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ADDENDUM

Mechanisms and consequences of gut commensal translocation in chronic diseases

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ABSTRACT

Humans and other mammalian hosts have evolved mechanisms to control the bacteria colonizing their mucosal barriers to prevent invasion. While the breach of barriers by bacteria typically leads to overt infection, increasing evidence supports a role for translocation of commensal bacteria across an impaired gut barrier to extraintestinal sites in the pathogenesis of autoimmune and other chronic, non-infectious diseases. Whether gut commensal translocation is a cause or consequence of the disease is incompletely defined. Here we discuss factors that lead to translocation of live bacteria across the gut barrier. We expand upon our recently published demonstration that translocation of the gut pathobiont *Enterococcus gallinarum* can induce autoimmunity in susceptible hosts and postulate on the role of *Enterococcus* species as instigators of chronic, non-infectious diseases.

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Introduction

Only a thin layer of highly specialized epithelium separates our internal organs from trillions of intestinal microbes.[1] This gut barrier functions as both a selective gatekeeper that keeps pathogens from invading as well as a site for immune cell education, nutrient absorption, and waste secretion. Although a thick mucus layer, antimicrobial peptides, and IgA serve to maintain the barrier function, perturbations in host defenses and alterations in microbial community composition can lead to pathologic breaches and subsequent disease states.[2-4] Our recent demonstration that a gut microbe, *Enterococcus gallinarum*, crosses the gut barrier in autoimmune-prone hosts to colonize internal organs and incite autoimmunity provides a model for gut commensal translocation leading to pathologic states.[5] In this addendum to our published study, we discuss these findings in the context of other investigations of translocation of whole bacteria across the gut barrier.

Epithelial barrier

The intestinal epithelia regulates the entry of micro- and macromolecules from the gut lumen into the host.[1] The movement of solutes across the barrier is controlled in part by tight junctions (TJs) that tether together epithelial cells and selectively control solute entry based on their size and charge. Passage of important molecules beneficial to the host usually occurs paracellularly or transcellularly through enterocytes, with larger macromolecules being actively transcytosed. Two major paths of paracellular passage exist: a high capacity pore pathway permeable up to ~10Å molecules and a low capacity leak pathway permeable to up to ~125Å macromolecules.[6,7] A third, unrestricted pathway becomes active at sites of erosive and ulcerative damage, such as that induced by dextran-sodium sulfate (DSS), where the loss of cellular integrity enables the passage of ions, metabolites, and even whole bacteria.[6,7]

Barrier integrity proteins are regulated by internal and external factors and can be direct targets of

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bacteria. Their expression varies depending on cell type and location. Table 1 summarizes selected barrier proteins-based on cellular and anatomical location as well as their respective contributions to barrier integrity.[1,6,8-11] TJs are comprised of multiple transmembrane proteins, including claudins, occludins, and junctional adhesion molecules, which interact with cytoskeletal components through plaque proteins such as zonula occludens (ZOs). Claudins generally regulate the paracellular pore pathway whereas ZOs, occludins, and tricellulins regulate the leak pathway.[7] While expression of many of these proteins leads to decreased permeability, up-regulation of some such as claudin-2 lead to increased pore pathway activity. Due to the importance of barrier integrity to the host, there is considerable compensation of barrier proteins if one of them has been disrupted.[7] TJ protein formation and disassembly are regulated by multiple signaling pathways including protein kinase C, mitogen-activated protein kinases, myosin light chain kinases, and Rho GTPases.[12] The activity of these enzymes is in turn modulated by cytokines and intraluminal molecules including bacterial metabolites and dietary factors.[1,12] Some beneficial bacteria exert barrier protective effects by inducing TJ formation and limiting permeability.[12] Others take advantage of these TJ regulatory mechanisms for invasion.[4]

Vascular barrier and enteric nervous system

Within the lamina propria beneath the epithelial layer lies the gut vascular barrier (GVB) and gut lymphatic barrier (GLB) comprised of endothelial cells that are held together by TJs and adherens junctions (AJs). The GVB barrier is permeable to 4 kD fluorescein isothiocyanate (FITC)-dextran,[8] so as with the epithelial barrier, restricts the passage of bacteria. The GVB integrity is influenced by the WNT-β-catenin signaling pathway with β-catenin as a major AJ component.[8] When β-catenin is constitutively active in endothelial cells, mice are less susceptible to enhanced GVB permeability and pathogen invasion normally seen with *Salmonella typhimurium* infection.[8]

The enteric nervous system is composed of enteric neurons and glial cells.[13] Enteric glial cells and pericytes are in immediate contact with the GVB and form the gut vascular unit.[8] Cells from the enteric nervous system produce substances that can also modulate barrier permeability. For example, the release of S-nitrosoglutathione by enteric glial cells induces TJ expression at the epithelium, and stimulation of the vagus nerve prevents burn-induced gut permeability.[14] Furthermore, mice lacking enteric glial cells are susceptible to epithelial permeability that leads to bacterial invasion and host death.[15]

Table 1. Gut barrier-related molecules & barrier function*.

