EXTRACELLULAR VESICLES IN BACTERIA-HOST COMMUNICATION

CHARACTERIZING THE ROLE OF EXTRACELLULAR VESICLES

IN BACTERIA-HOST COMMUNICATION

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LAY ABSTRACT

Recent research shows that the trillions of microorganisms residing in our gut influence our development, brain function, and immunity, through mostly unknown mechanisms. To learn more, we gave pregnant mice penicillin to kill bacteria in their gut and found that this altered the brain and behaviour of their adult offspring. However, it is unclear how these bacteria could influence fetal mice in the womb. To test a potential mechanism, we investigated one type of bacteria and found that it naturally produces nanoparticles that contain immune-modulating cargo and are internalized by cells of the gut lining. When we fed these bacteria to mice, we discovered that the same nanoparticles were present in their bloodstream within 2.5 hours. This suggests that gut bacteria can produce nanoparticles that traverse the gut lining into the circulatory system, which may be one mechanism by which bacteria influence the organism they inhabit.

ABSTRACT

The gastrointestinal tract contains trillions of symbiotic microorganisms (microbiota) that are critical for normal immunity, physiology, and development. Yet the extent to which these microbes influence neurodevelopment, and the mechanisms they use to do so, are poorly characterized. Using a mouse model, we show that perturbations of the maternal microbiota by treatment with low-dose penicillin during the last week of pregnancy alters behaviour and microbiota composition in adult offspring. These changes were sex-specific; female offspring had reduced anxiety-like behaviours, while males showed abnormal social behaviours, which correlated with altered hippocampal gene expression and reduced regulatory T cells. Microbiota composition was distinct between sexes and from untreated controls, suggesting that antibiotic exposure altered microbiota, which may have mediated other changes seen. To investigate a mechanism by which gut microbes may influence distal organ systems, we focused on the bacterium *Lacticaseibacillus rhamnosus* JB-1. We found that membrane vesicles (MV) produced by JB-1 contain lipoteichoic acid, which activates Toll-like receptor 2 (TLR2) and induces interleukin-10 production by dendritic cells. We further showed that JB-1 MV are internalized by human and mouse intestinal epithelial cell lines in a clathrin-dependent manner in culture, and by mouse intestinal epithelial cells *in vivo*. We then fed JB-1 bacteria to mice and showed that within 2.5 hours there are functional nanoparticles in their blood that reproduce effects associated with the fed bacteria. Plasma nanoparticles from fed mice had a size distribution distinct from that of saline-fed mice.

They also activated TLR2 and induced interleukin-10 production by dendritic cells via lipoteichoic acid. These nanoparticles are likely bacterial MV as they contained bacterial protein and DNA from a novel bacteriophage in the original fed bacteria. Altogether these experiments support a role for microbiota in neurodevelopment and demonstrate novel nanoparticulate mechanisms of bacteria-host communication that may underlie their systemic influence.

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A33	Cell surface A33 antigen
ANOVA	Analysis of variance
AVPR1A	Arginine vasopressin receptor 1A
AVPR1B	Arginine vasopressin receptor 1B
BDNF	Brain-derived neurotrophic factor
BMDCs	Bone marrow-derived dendritic cells
BMP-2	Bone morphogenetic protein 2
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFDA SE	Carboxyfluorescein diacetate succinimidyl ester
CFSE	Carboxyfluorescein succinimidyl ester
CNS	Central nervous system
DCs	Dendritic cells
DiO	3,3-Dioctadecyloxacarbocyanine perchlorate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EcN	*Escherichia coli* Nissle 1917
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nervous system
EV	Extracellular vesicles
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDR	False discovery rate
FMO	Fluorescence minus one
FOXP3	Forkhead box P3
GABA	γ-aminobutyric acid
GO	Gene ontology
GR	Glucocorticoid receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IEC	Intestinal epithelial cell
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IKca	Intermediate-conductance Ca^{2+}-dependent K^{+}
IL	Interleukin
JB-1	*Lacticaseibacillus rhamnosus* JB-1
LC-MS	Liquid chromatography-mass spectrometry
LDA	Linear discriminant analysis

LEfSe	Linear discriminate analysis effect size
LTA	Lipoteichoic acid
Ly6C	Lymphocyte antigen 6 complex
MAMPs	Microbe-associated molecular patterns
MHC	Major histocompatibility complex
MRS	Man-Rogosa-Sharpe medium
MV	Membrane vesicles
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa B
NTA	Nanoparticle tracking analysis
OMV	Outer membrane vesicles
OXTR	Oxytocin receptor
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PND	Postnatal day
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
qPCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPM	Revolutions per minute
rRNA	ribosomal ribonucleic acid
TBS-T	Tris-buffered saline with Tween
TCEP-HCl	Tris(2-carboxyethyl)phosphine hydrochloride
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
TRPV1	Transient receptor potential cation channel subfamily V member 1

DECLARATION OF ACADEMIC ACHIEVEMENT

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CHAPTER 1. Introduction

The human gastrointestinal tract is home to trillions of diverse microorganisms, collectively referred to as the gut microbiota. These microbes are not just passengers; rather, they are active participants in our physiology. For this reason it has been argued that animals are best thought of as *holobionts*: not independent organisms defined by a single genome, but rather evolving in concert with the bacteria, archaea, fungi, and other microorganisms that have accompanied them throughout evolutionary history (Zilber-Rosenberg & Rosenberg, 2008). Indeed, the gut microbiota have been called a *forgotten organ* (O'Hara & Shanahan, 2006), owing to the fact that gut microbes are crucial in digestion, metabolism, and even immunity from disease. However, despite the now well-recognized importance of gut microbes to health and well-being (Champagne-Jorgensen & McVey Neufeld, 2021), much remains unknown as to the mechanisms by which they influence their host and the impacts they have as a result.

1.1. Methods of studying gut-host interactions

Studying how the microbiota and host bidirectionally communicate is challenging. There are approximately as many bacteria in the gastrointestinal tract as there are human cells in the body (Sender, Fuchs, & Milo, 2016). These trillions of microorganisms encompass hundreds of species (The Human Microbiome Project Consortium, 2012), all of which are continually competing and cooperating with each other and their host within distinct ecological niches. This complexity renders it difficult

to disentangle which microbes, if any, are key in communicating with the host, and to determine what methods they use to do so.

1.1.1. Observational evidence

It is possible to infer microbiota influences by population-based correlational studies in humans or model organisms. By measuring a proxy of microbiota composition (e.g., bacterial 16S ribosomal RNA) in fecal material or using intestinal sampling methods, microbiota composition can be related to health outcomes in much the same way as is done for genome-wide association studies. Such research has demonstrated striking relationships between human microbiota composition and obesity (Kalliomäki, Carmen Collado, Salminen, & Isolauri, 2008; Korpela et al., 2017), asthma (Arrieta et al., 2015; Stiemsma et al., 2016), and even neurological disorders such as multiple sclerosis, Parkinson's disease, and autism spectrum disorder (Cryan, O'Riordan, Sandhu, Peterson, & Dinan, 2019). A major difficulty with this approach, however, is that it is generally not possible to infer causality. Not only can microbiota influence the host, but in turn microbiota composition is altered by host genetics and life history (Blaser & Falkow, 2009). Moreover, both microbiota composition and host physiology can be directly or indirectly influenced by external factors, including diet, environment, and disease (Blaser & Falkow, 2009). For these reasons, most informative studies have been experimental animal work, using methods described below.

1.1.2. Germ-free animal models

One approach is to simplify the problem. Instead of studying organisms with typical gut ecologies, animals have been generated in sterile environments without colonization by any microbes. This approach represents a baseline to reveal aspects of host physiology that microbiota affect, and has been used to demonstrate that mammals require microbes for normal development. Germ-free mice have underdeveloped immune tissues, reduced immune responsivity, and altered structure and function of the gastrointestinal epithelium (Luczynski, McVey Neufeld, et al., 2016). Surprisingly, they also have abnormal brain and behaviour, including exaggerated stress responsivity (Sudo et al., 2004), reduced anxiety-like behaviour (Heijtz et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011), and altered social behaviours (Arentsen, Raith, Qian, Forssberg, & Heijtz, 2015; Desbonnet, Clarke, Shanahan, Dinan, & Cryan, 2014), among others (Luczynski, McVey Neufeld, et al., 2016).

A benefit of the germ-free animal model is that it is possible to introduce them to microorganisms at defined periods of time, and thereafter measure which abnormalities are ameliorated. Some such changes can be normalized even in adulthood, while for others there appear to be developmental windows or critical periods during which these systems develop and after which changes do not occur (Borre et al., 2014). For example, Sudo and colleagues (2004) found that bacterial colonization of germ-free mice resolved abnormal stress-induced corticosterone release, but only if done prior to 6 weeks of age. Similarly, Desbonnet and colleagues (2014) demonstrated that exposure of germ-free

mice to bacteria after weaning normalized some aspects of abnormal social behaviours, but not all, indicating that some microbial influences on brain and behaviour occur chronically, while others are mediated by induced developmental changes.

However, a major limitation of the germ-free model is that it represents an ecologically unrealistic physiological state, with developmental abnormalities spanning bodily systems and likely compounding one another. This renders it difficult, if not impossible, to disentangle the mechanisms underlying germ-free phenotypes. It does, however, underline the fundamental connectivity between bodily systems previously considered unrelated, particularly the interface between gut microbes, immunity, and the central nervous system (CNS), as elaborated in later sections.

1.1.3. Antibiotics for temporally specific disruptions

It is not necessary to eliminate all contact with microorganisms to study their influence on animals. One alternative approach is to use anti-microbial agents to perturb the microbiota at different stages of life and examine how this influences the host. This allows the host to develop normally until antibiotic treatment, avoiding serious developmental abnormalities that otherwise render it difficult to test the role of microbes at specific life stages. It is also possible to tailor antibiotics used toward certain types of microbes, enabling more targeted research (though this approach has seen little use). However, there are also challenges to using antibiotics to study gut microbial influence, as we have previously argued (Champagne-Jorgensen, Kunze, Forsythe,

Bienenstock, & McVey Neufeld, 2019). For example, most antibiotics used are broad-spectrum, and can have long-term impacts on community composition that render experiments difficult to interpret. Moreover, antibiotics themselves can have direct impacts on host tissues. Exposure to individual antibiotics rapidly alters intestinal motility *ex vivo* in the absence of microbes (Delungahawatta et al., 2017). Moreover, many antibiotics can cross the blood-brain barrier (Nau, Sörgel, & Eiffert, 2010) and have been associated with wide-ranging, though limited, neurotoxicity and psychiatric effects in humans (Zareifopoulos & Panayiotakopoulos, 2017) that are likely independent of their antimicrobial effects (Champagne-Jorgensen et al., 2019).

Despite these caveats, research using antibiotics to probe the microbiota-gut-brain axis in animal models has been fruitful. Well-designed studies have shown that antibiotic treatment can produce effects similar to those seen in germ-free animals, including altered corticosterone activity (Fröhlich et al., 2016), anxiety-like behaviours (Bercik et al., 2011; Desbonnet et al., 2015), and social behaviours (Guida et al., 2018), among others. While direct influence of antibiotics on these systems may contribute, the similarities with germ-free phenotypes and the use of extensive negative controls (e.g., (Bercik et al., 2011)) suggests that perturbation of the microbiota is involved.

1.1.4. Supplementation with defined bacteria

It is also possible to study the influence of specific microorganisms in the context of a normal microbiota, by introducing bacteria of interest into the gastrointestinal tract

and measuring outcomes. While it can be difficult to infer exactly what mediates any changes seen (e.g., direct effects on the host versus indirect effects through microbiota modulation), such studies can still conclude that bacteria influence their host in defined experimental systems. Bacteria introduced can be pathogenic, neutral, or beneficial. In the latter case, and when used clinically, these organisms are often referred to as probiotics. Probiotics have been defined as live microbes that confer a health benefit on the host (Hill et al., 2014). The extent to which particular bacteria meet these criteria, especially in humans (Suez, Zmora, Segal, & Elinav, 2019), is contentious, however.

Experiments treating animals with specific microbes have demonstrated that bacteria can have significant effects on the host even in the presence of a normal microbiota. Some of the first evidence for this was suggested by Elie Metchnikoff in the early 1900s, who suspected that consumption of fermented dairy products was responsible for increased longevity in some populations (Gasbarrini, Bonvicini, & Gramenzi, 2016). Since then, consumption of defined strains of bacteria has been shown to reduce intestinal inflammation, promote systemic immune regulation, and even attenuate visceral pain (Bienenstock, Kunze, & Forsythe, 2015; Sommer & Bäckhed, 2013). Individual bacteria can also modulate brain and behaviour, including stress responsivity, anxiety- and depression-like behaviours, and learning and memory (Sarkar et al., 2016). Altogether, it is now well-accepted that consumption of individual strains or defined communities of bacteria is a valuable tool for assessing bacterial influence.

1.2. Influence of gut microbes on development

Mammalian development assumes the presence of a microbiota, but precisely when gut microbial communities are established is controversial. Until recently, it was assumed that infants are first exposed to live microbes only after leaving the birth canal. Microbiota development is strongly associated with birth route (Dominguez-Bello et al., 2010) and several studies have failed to find evidence for cultivable bacteria (Perez-Muñoz, Arrieta, Ramer-Tait, & Walter, 2017) or even bacterial nucleic acids (de Goffau et al., 2019; Kuperman et al., 2020) in fetal samples. Moreover, the fact that microbe-free mammals, including humans, can be delivered by caesarian section convincingly argues against the possibility of *in utero* intestinal colonization (Hornef & Penders, 2017; Perez-Muñoz et al., 2017). Despite this, over the past decade several studies have questioned the sterility of the womb, finding either cultivable bacteria (Collado, Rautava, Aakko, Isolauri, & Salminen, 2016; Jiménez et al., 2005, 2008) or microbial nucleic acids (Aagaard et al., 2014; Collado et al., 2016) in fetal and associated tissues. Though it has been argued that these results can be better explained by contamination or non-live microbial components (de Goffau et al., 2019; Perez-Muñoz et al., 2017), the topic remains contentious. Regardless of outcome, what is certain is that specific types of microbes are acquired from the mother during birth (Dominguez-Bello, Godoy-Vitorino, Knight, & Blaser, 2019). In mammals, these include bacteria that are specialized for digesting milk, selectively nurturing a subset of organisms that lay the foundation for

future microbiota development and concomitantly influence development of gut, immune, and nervous systems (Dominguez-Bello et al., 2019).

1.2.1. Gut microbes influence immune development

Research with germ-free animals has demonstrated widespread developmental influences of gut microbes. Mice raised in sterile environments have immature immune systems, with decreased expression of immune receptors, limited intestinal lymphoid tissues, and dampened immune responses to pathogen signals (Luczynski, McVey Neufeld, et al., 2016; Sommer & Bäckhed, 2013). Gastrointestinal function is also abnormal, with lower intestinal surface area for absorption, deficient digestion and metabolism, and altered enteric nervous system (ENS) activity (Luczynski, McVey Neufeld, et al., 2016), among others (Sommer & Bäckhed, 2013). Microbiota also influence the development and function of systemic immune components. For example, germ-free mice have fewer and less active regulatory T cells (Östman, Rask, Wold, Hultkrantz, & Telemo, 2006), which are important for moderating immune responses to microbial components.

1.2.2. Gut microbes influence neurodevelopment

Microbiota also have a profound influence on nervous system development. In the enteric nervous system, germ-free mice have deficient glial populations (Kabouridis et al., 2015), decreased nerve density (Collins, Borojevic, Verdu, Huizinga, & Ratcliffe,

2014), and immature neuronal network anatomy (De Vadder et al., 2018). Functionally, they have decreased excitability of some myenteric neurons (McVey Neufeld, Perez-Burgos, Mao, Bienenstock, & Kunze, 2015; Neufeld, Mao, Bienenstock, Foster, & Kunze, 2013) and altered intestinal motility (Abrams & Bishop, 1967). Moreover, in adult animals, bacteria continually promote intestinal neurogenesis via Toll-like receptor 2 (TLR2) signalling (Yarandi et al., 2020), and conventionalization of mice with a normal microbiota later in life can reverse many of these deficits (De Vadder et al., 2018; McVey Neufeld et al., 2015).

Intestinal microbes also appear to influence the development of central nervous system structure and function. This was first suggested in a landmark paper by Sudo and colleagues, who found that germ-free mice have an exaggerated hypothalamic-pituitary-adrenal stress response that is reversible if animals are exposed to a normal microbiota at only a young age (Sudo et al., 2004). Subsequent work has shown that adult germ-free mice have altered brain morphology and size (Luczynski, Whelan, et al., 2016), enhanced hippocampal neurogenesis (Ogbonnaya et al., 2015) and increased blood-brain barrier permeability (Braniste et al., 2014). They are also behaviourally distinct from microbiota-containing mice. Germ-free mice have increased hypothalamic-pituitary-adrenal axis activity (Neufeld et al., 2011; Sudo et al., 2004), but reduced anxiety-like behaviours (Heijtz et al., 2011; Neufeld et al., 2011). They also have altered social behaviours (Arentsen et al., 2015; Desbonnet et al., 2014), though the direction of changes is inconsistent between studies.

Similar results have been found using transient antibiotic-induced microbiota perturbations throughout defined developmental periods in mice. Degroote and colleagues demonstrated that antibiotic treatment of pregnant dams until the last week before birth resulted in offspring with reduced social behaviour and possibly altered anxiety-like behaviours (Degroote, Hunting, Baccarelli, & Takser, 2016). Tochitani and colleagues found altered anxiety-like behaviour of offspring from mice exposed to antibiotics mid-gestation (Tochitani et al., 2016). Our group found similar results after treating pregnant mice with low-dose penicillin from the last week prenatal until weaning, in that adult offspring demonstrated reduced social behaviours and altered anxiety-like behaviours (Leclercq et al., 2017), and subsequent studies in mice have furthered this line of inquiry (Champagne-Jorgensen et al., 2020; Kayyal et al., 2020).

1.3. Bacteria-host interactions in steady-state

In conventionally colonized animals, gut microbiota are constantly interacting and communicating with each other and their host. Most microbes reside in the lumen of the gastrointestinal tract, prevented from proximity to intestinal epithelial cells by a layer of polymerized mucin glycoproteins (Burgueño & Abreu, 2020). The gut epithelium itself acts as a gate to allow diffusion and selective transport of nutrients, water, and ions into the intestinal tissue while restricting other macromolecules and microbes to the lumen (Sharkey, Beck, & McKay, 2018). It also directly modulates the microbiota by secreting or transporting antimicrobial peptides, antibodies such as IgA, and other

compounds (Abbas, Lichtman, & Pillai, 2011). In homeostasis, this results in relatively stable populations of mutualistic gut microbes that aid in digestion, produce nutrients, and contribute to immunity by outcompeting opportunistic pathogens and promoting immune tolerance (O'Hara & Shanahan, 2006).

1.3.1. Immune tolerance of gut microbes

The gastrointestinal tract is the dominant immune organ in mammals. It contains a substantial fraction of bodily immune cells and is constantly exposed to non-self *antigens* (foreign compounds) from ingested substances, environmental microbes, and the indigenous microbiota (Yoo & Mazmanian, 2017). A major challenge therefore is to distinguish between harmless ingested substances and those that are potentially dangerous. Part of this is due to immunological compartmentalization, in that gut-derived antigens are primarily processed in local immune tissue that is biased towards a non-inflammatory tolerant phenotype (Konrad, Cong, Duck, Borlaza, & Elson, 2006; Macpherson & Uhr, 2004; Mowat, 2018). By contrast, gut bacterial antigens that pass the gut epithelium and enter systemic circulation can induce an inflammatory immune response as it would to a pathogen (Konrad et al., 2006). It is also possible for certain antigens, such as those from food, to pass into systemic circulation and yet still not elicit an immune response. This concept is termed *immune tolerance* (Abbas et al., 2011).

The mammalian immune system can be divided into two domains. Innate immunity involves germline-encoded mechanisms to rapidly detect and respond to

evolutionarily conserved signals associated with broad categories of microbes (termed microbe-associated molecular patterns [MAMPs] or pathogen-associated molecular patterns [PAMPs]), while adaptive immunity refers to immune mechanisms, mediated by B and T lymphocytes, that learn to recognize and target idiosyncratic features of specific pathogens (Abbas et al., 2011). This latter feature is key to immune tolerance. As lymphocytes are trained to recognize a unique pathogen-associated feature (antigen), they are also screened to ensure that they do not react to any antigens considered harmless, including the body's own proteins. Precisely how non-self but innocuous antigens, such as those from food and microbiota, are granted immune tolerance, while those of pathogens are not, is complex and remains incompletely understood.

It is important to note that microbes are not purely passive in this process, however. Gut microbiota influence both development and maintenance of immunoregulatory lymphocyte subsets, thereby improving tolerance not only of themselves, but of other innocuous antigens. Indeed, Rook and colleagues have proposed that childhood exposure to diverse microbes is crucial for healthy development of the immune system (Rook, Raison, & Lowry, 2014). In addition to work with germ-free mice described previously, evidence for this comes from the fact that some bacteria have been associated with the presence of specific immune subsets. For example, Ivanov and colleagues demonstrated that the presence of segmented filamentous bacteria in the microbiota of C57BL/6 mice from one supplier, but not those of a different supplier, was sufficient to explain differences in number of a subset of

effector T cells (T_H17) cells between those two groups of mice (Ivanov et al., 2009). And interestingly, while T_H17 cells induced by pathogens are often inflammatory, those induced by segmented filamentous bacteria are not (Omenetti et al., 2019). This distinction of different cellular responses to different bacterial taxa appears to be common between gut microbes and pathogens, but the underlying mechanisms are still actively investigated.

1.3.2. Gut microbes and immunity

Evidence suggests that innate immune processes may be involved in differentiating between beneficial microbes and harmful ones. Innate immunity is evolutionarily ancient; pattern-recognition receptors (PRRs) are found in organisms as simple as corals (Fitzgerald & Kagan, 2020), and evidence is accumulating that they evolved, at least in part, to enable symbioses between microbes and host (Chu & Mazmanian, 2013).

The relationship between microbes and PRRs is complicated. As initially hypothesized by Janeway (1989), PRRs are crucial as an early warning or danger signal to alert the immune system to pathogenic microbes and produce immediate anti-microbial responses. Yet stimulation of PRRs is also required to enable intestinal colonization by commensal microbes and promotes immune tolerance towards the same (Chu & Mazmanian, 2013).

The most thoroughly studied PRRs in mammals are the Toll-like receptors (TLRs). In humans there are 10 TLRs, while mice have 12 (Fitzgerald & Kagan, 2020). Each TLR detects a different set of ligands, with a broad range of specific agonists. All TLRs, except for TLR3, signal via the same general mechanism. TLR interaction with its ligand causes dimerization with a second TLR, resulting in coordination of their cytosolic tails and inducing downstream signalling via a MyD88-dependent supramolecular organizing centre, eventually inducing NF-κB transcription factor activity and promoting production of appropriate cytokines and other mediators (Fitzgerald & Kagan, 2020). Nonetheless, despite this conserved signalling cascade, TLR activation does not necessarily result in the same cellular effects for every agonist.

One of the best-studied examples of this is TLR2, a cell surface PRR with specificity for a broad range of ligands, including some gram-negative lipoproteins, gram-positive lipoteichoic acids, peptidoglycan, and others (Kawai & Akira, 2010). TLR2 forms heterodimers with TLR1 to interact with triacylated gram-negative lipopeptides, and dimerizes with TLR6 to recognize diacylated lipopeptides and lipoteichoic acids from gram-positive bacteria (Kawai & Akira, 2010; Oliveira-Nascimento, Massari, & Wetzler, 2012). TLR2 plays important roles in anti-bacterial immunity, but it is also crucial for promoting immune tolerance by gut bacteria and thereby permitting their intestinal colonization (Chu & Mazmanian, 2013; Round et al., 2011).

It is unclear how TLR2 signalling has such divergent effects, but its outcome appears to depend on the nature of the agonist and presence of coreceptors (Oliveira-

Nascimento et al., 2012). A major class of ligands for TLR2 is gram-positive lipoteichoic acid (LTA). LTAs are membrane-bound polymers whose structures are variable between different bacterial species (Shiraishi, Yokota, Fukiya, & Yokota, 2016). They are associated with the peptidoglycan cell wall and may contribute to its structure (Kleerebezem et al., 2010). LTAs were initially studied as a gram-positive analogue to the highly inflammatory gram-negative lipopolysaccharide (Lebeer, Claes, & Vanderleyden, 2012), but evidence in recent years suggests that this is an incomplete understanding. While LTA from some pathogens, such as *Staphylococcus aureus*, has significant pro-inflammatory effects, this appears to depend on LTA concentration and the presence of other MAMPs (Kim, Jung, Kim, & Chung, 2014; Saito, Lin, & Wu, 2019; Saito et al., 2020). Effects may also depend on LTA structure; genetically altered LTA has been shown to influence its immunogenicity (Claes et al., 2012; Grangette et al., 2005; Smelt et al., 2013), and LTAs from beneficial microbes, such as *Lactiplantibacillus plantarum*, appear to inhibit the pro-inflammatory effects of other LTA (Kim, Park, Kim, & Chung, 2020) and may have anti-inflammatory effects on their own (Kim, Lee, et al., 2020; Saito et al., 2020).

1.3.3. Gut microbes and the enteric nervous system

The gastrointestinal tract is innervated and encompassed by the ENS, a network comprising about as many neurons as the spinal cord and capable of functioning without CNS input (Furness, 2012). The ENS regulates peristalsis, digestion, and fluid balance (Furness, 2012). It is also in direct communication with gut immune and epithelial cells, and directly and indirectly with the gut microbiota (Sharkey et al., 2018). These connections are bidirectional. Enteroendocrine cells (which secrete hormones in response to luminal stimuli) form synapse-like processes that functionally synapse onto enteric neurons, which may be involved in transmitting precise gut sensory signals to adjacent neurons (Bohórquez et al., 2015). Moreover, gut microbiota can influence enteric neurons via immune cells; microbes induce production of BMP-2 by macrophages, which activates enteric neurons and modulates intestinal peristalsis (Muller et al., 2014). In turn, neurons can influence immune cells such as mast cells (MacQueen, Marshall, Perdue, Siegel, & Bienenstock, 1989; Stead et al., 1987) and lymphocytes (Felten, Ackerman, Wiegand, & Felten, 1987) via synapse-like processes (Stead et al., 1987; Suzuki et al., 1999). A seminal example of neuronal modulation of immunity is the description of the cholinergic anti-inflammatory pathway, whereby acetylcholine release by the efferent vagus nerve attenuates pro-inflammatory cytokine release by immune cells and can thereby prevent inflammation *in vivo* (Borovikova et al., 2000).

Gut microbiota can also influence enteric neurons in ways thought to be independent of immune cells. For example, *Lacticaseibacillus rhamnosus* JB-1 can increase excitability of afterhyperpolarization neurons in the myenteric plexus by inhibiting a calcium-dependent potassium channel (IK_{Ca}) (Kunze et al., 2009). Importantly, this was not a direct effect of JB-1 on neurons as bacterial components alone only influenced neuronal activity when exposed to the adjacent epithelium but not when directly applied to the same neurons (Al-Nedawi et al., 2015). Indeed, these afterhyperpolarization neurons project to the epithelium, but do not penetrate it, suggesting that bacterial signalling to these nerves may be mediated by intestinal epithelial cells (Sharkey et al., 2018). Bacterial signals may also directly modulate neuronal activity. This has been well-explored with the bacterium *Limosilactobacillus reuteri* DSM17938, which, through an unknown component, appears to reduce visceral pain in rodents by inhibiting the TRPV1 receptor on dorsal root ganglion neurons (Perez-Burgos et al., 2015).

1.3.4. Gut microbes and nervous communication

The influence of gut microbes is not limited to local enteric nerves. The ENS is innervated by extrinsic nerve endings including spinal nerves and the vagus nerve, which transmit sensory information and are involved in intestinal homeostasis (Ratcliffe, 2011). Of these, the vagus nerve has been most extensively studied as a conduit of communication between the brain, gut, and microbiota. The vagus comprises both

afferent (sensory) and efferent (motor) axons, and projects from the medulla to innervate most of the gastrointestinal tract (Furness, Callaghan, Rivera, & Cho, 2014). Vagal afferents include primary sensory nerve endings, but also synapse onto ENS myenteric neurons (Perez-Burgos, Mao, Bienenstock, & Kunze, 2014). It has been known for decades that components of bacterial pathogens can signal via the vagus nerve to influence host behaviour independent of systemic inflammation (Konsman, Luheshi, Bluthé, & Dantzer, 2000; Lyte, Li, Opitz, Gaykema, & Goehler, 2006). For example, Lyte and colleagues demonstrated that inoculation of mice with the pathogen *Citrobacter rodentium*, at doses too low to elicit an observable immune response, increased anxiety-like behaviour in infected mice in a manner that may have been vagal-dependent (Lyte et al., 2006). Subsequent work has shown that other stimuli may also signal via the vagus nerve. For example, we have recently shown that the vagus nerve is required for the efficacy of anti-depressive selective serotonin reuptake inhibitors in mice (Neufeld et al., 2019). Moreover, while mice fed with the bacterium *L. rhamnosus* JB-1 have regional brain activation within 2.5 hours and develop an anxiolytic-like and anti-depressant-like phenotype after long-term feeding, these effects are mostly absent in animals where the vagus has been severed (Bharwani et al., 2020; Bravo et al., 2011).

1.3.5. Neuro-immune interactions in the central nervous system

Neuro-immune crosstalk extends beyond the ENS and its local immune cells. Research is beginning to uncover numerous unexpected roles for immune cells in brain

function (Kipnis, 2016). This has come as a surprise, because the brain is thought to be free of microorganisms (though some research questions this, e.g., Branton et al., 2013) and for years was thought to be an immune-privileged site incapable of inducing an immune response (Medawar, 1948). This is in part because the brain is separated from systemic circulation by the blood-brain barrier, which typically prevents immune cells from translocating into brain tissue. During infection only a certain subset of immune cells can enter the parenchyma, and their responses are relatively suppressed relative to other tissues (Klein & Hunter, 2017). Conceptually, this makes sense, as neurons of the CNS are fragile and have a limited capacity for regeneration (Roig-Puiggros et al., 2020). Given that they are responsible for crucial aspects of host function, inflammatory responses in brain tissue can lead to widespread incidental neuron death with drastic consequences for the organism (Prinz & Priller, 2017).

Nonetheless, research has begun to demonstrate that, rather than being absent of immunity, the brain is instead a highly immune-specialized site. The brain is protected in the steady-state by specialized macrophage-like cells termed microglia. These cells not only monitor for infection, but also are crucial for neurodevelopment, maintenance, and learning (York, Bernier, & MacVicar, 2018). They are also influenced by gut microbiota, and require exposure to circulating bacterial signals such as short chain fatty acids to develop and function normally (Erny et al., 2015). Non-immune brain cells are also active participants in neuroimmunity. Astrocytes and neurons express many PRRs (Kigerl, de Rivero Vaccari, Dietrich, Popovich, & Keane, 2014) and have distinct anti-

microbial responses. For example, astrocytes form dense networks termed *glial scars* (analogous to granulomas) to prevent pathogen spread and reduce tissue damage (Stenzel, Soltek, Schlüter, & Deckert, 2004), while neurons have low or absent expression of MHC I and use non-canonical non-apoptotic responses to intracellular infections (Miller, Schnell, & Rall, 2016). Indeed, microbial signalling influences brain development, as circulating peptidoglycan alters neuroproliferation (Humann et al., 2016) and influences behaviour in mice (Arentsen et al., 2017).

While the blood-brain barrier prevents many substances from entering the brain, it is not impermeable and includes active transport mechanisms for some substances (Nau et al., 2010). Moreover, while the brain lacks conventional lymphatics, it instead contains a *glymphatic* system, where feet-like processes of astroglia induce directional flow of cerebrospinal fluid through the brain parenchyma and allow fluid efflux into paravenous drainage pathways (Iliff et al., 2012). This fluidic system thus allows certain blood components to circulate through the brain and additionally ensures that brain-derived antigens can be absorbed into lympatic vessels that were recently discovered in the dura mater (Aspelund et al., 2015; Louveau et al., 2015) and eventually drain into the deep cervical lymph nodes for immune surveillance.

