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ANNUAL REVIEWS

Annual Review of Food Science and Technology

Nano in Micro: Novel Concepts in Foodborne Pathogen Transmission and Pathogenesis

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Keywords

bacteriophage, extracellular vesicle, bacterial microcompartment, carbon source, pathogen–microbiota–host interactions, risk assessment

Abstract

In this article, we highlight novel components of foodborne pathogens that influence their response, physiology, adaptation, and survival in the face of diverse stresses, and consequently have implications for their transmission in the food chain and their pathogenesis. Recent insights into the role of bacteriophages/prophages, bacterial extracellular vesicles, and bacterial microcompartments, which make up the emerging field we coined as "nano in micro," are presented, together with the role of understudied food-relevant substrates in pathogen fitness and virulence. These new insights also lead to reflections on generally adopted laboratory conditions in the long-standing research field of adaptive stress response in foodborne pathogens. In addition, selected examples of the impact of diet and microbiota on intestinal colonization and host invasion are discussed. A final section on risk assessment presents an overview of tools for (kinetic) data modeling and perspectives for the implementation of information derived from whole-genome sequencing, combined with advancements in dose-response models and exposure assessments.

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1. INTRODUCTION

Foodborne pathogens pose a significant threat to public health. The ability of pathogens to adapt and survive under diverse environmental stresses is critical to their transmission within the food chain and subsequent pathogenesis in humans. Therefore, a comprehensive understanding is essential for developing effective control strategies. In this article, studies on *Listeria monocytogenes* are referred to as the primary examples to demonstrate these concepts, with an extension to other foodborne pathogens. Among other reasons, our focus on *L. monocytogenes* is based on the increasing number of listeriosis cases and on a recent European Food Safety Authority (EFSA) report (EFSA Panel Biol. Hazards et al. 2024) on biological hazards that identified this notorious foodborne pathogen as the most relevant microbiological hazard associated with persistence in meat, fish and seafood, dairy, and fruit and vegetable processing environments.

Recent research has identified several novel players that significantly influence the physiology and virulence of pathogens, including microbial nano-structures, substrate utilization under different environmental conditions, and pathogen–diet–microbiota interplay. These new insights also contribute to risk assessment and ultimately to food safety management strategies.

2. NANO IN MICRO: AN EMERGING FIELD IN FOOD MICROBIOLOGY

Bacteriophages and prophages, bacterial extracellular vesicles (BEVs), and bacterial microcompartments (BMCs) (see Section 3.4) have emerged as key factors in what we refer to as the "nano-in-micro" field. These components play crucial roles in modulating pathogen response and resilience under stress conditions, which can affect their physiology and virulence.

2.1. Bacteriophages/Prophages

The ecophysiology of pathogens cannot be discussed without addressing the role of bacteriophages (phages), viruses that infect bacteria. Phages are nanosized protein capsids encapsulating their genetic material and are the most abundant biological entities on earth, found in large quantities wherever the bacterial hosts exist, including the human microbiota (Dion et al. 2020, Tobin et al. 2023).

As obligate parasites, phages can only complete their life cycles via mechanisms dependent on bacterial hosts. Phages are best known for their bacteria-killing action via the lytic cycle, where phages inject genetic material into the host bacterium and multiply using the host's machinery. Eventually, phage-encoded lytic enzymes are expressed to lyse the host, resulting in lytic death of the bacterium and the liberation of phage progeny that can infect other bacterial cells. In medical or food microbiology fields, there exist several applications for use of lytic phages: Phage therapy is regaining attention as a treatment for bacterial infections, replacing antibiotics, and phages are applied to food products to control foodborne pathogens like *L. monocytogenes* as a minimal processing strategy (Lenneman et al. 2021).

Temperate phages can also adopt the lysogenic cycle: The phage genome integrates into the bacterial host chromosome, becoming a prophage, which is passed down to the daughter cells. When the bacterium encounters certain stress factors, the prophage excises from the bacterial chromosome, switching to the lytic cycle. This process is known as prophage induction/activation. Prophages are very common in bacterial genomes. Their presence, composition, and induction can influence the ecophysiology, transmission, and virulence of pathogens.

2.1.1. Stresses related to prophage induction. Prophage induction can determine the fate of the bacterial host, and thus it is highly relevant to examine the presence and inducing factors of prophages (**Figure 1**).

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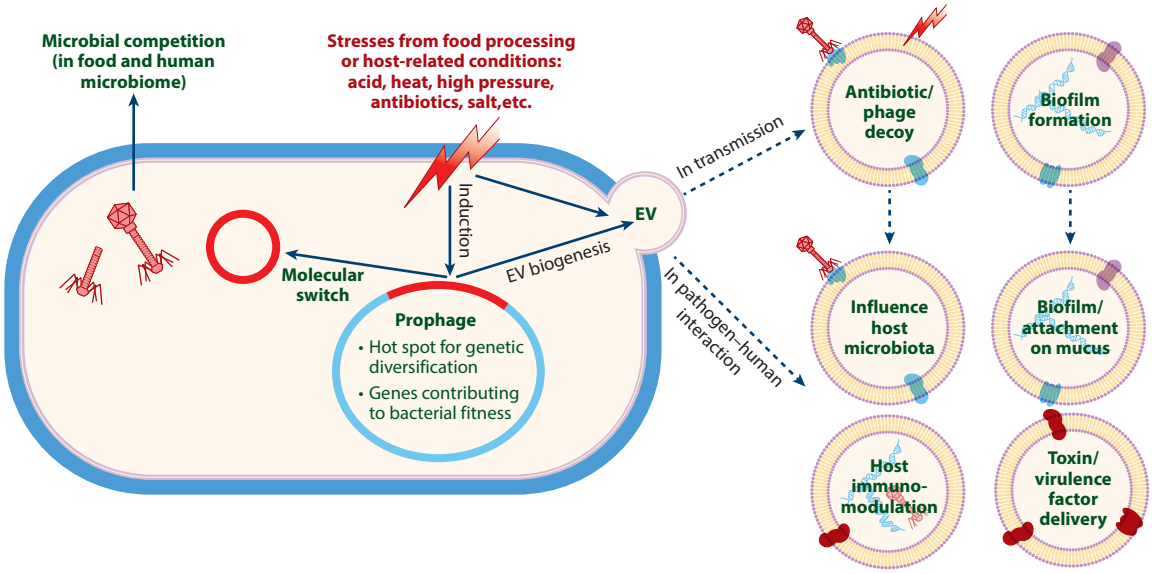


Figure 1

Nano in micro: (pro)phages and bacterial extracellular vesicles (EVs) in foodborne pathogen transmission and pathogen–host interactions. Summary from examples of primarily *Listeria monocytogenes* but also *Staphylococcus aureus* and *Bacillus cereus*. Stresses from food processing or host conditions can trigger prophage induction, where a prophage excises from the bacterial chromosome, expresses phage-encoded proteins, and forms phage particles. Prophage excision and reintegration can restore or disrupt genes at insertion sites, acting as a molecular switch. The phage particles, including phage-tail-like bacteriocins, contribute to microbial competition in food and the human microbiome. Stress also stimulates EV formation through both prophage-dependent and -independent pathways. EVs play roles in bacterial transmission and pathogen–human interactions. By acting as antibiotic or phage decoys and influencing biofilm formation, EVs also influence host microbiota and attachment on mucus and modulate host immune response by delivering nucleic acids, phage particles, toxins, and virulence factors.

The bacterial SOS response, triggered by DNA damage, is the canonical cue for prophage induction. This response inactivates the phage repressor via the host RecA protein or phage-encoded antirepressor (Silpe et al. 2023). DNA-damaging agents like antibiotic mitomycin C, acid stress, and high pressure can induce prophages in *L. monocytogenes*, most likely via the SOS response (Argov et al. 2019, Bowman et al. 2012, Duru et al. 2021).

