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Outer membrane vesicles from commensal and pathogenic anaerobic bacteria: a systematic review of literature reviews

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10 **Abstract**

11 Outer membrane vesicles (OMVs) are constitutively produced by Gram-negative
12 bacteria (GNB), those from pathogenic bacteria play key roles in pathogen-host
13 interactions, modulating host's immune response and promoting virulence. OMVs of
14 commensal bacteria are fundamental in the maturation of the host immune system and to
15 maintain intestinal homeostasis.

16 The objective of this systematic review was to synthesize the knowledge available
17 on literature reviews on OMVs from anaerobic GNB. The information was classified into
18 categories: induction and biogenesis, OMVs liberation, internalization by host cells, and
19 their interaction. The most studied OMVs are from *Porphyromonas gingivalis* and
20 *Bacteroides* spp.

21

22 **Keywords**

23 Outer membrane vesicles, Anaerobic bacteria, *Porphyromonas gingivalis*,
24 *Bacteroides*, Systematic review

25 **Introduction**

26 Extracellular Vesicles (EVs) are a heterogeneous group of proteolipid bi-layered
27 particles, 20-500 nm in size (1–7), secreted by most eukaryotic cells (fungi (8), protozoa
28 (9), plants (10) and mammals (11)); archaea (12); and bacteria. EVs are released under both
29 normal and pathological conditions by metabolically active cells (13).

30 Bacterial Extracellular Vesicles (BEVs) range between 20 and 500 nm in diameter,
31 similarly to other EVs (13). BEVs were first identified in the Gram-negative bacterium
32 (GNB), *Escherichia coli*, and later, found in the Gram-positive *Staphylococcus aureus*
33 (14,15), and in mycobacteria (16). At first considered an artifact of lysis or cell wall
34 turnover, their release is now recognized as a secretion system that can transfer an
35 extremely diverse range of cargo and establish cell-to-cell communication (17,18).

36 To study BEVs, isolation and purification are done by low-speed centrifugation and
37 supernatant filtration to remove cells and debris. Then, ultracentrifugation (100 000 to 200
38 000g for 1-4 h) and/or precipitation (using different concentrations of salts or polymers) are
39 performed to separate BEVs from soluble components released during bacterial lysis. To
40 increase purity and concentrate BEVs, other purification steps may be necessary; for
41 example, by density-gradient centrifugation (in iodixanol, sucrose or dextran), size-
42 exclusion chromatography (gel filtration), ultrafiltration (using a 50-100 kDa cutoff,
43 cellulose or polyethersulfone membrane filters and applying pressure, vacuum or
44 centrifugal force) or tangential flow filtration (7,13,15,19–22). Some steps in the isolation
45 and purification protocol might affect the BEVs' integrity and/or activity. Thus, quality
46 control of isolates is key for research (22,23).

47 There is not a consensus about EVs nomenclature and the term itself is a generic
48 one that encompasses several vesicles produced by cells (24,25). Based on their

49 biotechnical production process, BEVs could have several names, including “bacterial
50 double-layered membrane vesicles” (26) or “protoplast-derived nanovesicles” (27), to cite
51 only a few.

52 Naturally occurring BEVs can be classified into at least three types: Cytoplasmic
53 Membrane Vesicles (CMVs or MVs) produced by monoderm (Gram-positive) bacteria,
54 Outer-Inner Membrane Vesicles (OIMVs) produced by diderm (Gram-negative) bacteria,
55 and Outer Membrane Vesicles (OMVs) also produced by diderm bacteria (15,18,28,29).
56 Another term “explosive outer-membrane vesicles” (EOMVs) has been used for OMVs
57 produced by “explosive cell lysis” (15,22,30).

58 MVs (or CMVs) seem to be produced at lower quantities than the other BEVs and
59 are implicated in stress resistance, biofilm formation and immunity (31). OIMVs are
60 produced at very low quantities, representing for many Gram-Negative species around 1%
61 of BEVs production. The possession of outer membranes, plasma membranes, and
62 cytoplasmic content characterizes OIMVs. These BEVs seem to be involved in horizontal
63 gene transfer (6,29,32). Finally, OMVs derive from the outer membrane (OM) (28).

64 As the study of BEVs is still challenging in terms of isolation, purification, and
65 individual analysis (19), its classification and nomenclature is not congruent throughout
66 literature (25,33). In this review, all BEVs derived from GNB are termed OMVs and their
67 biological features will be discussed. A special emphasis will be provided on OMVs
68 derived from anaerobic GNB. To do so, information derived from review articles about
69 anaerobic GNB’s OMVs will be analyzed, and examples will be commented, to introduce
70 the uninitiated reader to the subject, as a first educational approach to a topic in full
71 expansion.

72

73 **Materials and methods**

74 The recommendations of PRISMA (34) were followed for the writing of this
75 systematic review:

76

77 1. Search

78 PubMed and ScienceDirect databases were searched from the oldest records to
79 10/31/2023. The search was performed using the following constructs: "Outer Membrane
80 Vesicles", "Extracellular Vesicles" or "Bacterial Membrane Vesicles" AND "Anaerobic
81 bacteria", "Bacteroides", "Porphyromonas".

82

83 2. Selection criteria

84 Inclusion criteria were: (1) literature reviews, (2) written in English, (3) published in
85 a peer-reviewed journal, and (4) full-text available. The exclusion criteria were: (1) it did
86 not contain relevant information or (2) the OMVs were not a central topic in the article.
87 Results were downloaded and duplicate records were removed.

88

89 3. Data extraction

90 The information was organized in summary tables (Table S1) that included the
91 metadata of each bibliographic review and the relevant information for the present study.
92 Each article was evaluated independently.

93

94 4. Data analysis

95 The information obtained from each study was organized into five categories: (1)
96 induction of vesiculation and biogenesis, (2) release, (3) content, (4) internalization of

97 OMVs by a host cell, and (5) interaction of the OMV with the host cell. Based on this
98 information, a synthesis of knowledge was written in each of the sections described above
99 (Figure 1).

