Gut Microbiota Extracellular Vesicles as Signaling Carriers in Host-Microbiota Crosstalk

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Abstract

Microbiota-released extracellular vesicles (MEVs) have emerged as key players in intercellular signaling in host-microbiome communications. However, their role in gut-brain axis signaling has been poorly investigated. Here, we performed deep multi-omics profiling of MEVs generated ex-vivo and from stool samples to gain insight into their role in gut-brain-axis signaling. Metabolomics unveiled a wide array of metabolites embedded in MEVs, including many neurotransmitter-related compounds such as arachidonyl-dopamine (NADA), gabapentin, glutamate, and N-acylethanolamines. To test the biodistribution of MEVs from the gut to other parts of the body, Caco-2, RIN-14B, and hCMEC/D3 cells showed the capacity to internalize labeled MEVs through an endocytic mechanism. Additionally, MEVs exhibited dose-dependent paracellular transport through Caco-2 intestinal cells and hCMEC/D3 brain endothelial cells. Overall, our results revealed the capabilities of MEVs to cross the intestinal and blood-brain barriers to delivering their cargo to distant parts of the body.

Keywords: gut microbiome, extracellular vesicles, multi-omics characterization, gut-brain axis

Résumé

Les vésicules extracellulaires secrétées par le microbiote (MEV) sont devenues un acteur clé de la signalisation intercellulaire entre le microbiome intestinal et l'hôte. Cependant, leur rôle dans la signalisation de l'axe intestin-cerveau est peu étudié. Dans cette étude, nous avons effectué un profilage multi-omique approfondi des MEV générées ex vivo et à partir d'échantillons de selles afin de mieux comprendre leur rôle dans la signalisation de l'axe intestin-cerveau. La métabolomique a révélé un large éventail de métabolites présents dans les MEV, y compris de nombreux composés liés aux neurotransmetteurs tels que l'arachidonyl-dopamine (NADA), la gabapentine, le glutamate et les N-acyléthanolamines. Pour tester la biodistribution des MEV traversant l'intestin vers d'autres parties du corps, les cellules Caco-2, RIN-14B et hCMEC/D3 ont montré une capacité à internaliser les MEV via un mécanisme endocytaire. De plus, les MEV présentaient un transport paracellulaire dose-dépendant à travers les cellules intestinales Caco-2 et les cellules endothéliales cérébrales hCMEC/D3. Dans l'ensemble, nos résultats démontrent la capacité des MEV à traverser les barrières intestinale et hémato-encéphalique pour acheminer leurs cargaisons vers les organes du corps.

Mots-clés : microbiome intestinal, vésicules extracellulaires, caractérisation multi-omique, axe intestin-cerveau.

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CHAPTER 1: General introduction

Accumulating evidence supports communication between gut microbiota and mental health [1, 2]. Gut microbiota dysbiosis has been linked to many behavioral and mood disorders, such as autism spectrum disorders, anxiety, and depression [1, 3, 4]. Therefore, the modulation of dysbiotic microbiota via microbial therapeutics may help restore or maintain normal microbiota composition and activity in these disorders [5, 6]. Many strategies have been developed to manipulate gut microbiota composition, such as fecal microbiota transplantation (FMT), prebiotics, and probiotics [6]. The effect of FMT is still controversial [7], with many limitations, including resistance to colonization, adverse effects, donor selection, sample handling, route of administration, and cost-effectiveness [8]. However, probiotic and prebiotic approaches have shown some success as microbiota management tools in various diseases [9, 10]. For example, some psychobiotics (probiotics conferring mental health benefits), such as *Lactobacillus helveticus* and *Bifidobacterium longum*, have been reported to reduce anxiety-like behavior in animals and demonstrated positive psychological effects and decreased serum cortisol levels in humans [11]. However, the mechanistic understanding of this microbiota-nervous system crosstalk and how these microbes impact mental health or disease-related functions is still lacking. The gut microbiota produces a variety of neurotransmitters, including γ-aminobutyric acid (GABA), serotonin, and dopamine, that contribute to gut-brain axis signaling [12]. Recently, GABA was identified as an essential growth factor that solely can induce the growth of unculturable gut microorganisms [13]. The same study illustrated that depression brain signatures are

negatively correlated with known GABA producers, suggesting a potential influence of microbial-derived GABA on the host brain [13]. Another host-microbial communication route is the generation of extracellular vesicles, which are small vesicles of DNA, RNA, and proteins inside a phospholipid membrane [14]. These vesicles act as vectors that transfer information to target cells and are involved in processes such as quorum sensing, biofilm formation, relief of environmental stresses, and host immunomodulation [15]. The production and role of MEVs released by probiotic and commensal microbes in the gut environment are poorly investigated [16], as MEVs have been predominantly examined in pathogenic strains [13, 14, 17]. Kang et al. [18] reported an important shift in stool MEV composition compared to the microbiome in an IBD DSS mouse model. Moreover, the same authors observed an attenuating effect of *Akkermansia muciniphila*-derived MEVs on colitis severity [18], suggesting the potential of MEVs as biomarkers and therapeutic agents. Interestingly, the relative abundance of main MEVs producers, such as *Akkermansia* and *Faecalibacterium*, was negatively correlated with depressive-like behaviors in mice [19, 20]. A recent report on increased levels of systemic LPS-positive bacterial extracellular vesicles in patients with intestinal barrier dysfunction [21] provides some evidence of the capacity of MEVs to reach the systemic circulation and deliver and elicit a variety of immunological and metabolic responses in different organs, including the brain. Therefore, MEVs might be an important delivery vehicle for host-modulating metabolites and thus can be regarded as promising psychobiotic candidates compared to the clinical and regulatory limitations faced by fecal

transplantation. Harnessing the MEVs production ability of gut microbiota could help decipher their interaction with the gut–brain axis.

CHAPTER 2 : Gut Microbiota Extracellular Vesicles as Signaling Molecules Mediating Host-Microbiota Communications

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2.1. Introduction

Gut microbiota is the most significant microbial ecosystem in the human body. Its huge gene content and diversity enable this ensemble to exhibit many beneficial functions to the host, including nutritional, physiological, and immunological roles that collectively contribute to human health [22–24]. The host-gut microbiota crosstalk has been extensively reported for multiple health and disease statuses [25–32]. This bidirectional communication is thought to be mediated through metabolic, immunological, endocrine, and neuronal pathways [33]. Recently, a new communication channel through secreted microbiota extracellular vesicles (MEVs) started appearing. It is commonly believed that the communication between Gram-negative bacteria and the host is mediated by secreted vesicles known as outer membrane vesicles (OMVs) [34]. Gram-positive bacteria have also been reported to generate EVs [35]. In 2013, Kang et al. [36] characterized the microbiota-derived EVs in mice stools. They illustrated that stool MEVs from an IBD mouse model exhibited severe dysbiosis compared to the change in the microbiota composition between the inflammation and control phenotypes [36]. While it is unclear whether this dysbiosis is a consequence or a cause of inflammation, this study illustrates that EVs play a regulatory role in intestinal immunity and homeostasis [36]. For instance, the EVs of the gut microbe *Akkermansia muciniphila* protected the mice from developing colitis and lowered the production of the proinflammatory cytokine IL6 in response to *E. coli* treatment [36]. Additionally, the *A. muciniphila* EVs were reported to induce serotonin secretion in both the colon and hippocampus of mice, suggesting MEVs potential as signaling molecules in the gut-brain axis [37]. A more recent report has shown that MEVs may cross intestinal barriers and reach distal organs such as the liver and adipose tissues, inducing insulin resistance and glucose intolerance [38]. EVs derived from *Lactobacillus plantarum* have exhibited an antidepressant-like effect [39]. Collectively, this supports the hypothesis that gut

microbiota-derived EVs may act as inter-bacterial and host-microbe signaling pathways that regulate intestinal homeostasis and human health, even in distal organs (Figure 1). In this review, we discuss the biogenesis of both the host- and microbiota-derived EVs. We will focus on the microbiota-derived vesicles and their roles in inter-bacterial signaling and host-microbiota interactions. This will cover only one direction of communication from the microbiota towards the host. The other direction of the crosstalk will be covered in a future review.

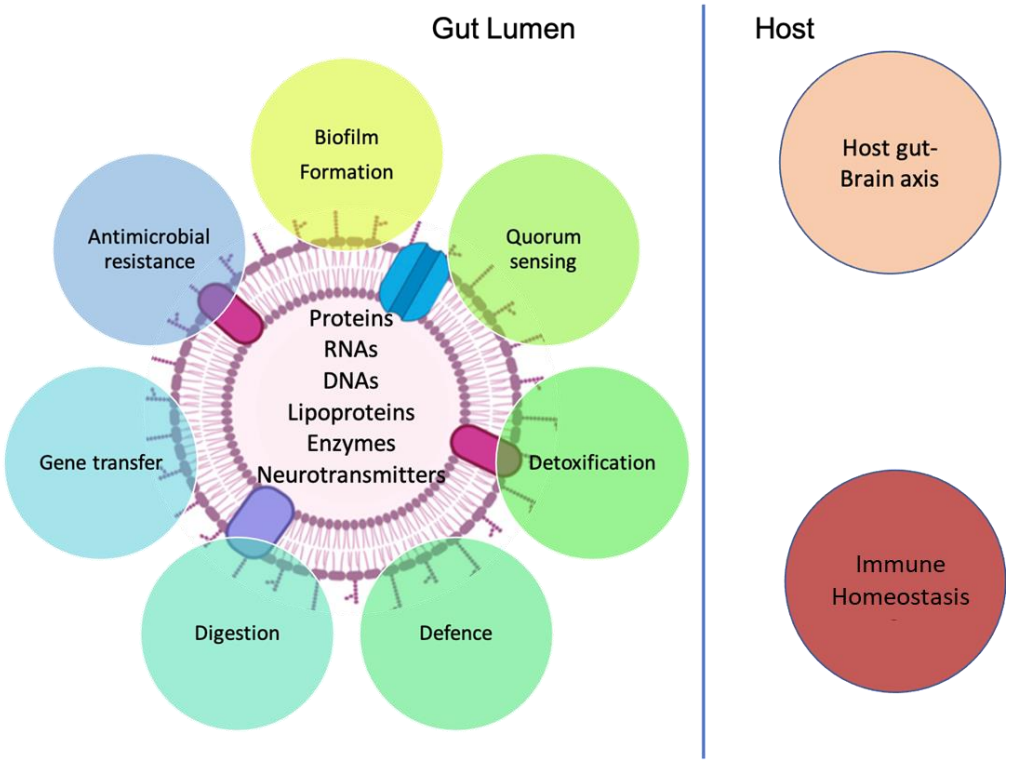

Figure 1. Roles of MEVs in interbacterial and microbiota-host signaling. Microbiota extra vesicles (MEVs) contribute to the communication between gut commensals including transfer of antimicrobial resistance genes [40], horizontal gene transfer [41], biofilm formation [42], quorum sensing [43], detoxification [44], and digestion. Also, MEVs and their cargoes induce immune homeostasis [45, 46] and act as a communication approach in gut-brain axis[47, 48].

2.2. Gut Microbiota

Gut microbiota refers to a collective complex, dynamic microbial community along the gastrointestinal tract's length (GIT) that reaches its maximum density at the colon [49]. This

ensemble of microbes includes bacteria, viruses, archaea, and eukaryotes [50, 51]. The gut microbial gene content was estimated to be 150-fold that of humans, and more than 99% of these genes belong to bacteria [50]. Approximately 1,150 bacterial species have been identified in the human gut, with an average of 160 species per individual [50]. Gut bacteria are dominated by the two phyla, Bacteroidetes and Firmicutes, which constitute more than 70% of the gut bacteria with low proportions of phyla like Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia [52].

Gut microbiota plays critical roles in human health. They perform as a significant factor in shaping and evolving the immune system [23]. They also metabolize indigestible plant fibers to generate essential metabolites such as short-chain fatty acids (SCFAs) [24]. Major SCFAs producers include Clostridial clusters IV and X1Va, *Bacteroides,* and *Bifidobacterium* [53]. The major microbiota-generated SCFAs include butyrate, propionate, and acetate, where colonocytes mainly utilize butyrate as the primary energy source while acetate and propionate act as substrates of lipogenesis and gluconeogenesis in peripheral tissues [54, 55]. Additionally, the integrity of the intestinal barrier is controlled by SCFAs. For example, the butyrate upregulates the expression of tight junction-associated proteins [56]. In addition to colonic fermentation of dietary fibers, gut microbiota interacts with other host metabolic processes such as regulation of bile acid metabolism, metabolism of choline, and insulin resistance [57].

Microbiota-host interaction involves not only host sensing of bacterial metabolites but also direct interaction with the bacteria. This last point is particularly confusing since most bacteria are physically separated from the host by the mucus layer. Moreover, live bacteria's effects are often different from those of heat-killed bacteria, suggesting that bacterial membrane components' recognition is more than just a passive interaction. Shen et al., 2012 first demonstrated the phenomenon by showing that commensal bacteria produce EVs [58].

They reported that the administration of EVs isolated from *Bacteroides fragilis* simulated similar benefits compared to administering the bacteria itself. This finding, soon followed by others, opens a new dimension in understanding how gut bacteria affect host homeostasis and, importantly, understand the systemic and distal impact of gut bacteria on the host. This review will discuss our current knowledge regarding the functions of microbiota-derived EVs on the host's health as a shuttle for transferring bioactive cargoes (i.e., proteins, mRNA, miRNA, DNA, carbohydrates, and lipids) and potential role as signaling pathways as well (Figure 1).

2.3. Gut Microbiota-Derived Extracellular Vesicles

As a part of the communication process between organisms, the gut microbiota produces small bodies called microbial extracellular vesicles (MEVs). They carry the message of antibiotics' resistance to the surrounding bacteria [40, 59]. Moreover, they act as an efficient system for detoxification of unfavourable components to bacterial growth[44]. Bryant, W.A, 2017 suggested that commensal bacteria-derived vesicles could contribute to colonization in the gastrointestinal tract [60].

2.3.1. Biogenesis

Bacteria are categorized into two classes according to their outer membrane nature: gram-negative (G-) and gram-positive (G+) bacteria. G- bacteria are characterized by a double plasma membrane separated by periplasm. Vesicles are arising from outer membrane blebbing of G- bacteria are called Outer-Membrane Vesicles (OMVs) [61]. They carry periplasmic contents such as lipoproteins, lipids, and outer membrane proteins [62]. Furthermore, some pathogenic G- bacteria produce another type of vesicles called Inner Outer Membrane Vesicles (IOMVs). They contain pieces from both cytoplasmic and periplasmic membranes and are enriched with ATPs and DNA [63]. Three models demonstrating the OMVs' production were reviewed by C. Volgers and his team in 2018

[64] (Figure 2). These models suggest that the production technique maintains the outer membrane homeostatic state. Accordingly, the OMVs are produced when the outer membrane asymmetry is achieved (Model A), misfolded proteins are condensed in the outer membrane (Model B), and lipopolysaccharides modification (Model C). The outer membrane of G- bacteria is characterized by asymmetric distribution of lipids with lipopolysaccharides in the outer side and phospholipids in the inner side of the membrane [65]. The defect in this asymmetric distribution will lead to increased microbial vesiculation (Model A). Also model A can be achieved by reduced interactions between the outer membrane lipids and the peptidoglycan layer [66]. Genes involved in this model include genes encoding the proteins associated with the peptidoglycan layer such as Oprl, OmpA, Pal or TolA in *Pseudomonas aeruginosa* [67], TolA, TolQ, and Tol/Pal in *E. coli* [68, 69], OmpA in *Acinetobacter baumannii* [70], and the ABC-transporter VacJ/YrbC in *Haemophilus influenzae* and *V. cholerae* [71] and its homolog Mla in *E. coli* [65]. Deletion or under-regulation of these genes will reduce the interaction between the peptidoglycan and the outer membrane that grows faster and increases the microbial vesiculation [71]. The second model (B) suggests that accumulation of misfolded protein or peptidoglycan fragments presses on the outer membrane and results in protrusion of the membrane and vesicle generation. This can be triggered by temperature stress or defect in cell wall remodeling [71]. The third model is specific to *Ps. aeruginosa* until now that assumes enrichment of membrane curvature inducing molecules such as B-band lipopolysaccharide and the quinolone PQS. PQS is hypothesized to induce anionic repulsion among the membrane lipopolysaccharides and form a stable salt bridge between the negatively charged B-band lipopolysaccharide and cationic salts which results in a membrane curvature and asymmetric expension of the outer leaflet of the membrane compared to the inner one [72].