Prophage induction may also be triggered by stressors via SOS-independent routes. Notably, bacteria often carry more than one prophage element that may respond differently to triggers or coordinate the lytic induction. For example, *L. monocytogenes* 10403S carries two prophage elements: One encodes infective phage particles, and the other is a cryptic element that encodes a bacteriocin (monocin) (Argov et al. 2019). Induction of both prophage elements is controlled by a metalloprotease from the monocin element, which responds to stresses encountered in mammalian cells during *L. monocytogenes* infection independently of the SOS response (Argov et al. 2019). Other stressors, including UV, oxidation, temperature change, nutrient limitation, quorum sensing, food/chemical additives, and plant extracts, have been shown to induce prophages in (food-associated) microbes (Miller-Ensminger et al. 2020).

2.1.2. Relevance of prophages in bacterial ecophysiology and virulence. Prophages are subjected to coevolution with the bacterial host. This coevolution often leads to genetic degradation of prophages, leading to incomplete or defective prophages that are no longer inducible or capable of forming infective particles (Bobay et al. 2014). Besides undergoing purifying selection from

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the bacterial host, prophages that stably inhabit bacterial chromosomes often contribute to the fitness of the host by supplying additional genes and functions or have adapted/cooperated to the lifestyle of the bacterial host (Argov et al. 2019).

The insertion and excision dynamics make prophages mobile genetic elements and hot spots for recombination enriched in polymorphisms, contributing to genetic diversity (**Figure 1**) (Brüssow et al. 2004). This drives the adaptation of pathogens in transmission and infection. It was reported that the evolution of *L. monocytogenes* in a food processing plant involves considerable diversification by gain and loss of prophages (Harrand et al. 2020); prophages are associated with strain persistence in food-associated environments, including retail markets and dairy farms (Castro et al. 2021, Wang et al. 2022); a prophage inserted in the *comK* gene has been proposed as a rapid adaptation island containing genes facilitating rapid niche adaptation of *Listeria*, biofilm formation, persistence, and subsequent transmission to food (Verghese et al. 2011). ComK is a major activator of the DNA uptake competence system that has historically been considered nonfunctional in *L. monocytogenes*, and strains associated with outbreaks of foodborne illness typically carry the *comK* prophage (Argov et al. 2019, Loessner et al. 2000). Prophage profiling, including assessment of polymorphisms in the *comK* prophage, is relevant for implementing genomics in advanced predictive modeling of growth and survival of *L. monocytogenes* in food and processing plant environments and in differentiating outbreak isolates (Palma et al. 2020).

Prophages contribute to the fitness of bacterial hosts by introducing beneficial gene elements (**Figure 1**). For example, prophages in persistent *L. monocytogenes* strains frequently carry antimicrobial resistance and heavy metal resistance genes, which are associated with strain persistence in dairy farms (Castro et al. 2021). Prophages also carry phage resistance genes, which protect the host from superinfection by other phages (Bondy-Denomy et al. 2016).

Prophages can affect the bacterial host by acting as a molecular switch and interrupting and recovering host genes during integration and excision (**Figure 1**). In *L. monocytogenes* serovar 1/2 strains, the prophage A118 integrates into the *comK* gene (Loessner et al. 2000, Rabinovich et al. 2012). During intracellular growth, A118 is induced to excise, restoring the functionality of the *comK* gene, which contributes to the escape of *L. monocytogenes* from the macrophage phagosome and promotes infection in mammalian cells (Argov et al. 2019, Rabinovich et al. 2012). Notably, prophage induction does not lead to formation of infective phage progenies or bacterial lysis, a nonclassical status referred to as active lysogeny (Pasechnek et al. 2020). This example illustrates the adaptation and cooperation between prophages and their pathogenic bacterial hosts, shaped by long-term coevolution.

Even when prophage induction results in lytic phage particles, the consequence for bacteria is not always negative. Although individual bacterial cells may lyse, the bacterial population can benefit from the released phages, which kill susceptible competitors in soil, food, or the human microbiome (**Figure 1**) (Wendling et al. 2021). The effect is seen not only with intact prophages but also with cryptic elements like the monocin loci in *L. monocytogenes*. Induction of this element releases a phage tail-like bacteriocin that can kill various competing *Listeria* strains and species (Argov et al. 2019).

When prophage activity leads to expression of phage-encoded lysis enzymes or bacterial lysis, the formation of BEVs may occur, adding another layer of complexity to bacterial ecophysiology and virulence, which is discussed in the next section.

2.2. Bacterial Extracellular Vesicles

BEVs are nano-sized, lumen-containing spheres enclosed by a lipid bilayer, originating from the bacterial membrane and released into the extracellular environment (Toyofuku et al. 2018). Similar

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to EVs from Eukarya and Archaea, BEVs carry diverse cargos, including lipids, nucleic acids, metabolites, and proteins. BEVs protect the cargo compounds from degradation and deliver them to destinations that whole-cell bacteria cannot always reach. Thus, BEVs play crucial roles in bacteria–environment, bacteria–bacteria, and bacteria–host interactions (**Figure 1**).

2.2.1. Stresses related to extracellular vesicle biogenesis. To date, several studies have provided evidence for a role of prophage activation in the biogenesis of EVs from Gram-positive bacteria (**Figure 1**). As demonstrated in *Staphylococcus aureus*, *Lactococcus lactis*, and *Bacillus subtilis* (Andreoni et al. 2019, Liu et al. 2021, Toyofuku et al. 2017), the induction of prophage and expression of prophage-encoded holin and endolysin results in peptidoglycan damage and protrusion of the cytoplasmic membrane, or cell lysis, both of which support release of EVs (Andreoni et al. 2019, Liu et al. 2021, Toyofuku et al. 2017). In *L. monocytogenes*, stressors activating prophages also increased EV release (Karthikeyan et al. 2020). In addition, the general stress response regulator Sigma B (SigB) (O'Byrne & Karatzas 2008) has been shown to play a role in *L. monocytogenes* EV release during the stationary growth phase with nutrient stress (Lee et al. 2013). Antibiotics that damage or inhibit the synthesis of the cell wall also promote EV formation conceivably independent from prophage activity, as demonstrated in *L. monocytogenes* with subinhibitory concentration of ampicillin (Woo et al. 2021) and in *S. aureus* with flucloxacillin and ceftaroline (Andreoni et al. 2019).

2.2.2. Relevance of bacterial extracellular vesicles in bacterial ecophysiology and virulence. Notably, EVs from *L. monocytogenes* can also offer protection against antibiotics such as trimethoprim and streptomycin by sequestering them (Karthikeyan et al. 2020). EVs have been shown to protect *S. aureus* against the membrane-targeting antibiotic daptomycin and antimicrobial fatty acids (Andreoni et al. 2019, Kengmo Tchoupa & Peschel 2020). Similarly, a decoy function of EVs to neutralize phages has been suggested (Zhao & Jones 2023). Roles of BEVs in horizontal gene transfer, nutrient scavenging, and inhibition/killing of competitors were also evidenced in a variety of bacterial species (**Figure 1**) (Toyofuku et al. 2023). These functions of EVs are relevant for both pathogen transmission and interaction with a human host by shaping the host microbiota (see Section 4).

EVs can enhance biofilm formation as they carry extracellular polysaccharides, proteins, and extracellular DNAs that contribute to the production of extracellular polymeric substances (**Figure 1**), as demonstrated for *Streptococcus mutans* (Jeong et al. 2024), although the link remains to be established in foodborne pathogens. An indirect link was noted when a mutation in *S. aureus* promoted EV production and biofilm formation simultaneously (Qiao et al. 2022). The role of EVs in biofilm formation is relevant not only for pathogen transmission but also in pathogen–host interactions with, for example, adhesion to mucus (**Figure 1**).