100

101 **Results**

102 The search returned 1400 articles for inclusion in this review. After the process of
103 selection, deduplication, and a manual review of each of the retrieved articles, a total of 53
104 review articles were used for this paper (Figure 2). The genera of Gram-negative anaerobic
105 bacteria (GNB) whose OMVs have been studied in these articles correspond to
106 *Porphyromonas* spp., *Bacteroides* spp., *Akkermansia* spp., *Aggregatibacter* spp.,
107 *Treponema* spp., *Fusobacterium* spp., *Tannerella* spp., *Odoribacter* spp. and *Prevotella*
108 spp., in decreasing order of literature sources. None of the articles found had information
109 on the OMVs of any other anaerobic GNB (Table 1). Notably, the only case for which an
110 anaerobic GNB OMV was the primary topic of a review was for *Porphyromonas gingivalis*
111 with 4 articles out of the 53 (7,5%) used in this review. The information collected from the
112 articles was categorized into the following topics: (1) vesiculation induction and biogenesis,
113 (2) release, (3) content, (4) trafficking and internalization of OMVs by host cells, and (5)
114 interaction of OMVs with host cells. The information collected is detailed below.

115

116 1. Induction of vesiculation and OMV biogenesis:

117 In GNB, vesicles are formed when a section of the outer membrane (OM)
118 selectively buds, producing vesicles that encapsulate periplasmic material and detach from
119 the OM. This results in the creation of spheroidal particles that consist of a single lipid

120 bilayer (an outer layer rich in lipopolysaccharide (LPS) and an inner layer with more
121 phospholipids) with a lumen (35–40).

122 OMVs share similar characteristics and biomolecular components to the precursor
123 cells from which they originated (33,41,42). They range in size from 20 to 250 nm (42,43)
124 and their composition reflects components of the OM and periplasm (33,41).

125 OMVs are produced constitutively, at low concentrations, during different growth
126 phases and under different conditions such as in pure culture (solid or liquid media) or in
127 natural environments, whether in biofilm or not (15,40,42,44–46). The rate of OMV
128 production varies between bacterial species, even between strains of the same species, and
129 can also be influenced by different environmental conditions (7,36,47). For example,
130 among “the red complex”, a microbial group that colonizes periodontal pockets, *P.*
131 *gingivalis* produces the most OMVs, followed by *Tannerella forsythia* and *Treponema*
132 *denticola* (40). As mentioned, vesicle production also varies considerably between strains,
133 for example in *Bacteroides fragilis*: whereas the OMVs were formed in great volumes by
134 some clinical isolates, they were virtually completely absent in others (47).

135 Some factors such as quorum sensing, population size, and hostile or stress-
136 generating external factors affect the properties of OMVs and increase their production
137 (38,41,43,48–51). For instance, under low iron/heme conditions, *P. gingivalis* OMVs
138 exhibited high levels of the protein HmuY, involved in heme acquisition (38). It is
139 noteworthy that the molecular composition of naturally produced OMVs could differ from
140 those induced by stress (42), and that OMVs can be isolated directly from the environment,
141 particularly from aquatic environments, and have been isolated from household dust (46).

142 Despite initially being considered the result of cell lysis, vesiculation occurs in
143 viable and metabolically active cells in which the integrity of the OM is not compromised
144 (35,48,49). Moreover, certain OM or periplasmic proteins are more abundant in OMVs
145 while others are completely absent (41). These observations support that vesiculation is
146 regulated and that it would depend on environmental factors, bacterial pathogenicity, and
147 the cellular metabolic state (36). However, experimental data to support the hypothesis that
148 OMV biogenesis is a regulated process is lacking. Some important biological mechanisms
149 that continue to be unknown are: genes and regulatory pathways involved in OMV
150 production, and whether or not bacterial populations coordinate OMV liberation, to cite a
151 few open questions.

152 Yet, OMV biogenesis is an energy-demanding process, so it should be a tightly
153 controlled process, well-regulated and coordinated, as it enables crucial functions for the
154 benefit of the producer (50). Some genes increase vesiculation rates by compromising
155 bacterial OM, such as mutations in the Tol-Pal system or in *ompA* (43,49,52); for example,
156 in *P. gingivalis* an *ompA* mutant showed hypervesiculation (53).

157

158 2. OMV release:

159 Given the OMVs heterogeneity in production rate and size, it is likely that multiple
160 biogenesis pathways occur in different stains and even in the same bacterial cell (50). It was
161 observed that different *P. gingivalis* strains had varied vesiculation levels, correlating to
162 fimbrial (FimA/FimR), OmpA-like protein and GalE expression (53,54).

163 OM microdomains formed during envelope biogenesis would exhibit compositional
164 differences resulting in there being certain areas prone to form OMVs (36). The sites where

165 OMV biogenesis would be favored would correspond to microdomains with specific
166 physical characteristics in terms of curvature, charge, fluidity, and/or affinity (36).

167 Induction of OMV formation results from increased OM curvature, where OM and
168 peptidoglycan (PG)-binding proteins are decreased, absent or broken (36,42,46,53,54). This
169 mechanism may be concomitant with others that increase turgor pressure and promote OM
170 curvature. For example, OMV production could be induced by a negatively charged
171 molecule that induces repulsion of the OM anionic charge (46,55,56) and/or the physical
172 force induced by accumulation of misfolded or overexpressed envelope proteins (46,53).

173 OM curvature is also favored in regions of low fluidity, where the proportion of
174 saturated fatty acids is higher, which is also consistent with higher proportion of fatty acids
175 in OMV envelopes (46). Additionally, the local curvature of bacterial OM is enhanced by
176 extracellular signals (53), related to the affinity for amphipathic substances in the
177 extracellular medium. This mechanism may be relevant to stress-induced responses (46).

178 Another factor that could promote OMV biogenesis is OM curvature increase,
179 favored by conformation of transmembrane proteins (36,46). Finally, decreased, or absent
180 expression of *vacJ* and/or *yrb* genes, due to iron and sulfate depletion, leads to the
181 accumulation of phospholipids in OM which induces a change in membrane curvature
182 (43,54,57).

183 As a consequence of the increase in OM curvature, by the mechanisms outlined
184 above, a budding arises and then the OM constricts forming a proteo-liposome and
185 encapsulating contents in its lumen (36,41,44). Different inductors of OMV production and
186 release should influence different biogenesis pathways and induce mechanisms for OMV
187 liberation from its parental cell. Knowledge about OMV surface biomarkers and their

188 differential expression is needed. Without this information, the study of biogenesis routes
189 or the mechanism of release would be extremely difficult.

190

191 3. OMVs content:

192 OMVs composition varies even within the same bacterial population and depends
193 on the growth phase and environmental conditions (48,52). However, detailed molecular
194 analysis of OMV contents supports the idea of selective enrichment or exclusion of specific
195 proteins, lipids and other biomolecules (46,49,58). Load selection mechanisms in OMVs
196 remain incompletely understood (38,58). A schematic representation of the content of the
197 OMVs is presented in Figure 3.