Since G+ bacteria exhibit a different nature of the cell membrane, one thick layer of peptidoglycans, they were not considered extracellular vesicles producers until the revelation of MEVs from *Staphylococcus aureus* by Lee EY et al., 2009 [73]. In addition, the studies by Rivera J. et al., 2010 and Jeon J. et al., 2017 have reported the production of MEVs by *Bacillus anthracis* and *Cutibacterium acnes* [74, 75]. Different mechanisms are utilized by G+ bacteria to release MEVs when compared to the G- bacteria (Figure 2). In a sense, to push the vesicles through the thick membrane by turgor pressure, protease lysis, or protein channels [76]. Genetic regulation of the vesiculation in G+ bacteria is proven for the general regulators *sigB* and two-component systems [77, 78]. The EVs formation in *Staphylococcus aureus* relies on phenol-soluble modulins which is an amphipathic alpha-helical peptide that disrupts the cytoplasmic membrane, in addition to a reduction in the peptidoglycan cross-linking [79]. The reduction in the peptidoglycan crosslinking suggest a role for cell wall modifying molecules such as penicillin binding protein and autolysins in EVs biogenesis. This is supported by detecting these molecules in EVs as revealed by mass spectrometry [80]. Finally, the differences between the phospholipids between the EVs and their parental cells indicates that EVs are generated at specific locations [81].

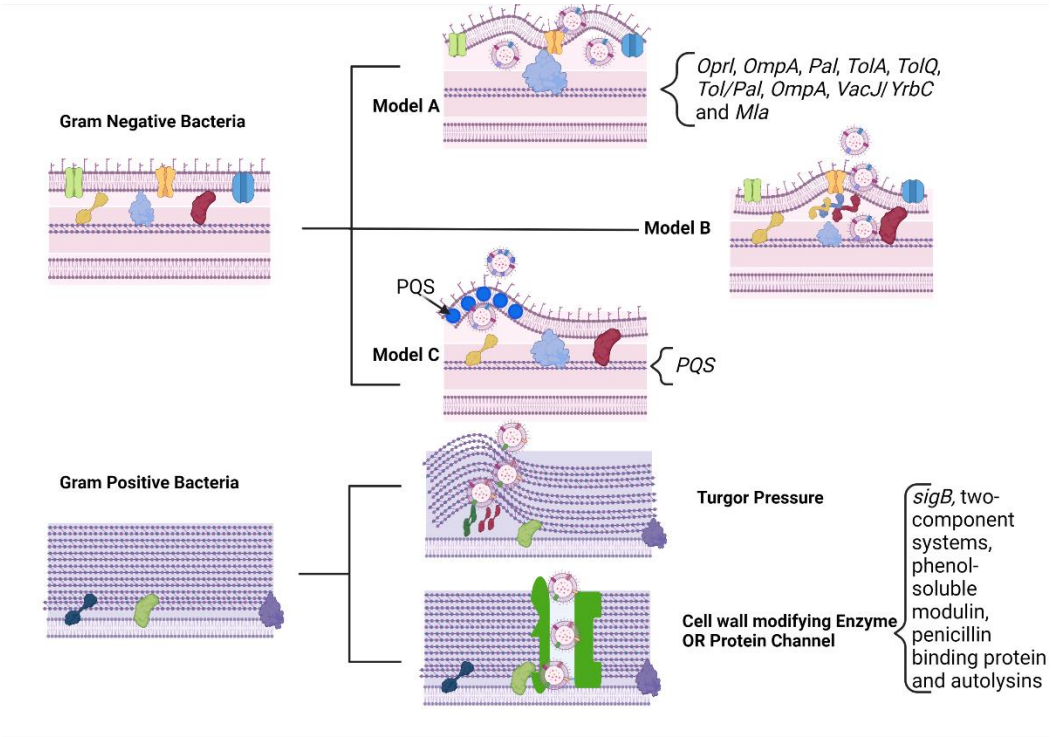

Figure 2. Biogenesis of EVs from both Gram-negative and Gram-positive bacteria. Gram negative OMVs are produced when the outer membrane asymmetry is achieved (Model A), misfolded proteins are condensed in the outer membrane (Model B), and lipopolysaccharides modification (Model C)[82]. On the other hand, Gram positive bacteria may vesiculate following a turgor pressure or via the action of cell wall modifying enzymes or protein channel [81] . Figure is created with BioRender.com

2.3.2. Biomarkers

Extracellular vesicles (EVs) are abundant in all body fluids, including plasma, saliva, urine, semen, cerebral spinal fluid (CSF), bronchial fluid, breast milk [83]. They can readily cross the physiological barriers due to their good stability and small dimensions [84]. That is why they are considered a beneficial source for biomarkers in circulation [85]. Since they are enriched by molecular contents such as nucleic acids, lipids, and a wide collection of proteins [86, 87], this features the MEVs as implications in many disorders including neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [88–90]. Combes et al. (2004) demonstrated the correlation between EVs and the occurrence of some neurological syndrome-like cerebral malaria [91]. Furthermore, the normalization of these vesicles during the recovery period suggests their potential as biomarkers of disease

intensity. More recently, carbohydrase 1 (CA-1) and S100A8 were identified through proteomics analysis as cargoes of EVs in cerebral malaria syndrome. They are specifically increased during the pathogenesis enforcing the concept of these molecules as biomarkers in malaria [92]. Cancer [93–95] and stroke [96] have also been reviewed to show the potential of EVs as biomarkers.

Notably, EVs' contents are present in different originating forms, either as components from the parent cells or membrane-associated particles. During the biogenesis process of the EVs, different cargoes (i.e., mRNA, DNA, proteins, lipids, etc.) are packed into the vesicles, and this could be used as surrogate indicators of parent cells to provide specific cell-origin biomarkers [97, 98].

Three main mechanisms, that can describe the way of MEVs interact with their host, were described by O'Donoghue et al. (2016) [99]: (i) full in corporation in the host's cytoplasm, (ii) activation of the host's receptors, and (iii) the delivery of their bacterial content (Figure 3). More focus was added to study the activation of the host's receptors by pathogen-associated molecular pattern (PAMP) induced by pathogenic bacteria other than commensal strains [100].

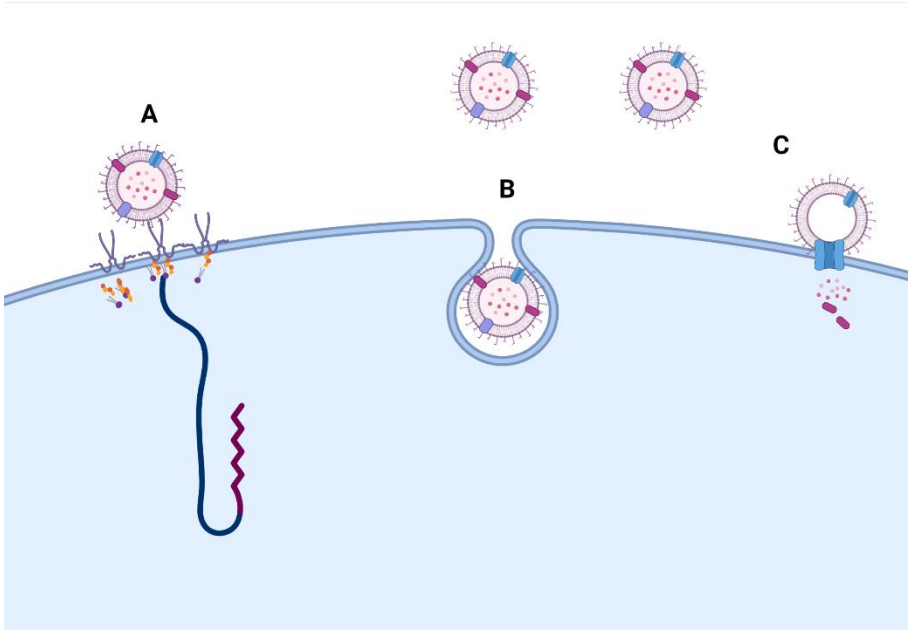

Figure 3. Routes of MEVs entry into the host cells. MEVs may interact with the host cells by either (A) binding with the cell receptor and activation of a cellular response; (B)Full incorporation with the cellular cytoplasm; or (C) Delivery of their content to the host cell [99]. Figure is created with BioRender.com

2.3.3. MEVs and cellular communication

2.3.3.1. Role in inter-bacterial signaling.

EVs have shown a variety of roles in bacterial crosstalk (Table 1). *Haemophilus influenza* generates and receives DNA-containing EVs in which EVs played a significant role in transferring DNA among bacteria by protecting it from nucleases [41], thus indicating a deep involvement of EVs in horizontal gene transfer in inter-bacterial communication. EVs released from *Bacteroides* possess β-lactamases that protect gut commensals and pathogens from β-lactam antibiotics [101]. Bacterial EVs also represent a means of detoxifying harmful molecules, including misfolded proteins, toxic materials, and viral particles [44, 102–104]. Additionally, bacteria EVs play essential roles in bacterial quorum sensing. For example, EVs from *Ps. aeruginosa* contain pseudomonas quinolone signal molecules, enabling *Ps. aeruginosa* to live in nutrient-poor environments [105].

Within the gut lumen, gut microbiota-derived EVs act as delivery vehicles for digestive enzymes, including glycosidases and proteases that hydrolyze the complex polysaccharides into simple nutrients for other commensals in the gut [106, 107].

EVs contribute to the formation of biofilms, such as *H. pylori* and *Ps. aeruginosa* [108, 109]. Moreover, EVs could be a valuable means of protecting other/neighbour strains of bacteria by enveloping toxic compounds inside vesicles. For instance, some strains of *Sulfolobus* release EVs containing sulfolobicin toxin that can kill other different strains even within the same genus [110]. In contrast, EVs derived from certain bacterial strains possess antimicrobial activities against competitor microbes. Wang and his co-authors have recently illustrated that *Burkholderia thailandensis* releases outer membrane vesicles (OMVs) with antimicrobial activities against drug-resistant and competitor microbial species, including methicillin-resistant *Staphylococcus aureus* (MRSA) [111]. A similar antimicrobial effect has been reported previously to the OMVs released by *Ps. aeruginosa* [112].

An important role of EVs in molecular exchange between bacterial cells is their role in phage transfer between bacterial cells [113]. Presence of prophages has been shown to induce *S. aureus* vesiculation compared to prophage-devoid cells [114]. Initially, EVs has been viewed as antiphage protector through lowering the phage concentration by adsorption. For instance, the efficiency of T4 bacteriophage infection was reduced by binding to OMVs of E. coli [115]. The same study has revealed the role of OMVs in innate bacterial defense by neutralizing antimicrobial peptides [115]. Additionally, EVs released by marine Cyanobacteria have been illustrated to defend marine bacteria against phage infection *through* sequestration of phages by EVs containing the phage receptors [116]. In contrast, a more recent study has illustrated that bacterial extra vesicles promote phage infection in phage resistant bacteria through sharing surface components including phage receptors or attachment molecules from phage sensitive cells to phage resistant cells [117].

Furthermore, bacteriophages were capable to inject their genetic materials in minicells that resemble EVs [118], indicating that EVs may facilitate the transfer of phage genetic materials between cells.

Table 1. Roles of bacteria EVs in inter-bacterial signaling.

Activity	Example source organism(s)	Example affected organism(s)	Reference
Horizontal gene transfer	*Haemophilus influenza*	*Haemophilus influenza*	[41]
Antimicrobial Resistance	*Bacteroides* spp. and *Haemophilus influenza* (β-lactamases)	Gut microbiota Group A streptococci	[40, 59]
Detoxification of harmful molecules and stress relief	*E. coli*, *Salmonella enterica* serovar Typhimurium		[44, 102–104]
Quorum sensing	*Ps. aeruginosa*	*Ps. aeruginosa*	[43, 105]
Digestive enzymes carrier	Gut microbiota	Gut microbiota	
Bacterial biofilm	*H. pylori* and *Ps. aeruginosa*	*H. pylori* and *Ps. aeruginosa*	[42, 108, 109]
Carrier of antimicrobial materials (survival)	*Sulfolobus spp.* *Burkholderia thailandensis*	Same species or drug-resistant and competitor species, including MRSA	[43, 110–112]

2.3.3.2. Role in interkingdom signaling.

Bacteria-derived EVs, especially those from gut microbiota, can cross eucaryotic cell membranes and intestinal cell walls [119]. Microbiota-derived vesicles can be phagocytosed by immune cells of lamina propria [58], and they can be detected in blood and urine [120]. DNA from bacterial origin has been detected in the serum of healthy subjects, which is known as DNAemia [121]. Indeed, bacterial DNA originated from bacteria membrane

vesicles was found in plasma [120]. This finding implies that the microbiota-generated vesicles can penetrate different barriers such as the intestinal epithelium and the vascular endothelium to reach distant locations inside the host. Two distinct pathways have been suggested for bacterial vesicles to cross the intestinal wall; the paracellular and transcellular pathways [119]. EVs can alter the composition of the tight junction through which they may enable the parental pathogen to invade the intestinal epithelium. For example, the vesicles from *Campylobacter jejuni* break down the junction proteins E-cadherin and occludin to enable *C. jejuni* invasion [122]. On the other hand, vesicles from commensal bacteria increase the expression of tight junction proteins to limit paracellular transport [123]. Also, the probiotic *Escherichia coli* Nissle 1917 strain generates outer membrane vesicles that regulate the expression of tight junction proteins, ZO-1 and ZO-2, in the intestinal epithelium cells [124]. Bacteria-generated vesicles can also enter the host cells through the endocytic pathway, as reviewed in O'Donoghue and Krachler (2016). It has been shown that bacterial outer membrane vesicles utilize the four types of endocytosis to invade the host cells, including clathrin-mediated, actin-dependent, caveolin-mediated, or clathrin-caveolin-independent endocytosis [125].

2.3.4. MEVs and immune homeostasis

Gut microbiota-derived EVs (MEVs) play a significant role in maintaining gut immune homeostasis. MEVs enclose multiple copies of microorganism associated molecular patterns, including periplasmic proteins, DNA, RNA, LPS, and peptidoglycan, which interacts with pattern recognition receptors such as NOD1 and NOD2, and Toll-Like Receptors (TLR) on the immune cells to start a cascade of immune signaling [126–129]. This EVs-immune cells interaction relies on the EVs cargo which vary according to the virulence of the source strain. For instance, proteomic analyses have illustrated that only EVs from virulent Mycobacterium strains carry the TLR 2 lipoprotein agonist [130].

Additionally, this TLRs-EVs interaction is selective for the receptor. EVs released by *Lactobacillus* and *Bifidobacterium* genera were found to have differential effects on TLRs where they enhanced the cellular responses of TLR 2/1 and TLR 4, while suppressed the responses of TLR 2/6 with no effect on TLR5 [131]. Also, EVs could suppress the immune system through their sRNA and miRNA content, which is the case of sRNA from the fungus Botrytis cinerea that suppress the plant immunity through gene silencing approach[132]. Moreover, microRNA (miRNA) generated by anopheline mosquitoes my interfere with the host miRNA and regulate some immune responses [133] indicating that pathogens may utilize EVs as a mean of suppressing the host immune system [134].

