Bacterial extracellular vesicles as bioactive nanocarriers for drug delivery: Advances and perspectives

Han Liu a, Qin Zhang a, Sicheng Wang a, b, Weizong Weng a, **, Yingying Jing a, ***, Jiacan Su a, *

a Institute of Translational Medicine, Shanghai University, Shanghai, 200444, China
b Department of Orthopedics, Shanghai Zhongye Hospital, Shanghai, China

A R T I C L E   I N F O

Keywords:
Bacterial extracellular vesicles
Bioactive nanocarriers
Isolation and purification
Drug delivery

A B S T R A C T

Nanosized extracellular vesicles derived from bacteria contain diverse cargo and transfer intercellular bioactive molecules to cells. Due to their favorable intercellular interactions, cell membrane-derived bacteria extracellular vesicles (BEVs) have great potential to become novel drug delivery platforms. In this review, we summarize the biogenesis mechanism and compositions of various BEVs. In addition, an overview of effective isolation and purification techniques of BEVs is provided. In particular, we focus on the application of BEVs as bioactive nanocarriers for drug delivery. Finally, we summarize the advances and challenges of BEVs after providing a comprehensive discussion in each section. We believe that a deeper understanding of BEVs will open new avenues for their exploitation in drug delivery applications.

1. Introduction

As one of the main groups of organisms, bacteria have formed a close relationship with human health and disease, including sepsis, inflammatory bowel disease, arthrits, and tumors [1-4]. The combination of synthetic biology technology with bacteria has been shown to improve characteristics such as chemotaxis and molecule secretion, thereby facilitating their clinical applications in a range of diseases [5]. Accordingly, in recent years, the application of engineered bacteria in drug delivery has received increasing interest [6]. Bacteria-based delivery systems have led to an ingenius synergy of interdisciplinary research for various biomedical applications [7,8]. However, the pathogenicity and immunogenicity of bacteria are major challenges for bacteria-based drug delivery systems. Although attenuated bacteria with the deletion of virulence genes improve the safety of bacteria-based delivery systems, the potential and unknown side effects remain a significant issue [9]. Recently, with the in-depth understanding of bacteria-based drug delivery systems, the use of bacterial extracellular vesicles (BEVs) for biomedical applications has become a new trend [10].

Bacteria release nanosized extracellular vesicles with diameters ranging from 20 to 400 nm, which carry a range of cargo, including lipopolysaccharide (LPS, also called endotoxin), peptidoglycan (PG), membranes, periplasmic and cytoplasmic proteins, toxins, and nucleic acids. BEVs affect a variety of biological processes, including virulence, horizontal gene transfer, phage infection, transport of cell metabolites, and bacterial-bacterial or bacterial-host interactions [11,12]. From a drug delivery perspective, BEVs are comparable to liposomes, given that both comprise biocompatible lipid bilayers [13]. Lipid-based nanocarriers offer a versatile platform for drug encapsulation, which has led to clinical translation of several formulations [14]. Similarly, cell membrane derived BEVs can transport various bioactive molecules to recipient cells, thereby changing the physiology of those cells. BEVs have been widely investigated as biotherapeutics due to their drug loading capacity, immunostimulatory capability, and ease of modification [15,16]. Such attractive properties have drawn a great deal of attention regarding the use of BEVs for drug delivery, which may overcome issues related to liposomes and other synthetic drug delivery systems.

In this review, we have extensively focused on the recent development of extracellular vesicles derived from gram-negative (G−) bacteria and gram-positive (G+) bacteria. Following a particular emphasis on the
2. Biogenesis and contents of bacterial extracellular vesicles

Based on their structure, morphology, and staining properties, bacteria are divided into G- or G+ types. G- bacteria have thick cell walls with abundant PG, while G+ bacteria have inner and outer membranes. The generation of extracellular vesicles is a spontaneous process that does not require energy consumption [17,18]. The differences in bacterial structure and physiology lead to distinct types of extracellular vesicles [19]. Moreover, the formation mechanisms and contents of BEVs are different between G- bacteria and G+ bacteria (Fig. 1).

2.1. Gram-negative bacteria

As with the history of mammalian extracellular vesicles (MEVs), BEVs have always been regarded as cellular debris from the decomposition of dead cells. The discovery that bacteria produce extracellular vesicles through metabolic activity and the fact that BEVs and MEVs have many similarities in structure and function strongly indicate that extracellular vesicles are released by living bacteria, especially G- bacteria [20–22]. G- bacteria are characterized by an outer membrane, cytoplasmic membrane, and periplasmic space [23]. The outer membrane contains an outer leaflet of LPS and an inner leaflet of phospholipids. The cytoplasmic membrane, which serves as an electrochemical barrier, is composed of a typical phospholipid bilayer. The periplasmic space contains a PG layer, lipoproteins, and periplasmic proteins [24]. The reticulated PG layer in the periplasm imparts bacterial morphology and protects the cell from absolute pressure and osmotic changes.

It has been observed that all types of G- bacteria generate BEVs in different environments, such as biofilms, planktonic cultures, fresh and salt water, and inside eukaryotic cells and mammalian hosts [25–27]. Extracellular vesicles were first reported in G- bacteria in the 1960s [28,29]. In 1995, Kadurugamuwa et al. provided obvious evidence that the G- bacterium Pseudomonas aeruginosa releases extracellular vesicles into medium during normal growth [30]. Transmission electron microscopy (TEM) demonstrated that these extracellular vesicles were spherical bilayers with a diameter of 50–150 nm. Moreover, immunoelectron microscopy and western blot analysis showed that these extracellular vesicles contain LPS and DNA [30]. The extracellular vesicles derived from Escherichia coli DH5α had a diameter of 15–100 nm [31]. Additionally, a total of 141 proteins were identified by using a proteomics approach in DH5α-derived extracellular vesicles, which were highly enriched in outer membrane proteins but lacking in inner membrane proteins. Other G- bacteria such as Salmonella sp. [32], Shigella sp. [33], Campylobacter jejuni [34], Helicobacter pylori [35], Vibrio sp. [36], Borrelia burgdorferi [37], Neisseria sp. [38], and Acinetobacter baumannii [39] et al. have also been reported to produce extracellular vesicles.

The extracellular vesicles derived from G- bacteria are generated by two main models: blebbing of the outer membrane and explosive cell lysis [19]. The insertion of hydrophobic molecules or the instability of PG biosynthesis into the outer membrane cause blebbing of the outer membrane, which produces classical outer membrane vesicles (OMVs) (Fig. 1) [12,19]. Both genetic background and growth conditions strongly affect the production of BEVs. It has been reported that the use of polymyxin or gentamicin can influence lipid homeostasis to trigger cell outer membrane stress and generate OMVs through blebbing of the outer membrane [40,41]. Structurally, OMVs are spherical particles with a size of 20–250 nm, which consist of an inner leaflet of phospholipid and an outer leaflet of LPS [16,18,42,43]. In this case, the inner proteins of OMVs are thought to be expressed in a similar manner to those of the outer membrane, whereas the outer leaflet of the lipopolysaccharide and LPS [44].

