nature portfolio

Peer Review File

New insights into outer membrane vesicles in Gram-negative bacteria from biogenesis to applications

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
This manuscript presents a review of outer membrane vesicles (OMVs) as survival mediators of Gram-negative bacteria within the host. Furthermore, the authors cover OMV biogenesis, host-cell uptake pathways, pathogenic and commensal effects, and translational applications including vaccine development and cancer immunotherapy. The authors should either narrow the manuscript more tightly around host survival/persistence, or change the title to reflect a broader review of OMV biology and applications. At this stage the review feels unfocused and lacks key information in several parts e.g. OMV biogenesis as adaptation strategy to the host, and the Mla (VacJ/Yrb) lipid asymmetry pathway as a way of OMV biogenesis.

Besides that, the manuscript is well organized, clearly written, and timely. However, several important mechanistic and conceptual aspects of OMV regulation and function are either underdeveloped or missing. Addressing these points would substantially strengthen the manuscript and enhance its impact.

Major Comments

1. The review would benefit from including studies that directly demonstrate OMV biogenesis as an adaptive mechanism during host colonization. For example, work in Vibrio cholerae has shown that repression of the VacJ/Yrb phospholipid transporter during infection induces hypervesiculation, enabling rapid outer membrane remodeling and increasing resistance to host-derived stresses, which enhances intestinal colonization fitness.

2. One of the most conserved and mechanistically defined pathways regulating OMV biogenesis is not discussed. The Mla pathway provides a direct mechanistic link between envelope homeostasis and vesiculation. Host-relevant signals such as iron limitation and bile salts have in certain pathogens been shown to downregulate vacJ/mla expression, leading to increased OMV production.

3. The manuscript describes multiple mechanisms contributing to OMV biogenesis (Tol–Pal, Lpp/OmpA tethering, PG remodeling, lipid changes), but these are presented largely as independent phenomena. How are these mechanisms connected to the host?

4. Although antibiotic-degrading OMVs are discussed, the broader concept of OMVs functioning as extracellular decoys is underdeveloped. OMVs are also well established to function as extracellular decoys, including sequestration of complement components, binding of antimicrobial peptides, antibody absorption, and neutralization of bacteriophages. These functions are central to the “survival strategy” framing and deserve explicit synthesis. Even vesicles lacking specialized cargo can serve protective roles via surface interactions alone.

5. Lack of distinction between vesicle subtypes
The manuscript discusses cytosolic cargo in OMVs but does not clearly distinguish between:
Classical outer membrane blebs (OMVs)
Outer–inner membrane vesicles (OIMVs)
Lysis-derived vesicles
A short clarifying section on vesicle heterogeneity and methodological considerations (e.g., purification controls) could improve the manuscript.

Minor Comments

In vivo context could be expanded on estimated production rates during infection as well as how OMVs can disseminate through host tissue

Add discussion of evolutionary trade-offs as hypervesiculation may reduce membrane integrity, increase immune detection or impose metabolic cost.

Some mechanistic statements regarding lipid curvature (e.g., cardiolipin-mediated bending) should be softened to reflect species-dependent variability.

The discussion of OMV-mediated host immune responses could benefit from inclusion of additional primary studies examining epithelial inflammatory responses to bacterial membrane vesicles.

Uptake pathway conclusions relying solely on pharmacological inhibitors should be presented with caution, noting limitations of inhibitor specificity.

The pathogen-versus-commensal dichotomy should acknowledge context dependence (host genotype, barrier integrity, inflammatory state).

In the section on OMV-based therapeutic applications, the authors may wish to include additional examples of experimental studies exploring OMV-based vaccine strategies against enteric pathogens.