	Cell Types	Cellular Location	Anatomical Location in SI/Colon	Barrier Formation	Pore Formation
Claudin-2	IECs, vascular ECs	TJ	Crypt base	N/A	Na+, water
Claudin-3	IECs, vascular ECs	TJ & basolateral membrane	Crypt; villus (SI only)	+	N/A
Claudin-5	IECs, lymphatic ECs	TJ & basolateral membrane	Crypt; villus (SI only)	+	N/A
Zonulin	IECs (secreted)	Extracellular space, gut lumen	Serum (soluble form; precursor pre-haptoglobin in IECs)	N/A	Opening paracellular pathway
Occludin	IECs, vascular ECs	TJ	Epithelium and vasculature	+ (Epithelial barrier; GVB)	N/A
JAM-A	IECs, lymphatic and vascular ECs	TJ	Epithelium and Vasculature	+ (Epithelial barrier; GVB)	N/A
VE-cadherin	Vascular and lymphatic ECs	AJ (together with β-catenin)	Vasculature	+ (GVB)	N/A

* Modified after Luissint et al, ref. 1. Individual molecules reviewed in ref. 1,6,8-11. Listed are selected gut barrier molecules that may also be expressed beyond the gut (not addressed here). They either strengthen (green) or reduce (red) gut epithelial and vascular barrier function. Abbreviations: SI - small intestine; IEC - intestinal epithelial cell; EC - endothelial cell; TJ - tight junction; AJ - adherens junction; JAM - junctional adhesion molecule; ZO -zonulaoccludens; GVB - gut vascular barrier; N/A - not applicable.

Mechanisms of microbial translocation

Bacteria may translocate past the gut epithelial layer through both physiologic and pathologic means depending on the context and type of bacteria.[16,17] Bacterial size, virulence factors, defects in host barrier integrity, and uptake by antigen-presenting cells are all factors that influence entry into the host organism.[4,8,16,18,19] Inert particles of the size of bacteria are passively taken up in the gastrointestinal tract,[20] suggesting that entry of some bacteria may naturally occur because of mass and quantity if they can avoid a sterilizing immune response. Under steady state, M cells, $CXCR3R1^+$ macrophages and $CD103^+$ dendritic cells (DCs) extend protrusions that sample luminal contents and enable some commensals and pathogens to gain access beyond the epithelial layer without causing damage.[2,3,21-23] These mechanisms are important for normal induction of IgA and T cell tolerance,[18,24] as well as bacterial clearance.[22] For example, live commensal *E. cloacae* are taken in by M cells to Peyer's patches (PP) and carried by DCs to mesenteric lymph nodes (MLNs) to mount IgA responses.[18] Other commensals, such as *Alcaligenes*, naturally inhabit DCs within host PPs where they induce broadly mucosal IgA in a non-inflammatory context.[25,26] These unique bacteria have likely co-evolved for millennia with the host because their levels drop in an IgA-deficient host, suggesting that IgA binding may be needed for their uptake and growth within gut-associated lymphoid tissue.[25] Additional commensals within lymphoid tissues promote tolerance by inducing interleukin (IL)-10 and IL-22 by DCs and innate lymphoid cells type 3, respectively, which collectively inhibit Th17 responses and facilitate bacterial colonization.[27] In the absence of host defects or virulence and in the presence of intact MLNs, these commensals are prevented from further dissemination, in part due to efficient killing by macrophages.[18]

On the other hand, pathogens have developed the machinery to exploit these sampling cells and facilitate their invasion, including pili that mediate adhesion and Type III and IV secretion systems that inject effector proteins into host cells. *Salmonella typhimurium* targets M cells, leading to their destruction and disruption of the intestinal epithelium.[2] *Shigella flexneri*, on the other hand, invades M cells and subsequently reenters enterocytes basolaterally, triggering cell death and inflammation that leads to additional bacterial translocation.[3] Mechanisms of invasion by these and other infectious agents including *Yersinia enterocolitica* and *Salmonella enterica* have been described in detail previously.[2,4]

Paracellular passage is achieved by certain pathogens like *Entamoeba histolytica, Toxoplasma gondii, Streptococcus agalactiae*, and group A *Streptococcus*, which disrupt TJs and AJs to enhance permeability and facilitate their translocation.[2,3,28] Commensals may also gain access to the host paracellularly as a consequence of barrier disruption caused by pathogens or irritants.

The dissemination of bacteria to various organs beyond the intestinal epithelium also depends on whether the GVB or GLB is breached. Some bacteria may breach only lymphatic vessels and are carried to the MLNs. *Serratia marcescens*, for example, was reported to reside only in lymph and not blood, paralleling translocation of a bacteriophage.[29] Group A *Streptococcus* exhibits tropism for lymphatics as its hyaluronan capsule binds to lymphatic vessel endothelial receptor-1. Disruption of this interaction, interestingly, impairs lymphatic dissemination and leads to blood vessel invasion.[30]

Bacteria that breach the capillary system invade the enterohepatic circulation and travel to the liver via the portal vein. Thus, the liver represents a firewall after a breach of the GVB whereas the MLNs limit bacteria that break through the GLB.[8,31] Particularly pathogenic bacteria, like *Salmonella*, are able to breach both GVB and GLB.[8,18] Additionally, *E. gallinarum*, a gut pathobiont that induces autoimmunity, sequentially colonizes mesenteric veins, MLN, livers, and spleens of monocolonized mice, suggesting it can breach both lymphatic and blood vessels (Figure 1).[5] This sequence of colonization contrasts with direct inoculation of bacteria into the peritoneal cavity, which are recovered in the spleen rather than the MLNs.[18]