Fig. 1. The biogenesis and composition of bacterial extracellular vesicles. The G- BEVs are generated by two main models, blebbing of the outer membrane and explosive cell lysis. The insertion of hydrophobic molecules or the instability of PG biosynthesis into the outer membrane cause blebbing of the outer membrane, which produces classic OMV. The weakness of PG layer by endolysin leads the inner membrane protrudes into the periplasm, which generate explosive EOMV or OIMV. On the other hand, the biogenesis mechanism of G+ BEVs release is bubbling cell death. The endolysin degrades the PG layer and triggers bubbling cell death in G+ bacteria and produces CMVs. The difference in contents of G- and G+ BEVs goes beyond the presence of LPS and includes other molecules, such as nucleic acids, proteins, lipids, and metabolites. LPS: lipopolysaccharide, OM: other membrane, PG: peptidoglycan, IM: Inner membrane.
membrane is not damaged, and the components in the cytoplasm cannot access OMVs. Therefore, these OMVs contain abundant outer membrane proteins and lipids. Growth conditions such as the temperature, oxidation state, nutrient availability, quorum sensing, and envelope-targeting antibiotics, etc., also affect the contents of cargo in OMVs [44,45]. As growth conditions have significant influence on the BEV process, late-stage cells display maximum OMV yields [46]. However, unavoidable cell death in later stages causes contamination with cytosolic proteins and membrane components. Moreover, nutrient deficiency and waste accumulation at later life stages of cells affect the composition profile of BEVs [47,48]. Notably, many studies have shown that OMVs contain cytosolic and periplasmatic proteins, DNA and RNA [49,50]. The selectivity of OMV cargo indicates that biogenesis of extracellular vesicles is a deliberate process, rather than a random event. However, the biogenesis mechanism of cargo selection is still ambiguous. In waste accumulation at later life stages of cells affect the composition ranges from 20 nm to 100 nm. Those BEVs goes beyond the presence of LPS and -bacteria do not contain LPS, which is a compound that may cause an innate immune response [17]. However, the difference in the contents of G+ and G− BEVs goes beyond the presence of LPS and includes other molecules such as DNA, RNA, proteins, lipids, and metabolites, ultimately resulting in the different biological functions of these BEVs. Functionally, BEVs have been determined to have diverse roles, resulting in distinct host immune responses [11,16,17,19,75]. In general, it appears that bacteria can use extracellular vesicles as active nanocarriers to communicate with other bacteria and hosts. Recent development of nanostructured BEVs for drug delivery and therapeutics will be introduced in detail in subsequent chapters.

### 3. Isolation and purification of bacterial extracellular vesicles

Here, effective BEV isolation and purification techniques are provided in this review (Fig. 2). After culturing for an appropriate amount of time, bacteria in the fermentation broth are removed by low-speed centrifugation (usually 2000 × g ~ 10,000 × g) [31,63,65]. The supernatant is then filtered through a sterile filter (usually 0.22 µm or 0.45 µm, depending on the size of bacteria) to remove residual bacteria [76,77]. Subsequently, ultrafiltration membranes (usually 50 KDa ~ 100 KDa) are required to concentrate BEVs and remove non-BEV-associated proteins. The ultrafiltration membrane technique is the commonly used method in the concentration step of BEVs. The choice of ultrafiltration membrane is critical for successful concentration. Regenerated cellulose membranes rather than polyether sulfone membranes are the first choice for dilute solutions. However, polyether sulfone membranes are preferred for high protein concentrations, such as porcine mucin for Akkermansia muciniphila (A. muciniphila) [17,78]. Furthermore, the retentate is subjected to ultracentrifugation, which is the most conventional approach for extracellular vesicle isolation, to collect the BEVs [11,31,79]. However, unpurified BEVs can mix with bacteria flagella or large protein complexes, which may affect the subsequent experiments and lead to unexpected immunomodulatory effects [42]. Therefore, non-BEVs related materials are often separated by density gradient centrifugation, which is a more stringent form of ultracentrifugation [80]. Usually, density gradient centrifugation and ultracentrifugation are used in combination to obtain purified BEVs. The most frequently applied media for density gradient centrifugation are sucrose [31] and iodixanol [79]. Compared with other media, iodixanol has obvious advantages, and its solutions are isosmotic, protecting subtle membraneous structures [81]. Unpurified BEVs are typically mixed with 60% w/v iodixanol and then stacked with 40% w/v, 20% w/v, and 10% w/v iodixanol [79,82,83]. BEVs migrate into an
equilibrium position according to their density during ultracentrifugation. Then, the BEV-rich fraction is diluted to 50 mL PBS and centrifuged at 100,000 × g for 3 h to collect BEV pellets [31,79]. Although gel filtration is another choice for BEV purification, this method requires special equipment and fillers, and it is not suitable for multiple sample operations at the same time.

After obtaining purified BEVs, their quantification and quality determination are vital steps for further application. TEM is a well-established and commonly used technique to demonstrate the presence of BEVs and to observe their sizes, shapes, and purity [84]. Moreover, dynamic light scattering (DLS) is usually used to measure the size distribution of extracellular vesicles in liquid suspension [79,85–87]. Liu et al. used both TEM and DLS to characterize the sizes and shapes of isolated extracellular vesicles derived from gut microbiota [79]. The TEM and DLS results showed that the extracellular vesicles derived from child gut microbiota, elderly gut microbiota, and A. muciniphila were spherical structures with diameters of nearly 200 nm. However, when DLS is used to measure smaller particles, the measured particle size has a large deviation from the actual size. To make up for the shortcomings of DLS, nanoparticle tracking analysis (NTA) technology was developed. NTA is another commonly used method to measure the size distribution and concentration of nanoparticles and has higher accuracy than DLS [88]. Therefore, it has become the most mainstream size measurement method in extracellular vesicles. Hu et al. applied both TEM and NTA to evaluate hybrid nanoparticles (liposomes and exosomes) [89]. The TEM and NTA results showed that the nanoparticles were spherical with a diameter of nearly 150 nm, accounting for 99.2% of the total amount [89]. Although the tunable resistive pulse sensing (TRPS) method is one option to measure the size distribution and concentration of extracellular vesicles, for small nanoparticles (usually <150 nm), NTA always detects more extracellular vesicles than TRPS [90].

4. Application of bacterial extracellular vesicles

Conventional synthetic nanomaterials, such as liposomes, metal-based nanoparticles, and polymers, have been widely used as drug carriers [91]. However, the simple conjugation of synthetic nanomaterials is not efficient in replicating intercellular interactions that facilitate nanoparticle trafficking and delivery [18]. Due to the ability to contain and deliver various bioactive molecules, nanosized BEVs have great potential as a new type of drug delivery carrier. Moreover, it has been demonstrated that BEVs can enter distant organs by systemic circulation through the disrupted tight junction under microbial disorder and inflammatory environment (Fig. 3) [92]. Here, we summarize the current applications of BEVs in gut, brain, bone, and tumor diseases (Fig. 4).

4.1. Gut

The gut microbiota, which is the basis for regulating human health, actively promotes the functions of the intestine and the distal organs [93]. The intestinal microbiota and host cells have established a complex and dynamic relationship, also known as interkingdom crosstalk [93]. Epithelial cells from both the gastrointestinal tract, lung, cornea, and oral cavity are besieged by numerous pathogens, opportunistic pathogens, and probiotics. In an in vivo experiment, both Porphyromonas gingivalis cells and their vesicles increased IL-8 expression, but monocytes were more likely to be recruited by extracellular vesicles, indicating a vesicle-induced innate immune response in the epithelial system [94]. Moreover, excessive IL-8 production was observed in primary human bronchial and intestinal epithelial cells cocultured with P. aeruginosa and E. coli O157 extracellular vesicles [95,96]. The immunostimulatory functions of BEVs have also been appreciated in mouse models, represented by other cytokines including IL-1β, IL-6 and CCL2. For example, mitochondrial apoptosis and NLRP3 inflammasome activation were induced by extracellular vesicles from Neisseria
Fig. 3. BEVs achieve access to systemic circulation through the disrupted tight junction under microbial disorder and inflammatory environment. Reprinted with permission [92]. Copyright 2020, Springer.