198 In the intravesicular lumen, the content or cargo is mostly composed of OM and
199 periplasmic proteins, and to a minor degree, inner membrane (IM) and cytoplasmic
200 proteins, including soluble proteins, integral membrane proteins, and lipoproteins
201 (36,38,40,41,51,54,59,60). For instance, *Aggregatibacter actinomycetemcomitans* exports
202 leukotoxin, a Type I-secreted, highly conserved repeat toxin (RTX) family-member,
203 through OMVs (36), while TonB-dependent receptors have been found in vesicles of *T.*
204 *forsythia* (40) and Type V secretion system proteins have been identified in OMVs from
205 *Fusobacterium nucleatum* (40). It has also been described, in the lumen, genetic material
206 including chromosomal DNA, rRNA, mRNA, miRNA lncRNA, circRNA and other small
207 RNAs (35,39,48,54,55,58,61,62). For example, small RNAs have been found in vesicles
208 from *A. actinomycetemcomitans*, *P. gingivalis* and *T. denticola* (39). In the case of *P.*
209 *gingivalis*, chromosomal DNA, mRNA and rRNA are present in OMVs (48). Some of these
210 biomolecules could be biologically active (58,63), and misfolded proteins or exogenous
211 components such as antibiotics and metal ions have also been reported (48). OMVs from *B.*

212 *fragilis* exhibited inhibitory activity against other Bacteroidales through the intravesicle
213 antimicrobial peptide Bacteroidales secreted antimicrobial proteins (BSAP) (55).

214 Abundant structural proteins and porins, as well as enzymes, ion channels,
215 transporters, and proteins related to stress responses are found in most OMVs in both the
216 lumen and outer layers (55). For instance, several hydrolytic enzymes such as glycosidases,
217 sulfatases and proteases are contained in vesicles derived from *Bacteroides* spp. (55).
218 Moreover, OMV envelopes may contain a specific lipid profile that differs from OM, with
219 distinctive differences in the LPS profile (46,50). *P. gingivalis* synthesizes anionic LPS and
220 neutral LPS, both of which are present in OMVs; nonetheless, lipid A was deacylated in
221 vesicles in comparison to the OM and cardiolipin was enriched in OMVs from *A.*
222 *actinomycetemcomitans* (46,52). Likewise, OMVs contain pathogen-associated molecular
223 patterns (PAMPs) present in the outer membranes of bacteria (49,64). For instance,
224 polysaccharide derived from OMVs shed by *B. fragilis* is sensed by gut dendritic cells
225 through Toll-Like Receptor 2 (TLR2) and leads to Interleukin 10 (IL-10) expression (59).
226 These interactions with host immune cells through PAMPs provide OMVs with
227 immunomodulatory properties.

228 In this sense, virulence factors are associated with the OMVs released by pathogens
229 (44,65). Thus, OMVs are a rich source of antigenic determinants and other bioactive
230 components (66–68). Said biomolecules would play various roles in adherence and
231 invasion to host cells, resistance to antibiotics, biofilm formation, promotion of virulence,
232 or have immunomodulatory effects (7,29,36,63). Table 2 provides more details on the
233 virulence factors, mainly proteases and adhesins, described in anaerobic GNB's OMVs.

234 Other OMV-associated proteins may play roles in interspecies cooperation and
235 intercellular communication (36). For example, OMVs from *Bacteroides* spp. contain

236 proteins involved in carbohydrate (e.g. cellulase and xylanase) and amino acid metabolism,
237 the citrate cycle, and cell division (45,60).

238 Many scientific teams, using proteomics, have extensively studied the presence of
239 proteins from different cell compartments. However, protein sorting for cargo into the
240 OMV lumen is poorly understood.

241 Additionally, the reported presence of nucleic acids in OMV's lumen is more
242 controversial since the biological mechanism is unknown and may be the result of
243 contamination or a random event, rather than of active transport and packaging. It has been
244 proposed that nucleic acids could be present in OMV's lumen through the cytoplasmic
245 route (where cytoplasmic content is trapped into OIMVs), through the periplasmic route
246 (where nucleic acids are exported from the cytosol to the periplasm, and then packed into
247 OMVs), and/or through a extracellular route (where OMVs reassemble in extracellular
248 media and incorporate nucleic acids, and/or phages inject DNA into OMVs).

249 DNA and/or RNA sequencing from individual OMVs in a population might shed
250 light on this controversy. Moreover, whether or not these nucleic acids are biologically
251 significant and can contribute in the adaptation and response from host cells interacting
252 with anaerobic GNB OMVs should be further explored.

253

254 4. Internalization of OMVs by a host cell:

255 Once released, OMVs can travel to distant sites and interact with various cells; this
256 has been studied mainly in pathogenic anaerobic GNB. OMVs lack a mechanism to self-
257 target cells and the medium they are in would affect their transport (36,46). Several factors
258 have been identified to promote OMVs adhesion to eukaryotic cells, such as LPS-binding

259 proteins (46). In the case of *P. gingivalis*, fimbriae confer adhesive abilities to OMVs, and
260 *T. denticola* OMVs also contain adhesins such as dentilisin (69).

261 A few routes for OMV entry into host cells have been proposed: (1) endocytosis, in
262 eukaryotic cells it can be clathrin- or caveolin-dependent, (2) pinocytosis, an actin-induced
263 process where nonspecific uptake of extracellular components occur, (3) by lipid rafts in
264 the host plasma membrane via caveolins or GTPases, (4) phagocytosis, and (5) by direct
265 membrane fusion (18,22,51,53,54,56,59,62–65,70,33,71–73,37–40,43,46,50). In that sense,
266 OMVs have an intrinsic ability to fuse with the cell and spill their contents into the host's
267 cytoplasm (39,46,64,72,74).

268 Importantly, OMVs size and content have been shown to influence the route and
269 speed of uptake, with potentially several endocytic pathways in simultaneous use
270 (6,54,70,71,73). For example, rough OMVs with shorter LPS will rapidly fuse to the host
271 membrane surface, whereas smooth OMVs, containing LPS with O-terminal antigen-
272 terminal chains, tend to retain their spherical shape during interaction (71).

273 Importantly, for pathogens with an intracellular (facultative) lifestyle, OMVs can be
274 released directly into the host cell without the need to overcome a cytoplasmic membrane.
275 Therefore, host responses are likely to differ according to pathogen lifestyle and interaction
276 pathway (39).