Reviewer #3

(Remarks to the Author)
The authors provided a comprehensive and well-structured review of current knowledge on outer membrane vesicles (OMVs), including their biogenesis, mechanisms of host cell entry, and their roles in modulating immune responses. The authors also described current therapeutic applications involving OMVs, such as vaccine development. Overall, the manuscript is clearly written, easy to follow, and provides useful summary of the field. I have a few minor comments and suggestions:
1) The introduction and title stated that OMVs originate from Gram-negative bacteria; however, in section 4 the authors noted that some Gram-positive commensal bacteria can also produce extracellular vesicles with anti-inflammatory activity. Are OMVs strictly limited to Gram-negative bacteria, with Gram-positive bacteria producing a different class of extracellular vesicles? It would be helpful if the authors clarify the terminology and distinctions.
Additionally, are there established mechanistic differences in vesicle biogenesis, cargo selection, or membrane composition between Gram-negative OMVs and Gram-positive vesicles?
2) The article thoroughly described how OMVs can enter host cells through various mechanisms, but their roles in bacteria-bacteria interaction could be further elaborated. The authors briefly mentioned OMV-mediated gene transfer (lines 71–73) and roles in cell-cell communication under nutrient-limiting conditions (lines 76–78), as well as how OMVs derived from pathogenic bacteria can reshape host-associated microbiota therefore indirectly affect disease progression in later sections. It might be helpful to elaborate more on the mechanisms by which OMVs interact with or are taken up by other bacteria (e.g., membrane fusion, adhesion, enzymatic delivery), the types of cargo involved in interbacterial transfer, and the functional consequences of such inter-bacteria OMV transfer.
3) In line 597, the authors introduced inner membrane vesicles (IMVs). It would be helpful to briefly explain their biogenesis and clarify whether they are naturally produced under physiological conditions or primarily arise under stress or specific experimental manipulation.
4) A couple of small typos:
a. Line 475: ROS generation response
b. Line 478: such as TLR2, TLR4, or NOD1
c. Line 537: ‘resulted in gut dysbiosis’ is repeated

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed my concerns raised and I recommend acceptance of the manuscript in its current form.

Reviewer #3

(Remarks to the Author)
The authors have addressed my comments and concerns.

Dear Editors,

We would like to thank the editors for the encouraging comments and valuable suggestions. We are submitting the revised version of our manuscript (Manuscript Number: COMMSBIO-25-10786) to *Commun Biol*. We found the editor's comments very helpful in improving our manuscript. As suggested by the editor, we revised all sections of the manuscript.

1) We revised all parts of the manuscript to make the manuscript clearer.
2) We carefully verified that all cited references accurately correspond to the information described throughout the manuscript.
3) We reorganized the former Section 3 ("OMVs as strategic tools for bacterial survival in host environments") into Section 2 ("Stress-associated differences in OMV cargo composition"), revising the content to emphasize stress-dependent selectivity in OMV protein cargo and its contribution to bacterial fitness under host stress.
4) Figure 4 has been split into two separate figures, now presented as Figures 4 and 5.

We hope you find these improvements sufficient for publication in *Commun Biol*. All authors agree to submit the revised manuscript. The authors declare that there are no conflicts of interest.

Editor #1

Your manuscript entitled "Outer membrane vesicle as a survival strategy of Gram-negative bacteria within a host" has now been seen and our in-house editorial team. We are interested in the possibility of publishing your study in Communications Biology, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

Generally, while we believe this review is timely and interesting, we find a significant part of the content has been covered or partially covered by existing reviews. We therefore hope that this review could involve more discussion on how the mechanisms of OMVs highlighted here could be targeted in the case of pathogenic Gram-negative bacterial infection, or more generally, what such mechanisms of OMVs are implied for in tackling infectious disease apart from sustaining the microbes themselves, which are aspects hardly being discussed by existing reviews.

We therefore invite you for an editorial revision, taking into account this general concern and the specific points raised as follows:

1) Please pay more attention to less covered OMV proteome and their functon(e.g. https://journals.asm.org/doi/full/10.1128/mbio.00644-25 and similar) and shorten the more "general" OMV biogenesis and general role in pathogenesis part and pay attention to differentiate the discussion compared to existing reviews such as (doi: 10.3390/antibiotics13010032).

We agree with the editor's suggestion. We restructured the manuscript to emphasize stress-dependent OMV proteome remodeling. The former Section 3 (“OMVs as strategic tools for bacterial survival in host environments”) was restructured and moved to Section 2 (“Stress-associated differences in OMV cargo composition”), with a focus on stress-dependent OMV protein composition and how specific OMV cargo supports bacterial fitness under stress. We described this information in lines 169-252.