Fig. 4. The application of bacterial extracellular vesicles in drug delivery. As a nano-scale bioactive material, BEVs deliver a variety of active molecules such as nucleic acids, protein, metabolisms etc. for the treatment of gut, brain, bone, and tumors.
gonorrhoeae, E. coli and P. aeruginosa, thus activating and releasing IL-1β [97]. After injection of A. baumannii-derived extracellular vesicles, the expression of proinflammatory cytokines, including IL-6, macrophage inflammatory protein-1α, Ccl2 and Ccl3, was upregulated in mouse lung epithelial cells [98]. Despite this evidence about BEV-induced epithelial immune reactions, the specific mechanism and promising application are not fully understood. Under the threat of millions of microbiomes, how epithelial cells maintain the gastrointestinal barrier remains unknown.

There are two layers of mucus gel in vivo: an outer layer that is easy to remove by simple suctioning and an inner layer that is free of bacteria [99, 100]. Muc2 mucin from the outer sparse layer not only provides an anchor point for bacterial adhesion but also acts as a source of carbon and energy or even an anoxic environment if necessary [101]. Pathogenic alterations or genetic defects such as Muc2−/− lead to direct contact between bacteria and epithelia and trigger an immune response, chronic inflammation or even cancerization [102]. BEVs from C. jejuni proteolytic activity in vitro were able to cleave both E-cadherin and occludin, thus disrupting both tight junctions and adherens junctions [103]. On the other hand, probiotics can help maintain the physiologic barrier via membrane proteins or BEVs. A special membrane protein named Amuc_1100 increased the expression level of tight junction proteins including occludin and claudin3 through TLR2 activation [104]. The extracellular vesicles from probiotic E. coli Nissle 1917 enhanced the intestinal mucosal barrier by upregulating the tight junction proteins ZO-1, ZO-2 and claudin-14 [105]. Moreover, these vesicles were shown to rescue intestinal epithelial barrier dysfunction induced by enteropathogenic E. coli [106]. The extracellular vesicles secreted by probiotic A. muciniphila improved tight junctions and gut permeability by activating AMP-activated protein kinase (AMPK) [107]. Although protein differences have been shown to influence AMPK activation, it has not been determined which specific protein of A. muciniphila extracellular vesicles is responsible. Moreover, proteins are not the only bioactive components of extracellular vesicles. It has been reported that the extracellular vesicles derived from B. thetaiotaomicron contains inositol polyphosphates phosphatases, which could promote polysaccharide digestion [108]. Similarly, another common intestinal colonizing bacteria, Bacteroides spp. can release extracellular vesicles that can deliver a variety of hydrolase to participate in the decomposition of glycans and mucins (Fig. 4) [109].

4.2. Cancer

4.2.1. Gut microbiome, BEVs and cancer development

The close relationship of gut microbiota and cancer in organs has become fully appreciated in recent decades [110]. To date, microbiome dysbiosis has been identified in colorectal cancer [111, 112], breast cancer [113], lung cancer [114], liver cancer [115], and melanoma [116]. In addition, bacteria inside colorectal cancer travel along with tumor cells during liver metastasis, and metronidazole treatment reduces the Fusobacterium load and overall tumor growth [117]. However, it remains unknown what role BEVs play in the complex crosstalk between gut microbiota, intratumoral microra and host cells, and their effects may be subtle and context dependent.

As illustrated in the last chapter, BEVs can gain access to systemic circulation through disrupted tight junctions under microbial disorders and an inflammatory environment (Fig. 3). Microbe-associated molecular patterns (MAMPs) can be delivered by BEVs, thus triggering various immune responses (Fig. 5). MAMPs are recognized by pattern recognition receptors (PRRs), which are key components of innate immunity (Fig. 5). Interestingly, extracellular vesicles from different bacteria lead to disparate host immune responses, depending mainly upon their

Fig. 5. Recognition of BEV-associated molecular patterns by host immune receptors. TLR1/6, TLR2, TLR4, and TLR5 located at the host cell membrane, TLR3, TLR7/8, TLR9 located at the endosomal membranes. The downstream signaling pathways that lead to activation of NF-kB and inflammatory genes. Adaptor molecules: MyD88, TRAP, TRIF, TRAM. TLRs: toll-like receptors. Reprinted with permission [93]. Copyright 2021, Wiley-VCH GmbH.
relationship with host cells [92]. For example, extracellular vesicles derived from enterotoxigenic *Bacteroides fragilis* (ETBF) and non-toxigenic *Bacteroides fragilis* (NTBF) present different enzyme systems and biological functions, despite their genetic similarity. Compared with polysaccharide utilization enzymes in NTBF, many proteins contributing to stability, capability and virulence factor delivery were identified in ETBF [118].

### 4.3.2. From gut to brain: BBB penetration

Targeted delivery. Furthermore, bioengineered BEVs with kinesin spin (γ) production and antitumor immune responses [120]. Polysaccharide utilization enzymes in NTBF, many proteins contributing to stability, capability and virulence factor delivery were identified in ETBF [118].

**BEVs and cancer therapy**

Compared to live or weakened bacteria, BEVs have been thought to be safe, as they cannot be autonomously duplicated in vivo. BEVs exhibit great stability against temperature and carry multiple immunogenic membrane-associated or cytoplasm-associated components, and these intrinsic characteristics make them ideal candidates for both vaccines or immune modulators in novel cancer therapy [119]. Extracellular vesicles from LPS-depleted *E. coli* were reported to target cancer cells in vivo and reduce tumor burden by constant CXCL10 and interferon gamma (IFN-γ) production and antitumor immune responses [120]. Antigen-displaying BEVs were introduced to induce dendritic cell (DC) maturation and antigen presentation to T cells and yield tumor-specific antibodies like vaccines [121]. To expand the efficiency and application area of BEVs, Chen et al. combined melanoma cytomegamovirus, *Salmonella* vesicles and photothermal modules to suppress tumorigenesis [122].

BEV-derived nano-therapy has received wide attention as an antitumor vaccine or targeting vehicle [123]. The protein-rich membrane structure of BEVs makes them attractive biological components for hybrid nanoparticles when combined with traditional synthetic materials. Nanoparticles coated with *S. aureus*-derived membrane vesicles were shown to present homologous macrophage affinity, release anti-biotics, and eliminate intracellular *S. aureus* efficiently [124]. BEV-coated gold nanoparticles demonstrated enhanced biostability and elevated IFN-γ and IL-17 production, indicating a faster immune response, which could be applied in cancer cell-targeted therapy [125]. Chemotherapeutic-loaded BEVs enabled increased doxorubicin infiltration and tumor cell apoptosis [126], indicating a promising method for targeted delivery. Furthermore, bioengineered BEVs with kinesin spindle protein (KSP) siRNA were able to target and kill cancer cells [127].

### 4.3. Brain

#### 4.3.1. BEV-related neurologic abnormality

It is well recognized that bacteria-induced immune responses could be responsible for brain disorders, such as meningitis. Recent evidence has shown that BEVs not only activate the immune response via inherited components such as LPS and other antigens but also manipulate brain disorders through hereditary substance delivery [128]. BEVs from Alzheimer’s disease (AD) patients were reported to affect blood-brain barrier (BBB) permeability and impair the learning memory ability of mice [129]. It has been confirmed that *Paenelcaligenes hominis*-derived extracellular vesicles can deliver cargo to the brain through the blood and vasa nervus and result in AD [130]. In addition, enterohemorrhagic *E. coli* (EHEC)-derived extracellular vesicles induce permeabilization of the mitochondrial membranes and trigger the mitochondrial apoptotic pathway in human brain microvascular endothelial cells; this result might suggest an underlying regulatory mechanism between the gut microbiota and central nervous system [131].