1.3.6. Gut microbiota influence brain cells

These brain immune mechanisms suggest a potential route by which gut microbiota-derived signals may influence CNS development and function. Microbial

components may translocate the gut epithelium, enter systemic circulation, then cross the blood-brain barrier via diffusion or active uptake. Once in the parenchyma, these components can interact with specific receptors that determine their outcome. An example of one potential signalling pathway involves short chain fatty acids, by-products produced by bacterial fermentation of otherwise indigestible fibers (Dalile, Van Oudenhove, Vervliet, & Verbeke, 2019). Short chain fatty acids can be transported across the blood-brain barrier into the brain, where they appear to alter levels of neurotransmitters (Frost et al., 2014; Yano et al., 2015) and may influence behaviour (Sun et al., 2016). Some bacteria can also produce neurotransmitters, including serotonin, acetylcholine, and γ-aminobutyric acid (GABA; Caspani & Swann, 2019). While it is unknown whether bacterially produced neurotransmitters can enter the brain, it is possible. For example, oral GABA supplementation appears to influence brain and behaviour (Ko, Lin, & Tsai, 2013; Yoto et al., 2012) and may cross the blood-brain barrier (Boonstra et al., 2015). The bacterium *L. rhamnosus* JB-1, which can produce GABA, also increases levels of GABA in the brain (Janik et al., 2016) in addition to GABA receptor expression and behavioural changes (Bravo et al., 2011), suggesting that its production of this neurotransmitter may be involved in its CNS effects.

1.4. Lacticaseibacillus rhamnosus JB-1

Our laboratory has approached the study of bacteria-host communication by focusing on the beneficial bacterium *Lacticaseibacillus rhamnosus* JB-1 (JB-1), which was

previously of the genus *Lactobacillus* until a recent taxonomic restructuring (Zheng et al., 2020). This strain is closely related to the *L. rhamnosus* strain GG (ATCC 53103), which has been a commercial probiotic for decades used to improve gastrointestinal symptoms (Capurso, 2019). *L. rhamnosus* GG was isolated from the intestine of a healthy human; by contrast, JB-1 was initially purchased as a *L. reuteri* strain that was apparently misattributed, and its provenance as such is unknown. Despite this, JB-1 as a model organism has significantly advanced our understanding of bacteria-host communication, as indicated throughout this chapter, and as briefly summarized below.

1.4.1. Immune activity of *L. rhamnosus* JB-1

JB-1 was first studied as a potential probiotic bacterium with immunoregulatory effects. Ma and colleagues found that it had immunosuppressive effects in an intestinal epithelial cell line, as it inhibited TNF-induced IL-8 production (Ma, Forsythe, & Bienenstock, 2004). Soon afterwards, Forsythe and colleagues showed that its immunoregulatory effects extended to *in vivo* disease models, where JB-1 treatment inhibited allergic asthma (Forsythe, Inman, & Bienenstock, 2007) by inducing functional regulatory T cells (Karimi, Inman, Bienenstock, & Forsythe, 2009) and immunoregulatory dendritic cells (Karimi, Kandiah, Chau, Bienenstock, & Forsythe, 2012). It also reduced systemic mast cell activation in a manner that appeared to involve inhibition of the calcium-dependent potassium channel IK_{Ca} (Forsythe, Wang, Khambati, & Kunze, 2012). Moreover, JB-1 treatment was shown to protect against intestinal inflammation in mice

(van der Kleij, O'Mahony, Shanahan, O'Mahony, & Bienenstock, 2008), and, interestingly, protected against intestinal pathogen infections, including with *Salmonella enterica* serovar Typhimurium (Mian et al., 2016) and the nematode *Trichuris muris* (McClemens et al., 2013).

1.4.2. Neuroactivity of *L. rhamnosus* JB-1

Indications that JB-1 may have some neuroactivity came from the demonstration of Kamiya and colleagues that its oral consumption inhibited visceral pain in rats (Kamiya et al., 2006). JB-1 was then shown to alter colonic motility and enteric neuronal excitability via inhibition of the IK_{Ca} potassium channel (Wang, Mao, Diorio, Pasyk, et al., 2010; Wang, Mao, Diorio, Wang, et al., 2010), demonstrating that JB-1 is neuroactive in the ENS. Soon afterwards, the landmark paper of Bravo, Forsythe, and colleagues demonstrated that oral consumption of JB-1 reduced stress-response, anxiety-like behaviour, and depressive-like behaviour in mice, along with altered brain neurochemistry, in a vagal-dependent manner (Bravo et al., 2011). Since then, JB-1 has been shown to protect against antibiotic-induced neurodevelopmental changes (Kayyal et al., 2020; Leclercq et al., 2017) and against brain and behavioural changes following chronic stress (Bharwani, Mian, Surette, Bienenstock, & Forsythe, 2017). It induces regional brain activation via vagal dependent and independent pathways within 2.5 hours of oral consumption (Bharwani et al., 2020). It also appears to influence behaviour via the induction of regulatory T cells, as adoptive transfer of these cells from JB-1-fed

mice to naïve mice resulted in the transfer of behavioural phenotype as well (Liu, Mian, McVey Neufeld, & Forsythe, 2020). While the mechanisms by which JB-1 exerts these varied influences are largely unknown, they appear at least in part to involve its production of MV (Al-Nedawi et al., 2015), as described above and as argued in subsequent chapters.

1.5. Nanoparticulate mechanisms of microbe-host communication

While most work investigating bacteria-host communication pathways has focused on soluble mediators, research is beginning to show that nanoparticles, primarily extracellular vesicles (EV), are involved. EV are lipid bilayer-encapsulated nanoparticles that are produced by all known forms of life (Brown, Wolf, Prados-Rosales, & Casadevall, 2015). In prokaryotes, EV are produced when a region of the plasma membrane bunches together and buds off into the extracellular space; EV produced in this manner are often referred to as microvesicles or membrane vesicles (MV). Eukaryotic organisms too produce microvesicles, but also possess a mechanism for constructing vesicles intracellularly, then releasing these to the environment (termed exosomes).

EV were first recognized as physiologically relevant cellular derivatives in the 1970s, but their functions were generally unclear (Witwer & Théry, 2019). Interest in mammalian EV has dramatically increased in recent years, driven in large part by seminal findings in cancer research. In one major example, Hoshino and colleagues found that EV

from cancers express membrane markers that direct them to interact with specific target organs, and that this determined the location for subsequent cancer metastasis (Hoshino et al., 2015). Cancer EV are also involved in immune modulation, angiogenesis, and generally supporting tumour progression (Becker et al., 2016), and as such are an active area of cancer research. More generally, these findings have suggested that a more general system of specific EV targeting may be functional in non-disease states, leading to extensive and mounting current research interest.

1.5.1. Bacterial Membrane Vesicles

Recent interest in EV has extended to those produced by microorganisms. Initial studies focused on EV from gram-negative bacteria, which have both inner and outer cell membranes sandwiching a thin peptidoglycan layer. Outer membrane vesicles (OMV) from such bacteria were first investigated in the context of pathogenesis, as they can contain toxins and virulence factors and appear to improve pathogen success (Ellis & Kuehn, 2010). Later work suggests that they are also important in host-microbiota interaction. One of the earliest demonstrations by this was that of Shen and colleagues, who were investigating OMV produced by the commensal bacterium *Bacteroides fragilis* (Shen et al., 2012). They found that these OMV had immunoregulatory effects *in vitro* via associated polysaccharide A interacting with TLR2, and that treatment with *B. fragilis* OMV prevented experimentally induced colonic inflammation in mice. Subsequent work

has supported the idea that OMV are important signalling mediators of the gut microbiota (reviewed in: Badia & Baldomà, 2020; Haas-Neill & Forsythe, 2020).

Despite significant progress in OMV-related research, for decades it was not thought that gram-positive bacteria could produce MV except as an artefact of cell death (Brown et al., 2015). This is because gram-positive bacteria comprise a single cellular membrane, which is surrounded by a thicker cell wall of rigid peptidoglycan thought to preclude release of MV. Only very recently has it been widely accepted that gram-positive bacteria can produce MV physiologically and that these are relevant for cellular functioning (Brown et al., 2015). As a result, the mechanisms underlying their production are poorly characterized. In some bacteria MV formation is thought to be mediated by endolysins, which are enzymes encoded by bacterial viruses to break the cell wall and allow viral progeny to escape. Toyofuku and colleagues showed that endolysin expression in a *Bacillus subtilis* created pores in its peptidoglycan cell wall and induced MV production, though the perturbed cell wall integrity eventually led to cell death (Toyofuku et al., 2017). Other methods, which may avoid cell death, have been proposed, but not demonstrated (Brown et al., 2015).

Research is now showing that gram-positive bacterial MV are important signalling mediators not only between microorganisms, but also between beneficial microbes and their host. Perhaps the first demonstration of this was by López and colleagues (López et al., 2012), who isolated MV from the beneficial bacterium *Bifidobacterium bifidum* LMG13195 and showed that coculture with dendritic cells led to

their induction of regulatory T cells *in vitro*. Our laboratory has found similar effects of MV from other gram-positive beneficial bacteria. For example, *L. rhamnosus* JB-1 MV were shown to activate TLR2 and promote immunoregulatory phenotypes in dendritic cells and regulatory T cells (Al-Nedawi et al., 2015), as has previously been shown for the parent bacterium (Karimi et al., 2009, 2012). Similarly, we have recently shown that MV from the commercial probiotic *Limosilactobacillus reuteri* DSM-17938 reproduced effects on gut motility *ex vivo* in mice (West et al., 2020), suggesting that they might be involved in this bacterium's clinical effects.

MV from some beneficial bacteria are known to interact with the intestinal environment. Cañas and colleagues demonstrated that OMV from the gram-negative probiotic *E. coli* Nissle 1917 were internalized in a clathrin-mediated manner by intestinal epithelial cells (IEC) (Cañas et al., 2016). In similar IEC systems these internalized OMV activated intracellular NOD1 signaling (Cañas, Fábrega, Giménez, Badia, & Baldomà, 2018) and appeared to have immunomodulatory effects when cocultured with peripheral blood mononuclear cells (Fábrega et al., 2016). This suggests that internalization of MV by intestinal epithelial cells may be important in their effect.

Until very recently, no evidence for similar internalization of MV from gram-positive beneficial microbes had been published. As this thesis was being prepared, Bajic and colleagues demonstrated clathrin-mediated internalization of MV from the lactic acid bacterium *Lactiplantibacillus plantarum* strain BGAN8 by a human gut epithelial cell line (Bajic et al., 2020), showing that similar mechanisms may be involved in

internalization of both MV and OMV. Similarly, Rubio and colleagues found that MV from the gram-positive *Bacillus subtilis* were internalized by a different human IEC line (Rubio et al., 2020). Interestingly, using time series z-stacking microscopy and nanoparticle tracking analysis in a transwell system, these authors found evidence to suggest that some internalized MV were in fact transcytosing the epithelial cells to be released on the basal side. While the functional significance of this is unclear, it reinforces the idea that MV are important but as-yet poorly characterized mediators of bacteria-host communication.

1.5.2. Bacteriophages as putative host modulators

Another type of nanoparticle associated with bacteria are bacteriophages (phages), viruses that infect bacteria and are released as lipid- or protein-encapsulated particles that are similar in size to MV. Discovered independently by Frederick Twort in 1915 (Twort, 1915) and by Félix d'Hérelle in 1917 (D'Herelle, 2007), phages were initially used therapeutically with some success, but were supplanted in most countries after the development of easier-to-use antibiotics (though phage use continued to some extent in the former Soviet Union and elsewhere in Eastern Europe; Salmond & Fineran, 2015). With increasing concern about antibiotic resistance, phage research has recently again risen to prominence. Phages are often highly specialized to infect only one bacterial species or strain (Sausset, Petit, Gaboriau-Routhiau, & De Paepe, 2020). Once inside, phages can replicate rapidly and cause lysis of their host, or integrate into the host

genome and either lay dormant or produce viral progeny at a lower rate (Sausset et al., 2020). Most known phages are of the order *Caudovirales*, which comprise a DNA genome encapsulated by a protein capsid (Sausset et al., 2020).

While historically ignored in microbiota research, there are estimated to be approximately as many free phage particles in the gut as there are bacteria (Shkoporov et al., 2019), not including inactive *prophages* that most bacteria tend to be infected with (Sausset et al., 2020). The impact of these phages is hard to quantify. Phage populations are stable in organisms across time, yet highly distinct between individuals (Shkoporov et al., 2019), and whether phage community composition reflects phage-induced modulation of the resident gut microbiota or instead is the passive result of extant bacterial communities is unknown (Wahida, Tang, & Barr, 2021). Nonetheless, phages are highly relevant from an evolutionary perspective. Phage infection involves the transfer of genetic material from one bacterium to another, likely genetically different, one. Given the number of phage-bacteria interactions, it is estimated that at least 10^{16} gene transfer events are mediated by phages every second (Salmond & Fineran, 2015). It seems therefore certain that intestinal phages have a profound influence on bacterial ecology and community composition in the microbiota. However, because the vast majority of phages are unknown and highly diverse, most phage nucleic acids sequenced from intestinal microbiota have no known homology to current viral databases (Aggarwala, Liang, & Bushman, 2017), and thus the number of distinct genomes present is not currently knowable. The situation is rendered even more

complex given that most bacteria present in the intestine are themselves poorly characterized and have not yet been cultured *ex vivo*. Since phages tend to be highly specific to a certain host, characterizing this "viral dark matter" is an enormous challenge (Sausset et al., 2020). Altogether, this highly complex and relatively nascent area of study ensures that the extent to which phage are involved in microbiota ecology and host interactions will be unclear at least in the short term.

Surprisingly, research is beginning to uncover interactions between phage and host cells, despite current understanding that phages cannot replicate in mammalian cells (Huh, Wong, St. Jean, & Slavcev, 2019). Bacterial nucleic acids have components not present in nucleic acids of mammals and trigger intracellular innate immune receptors when present in mammalian cells (Tan, Sun, Chen, & Chen, 2015). Since phages, including their nucleic acids, are constructed within bacteria, they induce a similar response. This was recently shown by Gogokhia and colleagues, who found that therapeutic phage use to clear a bacterial infection in mice also induced a stronger immune response against the bacteria (Gogokhia et al., 2019). This appeared to be due to dendritic cells phagocytosing the therapeutic phage, detecting its microbial DNA via TLR9, and ultimately producing the proinflammatory anti-viral cytokine interferon-γ (Gogokhia et al., 2019). Phage may also assist bacteria in evading the immune response, however. Sweere and colleagues showed that phage-infected *Pseudomonas aeruginosa* produced a more persistent infection than a phage-free strain (Sweere et al., 2019). They found that endocytosis of a phage by immune cells resulted in transcription of its

single-stranded DNA genome into RNA, and that this RNA induced production of type I interferon via TLR3 activation, ultimately inhibiting tumour necrosis factor production and thereby reducing phagocytosis of *P. aeruginosa* by immune cells (Sweere et al., 2019).

Phages also interact with the intestinal epithelium, where they can be internalized by intestinal epithelial cells and be transcytosed (Nguyen et al., 2017) or remain internalized and protected against degradation for a prolonged period (Bichet et al., 2021). Moreover, phages of gut origin are known to circulate in healthy organisms and remain active (Blanco-Picazo et al., 2020; Keller & Engley, 1958). While the functional consequences of this remain unknown, the multiple effects associated with phage and significant remaining unknowns of phage-mammalian interaction suggests that they may prove to play an important role in host modulation by gut microbes.

1.6. Research Aims

Given the mounting evidence for microbiota influence in both neurodevelopment and normal physiology, we first aimed to (1) determine whether microbiota influence neurodevelopment during late pregnancy in mice by perturbing the maternal microbiota by a physiologically relevant dose of penicillin. As the mechanism by which the maternal microbiota could influence fetal development is unclear, we sought to (2) characterize MV as one potential mediator of bacterial activity, focusing on a single well-described bacterial strain (*L. rhamnosus* JB-1) for simplicity. Finally, with a

better understanding of the composition of JB-1 MV and their interaction with the intestinal epithelium, we then aimed to (3) investigate whether MV from JB-1 could mediate systemic influence associated with consumption of the bacterium.

1.7. Specific Objectives

Through the works described in this thesis, we aimed to:

1. Investigate whether disturbance of the maternal mouse microbiota via penicillin consumed only during the last prenatal week would alter brain gene expression and behavioural phenotypes in adult offspring.
2. Characterize specific cargo of JB-1 MV that is involved in its physiological effects.
3. Determine the nature of interaction between JB-1 MV and the intestinal epithelium.
4. Investigate whether circulating nanoparticles such as MV are involved in systemic influence of beneficial bacteria on their host.

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CHAPTER 2. Prenatal low-dose penicillin results in long-term sex-specific changes to murine behaviour, immune regulation, and gut microbiota

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2.1. Abstract

Growing evidence suggests that environmental disruptors of maternal microbes may have significant detrimental consequences for the developing fetus. Antibiotic exposure during early life can have long-term effects on neurodevelopment in mice and humans. Here we explore whether exposure to low-dose penicillin during only the last week of gestation in mice has long-term effects on offspring behaviour, brain, immune function, and gut microbiota. We found that this treatment had sex-specific effects in the adult mouse offspring. Female, but not male, mice demonstrated decreased anxiety-like behaviours, while male, but not female, mice had abnormal social behaviours which correlated with altered brain expression of *AVPR1A*, *AVPR1B*, and *OXTR*, and decreases in the balance of splenic FOXP3+ regulatory T cells. Prenatal penicillin exposure also led to distinct microbiota compositions that clustered differently by sex. These data suggest that exposure of pregnant mice to even a low dose of penicillin through only the last week before birth is nonetheless sufficient to induce long-term sex-specific developmental changes in both male and female offspring.

2.2. Introduction

The fetal origins of adult disease hypothesis postulates that environmental challenges in pregnancy might disorder fetal development and induce diseases which manifest later in life (Barker, 1997). There is considerable support for this hypothesis both clinically (Ravelli, Stein, & Susser, 1976; Skogen & Øverland, 2012) and experimentally (Padmanabhan, Cardoso, & Puttabyatappa, 2016). A variety of prenatal insults can lead to long-term physiological effects in adult offspring, including maternal stress (Caldji, Diorio, & Meaney, 2000), immune activation (Smith, Li, Garbett, Mirnics, & Patterson, 2007), and infection (Boksa, 2010). Recent evidence suggests that perturbation to the gut microbiota in early life may also have long-term developmental consequences (Leclercq et al., 2017; Lydholm et al., 2019; Slykerman et al., 2017).

There is now substantial evidence that the gut microbiota play an important role in brain function and development (Bienenstock, Kunze, & Forsythe, 2015; Dinan, Cryan, & Stanton, 2018; Erny et al., 2015; Forsythe, Kunze, & Bienenstock, 2016; Heijtz et al., 2011; Luczynski et al., 2016; Yano et al., 2015). Much comes from studies of germ-free animals, which develop abnormal stress responses (Sudo et al., 2004) and social (Desbonnet, Clarke, Shanahan, Dinan, & Cryan, 2014) and anxiety-like (Neufeld, Kang, Bienenstock, & Foster, 2011) behaviours. Germ-free mice also have altered brain physiology throughout life, including abnormal myelination in the prefrontal cortex (Hoban et al., 2016), increased blood-brain barrier permeability (Braniste et al., 2014),

and modified microglia activity (Castillo-Ruiz et al., 2018; Erny et al., 2015; Thion et al., 2018), among others (Luczynski et al., 2016).

While germ-free animals are useful for exploring the importance of the microbiota in development, they are not a clinically-relevant model and cannot prove direct causality. For this reason, many investigators have used antibiotics to perturb the microbiota during specific periods. Such studies have shown similar phenotypes as seen in germ-free mice (Bercik, Denou, et al., 2011), and developmental studies have shown that prenatal exposure to antibiotics can result in altered social (Degroote, Hunting, Baccarelli, & Takser, 2016; Lebovitz et al., 2019; Leclercq et al., 2017) and anxiety-like (Leclercq et al., 2017; Zhang et al., 2017) behaviours in mature rodents. Until recently, however, most experimental studies have used antibiotics in high doses or combinations that do not reflect clinical use, which may have increased off-target activity (Champagne-Jorgensen, Kunze, Forsythe, Bienenstock, & McVey Neufeld, 2019).

We previously sought to explore the effects of a more clinically-relevant antibiotic treatment (Cox et al., 2014), in which pregnant mice are exposed to low-dose penicillin from one week before birth until weaning. This model has previously been shown to affect long-term body weight and obesity (Cox et al., 2014), and in our experiment resulted in long-term changes to offspring anxiety-like, social, and aggressive behaviours, with concomitant changes in brain gene expression and microbiota composition (Leclercq et al., 2017). Since developmental windows of vulnerability have been ascribed to environmental changes in pregnancy, we have undertaken the present

exploratory study. We have refined our previous treatment window, and thus limited the same low-dose penicillin exposure to only the last prenatal week. Given that perinatal penicillin treatment altered social behaviours in both our current and previous studies, we chose to additionally examine whether the treatment would influence aspects of the arginine vasopressin and oxytocin systems that are known to be involved in social cognition (Bielsky & Young, 2004). In particular, we measured the mRNA expression of several neuropeptide receptors involved in this system, specifically *AVPR1A* (Bielsky & Young, 2004), *AVPR1B* (Wersinger, Ginns, O'Carroll, Lolait, & Iii, 2002; Wersinger et al., 2004), and *OXTR* (Lazzari et al., 2017) in the frontal cortex (Leclercq et al., 2017) and hippocampus (Lazzari et al., 2017). We also chose to study splenic immune cell population balances given the well-established link between immunological environment and psychiatric disturbances (Reber, Siebler, Donner, Morton, Smith, Kopelman, Lowe, Wheeler, Fox, Hassell, Greenwood, et al., 2016; Syed et al., 2018; Wohleb, Powell, Godbout, & Sheridan, 2013). We found that prenatal-only penicillin treatment led to some, albeit mostly different, changes to the adult offspring's behaviour, brain mRNA expression, immune cell populations, and gut microbiota in a sex-specific manner.

2.3. Materials and methods

2.3.1. Study design

All mice (specific pathogen free) were obtained from Charles River (Montreal, QC, Canada), acclimatized for at least one week, and maintained on a 12h light-dark cycle with free access to chow and water. Male and female BALB/c mice (8-10 weeks old) were housed 3-4 per cage, and male CD-1 retired breeders were singly-housed. Breeding was performed as previously described (Leclercq et al., 2017), and the day of pairing was assumed to be gestational day 0. Low-dose penicillin (phenoxymethylpenicillin; Sigma-Aldrich, MO, USA) was given in water from gestational day 15 to birth (average 6 days) at a dose of 50000 U/kg/day (31 mg/kg/day) (Leclercq et al., 2017), which was based on doses used in clinical practice (Heikkilä, 1993; Leclercq et al., 2017). Penicillin concentration in water was changed daily for each dam based on current weight and water consumption. All protocols adhered to the Canadian Council on Animal Care guidelines and were approved by the McMaster Animal Research Ethics Board. A total of 59 pups were included in this experiment (29 antibiotic-treated [14 males]; 30 controls [15 males]), with an average of 2 pups per sex from each litter (see Table S1).

2.3.2. Maternal behaviour

Maternal behaviour was monitored daily from postnatal day 1 (PND 1) to PND 7 following a simplified protocol (Champagne, Curley, Keverne, & Bateson, 2007). Briefly, dams were observed twice daily during the light phase for 30 minutes, with one observation per dam every three minutes. For each observation the assessor recorded

whether the dam was on or off her pups. The assessor was blind to treatment group status while scoring.

2.3.3. Weaning

On PND 21 pups were weaned and randomly assigned to non-littermate same-sex and same-treatment cages of 3-4 mice each. The experimenter was then blinded to treatment groups for the remainder of the study.

2.3.4. Behavioural testing

Behavioural testing began on approximately PND 43. Animals were tested in a partially-counterbalanced sequence for each test. Prior to each behavioural test the animals were habituated for at least one hour to the testing room, and habituation began no earlier than one hour after the light-cycle change.

2.3.4.1. Open field

The open field test was performed during the dark phase and under dim-light conditions (80 lux), as previously described (Leclercq et al., 2017). Mice were placed in the open field enclosure for a period of 30 min and allowed to explore undisturbed. Movement was measured by infrared beam breaks and automatically recorded in time bins with Motor Monitor software (Kinder Scientific, Poway, CA).

2.3.4.2. Light dark box

The light dark test was performed during the dark phase and under dim-light conditions (80 lux), as previously described (Bharwani, Mian, Surette, Bienenstock, & Forsythe, 2017). Mice were placed in the dark chamber to start the test and were then allowed to explore undisturbed for 10 min. Movement was measured by infrared beam breaks in both sections of the enclosure and automatically recorded in time bins with Motor Monitor software (Kinder Scientific, Poway, CA).

2.3.4.3. Three chamber social behaviour

The 3-chamber tests were performed during the light phase and under regular lighting (800 lux), as previously described (Leclercq et al., 2017). The test mouse was first allowed to explore the centre chamber for 5 min to habituate to the environment. An unfamiliar same-sex probe mouse was then placed under an inverted wire cup in one of the side chambers, while an empty identical wire cup was placed in the other chamber. The test mouse was then allowed to explore the entire apparatus for 10 min. Finally, a second unfamiliar probe mouse was placed in the previously empty wire cup, and the test mouse was again allowed to explore the entire apparatus for 10 min. The test was recorded by a video camera, and EthoVision XT software (v. 8.5; Noldus, Leesburg, VA) was used to automatically track mouse movements from the footage.

2.3.4.4. Elevated plus maze

The elevated plus maze test was performed during the dark phase and under dim-light conditions (80 lux), as previously described (Leclercq et al., 2017). Mice were placed in the intersection of the 4 arms facing towards an open arm to start the test and then explored undisturbed for 5 min. Movement was measured by infrared beam breaks throughout the enclosure and automatically recorded with Motor Monitor software (Kinder Scientific, Poway, CA).

2.3.4.5. Microdefeat

The microdefeat procedure is only validated for use with males, and was performed as previously described (Leclercq et al., 2017) during the light phase under regular lighting (800 lux). BALB/c test mice were individually placed in the home cage of a CD1 retired breeder (previously screened to be aggressive) for 3 trials of 3 min each. Trials were separated by 15 min of rest, and each involved a novel BALB/c to CD1 pairing. CD1 mice were pseudo-randomly assigned to test mice, such that each CD1 mouse was equally distributed between groups, was allowed at least 30 min of rest between exposures, and did not encounter the same mouse more than once. After the last exposure trial, test mice were singly housed for the remainder of the experiment. This procedure reliably provokes CD1 aggression towards the BALB/c intruder, and either submissive or retaliatory behaviour by the BALB/c. Each trial was video recorded (Canon VIXIA HF R700) and the blinded footage later scored for the amount and duration of CD1 attacks, BALB/c submissive posture, agonistic tail-rattles, and retaliatory

attacks. Scoring was done using the event-logging open-source software BORIS (v. 4.1.11, (Friard & Gamba, 2016)), and scores were summed across trials for analysis.

2.3.4.6. Aggressor avoidance

The aggressor avoidance test was performed 24h after the microdefeat procedure under regular lighting (800 lux) as described previously (Leclercq et al., 2017). During the first phase of the test, an empty wire cup was placed in the centre of one half of a rectangular chamber. The test mouse was placed in the half of the chamber opposite to the empty cup and then explored the chamber for 2.5 min. For the second phase, an unfamiliar CD1 aggressor mouse was placed under the previously empty wire cup. The test mouse then explored the chamber for 2.5 min. Both phases were recorded by a video camera and EthoVision XT software (v. 8.5; Noldus, Leesburg, VA) was used to automatically track mouse movements from the footage.

2.3.5. Sacrifice and tissue collection

Animals were sacrificed by decapitation 24h following the aggressor avoidance test. Brains were immediately removed, bisected, flash-frozen in dry ice-cooled isopentane, then stored at -80°C. Spleens were also immediately removed and placed into ice-cold sterile phosphate-buffered saline (PBS).

2.3.6. Brain gene expression analysis

2.3.6.1. Tissue dissection

Left- or right-side hemispheres (side systematically varied between animals) were transferred on dry ice into cooled RNA*later*-ICE (Invitrogen, CA, USA) and soaked at -20°C for at least 24h. Hemispheres were then dissected to isolate frontal cortex and hippocampus, which were immediately stored in RNA*later* (Invitrogen) at -20°C until further processing. Tissue from 12 animals per group was randomly selected for use downstream in real-time quantitative polymerase chain reaction (qPCR).

2.3.6.2. RNA isolation and cDNA synthesis

RNA from frontal cortex tissue was isolated using the RNeasy Lipid kit (Qiagen, NRW, Germany). RNA from hippocampal tissue was isolated using the *mir*Vana kit (Invitrogen) for total RNA isolation. Isolated RNA was immediately stored at -80°C. RNA was reverse transcribed to cDNA using the SuperScript IV VILO kit (Invitrogen) with genomic DNA degradation step. The resulting cDNA was then stored at -20°C until further processing.

2.3.6.3. Real-time quantitative PCR

Diluted cDNA was used as a template for real-time qPCR using PowerUp SYBR Green Master Mix (Applied Biosystems, CA, USA) following manufacturer's directions for fast cycling mode. Reactions were run in triplicate (500 nM each primer) in a QuantStudio 3 Real-Time PCR System (Applied Biosystems). Data were analyzed by the comparative CT method (Schmittgen & Livak, 2008) using *GAPDH* as the endogenous control. *GAPDH* mRNA expression did not vary between groups and primers were

validated for efficiency within 90-110% (data not shown). Primer sequences are listed in Table S2.

2.3.7. Fluorescence-activated cell sorting

2.3.7.1. Splenocyte isolation

Splenocyte isolation and flow cytometry were done as previously described (Bharwani et al., 2016). Spleen tissue was homogenized in cold sterile PBS and washed by centrifugation at 500 x g for 10 min at 4°C. Cells were then lysed with red blood cell lysis buffer for 2 min before again being washed. The resulting pellet was resuspended in 5 mL of complete RPMI medium (containing 10% fetal bovine serum, 100 U/mL penicillin, 100 ug/mL streptomycin, 2 mM L-glutamine, and 0.01% β-mercaptoethanol). Viable cells were then counted by trypan blue exclusion and diluted to 10^7 cells/mL in RPMI as above.

2.3.7.2. Flow cytometry

To identify FOXP3$^+$ regulatory T cells, 10^6 splenocytes per well in a 96-well plate were washed twice in cold PBS and resuspended in FACS buffer (2% FBS in PBS). Nonspecific binding was blocked with 50 µL anti-mouse CD16/CD32 antibody (1:50 dilution; clone 2.4G2; BD Biosciences, ON, Canada) for 15 min on ice, then samples were washed in FACS buffer. For extracellular markers, splenocytes were then stained with CD4-FITC, CD3-APC, and CD25-PE-Cy7 antibodies (Invitrogen; 50 µL/well at 1:200 dilutions) for 30 min on ice in darkness before being washed twice in FACS buffer to

remove unbound antibodies. To detect intracellular markers, splenocytes were permeabilized in Cytofix/Cytoperm buffer from the eBioscience Foxp3 / Transcription Factor Staining Buffer Set (Invitrogen), then washed twice with Cytoperm wash buffer. Finally, cells were stained with FOXP3-PerCP-Cy5.5 antibody (1:200 dilution; Invitrogen) for 30 min on ice in darkness, then washed twice in Cytoperm wash buffer and resuspended in 1% formaldehyde-containing FACS buffer for analysis.

To identify IL-10-, IL-12-, or Ly6C-expressing monocytes, 10^6 splenocytes washed and blocked as described above (without restimulation) were surface stained with CD11b-PerCP-Cy5.5, F4/80-APC-Cy7, and Ly6C-FITC antibodies (1:200 dilution; Invitrogen). After permeabilization as described above, cells were stained for intracellular markers using IL-12p40-PE (BD Biosciences), and IL-10-PE-Cy7 (Invitrogen) antibodies (1:200 dilutions), then resuspended in 1% formaldehyde-containing FACS buffer for analysis. All samples were run on a BD FACSCelesta flow cytometer (BD Biosciences), and data analyzed using FlowJo Software (Tree Star, OR, USA). See supplemental results for details on the gating strategies used (Figure S1).

2.3.8. 16s rRNA gene analysis

2.3.8.1. Fecal sample collection

Fecal pellets for 16S rRNA gene sequence analysis were collected from a clean surface into sterile tubes on ice, then frozen at -80°C within 2h. Samples were collected

at PND 21 (weaning) and approximately PND 40 (beginning of adulthood) prior to beginning behavioural testing.

2.3.8.2. Bacterial DNA extraction, amplification and sequencing

DNA was extracted from all mouse fecal samples using Quick-DNA Fecal/Soil Microbe 96 Kit (Zymo Research, CA, USA). The V4 region of the bacterial 16S rRNA gene was amplified by PCR using the 515F (AATGATACGGCGACCACCGAGATCTACACGCT) barcoded and 806R (TATGGTAATTGTGTGYCAGCMGCCGCGGTAA) primers. For each PCR tube the following materials were added: 2 µL 515F (forward, 10µM) primer, 2 µL 806R (reverse, 10 µM) primer, 25 µL PrimeSTAR Max PCR mix (Takara Bio, Shiga Prefecture, Japan), 17 µL ultra-pure water, and 4 µL of sample DNA. PCR reactions were carried out by 30 cycles of denaturation (95°C), annealing (55°C), and extension (72°C), with final elongation at 72°C. PCR products were purified using AMPure magnetic beads (Beckman Coulter, CA, USA) and quantified using a Quant-iT PicoGreen dsDNA quantitation kit (Invitrogen). Samples were then pooled at equal amounts (50 ng), loaded on 2% agarose E-Gel (Invitrogen), purified, and sent for sequencing using the Illumina MiSeq platform (Genomic Center, Azrieli Faculty of Medicine, Bar Ilan University, Israel).