Induced gut barrier disruption and translocation

Bacterial translocation can occur after gut barrier disruption due to certain drugs, radiation, epigenetic changes, alcohol, hyperglycemia, ischemia, and autonomic dysfunction after stroke, among other

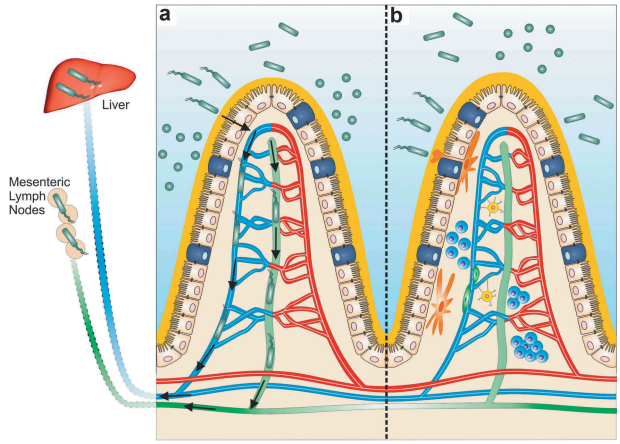

Figure 1. Gut barrier breach by the gut pathobiont *E. gallinarum* in autoimmune-prone hosts. Beyond the intestinal epithelial barrier (IEB), the gut vascular (GVB) and gut lymphatic barriers (GLB) shield the internal organs from colonization by commensals breaching the inner mucus layer and epithelial lining. (a) In (NZW x BXSB)F_1 mice, a leaky gut barrier allows *E. gallinarum* to translocate beyond the intestinal epithelium. In monocolonized mice, *E. gallinarum* downregulates barrier molecules related to both the GVB and GLB,[5] suggesting that it can breach all barrier components in the gut. Alternatively, it could be carried by host cells into host tissues. Once past the IEB and inside the lamina propria, *E. gallinarum* travels via the mesenteric veins (blue) and lymphatics (green) to the so-called "firewalls", the liver and mesenteric lymph nodes, respectively. Within these organs, *E. gallinarum* interacts with host immune and epithelial cells to promote autoimmunity.[5] (b) In a non-autoimmune-prone host, the intestinal epithelia, GVB, and GLB are intact. At steady state, antigen-presenting cells (orange) sample luminal bacteria to induce homeostatic IgA responses. T- and B-lymphocytes (blue) participate in this process leading to T cell-dependent and -independent IgA. The GVB is supported by enteric glial cells (yellow) and pericytes (dark green) surrounding a fenestrated endothelium sealed by tight junctions.[8.]

factors.[32-39] Chemotherapy and radiation alter the integrity of the gut barrier and allow for the passage of macromolecules and bacteria,[38] a process that enhances antitumor immunity.[32,34] Treatment of mice with cyclophosphamide enhances gut permeability as shown by the leakage of orally administered FITC-dextran into the blood. Commensals such as *Lactobacillus johnsonii, L. murinus*, and *Enterococcus hirae* could be cultured in this setting from lymphoid organs including MLN and spleen.[34] Daily injections of low doses of the chemotherapeutic drug streptozosin lead to bacterial translocation to the pancreatic lymph node, innate immune activation, and type I diabetes.[33] Bacterial translocation to MLNs, blood, and adipocytes is also seen following DSS-induced colitis as well as indomethacin-induced ileitis.[40,41] Furthermore, chronic alcohol consumption, proton pump inhibitors (PPIs), and other non-steroidal anti-inflammatory drugs (NSAIDs) induce intestinal permeability to certain molecules.[4,7,36] Interestingly, translocation of *Enterococcus* spp., in particular, *E. faecalis*, has been linked to gastric acid suppression in alcoholic liver disease, which leads to liver inflammation and hepatocyte death.[36]

Besides PPIs and NSAIDs, treatment with antibiotics can enable bacterial translocation.[42] Antibiotic-treated mice are more susceptible to DSS-induced epithelial injury, translocation of live bacteria, and inflammasome-mediated damage.[43] In addition to eradicating beneficial bacteria that contribute to barrier integrity, antibiotic use can lead to overgrowth of pathogenic bacteria. Ampicillin treatment, for example, promotes colonization with vancomycin-resistant *Enterococcus* (VRE) in mice and humans prior to bloodstream infections.[44] Metronidazole and streptomycin treatment enable *E. faecalis* overgrowth and approximation to the epithelial border, where it can be visualized inside intestinal epithelial cells, deeper in the lamina propria and beyond the mucosa.[45] Similarly, a 2-day course of ceftriaxone enabled *E. faecalis* and *Lactobacillus* spp. overgrowth and systemic dissemination to the liver, spleen, and MLN within 3–4 days of exposure, with subsequent clearance by 14 days. Of note, exposure to ceftriaxone did not affect TJ protein expression, fecal albumin, or permeability to FITC-dextran.[46] Oral antibiotics were shown to induce colonic goblet cell-associated antigen passages that enabled translocation of live bacteria to MLN, which continued for ~5 days after antibiotic withdrawal.[47] Thus, antibiotics may disrupt colonization resistance and physiological homeostatic processes, allowing for transient dissemination of bacteria even without overt intestinal pathology.

