CHAPTER 9

Membrane Vesicles from the Gut Microbiota and their Interactions with the Host

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Abstract

Gut microbiota plays an essential role in maintaining intestinal homeostasis and human health. Microbiota establishes a complex network of dynamic and reciprocal interactions with the intestinal epithelium and immune system. The mucin layer that covers the epithelium prevents luminal bacteria from accessing host cells. Thus, microbiota-host communication mainly relies on secreted factors and membrane vesicles (MVs), which can cross the inner mucus layer and reach the epithelium. This chapter focuses on the role of microbiota-secreted MVs as key players in signalling processes in the intestinal mucosa. This is an emerging research topic, with the first reports dating from 2012. Microbiota-derived MVs are involved in inter-species communication in the gut, between bacteria and between microbiota and host. Here we present current knowledge on the mechanisms used by microbiota MVs to assist and control the gut microbial community and to modulate host immune and defence responses. Constant stimulation of immune receptors by microbiota MVs results in tightly controlled inflammation that contributes to tolerogenic responses essential to maintaining intestinal homeostasis. Moreover, gut microbiota MVs are emerging as physical vehicles for distribution and delivery of bacterial effectors to distal tissues in human health and disease.

Key words: Escherichia, Akkermansia, Bacteroides, probiotic, gut colonization, immune modulation, epithelial barrier, microbiota-host communication.

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1 Gut Microbiota

Gut microbiota refers to the entire set of microbial communities that colonize the human gastrointestinal (GI) tract. This community is mainly composed of bacteria, but other groups such as archaea, fungi, protozoa and viruses are also represented. Recent advances in omics technologies such as metagenomics, transcriptomics and proteomics are currently being applied to study intestinal microbial ecology at a molecular level. From these studies, we have learned that the gut microbiome comprises more than three million genes, which greatly exceed and complement the genetic information encoded by the human genome (Qin et al. 2010). Microbiota-encoded products provide trophic, metabolic and protective signals that are beneficial to the host. In fact, the gut microbiota is considered a "hidden organ" as it plays fundamental roles in intestinal homeostasis and human health (Jandhyala et al. 2015). There is strong scientific evidence that the gut microbiota exerts pivotal functions by regulating food digestion, maintaining the intestinal epithelial barrier and contributing to immune system functions and development (Thursby and Juge 2017; Cani 2018). Moreover, commensal bacteria help to protect the host against pathogens through mechanisms that include secretion of antimicrobial factors, competition for binding sites, reduction of intraluminal pH and enhancing host immune responses (Llewellyn and Foey 2017). Alterations in normal gut microbiota biodiversity (dysbiosis) have been associated with a wide range of illnesses including inflammatory bowel disease (IBD), allergic and immune disorders, metabolic diseases (insulin resistance and obesity) and cancer (Vindigni et al. 2016; Baothman et al. 2016; Gopalakrishnan et al. 2018). The high plasticity of the human microbiome provides new opportunities for therapeutic strategies aimed at modulating the composition of the gut microbiota that is altered in certain pathologies (Shanahan 2011; Maguire and Maguire 2018). One such approach to modulate the host microbiota is the administration of probiotics.

The intestinal ecosystem is characterized by dynamic and reciprocal interactions between the microbiota, the epithelium and the host immune system. The capacity of cells of the intestinal mucosa to discriminate between pathogens and commensal bacteria is a key issue. The host response to pathogens is characterized by rapid recognition of the pathogen combined with innate (inflammatory) and adaptive immune responses. This leads to pathogen eradication, at the cost of significant tissue damage. The response to symbiotic microbiota is known as tolerance: a process that encompasses a complex combination of microbe recognition and highly regulated innate and adaptive immune responses. This dichotomy in the host response is fundamental on the surface of the intestinal mucosa that is massively colonized by a diverse population of bacteria (Bron et al. 2011).

The gastrointestinal epithelial layer is the surface where the host interacts with microbiota. This epithelium creates a physical and biochemical barrier between gut microbiota and host. Several mechanisms are involved in the epithelial barrier function such as: (i) secretion of the mucin layer that covers the epithelial surface and avoids direct contact with gut microbes, (ii) secretion of antimicrobial peptides, and (iii) formation of tight junctions between intestinal epithelial cells that separate the host tissue from the luminal ecosystem.

Intestinal epithelial cells (IECs) play an important role in sensing microbial signals. Upon activation, these cells release signalling molecules that communicate the information to the intestinal immune cells, which trigger appropriate immune responses (reviewed by Turner 2009; Wells et al. 2011;

Peterson and Artis 2014). Detection of gut microbes by IECs depends on specific immune receptors, known as pattern recognition receptors (PRRs) that specifically recognize conserved microbial-associated molecular patterns (MAMPs). Toll-like receptors (TLRs) are transmembrane proteins. TLRs located at the plasma membrane (TLR1, TLR2, TLR4 TLR5) recognize extracellular components of bacteria, viruses or fungi whereas intracellular TLRs (TLR3, TLR7, TLR8, TLR9), mainly found in endosomes and lysosomes, recognize internalized microbial DNA or RNA (Kawai and Akira, 2010). There are also cytosolic receptors such as nucleotide-binding oligomerization domain protein 1 (NOD1) and NOD2 that are activated by peptidoglycan (PGN) fragments of Gram-negative and/or Gram-positive bacteria (Philpott et al. 2014). Other cytosolic receptors are part of the protein complex known as inflammasome, whose main function is the activation of caspase-1, which in turn activates the inflammatory cytokines IL-1β and IL-8 (reviewed in Thaiss et al. 2014). Interaction of PRRs with their specific ligand activates signalling cascades that lead not only to the release of chemokines and cytokines that communicate the information to the intestinal immune cells, but also to the secretion of antimicrobial peptides that help to control the gut microbial population. This feedback control is essential in limiting immune activation and maintaining mutualistic associations between bacteria and the host.

In addition to the intestinal epithelium, other cells of the innate immune system such as dendritic cells (DCs) of the lamina propria contribute to the sampling of gut microbes. DCs are antigen-presenting cells that can contact the luminal environment through the inner mucosal lining. These phagocytic cells sense intestinal microbes through their PRRs and act as messengers for the rest of the immune system through antigen presentation and release of immune mediators. DCs ensure intestinal homeostasis by tuning host immune responses in the gut and they are involved in immunological tolerance to gut microbiota (reviews of the topic in Belkaid and Hand 2014; Caballero and Pamer 2015). In response to beneficial gut microbes, DCs induce proliferation of anti-inflammatory regulatory FoxP3 T cells (Treg), which contribute to maintaining immune intestinal homeostasis (Geuking et al. 2011; Jia et al. 2018). Alterations in microbiota may have an impact on immune mucosal tolerance by negatively affecting the Treg response, which in turn leads to the development of intestinal inflammatory pathologies.

Given the complexity of the gut microbiota and its interaction with host intestinal cells, elaborated regulatory mechanisms are required to ensure symbiosis and avoid aberrant responses that lead to pathological states. Many studies have focused on the signalling pathways, regulatory proteins and transcription factors activated by microbiota to modulate intestinal homeostasis (Caballero and Pamer 2015). An emerging topic is the role of host microRNAs (miRNAs) as key players in the host-microbiota interplay and in cell-to-cell communication. The miRNAs are small non-coding RNAs (20-25 nucleotides) that, after maturation, associate with the RNA-induced silencing complex and regulate the expression of target mRNAs through binding to sequences at the 3'-UTR region. This interaction triggers mRNA degradation or blocks translation. Therefore, miRNA are post-transcriptional regulators that allow signalling pathways to be tightly controlled. The miRNAs are involved in the control of multiple cellular processes, including the immune response. In this context, many studies indicate that microbiota and miRNAs regulate each other. Gut bacteria (either commensal or pathogens) have a great impact on miRNAs expression, and host miRNAs shape and regulate gut microbiota (Masotti 2012; Runtsch et al. 2014; Celluzzi and Masotti 2016; Feng et al. 2018; Aguilar 2018).

2. Role of Microbiota-Secreted Membrane Vesicles in Interspecies Communication in the gut

The gut microbiota is not in direct contact with the epithelium. Both cell types are physically separated by the mucus layer, which is structured in two sections. The inner dense mucus layer is closely linked to IECs and acts as a highly efficient barrier that prevents bacteria from reaching the intestinal epithelium (Johansson et al. 2011; Vaishnava et al. 2011). In addition to this protective role, the inner mucus layer also contributes to maintaining the outer mucus layer, which is highly dynamic and in close contact with the microbiota. The external mucus layer can be degraded by specific bacteria of the gut microbiota and thus needs to be constantly renewed. Goblet cells are the main source of secreted mucin (MUC2), whose production is upregulated by TLR signalling in response to its degradation by commensals or other mechanical sources (Faderl et al. 2015). In addition to the MUC2 structure, spatial separation between the microbiota and the intestinal epithelium is maintained by soluble factors with antimicrobial activity that are secreted by the epithelium, such as β-defensin which is active against Gram-negative bacteria, RegIIIγ lectin that is active against Gram-positive bacteria and IgA that is secreted by immune cells. Since access of the microbiota to the intestinal epithelium is limited by the inner mucin layer, communication with the host mainly depends on microbiota-secreted factors (metabolites, proteins and vesicles) that can go through the mucin layer and reach host cells at the intestinal mucosal surface.