EVs from foodborne pathogens can carry virulence factors and host immunomodulation molecules that are crucial in pathogen–host interactions (**Figure 1**). Toxins, lipoproteins, DNA, and RNA contained by *S. aureus* EVs (Wang et al. 2020, Woo et al. 2021) and noncoding small RNAs contained by *L. monocytogenes* EVs (Frantz et al. 2019) are examples of potent activators of the host immune response. Moreover, phage particles are often found to be released in or together with BEVs, as prophage activity contributes to BEV biogenesis (Liu et al. 2021, Toyofuku et al. 2017), and the phages can also induce an immune response in mammalian cells (Champagne-Jorgensen et al. 2023, Kan & Barr 2023).

Notably, EVs from *L. monocytogenes* are often enriched in important virulence factors, including internalin B, as well as listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase C (Coelho et al. 2019, Karthikeyan et al. 2019). These virulence factors promote *L. monocytogenes* infection of host cells by inducing bacterial uptake and facilitating bacterial escape from

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the phagosomal vacuole to the cytoplasm. Bioactive LLO associated with *L. monocytogenes* EVs caused cytotoxicity in human intestinal epithelial cells (Karthikeyan et al. 2020) and murine mouse macrophages (Coelho et al. 2019). In the same study, preincubation of mouse embryonic fibroblasts with EVs also promoted intracellular survival by *L. monocytogenes*.

Similarly, EVs from enteropathogenic *B. cereus* strain NVH0075-95 act as shuttles for virulence-associated factors, including sphingomyelinase (SMase), phospholipase C, and the multicomponent enterotoxin Nhe, and elicit a cytotoxic effect in vitro on human intestinal epithelial cells (Buchacher et al. 2023). In particular, the essential subunit NheC of the pore-forming enterotoxin was exclusively detected in EVs instead of a vesicle-free supernatant, likely because of a hydrophobic domain that is required for membrane integration of the multicomponent toxin. Of note, the presence of Nhe and SMase in *B. cereus* EVs also showed increased hemolysis in human cells as compared to vesicles containing each factor alone, pointing to the unique function of EVs to allow simultaneous loading of multiple virulence factors to achieve a synergistic effect (Buchacher et al. 2023). An intriguing question yet to be answered is whether EVs from emetic-type *B. cereus* carry the highly hydrophobic toxin cereulide (Agata et al. 1994, Huang et al. 2022b), as EVs would provide a plausible route for this non-water-soluble biomass-associated toxin to be delivered to host intestinal epithelial cells to exert toxicity.

3. PATHOGEN METABOLISM AND ITS IMPLICATIONS IN TRANSMISSION AND VIRULENCE

The use of specific carbohydrates can affect the phenotype of *L. monocytogenes*, influencing biofilm formation, stress resistance, and virulence, underscoring the importance of considering both genetic variation and environmental factors when studying the metabolic and phenotypic responses of *L. monocytogenes*. Notably, most studies utilize rich culture media such as brain heart infusion (BHI) or defined media with glucose as the carbon source. However, a range of other compounds may serve as growth substrates in specific niches, including food, food processing environments, and the human host.

3.1. Lactose and Cellobiose

Lactose is a primary carbon source in milk and dairy products, and cellobiose, naturally produced from hydrolyzed cellulose, was recently approved as a novel food in the European Union (EU) [Regulation (EU) 2023/943]. In *L. monocytogenes*, lactose and cellobiose can enter the cell via three phosphoenolpyruvate sugar phosphotransferase systems (PTSs) (**Figure 2**). The transcriptional activator LacR (Lmo1721/CsrA/CelR) controls two lactose/cellobiose PTSs, one formed by LpoA, LpoB, and putative Lmo2708, and the other by Lmo2683-Lmo2685 (Dalet et al. 2003, Ma et al. 2024, Stoll & Goebel 2010). A third lactose PTS was recently identified and is composed of Lmo2762, Lmo2763, and Lmo2765 and controlled by Lmo2766 (Ma et al. 2024). These PTSs transport and phosphorylate lactose and cellobiose into lactose-6-P and cellobiose-6-P, respectively. The cellobiose-6-P is hydrolyzed intracellularly to glucose-6-P and glucose, and the lactose-6-P is hydrolyzed to galactose-6-P and glucose. Next, galactose is secreted from the cells after dephosphorylation of galactose-6P, and glucose is phosphorylated by ATP yielding glucose-6-phosphate that enters glycolysis (**Figure 2**). The consumption of one lactose can yield three ATPs aerobically with acetate as the final product but only one ATP anaerobically, with lactate as the final product. This exemplifies that utilization of different carbon sources under different environmental conditions influences ATP yield and conceivably cell physiology (Ma et al. 2024).

Notably, lactose was recently shown to induce the SigB-dependent stress defense in *L. monocytogenes* EGDe, resulting in resistance to heat and acid stress, increased biofilm formation, and

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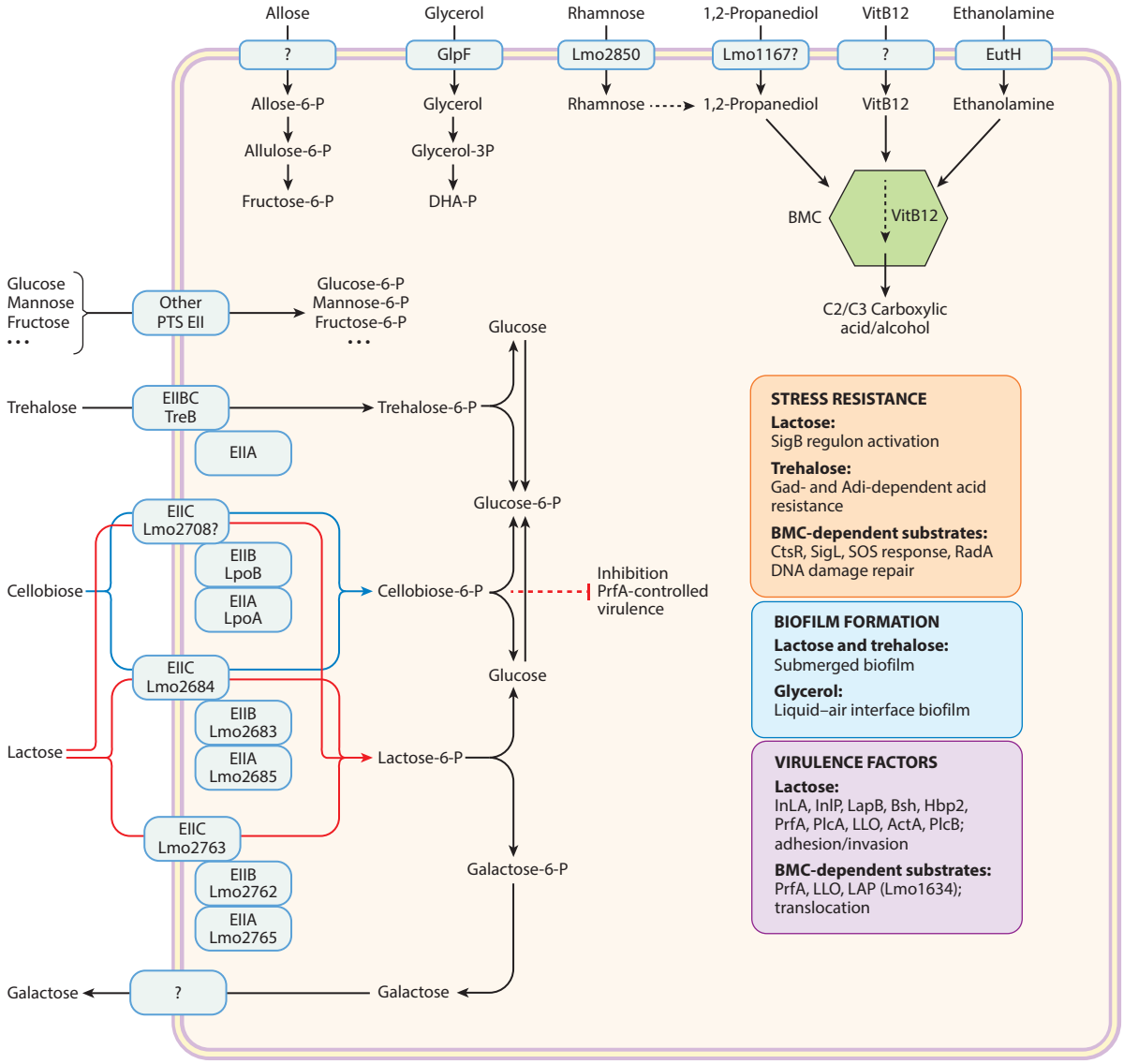