#### 4.3.2. From gut to brain: BBB penetration

Various studies have identified BEVs as BBB breakers and have found that BEVs play a critical role in gut-brain communication [132]. Two papers recently published by Lee et al. introduced periodontopathogenic extracellular vesicles to deliver RNA cargo to intracranial immune cells such as macrophages and induce neuroinflammatory diseases [133, 134]. For instance, BEVs from *Aggregatibacter actinomycetemcomitans* can travel across the BBB and successfully upregulate TNF-α expression. Due to this natural penetration ability, BEVs are valued as potential players in intracranial delivery. Nanogold and bacterial vesicle hybrid particles were introduced by Chen et al. [135] to augment chemoradiotherapy against glioblastoma and achieve longer survival of experimental mice. 4CMenB (Bexsero; GSK Biologicals), a vaccine against *Neisseria meningitidis* serogroup B (MenB), was introduced in 2013 and was confirmed to protect infants and children from MenB [136]. Porin protein A displays BEVs and is one of the four constituents in the vaccine [137,138], indicating enormous potential in both pharmacology and materials science. Recently, Bittel et al. visualized the process of transfer the microbial biomolecules (Cre/RNA) by BEVs in vivo via Cre/LoxP system (Fig. 4) [139].

### 4.4. Bone

The gut microbiome plays a critical role in bone metabolism during skeletal development and dynamic homeostasis [140]. Insulin-like growth factor 1 (IGF-1) was the first metabolite identified to be a messenger between intestine and bone, and antibiotic administration decreased serum IGF-1 levels and thus inhibited bone formation [141]. Recently, microbiota-derived short-chain fatty acids (SCFAs) were confirmed to connect the intestinal tract and bone metabolism [142]. Oral administration of lactulose increased SCFA production and attenuated ovarioectomy-induced osteoporosis, indicating a close relationship between the gut and bone [143]. Claire et al. declared that warmth enhances bacterial polymame biosynthesis, thus increasing bone strength [144]. Despite all these studies on the gut-bone axis, the role of BEVs in the close communication between the microbiota, gut epithelium and bone is still a mystery. According to the gut-bone axis and BEV behavior in other systems such as the brain and cancer, we hypothesize that BEVs participate in bone metabolism in both intestinal-mediated and bone-targeting manners, and we are currently conducting experiments to prove this hypothesis.

The latest research has reported that specific BEVs from the gut microbiome travel across the gut mucosal barrier and arrive at the marrow space. For example, BEVs isolated from *A. muciniphila* were found to reach the marrow space in 1 h via oral, rectal, and intravenous administration. The study identifies that the gut bacterium *A. muciniphila* mediates the antiosteoporotic effects of the gut microbiota of children and presents a novel mechanism underlying the exchange of signals between the gut microbiota and host bone [79]. Moreover, it has been reported that extracellular vesicles from *A. muciniphila* promoted osteoblasts and inhibited osteoclasts (Fig. 4) [79]. Based on rapid industrial production, bone-targeting engineering techniques reported for exosomes could be translated to BEVs to generate novel nanocarriers. For instance, stromal cell-derived factor 1 (SDF1) is mainly provided by bone marrow stromal cells (BMSCs) and brings CXCR4-positive cells back to bone marrow, including hematopoietic stem cells (HSCs) [145] and breast cancer cells [146]. Our group constructed a bone-targeting hybrid exosome-based nanocarrier system for bone microenvironment regulation [89].

### 5. Advantages and challenges

Bacteria and humans have formed a close and inseparable relationship during the long history of evolution. The extracellular vesicles secreted by bacteria offer various ingenious synergies for biomedical applications. Due to their proven immunomodulatory properties, BEVs have been used as viable tools for drug delivery, vaccine development, disease diagnosis, etc. Although the study of BEVs is less developed than that of eukaryotic extracellular vesicles (such as exosomes), the number of BEV studies has continuously increased in recent years [17]. Here, we summarize the advantages and challenges associated with BEVs (Fig. 6).
5.1. Advantages

5.1.1. Ease of industrialization

Compared with eukaryotic cells, bacteria show remarkable advantages including rapid proliferation abilities, mature high cell density culture techniques, and easy gene editing methods [147], which have the opportunity to produce more extracellular vesicles. Moreover, bacterial fermentation has always been a more viable production process because it offers the advantages of cost effectiveness and scalability [148, 149]. Therefore, bacterial-derived extracellular vesicles have greater opportunities for industrialization. For example, Liu and colleagues were granted a national invention patent on the anti-osteoporotic effect of probiotic *A. muciniphila* and its extracellular vesicles [79], which lays the foundation for the future industrial application of probiotics or their functional extracellular vesicles to prevent and treat osteoporosis.

5.1.2. High efficiency of drug delivery

As nanosized materials, BEVs can enhance the efficiency of drug uptake and delivery. In addition, BEVs present great biostability, which can ensure efficient drug delivery. BEVs can protect bioactive molecules from degradation, which enhances the stability of cargo and enables delivery of cargo under functional conditions to target cells [150]. For example, RNA, an unstable bioactive molecule, cannot survive without shelter during systemic circulation. However, bacterial RNA has been found in the brains of patients with AD [151, 152], suggesting that BEVs deliver RNA throughout the microbiota-gut-brain axis.

5.1.3. Ease of modification

Moreover, the loading of bioactive molecules in BEVs can occur in vivo or in vitro. *In vivo*, molecules of interest can be loaded in the generated extracellular vesicles when cells are treated with these molecules. For example, the strain *P. aeruginosa* PAO1 produced extracellular vesicles containing gentamicin when it was cultured with gentamicin [153]. This method may be used to produce extracellular vesicles carrying the compound of interest. *In vitro*, advances in genetic engineering have enabled the use of BEVs to deliver specific drugs and agents as a strategy to circumvent biocompatibility and large-scale production issues associated with synthetic nanomaterials [154]. Puriﬁed extracellular vesicles could also be encapsulated with different types of compounds through electroporation. Gujrati et al. developed a simple electroporation method to load siRNA into BEVs and showed that the established protocol was effective [155]. Similarly, Ayed et al. established a novel drug delivery system by loading BEVs with gold nanoparticles via electroporation [156]. The capacitance on the membrane creates pores in the lipid bilayer of BEVs, which allows any nanoparticle smaller than 10 nm to enter the vesicles. Subsequently, the closure of the BEV membrane captures the nanoparticles as cargo.
5.1.4. Ease of bacterial infections diagnose

BEVs can be used for the diagnosis of certain bacterial infections [157]. Since BEVs spread with intestinal microorganisms and are easily found in body fluids, diagnosis based on extracellular vesicles has significant advantages. Therefore, BEVs provide new insights into the links between microorganisms and the health status of the host. In addition, BEVs are a promising candidate for vaccine development against bacterial infections [34,83,84,95,158]. One of the advantages of using BEVs for vaccination is their ability to present native antigens, thus inducing effective immune responses.

5.2. Challenges

5.2.1. Ambiguous mechanism

Although BEVs have been proven to be a new drug delivery system for targeted drug delivery [18], there are still many areas for exploration of BEVs. For example, the mechanism of BEV internalization by host cells remains ambiguous. Several internalization pathways such as clathrin-mediated endocytosis [84,159], micropinocytosis [16], non-clathrin-mediated endocytosis [160], and membrane fusion [161] have been found. It is necessary to address which receptors mediate the internalization of BEVs. In addition, the mechanism of packaging these components in BEVs is not yet fully understood [17]. Is a molecule specific to certain strains and extracellular vesicles? Is it specific to certain environmental conditions? These questions remain to be elucidated in the future.