Additionally, we condensed Section 1 (“Mechanisms of OMV biogenesis in Gram-negative bacteria”) to a concise summary, removing detailed descriptions that are extensively covered in previous reviews.

2) Please try to reduce the length to around 7-8k, and references should be limited to (at most) 200 studies (vs. the 253 currently)

We reduced the main text to approximately 7,000 words, and decreased the number of references from 253 to 172, thereby complying with the requested length and citation limits.

3) Figures are nice but over-detailed in their current form. Unless each of the individual pathways/components that are outlined in each figure are described or focused on, authors should simplify these items. For instance, Figure 3 does not really need the chemical structures of antibiotics, as these aren't described in the text.

We agree with this comment. Accordingly, Figure 1 was simplified to present a concise overview, and Figure 3 was removed to improve clarity and ensure consistency between the figures and the text.

Dear Editor and Reviewers,

We thank the Editors and Reviewers for their constructive comments and valuable suggestions. We are pleased to submit the revised manuscript entitled (**Manuscript Number: COMMSBIO-25-10786A**) to *Commun Biol*. We found the reviewer's comments very helpful in improving our manuscript. As suggested by the editor and reviewers, we revised all sections of the manuscript.

1) The title and abstract were revised to better reflect the overall focus of the manuscript.
2) We carefully revised the manuscript for clarity and consistency.
3) We verified that all cited references accurately correspond to the information described throughout the manuscript.
4) We clarified the terminology used for bacterial vesicles and explicitly distinguished outer membrane vesicles produced by Gram-negative bacteria from extracellular vesicles released by Gram-positive bacteria.

We hope that the revised manuscript is now suitable for publication in *Commun Biol*. All authors have approved the revised manuscript and its submission. The authors declare that there are no conflicts of interest.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript presents a review of outer membrane vesicles (OMVs) as survival mediators of Gram-negative bacteria within the host. Furthermore, the authors cover OMV biogenesis, host-cell uptake pathways, pathogenic and commensal effects, and translational applications including vaccine development and cancer immunotherapy. The authors should either narrow the manuscript more tightly around host survival/persistence, or change the title to reflect a broader review of OMV biology and applications. At this stage the review feels unfocused and lacks key information in several parts e.g. OMV biogenesis as adaptation strategy to the host, and the Mla (VacJ/Yrb) lipid asymmetry pathway as a way of OMV biogenesis. Besides that, the manuscript is well organized, clearly written, and timely. However, several important mechanistic and conceptual aspects of OMV regulation and function are either underdeveloped or missing. Addressing these points would substantially strengthen the manuscript and enhance its impact.

We appreciate this helpful suggestion. We agree that our original title did not fully reflect the broader scope of the manuscript. We changed the title to better reflect the broader scope of our manuscript: **"New insights into outer membrane vesicles in Gram-negative bacteria from biogenesis to applications"**.

Major Comments

1. The review would benefit from including studies that directly demonstrate OMV biogenesis as an adaptive mechanism during host colonization. For example, work in Vibrio cholerae has shown that repression of the VacJ/Yrb phospholipid transporter during infection induces hypervesiculation, enabling rapid outer membrane remodeling and increasing resistance to host-derived stresses, which enhances intestinal colonization fitness.

We appreciate this valuable suggestion. Including studies that demonstrate OMV biogenesis as a host-adaptive mechanism strengthens the overall framework of the review. We incorporated a representative example from *Vibrio cholerae*, which provides direct in *vivo* evidence linking OMV production to bacterial fitness during host colonization. We revised the manuscript in lines 278-286 with a reference.

(Line 278-286) "Hypervesiculation in *V. cholerae* provides a clear example of OMV

biogenesis functioning as an adaptive mechanism during host colonization (51).....”

Zingl F et al., Outer membrane vesiculation facilitates surface exchange and in vivo adaptation of *Vibrio cholerae*. *Cell Host Microbe*. 2020; 2:225-237.e8.

2. One of the most conserved and mechanistically defined pathways regulating OMV biogenesis is not discussed. The Mla pathway provides a direct mechanistic link between envelope homeostasis and vesiculation. Host-relevant signals such as iron limitation and bile salts have in certain pathogens been shown to downregulate *vacJ*/*mla* expression, leading to increased OMV production.