Figure 2

Schematic presentation of selected substrate metabolism and related implications in *Listeria monocytogenes*. EIIABC transporter components of selected phosphotransferase systems (PTSs) are shown, and the respective transported carbohydrates are indicated. Rhamnose, 1,2-propanediol, and ethanolamine are transported via Lmo2850, conceivably Lmo1167, and EutH, respectively, and further metabolized via bacterial microcompartment (BMC)-specific, vitamin B12 (VitB12)-dependent pathways in anaerobic conditions. Corresponding stress resistance, biofilm formation, and in vitro virulence phenotypes are indicated for selected substrates.

enhanced adhesion/invasion of Caco-2-derived C2Bbe1 cell lines (Tapia et al. 2020). Furthermore, in contrast to utilization of monosaccharides, cellobiose utilization strongly represses virulence gene expression by inhibiting the virulence master regulator PrfA (Johansson & Freitag 2019, Milenbachs et al. 1997), and *L. monocytogenes* EGDe grown in nutrient broth (NB) with lactose was shown to induce expression of virulence factors, including internalin genes *inlA* and *inlP* (see data published in BioProject PRJNA1107127) (Ma et al. 2024). Previously, PrfA activity has been

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suggested to correlate with the phosphorylation state of PTS permeases via the phosphocarrier HPr protein of the PTS (Johansson & Freitag 2019, Joseph et al. 2008). Whether this underlies the observed differences in expression of PrfA and other virulence factors in cells grown with cellobiose and lactose requires further study.

A comparative whole-genome sequence (WGS) analysis of a collection of *L. monocytogenes* strains showed that the F2365 strain, isolated from the 1985 Jalisco cheese outbreak and associated with a high percentage (65.5%) of pregnancy-related cases (Linnan et al. 1988), had reduced capacity to utilize lactose because of a frameshift mutation ($lacR^{887del}$) resulting in truncation of the lacR activator (Ma et al. 2024). Gene expression analysis of F2365 cells grown in NB with glucose or lactose showed that lactose-grown cells activated the expression of a set of virulence genes, including pathogenicity island LIPI-1 genes *prfA*, *plcA*, *hly*, *actA*, and *plcB*, and, additionally, *inlP*-encoding internalin P (InlP) (Ma et al. 2024). InlP was recently shown to facilitate the movement of *L. monocytogenes* from the cytotrophoblast layer to the stroma and to promote placental infection (Faralla et al. 2016, 2018). These results seemingly contrast with previous studies that showed reduced virulence of BHI-grown F2365 cells in adhesion/invasion studies with human Caco-2 cells as compared to other *L. monocytogenes* serotype 4b strains (Nightingale et al. 2007). Combined with the observed impact of lactose on growth and gene expression and taking into account *L. monocytogenes* strain diversity in lactose utilization capacity, it cannot be excluded that these factors affect the ecology and persistence of *L. monocytogenes* in dairy-processing environments and dairy-derived products, especially fresh soft cheeses that contain high levels of lactose (Ibarra-Sánchez et al. 2017). Comparative WGS analysis surprisingly showed truncated LacR to be over-represented in *L. monocytogenes* isolated from dairy products, suggesting that LacR-mediated lactose utilization capacity does not necessarily confer a fitness advantage in these environments (Ma et al. 2024). Additional studies using culture conditions mimicking that of relevant foods and processing environments will further add to our understanding of *L. monocytogenes* strain-specific features of environmental and clinical strains, including, for example, recently isolated dairy product–associated outbreak isolates (EFSA Panel Biol. Hazards et al. 2018, FDA 2024).

3.2. Trehalose and Other Phosphotransferase System Carbon Sources

Trehalose is a disaccharide abundant in bacteria, plants, fungi, and insects (Iturriaga et al. 2009). In *L. monocytogenes*, the trehalose-specific PTS EIIBC subunit TreB (Lmo1255) is responsible for trehalose uptake (**Figure 2**) (Wu et al. 2023). Notably, *L. monocytogenes* trehalose metabolism activates amino acid–dependent acid resistance mechanisms and affects biofilm formation (Wu et al. 2023).

Carbon source utilization can also affect resistance to bacteriocins, which are antimicrobial peptides (AMPs) produced by bacteria. Notably, resistance to class IIa bacteriocins has been correlated with the downregulation of genes associated with the mannose PTS (Man-PTS) involved in the uptake of mannose, glucose, and fructose (Dalet et al. 2001, Gravesen et al. 2002), and cellobiose and sucrose can increase *L. monocytogenes* class IIa bacteriocin resistance (Balay et al. 2018). Reduced bacteriocin action was linked to the loss of the Man-PTS components IIC and IID, which facilitate membrane insertion of class IIa bacteriocins and subsequent inactivation of target cells (Kjos et al. 2010, 2011). These results indicate that utilization of specific PTS-dependent carbohydrates can influence the ability of *L. monocytogenes* to grow in the presence of bacteriocins, which may affect their efficacy in food preservation applications.

Along with trehalose, glucose, fructose, and maltose are rapidly metabolizable PTS carbon sources that can repress several virulence genes of *L. monocytogenes* (Milenbachs et al. 1997) (**Figure 2**). Metabolism of non-PTS sugars like glucose-6-P and three-carbon compounds such

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as glycerol does not repress virulence gene expression, and they serve as the main carbon sources for *L. monocytogenes* during intracellular growth (Eylert et al. 2008). Glycerol is also present in food or food-processing environments as a common fermentation product and widely used food additive (Pagliaro & Rossi 2010), and its metabolism and impact on *L. monocytogenes* growth and phenotypes are discussed in the next section.

3.3. Glycerol and Allose

The main glycerol metabolic pathway of *L. monocytogenes* includes the GlpF facilitator, the GlpK kinase, and the GlpD glycerol-3P dehydrogenase (**Figure 2**) (Deutscher et al. 2014). A more recent study found that *L. monocytogenes* EGDe requires oxygen to increase the expression of *glpF*, *glpK*, and *glpD* involved in the import and utilization of glycerol (Tapia et al. 2018). The presence of glycerol in the medium under aerobic conditions induces planktonic growth and air–liquid interface biofilm formation, conceivably linked to aerotaxis toward the surface of the culture. Previously, utilization of glycerol under both aerobic and anaerobic conditions has been reported for *L. monocytogenes* ATCC 15313 and other isolates (Muchaamba et al. 2019, Pine et al. 1989). Based on the observed growth and biofilm formation effects, the impact of specific features of *L. monocytogenes* isolates on glycerol utilization capacity and corresponding phenotypes requires further study.

The use of phenotype microarrays also showed significant variability in the efficacy of the utilization of other carbon sources by *L. monocytogenes* strains (Muchaamba et al. 2019, Sharar et al. 2018). One notable genetic variation impacting *L. monocytogenes* carbon source utilization involves the allose metabolism-associated *lmo0734-0739* gene cassette found in genetic lineage II but not in lineage I or III strain (Zhang et al. 2018). Interestingly, substituting allose as the primary carbon source during enrichment improved detection and isolation of lineage II *L. monocytogenes* from food (Upham et al. 2023), thus providing an approach to target, for example, isolates of this specific subtype in outbreak investigations.