Commensals-derived MEVs have been shown to regulate gut immune homeostasis. EVs released by *Bacteroides fragilis* have induced the secretion of anti-inflammatory cytokines while reduced the secretion of proinflammatory cytokines [135]. Additionally, it mediated regulatory Treg responses that suppressed the mucosal inflammation in DSS model of colitis [58]. Likewise, the MEVs from *Lactobacillus rhamnosus* induced the expression of IL-10 and enhanced Treg responses in mice mesenteric lymph nodes and Peyer's patches[47]. Similarly, Kang and his coauthors [36] reported an important shift in stool MEVs composition in a DSS mice model of IBD compared to controls. In addition, EVs derived from *Akkermansia muciniphila* have been reported to reduce body weight loss, increase colon length, improve epithelial stability, and reduce inflammatory cells infiltration to the colon wall of DSS-treated mice [36]. The same study reported an inverse relationship between the severity of colitis and *A. muciniphila* EVs [36]. Together, this indicates that gut microbiota-derived vesicles possess a potential role in maintaining gut immune homeostasis.

Table 2. Evidence and summary of MEVs contribution to maintaining gut immune homeostasis.

Model system/Host organism	Microbial species	Experimental setup/clinical context	MEV gene/proteins/lipids involved	Reference
Mice/epithelial cells	*Helicobacter pylori*, *Pseudomonas aeruginosa* and *Neisseria gonorrhoea*	Measurement of immune responses and antibody production	Peptidoglycan within OMVs	[45]
Human umbilical endothelial cells	non-pathogenic or pathogenic *E. coli*	Adhesion protein synthesis, cytokine production, and necrosis factor (NF)-κB translocation.	OMVs	[136]
Caco-2, HCT-8, and HT-29 intestinal epithelial cell lines	Enterohemorrhagic *Escherichia coli* O157	Interleukin 8 production and Toll-like receptors TLR4, TLR5 and the nuclear factor (NF-κB) activation.	H7 flagellin, cytolethal distending toxin V and O157 lipopolysaccharide (LPS).	[137]
Mice/ airway epithelial cells, THP-1-monocytes and -macrophages	Dust EVs	Measuring lung neutrophilic infiltration and inflammation markers such as IL-8, IL-6, ICAM-1, proIL-1β, and TNF-α levels.	EVs	[46]
Mice/alveolar	Mycobacteria	Proteomic analyses of EVs, H&E staining/confocal fluorescence microscopy and flow cytometry.	TLR2 lipoprotein agonists	[130]
Human derived dendritic cells, THP-1 Blue-CD14 and HEK293 cell lines	Lactobacilli and Bifidobacterium species	Bacterial phagocytosis, Bacterial aggregation, and induction of TLRs pathways	Serum derived EVs	[138]
Human Intestinal Epithelial Cells (Caco-2)	*Bacteroides fragilis*	Toll Like Receptor 2, Toll Like Receptor 4 Genes Expression (qRT-PCR) and Pro-inflammatory (IFN_{γ}) and anti-inflammatory	Isolated OMVs	[139]

		(IL- 4 and IL-10) cytokines Concentration (ELISA)		
Mice/ ex vivo model of peristalsis/ in situ patch-clamped enteric neurons	*Lactobacillus rhamnosus* JB-1	Proteomic analyses (EVs), flow cytometry, Intracellular cytokines staining in presence and absence of receptor inhibitors.	Isolated EVs	[140]
dextran sulfate sodium (DSS)- treated C57BL/6 mice and colon epithelial cells induced by *Escherichia coli* EV	Gut microbiota and *A. muciniphila*-derived EV	Metagenome sequencing and measuring weight loss, colon length, inflammatory cell infiltration of colon wall and cytokines level.	Isolated EVs	[36]

2.3.5. MEVs and the gut-brain axis

The contribution of the microbiota-gut-brain axis to the host's mental health and neural development has increased focus over the past decade. The term microbiota-gut-brain axis refers to the interactions between the gut microbiota and the central nervous system (CNS) through neural, endocrine, and immune signalling pathways [141]. Sudo et al. (2004) [142] reported that germ-free mice have a hyperactive hypothalamus-pituitary (HPA) axis with a noticeable level of stress-associated hormones compared to mice with conventional microbiota. Various studies showed the gut microbiota play a critical role in modulation anxiety [143–145] and memory processing [146]. The diversity in gut microbiota has been linked to behavioural disorders. At the same time, exposure to non-pathogenic bacteria can harmonize adult animals' behaviours [143] and anxiety symptoms in human subjects [147, 148]. Additionally, CNS development is directly related to exposure to certain commensal bacteria in early life [149–152]. Although many studies support the microbiota-gut-brain axis's existence, there is a limited understating of how signals are transferred from the gut to the brain. However, there is evidence that the gut can modulate the CNS through some

pathways (Table 3, Figure 4): (i) The gut microbiota can captivate the neural signaling between the brain and the gut through the interaction between the vagal nerve and the enteric nervous system (ENS) [153–157]. (ii) The endocrine response of the host can communicate the gut microbes signal to the brain through circulation [158, 159]. (iii) The gut microbe can modulate the central and peripheral immune cells resulting in changes in stress and behaviours responses [160–165]. (iv) Gut microbes release metabolites, such as a neurotransmitter, that can travel through the circulation of the CNS [16, 166].

A recent report on increased levels of systemic LPS-positive bacterial extracellular vesicles in patients with intestinal barrier dysfunction provides some evidence on the capacity of MEVs to reach the systemic circulation [167] and deliver and elicit a variety of immunological and metabolic responses in different organs, including the brain. Recently, gut microbiota-generated MEVs were illustrated to correlate with the inhibition of energy metabolism in the hypothalamus of MDD patients [168]. Another recent study showed that MEVs derived from *Lactobacillus plantarum* induced antidepressant-like behaviour in mice [39], which supports the potential of MEVs as biotherapeutics in MDD. Al-Nedawi and his coauthors have illustrated that EVs from *Lactobacillus rhamnosus* can stimulate the afferent neurons of the enteric nervous system [47]. *L. rhamnosus* is known to spike the vagus nerve, which is an essential signalling pathway in the gut-brain axis [169]. Other investigators have illustrated that the EVs from the gut member, *Paenalcaligenes hominis*, cause vagus nerve-dependent cognitive impairment reduced by vagotomy [170]. Recently, EVs from *Akkermansia muciniphila* have been reported to induce the secretion of serotonin in mice colon and hippocampus, and in the Caco-2 cell line [37]. Together, this supports the hypothesis that MEVs are signaling molecules that could control brain activities.

In addition to being a signaling molecule in the enteric nervous system, MEVs have been demonstrated as cargoes that package psychoactive molecules and shuttle them to

distant locations from the gut. Analyzing the EVs released by *Bacteroides fragilis* have revealed their histamine and gamma-amino-butyric acid (GABA) content, the two neurotransmitters that could affect brain functions [48]. RNA in MEVs could also mediate gut-brain communications. Assessing the bacteria RNA content in post-mortem brains of patients with Alzheimer's illustrated the prevalence of RNA related to Proteobacteria, Firmicutes, *Staphylococcaceae*, *Corynebacteriaceae*, and *Propionibacteriaceae* [171]. Actinobacteria and Firmicutes dominated Alzheimer's brains along with depletion of Proteobacteria and Bacteroidetes compared to controls [171].

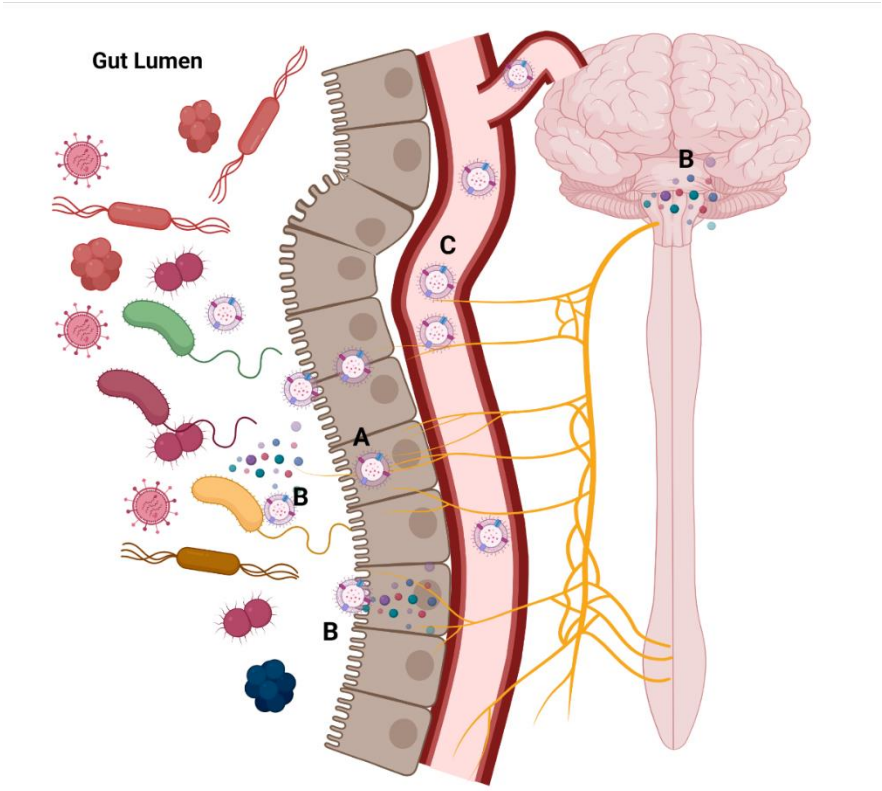

Figure 4. Microbiota generated extracellular vesicles (MEVs) and gut brain axis communication. MEVs facilitate gut brain axis communication through 3 hypothesized pathways; (A) Vagal nerve stimulation [47, 169]; (B) Endocrine release modulation from gut bacteria, enterocytes, and hippocampal neurons [37, 39]; or (C) delivery of cargoes to the CNS through the blood circulation [48]. Figure is Created with BioRender.com

Table 3. Roles of MEVs in microbiota gut-brain axis communications.

Activity	Evidence	Refs
Vagal nerve stimulation	EVs of *Lactobacillus rhamnosus* can stimulate the afferent neurons of the enteric nervous system	[47, 169]
	EVs of *Paenalcaligenes hominis*, cause vagus nerve-dependent cognitive impairment.	[170]
Endocrine modulation	EVs from *Akkermansia muciniphila* have been reported to induce the secretion of serotonin in mice colon and hippocampus, and in the Caco-2 cell line	[37]
	Extracellular vesicles derived from *Lactobacillus plantarum* increase Brain Derived Neurotrophic Factor (BDNF) expression in cultured hippocampal neurons and produce antidepressant-like effects in mice.	[39]
Cargoes carrier	EVs released by *Bacteroides fragilis* includes histamine and gamma-amino-butyric acid (GABA) as part of their content.	[48]
	Patients with Alzheimer's exhibited a prevalence of RNA related to Proteobacteria, Firmicutes, *Staphylococcaceae*, *Corynebacteriaceae*, and *Propionibacteriaceae* in their brains.	[171]

2.4. Conclusions and perspectives

Accumulating evidence supports the role of MEVs as signaling molecules that mediate microbiota-host communications. MEVs are representatives of their parental microbes in many communicative activities. In contrast to their microbial origins, they have more accessibility to blood circulation, and they can shuttle their contents to distant locations from the gut, such as the brain. In contrast to individual metabolites and secreted proteins (secretome), the MEVs contents are enclosed in a bilayer membrane that protects them from lytic enzymes and RNases in the extracellular environment [47] and facilitate their diffusion to distant organs [38]. Still, MEVs are underestimated as a way of communication with the host. Previous studies have focused on characterization of their proteomic and/or RNA contents or investigating the correlation of EVs from a specific microbe with certain body response[48, 140, 169–172]. This may be attributed to lack of standard methods for isolation and identification of MEVs contents, lack of well-defined biomarkers isolated from MEVs. Additionally, current methods do not separate the host EVs from MEVs. recently some approaches have been described to separate bacterial EVs from human body

fluids through implementation of ultrafiltration, density gradient centrifugation and size exclusion chromatography [167]. Another obstacle is the lack of a reliable method to identify the mother bacterial origin of different MEVs or their identified content in a heterogenous microbial community such as the gut microbiota [173]. Future research is required to illustrate how the variability of the parent microbiome correlates with the variability of MEVs contents and production. Also, additional research is required to assess how MEVs are packaged by microbial cells, why this specific molecules are packed, are they targeted to specific cells, how they are targeted to the host cells, how they release their cargoes, and whether they can cross biological barriers such as intestinal barrier and blood-brain barrier. Despite the several hurdles that must be overcome for the potential exploitation of MEVs as a drug delivery platform for biologics to targeted body locations, the recent developments discussed in this review offer a taste of their emerging role as mediators of host-microbiota interplay.

CHAPTER 3 : Hypothesis, Research Questions, Specific Aims, and Objectives

3.1. Hypothesis

Gut microbiota extracellular vesicles may play a critical role in the gut-brain-axis by serving as a signaling pathway through which the gut microbiota modulates host brain functions.

3.2. Research Questions and objectives

This thesis aims to answer the following research question: Could microbiota-released extracellular vesicles be involved in the communication between the gut microbiota and the brain? The objectives of this study are as follows:

Objective I. Isolate and characterize molecular content of MEVs isolated from stool samples obtained from healthy adults and *ex vivo*-developed microbiota (Chapter 4).

Objective II. Investigate the ability of characterized MEVs in crossing the gut epithelial and blood-brain barriers *in vitro* (Chapter 5).

CHAPTER 4: Comprehensive Multi-Omics Characterization of Gut Microbiome Extracellular Vesicles

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4.1. Introduction

Appreciable evidence suggests a connection between the microbiota-gut-brain axis and the host's brain activity [174, 175]. For instance, germ-free mice exhibited a hyperactive hypothalamus-pituitary axis and higher levels of stress hormones than conventional mice[142]. In addition, the gut microbiota has been shown to regulate emotional behaviors and modulate anxiety and memory processing [144, 146, 176]. Some probiotics, known as "psychobiotics", have psychotropic-like activities and have been suggested to modulate mental and behavioral disorders. While some studies have reported mitigated efficacy [177, 178], several trials support a role for psychobiotics in normalizing brain processes related to stress responses and mood improvements [179–182]. However, the mechanisms by which psychobiotics and the gut microbiota interact with the gut–brain axis and modulate mental health remain hypothetical.

Microbiota-released extracellular vesicles (MEVs) have recently emerged as signaling molecules that mediate host-microbiota crosstalk[15]. MEVs are small membrane-bound phospholipid vesicles that range from 30 nm to 1 mm in size, with larger vesicles originating from the cell surface (microvesicles/ectosomes) and those on the smaller side derived from either the plasma membrane or the endosomal system (exosomes) [183]. MEVs contain bacterial components such as nucleic acids, proteins, and cell wall components and are involved in quorum sensing, biofilm formation, relief of environmental stresses, and host immunomodulation [184]. MEVs encase a spectrum of biologically active proteins, mRNA, miRNA, DNA, carbohydrates, and lipids, thus propagating the horizontal transfer of their

cargo across both short and long distances [15]. The production and role of MEVs released by probiotic and commensal microbes in the gut environment are poorly investigated [185], as MEVs have been predominantly examined in individual strains [184, 186, 187]. Previously, Kang et al. [188] reported an important shift in stool MEV composition compared to the microbiome in an IBD DSS mouse model. Moreover, the same authors observed an attenuating effect of *Akkermansia muciniphila*-derived MEVs on colitis severity[188], suggesting the potential of MEVs as biomarkers and therapeutic agents. A recent report on increased levels of systemic LPS-positive bacterial extracellular vesicles in patients with intestinal barrier dysfunction provides some evidence of the capacity of MEVs to reach the systemic circulation[189] and deliver and elicit a variety of immunological and metabolic responses in different organs, including the brain. Therefore, MEVs should be considered of utmost importance as delivery vehicles for host-modulating metabolites, and thus can be regarded as promising psychobiotic candidates compared to the clinical and regulatory limitations faced by fecal transplantation [190, 191]. Harnessing the ability of the gut microbiota to produce MEV could decipher interactions with the gut–brain axis. In the current study, we postulate that microbiota interplay with the gut–brain axis involves microbiota extracellular vesicles as a communication-based mechanism. We investigated MEVs generated *ex vivo* and from stool samples of healthy adults as a potential cargo mechanism by which the gut microbiota may exert psychobiotic effects.