All bacteria release extracellular membrane vesicles (MVs) as a mechanism of communication between species. MVs are nano-scale bilayer structures derived from the bacterial membranes (see chapters 2 and 3). They are part of a secretion mechanism that allows long-distance delivery of bacterial active compounds in a protected environment, avoiding direct intercellular contact. MVs comprise components of the bacterial membrane, cytosolic proteins, metabolites, DNA and RNA. MVs from Gram-negative bacteria also include outer membrane and periplasmic biomolecules (Guerrero-Mandujano et al. 2017). The functions of MVs are versatile, including bacterial response to stress, quorum sensing, biofilm formation and inter-species communication (bacteria-bacteria and bacteria-host dialogue) (Schwechheimer and Kuehn, 2015). In the last twenty years, numerous studies have focused on MVs from Gram-negative pathogens, showing that these structures act as vehicles for the delivery of cytotoxic/virulence factors and mediators that alter the host immune response (reviewed in Kaparakis-Liaskos and Ferrero 2015).

In this chapter, we will focus on gut microbiota-derived vesicles. This is an emerging topic, with the first reports of microbial derived MVs dating from 2012 (Shen et al. 2012; López et al. 2012). Most studies deal with Gram-negative commensals of the genus Bacteroides (*B. fragilis* and *B. thetaiotaomicron*), *Akkermansia muciniphila*, and intestinal *Escherichia coli* isolates including the probiotic strain *E. coli* Nissle 1917 (EcN). Studies on MVs from Gram-positive beneficial gut microbes are limited and mostly centred on probiotics of the genus Lactobacillus and Bifidobacterium (reviewed in Liu et al. 2018). Due to the membrane structure of Gram-positive bacteria, their MVs lack lipopolysaccharyde (LPS) and periplasmic components, although they carry similar types of cargo molecules as Gram-negative MVs including PGN, lipids, proteins and nucleic acids (Brown et al. 2015).The effects exerted by microbiota derived MVs depend on their bacterial origin and cargo. Therefore, some of their effects are strain-specific. Proteomic studies have revealed that vesicles released by probiotic and commensal strains

contain proteins that contribute to intestinal barrier and immune modulation and proteins that help to compete for colonization and bacterial survival in the harmful environment of the GI tract (Lee et al. 2007; Aguilera et al. 2014; Elhenawy et al. 2014; Dominguez-Rubio et al. 2017; Li et al. 2017; Zakharzhevskayaet al. 2017). The first proteomic analysis of MVs released by beneficial gut bacteria was performed with the probiotic EcN (Aguilera et al. 2014). Some of the identified proteins are encoded by strain-specific genes and most of them are fitness factors that contribute to adhesion to host tissues and to bacterial survival in the GI tract. In this context, EcN vesicles harbour the porin NanC, whose expression is induced by N-acetylneuraminic acid, one of the most abundant sialic acids of eukaryote cell membranes. Sialic acids can be used by enteric bacterial pathogens as carbon and nitrogen sources (Vimr et al. 2004; Severi et al. 2007). By this mechanism EcN MVs could compete with enteric pathogens for colonization of the intestinal tract. Furthermore, there is a set of identified proteins that are common to MVs secreted by Gram-negative pathogens and probiotic bacteria. The main group of these common probiotic/pathogen vesicular proteins are cytoplasmic proteins, and a high number of them are metabolic enzymes classified as moonlighting proteins that have different functions depending on the cell location (Aguilera et al. 2014). Examples of these moonlighting proteins are enolase, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase or succinyl-CoA synthase.

Regarding the presence of metabolic enzymes in bacterial MVs, a comparative study based on proteomics and metabolomics approaches has found great differences between vesicles isolated from genetically related pathogenic and commensal *B. fragilis* strains (Zakharzhevskaya et al. 2017). MVs from the non-pathogenic strain are enriched in enzymes required for polysaccharide utilization. In contrast, MVs from the pathogenic strain contain, in addition to virulence factors, a larger number of enzymes involved in energy-producing metabolic pathways such as glycolysis and the tricarboxylic acid cycle. The activity of the vesicular enzymes and transporters was validated by fluxomic experiments with isotope-labeled glucose (^{13}C-glucose), thus confirming that these pathways are fully operative in pathogen-derived MVs. The associated metabolic activity provides vesicles released by pathogens with energy for long persistence in the human GI tract (Zakharzhevskaya et al. 2017). The proteomic analysis of MVs isolated from the Gram-positive probiotic *Lactobacillus casei* BL23 corroborates the presence of numerous proteins that fit the profile of Gram-negative derived vesicles, including envelope associated proteins, metabolic enzymes, transporters and ribosomal components. The presence of adhesins and proteins known to mediate the effects of this probiotic reinforces the role of MVs in the bacteria-gut interaction (Dominguez Rubio et al. 2017).

2.1 Contribution of Gut Microbiota-Derived MVs to the Intestinal Ecosystem

Several studies highlight the role of enzymes released through microbiota MVs in the colonization of the human gut (Donaldson et al. 2016). Once released into the intestinal lumen, MVs can affect the surroundings distantly from their parent cells. These MV triggered changes can be used by the parental bacterium to their own benefit or even to help other members of the microbiota community. As an example, MVs secreted by commensals of the Bacterioides genus deliver to the intestinal environment enzymes of the hydrolase class (glycosylases and proteases) that catalyse the breakdown of complex polysaccharides (**Figure 1a)**. This metabolic activity generates products that can be used as a source of nutrients by other

members of the gut microbiota, which in turn produce short-chain fatty acids that are beneficial to the host (Ethenawy et al. 2014). MVs from certain Bacteroides strains can also distribute sulfatases that help in the degradation of mucin glycans by other bacterial hydrolases (Hickey et al. 2015). As Bacterioides MVs are equipped with a wide range of hydrolytic enzymes, they can assist the gut microbial community in the acquisition of nutrients and favour symbiosis between the gut microbiota and the host (Rakoff-Nahoum et al. 2014; Elhenawy et al. 2014). Other vesicle associated-enzymes can be detrimental to the host. For instance, packaging of β-lactamases into Bacteroides MVs provides a mechanism for spreading antibiotic resistance to other microbiota members and to enteric pathogens (Stentz et al. 2015).

Studies performed in *B. thetaiotaomicron* showed that released MVs carry enzymes involved in the assimilation of dietary inositol polyphosphates (InsP6) present in vegetables (Stentz et al. 2014). In the luminal GI tract, InsP6 chelate divalent cations and inhibit polysaccharide digestion. The human gut lacks enzymes (phytases) that can dephosphorylate this kind of molecules. Therefore, assimilation of luminal InsP6 depends on enzymes provided by the gut microbiota (**Figure 1a**). These bacterial enzymes are homologues of the mammalian InsP6 phosphatase and are widely distributed among resident gut bacteria (Stentz et al. 2014). In addition to this metabolic role in dietary InsP6, the bacterial enzyme also participates in inter-kingdom signalling (see Section 2.2).

Besides metabolic roles, microbiota derived MVs can promote competitive interference among related bacterial species. It is known that gut microbiota releases a wide range of antimicrobial peptides as a mechanism to persist and compete with other members of the microbial community. A study performed with *B. fragilis* strains showed that secretion of the antimicrobial peptide known as BSAP-1 (Bacteroidales secreted antimicrobial protein 1), which displays inhibitory activity against other Bacteroidales of the human gut, is mediated by MVs (Chatzidaki-Livanis et al. 2014). This peptide contains the membrane attack complex/perforin (MACPF) domain present in host immune mediators that can kill pathogens and virus-infected cells. Proteins with the MACF domain generate pores in the membrane of sensitive target cells, causing increased membrane permeability (**Figure 1a**). Furthermore, MVs secreted by pathogens also serve as a vehicle to secrete hydrophobic quorum sensing molecules (Brameyer et al. 2018). However, no studies have been reported to date concerning this role for microbiota derived MVs.