3.4. Bacterial Microcompartment-Mediated Metabolism

BMCs are nano-sized, self-assembling organelles that consist of an enzymatic core encapsulated by a selectively permeable protein shell (Kerfeld et al. 2010), and these nanostructures enable *L. monocytogenes* to utilize selected carbon sources that are internalized via dedicated transporters. These carbon sources are abundant in foods and in the human gastrointestinal (GI) tract and include ethanolamine and 1,2-propanediol derived from membrane phospholipid degradation and metabolism of rhamnose and fucose, respectively (Anast et al. 2020, Zeng et al. 2022). Moreover, 1,2-propanediol is an authorized food additive in the EU in various food categories, including dairy (EFSA Panel Food Addit. Flavour. et al. 2018). The BMC-specific, vitamin B12–dependent metabolic pathways involve transient production of toxic volatile aldehydes, which are sequestered by BMCs to minimize interference with other cellular functions, whereas corresponding C2 and C3 alcohols and carboxylic acids are formed as the end products, concomitant with the production of ATP required for growth (Kerfeld et al. 2010, Zeng et al. 2022).

In *L. monocytogenes*, the *pdu* and *eut* operons encode the proteins that constitute propanediol utilizing (PDU) and ethanolamine utilizing (EUT) BMCs, respectively (**Figure 2**) (Zeng et al. 2021b). Previously, vitamin B12 biosynthesis as well as ethanolamine and 1,2-propanediol utilization clusters were found to be significantly more highly expressed in *L. monocytogenes* H7858 cells growing on vacuum-packaged cold-smoked salmon than in BHI broth (Tang et al. 2015). BMC activation and utilization of ethanolamine and (rhamnose-derived) 1,2-propanediol stimulated the growth of *L. monocytogenes* only under anaerobic conditions (Zeng et al. 2021a,b), and

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stress defense proteins were found to be overexpressed in these conditions (Zeng et al. 2024). In addition, glutathione synthase GshAB, previously linked to the activation of the virulence response in *L. monocytogenes*, was overexpressed in PDU-BMC-induced cells (Zeng et al. 2021b), whereas EUT-BMC-induced cells showed significantly higher levels of PrfA and LLO (Zeng et al. 2021c). Furthermore, *L. monocytogenes* with active EUT or PDU BMCs showed significantly enhanced translocation efficacy compared to non-BMC-induced cells, and adhesion and invasion capacity remained similar in Caco-2 cell studies (Zeng et al. 2021b,c) (see **Figure 2** and Section 4). These findings point to the roles of BMC-dependent metabolism in *L. monocytogenes* growth, survival, competition, and pathogenicity in respective food- and host-related anaerobic environments.

4. PATHOGEN–MICROBIOTA–HOST INTERACTIONS AND INFLUENCES ON PATHOGENESIS

The GI tract is a complex environment where microbiota-mediated competition, epithelial barrier function, and local immune responses work together to exclude pathogens and limit foodborne infection. Dietary components such as fatty acids, carbohydrates, and proteins can directly influence gut microbiota community structure, epithelial barrier function, and systemic immunity with likely consequences for susceptibility to foodborne infection (**Figure 3**) (Las Heras et al. 2022). Understanding such diet–microbiota–pathogen interactions is crucial for developing food or microbial interventions to limit or prevent foodborne infections. Furthermore, the prior history of the pathogen (related to growth conditions in foods and the food processing environment) has the capacity to influence the physiology of the pathogen and the initial capacity to infect the intestine through regulation of motility and virulence/stress factors (see Section 4.3).

4.1. Pathogen–Microbiota Dynamics

The gut microbiota plays a pivotal role in regulating local and systemic immune processes as well as maintaining GI barrier integrity (Kreuzer & Hardt 2020). Germ-free mice, lacking a resident microbiota, generally demonstrate increased susceptibility to foodborne pathogens relative to conventionally colonized animals, indicating that the microbiota plays a role in resisting infection (Herzog et al. 2023). This resistance, termed colonization resistance, involves mechanisms such as the production of inhibitory short-chain fatty acids (SCFAs) and bacteriocins, bile acid modification, and immune regulation (Herzog et al. 2023). Host cells (including gut epithelial cells and immune cells) are equipped with cellular receptors for microbiota-associated metabolites (such as SCFAs, bile acid derivatives, and vitamins), which thereby act as mediators of host homeostatic responses (Vinolo et al. 2011). Disruption of microbiota (often termed dysbiosis), through antibiotics, diet, age, or immune status, can impair host physiological processes and resistance to pathogens.

In turn, gut pathogens are equipped with mechanisms to attempt to overcome host colonization resistance mechanisms. For instance, *L. monocytogenes* (but not the nonpathogenic species *Listeria innocua*) is equipped with a bile salt hydrolase (BSH) enzyme that contributes to bile tolerance and plays a role in GI colonization (Begley et al. 2005, Dussurget et al. 2002). The *L. monocytogenes* BSH system is regulated by PrfA and SigB, suggesting complex regulation in response to conditions encountered during infection (Begley et al. 2005). Indeed, analysis of transcriptional profiles from *L. monocytogenes* within the germ-free mouse gut or in mice monocolonized by *Lactobacillus* species demonstrated that the pathogen can adapt to the gut environment through expression of a gut-specific stress regulon (which includes BSH and various stress/environmental response systems), the expression of which is enhanced by the presence of resident lactobacilli (Archambaud et al. 2012). However, despite the adaptation of the pathogen,

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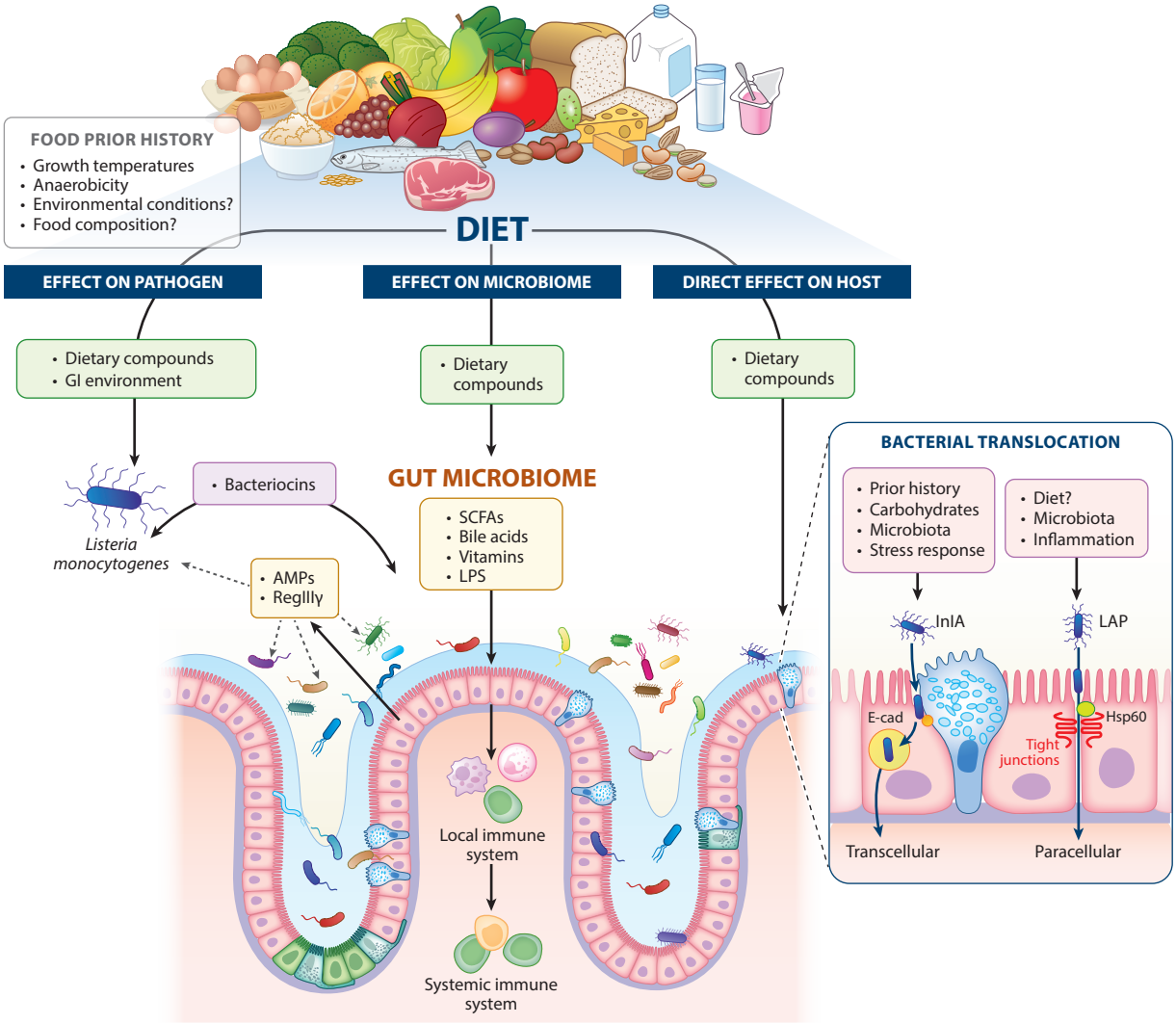