Spontaneous gut commensal dissemination

Spontaneous translocation of commensal bacteria beyond physiologic uptake by host cells can occur as a result of inherent alterations in the mucosal barrier, the immune system, or the microbial community structure. Mice grown in germ-free facilities exhibit greater translocation due to an immature immune system, a permeable mucus

layer, and the absence of an intact commensal community.[48,49] Germ-free mice are impaired in forming PPs and MLNs, and in secreting IgA, which is reversible by colonization with commensal bacteria.[50-52] Viable *E. coli* and *L. acidophilus* could be recovered from MLNs, spleens, and livers of germ-free mice inoculated with whole cecal content from specific pathogen-free (SPF) mice, but could not be cultured from those organs in SPF mice with intact microbiota.[48] Similarly, our laboratory found bacterial translocation and colonization of mesenteric veins, followed by MLNs, livers, and spleens of C57BL/6 mice with *E. gallinarum* in the monocolonized setting, but not under normal housing conditions, suggesting that the resident microbiota of a healthy host prevent either colonization or translocation of this *Enterococcus* species. We observed consistent translocation of *E. gallinarum* to these organs under SPF conditions only in an autoimmune-prone host, the male (NZW x BXSB)F_1 mouse, which carries a toll-like receptor 7 (*tlr7*) gene duplication.[5] In addition, lactobacilli translocate in a host predisposed to excessive TLR7 signaling induced by transgenic overexpression of *tlr7*.[53] Specifically, *L. reuteri* was shown to drive innate inflammation in TLR7 transgenic mice kept under SPF conditions. Translocation of *L. reuteri* to the MLN or liver was observed in non-transgenic C57BL/6 wild-type (WT) mice only after stimulation with the TLR7 agonist imiquimod. TLR7 signals were needed both under SPF conditions and in *L. reuteri*-monocolonized C57BL/6 mice for translocation to occur. WT but not TLR7 KO C57BL/6 mice displayed increased gut leakiness to FITC-dextran when exposed to TLR7 transgenic mouse-derived microbiota. In addition, C57BL/6 mice treated with imiquimod have increased permeability to FITC-dextran, further supporting a role for TLR7 in barrier integrity.[53] The mechanisms of how TLR7 signals regulate barrier integrity remain to be determined, as the receptor is not expressed in the gut epithelium itself.[53,54] In addition, factors that regulate the translocation of lactobacilli across the gut barrier are incompletely understood. Interestingly, deletion of tet2, an enzyme involved in DNA demethylation, also leads to translocation of *L. reuteri* and related species to the MLN and spleen.[39] Overall, enterococci and lactobacilli appear to be predominant genera translocating under pathologic conditions.[46]

Functional consequences of spontaneous bacterial translocation

A functional consequence of barrier breach and bacterial dissemination, whether induced or spontaneous, is a systemic immune response with the production of systemic IgG in addition to mucosal IgA.[41] In genetically susceptible hosts, this systemic immune response, perpetuated by continuous bacterial translocation, may propagate autoimmunity and other non-infectious chronic diseases (Figure 2). Our laboratory has recently shown that spontaneous bacterial translocation of *E. gallinarum* plays a causative role in the induction of autoimmune disease.[5] In the (NZW x BXSB)F_1 model, male mice carry a duplication of the *tlr7* gene on the BXSB background and additional autoimmune genes on the NZW background, which lead to heightened levels of type I interferon and spontaneous features of systemic autoimmunity. These mice develop impairment of the intestinal barrier starting in adolescence as measured by FITC-dextran, and exhibit translocation of several organisms, among them predominantly and most consistently *E. gallinarum*. Monocolonization of non-autoimmune-prone C57BL/6 mice with *E. gallinarum* induced gut permeability to FITC-dextran, suggesting that both microbial, as well as host-related factors (e.g., TLR7-mediated signals as above), contribute to loss of barrier integrity. *E. gallinarum* reduced gut barrier-tightening molecules (such as those listed in Table 1) in (NZW x BXSB)F_1 mice and in monocolonized non-autoimmune C57BL/6 mice.[5] In addition, *E. gallinarum* downregulates RNA of claudin-3 and -5 in gut organoids derived from small intestines of (NZW x BXSB)F_1 mice (Figure 3). In long-term-monocolonized C57BL/6 mice, claudin-3 and -5 proteins were also reduced, as assessed by confocal imaging, whereas short-term exposure led to claudin-3 RNA upregulation. These findings likely reflect dynamic kinetics following epithelial cell interactions as seen also for claudin-5 in (NZW x BXSB)F_1 ileum-derived organoids (Figure 3).[5] Importantly, *E. gallinarum*