We appreciate this important point. We agree that the Mla (VacJ/Yrb) pathway represents one of the most conserved and mechanistically well-defined systems linking outer membrane homeostasis to OMV biogenesis. We added this discussion in lines 182-188 with references.

(Line 182-188) “In addition to LPS remodeling, disruption of phospholipid asymmetry represents another key lipid-driven mechanism of OMV biogenesis (50, 51).....”

Roier S et al., A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. *Nat Commun*. 2016;7:10515.

Zingl F et al., Outer membrane vesiculation facilitates surface exchange and in vivo adaptation of *Vibrio cholerae*. *Cell Host Microbe*. 2020;2:225-237.e8.

Additionally, we included a representative example under iron-limiting conditions describing how OMV production enhances resistance to host-derived stresses and improves bacterial colonization, thereby directly linking OMV biogenesis to host adaptation and survival (lines 279-286 with a reference).

(Line 279-286) “Under iron limitation conditions induced by the iron chelator 2,2′-bipyridyl, transcription of the VacJ/Yrb (Mla) phospholipid transporter is repressed, leading to phospholipid accumulation in the outer leaflet of the OM and then increased vesiculation (51).....”

Roier S et al., A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. *Nat Commun*. 2016;7:10515.

3. The manuscript describes multiple mechanisms contributing to OMV biogenesis (Tol–Pal, Lpp/OmpA tethering, PG remodeling, lipid changes), but these are presented largely as independent phenomena. How are these mechanisms connected to the host?

Thank you for this helpful suggestion. The mechanisms mentioned (e.g., Tol–Pal, Lpp/OmpA tethering, PG remodeling, and lipid asymmetry) primarily serve as determinants of OMV biogenesis, rather than functioning as active cargo components within OMVs. Notably, disruption or reduced activity of these envelope-stabilizing systems is itself a prerequisite for vesicle formation. These factors are therefore unlikely to function as active OMV components. Instead, under host-associated stresses, perturbation of envelope homeostasis frequently leads to increased OMV production, and these vesicles predominantly contribute to bacterial survival by acting as extracellular decoys that physically intercept antimicrobial factors. We added this information in lines 287-322 with references.

(Line 287-322) “Although envelope-stabilizing systems (e.g., the Tol–Pal complex, Lpp/OmpA-mediated tethering, PG remodeling, and lipid asymmetry) are key determinants of OMV biogenesis, their depletion or functional impairment during vesiculation makes their presence within OMVs as functional components unlikely……..”

Park J et al. A novel decoy strategy for polymyxin resistance in *Acinetobacter baumannii*. *eLife*. 2021;10:e66988.

Roier S et al., A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. *Nat Commun*. 2016;7:10515.

Marchant P et al., "One for All": Functional Transfer of OMV-Mediated Polymyxin B Resistance From *Salmonella enterica* sv. Typhi Δ*tolR* and Δ*degS* to Susceptible Bacteria. *Front Microbiol*. 2021;12:67246.

Davies C et al., Sodium Taurocholate Stimulates *Campylobacter jejuni* Outer Membrane Vesicle Production via Down-Regulation of the Maintenance of Lipid Asymmetry Pathway. *Front Cell Infect Microbiol.* 2019;9:177.

However, OmpA, an OM–PG tethering protein that contributes to cell envelope stability by mediating hydrophobic interactions between the OM and PG, has been reported to directly modulate host immune responses by triggering inflammatory signaling pathways. We have addressed the effects of OmpA on host cells in lines 577-586 and 624-626.

(Line 577-586) “Because *A. baumannii* OmpA interacts with fibronectin on lung epithelial A549 cells, the release of OMVs carrying OmpA may further enhance bacterial adhesion and invasion by increasing fibronectin engagement (Fig. 3A) (140-142).....”