277 Additionally, OMVs from intestinal bacteria can be detected in host's blood and
278 urine. This suggests that OMVs can cross the intestinal epithelium and vascular
279 endothelium to reach sites beyond the gastrointestinal tract (65). To do this, OMVs can use
280 two distinct pathways: the paracellular or transcellular pathways (37,65). The paracellular
281 pathway works through the disruption of tight junctions (TJ) that form the intercellular
282 barrier between epithelial cells; and the transcellular pathway consists in OMVs being

283 internalized through endocytosis, transported through the cell body and released at the
284 basolateral cell surface (75,76). For example, OMVs produced by *Bacteroides*
285 *thetaiotaomicron* have been reported to travel by both pathways, with imaging techniques
286 showing that after hours of oral administration of OMVs, they can be detected in tissues
287 such as the liver (76). These results support the possibility that OMVs can act as a long-
288 distance communication system between microbiota and the host.

289 As for specific examples, it has been described that OMVs from *P. gingivalis* can be
290 internalized via receptors, such as integrin $\alpha 5\beta 1$, via lipid rafts and via caveolin/clathrin-
291 independent endocytosis (40,50,53,54,64,71). OMVs from *P. gingivalis* have been shown
292 to enter cells such as human oral keratinocytes and gingival fibroblasts more efficiently
293 than parental bacterial cells (72). Moreover, the content of *T. denticola* OMVs can be
294 sensed by the absent in melanoma 2 (AIM2) inflammasome and consequently trigger an
295 inflammatory cell death (59).

296 Limited information about receptors for internalization and the actual entry route
297 skew our understanding on anaerobic GNB OMVs entry into cells. Moreover, a bias exists
298 in the lack of information for almost all anaerobic GNB (*P. gingivalis* being perhaps the
299 exception), as they are not usually recognized as bacterial models of study, and therefore
300 their particularities are poorly understood.

301

302 5. Interaction of OMVs with host cells

303 Host cells can internalize OMVs released by an anaerobic GNB. Host cells can be
304 distant from where the OMVs parental cell is located. In animals, OMVs could be dispersed
305 in body fluids (36,65,67). The host cells that internalize OMVs can be bacterial or
306 eukaryotic, as detailed below.

307

308 5.A. OMVs-bacteria interactions

309 OMVs play an important role in competitive and cooperative functions for the
310 microorganisms that secrete them, and for the microbial community by participating in
311 events such as: i. biofilm formation, ii. gene transfer, iii. antibiotic resistance, and iv. phage
312 neutralization (35,36,38–40,42,55,70,71,77,78).

313 i. OMVs play their role in biofilm formation and stabilization by participating in
314 nutrient acquisition, protection against antimicrobial effects, nucleation, and intercellular
315 communication (35,41,70). It has even been reported that OMVs can be part of the biofilm
316 matrix and are one of the most important protein sources for the matrix of certain biofilms
317 (35,46,71). For example, under starvation conditions, quorum sensing and the HmuY
318 protein detected in OMVs of *P. gingivalis* aid in cell survival and biofilm formation
319 (36,70).

320 ii. OMVs could participate in the lateral transfer of plasmids and genomic DNA to other
321 bacteria (35,37,46,55,70,71). Gene transfer via OMVs contributes to the propagation of
322 factors necessary for bacterial adaptation, such as antimicrobial resistance genes or
323 virulence factors (35,48,55,58,70). For example, chromosomal DNA transfer by OMVs
324 from two different strains of *P. gingivalis* with subsequent integration by homologous
325 recombination and gene expression in the recipient strain has been demonstrated (48).

326 iii. OMVs can protect bacteria from antibiotics in several ways: (1) by carrying β -
327 lactamases (36,63,65,70,71), (2) by binding antibiotics in the extracellular environment,
328 serving as a decoy, and preventing cellular entry (38,41,55,70,71), and (3) by exhibiting a
329 high content of drug-binding proteins (70). In a mixed bacterial population, OMVs may
330 provide antibiotic protection not only to parental cells, but to the whole bacterial

331 population; thus, OMVs may be involved in the emergence of drug-resistant strains (36,70).
332 For example, β -lactamases associated with OMVs from *Bacteroides* sp. provide antibiotic
333 resistance to parental cells, but also to other commensals and pathogens against β -lactam
334 antibiotics (55,63).

335 iv. Since OMVs have similar surface structures to the parental bacteria, the release of
336 OMVs may act as a trap to prevent phage adsorption (70). Phages form complexes with
337 OMVs and avoid binding to bacterial cells, preventing harmful effects on bacteria (55,70).

338 Other functions of OMVs have been described; for example, they could be a removal
339 system for unnecessary or harmful substances that accumulate in microbial cells, such as
340 misfolded proteins and xenobiotics (46,71). A graphical summary of these and other
341 functions performed by OMVs is presented in Figure 4.

342

343 5. B. OMVs-eukaryotic cell interactions

344 OMVs of pathogens and commensals play a pivotal role in the communication
345 between bacteria and their eukaryotic hosts (51,55,57,79). Generally, OMVs of pathogenic
346 and commensal bacteria are related to distinct interactions with the host: i. OMVs of
347 pathogenic bacteria participate in the pathological process, and ii. OMVs of commensal
348 bacteria promote host homeostasis (47,55,79,80).

349

350 5. B.1. OMVs from pathogenic bacteria-eukaryotic cell interactions

351 The content of OMVs of pathogenic bacteria is involved in host cell invasion,
352 nutrient acquisition, antibiotic resistance, immune modulation, virulence, biofilm formation
353 and intracellular communication (35,36,51,53–56,70,71). In addition to transferring toxins
354 to host cells, OMVs contribute to pathogen survival and maintenance of pathogenesis by

355 evading host immunity, aiding adaptation to the stressful environment, and nutrient capture
356 (70). Some examples of virulence factors and their interaction with the host are cited in
357 Table 2.

358 OMVs from different bacteria, and their components, induce distinct immune
359 responses (78). LPS derived from OMVs activates toll-like receptor 4 (TLR4), which
360 triggers inflammation through the nuclear factor kappa-light-chain-enhancer of activated B
361 cells (NF- κ B), proinflammatory cytokine production, caspase-11-dependent effector
362 responses and cell death (35,59,64,70,73). Moreover, OMVs polysaccharides activate
363 TLR2 and cytosolic nucleotide-binding oligomerization domain-containing protein 2
364 (NOD2), which induces phagocytosis and anti-inflammatory responses associated with
365 autophagy (43,59). Finally, flagellin in OMVs is detected by the nucleotide-binding domain
366 leucine-rich repeat (NLR) family, apoptosis inhibitory protein 5 (NAIP5) and activates the
367 NLR family CARD domain-containing protein 4 (NLRC4) inflammasome, while OMV-
368 derived dsDNA promotes the formation of the AIM2 inflammasome; both inflammasomes
369 trigger caspase-1-mediated pyroptosis and inflammation (43).