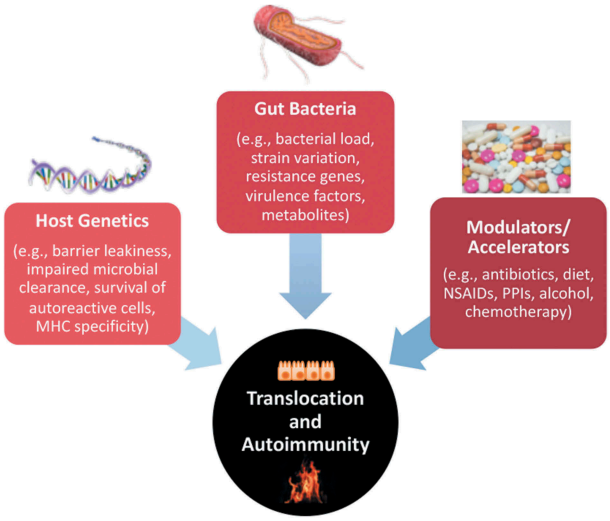

Figure 2. Factors influencing translocation of bacteria and extraintestinal autoimmunity. Autoimmunity arises in genetically susceptible hosts that are exposed to environmental stimuli that accelerate loss of self-tolerance. Barrier leakiness can either be intrinsic to the host or induced by external factors such as diet or medications. Bacteria that are normally contained in the gut lumen can gain access to extraintestinal tissues in response to enhanced intestinal permeability and via intrinsic bacterial mechanisms. The capability of bacteria to translocate depends on virulence factors, their abundance and proximity to the epithelial barrier, and their ability to compete within the gastrointestinal niche and evade immune defenses. Once translocated, bacteria such as *E. gallinarum* colonizing internal organs can directly induce autoimmunity by interacting with host tissues or indirectly via metabolites and their influence on the innate and adaptive immune system. The examples listed for each factor (host genetics, gut bacteria, modulators/accelerators) can impact translocation, autoimmunity or both (for instance, diet can influence barrier function as well as autoimmune responses). MHC, major histocompatibility complex; NSAIDs, non-steroidal anti-inflammatory drugs; PPIs, proton pump inhibitors.

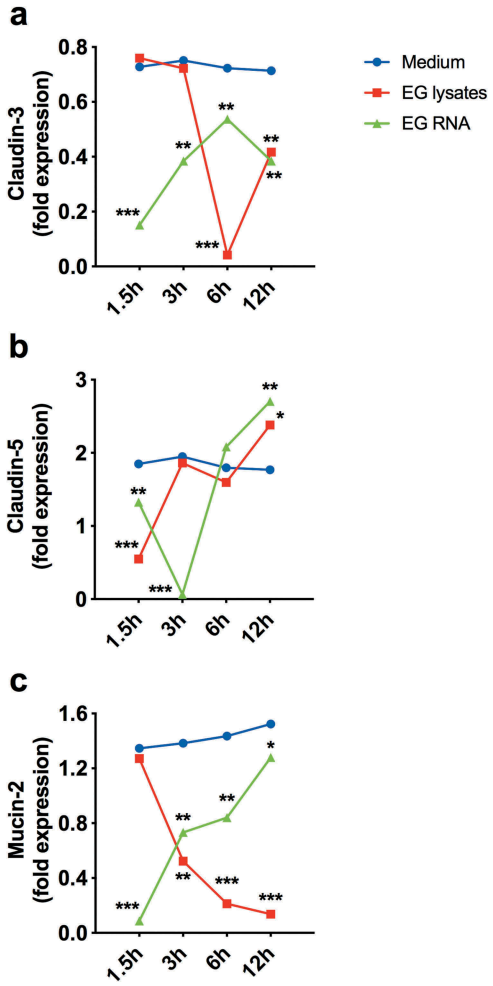

Figure 3. Small intestinal organoids from autoimmune-prone mice downregulate gut barrier molecules upon exposure to *E. gallinarum*. Ileum tissue was dissected from 12-week-old (NZW x BXSB)F_1 mice. Crypts were isolated and cultured for 7 days in IntestiCult Organoid Growth Medium (STEMCELL Technologies) and Matrigel Matrix (Corning). On day 7, heat-killed *E. gallinarum* (EG) or EG RNA, as prepared in ref. 5, or medium was added to the organoid cultures (n = 3) for 1.5, 3, 6, and 12 h. RNA was extracted and RT-qPCR performed as described in ref. 5. RNA expression of the barrier molecules claudin-3 and claudin-5, as well as the mucus protein mucin-2, were quantified in relation to actin. Blue lines, media alone; red lines, EG lysate; green lines, EG RNA. Data are presented as mean ± SD in (a) to (c); $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ANOVA followed by Bonferroni multiple-comparisons test.

functionally breached the gut barrier and migrated to MLNs, liver, and spleen. This process was linked to the progression of autoimmunity, including liver inflammation, systemic autoantibody production (against dsDNA, RNA, β_2-glycoprotein I, and endogenous retroviral proteins), signs of lupus nephritis, and widespread thrombi formation as seen in lupus-related antiphospholipid syndrome.[5] Notably, the portal inflammation in the liver is reminiscent of autoimmune hepatitis (AIH), an organ-specific autoimmune disease also characterized by anti-dsDNA antibodies in a subset of AIH patients.[55] Continuous oral vancomycin treatment depleted *E. gallinarum* in the gut microbiome of (NZW x BXSB)F_1 mice and prevented autoimmune manifestations.[5] Ongoing chronic treatment with vancomycin in our animal facility, however, created a niche for the development of vancomycin-resistant strains of *Enterococcus* (VRE). *E. gallinarum* is naturally resistant to low-dose vancomycin due to a *vanC* gene cassette.[56] When *E. gallinarum* acquires the *vanB* operon via horizontal transfer, however, it