5.2.2. Potential biosafety

BEVs, especially the extracellular vesicles derived from G⁺ bacteria, have been shown to contain many virulence factors, such as LPS and virulent proteins [32,84]. Therefore, regarding safety issues, caution should be exercised when BEVs are used as drug delivery nanocarriers because LPS could cause an innate immune response [97]. This immunotoxicity of BEVs will be a challenging aspect in drug delivery applications. For G⁻ bacteria, LPS is one of the most abundant components of BEVs and is displayed on the outer surface of these vesicles. There are also mutant G⁻ bacteria that do not contain LPS, such as E. coli EMKV15. Alternatively, the antibiotic polymyxin B can be used to neutralize LPS [162]. In principle, extracellular vesicles derived from G⁻ bacteria, which do not contain LPS and thus are generally less toxic than G⁺ bacteria-derived extracellular vesicles, may be a better choice in drug delivery [119].

5.2.3. Time-consuming isolation

Although an overview of BEV isolation and purification techniques has been provided, the methods are still complicated and time-consuming. A timesaving, cost-effective, and efficient isolation method from a wide range of biological matrices needs to be proposed. Moreover, the existing techniques still cannot successfully separate BEVs, and prokaryotic extracellular vesicles exist together in the same sample. The human body is inhabited by approximately 10^{13} microbes composing a multicompartment system, which is strongly involved in the regulation and maintenance of homeostasis. BEVs can be found in several human biofluids (such as blood, plasma, and urine) and can be used as tools for bacterial detection and identification [163]. In addition, the specific (surface) markers present on BEVs from different sources are still unknown. Vanaja et al. used Omfp, an outer membrane protein of E. coli, as a marker for E. coli-derived extracellular vesicles [84]. The separation of BEVs and prokaryotic extracellular vesicles may be one of the most promising methods to discover specific markers and reveal the pathophysiological mechanisms of diseases.

5.2.4. Complex contents

Although BEVs are rich in a variety of effective bioactive and functional molecules, the composition is still complex and ambiguous. Notably, the contents and sizes of BEVs can change drastically when growth conditions or strains change [164]. In addition, the mechanism of packaging these components into BEVs is not yet fully understood and needs to be addressed in the future. A better characterization of these extracellular vesicles will increase our understanding of the possible functions and applications. With the development of multomics technology, the composition of extracellular vesicles will be clearly identified.

6. Concluding remarks and outlook

We have shown that both G⁺ and G⁻ bacteria can produce BEVs. Bacteria have two types of vesicle formation mechanisms, namely, membrane blebbing and endolysin-triggered cell lysis. Both mechanisms generate various extracellular vesicles, such as OMVs, IMVs, EMVs, and CMVs. The difference in the contents of G⁺ and G⁻ BEVs goes beyond the presence of LPS and includes other molecules, such as nucleic acids, proteins, lipids, and metabolites. Isolation and purification of BEVs are essential steps for subsequent applications. Moreover, the lack of standard methods of isolation and purification and the lack of well-defined identification of individual markers present on BEVs are obstacles to the progress of BEV research [165]. At present, information on achieving a high production of BEVs from fermentation broth including a complex medium composition and metabolites is limited. Here, we provide an effective protocol for BEV isolation and purification.

BEVs have been exploited as a new drug delivery platform. As the gene editing technology has been widely used in the bacteria, the overexpression or knockout of certain genes can influence the secretion and contents of extracellular vesicles. We hope that this review will raise the awareness that the drug delivery applications of BEVs are far beyond treating the gut, the brain, bone, and tumors. The intestinal microbiota contributes to the metabolic health of the human host [3,4,166]. Therefore, we hypothesize that these intestinal microbiota-derived extracellular vesicles may be involved in the metabolism of various tissues and result in different health statuses.

BEVs display many desirable qualities such as ease of industrialization, highly efficient drug delivery, ease of modification, and ease of bacterial infections diagnosis. On the other hand, there are still significant challenges for the clinical translation of BEVs as delivery systems or therapeutics, including potential biosafety, complicated and time-consuming isolation, complex and ambiguous contents, and mechanism (Fig. 6). While research on BEVs is in the early stage, their unique nanosized structure and biofunctions make them promising platforms in nanomedicine.

Authorship contribution statement

Han Liu, Qin Zhang, and Sicheng Wang contributed equally to this work. Han Liu, Qin Zhang, and Sicheng Wang drafted and wrote the manuscript of this review. Weizong Weng, Yingying Jing, and Jiacan Su guided and revised the manuscript of this review.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Key R&D Program of China (2018YFC0201500); National Natural Science Foundation of China (NSFC) Key Research Program in Aging (91749204); National Natural Science Foundation of China (82172098, 81771491, 81972254).
Abbreviations Used

- Lipopolysaccharide (LPS)
- Gram-negative G
- Gram-positive G
- Bacteria extracellular vesicles (BEVs)
- Mammalian extracellular vesicles (MEVs)
- Peptidoglycan (PG)
- Outer membrane vesicles (OMVs)
- Transmission electron microscopy (TEM)
- Dynamic light scattering (DLS)
- Nanoparticle tracking analysis (NTA)
- Tunable resistive pulse sensing (TRPS)
- Alzheimer’s disease (AD)
- AMPPK (AMP-activated protein kinase)
- MAMPs (Microbe-associated molecular patterns)
- PRRs (Pattern recognition receptors)
- TEM (Transmission electron microscopy)
- RT-PCR (Reverse transcription-polymerase chain reaction)
- TRAP (Tumor necrosis factor-related apoptosis-inducing ligand)
- IGF-1 (Insulin-like growth factor 1)

References

1. P.O. Nguyen, N.D. Courchesne, A. Duraj-Thatte, P. Praveschotinunt, N.S. Joshi, Engineered living materials: prospects and challenges for using biological systems to direct the assembly of smart materials, Adv. Mater. 30 (39) (2018), e1704877.

2. D. Nejman, I. Livyatan, G. Fuks, N. Gavert, Y. Zwang, L.T. Geller, A. Rotter, Bacteroides fragilis 41.

3. H. Liu et al., K.A.Y. Defourey, E.J. Smid, T. Abe, Gram-positive bacterial extracellular vesicles and their impact on health and disease, Front. Microbiol. 9 (2018) 1502.

4. S. Liao, M.I. Klein, K.P. Heim, Y. Fan, J.P. Bitoun, S.J. Ahn, R.A. Burne, H. Koo, L. Brady, Z.T. Wen, Streptococcus mutans extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery, J. Bacteriol. 196 (13) (2014) 2355-2366.

5. T.J. Silhavy, D. Kahn, S. Walker, The bacterial cell envelope, Cold Spring Harbor Perspect. Biol. 2 (5) (2010) a004141.

6. K. Koester, J. Kriemler, Balancing oxidative protein folding: the influences of reducing pathways on disulfide bond formation, Biochem. Biophys. Acta 1844 (8) (2014) 1383-1390.

7. S. Jilici, P. Schmitz, S.E. Roggensack, A.W. Thompson, R.E. Summons, S.W. Chinholm, Bacterial vesicles in marine ecosystems, Science 343 (6167) (2014) 183-186.

8. E. Andaloussi, P. Vader, Extracellular vesicles as drug delivery systems: why and how? Adv. Drug Deliv. Rev. 159 (2020) 332.

9. C.A. Hickey, M.J. Kuehn, Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions, Nat. Rev. Microbiol. 13 (10) (2015) 605.

10. Z. Li, Y. Wang, J. Liu, P. Rawding, J. Bu, S. Hong, Q. Hu, Chemically and biologically engineered bacteria-based delivery systems for emerging cancer diagnosis and advanced therapy, Adv. Mater. (2021), e2102580.