2.2 Contribution of Gut Microbiota-Derived MVs to Inter-kingdom Signalling

Microbiota MVs enclose biological components that exist in their parent bacteria. In particular these vesicles contain a high number of MAMPs that are recognized by PRRs expressed by epithelial and immune cells. As stated above, these receptors are key components of innate immunity as they sense gut microbes and trigger appropriate immune responses (Turner, 2009). In the intestinal lumen, microbiota-released vesicles diffuse through the mucus layer and reach the intestinal epithelium. Surface-associated MAMPS (LPS, lipoproteins and extracellular polysaccharides) can interact with extracellular PRRs to trigger the activation of signalling cascades that ultimately regulate defence and immune responses (Kawai and Akira, 2010; Kaparakis-Liaskos and Ferrero, 2015). In addition, bacterial MVs are internalized by IECs through endocytic pathways (O'Donoghue and Krachler, 2016). By this mechanism MVs allow the intracellular delivery of MAMPs (DNA, RNA and PGN) that bind intracellular receptors activating signalling pathways

that control host responses (Kaparakis-Liaskos and Ferrero, 2015). Microbiota MVs can also interact with cells of the innate immune system, especially DCs, which in turn coordinate appropriate immune responses transmitting the information to cells of the adaptive immune system (Belkaid and Hand 2014; Caballero and Pamer 2015) (**Figure 1b**).

Modulation of the innate immune system by gut microbiota plays an essential role in maintaining gut homeostasis by favouring quick responses against pathogens in addition to preserving tolerance to commensal bacteria. MVs released by resident gut microbiota share a number of MAMPs with vesicles secreted by enteric pathogens, and hence can activate the same downstream signalling pathways. Therefore, inflammatory responses should be tightly controlled to avoid aberrant responses against commensal microbiota. Some specific microbiota vesicular components, such as *B. fragilis* polysaccharide A (PSA) trigger TLR-mediated signalling events that restrain host immune responses and allow commensal gut colonization (Round et al. 2011). Concerning cytosolic immune receptors, NOD receptors were first discovered as a defence mechanism against bacterial pathogens and the subsequent host inflammatory responses. However, it is suggested that NODs may also play a role in maintaining intestinal homeostasis as mutations that impair NOD2 activity or expression have been associated with chronic inflammatory and autoimmune diseases (Philpott et al. 2014; Feerick and McKernan 2016; Chu 2017).

The integrity of the epithelial barrier is also critical in maintaining homeostasis in the body. Several diseases are associated with the increased intestinal permeability that follows the disruption of gut epithelial tight junctions (Suzuki 2013). This mechanism is also used by certain enteric pathogens, either bacteria or viruses, to help their dissemination into the host tissues (Lu et al. 2013). It is well known that the gut microbiota plays a relevant role in maintaining the intestinal barrier, by either modulating epithelial tight junctions or by enhancing host defence mechanisms (Jandhyala et al. 2015). The ability of microbiota MVs to reinforce epithelial tight junctions has been reported for certain commensal *E. coli* and *A. muciniphila* strains (**Figure 1b**) (Alvarez et al. 2016; Chelakkot et al. 2018). Moreover, MVs released by commensal *E. coli* and *L. plantarum* strains trigger upregulation of host defence genes that encode secreted peptides with antimicrobial activity, such as β-defensin (Fábrega et al. 2016) or C-type lectins (Li et al. 2017). In the intestinal tract, microbiota derived MVs allow the sensing and delivery of microbial products that steadily prime the host innate immune system (Shen et al. 2012; Fábrega et al. 2016; Cañas et al. 2018). In this context, constant stimulation of immune receptors by MVs released by beneficial gut microbes may result in controlled basal inflammation that contributes to appropriate defence and immune responses against pathogens and, ultimately, to intestinal homeostasis (Cañas et al. 2018; Molina-Tijeras et al. 2019).

Components of microbiota MVs other than MAMPs can also modulate signal transduction pathways. As stated above, MVs from the commensal *B. thetaiotaomicron* carry InsP6 phosphatase. In addition to a role in dietary InsP6 metabolism, the bacterial enzyme can also modulate cellular functions and gastrointestinal physiology (**Figure 1b**). Upon internalization in IECs, MVs intracellularly deliver InsP6 phosphatase that interacts with the inositol signalling pathway of the host cell, leading to an increase in inositol-3-phosphate levels and the subsequent release of Ca^{2+} from intracellular stores. Thus, OMVs contribute to interkingdom cell-to-cell crosstalk triggering intracellular Ca^{2+}-signalling pathways in intestinal epithelial cells (Stentz et al. 2014).

The connection between gut commensal MVs and the host enteric nervous system has been reported for *L. rhamnosus* JB-1. In addition to immunomodulatory effects, released *L. rhamnosus* MVs mediate the functional effects of this commensal on peristalsis through nerve-dependent regulation of colon migrating motor complexes (**Figure 1b**). The modulation of this neuronal response by *L. rhamnosus* JB-1 MVs was observed in *ex vivo* experiments performed with colonic explants but not by direct neuronal stimulation. This finding highlights the role of IECs in inter-kingdom signalling between bacterial MVs and the enteric neuronal system (Al-Nedawi et al. 2015). The study reveals the ability of certain gut commensal MVs to communicate with local neurons indirectly through signals released by the intestinal epithelium. The nature of both the vesicular bacterial effector and the epithelium-derived signal remain unknown.

Other studies also point to the connection of microbiota MVs with the host metabolism. As stated above, metabolomic approaches have revealed that bacterial MVs contain a set of metabolites (Zakharzhevskaya et al. 2017). The metabolite content of *B. thetaiotaomicron* MVs was analysed and the putative role of the packaged metabolites were predicted by *in silico* approaches. This study revealed that *B. thetaiotaomicron* MVs are enriched with metabolites known to facilitate intestinal colonization, and interestingly with metabolites that can be incorporated into mouse metabolic pathways (Bryant et al. 2017). This is the first study showing that vesicles from a prominent gut commensal selectively contain metabolites that are useful for the mammalian host, although the specific effects on the host metabolism have yet to be confirmed (**Figure 1b**).

The RNA content of microbiota-derived MVs has been linked with regulatory functions affecting host epigenome and gene expression (Celluzzi and Masotti 2016). The first data on RNA sequences found in *E. coli* MVs revealed that the associated RNA is enriched in non-coding small RNA molecules (ncRNAs), which differ from bacterial intracellular RNAs (Ghosal et al. 2015). Interestingly, many extracellular ncRNA sequences align to the human genome, mostly in regions related with epigenetic mechanisms such as histone modification and chromatin remodelling or with cell-specific transcriptional control (Celluzzi and Masotti 2016). Changes in the epigenetic profile induced by the environment (diet, physical activity, drugs, etc) have a great impact on gene expression and disease development. In this context, delivery of bacterial ncRNA through MVs might be used as a mechanism to exert multiple effects on host gene expression, contributing to host health in the case of gut beneficial microbes, or the onset of diseases in the case of pathogens or imbalanced microbiota (**Figure 1b**). The role of bacterial small RNAs secreted through MVs in the dysregulation of host immune responses has been reported in pathogenic bacteria (Koeppen et al. 2016; Choi et al. 2017), suggesting that this may also be possible for microbiota derived MVs.

3 MVs in Microbiota-Host Interaction at the Intestinal Mucosa

3.1 Interaction with Intestinal Epithelial Cells

Internalization Pathway

Direct and indirect evidence proves that bacterial MVs are taken up by host epithelial cells. Most studies on this topic have been performed with MVs from Gram-negative pathogens. The uptake of pathogen-derived MVs into epithelial cells is mainly driven by endocytosis. This process involves invagination of the cell membrane and occurs through different pathways depending on the surface and cargo of the vesicles. Endocytic pathways can be classified into two main groups: clathrin-mediated endocytosis (CME) and clathrin-independent pathways that include lipid raft-mediated processes (O'Donoghue and Krachler, 2016).These pathways involve endosomal compartments with different surfaces that allow sorting of internalised vesicle cargo to various subcellular locations. CME depends on a complex protein network including clathrin and dynamin as key components (Vercauteren et al. 2010). Lipid rafts are dynamic membrane microdomains rich in cholesterol, sphingolipids and proteins such as caveolin or flotillin, and are associated with distinct internalization pathways that are cholesterol sensitive (O'Donoghue and Krachler 2016). The endocytic pathway of MVs depends on the presence of vesicular components (proteins, toxins or surface structures) that target the MVs to specific receptors in the host cell membrane (Kesty et al. 2004; O'Donoghue et al. 2017). More than one pathway mediates the internalization of some MVs, like those from *Helicobacter pylori* (Olofsson et al. 2014; Turner et al. 2018). Membrane fusion and micropinocytosis have also been implicated in the uptake of certain bacterial MVs (O'Donoghue and Krachler 2016). Micropynocitosis is an actin-driven process shown to be involved in the uptake of large MVs from *H. pylori* (Turner et al. 2018). Despite the structural differences between the membrane of bacterial vesicles and that of host eukaryotic cells, membrane fusion has been shown to direct entry of *Listeria monocytogenes* MVs into host cells (Jager et al. 2014). This mechanism has also been described for Pseudomonas aeruginosa MVs (Bomberger et al., 2009). An assay based on fluorescence resonance energy transfer has been used to study bacterial and host factors that determine the vesicle internalization pathway, kinetics and efficiency (O´Donoghue et al. 2017). One factor that has a great impact on the selection and kinetics of the entry route is the lipopolysaccharide O antigen present in the MV surface. Gram-negative MVs lacking the O antigen need surface protein receptors in the host cell membrane for their entry, whereas the presence of the O antigen allows MVs entry through receptor-independent uptake pathways (O'Donoghue et al. 2017).