2.3.8.3. Microbiota analysis

Data analysis was performed using QIIME2 (Bolyen et al., 2018). Sequence reads were demultiplexed by per-sample barcodes and Illumina-sequenced amplicon read errors were corrected using DADA2 (Callahan et al., 2016). A phylogenetic tree was

generated. All analyses for mouse fecal samples were calculated based on a feature table containing features observed in at least 50% samples in each group and rarefied at 23,500 sequences. Richness was calculated using Faith's Phylogenetic Diversity (Faith, 1992; Faith & Baker, 2006). Beta diversity (between-sample diversity) was analyzed using unweighted UniFrac distances (Lozupone & Knight, 2005). We also performed linear discriminate analysis effect size (LEfSe) (Segata et al., 2011), which determines the features that are significantly different between samples according to relative abundances.

2.3.9. Statistical analyses

Behavioural data where appropriate were analyzed by type III analysis of variance (ANOVA; termed ***F***), with group and sex as between-subject factors. ANOVA effect magnitudes were calculated as the generalized eta-squared (termed $\boldsymbol{R^2}$). Because only male animals were subject to the microdefeat and aggressor avoidance procedures, data collected from mice post-sacrifice were only analyzed by pairwise comparisons between groups within each sex. Moreover, because the sexes in our experiment were not treated identically, and given recent recommendations to maximize the ability of exploratory experiments to detect sex effects (e.g. (Miller et al., 2016)), we chose to use *a priori* non-directional groupwise contrasts disaggregated by sex for other behavioural data. Pairwise comparisons between data that were expected to be normal were computed by a non-directional t-test with Welch's correction for unequal variances (***t***),

and the magnitude of the difference is reported as Hedge's g (termed ***d***). Pearson correlations were calculated where indicated across both groups (***r***). Where data were expected to be non-normal (skewed towards zero), pairwise comparisons were computed by Mann-Whitney U-test (***U***), with the magnitude of the difference computed by Cliff's delta (also termed ***d***). All data preparation and analyses were conducted in R 3.4.4 (R Core Team, 2018) using the packages *ez* (Lawrence, 2016), *effsize* (Torchiano, 2017), and *Hmisc* (Harrell, 2018). Graphs were created in Prism (v. 6.01, GraphPad, CA, USA) or in R using the package *ggplot2* (Wickham, 2016). No outliers were removed from data analysis, and litter effects were not investigated. The number of animals required to obtain 80% power was estimated using G*Power software (v. 3.1.9.2; (Faul, Erdfelder, Lang, & Buchner, 2007)) based on behavioural effects seen previously (Leclercq et al., 2017).

2.3.10. Data availability

The 16S rRNA gene sequence data have been deposited in the European Bioinformatics Institute (EBI) database with accession code ERP117866.

2.4. Results

2.4.1. Gestational penicillin treatment did not alter dam weight or water intake

No significant effect of group was observed throughout the treatment period in either the weight of dams ($F_{1,61}$ = 0.05, p = 0.81) or amount of water consumed ($F_{1,61}$ = 0.54, p = 0.47).

2.4.2. Gestational penicillin treatment did not alter maternal behaviour

We found no significant differences between treatment groups in the amount of time spent by dams out of their nests over the first postnatal week ($t_{8.93}$ = 0.73, p = 0.48, d = 0.35; Figure S2).

2.4.3. Prenatal penicillin treatment decreased anxiety-like behaviours in female mice

2.4.3.1. Open field test

We found no significant main effects of group ($F_{1,55}$ = 0.11, p = 0.74, R^2= 0.002) or sex ($F_{1,55}$ = 1.691, p = 0.20, R^2 = 0.03) on total distance travelled in the open field (Figure S3, nor any significant interaction ($F_{1,55}$ = 1.690, p = 0.20, R^2 = 0.03). Regarding time spent in the centre of the arena, we found no significant effects of group ($F_{1,55}$ = 0.65, p = 0.42, R^2 = 0.01) or sex ($F_{1,55}$ = 3.43, p = 0.069, R^2 = 0.059), nor any significant interaction ($F_{1,55}$ = 0.63, p = 0.43, R^2 = 0.01).

2.4.3.2. Light-dark test

We found significant main effects of group ($F_{1,54}$ = 7.28, p = 0.009, R^2 = 0.12) and sex ($F_{1,54}$ = 14.67, p < 0.001, R^2 = 0.21) on time spent in the light zone, but no significant interaction ($F_{1,54}$ = 2.50, p = 0.12, R^2 = 0.04). Planned comparisons between groups of each sex (Figure 1A) revealed a large increase in time spent in the light zone by treated females as compared with control females ($t_{27.99}$ = 3.11, p = 0.004, d = 1.10), with no

differences detected in males ($t_{24.9}$ = 0.77, p = 0.45, d = 0.28). Similar results were found for distance travelled in the light zone (data not shown).

2.4.3.3. Elevated plus maze

We found no significant main effects of group ($F_{1,55}$ = 0.03, p = 0.86, R^2 < 0.001) or sex ($F_{1,55}$ = 2.45, p = 0.12, R^2 = 0.04), nor any significant interaction ($F_{1,55}$ < 0.01, p = 0.99, R^2 < 0.001), on time spent in the open arms (Figure 1B). Planned comparisons showed no significant differences between either male ($t_{25.9}$ = 0.12, p = 0.91, d = 0.04) or female ($t_{26.4}$ = 0.13, p = 0.90, d = 0.05) groups. We then ran post-hoc comparisons to examine whether differences between groups existed at any timepoint. These tests revealed an increase in time spent in the open arms by treated females as compared to control females during the first minute of the test ($t_{23.7}$ = 2.14, p = 0.043, d = 0.76; Figure 1C), but no significant differences at any other time point nor in males. Similar results were found for entries into the open arms of the maze (data not shown).

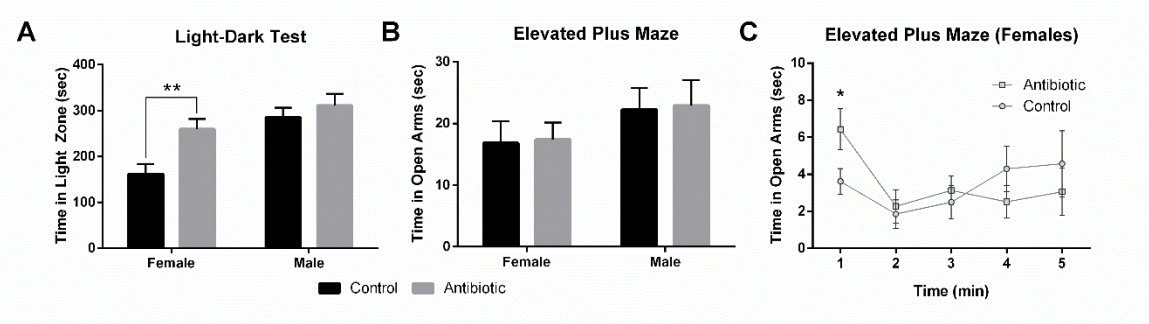

Figure 1: Prenatal penicillin exposure decreased anxiety-like behaviours in female mice. A) Antibiotic-treated females spent significantly more time in the light-zone of the light-dark test than control females. B) No significant differences were detected between groups of either sex in time spent in the open arms of the elevated plus maze when summed over the duration of the test. C) Antibiotic-treated females spent significantly more time in the open arms of the elevated plus maze during the first minute of the test. n =

14 antibiotic-treated males (13 in light-dark test), 15 in each other group. Graphs depict mean ± standard error. *$p < 0.05$, **$p < 0.01$.

2.4.4. Prenatal penicillin treatment altered social behaviours in male mice

2.4.4.1. Three-chamber sociability test

In the sociability test it is expected that the test mouse will prefer to investigate the mouse-containing chamber over the empty chamber. Planned pairwise comparisons to this end revealed a strong preference for the novel mouse chamber over the empty chamber for control ($t_{27.9} = 2.2$, $p = 0.035$, $d = 0.79$), but not treated male animals ($t_{24.0} = 0.36$, $p = 0.72$, $d = 0.13$) (Figure 2A). We found no significant preference for the mouse-containing chamber in control ($t_{27.2} = 0.74$, p = 0.46, $d = 0.26$) or treated ($t_{28.0} = 1.9$, p = 0.067, $d = 0.68$) females (Figure 2C). When looking at overall between-group differences in time spent in the mouse-containing chamber, we found no significant main effect of either group ($F_{1,55} = 0.23$, $p = 0.63$, $R^2 = 0.004$) or sex ($F_{1,55} = 0.30$, $p = 0.58$, $R^2 = 0.005$), nor group × sex interaction ($F_{1,55} = 2.29$, $p = 0.13$, $R^2 = 0.040$).

2.4.4.2. Three-chamber social novelty test

In the social novelty test it is expected that the test mouse will prefer to investigate the novel mouse over the familiar mouse. Planned pairwise comparisons to this end showed strong preferences for the novel mouse over the familiar mouse chamber for both control ($t_{27.9} = 3.4$, $p = 0.002$, $d = 1.21$) and treated ($t_{25.2} = 3.4$, $p = 0.002$, $d = 1.24$) male animals (Figure 2B), but no significant preference in either control ($t_{26.6} = 0.61$, p = 0.54, $d = 0.22$) or treated ($t_{27.6} = 1.1$, p = 0.27, $d = 0.40$) females (figure

2D). Examining overall between-group differences in time spent in the chamber containing the novel mouse revealed no significant main effects of group ($F_{1,55}$ = 0.86, p = 0.36, R^2 = 0.015) or sex ($F_{1,55}$ = 2.4, p = 0.13, R^2 = 0.042), nor group × sex interaction ($F_{1,55}$ = 0.15, p = 0.70, R^2 = 0.003).

2.4.4.3. Microdefeat and aggressor avoidance tests

These tests were only performed in males. Antibiotic-treated mice were attacked by the CD1 aggressor for significantly more time than were control males ($t_{19.13}$ = 2.26, p = 0.035, d = 0.86; Figure 2E), while control animals displayed agonistic tail rattles for significantly more time than antibiotic-treated animals (U = -157, p = 0.021, d = -0.50; Figure 2F). We detected no significant differences between groups in the time spent by the test mice retaliating against the CD1 aggressor (U = -137.5, p = 0.13, d = -0.31; Figure S4) or displaying a submissive posture (U = -96, p = 0.68, d = -0.095), nor in the time they spent next to the novel aggressor in the aggressor avoidance test the following day (U = -127.5, p = 0.33, d = -0.21; Figure 2G).

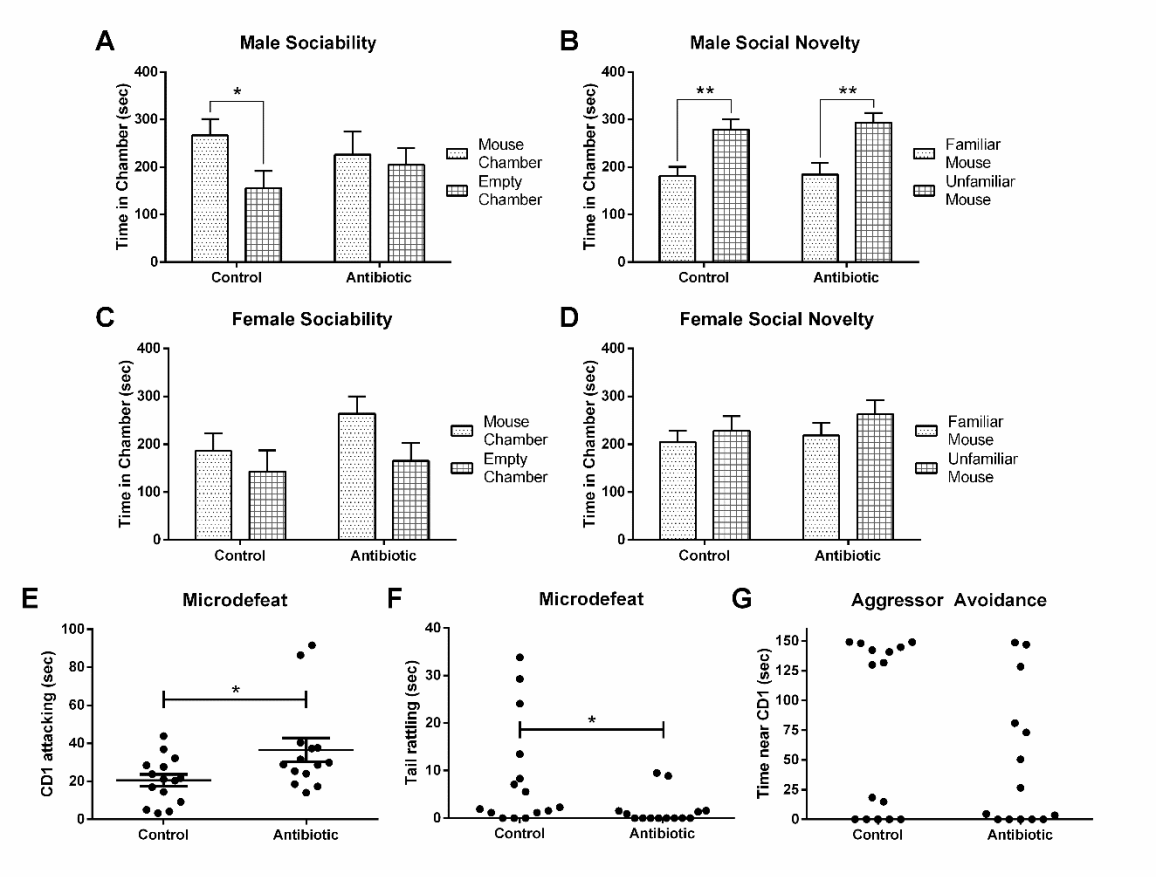

Figure 2: Prenatal penicillin exposure altered social-related behaviours in male mice. A) Male antibiotic-treated mice showed no significant preference for the chamber containing a novel mouse in the sociability test. B) Both male groups preferred the chamber containing the novel mouse in the social novelty test. C) Neither female group showed a significant preference for the mouse-containing chamber. D) Neither female group showed a significant preference for the chamber containing the novel mouse. E) In the microdefeat test, male antibiotic-treated mice were attacked for significantly more time than control males and F) spent significantly less time displaying rapid tail rattles than control males. G) No significant differences were detected in the amount of time male animals of either group spent near an unfamiliar aggressive CD1 in the aggressor avoidance test. n = 14 antibiotic-treated males, 15 in each other group. Graphs depict mean ± standard error. *$p < 0.05$, **$p < 0.01$.

2.4.5. Prenatal penicillin treatment may influence social-related gene expression in the male hippocampus

We examined the frontal cortex and hippocampus for differences in social-behaviour-related gene expression. In the hippocampus of treated males relative to

control males (Figure 3), we found large and near-significant decreases in arginine vasopressin receptor 1A (*AVPR1A*; $t_{20.2}$ = -1.8*,* p = 0.089, d = -0.70) and *AVPR1B* ($t_{16.1}$ = -2.1*,* p = 0.056, d = -0.81) expression, and a moderate but non-significant difference in oxytocin receptor (*OXTR*; $t_{19.8}$ = -1.32*,* p = 0.20, d = -0.52) expression. No significant or appreciable differences were detected between female groups, nor in expression of brain derived neurotrophic factor (*BDNF)* or glucocorticoid receptor (*GR*) between groups of either sex (Table S3). In the male frontal cortex we detected no significant differences between groups in the expression of interleukin-6 (*IL6*; $t_{14.3}$ = 1.32*,* p = 0.21, d = 0.52), *IL10* ($t_{14.98}$ = 1.42*,* p = 0.18, d = 0.56), or tumor necrosis factor α (*TNFα*; $t_{13.5}$ = 1.6*,* p = 0.13, d = 0.64), with no appreciable or significant effects in females or on the expression of other genes (Table S3).

Given our suggestive evidence for differences in social-related gene expression in the hippocampus, we wondered whether expression would correlate with particular behaviours in the 3-chamber and microdefeat tests (Figure S5). In the 3-chamber test we found a significant positive correlation between time spent with the first novel mouse and hippocampal *AVPR1B* mRNA expression in male (r = 0.41, p = 0.04), but not female (r = -0.32, p = 0.012), animals. In the microdefeat test (done only in males), we found significant positive correlations between hippocampal *AVPR1B* expression and retaliatory behaviour (r = 0.64, p < 0.001) and agonistic tail rattles (r = 0.55, p = 0.006). There were also significant negative correlations between submissive posture and

expression in the frontal cortex of *AVPR1A* (r = -0.42, p = 0.04) and *OXTR* (r = -0.43, p = 0.04), as well as a positive correlation with hippocampal *AVPR1A* (r = 0.44, p = 0.03).

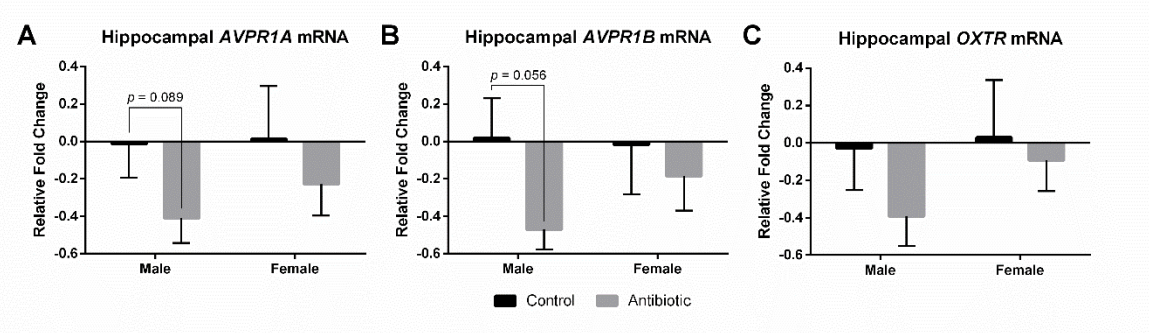

Figure 3: Exposure to prenatal penicillin may alter social-related gene expression in the male hippocampus. Male antibiotic-treated mice had near-significant decreases in hippocampal A) AVPR1A and B) AVPR1B mRNA, and C) a non-significant decrease in OXTR mRNA. No significant changes were seen between female groups. Fold change is relative to the median control-group value. n = 12 per group. Graphs depict mean ± standard error.

2.4.6. Prenatal penicillin treatment induced long-term altered immune regulation in male mice

Antibiotic-treated males had a significant decrease in forkhead box P3-positive (FOXP3$^+$) T_{reg} cells ($t_{21.3}$ = -3.4, p = 0.002, d = -1.26; Figure 4A) as a proportion of CD3$^+$ CD4$^+$ CD25$^+$ lymphocytes. These mice also showed a near-significant increase in cluster of differentiation 11b-positive (CD11b$^+$) lymphocyte antigen 6 complex-low monocytes (Ly6C^{lo}; $t_{26.9}$ = 1.9, p = 0.073, d = 0.67; Figure 4B), and F4/80$^+$ CD11b$^+$ IL-12$^+$ monocytes ($t_{21.7}$ = 1.92, p = 0.068, d = 0.70; Figure 4D). No significant differences were found in CD11b$^+$ Ly6C^{hi} ($t_{21.1}$ = 0.61, p = 0.55, d = 0.22; Figure 4C) or F4/80$^+$ CD11b$^+$ IL-10$^+$ ($t_{22.9}$ = 0.22, p = 0.83, d = 0.08; Figure 4E) monocytes in males, nor were any significant differences detected between female groups (T_{reg}: $t_{26.3}$ = -0.004, p = 0.99, d = -0.001;

Ly6C^{lo}: $t_{26.2}$ = -1.14, p = 0.26, d = -0.41; Ly6C^{hi}: $t_{27.9}$ = -0.93, p = 0.36, d = -0.36; IL-12^{+}: $t_{28.0}$ = 1.24, p = 0.22, d = 0.44; IL-10^{+}: $t_{26.7}$ = 0.15, p = 0.88, d = 0.05).

The decrease in relative proportion of T_{reg} cells in the spleens of treated male mice led us to wonder whether there was any relationship to the social-related brain or behavioural changes seen. We found a significant positive correlation between the amount of FOXP3^{+} T_{regs} and expression of *AVPR1B* in the hippocampus in male (r = 0.46, p = 0.025) but not female (r = -0.15, p = 0.48) animals (Figure S5), suggesting a relationship between immunological and social-related brain gene expression changes.

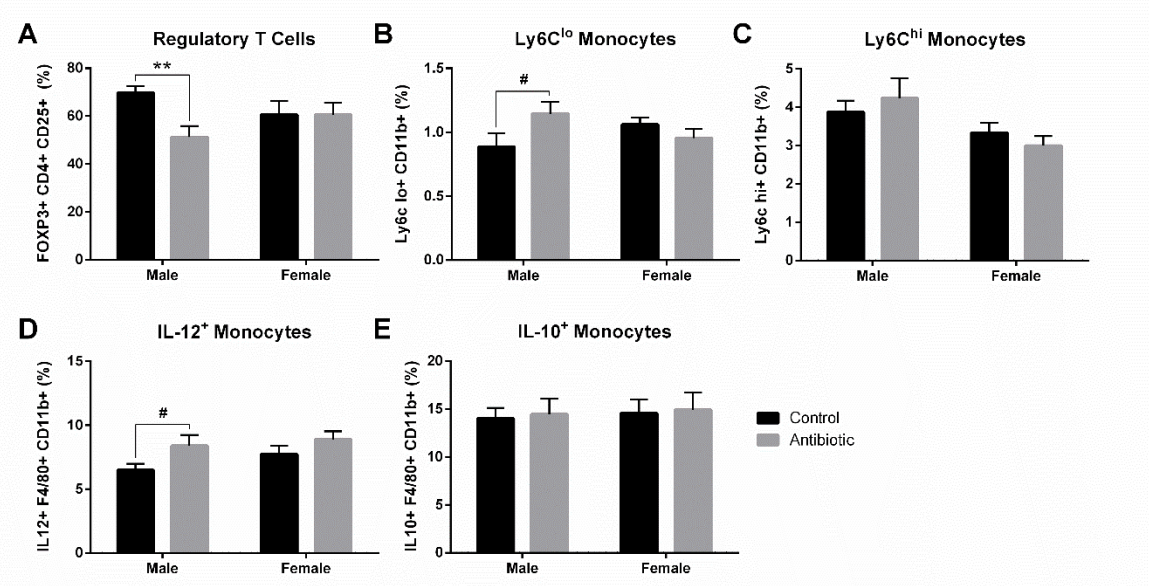

Figure 4: Exposure to prenatal penicillin influences immune parameters in male animals. A) Antibiotic-treated male mice had a significant decrease in the relative proportion of FOXP3^{+} regulatory T cells relative to control males. They also had near-significant increases in the proportions of CD11b^{+} B) Ly6C^{lo} and D) F4/80^{+} IL-12^{+} monocytes, but no apparent differences in C) Ly6C^{hi} or E) F4/80^{+} IL-10^{+} monocytes. No significant differences were seen between female groups. n = 14 antibiotic-treated males, 15 of each other group. Graphs depict mean + standard error. $^{\#}p < 0.08$, $^{**}p < 0.01$.

2.4.7. Prenatal antibiotics resulted in long-term microbial community changes in the offspring

We found a large sex-specific effect of prenatal penicillin exposure on microbiota composition. In particular, antibiotic-treated males had significantly increased bacterial diversity (alpha diversity) at PND 40 relative to PND 21 and to control mice (Figure 5A). No such difference was found in females (Figure 5B). We next wished to examine differences in between-sample diversity (beta diversity). Clear clustering was seen among all treatment groups (Figure 5C, D) and between sexes (Figure S6), indicating that microbiotas sampled from the same treatment group were more similar to each other than to microbiota from other treatment groups (Figure S7).

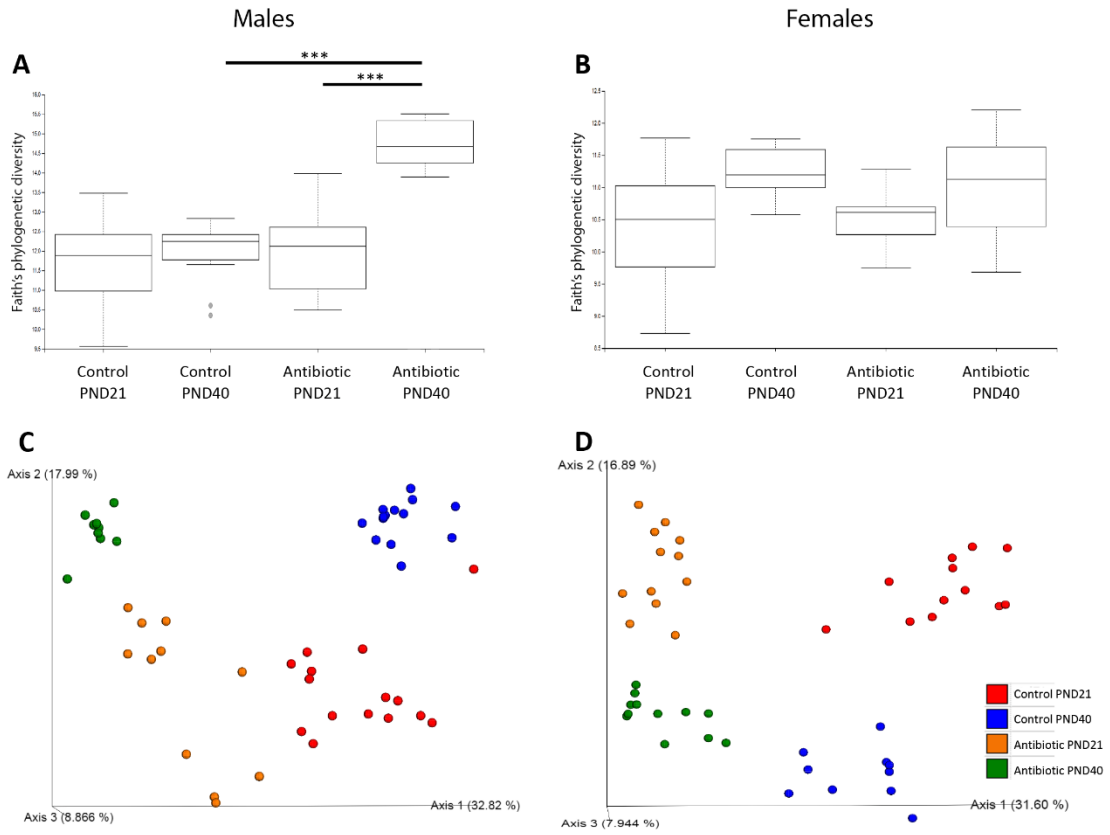

Figure 5: A) Prenatal penicillin-treated male mice had increased alpha diversity at PND 40 relative to PND 21 and controls at PND 40, and C) both groups of male mice clustered distinctly by beta diversity at each time point. B) No differences in alpha diversity were seen between female groups at either time point, though D) each group still clustered distinctly at each time point by beta diversity. *** $p < 0.001$.

To assess which features differed between mice groups and sampling times, we used LEfSe. Many more bacterial features were overrepresented in the treated males at PND 21 and PND 40 compared to untreated males at the same timepoints. These included features such as *Staphylococcus, Parabacteroides, Jeotgalicoccus,* and *Enterococcus* in males at PND 21, and *Adlercreutzia, Streptococcus*, Candidatus Arthromitus, *Dehalobacterium*, and *Blautia* in treated males at PND 40. The untreated male groups had significantly more *Prevotella*, *Bacteroides,* and *Mucispirillum*. The

overrepresentation of *Adlercreutzia* (PND 40), *Jeotgalicoccus* and *Enterococcus* (PND 21) in the antibiotic-treated male groups, and *Mucispirillum* in the untreated male group (PND 21), were also seen in the treated and untreated female groups, respectively (Figure S8).

2.5. Discussion

Our data suggest that prenatal-only low-dose penicillin treatment has long-term and sex-specific effects on mouse behaviour, brain mRNA expression, immune regulation, and gut microbiota composition of adult offspring. Specifically, we found evidence for decreased anxiety-like behaviours in females, and abnormal immune regulation, brain gene expression, and social-related behaviours in males. We also found sex-specific and long-term changes in microbiota composition in both sexes. While we make no claim in this respect, it is interesting to note that our behavioural results correspond with reported clinical gender disparities in incidence of anxiety disorders in females (Eaton et al., 2012; McLean, Asnaani, Litz, & Hofmann, 2011) and social disorders in males (Eaton et al., 2012; Werling & Geschwind, 2013).

In the light-dark test and elevated plus maze, female offspring from penicillin-treated dams had a greater willingness to explore exposed areas, indicating decreased anxiety-like behaviour. This finding was different from our previous longer-duration perinatal treatment study, which found antibiotic-induced decreased anxiety-like behaviour in only male animals (Leclercq et al., 2017). Others have also found a

differential effect of prenatal insult on male and female offspring: prenatal maternal stress induced altered microbiota and anxiety-like behaviour in female (Gur et al., 2017) but not male (Gur et al., 2019) mice.

In the three-chamber test we found evidence for abnormal social behaviour in male offspring from penicillin-treated dams, in that they did not show the normal preference for proximity to a novel mouse versus an empty chamber. However, they did show a preference for a novel mouse over the initial mouse in the next phase of the test, suggesting that social recognition was not affected. We did not detect any difference in social preferences in either female group. Previous work has also shown that prenatal insults can alter social behaviours in male, but not female, animals (Gur et al., 2019, 2017). It is important to note, however, that we cannot conclude an absence of effect on female animals, as we did not see preferences in either phase of the test in female control animals. Other work has also found social interaction to be decreased in female mice relative to males (Netser, Haskal, Magalnik, & Wagner, 2017), which may have contributed here.

In the microdefeat procedure we found additional evidence for abnormal social behaviour, in that penicillin-treated males spent less time displaying agonistic tail rattles and were attacked by the CD1 aggressor for more time than were control males. Control males also tended to retaliate in attacks against the aggressor more than did treated males, though this did not reach statistical significance. These findings are particularly interesting in light of our previous perinatal penicillin treatment experiment, where we

observed increased aggressive-related behaviours in treated male animals (Leclercq et al., 2017).

Given the observed abnormal male social behaviour, we wondered whether the expression of related genes in the brain might also be altered. In the hippocampus of treated male animals we found decreases in the expression of *AVPR1A* and *AVPR1B*, though these were just shy of statistical significance. Nonetheless, given that the largest effect was in *AVPR1B* expression, and that *AVPR1B* knock-out mice show reduced social motivation (Wersinger et al., 2004) and aggressive behaviours (Wersinger et al., 2002), these changes may be functionally relevant. To further examine this possibility, we determined the extent to which their expression correlated with measured social behaviours. We found that, in only male animals, the expression of *AVPR1A* and *AVPR1B* in the hippocampus correlated positively with markers of social behaviors, while negative correlations were seen for *AVPR1A* and *OXTR* in the frontal cortex.

We also found changes in the immune regulatory status of male (but not female) offspring from antibiotic-treated dams, which showed a relative decrease in $FOXP3^+$ T_{reg} cells as a proportion of $CD3^+$ $CD4^+$ $CD25^+$ lymphocytes, along with suggestive evidence for proinflammatory changes in proportions of IL-12^+ and $Ly6C^+$ monocytes. We did not, however, find significant evidence for inflammatory gene expression in the frontal cortex by PCR, as had been seen after perinatal penicillin treatment (Leclercq et al., 2017). Interestingly, we also found a strong positive correlation between proportion of T_{reg} cells and hippocampal *AVPR1B* expression in male animals only. The immune

abnormalities seen in males in the present study are interesting and may suggest that inflammatory changes and associated decreased immune regulation at least accompany, and may even be causal of, altered social but not anxiety-like behaviour. Behavioural abnormalities seen in chronically stressed mice appear to be caused by immune dysregulation (Reber, Siebler, Donner, Morton, Smith, Kopelman, Lowe, Wheeler, Fox, Hassell, & others, 2016) and studies of immune function in treated and untreated depressed patients show a clear association between lack of treatment response and inadequate immune regulation (Syed et al., 2018).

Prenatal penicillin treatment affected microbiota diversity and composition in a sex- and time-specific manner. In terms of species richness, antibiotics did not significantly change the diversity over time in female mice. However, antibiotic-treated males at PND 40 showed a dramatic and significant increase in diversity even when compared to the control groups. This increase could be explained by the fact that the mice were weaned and thus exposed to diverse microorganisms present in the diet. The differences found in alpha diversity between males and females could be explained by hormonal and immune changes between sexes. Progesterone, a female hormone, reduces bacterial diversity (Nuriel-Ohayon et al., 2019). Changes in the beta diversity persisted over time in both males and females, demonstrating the long-term effect of the antibiotic treatment during the late prenatal period. Interestingly, different bacterial taxa were impacted by antibiotic treatment according to sex.