4.2. Materials and methods

4.2.1. Isolation of MEVs from stools and ex vivo developed microbiota

Microbiota communities from healthy donors (n=6 males and 6 females) were developed in an *ex vivo* model mimicking the large intestine[192]. The donors did not receive antibiotics or probiotic supplementation for at least 3 months before donation. Microbiota was immobilized by inoculating fecal samples into gellan (2.5 %, w/v) and xanthan (0.25 %, w/v) gum beads under anaerobic conditions, as described previously [192]. Gel beads (30%) were transferred into a stirred glass reactor containing fresh MacFarlane culture medium [193]. The fermentation conditions have been described previously [192]. A total of 100 ml of the bioreactor culture 15 days post-inoculation (microbiota stabilization period) was centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was sterilized by filtration through a 0.22 µ filter. MEVs were isolated from the sterile supernatant by ultracentrifugation at 100,000 × g for 70 min. The size of the MEVs was determined using a Malvern Zetasizer Nano ZS instrument. The morphology of the isolated MEVs was inspected using transmission electron microscopy (TEM) as described previously[172]. Briefly, the isolated MEVs in PBS were diluted 1:100 and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The pellets were vortexed thoroughly and 10 µl of the suspension was placed on a carbon grid. Five minutes later, the grid was washed with a drop of distilled water. After drying, 10 µl uranyl-less stain was added to the grid and incubated for 1 min before removing the excess stain and drying at room temperature before imaging with TEM.

4.2.2. DNA extraction for microbiome characterization through 16S rRNA sequencing

DNA was extracted from stool or a 2 ml sample of the 15-day-old ex vivo microbiome using a FastDNA Spin Kit (MO BIO Laboratories Inc.) according to the manufacturer's instructions, with an additional cycle of mechanical homogenization and 5 min of cooling on ice between the two cycles. The extracted DNA was quantified using a Qubit fluorometer (Invitrogen; Carlsbad, CA, USA), and its quality was checked using a Bioanalyzer 2100 (Agilent) and stored at −20 °C until further analysis.

4.2.3. DNA Library preparation for 16S rRNA sequencing

The microbiome diversity was determined by sequencing the V3-V4 regions of the 16S rRNA gene using the Illumina MiSeq platform (N*u*GUT Laboratory), using the Illumina standard protocol as described in [192]. Raw paired-end sequences were fed to the Quantitative Insights Into Microbial Ecology pipeline 2 (QIIME 2) [194] for quality preprocessing and determination of microbial composition and diversity indices. Alpha-diversity was calculated as Shannon index. Beta-diversity analyses was conducted via statistical analysis [metadata table] module of MetaboAnalyst 5. Principle component analyses based on Bray-Curtis distances and the top 5 components were plotted against each other. Linear mix model were used for statistical comparisons between stool and ex-vivo predominant microbiota features (prevalent in more than 50% of samples) using donor as cofounding variable.

4.2.4. Extraction of metabolites from MEVs

To minimize contamination by soluble metabolites, 500 µL of MEVs diluted in PBS were resuspended in PBS (7 mL) and re-centrifuged at 100,000 × g for 70 min (4°C) to remove media traces from the samples. The tube containing MEVs at the bottom was kept upside down for a few minutes to remove any remaining PBS. Next, 990 µL of LC-MS-grade methanol and 10 µL of ^{13}C-phenylalanine (1 mg/mL, internal standard) were added to the tube containing dried MEVs and gently mixed using a pipette to extract metabolites in MEVs. The result was transferred to a 1.5 mL Eppendorf tube, and the insolubilized particles, such as proteins formed in the extraction process, were separated by centrifugation for 5 min at 16,000*g*. The clear supernatant was passed through a 0.2 µm polytetrafluoroethylene (PTFE) syringe filter (Millipore Corporation, Billerica, MA) to remove any remaining fine particles, followed by evaporation of the solvent under vacuum using a SpeedVac concentrator, and the dried samples were stored at -20°C until used for mass spectrometry analysis.

4.2.5. nLC-nESI-MS/MS analysis

The extracted metabolites from gut microbiota-derived MEVs were analyzed using nano-liquid chromatography coupled online with nanoelectrospray ionization and mass spectrometry (nLC-nESI-MS/MS) (Thermo Fisher Scientific, Waltham, Massachusetts, US). The metabolites were separated on an EASY-Spray™ HPLC column (Thermo Scientific) with 15 cm × 75 µm ID, C18, 3 µm, 100 Å, using a water/acetonitrile/0.1% formic acid gradient. Samples (1µL) were injected into the autosampler and loaded onto the column for

60 min at a flow rate of 0.23 μL/min. The metabolites were then separated using a linear gradient from 10 to 100% acetonitrile for 35 min, maintained for 10 min, followed by a gradient from 100 to 0% acetonitrile for 5 min, and then increased to 10% acetonitrile for 10 min.

The eluted metabolites were directly sprayed and transferred to a mass spectrometer using electrospray ionization (ESI). Full-scan MS spectra (m/z 100–1000) were acquired at a resolution of 70,000. The automatic gain control settings were 3×10^6 for full MS and 1×10^5 for MS/MS scans, and fragmentation of molecules occurred in the collision-induced dissociation (CID) cell in the linear ion trap. The precursor ions were separated using a 2 m/z isolation window and fragmented with a normalized collision energy of 35%.

4.2.6. nLC-nESI-MS/MS Data processing and analysis

Mass spectrometry data were processed and analyzed using MS dial version 4.60 along with the Massbank and Human Metabolome Database (HMDB) to facilitate the identification of target molecules. Default parameters were used to identify individual compounds in the samples, and contaminants detected in the blank were excluded from the list of identified compounds.

4.2.7. Preparation of samples for proteomics analysis

Protein concentrations were measured by the Bradford protein assay, and 50 μg of protein was processed using a filter-aided sample preparation (FASP) protocol. Exosomal fractions of approximately 50 mg were adjusted to 50 mM TRIS, 8 M urea, 5% (v/v) glycerol, and either 0.1% DDM or SDS (pH 8.0) and vortexed for 30 s. The suspension was centrifuged for 5 min at 10,000 × g, and the supernatant was separated from the insoluble

debris and passed through a 10 kDa MWCO filter (Millipore). Sample volumes were reduced to approximately 20 µL by vacuum centrifugation for 25 min at 14,000 × g, and proteins were reduced by the addition of 4 mM TCEP in 100 µL denaturation buffer for 45 min (30 min incubation + 15 min centrifugation) at room temperature (RT). Buffer was exchanged to alkylate proteins with 20 mM iodoacetamide in 100 µL denaturation buffer for 1 h at RT in the dark. The filter was washed once with digestion buffer (50 mM TRIS, 0.6% glycerol, pH 8) before being transferred to a clean collection tube. Digestion was performed with 300 ng trypsin/Lys-C (Promega, V5072) in 100 µL digestion buffer at 37°C for 12 h. Peptides were eluted from the filter by centrifugation at 14,000 × g for 10 min, and an additional centrifugation step was performed after adding 40 µL digestion buffer. Eluted peptides were treated with formic acid at a final concentration of 1%. The peptides were desalted on TopTip C-18 micro-spin columns (Glygen, # TT2C18) and dried by vacuum centrifugation.

4.2.8. Sample preparation & nano-LC-MS/MS

Samples were analyzed using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Mississauga, ON, Canada) coupled to an UltiMate 3000 nanoRSLC (Thermo Fisher Scientific, Mississauga, ON, Canada), as described previously [195].

4.2.9. Protein identification, quantification, and profiles

Peptide/protein identification and quantification, peptide taxonomic assignment, and functional protein annotation were performed using MetaLab software (version 1.2.0) [196] using a database based on the integrated gene catalog (IGC), which contains a complete set of

genes for gut microbiota members [197]. We employed the MaxQuant search engine in the MetaLab workflow for peptide/protein identification [198]. Carbamidomethyl (C) was set as a fixed modification, while protein N-terminal acetylation (protein N-term) and oxidation (M) were set as variable modifications. Peptide and protein identification was conducted with a false discovery rate (FDR) of 0.01. Proteins were grouped into Clusters of Ortholog Groups (COG) based on their KEGG Orthology (KO) Ids.

4.3. Results

4.3.1. Physical characteristics of the isolated MEVs

Transmission electron microscopy (TEM) and Zetasizer showed that the isolated MEVs were intact, the membrane was enclosed, and their size fell within the range of exosomes, microvesicles, and bacterial outer membrane vesicles[199] (Figure 5A-B).

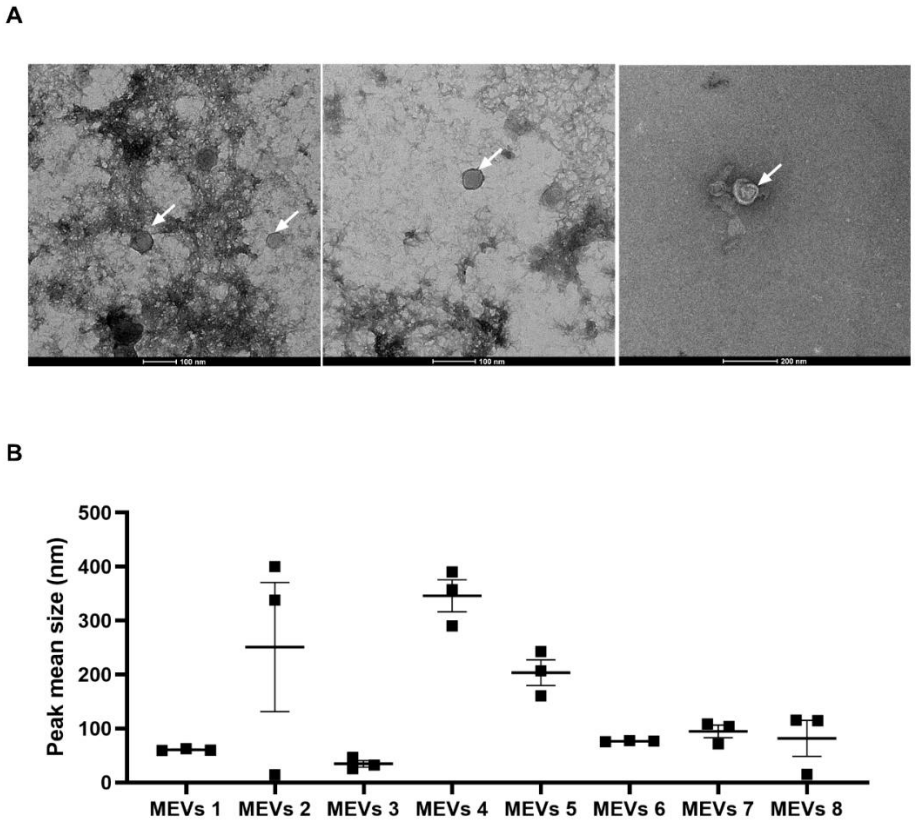

Figure 5. Transmission electron microscopy (TEM) and size of isolated vesicles. (A) TEM of the isolated vesicles. (B) Size range of the isolated MEVs as determined by a Malvern Zetasizer Nano ZS.

4.3.2. Metabolic content of MEVs determined by nLC-ESI-MS/MS analysis in the positive mode.

The lipid species identified in the gut microbiota-derived MEVs in positive mode are summarized in **Figure 6**. More than 40 lipid species were identified in the MEVs isolated from stool and fermented samples in positive mode. The major lipid classes found in the samples were sphingoid bases (SPB), acyl carnitines (CAR), lyso-phosphatidylethanolamine (LPE), and lyso-phosphatidylcholine (LPC), which possess only one acyl chain in their chemical structures. Given that a large portion of lipid species found in MEVs is the primary source for the construction of lipid bilayer membranes of MEVs owing to their amphiphilic nature, it is expected that the high proportion of lipids in the membrane of MEVs consists of

single acyl chain lipids such as SPB, CAR, LPE, and LPC. A few phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which have two acyl chains, were detected in positive mode, but these are not included in **Figure 6** as they require more data processing for identification. Overall, MEVs isolated from fermented samples showed a higher abundance of ion intensity than that of the stool sample.

In addition to lipid species, the metabolite profiles of MEVs isolated from stool and fermented samples were also determined in positive mode, as shown in **Figure 7**. A wide spectrum of molecules was identified, including carbohydrates, amino acids, and steroid derivatives, in this limited dataset. **Figure 7** shows that among the metabolic contents of MEVs is a set of neuroactive molecules, including choline, gabapentin, phenylalanine, tyrosine, arachidonyl-dopamine (NADA), L-glutamic acid, and N-acylethanolamines.

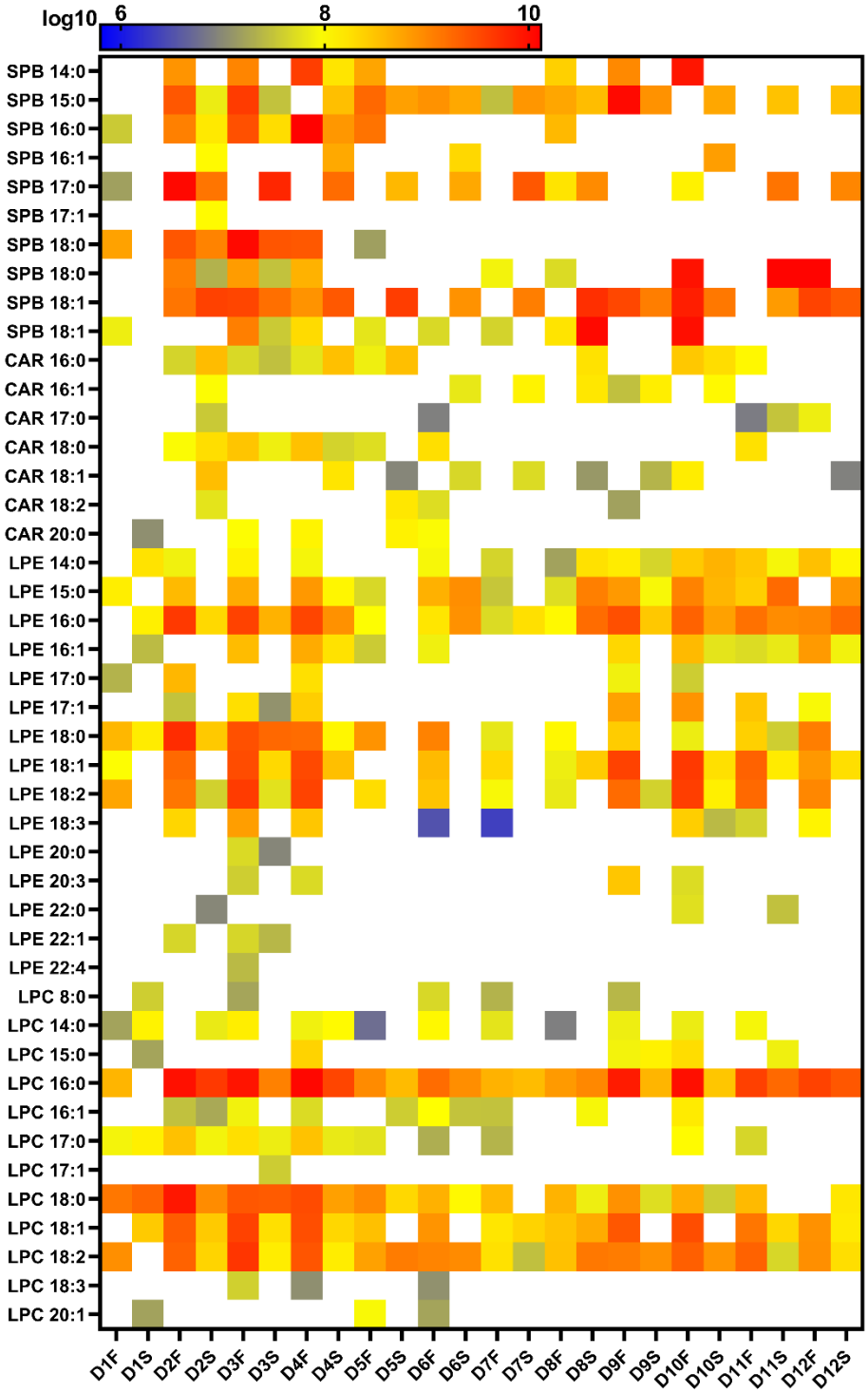

Figure 6. Mass spectrometry-based lipidomics in the determination of lipid profiles of gut microbiota-derived MEVs in the positive mode. The heat map (a) describes the relative abundances of individual lipid species found in isolated MEVs. SPB; Sphingoid bases, CAR; Acyl Carnitines, LPE; Lyso-phosphatidylethanolamine and LPC; Lyso-phosphatidylcholine**.**