(Line 624-626) “Bacterial OmpA displayed on OMVs induces lung alveolar epithelial A549 cell death by mitochondrial fragmentation through accumulation of dynamin-related protein 1 (Drp1) and evaluation in ROS in mitochondria (8)”

4. Although antibiotic-degrading OMVs are discussed, the broader concept of OMVs functioning as extracellular decoys is underdeveloped. OMVs are also well established to function as extracellular decoys, including sequestration of complement components, binding of antimicrobial peptides, antibody absorption, and neutralization of bacteriophages. These functions are central to the “survival strategy” framing and deserve explicit synthesis. Even vesicles lacking specialized cargo can serve protective roles via surface interactions alone.
Thank you for this helpful suggestion. Given that the extracellular decoy functions of OMVs have been extensively reported, this review focuses on stress-associated changes in OMV cargo composition. Nevertheless, we added a brief description of OMVs as extracellular decoys in response to the reviewer’s suggestion. This point is now addressed in lines 308-322 with references.

(Line 308-322) “While the precise determinants of OMV formation are not fully understood, numerous studies have reported that OMVs can function as extracellular decoys that protect bacterial cells against a broad range of antimicrobial agents.……..”

Balhuizen MD et al., Outer membrane vesicles protect Gram-negative bacteria against host defense peptides. *mSphere*. 2021;6:e0052321.
Reyes-Robles T et al., *Vibrio cholerae* Outer Membrane Vesicles Inhibit Bacteriophage

Infection. *J Bacteriol*. 2018;200:e00792-17.

5. Lack of distinction between vesicle subtypes

The manuscript discusses cytosolic cargo in OMVs but does not clearly distinguish between:

Classical outer membrane blebs (OMVs)

Outer–inner membrane vesicles (OIMVs)

Lysis-derived vesicles

A short clarifying section on vesicle heterogeneity and methodological considerations (e.g., purification controls) could improve the manuscript.

Thank you for your comment. We agree that clearly distinguishing between the different subtypes of cytoplasm-containing vesicles in Gram-negative bacteria is important. We revised the manuscript in lines 130-154 with references.

(Line 130-154) “OMVs have been classified into two types based on the underlying mechanisms: explosive cell lysis (lytic OMVs) and OM blebbing (non-lytic OMVs) in Gram-negative bacteria (29).....”

Toyofuku M et al., Types and origins of bacterial membrane vesicles. *Nat Rev Microbiol.* 2019;17:13-24.

Turnbull L et al., Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat Commun.* 2016;7:11220.

Liang A et al., The emerging role of bacterial extracellular vesicles in human cancers. *J Extracell Vesicles.* 2024;13:e12521.

Park Y et al., Alleviation of H_2O_2 toxicity by extracellular catalases in the phycosphere of Microcystis aeruginosa. *Harmful Algae*. 2024;137:102680.

Minor Comments

6. *In vivo* context could be expanded on estimated production rates during infection as well as how OMVs can disseminate through host tissue.

Thank you for your comment. Direct evidence for OMV production and systemic dissemination under host conditions is still limited, particularly in human settings. While several studies suggest that OMVs can be detected *in vivo*, most available evidence is derived

from animal models. We added this discussion in lines 535-543 with references.

(Line 535-543) “During host infection, exposure to diverse host-associated stresses is known to increase OMV production compared to non-stress conditions (126, 127).....”

Li, Q et al., Specific labeling of outer membrane vesicles with antibiotic-conjugated probe reveals early bacterial infections in blood. *Nat Commun.* 2025;16:3535.
Ou Z et al., Single-particle analysis of circulating bacterial extracellular vesicles reveals their biogenesis, changes in blood and links to intestinal barrier. *J Extracell Vesicles*. 2023;12:e12395.

7. Add discussion of evolutionary trade-offs as hypervesiculation may reduce membrane integrity, increase immune detection or impose metabolic cost.
We appreciate the reviewer’s thoughtful comment. We added a brief discussion in the **“Conclusion”** section on the evolutionary trade-offs associated with OMV production, incorporating our perspective on the balance between its adaptive advantages and associated costs in lines 792-813 with a reference.

(Line 792-813) “Although OMV production imposes energetic and structural costs, accumulating evidence suggests that these are balanced by significant functional advantages, including alleviation of membrane stress, immune evasion, and membrane remodeling. OMV formation can occur rapidly in response to environmental perturbations, indicating a dynamic process shaped by both physical forces and regulated cellular responses…….”