The timing of exposure to environmental insults clearly influences the long-term effects in the offspring (Padmanabhan et al., 2016). For example, Humann and colleagues showed that prenatal exposure to bacterial peptidoglycan had specific neurochemical and behavioural effects on the offspring that were dependent on timing, since beyond a certain date of pregnancy the neurotoxic effects were not seen (Humann et al., 2016). Similar timing-dependent effects of antibiotics have been found in human cohorts. Antibiotic consumption by pregnant human mothers has been correlated with increased risk for offspring mental disorders (Lydholm et al., 2019), and perinatal exposure has been associated with infant brain electroencephalography power and early childhood attentional deficits (Firestein et al., 2019). In contrast, antibiotic treatment of human infants (but not during pregnancy) has been associated with subsequent behavioural abnormalities in children and adolescents (Slykerman et al., 2019, 2017). It is important to stress, however, that in human situations antibiotics are prescribed on a background of infection and thus causal relationships, if any, are not established (Lydholm et al., 2019).

While we did not attempt to determine the mechanisms underlying changes seen in this exploratory study, previous work suggests that numerous possibilities exist. The gut microbiota are known to influence the host through multiple pathways, including immunological (Reber, Siebler, Donner, Morton, Smith, Kopelman, Lowe, Wheeler, Fox, Hassell, Greenwood, et al., 2016), neuronal via the vagus nerve (Bercik, Park, et al., 2011; Bravo et al., 2011), and humoral through secreted products (Lyte,

2014), among others (Forsythe et al., 2016). The relative importance of each of these mechanisms throughout development and in adult animals remains largely unknown, however (Dinan & Cryan, 2017). As such, we are not prepared to speculate on what contributed to the effects observed in this study, though we are attempting to address this in further experiments. It is also important to stress that, though our microbiota data are consistent with the hypothesis that penicillin-induced changes to gut microbiota composition mediated the changes we observed, we cannot rule out possible influence by penicillin-related neurotoxicity (Champagne-Jorgensen et al., 2019; Delungahawatta et al., 2017).

2.6. Conclusion

Overall, we found that prenatal-only exposure to low-dose penicillin has sex-specific effects on anxiety-like and social behaviours in adult mouse offspring. These behavioural changes were accompanied by and correlated with changes in brain gene expression and immune regulation, which occurred alongside significantly altered microbiota composition. These data suggest that exposure of mice to therapeutically-relevant doses of penicillin during late pregnancy influences neural and immunological development to produce long-term effects in the offspring.

Declarations of interest: None

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2.7. Supplemental Results

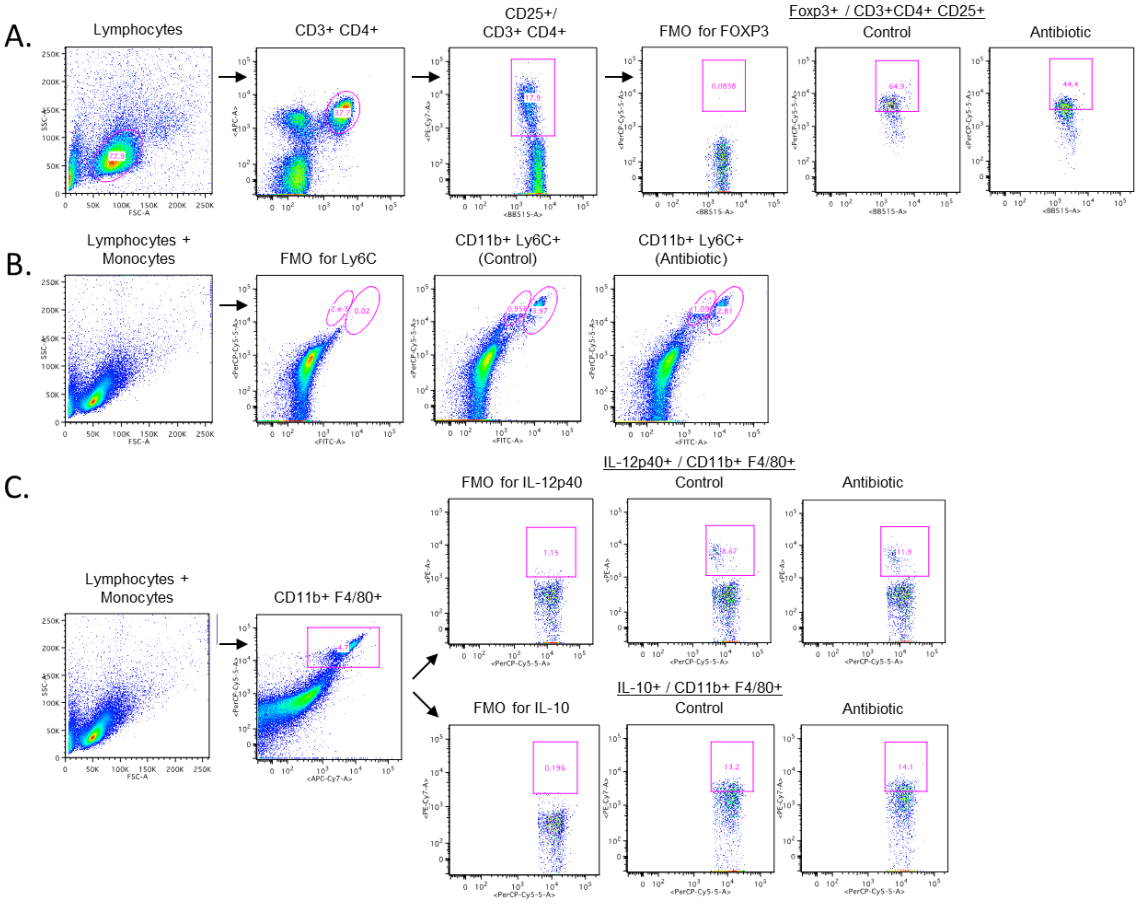

Figure S1: Gating strategies used to detect splenocyte subtypes by FACS. A) To evaluate FOXP3+ T_{reg} cells, splenic lymphocyte populations were gated for CD3+ CD4+ fluorescence, which were sub-gated for CD25+ fluorescence, which were then sub-gated for FOXP3+ fluorescence. B) For Ly6C-expressing monocyte analyses, mixed lymphocyte-monocyte populations were gated for CD11b+ Ly6C+ fluorescence; Ly6C-high (right circles) and Ly6C-low (left circles) are marked as circular gates. C) To evaluate IL-12- and IL-10-expressing monocytes, mixed lymphocyte-monocyte populations were gated for CD11b+ F4/80+ fluorescence. These were then sub-gated for IL-12 or IL-10 fluorescence, as appropriate.

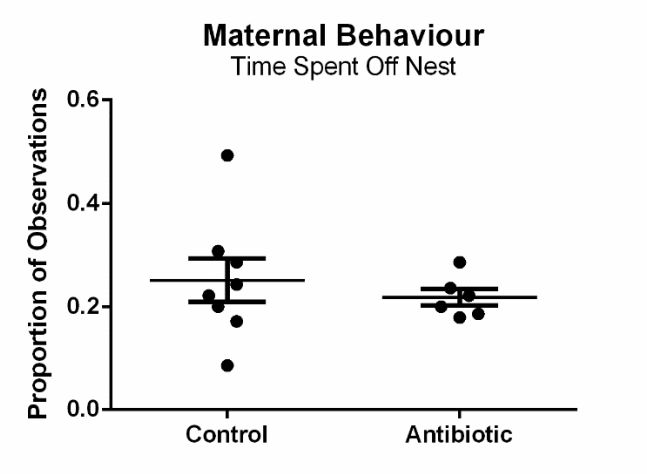

Figure S2: The proportion of time spent away from the nest for treated or control dams. We detected no differences between groups. Scores represent the average proportion of observations from PND 1 to PND 7. Graphs depict mean ± standard error.

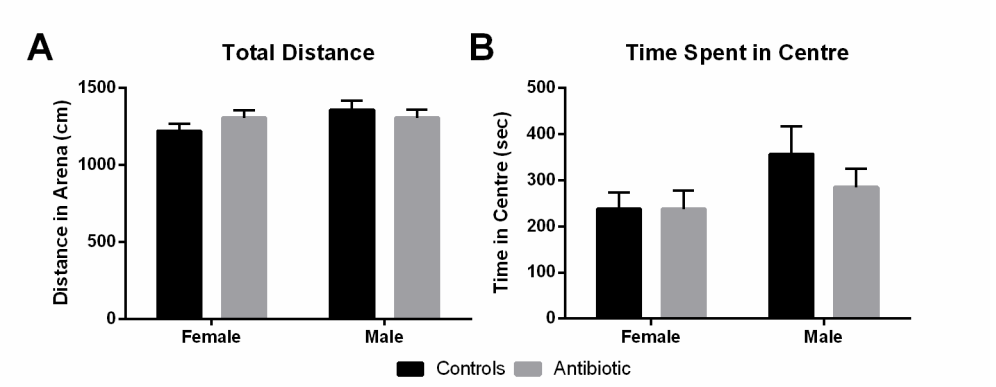

Figure S3: Results from the open field test. No differences were detected in A) the distance travelled in the arena nor B) the time spent in the centre of the arena between groups of either sex. Graphs depict mean ± standard error.

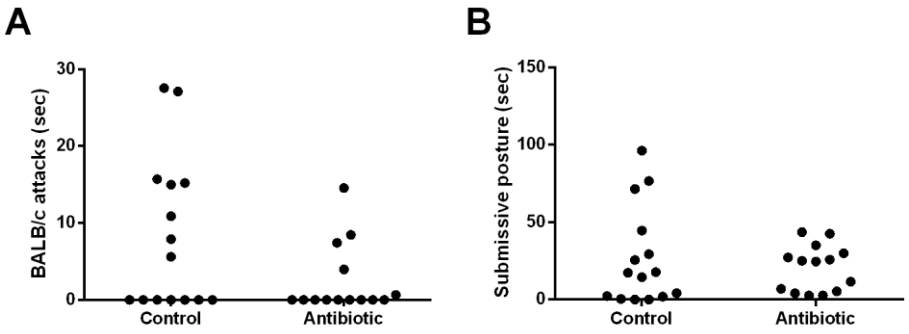

Figure S4: Additional results from the microdefeat procedure, described in the main paper. Each point represents the sum total of all three microdefeat trials. A) Time spent by each BALB/c retaliating against CD1 attacks. B) Time spent by each mouse in a submissive posture.

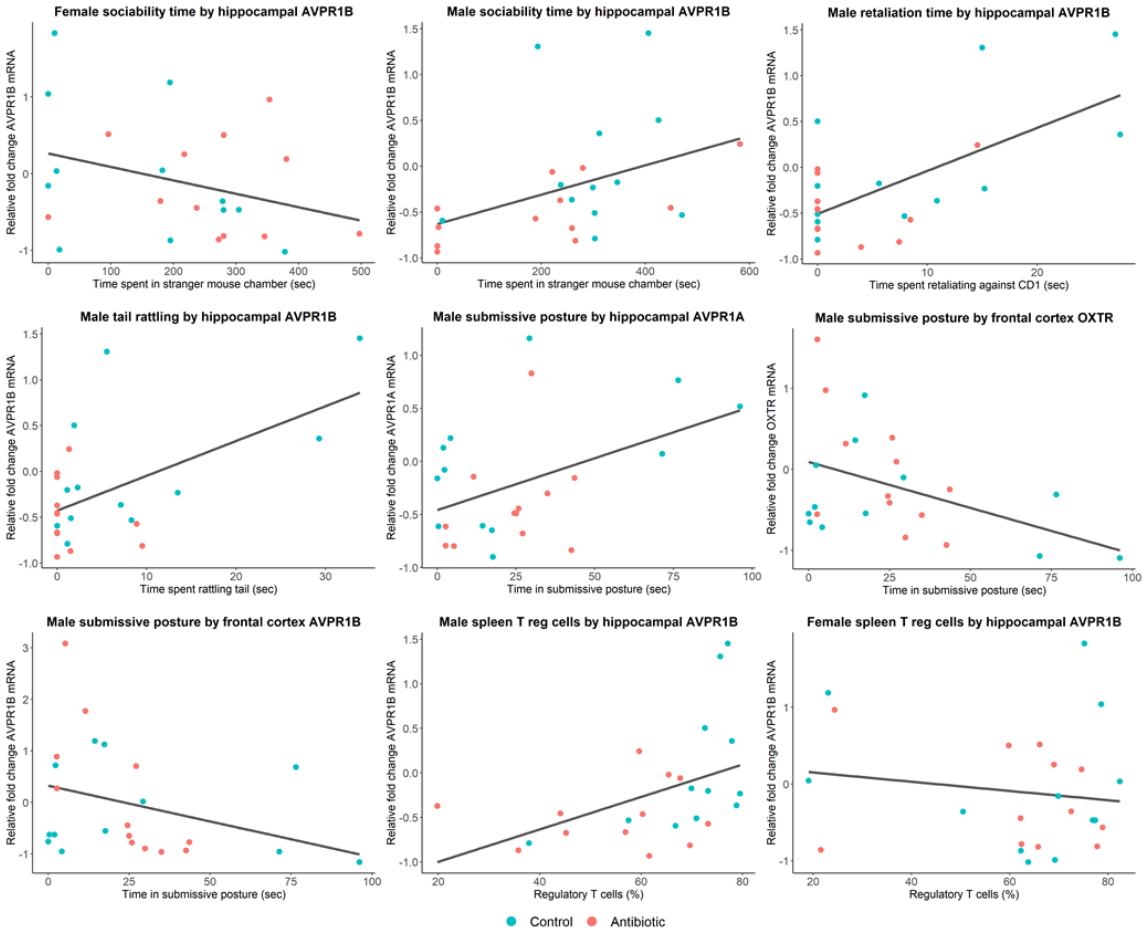

Figure S5: Graphs for Pearson correlations described in the main text. Trendlines are calculated across both groups depicted.

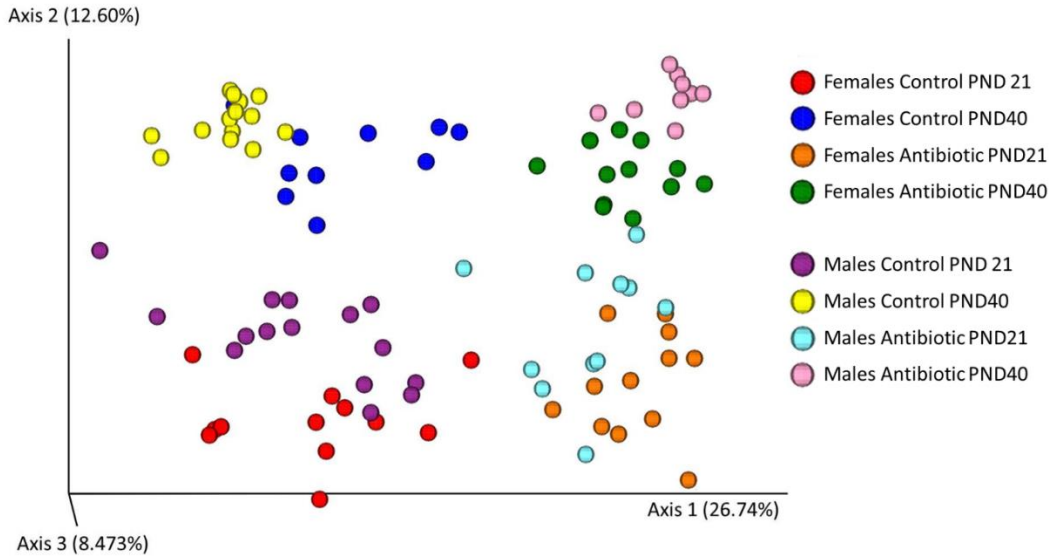

Figure S6: Gut microbiota compositions clustered distinctly between sexes and treatment groups by beta diversity.

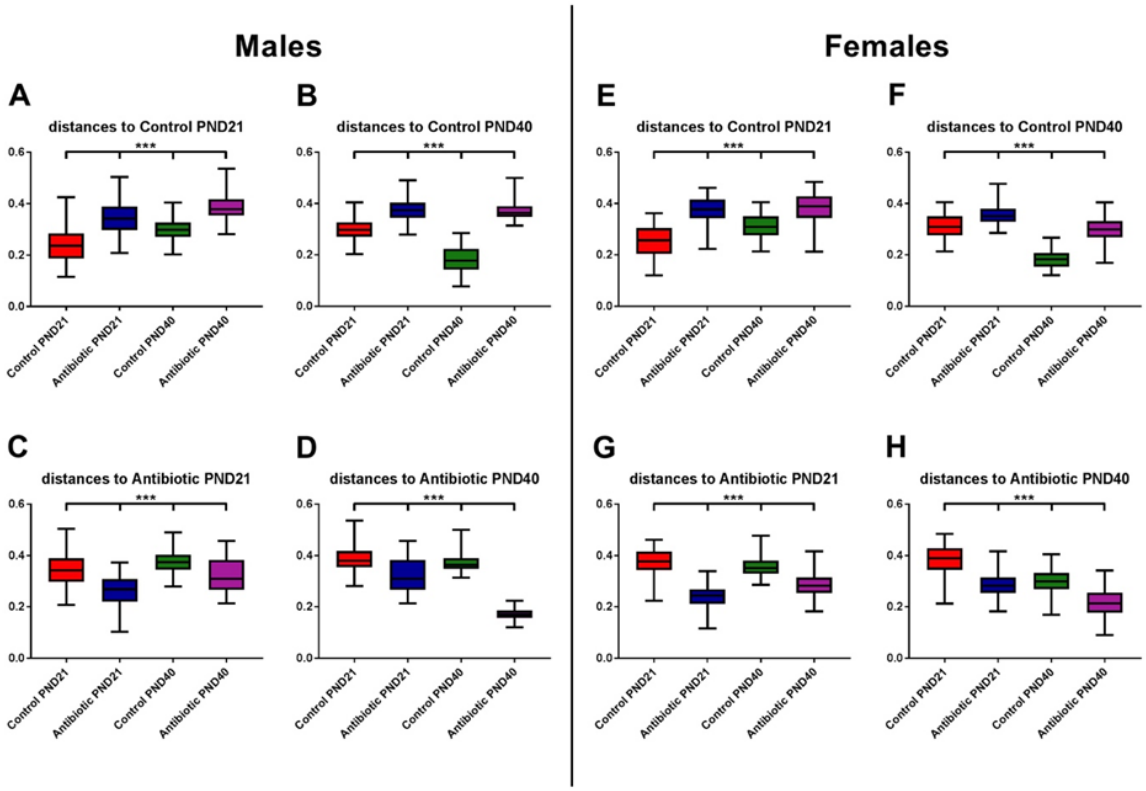

Figure S7: Box plots showing mean unweighted UniFrac distances for male (A-D) and female (E-H) mouse fecal samples, for the control PND21, control PND40, antibiotic PND21 and antibiotic PND40 categories. ***$p < 0.001$.

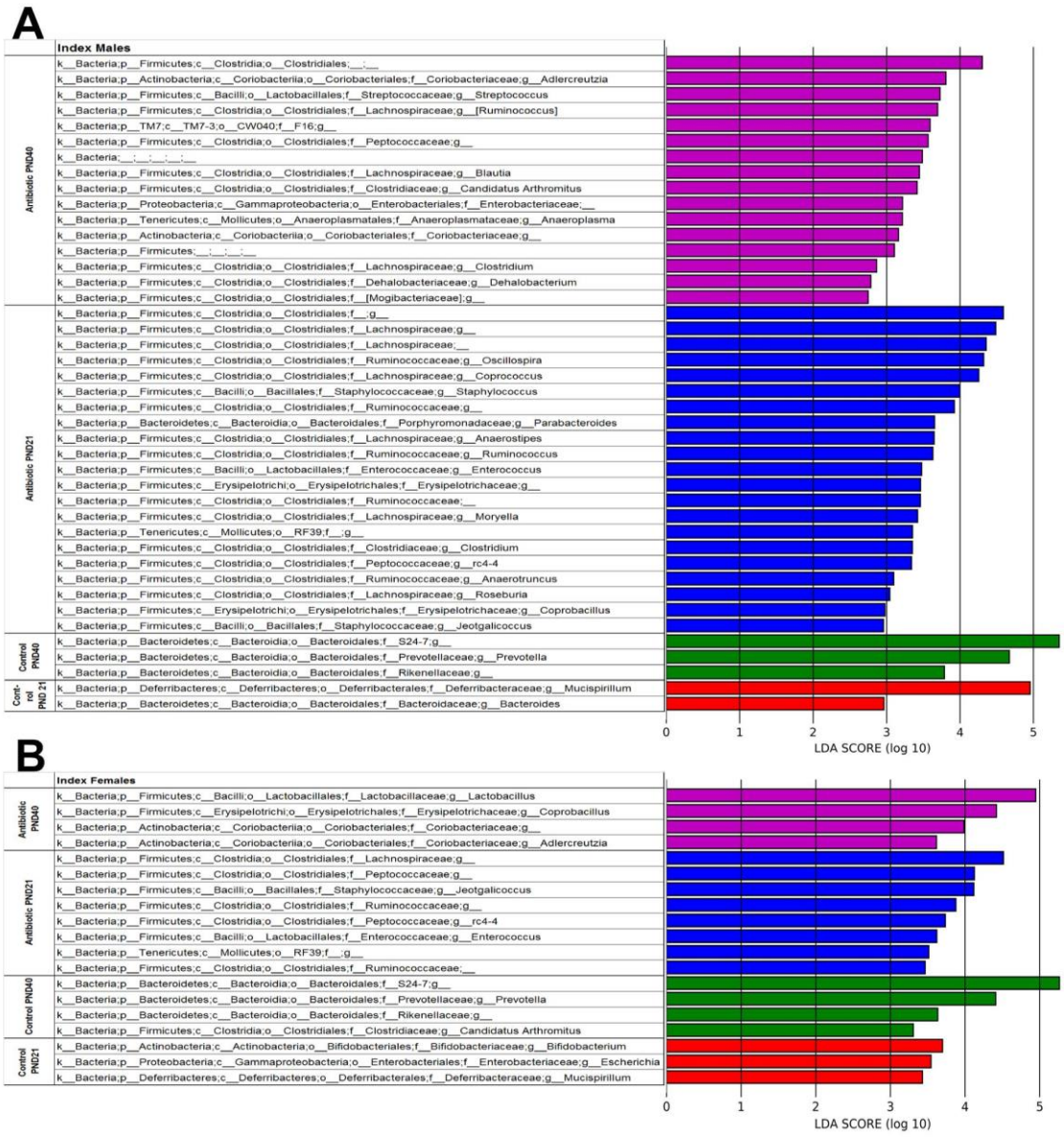

Figure S8: Bacterial taxa which differ significantly between treatment groups in (A) males and (B) females. Analysis was carried out using LEfSe with a cut off of LDA score >2.5.

Table S1: Offspring and treatment information for dams in experiment

Dam	Group	Treatment days	Female Pups	Male Pups
1	Control	0	3	3
2	Control	0	1	1
3	Control	0	2	3
4	Control	0	2	0
5	Control	0	3	1
6	Control	0	2	4
7	Control	0	1	3
8	Control	0	3	0
9	Antibiotic	6	4	2
10	Antibiotic	7	1	4
11	Antibiotic	6	2	1
12	Antibiotic	6	3	1
13	Antibiotic	6	3	3
14	Antibiotic	5	3	3

Table S2: Primer sequences used for real-time qPCR

Gene	Accession Number	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
gapdh	NM_001289726	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG
avpr1a	NM_016847	GGGATACCAATTTCGTTTGG	AAGCCAGTAACGCCGTGAT
avpr1b	NM_011924	TCTACTCTCCGTCTTAGCCTTAACCT	CTCCATCCACCTGCTCCAA
oxtr	NM_001081147	GGAGCGTCTGGGACGTCAAT	AGGAAGCGCTGCACGAGTT
gr (nr3c1)	NM_008173	AAAGGTGGCGCTTATGTACTTAGAG	CGTGCGGAGGCTGCAT
bdnf	NM_007540	GGCACTGGAACTCGCAATG	ATGAATCGCCAGCCAATTCT
il6	NM_031168	CTGCAAGAGACTTCCATCCAGTT	GAAGTAGGGAAGGCCGTGG
il10	NM_010548	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
tnfα	NM_013693	CCACCACGCTCTTCTGTCTAC	TGGGCTACAGGCTTGTCACT

Table S3: Additional qPCR analyses

Sex	Region	Transcript	*t* (df)	*p*	*d*
Female	Hippocampus	AVPR1A	0.73 (17.9)	0.47	0.29
Female	Hippocampus	AVPR1B	0.52 (19.6)	0.61	0.21
Female	Hippocampus	OXTR	0.35 (16.8)	0.73	0.14
Female	Hippocampus	BDNF	-0.13 (20.3)	0.89	-0.05
Female	Hippocampus	GR	-0.68 (18.4)	0.51	-0.27
Male	Hippocampus	BDNF	-0.96 (20.5)	0.35	-0.38
Male	Hippocampus	GR	-0.80 (19.6)	0.44	-0.31
Female	Frontal Cortex	IL6	-0.30 (15.5)	0.77	-0.12
Female	Frontal Cortex	IL10	-0.10 (19.1)	0.92	-0.04
Female	Frontal Cortex	TNFα	-0.19 (16.7)	0.85	-0.08

Female	Frontal Cortex	AVPR1A	-0.09 (21.6)	0.93	-0.04
Female	Frontal Cortex	AVPR1B	-0.12 (21.8)	0.91	-0.05
Female	Frontal Cortex	OXTR	-0.51 (21.9)	0.61	-0.20
Female	Frontal Cortex	GR	0.33 (17.9)	0.74	0.13
Male	Frontal Cortex	AVPR1A	-0.61 (16.8)	0.55	-0.24
Male	Frontal Cortex	AVPR1B	0.59 (19.2)	0.56	0.23
Male	Frontal Cortex	OXTR	1.11 (20.6)	0.28	0.44
Male	Frontal Cortex	GR	0.55 (21.9)	0.58	0.22

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CHAPTER 3. Membrane vesicles of *Lacticaseibacillus rhamnosus* JB-1 contain immunomodulatory lipoteichoic acid and are endocytosed by intestinal epithelial cells

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3.1. Abstract

Intestinal bacteria have diverse and complex influence on their host. Evidence is accumulating that this may be mediated in part by bacterial extracellular membrane vesicles (MV), nanometer-sized particles important for intercellular communication. Little is known about the composition of MV from gram-positive beneficial bacteria nor how they interact with intestinal epithelial cells (IEC). Here we demonstrate that MV from *Lacticaseibacillus rhamnosus* JB-1 are endocytosed in a likely clathrin-dependent manner by both mouse and human IEC *in vitro* and by mouse IEC *in vivo*. We further show that JB-1 MV contain lipoteichoic acid (LTA) that activates Toll-like receptor 2 (TLR2) and induces immunoregulatory interleukin-10 expression by dendritic cells in an internalization-dependent manner. By contrast, neither LTA nor TLR2 appear to be required for JB-1 MV endocytosis by IEC. These results demonstrate a novel mechanism by which bacterial MV can influence host physiology and suggest one potential route for beneficial influence of certain bacteria and probiotics.

3.2. Introduction

Intestinal microbes (microbiota) have complex bidirectional relationships with their host. In mammals, gut bacteria profoundly influence intestinal homeostasis, metabolism, and immunity, both developmentally (Dominguez-Bello, Godoy-Vitorino, Knight, & Blaser, 2019) and in adults (Schroeder & Bäckhed, 2016). As evidence for their diverse and often beneficial influence has increased, so too has interest in developing probiotics, bacterial supplements to improve intestinal health, treat disease, and even modulate mood (Forsythe, Kunze, & Bienenstock, 2016; Suez, Zmora, Segal, & Elinav, 2019). Various mechanisms by which gut bacteria may influence their host have been proposed, including interactions between conserved bacterial components and host receptors, and secretion of bacterially-produced neurotransmitters, short-chain fatty acids, and other products (Fung, Olson, & Hsiao, 2017; Neuman, Debelius, Knight, & Koren, 2015; Schroeder & Bäckhed, 2016). However, despite strong commercial and academic scientific research interest, studies of these candidates have had limited success in fully uncovering the pathways involved.

One potential communication system, currently understudied in this context, involves extracellular vesicles, membrane-bound nanoparticles that are ubiquitously produced by both prokaryotes and eukaryotes (Gill, Catchpole, & Forterre, 2019). In bacteria, these are often referred to as microvesicles or membrane vesicles (MV), or more specifically outer membrane vesicles (OMV) in the case of gram-negative bacteria. While OMV have been recognized for decades, it is only recently that release of MV

through the thick gram-positive peptidoglycan cell wall was considered plausible (Brown, Wolf, Prados-Rosales, & Casadevall, 2015). As a result, while OMV have been well characterized for their roles in bacterial survival, pathogenesis, and immune modulation (Kaparakis-Liaskos & Ferrero, 2015), relatively little is known about the physiological relevance of gram-positive MV. In general, bacterial MV are known to contain active cargo reflecting the parent bacterium including toxins, virulence factors, nucleic acids, and other components (Toyofuku, Nomura, & Eberl, 2019), which play an important role in interbacterial (Toyofuku & Nomura, 2017) and bacteria-host communication (Caruana & Walper, 2020).

A role for bacterial MV in activity related to beneficial bacteria was demonstrated by Shen and colleagues (Shen et al., 2012), who found that OMV from the gram-negative commensal *Bacteroides fragilis* had immunoregulatory effects similar to the parent bacterium, and that these were mediated by OMV-associated polysaccharide A. Similarly, we have previously shown that the gram-positive bacterium *Lacticaseibacillus rhamnosus* JB-1 (JB-1; recently reclassified from *Lactobacillus* (Zheng et al., 2020)) and its MV can promote the number and functions of regulatory T cells, activate TLR2, and induce an immunoregulatory phenotype in dendritic cells (DCs) (Al-Nedawi et al., 2015; Karimi, Inman, Bienenstock, & Forsythe, 2009; Karimi, Kandiah, Chau, Bienenstock, & Forsythe, 2012). Substantial evidence now exists that MV from beneficial bacteria can independently influence the host (Haas-Neill & Forsythe, 2020), though their mechanisms of action in many cases remain unclear.

Bacteria express conserved features (microbe-associated molecular patterns; MAMPs) that are recognized by pattern recognition receptors (PRRs) of many host cells, including epithelial, endothelial, and immune (Fitzgerald & Kagan, 2020). These MAMPs are thought to be important signalling molecules also present on MV, which can modulate responses in host intestinal epithelial cells (IEC) and immune cells (Kaparakis-Liaskos & Ferrero, 2015) and may be involved in their immunoregulatory influence. Indeed, interactions between PRRs and MAMPs from commensal microbes are necessary to maintain homeostasis between host and microbiota, and thus contribute to healthy host development and immune responses (Chu & Mazmanian, 2013). Whether MAMPs are involved in activity associated with gram-positive MV is unknown.

Since we remain uncertain as to the exact mechanisms whereby gut bacteria influence the host, we sought to extend our previous work with JB-1 MV to learn more about their interaction with the gut epithelium and to further characterize their influence on dendritic cells. Here, we show that MV from JB-1 are endocytosed in a likely clathrin-mediated manner by both mouse and human IEC lines *in vitro* and by mouse IEC *in vivo*, using OMV from *Escherichia coli* Nissle 1917 (EcN) as a positive control as they are known to be endocytosed by IEC (Cañas et al., 2016). We further demonstrate that JB-1 MV contain lipoteichoic acid (LTA), which is recognized by TLR2 and induces interleukin 10 (IL-10) expression in DCs *in vitro*. Moreover, our data suggest that internalization of JB-1 MV by DCs is involved in their induction of IL-10 expression.

3.3. Results

3.3.1. Physical characterization of bacterial membrane vesicles

Membrane vesicles from *L. rhamnosus* JB-1 (JB-1) and *E. coli* Nissle 1917 (EcN) were collected by ultracentrifugation of cell-free culture broth and characterized by nanoparticle tracking analysis, transmission electron microscopy, and protein content. JB-1 MV preps had approximately 200 µg/mL protein and $3.1x10^{11}$ particles/mL, with a mean size of 130 nm (mode: 145 nm) (Fig. 1a). By contrast, EcN OMV had approximately 100 µg/mL protein and $1.2x10^{10}$ particles/mL, with a mean size of 145 nm (mode: 120 nm) (Fig. 1b).