Figure 3

Influence of microbiome, diet, and prior growth history on *L. monocytogenes* pathogenesis. Growth conditions in foods have the potential to influence *L. monocytogenes* stress resilience, expression of EVs, motility, and virulence factor expression, which potentially impacts virulence. The gut microbiome influences production of antimicrobial peptides (AMPs), and both the pathogen and the microbiota produce bacteriocins that influence early colonization and pathogenesis. Dietary components such as lipids, carbohydrates, and proteins influence the composition of the microbiome with consequences for microbiome–host regulatory processes. Constituents of the diet can also directly influence local and systemic immune responses to the pathogen. At the host–pathogen interface, local nutritional and physicochemical factors influence virulence gene expression and, potentially, invasion of the host, subsequent pathogenesis, and spread. Abbreviations: GI, gastrointestinal; LAP, *Listeria* adhesion protein; LPS, lipopolysaccharide; SCFAs, short-chain fatty acids.

Lactobacillus species also enhanced the anti-*Listeria*, interferon-mediated immune response to promote systemic clearance of the pathogen. The microbiota is also known to regulate the expression of local AMPs, including RegIIIg, which was demonstrated to kill *L. monocytogenes* in the small intestine (Brandl et al. 2007). A more recent study utilized antibiotic treatment of mice to reduce colonization resistance against *L. monocytogenes* and subsequently identified commensal

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species (including *Clostridium saccharogumia*, *Clostridium ramosum*, *Clostridium hathewayi*, and *Blautia*) that can reinstate colonization resistance in model systems (Becattini et al. 2017).

A separate study demonstrated that some *Lactobacillus* species produce a bacteriocin that inhibits *L. monocytogenes* infection in mice (Corr et al. 2007). Conversely, *L. monocytogenes* produces bacteriocins like Lmo2776 to target *Prevotella copri* in the gut to permit successful infection by the pathogen (Rolhion et al. 2019). Similarly, the function of Listeriolysin S appears to modulate the microbiota by targeting *Streptococcus*, *Alloprevotella*, and *Allobaculum* species to favor colonization by the pathogen (Quereda et al. 2017).

Understanding colonization resistance mechanisms is essential for addressing interindividual susceptibility to foodborne infections. Studies have shown a reduction of microbiota diversity in the elderly (Claesson et al. 2012), an age group that is particularly sensitive to listeriosis and other GI pathogens. A recent study demonstrated that older mice (∼20 months of age) are more susceptible to oral *L. monocytogenes* infection compared to younger animals (∼2 months of age) (Alam et al. 2021). The authors showed an age-related reduction in *Lactobacillus*, *Clostridiales*, and *Akkermansia* spp. that correlated with increased susceptibility to listeriosis in older mice and highlighted that further functional characterization of representative taxa will be necessary to determine direct links to colonization resistance in this model (Alam et al. 2021).

Similarly, the barrier properties of the infant microbiota are relatively underdeveloped, and microbiota diversity increases over time, maturing as late as four years of age (Fouhy et al. 2019). Infants are clearly at increased risk of developing listeriosis (contracted either intrapartum or postpartum) and, before the maturation of the microbiota, are also at risk of other foodborne and waterborne infections (Panigrahi 2024). There is therefore significant potential for the use of live microbial interventions in infants to improve colonization resistance and enhance immune development. In a large randomized double-blind placebo-controlled trial (involving 4,556 neonatal infants) the use of a synbiotic made up of a combination of live *Lactiplantibacillus plantarum* and fructooligosaccharides significantly reduced the incidence of sepsis and death, with significant reductions in sepsis alone and in lower respiratory tract infections (Panigrahi 2024). Although the elderly and neonatal gut may be relatively amenable to manipulation, it may be more challenging to alter the adult microbiome.

Additionally, the roles of phages and EVs in bacterial ecophysiology (discussed in Section 2) may also provide insights into their impacts on human microbiota–pathogen interactions (**Figure 1**) and, consequently, host physiology.

4.2. Host Physiology and Diet

Dietary composition directly impacts several aspects of host physiology, including GI and systemic immune function (Las Heras et al. 2022). These effects may be mediated directly or through alterations to microbiota community profiles that, in turn, impact immune and barrier functions (Las Heras et al. 2022, Wolter et al. 2021). Individual food constituents, including fatty acids, carbohydrates, and proteins, can influence host immunity and barrier function through discrete interactions with the host (Las Heras et al. 2022). For instance, long-chain saturated fatty acids such as palmitic acid (C16:0) that are associated with animal fats engage the NLRP3 inflammasome to promote adiposity (Las Heras et al. 2022). An emerging area of interest is how food components impact susceptibility to infectious disease. Resistance to *Salmonella* Typhimurium (Wotzka et al. 2019) and *L. monocytogenes* (Las Heras et al. 2019) is significantly reduced in mice fed a Western diet enriched in animal fats. For *Salmonella*, this effect is linked to elevated bile acid production in high-fat-diet hosts, providing *S.* Typhimurium (inherently bile tolerant) with a competitive advantage over the microbiota. Protection against diet-induced susceptibility to *Salmonella* could

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be increased by administering live commensal *Escherichia coli* strains, which are also inherently bile tolerant and compete with *Salmonella* under high-fat-diet conditions (Wotzka et al. 2019). In the *Listeria* infection model, a high-fat diet influenced multiple physiological parameters (including microbiota, goblet cell number, and inflammatory profiles), affecting sensitivity to infection. Notably, dietary effects influenced susceptibility to both GI and systemic infections, highlighting the systemic effects of diet on the host immune system (Las Heras et al. 2019). Interestingly, the high-fat-diet-induced sensitivity to *L. monocytogenes* infection could be significantly reduced by administering the next-generation probiotic species *Akkermansia muciniphila* (Keane et al. 2023).

Effects on outcome of infection are likely to reflect the precise constituents of the diet (Las Heras et al. 2022). Cruz-Chamorro et al. (2011) showed that fish oil, but not olive or sunflower oil, enhanced bacterial clearance during secondary *L. monocytogenes* infection.

In vitro analyses have demonstrated that growth media supplemented with fatty acids commonly present in milk directly decreased *L. monocytogenes* survival (Wang & Johnson 1992) and invasion of epithelial cells (Petrone et al. 1998). Unsaturated long-chain fatty acids, such as palmitoleic acid and γ-linolenic acid, exert an antimicrobial effect on *L. monocytogenes* in rich medium and reduce expression of PrfA-activated virulence genes such as *plcA*, *plcB*, *hly*, and *inlA* (Sprong et al. 2002). Another study demonstrated that similar milk-associated fats may be active in vivo against *L. monocytogenes* (Sprong et al. 2012).

In addition to long-chain fatty acids, carbohydrates regulate the virulence-associated PrfA regulon in *L. monocytogenes*. Rapidly metabolized carbohydrates, including glucose and cellobiose, commonly encountered outside the host downregulate PrfA and influence virulence potential. Other compounds such as the complex aminopolysaccharide chitin have also been shown to downregulate the virulence regulon (Villoria Recio et al. 2020). These findings suggest that dietary carbohydrates (e.g., a plant-based diet containing cellobiose) or dietary supplements (e.g., chitin) may influence *Listeria* infection in the host (Kallipolitis 2017) (see Section 3).

