diseases. In the past, traditional organismal drug delivery methods lacked tissue specificity and had limited bioavailability, whereas PEVs, as nanoscale materials, not only improve the efficiency of substance uptake and delivery, but also their own powerful anti-inflammatory function can further enhance drug activity. For example, Qi et al. [164] skillfully compounded TA-Mg, which has antimicrobial and antioxidant properties, with *Lactobacillus druckerii* CMVs, which has anti-inflammatory functions, to form a patch, which significantly accelerates wound healing and reduces scar formation when compared with conventional drugs. In addition, PEVs can also be used as active components or carriers, loaded with drugs into other delivery vehicles, such as metal-organic frameworks (MOFs), which have been widely used in manufacturing and targeted drug delivery due to their excellent loading capacity, biodegradability, and tunability [170]. Recently, Li et al. loaded antibiotics and *Lactobacillus animalis* CMVs onto MOFs, which ultimately formed a surface coating for titanium implants, alleviating inflammation and accelerating bone healing while eliminating potential bacterial infections in a non-invasive manner [163]. In addition, Qin et al. [171] effectively suppressed the immunosuppression induced by tumour-derived EVs by engineering *ECN* to co-express anti-PD-L1 and anti-CD9 antibodies and load them into MOFs. However, given the potential threat of *ECN* and the immunocompromised status of tumour patients, the use of *ECN* OMVs may not be optimal, and the function of *ECN* OMVs remains to be further clarified.

Future challenges and perspectives of PEVs

Carrier application limitations of PEVs

After exploring the powerful biological activities of PEVs themselves, in recent years, there has been a focus on PEVs in translation and practice. As mentioned above, PEVs are added as immune adjuvants to implant carriers, wound dressings, and even as helpers in antitumor therapy, synergistically enhancing antitumor efficacy by modulating intestinal bacterial metabolites [109, 110]. In addition, PEVs can also be used as carriers loaded with fucoidan for the effective treatment of colitis and NAFLD. However, the research on PEVs as carriers for drug loading has just started, and the efficiency, cost, potential PEVs damage, and even the selectivity of the loaded cargoes for traditional cargo loading strategies (e.g., electroporation, incubation, sonication, etc.) [172] in PEVs application are still unknown. In the future practice of PEVs drug carrier development, while choosing the appropriate loading strategy, it is also necessary to evaluate the drug loading efficiency of PEVs and consider the maximum loading capacity of PEVs, as well as reevaluate the size, biocompatibility, membrane penetration, and other surface modification aspects of the loaded PEVs. In addition, when the drug-loaded PEVs are applied to organisms, the safety and bioavailability of the PEVs to the host should be reassessed, as well as the appropriateness of the drug concentration in the target cells.

In addition, we believe that the following questions may need to be addressed and answered in the future when it comes to engineered PEVs: whether PEVs screen out some of the larger PEV particles after they enter the circulation through different modes of delivery (orally, intravenously, and topically); and whether there is a difference in the bioactivity or carrying capacity of the same batch of PEVs based on the difference in their sizes; what are the differences in the bioactivities or carrying capacity of the same batch of PEVs when they pass through the circulation to the liver and kidney, what is their clearance capacity; and whether there are differences in tissues and organs based on particle size, surface marker proteins, etc., leading to selective uptake of PEVs. This places greater demands on PEVs preparation and processing, engineering efficiency, choice of delivery method, dose control, and future biosafety. In addition, there is no evidence to support whether the surface properties of PEVs change after engineering, and specific questions include whether the physical and biological properties of the PEVs bilayer membrane structure are altered, whether the active components of PEVs are missing or inhibited, and whether the biosafety and utilization of engineered PEVs need to be reassessed. Therefore, we believe that PEVs, as a new type of nanoparticle, has a long way to go in the future.

The potential biosafety of PEVs

It is worth noting that while exploring the beneficial effects of PEVs, we cannot ignore the potential threats of PEVs. In particular, gram-negative probiotics, such as *ECN* [27] and *A. muciniphila* [85] OMVs, did not cause LPS-induced activation of the inflammatory pathway by the TLR4 pathway, but instead inhibited DSS-induced colonic inflammation, despite the fact that their contents include LPS, which may be based on the fact that probiotics and their OMVs carry certain secreted proteins or membrane proteins that exert stronger anti-inflammatory effects, although their exact composition

is unknown. Therefore, although PEVs reduce the toxicity of probiotics to some extent and moderate intake is beneficial for maintaining or activating innate immune responses [173], we cannot deny that PEVs still have a certain immunogenicity, especially OMVs, which may not be suitable for infants, severely immunocompromised individuals, and those with other diseases. Therefore, the development of a reasonable dosage criterion for the administration of PEVs is also a key focus in future research. In addition, we should also determine the applicable population of PEVs. Wei et al. first found that *Lacticaseibacillus rhamnosus(Lactobacillus rhamnosus)*GG CMVs [174] could exacerbate the degree of vascular calcification in rats with chronic kidney disease through the PI3K/AKT signaling pathway, suggesting that the use of PEVs in specific populations could increase the potential risk.