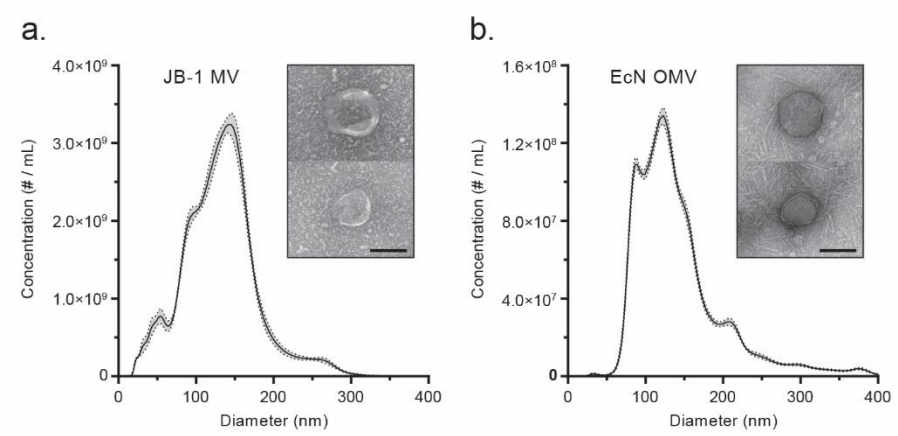

Figure 1. Characterization of bacterial MV. Membrane vesicles from (**a**) *L. rhamnosus* JB-1 and (**b**) OMV from *E. coli* Nissle 1917 were enumerated by nanoparticle tracking analysis (graphs) and visualized by transmission electron microscopy (inset images). Scale bars represent 100 nm. Ribbon represents ± 1 standard error of 5 technical replicates.

3.3.2. *L. rhamnosus* JB-1 MV are internalized by gut epithelial cells *in vitro*

To test whether JB-1 MV are internalized by cells *in vitro*, we first examined whether CFSE-labelled MV and OMV were successfully stained and retained activity by incubating them with bone marrow-derived dendritic cells (BMDCs) for 1 h. We assessed MV and OMV uptake by measuring CFSE signal in flow cytometry and found that >85% of BMDCs were CFSE-positive for both EcN OMV and JB-1 MV (Supplementary Fig. S1), consistent with phagocytosis and suggesting that vesicles remained immunologically active after CFSE labelling.

Since EcN OMV are known to be endocytosed by HT-29 cells (a human IEC line) (Cañas et al., 2016), we used this as a positive control in exploring whether JB-1 MV would also be internalized by IEC *in vitro*. We incubated HT-29 cells with CFSE-labelled JB-1 MV or EcN OMV and measured internalization by association with CFSE fluorescence. Flow cytometry analyses revealed >96% of cells were positive for MV-related fluorescence (Fig. 2a, left panels) and clear puncta were visible within cells when viewed with fluorescence microscopy (Fig. 2a, right panels). We repeated these experiments using the mouse duodenal cell line MODE-K, and again found that both JB-1 and EcN MV were internalized to similar extents (Fig. 2b). These MV appear to be intracellular as CFSE fluorescence was consistently found near nuclei and dispersed through the cell when examined by z-stacking (Fig. 2c).

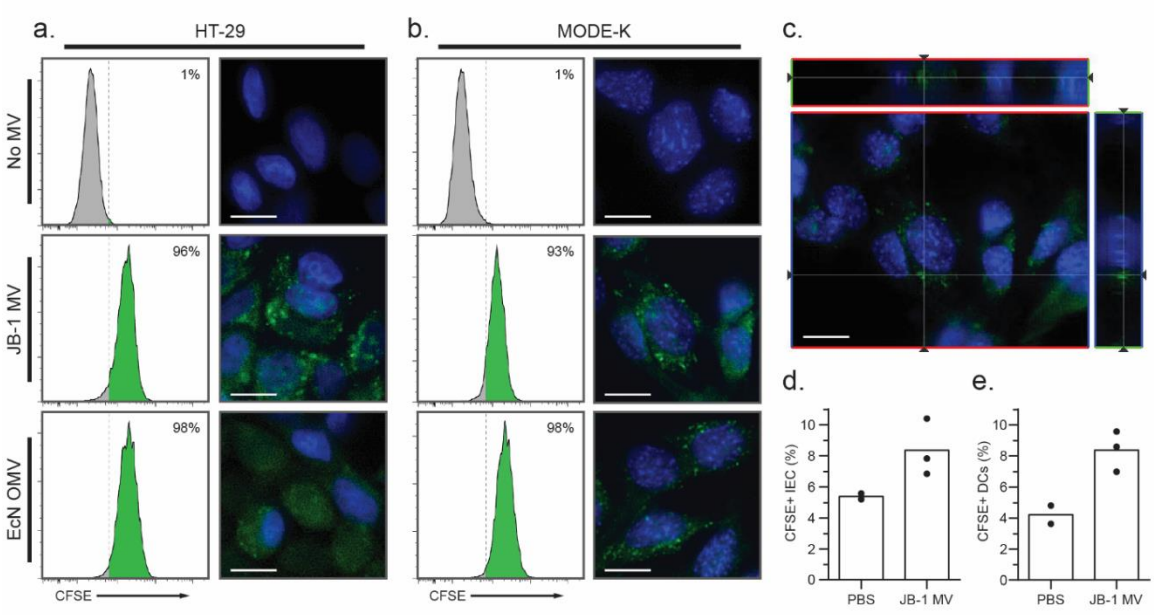

Figure 2. *L. rhamnosus* MV are internalized within 2 hours by intestinal epithelial cells. (**a**-**c**) Approx. $3x10^{10}$ CFSE-labelled JB-1 MV or EcN OMV were incubated at 37°C for 2 h with HT-29 cells (**a**) or MODE-K cells (**b**, **c**), and cell-associated fluorescence was measured by flow cytometry (left) or fluorescence microscopy (right). (**c**) Representative z-stack demonstrating CFSE fluorescence adjacent to a MODE-K nucleus after incubation with CFSE-labelled JB-1 MV. Crosshairs represent the reconstructed slices shown on the upper and right side of the image, while lines through the slices represent the z-plane shown in the main image. (**d**, **e**) Approx. $3x10^{10}$ CFSE-labelled JB-1 MV or phosphate-buffered saline (PBS) vehicle were orally gavaged to BALC/c mice, then 2 h later jejuna were isolated, and fluorescence was measured by flow cytometry of (**d**) $A33^{+}$ $CD45^{-}$ intestinal epithelial cells or (**e**) $CD11c^{+}$ MHC II^{+} lamina propria dendritic cells. Scale bars represent 10 µm. Green colour represents CFSE signal, while blue represents nuclear stain (Hoechst 33342).

3.3.3. *L. rhamnosus* JB-1 MV are internalized by gut epithelial cells *in vivo*

Given that MV are internalized *in vitro*, we wished to see if this also occurred *in vivo*. We gavaged BALB/c mice with CFSE-labelled JB-1 MV, then collected jejuna after 2 h. To differentiate IEC from phagocytes, we physically separated and independently isolated IEC and lamina propria cells by differential gradient separation. By flow cytometry we identified IEC as CD45-negative (marker of differentiated hematopoietic cells) and A33-positive (intestinal epithelial cell marker (Johnstone et al., 2000)). We

identified lamina propria DCs by positivity for CD11c and MHC II. Analyses of these populations indicated appreciable and similar fluorescence in both IEC (Fig. 2d) and lamina propria DCs (Fig. 2e), suggesting that each internalized MV. This is consistent with active internalization of JB-1 MV by IEC and DCs *in vivo*.

3.3.4. *L. rhamnosus* JB-1 MV are likely internalized by clathrin-mediated endocytosis

OMV may be internalized by epithelial cells through a variety of mechanisms (O'Donoghue & Krachler, 2016). When this work was undertaken, one published study had shown that MV from the gram-positive bacterium *Staphylococcus aureus* are internalized by HeLa cells via cholesterol-dependent membrane fusion (Thay, Wai, & Oscarsson, 2013). Since EcN OMV are internalized by HT-29 in a clathrin-dependent manner (Cañas et al., 2016), we tested if the same mechanism is active here and focused on JB-1 MV.

We pre-incubated cells with the dynamin inhibitor dynasore, which blocks phagocytosis (Marie-Anaïs, Mazzolini, Herit, & Niedergang, 2016) and clathrin-mediated endocytosis (Macia et al., 2006), then added CFSE-labelled MV as before. We encountered an unexplained interaction between dynasore and CFSE causing an artifactual increased fluorescent signal that we could not prevent. We therefore labelled the MV with DiO, a lipid-soluble membrane marker previously used with dynasore in analogous experiments (Kunsmann et al., 2015). DiO-labelled MV were internalized by 80% of BMDCs, and this was prevented by preincubation with dynasore (Fig. 3a).

Similarly, both HT-29 (Fig. 3b) and MODE-K cells (Fig. 3c) were prevented from internalization of DiO-labelled JB-1 MV by dynasore, suggesting that internalization of JB-1 MV by IEC is an active and likely clathrin-mediated process.

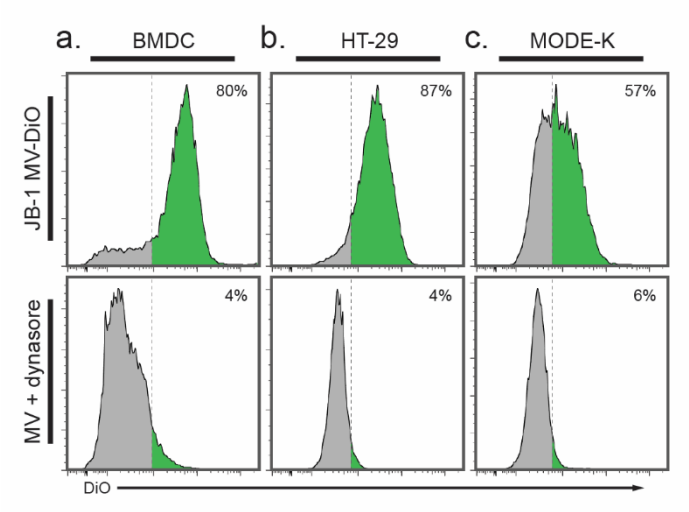

Figure 3. *L. rhamnosus* JB-1 are endocytosed in a likely clathrin-dependent manner. (**a**-**c**) Approx. $3x10^{10}$ DiO-labelled JB-1 MV were incubated with (**a**) BMDCs for 1 h or (**b**) HT-29 or (**c**) MODE-K cells for 2 h, either after preincubation with the dynamin inhibitor dynasore (bottom panels) or without preincubation (top panels).

3.3.5. *L. rhamnosus* JB-1 MV contain immunologically active lipoteichoic acid

Recent work suggests that MV from some lactic acid bacteria contain LTA (Shiraishi et al., 2018), a known ligand for TLR2 that can induce IL-10 production by DCs (Saito, Lin, & Wu, 2019). As we previously showed JB-1 MV to have these same effects (Al-Nedawi et al., 2015), we questioned whether LTA could mediate them and additionally serve as a ligand to induce receptor-mediated endocytosis. Using western blot, we first demonstrated the presence of LTA in JB-1 and its MV (Fig. 4a). We then

performed antibody neutralization experiments to determine whether LTA is involved in MV-related effects *in vitro*. Anti-LTA antibodies, but not isotype control, inhibited MV interaction with TLR2 in a reporter cell assay (t = 10.1, d = 2.0, p = 0.0048; Fig. 4b). They also inhibited internalization of DiO-labelled JB-1 MV to a similar extent as dynasore (Fig. 4c), and abolished MV-induced production of IL-10 (Fig. 4d). Interestingly, decreased IL-10 correlated with decreased internalized MV in experiments with both anti-LTA and dynasore (Fig. 4e), suggesting that internalization of whole MV is involved in the induction of IL-10.

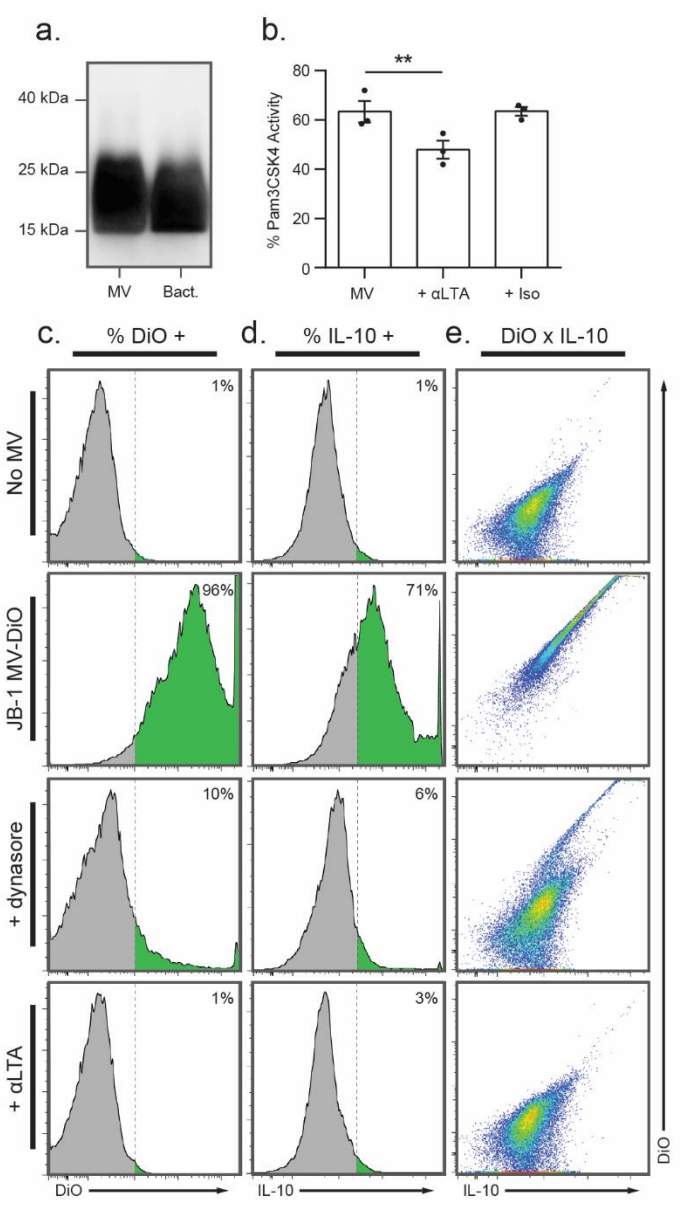

Figure 4. Lipoteichoic acid in *L. rhamnosus* JB-1 MV is responsible for immunomodulatory effects. (**a**) Western blot analysis was used to measure LTA associated with JB-1 MV (MV) or in lysates of whole JB-1 bacteria (Bact.). (**b**) Independent preparations of JB-1 MV were incubated with a TLR2 reporter cell line with or without preincubation with anti-LTA antibody (+ αLTA) or isotype control (+ Iso), and TLR2 activity was expressed as a percentage of that measured for the synthetic TLR2 ligand Pam3CSK4 (300 ng/mL). (**c**-**e**) DiO-labelled MV or vehicle were incubated with BMDCs for 18 h either with or without preincubation with dynasore or anti-LTA antibody, then (**c**) DiO-related or (**d**) IL-10-related fluorescence were measured by flow cytometry. (**e**) The extent to which MV internalization was associated with IL-10 expression was assessed by plotting cells as a function of DiO and IL-10 signal. Error bars represent ±1 standard error. **= $p < 0.01$. Full-length blot is presented in Supplementary Fig. S3.

Given that LTA mediates immune phenotypic change in DCs, we tested possible involvement of LTA in internalization by MODE-K cells but found no effect of anti-LTA antibodies (Supplementary Fig. S2). We further attempted to block the mouse pattern recognition receptors TLR2 and SIGN-R1 with neutralizing antibodies as we have done previously with JB-1 MV in DCs (Al-Nedawi et al., 2015), but again found no effect (Supplementary Fig. S2). This suggests that some other ligand-receptor systems are involved in inducing clathrin-mediated endocytosis of JB-1 MV.

3.4. Discussion

Membrane vesicles are promising mediators of bacterial-host communication because they enable the delivery of diverse signaling molecules, including proteins, lipids, carbohydrates, and nucleic acids, to diverse recipient cells, potentially allowing for more complex signalling and protecting contents from degradation (Caruana & Walper, 2020). Though well-studied in multicellular eukaryotes, only recently have MV been considered as possible mediators of communication between beneficial bacteria and their host.

MV from intestinal microbes are thought to affect the local gut environment. Most work thus far has examined the role of MV in pathogenic effects of especially gram-negative bacteria (O'Donoghue & Krachler, 2016), while more recent work has considered the interaction between intestinal epithelial cells and MV from beneficial bacteria. Some of the first work to this end focused on the gram-negative beneficial

bacterium *E. coli* Nissle 1917 (EcN), whose OMV were shown to be endocytosed by IEC in a clathrin-dependent manner (Cañas et al., 2016). While the current paper was in preparation, two recent reports found similar results in gram-positive non-pathogenic bacteria. Rubio and colleagues found that *Bacillus subtilis* MV were internalized and apparently transcytosed by Caco-2 cells *in vitro* (Rubio et al., 2020), while Bajic and colleagues demonstrated that MV from *Lactiplantibacillus plantarum* BGAN8 are endocytosed by HT-29 cells in a clathrin-dependent manner (Bajic et al., 2020).

Here we show that CFSE-labelled MV from the gram-positive beneficial bacterium *L. rhamnosus* JB-1 are internalized by both murine and human IEC within 2 h *in vitro*, as evidenced both by flow cytometry experiments and by the presence of distinct puncta when viewed under routine fluorescence microscopy and z-stacking. We further showed evidence of JB-1 MV being internalized *in vivo* within 2 h after oral consumption of CFSE-labelled MV by both small intestinal gut epithelial cells and mononuclear dendritic cells in the lamina propria. While we have shown in a previous publication that labelled MV were internalized by cells in Peyer's patches 18 h after feeding (Al-Nedawi et al., 2015), in the present study visible Peyer's patches were excised prior to isolation of cells for analysis and flow cytometric analysis revealed their dendritic cell nature.

Classically, internalization of MV by IEC is thought to occur via several different mechanisms, including macropinocytosis, clathrin-dependent endocytosis, clathrin-independent endocytosis, and membrane fusion (O'Donoghue & Krachler, 2016).

Interestingly, though rarely acknowledged, phagocytosis (e.g., of pathogens like *Salmonella typhimurium* and *Staphylococcus aureus*) can also occur in IEC (Veiga et al., 2007), though there is no evidence for this with non-pathogenic bacteria. Other investigations of the mechanism of internalization of MV from potentially beneficial bacteria have concluded that endocytosis is primarily clathrin-mediated, as internalization was inhibited by dynasore and chlorpromazine but not filipin III or nystatin (Bajic et al., 2020; Cañas et al., 2016).

In microscopy experiments, we found that internalization of JB-1 MV was indistinguishable from that of EcN OMV, which suggested that similar mechanisms might be involved. Indeed, pre-incubation of both human and mouse IEC lines with the dynamin inhibitor dynasore almost entirely abolished endocytosis of JB-1 MV, suggesting that their endocytosis is clathrin-mediated. It is important to note, however, that in addition to clathrin-mediated endocytosis, dynasore also inhibits activity of dynamin required for phagocytosis (Marie-Anaïs et al., 2016). Thus, we cannot distinguish between these mechanisms. We were also unable to determine which ligand-receptor systems were involved in internalization, as antibodies to LTA, TLR2, and SIGN-R1 were without inhibitory effect in MODE-K cells.

MV from several lactic acid bacteria have been shown to contain distinct cargo (Bajic et al., 2020; Dean, Leary, Sullivan, Oh, & Walper, 2019), which in some cases associate with their functional effects (Al-Nedawi et al., 2015). LTA is one promising candidate, as it is present on MV of some lactic acid bacteria (Shiraishi et al., 2018),

including the *L. rhamnosus* strain ATCC 7469 (Pollack, Ntamere, & Neuhaus, 1992). Using western blot, we found that LTA is also present on MV from *L. rhamnosus* JB-1, and that it appears to be a major TLR2 agonist associated with MV as antibody neutralization experiments with anti-LTA reduced TLR2 activation in a reporter cell line. Moreover, anti-LTA inhibited internalization of JB-1 MV by BMDCs and simultaneously reduced their induction of IL-10 expression, suggesting that LTA is involved in immunoregulatory effects of JB-1 MV and that these are magnified by MV internalization.

Lipoteichoic acids are amphiphilic membrane-anchored polymers associated with the cell wall of gram-positive bacteria (Shiraishi, Yokota, Fukiya, & Yokota, 2016). They are important for bacterial physiology and host-bacteria interaction, and are commonly considered analogous to the gram-negative lipopolysaccharide, as both are studied as highly immunologically active molecules in host-bacteria interactions (Lebeer, Claes, & Vanderleyden, 2012). LTA are structurally variable between species (Shiraishi et al., 2016), and evidence is accumulating that LTA from some bacteria are immunoregulatory (Kim et al., 2012; Kim, Park, Kim, & Chung, 2020; Saito et al., 2019, 2020; Volz et al., 2018).

The most extensively studied receptor for LTA is TLR2, though interactions with other receptors have been documented (Shiraishi et al., 2016). Interestingly, structurally distinct LTAs appear to interact with TLR2 to different extents and are thought to contribute to varied response magnitudes associated with different bacteria (Kang et al., 2009). The potential role for TLR2 signalling in MV-associated LTA is interesting

considering this receptor's known role in microbiota-host homeostasis. Round and colleagues demonstrated that colonization of the gut by *Bacteroides fragilis* requires TLR2 stimulation by polysaccharide A resulting in mucosal tolerance to the bacterium (Round et al., 2011). Subsequent experiments found that similar effects were seen using B fragilis OMV alone, which induced IL-10 production by DC via OMV-associated polysaccharide A interacting with TLR2 (Shen et al., 2012).

JB-1 MV *in vitro* and *in vivo* have immunoregulatory activity through their effect on the generation and function of both regulatory T cells and dendritic cells (Al-Nedawi et al., 2015). We show here that this may be largely dependent on their content of LTA. Indeed, the endocytosis of JB-1 MV suggests that transcytosis may also occur *in vivo*, and circulating MV with LTA could thus explain some systemic effects (Al-Nedawi et al., 2015; Forsythe, Wang, Khambati, & Kunze, 2012; Karimi et al., 2009, 2012) associated with JB-1 treatment. Overall, these observations have added to our understanding and the characterization of many factors responsible for some of the immune effects of *Lacticaseibacillus rhamnosus* JB-1.

The gram-positive organism we have used in the present experiments is a useful model for exploring mechanisms of action of beneficial bacteria and possibly predicting the importance of similar molecular pathways for candidate and actual probiotics. We have underlined the importance of bacterial membrane vesicles in communication with the host. Transcytosis of epithelium in the gut and other tissue sites by bacterial MV may

help explain some of the distant and systemic effects of many bacteria including probiotics.

3.5. Methods

3.5.1. Animals

Male 8- to 10-week-old specific pathogen-free BALB/c mice were purchased from Charles River (Montreal, Canada) and maintained on a 12-hour light-dark cycle free access to food and water. Mice were euthanized by decapitation. All experiments involving mice were approved by the McMaster Animal Research Ethics Board and followed both the Canadian Council on Animal Care guidelines and the ARRIVE guidelines.

3.5.2. Bacteria and MV preparation

Lacticaseibacillus rhamnosus JB-1 (JB-1) was grown from stock in Man-Rogosa-Sharpe (MRS) medium at 37°C in anaerobic conditions. *Escherichia coli* strain Nissle 1917 (EcN) was a gift from Ardeypharm GmbH (Herdecke, Germany) and was grown in LB (Lennox) in aerobic conditions at 37°C with shaking. After 24 h, cultures were centrifuged at 4°C and 1900 × g for 45 min to pellet bacteria. Supernatants were vacuum filtered through 0.20 µm filter units. The resulting filtrates were ultracentrifuged at 42,000 RPM (138,000 × g) for 3h at 4°C in a Type 45 Ti fixed-angle rotor (Beckman Coulter, Mississauga, Canada), pellets resuspended in cold PBS, then ultracentrifuged

again at 42,000 RPM (121,000 × g) for 3h at 4°C in a Type 70 Ti fixed-angle rotor (Beckman Coulter). Pellets were finally resuspended in 5 µL PBS for every 1 mL of ultracentrifuged supernatant (i.e., concentrated 200x), aliquoted, and frozen at -80°C until further use. Protein concentrations of MV preparations were determined using the Pierce Rapid Gold bicinchoninic acid assay (Thermo Scientific, Mississauga, Canada).

To fluorescently label them, MV were incubated with 20 µm CFSE (CFDA SE; Invitrogen, Burlington, Canada) or 20 µm DiO (Invitrogen) in the dark for 20 min at 37°C. Samples were diluted in cold PBS, ultracentrifuged to wash, then resuspended in equal volume PBS and stored at -80°C. To ensure the absence of nanoparticles in the original dye stocks, negative controls for all experiments were created by incubating the same concentration of CFSE or DiO with an equal volume of sterile PBS, then ultracentrifuging and resuspending as above. These negative controls did not produce fluorescence when incubated with any cell lines.

3.5.3. Nanoparticle tracking analysis

MV were characterized by nanoparticle tracking analysis (NTA) using a NanoSight NS300 (Malvern Panalytical, Montreal, Canada) at the Structural & Biophysical Core Facility at the Hospital for Sick Children (Toronto, Canada). MV were diluted in PBS to 30-100 particles per frame then continuously flowed by syringe pump through a 532 nm laser. Five 60 sec recordings (camera level 16) were analysed using NTA software (v. 3.2; Malvern Panalytical) with a detection threshold of 5.

3.5.4. Electron microscopy

Electron microscopy was performed by the Canadian Centre for Electron Microscopy (McMaster University, Hamilton, Canada). Samples were deposited (3.5 µL) onto formvar-coated copper grids and incubated for 10 min. Excess liquid was blotted, samples were negatively stained with 1% aqueous uranyl acetate (3.5µL) to each grid, incubated for 1 min, then blotted and dried by evaporation. Grids were viewed in a 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, USA) operating at an accelerating voltage of 80 kV. Images were acquired with a 4-megapixel digital camera (Advanced Microscopy Techniques, Woburn, USA).

3.5.5. Cell culture

All cell lines were grown in 5% CO_2 at 37°C, passaged at 80% confluence, and discarded after 20 passages. HT-29 (human colonic epithelial) cells and MODE-K (mouse duodenal epithelial) cells were a gift from Dr. Ali Ashkar (McMaster University). HT-29 cells were cultured in DMEM/F-12 with L-glutamine, HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (FBS). MODE-K cells were cultured in DMEM with L-glutamine, HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS. HEK-Blue mTLR2 cells were obtained from InvivoGen (San Diego, USA) and cultured in DMEM with L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL Normocin, and 10% FBS.

BMDCs were derived as previously described (Al-Nedawi et al., 2015; Lutz et al., 1999) using tibia and femurs from BALB/c mice. Cells were plated in 100 mm dishes at 10^6/mL in 20 mL growth medium (day 0), refreshed on days 2 and 6, and harvested on day 7.

3.5.6. *In vitro* internalization assays

Cells were cultured to 80-90% confluence on glass coverslips or in cell culture plates. Where appropriate, cells were preincubated with anti-LTA (5 µg/mL; clone G43J, Invitrogen), anti-SIGN-R1 (10 µg/mL; Invitrogen), or anti-mouse TLR2 (2 µg/mL; InvivoGen) for 1h, or dynasore (80 µM; Abcam, Toronto, Canada) for 30 min, then incubated with approx. $3x10^{10}$ CFSE-labelled JB-1 MV or EcN OMV for 2h. Cells were then washed twice in PBS. For flow cytometry analysis cells were dissociated in Accutase for 15 min, washed once, and resuspended in 2% FBS in PBS. Cells were then analysed by flow cytometry using a FACSCelesta flow cytometer (BD Biosciences, San Jose, USA), and data analysed in FlowJo (v. 9.4; BD Biosciences).

For fluorescence microscopy, cells on coverslips were fixed for 15 min in 4% formaldehyde in PBS, washed twice, mounted with ProLong Glass antifade mountant with NucBlue nuclear stain (Hoechst 33342; Invitrogen), allowed to cure for 24 h, then imaged using a Zeiss Axio Imager Z1 microscope and processed using AxioVision software (v. 4.8; Zeiss, Toronto, Canada).

3.5.7. TLR2 assay

TLR2 ligand presence was determined using a mouse TLR2 reporter cell line (HEK-Blue-mTLR2; InvivoGen) following manufacturer's directions and as described previously(Al-Nedawi et al., 2015). Where appropriate, cells were pre-incubated with anti-LTA antibody (5 μg/mL) for 1 h then incubated with 10 μL sample in 90 μL media at 37°C for 20 h. Positive control wells were incubated with the TLR2 agonist Pam3CSK4 (300 ng/mL). Cell-free supernatants (20 μL) were then added to the detection reagent (180 μL), incubated at 37°C for 1 h, and measured spectrophotometrically at 650 nm.

3.5.8. BMDC flow cytometry analysis for IL-10 expression

BMDCs were seeded in 6-well plates at 10^6 cell/well in 450 μL antibiotic-free RPMI. Where appropriate, cells were preincubated with anti-LTA antibody (5 μg/mL) for 1 h, then 50 μL sample were added to BMDCs and incubated at 37°C for 18 h. Cells were suspended by cell scraper, washed, Fc blocked for 15 min, then incubated for 30 min with anti-CD11c-PerCP-Cy5.5 (1:200; Invitrogen). After washing, cells were fixed and permeabilized with BD Cytofix/Cytoperm kit (BD Biosciences) per manufacturer's directions. Finally, BMDCs were incubated for 30 min with anti-IL-10-PE (1:200; Invitrogen) to label intracellular cytokines and analysed by flow cytometry.

3.5.9. Western blot

JB-1 bacteria were lysed with 2x B-PER lysis reagent (Thermo Scientific). JB-1 lysates (approx. 600 µg/mL protein) or unlysed JB-1 MV (approx. $3x10^{11}$ MV/mL) were then heated at 95°C for 15 min in β-mercaptoethanol-containing sample buffer. Samples were cooled and 10 µL electrophoresed in a 10% acrylamide gel, then transferred to a PVDF membrane. Blots were blocked in 5% BSA in TBS-T, then labelled with mouse IgG1 anti-LTA (G43J; Invitrogen) at 1:100. After washing, blots were then incubated with goat anti-mouse IgG1-HRP (Abcam) at 1:2000, washed again, then imaged with chemiluminescent substrate in a ChemiDoc Touch Imaging System (Bio-Rad). The full-length blot is shown in Supplementary Fig. S3.

3.5.10. Measuring JB-1 MV-CFSE internalization by IEC and DCs *in vivo*

Mice were gavaged with approx. $3x10^{10}$ CFSE-labelled JB-1 MV in 200 µL PBS or with PBS alone, then 2 h later were euthanised and jejuna removed. Single-cell suspensions of IEC and lamina propria DC were then prepared as previously described (Bogunovic et al., 2009; Jeon et al., 2012) and kept dark where possible to limit photobleaching. Tissues were stripped of mesentery and visible Peyer's patches excised, flushed with cold PBS, cut into segments, suspended in 30 mL Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free) with 5% FBS, 1 mM DTT, and 5 mM EDTA, then incubated in a shaking water bath for 30 min at 37°C to dissociate epithelial cells. Suspensions were then filtered successively through 70 µm and 40 µm cell strainers, separating dissociated IEC from lamina propria. IEC suspensions were washed twice then incubated

with rabbit anti-mouse A33 polyclonal antibody (1:100; Invitrogen) for 1 h and goat anti-rabbit IgG (APC, 1:50; Invitrogen) and anti-mouse CD45-APC-Cy7 (1:100; Invitrogen) for 45 min, then analysed by flow cytometry.

To isolate lamina propria, non-dissociated jejunal segments were collected from the first 70 µm strainers and cut into smaller pieces. These were then suspended in 20 mL RPMI with 5% FBS, 1 mg/mL collagenase IV/dispase (Invitrogen), and 40 µg/mL DNAse I (Roche, Mississauga, Canada) and incubated in a shaking water bath at 37°C for 45 min. Resultant suspensions were filtered though a 40 µm strainer, washed once with cold PBS, then applied to a Percoll (GE Healthcare, Mississauga, Canada) gradient (top layer 30%, bottom layer 75%) and centrifuged at 540 × g for 20 min at room temperature. The cells at the interface were collected, washed twice with PBS, then antibody-labelled with anti-mouse CD11c-PerCP-Cy5.5 (1:200; Invitrogen) and MHC II-APC (1:200; Invitrogen) for 30 min. Cells were fixed and permeabilized with BD Cytofix/Cytoperm kit, intracellular cytokines labelled with anti-mouse IL-10-PE (1:200; Invitrogen) for 30 min, and finally analysed by flow cytometry.

3.5.11. Data analysis

Data were analysed in R (v. 3.4.4) (R Core Team, 2018) using the effsize package (Torchiano, 2017). Pairwise comparisons were done by directional t-test with Welch's correction for unequal variances (*t*) with effect size reported as Hedge's g (*d*), with each datapoint corresponding to data from independent MV preparations. Graphs were

created using the R package ggplot2 (Wickham, 2016), GraphPad Prism (v. 6.01), or FlowJo (v. 9.4).