In the first decade of the 21st century there have been numerous studies on the internalization pathway of pathogen-derived MVs in epithelial cells. The study of microbiota-derived MVs uptake started later and was principally focused on *Escherichia coli*, which is found as part of normal human gut microbiota. The first study on this topic was performed with MVs from the probiotic *E. coli* Nissle 1917 (EcN) and the commensal ECOR12 strains (Cañas et al. 2016). EcN is a good colonizer of the human gut with beneficial effects on intestinal homeostasis and microbiota balance. This probiotic strain belongs to the phylogenetic group B2, associated with virulent strains that cause extra-intestinal infections. In fact, EcN shares a common ancestor with *E. coli* uropathogenic strains. During evolution, EcN lost virulence

factors but preserved fitness factors that confer competence to survive in the human gut (Vejborg et al. 2010; Toloza et al. 2015). ECOR12 is an intestinal isolate that belongs to phylogenetic group A, which is mostly associated with non-pathogenic *E. coli* strains (Ochman and Selander 1984). Uptake analysis of fluorescent-labelled MVs in human intestinal epithelial cell lines in the presence of endocytosis inhibitors showed that EcN and ECOR12 MVs enter epithelial cells via CME (**Figure 2**). Disruption of lipid rafts and caveolae domains by cholesterol-sequestering agents have no effect on the vesicle uptake by HT-29 or Caco-2 cells, whereas vesicle internalization is severely impaired by CME inhibitors (Cañas et al. 2016). Following the intracellular trafficking through CME pathway, internalized MVs reach lysosomal compartments. Consistently intracellular MVs colocalize with clathrin and specific markers of early endosomes and lysosomes (Cañas et al. 2016). A study performed with an EcN *tolR* mutant that displays a hypervesiculating phenotype evidenced that typical MVs are internalized by epithelial cells, whereas aberrant membranous structures are not. Cryo-transmission electron microscopy analysis of isolated MVs from wild-type EcN and the *tolR* isogenic mutant showed substantial structural heterogeneity in EcN *tolR* samples. In addition to common MVs (outer membrane vesicles and outer-inner membrane vesicles) aberrant vesicular structures were observed. Analysis of MV uptake in Caco-2 cells evidenced that mutant-derived MVs exhibit lower internalization levels than wild-type MVs due to the reduced capacity of tolR-derived MVs to bind host cell membrane (Pérez-Cruz et al. 2016). These findings prove that EcN MVs interact with their target(s) in the host cell membrane, a key step before being taken up by epithelial cells through CME. In contrast, circularized broken membranes or artefacts generated during bacterial cell lysis are not properly targeted to these sites, and therefore cannot mediate the functional effects attributed to conventional MVs.

Effects on Cell Viability

In contrast to MVs from pathogenic *E. coli* strains MVs derived from EcN and ECOR12 do not affect cell viability nor promote oxidative stress, but they do reduce cell proliferation of intestinal epithelial cells. Flow cytometry analysis of epithelial cells showed that microbiota *E. coli* MVs promote S/G2 cell cycle arrest in HT-29 cells, an effect that is consistent with their inhibition effect on cell growth. Although these MVs have not been observed in the nuclei, EcN MVs specifically promote double-stranded breaks in host cell DNA. MVs from the commensal strain ECOR12 do not induce such lesions (Cañas et al. 2016). In the probiotic EcN, both genotoxic and immunomodulatory effects have been attributed to the non-ribosomal peptide colibactin (Olier et al. 2012). It is not known how this bacterial mediator is exported and targeted to the host cell. The fact that EcN MVs induce the same type of DNA lesions as colibactin suggests that colibactin could be delivered to mammalian cells by MVs. This emphasises the role of MVs derived from beneficial gut microbes in communication with the host. Secretion of colibactin through MVs is an open question that requires further study.

Activation of Cytosolic NOD Receptors

NOD1 and NOD2 cytosolic receptors sense PGN, a component of the bacterial cell wall. NOD1 detects D-glutamyl-meso-diaminopimelic acid, which principally exist in PGN of Gram-negative bacteria while

NOD2 detects muramyl dipeptide, which is common to Gam-negative and Gram-positive bacteria (Girardin et al. 2003a; Girardin et al. 2003b; Chamaillard et al. 2003). PGN interaction with NOD triggers receptor oligomerization, the initial step in the downstream signalling cascade that leads to recruitment of the specific kinase RIP2 (receptor-interacting protein 2) and the subsequent activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways that induce the expression of inflammatory genes (Inohara et al. 2000; Hasegawa et al. 2008; Allison et al. 2009). As indicated above, NOD receptors are essential in maintaining intestinal homeostasis. They are involved in defence responses against bacterial infection and in regulation of the intestinal inflammatory response to microbiota (reviewed Kaparakis-Liaskos 2015). Since gut microbiota is composed of non-invasive bacteria, a key matter is to decipher the mechanisms for PGN delivery into host cells. One mechanism involves PGN fragments released into the gut lumen during bacterial cell division, which can be internalized in epithelial cells by endocytosis or through oligopeptide transporters (Swaan et al. 2008; Philpott et al. 2014). Another pathway for intracellular PGN delivery is through MVs. This is a well-studied mechanism in Gram-negative pathogens. Studies performed with *H. pylori* revealed that MVs internalized through CME reach endosomal compartments, and that interaction of vesicle-associated PGN with NOD1 occurs at early endosomes. (Irving et al. 2014). Studies performed with EcN and ECOR12 MVs showed that this pathway is also effective for microbiota-derived MVs, which activate NOD1 signalling pathways in IECs (Cañas et al. 2018). In Caco-2 cells, both RIP2 inhibition and NOD1-specific siRNA knockdown (but not that of NOD2) decrease vesicle-mediated activation of NF-κB and subsequent expression of the pro-inflammatory cytokines IL-6 and IL-8 (**Figure 2**). Results concerning IL-8 secretion revealed that in addition to PGN, other MAMPs included in MVs can activate signalling pathways that lead to expression of this cytokine (Cañas et al. 2018). EcN and ECOR12 internalized MVs colocalize with NOD1, activate NOD1 aggregation, and promote association of NOD1 with early endosomes. Although MVs from both strains activate the NOD1 pathway, kinetics of NOD1 aggregation and NF-κB activation reveal significant differences between them. The cell response to internalized MVs is faster for the probiotic EcN than for the commensal ECOR12 strain (Cañas et al. 2018). This study reveals that MVs released by beneficial gut bacteria modulate NOD-mediated host immune responses and contribute to priming of the immune system.

Immune Modulation Through the Intestinal Epithelial Barrier

Studies performed with EcN and ECOR12 MVs also corroborate the role of MVs as an active mechanism used by beneficial gut bacteria to activate signalling pathways through the intestinal epithelial barrier, which result in modulation of immune responses at the intestinal mucosa (Fábrega et al. 2016). In this study several models were used to assess the crosstalk between bacterial MVs, intestinal epithelial cells and immune cells. This involved: (i) stimulation of human peripheral blood mononuclear cells (PBMCs) as a model of intestinal barrier disruption, where microbiota MVs interact directly with immune cells, (ii) apical stimulation of polarized Caco-2/PMBCs co-cultures as a model of healthy, undamaged intestinal mucosa, and (iii) normal human colon tissue as an *ex vivo* model that is more similar to *in vivo* gut conditions. To prove that MVs exert specific effects, stimulations with bacterial lysates were also performed in all these models. Analysis of cell responses revealed that bacterial MVs and lysates trigger the expression of immune

mediators in PBMCs. In contrast, only MVs induce expression and secretion of cytokines to the basolateral compartment in Caco-2/PBMCs co-cultures. In this model, the epithelial barrier made by differentiated Caco-2 cells prevents direct access of bacterial effectors to PBMCs. Under these conditions, MVs taken up by epithelial cells interact with immune receptors and activated epithelial cells in turn release soluble mediators that elicit a response in immune cells (**Figure 2**). This intercellular cross-talk was corroborated in human colonic explants. In this *ex vivo* model MVs trigger upregulation of MIP1α, IL-10, IL-8, IL-6 and TNF-α and downregulation of the pro-inflammatory cytokine IL-12 (Fábrega et al. 2016). Remarkably, values of the IL-10/TNF-α and IL-10/IL-12 expression ratio indicate that MVs from the probiotic EcN elicit better anti-inflammatory balance than ECOR12 MVs.