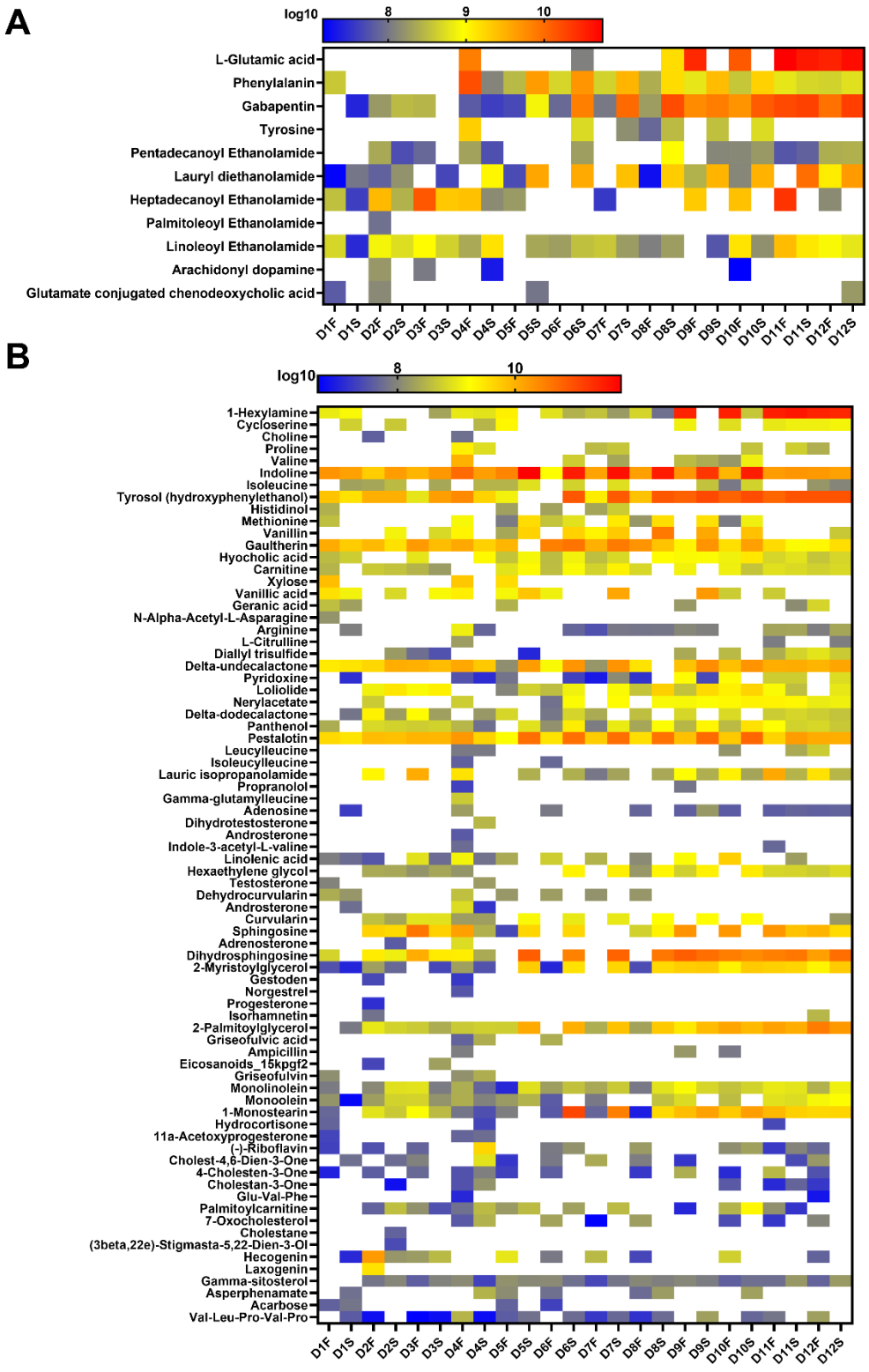

Figure 7. Mass spectrometry-based metabolomics in the determination of cargoes inside MEVs in the positive mode. The heat maps describe the relative abundances of individual metabolites identified in isolated MEVs. (A) Potentially neuro-related metabolites; (B) Other metabolites.

4.3.3. Metabolic content of MEVs determined by nLC-ESI-MS/MS analysis in the negative mode.

The lipid species identified in the gut microbiota-derived MEVs in the negative mode are summarized in **Figure 8**. In total, 54 lipid species were identified in the MEVs isolated from stool and fermented samples in negative mode. The dominant lipid class found in the MEVs was lyso-phosphatidylethanolamine (LPE), and several species of N-acetylglycine (NAGly), N-acyl glycylserine (NAGlySer), phosphatidylethanolamines (PE), and phosphatidylinositol (PI) were also identified in the negative mode. Overall, different classes of lipid species were found in the negative mode compared to the positive mode, which might be due to the discrepancy in the ionization properties of each lipid class. Several lipid species conjugated with high unsaturated fatty acids (HUFAs), such as linoleic acid (18:3) and arachidonic acid (20:4), were identified in the negative mode.

The metabolite profiles, other than lipid species, in MEVs isolated from stool and fermented samples were also determined in negative mode and are summarized in **Figure 9**. A wide range of metabolites were found in MEVs in the negative mode, including carbohydrates, amino acids, vitamins, and organic acids, and some neurotransmitter-related compounds were identified. For instance, 3,4-dihydroxymandelic acid (DOMA), a metabolite of norepinephrine, referred to as a representative neurotransmitter, was identified at m/z $[M-H]^-$ 183.0 in the negative mode. Arachidonyl dopamine at m/z $[M-H]^-$ 438.2, found in the positive mode, was also identified in the negative mode. Dopamine, a representative human neurotransmitter, was found in gut microbiota-derived MEVs in a conjugated form by association with arachidonic acid (20:4).

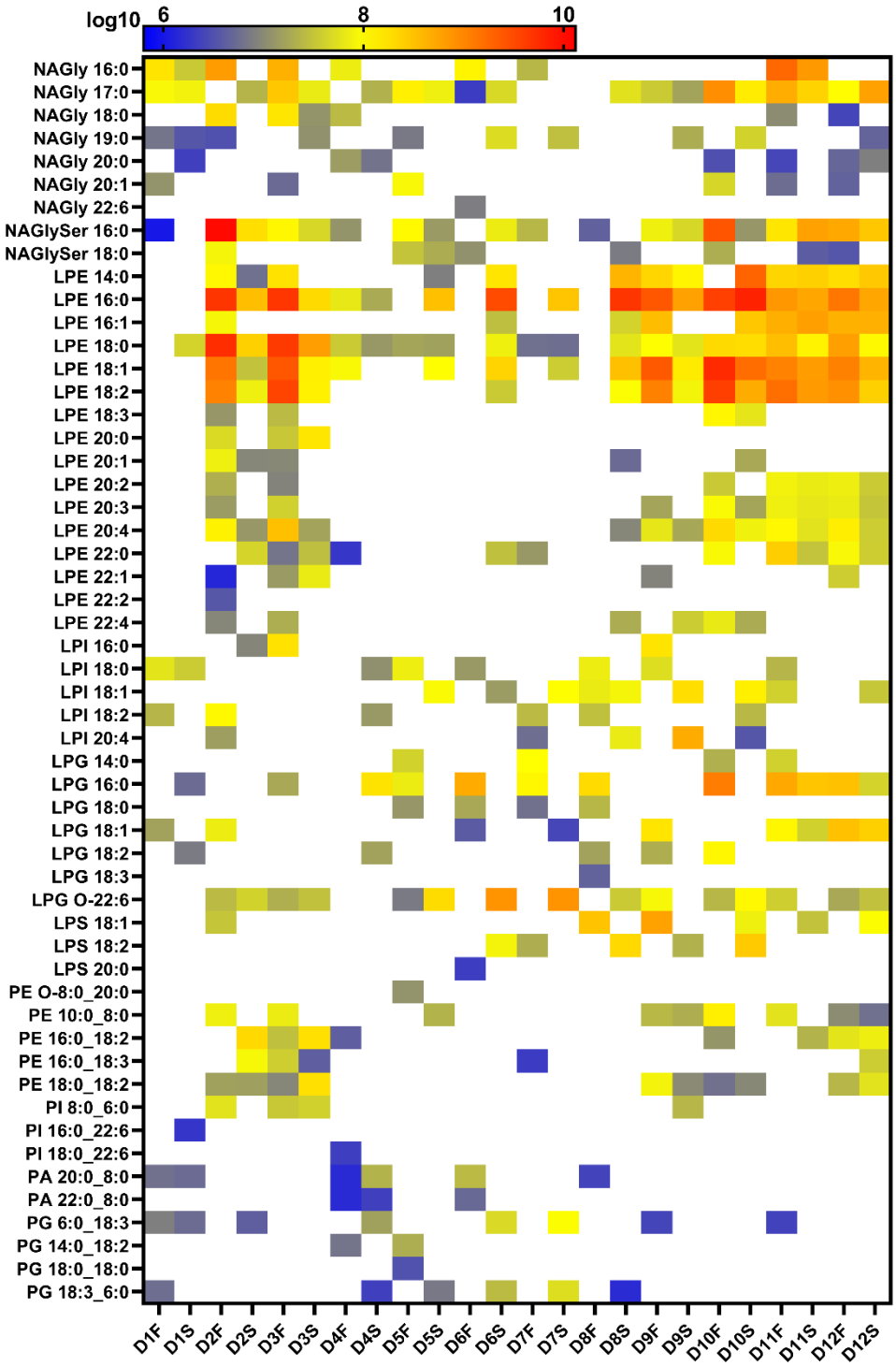

Figure 8. Mass spectrometry-based lipidomics in the determination of lipid profiles of gut microbiota-derived MEVs in the negative mode. The bar describes the relative abundances of individual lipid species found in isolated MEVs. NAGly: N-Acetylglycine; NAGlySer: N-acyl glycylserine; PE: Phosphatidylethanolamines; PI: Phosphatidylinositol.

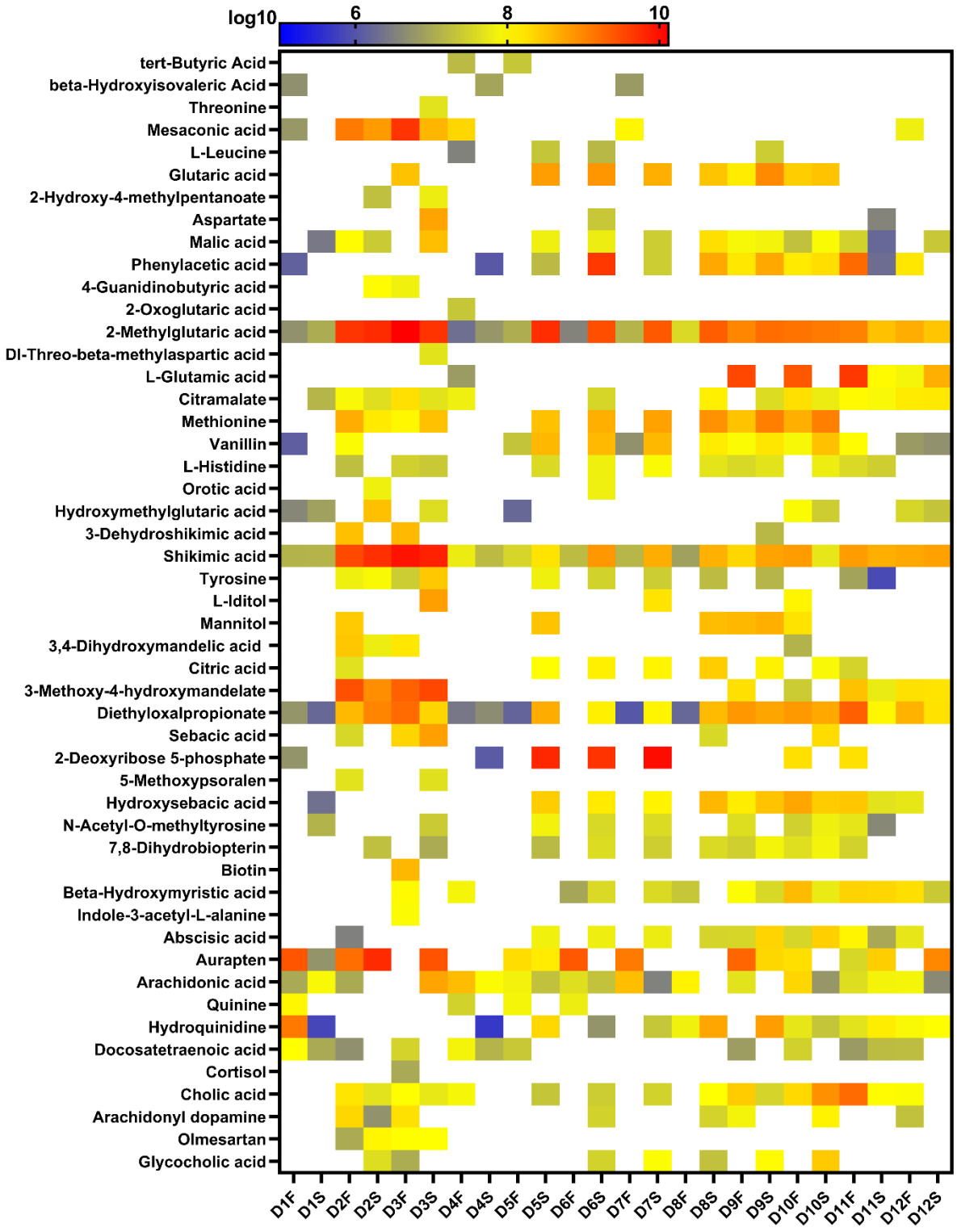

Figure 9. Mass spectrometry-based metabolomics in the determination of cargoes inside MEVs in the negative mode. The bar describes the relative abundances of individual metabolites identified in isolated MEVs**.**

4.3.4. Metaproteomics Characterization of MEVs

We identified the protein content of the isolated MEVs from both donor stool samples and their bioreactor-developed microbiome using nano-LC-MS/MS. We identified 9425 protein groups, 197 of which had no intensity information. Bioreactor-generated MEVs generated more protein groups (7,847) than stool MEVs (1356), with 676 protein groups shared between both groups (**Figure 10A**). The identified proteins were categorized into 15 COG categories, with categories G (carbohydrate transport and metabolism), N (cell motility), P (inorganic ion transport and metabolism), and M (cell wall/membranes/envelope biogenesis) being the predominant proteins (**Figure 10B-C**). **Figure 11** shows the identified proteins linked to neuroactivities, such as the glutamine/glutamate/GABA pathway, biosynthesis of inositol, microbiota-derived trimethylamine (TMA), and cellular energy production.