Data Availability

Data generated during the current study are present in the supplementary section or are available from the corresponding author on request.

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Contributions

KCJ designed the experiments and wrote the first drafts. JB helped with design and editing. KCJ and MFM performed the experimental work. AMS and KAMN assisted with

material preparation and advised on methodologies used. KCJ analysed the data and prepared figures. All authors reviewed the manuscript.

Additional Information

The authors declare no competing interests.

3.6. Supplementary Results

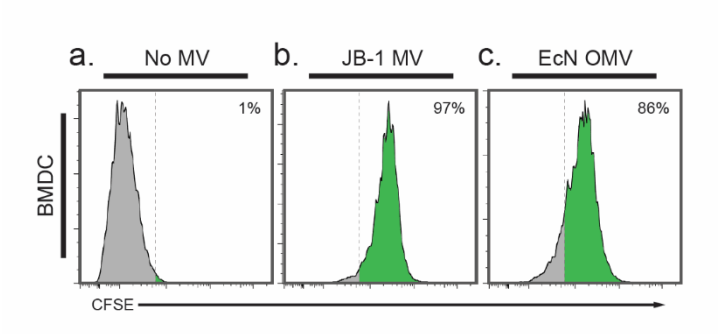

Figure S1. *L. rhamnosus* JB-1 MV are internalized within 1 hour by bone marrow-derived dendritic cells. (**a**) Either PBS vehicle or approx. $3x10^{10}$ (**b**) CFSE-labelled JB-1 MV or (**c**) EcN OMV were incubated at 37°C for 1 h with BMDCs and cell-associated fluorescence was measured by flow cytometry.

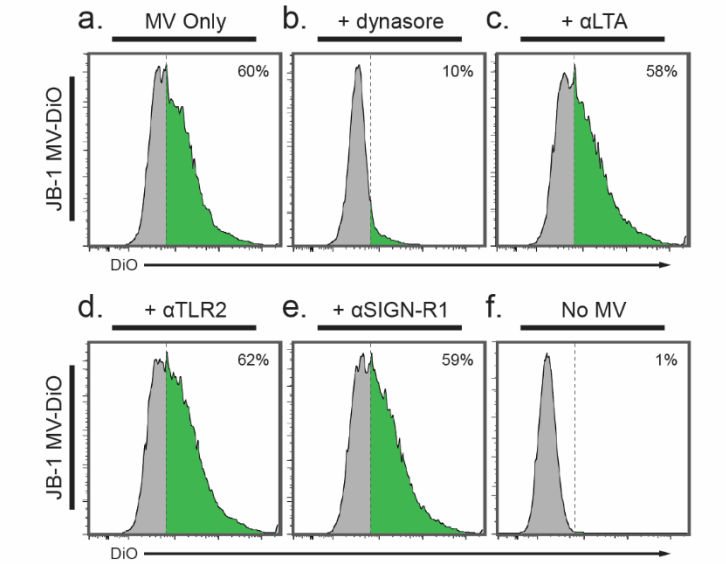

Figure S2. *L. rhamnosus* JB-1 MV are endocytosed independent of LTA, TLR2, and SIGN-R1. (**a**-**e**) Approx. $3x10^{10}$ DiO-labelled JB-1 MV were incubated with MODE-K cells for 2 h after preincubation with (**a**) nothing added, (**b**) dynasore, (**c**) anti-LTA antibody, (**d**) anti-mouse TLR2 antibody, or (**e**) anti-SIGN-R1 antibody. (**f**) Cells were incubated only with PBS vehicle.

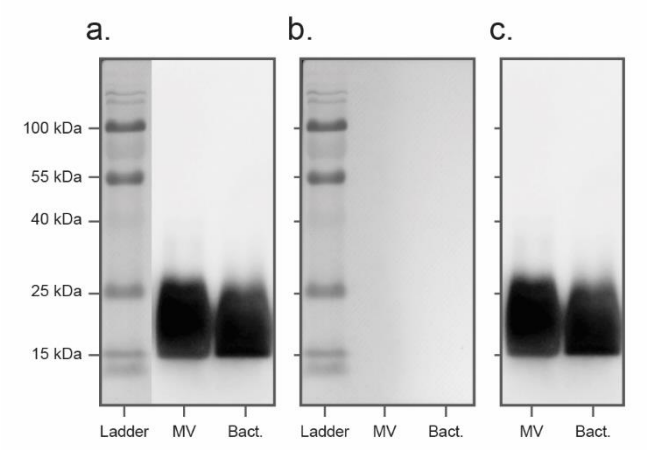

Figure S3. *L. rhamnosus* JB-1 MV contain lipoteichoic acid. (**a**-**c**) Full-length western blot used to measure LTA associated with JB-1 MV (MV) or in lysates of whole JB-1 bacteria (Bact.). Images show (**a**) colorimetric ladder merged with chemiluminescent LTA signal, (**b**) colorimetric blot alone, or (**c**) chemiluminescent signal from sample-containing wells.

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CHAPTER 4. Bacterial membrane vesicles and phage in blood after consumption of *Lacticaseibacillus rhamnosus* JB-1

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4.1. Abstract

Gut microbiota have myriad roles in host physiology, development, and immunity. Though confined to the intestinal lumen by the epithelia, microbes influence distal systems via poorly characterized mechanisms. Recent work has considered the role of extracellular membrane vesicles in interspecies communication, but whether they are involved in systemic microbe-host influence is unknown. Here, we show that nanoparticles isolated from the blood of mice within 2.5 h of consuming *Lacticaseibacillus rhamnosus* JB-1 have a distinct size distribution and reproduce the original bacterial activity. These nanoparticles activate TLR2 and increase IL-10 expression by dendritic cells through bacterial lipoteichoic acid and reduce IL-8 induced by TNF in an intestinal epithelial cell line. While they are enriched for neuronal proteins, they also contain protein of fed bacterial origin and DNA from a bacteriophage in the bacterium's genome. Altogether, these data strongly suggest that specific bacterial membrane vesicles circulate in blood soon after oral consumption of live bacteria and therefore, together with their phage, mediate systemic communication with the host. This pathway may have broader implications that underlie how orally consumed beneficial bacteria and probiotics affect their hosts.

4.2. Introduction

Intestinal microbiota have extensive and diverse roles in mammalian physiology. Consumption of beneficial microbes, probiotics, and complex communities thereof has been shown to modulate metabolism, immunity, and nervous system function, both locally in the gut and throughout the wider organism (Forsythe, Kunze, & Bienenstock, 2016; Schroeder & Bäckhed, 2016). Though several candidate mediators of bacterial activity have been proposed, including conserved molecular features, short chain fatty acids, and bacteria-produced hormones, among others (Neuman, Debelius, Knight, & Koren, 2015; Schroeder & Bäckhed, 2016), the mechanisms by which bacteria exert systemic influence are poorly understood.

Recent work has begun to examine the role of extracellular vesicles (EV) in bacteria-host communication. EV are membrane-bound nanoparticles that are released by all domains of life (Brown, Wolf, Prados-Rosales, & Casadevall, 2015). EV from prokaryotic organisms are produced from the cellular membrane and are often termed microvesicles or membrane vesicles (MV), while eukaryotic EV can be produced by budding off the plasma membrane or by release of intracellular endosomes to form exosomes (Théry et al., 2018). EV regardless of source can contain specific cargo and are considered important mediators of intercellular communication (Caruana & Walper, 2020).

Bacterial MV appear to play an important role in intestinal bacteria-host interactions. MV from pathogens can deliver immunostimulatory and toxin cargo to host

cells and directly support bacterial pathogenesis (Bomberger et al., 2009; Codemo et al., 2018; Furuta, Takeuchi, & Amano, 2009; Johnston, Kufer, & Kaparakis-Liaskos, 2020). MV from commensal and beneficial bacteria can influence host metabolism (Choi et al., 2015), protect against intestinal inflammation (Fábrega et al., 2017; López et al., 2012; Shen et al., 2012), and modulate intestinal barrier strength (Alvarez, Badia, Bosch, Giménez, & Baldomà, 2016), among others (Badia & Baldomà, 2020).

The bacterium *Lacticaseibacillus rhamnosus* JB-1 has wide-spread immune- and neuro-activity after oral consumption by mice. It promotes a regulatory phenotype in dendritic cells (DCs) and increases the number of functional regulatory T cells (Karimi, Inman, Bienenstock, & Forsythe, 2009; Karimi, Kandiah, Chau, Bienenstock, & Forsythe, 2012), and is protective in murine models of asthma including systemic inhibition of mast cell activation (Forsythe, Wang, Khambati, & Kunze, 2012). It also inhibits hypothalamic-pituitary-adrenal axis stress responses and reduces anxiety-like behaviour while altering GABA receptor expression in the brain (Bravo et al., 2011). Many of these behavioural effects appear to be mediated by regulatory T cells (Liu, Mian, McVey Neufeld, & Forsythe, 2020) or the vagus nerve (Bravo et al., 2011), with regional brain activity occurring via both vagus-dependent and -independent mechanisms within 2.5 h of feeding (Bharwani et al., 2020). Thus, JB-1, like many other non-pathogenic bacteria, communicates systemically from the gut lumen through unknown mechanisms.

Many local effects of JB-1 are recapitulated by its MV, including activation of Toll-like receptor 2 (TLR2), induction of an immunoregulatory phenotype in DCs, and

increased excitability of intestinal neurons (Al-Nedawi et al., 2015). We have recently shown that MV from JB-1 contain immunomodulatory lipoteichoic acid (LTA) and are endocytosed by intestinal epithelial cells both *in vitro* in a clathrin-dependent mechanism as well as *in vivo* (Chapter 3). Internalized MV from some beneficial bacteria appear to be protected against intracellular destruction (Al-Nedawi et al., 2015) and may even transcytose the epithelium (Rubio et al., 2020). This suggests the possibility that bacterial MV may circulate from the gut and mediate some systemic effects associated with the parent bacterium. Here, we show that nanoparticles isolated from the plasma of mice fed with JB-1 are TLR2-active and immunomodulatory *in vitro*. This activity is inhibited by neutralizing antibodies against LTA, while isolated particles contain labelled proteins and bacteriophage DNA of JB-1 origin. These data suggest that MV from a beneficial gram-positive bacterium circulate systemically shortly after its consumption and are strong candidates to mediate its systemic influence.

4.3. Results

4.3.1. Physical characterization of extracellular vesicles

BALB/c mice were gavaged with $2x10^9$ JB-1 bacteria or saline vehicle, then 2.5 h later plasma was collected and ultracentrifuged to obtain circulating nanoparticles (which we refer to as EV). By nanoparticle tracking analysis (Fig. 1) we found that EV from JB-1-fed mice had a mean diameter of 134 nm (mode 105 nm), while those from PBS-fed mice had a mean diameter of 130 nm (mode 103 nm). The difference between

means was not statistically significant (t_{11} = 1.73, d = 0.34, p = 0.11). The average concentrations of EV were 2.6x10^{10} EV/mL from JB-1-fed mice and 2.3x10^{10} EV/mL from PBS-fed mice, but this was not a statistically significant difference (t_{11} = 0.97, d = 0.15, p = 0.35). An analysis of variance to compare normalized size distributions between EV from JB-1- and PBS-fed mice revealed a significant group by size interaction (F = 9.3, η^2_G = 0.002, p = 0.002). Follow-up pairwise comparisons revealed significant differences (ps < 0.05) from 234 nm to 254 nm (ds = 0.51 - 0.61). These data suggest that feeding with JB-1 influences the size composition of circulating EV within 2.5 h.

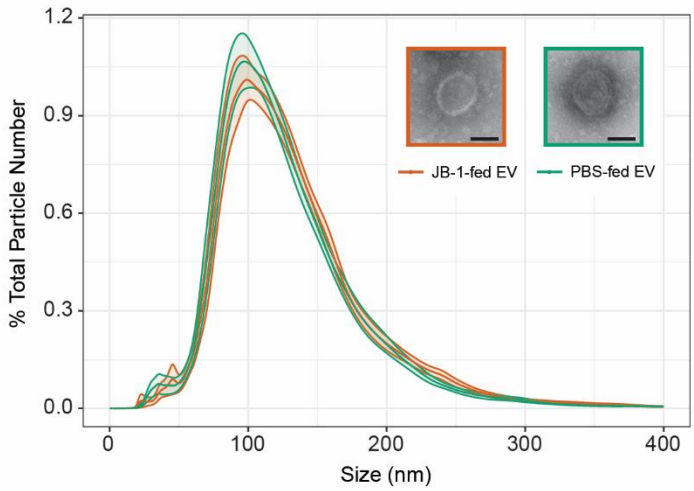

Figure 1. Plasma EV from *L. rhamnosus* JB-1 fed mice have a distinct size distribution. Nanoparticle tracking analysis (graph) was used to characterize the size distribution of EV from mice fed with *L. rhamnosus* JB-1 or PBS vehicle, while transmission electron microscopy (inset images) was used to visualize them. Scale bars represent 50 nm. Ribbon represents ± 1 standard error of 12 EV preparations (6-12 mice pooled per preparation).

4.3.2. EV from JB-1-fed mice reproduce some JB-1-related activity *in vitro*

We hypothesized that effects seen in mice after consumption of JB-1 are mediated by circulating nanoparticles, and thus tested them for effects seen with JB-1

treatment *in vitro*. Using a TLR2 reporter cell line, we found that EV from mice fed with JB-1 activated TLR2 to a greater extent than did those from mice fed with PBS (t_{10} = 5.69, d = 1.78, p < 0.0001; Fig. 2A). When incubated with bone marrow-derived dendritic cells (BMDCs), these EV induced higher IL-10 expression (t_6 = 3.2, d = 0.60, p = 0.0094; Fig. 2B). And in a model of TNF-induced inflammation in a human intestinal epithelial cell line (T84), EV from JB-1-fed mice inhibit IL-8 release to a greater extent than those from PBS-fed mice (t_3 = -5.11, d = -0.96, p = 0.007; Fig. 2C). These results demonstrate that nanoparticles with immune-modulating activity are present in plasma of mice within 2.5 h of consumption of a beneficial bacterium.

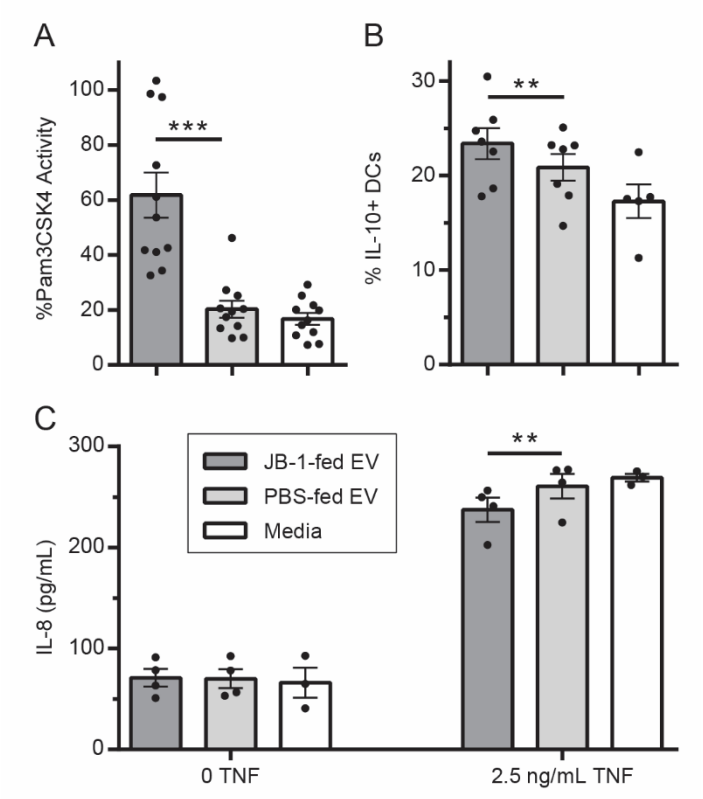

Figure 2. EV from mice fed with *L. rhamnosus* JB-1 reproduce *in vitro* activity of the original bacterium. (**A**) TLR2 activation by EV was quantified by colorimetric assay using a reporter cell line, with data

expressed as a percentage of activity measured for the synthetic TLR2 ligand Pam3CSK4 (300 ng/mL). (**B**) EV were incubated with BMDCs and subsequent IL-10 expression was measured by flow cytometry. (**C**) EV were preincubated with T84 cells for 2 h, exposed to 0 or 2.5 ng/mL TNF for 2 h, then IL-8 secretion was measured by ELISA. Error bars represent ± 1 standard error. Each point represents one EV preparation (6-12 mice pooled). ** $p < 0.01$, *** $p < 0.001$.

4.3.3. Neuronal proteins are relatively enriched in EV from JB-1-fed mice

To investigate whether JB-1 consumption influenced the mouse plasma EV proteome, we performed proteomic analyses. We detected 3421 proteins between JB-1- and PBS-fed EV samples. Of these, 19 were significantly increased in EV from JB-1-fed mice, while 6 were significantly decreased (Fig. 3A; Table S1). In addition, we found 19 proteins that were exclusively detected in all EV preps from JB-1-fed mice, and 17 that were exclusively detected in those from PBS-fed mice (Table S2). We used Gene Ontology (Ashburner et al., 2000; Gene Ontology Consortium, 2021) enrichment analysis to determine whether enriched proteins related to any particular processes. Interestingly, proteins upregulated in EV from JB-1-fed mice were enriched in terms related to neuronal structure and function, along with terms associated with more general cellular processes (Fig. 3B; Table S3). Analysis of proteins downregulated in EV from JB-1-fed mice did not reveal any significantly enriched terms. These results did not indicate any particular proteins or pathways that seemed likely to be involved in activities associated with these EV preparations.

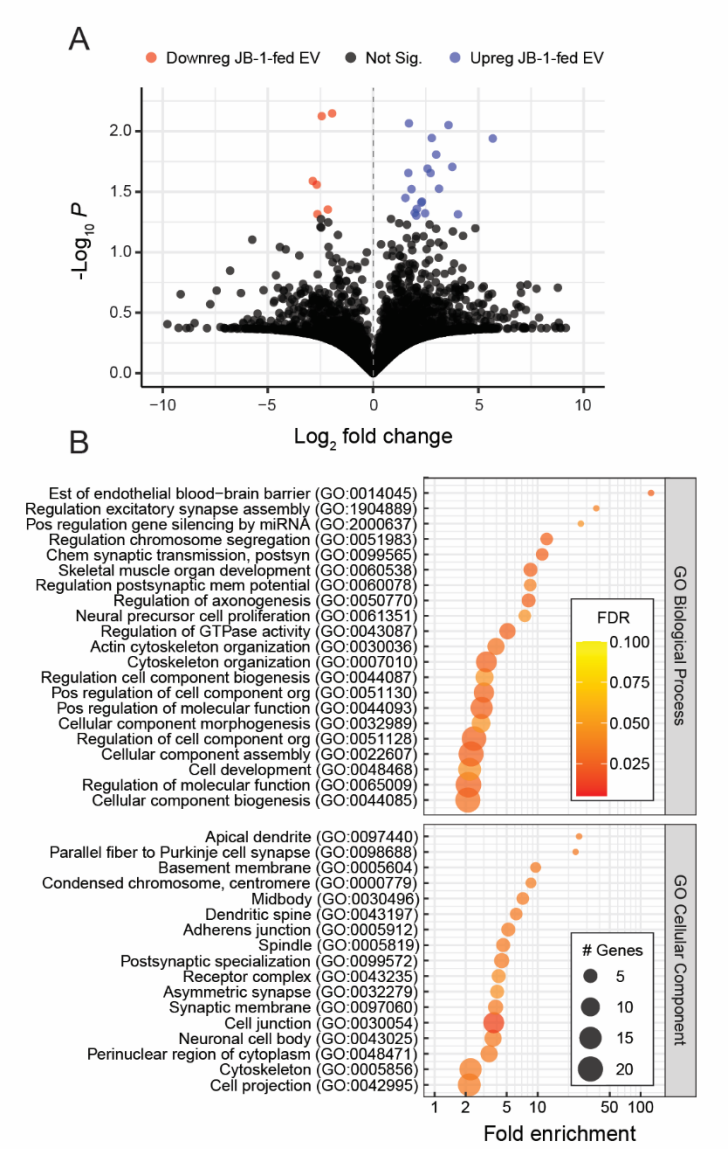

Figure 3. EV from *L. rhamnosus* JB-1-fed mice are relatively enriched in neuronal proteins. (**A**) Volcano plot comparing relative intensities of individual proteins detected in EV from JB-1- or PBS-fed mice. (**B**) Gene Ontology enrichment analysis of EV proteins that were enriched in EV from JB-1-fed mice relative to those of PBS-fed mice, showing terms with FDR < 0.05. Data are from 3 EV preparations per group.

4.3.4. Activity of EV from JB-1-fed mice is mediated by lipoteichoic acid

We have recently shown that JB-1 MV contain immunomodulatory LTA (Chapter 3). To determine whether the same is present in EV from JB-1-fed mice, we used

antibody neutralization experiments. Preincubation with anti-LTA antibodies significantly decreased both TLR2 activation in a reporter cell line (t_3 = -3.22, d = -0.86, p = 0.024; Fig. 4A) and IL-10 expression by BMDCs (t_3 = -3.00, d = 1.81, p = 0.029; Fig. 4B) of EV from JB-1-fed mice. We did not test this effect in TNF-induced IL-8 release as LTA from JB-1 MV is not known to have effect in this system. Altogether, these experiments suggest that LTA contained on bacterial membrane is present in EV isolated from JB-1-fed mice.

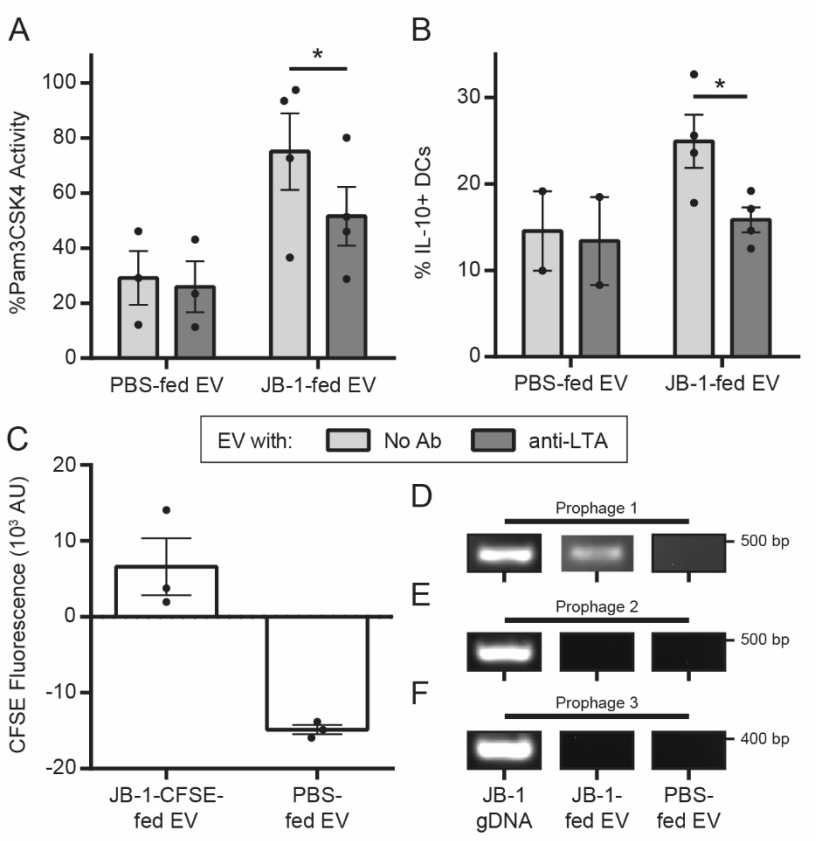

Figure 4. EV isolated from plasma of mice fed *L. rhamnosus* JB-1 contain bacterial LTA, labelled protein, and phage DNA. (**A**) EV were assessed for TLR2 activity in a reporter cell line after pre-incubation with or without anti-LTA antibody. Data are expressed as a percentage of activity measured for the synthetic TLR2 ligand Pam3CSK4 (300 ng/mL). (**B**) BMDCs expressing IL-10 were counted by flow cytometry after pre-incubation with or without anti-LTA antibody and incubation with EV samples. (**C**) Plasma EV from mice fed with CFSE-labelled JB-1 or PBS vehicle was assessed for CFSE-related fluorescence using a plate reader.

Data are shown after subtraction of PBS blank wells. (**D**-**E**) DNA electrophoresis of qPCR products showing (**D**) Prophage 1 DNA detected in JB-1 genomic DNA (gDNA) and EV from JB-1-fed mice, but not EV from PBS-fed mice, whereas (**E**) Prophage 2 and (**F**) Prophage 3 were detected only in JB-1 genomic DNA. Full-length gels shown in Supplemental Fig. S5.

4.3.5. EV contain bacterial protein

To test whether EV from JB-1-fed mice contain protein of JB-1 origin, we labelled JB-1 with carboxyfluorescein succinimidyl ester (CFSE), a stable fluorophore that covalently binds intracellular amines that we have previously used to label JB-1 MV (Al-Nedawi et al., 2015) (Chapter 3). We confirmed that CFSE did not impair bacterial growth (Supplemental Fig. S1), then gavaged CFSE-labelled JB-1 to mice and collected plasma EV as usual. Using a fluorescent plate reader, we found detectable fluorescence in EV preparations from CFSE-labelled JB-1-fed mice (Fig. 4C).

4.3.6. EV contain bacteriophage DNA

The presence of CFSE-labelled protein led us to wonder whether bacterial proteins were detectable in EV from JB-1-fed mice. We thus re-analyzed our proteomics data using the database for the closely related *L. rhamnosus* GG. While 95 proteins were detected across both groups, we suspect most are the result of homology with mitochondrial proteins or microbiota bacterial proteins, as only four were absent in all PBS-fed EV. Of those, however, only two were detected in at least two of the three JB-1-fed EV preparations: a predicted phosphoglucosamine mutase (LGG_00981) and a predicted phage tail related component (LGG_01524). The latter caused us to

investigate whether JB-1 might be carrying a functional bacterial virus (phage) integrated in its genome in a dormant state (prophage), which could also be a component of the EV preparations.

Leveraging three prophage prediction tools on the genome of the closely related *L. rhamnosus GG* (Accession: FM179322.1) we predicted the existence of three seemingly intact prophages (Supplemental Fig. S2), potentially capable of induction (awakening), lysing its host bacterium, and releasing phage progeny. Prophage 1 (~42 kb) and 2 (~33 kb) share clear homology with portions of bacteriophage iLp84 (NC_028783) and Lactobacillus phage Lc-Nu (NC_007501), respectively. In contrast, prophage 3 is much smaller (~15 kb), with no close phage homologues. According to another analysis by ViPTree (Nishimura et al., 2017), all 3 cluster with viruses of family *Siphoviridae*.

To validate those predictions, we performed classical phage induction experiments, exposing *L. rhamnosus* JB-1 to the DNA-damaging agent Mitomycin C, filtering out bacteria components, treating with DNAse to remove any free-floating DNA, and using primers specific to each of these phage regions to detect the presence of protein-protected phage DNA, consistent with a functional phage particle. All three predicted prophages were detectable in extractions from concentrated filtrates (Supplemental Fig. S3), confirming they are intact phages that can exist as particles independently of the JB-1 bacterium.

To determine whether these phages were naturally associated with JB-1 MV, in the absence of the stressor, we applied an identical DNAse-treatment followed by PCR to detect phage DNA within the preparations. While DNA from all three phages was detectable (even after DNAse treatment), only Prophage 1 was consistently detectable at a high level (Supplemental Fig. S4).

The association of Prophage 1 with MV suggested that its DNA could serve as a barcode for the presence of JB-1-origin nanoparticles in EV from JB-1-fed mice. We thus examined whether phage DNA was detectable by qPCR, and found Prophage 1 DNA in JB-1-fed EV preparations (average 33 cycles) but not those from PBS-fed mice (Fig. 4D). We did not detect DNA from Prophages 2 or 3 (Fig. 4E-F), suggesting an enrichment in replicated Prophage 1 DNA and not chromosomal DNA. These data show that nucleic acids from JB-1 are present in nanoparticulate isolates from the blood of mice fed with JB-1.

4.3.7. Estimated number of bacteria-derived particles in plasma

We compared standard curves of characterized JB-1 MV to estimate the number of bacterial MV in JB-1-fed EV samples. By combining these three independent methods we estimate that between 10^8 to 10^9 MV/mL are present in the plasma of mice fed with JB-1 (Fig. 5 A-C). Given an average of $2.6x10^{10}$ EV/mL measured by nanoparticle tracking analysis described above, this suggests that 0.4%-4% of particles collected from JB-1-fed mice are bacterial in origin.

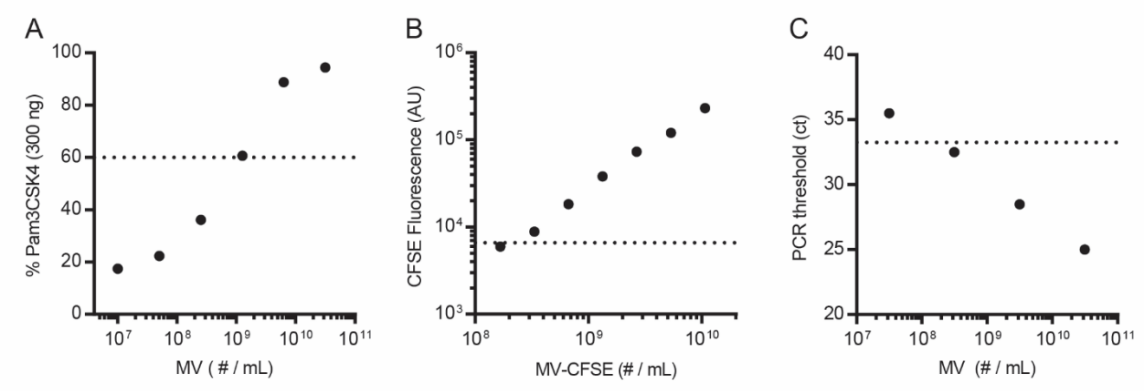

Figure 5. Estimation of bacterial MV in plasma EV isolated from mice fed *L. rhamnosus* JB-1. We enumerated JB-1 MV by nanoparticle tracking analysis and used these to produce standard curves for (**A**) MV activation of TLR2 in a reporter cell line, (**B**) CFSE fluorescence after labelling MV with CFSE, and (**C**) qPCR cycle number at which Prophage 1 DNA was detected (ct). Horizontal dotted lines show the average values of EV from JB-1-fed mice in the same assays. Where the horizontal line and trend of response intersect is an estimate of the number of MV in an average EV preparation.

4.4. Discussion

Bacterial MV are promising mediators of bacteria-host communication, but whether this is confined to the intestinal lumen or relevant to systemic influence is unclear. Here we show that within 2.5 h of consumption by mice of the bacterium *L. rhamnosus* JB-1 there are functional EV circulating in blood that contain activity associated with the fed bacterium. These EV appear to be bacterial MV as their activity was in large part mediated by LTA and the particles contained both fluorescent protein and phage of JB-1 origin.

The differences found between plasma EV from JB-1-fed and PBS-fed mice are notable for several reasons. We recently characterized MV from JB-1 by nanoparticle tracking analysis and found that they have a relatively wide size distribution with a mode

size of 145 nm (Chapter 3), which is larger than the mode size of mouse EV measured here. Interestingly, the size distribution of EV from JB-1-fed mice differed from those of PBS-fed mice, with specific differences detected between 234 nm to 254 nm, which is within the size range associated with JB-1 MV (Chapter 3).

We have previously shown that JB-1 inhibits TNF-induced IL-8 release by gut epithelial cells (Ma, Forsythe, & Bienenstock, 2004), activates TLR2 (Al-Nedawi et al., 2015), and induces an immunoregulatory phenotype in DCs (Karimi et al., 2012). We also recently demonstrated that JB-1 MV contain LTA, which activates TLR2 and induces IL-10 production by DCs *in vitro* (Chapter 3). These previous findings are all consistent with the activities associated with EV from blood of JB-1- but not PBS-fed mice in the current study, including specific inhibition by anti-LTA antibody.

To better characterize these active particles, we fluorescently labelled *L. rhamnosus* JB-1 with CFSE (CFDA SE), a cell-permeable pro-fluorophore that is modified by intracellular esterases to fluoresce (Parish, 1999). It then covalently binds to intracellular primary amines, allowing long-term highly stable fluorescence of labelled cytosolic proteins for prolonged time periods *in vivo* (Parish, 1999). Importantly, this implies that the CFSE-associated fluorescence that we detected in EV from mice fed with CFSE-labelled JB-1 reflects cytosolic JB-1 proteins that were packaged into MV *in situ*. This is also consistent with the detection of JB-1 bacteriophage DNA in these EV preps, which would be unique to JB-1 and not expected to exist in the indigenous microbiota.