Additional analysis in human colonic explants revealed that EcN and ECOR12 MVs also promote upregulation of IL-22 and the antimicrobial peptide β-defensin-2 (Fábrega et al. 2016). These two mediators are interconnected. Epithelial cells are targets of IL-22, a cytokine mainly expressed by immune cells. In the gut, the local innate lymphoid cells in the lamina propria integrate microbiota-derived signals and regulate adaptive immune responses. In particular, IL-22 released by intestinal lymphoid cells helps to maintain the integrity of the epithelial barrier through several mechanisms, one of which is the induction of β-defensins (Nikoopour et al. 2015). In addition, these bacterial MVs elicit downregulation of genes encoding TGF-β and the membrane-anchored mucin 1 (MUC-1), both linked to IL-17 responses. TGF-β is a pleiotropic cytokine, whose inflammatory and regulatory activities depend on other cellular factors. It is known that TGF-β triggers differentiation of Treg cells (anti-inflammatory), but in the presence of IL-6 this factor can promote differentiation of T helper 17 (Th17) cells (pro-inflammatory) (Sanjabi et al. 2009). Cooperation between Th17 and Treg cells is essential to preserve intestinal homeostasis. Imbalances in these cell populations towards high production of Th17 cells results in IBD. Since the pro-inflammatory effects of TGF-β are linked to Th17 cell differentiation, downregulation of TGF-β promoted by the probiotic EcN is consistent with its effectiveness in the remission of ulcerative colitis by restoring the Th17/Treg balance (Fábrega et al. 2016). MUC-1 and TGF-β are overexpressed in several cancer types (Apostolopoulos et al. 2015). In this context, the ability of EcN and ECOR12 MVs to downregulate these markers links the beneficial effects of certain gut bacteria to cancer progression or treatment effectiveness, especially in immunotherapy-based strategies in which the individual response depends on gut microbiota (Vétizou et al. 2015, Gopalakrishnan et al. 2018).

Modulation of Tight Junction Proteins

The integrity of the intestinal epithelial barrier is critical in maintaining homeostasis. Adjacent IECs are connected by a set of proteins that establish tight junctions. The tight junction complexes include integral membrane proteins (occludin, several claudins, tricellulin and junctional adhesion molecules) and peripheral membrane proteins of the zonula occludens (ZO), which bind to claudins and act as scaffolds anchoring the transmembrane proteins to the actin cytoskeleton (Turner 2009). Claudins are a large family of tight junction proteins that regulate barrier integrity and paracellular permeability. Besides claudins that have a sealing function (like claudin-1 or claudin-14), other act as selective channels for ions or water (like claudin-2). Certain beneficial gut microbes positively influence barrier integrity by strengthening tight

junctions and reducing gut permeability. In some cases, these effects are mediated, at least partially, by secreted bacterial metabolites (Ewaschuck et al. 2008), proteins (Hering et al. 2014) or MVs (Alvarez et al. 2016, Chelakkot et al. 2017).

The probiotic EcN reinforce the intestinal epithelial barrier through upregulation and redistribution of the tight junction proteins ZO-1, ZO-2 and claudin-14 (Ukena et al. 2007, Zyreck et al. 2007, Hering et al. 2014). Induction of claudin-14 is mediated by the secreted protein TcpC, an immunomodulatory protein that inhibits the TLR4 signaling cascade (Hering et al. 2014). The contribution of EcN MVs to the regulation of tight junction proteins was studied in the intestinal epithelial cell lines T-84 and Caco-2 (Alvarez et al. 2016). This study included other *E. coli* strains of human intestinal origin such as ECOR12 (*tcpC* negative) and ECOR63 (*tcpC* positive). Secreted MVs and soluble factors were separated from culture supernatants of the wild-type strains and the isogenic EcN and ECOR63 *tcpC* mutants, and their effect on the epithelial barrier was assessed by measuring transepithelial resistance, and gene and protein expression analyses of several tight junction proteins in polarized cell monolayers. This analysis revealed that MVs from the commensal ECOR12 do not have any positive effect on these parameters. In contrast, both extracellular fractions (MVs and soluble factors) from EcN and ECOR63 promote upregulation of ZO-1 and claudin-14 without affecting the expression of ZO-2 (**Figure 3**). The strengthening barrier effects mediated by EcN and ECOR63 MVs are independent of TcpC, which is secreted as a soluble protein. In addition to tight junction proteins known to be regulated by EcN, this study also examined the leaky protein claudin-2. It is known that regulation of claudin-2 results in increased intestinal permeability (Luettig et al. 2015). Pathogens like Salmonella or microbial toxins increase claudin-2 expression to disrupt the intestinal epithelial barrier and facilitate bacterial invasion (Zhang et al. 2013; Liu et al. 2013), whereas some probiotic strains enhance the intestinal barrier by downregulating claudin-2 (Ewaschuk et al. 2008). The probiotic EcN and the intestinal isolate ECOR63 also exploit this mechanism to improve barrier function (**Figure 3**). In these strains, downregulation of claudin-2 is mediated by released MVs and soluble factors that have not been identified yet. The negative regulation of claudin-2 together with the positive regulation of the sealing proteins ZO-1 and claudin-14 exerted by EcN-secreted MVs contribute to the efficacy of this probiotic in the treatment of inflammatory diseases and intestinal infections.

The beneficial gut bacterium *Akkermansia muciniphila* also improves intestinal barrier function. *A. muciniphila* MVs have been shown to prevent barrier disruption and neutralize the increased gut permeability induced by LPS in Caco-2 cell monolayers (Chelakkot et al. 2018). Immunoblotting analysis revealed that these vesicles increase the expression of the tight junction protein occludin (**Figure 3c**). The mechanism involved in these vesicle-mediated effects is the phosphorylation of AMPK, a kinase involved in the regulation of tight junction assembly (Chelakkot et al. 2018). The ability of *A. muciniphila* MVs to improve gut permeability *in vivo* has also been evaluated in mouse models (see Section 3.3).

3.2 Interaction with Immune Cells

Direct interaction of bacterial MVs with cells of the host immune system has been widely studied for pathogen-derived vesicles. These vesicles activate immune cells via PRRs and, depending on the producing strain, act as pro- or anti-inflammatory mediators and, in some cases, subvert the immune system to promote

pathogen survival in the host. Although there is extensive information on the interactions and effects of beneficial gut microbes in immune cells, few studies are focused on their MVs. Strains of the genus *Bifidobacterium* elicit strain-specific effects on the maturation of DCs and their ability to polarize naïve $CD4^+$ T cells to an effector response (López et al. 2010). Specifically, the probiotic *Bifidobacterium bifidum* LMG13195 elicits a T regulatory (Treg) response. Analysis of several probiotic subcellular fractions on human DCs showed that isolated MVs activate DCs to promote differentiation of Treg functional cells ($FoxP3^+$) with a suppressor balance, supported by the highest IL-10/pro-inflammatory cytokines ratio (López et al. 2012).

The gut symbiont *Bacteroides fragilis* also induces Treg cells and mucosal tolerance. In this case the immunomodulatory molecule is PSA, which is secreted through MVs. Extracellular vesicles from this commensal are taken up by DCs in an actin-dependent manner and induce anti-inflammatory immune responses in cell co-cultures of mouse bone marrow-derived DCs (BMDCs) and naïve $CD4^+$ T cells (Shen et al. 2012). PSA delivered through MVs is sensed by DCs via TLR2. Activation of this receptor triggers signal transduction events that induce tolerogenic DCs, which promote development of IL-10 secreting Treg cells (**Figure 2**). MVs isolated from a defective PSA mutant failed to mediate these effects. A microarray-based transcriptomic analysis in BMDCs challenged with MVs from the wild-type or the ΔPSA mutant strain revealed that transcripts specifically regulated by PSA essentially correspond to genes known to be regulated in a TLR2 dependent manner. In addition, *B. fragilis* MVs elicit gene expression changes that are PSA-independent (Shen et al. 2012).