370 An example is *P. gingivalis* OMVs which can induce inflammasome activation via
371 caspase-1 activation and IL-1 β and IL-8 production (54,59,72), and induce the production
372 of tumor necrosis factor alpha (TNF- α), IL-12p70, IL-6, IL-10, interferon beta (IFNB) and
373 nitric oxide in macrophages (54,72). In addition, gingipains in *P. gingivalis* OMVs induce
374 IL-8 scission and activation, leading to neutrophil recruitment (78). Importantly, it has been
375 reported that inflammation may be enhanced in macrophages exposed to *P. gingivalis*
376 OMVs compared to inflammation induced by exposure to *P. gingivalis* cells (72).

377 The involvement of *P. gingivalis* OMVs in several systemic diseases has been
378 described, for example in: (1) periodontitis: OMVs induce inflammation and tissue

379 destruction, (2) diabetes: OMVs translocate to the liver and attenuate insulin sensitivity and
380 glycogen synthesis, (3) arteriosclerosis: OMVs possess potent platelet aggregation activity,
381 stimulate foam cell formation and low-density lipoprotein (LDL) aggregation, (4)
382 rheumatoid arthritis: OMVs induce production of anti-citrullinated protein antibodies and
383 loss of tolerance to citrullinated proteins, (5) Alzheimer's disease: OMVs induce brain
384 inflammation (22,40,54,72).

385 For other pathogens, the amount of information found is more limited. In the case of
386 *T. denticola*, its OMVs could destroy the integrity of the epithelial cell barrier (64,66). *F.*
387 *nucleatum* OMVs activate TLR2-MyD88-NF- κ B signaling through the FomA porin, and
388 thus exert proinflammatory effects and regulate innate immunity (43). Finally, *A.*
389 *actinomycetemcomitans* OMVs contain cytolethal distending toxin and leukotoxin A, the
390 latter of which is lethal to human monocytes and polymorphonuclear leukocytes, and the
391 genetic material contained in OMVs could regulate human macrophage gene expression,
392 promoting TNF- α production (40,44,81).

393 Limited information about OMVs cargo is presented in the literature that presents
394 regulation of eukaryotic cells after OMVs content's internalization. Besides the examples
395 presented, and to add to the controversy about the presence of nucleic acids in OMVs,
396 several studies presented downregulation of immune effectors after delivery of small RNAs
397 into host cells by OMVs from *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola*,
398 and/or *T. forsythia*. The lack of genetic tools to inhibit vesiculation in order to understand
399 OMVs implication in infections is a difficulty that should be addressed.

400

401 5. B.2. OMVs from commensal bacteria-eukaryotic cell interactions

402 OMVs from commensal bacteria can generate positive effects on host immune
403 tolerance by regulating the host innate immune system, assisting in the maturation of the
404 immune system, and promoting intestinal homeostasis (48,55,57,70,80,82). For example,
405 OMVs would serve to control bacterial populations by loading with toxic molecules, such
406 as peptidoglycan hydrolases, murein hydrolases, endopeptidases, and other types of
407 enzymes that induce bacterial lysis, thus controlling bacterial overgrowth (45,55,57,71). In
408 addition, OMVs from commensal intestinal bacteria promote regulatory T cells and anti-
409 inflammatory cytokine secretion through activation of host dendritic cells (DCs) (57,66,73).

410 For example, *Akkermansia muciniphila* OMVs have been associated with intestinal
411 barrier fortification and reduced inflammation by enhancing TJ expression and
412 downregulating TLR expression in intestinal epithelial cells (6,37,64,81,83). In mice
413 models, *A. muciniphila* OMVs have been described to improve TJ function and glucose
414 tolerance (37,64,70,81,82), colitis symptoms and enhance intestinal epithelial barrier
415 integrity (51,55,64), and provided protection against inflammatory bowel disease (IBD)
416 phenotypes (70). Furthermore, direct intervention of mice with *A. muciniphila*-derived
417 OMVs had exhibit an anti-osteoporotic effect (62,78). In addition, *Odoribacter*
418 *splanchnicus*-derived OMVs have exhibited immunomodulatory properties, mitigating IL-8
419 production in enterocyte cultures treated with LPS from *E. coli* (18,81).

420 Moreover, polysaccharides associated with *B. fragilis* OMVs are detected by mouse
421 intestinal DCs via TLR2 to induce IL-10 expression, which in turn enhances Treg function
422 and decreases intestinal inflammation (48,51,55,59,65,81). Noticeably, *B. fragilis* OMVs
423 have been reported to have a protective effect on some types of colitis (7,64); for example,
424 they prevented the development of colitis in mice by inducing immune tolerance via DC
425 activation (81). *B. fragilis*-derived OMVs are rich in polysaccharide A, which ameliorates

426 human inflammatory disorders such as inflammatory bowel disease and multiple sclerosis.
427 These OMVs show comparable therapeutic activity to their parental bacteria in terms of
428 immune regulation (57,61).

429 *B. thetaiotaomicron* and *Phocaeicola vulgatus* (previously known as *Bacteroides*
430 *vulgatus*) OMVs have also been reported to have immunomodulatory effects by controlling
431 DC maturation, suppressing inflammatory cytokine secretion and promoting anti-
432 inflammatory cytokine production (6,37,55,64). Specifically, *B. thetaiotaomicron*-derived
433 OMVs contain enzymes involved in polysaccharide digestion (7,29,65,78) and mediate
434 cholesterol uptake into enterocytes through positive regulation of transporters (55), but can
435 also be taken up by intestinal macrophages and elicit an inflammatory response by inducing
436 the production of proinflammatory cytokines and driving the development of colitis in
437 susceptible mice (81).

438 The interaction between anaerobic GNB OMVs and eukaryotic cells is not fully
439 known, many aspects need clarification, as stated throughout this review. In this sense,
440 OMVs potentialities, especially for those produced by commensal anaerobic GNB, as drug-
441 delivery systems or other biotechnological/biomedical uses are still too poorly understood
442 for clinical applications.