We reasoned that, if the circulating nanoparticles we detect here are indeed MV produced by JB-1 in the gut lumen, then their properties are likely similar to those of MV produced in culture. We therefore performed standard curves of JB-1 MV that had been characterized by nanoparticle tracking analysis, allowing us to directly compare results with those found of EV from mice. By doing so and measuring CFSE fluorescence, TLR2 activation, and phage DNA concentration, we estimate that between 0.4%-4% of the particles isolated from the blood of mice fed with JB-1 are bacterial in origin, which is within the range of concentration differences between JB-1-fed and PBS-fed EV as measured by nanoparticle tracking analysis. Since JB-1 MV can be endocytosed by mouse intestinal epithelial cells (Chapter 3), our data suggest a mechanism by which LTA-containing MV are internalized by gut epithelial cells, transcytose the epithelium, and circulate systemically, thereby influencing systems distal from the gut as has been shown for JB-1 bacteria (Forsythe et al., 2012; Karimi et al., 2009, 2012).

There is some support for this possibility. Rubio and colleagues recently reported that *Bacillus subtilis* MV are rapidly transcytosed by the human colonic epithelial cell line Caco-2 (Rubio et al., 2020), while Tulkens and colleagues demonstrated that lipopolysaccharide from gram-negative MV is detectable in human blood, especially in individuals with intestinal barrier dysfunction (Tulkens et al., 2020). In mice, consumption of *Bacteroides thetaiotaomicron* OMV labelled with a lipid-soluble fluorophore resulted in fluorescence detectable in various organs within 8 hours, most notably in the liver (Jones et al., 2020). And from a functional perspective, Aoki-Yoshida

and colleagues demonstrated that nanoparticles precipitated from blood of mice fed with *Lactobacilli* for 7 days had anti-inflammatory effects on phagocytes *in vitro* (Aoki-Yoshida et al., 2017). While the authors inferred that these effects were due to immunomodulatory exosomes in circulation, we suggest that they may also have been driven by the mechanism we propose here.

We did not find evidence to suggest that host EV are involved in the nanoparticulate effects seen in this study. Proteomic analyses did not indicate any functionally relevant mouse proteins, though we did see an enrichment in proteins related to neuronal physiology. Both JB-1 and its MV influence nervous system function (Al-Nedawi et al., 2015; Bravo et al., 2011; Perez-Burgos, Mao, Bienenstock, & Kunze, 2014; Wang et al., 2010). As such, the enrichment seen may be explained by a relative increase in neuronal activity in the enteric nervous system or vagus, leading to heightened release of neuronal EV into circulation. We also did not detect any significantly enriched bacterial proteins in this analysis. Of all detected proteins, only two (a phosphoglucosamine mutase and a phage tail related component) were not found in any PBS-fed samples but were found in two of three samples from JB-1-fed mice. However, as many hits appear to have homology with mitochondrial proteins and were found equally in EV from both JB-1- and PBS-fed mice, it is possible that any proteins of JB-1 origin would be diluted out of detection range or obscured by host homologues.

In this experiment we examined plasma nanoparticles 2.5 h after feeding JB-1. We based this time on our recent experiment showing that JB-1 MV produces regional brain activation within 2.5 h of feeding in a manner that was partially independent of the vagus nerve (Bharwani et al., 2020). While brief, this time period is also consistent with findings of Karlsson and colleagues, who described the production of blood nanoparticles termed *tolerosomes* by intestinal epithelial cells within 2 h of consumption of a large dose of ovalbumin (Karlsson et al., 2001), which mediated antigen-specific tolerance in an MHC II-dependent manner (Östman, Taube, & Telemo, 2005). Moreover, we have recently shown that labelled MV from JB-1 are endocytosed by gut epithelial cells within 2 h both *in vivo* and *in vitro* (Chapter 3), suggesting that rapid transcytosis is plausible.

Bacteriophages are diverse and target only specific hosts (Sausset, Petit, Gaboriau-Routhiau, & De Paepe, 2020). We thus reasoned that the phage detected in JB-1 would not be present in the indigenous microbiota and could serve as a unique genomic marker of the bacterium. We propose that the most parsimonious explanation for these data is that JB-1 in the gut lumen produces MV that contain phage DNA and transcytose into circulation, and these are the active nanoparticles that we characterize here. This is consistent with previous work showing that phage nucleic acid (Biller et al., 2014) and even entire phages (Toyofuku, Nomura, & Eberl, 2019) can be contained within bacterial MV. Since phage-encoded endolysins are thought to be involved in production of MV by some gram-positive bacteria (Toyofuku et al., 2017), MV

production should be increased during active phage replication, suggesting that MV may be enriched for phage DNA.

Nonetheless, it is also possible that the phage DNA detected represents an independent set of particles in circulation. Phage can be endocytosed by gut epithelial cells and remain viable for prolonged periods (Bichet et al., 2021) and they may also transcytose the gut epithelium (Nguyen et al., 2017). Active gut bacterial phages can be found in blood samples from healthy individuals (Blanco-Picazo et al., 2020; Keller & Engley, 1958). Moreover, phage may directly modulate mammalian systems (Van Belleghem, Dąbrowska, Vaneechoutte, Barr, & Bollyky, 2018). Recent work has shown that phages, despite being unable to replicate within mammalian cells, can nonetheless modulate host interferon responses (Gogokhia et al., 2019; Sweere et al., 2019). Importantly, our use of CFSE could also have labelled phage proteins in bacteria resulting in labelled phage in blood samples. It is not known if phage can activate TLR2, but proteins of other viruses have been shown to do so (Oliveira-Nascimento, Massari, & Wetzler, 2012). It seems unlikely, however, that anti-LTA would inhibit TLR2-activating phage protein. We do not know whether the identified nanoparticulate phage DNA represents active phage, but if this occurs with other beneficial strains such as probiotics it may represent an additional novel pathway whereby such bacteria may have systemic effects.

In summary, we have shown that oral consumption of a beneficial bacterium leads to circulating nanoparticles containing bacterial components that are likely

bacteria-produced membrane vesicles. That these particles were active in multiple systems and reproduced activity associated with the parent bacterium supports the possibility that circulating bacterial MV can mediate their systemic influence. Our data further suggest that bacterial phages may be involved in their systemic communication and opens the door to further study of the role of MV and phages in host-bacterial communication and probiotic efficacy.

4.5. Methods

4.5.1. Animals

Male BALB/c mice (8-10 weeks old) were obtained from Charles River (Montreal, Canada) and maintained on a 12-hour light-dark cycle in a specific pathogen-free facility with ad libitum access to food and water. Mice were acclimatized for at least one week after arrival and were used in experiments within 4-8 weeks. All experiments involving mice followed the Canadian Council on Animal Care guidelines and were approved by the McMaster Animal Research Ethics Board.

4.5.2. Bacterial culture

Lacticaseibacillus rhamnosus JB-1 (JB-1) was grown anaerobically in Man-Rogosa-Sharpe (MRS) medium for 24 h, then centrifuged at 4°C and 1900 × g for 30 min to pellet bacteria. Bacterial pellets were resuspended in PBS and diluted to 10^{10} JB-1/mL according to optical density, then frozen at -80°C until use. To obtain bacterial MV,

supernatants from bacterial cultures were filtered, ultracentrifuged, and fluorescently labelled (where necessary) as previously described (Chapter 3). When appropriate, JB-1 was fluorescently labelled with 20 µm CFSE (CFDA SE; Invitrogen, Burlington, Canada) for 20 min at 37°C in the dark, washed in cold PBS, then used immediately as below in equivalent dose to unlabelled JB-1.

4.5.3. Plasma EV isolation

Mice were orally gavaged in groups of 6-12 animals with 200 µL PBS or approx. $2x10^9$ JB-1 in PBS, then 2.5 h later were exsanguinated by decapitation. Blood was collected from individual mice in EDTA-containing tubes, then sequentially centrifuged at 6000 × g and 9000 × g for 15 min at 4°C to remove cells and platelets. Plasma from individual animals of each group were then pooled together, diluted in cold PBS, and ultracentrifuged at 42,000 RPM (121,000 × g) at 4°C in a Type 70 Ti fixed-angle rotor (Beckman Coulter, Mississauga, Canada) for 16 h followed by 3 h, with washing in PBS between spins. EV were resuspended at a final volume of 200 µL per contributing mouse, then aliquoted and stored at -80°C until further use. Protein concentration was measured by Pierce Rapid Gold bicinchoninic acid assay (Thermo Fisher, Mississauga, Canada).

4.5.4. Nanoparticle tracking analysis

Particle concentration and size distributions were determined by nanoparticle tracking analysis (NanoSight NS300; Malvern Panalytical, Montreal, Canada) at the Structural & Biophysical Core Facility at the Hospital for Sick Children (Toronto, Canada). EV or MV preps were diluted in sufficient PBS to measure 30-100 particles per frame, then run with a continuous flow syringe pump through a 532 nm laser. Five 60-second videos were captured and analyzed using NTA software (v. 3.2, build 3.2.16; Malvern Panalytical) with camera level 16 and detection threshold of 5.

4.5.5. Transmission electron microscopy

Electron microscopy of EV samples was performed as recently described (Chapter 3).

Briefly, EV were deposited onto formvar-coated copper grids, negatively stained with 1% aqueous uranyl acetate, then viewed in a JEOL 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, USA) operating at 80 kV. Micrographs were taken with a 4-megapixel digital camera (Advanced Microscopy Techniques, Woburn, USA).

4.5.6. Cell culture

T84 cells were a gift from Dr. Ali Ashkar (McMaster University) and were cultured in a 5% CO_2 incubator at 37°C in DMEM/F-12 with L-glutamine, HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS). HEK-Blue mTLR2 cells were obtained from InvivoGen (San Diego, USA) and cultured as per manufacturer's

directions and as recently described (Chapter 3) in supplemented DMEM. BMDCs were derived as previously described from BALB/c mouse tibia and femurs (Al-Nedawi et al., 2015; Lutz et al., 1999)(Chapter 3).

4.5.7. TLR2 assay

TLR2 ligand presence was determined using a mouse TLR2 reporter cell line (HEK-Blue-mTLR2; Invivogen) following manufacturer's directions and as recently described (Chapter 3). Where indicated, cells were pre-incubated with anti-LTA antibody (5 µg/mL; clone G43J, Invitrogen) for 1 h prior to sample addition. Positive control wells were incubated with the TLR2 agonist Pam3CSK4 (300 ng/mL). Standard curves of JB-1 MV were performed by serial dilution of characterized MV preparations. After sample incubation, cell-free supernatants were added to the detection reagent and incubated at 37°C for 1 h, then measured spectrophotometrically at 650 nm.

4.5.8. BMDC flow cytometry analysis for IL-10 expression

BMDC IL-10 expression was assessed as previously described (Chapter 3). Briefly, 10^6 BMDCs were seeded per well in 6-well plates in 450 µL complete RPMI medium. If necessary, cells were pre-incubated with anti-LTA antibody (5 µg/mL) for 1 h, then incubated with 50 µL sample at 37°C for 18 h. Cells were then resuspended, washed, Fc blocked, then incubated with anti-CD11c-PerCP-Cy5.5 (1:200; Invitrogen) for 30 min. Cells were then fixed and permeabilized using a BD Cytofix/Cytoperm kit (BD

Biosciences, San Jose, USA), then incubated with anti-IL-10-PE (1:200; Invitrogen) for 30 min. After washing, fluorescence was measured by flow cytometry in a FACSCelesta flow cytometer (BD Biosciences) and analyzed in FlowJo (v. 9.4; BD Biosciences, Ashland, USA).

4.5.9. TNF-induced IL-8 expression by T84 cells

T84 cells were plated on 6-well cell culture plates at 10^6 cells/well in 4 mL normal growth medium. Three days later, cells were washed and media replaced with total 2 mL antibiotic-free growth medium containing 200 µL EV samples or PBS. Cells were incubated with samples for 2 h, then 2.5 ng/mL recombinant human TNF (Gibco), was added as appropriate and incubated for an additional 2 h. Supernatants were collected and immediately frozen at -80°C until further processing. To measure secreted IL-8 concentrations, supernatants were thawed, centrifuged (500 × g, 7 min) to pellet debris, then analyzed by IL-8 ELISA (Invitrogen) according to manufacturer's directions.

4.5.10. Proteomics

Proteomics analyses were performed by the Center for Advanced Proteomics Analyses, a Node of the Canadian Genomic Innovation Network that is supported by the Canadian Government through Genome Canada. Briefly, samples in PBS (approx. 200 µg protein; 3 paired samples per group) were dried then reconstituted in 100 µL 50 mM ammonium bicarbonate with 10 mM TCEP-HCl and vortexed for 1 h at 37°C.

Chloroacetamide (55 mM) was added for alkylation and samples vortexed for 1 h at 37°C. Trypsin (1 µg) was then added and samples digested for 8 h at 37°C. Samples were dried and solubilized in 5% acetonitrile and 0.2% formic acid, then loaded on a 1.5 µL pre-column (Optimize Technologies, Oregon City, USA). Peptides were separated on a home-made reversed-phase column (150 µm inner diameter by 200 mm) with a 56 min gradient from 10% to 30% acetonitrile with 0.2% formic acid and a 600 nL/min flow rate on an Easy nLC-1000 connected to a Q Exactive HF Orbitrap LC-MS/MS System (Thermo Scientific, San Jose, USA). Each full MS spectrum, acquired at a resolution of 60,000, was followed by tandem-MS spectra acquisition on the 15 most abundant multiply charged precursor ions. Tandem-MS experiments were performed using higher energy collision dissociation at a collision energy of 27%. Data were processed using PEAKS X (Bioinformatics Solutions, Waterloo, Canada) and the UniProt mouse and *L. rhamnosus* GG (ATCC 53103) databases. Mass tolerances on precursor and fragment ions were 10 ppm and 0.01 Da, respectively. Fixed modification was carbamidomethyl. Variable selected posttranslational modifications were oxidation, deamidification, and phosphorylation. The data were visualized with Scaffold (v. 4.3.0; Proteome Software, Portland, USA), protein threshold 99%, with at least 2 peptides identified and a false-discovery rate of 1% for peptides). We used the average intensity of the 3 highest-intensity peptides for further analyses.

4.5.11. Bioinformatics

Pairwise comparisons of all proteins detected in at least 1 sample per group were conducted by Welch's t-test and plotted using the EnhancedVolcano package (v 1.8.0) (Blighe, Rana, Turkes, Ostendorf, & Lewis, 2021) in R (v. 3.4.4) (R Core Team, 2018). For Gene Ontology analysis (Ashburner et al., 2000; Gene Ontology Consortium, 2021), proteins were filtered to include only those consistently enriched across samples (e.g., all 3 JB-1-fed EV samples higher than their paired PBS-fed EV sample) and with $p < 0.25$. Proteins that were exclusively found in all 3 samples of one group and none of the other group were also included. The resulting dataset was then split by whether proteins were upregulated or downregulated in EV from JB-1-fed mice and analyzed separately. Gene ontology enrichment analysis was performed using ShinyGO (v. 0.61) (Ge, Jung, & Yao, 2020). Results were converted to GO IDs using the GOfuncR package (v. 1.10.0) (Grote, 2020) then reduced by removing redundant GO terms with the rrvgo package (v. 1.2.0) (Sayols, 2020) with a medium similarity threshold (0.7). Reduced terms were plotted with ggplot2 (v 3.3.2) (Wickham, 2016) as adapted from published protocols (Bonnot, Gillard, & Nagel, 2019).

4.5.12. Fluorescence measurement

To measure CFSE fluorescence, samples were added at 50 µL per well to a black opaque 96-well plate and fluorescence measured in a SpectraMax i3x plate reader (Molecular Devices, San Jose, USA) with excitation at 492 nm and emission measured at 517 nm. Standard curves of JB-1 MV were performed by serial dilution of characterized

CFSE-labelled MV preparations. Raw fluorescence values were corrected by subtracting values from PBS-containing negative control wells.

4.5.13. Prophage prediction, annotation, and primer design

We combined the output of PHASTER (Arndt et al., 2016), PhiSpy (Akhter, Aziz, & Edwards, 2012) and VirSorter (Roux, Enault, Hurwitz, & Sullivan, 2015) predictions on the genome of *L. rhamnosus* GG (Accession: FM179322.1), aligning them to look for consensus predictions. We then manually curated the predictions based on the presence of signature phage genes, such as capsid, terminase, and tail structural modules. Following this prediction, the designated prophages were then annotated in detail. An analysis of each prophage region was conducted with ViPTree, an online server that generates a proteomic tree of viral genome sequences based on genome-wide sequence similarities computed by tBLASTx. We then designed primers using Geneious Prime (v.2020.0.04, https://www.geneious.com), internal to recognizable phage genes to ensure that even with phage recircularization/excision, the primers would still amplify the targeted phage. The primers and the gene they target are found in Table S4.

4.5.14. Phage induction

L. rhamnosus JB-1 was grown aerobically to an OD600nm of 0.1-0.2 in 1 L of MRS broth. Mitomycin C was added to a final concentration of 2 μg/mL and incubated for 6 h, then bacteria centrifuged at 16,300 × g for 15 min. The supernatant was collected and

filtered through a 0.45 µm PES filter. Filtrates were concentrated by adding 29.2 g/mL NaCl and 100 g polyethylene glycol 8000 (PEG; final concentration of 10% w/v) and incubating overnight at 4°C with stirring. The precipitate was then centrifuged at 16,300 × g for 15 min at 4°C and the pellet resuspended in 5 mL phage buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 8 mM MgSO4). The resulting phage concentrate was re-filtered through a 0.45 µm syringe filter and used for DNA extraction.

4.5.15. Phage DNA extraction

Phage DNA was extracted from concentrates by phenol-chloroform treatment. Briefly, 1 mL of phage concentrate was incubated at 37°C for 30 min with 20 µg/mL RNAse A (New England BioLabs, 20 mg/ml stock) and 2 U/mL DNAse I (New England BioLabs) in DNAse buffer (New England BioLabs). Nucleases were inactivated with 5 mM EDTA (65°C for 10 min). Proteinase K (200 µg/mL; New England BioLabs) and 200 µL 10% SDS were then added and incubated for 1h at 37°C to increase yield from phage capsids. Finally, one volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortexed, then samples centrifuged at 16,000 × g for 10 min. DNA was recovered by alcohol precipitation from the aqueous phase and resuspended in 20 µL elution buffer (10 mM Tris, 0.1 mM EDTA, pH 8.5).

4.5.16. EV nucleic acid isolation

Nucleic acids were extracted from samples using TRIzol Reagent (Invitrogen) as per manufacturer's directions. Briefly, 50 µL of samples in PBS vehicle were lysed in 450 µL TRIzol then mixed with 90 µL chloroform and centrifuged. The aqueous phase was removed, then DNA was precipitated by adding 100% ethanol to the organic phase, washed twice in 0.1 M sodium citrate in 10% ethanol, washed once in 75% ethanol, then resuspended in 50 µL 8 mM NaOH in nuclease-free water and frozen at -20°C.

4.5.17. Real-time quantitative PCR

Isolated DNA was measured by real-time qPCR using PowerUp SYBR Green Master Mix (Applied Biosystems). Standard curves of phage DNA were made by serial dilution of DNA isolated from characterized JB-1 MV preparations. Forty cycles were run with 15 sec denaturing at 95°C, 15 sec annealing at 60°C, and 1 min extending at 72°C. Primers used are shown in Supplementary Table S4. PCR products were electrophoresed in a 1.5% agarose gel and visualized using a ChemiDoc Touch Imaging System (Bio-Rad, Mississauga, Canada). Full length gels are shown in Supplementary Figure S5.

4.5.18. Data analysis

Data were analysed in R (v. 3.4.4) (R Core Team, 2018) with the effsize package (Torchiano, 2017) or others as described in earlier sections. Pooled EV isolated from JB-1-fed or PBS-fed mice from the same batch (received, treated, and euthanized at the same time) were considered matched controls to reduce extraneous between-batch

variability. Thus, where indicated, pairwise comparisons were done by directional t-test with Welch's correction for unequal variances (*t*) with effect size reported as Hedge's g (*d*). Graphs were created using the R package ggplot2 (Wickham, 2016) or GraphPad Prism (v. 6.01) and assembled in Adobe Illustrator.

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4.6. Supplemental Results

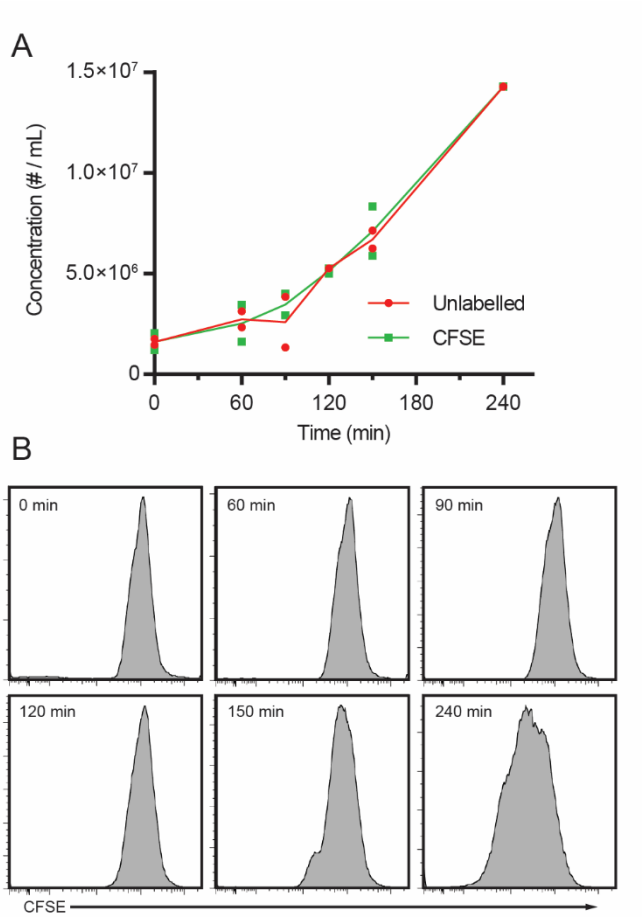

Figure S1. Fluorescence labelling with CFSE does not alter growth of *L. rhamnosus* JB-1. Bacteria were labelled with 20 µM CFSE, washed, then cultured in duplicate in 45 mL MRS broth. Duplicate unstained control JB-1 cultures were initiated identically. Cultures were sampled after 0, 60, 90, 120, 150, and 240 minutes in culture, fixed with 4% formaldehyde, then enumerated by flow cytometry with a flow rate of 60 µL/min. (**A**) Concentrations of bacteria at each time point were estimated by the amount of time it took to count 100,000 particles and are plotted above. (**B**) CFSE fluorescence across time for one representative replicate.

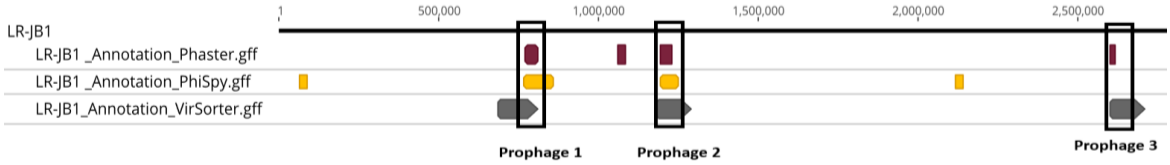

Figure S2. Bioinformatic tools predict 3 prophages in *L. rhamnosus* JB-1. Analysis with Phaster, PhiSpy, and Virsorter is used to identify prophages within bacterial genomes. Regions that have consistency with all three software are identified as a prophage region for further analysis.

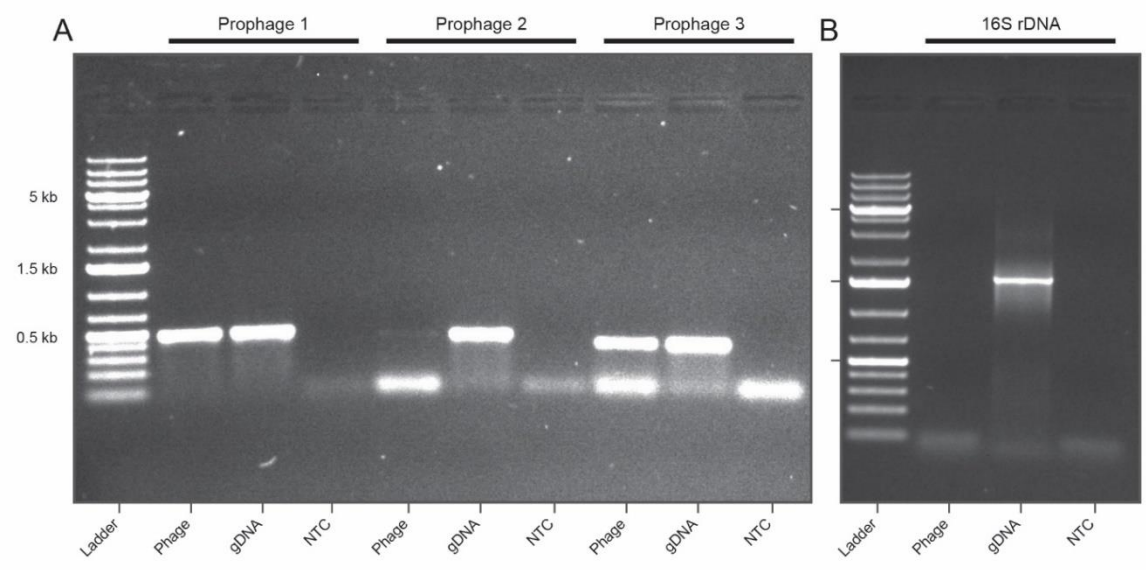

Figure S3. Three prophages were released after induced DNA damage. Mitomycin C was used to damage bacterial DNA and induce phage release. Phages were then precipitated from the culture supernatant by polyethylene glycol. Precipitates were sequentially treated with nucleases and proteinase K prior to DNA extraction. Precipitated DNA extracts (Phage), L. rhamnosus JB-1 genomic DNA extract (gDNA), or no template controls (NTC) were then amplified by PCR for (**A**) indicated prophage sequences or (**B**) universal 16S rDNA sequence, then were visualized after electrophoresis.

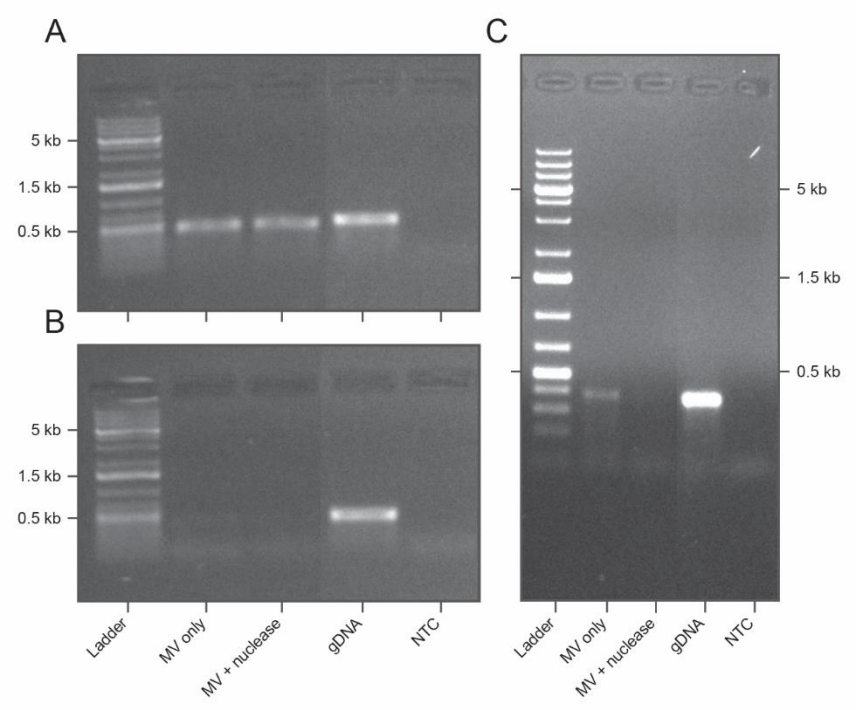

Figure S4. Prophage 1 is released by JB-1 in the absence of applied stressors. JB-1 MV were collected from ultracentrifuged culture supernatants (MV only) and treated with DNase and RNase (MV + nuclease). Extracted DNA from these, JB-1 bacteria (gDNA), or no template control (NTC) were then tested by PCR for (**A**) Prophage 1 DNA, (**B**) Prophage 2 DNA, and (**C**) Prophage 3 DNA, then visualized after DNA electrophoresis. Note that gels are cropped to remove unrelated wells between nuclease and gDNA wells from the same gel.

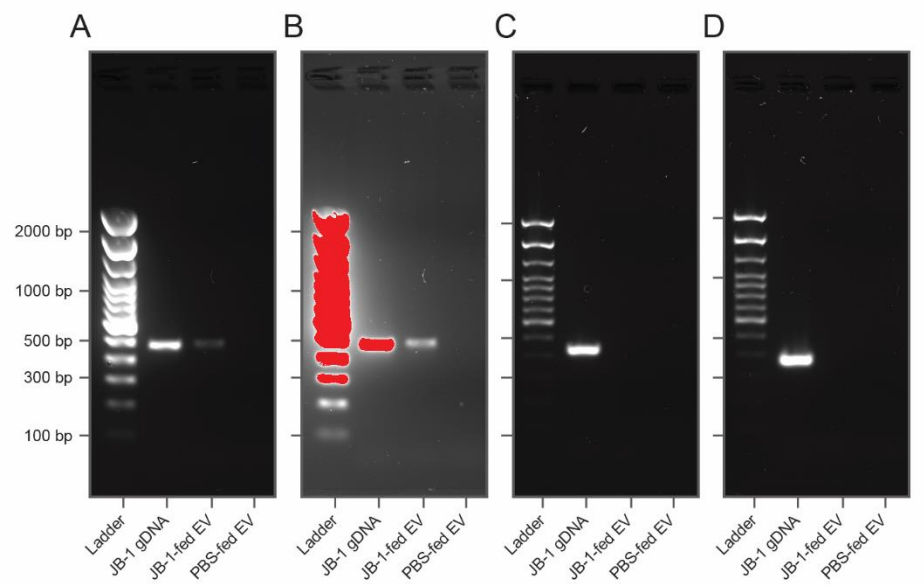

Figure S5. EV isolated from plasma of mice fed *L. rhamnosus* JB-1 contain phage DNA (full-length gels). DNA isolated from EV was amplified by qPCR and products electrophoresed. (**A**) Short (1 s) exposure and (**B**) long (3 s) exposure of electrophoresed qPCR products after amplification with Prophage 1 primers. (**C-D**) Short (500 ms) exposure of electrophoresed qPCR products after amplification with (**C**) Prophage 2 or (**D**) Prophage 3 primers. Red colour denotes overexposed pixels.