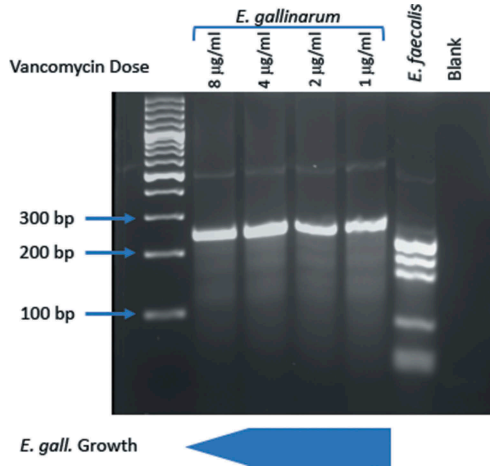

Figure 4. Vancomycin resistance gene restriction fragment length patterns for *E. gallinarum and E. faecalis*. *E. gallinarum* was grown in increasing concentrations from 1 µg/ml to 8 µg/ml of vancomycin. Restriction digest with MspI and multiplex PCR was performed as described in ref. 56 to determine vancomycin resistance genes. *E. gallinarum* normally carries the low-level vancomycin resistance *vanC-1* operon (fragment sizes at 230/237 bp). *E. faecalis* carries the high-level vancomycin resistance *vanB* operon (fragment sizes at 136, 160, 188/189 bp). Horizontal transfer of this operon to *E. gallinarum* can occur within gut microbiomes (not shown). Depicted below the gel is the in vitro growth of *E. gallinarum* without the *vanB* operon. Normal growth at concentrations between 1 and 4 µg/ml is noted with diminishing growth starting at 8 µg/ml and little to no growth at 20 µg/ml and beyond (not shown).

becomes VRE with high-level resistance that overcomes the high oral doses we applied to prevent autoimmunity and normally suppress the growth of *E. gallinarum* (Figure 4). Remarkably, the mouse colonized with the VRE strain of *E. gallinarum* progressed to full-blown autoimmunity with VRE growing in internal organs. Along with the host-microbial interaction studies we performed, this observation supports that autoimmunity was induced by *E. gallinarum* and not any of the other diverse bacteria sensitive to vancomycin in the gut of (NZW x BXSB)F_1 mice. In addition to monocolonization studies, vaccination against *E. gallinarum* provides evidence that this bacterium alone was the main driver of organ-specific and systemic autoimmunity. Intramuscular vaccination against *E. gallinarum*, but not against phylogenetically related *E. faecalis* or other less related gut bacteria, prevented autoimmunity and associated mortality.[5] When introduced into germ-free non-autoimmune-prone C57BL/6 mice by oral gavage, *E. gallinarum* induced Th17 responses not only in the lamina propria, as has been seen with gut resident segmented filamentous bacteria,[57] but also in MLNs, supporting immunologic effects beyond the gut. This phenomenon was linked to *E. gallinarum* translocation to MLNs, followed by liver and spleen, and induction of serum autoantibodies directed against dsDNA and RNA, which are hallmarks of human SLE.

These findings have relevance to human disease as *E. gallinarum* DNA can be detected by PCR in liver biopsy samples from patients with AIH and SLE, but not from healthy controls.[5] We found that healthy livers contained other *Enterococcus* spp., suggesting that bacteria of this genus are prone to gut barrier translocation like lactobacilli, which were also present in human liver samples.[5,53] Sera from patients with AIH and SLE have greater antibody titers to *E. gallinarum* RNA in comparison to RNA from *E. faecalis* or *B. thetaiotaomicron*, whereas antibody titers to these three bacterial RNAs occur at similar levels in healthy controls.[5] Of note, *E. gallinarum* RNA antibodies correlated highly with autoantibodies against human RNA, possibly due to shared immunogenic sequences.

Strain comparisons and further gnotobiotic studies should help elucidate if only particular *E. gallinarum* strains promote autoimmunity or if genetic predisposition of the host is needed for penetrance of autoimmune manifestations independently from the *E. gallinarum* strain. In addition, the duration of translocation and microbial burden in tissues likely matters in eliciting disease phenotypes. In the autoimmune-prone model, we noted persistent and progressive translocation overtime but could not detect naturally colonizing *E. gallinarum* in feces compared to very high fecal levels after exogenous reconstitution in a pancreatic sepsis model.[5,58] In that model, *E. gallinarum* translocation to blood, pancreas, and spleen was linked to sepsis that was partly TLR2 dependent.[58] Another open question is how enterococci travel and persist within the host. It remains to be determined if *E. gallinarum* migrates freely in the extracellular space or hijacks antigen-presenting cells or other host cells to reach internal organs and persist in host tissues. *E. faecalis* is well known to persist within macrophages, which is one of many possibilities for *E. gallinarum* persistence within its host that need to be tested.[59]

Similarly, it would be important to know if the closely related *E. casseliflavus* is also capable of inducing autoimmunity in susceptible models, as *E. faecalis* is not capable of doing so but is more distantly related to *E. gallinarum*. Although *E. faecalis* induced barrier leakiness and translocation similar to *E. gallinarum*, it did not induce signs of systemic autoimmunity. In contrast to monocolonization of C57BL/6 mice with *E. gallinarum, E. faecalis* did not induce Th17 cells or systemic autoantibodies.[5] Biosynthetic gene clusters are markedly different in *E. faecalis* compared to *E. gallinarum/E. casseliflavus* (Figure 5),[60] suggesting different virulence factors may contribute to autoimmunity versus translocation. Clearly, different translocating species within the *Enterococcus* genus promote diverse host phenotypes depending on distinct pathogenicity and host factors, respectively (Table 2 and Figure 2). The commonality of translocating into host tissues may be related to its evolutionary history.