443

444 **Concluding remarks and perspectives**

445 OMVs are unique delivery systems used by GNB that fulfill several roles in
446 bacteria-bacteria interactions and bacteria-eukaryotic interactions. This systematic review
447 offers a synthesis of current knowledge available for the OMVs produced by anaerobic
448 GNB. Induction of OMVs production, its biogenesis, contents, liberation from the parental
449 cell, spread to the environment, internalization by either bacterial or eukaryotic host cells

450 and the interactions with host cells are discussed. A graphic summary of the contents of the
451 review is presented in Figure 1. An important limitation in literature is the lack of
452 consistent nomenclature for BEVs produced by GNB as at least two different types have
453 been described: OIMVs, and OMVs. In this review, we termed OMVs both types, as almost
454 all analyzed articles named them as such, and did not state which type of BEV was being
455 discussed. This is the fundamental source of confusion in the understanding of important
456 mechanisms for OMVs cargo loading. Additionally, many aspects of OMVs biology are
457 still poorly described: biogenesis pathways, signaling for release, receptors in the target
458 cell, and mechanism for host cells to respond. This hinders concluding whether or not a
459 specific OMV cargo has meaningful biological value to explore or is the result of
460 contamination. As a result, contradictory results between different working groups exist,
461 and several authors have clear positions on particular aspects regarding OMVs. This article
462 is not intended to be a critical review, but rather to present the information as it is stated,
463 and to be an invitation for the curious and informed reader to draw their own conclusions.

464 Nonetheless, literature describes that OMVs derived from pathogenic bacteria, like
465 *P. gingivalis*, contain virulence factors and have inflammatory and immunomodulatory
466 properties. Those shed by commensal bacteria, such as OMVs from *B. fragilis* or *A.*
467 *muciniphila*, play mostly an immunomodulatory role.

468 However, knowledge available for different species is different. Some genera have
469 very limited information (e.g. *Odoribacter* sp.) while other dominates literature (*P.*
470 *gingivalis*). Given the heterogeneity of OMVs, the characteristics of every species' OMVs
471 should be studied independently; therefore, there is still a broad field of research to be
472 developed. Considering that several of the included studies were published in recent years,

473 this subject is in constant development, and it would be worth updating the knowledge
474 synthesis often.

475 The understanding of OMVs, their nature and their interactions in a biosystem not
476 only deepens our comprehension of the bacterial reality, but also clears the path to their
477 biotechnological use. The development of the field has the potential for vaccine production,
478 adjuvant platforms, antibacterial treatment, biofilm studies, drug delivery, and more.
479 Further studies are necessary to assure safe use of OMVs in the prevention and treatment of
480 infection and disease. Finally, the study of OMVs produced by anaerobic GNB is also
481 important for broadening our comprehension of mucosal immunity as they are produced by
482 commensal and pathogenic bacteria.

483

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488

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490 The authors declare that they have no competing interests.

491

492 **Author contributions**

493 **P.C.-V.:** Formal analysis, Data Curation, Writing - Original Draft, Visualization; **F.B.H.:**
494 Data Curation, Writing - Review & Editing, Supervision; **L.A.-A.:** Conceptualization,
495 Methodology, Validation, Writing - Review & Editing, Supervision.

496

497 **Data availability**

498 Data will be made available on request.

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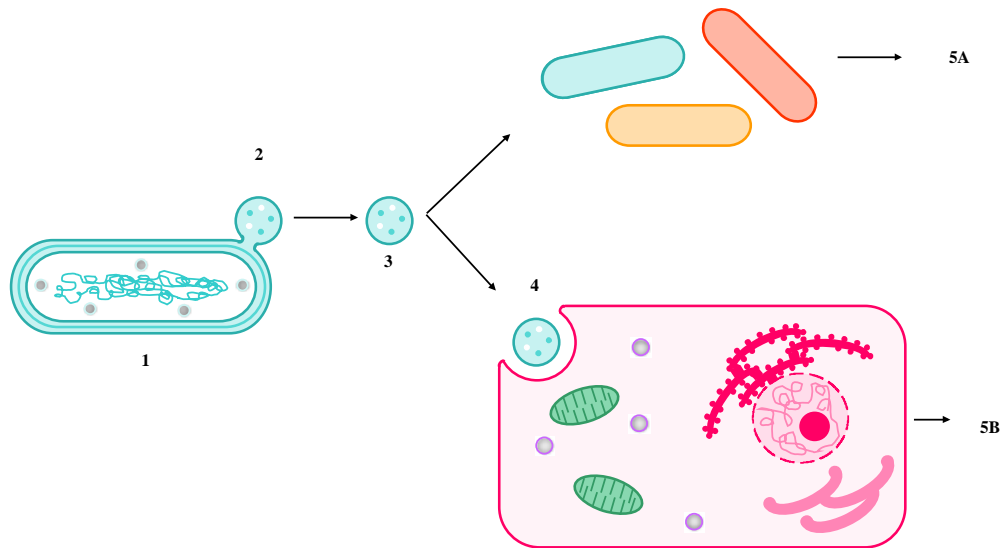
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725 **Figures, Tables and Supplementary data**



726

727 **Figure 1. Summary of the paper's sections.** (1) Induction of vesiculation and biogenesis
728 of OMVs. (2) Release of OMVs. (3) OMVs content. (4) Trafficking and internalization of
729 OMVs. (5) Functions: (5.A.) Bacteria-bacteria interactions and (5.B.) Bacteria-eukaryotic
730 interactions.