11. L. Brown, J.M. Wolf, R. Pradossi-Rosas, A. Casadella, Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi, Nat. Rev. Microbiol. 13 (10) (2015) 620-630.

12. C. Schwechheimer, M.J. Kuehn, Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions, Nat. Rev. Microbiol. 13 (10) (2015) 605-619.

13. M.O. Elsharkasy, J.Z. Nordin, D.W. Hagey, O.G. de Jong, R.M. Schiffelers, S. van Soest, Membrane vesicle formation is associated with pyocin production under stress conditions, Cell Microbiol. 18 (11) (2016) 1525.

14. R.J. Shoberg, D.D. Thomas, Specific adherence of Shigella flexneri to mammalian epithelial cells mediated by outer membrane vesicles, Cell Microbiol. 1 (3) (2000) 177-185.

15. D. Chatterjee, K. Kadam, A.J. Clarke, T.J. Beveridge, Cell-free DNA from Gram-negative bacteria induces epigenetic changes in human intestinal epithelial cells, Infect. Immun. 80 (12) (2012) 4089-4098.

16. J. Turner, J. Prazsikler, M.I. Hutton, D. Steer, G. Ram, M. Kaparasik-Laskos, R.L. Stewart, Increased outer membrane vesicle formation in a Helicobacter pylori cagA mutant, Helicobacter 20 (4) (2015) 269-283.

17. R.J. Shoberg, D.D. Thomas, Specific adherence of Bordetella pertussis extracellular vesicles to human endothelial cells in culture, Infect. Immun. 69 (1) (1997) 392-3960.

18. D. Nejman, I. Livyatan, G. Fuks, N. Gavert, Y. Zwang, L.T. Geller, A. Rotter, Bacteroides fragilis 41.

19. J.L. Kadurugamuwa, T.J. Beveridge, Membrane vesicles derived from Pseudomonas aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion, J. Bacteriol. 177 (14) (1995) 4998-5008.

20. E. Andaloussi, P. Vader, Extracellular vesicles as drug delivery systems: why and how? Adv. Drug Deliv. Rev. 159 (2020) 332.

21. C.A. Hickey, M.J. Kuehn, Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions, Nat. Rev. Microbiol. 13 (10) (2015) 605-630.

22. S. Liao, M.I. Klein, K.P. Heim, Y. Fan, J.P. Bitoun, S.J. Ahn, R.A. Burne, H. Koo, L. Brady, Z.T. Wen, Streptococcus mutans extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery, J. Bacteriol. 196 (13) (2014) 2355-2366.

23. T.J. Silhavy, D. Kahn, S. Walker, The bacterial cell envelope, Cold Spring Harbor Perspect. Biol. 2 (5) (2010) a004141.

24. K. Koester, J. Kriemler, Balancing oxidative protein folding: the influences of reducing pathways on disulfide bond formation, Biochem. Biophys. Acta 1844 (8) (2014) 1383-1390.

25. S. Jilici, P. Schmitz, S.E. Roggensack, A.W. Thompson, R.E. Summons, S.W. Chinholm, Bacterial vesicles in marine ecosystems, Science 343 (6167) (2014) 183-186.

26. E. Andaloussi, P. Vader, Extracellular vesicles as drug delivery systems: why and how? Adv. Drug Deliv. Rev. 159 (2020) 332-343.

27. E.K. Lee, J.Y. Bang, G.C. Smith, N.S. Joshi, H.K. Kim, K.S. Park, J.O. Lee, Y.K. Kim, H.J. Kwon, K.P. Kim, Y.S. Gho, Global proteomic profiling of native outer membrane vesicles derived from Escherichia coli, Proteomics 7 (2007) 3143-3153.

28. E. Andaloussi, P. Vader, Extracellular vesicles as drug delivery systems: why and how? Adv. Drug Deliv. Rev. 159 (2020) 332-343.

29. C.A. Hickey, M.J. Kuehn, Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions, Nat. Rev. Microbiol. 13 (10) (2015) 605-619.

30. S. Liao, M.I. Klein, K.P. Heim, Y. Fan, J.P. Bitoun, S.J. Ahn, R.A. Burne, H. Koo, L. Brady, Z.T. Wen, Streptococcus mutans extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery, J. Bacteriol. 196 (13) (2014) 2355-2366.

31. T.J. Silhavy, D. Kahn, S. Walker, The bacterial cell envelope, Cold Spring Harbor Perspect. Biol. 2 (5) (2010) a004141.

32. K. Koester, J. Kriemler, Balancing oxidative protein folding: the influences of reducing pathways on disulfide bond formation, Biochem. Biophys. Acta 1844 (8) (2014) 1383-1390.

33. S. Jilici, P. Schmitz, S.E. Roggensack, A.W. Thompson, R.E. Summons, S.W. Chinholm, Bacterial vesicles in marine ecosystems, Science 343 (6167) (2014) 183-186.

34. E. Andaloussi, P. Vader, Extracellular vesicles as drug delivery systems: why and how? Adv. Drug Deliv. Rev. 159 (2020) 332-343.

35. C.A. Hickey, M.J. Kuehn, Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions, Nat. Rev. Microbiol. 13 (10) (2015) 605-619.

36. O.M. Elsharkasy, J.Z. Nordin, D.W. Hagey, O.G. de Jong, R.M. Schiffelers, S. van Soest, Membrane vesicle formation is associated with pyocin production under stress conditions, Cell Microbiol. 1 (3) (2000) 177-185.

37. R.J. Shoberg, D.D. Thomas, Specific adherence of Bordetella pertussis extracellular vesicles to human endothelial cells in culture, Infect. Immun. 69 (1) (1997) 392-3960.

38. D. Nejman, I. Livyatan, G. Fuks, N. Gavert, Y. Zwang, L.T. Geller, A. Rotter, Bacteroides fragilis 41.
Y. Jiang, Q. Kong, K.L. Roland, R. Curtiss 3rd, Membrane vesicles of Pseudomonas aeruginosa PA01 and their genetic transformation potential, Microbiology (Read.) 150 (Pt 7) (2004) 2161–2169.

L. Turnbull, M. Toyohuku, A.L. Hynen, M. Kurosawa, G. Peski, N.R. Petty, S. R. Orvath, G. Carcamo-Guerra, E.S. Glog, H. Shimoni, U. Omutis, S. Ito, X. V. Lan, M.H. Monahan, R. Cavaliere, C.H. Ahrens, I.G. Charles, N. Nomura, Eberl, C. B. Whitchurch, Explorative cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms, Nat. Commun. 7 (2016) 11220.

A. Baswens, L. Kuhn, H.K. Han, A. Gu, I. M. Bielanszewska, Antibiotic-mediated modulations of outer membrane vesicles in Enterobacteriaceae, Escherichia coli O144:H4 and O157:H7, Antimicrob. Agents Chemother. 61 (9) (2017).

C. Pérez-Cruz, L. Delgado, C. López-Iglesias, E. Mercade, Outer-inner membrane vesicles naturally secreted by Gram-negative pathogenic bacteria, PLoS One 10 (1) (2015), e0116896.

J. Li, F. Zaam, S. Zhang. Outer membrane vesicles containing signalling molecules and extracellular DNA from adjacent cells induce the mitochondrial membrane potential in human liver cancer HepG2 cells, Sci. Rep. 7 (1) (2017) 17092.

M. Renelli, V. Matias, R.Y. Lo, T.J. Beveridge, DNA-containing membrane vesicles of Pseudomonas aeruginosa PA01 and their genetic potential transformation, Microbiology 167 (2011) 1120–1132.