3.3 Immunomodulatory and Barrier Protective Effects in Animal Models of Human Diseases

Several *in vivo* studies in mouse models of IBD and food allergy have confirmed the immunomodulatory and barrier protective effects of gut microbiota-derived MVs (Shen et al. 2012; Kang et al. 2013; Kim et al. 2015; Fábrega et al. 2017). The term IBD encompasses chronic inflammatory disorders of the intestinal tract such as ulcerative colitis or Crohn's disease. These are multifactorial diseases that involve an imbalanced immune response to commensal gut microbes in genetically susceptible individuals, leading to inflammation and reduced intestinal epithelial integrity (Zhang and Li 2014). Dysbiosis is a common feature in IBD. For this reason, therapeutic approaches targeting modulation of the gut microbiota have been explored, and the therapeutic potential of certain beneficial gut bacteria has been confirmed in clinical trials or animal models of experimental colitis. Experimental colitis in mice can be chemically induced by rectal administration of trinitrobenzene sulphonic acid (TNBS) or oral treatment with dextran sulphate sodium (DSS).

Concerning *B. fragilis*, the immunomodulatory potential of PSA has been proved in the TNBS-induced colitis model. Oral feeding of purified PSA (Mazmanian et al. 2008) or *B. fragilis* MVs (Shen et al. 2012) ameliorates colitis progression by reducing animal weight loss, histological damage and inflammation. Consistently, mice treated with PSA-containing MVs display reduced leukocytic infiltration at the intestinal mucosa, downregulation of pro-inflammatory cytokines and increased IL-10 expression. In contrast, MVs isolated from a PSA-defective mutant fail to protect mice against TNBS-induced colitis (Shen et al. 2012). Since the effect of PSA on DCs is mediated through TLR2, the function of Gadd45α (Growth arrest and DNA-damage-inducible protein), a downstream factor of the TLR2 signalling pathway

that stimulates T cell responses, was analysed in *Gadd45α*$^{-/-}$ knockout mice. In contrast to wild-type mice, *B. fragilis* MVs do not protect *Gadd45α*$^{-/-}$ knockout mice from TNBS-induced colitis. Therefore, this study showed that MVs from this resident gut microbe prevent colitis development by essentially activating tolerogenic DCs (Shen et al. 2012).

The therapeutic efficacy of EcN on the remission of ulcerative colitis has been supported in several clinical assays (Losurdo et al. 2015) and experimental colitis models. Like viable probiotic suspensions, daily oral administration of EcN MVs significantly reduces DSS-induced weight loss and ameliorates clinical symptoms, mucosal injury and inflammation in the gut (Fábrega et al. 2017). Treatment with EcN MVs counteracts altered expression of cytokines and markers of intestinal barrier function. Several mechanisms are exploited by EcN vesicles to ameliorate disease progression. Regarding inflammatory markers of colitis, EcN MVs decrease the expression of several pro-inflammatory cytokines (IL-6, IL-8, IL-1β, TNF-α, IL-12 and IL-17) and counteracts the lower expression of IL-10 associated with DSS treatment. In addition, EcN MVs elicite compensatory effects on expression of the inflammatory enzymes cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) (Fábrega et al. 2017). Increased iNOS expression in infiltrating macrophages in the intestinal mucosa is a common feature in clinical IBD. The consequent excessive nitric oxide production results in tissue injury in IBD patients and colitic mice (Palatka et al. 2005). Consequently, reduction of iNOS expression by EcN MVs helps to attenuate colitis in MVs-treated mice (Fábrega et al. 2017). Concerning markers of intestinal barrier function, EcN MVs cannot counteract DSS-induced downregulation of ZO-1. These findings do not match the effects observed in *in vitro* models of epithelial barrier integrity (Alvarez et al. 2016), indicating that different regulatory mechanisms could be activated by EcN MVs in the presence of highly expressed inflammatory mediators. Reinforcement of the epithelial barrier can also be triggered by post-translational modification mechanisms that direct ZO-1 to the cell boundaries and allow its association with tight junction structures. The intestinal trefoil factor 3 (TFF-3), a bioactive peptide involved in epithelial protection and repair, is one of the mediators that promote ZO-1 redistribution from the cytosol to intercellular tight junctionsin intestinal epithelial monolayers without changes in ZO-1 protein levels. Expression of TFF-3 is downregulated in DSS-inflamed colonic tissue. Oral administration of EcN MVs restore the mRNA levels of TFF-3 to values similar to those of healthy mice, thus preserving the colonic mucosa against DSS-induced damage (Fábrega et al. 2017). In the context of tissue remodelling, the matrix metalloproteinases (MMPs) MMP-9 and MMP-2 are also relevant. Upregulation of MMP-9 in DSS-treated mice promotes tissue injury by disrupting intestinal tight junctions, which in turn results in increased intestinal permeability and subsequent inflammation (Nighot et al. 2015). In contrast, MMP-2 plays a protective role in maintenance of gut barrier function. Treatment with EcN MVs downregulates MMP-9 and tends to preserve MMP-2 expression, thus pointing to another mechanism used by EcN vesicles to protect intestinal barrier function in DSS-experimental colitis (Fábrega et al. 2017).

MVs released by the beneficial gut bacterium *A. muciniphila* also protect DSS-induced experimental colitis in mice. The beneficial effects of these vesicles have been evidenced by the preservation of body weight and colon length, and reduced infiltration of immune cells in the colonic tissue (Kang et al. 2013). The role of *A. muciniphila* MVs in the modulation of gut permeability has been examined in a mouse model of diabetes induced by a high-fat diet (HFD) (Chelakkot et al. 2017). There is

mounting evidence of a close association between intestinal permeability and metabolic diseases. Metagenomics studies have found diminished abundance of *A. muciniphyla* in patients with obesity and type 2 diabetes, and their fecal samples also contain less *A. muciniphila*-derived MVs than healthy controls (Chelakkot et al. 2017). The impact of orally administered MVs isolated from this gut symbiont in HFD-fed mice was compared with control mice fed a normal diet. Treatment with *A. muciniphila* MVs improved all the alterations caused by the HFD. Specifically, MVs reduced body weight gain and HFD-increased intestinal permeability, restored the intestinal barrier from HFD-induced damage, reduced the subsequent recruitment of immune cells, and increased the expression of the tight junction proteins downregulated by HFD (Chelakkot et al. 2017). Consistently, improvement of the intestinal barrier function by *A. muciniphila* MVs results in less endotoxemia and improved glucose tolerance in HFD-fed mice.

Food allergy, a disease with increasing incidence over the last decade, results from abnormal immune responses to food components. Apart from avoiding allergenic foods, administration of probiotics has been proposed as an alternative treatment for patients with food allergy. In this context, the therapeutic potential of by *Bifidobacterium longum* has been evaluated in a mouse model of allergen-induced food allergy that causes acute diarrhoea. Allergy was induced by intraperitoneal injection of ovalbumin in aluminium potassium sulphate adjuvant followed by oral administration of the allergen for several days (Kim et al. 2015). During the induction of food allergy, animals were orally treated with two resident gut bacteria, either *B. longum* or *Enterococcus faecalis*. Only *B. longum* alleviated diarrhoea without counteracting the allergen-induced Th2 response. The specific mechanism used by this probiotic to limit inflammation is the reduction of mast cell number in the small intestine. *B. longum*-derived MVs selectively induce apoptosis of bone marrow-derived mast cells. The effector molecule that triggers cell-death is the main vesicular protein ESBP (family 5 extracellular solute-binding protein) (Kim et al. 2015).

4 Spreading of Microbiota MVs Through the Body

As stated above, gut dysbiosis has been associated with a wide range of diseases that affect distal body tissues (Maguire and Maguire 2018). Pathohistological effects have traditionally been attributed to the increased gut permeability that follows an imbalance in the microbiota. This condition, known as leaky gut, impairs epithelial barrier function, leading to the translocation of microbiota-derived products or luminal bacteria to the bloodstream. Once in the general circulation, these bacterial effectors can be distributed throughout the body and reach any tissue. First studies in this field connected LPS in human blood with endotoxemia, a condition that has been associated with obesity and insulin resistance (Cani et al. 2007). However, recent reports prove that gut microbiota-derived compounds are also found in blood, urine and distal tissues in healthy subjects (Clarke et al. 2010, Païssé et al. 2016, Lee et al. 2017). One example is bacterial PGN derived from gut microbiota, which crosses the blood-brain barrier and regulates brain development and behaviour through specific sensing molecules and activation of NOD signalling pathways in normal mice (Arentsen et al. 2017). Moreover, perturbation of gut microbiota leads to dysregulation of proteins involved in PGN detection in the developing brain (Arentsen et al. 2017). Bacterial genomic DNA has also been found in blood samples of healthy donors (Nikkari et al. 2001, Païssé et al. 2016). The mechanism by which this bacterial DNA reaches the bloodstream has been recently revealed in mice (Park

et al. 2017). This study showed that blood-associated bacterial DNA is transported by MVs, predominantly from gut microbiota. To reach the bloodstream, gut microbiota MVs have to cross intestinal epithelium and the vascular endothelium. This passage has been proposed to be mediated by paracellular or transcellular pathways (Stentz et al. 2018).