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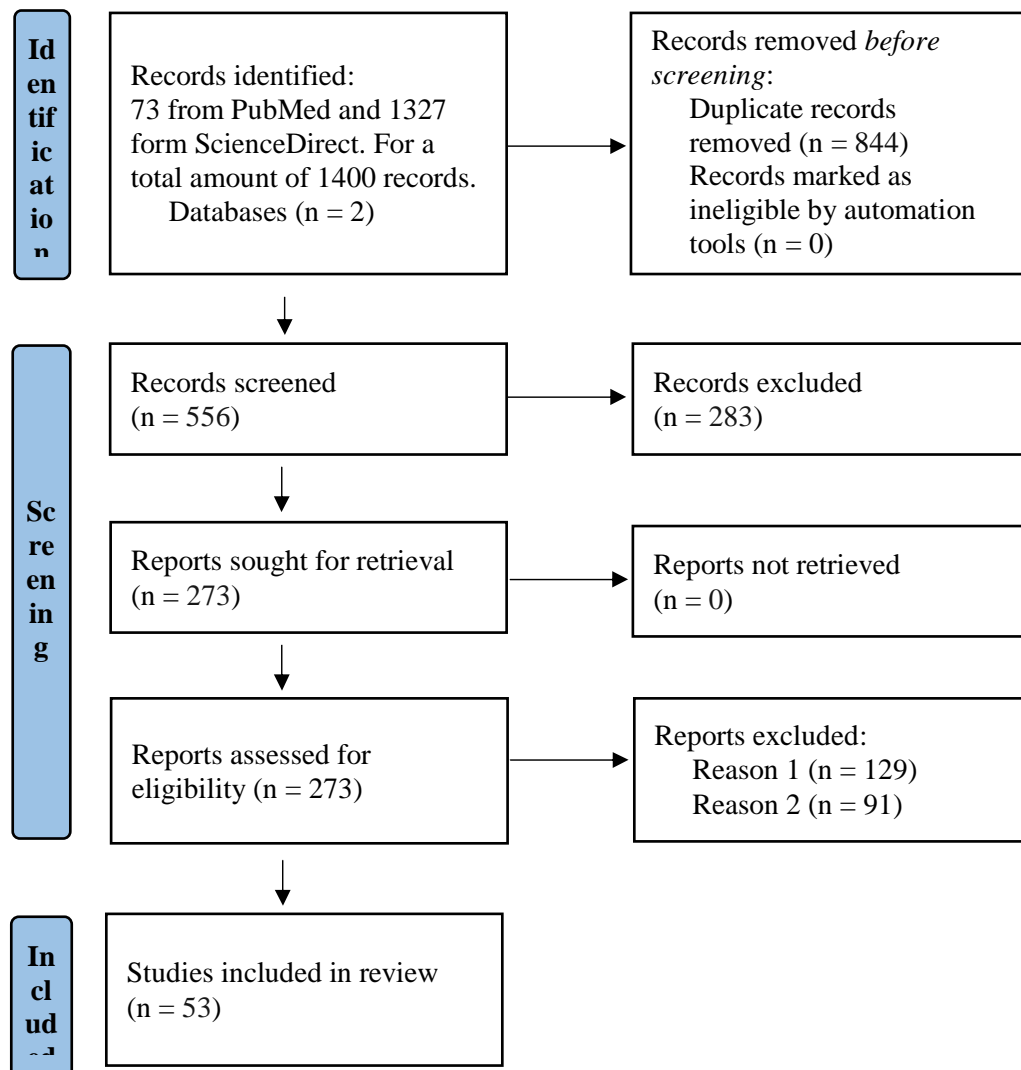
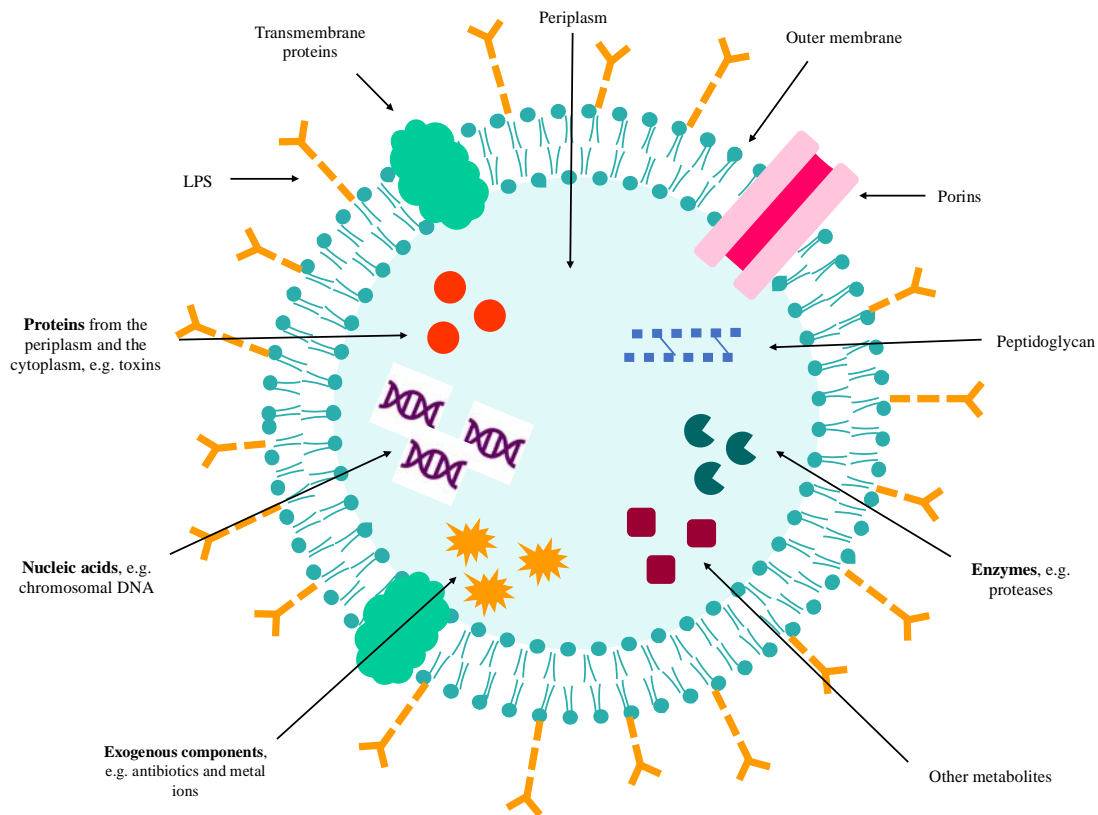


Figure 2. Flow chart based on PRISMA. Information on the article selection process for the systematic review is presented, along with the reasons for exclusion. Reason 1 for exclusion was that the article did not contain relevant information and Reason 2 was that OMVs were not a central theme in the article.