A.E. Sjostrom, L. Sandblad, B.E. Uhlin, S.N. Wei, M. Hynen, M. Kurosawa, G. Peski, N.R. Petty, S. Orvath, G. Carcamo-Guerra, E.S. Glog, H. Shimoni, U. Omutis, S. Ito, X. V. Lan, M.H. Monahan, R. Cavaliere, C.H. Ahrens, I.G. Charles, N. Nomura, Eberl, C. B. Whitchurch, Explorative cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms, Nat. Commun. 7 (2016) 11220.

A. Baswens, L. Kuhn, H.K. Han, A. Gu, I. M. Bielanszewska, Antibiotic-mediated modulations of outer membrane vesicles in Enterobacteriaceae, Escherichia coli O144:H4 and O157:H7, Antimicrob. Agents Chemother. 61 (9) (2017).

C. Pérez-Cruz, O. Carrion, L. Delgado, G. Martinez, C. Lopez-Iglesias, E. Mercade, New type of outer membrane vesicle produced by the Gram-negative bacterium Shewanella vesiculosa, FEMS Microbiol. Lett. (2019) 360–366, Microbiol. 79 (6) (2013) 1874–1881.

S. Hagemann, L. Stoger, M. Kappelmann, I. Hasel, A. Ellinger, B. Velmironov, DNA-bearing membrane vesicles produced by Aeromonas caviae and Pseudomonas aeruginosa, J. Bacteriol. 194 (8) (2012) 1062–1072.

D.W. Dorworth, C.F. Garon, DNA is packaged within membrane-derived vesicles of Gram-negative but not Gram-positive bacteria, Appl. Environ. Microbiol. 56 (6) (1990) 1483–1487.

G.D. Shockman, J.F. Barrett, Structure, function, and assembly of cell walls of D.W. Dorward, C.F. Garon, DNA is packaged within membrane-derived vesicles of Gram-negative but not Gram-positive bacteria, Appl. Environ. Microbiol. 56 (6) (1990) 1483–1487.

A. Baswens, L. Kuhn, H.K. Han, A. Gu, I. M. Bielanszewska, Antibiotic-mediated modulations of outer membrane vesicles in Enterobacteriaceae, Escherichia coli O144:H4 and O157:H7, Antimicrob. Agents Chemother. 61 (9) (2017).

C. Pérez-Cruz, O. Carrion, L. Delgado, G. Martinez, C. Lopez-Iglesias, E. Mercade, New type of outer membrane vesicle produced by the Gram-negative bacterium Shewanella vesiculosa, FEMS Microbiol. Lett. (2019) 360–366, Microbiol. 79 (6) (2013) 1874–1881.

S. Hagemann, L. Stoger, M. Kappelmann, I. Hasel, A. Ellinger, B. Velmironov, DNA-bearing membrane vesicles produced by Aeromonas caviae and Pseudomonas aeruginosa, J. Bacteriol. 194 (8) (2012) 1062–1072.

D.W. Dorworth, C.F. Garon, DNA is packaged within membrane-derived vesicles of Gram-negative but not Gram-positive bacteria, Appl. Environ. Microbiol. 56 (6) (1990) 1483–1487.
M. Song, A.T. Kim, S.I. Kim, T.I. Park, J.C. Lee, Y.C. Lee, Acinetobacter baumannii outer membrane vesicles elicit potent innate immune response via membrane proteins, PLoS One 8 (8) (2013), e17571.

M.E. Johansson, M. Phillipson, J. Peterson, A. Velcich, G.C. Hansson, Mucins and the microbiome, Annu. Rev. Biochem. 89 (2020) 769–793.

K.E. Lee, J.H. Lee, S.H. Hong, H.J. Lee, Extracellular RNAs in gut microbiota as mediators of host-microbial communication, Front. Cell. Infect. Microbiol. 7 (2017) 1–19.

C. Sun, C. Cuesta, C. Guerri, J. Ureña, E. Cánovas, M. Cuesta, J.A. Berzofsky, V. Fako, T. Ritz, T. Longerich, C.M. Theriot, J.A. McCulloch, J. Emery, J. Li, B.T. Kile, J.E. Vince, K.E. Lawlor, T. Naderer, Mitochondrial dysfunction caused by outer membrane vesicles from Gram-negative bacteria activates intrinsic apoptosis and inflammation, Nat Microbiol 5 (11) (2020) 1418–1427.

S.H. Jun, J.H. Lee, B.R. Kim, S.I. Kim, T.I. Park, J.C. Lee, Y.C. Lee, Acinetobacter baumannii outer membrane vesicles act as a host pathogen interaction model to study innate immune responses, Sci. Rep. 7 (1) (2017) 1–12.

E. Sirmans, J. Hu, A.J. Lazar, T. Tsujikawa, A. Diab, H. Tawbi, I.C. Glitza, W. F. McAllister, E. Marcelo Riquelme Sanchez, Y. Zhang, E. Le Chatelier, L. Zitvogel, C.W. Hudgens, D.S. Hutchinson, T. Manzo, M. Petaccia de Macedo, T. Cotechini, G. Trinchieri, T.F. Greten, Gut microbiome-mediated bile acid metabolism regulates colon cancer immunity, Cancer Discov. 11 (5) (2021) 1138–1150.

A. Wargo, Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients, Science 359 (6371) (2018) 97–100.

C. E. Han, S.Y. Choi, Y. Lee, J.W. Park, S.H. Hong, H.J. Lee, Extracellular RNAs in gut microbiota as mediators of host-microbial communication, Front. Cell. Infect. Microbiol. 7 (2017) 1–19.

C. Sun, C. Cuesta, C. Guerri, J. Ureña, E. Cánovas, J.A. Berzofsky, V. Fako, T. Ritz, T. Longerich, C.M. Theriot, J.A. McCulloch, J. Emery, J. Li, B.T. Kile, J.E. Vince, K.E. Lawlor, T. Naderer, Mitochondrial dysfunction caused by outer membrane vesicles from Gram-negative bacteria activates intrinsic apoptosis and inflammation, Nat Microbiol 5 (11) (2020) 1418–1427.

C. Sun, C. Cuesta, C. Guerri, J. Ureña, E. Cánovas, J.A. Berzofsky, V. Fako, T. Ritz, T. Longerich, C.M. Theriot, J.A. McCulloch, J. Emery, J. Li, B.T. Kile, J.E. Vince, K.E. Lawlor, T. Naderer, Mitochondrial dysfunction caused by outer membrane vesicles from Gram-negative bacteria activates intrinsic apoptosis and inflammation, Nat Microbiol 5 (11) (2020) 1418–1427.

A. Wargo, Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients, Science 359 (6371) (2018) 97–100.

C. E. Han, S.Y. Choi, Y. Lee, J.W. Park, S.H. Hong, H.J. Lee, Extracellular RNAs in gut microbiota as mediators of host-microbial communication, Front. Cell. Infect. Microbiol. 7 (2017) 1–19.

C. Sun, C. Cuesta, C. Guerri, J. Ureña, E. Cánovas, J.A. Berzofsky, V. Fako, T. Ritz, T. Longerich, C.M. Theriot, J.A. McCulloch, J. Emery, J. Li, B.T. Kile, J.E. Vince, K.E. Lawlor, T. Naderer, Mitochondrial dysfunction caused by outer membrane vesicles from Gram-negative bacteria activates intrinsic apoptosis and inflammation, Nat Microbiol 5 (11) (2020) 1418–1427.

C. Sun, C. Cuesta, C. Guerri, J. Ureña, E. Cánovas, J.A. Berzofsky, V. Fako, T. Ritz, T. Longerich, C.M. Theriot, J.A. McCulloch, J. Emery, J. Li, B.T. Kile, J.E. Vince, K.E. Lawlor, T. Naderer, Mitochondrial dysfunction caused by outer membrane vesicles from Gram-negative bacteria activates intrinsic apoptosis and inflammation, Nat Microbiol 5 (11) (2020) 1418–1427.