The blood-brain barrier efficiently regulates cellular and molecular trafficking between the blood and the neural tissue. The permeability of this barrier to circulating compounds increases under inflammatory conditions. In this context, studies based on sequencing approaches provide evidence on the presence of ribosomal DNA from commensal bacteria in the brain (Zhan et al. 2016; Emery et al. 2017). How this bacterial DNA comes from translocated bacteria or circulating MVs is an open question (Stentz et al. 2018).

The role of gut microbiota-derived MVs as vehicles for the distribution and delivery of many bacterial effectors to distal tissues is an emerging topic. Due to the existence of the gut-brain axis and the great impact of gut microbiota on neurological diseases (Marin et al. 2017, Van Den Elsen et al. 2017) such as depression, stress, anxiety, autism or Alzheimer's, studies on circulating microbiota-derived vesicles are principally focused on human patients or animal models of such diseases (Park et al. 2017; Lee et al. 2017). In this context, metagenomic analysis of MVs isolated from human urine samples has been proved an efficient method to assess changes in gut microbiota composition in autism. Sequencing of 16S rRNA gene variable regions in DNA samples extracted from bacterial MVs isolated from urine have revealed markedly altered microbiota profiles in people with autism disorder relative to healthy control individuals, even at the genus level (Lee et al. 2017). These differences correlate with the changes in gut microbiome previously described in autism, thus reinforcing the utility of urine MVs for diagnostic purposes. Likewise, in a mouse model of Alzheimer's disease great differences in the genomic profile of bacterial MVs isolated from blood have also been found compared to wild-type mice (Park et al. 2017).

There is growing evidence of the gut microbiome's influence on cancer onset, development and treatment (reviewed in Gopalakrishnan et al. 2018). However, very few studies have focused on the impact of microbiota-derived MVs. As stated above, MVs released in the GI tract by resident gut bacteria can be disseminated throughout the body, reach any tissue and induce either beneficial or harmful effects depending on the producer bacterial strain (Liu et al. 2018). The beneficial effects of MVs secreted by *Lactobacillus rhamnosus* on hepatic cancer cells have been assessed in HepG2 cell cultures. Specifically, MVs from the probiotic *L. rhamnosus* induce apoptosis in this cancer cell line (Behzadi et al. 2017).

5 Conclusions

We are currently living in the microbiome age. Clinical studies have provided insight into the influence of the microbiome on immunity and a wide range of diseases. However, there is still much to learn about the intrinsic mechanisms of this action. Further research is also required to develop optimal strategies to modulate gut microbiota for therapeutic purposes. Microbiota secreted MVs can deliver functional molecules to distant cells and, nowadays, are being revealed as key players in microbiota-host communication. Due to their nano-size structure and the great variety of cargo molecules, microbiota-derived vesicles can diffuse through the intestinal mucus layer and modulate host metabolism, immune and

defence responses. Consequently, microbiota derived MVs may have great influence on health and disease. Gut microbiome profiling is also seen as a potentially useful tool for diagnosis or improvement of the immunotherapy response. However, the method selected for sampling (stools or gut luminal content) and sequencing (metagenomics or 16SRNA sequencing) greatly affect the final output and reproducibility of the results. The recent discovery of gut-derived MVs in blood and urine samples from either patients or healthy individuals with a profile that strongly correlates with the real gut microbiome opens novel strategies for diagnostic purposes.

List of abbreviations

BMCDs: bone marrow-derived dendritic cells, CME: clathrin-mediated endocytosis, DCs: dendritic cells, DSS: dextran sodium sulphate, Gadd45α: growth arrest and DNA-damage-inducible protein, GI: gastrointestinal, HFD: high-fat diet, IBD: inflammatory bowel disease, IECs: intestinal epithelial cells, iNOS: inducible nitric oxide synthase, LPS: lipopolysaccharide, MAMPs: microbial-associated molecular patterns, miRNAs: micro-RNAs, MMP: matrix metalloprotease; MUC: mucin; MVs: membrane vesicles, ncRNAs: non-coding RNAs, NOD: nucleotide-binding oligomerization domain protein, PGN: peptidoglycan, PRRs: pattern-recognition receptors, PSA: polysaccharide A, RIP2: receptor-interacting protein 2, TFF-3: trefoil factor 3, TLRs: tol-like receptors, TNBS: 2,4,6-trinitrobenzene sulfonic acid, Th: T helper, Treg: regulatory T cells; ZO: zonula occludens

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Figure captions

Fig. 1 Overview of interspecies interaction in the gut mediated by microbiota-derived MVs. MVs are represented by circles and the specific molecules responsible for the known effects on (a) gut colonization and (b) microbiota-host communication are indicated inside. *Arrows* link each vesicular mediator with the specific role and the producer bacterial strain (in red). *Bacteria* are represented by elipses and *complex polysaccharides* by chain-connected triangles. A *cross* inside a bacterium indicates inhibitory activity on cell growth. *IECs*: intestinal epithelial cells, *SCFA*: short-chain fatty acids, *InsP6*: inositol phosphates, *MAMPs*: microbial-associated molecular patterns, *PRRs*, pattern-recognition receptors, *sRNAs*: small RNAs, *EcN*: *E. coli* Nissle 1917, AM: *Akkermansia muciniphila, LP: Lactobacillus plantarum*

Fig. 2 Schematic representation of immunomodulatory effects elicited by microbiota-derived MVs in the gut. The drawing shows the structure of the intestinal barrier in which the mucin layer maintains segregation between luminal microbes and the intestinal epithelium. *Gut microbiota* (represented by elipses) reside in the outer mucus layer. *MVs* (small black circles) can diffuse through the *inner mucus layer* (dotted lines) and reach the epithelium. Immune cells (*lymphocytes, macrophages and dendritic cells*) in the lamina propria are shown below the epithelial monolayer. Microbiota MVs exert immune modulation through two main mechanisms. Undirect activation of inmmune cells through the intestinal epithelium. MVs from EcN and ECOR12 are taken up by intestinal epithelial cells (*IECs*) and trigger secretion of *epithelial mediators* (asterisks), which in turn activate underlying immune cells that secrete a wide range of cytokines. Activation of the NOD1 signalling pathway by internalized EcN and ECOR12 MVs is presented in more detail. MVs enter IECs by clathrin mediated endocytosis and recruit *NOD1* (dark blue cylinders) to early endosomes. Activated NOD1 interacts with the specific kinase *RIP2* (red hexagons), leading to NF-kB activation and its translocation into the nucleus. This transcription factor upregulates host genes involved in the inflammatory response (IL-6, IL-8). Direct activation of dendritic cells (*DCs)* by MVs from *B. bifidum* and *B. fragilis* promotes a Treg response that contribute to immune tolerance. Activation of IECs and immune cells by microbiota MVs provides a controlled physiological mechanism for priming of the innate immune system.

Fig. 3 Modulation of the gut epithelial barrier by microbiota MVs. The drawing shows the structure of the intestinal barrier in which the mucin layer maintains segregation between luminal microbes and the intestinal epithelium. *Gut microbiota* (represented by elipses) reside in the outer mucus layer. *MVs* (small black circles) can diffuse through the *inner mucus layer* (dotted lines) and reach the epithelium. Interaction of MVs from EcN, ECOR63 and *A. muciniphila* with intestinal epithelial cells (*IECs*) elicit transcriptional regulation of genes encoding tight junction (*TJ*) proteins. Upregulation is indicated by (+) and downregulation by (-). MVs from EcN also induce expression of the antimicrobial peptide *β-defensin*. The regulatory effects mediated by microbiota MVs on TJ proteins lead to the reinforcement of gut epithelial barrier and, consequently, to a reduction in paracellular permeability.