Min J et al., Sphingolipid-mediated vesiculation in multidrug-resistant *Sphingobacterium detergens u*nder polymyxin B stress. *Appl Environ Microbiol.* 2025;91:e0172625.

8. Some mechanistic statements regarding lipid curvature (e.g., cardiolipin-mediated bending) should be softened to reflect species-dependent variability.
We revised statements regarding lipid curvature to better reflect species- and condition-dependent variability. We revised it in lines 102-106.

(Line 102-106) “CL enrichment can facilitate membrane curvature owing to its cone-like molecular geometry, inducing membrane bending and contributing to OM remodeling and vesicle formation (20, 23). However, the extent to which such lipid-driven curvature directly contributes to OMV biogenesis likely varies depending on species and environmental conditions.”

9. The discussion of OMV-mediated host immune responses could benefit from inclusion of additional primary studies examining epithelial inflammatory responses to bacterial membrane vesicles.
Thank you for this suggestion. We described additional primary studies that specifically examine epithelial inflammatory responses to bacterial membrane vesicles in lines 584-589 and 600-607 with references.

(Line 584-589) “OMVs from enterotoxigenic *E. coli* show markedly reduced internalization into intestinal epithelial cells when OmpA or OmpF is absent, indicating that these porins are required for efficient uptake (146).……”

(Line 600-607) “In addition to immune activation, OMVs can deliver enzymatically active factors that directly damage host cells. For instance, *V. cholerae* OMVs transport active proteases such as VesC and hemagglutinin/protease (HAP) into epithelial cells via lipid raft–dependent internalization (150).....”

Thapa HB et al., Characterization of the inflammatory response evoked by bacterial membrane vesicles in intestinal cells reveals an RIPK2-dependent activation by enterotoxigenic *Escherichia coli* vesicles. *Microbiol Spectr*. 2023;11:e0111523.
Mondal A et al., Cytotoxic and inflammatory responses induced by outer membrane vesicle-associated biologically active proteases from *Vibrio cholerae*. *Infect Immun*. 2016;84:1478-1490.

10. Uptake pathway conclusions relying solely on pharmacological inhibitors should be presented with caution, noting limitations of inhibitor specificity.
We added a statement highlighting the need for caution when interpreting inhibitor-based

conclusions, due to their limited specificity and potential off-target effects. This point is now addressed in lines 527-532.

(Line 527-532) “It should be noted, however, that conclusions regarding OMV internalization pathways derived solely from pharmacological inhibitor studies should be interpreted with caution……”

11. The pathogen-versus-commensal dichotomy should acknowledge context dependence (host genotype, barrier integrity, inflammatory state).
Thank you for this comment. We revised the manuscript to clarify that the commensal–pathogen distinction is not strictly dichotomous. We described this information in lines 550-555.

(Line 550-555) “Importantly, the distinction between commensal and pathogenic bacteria is not strictly dichotomous but is determined by host physiological and immunological conditions.……”

12. In the section on OMV-based therapeutic applications, the authors may wish to include additional examples of experimental studies exploring OMV-based vaccine strategies against enteric pathogens.
We added an example of an experimental study investigating OMV-based vaccine strategies against *Shigella* and *Campylobacter*. We revised the manuscript in lines 739-744 and 749-755 with references.

(Line 739-744) “The *S. flexneri* 2a O-polysaccharide antigen was biosynthesized in *Salmonella* Typhimurium and enzymatically ligated by WaaL to the *Salmonella* LPS core–lipid A, resulting in the display the heterologous antigen on the OM (178).....”

(Line 749-755) “Similarly, OMV-based vaccines have been developed against *C. jejuni* by displaying protective glycan antigens on the vesicle surface (181). Their heptasaccharide N-linked glycan antigen, normally associated with protein glycosylation in *C. jejuni*, was expressed in engineered *E. coli* and displayed on the outer membrane. OMVs presenting the

Campylobacter-derived glycans were subsequently purified and conferred protective immunity in chickens, resulting in a substantial reduction in intestinal colonization following *C. jejuni* challenge."