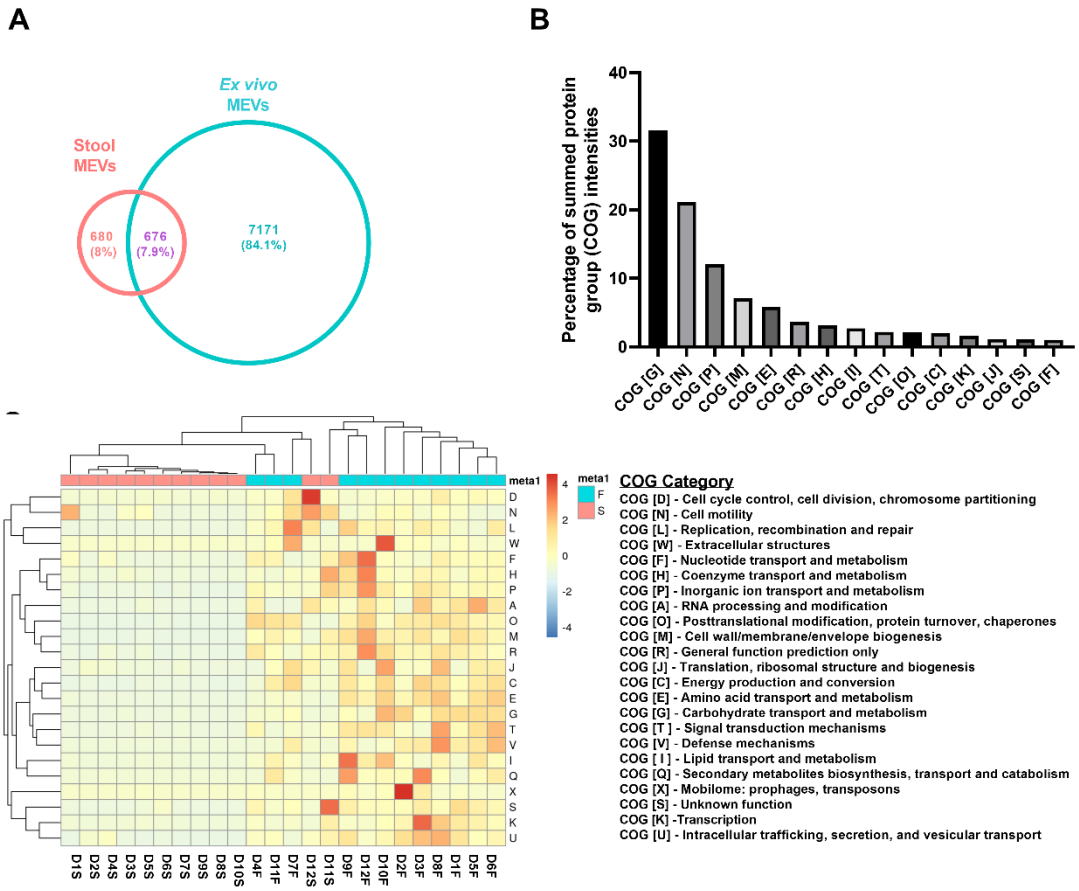

Figure 10. Metaproteomics characterization of MEVs contents. (A) the number of proteins identified in both stool MEVs and ex vivo generated MEVs. B-C: predominance of the identified proteins at the level of COG categories (B) and their distribution in every sample**.**

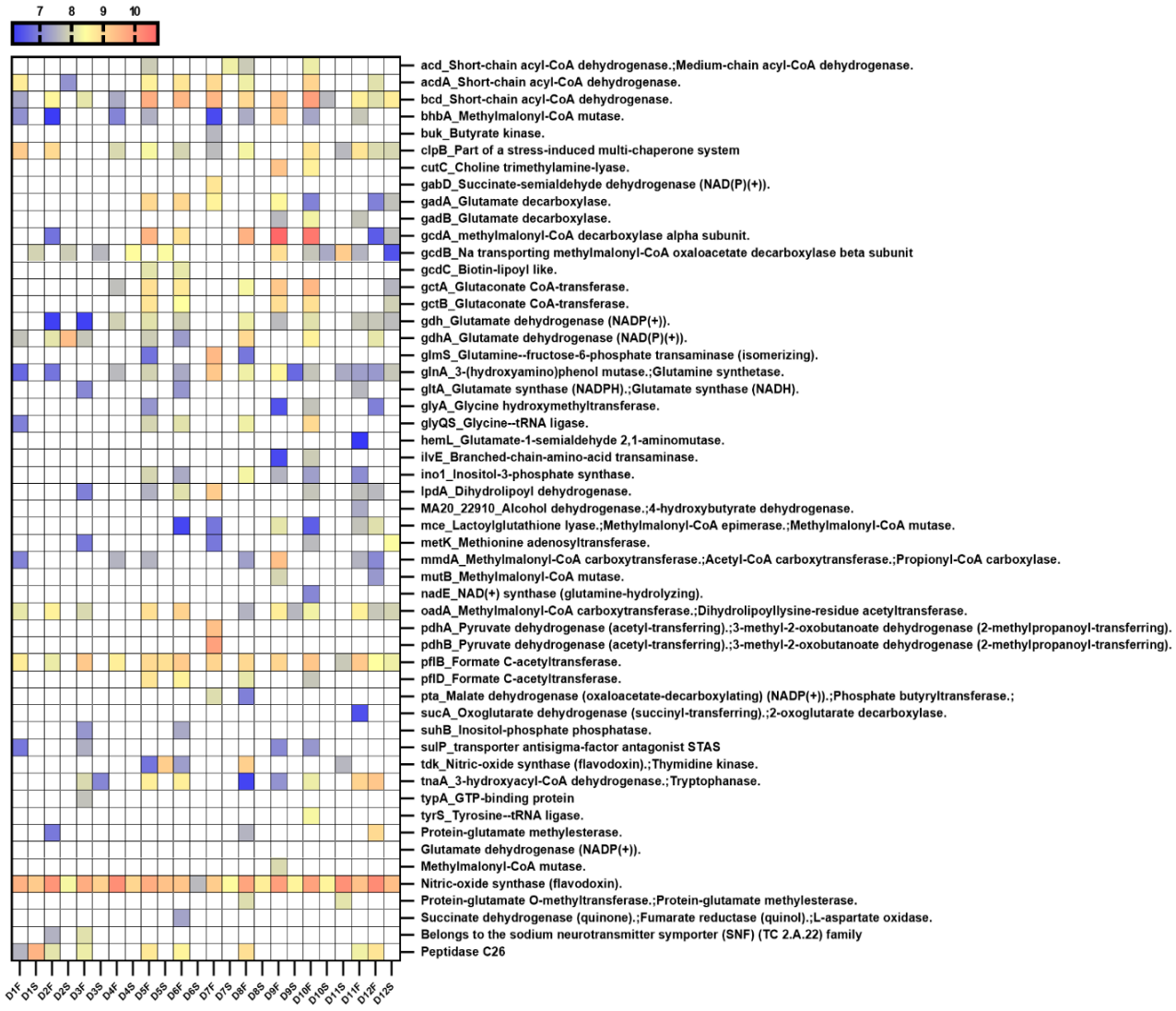

Figure 11. Protein Content of MEVs with neuroactive potential. The heatmap shows the log10 of summed protein intensities in the tested samples.

4.3.5. Gut Microbiota Structure

We characterized the microbiota structure from which we extracted MEVs via 16S rRNA sequencing. Stool samples showed higher diversity than the *ex vivo-developed* microbiome (**Figure 12A**). The microbiota from the stool and *ex vivo* samples followed the common structure of the microbiome, which was dominated by Bacteroidetes and

Firmicutes, with a lower abundance of Proteobacteria and Actinobacteria and low percentages for other phyla (**Figure 12B**). The *ex vivo*-developed microbiome showed depletion of Ruminococcaceae and Lachnospiraceae along with the expansion of Enterobacteriaceae and Veillonellaceae (**Figure 12B-C**) compared to the stool samples. Nevertheless, the effect of the donor on the microbiome structure is evident, where PCA analyses showed that the samples clustered mainly by sample type (stool vs. *ex vivo*) and then by the donor (**Figure 12D**). A total of 102 taxonomic features showed differential abundances between the stool and *ex vivo* microbiota (Figure 12E). These features were dominated by *Faecalibacterium* spp. (046cb7522f5d3589a6dcbcd1f2cda95d), Ruminococcaceae family (70392e48fb80c7c9fd7adeae40a3585e) mainly *Gemmiger formicilis* (b1ffa316da920a0242cfa65f45395023) and Lachnospiraceae (0cfab271db3ec8f652b4159488aab51f), mainly the *Clostridium* genus (db70b8d89735474e9c237ba7be41c449).

We tested the correlation between predominant gut bacterial species and neuroactive metabolites. We identified positive correlations between *Bacteroides* spp. and glutamic acid, phenylalanine, gabapentin, pentadecenoyl ethanol amide, and linoleoyl ethanol amide. In addition, *Alistipes* spp. showed a positive correlation with glutamic acid, gabapentin, and lauryl diethanolamide levels (**Figure 13**). In contrast, *Ruminococcus* and *Clostridium* spp. were negatively correlated with the identified metabolites (**Figure 13**).

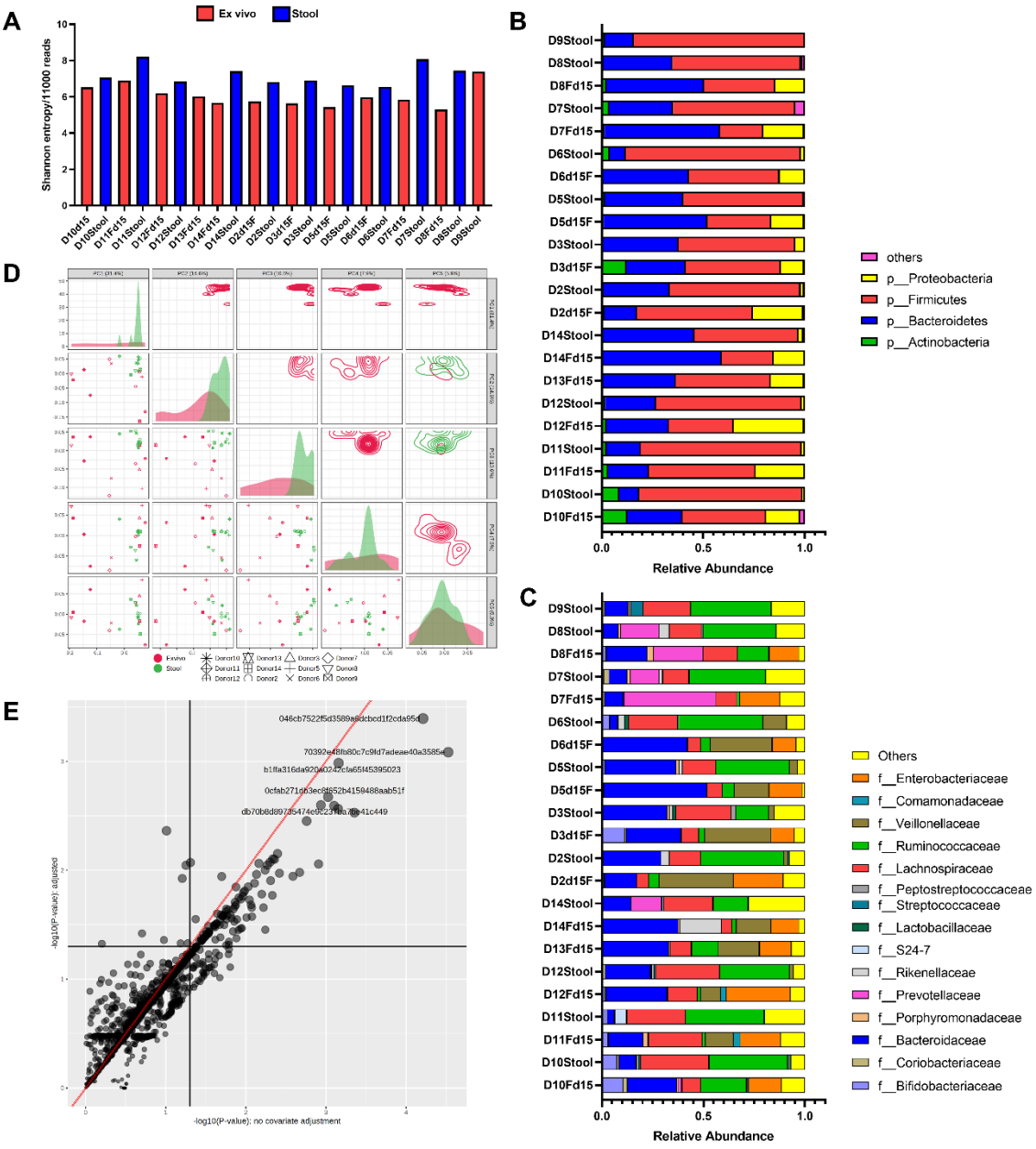

Figure 12. Gut microbiota structure from which MEVs were generated. (A) Diversity of gut microbiota in each sample as indicated by Shannon indices. (B) and (C) Composition of the gut microbiota at the phyla and family level. (D) Principal component analyses of different samples based on the relative abundance of different features identified in each sample generated by Metaboanalyst 5.0 using the features table generated by QIIME 2.6. (E) Linear model showing the variable features between stool and ex vivo generated microbiome using donor as a covariate for adjustment.