Figure 1

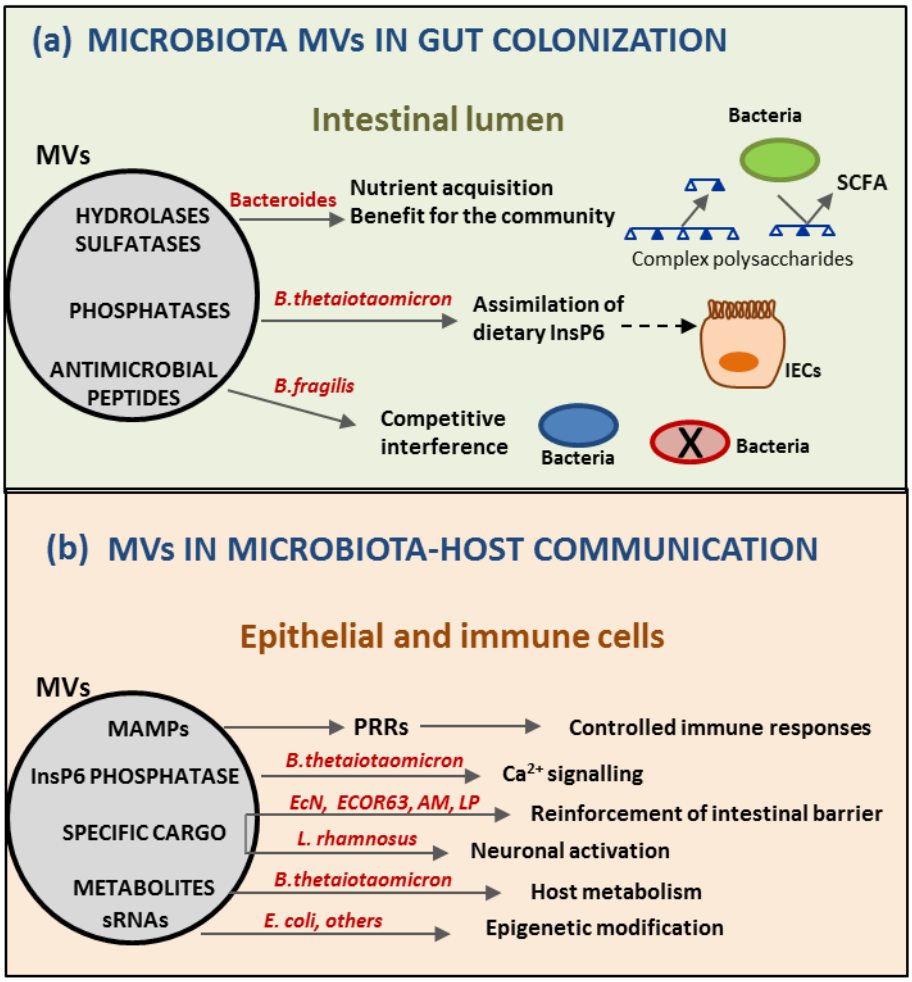

(a) MICROBIOTA MVs IN GUT COLONIZATION
Intestinal lumen
MVs
HYDROLASES
SULFATASES
PHOSPHATASES
ANTIMICROBIAL
PEPTIDES
Bacteroides
Nutrient acquisition
Benefit for the community
Bacteria
SCFA
Complex polysaccharides
B.thetaiotaomicron
Assimilation of
dietary InsP6
IECs
B.fragilis
Competitive
interference
Bacteria
Bacteria
(b) MVs IN MICROBIOTA-HOST COMMUNICATION
Epithelial and immune cells
MVs
MAMPs
PRRs
Controlled immune responses
InsP6 PHOSPHATASE
B.thetaiotaomicron
Ca2+ signalling
SPECIFIC CARGO
EcN, ECOR63, AM, LP
Reinforcement of intestinal barrier
L. rhamnosus
Neuronal activation
METABOLITES
B.thetaiotaomicron
Host metabolism
sRNAs
E. coli, others
Epigenetic modification

Figure 2

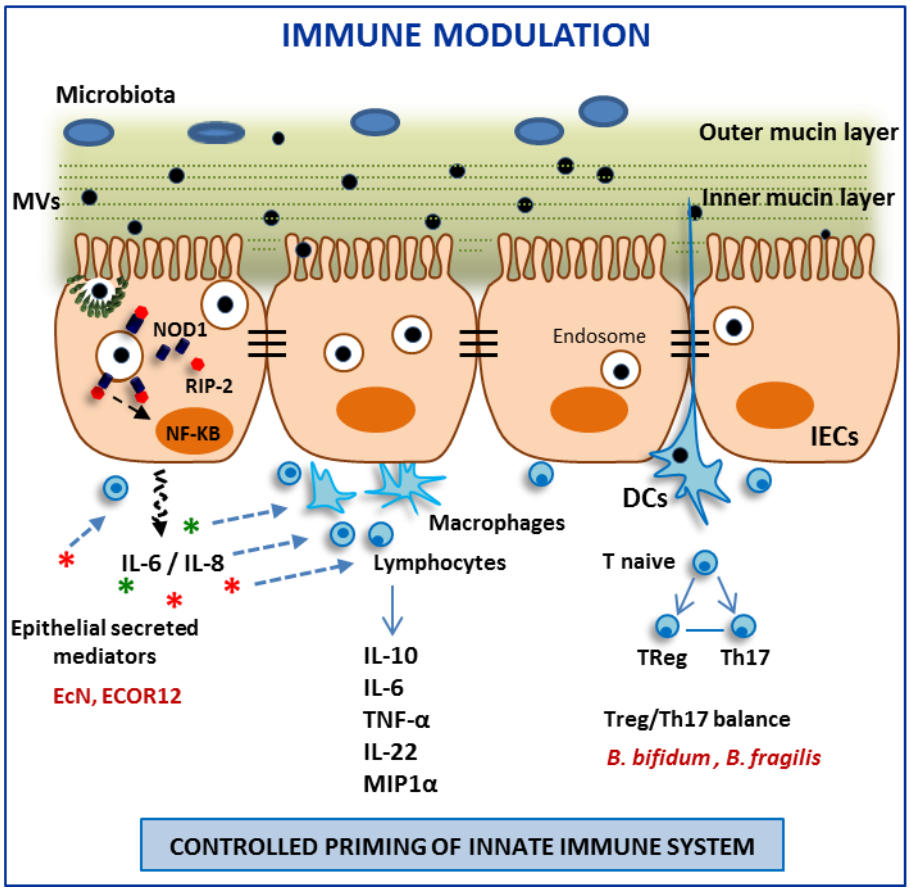
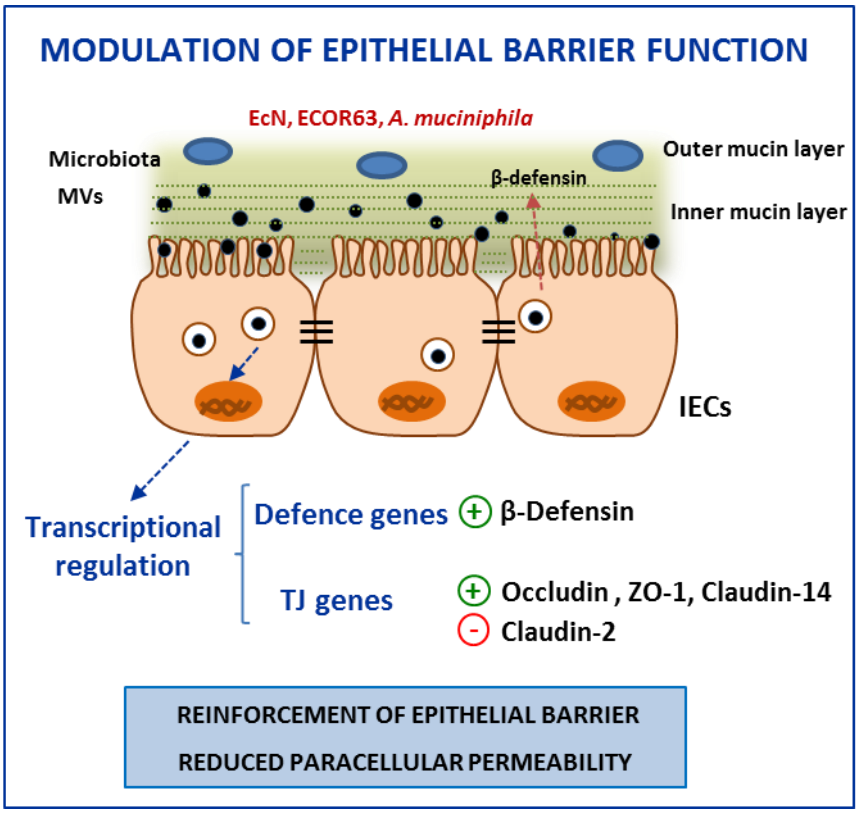

IMMUNE MODULATION
Microbiota
Outer mucin layer
MVs
Inner mucin layer
NOD1
RIP-2
NF-KB
Endosome
IECs
DCs
Macrophages
IL-6 / IL-8
Lymphocytes
T naive
Epithelial secreted mediators
EcN, ECOR12
IL-10
IL-6
TNF-α
IL-22
MIP1α
TReg
Th17
Treg/Th17 balance
B. bifidum , B. fragilis
CONTROLLED PRIMING OF INNATE IMMUNE SYSTEM

Figure 3


MODULATION OF EPITHELIAL BARRIER FUNCTION
EcN, ECOR63, A. muciniphila
Microbiota
MVs
Outer mucin layer
β-defensin
Inner mucin layer
IECs
Transcriptional regulation
Defence genes
β-Defensin
TJ genes
Occludin , ZO-1, Claudin-14
Claudin-2
REINFORCEMENT OF EPITHELIAL BARRIER
REDUCED PARACELLULAR PERMEABILITY