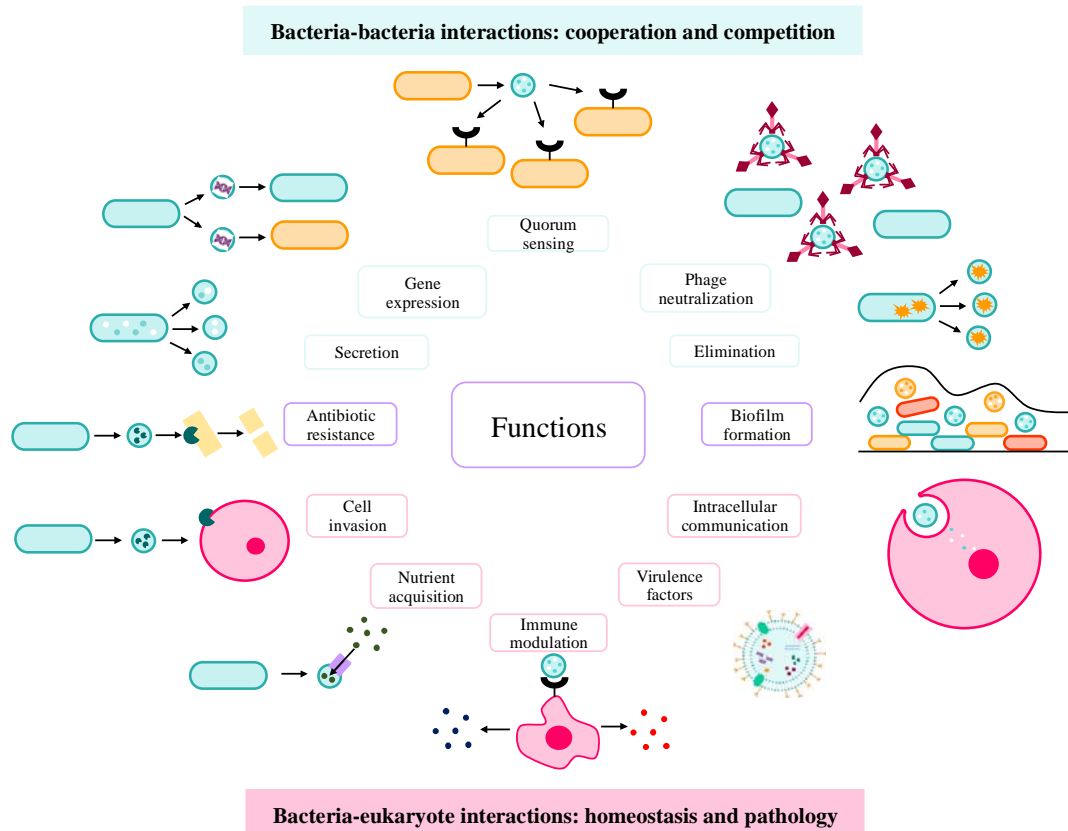
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771 **Figure 3. OMVs content and structure.** OMVs are enveloped by an outer membrane
772 bilayer of Gram-negative bacteria (GNB). They contain LPS, porins, transmembrane
773 proteins, peptidoglycan, enzymes, proteins, nucleic acids, exogenous components, and
774 other metabolites.

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777 **Figure 4. OMVs functions.** OMVs play several roles in bacteria-bacteria interactions in
778 processes of cooperation and competition, and in bacteria-eukaryotic interactions either in
779 homeostasis or pathology.

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781 **Table 1.** Articles included in this review (number and percentage) with information on
782 anaerobic GNB, presented by genus and with the reference number used throughout the
783 main text of the review.

Bacterium	Article number	Article percentage	Reference in main review text
<i>Porphyromonas</i> spp.	36	67,9%	(15,18,41–44,46–51,22,53,54,56,58,59,61,64,66–68,29,70–73,78,79,33,35,36,38–40)
<i>Bacteroides</i> spp.	33	62,3%	(6,7,47,50,51,55–57,59–61,63,15,64–66,68,71,73,74,77,78,81,18,82–84,29,37,43–46)
<i>Akkermansia</i> spp.	16	30,2%	(6,7,64,72,78,79,81,83,15,18,22,39–41,44,50)
<i>Aggregatibacter</i> spp.	13	24,5%	(15,22,79,81,82,37,51,55,56,62,64,70,78)
<i>Treponema</i> spp.	7	13,2%	(39,40,44,58,59,64,66)
<i>Fusobacterium</i> spp.	5	9,5%	(6,40,43,52,56)
<i>Tannerella</i> spp.	2	3,8%	(40,59)
<i>Odoribacter</i> spp.	2	3,8%	(18,81)
<i>Prevotella</i> spp.	2	3,8%	(56,81)
Other anaerobic GNB	0	0%	-

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786 **Table 2.** OMV-associated contents in anaerobic Gram-negative bacteria (GNB).

Bacterium	Contents	Associated effect	References
<i>Aggregatibacter</i> sp.	Leukotoxin	Kills human leukocytes	(36,40,41,44,56)
	Cytolethal distending toxin	Cytolethal distension in eukaryotic cells	(36,40)
	Peptidoglycan-associated lipoprotein	N/A	(44)
	LPS	N/A	(40)
	Phosphatidylethanolamine	N/A	(40)
	Cardiolipin	N/A	(40)
	RNA	Enhanced macrophage TNF- α production	(40)
<i>Bacteroides</i> sp.	Hemagglutinin	N/A	(44)
	Cellulase	Provide a source of carbon from digestion of non-metabolizable carbohydrate polymers	(45)
	Xylanase		
	Antimicrobial peptide BSAP	Inhibitory activity against other Bacteroidales	(33,55)
	Capsular polysaccharide A	Immune modulation	(33,51,63)
	β -lactamases	Degradation of antibiotics	(33)
	Histamine and γ -aminobutyric acid (GABA)	N/A	(83)
<i>Fusobacterium</i> sp.	Type V secretion system protein	N/A	(40)
<i>Porphyromonas</i> sp.	Gingipains	Cytokine degradation,	(33,40,41,44,46,54,56,72)

	(Cysteine proteases)	tissue destruction, bacterial colony formation	
	Fimbriae	Mediation of bacterial adherence to and entry to cells	(40,44,72)
	Peptidylarginine deiminase	Citrullination of bacterial and host proteins	(33,40)
	Anionic LPS and neutral LPS	N/A	(44,46,50,52–54,72)
	Muramic acid	N/A	(44,72)
	Capsule	N/A	(44,72)
	Hemagglutinin	N/A	(53)
	Heme binding and transporting proteins	Heme acquisition	(40,50,54)
	DNA fragments encoding fimbriae, superoxide dismutase, and gingipains	N/A	(53)
	RNA	N/A	(39,40,48,53,54,72)
<i>Tannerella</i> sp.	Glycoproteins	N/A	(40)
	Type IX secretion system substrates	N/A	(40)
	TonB-dependent receptor	N/A	(40)
	RNA and DNA	N/A	(40)
<i>Treponema</i> sp.	Dentilisin	Proteolysis and adhesion	(44)
	RNA and DNA	N/A	(40)

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788 **BSAP: Bacteroidales secreted antimicrobial proteins; DNA: Deoxyribonucleic acid;**

789 **LPS: Lipopolysaccharides; RNA: Ribonucleic acid; TNF: Tumor necrosis factor;**

790 **N/A: not available.**

791 **Table S1.** Summary sheet used to extract and organize the information from the articles
792 included in the review.

Article n°
Authors:
Publication year:
Title:
DOI:
Main objective:
Obtained information: <ol style="list-style-type: none">1. Definition:2. Production signals:3. Biogenesis:4. Content:5. Exportation mechanisms:6. Intracellular traffic:7. Functions:8. Study methods:9. Other information:
Pending issues:

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