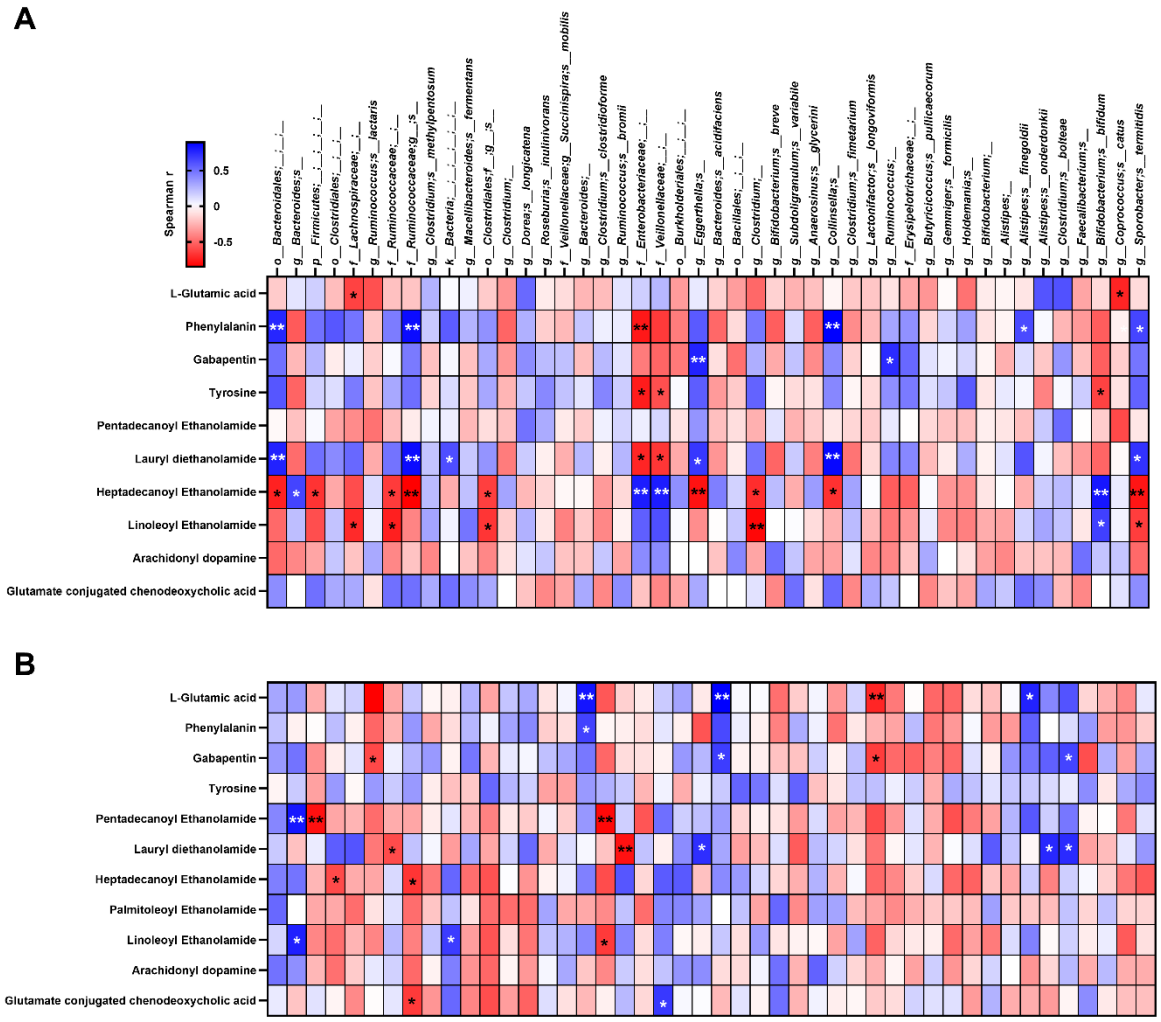

Figure 13. Correlation between predominant microbiota bacterial species and neuroactive metabolites. The relative abundances of microbiota bacterial species detected in >50% of the tested samples were correlated with the identified neuroactive metabolites from the same samples. A) The correlation of stool-MEVs. B) The correlation of fermented MEVs. Spearman correlation analyses were applied, where the color scale represents the Spearman's r calculated while the asterisks denote significance; *p<0.05, **p<0.01.

4.4. Discussion

Dysbiosis of the intestinal microenvironment has been linked to many health disorders, including mental and behavioral disorders. Microbiome-based approaches have shown promise as potential disease modulating strategies. However, the mechanism by which the gut microbiome contributes to these disorders is still hypothetical. In order to achieve precise

modulation of the gut microbiome with clinical effectiveness, it is essential to identify the regulatory mechanisms that control host-microbiome interactions. Recently, microbiome-released extracellular vesicles (MEVs) have emerged as a key delivery mechanism for controlling the intestinal microenvironment and bacteria-host communication [15, 200]. MEVs are small membrane-bound phospholipid vesicles that encase a spectrum of biologically active molecules (i.e., proteins, mRNA, miRNA, DNA, carbohydrates, and lipids), protect them from lytic enzymes and RNases in the extracellular environment[47], and facilitate their transfer across both short and long distances, such as to the brain[38, 201]. MEVs are involved in numerous processes, such as quorum sensing, biofilm formation, relief of environmental stresses, and host immunomodulation [184]. The production and role of MEVs released by probiotic and commensal microbes in the gut environment have been poorly investigated [185, 201] and predominantly examined in pathogenic strains [184, 186, 187].

MEVs possess a potential intra- and inter-kingdom signaling mechanism. It is commonly believed that communication between gram-negative bacteria and the host is mediated by secreted vesicles, known as outer membrane vesicles (OMVs)[34]. Gram-positive bacteria have also been reported to generate EVs[35]. In 2013, Kang et al.[36] reported an important shift in stool MEVs composition compared with the microbiome in a dextran sodium sulfate (DSS)-induced colitis mouse model. While it was unclear whether this dysbiosis was a consequence or cause of inflammation, this study illustrates that EVs play a regulatory role in intestinal immunity and homeostasis [36]. Moreover, *Akkermansia muciniphila*-secreted EVs

protected mice from developing colitis and lowered the production of the proinflammatory cytokine IL6 in response to *E. coli* treatment[36]. Additionally, *A. muciniphila*-EVs were reported to induce serotonin secretion in both the colon and hippocampus of mice, suggesting MEVs' potential as signaling molecules in the gut–brain axis[37]. Likewise, *Lacticaseibacillus rhamnosus* GG was shown to produce EVs with immune regulatory activity[202].

Our study revealed the presence of a wide range of metabolites embedded in MEVs, including carbohydrates, amino acids, vitamins, and organic acids[203]. Interestingly, we identified many neurotransmitter-related compounds or their precursors inside MEVs, including arachidonyl-dopamine (NADA), gabapentin, and N-acylethanolamines[203]. Dopamine, a representative human neurotransmitter, was found in gut microbiota-derived MEVs in a conjugated form with arachidonic acid. N-acylethanolamines (NAEs), such as palmitoyl-ethanolamide (PEA) and linoleoyl-ethanolamide (LEA), have been reported as effective neuroprotective agents [204, 205]. Also, NADA is an endocannabinoid with widespread physiological and pharmacological activities, including modulation of neuropathic pain, inflammatory hyperalgesia, and immune and vascular systems [206]. In accordance with this, a previous report showed that EVs released by *Bacteroides fragilis* contain GABA and its intermediates α-ketoglutarate and glutamate [207]. Oral administration of *B. fragilis* reduced gut permeability, microbiome dysbiosis, and several behavioral abnormalities in a mouse model of autism spectrum disorder (ASD), thus highlighting the potential of microbial interventions in modulating gut microbiome-mediated neurological

disorders[208]. In addition, *Bacteroides*, a major GABA-producing genus in the gut, has been linked to higher levels of serotonin and myoinositol, which are pivotal in maintaining signaling between the enteric and central nervous systems [209]. The relative abundance of *Bacteroides* was negatively correlated with depression-associated brain signatures[13], indicating a significant role of microbiome-secreted GABA in brain functionality. Similarly, Mason et al.[210] reported the depletion of *Bacteroides* in depression and anxiety. Finally, several lipid species in our dataset were conjugated with highly unsaturated fatty acids (HUFAs), such as linoleic acid and arachidonic acid. HUFAs are key molecules for the development, maintenance, and performance of the nervous system as well as brain functionality, by improving the fluidity of the cell membrane[211]. Collectively, these results indicate that gut microbiota-derived MEVs contain neurotransmitter-like molecules proposing MEVs as signaling shuttles in the gut microbiota-brain axis.

Neurochemicals, such as GABA, serotonin, dopamine, and their precursors and derivatives, are microbially metabolized by gut commensals and are considered major modulators of the gut environment, including the enteric nervous system[212]. We found 3,4-dihydroxymandelic acid (DOMA), a metabolite of norepinephrine, to be part of the MEVs' content. Sule et al. reported that DOMA is produced by the metabolic activity of *E. coli* [213]. Also, *Bifidobacterium dentium*, a GABA-producing bacterium, modulated sensory neuron activity in a rat fecal retention model of visceral hypersensitivity. Additionally, GABA has been detected in the cytoplasm and brush border of epithelial cells in the rat jejunum and colon[214]. The exposure of GABA to epithelial cells selectively stimulated

MUC1 expression in isolated pig jejunum[215] and increased the expression of tight junctions and transforming growth factor beta (TGF-β)[216] while decreasing IL-1β-mediated inflammation *in vitro*[216], providing a protective effect against the disruption of the intestinal barrier. Importantly, GABA has also been identified as an essential growth factor that solely can induce the growth of unculturable gut microbes[13]. Together, these results indicate that metabolites embedded in MEVs may also modulate the gut microenvironment.

4.5. Ethical Statement

This study was conducted in accordance with the Declaration of Helsinki and approved by the University of Ottawa Research Ethics Board and Integrity (protocol code H-02-18-347 and approved on July 29, 2019).

CHAPTER 5: Efficiency of the characterized MEVs in crossing the gut epithelial and blood-brain barriers *in vitro*

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5.1. Introduction

MEVs are lipid bilayer-delineated, nanometer-sized particles that incorporate nucleic acids, metabolites, and proteins. A recent report has shown increased levels of systemic LPS-positive bacterial extracellular vesicles (EVs) in patients with intestinal barrier dysfunction, providing some evidence of the capacity of MEVs to reach the systemic circulation [189] and deliver their cargo to elicit a variety of immunological and metabolic responses in different organs. Bacterial EVs may penetrate the host tissue barriers, including the intestinal barrier and blood-brain barrier, through three main mechanisms: (1) paracellular transport, (2) the transcellular pathway, and/or (3) immune cells infected with the vesicles [217]. The transcellular route can be achieved through four endocytosis pathways: clathrin-mediated micropincytosis and endocytosis, caveolin- and clathrin-mediated endocytosis, and clathrin- and caveolin-independent mechanisms, such as lipid raft formation or membrane fusion [218]. This discrepancy in the delivery route of EVs to the circulation and distant organs may be attributed to the heterogeneity of EVs secreted by different bacteria.

Once bacterial EVs reach systemic circulation, they disseminate to different organs, including the kidney, liver, and spleen [219]. Additionally, outer membrane vesicles secreted by the periodontopathogen *Aggregatibacter actinomycetemcomitans* have been detected in brain monocytes and microglial cells through their uptake and transfer by macrophages [220]. In addition, mice gavaged with *Paenalcaligenes hominis* or its secreted EVs developed cognitive impairment and showed increased levels of 16S rDNA in the hippocampus [218]. This supports the ability of bacterial EVs to cross the blood-brain barrier and deliver their

cargo to the brain. However, the degree of penetration of microbiota extracellular vesicles (MEVs) into the brain from both the intestine and bloodstream remains to be determined. Therefore, in this study, we used in vitro tissue cell line models of the intestinal epithelial and blood-brain barriers to assess the capacity of isolated MEVs to cross and/or be endocytosed by these host tissue barrier models.

5.2. Materials and Methods

Unless otherwise specified, all the reagents were purchased from Millipore Sigma (Toronto, ON). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, L-glutamine, penicillin/streptomycin, chemically defined lipid concentrate, HEPES, trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA), trypan blue, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Thermo Fisher Scientific (Gibco, Nepean, ON). Tissue culture well plates (12, 24, and 48), flasks (25 and 150 cm2), and inserts (0.4µm, 12 well plates) were purchased from Corning (Maine, USA). Tissue Culture Inserts, 0.4 µm (12 well plates) were purchased from ThinCert (Greiner Bio-One, Monroe, NC). Rat collagen I and recombinant human basic fibroblast growth factor (FGF-2) were purchased from R&D Systems (Toronto, ON). Endothelial cell growth basal medium-2 (EBM-2) was purchased from Lonza (Kingston, ON).

5.2.1. Endocytosis activity

RIN-14 cells (ATCC® CRL 2059) were grown to confluence in complete culture media (CCM) consisting of RPMI-1640 supplemented with 10% heat-inactivated FBS (HI-FBS), 100U/mL penicillin, and 100 mg/mL streptomycin [221]. Caco-2 cells (ATCC HTB

37) were grown to confluence in CCM consisting of DMEM supplemented with 10% HI-FBS, 100U/mL penicillin, and 100 mg/mL streptomycin [222], while hCMEC/D3 cells (Cedarlane Labs, CLU 512) were grown on collagen-coated flasks using CCM constituted of endothelial basal medium (EBM-2) supplemented with 5% HI-FBS, 100U/mL penicillin, 100 mg/mL streptomycin, hydrocortisone (1.4μM), ascorbic acid (5μg/mL), chemically defined lipid concentrate (1%), HEPES (10mM), and basic fibroblast growth factor (1 ng/mL)[223]. All cells were maintained at 37 °C in a 5% CO_2 humidified incubator (VWR, Mississauga, Ontario, Canada). For subculturing, different cells were dissociated using 0.05% trypsin–EDTA, centrifuged, and washed twice with DPBS (pH 7.4) to remove traces of trypsin [224]. Caco-2 cells at a density of 2×10^5 [225] and RIN-14B cells at 2.5×10^5 cells were seeded on 12 well plates [226]. In contrast, hCMEC/D3 cells were seeded at a density of 2 × 105 on a collagen-coated 12-well plate using rat tail collagen (150 μg/mL) [227] and maintained at 37 °C in a 5% CO2 humidified incubator for 2 days (Caco-2 and hCMEC/D3 cells) and 5 days (RIN-14B), with refreshing media every 3-4 days. Microbiota extracellular vesicles (MEVs) equivalent to 2 g of stool were labeled with Cyanine 7 (Cy7), added to each well of different cells, and incubated for 24 h. The conditioning medium containing unbound MEVs was discarded. Cells grown in the absence of labeled MEVs served as negative controls. The cells were washed three times, mounted in DPBS, and imaged using an inverted Zeiss AxioObserver 7 microscope (Carl Zeiss Canada Ltd., Toronto, ON).

5.2.2. Transport activity

5.2.2.1. Caco-2 cells

Caco-2 cells were seeded at a density of $5x10^4$ (P8) onto ThinCert™ tissue culture inserts (12 well plates, 0.4 µm, 108 pores, 1.131 cm2) and then maintained in CCM [228]. The culture media were refreshed every 2 days. The cells were allowed to grow and differentiate for 28 days. On day 28, CCM was replaced with phosphate-buffered saline (PBS), and resistance values (Ω) were measured for all tissue culture inserts, including the blank, using Millicell ERS-II voltohmmeter[229] (MilliporeSigma, Toronto, ON). The TEER values for each insert were calculated using the following equation:

TEER = (R (Cells) - R (Blank)) x tissue culture inserts surface area ($\Omega.cm^2$) [229]

MEVs labeled with fluorescein isothiocyanate (FITC)[230, 231] (equivalent to 0.9, 1.8, and 3.6-grams stool) were suspended in CCM and added to the apical side of the tissue culture inserts. Fresh CCM was added to the basolateral side and the conditioned culture supernatant was collected from the basolateral side at 0, 3, and 24 h. Fluorescence intensity was measured using a TECAN plate reader at an excitation/emission wavelength of 485/535 nm. Conditioned media in the apical and basolateral compartments were replaced with PBS, and TEER values were reported for various treatment groups.

5.2.2.2. hCMEC/D3 cells

For the blood-brain barrier transport study, hCEC/D3 cells were seeded at a density of 5×10^4 onto collagen-coated inserts (12 well plates)[223] using CCM and allowed to differentiate for 4 days. The TEER resistance values were recorded daily. The TEER values

for each insert were calculated by using the aforementioned equations. After identifying the time point showing the peak TEER value, MEVs labeled with Cy7[232] (equivalent to 0.9, 1.8, and 3.6-grams stool) were suspended in CCM and added to the apical side of the tissue culture insert (Corning). Fresh CCM was added to the basolateral side and the conditioned culture supernatant was collected from the basolateral side at 0, 3, and 24 h. The fluorescence intensity was measured using a TECAN plate reader at an excitation/emission wavelength of 743/780 nm. PBS-conditioned media were replaced with PBS in the apical and basolateral compartments, and TEER values were measured for various treatment groups.

5.2.3. Cell Culture Statistical Analysis

To compare the mean values of different results, one-way ANOVA (SPSS, version 23) followed by Tukey's test as a post-hoc test was used to determine the statistically significant difference for data involving more than two experimental groups. A paired sample t-test was used to identify the statistically significant difference between the mean TEER values of Caco-2 and hCMEC/D3 cells before and after MEVs treatment compared to the control. All results are expressed as the Mean ± Standard Error. All figures were designed using Microsoft 365® Apps for enterprise (Excel and PowerPoint) and Adobe Photoshop CS (version 8).