4.3. Crossing the Epithelial Barrier

Following foodborne transmission, *L. monocytogenes* adapts to conditions in the GI tract (Archambaud et al. 2012) and initiates a process whereby it crosses the intestinal barrier through key virulence proteins: internalin A (InlA) and *Listeria* adhesion protein (LAP) (Drolia & Bhunia 2019). InlA mediates transcytosis by interacting with host E-cadherin, which is exposed through cell extrusion at the tips of the villi or goblet cells during mucus exocytosis (Bonazzi et al. 2009). This interaction initiates a series of host cellular trafficking events, resulting in the pathogen's internalization. The process is host specific, as *L. monocytogenes* InlA interacts efficiently with human E-cadherin but not with murine E-cadherin (Bonazzi et al. 2009). The systemic dissemination of ΔinlA-mutant strains, which are LAP-positive, suggested a second mechanism for crossing the epithelial barrier in mice and other model systems (via a mechanism known as translocation) (Drolia et al. 2024, Koo et al. 2012). Translocation depends on LAP expression, which initially interacts with epithelial Hsp60 to trigger endocytosis of tight junctions and permit *Listeria* translocation between epithelial cells (see **Figure 3**). This process also exposes more E-cadherin, potentially enhancing InlA-mediated transcytosis, indicating that the two processes may be complementary (Drolia et al. 2024).

Such information may be beneficial in developing targeted therapeutic or preventative approaches that can block these mechanisms. Koo et al. (2012) developed a *Lactobacillus paracasei* strain expressing LAP from *L. monocytogenes* (LbpLAP), which demonstrated enhanced interaction with Hsp60, resulting in increased adhesion to Caco-2 cells and a notable reduction in *L. monocytogenes* translocation in coculture, suggesting that LbpLAP can competitively prevent

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L. monocytogenes translocation across epithelial barriers. In addition, it is interesting that diet and the community structure of the microbiota may also influence the GI environment to impact barrier function and, potentially, resistance to bacterial translocation. For instance, preliminary evidence suggests that a high-fat diet can influence the number of goblet cells in the murine GI tract as well as the expression of genes encoding tight junction proteins (Las Heras et al. 2019). Future research should explore how these luminal influences affect molecular pathogenesis and their modulation by dietary factors.

Additionally, the pathogen's prior history and the nature of the food matrix are important as *L. monocytogenes* enters the GI tract. Molecular systems necessary for stress survival in foods may potentially aid initial survival in the GI environment (Gahan & Hill 2014). The pathogen expresses systems for low pH survival (arginine deiminase and glutamate decarboxylase pathways) in response to the GI environment (Archambaud et al. 2012), which are also important for survival in low-pH food environments (Feehily et al. 2014, Gahan & Hill 2014). It is interesting to note that growth of *L. monocytogenes* under anaerobic conditions induces InlA and greatly enhances oral infectivity in the guinea pig model (Bo Andersen et al. 2007). Although flagellar biosynthesis is downregulated at body temperature (~37°C), prior growth at room temperature induces flagellar motility that is retained during initial small intestine infection and is important for pathogenesis (Abdulkadieva et al. 2023, Wortel et al. 2022).

5. RISK ASSESSMENT

5.1. Quantitative Assessments of Microbial Behavior

A quantitative microbiological risk assessment (QMRA) aims to estimate the health risk from pathogen exposure via food, water, or other transmission routes and integrates available quantitative data on microbial behavior. A QMRA typically includes four steps: (*a*) hazard identification, (*b*) exposure assessment, (*c*) hazard characterization, and (*d*) risk characterization. In the hazard identification step, the target pathogen is selected. In the exposure assessment, the pathways of exposure are quantitively described to estimate the exposure dose (i.e., frequency and the level of exposure) of the consumers. The hazard characterization step defines the correlation between the exposure dose and the probability of the health risk outcome (i.e., the dose–response correlation). In the final risk characterization step, this dose–response correlation is used to estimate the health risk based on the exposure dose.

A QMRA can be used to compare risks between groups of individuals that differ in susceptibility and evaluate the efficacy of interventions that may reduce the transmission and survival of pathogens to improve food safety control. Available quantitative data to support the hazard characterization step are often very limited in comparison to the exposure assessment. This is mainly due to the fact that it is typically impossible to carry out relevant experiments to determine the dose–response correlation where consumers are intentionally exposed to a pathogen, and, therefore, the dose–response correlation includes large uncertainty and variability (Haddad et al. 2018, Pouillot et al. 2016). For *L. monocytogenes*, an ice cream outbreak in the United States in 2015 provided an unintended but unique opportunity to assess exposure levels from implicated products (Pouillot et al. 2016) because ice cream has a long shelf life and *L. monocytogenes* does not grow in the frozen product, allowing investigators to estimate the number of ingested cells. The outbreak data were used to estimate the so-called r-value (i.e., probability of illness after ingestion of one cell) for populations that differ in susceptibility (the overall population, aged populations, and highly susceptible population) by dividing the number of cases by the number of ingested cells by the subpopulation. The r-value is a critical parameter in hazard characterization because it defines the dose–response correlation for each of the subpopulations (discussed in Section 5.2).

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In the exposure assessment step, various models can be used to quantitively describe the growth, inactivation, and survival of the pathogen along the exposure route. Predictive models can be grouped into probabilistic and kinetic models. Growth/no growth (G/NG) models are probabilistic models that predict the (combined) effect of growth-limited factors on the growth probability of the target pathogen. G/NG models are used to evaluate suitable preservation conditions to suppress growth (see e.g., Huang et al. 2022a, Koutsoumanis et al. 2004, Valero et al. 2006, Vermeulen et al. 2007). The output of a G/NG model shows the interface of conditions where growth can or cannot occur with a certain probability (expressed as, e.g., 90%, 50%). The number of replicates for each of the tested conditions affects the uncertainty in the probability estimation (Tsuruma et al. 2021), and the higher the number of replicates, the lower the uncertainty in the probability estimation. Kinetic models describe the number of microorganisms over time. Kinetic growth models are used, for example, to calculate the microbial concentration at the moment of consumption, whereas kinetic inactivation models can be used, for example, to estimate the achieved reduction of the pathogen. As kinetics are not always log-linear over time, models with increasing complexity have been developed (for an overview, see, e.g., den Besten et al. 2018; Garre et al. 2023; Geeraerd et al. 2000, 2005). To ease the use of models, various user-friendly, freely available tools have been developed (Tenenhaus-Aziza & Ellouze 2015), not only for kinetic modeling but also to perform a QMRA (**Table 1**). The choice of the best kinetic model for data fitting is not trivial, and selection criteria are, among others, statistical acceptability (i.e., model fit of the data is statistically acceptable), model parameters with a biological interpretation (e.g., lag phase, specific growth rate, inactivation rate), and lowest possible number of model parameters following the principle of parsimony. Models are often developed using a nutrient-rich laboratory medium, and food factors (e.g., acidity, low water activity) are simulated by artificially modifying the test medium. This approach allows wide application of the models in foods with different stress factors and can serve as part of the scientific evidence on the fate of the pathogen in the food but may not fully cover the behavior in the actual food product. A meta-analysis is a systematic analysis of a large collection of data from individual studies aiming to integrate the findings and produce a global estimate of a kinetic parameter (e.g., specific growth rate or inactivation rate) with its variability (den Besten & Zwietering 2012). The ComBase database (**https://www.combase.cc/**) is a rich data depository that can be used as input for a meta-analysis and subsequent parameter estimation for the exposure assessment of a QMRA (for some recent examples, see Abe et al. 2023, Van der Vossen-Wijmenga et al. 2024). The strong point of using data from a meta-analysis in a QMRA is that the variability of the kinetic parameter is estimated based on multiple studies, including microbial and product variability. This may overestimate the variability of a parameter, but one may proceed to a more product-specific estimation when these data are available. Although when available growth or inactivation data in the specific product of interest are not available, additional validation in the food of interest is needed. Documents of, for example, the International Organization for Standardization specify procedures for conducting challenges to test for growth and inactivation (ISO 2019, 2022).