A. Wargo, Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients, Science 359 (6371) (2018) 97–100.

C. E. Han, S.Y. Choi, Y. Lee, J.W. Park, S.H. Hong, H.J. Lee, Extracellular RNAs in gut microbiota as mediators of host-microbial communication, Front. Cell. Infect. Microbiol. 7 (2017) 1–19.
[141] J. Yan, J.W. Herzog, R. Tsang, C.A. Brennan, M.A. Bower, W.S. Garrett, B. R. Sartor, A.O. Allgrants, J.F. Charles, Gut microbiota induce IGF-1 and promote bone formation and growth, Proc. Natl. Acad. Sci. U. S. A. 113 (47) (2016) E7554–e7563.

[142] S. Lucas, Y. Omata, J. Hofmann, M. Bottcher, A. Ilijazovic, K. Sartor, O. Albrecht, O. Schulz, B. Krishnacumar, G. Kronke, M. Herrmann, D. Mougiakakos, T. Strowig, G. Schett, M.M. Zains, Short-chain fatty acids regulate systemic bone mass and protect from pathological bone loss, Nat. Commun. 9 (1) (2018) 55.

[143] X. Chen, Z. Zhang, Y. Hu, J. Cui, X. Zhi, H. Jiang, R. Wang, Z. Gu, Z. Qiu, X. Dong, Y. Li, J. Su, Lactulose suppresses osteoclastogenesis and ameliorates estrogen deficiency-induced bone loss in mice, Aging Dis 11 (5) (2020) 629–641.

[144] C. Chevalier, S. Kieser, M. Špoláková, N. Hadadi, J. Brun, D. Rigo, N. Suárez-Zamorano, M. Spijjar, S. Fabbiano, B. Bunne, J. Ivaniičević, A. Macpherson, H. Liu, Y. Hou, Y. Wang, Z. Li, Enhancement of sulfur conversion rate in the microbiota-gut-brain axis, Int. J. Mol. Sci. 21 (23) (2020).

[145] A. Müller, B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, S. Zehentmeier, J.P. Pereira, Cell circuits and niches controlling B cell development, Immunol. Rev. 289 (1) (2019) 142–157.

[146] A. Müller, B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verástegui, A. Zlotnik, Involvement of chemokine receptors in breast cancer metastasis, Nature 410 (6824) (2001) 50–56.

[147] D. Yang, S.Y. Park, Y.S. Park, Y. Eun, S.Y. Lee, Metabolic engineering of Escherichia coli for natural product biosynthesis, Trends Biotechnol. 38 (7) (2020) 745–765.

[148] H. Liu, Y. Hou, Y. Wang, Z. Li, Enhancement of sulfur conversion rate in the production of L-Cysteine by engineered Escherichia coli, J. Agric. Food Chem. 68 (1) (2020) 250–257.

[149] H. Liu, Y. Wang, Y. Hou, Z. Li, Fitness of chassis cells and metabolic pathways for L-cysteine overproduction in Pseudomonas aeruginosa PAO1, J. Agric. Food Chem. 68 (50) (2020) 14928–14937.

[150] P. Väder, E.A. Möl, G. Pasterkamp, R.M. Schiffeleers, Extracellular vesicles for drug delivery, Adv. Drug Deliv. Rev. 106 (Pt A) (2016) 148–156.

[151] D.C. Emery, D.K. Shoemak, T.E. Batstone, C.M. Waterfall, J.A. Coghill, T. Jass, A. Richter-Dahlfors, Y. Mizunoe, B.E. Uhlin, Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin, Cell 115 (1) (2003) 25–35.

[152] S. Haas-Neill, P. Fereyhte, A budding relationship: bacterial extracellular vesicles in the microbiota-gut-brain axis, Int. J. Mol. Sci. 21 (23) (2020).

[153] N.D. Allan, T.J. Beveridge, Gentamicin delivery to Burkholderia cepacia group II A strains via membrane vesicles from Pseudomonas aeruginosa PAO1, Antimicrob. Agents Chemother. 47 (9) (2003) 2962–2965.

[154] V. Gujrati, J. Prakash, J. Malekzadeh-Najafabadi, A. Stiel, U. Klemm, G. Mettenleiter, M. Aichler, A. Walch, V. Nitzschchrist, Bioengineered bacterial vesicles as biological nano-heaters for optoacoustic imaging, Nat. Commun. 10 (1) (2019) 1114.

[155] V.B. Gujrati, S. Jon, Bioengineered bacterial outer membrane vesicles: what is their potential in cancer therapy? Nanomedicine 9 (7) (2014) 933–935.

[156] Z. Ayed, L. Cuvillier, G. Dobhal, R.V. Goreham, Electroporation of outer membrane vesicles derived from Pseudomonas aeruginosa with gold nanoparticles, SN Appl. Sci. 1 (12) (2019) 1600.

[157] Y. Lee, J.Y. Park, E.H. Lee, J. Yang, B.R. Jeong, Y.K. Kim, J.Y. Seoh, S. Lee, P. L. Han, E.J. Kim, Rapid assessment of microbiota changes in individuals with autism spectrum disorder using bacteria-derived membrane vesicles in urine, Exp Neurobiol. 26 (5) (2017) 307–317.

[158] Y. Peng, S. Yin, M. Wang, Extracellular vesicles of bacteria as potential targets for immune interventions, Hum. Vaccines Immunother. 17 (3) (2021) 897–903.

[159] H. Parker, K. Chitcholtan, M.B. Hampton, J.I. Keenan, Uptake of Helicobacter pylori outer membrane vesicles by gastric epithelial cells, Infect. Immun. 78 (12) (2010) 5054–5061.

[160] N. Furuta, H. Takeuchi, A. Amano, Entry of Porphyromonas gingivalis outer membrane vesicles into epithelial cells causes cellular functional impairment, Infect. Immun. 77 (11) (2009) 4761–4770.

[161] J.M. Bamberger, D.P. Macneachran, B.A. Coutermarch, S. Ye, G.A. O’Toole, B. A. Stanton, Long-distance delivery of bacterial virulence factors by Pseudomonas aeruginosa outer membrane vesicles, PLoS Pathog. 5 (4) (2009), e1000382.

[162] S.N. Wai, B. Lindmark, T. Soderblom, A. Takade, M. Westmark, J. Oscarson, J. Jasa, A. Richter-Dahlfors, Y. Mizunoe, B.E. Uhlin, Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin, Cell 115 (1) (2003) 25–35.

[163] V. Ricci, D. Carcione, S. Messina, G.I. Colombo, Y. D’Alessandra, Circulating 16S RNA in biofluids: extracellular vesicles as mirrors of human microbiome? Int. J. Mol. Sci. 21 (23) (2020).

[164] H. Taboada, N. Meneses, M.F. Dunn, C. Vargas-Lagunas, N. Buchs, J.A. Castro-Mondragon, M. Heller, S. Encarnacion, Proteins in the periplasmic space and outer membrane vesicles of Rhzobium etli CE3 grown in minimal medium are largely distinct and change with growth phase, Microbiology (Read.) 165 (6) (2019) 638–650, e739.

[165] L. Macia, R. Nanan, E. Hosteini-Beheshti, G.E. Grau, Host-and microbiota-derived extracellular vesicles, immune function, and disease development, Int. J. Mol. Sci. 21 (1) (2019).

[166] E.N. DeJonq, M.G. Surette, D.M.E. Bowdish, The gut microbiota and unhealthy aging: disentangling cause from consequence, Cell Host Microbe 28 (2) (2020) 180–189.