5.3. Results

5.3.1. Endocytic internalization of MEVs by different cell lines

The endocytic activity of different cell lines that model intestinal transport (Caco-2), secretory (RIN-14B), and blood-brain barrier activities (hCMEC/D3) was evaluated by the

addition of Cy7- labeled MEVs to the monolayer culture and compared to the non-treated negative control. Caco-2, RIN-14 B, and hCMEC/D3 cells showed the capacity to internalize MEVs through an endocytic mechanism (**Figure 14**).

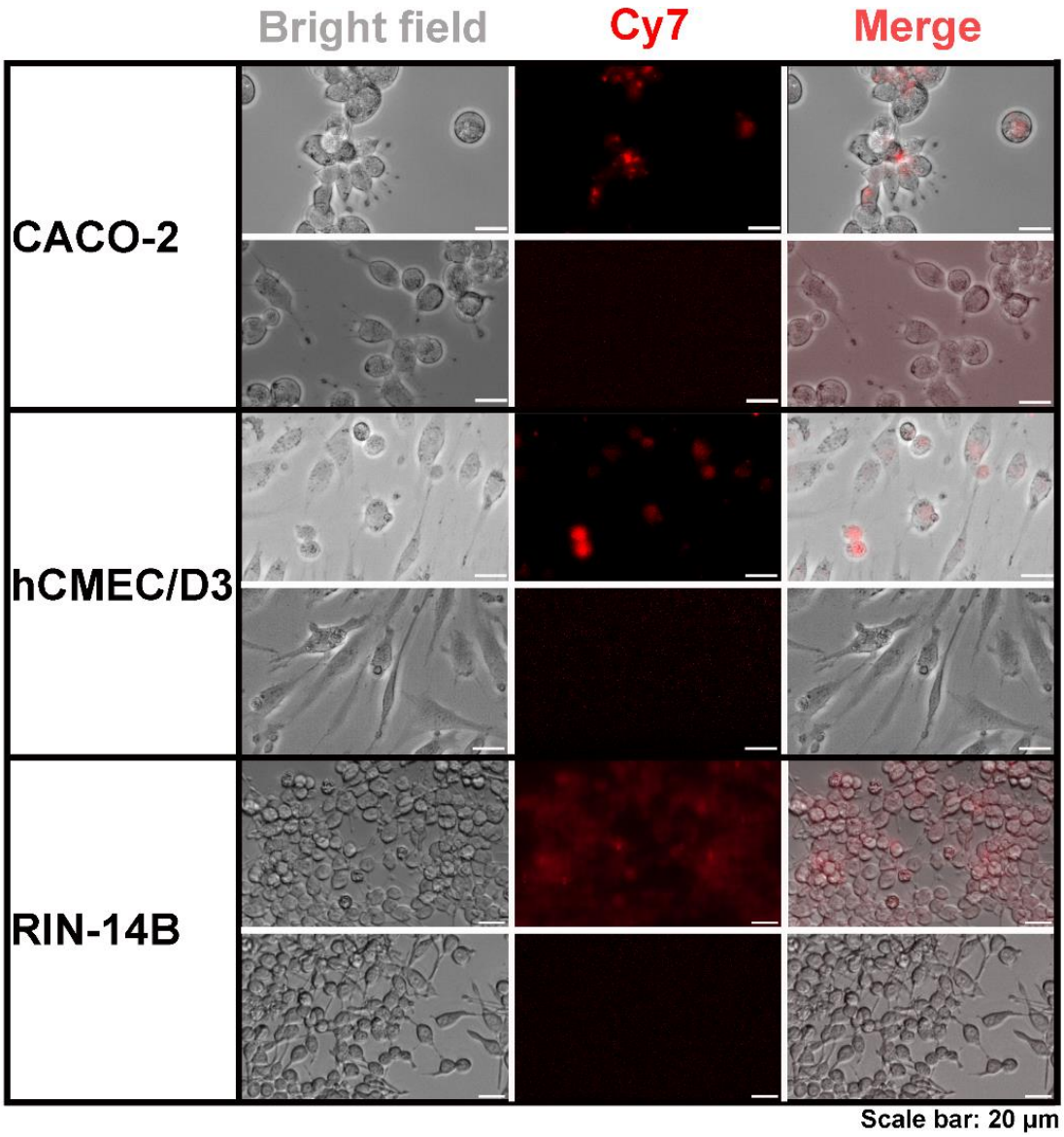

Figure 14. Endocytosis of Cy7-labeled MEVs by various cell types, including CACO-2 (Model of intestinal transport activities), hCMEC/D3 (Model of blood-brain-barrier activities), and RIN-14B (Model of secretory activity).

5.3.2. Transport of MEVs across CACO-2 and hCMEC/D3 cells

FITC-labeled MEVs showed a dose-dependent transport (expressed as paracellular transport (A.U.)) across the intestinal transport model system, CACO-2 cells. The transport of FITC-labeled MEVs (equivalent to 3.6 g stool, 9±1.6 A.U.) was significantly higher after 24 hours as compared to labeled MEVs (equivalent to 1.8 g stool, 4±0.4 A.U., $P<0.01$) and labeled MEVs (equivalent to 0.9 g stool, 2.8±0.5 A.U., $P<0.05$) (**Figure 15**). Exposure of CACO-2 cells to different concentrations of FITC-labeled MEVs for 24 h significantly decreased TEER values. (**Figure 15** and **Table 4**). The hCMEC/D3 monolayer cells grown over the tissue culture inserts reached the maximum TEER value after 48 h. Cy7-labeled MEVs showed dose-dependent transport activity (expressed as paracellular transport (A.U.)) across the blood-brain-barrier model system, hCMEC/D3 cells. The transport of Cy7-labeled MEVs (equivalent to 3.6 g stool, 34.7±0.3 A.U.) was significantly higher after 24 hours as compared to labeled MEVs (equivalent to 1.8 g stool, 17.0±1.2 A.U., $P<0.001$) and labeled MEVs (equivalent to 0.9 g stool, 7.7±0.3 A.U., $P<0.001$). In addition, the transport of Cy7-labeled MEVs (equivalent to 1.8 g stool) was significantly higher after 24 h than that of labeled MEVs (equivalent to 0.9 g stool, $P<0.001$). Exposure of hCMEC/D3 cells to different concentrations of Cy7-labeled MEVs for 24 h did not alter the TEER values (**Figure 15**).

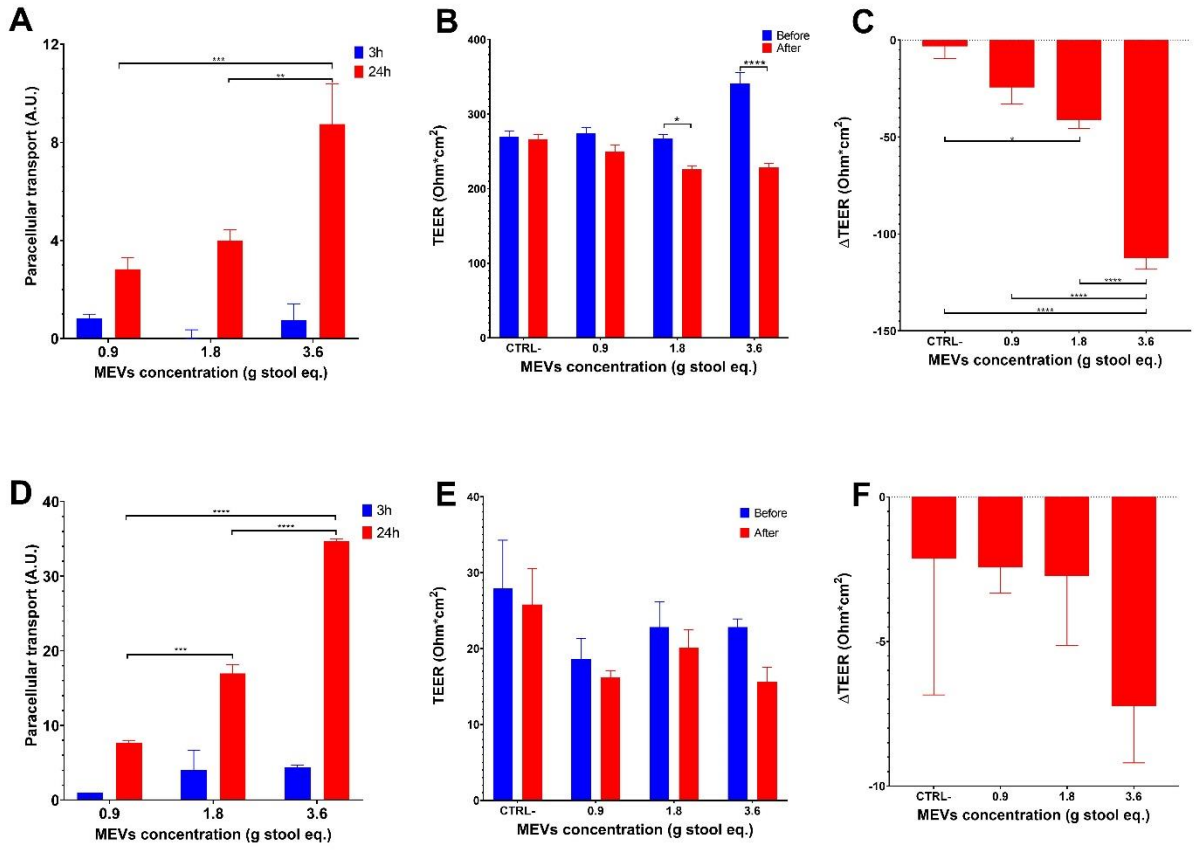

Figure 15.Transport of various concentrations of FITC-labeled MEVs across different cell lines, Caco-2 cells (expressed as paracellular transport (A.U.)). *: P<0.05, **: P<0.01 (A), and TEER values of CACO-2 cells before and after the addition of MEVs (B & C). Transport of Cy7-labeled MEVs across hCMEC/D3 cells (expressed as paracellular transport (A.U.)). ***: P<0.001 (D), TEER values of hCMEC/D3 before and after the addition of MEVs (E & F).

Table 4. TEER values before and after the addition of MEVs to Caco-2 cells compared to the non-treated negative control.

FITC-labeled MEVs	3.6 g stool	1.8 g stool	0.9 g stool	Negative control
TEER values before treatment	354±5.9	270±3.7	277±5.8	277±4.7
TEER values after treatment	230±5.5	229±4.6	249±7.0	263±4.9
Statistical significance	P<0.001	P<0.01	P<0.05	P>0.05

5.4. Discussion

We first tested the capacity of MEVs to cross the epithelial and blood-brain barriers [203]. Our results illustrate the capacity of MEVs to cross and be endocytosed by the intestinal and BBB cell lines. MEVs may cross intestinal barriers and reach distal organs such as the liver and adipose tissues, inducing insulin resistance and glucose intolerance [38]. We found that MEVs reduced TEER resistance in CACO-2 cells. This may explain the reported increased levels of systemic LPS-positive bacterial EVs in humans with intestinal barrier dysfunction, which provides evidence of the capacity of MEVs to reach systemic circulation [167] and deliver and elicit various immunological and metabolic responses in different organs, including the brain. MEVs penetrate hCMEC/D3 human brain endothelial cells as a model of paracellular permeability of the human blood-brain barrier [233]. Similarly, lipophilic EVs, when orally gavaged, injected intravenously or intraperitoneally, were detected in different organs, including the brain [234]. In addition, EVs secreted by *Aggregatibacter actinomycetemcomitans* have been detected in brain monocytes and microglial cells and in their cargo for RNA-induced neuroinflammation [220]. This indicates that MEVs that reach the blood circulation can cross the BBB and deliver their cargo to the brain, thus modulating its functionalities. For instance, *Lactiplantibacillus plantarum*-secreted EVs have been reported to suppress the stress-induced reduced hippocampal expression of proBDNF and BDNF (Brain-derived neurotrophic factor) in chronic restraint stress-treated (CRST) mice [39]. The same study postulated that intraperitoneally injected *L. plantarum EVs* may reach the brain and induce direct genomic changes in brain cells [39].

Additionally, EVs of the gut bacterium *Paenalcaligenes hominis* have been reported as a risk factor for vagus-nerve-mediated cognitive impairment [235]. Collectively, these findings indicate that MEVs employ different pathways to cross the host barriers, including the intestinal and blood-brain barriers, to reach distant organs where they may release their cargo that modulates organ functionality.

CHAPTER 6 : General Discussion and Conclusions

Microbiota extracellular vesicles (MEVs) are nanoparticles with lipid membrane boundary secreted by gut microbial residents. They attracted the research attention due to their role in cell-cell communication either among microbial cells or between host and microbes [15]. This research project characterized extracellular vesicles released by stool microbiota and *ex vivo*-developed microbiota using multi-omics approaches. Metabolomic characterization identified a broad set of metabolites, including four categories of lipids and metabolites known to be involved in neuronal processes, such as precursors and regulators of neural activities. For example, arachidonyl-dopamine (NADA), gabapentin, glutamate, and N-acylethanolamines have been detected in the cargos of MEVs. NADA, for example, is a widespread endocannabinoids with the ability to manage neuroinflammation beside other physiological and pharmacological effects [206]. The identified neuroactive metabolites positively correlated with *Bacteroides* spp. Previous studies have detected GABA in the cargo of EVs released by *Bacteroides fragilis* [207]. In agreement with this, our proteomic analyses detected that *Bacteroides* generated *gadB* protein among the total protein content of MEVs. A previous transcriptomic analyses of human stool samples have reported that GABA metabolic pathways are actively expressed by mainly *Bacteroides* and *Parabacteroides* spp. [13]. In addition, the relative abundance of *Bacteroides* in stool were detected to be negatively correlated with depression associated brain signatures [13]. Metaproteomic analyses also detected many proteins linked to neuronal processes, such as the glutamine/glutamate/GABA pathway, inositol biosynthesis, microbiota-derived

trimethylamine (TMA), and cellular energy production. Proteins involved in these neuronal processes have arisen from a wide array of microbiota members, including Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria.

Next, we tested the routes by which MEVs could penetrate the host tissue barriers. Caco-2, RIN-14B, and hCMEC/D3 cells have the capacity to internalize MEVs through an endocytic mechanism. MEVs also exhibited dose-dependent paracellular transport through Caco-2 intestinal cells and hCMEC/D3 brain endothelial cells. In agreement with this, the outer membrane vesicles secreted by *Aggregatibacter actinomycetemcomitans* have been shown to cross blood brain barrier to deliver it's RNA content to brain monocytes and induce the secretion of proinflammatory cytokines [220]. Lee and coauthors have also shown that the exracellular vesicles of *Paenalcaligenes hominis* is a risk factor for cognitive impairment [235]. Together, this indicates that MEVs are capable to reach the brain and deliver their cargo to the brain where it could affect the cognitive and mental health. Previous reports have shown that EVs released by different bacterial pathogens exhibit different routes of crossing host barriers [217, 218]. Therefore, the heterogeneous nature of MEVs may explain the employment of different transmission routes across host barriers.

Overall, our results provided pioneering and significant insights into MEVs' capacity to transfer neuroactive metabolites to the host intestine and other organs, including the brain, filling some gaps in knowledge of the mechanisms underlying microbiome-gut-brain interactions.

Future directions in this area of research would be:

- *In vivo* trafficking of biodistribution of orally gavaged and IV administered MEVs in mice organs.
- Investigating the difference in the contents of MEVs between a healthy microbiome cohort and the microbiome of subjects with mental health disorders.
- Testing the capacity of MEVs isolated from healthy subjects to modulate the microbiome of a cohort of mental health disorders.
- Isolate EVs of potentially neuroactive strains and compare them to non-neuroactive control strains regarding their content, host barriers crossings, impact on behavior, and microbiome of mice models of mental disorders.

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