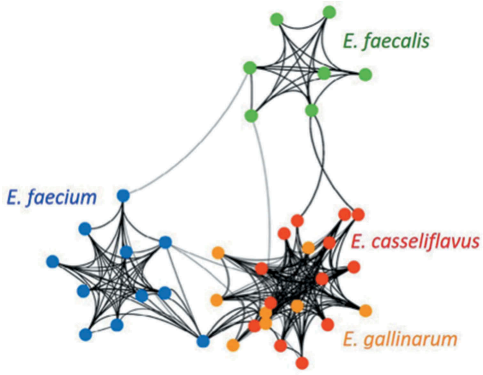

Figure 5. Network of predicted biosynthetic gene clusters (BGCs) from *E. faecium, E. faecalis, E. gallinarum*, and *E. casseliflavus* genomes according to Rashidi et al. Figure reproduced with permission from ref. 60. Unique strains are represented by colored nodes. Connecting lines indicate at least 75% similarity of BGCs between two strains. The weight of each line is proportional to the number and strength of BGC connections between two strains. Thicker lines represent more frequent higher-similarity pathways. Secondary metabolite profile predictions revealed distinct clusters. Intrinsically vancomycin-resistant *E. gallinarum* (orange) and *E. casseliflavus* (red) cluster together whereas *E. faecalis* (green) and *E. faecium* (blue) cluster independently from each other.

Evolution of enterococci as instigators of chronic human diseases

In the antibiotic era, enterococci have evolved from commensals to hospital-endemic human pathogens, now becoming a major cause of nosocomial infection.[61] By nature, enterococci are ubiquitous bacterial residents of the gastrointestinal tracts of mainly land animals, and are highly adapted to terrestrialization and the selective pressures of desiccation, starvation, and isolation. Their intrinsic-hardened outer cell walls and ability to exist in an environmentally persistent state naturally confer resistance to many antibiotics and disinfectants.[61] The introduction of antibiotics over the last 80 years selected for lineages of *E. faecalis* and *E. faecium* that lack CRISPR protection of their genomes.[62] This leads to a destabilization of the coevolved relationship with the human host. As a result of the easier entry of mobile genetic elements (MGE), hospital-adapted lineages possess "swollen genomes" replete with MGEs including a pathogenicity island, lysogenic phages, and many plasmids and transposons that confer new metabolic abilities and resistance to even last-line antibiotics.[62-64] This leads to a destabilization of the coevolved relationship with the human host. The combination of long evolved and recently acquired traits make them well suited for persistent residence in hospitals and transmission among antibiotic-treated individuals. These processes result in nosocomial infections, especially bacteremia, surgical site, and urinary tract infections.

Even more so, enterococci are becoming recognized as contributors of diseases not classically considered infectious in origin (Table 2).[65] Their broad living conditions may make them particularly suitable to detect in disease states, especially compared to bacteria that are harder to culture or propagate. Within the intestine, *Enterococcus* has been considered a driver of both inflammatory bowel disease and neoplasm.[66-71] Beyond the intestine, *Enterococcus* expansion is linked to hepatic inflammation in hepatitis B virus-related cirrhosis and *E. faecalis* enhances inflammation in mouse models of alcoholic cirrhosis with gastric acid suppression.[36,72] *Enterococcus* spp. among other species were also recovered from MLNs of cirrhotic patients and this genus is enriched in the gut mucosa of encephalopathic patients with cirrhosis.[73,74] Furthermore, *Enterococcus* is enriched in the fecal microbiome of patients with primary sclerosing cholangitis (PSC),[75] another liver

Table 2. Translocation of enterococci in non-infectious diseases*.