Tian H et al., Outer membrane vesicles derived from *Salmonella enterica* Serotype Typhimurium can deliver *Shigella flexneri* 2a O-polysaccharide antigen to prevent *Shigella flexneri* 2a infection in mice. *Appl Environ Microbiol* 2021;87:e00968-21.
Price NL et al., Glycoengineered Outer Membrane Vesicles: A Novel Platform for Bacterial Vaccines. *Sci Rep.* 2016;6:24931.

Reviewer #2 (Remarks to the Author):
The authors provided a comprehensive and well-structured review of current knowledge on outer membrane vesicles (OMVs), including their biogenesis, mechanisms of host cell entry, and their roles in modulating immune responses. The authors also described current therapeutic applications involving OMVs, such as vaccine development. Overall, the manuscript is clearly written, easy to follow, and provides useful summary of the field. I have a few minor comments and suggestions:
1) The introduction and title stated that OMVs originate from Gram-negative bacteria; however, in section 4 the authors noted that some Gram-positive commensal bacteria can also produce extracellular vesicles with anti-inflammatory activity. Are OMVs strictly limited to Gram-negative bacteria, with Gram-positive bacteria producing a different class of extracellular vesicles? It would be helpful if the authors clarify the terminology and distinctions. Additionally, are there established mechanistic differences in vesicle biogenesis, cargo selection, or membrane composition between Gram-negative OMVs and Gram-positive vesicles?
Thank you for this important point. OMVs are classically defined as vesicles derived from the outer membrane of Gram-negative bacteria. In contrast, Gram-positive bacteria, which lack an outer membrane, produce vesicles that are more generally referred to as extracellular vesicles (EVs). To avoid confusion, we clarified this terminology in lines 153-154 with references.

(Line 153-154) "In contrast, Gram-positive bacteria, which lack an OM, produce vesicles that

are more generally referred to as extracellular vesicles (EVs) (6).”

McMillan HM and Kuehn MJ. The extracellular vesicle generation paradox: a bacterial point of view. *EMBO J*. 2021;40:e108174.

2) The article thoroughly described how OMVs can enter host cells through various mechanisms, but their roles in bacteria-bacteria interaction could be further elaborated. The authors briefly mentioned OMV-mediated gene transfer (lines 71–73) and roles in cell-cell communication under nutrient-limiting conditions (lines 76–78), as well as how OMVs derived from pathogenic bacteria can reshape host-associated microbiota therefore indirectly affect disease progression in later sections. It might be helpful to elaborate more on the mechanisms by which OMVs interact with or are taken up by other bacteria (e.g., membrane fusion, adhesion, enzymatic delivery), the types of cargo involved in interbacterial transfer, and the functional consequences of such inter-bacteria OMV transfer.
Thank you for this insightful suggestion. Because this review focuses on host-associated functions of OMVs, we did not expand this section further. While OMV-mediated interbacterial interactions are indeed important, they have been extensively covered in several dedicated reviews. Accordingly, we clarified this scope in **Section 1** (lines 193–198).

(Line 193-198) “While numerous recent reviews have extensively addressed the roles of OMVs in interbacterial communication—where OMV-mediated cell–cell interactions support bacterial survival under stressful conditions such as antibiotic exposure, nutrient limitation, and host-associated biofilms—their functions in host-associated contexts remain less well explored (1, 4, 6).....”

3) In line 597, the authors introduced inner membrane vesicles (IMVs). It would be helpful to briefly explain their biogenesis and clarify whether they are naturally produced under physiological conditions or primarily arise under stress or specific experimental manipulation.
Thank you for this important comment. Inverted inner membrane vesicles refer to vesicles generated through specific experimental manipulation, namely high-pressure microfluidization–mediated cell lysis, which produces inverted vesicles derived from the

inner membrane. This mechanism is distinct from the biogenesis of classical OMVs, which are naturally produced under physiological conditions via outer membrane blebbing. This point has been clarified in lines 698–704.

(Line 698-704) "Notably, inverted inner-membrane vesicles were experimentally generated by high-pressure microfluidization–mediated cell lysis, which induces membrane inversion and vesicle reassembly (170)……."

4) A couple of small typos:

a. Line 475: ROS generation response

b. Line 478: such as TLR2, TLR4, or NOD1

c. Line 537: 'resulted in gut dysbiosis' is repeated

These typographical errors have been corrected in the revised manuscript.