Table S1. Proteins differentially expressed in plasma EV from mice fed *L. rhamnosus* JB-1 relative to EV from mice fed PBS

Fold Change*	p-value	Accession	Gene	Description
51.38	0.0115	Q5XK03	Serinc4	Serine incorporator 4
16.34	0.0486	P06795	Abcb1b	Multidrug resistance protein 1B
13.55	0.0197	E9PZQ0	Ryr1	Ryanodine receptor 1
11.95	0.0089	Q9JJR8	Tmem9b	Transmembrane protein 9B
8.75	0.0298	Q80Y19	Arhgap11a	Rho GTPase-activating protein 11A
7.98	0.0156	Q80X50	Ubap2l	Ubiquitin-associated protein 2-like
6.89	0.0114	Q7TSQ1	Clec18a	C-type lectin domain family 18 member A
6.63	0.0221	P19070	Cr2	Complement receptor type 2
5.98	0.0204	P97499	Tep1	Telomerase protein component 1
5.53	0.0476	Q9EQJ0	Tpcn1	Two pore calcium channel protein 1
4.98	0.0381	Q8C0R0	Usp37	Ubiquitin carboxyl-terminal hydrolase 37
4.89	0.0388	Q9ESG9	Pkmyt1	Myt1 kinase
4.21	0.0438	P35922	Fmr1	Synaptic functional regulator FMR1
4.15	0.0497	P01865	Igh-1a	Ig gamma-2A chain C region membrane-bound form
3.94	0.0473	Q80WJ6	Abcc12	Multidrug resistance-associated protein 9
3.54	0.0301	Q6ZQF0	Topbp1	DNA topoisomerase 2-binding protein 1
3.26	0.0086	P55066	Ncan	Neurocan core protein
3.19	0.0221	Q9EPQ2	Rpgrip1	RPGR-interacting protein 1
2.89	0.0356	P01869	Ighg1	Ig gamma-1 chain C region membrane-bound form
-3.85	0.0071	P33622	Apoc3	Apolipoprotein C-III
-4.44	0.0443	P11859	Agt	Angiotensinogen
-5.44	0.0075	Q6GQT1	A2m	Alpha-2-macroglobulin-P
-6.26	0.0484	Q8BHB0	Nod1	Nucleotide-binding oligomerization domain-containing protein 1
-6.40	0.0276	Q80YT7	Pde4dip	Myomegalin
-7.29	0.0258	O70279	Ess2	Splicing factor ESS-2 homolog

*Fold change expressed as average protein intensity in JB-1-fed EV divided by average protein intensity in PBS-fed EV; if less than 1, then result is multiplied by the negative reciprocal

Table S2. Proteins found exclusively in plasma EV from mice fed either *L. rhamnosus* JB-1 or PBS

Found in	Accession	Gene	Description
JB-1 EV	Q8VEC3	Adgrf1	Adhesion G-protein coupled receptor F1
JB-1 EV	Q766D5	B4galnt4	Beta-1,4-N-acetylgalactosaminyltransferase IV
JB-1 EV	Q8K2J9	Btbd6	BTB/POZ domain-containing protein 6
JB-1 EV	P10287	Cdh3	Cadherin-3
JB-1 EV	O70566	Diaph2	Protein diaphanous homolog 2
JB-1 EV	Q69ZL1	Fgd6	FYVE RhoGEF and PH domain-containing protein 6
JB-1 EV	P52187	Kcnj12	ATP-sensitive inward rectifier potassium channel 12
JB-1 EV	Q6P5G3	Mbtd1	MBT domain-containing protein 1
JB-1 EV	Q0II04	Nebl	Nebulette
JB-1 EV	Q923S6	Neurl1	E3 ubiquitin-protein ligase NEURL1
JB-1 EV	O54803	P2rx6	P2X purinoceptor 6
JB-1 EV	Q1EHW4	Sap25	Histone deacetylase complex subunit SAP25
JB-1 EV	Q6ZPE2	Sbf1	Myotubularin-related protein 5
JB-1 EV	Q61420	Slc35a1	CMP-sialic acid transporter
JB-1 EV	Q8R1X6	Spart	Spartin
JB-1 EV	Q8K2X3	Stn1	CST complex subunit STN1
JB-1 EV	Q9ES63	Usp29	Ubiquitin carboxyl-terminal hydrolase 29
JB-1 EV	P24383	Wnt7a	Protein Wnt-7a
JB-1 EV	Q3V0E1	N/A	Uncharacterized protein C9orf131 homolog
PBS EV	Q9JIY2	Cbll1	E3 ubiquitin-protein ligase Hakai
PBS EV	Q8C7V8	Ccdc134	Coiled-coil domain-containing protein 134
PBS EV	Q8BWD8	Cdk19	Cyclin-dependent kinase 19
PBS EV	Q8BWY9	Cip2a	Protein CIP2A
PBS EV	Q2UY11	Col28a1	Collagen alpha-1(XXVIII) chain
PBS EV	Q9WVJ3	Cpq	Carboxypeptidase Q
PBS EV	Q6P5D3	Dhx57	Putative ATP-dependent RNA helicase DHX57
PBS EV	Q99MS7	Ehbp1l1	EH domain-binding protein 1-like protein 1
PBS EV	P63017	Hspa8	Heat shock cognate 71 kDa protein
PBS EV	Q9WV04	Kif9	Kinesin-like protein KIF9
PBS EV	Q9JHQ5	Lztfl1	Leucine zipper transcription factor-like protein 1
PBS EV	P13297	Msx1	Homeobox protein MSX-1
PBS EV	Q9CQH3	Ndufb5	NADH-ubiquinone oxidoreductase SGDH subunit
PBS EV	Q60890	Olfr11	Olfactory receptor 11
PBS EV	Q63836	Selenbp2	Selenium-binding protein 2
PBS EV	Q9D7D2	Serpina9	Serpin A9
PBS EV	Q9QUK6	Tlr4	Toll-like receptor 4

Table S3. GO terms enriched in proteins upregulated in EV from mice fed *L. rhamnosus* JB-1.

*	GO ID	GO Term	FDR	# Genes	Total Genes	Genes
BP	0099565	Chemical synaptic transmission, postsynaptic	0.022	4	114	GLRA4, P2RX6, WNT7A, GRIN1
BP	0007010	Cytoskeleton organization	0.022	13	1288	NEK2, RHOH, NEBL, TRF, DIAPH2, SORBS1, ARHGAP26, CEP250, SIPA1L1, CENPE, NPHS1, SPAG5, NEURL1A
BP	0014045	Establishment of endothelial blood-brain barrier	0.022	2	5	WNT7A, ABCB1B
BP	0022607	Cellular component assembly	0.022	20	2792	CLU, NEK2, NEBL, CDH3, NEURL1A, WNT7A, DIAPH2, FMR1, P2RX6, LAMC1, ICE1, CEP250, CENPE, NPHS1, GRIN1, ADGRF1, SPAG5, OPTN, KCNJ12, SIPA1L1
BP	0043087	Regulation of GTPase activity	0.022	7	433	PLXNA1, FGD6, ARHGAP26, SBF1, ARHGAP11A, SIPA1L1, ARHGAP8
BP	0044093	Positive regulation of molecular function	0.022	15	1654	TRF, ADGRF1, FMR1, PSMA6, CLU, NEK2, ARHGAP26, ARHGAP11A, SIPA1L1, CENPE, CDH3, ARHGAP8, MAGI3, NEURL1A, GRIN1
BP	0050770	Regulation of axonogenesis	0.022	5	193	PLXNA1, WNT7A, SIPA1L1, SPG20, GRIN1
BP	0051128	Regulation of cellular component organization	0.022	19	2487	WNT7A, CLU, NEK2, RHOH, PLXNA1, STN1, NEURL1A, SIPA1L1, FMR1, OPTN, ICE1, CEP250, CENPE, NPHS1, SPG20, GRIN1, SPAG5, TRF, SBF1
BP	0051983	Regulation of chromosome segregation	0.022	4	103	SPAG5, NEK2, PUM1, CENPE
BP	0060538	Skeletal muscle organ development	0.022	5	185	NEURL1A, NPHS1, RBFOX1, MYT1, PSMA6
BP	0065009	Regulation of molecular function	0.022	21	3108	PLXNA1, SERPINA3M, NEK2, TRF, ADGRF1, FMR1, FGD6, PSMA6, CLU, RHOH, WNT7A, ARHGAP26, SBF1, ARHGAP11A, SIPA1L1, CENPE, CDH3, ARHGAP8, MAGI3, NEURL1A, GRIN1
BP	0051130	Positive regulation of cellular component organization	0.024	12	1258	PLXNA1, NEURL1A, WNT7A, FMR1, CLU, NEK2, OPTN, ICE1, NPHS1, SPAG5, GRIN1, TRF
BP	0044085	Cellular component biogenesis	0.030	20	3000	CLU, NEK2, NEBL, CDH3, NEURL1A, WNT7A, DIAPH2, FMR1, P2RX6, LAMC1, ICE1, CEP250, CENPE, NPHS1, GRIN1, ADGRF1, SPAG5, OPTN, KCNJ12, SIPA1L1
BP	0030036	Actin cytoskeleton organization	0.030	8	640	RHOH, NEBL, TRF, DIAPH2, SORBS1, ARHGAP26, SIPA1L1, NPHS1
BP	1904889	Regulation of excitatory synapse assembly	0.038	2	17	WNT7A, SIPA1L1
BP	0060078	Regulation of postsynaptic membrane potential	0.038	4	149	GLRA4, P2RX6, WNT7A, GRIN1
BP	0048468	Cell development	0.046	16	2307	PLXNA1, NEBL, NEURL1A, WNT7A, ADGRF1, SIPA1L1, FMR1, NPHS1, CLU, LAMC1, DIAPH2, RPGRIP1, SPG20, GRIN1, ABCB1B, TRF
BP	0061351	Neural precursor cell proliferation	0.046	4	168	WNT7A, RPGRIP1, OPTN, TRF
BP	2000637	Positive regulation of gene silencing by miRNA	0.047	2	24	FMR1, PUM1
BP	0032989	Cellular component morphogenesis	0.047	10	1118	RHOH, PLXNA1, NEBL, CDH3, WNT7A, SIPA1L1, CLU, LAMC1, SPG20, GRIN1
BP	0044087	Regulation of cellular component biogenesis	0.047	9	931	CLU, NEURL1A, WNT7A, FMR1, ICE1, NPHS1, GRIN1, SPAG5, SIPA1L1
CC	0030054	Cell junction	0.006	13	1095	MAGI1, MAGI3, CDH3, NPHS1, ABCB1B, NEURL1A, GLRA4, P2RX6, SORBS1, GRIN1, ARHGAP26, SIPA1L1, PRR12
CC	0005604	Basement membrane	0.034	3	99	FREM2, LAMC1, TRF
CC	0005819	Spindle	0.034	5	341	SPAG5, NEK2, CEP250, TOPBP1, CENPE

CC	0005856	Cytoskeleton	0.034	15	2123	NEK2, SPAG5, CEP250, RPGRIP1, FGD6, CLU, SORBS1, TOPBP1, ARHGAP26, KRT80, STN1, SIPA1L1, CENPE, KRT42, NEBL
CC	0005912	Adherens junction	0.034	5	304	CDH3, NPHS1, MAGI1, SORBS1, ARHGAP26
CC	0030496	Midbody	0.034	4	176	NEK2, SPAG5, SPG20, CENPE
CC	0042995	Cell projection	0.034	16	2328	GLRA4, RHOH, NEURL1A, NPHS1, GRIN1, RPGRIP1, FMR1, CLU, P2RX6, CEP250, SIPA1L1, MAGI1, PRR12, ALS2CR12, OPTN, KCNJ12
CC	0043025	Neuronal cell body	0.034	8	684	NEURL1A, FMR1, GLRA4, P2RX6, OPTN, GRIN1, KCNJ12, SIPA1L1
CC	0043197	Dendritic spine	0.034	4	204	NEURL1A, GRIN1, P2RX6, SIPA1L1
CC	0048471	Perinuclear region of cytoplasm	0.034	8	748	OPTN, FMR1, NEURL1A, CLU, DOCK6, SBF1, CEP250, TRF
CC	0097060	Synaptic membrane	0.034	6	485	GLRA4, GRIN1, NEURL1A, P2RX6, SIPA1L1, PRR12
CC	0097440	Apical dendrite	0.034	2	25	NEURL1A, CLU
CC	0098688	Parallel fiber to Purkinje cell synapse	0.034	2	27	P2RX6, GRIN1
CC	0099572	Postsynaptic specialization	0.034	6	423	NEURL1A, GLRA4, GRIN1, P2RX6, SIPA1L1, PRR12
CC	0000779	Condensed chromosome, centromeric region	0.036	3	110	SPAG5, NEK2, CENPE
CC	0043235	Receptor complex	0.044	5	377	GRIN1, PLXNA1, SORBS1, CR2, TRF
CC	0032279	Asymmetric synapse	0.045	5	390	NEURL1A, GRIN1, P2RX6, SIPA1L1, PRR12

*GO Aspect: PB = Biological Process; CC = Cellular Component

Table S4. Primers used in this study

Entity	Gene	Forward Primer Sequence	Reverse Primer Sequence
Prophage 1	Terminase large subunit	TAGACTGGATGAGGTGCCAA	ACGTGTGGATCGTCAGAATG
Prophage 2	Phage portal protein	GGAACACCAAAAACCTTGCC	GCAGACAGCAGTGCAGATAT
Prophage 3	Phage capsid protein	AAGAAACAGGACGACACCAC	TACGAATGACTTGTCCGCTG
Bacterial 16S	16S rRNA	AGAGTTTGATCCTGGCTCAG	GGTTACCTTGTTACGACTT

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CHAPTER 5. Discussion

5.1. Summary of findings

Gut microbes hold substantial influence over the physiology of their host, but the repercussions of this, and the underlying mechanisms of action, are in many respects poorly understood. This dissertation examined the influence of prenatal bacteria on mouse brain and behaviour and probed whether bacteria communicate with their host via nanoparticulate mediators.

In Chapter 2, we aimed to delineate the influence of bacteria during a defined period of prenatal development by exposing pregnant mice to a physiologically relevant dose of penicillin during only the last prenatal week. We found that this brief treatment resulted in long-term and sex-specific alterations to behaviour, immune cells, and microbiota composition, suggesting that even a brief perturbation of the maternal microbiota is sufficient to influence offspring development. In Chapter 3, we sought to characterize one potential mediator of bacterial activity. Using *Lacticaseibacillus rhamnosus* JB-1 as a model beneficial gram-positive bacterium, we isolated the membrane vesicles (MV) it produces and determined that they contain lipoteichoic acid (LTA), an immunoregulatory membrane-linked polymer that we showed to activate TLR2 and induce IL-10 production in dendritic cells. We further showed that these MV are endocytosed in a clathrin-dependent manner by human and murine intestinal epithelial cell lines both *in vitro* and in mice *in vivo*. Finally, in Chapter 4 we assessed the physiological relevance of MV production by JB-1 by investigating whether they are

involved in systemic activity related to the bacterium. We found that within 2.5 hours of oral consumption by mice, functional nanoparticles are present in blood that reproduce some *in vitro* bacterial immune activity in an LTA-dependent manner. These nanoparticles appear to be bacterial MV as they additionally contained fluorescently labelled protein and bacteriophage DNA of JB-1 origin. These findings demonstrate that MV produced by gut lumenal bacteria can translocate the intestinal epithelium and circulate through the blood, which may be one mechanism by which bacteria from a pregnant mouse can influence the development of fetuses in a sterile womb.

5.2. Maternal microbiota influence prenatal development

There is increasing concern that exposure to antimicrobial agents during critical periods in life may have long-term detrimental effects on an organism's development (Neuman, Forsythe, Uzan, Avni, & Koren, 2018). Given that up to 40% of infants are exposed to perinatal antibiotics in Canada (Persaud et al., 2015), a better understanding of possible repercussions is required. In Chapter 2 (Champagne-Jorgensen et al., 2020), we treated pregnant mice with a clinically relevant dose of penicillin in drinking water through only the last week prior to birth, then assessed the adult offspring for differences from control mice who did not receive penicillin. We found that prenatal penicillin exposure influenced animals in adulthood in several sex-specific manners. Female mice exposed to prenatal penicillin showed reduced anxiety-like behaviours, while male mice had abnormal social behaviour that correlated with altered

hippocampal expression of mRNA for *AVPR1A*, *AVPR1B*, and *OXTR*. Interestingly, male mice also had relatively fewer regulatory T cells, and both sexes had strikingly distinct microbiota diversity from each other and from their control counterparts.

While this study was the first to investigate a prenatal-only exposure to a physiologically relevant antibiotic dose, its results are consistent with analogous work done by others. Early-life exposure to antibiotics has been variably shown to alter anxiety-like (Leclercq et al., 2017; Zhang et al., 2017) and social behaviours (Degroote, Hunting, Baccarelli, & Takser, 2016; Kayyal et al., 2020; Lebovitz et al., 2019; Leclercq et al., 2017), which associates with changes in brain gene expression (Kayyal et al., 2020; Leclercq et al., 2017) and regulatory T cell populations (Kayyal et al., 2020). Moreover, recent epidemiological studies suggest that similar neurocognitive effects may be seen after early life exposure to antibiotics in humans (Firestein et al., 2019; Lydholm et al., 2019; Slykerman et al., 2019, 2017). While it is difficult in humans to disentangle effects of antibiotics used relative to the underlying infections they aim to treat, such studies nonetheless suggest that our data have clinical relevance.

As we have previously argued, experimental use of antibiotics is complicated by wide-ranging off-target interactions of these drugs with gastrointestinal and nervous tissues (Champagne-Jorgensen, Kunze, Forsythe, Bienenstock, & McVey Neufeld, 2019), and we cannot exclude a potential direct influence of penicillin in this system. Nonetheless, the fact that the dose of penicillin used in this experiment was relatively

low and differentially perturbed microbiota composition in treated animals is consistent with a microbiota-mediated developmental influence.

The effect of a prenatal microbiota on fetal development is puzzling, given current evidence suggesting that the uterine environment is sterile (Dominguez-Bello, Godoy-Vitorino, Knight, & Blaser, 2019). Some evidence suggests that bacterial components such as peptidoglycan can enter fetal circulation and thereby influence development (Arentsen et al., 2017; Humann et al., 2016), but how such components would reach the fetus from the maternal microbiota is unknown. Indeed, this is part of a broader question as to how an animal's intestinal microbiota, largely thought to be confined to the gut, are nonetheless able to systemically influence their host. While some soluble mediators may enter the blood, it is unclear how larger and immunogenic components (such as peptidoglycan) can do so non-pathologically.

5.3. *L. rhamnosus* JB-1 membrane vesicles contain lipoteichoic acid and are endocytosed by intestinal epithelial cells

Most research examining modes of communication between gut bacteria and host has focused on small soluble mediators, including short chain fatty acids, neurotransmitters, and immunologically active compounds (Forsythe, Kunze, & Bienenstock, 2016; Schroeder & Bäckhed, 2016). Recent work has begun to investigate the role of bacterial MV (Haas-Neill & Forsythe, 2020). In Chapter 3, we sought to better-characterize MV produced by the bacterium *Lacticaseibacillus rhamnosus* JB-1, using this

model organism as our group has previously shown it to have wide immunoregulatory and neuroactive effects when administered to mice (Bravo et al., 2011; Kamiya et al., 2006; Karimi, Inman, Bienenstock, & Forsythe, 2009), several of which can be reproduced by its MV alone (Al-Nedawi et al., 2015). We used fluorescence microscopy and flow cytometry to show that fluorescently labelled JB-1 MV are endocytosed in a clathrin-dependent manner by human and mouse intestinal epithelial cells *in vitro* and by mouse gut epithelial cells *in vivo*. We also demonstrated by western blot and antibody neutralization experiments that JB-1 MV contain LTA, which activates TLR2 and induces an immunoregulatory IL-10-positive phenotype in cultured dendritic cells. Interestingly, these experiments also suggest that LTA is required for endocytosis of JB-1 MV and their subsequent induction of IL-10 by dendritic cells, as anti-LTA antibody blocked both effects.

Bacteria can broadly be classified as gram-positive (with a single cellular membrane surrounded by a thick peptidoglycan cell wall) or gram-negative (with an inner and an outer membrane sandwiching a thinner peptidoglycan layer). Until recently it was assumed that the cell wall of gram-positive bacteria precluded MV formation (Brown, Wolf, Prados-Rosales, & Casadevall, 2015). As such, most research into bacterial MV thus far has focused on gram-negative outer membrane vesicles (OMV), especially in the context of pathogenesis. OMV can contain immunogenic cargo including lipopolysaccharide, nucleic acids, and cytotoxic compounds, and can increase

pathogenicity by contributing to biofilm formation and disrupting host barriers, among others (Johnston, Kufer, & Kaparakis-Liaskos, 2020).

The composition and functions of gram-positive bacterial MV are substantially less characterized. As such, the finding in Chapter 3 that JB-1 MV contain immunoregulatory LTA is a valuable indicator of a potential molecular mediator of other bacterial MV effects. LTAs are membrane-linked amphiphilic polymers involved in gram-positive bacterial physiology and host interaction (Shiraishi, Yokota, Fukiya, & Yokota, 2016). They are especially interesting in this context as they are structurally variable between bacteria and this variability appears to influence their immune activities (Lebeer, Claes, & Vanderleyden, 2012; Shiraishi et al., 2016). While LTA from some bacteria, like *Staphylococcus aureus*, is proinflammatory in most experiments (Saito, Lin, & Wu, 2019), this is influenced by concentration and context (Kim, Jung, Kim, & Chung, 2014; Saito et al., 2019, 2020), and slight changes to LTA structure can alter its function in a single organism (Claes et al., 2012; Grangette et al., 2005; Smelt et al., 2013). More recent work has examined LTA as a possibly immunoregulatory molecule of beneficial bacteria (Kim et al., 2020; Saito et al., 2020; Shiraishi et al., 2016), which is consistent with the results described in Chapter 3.

When this work was conducted, it had been shown that MV from only some gram-negative bacteria could be actively endocytosed by gut epithelial cells *in vitro* (O'Donoghue & Krachler, 2016), which is thought to play a role in their associated effects. Whether this occurs with MV from gram-positive beneficial bacteria was only

described in the past year, when MV from bacteria such as *Lactiplantibacillus plantarum* and *Bacillus subtilis* were shown to be endocytosed by cultured gut epithelial cells (Bajic et al., 2020; Rubio et al., 2020). These findings are consistent with what we report in Chapter 3, where we used flow cytometry and fluorescence microscopy experiments to show a clathrin-mediated mechanism of internalization.

Clathrin-mediated endocytosis is a highly-studied active non-phagocytic internalization method used by many cell types, which nonetheless is incompletely understood (Kaksonen & Roux, 2018). It is induced when certain membrane proteins (usually extracellular receptor proteins and their ligands) cluster and recruit coat proteins, including clathrin, which ultimately invaginate the membrane and form a new endocytic vesicle (Kaksonen & Roux, 2018). Thus, the finding that JB-1 MV are internalized in a clathrin-mediated manner implies that there is a ligand on MV that binds to a receptor on host cells. In our experiments, we were not able to determine the nature of this ligand-receptor system, as antibody neutralization experiments against LTA had no effect, nor did blocking the host pattern recognition receptors TLR2 or SIGN-R1, which our group has previously shown to be activated by JB-1 MV (Al-Nedawi et al., 2015). Further work is required to elucidate the MV membrane components involved in their internalization.

5.4. Bacterial membrane vesicles may mediate systemic bacterial influence

The discovery in Chapter 3 that *L. rhamnosus* JB-1 MV contain LTA and are internalized by gut epithelial cells led us to wonder if these could be involved in effects associated with consumption of the bacterium by mice. Our group has previously shown that JB-1 can modulate rodent immune function by promoting a regulatory phenotype in dendritic cells and increasing the number of functional regulatory T cells (Karimi et al., 2009; Karimi, Kandiah, Chau, Bienenstock, & Forsythe, 2012). It also regulates intestinal neuron function including inhibition of a calcium-dependent potassium channel (IK_{Ca}) on enteric neurons (Kunze et al., 2009). Allergic manifestations in murine models of asthma (Forsythe, Inman, & Bienenstock, 2007; Karimi et al., 2009), such as systemic mast cell activation and degranulation, are also reduced through inhibition of this same ion channel (Forsythe, Wang, Khambati, & Kunze, 2012). After oral consumption, JB-1 inhibits hypothalamic-pituitary-adrenal axis stress responses and reduces anxiety-like behaviour while altering neurotransmitter (GABA) receptor expression in the brain (Bravo et al., 2011). These behavioural effects are largely mediated by regulatory T cells (Liu, Mian, McVey Neufeld, & Forsythe, 2020) and the vagus nerve (Bravo et al., 2011) and occur only after several days of treatment, though regional brain activity via vagal dependent and independent pathways occurs within 2.5 hours of feeding (Bharwani et al., 2020). In most cases, the molecular mediator of these effects by JB-1 is unknown.

In Chapter 4, we show that, within 2.5 hours of oral consumption of JB-1, there are active nanoparticles circulating in the blood of fed mice that reproduce activity

associated with JB-1 itself, including activation of TLR2, induction of IL-10 expression by dendritic cells, and inhibition of the inflammatory mediator IL-8 induced by TNF in a gut epithelial cell line. Interestingly, these effects were largely abolished by neutralizing antibodies against LTA, similar to what was shown in Chapter 3 for JB-1 MV. When fluorescently labelled JB-1 bacteria were fed to mice and experiments repeated, we found that recovered nanoparticles contained fluorescence. And by extracting nucleic acids from plasma nanoparticles of JB-1-fed mice, we showed that they contained DNA of a specific bacteriophage that is present in JB-1. These data strongly suggest that the active circulating nanoparticles we recovered are in fact JB-1 MV, with or without free phage derived from the parent bacteria. Using standard curves of enumerated JB-1 MV, we estimate that between 0.4 – 4% of the nanoparticles circulating in blood of mice fed JB-1 are MV of JB-1 origin.

The idea that gut microbial MV may circulate through the body and influence host physiology has been suggested previously (Haas-Neill & Forsythe, 2020; Stentz, Carvalho, Jones, & Carding, 2018), but has never convincingly been demonstrated. Tulkens and colleagues detected circulating lipopolysaccharide-positive OMV in the plasma of humans with intestinal barrier dysfunction, and to a minor extent in healthy individuals (Tulkens et al., 2020). In mice fed with fluorescently-labelled OMV, associated fluorescence was detected in organs including the liver (Jones et al., 2020) and hippocampus (Lee et al., 2020), but whether these contained intact OMV or reflected breakdown products or free dye is unclear.

It has recently been shown that MV from *Bacillus subtilis* can by transcytosed *in vitro* by a human gut epithelial cell line (Rubio et al., 2020). While we did not determine if this occurs with JB-1 MV, we did find in Chapter 3 that they are actively and rapidly endocytosed by a different human gut epithelial cell in culture and additionally by mouse epithelial cells both in culture and *in vivo*, supporting the idea that JB-1 MV could transcytose the epithelium and circulate in mice after consumption of the bacterium. Indeed, circulating MV would be consistent with systemic effects associated with JB-1. For example, JB-1 promotes the generation of regulatory T cells in mice (Karimi et al., 2009). Regulatory T cells express TLR2 (Sutmuller, Morgan, Netea, Grauer, & Adema, 2006), which, when activated, increases their proliferation and survival (Chen, Davidson, Huter, & Shevach, 2009). Moreover, systemic injection of a TLR2 agonist has been shown to increase IL-10 production by DCs and increase numbers of regulatory T cells (Yamazaki et al., 2011). Since JB-1 MV activate TLR2 via LTA (Chapter 3), they may have similar effects in circulation.

It is also possible that bacteriophage of JB-1 origin is involved in JB-1-related effects. In Chapter 4, we discovered an inducible prophage in the genome of JB-1, which is released in MV preparations. Evidence suggests that enzymes of phage origin are involved in MV formation by creating pores in the cell wall (Toyofuku et al., 2017). This implies that MV production would be heightened during times of phage replication, and as a result MV may contain phage nucleic acid or complete phages as has been previously demonstrated (Biller et al., 2014; Toyofuku, Nomura, & Eberl, 2019). Because

bacterial phages are highly diverse and tend to only infect one specific bacterial species (Sausset, Petit, Gaboriau-Routhiau, & De Paepe, 2020), we reasoned that phage DNA could serve as a unique semi-quantitative genetic barcode for JB-1 MV. By qPCR, we demonstrated that phage DNA is present in active nanoparticles isolated from the plasma of mice fed JB-1, but not mice fed PBS, supporting the possibility that the nanoparticles are in fact JB-1 MV.

Nonetheless, phage of JB-1 origin could represent an independent population of particles present in recovered blood nanoparticles, which may on their own influence host physiology in ways distinct from, or additive to, those of JB-1 MV. Though bacterial phages are unable to replicate in mammalian cells (Huh, Wong, St. Jean, & Slavcev, 2019), recent evidence suggests that internalization of phage particles can induce interferon responses (Gogokhia et al., 2019; Sweere et al., 2019), influencing the immune response to their bacterial hosts. Moreover, phage have been shown to be endocytosed by gut epithelial cells (Bichet et al., 2021) and transcytose them (Nguyen et al., 2017), possibly explaining why active phage can be found in circulation after oral treatment (Blanco-Picazo et al., 2020; Keller & Engley, 1958). While it is unlikely that JB-1 phage could activate TLR2 or be inhibited by anti-LTA antibodies, some other viruses have been shown to activate TLR2 (Oliveira-Nascimento, Massari, & Wetzler, 2012). In any event, release of active phage from administered gut bacteria is a novel potential explanation for systemic effects associated with beneficial bacteria and probiotics.

When beginning these studies, we anticipated that we would find a role for mammalian extracellular vesicles (EV), rather than MV, in systemic bacterial effects. Mammalian EV have been heavily researched in the past decade as they have been shown to traffic via blood from origin to target cells in distal locations (Becker et al., 2016). Distinct surface EV markers are thought to promote interactions with specific target cell types, which can then signal via ligand-receptor interactions or induce internalization of EV and thereby deposit their cargo (Tkach & Théry, 2016). This cargo, which can include proteins, lipids, and nucleic acids, can then influence the physiology of the recipient cell (Tkach & Théry, 2016). Mammalian EV are also thought to play a role in immunoregulation, including by delivery of RNA-silencing microRNA, antigen-loaded MHC together with costimulatory molecules, and immunoregulatory proteins, among others (Kalluri & LeBleu, 2020).

Interestingly, this system can be hijacked. For example, some viruses, including hepatitis A (Feng et al., 2013) and hepatitis C (Ramakrishnaiah et al., 2013) have been shown to circulate via host EV in what has been termed a “Trojan exosome” method (Gould, Booth, & Hildreth, 2003). Other pathogens, including Epstein-Barr virus (Pegtel et al., 2010) and the nematode *Heligmosomoides polygyrus* (Buck et al., 2014) can transfer immunoregulatory microRNA to host cells via EV. We wondered whether beneficial or commensal bacteria may similarly hijack host EV, either by inserting endocytosed MV or other components within exosomes, or by influencing host EV production (e.g., after internalization by host gut epithelial cells).

However, as outlined in Chapter 4, we did not find evidence to support these hypotheses. Though proteomic analysis suggests that oral consumption of JB-1 alters the host EV proteome within 2.5 hours, this likely reflects a change in the types of circulating EV, rather than JB-1-induced changes to cellular translation. Indeed, by Gene Ontology analysis we found a significant overrepresentation in apparent neuronal proteins. Since JB-1 is neuroactive in the enteric nervous system (Wang, Mao, Diorio, Pasyk, et al., 2010; Wang, Mao, Diorio, Wang, et al., 2010) and in the brain via the vagus (Bharwani et al., 2020; Bravo et al., 2011), we suggest that increased neuronal activity led to increased release of neuronal EV, which we detected in Chapter 4.

5.5. Significance

As awareness of the influence of microbiota on host physiology has grown, so too have concerns about collateral damage after use of antibiotics (Blaser & Falkow, 2009). Here, we provide evidence that maternal microbiota influence fetal development during the late prenatal period. Our experiments in mice suggest that even a low dose of penicillin for a single week prior to birth can detectably perturb neurodevelopment. While it is possible that these effects reflected a direct influence of penicillin on the developing fetus, we argue in Chapter 2 that an effect mediated by microbiota is more compelling, especially given long-term alterations to microbiota composition measured across life. Though mice differ in many ways from humans, these findings add to a growing literature cautioning against unnecessary antibiotic use in early life (Schulfer &

Blaser, 2015). Further work is necessary to ascertain whether these findings extend to human clinical practice, and additional mechanistic studies are required to determine the mechanisms underlying microbiota influence on fetal development.

There is substantial recent interest in bacterial MV as inter-organismal and inter-species communication vessels, but this field is nascent especially regarding gram-positive and non-pathogenic organisms. In Chapter 3, we show for the first time that MV from a gram-positive beneficial bacterium are internalized by intestinal epithelial cells not only in culture, but also after consumption by mice. Moreover, we link several immune effects of JB-1 MV to its content of LTA. Though recent work has begun to investigate immunoregulatory influence of gram-positive MV, the responsible molecular mediators have to our knowledge never been characterized. Our results suggest that LTA, as a structurally variable membrane component, may explain the varied pro- and anti-inflammatory effects that have been associated with MV from different gram-positive bacteria.

After better characterizing JB-1 MV in Chapter 3, we provide evidence that these MV are in fact relevant for long-distance communication by gut bacteria. While previous work has hinted at this possibility, we are the first to show that functional nanoparticles appear rapidly in circulation after consumption of a bacterium, and that these reproduce bacterial activity via LTA and additionally contain protein and phage DNA originating from the fed bacteria. Together, these data suggest that JB-1 MV circulate within the fed organism and may be in part responsible for systemic activities such as

immunoregulation. However, we are unable to ascertain this from the single time point we investigated. Future experiments are required to delineate the timecourse of circulating MV after JB-1 feeding. Our data also hint at a possible role for bacteriophage in effects associated with JB-1 or other bacteria. Though we do not show the existence of complete (active and infectious) phage in blood after feeding with JB-1, it nonetheless opens the door for future study of gut bacteriophages in bacteria-host communication.

5.6. Conclusion

The past two decades have seen substantial increases in our understanding of mammalian biology. Yet, despite technological innovations, seminal discoveries, and massive commercial and scientific research interest, we still have only a surface understanding of the incredible complexity underlying our relationship with our trillions of symbiont microorganisms. In this thesis, we have bolstered the case for a microbial influence on neurodevelopment and have provided compelling evidence for novel mechanisms by which bacteria might exert such influence over their host. These results provide a clear starting point for subsequent studies to examine whether similar mechanisms are involved in wider microbiota-host communication.

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