5.2. Differences Among Hosts and Strain Virulence Classes: Perspectives for Quantitative Microbiological Risk Assessments

It is well recognized that the disease outcome and the severity of the symptoms and incidence rate of an *L. monocytogenes* infectious event differ between hosts (see Section 4). In most published QMRAs, the risk of listeriosis is predicted for two groups of people: the general (i.e., healthy) population and the susceptible population (including elderly, infants, pregnant women, and immunocompromised people) following the FAO/WHO (2004) approach, and the

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Table 1 Tools for kinetic data modeling (fitting, prediction) and/or performing a (semi)- quantitative microbiological risk assessment

Tool	Application	Reference
Biogrowth, bioinactivation	Fitting and prediction (growth, inactivation), with model selection and comparison, R-packages	Garre et al. 2023, 2017
ComBase	Database with option to donate data, prediction (growth, inactivation, survival) for broth, food, and DMfit tool	**https://combase.errc.ars.usda.gov/**
DMfit	Fitting (growth and inactivation), with model selection and comparison, ComBase online version and Excel add-in	**https://combase.errc.ars.usda.gov/**
Danish Meat Research Institute Predict	Prediction (growth, inactivation, survival) for (fermented) meat	**https://dmripredict.dk**
FDA-iRisk	Risk ranking based on QRA for (microbial and chemical) food-hazard pairs, and comparison of impact of interventions on health risk for population groups	Chen et al. 2013, **https://irisk.foodrisk.org**
Food Spoilage and Safety Predictor	Prediction (growth, G/NG interface, histamine formation) for various foods	**http://fssp.food.dtu.dk**
GinaFit	Fitting (inactivation), with model selection and comparison, Excel add-in	Geeraerd et al. 2005, **https://cit.kuleuven.be/biotec/software/GinaFit**
GroPIN	Prediction (growth, inactivation, G/NG interface) for various foods, Excel-VBA	**https://www.foodsctech.com/gropin**
Microbial Responses Viewer	Prediction (G/NG interface) for food-hazard pairs based on ComBase	Koseki 2009, **http://mrviewer.info/**
MicroHibro	Fitting and prediction (growth, inactivation, shelf life) for various foods, and risk assessment and sampling plan modules	**https://microhibro.com/**
MiId, MiRa	Hazard identification and risk ranking based on semi-QMRA for infant foods, R-packages	Yeak et al. 2024a,b
MLA Refrigeration Index calculator	Prediction (growth) of *Escherichia coli* for meat	Ross et al. 2003, **https://ricalculator.mla.com.au/**
Pathogen Modeling Program	Prediction (growth, inactivation, survival) for broth and various foods	**https://pmp.errc.ars.usda.gov/PMPOnline.aspx**
Risk Ranger	Risk ranking based on semi-QMRA for food-hazard pairs, and comparison of impact of interventions on health risk for population groups	**https://foodsafetyportal.eu/riskranger/rr_riskranger.html**
Sym'Previus	Fitting and prediction (growth and inactivation), paid premium version has extended modules (e.g., fungal germination)	**https://symprevius.eu/en/**

Abbreviations: G/NG, growth/no growth; MLA, Meat & Livestock Australia; QMRA, quantitative microbiological risk assessment; QRA, quantitative risk assessment; VBA, visual basic application.

susceptible population is accountable for the majority of the cases in a risk estimation (see, e.g., EFSA Panel Biol. Hazards et al. 2018, FAO/WHO 2004). The exponential dose–response model is frequently used for hazard characterization, with an r-value for the two groups of people, considering that the r-value is constant for all cells and all individuals within a subpopulation. More recent QMRAs attempt to refine the hazard characterization and differentiate within a subpopulation

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and between more subpopulations that differ in susceptibility (EFSA Panel Biol. Hazards et al. 2018, Pouillot et al. 2015).

Fritsch et al. (2018) refined the hazard characterization from the microbial virulence perspective. It is commonly recognized that no *L. monocytogenes* strains are avirulent (Pouillot et al. 2024), but some recent studies demonstrated that some clonal complexes or sequence types (CCs/STs) are overrepresented in disease cases. Fritsch et al. (2018) classified *L. monocytogenes* strains of different CCs/STs into three classes of virulence, namely, hypovirulent, medium virulent, and hypervirulent CCs/STs, based on clinical frequencies of different CCs/STs reported by Maury et al. (2016). The authors also addressed strain differences in the exposure assessment step. Strains were grouped into two groups with respect to the minimum growth temperature (i.e., T_{min}), resulting in a higher growth at a given temperature for strains with a lower T_{min}. The authors predicted that for cold salmon–related listeriosis cases, 95% were attributed to the hypervirulent strains with high growth, despite the relatively low frequency (i.e., 12%). As a follow-up, Pouillot et al. (2024) integrated the subgrouping proposed by the EFSA Panel on Biological Hazards et al. (2018) and the virulence classification proposed by Fritsch et al. (2018) and came to a set of 42 (i.e., 7 age groups, 2 sexes, 3 virulence classes) $\log_{10}$ r-values (mean and standard deviation). This allowed them to compare risks between various subpopulations when exposed to different classes of strains at a similar dose by calculating the relative risks. This resulted in a maximum difference in relative risk of 655, meaning that the highest susceptible group (i.e., females >75 years old) exposed to the most virulent strains has a 655 times higher risk than the least susceptible group (males between 15 and 24 years old) exposed to the less virulent strains.

Scenario analysis of QMRAs highlights the need for representative data of *L. monocytogenes* along the chain for occurrence and strain variability, as well as for data on actual consumer practices (see, e.g., Abe et al. 2023, Chen et al. 2022, Van der Vossen-Wijmenga et al. 2024), to support the exposure assessment step and realistically compare the impact of factors on the final risk estimation to optimize advice for risk control. Often collection of this type of data is time-consuming, and the data are not always representative. Also, recent advancements in the field of dose–response modeling and exposure assessment demonstrated the added value of information on the genetic background of strains implicated in the disease and occurring in the chain. This underlines the necessity of close collaboration between food inspection agencies and national health institutes when outbreaks or incidents occur for root-cause analysis and follow-up actions. The authorities may also keep the opportunity open together with food producers to extend sampling practices when contaminated batches implicated in cases become available with microbial counts that are deemed stable. Joining of forces to collect information on the actual ingested doses when people fall ill, which is one of the critical steps in a QMRA, improves the knowledge of the virulence potential of CCs and helps to improve dose–response models (Chen et al. 2017, Fritsch et al. 2018, Pouillot et al. 2016) although still subject to a high degree of uncertainty.

6. CONCLUDING REMARKS

In this review, we raise awareness of the emerging research field of "nano in micro," where the genetics, occurrence, and functionality of bacteriophages/prophages, BEVs, and BMCs in foodborne pathogens are addressed, and their relevance in transmission and pathogenesis is discussed. Insights obtained in *L. monocytogenes* substrate utilization capacity, including strain-specific effects on stress resistance and virulence, may provide further leads and targets for the development of novel and improved strategies to control transmission and growth of this pathogen on selected (ready-to-eat) foods. These aspects in combination with a critical reflection on factors in food processing and host-related environments highlight the necessity to study stress response, physiology,

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adaptation, and survival of foodborne pathogens in laboratory conditions beyond historically adopted norms. These new insights and proposed efforts represent valuable inputs for optimized risk assessment of foodborne pathogens, where the updated knowledge on pathogen growth and persistence in different environments, transmission, and pathogenesis, including impact of host diet and intestinal microbiota and the link between genotype and phenotype, will be key.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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