Disease	Genus/spp.	Main Study Findings	Translocation	Reference
Systemic and Organ-Specific Autoimmunity				
• Monocolonized C57BL/6 mice	*E. gallinarum*	*E. gallinarum* translocates and induces autoimmunity in a spontaneous model of lupus and non-autoimmune prone monocolonized mice. Features of SLE, antiphospholipid syndrome, and AIH are induced by *E. gallinarum*. Vaccination against *E. gallinarum* prevents translocation and autoimmunity.	Mesenteric veins, MLN, Liver, Spleen	Manfredo Vieira et al. (5)
• (NZW x BXSB)F_1 mice		*E. gallinarum* detected in liver biopsies of SLE and AIH subjects but not healthy controls.		
• SLE and AIH patients		SLE and AIH patients have greater IgG response to *E. gallinarum* RNA compared to controls.		
• PSC patients	*E. gallinarum*	*E. gallinarum* among other species (*Klebsiella pneumoniae, Proteus mirabalis*) is enriched in feces of patients with PSC and shown to translocate to MLN and promote Th17 responses related to PSC in a humanized gnotobiotic model.	MLN	Nakamoto et al. (75)
Viral and Alcoholic Liver Disease				
• Cirrhosis patients (mainly due to viral and alcoholic liver disease)	*Enterococcus*	*Enterococcus* spp. among others detected in MLN of patients with cirrhosis. Bacterial translocation increased with increasing Child-Pugh Score.	MLN	Cirera et al. (73)
• Gastric acid suppression ($Atp4a^{SI/SI}$) in ethanol or high fat diet-treated mice • Patients with alcoholic hepatitis	*E. faecalis*	*E. faecalis* intestinal expansion linked to translocation, hepatic inflammation and hepatocyte death in a TLR2-dependent manner.	MLN, Liver	Llorente et al. (36)
Hematopoietic Stem Cell Transplantation				
• Allogeneic stem cell transplant patients	VRE	VRE colonization is a surrogate marker but not independent predictor of worse outcomes after HCT.	Blood	Hefazi et al. (81)
Stroke				
• Stroke patients	*Enterococcus*	*Enterococcus* spp. most commonly detected in bloodstream post-stroke in patients.	Blood, Lung	Stanley et al. (37)
• Induced stroke mouse model		*E. faecalis* translocation after stroke in monocolonized setting.		
Pancreatitis				
• Acute pancreatitis in mice with bile duct obstruction pretreated with meropenem	*E. gallinarum*	*E. gallinarum* translocation linked to sepsis and mortality in mice with acute pancreatitis in a TLR2-dependent process.	Blood, Pancreas, Spleen	Soares et al. (58)
Inflammatory Bowel Disease				
• Crohn's disease patients	*E. faecalis*	*E. faecalis* detected in mesenteric fat and omentum of patients with Crohn's disease. *E. faecalis* linked to adipocyte proliferation and reduction in lipid content in vitro.	Mesenteric fat, Omentum	Zulian et al. (69)
• $IL\text{-}10^{-/-}$ monocolonized mice	*E. faecalis*	*E. faecalis*-monoassociated $IL\text{-}10^{-/-}$ mice develop distal colitis at 10–12 weeks of age and later duodenal inflammation and obstruction. *E. faecalis* induces IFN-γ- and IL-4-producing $CD4^+$ T cells, which precedes histological inflammation in colon.	MLN	Kim et al. (71)
Cancer and Cancer Therapy				
• Oral cancer patients	*E. faecalis*	*E. faecalis*, among other microbes, detected in lymph nodes excised from patients with oral cancer, most commonly in metastatic lymph nodes.	LN	Sakamoto et al. (65)
• $Nod2^{-/-}$ mice with sarcoma treated with cychlophosphamide	*E. hirae*	NOD2-dependent translocation of *E. hirae* to secondary lymphoid organs increased intratumoral $CD8^+$ T cell/Treg ratio, thereby facilitating chemotherapeutic effects mediated by cyclophosphamide.	MLN, spleen	Daillere et al. (34)

* Summary of studies demonstrating enterococcal translocation either alone or together with other bacteria (not addressed here) in chronic, non-infectious diseases or their treatment. Abbreviations: SLE – systemic lupus erythematosus; AIH – autoimmune hepatitis; MLN – mesenteric lymph node; LN – lymph node; TLR – toll-like receptor-2; VRE – vancomycin resistent *Enterococcus*; PSC – primary sclerosing cholangitis; HCT – hematopoietic cell transplantation

autoimmune disease related to AIH and associated with bacterial RNA in the portal tracts of the liver.[76] Consistent with these findings, *Enterococcus* abundance in the stool of PSC patients correlates with alkaline phosphatase, a marker of PSC progression.[75] In addition, very recent work from Japan found specifically *E. gallinarum* translocated to the MLN in humanized gnotobiotic mice along with *Klebsiella pneumoniae* and *Proteus mirabilis*, which together promoted Th17 responses in PSC.[77] Similarly, another recent study showed anti-tumor properties of an *E. gallinarum* strain mediated by its flagellin,[78] following human cancer microbiome studies which revealed *E. hirae, E. durans*, and *E. gallinarum* among the most enriched species in the stool of patients responding to checkpoint immunotherapy.[79] In a study with transplant patients, *Enterococcus* spp. including *E. faecium* and *E. gilvis* expanded after allogeneic stem cell transplantation, especially in those patients with graft-versus-host disease.[80] Patients with the outgrowth of these bacteria also have lower urinary 3-indoxyl sulfate, which correlates with poor early and long-term outcomes.[80,81] Finally, *E. gallinarum* translocates to internal organs in autoimmune-prone hosts and interacts with host cells in tissues, contributing to autoimmunity related to SLE, AIH, and PSC as detailed above. Further research is needed to fully assess *E. gallinarum* as a target in human autoimmune diseases, but the evidence for *Enterococcus* spp. in promoting non-infectious, chronic disease states is growing. More broadly, disease-promoting candidates across different genera will likely be discovered as the "dark matter" within the human microbiome is being revealed.[82,83]

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Disclosure of Potential Conflicts of Interest

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