In addition, to a comprehensive assessment of the safety of PEVs for host cells when administered, consideration should be given to whether the dose of PEVs achieves an interventional effect and whether an overdose may have negative effects or dependence. In a study on *Limosilactobacillus reuteri (Lactobacillus reuteri)* CMVs for the treatment of skin healing [36], the authors explicitly mentioned that 50–100 μg/mL of CMVs had a significant therapeutic effect, whereas either too high or too low a dose was ineffective, and that this was only done by cellular experiments to determine the optimal concentration of PEVs. In addition to the distribution and metabolic time of PEVs in the body, when conducting animal experiments, especially oral administration, the optimal dosage, frequency, and duration of administration of PEVs should also be determined, as well as whether PEVs may cause potential damage or develop dependence upon long-term ingestion. Chen et al. [57] discuss the limitations of the dosing regimen when administering *Lacticaseibacillus rhamnosus* CMVs to mouse animals for the treatment of osteoporosis. In addition, the survival and attrition of PEVs in the gastrointestinal (GI) tract is also an issue of concern. In fact, it has been shown that PEVs can resist the attrition of GI digestive juices after oral administration [175], however, we also need to consider the possible dilution effect of GI digestive juices on PEVs, the rate of intestinal uptake of PEVs, and the attrition of PEVs by other host microorganisms, in view of this, Kuhn et al. designed a particulate molecule coupled to the membrane proteins of PEVs, which somewhat improved the adaptability and survival of PEVs in the GI tract [38].

Technical challenges in the production and application of PEVs

PEVs still face technical challenges such as large-scale production, separation and purification. In the production stage of PEVs, the optimal culture conditions and nutrients take the lead in determining the quality and purity of PEVs. At present, the culture of probiotics usually uses a high concentration of broth medium, it is inevitable that a large number of animal-derived EVs will affect the production results of PEVs, perhaps giving a lower concentration ratio of broth medium or artificial medium will reduce the impact [176]. In addition, the selection of culture conditions for bacteria, such as temperature, pH, and number of passages is particularly important, as it may affect the viability of the bacteria, and poor culture conditions may affect the active components of the bacteria [177]. In addition, regarding the separation and purification of PEVs, there is a lack of mature technology and standardized application procedures. As mentioned above, separation methods such as ultracentrifugation, volumetric exclusion chromatography, and ultrafiltration have been applied to the separation of PEVs, and each of these techniques has its own advantages and disadvantages, and the different methods of separation and purification lead to the separation of the same kind of or even the same strain of PEVs that are significantly different at least in terms of size, and further investigations are needed regarding whether their components are differences need to be further explored, so it is also important to see whether uniform separation techniques and procedures can be developed in the future based on the characteristics of various PEVs. In addition, the characterization of PEVs in terms of their morphology, size, and physicochemical properties is the primary task in the study of PEVs. Currently, most studies are limited to demonstrating the morphology and size of PEVs using TEM, NTA, etc., and labeled proteins by Western blotting, etc., and only a small number of studies have comprehensively analyze specific components of proteins and RNAs to characterize the same PEVs in order to support subsequent scientific studies [23, 56].

Conclusion

In recent decades, the strong biological activity and carrier potential of PEVs have been gradually demonstrated, and they have shown similar functional activities to probiotics in host organs such as the gut, bone, and brain, which also provides new ideas for future studies on the intrinsic mechanisms of probiotics maintaining host homeostasis and their emergence, and their emergence, to a certain extent, circumvents the potential risks associated with the use of active probiotics in children and immunocompromised individuals. Based on their many idealized qualities, PEVs may also become an emerging therapeutic or drug delivery platform in future clinical and industrial practice. However, because research on PEVs is still in its infancy, there is still limited exploration in the isolation and characterization of PEVs, biosafety

and potential risks, as well as the intrinsic mechanisms of microbiota-host interactions mediated by PEVs. Further exploration of the potential of PEVs as therapeutic and pharmaceutical carriers of probiotic metabolites could contribute to the understanding of PEVs-mediated microbiota-host communication and disease control.

Abbreviations

EVs	Extracellular vesicles
PEVs	Probiotic-derived extracellular vesicles
OMVs	Outer membrane vesicles
ECN	*E. coli* Nissle1917
A. muciniphila	*Akkermansia muciniphil*a
O-IMV	Outer-inner membrane vesicle
CMVs	Cytoplasmic membrane vesicles
TRPS	Tunable resistive pulse sensing
NTA	Nanoparticle tracking analysis
SEC	Size exclusion chromatography
DLS	Dynamic light scattering
Cryo-TEM	Cryogenic transmission electron microscopy
SEM	Scanning electron microscope
TEM	Transmission electron microscopy
LTA	Lipophosphatidic wall acids
AFM	Atomic force microscopy
DSS	Dextran sulfate sodium
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
PG	Prostaglandin
ROS	Reactive oxygen species
AFB1	Aflatoxin B1
EPEC	*Enteropathogenic Escherichia coli*
ETEC	*Enterotoxigenic Escherichia coli*
CAT	Catalase
SOD	Superoxide dismutase
GSH	Glutathione peroxidase
ER	Endoplasmic Reticulum
HIF-1α	Hypoxia-inducible transcription factor-1α
GLUT1	Glucose transporter 1
CTLs	Cytotoxic $CD8^+$ T lymphocytes
Anti-PD-1	Anti-programmed cell death 1
CNS	Central nervous system
Treg	Regulatory T-cell
BDNF	Brain-derived neurotrophic factor
ENS	Enteric nervous system
5-HT	Serotonin
OP	Osteoporosis
3-HPA	Royale protein
Hsp70	Chaperonin DnaK
EGFR	Epidermal growth factor receptor
SCFAs	Short-chain fatty acids
PE	Pre-eclampsia
TNZ	Tinidazole
MOFs	Metal-organic frameworks
TA-Mg	Magnesium tannate
GI	Gastrointestinal
BMSCs	Bone marrow mesenchymal stem cells
ASD	Autism spectrum disorder

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X.Z., as a major contributor, wrote the original manuscript. Y.W.and X.Z. draw the figures. M.L., Q.E. and M.N. participated in part writing the manuscript. X.W., Y.L. and M.L. provided the funding and reviewed this manuscript. All the authors revised the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

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